A Molecular Window into Parkinson's Disease

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A molecular window into Parkinson's disease

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“Das Wahre ist das Ganze”

(Georg Wilhelm Friedrich Hegel, 1770-1831)

CONTENTS

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General introduction and outline of the thesis

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A disease can be described at various levels, based on its etiology, pathogenesis, pathophysiology and phenotype. The etiology and pathogenesis describe the cause and disease-specific processes, respectively, that lead to the pathophysiology, i.e. the functional changes that accompany a disease. These physiological changes, in turn, lead to clinical symptoms, i.e. the phenotype of the disease. Clinical and biological research aims at advancing our insights into the etiology, pathogenesis, pathophysiology and phenotype of a disease. In this introduction, the increasing understanding of Parkinson's disease (PD) will be summarized according to these four levels of description, starting with the clinical description (phenotype; section 1.1), followed by the pathophysiology (section 1.2), and the etiology and pathogenesis (section 1.3) of PD. This view into knowledge development over time not only gives insights into the workings of science, but also provides us an overview of all currently known factors involved in PD and enables us to appreciate the complexity of the disease.

In the research described in this thesis, we use (animal) models, generate hypotheses, and seek to uncover and further advance the knowledge of the core molecular processes underlying PD. It is therefore of crucial importance that we compare the model that we apply, or the hypothesis that we pose, with the real-life situation, in this case PD. Is our model sufficiently simulating the disease that we want to understand? What are the limitations and caveats of the model used? Is our hypothesis in accordance with the human situation? To allow such considerations, I believe it is essential to have at least a basic understanding of the clinical and biological aspects of the disease.

Further, I shortly discuss current treatments of PD (section 1.4), explain the landscape building approach – the method that is used throughout this thesis to integrate and interpret large datasets – (section 1.5), and present the specific aims of this thesis and a brief outline of each chapter (section 1.6).

1.1 A HISTORICAL WINDOW INTO THE CLINICAL DESCRIPTION OF PD

1.1.1 The cardinal motor features of PD

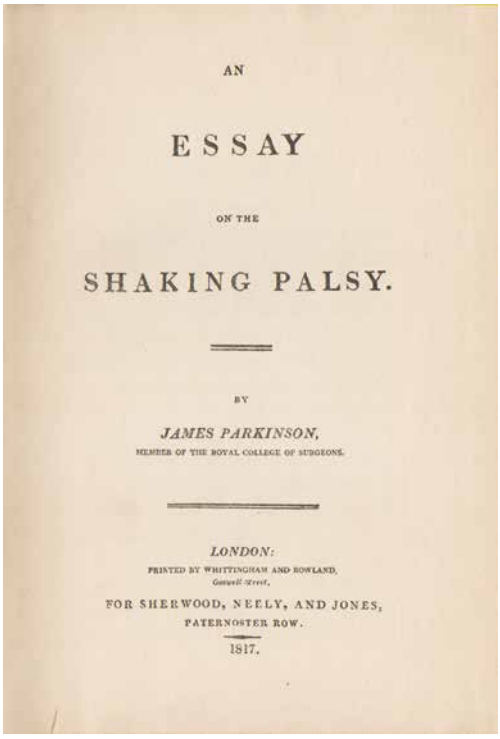
Until the early 19th century, diseases such as PD, multiple sclerosis, progressive muscle atrophy and amyotrophic lateral sclerosis (ALS) were all described as 'palsies'. A palsy referred to any type of paralysis, which was defined by the nosologist Philippe Pinel (1745-1826) as:

> *"...a total or a partial decrease of voluntary movement. The arms and legs could be in various states: without any tension, with tremor or in a state of contraction."*[1, 2]

Therefore, basically all diseases with involuntary motor symptoms were categorized in this group, which asked for a more specific definition of the disorders. In 1817, the English surgeon James Parkinson (1755-1824) published "An essay on the shaking

palsy" (**Figure 1**). In this medical essay, Parkinson described six cases with the shaking palsy of which he observed three in his practice and three others from a distance on his walks around the neighborhood. In 66 pages, Parkinson described his definition of the shaking palsy (or 'paralysis agitans') and thereby set it apart from other palsies:

> *"Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellects being uninjured."*


AN

ESSAY

ON THE

SHAKING PALSY.

BY

JAMES PARKINSON,

MEMBER OF THE ROYAL COLLEGE OF SURGEONS.

LONDON:

PRINTED BY WHITTINGHAM AND ROWLAND,

Goswell Street,

FOR SHERWOOD, NEELY, AND JONES,

PATERNOSTER ROW.

1817.

Figure 1. Front page of Parkinson's essay on the shaking palsy[3].

In this way, Parkinson described two of the four cardinal features of PD, the resting tremor and postural instability. The latter causes the propulsion observed in PD patients, caused by their inability to maintain their balance. In addition to these features, Parkinson described non-motor symptoms such as constipation:

> *"The bowels, which had been all along torpid, now, in most cases, demand stimulating medicines of very considerable power"*

and drooling:

> *"the saliva fails of being directed to the back part of the fauces, and hence is continually draining from the mouth, mixed with the particles of food, which he is no longer able to clear from the inside of the mouth."*

Furthermore, Parkinson made a distinction between acute cases of palsy and the cases of shaking palsy he described in his essay. He defined an acute palsy as a sudden decrease in voluntary muscle action, sometimes in combination with a 'lessened sense of feeling' and caused by a trauma, or as he put it:

> *"compression of the brain, or dependent on partial exhaustion of the energy of that organ"*

In contrast, in the six cases of shaking palsy described by Parkinson, he saw a longer

duration of disease onset and progression, eventually resulting in reduced mobility with increased speech problems, but without a loss of feeling as seen in the acute cases:

> *"...the diminution of the influence of the will on the muscles comes on with extreme slowness, is always accompanied, and even preceded, by agitations of the affected parts, and never by a lessened sense of feeling. (...) he began to find a small impediment in uttering some words (...) the difficulty in speaking (...) considerably increased (...) a spitting began, and now it was with difficulty that he uttered a few words."*

Parkinson therefore noted that the cases of shaking palsy had a slower progression of the disease and other symptoms than was observed in patients with acute palsies.
Lastly, Parkinson defined the tremor and the propulsion as pathognomonic (occurring in only one disease) for shaking palsy, distinguishing it from other patients with palsies[3]. However, nowadays these symptoms are not considered to be pathognomonic anymore and can overlap with other neurodegenerative diseases such as multiple system atrophy and progressive supranuclear palsy[4, 5].

Although Parkinson tried to excite his fellow researchers, it was not until the second half of the 19th century that the French internist Armand Trousseau (1801-1867) in his lectures at the Hôtel-Dieu in Paris and the neurologist Jean-Martin Charcot (1825-1893) during his teaching at the Salpêtrière in Paris further defined and elucidated the clinical features of the shaking palsy. Trousseau described rigidity in PD patients, the third cardinal motor symptom of PD, and also described a progressive slowing of repeated hand opening, which is the first account of bradykinesia[6] and the fourth cardinal feature of PD. Moreover, and in contrast to Parkinson, Trousseau noted that PD patients show a cognitive decline:

> *"The intellect is at first unaffected, but gets weakened at last; the patient loses his memory, and his friends soon notice that his mind is not so clear as it was: precocious caducity sets in."*[6]

Charcot contributed to the systematic examination of neurological diseases, which made it possible to distinguish PD from other diseases such as multiple sclerosis[7]. He was the first to provide a clear description of ALS[8], and to give a complete overview of the clinical symptoms of PD in the 1870s and 1880s, which was eventually published in the 'Leçons sur les maladies du système nerveux, faites à la Salpêtrière'. In this work, Charcot, like Parkinson, described the cardinal PD motor features resting tremor:

> *"the patient closes the fingers on the thumb as though in the act of spinning wool (...) The thumb moves over the fingers as when a pencil or paper-ball is rolled between them; in others the movements are more complicated and resemble what takes place in crumbling a piece of bread."*

and postural instability:

> *"a tendency to propulsion or to retropulsion (...) the individual is, in the first case, propelled forward, and, as it were, compelled to adopt a quick pace; the individual is unable, without extreme difficulty, to stop – being apparently forced to follow a flying centre of gravity."*

but also recognized, like Trousseau, the rigidity in the neck, trunk and extremities. Moreover, Charcot described slowness in the execution of movement (bradykinesia) as a cardinal feature of the disease and noted that this was independent of the rigidity in the limps[7]. In 1925, Jean-René Cruchet (1875-1959) was the first to use the term 'bradykinesia' for the slow execution of movement and defined this as the most important symptom of PD[9, 10]. In the 1920s, the French Neurologist Jules Froment (1878-1946) showed that rigidity in PD depends on the static posture of the body and that in PD 'maintenance stabilization' of the body is impaired and is compensated by 'rigidification' of the body[11].

Charcot also noted the 'poker face' of PD patients:

> *"a fixed look, and immobile features"*

Further, he acknowledged PD patients' complains of pain and sensations:

> *"They complain of cramps, or rather of a nearly permanent sensation of tension and traction in most of the muscles."*

but also their problems with speaking:

> *"the utterance is slow, jerky and short of phrase: the pronunciation of each word appears to cost a considerable effort (...)the utterance will be tremulous, broken, jolted out as it were like that of an inexperienced rider on horseback, when the animal is trotting."*

and he studied the problems PD patients have with writing (micrographia) (**Figure 2**):

> *"The strokes forming the letters are very irregular and sinuous, whilst the irregularities and sinuosities are of a very limited width. (...) the down-strokes are all, with the exception of the first letter, made with comparative firmness and are, in fact, nearly normal – the finer up-strokes, on the contrary, are all tremulous in appearance, and it is to the unsteadiness of these lines that the peculiar character of the writing here is principally due."*

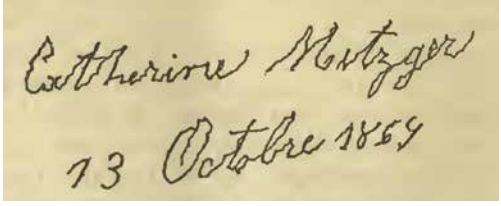


Figure 2. Handwriting of a PD patient, adapted from ref. [7].

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In describing the cardinal features of PD, Charcot recognized the early contributions of James Parkinson and therefore proposed to name the disease "Parkinson's disease"[7], a name that has been used ever since.

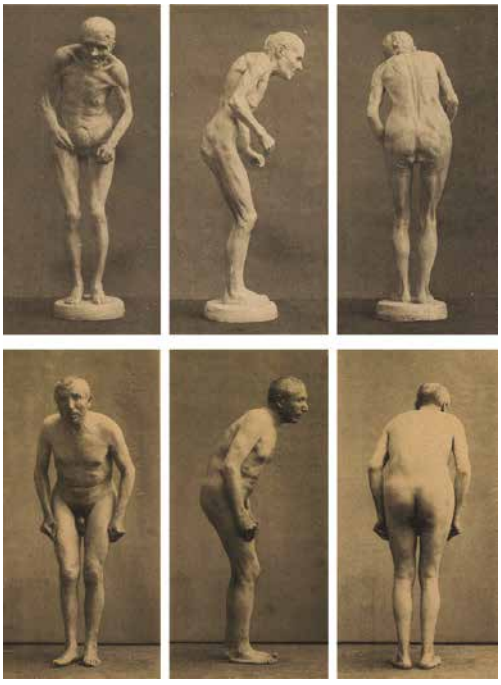


Figure 3. Photographs of a statue (46 cm tall) of a female PD patient sculpted by Richer (upper panels) and of a male PD patient (lower panels). Photographs are adjusted from ref. [13].

In the 1870s, the anatomist Paul Richer (1849-1933) started as an assistant of Charcot and in 1882 became the head of the Salpêtrière's museum of pathological anatomy. Charcot founded this museum that was focused on the visual representation of different (neurological) diseases[12]. Charcot and Richer had a shared interest in the representation of diseases in art and wrote two books, 'Les Démoniaques dans l'art' (1887) and 'Les Difformes et les Malades dans l'Art' (1889), in which they diagnosed patients depicted in artwork[12]. By creating and collecting sculptures and other visual representations of pathologies, Charcot and Richer meant to provide an objective, three-dimensional alternative for photography[12]. For the Salpêtrière's museum of pathological anatomy, Richer sculpted a series of very accurate representations of patients suffering from e.g. myopathy, hypothyroidism and PD[12]. In 1895, Richer published *'Étude morphologique sur la maladie de Parkinson*', which contains photographs of a statue of a 58-year-old female PD patient he had sculpted that were compared with photos of a male PD patient photographed in the same angles and lighting conditions as the statue[13] (**Figure 3**). In this way, Richer confirmed the pathological likeness and objectivity of his 'scientific artworks'[12, 13]. This work by Richer is one of the first known depictions of PD. Another iconic illustration of a PD patient was published in 1888 by the British neurologist William Gowers (1845-1915) in his book '*A Manual of Diseases of the Nervous System*' (**Figure 4**).

Figure 4. Illustration of a PD patient by William Gowers, adapted from ref. [14].

Gowers was the first to recognize the predominance of male PD patients and he also reported the high age of onset (50-60 years of age), the joint deformities in PD and an increased sense of heat and perspiration. On the treatment of PD patients Gowers said:

> *"My own experience is to the effect that arsenic and Indian hemp, the latter sometimes combined with opium, are of most use"* [14]

and he also notes that:

> *"Electricity in all forms is useless."* [14]

These statements probably made him – in comparison to all other used practices such as nerve stretching (Westphal), static electricity (Charcot) and voltaic electricity (Berger)[14] – an attractive physician to consult at that time.

1.1.2 Cognition and psychiatric symptoms in PD

Although some neurologists have reported a cognitive decline in the end stages of the disease, the psychiatric symptoms of PD have been less well described. In 1881, the first professor of psychiatry in Paris, Benjamin Ball (1833-1893), stated on a medical congress in London that:

> *"(…) a large number of Parkinsonian patients present psychological disorders extending from simple irritability to psychosis; far from being an exception I would say that a slight degree of cognitive impairment is the rule"* [15].

By doing so, Ball was the first to describe psychiatric symptoms, like depression, in association with PD and started a debate among neurologists about psychiatric symptoms as an integral part of PD. Charcot and his (former) student Brissaud (1852-1909) still believed that these symptoms were not part of the disease and irrelevant. Moreover, the British neurologists Gowers and Samuel Wilson (1878-1937) believed that the depression and irritability observed in PD patients should be regarded a *"natural outcome, perhaps, of an incurable disease"*. This implied that these symptoms would have been caused by the frustration of being incurably ill and that these and other psychiatric symptoms could be ascribed to other diseases or were incidental[15, 16]. Nevertheless, later Gowers also stated that PD patients had a propensity for mental weakness, memory loss and delusions:

> *"Pronounced mental symptoms are occasionally present, however, in the later stages of the disease, commonly limited to mental weakness and loss of memory, but sometimes accompanied by a tendency to delusions."* [14]

Therefore, although not fully accepted as part of the disease, the psychiatric symptoms were included more and more into the clinical description of PD. However, it was not until the mid-twentieth century before psychiatric symptoms were accepted as part of

PD[15, 16].

In summary, PD is defined primarily as a motor disorder, exhibiting the cardinal features resting tremor, postural instability, rigidity and bradykinesia and is, especially in the later stages of disease progression, associated with cognitive decline and psychiatric symptoms. In addition, although the clinical depiction of PD has changed over time, the initial descriptions by Parkinson and Charcot still form the basis for defining the disease.

1.1.3 Categorization of disease progression

Parkinson described the disease as extremely slow and gradually progressing over the course of multiple years. In one of his case descriptions, Parkinson reported the progression of the disease as starting with a tremor in the left hand and arm, extending three years later to the right arm and hand, and another three years later also to the legs[3]. Charcot gave a more elaborate description of the average clinical progression of PD and recognized three disease phases[7]. The first phase is a 'slow invasion' of disease symptoms:

> *"At this stage of the disease the tremor may be merely passing and transitory. It breaks out when least expected, the patient enjoying complete repose of mind and body, and it frequently occurs without his being conscious of it. (…) Later on, it will be no longer so. Moreover, as it augments in intensity and persistence, the tremor invades little by little (…)".*

The second phase is a stationary period in which the characteristics of the disease such as the tremor are *"displayed in all their fullness"* and the third phase is the terminal period, for which he described the symptoms of the patient as:

> *"(…) difficulty of movement increases, and the patients are obliged to remain, the whole day long, seated on a chair, or are altogether confined to the bed. Then, nutrition suffers, especially the nutrition of the muscular system. (…) the patients succumb to the mere progress of their disease, by a sort of exhaustion of the nervous system".*

In addition, Gowers noted that the disease is *"always chronic, and usually progressive, in its course."* and he described the extension of motor symptoms from one side of the body to the opposite site taking place *"six months to three or four years after the onset."* [14]. In 1967, Hoehn and Yahr updated this motor symptoms-based description[17], and categorized PD patients according to five disease stages:

Stage I:	Unilateral motor symptoms;
Stage II:	Bilateral motor symptoms;
Stage III:	Difficulties with physical movement and postural stability,

but the patient can still cope without assistance;

Stage IV: Severe and disabling motor symptoms, assistance is often necessary during physical movement;

Stage V: Most severe motor symptoms, the patient is unable to perform physical movement without assistance, and is confined to a bed or wheelchair.

However, acknowledging the shortcomings of categorizing PD patients solely on their motor symptoms, the Movement Disorder Society - Unified Parkinson's Disease Rating Scale (MDS-UPDRS) is currently used to describe PD progression. This detailed rating scale describes the various aspects of motor dysfunction and includes the Hoehn and Yahr classification of progression as well, but it also takes into account the non-motor symptoms seen in (especially late-stage) PD e.g. (impaired) mental functioning, mood, pain and other sensations, sleep problems and daily living activities[18]. As such, the categorization of PD disease progression is – by including the non-motor symptoms of PD – (again) in accordance with the clinical description of PD.

1.2 TOWARDS THE PATHOPHYSIOLOGY OF PD

In the 19th century, the concept of networks between different brain areas was not known yet, and therefore various pathological origins of PD were proposed, and a discussion arose about which brain area would cause PD. Parkinson and later also the English physiologist Marshall Hall thought that lesions in the medulla caused Parkinson's disease[19, 20]. Charcot on the other hand thought that PD was caused by neurosis *"in this sense that it possesses no proper lesion"* [7], and the German neuropathologist Oskar Berger (1844-1885) suggested that sudden intense emotions (especially sudden terror), long exposure to damp cold, or trauma to the peripheral nerves were initiators of PD[21]. It was in 1895 that Brissaud commented in his lesson *'Nature et Pathogénie de la Maladie de Parkinson'* at the Salpêtrière that:

> *"a lesion of the locus niger may be the anatomical substrate of Parkinson's disease"* [22] (translated from French).

Brissaud made this clinicopathological association based on a case report by Blocq and Marinesco in which they described a patient with unilateral Parkinsonism caused by a tumor in the substantia nigra (SN)[23]. In 1919, the Russian neuropathologist Konstantin Tretiakoff (1892-1958) confirmed Brissaud's remark in his thesis, i.e. Tretiakoff reported a loss of pigmented neurons in the SN of six patients with PD and also noted the presence of 'Lewy bodies' in the remaining SN neurons[24]. The latter he did in recognition of earlier research by Friedrich Lewy (1885-1950) who reported similar inclusions in neurons of PD patients[25]. Tretiakoff ended his thesis with the statement:

> *"Thus, the results of our research lead us to say that, between lesions in the*

> *substantia nigra and Parkinson's disease, there is a very close relation. This is most likely a causal relation."* [24] (translated from French).

However, others claimed that lesions in the cerebral cortex[26], globus pallidus and striatum[27, 28] were more relevant. Moreover, although the degeneration of pigmented neurons of the SN was consistently found in patients with PD[29, 30], Tretiakoff's findings were not widely accepted until they were confirmed by the German pathologist Rolf Hassler (1914-1984) in 1938 and by Greenfield and Bosanquet in 1953. Hassler showed that all PD patients had a loss of pigmented neurons in the SN, that surviving neurons contained Lewy bodies, and that lesions in the striatum or globus pallidus were not always present[31], whereas Greenfield and Bosanquet provided an extensive pathological overview of the lesions observed in PD[32].
In subsequent years, the concept of neuronal circuits took hold, with the SN, striatum and globus pallidus (all part of the basal ganglia) as key brain nuclei involved in the clinical manifestation of PD[33].

In the 1950s, the biochemical changes underlying this neuronal circuit in the basal ganglia were identified by, among others, the Swedish researcher Arvid Carlsson. Carlsson showed that dopamine (DA) was a neurotransmitter in the brain that could be depleted by reserpine – a cardiovascular drug that also has an akinetic effect – and restored by levodopa (L-DOPA), a precursor of DA[34]. Moreover, Bertler and Rosengred, medical students in the lab of Carlsson, showed that DA concentrated in the striatum of a dog brain[35, 36] and they concluded that:

> *"The results favour the assumption that dopamine is concerned with the function of the corpus striatum and thus with the control of motor function."*

These observations were also replicated in humans[37] and in 1959, Carlsson suggested that DA plays a role in PD[38]. In 2000, Carlsson received the Nobel Prize in Physiology or Medicine for his work on signal transduction in the nervous system[39].

In 1960, Ehringer and Hornykiewicz found that depletion of striatal DA was specific for PD patients and not observed in patients with Huntington's disease or exhibiting extrapyramidal signs – such as tremor, rigidity and hyperkinesias – of unknown origin, i.e. neurological diseases that exhibit motor symptoms similar to PD[40]. Their first clinical trial showed that intravenous administration of L-DOPA reduces the motor symptoms of PD patients[41]. In 1967, George Cotzias demonstrated that starting with very low doses of L-DOPA that were gradually increased over time not only reduced the motor symptoms of PD but also the side-effects of high dosages of L-DOPA[42].
The link between striatal DA levels and SN degeneration was confirmed in 1964 by Nils-Erik Andén (and Carlsson as a co-author) using a fluorescence technique developed by Falck and Hillarp two years before to visualize DA neurons[43, 44]. Andén mapped the

axons of the SN neurons and showed that an SN lesion causes a loss of fluorescence in the striatum and thus reduced striatal DA[45].
In conclusion, the pathophysiology of PD is characterized by lesions in basal ganglia nuclei, and especially by the loss of DA-producing neurons in the SN, resulting in reduced DA release in the striatum. Treatment of PD patients with the DA precursor L-DOPA attenuates the motor symptoms caused by low striatal DA.

The presence of non-motor symptoms in PD supports the involvement of neurotransmitters other than DA that regulate processes such as memory and learning (e.g. glutamate, acetylcholine)[46, 47], and mood and sleep (e.g. serotonin)[48], or pain (e.g. neuropeptides)[49, 50]. Moreover, the neurological substrate of PD is not limited to the SN and involves the degeneration of multiple other brain areas and non-DA pathways that contribute to both the motor and non-motor features of PD. The establishment of PD as a disease spanning multiple brain areas was further emphasized by a study by Braak in 2003. In this study, the pathological disease pattern of PD was described in six stages starting in the pre-motor period of PD with lesions in the anterior olfactory nucleus and brain stem (stages 1-2), ascending to the basal mid- and forebrain nuclei (among which the SN) and paralimbic cortex (stages 3-4) and in the final stages (5-6) to the neocortex[51, 52]. Lesions in all these areas, other than the basal ganglia nuclei, may contribute to the non-motor symptoms of PD and affect e.g. olfactory function, rapid eye movements and mood, and this even before the clinical manifestation of motor symptoms. Nevertheless, until today, the biochemical changes underlying the pre-motor symptoms in PD have been under-investigated compared to such changes underlying the motor symptoms and are therefore still largely unresolved[53].

To better understand how decreased levels of DA in specific brain areas could lead to the diverse clinical manifestations as seen in PD, new models were developed taking connections between different brain areas into account. The classic model of basal ganglia circuitry is based on a loop between the cortex and the basal ganglia nuclei, which is thought to be dysregulated in PD (**Figure 5**). In this model, the reduced input from the SN pars compacta (SNpc) to the striatum results in increased activation of the globus pallidus pars interna (GPi) and SN pars reticulata (SNr), and subsequent inhibition of the thalamocortical projection. Increased activation of the GPi may occur either through the direct pathway (striatum > GPi) or indirect pathway (striatum > globus pallidus pars externa > subthalamic nucleus > GPi/SNr). In this way, activation of the direct pathway reduces the activity of the GPi and facilitates movement, whereas activation of the indirect pathway increases GPi activity and suppresses movement. Nevertheless, this model is now regarded as limited, because it cannot explain multiple clinical features of PD[54]. For example, the origin of a rest tremor and rigidity as seen in PD is not explained by an increased inhibition of the thalamocortical projection. Further,

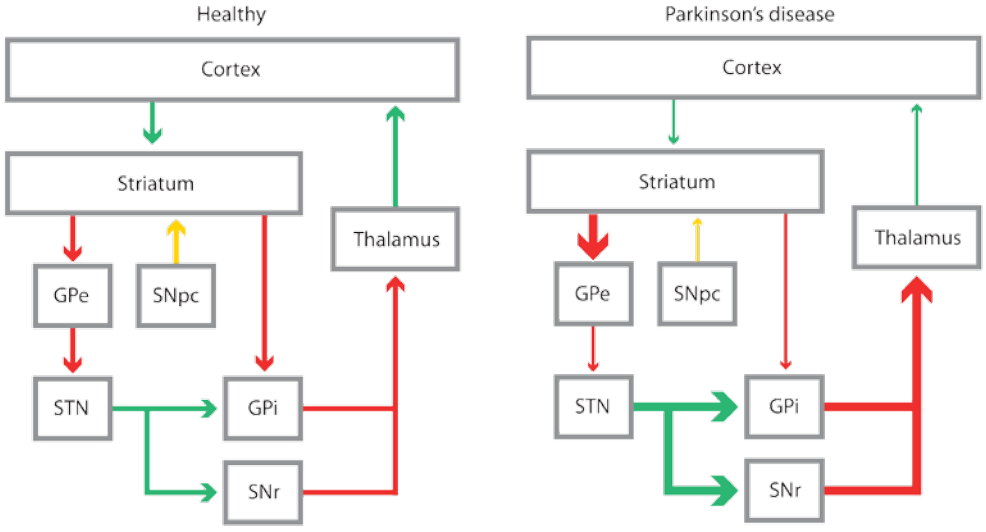


Figure 5. The classic pathophysiological model of the basal ganglia circuit in a healthy person (left) and in a PD patient (right). In PD, a reduced DAergic innervations of the striatum (yellow arrow) increases the activity of the indirect pathway (striatum-GPe-STN-GPi/SNr) and decreases the activity of the direct pathway (striatum-GPi). Green arrows show the excitatory glutamatergic connections and red arrows show the inhibitory GABAergic connections between the brain areas. GPe, globus pallidus pars externa; GPi, globus pallidus pars interna; SNpc, substantia nigra pars compacta; SNr, substantia nigra pars reticulate; STN, subthalamic nucleus. Figure modified from refs. [54] and [55].

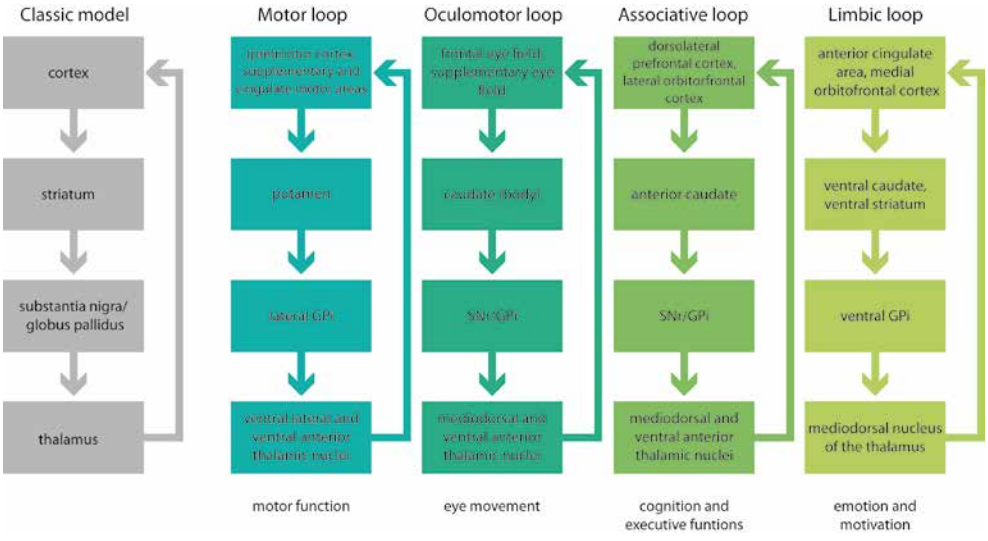


Figure 6. The basal ganglia circuitry subdivided in motor, oculomotor, associative and limbic loops. As an extension of the classic basal ganglia model (shown left) the basal ganglia circuit is subdivided in four loops that each regulate a subset of functions (shown below each loop). These loops are located in more precisely defined areas of the cortex and basal ganglia nuclei that are also part of the classic model. GPi, globus pallidus pars interna; SNr, substantia nigra pars reticulate. Figure is adapted from refs. [55-57].

lesions of the global pallidus or thalamus do not aggravate the motor function of PD patients, and GPi lesions abolish L-DOPA-induced dyskinesias, which is incompatible with a model that associates increased inhibition of the GPi with the facilitation of movements such as dyskinesias[54]. Therefore, in the most recent basal ganglia model, the basal ganglia circuitry is subdivided into motor, oculomotor, associative and limbic loops (**Figure 6**) that each regulate a subset of functions such as learning, planning, memory and emotions. In this respect, the dysregulation of the motor loop in the form of abnormal synchronized oscillatory activity – i.e. repetitive activation of groups of neurons – results in motor impairment in PD[58], whereas the oculomotor loop is involved in the control of eye movements which are affected in PD patients[59]. Further, defects in the associative loop result in cognitive inertia and executive dysfunction in PD[60], and dysregulation of the limbic loop in PD contributes to motivational and emotional processes such as impulse control disorders and emotional blunting[60, 61]. Although this current model is still oversimplified, the loops between basal ganglia areas and the cortex nevertheless provide a better model for the pathophysiology of PD than the classic basal ganglia model, and also better explain the occurrence of non-motor symptoms in PD.

1.3 THE ETIOLOGY AND PATHOGENESIS OF PD

Up until now, research on patients with familial PD – i.e. the Mendelian form that is passed on from parent to child – has resulted in the identification of 23 genetic risk loci and 17 genes that, when mutated, cause PD. The list of familial PD genes, their mode of inheritance, and the proteins that they encode can be found in **Table 1**. The involvement of these genes in PD provided an initial idea of the processes and pathways leading to the degeneration of neurons in the SN, e.g. the protein encoded by the alpha-synuclein (SNCA) PD-familial gene is part of the earlier identified Lewy bodies, while *PARK2*, *PARK7* and *PINK1* encode proteins that regulate mitochondrial quality control and oxidative stress, and the proteins encoded by *LRRK2* and *VPS35* are involved in autophagy and protein trafficking, respectively. However, as only 5-10% of the patients have the Mendelian forms, PD is predominantly considered a sporadic disease[62, 63], i.e. occurring 'spontaneously' in a patient without a family history of PD. In contrast to a 'one-hit model' like familial PD, sporadic PD is considered to be a multifactorial disease that requires 'multiple hits' – i.e. by a combination of genetic and environmental risk factors – for the disease to manifest itself[64, 65]. Because these risk factors all contribute to the onset of PD for only a small part and they can differ between and within populations, it is more difficult to pinpoint them than familial mutations. However, since the completion of the human genome project in 2001[66, 67] the use of genome-wide screening methods such as genome-wide association studies (GWASs) and genome-wide expression profiling became possible. Using these methods, instead of studying single candidate genes, an unbiased genome-wide analysis can be performed to identify genetic risk

Table 1. PD risk loci and associated familial PD genes. For each PD gene, its protein product and its mode of inheritance is shown. The references provide for each gene (or locus when the causative gene in the locus is unknown), the study that firstly associated the gene/locus with PD is referred to. AD: Autosomal dominant; AR: Autosomal recessive.

Locus	Location	Gene	Protein	Inher.	Ref.
PARK1	4q22.1	*SNCA* (mutation)	Alpha-synuclein	AD	68
PARK2	6q26	*Parkin*	E3 ubiquitin-protein ligase parkin	AR	69
PARK3	2p13	Unknown	-	AD	70
PARK4	4q22.1	*SNCA* (triplication)	Alpha-synuclein	AD	71
PARK5	4p13	*UCHL1*	Ubiquitin carboxyl-terminal hydrolase isozyme L1	AD	72
PARK6	1p36.12	*PINK1*	Serine/threonine-protein kinase PINK1	AR	73
PARK7	1p36.23	*DJ-1*	Protein deglycase DJ-1	AR	74
PARK8	12q12	*LRRK2*	Leucine-rich repeat serine/ threonine-protein kinase 2	AD	75
PARK9	1p36.13	*ATP13A2*	Probable cation-transporting ATPase 13A2	AR	76
PARK10	1p32	Unknown	-	-	77
PARK11	2q36-q37	*GIGYF2* [1]	PERQ amino acid-rich with GYF domain-containing protein 2	AD	78
PARK12	Xq21-q25	Unknown	-	-	79
PARK13	2p13.1	*HTRA2*	Serine protease HTRA2	AD	80
PARK14	22q13.1	*PLA2G6*	85/88 kDa calcium-independent phospholipase A2	AR	81
PARK15	22q12.3	*FBXO7*	F-box only protein 7	AR	82
PARK16	1q32	Unknown	-	-	83
PARK17	16q11.2	*VPS35*	Vacuolar protein sorting-associated protein 35	AD	84
PARK18	3q27.1	*EIF4G1* [2]	Eukaryotic translation initiation factor 4 gamma 1	AD	85
PARK19	1p31.3	*DNAJC6*	Putative tyrosine-protein phosphatase auxilin	AR	86
PARK20	21q22.11	*SYNJ1*	Synaptojanin-1	AR	87
PARK21	3q22	*DNAJC13*	DnaJ homolog subfamily C member 13	AD	88
PARK22	7p11.2	*CHCHD2* [3]	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2	AD	89
PARK23	15q22.2	*VPS13C* [4]	Vacuolar protein sorting-associated protein 13C	AR	90

[1] It is questionable whether GIGYF2 mutations actually cause PD. Mutations in GIGYF2 are provisionally designated as risk factors for PD[91].
[2] Confirmed as a risk factor for PD[92], but not as a familial, causative gene[92, 93].
[3] The mutations in the initial study have not been confirmed, but other putative pathogenic mutations have been reported[94].
[4] Not independently confirmed by other studies.

factors or expression differences. GWASs determine if there is an association between common variations in the genome, so called single nucleotide polymorphisms (SNPs), and a specific trait or disease[95]. Further, mRNA expression profiling using microarrays or RNA sequencing (RNAseq) made it possible to identify genes – or rather the mRNAs transcribed by these genes – that show a dysregulated expression and reflect the causative and compensatory processes that are involved in the disease. In this way, an enormous amount of genetic and expression data for PD has been collected in the past years. GWASs identified multiple susceptibility genes for sporadic PD (e.g. *SNCA*, *WNT3*, *FAM190A*, *GBA*, *NSF*)[96-99], whereas genome-wide expression studies showed the dysregulation of genes/mRNAs involved in (among others) the ubiquitin-proteasome system, oxidative stress, mitochondrial function, vesicular transport, axon guidance, synaptic function and the immune response[100-105]. In addition to the findings from genetic and expression studies, environmental risk factors for PD have been thoroughly researched, i.e. increased risk for PD has been attributed to pesticides, head injury and use of beta-blockers, whereas smoking and caffeine intake have been associated with a decreased risk[106]. Although these different approaches resulted in large datasets and have led to the identification of multiple PD candidate genes and proteins exhibiting a wide variety of functions, it has proved difficult to integrate these data and find a common mechanism leading to sporadic PD. However, as variants within familial PD genes (e.g. *SNCA*, *LRRK2*) were also identified as susceptibility factors for sporadic PD[107, 108], the underlying causal mechanisms of familial and sporadic PD are expected to – at least partially – overlap. Nevertheless, the absence of clear understanding of the underlying molecular pathways hampers the development of effective new therapies and drugs to treat PD. This is made clear by the fact that, despite all the research of the past decades, symptomatic treatment with L-DOPA is still the gold standard. To develop true disease-modifying therapies that not only provide symptomatic relief but also slow – or even stop – the progression of the disease, the underlying biological processes and causal mechanisms of the disease must be targeted. Therefore, further and more in-depth knowledge about the etiology and pathogenesis of PD is essential.

1.4 CURRENT TREATMENTS OF PD

Treatment of PD may consist of dopaminergic medication, neurosurgical methods such as deep brain stimulation (DBS) and supportive care such as physiotherapy[109]. Dopaminergic therapy aims to increase DA signaling in the brain by increasing its production through the administration of L-DOPA, by using DA agonists that activate DA receptors, or by using drugs that inhibit the proteins involved in DA degradation. Often these compounds are administrated in different combinations, depending on the stage of the disease and side effects experienced by the patient. Dopaminergic therapy works very effectively against the motor symptoms of PD, but does not slow down the progression of PD. Moreover, in later disease stages, when more DA neurons have

degenerated and higher dosages are needed, L-DOPA may induce dyskinesias[110-112] and DA agonists potentially cause hallucinations[113]. Therefore, especially in the late stages of PD, dopaminergic therapy is complex and needs to be tailored per patient.

DBS is an alternative, established therapy for PD. Usually an electrode is placed in the subthalamic nucleus (STN) or the GPi, two hyperactive brain regions in the basal ganglia of PD patients[109, 114] (see also Figure 5). The electrical stimulation from the electrode is thought to disrupt the signaling of these brain areas and block the abnormal signals through the basal ganglia circuit[115]. DBS is used to treat the motor symptoms of PD, particularly motor fluctuations – rapid changes between good and poor response to dopaminergic therapy – and resting tremor that is resistant to pharmacotherapy. The effects of DBS on non-motor symptoms in PD is controversial, i.e. were some studies show worsening of cognitive function – i.e. measures of reasoning, memory, executive functions and language – after DBS surgery[116-119], others suggest that this is due to worsening of the disease, and reduction of medication and not as a result of the DBS[120-123].

Of interest, aerobic exercise (e.g. treadmill exercise, cycling or dancing) or strength training (e.g. using a modified fitness counts program or progressive resistance exercising) that is used as supportive therapy to treat PD have been demonstrated to improve DA signaling in a mouse model[124, 125], and motor dysfunction – including bradykinesia, rigidity and tremor – in PD patients[126-131]. Further, physical exercise has also been reported to improve mood, cognitive function and sleeping problems in PD patients[132-134] and therefore improves – in contrast to dopaminergic therapy and DBS – not only the motor, but also certain non-motor symptoms of PD.

Although therapeutic interventions based on the main pathophysiology – e.g. improving DA signaling with L-DOPA treatment – or main clinical symptoms – e.g. improvement of motor symptoms with physical exercise – may not modulate the underlying disease-causing processes, they do however provide symptomatic relief. Moreover, further insight into the molecular and cellular effects of these treatments may improve our understanding of the pathophysiology of PD, and may also enable us to develop more effective therapies.

1.5 THE LANDSCAPE BUILDING APPROACH

In this thesis, large genetic and expression datasets are integrated and interpreted using the landscape building approach to gain insight into the molecular pathways underlying 1) the etiology and pathogenesis of PD, and 2) the beneficial effects of physical exercise therapy. An unbiased dataset is used as starting point for the landscape building approach – e.g. GWAS or genome wide expression data – to identify a list of disease-associated and protein-coding candidate genes. In **Figure 7**, a GWAS

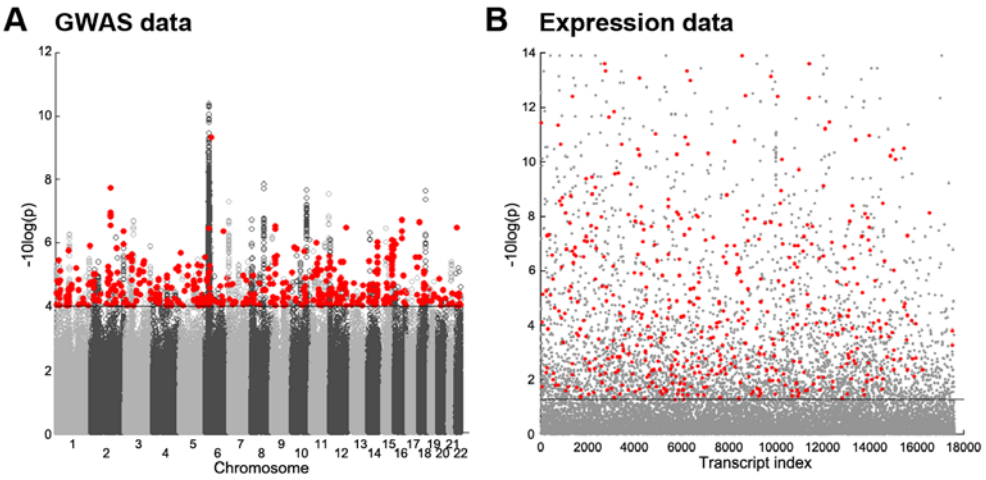


Figure 7. Visualization of GWAS (A) and genome wide expression (B) data. The SNPs respectively the transcripts that meet the inclusion criteria – and therefore represent the candidate genes – are indicated in red.

and expression dataset are visualized, and respectively the SNPs and transcripts that represent the candidate genes are indicated. These SNPs and transcripts meet certain inclusion criteria that are presented below and are also shown in **Figure 8A** as the first part of the landscape building approach:

When GWAS data is used as a starting point, only the SNPs with a p-value <0.0001, often employed to designate 'suggestive' association[135-137], are considered. The candidate genes either contain one or more of these SNPs within their exonic, intronic or untranslated region, or are the nearest gene downstream, or the nearest gene upstream within 100 kilobases (kb) of a SNP. The latter is based on the fact that the vast majority of expression quantitative trait loci (eQTL; SNPs that are not located in a gene but regulate the expression of one or more genes in their (relative) vincinity) for a given gene are located within 100 kb down- and/or upstream of this gene[138-140] and because trait-associated SNPs are more likely to be eQTL[141]. When candidate gene selection is based on expression data e.g. resulting from RNAseq, the transcripts are subjected to a cut-off for the RPKM (Reads Per Kilobase of transcript per Million mapped reads; a correction for gene length), the adjusted p-value and the fold-change between the healthy and the diseased state. In the case of our RNAseq analysis (chapter 4), these cut-offs were chosen based on qPCR measurements that indicated which transcripts could be detected in the individual samples, and as such, which cut-offs should be used as a detection threshold.

In the second stage of the landscape building approach (**Figure 8B**), the resulting list of candidate genes is subjected to an elaborate literature study to establish:

1) the interactions between the proteins encoded by these genes, and other proteins that function as key interactors (e.g. by forming a functional hub),
2) the interactions with additional proteins that are associated with the disease through candidate gene, functional, animal and/or cell line studies,
3) functional themes, signaling pathways and/or cascades within the set of interacting proteins, and
4) identification of putative druggable targets.

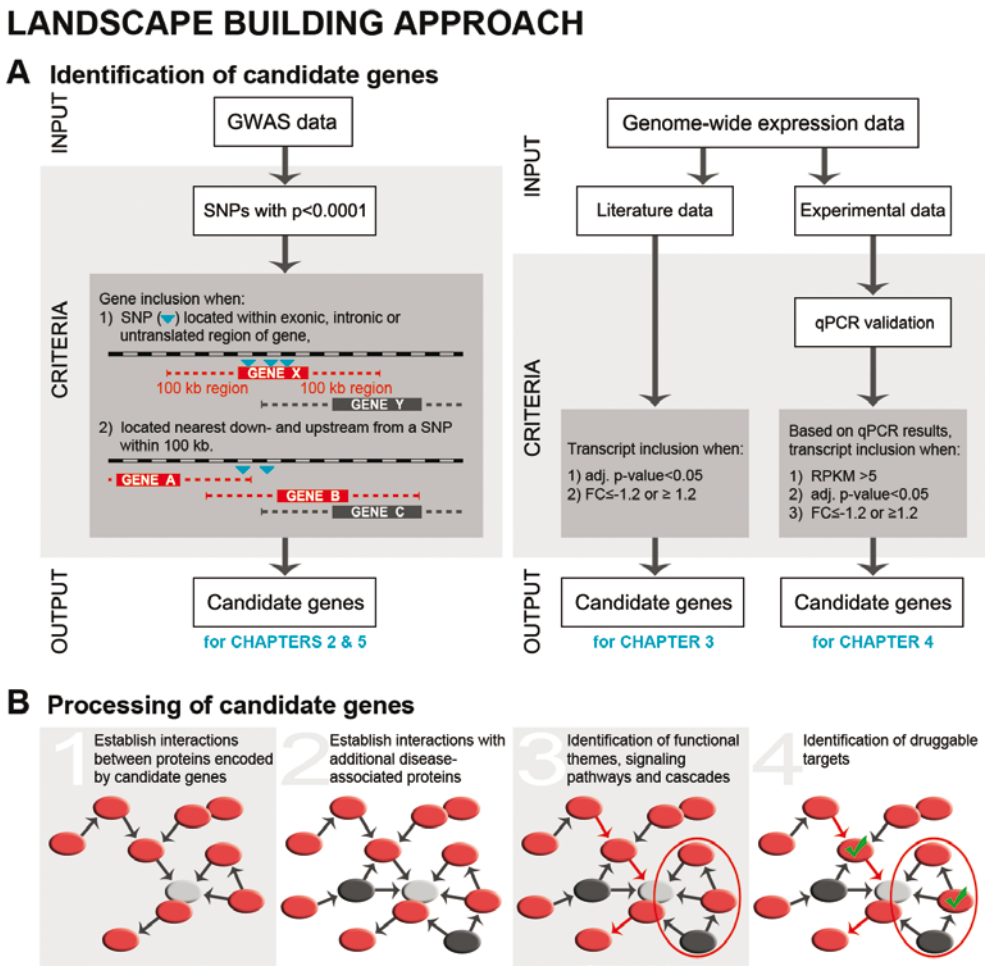


Figure 8. Overview of the landscape building approach. Candidate genes potentially involved in the studied disease are identified from genome wide association studies (GWASs) or genome wide expression data (A). Subsequently, the interactions between the proteins encoded by these candidate genes (red), but also the interactions with proteins that function as key interactors (light gray) and/or are disease-associated (dark gray) are established. Combined, all these interactions form the molecular landscape in which functional themes (red circle), signaling pathways and cascades (red arrows), and putative druggable targets (green marks) can be identified (B). FC, fold-change; qPCR, quantitative real-time polymerase chain reaction; RPKM, reads per kilobase of transcript per million mapped reads; SNPs, single nucleotide polymorphisms.

These interactions, themes and pathways result in a molecular landscape that shows a comprehensive overview of biological processes and cascades that may be relevant to disease etiology and/or pathophysiology. As such, the molecular landscape generates (more) in-depth and new insights into disease etiology and provides hypotheses for the development of novel treatments. This landscape building approach has previously also been applied to neurodevelopmental disorders such as ADHD[142], autism[143] and OCD[144].

1.6 AIMS AND OUTLINE OF THIS THESIS

This thesis aims to:

(1) provide insights into the molecular pathways underlying PD etiology and as such generate clues as well as hypotheses for the future development of novel PD treatment options;

(2) improve the understanding of the molecular pathways underlying the beneficial effects of physical exercise on PD;

(3) further establish the landscape building approach as a useful tool to identify the molecular pathways underlying complex neurodegenerative diseases.

In **chapter 2**, the landscape building approach is used to construct a molecular landscape of PD based on PD GWAS data, and other genes/proteins implicated in PD through e.g. familial candidate gene association and functional studies. This molecular landscape gives novel insights into the etiology of PD.

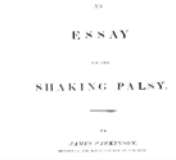
In **chapter 3**, the validity of the 1-methyl-4-pheynl-1,2,3,6-tetrahydropyridine (MPTP) mouse as a model for the molecular mechanisms that underlie human PD is assessed. MPTP, a drug that is transported into the brain and subsequently converted to the neurotoxin MPP+, causes the degeneration of DA neurons, especially in the SN. The MPTP-treated mouse is therefore a widely-used model for PD that enables us to perform *in vivo* studies on a pathology similar to PD. However, although the pathophysiology is similar, the underlying molecular pathways have never been directly compared. By comparing genome-wide expression data from MPTP-treated mice with expression data from both the SN and striatum of human PD patients, the validity of the MPTP-model as a model for the molecular pathways underlying human PD is assessed. Subsequently, the MPTP mouse model enables us to identify in **chapter 4** the underlying molecular pathways and therapeutic effects of physical exercise on PD in a preclinical setting. In both chapters 3 and 4, the landscape building approach is used as a tool to elucidate the underlying molecular pathways and interpret the datasets.

In **chapter 5**, I analyze whether it is possible to elucidate distinct, disease-specific and substrate-specific mechanisms for another neurodegenerative disease – i.e. ALS – by applying the landscape building approach. In ALS, the upper and lower motor neurons

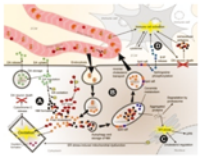
degenerate, which results in muscle weakness and ultimately respiratory failure within 2-5 years after onset of the first symptoms. As such, ALS exhibits – similar to PD – motor symptoms that are caused by the degeneration of a specific neuron population.

Lastly, in **chapter 6** the main results and conclusions from this thesis will be summarized, compared and discussed.

A graphical overview of the chapters in this thesis is provided in **Figure 9**.

1 

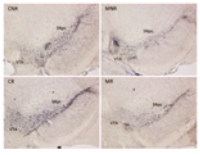
General introduction on Parkinson's disease (PD) and outline of the thesis.

2 

The molecular landscape for PD shows the underlying pathways and processes that lead to PD.

3 

The MPTP-treated mouse is validated as a model for PD by comparing its gene expression to the expression in human PD.

4 

The MPTP mouse model is used to determine the molecular pathways underlying the beneficial effects of physical exercise as seen in PD patients.

5 

The molecular landscape for amyotrophic lateral sclerosis (ALS) shows, compared to the PD landscape, other pathways and processes that lead to neurodegeneration.

6 

General discussion

Appendices

Summary
Samenvatting (summary in Dutch)
Dankwoord (acknowledgements in Dutch)
Curriculum vitae
List of publications

Figure 9. Graphical overview of this thesis.

1

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2

Integrated molecular landscape of Parkinson's disease

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2.1 ABSTRACT

Parkinson's disease (PD) is caused by a complex interplay of genetic and environmental factors. Although a number of independent molecular pathways and processes have been associated with familial PD, a common mechanism underlying especially sporadic PD is still largely unknown. In order to gain further insight into the etiology of PD, we here conducted genetic network and literature analyses to integrate the top-ranked findings from thirteen published genome-wide association studies (GWASs) of PD (involving 13.094 cases and 47.148 controls) and other genes implicated in (familial) PD, into a molecular interaction landscape. The molecular PD landscape harbors four main biological processes – oxidative stress response, endosomal-lysosomal functioning, endoplasmic reticulum stress response, and immune response activation – that interact with each other and regulate dopaminergic (DA) neuron function and death, the pathological hallmark of PD. Interestingly, lipids and lipoproteins are functionally involved in and influenced by all these processes, and affect DA neuron-specific signaling cascades. Furthermore, we validate the PD-lipid relationship by GWAS data-based polygenic risk score analyses that indicate a shared genetic risk between lipid/lipoprotein traits and PD. Taken together, our findings provide novel insights into the molecular pathways underlying the etiology of (sporadic) PD and highlight a key role for lipids and lipoproteins in PD pathogenesis, providing important clues for the development of disease-modifying treatments of PD.

KEYWORDS: Parkinson's Disease, Molecular Landscape, Etiology, Lipids, Lipoproteins, GWAS

2.2 INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease, with an estimated prevalence of 0.3%, affecting 1-2% of people over 60 years of age[1, 2]. The pathological hallmark of PD is loss of dopaminergic (DA) neurons in the substantia nigra (SN), and the presence of protein aggregates (i.e. Lewy bodies) involving synuclein alpha (SNCA) in the residual DA neurons[3]. A number of biological processes that contribute to the pathogenesis of PD have been identified, including defects in mitochondrial function[4], oxidative stress[5] and protein aggregation[6-8]. However, detailed insights into the molecular mechanisms underlying these processes, and how they interact with each other, are essentially lacking. In many studies exploring PD pathogenesis, familial PD genes served as starting point. Thus far, at least eighteen genetic loci for familial PD have been found, and twelve familial PD candidate genes have been identified (*ATP13A2*, *DJ-1*, *DNAJC6*, *EIF4G1*, *FBXO7*, *LRRK2*, *PARK2*, *PINK1*, *PLA2G6*, *SNCA*, *SYNJ1* and *VPS35*)[9, 10]. However, as a mutation in one of these familial genes is found in only 5-10% of the cases, PD should be considered a predominantly sporadic disease[11, 12], with both genetic and environmental contributing risk factors. In recent years, 15 genome-wide association studies (GWASs) have investigated genetic

risk factors for sporadic PD[13-26] but the functional coupling of the proteins encoded by the GWAS-identified candidate genes to PD pathophysiology is often not clear. In the present study, we aimed to identify the core mechanisms underlying PD pathogenesis by using bioinformatics and extensive literature analyses to integrate (1) the genes corresponding to the top-ranked single-nucleotide polymorphisms (SNPs) found in published GWASs of sporadic PD, and (2) other PD candidate genes (e.g. familial PD genes) into a protein interaction landscape. This molecular landscape allowed us to identify the specific biological processes that are key in PD pathogenesis and provides clues for the development of novel PD treatment strategies.

2.3 METHODS

2.3.1 PD GWAS gene selection

The first step of our molecular landscape building approach[27-29] is the selection of candidate genes based on GWAS single nucleotide polymorphisms (SNPs) and their corresponding p-values. All 15 PD GWASs published to date were considered. Criteria for inclusion were a publicly available GWAS discovery sample with all SNPs associated at $p<0.0001$. From the GWASs for which these data were available, we then selected the SNPs that were associated with PD at $p<0.0001$ to compile a list of associated genes. The selected genes either contained a SNP that was located within an exonic, intronic or untranslated region of the gene or were found within 100 kilobases (kb) downstream and upstream of the SNP. The latter was based on the fact that the vast majority of expression quantitative trait loci (eQTL) for a given gene are located within 100 kb downstream and/or upstream of a gene[30-32] and because trait-associated SNPs are more likely to be eQTL[33]. The chosen cut-off for association ($p<0.0001$) is often employed to designate 'suggestive' association and has been used in GWASs of multiple disorders[34-36]. Subsequently, the literature was searched for additional (genetic) evidence linking the selected GWAS candidate genes to PD.

2.3.2 Genetic network enrichment analysis

To identify enriched protein networks in the PD GWAS candidate genes, a network analysis using the Ingenuity Pathway Analysis (IPA) software package (http://www.ingenuity.com) was performed, using default parameters. In this respect, the analysis used the so-called reference set of known genes and endogenous chemicals, which is accessible through the 'Ingenuity Knowledge Base', a repository of extensive information from manually curated published literature as well as many other sources, including gene expression databases. In addition, only functional relationships that are corroborated by experimental evidence were included in the networks. For each network, the Ingenuity software also generates an enrichment score that takes into account the number of eligible molecules/proteins in the network and its size, as well as the total number of network-eligible molecules analyzed and the total number

of molecules in the Ingenuity Knowledge Base that could potentially be included in networks. This score is the negative logarithm of the right-tailed Fisher's exact test result.

2.3.3 Molecular landscape building

Following the network enrichment analysis, the literature was extensively searched for the functions and interactions of all proteins encoded by the candidate genes implicated through PD GWASs as well as other PD candidates implicated via other evidence, including genetic association studies, mRNA/protein expression studies and/or functional studies. First, we used the UniProt Protein Knowledge Base (http://www.uniprot.org/uniprot)[37] to gather basic information on the functions of all candidate genes and their encoded proteins. Subsequently, and starting with the interactions in the most enriched genetic network, we used PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) to search for all functional, experimental evidence-based interactions between all PD candidate genes/proteins. While building the landscape in this way, we also included genes/proteins and metabolites that have no known link with PD, but have multiple – i.e. at least two different – functional interactions with PD-implicated proteins. Based on all gathered information, we generated a protein interaction landscape. The figures depicting this landscape were made using the drawing program Serif DrawPlus version 4.0 (www.serif.com)."

2.3.4 Polygenic risk score (PRS) analyses

Our molecular landscape pointed towards an important role for lipids and lipoproteins in PD etiology (see below). Therefore, we conducted PRS analyses using the tool PRSice[38], with summary statistics data from genome-wide association studies (GWASs) of blood levels of total cholesterol, total triglycerides, total high density lipoprotein (HDL) and total low density lipoprotein (LDL) as 'base' samples (GWAS data for 188577 European-ancestry individuals from the general population)[39] and summary statistics data from a meta-analytic PD GWAS by the International Parkinson Disease Genomics Consortium (IPDGC) as 'target' sample (GWAS data for 5333 PD cases and 12019 healthy control subjects, all of European ancestry)[40]. Using the default settings in PRSice we calculated the shared genetic etiology between the four lipid/lipoprotein traits and PD at seven broad p-value thresholds (indicated by p_T) which were used to select the SNPs from the base sample that were included in the PRS analysis, i.e. $p_T < 0.001$, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5. As such, the seven p_T thresholds led to the selection of all SNPs that were associated with the base lipid/lipoprotein phenotype at $p< 0.001$, 0.05, 0.1 etc.

The calculated p-values indicating the significance of a shared genetic etiology between each lipid/lipoprotein trait and PD were aggregated and corrected for multiple comparisons using the false discovery rate (FDR) method, incorporating

potential dependencies between p-values[41]. To calculate the FDR, we used the mafdr function in MATLAB (R2012a, The Mathworks, Natick, MA, USA) using the bootstrap selection method for the FDR parameter lambda. FDR was set to not lower p-values below uncorrected p-values, which would have occurred due to overall (relatively) low uncorrected p-values.

In addition to calculating the shared genetic etiology between the four lipid/lipoprotein traits and PD, we performed similar analyses using four 'combined lipoprotein traits'. In order to do this, we first divided the HDL and LDL level summary statistics GWAS data into four groups of SNPs, i.e. all SNPs associated with (1) increased HDL levels, (2) increased LDL levels, (3) decreased HDL levels and (4) decreased LDL levels (where 'increased' and 'decreased' refer to all SNPs that had an effect size-indicating beta > 0 and beta < 0, respectively). Subsequently, we conducted PRSice analyses with four combined data sets as base sample, i.e. (1) all SNPs increasing HDL and LDL levels, (2) all SNPs decreasing HDL and LDL levels, (3) all SNPs increasing HDL and decreasing LDL levels, and (4), all SNPs decreasing HDL and increasing LDL levels. Before the data sets were fed into PRSice, an equivalent, weighted p-value for each SNP in each of the four combined HDL/LDL data sets was calculated as:

$$\frac{2}{p_{eq}} = \frac{1}{p_{HDL}} + \frac{1}{p_{LDL}}$$

2.4 RESULTS

2.4.1 Selected PD GWAS genes and genetic network enrichment analysis

Thirteen of the fifteen published PD GWASs met our inclusion criteria (**Supplementary Table 1**) and were used to select a total of 451 PD GWAS candidate genes based on SNPs with P <0.0001 (**Supplementary Table 2**). Of the five most significantly enriched IPA networks (**Supplementary Table 3**), the network with the highest enrichment score (P=1.00E-44) and the highest number of PD GWAS candidate gene-encoded proteins (28 proteins) served as the starting point for the building of the molecular landscape (**Supplementary Figure 1**).

2.4.2 The molecular landscape of PD

Guided by the most significantly enriched genetic network and extensive literature searches, we built a molecular landscape consisting of 260 interacting proteins (i.e. encoded by approximately 58% of the 451 PD GWAS genes, **Supplementary Table 2**), 128 proteins implicated in PD through other evidence (**Supplementary Table 4**) and 49 proteins that have not been directly linked to PD (yet) but have multiple functional interactions within the landscape (**Supplementary Table 4**). Approximately one in three landscape proteins are implicated in PD etiology through at least two types of

evidence.

Supplementary Figures 2 and **3** show all relevant protein interactions in the PD landscape that are functionally involved in four main biological processes: oxidative stress response, endosomal-lysosomal functioning, endoplasmic reticulum (ER) stress response, and neuron death and immune response. The **Supplementary Information** provides a detailed and referenced description of the evidence linking all the proteins in the landscape. In **Supplementary Table 5**, we have indicated in which process(es) each landscape protein exerts its main effect and where it is located in **Supplementary Figures 2** and/or **3**.

The above being said, we here give a succinct description of the four main biological processes and signaling cascades in the PD landscape that are depicted in **Figure 1**. Central in the landscape is signaling involving lipoproteins – i.e. low density lipoprotein (LDL), high density lipoprotein (HDL) and very low density lipoprotein (VLDL) – and their component lipids and metabolites (e.g. cholesterol, oxysterols, sphingolipids such as ceramide and sphingosine, and triglycerides). Lipid and lipoprotein signaling represents the 'common denominator' that functionally integrates, regulates and is regulated by the four landscape processes (**Fig. 1A-D**). Either by themselves or in combination, deficits or impairments in any of these four processes – each composed of multiple signaling cascades – can contribute to the degeneration and ultimately death of DA neurons.

First, deficits or impairments in dopamine synthesis and - linked to this - iron metabolism can cause an increased **oxidative stress response** (**Fig. 1A**). Dopamine can be either taken up through active transport or is newly synthesized in neurons and can subsequently be re-released (through vesicular exocytosis), degraded or (auto-)oxidized into neuromelanin (NM). Further, like erythrocytes (see below), SN DA neurons have a high oxygen demand and express oxygen-carrying hemoglobin.

Through oxidation, cytotoxic heme is released from hemoglobin and then converted in DA neurons to ferrous iron, Fe(II). Fe(II) increases oxidative stress and together with free cholesterol – that is taken up by neurons through lipoproteins (see below) – induces mitochondrial oxysterol formation. In turn, this causes mitochondrial dysfunction and triggers the release of pro-apoptotic cytochrome c and, eventually, neuron death.

The second main landscape process centers around the (dys)regulation of **endosomal-lysosomal functioning** (**Fig. 1B**). Neuronal uptake of cholesterol occurs through the endosomal system, i.e. after neuronal uptake through vesicular endocytosis, LDL particles are processed into their composite parts: proteins, free cholesterol and other lipids. Free cholesterol and Fe(II) are bound in complexes by NM, which are then

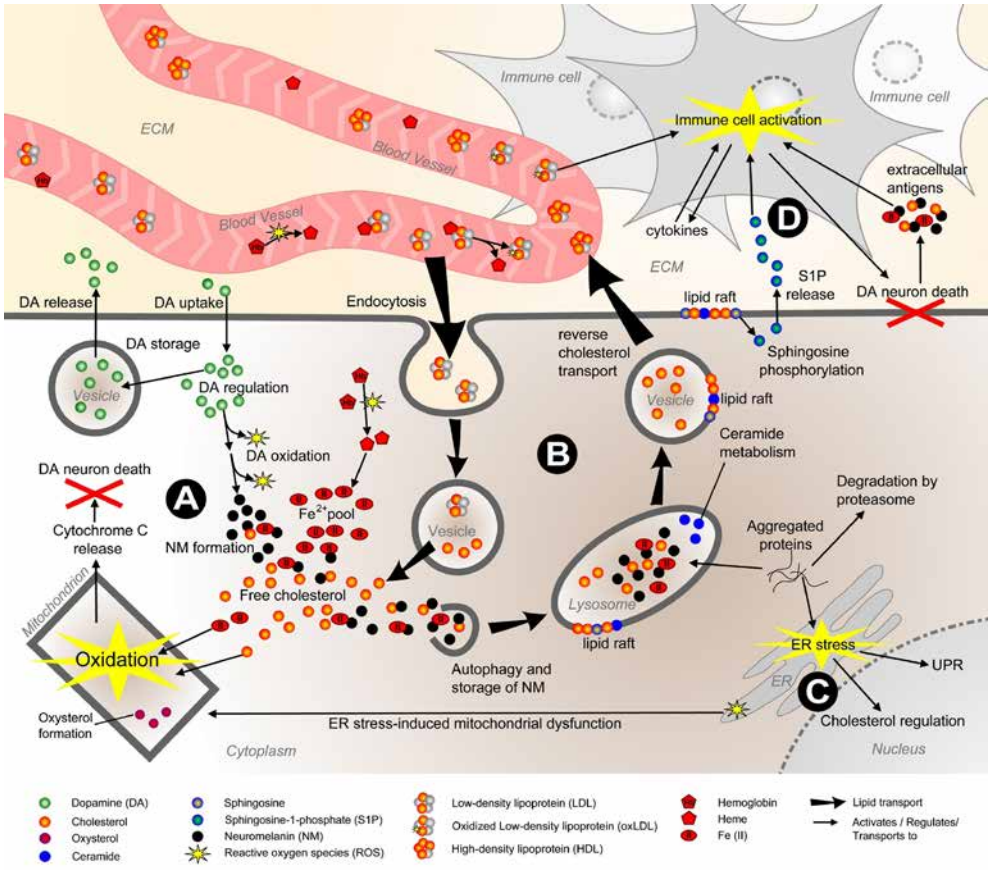

Figure 1. Overview of the molecular landscape of PD. The four main biological processes in the PD landscape – oxidative stress response (**Fig. 1A**), endosomal-lysosomal functioning (**Fig. 1B**), endoplasmic reticulum (ER) stress response (**Fig. 1C**), and neuron death and immune response (**Fig. 1D**) – are depicted. ECM, extracellular matrix; ER, endoplasmic reticulum; UPR, unfolded protein response.

stored in lysosomes through autophagy. Hence, NM complex formation prevents the above described Fe(II)- and cholesterol-induced oxidative stress response. Moreover, their ageing-related increase in NM content and the associated increased demands on lysosomal function renders DA neurons particularly vulnerable to lysosomal defects. Other important lysosomal functions include the degradation of misfolded or aggregated proteins (such as pathological SNCA aggregates), the regulation of ceramide metabolism and reverse cholesterol transport, i.e. the vesicle-mediated transport and exocytosis of cholesterol into HDL particles in the bloodstream (and back to the liver). As such, a defect in any of these endosomal-lysosomal system components results in disturbed levels of lipids such as cholesterol and ceramide. In turn, these disturbed lipid levels affect membrane function in general and more specifically the functioning of so-called lipid rafts – microdomains of the vesicular, lysosomal and plasma membrane

containing high amounts of cholesterol and sphingolipids and crucial for membrane function – and hence processes such as autophagy, endo- and exocytosis. Deficient lysosomal function together with reduced degradation by the proteasome also leads to misfolded or aggregated protein formation.

Misfolded/aggregated proteins trigger the **ER stress response** (**Fig. 1C**), the third main landscape process, and subsequent activation of the protective unfolded protein response (UPR) as well as stimulation of cholesterol influx through upregulating the expression of key lipoprotein receptors. Prolonged ER stress that can no longer be counteracted by the UPR induces mitochondrial dysfunction, which eventually results in DA neuron death.

Lastly, apart from or in addition to dysregulated processes *within* DA neurons (as described above), DA neuron death can be the consequence of *external* factors such as an exaggerated **immune response** (**Fig. 1D**), the fourth landscape process. In this respect, immune cells are activated and attracted to damaged or already dying DA neurons by extracellular factors such as the sphingolipid-derived sphingosine-1-phosphate (S1P), triglyceride-rich extraneuronal VLDL particles (not shown), heme-oxidized LDL (oxLDL, see above), and various cytokines. Subsequently, the damaged/dying DA neurons are removed by the activated immune cells, an essentially normal and adequate response that is exaggerated in PD by DA neuron-specific antigens such as SNCA aggregates and NM complexes – released by dying DA neurons – creating a vicious cycle of DA neuron death and immune cell activation.

2.4.3 PRS analyses

Because our molecular landscape pointed towards an important role for lipids and lipoproteins in PD etiology (see above), we conducted polygenic risk score (PRS) analyses using the tool PRSice[38], with GWAS data for the blood levels of various lipids and lipoproteins[39] as base samples and meta-analytic PD GWAS data from the International Parkinson Disease Genomics Consortium (IPDGC)[40] as target sample. We found statistically significant evidence (FDR-corrected $p<0.05$) for a shared genetic etiology between the lipid traits 'total cholesterol levels' and 'total triglyceride levels' and PD, with the most predictive p-value threshold (p_T) at 0.001 and 0.05, respectively (**Figure 2**). In contrast, the lipoprotein traits 'total HDL levels' and 'total LDL levels' yielded no evidence for a shared genetic risk with PD (**Figure 2**). For the various combinations of increased or decreased HDL and LDL levels, we found significant evidence for a shared genetic etiology between PD and the combined trait 'increased HDL + increased LDL' (most predictive $p_T=0.05$) (**Supplementary Figure 4**).

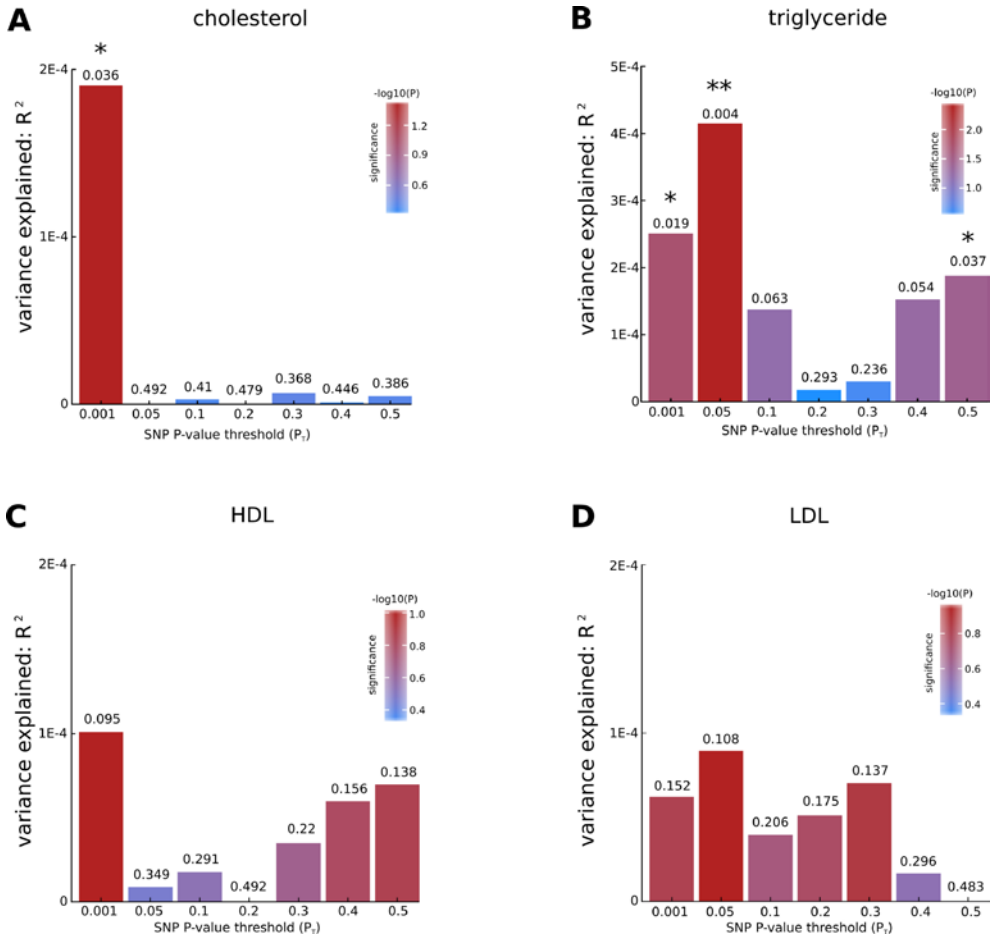

Figure 2. Bar plots from PRSice for shared genetic etiology between four lipid and lipoprotein traits (cholesterol, triglyceride, HDL and LDL levels) and PD showing results at broad p-value thresholds (p_T). The numbers above the bars indicate the p-values for shared genetic etiology, and these p-values were corrected using the false discovery rate (FDR) method; * denotes FDR-corrected p<0.05, ** denotes FDR-corrected p<0.01

2.5 DISCUSSION

In this study, we integrated the available genetic and expression data with data about environmental risk factors into a protein interaction landscape that reveals the main biological processes and signaling cascades that are affected in PD and occur in DA neurons and immune cells. Our PD landscape represents processes and cascades that are affected in both the monogenic, familial and the more prevalent polygenic, sporadic forms of PD. In this respect, the molecular landscape includes the 'classic' processes and cascades known to be affected in PD and based on the familial PD candidate genes (e.g. SNCA, PARK2, LRRK2): mitochondrial function, oxidative stress and protein aggregation. In addition, the landscape harbors more novel processes that have been less well studied in relation to PD pathogenesis yet, such as impairments in lysosomal

function and immune response regulation. The landscape does not imply a 'sequence of events' that leads to DA neuron loss, i.e. a number of (impaired) biological processes and cascades that occur in a temporally and/or spatially distinct order. Instead, deficits in any of the main landscape processes/cascades, either by themselves or in combination with deficits of other processes/cascades, may cause DA neurons to die. Moreover, an aging-related decline in the functionality and/or efficiency of landscape processes/cascades may play a role in PD onset and progression. For example, a gradual buildup of NM or aggregated proteins may disturb lysosomal function in DA neurons[42] or an age-related decrease in the expression and activity of ER folding enzymes can compromise proper protein folding[43].

Lipid and lipoprotein signaling functionally integrates, regulates and is regulated by the key landscape processes and cascades. Any disturbance of these processes and cascades can (eventually) result in DA neuron death, which is further aggravated or initiated by an increased (auto-)immune response[44]. The involvement of deficient lipid and lipoprotein signaling in PD pathophysiology is corroborated by a number of environmental risk factor studies. Increased plasma levels of total cholesterol are associated with a lower PD risk[45-47]. Nevertheless, a recent meta-analysis did not show an effect of higher or lower dietary cholesterol intake on PD risk[48], suggesting that direct cholesterol intake through food may not play a major role in PD etiology. Further, low plasma levels of LDL are linked to a higher PD risk[49, 50], whereas high plasma HDL and CSF oxysterol levels are associated with increased PD risk and duration[51, 52]. In addition, the levels of *oxidized* LDL, oxysterols, sphingolipids are increased in the plasma of PD patients[53-55]. Thus, PD patients have a lower LDL:HDL ratio that is associated with a lower risk of cardiovascular disease (CVD)[56] and could at least to some extent explain why the PD population is indeed less susceptible to developing CVD[51]. Apart from the observed dysregulated levels of cholesterol (metabolites) and cholesterol-containing lipoproteins in PD patients, lower serum levels of triglycerides – which are highly enriched in VLDL particles – associate with an increased PD risk[57, 58].

Intriguingly, we found a significant overlap between the polygenic risk associated with total cholesterol and triglyceride levels and PD. We also identified a shared genetic etiology between the combined lipoprotein trait 'increased HDL + increased LDL' and PD. To our knowledge, we are the first to find a shared genetic risk between quantitative traits and a neurodegenerative disease but these findings need to be replicated in larger data sets, especially for the target sample, i.e. the PD GWAS data set. Together, the epidemiological and our PRS analysis findings indicate that the link between specific lipid/lipoprotein traits and PD may be the result of both shared environmental and genetic risk factors.

Given the converging evidence for lipid and lipoprotein signaling playing a key role in PD etiology, compounds that modulate lipid/lipoprotein levels could represent effective novel PD treatments. In this respect, statins – inhibitors of peripheral cholesterol synthesis that are used to treat hypercholesterolemia and hence prevent cholesterol-associated CVD – have a neuroprotective effect in the rat brain[59], but their effect on PD risk remains unclear[60-67]. Interestingly, the only published prospective study that has adjusted for baseline cholesterol levels before statin treatment has found that statin use is associated with a significantly higher PD risk[47], which is in keeping with the observation that higher total plasma cholesterol levels – which are lowered by statins – are protective against PD. Other signaling molecules from the landscape that affect cholesterol and lipoprotein levels are testosterone and vitamin D3. Caucasian male PD patients show significantly reduced testosterone levels[68-70], and free testosterone levels are positively correlated with LDL, HDL and total cholesterol levels[71]. Therefore, decreased testosterone levels may impact on several key PD landscape processes, as testosterone regulates the efflux of LDL and HDL to the circulation[72]. Hence, testosterone could be used for treating PD in male patients and indeed, testosterone treatment has some modest beneficial effects in men with PD[73, 74]. Deficiency of vitamin D3 – which affects cholesterol metabolism through downregulating SREBF1[75], the main transcriptional activator of lipid homeostasis and key landscape protein – has been consistently associated with an increased PD risk[76] and its supplementation may stabilize PD symptoms[77].

Lastly, a number of landscape proteins that both regulate lipid/lipoprotein signaling and landscape cascades involved in DA neuron death represent attractive (novel) drug targets for PD. Examples include HMOX1 that prevents oxidative stress by heme, PSAP and its receptor GPR37 that mediate ceramide metabolism, the immunity-related ICAM1 that is regulated by extracellular lipids and (oxidized) lipoproteins, and plasmin that regulates the degradation of extracellular SNCA and (lipo)proteins.

In conclusion, our integrated molecular landscape yields detailed insights into the mechanisms underlying PD pathogenesis, and highlights the involvement of deficient lipid and lipoprotein signaling. These findings warrant future rigorous perturbation experiments in PD cell and animal models that may eventually provide validated drug target 'leads' for the development of novel disease-modifying PD treatments.

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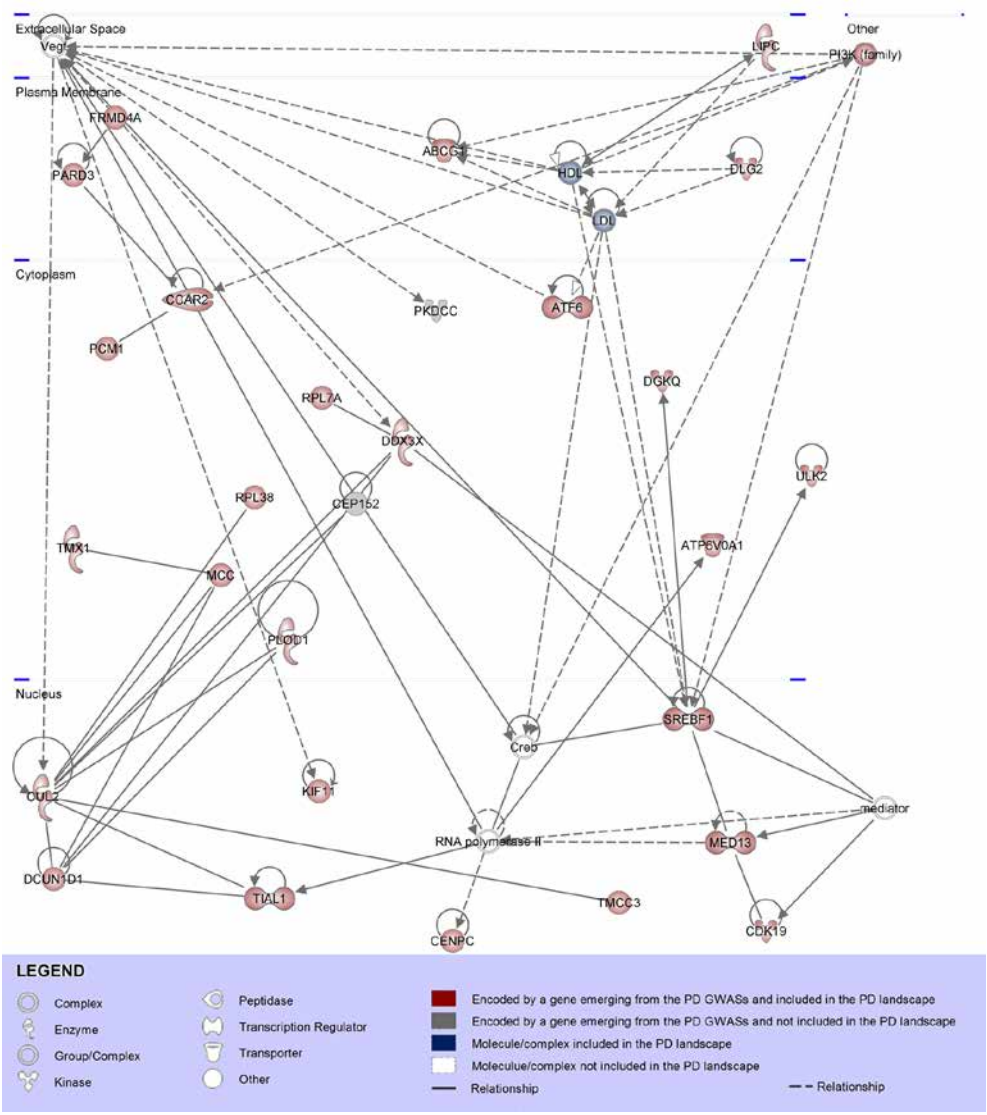
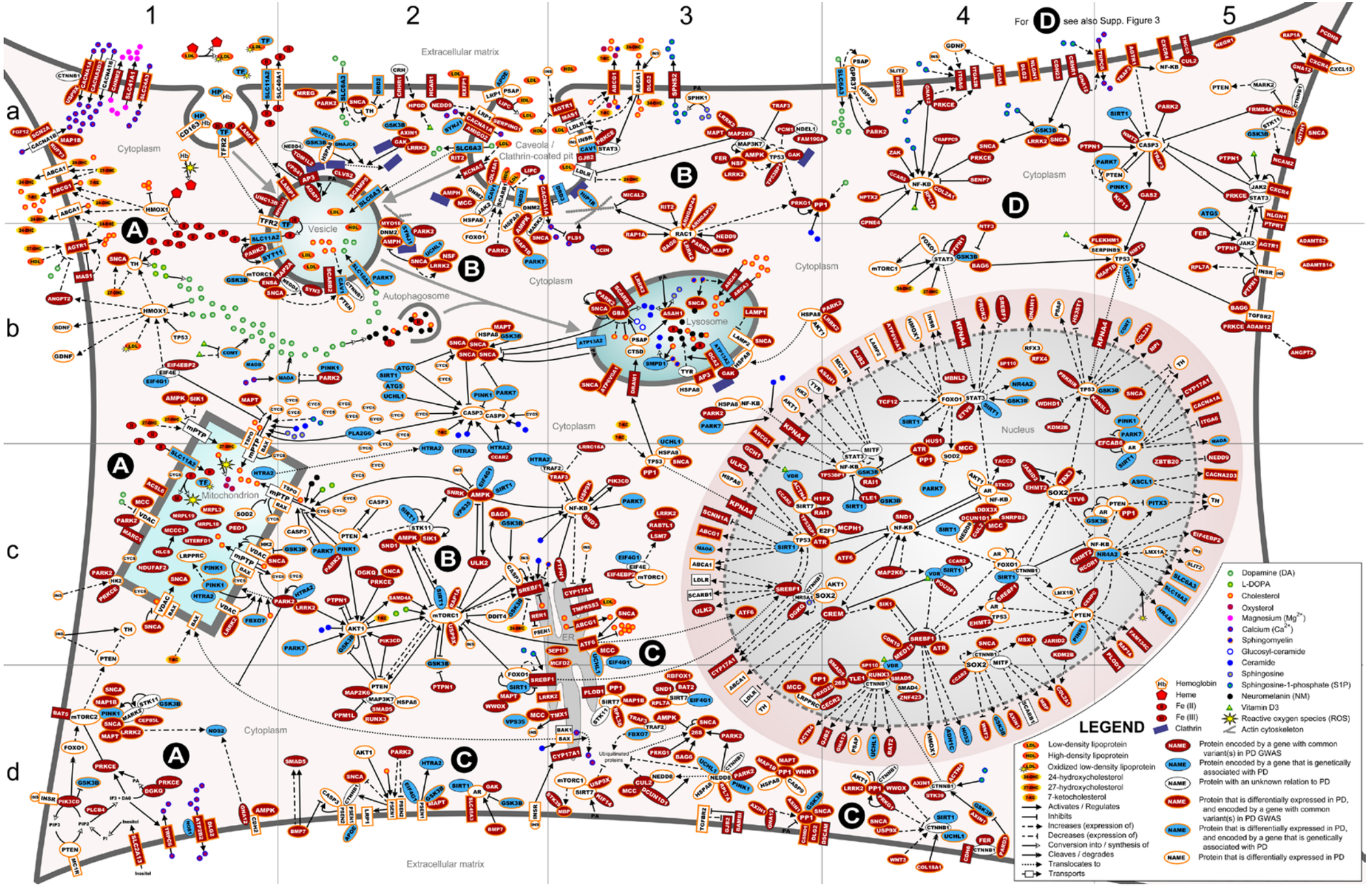
2.9 SUPPLEMENTARY INFORMATION

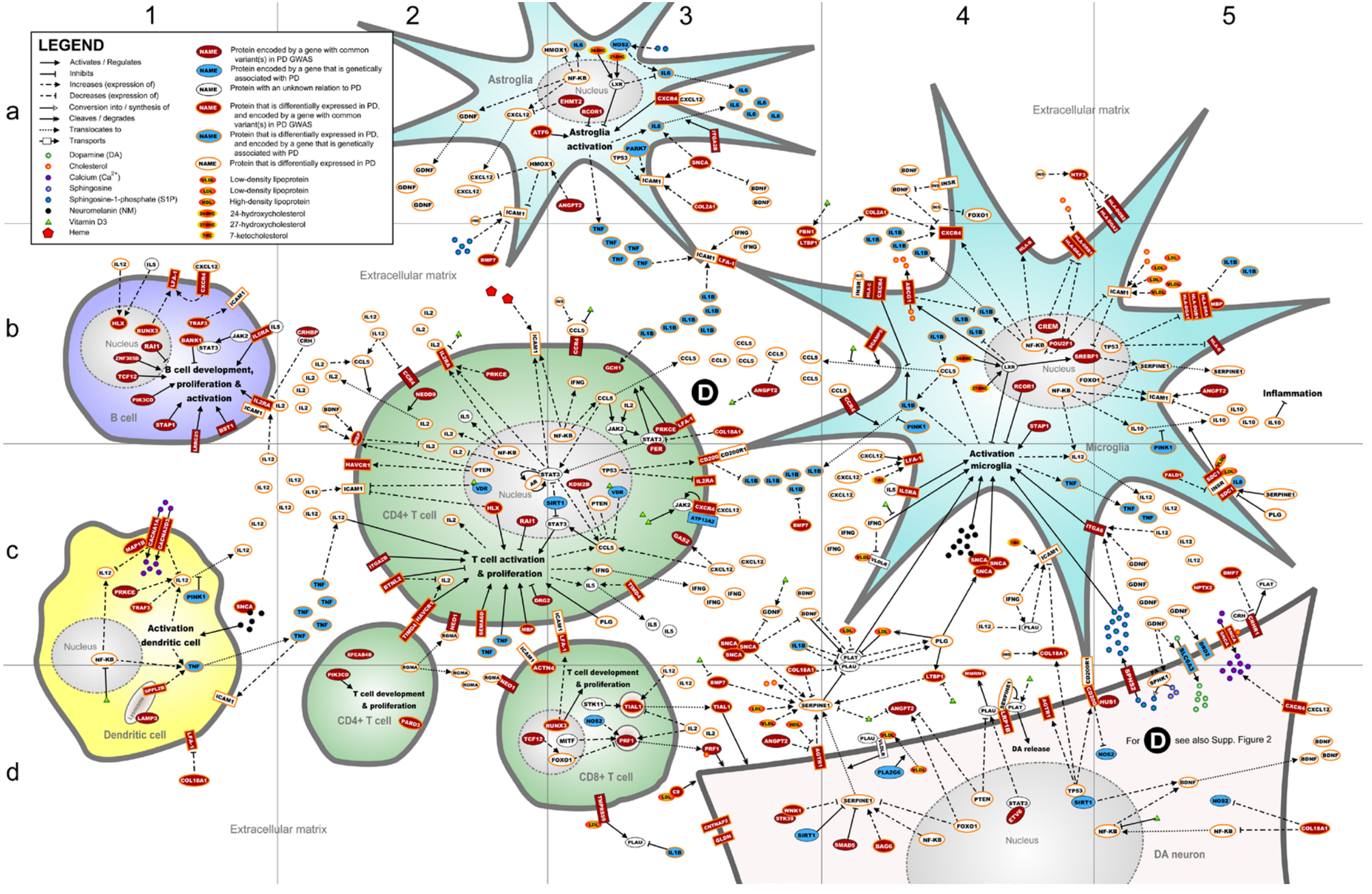
CONTENTS



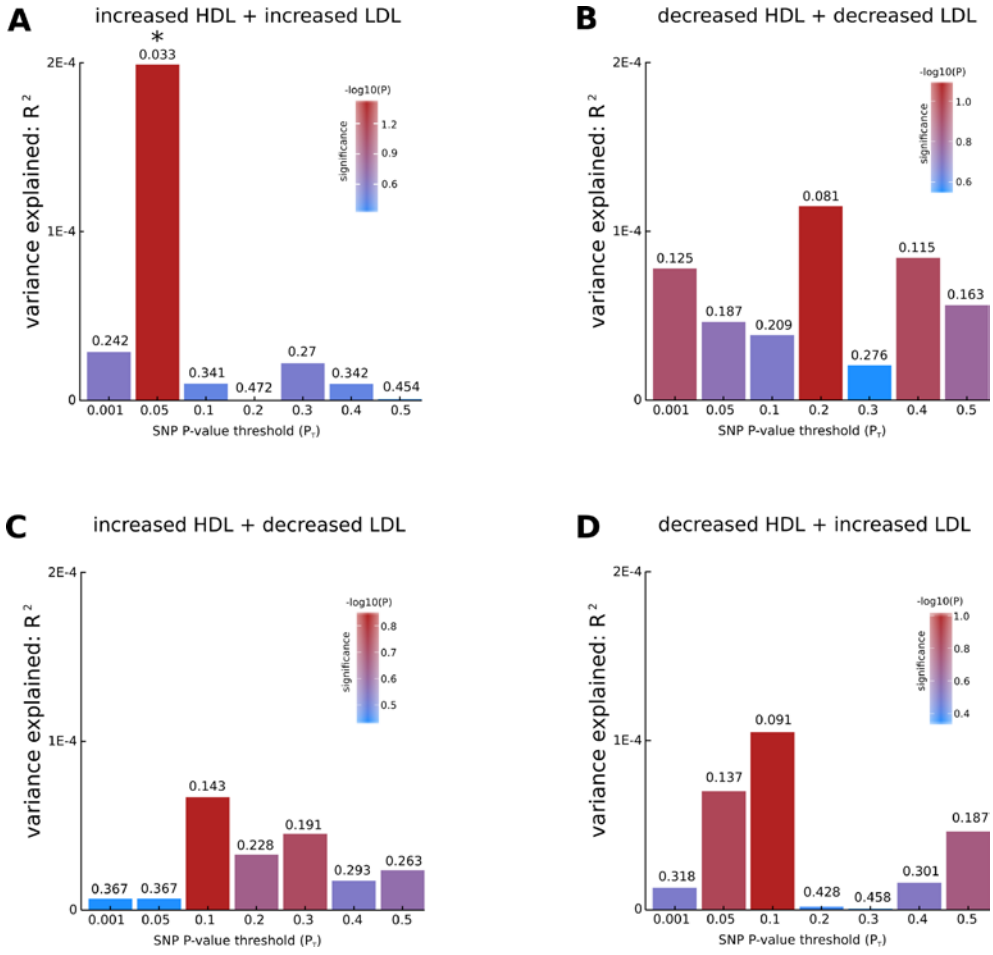
Supplementary Figure 1. Top enriched Ingenuity genetic network. The network with the highest score (P=1.00E-44) and containing the highest number of proteins (28 proteins) obtained by a network enrichment analysis of the PD GWAS candidate genes from **Supplementary Table 2** using Ingenuity pathway software (www.ingenuity.com) (**Supplementary Table 3**). The proteins encoded by a gene that emerged from (at least) one of the PD GWASs and that were included in the PD landscape are indicated in red, whereas the PD GWAS gene-encoded proteins that are not included in the landscape are depicted in grey. Of note, two molecules – HDL and LDL – did not emerge from one of the GWASs, but are included in the landscape (in blue).



Supplementary Figure 2: PD landscape; intracellular pathways constituting processes A-D. The functional interactions between proteins functioning within the processes oxidative stress response (A), endosomal-lysosomal functioning (B), ER stress response (C) and neuron death and immune response (D) are shown. The transcription factors and transcription regulators in the nucleus regulate the expression of proteins that are involved in all four processes. These expressed proteins are shown in the vicinity of the nucleus (in the light brown band surrounding the nucleus) and will translocate to their cellular destination, i.e. either the cytoplasm, one of the organelles, or the cell membrane. The functional interactions within process D that are more directly related to immune cell regulation are shown in **Supplementary Figure 3**. For each individual protein in this figure, **Supplementary Table 5** shows the location coordinates and the main process(es) (A-D) in which they exert their main effect.



Supplementary Figure 3: PD landscape; immune cell regulation-related pathways of process D. The functional interactions within process D: ´neuron death and immune response´ that regulate the activation of astroglia, microglia, T cells, B cells and dendritic cells are shown. For each individual protein in this figure, **Supplementary Table 5** shows the location coordinates and the main process(es) (A-D) in which they exert their main effect.



Supplementary Figure 4. Additional Polygenic Risk Score Analyses. Bar plots from PRSice showing results at broad P-value thresholds (P_T) for shared genetic etiology between four 'combined lipoprotein traits' (increased HDL + increased LDL, decreased HDL + decreased LDL, increased HDL + decreased LDL, decreased HDL + increased LDL) and PD. The numbers above the bars indicate the P-values for shared genetic etiology, and these P-values were corrected using the false discovery rate (FDR) method; * denotes FDR-corrected P < 0.05

Supplementary Table 1. Overview of the fifteen published GWASs for PD. The thirteen GWASs used in our analysis are shown in bold (in total 13044 cases and 47148 controls).

GWAS	Discovery sample		Population	Genotyping platform	Phenotype	Diagnosis	Notes
	Patients	controls					
Maraganore et al., 2005 [1]	443	443	Primarily European origin	Perlegen genotyping platform	IPD	Standardized clinical assessment performed by a neurologist specialized in movement disorders	Sibling pairs
Fung et al., 2006 [2]	267	270	Caucasian	Illumina Infinium I and Infinium HumanHap300 SNP chip	IPD (>55yrs)	N/A	Samples derived from NINDS Neurogenetics repository
Pankratz et al., 2009 [3]	857	867	Caucasian, non-hispanic	Illumina HumanCNV370 version1_C BeadChips	FPD	Neurological evaluation based on a modified version of the UK PD society Brain Bank Criteria	Known disease producing mutations were excluded [1].
Latourelle et al., 2009 [4] [2]	857	N/A	Caucasian, non-hispanic	Illumina HumanCNV370 version1_C BeadChips	FPD	Neurological evaluation based on a modified version of the UK PD society Brain Bank Criteria	Known disease producing mutations were excluded [1].
Latourelle et al., 2009 [4] [3]	440	N/A	Primarily European origin	Perlegen genotyping platform and Illumina HumanCNV370Duo array	IPD	Standardized clinical assessment performed by a neurologist specialized in movement disorders	-
Satake et al., 2009 [5]	1078	2628	Japanese	Illumina Infinium HumanHap550 array	IPD [4]	At least two cardinal PD symptoms without secondary cause, levodopa responsive, and without other neurological problems.	Known disease producing mutations were excluded [5]. Not, included; not all data online available.
Simon-Sanchez et al., 2009 [6]	1713	3978	Caucasian; European ancestry	Illumina Infinium HumanHap550 array	IPD	Diagnosed according to the UK Brain Bank criteria	-
Edwards et al., 2010 [7]	604	619	Caucasian	Illumina Infinium 610-quad BeadChip / Illumina HumanHap 550 BeadChip	IPD	At least two cardinal PD symptoms without secondary cause and without other neurological problems.	-
Hamza et al., 2010 [8]	2000	1986	Ashkenazi Jewish and European origin	Illumina HumanOmni1-Quad_v1-0_B BeadChips	IPD (435 FPD)	Diagnosed according to the UK Brain Bank criteria	-
Spencer et al., 2011 [9]	1705	5175	UK	Illumina Human660-Quad array / Illumina 1.2 M Duo array	IPD	Diagnosed according to the UK Brain Bank criteria	-

Saad et al., 2011 [10]	1039	1984	French	Illumina Human610-Quad BeadChip	FPD	At least two cardinal PD symptoms, levodopa responsive, and without other neurological problems.	Known disease producing mutations were excluded [5].
Simon-Sanchez et al., 2011 [11]	772	2024	Dutch	Illumina Human660W-Quad beadchips / Illumina Human610K beadchips	PD	N/A	-
Do et al., 2011 [12]	3426	29624	Primarily European ancestry	Illumina HumanHap550+ BeadChip + approximately 25.000 custom SNPs	PD	Patients who stated they had been diagnosed with PD and who gave complete, non-suspicious answers to the other questions were included	Cases and controls were selected based on online questionnaires.
Liu et al., 2011 [13]	268	178	Ashkenazi Jewish	Illumina Human 610-quad bead arrays / Illumina Human 660-quad bead arrays	PD	Patients and controls were evaluated using the Unified Parkinson's Disease Rating Scale (UPDRS) and the Mini Mental State Exam (MMSE).	Enriched for cases with an age of onset of 50 or younger.
Hernandez et al., 2012 [14]	387	496	Finish	Illumina Human660W v1 BeadChip / Illumina HumanCNV370 BeadChip	PD (<55yrs)	The diagnosis fulfilled international criteria for PD	Not, included; not all data online available.

[1] All cases were negative for LRRK2 (G2019S) and most were screened for SNCA, PARK2 and PARK7 mutations.
[2] GWAS for age of PD onset. PD cases from Pankratz et al., 2009 were used.
[3] GWAS for age of PD onset. PD cases from Maragnore et al., 2005 were used.
[4] 39 cases had a relative with PD.
[5] Subjects diagnosed genetically with known PARK mutations (SNCA, LRRK2, PARK2 and PINK1) were excluded.

Supplementary Table 2. PD candidate genes. Top single SNPs located in gene regions (including 100 kb of flanking downstream and/or upstream sequences) and with *P* < 1.00E-04 for association with Parkinson's disease (PD) from the genome-wide association studies (GWASs) reported by Maraganore et al.[1] (GWAS 1), Fung et al.[2] (GWAS 2), Pankratz et al.[3] (GWAS 3), Latourelle et al.[4] (GWAS 4), Latourelle et al.[4] (GWAS 5), Simon-Sanchez et al.[6] (GWAS 6), Edwards et al.[7] (GWAS 7), Hamza et al.[8] (GWAS 8), Spencer et al.[9] (GWAS 9), Saad et al.[10] (GWAS 10), Simon-Sanchez et al.[11] (GWAS 11), Do et al.[12] (GWAS 12), and Liu et al.[13] (GWAS 13). The genes encoding proteins that could be directly placed in the PD landscape (Figures 1, S2 and S3) are indicated in **bold**. If these genes or their protein products are linked to PD, either by genetic or expression and immunohistological studies (in PD patients), this is shown in the column 'Corroborating evidence'. Single underlined genes are genetically associated with PD, dotted underlined genes encode proteins that are differentially expressed in PD patients and double underlined genes are both genetically associated with PD and encode a protein that is differentially expressed in PD.

GWAS 1 (Maraganore et al.)

SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs3746736	1.30E-05	20p11.21	*CSTL1*	non-syn coding	-	-
rs17463995	3.50E-05	15q21.1	*CEP152*	1.4 kb downstream	-	-
rs17463995	3.50E-05	15q21.1	***FBN1***	66 kb upstream	-	FBN1 mRNA is increased in blood[15] and FBN1 protein is increased in CSF[16] of PD patients compared to controls.
rs16887478	3.64E-05	8p11.22	*C8orf86*	56 kb upstream	-	-
rs11887431	3.66E-05	2p21	*PKDCC*	7.7 kb upstream	-	-
rs1984279	3.89E-05	20p11.21	*NAPB*	42 kb downstream	-	NAPB mRNA is decreased in the SN[17,18] and NAPB mRNA[19] and protein[20] is increased in the striatum of PD patients compared to controls.
rs1984279	3.89E-05	20p11.21	*NXT1*	18 kb upstream	-	-
rs10815285	4.64E-05	9p24.1	*ERMP1*	intronic	-	-
rs960190	4.97E-05	Xp22.13	*GPR64*	69 kb upstream	-	-
rs7694392	5.56E-05	4q24	***BANK1***	intronic	-	BANK1 mRNA is increased in the striatum[20] and BANK1 protein is decreased in the CSF[16] of PD patients compared to controls.
rs7180500	6.35E-05	15q12	*GABRG3*	intronic	-	GABRG3 mRNA is decreased in the SN of PD patients compared to controls[20].
rs723268	7.15E-05	8q24.11	*EXT1*	43 kb upstream	-	-
rs723268	7.15E-05	8q24.11	*SAMD12*	35 kb downstream	-	-
rs6039424	8.04E-05	20p12.2	***PLCB4***	intronic	-	-
rs7686646	8.38E-05	4q13.3	***RUFY3***	intronic	-	RUFY3 mRNA is increased in the blood[15] and in the SN (female patients)[21] of PD patients or decreased in the SN[17,22,23] of PD patients compared to controls.
rs4752662	8.94E-05	10q26.13	***TACC2***	intronic	-	-
rs6802211	8.99E-05	3q22.3	***ARMC8***	34 kb downstream	-	ARMC8 protein is decreased in the CSF of PD patients compared to controls[16].
rs6802211	8.99E-05	3q22.3	*NME9*	2.7 kb upstream	-	-
rs17719492	9.12E-05	4p16.3	*ZNF141*	intronic	-	ZNF141 mRNA is increased in the blood of PD patients compared to controls[15].

GWAS 2 (Fung et al.)

SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs2242330	1.70E-06	4q13.2	*STAP1*	intronic	-	-
rs10501570	7.30E-06	11q14	***DLG2***	intronic	-	DLG2 mRNA is decreased in the blood[24], DLG2 protein is decreased in the SN[25] and increased in the striatum[20] of PD patients compared to controls.

rs281357	9.80E-06	17p11.2	***ULK2***	intronic	-	-
rs988421	4.90E-05	1p13	***NEGR1***	intronic	-	NEGR1 protein is decreased in the SN[26] and CSF[16] and increased in the striatum[20] of PD patients compared to controls.
rs1912373	5.60E-05	11q11	*OR9G1*	15 kb downstream	-	-
rs1912373	5.60E-05	11q11	*OR5AP2*	74 kb upstream	-	-
rs1887279	5.70E-05	1q25	*COLGALT2*	intronic	-	COLGALT2 mRNA is increased in the SN of PD patients compared to control[18].
rs6125829	6.60E-05	20q13.13	*RNF114*	3' UTR	-	RNF114 mRNA is decreased in the SN of PD patients compared to controls[23].
rs355477	7.90E-05	4q13.2	***CENPC***	intronic	-	
GWAS 3 (Pankratz et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs11248060	3.40E-06	4p16.3	***DGKQ***	intronic	Genome-wide significance was reached for the intronic SNP rs11248060 in DGKQ (OR=1.21; P=3.04E-12)[27] (OR=1.35; P=2.0E-9)[28], a SNP that also increases the risk for PD in a Chinese population (P<0.05)[29].	-
rs11248051	5.20E-06	4p16.3	***GAK***	intronic	SNPs in GAK rs1564282 (Caucasian; OR=1.61; P=0.0151)[30], rs1564282 (Chinese)[29], rs11248051 (Taiwanese; CT/TT vs CC genotypes OR=1.37; P=0.03)[31], rs11248051 (meta-analysis; OR=1.35; P=8.2E-09)[28] are associated with an increased PD risk. Based on motor Unified Parkinson's Disease Rating Scale subscores GAK (rs1564282) is associated with tremor in PD (P=0.03)[32].	-
rs1997791	1.90E-05	20q13.13	***PTPN1***	30 kb upstream	-	-
rs1724425	2.00E-05	17q21.31	***CRHR1***	79 kb upstream	-	-
rs4240910	3.90E-05	1p36.22	***PIK3CD***	intronic	-	-
rs10094981	4.80E-05	8p11.21	*ZMAT4*	76 kb upstream	-	ZMAT4 mRNA is increased in the SN of PD patients compared to controls[20].
rs898528	4.90E-05	17q25.3	*RBFOX3*	intronic	-	-
rs12871648	5.00E-05	13q34	***LAMP1***	intronic	-	LAMP1 mRNA is decreased in the SN of female PD patients compared to controls[23]. LAMP1 expression is decreased in SN neurons of PD patients[33,34] and even further decreased when these neurons contained SNCA inclusions[33].
rs4670322	5.10E-05	2p22.3	***LTBP1***	intronic	-	LTBP1 mRNA is increased in the blood of PD patients compared to controls[15].
rs10937194	5.90E-05	3q27.2	*VPS8*	intronic	-	-
rs3775478	6.10E-05	4q22.1	***MMRN1***	intronic	-	MMRN1 mRNA is increased in the striatum of PD patients compared to controls[20].
rs1519686	7.10E-05	6q21	*HS3ST5*	intronic	-	-
rs4890430	7.10E-05	18q12.3	***RIT2***	1.9 kb upstream	In a meta-analysis of GWASs RIT2 was identified as a novel susceptibility locus (rs12456492, OR=1.19, P=2E-10 (combined sample))[28].	RIT2 mRNA is decreased in the SN of PD patients c-ompared to controls[17].
rs4901519	7.60E-05	14q22.2	*CGRRF1*	13 kb downstream	-	-
rs2083482	7.60E-05	2q24.3	*FIGN*	12 kb downstream	-	-

rs4901519	7.60E-05	14q22.2	***SAMD4A***	15 kb upstream	-	SAMD4A mRNA is increased in the blood of PD patients compared to controls[15, 24].
rs1159220	8.20E-05	22q12.3	***SYN3***	intronic	-	-
rs12638253	8.30E-05	3q25.31	*LEKR1*	intronic	-	-
rs1504489	8.40E-05	4q22.1	*GPRIN3*	29 kb upstream	-	-
rs356188	8.40E-05	4q22.1	***SNCA***	intronic	The mutation G209A (Ala53Thr) in SNCA was found in one Italian and three Greek families with PD, but not in controls[35] and the G88C (Ala30Pro) mutation was found in a German family[36]. SNCA gene duplication[37, 38] and triplication[39] causes familial PD. Based on motor Unified PD Rating Scale subscores SNCA (rs356220) is associated with rigidity in PD (P=0.04)[32].	SNCA mRNA is decreased in the SN of PD patients compared to controls[17, 22, 23]. LB immunoreactive for SNCA were found in the SN of PD patients[40, 41].
rs10859725	8.50E-05	12q22	*CEP83*	90 kb upstream	-	-
rs10859725	8.50E-05	12q22	***TMCC3***	17 kb downstream	-	-
rs11012	8.80E-05	17q21.31	***PLEKHM1***	3' UTR	-	PLEKHM1 mRNA is decreased in the SN of female PD patients compared to controls[23] and is increased in the blood of PD patients compared to controls[15].
rs9655034	8.80E-05	7p14.1	*POU6F2*	intronic	-	-
rs1197313	8.90E-05	3q22.1	*TMEM108*	intronic	-	-
rs6912010	9.20E-05	6q21	***CDK19***	35 kb downstream	-	CDK19 mRNA is increased in the SN[21] and blood[15] of (female) PD patients compared to controls.
rs6912010	9.20E-05	6q21	*SLC22A16*	99 kb upstream	-	-
rs7312607	9.30E-05	12q23.1	*CDK17*	32 kb upstream	-	-
rs1355095	9.40E-05	5q31.1	***ACSL6***	intronic	-	ACSL6 mRNA is decreased in the SN of PD patients compared to controls[18].
rs9859577	9.90E-05	3q28	***FGF12***	intronic	-	FGF12 mRNA is decreased in the SN of PD patients compared to controls[17, 18].
GWAS 4 (Latourelle et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs10952735	6.90E-08	7q36.1	***CNTNAP2***	intronic	-	CNTNAP2 mRNA is decreased in the SN[23] and increased[15] or decreased[24] in the blood of PD patients compared to controls.
rs12261736	1.80E-07	10q21.1	***PRKG1***	intronic	-	-
rs7954006	7.10E-07	12q24.31	***HCAR1***	intronic	-	-
rs11974194	1.20E-06	7q36.3	*LMBR1*	intronic	-	LMBR1 mRNA is decreased in the SN of PD patients compared to controls[18].
rs17565841	2.70E-06	15q12	***OCA2***	2.8 kb downstream	-	OCA2 mRNA is increased in the striatum of PD patients compared to controls[20].
rs9904572	4.30E-06	17p12	***ARHGAP44***	intronic	-	ARHGAP44 mRNA is decreased in the SN of PD patients compared to controls[18].
rs12504099	7.70E-06	4p14	*TBC1D1*	intronic	-	-
rs7076519	9.30E-06	10q26.2	***ADAM12***	intronic	-	-
rs10767971	9.30E-06	11p13	*QSER1*	19 kb upstream	-	-
rs7828992	1.00E-05	8q24.3	***TRAPPC9***	intronic	-	-
rs1843604	1.20E-05	3q22.1	***CPNE4***	15 kb downstream	-	-

rs1843604	1.20E-05	3q22.1	***MRPL3***	15 kb upstream	-	MRPL3 mRNA is decreased in the SN of PD patients compared to controls[22, 23].
rs4791571	1.30E-05	17p12	*HS3ST3B1*	intronic	-	-
rs12146113	1.30E-05	1q32.1	***KIF14***	78 kb downstream	-	-
rs6069640	1.30E-05	20q13.2	*MC3R*	50 kb upstream	-	-
rs12146113	1.30E-05	1q32.1	*ZNF281*	64 kb upstream	-	-
rs11062784	1.70E-05	12p13.32	***EFCAB4B***	intronic	-	-
rs2826833	1.80E-05	21q21.1	***NCAM2***	intronic	-	-
rs7556447	2.10E-05	1p36.32	*RER1*	11 kb downstream	-	RER1 mRNA is decreased in the blood of PD patients compared to controls[15].
rs7556447	2.10E-05	1p36.32	*PEX10*	863 bp upstream	-	
rs10918270	2.90E-05	1q23.3	***ATF6***	intronic	-	ATF6 mRNA is increased in the blood of PD patients compared to controls[15].
rs12328510	3.10E-05	2p21	***PRKCE***	intronic	-	-
rs956322	3.10E-05	11p13	*PRRG4*	intronic	-	PRRG4 mRNA is increased in the SN of PD patients compared to controls[18].
rs4827256	3.50E-05	Xp11.4	***DDX3X***	99 kb upstream	-	DDX3X is increased in the striatum of PD patients compared to controls[42].
rs4827256	3.50E-05	Xp11.4	***USP9X***	1.2 kb downstream	-	USP9X protein localizes to LB and is lower expressed in the SN of PD patients[43].
rs11655490	3.80E-05	17q25.1	*FAM104A*	90 kb downstream	-	-
rs11655490	3.80E-05	17q25.1	*SLC39A11*	25 kb upstream	-	-
rs17817190	3.90E-05	2q21.2	*NCKAP5*	26 kb upstream	-	-
rs1572662	4.10E-05	6q25.2	*CNKSR3*	intronic	-	CNKSR3 mRNA is increased in the SN of PD patients compared to controls[18].
rs11127593	5.10E-05	3p12.3	***ROBO2***	intronic	-	ROBO2 mRNA is decreased in the SN[17, 18, 44] and ROBO2 protein is decreased in the CSF[16] of PD patients compared to controls.
rs10420134	5.80E-05	19q13.12	*C19orf55*	1.9 kb downstream	-	-
rs10420134	5.80E-05	19q13.12	***ARHGAP33***	4.5 kb upstream	-	ARHGAP33 mRNA is decreased (in female)[23] or increased[18] in the SN of PD patients compared to controls.
rs2667648	7.00E-05	16q23.1	***WWOX***	intronic	-	-
rs9916114	7.30E-05	17q11.2	***PSMD11***	4.6 kb upstream		Proteasomal function is impaired in the PD SN[45].
rs9916114	7.30E-05	17q11.2	*ZNF207*	52 kb downstream	-	-
rs11899121	8.00E-05	2p24.1	***SDC1***	33 kb downstream	-	SDC1 mRNA is decreased in the SN of (female) PD patients compared to controls[18, 23].
rs4764854	8.20E-05	12q23.2	***DRAM1***	intronic	-	-
rs1234326	8.70E-05	10q22.1	***CDH23***	intronic	-	-
GWAS 5 (Latourelle et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs887458	1.90E-06	7p14.1	***AMPH***	61 kb downstream	-	AMPH mRNA is decreased in the SN of PD patients compared to controls[17, 46, 47]. AMPH protein is increased in the striatum of PD patients compared to controls[20].
rs1467751	1.30E-05	21q22.2	***DSCAM***	intronic	-	-

rs1467751	1.30E-05	21q22.2	***TMPRSS3***	intronic	-	-
rs6440565	1.50E-05	3q24	***AGTR1***	57 kb upstream	-	AGTR1 mRNA is decreased in the SN of (male) PD patients compared to controls[17, 18, 20, 21].
rs2550401	2.00E-05	16p13.3	*ZNF200*	6 kb downstream	-	-
rs2550401	2.00E-05	16p13.3	*ZNF263*	48 kb upstream	-	-
rs1355637	4.10E-05	1q21.3	***ENSA***	intronic	-	-
GWAS 6 (Simon-Sanchez et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs2736990	**5.69E-09**	4q22.1	***SNCA***	intronic	The mutation G209A (Ala53Thr) in SNCA was found in one Italian and three Greek families with PD, but not in controls[35] and the G88C (Ala30Pro) mutation was found in a German family[36]. SNCA gene duplication[37, 38] and triplication[39] causes familial PD. Based on motor Unified PD Rating Scale subscores SNCA (rs356220) is associated with rigidity in PD (P=0.04)[32].	SNCA mRNA is decreased in the SN of PD patients compared to controls[17, 22, 23]. LB immunoreactive for SNCA were found in the SN of PD patients[40, 41].
rs415430	**4.50E-08**	17q21.31	***WNT3***	intronic	-	WNT3 mRNA is decreased in the SN of PD patients compared to controls[18].
rs199533	5.05E-08	17q21.31	***NSF***	intronic	-	NSF mRNA is decreased in the SN[17, 18, 22, 23, 46-48] and striatum[46] of PD patients compared to controls.
rs393152	1.42E-07	17q21.31	***CRHR1***	intronic	-	-
rs12185268	1.90E-07	17q21.31	*SPPL2C*	non-syn coding	-	-
rs1981997	2.02E-07	17q21.31	***MAPT***	intronic	Genome-wide significant association (P<5E-08) of MAPT with PD was found in multiple meta-analyses (H1/H2 haplotype; OR=0.78, P=7.97E-52[27], rs242559; OR=0.78, P=1.5E-10)[28].	MAPT mRNA is decreased in the SN[21-23] and MAPT protein is decreased in the CSF[49] of PD patients compared to controls.
rs2532274	2.22E-07	17q21.31	***KANSL1***	intronic	-	-
rs11648673	4.77E-07	16p13.3	***AXIN1***	intronic	-	AXIN1 mRNA is increased in the SN of PD patients compared to controls[18].
rs239748	1.17E-06	Xp22.13	*PHKA2*	27 kb downstream	-	-
rs7013027	1.85E-06	8p23.2	***CSMD1***	intronic	-	CSMD1 mRNA is decreased in the SN of PD patients compared to controls[18].
rs11012	2.85E-06	17q21.31	***PLEKHM1***	3' UTR	-	PLEKHM1 mRNA is decreased in the SN of female PD patients compared to controls[23] and is increased in the blood of PD patients compared to controls[15].
rs10857899	3.06E-06	1p13.2	***RAP1A***	intronic	-	RAP1A protein is increased in the SN of PD patients compared to controls[26].
rs6542651	3.34E-06	2p25.3	*DCDC2C*	intronic	-	-
rs2285459	3.38E-06	16p11.2	***ITGAL***	intronic	-	(Part of LFA-1 complex). The number of LFA-1 (ITGAL) positive microglia in the SN and striatum increased during degeneration of the SN[50].
rs2492448	3.84E-06	10p11.21	***PARD3***	91 kb upstream		PARD3 mRNA is increased in BA9 in PD patients compared to controls[51].
rs4957473	4.24E-06	5p13.1	***C9***	intronic	-	C9 protein is decreased in the CSF of PD patients compared to controls[16]. Intra- and extraneuronal LB and dendritic spheroid bodies were immunoreactive for C9 in PD SN, but not in controls[52].

rs2896905	5.03E-06	12q12	***SLC2A13***	intronic	An interaction between the SNP rs2896905 in SLC2A13 and caffeine intake or smoking and caffeine intake combined is associated with PD risk i.e., high caffeine intake reduces PD risk (OR=0.81, P=0.049), never smokers with low caffeine intake have a higher PD risk (OR=1.35, P=0.04) and smokers with high caffeine intake have a lower PD risk (OR=0.68, P=0.007)[53].	SLC2A13 mRNA is decreased in the SN of PD patients compared to controls[18].
rs817097	6.22E-06	17q24.3	***MAP2K6***	33 kb upstream	-	-
rs2856336	7.69E-06	12p13.2	***ETV6***	intronic	-	-
rs764660	7.83E-06	2q24.3	***SCN2A***	intronic	-	SCN2A mRNA is decreased in the SN[17,18] and increased in the striatum[19] of PD patients compared to controls.
rs11244079	8.66E-06	9q34.2	***RPL7A***	31 kb upstream	-	RPL7A mRNA is decreased in male PD patients compared to controls[23].
rs11244079	8.66E-06	9q34.2	*SURF6*	13 kb downstream	-	-
rs2733333	9.31E-06	15q21.3	***TCF12***	intronic	-	-
rs11878803	1.07E-05	19q13.41	*ZNF615*	intronic	-	-
rs17654531	1.16E-05	1p13.2	*CHI3L2*	14 kb downstream	-	CHI3L2 is increased in the striatum of PD patients compared to controls[20].
rs7923172	1.43E-05	10p11.21	***CUL2***	intronic	-	-
rs163321	1.49E-05	5q35.3	***ADAMTS2***	intronic	-	ADAMTS2 mRNA is increased in the SN[23] and blood[24] of (female) PD patients compared to controls.
rs869714	1.54E-05	1q24.1	*DUPD1*	28 kb downstream	-	-
rs869714	1.54E-05	1q24.1	***POU2F1***	64 kb upstream	-	-
rs10827492	1.69E-05	10p11.21	***CREM***	intronic	-	-
rs13139027	1.75E-05	4p16.2	***MSX1***	61 kb downstream	-	MSX1 mRNA is increased in the SN of PD patients compared to controls[18].
rs2491015	1.76E-05	10q22.1	*KIAA1279*	intronic	-	-
rs3792738	2.19E-05	5q13.3	***CRHBP***	754 b upstream	-	-
rs3792738	2.19E-05	5q13.3	*S100Z*	30 kb downstream	-	S100Z mRNA is increased in the striatum of PD patients compared to controls[20].
rs7651825	2.20E-05	3p25.1	*C3orf20*	intronic	-	-
rs17115100	2.46E-05	10q24.32	***CYP17A1***	intronic	-	-
rs558076	2.53E-05	17q24.3	***ABCA5***	12 kb upstream	-	-
rs4247113	2.62E-05	17p13.3	*FAM101B*	61 kb downstream	-	FAM101B mRNA is increased in the SN of PD patients compared to controls[18].
rs4247113	2.62E-05	17p13.3	***RPH3AL***	26 kb upstream	-	-
rs1793949	3.14E-05	12q13.11	***COL2A1***	intronic	-	COL2A1 mRNA is decreased in the SN[20] and COL2A1 protein is increased in the CSF[16] of PD patients compared to controls.
rs859522	3.41E-05	7p14.1	***VPS41***	intronic	-	-
rs2708909	3.44E-05	7p12.3	*SUN3*	intronic	-	-
rs1395993	3.49E-05	3p24.3	***ZNF385B***	90 kb downstream	-	-
rs10849446	3.66E-05	12p13.31	***SCNN1A***	intronic	-	-
rs3824754	3.92E-05	10q24.32	*C10orf32*	intronic	-	-
rs12261843	4.05E-05	10p11.21	*CCNY*	intronic	-	CCNY mRNA is increased in the SN of male PD patients compared to controls[21].

rs4563067	4.11E-05	17q25.1	***RPL38***	11 kb upstream	-	RPL38 mRNA is decreased in the SN of PD patients compared to controls[23].
rs6794137	4.27E-05	3q22.1	*TMEM108*	intronic	-	-
rs9458499	4.27E-05	6q26	***PARK2***	intronic	Exon deletions, duplications and triplications in the PARK2 gene cause autosomal recessive juvenile parkinsonism[54-59]. Multiple mutations in the PARK2 gene are associated with early-onset autosomal recessive PD[60-63]. The GG genotype of the -258 T/G PARK2 polymorphism is associated with a lower age of onset of PD compared with the common TT genotype[64].	A PARK2 splice variant (resulting in exon 4 deletion and a truncated protein) is increased in PD SN and lymphocytes and the PARK2 splice variant/wild type ratio increases with age in PD patients[65].
rs6044224	4.37E-05	20p12.1	***SNRPB2***	52 kb upstream	-	-
rs11778693	4.45E-05	8p21.3	***CCAR2***	5' UTR	-	-
rs7707022	4.58E-05	5p13.2	*WDR70*	13 kb downstream	-	-
rs11183395	4.59E-05	12q13.11	*SLC38A1*	intronic	-	SLC38A1 mRNA is increased in the striatum of PD patients compared to controls[19,20].
rs935378	4.63E-05	2p21	***MCFD2***	20 kb downstream	-	MCFD2 mRNA is decreased in the SN of PD patients compared to controls[23].
rs1005511	4.68E-05	11q12.1	***SERPING1***	intronic	-	SERPING1 protein is decreased in the blood of PD patients compared to controls[66].
rs12413409	4.69E-05	10q24.32	***CNNM2***	intronic	-	-
rs17071181	4.86E-05	18q21.33	***SERPINB5***	intronic	-	-
rs2227928	5.11E-05	7p13.2	***ATR***	non-syn coding	-	ATR mRNA is decreased in the SN of PD patients compared to controls[23].
rs10246477	5.25E-05	7q21.11	***SEMA3E***	intronic	-	SEMA3E mRNA is decreased in the SN of PD patients compared to controls[18].
rs10894203	5.46E-05	11q24.3	*ADAMTS15*	intronic	-	-
rs9525776	5.53E-05	13q14.11	*ENOX1*	intronic	-	-
rs7454430	5.54E-05	6q22.31	***CLVS2***	27 kb upstream	-	CLVS2 mRNA is decreased in the SN of PD patients compared to controls[18].
rs9924026	5.94E-05	16q12.1	***ZNF423***	intronic	-	-
rs1934828	6.08E-05	13q22.2	*LMO7DN*	75 kb downstream	-	-
rs6599389	6.28E-05	4p16.3	*TMEM175*	intronic	-	-
rs265120	6.38E-05	1q41	*GPATCH2*	7 kb downstream	-	-
rs3740484	6.57E-05	10p24.31	***PEO1***	intronic	The mutations c.G1121A (Arg374Gln) and c.G1750A (Ala359Thr) in PEO1 are associated with familial parkinsonism and ophthalmoplegia and segregate with the disease phenotype[67,68].	-
rs636508	6.65E-05	9q21.32	***TLE1***	88 kb upstream	-	-
rs6812193	6.67E-05	4q21.1	*FAM47E*	intronic	-	-
rs6812193	6.67E-05	4q21.1	*STBD1*	intronic	-	-
rs6959225	6.84E-05	7p21.3	*NXPH1*	intronic	-	-
rs8111509	6.84E-05	19q13.41	***FPR3***	intronic	-	FPR3 mRNA is increased in the SN[18] and striatum[20] of PD patients compared to controls.
rs7077361	6.88E-05	10p13	***ITGA8***	intronic	A meta-analysis on GWAS data showed genowe-wide association of a SNP (rs7077361) in ITGA8 with PD (OR=0.88, P=1.3E-08)[27].	ITGA8 mRNA is decreased in the SN and increased in the striatum of PD patients compared to controls[20].
rs9839984	6.92E-05	3q26.1	***PPM1L***	intronic	-	-

rs2240914	6.98E-05	9q34.11	*GPR107*	3' UTR	-	-
rs4661747	7.02E-05	1p36.13	*SPATA21*	intronic	-	-
rs4584384	7.09E-05	1q21.3	*TDRD10*	intronic	-	-
rs595046	7.27E-05	21q22.3	*C21orf125*	64 kb upstream	-	-
rs595046	7.27E-05	21q22.3	***SIK1***	32 kb downstream	-	SIK1 mRNA is decreased in the SN of PD patients compared to controls[18].
rs2686831	7.31E-05	7p12.3	***HUS1***	intronic	-	-
rs4242434	7.31E-05	8p21.3	*BIN3*	intronic	-	BIN3 mRNA is increased in the SN of PD patients compared to controls[18].
rs6582668	7.41E-05	12q12	*ALG10B*	43 kb downstream	-	-
rs6780193	7.93E-05	3p13	***PROK2***	72 kb upstream	-	PROK2 mRNA is increased in the striatum of PD patients compared to controls[20].
rs16944593	7.95E-05	12q24.21	***TBX3***	35 kb upstream	-	TBX3 mRNA is increased in the SN of PD patients compared to controls[18].
rs7436941	8.13E-05	4q32.1	***RXFP1***	intronic	-	RXFP1 mRNA is increased in the striatum of PD patients compared to controls[20].
rs7911697	8.19E-05	10p13	***FRMD4A***	intronic	-	-
rs7903802	8.38E-05	10p13	*CCDC3*	intronic	-	-
rs699038	8.53E-05	12p12.1	*C12orf77*	9 kb upstream	-	-
rs2515501	8.85E-05	8p23.1	***ANGPT2***	intronic	-	-
rs2515501	8.85E-05	8p23.1	***MCPH1***	intronic	-	-
rs9480154	9.25E-05	6q25.1	*IYD*	79 kb upstream	-	-
rs9480154	9.25E-05	6q25.1	***PPP1R14C***	39 kb downstream	- (Part of the PP1-complex).	-
rs2470179	9.27E-05	15q21.2	***GLDN***	intronic	-	GLDN mRNA is decreased in the striatum[20] and increased in the blood[24] and GLDN protein is increased in the CSF[16] of PD patients compared to controls.
rs12777747	9.30E-05	10q26.13	***TACC2***	intronic	-	-
rs1224671	9.32E-05	15q21.1	***SEMA6D***	intronic	-	SEMA6D mRNA is decreased in the SN[18] and SEMA6D protein is decreased in CSF[16] of PD patients compared to controls.
rs2708851	9.33E-05	7p12.3	*C7orf57*	intronic	-	C7orf57 mRNA is increased in the striatum of PD patients compared to controls[20].
rs8014371	9.34E-05	14q13.1	*NPAS3*	intronic	-	-
rs1605527	9.41E-05	3p24.3	*ZNF385D*	83 kb downstream	-	ZNF385D mRNA is decreased in the SN of PD patients compared to controls[18].
rs6440096	9.52E-05	3q23	***PLS1***	intronic	-	-
rs748088	9.66E-05	21q22.13	*DSCR4*	intronic	-	-
rs6596287	9.72E-05	5q31.1	***SMAD5***	intronic	-	-
GWAS 7 (Edwards et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs356220	2.67E−06	4q22.1	***SNCA***	5 kb downstream	The mutation G209A (Ala53Thr) in SNCA was found in one Italian and three Greek families with PD, but not in controls[35] and the G88C (Ala30Pro) mutation was found in a German family[36]. SNCA gene duplication[37,38] and triplication[39] causes familial PD. Based on motor Unified	SNCA mRNA is decreased in the SN of PD patients compared to controls[17,22,23]. LB immunoreactive for SNCA were found in the SN of PD patients[40,41].

