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Des Fachbereichs Medizin der Philipps-Universität Marburg

The Locus coeruleus in Parkinson's disease
– from basic research to new translational perspectives –

Kumulative-Dissertation zur Erlangung des Doktorgrades
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This cumulative dissertation represents a summary of the research results published in the following three peer-reviewed articles:

[1] **Henrich MT***, Geibl FF*, Lee B, Chiu W-H, Koprich JB, Brotchie JM, Timmermann L, Decher N, Matschke LA, Oertel WH (2018) A53T-alpha-synuclein overexpression in murine locus coeruleus induces Parkinson's disease-like pathology in neurons and glia. Acta Neuropathol Commun 6(1):39. doi:10.1186/s40478-018-0541-1 (* shared first authors)

[2] Oertel WH, **Henrich MT**, Janzen A, Geibl FF (2019) The locus coeruleus: Another vulnerability target in Parkinson's disease. Movement Disorders 24(2):197. doi:10.1002/mds.27785

[3] Geibl FF*, **Henrich MT***, Oertel WH (2019) Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease. J. Neural Transmission 126(4):377–396. doi:10.1007/s00702-019-01970-9 (* shared first authors)

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1. List of abbreviations

6-OHDA	6-hydroxydopamine
AADC	aromatic L-amino acid decarboxylase
aSYN	α-synuclein
BGH	bovine growth hormone
CBA	chicken β-actin
CMV	cytomegalovirus
CNS	central nervous system
DA	dopamine
DAT	dopamine transporter
ENS	enteric nervous system
GBA	glucocerebrosidase A
GFAP	glial fibrillary acidic protein
IbA1	ionized calcium binding adaptor molecule
IHC	immunohistochemical
LC	locus coeruleus
L-DOPA	3,4-hihydroxy-L-phenylalanine
LRRK2	leucine rich repeat kinase 2
MDS	Movement Disorders Society
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	magnetic resonance imaging
NA	noradrenaline
p62	sequestosome-1
pA	polyadenylate tail
PD	Parkinson's disease
PDGF	platelet derived growth factor
PET	positron emission tomography
PNS	peripheral nervous system
PRION	prion promotor
rAAV	recombinant adeno-associated viral
SNc	substantia nigra pars compacta
SNCA	gene of α-synuclein
SPECT	single photon emission computed tomography
TH	tyrosine hydroxylase
Thy1	Thy-1 cell surface antigen promotor
Ubi-1	ubiquitin
VMAT2	vesicular monoamine transporter 2
WPRE	woodchuck posttranscriptional regulatory element

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3. Abstract – Zusammenfassung

3.1. Abstract

This cumulative dissertation summarizes three peer-reviewed publications addressing different aspects of the prodromal and manifest phase of Parkinson's disease with special emphasis on the vulnerability of the noradrenergic locus coeruleus. The first publication represents an original article describing the establishment and characterization of the first ever α-synuclein overexpression mouse model for the locus coeruleus. Narrative articles two and three discuss the importance of the locus coeruleus in context of prodromal Parkinson's disease, and the heterogeneity of the affected mesencephalic and extramesencephalic dopaminergic systems in manifest Parkinson's disease.

The first publication entitled **"A53T-α-synuclein overexpression in murine locus coeruleus induces Parkinson's disease-like pathology in neurons and glia"** describes the establishment of the first locus coeruleus α-synucleinopathy mouse model. The data show that viral vector mediated focal overexpression of human A53T-α-synuclein triggered time-dependent neurodegeneration of noradrenergic locus coeruleus neurons, accompanied by progressive α-synuclein phosphorylation, formation of proteinase K-resistant α-synuclein-aggregates, accumulation of Ubi-1- and p62-positive inclusions in microglial cells and induction of progressive micro- and astrogliosis. Apart from this local pathology, we observed abundant α-synuclein positive axons in LC output regions, indicating rapid anterograde axonal transport of A53T-α-synuclein.

The second publication entitled **"The locus coeruleus – another vulnerability target in Parkinson's disease"** addresses the role of the locus coeruleus noradrenergic system in prodromal and manifest Parkinson's disease. Within this review we provide a comprehensive description of the neuroanatomical basis of the locus coeruleus system and its implication in Parkinson's disease, summarize highly relevant vulnerability factors, and list all animal studies conducted so far investigating locus coeruleus pathology in experimental research. Further, we provide a therapeutic outlook on how noradrenergic replacement therapy has already been successfully tested in manifest Parkinson's disease patients and how locus coeruleus dysfunction can be of use for the development of disease modifying therapy approaches and disease progression biomarkers.

Within the third publication entitled **"Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease"**, we provide a historical overview over the key milestones of Parkinson's disease pathogenesis and therapy, dissect the dopaminergic basis of the cardinal parkinsonian motor symptomatology, summarize the anatomical features of the ten dopaminergic systems of the mammalian central nervous system and their involvement in Parkinson's disease, illustrate how the advanced dopaminergic imaging techniques contribute to optimized differential diagnosis and pathogenetic knowledge, and explain how dopaminergic replacement therapy improves the cardinal motor symptomatology while simultaneously inducing a new set of symptoms based on a hyperdopaminergic status.

3.2. Zusammenfassung

Diese kumulative Dissertation fasst drei von Fachleuten begutachtete Veröffentlichungen zusammen, die sich mit verschiedenen Aspekten der prodromalen und manifesten Phase der Parkinson-Krankheit befassen, wobei der Schwerpunkt der Dissertation auf der Betroffenheit des noradrenergen locus coeruleus liegt. Die erste Veröffentlichung stellt einen Originalartikel dar, der die Etablierung und Charakterisierung des weltweit ersten α-Synuclein-Überexpressionsmodells im noradrenergen locus coeruleus der Maus beschreibt. In den Veröffentlichungen zwei und drei wird die Bedeutung des locus coeruleus im Kontext der prodromalen Krankheitsphase und die Heterogenität der betroffenen mesencephalen und extramesencephalen dopaminergen Systeme im Rahmen der manifesten Parkinson-Krankheit erörtert.

Die erste Veröffentlichung mit dem Titel **"A53T-α-synuclein overexpression in murine locus coeruleus induces Parkinson's disease-like pathology in neurons and glia"** beschreibt die Etablierung des ersten α-Synucleinopathie-Mausmodells im noradrenergen locus coeruleus. Die gewonnenen Daten zeigen, dass eine viral vermittelte fokale Überexpression von humanem A53T-α-Synuclein im murinen locus coeruleus eine zeitabhängige Neurodegeneration noradrenerger locus coeruleus Neurone auslöste, begleitet von einer zunehmenden Phosphorylierung des überexprimierten α-synuclein und Bildung von Proteinase-K resistenten α-synuclein-Aggregaten. Es zeigte sich zudem eine Akkumulation von Ubi-1- und p62-positiven Einschlüssen in Mikrogliazellen sowie eine Induktion einer fortschreitenden Mikro- und Astrogliose. Abgesehen von dieser lokalen Pathologie beobachteten wir zahlreiche α-synuclein-positive Axone in locus coeruleus-Projektionsregionen, was auf einen schnellen anterograden axonalen Transport von A53T- α-synuclein hinweist.

Die zweite Veröffentlichung mit dem Titel **"The locus coeruleus – another vulnerability target in Parkinson's disease"** befasst sich mit der Rolle des noradrenergen locus coeruleus-Systems im Rahmen der prodromalen und manifesten Parkinson-Krankheit. Im Rahmen dieser Publikation stellen wir eine umfassende Beschreibung der neuroanatomischen Grundlagen des locus coeruleus-Systems und seiner Beteiligung im Rahmen der Parkinson-Krankheit bereit, fassen die relevantesten Vulnerabilitätsfaktoren zusammen und listen alle Tierstudien, die bisher zur Untersuchung der locus coeruleus-Pathologie in der experimentellen Parkinson-Forschung durchgeführt wurden. Darüber hinaus geben wir einen therapeutischen Ausblick darauf, wie die noradrenerge Substitutionstherapie bereits an manifesten Parkinson-Patienten erfolgreich getestet wurde und wie eine locus coeruleus-Dysfunktion für die Entwicklung krankheitsmodifizierender Therapieansätze und Progressionsmarker von Nutzen sein kann.

In der dritten Veröffentlichung mit dem Titel **"Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease"** gebe ich zunächst einen historischen Überblick über die wichtigsten Meilensteine der Pathogenese und Therapie der Parkinson-Krankheit, analysiere dann die dopaminerge Grundlage der kardinalen motorischen Symptomatik und fasse die

anatomischen Merkmale der zehn dopaminergen Systeme und ihre Beteiligung an der Parkinson-Krankheit zusammen. Zudem veranschauliche ich wie neue dopaminerge Bildgebungstechniken zur Optimierung der Differentialdiagnose und des pathogenetischen Wissens beigetragen haben, und erläutere, wie die dopaminerge Substitutionstherapie die kardinalmotorische Symptomatik verbessert und gleichzeitig eine Reihe neuer hyperdopaminerger Symptome hervorruft.

4. Theoretical background

4.1. Parkinson's disease

4.1.1. Clinical presentation

In 1817, James Parkinson described the clinical symptomatology of six patients which suffered from a movement disorder that presented with involuntary trembling of one or more body parts, general slow- and weakness of the limb muscles, the inability to walk with normal pace, and an altered bending forward posture (Parkinson, 1817). In honor of James Parkinson, Jean-Martin Charcot suggested the term Parkinson's disease (PD). In retrospective, Parkinson's "An Essay on the Shaking Palsy" marks the starting point for the long history of PD. Over 200 years later, idiopathic PD represents the most common movement disorder worldwide affecting over 1% of those individuals older than 65 years of age with a slight preference for the male gender (Benito-León et al., 2003; Samii et al., 2004; Lau and Breteler, 2006). Still today PD represents a clinical diagnosis relying on the presence of the classical motor symptomatology: 1) brady- or hypokinesia, 2) muscular rigidity, 3) rest tremor, and 4) postural instability (Kalia and Lang, 2015). Specified diagnostic criteria containing defined inclusion and exclusion criteria were recently ratified by the Movement Disorder Society (MDS) (Postuma et al., 2015). Importantly, PD motor symptomatology is not limited to the aforementioned disease defining symptoms, but can include several other heterogeneous motor manifestations like reduced arm swinging, short stride length, decreased blinking rate, reduced facial expressions, or a general reduction of daily life movements (Rodriguez-Oroz et al., 2009). Despite the fact that the parkinsonian motor symptoms represent core features of PD, it is noteworthy to highlight that PD patients exhibit numerous daily life impairing non-motor symptoms including but not limited to, reduced gastro-enteral motility, hyposmia, sleep disturbances, autonomic dysfunction, cognitive decline, depression, and apathy (Martinez-Martin et al., 2007; Chaudhuri and Schapira, 2009; Schapira et al., 2017).