					PD Rating Scale subscores SNCA (rs356220) is associated with rigidity in PD (P=0.04)[32].	
rs1543467	2.97E−06	1p22.3	*CLCA4*	intronic	-	-
rs12063142	5.02E-06	1p36.13	*TAS1R2*	27 kb downstream	-	-
rs9513249	5.92E-06	13q32.1	***RAP2A***	39 kb downstream	-	-
rs976683	1.04E-05	3q26.31	***NLGN1***	intronic	-	NLGN1 mRNA is decreased in the SN of PD patients compared to controls[18].
rs1816879	1.16E-05	15q21.3	***LIPC***	92 kb upstream	-	-
rs1406968	1.16E-05	20p11.23	***SLC24A3***	intronic	-	SLC24A3 mRNA is decreased in the SN of (female) PD patients compared to controls[18, 23].
rs11625012	1.61E-05	14q22.1	***TMX1***	80 kb downstream	-	-
rs135066	1.91E-05	22q13.2	*MPPED1*	intronic	-	MPPED1 mRNA is increased in the SN of PD patients compared to controls[69].
rs9457743	2.03E-05	6q25.3	***MAS1***	54 kb upstream	-	-
rs9457743	2.03E-05	6q25.3	*PNLDC1*	32 kb downstream	-	-
rs1159278	2.21E-05	13q32.1	***MBNL2***	39 kb downstream	-	-
rs12142266	2.31E-05	1p22.3	***SEP15***	intronic	-	SEP15 mRNA is downregulated in the SN of PD patients[70].
rs358079	2.99E-05	3p14.3	***CACNA2D3***	1.7 kb downstream	-	CACNA2D3 mRNA is decreased in the SN[18] and striatum[42] of PD patients compared to controls. CACNA2D3 protein is increased in the CSF of PD patients compared to controls[16].
rs13411180	3.09E-05	2q31.2	***ZNF385B***	intronic	-	-
rs6930229	3.14E-05	6q25.3	***MRPL18***	42 kb downstream	-	-
rs13157	3.52E-05	1p36.11	***RUNX3***	3' UTR	-	RUNX3 mRNA is increased in the SN of PD patients compared to controls[18].
rs929708	3.70E-05	3p25.3	***ATP2B2***	intronic	-	ATP2B2 mRNA is decreased in the SN of PD patients compared to controls[18, 22, 23].
rs10882088	3.89E-05	10q23.33	***KIF11***	intronic	-	-
rs11833635	4.09E-05	12q15	*PTPRR*	intronic	-	-
rs4777585	4.57E-05	15q24.1	***NEO1***	intronic	-	-
rs12938031	4.61E-05	17q21.31	***CRHR1***	7 kb upstream	-	-
rs6598020	4.63E-05	11p15.5	*ANO9*	2.6 kb upstream	-	-
rs6598020	4.63E-05	11p15.5	*PKP3*	40 kb downstream	-	-
rs4927602	4.79E-05	2p25.3	***TPO***	intronic	-	-
GWAS 8 (Hamza et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs356220	**3.40E-11**	4q22.1	***SNCA***	5 kb downstream	The mutation G209A (Ala53Thr) in SNCA was found in one Italian and three Greek families with PD, but not in controls[35] and the G88C (Ala30Pro) mutation was found in a German family[36]. SNCA gene duplication[37, 38] and triplication[39] causes familial PD. Based on motor Unified PD Rating Scale subscores SNCA (rs356220) is associated with rigidity in PD (P=0.04)[32].	SNCA mRNA is decreased in the SN of PD patients compared to controls[17, 22, 23]. LB immunoreactive for SNCA were found in the SN of PD patients[40, 41].
rs1350855	**1.30E-09**	4q22.1	***FAM190A***	intronic	-	-
rs3129882	**2.90E-08**	6p21.32	***HLA-DRA***	intronic	The PD associated SNP rs3129882 is an eQTL for HLA-	HLA-DRA mRNA is increased in the striatum of PD

					DRA and affects its expression[71,72]. HLA-DRA intronic variant rs3129882 is associated with late-onset sporadic PD in Chinese Han patients[73].	patients compared to controls[20].
rs199533	1.30E-06	17q21.31	***NSF***	intronic	-	NSF mRNA is decreased in the SN[17,18,22,23,46-48] and striatum[46] of PD patients compared to controls.
rs199528	1.40E-06	17q21.31	***WNT3***	intronic	-	WNT3 mRNA is decreased in the SN of PD patients compared to controls[18].
rs7915262	2.80E-06	10p13	***NMT2***	intronic	-	-
rs3117098	2.90E-06	6p21.32	*C6orf10*	19 kb upstream	-	-
rs10741569	3.20E-06	11p15.3	***MICAL2***	intronic	-	-
rs3129955	3.80E-06	6p21.32	***BTNL2***	intronic	-	BTNL2 mRNA is decreased in the SN of PD patients compared to controls[20].
rs17651549	4.80E-06	17q21.31	***MAPT***	non-syn coding	Genome-wide significant association (P<5E-08) of MAPT with PD was found in multiple meta-analyses (H1/H2 haplotype; OR=0.78, P=7.97E-52[27], rs242559; OR=0.78, P=1.5E-10)[28].	MAPT mRNA is decreased in the SN[21-23] and MAPT protein is decreased in the CSF[49] of PD patients compared to controls.
rs4790246	5.30E-06	17p13.2	*ZFP3*	10 kb upstream	-	-
rs4790246	5.30E-06	17p13.2	*ZNF232*	37 kb downstream	-	-
rs12373142	5.50E-06	17q21.31	*SPPL2C*	non-syn coding	-	-
rs241041	5.80E-06	17q21.31	***CRHR1***	intronic	-	-
rs4678550	6.40E-06	3p22.2	*DCLK3*	23 kb upstream	-	-
rs4678550	6.40E-06	3p22.2	*TRANK1*	64 kb downstream	-	-
rs36076725	6.50E-06	17q21.31	***KANSL1***	intronic	-	-
rs2957316	9.70E-06	17q21.31	*LRRC37A*	39 kb upstream	-	-
GWAS 9 (Spencer et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs10447854	**3.11E-09**	7q32.1	***SND1***	63 kb downstream	-	-
rs356220	**5.18E-09**	4q22.1	***SNCA***	5 kb downstream	The mutation G209A (Ala53Thr) in SNCA was found in one Italian and three Greek families with PD, but not in controls[35] and the G88C (Ala30Pro) mutation was found in a German family[36]. SNCA gene duplication[37,38] and triplication[39] causes familial PD. Based on motor Unified PD Rating Scale subscores SNCA (rs356220) is associated with rigidity in PD (P=0.04)[32].	SNCA mRNA is decreased in the SN of PD patients compared to controls[17,22,23]. LB immunoreactive for SNCA were found in the SN of PD patients[40,41].
rs7215239	**1.49E-08**	17q21.31	***CRHR1***	intronic	-	-
rs8070723	5.21E-08	17q21.31	***MAPT***	intronic	Genome-wide significant association (P<5E-08) of MAPT with PD was found in multiple meta-analyses (H1/H2 haplotype; OR=0.78, P=7.97E-52[27], rs242559; OR=0.78, P=1.5E-10)[28].	MAPT mRNA is decreased in the SN[21-23] and MAPT protein is decreased in the CSF[49] of PD patients compared to controls.
rs4522464	9.84E-07	17q23.2	***MED13***	56 kb upstream	-	-
rs4457092	4.05E-06	5p13.3	***CDH6***	intronic	-	-
rs2033884	7.33E-06	7p15.3	***DNAH11***	intronic	-	DNAH11 mRNA is increased in the striatum of PD patients compared to controls[20].
rs252761	7.85E-06	5q14.1	***AP3B1***	intronic	-	AP3B1 mRNA is increased in the blood of PD patients compared to controls[15].

rs11256442	8.30E-06	10p15.1	***IL2RA***	intronic	-	IL2RA mRNA is increased in the striatum of PD patients compared to controls[20].
rs2642444	8.94E-06	1q41	***MARC1***	intronic	-	-
rs10744675	2.51E-05	12p13.32	***KCNA5***	8 kb upstream	-	-
rs12674264	2.95E-05	7q21.13	*ZNF804B*	intronic	-	-
rs17183533	3.83E-05	16p13.3	***ABCA3***	intronic	-	-
rs11759658	4.38E-05	6p22.2	***LRRC16A***	intronic	-	-
rs1320163	4.73E-05	3p22.1	*ANO10*	93 kb downstream	-	-
rs1320163	4.73E-05	3p22.1	***SNRK***	25 kb upstream	-	-
rs10477933	4.78E-05	5q21.3	***FER***	intronic	-	-
rs11118618	4.86E-05	1q41	***HLX***	41 kb upstream	-	HLX mRNA is increased in the blood of PD patients compared to controls[15].
rs7748486	5.15E-05	6p24.2	***NEDD9***	intronic	The intronic SNP rs760678 in NEDD9 is associated with susceptibility to PD (OR=1.26, P=0.0017)[74].	-
rs764606	5.18E-05	12q13.12	*METTL7A*	5 kb upstream	-	METTL7A is alternatively spliced in the blood of PD patients compared to controls[75].
rs2242565	5.19E-05	11p15.5	*ATHL1*	3' UTR	-	ATHL1 mRNA is increased in the blood of PD patients compared to controls[15].
rs12831858	8.35E-05	12q13.12	*TMPRSS12*	intronic	-	-
GWAS 10 (Saad et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs2736990	**2.88E-08**	4q22.1	***SNCA***	intronic	The mutation G209A (Ala53Thr) in SNCA was found in one Italian and three Greek families with PD, but not in controls[35] and the G88C (Ala30Pro) mutation was found in a German family[36]. SNCA gene duplication[37,38] and triplication[39] causes familial PD. Based on motor Unified PD Rating Scale subscores SNCA (rs356220) is associated with rigidity in PD (P=0.04)[32].	SNCA mRNA is decreased in the SN of PD patients compared to controls[17,22,23]. LB immunoreactive for SNCA were found in the SN of PD patients[40,41].
rs12294719	5.42E-07	11p12	*C11orf74*	47 kb downstream	-	-
rs17690703	3.94E-06	17q21.31	*SPPL2C*	859 bp downstream	-	-
rs17690703	3.94E-06	17q21.31	***MAPT***	46 kb upstream	Genome-wide significant association (P<5E-08) of MAPT with PD was found in multiple meta-analyses (H1/H2 haplotype; OR=0.78, P=7.97E-52[27], rs242559; OR=0.78, P=1.5E-10)[28].	MAPT mRNA is decreased in the SN[21-23] and MAPT protein is decreased in the CSF[49] of PD patients compared to controls.
rs9899558	5.04E-06	17p13.2	***SPNS2***	intronic	-	-
rs621341	5.11E-06	2q21.3	*TMEM163*	intronic	-	-
rs26990	6.67E-06	5q22.2	***MCC***	intronic	-	MCC mRNA is decreased in the SN of PD patients compared to controls[18].
rs4698412	6.88E-06	4p15	***BST1***	intronic	The SNP rs11724635, intronic in BST1, is associated with an increased risk of PD[27,76], whereas this association is stronger in Asian than in Caucasian populations[77]. Further, interaction between this SNP and ever drinking well water was associated with increased PD risk in Taiwanese (heterozygous; OR=1.45, P=0.024 and homozygous OR=1.623, P=0.008), possibly due to heavy metal (arsenic) contamination of well water in Taiwan[78].	-

rs6741233	9.34E-06	2q35	***MREG***	52 kb downstream	-	MREG mRNA is decreased in the SN of PD patients compared to controls[17].
rs4954564	1.21E-05	2q22.1	***CXCR4***	20 kb upstream	-	CXCR4 mRNA is increased in the SN[44] and striatum[20, 42] and CXCR4 is alternatively spliced in the blood[75] of PD patients compared to controls. CXCR4 expression is increased in the SN of PD patients[79].
rs1035767	1.50E-05	12q23.3	***RFX4***	intronic	-	RFX4 mRNA is decreased in the SN[18] and increased in the striatum[20] of PD patients compared to controls.
rs368039	1.52E-05	4p15.33	***HS3ST1***	14 kb upstream	-	-
rs6729702	1.75E-05	2q21.3	***ACMSD***	intronic	A meta-analysis on GWAS data identified ACMSD (rs6710823) as a new risk locus for PD (OR=1.38, P=1.35E-09)[76].	-
rs2532269	1.90E-05	17q21.31	***KANSL1***	intronic	-	-
rs1423326	2.10E-05	5q12.1	*ZSWIM6*	18 kb downstream	-	-
rs393152	2.68E-05	17q21.31	***CRHR1***	intronic	-	-
rs4964469	2.73E-05	12q23.3	*POLR3B*	46 kb downstream	-	POLR3B protein is increased in the striatum of PD patients compared to controls[20].
rs11064524	2.80E-05	12p13.33	***WNK1***	intronic	-	WNK1 mRNA is increased in the striatum[20] and decreased in the blood[15] of PD patients compared to controls
rs9608247	2.99E-05	22q11.23	*SUSD2*	2.2 kb downstream	-	-
rs2259599	3.74E-05	13q34	*TMCO3*	1.4 kb downstream	-	-
rs12724129	4.35E-05	1p36.22	*KIAA2013*	14 kb downstream	-	-
rs12724129	4.35E-05	1p36.22	***PLOD1***	37 kb upstream	-	-
rs12295401	4.40E-05	11q13.5	***PRKRIR***	29 kb downstream	-	-
rs10902724	5.13E-05	1p36.11	*CEP85*	intronic	-	CEP85 mRNA is increased in the SN of PD patients compared to controls[18].
rs9360414	5.34E-05	6q13	*COL19A1*	intronic	-	COL19A1 mRNA is decreased in the blood of PD patients compared to controls[24].
GWAS 11 (Simon-Sanchez et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs7918386	6.39E-06	10q22.1	***EIF4EBP2***	intronic	-	-
rs2412777	1.51E-05	15q15.2	*TGM7*	intronic	-	-
rs2736990	1.63E-05	4q22.1	***SNCA***	intronic	The mutation G209A (Ala53Thr) in SNCA was found in one Italian and three Greek families with PD, but not in controls[35] and the G88C (Ala30Pro) mutation was found in a German family[36]. SNCA gene duplication[37, 38] and triplication[39] causes familial PD. Based on motor Unified PD Rating Scale subscores SNCA (rs356220) is associated with rigidity in PD (P=0.04)[32].	SNCA mRNA is decreased in the SN of PD patients compared to controls[17, 22, 23]. LB immunoreactive for SNCA were found in the SN of PD patients[40, 41].
rs10497310	2.22E-05	2q24.3	*XIRP2*	intronic	-	-
rs10504139	2.65E-05	8q11.23	*FAM150A*	1.7 kb downstream	-	-
rs6057657	2.77E-05	20q11.21	*DEFB119*	1.1 kb downstream	-	-
rs6057657	2.77E-05	20q11.21	*DEFB123*	64 kb upstream	-	-
rs12704998	3.31E-05	7q22.1	***NPTX2***	40 kb downstream	-	NPTX2 mRNA is increased in the SN[18, 20] and decreased in the striatum[42] of PD patients compared to controls. NPTX2 is highly upregulated in PD SN and a component

						of LB[80].
rs2383025	3.61E-05	9p22.2	*CNTLN*	intronic	-	CNTLN mRNA is increased in the blood of PD patients compared to controls[15].
rs4248166	4.39E-05	6p21.32	***BTNL2***	intronic	-	BTNL2 mRNA is decreased in the SN of PD patients compared to controls[20].
rs1217770	4.44E-05	5q13.2	***MAP1B***	96 kb upstream	-	MAP1B mRNA is decreased in the SN of PD patients compared to controls[22, 23].
rs17782975	4.46E-05	15q15.3	***TP53BP1***	intronic	-	-
rs8446	5.17E-05	2q31.1	***ZAK***	intronic	-	ZAK mRNA is decreased in the SN of female PD patients compared to controls[21].
rs524908	5.52E-05	15q15.3	*FRMD5*	intronic	-	FRMD5 mRNA is increased in the SN of PD patients compared to controls[18].
rs17202259	6.22E-05	6p21.32	*C6orf10*	18 kb upstream	-	-
rs8132225	6.49E-05	21q22.13	***HLCS***	intronic	-	-
rs2250175	6.50E-05	4q34.1	*GLRA3*	55 kb downstream	-	GLRA3 mRNA is decreased in the SN[20] and increased in the blood[15] of PD patients compared to controls.
rs2250175	6.50E-05	4q34.1	***HPGD***	59 kb upstream	-	HPGD mRNA is decreased in the SN of PD patients compared to controls[17].
rs12184950	6.91E-05	14q32.32	***RCOR1***	3.2 kb downstream	-	-
rs12184950	6.91E-05	14q32.32	***TRAF3***	44 kb upstream	-	TRAF3 mRNA is decreased in the blood of PD patients compared to controls[15].
rs6693597	7.01E-05	1p31.1	*NFIA*	intronic	-	-
rs2255663	7.15E-05	15q15.3	*PPIP5K1*	intronic	-	-
rs11177355	8.95E-05	12q15	*SLC35E3*	intronic	-	-
rs6832140	9.42E-05	4q22.1	***FAM190A***	intronic	-	-
rs4819594	9.52E-05	22q11.21	***CECR2***	intronic	-	-
GWAS 12 (Do et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs34637584	**1.82E-28**	12q12	***LRRK2***	non-syn coding	The mutations c.4322G>A (Arg1441His), c.4321C>G (Arg1441Gly), c.4321C>T (Arg1441Cys), c.5096A>G (Tyr1699Cys), c.6055G>A (Gly2019Ser) and c.6059T>C (Ile2020Thr) in LRRK2 cause late-onset PD and segragate with disease in PD families[81-85].	LRRK2 protein is increased in the CSF of PD patients compared to controls[16].
i4000416 / rs76763715	**5.17E-21**	1q22	***GBA***	non-syn coding / splice site	GBA mutations are associated with (early-onset) parkinsonism[86] and (early-onset) PD in multiple ethnic populations[87] (Ashkenazi Jews, P<0.001[88]; Brazillians, P=0.0379[89]; Italians, P=0.0018[90], caucasian P=0.0001[91]; Chinese, P=0.001[92]; Brazillians, P=0.0047[93]; Greek, P=0.006[94]; Koreans, P<0.01[95]; Serbians, P=0.0041[96]; Mexicans, P=0.014[97].	GBA enzyme activity is lower in the SN of both PD patients with a GBA mutation (P<0.01) and sporadic PD patients (P<0.05)[98].
rs356219	**7.91E-21**	4q22.1	***SNCA***	9 kb downstream	The mutation G209A (Ala53Thr) in SNCA was found in one Italian and three Greek families with PD, but not in controls[35] and the G88C (Ala30Pro) mutation was found in a German family[36]. SNCA gene duplication[37, 38] and triplication[39] causes familial PD. Based on motor Unified PD Rating Scale subscores SNCA (rs356220) is associated	SNCA mRNA is decreased in the SN of PD patients compared to controls[17, 22, 23]. LB immunoreactive for SNCA were found in the SN of PD patients[40, 41].

					with rigidity in PD (P=0.04)[32].	
rs1876828	**1.14E-14**	17q21.31	***CRHR1***	intronic	-	-
rs12185268	**2.72E-14**	17q21.31	*SPPL2C*	non-syn coding	-	-
rs1918798	**3.54E-14**	17q21.31	***KANSL1***	intronic	-	-
rs17563986	**4.85E-14**	17q21.31	***MAPT***	intronic	Genome-wide significant association (P<5E-08) of MAPT with PD was found in multiple meta-analyses (H1/H2 haplotype; OR=0.78, P=7.97E-52[27], rs242559; OR=0.78, P=1.5E-10)[28].	MAPT mRNA is decreased in the SN[21-23] and MAPT protein is decreased in the CSF[49] of PD patients compared to controls.
rs415430	**1.71E-13**	17q21.31	***WNT3***	intronic	-	WNT3 mRNA is decreased in the SN of PD patients compared to controls[18].
rs199533	**1.90E-13**	17q21.31	***NSF***	syn coding	-	NSF mRNA is decreased in the SN[17, 18, 22, 23, 46-48] and striatum[46] of PD patients compared to controls.
rs11012	**7.74E-11**	17q21.31	***PLEKHM1***	non-syn coding, 3' UTR	-	PLEKHM1 mRNA is decreased in the SN of female PD patients compared to controls[23] and is increased in the blood of PD patients compared to controls[15].
rs10513789	**2.67E-10**	3q27.1	***MCCC1***	intronic	The intronic SNP rs11711441 in MCCC1 is in a GWAS meta-analysis associated with PD (OR=0.82, P=1.17E-08[76]; OR=0.86, P=9.20E-10[27]) and also with a lower risk for PD in Han Chinese (OR=0.82, P=0.043[99]; OR=0.43, P<0.001[100]).	-
rs6812193	**7.55E-10**	4q21.1	*FAM47E*	intronic	-	-
rs6812193	**7.55E-10**	4q21.1	*STBD1*	intronic	-	-
rs6599389	**3.87E-08**	4p16.3	*TMEM175*	intronic	-	-
rs11868035	5.61E-08	17p11.2	***SREBF1***	splice site, intronic	Based on motor Unified PD Rating Scale subscores SREBF1 (rs11868035) is associated with gait impairment in PD (P=0.005)[32].	-
rs823156	1.27E-07	1q32.1	***SLC41A1***	intronic	Three mutations c.436A>G (Lys146Glu), c.1049C>T (Ala350Val) (gain of function[101]) and c.1440A>G (Pro480Pro) were identified in the SLC41A1 gene in PD patients, but not in controls[102, 103].	-
rs11724804	2.43E-07	4p16.3	***DGKQ***	intronic	Genome-wide significance was reached for the intronic SNP rs11248060 in DGKQ (OR=1.21; P=3.04E-12) [27] (OR=1.35; P=2.0E-9)[28], a SNP that also increases the risk for PD in a Chinese population (P<0.05)[29].	-
rs4130047	2.44E-07	18q12.3	***RIT2***	intronic	In a meta-analysis of GWASs RIT2 was identified as a novel susceptibility locus (rs12456492, OR=1.19, P=2E-10 (combined sample)[28].	RIT2 mRNA is decreased in the SN of PD patients compared to controls[17].
rs482912	5.17E-07	3q27.1	***LAMP3***	non-syn coding	-	-
rs4925114	6.75E-07	17p11.2	***RAI1***	intronic	-	-
rs9878775	1.00E-06	3q26.33	***DCUN1D1***	3' UTR	-	-
rs278901	1.17E-06	12q12	***CNTN1***	intronic	-	CNTN1 mRNA and protein[20] is increased in the striatum and decreased in the CSF[16] of PD patients compared to controls.
rs4925119	1.55E-06	17p11.2	***TOM1L2***	7 kb downstream	-	-
rs10999435	1.81E-06	10q22.1	***PALD1***	48 kb downstream	-	PALD1 mRNA is increased in the SN of PD patients compared to controls[18].
rs10999435	1.81E-06	10q22.1	***PRF1***	13 kb upstream	-	-
rs823114	2.12E-06	1q32.1	*NUCKS1*	regulatory region	-	-

rs660895	2.13E-06	6p21.32	***HLA-DRB1***	intronic	The PD associated SNP rs2395163 is an eQTL for HLA-DRB1 and affects its expression[72].	HLA-DRB1 mRNA is increased in the striatum of PD patients compared to controls[20].
rs7451962	2.49E-06	6p21.32	***HLA-DQB1***	45 kb downstream	The alleles HLA-DQB1*03:02 (OR=0.75, P=3E-04) and HLA-DQB1*06:02 (OR=1.26, P=7E-04) are associated with PD[72]. The PD associated SNP rs3129882 is an eQTL for HLA-DQB1 and affects its expression[71, 72].	HLA-DQB1 mRNA is increased in the striatum of PD patients compared to controls[20].
rs1564282	2.56E-06	4p16.3	***GAK***	intronic	SNPs in GAK rs1564282 (Caucasian; OR=1.61; P=0.0151)[30], rs1564282 (Chinese)[29], rs11248051 (Taiwanese; CT/TT vs CC genotypes OR=1.37; P=0.03)[31], rs11248051 (meta-analysis; OR=1.35; P=8.2E-09)[28] are associated with an increased PD risk. Based on motor Unified Parkinson's Disease Rating Scale subscores GAK (rs1564282) is associated with tremor in PD (P=0.03))[32].	-
rs9379968	2.59E-06	6p22.1	*POM121L2*	9 kb downstream	-	-
rs9379968	2.59E-06	6p22.1	*ZNF391*	98 kb upstream	-	ZNF391 mRNA is increased in the striatum of PD patients compared to controls[20].
rs2894181	3.32E-06	6p21.33	*HCG27*	2.8 kb downstream	-	-
rs2894181	3.32E-06	6p21.33	*PSORS1C3*	29 kb upstream	-	-
rs2837740	3.62E-06	21q22.2	***DSCAM***	intronic	-	-
rs1467751	3.62E-06	21q22.2	***TMPRSS3***	intronic	-	-
rs7080373	3.66E-06	10q22.1	***ADAMTS14***	76 kb upstream	-	ADAMTS14 mRNA is decreased in the SN of PD patients compared to controls[20].
rs9917256	3.72E-06	2q24.3	***STK39***	39 kb upstream	A meta-analysis on GWAS data identified STK39 (rs2102808) as a new risk locus for PD (OR=1.28, P=3.31E-11)[76], which was replicated in a Caucasian (OR=1.21, P<0.001, Sharma, 2012) and a Scandinavian PD group (OR=1.34, P=0.0005)[104]. A meta-analysis on GWAS data also showed association of rs2390669 intronic in STK39 with PD (OR=1.19, P=1.37E-09)[27].	-
rs3763309	4.70E-06	6p21.32	***BTNL2***	intronic	-	BTNL2 mRNA is decreased in the SN of PD patients compared to controls[20].
rs11878694	5.59E-06	19p13.2	*MBD3L4*	41 kb downstream	-	-
rs11878694	5.59E-06	19p13.2	*MBD3L5*	34 kb upstream	-	-
rs10886515	5.86E-06	10q26.11	***TIAL1***	intronic	-	TIAL1 mRNA is decreased in the SN of PD patients compared to controls[23].
rs1882642	6.27E-06	2q22.1	***LRP1B***	intronic	-	LRP1B mRNA is decreased in the SN of PD patients compared to controls[20].
rs35883	6.67E-06	3p24.1	*RBMS3*	intronic	-	-
rs659445	7.20E-06	6p21.33	***EHMT2***	intronic	-	-
rs1471738	8.60E-06	3q12.3	*IMPG2*	29 kb downstream	-	-
rs3957148	8.83E-06	6p21.32	***HLA-DQA2***	27 kb upstream	The PD associated SNPs rs3129882 and rs2395163 are eQTLs for HLA-DQA2 and affect its expression[71, 72].	-
rs3957148	8.83E-06	6p21.32	***HLA-DQB2***	42 kb downstream	-	-
rs11097338	9.03E-06	4q21.1	*CCDC158*	intronic	-	-
rs4925138	9.22E-06	17p11.2	***DRG2***	intronic	-	-
rs9275184	1.09E-05	6p21.32	***HLA-DQA1***	40 kb downstream	The alleles HLA-DQA1*01:02 (OR=1.15, P=0.02) and HLA-DQA1*03:01 (OR=0.77, P=1E-04) are associated with PD[72]. The PD associated SNP rs2395163 is an eQTL for HLA-	HLA-DQA1 mRNA is increased in the striatum of PD patients compared to controls[20].

					DQA1 and affects its expression[72].	
rs1568069	1.24E-05	2q37.2	***AGAP1***	intronic	-	-
rs2139950	1.30E-05	3q13.31	***ZBTB20***	5 kb upstream	-	-
rs7251424	1.36E-05	19p13.3	***LSM7***	92 bp downstream	-	-
rs7251424	1.36E-05	19p13.3	***SPPL2B***	7 kb upstream	-	SPPL2B protein is decreased in the CSF of PD patients compared to controls[16].
rs7803999	1.37E-05	7p12.3	*ABCA13*	intronic	-	ABCA13 protein is increased in the CSF of PD patients compared to controls[16].
rs2072369	1.51E-05	7p15.3	*KLHL7*	5' UTR	-	KLHL7 mRNA is decreased in the SN of PD patients compared to controls[18].
rs10519001	1.55E-05	15q22.2	***MYO1E***	intronic	-	MYO1E mRNA is increased in the SN of PD patients compared to controls[18].
rs12694823	1.55E-05	2q36.3	***FBXO36***	74 kb downstream	-	-
rs12694823	1.55E-05	2q36.3	*SLC16A14*	18 kb upstream	-	SLC16A14 mRNA is decreased in the SN of PD patients compared to controls[47].
rs3905495	2.01E-05	6p21.33	***HLA-B***	56 kb downstream	The alleles HLA-B*07:02 (OR=1.20, P=8E-03) and HLA-B*40:01 (OR=0.79, P=0.02) are associated with PD[72].	-
rs3905495	2.01E-05	6p21.33	***HLA-C***	26 kb upstream	The alleles HLA-C*03:04 (OR=0.76, P=2E-03) and HLA-C*07:02 (OR=1.22, P=3E-03) are associated with PD[72].	-
rs10992619	2.05E-05	9q22.31	*SUSD3*	intronic	-	SUSD3 mRNA is increased in the SN[18] and striatum[20] of PD patients compared to controls.
rs3792424	2.12E-05	3p26.2	***IL5RA***	intronic	-	IL5RA mRNA is increased in the striatum of PD patients compared to controls[20].
rs11946079	2.16E-05	4q21.1	***SCARB2***	intronic	The SNP rs6825004, intronic in SCARB2 (OR=0.71, P=0.03), as well as a haplotype of 5 intronic SNPs in SCARB2 is associated with PD in Greek patients (OR=1.75, P=0.004)[105].	-
rs9461362	2.26E-05	6p22.1	*PRSS16*	80 kb downstream	-	-
rs8012152	2.33E-05	14q22.2	***WDHD1***	intronic	-	-
rs34255679	2.35E-05	16p13.3	***RBFOX1***	intronic	-	RBFOX1 mRNA is increased in the striatum of PD patients compared to controls[19].
rs6735555	2.37E-05	2q37.1	***SP110***	11 kb downstream	-	SP110 is alternatively spliced[75] and SP110 mRNA is increased[15] in the blood of PD patients compared to controls.
rs6735555	2.37E-05	2q37.1	*SP140*	69 kb upstream	-	-
rs4698412	2.41E-05	4p15.32	***BST1***	intronic	The SNP rs11724635, intronic in BST1, is associated with an increased risk of PD[27, 76], whereas this association is stronger in Asian than in Caucasian populations[77]. Further, interaction between this SNP and ever drinking well water was associated with increased PD risk in Taiwanese (heterozygous; OR=1.45, P=0.024 and homozygous OR=1.623, P=0.008), possibly due to heavy metal (arsenic) contamination of well water in Taiwan[78].	-
rs1688609	2.45E-05	6q22.31	***CEP85L***	3.9 kb upstream	-	CEP85L mRNA is decreased in the SN of PD patients compared to controls[18].
rs1688609	2.45E-05	6q22.31	*MCM9*	99 kb downstream	-	-
rs947211	2.55E-05	1q32.1	***RAB7L1***	8 kb upstream	A RAB7L1 mutation (c.379-12insT) is associated with PD (OR=3.3, P=0.0399) and a novel mutation (Lys157Arg) was found in one PD patient[102]. The SNPs rs1572931 and	-

					rs823144 are localized in the putative RAB7L1 promotor and are associated with a reduced PD risk in Ashkenazim Jews (OR=0.64, P=0.0002; OR=0.72, P=0.002)[106].	
rs2736994	2.56E-05	4q22.1	***MMRN1***	16 kb upstream	-	MMRN1 mRNA is increased in the striatum of PD patients compared to controls[20].
rs854791	2.57E-05	17p11.2	*MYO15A*	intronic	-	-
rs11065598	2.66E-05	12q24.31	***KDM2B***	intronic	-	-
rs708382	2.81E-05	17q21.31	*FAM171A2*	1.1 kb upstream	-	-
rs708382	2.81E-05	17q21.31	***ITGA2B***	7 kb downstream	-	-
rs11564252	2.83E-05	12q12	*MUC19*	intronic	-	-
rs7018431	2.89E-05	8p23.3	*FAM87A*	45 kb downstream	-	-
rs7018431	2.89E-05	8p23.3	***FBXO25***	76 kb upstream	-	-
rs138054	2.95E-05	22q13.31	***EFCAB6***	11 kb upstream	-	-
rs138054	2.95E-05	22q13.31	*SULT4A1*	817 bp downstream	-	SULT4A1 mRNA is decreased in the SN of PD patients compared to controls[18].
rs12132270	3.03E-05	1q32.1	***SLC45A3***	11 kb upstream	-	SLC45A3 mRNA is increased in the SN of PD patients compared to controls[18].
rs2801943	3.17E-05	10q11.21	*CCNYL2*	intronic	-	-
rs6140909	3.43E-05	20p12.2	***PLCB4***	intronic	-	-
rs16860458	3.44E-05	2q31.1	***ITGA6***	intronic	-	-
rs17763599	3.50E-05	19p13.3	***TMPRSS9***	20 kb upstream	-	-
rs11727049	3.66E-05	4q28.2	*LARP1B*	intronic	-	-
rs12464032	3.79E-05	2q14.3	*CNTNAP5*	intronic	-	CNTNAP5 mRNA is increased in (female) SN[21] or decreased in SN PD patients compared to controls[18].
rs8116325	3.90E-05	20q12	***PTPRT***	intronic	A copy number variant study showed association of a heterozygous deletion in the intron of PTPRT with PD[107].	-
rs1867153	3.91E-05	15q24.2	***MPI***	79 kb downstream	-	-
rs1867153	3.91E-05	15q24.2	***SCAMP5***	17 kb upstream	-	SCAMP5 mRNA is decreased in the SN of PD patients compared to controls[18].
rs10819174	3.95E-05	9q33.3	*MVB12B*	3' UTR	-	MVB12B mRNA is increased[18] or decreased[108] in the SN of PD patients compared to controls.
rs9948128	4.03E-05	18p11.31	*L3MBTL4*	62 kb downstream	-	-
rs9948128	4.03E-05	18p11.31	*TMEM200C*	414 bp upstream	-	TMEM200C mRNA is decreased in the SN of PD patients compared to controls[17].
rs8054636	4.21E-05	16p12.3	*SYT17*	intronic	-	SYT17 mRNA is decreased in the SN of PD patients compared to controls[18].
rs1265093	4.22E-05	6p21.33	*PSORS1C1*	intronic	-	-
rs4076437	4.22E-05	3q21.3	***HIFX***	829 bp upstream	-	-
rs2553427	4.35E-05	3q12.3	***SENP7***	intronic	-	-
i4000434 / rs80338939	4.47E-05	13q12.11	***GJB2***	Deletion / frameshift	-	-
rs2968538	4.58E-05	7q11.22	*CALN1*	intronic	-	CALN1 mRNA is decreased in the SN of PD patients compared to controls[17].
rs17615676	4.73E-05	7p21.3	***ARL4A***	8 kb upstream	-	ARL4A mRNA is increased in the SN of PD patients compared to controls[18].
rs17615676	4.73E-05	7p21.3	***SCIN***	25 kb downstream	-	SCIN mRNA is increased in the striatum of PD patients

						compared to controls[19, 20].
rs10038927	4.80E-05	5q12.3	*ADAMTS6*	intronic	-	-
rs17126237	4.92E-05	8p22	***ASAH1***	39 kb upstream	-	ASAH1 mRNA is decreased in the SN[23] and increased in the blood[15] and ASAH1 protein is increased in the striatum[20] and CSF[16] of PD patients compared to controls. ASAH1 is alternatively spliced in the blood of PD patients compared to controls[75].
rs17126237	4.92E-05	8p22	***PCM1***	94 kb downstream	-	
rs9897702	5.00E-05	17q21.2	***ATP6V0A1***	1.5 kb upstream	-	ATP6V0A1 mRNA is decreased in the SN of PD compared to controls[22, 108].
rs10074991	5.11E-05	5p13.1	***PRKAA1***	Intronic	- (Part of the AMPK-complex).	-
rs909626	5.19E-05	6p22.3	***JARID2***	intronic	-	-
rs11675641	5.27E-05	2p12	*GCFC2*	39 kb upstream	-	-
rs11675641	5.27E-05	2p12	***MRPL19***	60 kb downstream	-	-
rs1920650	5.27E-05	3q25.33	***KPNA4***	52 kb upstream	-	-
rs902910	5.45E-05	4q35.2	*TRIML2*	intronic	-	-
rs17529642	5.55E-05	2q33.1	*TYW5*	97 kb downstream	-	-
rs17529642	5.55E-05	2q33.1	*C2orf69*	78 kb upstream	-	-
rs755690	5.82E-05	19q13.2	***ACTN4***	intronic	-	ACTN4 mRNA[18] and protein[26] is increased in the SN of PD patients compared to controls.
rs624032	6.01E-05	10p12.1	***BAMBI***	8 kb upstream	-	-
rs624032	6.01E-05	10p12.1	*WAC*	47 kb downstream	-	-
rs716409	6.23E-05	13q14.3	***PCDH8***	20 kb upstream	-	PCDH8 mRNA is decreased in the SN of PD patients compared to controls[17, 20].
rs4793039	6.46E-05	17q21.2	***FAM134C***	intronic	-	-
rs11780980	6.53E-05	8p23.1	*C8orf12*	intronic	-	-
rs11780980	6.53E-05	8p23.1	*FAM167A*	intronic	-	-
rs11145739	6.70E-05	9q21.2	*CEP78*	43 kb upstream	-	-
rs2270822	6.98E-05	5q12.1	*SMIM15*	739 bp upstream	-	-
rs2270822	6.98E-05	5q12.1	***NDUFAF2***	10 kb downstream	-	-
rs7402147	7.04E-05	15q12	*GABRG3*	intronic	-	GABRG3 mRNA is decreased in the SN of PD patients compared to controls[20].
rs10934878	7.08E-05	3q21.3	*HMCES*	intronic	-	HMCES mRNA is decreased in the SN of female PD patients compared to controls[23].
rs2242656	7.12E-05	6p21.33	***BAG6***	intronic	-	BAG6 mRNA is decreased in the blood of PD patients compared to controls[15].
rs2395157	7.12E-05	6p21.32	*C6orf10*	8 kb upstream	-	-
rs1266071	7.18E-05	6p21.33	***BAT5***	intronic	-	-
rs1266071	7.18E-05	6p21.33	*LY6G6E*	intronic	-	-
rs1901632	7.20E-05	10p15.1	*AKR1E2*	16 kb upstream	-	-
rs7320516	7.27E-05	13q13.3	*PROSER1*	31 kb upstream	-	-
rs7320516	7.27E-05	13q13.3	*NHLRC3*	18 kb downstream	-	-
rs6576808	7.33E-05	1p22.3	*COL24A1*	16 kb upstream	-	-
rs7147286	7.55E-05	14q22.2	***GCH1***	intronic	-	-
rs6905949	7.58E-05	6p22.1	*TRIM10*	12 kb upstream	-	-

rs6905949	7.58E-05	6p22.1	*TRIM15*	52 bp downstream	-	-
rs1406773	8.24E-05	3q22.1	***CPNE4***	intronic	-	-
rs4255064	8.44E-05	7q22.1	*KPNA7*	75 kb upstream	-	-
rs2721819	8.47E-05	7p15.3	*DFNA5*	3' UTR	-	-
rs3132453	8.76E-05	6p21.33	***BAT2***	non-syn coding	-	-
rs17632029	8.92E-05	10p13	***FRMD4A***	intronic	-	-
rs6873137	9.01E-05	5q33.3	***HAVCR1***	22 kb downstream	-	HAVCR1 mRNA is decreased in the SN and increased in the striatum of PD patients compared to controls[20].
rs6873137	9.01E-05	5q33.3	***TIMD4***	44 kb upstream	-	TIMD4 mRNA is increased in the striatum of PD patients compared to controls[20].
rs979316	9.14E-05	20q13.31	***BMP7***	87 kb downstream	-	BMP7 mRNA is decreased in the blood of PD patients compared to controls[15].
rs6464536	9.27E-05	7q34	*KEL*	30 kb upstream	-	KEL mRNA is increased in the SN of PD patients compared to controls[18].
rs6464536	9.27E-05	7q34	*OR9A2*	34 kb downstream	-	-
rs10928374	9.31E-05	2q23.1	*MBD5*	intronic	-	-
rs10211158	9.40E-05	2q14.3	*GYPC*	72 kb upstream	-	-
rs11174631	9.64E-05	12q12	***SLC2A13***	intronic	An interaction between the SNP rs2896905 in SLC2A13 and caffeine intake or smoking and caffeine intake combined is associated with PD risk i.e., high caffeine intake reduces PD risk (OR=0.81, P=0.049), never smokers with low caffeine intake have a higher PD risk (OR=1.35, P=0.04) and smokers with high caffeine intake have a lower PD risk (OR=0.68, P=0.007)[53].	SLC2A13 mRNA is decreased in the SN of PD patients compared to controls[18].
rs7297212	9.89E-05	12p13.31	***NTF3***	intronic	-	In the PD SN increased numbers of NTF3 immunoresponsive ramified glia cells surrounded fragmented neurons[109].
rs4072739	9.96E-05	17p11.2	*LRRC48*	intronic	-	-
GWAS 13 (Liu et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence**	
					Genetic studies	**Expression and immunohistological studies** [2]
rs151358	2.24E-06	20q13.32	*SLMO2*	3' UTR	-	-
rs1879512	3.04E-06	3q13.2	*SLC9A10*	81 kb upstream	-	-
rs1916642	3.41E-06	5q13.2	*TMEM171*	25 kb downstream	-	TMEM171 mRNA is decreased in the striatum of PD patients compared to controls[20].
rs1916642	3.41E-06	5q13.2	*TMEM174*	16 kb upstream	-	-
rs12613026	6.49E-06	2p21	*HAAO*	intronic	-	-
rs1684524	7.74E-06	3p24.3	*ZNF385D*	intronic	-	ZNF385D mRNA is decreased in the SN of PD patients compared to controls[18].
rs10999501	9.75E-06	10q22.1	***ADAMTS14***	intronic	-	ADAMTS14 mRNA is decreased in the SN of PD patients compared to controls[20].
rs11661054	1.01E-05	18q23	***MBP***	intronic	-	MBP mRNA is increased in the SN[70,108] and MBP mRNA[42] and protein[20] are decreased in the striatum of PD patients compared to controls. Serum IgM autoantibodies against MBP are increased in PD patients (P<0.0001) and increase during disease progression (P<0.05)[110].
rs7464066	1.35E-05	8q24.3	*ZFP41*	intronic	-	-

rs8030609	1.78E-05	15q13.3	*TMCO5B*	4 kb upstream	-	-
rs9867544	3.08E-05	3q13.2	***CD200***	intronic	-	CD200 mRNA is decreased in the striatum[46] and BA9[46] of PD patients compared to controls. CD200 protein is decreased in the SN[26] and CSF[16] of PD patients compared to controls.
rs10415765	3.16E-05	19p13.11	***LRRC25***	54 kb downstream	-	-
rs10415765	3.16E-05	19p13.11	*PGPEP1*	3 kb upstream	-	-
rs7171137	3.79E-05	15q26.1	***SLCO3A1***	intronic	-	SLCO3A1 mRNA is increased in the SN[18, 21] and blood[15] of PD patients compared to controls.
rs12469652	3.95E-05	2p22.2	*VIT*	intronic	-	-
rs12734001	4.03E-05	1q32.1	***PPP1R12B***	Intronic	- (Part of the PP1-complex).	PPP1R12B mRNA is decreased in the SN of female PD patients compared to controls[21] and is increased in the blood of PD patients compared to controls[15].
rs225376	4.30E-05	21q22.3	***ABCG1***	intronic	-	ABCG1 protein is increased in the CSF of PD patients compared to controls[16].
rs7129006	4.61E-05	11p14.3	***GAS2***	intronic	-	-
rs2186580	4.69E-05	11q22.1	***TRPC6***	70 kb upstream	-	TRPC6 mRNA is decreased in the blood of PD patients compared to controls[24].
rs10121009	5.32E-05	9p13.3	***UNC13B***	intronic	-	-
rs3808386	5.37E-05	8q22.1	***MTERFD1***	intronic	-	-
rs2183593	5.43E-05	21q22.3	***COL18A1***	33 kb upstream	-	COL18A1 mRNA is increased in the SN[18] and COL18A1 protein is decreased in the CSF[16] of PD compared to controls.
rs4745122	5.48E-05	9q21.13	*ABHD17B*	85 kb downstream	-	ABHD17B mRNA is increased in the SN of PD patients compared to controls[18].
rs4745122	5.48E-05	9q21.13	***TMEM2***	9 kb upstream	-	TMEM2 mRNA is increased in the SN of PD patients compared to controls[18].
rs2843518	5.63E-05	Xq25	*TENM1*	intronic	-	-
rs1194491	5.64E-05	10q21.1	***PRKG1***	intronic	-	-
rs2266920	5.76E-05	7p22.3	*AMZ1*	intronic	-	-
rs2266920	5.76E-05	7p22.3	***GNA12***	intronic	-	-
rs2158133	6.19E-05	12q13.11	***AMIGO2***	19 kb downstream	-	-
rs2158133	6.19E-05	12q13.11	*PCED1B*	23 kb upstream	-	-
rs4976493	6.66E-05	5q31.1	*SLC25A48*	intronic	-	SLC25A48 mRNA is increased in the striatum[20] and BA9[51] of PD patients compared to controls.
rs183211	7.16E-05	17q21.31	***NSF***	intronic	-	NSF mRNA is decreased in the SN[17, 18, 22, 23, 46-48] and striatum[46] of PD patients compared to controls.
rs11714053	7.98E-05	3q22.1	***CPNE4***	intronic	-	-
rs415430	8.45E-05	17q21.31	***WNT3***	intronic	-	WNT3 mRNA is decreased in the SN of PD patients compared to controls[18].
rs4678649	8.54E-05	3p22.3	***CCR4***	19 kb downstream	-	-
rs12985786	8.86E-05	19p13.2	***CACNA1A***	intronic	-	CACNA1A protein is increased in the CSF of PD patients compared to controls[16].
rs13003114	8.92E-05	2q31.2	***ZNF385B***	intronic	-	-

[1] Genetic position according to the Ensembl Human Genome Browser (http://www.ensembl.org/Homo_sapiens/).
[2] Expression data from genome wide expression studies in PD-associated brain areas (SN, striatum, BA9), CSF or blood were included if they met the following criteria; *with* an adjusted p-value for multiple comparisons <0.05, mRNAs should have a fold change of <-1.5 or >1.5 and proteins <-1.2 or >1.2, and *without* a published adjusted p-value, mRNAs and proteins were only included with a p-value <0.05 and a fold change of <-2.0 or >2.0 and of <-1.5 or >1.5 respectively.

Supplementary Table 3. Network enrichment analysis of the PD GWAS candidate genes from **Supplementary Table 2** using Ingenuity pathway software (www.ingenuity.com). Separate networks of interacting proteins were generated and ranked by their enrichment score (see [1]). Network 1, the network with the highest score and also containing the highest number of molecules/proteins, is shown in **Supplementary Figure 1**. The five most significantly enriched networks are shown. In addition, the PD GWAS genes encoding proteins that could be directly placed in the molecular PD landscape are indicated in bold.

Rank	PD GWAS genes in network	Score [1]	*P* [1]
1	**ABCG1**, **ATF6**, **ATP6V0A1**, **CCAR2**, **CDK19**, **CENPC**, CEP152, **CUL2**, **DCUN1D1**, **DDX3X**, **DGKQ**, **DLG2**, **FRMD4A**, **KIF11**, **LIPC**, **MCC**, **MED13**, **PARD3**, **PCM1**, PKDCC, **PLOD1**, **RPL38**, **RPL7A**, **SREBF1**, **TIAL1**, **TMCC3**, **TMX1**, **ULK2**	44	1.00E-44
2	**ARL4A**, **ASAH1**, **AXIN1**, **BTNL2**, **EIF4EBP2**, **GJB2**, **HLCS**, **HPGD**, **JARID2**, **KCNA5**, **MSX1**, **NTF3**, **PROK2**, **ROBO2**, **RUNX3**, **SEMA6D**, SLC22A16, **SP110**, SP140, **TBX3**, **TCF12**, **TLE1**, **TRPC6**, **WNT3**	37	1.00E-37
3	**ADAMTS2**, **ADAMTS14**, **BMP7**, **CD200**, **CNTN1**, **COL18A1**, **COL2A1**, **ENSA**, EXT1, **FGF12**, **GAK**, **ITGA6**, **ITGA8**, **LAMP1**, **LRP1B**, **NLGN1**, **RAP2A**, **RER1**, **SCN2A**, **SDC1**, **SERPINB5**, **SERPING1**, **SMAD5**	35	1.00E-35
4	**CYP17A1**, **DSCAM**, **FBN1**, **GNA12**, **SIK1**, **LRRK2**, **LTBP1**, **MAP2K6**, **MBP**, MYO15A, **PIK3CD**, **PPM1L**, **PRKAA1**, **PSMD11**, **SEMA3E**, **SND1**, **SNRK**, **STK39**, **WNK1**, XIRP2, **ZAK**	29	1.00E-29
5	**AGAP1**, **AMIGO2**, **AP3B1**, **CPNE4**, **GAS2**, **ITGA2B**, **ITGAL**, **KPNA4**, **MAP1B**, **MYO1E**, **OCA2**, **PPP1R12B**, **PRKG1**, **RAP1A**, **SCIN**, SYT17, **TPO**, **TRAPPC9**, **VPS41**, ZNF207	29	1.00E-29

[1] This score takes into account the number of eligible molecules/proteins in the network and its size, as well as the total number of network-eligible molecules analyzed and the total number of molecules in the Ingenuity Knowledge Base that could potentially be included in networks. The score is calculated with the right-tailed Fisher's exact test and displayed as the negative logarithm of the Fisher's exact test result.

Supplementary Table 4. Additional genes that encode proteins located in the PD landscape. If these genes or their protein products are linked to PD, either by genetic or by expression and immunohistological studies (in PD patients), this is shown in the column 'Corroborating evidence'. Single underlined genes are genetically associated with PD, dotted underlined genes encode proteins that are differentially expressed in PD patients and double underlined genes are both genetically associated with PD and encode a protein that is differentially expressed in PD.

Gene	Locus	Corroborating evidence	
		Genetic studies	**Expression and immunohistological studies** [1]
ABCA1	9q31.1	-	ABCA1 protein is increased in CSF of PD patients compared to controls[111].
ADH1C	4q23	A mutation in *ADH1C* c.232G>T (rs283413), introducing a stop codon, increases the risk for PD (OR=3.25, P=0.007)[112].	-
AICDA	12p13.31	-	-
AKT1	14q32.33	-	Levels of total and Ser473-phosphorylated AKT1 is lower in the PD brain, but are increased in glia cells in the SN of PD patients[113].
AKT1S1	19q13.33	- (Part of the mTORC1-complex).	-
APOE	19q13.32	In Thai PD patients the ApoE-ε2 allele shows an increased risk (OR=2.309)[114], in non-Hispanic Caucasians PD patients the ApoE-ε4 allele shows a lower risk (OR=0.75)[115], whereas in mexican PD patients the ApoE-ε3 allele shows a protective risk effect (OR=0.36) and the ApoE-ε4 allele shows an increased risk for PD (OR=2.57)[116].	APOE protein is higher expressed in melanized neurons of the SN of early PD patients[117], higher expressed in the striatum of PD patients[20] and dysregulated in the CSF of PD patients compared to controls[16, 49, 118].
AR	Xq12	-	AR mRNA is increased in the striatum[20] and decreased in the SN of PD patients compared to controls[18].
ASCL1	4q35.1	Polyglutamine length variants in the MASH1 gene are associated with PD risk[119].	ASCL1 mRNA is increased in the straitum of PD patients compared to controls[42].
ATG5	6q21	A variant in the *ATG5* promotor (106774459T>A) enhancing its transcriptional activity (P<0.01) was identified in one patient, but not in controls[120].	ATG5 mRNA expression is increased in nucleated blood cells[15] and in leukocytes[120] of PD patients compared to controls.
ATG7	3p25.3	Four variants (11313449G>A, 11313811T>C, 11313913G>A and 11314041G>A) that decrease the transcriptional activity of the *ATG7* gene promoter (P<0.01) were identified in PD patients, but not in the controls[121].	ATG7 mRNA expression is decreased in the SN of PD patients compared to controls[18, 122].
ATP13A2	1p36.13	*ATP13A2* missense mutations c.35C>T (Thr12Met) and c.1597G>A (Gly533Arg) were found in Italian young onset PD patients[123] and the missense mutation c.2236G>A (Ala746Thr) was found to be more frequent in Chinese PD patients (Relative risk=4.3, P=0.01)[124].	-
BAK1	6p21.31	-	-
BAX	19q13.33	-	PD patients show increased BAX immunoreactivity in neuromelanin containing neurons[125] and differences in aggregation of BAX-rich inclusions[126].
BDNF	11p14.1	The *BDNF* polymorphisms C270T (in familial PD)[127] and G196A (Val66Met; cognitive impairment in PD)[128, 129] are associated with PD.	BDNF protein expression is decreased in neurons of the SN[130], decreased[131] or increased[132] in the serum and decreased[49] or increased[133] in the CSF of PD patients. BDNF serum levels have been shown to positively correlate with PD motor impairment[131]. BDNF mRNA is decreased in the striatum of PD patients compared to controls[48].
CACNA1B	9q34.3	-	-
CACNA1C	12p13.33	-	The number of CACNA1C-positive stained cells in the SN is lower in PD patients than in controls[134].
CACNA1D	3p21.1	-	CACNA1D is higher expressed in the PD SN than in controls[135]. The number of CACNA1D-positive stained cells in the SN is lower in PD patients, but their intensity is higher than in controls[134].
CACNA1E	1q25.3	-	CACNA1E mRNA is increased in the striatum[19] and decreased in the SN[18] of PD patients compared to controls.
CACNA1F	Xp11.23	-	-

CACNA1S	1q32.1	-	CACNA1S mRNA is increased in the striatum of PD patients compared to controls[20].
CASP3	4q35.1	-	CASP3 expression is increased in the SN of PD patients[125, 136].
CASP9	1p36.21	-	CASP9 protein is activated in the SN of PD brains[137] and immunologically detectable within TH+ SN neurons of late-onset sporadic PD patients[138].
CAV1	7q31.2	Motif lengths in *CAV1* haplotypes that increase CAV1 expression were found in PD patients, but not in controls (P<0.002)[139].	CAV1 mRNA is increased in the SN[18] and striatum[42] of PD patients compared to controls.
CCL5	17q12	-	CCL5 protein expression is increased in the circulation of PD patients (P<0.001)[140-142]. CCL5 is alternatively spliced in the blood of PD patients compared to controls[75].
CD163	12p13.31	-	The number of CD163-positive microglia is increased in the SN of PD patients[143]. CD163 mRNA is increased in the striatum[20] and the blood[24] of PD patients compared to controls.
CD200R1	3q13.2	-	Induction of CD200R expression on monocyte-derived macrophages is reduced in PD patients[144].
CDH2	18q12.1	-	CDH2 protein is increased in the striatum of PD patients compared to controls[20].
COMT	22q11.21	The *COMT* missense variant rs4680 (Val158Met) lowers enzyme activity and is associated with an increased risk of PD (AA compared to GG genotype; OR = 1.86, P=0.044)[145] and associated with age of onset in males (P=0.007)[146].	COMT mRNA is decreased in the blood of PD patients compared to controls[15].
CRH	8q13.1	-	-
CTNNB1	3p22.1	-	-
CTSD	11p15.5	-	CTSD protein is lower expressed in PD SN neurons[33], but is increased in the CSF of PD patients[16] compared to controls.
CXCL12	10q11.21	-	CXCL12 protein expression is increased in the SN of PD patients[79] and CXCL12 mRNA expression is increased in the striatum[42] and blood of PD patients[24] compared to controls.
CYCS	7p15.2	-	CYCS mRNA is decreased in the SN of PD patients compared to controls[23].
DDIT4	10q22.1	-	DDIT4 protein expression is increased in the SN of PD patients[147] and DDIT4 mRNA is increased[46] or decreased[23] in the SN and increased[20, 46] in the striatum and BA9[46] of PD patients compared to controls.
DEPTOR	8q24.12	- (Part of the mTORC1- and mTORC2-complex).	-
DNAJC13	3q22.1	Missense variant rs387907571 (Asn855Ser) in the *DNAJC13* gene increases late-onset PD[148].	DNAJC13 mRNA is decreased in the blood of PD patients compared to controls[15].
DNAJC6	1p31.3	The splice acceptor variant rs398122404 (c.801-2A>G) in *DNAJC6*, decreases its expression and is associated with juvenile parkinsonism[149].	DNAJC6 mRNA is decreased in the SN of PD patients compared to controls[23].
DNM2	19p13.2	-	DNM2 mRNA is increased in the SN of PD patients compared to controls[18].
DRD2	11q23.2	The *DRD2* Taq1A SNP (rs1800497) is associated with PD (P=0.025; OR=2.2)[150]. Among non-Hispanic whites, homozygous carriers of this SNP have an increased risk of PD compared to homozygous wildtype carriers (OR=1.5), whereas African-Americans, showed an inverse association with PD risk (OR=0.10)[151].	DRD2 mRNA is decreased in the SN of PD patients compared to controls[18, 20].
DRD3	3q13.31	In white Hispanics homozygeous carriers of the *DRD3* missense variant rs6280 (Ser9Gly) were associated with a decreased risk of PD (OR=0.4)[151].	-
E2F1	20q11.22	-	DA SN neurons show high cytoplasmic E2F1 protein expression compared to controls[152].
EIF2A	3q25.1	-	-
EIF4E	4q23	-	-
EIF4G1	3q27.1	The c.3614G>A (Arg1205His) mutation in the *EIF4G1* gene segregates with disease in families with PD and the mutations c.1505C>T (p.Ala502Val) and c.2056G>T	-

		(p.Gly686Cys) were found in PD patients but not in controls. The c.3614G>A and c.1505C>T mutations impair EIF4G1 protein function[153].	
FBXO7	22q12.3	The homozygous mutation c.1132C>G (Arg378Gly) in *FBXO7* is associated with parkinsonian-pyramidal syndrome[154]. In an Italian family a *FBXO7* homozygous truncating mutation (Arg498Stop) was found in an Italian family, while in a Dutch family a splice-site mutation (IVS7 + 1G/T) and a missense mutation (Thr22Met) were associated with early-onset parkinsonian-pyramidal syndrome[155].	FBXO7 mRNA is decreased in the SN of female PD patients compared to female controls[23].
FOXO1	13q14.11	-	FOXO1 expression is increased in the BA9 of PD patients compared to controls[51].
GDNF	5p13.2	-	GDNF expression is decreased in neurons of the SN of PD patients (P<0.0001)[130].
GPR37	7q31.33	-	GPR37 accumulates in PD lewy bodies[156] and in the brains of juvenile Parkinsonism patients, and may cause DA neuron death[157]. GPR37 mRNA is increased in the SN of female PD patients compared to female controls[21].
GSK3B	3q13.33	The *GSK3B* polymorphisms rs334558 and rs6438552 are associated to PD and can alter *GSK3B* transcription and splicing respectively[158].	GSK3B protein is localized in lewy bodies[159] and the active form of GSK3B (phosphorylated on Tyr216) is elevated in the striatum of PD patients compared to controls[160]. GSK3B mRNA is decreased in the SN of PD patients compared to controls[18].
HBA1	16p13.3	- (Part of the hemoglobin complex).	After adjustment for age and concomitant risk factors, PD incidence was increased from 10.3 to 34.9/10,000 person-years as Hb increased from <14 to ≥16 g/dL (p=0.022)[161]. The SN of PD brains shows that the few remaining nigral neurons have increased quantity of HBA containing mitochondria compared to controls, however, reduced mitochondrial/cytoplasmic ratios are suggested for HBA (P=0.06)[162]. HBA1 mRNA is decreased in the SN[23] and HBA1 protein is decreased in the striatum[20], but increased in the CSF[16] of PD patients compared to controls.
HBA2	16p13.3	- (Part of the hemoglobin complex).	HBA2 mRNA is decreased in the blood[15] and HBA2 protein is increased in the CSF[16] of PD patients compared to controls.
HBB	11p15.4	- (Part of the hemoglobin complex).	After adjustment for age and concomitant risk factors, PD incidence was increased from 10.3 to 34.9/10,000 person-years as Hb increased from <14 to ≥16 g/dL (p=0.022)[161]. The SN of PD brains shows that the few remaining nigral neurons have increased quantity of HBA containing mitochondria compared to controls, however, mitochondrial/cytoplasmic ratios are reduced for HBB in PD SN neurons (P=0.038)[162]. HBB protein is increased[108] or decreased in the SN[20] and decreased in the striatum[20] of PD patients compared to controls. HBB protein is increased in the SN[135] and blood[163] and increased[16] or decreased in the CSF[164] of PD patients compared to controls.
HBD	11p15.4	- (Part of the hemoglobin complex).	HBD mRNA is decreased in the striatum[20] and blood[165] of PD patients compared to controls. HBD protein is decreased in the striatum of PD patients compared to controls[20].
HBE1	11p15.4	- (Part of the hemoglobin complex).	-
HBG1	11p15.4	- (Part of the hemoglobin complex).	HBG1 mRNA is decreased in the SN[20] and the blood[24] of PD patients compared to controls.
HBG2	11p15.4	- (Part of the hemoglobin complex).	-
HBM	16p13.3	- (Part of the hemoglobin complex).	HBM mRNA is decreased in the striatum[20] and blood[24,165] of PD patients compared to controls.
HBQ1	16p13.3	- (Part of the hemoglobin complex).	HBQ1 mRNA is decreased in the SN of PD patients compared to controls[18].
HBZ	16p13.3	- (Part of the hemoglobin complex).	-
HIP1R	12q24.31	The SNP rs10847864 in *HIP1R* is associated with an increased risk for PD (OR=1.15; P=9.06E-07)[77].	HIP1R mRNA is increased in the SN of PD patients compared to controls[17].

HK2	2p12	-	HK2 mRNA is increased in the striatum of PD patients compared to controls[20].
HMOX1	22q12.3	-	Lewy bodies in PD SN neurons exhibit intense HMOX1 staining in their peripheries[166] and the number of HMOX1-positive astroglia is increased in the PD SN[166]. Further, HMOX1 is increased in the serum of PD patients[167].
HMOX2	16p13.3	The homozygous G/G genotype of SNP rs2270363 in *HMOX2* is associated with PD (OR=1.38, P=0.015)[168].	-
HP	16q22.2	The Hp 2-1 genotype is associated with increased PD risk in female, moreover in never-smokers (adjusted for gender) the Hp 2-1 and Hp 1-1 genotypes were associated with an increased PD risk (OR=1.79 and 1.62 respectively) (P=0.034)[169].	HP protein is increased in the striatum[20], decreased in the blood[66, 170] and decreased[118] or increased[16, 111] in the CSF of PD patients compared to controls.
HSPA5	9q33.3	-	HSPA5 mRNA and protein are increased in the SN[108] and striatum[20] respectively of PD patients compared to controls.
HSPA8	11q24.1	-	HSPA8 mRNA is decreased in the (male) SN[22, 23, 69] and blood[75] of PD patients compared to controls. HSPA8 protein is increased in the striatum[20] and decreased in the SN[171] and CSF[16] of PD patients compared to controls.
HTRA2	2p13.1	The loss of function mutation c.1195G>A (Gly399Ser) in *HTRA2* was identified in PD patients and another mutation c.421G>T (Ala141Ser) was associated with PD risk in Germans (OR=2.15, P=0.039)[172]. The loss of function mutation c.1210C>T (Arg404Trp) in *HTRA2* and six patient-specific variants that might affect *HTRA2* expression were identified in Belgians[173]. The c.427C>G (Pro143Ala) mutation in *HTRA2* induces its hyperphosphorylation in mitochondria and is associated to PD susceptibility in Taiwanese (Relative risk=2.3; P = 0.04)[174]. In Han Chinese the *HTRA2* IVS5+29T>A variant may be a risk factor for PD (OR=7.53, P<0.05)[175].	HTRA2 is a component of LB in the PD brain[172].
ICAM1	19p13.2	-	ICAM1 expression is increased in astroglia cells in the SN of PD patients[176] and activated, ICAM1 positive, microglia are increased in the SN and striatum of PD patients[50]. Plasma ICAM1 protein levels are increased in stage 1 and 2, but not stage 3 and 4 PD patients compared to controls[177].
IFNG	12q15	-	IFNG protein serum levels[178, 179] and IFNG mRNA[180] are increased in the blood of PD patients compared to controls.
IL10	1q32.1	-	IL10 is increased in the serum PD patients[179, 181], whereas in the SN of PD patients IL10 mRNA is decreased (P<0.01)[182].
IL12A	3q24.33	- (Part of the IL12-complex).	IL12A mRNA is increased in the blood of PD patients compared to controls[15].
IL12B	5q33.3	- (Part of the IL12-complex).	-
IL1B	2q14.1	The *IL1B* (-511) allele (rs16944) is associated with PD in Finish patients[183].	IL1B protein is increased in the CSF[184, 185] and peripheral blood mononuclear cells[141], whereas IL1B mRNA is decreased in the SN[20, 182] and increased in the striatum[20] of PD patients compared to controls.
IL2	4q27	-	IL2 is increased in the serum and CSF of PD patients[179, 185, 186].
IL4	5q31.1	-	IL4 is increased in the serum and CSF of PD patients[179, 185].
IL5	5q31.1	-	-
IL6	7p15.3	The SNP rs1800795 in the promotor of *IL6* is associated with PD in Ashkenazi Jews and caucasians[187].	IL6 is increased in the serum and CSF in PD patients[179, 184], whereas in the SN of PD patients IL6 mRNA is decreased (P<0.01)[182].
IL8	4q13.3	Different genotypes of the SNP c.251A>T in the gene promotor of *IL8* are associated with PD in Irish patients[188].	IL8 is increased in the blood of PD patients[141].
INS	11p15.5	-	The autoimmune reaction towards serum insulin (INS) is increased by 70% in PD patients[189].
INSR	12p13.2	-	INSR protein and mRNA is decreased in the PD SN[190, 191] and INSR protein is increased in the CSF of PD patients[16] compared to controls.
ITGB2	21q22.3	- (Part of the LFA-1 complex).	ITGB2 mRNA expression is increased in the angular cortex in PD from Braak stage 3 onwards[182] and is increased in the striatum of PD patients compared to controls[20].

JAK2	9p24.1	-	-
LAMP2	Xq24	-	PD patients show a decreased LAMP2 protein expression in the SN[171], in peripheral leukocytes[192] and in CSF[16] compared to controls, whereas LAMP2 mRNA is increased in the SN[18] and blood[15] and decreased in peripheral leukocytes[192] of PD patients compared to controls.
LDLR	19p13.2	-	-
LMX1A	1q23.3	-	LMX1A mRNA is decreased in the SN of PD patients compared to controls[20].
LMX1B	9q33.3	-	LMX1B protein is decreased in midbrain DA neurons of PD patients compared to controls (P<0.0005)[193].
LRP1	12q13.3	-	LRP1 expression in melanized neurons of the SN is increased early in PD[117].
LRPPRC	2p21	-	LRPPRC mRNA is decreased in the SN of PD patients compared to controls[17, 22, 23].
LXRA	11p11.2	- (LXR isoform).	-
LXRB	19q13.33	- (LXR isoform).	-
MAOA	Xp11.3	The variation c.941T>G (rs1799835) in *MAOA* is associated with early onset PD[194] and *MAOA* variations seem to be more associated with the male gender in PD patients[194-196].	MAOA mRNA is increased in the SN of PD patients compared to controls[122].
MAOB	Xp11.3	A GT repeat variation (> or =188 bp) in *MAOB* is associated with PD (OR=4.60, P<0.00005)[197]. The synonymous SNP rs1799836 (G>A) in *MAOB* is associated with PD in females[198], but also with an overal increased PD risk (AA vs AG/GG OR=1.70, P=0.016)[145].	MAOB mRNA is increased in the striatum of PD patients compared to controls[19, 20].
MAP3K7	6q15	-	-
MAPKAP1	9q33.3	- (Part of the mTORC2-complex).	-
MARK2	11q13.1	-	-
MC1R	16q24.3	-	MC1R mRNA is decreased in the SN of male PD patients compared to controls[23]. MC1R protein is upregulated in the striatum of PD patients compared to controls[20].
MITF	3p13	-	-
MLST8	16p13.3	- (Part of the mTORC1- and mTORC2-complex).	-
MTOR	1p36.22	- (Part of the mTORC1- and mTORC2-complex).	MTOR protein is increased in the CSF of PD patients compared to controls[16].
NDEL1	17p13.1	-	-
NEDD4	15q21.3	-	-
NEDD8	14q12	-	NEDD8 increases PARK2 activity and stabilizes PINK1 and its accumulation is observed in LB in DA neurons in the SN of PD patients[199, 200].
NEUROG2	4q25	-	-
NFKB1	4q24	- (Part of the NF-KB complex).	NFKB1 mRNA is increased in the SN of PD patients compared to controls[69]. NFKB levels are elevated in the striatum (P<0.05) and SN of PD patients (P<0.01)[201].
NFKB2	10q24.32	- (Part of the NF-KB complex).	NFKB levels are elevated in the striatum (P<0.05) and SN of PD patients (P<0.01)[201].
NOS1	12q24.22	The SNP in exon 29 of *NOS1* is associated with PD (OR for T allele carriers=1.53, P=0.02)[202]. Further, the SNPs rs3782218, rs11068447, rs7295972, rs2293052, rs12829185, rs1047735, rs3741475, and rs2682826 in *NOS1* were associated with early-onset PD families (range of P=0.00083-0.046)[203].	-
NOS2	17q11.2	The SNP in exon 22 of *NOS2* is associated with PD (OR for AA carriers=0.50, P=0.01)[202]. Further, the SNPs rs2072324, rs944725, rs12944039, rs2248814, rs2297516, rs1060826, and rs2255929 in *NOS2* were associated with early-onset PD families (range of P=0.0000040-0.047)[203].	-

NR4A2	2q24.1	Homozygous insertion of a single nucleotide (7048G7049) in intron 6 of the *NR4A2* gene is associated with PD (OR=8.4, P<0.005)[204]. The 3G/2G genotype of the IVS6+ 18insG polymorphism is associated with early-onset PD (OR=1.91, P=0.011)[205]. Three variations in the 5UTR of *NR4A2* (c.-309C>T, c.-291Tdel, c.-245T>G) are associated with PD and decrease *NR4A2* expression[206, 207].	NR4A2 mRNA is decreased in the SN[17, 18, 20] and peripheral blood lymphocytes[208, 209] of PD patients compared to controls. NR4A2 protein is decreased in SNCA-positive nigral neurons of PD patients compared to controls[210].
NR5A1	9q33.3	-	-
PARK7	1p36.23	Exon deletions and duplications and a c.497T>C (Leu166Pro) mutation of the *PARK7* gene are associated with autosomal recessive early-onset PD [211, 212].	PARK7 mRNA is decreased in (male) PD patients compared to controls[22, 23]. PARK7 protein is decreased in CSF of PD patients compared to controls[16].
PINK1	1p36.12	Exon deletions and multiple mutations in *PINK1* are associated with early-onset PD[213-217].	PINK1 mRNA is decreased in the SN of PD patients compared to controls[22, 23].
PITX3	10q24.32	The *PITX3* promoter SNP rs3758549 (C>T) is associated with PD susceptibility (OR=1.42, P=0.004)[218]. In a meta-analysis the A allele of the SNP rs4919621 was significantly associated with increased risk of PD in a Caucasian population (OR=1.15, P=0.04) and both the C allele of rs2281983 (OR=1.62, P=0.0001) and the A allele of rs4919621 (OR=1.70, P<0.0001) are associated with early-onset PD[219]. Another meta-analysis showed association of the *PITX3* SNP rs3758549 with PD risk (OR=1.21, P=0.019) and early-onset PD in an Asian population (OR=1.44, P=0.004)[220]. The T allele of the SNP rs3758549 is associated with PD in the Asian population (P=0.019) and early-onset PD (P=0.004)[220].	PITX3 mRNA is decreased in the SN[18] and peripheral blood lymphocytes[209] of PD patients compared to controls.
PLA2G6	22q13.1	The mutations c.2222G>A (Arg741Gln) and c.2239C>T (Arg747Trp) in the *PLA2G6* gene are associated with dystonia-parkinsonism[221]. The nonsyn SNPs c.2339A>G (Asn780Ser) and c.2341G>A (Ala781Thr) were found in patients with sporadic early-onset PD but not in controls[222]. The SNP c.1959T>A, two nonsyn c.1966C>G (Leu656Val) and c.2077C>G (Leu693Val) and a frameshift (His597fx69) were identified in Chines PD patients but not in controls. The two nonsyn SNPs and the frameshift reduce PLA2G6 enzymatic activity[223].	-
PLAT	8p11.21	-	-
PLAU	10q22.2	-	-
PLAUR	19q13.31	-	-
PLG	6q26	- (Proenzym of plasmin).	PLG protein is increased in the blood of PD patients compared to controls[66].
PPP1CA	11q13.2	- (Part of the PP1-complex).	-
PPP1CB	2p23.2	- (Part of the PP1-complex).	-
PPP1CC	12q24.11	- (Part of the PP1-complex).	-
PPP1R12A	12q21.31	- (Part of the PP1-complex).	PPP1R12A is alternatively spliced in the blood of PD patients compared to controls[75]. PPP1R12A protein is decreased in the CSF of PD patients[224].
PRKAA2	1p32.2	- (Part of the AMPK-complex).	-
PRKAB1	12q24.23	- (Part of the AMPK-complex).	-
PRKAB2	1q21.1	- (Part of the AMPK-complex).	-
PRKAG1	12q13.12	- (Part of the AMPK-complex).	-
PRKAG2	7q36.1	- (Part of the AMPK-complex).	PRKAG2 mRNA is increased in the SN of female PD patients compared to controls[21].
PRKAG3	2q35	- (Part of the AMPK-complex).	-
PRR5	22q13.31	- (Part of the mTORC2-complex).	-
PSAP	10q22.1	-	PSAP mRNA is increased[48] or decreased[22, 23, 69] in the SN of PD patients compared to controls. PSAP is alternatively spliced in the blood of PD patients compared to controls[75].
PSEN1	14q24.2	-	PSEN1 mRNA is increased in the SN of PD patients compared to controls[18, 22, 23].
PSEN2	1q42.13	-	PSEN2 mRNA is decreased in the SN of PD patients compared to controls[18].

PTEN	10q23.31	-	PTEN is alternatively spliced in the blood of PD patients compared to controls[75].
RAC1	7p22.1	-	RAC1 mRNA is decreased in the SN of (male) PD patients compared to controls[22, 23].
REL	2p16.1	- (Part of the NF-KB-complex).	NFKB levels are elevated in the striatum (P<0.05) and SN of PD patients (P<0.01)[201].
RELA	11q13.1	- (Part of the NF-KB-complex).	RELA mRNA is increased in the SN of PD patients compared to controls[18]. NFKB levels are elevated in the striatum (P<0.05) and SN of PD patients (P<0.01)[201] and the number of NFKB (RELA) immunoreactive nuclei of DA neurons in PD patients was increased over 70-fold compared to control subjects[225].
RELB	19q13.32	- (Part of the NF-KB-complex).	NFKB levels are elevated in the striatum (P<0.05) and SN of PD patients (P<0.01)[201].
RFX3	9p24.2	-	RFX3 mRNA is increased in the striatum of PD patients compared to controls[20].
RGMA	15q26.1	-	RGMA mRNA is increased in the SN[17, 69] and striatum[20] of PD patients compared to controls.
RHOA	3p21.31	-	RHOA is alternatively spliced in the blood of PD patients compared to controls[75].
RICTOR	5p13.1	- (Part of the mTORC2-complex).	-
RPTOR	17q25.3	- (Part of the mTORC1-complex).	-
SCARB1	12q24.31	-	-
SERPINB9	6p25.2	-	SERPINB9 mRNA is decreased in the blood of PD patients compared to controls[15].
SERPINE1	7q22.1	-	SERPINE1 mRNA is increased in the SN of PD patients compared to controls[20].
SIRT1	10q21.3	Three novel heterozygous sequence variants in the *SIRT1* promotor, g.69644133C>G, g.69644213G>A and g.69644351G>A were identified in PD patients, but not in controls and may reduce *SIRT1* expression[226].	-
SIRT7	17q25.3	-	SIRT7 mRNA is increased in the SN of PD patients compared to controls[18].
SLC11A2	12q13.12	The C allele of rs150909 in *DMT1* (*SLC11A2*) is associated with 3.09 (95% CI 0.13-6.06) years older age at PD diagnosis (P=0.03)[227].	The DMT1+IRE isoforms are upregulated (P<0.01) whereas the DMT1-IRE isoforms are downregulated in PD SNpc compared to controls (P<0.01)[228].
SLC18A2	10q25.3	Gain of function haplotypes in the *SLC18A2* promotor are associated with lower PD risk in females (homozygeous vs wt OR=0.38, P=0.01)[229]. The SNP rs363371 in the *SLC18A2* promotor is associated with lower PD risk in Italians (dominant model OR=0.72, P=0.03)[230].	SLC18A2 mRNA is decreased in the SN of PD patients compared to controls[17, 18, 20].
SLC40A1	2q32.2	-	SLC40A1 (Ferroportin) is alternatively spliced in the blood of PD patients compared to controls[75] and SLC40A1 protein is 1.4 fold increased in PD SN compared to controls (P<0.05)[231].
SLC6A3	5p15.33	The 11-copy allele of a variable number tandem repeat polymorphism in *SLC6A3* was more frequent present in PD (OR=4.08, P=0.008[232]; OR=2.5, P<0.02[233]). The 10-copy allele of this polymorphism is neuroprotective in East Asians (OR=0.78, P=0.009)[234], whereas the *SLC6A3* promoter SNP rs2652510 (A>G) is associated with increased PD risk in Caucasians (OR=1.26, P=0.018)[234].	SLC6A3 mRNA is decreased in the SN of PD patients compared to controls[18, 20, 48].
SLIT2	4p15.31	-	SLIT2 is decreased in the SN[18, 44] and increased in the blood[24] of PD patients compared to controls.
SMAD4	18q21.2	-	SMAD4 mRNA is increased in the blood of PD patients compared to controls[15].
SMPD1	11p15.4	The *SMPD1* mutations, Arg591Cys (OR=not applicable, P=0.009)[235], Pro533Leu (OR=1.76, P=0.047)[235] and Leu302Pro (OR=9.4, P<0.0001)[236] are associated with an increased PD risk.	-
SOD2	6q25.3	-	SOD2 protein levels are lower in the SN (P<0.05) and striatum (P<0.05) of PD patients, but its activity is nevertheless elevated in the straitum (P<0.05)[237]. SOD2 immunoreactivity is affected in the lateral, but not in the medial and central part of the SN of PD patients[238]. SOD2 protein is increased in the SN[25] and decreased

			in the CSF of PD patients compared to controls[16]. Reactive dopamine quinones reduce enzymatic activity of SOD2 up to 50% by promoting its aggregation[239].
SOX2	3q26.33	-	SOX2 mRNA is increased in the SN of PD patients compared to controls[17, 46].
SPHK1	17q25.1	-	SPHK1 mRNA is increased in the striatum of PD patients compared to controls[20].
STAR	8p11.23	- (Part of the mPTP-complex).	-
STAT3	17q21.2	-	-
STK11	19p13.3	-	-
SYNJ1	21q22.11	Mutation c.773G>A (Arg258Gln) in the *SYNJ1* gene is associated with autosomal recessive, early-onset Parkinsonism in an Italian[240] and Iranian family[241].	SYNJ1 mRNA is decreased in the SN of PD patients compared to controls[18].
SYT11	1q22	A meta-analysis on GWAS data identified *SYT11* (chr1:154105678) as a new risk locus for PD (OR=1.67, P=5,70E-09)[242] and confirmed by another meta-analysis (OR=1,73, P=2.35E-12)[27] and replicated in a Caucasian (OR=1,43, P=0,001)[77] and a Scandinavian PD group (OR=1,46, P=0.011)[104].	SYT11 mRNA is decreased in the SN of PD patients compared to controls[108].
TF	3q22.1	The AT haplotype of the SNPs rs1880669/rs1049296 in transferrin (*TF*) is protective for PD (OR=0.83, 95% CI: 0.71-0.96) (P=0.01)[243].	TF mRNA is increased in the SN[46, 47] and CSF[16, 164], but decreased in the blood[66] of PD patients compared to controls. Also, there is increased oxidized TF in the PD SN[244].
TFR2	7q22.1	-	The number of [125I]-Tf(Fe)2 binding sites, as a measure for the number of TFRs, was lower on the perikarya of melanized neurons in the PD SNpc compared to controls (P=0.01)[245]. TFR2 protein expression is highly specific for DA neurons in the SNpc[244].
TGFBR2	3p24.1	-	TGFBR2 mRNA is decreased in the SN of PD patients compared to controls[21].
TH	11p15.5	-	TH mRNA is decreased in the SN of PD patients compared to controls[18, 20]. TH protein and mRNA is reduced in the SN of PD patients, but protein:mRNA ratio is unaffected[246].
TNF	6p21.33	The homozygous genotype of the c.1031T>C mutation in the *TNF* promotor is associated with PD (OR=3.5, P=0.032[247]; OR=2.96, P=0.0085[248]) and even stronger associated with early-onset PD (OR=5.0, P=0.0039)[247]. Also the homozygous mutation of c.308G>A in the *TNF* promotor is associated with early-onset PD (OR=1.86, P=0.0037)[249].	TNF protein is higher expressed in the striatum and CSF of PD patients[250], whereas its mRNA is lower expressed in the PD SN (P<0.001) and striatum (P<0.05) of PD patients[182]. Furthermore, TNF protein is increased in the serum[179] and peripheral blood mononuclear cells[141] of PD patients.
TP53	17p13.1	-	TP53 protein expression is higher in the caudate nucleus, but not in the SN of PD patients (P<0.05)[201], however, an increase in (Ser15-)phosphorylated TP53 in the PD SN was shown (P<0.001)[251].
TRAF2	9q34.3	-	-
TSPO	22q13.2	-	Increased TSPO expression (measured with positron emission tomography imaging) in the midbrain and striatum of PD patients correlates with PD motor severity[252].
TYR	11q14.3	-	-
UCHL1	4p13	A dominant mutation (Ile93Met) in *UCHL1* is identified in a German PD family[253] and a heterozygeous c.54C>A (Ser18Tyr) mutation is associated with a decreased PD risk (OR=0.55, P<0.05, 10563640; OR=0.54, P=0.033[253]; OR=0.66, P=0.02[254]).	UCHL1 mRNA is decreased in the SN[22, 23, 255] and striatum[48] of PD patients compared to controls. UCHL1 protein is increased in the striatum of PD patients compared to controls[20].
VDAC1	5q31.1	-	VDAC1 mRNA is decreased in the blood of PD patients[15], and VDAC1 protein immunoreactivity is decreased in SN PD NM-positive neurons (and significantly greater in neurons with SNCA inclusions) compared to controls[256].
VDAC2	10q22.2	-	VDAC2 mRNA is decreased in the SN[69], and VDAC2 protein is increased in the striatum[20] of PD patients compared to controls.
VDAC3	8p11.21	-	VDAC3 protein is increased in the striatum of PD patients compared to controls[20].
VDR	12q13.11	The frequency of the C allele of the FokI C/T polymorphism (rs10735810) was significantly higher in PD patients than in controls (OR=1.34, P=0.024)[257]; OR=1.615,	-

		P=0.017[258]) and also a genotype with the C allele was more frequent in patients (OR=2.164, P=0.004[257]; OR=2.677, P=0.015[258]). The intronic SNPs rs4334089 and rs7299460 in *VDR* were associated with PD age of onset (P= 0.0008 and 0.0016 respectively)[259].	
VLDLR	9p24.2	-	-
VPS35	16q11.2	The c.1858G>A (Asp620Asn) mutation in *VPS35* was found to co-segragate with PD in multiple families[260, 261].	VPS35 mRNA is decreased in the SN of (male) PD patients compared to controls[23, 108].

[1] Expression data from genome wide expression studies in PD-associated brain areas (SN, striatum, BA9), CSF or blood were included if they met the following criteria; *with* an adjusted p-value for multiple comparisons <0.05, mRNAs should have a fold change of <-1.5 or >1.5 and proteins <-1.2 or >1.2, and *without* a published adjusted p-value, mRNAs and proteins were only included with a p-value <0.05 and a fold change of <-2.0 or >2.0 and of <-1.5 or >1.5 respectively.

Supplementary Table 5. Landscape proteins, the main process(es) they are functionally involved in and their location within the figures. For each protein (complex) in the landscape, the process(es) oxidative stress response (A), endosomal-lysosomal functioning (B), endoplasmic reticulum (ER) stress response (C), and neuron death and immune response (D) in which they exert their main effect is indicated in columns A to D. The location of each protein (complex) in the landscape is indicated in the column 'Location coordinates in Figures S2 and S3' with these coordinates corresponding to the location of the protein (complex) in Figure S2 and/or S3. A total of 113, 152, 131 and 208 proteins were assigned to processes A, B, C and D, respectively. Between parentheses, protein complex subunits (csu) or protein isoforms (iso) that function within the landscape are indicated. NS; not shown.

Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3	Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3
26S (PSMD11)			X	X	**S2**-3d	BDNF	X			X	**S2**-1b; **S3**-2b, 3a, 3c, 4a, 5d
ABCA1	X	X	X		**S2**-1a, 3a, 3c, 3d	BMP7	X	X	X	X	**S2**-2d; **S3**-2b, 3c, 3d, 5c
ABCA3		X			**S2**-3b	BST1				X	**S3**-1b
ABCA5		X			**S2**-3b	BTNL2				X	**S3**-2c
ABCG1	X	X	X	X	**S2**-1a, 3a, 3b, 3c; **S3**-4b	C9				X	**S3**-3d
ACMSD	X				NS	CACNA1A	X				**S2**-1a, 2a, 5b; **S3**-1c
ACSL6			X		**S2**-1c	CACNA1B	X				**S2**-1a
ACTN4		X	X	X	**S2**-3c, 3d, 4d; **S3**-2d	CACNA1C	X				NS
ADAM12				X	**S2**-5b	CACNA1D	X				NS
ADAMTS14				X	**S2**-5b	CACNA1E	X				NS
ADAMTS2				X	**S2**-5b	CACNA1F	X				NS
ADH1C			X		**S2**-4d	CACNA1S	X				NS
AGAP1	X	X			**S2**-2a	CACNA2D3	X				**S2**-1a, 5c; **S3**-1c
AGTR1	X	X		X	**S2**-1b, 3a, 5a, 5b; **S3**-3d, 4d	CASP3	X		X	X	**S2**-2b, 2c, 2d, 5a
AICDA				X	NS	CASP9	X		X	X	**S2**-2b, 3d
AKT1	X	X	X	X	**S2**-2c, 2d, 3b, 4c, 4d	CAV1		X	X		**S2**-2a, 2b, 3a
AMIGO2	X			X	**S2**-2a	CCAR2			X	X	**S2**-2c, 3c, 4a, 4c, 4d
AMPH		X			**S2**-2a, 2b	CCL5				X	**S3**-2b, 3b, 3c, 4b
AMPK (PRKAA1, PRKAA2, PRKAB1, PRKAB2, PRKAG1, PRKAG2, PRKAG3)	X	X			**S2**-1b, 1d, 2a, 2c, 3a, 3d	CCR4				X	**S3**-2b, 3b, 4b
ANGPT2	X	X		X	**S2**-1b, 5b; **S3**-3a, 3b, 3d, 4d, 5b	CD163	X				**S2**-1a
AP3 (AP3B1)		X			**S2**-2a, 3b	CD200				X	**S3**-3c, 4d
APOE		X	X		**S2**-2a, 2d	CD200R1				X	**S3**-3c, 4d
APP			X		NS	CDH2				X	**S2**-1d
AR			X		**S2**-2d, 4c, 5c; **S3**-2c	CDH23				X	**S2**-4a
ARHGAP33		X			**S2**-3a	CDH6				X	**S2**-4d
ARHGAP44		X			**S2**-3a	CDK19			X		**S2**-4c
ARL4A			X		NS	CECR2	X				**S2**-4d
ARMC8			X		NS	CENPC				X	**S2**-4c
ASAH1		X			**S2**-3b, 4b	CEP85L		X			**S2**-1d
ASCL1				X	**S2**-5c	CLVS2		X			**S2**-2a
ATF6			X	X	**S2**-3c, 4c; **S3**-2a	CNNM2	X				**S2**-1a
ATG5	X	X			**S2**-2b, 5a	CNTN1				X	**S2**-5a
ATG7	X	X		X	**S2**-2b	CNTNAP2				X	**S3**-3d
ATP13A2	X	X		X	**S2**-3b; **S3**-3c	COL18A1	X	X	X	X	**S2**-2a, 4d; **S3**-1d, 3b, 3d, 4c, 5d
ATP2B2	X		X		**S2**-1d	COL2A1		X	X	X	**S2**-4a, 4d, 5b; **S3**-3a, 4a
ATP6V0A1		X			**S2**-3b, 4b	COMT	X				**S2**-1b, 5b
ATR			X	X	**S2**-3c, 4c	CPNE4				X	**S2**-4a
AXIN1			X		**S2**-2a, 3d, 4d	CREM	X	X	X	X	**S2**-4c; **S3**-4b
BAG6		X	X	X	**S2**-2c, 3b, 3d, 4b, 5b; **S3**-4d	CRH	X		X	X	**S2**-2a; **S3**-2b, 5c
BAK1	X		X		**S2**-3d	CRHBP				X	**S3**-2b
BAMBI		X	X	X	**S2**-3d	CRHR1	X		X	X	**S2**-2a, 4a; **S3**-5c
BANK1				X	**S3**-1b	CSMD1			X		**S2**-3d
BAT2			X		**S2**-3d, 4d	CTNNB1		X	X	X	**S2**-1a, 2b, 2d, 3c, 3d, 4c, 4d, 5a
BAT5	X				**S2**-1d	CTSD		X			**S2**-3b
BAX	X		X		**S2**-1c, 2c, 3d	CUL2			X	X	**S2**-3d, 4c, 5a

Supplementary Table 5. (continued)

Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3	Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3
CXCL12				X	**S2**-5a; **S3**-1b, 2a, 3a, 3c, 4c, 5d	GPATCH2			X		NS
CXCR4		X		X	**S2**-5a; **S3**-1b, 3a, 3c, 4b, 5d	GPR37		X			**S2**-4a
CYCS	X			X	**S2**-1b, 1c, 2b, 2c	GSK3B	X	X	X	X	**S2**-1b, 1d, 2a, 2b, 2c, 2d, 3d, 4a, 4b, 4c, 4d, 5a, 5b, 5c
CYP17A1			X		**S2**-3c, 3d, 5b	H1FX			X		**S2**-3c
DCUN1D1		X	X		**S2**-3d, 4c	HAVCR1				X	**S3**-2c
DDIT4		X		X	**S2**-2c	Hb (HBA1, HBA2, HBB, HBD, HBE1, HBG1, HBG2, HBM, HBQ1, HBZ)	X				**S2**-1a
DDX3X				X	**S2**-4c	HCAR1	X				**S2**-2a
DGKQ	X		X		**S2**-1d, 2c, 3c	HIP1R		X			**S2**-3a
DLG2			X	X	**S2**-1d, 3a, 3d, 4a	HK2	X				**S2**-1c, 3b
DNAH11		X			**S2**-4b	HLA-B				X	**S3**-4b, 5b
DNAJC13		X			**S2**-2a	HLA-C		X		X	**S3**-4b
DNAJC6		X			**S2**-2a	HLA-DQA1				X	**S3**-5b
DNM2		X	X		**S2**-2a, 2b	HLA-DQA2				X	**S3**-5a
DRAM1		X			**S2**-3b	HLA-DQB1				X	**S3**-5b
DRD2	X	X		X	**S2**-2a; **S3**-5c	HLA-DQB2				X	**S3**-5a
DRD3	X				**S2**-3a	HLA-DRA				X	**S3**-4b, 5b
DRG2				X	**S3**-2c	HLA-DRB1				X	**S3**-4b
DSCAM			X	X	**S2**-4d	HLCS			X		**S2**-1c
E2F1				X	**S2**-4c	HLX				X	**S3**-1b, 2c
EFCAB4B	X				**S3**-2c	HMOX1	X			X	**S2**-1a, 1b, 4b, 4d; **S3**-3a, 2a
EFCAB6			X		**S2**-5b	HMOX2	X				NS
EHMT2	X			X	**S2**-4c; **S3**-3a	HP	X				**S2**-1a
EIF2A			X		NS	HPGD		X			**S2**-2a
EIF4E			X		**S2**-1b, 3c	HS3ST1				X	**S2**-4b
EIF4EBP2			X		**S2**-1b, 3c, 5c	HSPA5			X		NS
EIF4G1			X		**S2**-1b, 2c, 2d, 3c, 3d	HSPA8		X			**S2**-2a, 2b, 2d, 3b, 3c, 3d, 4a
ENSA		X	X		**S2**-1b	HTRA2	X		X	X	**S2**-1c, 2c, 2d
ETV6				X	**S2**-4b, 4c; **S3**-4d	HUS1	X			X	**S2**-4b; **S3**-5d
FAM134C				X	**S2**-5c	ICAM1				X	**S3**-1b, 1d, 2a, 2b, 2c, 3a, 3b, 3c, 4c, 5b
FAM190A		X			**S2**-3a	IFNG				X	**S3**-3b, 3c, 4c
FBN1		X		X	**S3**-3b	IGF1			X		NS
FBXO25			X		**S2**-3d	IL10				X	**S3**-5b
FBXO36			X		NS	IL12 (IL12A, IL12B)				X	**S3**-1b, 1c, 2b, 2c, 3d, 4c, 5c
FBXO7	X		X		**S2**-1c, 3d	IL1B				X	**S3**-3b, 3c, 3d, 4b, 5b
FER		X		X	**S2**-3a, 4d, 5b; **S3**-3c	IL2				X	**S3**-2b, 2c, 3b, 3d
FGF12	X				**S2**-1a	IL2RA				X	**S3**-1b, 2b, 3c
FOXO1	X	X	X	X	**S2**-1d, 2b, 2d, 4b, 4c; **S3**-3d, 4a, 4b, 4d	IL4				X	NS
FPR3	X				NS	IL5				X	**S3**-1b, 2b, 3c, 4c
FRMD4A				X	**S2**-5a	IL5RA				X	**S3**-1b, 4c
GAK		X	X		**S2**-2a, 2d, 3a, 3b	IL6				X	**S3**-3a
GAS2				X	**S2**-5a; **S3**-3c	IL8				X	**S3**-5c
GBA		X			**S2**-3b	INS	X	X	X	X	**S2**-1c, 1d, 2c, 2d, 3a, 3c, 4a, 5b, 5c; **S3**-2a, 2b, 3b, 4a, 4b, 4c
GCH1	X	X			**S2**-3c; **S3**-3b	INSR	X	X	X	X	**S2**-1d, 2d, 3a, 4b, 5b; **S3**-4a, 4b, 5c
GDNF	X			X	**S2**-1b, 4a; **S3**-2a, 3c, 5c	ITGA6				X	**S2**-4a, 5b; **S3**-5c
GJB2		X		X	**S2**-3a, 3d, 4b, 4d	ITGA8				X	**S2**-4a
GLDN				X	**S3**-3d	ITGA2B				X	**S2**-4a; **S3**-2c, 3a
GNA12	X	X	X	X	**S2**-1d, 3d, 4a, 4d, 5a	JAK2				X	**S2**-2a, 5a, 5b; **S3**-1b, 3b, 3c

Supplementary Table 5. (continued)

Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3	Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3
JARID2				X	**S2**-4c	mPTP (STAR)	X				**S2**-1b, 1c, 2c
KANSL1				X	**S2**-5b	MREG		X			**S2**-2a
KCNA5		X			**S2**-2a	MRPL18	X				**S2**-1c
KDM2B				X	**S2**-4b, 4c; **S3**-3c	MRPL19	X				**S2**-1c
KIF11				X	**S2**-5a	MRPL3	X				**S2**-1c
KIF14			X		**S2**-3d	MSX1			X	X	**S2**-4c
KPNA4		X		X	**S2**-3b, 3c, 4b, 5b	MTERFD1	X				**S2**-1c
LAMP1		X		X	**S2**-1a, 2a, 3b	mTORC1 (AKT1S1, DEPTOR, MLST8, MTOR, RPTOR)	X	X	X		**S2**-1b, 2c, 3c, 3d, 4b
LAMP2		X			**S2**-3b, 4b	mTORC2 (DEPTOR, MAPKAP1, MLST8, MTOR, PRR5, RICTOR)	X	X			**S2**-1d
LAMP3		X		X	**S3**-1d	MYO1E		X			**S2**-2a
LDLR		X	X		**S2**-3a, 3c, 3d	NAPB		X			NS
LFA-1 (ITGAL, ITGB2)				X	**S3**-1b, 1d, 3b, 3c, 4c	NCAM2				X	**S2**-5a
LIPC	X	X			**S2**-2a	NDEL1		X			**S2**-3a
LMX1A				X	**S2**-5c	NDUFAF2	X				**S2**-1c
LMX1B			X	X	**S2**-4c	NEDD4		X			**S2**-2a, 2b
LRP1		X	X		**S2**-2a, 2d	NEDD8		X	X		**S2**-3d, 4c
LRP1B				X	**S3**-4d	NEDD9		X		X	**S2**-2a, 3b, 5c; **S3**-2b
LRPPRC	X				**S2**-1c, 3d	NEGR1				X	**S2**-5a
LRRC16A			X		**S2**-3c	NEO1				X	**S3**-2c, 2d
LRRC25				X	**S3**-1b	NEUROG2				X	NS
LRRK2	X	X	X	X	**S2**-1c, 1d, 2a, 2b, 2c, 3a, 3b, 3c, 3d, 4a, 4b, 4d	NF-KB (NFKB1, NFKB2, REL, RELA, RELB)		X		X	**S2**-3b, 3c, 4a, 4c, 5a, 5c; **S3**-1c, 2c, 3a, 3b, 4b, 4d, 5d
LSM7			X		**S2**-3c	NLGN1				X	**S2**-4a, 5a
LTBP1				X	**S3**-3b, 4d	NMT2				X	**S2**-5a, 5c
LXR (LXRA, LXRB)		X		X	**S3**-3a, 4b	NOS1	X				**S2**-1d
MAOA	X				**S2**-2b, 3c, 5b	NOS2	X			X	**S2**-1d, 4d; **S3**-3a, 3d, 5d
MAOB	X				**S2**-1b	NPTX2				X	**S2**-4a; **S3**-5c
MAP1B	X		X	X	**S2**-1a, 1d, 2b, 3d, 5b; **S3**-1c	NR4A2				X	**S2**-4b, 5c
MAP2K6		X	X	X	**S2**-2d, 3a, 4c	NR5A1	X		X	X	**S2**-3c
MAP3K7		X	X		**S2**-2d, 3a	NSF		X			**S2**-2b, 3a
MAPT		X	X		**S2**-1b, 1d, 2b, 2d, 3a, 3b, 3d	NTF3	X	X		X	**S2**-4b; **S3**-4a
MARC1	X				**S2**-1c	OCA2		X			**S2**-3b
MARK2		X		X	**S2**-1d, 2a, 5a	PALD1		X			**S3**-5c
MAS1	X				**S2**-1b, 3a	PARD3		X	X	X	**S2**-3d, 5a; **S3**-2d
MBNL2		X			**S2**-4b	PARK2	X	X	X	X	**S2**-1b, 1c, 2a, 2b, 2c, 2d, 3b, 3d, 4a, 4b, 5a
MBP				X	**S2**-3d, 4d; **S3**-2b, 2c, 5b	PARK7	X	X	X	X	**S2**-2b, 2c, 3b, 3c, 4c, 5a, 5b; **S3**-3a
MC1R		X		X	**S2**-1d, 4b	PCDH8				X	**S2**-5a
MCC	X	X	X		**S2**-1c, 2a, 2d, 3c, 3d, 4c	PCM1		X			**S2**-3a
MCCC1			X		**S2**-1c	PEO1	X				**S2**-1c
MCFD2			X		**S2**-3c	PIK3CD	X			X	**S2**-1d, 2c, 3c; **S3**-1b, 2d
MCPH1				X	**S2**-4c	PINK1	X	X	X	X	**S2**-1c, 1d, 2b, 2c, 3d, 4c, 5a, 5b; **S3**-1c, 4b, 5c
MED13		X	X		**S2**-4c	PITX3	X			X	**S2**-5c
MICAL2		X			**S2**-3a	PLA2G6	X	X	X		**S2**-2b; **S3**-4d
MITF		X	X	X	**S2**-4c; **S3**-3d	PLAT				X	**S3**-4c, 4d, 5c
MMRN1				X	**S3**-4d	PLAU				X	**S3**-3d, 4c, 4d
MPI				X	**S2**-5b	PLAUR				X	NS

Supplementary Table 5. (continued)

Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3	Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3
PLCB4	X				**S2**-1d	SEMA3E				X	NS
PLEKHM1		X		X	**S2**-5b	SEMA6D				X	**S3**-2c
PLG				X	**S3**-3c, 4c, 5c	SENP7				X	**S2**-4a
PLOD1			X	X	**S2**-3d, 5c	SEP15			X		**S2**-3c
PLS1		X			**S2**-3b	SERPINB5			X		NS
POU2F1				X	**S2**-4c; **S3**-4b	SERPINB9				X	**S2**-5b
PPM1L			X		**S2**-2d	SERPINE1				X	**S3**-3d, 4d, 5b, 5c
PP1 (PPP1CA, PPP1CB, PPP1CC, PPP1R12A, PPP1R12B, PPP1R14C)			X		**S2**-3a, 3c, 3d, 4c, 4d, 5c	SERPING1		X		X	**S2**-2a
PRF1				X	**S3**-3d	SIK1		X	X		**S2**-1b, 2c, 4c
PRKCE	X	X		X	**S2**-1c, 1d, 2c, 3a, 4a, 5a, 5b; **S3**-1c, 2b, 3b	SIRT1	X	X	X	X	**S2**-2b, 2c, 2d, 3c, 4a, 4c, 4d, 5a, 5c; **S3**-3c, 3d, 4d
PRKG1			X		**S2**-3a, 3d, 4d	SIRT7			X		**S2**-3c, 3d
PRKRIR		X		X	**S2**-4b	SLC11A2	X				**S2**-1a, 1b, 1c
PROK2				X	**S2**-4b	SLC18A2	X	X			**S2**-2b, 5c
PSAP		X			**S2**-2a, 3b, 4a, 4b, 4d	SLC24A3	X				**S2**-1a
PSEN1			X		**S2**-2c, 2d	SLC2A13	X				**S2**-1d
PSEN2			X		**S2**-2d	SLC40A1	X				**S2**-1a
PTEN	X	X	X	X	**S2**-1c, 1d, 2b, 2c, 2d, 4c, 5a, 5c; **S3**-2c, 3c, 4d	SLC41A1	X				**S2**-1a
PTPN1			X		**S2**-2c, 2d, 3c, 4a, 4b, 5a, 5b	SLC45A3			X		**S2**-2d
PTPRT				X	**S2**-5b	SLC6A3	X	X			**S2**-2a, 4a, 5c; **S3**-5c
RAB7L1		X			**S2**-3c	SLCO3A1				X	NS
RAC1		X		X	**S2**-3b	SLIT2				X	**S2**-4a, 5c
RAI1				X	**S2**-3c, 4c; **S3**-1b, 2c	SMAD4			X		**S2**-4d
RAP1A		X		X	**S2**-2c, 3b, 5a, 5c	SMAD5		X	X		**S2**-2d, 4d; **S3**-4d
RAP2A		X		X	**S2**-2b	SMPD1		X			**S2**-3b
RBFOX1			X		**S2**-3d	SNCA	X	X	X	X	**S2**-1b, 1c, 1d, 2a, 2b, 2c, 2d, 3b, 3c, 3d, 4a, 4c, 4d, 5a; **S3**-1c, 3a, 3c, 4c, 5c
RCOR1				X	**S2**-4c; **S3**-3a, 4b	SND1		X	X		**S2**-2c, 3c, 3d, 4c
RER1		X	X		**S2**-2c	SNRK		X			**S2**-2c
RFX3		X			**S2**-4b	SNRPB2			X		**S2**-4c
RFX4		X			**S2**-4b	SOD2	X				**S2**-1c, 4c
RGMA				X	**S3**-2c, 2d	SOX2				X	**S2**-4c
RHOA				X	NS	SP110				X	**S2**-4b, 4c
RIT2		X			**S2**-2a, 3a	SPHK1		X		X	**S2**-3a; **S3**-5d
ROBO2				X	**S2**-4a	SPNS2		X		X	**S2**-3a; **S3**-5d
RPH3AL		X			**S2**-1a	SPPL2B				X	**S3**-1d
RPL38			X		**S2**-3d	SREBF1		X	X		**S2**-2c, 2d, 3c, 4b, 4c; **S3**-4b
RPL7A			X		**S2**-3d, 4a, 5b	STAP1				X	**S3**-1b, 4b
RUFY3	X				**S2**-1a	STAT3				X	**S2**-3a, 4b, 4c, 5a; **S3**-1b, 3b, 3c, 4d
RUNX3			X	X	**S2**-2d, 4d; **S3**-1b, 3d	STK11		X			**S2**-1d, 2c, 3d, 5a; **S3**-3d
RXFP1		X			**S2**-2a	STK39			X	X	**S2**-3d, 4c, 4d; **S3**-3d
RXRA			X		NS	SYN3		X			**S2**-2b
SAMD4A		X			**S2**-2c	SYNJ1		X			**S2**-2a, 2b
SCAMP5		X			**S2**-2a; **S3**-4b	SYT11		X			**S2**-1b
SCARB1		X	X		**S2**-2a, 3c, 4d	TACC2			X		**S2**-4c
SCARB2		X			**S2**-2b, 3b	TBX3				X	**S2**-4c
SCIN		X		X	**S2**-3b	TCF12	X			X	**S2**-4b; **S3**-1b, 2d
SCN2A	X				**S2**-1a	TF	X				**S2**-1a, 1c, 2b
SCNN1A	X				**S2**-3c	TFR2	X				**S2**-1a
SDC1				X	**S3**-5c	TGFBR2			X	X	**S2**-3d, 5b

Supplementary Table 5. (continued)

Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3	Protein (csu/iso)	A	B	C	D	Location coordinates in Figures S2 and S3
TH	X	X			**S2**-1b, 1c, 2a, 3d, 5a, 5c	TYR		X			**S2**-3b
TIAL1				X	**S3**-3d	UCHL1	X	X	X	X	**S2**-2b, 3b, 3d, 4d, 5b
TIMD4				X	**S3**-2c, 3c	ULK2		X	X		**S2**-2c, 3c
TLE1			X	X	**S2**-4c, 4d	UNC13B		X			**S2**-1a
TMCC3				X	**S2**-5a	USP9X			X		**S2**-1a, 2c, 3c, 3d, 4d
TMEM2			X		NS	VDAC (VDAC1, VDAC2, VDAC3)	X				**S2**-1c
TMPRSS3		X	X		**S2**-3c	VDR	X	X		X	**S2**-3c, 4c; **S3**-2c, 3c
TMPRSS9		X		X	**S3**-3d	VLDLR		X		X	**S3**-4c, 4d
TMX1		X	X		**S2**-3d	VPS35		X	X		**S2**-2c, 2d
TNF				X	**S3**-1d, 2c, 3b, 4c, 5c	VPS41		X			**S2**-2a
TOM1L2		X			**S2**-2a	WDHD1				X	**S2**-4b
TP53				X	**S2**-1b, 3a, 3c, 4b, 4c, 5a; **S3**-3a, 3c, 4d, 5b	WNK1			X	X	**S2**-3d; **S3**-3d
TP53BP1				X	**S2**-3a, 3c, 4c	WNT3			X		**S2**-4d
TPO	X				NS	WWOX			X		**S2**-2d, 4d
TRAF2			X		**S2**-3c, 3d	ZAK		X		X	**S2**-4a
TRAF3			X	X	**S2**-3a, 3c, 3d, 5a; **S3**-1b, 1c	ZBTB20		X			**S2**-5c
TRAPPC9				X	**S2**-4a	ZNF385B				X	**S3**-1b
TRPC6		X			**S2**-1d, 5a	ZNF423			X		**S2**-4d
TSPO	X				**S2**-1b, 2c						

DETAILED DESCRIPTION OF THE MOLECULAR LANDSCAPE FOR PARKINSON'S DISEASE

CONTENTS

INTRODUCTION

In **Supplementary Figures 2** and **3** all relevant protein interactions that constitute the molecular landscape of Parkinson's disease (PD) are shown. In this description, the various interactions operational within the molecular landscape for PD are grouped into four main processes, (A) oxidative stress response, (B) endosomal-lysosomal functioning, (C) endoplasmic reticulum (ER) stress response, and (D) neuron death and immune response. Obviously, these processes overlap, and some of the proteins and protein signaling cascades are involved in multiple processes. Nevertheless, recurrent description of processes and interactions is avoided as much as possible. In **Supplementary Figures 2** and **3**, these processes are designated with the letters A-D. **Supplementary Table 5** gives an overview of all the proteins in the PD landscape, where they are located in **Supplementary Figures S2** and **S3** and in which process(es) they exert their main effect.

In all descriptions of the PD landscape, names of proteins derived from the genome-wide association studies (GWASs) are in **bold**, single-underlined proteins are genetically associated with PD, dotted underlined genes encode proteins that are differentially expressed in PD and double underlined genes are genetically associated with PD *and* encode a protein that is differentially expressed in PD. **Supplementary Table 2** gives an overview of all GWAS gene-encoded proteins per GWAS and the corroborating evidence for their associations with PD. **Supplementary Table 4** shows all other landscape proteins and the corroborating evidence for their associations with PD.
Not all GWAS gene-encoded proteins were placed in the landscape, either because there were no connections with other landscape proteins or due to lack of annotation, or both. However, this does not necessarily mean that they are not involved in PD pathophysiology. For example, *SPPL2C* is one of the genes that is not included in the landscape, but is still on the shortlist of highly interesting genes in relation to PD. Namely, *SPPL2C* is one of the most significantly PD-associated genes in four different PD GWASs[6, 10, 12, 262], which includes association with two non-synonymous coding SNPs in *SPPL2C*. However, due to lack of annotation and knowledge about its protein function[263] it could not be placed in the landscape. Future research should clarify if this highly associated gene indeed encodes a functional active protein[263, 264] that is of importance in PD pathophysiology.

Overall, two generalization have been made. First, when a knock-out of protein A in a cell or animal model *increases* the expression of protein B, we assume that endogenous expression of protein A leads to the opposite effect and *decreases* the expression of protein B. Second, we assumed that all identified protein interactions (in any organism and/or cell type) can be extrapolated to the interactions in human DA neurons and immune cells.