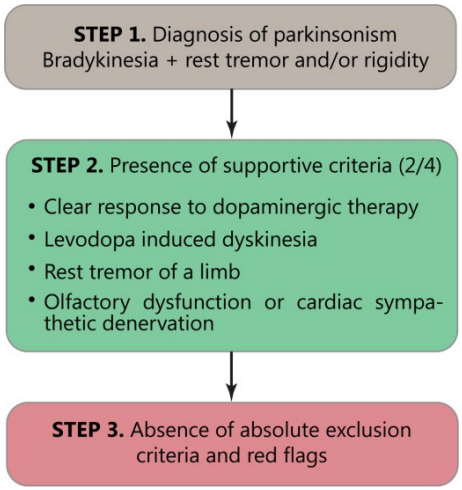
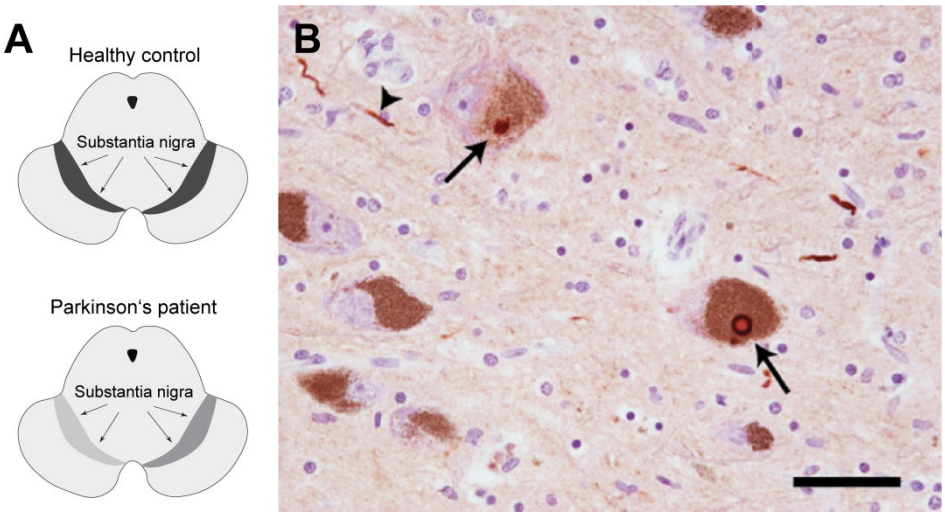


Figure 1 | Scheme depicting PD diagnostic steps defined by the MDS criteria

4.1.2. Neuropathological hallmarks

On a neuropathological basis, PD represents a progressive neurodegenerative disorder which is predominantly characterized by degeneration of dopaminergic midbrain neurons in the Substantia nigra pars compacta (SNc) leading to dopamine (DA) deficiency in the nigrostriatal network (Carlsson et al., 1957; Hornykiewicz, 1963; Trétiakoff, 1919; Kordower et al., 2013). Apart from the characteristic nigrostriatal degeneration, histological analysis of postmortem PD brain tissue has revealed another important neuropathological hallmark of PD; the formation of eosinophilic intracytoplasmic protein inclusions, termed Lewy-bodies in honor of their first describer Friedrich H. Lewy (Lees et al., 2009; Wakabayashi et al., 2013). These proteinaceous deposits can be found in cell somata (Lewy-bodies) and neuronal axons (Lewy-neurites), and consist predominantly of aggregated α-synuclein (aSYN) (Spillantini et al., 1997), a physiologically presynaptic protein involved in neurotransmitter release at the synaptic cleft (Bartels et al., 2011; Wang and Hay, 2015). During the pathogenesis of PD misfolded aSYN starts to accumulate forming insoluble intracytoplasmic protein aggregates (Peelaerts et al., 2015). Interestingly, first signs of Lewy pathology are not observed within the SNc, but in regions of the caudal medulla, olfactory bulb and the peripheral nervous system (PNS) (Braak et al., 2003; Beach et al., 2009; Hawkes et al., 2009; Beach et al., 2010). Based on the characteristic distribution of Lewy-bodies and neurites in post mortem brain samples of over 160 individuals, Braak et al. (Braak et al., 2003) developed a neuropathological staging system (Braak stage I – VI) which depicts the temporospatial progression pattern of Lewy pathology from early involved structures in the brainstem to late stage pathology in the neocortex.

Figure 2 | Neuropathological hallmarks of PD



A. Midbrain sections depicting the SNc of a PD patient and a healthy control.

B. SNc of a PD patient containing Lewy bodies (arrows) and Lewy neurites (arrowhead), stained with an antibody directed against aSYN. Scale bar = 50μm. Extracted from Ingelsson et al. (Leire Almandoz Gil, CC BY)

One important conclusion drawn from the Braak staging scheme is that Lewy pathology is not limited to the SNc or the basal ganglia, but affects multiple neurotransmitter systems in different brain regions over time in an ascending caudo-rostral hierarchical pattern. The Braak scheme implies that Lewy pathology evolves within defined brain regions and spreads over time to connected brain structures. However, without an available aSYN tracer or other valid biomarkers this is currently not testable in PD patients. Despite of the great value for PD, three aspects of the Braak scheme should be considered: 1) the pattern of Lewy-pathology in PD is much more variable than Braak's staging predicts, i.e. only half of the PD patients possess a distribution of Lewy-pathology that clearly matches the Braak staging (Kalaitzakis et al., 2008; Halliday et al., 2012), 2) the mere presence of aSYN inclusions in a given brain region does not correlate well with loss of neurons in that brain region (Surmeier et al., 2017b), and 3) Lewy pathology is not limited to the central nervous system (CNS), but affects also the enteric nervous system (ENS), sympathetic ganglia, autonomic nervous system of the heart, salivary glands and cutaneous nerve fibres (Beach et al., 2010; Donadio et al., 2014; Doppler et al., 2014).

4.1.3. Pathogenesis

While the knowledge on motor and non-motor symptomatology, dopaminergic replacement therapy, and neuropathology expanded enormously within the last decades, the etiology and pathogenesis of PD remain unsolved problems (Kalia and Lang, 2015). Over the last decades several risk factors have been identified including but not limited to aging, pesticide or herbicide exposure, melanoma, traumatic brain injury, methamphetamine consumption, and postmenopausal hormones (Lees et al., 2009; Ascherio and Schwarzschild, 2016). Factors which are associated with a decreased risk for developing PD are smoking, caffeine intake, high plasma urate concentration, physical activity, and calcium channel blockers (Ascherio and Schwarzschild, 2016). Apart from these factors which in- or decrease the risk for sporadic PD, several genetic alterations causing inherited forms of PD have been discovered (Polymeropoulos et al., 1997; Warner and Schapira, 2003). Mutations in the leucine rich repeat kinase 2 (LRRK-2) gene or a heterozygous loss of function mutation of the glucocerebrosidase (GBA) gene are clearly linked to hereditary PD. Furthermore, duplications, triplications or missense mutations of the aSYN gene (SNCA) can cause genetic forms of PD (Farrer, 2006; Ascherio and Schwarzschild, 2016; Schneider and Alcalay, 2017). The observation that mutations of the aSYN gene can cause PD in combination with experimental *in vitro* and *in vivo* findings of retro- and anterograde transport of defined aSYN protein species has fueled the idea that PD progression might rely on prion-like spreading of Lewy pathology from one affected brain region to another synaptically connected brain region (Brundin et al., 2016; Brundin and Melki, 2017). In contrast to this, a second group of authors (Surmeier et al., 2017a, 2017b; Giguere et al., 2018) proposes that not neuronal connectivity but rather certain shared cell-autonomous factors, like thin myelinated but extensively branched axons, constant autonomous pacemaking, or low intrinsic

calcium buffering capacity, render these neuronal populations particularly vulnerable to the disease process.

4.1.4. Therapy

The finding of dopaminergic deficiency in PD had and still has enormous implications for the employed therapeutic strategies. In the absence of any neuroprotective approaches, PD therapy is primarily focused on the reduction of motor symptomatology by restoring dopaminergic neurotransmission. In 1961 L-DOPA (L-Di-HydrOxy-Phenyl-Alanine), the direct precursor of DA, was introduced as the first rationally derived PD therapy (Birkmayer and Hornykiewicz, 1961). Notably, still today administration of L-DOPA, in combination with peripheral dopamine decarboxylase inhibitors, represents a key approach for treatment of PD motor symptomatology as it is recommended in all stages of the disease regardless of the presence or absence of motor fluctuations or dyskinesia (Oertel and Schulz, 2016). Apart from the gold standard L-DOPA, several DA agonists were developed and successfully implemented. Out of those, the non-ergot DA agonists (pramipexole, ropinirol, piribedil, apomorphine, and rotigotine) are in common use, whereas the ergot agonists (bromocriptine, cabergoline, lisuride, pergolide, and α-dihydroergocriptine) are hardly used anymore due to their adverse effect profile (e.g. cardiac and non-cardiac fibrotic reactions). DA agonists are used as a monotherapy in early PD patients, as an ad on approach with L-DOPA in fluctuating and non-fluctuating patients, and in the advanced stages of PD (Oertel and Schulz, 2016). However, based on the side effect profile and the tendency to require supplementary L-DOPA to achieve sufficient symptomatic relief, monotherapy with DA agonists is not considered superior compared to L-DOPA monotherapy. Other valuable approaches to enhance and restore dopaminergic neurotransmission include monoamine-oxidase-B inhibitors (rasagiline, safinamide, and selegiline) and catechol-O-methyltransferase inhibitors (entacapone, opicapone, and tolcapone), which delay the degradation of DA and L-DOPA thereby increasing dopaminergic neurotransmission (Oertel, 2017). In contrast to the well-established symptomatic treatment options for PD motor symptomatology, therapy options for the numerous non-motor symptoms remain a major challenge. Due to the little number of randomized-controlled trials which address non-motor symptomatology in the therapeutic context evidence based recommendations are sparse (Seppi et al., 2011). The current individual treatment options for the broad non-motor symptomatology have recently been reviewed elsewhere (Seppi et al., 2011; Sauerbier et al., 2017).

4.2. The concept of prodromal PD

It becomes increasingly clear that PD affects multiple transmitter systems, in different brain regions or even outside of the CNS, years before pathology reaches the SNc and the characteristic motor phenotype becomes overt. This latency, in which affected individuals present with early non-motor or subtle motor signs not yet qualifying as PD, is called the prodromal phase of PD (Mahlknecht et al., 2015). Based on the MDS criteria for prodromal PD (Berg et al., 2015) this phase is characterized by initial neurodegeneration of structures other than the SNc, detectable non-motor and or subtle motor symptomatology, and the tendency to progress slowly over many years till the prodromal patient converts to manifest PD (Goldman and Postuma, 2014). Frequently observed non-motor symptoms during the prodromal phase are hyposmia, constipation, depression, anxiety and REM-sleep-behavior disorder (Postuma and Berg, 2016). As prodromal PD may take up to 20 years, it is highly relevant for disease modifying therapeutic approaches, which are aimed on decreasing or stopping the underlying neurodegeneration. At the moment, prodromal PD patients are considered to represent an ideal study population given that they have a broader therapeutic window and do not yet receive symptomatic treatment (Postuma and Berg, 2016). However, the neurobiological correlates of the prodromal symptomatology are still largely unclear. If we apply the Lewy pathology staging of Braak (Braak et al., 2003) on the prodromal setting, prodromal PD patients should be located in Braak stage 1 (olfactory bulb, dorsal motor nucleus of the vagus, intermediate reticular zone) and 2 (locus coeruleus, gigantocellular nucleus, caudal raphe), since stage 3 is already characterized by beginning Lewy pathology in the SNc.

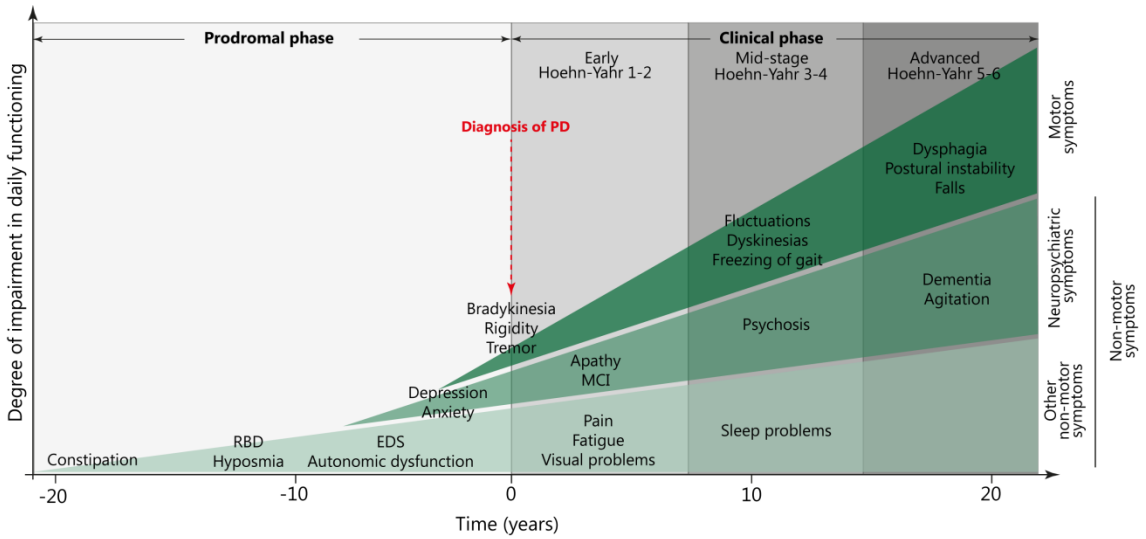


Figure 3 | Time course of PD and clinical symptomatology [Adapted from (Kalia and Lang 2015)].