In the descriptions below, the gene name abbreviations refer to both the gene and the protein. Furthermore, the terms 'activates' and 'inhibits' are used to indicate the activation of a protein (e.g. by (de)phosphorylation) by another protein. To describe a difference in abundance of a protein (directly or indirectly) induced by another protein, the terms 'increases the expression of' or 'decreases the expression of' are used. And lastly, interactions in the PD landscape that are not shown in the figures are indicated with '(not shown)' in the text.

A. OXIDATIVE STRESS RESPONSE

Oxidative stress in DA neurons is increased by cell-specific processes (A1), dysregulation of mitochondria and oxidative phosphorylation (A2) and by dysregulation of regulators of oxidative stress (A3). The functional interactions between the proteins within process A, 'oxidative stress response', are shown in **Supplementary Figure 2**.

A1. Dopamine (DA) neuron specificity

The processes that have been associated with PD, as discussed in this and the next sections – such as autophagy, lysosomal degradation, ER stress, mitochondrial destabilization and apoptotic cascades – are often generic processes that can occur in virtually every cell in the body. However, midbrain dopamine (DA) neuron death is a hallmark of PD, implying a cell-specific vulnerability for dysregulation of these processes. Therefore, sections A1.1 - A1.3 describe processes that are (highly) specific to DA neurons and may play a crucial role in their vulnerability. Subsequently, a genetic predisposition for defects in more generic processes such as autophagy, lysosomal degradation, ER stress, mitochondrial destabilization or proteasomal degradation (as discussed in the next chapters) would increase the vulnerability of these neurons beyond that of neurons that do not synthesize DA or other monoaminergic neurotransmitters. In this way, a relatively small increase in protein aggregation, mitochondrial destabilization and/or lysosomal dysfunctioning may lead to specific DA neuron death.

A1.1 DA synthesis, storage and degradation, and neuromelanin (NM) production

In PD patients, monoaminergic neurotransmitter-synthesizing neurons are vulnerable and degenerate[265]. In DA neurons, DA can be spontaneously oxidized to the NM precursor aminochrome, an oxidation process that produces oxygen radicals and increases oxidative stress[266]. DA oxidation results in increased reactive oxygen species (ROS), that are involved in the formation of oxysterols and in lipid peroxidation. Indeed, PD patients show increased lipid peroxidase activity, indicated by the elevated presence of the lipid peroxidation end products malondialdehyde[179, 267-269] and 4-hydroxynonenal[270, 271].

Monoamine oxidase A (MAOA) increases the expression of SLC18A2[272], that sequesters DA into secretory vesicles. SLC18A2 (that binds to PARK7[273]) thereby prevents DA oxidation[274], as is suggested from the inverse association between SLC18A2 expression and both NM production in the SN and vulnerability to neuronal degradation in the human postmortem brain[274], reduced vesicular storage of DA by SLC18A2 causing nigrostriatal neurodegeneration in mice[275] and association studies showing that gain of function polymorphisms in the SLC18A2 gene promotor are protective against PD in females[229, 230]. Taken together increased sequestration of DA in secretory vesicles by SLC18A2 appears to have neuroprotective properties against PD.

Apart from DA loading into secretory vesicles, DA oxidation can also be prevented by monoamine oxidase (MAO)-dependent (occurring in two forms, MAOA and MAOB) or catechol ortho-methyl transferase (COMT)-dependent degradation of DA[266] (all three genetically associated with an increased PD risk[145, 194, 196, 197]). More specifically, MAOA variations are associated with PD in males[194-196], MAOB variations are associated with PD in females[198] and a COMT polymorphism with lower age of onset in males[146], showing that altered DA degradation affects the risk for PD. **PARK2** decreases the expression and inhibits the activity of both MAOA and MAOB[276], whereas AR[277] and SIRT1[278] increase MAOA expression, calcium (Ca^{2+}) increases MAOA activity[279] and vitamin D3 decreases MAOB expression and activation[280]. Further, COMT expression is increased by **ANGPT2**[281] (not shown) and TP53[282]. Thus, the expression of the DA degradating enzymes MAOA, MAOB and COMT is regulated by proteins in the PD landscape.

Taken together, decreased transport into secretory vesicles or decreased degradation of DA increases DA auto-oxidation and thus cellular aminochrome levels, which can have major effects on cellular processes by inhibiting the proteasome[283], increasing cellular NM levels[266] and binding to the familial PD proteins **SNCA**[284], UCHL1[285], **PARK2** (and aminochrome also inhibits **PARK2**[286]) and PARK7[287]. Aminochrome is speculated to affect chaperone mediated autophagy (CMA) function (for more on CMA see section B3.4) by binding to **SNCA** and UCHL1[266], by inducing formation of **SNCA** protofibrils[284] that inhibit CMA just like mutated **SNCA**[266] and UCHL1[288].

The mRNA and protein levels of tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis in the cytoplasm of DA neurons[289], are decreased in PD SN neurons[246]. TH expression is normally increased by PARK7[290], **CRHR1**[291], **NTF3**[292] (not shown), the DA transporter SLC6A3[293] and the transcription factor PITX3[294], and decreased by **SNCA**[295, 296] (accumulates in lewy bodies[40, 41]), PTEN[297] and **CREM**[298], and inhibited by the DA receptor DRD2[299]. Cytoplasmic TH binds to **SNCA**[300] and SLC18A2[301] (not shown), suggesting a direct regulation of TH by **SNCA**. Further, SLC18A2 is found in a complex together with the DA transporter SLC6A3[302] (not shown), i.e. in close contact with the DA transporter

responsible for DA influx in the cell. SLC6A3, in turn, binds to the familial PD proteins **SNCA**[303] and **PARK2**[304].

In summary, monoaminergic neurotransmitter-synthesizing neurons are more vulnerable due to aminochrome production that increases the cellular burden by inhibiting the proteasome, increasing cellular NM levels and affecting familial PD proteins that regulate autophagy, CMA, mitochondrial and lysosomal stabilization and proteasomal degradation.

A1.2 Hemoglobin, iron homeostasis and lipoprotein oxidation

Hemoglobin (Hb) is an iron-containing metalloprotein that in the blood transports oxygen from the lungs to the rest of the body. Hb is a tetramer of globin subunits (HBA1, HBA2, HBB, HBD, HBE1, HBG1, HBG2, HBM, HBQ1 and HBZ) that changes its composition during embryonic and fetal development. During adult life the most common Hb is a tetramer of two alpha (HBA1 or HBA2) and two beta (HBB) subunits[305]. Higher levels of late-life Hb[161] and genotypes of the Hb-binding protein haptoglobin (HP) are associated with increased PD risk[169]. PD patients show an increased number of HBA1 containing mitochondria, reduced mitochondrial/cytoplasmic HBB ratios in SN neurons[162]. HP, which also binds HDL[306, 307], prevents oxidation of Hb and thereby ensures its transport into the cell by the scavenger receptor CD163[308]. SN DA neurons are (next to red blood cells) one of the few cell types in the body that express Hb[309], where Hb may be involved in iron metabolism, oxygen supply and mitochondrial function[309]. Oxidation of hemeproteins (e.g. Hb) can result in the release of their heme group[310], which can become cytotoxic in the presence of multiple inflammatory factors[310]. Heme is also a cofactor for the thyroid peroxidase **TPO** (not shown; necessary for thyroid hormone synthesis and reducing hydrogen peroxide to water)[311, 312] and essential for NOS1 activation[313] and therefore affects (anti)oxidative reations. The lipoproteins HDL and LDL are the initial heme scavengers in the circulation and get oxidized (to oxHDL and oxLDL, respectively) by binding to heme[310], which also renders them toxic. Of note, oxLDL is increased in the plasma of (L-DOPA treated) PD patients[177]. Clearance of heme by its conversion into biliverdin by the heme oxygenases HMOX1 and HMOX2[312] prevents the oxidation of HDL and LDL by oxidized Hb. The inducibe heme oxygenase HMOX1 is increased in PD serum[167], the number of HMOX1-positive astroglia (see main process D) is increased in the PD SN[166] and lewy bodies in the SN neurons show intense HMOX1 expression[166]. Also, the constitutively active heme oxygenase HMOX2 is genetically associated with PD[168]. EIF4E increases HMOX1 translation[314] and HMOX1 expression is increased by oxLDL[315], **ANGPT2**[316], STAT3[317] (not shown), AKT1[318, 319] (not shown), FOXO1[320], RAC1[321] (not shown) and **SREBF1**[322] and decreased by ferrous iron (Fe(II))[323] and NF-KB[324] (not shown). Further, HMOX1 activation is increased by TP53[325], ER stress[326] (not shown), hypoxia[327] (not shown), superoxide[328] (not shown) and DA[329] and inhibited by SOD2[330] (not shown).

Downregulation of HMOX1 results in increased **SNCA** aggregation[323]. Moreover, HMOX1 increases the expression of BDNF[331], GDNF[331], TH[332], ABCA1[333], **ABCG1**[333], PITX3[332] (not shown), **ANGPT2**[334] and DA[332], decreases the expression of CXCL12[335] and ICAM1[336, 337] (both part of main process D) and inhibits the accumulation of cholesterol[333].

Conversion of heme into biliverdin by HMOX1 results in the production of carbon monoxide and the highly oxidative ferrous iron (Fe(II))[312]. NM binds Fe(II) and is in DA neurons the main Fe(II) store[338, 339]. Fe(II) is also a cofactor for TH in the DA synthesis pathway[340], but highly cytotoxic and its cytoplasmic levels should therefore be tightly controlled. In the SN of PD patients the total iron content is higher than in controls[228], whereas increased *serum* iron levels are associated with a *decreased* risk of developing PD[341]. SLC40A1 transports Fe(II) out of the cell and is upregulated in the PD SN[231]. SLC11A2, another iron transporter, is genetically linked to PD risk[227] and its different isoforms are differentially expressed in PD[228]. SLC11A2 isoforms transports Fe(II) from endosomes into the cytoplasm and also into the mitochondria[342]. **PARK2** increases proteasomal degradation of SLC11A2[343], putatively decreasing cytoplasmic and mitochondrial Fe(II). Ferric iron (Fe(III)) can be transported into the cell by binding to transferrin (TF[344]; part of the HDL-complex[345]). TF binds to the transferrin receptor 2 (TFR2, the number of TF binding places, as a measure for transferring receptor expression, is lower on PD DA SN neurons[245] and TFR2 expression is highly specific for SN DA neurons[244]), and is transported into an endosome where the low pH releases Fe(III) from TF[346]. The PD SN shows a dramatic increase of oxidized TF[244]. This oxidation of TF results in the reductive release of Fe(II) from TF[244] and increases the oxidative cross-linking of TF to other proteins by formation of a disulfide bond, which likely impairs normal trafficking of TF[244] and thus affects the localization of (cytotoxic) iron ions.

In summary, Hb and iron homeostasis increase the 'oxidative burden' of the DA neuron, and may also result in an increased oxidation of lipoproteins. Of note, low cholesterol intake, especially in combination with high dietary iron intake, increases PD risk[347], perhaps due to insufficient (unoxidized) LDL (the major blood cholesterol carrier) in the PD brain, affecting the downstream targets and regulation of lipoproteins.

A1.3 Interaction of the DA- and angiotensin-system

The angiotensin II receptor **AGTR1** is involved in the renin-angiotensin system (RAS), that regulates water homeostasis and hypertension, of which the latter is associated with PD[348-350]. Moreover, overactivation of the RAS results in hypertension, increases superoxide formation by activating NADPH-dependent oxidases, increases microglial activation and increases oxidative stress as shown in PD animal models[351]. The DA and angiotensin II systems directly counterregulate each other in the 'classical' RAS in the renal cells[352], but also in local RAS in the brain. Namely, in the striatum and

SN angiotensin II exacerbates toxin-induced DA neuron death via **AGTR1** activation, whereas DA depletion increases the expression of the angiotensin receptors[353]. DA depletion may therefore result in increased RAS activation and exacerbate the angiotensin II-regulated oxidative stress and microglial inflammatory responses[354, 355] and thereby contribute to the degeneration of DA neurons[351].
Aging in general is associated with an increase in RAS activation, which can be reduced by angiotensin II antagonists and subsequently prevent SN DA neuron degeneration in PD models[351]. **AGTR1** regulates the JAK/STAT pathway[356-358], caspase[359] and NF-KB signaling[360, 361], E3 ubiquitin-protein ligase complexes[362], coagulation factors (for more on coagulation factors see section D2.2.3)[363, 364], increases cholesterol esterification[365], mobilization of intracellular Ca^{2+} and the production of inositol phosphates[366], ER stress factors[367, 368], autophagy[369, 370] and endocytosis[371]. Thus, dysregulation of the RAS-dopamine system interaction can affect many cellular processes in the PD landscape. The angiotensin 1-7 receptor **MAS1** upregulates[372], binds and is a functional antagonist of **AGTR1**[366]. Further, **AGTR1** expression is decreased by the secreted vascular remodeling protein angtiopoietin-2 (**ANGPT2**)[373] and HDL-cholesterol[374] (which levels are positively correlated with the duration of PD[375]) and increased by the oxysterols 24-hydroxycholesterol (24-OHC, which CSF-levels correlated with the duration of PD[376]) and 27-hydroxycholesterol (27-OHC)[377] (for more on 24-OHC and 27-OHC see section B4).

In summary, the RAS and DA system counterregulate each other, indicating that the regulation of local brain RAS is important for a normal DA system. DA depletion, aging and oxysterols increase RAS activation, that in turn regulates multiple pathways in the PD landscape leading to increased oxidative stress, microglial activation and DA neuron death.

A2. Mitochondrial dysfunction

Mitochondrial dysfunction, increased oxidative stress and release of the apoptosis-inducing cytochrome c may be caused by imperfect functioning mitochondrial proteins e.g., dysregulation of the mitochondrial transcription factor **MTERFD1**[378] (its inactivation leads to respiratory complex deficiency[379]), the mitochondrial DNA regulator **PEO1**[312], the mitochondrial complex I assembly chaperone **NDUFAF2**[380] or the mitochondrial 39S ribosome complex proteins **MRPL3**, **MRPL18** and **MRPL19**[312], which may impair mitochondrial protein translation.

The superoxide dismutase SOD2 (differentially expressed in the PD brain[237, 238, 381, 382]) neutralizes superoxide, the toxic byproduct of the respiratory chain[239] and decreases release of cytochrome c from the mitochondria[383]. Further, SOD2 inhibits **ATF6**[384] (not shown), regulates expression of **LIPC**[385] (not shown), increases expression of the

GTP cyclohydrolase **GCH1**[386] (not shown) and binds **MCC**[387]. And, SOD2 expression is decreased by PARK7[388], increased by FOXO1[389] and **ANGPT2**[281] (not shown) and enzymatic activity of SOD2 is attenuated (up to 50%) by DA neuron specific reactive dopamine-quinones[239]. Further, LRPPRC is an essential posttranscriptional regulator of (mitochondrial) mRNA[390], localizes predominantly to mitochondria but also to the nuclear membrane[312]. LRPPRC interacts with and its expression is increased by PINK1[391, 392], interacts with **PARK2**[393] (not shown) and binds to **MCC**[387], SIRT7[394] (not shown), **FBXO25**[395] and **CECR2**[396]. Dysregulation of LRPPRC results in defective regulation of cytochrome c oxidase subunits[392]. Thus, both SOD2 and LRPPRC may affect cytochrome c production in the mitochondria.

In addition to self-regulation, the mitochondria are also highly regulated by external factors from the cytoplasm. The next sections will discuss the regulation of mitochondrial (dys)function by familial PD proteins and the mitochondrial permeability transition pore (A2.1), by Ca^{2+} (A2.2) and by cholesterol and oxysterols (A2.3).

A2.1 Regulation of mitochondrial membrane permeability and cytochrome c release

Mitochondrion integrity and degradation is highly regulated by familial PD proteins, suggesting a key role in (familial) PD pathogenesis. Loss of the mitochondrial transmembrane potential ($\Delta\Psi m$) results in activation of caspase-independent cell death pathway that is controlled by the serine protease HTRA2[397] and mitochondrial accumulation of PINK1[398]. HTRA2 degradates denatured mitochondrial proteins and promotes apoptosis when released into the cytoplasm[399] and accumulated mitochondrial PINK1 increases mitochondrial Ca^{2+} levels and opening of the mitochondrial permeability transition pore (mPTP)[400] and subsequent cytochrome c release to the cytoplasm[401]. Further, accumulated PINK1 recruits **PARK2** to mitochondria to initiate mitophagy[398, 402, 403]. This recruition of **PARK2** to mitochondria is disturbed in FBXO7 deficient cells[404]. Further, HTRA2 binds and cleaves **PARK2** and thereby inhibits its E3 ubiquitin ligase activity[405] and **PARK2** is bound and inhibited (via ubiquitination) by **LRRK2**[406] (binds **SNCA**[407]). Thus, PINK1, FBXO7, HTRA2 and **LRRK2** regulate **PARK2** that, by ubiquinating BAX, functions as a 'gateway' for BAX transport to the mitochondrial membrane and subsequent cytochrome c release[408].

Cytochrome c (endoded by CYCS) is an essential component in the mitochondrial electron transport chain, but is also involved in the initiation of apoptosis when release from the mitochondria into the cytoplasm. Cytochrome c release is increased by **ATP2B2**[409] (not shown), PTEN[410], **AMPK**[411], RAC1[412] (not shown), TP53[413] (not shown), CASP3[414], CASP9[415] (not shown; activated in TH+ SN neurons of late-onset PD patients[138]), cholesterol[416], ceramide[417], sphingosine[418], NM[419] and L-DOPA[420] and decreased by EIF4E[421], SOD2[383], **PRKCE**[422] (not shown), **MAPT**[423], ATP13A2[424] (not shown), PINK1[425], PARK7[426]

(inhibits **SNCA** aggregation[427]) and **PARK2**[428]. Cytoplasmic cytochrome c increases the aggregation of **SNCA**[429] and activates the apoptotic caspase pathway by activating CASP9 and subsequently CASP3[430-433], which are also regulated by the familial PD proteins, i.e. CASP3 is activated by UCHL1[434] and HTRA2[435] and inhibited by PARK7 and PINK1[436, 437], whereas CASP9 is activated by HTRA2[438] and inhibited by PARK7[436].

The proapoptotic proteins BAX and BAK1 bind to the voltage-dependent anion channel (VDAC), which results in loss of ΔΨm, mPTP opening, release of cytochrome c in the cytoplasm and activation of the caspase pathway by activation of CASP9 and CASP3[439-444]. The VDAC (there are three VDAC isoforms present in humans; VDAC1-3) is the major mitochondrial outer membrane channel, that allows ATP/ADP exchange between cytoplasm and mitochondria and allows the transport of ions (e.g. Ca^{2+}), lipids (e.g. cholesterol) and metabolites over the mitochondrial outer membrane[312, 445, 446]. The VDAC opens at low membrane potentials and closes at potentials above 30mV[447-449] and regulates, but is not necessary for mPTP formation[450-453]. Of note, in yeast cells **SNCA**-toxicity is dependent on VDAC. More precisely, the VDAC is able to transport monomeric **SNCA** into mitochondria and is blocked by **SNCA**. In this way, **SNCA** disrupts the VDAC-mediated ATP/ADP exchange, decreases ΔΨm and impairs oxidative phosphorylation[454] (not shown). In addition to BAX and BAK1, VDAC also binds to CAV1[455] (not shown), cholesterol (may also affect VDAC functioning)[456], **HLA-B**[387] (not shown), **LRRK2**[457], **MCC**[387], **PARK2**[458, 459], PLAT (on the plasma membrane)[460] (not shown), **PRKCE**[422] (not shown), SIRT7[394] (not shown), **SNCA**[461] and the mPTP components STAR[462] and TSPO[463]. Further, VDAC accumulation[440] and oligomerization induces apoptosis[464, 465] and its expression is increased by **COL18A1**[440] (not shown), IL2[466] (not shown) and vitamin D[467] (not shown) and decreased by TSPO[468] (not shown). VDAC recruits **PARK2** to dysfunctioning mitochondria to induce mitophagy[458]. Ubiquitinating of VDAC by **PARK2** is PINK1-dependent[469], and necessary for **PARK2**-mediated mitophagy[470]. Apoptotic pathways are activated via regulation of VDAC by BAX[471], **COL18A1**[440] and GSK3B[472] and inhibited by **PRKCE**[422]. Of note, DA decreases the ΔΨm and *reduces* VDAC levels on the mitochondrial outer membrane[473, 474], but this DA toxicity is not counteracted by (VDAC-dependent[458, 470]) mitophagy and thereby enhances oxidative stress, which may possibly explain DA neuron death in PD[474].

Binding and stabilization of hexokinase 2 (HK2) to VDAC and the mitochondrial outer membrane suppresses (BAX-induced) cytochrome c release, CASP3 activation and apoptosis[475-477], by preventing mitochondrial VDAC accumulation[440]. HK2 locates to the outer membrane of mitochondria and is involved in glucose metabolism by phosphorylating glucose to glucose-6-phosphate[312]. HK2 also protects against neurodegeneration in rotenone and MPTP mouse models of PD[478] and it is proposed that the VDAC-HK2 complex is necessary to generate the ΔΨm[479]. **COL18A1** and GSK3B both

increase phosphorylation of VDAC, which disrupts binding of the hexokinase HK2 to VDAC and decreases ΔΨm[440, 472], whereas GSK3B inhibition increases the accumulation of HK2 in mitochondria, enhances glycolysis in the cell and increases neuronal survival[480].

HK2 expression is regulated by binding of STAT3 and **SREBF1** to the HK2 gene promotor[481, 482] and is increased by AKT1[483] (not shown), mTORC1[484, 485] (not shown), CAV1[486] (not shown), STAT3[487], IFNG[488] (not shown), IL1B[489] (not shown), insulin (INS)[490] (not shown) and mutant TP53[491] (not shown) and decreased by **COL18A1**[440] (not shown). HK2 binds to **PRKCE**[422] and **PARK2**[459] and INSR activation results in HK2 translocation to mitochondria (via the AKT1 pathway)[492] (not shown).

Thus, by regulating VDAC and HK2, cytochrome c release by the mPTP and subsequent activation of the caspase pathway can be suppressed. Of note, prevention of apoptosis by HK2 stabilization to VDAC and the mitochondrial outer membrane also favours the glycolysis pathway followed by lactic acid fermentation and reduces the oxidation of pyruvate in mitochondria[475, 493]. This change in energy production, from aerobic to anaerobic, is called the Warburg effect and reduces the production of ROS and is often seen in cancer cells[494]. Further, **PARK2** deficiency activates glycolysis and reduces aerobic respiration by the mitochondria[495].

A2.2 Ca^{2+}-induced mitochondrial dysfunction

The VDAC (as seen in A2.1) regulates transport of **SNCA**, metabolites and ions, such as Ca^{2+}, over the mitochondrial membrane. However, the rate of their transport into the mitochondria also depends on their cytoplasmic levels. High cytoplasmic Ca^{2+} levels increase the transport of Ca^{2+} into the mitochondria. Therefore, regulation of cytoplasmic **SNCA** and Ca^{2+} levels is important to maintain homeostasis.

Of note, SN DA neurons are able to generate action potentials in the absence of synaptic input. They are autonomously active, which is regulated via Ca^{2+} entry through L-type calcium channels (composed of four subunits; CACNA1C, CACNA1D, CACNA1S and CACNA1F[312])[496]. Ca^{2+} influx is therefore an important factor in regulating the basal activity of DA neurons. Further, L-type calcium channel-mediated Ca^{2+} influx enhances the production of DA from L-DOPA[497], the use of L-type calcium channel blockers has been shown to reduce the risk of developing PD in a Danish population[498]. Furthermore, in the PD SN the number of cells that express CACNA1C and CACNA1D is lower[134], but in these cells CACNA1D is higher expressed than in controls[134]. And, the CACNA1D subunit is also shown to be higher expressed in the PD SN[135]. Lastly, also the CACNA1D to CACNA1C ratio is increased in PD brains[134]. Thus, by regulating the intrinsic tonic firing typical for DA neurons, Ca^{2+} ensures that there is a continuous DA supply to target areas such as the striatum. This however exposes SN neurons to a higher Ca2+ influx than other neurons and it is hypothesized that together with the low Ca^{2+} buffering capacity

of SN neurons, this influx is directly responsible for mitochondrial stress and increased ROS production, which makes them more vulnerable[499, 500]. Increased intracellular Ca^{2+} levels disrupt mitochondrial membrane integrity, which results in cytochrome c release and apoptosis[501].

Secreted **SNCA**-toxicity is mediated by an increased Ca^{2+} influx and deregulation of the cellular Ca^{2+} homeostasis[503]. And, intracellular Ca^{2+} levels can also be increased by **CXCR4**, by **AGTR1** via activation of G proteins (e.g. **GNA12**)[312], by influx through or mediated by the $Na^{+}/K^{+}/Ca^{2+}$-exchanger **SLC24A3**[312] (highly expressed in nigral DA neurons, and a potential role in DA neuron survival[504]), by the Ca2+-binding protein **EFCAB4B** (plays a key role in store-operated Ca2+ entry in T-cells[312]) and the voltage-dependent calcium channels (such as the L-type calcium channel subunits mentioned above, but also by other types of channels consisting of alpha-1 (**CACNA1A**), alpha-2 (**CACNA2D3**), beta and delta subunits[312]). These calcium channels are mediated by multiple proteins in the landscape i.e., **CACNA1A** binds to **USP9X**[505], **MAP1B**[505], **EHMT2**[506] (not shown), LRP1[506], SYNJ1[505], CTNNB1[505] and **AMIGO2**[506] (activates the NF-KB complex[507] (not shown)) and is inhibited by the dopamine receptor DRD3[508] (not shown). Further, DA decreases the expression of **CACNA2D3**[509] (not shown), which is high and highly specific expressed in the rat SN and is decreased after 6-OHDA treatment [504].

Other alpha-1 calcium channel subunits are also regulated by proteins in the landscape. CACNA1B (increases blood INS level[510]) binds **SCN2A**[505] (**SCN2A** also binds to **FGF12**[511]), **CNNM2**[505], **MAP1B**[505] and the regulator of axonogenesis **RUFY3**[312, 505] and is regulated by the dopamine receptor DRD2 (not shown)[512]. Further, CACNA1E also binds to **RUFY3**[505] (not shown), CACNA1C binds SIRT1[513] and alternative splicing of CACNA1D and CACNA1S (both L-type calcium channel subunits) is regulated by **RFBOX1**[514] (not shown).

Administration of Mg^{2+} reduces Ca^{2+} mediated microglial DA neurotoxicity in PD[515] and increases the kinase activity of **LRRK2**[516]. Mg^{2+} is transported out of the cell by the transporters **CNNM2**[517] and **SLC41A1**[518]. Moreover, the magnesium dependent ATPase **ATP2B2** couples ATPase activity to Ca^{2+} efflux[312] and its overexpression depletes intracellular Ca^{2+} stores and triggers apoptosis[409].

Thus, voltage-gated calcium channels regulate Ca^{2+} levels in the neuron, which is important for the intrinsic tonic firing of DA neurons and DA release, and are themselves regulated by multiple landscape proteins (e.g. the DA receptors DRD2 and DRD3). Increased Ca^{2+} influx increases mitochondrial dysfunction and increases **SNCA**-toxicity.

A2.2.1 ER stress-induced mitochondrial dysfunction

Oxidative stress increases the influx of Ca^{2+} into the cytoplasm from the ECM, but also increases release of Ca^{2+} from the ER Ca^{2+}-store[519]. Acute release of Ca^{2+} from the ER triggers Ca^{2+}-mediated mitochondrial cell death[520]. Prolonged ER stress (disruption of

normal ER function, resulting in activation of the unfolded protein response (UPR; see also section C1)) results in a perturbed mitochondrial function through a disturbed ER-mitochondrial Ca^{2+} homeostasis[521-523], i.e. BAX and BAK1 oligomerize in the ER membrane and allow Ca^{2+} release to the cytoplasm[524], which is taken up by the mitochondria resulting in loss of the $\Delta\Psi m$[522, 524]. Further, during ER stress BAX translocates to the mitochondrial membrane (increased by mitochondrial lipid rafts[525]) where it interacts with the mPTP leading, together with the loss of $\Delta\Psi m$, to cytochrome c release in the cytoplasm[439, 521, 524, 526], which results in caspase activation and apoptosis[397].

The relevance of these processes in PD is illustrated by the increased BAX immunoreactivity in NM-containing neurons[125] and differences in aggregation of BAX-rich inclusions in PD[126]. Moreover, BAK1-deficient mice were resistant to paraquat neurotoxicity, a model for PD[527] and the receptor for humanin, **FPR3** (not shown), that mediates the anti-apoptotic activity of humanin and suppresses BAX-dependent apoptosis[312], was found in the GWASs[6].
Hence, ER stress results in BAX-dependent apoptosis, through Ca^{2+} release from the ER that is taken up by mitochondria, leading to a loss of $\Delta\Psi m$, mitochondrial dysfunction, cytochrome c release and activation of apoptotic pathways.

A2.2.2 Intracellular Ca^{2+} release and regulation of inositol phosphates

Ca^{2+} release from intracellular stores (including the ER) is also increased by the secondary messenger inositol triphosphate (IP3)[528], which is produced together with diacylglycerol (DAG) by cleaving of phosphatidylinositol 4,5-bisphosphate (PIP2) by **PLCB4**[312]. Myo-inositol serves as an important component of inositol phosphates (e.g. PI, PIP2, PIP3 and IP3) and is transported into the cell by the proton myo-inositol cotransporter **SLC2A13**[529] (genetically associated with PD[53]). **PIK3CD** generates the AKT1 activating PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) by phosphorylating PIP2[312] and increases intracellular Ca^{2+}[530]. Further, **PIK3CD** also inhibits PTEN[531], that generates PIP2 by dephosphorylating PIP3[312] and thereby functions as an antagonist for the AKT1 signaling pathway[312]. The **SNCA**-binding[532] serine/threonine-protein kinase **PRKCE** binds to diacylglycerol kinase theta (**DGKQ**, associated to PD[27-29]) and increases its translocation to the plasma membrane[533]. **DGKQ** phosphorylates DAG and thereby produces phosphatidic acid (PA)[312]. PA quantity is also increased by S1P[534] (not shown). PA and DAG are both essential for **PRKCE** activation and translocation to the plasma membrane[535]. Further, PA also activates, and is necessary for mTORC1 signaling[536-538], binds NR5A1[539] (not shown) and increases NR5A1-dependent expression[539], inhibits PPP1CA[540], activates SPHK1[541] (and increases its translocation to the plasma membrane[542]), activates **AGAP1**[543] (GTPase activity stimulated by PIP3 and PIP2, whereas PA potentiates PIP2 activation[543]), activates the reverse activity of **ASAH1** (resulting in ceramide production)[544] (part of main process B), increases **MAPT** phosphorylation (via

MTOR activation)[545] (not shown), binds HIP1R[546] (not shown) and **MBP**[547] (not shown) and induces fibrillization of **SNCA**[548]. Thus, inositol phosphate regulation affects Ca^{2+} release and production of PA affects among others, energy and redox sensing (mTORC1), sphingosine regulation (**ASAH1**, SPHK1) and **SNCA** aggregation as seen in PD.

A2.3 Cholesterol- and oxysterol-induced mitochondrial dysfunction

Several findings suggest that lipid metabolism is involved in PD pathogenesis. For example, high dietary intake of (poly)unsaturated fatty acids and plasma hypercholesterolemia are associated with lowering the risk of PD[350, 549, 550], low plasma levels of LDL-cholesterol and total cholesterol are associated with an increased PD risk[551-554] and PD disease duration is positively correlated with plasma HDL-cholesterol[375]. Dietary cholesterol increases nicotinamide adenine dinucleotide (NAD) synthesis from tryptophan by inhibiting the decarboxylase **ACMSD**[555] (not shown), that is also downregulated by long chain fatty acids[556]. NAD+ is reduced to NADH in the citric acid cycle or during β-oxidation or glycolysis, which is subsequently used during ATP production through oxidative phosphorylation in the mitochondria[557]. Activation of the lactate receptor **HCAR1** inhibits lipolysis and thus the hydrolysis of triglycerides into glycerol and fatty acids[558, 559] (its expression is inhibited by inflammation[560]). The mitochondrial oxidoreductase **MARC1** catalyzes the NADH-dependent nitrite reduction to nitric oxide (NO) under anaerobic conditions[561], is associated with LDL cholesterol levels[562] and binds **PARK2**[459]. Maintaining a balance between (oxLDL-generated) reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as NO is important in preventing apoptosis[563].

Plasma levels of oxidized cholesterol derivates are associated with PD. Patients have higher plasma oxLDL[177] and 7-ketocholesterol (7-KC)[564] (the main cholesterol oxidation product in oxLDL[565]). Statins, inhibitors of cholesterol synthesis, decrease oxysterol levels in the brain[566] and reduce the aggregation of **SNCA** in vitro and in **SNCA** transgenic mice[567, 568]. Whereas high plasma LDL-cholesterol levels are protective, cellular LDL is oxidized in mitochondria[569], increases ROS formation[570] and impairs the activity of the mitochondrial oxidative complexes[571]. OxLDL consists of multiple oxysterols[572, 573] and results in $\Delta\Psi m$ disruption and release of the pro-apoptotic proteins cytochrome c and HTRA2[574]. Further, high cytoplasmic free cholesterol levels are toxic[575] and can cause mitochondrial dysfunction[416, 576], i.e. increased mitochondrial cholesterol levels increase oxidative stress[577], resulting in increased oxysterol formation[578]. Oxysterols can be generated by either auto-oxidation or by ER or mitochondrial cholesterol hydroxylases[579] and regulate lipid metabolism (via **SREBF1**, see also main process C), receptor function, immune response and apoptosis[579] and cause **SNCA** aggregation[568, 580, 581] and lysosomal and mitochondrial destabilization[582-584].

Mitochondrial function is affected by ER-stress-related components, e.g. BAX

(see section A2.2.1) but also by cholesterol regulation in the ER. Namely, **SREBF1** inhibition or knockdown reduces cholesterol-dependent stabilization of PINK1 on the mitochondrial membrane, **PARK2** translocation to the mitochondria and subsequent mitophagy[585]. Further, the familial PD protein PLA2G6[222, 223] catalyzes fatty acids release from phospholipids and associates with mitochondria during cholesterol-induced ER stress[586], whereas sustained PLA2G6 activation leads to disruption of the mitochondrial outer membrane and cytochrome c release[587].

Expression of the outer mitochondrial membrane translocator protein (TSPO) is increased in PD striatum and midbrain, and correlates with motor disease serverity[252]. It is uncertain if TSPO is part of the mPTP or only associated to this complex[578]. TSPO transports cholesterol into the mitochondria[578, 588, 589]. TSPO expression is increased by S1P[590] and in a 6OHDA rat PD model[591] and is activated during microglia activation[252]. In addition to TSPO, also the steroidogenic acute regulatory protein (STAR; part of the mPTP) binds cholesterol[592] and increases cholesterol transport into the mitochondria[593, 594]. STAR expression is increased by angiotensin II[595, 596] (not shown), NR5A1[597, 598] (not shown), **CREM**[599] (not shown), LDL[600] (not shown), HDL[600] (not shown), 27-OHC[601, 602], S1P[590] (not shown) and **SREBF1**[603, 604] (not shown) and decreased by **AMPK**[605], **SIK1**[606], **BMP7**[607, 608] (not shown), NF-KB[609] (not shown), LXRA/RXRA[610] (not shown) and **ASAH1**[611] (not shown). Furthermore, STAR binds to the vitamin D receptor (VDR)[612] (not shown) and **PRKG1**[613] (not shown) and increases transport of VDR into mitochondria [612] (not shown). Thus, cellular lipoprotein (LDL, HDL), cholesterol-related (27-OHC, **SREBF1**) and sphingosine-related (S1P, **ASAH1**) proteins affect cholesterol uptake by the mitochondria. STAR-mediated mitochondrial cholesterol transport increases mitochondrial accumulation[614] of the apoptotic cholesterol derivate 27-OHC[615]. 27-OHC is increased in the plasma of PD patients[564], decreases TH expression and increases **SNCA** expression[616, 617] and is involved in reverse cholesterol transport (see section B4).

Cholesterol transport into mitochondria is required for the formation of steroid hormones. TSPO and STAR transport cholesterol into the mitochondria for conversion to pregnenolone and eventually to testosterone[594, 618-622] by **CYP17A1** in the ER[623, 624]. Thus, TSPO and STAR are indirectly important in production of testosterone. Testosterone decreases the expression of STAR[620] (not shown), and thereby functions as a feedback loop on cholesterol transport into the mitochondria and subsequent testosterone synthesis, but may also increase the formation of 27-OHC and oxysterols. For more on testosterone function in PD pathogenesis see section C4.

A3. FOXO1 and SIRT1: Transcriptional regulators of oxidative stress

The transcription factor FOXO1 mediates cellular homeostasis during oxidative stress[312], is inhibited by INS signaling[312] and promotes neuronal cell death[312]. FOXO1

has a key role as it is connected with many proteins in the landscape. FOXO1 binds STAT3[625], is activated by CXCL12[626] (not shown), its expression is increased by **TCF12**[627] and **PIK3CD** increases FOXO1 degradation[628]. Further, FOXO1 increases expression of NF-KB[629], **ATP6V0A1**[630], **HUS1**[629], SOD2[389], INSR[631], SCARB1[632], the mTORC2 complex[633] (not shown; mTORC2 consists of MTOR, RICTOR, MLST8, PRR5, MAPKAP1 and DEPTOR[312], binds PINK1[634], **EHMT2** and **BAT5**[635] and increases the expression of PTEN[636]), LAMP2[632], SIRT1[637] and decreases the expression of **SREBF1**[632], **ANGPT2**[638], **PRF1**[630] and SERPINE1[639] and regulates CCL5 release[640]. Furthermore, deacetylated FOXO1 increases expression of ICAM1[641]. Thus, FOXO1 regulates DNA repair (**HUS1**), oxidative stress (SOD2), acidification of intracellular compartments (**ATP6V0A1**), INS metabolism (INSR), cholesterol metabolism (SCARB1, **SREBF1**), chaperone-mediated autophagy (LAMP2) and the immune response (**PRF1**, ICAM1, CCL5).

The PD-linked[226] deacetylation factor SIRT1 is a sensor for cellular energy status and is activated by an increased NAD/NADH$^+$ ratio, and is subsequently involved in regulation of cell cycle, apoptosis and autophagy, and shuttles between the cytoplasm and the nucleus[312]. SIRT1 is, like FOXO1, an important regulator of the main transcriptional pathways in the PD landscape (see below) and is involved in cholesterol homeostasis[642]. Mutations in the SIRT1 promotor that may decrease SIRT1 transcription were found in some PD patients, but not in controls[226]. Cytoplasmic SIRT1 binds mTORC1[643], **MAPT**[644], CTNNB1[645], ATG5[646], ATG7[646], CACNA1C[513] and **SREBF1**[647] and binds to and activates **AMPK**[648] (another energy sensor, activated in response to low cellular ATP levels, see section B3.1). In the nucleus, SIRT1 binds to NF-KB[649], TP53[650], STAT3[651], **TLE1**[652], AR[653], VDR[654], **CCAR2**[655] (inhibits SIRT1[655]) and NEDD8[656]. SIRT1 activates PTEN[657, 658] and **AMPK**[659], and inhibits CASP3[660], NF-KB[652], SERPINE1[661], TP53[650] and **SREBF1**[662]. Although SIRT1 decreases **SREBF1** stability via deacetylation[662], it also increases the expression of the cholesterol transporters ABCA1[663] and **ABCG1**[663] and thereby increases reverse cholesterol transport[663, 664] (see also section B4).
Further, SIRT1 increases expression of MAOA[278], regulates expression of SOD2[665, 666] and decreases expression of the sodium channel **SCNN1A**[667], STAT3[668], TP53[651], AR[653] and the production of testosterone[669].
Thus, in response to cellular energy levels, SIRT1 regulates autophagy (**AMPK**, mTORC1, ATG5, ATG7), transcription (FOXO1, STAT3, **SREBF1**, VDR, TP53), and is involved in the regulation of oxidative stress (SOD2) and apoptosis (CASP3, TP53). Finally, FOXO1 and SIRT1 tightly regulate each other. They bind[670], FOXO1 activates[671] and increases expression of SIRT1[637] and SIRT1 inhibits FOXO1[672].

A4. Concluding remarks

The uptake, processing and signaling of lipoproteins and their components (e.g. cholesterol, sphingolipids, triglycerides) and their subsequent metabolites and

derivatives (e.g. oxysterol, ceramide, sphingosine, S1P, fatty acids) appear to play a crucial role in the PD landscape. Dysregulation of cellular cholesterol levels increase oxidative- and mitochondrial stress, which - given the increased oxidative state of DA neurons (e.g. due to iron and DA metabolism) – may just tip the scales in DA neurons and result in (ER stress-induced) mitochondrial dysfunction, increased cellular stress and apoptosis. Of note, familial PD proteins are directly involved in mitochondrial membrane quality control and cellular apoptosis and are therefore located at the 'end of the funnel' of interactions ultimately leading to mitochondrial-mediated DA neuron death. Single sporadic variations have a lower impact on the same pathways, but when accumulated, will also lead to mitochondrial dysfunction and DA neuron death.

B. ENDOSOMAL-LYSOSOMAL FUNCTIONING

Many of the PD-GWAS-associated proteins regulate endocytosis, autophagy and lysosomal function. This section discusses these processes and pathways in detail, starting with sphingolipids (B1), as they are important for membrane (and lipid raft) function and thus crucial for the regulation of endocytosis, autophagy and lysosomal function. Further, section B2 discusses the endocytosis of extracellular proteins and lipids into the intracellular endosome-lysosome system, and B3 elaborates on the function of the lysosome in autophagy and protein degradation. Lastly, section B4 covers the role of reverse cholesterol transport and (systemic) regulation of lipoproteins and INS in PD. The functional interactions between the proteins within process B, 'endosomal-lysosomal functioning', are shown in **Supplementary Figure 2**.

B1. Sphingolipids

Sphingolipids are a class of bioactive lipids containing a hydrophobic backbone of a long-chain spingoid base that is linked to a fatty acid and an hydrophilic head group than may contain hydroxyl groups, phosphates or sugar residues. Simple spingolipids include sphingosine (sphingoid base) and ceramide (sphingosine linked to fatty acid without additional head groups). More complex sphingolipids include sphingomyelin (ceramide with a phosphocholine or phosphoethanolamine head group), cerebrosides (ceramide with a single glucose or galactose head group) and gangliosides (ceramide with at least three sugars, one of which must be sialic acid). Ceramide, sphingomyelin, sphingosine, cerebrosides and gangliosides are discussed in more detail in the next paragraphs.

B1.1 Ceramide and sphingomyelin

Ceramide is important for the functioning of cellular membranes and plays a role in apoptosis[673]. Further, plasma ceramide is higher in sporadic PD patients versus controls and in PD patients with versus without cognitive impairment[674], whereas

sphingomyelin is reduced in PD frontal cortex lipid rafts compared to controls[675]. In addition to the de novo synthesis from less complex molecules, ceramide can be generated through hydrolysis from sphingomyelin by the lysosomal sphingomyelinase SMPD1. SMPD1 is activated by cholesterol[676] and induces translocation of **PRKCE** to the cytosol and NF-KB to the nucleus[677, 678] (not shown), this is presumably resulting from a change in ceramide/sphingomyelin ratio by SMPD1, for ceramide increases cytosolic accumulation of **PRKCE**[677, 679] (not shown) and translocation of NF-KB to the nucleus[678]. Moreover, ceramide also increases activation of NF-KB[680-683], CASP3[684-686], CASP9[687] and BAX[418, 688], inhibits AKT1[689-691] and increases ER- Ca^{2+} and mitochondrial cytochrome C release and apoptosis[528, 692, 693]. The lysosomal glucosylceramidase **GBA** and ceramidase **ASAH1** respectively increase and decrease ceramide levels, i.e. **GBA** converts glucosylceramide (a cerebroside, and also called glucocerebroside) to ceramide and **ASAH1** catalyzes the conversion of ceramide to sphingosine[694, 695]. **GBA** also binds to **PARK2**[696] and **SNCA**[697] and mutations in, or knockdown of **GBA**[698] increases **SNCA** aggregation, whereas **SNCA** in turn inhibits **GBA** activity[698].

The transporter **ABCG1** is located on the plasma membrane and increases secretion of sphingomyelin from the cell[699]. Further, sphingomyelin may be a substrate for and increases the expression of the late-endosomal/lysosomal cholesterol transporter **ABCA5**[700] (mRNA expression is increased in the amygdala of PD patients[700]). Moreover, sphingomyelin also increases expression of **SNCA**[700], and decreases expression of the cellular cholesterol homeostasis-controlling transcription factor **SREBF1**[701]. On the other hand, ceramide increases activation and nuclear translocation of **SREBF1**[702], suggesting that sphingomyelin and ceramide have opposite effects on cholesterol regulation. In turn, cellular sphingolipid levels are affected by lipoproteins, e.g. HDL increases ceramide levels[703] by binding to SCARB1[704], and oxLDL and 7-KC can increase ceramide accumulation[705] (not shown), but 7-KC in oxLDL can also inhibit lysosomal sphingomyelinase[706] (not shown).

B1.2 Sphingosine

The sphingosine synthesis pathway is regulated by the precursor protein prosaposin (PSAP), whose uptake and transport into endosomal-lysosomal compartments[707] and to the lysosome is regulated by its binding to LRP1[708, 709] and sphingomyelin[710] and to the PSAP receptor GPR37.[711]. PSAP is increased by CTNNB1[712], TP53[713] and in the striatum by the DA transporters SLC6A3[714] and SLC18A2[714] (not shown). In the lysosome, PSAP binds CTSD[715], that regulates the proteolytic processing of PSAP into saposins A, B, C and D[716]. These saposins are associated to PD-related mechanisms i.e. saposin A and B deficient mice show altered autophagy[717], saposin A and B knockout mice show increased number of foot slips in the narrow bridge test (a behavioral test to assess motor balance and coordination) and develop a tremor[718]. Further, saposin C attenuates MPTP toxicity[719]

and binds[720] and activates **GBA**[721-723], whereas saposin D increases **ASAH1** activity[723, 724]. Thus, saposin C and D stimulate the conversion of glucocerebroside to ceramide (via **GBA**), to sphingosine (via **ASAH1**) respectively.
Of note, the PSAP receptor, GPR37 is associated with juvenile parkinsonism[157], is accumulated in PD lewy bodies[156] and its overexpression induces macroautophagy[725]. GPR37 increases ER stress and expression of HSPA5[725,726] (not shown) and **PARK2**[726]. GPR37 binds HSPA8[727] and SLC6A3[301, 728]. Further, **PARK2** binds, increases the ubiquitination and increases the degradation of GPR37 and thereby prevents its aggregation and subsequent ER stress-mediated neuron death[157, 727, 729, 730]. GPR37 is involved in the expression of SLC6A3[301] and the uptake and quantity of DA in the striatum[728, 731, 732]. So, overall, PSAP regulation is important for activation of the sphingosine synthesis pathway and in maintaining normal DA levels in the striatum.

B1.2.1 Sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P), formed through phosphorylation of sphingosine by sphingosine kinase 1 (SPHK1)[733], is an immune attractant (see section D2.2.2.1) and is transported out of the cell by the transporter **SPNS2**[734]. Further, S1P activates the UPR and ER stress[735, 736] and is, similarly to cholesterol, transported by lipoproteins and involved in lipid raft functioning[590, 735-737]. Sphingosine and cholesterol metabolism are linked, illustrated by S1P activation of **SREBF1**[737] (the cholesterol uptake proteins SCARB1 and LDLR are upregulated by S1P[590]) and the regulation of **GBA** activity by cholesterol[707].

The S1P receptors bind and increase activation (after activation by S1P) of the G protein **GNA12** to regulate cell shape and motility[738, 739]. **GNA12** transduces extracellular signals over the membrane[312] and also binds to CDH2[740], **CXCR4**[741], **LRRK2**[742] and the cytoplasmic proteins **PRKCE**[743] and **AXIN1**[744]. Thus, S1P activates **GNA12**[739] and activates CTNNB1[740], GSK3B[745] and NF-KB[746], increases the expression of the immune regulator **ITGA6**[747], the diacylglycerol (DAG) activated calcium channel **TRPC6** (increasing Ca^{2+} influx)[748] and NOS2[749]. NOS2 expression is also increased by **LRRK2**[750], and **SREBF1**[751] and decreased by **COL18A1**[752] (not shown) and NOS2 itself increases the expression of perforin (**PRF1**)[753] and IL6[312], indicating a role in the immune response (part of main process D).

B1.3 Cerebrosides and gangliosides

Cerebrosides are primarily found in nervous tissue and are reduced in lipid rafts of the frontal cortex of PD patients[675], but increased in the plasma of PD patients compared to controls[674]. Further, **GBA**, the glucocerebrosidase that converts glucocerebroside to ceramide, is downregulated in PD patients compared to controls[98, 754]. Glucocerebroside can be converted into globoside by addition of a galactose, further addition of the sugars sialic acid, N-acetylgalactosamine and galactose results in the synthesis of

respectively the gangliosides GM3, GM2 and GM1. GM1 is the most common ganglioside in the brain and is involved in neuronal plasticity and repair as seen by its protective effects after a mechanical lesion of the dopaminergic nigro-striatal system[755]. Development of parkinsonistic features, i.e. motor impairment, striatal DA depletion, loss of TH positive neurons and **SNCA** aggregation were seen in mice devoid of the GM1[756, 757]. Whereas GM1 administration in mice treated with MPTP (a model for PD) resulted in partial restoration of DA neurons in the SN[758, 759]. Further, in a cell model for lysosomal disease, characterized by reduced lysosomal activity, lysosomal cytotoxicity, and inhibition of the autophagy-lysosomal pathway resulting in **SNCA** accumulation, GM1 administration reversed the phenotype[760]. Moreover, use of ganglioside GM1 by PD patients improves their motor symptoms and slows down symptom progression[761], even over a five-year period[762]. And, anti-GM1 ganglioside antibodies are increased in the serum of PD patients compared to controls[763]. Lastly, GM1 content in the brain decreases with age, while GM3, a minor brain ganglioside, is increasing[764]. GM3 has a higher binding affinity to **SNCA** than GM1[765] and can specifically regulate **SNCA**-induced pore formation[766, 767] (not shown). **SNCA** membrane association, by binding to GM3 or GM1, induces folding of an alpha-helix domain that displays a high affinity for cholesterol enabling it to get inserted in a cholesterol rich part of the plasma membrane (lipid raft) and form an oligomeric ion channel[765]. In this way, **SNCA** can form pores in the neuronal plasma membrane[767] that increase Ca^{2+} influx, increasing synaptic vesicle release and increase synaptotoxicity[768]. Of note, defects in the endocytic pathway and membrane trafficking to the lysosome results in accelerated release of exosome-associated GM1[769] and these exosomes (extracellular vesicles secreted by the cell) containing GM1 or GM3 accelerate the aggregation of **SNCA**[770] (not shown). Moreover, GM1 binds **MBP**[771] (not shown) and GM3 binds the INSR[772] (not shown) and decreases expression of ICAM1[773] (not shown). Further, the proprotein PSAP binds GM1 and GM3 and may function as a ganglioside transport protein for transport into the cell[774] (not shown), whereas its proteolytic cleavage product saposin B increases the degradation of GM1 in lysosomes[723, 775] (not shown).

Thus, ganglioside content in plasma or exosome membranes, which is regulated by among others the endosomal-lysosomal pathway and PSAP, may affect membrane function, but also **SNCA** membrane association and thereby be important in PD pathophysiology.

B2. The endosome-lysosomal system

The endosome-lysosomal system recycles and catabolizes material taken up by endocytosis, from the external milieu or from the cytosol by autophagy. The endosomal-lysosomal system regulates protein trafficking, sorting, recycling and degradation, and microtubular motor transport. Moreover, in immune cells, the endosomal-lysosomal system also functions in protein processing for antigen presentation. Its main

subprocesses include clathrin- and caveolae-mediated endocytosis, the trafficking, targeting and recycling of vesicles, as well as lipoprotein uptake and processing. Particularly, the organization of membrane components in lipid rafts, such as cholesterol and sphingolipids (including sphingomyelin and ceramide), regulates the fluidity of the membrane[676, 776, 777], and is essential for the clustering of receptor molecules, recruitment of intracellular signaling molecules[778-780], endocytosis, membrane trafficking and activation of the immune response[781, 782]. Lipid rafts, i.e. cholesterol-rich microdomains, are located on plasma, endosomal, lysosomal, ER and mitochondrial membranes. Interestingly, oxysterols can regulate membrane fluidity[783] and the formation of lipid rafts[777]. Further, also the familial PD protein **LRRK2** and cholesterol affect lipid raft function[784-787]. Moreover, membrane cholesterol affects the DA uptake/efflux function of the PD-associated[232-234, 788] DA transporter SLC6A3[789-792] that is located in both lipid rafts and non-raft membrane regions[789] and undergoes clathrin-mediated endocytosis[793, 794]. In lipid rafts SLC6A3 binds to the PD associated[28] and SN specific[504] **RIT2** that regulates SLC6A3 internalization and functional downregulation[795], and may be involved in the survival of DA neurons[504].

The next paragraphs will discusse clathrin-independent (B2.1) and -dependent (B2.2) endocytosis and their role in cholesterol and lipoprotein uptake, vesicle trafficking and sorting.

B2.1 Clathrin-independent endocytosis

Clathrin-independent endocytosis occurs by caveolae, i.e. specialized lipid rafts in caveolin-1 (CAV1)-enriched plasma membrane invaginations[796, 797]. **SNCA** and **PRKCE** increase the expression of CAV1[798, 799] and thus may promote the formation of caveolae. CAV1 binds to and increases lipid raft localization of the gap junction protein **GJB2**[800]. CAV1 also binds to PTEN[801], INSR[802], **SCARB2**[803], RAC1[804] (not shown), SCARB1[805] (stabilization by **PARK2** prevents its degradation[806, 807]), LRP1[808], CTNNB1[809], JAK2[810] and STAT3[811], indicating that CAV1 and thus caveolae regulate the JAK2/STAT3 pathway as well as cholesterol (SCARB1, **SCARB2** and LRP1) and INS (INSR) signaling. Moreover, CAV1 increases the cholesterol content of lipid rafts[812], decreases cholesterol esterification[813] and increases cholesterol efflux[814]. In turn, cholesterol increases CAV1 expression[815, 816] (not shown), indicating a complex interaction between CAV1, lipid rafts and cholesterol. Further, in lipid rafts sphingosine can be converted into sphingosine-1-phosphate (S1P) by sphingosine kinase 1 (SPHK1)[733]. S1P increases the localization of CAV1 and actin cytoskeleton-regulating proteins, such as the CAV1-binding **COL18A1** (endostatin)[817], to lipid rafts[818], enabling cytoskeleton regulation that is necessary for caveolae-mediated endocytosis. Defective sphingosine production (as regulated by the enzymes **GBA** and **ASAH1**) can therefore have major effects on lipid raft function and (caveolae-mediated) endocytosis by the cell. The CAV1-binding[819, 820] microtubule-

associated protein DNM2 mediates endocytosis and vesicle budding in caveolae[821] and further binds to **CACNA1A**[505], **MCC**[387], **AMPH**[822], **AMPK**[387] and DRD2[823], and binds to and colocalizes with the myosin motor protein **MYO1E**[824] that is involved in freshly-budded vesicle trafficking. The caveolae-localized potassium voltage-gated channel subunit Kv1.5 (**KCNA5**) is regulated by angiotensin II[825], cholesterol, sphingolipid and oxLDL[825-827] and linked to apoptosis[828]. And lastly, the transcription factor **RFX4** heterodimerizes with RFX3[829], a transcription factor that binds to the promotor of **DNAH11**[830]. **DNAH11** is a protein that is involved in microtubule motor activity and genetically associated with LDL levels[831].

B2.2 Clathrin-dependent endocytosis

Clathrin-mediated endocytosis is regulated by the clathrin- and dynamin-binding protein amphiphysin (**AMPH**)[822, 832-837] and by the adaptor protein 3-complex (**AP3**, subunit **AP3B1**) that binds and sorts proteins to endosomes and lysosomes[838]. Association of the endocytic clathrin-coat with the actin cytoskeleton is regulated by the genetically PD-linked HIP1R[77, 839, 840]. The myosin motor protein **MYO1E** (required for actin assembly during clathrin-mediated endocytosis)[841], **AMPH**[842], but also the cytoplasmic actin-bundling and calcium-sensitive protein fimbrin (**PLS1**)[843] and RAC1 (necessary for actin polymerization during endocytic clathrin-coated pit formation)[844] are involved in actin formation during endocytosis, whereas the cytoplasmic monooxygenase **MICAL2** promotes depolymerisation of F-actin[312]. RAC1 binds to and is activated by the familial PD proteins **LRRK2** (changes the cellular localization of membrane-bound RAC1)[845] and **PARK2**[846], the Rho GTPases **ARHGAP33** and **ARHGAP44**[312, 847, 848], binds to the nuclear importin **KPNA4**[848] (not shown), and is activated by **RAP1A**[849], **RIT2**[850] and **NEDD9**[851] and inhibited by PTEN[852].

In addition to endocytosis, clathrin is also used for protein sorting towards lysosomes[853, 854]. The clathrin-binding proteins **CLVS2** and **TOM1L2** are required for normal endosome/lysosome morphology[855] and protein trafficking to the lysosome[856] respectively. Of note, the ubiquitin ligase NEDD4 promotes degradation of **SNCA** by the endosomal-lysosomal pathway and is located in lewy bodies[857, 858]. NEDD4 binds to **SMAD5**[859] (not shown), **RAP2A**[860], **ZAK**[861] (not shown), **GBA**[861] (not shown), **TOM1L2**[861], **MRPL19**[861] (not shown) and binds and increases mono-ubiquitination of **DCUN1D1**[862] (not shown).

To uncoat clathrin-coated vesicle – necessary for fission with the target membrane – DNAJC6 or its homologue **GAK** (also referred to as auxilin 1 and 2, respectively) recruit the (in the PD SN downregulated[171]) clathrin uncoating ATPase Hsc70 (HSPA8)[863-865]. Both **GAK** and HSPA8 bind to the familial PD lysosomal protein ATP13A2[866] (found in the lewy bodies of remaining PD DA neurons[867]), which deficiency leads to lysosomal dysfunction and **SNCA** aggregation[868]. Further, depletion of either **GAK** or HSPA8 inhibits clathrin-

mediated endocytosis[869] and the **GAK**-**LRRK2** complex promotes golgi-derived vesicle clearance through the autophagy–lysosome system[870].

B2.2.1 Vesicle trafficking and recycling

PCM1 and **FAM190A** are both involved in early vesicle trafficking, by binding the dynein-mediated organellar transport regulator NDEL1, the dysfunctioning of which results in delayed endocytic-lysosomal compartment formation[871]. Further, the lysosomal trafficking protein **VPS41** binds the **AP3**-complex[872, 873], reduces **SNCA** accumulation and caspase activation, and is protective against **SNCA** overexpression and the neurotoxins 6-OHDA and rotenone in PD models[874]. Lysosomal trafficking by **AP3B1** (**AP3**-complex)[875] includes the membrane protein **SCARB2**[876] that regulates lysosomal targetting of **GBA**[877], enabling **GBA** to convert glucocerebroside into ceramide (see section B1). Further, Localization of **LAMP1** (lower expressed in DA neurons in the PD SN[34]) to the endosomal / lysosomal membrane is regulated by **AP3**[875, 878]. **LAMP1** decreases expression of LAMP2[879] and both **LAMP1** and LAMP2 regulate cholesterol traffic and decrease cholesterol accumulation[880].

Recycling of (endosomal) vesicles is regulated by multiple proteins in the landscape. The familial PD protein[260] VPS35 is part of the retromer complex for endosome/trans-golgi network transmembrane receptor recycling and the sorting of cargo proteins[881, 882], e.g. for sorting of the lysosomal acid protease cathepsin-D (CTSD)[883]. The retromer complex interacts with the PD-linked DNAJC13[148], an endosomal recycling component regulating early-endosome clathrin-coat dynamics[148]. DNAJC13 binds to GSK3B[884] and HSPA8[885]. Further, **RER1** regulates retrograde vesicle-mediated transport of proteins from the golgi to the ER[886]. **RER1** binds the γ-secretase complex (by bind to e.g. PSEN1) and thereby increases the retention and retrieval of this complex and its subunits in the ER, preventing γ-secretase activity[887-889] (for more on the γ-secretase complex see section C2.4).

The membrane fusion protein **NSF** is required for endocytic vesicle/golgi fusion[890], vesicle-mediated ER-golgi transport[891], clathrin-coated vesicle/target membrane fusion[892, 893], thus regulating endocytic recycling[894] and exocytosis[895]. **LRRK2** binds to **NSF** (thus controling vesicle recycling)[896] and interacts with **SNCA** ([406, 897, 898] that inhibits vesicle recycling[899]. The cytoplasmic protein **SYN3** localizes to the membrane of cytoplasmic vesicles and decreases release of DA in the striatum[900] and is downregulated by **SNCA**[901]. **FER** also binds to and phosphorylates **NSF**, thus inhibiting subsequently vesicle fusion[902]. **NSF**-attachement protein beta (**NAPB**) helps **NSF** binding to the SNARE complex[903] and is thus involved in vesicle fusion and exocytosis. Other exocytosis-regulating proteins are **RPH3AL**[904] (involved in INS secretion[905]) that binds to **UNC13B**[312, 906] and the ER-stress-induced[907], CCL5-regulating[908] clathrin-coated vesicle protein[909] **SCAMP5**[908]. The PD associated protein SYT11[76, 77, 104] regulates Ca^{2+}-

dependent exocytosis of secretory vesicles[312] (the Ca^{2+}-dependent protein **SCIN**[312] also regulates exocytosis by regulating the actin cytoskeleton during exocytosis[502]). SYT11 binds ATP13A2[866] (not shown) and **PARK2**, that also increases SYT11 degradation[910]. The early-onset Parkinsonism-associated SYNJ1[240, 241] is involved in synaptic vesicle recycling[240] and components of this machinery, i.e. it binds to **AMPH**[911], **MYO1E**[824], **PARK2**[912], clathrin[913] and the calcium-channel subunit **CACNA1A**[505]. The recycling endosome membrane protein **RAP2A**[914] binds to the mTORC1 complex[915] and the **SNCA**-binding phosphoprotein **ENSA**[916-918]. Dysfunctioning of the **AP3** complex regulator **AGAP1** (that binds **AP3** and regulates **AP3**-dependent trafficking)[919], affects striatal DA release, linking endocytic recycling to DA release[919, 920].

B2.2.2 Lipoprotein uptake and processing

Lipoproteins are composed of lipids, triglycerides and proteins. Very low-density lipoprotein (VLDL) particles are enriched in triglycerides and the removal of triglycerides from VLDL results in the formation of intermediate density lipidprotein (IDL)[921]. IDL is enriched in cholesterol and can subsequently be converted by **LIPC** into LDL[312], which is highly enriched in cholesterol. The clathrin-mediated endosomal-lysosomal system facilitates lipoprotein (e.g. VLDL, LDL) uptake via (among others) the VLDL receptor (VLDLR) and LDL receptor (LDLR)[312]. Early endocytic vesicle acidification by the V-ATPase (**ATP6V0A1**) releases LDL from the LDLR. LDL is then degraded in the lysosome, and cholesterol is salvaged for cellular use[922] and increases the expression of the transporter **ABCG1**[923-925] that subsequently exports cholesterol to HDL[926, 927] and thereby also inhibits plasma membrane lipid raft formation[928]. LDLR expression is in turn increased by **PRKCE**[929] and its activation inhibited by **MAP2K6**[930] that is activated by **LRRK2**[931]. Uptake of IDL and HDL is regulated by the receptors SCARB1 (upregulated by FOXO1)[632] and **SREBF1**[932], and downregulated by **ASAH1**[590] and LRP1 respectively, both of which are affected by the extracellular matrix (ECM) lipase **LIPC**[933-935]. **LIPC** deficiency increases serum HDL-cholesterol[936]. The expression of the APOE receptor LRP1 (binds to **SERPING1**[708]) and APOE itself is increased in early PD[117], which may indicate a defect in lipoprotein regulation in early PD.

The ER serine protease **TMPRSS3**, the plasma membrane serine protease **TMPRSS9**, the anti-apoptotic protein[937, 938] **RXFP1**, the pore forming complement system member **C9**, and the lipoprotein uptake receptors VLDLR, LRP1 and LDLR all contain a LDLR class A domain that can bind LDL[312].

B3. The lysosome-autophagosomal system

B3.1 AMPK and mTORC1 regulated autophagy

The AMP-activated protein kinase (**AMPK**)-complex and the mTORC1-complex are both essential for autophagy functioning[939, 940]. The mTORC1-complex (composed of MTOR, RPTOR, MLST8, AKT1S1 and DEPTOR) is a nutrient sensor that controls protein

synthesis for cell growth and proliferation and is activated by INS, growth factors and oxidative stress[941, 942]. mTORC1 is involved in DA neuron survival, whereas the mTORC1 inhibitor rapamycin is neuroprotective in *in vitro* and *in vivo* PD models[943] and blocks translation of the MTOR inhibitor DDIT4, a protein that is elevated in PD SN neurons and mediates cellular death in PD models[147], indicating that inhibition of only certain mTORC1 functions is beneficial for neuronal survival. Of note, prolonged treatment of PD patients with the DA precursor L-DOPA results in L-DOPA-induced dyskinesia, which is associated with persistent activation of mTORC1[944].

The **AMPK**-complex – consisting of an α-subunit (**PRKAA1** or PRKAA2), a β-subunit (PRKAB1 or PRKAB2) and a γ-subunit (PRKAG1, PRKAG2 or PRKAG3)) – is a cellular energy sensor that is activated when intracellular ATP levels are low[940] and subsequently tries to restore cellular energy (ATP) levels by stimulation of fatty acid oxidation, glycolysis, glucose uptake and ketogenesis and inhibition of synthesis pathways for proteins, glycogens, fatty acids and cholesterol[945-948]. **AMPK** activation results in increased oxidative stress, release of mitochondrial cytochrome c and mitochondrial caspase pathway activation[949]. The mTORC1-complex is regulated by **AMPK**, i.e. **AMPK** binds mTORC1[950] and inhibits mTORC1 activation[940, 950], thereby preventing mTORC1-mediated autophagy inhibition[940] and mTORC1-mediated **SREBF1** activation[951]. Following mTORC1 inhibition (by **AMPK**), autophagosomes form[952] and fuse with the lysosome to degrade their cargo[953]. The PD-associated proteins ATG5 en ATG7[120, 121] are involved in autophagosome assembly[954]. ATG5 binds to the familial PD protein UCHL1[387] and SIRT1[646], activates JAK2[955], and is essential in T- and B- lymphocyte survival and proliferation[312] (not shown; part of main process D). Both ATG5 and ATG7 are involved in mitochondrial quality control following oxidative damage[312]. ATG7 increases autophagy[956], decreases **SNCA**[957] and **LRRK2**[957] aggregation, activates CASP3[958], JAK2[955] and STAT3[959] and, like ATG5, binds to and is deacetylated by SIRT1[646, 960]. In a PD mouse model, conditional deletion of ATG7 results in age-related loss of DA neurons and loss of striatal dopamine[961]. Together, these results indicate that proper autophagy is important to prevent DA neuronal loss and PD.

In addition to inhibition by **AMPK**, mTORC1 activity is also inhibited by **ULK2**[962], GSK3B[963], inhibited and decreased by PTEN[964, 965] and FOXO1[633, 966] and activated by **ATF6**[967] and INS[968, 969]. mTORC1 in turn inhibits GSK3B[970, 971] and CASP3[972], increases the expression of PTEN[636], activates and increases the nuclear expression of **SREBF1**[973, 974], regulates **BAMBI**[975] (not shown), binds the deubiquitinase **USP9X**[976], the NAD-dependent deacetylases SIRT1[643] (not shown) and SIRT7[394], the transcription factor EIF4E[977] and binds and phosphorylates the repressor of translation initiation **EIF4EBP2**[977, 978]. Hypophosphorylated **EIF4EBP2** competes with the familial PD protein EIF4G1 to interact with the translation initiation factor EIF4E[312, 979]. Thus, mTORC1 favors the binding

of EIF4E to EIF4G1[979], which results in recognition of the mRNA cap and initiation of translation[312, 980] (e.g. **LSM7** mRNA translation[314]) (part of main process C).

STK11 is a master upstream kinase that increases the activity of **AMPK** and **AMPK**-related kinases[981]. Deacetylation of STK11 by SIRT1 increases STK11 activation and subsequent **AMPK** activation[982]. In addition, STK11 also binds **AMPK**[983-987], the kinase **SIK1**[988, 989], **SNRK**[990] (a distant **AMPK** relative[981]), **PARD3**[991], GSK3B[992], PTEN[993], inhibits MTOR[994], increases the expression of PTEN[995], **TIAL1**[995] and **NCAM2**[995], decreases the expression of **CNTN1**[995] (**CNTN1** binds **SNCA**[461]) and binds and activates both TP53[996, 997] and MARK2[988, 989]. MARK2 is an **AMPK**-related kinase[981] that binds to **AMPK**[998], **CEP85L**[999], **SNCA**[461], **CACNA1A**[505], binds and activates PINK1[1000] and is inactivated by **PARD3**[1001]. Further, **AMPK** also binds to and inhibits NF-KB[1002, 1003], binds to **SIK1**[998] (inhibits nuclear **SREBF1**[1004]), **SNRK**[998] and **SND1**[954], regulates **BAMBI**[975] (not shown) and binds to, inhibits and increases expression of SIRT1[648, 1005, 1006]. Furthermore, familial PD proteins VPS35[387] and EIF4G1[387] directly bind to **AMPK**, while **LRRK2** (mutations in **LRRK2** have been associated with autophagy impairment[1007]) activates **AMPK**[1008], indicating that altered **AMPK** function may be important in PD. The serine/threonine-protein kinase and **AMPK** inhibitor[1009] **ULK2** is involved in autophagy and is activated following phosphorylation by **AMPK**[1010]. **ULK2** is also both a downstream effector[952] and a negative regulator of mTORC1 signaling[962] and **ULK2** expression is increased by **SREBF1**[1011]. Thus, **AMPK** and **SREBF1** regulate autophagy and mTORC1 activation via regulation of **ULK2**. Moreover, **ULK2** functions as a negative feedback loop for both **AMPK** (directly) and **SREBF1** (via inhibition of mTORC1).

Lastly, after Ser473-phosphorylation, AKT1 activates the mTORC1 activator **SAMD4A**[1012], that is necessary for mTORC1 activation. In control brains, AKT1 and Ser473-phosphorylated AKT1 are expressed at high levels in DA neurons in the SN, whereas PD patients show diminished brain levels of both total and Ser473-phosphorylated AKT1[113]. In contrast to neuronal loss of AKT1 in PD, both phosphorylated and unphosphorylated AKT1 are increased in glia cells in the PD brain SN[113]. Further, AKT1 Ser473-phosphorylation is increased by the familial PD proteins PARK7 (by binding and inhibiting the negative AKT1-regulator PTEN)[1013, 1014], PINK1 (by activating the mTORC2-**PRKCE**-AKT1 pathway)[634, 1015] and **LRRK2** (that also binds to AKT1)[1016]. AKT1 is also activated by **PRKCE**[1017] (binds **SNCA**[532]), **PIK3CD**[1018, 1019], inhibited by **PTPN1**[1020, 1021] (which is again inhibited by AKT1[1022]) and bound and inhibited by the **LRRK2** phosphatase PPP1CA (**PP1**)[1023, 1024].

In summary, activation of the mTORC1 complex is regulated by multiple proteins in the PD landscape, either indirectly (via **AMPK** and AKT1) or directly (via DDIT4, **ULK2** and **SAMD4A**). mTORC1 subsequently regulates autophagy, cholesterol homeostasis (via

SREBF1), apoptosis (via CASP3) and translation (via EIF4E, EIF4G1 and **EIF4EBP2**).

B3.2 Lysosomal acidification

Lysosomal function and autophagy require organellar acidification that involves **PLEKHM1** via its Rab7-binding domain RUN[1025, 1026]. The PD-associated Rab7-like protein **RAB7L1**[102, 106] interacts with **LRRK2** to modify intraneuronal protein sorting[1027] and binds to the pre-mRNA splicing factor **LSM7**[1028]. The familial PD lysosomal ATPase ATP13A2[1029-1031] is decreased in PD SN[867] and its dysfunctioning leads to lysosomal membrane instability, reduced processing of lysosomal proteins, diminished degradation of lysosomal substrates, reduced clearance of autophagosomes and impaired lysosomal acidification which subsequently may contribute to the formation of Lewy bodies, a hallmark of PD[867, 1007]. **ATP6V0A1** is a subunit of the V-ATPase, a proton pump essential for lysosomal acidification[1032], and binds to **SNCA**[461]. Lysosomal V-ATPase activity is regulated by **DRAM1**[1033], which is activated by TP53[1034], and activates the lysosomal acid protease cathepsin-D (CTSD)[1035] (lower expressed in PD SN neurons)[33] and autophagy following mitochondrial dysfunction[1033]. CTSD is necessary for lysosomal protease activity, but e.g. also for proteolytic cleavage of PSAP into saposin C and D, peptides that activate **GBA** and **ASAH1** (see also section B1.1.2). Conversion of ceramide into sphingosine and *vice versa* by **ASAH1** in the lysosomes is pH-dependent[544, 1036]. Therefore, lysosomal pH, controlled by the proton V-ATPase (including subunit **ATP6V0A1**), affects celllular ceramide and sphingosine levels, that may affect cellular membrane regulation and apoptosis (see section B1.1). Of note, lysosomal acidification (and thus stability) is also dependent on lysosomal cholesterol membrane content[1037, 1038], again illustrating the importance of proper cholesterol regulation for normal lysosomal functioning.

B3.3 (Neuro)melanin regulation

PD is characterized by selective death of SN DA neurons containing neuromelanin (NM), suggesting involvement of NM in PD pathogenesis. There is an age-related increase in NM in the SN[1039], and NM production, through oxidation of DA, increases oxidative stress and lipid peroxidation (see section A1.1). NM is cytotoxic[419, 1040-1042] and results in collapse of mitochondrial transmembrane potential, cytochrome c release and CASP3 activation[419]. Therefore, DA neurons need an optimal autophagy and lysosomal function to store NM and prevent cytoplasmic NM-toxicity[1040]. Moreover, cellular increase in NM might eventually interfere with the endosomal-lysosomal pathway and lysosomal function[1040]. Release of NM in the ECM, e.g. as a consequence of DA neuron death, increases immune cell activation[1043, 1044] (see also section D2.2). Nevertheless, due to its ability to chelate ferrous iron[338, 339] and free radicals, NM may in addition to its toxic properties also have neuroprotective functions[1043, 1044].

Of note, NM has the ability to absorb lipids, e.g. cholesterol[1045] and associates with **SNCA**[1046, 1047] that itself contains two cholesterol-binding domains[1048]. Further, **SREBF1**

increases the production of both cholesterol and isoprenoid dolichol[1049, 1050], both lipid components of neuromelanin[1051]. This indicates that there may be a complex interaction between NM, **SNCA** and lipid accumulation in DA neurons. Dysregulation of either NM, **SNCA** or, for example, cholesterol may increase their aggregation and cytotoxicity.

Proteins regulating the melanin producing melanocytes in the periphery, like MC1R, MITF, **MREG** and TYR, might also be involved in the regulation of neuromelanin in DA neurons. Of note, mutations in the melanocyte-stimulating hormone receptor MC1R may increase the risk for PD[1052, 1053] and MC1R binds to the DA neuron determinant[1054] **MSX1**[1055] (not shown, see also section section D1.1). Activation of MC1R triggers transcription of MITF[1056], a transcription factor involved in melanocyte development and melanin production[1057]. MITF expression is increased by cholesterol[1058] and decreased by STAT3[1059]. Further, MITF is activated by GSK3B[1060], cleaved by CASP3 (this cleavage is essential in apoptosis of melanocytes)[1061] (not shown), binds to CTNNB1[1062] and STAT3[1063] and increases the transcription of **ASAH1**[1064], SCARB1[1064], **PRF1**[1065], **MBP**[1064], **COL2A1**[1066] (also increased by **MSX1**[1067] and **KDM2B**[1068]), MC1R[1069] (binds to PTEN[1070] and its expression is decreased by retinoic acid[1071] (not shown)) and the melanin producing[1072] TYR[1064, 1073]. Subsequently, the lysosomal maturation protein melanoregulin (**MREG**) is involved in the transfer of melanin-containing melanosomes from melanocytes to keratinocytes, and as such drives skin and hair pigmentation[1074-1076]. **MREG** dysfunction results in an increased secretion of CTSD[1077]. The **AP3**-complex-regulated[1078, 1079] membrane protein **OCA2** is involved in melanin synthesis[1080] and regulates trafficking of TYR[1081] that is mediated by the **AP3**-complex[1082]. Further, **OCA2** binds to the familial PD protein ATP13A2[866] (ATP13A2 inhibits **SNCA** aggregation[868]) and loss of **OCA2** disrupts the unfolded protein response (UPR) and increases resistance to ER stress in melanocytes[1083]. TYR mRNA and protein are expressed in the SN[1084-1086] and increased TYR levels are toxic for DA neurons and can exacerbate the toxic effect of mutant **SNCA**[1086]. Of note, decreasing hair color darkness is associated with an increased PD risk[1052] and familial grey hair frequency is higher in PD patients[1087], underscoring a possible association between peripheral melanin regulation (e.g. by MITF, MC1R, **MREG** and TYR) and PD.

B3.3.1 Vitamin D3

Active vitamin D3 (calcitriol) increases melanocyte maturation, inhibits their proliferation and increases TYR activity (by inducing MITF expression[1088]) and melanin production[1088, 1089]. Vitamin D3 is synthesized from a cholesterol precursor in the skin under influence of UV light[1090]. Vitamin D3 is lower expressed in PD patients[1090-1092] and its supplementation may stabilize PD for a short period[1093]. Vitamin D3 regulates the expression of PPP1CA[1094] (not shown) and **NEDD9**[1094] (expression decreased by **ZBTB20**[1095]), increases the expression of **HPGD**[1096], **FBN1**[1094], **GCH1**[1097], PLAT[1098], GDNF[1099]

and SERPINB9[1100] and decreases the expression of **ANGPT2**[1101], **IL2RA**[1102], **BMP7**[1103] (not shown), **LTBP1**[1094] and **SREBF1**[1104]. Vitamin D3 also activates JAK2[1105], inhibits SERPINE1 expression by inhibiting NF-KB activation[1106] and inhibits the immune response by reducing the production of CCL5[1107] and IL8[1107], by inhibiting NF-KB-mediated IL12 expression[1108] and IFNG-activation of macrophages[1109].

The vitamin D3 receptor (VDR) is a nuclear transcription factor that is associated with PD[257-259] and binds to **ACTN4**[1110], the transcription factors **POU2F1**[1111] and **RUNX3**[1112] and the transcription regulators SIRT1[654] and **MED13** (also activates VDR)[1113]. The VDR is transported into the mitochondria by the mPTP[612] and its expression is increased by **MAP2K6**[1114]. The active vitamin D3, binds to the VDR and thereby regulates gene expression in the nucleus of the cell. The VDR (bound by vitamin D3) inhibits **CREM**[1117] and decreases the expression of the immune response-associated proteins CCL5[1115] and ICAM1[1116].

Thus, vitamin D3 and its receptor VDR regulate transcription (via NF-KB, **POU2F1**, SIRT1, **SREBF1, RUNX3**, **MED13**), pigmentation (via MITF, cholesterol homeostasis (via **SREBF1**), coagulation (via **BMP7**, **LTBP1**, SERPINE1, PLAT), and the immune response (e.g. via JAK2/STAT3, NF-KB, CCL5, ICAM1). Moreover, as vitamin D3 and its receptor VDR can regulate production of melanin in the skin, they might also affect neuromelanin production and indirectly DA production in DA neurons in PD as indicated by decreased DA neuron death[1118] and partially restored TH expression[1099] by vitamin D3 in PD models.

B3.4 Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) is the targeting of cytosolic proteins to the lysosomal membrane by chaperone HSPA8. The HSPA8-substrate complex binds to the lysosomal-associated membrane protein type 2A (LAMP2), so they can be translocated over the lysosomal membrane and subsequently degraded[1119]. LAMP2 is reduced in peripheral leukocytes of PD patients[192] and both LAMP2 and HSPA8 are reduced in the PD SN[171]. LAMP2 is degraded in cholesterol-rich lipid rafts[1119, 1120]. Cholesterol depletion of lysosomal lipid rafts therefore enhances CMA activity, whereas lysosomal cholesterol loading reduces CMA activity[1119, 1120]. LAMP2 also decreases cholesterol accumulation[880] and its reduced expression may thus affect cholesterol regulation in PD patients. Moreover, reduced levels of LAMP2 and HSPA8 affect MHC class II molecules[1121] and therefore immune cell activity. Of note, the lysosomal membrane protein **LAMP3**, is involved in adjusting lysosomal function after the transfer of peptide-MHC class II molecules to the surface of dendritic cells[1122] and increases autophagy[1123].

HSPA8 also binds to (and increases degradation of) ATP13A2[866], **LRRK2**[1124], **SNCA**[918, 1125], **PARK2**[727, 1126], UCHL1[1127], **MAPT**[1128], TP53[1129], AKT1[1130], **AMPK**[1131], the NF-KB-complex subunits REL, RELA, RELB and NFKB1[1132], **LIPC**[1133], NEDD8[1134] (is associated with PD lewy bodies[200], binds **RPL7A**[1135] and UCHL1[846] and binds **PARK2** and PINK1 and increases their

ligase activity and stabilization respectively[200]), DNAJC6[1136], **FBXO25**[395] (not shown), **HLA-DRA**[1137], AR[1138], **AP3**-complex[1139], **BAG6**[1140], JAK2[1141], **GAK**[863, 1142], MAP3K7[1132], GSK3B[1131], **CCAR2**[1143], BAX[1144] (not shown) and LAMP2[1145]. LAMP2 also binds to UCHL1[288], TP53[1146] and **SNCA**[1147], and its expression is increased by FOXO1[632] and the **AP3**-complex[875] (not shown) and is mediated by the NF-KB-complex[1148] (not shown). Thus, HSPA8 and LAMP2 bind to at least five familial PD proteins and interacts with several key landscape proteins. Overall the CMA proteins HSPA8 and LAMP2 are required for a normal degradation of (aggregated) **SNCA**[1149, 1150]. This notion is underscored by mutant **LRRK2**[1124] and mutant UCHL1[288] that both inhibit CMA, which in turn results in increased **SNCA** aggregation[288, 1124]. Of note, in contrast to the familial PD proteins **LRRK2** and UCHL1, mutant TP53 is normaly degraded by CMA[1146].

B4. Reverse cholesterol transport

Reverse cholesterol transport is the routing of excess cholesterol and oxysterols back to the liver and the subsequently excretion from the body. Several key components of the landscape are involved in these processes, which contribute to cholesterol homeostasis. First, in the liver, the familial PD protein **PARK2** is a lipid-responsive regulator of fat uptake and thereby increases hepatic fat uptake[806]. Second, the neuronal ATP-binding cassette transporters **ABCA3** and **ABCA5** (located on lysosomal/late endosomal membranes[1151-1153]) and **ABCG1** (located on ER, golgi, late endosome and plasma membrane[1154-1156]) are involved in reverse transport, by transporting cholesterol into the lysosomes (**ABCA3**)[1152] and increasing cholesterol efflux to HDL (**ABCA5** and **ABCG1**)[926, 927, 1153]. Of note, the prospective studies that found association of low plasma LDL-cholesterol and low total cholesterol with increased PD risk[552-554] and the positive correlation between PD disease duration and plasma HDL-cholesterol[375], indicate that lipoprotein-cholesterol levels are affected in PD, which may be due to dysregulation of reverse cholesterol transport regulated by these transporters and **PARK2**.

PD patients have higher plasma oxLDL[177] and 7-KC[564], the main cholesterol oxidation product in oxLDL[565]. Statins, inhibitors of cholesterol synthesis, also decrease oxysterol levels in the brain[566] and reduce the aggregation of **SNCA** in vitro[567] and in **SNCA** transgenic mice[568]. Statin use has been associated with lower PD risk[1157, 1158] and even prospective studies found lower PD risk when using statins[1159, 1160]. However, statin use has also been reported not to affect PD risk[1161-1164], and it is unclear if the PD risk lowering is due to statin use, or merely due to high baseline cholesterol levels of these statin users. The only prospective study, that did took baseline cholesterol levels before statin use and during PD treatment into account, showed association between statin use and *higher* PD risk[554].

OxLDL increases expression of **ABCA5** and **ABCG1**[1153]. **ABCG1** protects against oxLDL-induced apoptosis by promoting efflux of 7-KC to HDL[565, 1165], as 7-KC activates apoptotic cascades by increasing the amount of cytosolic BAX[1166, 1167], activating CASP3[1167] and

increasing the expression of TP53[1167]. Further, 7-KC inhibits AKT1[1168], induces ER stress by increasing the expression of the **ATF6**-dependent ER stress chaperone HSPA5[967, 1166] (not shown), increases the expression of ICAM1 and **ITGAL** on microglia[1169] and is involved in inhibition of inflammatory responses[1170]. Thus, maintaining low intracellular 7-KC levels by the regulation of 7-KC efflux by (among others) **ABCG1** is therefore important in prevention of ER stress, the activation of apoptotic and regulation of immune responses.

As opposed to cholesterol, its derivates hydroxycholesterol (24-OHC) and 27-hydroxycholesterol (27-OHC) can cross the blood brain barrier. 24-OHC originates primarily from the brain and is the main cholesterol elimination product of the brain[1171]. 27-OHC is the major oxysterol in the circulation that can cross the blood brain barrier and has under normal physiological conditions a steady influx into the brain[1172]. Both 24-OHC and 27-OHC levels are associated with PD, i.e. 24-OHC levels in the CSF of PD patients correlate with the duration of PD[376], whereas 27-OHC is increased in the plasma of PD patients[564]. An increased 27-OHC flux into the brain, e.g. due to hypercholesterolemia, potentially has implications for PD pathogenesis, as 27-OHC decreases TH and increases **SNCA**[616, 617] expression. Healthy men with low HDL-cholesterol have a high 27-OHC/total cholesterol ratio, indicating that the production of 27-OHC and its transport to the liver may represent an alternative pathway for reverse cholesterol transport by HDL[1173].
Further, high 24-OHC and 27-OHC concentrations induce apoptosis of neuronal cells[616, 1174] (low 27-OHC concentrations trigger survival and high concentrations apoptosis[615]) and should therefore be thighty controlled. One of these control mechanisms is the transport of 24-OHC out of the cell to HDL by the transporter ABCA1[1174]. 24-OHC increases the expression of ABCA1, but also that of **ABCG1**[1175] and decreases the expression of LDLR and **SREBF1**[1175]. 27-OHC also increases the expression of ABCA1[1176, 1177] and **ABCG1**[1177] and is involved in the expression of **SREBF1**[1178, 1179] (not shown). Thus, 24-OHC and 27-OHC increase the export and decrease the uptake of cholesterol by the cell.
In addition to regulation of cholesterol homeostasis, 24-OHC and 27-OHC both increase activation of STAT3 and increase the expression of the angtiotensin II receptor **AGTR1** via the liver X receptors (LXR)[377] (not shown), indicating that there is a relation between oxysterol regulation and the brain renin-angiotensin system, that is again known to interact with the DA system (see section A1.3). 24-OHC and 27-OHC are both endogenous activators of the LXR[1178, 1180]. LXR, having 2 isoforms, LXRA and LXRB[312] is activated by 27-OHC in response to cholesterol overload in the cell[1178] and subsequently inhibits the production of IL1B and IL6 and the inflammatory response of microglia and astroglia[1181] (part of process D). 27-OHC also induces production of TNF from macrophages[1182] (not shown), a factor involved in T cell activation (part of process D), and the LXR also activates **SREBF1**[1183] and redistributes **ABCG1** to the plasma membrane where

it transports cholesterol out of the cell[1154]. Further, administration of an LXR agonist prevented DA neuron loss in a PD mice model, whereas knockout of LXR-beta increased damaged to the DA neurons in the SN. Interestingly, LXR-beta was not expressed in the DA neurons, but in the microglia and astroglia of the SN, indicating that LXR-beta activation inhibits activation of the microglia and astroglia in the SN[1184]. Thus, LXR is a mediator of cholesterol homeostasis in the cell, and involved in immune cell regulation that is critical for DA neuron survival.

In summary, oxysterols and cholesterol oxidation products are tightly regulated in the brain, any disturbance in the levels of 7-KC, 24-OHC and/or 27-OHC can therefore induce immunological or apoptotic responses that decrease the viability of DA neurons. More precisely, **ATF6**, **SREBF1**, STAT3, **AGTR1** and expression of TH and **SNCA** itself are regulated by cholesterol derivates, showing that they are regulating the main cascades in the PD landscape and fullfil a very important modulating role in PD.

B4.1 Fat uptake and insulin (INS)

Diabetics show an increased risk of developing PD[1185], and the frequency of diabetics among PD patients is higher than normally expected[1087], suggesting an influence of glucose metabolism on PD pathogenesis. Indeed, INS, is increased by the familial PD protein PLA2G6 (increases INS secretion; not shown)[1186,1187], whereas the ER protein **TMX1** increases cleavage of INS[1188] and **ZBTB20** increases INS blood levels[1095] (not shown). INS itself has effects on many components of the PD landscape. First, it activates JAK2[1189], **ACTN4**[1190] (not shown), mTORC1[968, 969, 978, 1191], TH[1192] and **SREBF1**[1193] and inhibits **AMPK**[1194]. Furthermore, INS decreases expression of FOXO1[1195], PTEN[1196] and CCL5[1197] and increases expression of **AGTR1**[1198], **COL18A1**[1199], **CYP17A1**[1200, 1201], SCARB1[1202, 1203] (not shown), the thyroid peroxidase **TPO**[1204] (not shown; binds heme[312]), **NTF3**[1205], **MBP**[1206], **FER**[1207] (not shown), **ITGA6**[1208] and **SREBF1**[1201, 1209]. In addition to this, INS increases degradation of ABCA1[1210] and increases binding of **FER** and STAT3[1207] and binding of STAT3 to the ICAM1 promotor[1211] (not shown). Moreover, INS increases binding of INSR and **SDC1**[1212] and binding of EIF4E to **EIF4EBP2**[1213] and to EIF4G1[1214] (not shown).

The INS receptor (INSR) is a tyrosine kinase receptor that binds INS and regulates glucose homeostasis. The INSR is decreased in the SN of PD patients[190, 191]. The INSR is regulated by **MBNL2** (by regulating alternative splicing)[1215], **PRKCE** (binds and inhibits)[1216], FOXO1 (increases expression)[631], **PALD1** (decreases expression)[1217], **HLA-C** (binds and translocates INSR to the membrane)[1218, 1219] and **PTPN1** (inhibits)[1020, 1220, 1221]. So, the expression of INSR, its alternative splicing and its localization to the membrane is part of the PD landscape. In turn, INSR increases the expression of **RPL7A**[1222] and SCARB1[1223] (not shown), regulates expression of SIRT1[1223, 1224] (not shown), **ULK2**[1225] (not shown), **CXCR4**[1225] (not shown), **PRKRIR**[1226] (not shown) and **ACTN4**[1227] (not shown) and

activates **CYP17A1**[1200]. Further, INSR binds **SDC1**[1212], **PIK3CD**[1228] and CAV1[802], and binds and activates JAK2[1229, 1230] and **PTPN1**[1231, 1232].

Of note, INS metabolism has profound effects on fat uptake. INS inhibits HDL-mediated cholesterol reverse transport by inhibiting expression of **ABCG1**[1233]. Further, INS (resistance) disturbs cholesterol regulation in the periphery[1234, 1235], a high-fat diet and INS resistance have been shown to impair nigrostriatal functioning (they attenuated release and clearance of DA in the striatum and increased iron deposition in the SN)[1236] and PD patients show an increased autoimmune reaction towards serum INS[189], indicating that INS function may affect PD pathogenesis.

In conclusion, INS and its receptor regulate in the PD landscape, among others, four important transcription regulators (PTEN, FOXO1, **SREBF1** and STAT3), cholesterol metabolism (via SCARB1, ABCA1, **SREBF1**), the rennin-angiotensin system (**AGTR1**), steroid production (**CYP17A1**), immune responses (**CXCR4**, **SCD1**, ICAM1) and mRNA translation (EIF4E, **EIF4EBP2**, EIF4G1).

B5. Concluding remarks

Cholesterol and sphingolipids are important for membrane function and therefore crucial for normal functioning of the endosome-lysosomal and the lysosome-autophagosomal system that depend heavily on membrane lipid rafts, membrane fission, fusion and trafficking. In return, the endosomal-lysosomal and lysosome-autophagosomal system are important for normal cholesterol and sphingolipid transport and synthesis. The proprotein PSAP regulates the sphingosine synthesis pathway and the trafficking of gangliosides and is therefore an important regulator of (membrane) lipids in the cell. The endosomal-lysosomal system regulates clathrin-(in)dependent endocytosis and recycling and trafficking of vesicles in the cytoplasm. Thereby, the endosomal-lysosomal system regulates the uptake and the processing of lipoproteins, but also the reverse transport of excess lipids out of the cell. Moreover, it also regulates the uptake, degradation or recycling of membrane (receptor) proteins and is in immune cells required for the uptake and processing of proteins for antigen presentation.

During life NM content increases in the DA neurons, hindering the function of the lysosomal compartments and making DA neurons especially vulnerable for defects in the endosomal-lysosomal and lysosome-autophagosomal system. Autophagy-related pathways (e.g. the AMPK-mTORC1 pathway and chaperone-mediated autophagy) regulate protein degradation by the lysosome, reverse cholesterol transport and NM storage. Any defect in the endosomal-lysosomal or lysosome-autophagosomal system – e.g. due protein or NM aggregation, due to mutations or SNPs in genes coding for proteins important for these systems, or due to a dysbalance of membrane lipids – can result in missorting, (further) aggregation of proteins, differential endocytic uptake of lipoproteins and changed cholesterol levels in the cell, affecting cellular function and viability.

Thus, either aggregation of NM or proteins and/or a dysbalance of membrane lipids can create a vicous cycle of lysosomal dysfunction and increased protein aggregation and/or a further dysbalance in cellular lipid levels.
Lastly, systemic regulation of lipoproteins, cholesterol, INS and vitamin D3 are associated with PD and might prove valuable targets for future PD therapies. Moreover, these factors regulate multiple major cascades in the PD landscape and balancing of these factors in the periphery can possibly indirectly improve the viability of DA neurons in PD.

C. ER STRESS RESPONSE

The ER has a broad range of functions, including protein folding, lipid biosynthesis and Ca^{2+} storage[1237]. The balance of synthesis, folding and degradation of proteins is perturbed as we age, resulting in the production and accumulation of misfolded proteins[1238]. Aging-linked declines in the expression and activity of ER molecular chaperones and folding enzymes compromise proper protein folding and the unfolded protein response (UPR)[1237]. And, as PD incidence is higher among older individuals, with most cases older 50 years of age[1239, 1240] and PD prevalence rises with age[1241], it is possible that aging-linked decline of ER function and increase of protein aggregation, resulting in ER stress, may play a role in onset of PD pathology.

Section C1 discusses the pathways and proteins that regulate the UPR and section C2 discusses the pathways and mechanisms that (when dysregulated) can increase protein aggregation and subsequently ER stress. Section C3 shows the interaction between ER stress regulation and cholesterol-regulated gene expression and section C4 discusses the role of the – in the ER synthesized – male hormone testosterone in PD. The functional interactions between the proteins within process C, 'ER stress response', are shown in **Supplementary Figure 2**.

C1. Activation of the UPR

Disturbances in normal ER function, e.g. by unfolded or misfolded proteins in the ER, cause ER stress, which activates the UPR, a stress response that tries to restore normal ER function by degrading misfolded proteins, increasing production of ER chaperones that regulate protein folding and halting protein translation. The other way around, dysregulation of proteins involved in protein degradation, folding or synthesis may result in the accumulation of misfolded proteins that induce ER stress and activate the UPR[1242, 1243]. Misfolded proteins and protein aggregates, e.g. **SNCA** and CTNNB1 aggregates, thus induce ER stress[1244-1246] and thereby activate site-1 and site-2 protease that in turn activate the transmembrane transcription factor **ATF6**[1247]. **ATF6** activation increases expression of genes controlled by ER stress elements, resulting in

the UPR[1248, 1249], i.e. ER stress increases the expression of **MCFD2**, the cargo receptor for ER-to-golgi transport[1250] and the selenoprotein **SEP15**, which both may be involved in ER quality control of protein folding[312, 1250]. Further, also **TMX1** is involved in ER quality control[1251] and binds to **MCC**[387], that in turn binds to **CUL2**[387], **DCUN1D1**[387], **SNRPB2**[387] and the familial PD proteins VPS35[387], UCHL1[387] (associated with the ER membrane[1252]) and EIF4G1[387]. Activation of **ATF6** also increases MTOR activity[967] that in turn causes ER stress by inhibiting autophagy and increasing protein synthesis[1253, 1254]. Thus, MTOR activation increases the amount of misfolded proteins and creates a separate reinforcing feedback loop of UPR/**ATF6**- and MTOR- activation. Nevertheless, mild ER stress induces autophagy and inhibits neuronal death[939, 1243], whereas prolonged ER stress decreases **ATF6** activation[1255], results in increased **SNCA** aggregation[1245] and causes neuronal death due to opening of the mPTP for cytochrome c release[1256].

Of note, **LRRK2** is associated with the ER in DA neurons[1257] and prevents DA neurodegeneration by supporting the upregulation of the **ATF6**-dependent molecular chaperone HSPA5 during ER stress[1258]. HSPA5 (also upregulated by CRH-activation of **CRHR1**, i.e. CRH-induced ER stress[1259]) activates the UPR and diminishes **SNCA** neurotoxicity in a PD rat model[967, 1260].

C1.1 UPR feedback inhibition

The protein phosphatase-1 complex (**PP1**) dephosphorylates proteins and thereby regulates multiple processes in the cell, such as cell division, glycogen metabolism, muscle contractility and protein synthesis[312]. A hallmark of UPR activation is the phosphorylation of the translation initiator EIF2A[1261] (EIF2 signaling is associated with PD[165]). This attenuates protein synthesis and enables the cell to remove misfolded proteins from the ER[1262]. During ER stress, there is feedback inhibition through **PP1**-dependent dephosphorylation of EIF2A[312, 1263-1265] (not shown). **PP1** thereby reinitiates protein synthesis and facilitates the recovery of cell from stress[1263]. However, overactivation of the **PP1** complex would shut down the UPR too early and increase ER stress. Selective inhibition of the **PP1** complex can therefore protect cells from ER stress[1265].

Two inhibitory subunits of the **PP1** (**PPP1R12B** and **PPP1R14C**[312]) were associated with PD in the GWASs[6, 13]. **PPP1R12B** is part of the myosin phosphatase complex, binds the catalytic **PP1** subunit PPP1CB[1266] and thereby regulates myosin activity[312]. Further, another member of the myosin phosphatase complex PPP1R12A is downregulated in the CSF of PD patients[224], is activated by **PRKG1**[1267] and binds PPP1CB[387] and **LRRK2**[742]. PPP1CA, PPP1CB and PPP1CC are the catalytic subunits of **PP1**, whereas PPP1CA is the physiological **LRRK2** phosphatase and is inhibited by the membrane phospholipid PA[540] and activated by ceramide[540]. Pathogenic PD mutations in **LRRK2** mutations

are associated with a decreased phosphorylation state of **LRRK2**[1268], implying that phosphatase acitivity of PPP1CA is important in PD. PPP1CA also binds to **SNCA**[461], EIF2A[1269] (not shown), AKT1[1023] (is inhibited by PPP1CA[1023, 1024]), **ATR**[1269], **AXIN1**[1270], CAV1[1023] (not shown) (inhibits PPP1CA[1023] (not shown)), **CNTN1**[1271] (not shown), **CSMD1**[1272], CASP9[1273], **DLG2**[1272] (binds NOS1[1274] (not shown), **DSCAM**[1275] and **ATP2B2**[1276]), **GPATCH2**[1272] (not shown), GSK3B[1277], HSPA8[1278], **MAP1B**[1272], **MAPT**[1269], PTEN[1269] , SIRT7[394], TP53[859, 1269], **WNK1**[1272] and **WWOX**[1279]. PPP1CA expression is increased by **GNA12**[747], regulated by vitamin D3[1094] (not shown) and TP53[1280] (not shown) and decreased by chronic DA depletion[509].

In summary, a disturbance in **PP1** activity, e.g. due to dysregulation of PA, ceramide, vitamin D3 or DA, affects ER stress regulation and the regulation of multiple proteins in the landscape, including the familial PD proteins **LRRK2** and **SNCA**.

Another phosphatase, the **PP1**-like phosphatase **PPM1L**, inhibits binding of **MAP2K6** and MAP3K7[1281] and thereby prevents activation of **MAP2K6** by MAP3K7[1282]. **MAP2K6** increases NF-KB complex expression[1283-1285] and binds and regulates the expression of **LRRK2**[1286], but **MAP2K6** is also phosphorylated by **LRRK2**[931]. Further, MAP3K7 activates the **AMPK** complex[1287], is inhibited by **TRAF3**[1288] and binds to **SMAD5**[1289]. **SMAD5** is activated by **BMP7**[1290] and binds to SMAD4[859] (binds to **ZNF423**[1291]), **RUNX3**[1292] and the **26S** proteasome[859] (binds **FBXO25**, that again binds to HSPA8[395]).

C2. Protein aggregation

Timely detection of misfolded proteins by E3 ubiquitin-protein ligase complexes and subsequent degradation by the proteasome prevents protein aggregation and ER stress. Section C2.1 discusses shortly proteins that affect protein translation, section C2.2. discusses the targeting of proteins to the proteasome by the small protein ubiquitin and section C2.3 discusses the proteins in the PD landscape that directly regulate proteasome function. Section C2.4 shows the involvement of presenilins and protein cleavage and section C2.5 discusses the effects of beta-catenin (CTNNB1) aggregation on (DA) neuron function and how its levels are controlled in the cell.

C2.1 Protein translation

Incorrect protein translation can result in abnormal protein function, mislocalized proteins and protein aggregation. The ribosomal DNA transcription factor SIRT7 binds to proteins that are involved in the regulation of protein synthesis, e.g. the histone protein **H1FX**[394], the RISC complex component **SND1**[394], pre-mRNA splicing factor **BAT2**[394] (binds the RNA-binding and alternative splicing regulator **RBFOX1**[1293]), the 60S ribosomal proteins **RPL7A**[394] and **RPL38**[394] and the familial PD protein EIF4G1[394] (involved in mRNA cap recognition and recruitment to the ribosome[1294]). Further, SIRT7 also binds **USP9X**[394]

and **PLOD1** (involved in the formation and stabilization of collagens[1295], such as **COL2A1** and **COL18A1**[394]) and regulates autophagic (via binding to MTOR, STK11) and cytoskeletal (via binding to **KIF14**, **MAP1B**, DNM2, **ACTN4**) processes[394]. Thus, the PD landscape contains multiple proteins that regulate protein transcription and translation.

C2.2 Ubiquitin

Ubiquitin is a small regulatory protein that, by binding to substate proteins, is able to regulate their cellular localization, protein-protein interaction or degradation[1296, 1297]. E3 ubiquitin-protein ligase complexes catalyze the ubiquitination of proteins and targets them for proteasomal degradation. **TRAF3** is an essential constituent of several E3 ubiquitin-protein ligase complexes[312], regulates the NF-KB complex (see above) and binds to **AGTR1**[362]. Also, TRAF2 is part of the E3 ubiquitinase complex, binds **TRAF3**[1298] and like **TRAF3** decreases the expression of the actin polymerization protein[1299] **LRRC16A**[1300]. Further, TRAF2 ubiquitin ligase activity is strongly activated by S1P[1301], TRAF2 mediates **SREBF1** activity[1302] (not shown) and binds to **AMPK**[1303] and the familial PD proteins HTRA2[954] and FBXO7[1304]. Upon cellular stress, HTRA2 is released from mitochondria and induces apoptosis[405] (part of process A) – e.g. by binding to the cell cycle and apoptosis regulator **CCAR2**[1305] – and inhibits the E3 ubiquitin ligase activity of **PARK2**[405]. FBXO7 on the other hand, is together with the F-box proteins **FBXO25** (binds to a complex consisting of PPP1R12A and **PPP1R12B**[395]) and **FBXO36** part of an ubiquitin-protein ligase complex[312, 1306, 1307].
Cullin-2 (**CUL2**) is a core component of E3 ubiquitin-protein ligase complexes[312] and binds to **DCUN1D1**[1308] and NEDD8[1309]. **DCUN1D1** activates the E3 ubiquitin-ligase complex by recruiting a NEDD8-charged E2 enzyme to the cullin component[312]. Further, NEDD8 also binds to SIRT1[656], **RPL7A**[1135], HSPA8[1134], UCHL1[846] and binds **PARK2** and increases its ubiquitinase activity[200, 1310] (activates the **26S** proteasome[1310]) and binds and stabilizes PINK1[200]. Moreover, NEDD8 accumulation is observed in LB in DA neurons in the SN of PD patients[199, 200].

The deubiquitinases UCHL1 and **USP9X** affect, by their ability to deubiquitinate, proteasomal degradation of misfolded proteins[43, 1252]. Of note, **USP9X** deubiquitinates **SNCA** and is lower expressed in the SN of PD patients, which may contribute to higher levels of monoubiquitinated **SNCA**[43]. **USP9X** thereby determines if **SNCA** is degraded by the proteasome (monoubiquitinated **SNCA**) or by autophagy (deubiquitinated **SNCA**)[43, 1311]).

Thus, proteasomal targeting of proteins by ubiquitin is highly regulated in the PD landscape, either by ubiquitin-protein ligase complexes (composed of among others **CUL2**, **DCUN1D1**, FBXO7, **FBXO25**, **FBXO36**, **PARK2** and **TRAF3**) or deubiquitinases (e.g. UCHL1, **USP9X**).

C2.3 Proteasomal degradation

Proteasomal function is impaired in the PD SN and results in aggregation of (misfolded) proteins[45]. Degradation of misfolded proteins requires retrograde transport of these proteins across the ER membrane and subsequent degradation by the ubiquitin-proteasome system, which includes the **26S** proteasome[1312]. **PSMD11** is a regulatory subunit of the **26S** proteasome[1313]. The **26S** proteasome is inhibited by **AMPK**[1314], whereas aggregated **SNCA** binds and decreases its activity[532, 1315, 1316]. The protein **BAG6** is part of a ubiquitination-complex that prevents aggregation of mislocalized proteins, by targeting them for proteasomal degradation[1317]. Further, **BAG6** has a key role in assembly of the **26S**-proteasome complex[1318] and cleavage of **BAG6** by CASP3 induces apoptosis[1319]. Further, the cGMP-dependent protein kinase **PRKG1** (also regulating actin cytoskeleton and myosin-mediated trafficking by binding to **PPP1R12B**[1320]) increases activity of the proteasome and increases proteasome-mediated degradation of misfolded proteins[1321]. Thus, proteasome function is regulated in the PD landscape (via **BAG6**, **PRKG1**, **PSMD11**) and defects in this system may result in protein aggregation and neuron death.

C2.4 Presenilins and protein cleavage

Activation of the UPR by ER stress increases the activation of γ-secretase to increase proteolytic cleavage of unfolded proteins in the ER[1322]. The γ-secretase is a protease complex (its catalytic core consisting of the presenilin PSEN1 or PSEN2) that cleaves single-pass transmembrane proteins within their transmembrane domain. γ-Secretase is well known for cleaving of APP[1323] (not shown), resulting in amyloid-beta aggregation in Alzheimer's disease[1324]. In addition to APP, γ-secretase also cleaves and thereby decreases CTNNB1 levels in the cell[1325-1327], cleaves LDLR[1328] (not shown), **SDC1**[1328] (not shown), LRP1 (is in competition with APP for γ-secretase activity)[1329, 1330] and increases the expression of **IL2RA** on the plasma membrane[1331] (not shown). AKT1 is necessary for γ-secretase activation[1332], cholesterol increases γ-secretase activation[1333] and **RER1** increases the retention and retrieval of γ-secretase (subunits) in the ER, preventing its activity[887-889]. **PARK2** regulates the promoter activity of PSEN1 and PSEN2 and thereby increases PSEN1-associated γ-secretase activity and reduces PSEN2-associated CASP3 activation[1334]. The presenilins PSEN1 (activated by ER stress[1335]) and PSEN2 also have γ-secretase-independent functions[1336], i.e. they decrease INSR expression and thereby inhibit INS-signaling[1337] and are necessary for protein degradation by the lysosome-autophagosomal system (part of process B) by regulating (among others) MTOR and LAMP2[1338]. Of note, PSEN1 knockout mice show increased lysosomal **SNCA** aggregation[1339]. PSEN1 binds CTNNB1[1340], **MAPT**[1341], APOE[1342], GSK3B[1343], HSPA8[1344] (not shown), **ENSA**[1342] (not shown), EIF4G1[1345], HTRA2[1346] (not shown) and **ATP6V0A1**[1347] (not shown) and activates HTRA2[1348]. Furthermore, PSEN2 binds CTNNB1[1349], **ATP6V0A1**[1344] (not shown) and CASP3[1350], increases expression of TP53[1351] (not shown) and CASP3 (not

shown; and increases activation of CASP3)[1351, 1352], decreases expression of PSEN1[1351] and inhibits activation of PLA2G6[1353] (not shown).

Thus, the presenilins PSEN1 and PSEN2 are important for γ-secretase-dependent *and* -independent cleavage and degradation of proteins. Dysregulation of these presenilins or γ-secretase (e.g. due to **RER1**- and **PARK2**-dependent regulation or via activation by AKT1 and cholesterol) may result in activation of apoptotic processes (via HTRA2, TP53, CASP3), dysregulation of the immune response (via **SDC1**, **IL2RA**) and of cellular cholesterol homeostasis (via LDLR and LRP1) and CTNNB1 and **SNCA** aggregation.

C2.5 Beta-catenin aggregation and -dependent transcription

Protein aggregation is not always bad and is sometimes even crucial for cell development, i.e. stabilization and subsequent aggregation of cytoplasmic beta-catenin (CTNNB1) in ventral midbrain precursor cells increases their differentiation into DA neurons[1354]. Aggregation of CTNNB1 in the cytoplasm causes it to translocate into the nucleus to function as a coactivator of transcription factors[1355], e.g. CTNNB1 increases expression of UCHL1[1356] and LMX1B[1357]. This implies that cytoplasmic CTNNB1 aggregation may regulate DA neuron development and maintenance (see also section D1.1) and could play a role in PD pathology. Nevertheless, cytoplasmic CTNNB1 aggregation also results in ER stress and activation of the UPR[1246].

CTNNB1 expression and aggregation is decreased by CAV1[1358] (not shown), **COL18A1**[1359-1361], **PRKG1**[1362, 1363], PTEN[1364], **WWOX**[1365] and γ-secretase (see section C2.4). Moreover, in a healthy cell, cytoplasmic CTNNB1 is degraded by the beta-catenin destruction complex, that consists of, among others, **AXIN1** and GSK3B[1366]. **AXIN1** binds CTNNB1[1366] and degrades excessive cytoplasmic CTNNB1[1367], whereas inhibition of GSK3B stabilizes cytoplasmic CTNNB1[1354]. Further, **AXIN1** also binds to **GAK**[846] and GSK3B[1368], and **AXIN1** inhibits GSK3B-dependent phosphorylation of **MAPT** (and thus may prevent against **MAPT** hyperphosphorylation and tau aggregation[1369]). **MAPT** also binds to **SNCA**[1370], **LRRK2**[1371] and **PARK2**[1372]. The wnt pathway inhibits degradation of CTNNB1 by the beta-catenin destruction complex[1373] causing CTNNB1 accumulation – e.g. because **WNT3** is involved in a signaling cascade that stabilizes and increases the expression of CTNNB1[1365, 1374] – and is involved in DA neuroprotection, development and repair[1375, 1376]. Further, under apoptotic-conditions (e.g. Ca^{2+} influx), the γ-secretase complex promotes disassembly of the E-cadherin/catenin-complex and thereby increases the pool of cytoplasmic CTNNB1[312].

Other proteins that increase CTNNB1 aggregation or decrease its degradation are the deubiquitinase **USP9X** (binds CTNNB1, close to the **AXIN1**-binding site, and inhibits CTNNB1 degradation)[1377], the familial PD protein UCHL1 (binds and stabilizes CTNNB1)[1356] and **GNA12** (by decreasing the inhibitory function of cadherins on active CTNNB1[740]. **GNA12** also binds and is regulated by **AXIN1**[744]). Further, CAV1 binds CTNNB1[809] and

nuclear CTNNB1 is increased in a mouse CAV1-knockout[1358], indicating a role for the endocytic/autophagic pathway in maintaining CTNNB1 levels.
Other proteins that bind to CTNNB1 are **CACNA1A**[505], **ACTN4**[1378], **ARMC8**[1379] (not shown), SIRT1[645], **CDH6**[1380], **STK39**[1367], **FER**[1381], **PARD3**[1382], UCHL1[1356] and **SNCA**[461]. The latter indicating that CTNNB1 and **SNCA** aggregation may be able to affect each other.

Nuclear translocation of CTNNB1 increases the expression of **ACTN4**[1383], **BAT2**[1383], **GJB2**[1383], **GNA12**[1383], **BMP7**[1357] (not shown), **TMEM2**[1384] (not shown), **MSX1**[1385], **BAMBI**[1386] (not shown), **ARL4A**[1387] (not shown) and UCHL1[1356]. Nuclear CTNNB1 binds to the transcription regulators **RUNX3**[1388], FOXO1[1389], MITF[1062], SIRT1[645], **TLE1**[1390] and also to **SNCA**[461] and may affect their function. Controlling CTNNB1 levels in the cell may therefore be important in DA neuron homeostasis (**MSX1**), regulation of familial PD proteins (UCHL1, **SNCA**) and multiple (PD associated) transcription regulators (**RUNX3**, FOXO1, MITF, SIRT1 and **TLE1**).

C3. ER stress- and cholesterol-regulated gene expression

During ER stress **ATF6** is cleaved and translocated to the nucleus where it activates the transcription of genes involved in the UPR[1248, 1249], e.g. HSPA5[1248] (diminishes **SNCA** neurotoxicity in a rat PD model[1260]). Further, **ATF6** activates NF-KB[1391] and is involved in astroglia activation and neuronal survival (in a PD mice model)[1392].

ER stress not only activates the UPR, but also increases cholesterol uptake by increasing **SREBF1** activity[1393, 1394], as the same site-1 and site-2 proteases that activate the transmembrane transcription factor **ATF6** also splice and activate **SREBF1**[1247, 1395]. Mature, cleaved **SREBF1**, enters the nucleus and increases the transcription of proteins involved in cholesterol metabolism. Of note, a SNP (rs11868035; which was also associated with PD via the GWASs[12]) located in the splice site of **SREBF1** is associated with gait impairment in PD[32]. **SREBF1** increases transcription of proteins involved in lipid and cholesterol regulation, e.g. SCARB1[932], LDLR[1393, 1396, 1397], ABCA1[925] and STAR[603, 604] (not shown) and **SREBF1** activation results in activation of the mevalonate pathway and synthesis of cholesterol[1398]. Cholesterol is de-novo synthesized in the ER (in small amounts) and transported to the cytoplasm by **ABCG1**[1154, 1399]. Other proteins that regulate the mevalonate pathway are the mitochondrial **MCCC1** and **ACSL6**. **MCCC1** is a 3-methylcrotonyl-CoA carboxylase (activated via biotinylation by **HLCS**[312]) involved in the leucine metabolism eventually resulting in the production of HMG-CoA, an intermediate of the mevalonate pathway[1400]. **ACSL6** is an acyl-Coa synthase that is located in membranes and activates long-chain fatty acids so they can subsequently be degraded by β-oxidation in the mitochondria and produce acetyl-CoA[312]. Acetyl-CoA can be oxidized in the citric-acid cycle or can enter the mevalonate pathway for the production of cholesterol[312].

In addition to ER stress, **SREBF1** expression is also regulated by cholesterol[1401, 1402], increased by LDLR[1403] and the tyrosine-protein phosphatase and regulator of the UPR **PTPN1**[312, 1404]. Moreover, **SREBF1** is decreased by vitamin D3[1104], FOXO1[632], PTEN[1405, 1406], the serine/theronine-protein kinase **SIK1**[1004], TP53[1407], STAT3[1408], IL1B[1409] (not shown) and ICAM1[1410] and binds **ATR**[1411], **MED13**[1412] (binds also to **CDK19**[1413]) and SIRT1[647]. The transcriptional regulator **CREM** dysregulates cholesterol homeostasis by increasing ABCA1 expression[1414], decreasing LDLR expression[1414] and increasing the expression of the **SREBF1**-inhibiting kinase **SIK1**[1414]. **CREM** also decreases expression of **HLA-DRA**[1415] and TH[298] and thereby not only affects cholesterol homeostasis in the cell, but also immune function and dopamine production. Next to cholesterol-linked proteins, **SREBF1** also increases the transcription of SERPINE1[1416], **ULK2**[1011], TP53[1417], NOS2[751] and AR[1418] and decreases the expression of ADH1C[1419], a cytoplasmic enzyme involved in the production of retinoic acid[1420]. A mutation in ADH1C is associated with PD susceptibility[112] and its expression is, in addition to **SREBF1**, also decreased by RXRA[1421] (not shown) and INS[1419] (not shown) and increased by angiotensin II[1422] (not shown).

Of note, whereas ER stress increases **SREBF1** activity, dysregulation of cholesterol, oxLDL, sphingomyelin and **SREBF1**, and cellular lipid accumulation have been associated with ER stress and UPR activation by **ATF6**[523, 778, 1166, 1423-1426].

In conclusion, both ER stress and dysregulation of lipid homeostasis can activate **ATF6** and **SREBF1**, and thereby show a complex interaction between ER stress, lipid homeostasis and activation of the UPR.

C4. Testosterone metabolism

Overall, in Caucasian populations the incidence rate of PD is 1.5-2.0 times higher among men than women[1239, 1427, 1428], making the male gender a risk factor for developing sporadic PD[23, 1239, 1429] and suggesting that sex hormone levels may play a role in PD etiology. Of note, in Asian PD patients such a male predominance was not found[1239, 1428]. Healthy Asian males have comparable estradiol levels with healthy non-Asian males, but show lower testosterone levels[1430, 1431]. The higher testosterone levels in Caucasian males may make them more susceptible for the effects of a testosterone drop than females. Due to their lower testosterone levels, there is no such drop in Asian males, which may explain the absence of the male gender as a risk factor in the Asian population. Indeed, male PD patients show significantly reduced testosterone levels compared to healthy controls[1432-1434], and their possible contribution to PD pathogenesis may be illustrated by the inverse correlation of testosterone level and apathy in PD patients[1432]. Further, other indicators of testosterone involvement in PD-related mechanisms are; the lower testosterone levels by inhibition of mitochondrial complex I in the rotenone-treated rat, a model for PD[1435], and the increase in **SNCA** levels accompanying a decrease in

TH-positive neurons and fibers in the SN and striatum respectively after castration of young male mice[1436].

C4.1 Regulation of testosterone and cholesterol

CYP17A1 dysregulation could attribute to the lower testosterone levels seen in male PD patients, namely, **CYP17A1** is one of the enzymes involved in the conversion of pregnenolone and progesterone (both synthesized from cholesterol) to testosterone[623, 1437]. **CYP17A1** is located on the mitochondrial and ER membrane[312] and its expression is increased by INS[1200, 1201], AR[1438], angiotensin II[1439], MTOR and the mTORC1[1440], S1P (via activation of **SREBF1**)[737] and decreased by RELA[609], AR[1441], **BMP7**[1442] (not shown) and the acid ceramidase **ASAH1** (hydrolyzes ceramide into sphingosine)[1443]. The role of **ASAH1** can be explained by the inhibiting effect of sphingosine on **CYP17A1** expression by binding to NR5A1, a nuclear receptor that activates (synergistically with **DGKQ**[312]) **CYP17A1** transcription[1444]. NR5A1 also binds CTNNB1[1445], NF-KB[1446] (not shown), AR[1447] (not shown), **SREBF1**[1448] and **DGKQ** ([539] and increases the expression of SCARB1[1449] (not shown), **DGKQ**[1448] and the mitochondrial cholesterol transporter protein STAR[597, 598] (not shown). Dysregulation of the sphingosine-S1P balance in the cell can therefore have major consequences on (mitochondrial) cholesterol levels, **CYP17A1** expression and subsequently testosterone levels. See for details on the role of sphingosine and S1P in the PD landscape section B1.2.

Dysregulation of more upstream processes of the testosterone synthesis cascade, i.e. the cholesterol metabolism, could also affects testosterone levels. For example, the transporters STAR and TSPO regulate import of cholesterol into the mitochondria for conversion into pregnenolone and subsequently testosterone[619, 620, 1450]. Indeed, inhibition of TSPO results in a decreased testosterone production[1450] and testosterone itself functions as a feedback inhibition by decreasing the expression of STAR[620]. Furthermore, the cholesterol transporter ABCA1 and also INS increase the quantity of testosterone[1201, 1451, 1452].
In healthy adult men, free testosterone levels are positively correlated with LDL-, HDL- and total cholesterol levels[1453]. A decreased testosterone level could influence several processes in the PD landscape (not shown), as testosterone regulates efflux of LDL- and HDL-cholesterol to the blood[1454-1456], increases the expression of INS[1457], MHC class II proteins[1458], CAV1[1459], CASP3[1460], SYT11[1461], PSEN1[1461], SIRT1[1462], SOD2[1463] and decreases expression of PLAU[1464], TGFBR2[1465] and **CRHR1**[1466].

In summary, dysregulation of the cholesterol metabolism (e.g. due to ER stress) may result in lower testosterone levels as seen in PD patients. And, in return, changes in testosterone levels can affect cholesterol homeostasis and multiple pathways in the PD landscape, and may play a role in PD pathology.

C4.2 Androgen receptor (AR)

In the nucleus, testosterone binds and activates the AR, increasing AR-dependent transcription[1467]. This results in a positive feedback loop, as **CYP17A1** expression is increased by the AR[1438]. Regulation of the AR by other pathways and proteins in the PD landscape can thus indirectly also affect testosterone levels. In the PD landscape, AR expression is increased by **SREBF1**[1418] and NF-KB[1468] and decreased by SIRT1[653], TP53[1469] and regulated by MTOR[1470, 1471], **SLC45A3**[1472] and CTNNB1[1473, 1474]. Moreover, multiple PD landscape proteins bind to the AR, i.e. **SREBF1**[1475], **EFCAB6**[1476], CAV1[1477] (not shown), FOXO1[1478], PARK7[1479], AKT1[1480], GSK3B[1481], STAT3[1482], **POU2F1**[1483] (not shown), NF-KB[1484], SIRT1[653], HSPA5[1138] (not shown), PTEN[1485], CASP3[1486] (not shown), TP53[1487], **GAK**[1488] and **STK39**[1489]. Most of these physical interactions are often accompanied by an inhibition or activation of one or both of the binding partners, i.e. AR is activated by **BMP7**[1490] and PARK7[1479, 1491] (by binding and inhibition of **EFCAB6**[1476]), activates AKT1[1492] (not shown) and activates and is activated by STAT3[1482]. Further, AR is inhibited by **SREBF1**[1475], FOXO1[1493], AKT1[1480], GSK3B[1481] and PTEN[1485], inhibits and is inhibited by NF-KB[1484] and is cleaved by CASP3[1494] (not shown).

AR activation increases the expression of **TACC2**[1495], CTNNB1[1496] (increases nuclear CTNNB1[1497] and CTNNB1-mediated transcription[1498]), MAOA[277], PLAT[1499] (not shown), **CACNA1A**[1499], HSPA5[1500] (not shown), IGF1[1501] (not shown), PTEN[1502] and STAT3[1482]. Further, AR decreases the expression of **ITGA6**[1503], SERPINE1[1504] (not shown) and **SERPINB5**[1505] (not shown), is required for BAX translocation to mitochondria[1506] (not shown) and decreases nuclear TP53 accumulation[1507].

In summary, the AR is regulated by several major pathways in the PD landscape, is regulated by testosterone and by the familial protein PARK7. Moreover, AR itself regulates CTNNB1-mediated transcription, ER stress responses (via HSPA5), coagulation (via PLAT, SERPINE1), testosterone levels (via **CYP17A1**), intracellular Ca^{2+} levels (via **CACNA1A**), INS (via IGF1), immune (via STAT3, **ITGA6**) and apoptotic (via BAX, TP53) pathways. A slight change in testosterone level, as seen in PD patients can therefore have a huge effect on the regulation of these pathways.

C5. Concluding remarks

Dysfunction of E3 ubiquitin ligase complexes, the proteasome or processes involved in protein translation, modification, cleavage and degradation (by presenilins, the proteasome and/or lysosome) result in protein misfolding and/or aggregation that leads to ER stress, activation of **ATF6** and the subsequent induction of the UPR. ER stress activates presenilins, that (either γ-secretase-dependent or –independent) decrease protein aggregation, but also regulate activation of apoptotic and immune pathways. The site-1 and -2 proteases necessary for **ATF6** activation during ER stress, also activate **SREBF1** and thereby affect the cellular cholesterol homeostasis. In turn, cholesterol, and (oxidated) lipids not only affect **SREBF1** activation, but also induce ER stress and

activation of the UPR via **ATF6**. This shows that ER stress and cholesterol metabolism are highly interconnected, indicating that their dysregulation can lead to ER stress-induced mitochondrial dysfunction (as discussed in section A2.2.1) and neuron death.

Moreover, testosterone metabolism is affected by both cholesterol regulation and ER (dys)function, i.e. it is synthesized from cholesterol and produced in the ER. Lower testosterone levels as seen in male PD patients can result from a disbalance in either cholesterol metabolism or ER function and may play a role in PD pathology.

D. NEURON DEATH AND IMMUNE RESPONSE

DA neuron death can occur due to dysregulation of intracellular pathways and processes, immune responses targeting DA neurons, or a combination of the two. This section discusses pathways that regulate DA neuron determination, neuron survival and death (section D1) and pathways that regulate the immune response and immune cell activation (section D2). The functional interactions between the proteins within process D, 'neuron death and immune response', are shown in **Supplementary Figures 2** and **3**.

D1. DA neuron determination, survival and death

The following subsections discuss the regulation of a small set of transcription factors necessary for differentiation into and maintenance of DA neurons (D1.1), the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived factor (GDNF) that support the survival of neurons (D1.2) and the pro-apoptotic factors (GSK3B, PTEN, TP53 and CASP3) that regulate cellular death (D1.3).

D1.1 Regulation of the "DA signature"

The transcription factors ASCL1, **MSX1**, LMX1A, LMX1B, NEUROG2, NR4A2, PITX3 and SOX2 are implicated in DA neuron development and are sufficient for reprogramming of fibroblasts into DA neurons[1054, 1508-1510]. These transcription factors are therefore important in maintaining an expression pattern typically for DA neurons. ASCL1 increases the expression of TH[1511] and **CACNA2D3**[1512] and is itself regulated by active AKT1[1513]. SNPs in LMX1A and LMX1B may increase PD risk[1514]. LMX1A increases the expression of NR4A2[1509], SLC6A3[1515], NEUROG2[1516] (not shown), SLIT2[1517] and INS[1518]. LMX1B is required for normal functioning of the lysosomal-autophagosomal system and DA neuron survival and is decreased in PD DA neurons[193]. LMX1B expression is increased by CTNNB1[1357] and decreased by PTEN[297]. **MSX1** increases expression of **COL2A1**[1067]. NEUROG2 expression is increased by PINK1[1519] (not shown) and PITX3[1516] (not shown), decreased by SIRT1[1520] (not shown) and is inhibited by GSK3B[1521] (not shown). NR4A2 increases the expression of DA transporters SLC6A3[1508, 1522] and SLC18A2[1522],

TH[1522], LMX1B[1522], PITX3[1522], **EIF4EBP2**[1523] and PRKAA2[1523], regulates oxLDL[1524] (not shown) and binds to RELA[1525] and **RCOR1**[1525]. NR4A2 expression is increased by HMOX1[332] (not shown), MC1R[1526] (not shown), STAT3[1527], IL1B[1528] (not shown) and TNF[1528] (not shown) and decreased by oxLDL[1529] (not shown), PTEN[297] and **ASAH1**[611] (not shown). Mutant **SNCA** increases the degradation of NR4A2, which is dependent on th **26S** proteasome complex[1530] (not shown). SOX2 increases expression of **JARID2**[1531], **TBX3**[1532], ASCL1[1532, 1533], GSK3B[1534], binds[1534-1537] and increases expression of CTNNB1[1538], decreases expression of NFKB2[1532], **WNT3**[1532, 1533] and **AXIN1**[1533] and phosphorylates TP53[1539] (not shown). CAV1[1540] (not shown), FOXO1[1541] and CTNNB1[1542-1544] increase the expression of SOX2. AKT1 binds, phosphorylates and stabilizes SOX2, which increases SOX2 expression[1545, 1546] (not shown). Further, SOX2 binds **CCAR2**[1536], **EHMT2**[1536], **ETV6**[1536], **TBX3**[1536], **TIAL1**[1536], **USP9X**[1536], the nuclear transporter **KPNA4**[1536] (not shown) and NR5A1[1547]. CTNNB1 also increases expression of **MSX1**[1385].

In summary, normal transcriptional activity of ASCL1, **MSX1**, NEUROG2, NR4A2, PITX3 and SOX2 is necessary to maintain a dopaminergic phenotype. Multiple proteins in the landscape regulate and are regulated by these transcription factors, whose dysregulation would affect expression of proteins that are crucial for DA signaling and regulation e.g., TH, SLC6A3 and SLC18A2. Interestingly, oxLDL decreases NR4A2 expression, and AKT1 – which is highly regulated in the PD landscape – regulates both ASCL1 and SOX2.

D1.2 Neurotrophic factors

BDNF is an extracellular protein that supports the survival, growth and differentiation of neurons[1548, 1549]. Further, BDNF is associated with cognitive and motor impairment in PD[128, 131], is decreased in PD SN neurons[130] and dysregulated in PD serum[131, 132] and CSF[133]. BDNF expression is increased by SIRT1[1550, 1551] and NF-KB[1552] and decreased by INS[1553], IFNG[1554] (not shown), PLAT[1555] and **SNCA**[1556, 1557]. BDNF activates PLAT[1558], NF-KB[1559] (increases NF-KB translocation to the nucleus[1560]) (not shown), STAT3[1561] (not shown), RAC1 (via **PARD3**)[1562] (not shown), MTOR[1563, 1564] (not shown), **SIK1** (and increases its translocation from the cytoplasm to the nucleus)[1565] (not shown) and AKT1[1566] (not shown) and inhibits CASP3[1567] (not shown). Further, BDNF regulates activity of DRD2[1568] (not shown), increases cholesterol synthesis[1569] (not shown), decreases INS levels in the blood[1570] (not shown) and binds clathrin in the cytoplasm[1571] (not shown). BDNF increases the release of DA[1571, 1572] (not shown) but can also decrease the expression of TH[1573] (not shown). Furthermore, BDNF decreases expression of **CXCR4**[1574] and increases expression of DA receptors[1571, 1575, 1576] (not shown), AR[1577] (not shown), PTEN[1578] (not shown), SOD2[1579] (not shown) and **MBP**[1580]. **MBP** is the major component of myelin sheats, allowing the axon to rapidly propagate action potentials[312]. Further, **MBP** binds to PA[547], **HLA-DRA**[1581] and binds and is phosphorylated by **STK39**[1582] and **LRRK2**[1583, 1584]. Antibodies

against **MBP** are increased in the serum of PD patients[110], indicating a dysregulation of **MBP**, affecting signal transduction between cells. Moreover, **GLDN** is probably involved in the formation the nodes of Ranvier[312] and **CNTNAP2** is localized at juxtaparanodes of myelinated axons and mediates interactions between neurons and glia[1585, 1586], indicating that signaling between DA neurons and glia cells may be disturbed in PD.
Thus, BDNF is regulated by and regulates the immune response (**CXCR4**, NF-KB, IFNG, STAT3), regulates the activity and expression of DA receptors and DA (TH), affects signaltransduction (**MBP**) and regulates lipogenesis (MTOR, **SIK1**), oxidative stress (SOD2) and apoptosis (CASP3).

The neurotrophic factor GDNF promotes survival and differentiation of DA neurons and increases their DA uptake[1587] and is decreased in neurons of the SN of PD patients[130]. GDNF expression is increased by **ITGA8**[1588], NF-KB[1552], regulated by CTNNB1[1589] (not shown) and decreased by **SNCA**[1556]. GDNF activates STAT3[1590] (not shown), RAC1[1591, 1592] (not shown), AKT1[1591, 1593, 1594] (not shown) and TH[1595] (not shown). Further, GDNF increases secretion of DA[1596] (not shown) and increases the expression of TH[1597, 1598] (not shown), SLC6A3[1598-1600], DRD2[1572], SPHK1[418] (increasing synthesis of S1P[418]), **ITGA6**[1601] and decreases the expression of CASP3[1602] (by decreasing cleavage of pro-CASP3[1603]) (not shown) and cytochrome c[1602] (not shown). However, chronic upregulation of GDNF decreases TH expression[1604] (not shown).
Thus, GDNF regulates DA homeostasis (TH, SLC6A3, DRD2) is regulated by and regulates the immune response (NF-KB, **ITGA6**, **ITGA8**, S1P) and apoptotic processes (CASP3, cytochrome c).

D1.3 Pro-apoptotic proteins

Cellular death is regulated by a variety of proteins, the main proteins that regulate apoptotic pathways in the PD landscape, GSK3B (D1.3.1), PTEN (D1.3.2), TP53 (D1.3.3) and CASP3 (D1.3.4) are discussed in this section.

D1.3.1 GSK3B

PD-associated polymorphisms alter the transcription and splicing of GSK3B[158] and affect it functions in the cell. For example, GSK3B is part of the CTNNB1 destruction complex (see also section C2.5), regulates cellular energy levels, inhibits mitochondrial complex I, which affects mitochondrial function and increases ROS production[1605, 1606] and increases accumulation of **SNCA**[1607]. In turn, **SNCA** can activate GSK3B[1606, 1607], which may lead to a vicous cycle of GSK3B activation and **SNCA** aggregation. Further, GSK3B is also activated by **CRHR1**[1608], IFNG[1609], mTORC1[971], PTEN[1610] and inhibited by **PARD3**[1611], **PTPN1**[1612], **PIK3CD**[628, 1613], AKT1[1614], PTEN[1615], INSR[1616], **NTF3**[1617], **ASAH1**[1618] (not shown), **ATR**[1619] (not shown), **PRKCE**[1620], TP53[1280] and HDL-S1P[1621]. Further, GSK3B binds to **SREBF1**[1622], **MAPT**[1623], **BAG6**[884], HSPA8[1131], **HLA-DQA1**[1624] (not shown), DDIT4[1625], AKT1[1626], PPP1CA[1277],

PTPN1[1612], **AXIN1**[1368], NF-KB[1627], mTORC1[884], STAT3[1628] and the PD proteins **LRRK2**[1629] and **SNCA**[1630].

GSK3B increases the phosphorylation of **MAP1B**[1631] (binding to both **SNCA**[1632] and PINK1[1633]) and **MAPT**[312] (dephosphorylated by **AXIN1**[1369]), affecting microtubule binding and stabilization. GSK3B activation also increases accumulation of cholesterol[1634], increases the accumulation and activation of TP53[1635, 1636] and the activation of CASP3[1637, 1638] and STAT3[1609]. Further, GSK3B inhibits **SREBF1**[1639], inhibits lysosomal acidification and regulates autophagy by regulating mTORC1 activity[963, 1640] and regulates NF-KB complex activation (in the nucleus)[1641-1645] and TP53 expression[1646-1648]. Therefore, GSK3B can play a crucial role in **SNCA** aggregation, autophagy, cholesterol regulation and activation of the apoptotic factors TP53 and CASP3.

D1.3.2 PTEN

The phosphatase and tumor suppressor PTEN is located in the cytoplasm when it is nonubiquitinated and located in the nucleus when monoubiquitinated[312]. PTEN knock down in DA neurons is neuroprotective in PD models[297]. PTEN interacts with the familial PD proteins PARK7 and PINK1. (Oxidized) PARK7 binds PTEN[1649], decreases its expression[428] and inhibits its activity[1649], whereas PINK1 is bound and regulated by PTEN[1650, 1651]. Further, PINK1 also binds to **KIF11**[1633], the familial PD proteins HTRA2[1652], **PARK2**[1653], PARK7[1654] and **SNCA**[1655] and binds and is activated by MARK2[1000] (MARK2 increases the expression of the vasopressin transporter[1656] **SLCO3A1**[1657] (not shown) and vasopressin levels are altered in PD[1658, 1659]). Thus, PTEN can affect multiple familial PD proteins via PINK1 regulation. PTEN expression is increased by **JARID2**[1660], whereas **COL18A1** decreases tyrosine phosphorylation of PTEN[752]. In addition to PINK1 regulation, PTEN also regulates STAT3 expression[1661], increases the expression of **FAM134C**[1405], **HAVCR1**[1405], **MBP**[1662] and **RAP1A**[1663] and decreases the expression of **PLOD1**[1405], NF-KB[1664], **SREBF1**[1405, 1406], MTOR[965], CTNNB1[1364], **ANGPT2**[1665], PITX3[297], PLAU[1666], CCL5[1667] and TH[297]. Further, PTEN activates CASP3[1668], inhibits RAC1[852], and binds **MAP2K6**[1650], **CENPC**[1650], AR[1485], PPP1CA[1269], CAV1[801] and MC1R[1070].

PTEN also decreases FOXO1 phosphorylation[1669], increases cytochrome c release[410] and increases mislocalization of the adaptor protein **PARD3**[850] (not shown; **PARD3** binds to **FRMD4A**[1670]). PTEN is therefore involved in regulation of apoptosis (CASP3, cytochrome c), autophagy (MTOR) protein modification (**PLOD1**), cholesterol homeostasis (**SREBF1**, CAV1), the immune response (STAT3, NF-KB, CCL5, **HAVCR1**; see also section D2), DA metabolism (TH, PITX3), the MAP kinase pathway (**MAP2K6**) and beta-catenin aggregation (CTNNB1).

D1.3.3 TP53

TP53 is activated in response to, among others, oxidative stress and DNA damage. Phosphorylated (i.e. activated) TP53 levels are increased in the SN of the PD brain[251]. TP53 expression and thus the susceptibility to TP53-dependent apoptosis is increased by **SREBF1**[1417], **GAS2**[1671], the familial PD protein UCHL1 (increases TP53 accumulation)[434] and histon demethylase **KDM2B**[1672] and decreased by the transcriptional repressor **TBX3**[1673]. TP53 is activated by PARK7[1674] (not shown), GSK3B[1636], STK11[997], **CXCR4**[1675] and **BAG6**[1676, 1677] and binds **MAP1B**[1678], SERPINB9[846] (not shown) and UCHL1[1679]. TP53 increases the cleavage of familial PD protein EIF4G1[1680] (not shown).

Activated TP53 translocates into the nucleus by binding to **KPNA4**[1681], where TP53 subsequently binds to **EHMT2**[1682], **KANSL1** [1683] (suggested to be involved in PD risk[1684]), **ATR**[1685] (**ATR** binds to **HUS1**[1686], **TP53BP1**[1687] and transcription factor E2F1[1685] and E2F1 binds to **MCPH1**[1688]), **TP53BP1**[1689] (binds to E2F1[1690]), **PRKRIR**[1691], GSK3B[1692], SIRT1[650] and PARK7[436]. Inhibition of the deacetylase activity of SIRT1 by **CCAR2**[655] increases acetylated TP53 levels and TP53-mediated apoptosis[651], whereas oxidized PARK7 binds to the TP53 DNA-binding region and thereby inhibits TP53-dependent gene transcription[1693].
TP53 increases the expression of **AGTR1**[1694], **COL2A1**[1695], **COL18A1**[1696, 1697], **ULK2**[1695], **MPI**[713], **PRKG1**[1698], **FRMD4A**[1699] (not shown), COMT[282], **CD200**[1700] and ICAM1[1701, 1702] and decreases the expression of **HLA-DQA1**[1703], **HLA-B**[1704], **HS3ST1**[1703], **WDHD1**[1705], **IL2RA**[1706], HSPA8[1703] and **SREBF1**[1407], showing involvement in regulation of, among others, DA degradation (via COMT), the angiotensin system (via **AGTR1**), autophagy (via **ULK2**), the immune response (via **CD200**, **HLA-DQA1**, **HLA-B**, **IL2RA**), protein degradation pathways (via HSPA8, **PRKG1**) and cholesterol regulation (via **SREBF1**).

D1.3.4 CASP3

CASP3 induces apoptosis and is increased in the SN of PD patients[125,136]. CASP3 is activated by UCHL1[434], ATG7[958], **PTPN1**[1707] (also activates CASP9[1707]), HTRA2[435, 1708] and CASP9[433] and inhibited by the TGF-beta propeptide **BMP7**[1709], JAK2[1710], SIRT1[660], **FRMD4A**[1711], **PRKCE**[1712], PARK7[436, 1713] and **SNCA**[1714], whereas **AGTR1** activation increases CASP3 expression[359]. CASP3 activates the cell death substrate **GAS2**[1715], which is associated to the cytoskeleton and during apoptosis cleaved by CASP3 causing rearrangements of the cytoskeleton[1715]. The myristoyltransferase **NMT2** binds to both CASP3 and TP53[1716]. Myristoylation, the transfer of a myristoyl-group, a 14-carbon saturated fatty acid, to a protein, typically promotes membrane binding, which is essential for protein localization and function[1717]. During apoptosis CASP3 mediated cleavage of **NMT2**, results in a relocalization of **NMT2** from the cytoplasm to the plasma membrane[1718] and CASP3-mediated cleavage of intracellular proteins enables **NMT2** to myristoylate many of these proteins[1717], resulting in the translocation of these caspase-cleaved and myristoylated proteins to their new membrane locations to affect apoptosis[1719].

D2. Immune regulation

The PD landscape includes the regulation of immune cells, that may become activated by the death of DA neurons and/or cause the induction of DA neuron death. More specifically, the main immunological cascades in PD in a dendritic cell, astroglia, microglia, CD4+ T(helper) cell, CD8+ T(helper) cell, B cell and the DA neuron are presented. The next sections discuss the immune cell-specific processes. Yet, note that multiple pathways that are described below are not per se limited to immune cells and may also fulfill functions in DA neurons. Section D2.1 discusses the immune-related pathways and proteins in the PD landscape and section 2.2 discusses the activation of the immune cells of the innate and adaptive immune response.

D2.1 Immune-related pathways

STAT3 and the NF-KB-complex both regulate cellular responses to (extracellular) stimuli and both regulate activation of immune-related processes. More specifically, STAT3 and NF-KB are stress-responsive transcription factors that after activation in the cytoplasm bind to, and are transported into the nucleus by nuclear importin alpha 3 (**KPNA4**)[1720-1722]. In the nucleus they regulate transcription together with (among others) earlier mentioned transcription regulators SIRT1, PTEN, GSK3B and CTNNB1, the transcription factors **SREBF1**, **ATF6**, AR, VDR, FOXO1, and MITF and the – in DA neuron development implicated – transcription factors ASCL1, **MSX1**, NEUROG2, NR4A2, PITX3 and SOX2.

D2.1.1 JAK2/STAT3

The JAK2-STAT3 pathway transduces extracellular signals over the cell membrane into the cell by binding of JAK2 to multiple cell surface receptors. JAK2 binds to **CXCR4**[1723], **AGTR1**[358], the interleukin 5 receptor **IL5RA**[1724] and binds and is inhibited by **PTPN1**[1725] and is activated by **FER**[1726], vitamin D3[1105], the immune factors IL2[1727], IL12[1728, 1729] (not shown) and CCL5[1730] and the receptors **CXCR4**[1731], **AGTR1**[356, 357] and the INS receptor (INSR)[1230]. Subsequently, JAK2 increases activation of NF-KB complexes[1732] and binds[1733] and activates the transcription factor STAT3[1734-1736], which is involved in cell growth, immune response[312], DA neuronal apoptosis[1737] and extracellular **SNCA**-mediated neurotoxicity[1738]. STAT3 binds (in addition to JAK2) also to FOXO1[625], RAC1[1739] (not shown), **CXCR4**[1723] (is increased by JAK2[1723]), **PTPRT**[1740], **PTPN1**[1741] and **PRKCE**[1742] and is activated (via JAK2) by **FER**[1726], IL2[1743] (not shown), IL6[1744] (not shown), IL10[1745] (not shown), IFNG[1746] (not shown), CXCL12[1747] (not shown) and mTORC1[1748] and inhibited (via JAK2 inhibition) by **PTPN1**[1725, 1749] and **PTPRT**[1740].
Moreover, STAT3 expression is increased by CCL5[1750] and decreased by **COL18A1**[752] and SIRT1[668] and is indirectly affected by **CCAR2** that binds and inhibits SIRT1 activity[655].

STAT3 increases the proliferation of T cells by preventing apoptosis[1751] by limiting their

production of IL2 (via upregulation of FOXO1[1752]). Activation of STAT3 results in its nuclear translocation where it acts as a transcription activator[1720]. This translocation of STAT3 is an active process that requires receptor-mediated endocytosis[1753], e.g. dysregulation of the clathrin-mediated endocytosis regulator **AMPH** disrupts co-localization of STAT3 with endocytic vesicles and subsequent transport to the perinuclear region[1753]. In the nucleus STAT3 binds to **ETV6**[1754], SIRT1[651], FOXO1[625], MTOR[1755] (not shown), MITF[1063] and NF-KB[1756-1758]. By binding, STAT3 and NF-KB can influence each other's transcriptional activity[1758, 1759] or can collaboratively induce gene expression[1760]. STAT3 also binds to the promoter of IFNG[1761], IL5[1762], IL6[1760] and CCL5[1763], increases the expression of **IL2RA**[1764], AKT1[1765] (not shown), PLAU[1766], SOD2[1767], **PROK2**[1768], CCL5[1760] (and also CCL5 release[1763]), ITGB2[1769, 1770] and ICAM1[1211, 1769, 1771], regulates the expression of **HLA-DRB1**, **HLA-DQA1** and **HLA-C**[1772] and decreases the expression of transcription factor **SP110**[1766], MITF[1059] and **SREBF1**[1408].

Thus, STAT3 regulates (or is regulated by) the immune response (via NF-KB, IFNG, IL2, IL5, IL6, CCL5, ITGB2, **IL2RA**, ICAM1, **HLA-DRB1**, **HLA-DQA1**, **HLA-C**), regulates gene transcription with multiple other transcription regulators (SIRT1, FOXO1, MITF, NF-KB, **SREBF1**), is affected by the ER UPR (via **PTPN1**) and regulates the AKT1/mTORC1/**SREBF1** pathway (see also [973]).

D2.1.2 NF-KB

The cytoplasmic NF-KB complex (consisting of NFKB1, NFKB2, REL, RELA and RELB) functions as a transcription factor that rapidly acts upon cell stimulation by extracellular stimuli[312] and regulates autophagy[1773], immune responses, inflammation, cell growth and apoptosis[312]. PD patients show increased NF-KB levels in the nigrostriatal DA region[201] and in the nuclei of DA neurons[225].

The NF-KB-complex is activated by **CPNE4**[507], **CUL2**[507] (not shown), **GNA12**[746], **PRKCE**[1774, 1775], **PIK3CD**[1776], **TRAPPC9**[1777], **PARK2**[1778], **ZAK**[1779] and **AGTR1**[360, 361], regulated by **TRAF3** (inhibits NFKB2[1300], REL[1780], RELA[1780], RELB[1780] and activates NFKB1[1781]) and inhibited by **PTPN1**[1782], **SENP7**[1783] and **NPTX2** (inhibits NF-KB through a TP53-PTEN-PI3K-AKT1 pathway[1784], regulates excitatory synapse formation, is highly upregulated in PD and is a component of Lewy bodies[80]). Further, NF-KB binds to **CXCR4**[1785], **CCAR2**[1132], **RPL7A**[1132], **CUL2**[1786] (also binds to **TMCC3**[1134]), **USP9X**[1132] and **SND1**[1002], activates **PIK3CD**[1776] and NF-KB transport into the nucleus is increased by PARK7[1787]. These NF-KB-regulating proteins are involved in a variety of processes, such as signal transduction (**GNA12**, **CXCR4**, **PRKCE**, **PIK3CD**), angiotensin system (**AGTR1**) protein synthesis (**RPL7A**, **SND1**), post-translational modification (**SENP7**), protein ubiquitination and degradation by the E3 ubiquitin ligase complex (**PARK2**, **TRAF3**, **CUL2**, **USP9X**), (apoptotic) transcription (**CCAR2**) and the ER UPR (**PTPN1**). Interestingly, many of these proteins are related to

protein synthesis, modification and degradation. Dysregulation of these processes may result in misfolded proteins in the cytoplasm, resulting in increased ER stress and UPR activation.

Further, NF-KB expression is increased by FOXO1[629] and **MAP2K6**[1284, 1285] and decreased by PTEN[1664] and the transcription factor **POU2F1**[1788] (that also decreases **HLA-DRA**[1789] and ICAM1[1790] expression).
Nuclear NF-KB binds to **POU2F1**[1788, 1791], the histon methyltransferase **EHMT2**[1792] (that also decreases CXCL12 expression[1793] and binds to histon modificator **RCOR1**[1794]), GSK3B[1627], **TP53BP1**[1795], **SND1**[1002], **TLE1**[1796] (not shown), **DDX3X**[1132], STAT3[1756-1758] and CCL5[1797] (not shown). Further, NF-KB increases the expression of the cytokines CCL5[1798, 1799], CXCL12[1800], IFNG[1801], IL1B[1802], IL2[1803], IL5[1804], IL6[1805, 1806], IL10[1807], IL12[1108, 1806] and TNF[1802, 1806] and of **ABCG1**[1808], **CREM**[1809], **CXCR4**[1810, 1811], **HLA-B**[1812], **IL2RA**[1803, 1813-1816] and ICAM1[1798, 1817]. Further, NF-KB decreases the expression of **CYP17A1**[609] and regulates **PRF1** expression[1818] (not shown). Thus, NF-KB regulates histon regulation (via **EHMT2**, **RCOR1**), many immune related processes (via CXCL12, IFNG, IL1B, IL2, IL5, IL6, IL10, IL12, TNF STAT3, CCL5, **HLA-B**, **CXCR4**, **IL2RA**, ICAM1, **PRF1**), testosterone production (via **CYP17A1**) and cholesterol regulation (via **ABCG1**).
Also multiple nuclear proteins regulate NF-KB activity, i.e. NF-KB is activated by **MAP2K6**[1283] and GSK3B[1643] and inhibited by CTNNB1[1819] and by the kinase and DNA damage sensor **ATR**[1820] (inhibits GSK3B-mediated NF-KB activation[1619]) and by a complex consisting of the transcriptional corepressor **TLE1** and SIRT1[652]. Moreover, **TLE1** also binds to GSK3B[884], the **26S** proteasome[846] and the transcription factor **RUNX3**[1390] (decreases expression of AKT1[1821] and increases expression of IFNG[1822] (not shown) and **ITGAL**[1823] (not shown)).

Thus, NF-KB is regulated by signal transduction, protein synthesizing and modifying pathways in the PD landscape, resulting in activation of multiple immune response pathways. Increase in NF-KB, as seen in the DA neurons of PD patients, mirrors the increased stress and the inflammatory state in DA neurons resulting in cell death.

D2.1.3 Cell adhesion, cell-cell interaction, axon guidance and immune regulation

Many proteins in the PD landscape are involved in cell adhesion, cell-cell interaction and axon guidance and are hereby also often involved in regulation of immune cells, for immune cells require these same mechanisms for e.g. chemotaxis, co-stimulation and activation. Members of the immunoglobulin superfamily are often associated with the immune system and include proteins involved in antigen presentation, cytokine binding, cell adhesion and co-stimulation. The immunoglobulin-like domains of the immunoglobulin superfamily proteins can be classified as variable (IgV), constant (IgC1 and IgC2) or intermediate (IgI). All PD-GWAS-associated HLA proteins have the IgC1

domain[312] and can therefore interact with the T cell receptor. Further, **AMIGO2**, **CD200**, **CNTN1**, **DSCAM**, **NEO1**, **NCAM2**, **NEGR1**, **PTPRT**, **ROBO2** and **SEMA3E** all have one or more IgC2 domains[312], the same domain that is found primarily in the mammalian T cell surface antigens CD2, CD4 and CD80 and the intercellular cell adhesion molecule ICAM1[312]. Furthermore, **HAVCR1** and **BTNL2** have the IgV domain that is also located on the T cell surface antigens CD2 and CD4[312]. Indicating that all these proteins may have a function in the immune system. And, apart from **AMIGO2**, **CNTN1** and **NEGR1** (that are involved in cell-adhesion and axon guidance[1824-1826]) all of these proteins indeed have a known function in the immune response. For instance, **CD200** is involved cell adhesion and in the activation of the immune response (see also section D2.2.1). The axon guidance receptor **DSCAM** is involved in neuronal self-avoidance[312] and is part of the innate immune system in invertebrates[1827, 1828]. (However, it is unknow if **DSCAM** has a function in the human immune system.) RGMA is expressed by T- and B-cells and inhibits migration of these cells by contact- and chemo-repulsion via its receptor **NEO1**[1829, 1830], activating RHOA[1831] (not shown), which regulates the actin cytoskeleton in migration, axon guidance and cell adhesion[312]. **NCAM2** is a cell adhesions molecule that regulates neurite outgrowth[1832], its expression is increased by IL2 and essential for survival and differentiation of natural killer cells[1833] (not shown). **PTPRT** is involved in cellular adhesion[312] and increases dephosphorylation of STAT3 (not shown)[1740], which is essential in the differentiation of T cells[1834]. SLIT2, the ligand for **ROBO2**[1835, 1836] has a role in axon guidance and regulates cell migration[312], but also inhibits CCL5-induced[1837] and CXCL12-**CXCR4**-induced[1838] leukocyte chemotaxis (not shown). The secreted semaphorin **SEMA3E** (not shown) inhibits the CXCL12 regulated migration of thymocytes in the thymus[1839]. (**SEMA6D**, the other semaphorin in the GWASs, has no immunoglobulin-like domain, but is nevertheless required for late-phase activation of T cells, and its expression on the membrane of CD4+ T cells is increased after their activation[1840].) And lastly, **HAVCR1** is expressed on CD4+ T cells and amplifies their activation[1841], whereas **BTNL2** is a negative regulator of T cell proliferation[312]. As a result, expression of IL2 by T cells is increased by **HAVCR1**[1842] and decreased by **BTNL2**[1843].

Other PD-GWAS-associated proteins involved in cell adhesion, cell-cell interaction and/or axon guidance, but without immunoglobulin-like domain, are the integrins **ITGA2B**, **ITGA6** and **ITGA8**, the cadherins **CDH6**, **CDH23** and **PCDH8**, the cell-surface protein **NLGN1**, the contactin **CNTNAP2** and the metalloproteases **ADAM12**, **ADAMTS2** and **ADAMTS14**. The integrines **ITGA2B** (binds **COL2A1**[1844], is increased by **PRKCE**[1845] and **SCIN**[1846]), **ITGA6** and **ITGA8** mediate cell-cell interactions, neurite outgrowth and adhesion to the ECM[312]. The Ca^{2+}-dependent cadherins CDH2, **CDH6**, **CDH23** and **PCDH8** form adherends junctions for cell-cell interactions. CDH2 binds to **GNA12**[740] and **AMPK**[1847], and is probably internalized by interaction with **PCDH8**[312]. **NLGN1** binds **PTPRT**[1848] and **DLG2**[1849] and is a postsynaptic cell-adhesion molecule that regulates the

formation of excitatory synapses[1850]. **CNTNAP2** regulates the myelination of axons by glia cells[1851]. The metallopeptidase **ADAM12** is involved in amyloid-beta neurotoxicity and neuronal death[1852]. **PRKCE** binds to **ADAM12**[1853] and increases, like **ANGPT2**[1854], expression of **ADAM12**[1855] and increases, like **COL2A1**[1853], translocation of **ADAM12** to the cell membrane[1856]. At the cell membrane **ADAM12** binds to TGFBR2[1857] a growth factor receptor that decreases the expression of gap junction protein **GJB2**[1858], binds to **BAG6**[1859] and is inhibited by **BAMBI**[1386]. The extracellular pro-collagen N-propeptidases **ADAMTS2** and **ADAMTS14** process procollagens into mature collagens that are able to form collagen fibrils and thereby regulate ECM organization[312, 1860].

Of all these proteins, only **ITGA2B**, **ITGA6**, **ADAM12** and **ADAMTS2** have explicitly been linked to immune regulation. For **ITGA2B** increases proliferation of T cells[1861] and secretion of IL6[1862], **ITGA6** increases microglia activation[1863], **ADAM12** regulates differentiation of T cells[1864] and **ADAMTS2** mRNA is increased specifically in monocytes and macrophages after glucocoricoid stimulation[1865].

Thus, multiple proteins in the landscape regulate cell adhesion and axon guidance and many of those proteins, especially when containing an immunoglobulin-like domain, also have explicit functions in immune cell regulation. The cell adhesion proteins, without an immunoglobulin-like domain (the integrins, cadherins and metalloproteases) are only sporadically linked to immune regulation. However, increasing knowledge about their protein function may show that they are also involved in adhesion, activation and/or migration of immune cells. Overall, dysregulation of these proteins may affect immune cell regulation and ofcourse also neuronal axonal and synaptic functioning.

D2.2 Immune cell activation

PD is associated with immune system alterations, such as ratio changes in lymphocyte populations[1866-1869], immunoglobulin[1870] and cytokine levels[185, 1871]. Moreover, PD patients show a lower incidence of infections and cancer, suggesting an (over)active immune system[1872]. PD pathogenesis is associated with multiple interleukines, inflammatory factors and cytokines. For example, SNPs in the gene or gene promotor of IL1B[183], TNF[247-249], IL6[187] and IL8[188] are associated with PD. And, IFNG[178, 179], CCL5[140, 141], CXCL12[79], IL1B[141, 184, 185], IL2[186 185], IL4[179, 185] (not shown), IL6[184], IL8[141], IL10[181] and TNF[141, 179, 250] all show increased expression levels in PD patients. Further, the receptors (subunits) for IL2, IL5, CCL5 and CXCL12, respectively **IL2RA**, **IL5RA**, **CCR4** and **CXCR4** were found in the GWASs.

Upon cytokine activation the expression of intercellular adhesion molecule 1 (ICAM1) is increased (by IL1B[1873, 1874], IFNG[1874, 1875], TNF[1874, 1876] that increase binding of NF-KB to the ICAM1 promotor and by IL2 that increases binding of STAT3 to the ICAM1 promotor[1877]). ICAM1 regulates cell-cell interactions, adhesion and proliferation and is

expressed on many (immune) cell types, i.e. it is expressed on astroglia[176], dendritic cells[1878], microglia[1879], T cells[1880-1882], B cells[1883, 1884] and vascular endothelial cells[1885] and subsequently functions as a ligand for the integrin lymphocyte function-associated antigen-1 **LFA-1**[1886-1889] and thereby attracts immune cells[1887, 1889, 1890]. **LFA-1** is a complex of integrin alpha-L (**ITGAL**) and integrin beta-2 (ITGB2)[1891, 1892] that is expressed on circulating leukocytes and mediates rolling on ICAM1, which makes leukocytes bind to vascular endothelial cells, so they can enter the target tissue[1893]. The actin anchor **ACTN4** binds to ICAM1[1894] and is required for leukocyte extravasation[1894].

In addition to cytokines, ICAM1 expression is also increased by **ANGPT2**[1895], TP53[1701], FOXO1[641], **COL2A1**[1896], familial PD protein PARK7[1787], JAK2 activation[1897, 1898] and by INS that increases binding of STAT3 to the ICAM1 promotor[1211]. Further, ICAM1 expression is decreased by **HLX**[1899], **BMP7**[1900], **POU2F1**[1790], **COL18A1**[752] and IL10 (decreases NF-KB binding to the ICAM1 promotor[1875]). ICAM1 itself increases expression of **IL2RA** on CD8+ T cells[1901] (not shown), decreases the expression of **SREBF1**[1410] and binds **IL2RA**[1902] and **ACTN4**[1894]. Furthermore, cholesterol[1903, 1904], VLDL[1905], 7-ketocholesterol[1169] (not shown), LDL and oxLDL[1906] increase expression of ICAM1, showing a role for lipids in activating the immune response.

(Sphingo)lipids (e.g. cholesterol, ceramide and sphingomyelin) are important in regulation of the immune response, i.e. shingomyelinase and ceramide inhibit IL2 production[1907], ceramide increases expression and secretion of the proinflammatory interleukin IL8[1908, 1909], expression of intercellular adhesion molecule 1 (ICAM1)[1910] and is involved in the secretion of IL6[1911]. Ceramide and sphingomyelin are themselves also regulated by cytokines that are elevated in PD, i.e. IL4 decreases the quantity of ceramide[1912] (not shown) and IFNG decreases the quantity of sphingomyelin[1913] (not shown). Lipid rafts on the membrane also regulate clathrin-independent constitutive endocytosis of the lipid raft-associated interleukin-2 receptor (IL2R, subunit **IL2RA** in GWASs)[1914], recycling of the immune related integrin complex **LFA-1** to the plasma membrane[1915] and **LFA-1**-mediated adhesion of T cells and primary T cells[1916].

Therefore, a proper balance of sphingolipids and cholesterol in the cell is mandatory to maintain a normal immune regulation, for their dysregulation directly affects the regulation of cytokines and migration of immune cells. Moreover, membrane organization is of crucial importance for antigen-presenting cells to capture and present antigens, i.e. membrane lipid rafts are involved in regulation of endocytosis, membrane trafficking and activation of the immune response[781, 782]. Discrimination between 'self' and 'non-self' antigens during antigen presentation is one of the key elements of the adaptive immune system (see section D2.2.2). The proteins required for antigen presentation, the MHC class I proteins **HLA-B** and **HLA-C** and the MHC class II proteins

HLA-DQA1, **HLA-DQA2**, **HLA-DQB1**, **HLA-DQB2**, **HLA-DRA**, **HLA-DRB1**, were found in the PD GWASs, and the HLA gene region has been associated to PD[71-73]. Whereas MHC class I peptides for antigen presentation to CD8+ T cells are derived from **26S**-processed cytoplasmic proteins, MHC class II peptides for presentation to CD4+ cells are acquired via endocytosis of antigens[1917]. This shows the importance of the proteasome and the endosomal system in immune cell activation. For example, disruption of lipid rafts inhibits internalization of **SNCA** by microglia[1918] and affects localization of MHC class II proteins on the plasma membrane of dendritic and B cells[782, 1919], which again affects T cell activation[1919].

In summary, activation of the immune response, production of cytokines, immune cell migration and regulation of antigen presentation (which requires the endosomal/lysosomal system (see also section B2) for antigen processing) are regulate by (sphingo) lipids and are highly associated with PD. The next sections discuss the activation of the innate (D2.2.1) and adaptive (D2.2.2) immune response and the involvement of coagulation factors in immune regulation (D2.2.3).

D2.2.1 Activation of the innate immune response

The innate immune system is the first line of defense against infection, and consists of epithelial barriers, astroglia[1920], phagocytes (e.g. microglia, denritic cells) and the complement system. Activated astroglia secrete cytokines (e.g. IL6) that regulate both innate and adaptive immune responses[1920]. **SNCA** stimulates astroglia to produce IL6 and ICAM1[1921], and together with the ability of **SNCA** to activate microglia, this can attract microglia to the site of inflammation[1922]. Indeed, the astroglia cells in the SN of PD patients, show increased ICAM1 expression, which is associated with increased **LFA-1** positive microglia in the SN[176]. Moreover, activated, ICAM1 and **LFA-1** positive, microglia are increased in the SN and striatum of PD patients[50, 1923] and based on *in vitro* studies contribute to the death of DA neurons[1924-1926]. Multiple factors such as (extracellular) **SNCA**, NM, S1P and cholesterol activate microglia [1927-1930], whereas oxLDL/oxysterols ameliorates microglia activation via the LXR receptor[1170, 1930]. **SNCA**-mediated microglia activation increases the expression of MHC class II proteins, leading to CD4+ Th cell proliferation (part of the adaptive immune response) and DA neurodegeneration[1931]. Further, NM-mediated microglia activation is NF-KB-dependent[1927, 1932] and results in the production of pro-inflammatory cytokines[1932]. The interaction of glycoprotein **CD200**, located on the plasma membrane of a neuron, with its receptor CD200R1 (reduced expression in PD patients[144]) on the microglia holds the microglia in a quiescent state[1933]. In a PD rat model impaired **CD200**-CD200R1 function resulted in an increase in microglia activation and increased degeneration of DA neurons[1933, 1934]. **CD200** expression is inversely correlated with expression of ICAM1[1933] and **CD200** decreases the expression of **ITGAL** and thus **LFA-1**[1935] and thereby controls

the regulation and signaling of immune cells. Microglia activation is also increased by **ITGA6**[1863], IL5 and **IL5RA**[1936], **STAP1**[1937] and IFNG[178] and inhibited by **RCOR1**, that thereby prevents inflammation-induced death of DA neurons[1525]. In contrast, **RCOR1** also inhibits astroglia activation[1525], whereas astroglia activation (by **ATF6**) results in neuronal survival in an MPTP mice model[1392]. This indicates that a delicate balance in microglia and astroglia activation and inhibition is necessary for DA neuron survival. Moreover, extracellular NM also stimulates the maturation of dendritic cells[1938]. Dendritic cell levels are reduced in blood of PD patients (maybe due to recruitment to inflammatory sites) and negatively associated with motor symptom severity[1939].

Activated microglia and dendritic cells start secreting the pro-inflammatory factors IL1B, IL12 (IL12 is a heterodimeric protein, encoded by IL12A and IL12B[312]), TNF and CCL5[1929, 1940-1942]. IL1B increases NF-KB-dependent ICAM1 expression on glial cells[1873], increases the expression of NR4A2[1528] (not shown) and CCL5[1941] and decreases the expression of **HLA-DRA**[1943]. IL12 secretion by microglia is inhibited by IL10 and astroglia[1940] (not shown) and its expression is decreased by **TRAF3**[1780, 1944] and blockage of Ca^{2+} influx[1108] (e.g. via **CACNA1A** and **CACNA2D3**), but increased by **PRKCE**[1945]. In dendritic cells the intramembrane-cleaving aspartic protease **SPPL2B** promotes proteolysis of TNF to trigger IL12 production[1946]. Secreted IL12 increases the expression of **IL2RA**[1947], **ITGA6**[1948], **HLX**[1949] and CCL5[1950] and decreases the expression of the CCL5 receptor **CCR4**[1951].

Lastly, the complement protein **C9** is located in lewy bodies in PD SN[52] and is a subunit of the membrane attack complex that forms pores in the plasma membrane of target cells[312]. The plasma protease C1 inhibitor (**SERPING1**) inhibits the complement system[1952] (not shown).

Thus, the innate immune response is activated in PD pathology and NM- and **SNCA**-dependent activation of dendritic cells and microglia results in the presentation of SN antigens to the adaptive immune system[1938, 1953]. This indicates that autoimmune responses to DA neuron-specific antigen may play a role in the pathology of PD. Of note, whereas cholesterol and oxysterols both increase ICAM1 expression, they have opposing effects on microglia activation.

D2.2.2 Activation of the adaptive immune response

The adaptive immune system is unlike the innate immune system highly specific to the invading pathogens and is activated when the innate immune system is not sufficient. Cytokines secreted by cells of the innate immune system recruit and activate the cells of the adaptive immune response, (CD4+ and CD8+) T cells and B cells, to the site of inflammation, i.e. IL1B stimulates T and B cell maturation and proliferation[1954], IL12 regulates differentiation of naive T cells into CD4+ T cells[1955] and enhances the activity

of naïve CD8+ T cells[1956], whereas TNF activates CD4+ T cells[1957, 1958]. And lastly, CCL5 is a chemoattractant for T cells[312] and increases the expression of IL1B[1959] and STAT3[1750].

The next sections will show in more detail the processes involved in migration and chemotaxis of T and B cells (D2.2.2.1) and the activation of T (D2.2.2.2) and B cells (D2.2.2.3).

D2.2.2.1 Migration and chemotaxis of T and B cells

In addition to ICAM1-mediated attraction of immune cells (see section D2.2), B and T cell migration is inhibited via contact repulsion by the repulsion guidance molecule RGMA together with its receptor **NEO1** [1829]. They thereby regulate lymphocyte infiltration in the tissue and suppress the inflammatory response [1829]. Moreover, chemotaxis is also regulated by **PARD3**, **CCR4**, CXCL12-**CXCR4** interaction and S1P, i.e. **PARD3** regulates polarization and chemotaxis of T cells[1960] and **CCR4** is a chemoattractant homing receptor on circulating T cells[312] and microglia[1961], for the T cell attractant CCL5[1750].

CXCL12 activates **RAP2A** (not shown), which is essential for B cell migration[1962]. Cholesterol increases the binding of CXCL12 to **CXCR4**[1963], which activates **CXCR4** resulting in increased intracellular Ca^{2+} levels[312], activation of the JAK2/STAT3 and NF-KB pathways (see sections D2.1.1 and D2.1.2) and an increased expression of **LFA-1**, which increases adhesion of B cells to ICAM1[1731, 1887, 1964, 1965] (not shown). Interestingly, **CXCR4** is increased in the nigro-striatal system of PD patients[79] and CXCL12 and **CXCR4** are both increased in a mouse model preceding DA neuron loss[79]. Moreover, **CXCR4** binds the familial PD protein ATP13A2[866] and **CXCR4** distribution on the cell membrane is regulated by the CXCL12-activated[1966] **RAP1A**[1967].

And lastly, S1P is transported out of the cell by **SPNS2**[734], to create a S1P gradient that attracts T and B cells[1968]. S1P is transported by the lipoproteins LDL and HDL[1969] (HDL is the major S1P carrier[1970] and has a 9-fold higher S1P content than LDL[1969]). HDL-S1P inhibits GSK3B[1621], activates STAT3 and increases cell migration[1971]. STAT3 increases the expression of the S1P receptor (not shown), creating a positive feedback loop[1972]. S1P activates both STAT3 and NF-KB[1973].

In summary, migration of T and B cells to their target tissue is regulated by ICAM1-**LFA1**, RGMA-**NEO1**, CCL5-**CCR4** and CXCL12-**CXCR4** ligand-receptor interactions and S1P gradients. Further, cholesterol, oxysterols and lipoproteins regulate the activation and expression of ICAM1 and **LFA1** and are therefore important in astroglia and microglia mediated activation of T and B cells. An increase of these factors in the PD SN indicates that T and B cell activation may be increased.

D2.2.2.2 T cell activation

In the blood of PD patients, CD4+ T cells subsets[1866-1868] and CD4+:CD8+ T cell ratios[1867] are decreased, and may reflect T cell alterations in brain. CD4+ T cell activation by peptides presented on MHC Class II proteins[1917], results in expression and secretion of IL2[1974, 1975], IL5[1974], CCL5[1976] and IFNG[1974]. IL2[1977, 1978] activates T cells and is together with **IL2RA** required for T cell proliferation[1979]. IL5 stimulates differentiation of B cells into immunoglobulin secreting cells[312] and is involved in microglia activation and proliferation[1936, 1980]. Further, CCL5 and also **NEDD9** increase secretion of IL2[1981, 1982] and IFNG activates microglia-mediated DA neuron death[178]. Thus, IL2, IL5, CCL5 and IFNG are involved in several reinforcing feedback loops of T cell and microglia activation and proliferation. T cell activation and proliferation is also amplified by the interaction between **TIMD4** (expressed on antigen presenting cells) and its ligand **HAVCR1** (expressed on T cells early after their activation[1841, 1983]. And, **PIK3CD** activation contributes to T cell development, migration and differentiation[312].

MHC Class I proteins (e.g. **HLA-B** and **HLA-C**) are located on almost all cells and present peptides to the immune system. CD8+ T cells become activated when these peptides are not recognized as endogenous and subsequently destroy the antigen presenting cell. The nuclear protein **RUNX3** regulates development of CD8+ T cells[1984] and increases CD8+ T cell proliferation[1985]. (Activated) CD8+ T cells contain **TIAL1**[1986] and **PRF1**[1987] (binds cholesterol[1988]) in their cytotoxic granules which are secreted into the cleft between the T-cell and its target cell. **LAMP1** regulates **PRF1** quantity and localization to these granules[1989]. **PRF1** makes pores in the plasma membrane of the target cell[1990] and **TIAL1** induces DNA fragmentation[1986]. Membrane permeabilization by **PRF1** makes it possible for granozymes and factors like **TIAL1** to enter the target cell and induce DNA fragmentation and apoptosis[1986, 1991]. **TIAL1** and **PRF1** are both regulated by IL2, i.e. it decreases **TIAL1**[466] and increases **PRF1**[1818] expression. Further, **PRF1** expression is increased by IL12[1992], which also increases activation of MTOR in CD8+ T cells[1993].

SERPINB9 (not shown) is a factor that protects cells against cytotoxic T cell-mediated apoptosis[1994] and is upregulated by STAT3[1766], IL1B[1995] and IFNG[1996] and binds to TP53[846], **TLE1**[846] and **PLEKHM1**[846] (**PLEKHM1** regulates vesicle acidification[1025] and the endocytic and autophagic pathway[1026]).

In the PD landscape, several nuclear proteins regulate T cell proliferation and activation, i.e. **TCF12** increases differentiation of T cells[1997], **RAI1** protects against auto-immune reactions by inhibiting lymphocyte activation[1998] and differentiation of T cells into Th1 and Th17 subsets[1999] and STAT3 activation is essential for the differentiation of helper T cells[1834, 2000]. Further, **HLX** is involved in the maturation of Th1 cells[2001] and regulates expression of ICAM1 and **LFA-1**[1899] (which are important for T cell-T cell

interaction, adhesion and proliferation[1882]) and Th1-specific gene expression[2002]. **DRG2** overexpression on the other hand, suppresses T cell growth[2003].

In summary, proliferation and activation of T cells, especially CD4+ and CD8+ T cells, is regulated by multiple proteins in the PD landscape. CD4+ T cells assist in B cell activation (see D2.2.2.3) and CD8+ cells destroy their target cell. Dysregulation in the activation or functioning of these cells may increase inflammation or autoimmune responses that increase DA neuron death.

D2.2.2.3 B cell activation

B cell activation occurs directly via antigen recognition by the B cell receptor (BCR) or with the assistance of CD4+ T cells. After antigen recognition the B cell presents a peptide of the antigen on MHC Class II proteins, which can be recognized by CD4+ T cells to provide costimulation and trigger B cell activation and proliferation. Now, the B cells migrate out of the lymphoid follicle (B cell zone) and a part of the activated B cells migrate into the germinal center for affinity maturation[2004]. Circulating B cells levels are reduced in the blood of PD patients[1866], and activation, differentiation, affinity maturation and migration of B cells is regulated by multiple proteins in the PD landscape. Activation of naive B cells is inhibited by **BANK1**, a protein that is highly expressed in B cells[2005, 2006]. After activation of the BCR, **BANK1** is phosphorylated and increases the mobilization of Ca^{2+} in B cells[2005] (not shown), inhibits AKT1 activation[2006] (not shown) and binds to STAT3[2007]. Also, the transcriptional regulator **RAI1** inhibits B cell activation and proliferation, by inhibiting BCR signaling[1998]. Activation of the BCR increases the activation of **STAP1**[2008, 2009]. **PIK3CD** is required for BCR signaling and regulates B-cell development, proliferation, migration[312]. Further, the proteins **LRRC25**, **BST1** and **TCF12** activate and regulate development and growth of B cells[2010-2014]. Furthermore, **TCF12** increases B cell differentiation[2015], increases the expression of FOXO1[627] and decreases the expression of gap junction protein **GJB2**[2016]. FOXO1 deficiency impairs B-cell development[2017] and is important in regulation of the PI3K-AKT1 axis in B-cell development[2017], the same axis that is inhibited by **BANK1** (see above).

During co-stimulation the CD4+ T cell secretes the cytokines IL2, IL4 and IL5 that bind to their receptors (e.g. **IL2RA** and **IL5RA**) on the B cell and trigger B cell activation and proliferation[2018-2022]. CRH decreases the expression of **IL2RA**[2023], but is also bound and inactivated by secreted **CRHBP**[312]. JAK2 binds to and is activated by the IL5 receptor subunit **IL5RA**[1724, 2022]. Further, IL5 and IL12 increase the expression of **HLX**[1949, 2024], which is low expressed in inactive B (and T) cells, but high in activated lymphocytes[2025], is involved in differentiation of B cells[2026] and regulates IFNG expression[1949, 2027].

B cell trafficking and recirculation through lymphoid tissues, which is required for

efficient antigen presentation and subsequent activation of B cells, is regulated by CXCL12 and **CXCR4**[2028]. **CXCR4** is expressed on B cells and quickly downregulated after CXCL12 binding[2029]. CXCL12 is produced in the cell layers surrounding the germinal center and attracts naive and memory B cells, but not germinal center B cells[2029]. This shows that the responsiveness to the chemoattractant CXCL12 is regulated during activation of B cells, to increase their trafficking to germinal centers[2029]. Moreover, adherens of B cells to ICAM1 is impaired in **NEDD9** knockout B cells, resulting in a reduced migration to secondary lymphoid organs[2030] for affinity maturation. B cells have to interact with antigen presenting cells in germinal centers to survive[2004] and the **LFA-1**-ICAM1 interaction contributes to B cell selection and promotes their survival[2031]. **RUNX3** is expressed in the late stages of B cells development into plasma cells[2032, 2033] and increases expression of **ITGAL** resulting in an increased **LFA-1** surface expression[1823], which would therefore be beneficial in B cell selection and survival. **ZNF385B** (associated with PD in three GWASs) is expressed in germinal center B cells and binds to TP53[2034] (not shown). Depending on the **ZNF385B** isoform, **ZNF385B** can have both pro- and anti-apoptotic actions in the B cell and can therefore affect B cell selection[2034].

B cell isotope class switching occurs in germinal centers. MTOR deficiency in B cells leads to a reduced high-affinity antibody production by decreasing the activity of activation-induced (DNA-cytosine) deaminase (**AICDA**, not shown). This enzyme causes DNA mutations and thereby leads to antibody diversity[2035] and thus is necessary for isotope class switching. **TRAF3** increases the expression of **AICDA**[2036] (not shown), but also inhibits (together with TRAF2) mature B cell survival and suppresses expression of NF-KB[1300, 2037]. NF-KB binds to the AICDA promotor and is required for its expression[2038] (not shown). This discrepancy with inhibition of NF-KB expression by **TRAF3** is explained by **TRAF3**-mediated inhibition of NF-KB inhibitors[2039, 2040]. Further, to induce isotope class switching, AICDA is actively transported into the nucleus of B cells by the importin **KPNA4**[2041] (not shown).

S1P induces adhesion to ICAM1 and thereby allows B cells and plasma (B) cells to exit the germinal centers and re-enter the circulation[2042]. Circulating B cells not only bind to ICAM1 on other cells, but can also express ICAM1 themselves and thereby costimulate T cells[2043].

In conclusion, the reduced levels of circulating B cells in PD and the regulation of their activation, differentiation, affinity maturation and migration by proteins in the PD landscape indicate that their regulation is part of the PD pathology. Changes in B cell activation by CD4+ T cells or by changes in their selection and maturation may affect the immune response in PD.

D2.2.3 Coagulation factors, lipoproteins and immune cell activation

Coagulation factors regulate the formation of a platelet and fibrin clot in a damaged vessel wall and dysregulation of this process can lead to increased clotting (thrombosis) or increased bleeding (hemorrhage). PD patients show a decrease in the serum coagulation factors α-2-antiplasmin and factor V[66]. Factor V deficiency leads to reduced clotting and hemorrhage[2044], whereas a decrease in α-2-antiplasmin would increase plasmin activity[2045]. The PLG gene encodes the protein plasminogen, that is converted into plasmin by the plasminogen activators PLAU and PLAT[312]. Extracellular **SNCA** regulates the plasmin system by increasing SERPINE1 expression[2046] (a serine protease inhibitor that mediates diabetic vascular complications and is increased in the plasma of patients with diabetes, obesity and hypertension[2047-2050]). SERPINE1 binds and inhibits both PLAU[2051-2053] (PLAU increases proteolysis of **MMRN1**[2054]) and PLAT[2045, 2055]. SERPINE1, PLAT and PLAU bind and are internalized by the low-density lipoprotein receptor-related protein 1B (**LRP1B**)[2056] and VLDLR binds to plasminogen and PLAU and mediates their endocytosis[2057]. Further, SERPINE1 increases cholesterol in the blood[2050] and plasmin increases degradation of LDL[2057, 2058], whereas plasminogen knockout mice show decreased HDL-cholesterol levels[2059]. Furthermore, SERPINE1 expression and secretion is increased by VLDL[2060, 2061], VLDLR[2061, 2062], LDL[2061] and OxLDL[2061] (enhances SERPINE1 expression compared to LDL induced expression[2061]) and PLAT expression is decreased by LDL[2063]. Thus coagulation factors and lipoproteins regulate each other. Knockout of PLAT in a mouse model decreased levels of dopamine, indicating a role for the PLAT/plasmin system in regulating dopamine release[2064].

In addition to lipoproteins and **SNCA**, SERPINE1, PLAT and PLAU expression is regulated by multiple other proteins in the PD landscape. Namely, SERPINE1 expression is also increased by **SREBF1**[1416, 2065], **ADAM12**[1857] (not shown), **BAG6**[1859], **BMP7**[608, 2066], STK11[995] (not shown), INS[639] (not shown), **NEDD9**[2067] (not shown) and **AGTR1**[363], and is decreased by **COL18A1**[2068], **PRKG1**[2069] (not shown), FOXO1[639], **SMAD5**[2070], vitamin D3 (via inhibition of NF-KB)[1106] and the cytoplasmic serine-threonine kinase **WNK1**[2071] (that binds and is phosphorylated by **STK39**[2072]). Further, SERPINE1 expression is increased by mRNA stabilization by TP53[2073] (not shown), SERPINE1 accumulation is decreased by **BMP7**[2070] and SERPINE1 activation inhibited by SIRT1[661]. Thus, SERPINE1 expression is regulated by lipoproteins (VLDL, LDL, oxLDL, **LRP1B**), the angiotensin system (**AGTR1**) and by four (**SREBF1**, FOXO1, NF-KB and TP53) of the main transcription regulatory pathways in the PD landscape, which therefore can affect SERPINE1-mediated inhibition of PLAU and PLAT. Moreover, the secreted latent-transforming growth factor beta-binding protein 1 (**LTBP1**) binds the extracellular protein Fibrillin-1 (**FBN1**)[2074], is activated by plasmin[2045] and increases the expression of **COL2A1**[2075] and SERPINE1[2076] and is thus an important regulator in the ECM.

PLAU is inhibited by IL1B[2077] and activated by a splice variant of **TMPRSS9** (highly expressed in CD8+ T cells)[2078], whereas PLAT is inhibited by LDL[2079] and activated by CRH via its receptor **CRHR1**[2080]. PLAU expression is decreased by **COL18A1**[2068], IL12[2081], PTEN[1666], mediated by CXCL12[2082] (not shown) and increased by **MAP2K6**[2083] (not shown), **AGTR1**[364], STAT3[1766], NF-KB[2084] (not shown), IFNG[2085], IL1B[2085] and TNF[2085] (not shown). PLAT expression is decreased by IL1B[2086] and increased by vitamin D3[1098]. So, in addition to SERPINE1, also PLAU and PLAT are directly regulated by PD landscape proteins, affecting plasminogen / plasmin levels.

Of note, coagulation factors also have a role in immune cell regulation. PLAT activates microglia[2087, 2088] and PLAU increases the expression of ICAM1[2089]. Moreover, PLG is expressed by microglia[2090], and plasmin cleaves and degrades both aggregated and monomeric forms of **SNCA**[2046] and thereby inhibits the translocation of extracellular **SNCA** into neighboring cells and the activation of microglia and astroglia by extracellular **SNCA**[2046]. Plasmin also triggers chemotaxis of dendritic cells triggering a T cell response[2091] and induces the release of IL8 from **SDC1** (a cell surface proteoglycan that stabilizes the chemoattractant form of IL8 at the cell surface)[2092]. SERPINE1 (by inhibiting plasmin production) stabilizes the chemoattractant function of IL8 by stabilizing its binding to **SDC1**[2092]. Further, PARK7 increases expression of IL8[1787] and PD patients with a **GBA** mutation have increased IL8 plasma levels compared to PD patients without a **GBA** mutation[2093]. The latter indicates an involvement of sphingosine, which is indeed shown by a S1P-mediated suppression of IL8 secretion by T cells[2094] (not shown). PLAT triggers activation of SPHK1 by binding to its receptor PLAUR[2095] (not shown) and thereby increases the phosphorylation of sphingosine to S1P, a major regulator of T- and B-cell traficking[2096].

In summary, coagulation factors, regulated by cytokines, lipoproteins and **SNCA**, regulate the chemotaxis of immune cells via IL8- and S1P-regulation and prevent **SNCA**-mediated activation of astroglia and microglia. Moreover, coagulation factors regulate lipoproteins, sphinglipids and cholesterol and can therefore modify their effects on immune cell activation.

D3. Concluding remarks

The viability of DA neurons is maintained by DA neuron specific transcription factors, increased by neurotrophic factors and is decreased by activation and upregulation of pro-apoptotic proteins. The transcription factors ASCL1, **MSX1**, NEUROG2, NR4A2, PITX3 and SOX2 are necessary to maintain a dopaminergic phenotype. The neurotrophic factors BDNF and GDNF support the survival of DA neurons by regulating apoptotic pathways, whereas GSK3B, PTEN, TP53 and CASP3 increase apoptotic signaling and neuron death. Further, these neurotrophic and apoptotic proteins regulate, together

with the transcription factors STAT3 and NF-KB, immune cell activation. In PD patients, cytokines and cytokine receptors are dysregulated and both the innate and adaptive immune response are affected. The innate immune response is activated by NM, **SNCA** and cholesterol, and results in the presentation of SN-specific antigens to the adaptive immune system that subsequently targets these cells for destruction. Migration, maturation, proliferation and activation of T and B cells (cells of the adaptive immune system) is regulated by proteins in the PD landscape. Moreover, almost all transcription factor pathways in the PD landscape regulate the immune regulator ICAM1, which stresses the importance of this protein in (dysfunctional) immune regulation in PD. Changed (oxidized) lipoprotein levels affect the immune response, directly, by regulating immune cell activation (and ICAM1 expression), or indirectly due to changes in membrane lipid composition, affecting (among others) internalization of antigens and localization of receptors responsible for chemotaxis or immune cell activation. Lastly, coagulation factors (regulated by cytokines, lipoproteins and **SNCA**) regulate the chemotaxis of immune cells, prevent **SNCA**-induced immune cell activation, but also regulate lipoproteins, sphingolipids and cholesterol and thereby mediate their effects on the immune response.

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LIST OF ABBREVIATIONS

ΔΨm, mitochondrial transmembrane potential;
Ca^{2+}, calcium ion;
CSF, cerebral spinal fluid;
DA, dopamine;
eQTL, expression quantitative trait locus;
ECM, extracellular matrix;
ER, endoplasmic reticulum;
FPD, amilial Parkinson's disease;
GWAS, genome-wide association study;
GWASs, genome-wide association studies;
INS, insulin;
IPD, idiopathic Parkinson's disease;
K^{+}, potassium ion;
LB, Lewy bodies;
LD, linkage disequilibrium;
Mg^{2+}, magnesium ion;
mPTP, mitochondrial permeability transition pore;
Na^{+}, sodium ion;
NM, neuromelanin;
nonsyn, nonsynonymous;
NS, not shown;
OR, odds ratio;
PD, Parkinson's disease;
RAS, renin-angiotensin system;
SN, substantia nigra;
SNP(s), single nucleotide polymorphism(s);
syn, synonymous;
UTR, untranslated region.

3

Validity of the MPTP-treated mouse as a model for Parkinson's disease

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3.1 ABSTRACT

Parkinson's disease (PD) is characterized by dopaminergic (DA) neuron death in the substantia nigra (SN) and subsequent striatal adaptations. Mice treated with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimidine (MPTP) are widely used as a model for PD. To assess the validity of the MPTP mouse model for PD pathogenesis, we here identify the biological processes that are dysregulated in both human PD and MPTP-treated mice. Gene enrichment analysis of published differentially expressed mRNAs in the SN of PD patients and MPTP-treated mice revealed an enrichment of gene categories related to motor dysfunction and neurodegeneration. In the PD striatum, a similar enrichment was found, whereas in the striatum of MPTP mice acute processes linked to epilepsy were selectively enriched shortly following MPTP treatment. More importantly, we integrated the proteins encoded by the differentially expressed mRNAs into molecular landscapes showing PD pathogenesis-implicated processes only in the SN, including vesicular trafficking, exocytosis, mitochondrial apoptosis and DA neuron-specific transcription, but not in the striatum. We conclude that the current use of the MPTP mouse as a model for studying the molecular processes in PD pathogenesis is more valid for SN than striatal mechanisms in PD. This novel insight has important practical implications for future studies using this model to investigate PD pathogenesis and evaluate the efficacy of new treatments.

KEYWORDS: Parkinson's disease, MPTP mouse model, Genome-wide mRNA expression, Molecular landscape

3.2 INTRODUCTION

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxic impurity that may occur during the synthesis of the opioid drug desmethylprodine, causes an irreversible parkinsonian syndrome in humans almost indistinguishable from Parkinson's disease (PD)[1]. Therefore, MPTP toxicity in monkeys, rats and mice has been studied to elucidate the pathogenic mechanisms implicated in PD. MPTP-treated mice are advantageous to explore the molecular background of MPTP toxicity, because lines of genetically engineered animals allow high levels of control of the experimental conditions. Mice treated with MPTP share specific biological features with PD, including loss of dopaminergic (DA) neurons in the substantia nigra (SN) and dopamine depletion in the striatum[2]. However, their pathogenetic backgrounds are different, being a toxic nature in a mouse model and a neurodegenerative process in human PD. Moreover, not all PD phenomenology is reproduced in MPTP-treated mice[3]. Therefore, the construct validity of the MPTP mouse as a model to study and elucidate the pathogenesis of PD remains unclear.

In order to identify the biological processes that are dysregulated in MPTP toxicity and their relationship to PD pathogenesis, differentially expressed mRNAs from

postmortem SN and striatum of PD patients, as well as differentially expressed mRNAs in the SN and striatum of MPTP-treated mice, were analyzed. Furthermore, based on proteins encoded by the mRNAs that were differentially expressed in both PD patients and MPTP mice, molecular landscapes of interacting proteins were built for both the SN and striatum. These landscapes represent molecular mechanisms that are shared between PD and MPTP toxicity. Together, these analyses will help to understand and value experimental findings in the MPTP mouse in the light of human PD pathogenesis.

3.3 METHODS

3.3.1 Genome-wide mRNA expression data

Available genome-wide mRNA expression data from multiple previously published studies were used to generate a list of differentially expressed transcripts in the postmortem SN and striatum of PD patients and MPTP treated mice, studied at various time points following MPTP treatment. If raw expression data was available at the Gene Expression Omnibus (GEO) site, this data was re-analyzed in GeneSifter (www.genesifter.com) using robust microarray analysis (RMA). The Benjamini-Hochberg method was then used to correct for multiple comparisons, and only mRNAs with a fold change (FC) of ≥ 1.2 or ≤-1.2 and a corrected p-value <0.05 were considered to be differentially expressed and used for the subsequent gene enrichment analysis, as described below. If no raw data was available, our inclusion criteria were: 1) correction for multiple testing was performed, with a corrected p-value <0.05 and the correction method was explicitly mentioned; and 2) an mRNA expression FC of ≥ 1.2 or ≤-1.2. Only protein-coding mRNAs were included in our analyses.

3.3.2 Enrichment analysis

The Ingenuity pathway analysis software package (www.ingenuity.com) was used to identify enriched gene categories in the lists of differentially expressed mRNAs in the SN and striatum of both human PD patients and MPTP-treated mice, and in the lists of overlapping mRNAs that were differentially expressed in the SN or striatum of both PD patients and MPTP mice. Ingenuity assigns genes and their corresponding mRNAs/proteins to (sub) categories of functional classes, e.g. 'diseases and disorders' and 'molecular and cellular functions'. For these analyses, only functional categories and pathways with significant enrichment (i.e. Benjamini-Hochberg corrected $p < 0.05$) and containing two or more genes were taken into account.

3.3.3 Molecular landscape building

Subsequently, the mRNAs that were differentially expressed in the SN and striatum of both PD patients and MPTP-treated mice were analyzed in more depth. Guided by the results of the Ingenuity enrichment analyses, the literature was searched for the (putative) function of all the proteins encoded by the mRNAs overlapping between

human PD and the MPTP mouse, as well as their functional interactions, using the UniProt Protein Knowledge Base (http://www.uniprot.org/uniprot)[4] and PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez). Based on these findings and applying an approach similar to the one we used previously to build landscapes based on genome-wide association data[5,6], we then built two molecular landscapes comprising interacting proteins encoded by the overlapping mRNAs in the SN and striatum, respectively. To complement these protein interaction cascades, we also added a number of proteins that were not encoded by the overlapping differentially expressed mRNAs but that have been implicated in PD etiology through other lines of (genetic) evidence. In this respect, proteins encoded by familial PD candidate genes were included if they have at least one functional interaction with one or more other landscape proteins. Additional proteins were included when having at least two interactions with other landscape proteins. Serif Drawplus 4.0 (www.serif.com) was used to draw the landscape figures.

3.4 RESULTS

In this study, we analyzed with gene enrichment approaches and systematic literature searches published datasets of differentially expressed transcripts in SN and striatum of PD patients and MPTP-treated mice that met our criteria for inclusion (see **Table 1** for dataset details).

3.4.1 Enrichment analysis of SN mRNA expression data

Human PD. Ingenuity enrichment analysis of the mRNAs that, compared to healthy controls, were differentially expressed in the SN of human PD patients, revealed the subcategories that were most significantly enriched within the two main functional classes, "diseases and disorders" and "molecular and cellular functions" (**Table 2**). When analyzing all differentially expressed SN mRNAs, the most significantly enriched diseases and disorders were predominantly in the movement disorders domain. Similar annotations were found for the top-5 enriched categories within the downregulated mRNAs, while the enriched annotations within the up-regulated mRNAs were not specifically related to (any) neurological function (data not shown). At a more functional level (i.e. the 'molecular and cellular functions' category), the enriched annotations were all related to neuronal and/or synaptic function.

MPTP mouse. Similar Ingenuity analyses revealed the most enriched functional categories within the mRNAs that were differentially expressed in the SN of MPTP-treated mice compared to untreated animals (**Table 2**). Analyzing mRNA expression profiling data at different intervals following MPTP treatment assessed temporal aspects of MPTP-induced neurotoxicity. More specifically, mice were injected four times within an eight-day period, and subsequently sacrificed for analysis one and seven days after the last treatment[17]. The enriched diseases and disorders were, at both intervals,

Table 1. Datasets of differentially expressed transcripts in SN and striatum of PD patients and MPTP-treated mice that met the criteria for inclusion. B&H, Benjami and Hochberg; B-Y, Benjami-Yekutieli; DA, dopamine; F, female; FC, fold change; GEO, gene expression omnibus; M, male; LCM, laser capture microdissecton; SAM, significance analysis of microarrays; SN, substantia nigra. For each study, the corresponding reference is indicated between brackets.

Species	Gender	Reference (GEO accession)	Cases / controls	Substrate	Microarray platform	FC cut off (up/down)	Statistics	Number of significant genes
Human	M/F	Zhang, 2005 [7]	11/18	SN	Affymetrix Human Genome U133A Array	1.2	B&H p<0.05	26
Human	M/F	Moran, 2006 [8]	15/7	Medial SN	Affymetrix Human Genome U133A Array	1.2	B&H p<0.05	600
		(GSE8397)	15/7	Medial SN	Affymetrix Human Genome U133B Array	1.2	B&H p<0.05	310
			9/6	Lateral SN	Affymetrix Human Genome U133A Array	1.2	B&H p<0.05	170
			9/6	Lateral SN	Affymetrix Human Genome U133B Array	1.2	B&H p<0.05	95
Human	M/F	Cantuti-Castelvetri, 2007 [9]	8/8	SN (LCM DA neurons)	Affymetrix Human X3P	2.0	SAM q<0.05	31
Human	M/F	Lesnick, 2007 [10] (GSE7621)	16/9	SN	Affymetrix Human Genome U133 Plus 2.0 Array	1.2	B&H p<0.05	42
Human	M/F	Bossers, 2009 [11]	4/4	SN	Agilent 22k 60mer oligonucleotide array	1.4	Bonferoni p<0.05	259
Human	M/F	Zheng, Liao, 2010 [12] (GSE20141)	10/8	SN (LCM DA neurons)	Affymetrix Human Genome U133 Plus 2.0 Array	1.2	B&H p<0.05	0
		(GSE20163)	8/9	SN	Affymetrix Human Genome U133A Array	1.2	B&H p<0.05	0
		(GSE20164)	6/5	SN	Affymetrix Human Genome U133A Array	1.2	B&H p<0.05	0
Human	M/F	Elstner, 2011 [13]	8/9	SN (LCM DA neurons)	Illumina WG6v1 expression chip	1.2	B&H p<0.05	1037
Human	M/F	Diao, 2012 [14] (GSE20333)	6/6	SN	Affymetrix Human HG-Focus Target Array	1.2	B&H p<0.05	0
Human	M/F	Zhang, 2005 [7]	15/20	Putamen	Affymetrix Human Genome U133A Array	1.2	B&H p<0.05	1
Human	M/F	Vogt, 2006 [15]	8/8	Putamen	Affymetrix Human Genome U133A Array	2.0	B-Y p<0.05	78
Human	M/F	Botta-Orfila, 2012 [16]	5/5	Putamen	Affymetrix 1.0 Exon	2.0	B&H p<0.05	186
Mouse	M	Miller, 2004 [17] (GSE4788)	24/12	SN	Affymetrix Murine Genome U74A Array	1.2	B&H p<0.05	608
Mouse	F	Pattarini, 2008 [18]	3/6	Striatum	Affymetrix Mouse Genome 430 2.0 Arrays	1.5	B&H p<0.05	430

Table 2. Gene enrichment analysis of the substantia nigra. Ingenuity annotations of genes dysregulated by MPTP in the mouse substantia nigra, after short and longer intervals between treatment and analyses (608 genes for intervals combined), annotations of all dysregulated genes in the substantia nigra of PD patients (2027 genes), and those that are dysregulated in both the MPTP mouse model and human PD (i.e. 116 'overlapping' genes). Data are extracted from references in table 1. The top 5 Ingenuity annotations of the categories 'Diseases and disorders' and 'Molecular and cellular functions' are displayed, as well as their respective p-value and number of genes involved (#). All p-values are corrected for multiple testing by the Benjamini-Hochberg false discovery rate. Categories with only 1 (target) gene were discarded. See text for further details.

Category: Diseases and disorders

	MPTP Mouse						*Human PD*			*Overlap*			
Rank	*shorter interval*	*p-value*	*#*	*longer interval*	*p-value*	*#*		*p-value*	*#*		*p-value*	*#*	*Overlapping genes*
1	Movement disorders	1.54E-16	89	Movement disorders	3.41E-10	58	Disorder of basal ganglia	2.35E-22	203	Dyskinesia	1.91E-07	24	ACHE, ATP5C1, BCL2, CDH2, CDK5, DDX1, FAM3C, GABRG2, GRIN1, MAP2K4, NDRG1, PFKM, RAB11A, RAB6A, RGS4, RTN2, SLC6A3, SNAP25, SOX2, ST8SIA3, TH, VAMP2, VSNL1, YWHAZ
2	Disorder of basal ganglia	1.30E-12	66	Neuromuscular disease	2.48E-07	45	Movement disorders	4.33E-20	250	Disorder of basal ganglia	2.86E-07	26	ACHE, ATP5C1, BCL2, CDH2, CDK5, DDX1, EIF4G1, FAM3C, GABRG2, GRIN1, MAP2K4, NDRG1, NR4A2, PFKM, RAB11A, RAB6A, RGS4, RTN2, SLC6A3, SNAP25, SOX2, ST8SIA3, TH, VAMP2, VSNL1, YWHAZ
3	Dyskinesia	3.02E-12	57	Neurological signs	1.26E-06	36	Neuromuscular disease	6.42E-19	211	Neuromuscular disease	5.35E-07	27	ACHE, ATP5C1, BCL2, CDH2, CDK5, DDX1, EIF4G1, FAM3C, GABRG2, GRIN1, MAP2K4, NDRG1, NR4A2, PFKM, RAB11A, RAB6A, RGS4, RPL5, RTN2, SLC6A3, SNAP25, SOX2, ST8SIA3, TH, VAMP2, VSNL1, YWHAZ
4	Neurological signs	3.02E-12	58	Disorder of basal ganglia	1.63E-06	40	Chorea	1.24E-16	154	Huntington's disease	5.78E-07	22	ATP5C1, BCL2, CDH2, CDK5, DDX1, FAM3C, GABRG2, GRIN1, MAP2K4, NDRG1, PFKM, RAB11A, RAB6A, RGS4, RTN2, SLC6A3, SNAP25, SOX2, ST8SIA3, VAMP2, VSNL1, YWHAZ
5	Huntington's disease	1.11E-11	54	Dyskinesia	1.74E-06	35	Neurological signs	1.41E-16	163	Movement disorders	1.70E-06	28	ACHE, ATP5C1, ATXN10, BCL2, CDH2, CDK5, DDX1, EIF4G1, FAM3C, GABRG2, GRIN1, MAP2K4, NAPB, NDRG1, NR4A2, PFKM, RAB11A, RAB6A, RGS4, RTN2, SLC6A3, SNAP25, SOX2, ST8SIA3, TH, UGT8, VAMP2, VSNL1, YWHAZ

Table 2. (continued)

Category: Molecular and cellular functions

	MPTP Mouse					*Human PD*			*Overlap*			
Rank	*shorter interval*	*p-value*	*#*	*longer interval*	*p-value*	*#*		*p-value*	*#*		*p-value*	*#*	*Overlapping genes*
1	Cell death	1.11E-12	170	Proliferation of cells	2.28E-11	128	Transport of vesicles	9.27E-11	40	Neuronal cell death	5.01E-05	20	ACHE, AKT1S1, BCL2, CDK5, FYN, GRIN1, KIFAP3, L1CAM, MAGED1, MAP2K4, MAPK8, NFKBIA, NR4A2, RET, SLC6A3, SNAP25, SOX11, SRPK2, STXBP1, YWHAZ
2	Microtubule dynamics	1.30E-12	77	Cell death	3.41E-10	118	Formation of plasma membrane projections	6.45E-08	112	Microtubule dynamics	9.35E-05	25	ACTG1, ATXN10, BCL2, CDH2, CDK5, CHP1, CRMP1, FYN, GRIN1, IFT20, KLC1, L1CAM, LPAR1, MAP2K4, MAP4, MAPK8, MARK2, NDRG1, NFIB, NFKBIA, RAB11A, RANBP9, RET, TNK2, UGT8
3	Organization of cytoskeleton	1.30E-12	85	Proliferation of tumor cell lines	8.64E-09	67	Microtubule dynamics	8.73E-08	195	Synthesis of neuro-transmitter	9.67E-05	6	BCL2, NR4A2, SLC6A3, SNAP25, TH, YWHAZ
4	Organization of cytoplasm	4.30E-12	88	Apoptosis	1.13E-08	97	Organization of cytoplasm	1.09E-07	237	Exocytosis by cells	1.05E-04	8	CDK5, GNAI2, NAPB, NSF, RAB11A, SNAP25, STXBP1, VAMP2
5	Proliferation of cells	1.96E-11	173	Degeneration of cells	2.24E-08	24	Formation of cellular protrusions	1.47E-07	149	Production of catecholamine	1.54E-04	5	BCL2, NR4A2, SLC6A3, TH, YWHAZ

predominantly in movement disorders-related domains. The enriched molecular and cellular functions categories were also similar over time, relating mainly to cell death, proliferation and development (both intervals), as well as to structural organization of the cell (short interval only).

Overlap between human PD and MPTP mouse. The most significantly enriched functional categories within the mRNAs that were differentially expressed in the SN of *both* PD patients and MPTP-treated mice are also shown in **Table 2**. Similar to the human and mouse results mentioned above, the enriched diseases and disorders encompass movement disorders-related domains, while the more functional categories related to neuronal cell death, microtubule dynamics and cellular functions, including neurotransmitter synthesis and exocytosis, as well as (neuronal) cell growth and death.

3.4.2 Molecular landscape of shared processes in the SN

Figure 1 shows a molecular landscape of interacting proteins encoded by the mRNAs that are differentially expressed in the SN of *both* human PD patients and MPTP-treated mice. These proteins form signaling cascades that are located in the SN neuron presynapse, cell body or nucleus. The main cascades in the presynaptic landscape regulate DA synthesis, autophagy, calcium signaling, vesicle trafficking and exocytosis (**Fig. 1A**). In the cell body and nucleus,

particularly mitochondrial (dys)function and transcriptional regulation through histone and nucleosome modification and its reciprocal effect on pre-mRNA splicing are present (**Fig. 1B**). More specifically, NR4A2 and SOX2, two of the five transcription factors that are required for a DA neuron-like expression pattern, bind to HDAC1, a histone deacetylase that interacts with many proteins in the landscape. Therefore, dysregulation of any of these processes affects DA neuron-specific expression and reduces the number of neurons with a DA phenotype. In the **Supplementary Information**, the landscape is described in full detail, and the current knowledge about the functions of all landscape proteins is summarized.

3.4.3 Enrichment analysis of striatal mRNA expression data

Human PD. Similar to the SN data described above, the disease/disorder categories that are directly related to PD-like movement disorders and motor symptoms were significantly enriched within the mRNAs that were found to be differentially expressed in the striatum of PD patients (**Table 3**). Furthermore, the enriched functional categories were mainly related to neuronal functions such as (synaptic) transmission, and molecular/metal ion transport.

MPTP mouse. Again, the most significantly enriched categories were determined at various time points following MPTP treatment. However, the respective study used a timing regimen different from the study focusing on the SN mentioned above. At 5 and 24 hrs after injection, the predominant functional categories were implicated in (neuronal) cell death and other acute, MPTP toxicity-related processes, including (dys) regulation of inflammatory responses and immunity-related cells, and endometriosis. At 72 hrs after MPTP injection, the most significantly enriched categories shift towards those enriched within the PD patient striatal data, i.e. categories related to PD-like motor symptoms and neuronal/synaptic function (**Table 3**).

Overlap between human PD and mouse MPTP. The most significantly enriched categories within the mRNAs that were differentially expressed in the striatum of *both* human PD patients and the MPTP mouse model comprise a combination of the enriched 'diseases and disorders' categories identified in human PD and MPTP-treated mice as summarized above, i.e., relating to both movement disorders and epilepsy (**Table 3**). Indeed, the 'cellular and molecular functions' categories involved in both neuronal/synaptic function and neuronal cell growth/death are enriched within the overlapping PD patient/MPTP mouse striatal data.

3.4.4 Molecular landscape of shared processes in the striatum

Figure 2 shows a molecular landscape of interacting proteins encoded by the mRNAs differentially expressed in the striatum of *both* human PD patients and MPTP-treated

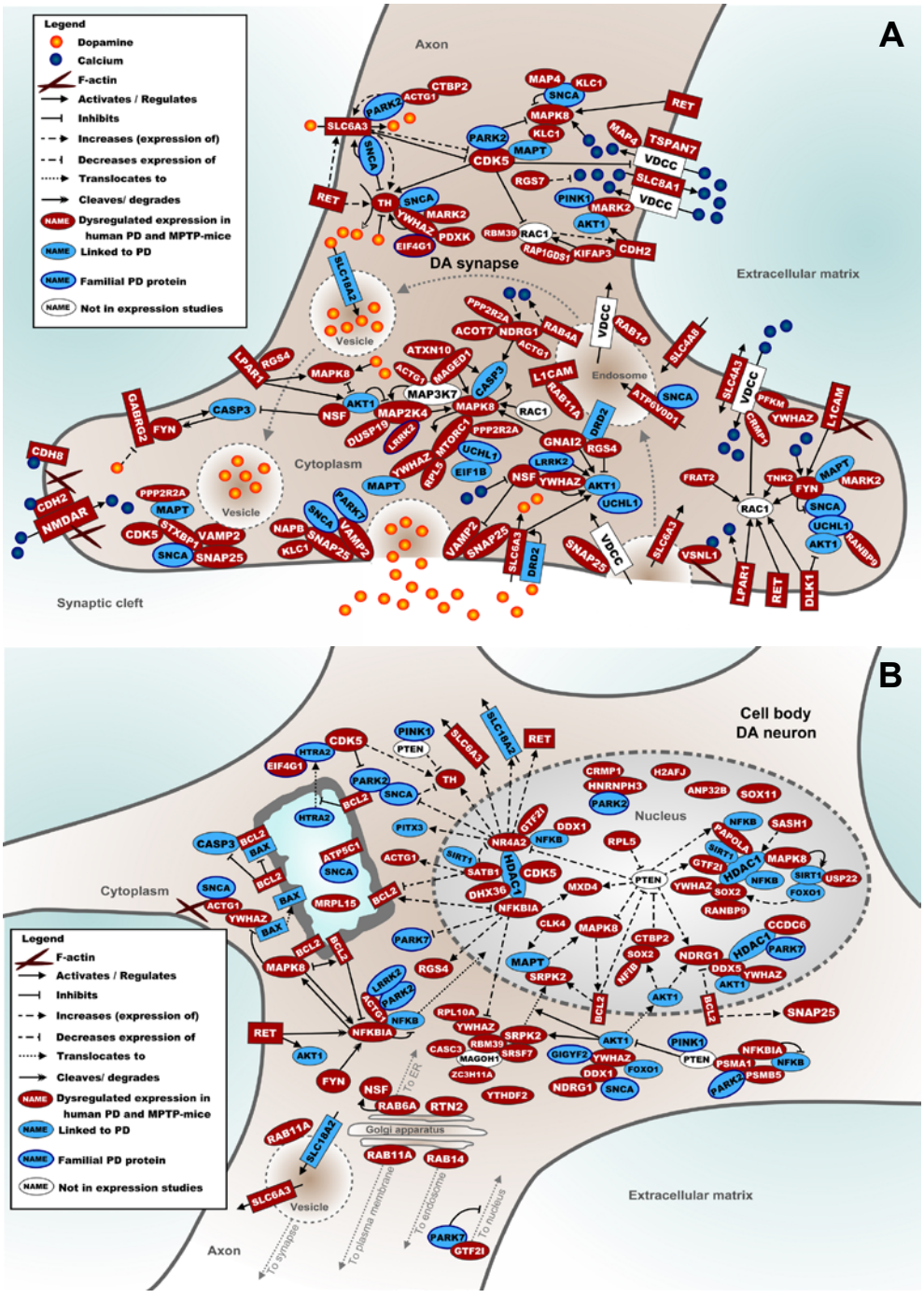

Figure 1. Molecular landscape of interacting proteins, encoded by the mRNAs that are differentially expressed in the SN of *both* human PD patients and MPTP-treated mice, located primarily in the (pre) synapse and axon of the DA neuron (**A**) and in the cell body and nucleus of the DA neuron (**B**). See text and Supplementary Information for details

Table 3. Gene enrichment analysis of the striatum. Ingenuity annotations of genes dysregulated by MPTP in the mouse striatum, after 5, 24 and 72 hr between treatment and analyses (430 genes for time points combined), annotations of all dysregulated genes in the striatum of PD patients (259 genes), and those that are dysregulated in both the MPTP mouse model and human PD (i.e. 14 'overlapping' genes). Data are extracted from references in Table 1. The top 5 Ingenuity annotations of the categories 'Diseases and disorders' and 'Molecular and cellular functions' are displayed, as well as their respective p-value and number of genes involved (#). All p-values are corrected for multiple testing by the Benjamini-Hochberg false discovery rate. Categories with only 1 (target) gene were discarded. See text for further details.

Category: Diseases and disorders

	MPTP Mouse								*Human PD*			*Overlap*			
Rank	*5 hr*	*p-value*	*#*	*24 hr*	*p-value*	*#*	*72 hr*	*p-value*	*#*		*p-value*	*#*		*p-value*	*#*	*Overlapping genes*
1	Epileptic seizure	2.55E-21	21	Psoriasis	6.98E-14	39	Movement disorders	1.05E-09	40	Neurological signs	5.52E-28	64	Seizures	4.14E-03	5	CHGB, ENC1, KCNQ5, NPTX2, TGM2
2	Seizures	3.94E-18	25	Glucose metabolism disorder	3.37E-11	50	Schizophrenia	2.52E-08	25	Dyskinesia	5.52E-28	63	Abnormal secretion by adrenal gland	5.71E-03	2	CHGB, ITSN1
3	Epilepsy	7.14E-18	22	Inflammation of organ	3.98E-11	51	Amyloidosis	7.20E-08	25	Disorder of basal ganglia	8.34E-28	69	Epilepsy	9.93E-03	4	CHGB, ENC1, KCNQ5, NPTX2
4	Dyskinesia	1.07E-06	18	Vascular disease	7.16E-11	45	Dementia	2.41E-07	24	Chorea	7.07E-27	60	Dyskinesia	1.34E-02	5	CHGB, DIRAS2, FABP7, S100A10, TGM2
5	Endometriosis	2.70E-06	14	Inflammatory response	3.45E-10	37	Quantity of phagocytes	6.73E-07	20	Movement disorders	8.28E-27	79	Movement disorders	1.34E-02	6	CHGB, DIRAS2, FABP7, S100A10, TGM2, TMEM176B

Category: Molecular and cellular functions

	MPTP Mouse								*Human PD*			*Overlap*			
Rank	*5 hr*	*p-value*	*#*	*24 hr*	*p-value*	*#*	*72 hr*	*p-value*	*#*		*p-value*	*#*		*p-value*	*#*	*Overlapping genes*
1	Apoptosis	5.80E-08	39	Proliferation of cells	3.23E-17	115	Morphology of cells	6.61E-11	59	Neuro-transmission	1.44E-13	35	Proliferation of endothelial cell lines	2.37E-02	2	ADAMTS1, ITSN1
2	Differentiation of cells	1.07E-06	31	Morphology of cells	7.55E-08	79	Organization of cytoskeleton	6.61E-11	45	Synaptic transmission	2.90E-11	29	Apoptosis of neuroblastoma cell lines	3.02E-02	2	ITSN1, TGM2
3	Proliferation of cells	1.89E-06	43	Necrosis	3.61E-15	91	Organization of cytoplasm	1.93E-10	46	Transport of molecule	3.95E-09	63	Cell death of cortical neurons	4.08E-02	2	ITSN1, TGM2
4	Cell death	2.58E-06	41	Apoptosis	2.05E-14	91	Formation of cellular protrusions	8.34E-10	33	Transport of metal ion	1.02E-08	24	Neuritogenesis	4.08E-02	3	DCLK1, ENC1, ITSN1
5	Cell cycle progression	4.27E-06	21	Cell movement	2.81E-14	77	Apoptosis	8.34E-10	67	Morphology of neurites	2.10E-08	17	Apoptosis of endothelial cells	4.34E-02	2	ADAMTS2, ITSN1

mice. PD as well as MPTP treatment result in the degeneration of nigrostriatal DA neurons, which decreases DA release in the striatum and results in diminished activation of the DRD2 and DRD3 DA receptors, in turn affecting postsynaptic striatal protein expression and calcium signaling. Furthermore, the majority of the proteins in this landscape regulate CREB1 - a transcription factor that is essential for DA-dependent gene expression in the striatum - either directly via calcium signaling or through activation of the ERK1/2 kinases. In the **Supplementary Information**, the landscape is described in full detail, and the current knowledge about the functions of the landscape proteins is presented.

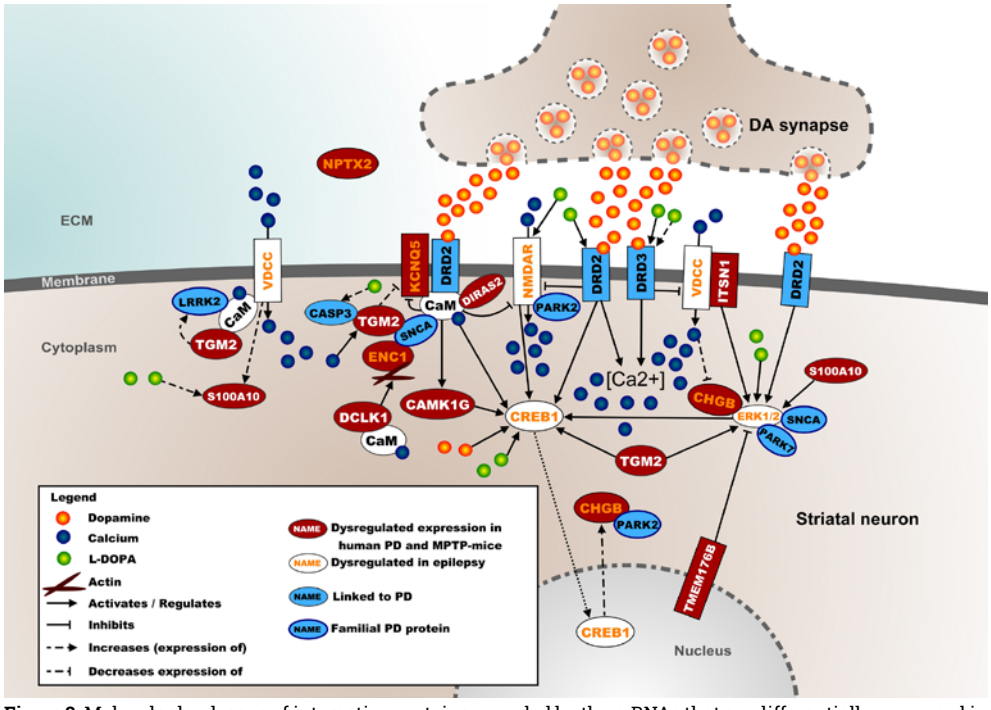

Figure 2. Molecular landscape of interacting proteins, encoded by the mRNAs that are differentially expressed in the striatum of *both* human PD patients and MPTP-treated mice located in the post-synapse of a striatal neuron. See text and Supplementary Information for details.

3.5 DISCUSSION

This study aimed to determine the construct validity of the MPTP mouse as a model to study human PD pathogenesis. First, the most important dysregulated biological processes underlying both human PD and MPTP toxicity were identified by enrichment analyses of published genome-wide mRNA expression data from postmortem SN and striatum of PD patients and MPTP-treated mice. Second, proteins encoded by the mRNAs that were differentially expressed in both PD patients and MPTP-treated mice were integrated into molecular landscapes representing the main biological processes

that are shared by human PD and mouse MPTP toxicity. Our findings demonstrate that, at the level of the SN, MPTP toxicity has substantial relevance for PD pathogenesis. This is less obvious for the striatum, in which important temporal effects of MPTP toxicity were noted.

Because categories related to basal ganglia-based motor dysfunction and neurodegeneration were enriched in the SN of both PD patients and MPTP-treated mice, the effects of MPTP toxicity on gene expression in the mouse SN appear to have similar phenotypic consequences as human PD. However, differences exist between PD and MPTP toxicity regarding the specific (dysregulated) biological processes involved. While in the SN of PD patients, enriched molecular and cellular functions relate to neuronal and synaptic functions, functional themes pertaining to cell growth and death predominate in the MPTP mouse model. This discrepancy could well reflect the differences between the protracted processes of neurodegeneration in PD, as well as simultaneous compensatory neuroplastic mechanisms, compared to the acute MPTP toxicity in mice. Moreover, the biological processes that overlap between the SN of both PD patients and MPTP-treated mice mainly relate to neuronal/synaptic function and (neuronal) cell death, while the molecular signaling cascades involved regulate DA synthesis and recycling, endocytosis and exocytosis of (DA-containing) synaptic vesicles, and cytoskeleton-dependent synaptic remodeling. These biological processes have been implicated in the pathogenesis of PD before[19-21]. Proteins encoded by other differentially expressed mRNAs are important players in other processes that have been implicated in DA neuronal dysregulation and death, including cytoplasmic and nuclear cascades regulating (vesicular) trafficking[22], mitochondrial function and apoptosis[23], proteosomal degradation (including the degradation of DA neuron-specific transcription factors)[24], as well as transcriptional, post-transcriptional and translational processes such as histone regulation[25] and pre-mRNA splicing[26].

As opposed to the SN findings, the categories that were most significantly enriched within the differentially expressed striatal mRNAs did not unequivocally overlap between human PD and mouse MPTP-induced toxicity. In PD, they are related to PD-associated motor symptoms, but in the MPTP-treated mouse striatum, the enriched categories depend on the length of the time period between MPTP treatment and transcriptional profiling. Early (i.e., 5 hours) after MPTP injection, the most significantly enriched disease categories within the mouse striatal mRNA expression data are not related to motor dysfunction, but to epilepsy. Epilepsy is a known acute side-effect of MPTP injection in mice[27] and is directly linked to the temporary presence of the active MPTP metabolite MPP+[28]. Although observational studies have reported an association between epilepsy and PD[29], an acute side effect of MPTP is more likely, as MPTP treatment does not seem to have long-lasting epileptogenic effects[30]. Indeed, in

line with a gradual reduction of MPP+ levels in the mouse brain over time, at 24 hours after injection, some of the significantly enriched disease categories point towards an inflammatory response, while at 72 hours, they are related to motor dysfunction and neurodegeneration. A similar pattern is observed for the molecular and cellular functions, where at 5 hours after injection, the enriched functions are mainly related to cell growth and death, shifting to cellular organization- and morphology-related functions at later time points. Although direct comparison of studies is challenging due to different injection regimens, these findings may suggest that in the striatum – more clearly than in the SN – the MPTP-induced expression changes and the molecular signaling cascades that are affected by these expression changes are not consistent but change towards more PD-relevant processes over time. The overlap between PD and MPTP-treated mice in biofunctions of striatal mRNAs, encompassing both 'acute' toxicity-related and 'chronic' PD-related categories, is corroborated by the molecular landscape. In this landscape, part of the affected biological processes and functions appears directly related to diminished DA striatal innervation through postsynaptic DA receptors. In addition, there seems to be a strong convergence on the regulation of intracellular calcium levels as well as CREB1-related signaling, which have both been functionally linked to epilepsy before[31]. Again, it remains unsure whether these signaling cascades are affected by the reduced DA innervation from the presynaptic SN neuron, or that they merely reflect an acute 'side-effect' of MPTP rather than having much direct relevance for PD pathogenesis. The finding of endometriosis-related enrichment in mice may be related to the fact that only female mice were used, while for the human studies, both male and female subjects were included. However, since 8 out of the 14 proteins with the annotation 'endometriosis' are also present in the annotated category 'dyskinesia', the 'endometriosis' enrichment may be due to either a true gender effect, an enrichment of genes involved in movement dysfunction-related processes, or both.

Thus, our transcriptome analyses and molecular landscapes indicate that the MPTP mouse constitutes a valid model for the chronic molecular and pathological changes that occur in the SN of PD patients, and hence of the PD phenotype that is associated with these changes. However, this is less obvious for the striatum, because early after MPTP injection, enriched categories and functions encompass mainly processes that are not directly related to PD. It appears that human PD pathogenesis in the striatum is better recapitulated at the molecular level in the MPTP-treated mouse model 72 hours post-injection, and perhaps even later. Effects of the level of chronicity of the dosing regimen on markers of DA neurotransmission – e.g. TH expression and striatal DA levels – and behavioral outcome have been reported before[32-34], but specific effects on the transcriptome have not been addressed. In this respect, assessing the relationship between the temporal expression patterns in human PD patients and their disease

duration would be of interest, similar to the analyses that were performed on the mouse-MPTP data. Unfortunately, for the reported human expression studies, disease duration data were not available for all cases.

In addition to the acutely toxic nature of early MPTP-induced pathology, there may be several other explanations for the apparent discrepancy between the striatal PD pathogenesis and MPTP toxicity. First, presynaptic DA denervation may result in enriched processes that are different between humans and mice due to species specificity. For example, similar degrees of DA degeneration in humans and mice do not result in similar phenotypic severity[35] and clinical phenotypes differ as mice do not show the tremor often seen in patients. Second, chronic compensating processes including adaptive neuroplasticity could play an important role in PD, but less in MPTP-treated mice. In PD, these processes may be linked to the synaptic transmission- and molecular transport-related functions that are enriched in striatal mRNAs. The acutely toxic nature of MPTP would not allow for such an adaptation. Finally, it should be noted that, despite the high degree of overlap, the absolute number of differentially expressed mRNAs that overlap between human PD striatum and MPTP mouse striatum is low, perhaps prohibiting the detection of statistically relevant enrichment.

The principal differences between the chronological orders of events in PD-linked neurodegeneration versus MPTP-induced toxicity are summarized in **Figure 3**. In PD, a number of molecular mechanisms in presynaptic SN neurons – including vesicular trafficking and exocytosis, mitochondrial apoptosis, as well as several transcriptional and translational processes – cause neuronal / synaptic dysfunction and cell death, which is followed by chronic, postsynaptic compensatory mechanisms in the striatum. In contrast, MPTP is taken up readily as MPP+ through the DA transporter (DAT) in presynaptic SN neuron terminals[36], causing toxicity and sequestration of MPP+ into synaptic vesicles[37]. MPP+ also reaches the cell body of presynaptic SN neurons through retrograde axonal transport[38], which in turn causes neuronal death through mitochondrial accumulation and electron transport chain inhibition, inducing neuronal apoptosis[39]. This relatively rapid cell death causes more acute compensatory effects in the postsynaptic striatal neurons[40].

Together, our findings provide further evidence that the molecular changes in the SN of MPTP-treated mice correspond to the observed alterations in the SN of PD patients. However, for a proper reflection of the molecular changes occurring during PD pathogenesis in the striatum, the time point of studying the changes following MPTP treatment is crucial. Further transcriptome studies are needed to determine whether waiting longer than 72 hours would indeed provide a better construct validity for human PD pathogenesis and whether there is an optimal time frame following MPTP

injection to assess striatal PD pathology in the MPTP mouse model. Such knowledge will have important practical implications for the use of the MPTP mouse as a model for PD and for PD drug testing.

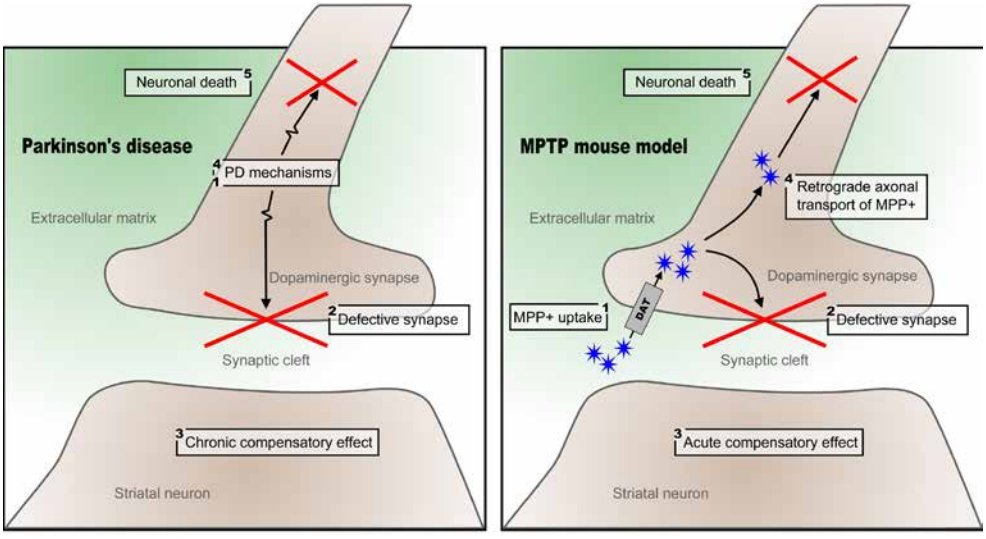

Figure 3. Proposed fundamental mechanisms underlying the degeneration of dopaminergic neurons in Parkinson's disease and mouse MPTP toxicity. The numbers denote the sequence of events in Parkinson's disease and the MPTP mouse respectively. See text for further details.

3.6 ACKNOWLEDGEMENTS

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3.8 SUPPLEMENTARY INFORMATION

CONTENTS

INTRODUCTION

Parkinson's disease (PD) as well as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment result in the degeneration of dopaminergic neurons in the substantia nigra (SN), leading to a decrease of dopamine (DA) release to the striatum. Below, the molecular landscapes of biological processes shared between PD and MPTP treatment in the substantia nigra (SN) (**Figures 1A and 1B**) and striatum (**Figure 2**) are described in full detail, and the current knowledge about the functions and interactions of all landscape proteins is presented. In these descriptions, proteins that appear in **bold** are dysregulated in both human PD and the MPTP mouse model. Underlined proteins are associated with PD through either expression or genetic data from PD patients, and familial PD proteins are double underlined.

1. DETAILED DESCRIPTION FIGURE 1A

Figure 1A shows a molecular landscape of interacting proteins, encoded by the mRNAs that are differentially expressed in the SN of *both* human PD patients and MPTP-treated mice, located primarily in the (pre) synapse and axon of the DA neuron. The biological processes depicted in the figure are described below:

1.1 DA synthesis

TH catalyzes the rate-limiting step in DA synthesis, i.e. the conversion of the amino acid L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA)[1, 2]. **TH** expression is increased by the receptor tyrosine-protein kinase **RET**[3] and **SLC6A3**[4]. **TH** is activated by the adaptor protein **YWHAZ**[5, 6] and the cyclin-dependent kinase **CDK5**[7], and DA itself inhibits **TH** activity in a negative feedback loop[8, 9]. **YWHAZ** also binds to the kinases **MARK2**[10], involved in microtubule regulation[11], and **PDXK**[12], required for the synthesis of pyridoxal-5-phosphate (PLP) from vitamin B6[11]. PLP, in turn, is an essential cofactor for the conversion of L-DOPA into DA by dopa decarboxylase[13].

1.2 (DA) release

Soluble NSF Attachment Protein Receptor (SNARE) proteins form a complex that is required for synaptic vesicle docking and subsequently the release of their contents (e.g., DA) into the synaptic cleft[11]. **SNAP25** and **VAMP2**, core components of the SNARE complex, physically interact with each other[14] and form a complex together with **STXBP1**[15], a protein that is also involved in synaptic vesicle fusion and docking[11]. **STXBP1** binds to **CDK5**[16], a protein involved in cytoskeleton regulation, synapse plasticity, exocytosis and endocytosis (see more on **CDK5** below). The vesicle fusion ATPase **NSF** increases the dissociation of the SNARE complex (dissociation of **SNAP25** and **VAMP2**) and thereby enables the vesicle to fuse with the plasma membrane[17]. **NSF** binds to **YWHAZ**[18] and AKT1[19], and inhibits CASP3 activity[20].

The DA – acetylcholine (ACh) balance may be involved in PD pathology[21] and ACh regulates DA release[22]. The extracellular protein **ACHE** hydrolyzes ACh that is released into the synaptic cleft[11]. Although **ACHE** does not directly interact with other landscape proteins, its function may well be linked to PD since its activity is reduced in the cerebral cortex and the medial occipital cortex of PD patients[23]. Moreover, **ACHE** is inactivated by MPTP[24] and **ACHE** deficiency is neuroprotective in the MPTP mouse model[25].

1.3 DA reuptake

In response to an action potential, DA is released from vesicles in the synaptic cleft by exocytosis and reuptake of extracellular DA occurs by **SLC6A3** to terminate the signal[26, 27]. **SLC6A3** binds to the familial PD proteins PARK2[28] and SNCA[29], which both enhance its activity. PARK2 and **RET** increase the expression of **SLC6A3**[3, 28, 30], while SNCA increases **SLC6A3** translocation to the plasma membrane[29]. In addition to increasing the expression of **TH**, **SLC6A3** activates AKT1[31] and both inhibits the activity[32] and decreases the expression of **CDK5**[33]. Cytoplasmic DA (either synthesized by **TH** or after reuptake by **SLC6A3**) is transported into cytoplasmic vesicles by SLC18A2[11]. These vesicles are subsequently translocated via axoplasmic transport to the synapse for exocytosis.

1.4 Recycling of membrane-bound synaptic components

To further control DA levels in the synaptic cleft, proteins including **SLC6A3**, DRD2 and the voltage-dependent calcium channel (VDCC) are endocytosed, transported to the endosome and either recycled back to the membrane or degraded. **ATP6V0D1** is a subunit of the proton V-ATPase and increases the acidity of the endosome[11], which is required for a normal endosomal function. DRD2 binds to **SLC6A3**[34] and increases its localization in the plasma membrane[35], and activates the transport activity of **SLC6A3**[36]. **SNAP25** is also involved in this process, by binding to **SLC6A3**[37] and VDCC[38, 39]. Correct regulation of the recycling and degradation of DRD2 and **SLC6A3** is essential for normal neuronal DA signaling and deregulation of the proteins that regulate their endocytosis and/or expression at the plasma membrane can disturb this signaling. The G protein **GNAI2** regulates the expression of, binds to, and prevents the plasma membrane translocation of, DRD2[40]. **RGS4** binds to both DRD2[41] and **GNAI2**[42], and increases the GTPase activity of **GNAI2** (not shown)[43]. In turn, **GNAI2** increases the recruitment of **RGS4** to the plasma membrane (not shown) [44], where it binds – among others – to **LPAR1**[41]. The Rab GTPases **RAB4A**, **RAB6A**, **RAB11A** and **RAB14** are also involved in (vesicular) recycling and endosomal function; see the section '(Vesicle) trafficking and exocytosis' in the detailed description of figure 1b below for their functions within the landscape.

1.5 Cytoskeleton / cytoplasmic cascades

Recycling, trafficking and exocytosis require cytoskeletal regulation. A substantial number of landscape proteins control cytoskeleton dynamics, e.g., by regulating RAC1

activity. RAC1 is activated by **FRAT2**[45], **TNK2**[46], **FYN**[47], **DLK1**[48], **RET**[49, 50], **LPAR1**[51], and **KIFAP3**[52], and inhibited by **CDK5**[53] and **CRMP1**[54]. Further, RAC1 binds to **RBM39**[55] and **RAP1GDS1**[56]. The latter also binds to **KIFAP3**[57] and regulates the GDP/GTP exchange of GTP-binding proteins such as RAC1[58].

RAC1 has multiple functions. First, it increases the expression of **CDH2**[59] and activates **MAPK8**[60], a serine/threonine-protein kinase that is involved in cell proliferation, differentiation, migration, transformation and programmed cell death (see also the section 'Mitochondrial function and apoptosis' in the description of figure 1b for the role of **MAPK8** in apoptosis)[11]. **MAPK8** binds to MAP3K7[61], CASP3[62], **mTORC1**[63] and **KLC1**[64]. Apart from RAC1 activation, **MAPK8** is also activated by **MAGED1**[65], **GNAI2**[66], **MAP2K4**[67], **RET**[49], **LPAR1**[68] and DA[69], and inhibited by AKT1[70]. **MAGED1** also binds to MAP3K7[71] and **ATXN10**[72], and activates CASP3[65]. **MAPK8** also activates CASP3[73, 74] and **mTORC1**[63], and inhibits AKT1[75, 76]. **MAP2K4** is an essential part of the MAP kinases signaling pathway and is bound to the phosphatase **DUSP19**[77], AKT1[70] and MAP3K7[78], whereas AKT1 inhibits[70] and MAP3K7 activates **MAP2K4**[79]. Multiple proteins in the landscape regulate AKT1. AKT1 binds to **NSF** and **MAP2K4**, as mentioned above, as well as to **YWHAZ**[80] and **MARK2**[81], is activated by **SLC6A3** (see above), **YWHAZ**[82], **GNAI2**[83], **LPAR1**[68], **TNK2**[84] and **CDH2**[85], and inhibited by **MAPK8** (see above), **RGS4**[86] and **DLK1**[87]. Second, RAC1 increases the polymerization of globular actin (**ACTG1**)[88] to filament actin (F-actin)[11]. **ACTG1** binds to **CTBP2**[89], MAP3K7[90] and **NDRG1**[91] (see Figure 1b and its description for more **ACTG1** interactions). Moreover, **CDH2**[92], **GNAI2**[93] (not shown), **VSNL1**[94] and the **NMDAR**[95] bind to actin, and **CDK5** increases actin polymerization[53].

1.6 Microtubule-dependent trafficking

In addition to RAC1, **FYN** is a non-receptor tyrosine-protein kinase that is involved in cell growth and survival, cell adhesion, cytoskeletal remodeling and axon guidance[11]. **FYN** is activated by **L1CAM**[96] and is itself an activator of RAC1 (see above), **TNK2**[97] and CASP3[98]. **FYN** is cleaved by CASP3[99, 100]and binds to **TNK2**[97], **GABRG2**[101] and MAPT[102]. Like **FYN, TNK2** is a non-receptor tyrosine-protein kinase and is involved in cell survival, proliferation and endocytosis[11], whereas **GABRG2** is a subunit of the GABA receptor and regulates neuronal inhibition[11]. Moreover, **FYN** phosphorylates MAPT[103, 104], which is a susceptibility gene for idiopathic PD[105-109] that promotes assembly and stability of microtubules[11]. Microtubule-dependent trafficking is affected in PD and, among others, affects axonal transport of autophagosomes that contain damaged mitochondria and aggregated proteins, which can lead to SNCA accumulation and synaptic dysfunction[110, 111]. In the landscape, MAPT binds to **MARK2**[112], **YWHAZ**[10], **KLC1**[113], **CDK5**[114], **STXBP1**[16] and **PPP2R2A**[115]. In addition, MAPT is phosphorylated by **CDK5**[116] and **FYN**[104], whereas **PPP2R2A** dephosphorylates MAPT[117]. Furthermore, **MARK2** phosphorylates both MAPT and **MAP4** which causes microtubule detachment and disassembly[11]. **CHP1** binds to microtubules and mediates the binding of the endoplasmic reticulum (ER) and the Golgi

apparatus with microtubules (not shown)[11]. Other proteins in the landscape that bind microtubules are **MAPRE2** (not shown)[118], **NDRG1** (not shown)[119], **KLC1** (not shown)[11] and **MAP4** (not shown)[120]. **KLC1** is a kinesin that regulates microtubule-associated transport of organelles[11] and **MAP4** promotes the assembly of microtubules[121]. In addition, **MAPK8** is known to increase microtubular assembly[122, 123].