4.3. The noradrenergic locus coeruleus

4.3.1. Physiological role of LC neurons

The human noradrenergic locus coeruleus (LC), a small structure located in the formatio reticularis of the pontine brainstem close to the 4th ventricle, provides the major source of noradrenaline (NA) for vast parts of the human brain (Berridge and Waterhouse, 2003; Benarroch, 2009, 2017). Despite the small size, on average 35 000 neurons per hemisphere in a healthy human individual (Aston-Jones and Cohen, 2005; Espay et al., 2014) and 1500 neurons per hemisphere in mice (Berridge and Waterhouse, 2003), the LC system possesses an enormous axonal projectome (Jones and Moore, 1977; Aston-Jones and Cohen, 2005). Tract tracing studies in mice revealed ascending LC noradrenergic projections into the periaqueductal grey, superior colliculus, ventral tegmental area, thalamus, hypothalamus, hippocampus, basal forebrain, amygdala, olfactory bulb and the complete neocortex. Descending projections were observed in the cerebellum, caudal medulla and spinal cord. The LC targets almost all brain regions from the olfactory bulb to the spinal cord. Exceptions from this are the striatum, globus pallidus, nucleus accumbens, and the substantia nigra which receive almost no noradrenergic innervation from the LC (Szabadi, 2013; Schwarz and Luo, 2015). Interestingly, NA cannot only be released at LC synaptic terminals but also at non-synaptic release sites, termed varicosities, along LC axons. Comparable to the LC output connectome, the projection pattern for incoming afferent input is similarly broad. The murine LC receives information from over 100 different brain regions (Schwarz et al., 2015) spanning the complete rostro-caudal extent of the neuroaxis. It has been suggested that the anatomical organization of the LC noradrenergic system provides the basis for a working neuromodulatory system, in which incoming environmental information can be broadcasted to distinct target nuclei to evoke behavioral and autonomic adaptations (Sara, 2009; Sara and Bouret, 2012). In addition, the LC is involved in several highly preserved brain functions like generation of arousal and vigilance, facilitation of behavioral adaptions following new sensory information or environmental stress, memory consolidation, learning, modulation of motor control, and regulation of local blood flow in the brain (Benarroch, 2009; Weinshenker, 2018).

4.3.2. LC dysfunction and degeneration in PD

Loss of LC neurons is a prominent feature of several neurodegenerative disorders, including PD, progressive supranuclear palsy and corticobasal degeneration, but also dementia with Lewy bodies, Alzheimer's disease, and Down syndrome (Vermeiren and Deyn, 2017). In PD, first aSYN positive inclusions are found in LC neurons during Braak stage 2 (Braak et al., 2003), implying that the initial pathological alterations occur already in the prodromal phase of PD. Moreover, occurring Lewy pathology was found to be associated with axonal loss of noradrenergic projections and altered synaptic morphology in LC output targets, going along with decreased noradrenergic neurotransmission (Delaville et al., 2011; Espay et al., 2014; Weinshenker, 2018). Notably, despite these early alterations the majority of LC neurons can survive the pathological process for many years,

thereby even outliving the loss of other vulnerable brain regions like the SNc (Halliday et al., 1990). Post mortem histological studies report 21-93% cell loss of LC neurons in late stage PD (Halliday et al., 1990; Paulus and Jellinger, 1991; German et al., 1992). However, sufficient postmortem data of prodromal or de novo PD patients is still lacking. The resulting noradrenergic deficiency in PD is thought to be associated with several important non-motor symptoms of PD, including cognitive impairment, depression, anxiety, apathy, fatigue, and REM-sleep-behavior-disorder (Benarroch, 2009; Weinshenker, 2018). Furthermore, dysfunctional noradrenergic neurotransmission is also implicated in impaired motor control and freezing of gait (Espay et al., 2014). While loss of LC neurons has been commonly reported over the past decades, the mechanisms and etiology are still largely unknown. Compared to other vulnerable brain regions known to degenerate in PD, LC cells are thought to belong to a group of brain nuclei which possess a shared phenotype which could mediate the observed vulnerability (Surmeier et al., 2017b). Increasing evidence, mainly from research on dopaminergic SNc cells, suggests that certain cell-autonomous factors function as vulnerability traits increasing the basal rate of cellular stress and mediating the neurodegenerative process in PD. For the LC noradrenergic system these include an extensive axonal arborization with multiple synaptic and paracrine neurotransmitter release sites, the electrophysiological phenotype of a pacemaker neuron continuously generating slow tonic spiking, the burden to generate and metabolize a highly reactive neurotransmitter, high amounts of intracellular neuromelanin, and its location directly next to the 4th ventricle (Sanchez-Padilla et al., 2014; Weinshenker, 2018). However, it is still largely unclear if the aforementioned vulnerability factors are cause or bystander of LC degeneration in PD. Compared to dopaminergic SNc neurons LC cells exhibit a considerable time lag between initial Lewy pathology in the prodromal phase of PD and final cell loss in the advanced PD stages (Halliday et al., 1990), leaving the LC for many years in a dysfunctional state. Further, experimental research revealed that toxin-induced LC cell loss sensitizes dopaminergic SNc neurons for neurodegeneration whereas noradrenergic hyperinnervation resulted in neuroprotective effects (Bing et al., 1994; Fornai et al., 1996; Kilbourn et al., 1998; Rommelfanger and Weinshenker, 2007). Similar observations were made in Alzheimer's disease animal models (Jardanhazi-Kurutz et al., 2010; Kummer et al., 2014; Bharani et al., 2017; Chalermpalanupap et al., 2018). This implies that LC cell loss in PD could play a double role by firstly being responsible for several non-motor symptoms, and secondly for accelerating the progression of the disease (Gesi et al., 2000). Based on the histological data indicating early occurring LC Lewy pathology in the course of PD, the profound noradrenergic cell loss in manifest PD, and the increasing evidence for a causative role of noradrenergic deficiency in context of non-motor symptomatology, noradrenergic replacement therapy has been discussed as a new therapeutic target and clinical trials in manifest PD patients have been conducted (Espay et al., 2014). Despite promising effects showing symptomatic improvement of cognitive symptoms, mood, and distinct motor complications, noradrenergic replacement therapy has not become a topic of major interest, yet.

4.4. Classical and new animal models of PD

4.4.1. Neurotoxin based animal models

Over the last two decades several animal models have been established to mimic the core pathology of PD, i.e. aSYN aggregation, progressive neurodegeneration, and evolvement of behavioral motor or non-motor alterations (Przedborski, 2017). Since PD has been traditionally considered a disease of the nigrostriatal system, the first characterized animal models were designed to reproduce neurodegeneration of the SNc resulting in striatal DA deficiency. In regard to this the most extensively studied models are based on the administration of neurotoxins which cause cell loss of dopaminergic SNc neurons (Blesa et al., 2012). Substances frequently used to induce dopaminergic deficiency include 6-OHDA (6-hydroxydopamine) (Ungerstedt, 1968; Sachs and Jonsson, 1975) and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Langston et al., 1983), while rotenone, paraquat, isoquinoline derivates, and methamphetamine are less often used (Bezard et al., 2013). The advantages of neurotoxin models are the reliable reproduction of severe nigrostriatal dopaminergic denervation in combination with the evolvement of robust parkinsonian behavioral alterations (Bove and Perier, 2012). Moreover, they offer a simple way to study dopaminergic replacement therapy and L-DOPA induced dyskinesia (Bezard et al., 2013). The major limitation of these models lies within the lack of mimicking the etiology of PD, as intoxication, e.g. with MPTP, might only account for rare cases of parkinsonism. Neurotoxin based models have been widely used to test neuroprotective compounds which protected SNc neurons from toxin induced cell loss. However, none of these substances has shown efficacy in clinical trials (Athauda and Foltynie, 2015). Another important point is that neurotoxin based models do not model the multisystem disease aspect of PD, i.e. neurodegeneration and Lewy pathology outside of the SNc.

4.4.2. Genetic animal models

With the growing demand to develop disease modifying therapy, experimental PD research has seen major changes regarding the employed animal models. It has become evident that the absence of understanding the mechanisms leading to the neurodegenerative process in PD presents a crucial limitation for the development of new therapeutic approaches (Bezard et al., 2013). Based on this and the general interest to understand the cause of PD, experimental PD research has shifted its focus to etiologic animal models (Dawson et al., 2010; Blesa et al., 2012). The discovery that genetic alterations can cause familial forms of PD (Polymeropoulos et al., 1997), and that aSYN constitutes a major component of the observed Lewy pathology (Spillantini et al., 1997), has formed the opinion that genetic aSYN models might better mimic the pathophysiological mechanisms of PD. Various transgenic mouse models have therefore been developed using different promotors (Thy1, PRION, PDGF, TH) to conditionally overexpress human wild-type or different mutated forms of aSYN in the mouse brain (Bezard et al., 2013). The main novelty of these models at that time was that the role of aSYN aggregation but also its physiological function played a central role. Furthermore, they provided new aspects on induced toxicity by the different mutated aSYN forms and offered new

possibilities for drug development (Visanji et al., 2016). Based on the employed promotor but also on the characteristics of the transgene model, severe differences in the degree of aSYN overexpression, local protein distribution and behavioral phenotype have been observed (Fernagut and Chesselet, 2004). Notably, no transgene mouse line has fulfilled all criteria of a proper PD animal model, i.e. aSYN aggregation, progressive neurodegeneration, and evolvement of behavioral motor or non-motor alterations. While most transgene mouse models have shown progressive accumulation of aggregated aSYN without dopaminergic cell loss, some models report DA degeneration in the absence of aSYN aggregation. Another major limitation of these models is that they do not replicate the stereotyped propagation pattern of the Braak staging scheme, as the aSYN overexpression is highly dependent on the used promotor.