1.7 Cell adhesion

The proteins **L1CAM**, **FYN**, **CDH2**, **CDH8** and **RET** regulate cell adhesion[11]. **L1CAM** regulates neuron-neuron adhesion and is found in axon terminals[11] and **FYN** regulates synapse formation[124]. The cadherins **CDH2** and **CDH8** are calcium-dependent adhesion molecules[11]. **CDH2** binds **KIFAP3**[125], **NMDAR**[95] and actin[92]. Cleavage of **RET** by caspases results in a fragment that functions as a cadherin accessory protein that potentiates cadherin-mediated cell aggregation[126].

1.8 Autophagy

As indicated above, microtubule-dependent trafficking is necessary for the transport of autophagosomes and degradation of their cargo. Autophagy dysregulation is also directly implicated in PD via the familial PD proteins[127]. In the landscape, **MAPK8**, **CDK5** and **AKT1S1** (**mTORC1**) regulate autophagy, e.g., **MAPK8** phosphorylates **BCL2**, which induces dissociation of **BCL2** from Beclin-1 and autophagy activation (not shown)[128]. In addition, **mTORC1** binds to[63], and is activated by, **MAPK8**[63], and has been shown to inhibit autophagy[129, 130]. Furthermore, **mTORC1** binds to **PPP2R2A**[131], **YWHAZ**[12] and **RPL5**[132], whereas **YWHAZ** and **RPL5** also bind to each other[12]. Lastly, the kinase **CDK5** has been shown to be required for autophagy in multiple PD models[133-135].

1.9 Calcium

Calcium channels regulate neuron excitability and release of neurotransmitters such as DA. In PD, nigral DA neurons show a dysregulation of calcium[136]. In the landscape, calcium is transported into the cell by the **NMDAR**[137] and the VDCC[138], and transported out of the cell by **SLC8A1**[139, 140]. The **NMDAR** binds to **CDH2**[95] and the VDCC binds to **SLC8A1**[39, 141], **TSPAN7**[142], **MAP4**[39], **MARK2**[39], **SLC4A3**[39], **CRMP1**[142], **PFKM**[39], **RAB14**[39] and, as already mentioned above, **SNAP25**[38, 39]. These proteins have a wide range of functions. **SLC8A1** rapidly transports Ca2+ out of the cell to prevent overloading of intracellular stores[139]. **TSPAN7** is a surface glycoprotein that may have a role in neurite outgrowth[143]. **MAP4** and **MARK2** regulate microtubular dynamics (see above). **SLC4A3** is an anion exchanger that exchanges $HCO3^-$ for Cl^- and thereby regulates the intracellular pH[11]. Another protein in the PD landscape that regulates neuronal pH by transporting HCO3- into the cell is **SLC4A8**[11]. **CRMP1** regulates remodeling of the cytoskeleton[11]. **PFKM** binds to **YWHAZ**[18] and catalyzes the conversion of D-fructose 1,6-phosphate to D-fructose 1,6-biphosphate[11]. Binding of D-fructose 1,6-bisphosphate to soluble Fe2+ prevents its

conversion to the insoluble Fe3+, an oxidation step that produces oxygen radicals. The availability of D-fructose 1,6-biphosphate may therefore affect iron content and oxygen radical levels[144] in the SN of PD patients. Lastly, **RAB14** and **SNAP25** are involved in intracellular trafficking (see above). Furthermore, VDCC function and thus calcium influx is inhibited by **CDK5**[145, 146]. **LPAR1** increases calcium mobilization in the cytosol[147], whereas **RGS4** and **RGS7** both decrease mobilization of calcium[148, 149]. In addition, **RAB4A** and **RAB11A** (not shown) increase the intracellular calcium concentration[150]. Calcium in turn activates **MAPK8**[151], **FYN**[96] and **VSNL1**[152, 153], and inhibits **NSF**[154]. Moreover, calcium increases the expression of **NDRG1**[155] and binds to **VSNL1**[156], **CHP1** (not shown)[157], **CDH2**[11] and **CDH8**[11].

1.10 Familial PD proteins

The familial PD proteins have many interactions with components within the landscape. SNCA, the primary component of Lewy bodies in PD DA neurons, binds to **SLC6A3**[29], **TH**[158], **YWHAZ**[159], **MARK2**[159], **MAPK8**[160], **KLC1**[161], **MAP4**[159], **ATP6V0D1**[159], **FYN**[162, 163], **STXBP1**[159], **SNAP25**[164] and **VAMP2**[165]. Further, SNCA activates **SLC6A3**[29], decreases **TH** expression (not shown)[166], inhibits **TH**[158, 167] and **MAPK8**[168], and is inhibited itself by **FYN**[162]. Interestingly, SNARE (**SNAP25** and **VAMP2**) dysfunction results in mislocalization and accumulation of SNCA and could be an important pathomechanism of PD[169], which emphasizes the importance of the normal functioning of the SNARE complex. Furthermore, binding of PARK7 to **VAMP2**[170] and of LRRK2 to **NSF**[171] shows that other familial PD proteins also have a direct impact on SNARE complex function. LRRK2 also binds to **MAP2K4**[172], **GNAI2**[173] and **YWHAZ**[174], and activates AKT1[175]. PARK2, UCHL1, EIF4G1 and PINK1 are four other familial PD proteins that have interactions with proteins in the landscape, i.e., PARK2 binds to and is phosphorylated by **CDK5**[176], binds to **SLC6A3**[28] and **ACTG1**[177], inhibits **MAPK8**[178], and increases expression of **SLC6A3**[30]. UCHL1 binds to AKT1[19], SNCA[179], **RANBP9**[180], **mTORC1**[181] and **EIF1B**[72], while EIF4G1 binds to **YWHAZ**[182] and **MARK2** binds to[183], and activates, PINK1[183].

2. DETAILED DESCRIPTION FIGURE 1B

Figure 1B shows a molecular landscape of interacting proteins, encoded by the mRNAs that are differentially expressed in the SN of *both* human PD patients and MPTP-treated mice, located primarily in the cell body and axon of the DA neuron. The biological processes depicted in the figure are described below:

2.1 Transcriptional and translational regulation

2.1.1 Histone regulation

HDAC1 deacetylates core histones and thereby represses gene transcription[11]. HDAC1 expression is increased in the SN of PD patients[184] and is one of the central proteins

in the landscape of SN mechanisms overlapping between PD and MPTP-treated mice. HDAC1 expression is increased by **SASH1**[185] and HDAC1 binds to **CDK5**[11], **DHX36**[186], **CCDC6**[187], PARK7[188], **MAPK8**[189], **PAPOLA**[190], the transcriptional regulators SIRT1[191], **GTF2I**[192], **DDX5**[193] and **NFKBIA**[194], and the transcription factors **SOX2**[195], **NR4A2**[196], **SATB1**[197] and NFKB[198]. HDAC1 itself activates AKT1[199], decreases the expression of BAX[200] and binds to the promoters of the genes encoding **SLC8A1**[201] and **TH**[196]. The HDAC1-associated transcriptional regulators SIRT1, **GTF2I**, **DDX5** and **NFKBIA** have multiple other landscape interactors. Variants in the SIRT1 gene promoter contribute to PD risk[202], and SIRT1 deacetylates HDAC1 and thereby increases its enzymatic activity (not shown)[191]. Further, SIRT1 binds to **USP22**[203,204], a histone deubiquitination protein that inhibits SIRT1 degradation[204] and, by removing ubiquitin from H2A and H2B, functions as a coactivator of histones[11]. Furthermore, SIRT1 binds to **SATB1**[205], **PAPOLA**[190], **GTF2I**[206], **MAPK8**[207] and the PD-associated[208] FOXO1[209]. SIRT1 inhibits FOXO1 (not shown)[210], whereas FOXO1 increases SIRT1 expression[211]. Further, **MAPK8** increases the degradation of SIRT1[212]. The familial protein PARK7 binds in the cytoplasm to **GTF2I** and thereby prevents its translocation to the nucleus in which **GTF2I**[213] is together with HDAC1 part of the deacetylation complex[11]. In addition to binding to HDAC1, **DDX5** binds to **NDRG1**[91], AKT1[214] and **YWHAZ**[182]. Expression of NFKB is increased in the PD brain[215]. **NFKBIA** binds to NFKB and thereby prevents its activation and translocation to the nucleus[216]. **NFKBIA** degradation is increased by **RET**[217], **FYN**[218] and **MAPK8**[219], and inhibited by **BCL2**[220]. Increased degradation or inhibition of **NFKBIA** increases NFKB activation and translocation to the nucleus[216]. **NFKBIA** binds to **ACTG1**[90] and **PSMA1**[221], and activates **MAPK8**[222]. Further, **NFKBIA** increases the expression of the transcriptional repressor **MXD4**[223] and **RGS4**, a regulator of G proteins[224]. Furthermore, **NFKBIA** decreases the expression of the familial PD protein PARK7[223] and of adaptor protein **YWHAZ**[225]. Regulation of the expression by **NFKBIA** is probably an indirect effect of its inhibitory function on the NFKB complex. Like **NFKBIA,** NFKB binds also to **ACTG1**[90], **PSMB5**[90], **PAPOLA**[90] and **DDX1**[90]. HDAC1 deacetylates the NFKB subunit RELA and in this way inhibits the transcriptional activity of NFKB[11].

In addition to binding to **NFKBIA** and NFKB (see above), **ACTG1** also binds to LRRK2[173], PARK2[177], SNCA[159] and **YWHAZ**[182]. The adaptor protein **YWHAZ** binds (in addition to the proteins mentioned above) to the 60S ribosomal protein **RPL10A**[12], the ATP-dependent RNA helicases **DDX1**[12] and **DDX5**[182], GIGYF2[12], AKT1[80] and FOXO1[226]. GIGYF2, AKT1 and FOXO1 are all associated with PD[208,227,228] and dysreglation of **YWHAZ** may interfere with their function. On its turn, **DDX1** binds to **NDRG1**[91] and SNCA[229] and acts as a coactivator to enhance NFKB-mediated transcriptional activation[11].

2.1.2 DA neuron signature

NR4A2 and **SOX2** are important transcription factors for establishing and maintaining

a DA-neuron-like expression pattern,[230] as is also apparent from their requirement for reprogramming fibroblasts towards a dopaminergic phenotype[231]. **NR4A2** increases the expression of **TH**[232], **SLC6A3**[232], PITX3[232], **RET**[233] and SLC18A2[232], and decreases the expression of SNCA[234]. In addition to binding to HDAC1, **NR4A2** binds to **GTF2I**[235] and NFKB[236]. **SATB1** decreases expression of **NR4A2**[237] and increases the expression of **ACTG1**[237]. The HDAC1-binding **SOX2** (see above)[195] also binds to **YWHAZ**[238], **RANBP9**[238], **CTBP2**[195] and **NFIB**[195], and its expression is increased by AKT1[239] and FOXO1[240].

2.1.3 Other transcriptional regulators

PTEN is a phosphatase that dephosphorylates PIP3 to PIP2 and hence inhibits AKT1 signaling[11, 241]. **RET** activates AKT1[242] that subsequently translocates to the nucleus[11] and increases the expression of **NDRG1**[243] and **SOX2** (see above)[239]. Furthermore, PTEN binds to and activates the familial PD protein PINK1[244] and affects the expression of multiple proteins in the landscape by increasing the expression of **PAPOLA**[245], **GTF2I**[245], **MXD4**[245] and **NDRG1**[246], and decreasing the expression of **NR4A2**[247], **MAPK8**[248] and **TH**[247]. The expression of PTEN itself is decreased by the 60S ribosomal protein **RPL5**[249] and the transcriptional repressor **CTBP2**[250].

2.1.4 Alternative pre-mRNA splicing

The polymerase **PAPOLA** creates the 3'-poly(A) tail of mRNAs[11], is required for endoribonucleolytic cleavage at poly(A) sites[11] and binds to HDAC1 (see above). **YTHDF2** has also a role in mRNA stability and splicing, by binding to N6-methyladenosine[11]. Of note, multiple other proteins involved in mRNA splicing are dysregulated in both human PD and the MPTP mouse model. MAGOH1 and **CASC3** are core components of the exon junction complex that is deposited at splice junctions on mRNAs, regulating mRNA splicing, nuclear export, cellular localization and translation efficiency[11]. MAGOH1 binds to **CASC3**[251], **ZC3H11A**[251], **SRSF7**[251], **RBM39**[251], and **SRPK2** (not shown)[252]. **RBM39** also binds to **SRSF7**[253] as well as **YWHAZ**[182] and **SRPK2**[254]. **SRPK2** is required for spliceosome complex formation[255] and, together with MAGOH1 and **RBM39**, binds to **SRSF7**[252] and MAPT[256], and increases the phosphorylation of **RBM39** (not shown)[252], **SRSF7** (not shown)[252] and MAPT (not shown)[256]. Phosphorylation of **SRPK2** at Thr-492 by AKT1 promotes its nuclear translocation and enhances its activity[11]. Like **CASC3**, MAGOH1 and **SRPK2, RBM39** and **SRSF7** are involved in pre-mRNA splicing[257, 258]. For instance, **SRSF7** is involved in mRNA export out of the nucleus[259] and is known to prevent splicing of exon 10 of MAPT (not shown)[260]. **CLK4** phosphorylates proteins of the spliceosome complex[11] and regulates the alternative splicing of MAPT[261]. MAPT itself increases the expression of **MAPK8**[262].

Other proteins that also affect alternative splicing and are involved in nucleosome/histone regulation are **HNRNPH3**, **CRMP1**, **H2AFJ** and **ANP32B**. **HNRNPH3** associates with pre-mRNA in the nucleus[11], and binds to PARK2[263] and **CRMP1**[264]. **H2AFJ** is a H2A

histone variant and core component of the nucleosome[11] and **ANP32B** stimulates core histones to assemble into a nucleosome[11]. Nucleosomes define the exon-intron border and since pre-mRNA splicing occurs co-transcriptionally, nucleosome organization, transcription elongation rate or epigenetic marks can affect pre-mRNA splicing[265, 266]. Moreover, histone deacetylation by HDAC1 affects pre-mRNA splicing, resulting in local repression of transcription[265, 267, 268]. HDAC1 is up-regulated in the SN of human PD patients and interacts with multiple proteins in the landscape (see also above).

Taken together, the central position of HDAC1 and the occurrence of multiple proteins involved in histone regulation and pre-mRNA splicing in the SN landscape suggest that dysregulation of nucleosome organization and the splicing machinery are important factors in the biological processes that overlap between PD and the MPTP mouse model.

2.2 (Vesicle) trafficking and exocytosis

In **Figure 1A**, the involvement of the SNARE complex in (DA) exocytosis is shown, however, the SNARE complex also regulates intracellular transport, as is apparent from the binding of **SNAP25** to both **NAPB**[269] and **KLC1**[270]. **NAPB** is required for vesicular transport between the ER and the Golgi apparatus[11], and **KLC1** is a microtubule-associated protein that regulates the transport of organelles such as mitochondria. Like the SNARE complex, the familial PD protein SNCA may be involved in DA release and transport[11], but also in ER-to-Golgi vesicle trafficking[271, 272]. SNCA modulates vesicle trafficking by binding to RABAC1 (not shown)[273], a protein that regulates the interaction between Rab GTPases and the SNARE complex[274]. Overexpression of SNCA disrupts vesicle trafficking and increases accumulation of vesicles in the cytoplasm[273]. Four Rab GTPases (**RAB4A**, **RAB6A**, **RAB11A** and **RAB14**) are overlapping between PD and the MPTP mouse. These proteins are involved in vesicular trafficking between compartments of the cell. **RAB4A** regulates localization of **VAMP2** to early endosomes and vesicles[275] and the membrane-bound form of **RAB4A** binds to **NDRG1**[276], a protein that is required for vesicular recycling[11]. **NDRG1** binds to actin filaments by binding to **ACTG1**[91] as well as to **ACOT7**[277] and **PPP2R2A**[91], and activates CASP3[278]. The RAB proteins **RAB6A**, **RAB11A** and **RAB14** are located in the Golgi complex and regulate protein trafficking to other organelles and the plasma membrane of the cell. Dysfunctioning of these proteins results in defective protein trafficking and membrane fusion, which can result in protein aggregation. **RAB6A** is located at the Golgi[279] and regulates vesicular transport from early and recycling endosomes to the Golgi (not shown)[280] but also transport from the Golgi to the ER[281]. Furthermore, **RAB6A** affects release of the SNARE (**SNAP25** and **VAMP2**) complex, which itself is involved in membrane fusion (see also Figure 1a) by binding and activating **NSF**[282]. **RAB11A** is located in recycling endosomes, the Golgi complex and on the cytoplasmic side of cytoplasmic vesicles, and regulates transport from the Golgi to the endosome[283] and from the Golgi to the plasma membrane[283]. **RAB11A** binds

to the neuronal cell adhesion protein **L1CAM**[284] and therefore is probably involved in its trafficking. The RAB protein **RAB14** regulates vesicular transport between the Golgi and early endosomes, and is involved in **CDH2** shedding (not shown)[285] and as such affects cell-cell adhesion (not shown)[285]. Lastly, also the ER-shaping protein **RTN2**[286] is involved in vesicular ER to Golgi transport[287].
Dysregulated (vesicle) trafficking affects exocytosis, receptor trafficking, (membrane) recycling and ultimately decreases the viability of the neuron.

2.3 Proteasomal degradation

The proteasome is involved in removal of unwanted, damaged or aggregated proteins[288, 289]. **PSMA1** and **PSMB5** are both part of the 26S proteosomal complex[290], bind to each other[291] and both bind to PARK2[292, 293]. Moreover, **PSMA1** binds to PTEN[294] and **NFKBIA**[221], whereas **PSMB5** binds to the NFKB complex[291]. Dysregulation of the 26S proteasome can heavily affect the PD protein landscape, for it degrades **RGS7**[295], **NR4A2**[296], **GRIN1** (NMDAR)[297], **NFKBIA**[298], SNCA[299], NFKB[300, 301], SIRT1[302], HDAC1[303], MAPT[304], MAP3K7[305], PTEN[306, 307] and **SOX2**[308]. Reduced activation of the proteosomal complex could therefore increase protein (e.g., SNCA) aggregation, which would affect neuronal functioning.

2.4 Mitochondrial function and apoptosis

Mitochondrial dysfunction is associated with both familial and sporadic PD[309]. **BCL2,** located in the nuclear membrane and in the mitochondrial outer membrane**,** is an important anti-apoptotic factor that binds to, inhibits and decreases the expression of the proapoptotic protein BAX[310-313]. BAX inhibition is mediated via the inhibition of **MAPK8** that inhibits the binding of **YWHAZ** and BAX, and in this way increases the translocation of BAX to the mitochondrial membrane[314]. **BCL2** is bound and regulated by multiple proteins in the landscape, i.e., **SATB1** decreases and **NFKBIA** increases **BCL2** expression[315, 316]. **MAPK8** also increases **BCL2** expression[317], but inhibits **BCL2** function[318, 319]. **BCL2** in turn inhibits **MAPK8**[319], decreases expression of **NFKBIA**[320], **NDRG1**[321] and PTEN[322], increases expression of **SNAP25**[321], and decreases cleavage of **SRPK2**[323]. **BCL2** binds **MAPK8**[324], CASP3[325] and PARK2[326], and inhibits apoptotic pathways in that it, in addition to inhibiting BAX, also inhibits CASP3[327] and HTRA2[328] and HTRA2 translocation out of mitochondria[329]. In the cytoplasm, HTRA2 binds EIF4G1[330], PARK2 (not shown)[331], PINK1 (not shown)[332] and **CDK5**[332]. **CDK5** in turn inhibits PARK2[176] and increases **TH** expression[7]. SNCA binds to PARK2[333] and, in contrast to **CDK5,** decreases **TH** expression[166, 334].
Other proteins in the landscape that affect mitochondrial function are **MRPL15**, **ATP5C1** and **RET**. The 39S ribosomal protein **MRPL15** is located in mitochondria and involved in mitochondrial-specific protein expression. Moreover, **MRPL15** binds to the transcription factor **SOX2** (not shown)[238] and as such may affect DA-neuron-specific expression (see paragraph 'DA neuron signature' in the section 'Transcriptional and

translational regulation'). The ATPase **ATP5C1** is part of complex V of the respiratory chain that uses the proton gradient across the mitochondrial membrane to produce ATP from ADP[11]. SNCA may also affect the respiratory chain directly by binding to **ATP5C1**[159]. Lastly, the tyrosine kinase **RET** increases the expression of **TH** and **SLC6A3** (Figure 1a), and ameliorates complex I dysfunction in a PD model[335].

3. DETAILED DESCRIPTION FIGURE 2

Figure 2 shows a molecular landscape of interacting proteins, encoded by the mRNAs that are differentially expressed in the striatum of *both* human PD patients and MPTP-treated mice located in the post-synapse of a striatal neuron. The biological processes depicted in the figure are described below:

As a result of the dysregulation of the biological processes constituting the molecular landscape of the processes shared in the SN (summarized in **Figure 1A** and **B**), the release of DA to the striatum is decreased. Due to the lower DA release into the synaptic cleft, affecting protein expression in the striatal post-synapse, the activation of the DA receptors DRD2 and DRD3 is diminished; these receptors are associated with PD[336, 337]. When activated, DRD2 (long variant) and DRD3 increase intracellular calcium[338], but they also inhibit the function of the NMDA receptor (NMDAR)[339] and the VDCC[340, 341]. The VDCC binds to **ITSN1**[39], a protein involved in actin reorganization and assembly[342, 343]. **DCLK1** and **ENC1** are also involved in actin regulation, i.e. **DCLK1** regulates the distribution of actin[344] and **ENC1** is an actin-binding protein[345] that also binds to SNCA[161].
DRD2 also binds to calmodulin (CaM)[346, 347] and thereby exerts influence on calcium signaling in the striatal neuron. Namely, CaM binds to the VDCC[142], the NMDAR (not shown)[348], SNCA[349], LRRK2[173], **TGM2**[350], **KCNQ5**[351], **DIRAS2**[352] and **DCLK1**[352], and can thereby affect multiple proteins in the landscape. Furthermore, CaM regulates **KCNQ5**[353] and inhibits calcium flux through the NMDAR into the cell[354, 355]. In addition, calcium-bound CaM activates CREB1[356, 357] and **CAMK1G**[358], and regulates **TGM2** function (not shown)[350]. **CAMK1G** also activates CREB1[358], and **TGM2** activates ERK1/2[359] and CREB1[360], but also binds to CASP3[361], decreases the expression of **KCNQ5**[362] and increases the expression of LRRK2[362]. **TGM2** is also activated by calcium[363], increases the efflux of calcium out of the cell[364], binds to SNCA[365] and increases its aggregation (not shown)[365, 366]. Calcium and CaM therefore affect the activity of ERK1/2 and CREB1 either directly or via the activation of **TGM2** or **CAMK1G**.
Activation of DRD2 by DA also results in the activation of ERK1/2[367] and CREB1[368]. ERK1/2 binds to **CHGB**[369] and the familial proteins SNCA[370] and PARK7[371]. Furthermore, in addition to DRD2 and **TGM2** (see above), **S100A10**[372] and **ITSN1**[373] activate ERK1/2, whereas the nuclear membrane protein **TMEM176B** inhibits ERK1/2 activation[374]. Of note, all these processes converge on CREB1. ERK1/2 activates CREB1[375, 376], and CREB1 is

activated by CaM, **CAMK1G**, **TGM2** and DRD2 (see above), but also by the NMDAR[356, 377] and the (L-type) VDCC (not shown)[356] due to their ability to increase calcium influx, which is necessary for CREB1 activation[378, 379]. Thus, CREB1 is regulated by the majority of the proteins in the striatal landscape, either directly or via ERK1/2 activation. Moreover, DA activates both ERK1/2[367] and CREB1 (via the DA receptors)[368, 380], suggesting that ERK1/2 and CREB1 activation (via phosphorylation) is reduced in PD or after MPTP treatment due to the absence of DA.

These pathways also play a role in the effect of L-DOPA, the mainstay of treatment in PD. L-DOPA administration activates ERK1/2 in the striatum[381]. DA-induced, CREB1-dependent transcription in the intact striatum in a PD model[382] is further potentiated by NMDAR activation[377]. The secretory granule protein **CHGB** is one of the proteins of which the expression is regulated by CREB1, i.e. CREB1 binds to the CRE element of the **CHGB** gene promoter[383]. Furthermore, calcium decreases the expression of **CHGB**[384] and **CHGB** binds to PARK2[263]. In addition to ERK1/2 and CREB1, L-DOPA also activates DRD2[385], DRD3[385] and NMDAR[385, 386], and increases the expression of DRD3[386], CASP3[387] and **S100A10**[388]. In a PD rat model, **S100A10** is involved in L-DOPA-induced abnormal involuntary movements[389]. The activation of striatal ERK1/2 by L-DOPA also appears involved in L-DOPA-induced dyskinesias[389], but not the L-DOPA induced CREB1 activation[381, 390, 391]. These processes could therefore not only give insights into the PD-related disease mechanisms in the striatum, but also in the beneficial, and adverse, effects of pharmacological treatment.
CREB1 and ERK1/2 are also known for their role in epilepsy. Brain areas prone to epileptic seizures show an increased activation of CREB1 and ERK1/2[382], and an up regulation of **CHGB**[392], CREB1[392], **ENC1**[356] and **NPTX2**[392]. **NPTX2** is thought to play a role in long-term plasticity[392] and increases apoptosis[11]. Further, **KCNQ5**[393], the NMDAR[394] and the VDCC[395] are associated with epileptic seizures. Therefore, the landscape cannot only give insight in treatment outcome, but can also explain the associations seen in functional studies with PD, in this respect with epilepsy[396].

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Physical exercise modulates L-DOPA-regulated molecular pathways in the MPTP mouse model of Parkinson's disease

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Submitted

4.1 ABSTRACT

Parkinson's disease (PD) is characterized by the degeneration of dopaminergic (DA) neurons in the substantia nigra (SN) pars compacta, resulting in motor and non-motor dysfunction. Physical exercise improves these symptoms in PD patients. To explore the molecular mechanisms underlying the beneficial effects of physical exercise, we exposed 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimidine (MPTP)-treated mice to a four-week physical exercise regimen, and subsequently explored their motor performance and the transcriptome of multiple PD-linked brain areas. MPTP reduced the number of DA neurons in the SN, whereas physical exercise improved beam walking, rotarod performance and motor behavior in the open field. Further, enrichment analyses of the RNA-sequencing data revealed that in the MPTP-treated mice physical exercise predominantly modulated signaling cascades that are regulated by the upstream regulators L-DOPA, RICTOR, CREB1, or bicuculline/dalfampridine, and involved in movement disorders, mitochondrial dysfunction and epilepsy. To elucidate the molecular pathways underlying these cascades in the various brain areas studied, we integrated the proteins encoded by the exercise-induced differentially expressed mRNAs for each of the top upstream regulators into a molecular landscape. Most notable was the opposite effect of physical exercise compared to previously reported effects of L-DOPA on the expression of mRNAs in the SN and the ventromedial striatum that are involved in – among other processes – circadian rhythm and signaling involving DA, neuropeptides and endocannabinoids. Altogether, our findings suggest that physical exercise can indeed improve motor function in PD, may counteract L-DOPA-mediated molecular mechanisms and ameliorate non-motor symptoms of PD, some of which may be the result of (chronic) L-DOPA use.

KEYWORDS: Parkinson's disease, physical exercise, MPTP, L-DOPA, motor function, non-motor function, molecular landscape

4.2 INTRODUCTION

Parkinson's disease (PD) is characterized by the degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). The clinical phenotype encompasses motor symptoms – including bradykinesia, rigidity, tremor, gait dysfunction and postural instability – and non-motor symptoms such as sleeping disturbances, pain, or cognitive deficits that affect executive functions, attention, mood and working memory[1-3]. Levodopa (L-DOPA), a precursor of DA, has been used since the 1960s to treat PD motor symptoms and is still considered the gold standard of therapy[4, 5]. In recent years, physical exercise – including intervention strategies such as aerobic exercise (e.g. treadmill exercise, cycling or dancing) or strength training (e.g. using a modified fitness counts program or progressive resistance exercising) – has been reported to improve DA signaling[6, 7] and motor dysfunction[8-10], including

bradykinesia[11, 12], rigidity[13] and tremor[11]. Physical exercise has also been reported to improve less dopamine-dependent symptoms involving postural control such as turning performance[6] and instability[14], as well as cognitive function[2, 15, 16] in PD patients. Although these beneficial clinical effects of exercise on PD symptoms are evident, the underlying molecular mechanisms are not well understood. A better understanding of these processes may ultimately lead to a more efficient treatment of these symptoms, through directly targeting the underlying pathways.

Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice results in the loss of nigrostriatal DA neurons, and is widely used to study the pathophysiological mechanisms underlying DA neuron degeneration in PD[17]. Moreover, similar to human PD, physical exercise improves motor behavior and reduces cognitive impairment in MPTP-treated mice[18-21]. In this study, we aimed to elucidate the molecular pathways underlying the beneficial effects of exercise in PD, using the MPTP mouse model of PD.

4.3 METHODS

4.3.1 Animals

Six month old male C57BL/6J mice were housed, five-to-a-cage, with *ad libitum* access to food and water and at a constant 12/12h light/dark cycle (lights on between 07:00 and 19:00h). Room temperature was controlled at 21°C and rooms were homogenously lighted by 60 LUX with controlled humidity. Following arrival, the mice were acclimatized to their new housing for one week, after which they were randomly assigned to one of four treatment groups: (1) saline-treated; (2) saline-treated with physical exercise; (3) MPTP-treated; and (4) MPTP-treated with physical exercise. MPTP-HCl (Sigma-Aldrich) dissolved in saline was administered via four intraperitoneal injections at 2h intervals, amounting to a total administered dose of 70mg/kg (free-base). The control mice underwent the same protocol using saline injections. Mice were allowed to recover from the injections for two weeks. All animal experiments were approved by the Animal Care Committee of the Radboud University Nijmegen Medical Center, The Netherlands, and performed according to the guidelines of the Dutch Council for Animal Care and the European Communities Council Directive 2010/63/EU.

4.3.2 Physical exercise

Physical exercise was initiated three weeks following MPTP or saline treatment and was performed daily. Mice ran 30 minutes twice a day during a training period of 28 consecutive days in individual, horizontal lanes on a 5-lane treadmill (Panlab Harvard Apparatus) at a speed of 20cm/s. Automated short air puffs were used to stimulate the mice to keep running when drifting too far to the back of the lane. Mice assigned to the groups without physical exercise were placed in the same experimental room, adjacent

to the treadmill, in their housing cage.

4.3.3 Behavioral testing

Behavioral testing commenced one week before the physical exercise regimen started (week 0), and was repeated each week during the exercise regimen (weeks 1-4): beam walk on the first, rotarod on the third and open field on the fifth day of each week, in each case performed between 08:00 and 13:00h. Prior to all behavioral tests, the animals of all four treatment groups were habituated to the experimental room for one hour. Mice from different treatment groups were tested concurrently on the rotarod and in the open field.

Open field

The mice were placed in a white plexiglass box (50x50x40cm) and video recorded from above for 30 minutes using Ethovision XT 7.0 software (Noldus Information Technology B.V., Wageningen, The Netherlands). Afterwards, the parameters 'total walking distance', 'total movement time', 'mean velocity' and 'mean angular velocity' were calculated by the software.

Rotarod

Mice were placed on the rotarod apparatus (IITC Inc.) with a rod diameter of 32 mm and an increasing speed of 4 to 38 rpm in 300s. Five mice were tested simultaneously on the rotarod and their latency to fall was measured. On each testing day, each mouse performed one pre-trial and three trials, each with a maximum duration of 300s and with a minimum of one hour of rest between the trials. The pre-trial enabled the mice to habituate (again) to the rotarod and was not included in the results. For each testing day, the latency times of the three trials were averaged per mouse.

Beam walk

The mice were placed on a white plasticized iron rod (full length 80cm, diameter 10mm) suspended at 40cm height and were trained to cross the beam to their home cage. Training of the mice occurred on the first day. During the training the distance to cross was increased each time they successfully reached their cage, until they were able to reach their home cage over the full length of the beam. For testing, the time it took for the mouse to cross the full beam to reach their home cage was measured each week in three trials, with at least one hour of rest between the trials. For each testing day, the times of the three trials were averaged per mouse.

4.3.4 Immunohistochemistry

Twenty-four hours following their last exercise training, mice were sacrificed by cervical dislocation and brains were dissected and fixated in 4% paraformaldehyde in PBS solution for three hours and subsequently cryoprotected by immersion in 30%

sucrose for twenty-four hours. After cryosectioning, DAB staining was performed on 20µm thick coronal slices, placed on gelatinized glass slides. For this, the sections were washed with PBS (3x10min), non-specific sites blocked with blocking buffer (2.5% normal donkey serum, 2.5% normal goat serum, 1% BSA, 1% glycine, 0.1% lysine and 0.4% Triton X-100 in PBS) for 30 minutes and incubated with rabbit anti-tyrosine hydroxylase (TH, 1:1000; Pel-Freez Biologicals #P40101-0; Lot.No.: 19335) for 16 hours at 4°C. This was followed by one hour incubations with biotinylated goat-anti-rabbit (1:200; Jackson Immuno Research; 711-065-152; Lot.No.:117858) and avidin-biotin-peroxidase complex (A and B 1:800; Vectastain Elite ABC kit, PK-6100 Standard), with PBS washing steps in between. To visualize antibody binding, the sections with SNpc and ventral tegmental area (VTA) areas were incubated for 30 minutes, and those with dorsolateral striatum (DL) and ventromedial striatum (VM) areas were incubated for 20 minutes, in a DAB/H_2O_2-solution potentiated by ammonium-nickel-sulphate. The sections were subsequently dehydrated and cover-slipped. For each mouse, every sixth section throughout the different brain areas was included in the counting procedure and for optimal comparison between groups, sections of different treatment groups were stained concurrently.

Images were captured by a Leica DM6000B microscope. TH-positive (TH+) cells were counted in the sections of the SNpc (-2.54 to -3.88mm to Bregma[22]) and VTA (-2.92 to -3.88mm to Bregma[22]), using a 20x magnification. The number of TH+ cells in each section (both the left and right side) were counted by a blinded assessor, and averaged over the total number of sections per animal. DA fiber density was estimated in the DL (1.18 to -0.10mm to Bregma[22]) and VM (1.54 to 0.62mm to Bregma[22]) by quantifying the optic density (OD) with FIJI[23], using a 5x magnification. In both areas, the OD per section was determined by averaging the OD of ten separate areas within the striatal matrix (i.e. in-between the striosomes). Subsequently, the OD in the DL and VM was normalized by subtracting the OD of respectively the corpus callosum (CC) and anterior commissure (AC) in the same section, and all sections were averaged per animal.

4.3.5 RNA isolation and sample preparation

Twenty-four hours following the last physical exercise training, brains of 8-10 mice per group – that were sacrificed by cervical dislocation – were dissected, immediately frozen on dry ice and stored at −80°C until further preparation. Specific brain areas, i.e. prefrontal cortex (PFC), DL, VM, VTA, SN, pedunculopontine nucleus (PPN), were then cryo-punched based on the stereotaxic atlas of the mouse brain[22] from 200µm thick coronal slices, using punch needles with a diameter of 0.5 and 0.75mm (see **Supplementary Figure 1** for the estimated punching locations per area). All specimens were kept at -20°C during processing. For RNA isolation, punched samples were homogenized with a TissueLyser (Retsch GmbH) in 800µL TRIzol reagent and RNA isolation was performed according to the manufacturer's instructions (Invitrogen). Total

RNA concentration was determined with a NanodropTM ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) and RNA quality was visually assessed by 1% agarose gel electrophoresis. Genomic DNA was removed by treatment with DNase I in the presence of RNAsin (Thermo Fisher) in 5x FSB buffer and RNAse-free water. Subsequently, total RNA samples were stored at -80°C until further use. For each treatment group and brain area, RNA samples of six mice were pooled for RNAseq analysis.

4.3.6 RNA sequencing and data processing

All RNA samples were subjected to RNA sequencing (RNAseq; HudsonAlpha Genomic Services Lab, Huntsville, AL). In short, total RNA concentration was estimated by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and RNA integrity by using the Agilent 2100 Bioanalyzer (Applied Biosystems, Carlsbad, CA, USA). RNAseq libraries were formed from approximately 500ng total RNA of each pooled sample, followed by poly(A)-enrichment. RNAseq was performed using paired end sequencing on Illumina HiSeqH2000 (Illumina, San Diego, CA, USA), at 50 base pairs, generating over 25 million paired reads per sample. Raw RNAseq FASTQ files were demultiplexed by bcl2fastq conversion software v1.8.3 (Illumina, Inc., San Diego, CA, USA) using default settings. RNAseq data was analyzed using GeneSifter software (VizX Labs, Seattle, WA). RNAseq reads were mapped to the *Mus musculus* reference genome build 37.2 and for this, the reads were trimmed by 15 base pairs at the 5-prime end. Subsequently, transcript abundance was calculated by estimating the reads per kilobase of exon per million mapped reads (RPKM) and normalization to the number of mapped reads was used for comparison of two mRNA sets. A t-test was used for pairwise comparison and a Likelihood Ratio Test to adjust for distribution probability.

4.3.7 qPCR validation

The RNAseq results were validated by comparing expression levels of at least eight mRNAs/genes per area with their expression as established by qPCR. These genes were chosen randomly, although there was one requirement, namely that genes from all three comparisons of interest, i.e. the comparisons to assess the effect of MPTP (group 3 vs. group 1), physical exercise (group 2 vs. group 1) and physical exercise in the MPTP-model of PD (group 4 vs. group 3), should be included. RNA from the same samples used for the RNAseq pools was reverse-transcribed to cDNA with random primers using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, #K1632 Lot No: 00167909) according to the manufacturer's protocol. Three-step qPCR (95°C for 10 min, followed by 45 three-step-cycles of 95°C for 5 sec, 65°C for 10 sec, and 72°C for 20 sec and the generation of melting curves from 70°C to 95°C; Rotor-Gene 6000 Series, Corbett Life Science Pty. Ltd.) was performed using the 2x SensiFAST SYBR No-ROX mix (Bioline Lot No: SF582-313209) and primers designed with NCBI Primer-Blast (www.ncbi.nlm.nih.gov/tools/primerblast/) and synthesized at Sigma Life Sciences (The

Netherlands) (For a complete overview of used primers see **Supplementary Table 1**). The housekeeping genes ACTB and YWHAZ were used as reference for normalization of gene expression. Based on the qPCR results, the minimum requirements to be included in the enrichment analysis – regarding fold change (FC) cut-off, maximum likelihood ratio value and minimal RPKM value – were adjusted so that at least 90% of the gene expression changes could be validated by qPCR. As there was insufficient remaining RNA available to perform the complete qPCR validation for the PPN RNAseq data, the same cut-off values were used as for the other brain areas.

4.3.8 Overlap of MPTP- and exercise-regulated genes

To determine the direct effect of exercise on MPTP-regulated genes we looked at the overlap between the genes regulated by MPTP (group 3 vs. group 1) and the genes regulated by exercise in the MPTP model (group 4 vs. group 3). To quantify this overlap we used the hypergeometric distribution test:

$$p(x|\, n, M, N) = \frac{\binom{M}{x}\binom{N-M}{n-x}}{\binom{N}{n}}$$

and determined the chance of observing exactly x overlapping genes from a total of n differentially expressed genes by exercise in the MPTP-model, with a total of M genes that were differentially expressed by MPTP and a total of N genes detected with RNAseq. The number of unique genes detected with RNAseq in each brain area (N), consists of genes detected in both comparisons (group 3 vs. group 1 and group 4 vs. group 3), irrespective of their FC or expression p-value. Of note, for all comparisons only protein-coding genes were considered.

4.3.9 Enrichment analysis and building of molecular landscapes

The Ingenuity pathway analysis software package (www.ingenuity.com) was used to identify enriched gene categories in the lists of differentially expressed protein-coding mRNAs in each of the brain areas. Again, we focused on the three main comparisons of interest (see above) – i.e. the comparisons that assess the effect of MPTP, physical exercise and physical exercise in the MPTP-model of PD – in the six brain areas. Ingenuity assigns genes or rather their corresponding mRNAs/proteins to functional (sub)-categories, i.e. 'canonical pathways' and 'biofunctions', with the latter including 'diseases and disorders' and 'molecular and cellular functions'. In addition, Ingenuity generates a list of 'upstream regulators', i.e. proteins or compounds that regulate multiple proteins/mRNAs from the input list. When possible, the program also calculates a z-score that is based on the expression changes of the input mRNAs and that is a measure for the directionality of the upstream regulator, canonical pathway or biofunction. A z-score <-2 or >2 is considered significant. For all analyses,

only functional categories and upstream regulators with significant enrichment (i.e. Benjamini-Hochberg corrected p<0.05) and containing at least two genes were taken into account.

Proteins/mRNAs regulated by the top upstream regulators were analyzed in more depth to identify their relation to physical exercise-induced processes in the MPTP-model of PD (i.e. the comparison of group 4 with group 3). Guided by the results of the Ingenuity enrichment analyses, an extensive literature search was performed for the (putative) roles of all the proteins encoded by the differentially expressed mRNAs as well as their functional interactions, using the UniProt Protein Knowledge Base (http://www.uniprot.org) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Based on these findings and applying an approach similar to the one we used previously for genome-wide association and expression data[24-26], we then built molecular landscapes containing interacting proteins encoded by the mRNAs that are differentially expressed by physical exercise and are known to be regulated by the top regulators for each brain area. To complement these protein interaction cascades, we added a number of proteins that were not encoded by the differentially expressed mRNAs but that have been implicated in PD etiology through other lines of (genetic) evidence. In this respect, proteins encoded by familial PD candidate genes were included if they have at least one functional interaction with one or more other landscape proteins. Additional proteins were included when having at least two interactions with other landscape proteins.

4.3.10 Statistics

Statistical comparisons of values between multiple treatment groups were carried out using a two-way ANOVA. For behavioral test data, with data at multiple time points, a linear mixed model was applied using SPSS (IBM, version 23), with 'week', 'physical exercise' and 'MPTP' as fixed factors to calculate the main effects of the training period, physical exercise, and the interaction between physical exercise and MPTP. The main effect of MPTP in the behavioral tests was assessed using a pair-wise comparison of saline-treated and MPTP-treated mice before the start of the exercise regimen. For pair-wise comparison, an F-test was used to determine if the distributions of the compared two groups have the same variance. Based on the F-test, a Student's t-test for equal or unequal variance was then used to evaluate the significance of the expression differences. For all comparisons, data are represented as mean with the standard error of the mean (SEM), and a p-value <0.05 was considered statistically significant.

The p-values calculated with the hypergeometric distribution test were adjusted for multiple testing using the Bonferroni correction.

4.4 RESULTS

In this study, we assessed the effects of physical exercise in the MPTP-treated mouse model of PD at the behavioral and molecular levels.

4.4.1 Physical exercise affects the motor function of MPTP-treated mice

At baseline, i.e. following recovery from MPTP treatment but before the exercise regimen started, MPTP-treated mice showed in the open field an increased total walking distance ($p<0.01$), total movement time ($p<0.005$) and mean velocity ($p<0.005$), and a decreased mean angular velocity ($p<0.005$) compared to saline-treated control mice. In contrast, their performance on rotarod and beam walk tests were not significantly different from controls (**Figure 1**).

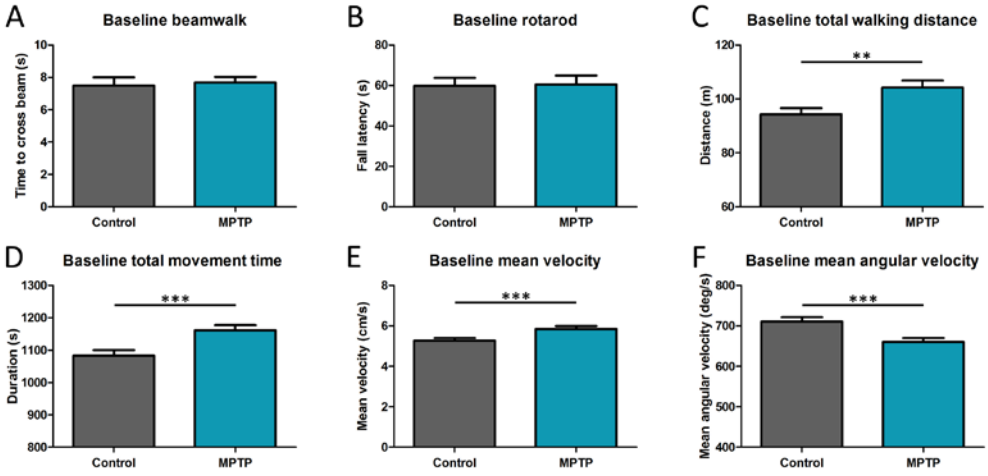

Figure 1. Effect of MPTP. Results of the behavioral tests in week 0 (set as the baseline for the effect of physical exercise see Figure 2). No effect of MPTP was shown for the beam walk (A) or rotarod (B) tests, but MPTP significantly affects the parameters in the open field (C-F). **P<0.01; ***P<0.005, mean+SEM, n=28 for controls (saline-treated) and n=23 for MPTP-treated mice.

In **Figure 2**, the effects of physical exercise during the course of the training period relative to baseline are shown for each of the four treatment groups. The beam walk task showed a clear training effect over time in all groups (main effect of 'week' $p<0.001$), and the test performance was improved by physical exercise in both the MPTP-treated and saline-treated mice, without significant differences between the groups (main effect of physical exercise $p<0.05$) and no significant interaction between physical exercise and MPTP-treatment (**Figure 2A**). Rotarod performance was also significantly improved by physical exercise ($p<0.01$), but no improvement over time or interaction with MPTP-treatment was found (**Figure 2B**). Of the tested parameters in the open field (total walking distance, total movement time, mean velocity, and mean angular velocity), the mean angular velocity was increased ($p<0.001$), and the total movement time showed a decreasing trend ($p=0.051$) for all treatment groups over time during the exercise regimen (i.e. main effect of 'week'). There was no significant (main) effect of physical exercise on any of the four tested open field parameters, only a trend towards a higher 'mean velocity' ($p=0.082$). However, for all four open field parameters, significant

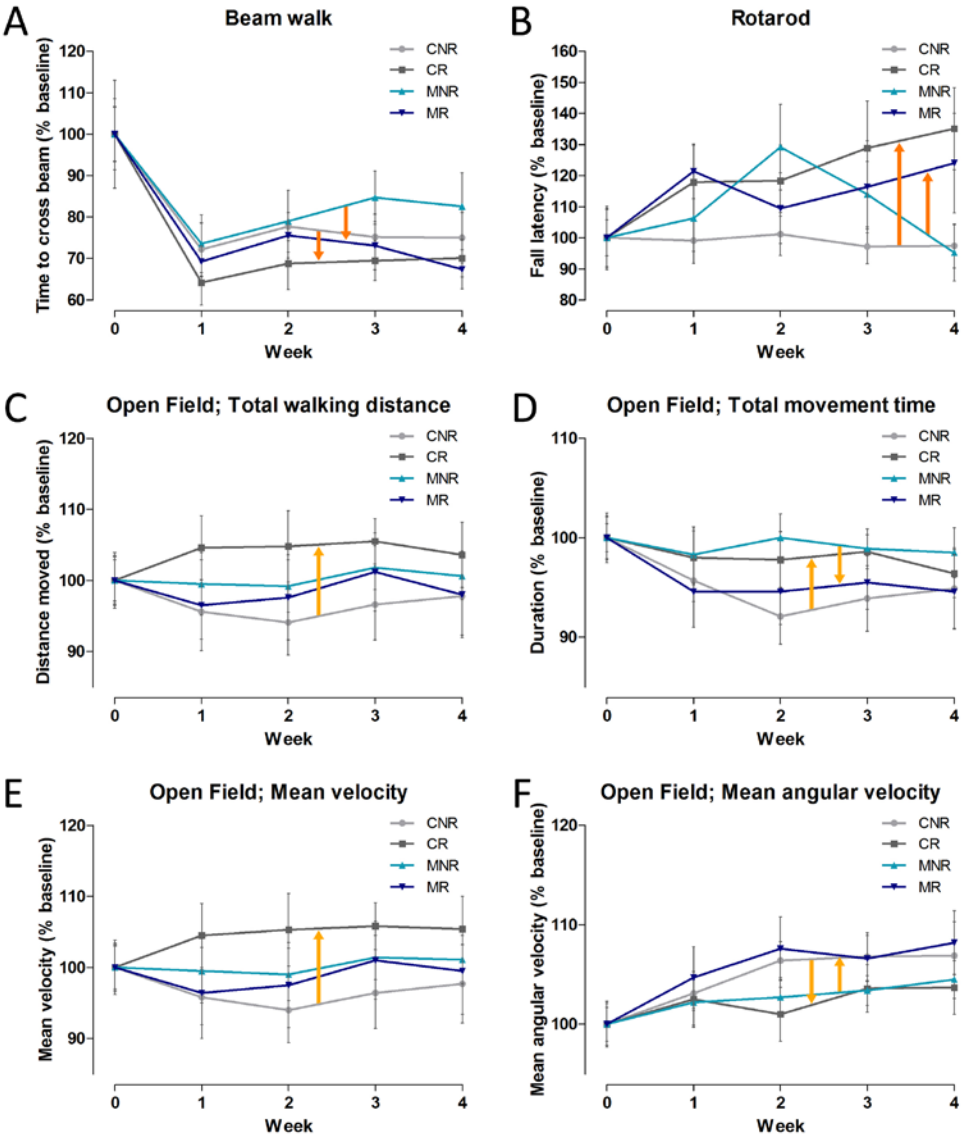

Figure 2. Effect of physical exercise and interaction with MPTP compared to the baseline (week 0); results of the behavioral tests in week 0-4. Measurements in week 1-4 were normalized to week 0 (100%). Dark orange arrows indicate the main effect of physical exercise (A-B) and light orange arrows show the effect of the interaction between physical exercise and MPTP treatment (C-F). mean±SEM. n=14 for both CNR and CR, n=10 for MNR and n=13 for MR. CNR, control not running; CR, control running; MNR, MPTP-treated but not running; MR, MPTP-treated and running.

interactions between physical exercise and MPTP-treatment were found ($p<0.05$). Physical exercise *increased* the walking distance and mean velocity of saline-treated mice, but *not* of MPTP-treated mice. Moreover, physical exercise *increased* the total movement time of saline-treated mice and *decreased* that of MPTP-treated mice. This opposite effect was also observed for mean angular velocity, i.e. a *decrease* by physical

exercise in saline-treated mice and an *increase* in MPTP-treated mice (**Figure 2C-F**).

4.4.2 TH depletion in the SNpc and striatum following MPTP treatment

The number of DA neurons in the SNpc and VTA of each treatment group, as well as an estimate of DA fiber density in striatal target areas (DL and VM, respectively) was determined by immunohistochemistry for TH – the rate-limiting enzyme in DA synthesis. These measures were primarily taken to confirm and estimate the degree of neuronal loss due to MPTP treatment, but they may also provide some insight into whether exercise could affect these structural changes. MPTP significantly reduced the number of TH+ cells in the SNpc ($p<0.005$), but not in the VTA. Pairwise comparison between the treatment groups revealed that the number of TH+ cells in the SNpc of MPTP-treated mice *without* and *with* physical exercise was reduced by 29% and 20%, respectively, compared to the saline-treated group *without* exercise (both $p<0.05$; **Figure 3**). There was no significant effect of physical exercise on the number of TH+ cells in either the SNpc or the VTA, and no interaction between MPTP and physical exercise.

In **Supplementary Figure 2**, the relative OD of TH+ fibers in the DL, the primary striatal target area of the SNpc, is shown. The OD of TH+ fibers was reduced by MPTP ($p<0.05$), without a main effect of physical exercise or an interaction between MPTP and physical exercise. Pairwise comparison showed that MPTP decreased the density of TH+ fibers in MPTP-treated mice *without* exercise by 33% ($p<0.005$) compared to saline-treated mice *without* physical exercise. There was a trend towards an increased TH+ OD by physical exercise in MPTP-treated mice, but this increase was not significant.
Supplementary Figure 3 shows the OD of TH+ fibers in the VM, the primary striatal target area of the VTA. Although all treatment groups (physical exercise, MPTP and MPTP + physical exercise) showed a reduced OD of TH+ fibers, no significant effects of MPTP, physical exercise or their interaction were found.

4.4.3 qPCR validation of the RNAseq data

The RNAseq data were obtained from pooled samples and in order to validate these data, the mRNA expression levels in each of the investigated brain areas were determined in individual samples by qPCR. The results of the qPCR experiments (**Supplementary Figure 4**) led us to adopt the following requirements for the inclusion of differentially expressed protein-coding mRNAs in the subsequent analyses: FC >1.2, likelihood ratio <0.05, RPKM >5.

4.4.4 A direct effect of physical exercise on MPTP-regulated genes

The overlap between the protein-coding mRNAs that are differentially expressed due to MPTP alone and due to exercise in MPTP-treated mice is represented in **Supplementary Figure 5**. In all brain areas the probability of this overlap was calculated by using the

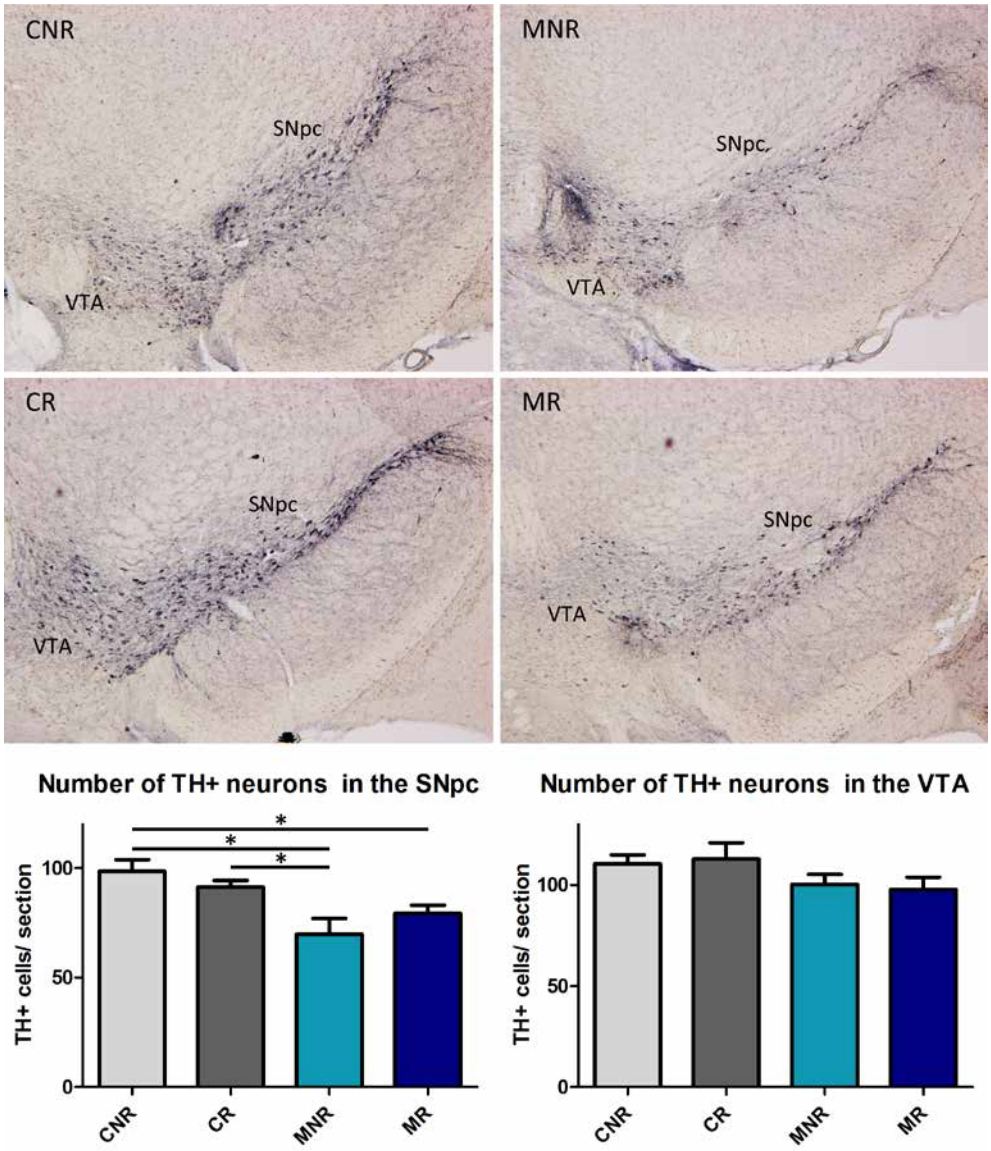

Figure 3. TH+ neurons in the SNpc and VTA. The upper panel shows a representative picture for each of the four treatment groups and the lower panel shows the average number of TH+ neurons in the SNpc and the VTA per treatment group. *P<0.05, mean+SEM, n=5 for CNR and MR and n=4 for CR and MNR for both brain areas. CNR, control not running; CR, control running; MNR, MPTP-treated but not running; MR, MPTP-treated and running; SNpc, substantia nigra pars compacta; VTA, ventral tegmental area.

hypergeometric distribution test, which showed that for all areas, the overlap is greater than would be expected based on random gene selection ($p<0.05$). Further, in all areas 82-99% of the overlapping mRNAs are regulated in opposite directions by MPTP and exercise. Enrichment analyses of mRNAs that overlap but are regulated in opposite directions are summarized in **Supplementary Table 2**. The VTA and PFC show the most

significant results, and are also the brain areas with the biggest absolute and relative overlap (i.e. the overlap in number and proportion of mRNAs). The analysis of the VTA displays a *downregulation* of the top regulator 'inosine', whereas the PFC and to a lesser extent also the DL show an *increase* in effect of dalfampridine and bicuculline.

4.4.5 Enriched regulators, pathways and biofunctions in the RNAseq data

Enrichment analysis of the differentially expressed mRNAs was performed for each of the brain area examined to investigate the effects of MPTP (i.e. comparing the MPTP-treated group *without* exercise to the saline-treated group *without* exercise), physical exercise (i.e. comparing saline-treated mice *with* exercise to saline-treated mice *without* exercise), and the effects of physical exercise in MPTP-treated mice (i.e. comparing the MPTP-treated mice *with* exercise to MPTP-treated mice *without* exercise). In **Tables 1-3**, a short overview of the main effects – the top regulator(s), canonical pathway(s) and biofunction(s) – of MPTP, physical exercise and physical exercise in MPTP-treated mice is provided for each brain area separately. A more elaborate overview of these enrichment analyses per brain area can be found in **Supplementary Tables 3-8**.

In all brain areas examined, MPTP treatment affected a set of mRNAs that is involved in epilepsy, which is reflected by the presence of the epilepsy-regulating transcription factor CREB1, the convulsants bicuculline and dalfampridine, and the biofunction 'epilepsy'. Other regulators and related functional themes enriched within the mRNAs affected by MPTP are RICTOR and its regulation of ribosomal and mitochondrial proteins, as well as L-DOPA and DA receptor signaling (**Table 1**).

Furthermore, in the various brain areas examined, physical exercise affected sets of mRNAs that are regulated by the upstream regulators CREB1, RICTOR, L-DOPA and dexamethasone. These regulators overlap to some extent with the upstream regulators for the MPTP-regulated mRNAs as mentioned above. However, the top canonical pathways and biofunctions due to physical exercise are not epilepsy-related, but rather associated with 'mitochondrial dysfunction' and 'movement disorder' (**Table 2**).

The top regulators of the mRNAs differentially expressed due to physical exercise in MPTP-treated mice are L-DOPA, RICTOR, bicuculline/dalfampridine, and CREB1. The top canonical pathways and biofunctions enriched in exercised MPTP-treated mice are 'mitochondrial dysfunction' and 'protein synthesis' in the VTA and DL, 'G-protein signaling', 'movement disorder', 'seizures and cytoskeleton dynamics' in the VM and are related to (cell) death in the PFC (**Table 3**).

Of note, the predicted direction of effect of the top regulators RICTOR and L-DOPA is changed in the VTA, DL and VM of exercised MPTP-treated mice compared to exercised saline-treated mice. More specifically, the predicted direction of effect of RICTOR is

Table 1. Main effects of MPTP (MPTP-treated mice *without* physical exercise vs. saline-treated mice *without* physical exercise) per brain area. For each of the effects, the corresponding z-score, a predicted direction of the effect, is displayed as increased (z-score ≥ 2; Λ), no significantly predicted direction (=) or decreased (z-score ≤ -2; V). "N/A": no significantly enriched canonical pathways or biofunctions for a brain area (p≥0.05).

Brain area	Regulator(s)		Canonical pathway(s)		Biofunction(s)	
SN	CREB1	Λ	Neuropathic pain signaling	V	Seizures Cognition	Λ V
VTA	CREB1 RICTOR	Λ V	Protein synthesis	Λ	Movement disorder	=
DL	bicuculline/dalfampridine RICTOR	V V	Mitochondrial dysfunction Protein synthesis	= Λ	Epilepsy	=
VM	CREB1 bicuculline/dalfampridine	= V	N/A		Epilepsy	=
PFC	bicuculline/dalfampridine	V	N/A		N/A	
PPN	L-DOPA	=	Dopamine receptor signaling	=	Epilepsy Cognition	= V

Table 2. Main effects of physical exercise (saline-treated mice *with* physical exercise vs. saline-treated mice *without* physical exercise) per brain area. For each of the effects, the corresponding z-score, a predicted direction of the effect, is displayed as increased (z-score ≥ 2; Λ), no significantly predicted direction (=), decreased (z-score ≤ -2; V) or very much decreased (z-score ≤ -6; VV). "N/A": no significantly enriched canonical pathways or biofunctions for a brain area (p≥0.05).

Brain area	Regulator(s)		Canonical pathway(s)		Biofunction(s)	
SN	CREB1	=	Dopamine receptor signaling	=	Movement disorder Neurotransmission	V V
VTA	CREB1 RICTOR	= V	Protein synthesis	=	Seizures	V
DL	RICTOR	VV	Mitochondrial dysfunction Protein synthesis	= Λ	Mitochondrial dysfunction	=
VM	L-DOPA	=	N/A		N/A	
PFC	Dexamethasone	V	N/A		Cell proliferation / cancer Mortality	V Λ
PPN	L-DOPA	=	Axonal guidance signaling	=	Movement disorder Development of neurons	= V

Table 3. Main effects of physical exercise in MPTP-treated mice (MPTP-treated mice *with* physical exercise vs. MPTP-treated mice *without* physical exercise) per brain area. For each of the effects, the corresponding z-score, a predicted direction of the effect, is displayed as very much increased (z-score ≥ 6; ΛΛ), increased (z-score ≥ 2; Λ), no significantly predicted direction (=), decreased (z-score ≤ -2; V) or very much decreased (z-score ≤ -6; VV). "N/A": no significantly enriched canonical pathways or biofunctions for a brain area (p≥0.05).

Brain area	Regulator(s)		Canonical pathway(s)		Biofunction(s)	
SN	L-DOPA	=	N/A		N/A	
VTA	RICTOR	ΛΛ	Mitochondrial dysfunction Protein synthesis	= =	Mitochondrial dysfunction	=
DL	RICTOR	=	Mitochondrial dysfunction Protein synthesis	= =	Mitochondrial dysfunction	=
VM	L-DOPA	VV	G-protein signaling	V	Movement disorder Seizures Cytoskeleton dynamics Learning	V V Λ Λ
PFC	bicuculline/dalfampridine	Λ	RAR Activation	=	Cell proliferation / cancer Epilepsy (cell) death	= = V
PPN	CREB1	=	N/A		N/A	

(strongly) *decreased* in the VTA and DL after exercise in saline-treated mice, but is strongly *increased* and has no significant predicted direction in the VTA and DL of exercised MPTP-treated mice, respectively. Further, L-DOPA shows a strongly *decreased* predicted direction of effect in the VM of exercised MPTP-treated mice, whereas this direction of effect was *absent* after exercise alone.

4.4.6 The main molecular pathways regulated by physical exercise

To elucidate the main molecular pathways regulated by physical exercise in MPTP-treated mice, the mRNA sets regulated by the top upstream regulators L-DOPA (in the SN and VM, **Supplementary Tables 9** and **10**), RICTOR (in the DL and VTA, **Supplementary Tables 11** and **12**), bicuculline/dalfampridine (in the PFC, **Supplementary Table 13**) and CREB1 (in the PPN, **Supplementary Table 14**) were studied in greater detail and used to build molecular landscapes for each top upstream regulator in the various brain areas. Here, we provide a short description of each of these molecular landscapes. In the **Supplementary Information**, all landscapes are described in full detail.

The molecular landscapes of interacting proteins encoded by the L-DOPA-regulated mRNAs that are differentially expressed in the SN and the VM due to physical exercise in MPTP-treated mice, are shown in **Figures 4** and **5**, respectively. In the SN landscape, G-coupled receptor signaling (involving the proteins ARRB2 and GRP39), glucose uptake and signaling (SLC2A1), DA signaling (PPP1R1B) and reactive oxygen species (ROS) regulation (HSPB6, FTL, ROMO1) converge on the activation of ERK1/2 (ACKR1, EDNRB, GPR39, IER3, TP53), apoptotic pathways (CASP3, TP53), CREB1, and circadian clock regulation (PER1, DBP, CIART) (**Figure 4**). In the VM landscape, the main molecular

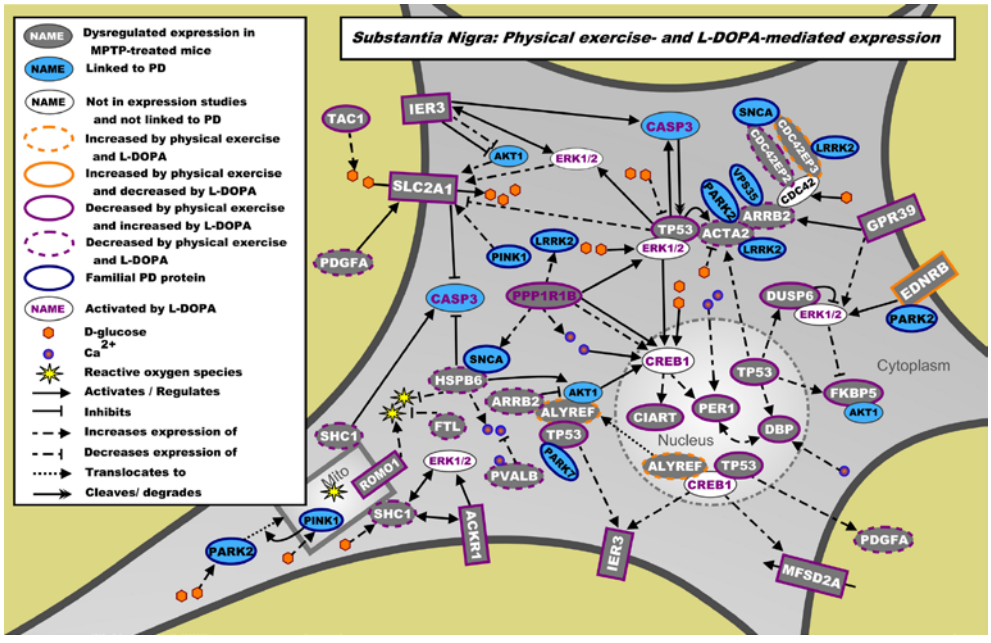

Figure 4. Landscape of proteins encoded by the mRNAs regulated by physical exercise and the upstream regulator L-DOPA in the SN. mRNAs differentially expressed in the SN due to physical exercise in MPTP-treated mice are shown in gray. Blue proteins are additional genes/proteins that are associated with PD through genetic and/or expression studies, whereas white proteins have no known link with PD. The direction of effect of physical exercise (measured) and L-DOPA (from literature) on the expression of these mRNAs is depicted through coloured borders. L-DOPA-activated proteins are shown with purple writing for the protein name, and familial PD proteins are shown with a blue border.

pathways are (interneuron-mediated) DA release (involving the proteins CHAT, DOC2B, SYN1 and TH) and signaling (DRD2, PPP1R1B), cannabinoid signaling (CNR1, FAAH) and neuropeptide signaling (PDYN, PENK, TAC1) that subsequently regulate/activate ERK1/2, CREB1 and CCND1 signaling. The latter is a cell cycle regulator that may also be involved in synaptic plasticity and learning[27] (**Figure 5**). Of note, almost all proteins in this landscape are regulated by physical exercise and L-DOPA in opposite directions.

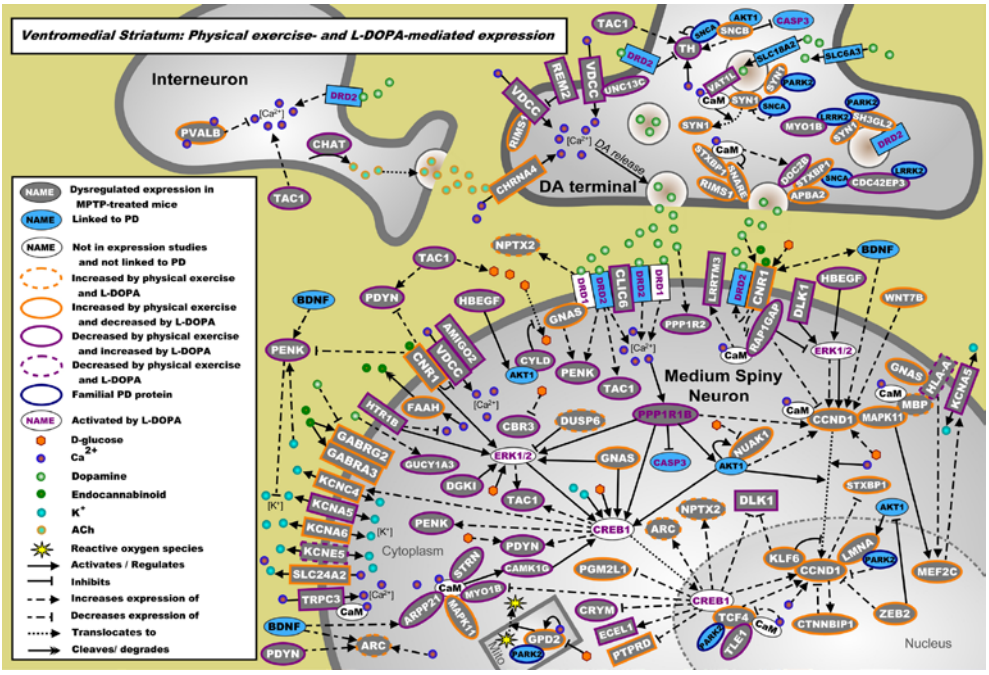

Figure 5. Landscape of proteins encoded by the mRNAs regulated by physical exercise and the upstream regulator L-DOPA in the VM. mRNAs differentially expressed in the VM due to physical exercise in MPTP-treated mice are shown in gray. Blue proteins are additional genes/proteins that are associated with PD through genetic and/or expression studies, whereas white proteins have no known link with PD. The direction of effect of physical exercise (measured) and L-DOPA (from literature) on the expression of these mRNAs is depicted through coloured borders. L-DOPA-activated proteins are shown with purple writing for the protein name, and familial PD proteins are shown with a blue border.

The RICTOR-regulated mRNAs that are differentially expressed in the DL and VTA due to physical exercise in the MPTP-treated mice encode proteins that are specifically involved in three cellular systems: the complex I-V of the electron transport chain, the 40S and 60S ribosomal subunits, and the proteasome (see **Supplementary Tables 11** and **12**). These are complexes that regulate cellular energy, protein translation and protein degradation, respectively (**Supplementary Figures 6** and **7**). Of note, physical exercise and RICTOR have an opposite effect on the expression of all differentially expressed mRNAs in the mitochondrial electron transport chain in the DL, whereas physical exercise and RICTOR exert the same direction of effect (i.e. a decreasing effect) on the expression of electron transport chain mRNAs in the VTA.

In the PFC, 8 out of 9 mRNAs differentially expressed due to physical exercise in MPTP-treated mice and regulated by bicuculline/dalfampridine have been linked to epilepsy (**Supplementary Table 13**). Immediate-early gene activation is one of the main processes regulated by these mRNAs e.g. via the early response genes/proteins FOS, FOSB and NR4A1, which in turn are regulated by insulin and low density lipoprotein. In **Supplementary Figure 8**, an overview of the interactions of the proteins encoded by these mRNAs and their regulation by bicuculline/dalfampridine and physical exercise is shown in a molecular landscape.

In the PPN, the proteins encoded by the mRNAs that were differentially expressed due to physical exercise in MPTP-treated mice and regulated by CREB1 have only a limited number of interactions in the built landscape (**Supplementary Figure 9**). Nevertheless, a few functional themes such as vascular remodeling, neuropeptide signaling, lipid metabolism, epilepsy/immediate early gene regulation and calcium signaling were identified, with CREB1 as their central regulator (**Supplementary Table 14**).

4.5 DISCUSSION

This study aimed to explore the molecular mechanisms underlying the beneficial effects of physical exercise on motor functioning in the MPTP-treated mouse model of PD. After validation of the model, through demonstrating significant nigral neuronal loss following MPTP treatment, the effects of a four-week physical exercise regimen on motor performance, and the accompanying molecular changes in multiple brain areas were assessed using behavioral tests and RNAseq analysis, respectively. The behavioral tests showed that physical exercise improved beam walk and rotarod performance in both MPTP-treated and control mice, but had a different and often opposite effect on the four tested open field parameters in these groups. Our RNAseq findings demonstrated that physical exercise in MPTP-treated mice mainly affects the expression of mRNAs involved in L-DOPA-mediated pathways in the SN and VM that regulate DA signaling, RICTOR-mediated pathways in the VTA and DL involved in energy metabolism and cellular stress[28, 29], and bicuculline/dalfampridine-mediated pathways in the PFC and CREB1-mediated pathways in the PPN that are both a measure of neuronal activity[30, 31]. To further elucidate the specific molecular mechanisms underlying the effects of physical exercise in MPTP-treated mice, the differentially expressed mRNAs regulated by these top regulators were integrated into molecular landscapes, depicting the main biological processes and signaling cascades affected.

Our animal model was validated by demonstrating a significant nigral DA neuronal loss following MPTP treatment. The observed moderate neuronal loss in the midbrain due to MPTP-treatment, i.e. a 29% reduction of TH-positive neurons in the SNpc without a statistical significant loss in the VTA, is in keeping with earlier studies using a similar

MPTP treatment regimen in 5-month old mice showing 33% loss in the SNpc and no significant loss in the VTA[32]. Other studies, in 8-10 week old mice, reported a neuronal loss of more than 50% in the SNpc[7, 33, 34]. Differences in level of neurodegeneration[35] and molecular effects[26] due to MPTP toxicity may be explained by age of the mice, MPTP dosing, and the duration between MPTP injection and sacrifice. We did not find a significant effect of physical exercise on the number of surviving DA neurons, but noted a trend towards an increased number of TH-positive neurons in the SNpc and an increased TH-positive fiber density in the DL and VM in MPTP-treated mice with physical exercise compared to MPTP-treated mice without exercise. From previous studies, it remains unclear whether physical exercise can protect against cellular loss in the MPTP mouse model. Preservation of SNpc neurons by physical exercise has been described before[34, 36], but the findings were inconsistent[7, 21].

Regarding motor function, MPTP treatment alone resulted in an increased activity in the open field, as reported before[32, 35, 37-39], but did not affect the performance on beam walk and rotarod. The effects of exercise on the motor performance included an improvement on the beam walk and rotarod in both saline and MPTP-treated groups. However, the effects of physical exercise on the open field parameters in saline-treated mice was either absent or opposite in MPTP-treated animals. These findings suggest that some effects of physical exercise may be dependent on the 'disease-state' (i.e. saline- or MPTP-treated). It could be argued, however, that the lack of effect of physical exercise in MPTP-treated mice on total walking distance and mean velocity (**Figure 2**) may be due to their MPTP-induced hyperactivity (**Figure 1**) that could have limited a further increase in motor performance due to physical exercise. Furthermore, the opposite effect of exercise on total movement time and mean angular velocity in MPTP-treated mice (**Figure 2**) compared to the effect of MPTP alone (**Figure 1**) suggests that physical exercise counteracts the effect of MPTP.

The RNAseq analysis showed that the level of overlap between MPTP-regulated genes and physical exercise-regulated genes differed between the brain areas studied and was particularly high in the PFC and VTA. These data suggest that in the PFC and VTA, physical exercise influences the processes affected by MPTP more directly than in the other areas in which more indirect mechanisms may prevail. Nevertheless, in all brain areas examined, the majority of overlapping genes (82-99%) were regulated in opposite directions by physical exercise compared to MPTP, suggesting counteracting effects of physical exercise on MPTP-regulated mechanisms. For example, the enrichment analysis of the overlapping genes in the PFC and DL (see **Supplementary Table 2**) shows a predicted activation of the top regulators dalfampridine, bicuculline and CREB1 – indicative for neuronal activation[30, 31] – whereas these are inactivated by MPTP.

The roles of the PD-related brain areas examined in this study can be summarized in a simplified basal ganglia circuitry model, wherein PPN, SN and DL are mainly involved in motor control, and the VTA, VM and PFC contribute particularly to the regulation of (complex) behavior and cognition (**Figure 6**)[40-45]. The top regulators – and to a lesser extent also the canonical pathways and biofunctions – regulated by physical exercise in the cognition-associated brain areas of MPTP-treated mice, showed highly significant predicted directions of effect, whereas these effects were less prominent in the motor-related areas. This implicates that, although physical exercise is able to improve motor function (as supported by the behavioral tests), it may also have strong effects on cognition and behavior. This is interesting from a therapeutic point of view, because non-motor symptoms in PD patients – including cognitive impairment, depression, pain and sleep disorders – are usually less responsive to dopamine replacement therapy and therefore treatment options are limited[46-48].

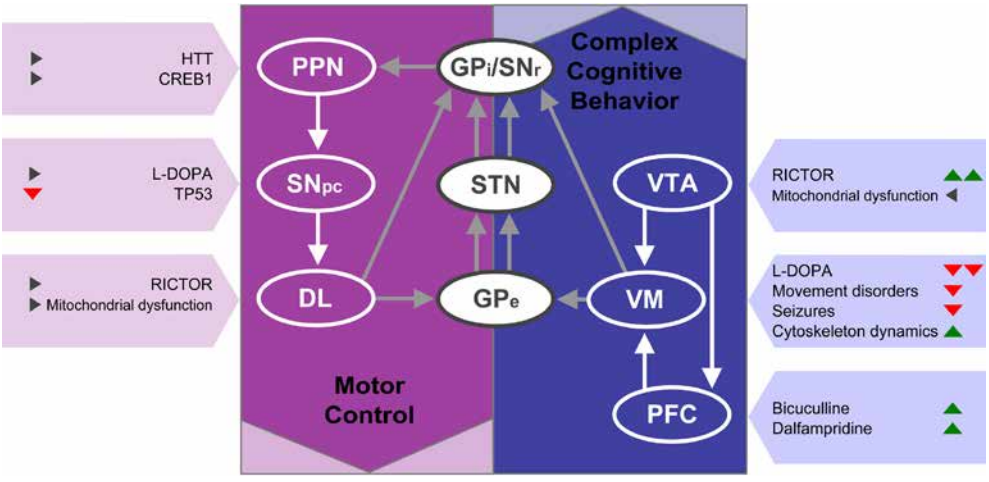

Figure 6. Overview of the brain areas analyzed, and the top upstream regulators and processes per area. The brain areas are shown in a simplified model of the basal ganglia circuitry. Green, red and gray triangles depict positive (>2), negative (<-2) or non-significant z-scores respectively from the enrichment analyses of the physical exercise-regulated mRNAs in MPTP-treated mice. DL, dorsolateral striatum; GPe, globus pallidus external; GPi, globus pallidus internal; PFC, prefrontal cortex; PPN, pedunculopontine nucleus; SNpc, substantia nigra pars compacta; SNr, substantia nigra reticularis; STN, subthalamic nucleus; VM, ventromedial striatum; VTA, ventral tegmental area.

Almost five decades after its introduction[4], the DA precursor L-DOPA is still the gold standard for symptomatic treatment to alleviate the motor symptoms of PD[5]. It should be noted, however, that chronic high-dose L-DOPA use is associated with complications such as dyskinesias[49-51]. Moreover, the effects of L-DOPA on non-motor symptoms in PD are even less predictable and L-DOPA use may even lead to deterioration of these symptoms, e.g. impaired reversal learning or motor sequence learning deficits[52-59]. It has been suggested that these adverse cognitive effects of L-DOPA may be due to a higher L-DOPA demand in the motor systems compared to cognitive areas, resulting in

a relative L-DOPA overdose in cognitive areas[60-62]. Therefore, novel 'add-on' treatments that can enable low-dose L-DOPA use and/or reduce the adverse effects of (long-term) L-DOPA use are desirable. In this respect, our study suggests that physical exercise is an attractive add-on treatment for PD, and that exercise combined with L-DOPA treatment may be more beneficial than treatment of PD patients with L-DOPA alone[9, 63]. Other findings that support this hypothesis include the reports indicating that physical exercise not only improves the motor symptoms of PD patients[8, 9], but also L-DOPA-induced dyskinesias in PD patients[64] and animal models[65], and cognitive function in PD patients[2, 15, 16].

Considering the above, it is worth noting that our landscapes revealed that physical exercise and L-DOPA regulate similar pathways in the SN and VM – often in an opposite direction – and that most of these pathways have been linked to sleeping problems (SN) and cognitive and/or motor dysfunctioning (VM) in PD. For example, the expression of clock proteins was affected by physical exercise and L-DOPA in the SN, a brain region known to be involved in the regulation of REM sleep[66, 67] and causing circadian rhythm irregularities when damaged by MPTP[68, 69]. Further, the use of L-DOPA can disturb REM sleep[70] and result in a delayed sleep onset in PD patients, which suggests an uncoupling of sleep and circadian regulation[71]. On the other hand, physical exercise can improve circadian rhythm regulation[72-74] and may therefore serve as a complementary therapy to strengthen circadian function in PD, as suggested earlier[75].
In the VM, both physical exercise and L-DOPA regulate DA, neuropeptide and endocannabinoid signaling, but in opposite directions. L-DOPA treatment results in sustained DA signaling in the striatum and can disrupt DA and (endo)cannabinoid receptor crosstalk[76, 77]. In contrast, physical exercise may rebalance DA signaling after sustained L-DOPA treatment (by reducing PPP1R1B activation)[65], attenuates depression-like behavior by decreasing the expression of neuropeptides[78] and activates the endocannabinoid system[79-81]. In turn, the endocannabinoid system modulates synaptic (DA) transmission in the striatum of PD patients[82-84], restores homeostasis following DA depletion[85, 86] and exerts beneficial effects on cognition, mood and nociception[80]. Therefore, physical exercise seems to exert a positive effect on the regulation of DA, neuropeptide and endocannabinoid signaling. Moreover, these three signaling pathways are not only associated with L-DOPA-induced dyskinesia[87-92], a process that is mainly due to dysregulation in the DL, but are also involved in regulating VM-associated cognitive functions and behaviors[78, 93-98], supporting the notion that the anatomical and neurophysiological boundaries of the striatal domains regulating control of movement (DL) and (more) cognition-related processes (VM) may functionally overlap[99, 100].

In summary, the molecular pathways that are regulated in the SN and VM by both physical exercise and L-DOPA can be directly linked to clinical features of PD.

Interestingly, the overall effects of physical exercise on these pathways seem to particularly improve the motor and behavioral clinical phenotype, whereas (chronic) L-DOPA-treatment can also cause adverse effects. Moreover, to our knowledge, physical exercise exerts – although it may counteract some L-DOPA-regulated pathways – no adverse effects on PD patients. To confirm the positive effects of physical exercise on cognitive function, future physical exercise studies in PD animal models and patients should include cognitive tests, e.g. reversal learning tasks. Furthermore, these studies should aim at further elucidating the molecular pathways underlying physical exercise in relation to (chronic) L-DOPA treatment in animal models.

Taken together, our findings provide further evidence that physical exercise improves motor function in PD, while it also affects the regulation of non-motor brain areas of MPTP-treated mice. We found that physical exercise and L-DOPA exert opposite effects on molecular pathways in several PD-associated brain areas, including those involved in sleeping and cognitive function. Overall, the present study suggests that physical exercise has therapeutic potential, not only to improve motor function but it may also improve non-motor symptoms of PD – and perhaps even alleviate detrimental effects associated with (chronic) L-DOPA use.

4.6 ACKNOWLEDGEMENTS

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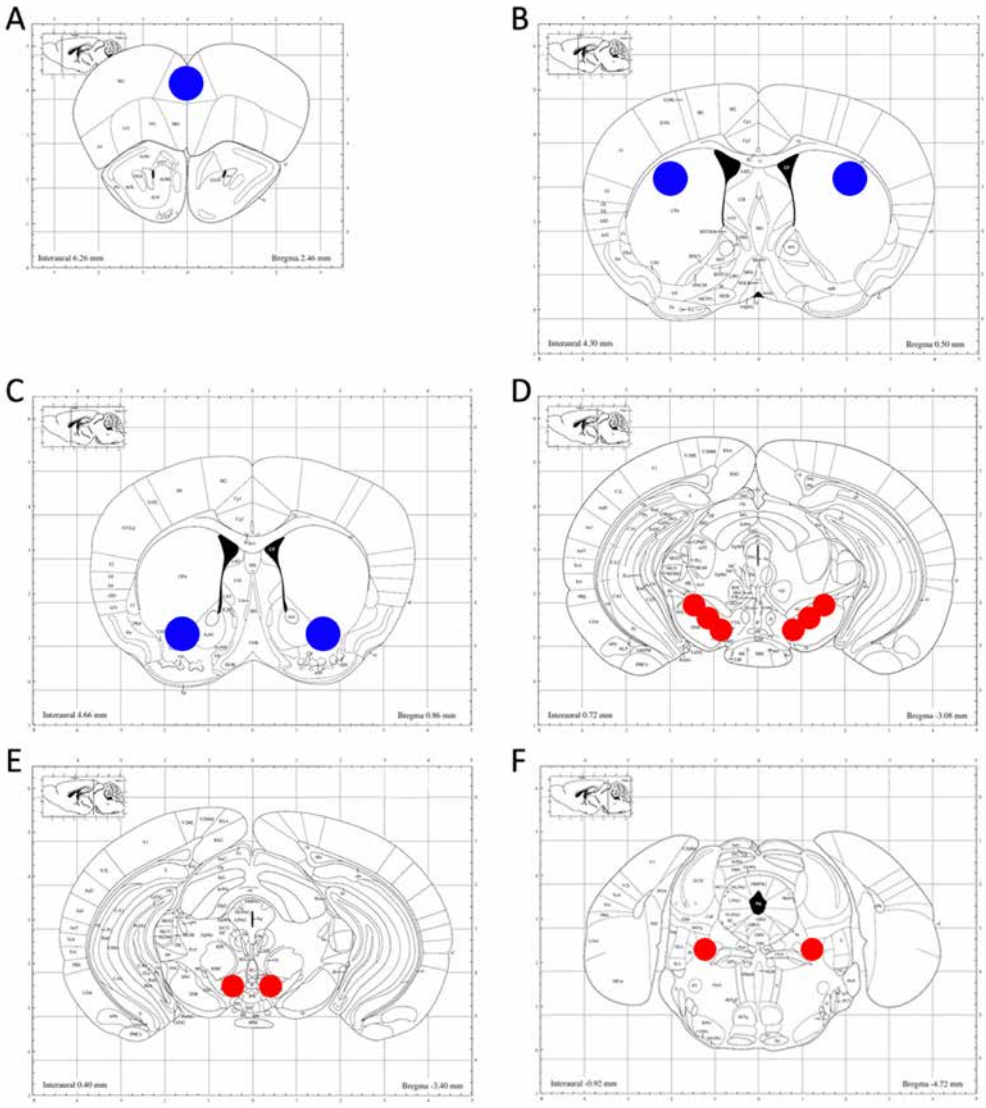
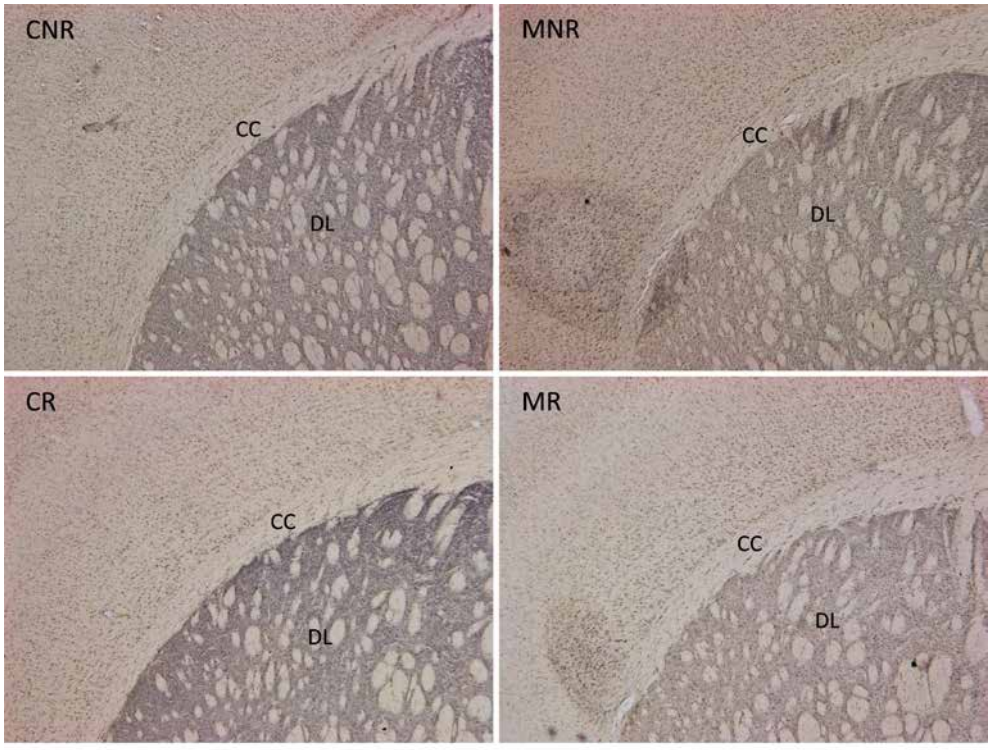
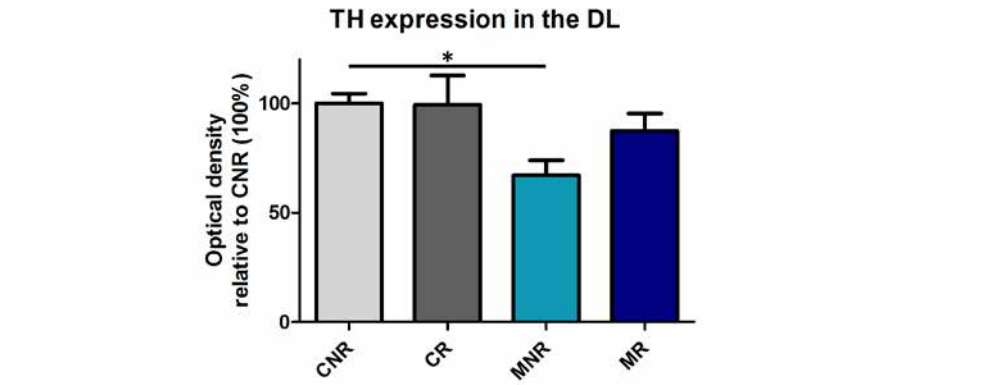
4.8 SUPPLEMENTARY INFORMATION

CONTENTS

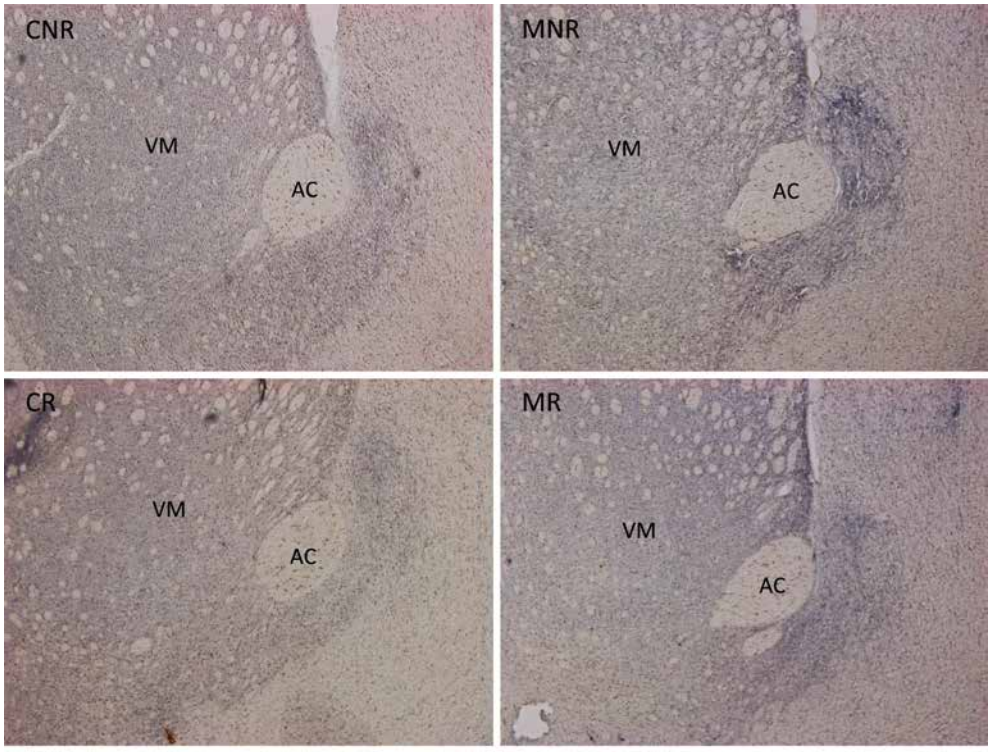


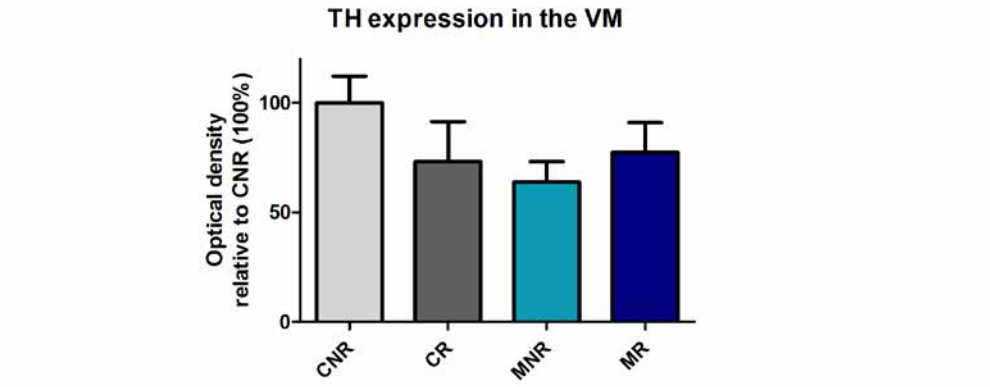
Supplementary Figure 1. Punching locations per brain area. For each brain area – PFC (A), DL (B), VM (C), SN (D), VTA (E) and PPN (F) – the punching locations are visualized in a cross section adapted from the Paxinos mouse brain atlas[1]. Punching locations with a punching needle of 0.5mm are shown with red circles, and blue circles indicate the location with 0.75mm punch needles.



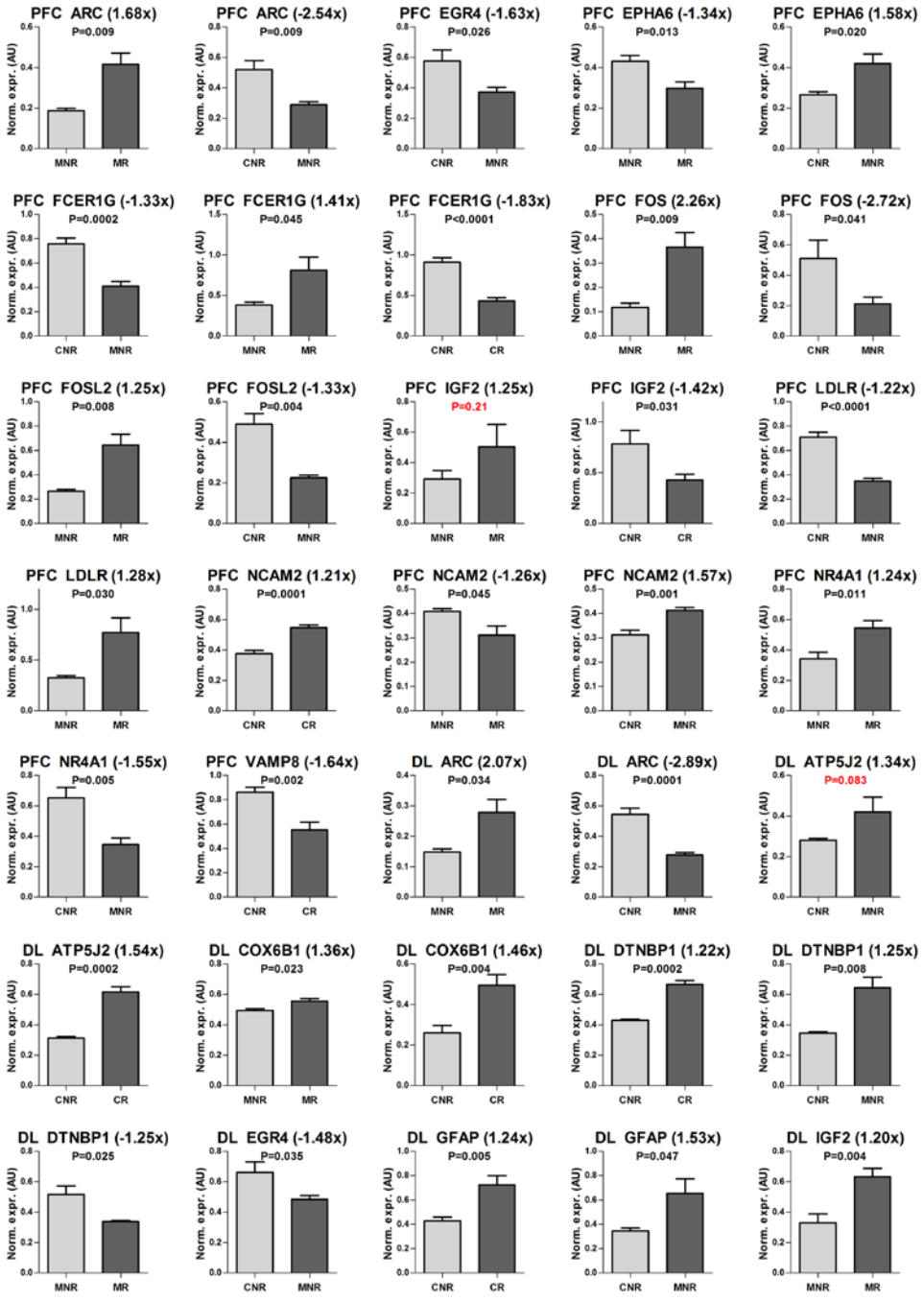


Supplementary Figure 2. Optical density of fibers in the DL. The upper panel shows a representative picture for each of the four treatment groups and the lower panel shows the optic density in the DL per treatment group. *P<0.05, means ± SEM, n=5 for CNR and MR and n=4 for CR and MNR. CC, corpus callosum; DL, dorsolateral striatum.

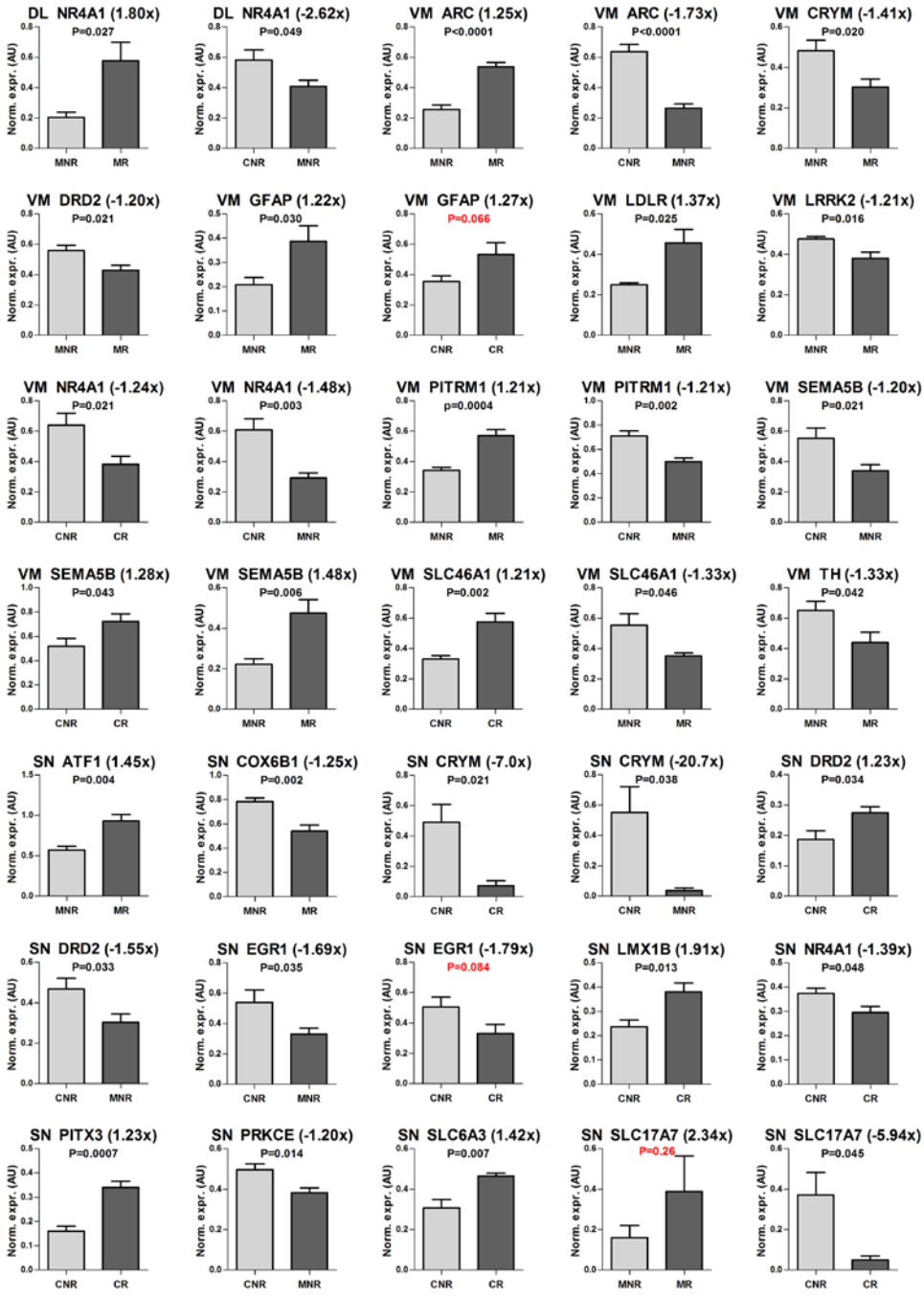




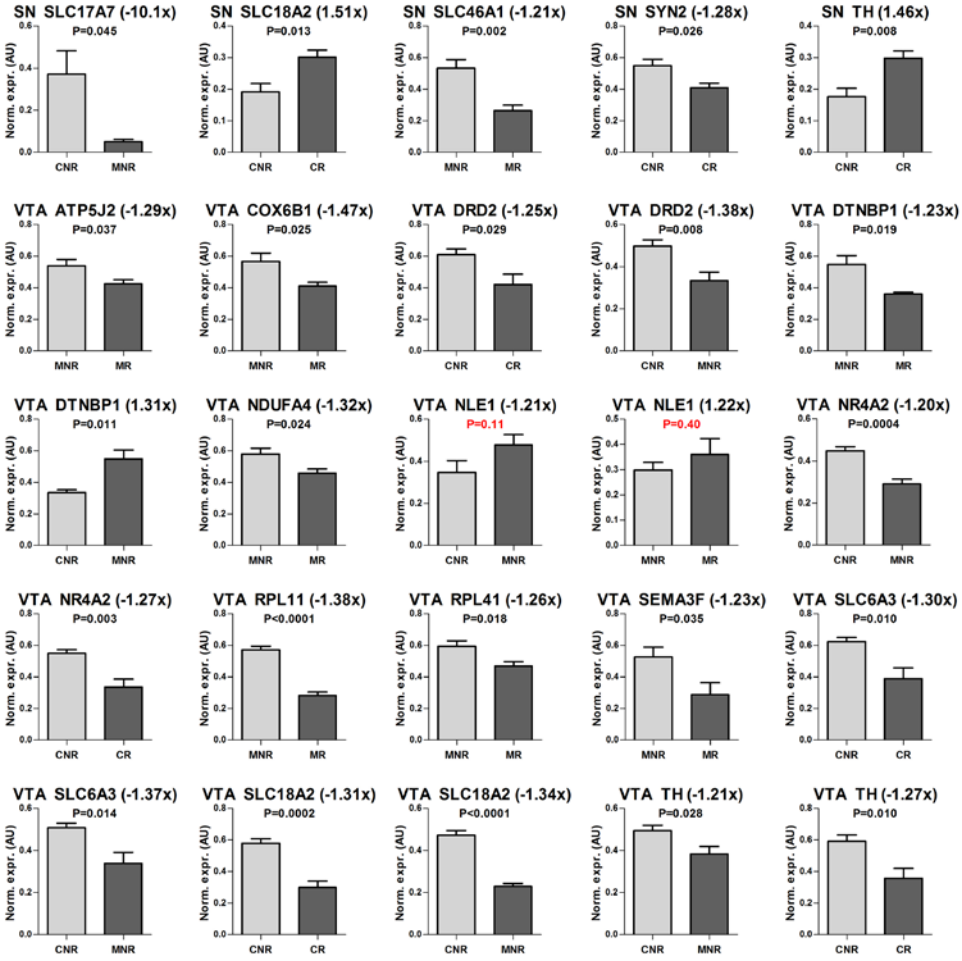
Supplementary Figure 3. Optical density of fibers in the VM. The upper panel shows a representative picture for each of the four treatment groups and the lower panel shows the optic density in the VM per treatment group. Means ± SEM, n=5 for CNR and MR and n=4 for CR and MNR. AC, anterior commissure; VM, ventromedial striatum.



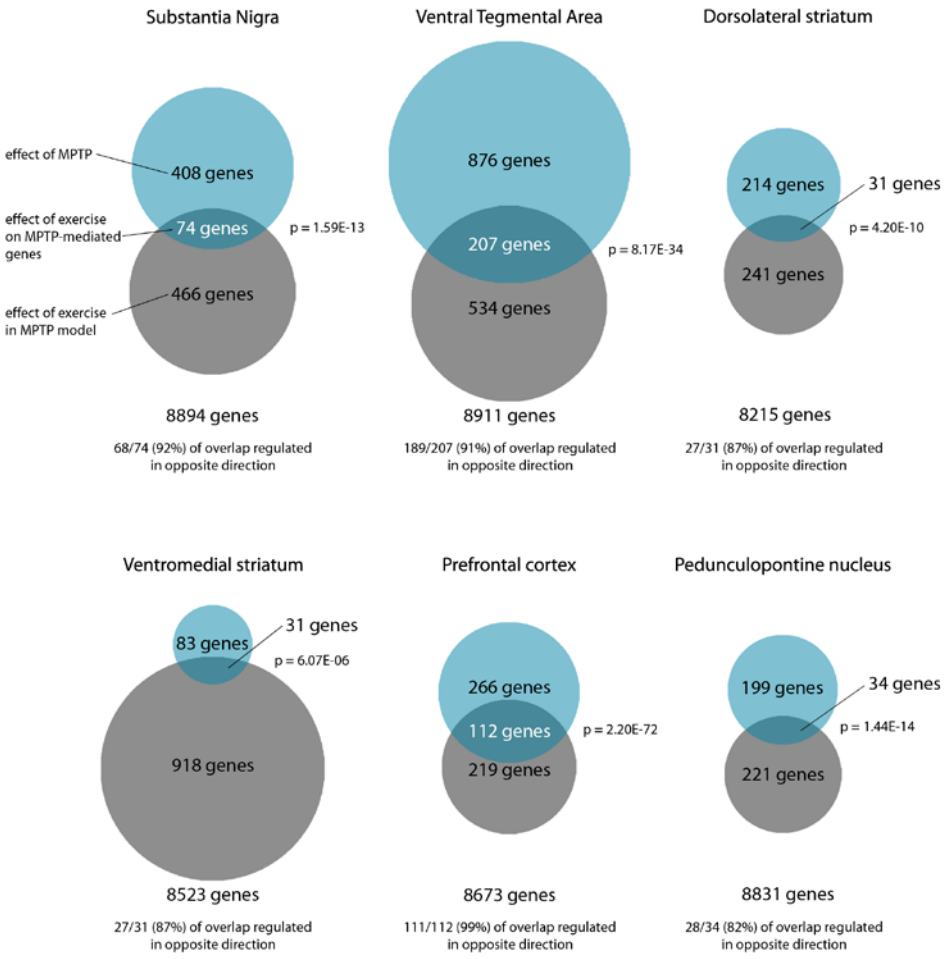
Supplementary Figure 4 (part 1/3). Validation of the RNAseq data using qPCR.



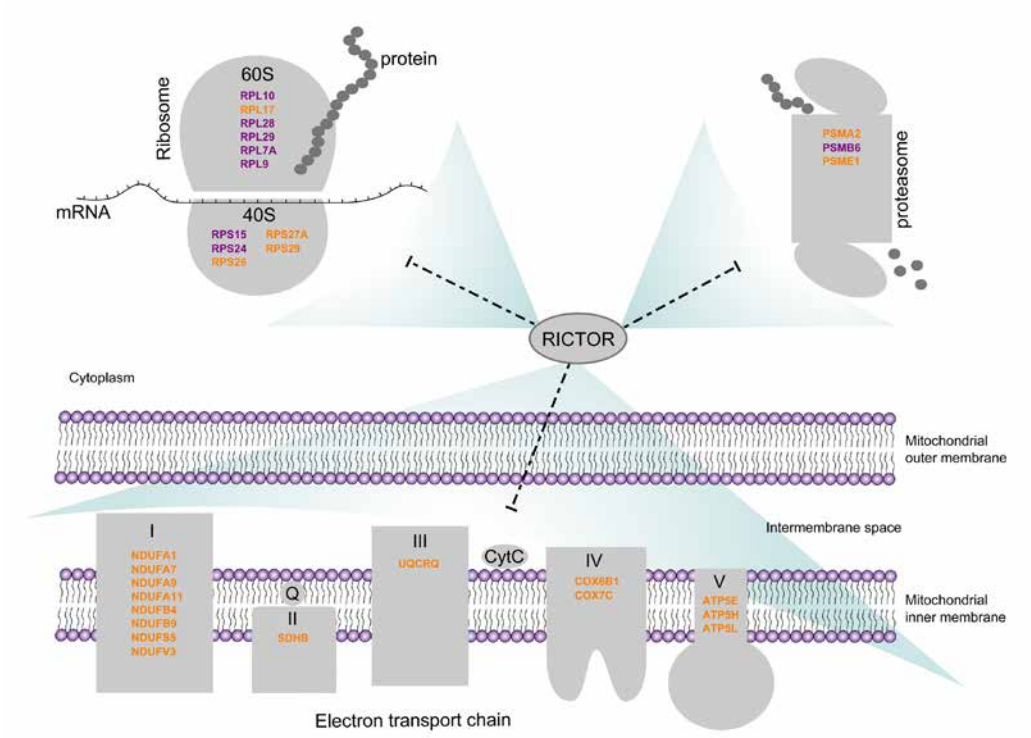
Supplementary Figure 4 (part 2/3). Validation of the RNAseq data using qPCR.



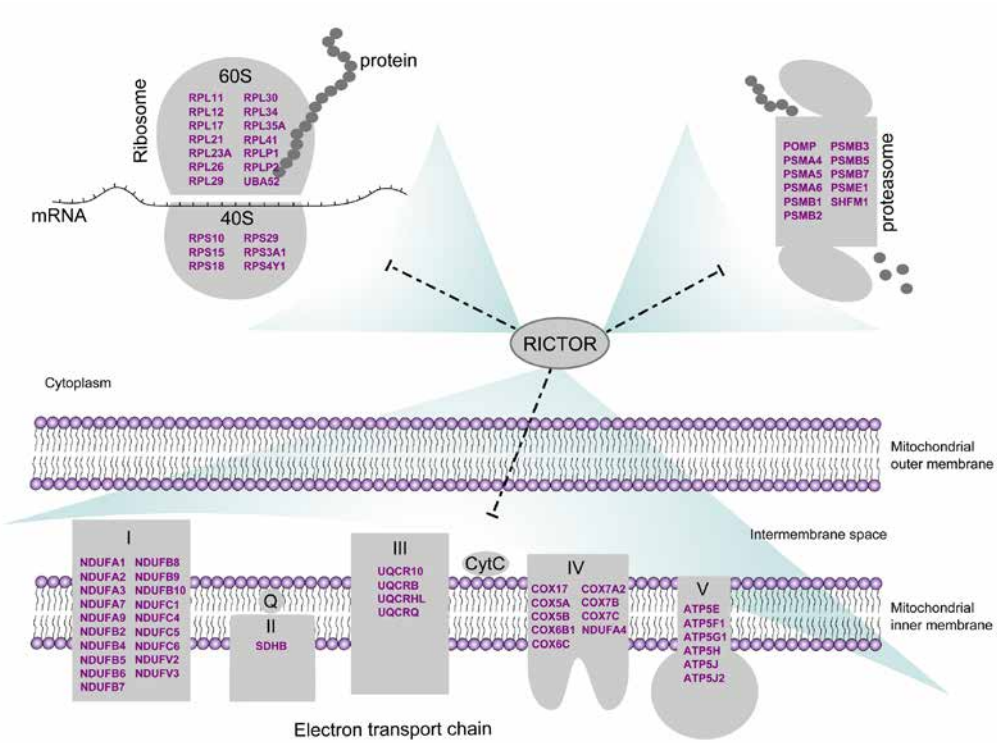
Supplementary Figure 4 (part 3/3). Validation of the RNAseq data using qPCR. The fold change of each mRNA in the RNAseq dataset and the brain area (PFC, DL, VM, SN, VTA) are shown above each graph, with the expression levels as measured by qPCR shown underneath. Expression levels are normalized to ACTB and YWHAZ and shown in arbitrary units (AU). Mean+SEM. The p-values are indicated in each graph (Student's T-test).



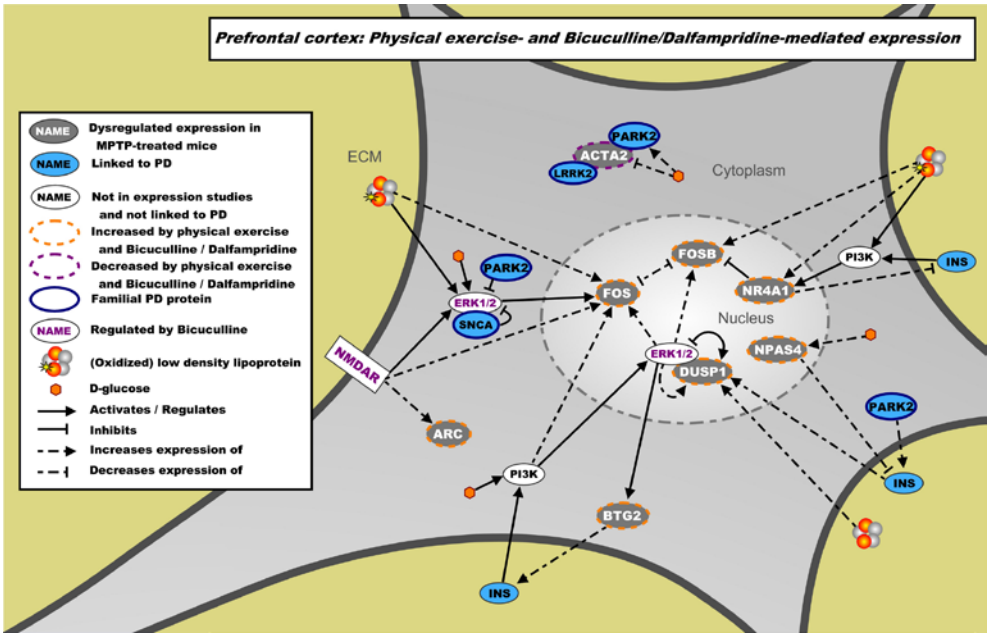
Supplementary Figure 5. The effect of physical exercise on MPTP-mediated genes. Per brain area the number of differentially expressed genes due to MPTP alone (in blue) or due to exercise in MPTP-treated mice (in gray), and their overlap are shown. The chance of observing this overlap is calculated with the hypergeometric distribution test and shown next to the overlapping area. Below the blue and gray circles, the total number of unique genes detected by RNAseq for each brain area is shown and also the number and percentage of overlapping genes that is regulated in opposite direction by MPTP and exercise in MPTP-treated mice.



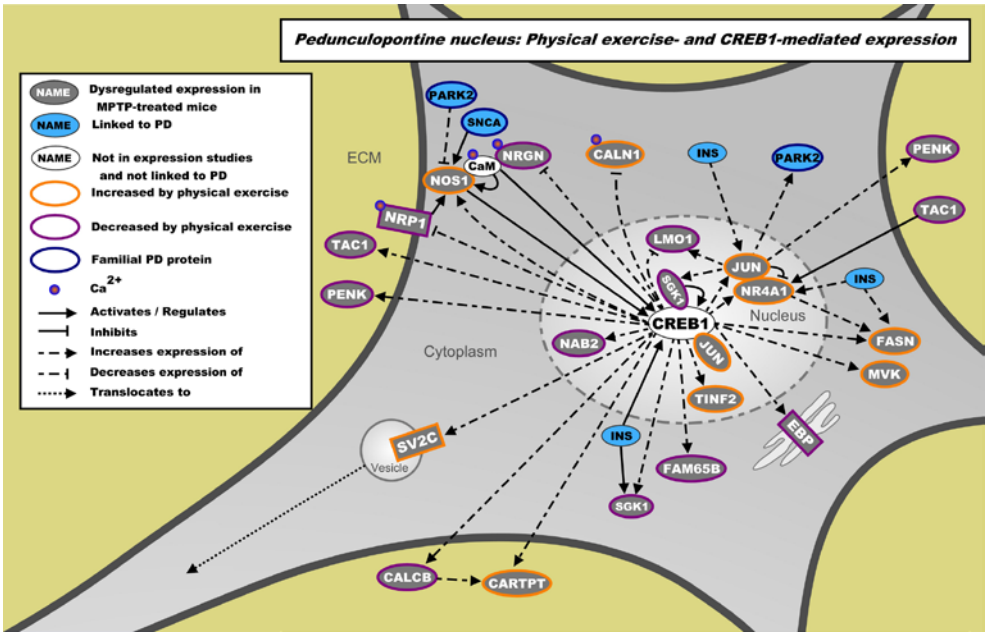
Supplementary Figure 6. mRNAs differentially expressed in the DL due to physical exercise in MPTP-treated mice and regulated by RICTOR. The expression of the purple mRNAs is decreased by both physical exercise and RICTOR. The expression of orange mRNAs is increased by physical exercise and decreased by RICTOR.