4.4.3. Viral vector mediated models

The development of new lentiviral or recombinant adeno-associated viral (rAAV) vectors allowing overexpression of aSYN in locally defined brain regions had a major impact on experimental research in PD. While the aforementioned transgene mouse lines used conditional overexpression of aSYN which generally required several months of survival time for the aSYN pathology to develop, stereotactic injection of viral vectors offered the advantage to induce the α-synucleinopathy within several weeks. Furthermore, it was now possible to restrict the initial overexpression to PD relevant brain regions, e.g. the striatum, SNc, or dorsal motor nucleus of the vagus. Since the first study of Kirik et al. (Kirik et al., 2002) numerous studies were published investigating wild-type or mutated aSYN forms (A30P, A53T, A56P) (Koprich et al., 2010; Taschenberger et al., 2012; Ulusoy et al., 2013; Helwig et al., 2016). One core observation made was that aSYN overexpression in SNc neurons resulted in prominent SNc neurodegeneration in combination with loss of striatal dopaminergic terminals and behavioral alterations like forepaw asymmetry or apomorphine-induced rotations (Kirik et al., 2002). Notably, synaptic abnormalities and axonal degeneration in the striatum preceded SNc cell loss, indicating a dying back mechanism. In some studies local neuron loss was additionally associated with signs of neuroinflammation, a prominent feature observed in postmortem brains of PD patients (Theodore et al., 2008; Thakur et al., 2017). Regarding the induced local pathology, AAV-mediated overexpression of aSYN has thereby proven to recapitulate many features of human PD. A completely new observation compared to all the other animal models before was that locally induced aSYN aggregation could develop with time into a brain-wide α-synucleinopathy affecting neuronal systems distant to the injection side (Ulusoy et al., 2013). While most studies reported that the overexpressed aSYN is only transported towards the synaptic terminals but does not spread to interconnected neurons (Uchihara and Giasson, 2016), some authors hypothesized that AAV mediated overexpression of aSYN in the vagal nerve could induce spreading of aSYN to other neuronal systems (Ulusoy et al., 2013; Helwig et al., 2016; Rusconi et al., 2018). Despite the promising results, there are also important limitations of the viral vector mediated model systems. First, the strength and extent of local aSYN overexpression depends highly on the accuracy of the stereotactic

injection of the AAV's, meaning a misplaced injection can cause a considerable amount of mouse to mouse variability. Depending on the employed serotype and promotor, aSYN overexpression cannot be fully limited to neurons and an unintended co-transduction of glial cells can occur. Furthermore, induced aSYN aggregates do not fully recapitulate all features of human Lewy-bodies or neurites (Volpicelli-Daley et al., 2016).

4.4.4. Preformed aSYN fibril model

In the first landmark study, Luk et al. (Luk et al., 2012) revealed that local injection of pre-formed aSYN fibrils into the striatum of C57BL6/C3H mice resulted in formation of Lewy-body like inclusions in the SNc but also in anatomically interconnected brain regions, suggesting cell-to-cell transmission of the aSYN pathology. The observed Lewy pathology was associated with neurodegeneration of the ipsilateral SNc, a decrease of striatal DA, and alterations of motor behavior. Subsequent studies revealed that the induced aSYN inclusions exhibited several features of those observed in PD patients, e.g. hyperphosphorylation, ubiquitination, and insolubility (Masuda-Suzukake et al., 2013; Holmqvist et al., 2014; Recasens et al., 2014; Rey et al., 2016). The aSYN fibril model has therefore been suggested to most accurately reproduce the human Lewy-pathology compared to all in vivo models discussed so far (Volpicelli-Daley et al., 2016). Furthermore, it is considered a relatively mild model, as it does not induced massive cell loss in a short time frame like the toxin models, or drives the cells to extreme protein translation, like the aSYN overexpression models. The observation of cell-to-cell transmission does not only offer the possibility to investigate the mechanisms of disease propagation it further opens up new targets for disease modifying therapy (Koprich et al., 2017), e.g. antibodies which bind and thereby prevent spreading of pathological aSYN species. Regarding *in vitro* models, aSYN fibrils offer the unique possibility to induce Lewy-body formation in primary neuron cultures (Volpicelli-Daley et al., 2014; Mao et al., 2016). While the aSYN fibril model has drastically improved the field of experimental PD research, there are important limitations to consider. First, high quality aSYN fibrils are currently (2019) not commercially available. Further, monomeric aSYN species are generally used as a control, but monomeric aSYN can start to form oligomeric and fibrillar aSYN under the influence of room temperature and thereby induce false positive results. Further, the inherent tendency to form insoluble aSYN aggregates which induce brain-wide pathology affords increased safety precautions for the person who performs the stereotactic surgeries and further handling of the mice (Polinski et al., 2018). While all of the aforementioned animal models generally require Biosafety Level 1 laboratories, production, handling and injection of aSYN fibrils has to be performed on Biosafety Level 2 standards.

5. Summary of the publications

5.1. A53T-α-synuclein overexpression in murine locus coeruleus induces Parkinson's disease-like pathology in neurons and glia

Henrich MT*, Geibl FF*, Lee B, Chiu W-H, Koprich JB, Brotchie JM, Timmermann L, Decher N, Matschke LA, Oertel WH: A53T-α-synuclein overexpression in murine locus coeruleus induces Parkinson's disease-like pathology in neurons and glia. (*shared first authors). Acta Neuropathol Commun. 2018 May 10;6(1):39. doi: 10.1186/s40478-018-0541-1

Impact factor: 5.883 (2018)

5.1.1. Aim of the study

PD is a multisystem disorder characterized by dopaminergic, serotonergic, and noradrenergic deficiency. Decreased noradrenergic neurotransmission is thought to result majorly from loss of noradrenergic LC neurons and accompanied Lewy pathology within the remaining LC cells (Espay et al., 2014). Degeneration of the noradrenergic LC is seen as a key event in the early prodromal phase of PD (Rommelfanger and Weinshenker, 2007). During this phase LC dysfunction and early cell loss play a crucial role firstly by being responsible for several non-motor symptoms (e.g. depression, reduced arousal, and REM-sleep-behavior-disorder) and secondly for accelerating the progression of PD (Gesi et al., 2000). Despite the comprehensive data set on nigral aSYN overexpression models (Kirik et al., 2002; Koprich et al., 2010; Ip et al., 2017; Thakur et al., 2017), LC function and dysfunction in PD has not been investigated in an aSYN overexpression mouse model, yet. The main aim of the presented original research article entitled "A53T-α-synuclein overexpression in murine locus coeruleus induces Parkinson's disease-like pathology in neurons and glia" was to establish and characterize the first aSYN overexpression model in the noradrenergic LC which should replicate cardinal morphological features of the human LC neuropathology, provide sufficient information about the time course of noradrenergic neurodegeneration and finally lead to robust histological markers which can be used to further test disease modifying therapy approaches in rodents or non-human primates.

5.1.2. Methods

Two different rAAV vectors of a mixed 1/2 serotype were stereotactically injected in the right LC of male C57BL/6N wild-type mice to overexpress human mutant-A53T-aSYN (rAAV1/2-CMV/CBA-human-A53T-aSYN-WPRE-BGH-pA) or luciferase (rAAV1/2-CMV/CBA-luciferase-WPRE-BGH-pA) (Koprich et al., 2010; He et al., 2015; Ip et al., 2017). To investigate the time dependent effects of the evolving neuropathology, mice were sacrificed at pre-defined time-points (1, 3, 6, and

9 weeks post-injection). Double immunofluorescence stainings against tyrosine hydroxylase (TH) and human aSYN or luciferase were employed to confirm successful protein overexpression after viral vector delivery. To assess potential aSYN induced LC cell loss TH positive LC neurons were quantified for each time point using the optical fractionator workflow (StereoInvestigator version 9, MicroBrightField Biosciences). Different immunofluorescence stainings were then used to investigate the development of aSYN phosphorylation and formation of Lewy-body like aggregates. To assess the induction of reactive micro- or astrogliosis a triple immunofluorescence staining for IbA1 (microglial marker), GFAP (astroglial marker) and TH was carried out and the intensity of fluorescence signal was quantified. To address the question whether a viral vector mediated focally induced α-synucleinopathy in the LC can trigger brain-wide propagation of aSYN, predetermined brain sections were stained against human aSYN or luciferase and the occurrence of aSYN or luciferase positive axons or cell bodies was rated. For all experiments differences were considered significant at $p < 0.05$. Statistical significance of differences between two groups was analyzed by Student's *t*-Test. Multiple comparisons were made by one-way or two-way ANOVA analysis followed by Tukey's or Sidak's multiple comparisons test. To calculate correlations scatterplots and Pearson's correlation coefficient with 95% confidence interval was used.

5.1.3. Results

First, we confirmed that both vectors entered LC neurons equally, validating a comparable infection efficacy of the applied viral vectors (Fig. 1a-d) (for A53T-aSYN group 85.17 ± 2.53% and for luciferase group 83.87 ± 3.31%; $p = 0.77$, unpaired t-test). Next, double immunofluorescence stainings against TH and human aSYN or TH and luciferase showed that both vectors induced protein expression with similar strength, as depicted by the respective transduction rates (Fig. 1e-h) (for A53T-aSYN group 59.89 ± 2.95% and for luciferase group 54.39 ± 3.57%; $p = 0.30$, unpaired t-test). While aSYN signal was mainly restricted to the LC region, structures directly next to the LC (ncl. parabrachialis, Barrington's nucleus, mesencephalic trigeminal nucleus and vestibular nuclei) also exhibited some aSYN. To assess the induction of LC cell loss as a consequence of protein overexpression unbiased stereological quantification of TH positive cells 1, 3, 6 and 9 weeks after viral vector delivery (Fig. 2 a,b) were carried out. This data revealed a progressive loss of noradrenergic LC neurons for the A53T-aSYN injected animals starting 3 weeks after viral vector delivery with 15.86 ± 2.09% cell loss compared to control side ($p < 0.01$, two-way ANOVA analysis followed by Tukey's post-hoc test), increasing up to 56.25 ± 5.19% after 9 weeks ($p < 0.001$, Two-way ANOVA analysis followed by Tukey's post-hoc test). Moreover, LC cell loss was accompanied by qualitative changes of neuronal morphology, including dystrophic axons and pyknotic perikarya (Fig. 2c). To assess posttranslational modifications of the overexpressed aSYN, like S129-phosphorylation or ubiquitination, several immunofluorescence stainings were carried out for the respective time points. Overexpression of A53T-aSYN resulted in strong and progressive phosphorylation of aSYN being evident as early as 1 week after viral vector delivery (Fig. 3). The

degree of aSYN phosphorylation correlated strongly with the degree of noradrenergic neurodegeneration ($r = 0.67$, $p < 0.05$, Pearson's correlation coefficient with 95% confidence interval). Further, proteinase K digestion experiments revealed formation of insoluble p62 and ubiquitin positive aggregates that were restricted to the ipsilateral LC region (Fig. 4). Notably, these small circular aggregates with an immuno-negative core were not located in LC neurons but IbA1 positive microglial cells (Fig. 5). As activation of micro- and astroglia are core features not only of clinical PD but also experimental animal models, we investigated the involvement of micro- and astroglia by triple immunofluorescence stainings for IbA1 (microglial marker), GFAP (astroglial marker) and TH. Notably, overexpression of A53T-aSYN lead to a strong increase of micro- and astroglial signal intensities in a time-dependent manner within the LC region (Fig. 6a-c). First glial reactions were observed already 3 weeks after viral vector delivery. 3D reconstructed high magnification confocal images revealed a dense glial network in A53T-aSYN overexpressing animals, in which the remaining TH positive LC neurons were embedded 3 weeks after rAAV injection (Fig. 6d). The degree of microgliosis was further found to correlate strongly with noradrenergic LC cell loss for the respective time points (Fig. 6f,g). Importantly, overexpression of luciferase was not associated with induction of micro- or astrogliosis at any time point when the injected side was compared against the non-injected side or when 1 week of luciferase overexpression was compared against 9 weeks of luciferase overexpression (Fig. 6a-d). After assessing the local histopathological alteration, we aimed to address the question if a locally induced aSYN pathology can induce a brain-wide propagation of aSYN. Therefore, we performed aSYN immunofluorescence stainings on predetermined brain sections and systematically analyzed all sections for signs of transported aSYN. Already one week after initiation of A53T-aSYN overexpression in the right LC region, abundant aSYN positive axons were observed in various brain regions which are known LC output regions, indicating rapid anterograde transport of the human aSYN (Fig. 7). Despite the increase of axonal aSYN signal at later time points, no aSYN positive cell bodies were detected outside of the LC region at any investigated time point. In contrast, luciferase overexpressing animals exhibited no aSYN signal at any time point. In addition, the luciferase staining pattern was limited to the injection side suggesting no axonal protein transport. Since dopaminergic SNc cells were densely surrounded by aSYN positive axons already 1 week after viral vector delivery we systematically quantified dopaminergic SNc cells after the 9 week time point (Fig. 8b). Notably, our stereological quantification revealed no significant difference of TH-immunoreactive neurons between A53T-aSYN overexpressing animals compared to luciferase control mice, neither for the left nor for the right SNc ($p > 0.05$, One-way ANOVA).