Supplementary Figure 7. mRNAs differentially expressed in the VTA due to physical exercise in MPTP-treated mice and regulated by RICTOR. The expression of the purple mRNAs is decreased by both physical exercise and RICTOR.



Supplementary Figure 8. mRNAs differentially expressed in the PFC due to physical exercise in MPTP-treated mice and regulated by Bicuculline/Dalfampridine. mRNAs differentially expressed in the PFC due to physical exercise in MPTP-treated mice are shown in gray. Blue proteins are additional genes/proteins that are associated with PD through genetic and/or expression studies, whereas white proteins have no known link with PD. The direction of effect of physical exercise (measured) and Bicuculline/Dalfampridine (from literature) on the expression of these mRNAs is depicted through coloured borders. Bicuculline-regulated proteins are shown with purple writing for the protein name, and familial PD proteins are shown with a blue border.



Supplementary Figure 9. mRNAs differentially expressed in the PPN due to physical exercise in MPTP-treated mice and regulated by CREB1. mRNAs differentially expressed in the PPN due to physical exercise in MPTP-treated mice are shown in gray. Blue proteins are additional genes/proteins that are associated with PD through genetic and/or expression studies, whereas white proteins have no known link with PD. The direction of effect of physical exercise (measured) on the expression of these mRNAs is depicted through orange (increase) or purple (decrease) borders. Familial PD proteins are shown with a blue border.

Supplementary Table 1. Primers used for validation of the RNAseq data by qPCR.

Gene	FW/RV + gene location	Sequence (5' to 3')
ABCA1	FW3607-3626	TCCTTGGGGACAGAATTGCC
	RV3801-3779	TCTGAGAAACACTGTCCTCCTTT
ACTB	FW1055-1079	AAGATCAAGATCATTGCTCCTCCTG
	RV1228-1209	CGCAGCTCAGTAACAGTCCG
ARC	FW1086-1105	ACCACTCGACCAGTTCCTCT
	RV1267-1248	CCTGCACTTCCATACCCCTC
ATF1	FW724-745	TGGTTGTACAGACTGCATCAGG
	RV821-802	AGGAGAAGTCATCACCACGG
ATP5J2	FW224-244	CATCAACGTTCGGAAAGGCAG
	RV382-363	TTATGCTCGGCCATGCAATG
COX6B1	FW223-242	CCGCTGTGAGAAGGCAATGA
	RV325-307	GGCTGAGACCCATGACACG
CREB1	FW728-748	ACATTGCCATTACCCAGGGAG
	RV921-901	TGAGGCAGCTTGAACAACAAC
CRYM	FW793-812	GGTGCTGTATGTGGACTCCC
	RV953-934	CCACTGCCATCCCCAAAGAT
DRD2	FW891-909	ACAGGCGGAGAATGGATGC
	RV1045-1026	GCTATGTAGACCGTGGTGGG
DTNBP1	FW603-625	AAAGTAAGAGGAAGGAGCTTGAA
	RV723-704	TCGAAGAACTTCTGCCGCTC
EGR1	FW570-590	CCTGACCACAGAGTCCTTTTC
	RV684-665	GAAGCGGCCAGTATAGGTGA
EGR2	FW512-531	GTGGCGGGAGATGGCATGAT
	RV660-640	GGGTACTGTGGGTCAATGGAG
EGR4	FW271-289	TCCTGGAGGCGACTTCTTG
	RV457-437	AGACATGAGGTTGAAGAGGGC
EPHA6	FW727-747	AAATGGGTGGGATGCCATTAC
	RV855-836	GCAGCATCACGAGAGATCCA
EPOR	FW717-736	GGACACAAAGGGTGGAGGTC
	RV842-823	TCCAGAATCCGCTGAAGCTC
ETS1	FW901-920	ACTGTGTGCCCTGGGTAAAG
	RV1083-1064	TGCTCGATACCGTAGCTGAT
FCER1G	FW434-454	CTCTGTGCTTTGAAGGTTGGC
	RV601-582	GAGTCGAGGATCAGGGAAGG
FOS	FW624-644	CAGATACACTCCAAGCGGAGA
	RV779-760	CTGGGAAGCCAAGGTCATCG
FOSL2	FW456-475	CCTATCCACGCTCACATCCC
	RV593-571	GAGACAGCTGCTCATCTCTCCTT
GFAP	FW808-827	TGGCCACCAGTAACATGCAA
	RV995-976	CTCTAGGGACTCGTTCGTGC
IGF2	FW903-922	CAAACGTCATCGTCCCCTGA
	RV1074-1055	TGTGGGACGTGATGGAACTG
LDLR	FW2591-2610	CCTATTGCACTGGTTGCCCT
	RV2737-2718	ATCCTGGCTTCGGCAAATGT
LMX1A	FW1199-1218	CCCTATGGTGCTGAACCTCT
	RV1326-1306	TCAATGGGGTTTCCCACTCTG
LMX1B	FW810-829	GGGCCAAGAGGTTCTGTCAA
	RV972-953	GGAGTCGTTCCCTGGCATTT

Supplementary Table 1. (continued)

Gene	FW/RV + gene location	Sequence (5' to 3')
LRRK2	FW591-611	CCAAAAACTGGGATGCAAAGC
	RV778-759	CATTGCTGCATGTAACCGCC
NCAM2	FW1849-1868	AGTACGCTCCCATGGAGTTC
	RV2043-2024	AAACTCTTGCCACTGCTTGG
NDUFA4	FW204-224	AGATGTCAGCTGGGACAGAAA
	RV388-369	GTGCGGATGGCTTCTGAAAG
NLE1	FW1074-1093	CTCTGGCTCAGACGACTTCA
	RV1265-1247	GAAGCTAGGTACTTGCCCG
NR4A1	FW1590-1609	AACATCCTGGCCTTCTCACG
	RV1691-1673	GAGCCCGTGTCGATCAGTG
NR4A2	FW1213-1232	TCCCTCCAATGAGGGTCTGT
	RV1319-1300	GCACCGTGCGCTTAAAGAAA
NR4A3	FW1322-1341	TTCTGACGGCCTCCATTGAC
	RV1471-1451	CAGCAGTGTTTGACCTGATGG
PITRM1	FW2536-2555	AATTGGTGACAGACCCCACC
	RV2652-2633	GGTCTGGATCAGCATACGGG
PITX3	FW452-470	CGTGCGGGTGTGGTTCAAG
	RV580-561	TACACCTCCTCGTAGGGTGG
PRKCE	FW2292-2311	TGTTCCAGATTCAGCGGTCC
	RV2407-2385	CAAATCCCTGTAGATCACTCCGT
PTGS2	FW1363-1382	TGGGGGAAGAAATGTGCCAA
	RV1523-1502	CAGCCATTTCCTTCTCTCCTGT
RPL11	FW351-371	ACACATTGACCTGGGCATCAA
	RV492-473	GCTGATTCTGTGTTTGGCCC
RPL41	FW245-263	CTGTGTGCTGCCATCGGTA
	RV350-330	GCAGAGGGACTGTTTTGGTTG
SEMA3F	FW1002-1021	CATCTGCCTCAACGATGACG
	RV1200-1179	AGAGCCTGAAGAGGTAAAGACA
SEMA5B	FW2240-2259	GGGAGGAGCGGTTCTGTAAT
	RV2411-2392	CAGGTCTTGAACTCCACGCC
SERPING1	FW1121-1140	TTGAAGGCCAAGGTGGGACA
	RV1299-1280	GGCATCGTCAGGTAAGTGGG
SLC17A7	FW1251-1270	CTTTTTGCGCAGTCGTCACA
	RV1433-1413	ACGTTAAACCCAGAGATGGCA
SLC18A2	FW1544-1474	GGGGTATGCTATCGGTCCCT
	RV1633-1614	TAATGGGGCAGTTGTGGTCC
SLC46A1	FW1151-1171	GTTCACAGGGTACGGATTGCT
	RV1333-1314	TTCAGAGTGGCCGGGTAGAT
SLC6A3	FW1835-1854	GGCTGGATCATTGCCACATC
	RV2003-1985	AATGGCGCAGCGTGAATTG
SYN2	FW945-964	CACCGAGAGATGCTTACGCT
	RV1084-1065	TAAGTTTGGGTGAGGGCCAC
TH	FW964-983	CACCTATGCACTCACCCGAG
	RV1121-1102	CCAGTACACCGTGGAGAGTT
VAMP8	FW342-362	TTGGAAGCCACGTCTGAACAC
	RV525-505	GAGGAGTAGGGTGGGATGGAA
YWHAZ	FW1176-1197	GCAAAAACAGCTTTCGATGAAG
	RV1345-1326	GCCGGTTAATTTTCCCCTCC

Supplementary Table 2. Enrichment analysis of MPTP-mediated mRNAs that are regulated in the opposite direction by exercise in the MPTP model (see also Supplementary Figure 5). Shown are the results of the analyses in the SN (68 genes), VTA (189 genes), DL (27 genes), VM (27 genes), PFC (111 genes) and PPN (28 genes). The top 10 upstream regulators, top 5 canonical pathways and top 5 annotations of the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are displayed, as well as their respective z-score, p-value and number of genes involved (#). The z-scores are based on the effect of exercise in the MPTP-treated mice (MPTP + physical exercise vs. MPTP), which means that they are in the opposite direction due to MPTP alone (MPTP vs. Control). All p-values are calculated using the Fisher's Exact Test, and the p-values for the canonical pathways and the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are corrected for multiple testing using the Benjamini-Hochberg correction. For the category 'Molecular and Cellular Functions', only the annotations with a significant z-score (i.e. <-2 (in red) or >2 (in green)) are shown. Annotations with only 1 (target) gene were discarded.

(1) Upstream Regulators

SN				VTA				DL			
Upstream Regulator	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**
NR3C1	0.055	4.22E-04	8	Inosine	-2.433	1.96E-06	6	Dalfampridine	2.000	3.48E-08	4
HOXA11	-	5.61E-04	2	KDM5A	2.828	5.42E-06	8	Bicuculline	1.941	7.92E-08	4
HMGA1	-	6.70E-04	4	RICTOR	3.162	2.60E-05	10	MECP2	-	6.75E-06	4
NRG2	-	7.52E-04	3	Dexamethasone phosphate	-1.000	2.65E-05	4	2-amino-5-phosphonovaleric acid	-1.960	1.45E-05	4
PTPN11	-	8.21E-04	3	Mt1		1.65E-04	3	Tacedinaline	-	1.61E-05	2
Plerixafor	-	1.35E-03	2	MYC	-2.540	1.81E-04	19	MAPK1	0.200	2.63E-05	5
GLI1	-1.000	2.15E-03	4	E. coli B4 lipopolysaccharide	-2.343	2.17E-04	9	Caffeine	-	4.41E-05	3
TFAP2A	-	2.25E-03	3	STAT4	-2.630	2.22E-04	8	pCPT-cAMP	-	4.83E-05	2
Beta-estradiol	0.841	2.65E-03	12	Interferon beta-1a	-	2.86E-04	6	Atorvastatin	0.849	5.66E-05	4
Propylthiouracil	-	2.98E-03	3	ZFHX3	-	4.32E-04	5	HNRNPAB	-	7.07E-05	2

(2) Canonical Pathways

SN				VTA				DL			
Canonical pathway	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**
-				-				Nur77 Signaling in T lymphocytes	-	4.80E-02	2
								Calcium-induced T lymphocyte apoptosis	-	4.80E-02	2

(3) Diseases and Disorders

SN				VTA				DL			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Tumorigenesis of tissue	-	3.58E-02	60	Severity of renal lesion	-	4.33E-02	2	Unstable hemoglobin disease	-	5.09E-04	2
Cancer	0.371	3.58E-02	62	Complement component C1q deficiency	-	4.33E-02	2	Epileptic seizure	-	5.93E-04	5
Epithelial cancer	-	3.58E-02	59	Degeneration of renal tubule	-	4.33E-02	2	Inflammation of organ	-0.847	1.72E-02	8
Abdominal cancer	-	3.58E-02	58	Formation of renal-cell carcinoma	-	4.33E-02	2	Vasculitis	-	3.14E-02	2
Malignant solid tumor	-1.000	3.58E-02	61	Nephromegaly	-	4.33E-02	2	Movement disorders	-	4.27E-02	6

(4) Molecular and Cellular Functions

SN				VTA				DL			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
-				Adhesion of endothelial cells	-2.049	4.33E-02	8	Differentiation of cells	2.299	3.20E-02	8
				Quantity of heavy metal	2.191	1.32E-03	5				
				Inflammation of body region	2.091	1.20E-02	19				
				Expression of RNA	-2.277	4.10E-02	32				

Supplementary Table 2. Enrichment analysis of MPTP-mediated mRNAs that are regulated in the opposite direction by exercise in the MPTP model. (continued)

(1) Upstream Regulators

VM				PFC				PPN			
Upstream Regulator	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**
Trichostatin A	-0.458	2.46E-05	6	Dalfampridine	2.828	1.21E-12	8	Alpha-amanitin	-	9.74E-05	2
Actin	-	8.35E-05	2	Bicuculline	2.774	6.87E-12	8	CBX2	-	9.74E-05	2
Brd4	-	9.74E-05	2	GNB2	-	6.40E-07	5	POMC	-	1.33E-04	3
L-dopa	1.432	6.17E-04	5	GNB1	-	8.40E-07	5	Propylthiouracil	-	1.84E-04	3
PSEN1	-	6.51E-04	4	CREB1	2.591	9.44E-07	13	ASAH1	-	3.72E-04	2
MBTD1	-	7.40E-04	2	RNF20	-	1.11E-06	4	KMT2D	-	8.07E-04	3
APLN	-	9.93E-04	2	tacedinaline	-	1.73E-06	3	CRH	-	1.90E-03	2
GLI1	-	1.19E-03	3	morphine	0.629	2.58E-06	7	25-hydroxycholesterol	-	1.96E-03	2
ARNT2	-	1.31E-03	3	atorvastatin	2.019	3.52E-06	8	GnRH-A	-	2.63E-03	2
BRD2	-	1.33E-03	2	2-amino-5-phosphonovaleric acid	-1.942	3.61E-06	7	5-N-ethylcarboxamido adenosine	-	5.51E-03	2

(2) Canonical Pathways

VM				PFC				PPN			
Canonical pathway	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**
-				-				-			

(3) Diseases and Disorders

VM				PFC				PPN			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Toxic epidermal necrolysis	-	1.62E-02	2	Gastrointestinal adenocarcinoma	-	4.57E-06	69	Aggregation of T lymphocytes	-	1.67E-02	2
Lichen planus	-	1.62E-02	3	Abdominal carcinoma	-	4.57E-06	81	Epithelial cancer	-	1.67E-02	23
Stevens-Johnson syndrome	-	1.62E-02	2	Epilepsy	-	4.57E-06	14	Erythrocytosis	-	1.96E-02	2
Transverse myelitis	-	1.62E-02	2	Seizures	0.664	1.06E-05	15	Abdominal cancer	-	2.07E-02	22
Malaria	-	2.17E-02	2	Seizure disorder	0.744	2.43E-05	16	Digestive system cancer	-	2.56E-02	21

(4) Molecular and Cellular Functions

SN				SN				SN			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
-				-				-			

Supplementary Table 3. Enrichment analysis of mRNAs differentially expressed in the SN. Ingenuity annotations of genes differentially expressed by running alone (CNR vs. CR; 514 genes), MPTP alone (CNR vs. MNR; 482 genes) and by running in MPTP-treated mice (MNR vs. MR; 540 genes). The top 10 upstream regulators, top 5 canonical pathways and top 5 annotations of the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are displayed, as well as their respective z-score, p-value and number of genes involved (#). All p-values are calculated using the Fisher's Exact Test, and the p-values for the canonical pathways and the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are corrected for multiple testing using the Benjamini-Hochberg correction. For the category 'Molecular and Cellular Functions', only the annotations with a significant z-score (i.e. <-2 (in red) or >2 (in green)) are shown. Annotations with only 1 (target) gene were discarded.

(1) Upstream Regulators

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Upstream Regulator	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**
CREB1	1.922	3.44E-19	49	CREB1	2.325	1.58E-14	42	L-dopa	-0.669	2.20E-07	36
HTT	-0.138	6.80E-17	53	HTT	1.618	2.75E-12	45	HNF4A	-1.320	4.09E-06	56
Beta-estradiol	-0.310	9.94E-15	85	L-dopa	-0.903	2.91E-12	44	TP53	-2.340	5.70E-06	74
L-dopa	-1.778	2.23E-14	48	BDNF	-2.313	1.06E-09	25	R5020	-2.397	8.98E-06	9
BDNF	-2.747	1.97E-12	29	ATN1	-	1.16E-09	17	ARHGDIG	0.447	3.09E-05	5
Dopamine	-0.033	2.20E-11	17	HDAC4	-	4.12E-08	14	Benzene	0.453	3.28E-05	8
Forskolin	-1.524	1.09E-10	41	FGF2	-0.194	8.65E-08	24	PEBP4	-	3.91E-05	3
Nicotine	1.300	1.11E-10	21	NR3C1	-1.329	9.38E-08	35	EIF4E	-2.863	4.29E-05	12
FGF2	-1.038	3.49E-10	28	Okadaic acid	0.067	1.28E-07	12	ACVRL1	-	5.47E-05	6
EGR1	0.724	1.48E-09	19	Cocaine	-0.443	6.80E-07	15	Bardoxolone	0.059	7.83E-05	8

(2) Canonical Pathways

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Canonical pathway	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**
Dopamine receptor signaling	1.000	9.84E-04	11	Neuropathic pain signaling in dorsalhorn neurons	-2.714	2.87E-03	11	-			
VDR/RXR activation	-	9.84E-04	10	Cholecystokinin/gastrin-mediated signaling	-2.530	9.97E-03	10				
Neuropathic pain signaling in dorsalhorn neurons	-1.508	1.31E-03	11	Fcγ receptor-mediated phagocytosis in macrophages and monocytes	-1.667	1.34E-02	9				
Axonal guidance signaling	-	5.16E-03	23	UVC-induced MAPK signaling	-2.449	1.34E-02	6				
Serotonin signaling	-	8.63E-03	7	VDR/RXR activation	-	1.34E-02	8				

(3) Diseases and Disorders

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Movement disorders	-1.827	4.11E-15	88	Seizures	2.661	1.58E-10	41	Abdominal cancer	0.349	1.91E-03	395
Dyskinesia	-1.961	2.45E-13	60	Movement disorders	-0.599	1.06E-09	74	Cancer	0.794	1.96E-03	429
Neurological signs	-1.029	2.45E-13	62	Neurological signs	-0.600	1.06E-09	54	Abdominal neoplasm	0.593	2.18E-03	396
Disorder of basal ganglia	-2.621	1.64E-11	65	Dyskinesia	-	1.06E-09	52	Tumorigenesis	1.283	2.18E-03	406
Huntington's disease	-	1.47E-10	52	Huntington's disease	-	1.86E-09	49	Epithelial cancer	1.141	2.28E-03	399

(4) Molecular and Cellular Functions

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Neurotransmission	-2.398	4.11E-15	51	Learning	-3.135	1.06E-09	41	-			
Cognition	-2.156	1.14E-12	49	Cognition	-3.183	1.20E-09	43				
Development of neurons	-2.001	8.80E-12	65	Development of neurons	-2.474	1.91E-07	54				
Learning	-2.159	9.41E-12	45	Synaptic depression	-2.193	8.46E-07	18				
Disorder of basal ganglia	-2.621	1.64E-11	65	Memory	-2.804	1.49E-06	26				

Supplementary Table 4. Enrichment analysis of mRNAs differentially expressed in the VTA. Ingenuity annotations of genes differentially expressed by running alone (CNR vs. CR; 1113 genes), MPTP alone (CNR vs. MNR; 1083 genes) and by running in MPTP-treated mice (MNR vs. MR; 741 genes). The top 10 upstream regulators, top 5 canonical pathways and top 5 annotations of the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are displayed, as well as their respective z-score, p-value and number of genes involved (#). All p-values are calculated using the Fisher's Exact Test, and the p-values for the canonical pathways and the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are corrected for multiple testing by the Benjamini-Hochberg correction. For the category 'Molecular and Cellular Functions', only the annotations with a significant z-score (i.e. <-2 (in red) or >2 (in green)) are shown. Annotations with only 1 (target) gene were discarded.

(1) Upstream Regulators

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Upstream Regulator	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**
CREB1	1.731	6.71E-15	66	CREB1	2.414	4.16E-14	65	RICTOR	8.429	9.76E-53	75
HTT	-0.218	1.98E-12	73	MYCN	2.011	6.24E-12	38	ST1926	5.568	6.31E-17	120
ATN1	-	2.57E-12	28	L-dopa	-0.667	1.26E-11	70	KDM5A	5.099	6.75E-15	31
RICTOR	-3.479	5.41E-11	38	HTT	0.560	2.69E-11	71	HNF4A	-1.134	2.57E-14	31
Dopamine	1.365	2.22E-09	21	RICTOR	-5.088	7.94E-11	38	CD 437	5.204	4.93E-14	28
MYCN	2.397	1.61E-08	32	ATN1	-	6.43E-10	25	MYCN	-3.838	1.09E-11	52
APP	1.336	2.54E-08	72	VHL	-0.181	6.46E-10	24	5-fluorouracil	4.811	1.10E-10	19
SOD1	-	2.94E-08	27	HNF4A	1.708	2.35E-09	143	HTT	1	7.77E-10	37
L-dopa	-0.791	1.66E-07	59	Tretinoin	2.455	1.32E-07	103	interferon beta-1a		2.58E-09	12
EN1	-1.461	3.57E-07	7	HMGA1	-0.469	3.16E-07	22	sirolimus	4.980	6.00E-09	31

(2) Canonical Pathways

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Canonical pathway	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**
EIF2 signaling	0.832	1.30E-07	31	mTOR signaling	-	9.34E-05	27	Oxidative phosphorylation	-	4.54E-26	38
mTOR signaling	-	1.25E-04	26	EIF2 signaling	3.464	9.34E-05	26	Mitochondrial dysfunction	-	2.07E-21	41
Dopamine receptor signaling	-	1.92E-03	15	Regulation of eIF4 and p70S6K signaling	-	2.37E-02	18	EIF2 signaling	-	5.83E-18	38
Regulation of eIF4 and p70S6K Signaling	-	4.48E-03	19	Oxidative phosphorylation	-	3.56E-02	15	Regulation of eIF4 and p70S6K signaling	-	1.09E-02	15
Mitochondrial dysfunction	-	9.32E-03	21								

(3) Diseases and Disorders

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Seizure disorder	-2.654	1.44E-12	77	Dyskinesia	1.091	4.56E-08	85	Mitochondrial disorder	-	4.45E-09	25
Movement disorders	-0.639	1.72E-10	131	Movement Disorders	-0.814	4.56E-08	127	Mitochondrial respiratory chain deficiency	-	4.45E-09	17
Seizure	-2.543	9.74E-10	62	Disorder of basal ganglia	1.477	1.73E-07	98	Mitochondrial complex I deficiency	-	2.22E-06	11
Epilepsy	-1.890	1.46E-09	51	Neurological signs	0.928	1.73E-07	86	Neurological signs	-	5.47E-04	55
Neurological signs	0.926	1.85E-08	86	Neuromuscular disease	1.131	8.60E-07	106	Dyskinesia	-	6.75E-04	52

(4) Molecular and Cellular Functions

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Development of central nervous system	2.819	1.32E-05	77	Size of body	5.539	5.45E-04	86	Cell death of osteosarcoma cells	5.099	3.10E-10	26
Size of body	4.217	5.07E-05	62	Release of neurotransmitter	-2.233	1.03E-03	27	Cell death of cancer cells	4.669	4.02E-02	32
Release of neurotransmitter	-2.522	1.66E-04	73	Morbidity or mortality	-6.049	2.09E-03	210				
Morbidity or mortality	-2.150	2.16E-03	88	Organismal death	-6.217	3.28E-03	206				
Organismal death	-2.261	2.67E-03	645	Secretion of neurotransmitter	-2.164	3.28E-03	22				

Supplementary Table 5. Enrichment analysis of mRNAs differentially expressed in the DL. Ingenuity annotations of genes differentially expressed by running alone (CNR vs. CR; 607 genes), MPTP alone (CNR vs. MNR; 245 genes) and by running in MPTP-treated mice (MNR vs. MR; 272 genes). The top 10 upstream regulators, top 5 canonical pathways and top 5 annotations of the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are displayed, as well as their respective z-score, p-value and number of genes involved (#). All p-values are calculated using the Fisher's Exact Test, and the p-values for the canonical pathways and the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are corrected for multiple testing by the Benjamini-Hochberg correction. For the category 'Molecular and Cellular Functions', only the annotations with a significant z-score (i.e. <-2 (in red) or >2 (in green)) are shown. Annotations with only 1 (target) gene were discarded.

(1) Upstream Regulators

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Upstream Regulator	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**
RICTOR	-7.230	1.72E-42	60	Dalfampridine	-3.317	6.20E-15	11	RICTOR	-2.191	6.91E-22	31
MYCN	2.809	2.42E-18	35	Bicuculline	-3.233	5.49E-14	11	HNF4A	-	4.46E-10	54
HNF4A	-1.463	1.91E-12	96	RICTOR	-4.472	1.52E-12	20	Sirolimus	-1.171	1.36E-07	20
Guanidinopropionic acid	3.308	9.81E-09	11	CREM	-2.844	2.36E-09	13	5-fluorouracil	-0.954	1.93E-07	14
MAP4K4	-3.873	4.69E-08	15	Cocaine	-3.472	6.60E-09	13	IGF1R	2.813	2.04E-07	14
INSR	4.094	5.61E-08	27	Kainic acid	-1.318	1.53E-08	12	MYCN	-0.898	6.61E-07	14
PSEN1	-1.035	6.17E-08	28	PSEN1	-1.755	4.24E-08	18	HTT	-	2.20E-06	24
MAPT	-	8.06E-08	22	MYCN	1.136	7.82E-07	13	INSR	2.157	1.48E-05	16
IGF1R	2.853	2.02E-07	20	CREB1	-2.521	1.16E-06	19	Guanidinopropionic acid	1.633	1.56E-05	6
RRP1B	-	4.25E-07	13	2-amino-5-phosphonovaleric acid	2.559	1.34E-06	10	CD 437	-0.302	2.48E-05	11

(2) Canonical Pathways

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Canonical pathway	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**
Oxidative phosphorylation	-	3.46E-20	30	Oxidative phosphorylation	-	1.00E-04	10	Oxidative phosphorylation	-	1.91E-08	14
Mitochondrial dysfunction	-	1.93E-15	31	EIF2 signaling	2.333	4.78E-04	11	Mitochondrial dysfunction	-	3.91E-06	14
EIF2 signaling	3.771	1.95E-09	24	Mitochondrial dysfunction	-	2.06E-03	10	EIF2 signaling	-0.816	3.32E-05	13
								mTOR signaling	-	7.31E-03	10
								Regulation of eIF4 and p70S6K signaling	-	2.48E-02	8

(3) Diseases and Disorders

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Mitochondrial disorder	-	1.84E-02	14	Epileptic seizure	-	2.07E-07	16	Cell death of osteosarcoma cells	-	1.05E-02	10
Mitochondrial respiratory chain deficiency	-	1.84E-02	9	Epilepsy	-	3.29E-04	17	Mitochondrial respiratory chain deficiency	-	1.31E-02	7
Diamond-Blackfananemia	-	1.84E-02	5	Congenital anemia	-	3.29E-04	8	Mitochondrial complex I deficiency	-	2.49E-02	5
Congenital anemia	-	2.65E-02	9	Seizures	-	3.09E-03	18	Mitochondrial disorder	-	3.12E-02	9
				Congenital aplastic anemia	-	3.09E-03	6	Unstable hemoglobin disease	-	4.75E-02	2

(4) Molecular and Cellular Functions

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
-				-				-			

Supplementary Table 6. Enrichment analysis of mRNAs differentially expressed in the VM. Ingenuity annotations of genes differentially expressed by running alone (CNR vs. CR; 304 genes), MPTP alone (CNR vs. MNR; 114 genes) and by running in MPTP-treated mice (MNR vs. MR; 949 genes). The top 10 upstream regulators, top 5 canonical pathways and top 5 annotations of the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are displayed, as well as their respective z-score, p-value and number of genes involved (#). All p-values are calculated using the Fisher's Exact Test, and the p-values for the canonical pathways and the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are corrected for multiple testing by the Benjamini-Hochberg correction. For the category 'Molecular and Cellular Functions', only the annotations with a significant z-score (i.e. <-2 (in red) or >2 (in green)) are shown. Annotations with only 1 (target) gene were discarded.

(1) Upstream Regulators

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Upstream Regulator	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**
L-dopa	-1.460	7.09E-06	23	CREB1	-1.066	1.31E-07	14	L-dopa	-8.463	2.28E-45	118
Alpha-amanitin	-	1.83E-05	4	Dalfampridine	-2.236	3.25E-07	5	HTT	1.820	4.23E-23	87
APP	1.963	3.38E-05	25	Bicuculline	-2.177	7.92E-07	5	CREB1	-0.960	3.27E-22	74
EPHB2	-	1.35E-04	3	Trichostatin A	0.864	1.58E-06	13	ATN1	-	3.69E-16	31
CREB1	-0.117	2.38E-04	17	Alpha-amanitin	-	3.22E-05	3	Beta-estradiol	1.885	1.26E-15	135
ADRB	-1.480	2.65E-04	8	2-amino-5-phosphonovaleric acid	0.896	4.79E-05	6	BDNF	2.971	3.25E-15	45
MYOD1	1.450	4.08E-04	9	Forskolin	-1.422	1.95E-04	11	Dopamine	0.076	9.48E-15	26
HIF1A	-1.009	4.70E-04	13	G2535	-	2.03E-04	2	MAPT	-0.896	1.05E-13	39
KLF1	-	6.25E-04	4	N-lauroyl-L-phenylalanine	-	2.03E-04	2	APP	0.170	7.12E-12	74
SIGMAR1	-	8.43E-04	2	Lactacystin	-0.342	2.51E-04	6	REST	-2.148	2.02E-10	23

(2) Canonical Pathways

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Canonical pathway	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**
-				-				Gai Signaling	-2.000	6.37E-08	24
								Breast cancer regulation by Stathmin1	-	1.05E-07	31
								cAMP-mediated signaling	-0.365	1.10E-07	32
								Role of NFAT in cardiac hypertrophy	2.558	6.48E-05	25
								G-Protein Coupled Receptor Signaling	-	9.00E-05	23

(3) Diseases and Disorders

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
-				Epilepsy	-	6.30E-05	13	Movement disorders	-2.334	1.57E-28	169
				Epileptic seizure	-	2.52E-03	8	Disorder of basal ganglia	0.415	1.49E-24	129
				Seizures	1.172	3.16E-03	12	Seizure disorder	-2.791	4.66E-23	93
				Unstable hemoglobin disease	-	7.81E-03	2	Seizures	-2.841	3.00E-22	81
				Alphathalassemia	-	1.40E-02	2	Neuromuscular disease	0.314	9.11E-22	135

(4) Molecular and Cellular Functions

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
-				-				Microtubule dynamics	2.824	2.94E-21	164
								Organization of cytoskeleton	2.640	5.54E-21	106
								Organization of cytoplasm	2.640	9.30E-21	103
								Learning	2.055	1.44E-14	72
								Quantity of neurons	2.680	1.89E-10	62

Supplementary Table 7. Enrichment analysis of mRNAs differentially expressed in the PFC. Ingenuity annotations of genes differentially expressed by running alone (CNR vs. CR; 367 genes), MPTP alone (CNR vs. MNR; 378 genes) and by running in MPTP-treated mice (MNR vs. MR; 331 genes). The top 10 upstream regulators, top 5 canonical pathways and top 5 annotations of the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are displayed, as well as their respective z-score, p-value and number of genes involved (#).All p-values are calculated using the Fisher's Exact Test, and the p-values for the canonical pathways and the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are corrected for multiple testing by the Benjamini-Hochberg correction. For the category 'Molecular and Cellular Functions', only the annotations with a significant z-score (i.e. <-2 (in red) or >2 (in green)) are shown. Annotations with only 1 (target) gene were discarded.

(1) Upstream Regulators

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Upstream Regulator	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**
Dexamethasone	-2.765	9.42E-12	58	Dalfampridine	-3.464	2.06E-14	12	Cycloheximide	1.398	6.15E-10	23
MYC	-1.471	1.65E-10	42	Bicuculline	-3.384	2.28E-13	12	Dexamethasone	0.679	1.43E-09	54
HRAS	-0.616	1.53E-09	29	CREB1	-3.969	1.15E-08	28	Bicuculline	2.946	1.67E-09	9
KRAS	2.829	1.71E-08	21	Pyridaben	3.000	1.29E-07	9	Dalfampridine	2.828	6.90E-09	8
TGFB1	-1.697	3.10E-08	51	Maneb	-2.256	4.26E-07	13	HRAS	0.425	1.22E-08	27
RICTOR	3.769	3.11E-08	18	6,7-dinitroquinoxaline-2,3-dione	3.000	4.32E-07	9	HTT	2.391	1.91E-08	30
Methylprednisolone	-0.652	3.33E-08	26	GnRH-A	-1.732	7.54E-07	4	TGFB1	0.708	2.33E-08	50
Tretinoin	-2.546	5.18E-08	46	Kainic acid	-2.946	1.38E-06	9	F2	1.657	5.06E-08	17
BMP7	-1.307	1.32E-07	12	Atipamezole	-2.901	1.79E-06	12	Atorvastatin	2.339	8.39E-08	15
SMARCA4	-2.892	1.43E-07	26	2-amino-3-phosphonopropionic acid	-	3.43E-06	3	N-lauroyl-L-phenylalanine	0	1.49E-07	4

(2) Canonical Pathways

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Canonical pathway	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**
-				-				RAR Activation	-	4.59E-02	11

(3) Diseases and Disorders

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Advanced malignant tumor	-1.854	2.87E-04	41	Epileptic seizures	-	6.87E-08	20	Cancer	0.101	9.84E-04	280
Metastasis	-1.854	4.77E-03	33	Epilepsy	0.625	1.46E-07	27	Malignant solid tumor	0.377	9.84E-04	276
Hypersensitive reaction	-2.214	4.77E-03	22	Seizures	-0.575	7.08E-05	27	Epilepsy	-	9.84E-04	21
Quantity of phagocytes	-0.418	4.77E-03	22	Seizure disorder	-0.587	1.87E-04	29	Hypersensitive reaction	1.858	9.84E-04	19
Infarction	0.444	4.77E-03	20	Alphathalassemia	-	3.02E-03	3	Tumorigenesis of tissue	0.158	1.81E-03	264

(4) Molecular and Cellular Functions

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Proliferation of cells	-3.071	1.17E-03	118	-				Morbidity or mortality	-2.177	1.17E-02	79
Morbidity or mortality	5.342	4.77E-03	81					Organismal death	-2.299	1.19E-02	78
Vasculogenesis	-2.127	4.77E-03	34					Development of abdomen	2.170	1.34E-02	26
Organismal death	5.577	4.77E-03	79					Development of epithelial tissue	2.009	2.32E-02	23
Migration of endothelial cells	-2.625	4.77E-03	19					Neuronal cell death	-2.067	2.33E-02	29

Supplementary Table 8. Enrichment analysis of mRNAs differentially expressed in the PPN. Ingenuity annotations of genes differentially expressed by running alone (CNR vs. CR; 501 genes), MPTP alone (CNR vs. MNR; 233 genes) and by running in MPTP-treated mice (MNR vs. MR; 255 genes). The top 10 upstream regulators, top 5 canonical pathways and top 5 annotations of the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are displayed, as well as their respective z-score, p-value and number of genes involved (#). All p-values are calculated using the Fisher's Exact Test, and the p-values for the canonical pathways and the categories 'Diseases and disorders' and 'Molecular and Cellular Functions' are corrected for multiple testing by the Benjamini-Hochberg correction. For the category 'Molecular and Cellular Functions', only the annotations with a significant z-score (i.e. <-2 (in red) or >2 (in green)) are shown. Annotations with only 1 (target) gene were discarded.

(1) Upstream Regulators

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Upstream Regulator	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**	**Upstream Regulator**	**z-score**	**p-value**	**#**
L-dopa	-1.820	2.50E-17	53	L-dopa	-0.525	1.75E-10	27	CREB1	0.510	2.20E-06	19
HTT	1.858	5.48E-16	52	Amphetamine	-1.249	4.49E-10	11	Haloperidol	-0.166	2.61E-06	8
CREB1	-0.435	1.05E-14	43	GDNF	-0.049	5.10E-10	10	HU-210	-	1.00E-05	3
Beta-estradiol	-2.207	3.66E-10	75	Alpha-amanitin	1.387	1.67E-09	6	PRKAA2	0.194	1.53E-05	8
BDNF	-2.024	4.23E-10	26	K+	-2.023	2.30E-09	8	NGF	0.671	1.73E-05	10
ATN1	-	1.90E-09	17	BMP2	-2.019	3.48E-09	14	Clozapine	0.186	3.49E-05	6
REST	0.693	2.63E-08	15	Quinolinic acid	-0.538	6.36E-09	8	Mek	1.793	4.39E-05	9
NGF	-1.675	3.44E-08	18	Pargyline	-	7.97E-09	5	Risperidone	-	6.44E-05	4
ADCYAP1	-1.070	7.53E-08	20	HTT	0.623	8.79E-09	25	Cadmium	0.816	9.67E-05	6
Amphetamine	-2.756	1.60E-07	12	Beta-estradiol	-1.262	9.11E-09	42	HSD17B13	-	1.02E-04	2

(2) Canonical Pathways

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Canonical pathway	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**	**Canonical pathway**	**z-score**	**p-value**	**#**
Axonal guidance signaling	-	3.92E-02	23	Dopamine receptor signaling	-1.000	8.13E-04	8	-			
				Dopamine-DARPP32 Feedback in cAMP signaling	-0.816	7.05E-03	9				
				Glutamate receptor signaling	-	3.29E-02	5				
				Breast cancer regulation by stathmin1	-	4.45E-02	8				

(3) Diseases and Disorders

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Neurological signs	1.091	6.74E-08	54	Purkinje cell degeneration	-	1.51E-07	8	Methemoglobinemia	-	4.23E-02	3
Movement disorders	1.806	1.64E-07	73	Epileptic seizure	-	1.05E-05	14				
Dyskinesia	-	2.45E-07	50	Epilepsy	-	5.03E-05	18				
Disorder of basal ganglia	-	2.98E-07	58	Neurological signs	0.600	1.98E-04	27				
Huntington's disease	-	4.07E-07	47	Seizures	-0.798	3.30E-04	19				

(4) Molecular and Cellular Functions

Physical exercise vs. Control				MPTP vs. Control				MPTP + Physical exercise vs. MPTP			
Annotation	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**	**Annotation**	**z-score**	**p-value**	**#**
Development of neurons	-2.394	6.74E-08	60	Cognition	-3.400	1.58E-04	22	-			
Formation of cellular protrusions	-2.172	2.70E-06	60	Learning	-2.940	3.30E-04	20				
Microtubule dynamics	-2.272	2.59E-05	70	Transport of molecule	-3.275	1.03E-02	42				
Coordination	-3.490	3.96E-05	21	Release of neurotransmitter	-2.333	1.86E-02	9				
Organismal death	4.315	1.32E-03	111	Differentiation of cells	-2.082	3.48E-02	49				

Supplementary Table 9. Differentially expressed mRNAs in the SN due to physical exercise in MPTP-treated mice, *and* regulated by L-DOPA. Proteins encoded by the genes are **bold** when located in the molecular landscape (**Figure 4**) and single-underlined when associated to PD via 'corroborating evidence', i.e. when the gene/protein is genetically associated to PD or differentially expressed in the SN of PD patients compared to controls.

Gene	Effect on mRNA expression by		Corroborating evidence
	Physical Exercise	L-DOPA	
ACKR1	-1.24	Increased	-
ACTA2	-1.27	Decreased	-
ALYREF	1.26	Increased	-
ARRB2	-1.23	Decreased	-
C14ORF166	1.21	Decreased	-
CASKIN2	-1.20	Increased	-
CDC42EP2	-1.23	Decreased	CDC42EP2 mRNA is increased in the SN of PD patients compared to controls[2].
CDC42EP3	1.29	Increased	-
CIART	-1.34	Increased	-
CLN6	-1.31	Decreased	-
DBP	-1.20	Increased	-
DGKB	1.31	Increased	DGKB mRNA is decreased in the SN of PD patients compared to controls[2].
DPP7	-1.20	Decreased	-
DUSP6	-1.22	Increased	-
EDNRB	1.21	Decreased	-
FCRLS	-1.26	Decreased	-
FKBP5	-1.29	Increased	-
FTL	-1.24	Decreased	FTL mRNA is decreased in the SN of PD patients compared to controls[3]. FTL protein is increased in the SN of PD patients compared to controls[4, 5].
GDF1	-1.21	Increased	GDF1 mRNA is decreased in the SN of PD patients compared to controls[6].
GPR39	-1.26	Increased	-
HSPB6	-1.24	Decreased	HSPB6 protein is increased in the SN of PD patients compared to controls[4].
HVCN1	-1.22	Decreased	-
IER3	-1.22	Increased	-
INO80E	-1.38	Increased	-
MFSD2A	-1.34	Increased	-
MRPL33	1.36	Increased	-
PDGFA	-1.22	Decreased	-
PER1	-1.24	Increased	-
PPP1R1B	-1.21	Increased	PPP1R1B protein is decreased by 66% in the SNpr, and 79% in the SNpc of PD patients compared to controls[7].
PVALB	-1.21	Decreased	PVALB mRNA is increased in the SN of PD patients compared to controls[8]. PVALB protein is increased in a subgroup of PD DA SN neurons[9].
REEP3	1.21	Decreased	-
ROMO1	-1.29	Increased	-
SHC1	-1.24	Decreased	-
SLC2A1	-1.22	Increased	-
TAC1	-1.31	Increased	TAC1 mRNA is decreased in the SN of PD patients compared to controls[8].
TP53	-1.24	Increased	Ser15-phosphorylated TP53 is increased in the PD SN ($p<0.001$)[10].

Supplementary Table 10. Differentially expressed mRNAs in the VM due to physical exercise in MPTP-treated mice, *and* regulated by L-DOPA. Proteins encoded by the genes are **bold** when located in the molecular landscape (**Figure 5**) and single-underlined when associated to PD via 'corroborating evidence', i.e. when the gene/protein is genetically associated to PD or differentially expressed in the striatum of PD patients compared to controls.

Gene	Effect on mRNA expression by		Corroborating evidence
	Physical Exercise	L-DOPA	
ACY1	-1.2	Increased	-
AMIGO2	-1.46	Increased	-
APBA2	1.28	Decreased	-
ARC	1.25	Increased	ARC mRNA is decreased in the striatum of PD patients compared to controls[8].
ARPP21	-1.2	Increased	ARPP21 is decreased in the striatum of PD patients compared to controls[11].
C14orf37	1.28	Decreased	-
C18orf21	-1.47	Increased	-
C4A/C4B	-1.32	Decreased	-
CACNA2D3	-1.23	Increased	(VDCC) CACNA2D3 mRNA is decreased in the striatum of PD patients compared to controls[12].
CAMK1G	-1.49	Increased	CAMK1G mRNA is increased in the striatum of PD patients compared to controls[13].
CBR3	-1.37	Increased	CBR3 protein is increased in the striatum of PD patients compared to controls[8].
CCND1	1.2	Decreased	-
CDC42EP3	-1.29	Increased	-
CHAT	-1.58	Increased	-
CHRNA4	1.92	Decreased	PD patients have a higher frequency of intron3+182 Del 22 bp (p=0.015)[14].
CLIC6	-1.27	Increased	CLIC6 mRNA is increased in the striatum of PD patients compared to controls[8].
CLN6	1.31	Decreased	-
CNIH3	2.8	Decreased	-
CNR1	1.49	Decreased	The presence of two CNR1 alleles with >16 AAT trinucleotide repeats in PD patients, is associated with a reduced prevalence of depression (p=0.003)[15]. CNR1 mRNA is increased in the striatum of PD patients compared to controls[13].
CRTAC1	1.79	Decreased	-
CRYM	-1.41	Increased	CRYM protein is increased in the striatum of PD patients compared to controls[8].
CTNNBIP1	1.31	Decreased	-
CYLD	-1.24	Increased	-
DDIT4L	-1.27	Increased	-
DGKB	-1.2	Increased	-
DGKI	-1.27	Increased	-
DLGAP1	1.73	Decreased	-
DLK1	-1.54	Increased	-
DOC2B	-1.3	Increased	-
DUSP6	1.24	Increased	-
ECEL1	-1.41	Increased	-
ERLIN1	-1.24	Increased	-
FAAH	2.15	Decreased	The synonymous rs324419 SNP and also the haplotype of rs324419 and rs2295633 (both SNPs are located in the FAAH gene) are both associated with musculoskeletal pain in PD patients p=0.006 and p=0.012 respectively[16].
FAM126A	-1.25	Decreased	-
FAM184b	-1.28	Increased	-
FAM81A	2.1	Decreased	-
FDPS	1.47	Increased	-
FNDC9	-1.3	Increased	-
FOXP1	-1.21	Increased	-
GABRA3	1.43	Decreased	GABRA3 mRNA is increased in the striatum of PD patients compared to controls[8].
GABRG2	1.24	Decreased	GABRG2 mRNA is increased in the striatum of PD patients compared to controls[13].
GFAP	1.22	Decreased	GFAP mRNA and protein is increased in the striatum of PD patients compared to controls[8].
GNAS	1.55	Decreased	-
GNG7	-1.22	Increased	-
GOLIM4	-1.26	Decreased	GOLIM4 mRNA is increased in the striatum of PD patients compared to controls[8].

Supplementary Table 10. (continued)

Gene	Effect on mRNA expression by		Corroborating evidence
	Physical Exercise	L-DOPA	
GPD2	1.24	Decreased	GPD2 mRNA is increased in the striatum of PD patients compared to controls[8].
GUCY1A3	-1.33	Increased	-
HAPLN4	3.14	Decreased	-
HBEGF	-1.24	Increased	-
HLA-A	-1.24	Decreased	-
HTR1B	-1.32	Increased	-
IDO1	-1.35	Increased	-
KCNA5	-1.28	Increased	-
KCNA6	1.65	Decreased	KCNA6 mRNA is increased in the striatum of PD patients compared to controls[8].
KCNC4	4.15	Decreased	-
KCNE5	-1.32	Decreased	-
KLF6	1.59	Decreased	-
LMNA	1.23	Decreased	-
LPPR1	-1.28	Increased	-
LRRTM3	-1.26	Increased	-
LYPD1	-1.22	Increased	-
LZTS3	-1.2	Increased	-
MAPK11	2.9	Decreased	-
MBP	1.22	Increased	MBP mRNA is decreased in the striatum of PD patients compared to controls[12]. MBP protein is decreased in the striatum of PD patients compared to controls[8].
MEF2C	1.29	Decreased	MEF2C mRNA is increased in the striatum of PD patients compared to controls[8].
MPP6	-1.33	Increased	-
MSMO1	1.24	Increased	MSMO1 mRNA is increased in the striatum of PD patients compared to controls[13].
MTURN	1.26	Decreased	-
MYO1B	-1.29	Increased	-
NAV1	1.31	Decreased	-
NPTX2	1.27	Increased	NPTX2 mRNA is decreased in the striatum of PD patients compared to controls[12].
NUAK1	1.24	Decreased	-
PARM1	1.53	Decreased	-
PDYN	-1.49	Increased	PDYN mRNA is increased in the striatum of PD patients compared to controls[8].
PDZD2	-1.32	Increased	-
PENK	-1.2	Increased	PENK mRNA is increased in the striatum of PD patients compared to controls[8].
PGM2L1	1.37	Decreased	PGM2L1 mRNA is increased in the striatum of PD patients compared to controls[13].
PLEKHA2	1.35	Decreased	-
PPM1L	1.68	Decreased	-
PPP1R1B	-1.2	Increased	PPP1R1B protein is decreased by 45% in the striatum of PD patients compared to controls[7].
PPP1R2	-1.24	Increased	-
PRICKLE1	1.55	Decreased	-
PTPRD	1.26	Decreased	-
PVALB	4.27	Decreased	-
RAP1GAP	-1.2	Increased	-
RAPGEFL1	1.24	Decreased	-
REM2	-1.23	Increased	-
RIMS1	1.52	Decreased	-
ROBO2	1.22	Decreased	-
SATB1	2.73	Decreased	-
SCN4B	-1.2	Increased	SCN4B protein is increased in the striatum of PD patients compared to controls[8].
SCUBE3	-1.25	Increased	-
SH3GL2	1.3	Decreased	SH3GL2 mRNA is increased in the striatum of PD patients compared to controls[13].
SLC10A4	-1.56	Increased	-
SLC24A2	1.61	Decreased	-

Supplementary Table 10. (continued)

Gene	Effect on mRNA expression by		Corroborating evidence
	Physical Exercise	L-DOPA	
SLC2A6	1.68	Decreased	-
SLC6A7	1.94	Decreased	-
SMUG1	1.21	Increased	-
SNCB	1.84	Decreased	SNCB mRNA is decreased in the striatum of PD patients compared to controls[17].
SOBP	1.83	Decreased	-
STRN	-1.27	Increased	-
STXBP1	1.2	Decreased	STXBP1 mRNA is decreased in the striatum of PD patients compared to controls[17].
SYN1	1.3	Decreased	SYN1 mRNA is decreased in the striatum of PD patients compared to controls[17]. SYN1 protein is increased in the striatum of PD patients compared to controls[8].
SYNDIG1L	-1.27	Increased	-
TAC1	-1.23	Increased	TAC1 mRNA is increased[8, 13], or decreased[11] in the striatum of PD patients compared to controls.
TCF4	1.25	Decreased	-
TCIRG1	1.34	Decreased	-
TH	-1.33	Increased	TH mRNA and protein is decreased in the terminals of nigro-striatal DA neurons of PD patients[18, 19].
TLE1	-1.21	Increased	-
TMEM141	1.55	Decreased	-
TRPC3	-1.49	Increased	-
UNC13C	-1.37	Increased	-
VAMP1	1.59	Decreased	(SNARE)
VAT1L	-1.32	Increased	VAT1L mRNA is increased in the striatum of PD patients compared to controls[13]. VAT1L protein is increased in the striatum of PD patients compared to controls[8].
WDR17	-1.23	Increased	WDR17 mRNA is increased in the striatum of PD patients compared to controls[13].
WNT7B	2.27	Decreased	-
ZCCHC12	-1.23	Increased	-
ZEB2	1.62	Decreased	-

Supplementary Table 11. Differentially expressed mRNAs in the DL due to physical exercise in MPTP-treated mice, *and* regulated by RICTOR. If the proteins that are encoded by these mRNAs are a subunit of a bigger protein complex, then the name of the protein complex is shown in the column 'part of'.

Gene	Effect on mRNA expression by		Part of[20]:
	Physical Exercise	RICTOR	
ATP5E	1.39	Decreased	Mitochondrial complex V
ATP5H	1.28	Decreased	Mitochondrial complex V
ATP5L	1.54	Decreased	Mitochondrial complex V
COX6B1	1.36	Decreased	Mitochondrial complex IV
COX7C	1.21	Decreased	Mitochondrial complex IV
NDUFA1	1.33	Decreased	Mitochondrial complex I
NDUFA11	1.24	Decreased	Mitochondrial complex I
NDUFA7	1.25	Decreased	Mitochondrial complex I
NDUFA9	1.23	Decreased	Mitochondrial complex I
NDUFB4	1.56	Decreased	Mitochondrial complex I
NDUFB9	1.22	Decreased	Mitochondrial complex I
NDUFS5	1.36	Decreased	Mitochondrial complex I
NDUFV3	1.22	Decreased	Mitochondrial complex I
PSMA2	1.23	Decreased	20S proteasome subunit α
PSMB6	-1.3	Decreased	20S proteasome subunit β
PSME1	1.28	Decreased	Proteasome activator complex
RPL10	-1.34	Decreased	60S ribosome subunit
RPL17	1.26	Decreased	60S ribosome subunit
RPL28	-1.61	Decreased	60S ribosome subunit
RPL29	-1.37	Decreased	60S ribosome subunit
RPL7A	-1.21	Decreased	60S ribosome subunit
RPL9	-1.34	Decreased	60S ribosome subunit
RPS15	-1.22	Decreased	40S ribosome subunit
RPS24	-1.25	Decreased	40S ribosome subunit
RPS26	1.23	Decreased	40S ribosome subunit
RPS27A	1.31	Decreased	40S ribosome subunit
RPS29	1.79	Decreased	40S ribosome subunit
SDHB	1.24	Decreased	Mitochondrial complex II
SGK1	-1.46	Decreased	-
UQCRQ	1.2	Decreased	Mitochondrial complex III
VCAM1	1.25	Increased	-

Supplementary Table 12. Differentially expressed mRNAs in the VTA due to physical exercise in MPTP-treated mice, *and* regulated by RICTOR. If the proteins that are encoded by these mRNAs are a subunit of a bigger protein complex, then the name of the protein complex is shown in the column 'part of'.

Gene	Effect on mRNA expression by		Part of[20]:
	Physical Exercise	RICTOR	
ATP5E	-1.64	Decreased	Mitochondrial complex V
ATP5F1	-1.29	Decreased	Mitochondrial complex V
ATP5G1	-1.42	Decreased	Mitochondrial complex V
ATP5G2	-1.28	Decreased	Mitochondrial complex V
ATP5H	-1.25	Decreased	Mitochondrial complex V
ATP5J	-1.25	Decreased	Mitochondrial complex V
ATP5J2	-1.29	Decreased	Mitochondrial complex V
COX17	-1.35	Decreased	Mitochondrial complex IV
COX5A	-1.28	Decreased	Mitochondrial complex IV
COX5B	-1.33	Decreased	Mitochondrial complex IV
COX6B1	-1.47	Decreased	Mitochondrial complex IV
COX6C	-1.45	Decreased	Mitochondrial complex IV
COX7A2	-1.22	Decreased	Mitochondrial complex IV
COX7B	-1.25	Decreased	Mitochondrial complex IV
COX7C	-1.25	Decreased	Mitochondrial complex IV
MRPL13	-1.3	Decreased	39S ribosome subunit (mitochondrial)
NDUFA1	-1.58	Decreased	Mitochondrial complex I

Supplementary Table 12. (continued)

Gene	Effect on mRNA expression by		Part of[20]:
	Physical Exercise	RICTOR	
NDUFA2	-1.24	Decreased	Mitochondrial complex I
NDUFA3	-1.33	Decreased	Mitochondrial complex I
NDUFA4	-1.32	Decreased	Mitochondrial complex IV
NDUFA7	-1.37	Decreased	Mitochondrial complex I
NDUFA9	-1.22	Decreased	Mitochondrial complex I
NDUFB10	-1.22	Decreased	Mitochondrial complex I
NDUFB2	-1.32	Decreased	Mitochondrial complex I
NDUFB4	-1.22	Decreased	Mitochondrial complex I
NDUFB5	-1.36	Decreased	Mitochondrial complex I
NDUFB6	-1.25	Decreased	Mitochondrial complex I
NDUFB7	-1.22	Decreased	Mitochondrial complex I
NDUFB8	-1.23	Decreased	Mitochondrial complex I
NDUFB9	-1.28	Decreased	Mitochondrial complex I
NDUFC1	1.23	Decreased	Mitochondrial complex I
NDUFS4	-1.26	Decreased	Mitochondrial complex I
NDUFS5	-1.31	Decreased	Mitochondrial complex I
NDUFS6	-1.42	Decreased	Mitochondrial complex I
NDUFV2	-1.21	Decreased	Mitochondrial complex I
NDUFV3	-1.22	Decreased	Mitochondrial complex I
POMP	-1.37	Decreased	Proteasome
PPA2	-1.24	Decreased	-
PSMA4	-1.24	Decreased	20S proteasome subunit α
PSMA5	-1.32	Decreased	20S proteasome subunit α
PSMA6	-1.3	Decreased	20S proteasome subunit α
PSMB1	-1.27	Decreased	20S proteasome subunit β
PSMB2	-1.31	Decreased	20S proteasome subunit β
PSMB3	-1.35	Decreased	20S proteasome subunit β
PSMB5	-1.23	Decreased	20S proteasome subunit β
PSMB7	-1.24	Decreased	20S proteasome subunit β
PSME1	-1.2	Decreased	Proteasome activator complex
RPL11	-1.38	Decreased	60S ribosome subunit
RPL12	-1.35	Decreased	60S ribosome subunit
RPL17	-1.5	Decreased	60S ribosome subunit
RPL21	-1.2	Decreased	60S ribosome subunit
RPL23A	-1.3	Decreased	60S ribosome subunit
RPL26	-1.41	Decreased	60S ribosome subunit
RPL29	-1.29	Decreased	60S ribosome subunit
RPL30	-1.43	Decreased	60S ribosome subunit
RPL34	-1.44	Decreased	60S ribosome subunit
RPL35A	-1.35	Decreased	60S ribosome subunit
RPL38	-1.69	Decreased	60S ribosome subunit
RPL41	-1.26	Decreased	60S ribosome subunit
RPLP1	-1.25	Decreased	60S ribosome subunit
RPLP2	-1.52	Decreased	60S ribosome subunit
RPS10	-1.2	Decreased	40S ribosome subunit
RPS15	-1.32	Decreased	40S ribosome subunit
RPS18	-1.25	Decreased	40S ribosome subunit
RPS29	-1.49	Decreased	40S ribosome subunit
RPS3A1	-1.2	Decreased	40S ribosome subunit
RPS4Y1	-1.36	Decreased	40S ribosome subunit
SDHB	-1.22	Decreased	Mitochondrial complex II
SGK1	-1.62	Decreased	-
SHFM1	-1.43	Decreased	26S proteasome
UBA52	-1.33	Decreased	60S ribosome subunit
UQCR10	-1.21	Decreased	Mitochondrial complex III
UQCRB	-1.23	Decreased	Mitochondrial complex III
UQCRHL	-1.36	Decreased	Mitochondrial complex III
UQCRQ	-1.25	Decreased	Mitochondrial complex III

Supplementary Table 13. Differentially expressed mRNAs in the PFC due to physical exercise in MPTP-treated mice, *and* regulated by Bicuculline and Dalfampridine. For each gene/mRNA their association to epilepsy and/or seizures is shown in the column 'Association to epilepsy / seizures'.

Gene	Effect on mRNA expression by		Association to epilepsy / seizures
	Physical Exercise	Bicuculline / Dalfampridine	
ACTA2	-1.58	Decreased [1]	The R179H mutation in ACTA2 results in neonatal stroke and progressive leukoencephalopathy[21].
ARC	1.68	Increased	Immediate early gene, increased in epilepsy (6.2x)[22].
BTG2	1.57	Increased	-
DUSP1	1.38	Increased	Increased in epilepsy (3.4x)[22].
FOS	2.26	Increased	Immediate early gene, increased in epilepsy (4.2x)[22].
FOSB	1.24	Increased	Immediate early gene, increased in epilepsy (3.0x)[22].
GADD45G	1.23	Increased	Increased in epilepsy (2.0x)[22].
NPAS4	1.85	Increased	Npas4 inhibits seizures in pilocarpine-induced epileptic rats[23].
NR4A1	1.24	Increased	Immediate early gene[24], increased in epilepsy (4.7x)[22].

Supplementary Table 14. Differentially expressed mRNAs in the PPN due to physical exercise in MPTP-treated mice, *and* regulated by CREB1. For each gene/mRNA the cellular process in which they exert an effect is described in the column 'Involved in'.

Gene	Effect on mRNA expression by		Involved in[20]:
	Physical Exercise	CREB1	
CALCB	-2.10	Increased	Vascular remodeling
CALN1	1.23	Decreased	Calcium signaling
CARTPT	1.47	Increased	Neuropeptide signaling
EBP	-1.24	Increased	Cholesterol/lipid signaling
FAM65B	-1.27	Increased	Cytoskeleton rearrangement
FASN	1.21	Increased	Cholesterol/lipid signaling
JUN	1.22	Regulated	Immediate early gene; Increased in epilepsy (1.6x)[22].
LMO1	-1.49	Decreased	Transcription regulator
MVK	1.27	Increased	Cholesterol/lipid signaling
NAB2	-1.27	Increased	Transcription regulator
NOS1	1.52	Regulated	Nitric oxide signaling
NR4A1	1.27	Increased	Immediate early gene[24]; Increased in epilepsy (4.7x)[22].
NRGN	-1.23	Decreased	Calcium signaling
NRP1	-1.22	Decreased	Vascular remodeling; Calcium signaling
PENK	-1.26	Increased	Neuropeptide signaling
SGK1	-1.54	Increased	Vascular remodeling
SV2C	1.35	Increased	Increased in epilepsy (2.1x)[22].
TAC1	-1.36	Increased	Neuropeptide signaling; Vascular remodeling; Increased in epilepsy (1.8x)[22].
TINF2	1.32	Increased	Telomere regulation

DETAILED DESCRIPTION OF THE MOLECULAR LANDSCAPES

CONTENTS

INTRODUCTION

Ingenuity analysis of the mRNA sequencing (RNAseq) data of the MPTP-treated mice *with* physical exercise compared to MPTP-treated mice *without* physical exercise revealed the top regulator in each of the PD-related brain areas. In the SN and the VM, L-DOPA is the top regulator, whereas RICTOR is the top regulator in the DL and VTA, Bicuculline/Dalfampridine are the top regulators in the PFC, and CREB1 is the top regulator in the PPN. For each brain area a molecular landscape was built that represents the interactions between the proteins encoded by the mRNAs regulated by the top regulator and physical exercise. **Figure 4** and **Figure 5** show the molecular landscapes of the SN and VM, respectively, and the landscapes for the DL, VTA, PFC and PPN are shown in **Supplementary Figure 6-9**. Below, a description of the interactions that are represented in the molecular landscape figures is given.

All names of proteins derived from the mRNAs that were differentially expressed in the RNAseq due to physical exercise are shown in **bold** and proteins that are associated with PD (via genetic evidence, or differentially expressed in PD patients compared to controls) are (also) single-underlined. For a complete overview of all proteins in the landscapes (and the corroborating evidence for their associations with PD), see **Supplementary Tables 9-14**. In the molecular landscape descriptions below, the gene name abbreviations refer to both the gene and the protein. Furthermore, the terms 'activates' and 'inhibits' indicate effects on protein function (e.g. by (de)phosphorylation) by another protein, while 'increases the expression of' or 'decreases the expression of' denote effects on the abundance of a protein (directly or indirectly) induced by another protein.

Not all proteins encoded by mRNAs from the RNAseq were placed in the landscape, either because there were no connections with other landscape proteins or due to lack of annotation, or both. However, this does not necessarily mean that they are not involved in physical exercise-mediated effects. Interactions in the landscape that are (for practical reasons) not shown in the figures are indicated with '(not shown)' in the text.

Overall, in order to interpret protein-protein interactions in the landscape, two generalizations have been made. First, when a knock-out of protein A in a cell or animal model *increases* the expression of protein B, we assume that endogenous expression of protein A leads to the opposite effect and *decreases* the expression of protein B. Second, we assumed that all identified protein interactions (in any organism and/or cell type) can be extrapolated to the interactions in human (DA) neurons, even when the specific interactions have not been studied in these specific substrates.

1. MOLECULAR LANDSCAPE OF THE SN

In **Figure 4** the physical exercise-mediated changes in expression in the SN are shown in combination with the L-DOPA-mediated expression of the same proteins. In **Supplementary Table 9** an overview of the regulatory effects of physical exercise and L-DOPA in the SN is given. First, in the description below, the interactions and pathways of these proteins in the landscape are discussed (independent of their regulation by physical exercise or L-DOPA). And, secondly, the last paragraph of this section discusses the differential effects of physical exercise and L-DOPA on these pathways.

The central themes in the landscapes that represent the protein interactions associated with the changes in expression due to physical exercise in the SN are G-coupled receptor signaling, the regulation of cell survival (including ERK1/2 signaling, ROS regulation, glucose uptake and signaling and CREB1 signaling), as well as circadian clock proteins. Here we describe concisely the proteins involved in each theme, and their interactions.

1.1 G-coupled receptor signaling and cytoskeleton regulation

ARRB2 mediates the signaling by G-protein coupled receptors and is e.g. activated by the G-coupled receptor **GRP39**[25]. **ARRB2** binds phosphorylated AKT1 (total and Ser473-phosphorylated AKT1 are lower in the PD brain, but are increased in glia cells in the SN of PD patients[26]) and facilitates the inactivation of AKT1[27], and binds to the cytoskeleton protein **ACTA2**[28], to the familial PD[29-33] proteins VPS35[28] and PARK2[34], to the nuclear export adapter **ALYREF**[28] (binds also to AKT1[35]), and to the small GTPase CDC42[36] that is known to control actin polymerization and thereby e.g. affects cell morphology and endocytosis. **ACTA2** also binds the familial PD[37-41] proteins LRRK2[42] and PARK2[43], and binds **TP53**[44]. **TP53**, in turn, increases **ACTA2** expression[45, 46]. LRRK2 and **CDC42EP3** bind[47], while **CDC42EP3** and **CDC42EP2** both bind CDC42[48, 49], thereby regulating assembly of actin filaments[20]. Furthermore, both **CDC42EP2** and **CDC42EP3** both bind to the familial PD[50-54] protein SNCA[55].

1.2 The regulation of cell survival

The ERK1/2 pathway is activated by a wide array of stimuli, including growth factors, cytokines and ligands for G protein-coupled receptors, and regulates neuron proliferation, survival and apoptosis. In the molecular landscape represented here, ERK1/2 is activated by **PPP1R1B**[56], **ACKR1**[57], **EDNRB**[58] (also binds to PARK2[59]), **IER3**[60] and **SHC1**[61, 62]. ERK1/2 also binds to and is regulated by **TP53**[63-65], and binds to and is inhibited by **DUSP6**[66-69]. Moreover, ERK1/2 expression is increased by **GPR39**[70]. These proteins are also known to affect pathways involved in cell death, i.e. **IER3**, **TP53** and **SHC1** activate CASP3[71-74] and thereby induce apoptosis. CASP3 expression is increased in the SN of PD patients[75, 76]. Moreover, **IER3** inhibits the activation and expression of AKT1[77], and **TP53** binds to **ALYREF**[78] and binds to[79, 80] and decreases the expression of PARK7[81], whereas

PARK7 activates **TP53**[81]. Further, **TP53** increases the expression of **DUSP6**[82], **FKBP5**[83, 84], **PDGFA**[85] and **IER3**[86-88]. **SHC1** is also important for the cellular response to oxidative stress[20] and functions downstream of **TP53** to induce apoptosis[20]. While reactive oxygen species (ROS) are necessary for normal cell functioning[89, 90], high levels are toxic and induce cell death[89, 91, 92]. The mitochondrial protein **ROMO1** increases the production of ROS[93]. The ferritin light chain protein **FTL**, on the other hand, reduces the formation of ROS by regulating iron homeostasis[94] and also the heat shock protein **HSPB6** decreases the production of ROS[95]. **HSPB6** binds SNCA[96], increases calcium levels[97], activates AKT1[95], and inhibits CASP3 signaling[95]. Cytoplasmic calcium levels are decreased by **PVALB**[98], that is increased in a subgroup of PD DA SN neurons[9].

ERK1/2 activation, in turn, increases the expression of the glucose transporter **SLC2A1**[99]. Glucose influx is necessary for maintenance of cellular energy levels and cell survival, and PD patients show increased metabolic rates of glucose in the SN[100]. The glucose transporter **SLC2A1** regulates the influx of glucose into the cell and inhibits the activation of CASP3[101]. **SLC2A1** expression is also increased by AKT1[102-104] and the familial PD[105-107] protein PINK1[108], but decreased by **TP53**[109-111]. Further, the growth factor complex PDGF (composed of **PDGFA** and PDGFB) activates **SLC2A1**[112] thus increases the uptake of glucose[113]. The neuropeptide **TAC1** inhibits glucose uptake[114, 115] and thereby also increases extracellular (blood) glucose levels[115, 116]. Moreover, glucose activates ERK1/2[117, 118] and CDC42[119, 120], increases the expression of the anti-apoptotic proteins PARK2[121], PINK1[121] and **SHC1**[122] and decreases the expression of **TP53**[123, 124] and **ACTA2**[125]. Thus, the maintenance of sufficiently high cellular glucose levels prevents ROS formation and activation of apoptotic signaling cascades.

Lastly, ERK1/2 also decreases the expression of **FKBP5**[126] (part of the steroid receptor complex[20] and binds AKT1[127]) and activates **SHC1**[128], **IER3**[129] and CREB1[130-132]. CREB1 is a transcription factor that binds to cAMP response elements on the DNA and thereby regulates gene transcription of e.g. TH (not shown)[133, 134]. The earlier mentioned **PPP1R1B** (DARPP-32) integrates the signals in response to extracellular DA and glutamate[135, 136] and activates CREB1 (in response to DRD2 activation)[137, 138], increases the expression of LRRK2[139], SNCA[139] and CREB1[140] and increases mobilization of calcium[141]. CREB1 is also activated by glucose[142], calcium[143] and AKT1[144-147], binds **TP53**[148-150] and binds to the nuclear exporter adapter **ALYREF**[151] that transports spliced mRNAs from the nucleus to the cytoplasm. CREB1 increases the expression of **MFSD2A**[152] and **IER3**[152] and regulates the expression of the circadian clock proteins **PER1** and **CIART** (see below).

1.3 Circadian clock proteins

Multiple studies have reported a dysregulation of the circadian clock in PD patients and e.g. sleep-wake disturbances and increased daytime sleepiness are widely studied[153-159]. Also degeneration of DA neurons in the SN of MPTP-treated mice cause circadian rhythm irregularities[160]. In the SN landscape, the proteins **CIART** (not shown), **DBP** and **PER1**

are involved in circadian clock regulation. CREB1 increases the expression of **PER1**[152, 161, 162] and is necessary for expression of **CIART**[162]. Calcium increases the expression of both **PER1**[163] and **DBP**[163], and **TP53** (a regulator of the circadian clock[164]) increases the expression of **DBP**[46], whereas **DBP** and **PER1** increases each other's expression[165]. Thus, multiple proteins involved in regulation of the circadian rhythm are regulated in the SN landscape, suggesting that physical exercise might affect the (dysregulated) circadian clock function in PD.

1.4 Regulation by physical exercise vs. L-DOPA

Physical exercise and L-DOPA have an opposite effect on the regulation of proteins involved in glucose homeostasis (**SLC2A1**), ERK1/2 activation (**ACKR1**, **EDNRB**, **GPR39**, **IER3**, **TP53**), the DARPP-32 pathway (**PPP1R1B**) and circadian clock-regulating proteins (**PER1**, **DBP**, **CIART**). Namely, physical exercise downregulates the expression of **SLC2A1**, **PPP1R1B** and clock proteins, while (chronic) L-DOPA administration results in increased **SLC2A1** expression and (over)activation of the neuron by L-DOPA-mediated activation of ERK1/2[56, 166], **PPP1R1B**[167] and CREB1[168] and the subsequent *activation* of clock proteins. Further, in addition to the proteins differentially expressed due to physical exercise, L-DOPA activates[166] and increases the expression of CASP3[166, 169].

2. MOLECULAR LANDSCAPE OF THE VM

In **Figure 5** the physical exercise-mediated changes in expression in the VM are shown, in combination with the L-DOPA-mediated expression of these same proteins. **Supplementary Table 10** gives an overview of the regulatory effects of physical exercise and L-DOPA. The central themes in the landscapes that represent the protein interactions associated with the changes in expression due to physical exercise in the VM are (interneuron mediated) DA release, cannabinoid signaling, neuropeptide signaling, calcium mobilization and subsequent activation of **PPP1R1B**, ERK1/2, CREB1 and **CCND1** signaling. Here we describe concisely the proteins involved in each theme, and their interactions. First, in the description below, the interactions and pathways of these proteins in the landscape are discussed (independent of their regulation by physical exercise or L-DOPA). Secondly, the last paragraph of this section discusses the differential effects of physical exercise and L-DOPA on these pathways.

2.1 DA- and interneuron-mediated MSN activation

Medium spiny neurons (MSNs) are GABAergic inhibitory cells and represent the majority of cells present in the striatum. There are two primary MSNs subtypes, the DRD1 and DRD2 expressing MSNs, representing the direct and indirect pathway respectively. MSNs of the direct pathway project to the globus pallidus internal segment (GPi) and substantia nigra pars reticularis (SNr), whereas the indirect pathway MSNs project

to the external segment of the globus pallidus (GPe), to the subthalamic nucleus and subsequently to the GPi and SNr. Outputs of the direct and indirect pathway, respectively, cause an excitation and inhibition of the upper motor neurons in the cortex[170, 171].

DA release in the striatum is not only dependent on SN neuron activation, but also depends on the activity of cholinergic interneurons. Cholinergic interneurons integrate synaptic signaling in the striatum and mediate DA-dependent striatal plasticity of MSNs[172-175]. Through the activation of both muscarinic and nicotinic cholinergic receptors on DA terminals and the co-release of glutamate, activated cholinergic interneurons trigger striatal DA release[176-183]. Of note, in PD and in dystonia, a reduced release of DA in the striatum leads to an increased acetylcholine (ACh) release by interneurons[184], whereas DA-dependent pauses in the tonic firing of cholinergic interneurons are hypothesized to function as a learning mechanism in reward- and motor-related learning[185-187]. Further, the differentially expressed potassium channels (**KCNA5**, **KCNA6**, **KCNC4**, **KCNE5**) may regulate DA release and presynaptic DRD2 function[188], or may regulate the firing patterns of cholinergic interneurons via hyperpolarization-activated potassium currents[189, 190] or may be involved in depolarization of MSNs after activation of muscarinic ACh receptors[191-194].
Thus, the interplay between striatal ACh and DA release, by respectively cholinergic interneurons and DA neurons, is important for learning and plasticity of MSNs. Another factor involved in memory and learning is the transcription factor **MEF2C**, that suppresses the number of excitatory synapses on neurons[20]. **MEF2C** increases the expression of **KCNA5**[195], and **MEF2C** itself is activated by **MAPK11**[196, 197] and **MEF2C** expression is increased by **HLA-A**[198] and BDNF[199, 200] (not shown; BDNF is associated with (cognitive impairment in) PD[201-203]).

In the VM landscape, the choline O-acetyltransferase **CHAT**, necessary for ACh synthesis in cholinergic synapses, is decreased by physical exercise. Moreover, activation of the neuronal acetylcholine receptor subunit **CHRNA4** by ACh leads to opening of an ion channel, influx of calcium and depolarization that facilitates activation of the **SNARE** complex and neurotransmitter (e.g. DA) release[204, 205]. Depolarization through calcium influx is also mediated by the voltage-dependent calcium channel (**VDCC**)[20], that binds to **RIMS1**[206, 207] and **UNC13C**[208] and is inhibited by **REM2**[209]. The **SNARE** complex binds **STXBP1**[210, 211] and **RIMS1**[206] and **SNARE** assembly is inhibited by calmodulin (CaM)[212]. **STXBP1** regulates synaptic vesicle docking and fusion and binds also to CaM[213], **DOC2B**[214] (**DOC2B** expression is increased by CaM[215]), **APBA2**[216, 217] and familial PD protein SNCA[218].

Cytoplasmic vesicles are recycled and are again filled with DA for release into the synaptic cleft. Removal of **SYN1** from the cytoplasmic side of cytoplasmic vesicles mobilizes the vesicles for neurotransmitter release[20]. **SYN1** binds **SH3GL2**[219], LRRK2[220],

PARK2[221], SNCA[222] and CaM[213, 223]. SNCA decreases the expression of **SYN1**[224], whereas CaM binds the synaptic vesicle membrane protein **VAT1L**[213] and induces the release of **SYN1** from cytoplasmic vesicles[20]. **SH3GL2** regulates synaptic vesicle endocytosis and binds in addition to **SYN1** also to DRD2[225], PARK2[226], LRRK2[227] and CaM[213]. DA for release into the synaptic cleft is either synthesized in the DA neuron, or is taken up by the DA transporter SLC6A3 (that is associated with PD[228-230]) from the extracellular matrix[231]. TH is the rate-limiting enzyme in DA synthesis and binds SNCA[232]. TH is activated by calcium[233, 234] and inhibited by SNCA[232, 235] and DRD2[236]. Further, the TH expression is increased by the neuropeptide **TAC1**[237] and the SNCA-homologue **SNCB**[238]. **SNCB** also binds to SNCA[238], AKT1[239] and inhibits CASP3 activation[240]. Cytoplasmic DA is transported into the cytoplasmic vesicles by the DA transporter SLC18A2[241] (that is associated with PD[242, 243]) so it can be released. The protein **MYO1B** is a motor protein involved in among others vesicular transport and binds to LRRK2[42]. **CDC42EP3** is another protein involved in cytoskeleton regulation and binds to SNCA[55] and LRRK2[47].
In summary, DA release in the striatum is regulated by controlling reuptake, storage and synthesis of DA and thereby controlling the availability of DA ready for release. However, it should be noted that the proteins involved in vesicle fusion and release of neurotransmitter are not only involved in DA release in the synaptic cleft, but may also mediate e.g. ACh, GABA or glutamate release.

DA released in the synaptic cleft activates the DRD1 and DRD2 receptors present on MSNs[244]. The PD-associated DRD2[245, 246] binds to DRD1[247] and also to the chloride ion channel **CLIC6**[248]. In turn, DA receptor activation results in increased cytosolic calcium concentration[249, 250]. Cytoplasmic calcium levels are also increased by **TAC1**[251-253], **VDCC**[254] (binds to **AMIGO2**[255]), **TRPC3**[256-258] and decreased by **SLC24A2**[20, 259], **HTR1B**[260] and **PVALB**[98, 261, 262]. Calcium increases the expression of **ARC**[263] and **CCND1**[264, 265] and activates **FAAH**[266], **PPP1R1B**[267], ERK1/2[268, 269] and CaM[270, 271]. CaM is a calcium sensor and transduces the calcium signal and thereby increases the expression of **LRRTM3**[215], binds and decreases the expression of **TCF4**[272, 273] (**TCF4** also binds to **TLE1**[274] and PARK2[275]), binds **TRPC3**[276], **CCND1**[277], **STRN**[278], **MYO1B**[279, 280], **MAPK11**[281] (binds also to **MBP**[282]), **ARPP21**[283], **MBP**[284, 285] (**MBP** expression is increased by BDNF (not shown)[286]) and **RAP1GAP**[213]. Moreover, CaM activates **CAMK1G**[287] that in turn activates CREB1[20, 287]. Calcium mobilization therefore increases the main signaling cascades in the VM landscape; **PPP1R1B**, ERK1/2, CREB1 and **CCND1** signaling.

2.2 Main signaling cascades

Increased phosphorylation (and thus activation) of **PPP1R1B** and ERK1/2 in striatal neurons after chronic L-DOPA treatment is associated with L-DOPA-induced dyskinesias[56, 288, 289]. **PPP1R1B** is also known as dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) and activates ERK1/2[56, 290] and CREB1[137, 140], inhibits CASP3[140,

[291] and regulates AKT1 activation[138, 292].
ERK1/2 are protein kinases that are signal transducers for e.g. growth factors, cytokines and G protein-coupled receptors. In addition to activation by **PPP1R1B**, ERK1/2 are activated by **HBEGF**[293-295] (regulates the survival of midbrain dopaminergic neurons[296]), **DLK1** [297, 298], **GNAS**[299-301], **DGKI**[302], **HTR1B**[303] and the endogenous cannabinoid receptor **CNR1**[304-307] (see also below), and are inhibited by **DUSP6**[67, 69, 308, 309] and **RAP1GAP**[310]. ERK1/2 increase the expression of neuropeptide **TAC1**[311] and activate CREB1[131, 132].
The transcription factor CREB1 is, in addition to **CAMK1G**, **PPP1R1B** and ERK1/2 (see above), also activated by glucose[142, 312] (that decreases the expression of **CBR3**[313]), potassium[314], AKT1[144-146, 315] and **GNAS**[316]. Subsequently, CREB1 regulates the expression of **GABRG2**[22], decreases the expression of **DLK1**[317] (also decreased by **KLF6**[318]), **PGM2L1**[152], **PTPRD**[152], **MYO1B**[152] and **CRYM**[152] and increases the expression of **ARC**[162], **NPTX2**[152], **ECEL1**[152], BDNF (not shown)[143, 152, 319], **KCNC4**[152], **HLA-A**[152] (not shown, **HLA-A** binds **MBP**[320] and **GNAS**[321], and its expression is decreased by ERK1/2 (not shown)[322]) and of the neuropeptides **TAC1**[22, 152], **PENK**[152, 323] and **PDYN**[324, 325].

All three, **PPP1R1B**, ERK1/2 and CREB1, increase the expression of the cell cycle regulator **CCND1** [152, 292, 326-328]. **CCND1** may be involved in synaptic plasticity and learning[329] and its expression is increased by BDNF[330], **WNT7B**[331], AKT1[332-334], glucose[335], calcium[264, 265] and **TCF4**[336, 337] and decreased by **RAP1GAP**[310], PARK2[338], **ZEB2**[339] (**ZEB2** inhibits AKT1)[340] and **CTNNBIP1**[336]. Nuclear translocation of **CCND1** is activated by AKT1[341] and calcium[264] and inhibited by **KLF6**[342]. **CCND1** decreases the expression of **STXBP1**[343] and increases the expression of **CTNNBIP1**[344]. Further, **CCND1** binds **KLF6**[342], **LMNA**[345] (AKT1 activates **LMNA** [346]) and **MAPK11**[347]. Thus, **CCND1** is regulated by all the main signaling cascades in the landscape (**PPP1R1B**, ERK1/2, CREB1) and may affect synaptic plasticity.

2.3 Endogenous cannabinoid signaling

Following DA depletion, the endocannabinoid system in the basal ganglia rearranges to restore homeostasis[348, 349]. The cannabinoid receptor **CNR1** is abundant within the basal ganglia[350], can repress the release of glutamate and GABA (e.g. by cortical synapses, not shown)[351, 352] and interacts with DA transmission in the striatum[353, 354], i.e. **CNR1** forms heterodimers with DA receptors[354] and **CNR1** activation increases DRD2 expression[355]. Further, indirect pathway signaling (via DRD2-positive MSNs) is rescued by endocannabinoid signaling and improves motor dysfunction in PD models[356, 357]. Paradoxically, both **CNR1** agonist and antagonists alleviate L-DOPA-induced dyskinesia in PD models[358]. This contradiction may be explained by the differential coupling of **CNR1** to G proteins, either due to interaction with DA receptors[353], or by the functional selectivity of the different agonists and antagonists for G proteins and **CNR1** subpopulations[358]. Moreover, DRD2 activation modulates coupling of different G proteins to **CNR1**[359] (not shown). **CNR1** expression is increased by DA[360], glucose[361] and

BDNF[362] (and **CNR1** increases BDNF expression[355, 363]). **CNR1** binds and decreases the expression of **RAP1GAP**[364] and binds and inhibits **VDCC**[208, 365, 366]. Further, **CNR1** decreases the expression of the neuropeptides **PDYN**[367] and **PENK**[367], regulates the activation of ERK1/2[304-306, 368, 369] and increases DA release[370, 371].

The two most abundant endocannabinoids, 2-arachidonoylglycerol (2-AG) and anandamide, bind and activate **CNR1**[372-374] and 2-AG also increases the expression of **CNR1**[375]. 2-AG and anandamide inhibit **FAAH** (not shown)[376], whereas **FAAH** in turn increases the hydrolysis and breakdown of both endocannabinoids[366, 377]. **GABRG2** and **GABRA3** form a GABA receptor complex[378, 379] and are activated by 2-AG[379]. Further, DA decreases the expression of **GABRG2**[360].
In summary, the endocannabinoid system interacts with and compensates for defects in the DA system and may therefore have a therapeutic potential in the treatment of PD.

2.4 Neuropeptides

PD and L-DOPA-induced dyskinesia are associated with abnormal expression of striatal precursor peptides, i.e. **PENK**, **PDYN** and **TAC1** derived peptides are increased in the globus pallidus and their production is DA-state dependent[380-382]. Neuropeptides are small peptides used for communication between neurons. As shown above, CREB1 increases the expression of the neuropeptides **PENK**, **PDYN** and **TAC1**. **PENK** decreases the excretion of K^+ (potassium)[383], whereas K^+ increases the expression of **PENK**[384]. **PENK** expression is increased by DA[385, 386] and BDNF[387] and decreased by DRD2[388]. **PDYN** decreases degeneration of DA neurons in the rat midbrain[389]. **PDYN** expression is increased by glucose[390], DRD1[391] and **TAC1**[392]. **TAC1** expression is increased by BDNF[393, 394] (not shown), DA[360], DRD2[388] and ERK1/2[311]. In turn, **TAC1** activates ERK1/2[395, 396] (not shown), increases plasma glucose levels (by decreasing its uptake in cells)[114-116], increases intracellular calcium[251-253], binds CaM[397] (not shown) and increases the expression of TH[237] and **GFAP**[398] (not shown; **GFAP** is a marker for astrocytes and its expression is increased by BDNF[399] (not shown) and decreased by glucose[313] (not shown)). The mitochondrial **GPD2** binds PARK2[43], and when activated, increases the release of ROS from the mitochondria[400]. Calcium binds and activates **GPD2**[20], whereas glucose inhibits **GPD2** activation[401] and may therefore regulate ROS levels in the cell.
In summary, the regulation of neuropeptides is DA-state dependent and may affect neuron communication and functioning in the striatum and globus pallidus.

2.5 Regulation by physical exercise vs L-DOPA

The main signaling themes of the landscape encompass ACh interneuron functioning, potassium signaling, vesicle release, cannabinoid and neuropeptide signaling, calcium mobilization and subsequent activation of **PPP1R1B**, ERK1/2, CREB1 and **CCND1** signaling. Strikingly, physical exercise and L-DOPA have opposite effects on the regulation of

almost all proteins in this landscape, i.e. physical exercise increases the expression of vesicle release proteins (**STXBP1**, **RIMS1**, **SNARE**, **APBA2**), **CCND1** and proteins involved in cannabinoid signaling (**FAAH**, **CNR1**) and decreases the expression of **PPP1R1B**, neuropeptides (**PDYN**, **PENK**, **TAC1**) or proteins involved in ERK1/2 regulation (**HTR1B**, **DGKI**, **HBEGF**, **DLK1**, **RAP1GAP**). L-DOPA exerts an opposite effect on the expression of all these proteins compared to the effect of physical exercise and also activates **PPP1R1B**[167], CREB1[168], ERK1/2[56, 166], DRD1 and DRD2[402], and activates and increases the expression of CASP3[166, 169]. Of note, L-DOPA disrupts the crosstalk of an **CNR1**-DRD2 complex[403] and an **CNR1** agonist reduces L-DOPA-induced motor dysfunction in a PD rat model[404, 405]. Further, L-DOPA-induced dyskinesia is associated with increased neuropeptide levels in the striatum and globus pallidus[381, 382, 406-408]. Furthermore, ablation of striatal cholinergic interneurons attenuates L-DOPA-induced dyskinesia in mice[409, 410]. Of interest, whereas physical exercise decreases the ACh synthase **CHAT** and increase the expression of the ACh receptor subunit **CHRNA4**, L-DOPA causes the exact opposite.
In summary, physical exercise has been shown to have an opposite effect on the regulation of proteins that are regulated by L-DOPA and associated with L-DOPA-induced dyskinesia. Physical exercise may therefore have therapeutic value in attenuating the side effects of chronic L-DOPA use.

3. MOLECULAR LANDSCAPE OF THE DL

In **Supplementary Figure 6** the physical exercise-mediated changes in expression in the DL are shown in combination with the RICTOR-mediated expression of the same proteins.

The proteins regulated by both physical exercise and RICTOR are part of complex I-V of the electron transport chain in the mitochondria, the 40S and 60S ribosomal subunits, or the proteasome. **Supplementary Table 11** shows the localization of each of the proteins in these complexes and also provides an overview of the regulatory effects of physical exercise and RICTOR.

3.1 Regulation by physical exercise vs. RICTOR

RICTOR decreases the expression of all proteins in the landscape, and thereby regulates cellular energy levels, protein translation and degradation. In contrast to RICTOR, physical exercise increases the expression of all proteins in the landscape located in the electron transport chain, and also increases the expression of the proteins that are part of the ribosome or proteasome.

4. MOLECULAR LANDSCAPE OF THE VTA

In **Supplementary Figure 7** the physical exercise-mediated changes in expression in the VTA are shown in combination with the RICTOR-mediated expression of the same proteins.

The proteins regulated by both physical exercise and RICTOR are part of complex I-V of the electron transport chain in the mitochondria, the 40S and 60S ribosomal subunits, or the proteasome. **Supplementary Table 12** shows the localization of each of the proteins in these complexes and also provides an overview of the regulatory effects of physical exercise and RICTOR.

4.1 Regulation by physical exercise vs. RICTOR

Physical exercise and RICTOR both decrease the expression of all mRNAs in the landscape of the VTA. Therefore, physical exercise and RICTOR exert the same direction of effect on the functioning of the electron transport chain, the ribosome and the proteasome. This is in contrast to the opposite regulation of mRNAs in the ribosome, proteasome and especially the electron transport chain by physical exercise and RICTOR in the DL (see above).

5. MOLECULAR LANDSCAPE OF THE PFC

In **Supplementary Figure 8** the physical exercise-mediated changes in expression in the PFC are shown in combination with the Bicuculline/Dalfampridine-mediated expression of the same proteins. The drugs Bicuculline and Dalfampridine induce epileptic seizures, and eight out of nine of the mRNAs regulated by physical exercise and these convulsants are associated with epilepsy or seizures (**Supplementary Table 13**).

The main theme in the PFC landscape is immediate early gene activation, namely three out of nine proteins in the landscape – **FOS**, **FOSB** and **NR4A1** – are encoded by immediate early genes. **FOS** and **FOSB** decrease each other's expression[411] and **NR4A1** inhibits activation of **FOSB**[412]. The activation of these immediate early genes is regulated by insulin and low density lipoprotein (LDL). Impaired insulin homeostasis is associated with PD[413] and oxidized LDL is increased in the plasma of (L-DOPA-treated) PD patients[414]. Oxidized LDL regulates the expression of **FOS**[415, 416], and increases the expression of **FOSB**[417] and **NR4A1**[417, 418] and both insulin and LDL increase the expression of **DUSP1**[419-421] and activate the PI3K complex[337, 422] in the cytoplasm. The PI3K complex activates **NR4A1**[423] and ERK1/2[424-426] and increases the expression of **FOS**[411, 427, 428]. Oxidized LDL activates the ERK1/2 pathway[429] that is also activated by insulin (via PI3K activation)[422, 425, 430-432]. Further, ERK1/2 is also regulated by the familial PD proteins, i.e.

both SNCA and PARK2 inhibit ERK1/2 activation[433, 434] and SNCA also binds to ERK1/2[433]. Furthermore, ERK1/2 is activated by the NMDA receptor (NMDAR)[435, 436] that also increases the expression of **FOS**[411] and **ARC**[437] and is involved in **DUSP1** regulation[438] (not shown). The phosphatase **DUSP1** binds to ERK1/2[439] and inhibits ERK1/2 activation[440, 441]. In turn ERK1/2 activates **DUSP1**[442, 443] and **FOS** [444, 445], regulates **BTG2**[446], and also increases the expression of **FOS**[447, 448], **FOSB**[449] and regulates the expression of **DUSP1**[450]. Insulin expression/secretion is regulated by PARK2[451], increased by **BTG2**[452] and decreased by **NPAS4**[453] and **NR4A1**[454]. The actin protein **ACTA2** binds to the familial PD proteins LRRK2[42] and PARK2[43]. Insulin increases the level of intracellular glucose, which is mediated by PI3K[113, 455, 456]. In turn, glucose decreases the expression of **ACTA2**[125], increases the expression of PARK2[121] and **NPAS4**[453], activates PI3K[457, 458] and activates ERK1/2[117, 118].

Thus, immediate early gene activation and the epilepsy-related signaling are the main pathways in the PFC regulated by both physical exercise and Bicuculline/Dalfampridine. Further, (oxidized) LDL, insulin, glucose and the ERK1/2 and PI3K pathways seem important for the regulation of these proteins.

5.1 Regulation by physical exercise vs. Bicuculline/Dalfampridine

Physical exercise and Bicuculline/Dalfampridine exert the same direction of effect on all mRNAs in the PFC landscape, i.e. they decrease the expression of ACTA2 and increase the expression of the other eight proteins. Bicuculline/Dalfampridine treatment and physical exercise may therefore (partially) have the same effects on the molecular pathways in the PFC. The mRNAs coding for NMDAR and ERK1/2 did not show a differential expression due to physical exercise, but are both regulated by Bicuculline[459, 460].

6. MOLECULAR LANDSCAPE OF THE PPN

In **Supplementary Figure 9** the physical exercise-mediated changes in expression in the PPN are shown in combination with the CREB1-mediated expression of the same proteins. These proteins show very limited interactions in the landscape, but a few themes could be distinguished such as vascular remodeling / angiogenesis (**CALCB**, **NRP1**, **SGK1**, **TAC1**), neuropeptide signaling (**CARTPT**, **PENK**, **TAC1**), lipid metabolism (**EBP**, **FASN**, **MVK**), epilepsy/immediate early response (**JUN**, **NR4A1**, **SV2C**, **TAC1**) and calcium singaling (**CALN1**, **NRGN**, **NRP1**, Calmodulin (CaM)) (see also **Supplementary Table 14**).

Central in the landscape is the transcription factor CREB1 that regulates the expression of **JUN**[461, 462] and **NOS1**[463], increases the expression of **NR4A1**[152, 464], **FASN**[465], **MVK**[162], **EBP**[152], **TINF2**[152], **FAM65B**[152], **SGK1**[152], **CARTPT**[152], **CALCB**[466], **SVC2C**[152], **NAB2**[162], **PENK**[152] and

TAC1[152] and decreases the expression of **CALN1**[152] (binds calcium[20]), **LMO1**[152], **NRGN**[152] and **NRP1**[152]. Further, CREB1 binds to **JUN**[467], **SGK1**[468] and is activated by **SGK1**[468], **NOS1**[469] and CaM[411]. The proteins of these CREB1-regulated mRNAs show only a small number of interactions with each other. The immediate early response proteins **JUN** and **NR4A1** bind[470] and **JUN** inhibits **NR4A1** activation[470]. Further, **JUN** regulates the expression of **LMO1**[471], increases the expression of **PENK**[472] and **SGK1**[473] and mediates alternative splicing of PARK2[474]. **NR4A1** regulates the expression of **FASN**[475] and is activated by **TAC1**[476]. Further, **CALCB** increases the expression of **CARTPT**[477]. Furthermore, **NOS1** binds to the calcium binding CaM[478, 479] that is binding to the calcium binding **NRGN**[480, 481]. **NOS1** is activated by CaM[479, 482], **NRP1**[483] (binds calcium[20]) and SNCA[484], and **NOS1** expression is decreased by PARK2[485]. Lastly, insulin increases the expression of **JUN**[486], **NR4A1**[475] and **FASN**[487] and activates **SGK1**[488] and **CREB1**[489] and may therefore be a modulating factor of the landscape.

6.1 Regulation by physical exercise vs. CREB1

Physical exercise increases the expression of mRNAs related to epilepsy/immediate early response, and decreases the mRNAs involved in neuropeptide signaling. However, whereas some of these proteins are increased by CREB1, others are decreased by CREB1 and no clear directional effect of CREB1 functioning due to physical exercise could be discerned.

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LIST OF ABBREVIATIONS

AC, anterior commissure;
ACh, acetylcholine;
CC, corpus callosum;
CNR, control (saline) not running;
CR, control (saline) running;
DA, dopamine;
DL, dorsolateral striatum;
FW, forward strand;
GPe, globus pallidus external;
GPi, globus pallidus internal;
L-DOPA, levodopa;
MNR, MPTP but not running;
MR, MPTP and running;
MSNs, medium spiny neurons;
PD, Parkinson's disease;
PFC, prefrontal cortex;
PPN, pedunculopontine nucleus;
RV, reverse strand;
SN, substantia nigra;
SNpc, substantia nigra pars compacta;
SNpr, substantia nigra pars reticularis;
STN, s ubthalamic nucleus;
VDCC, voltage-dependent calcium channel;
VM, ventromedial striatum;
VTA, ventral tegmental area

5

Integrated molecular landscape of amyotrophic lateral sclerosis provides insights into disease etiology

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5.1 ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a severe, progressive and ultimately fatal motor neuron disease caused by a combination of genetic and environmental factors, but its underlying mechanisms are largely unknown. In order to gain insight into the etiology of ALS, we here conducted genetic network and literature analyses of the top-ranked findings from six genome-wide association studies of sporadic ALS (involving 3589 cases and 8577 controls) as well as genes implicated in ALS etiology through other evidence, including familial ALS candidate gene association studies. We integrated these findings into a molecular landscape of ALS that allowed the identification of three main processes that interact with each other and are crucial to maintain axonal functionality, especially of the long axons of motor neurons, i.e. (1) Rho-GTPase signaling; (2) signaling involving the three regulatory molecules estradiol, folate and methionine; and (3) ribonucleoprotein granule functioning and axonal transport. Interestingly, estradiol signaling is functionally involved in all three cascades and as such an important mediator of the molecular ALS landscape. Furthermore, epidemiological findings together with an analysis of possible gender effects in our own cohort of sporadic ALS patients indicated that estradiol may be a protective factor, especially for bulbar-onset ALS. Taken together, our molecular landscape of ALS suggests that abnormalities within three interconnected molecular processes involved in the functioning and maintenance of motor neuron axons are important in the etiology of ALS. Moreover, estradiol appears to be an important modulator of the ALS landscape, providing important clues for the development of novel disease-modifying treatments.

KEYWORDS: Amyotrophic lateral sclerosis, Molecular Landscape, Etiology, Estradiol, RNP granule, Axon maintenance, GWAS

5

5.2 INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the gradual degeneration of upper motor neurons in the cerebral motor cortex, and lower motor neurons in the brainstem and spinal cord. The subsequent muscle weakness and atrophy leads to death from respiratory failure, usually within two to five years after the first symptoms arise[1-4]. ALS has a worldwide incidence of 1-3 per 100,000 person-years, a mean age of onset between 50 and 65 years[2, 5-7], and a male to female ratio of approximately 2:1[REF8, 9]. Based on the neuroanatomical substrate from which the initial symptoms originate, ALS can be categorized as of either bulbar- or spinal-onset, which are characterized by initial speech/swallowing problems or limb-related symptoms respectively[2].

Previous ALS research has mainly focused on glutamate toxicity, or deficits in protein degradation, oxidative stress, mitochondrial function, axonal transport of organelles

and RNA processing[10, 11] as underlying disease-causing mechanisms. The familial ALS genes that cause the disorder when mutated often served as a starting point for these studies. Thus far, at least twelve familial genes causing ALS have been unequivocally identified (*C9ORF72*, *CCNF*, *CHCHD10*, *FUS*, *OPTN*, *PFN1*, *SOD1*, *SQSTM1*, *TARDBP*, *TBK1*, *UBQLN2*, *VCP*), whereas mutations in a number of other genes (e.g. *ALS2*, *ANG*, *ATXN2*, *CHMP2B*, *HNRNPA1*, *HNRNPA2B1*, *NEFH*, *VAPB*) have also been associated with ALS[12, 13]. Approximately 10% of the ALS cases are classified as 'familial', i.e. following a Mendelian inheritance pattern. However, this classification is no longer so clear because mutations in 'familial' genes also explain up to 11% of the sporadic, non-inherited cases of ALS[11, 12]. Nevertheless, ALS is still considered to be mainly a sporadic disease that, together with environmental and lifestyle risk factors[14, 15], is associated with a large number of common genetic variants (typically single nucleotide polymorphisms or SNPs), each with a slightly increased disease risk[16, 17]. In recent years, genome-wide association studies (GWASs) of ALS have identified many of these SNPs for sporadic ALS[18-27].

In this study, we have integrated the most significant findings from six published GWASs of sporadic ALS, through genetic network and elaborate literature analyses, into a molecular landscape that also includes proteins encoded by familial genes and therefore covers both familial and sporadic ALS-linked signaling cascades. The constructed landscape reveals the involvement of deficits in the functioning and maintenance of motor neuron axons as well as estradiol signaling in ALS etiology, and provides important clues for new ALS treatments.

5.2 METHODS

5.2.1 ALS GWAS gene selection

ALS candidate genes were selected based on GWAS SNPs and their corresponding p-values. All GWASs of sporadic ALS published to date were considered. Criteria for study inclusion were a publicly available independent GWAS discovery sample, with (at least) all SNPs associated at $p<0.0001$. From the GWASs for which these data were available, SNPs were selected that were associated with ALS at $p<0.0001$ to compile a list of associated genes. The selected genes either contained a SNP that was located within an exonic, intronic or untranslated region of the gene, or were found within 100 kilobases (kb) downstream or upstream of the SNP. The latter was based on the fact that the vast majority of expression quantitative trait loci (eQTL) for a given gene are located within 100 kb downstream and/or upstream of a gene[28-30] and because trait-associated SNPs are more likely to be eQTL[31]. The chosen statistical cut-off for association ($p<0.0001$) has been employed to designate 'suggestive' evidence of association before[32-34]. Subsequently, the literature was searched for additional (genetic) evidence linking the proteins encoded by the selected GWAS candidate genes to ALS.

5.2.2 Genetic network enrichment analysis

To identify enriched protein networks in the ALS GWAS candidate genes, a network analysis using the Ingenuity Pathway Analysis (IPA) software package (http://www.ingenuity.com) was performed, using default parameters. For each network, the Ingenuity software generates an enrichment score, i.e. the negative logarithm of the right-tailed Fisher's exact test result.

5.2.3 Molecular landscape building

Guided by the results of the network enrichment analysis, the literature was extensively searched for the (putative) functions of all proteins encoded by the ALS GWAS candidate genes using the Uniprot Protein Knowledgebase (UniProtKB) (http://www.uniprot.org/uniprot)[35] and PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez). Further, the literature was searched for interactions between the ALS GWAS candidate gene-encoded proteins and additional ALS candidate genes implicated in the disease through other (genetic) evidence, as well as genes/proteins and metabolites that have no known link with ALS, but have extensive functional connections with other proteins in the landscape.

5.2.4 Analysis of data from a cohort of sporadic ALS patients

We used epidemiological data from a cohort of sporadic ALS patients that were diagnosed and followed at the Department of Neurology, University Hospital Gasthuisberg (KU Leuven, Belgium) until death to analyze possible gender effects, linked to the relative abundance of estradiol in pre-menopausal women. In this respect, the male:female ratios before and after the start of the menopause (corresponding to 51 years of age on average in Western European women[36-39]) were compared for the disease-related variables 'age at first symptoms' and 'age at death' for the whole cohort of ALS patients and separately for the cases with spinal and bulbar onset. For these comparisons, a Chi-square test was used and p-values <0.05 were considered statistically significant. Further, possible gender effects on the mean disease duration – the mean time in years from age at first symptoms to age at death – before and after the menopause was assessed for all ALS patients and separately for the cases with spinal and bulbar onset. A Student's t-test was used and p-values <0.05 were considered statistically significant.

5.3 RESULTS

5.3.1 Selected ALS GWAS genes and genetic network enrichment analysis

Six of the eleven published ALS GWASs met our inclusion criteria (**Supplementary Table 1**) and were used to compile a list of 197 unique ALS candidate genes (**Supplementary Table 2**). The most significantly enriched genetic network (p<1.00E-43; **Supplementary Figure 1**) served as a starting point for building the molecular landscape.

5.3.2 The molecular landscape of ALS

Guided by the most significantly enriched genetic network and extensive literature searches, we built a molecular landscape that contains interacting proteins encoded by 121 of the 197 GWAS genes (61%; **Supplementary Table 2**), 92 proteins (and protein complexes) implicated in ALS etiology through (familial) candidate gene, mRNA/protein expression and/or functional studies (**Supplementary Table 3**), and 12 proteins that have not been directly linked to ALS (yet) but have extensive functional interactions within the landscape (**Supplementary Table 3**).

Supplementary Figures 2 and **3** show all relevant protein interactions that constitute the landscape. In the **Supplementary Information**, a detailed description of the evidence linking all the proteins in the ALS landscape is provided, together with the respective references. The description here will be restricted to the overview of the ALS landscape as shown in **Figure 1**, depicting the main biological processes and signaling cascades in the landscape. Three interconnected main signaling cascades are present in the molecular ALS landscape: (1) regulation of Rho-GTPase signaling, (2) signaling involving three regulatory molecules (estradiol, folate and methionine) and their metabolites, and (3) ribonucleoprotein (RNP) granule functioning and axonal transport. These signaling cascades will be briefly discussed below.

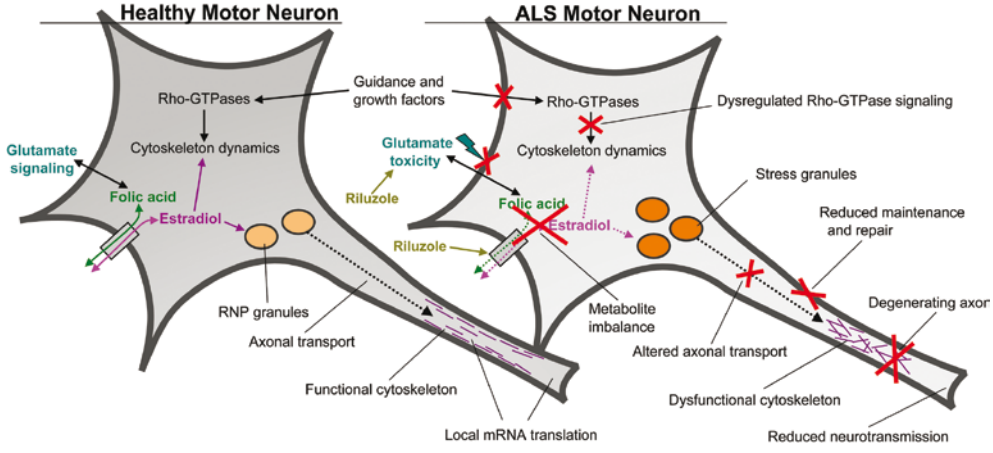

Figure 1. Overview of the molecular landscape of ALS. The different pathways and signaling cascades of the molecular landscape are shown in a healthy motor neuron (left) and a defective motor neuron from an ALS patient (right). Red crosses indicate the dysregulation of pathways and cascades that may result in motor neuron destabilization and death. See text for details.

The first cascade involves signaling through the Rho-GTPases (CDC42, RAC1 and RHOA), and controls cytoskeletal dynamics and neurite outgrowth, and is regulated by growth factors (e.g. EGF, NGF and VEGF), axonal guidance factors (e.g. CXCL12, netrin) and familial ALS proteins (e.g. SOD1 and TARDBP). In addition, the Rho-GTPase RAC1

is part of the NADPH oxidase complex that produces reactive oxygen species (ROS) and regulates neurite outgrowth. Therefore, dysregulated or deficient Rho-GTPase signaling affects cytoskeletal dynamics and neurite outgrowth of motor neurons and may increase ROS-mediated oxidative stress. Of note, two very recent genetic studies have identified novel ALS genes, *C21ORF2* and *NEK1* [40, 41]. The proteins encoded by these genes both fit within our ALS landscape, i.e. C21orf2 is a direct functional interactor of the NEK1 kinase and a regulator of the cytoskeleton. Moreover, NEK1 is also involved in regulating cytoskeletal dynamics, binds the kinesin-II motor complex and is a downstream target of the estradiol receptor ESR1.

Second, the folate and methionine cycles, linked with each other through vitamin B12, are implicated in ALS through multiple metabolites (e.g. carnitine, homocysteine and S-adenylmethionine) and affect estradiol metabolite levels and estradiol-mediated transcription (e.g. through transcriptional regulation by the methyltransferase MLL). The levels of folate and estradiol metabolites are regulated by the multidrug resistance transporters ABCB1 and ABCG2. Tetrahydrofolate (THF), the active form of folate, upregulates ABCG2 expression in the cell membrane and downregulates this transporter in intracellular organelles, which makes its localized expression dependent on THF availability. Furthermore, folate metabolites are involved in the synthesis of co-activators of the NMDA glutamate receptor, whereas polyglutamation – the binding of multiple glutamate groups – of folate metabolites affects their kinetics in the cell. Riluzole, the only FDA-approved drug to treat ALS, is an antiglutamatergic compound that reduces glutamate-induced excitotoxicity by inhibiting the NDMA receptor and increasing the expression of astrocytic glutamate transporters. However, riluzole itself is also transported by ABCB1 and ABCG2, and it increases the expression of ABCG2. Hence, riluzole not only affects and regulates glutamatergic signaling, but also the intracellular levels of estradiol and folate (metabolites). Estradiol metabolites have different affinities for ESR1 and their regulation (e.g. through efflux and conversion) affects ESR1-dependent transcription and activation. Taken together, a complex interaction exists between folate, methionine and estradiol metabolites, associated with glutamate-induced excitotoxicity and effects of riluzole.

Third, most of the proteins encoded by genetically linked ALS genes appear to be involved in RNA processing and transport. These proteins are located in RNPs, which are complexes of mRNAs and RNA-binding proteins. RNPs regulate the processing, transport and immediate local translation of their constituent mRNAs, enabling the neuron to quickly react to environmental cues and/or damage in the axon and/or distant synapse. Motor neurons have exceptionally long axons and therefore rely heavily on the RNP system to locally regulate protein expression far away from the cell body. Cellular stress causes RNPs to stop the translation of certain mRNAs and keep them dormant

until future demand. The formation and aggregation of these stress RNPs, or stress granules, are increased in ALS motor neurons. Moreover, mutations in e.g. the familial ALS genes *FUS* and *TARDBP* result in increased stress granule formation and affect (local) mRNA translation. The RNP system does also rely on the above-mentioned Rho-GTPase signaling, which is required for cytoskeletal maintenance and neuronal (out) growth. RNPs are transported via this 'cytoskeletal framework' to their site of action in/along the axon. Therefore, deficient Rho-GTPase signaling also negatively affects the axonal transport and function of RNPs.

Of note, estradiol and ESR1 control axonal (out)growth by regulating Rho-GTPases and their expression, physically interact with RNPs and familial ALS proteins, and affect the regulation of glutamate-induced excitotoxicity and the NADPH oxidase complex. Moreover, estradiol and ESR1 interact with and/or regulate (the expression of) multiple other proteins in the landscape. Therefore, estradiol-related signaling appears to be an important modulator within the ALS landscape, through regulating axonal function and maintenance and hence motor neuron function and survival.

5.3.3 Analysis of data from a cohort of sporadic ALS patients

Our molecular landscape of ALS pointed towards an important regulatory role of estradiol in the etiology of the disease. Therefore, we used epidemiological data of a cohort of sporadic ALS patients to analyze possible gender effects – linked to the relative abundance of estradiol in pre-menopausal women – on the age at onset, disease duration and age at death of bulbar- and spinal-onset ALS patients. An overview of the cohort of sporadic ALS patients is shown in **Supplementary Table 4**. The male:female ratio of all ALS patients is significantly lower *after* the start of the menopause – indicating a relatively increased number of affected females – for the disease-related variable 'age at first symptoms' (P=0.016; **Figure 2**). Further, the mean duration of

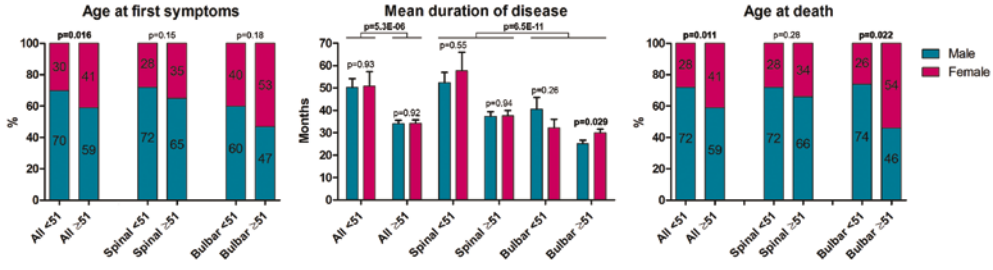

Figure 2. Analysis of data from a cohort of sporadic ALS patients for possible gender effects on the prevalence, onset and disease duration of (bulbar- and spinal-onset) ALS. The male:female ratio for the disease-related variable 'age at first symptoms' (left), the 'mean duration of disease' (middle) and ´age at death´ (right) was compared for all ALS cases, and separately for the cases with spinal or bulbar onset, before and after the start of the menopause (corresponding to 51 years of age in Western European women). A Chi-square test was used for the analyses of 'age at first symptoms' and 'age at death', and the Student's t-test was used for the analysis of 'mean duration of disease'. P-values are indicated above the bars and considered significant when p<0.05.

disease is longer in younger ALS patients (all <51 versus all ≥51 years old; p=5.3E-06), and in spinal-onset versus bulbar-onset ALS patients (p=6.5E-11), but is independent of gender for both. However, when analyzing bulbar- and spinal-onset separately, postmenopausal women with bulbar-onset ALS have a longer mean duration of disease compared to male bulbar-onset ALS patients (p=0.029). Furthermore, the parameter 'age at death' showed that the male:female ratio of all patients and especially that of bulbar-onset ALS patients is reduced (from a ratio of 3:1 to 1:1) after the start of the menopause (p=0.011 and p=0.022, respectively).

5.4 DISCUSSION

In this study, we integrated available ALS data into a molecular landscape that reveals the main biological processes that are affected in ALS, i.e. Rho-GTPase signaling, signaling involving estradiol, folate and methionine, and RNP granule functioning and axonal transport, that may contribute to motor neuron dysfunction and, ultimately, death. The molecular ALS landscape represents processes and cascades that may be affected in both the monogenic, familial and the more prevalent polygenic, sporadic forms of ALS. In this respect, the landscape includes processes and signaling cascades reported to be involved in familial ALS such as oxidative stress and RNA processing, as well as processes of 'classical' ALS theories such as glutamate toxicity. However, the landscape also comprises processes that have been less well studied before – e.g. growth- and guidance factor signaling and cytoskeletal dynamics – and also sheds further light on the functional relationships between the various ALS-linked processes. Interestingly, estradiol signaling is functionally involved in all main processes and as such an important modulator of the ALS landscape.

It is important to note that the molecular landscape is not intended to imply a fixed 'sequence of events' that eventually leads to motor neuron death in all patients, i.e. a number of biological processes that occur in a temporally and/or spatially distinct order. Instead, we propose that deficits in any of the main landscape processes, either by themselves or in combination with others, can cause motor neurons to die. Of note, the familial proteins are often involved in more than one of these main processes and/or of crucial importance to these processes. Consequently, mutations in the familial ALS genes have a functionally 'high impact' in the landscape and directly lead to ALS, whereas in sporadic ALS patients multiple functionally 'lower-impact' genetic variations are required to develop the disease. For example, mutations in the familial gene *SOD1* result in oxidative stress[42], affect Rho-GTPase signaling[43] and impair axonal transport of the enzyme choline acetyltransferase[44] that synthesizes acetylcholine, the main and essential neurotransmitter at the neuromuscular synapse[45]. Thus, both familial mutations and sporadic variations may result in disorganization of the cytoskeletion, defects in axon maintenance and motor neuron death.

Within our ALS landscape, Rho-GTPases are important regulators of neuronal development and survival as well as cytoskeleton dynamics[46], and as such they are crucial for axonal maintenance, regeneration and transport. This view is supported by reported defects in the regulation of motor neuron axonal regeneration of motor neurons in ALS patients[47, 48] and ALS mouse models[49, 50]. In addition, regulation of neurofilaments – the major building blocks of the cytoskeleton and crucial for axonal regeneration[51] – is affected in ALS patients[52] and in ALS mouse models[53, 54], and multiple studies have shown defects in axonal transport[55-58] and dysregulation of motor proteins[59-61] in motor neurons of ALS patients.

Of note, axonal regeneration is dependent on DNA methylation, which is regulated by the landscape's methionine and folate cycles and enhanced by folic acid (folate) supplementation[62-64]. Because the DNA methylation cascade regulates – through MLL activation – the transcriptional activity of the estradiol receptor ESR1[65], these findings together imply that methionine, folate and estradiol are important modulators of ALS pathogenesis.

The vitamin B12-linked folate and methionine cycles are also implicated in ALS through multiple previous studies. For example, homocysteine is increased in the plasma and CSF of ALS patients[66-68], whereas carnitine[69] and methionine[70] are decreased in the plasma of ALS patients. Furthermore, folic acid supplementation is neuroprotective[71], S-adenosylmethionine delays disease onset[72] and carnitine decreases disease progression and increases survival[73] in a mouse model for ALS. Carnitine supplementation may also be beneficial in ALS patients[74] and methyl-vitamin B12 may delay the motor symptoms of ALS[67, 75, 76]. Nevertheless, the benefits of supplementing these metabolites may not be universal, as ALS patients may have different nutritional deficiencies. For example, hyperhomocysteinemia has been noted in rats on a low methionine diet[77], as well as in human vegetarians and vegans along with decreased vitamin B12 and increased folate levels compared to controls on an omnivorous diet[78]. Another environmental factor that affects these pathways is endurance exercise, associated with increased homocysteine plasma levels[79, 80] together with low vitamin B12 and folate levels[79, 81, 82]. Vigorous physical activity may therefore – at least partially – affect pathways that are part of ALS etiology, and it is tempting to speculate that this may be the link between a highly active lifestyle and/or high level of physical fitness (e.g. athletes, blue-collar workers) and increased ALS incidence[14, 83-87]. Therefore, it is worthwhile to further investigate whether individuals on a specific diet, with or without an active lifestyle, have an increased risk to develop ALS due to dysregulation of their folate and methionine cycles.

Another important factor in the ALS landscape is estradiol signaling that functionally

integrates, regulates and is regulated by key landscape processes. Estradiol mediates cytoskeleton dynamics through interacting with Rho-GTPases and the NADPH oxidase complex, and as such may protect motor neurons against degeneration. This apparent critical role of estradiol and related signaling in ALS etiology is corroborated by several lines of evidence. First, epidemiological studies have demonstrated that men have an approximately three times higher risk to develop ALS before the age of 50 years than women[88], a gender difference that gradually decreases with increasing age[38]. Our epidemiological analysis of a large cohort of sporadic ALS patients confirms these findings and suggests that premenopausal women are – to a certain extent – protected against ALS. Further, for bulbar-onset – but not spinal-onset – ALS, the mean duration of disease is longer in postmenopausal women than in men, and also the male:female ratio for the age at death is drastically decreased after the menopause. This indicates that estradiol may exert a protective effect especially for bulbar-onset ALS and that the effect on the disease duration may be due to residual estradiol slowing disease progression. To our knowledge, this is the first study that shows an association between estradiol and the primary neuroanatomical substrate (spinal versus bulbar) from which the initial ALS symptoms originate.

The involvement of estradiol signaling in ALS pathogenesis is also in line with functional studies in ALS animal models showing that estradiol delays disease onset and progression, and increases survival[89-91]. Moreover, estradiol protects cultured spinal motor neurons against excitotoxicity and rescues these neurons from degenerating and dying[92-95]. Taken together, these findings imply that estradiol and estradiol-related signaling have an important modulatory role in motor neuron function and survival.

In this respect, it is of note that gender differences can also be observed at the genetic level, i.e. SNPs in the gene encoding the ESR1 co-activator PPARGC1A are associated with age of ALS onset and survival in males specifically[96]. Moreover, genetic variations in *MTHFR*[97, 98] – which encodes an important enzyme in the folate cycle – and in the promoter region of the growth factor *VEGFA*[99] have been associated with ALS in women. These notions imply that the presumed hormonal neuroprotective advantage of women could be counteracted by genetic variations in, for example, the folate cycle and/or VEGF signaling.

Currently, the antiglutamatergic drug riluzole is the only FDA-approved drug to treat ALS and extends the life expectancy of ALS patients by approximately 2-3 months[100]. Multiple other antiglutamatergic compounds have been tested in clinical trials – either by themselves or in combination with riluzole – but were unsuccessful[101-107]. These findings imply that the beneficial function of riluzole may not be limited to regulating glutamate toxicity only, as explained by our molecular landscape riluzole regulates and

is regulated by transporters that also control folate and estradiol metabolite levels in the cell[108-110], which may therefore be an additional mechanism through which riluzole conveys neuroprotection in ALS. It is tempting to speculate that such a multi-modal effect of riluzole explains its effectiveness in ALS, while other antiglutamatergic compounds fail.

In conclusion, our integrated molecular landscape of ALS highlights the involvement of processes that lead to deficient axonal functioning of motor neurons (i.e. axonal transport, local translation, regeneration and outgrowth) and points toward estradiol-related signaling as an important mediator of ALS pathological mechanisms. Consequently, the landscape not only yields in-depth insights into the etiology of ALS but also provides new clues for the development of disease-modifying ALS treatments.

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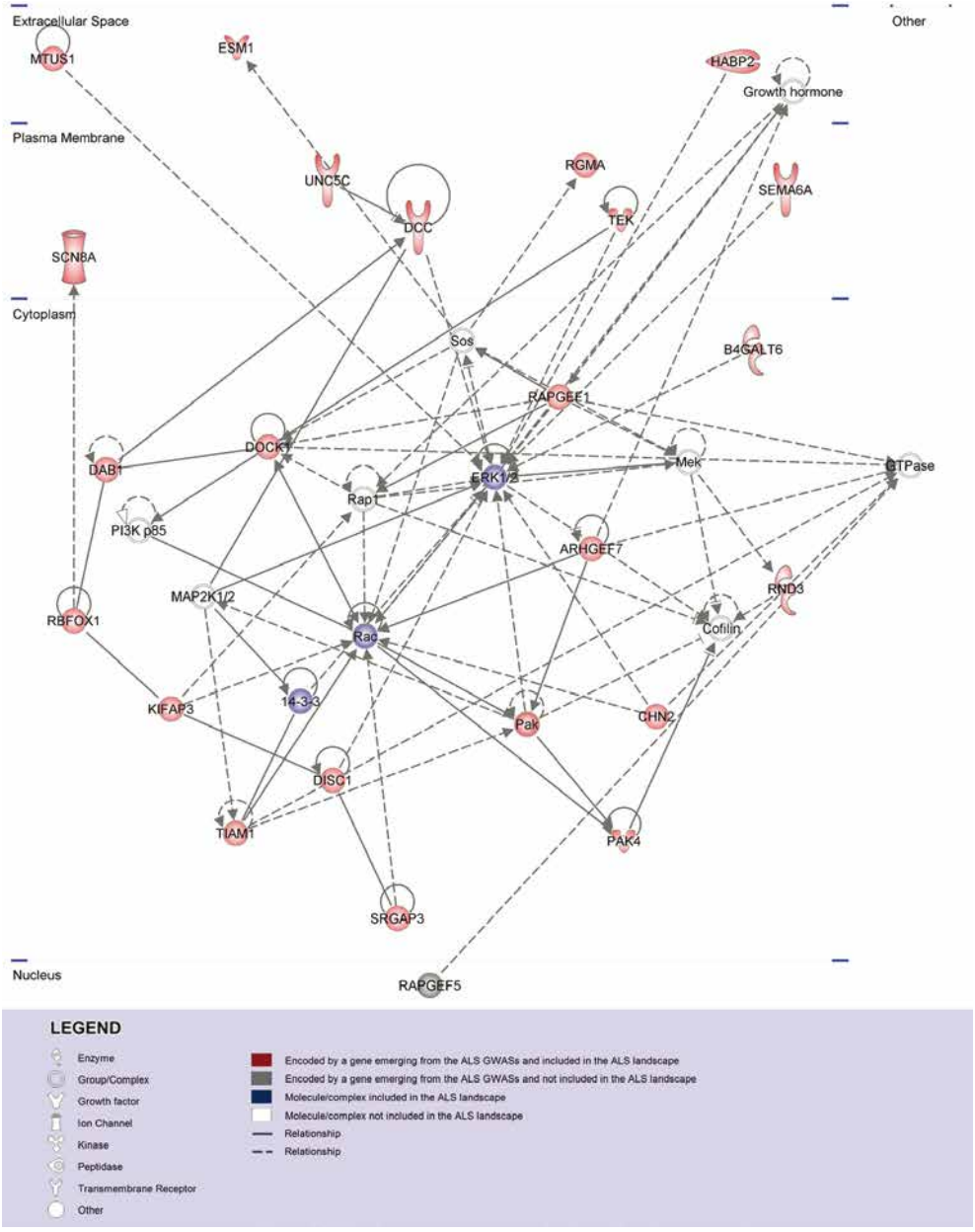
5.7 SUPPLEMENTARY INFORMATION

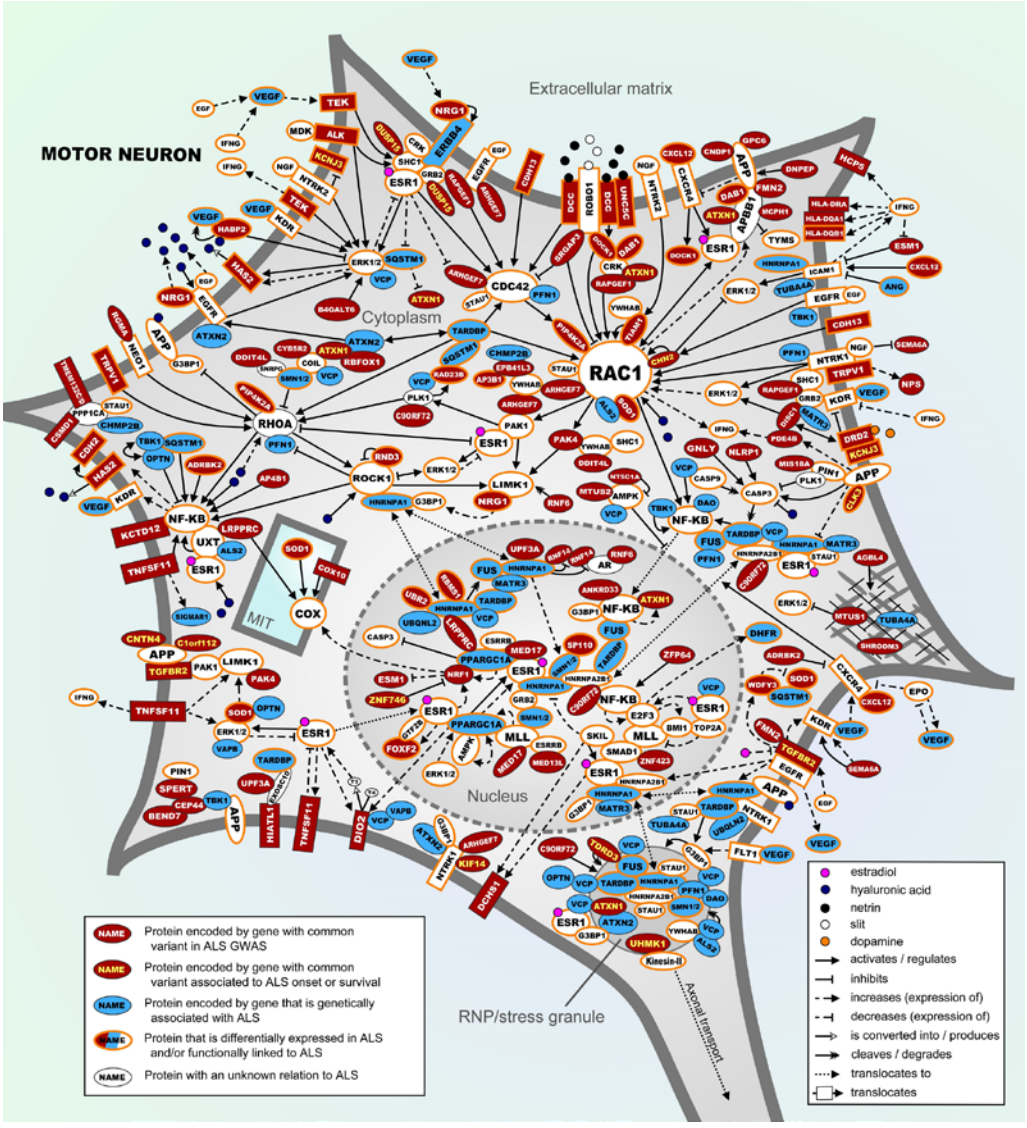
CONTENTS

FIGURES

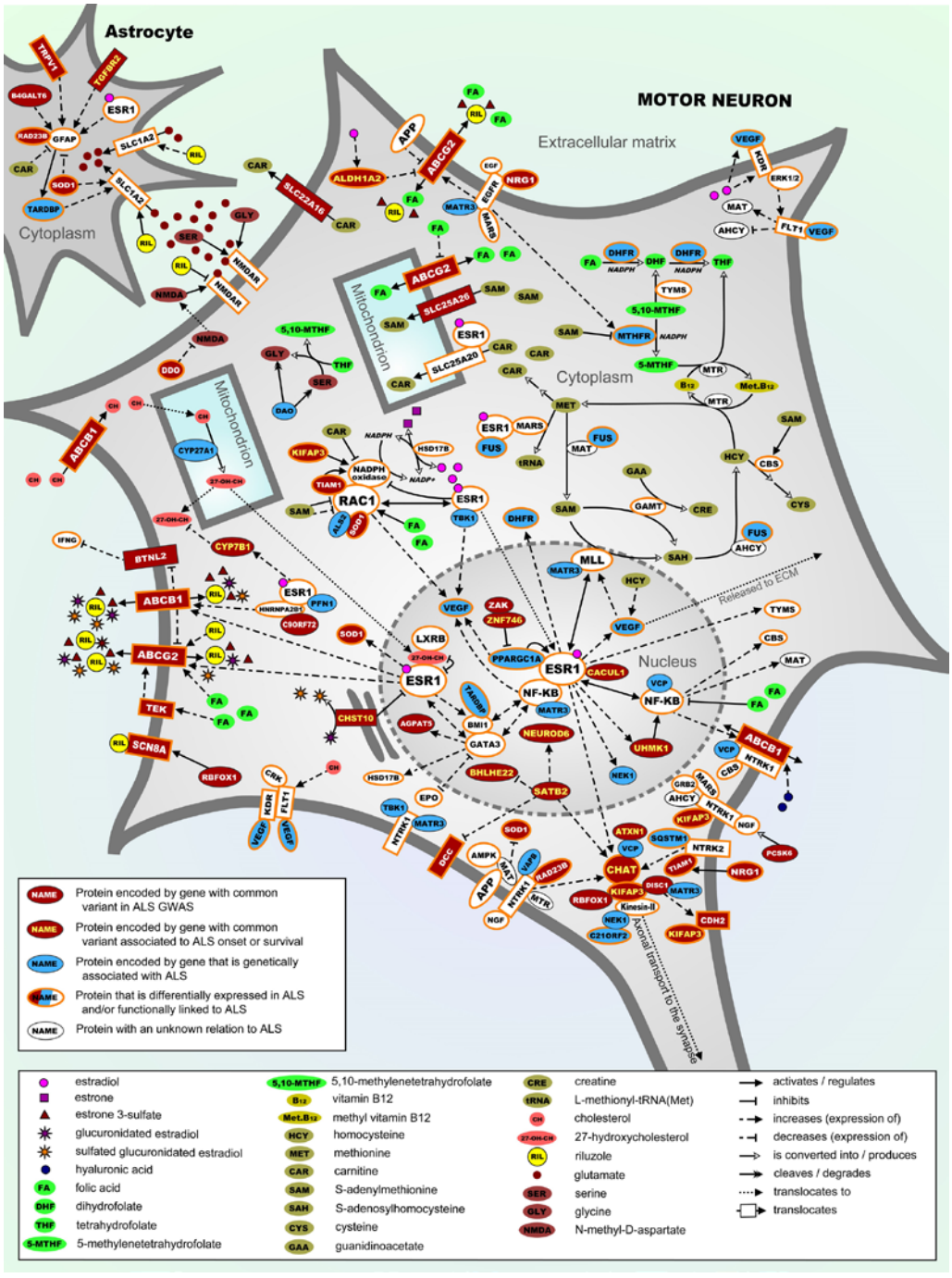
TABLES



Supplementary Figure 1. Top enriched Ingenuity genetic network. Shown is the network with the highest score (P=1.00E-43) and containing the highest number of proteins (23), as obtained by a network enrichment analysis of the ALS GWAS candidate genes from **Supplementary Table 2** using Ingenuity pathway software (www.ingenuity.com).



Supplementary Figure 2. Molecular landscape of ALS (1/2). The landscape is visualized in a motor neuron and especially shows Rho-GTPase signaling and RNP granule formation. MIT, mitochondrion; RNP, ribonucleoprotein particle



Supplementary Figure 3. Molecular landscape of ALS (2/2). The landscape is visualized in a motor neuron and an astrocyte, and it especially shows regulation of estradiol metabolites and the effects of the folic acid and methionine cycles on estradiol signaling.

Supplementary Table 1. Overview of the eleven published genome-wide association studies (GWASs) of amyotrophic lateral sclerosis (ALS). The six GWASs used in our analysis (in total 3588 cases and 8571 controls) are shown in bold; prerequisites for selection: the discovery data ($p<0.0001$) of the GWASs are available online and GWASs are based on independent sets of cases and controls.

GWAS	Discovery sample		Population	Genotyping platform	Phenotype	Diagnosis	Notes
	Patients	controls					
Van Es et al., 2007[1]	461	450	Dutch	Illumina Infinium II HumanHap300 SNP chips	sALS	Diagnosis according to the 1994 El Escorial criteria[2] by neurologists specialized in ALS.	Not, included; not all data online available. Patients with SOD1 mutations were excluded.
Schymick et al., 2007[3]	276	271	White, non-Hispanic	Illumina Infinium II HumanHap550 SNP chips	sALS	N/A	Samples derived from NINDS Neurogenetics repository. Cases had no family history of ALS.
Dunckley et al., 2007[4]	386	542	White	Affymetrix GeneChip Human Mapping 500K Array Sets / Illumina Infinium II HumanHap300 Genotyping BeadChip array	sALS	Diagnosis according to the 2000 El Escorial criteria[5].	Not, included; not all data online available.
Cronin et al., 2008[6]	221	211	Irish	Illumina Infinium HumanHap550 SNP chips	sALS	Diagnosis according to the 1994 El Escorial criteria[2] by neurologists specialized in ALS.	Cases had no family history of ALS.
Van Es et al., 2008[7]	461	450	Dutch	Illumina Infinium II HumanHap300 SNP chips	sALS	Diagnosis according to the 1994 El Escorial criteria[2] by neurologists specialized in ALS.	Not, included; not all data online available. Same dataset as used in Van Es et al., 2007.
Van Es et al., 2009[8]	2323	9013	Dutch/US/Irish/ Swedish/Belgian	Illumina 300K, 370K and 500K Beadchips	sALS	Diagnosis according to the 1994 El Escorial criteria[2] by neurologists specialized in ALS.	Combination of cohorts, overlap with Schymick et al. and Cronin et al.
Chio et al., 2009[9]	266	1190	Italian	Illumina Infinium HumanHap550 SNP chips	sALS	Diagnosis according to the 2000 El Escorial criteria[5].	-
Chio et al., 2009[9]	271	794	US	Illumina Infinium HumanHap550 SNP chips	sALS	N/A	Not included; same patients samples as used in Schymick et al.
Landers et al., 2009[10]	1821 [1]	2258	US/English/ French/Dutch	Illumina Infinium HumanHap300 SNP chips	sALS	Diagnosis according to the 1994 El Escorial criteria[2]	SNPs associated with survival and age of onset were also taken into account. Cases with known SOD1 mutations or family history of ALS were excluded.
Laaksovirta et al., 2010[11]	405	497	Finland	Illumina Infinium HumanHap370 BeadChips/ Illumina Infinium Human1M BeadChips	sALS / fALS	Diagnosis according to the 1994 El Escorial criteria[2] by neurologists specialized in ALS.	106 cases reported family history of ALS (n=93) and/or had a SOD1 mutation (n=40).
Shatunov et al., 2010[12]	599	4144	English	Illumina HumanHap550 BeadChips	sALS	Diagnosed with ALS by two consultant neurologists.	Cases had no family history of ALS.

[1] Dutch samples overlap with Van Es et al. 2007, 2008, 2009, but where not yet included in this analysis. US samples (187) obtained from the NINDS Neurogenetics repository possibly overlap with Schymick et al.

Supplementary Table 2. ALS candidate genes. Top single SNPs located in gene regions (including 100 kb of flanking downstream and/or upstream sequences) and with p<0.0001 for association with amyotrophic lateral sclerosis (ALS) from the genome-wide association studies (GWASs) reported by Schymick et al.[3] (GWAS 1), Cronin et al.[6] (GWAS 2), Chio et al.[9] (GWAS 3), Landers et al.[10] (GWAS 4), Laaksovirta et al.[11] (GWAS 5) and Shatunov et al.[12] (GWAS 6). The genes encoding proteins that could be directly placed in the ALS landscape (Figures 1 and 2) are indicated in **bold**. The column 'Corroborating evidence' indicates that these genes or their protein products are linked to ALS, either by genetic or expression studies in ALS patients or functional studies in animal or cell models of ALS. Single underlined genes are genetically associated with ALS, dotted underlined genes encode proteins that are differentially expressed in ALS patients or are functionally linked through animal and cell models of ALS and double underlined genes are both genetically associated with ALS and encode a protein that is differentially expressed and/or functionally linked.

GWAS 1 (Schymick et al.)

SNP	*P* value	Locus	Gene	Position ~ gene [1]	Corroborating evidence [2]	Shown in Supp. Fig.
rs4363506	6.80E-07	10q26.2	**DOCK1**	24 kb downstream	DOCK1 mRNA is increased in SMNs (1.34x) and in the VH (1.33x) of ALS patients compared to controls[13].	2
rs4363506	6.80E-07	10q26.2	**NPS**	73 kb upstream	-	2
rs16984239	1.70E-06	2p24.2	KCNS3	intronic	KCNS3 mRNA is increased in SMNs (1.28x) and in the VH (1.85x)[13] and decreased in IPS-MNs (-1.55x) of ALS patients compared to controls[14].	
rs6013382	4.70E-06	20q13.2	**ZFP64**	intronic	-	2
rs2782931	5.80E-06	9q31.3	SUSD1	intronic	-	
rs11099864	9.00E-06	4q31.3	FHDC1	intronic	-	
rs332389	1.40E-05	3p14.1	**SLC25A26**	intronic	-	3
rs4964213	1.80E-05	12q23.3	BTBD11	intronic	-	
rs3733242	2.20E-05	4q21.1	**SHROOM3**	nonsyn coding	-	2
rs1037666	2.30E-05	1q43	**FMN2**	intronic	-	2
rs1436918	2.50E-05	15q14	GOLGA8A	56 kb upstream	-	
rs852801	3.20E-05	1p32.2	**DAB1**	intronic	DAB1 is hypermethylated in brains of ALS patients compared to controls[15].	2
rs10459680	3.60E-05	15q26.1	**RGMA**	49 kb upstream	-	2
rs1752784	3.90E-05	9q22.32	**HIATL1**	intronic	-	2
rs5014235	5.90E-05	5q14.1	**AP3B1**	87 kb downstream	AP3B1 mRNA is increased in SMNs (1.27x), in the VH (1.54x)[13] and in IPS-MNs (1.45x) of ALS patients compared to controls[14].	2
rs5014235	5.90E-05	5q14.1	TBCA	45 kb upstream	TBCA mRNA is decreased in SMNs (-1.52x)[13] and in IPS-MNs (-3.63x) of ALS patients compared to controls[14].	
rs7201419	6.30E-05	16q23.3	**CDH13**	intronic	CDH13 mRNA is decreased in SMNs (-1.25x) and in the VH (-1.69x) of ALS patients compared to controls[13]. Further, CDH13 is hypermethylated in brains of ALS patients compared to controls[15].	2
rs11933187	6.30E-05	4q34.1	**CEP44**	intronic	-	2
rs10773543	6.90E-05	12q24.32	**TMEM132C**	intronic	-	2
rs7976059	7.00E-05	12q13.13	**ANKRD33**	30 kb upstream	-	2
rs7976059	7.00E-05	12q13.13	**SCN8A**	49 kb downstream	SCN8A mRNA is decreased in the spinal cord of symptomatic mutant SOD1 mice[16].	3
rs9608416	7.10E-05	22q12.1	**ADRBK2**	intronic	ADRBK2 mRNA is decreased in SMNs (-1.30x), in the VH (-1.30x)[13] and in IPS-MNs (-2.48x) of ALS patients compared to controls[14].	2
rs2272519	8.00E-05	2p24.2	RDH14	24 kb downstream	-	
rs4478530	8.60E-05	8p12	**NRG1**	99 kb upstream	Whereas membrane-bound NRG1 expression is decreases (together with motor neuron loss), secreted NRG1 expression is increased and associated with activation of glial cells in human ALS and mutant SOD1 mice spinal cords[17]. NRG1 protein is increased in spinal cords of ALS patients[18]. Further, NRG1 is neuroprotective in mutant SOD1 mice via restoration of C-boutons of spinal motor neurons[19,20]. NRG1 mRNA (isoforms) is differentially expressed in SMNs and in the VH of ALS patients compared to controls[13].	2,3
rs130110	8.80E-05	22q13.32	FAM19A5	intronic	-	
rs9510982	8.90E-05	13q12.12	C1QTNF9B	77 kb upstream	-	

GWAS 2 (Cronin et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence** [2]	**Shown in Supp. Fig.**
rs3813133	1.05E-05	13q34	CHAMP1	syn coding	-	
rs7316983	1.05E-05	13q34	**UPF3A**	3.6 kb downstream	-	2
rs1325803	1.57E-05	13q14.11	**TNFSF11**	23 kb downstream	-	2
rs1558878	3.50E-05	17q24.2	ARSG	nonsyn coding	-	
rs11208807	3.63E-05	1p31.3	**PDE4B**	intronic	-	2
rs10106208	3.88E-05	8q24.13	**HAS2**	92 kb upstream	HAS2 mRNA is decreased in SMNs (-1.59x) and increased in the VH (1.36x) of ALS patients compared to controls[13].	2
rs17724552	3.92E-05	9q31.2	**RAD23B**	45 kb upstream	RAD23B mRNA is decreased in SMNs (-1.35x) and increased in the VH (1.40x) of ALS patients compared to controls[13].	2,3
rs7245160	4.22E-05	18q22.3	**CNDP1**	15 kb downstream	-	2
rs7245160	4.22E-05	18q22.3	ZNF407	76 kb upstream	-	
rs6473902	4.29E-05	8q11.23	TCEA1	intronic	TCEA1 mRNA is increased in SMNs (2.21x) and decreased in the VH (-1.20x) of ALS patients compared to controls[13].	
rs409037	4.58E-05	5q31.3	GNPDA1	intronic	-	
rs252095	4.58E-05	5q31.3	**RNF14**	intronic	-	2
rs1551960	4.67E-05	16p13.3	**RBFOX1**	intronic	-	2,3
rs9328053	4.70E-05	6p25.3	**FOXF2**	48 kb downstream	FOXF2 mRNA is decreased in SMNs (-3.23x) and in the VH (-1.47x) of ALS patients compared to controls[13].	2
rs2408213	5.24E-05	13q14.3	RNASEH2B	31 kb upstream	-	
rs17105335	5.42E-05	1p33	**AGBL4**	intronic	-	2
rs873108	5.54E-05	11q13.5	TSKU	38 kb upstream	-	
rs9512144	6.15E-05	13q12.13	**RNF6**	intronic	-	2
rs4640677	6.62E-05	4q24	BANK1	intronic	-	
rs4798376	7.57E-05	18p11.31	**EPB41L3**	intronic	-	2
rs17527491	8.44E-05	10p12.2	**PIP4K2A**	24 kb upstream	PIP4K2A mRNA is increased in SMNs (1.74x) and in the VH (1.65x) of ALS patients compared to controls[13].	2
rs2374482	9.73E-05	2p21	THADA	88 kb downstream	-	
GWAS 3 (Chio et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence** [2]	**Shown in Supp. Fig.**
rs716730	4.91E-06	2q23.3	**RND3**	34 kb upstream	RND3 mRNA is differentiall expressed in SMNs (1.30x; -1.45x) and decreased in the VH (-1.37x) of ALS patients compared to controls[13].	2
rs7734164	5.66E-06	5q23.1	**SEMA6A**	intronic	-	2
rs7036709	8.00E-06	9q34.13	**RAPGEF1**	intronic	-	2
rs11792907	8.39E-06	9q34.13	UCK1	28 kb upstream	-	
rs6791324	1.03E-05	3p14.1	SUCLG2	intronic	-	
rs621341	1.34E-05	2q21.3	TMEM163	intronic	-	
rs8081956	2.21E-05	17p13.2	**NLRP1**	16 kb upstream	-	2
rs1505112	2.94E-05	16q12.1	**ZNF423**	intronic	-	2
rs11754231	3.56E-05	6p21.1	**UBR2**	intronic	UBR2 mRNA level in lymphocytes of ALS patients is inversely correlated with time from onset[21].	2
rs12162384	3.83E-05	2q36.3	SLC16A14	26 kb upstream	-	

rs12162384	3.83E-05	2q36.3	**SP110**	72 kb downstream	SP110 mRNA is decreased in SMNs (-2.00x) and differentially expressed in the VH (-1.35/1.43) of ALS patients compared to controls[13].	2
rs1371741	4.08E-05	8q12.3	CLVS1	intronic	-	
rs831768	4.10E-05	1q32.1	TMEM9	8 kb upstream	-	
rs329476	4.24E-05	5q35.1	KCNIP1	intronic	-	
rs514827	4.42E-05	18p11.22	CCDC165	intronic	-	
rs6903608	4.86E-05	6p21.32	**BTNL2**	49 kb upstream	-	3
rs6903608	4.86E-05	6p21.32	**HLA-DRA**	15 kb downstream	-	2
rs12364283	5.33E-05	11q23.2	ANKK1	76 kb downstream	-	
rs12364283	5.33E-05	11q23.2	**DRD2**	542 bp upstream	DRD2 mRNA is decreased in SMNs (-1.25x) and increased in the VH (1.76x) of ALS patients compared to controls[13].	2
rs2231142	5.41E-05	4q22.1	**ABCG2**	nonsyn coding	ABCG2 mRNA and protein are increased in the spinal cord of mutant SOD1 mice and transport activity is increased with disease progression. ABCG2 protein is increased in the spinal cord of ALS patients compared to controls[22]. ABCG2 mRNA is decreased in SMNs (-1.35x) and in the VH (-1.69x) of ALS patients compared to controls[13].	3
rs10264990	5.79E-05	7q21.12	**ABCB1**	intronic	ABCB1 mRNA and protein are increased in the spinal cord of mutant SOD1 mice and transport activity is increased with disease progression[22,23]. ABCB1 protein is increased in the spinal cord of ALS patients compared to controls[22]. ABCB1 mRNA is decreased in SMNs (-1.49x) and increased in the VH (1.33x) of ALS patients compared to controls[13].	3
rs2207356	5.94E-05	6q21	**DDO**	879 bp upstream	DDO mRNA is increased in SMNs (1.33x) and in the VH (1.21x) of ALS patients compared to controls[13].	3
rs2207356	5.94E-05	6q21	**SLC22A16**	4.3 kb downstream	-	3
rs532098	6.45E-05	6p21.32	**HLA-DQA1**	18 kb upstream	-	2
rs532098	6.45E-05	6p21.32	**HLA-DQB1**	49 kb downstream	HLA-DQB1 mRNA is increased in SMNs (1.33x) and in the VH (1.25x) of ALS patients compared to controls[13].	2
rs10014833	6.91E-05	4q32.3	SPOCK3	intronic	-	
rs524047	7.16E-05	18q12.1	**CDH2**	intronic	CDH2 mRNA is increased in SMNs (1.63x; 1.44x)[13] and decreased in IPS-MNs (-2.92x) of ALS patients compared to controls[14].	2,3
rs7560946	7.33E-05	2p21	**LRPPRC**	intronic	-	2
rs11218881	8.06E-05	11q22.1	**CNTN5**	intronic	CNTN5 is hypomethylated in brains of ALS patients compared to controls[15].	NS
rs6495788	8.09E-05	15q13.1	FAM189A1	13 kb upstream	-	
rs571250	9.23E-05	11q13.4	RNF169	intronic	-	
rs9585965	9.62E-05	13q33.1	CCDC168	29 kb downstream	-	
rs9585965	9.62E-05	13q33.1	METTL21C	6 kb upstream	-	
rs12999598	9.77E-05	2q37.3	AQP12A	44 kb upstream	-	
rs12999598	9.77E-05	2q37.3	GPR35	17 kb downstream	GPR35 mRNA is decreased in SMNs (-1.61x) and increased in the VH (1.42x) of ALS patients compared to controls[13].	
rs17464525	9.78E-05	1p13.2	**AP4B1**	syn coding	-	2
GWAS 4 (Landers et al.) (susceptibility)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence** [2]	**Shown in Supp. Fig.**
rs10438933	1.18E-06	18q12.1	**B4GALT6**	8 kb upstream	-	2,3
rs10438933	1.18E-06	18q12.1	MCART2	66 kb downstream	-	
rs16856202	7.98E-06	1q42.2	**DISC1**	intronic	-	2,3
rs873917	8.37E-06	1p34.2	**NT5C1A**	intronic	-	2
rs10192369	8.53E-06	2q24.2	**RBMS1**	31 kb upstream	RBMS1 mRNA is increased in SMNs (1.77x), in the VH (-1.53x)[13] and decreased in IPS-MNs (-7.63x) of ALS patients compared to controls[14].	2

rs1586030	2.22E-05	8p23.2	**CSMD1**	intronic	-	2
rs9923415	2.76E-05	16q23.2	CMC2	75 kb downstream	-	
rs9923415	2.76E-05	16q23.2	CDYL2	97 kb upstream	-	
rs6574333	3.56E-05	14q24.3	LRRC74A	intronic	-	
rs11258392	3.84E-05	10p13	**BEND7**	intronic	-	2
rs2505734	4.17E-05	10q11.21	**CXCL12**	3' UTR	CXCL12 mRNA (isoforms) is increased in SMNs (1.32x; 1.29x; 1.78x), dysregulated in the VH (-1.27x; 1.49x)[13] and increased in IPS-MNs (1.47x) of ALS patients compared to controls[14].	2
rs222741	5.00E-05	17p13.2	SHPK	3.0 kb downstream	-	
rs222741	5.00E-05	17p13.2	**TRPV1**	8 kb upstream	Activation of TRPV1 decreases the astrocyte number, increases the lifespan and improves motor function of mutant SOD1 mice[24].	2,3
rs993927	5.29E-05	18q21.2	**DCC**	intronic	DCC mRNA is increased in SMNs (1.82x; 3.13x) and in the VH (1.58x) of ALS patients compared to controls[13].	2,3
rs7319638	5.65E-05	13q22.3	**KCTD12**	79 kb downstream	-	2
rs2306933	6.22E-05	15q13.1	FAM189A1	nonsyn coding	-	
rs10516970	6.57E-05	4q22.3	**UNC5C**	intronic	UNC5C mRNA is increased in SMNs (1.42x) and in the VH (1.43x) of ALS patients compared to controls[13].	2
rs9555776	6.59E-05	13q34	**ARHGEF7**	intronic	ARHGEF7 is decreased in ALS patient-derived mesenchymal stem cells and reduces their ability to migrate[25].	2
rs3785833	6.64E-05	17q23.2	TBX4	intronic	-	
rs4381747	6.98E-05	2p23.2	**ALK**	intronic	ALK mRNA is increased in SMNs (1.68x; 2.29x) of ALS patients compared to controls[13]. Further, ALK is differentially methylated in brains of ALS patients compared to controls[15].	2
rs1998360	7.16E-05	13q12.3	**MTUS2**	52 kb upstream	-	2
rs7830863	7.26E-05	8p22	**MTUS1**	intronic	-	2
rs1179692	8.68E-05	2q35	TNP1	37 kb upstream	TNP1 mRNA is increased in SMNs (1.58x) and in the VH (1.22x) of ALS patients compared to controls[13].	
rs1005483	8.84E-05	5q31.1	KCT2	100 kb downstream	-	
rs3910444	8.98E-05	12q24.21	**MED13L**	20 kb downstream	-	2
rs2380902	9.41E-05	9p24.2	GLIS3	7 kb downstream	-	
rs2814707	9.54E-05	9p21.2	IFNK	10 kb downstream	-	
rs2814707	9.54E-05	9p21.2	MOB3B	7 kb upstream	-	
rs906236	9.68E-05	10p11.23	LYZL2	44 kb downstream	-	
rs2046402	9.74E-05	4q21.23	**WDFY3**	syn coding	WDFY3 (alfy) promotes autophagic removal of aggregated mutant SOD1 proteins[26].	2
rs7329006	9.95E-05	13q14.13	**SPERT**	25 kb upstream	-	2
GWAS 4 (Landers et al.) (survival) [3]						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence** [2]	**Shown in Supp. Fig.**
rs1541160	1.84E-08	1q24.2	**KIFAP3**	Intronic	Polymorphisms in the KIFAP3 gene are associated with upper motor neuron-predominant phenotype[27] and increased survival in ALS patients[10]. Further, mutant SOD1 impairs axonal transport by sequestering KIFAP3[28].	3
rs855913	4.02E-08	7q36.1	**ZNF746**	9 kb upstream	-	2,3
rs3099950	8.46E-06	2p22.1	MORN2	nonsyn coding	-	
rs4722094	1.06E-05	7p15.3	RAPGEF5	26kb downstream	-	
rs1062976	1.15E-05	1q24.2	SCYL3	syn coding	-	
rs9790230	1.99E-05	3q27.2	**LIPH**	intronic	-	NS
rs958706	4.19E-05	6q12	EYS	intronic	-	
rs10919242	4.26E-05	1q24.2	**C1orf112**	intronic	-	2

rs10919967	4.26E-05	1q32.1	**KIF14**	18kb downstream	-	2
rs10511494	4.27E-05	9p24.1	**PTPRD**	intronic	PTPRD mRNA is increased in SMNs (1.27x) and in the VH (1.28x) of ALS patients compared to controls[13].	NS
rs11072495	4.99E-05	15q24.1	**CLK3**	intronic	CLK3 mRNA is increased in SMNs (1.21x) and decreased in the VH (-1.23x) of ALS patients compared to controls[13].	2
rs2164847	5.16E-05	2q11.2	**CHST10**	50kb downstream	-	3
rs2164847	5.16E-05	2q11.2	LONRF2	20kb upstream	-	
rs3810947	6.18E-05	10q11.23	**CHAT**	5'UTR	CHAT activity and immunoreactivity are reduced in the spinal cord of ALS patients compared to controls[29, 30].	3
rs6493977	6.33E-05	15q21.3	**ALDH1A2**	intronic	ALDH1A2 is a marker of lateral motor column (LMC) motor neurons during differentiation[31, 32].	3
rs10043438	7.03E-05	5q15	MCTP1	intronic	-	
rs3863057	7.29E-05	3p24.1	**TGFBR2**	intronic	-	2,3
rs2107741	8.07E-05	7q34	KDM7A	intronic	-	
rs245857	8.41E-05	7p14.3	**CHN2**	intronic	-	2
rs245857	8.41E-05	7p14.3	**CPVL**	intronic	-	NS
rs3746599	8.93E-05	20q11.21	**DUSP15**	intronic	-	2
rs7519158	9.87E-05	1q43	**FMN2**	intronic	-	2
GWAS 4 (Landers et al.) (age of onset) [4]						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence** [2]	**Shown in Supp. Fig.**
rs697739	4.14E-06	6p22.3	**ATXN1**	Intronic	Intermediate-length PolyQ expansions (≥32) in ATXN1 are associated with ALS[33].	2,3
rs2619566	7.42E-06	3p26.3	**CNTN4**	Intronic	-	2
rs2451852	1.20E-05	5p15.1	**FAM134B**	Intronic	-	NS
rs2055593	1.39E-05	2q11.2	CNGA3	Intronic	CNGA3 is a potential biomarker for ALS[34]. CNGA3 mRNA is decreased in SMNs (-1.39x) and in the VH (-1.23x) of ALS patients compared to controls[13].	
rs6988825	1.44E-05	8p21.3	CSGALNACT1	Intronic	-	
rs2074388	1.69E-05	4q25	ALPK1	nonsyn coding	-	
rs2652442	2.39E-05	2q24.1	**KCNJ3**	Intronic	KCNJ3 mRNA is increased in SMNs (1.24x) and in the VH (1.30x) of ALS patients compared to controls[13].	2
rs6728825	2.50E-05	2q33.3	PLEKHM3	intronic	-	
rs7513662	3.15E-05	1q23.3	**UHMK1**	intronic	-	2,3
rs11198623	5.11E-05	10q26.11	**CACUL1**	57kb upstream	-	3
rs12351857	5.37E-05	9q22.1	NXNL2	intronic	-	
rs7338819	6.30E-05	13q21.2	**TDRD3**	68kb upstream	-	2
rs1316277	6.44E-05	1p36.21	TMEM51	intronic	-	
rs2375754	7.75E-05	1p31.3	**AK4**	1.6kb upstream	AK4 protein is increased in the spinal cords of mutant SOD1 mice[35].	NS
rs7210741	8.16E-05	17q25.3	RBFOX3	intronic	-	
rs7075318	8.29E-05	10q11.23	WDFY4	intronic	-	
rs10266898	8.53E-05	7p14.3	**NEUROD6**	48kb downstream	-	3
rs6981465	8.62E-05	8q12.3	**BHLHE22**	43kb upstream	-	3
rs6981465	8.62E-05	8q12.3	**CYP7B1**	50kb downstream	-	3
rs2287232	8.77E-05	2p16.3	**NRXN1**	intronic	-	NS
rs987281	9.05E-05	2q33.1	**SATB2**	3'UTR	-	3

GWAS 5 (Laaksovirta et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence** [2]	**Shown in Supp. Fig.**
rs3849942	9.11E-11	9p21.2	**C9ORF72**	3.3 kb downstream	A hexanucleotide repeat expansion in C9ORF72 is associated with fALS[36,37].	2,3
rs3849942	9.11E-11	9p21.2	MOB3B	14 kb upstream	-	
rs2814707	1.18E-10	9p21.2	IFNK	10 kb downstream	-	
rs13048019	2.58E-08	21q22.11	**TIAM1**	intronic	TIAM1 mRNA is increased in SMNs (1.92x; 1.51x) and decreased in the VH (-1.28x) of ALS patients compared to controls[13].	2,3
rs10511777	1.52E-06	9p21.2	CAAP1	57 kb downstream	-	
rs12627601	2.57E-06	21q22.11	SCAF4	95 kb downstream	-	
rs12627601	2.57E-06	21q22.11	**SOD1**	48 kb upstream	Mutations in SOD1 are associated with familial amyotrophic lateral sclerosis[38]. SOD1 mRNA is increased in SMNs (1.36x)[13] and in IPS-MNs (1.84x) of ALS patients compared to controls[14].	2,3
rs7390085	4.99E-06	9p21.2	IFT74	intronic	-	
rs7166898	1.77E-05	15q26.3	**PCSK6**	intronic	-	3
rs2043061	2.27E-05	14q31.1	**DIO2**	intronic	-	2
rs1873264	2.35E-05	3p14.1	FAM19A4	intronic	-	
rs3803478	2.42E-05	15q26.3	FAM169B	33 kb upstream	-	
rs2833752	3.97E-05	21q22.11	**MIS18A**	intronic	-	2
rs2063082	4.19E-05	11p15.4	**DCHS1**	intronic	-	2
rs911704	4.43E-05	10q25.3	**HABP2**	intronic	HABP2 mRNA is decreased in SMNs (-2.50x) and increased in the VH (1.38x) of ALS patients compared to controls[13].	2
rs4525696	4.45E-05	2p11.2	ATOH8	40 kb upstream	-	
rs4525696	4.45E-05	2p11.2	**GNLY**	12 kb downstream	-	2
rs2407759	4.50E-05	5q11.2	**ESM1**	76 kb downstream	-	2
rs1013741	6.93E-05	12q24.33	**TMEM132D**	intronic	-	2
rs1459826	7.50E-05	11p15.4	OR5P2	71 kb downstream	-	
rs7044842	7.95E-05	9p21.2	**TEK**	intronic	TEK mRNA is increased in SMNs (1.65x) and decreased in the VH (-1.45x) of ALS patients compared to controls[13].	2,3
rs6559175	7.99E-05	8p23.1	**AGPAT5**	32 kb upstream	-	3
rs6559175	7.99E-05	8p23.1	**MCPH1**	33 kb downstream	-	2
rs1600970	9.30E-05	11p15.4	**CYB5R2**	57 kb upstream	-	2
rs4674431	9.73E-05	2q35	**DNPEP**	46 kb upstream	-	2
rs178758	9.75E-05	14q11.2	RNF212B	intronic	-	
rs7758090	9.77E-05	6p21.33	**HCP5**	intronic	-	2
GWAS 6 (Shatunov et al.)						
SNP	***P* value**	**Locus**	**Gene**	**Position ~ gene** [1]	**Corroborating evidence** [2]	**Shown in Supp. Fig.**
rs903603	8.92E-08	9p21.2	MOB3B	intronic	-	
rs774359	1.09E-06	9p21.2	**C9ORF72**	3' UTR / intronic	A hexanucleotide repeat expansion in C9ORF72 is associated with fALS[36,37].	2,3
rs2866197	2.52E-06	4q24	**DDIT4L**	711 bp downstream	-	2
rs2814707	3.32E-06	9p21.2	IFNK	10 kb downstream	-	
rs7003470	8.91E-06	8p21.3	CSGALNACT1	intronic	-	
rs4684627	1.45E-05	3p25.3	**SRGAP3**	intronic	-	2

rs1494913	1.73E-05	8q23.1	RSPO2	23 kb downstream	RSPO2 protein is decreased in the spinal cord of sALS patients and of mutant SOD1 mice compared to controls[34].	
rs11669124	2.34E-05	19q13.2	**PAK4**	intronic	-	2
rs878765	2.84E-05	13q31.3	**GPC6**	intronic	-	2
rs8053509	4.33E-05	16p13.2	**ABAT**	intronic	-	NS
rs606087	4.60E-05	11q21	**MED17**	3' UTR	-	2
rs3736626	5.97E-05	7q32.2	**NRF1**	intronic	-	2
rs2836770	6.19E-05	21q22.2	TMPRSS3	intronic	-	
rs6004919	7.01E-05	22q12.1	MYO18B	28 kb downstream	-	
rs3769185	7.08E-05	2q31.1	**ZAK**	intronic	-	3
rs17680211	7.51E-05	17p12	CDRT15	32 kb upstream	-	
rs17680211	7.51E-05	17p12	**COX10**	57 kb downstream	-	2
rs1517166	8.11E-05	18q22.1	SERPINB8	25 kb downstream	SERPINB8 mRNA is differentially expressed in SMNs (-1.69x; 1.28x) of ALS patients compared to controls[13].	
rs1527307	9.00E-05	7q34	MGAM	intronic	-	
rs7298545	9.51E-05	12p13.32	DYRK4	intronic	-	
rs2726355	9.75E-05	11q22.1	**CNTN5**	intronic	CNTN5 is hypomethylated in brains of ALS patients compared to controls[15].	NS

[1] Genetic position according to the Ensembl Human Genome Browser (http://www.ensembl.org/Homo_sapiens/).
[2] Expression data from genome wide expression studies in ALS were only included when they were based on spinal cord, spinal ventral horn gray matter, laser-captured microdissected motor neurons or induced pluripotent stem cell-derived motor neuron from ALS patients[13, 14, 39, 40]. A gene was considered differentially expressed when it was reported, at least twice, on two different arrays with a fold change ≥1.2 or ≤1.2.
[3] SNPs associated to survival in ALS patients. Indicated separately in the figures by gray name letters.
[4] SNPs associated to age of onset in ALS patients. Indicated separately in the figures by gray name letters.

Supplementary Table 3. Additional genes that encode proteins located in the ALS landscape. The column 'Corroborating evidence' indicates that these genes or their protein products are linked to ALS, either by genetic, expression or immunohistological studies in ALS patients or functional studies in animal or cell models of ALS. Single underlined genes are genetically associated with ALS, dotted underlined genes encode proteins that are differentially expressed in ALS patients or are functionally linked through animal and cell models of ALS and double underlined genes are both genetically associated with ALS and encode a protein that is differentially expressed and/or functionally linked.

Gene	Locus	Corroborating evidence [1]	Shown in Supp. Fig.
AHCY	20q11.22	-	3
ALS2	2q33.1	ALS2 mutations are associated with fALS[41].	2,3
AMPK-complex	[2]	Cytoplastic mislocalization of TARDBP is mediated by PRKAA1 activation in motor neurons of ALS patients[42] and reduced AMPK activity prevents mutant SOD1-induced motor neuron death in a mutant SOD1 mouse model[43]. AMKP subunits are regulated in ALS, i.e. PRKAA1 mRNA is decreased in SMNs (-2.38x) and in the VH (-1.49x)[13] and in IPS-MNs (-1.46x) of ALS patients compared to controls[14]. Further, PRKAA2 is increased in the SMNs (1.26x) and in the VH (1.55x) of ALS patients compared to controls[13].	2,3
ANG	14q11.2	Mutations in ANG are associated with fALS and sALS[44]. ANG protein levels are dysregulated in the plasma of ALS patients compared to controls[45, 46].	2
APBB1	11p15.4	-	2
APP	21q21.3	APP is accumulated in the spinal cord motor neurons of ALS patients[47]. Soluble levels of APP are elevated in the CSF of ALS patients[48] and inhibition of APP cleavage or knock out of APP in a mutant SOD1 mice model decreased the levels of soluble APP, delayed disease onset and improved motor function and motor neuron survival[49, 50]. APP mRNA is increased in SMNs (1.39x) and decreased in the VH (-1.20x) of ALS patients compared to controls[13].	2,3
AR	Xq12	-	2
ATXN2	12q24.12	Intermediate-length polyQ expansions (27-33 glutamines, CAG repeats) in ATXN2 are significantly associated with ALS[51-56] and are a modifier for survival[57].	2
BMI1	10p12.2	-	2,3
C21ORF2	21q22.3	Nonsynonymous and loss-of-function mutations in C21ORF2 are associated with ALS risk[58]. C21ORF2 mRNA is decreased in SMNs (-1.32x) of ALS patients compared to controls[13].	3
CASP3	4q35.1	CASP3 mRNA is increased in SMNs (2.17x; 1.89x) of ALS patients compared to controls[13].	2
CASP9	1p36.21	CASP9 is activated in the spinal motor neurons of ALS patients[59] and is increased in the serum of ALS patients compared to controls[60]. CASP9 mRNA is increased in SMNs (1.89x)[13] and in IPS-MNs (4.20x) from ALS patients compared to controls[14].	2
CBS	21q22.3	CBS mRNA is increased in SMNs (1.46x) and in the VH (1.82x) of ALS patients compared to controls[13].	3
CDC42	1p36.12	CDC42 mRNA is increased in SMNs (1.50x) and in the VH (1.31x) of ALS patients compared to controls[13].	2
CHMP2B	3p11.2	Mutations in CHMP2B are associated with ALS[61, 62].	2
COIL	17q22	COIL mRNA is decreased in SMNs (-1.30x)[13] and in IPS-MNs (-5.00x) from ALS patients compared to controls[14].	2
COX	[3]	Cytochrome c oxidase / complex IV activity is decreased in spinal cords of ALS patients compared to controls[63, 64].	2
CRK	17p13.3	CRK mRNA is decreased in SMNs (-1.67x) and increased in the VH (1.38x) of ALS patients compared to controls[13].	2,3
CXCR4	2q22.1	Motor neurons in the spinal cord of mutant SOD1 mice are neuroprotected by LeX+ CXCR4+ neural stem cells[65]. In a SOD1 mouse model CXCR4 mRNA is increased starting from the onset of symptoms[66].	2
CYP27A1	2q35	Polymorphisms increasing CYP27A1 expression are associated with increased susceptibility to ALS[67].	3
DAO	12q24.11	A mutation in DAO is associated with fALS[68].	2,3
DHFR	5q14.1	A deletion in DHFR (c.594+59del19bp) is associated with bulbar onset ALS[69]. DHFR mRNA is increased in SMNs (1.41x) and in the VH (1.31x)[13] and decreased in IPS-MNs (-2.43x) of ALS patients compared to controls[14].	2,3
E2F3	6p22.3	E2F3 mRNA is increased in SMNs (1.75x)[13] and decreased in IPS-MNs (-3.82x) of ALS patients compared to controls[14].	2
EGF	4q25	EGF protein levels are reduced in the CSF of ALS patients compared to controls[70, 71]. EGF mRNA is increased in SMNs (1.27x) and in the VH (1.62x) of ALS patients compared to controls[13].	2,3
EGFR	7p11.2	Inhibition of EGFR delays disease progression, but does not improve survival in the SOD1 mouse model of ALS[72]. EGFR mRNA is increased in the spinal cord (>10x)[40], but decreased in SMNs (-1.43x) and in the VH (-1.49x) of ALS patients compared to controls[13].	2,3

EPO	7q22.1	EPO protein levels are lower in the serum and CSF of ALS patients[73,74] and seem to point towards a rapid progression of disease[73]. EPO delays disease onset in (female) mutant SOD1 mice[75,76] and recombinant EPO reduces the aggregation of mutant SOD1 in a mutant SOD1 cell model[77]. EPO mRNA is decreased in SMNs (-1.54x) and in the VH (-1.28x) of ALS patients compared to controls[13].	2,3
ERBB4	2q34	Mutations in ERBB4 reduce its ability to autophosphorylate upon NRG1 stimulation and causes ALS type 19[78]. ERBB4 protein expression is reduced in the motor neurons of mutant SOD1 mice[20]. ERBB4 mRNA is differentially regulated (1.81x; -1.21x) in the SMNs of ALS patients compared to controls[13].	2
ERK1/2	16p11.2/ 22q11.22	Aggregates of abnormal phosphorylated ERK1/2 were found in motor neurons of the spinal cord of ALS patients[79].	2
ESR1	6q25.1	ESR1 mRNA is increased in SMNs (1.23x) and decreased in the VH (-1.37x) of ALS patients compared to controls[13].	2,3
ESRRB	14q24.3	ESRRB mRNA is increased in SMNs (1.44x) and in the VH (1.29x)[13] and decreased in IPS-MNs (-1.51x) of ALS patients compared to controls[14].	2
EXOSC10	1p36.22	-	2
FLT1	13q12.3	(VEGFR-1) FLT1 mRNA is increased in SMNs (1.50x; 1.27x) of ALS patients compared to controls[13].	2,3
FLT4	5q35.3	(VEGFR-3) FLT4 mRNA is decreased in SMNs (-1.27x)[13] and increased in IPS-MN (1.53x) of ALS patients compared to controls[14].	NS
FPGS	9q34.11	-	NS
FUS	16p11.2	Mutations in the FUS are associated with fALS[80,81]. FUS mRNA is decreased in SMNs (-1.96x) and in the VH (-1.32x) of ALS patients compared to controls[13].	2,3
G3BP1	5q33.1	G3BP1 rescues the defective assembly of stress granules due to depletion of TARDBP[82].	2
GAMT	19p13.3	GAMT mRNA is decreased in SMNs (-5.56x) and in the VH (-1.41x) of ALS patients compared to controls[13].	3
GATA3	10p14	GATA3 mRNA is reduced in T-lymphocytes of ALS patients and inversely correlated with the disease progression rate[83].	3
GFAP	17q21.31	Acetylated GFAP and fragmented GFAP levels are increased in the spinal cords of ALS patients[84,85]. GFAP is increased in the CSF of patients with ALS compared to patients with other neurological diseases[86] and loss of GFAP accelerates disease progression in a mutant SOD1 mice model by enhancing glial cell activation[87].	3
GLE1	9q34.11	Mutations in GLE1 are associated with ALS[88].	NS
GRB2	17q25.1	GRB2 mRNA is increased in SMNs (1.48x; 2.04x) of ALS patients compared to controls[13].	2,3
GTF2B	1p22.2	GTF2B mRNA is increased in SMNs (2.94x; 1.76x) and decreased in the VH (-1.23x) of ALS patients compared to controls[13].	2
HNRNPA1	12q13.13	HNRNPA1 protein expression is decreased in spinal cord motor neurons of ALS patients compared to controls[89] and pathogenic mutations in HNRNPA1 were found in a dominant fALS patient and in a late-onset sALS patient[90].	2
HNRNPA2B1	7p15.2	HNRNPA2B1 mRNA is increased in SMNs (1.40x) and in the VH (1.36x)[13] and decreased in IPS-MNs (-2.99x) of ALS patients compared to controls[14].	2,3
HSD17B1	17q21.2	(HSD17B) HSD17B1 mRNA is decreased in SMNs (-1.92x) and in the VH (-1.92x) of ALS patients compared to controls[13].	3
HSD17B2	16q23.3	(HSD17B) HSD17B2 mRNA is increased in SMNs (1.37x) and in the VH (1.37x) of ALS patients compared to controls[13].	3
ICAM1	19p13.2	ICAM is increased in the spinal cord of a mutant SOD1 mouse[91,92].	2
IFNG	12q15	IFNG is increased in the spinal cord of sporadic ALS patients compared to compared to controls[93]. IFNG mRNA is decreased in SMNs (-5.26x) and in the VH (-1.28x) of ALS patients compared to controls[13].	2,3
ITGAL	16p11.2	-	NS
KDR	4q12	(VEGFR-2) KDR mRNA is increased in SMNs (2.18x)[13] and decreased in the VH (-1.33x)[13] and anterior horn cells[94] of ALS patients compared to controls.	2,3
Kinesin-II	[4]	Kinesin-II subunits are regulated in ALS, i.e. KIF3A mRNA and protein are decreased in the upper motor neurons of the motor cortex of sALS patients compared to controls[95], and KIF3C mRNA is increased in SMNs (1.51x) and in the VH (1.32x) of ALS patients compared to controls[13].	2,3
LIMK1	7q11.23	LIMK1 mRNA is increased in SMNs (1.28x) and decreased in the VH (-1.47x) of ALS patients compared to controls[13].	2
MARS	12q13.3	MARS mRNA is decreased in the VH (-1.47x)[13] and decreased in IPS-MNs (-2.60x) of ALS patients compared to controls[14].	3
MAT	[5]	In erythrocytes, MAT activity (Vmax) is 33% higher, but the affinity of MAT for methionine (Km) is 41% lower in male ALS patients compared to controls[96].	3
MATR3	5q31.2	Mutations in MATR3 are associated with ALS[97,98].	2,3
MDK	11p11.2	MDK mRNA is decreased in SMNs (-5.88x) and in the VH (-2.04x) of ALS patients compared to controls[13].	2
MLL-complex	[6]	MLL-complex subunits are regulated in ALS, i.e. KMT2A mRNA (core subunit) is decreased in SMNs (-1.39x) and increased in the VH (1.20x), HCFC1 mRNA (core subunit) is decreased in SMNs (-5.26x) and in the VH (-1.67x), RBBP5 mRNA (core subunit) is dysregulated in SMNs (2.63x; -2.56x), MAX (facultative subunit) is decreased in SMNs (-1.35x) and increased in the VH (1.30x) and MEN1 (facultative subunit) in increased in SMNs (1.46x), in the VH (1.56x) and in IPS-MNs (2.14x) of ALS patients compared to controls[13,14].	2,3

MTHFR	1p36.22	MTHFR polymorphism C677T is associated with bulbar onset sALS[69]. Further, the C677T polymorphism, the genotype T667T and the compound genotypes C677C/A1298A and T677T/A1298A are associated with (spinal onset in) sALS in female only, whereas the C1298C genotype and the C66T/C1298C compound genotype are associated with bulbar onset sALS in female patients only[99]. MTHFR mRNA is decreased in SMNs (-1.30x) and increased in the VH (2.04x) of ALS patients compared to controls[13].	3
MTR	1q43	-	3
NADPH-oxidase	[7]	NADPH-oxidase is activated in spinal cords of ALS patients compared to controls[100] and low NADPH-oxidase activity increases survival of ALS patients[101]. SOD1 inhibits RAC1-dependent activation of NADPH-oxidase, which is abolished in mutant SOD1 mice, leading to enhanced RAC1 and NAPDH oxidase activation[102]. NADPH subunits are regulated in ALS, i.e. CYBB (NOX2) mRNA is increased in the SMNs (2.15x) and in the VH (3.51x), NCF1 mRNA is increased in SMNs (1.53x) and in IPS-MNs (1.69x) of ALS patients compared to controls[13,14].	3
NEFH	22q12.2	Phosphorylated NEFH protein is increased in the CSF of ALS patients compared to controls[103].	NS
NEFL	8p21.2	NEFL mRNA is increased in the spinal cord of ALS patients compared to controls[104] and is a potential biomarker for ALS[34].	NS
NEFM	8p21.2	NEFM protein is increased in the plasma of ALS patients compared to controls[105].	NS
NEK1	4q33	NEK1 variants are associated with ALS risk[106].	3
NEO1	15q24.1	NEO1 mRNA is decreased in SMNs (-1.47x) and in the VH (-1.20x)[13] and increased in IPS-MNs (1.44x) of ALS patients compared to controls[14].	2
NF-KB-complex	[8]	NF-KB subunits are regulated in ALS, i.e. RELA mRNA and protein are increased in the spinal cords of ALS patients compared to controls[107]. Moreover, RELA protein is increased in microglia in the spinal cord of ALS patients and absent in motor neurons[108]. NFKB2 mRNA is increased in SMNs (1.71x) and decreased in the VH (-1.43x), RELA mRNA is increased in SMNs (3.99x; 5.05x) and in the VH (2.48x), RELB mRNA is dysregulated in SMNs (-1.89x; 1.38x) and increased in the VH (1.28x) of ALS patients compared to controls[13].	2,3
NGF	1p13.2	NGF protein is increased in the remaining motor neurons in ALS patients compared to controls[109] and plasma levels of NGF protein are positively correlated to disease duration[110].	2,3
NMDAR	[9]	NMDAR subunits are regulated in ALS, i.e. GRIN2A mRNA is decreased in the spinal cord (>-2x) of ALS patients compared to controls[111] and increased GRIN2B/GRIN2A ratios in motor neurons makes them selectively vulnerable to glutamate toxicity[112]. GRIN2B mRNA is increased in SMNs (1.28x) and in the VH (1.21x) of ALS patients compared to controls. GRIN2D mRNA is decreased in SMNs (-3.33x) and in the VH (-1.49x) of ALS patients compared to controls[13].	3
NR1H2	19q13.33	Inactivation of LXRB (encoded by NR1H2) leads to motor neuron degeneration in male mice[113].	3
NTRK1	1q23.1	NTRK1 protein is increased in the remaining motor neurons in ALS patients compared to controls[109].	2,3
NTRK2	9q21.33	NTRK2 protein and mRNA expression is increased, whereas phosphorylation of NTRK2 protein is decreased in the spinal cords of ALS patients compared to controls (10786708). Further, deletion of a truncated isoform of NTRK2 delays disease onset in a SOD1 mouse model[114]. NTRK2 mRNA increased in the spinal cord (2.40x)[40], dysregulated in SMNs (1.74x; -1.25) and decreased in the VH (-1.92x) of ALS patients compared to controls[13].	2,3
OPTN	10p13	Mutations in OPTN are associated with fALS[115].	2
PAK1	11q13.5	PAK1 mRNA is decreased in SMNs (-1.22x; -1.20) and in the VH (-1.25x) of ALS patients compared to controls[13].	2
PFN1	17p13.2	Mutations in PFN1 are associated with fALS[116].	2,3
PIN1	19p13.2	PIN1 mRNA is increased in SMNs (2.80x; 1.54x) and decreased in the VH (-1.43x) of ALS patients compared to controls[13].	2
PLK1	16p12.2	-	2
PPARGC1A	4p15.2	PPARGC1A mRNA is decreased in sALS and in mutant SOD1 mice[117]. Polymorphisms in the brain-specific promotor of PPARGC1A are associated with age of onset and survival in male ALS patients. Further, deficiency of full-length PPARGC1A leads to earlier age of onset and reduced survival in a male, but not female mutant SOD1 mice[118]. PPARGC1A is neuroprotective and slows down disease progression in mutant SOD1 mice[119-121].	2,3
PPP1CA	11q13.2	-	2
PRPH	12q13.12	Mutations in PHPR are associated with ALS[122-126]. Further, PRPH mRNA is increased in the spinal cord of ALS patients compared to controls[104] and a potential biomarker for ALS[34].	NS
RAC1	7p22.1	ALS2 regulates motor neuron survival en outgrowth in a RAC1-dependent manner, i.e. expression of constitutively active mutant RAC1 counteracts ALS2 knockdown, whereas expression of a dominant-negative RAC1 mutant mimicks ALS2 knockdown[127]. RAC1 mRNA is decreased in SMNs (-2.50x) and in the VH (-1.82x) of ALS patients compared to controls[13].	2,3
RHOA	3p21.31	-	2
ROBO1	3p21.3	ROBO1 mRNA is increased in SMNs (1.46x) and decreased in the VH (-1.47x) of ALS patients compared to controls[13]. ROBO1 is hypermethylated in brains of ALS patients compared to controls[15].	2

ROCK1	18q11.2	ROCK1 functions as a disease progression marker in the SOD1 mice model[128]. ROCK1 mRNA is increased in SMNs (1.48x) and decreased in the VH (-1.37x) of ALS patients compared to controls[13].	2
SHC1	1q21.3	SHC1 mediates onset, motor function and survival in mutant SOD1 mice by regulating RAC1 activation[129].	2
SIGMAR1	9p13.3	A mutation in SIGMAR1 is associated with juvenile fALS[130,131].	2
SKIL	3q26.2	SKIL mRNA is increased in SMNs (1.35x) and in the VH (1.50x) of ALS patients compared to controls[13].	2
SLC1A2	11p13	Editing of SLC1A2 pre-mRNA is increased in the spinal cord and motor cortex of ALS patients[132].	3
SLC25A20	3p21.31	SLC25A20 protein is increased in the plasma of ALS patients compared to controls[105]. SLC25A20 mRNA is increased in SMNs (1.89x) and in the VH (1.31x) of ALS patients compared to controls[13].	3
SMAD1	4q31.21	SMAD1 mRNA is increased in SMNs (1.21x) and in the VH (1.51x) of ALS patients compared to controls[13].	2
SMN1/2	5q13.2 / 5q13.2	SMN1 gene duplications are increased in sporadic ALS patients compared to controls[133,134], whereas homozygous SMN2 deletion is increased in sporadic ALS patients compared to controls[135]. SMN1 mRNA is increased in the VH (1.36x)[13] and decreased in IPS-MNs (-1.65x) of ALS patients compared to controls[14].	2
SNRPG	2p13.3	-	2
SQSTM1	5q35.3	Mutations in SQSTM1 are associated with fALS and sALS[136-139] and SQSTM1 accumulates and enhances formation of protein aggregates in the spinal cord of ALS patients and mutant SOD1 mice[140-142].	2,3
STAU1	20q13.13	STAU1 mRNA is decreased in SMNs (-1.47x) and increased in the VH (3.66x) [13] and increased in IPS-MNs (1.95x) of ALS patients compared to controls[14].	2
TARDBP	1p36.22	Mutations in TARDBP are associated with fALS and sALS[143] and result in loss of nuclear TARDBP and dysregulation of RNA processing in motor neurons[144].	2,3
TBK1	12q14.2	Mutations in TBK1 are associated with ALS[145,146].	2,3
TOP2A	17q21.2	TOP2A mRNA is dysregulated in SMNs (-5.88x; 2.58x) and increased in the VH (1.21x) of ALS patients compared to controls[13].	2
TUBA4A	2q35	Mutations in TUBA4A are associated with ALS[147,148].	2
TYMS	18p11.32	TYMS mRNA is decreased in SMNs (-1.28x) and increased in the VH (1.56x) of ALS patients compared to controls[13].	2,3
UBQLN2	Xp11.21	Mutations in UBQLN2 are associated with (juvenile) fALS[149].	2
UXT	Xp11.23	UXT mRNA is increased in SMNs (1.27x) and in the VH (1.30x) of ALS patients compared to controls[13].	2
VAPB	20q13.32	A mutation in VAPB is associated with fALS[150-152].	2,3
VCP	9p13.3	Mutations in VCP are associated with fALS[153].	2,3
VEGF	[10]	The haplotypes -2,578A/-1,154A/-634G and -2,578A/-1,154G/-634G in the VEGFA promotor are associated with an increased ALS risk[154]. VEGF CSF levels are reduced in early ALS patients compared to controls[155]. VEGF plasma levels are reduced by ~50% in sporadic ALS patients compared to healthy controls[154]. VEGFA mRNA is decreased in SMNs (-1.39x), VEGFB mRNA is increased in SMNs (2.02x; 1.98x) and in the VH (1.64x), VEGFC is decreased in SMNs (-3.13x) and in the VH (-1.33x), and PGF is decreased in SMNs (-1.79x; -2.86x) of ALS patients compared to controls[13].	2,3
YWHAB	20q13.12	Lewy body-like hyaline inclusions in the spinal cord of a fALS patient are immunopositive for YWHAB[156]. YWHAB mRNA is increased in SMNs (1.47x; 1.58x)[13] and decreased in IPS-MNs (-3.92x) of ALS patients compared to controls[14].	2

[1] Expression data from genome wide expression studies in ALS were only included when they were based on spinal cord, spinal ventral horn gray matter, laser-captured microdissected motor neurons or induced pluripotent stem cell-derived motor neuron from ALS patients[13,14,39,40]. A gene was considered differentially expressed when it was reported, at least twice, on two different arrays with a fold change >1.2 or <1.2.
[2] Complex consisting of (a combination of) the subunits PRKAA1, PRKAA2, PRKAB1, PRKAB2, PRKAG1, PRKAG2 and PRKAG3.
[3] Complex consisting of multiple proteins and part of the electron transport chain (complex IV).
[4] Complex consisting of (a combination of) the subunits KIF3A, KIF3B and KIF3C.
[5] Consists of the isoforms MAT1A, MAT2A and MAT2B.
[6] Complex consisting of (a combination of) the core subunits ASH2L, DPY30, HCFC1, HCFC2, KMT2A, RBBP5 and WDR5 and (among others) the facultative subunits MAX, MGA and MEN1.
[7] Complex consisting of (a combination of) the subunits CYBA, CYBB, NCF1, NCF2, NCF4 and RAC1.
[8] Complex consisting of (a combination of) the subunits NFKB1, NFKB2, REL, RELA and RELB.
[9] Complex consisting of (a combination of) the subunits GRIN1, GRIN2A, GRIN2B, GRIN2C, GRIN2D
[10] The VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD, PGF

Supplementary Table 4. Overview of ALS patient cohort			
	Spinal onset	**Bulbar onset**	**Total**
Male patients	341	114	455
Female patients	171	121	292
Mean age at first symptoms (years)	58.9	62.12	59.89
Mean age at death (years)	62.25	64.57	62.98
Mean duration of disease (months)	41.24	28.91	37.36

DETAILED DESCRIPTION OF THE MOLECULAR LANDSCAPE FOR AMYOTROPHIC LATERAL SCLEROSIS

CONTENTS

INTRODUCTION

In this supplementary section, the protein interactions and regulatory processes in the molecular landscape of amyotrophic lateral sclerosis (ALS) are described and divided into three themes: (1) Rho-GTPase signaling, (2) estradiol signaling, and (3) ribonucleoprotein particle (RNP) granules and intermediate filament functioning. These themes overlap functionally and are all involved in neurite outgrowth.

In the description of the ALS landscape, names of proteins derived from the genome-wide association studies (GWASs) are in **bold**, single-underlined proteins are genetically associated with ALS, dotted underlined genes encode proteins that are differentially expressed in ALS or functionally linked to ALS, and double underlined genes are genetically associated with ALS *and* encode a protein that is differentially expressed in or functionally linked to ALS. The gene name abbreviations refer to both the gene and the protein. Furthermore, the terms 'activates' and 'inhibits' are used to indicate the activation/inhibition of a protein (e.g. by (de)phosphorylation) by another protein. To describe a difference in abundance of a protein (directly or indirectly) induced by another protein, the terms 'increases the expression of' or 'decreases the expression of' are used. Finally, interactions in the ALS landscape that are not shown in the figures are indicated as '(not shown)' in the text.

Supplementary Figures 2 and 3 show all relevant protein interactions in the ALS landscape; the metabolites within the landscape are mostly presented in **Supplementary Figure 3**. **Supplementary Table 2** presents all GWAS gene-encoded proteins in the landscape and the corroborating evidence for their associations with ALS, and in **Supplementary Table 3** all other landscape proteins and the corroborating evidence for their associations with ALS are listed.

Not all GWAS gene-encoded proteins were placed in the landscape, either because they do not interact with any of the landscape proteins or due to lack of annotation, or both. However, this does not necessarily mean that they are not involved in ALS pathophysiology. For example, the genes coding for arylsulfatase G (*ARSG*), alpha-protein kinase 1 (*ALPK1*) and protein FAM189A1 (*FAM189A1*) contain (multiple) nonsynonymous single nucleotide polymorphisms (SNPs) that are associated with ALS and alter the amino acid sequence of these proteins. However, the functions of these proteins and their interactions with other proteins are essentially unknown and therefore these proteins could not be linked to processes or pathways in the ALS landscape. Nevertheless, future research may link these proteins to ALS pathophysiology and the processes and pathways in the current ALS landscape.

Overall, two assumptions have been made. First, when a knock-out of protein A in a cell

or animal model *increases* the expression of protein B, we presume that endogenous expression of protein A leads to the opposite effect and *decreases* the expression of protein B. Second, we assume that the protein interactions and regulatory processes observed in various organisms and/or cell types can be extrapolated to events occurring in human cells.

1. RHO-GTPase SIGNALING

Central in the ALS landscape is the regulation of the GTP-hydrolyzing (Rho-GTPase) proteins RAC1, CDC42 and RHOA, important mediators of neuronal functioning, including neurite outgrowth and apoptosis. The following findings indicate that the regulation of Rho-GTPase signalling and neurite outgrowth is indeed involved in the pathogenesis of ALS. First, (motor) neuronal cytoplasmic inclusions containing clusters of the familial ALS protein TARDBP have been found in the vast majority of familial and sporadic ALS patients[157, 158] and TARDBP activates the three GTPases RAC1, CDC42 and RHOA[159]. Second, the familial ALS, and cytoskeleton regulating protein profiling-1 (PFN1) binds to CDC42[160] and RHOA[161]. Third, RAC1 is bound, and regulated by the familial proteins **SOD1**[102] and ALS2[127, 162-164] that also bind to each other[165] and regulate neurite outgrowth[127, 164, 166].

In the next sections, the regulation of the three Rho-GTPases by guidance factors (section 1.1) and growth factors (section 1.2), and their link with ALS is described, followed by a description of the regulation of adaptor proteins and signalling cascades in the cytoplasm eventually leading to GTPase activation and regulation of the cytoskeleton (section 1.3).

1.1 Guidance factors

Netrin axonal guidance 'cues' bind to the netrin receptor **DCC** which activates CDC42[167] and RAC1[168] and (eventually) results in neurite attraction towards the netrin guidance cues[169, 170]. **DCC**-mediated RAC1 activation occurs through activation of the cytoplasmic guanine-nucleotide exchange factor **DOCK1**[171, 172]. **DCC** also binds and interacts with the netrin receptor **UNC5C**, which regulates axon guidance[173, 174] as well and is required for spinal motor neuron development[175]. In spinal motor neurons, **DCC** also binds and interacts with ROBO1 – a receptor for the SLIT family of axonal guidance cues that mediates neurite repulsion away from SLIT cues[176]. Upon binding to SLIT molecules, ROBO1 binds and interacts with the peripheral membrane protein **SRGAP3**[163] and **DOCK1**[177]. **SRGAP3** inhibits both CDC42 and RAC1[163], whereas **DOCK1** activates RAC1[172]. Further, **PIP4K2A** is a cytoplasmic kinase that binds to RAC1 and RHOA[178], is activated by CDC42 as well as RAC1 and RHOA (not shown)[178, 179] and catalyzes the formation of phosphatidylinositol 4,5-bisphosphate (PIP2)[180] that negatively regulates netrin/**DCC**-dependent neurite outgrowth of motor neurons (not shown)[181]. On the other hand,

DAB1-dependent signalling increases **DCC** expression[182] (not shown). The RHOA-ROCK1 signaling cascade, that is involved in neurite outgrowth inhibition, is activated and initiated through binding of **RGMA**, a repulsive axonal guidance membrane protein, to its receptor NEO1[183-186]. The cell surface receptor **SEMA6A** is another repulsive axonal guidance protein and its inhibitory function on axonal growth can be attenuated by NGF[187-189].

The extracellular chemoattractant **CXCL12** induces neurite outgrowth[190, 191] and binding of **CXCL12** to its membrane receptor CXCR4 activates **DOCK1**[172]; CXCR4 is neuroprotective in motor neurons derived from the **SOD1**-G93A transgenic mouse model of ALS[65]. Furthermore, **CXCL12**-CXCR4 signaling is inhibited by **ADRBK2**, a cytoplasmic kinase and negative regulator of cell growth[192,193]. **CXCL12** also increases the binding of adhesion membrane glycoprotein ICAM1 to its receptor ITGAL[194, 195] (not shown). ICAM1 binds TUBA4A[196], activates RAC1[197] (not shown) and RHOA[198] (not shown), and upregulates of the expression of interferon gamma (IFNG)[199, 200], an extracellular cytokine and sporadic ALS biomarker[93] that regulates the apoptosis of motor neurons[201]. In addition, ICAM1 competes with **ESM1** for binding to ITGAL[202] (not shown) and is inhibited by the motor neuron survival-promoting angiogenin (ANG)[92]. Conversely, IFNG induces the formation of the ICAM1-ITGAL complex through increasing the expression of ITGAL[203] and decreasing **ESM1** expression[204]. Further, IFNG can induce a neuronal (auto)immune response through increasing the expression of HLA class II membrane proteins **HLA-DQA1**, **HLA-DQB1** and **HLA-DRA**[205], and HLA class I membrane protein **HCP5**[206]. Thus, the GTPases RAC1, CDC42 and RHOA are regulated via netrin, SLIT and **RGMA** signalling, but also by the more immune related proteins **CXCL12**, ICAM1 and IFNG.

1.2 Growth factors

The main growth factors in the ALS landscape are epidermal growth factor (EGF), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF). In the next three paragraphs we describe how these growth factors regulate multiple (intracellular) cascades through activation of their receptors on the cell membrane, and how they are regulated themselves in the ALS landscape.

1.2.1 EGF

EGF binds to the EGF receptor (EGFR) and thereby stimulates the growth of cells and neurite outgrowth in motor neurons[207]. EGF increases the expression of the transporter **ABCG2**[208] (see also section 2.2.1) and decreases the expression of the estradiol receptor ESR1[209] (not shown). When bound/activated by EGF, EGFR binds to the Rho guanine nucleotide exchanged factors **RAPGEF1**[210, 211] and **ARHGEF7**[211] and activates the adapter protein GRB2[183] (not shown, see also section 1.3.1), the kinases ERK1/2[209, 212], the GTPase **CHN2**[213] – that regulates axon guidance[214], binds RAC1 and regulates RAC1

activation[213, 215-218] – and RAC1 itself[219] (not shown). In turn, RAC1 inhibits EGFR[220] (not shown). In addition, EGFR binds to the adenylate kinase **AK4**[221] (not shown), the serine carboxypeptidase **CPVL**[221] (not shown), the RNA-binding protein MATR3[222], the ESR1 coactivator PPARGC1A[223] (not shown), the kinase TBK1[221], the tubulin protein TUBA4A[223], and the TGF-beta receptor subunit **TGFBR2**[224] (that is increased by EGF[225], decreased by estradiol[226] and itself regulates the expression of the autophagy regulator **WDFY3** that increases the removal of aggregated **SOD1** proteins in mutant **SOD1** mice[227] and binds to the autophagy receptor SQSTM1[228, 229]). Further, EGFR increases the expression of MTHFR[230], **ABCG2**[231], E2F3[232] (not shown), and the hnRNP proteins HNRNPA1[233, 234] and HNRNPA2B1[233]. The ALS-associated protein ATXN2 functionally interacts with TARDBP[51], binds to EGFR[235], is involved in trafficking of EGFR and is a negative regulator of EGFR internalization[235].

The isoforms of pro-neuregulin-1 (**NRG1**) regulate neurite outgrowth and differentiation, induce the expression of the acetylcholine (ACh)-receptor, and may therefore play a role in motor neuron regulation[183, 236-238]. Moreover, **NRG1** binds to the EGFR[211, 239] and thereby inhibits EGF binding to the EGFR[211]. Nevertheless, **NRG1** activates RAC1 via transactivation of EGFR, and independently of the tyrosine kinase receptor ERBB4[219, 240], which is activated by **NRG1**[183, 241-243]. After binding **NRG1**, ERBB4 is internalized and promotes (neuronal) cell migration and reorganization of the actin cytoskeleton[183].

1.2.2 NGF

NGF is a ligand for its high-affinity receptors NTRK1 and NTRK2, and important for neuronal proliferation and survival by activating these receptors[183]. The extracellular proprotein convertase **PCSK6** produces NGF[244], and binding of NGF to NTRK1 promotes cell survival and differentiation by activating RAC1[163]. The GDP-dissociation stimulator protein **TIAM1** mediates NGF-induced NTRK1 and NTRK2 signaling and subsequent neurite outgrowth[245].

The NGF-NTRK1 complex binds and activates **TRPV1**[246], a non-selective cation channel that regulates apoptosis[247] and increases the release of the neuropeptide **NPS**[248]. Further, NTRK1 binds to the microtubule motor protein **KIF14**[249], the RNA-binding protein MATR3[249], the kinase PLK1[249], the ALS-associated proteins ATXN2[249], SQSTM1[250, 251] (not shown), TARDBP[249], UBQLN2[249] (binds and modulates TARDBP levels[252]), VAPB[249], PFN1[249], VCP[249] and TBK1[249], the RAC1-activator **ARHGEF7**[249], the transporter **ABCB1**[249], **RAD23B**[249], but also to enzymes involved in the methionine cyle (MTR[249], CBS[249], MARS[249], MAT[249] and AHCY[249]; see also section 2.2) and to the hnRNP proteins HNRNPA2B1[249] (not shown) and HNRNPA1[249].

Activation of NTRK2 results in the phosphorylation and inhibition of the potassium channel **KCNJ3**[253] (that also binds to and is activated by **DRD2**[254, 255] and upregulated by AR[256]; not shown). In addition, binding and activation of NTRK2 by NGF[257] results

in the activation of ERK1/2[258] and binding of NTRK2 to **TIAM1**[259]. **TIAM1** is activated by NTRK2[259] and **NRG1**[260], and subsequently binds RAC1 and stimulates RAC1 activation[259, 261-263]. Further, the familial ALS protein SQSTM1 binds to NTRK2[251], whereas ALS2 increases the endocytosis of NTRK2[264] (not shown) and activates PAK1[164] (not shown). Thus, NGF signaling is linked to major processes in the ALS landscape, namely to RAC1 signaling, neurite outgrowth, the methionine cycle by binding most of its regulating enzymes (see Figure 2), and the regulation of mRNA and RNP granules (see also section 3) by binding to multiple familial ALS and RNA-binding proteins.

1.2.3 VEGF

VEGF is originally described as an angiogenic factor, but has more recently also been shown to be involved in neurogenesis, nerve repair, neuronal survival, protection and growth[265, 266]. VEGF protects against hypoxia-induced motor neuron degeneration in mice[267] and delays disease onset and prolongs survival in a rat model of ALS[268]. The VEGF family consists of five proteins that are encoded by the genes VEGFA, VEGFB, VEGFC, VEGFD and PGF[183] (which are in the figures depicted together as VEGF) that bind and activate the VEGF receptors FLT1, KDR and FLT4[269]. The interactions and effects on protein expression and activation within the ALS landscape are described below for each VEGF family member and receptor.

VEGFA was genetically associated to ALS[154] and binds and activates the VEGF receptors FLT1[270-272] and KDR[273-276] and increases the expression of PPARGC1A[277] (not shown), HSD17B1[278] (not shown), **TEK**[279], **NRF1**[280] (not shown), **NRG1**[281], FLT4[282] (not shown), CXCR4[283-285] and KMT2A (the core subunit of the MLL-complex)[281], and decreases the expression of CASP3[286] (not shown) and EPO[287]. Further, VEGFA increases the activation of NF-KB[288, 289] (not shown), the kinase PAK1[290], the Rho-GTPases RHOA[291] (not shown), and activates and increases the expression of the Rho-GTPases RAC1[292-295] (not shown) and CDC42[295, 296] (not shown). In turn, VEGFA expression is increased by RAC1[297, 298], PAK1[299] (not shown), AR[300] (not shown), NF-KB[301, 302], **NRG1**[299, 303] (not shown), IFNG[304], EGF[305, 306], EGFR[307-309], TBK1[310], homocysteine[311], PPARGC1A[312], estradiol[301, 313-315] and ESR1[316-318], and regulated by NGF[319, 320] (not shown), **TEK**[321] (not shown) and **TGFBR2**[322, 323] (not shown). Lastly, VEGFA binds to **HABP2**[324] that increases the cleavage of VEGFA[324].
VEGFB binds and activates FLT1[325, 326], activates ERK1/2[327, 328] (not shown) and NF-KB[327] (not shown) and decreases the expression of CASP9[326] (not shown). **NRG1** decreases the expression of VEGFB[329], but increases the expression of VEGFC via NF-KB[330].
VEGFC binds, activates and increases the expression of KDR[274, 331-333] and FLT4[333-336]. Further, VEGFC activates ERK1/2[337], increases the expression of CXCR4[338], and increases the synthesis of hyaluronic acid (HA)[339]. VEGFC expression is increased by EPO[340], BMI1[341], EGF[342], IFNG[343], and decreased by ESR1[344].
VEGFD binds and increases the activation of KDR[345-347] and FLT4[345, 346, 348, 349]. VEGFD

expression is decreased by ERBB4[350].
PGF binds and activates FLT1[272, 351-354], activates ERK1/2[355, 356], inhibits CASP3[357] and increases the expression of FLT1[354] and CXCR4[358].
FLT1 binds and inhibits KDR[354, 359, 360], binds CRK[361], activates RAC1[362] (not shown) and ERK1/2[356, 363] (not shown). The soluble isoforms of FLT1 are secreted and sequester VEGF to block its access to the VEGF receptors on the cell membrane, and thereby regulate VEGF signaling[360]. Further, FLT1 increases the expression of G3BP1[362], MAT2A (isoform of MAT, see also section 2.2)[362], and decreases the expression of the homocysteine producing enzyme AHCY[362]. FLT1 expression is increased by ERK1/2[364] and cholesterol[365].
KDR binds ERK1/2[366], and the adaptor proteins SHC1[367, 368] (not shown) and GRB2[367] (see also 1.3.1). KDR activates ERK1/2[369] and mediates the activation of RAC1[370] (not shown) RHOA[370] (not shown) and SHC1[371] (not shown). Further, KDR activation is inhibited by **DRD2**[372] and DA increases (via **DRD2** activation) the endocytosis of KDR[373]. On the other hand, **SEMA6A** increases the activation of KDR[374], and also EPO increases KDR activation by increasing the expression of VEGF[375]. KDR expression is increased by estradiol[376-378], **TGFBR2**[323], **SEMA6A**[374] and NF-KB[379], and decreased by IFNG[380].
FLT4 binds to the adaptor proteins SHC1[381] (not shown) and GRB2[381] (not shown), and to KDR[382] (not shown). FLT4 expression is increased by IFNG[383] (not shown).

In summary, VEGF signalling regulates, and is regulated by (among others) Rho-GTPase signalling (through RAC1, CDC42, RHOA), estradiol signalling (through estradiol, HSD17B1, PPARGC1A), other growth factors (EGF, NGF), the methionine cycle (through AHCY, homocysteine, MAT) and HA signalling (through HA and **HABP2**). Thus, VEGF signalling interacts with multiple major ALS landscape processes and may in this way exert neuroprotective effects on motor neurons.

1.3 Intracellular signaling

The above-described signaling cascades that regulate the GTPases RAC1, CDC42 and RHOA require cytoplasmic adaptor proteins (section 1.3.1) and activate ERK1/2 signaling (section 1.3.2). Activation of these pathways eventually leads to the activation of the Rho-GTPases and subsequently the activation of the major downstream effector proteins ROCK1 and LIMK1 that regulate neurite outgrowth (section 1.3.3).

1.3.1 Adaptor proteins

Adaptor proteins such as SHC1, GRB2, CRK and YWHAB regulate the signal transduction processes of receptor-ligand complexes in the cytoplasm.

The adaptor protein SHC1 inhibits RAC1 and in this way mediates ALS-like motor neuronal death[129] (not shown), and increases the expression of SQSTM1[384] (not shown) that also binds to and interacts with the familial ALS proteins TARDBP[385, 386], **SOD1**[142],

OPTN[387] and TBK1[388]. Following activation, SHC1 binds to GRB2, another adaptor protein[183, 389, 390]. SHC1 as well as GRB2 bind to and are activated by (**NRG1**-activated) ERBB4[391-394], are recruited to, and bound and activated by the NTRK1-NGF complex[249, 395-398], and bind to the phosphatase **DUSP15**[399] and the membrane protein **TEK**[400-402] (not shown). **TEK** activates SHC1[403], increases the expression of IFNG[404] and regulates a number of processes, including cell survival and proliferation, reorganization of the actin cytoskeleton[183] and neurite outgrowth[405]. SHC1 is also activated by **ALK**[406], a neuronal receptor tyrosine kinase that is on its turn activated through binding to the extracellular growth factor midkine (MDK)[407], and promotes neuronal cell growth and neurite outgrowth[408, 409]. Further, GRB2 binds to MATR3[410] and to the RAC1-activators **ARHGEF7**[411] (not shown) and **DOCK1**[412, 413] (not shown). **ARHGEF7** is a guanine nucleotide exchange factor and binds to the kinase PAK1[412, 414, 415] and also binds and subsequently increases the activation of CDC42[416-418] and RAC1[417, 419, 420]. **ARHGEF7** expression is increased by AR[421] (that also decreases expression of PLK1[422] (not shown), and binds to SQSTM1[423] (not shown)) and is decreased by ESR1[424]. Like **ARHGEF7**, **DOCK1** is a guanine nucleotide exchange factor that binds to RAC1[425, 426] (not shown) and activates RAC1 by increasing the exchange of bound GDP for free GTP[172, 183, 427, 428]. The adaptor proteins SHC1 and GRB2 bind and interact with the guanine nucleotide exchange factor **RAPGEF1**[429-432], which in turn activates both CDC42[433] and RAC1[433, 434], and increases neurite outgrowth[435, 436].

The adaptor protein CRK binds to EGFR[211, 437] (not shown), **DAB1**[438, 439], GRB2[440, 441] (not shown), SHC1[442], ERBB4[392], FLT1[361], PPP1CA[443] (not shown), AR[444] (not shown), **ABAT**[399] (not shown), **ATXN1**[445] (that binds also to **RAPGEF1**[446]) and YWHAB[447] (not shown), binds to and activates **RAPGEF1**[429, 432, 448-450] and **DOCK1**[413, 441, 451], and activates RAC1[452, 453] (not shown). Further, **CXCL12** binding to its receptor CXCR4 activates CRK[454] (not shown) and CRK itself regulates the binding of **DAB1** to **DOCK1**[438]. Lastly, the adapter protein YWHAB forms functional complexes with the **PAK4** kinase[455] and the cytoplasmic proteins ALS2[455, 456], VCP[457], **EPB41L3**[456, 458] (a cytoskeleton adaptor that is involved in axoglial junction maintenance[459] and binds to **AP3B1**[460] and endosomal sorting protein CHMP2B[461] that also binds to RAC1[461]; not shown), **DDIT4L**[462], GFAP[463], the adaptor protein SHC1[464] and the Rho GTPase activators **TIAM1**[263] and **ARHGEF7**[465, 466].

1.3.2 ERK1/2 signaling

Aggregates of abnormally phosphorylated ERK1/2 have been found in spinal motor neurons of ALS patients[79], which indicates that ERK1/2-related signaling – that regulates many processes including neurite outgrowth[467] and (motor) neuronal survival[468] – is involved in ALS pathogenesis. ERK1/2 bind to the ALS-associated proteins VCP[457] and SQSTM1[469, 470]. Further, the ERK1/2 cascade is activated by EGF, NGF and VEGF signaling (see above), but also by **B4GALT6**[471], **TEK**[472], **ALK**[408, 472], **DCC**[473, 474] (not shown), **DISC1**[475]

(**DISC1** functionally interacts with the dopamine receptor **DRD2**[476, 477], binds to MATR3[478], and increases neurite outgrowth by increasing the expression of cell-cell adhesion protein **CDH2**[479] that is involved neurite outgrowth[480] and axonal guidance[481]), ESR1[482], folic acid (FA)[483] (not shown), **NRG1**[219] (not shown), **DRD2**[484] (not shown) and the familial ALS protein ANG[485]. Further, ERK1/2 is (positively or negatively) regulated by ICAM1[486, 487] and inhibited by the microtubule-associated protein **MTUS1**[488] and the GTPase **CHN2**[219]. Further, ERK1/2 expression is increased by **TNFSF11**[489], that also increases the expression of IFNG[490] and ICAM1[491], and interacts with ESR1 (see section 2.3). ERK1/2 binds to VCP[457], PRPH[492] (not shown) and VAPB[492], and interacts with **SOD1**[492]. In addition, ERK1/2 interacts with ROCK1 (see section 1.3.3.1), increases the expression of PPARGC1A[493] and RAC1[494], decreases the expression of ESR1[495, 496], and increases the activity of nuclear NF-KB[212, 497] (not shown). Thus, ERK1/2 is an important effector protein in multiple neurite outgrowth- and axonal guidance-regulating cascades, and thereby regulates RAC1, ESR1 and NF-KB function, i.e. main cascades in the ALS landscape.

Hyaluronic acid (HA) is increased in serum, skin and urine of ALS patients[498, 499]. Through binding HA activates the extracellular protein **HABP2**[183] that in turn activates RHOA[500] (not shown) and inhibits ERK1/2 activity[501]. HA also activates RAC1[502, 503], ESR1[504], NF-KB[505], ROCK1[506] and EGFR[507], inhibits CASP3[508], binds to APP[509], and increases the expression of RHOA[506] and **ABCB1**[510]. Further, HA production/synthesis is increased by EGF[511] and **NRG1**[512]. Furthermore, the HA-synthesizing enzyme[513] **HAS2** is activated[512] and its expression increased by ERK1/2[514], and regulated by NF-KB[515, 516]. **HAS2** itself regulates the distribution of **CDH2** in the plasma membrane[517]. Thus, HA and ERK1/2 signaling interact, and like ERK1/2 HA regulates the RAC1, ESR1 and NF-KB signaling cascades.

1.3.3 Regulation of neurite outgrowth

Multiple proteins in the ALS landscape regulate neurite outgrowth and guidance, e.g. the neurite outgrowth inhibitor **SHROOM3** reduces neurite outgrowth[518, 519], whereas the contactin **CNTN5** (not shown) increases neurite outgrowth[520] and the cadherin membrane protein **CDH13** regulates neuronal cell growth and guidance[180, 521] and activates both CDC42 and RAC1[522]. The regulation of neurite outgrowth by Rho-GTPase associated kinases and by amyloid beta will be discussed below (sections 1.3.3.1 and 1.3.3.2, respectively).

1.3.3.1 Rho-GTPase-associated kinases

Activation of the GTPases RHOA, CDC42 and RAC1, via guidance cues, growth factors or other signalling pathways, eventually leads to the activation of the NADPH-oxidase complex (see also section 2.1) and the kinases ROCK1 and LIMK1, essential for cytoskeleton regulation and neurite outgrowth[183, 523-526].

ROCK1 is a Rho-associated protein kinase that inhibits neurite outgrowth[526], whereas LIMK1 is a serine/threonine-protein kinase downstream of ROCK1 stabilizing the actin cytoskeleton and stimulating neurite outgrowth[183, 527]. ROCK1 is regulated by RHOA that binds to (not shown) and regulates the activity of ROCK1[528-532]. Further, ROCK1 expression is regulated by estradiol[533] (not shown) and decreased by EPO[534] (not shown). In addition, ROCK1 inhibits familial ALS protein PFN1[535] that through binding actin filaments, affects the structure of the cytoskeleton[183]. PFN1 also binds to RHOA[161] and CDC42[160] (also mentioned above) and the ALS-related proteins DAO[536] and SMN1/2[537]. ROCK1 binds to CASP3[538] (not shown), NTRK1[249] (not shown) and HNRNPA1[539], and is also bound by **RND3**[540]. **RND3** stimulates neurite outgrowth[541, 542] and inhibits ROCK1-mediated apoptosis[540]. Moreover, ROCK1 phosphorylates and hence activates ERK1/2[543, 544] and is itself inhibited by ERK1/2[545]. LIMK1 is activated by ROCK1[163, 530, 546], and binds to and is activated by the serine/threonin-protein kinases PAK1 and **PAK4**[547-549] that are both activated by CDC42[550, 551] (not shown) and RAC1[164, 550, 551]. In addition, PAK1 and ESR1 bind to[552] and activate each other[553, 554]. Furthermore, PAK1 binds the serine/threonine kinase receptor **TGFBR2**[555] (increased in expression by **DRD2**[556] (not shown)), and binds[557] (not shown) and regulates the activation of PLK1[558], which in turn is located in a complex with **C9ORF72**[559] and regulates activation of RHOA[560]. LIMK1 expression is increased by **TNFSF11**[561] and by mutant **SOD1**[562]. Further, LIMK1 is bound by **NRG1**[563] and degraded through polyubiquitination by the E3 ubiquitin-protein ligase **RNF6**[564] that by regulating local LIMK1 levels in axonal growth cones regulates neurite outgrowth[564]. Thus, there is a complex interaction between the Rho-GTPases and the kinases PAK1, **PAK4**, ROCK1 and LIMK1 to regulate cytoskeleton dynamics and neurite outgrowth.

1.3.3.2 Amyloid-beta precursor protein APP

The cell surface receptor APP regulates neurite outgrowth and the formation of axons[183]. APP accumulates in spinal motor neurons of ALS patients[47], is increased in the CSF of ALS patients compared to controls[48], and its inhibition or ablation (APP-KO mice cross-bred with mutant **SOD1** mice) delayed disease onset and improved motor function and motor neuron survival of the mutant **SOD1** mice[49, 50]. APP can be bound by the extracellular proteoglycan **GPC6**, which inhibits APP-induced neurite outgrowth[565]. In addition, APP binds to **C1orf112**[566], **TGFBR2**[566], the anti-apoptotic ER-anchored autophagy receptor **FAM134B**[566, 567] (not shown), the familial ALS protein TBK1[566], the mitochondrial **AK4**[566] (not shown), the contactin and regulator of axonal guidance and growth **CNTN4**[568-572] and **CNDP1**[566], an extracellular enzyme that degrades the dipeptide carnosine (not shown) which itself has been shown to be neuroprotective in **SOD1** mutation-dependent ALS[573]. Moreover, the cytoplasmic aminopeptidase **DNPEP** cleaves APP[574], and both APP[575] and the extracellular hormone erythropoietin (EPO)[576, 577] are involved in decreasing the expression of CXCR4. APP increases the expression of **PDE4B** [578], a cytoplasmic phosphodiesterase that increases the secretion and expression

of IFNG[579, 580] and binds **DISC1**[581].
RAC1 increases the expression of the APP-binding protein APBB1[582], an adapter protein forming a transcription complex with APP that translocates to the nucleus upon DNA damage to induce apoptosis[180, 183]. **DAB1** binds and modulates the degradation and clearance of both APP and APBB1[583]. In turn, APP retains **DAB1** in the cytoplasm and thereby antagonizes reelin-induced neurite outgrowth inhibition that depends on **DAB1**[584, 585]. APBB1 binds to **ATXN1**[445], **FMN2**[586] and **MCPH1**[587], whereas APBB1, **FMN2** and **MCPH1** are all three involved in (neuronal) DNA damage repair and apoptosis[588-591]. Moreover, **FMN2** binds to **TGFBR2**[592] and regulates growth cone stabilization, and axon guidance and outgrowth[593].

Motor neurons in which APP has accumulated display increased immunoreactivity of the cleaved pro-apoptotic caspase CASP3[47], i.e. APP activates CASP3[594] that subsequently triggers a cascade of caspases involved in the execution of (neuronal) apoptosis[183]. CASP3 is cleaved and thereby activated by the caspase CASP9[183] (that binds to SQSTM1[595]; not shown) and the cytoplasmic proteins **GNLY**[596] and **NLRP1**[597], and is inhibited by the cytoplasmic kinase PLK1[598]. PLK1 binds to the APP-regulating/associated protein PIN1[599, 600] and stabilizes PIN1 by inhibiting its ubiquitination[601]. **MIS18A** also binds to and functionally interacts with PIN1[602]. Lastly, CASP3-mediated cleavage of the familial ALS protein TARDBP attenuates its toxicity [603]. Therefore, dysregulation of APP and subsequent APP accumulation as seen in motor neurons of ALS patients may interfere with neurite outgrowth and eventually lead to motor neuron death.

2. ESTRADIOL SIGNALING

The female sex hormone estradiol is neuroprotective for motor neurons in cell and animal models[604-610]. Estradiol binds to and activates its receptor ESR1[183] and (activated) ESR1 is involved in many molecular signaling cascades in the landscape. First, ESR1 binds and regulates the adaptor proteins SHC1[611] and GRB2[612], and activates ERK1/2[613], which itself is involved in downregulating ESR1 expression[495, 496]. Further, **CXCL12** binding to CXCR4 increases ESR1 activity[614] and estradiol-bound/activated ESR1 is neuroprotective through inhibiting the translocation of APP to the nucleus by binding APBB1[615]. Furthermore, activated ESR1 increases the activity of CDC42 and RAC1 but decreases the activity of RHOA and in this way regulates neurite outgrowth[616]. Thus, estradiol signaling is involved in the pathways discussed in the previous section on Rho-GTPase signaling.

In the sections below, we describe the processes that are directly linked to, and are themselves also directly affected by, ESR1 signaling. First, we discuss the interaction between estradiol signaling and the NADPH-oxidase, of which RAC1 is a subunit[617] (section 2.1). Second, the effects of the folate and methionine cycles on ESR1 signaling

and glutamate toxicity are described (section 2.2). Third, the nuclear interactions between ESR1 and among others the NF-KB-complex and MLL-complex are discussed (section 2.3).

2.1 NADPH-oxidase

The NADPH-oxidase is an enzyme complex that generates reactive oxygen species (ROS) and thereby converts NADPH to NADP+[183], and is overactivated in spinal motor neurons from ALS patients and in a mouse model of ALS[100]. Inactivation of the NADPH-oxidase in an ALS mouse model delays neurodegeneration and extends the survival time of the mice[100]. The Rho-GTPases RAC1, CDC42 and RHOA increase the activation of the NADPH-oxidase complex[618-621]. Further, NADPH-oxidase subunit RAC1[617] increases the assembly, activation[618, 619, 622, 623] and stabilization of the NADPH-oxidase complex[624]. ALS2 and **SOD1** bind to and regulate RAC1 (see above). Mutations in **SOD1** disrupt RAC1-mediated regulation of the NADPH-oxidase complex[102], i.e. **SOD1** mutations keep RAC1 in a GTP-bound state, resulting in elevated NADPH-oxidase activity and ROS production[102]. ALS2 suppresses mutant **SOD1** toxicity by binding to mutant **SOD1**, but not wild-type **SOD1**[165] and also decreases activation of RAC1 which increased by mutant **SOD1**[625]. The NADPH complex is inhibited by AMPK[626, 627] (not shown) and activated by the RAC1-activating protein **TIAM1**[628]. Of note, whereas RAC1 increases neurite outgrowth[163, 629, 630], NADPH-oxidase activity negatively regulates NGF-NTRK1-induced neurite outgrowth[631]. Further, the physiological levels of ROS produced by NADPH-oxidase are necessary to maintain a dynamic cytoskeleton[524]. Furthermore, NADPH-oxidase is located in growth cones and its inhibition reduces F-actin content and neurite outgrowth[525]. Thus, there is a complex interaction between RAC1 and the NAPDH-oxidase complex regulating neurite outgrowth and cellular ROS levels.

Estradiol signaling interacts with and modulates the activation of both RAC1 and the NADPH-oxidase, i.e. estradiol, via the activation of ESR1, decreases the expression of RAC1 protein and mRNA[632] (not shown), and decreases the activation of the NADPH-oxidase complex[632, 633]. Nevertheless, estradiol increases neurite outgrowth by activating RAC1 and CDC42 and inhibiting RHOA[616] (RHOA inhibits the activity of ESR1[634]). In turn, RAC1 regulates the activity of ESR1, i.e. RAC1 has been reported to inhibit ESR1 transcriptional activity[634], but also to increases the activity of ESR1[635]. In addition, RAC1 binds to and activates PAK1[554], which in turn forms a mutual activation complex with ESR1[552-554]. Therefore, estradiol and ESR1 mediate neurite outgrowth by regulating RAC1 and NADPH-oxidase.

2.2 Folate cycle and methionine cyle

We next describe the regulation of the folate (FA) cycle and methionine (MET) cycle, and their metabolites, of which most have been linked to ALS pathology, i.e. FA is

neuroprotective[636], and the FA metabolite 5-methyl-tetrahydrofolate (5-MTHF) is decreased in an ALS mouse model[637]. Methyl vitamin B12 (Met.Vit.B12) increases survival time of ALS patients[638-640], MET is lower in the plasma of ALS patients[641], carnitine (CAR) is lower in plasma of ALS patients[642] and carnitine supplementation decreases disease progression and increases survival in an ALS mouse model[643] and in ALS patients[644], S-adenosylmethionine (SAM) supplementation delays the onset of disease in an ALS mouse model[645] and homocysteine (HCY) is increased in ALS patient's plasma and CSF[639, 646, 647]. First we discuss the regulation of these metabolites in the landscape, followed by their interaction with estradiol metabolites (section 2.2.1.1), glutamate toxicity and the effect of riluzole (2.2.1.2).

FA is an essential vitamin that mainly enters the body through food intake and is converted into dihydrofolate (DHF) and subsequently to tetrahydrofolate (THF) by DHFR, a cytoplasmic enzyme that also requires and converts NADPH into NADP+[183, 648]. Further, FA has a direct inhibitory effect on DHFR, to prevent intracellular FA levels from becoming too low[649] (not shown). In addition, the FA metabolite 5,10-methylene-tetrahydrofolate (5,10-MTHF) is converted into DHF by the cytoplasmic enzyme TYMS[183] (that is inhibited by APBB1[650]) and to 5-MTHF by MTHFR, a cytoplasmic enzyme that, like DHFR, also requires and converts NADPH into NADP+[183]. Both DHFR and MTHFR are genetically linked to ALS, i.e. a deletion in the gene encoding DHFR is associated with bulbar onset ALS[69], and polymorphisms in the MTHFR gene are associated with spinal and bulbar onset of ALS in female patients[99]. Activation of ESR1 increases the expression of the FA cycle enzymes, DHFR[651] and TYMS[652], which may therefore also play a role in gender-specific risk for developing ALS as has been observed for MTHFR[99]. Nuclear export of DHFR mRNA is regulated by the with ALS associated[88] GLE1[653] (not shown), a protein that shuttles between the nucleus and the cytoplasm to regulate mRNA export and translation, and stress granules (dis)assembly[654, 655].

The FA cycle is linked to the methionine cycle through MTR, a cytoplasmic enzyme that uses vitamin B12 (Vit.B12) as its cofactor, and converts 5-MTHF and HCY into THF and MET, respectively[183]. In addition, MET is converted into SAM by the cytoplasmic enzyme MAT[183] (of which three isoforms exist; MAT1A, MAT2A and MAT2B) that binds to NTRK1[249], AMPK[455] (not shown), APP[566] and the familial ALS protein FUS[656], and decreases the expression of **SOD1**[657]. SAM is converted into S-adenosylhomocysteine (SAH) by the methyltransferases GAMT and MLL, i.e. GAMT transfers the methyl group of SAM to guanidinoacetate, creating SAH and creatine, and MLL uses SAM as a methyl donor to regulate histon methylation and as such gene transcription[183, 658]. SAH is converted into HCY by AHCY[183], to 'close' the 'methionine' cycle. AHCY binds to NTRK1[249], the adaptor protein GRB2[410] and the familial ALS protein FUS[656].
HCY, which is increased in plasma and CSF of ALS patients (see above), can – in

addition to its conversion into MET – also be metabolized to cystathione (the precursor for cysteine, CYS) by the enzyme CBS, allowing the elimination of the neurotoxic HCY[183, 659]. Further, the methionyl-tRNA synthetase MARS can charge a tRNA with MET for translation[183]. MARS binds to EGFR[223], NTRK1[249], ESR1[660], AMPK[455] (not shown), NF-KB[661] (not shown) and GRB2[410]. MET can – apart from being converted into SAM, or used for translation – be metabolized to carnitine (CAR)[662]. CAR is neuroprotective[663] and has strong antioxidant properties, i.e. it inhibits the NADPH-oxidase complex[664, 665]. CAR is transported out of (neuronal) cells by the transporter **SLC22A16**[183] and into mitochondria by the transporter SLC25A20 (that binds to ESR1[666], and is activated and increased by the ESR1 coactivator PPARGC1A[667, 668]; not shown) where it is involved in the mitochondrial fatty acid-oxidation pathway[183]. The metabolite SAM is also transported into mitochondria by **SLC25A26**[183, 669] where it acts as a methyl donor and hence regulates mRNA and protein function. Mutations in **SLC25A26** lead to a deficiency of intra-mitochondrial methylation, which affects RNA stability, and protein modification and translation[670]. Furthermore, SAM inhibits the activity and decreases the expression of RAC1[671], and has an inhibitory effect on MTHFR[672], reflecting its participation in the regulation of the FA cycle. In turn, FA protects motor neurons against increased HCY and apoptosis in a mutant **SOD1** mouse model[636].

Thus, multiple metabolites of the FA and MET cycles are associated with ALS and their dysregulation affects RAC1 and NADPH-oxidase regulation, but also increases mitochondrial dysfunction and oxidative stress. Of note, also the NGF and EGF growth factor pathways heavily interact with MET cycle enzymes, showing the interactions of these enzymes with major pathways in the landscape.

2.2.1 Regulation of estradiol and cholesterol metabolites and riluzole

This section will elaborate on the interactions of landscape metabolites with the ABC transporters **ABCB1** and **ABCG2**, in particular the transport of estrone 3-sulfate (E3S), cholesterol (CH) and riluzole by these transporters. We already described that **ABCG2** transports FA and that its expression is regulated by FA (previous section).

2.2.1.1 Estradiol and cholesterol metabolites

E3S is the most prominent estrogen in postmenopausal women and men, and a long-lived estradiol derivative and as such acts as a reservoir that when necessary can be converted into the more active estradiol[673-675]. E3S is converted into estrone and subsequently into estradiol which is regulated by the enzyme estradiol 17-beta-dehydrogenase (HSD17B, that has multiple isoforms e.g. HSD17B1 and HSD17B2). HSD17B catalyzes the conversion of estradiol and NAD(P)+ into estrone and NAD(P)H and *vice versa*[183], i.e. HSD17B1 favors the conversion of estrogen into estradiol, whereas HSD17B2 favors the conversion of estradiol into estrone[183, 676-678]. EGF increases the expression of

HSD17B[679] (not shown).
The membrane transporters **ABCB1** and **ABCG2** regulate the uptake and secretion of E3S and glucuronidated estradiol (E2G), but not that of free estrogens[680-685]. **ABCG2** expression is increased by **TEK**[686], and decreased by APP[687] and **ALDH1A2**[688] (a cytoplasmic dehydrogenase that decreases CDC42 expression[689] (not shown) and its expression is increased by estradiol[690]). Further, **ABCB1** decreases the expression of **ABCG2**[691] and **BTNL2**[692] (that decreases the expression of IFNG[692]). Of note, activated ESR1 increases the expression of both **ABCB1**[693, 694] and **ABCG2**[695,696]. The sulfotransferase **CHST10** (located in the membrane of the Golgi apparatus) adds a sulfate group to E2G and thereby reduces the activity of estradiol by diminishing its binding affinity for ESR1[697]. Moreover, **CHST10** also downregulates estrogen levels, i.e. sulfation of glucuronidated steroids facilitates their export and elimination from the body[697]. Thus, **CHST10** and the transporters **ABCB1** and **ABCG2** regulate estradiol signaling by modulating estradiol metabolite activity and export from the cell.

The **ABCB1** transporter also transports CH into motor neurons[698, 699]. CH is converted into 27-hydroxycholesterol (27-OH-CH) by the mitochondrial membrane-located enzyme CYP27A1[700] that is associated with ALS[67]. 27-OH-CH is also associated with ALS[701] and directly competes with estradiol to bind ESR1[702] within the nucleus, which has a negative effect on the transcriptional activity of ESR1. Moreover, 27-OH-CH is the natural ligand of LXRB (encoded by NR1H2), a transcription factor that is upregulated by IFNG[203] and involved in regulating motor neuron death and survival[703, 704]. 27-OH-CH is further metabolized through 7alpha-hydroxylization of 27-OH-CH by the enzyme **CYP7B1**[705, 706], whose expression is increased by ESR1[707].

2.2.1.2 FA, glutamate toxicity and riluzole

Depending on its location, **ABCG2** transports FA out of the motor neuron or into intracellular organelles such as mitochondria. Low intracellular FA levels decrease expression of **ABCG2** in the cell membrane and increase **ABCG2** expression in mitochondrial membranes[683, 708-710]. In this way, the remaining intracellular FA is transported into mitochondria, and can still be used for serine-glycine metabolism, i.e. glycine is synthesized from serine by simultaneous conversion of THF to 5,10-MTHF – by cytoplasmic or mitochondrial serine hydroxymethyltransferase (SHMT) – and glycine is also cleaved by simultaneous conversion of THF into 5,10-MTHF[183, 711-714]. Glycine is an inhibitory neurotransmitter that is decreased in the spinal cord of ALS patients[715], whereas serine is increased[716, 717]. Moreover, glycine is a major component of collagen, and both are markedly decreased in the skin of ALS patients[718]. Intriguingly, the familial ALS protein DAO regulates the degradation of serine[717] and glycine[719], and dysregulation of this system or mutations in the DAO gene increase glutamate toxicity and motor neuron death, since serine and glycine are the endogenous co-agonists of

the N-methyl D-aspartate receptor (NMDAR), the receptor for glutamate[716, 717, 720-722]. Thus, regulation of FA levels in the cell is important for the regulation of serine and glycine levels, to prevent glutamate toxicity and motor neuron death.

NMDA is another agonist of the NMDAR and decreased via oxidation by **DDO**[183, 723], reducing potential glutamate toxicity in the synapse. The glial glutamate transporter EAAT2 (SLC1A2) removes excessive glutamate from the synapse to prevent an overstimulation of NMDAR on the postsynapse[723]. HNRNPA2B1 decreases SLC1A2 expression[724] (not shown) and mutant **SOD1** and mutant TARDBP inhibit the activity and decrease the expression of SLC1A2 [725-727]. Editing of SLC1A2 pre-mRNA is increased in the spinal cord and motor cortex of ALS patients[132]. Thus, in addition to NMDAR agonists (glycine, serine, NMDA), also dysregulation of glutamate transporters is associated with ALS and contributes to glutamate toxicity. Of note, folylpolyglutamate synthase (FPGS) catalyzes polyglutamation of folates (FA, DHF, THF)[728] and thereby increases the clearance of glutamate, but is also important for FA homeostasis by affecting the kinetics of the polyglutamated folates[729](not shown). The lipase **LIPH** binds to FPGS[592](not shown) and catalyzes the production of 2-acyl lysophosphatidic acid (LPA) that increases cell proliferation and regulates neurite retraction[730-732].

The anti-glutamatergic ALS drug riluzole – currently the only FDA-approved drug to treat ALS – inhibits activation of NMDAR complexes[733, 734], decreases the release of glutamate[727, 735, 736] and activates and increases the expression of glutamate transporters (e.g. SLC1A2)[737-739]. Further, riluzole may exert its anti-glutamatergic effects by inhibiting sodium channel activation and subsequent glutamate release in the synapse[740, 741]. Riluzole binds to and likely inhibits the sodium channel **SCN8A**[742, 743] that is involved in the regulation of voltage-dependent sodium ion permeability of excitable membranes[183] and regulates neurite outgrowth[744]. **RBFOX1**, an RNA-binding protein regulating alternative splicing[745], binds to the RNA-binding protein **ATXN1**[445] and the familial ALS protein ATXN2[445] (**ATXN1** and ATXN2 also bind to each other[445]) and regulates alternative splicing of the gene coding for **SCN8A**[746]. Of interest, riluzole is transported out of the motor neuron by both **ABCB1** and **ABCG2**, and increases the expression of **ABCG2**, which implies that riluzole decreases its own intraneuronal concentration when given over a longer period of time[747-749].

In summary, the regulation of the membrane transporters **ABCB1** and **ABCG2** by e.g. riluzole, FA and ESR1, but also by the growth factor receptors NTRK1 and EGFR (see section 1.2) may affect **ABCB1**- and **ABCG2**-mediated transport of E3S, (sulfated) E2G, FA, 27-OH-CH and riluzole (see also sections above) and may affect the viability of motor neurons. Since trials with other anti-glutamatergic drugs have been unsuccessful[678, 750-755], the beneficial actions of riluzole may not be limited to its regulation of the glutamate

system, but may also be the result of its regulation of **ABCB1** and **ABCG2**.

Of note, NADPH-oxidase, the estrone-estradiol converting enzyme HSD17B and the FA cycle enzymes DHFR and MTHFR all require NADPH for their activation. Hyperactivation of the NADPH-oxidase complex as found in spinal motor neurons from ALS patients and in a mouse model of ALS (see section 2.1) has a negative effect on NADPH availability and hence may affect the conversion and metabolism of FA and estradiol. Moreover, high NADP+ levels may favour the reverse reaction of HSD17B, i.e. the conversion of estradiol into the lesser active estrone.

2.3 Interaction between NF-KB, ESR1 and MLL-complex

NF-KB, ESR1 and the MLL-complex are the main transcriptional regulators in the ALS landscape. In this section, we describe their activation by proteins in the landscape, their interactions with each other and with other transcriptional regulators, and the subsequent effects on transcription and cellular function.

NF-KB is upregulated in the spinal cords of ALS patients[107, 756] and regulates motor neuron survival[757, 758]. In the cytoplasm, NF-KB is activated by HA (see above), the cytoplasmic proteins **AP4B1**[759], **ADRBK2**[760], ERK1/2 (see above), RAC1[761, 762], RHOA[763], ROCK1[544, 764], **TNFSF11**[765] and the familial ALS proteins TARDBP[107], SQSTM1[766, 767], VCP[768] and TBK1[769, 770], and inhibited by the familial ALS protein OPTN[771, 772] (OPTN and TBK1 bind and regulate each other's activation[773-777]). In addition, NF-KB binds to the familial ALS proteins DAO[778], **C9ORF72**[778], FUS[779], MATR3[661] and TBK1[661], the membrane protein **KCTD12**[661] and the NF-KB-associated cofactor UXT[780]. Following activation, NF-KB translocates to the nucleus where it functions as a transcription factor. AMPK – which itself is bound to and inhibited by **NT5C1A**[43, 781] – inhibits translocation of NF-KB to the nucleus[626]. In the nucleus, NF-KB is bound to and retained by **ANKRD33**[782], and is activated by the transcription factor **ZFP64**[759] and by the kinase **UHMK1**[759]. Subsequently, NF-KB increases the expression of the ABC transporter **ABCB1**[783], the methionine cycle enzymes MAT[784, 785] and CBS[786], and binds and increases the expression of PLK1[787] (not shown), **ATXN1**[778, 788] and the transcription factor E2F3[789, 790]. E2F3 also binds to the MLL-complex[791] and regulates the expression of BMI1[792] (that is also increased by PLK1[793] (not shown)), PIN1[794] (not shown), the FA cycle enzyme DHFR[795] and the transcription regulator UXT[796] (not shown). UXT binds ESR1[797] and AR[797], and together with its binding partner ALS2 activates NF-KB[798]. Further, UXT forms a functional complex with the mitochondrial protein **LRPPRC** that regulates mitochondrial aggregation and apoptosis[799]. **LRPPRC**[800], the mitochondrial membrane protein **COX10**[801] and **SOD1**[802] – that, when mutated like in familial ALS, aggregates and accumulates in mitochondria[183] – activate cytochrome-c oxidase (COX), the main enzyme involved in mitochondrial electron transport that has a decreased activity in spinal motor neurons from ALS

patients[63, 64].
Thus, NF-KB activation and function is regulated by multiple familial ALS proteins (TARDBP, VCP, OPTN, DAO, FUS, ALS2), Rho-GTPases and the transcriptional regulator UXT that also regulates mitochondrial function.

In addition to these processes, NF-KB and ESR1 bind to and activate each other[803-806]. ESR1 also binds to the familial ALS proteins PFN1[807], FUS[808], VCP[660] and TBK1[809], the hnRNP proteins HNRNPA1[660, 808] and HNRNPA2B1[808] (see also section 3) and TOP2A[808], and binds to and regulates ERBB4[424, 810, 811] (not shown). Estradiol potentiates NGF-induced neurite outgrowth[812] and estradiol-activated ESR1 upregulates the expression of the nuclear landscape proteins BMI1[792], E2F3[813], **FOXF2**[814], GATA3[815] and the transcription factor **NRF1**[816, 817] that positively regulates neurite outgrowth[818, 819], upregulates the expression of mitochondrial COX[820] and decreases the expression of **ESM1**[821] and autophagy receptor SQSTM1[424]. **TNFSF11** decreases the expression of ESR1[822] and increases the expression of SIGMAR1[561], a familial ALS protein that regulates mitochondrial function[823]. ESR1 increases the expression of **TNFSF11**[824], **UHMK1**[424], **DCHS1**[815], **SOD1**[825], GFAP[826] and the acetylcholine-synthesizing enzyme choline acetyltransferase **CHAT**[827]. **CHAT** is specific for motor neurons (for more on **CHAT** regulation, see section 3.4) and its expression is also increased by the transcription factor **SATB2**[828] that regulates neurite outgrowth by increasing the expression of the differentiation factor **NEUROD6**[829] (that stimulates mitochondrial mass, preceding axonal growth, to provide the necessary energy for cytoskeleton dynamics[830]), and decreasing the expression of netrin receptor **DCC**[831] and **BHLHE22**[832] (that forms a transcription repressor complex regulating the expression of genes that are important for guidance of motor neuron axons[833]).