5.1.4. Own contribution

All experimental data from Figs. 1-6 of the summarized publication were generated, analyzed and interpreted by me. This included the establishment of the framework (rAAV titer, rAAV volume, stereotactic coordinates) for the stereotactic surgeries to achieve a focal overexpression of A53T-aSYN or luciferase in the LC region, as well as handling and sacrificing of the experimental animals. For the data presented in Figs. 1-6 I established and performed the respective immunohistochemical (IHC) stainings, acquired all epifluorescence and confocal images, performed the complete data analysis, and carried out all parts of the statistical analysis. Regarding the manuscript, I compiled Figs. 1-6 and wrote the first draft of the manuscript and the revised version after peer-review. Dr. med. univ. Fanni F. Geibl contributed to data collection, analysis and interpretation of the brain-wide aSYN pathology presented in Figs. 7-8. Further, she wrote the first draft of the respective parts for the submitted manuscript and the revised version after peer-review. Dr. rer. nat. Bolam Lee conducted several experiments, which were finally not included in this publication but necessary for data validation. James B. Koprich PhD, and Jonathan M. Brotchie PhD, provided the viral vectors and technical expertise. Dr. rer. nat. Wei-Hua Chiu, Prof. Dr. med. Lars Timmermann, Prof. Dr. rer. nat. Niels Decher, Dr. rer. nat. Lina A. Matschke, and Prof. Dr. med. Wolfgang H. Oertel were involved in the conception, planning and supervision of the study. Prof. Dr. med. Wolfgang H. Oertel was the lead supervisor of this project.

Martin T. Henrich and Fanni F. Geibl are shared first authors on this publication.

5.2. The locus coeruleus – another vulnerability target in Parkinson's disease

Oertel WH, **Henrich MT**, Janzen A, Geibl FF: The locus coeruleus – another vulnerability target in Parkinson's disease. Mov Disord. 2019 24(2):197. doi:10.1002/mds.27785

Impact factor: 8.061 (2018)

5.2.1. Aim of the publication

Despite the central role of noradrenergic deficiency within the prodromal and motor phase of PD (Benarroch, 2009; Delaville et al., 2011; Weinshenker, 2018), the majority of clinical and basic PD research is still focused on the causes, consequences and therapeutic implications of dopaminergic cell loss in the SNc. Consequences of the missing awareness of LC dysfunction and cell loss in PD are e.g. a lack of treatment options for the majority of symptoms based on noradrenergic deficiency (Espay et al., 2014) and the absence of knowledge on the whereabouts of LC degeneration during the course of PD (Weinshenker, 2018). The main aims of the article entitled "The locus coeruleus – another vulnerability target in Parkinson's disease" were: to highlight the unique role of the LC in the prodromal and manifest phase of PD, to emphasize its contribution to the symptomatology and progression of PD, to point out the potential for the development of new therapeutic approaches, and to address findings and questions so far neglected in basic and clinical PD research.

5.2.2. Viewpoint

To address the aforementioned aims we firstly summarized the key neuroanatomical features of the LC noradrenergic system including its small size with only 35 000 neurons per hemisphere in the human (Aston-Jones and Cohen, 2005; Espay et al., 2014), the characteristic location next to the 4th ventricle, the extensive input output connectome (Aston-Jones and Cohen, 2005; Schwarz et al., 2015), and the involvement in highly preserved brain functions like generation of arousal, behavioral adaptations to incoming sensory information, and memory consolidation (Berridge and Waterhouse, 2003; Berridge, 2008; Sara, 2009). We then depicted the resulting consequences of noradrenergic deficiency in PD regarding several PD non-motor and motor symptoms, including but not limited to depression, anxiety, cognitive deficits, REM-sleep-behavior disorder, and freezing of gait (Espay et al., 2014). While keeping in mind that the causes of LC Lewy pathology and cell loss are still unclear, we summarized the most relevant vulnerability factors which are thought to mediate LC dysfunction and degeneration in PD (Weinshenker, 2018), and the so far employed animal models which investigated LC pathology in the experimental setting. Another major concern of this article was to emphasize that Lewy pathology within the LC and the clinical symptoms relating to decreased noradrenergic neurotransmission are not only features of the manifest motor phase of PD, but are also highly relevant in context to the early prodromal phase (Delaville et al., 2011; Weinshenker,

2018). Therefore, we discussed three key topics related to the involvement of the LC in prodromal and manifest PD: 1) new opportunities for improved symptomatic treatment over noradrenergic replacement therapy, 2) development of disease modifying therapy approaches (Qian et al., 2011; Feinstein et al., 2016; Mittal et al., 2017), and 3) identification and characterization of new disease progression biomarkers. To provide a comprehensive overview about the clinical research related to the LC noradrenergic system in PD, we summarized the most important clinical trials investigating different approaches for noradrenergic replacement therapy and their outcome (Fornai et al., 2007; Rommelfanger and Weinshenker, 2007). Since the LC exhibits Lewy pathology early in the prodromal phase of PD while neurodegeneration is commonly observed in manifest late stage PD, this noradrenergic structure seems to represent a suitable candidate for research on disease progression biomarkers. Therefore, we discussed three possible opportunities to use the involvement of the LC system in prodromal PD for the development of new disease progression markers. These include 1) neuromelanin-sensitive MRI approaches (Schwarz et al., 2017; Sulzer et al., 2018), 2) noradrenergic PET imaging (Pavese et al., 2011; Nahimi et al., 2018) and, 3) attentional set shifting (Owen et al., 1993) to monitor LC function *in vivo*.

5.2.3. Own contribution

For this publication I wrote the following sections of the first draft of the manuscript: "Introduction", "The noradrenergic LC – a structure to be rediscovered for PD research", and "Determinants of LC vulnerability in PD". I further assisted in the design and editing of Figure 1 and both tables, and contributed to the review and editing of the final manuscript. Prof. Wolfgang H. Oertel wrote the final manuscript and supervised the project. Dr. med. Annette Janzen assisted in the conception of the project and reviewed the final manuscript. Dr. med. univ. Fanni F. Geibl wrote the following sections for the first draft of the manuscript: "LC pathology in prodromal and manifest PD – opportunities for improved symptomatic treatment and neuroprotection" and "Kinetics of LC neurodegeneration – Potential for novel LC progression biomarkers?" She further compiled Tables 1 and 2 and created Figure 1.

5.3. Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease

Geibl FF*, **Henrich MT***, Oertel WH: Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease. (*shared first authors). J Neural Transm (Vienna). 2019 Apr;126(4):377-396. doi: 10.1007/s00702-019-01970-9

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5.3.1. Aim of the publication

Since Arvid Carlsson demonstrated in 1957 (Carlsson et al., 1957) that administration of reserpine resulted in manifest striatal DA depletion which was accompanied by parkinsonian symptomatology (Fig. 1), PD and its cardinal motor symptoms have been linked to DA deficiency in the nigrostriatal system (Rodriguez-Oroz et al., 2009). Despite the central role of DA in PD, it became evident that the neurodegenerative alterations do not affect all dopaminergic systems of the mammalian CNS (A8 – A17) to the same extent (Halliday et al., 1996; McRitchie et al., 1997; Seidel et al., 2015). This applies to the distribution of Lewy pathology within the different dopaminergic populations as well as to the distribution of dopaminergic cell loss. The narrative publication "Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease" dissects the cardinal motor symptomatology of PD, summarizes their neuroanatomical and neuropathological correlates within the different mammalian dopaminergic systems, and discusses the neurobiological diversity of the dopaminergic neurons and their susceptibility to the disease mechanisms in PD.

5.3.2. Review

Despite the fact that PD is now seen as a multisystem disease affecting the CNS, PNS (Comi et al., 2014; Del Tredici and Braak, 2016), and ENS (Wakabayashi et al., 1990; Derkinderen et al., 2011), it has traditionally been considered a disease of dopaminergic deficiency based on the degeneration of the SNc. To explain this monocentric view, we first summarized key milestones of the 200 years long history of PD, beginning with its first description by James Parkinson in 1817 (Parkinson, 1817), over the discovery of the characteristic Lewy body, and Arvid Carlsson's DA depletion studies (Carlsson et al., 1957) which paved the way for the dopaminergic replacement era. We then discussed the PD defining cardinal motor symptomatology (bradykinesia or akinesia, resting tremor, and rigidity) which are commonly seen as the consequence of nigrostriatal degeneration and following DA deficiency (Rodriguez-Oroz et al., 2009). The article highlights that, while bradykinesia and rigidity are clearly linked to decreased striatal DA, there is increasing evidence that tremor is not related to DA deficiency (Rinne et al., 1989; Politis, 2014), suggesting that even the cardinal motor symptomatology cannot be fully explained by single loss of DA. A major aim of this publication was to summarize the neuroanatomical knowledge on the ten different dopaminergic systems of the brain (A8-A17) (Björklund and Dunnett, 2007) and to systematically illustrate the neuropathological alterations of

each system in PD. Since dopaminergic systems outside of the midbrain have not been systematically investigated for Lewy pathology and neurodegeneration, the article aimed to clearly depict the existing evidence for PD pathology within each of the extramesencephalic dopaminergic systems, but also the mesencephalic complex. Based on the studies conducted so far we report a strong heterogeneity regarding Lewy pathology and occurring neurodegeneration. While the ventral tegmental area (Seidel et al., 2015) and the SNc (Hirsch et al., 1988; Halliday et al., 1996) exhibit moderate to severe cell loss, the dopaminergic retrorubral field neurons (McRitchie et al., 1997) or the dopaminergic cells in the olfactory bulb (Ubeda-Bañon et al., 2010) seem to be spared of PD pathology. Therefore, we suggested speaking of a spectrum of susceptibility, in which the dopaminergic SNc seems to be the most vulnerable structure followed by the dopaminergic ventral tegmental area neurons. The article highlights that PD is not simply a disease of dopaminergic deficiency and neither are all dopaminergic systems affected in PD. While most of the results presented here are based on postmortem histological analysis, new dopaminergic imaging approaches, like PET-, SPECT- or MRI-techniques, can now be applied to investigate alterations of the dopaminergic systems and metabolic changes caused by PD (Stoessl et al., 2011; Weingarten et al., 2015). Since dopaminergic neuroimaging gains increasing relevance, we discussed three key applications for dopaminergic imaging in PD: 1) clinical research on dopaminergic dysfunction in PD (Politis, 2014), 2) diagnosis of PD and its distinction from atypical parkinsonian syndromes (Scherfler et al., 2007), and 3) the identification of subclinical dopaminergic deficits in prodromal PD patients (Heller et al., 2017). Another opportunity which has proven useful for the identification of distinct symptoms associated with dysfunctional dopaminergic neurotransmission, are dopaminergic medication studies. Within the article we summarized the effect of dopaminergic medication with special emphasize on the resulting symptomatology in the "on and off state".

5.3.3. Own contribution

For this publication I wrote the following sections of the manuscript: "Parkinsonism as the core feature of PD", "The dopaminergic systems of the brain", "Neuropathological alterations of the dopaminergic systems in PD", and "Symptomatology 'off'/'on' dopaminergic medication: conclusions of clinical studies". I further compiled Table 1 and created Figures 2, 3, 6. Dr. med. univ. Fanni F. Geibl wrote the following sections of the manuscript: "Introduction", "A long road to go", "What we can learn from neuroimaging studies", and "Concluding remarks". She further designed and created Figures 1, 4, 5. Prof. Wolfgang H. Oertel supervised the project including review and editing of the final manuscript.

Martin T. Henrich and Fanni F. Geibl are shared first authors on this publication.