The cell cycle regulating protein **CACUL1** binds ESR1 and regulates its activity[666]. Estradiol-induced transcription is mediated by transcriptional activator and stress granule-associated protein **TDRD3**[834] (see section 3.2). Further, ESR1 is bound to and activated by the ESR1 coactivator PPARGC1A[835], and its expression is increased by PPARGC1A[835]. PPARGC1A mRNA levels are decreased in ALS patients compared to controls[117], and polymorphisms in the promoter region of the PPARGC1A gene are associated with an earlier age of onset in male ALS patients only[118]. In addition to its association with ESR1, PPARGC1A[836] binds to **NRF1**[837], EGFR[223], AMPK[838, 839], NF-KB[840, 841] (not shown), **LRPPRC**[842], **MED17**[843] and the MLL-complex[844]. PPARGC1A inhibits CASP3[845] and increases the expression of AMPK[846], **DIO2**[847] (an iodothyronine deiodinase[183]) and **NRF1**[837], and activates and increases the expression of SLC25A20[667, 668]. Further, PPARGC1A expression is increased by IFNG[848] (not shown), AMPK[849-851], ERK1/2[493] and the MLL-complex[852, 853]. The transcriptional repressor **ZNF746** binds **ZAK**[854], inhibits the activation of **NRF1**, and inhibits and represses the transcription of PPARGC1A[855, 856].

The MLL-complex methylates histone H3 lysine 4 (H3K4) (not shown) to activate gene transcription[183]. Activation of the MLL-complex is driven by the methionine cycle (see section 2.2). The MLL-complex binds the RNA- and DNA-binding protein MATR3[857, 858], and binds to and interacts with the nuclear transcription regulators BMI1[859] and E2F3[791, 860] that are also upregulated by ESR1 (see above). The DNA topoisomerase TOP2A binds to BMI1[861] and nuclear ERK1/2[862] (not shown), and downregulates the expression of MLL[863]. BMI1 binds to TARDBP[658], ATXN2[658] (not shown) and GATA3[864], a transcription factor that decreases the expression of IFNG[865] and **AGPAT5**[866], and regulates the expression of HSD17B[867]. Further, GATA3 and EPO decrease each other's expression[868, 869], whereas GATA3 and NF-KB[870-872], but also GATA3 and ESR1[318, 873], and NF-KB and BMI1[874, 875] increase each other's expression. Furthermore, ESR1 and the MLL-complex increase each other's transcriptional activity[876] and both bind to the transcriptional mediator **MED17**[877, 878]. Moreover, the nuclear receptor ESRRB may be an important modulator of this ESR1-MLL-interaction by binding to the MLL-complex[879], PPARGC1A[880], the mediator complex proteins **MED17**[879] and **MED13L**[879] – that regulate transcription of RNA polymerase II-dependent genes[183] – and inhibiting the transcriptional activity of ESR1 in the nucleus[881] (not shown).

In addition, SMAD1 binds to the MLL-complex[882], SKIL[883] and ESR1[884, 885], and is bound and regulated by **ZNF423**[886]. SKIL positively regulates axonal growth[887] and enhances the transcriptional activity of ESR1[888]. Moreover, SKIL upregulates the expression of **DCHS1**[889], a membrane protein and member of the cadherin protein family that regulates neuronal cell adhesion[183] and neuronal migration[890]. ESR1 is bound and modulated by the transcription initiation factor GTF2B[891], which itself binds to and interacts with the transcription factor **FOXF2**[892].

In summary, in the ALS landscape NF-KB, ESR1 and the MLL-complex as well as the ESR1 coactivator PPARGC1A interact with each other to regulate gene transcription and cellular processes, such as neurite outgrowth.

3. RNP GRANULES, INTERMEDIATE FILAMENTS AND AXONAL TRANSPORT

Motor neurons have long axons, i.e. upper motor neurons project from the motor cortex to the spinal cord, from where the lower motor neurons project to the muscles. Axonal transport over long distances is challenging and makes these neurons especially vulnerable for defects in axonal transport. To maintain their axons and distant synapses, motor neurons transport proteins to the required location, but are also able to quickly synthesize the necessary proteins locally in the axon or synapse. For this purpose, the cells transport translationally silent mRNA to the proper location in so-called ribonucleoprotein particle (RNP) granules. RNP granules are clusters of mRNA-binding proteins that regulate mRNA transport and translation, and initiate protein synthesis

upon various stimuli, such as injury, guidance cues and growth factors[893-896]. In the next sections the ALS landscape proteins involved in assembly, function (section 3.1) and clearance (section 3.2) of RNP granules will be discussed, followed by a description of the regulation of intermediate filaments that are important for axonal transport and neurite outgrowth (section 3.3). In section 3.4 the axonal transport of motor neuron-specific **CHAT** and the transport of RNP granules by motor complexes will be discussed.

3.1 Assembly and function of RNP- and stress granules

RNA-binding and -regulating proteins (e.g. HNRNPA1, HNRNPA2B1, TARDBP, FUS, YWHAB, ATXN2, PFN1, SMN1/2, MATR3) bind to RNA and form complexes, RNP granules, that can be classified into various types, e.g. transport RNP granules, stress granules and processing bodies (P-bodies). Transport RNP granules regulate mRNA transport and local mRNA metabolism and translation, e.g. in axon terminals, whereas cellular stress modifies the RNP granules and gives rise to either stress granules – that represses translation of mRNAs and holds mRNAs dormant until needed at a later time point – or to P-bodies in which the mRNA is degraded[897]. Mutations in FUS and TARDBP trap themselves and their mRNA targets (see below) in stress granules, resulting in pathological inclusions and subsequent impairment of RNA processing, axonal migration, protein synthesis by RNP granules in axon terminals and neurite outgrowth[897-904]. The RNA-binding protein MATR3 is associated to ALS[97, 98], binds to the familial ALS proteins FUS[858] and TARDBP[905, 906] and localizes to P-bodies and possibly also to stress granules[907]. **C9ORF72** localizes to P-bodies and is recruited to stress granules during cellular stress[908]. Further, stress granule assembly is mediated by **C9ORF72**[908] and by the stress granule assembly protein G3BP1[909, 910]. Hexanucleotide expansions upstream of **C9ORF72** dysregulates its expression and leads to spontaneous stress granule formation[908]. TARDBP is also required for stress granule assembly by activating and increasing the expression of G3BP1[82, 911]. Further, G3BP1 is inhibited by RAC1-mediated inhibition of RHOA[912]. G3BP1 expression is increased by **NRG1**[913] and FLT1[362], and G3BP1 binds to ATXN2 (a mediator of RNP assembly)[914], TARDBP[905] (not shown), VCP[457], NF-KB[915], BMI1[658] (not shown), AMPK[559] (not shown), HNRNPA1[916, 917], PIN1[918] (not shown), APP[566], GRB2[410] (not shown), NTRK1[249] and ESR1[808]. Thus, key proteins in the ALS landscape, such as familial ALS proteins, but also the Rho-GTPases (RAC1, CDC42, RHOA) and ESR1 bind to and regulate the assembly of stress granules that are important for RNA processing, local protein synthesis in the axons and neurite outgrowth.

The heterogeneous nuclear ribonucleoproteins (hnRNP) HNRNPA1 and HNRNPA2B1 regulate the packaging of pre-mRNA into RNP granules, their export from the nucleus and subsequent processing and translation[183]. Upon binding and activation of CXCR4 by **CXCL12**, HNRNPA2B1 is transported from the nucleus to the cytoplasm[919]. Pathogenic mutations in HNRNPA1 have been detected in ALS patients[90] and HNRNPA1 protein is

decreased in the spinal cord motor neurons of ALS patients[89]. HNRNPA1 and HNRNPA2B1 physically interact[917], and HNRNPA1 binds to APP[566] (that also binds to the pre-mRNA regulating **CLK3**[566, 920]), ESR1[660, 808], FUS[917], GRB2[410], ICAM1[196], **LRPPRC**[916, 921], MATR3 [858, 916], NTRK1[249], PFN1[917], **RBMS1**[592], **RNF14**[922], ROCK1[539], TARDBP[89, 539, 906, 916], **UBR2**[539], UBQLN2[923], **UPF3A** (that is a regulator of nonsense-mediated mRNA[924] and binds EXOSC10[925], that also binds to TARDBP[926] and the neuronal membrane protein **HIATL1**[926]) and VCP[457]. Further, HNRNPA1 decreases homodimerization of **RNF14**, and the interaction between **RNF14** and AR[922], and increases the degradation of FUS, but not mutant FUS[927]. Furthermore, HNRNPA1 expression is decreased by APP[928] and SKIL[889], and increased by EGFR[233, 234].
HNRNPA2B1 binds to GRB2[410] (not shown), ICAM1[196] (not shown) and TARDBP[906, 929], is located in a complex with **C9ORF72**[559] and binds to subunits of the MLL-complex[930, 931] (not shown) and, as previously mentioned, NTRK1[249] (not shown) and ESR1[808]. HNRNPA2B1 increases expression of IFNG[932, 933] and **ABCB1**[724], and regulates expression of **CPVL**[724] (not shown) and PPARGC1A[724] (not shown).

Of interest, both HNRNPA1 and HNRNPA2B1 bind to the 'survival motor neuron' genes SMN1 and SMN2 – together designated as SMN1/2 – and function as splicing silencers, resulting in the skipping of exon 7[934-937]. Overexpression of HNRNPA1 abrogates exon 7 inclusion in the mRNA[936], whereas reducing HNRNPA1 levels results in splicing of exon 7 and a non-functional SMN1/2 protein[934]. SMN1/2 binds to the transcription factor **SP110**[854], inhibits RHOA[938] and binds to and is regulated by coilin (COIL)[939]. COIL binds to **ATXN1**[940] (**ATXN1** degradation is increased by SQSTM1[941]), binds to and interacts with the enzyme **CYB5R2** [445], and is downregulated by the MLL-complex[942] (not shown). SMN1/2 bind to PFN1[938], BMI1[658] (not shown), GRB2[410], SNRPG[937, 943] (binds also to **DDIT4L**[944]) and the MLL-complex[945].

Thus, the hnRNP proteins HNRNPA1 and HNRNPA2B1 control the regulation of (pre-) mRNA processing by RNP granules, and bind to and interact with multiple familial ALS proteins, but also the MLL-complex and ESR1, and regulate estradiol signaling by modulating **ABCB1**, PPARGC1A and ESR1 signaling.

3.2 Stress granule clearance

The scaffolding protein **TDRD3** localizes to stress granules by binding dimethylarginine-containing proteins, binds to FUS and is involved in the disassembly of stress granules[183, 946, 947].

The familial ALS protein VCP increases the clearance of stress granules and as such prevents the aggregation of stress granules[948]. VCP binds to the other familial ALS proteins ALS2[457], VAPB[949], PFN1[950], OPTN[951] (OPTN also binds to **SOD1**[952] and its expression is regulated by FUS[953] (not shown)) and FUS[656] (that also binds to PFN1[656]). Further, VCP

binds NTRK1 (see section 1.2.2), NF-KB[457], **ATXN1**[954], **DIO2**[955], PLK1[457], COIL[457], AMPK[457, 956] (that also binds to VAPB[957]), ERK1/2[457], YWHAB[457], CASP9[457] and **RAD23B**[958] (that is in a complex together with UBQLN2[959] (not shown), binds SQSTM1[960] and its phosphorylation is increased by PLK1[961]) and activates NF-KB[768].
Thus, VCP has interactions with multiple proteins involved in RNP-granule functioning and prevents pathogenic aggregation of stress granules.

3.3 Intermediate filaments and RNP granules

Intermediate filaments, e.g. peripherin (PRPH) and neurofilaments (NEFL, NEFM, NEFH), are the 'building blocks' of the cytoskeleton and important for neurite outgrowth and maintenance of axons[962-966]. The Lewy body-like inclusions, as found in the motor neurons of ALS patients, contain PRPH[124, 967, 968], mutations in PRPH are associated with ALS[122-126] and overexpression of PRPH results in defective axonal transport of neurofilaments[969]. Furthermore, the RNP-granule associated proteins **SOD1**, TARDBP and YWHAB stabilize NEFL mRNA through interaction with its 3'UTR[970-972] (not shown) and TARDBP increases the transport of NEFL mRNA to the distal axon[903] (not shown). Mutations in **SOD1** and TARDBP result in neurofilament aggregates in motor neurons[727, 973-975], reduced levels of neurofilaments and reduced axonal length[976]. NEFL mRNA and protein is increased in the spinal cord[977] and serum and CSF[103, 978] of ALS patients, respectively. NEFL binds to the cytoplasmic protein phosphatase PPP1CA[979] (not shown) that on its turn binds to and interacts with the membrane proteins **TMEM132C** and **TMEM132D**[980], and the endosomal sorting protein CHMP2B[461], and binds to the membrane protein **CSMD1**[980] that is involved in neuronal growth cone stabilization[981, 982]. **PTPRD** also stabilizes growth cones and increases neurite outgrowth[982, 983], and its expression is regulated by estradiol[984] (not shown).
Further, the ALS-associated autophagy receptor SQSTM1 binds NEFM[559] (not shown). Phosphorylated neurofilament NEFH is increased in the CSF of ALS patients[103] and the ESR1 co-activator PPARGC1A increases the expression of NEFH mRNA[985] (not shown), whereas ERK1/2[986] and PIN1[987] increase phosphorylation of NEFH protein (not shown).

Thus, a defect in the regulation of RNP and stress granules – either due to upstream dysregulation or direct mutations in RNP-granule or intermediate-filament genes – cause stress granules to aggregate, resulting in defective local translation of intermediate filaments and subsequent (further) weakening of the axon, reduced outgrowth and motor neuron death. Proteins that require axonal transport to the synapse – e.g. **CHAT**[988] – are therefore unable to exert their function (see section 3.4 for more on **CHAT** regulation). Interestingly, estradiol is neuroprotective and increases neurite outgrowth via ESR1[989], resulting in the inhibition of RHOA and activation of the GTPases RAC1 and CDC42[616], which is the same pathway that inhibits stress granule assembly by G3BP1 (see above).

Of note, also the intermediate filament GFAP is dysregulated in ALS patients, i.e. GFAP is increased in the CSF of ALS patients[86], and acetylated and fragmented GFAP levels are increased in the spinal cords of ALS patients[84, 85]. However, GFAP is specifically expressed in, and a marker for, mature astrocytes[183]. Astrocytes interact with motor neurons, support their functioning and can affect motor neuron fate, i.e. degenerating motor neurons in ALS patients are surrounded by astrogliosis which is toxic to motor neurons[990, 991]. GFAP binds NEFL[992] (not shown), APP[566] (not shown), **RAD23B**[960] and YWHAB[463] (not shown). Further, GFAP is phosphorylated and thus inhibited by ROCK1[993, 994] (not shown), and GFAP expression is increased by ESR1[826], **B4GALT6**[471], **NRXN1**[995] (not shown; regulates neurite outgrowth[996, 997]), **TGFBR2**[998] and **TRPV1**[999], regulated by NF-KB[1000] (not shown), and decreased by **SOD1**[1001], AR[1002] (not shown) and carnitine[1003]. Furthermore, GFAP regulates the localization of TARDBP[1004] and is cleaved by CASP3[1005] (not shown). Thus, GFAP is regulated by ALS landscape genes, that as such may affect astrogliosis. ALS pathology is therefore not limited to motor neurons and at least part of the ALS landscape processes may take place in astrocytes or affect the functioning of astrocytes.

3.4 Axonal transport by motor complexes

To transport their cargo, axons require a functional microtubule network for motor proteins such as **KIF14**, Kinesin-II, **KIFAP3** and STAU1. Multiple proteins in the ALS landscape regulate microtubules, i.e. the non-selective calcium permeant cation channel **TRPV1** regulates stabilization of presynaptic microtubules and also regulates growth cone morphology, increases synaptic growth and regulates axonal guidance by activating the RHOA/ROCK1 pathway[1006-1008]. Further, microtubule dynamics is regulated by the microtubule organizing protein **CEP44** that binds to TBK1[1009], **BEND7**[778] and **SPERT**[944] (that also binds to PIN1[778]) and the cytoplasmic enzyme **AGBL4** that modulates microtubules and is thereby critical for neuronal survival[1010]. Furthermore, the microtubule-associated proteins **MTUS1** and **MTUS2** regulate microtubule stability and elongation[1011, 1012] (MTUS2 also binds to AMPK[778] and PIN1[778]; not shown). Lastly, TUBA4A is a major constituent of microtubules and mutations in the TUBA4A gene are associated with ALS[147, 148]. TUBA4A binds to the RNA-trafficking protein STAU1[1013], therefore, cytoskeletal defects through mutated TUBA4A may in this way affect axonal transport and local translation of RNA (see below).

CHAT synthesizes acetylcholine, the main and essential neurotransmitter at the neuromuscular synapse between motor neurons and skeletal muscle cells[1014-1016]. **CHAT** is produced in the cell body of the neuron and transported to the synapse[28], and therefore relies on axonal transport to exert its function. In mutant **SOD1** mice, axonal transport of **CHAT** is impaired and defects in axonal transport precede disease symptoms[28, 1017]. Of note, **CHAT** expression is lower in spinal motor neurons of ALS

patients[29, 30] and increased via the NGF pathway by NGF[1018, 1019], NTRK1[1020] and NTRK2[1020], and by estradiol[1021] and ESR1[827]. APP increases the activation of **CHAT** (not shown) and the binding affinity of **CHAT** for the familial ALS protein VCP[1022].

Mutant **SOD1** increases the sequestration of **KIFAP3**, a kinesin-associated protein that binds the Kinesin-II motor complex, and regulates microtubule-dependent transport of cargo proteins such as **CHAT**[28]. A polymorphism in the **KIFAP3** gene is associated with ALS and is thought to modify the ALS phenotype[10, 27]. In addition to **CHAT**, **KIFAP3** binds to **RBFOX1**[445], **DISC1**[478], AR[1023] (not shown), NTRK1[249] and **CDH2**[1024], and increases the activity of the Rho-GTPases RAC1, CDC42 and RHOA[216] (not shown) and NADPH-oxidase[1025]. **KIF14** is another microtubule motor protein in the landscape and binds to NTRK1[249]. Of interest, the Kinesin-II motor complex is involved in microtubule-dependent transport of cargo proteins such as **CHAT**, regulates the transport of RNP granules in the axons of neuronal cells[1026] and is required for neurite outgrowth[1027]. KIF3A, an RNP granule-associated subunit of the Kinesin-II motor complex, binds to the kinase NEK1[1028]. NEK1 is involved in microtubule dynamics[1029], binds to the ALS-associated[58] and cytoskeleton-regulating[1030] C21ORF2[858] and its expression is increased by ESR1[424]. Further, KIF3A also binds to RNA-binding protein **UHMK1** that regulates neurite outgrowth and is localized to RNP granules[1031]. Furthermore, the kinase activity of **UHMK1** stimulates protein translation and thereby enhances local axonal protein translation of e.g. actin[1031]. In addition, the RNA-trafficking and tubulin-binding protein STAU1 is also part of RNP granules and essential for the transport and local translation of mRNA[1032, 1033]. STAU1 binds to the hnRNP proteins HNRNPA1[1013] and HNRNPA2B1[1013], the familial ALS protein TARDBP[903], APP[566] (not shown), **LRPPRC**[1013] (not shown), PPP1CA[882, 1034], MATR3[1013], NTRK1[249] (not shown), ESR1[808], and the Rho-GTPases CDC42[1033] and RAC1[1033].

Thus, **CHAT**, the enzyme that is essential for motor neuron function, is regulated by the NGF and EGF growth factor pathways and by estradiol signaling. Further, both **CHAT** and RNP granules require a properly functioning cytoskeleton machinery to be transported from the cell body to the axon terminal, and defects or dysregulation of motor proteins (e.g. Kinesin-II, **KIFAP3** or STAU1) or of proteins regulating the cytoskeleton (e.g. TUBA4A) may affect their translocation and functioning.

4. CONCLUDING REMARKS

The ALS landscape converges on the regulation of Rho-GTPase signaling, and axonal outgrowth and maintenance. These processes are stimulated by guidance cues and growth factors (section 1) and mediated by estradiol-dependent signaling that interacts with the FA cycle, MET cycle and NADPH-oxidase activity (section 2). Under normal conditions, the factors mentioned in section 1 and 2 provide the necessary means for motor complex proteins and RNP granules to assemble and transport their

cargo through the axon to their target location and to allow the regulation of neurite outgrowth, axon maintenance or synaptic function (section 3). The familial ALS proteins directly regulate crucial pathways within the landscape, such as the regulation of growth factor pathways, Rho-GTPases and RNP granules. Consequently, the mutations in the familial genes directly lead to (familial) ALS, whereas in individual sporadic ALS patients multiple functionally 'lower-impact' genetic variations are involved in the development of the disease. Nevertheless, disorganization of the cytoskeleton, reduced neurite outgrowth and axon maintenance, caused by or causing RNP granule dysfunction, results in a defective synapse and motor neuron death in both familial and sporadic ALS. Furthermore, estradiol signaling is neuroprotective by regulating neurite outgrowth, and the activation of Rho-GTPases and the NADPH-oxidase complex as well as by mediating stress granule assembly and regulating **CHAT** expression. Hence, estradiol is a crucial modulator of ALS pathology.

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LIST OF ABBREVIATIONS

ALS,	amyotrophic lateral sclerosis;
fALS,	familial amyotrophic lateral sclerosis;
FA,	folic acid;
GWAS,	genome-wide association study;
GWASs,	genome-wide association studies;
IPS-MNs,	induced pluripotent stem cell-derived motor neurons;
MET,	methionine;
nonsyn,	nonsynonymous;
NS,	not shown;
sALS,	sporadic amyotrophic lateral sclerosis;
SMNs,	spinal motor neurons;
SNP(s),	single nucleotide polymorphism(s);
syn,	synonymous;
UTR,	untranslated region;
VH,	spinal ventral horn gray matter

6

General discussion

Parkinson's disease (PD) is caused by a complex interplay of genetic and environmental factors, and characterized by the degeneration of dopaminergic neurons in the substantia nigra (SN), resulting in motor and cognitive dysfunction. At present, there are no disease-modifying therapies for PD. To develop therapies that slow or even halt the progression of PD, knowledge about the dysregulated molecular pathways underlying the disease is essential. Currently, multiple candidate genes for familial PD – encompassing only 5-10% of the patients – have been identified, which provide general insights into the underlying pathogenesis of PD. For the other 90-95% of PD patients who have the so-called 'sporadic variant' of the disease, the underlying pathogenic mechanisms have barely been elucidated. However, the completion of the human genome project in 2001[1, 2] enabled researchers to perform genome-wide studies. Genome-wide association studies (GWASs) have yielded (putative) genetic risk factors for sporadic PD, but it has proven difficult to functionally couple the proteins encoded by these GWAS candidate genes to PD etiology, e.g. because they show a broad variety of functions and do not belong to a single pathway or biological process. Moreover, detailed insights into the interactions between the various GWAS- and familial candidate gene-associated processes are essentially lacking. Therefore, through building molecular landscapes, I focused in this thesis on the integration of all available GWAS data and other (genetic) PD data in order to further clarify the molecular mechanisms underlying PD.

In this General Discussion, I will describe how the molecular landscape building approach that I used (1) differs from systems biology and bioinformatics approaches, such as protein network analysis tools, gene ontology (GO) term analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and (2) which biases may be encountered or prevented when applying this approach (section 6.1). This is followed by a discussion of the unique features of the PD landscape (chapter 2) compared to the amyotrophic lateral sclerosis (ALS) landscape (chapter 5) (section 6.2) and a section on the putative druggable targets that were identified within these landscapes (section 6.3). Subsequently, I will discuss the differences in the interpretation of GWAS data and genome-wide expression data, and to what extent the genes/proteins and functional themes that are enriched within these two sets of data overlap (section 6.4). Then, I will determine the overlap between human PD brain expression data and expression data from the brains of MPTP-treated mice and exercised MPTP-treated mice, as follow-ups of chapters 3 and 4, respectively (section 6.5). Lastly, I will discuss how the molecular landscapes may contribute to personalized medicine (section 6.6) and will offer suggestions for future research directions (section 6.7). An overview of the sections in this General Discussion, and how they relate to the chapters in this thesis, is presented in **Figure 1**.

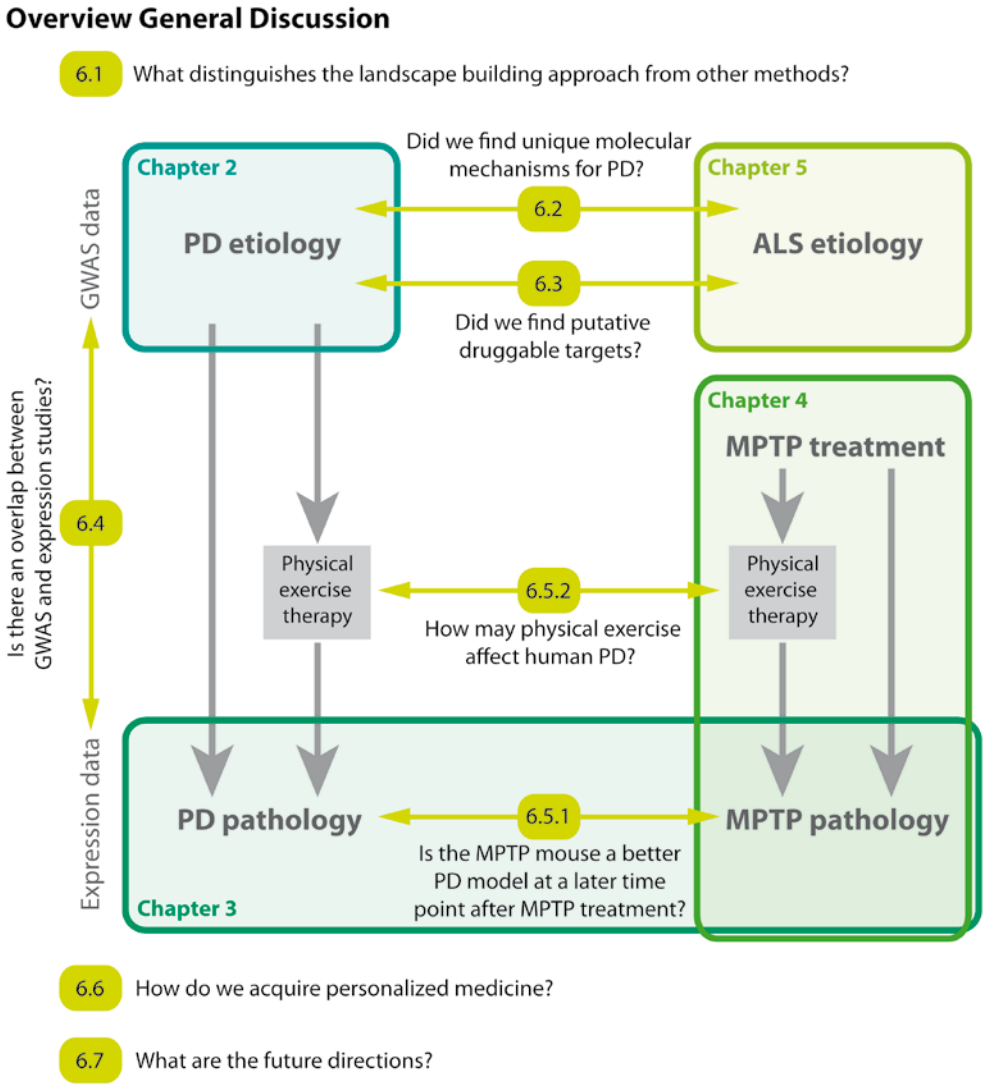

Figure 1. Graphical overview of the chapters in this thesis and their relationship with the sections in the General Discussion.

6.1 THE MOLECULAR LANDSCAPE BUILDING APPROACH

Systems biology, which could be defined as the computational and mathematical modeling of complex biological systems, is fundamentally different from the landscape building approach that underlies many of the studies in this thesis. Still, systems biology and the landscape building approach both aim to develop a model to describe a complex biological system based on as much relevant, unbiased information as possible, such as genomic, transcriptomic, proteomic, metabolomic and lipidomic

data, and the determination of the interactions between the individual components of the system (e.g. protein-protein interactions). However, while all interactions in 'networks' generated through systems biology approaches are mathematically quantifiable, the landscape building approach determines important 'hubs' or 'nodes' within the landscape on the basis of their biological relevance and not solely based on quantitative evidence (e.g. how quantifiably 'connected' or 'central' a hub is in the network). A seeming advantage of a network generated through systems biology is that it can also be interpreted without *a priori* biological knowledge, i.e. by only considering the most statistically significant outcomes (e.g. for the connectivity or centrality of a protein within the network). However, this has an important caveat in that, without biological knowledge, the interpretation and application of the findings are *only* based on statistical thresholds which may potentially be misleading[2]. For instance, a potential research bias – i.e. disease-associated proteins are generally studied in more detail than proteins with an unknown disease link and/or biological function – can have major repercussions for the interpretation of the results[3-5]. A few examples of these frequently studied, disease-associated proteins are tumor protein p53 (TP53), breast cancer 1 (BRCA1), interleukin-6 (IL-6), amyloid beta precursor protein (APP) and – for PD – alpha-synuclein (SNCA). For each of these proteins, thousands of interactions have been identified, leading them to be present as important regulators in virtually all computational analyses that are performed. Consequently, this may give the impression that these proteins are central in the regulation of a given disease, but in reality, they are overrepresented, and 1) mask the functional involvement of other, less well-documented, proteins in the analysis, and 2) create a circular reasoning for the diseases in which these proteins are already known and validated contributors. The latter would for example result in (not very innovative) interpretations such as "TP53 is central in cancer formation", "APP is crucial for the development of Alzheimer's disease", or "SNCA is important in PD". Thus, the well-documented proteins may be less involved in the disease than initially suggested by statistical analyses. Therefore, the qualitative aspects of a generated network – for example the involvement of generic vs. (more) disease-specific biological processes, the biological relevance of proteins for the system, and the degree of documentation of (i.e. literature about) the included proteins – are at least as important as the quantitative aspects (connectivity, centrality) to understand the biological system under investigation.

Freely available online bioinformatics-based tools can be used to generate protein interaction networks and analyze the enrichment of certain functional themes and processes within a set of proteins. Here, I will shortly discuss what the limitations of these tools are and how the landscape building approach is different from the bioinformatics-based generation of protein interaction networks. First, the landscape building approach distinguishes itself from protein network generation tools such

as STRING[6], GeneMania[7] or IMP[8] by manually curating all experimental evidence for the interactions in the landscape, but without assigning a confidence score or weight to these interactions. It should be noted that the number of interactions listed in these tools varies greatly depending on the databases they use, and/or are strictly separated per organism which makes it difficult to get a comprehensive overview of all possible interactions. Further, the score/weight they assign to these interactions is also dependent on these databases and on the algorithm they apply, and may not necessarily have any biological meaning due to a research bias (as discussed above). Nevertheless, these protein network generation tools have improved significantly in recent years (due to increasing content in the annotation/interaction-databases they use) and are useful tools to uncover protein-protein interactions. Second, in addition to protein-protein interactions, the landscape building approach adds interactions with/between molecules that are not proteins e.g. metabolites, endogenous chemicals, ions and lipids. Third, through the landscape building approach, protein complexes are identified whereas the protein networks generated through bioinformatics tools only contain bilateral interactions. The latter means that proteins that do not physically interact with each other but that are located in and regulate the same protein complex are not recognized as interacting proteins and hence, important information is missed. Lastly, candidate proteins that have the same function – e.g. the transport of calcium ions over the cell membrane – or belong to the same metabolic pathway – e.g. the folate cycle – are not necessarily functionally interacting with each other. Therefore, such proteins are not picked up by protein network generation tools, but are nonetheless relevant to understand the biological processes that are regulated by a set of candidate proteins and therefore included in the landscape building method.

This enrichment or overlap of functions, pathways and processes within a set of genes/proteins can also be assessed by using GO terms[9, 10] and/or KEGG pathways[11, 12]. GO terms are assigned to genes to provide a classification system for the cellular location, function, and involvement in biological processes of the proteins they encode. In the KEGG pathway database, proteins are assigned to a collection of pathways of various functions and (perturbed) biological systems within the cell. However, enrichment analyses on the basis of these GO terms/KEGG pathways should be used cautiously because the functional annotations these analyses use are often inconsistent, incomplete, too general, or sometimes incorrect[13-17]. Manually reviewing the functions and processes in which a protein is involved – as is done in the landscape building approach – is therefore necessary to achieve a more accurate, detailed and correct picture of the disease-underlying pathways/signaling cascades and processes on the basis of current knowledge. However, a functional annotation bias due to the use of (high-throughput) experimental assays that can only detect the abundant proteins[18], or only identify one or a small group of functions[19] seems inevitable. For example, a high-

throughput screening that identifies the involvement of proteins in tumor formation marks hundreds of proteins with the annotation 'cancer', while many of them are also involved in multiple other functions and diseases that are not annotated in this screening. But again, as with the protein network generation tools, the more complete these annotation databases become, the more useful they will be to identify enriched processes.

In summary, the landscape building approach focuses on the biological significance of the proteins and processes involved, and limits the effects of a potential research bias on the interpretation of the identified interactions. The landscapes enable us to interpret the functional/biological relevance of large, disease-linked gene/protein sets, provide an overview of the interconnectivity of multiple known and less known functional themes, and are hypothesis generating. That said, a functional annotation bias still seems unavoidable and only additional studies to get a more complete picture of all functions of these genes/proteins can decrease this bias.

A further aspect to consider is the personal bias of the researcher building the molecular landscape, as this researcher needs to decide e.g. which data sets can be used, which interactions have been sufficiently validated by experimental evidence, how the interactions are visualized within the landscape, which additional proteins are relevant for the molecular landscape, which functional themes and biological processes have biological relevance for the disease studied and, eventually, which hypotheses can be made based on the landscape. This personal bias can be minimized by 1) being as comprehensive as possible, i.e. including all known interactions, functions and themes from multiple data sets, and 2) staying hypothesis-free as long as possible to avoid a 'confirmation bias', i.e. only the evidence supporting the hypothesis is accepted and other evidence is dismissed. Finally, the hypotheses drawn from the landscape, and the important functional themes and key biological processes within the landscape should be tested/validated, which can be accomplished through a genetic, epidemiological and/or functional validation. For example, for the PD landscape (see chapter 2) we genetically validated the main landscape-derived hypothesis that 'lipids are important in the etiology of PD' by finding a shared genetic risk between lipid traits and PD. Moreover, for the ALS landscape (see chapter 5) we found corroborating epidemiological evidence in a cohort of ALS patients for our hypothesis that 'estradiol signaling is involved in the etiology of ALS'. However, these types of validation should be followed by a functional validation in which modulation of a landscape-derived pathway or process is demonstrated to directly affect PD symptoms and/or disease progression in a cell/animal model for PD or in PD patients, and as such provides the strongest evidence for corroborating the hypothesis.

6.1.1 Improving the molecular landscape building approach

Newer genetic studies often use bigger patient populations, better genotyping arrays and have a higher statistical power for detecting true genetic susceptibility variants. Therefore, more recent GWASs may yield more reliable results than older studies. In our landscape building method, we select our candidate genes based on set selection criteria – as described in the General Introduction – that are the same for all included GWASs. Therefore, we do not take into account the differences in power and reliability between these GWASs. In general this would mean that older, statistically less powered GWASs have a higher false positive rate than more recent GWASs. Using selection criteria that take into account these differences between GWASs would reduce the false positive rate and improve candidate gene selection.

The following two methods may improve candidate gene selection:

1) *Data-driven p-value threshold (DDPT).* The p-value threshold used for inclusion of SNPs is chosen in such a way that there is a false positive ratio of (for example) 0.25, that is calculated as:

$$false\ positive\ ratio = \frac{expected\ number\ of\ SNPs}{discovered\ number\ of\ SNPs}$$

This means that when one million (1.00E06) independent SNPs (i.e. SNPs in low linkage disequilibrium (LD)) have been genotyped in a GWAS, and an inclusion p-value of p<1.00E-04 is used, the expected number of SNPs with a p<1.00E-04 is 100 (i.e. 1.00E06 / 1.00E04). To obtain a false positive ratio of 0.25, this would mean that in the GWAS 400 SNPs should be found with a p<1.00E-04. When less SNPs are discovered with a p<1.00E-04, the p-value threshold has to be set to a lower threshold, and to a higher threshold when more SNPs are found. The resulting list of SNPs could then be used for candidate gene selection. In this way, rather than selecting SNPs/genes based on a single, pre-determined p-value cut-off, candidate SNPs/genes would be chosen based on the same false positive ratio, which (1) is independent of the size of the investigated patient population and, linked to this, the statistical power of the study and (2) can correspond to different p-value thresholds for different GWASs.

2) *Individual SNP-weighted p-value (ISWP).* The number of SNPs in a gene can vary and depends on the gene size and on the genetic variation in each gene. Moreover, in our landscape building method, we also select candidate genes based on SNPs that are not located in a gene itself but in the 100 kb upstream and downstream regions from each gene that likely contain expression quantitative trait loci (eQTL), SNPs that influence gene expression 'from a distance' (as described in the General Introduction). The chance to find a low

p-value-SNP in (the vicinity of) a large gene with many SNPs is therefore higher than in a small gene with only a few SNPs. Based on the results of a given GWAS, an ISWP can be calculated that takes into account the number of SNPs in a gene, with or without the SNPs in the 100 kb upstream/downstream regions from this gene, using this formula (for n SNPs):

$$\frac{n}{P_{ISWP}} = \frac{1}{P_{SNP1}} + \frac{1}{P_{SNP2}} + \frac{1}{P_{SNP3}} + \cdots + \frac{1}{P_{SNPn}}$$

A disadvantage of the ISWP is that it does not account for high LD between SNPs – which is by definition the case for imputed SNPs – and that all SNPs are assumed to be of equal functional importance. For example, when a high LD block in (the vicinity of) a gene contains a functional SNP with a low p-value, and another LD block in (the vicinity of) the same gene has no such SNPs, the latter LD block 'dilutes' the signal of the functional SNP, which could lead to the gene not being selected. Nevertheless, when a locus with multiple genes is associated to a disease, the ISWP-method makes it possible to indicate which gene in this locus is most likely to 'explain' the signal in the locus – i.e. the gene with the lowest ISWP – and can therefore be used as additional evidence for selecting a candidate gene. Of note, whereas the ISWP-method requires the summary statistics – i.e. the associated p-values for all genotyped SNPs – of a GWAS, the DDPT-method can already be applied to published GWAS data for which e.g. only the SNPs with a p<1.00E-04 are available.

6.2 HOW UNIQUE IS THE PATHOGENESIS OF PD?

The landscape building approach ideally results in a molecular landscape that encompasses pathways, biological processes and molecules that are disease specific, and as such, the landscape provides a unique molecular 'signature'. In this section, I will discuss to what extent the landscape building approach can distinguish between two neurodegenerative diseases and result in two landscapes containing unique molecular mechanisms (i.e. the landscapes for PD and ALS that are presented in chapters 2 and 5, respectively). Moreover, I will determine how these unique mechanisms are linked to disease-specific cellular and neuroanatomical substrates, i.e. what causes the degeneration of especially DA neurons in PD and motor neurons in ALS? First, I will describe the generic pathways, followed by the immune processes, and the main biological processes in the PD and ALS landscapes. Subsequently, I will discuss which of these pathways and processes can be linked to the specificity for DA- and motor neurons in PD and ALS, respectively.

Generic pathways – i.e. pathways that are common across cell types and hence not unique to a certain cell type – are often involved in a wide variety of (important) cellular processes. The processes regulated by these generic pathways are slightly different in

each landscape, i.e. common proteins such as the Rho-GTPases (RAC1, CDC42, RHOA) seem to particularly regulate vesicular transport, and endo- and exocytosis, in the PD landscape, whereas in the ALS landscape, their function is more related to growth factor signaling and neuronal outgrowth. This discrepancy is due to a different set of proteins that regulates the generic pathway in each landscape. For example, RAC1 is regulated by the familial PD proteins PARK2 and LRRK2, and by the familial ALS proteins SOD1, ALS2 and TARDBP. These five regulatory proteins have completely different functions. Moreover, the PD and ALS landscapes reveal that the components of the generic pathways seldom contain disease-linked genetic variations (i.e. SNPs) or more damaging mutations in these late-onset neurological diseases. In this respect, I assume that true genetic defects in generic proteins would manifest much earlier in life (e.g. such as *HPRT* mutations in Lesch-Nyhan disease) and would therefore have led to either a different disease or to a reduced viability resulting in premature death.
Thus, in our landscapes of (late-onset) neurodegenerative diseases, the generic pathways are often involved through a disease-specific set of proteins that regulates the pathway (e.g. through activation or inhibition) rather than through direct genetic variations/mutations in the genes constituting the generic pathway itself. In this way, not all processes regulated/modulated by the generic pathway are dysfunctional, and/or can be easily compensated for by other proteins/regulators in the cell.

The immune response is involved in both PD and ALS (see chapters 2 and 5, respectively), because when neuronal cells degenerate and die – which is the case in both diseases – they need to be cleaned up. However, an association of immune-related genes (and hence proteins) with the disease implies that the immune processes are not just a symptom, but may be causative for or (at least) contribute to the disease. Whereas multiple immune-related genes/proteins have been genetically linked to PD (e.g. *C9*, *CD200*, *CXCR4*, HLA-genes, *IL1B*, *IL2RA*, *IL5RA*, *IL6*, *PRF1*, *TIMD4*, *TNF*), the genetic basis for the involvement of the immune response in ALS is limited to only a few genes/proteins (*CXCL12* and the HLA-genes). Therefore, the immune response in PD may be an etiological factor, whereas in ALS it may be more likely that it is largely a secondary reaction to dying neurons. In this respect, the general scientific consensus is that the immune response in PD may be causative rather than responsive to the dying neurons[20-22], while in ALS it is unclear if the immune reaction is protective, contributes to the disease, or is initially protective and becomes neurotoxic over time[23-25]. Therefore, targeting the immune system as a treatment strategy may yield better results in PD than ALS to (positively) modulate a truly causative disease mechanism.

The main biological themes/processes in the PD landscape (lipid regulation and oxidative stress) are different from the main themes/processes in the ALS landscape (estradiol signaling and axonal function). Some of the pathways and signaling cascades

within these themes overlap between the two landscapes (e.g. cholesterol and the Rho-GTPases are present in both) but overall, the specific themes/processes provide a unique signature per landscape. Moreover, the landscapes show the involvement of specific cell organelles that have a remarkable mechanistic overlap with the main themes in each landscape. For example, compared to the ALS landscape the involvement of mitochondria is more pronounced and widely documented in PD[26, 27], and overlaps with oxidative stress pathways. Further, the lysosomes, the endocytic pathway and the endoplasmic reticulum are firmly represented in the PD landscape, whereas these organelles are barely present in the ALS landscape in which especially the ribonucleoprotein particle (RNP) granules have a central role. Interestingly, it appears that the – to some extent – disease-specific organelles are also the locations where the majority of processes involved in the landscape-specific main themes/processes occur, which provides a specific 'compartmentalization' of the landscapes within the neuronal cell.

In summary, we found that (1) generic pathways function in a slightly different context and are regulated by a different set of proteins in the PD versus ALS landscapes, (2) a dysregulated immune response is probably more of an etiological factor in PD than ALS, and (3) the main biological themes/processes within each landscape take place in distinct organelles.

A further question arising from our landscapes is which of the landscape pathways and processes are (most) disease-specific *and* (best) explain the substrate specificity of PD and ALS? The generic pathways do not provide this specificity because by default, they are present in virtually every cell type. In addition, the pathways of the immune response do not provide this specificity as they regulate the function of immune cells and not specifically DA neurons or motor neurons. Nevertheless, autoimmune processes towards neuron-specific antigens (e.g. neuromelanin generated by and accumulating in DA neurons) may explain a part of the substrate specificity. However, the strongest proof for the specific involvement of DA or motor neurons can be found in the cell-specific processes that are associated with the main biological processes in each landscape. DA neurons rely heavily on the regulation of reactive oxygen species that are produced in high amounts during to the production of DA, which also results in (and requires) the production, processing and storage of neuromelanin. A deficit in any of these processes, or related processes such as lipid homeostasis and SNCA regulation – that all heavily interact with neuromelanin processing and the regulation of reactive oxygen species – makes that the DA neurons are particularly sensitive. In PD, DA neurons in brain areas other than the SN are to a lesser extent subjected to neurodegeneration[28], indicating that SN DA neurons have specific characteristics that make them especially vulnerable to degeneration, e.g. the relatively high expression of

the DA transporter[29-32] and proteins involved in vesicle-mediated transport[33] in these neurons. As a result, SN DA neurons have the capacity to store more DA[32] than other DA neuron populations, which makes them even more sensitive to defects in mechanisms that protect them from cytosolic DA oxidation[34] and defects in related processes such as iron, neuromelanin and lipid regulation[35, 36].
The specificity for motor neurons in ALS may be explained by their very long axons (sometimes up to one meter), which makes that motor neurons, much more than other neurons, depend on axonal transport and local translation of proteins. Sensitivity to defects in these processes is in accordance with the main processes in the ALS landscape, such as axonal maintenance and RNP granule regulation.

In conclusion, the landscape building approach allowed for the identification of molecular pathways that are specific to PD and ALS. Moreover, some of these pathways are substrate specific and closely related to the main biological processes operating in the landscapes and hence provide an explanation why especially these specific types of cells degenerate. Of note, neurological diseases are often not as homogeneous as they are initially presented, e.g. late-stage PD patients often experience dementia and depression[37, 38]. It would therefore be interesting to find out to which degree the molecular landscapes of e.g. Alzheimer's disease and depression overlap with the processes, pathways and cell types in the PD and ALS landscapes. Ultimately, the availability of the complete landscapes of all neurological diseases would enable us to see their common pathways/processes, and understand why some of the diseases show clinical comorbidity and/or overlapping traits.

6.3 DID WE UNCOVER NEW POTENTIAL DRUGGABLE TARGETS?

As discussed above, the PD and ALS landscapes provide disease- and substrate-specific pathways and processes, and therefore offer opportunities for the identification and further development of novel druggable targets. New hypotheses can be generated based on the landscapes, and when these hypotheses turn out to be correct, e.g. 'lipids are important in PD', we can hypothesize that the proteins involved in e.g. lipid regulation are important players in PD pathogenesis and pathophysiology, and therefore represent potential druggable targets. The next step would then be to validate these targets in preclinical models, and assess if they are specific and can be – at least to some extent – specifically targeted by modulating drugs. Subsequently, these drugs should be tested in clinical trials to explore their effects. Below, I will elaborate on some of the possible druggable targets that we identified in the PD and ALS landscapes, and discuss how these proteins function within the landscape and what makes them potential targets. Note that these potential druggable targets do not have to be relevant for the entire patient population, but may be patient specific, depending on the pathways that are affected in each individual patient (see also section 6.6).

6.3.1 Druggable targets in the PD landscape

The molecular landscape of PD harbors four main biological themes, i.e. oxidative stress response, endosomal-lysosomal functioning, endoplasmic reticulum stress response, and immune response activation (see chapter 2). The main proteins per theme are summarized in **Figure 2 A-D** (a complete description of all proteins functioning in each theme can be found in chapter 2) and the most interesting putative druggable targets for each of the four themes (A-D) are discussed below:

A) Heme oxygenase 1 (HMOX1) represents a potential druggable target because it is involved in lipid regulation (the main overarching theme in the PD landscape), regulates DA-specific pathways and is protective in multiple cellular PD models[39-42]. HMOX1 is highly expressed in the periphery of Lewy bodies[43], increased in PD serum[44], regulated by DA[45] and degrades cytotoxic heme[46], and may therefore prevent oxidation of HDL and LDL, the initial heme scavengers in the circulation[47]. Further, HMOX1 regulates iron metabolism, reactive oxygen species formation, oxygen supply and mitochondrial function in the cell[46-48].

B) The propeptide PSAP, the four saposins (A-D), the LDL receptor LRP1 and the PSAP receptor GPR37 are potential druggable targets because they control lipid synthesis, are associated with DA-specific processes and regulate DA neuron survival. That is, PSAP is neuroprotective for DA neurons[49, 50] and exerts its effect by binding to LRP1 or GPR37 that both internalize PSAP upon binding[51, 52]. GPR37 binds to and regulates the expression of the DA transporter, is associated with juvenile PD, and a substrate of PARK2[53, 54] and hence strongly linked with PD. Further, uptake of PSAP results in its processing into four saposins (A-D) that regulate the ceramide/spingosine synthesis cascade (composed of SMPD1, GBA and ASAH1), but also SNCA aggregation, the immune response, apoptosis and membrane lipid composition[55-60].

C) Sterol regulatory element-binding protein 1 (SREBF1) is a putative druggable target, because it regulates lipid homeostasis, has an effect on ER stress[61] and mitophagy[62], and is associated with gait impairment in PD[63]. Through maintaining lipid homeostasis, SREBF1 is able to prevent cellular stress, but also the initiation of SNCA amyloid fibrils (the main component of Lewy bodies in PD)[64] and immune responses towards extracellular lipid and/or SNCA aggregation. SREBF1 is generic but the selective vulnerability of DA neurons to defects in lipid homeostasis may explain its specificity for these neurons. Nevertheless, SREBF1 targeting may also result in oncogenic growth[65], which possibly makes SREBF1 not specific enough for PD.

D) The angiotensin II receptor AGTR1, SERPINE1 and plasmin regulate the delicate balance between DA, lipids and the immune response, and are therefore

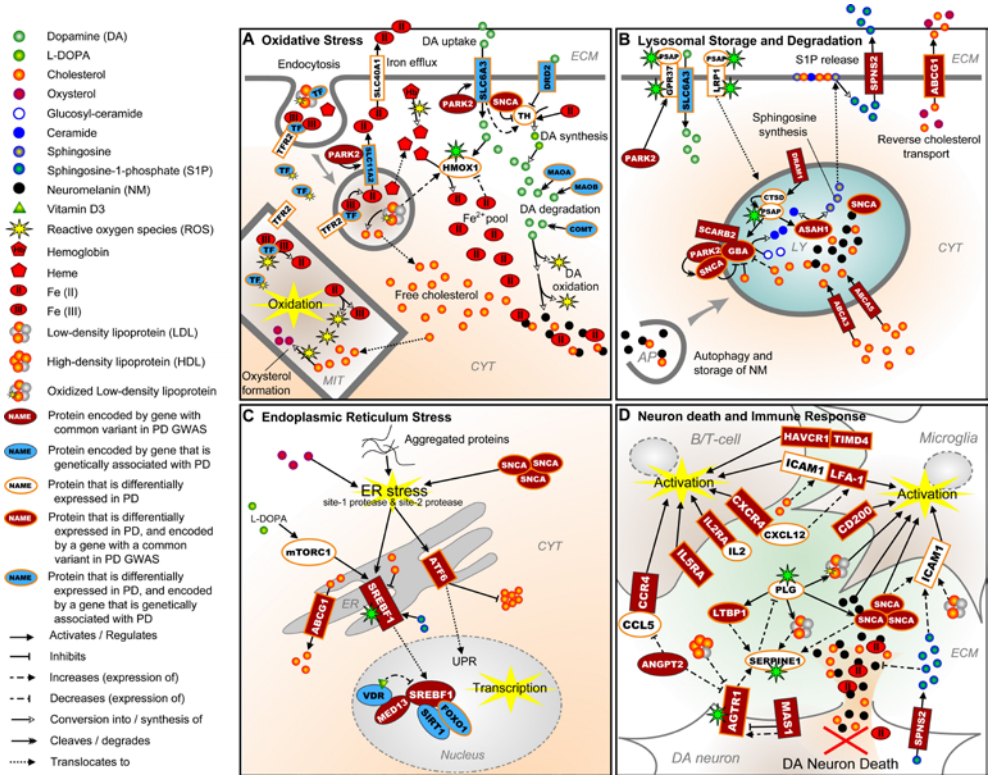

Figure 2. Putative druggable targets within each of the main themes (A-D) of the PD landscape are indicated with green stars. For each theme, the main processes are depicted, a full description of these processes can be found in chapter 2. AP, autophagosome; CYT, cytoplasm; ECM, extracellular matrix; ER, endoplasmic reticulum; LY, lysosome; MIT, mitochondrion; UPR, unfolded protein response.

potential druggable targets. Namely, loss of AGTR1 correlates with DA neuron death and CASP3 activation[66], and the DA and angiotensin II systems are known to counter-regulate each other[67, 68]. AGTR1 regulates intracellular cholesterol esterification[69] and increases the expression of SERPINE1[70], which in turn increases the risk for thrombosis and atherosclerosis[71]. The latter may occur due to a SERPINE1-mediated reduced splicing of plasminogen (PLG) into the active protease plasmin[72] that degrades blood plasma proteins to prevent atherosclerosis, but also degrades (aggregated) SNCA[72] and LDL (which increases extracellular lipid accumulation)[73, 74]. The (auto)immune response can be triggered by plasmin[75], SNCA[76, 77], lipids[78] and oxidized lipoproteins[79], which shows that the regulation of these factors may be crucial in PD. However, the specific targeting of AGTR1, SERPINE1 and plasmin may prove difficult because they also regulate e.g. hypertension, thrombosis, wound-healing and atherosclerosis[80-83].

The potential druggable mentioned above are all associated with core processes within the PD landscape and are directly or indirectly linked to substrate-specific processes such as the regulation of DA, reactive oxygen species and/or neuromelanin, which closely interact with SNCA, lipids and iron. In addition, an adjusted lipid diet in combination with targeting one or more of these druggable targets, may have the potential to alter the course and/or severity of PD.

6.3.2 Druggable targets in the ALS landscape

The ALS landscape points to an important modulatory role for estradiol, Rho-GTPases and RNP granules. The protective role of estradiol in women suggests that treatment with this steroid may be beneficial and can have protective effects. However, especially in males such a treatment will result in side effects. It is tempting to speculate that the beneficial effects of riluzole – the only FDA-approved life-prolonging ALS medication – are not only due to its effect on reducing glutamate toxicity – which was the initial reason for trialing this drug in ALS – but also to its effect (increase) on ABCG2 expression[84]. ABCG2 transports estradiol metabolites, folate and riluzole in and out of the cell[84-87]. ABCG2 is therefore a potential druggable target, not only because it interacts with riluzole, but also because it directly affects estradiol signaling.

The Rho-GTPases are probably too generic to serve as specific targets, but targeting them indirectly through activation of the NGF receptors – which are functional 'hubs' with multiple connections in the landscape – may prove beneficial. Of note, gambogic amide is a neuroprotective NGF receptor agonist that triggers neurite outgrowth, and strongly prevents glutamate-induced neuronal death[88], and may therefore be a potential drug to treat ALS. Other potential druggable targets within the ALS landscape are proteins that regulate RNP granule processing, e.g. the proteins TDRD3 and G3BP1, and most of the familial ALS proteins (see chapter 5).

6.4 INTERPRETATION OF GENOME-WIDE GENETIC AND EXPRESSION DATA

Genetic risk factors are the first dominos that set the causal mechanisms of the disease in motion by changing expression levels and/or by changing e.g. the activity, localization, folding or posttranslational modification of the encoded proteins. Even a subtle change in one gene and its encoded protein may result in a cascade of multiple other differentially expressed, (in)activated or abnormally localized proteins within the cell. The subsequent molecular changes may happen either directly – e.g. when the affected protein is a transcription factor or kinase and directly affects the expression and activation of other proteins – or indirectly, e.g. when compensatory mechanisms are put in place to counteract these changes and maintain homeostasis. In combination with the effects of aging and environmental risk factors, all these interactions represent the underlying biological processes that result in the pathological changes leading to the disease. Expression data may only partially reflect these changes, because other

alterations such as protein modification/(in)activation, binding affinity or cellular location are not included. Thus, overall, I do not expect a complete overlap between genetic and expression data, but do expect that some genetically associated proteins are also differentially expressed.

6.4.1 Overlap between genetic and expression data

The PD landscape (chapter 2) was based on genetic data, and expression data was solely used as corroborating evidence for the involvement of the GWAS candidate proteins and the additional proteins in the landscape. A total of 451 proteins were linked to PD through GWASs of which 158 (35%) were also found to be differentially expressed in the SN, striatum, CSF and/or blood of PD patients versus healthy controls in at least one study, and 60 proteins (13%) in at least two studies (**Table 1**). In addition to other signaling cascades, these 60 proteins are involved in vesicular transport (e.g. AMPH, NAPB, NSF, SNCA), sphingosine regulation (ASAH1), angiotensin regulation (AGTR1), cytoskeletal organization (RUFY3, MAPT) and the immune response (CXCR4, CD200, SDC1, SEMA6D) and as such represent some of the most prominent biological processes in the PD landscape. Of note, mRNAs encoding the vesicular transport-associated proteins AMPH, NAPB, NSF and SNCA are all consistently downregulated in the SN of PD patients. This could mean that (1) the turnover of these mRNAs is increased, (2) vesicular transport is greatly reduced in PD DA neurons and/or (3) that only the DA neurons with low vesicular transport survived. The latter implies a selective vulnerability of DA neurons with a high vesicular transport and/or SNCA expression, and is in accordance with the substrate specificity of SN DA neurons (see section 6.2).

Enrichment analysis of the complete set of GWAS candidate proteins compared to the smaller sets of 158 and 60 GWAS proteins that are also differentially expressed in PD patients reveals similar biological themes/processes among the three groups (**Table 2**). The most enriched canonical pathways are immune-related, and the enriched 'disease and disorders' categories are mainly cancer-/cell proliferation-related, but show also enrichment for 'diabetes mellitus' and 'movement disorders' in the group of 60 overlapping proteins. The enrichment for diabetes mellitus may be associated with the health problems that accelerate diabetes, such as dyslipidemia and high blood pressure[89], processes that, together with insulin signaling – the major pathway dysregulated in diabetes mellitus[90] – are present in the PD landscape. In contrast to the analysis of all GWAS candidate genes/proteins, the analyses of the differentially expressed genes/proteins show that the cancer-related themes are more specifically related to skin cancer and melanoma. Melanoma is a type of skin cancer that develops from the pigment-containing skin cells which represents an interesting functional overlap with the degenerating, pigment-containing DA neurons in the SN. Lastly, the group of 451 GWAS genes/proteins shows enrichment for nervous system morphology-

related functions in the category 'molecular and cellular functions', whereas the differently expressed GWAS candidate genes also show enrichment for synaptic transmission. The latter process is expected to be reduced when DA release (and therefore synaptic transmission) is decreased.

In summary, the observed overlap of genes/proteins associated with PD through GWASs *and* expression studies represents the most enriched processes from the total set of GWAS candidate genes, in particular vesicular transport and neurotransmission. These are processes that are expected to be decreased when DA release is reduced. However, to understand the pathological effects of all genetic risk factors and accomplish complete translatability of these factors to the underlying biological processes, additional studies are required that – in addition to expression changes – also map the proteins that are e.g. differentially phosphorylated, methylated, glycosylated or located within the cell.

6.4.2 Analysis of expression data

The analysis of PD patient expression data can help to – at least partially – elucidate the biological processes underlying the disease and are a reflection of the combination of genetic factors (i.e. the underlying causal molecular mechanisms) and environmental risk factors that lead to the disease. Therefore, analysis of expression data may provide additional information on top of the causal themes and processes derived from genetic studies. When we examine *all* the mRNAs/proteins that are differentially expressed in the SN, striatum, CSF or blood of PD patients (and not only those that overlap with GWAS candidate genes), other enriched themes/processes emerge (**Table 3**). The differentially expressed mRNAs/proteins in the SN are in particular related to mitochondrial dysfunction, disorders of the basal ganglia and movement disorders, which are 'classic PD themes'. In the striatum, we see similar enrichments in the category 'disease and disorders', whereas the enriched 'molecular and cellular functions' mainly relate to neurotransmission, which is in line with the reduced DA release in the striatum (see above). The CSF and blood analyses show enriched themes that are completely different from those from the SN and striatum analyses. Of note, in the CSF analysis, the 'liver X receptor (LXR)/retinoic X receptor (RXR)' pathway is enriched, in combination with 'atherosclerosis signaling', 'clathrin-mediated endocytosis signaling' and the enriched functions 'efflux of cholesterol' and 'concentration of lipid', which are all related to cholesterol and cholesterol signaling. In the blood of PD patients, the enriched canonical pathways and diseases and disorders are lined to immune regulation, and the enriched molecular and cellular functions are associated with cell death.

In summary, whereas the enriched functional categories from the analyses of the SN and striatum expression data are closely related to classic PD-linked processes/themes (movement disorders, neurotransmission), the analyses of PD CSF and blood

show enrichment for cholesterol and lipid regulation, the immune response and cell death, themes that are highly similar to the processes in the PD landscape. Therefore, these findings corroborate the findings from the PD landscape and indicate that lipid signaling is involved in PD.

6.5 THE EFFECTS OF MPTP AND PHYSICAL EXERCISE

In chapter 3, we established that, based on expression data, the MPTP-treated mouse accurately represents the underlying biological processes in human PD and therefore can be regarded as an adequate model for human PD. We also hypothesized that this validity of the MPTP mouse model may improve when a longer interval between MPTP treatment and tests is used. In chapter 4, we used this mouse model to explore how physical exercise affects the molecular pathways underlying MPTP toxicity and modulates the clinical outcome. We assume that these molecular pathways can be translated to the human situation. Here, I will discuss – as a follow-up of chapter 3 – the validity of the MPTP-treated mouse as a model for PD at a later time point, i.e. seven weeks after MPTP treatment (section 6.5.1). Further, to assess if physical exercise-mediated pathways may indeed regulate mechanisms underlying *human* PD pathophysiology, I will compare – as a follow-up of chapter 4 – the mRNAs that are induced by physical exercise in MPTP-treated mice with mRNAs from human PD expression data (section 6.5.2).

6.5.1 The validity of the MPTP model revisited

In chapter 3, we concluded that the MPTP mouse model is probably more valid for studying the SN-linked than the striatal mechanisms in PD. We noted that further transcriptome studies are needed to determine if waiting longer than 72 hours after MPTP injection would provide a better construct validity for human PD in the striatum. The MPTP-treated mice (without physical exercise; chapter 4) were sacrificed seven weeks after MPTP treatment and can therefore serve to study the effects of MPTP at a (much) later time point, as proposed in chapter 3. Here I compare the enrichment analysis of the differentially expressed mRNAs in the mouse SN and striatum seven weeks after MPTP-treatment to the human PD and MPTP-treated mouse mRNA expression analyses of chapter 3 (**Tables 4** and **5**).

Compared to the earlier time points, the enriched upstream regulators in the 'seven-week SN' are not only more epilepsy- (i.e. CREB1) but also more dopamine- (i.e. L-DOPA) related. CREB1 is predicted to be increased, which – when epileptic processes are considered to reflect neuronal activity[91, 92] – may point to an increased activation of the remaining neurons to compensate for the loss of DA neurons. The category 'diseases and disorders' is enriched for 'movement disorders', 'dyskinesia', and 'seizures', the latter indicating a relationship with epileptic processes. The molecular and cellular

functional annotations were predominantly cell death-related at the earlier time points, but are associated with neurotransmission and learning/cognition seven weeks after MPTP treatment, suggesting more chronic and perhaps adaptive processes following the death of a large portion of SN cells. These processes are also more similar to 'transport of vesicles', the main function that was found to be enriched in the SN expression data of human PD and that is related to neurotransmission and/or cell activity (see above).

In the striatum, seven weeks after MPTP treatment, the enriched annotations are mainly epilepsy-related for 'disease and disorders'. Further, the most enriched upstream regulators (Dalfampridine, Bicuculline, CREB1) are epilepsy-related and, together with the upstream regulator L-DOPA, are predicted to be decreased in activity (not shown). Again, when epileptic processes reflect neuronal activity[91, 92], the activation of the striatum is decreased, in accordance with a decreased activation of L-DOPA-mediated pathways and DA innervations as seen in human PD. As in the SN, cell death-related processes are enriched in the striatal expression data of the early time points, but absent seven weeks after MPTP treatment.

Overall, it seems that seven weeks after MPTP treatment (1) the SN may have to 'work harder', probably to compensate for neuronal loss (as indicated by an increased activation state), and (2) the striatum appears less active, probably due to a decrease in DA innervations. Moreover, especially because acute effects of MPTP, such as cell death, are not present anymore seven weeks after MPTP treatment, the seven-week MPTP model provides an even better construct validity for human PD and better mimics the slow deterioration seen in PD. Yet, one still has to wonder whether at this time point, the SN of the MPTP-treated mice is progressively degenerating while the striatum has to adapt continuously to a decreased DA release (as in human PD), *or* (maybe more likely) whether the SN is functioning less well and has reached a status quo and delivers a less-than-normal but continuous amount of DA to the striatum.

6.5.2 How would physical exercise affect human PD?

Physical exercise improves the motor function of PD patients[93, 94] and in my animal study (counter)regulates mRNAs that contribute to MPTP toxicity, which provides a direct 'knock-on' effect of physical exercise on the PD model (chapter 4). It would be of interest to see if physical exercise also may have the potential to regulate mRNAs that contribute to the pathophysiology of human PD, and as such function as a 'disease modifier'. To this end, I compared per brain region (SN, DL, VM) the mRNAs that were differentially expressed by physical exercise in the MPTP mice (from chapter 4) with the differentially expressed mRNAs in human PD[95-112]. This comparison shows that the number of overlapping mRNAs is larger in the SN (112 mRNAs) and VM (99 mRNAs) than in the DL (13 mRNAs). This indicates that – similar to the effects we observed in

the MPTP model (chapter 4) – physical exercise may especially have a direct effect on disease-related processes in the SN and VM of humans with PD. An enrichment analysis was performed on these overlapping mRNAs to assess which processes and themes are regulated by physical exercise as well as in human PD (**Table 6**).
The 112 overlapping mRNAs in the SN show enrichment for 'Parkinson's disease' and have microRNA-124-3p as top upstream regulator (and this microRNA is predicted to be increased in activity (not shown)). MicroRNA-124-3p is a regulator of apoptosis and autophagy, and prevents DA neuron loss[113, 114], and may therefore also be a potential target to treat PD. In the VM, the 99 mRNAs that overlap with human PD show L-DOPA as the top upstream regulator (which is predicted to be decreased in activity) but also show enrichment for movement disorder- and epilepsy-related diseases, and enriched molecular and cellular functions associated with synaptic transmission and behavior. Therefore, this analysis of VM-mRNAs that overlap with human PD shows enrichment for similar themes as the analysis of the total set of mRNAs regulated by physical exercised in the VM of MPTP-treated mice (see chapter 4). Lastly, in the DL enrichment analysis, epilepsy-related annotations predominate, which may indicate that physical exercise results in activation[91, 92] of the DL and in this way may compensate for the reduced basal DA release in the striatum. However, the total number of overlapping and epilepsy-related proteins is too low to draw a firm conclusion.

Overall, these analyses show that the effects of physical exercise as seen in the MPTP-treated mouse may well be translated to human PD, and may provide a direct 'knock-on' effect on molecular mechanisms of human PD that are markedly related to DA neuron loss and L-DOPA signaling.

6.6 MOLECULAR LANDSCAPES AND THE DEVELOPMENT OF PERSONALIZED MEDICINE

The medication for PD is still based on symptomatic treatment – e.g. L-DOPA – and is hence not directed at the core underlying disease mechanisms in order to slow down the progression or even cure PD. Moreover, the diversity in clinical (sub)phenotypes of PD suggests that the underlying molecular mechanisms may vary between individual PD patients, which implies that future disease modifying treatments should be tailored to the individual patient. Therefore, the development of more personalized medicine approaches for PD patients demands (increased) insight into the various subphenotypes of PD, both at a clinical level and a molecular level.

Large population-based analyses such as GWASs are necessary to uncover the genetic risk factors/variants and understand which pathways and biological themes/processes are involved in PD. By integrating all genetic risk factors/variants from multiple cohorts and populations (i.e. groups with different ethnicities and genetic

origins), the molecular landscape reflects the genetic variation spectrum of the disease. However, although PD is the end result in all cases, the (sub)phenotype of PD may vary greatly, and be dependent on a certain genetic predestination that is present, or more pronounced, within a specific population. In this respect, populations with a different genetic background – e.g. different ethnic groups – may not only have a different risk to develop PD, but may also have other genetic risk factors and affected pathways that lead to PD. Overall, three situations may occur:

1) genetic risk factors/variants overlap between populations, resulting in the same affected proteins and pathways,
2) different populations have genetic risk factors/variants in different proteins that are still functioning in the same pathways, or
3) genetic risk factors/variants within the genes/proteins from one or more pathways are more prominent in one population than another.

To some extent, all three scenarios may occur but the limited overlap between the observed top results from the various PD or ALS GWASs indicates that the overlap of genetic risk factors/variants between populations is limited. Large genetic studies that gain power by including multiple populations (e.g. meta- and mega-analyses) may therefore find the most generic disease-associated pathways that are shared by different populations, but miss the population-specific signals.

An individual sporadic PD patient may carry multiple common genetic variants – typically SNPs – that together with environmental risk factors and aging result in the onset of PD. These variants may be located within genes encoding proteins from one specific pathway or from multiple pathways, and in both instances, this will result in PD. Therefore, not all pathways within the landscape have to be affected for a patient to develop PD. Of course, when PD is caused by different combinations of (dysregulated) pathways, this also requires different treatment strategies rather than a 'one-size-fits-all' treatment. Ideally, the landscape can be used as a template on which the individual patient data is 'mapped' to divide the patients into different PD subcategories based on which patient-specific PD-associated molecular pathways are (genetically) affected. Subsequently, the treatment strategy could then be personalized with (disease-modifying) medication/therapy that specifically targets pathways affected in (subgroups of) individual patients (**Figure 3**).
Subcategorization of PD patients, not only on the basis of the molecular pathways that are (predicted to be) dysregulated, but also based on their (secondary) symptoms – e.g. the development of L-DOPA-induced dyskinesia and/or non-motor PD symptoms (cognitive dysfunction, depression, sleeping problems, etc.) – may help to further

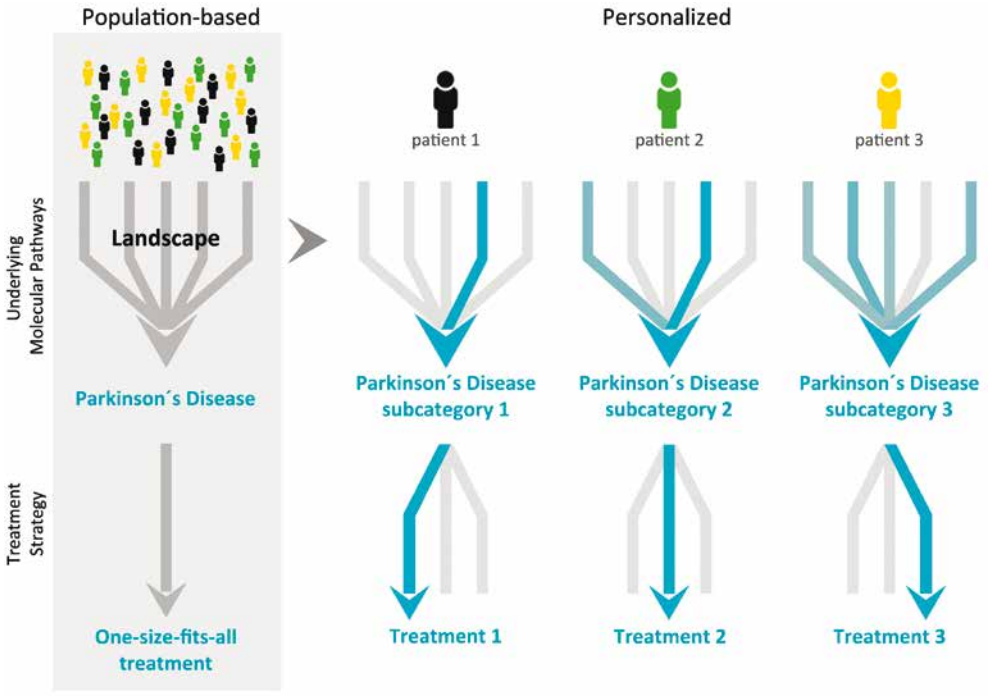

Figure 3. Subcategorization of PD patients by 'mapping' their individual data on the PD landscape, allowing personalized treatment based on the affected molecular pathways in each individual patient instead of a 'one size fits all' treatment.

personalize the treatment for each patient. Furthermore, a consistent link between the affected molecular pathway(s) and certain clinical symptoms may point to a genetic predisposition for these symptoms, and can again be used to improve PD treatment. For example, patients with a genetic predisposition to develop L-DOPA-induced dyskinesia could benefit (i.e. more than other PD patients) from starting with physical exercise therapy already early after disease onset, in order to minimize the side effects of chronic L-DOPA usage[115, 116].

Thus, although population-based research is initially necessary to map which processes are involved in PD, patients should subsequently be individually examined to determine which disease-associated pathways are affected. In this way, pathway-specific and personalized PD treatments can be developed, which increases treatment effectiveness and reduces side effects.

6.7 FUTURE DIRECTIONS

In this thesis, the landscape building approach was used to elucidate the molecular mechanisms underlying PD and ALS. In addition, the effect of physical exercise on the molecular mechanisms underlying PD was examined in a mouse model. The challenge

for the coming years will be to continuously update these molecular landscapes, and to (further) validate and use the landscapes to identify and further develop druggable targets in order to improve the treatment of patients. Moreover, the landscape building approach can be used to provide additional, in-depth insights into the effects of current therapeutic treatments at the molecular level, information that can then be used to improve these treatments and/or to develop better ones.

PD can be caused by a wide variety of genetic and environmental risk factors, and is characterized by a complex and diverse clinical picture. This diversity requires a treatment that can be tailored to the symptoms of each individual patient. In order to achieve such personalized medicine for PD patients, we first have to establish if we can (sub)categorize the patients based on their affected PD-related pathways. For this, a large number of PD patients should be screened individually to see if we can divide them into subgroups based on their genetic variants, affected proteins and/or pathways, in combination with their diagnosis, disease progression and/or clinical symptoms. Once we have reached this categorization of PD patients, we can start developing treatments (based on the putative druggable targets from the landscape) that are specifically targeting the affected pathways for each category, and accomplish a more accurate and effective PD treatment.

In addition to the data from the 'core' genetic and expression studies, we have used a broad array of other studies on a multitude of topics – including protein-protein interactions, druggable targets, environmental risk factors and enzymatic reactions – to construct the landscapes. Many of these individual studies did not have a direct link with a certain disease but investigated for example the function, intracellular location or interactions of one or more proteins. For the landscape building approach, particularly these studies provide a wealth of information and enable us to 'connect the dots' and generate a comprehensive picture of all disease-linked interactions. In this respect, the results of these fundamental, 'basic science', studies – that are not focused on unraveling a disease mechanism or developing a treatment – are critical for building a molecular landscape and elucidate the disease-related and -specific molecular pathways. Fundamental research is necessary to acquire and maintain a broad knowledge base in all kinds of research fields, whereas more applied research focuses on *known* disease processes and proteins and is therefore largely dependent on the fundamental research efforts to provide *revolutionary* new insights and to further advance the research field. The problem of applied research and the strength of fundamental research is clearly illustrated by a quote in a book by Robbert Dijkgraaf: "What is the shortest way from A to B if you do not know what B is or where it is?".
Large international research consortia that work towards a common goal – directed at providing answers to major research questions with a clearly defined societal

impact and/or practical use – are necessary. However, in order to let these consortia succeed, we should continue to cherish (and finance) research that does not have a direct practical use (yet) and of which the societal impact is only apparent to the researcher in question. Only when research in the fields of biology and neurology stays fundamental and thereby safeguards a continuous stream of knowledge on a multitude of new biological concepts, we will find the answers to major research questions. This will also be the only way in which methods such as the landscape building approach can be successful and result in the generation of new ideas, hypotheses and eventually the development of medicines and therapies to treat diseases, including PD.

Table 1. PD candidate genes/proteins associated with human PD through both GWASs and genome-wide expression studies. Only the genes that are differentially expressed in at least two different (protein and mRNA) expression studies of either the SN[95-110, 117-119], striatum[98, 100, 103, 104, 110-112], CSF[120-125] or blood[126-135] of PD patients are shown. Genes in **bold** are also located in the PD landscape (chapter 2). mRNAs were only included when their adjusted expression p-value was <0.05 and the corresponding fold change was ≥1.5 or ≤-1.5, or when the fold change was ≥2 or ≤-2. Proteins were only included when their adjusted expression p-value was <0.05 and the corresponding fold change was ≥1.2 or ≤-1.2, or when the fold change was ≥1.5 or ≤-1.5. SN, substantia nigra; STR, striatum; CSF, cerebral spinal fluid; BLD, blood.

Rank	Gene	SN	STR	CSF	BLD	#	Rank	Gene	SN	STR	CSF	BLD	#
1	**NSF**	7	1	0	0	8	31	**MAP1B**	2	0	0	0	2
2	**SNCA**	6	0	0	0	6	32	**MRPL3**	2	0	0	0	2
3	**AGTR1**	5	0	0	0	5	33	MVB12B	2	0	0	0	2
4	**NPTX2**	4	1	0	0	5	34	**SLC24A3**	2	0	0	0	2
5	**ROBO2**	4	0	1	0	5	35	**ACTN4**	2	0	0	0	2
6	**RUFY3**	4	0	0	1	5	36	CALN1	2	0	0	0	2
7	**ASAH1**	1	1	1	2	5	37	CNKSR3	2	0	0	0	2
8	**PCDH8**	4	0	0	0	4	38	LMBR1	2	0	0	0	2
9	**AMPH**	3	1	0	0	4	39	**SLC2A13**	2	0	0	0	2
10	**SCN2A**	3	1	0	0	4	40	SYT17	2	0	0	0	2
11	**MAPT**	3	0	1	0	4	41	TMEM200C	2	0	0	0	2
12	**SLCO3A1**	3	0	0	1	4	42	**GCH1**	2	0	0	0	2
13	**ADAMTS2**	3	0	0	1	4	43	**FPR3**	1	1	0	0	2
14	**NAPB**	2	2	0	0	4	44	**HAVCR1**	1	1	0	0	2
15	**CXCR4**	1	2	0	1	4	45	**ITGA8**	1	1	0	0	2
16	**ATP2B2**	3	0	0	0	3	46	**RFX4**	1	1	0	0	2
17	**FGF12**	3	0	0	0	3	47	SUSD3	1	1	0	0	2
18	**SDC1**	3	0	0	0	3	48	**COL18A1**	1	0	1	0	2
19	**SEMA6D**	2	0	1	0	3	49	**COL2A1**	1	0	1	0	2
20	**MBP**	1	2	0	0	3	50	**CDK19**	1	0	0	1	2
21	**CD200**	1	1	1	0	3	51	GLRA3	1	0	0	1	2
22	**CACNA2D3**	1	1	1	0	3	52	**PLEKHM1**	1	0	0	1	2
23	**NEGR1**	1	1	1	0	3	53	**PPP1R12B**	1	0	0	1	2
24	**DLG2**	1	1	0	1	3	54	**SCIN**	0	2	0	0	2
25	**CNTNAP2**	1	0	0	2	3	55	SLC38A1	0	2	0	0	2
26	**CNTN1**	0	2	1	0	3	56	**BANK1**	0	1	1	0	2
27	**GLDN**	0	1	1	1	3	57	**WNK1**	0	1	0	1	2
28	**ARHGAP33**	2	0	0	0	2	58	**FBN1**	0	0	1	1	2
29	**ATP6V0A1**	2	0	0	0	2	59	**SAMD4A**	0	0	0	2	2
30	CNTNAP5	2	0	0	0	2	60	**SP110**	0	0	0	2	2

Table 2. Enrichment analyses of PD GWAS candidate genes (541 genes) and GWAS candidate genes that are also differentially expressed in at least one or two studies (158 and 60 genes, respectively). Genes were defined as differentially expressed when either their mRNA or protein product was differentially expressed in the SN[95-110, 117-119], striatum[98, 100, 103, 104, 110-112], CSF[120-125] and/or blood[126-135] of PD patients. mRNAs were only included when their adjusted expression p-value was <0.05 and the corresponding fold change was ≥1.5 or ≤-1.5, or when the fold change was ≥2 or ≤-2. Proteins were only included when their adjusted expression p-value was <0.05 and the corresponding fold change was ≥1.2 or ≤-1.2, or when the fold change was ≥1.5 or ≤-1.5. All p-values are corrected for multiple testing by the Benjamini-Hochberg false discovery rate. Canonical pathways/annotations with only one (target) gene (#) were discarded.

(1) Canonical pathways								
GWAS (541)			**Overlap GWAS & human expression in at least 1 study (158)**			**Overlap GWAS & human expression in at least 2 studies (60)**		
Canonical pathway	**p-value**	**#**	**Canonical pathway**	**p-value**	**#**	**Canonical pathway**	**p-value**	**#**
Autoimmune Thyroid Disease Signaling	1.02E-03	8	B Cell Development	9.47E-03	4	-		
Antigen Presentation Pathway	1.02E-03	7	T Helper Cell Differentiation	9.47E-03	5			
Allograft Rejection Signaling	3.48E-03	9	Phagosome Maturation	9.47E-03	6			
Graft-versus-Host Disease Signaling	3.48E-03	7	Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	9.47E-03	5			
OX40 Signaling Pathway	3.81E-03	9	Autoimmune Thyroid Disease Signaling	9.47E-03	4			
(2) Diseases and disorders								
GWAS (541)			**Overlap GWAS & human expression in at least 1 study (158)**			**Overlap GWAS & human expression in at least 2 studies (60)**		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Tumorigenesis of Tissue	1.58E-15	385	Skin Cancer	4.65E-09	88	Diabetes Mellitus	9.80E-05	16
Cancer	1.58E-15	400	Melanoma	9.77E-09	87	Gastrointestinal Carcinoma	9.80E-05	38
Abdominal Neoplasm	1.83E-15	377	Malignant cutaneous melanoma cancer	1.41E-08	82	Melanoma	9.80E-05	34
Abdominal Cancer	1.83E-15	375	Abdominal Neoplasm	1.49E-08	139	Movement Disorders	2.85E-04	15
Digestive Organ Tumor	1.89E-15	361	Abdominal Cancer	2.37E-08	138	Malignant Cutaneous Melanoma Cancer	3.48E-04	31
(3) Molecular and Cellular Functions								
GWAS (541)			**Overlap GWAS & human expression in at least 1 study (158)**			**Overlap GWAS & human expression in at least 2 studies (60)**		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Formation of Brain	4.65E-05	34	Generation of cells	5.16E-06	46	Abnormal Morphology of Nervous System	2.30E-06	16
Morphology of Nervous System	7.97E-05	51	Morphology of nervous system	3.73E-05	26	Morphology of Nervous System	3.69E-06	17
Development of Central Nervous System	3.15E-04	38	Neuritogenesis	6.98E-05	20	Neurotransmission	4.81E-06	12
Abnormal Morphology of Nervous System	3.34E-04	42	Abnormal morphology of nervous system	1.12E-04	22	Synaptic Transmission	4.81E-06	11
Morphology of Brain	5.75E-04	32	Synaptic transmission	1.98E-04	14	Morphology of Central Nervous System	8.34E-06	13

Table 3. Enrichment analyses of differentially expressed mRNAs/proteins in the SN, striatum, CSF and blood of PD patients. Only the mRNAs/proteins that are differentially expressed in at least two expression studies of either the SN[95-110, 117-119], striatum[98, 100, 103, 104, 110-112], CSF[120-125] or blood[126-135] of PD patients are included in the analyses. A total of 872, 79, 34 and 67 mRNAs/proteins are differentially expressed in the SN, striatum, CSF and blood, respectively. mRNAs were only included when their adjusted expression p-value was <0.05 and the corresponding fold change was ≥1.5 or ≤-1.5, or when the fold change was ≥2 or ≤-2. Proteins were only included when their adjusted expression p-value was <0.05 and the corresponding fold change was ≥1.2 or ≤-1.2, or when the fold change was ≥1.5 or ≤-1.5. All p-values are corrected for multiple testing by the Benjamini-Hochberg false discovery rate. Canonical pathways/annotations with only one (target) gene (#) were discarded.

(1) Canonical pathways

SN (872)			Striatum (79)			CSF (34)			Blood (67)		
Canonical pathway	**p-value**	**#**	**Canonical pathway**	**p-value**	**#**	**Canonical pathway**	**p-value**	**#**	**Canonical pathway**	**p-value**	**#**
Oxidative Phosphorylation	4.84E-17	30	Ephrin B Signaling	2.55E-02	4	LXR/RXR Activation	1.65E-19	13	IL-6 Signaling	4.54E-04	6
Mitochondrial Dysfunction	9.90E-17	36	G-Protein Coupled Receptor Signaling	3.43E-02	6	FXR/RXR Activation	2.06E-19	13	FLT3 Signaling in Hematopoietic Progenitor Cells	5.91E-04	5
Axonal Guidance Signaling	2.25E-11	47	Neuroprotective Role of THOP1 in Alzheimer's Disease	3.43E-02	3	Atherosclerosis Signaling	2.95E-12	9	PPAR Signaling	6.13E-04	5
14-3-3-mediated Signaling	1.30E-10	24				Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	2.95E-12	10	Acute Phase Response Signaling	6.32E-04	6
Breast Cancer Regulation by Stathmin1	5.73E-10	29				Clathrin-mediated Endocytosis Signaling	2.95E-12	10	NF-KB Signaling	6.77E-04	6

(2) Diseases and disorders

SN (872)			Striatum (79)			CSF (34)			Blood (67)		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Disorder of Basal Ganglia	4.95E-53	149	Movement Disorders	1.87E-22	40	Amyloidosis	1.14E-12	17	Rheumatoid Arthritis	4.93E-04	13
Movement Disorders	3.09E-52	176	Disorder of Basal Ganglia	3.02E-22	35	Familial Amyloidosis	1.37E-12	8	Abnormal Morphology of Immune System	4.95E-04	9
Neuromuscular Disease	5.97E-48	153	Neuromuscular Disease	2.93E-20	35	Alzheimer's Disease	1.09E-10	15	Inflammation of Joint	6.55E-04	15
Neurological Signs	3.93E-35	113	Dyskinesia	1.62E-17	28	Formation of Amyloid Fibrils	8.22E-09	6	Viral Infection	7.17E-04	20
Dyskinesia	5.67E-34	107	Huntington's Disease	2.44E-17	27	Creutzfeldt-Jakob Disease	1.75E-08	4	Rheumatic Disease	7.37E-04	16

(3) Molecular and Cellular Functions

SN (872)			Striatum (79)			CSF (34)			Blood (67)		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Development of Neurons	3.14E-27	115	Organization of Cytoskeleton	2.58E-08	28	Transport of Steroid	3.16E-10	10	Cell Death of Tumor Cell Lines	1.01E-05	25
Microtubule Dynamics	7.21E-27	145	Neurotransmission	2.25E-07	15	Efflux of Cholesterol	4.31E-10	9	Cell Death	1.67E-04	35
Organization of Cytoskeleton	8.31E-26	158	Necrosis	7.37E-07	37	Export of Molecule	4.31E-10	12	Apoptosis of Tumor Cell Lines	1.67E-04	20
Formation of Plasma Membrane Projections	5.70E-24	93	Synaptic Transmission	1.17E-06	13	Transport of Molecule	9.71E-10	21	Apoptosis of Prostate Cancer Cell Lines	1.81E-04	8
Neuritogenesis	2.43E-23	90	Transport of Molecule	1.43E-06	28	Concentration of Lipid	3.15E-09	16	Morphology of Blood Cells	3.16E-04	11

Table 4. Enrichment analyses of differentially expressed mRNAs in the SN of MPTP-treated mice and in PD patients. mRNAs were only included when their adjusted expression p-value was <0.05 and the corresponding fold change was ≥1.2 or ≤-1.2. All p-values are corrected for multiple testing by the Benjamini-Hochberg false discovery rate. Canonical pathways/annotations with only one (target) gene (#) were discarded. The number of differentially expressed mRNAs at each time point after MPTP treatment and in human PD are indicated in parentheses.

(1) Upstream Regulators

Effect MPTP in SN 1 day after last treatment (431) [1]			Effect MPTP in SN 7 days after last treatment (289) [2]			Effect MPTP in SN 7 weeks after last treatment (482)			Human PD SN (2027)		
Upstream Regulator	**p-value**	**#**	**Upstream Regulator**	**p-value**	**#**	**Upstream Regulator**	**p-value**	**#**	**Upstream Regulator**	**p-value**	**#**
APP	8.68E-18	54	APP	5.27E-13	37	CREB1	1.58E-14	42	HNF4A	8.30E-24	297
HTT	1.43E-14	48	HTT	1.40E-09	31	HTT	2.75E-12	45	5-fluorouracil	1.36E-17	62
MAPT	2.99E-13	27	Lipopolysaccharide	2.39E-09	50	L-dopa	2.91E-12	44	MAPT	1.83E-15	63
PSEN1	8.58E-12	31	Lh	9.82E-08	16	BDNF	1.06E-09	25	APP	5.62E-14	124
Lh	2.58E-07	19	Benzo(a)pyrene	9.89E-08	15	ATN1	1.16E-09	17	HTT	9.14E-14	120
ATN1	3.04E-07	14	D-glucose	6.10E-07	21	HDAC4	4.12E-08	14	1,2-dithiol-3-thione	1.02E-12	52
Beta-estradiol	1.32E-06	58	miR-1-3p	1.49E-06	13	FGF2	8.65E-08	24	CD 437	1.68E-12	51
Forskolin	2.39E-06	29	BDNF	1.67E-06	13	NR3C1	9.38E-08	35	NFE2L2	2.09E-12	77
Lipopolysaccharide	3.75E-06	57	MYC	1.76E-06	29	Okadaic acid	1.28E-07	12	MYC	2.15E-12	136
PTEN	4.40E-06	22	MAPT	3.51E-06	14	Cocaine	6.80E-07	15	PSEN1	3.12E-12	75

(2) Diseases and Disorders

Effect MPTP in SN 1 day after last treatment (431) [1]			Effect MPTP in SN 7 days after last treatment (289) [2]			Effect MPTP in SN 7 weeks after last treatment (482)			Human PD SN (2027)		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Movement Disorders	1.54E-16	89	Movement Disorders	3.41E-10	58	Seizures	1.58E-10	41	Disorder of Basal Ganglia	2.35E-22	203
Disorder of Basal Ganglia	1.30E-12	66	Neuromuscular Disease	2.48E-07	45	Movement disorders	1.06E-09	74	Movement Disorders	4.33E-20	250
Dyskinesia	3.02E-12	57	Neurological Signs	1.26E-06	36	Neurological signs	1.06E-09	54	Neuromuscular Disease	6.42E-19	211
Neurological Signs	3.02E-12	58	Disorder of Basal Ganglia	1.63E-06	40	Dyskinesia	1.06E-09	52	Chorea	1.24E-16	154
Huntington's Disease	1.11E-11	54	Dyskinesia	1.74E-06	35	Huntington's disease	1.86E-09	49	Neurological Signs	1.41E-16	163

(3) Molecular and Cellular Functions

Effect MPTP in SN 1 day after last treatment (431) [1]			Effect MPTP in SN 7 days after last treatment (289) [2]			Effect MPTP in SN 7 weeks after last treatment (482)			Human PD SN (2027)		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Cell Death	1.11E-12	170	Proliferation of Cells	2.28E-11	128	Neurotransmission	1.41E-13	48	Transport of vesicles	9.27E-11	40
Microtubule Dynamics	1.30E-12	77	Cell Death	3.41E-10	118	Synaptic Transmission	2.03E-12	41	Formation of plasma membrane projections	6.45E-08	112
Organization of Cytoskeleton	1.30E-12	85	Proliferation of Tumor Cell Lines	8.65E-09	67	Learning	1.06E-09	41	Microtubule dynamics	8.73E-08	195
Organization of Cytoplasm	4.30E-12	88	Apoptosis	1.13E-08	97	Cognition	1.20E-09	43	Organization of cytoplasm	1.09E-07	237
Proliferation of Cells	1.96E-11	173	Degeneration of Cells	2.24E-08	24	Long-term potentiation	1.56E-09	30	Formation of Cellular Protrusions	1.47E-07	149

[1] In chapter 3 this enrichment analysis is referred to as 'Shorter interval'.
[2] In chapter 3 this enrichment analysis is referred to as 'Longer interval'.

Table 5. Enrichment analyses of differentially expressed mRNAs in striatum of PD patients and in the striatum of mice 5, 24, 72 hours or 7 weeks after MPTP treatment. mRNAs were only included when their adjusted expression p-value was <0.05 and the corresponding fold change was ≥1.2 or ≤-1.2. All p-values are corrected for multiple testing by the Benjamini-Hochberg false discovery rate. Canonical pathways/annotations with only one (target) gene (#) were discarded. The number of differentially expressed mRNAs at each time point after MPTP treatment and in human PD are indicated in parentheses. In human studies, there has been made no distinction between the dorsolateral and ventromedial striatum, therefore the differentially expressed mRNAs in these areas of MPTP-treated mice are combined and as such compared with the mRNAs that are differentially expressed in the whole striatum of human PD patients.

(1) Upstream Regulators

MPTP 5h Striatum (83)			MPTP 24h Striatum (220)			Effect MPTP 72h Striatum (163)			Effect MPTP 7 weeks Striatum (339)			Human PD Striatum (259)		
Upstream Regulator	**p-value**	**#**	**Upstream Regulator**	**p-value**	**#**	**Upstream Regulator**	**p-value**	**#**	**Upstream Regulator**	**p-value**	**#**	**Upstream Regulator**	**p-value**	**#**
CREB1	2.05E-19	18	TGFB1	8.52E-28	69	Dihydrotestosterone	1.88E-11	22	Dalfampridine	2.19E-13	11	Beta-estradiol	5.92E-15	57
Forskolin	4.74E-17	22	Lipopolysaccharide	2.82E-25	66	Dexamethasone	7.79E-11	37	Bicuculline	2.62E-12	11	HDAC4	4.07E-14	16
Cycloheximide	6.10E-15	17	IL1B	3.16E-25	48	Tretinoin	1.12E-10	34	RICTOR	7.92E-11	21	HTT	2.05E-13	35
Cocaine	1.49E-14	13	TNF	5.77E-25	64	HRAS	4.65E-10	21	PSEN1	5.89E-08	21	BDNF	1.72E-11	18
U0126	2.42E-14	18	Tretinoin	1.93E-24	60	MYC	5.30E-10	26	CREB1	9.92E-08	25	FSH	6.84E-10	22
CREM	1.02E-13	12	IFNG	2.06E-23	56	TNF	5.76E-10	35	CREM	1.20E-07	13	APP	1.26E-09	30
FOXO3	1.55E-13	12	Inosine	1.33E-20	16	PPP3CA	1.01E-09	9	Kainic acid	6.04E-07	12	TAZ	2.17E-08	9
Kainic acid	1.74E-12	11	Beta-estradiol	7.78E-19	57	HTT	1.08E-09	23	Cocaine	6.32E-07	13	NGF	1.25E-07	13
Dexamethasone	1.94E-12	27	Doxorubicin	6.09E-18	28	KRAS	1.59E-09	16	L-DOPA	1.62E-06	26	Dopamine	2.91E-07	10
Ca2+	3.05E-12	13	EGF	1.34E-17	31	Lipopolysaccharide	2.29E-09	35	MYCN	1.66E-06	15	MAPT	1.09E-07	14

(2) Diseases and Disorders

MPTP 5h Striatum (83)			MPTP 24h Striatum (220)			Effect MPTP 72h Striatum (163)			Effect MPTP 7 weeks Striatum (339)			Human PD Striatum (259)		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Epileptic Seizure	2.55E-21	21	Psoriasis	6.98E-14	39	Movement Disorders	1.05E-09	40	Epileptic Seizure	9.97E-07	18	Neurological Signs	5.52E-28	64
Seizures	3.94E-18	25	Glucose Metabolism Disorder	3.37E-11	50	Schizophrenia	2.52E-08	25	Epilepsy	5.33E-05	23	Dyskinesia	5.52E-28	63
Epilepsy	7.14E-18	22	Inflammation of Organ	3.98E-11	51	Amyloidosis	7.20E-08	25	Seizures	2.92E-03	23	Disorder of Basal Ganglia	8.34E-28	69
Dyskinesia	1.07E-06	18	Vascular Disease	7.16E-11	45	Dementia	2.41E-07	24	Seizure Disorder	2.58E-02	24	Chorea	7.07E-27	60
Endometriosis	2.70E-06	14	Inflammatory Response	3.45E-10	37	Quantity of Phagocytes	6.73E-07	20	Congenital Anemia	4.01E-02	7	Movement Disorders	8.28E-27	79

(3) Molecular and Cellular Functions

MPTP 5h Striatum (83)			MPTP 24h Striatum (220)			Effect MPTP 72h Striatum (163)			Effect MPTP 7 weeks Striatum (339)			Human PD Striatum (259)		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Apoptosis	5.80E-08	39	Proliferation of Cells	3.23E-17	115	Morphology of Cells	6.61E-11	59	-			Neurotransmission	1.44E-13	35
Differentiation of Cells	1.07E-06	31	Morphology of Cells	7.55E-16	79	Organization of Cytoskeleton	6.61E-11	45				Synaptic Transmission	2.90E-11	29
Proliferation of Cells	1.89E-06	43	Necrosis	3.61E-15	91	Organization of Cytoplasm	1.93E-10	46				Transport of Molecule	3.95E-09	63
Cell Death	2.58E-06	41	Apoptosis	2.05E-14	91	Formation of Cellular Protrusions	8.31E-10	33				Transport of Metal Ion	1.02E-08	24
Cell Cycle Progression	4.27E-06	21	Cell Movement	2.81E-14	77	Apoptosis	8.34E-10	67				Morphology of Neurites	2.10E-08	17

Table 6. Enrichment analysis of overlapping mRNAs in the SN and striatum between exercising MPTP-treated mice and human PD. mRNAs were only included when their adjusted expression p-value was <0.05 and the corresponding fold change was ≥1.2 or ≤-1.2 in at least one study[95-112]. In human studies, there has been made no distinction between the dorsolateral and ventromedial striatum, therefore the differentially expressed mRNAs in the striatum of PD patients are compared with the mRNAs that are differentially expressed in the either the DL or the VM of exercised MPTP-treated mice. A total of 540, 272 and 949 mRNAs are differentially expressed in the SN, DL and VM of exercised MPTP-treated mice, and in the SN and striatum of PD patients 3277 and 1215 mRNAs are differentially expressed, respectively. Between these mRNAs, there is an overlap of 112, 13 and 99 mRNAs in the SN, DL and VM, respectively (indicated in parentheses in the table). All p-values are corrected for multiple testing by the Benjamini-Hochberg false discovery rate. Canonical pathways/annotations with only one (target) gene (#) were discarded.

(1) Upstream Regulators								
SN (112)			**DL (13)**			**VM (99)**		
Upstream Regulator	**p-value**	**#**	**Upstream Regulator**	**p-value**	**#**	**Upstream Regulator**	**p-value**	**#**
miR-124-3p	1.05E-06	9	HNRNPAB	1.70E-05	2	L-DOPA	1.49E-17	25
RUNX1	9.50E-06	7	LAMC1	2.35E-05	2	HTT	3.88E-17	25
GATA3	3.27E-04	6	TET1	5.94E-05	2	BDNF	4.06E-15	17
ACOX1	5.04E-04	5	EGR1	9.33E-05	3	APP	2.95E-12	22
Dopamine	1.24E-03	4	HOXA7	1.04E-04	2	CREB1	5.51E-11	17
RICTOR	1.26E-03	6	GLI1	1.41E-04	3	PSEN1	4.26E-10	14
HDAC9	1.53E-03	2	CREB1	1.42E-04	4	MAPT	4.61E-10	12
CRY1	1.53E-03	2	AKT2	1.90E-04	2	CPE	7.95E-10	6
DSP	1.78E-03	2	NRG1	1.92E-04	3	Beta-estradiol	9.22E-10	27
CEBPB	2.24E-03	7	Laminin	2.10E-04	2	REST	1.63E-09	9
(2) Diseases and disorders								
SN (112)			**DL (13)**			**VM (99)**		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Hyperphagia	1.29E-02	6	Epilepsy	8.92E-03	4	Movement Disorders	1.49E-11	33
Parkinson's Disease	1.31E-02	9	Epileptic Seizure	8.92E-03	3	Disorder of Basal Ganglia	1.81E-11	28
Eating Disorders	1.31E-02	7	Seizures	8.92E-03	4	Neuromuscular Disease	5.64E-11	29
Adenocarcinoma	1.63E-02	87	Neurodegeneration	1.34E-02	3	Dyskinesia	9.01E-11	24
Viral Infection	2.59E-02	25	Alzheimer's Disease	2.69E-02	3	Seizure Disorder	2.42E-10	21
(3) Functions								
SN (112)			**DL (13)**			**VM (99)**		
Annotation	**p-value**	**#**	**Annotation**	**p-value**	**#**	**Annotation**	**p-value**	**#**
Organization of Cytoplasm	2.61E-03	29	Behavior	8.92E-03	6	Neurotransmission	1.49E-11	22
Migration of Cells	5.02E-03	32	Growth of Neurites	8.92E-03	4	Synaptic Transmission	1.49E-11	20
Organization of Cytoskeleton	5.02E-03	26	Synthesis of Prostaglandin E2	8.92E-03	3	Excitatory Postsynaptic Potential	4.67E-11	13
Microtubule Dynamics	7.23E-03	23	Cued Conditioning	8.92E-03	2	Morphology of Nervous Tissue	5.64E-11	23
Development of Cytoplasm	7.23E-03	13	Regeneration of Neurons	1.34E-02	2	Behavior	5.64E-11	31

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Appendices

SUMMARY

Parkinson's disease (PD) is the second most common neurodegenerative disease, after Alzheimer's disease, affecting 1-2% of people over 60 years of age. PD is characterized by the death of dopaminergic (DA) neurons in the substantia nigra (SN) that is located in the midbrain. Degeneration of these neurons in PD reduces the DA input to the striatum and results in motor symptoms such as tremor, bradykinesia, rigidity and postural instability. Although we have known for decades that DA neurons in the SN degenerate in PD, we still do not know why exactly these neurons are prone to degenerate in PD patients and which molecular pathways are responsible for this degenerative process. Due to this limited knowledge of the disease mechanisms underlying PD, it has thus far been impossible to develop disease-modifying treatments. While current treatments – e.g. L-DOPA administration or physical exercise – can improve the motor symptoms of PD, they do not stop or slow down disease progression. In this thesis, I aimed to further elucidate the molecular pathways underlying PD and the effects of physical exercise on PD. By doing so, I wanted to increase our knowledge of disease etiology and provide new insights into how physical exercise can alleviate PD symptoms, and thereby move the field forward towards the development of disease-modifying treatments.

In **chapter 1**, a general introduction to PD is provided by presenting a historical overview of the advancing insights into the clinical description and pathophysiology of PD through the years. This is followed by an outline of the genetic and environmental factors that are known to be involved in the etiology of PD. Lastly, the landscape building approach – the method used throughout this thesis to interpret large data sets – is explained.

In **chapter 2**, the landscape building approach was used to build a molecular landscape of PD, based on genome-wide association study (GWAS) data and other genes/proteins implicated in PD through e.g. familial candidate gene associations and functional studies. In this PD landscape, we identified four main biological processes that interact with each other and regulate DA neuron function and death, i.e. oxidative stress response, endosomal-lysosomal functioning, endoplasmic reticulum stress response, and immune response activation. Lipids and lipoproteins are functionally involved in all four processes and we therefore hypothesized that lipids are involved in PD pathogenesis. Subsequently, this hypothesis was confirmed by showing a shared genetic risk between lipid/lipoprotein traits and PD.

In **chapter 3**, we validated at a molecular level the use of mice treated with the neurotoxin 1-methyl-4-pheynl-1,2,3,6-tetrahydropyridine (MPTP) as a model of PD. We compared the genome-wide mRNA expression data of the SN and striatum from MPTP-treated mice with those from PD patients, and found pathways and processes – e.g. vesicular

trafficking, exocytosis, mitochondrial apoptosis and DA neuron-specific transcription – that were dysregulated in both the mouse model and PD patients. It was concluded that the MPTP-mouse represents a valid model to study PD, especially when studying the SN. In addition, we suggested that the construct validity of the MPTP-model may improve when the mice are used at a later time point following MPTP treatment.

In **chapter 4**, a treadmill device was used to physically exercise MPTP-treated mice, in order to study the effects of physical exercise on this PD-model. First, we confirmed that MPTP treatment reduces the number of DA neurons in the SN and DA fibers in the striatum. Second, we showed that physical exercise is able to improve the motor function of MPTP-treated mice. And third, we performed RNA sequencing of six PD-associated brain areas to study the effects of physical exercise on the transcriptome. We found that physical exercise particularly regulates CREB1- and bicuculine/dalfampridine-mediated pathways in the prefrontal cortex and pedunculopontine nucleus, RICTOR-mediated pathways in the ventral tegmental area and dorsolateral striatum, and L-DOPA-mediated pathways in the SN and ventromedial striatum. Of note, physical exercise and L-DOPA generally exerted an opposite direction of effect on the expression of the mRNAs in the L-DOPA-mediated pathways. This indicated that physical exercise may counteract certain L-DOPA-mediated pathways, including those involved in sleep and cognitive function. Although L-DOPA administration greatly improves PD motor symptoms, chronic and/or high-dose L-DOPA use has been associated with L-DOPA-induced dyskinesia and may also negatively affect cognitive function. On the other hand, moderate physical exercise has no known detrimental effects, and has been shown to improve both motor and cognitive functions. Therefore, we speculated that physical exercise not only improves motor function, but may also alleviate some of the adverse effects associated with (chronic) L-DOPA use.

In **chapter 5**, we built a molecular landscape of amyotrophic lateral sclerosis (ALS) to provide insights into the molecular pathways underlying ALS etiology. In addition, we wanted to assess if the landscape building approach can be used to identify which underlying molecular pathways are unique to PD and ALS. We integrated the available GWAS data of sporadic ALS patients with other genes/proteins implicated in ALS etiology through other evidence, including familial ALS candidate gene association studies. We identified three main processes that interact and are crucial to maintain axonal functionality of motor neurons (the pathological substrate of ALS), i.e. (1) Rho-GTPase signaling, (2) signaling involving estradiol, folate and methionine, and (3) ribonucleoprotein granule functioning and axonal transport. Estradiol is functionally involved in all three processes, and epidemiological findings together with our analysis of possible gender effects in a cohort of ALS patients indicated that estradiol may be protective. Overall, we concluded that the landscape provides in-depth insights into

the mechanisms underlying motor neuron degeneration in ALS, and that estradiol is an important modulator of these pathological mechanisms. Moreover, we saw that the landscape building approach resulted in different, unique mollecular pathways for ALS, compared to the PD landscape in chapter 2.

In **chapter 6**, the landscape building approach is discussed and the main findings of this thesis are put in a broader context. I have compared the PD and ALS landscapes, discussed the use of the landscape building approach in the identification of disease-specific pathways and processes, and explored the putative druggable targets within the landscapes. I also explained the differences in interpretation of genetic and expression data, and analyzed the genes/proteins that overlap between the genetic and expression data sets. Further, by using our expression data of the MPTP-treated mice (chapter 4), I discussed if the hypothesis posed in chapter 3 (i.e. the construct validity of the MPTP model may improve when used at a later time point following MPTP treatment) still holds. Moreover, I explored if physical exercise not only has an effect on mRNAs that contribute to MPTP toxicity (chapter 4), but also has the potential to regulate mRNAs that contribute to the pathophysiology of PD. Lastly, I discussed how the landscape building approach can be used to achieve personalized medicine and I provided suggestions for future research.

Taken together, the research described in this thesis yields in-depth insights into the molecular mechanisms underlying PD and ALS, and as such contributes to our understanding of the etiology of these diseases and the future development of disease-modifying treatments. Moreover, our results provide knowledge of the molecular pathways underlying the beneficial effects of physical exercise on PD, and further establish the landscape building approach as a useful tool to study complex neurodegenerative diseases.

SAMENVATTING

De ziekte van Parkinson komt voor bij 1-2% van de mensen boven 60 jaar en is daarmee, na de ziekte van Alzheimer, de meest voorkomende neurodegeneratieve ziekte. De ziekte van Parkinson wordt gekenmerkt door het doodgaan van dopaminerge (DA) neuronen in de substantia nigra (SN), een hersengebied dat zich in de middenhersenen bevindt. De degeneratie van deze neuronen vermindert de DA signalen naar het striatum en veroorzaakt daardoor motorische symptomen, zoals onvrijwillige trillingen (tremor), trage bewegingen (bradykinesie), stijfheid en posturale instabiliteit. Hoewel we al tientallen jaren weten dat in Parkinson patiënten DA neuronen in de SN dood gaan, weten we nog steeds niet precies waarom juist deze neuronen degenereren en welke moleculaire mechanismen verantwoordelijk zijn voor dit degeneratieve proces. Vanwege deze beperkte kennis van de mechanismen die ten grondslag liggen aan de ziekte van Parkinson is het tot nu toe onmogelijk geweest om een behandeling te ontwikkelen die de ziekte daadwerkelijk verandert. Hoewel de huidige behandelingen – bijv. L-DOPA toediening of lichaamsbeweging – de motorische symptomen van Parkinson kunnen verbeteren, wordt de progressie van de ziekte niet gestopt of vertraagd. In dit proefschrift heb ik mij gericht op het ontrafelen van de moleculaire mechanismen die onderliggend zijn aan de ziekte van Parkinson, en de mechanismen die betrokken zijn bij de effecten van lichaamsbeweging op een model voor Parkinson. Met dit onderzoek wilde ik onze kennis over de etiologie van Parkinson vergroten en nieuwe inzichten verschaffen in de werking van lichaamsbeweging op symptoomverlichting bij de ziekte Parkinson, om daarmee het onderzoeksveld een stap dichter bij de ontwikkeling van ziektemodificerende behandelingen te brengen.

In **hoofdstuk 1** is een algemene inleiding op de ziekte van Parkinson gegeven waarin een historisch overzicht van de voortschrijdende inzichten in de klinische beschrijving en de pathofysiologie van de ziekte van Parkinson is beschreven. Daarna volgt een overzicht van de genetische- en omgevingsfactoren waarvan bekend is dat ze betrokken zijn bij het ontstaan van Parkinson. Tenslotte, is de methode uitgelegd die in dit proefschrift is gebruikt om grote datasets te interpreteren en te verwerken tot een zogenaamd moleculair landschap.

In **hoofdstuk 2** werd de landschap-bouwmethode gebruikt om een moleculair landschap voor de ziekte van Parkinson te maken, uitgaande van genen / eiwitten die betrokken zijn bij Parkinson op grond van genoomwijde associatiestudies (GWASs), familiale kandidaat-genassociaties en functionele studies. In het moleculaire landschap van de ziekte van Parkinson konden we vier belangrijke biologische processen identificeren die met elkaar interageren en DA neuronfunctie en -dood reguleren, namelijk oxidatieve stressreactie, endosomaal-lysosomaal functioneren, endoplasmatisch reticulum stressreactie en activatie van een immuunreactie. Lipiden en lipoproteïnen

zijn functioneel betrokken bij alle vier processen en daarop baseerden we de hypothese dat lipiden betrokken zijn bij de pathogenese van Parkinson. Vervolgens werd deze hypothese bevestigd doordat aangetoond kon worden dat er een gedeeld genetisch risico is op zowel lipide- / lipoproteïne-eigenschappen als de ziekte van Parkinson.

In **hoofdstuk 3** hebben we het gebruik van muizen behandeld met de neurotoxine 1-methyl-4-pheynl-1,2,3,6-tetrahydropyridine (MPTP), als een model voor de ziekte van Parkinson, op moleculair niveau gevalideerd. We vergeleken de genoomwijde mRNA expressie in de SN en het striatum van MPTP-behandelde muizen met die van parkinsonpatiënten, en vonden processen – bijv. vesiculair transport, exocytose, mitochondriale apoptose en DA neuron-specifieke transcriptie – die werden ontregeld in zowel het muismodel als in de parkinsonpatiënten. We concludeerden dat de MPTP-behandelde muis een valide model is voor parkinsononderzoek, vooral met betrekking tot het bestuderen van de SN. Bovendien stelden we dat de constructvaliditeit van het MPTP-model mogelijk kan verbeteren wanneer de muizen worden gebruikt op een later tijdstip na MPTP-behandeling dan dat nu veelal gebruikelijk is.

In **hoofdstuk 4** werd een loopband gebruikt om de effecten van lichaamsbeweging op MPTP-behandelde muizen te bestuderen. Eerst bevestigden we dat de behandeling met MPTP het aantal DA neuronen in de SN en de DA vezels in het striatum vermindert. Vervolgens hebben we aangetoond dat lichaamsbeweging de motorische functie van MPTP-behandelde muizen kan verbeteren. En als laatste hebben we een RNA sequentie-analyse uitgevoerd om het effect van lichaamsbeweging op het transcriptoom van zes Parkinson-geassocieerde hersengebieden te bestuderen. We vonden dat lichaamsbeweging in het bijzonder CREB1- en bicuculine / dalfampridine-gereguleerde processen in de prefrontale cortex en pedunculopontinekern, RICTOR-gereguleerde processen in het ventrale tegmentale gebied en dorsolaterale striatum, en L-DOPA-gereguleerde processen in de SN en het ventromedial striatum beïnvloedt. Van belang was de vinding dat lichaamsbeweging en L-DOPA over het algemeen een tegengestelde invloed hebben op de expressie van de mRNA's in de door L-DOPA gereguleerde processen. Dit wijst erop dat lichaamsbeweging bepaalde L-DOPA-gereguleerde processen kan tegengaan, met inbegrip van processen die betrokken zijn bij slaap en cognitieve functie. Hoewel toediening van L-DOPA de motorische symptomen bij de ziekte van Parkinson sterk verbetert, wordt chronisch gebruik en / of gebruik van een hoge dosis L-DOPA geassocieerd met L-DOPA-geïnduceerde dyskinesie, en zou het mogelijk ook een nadelig effect hebben op cognitieve functie. Matige lichaamsbeweging heeft daarentegen geen bekende nadelige effecten en verbetert zowel motorische als cognitieve functies. Daarom speculeerden wij dat lichaamsbeweging niet alleen de motorische functies verbetert, maar mogelijk ook enkele van de bijwerkingen geassocieerd met (chronisch) L-DOPA-gebruik kan verlichten.

In **hoofdstuk 5** bouwden we een moleculair landschap voor amyotrofe laterale sclerose (ALS) om inzicht te krijgen in de moleculaire mechanismen onderliggend aan de etiologie van ALS. Daarnaast wilden we bepalen of de methode voor het bouwen van landschappen gebruikt kan worden om unieke moleculaire mechanismen te identificeren onderliggend aan de degeneratie van neuronen in ofwel Parkinson of ALS. We integreerden de beschikbare GWAS-gegevens van sporadische ALS-patiënten met andere genen / eiwitten die geassocieerd zijn met de etiologie van ALS via andere bewijzen, met inbegrip van familiale ALS kandidaat-gen associatiestudies. We konden drie hoofdprocessen identificeren die interageren en cruciaal zijn voor de functionaliteit van axonen van motorneuronen (het pathologische substraat van ALS), namelijk (1) Rho-GTPase signalering, (2) signalering met estradiol, folaat en methionine, en (3) het functioneren van ribonucleoproteïne deeltjes en axonaal transport. Estradiol is functioneel betrokken bij alle drie processen, en epidemiologische bevindingen, samen met onze analyse van mogelijke geslachtseffecten in een cohort van ALS patiënten, duiden er op dat estradiol beschermend kan zijn. Kortom, we concludeerden dat het landschap diepgaande inzichten biedt in de mechanismen die ten grondslag liggen aan de degeneratie van motorneuronen in ALS en dat estradiol een belangrijke modulator van deze pathologische mechanismen is. Bovendien zagen we dat de methode voor het bouwen van moleculaire landschappen resulteerde in andere, unieke moleculaire mechanismen in vergelijk met het Parkinson landschap in hoofdstuk 2.

In **hoofdstuk 6** worden de belangrijkste bevindingen van dit proefschrift in een bredere context geplaatst. Ik heb de landschappen van Parkinson en ALS vergeleken, het gebruik van de landschap-bouwmethode voor de identificatie van ziektespecifieke mechanismen en processen bediscussieerd, en de mogelijke doelen binnen de landschappen onderzocht die in aanmerking kunnen komen voor verder onderzoek naar de ontwikkeling van geneesmiddelen. Ik heb ook de verschillen in interpretatie van genetische- en expressiedata besproken, en de genen / eiwitten die overlappen tussen de genetische- en expressiedatasets geanalyseerd. Door gebruik te maken van onze expressiegegevens van de MPTP-behandelde muizen (hoofdstuk 4), heb ik tevens bediscussieerd of de in hoofdstuk 3 gestelde hypothese (namelijk dat de constructvaliditeit van het MPTP-model kan verbeteren bij gebruik op een later tijdstip na de MPTP behandeling) nog steeds geldig is. Verder ben ik nagegaan of lichaamsbeweging niet alleen gevolgen heeft voor mRNA's die bijdragen aan MPTP toxiciteit (hoofdstuk 4), maar ook het potentieel heeft om mRNA's die bijdragen aan de pathofysiologie van Parkinson te beïnvloeden. Tenslotte besprak ik hoe de landschap-bouwmethode gebruikt kan worden om gepersonaliseerde geneeskunde te bewerkstelligen en heb ik suggesties verstrekt voor toekomstig onderzoek.

Samengevat kan worden gesteld dat het onderzoek beschreven in dit proefschrift

diepgaand inzicht levert in de moleculaire mechanismen die betrokken zijn bij de ziekte van Parkinson en ALS, en als zodanig bijdraagt aan ons begrip van het ontstaan van deze ziekten en de toekomstige ontwikkeling van ziektemodificerende behandelingen. Daarnaast verschaffen onze resultaten kennis over de moleculaire processen die ten grondslag liggen aan de gunstige effecten van lichaamsbeweging op de ziekte van Parkinson, en is verder vastgesteld dat de landschap-bouwmethode een nuttig instrument is om complexe ziekten te bestuderen.

DANKWOORD

Promoveren doe je alleen, maar wel met de steun en inzet van anderen, welke ik dan ook uitdrukkelijke dank verschuldigd ben.

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April, 2017

CURRICULUM VITAE

Cornelius Jacobus Hendricus Maria Klemann (roepnaam: Koen) werd geboren op 28 januari 1986 in Tilburg en groeide op in het Brabantse Oisterwijk. Na het behalen van zijn VWO diploma in 2004 (Durendael, Oisterwijk) met het profiel Natuur & Techniek is hij begonnen met de studie Natuurwetenschappen aan de Radboud Universiteit in Nijmegen. Na vier jaar is Koen alsnog van studie veranderd en heeft in 2011 *cum laude* de master van Moleculaire Levenswetenschappen behaald. Tijdens zijn studie liep Koen twee onderzoeksstages. Zijn eerste onderzoeksstage was op de afdeling Cellulaire Dierfysiologie (Prof. Eric Roubos) onder begeleiding van Dr. Tom Rouwette. Hier deed hij onderzoek naar de dynamische veranderingen in het brein na acute pijnstress. Dit onderzoek, samen met een scriptie op de afdeling Cellulaire Dierfysiologie resulteerden in zijn eerste twee wetenschappelijke publicaties. Zijn eindstage liep Koen onder begeleiding van Dr. Leonie Waanders bij Philips in Eindhoven, waar hij onderzoek deed naar de optimalisatie van een biomarker assay voor prostaatkanker. Ondanks dit uitstapje naar het bedrijfsleven lonkte de academische wereld en startte Koen eind 2011 zijn promotieonderzoek aan de afdeling Moleculaire Dierfysiologie (Prof. Gerard Martens) op de Radboud Universiteit resulterend in dit proefschrift. Sinds april 2016 is Koen werkzaam bij DrugTarget ID, een spin-out van de Radboud Universiteit gespecialiseerd in het interpreteren van grote biologische datasets en het maken van moleculaire landschappen.

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