6. Discussion

6.1. A53T-α-synuclein overexpression in murine locus coeruleus induces Parkinson's disease-like pathology in neurons and glia

In this study, we present the first targeted LC α-synucleinopathy mouse model that replicates cardinal histopathological features of human PD pathology. Firstly, we confirmed that LC cells are susceptible to viral vector mediated overexpression of mutant A53T-aSYN, a feature which has been commonly shown for other vulnerable cell groups, like dopaminergic SNc neurons (Koprich et al., 2010; Ip et al., 2017). In contrast to most of the previously published models, we observed strong overexpression of aSYN already a few days after viral vector delivery, indicating a high transcription rate of the injected genomic particles. Since the employed viral vector system was based on a chicken β-actin promoter hybridized with a CMV immediate early enhancer sequence (CMV/CBA), it was not possible to restrict the evolving proteinopathy completely to TH positive LC neurons and adjacent structures like the ncl. parabrachialis or Barrington's nucleus also exhibited some aSYN positive cells (van der Perren et al., 2015). However, the majority of aSYN positive cells were clearly located within the LC region. In addition, co-transduction of adjacent neuronal populations is a known phenomenon when non-neurotransmitter specific vector systems are applied. After initiation of the α-synucleinopathy in the murine LC, we characterized the development of aggregated aSYN, a core hallmark of human Lewy pathology. We therefore conducted immunofluorescence stainings to detect phosphorylated aSYN, a posttranslational modification characteristic for pathological aSYN forms (Fujiwara et al., 2002; Anderson et al., 2006), and performed proteinase K digestion experiments (Fernagut et al., 2007; Taschenberger et al., 2012) to visualize aggregated aSYN. While LC neurons exhibited extensive amounts of phosphorylated-aSYN, proteinase K-resistant and Ubi-1 and p62 positive aSYN aggregates were only found in microglial cells. Two important conclusions can be drawn from these experiments. First, the observed discrepancy between strong phosphorylation of aSYN and the relative sparse number of proteinase K-resistant aggregates suggest that S129-phosphorylation of aSYN does not necessarily indicate aggregation or insolubility of aSYN (Uchihara and Giasson, 2016). This is of high importance since aSYN phosphorylation is commonly equated with formation of insoluble aSYN aggregates. Second, neuronal excretion and glial uptake of pathological aSYN seem highly relevant for the clearance of excessive intracellular aSYN (Zhang et al., 2005; Bruck et al., 2016). Supporting this hypothesis we observed direct physical contacts between LC cells and micro- and astroglia being evident 3 weeks after rAAV injection. The presence of aSYN within micro- and astroglia implies that glial dysfunction or failure could be a potential contributor of PD progression, once the local glial protein degradation system is overburdened (Halliday and Stevens, 2011). We observed a loss of TH positive LC cells starting 3 weeks after injection of the A53T-aSYN-gene containing rAAV's. The quantified neurodegeneration progressed continuously over the investigated time points and affected the entire length of the LC. In contrast, overexpression of our control protein luciferase did not result in LC cell loss at any investigated time point. This

clearly indicates that LC cells are vulnerable to artificially increased amounts of intracellular A53T-aSYN, an observation so far not reported. Our model thereby replicates characteristic features of the human PD pathology. Since recent evidence suggested that pathological aSYN species can be transported to interconnected brain regions (Desplats et al., 2009; Freundt et al., 2012; Volpicelli-Daley et al., 2014; Rey et al., 2016) mediating the progression of the disease, we performed a whole-brain aSYN distribution analysis which revealed massive axonal aSYN signal in LC output target regions in combination with lack of aSYN in cell somata of interconnected brain regions. This finding suggests that the overexpressed human A53T-aSYN, once produced in the cytoplasm of LC neurons, is transported axonally in the anterograde direction towards the synaptic terminals but does not spread over the synapse to interconnected brain regions within the investigated time frame of 9 weeks. This is in line with previous studies (Kirik et al., 2002; Maingay et al., 2006; Uchihara and Giasson, 2016) and stands in clear contrast to the aSYN fibril model, in which injection of pre-formed aSYN fibrils leads to trans-synaptic spreading of aSYN pathology to anatomically interconnected brain regions (Brundin et al., 2016; Rey et al., 2016; Rey et al., 2018). Notably, based on the intention to characterize the initial histopathological alterations of the noradrenergic LC, we have limited our analysis to the first 9 weeks after onset of aSYN overexpression. Increasing evidence (Rusconi et al., 2018) suggests that longer survival times might allow trans-synaptic spreading and consequent neurodegeneration of interconnected neuronal systems also in rAAV based aSYN mouse models. Therefore, it would be of interest to reevaluate the established histological markers after 6 or 12 months of A53T-aSYN overexpression. Another aspect which should be considered during the interpretation of this study is that we have used an rAAV system which leads to overexpression of the mutant-human-A53T-form of aSYN and not human-wildtype-aSYN, thereby probably inducing a more aggressive α-synucleinopathy (Li et al., 2001; Coskuner and Wise-Scira, 2013).

Taken together, degeneration of the LC noradrenergic system occurs early in PD and a notable amount of the PD non-motor symptoms are associated with dysfunction or degeneration of neurons in the LC (Espay et al., 2014; Weinshenker, 2018). The current study is the first to describe the occurrence of PD-like pathology in a murine model in which human A53T-aSYN is acutely overexpressed in the LC region. Furthermore, our data shed the first light on the vulnerability of noradrenergic LC neurons in an aSYN overexpression rodent model, provide neuronal and glial markers which allow testing of potentially neuroprotective substances, and represent the first *in vivo* evidence of p62- and Ubi1-positive inclusions in microglial cells.

6.2. The locus coeruleus – another vulnerability target in Parkinson's disease

Compared to the dopaminergic SNc the noradrenergic LC represents a neglected research target in PD. A pubmed literature search conducted in 2019 revealed 11,221 search results for "Parkinson's disease AND substantia nigra", but only 500 results for "Parkinson's disease AND locus coeruleus". Despite the increasing interest in prodromal PD and the clear evidence for noradrenergic deficiency in the early phase of PD, research on the LC in context of PD remains sparse with only around 20 publications per year (Fig. 4).

Data from human post mortem samples indicate early and profound Lewy pathology within the LC noradrenergic system which is accompanied by early loss of noradrenergic axons in output projection targets (Braak stage 2) (Braak et al., 2003; Braak et al., 2004). In addition, most studies investigating loss of LC neurons reported LC cell death from 21 to 93% (Hirsch et al., 1988; Chan-Palay and Asan, 1989; Paulus and Jellinger, 1991). Notably, decreased LC cell numbers were predominantly observed in advanced PD stages, indicating that the LC may possess specific cellular characteristics which facilitate partial resilience to the disease process and thereby provide the capacity to survive the pathological processes for many years. Importantly, this stands in clear contrast to the dopaminergic SNc, where nigral Lewy pathology is promptly followed by loss of SNc neurons (Braak et al., 2003). Based on this assumption, noradrenergic symptomatology evolving during the prodromal phase is likely mediated by cellular dysfunction of the affected LC system and not based on mere cell loss. Noradrenergic deficiency contributes to several non-motor and motor symptoms e.g. cognitive impairment, depression, anxiety, apathy, fatigue, REM-sleep-behavior-disorder, impaired motor control, and freezing of gait (Espay et al., 2014). While Lewy pathology seems to be one factor for LC vulnerability in PD, most neuronal groups at risk share a common anatomical and electrophysiological phenotype including several intrinsic cellular factors that are thought to mediate the vulnerability to the disease process. LC neurons have been identified to possess several of those vulnerability features which are thought to render them susceptible to PD: 1) extensively branched and thinly myelinated axons composing a huge output projectome, 2) intrinsic pacemaking activity (3-4 Hz), generating continuously action potentials, 3) low Ca^{2+} buffering capacity, 4) high amount of intracellular heavy metals and neuromelanin, and 5) the burden to generate the highly reactive neurotransmitter (Surmeier et al., 2017b; Weinshenker, 2018). Potential vulnerability factors which are not shared with dopaminergic SNc neurons include: 1) extensive varicosities for paracrine signaling, 2) dense innervation of blood vessels, and 3) close proximity to the 4th ventricle (Fig. 1) (Weinshenker, 2018). Animal models conducted so far reveal that LC cells are additionally susceptible to administration of neurotoxins which are commonly used to ablate the nigrostriatal system and overexpression of aSYN (Table 1). Apart from the contribution to the pathophysiology and symptomatology of PD, research on the LC offers further opportunities. While dopaminergic replacement therapy is the gold standard treatment for PD, there is mounting evidence that noradrenergic replacement or enhancement of noradrenergic neurotransmission leads to

improvement of several non-motor and motor complications of PD, e.g. improvement of global cognition and executive functions or gait and motor symptoms (Table 2). Importantly, these studies were conducted in *de novo* or manifest PD patients where noradrenergic neurodegeneration is generally advanced. Since several clinical manifestations of decreased noradrenergic neurotransmission, such as depression, anxiety, or cognitive impairment are already present in the prodromal phase of PD, based on early loss of noradrenergic axons in output projection targets, we suggest that trials employing noradrenergic replacement therapy should also be conducted in prodromal PD patient cohorts. The presence of LC pathology during the prodromal and manifest phase of PD offers another intriguing possibility. Based on the kinetic of LC dysfunction and cell loss we argue that the LC represents a suitable structure for characterization of new disease progression biomarkers which allow monitoring the ongoing neurodegenerative alterations from the prodromal phase to manifest motor and late stage PD. First promising attempts made in *de novo* or manifest PD patients include structural neuromelanin-sensitive MRI of the LC region, PET imaging to measure noradrenergic LC function, or attentional set shifting assessed with the Wisconsin Card Sorting Test or the Intra-/Extra-Dimensional Attentional Set-Shifting Task.

Taken together, this narrative publication summarizes key histopathological features of LC cell loss in PD patients, discusses specific vulnerability factors likely implicated in noradrenergic neurodegeneration, recapitulates the experimental and clinical studies conducted so far, and suggests new opportunities for improved symptomatic treatment and development of biomarkers to monitor the progression of PD.

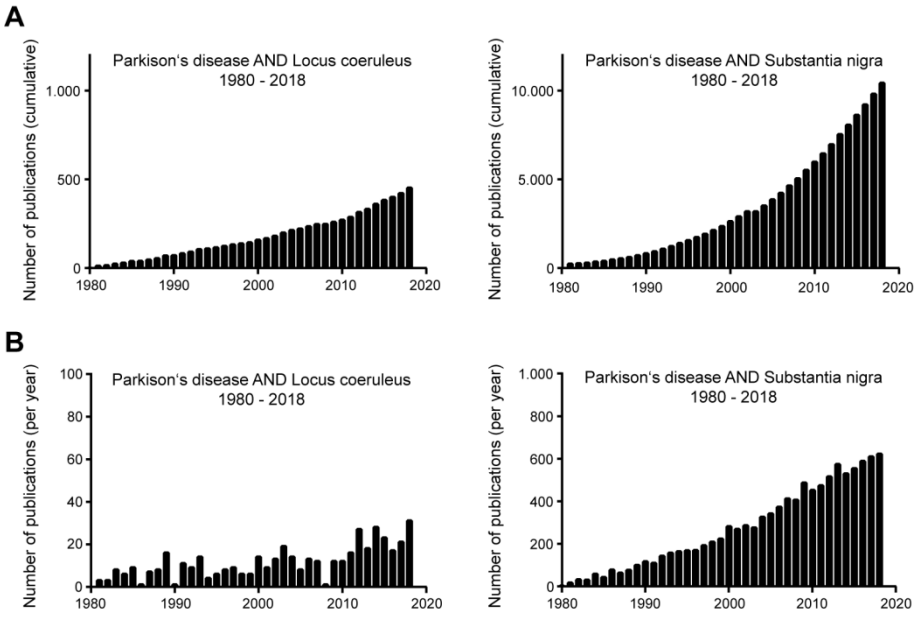

Figure 4 | Overview of the publication history for LC and SN in PD

A. Number of search results for "Parkinson's disease AND locus coeruleus" and "Parkinson's disease AND substantia nigra" *cumulated* for the years 1980-2018.

B. Number of search results for "Parkinson's disease AND locus coeruleus" and "Parkinson's disease AND substantia nigra" *per year* for the years 1980-2018.

6.3. Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease

Loss of dopaminergic SNc neurons and consequent striatal dopamine deficiency are core features of PD and clearly linked to the cardinal parkinsonian motor abnormalities. Furthermore, the presence of nigrostriatal neurodegeneration and its consequences are central for PD diagnosis (Table 1) and onset of DA replacement therapy (Postuma et al., 2015). PD pathology is not observed in all neuronal populations of a patients' CNS, neither is the pathology randomly distributed. Lewy pathology and associated neurodegeneration appears to be limited to distinct dopaminergic, noradrenergic, serotonergic and cholinergic neuronal systems. Even within these affected neurotransmitter systems only distinct cell groups exhibit Lewy pathology or cell loss. This spectrum of susceptibility to the disease process has been firstly shown for the dopaminergic systems. Within the mammalian CNS dopaminergic neurons are clustered into ten dopaminergic systems (A8-A17) which are distributed over the ventral mesencephalon, diencephalon, olfactory bulb and retina (Fig. 2). In regard to PD, the most extensively studied dopaminergic systems include the retrorubral field (A8), the SNc (A9), and the ventral tegmental area (A10). All three of them form a continuum of morphologically indistinguishable dopaminergic neurons referred to as the ventral mesencephalic dopaminergic complex (A8-A10) (Fig. 3). While the SNc (A9) exhibits cell loss between 41% and 79% across studies, on average 67% with disproportionately high neurodegeneration in the ventrolateral and caudal subregion (70-90% cell loss) (Bogerts et al., 1983; Javoy-Agid et al., 1984; Hirsch et al., 1988; Waters et al., 1988; German et al., 1989; Kempster et al., 1989; Gibb and Lees, 1991; Halliday et al., 1996; Damier et al., 1999; Zarow et al., 2003; Alberico et al., 2015), dopaminergic neurons in the retrorubral field (A8) show no or only minor neurodegeneration (McRitchie et al., 1997). In contrast, dopaminergic neurons in the ventral tegmental area (A10) exhibit a moderate degree of Lewy pathology and neurodegeneration (Seidel et al., 2015), on average 53% which is consistently below the observed neurodegeneration in the SNc (Javoy-Agid and Agid, 1980; Bogerts et al., 1983; Javoy-Agid et al., 1984; Uhl et al., 1985; Hirsch et al., 1988; Waters et al., 1988; German et al., 1989; McRitchie et al., 1997; Damier et al., 1999; Alberico et al., 2015). The post mortem histopathological reports uniformly suggest a spectrum of susceptibility, in which the dopaminergic cells of the ventral SNc (A9) are the most vulnerable, followed by the ventral tegmental area neurons (A10), the dorsal tier of the SNc (A9), and the dopaminergic retrorubral field neurons (A8). On a pathophysiological level the observed heterogeneity could be based on the neurobiological diversity of the cellular subgroups, taking into account that there is not one general type of dopaminergic neuron but rather a spectrum of different dopaminergic phenotypes. Classically a dopaminergic neuron is characterized by several cellular features (Fig. 4): (a) DA as the main neurotransmitter, (b) a DA synthetizing machinery (tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC)), (c) DA degrading enzymes, (d) DA transporters (i.e. vesicular monoamine transporter 2 (VMAT2), DA transporter (DAT)), and (e) autoreceptors (i.e. D_2-receptor) (Vernier et al., 2004). Notably, not all dopaminergic populations possess all of the aforementioned features thereby only partially fulfilling

all criteria for a classical dopaminergic neuron, e.g. A11 neurons lack AADC or DAT expression, suggesting that L-DOPA is not converted to DA, making these neurons L-DOPAergic rather than dopaminergic (Barraud et al., 2010). Apart from the observed neurotransmitter related alterations there are several other proteomic and metabolic differences between the different dopaminergic systems. The highly vulnerable ventral tier of the SNc (A9) exhibits a significantly higher intracellular Ca^{2+}-burden compared to the dorsal SNc (A9) or the ventral tegmental area (A10). It has been hypothesized that this could mainly result from lower expression of parvalbumin and calretinin, two calcium binding proteins (Yamada et al., 1990; McRitchie et al., 1996; Parent et al., 1996; Chung et al., 2005). In addition, SNc (A9) neurons show an almost 3-fold increased basal oxidative phosphorylation rate leading to a significantly lower respiratory reserve compared to ventral tegmental area neurons (A10) (Pacelli et al., 2015). The studies conducted so far suggest that there is neither one general type of dopaminergic neuron but rather a spectrum of different dopaminergic phenotypes, nor is the PD pathology distributed homogenously over the different dopaminergic systems. This also brings important implications for DA replacement therapy. Based on the inhomogeneous loss of dopaminergic neurons within the different dopaminergic systems, doses of L-DOPA which are needed to replace the dopaminergic deficit in the severely affected SNc (A9) simultaneously 'overdose' the better preserved dopaminergic networks, resulting in symptoms of hyperdopaminergism such as dyskinesia, impaired learning, impulse control disorders, or mania (Fig. 6) (Gotham et al., 1988; Swainson et al., 2000; Vaillancourt et al., 2013; Vriend et al., 2014; Joutsa et al., 2015; Voon et al., 2017). Especially in the advanced disease stages, PD symptomatology fluctuates between hypodopaminergic states as a consequence of disease progression and hyperdopaminergic states as a side effect of DA replacement therapy.

Taken together, neurodegeneration of the nigrostriatal dopaminergic system and concurrent DA deficiency in the basal ganglia represent core hallmarks of PD with implications for PD diagnosis, DA replacement strategies, and therapeutic complications. The conducted studies so far, suggest a spectrum of susceptibility, in which the dopaminergic neurons of the ventral SNc (A9) are the most vulnerable, followed by ventral tegmental area neurons (A10), the dorsal SNc (A9), and the retrorubral field cells (A8). The degree of susceptibility is associated with a rich neurobiological diversity of the different dopaminergic systems, suggesting that there is not one general type of dopaminergic neuron but rather a spectrum of different dopaminergic phenotypes. However, despite the notable amount of data on the midbrain dopaminergic systems (A8-A10), the diencephalic, olfactory bulbar and retinal dopaminergic systems have not been thoroughly investigated in regard to Lewy pathology or neurodegeneration, yet.

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8. Appendix

8.1. Publication 1

A53T-α-synuclein overexpression in murine locus coeruleus induces Parkinson's disease-like pathology in neurons and glia

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Abstract

Degeneration of noradrenergic locus coeruleus neurons occurs during the prodromal phase of Parkinson's disease and contributes to a variety of non-motor symptoms, e.g. depression, anxiety and REM sleep behavior disorder. This study was designed to establish the first locus coeruleus α-synucleinopathy mouse model, which should provide sufficient information about the time-course of noradrenergic neurodegeneration, replicate cardinal histopathological features of the human Parkinson's disease neuropathology and finally lead to robust histological markers, which are sufficient to assess the pathological changes in a quantitative and qualitative way. We show that targeted viral vector-mediated overexpression of human mutant A53T-α-synuclein in vivo in locus coeruleus neurons of wild-type mice resulted in progressive noradrenergic neurodegeneration over a time frame of 9 weeks. Observed neuronal cell loss was accompanied by progressive α-synuclein phosphorylation, formation of proteinase K-resistant α-synuclein-aggregates, accumulation of Ubi-1- and p62-positive inclusions in microglia and induction of progressive micro- and astrogliosis. Apart from this local pathology, abundant α-synuclein-positive axons were found in locus coeruleus output regions, indicating rapid anterograde axonal transport of A53T-α-synuclein. Taken together, we present the first model of α-synucleinopathy in the murine locus coeruleus, replicating essential morphological features of human Parkinson's disease pathology. This new model may contribute to the research on prodromal Parkinson's disease, in respect to pathophysiology and the development of disease-modifying therapy.

Keywords

Parkinson's disease; locus coeruleus; alpha-synuclein; adeno-associated viral vectors; prodromal mouse model; microglia, noradrenergic neurons

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder [1] characterized by progressive degeneration of dopaminergic (DA) substantia nigra (SN) neurons and their striatal axon terminals [2, 3]. One characteristic neuropathological hallmark of PD are intracytoplasmic eosinophilic inclusions, the so-called Lewy bodies, which develop in specific brain regions in a spatio-temporal pattern and consist predominantly of misfolded α-synuclein (aSYN) [4, 5]. The finding that duplications, triplications or missense mutations (e.g. A53T, A30P or G46L) of the aSYN gene (SNCA) cause familial forms of PD [6, 7] has justified the assumption that aSYN plays a crucial role in the pathogenesis of PD.

Only within the last 20 years it is accepted that PD cannot solely be understood as a disease associated with the degeneration of DA SN neurons, as the PD pathology involves the central, peripheral, autonomic and enteric nervous system [5, 8–11]. The degeneration of DA SN neurons and the onset of motor dysfunction are preceded by a latency of several years, if not decades, in which the PD pathology develops in brain regions outside the DA SN. This phase, termed prodromal PD, is clinically characterized by the occurrence of certain non-motor symptoms, e.g. hyposmia, constipation, depression and idiopathic REM sleep behavior disorder [12–14]. Since the prodromal phase is seen as the ideal time window for applying disease-modifying therapy [15, 16], it is of high importance to establish animal models, which allow testing of new and future therapeutic approaches on brain structures that are affected during prodromal PD. The noradrenergic locus coeruleus (LC), a monoaminergic nucleus located in the pontine brainstem [17, 18], plays a crucial role during the prodromal phase of PD and represents therefore an ideal brain structure for such in-depth characterization in an experimental animal model [19]. Dysfunction and degeneration of neurons in the LC region are associated with several of the above listed non-motor symptoms, including depression, signs of reduced arousal, anxiety and REM sleep behavior disorder (RBD) [20–22]. Neuropathological analysis of human PD brain samples revealed up to 80% LC neuronal cell loss in PD patients, thereby exceeding the degree of SN neurodegeneration in the same individuals [23, 24]. Moreover, experimental evidence indicates that toxin-induced LC cell loss sensitized DA SN neurons for neurodegeneration [25, 26], whereas noradrenergic hyperinnervation resulted in neuroprotective effects [27]. This data implies that LC neurodegeneration itself plays a double role by firstly being responsible for several non-motor symptoms and secondly for accelerating the progression of PD at the nigral level [21]. LC cells exhibit a common at-risk phenotype compared to other neuronal populations such as the DA nigral neurons and the cholinergic neurons of the dorsal motor nucleus of the vagal nerve which undergo neurodegeneration in PD [28, 29]. LC neurons integrate information from a broad range of different brain regions and broadcast information with extensively branched and thinly myelinated axons throughout the complete neuroaxis [18, 30]. Furthermore, they exhibit an intrinsic pacemaking activity, generating action potentials continuously [31] thereby raising their basal metabolic stress level [28].

In this study, we have characterized the first model of α-synucleinopathy in the murine LC. We show that targeted viral vector-mediated overexpression of human mutant A53T-aSYN in vivo in LC neurons of wild-type mice resulted in progressive LC neurodegeneration over a time frame of 9 weeks. Observed LC cell loss was accompanied by prominent and over time increasing micro- and astrogliosis. In addition, our data revealed accumulation of phosphorylated aSYN, progressive aggregation of aSYN as demonstrated by proteinase K-resistant aSYN aggregates and Ubi-1- and p62-positive inclusions comparable with findings from human PD samples. Co-staining with different cellular markers revealed that the p62- and Ubi-1-positive aggregates were found exclusively in microglial cells, while being absent in neurons, astrocytes and oligodendrocytes. Beside this local LC pathology, we observed abundant aSYN-positive axons in a high number of LC output regions, indicating rapid anterograde axonal transport of the human aSYN. In conclusion, our new murine LC model replicated cardinal morphological features of human PD pathology.

Methods

Animals

A total of 70 wild-type male C57BL/6N mice (Charles River, Sulzfeld, Germany), 8 weeks old at the beginning of the experiment, were used. Mice were housed in individually ventilated cages with ad libitum access to food and water under a 12 h/12h light-dark cycle. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted (Regierungspräsidium Giessen, Germany V54-19 c 20 15 h 01 MR 20/15 Nr. 66/2015).

Recombinant adeno-associated viral (rAAV) vectors and stereotactic injection

Two different recombinant adeno-associated viral (rAAV) vectors of a mixed 1/2 serotype were used to overexpress human mutant-A53T-aSYN (rAAV1/2-CMV/CBA-human-A53T-aSYN-WPRE-BGH-pA (rAAV1/2-A53T-aSYN); viral titer 5.1 x 1012 gp/ml, purchased from GeneDetect) or luciferase (rAAV1/2-CMV/CBA-luciferase-WPRE-BGH-pA (rAAV1/2-Luc), viral titer 5.0 x 1012 gp/ml, purchased from GeneDetect). Each of the two vectors was driven by a chicken beta actin (CBA) promoter combined with a cytomegalovirus (CMV) immediate early enhancer sequence and a woodchuck post-transcriptional regulatory element (WPRE) to assess a high transcription rate [32, 33]. For stereotactic delivery of the rAAV vectors, mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine via intraperitoneal injection. A volume of 1.25 µl of rAAV1/2-A53T-aSYN or rAAV1/2-Luc was stereotactically injected in the right LC region using a microinjector (UltraMicro Pump UMP3, World Precision Instruments) with a velocity of 125 nl/min based on the following coordinates: ML -0.9 mm, AP -5.4 mm and DV -3.65 mm relative to Bregma [34].

Tissue preparation

Mice were sacrificed through transcardial perfusion with 0.1 M phosphate-buffered saline (PBS) for 5 minutes followed by 4% ice-cold paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) (pH 7.4) for 5 minutes using a supply pump at a rate of 10 ml/min. Brains were carefully removed and post-fixed in 4% PFA for 3 days and then transferred to 30% sucrose solution for 3 days for cryoprotection. Brains were cut into 20 µm thick coronal sections using a cryostat microtome (Leica CM3050 S, Nussloch, Germany). Sections were then stored at 4 °C in cryoprotect-solution (1:1:3 volume ratio of ethylenglycol, glycerol and 0.1 M PB) until further processing.

Immunohistochemistry with 3,3-diaminobenzidine (DAB)

Free-floating sections containing the LC/SN region were washed in 0.1 M PB and quenched with 3% H2O2 and 10% methanol for 15 minutes. After a second wash, sections were blocked in 5% normal donkey serum with 0.3% Triton X-100 in 0.1 M PB for 1 hour before incubating them overnight with primary antibodies against TH, p-aSYN, Ubi-1 or p62 (Table 1) at 4 °C in the same blocking solution. On the second day, sections were washed in 0.1 M PB for 20 minutes and then incubated with the appropriate biotinylated secondary antibody (Table 1) for 1 hour, followed by incubation in avidin-biotin-peroxidase solution (ABC Elite, Vector Laboratories) for 1 hour before initiating the color reaction with 5% DAB (Serva), diluted in 0.1 M PB with 0.02% H2O2. All DAB-stained sections were mounted, dried, counterstained with cresyl-violet and coverslipped with mounting gel (Corbit-Balsam, Eukitt). Brightfield images were acquired using an AxioImager M2 microscope (Zeiss) equipped with an Axiocam 506 color camera (Zeiss).

Immunofluorescence staining

Sections were washed in 0.1 M PB, then blocked in 10% normal donkey serum with 0.3% Triton X-100 in 0.1 M PB for 1 hour before incubating them with primary antibodies (Table 1) at 4 °C in the same blocking solution overnight. On the second day, sections were washed in 0.1 M PB containing 0.3% Triton X-100 and then incubated with fluorophore-conjugated, species-specific secondary antibodies (Table 1) for 2 hours at room temperature in 0.1 M PB containing 0.3% Triton X-100 and 10% normal donkey serum. Before mounting sections were washed for 25 minutes in 0.1 M PB containing 0.3% Triton X-100. Exceptions from this general protocol were made for staining luciferase, p-aSYN, IbA1 and Olig2, where after primary antibody incubation a biotinylated species-specific secondary antibody was used to further improve signal to noise by conjugation with streptavidin. Images were acquired using an AxioImager M2 microscope (Zeiss) equipped with an ORCA-Flash4.0 LT CMOS camera (Hamamatsu C11440-42U). For confocal images, a TCS SP8 microscope (Leica) was used. Images were processed with FIJI image software [35] to enhance signal-to-noise. Image data for 3D reconstructions were obtained with a Zeiss Spinning Disc Microscope

(Axio Observer Z1) equipped with an Axiocam MRm (Zeiss) and an Evolve 512 EMCCD Camera (Photometrics) and post-processed with ZEN 2012 software (Zeiss).

Antigen	Host	Cat. No.	Manufacturer	Dilution
Tyrosine Hydroxylase	Rabbit	AB152	Merck Millipore	1:1000
Tyrosine Hydroxylase	Sheep	AB1542	Merck Millipore	1:1000
AAV VP1/VP2/VP3	Rabbit	61084	Progen	1:250
Alpha-synuclein (p-S129)	Rabbit	ab51253	Abcam	1:2000
Alpha-synuclein (syn211)	Mouse	AHB0261	ThermoFisher	1:1000
Luciferase	Goat	NB100-1677	Novus Biologicals	1:250
GFAP	Chicken	ab4674	Abcam	1:2000
IbA1	Rabbit	019-19741	Wako	1:500
Ubiquitin (Ubi-1)	Mouse	ab7254	Abcam	1:2000
SQSTM1/p62	Mouse	ab56416	Abcam	1:2000
Olig2	Rabbit	ab109186	Abcam	1:500
MAP2	Chicken	ab5392	Abcam	1:2000
Anti-rabbit AlexaFluor488	Donkey	A-21206	Invitrogen	1:1000
Anti-goat AlexaFluor488	Donkey	A-11055	Invitrogen	1:1000
Anti-mouse AlexaFluor488	Donkey	A-2102	Invitrogen	1:1000
Anti-chicken Cy3	Donkey	703-165-155	Jackson ImmunoResearch	1:1000
Anti-mouse Cy3	Donkey	715-165-150	Jackson ImmunoResearch	1:1000
Anti-goat Cy3	Donkey	705-165-147	Jackson ImmunoResearch	1:1000
Biotinylated anti-rabbit	Donkey	711-065-152	Jackson ImmunoResearch	1:1000
Biotinylated anti-mouse	Donkey	715-065-151	Jackson ImmunoResearch	1:1000
Biotinylated anti-goat	Donkey	705-065-147	Jackson ImmunoResearch	1:1000
Streptavidin AlexaFluor647	Donkey	016-600-084	Jackson ImmunoResearch	1:1000

Table 1 | Characteristics of the primary and secondary antibodies

Proteinase K treatment

To analyze the formation of insoluble aggregates, sections were digested with Proteinase K (PK) using a modified protocol described elsewhere [36, 37]. 20 µm thick sections with 120 µm interslice distance containing the complete LC region were washed in 0.1 M PB and subsequently digested in 0.1 M PB containing 0.3% Triton X-100 and 12 µg/ml PK (Cat. No. 4333793, Invitrogen) at 65°C for 10 min. To visualize insoluble aggregates, digested sections were double stained against human aSYN, p62, Ubi-1 or luciferase in combination with TH (Table 1), following the fluorescence staining protocol described above. Complete absence of TH immunoreactivity served as an indicator for successful PK digestion, thus sections in which TH immunoreactivity was still visible were excluded from analysis implicating an incomplete protein digestion. Images were acquired using an AxioImager

M2 microscope (Zeiss) equipped with an ORCA-Flash4.0 LT CMOS camera (Hamamatsu C11440-42U).

Stereology

To quantify TH-positive LC and SN neurons, the optical fractionator workflow (StereoInvestigator version 9, MicroBrightField Biosciences) was used. Therefore, tissue sections were stained against TH with DAB and counterstained with cresyl-violet as described above. To quantify LC cell numbers, five sections per animal containing the complete rostro-caudal extent of the LC region, separated by 120µm, were selected. Contours including all TH-positive neurons of the LC were drawn, excluding neurons of the SubLC region. For quantification of TH-positive SN neurons, seven sections separated by 240 µm covering the complete caudo-rostral extent of the SN were used. Contours were drawn based on the cytoarchitectonic distribution of SN neurons [38] including SN pars compacta but excluding SN pars reticulata or ventral tegmental area neurons. Parameters used for counting were: grid size 100 × 100 µm, counting frame 85 × 85 µm, and 2 µm guard zones.

Quantification of reactive micro- and astrogliosis

Triple immunofluorescence stainings were performed to visualize astro- and microgliosis using antibodies directed against GFAP for astroglia, IbA1 for microglia and TH to label LC neurons (Table 1). To quantify signs of reactive gliosis, we evaluated 5 LC sections of 6 animals per time point by measuring the optical density (OD) of the injected versus the non-injected side using FIJI. First, greyscale images were converted to 8 bit and the LC region was outlined with a rectangular contour (1200px x 800px). Then, OD was measured and lastly a background correction was performed by subtracting the mean background signal for every section. The background corrected OD values of all 5 sections of the injected side were summed and compared to the summed value of the non-injected side.

Quantification of S129-phosphorylated aSYN

To analyze the degree of p-aSYN, a triple immunofluorescence staining against p-aSYN, human aSYN and TH was performed (Table 1). Five sections of 4 animals per time point, containing the complete rostro-caudal extent of the LC region, were selected for analysis. First, images were converted to 8 bit before making them binary. By using a preset intensity threshold, pixels were given either an intensity value of 255 (when positive for p-aSYN) or 0 (when negative for p-aSYN). The resulting p-aSYN signal intensity value was then divided by the area positive for non-phosphorylated aSYN. This ratio was calculated for all five sections and averaged per animal.

Quantification of aSYN transport

Seven coronal sections (Bregma: +4.28, +2.86, +1.18, +0.38, -0.58, -3.16 and -7.56) covering the complete mouse brain were stained against human aSYN (Syn 211) or Luc (Table 1) and the degree of aSYN accumulation was assessed by scoring human aSYN positive axons/cell bodies as follows: – no positive axons; + sparse (few positive axons); ++ mild (more positive axons); +++ moderate (many positive axons, covering almost the complete brain region) and ++++ severe pathology (large number of positive axons densely covering the complete brain region). (+) describes an intermediate state. Six animals per time point were analyzed and the scores for each brain region were averaged.

Statistical analyses

In general, all data values are expressed as mean ± SEM or mean ± min/max. Differences were considered significant at $p < 0.05$. Multiple comparisons were made by one-way or two-way ANOVA analysis followed by Tukey's or Sidak's multiple comparisons test. To calculate correlations, Pearson's correlation coefficient with 95% confidence interval was used. All statistical analyses were performed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA). Figures were created with Adobe Illustrator version 21.1 (Adobe Systems).

Results

rAAV vector-mediated overexpression of human A53T-aSYN in LC neurons

To determine whether and in which time frame aSYN overexpression induces PD-like pathology in LC neurons we chose to overexpress human mutant A53T-aSYN by injecting rAAV1/2-A53T-aSYN [32, 33] unilaterally in the right LC region of wild-type mice (Fig. 1a, b). To verify that the resulting cellular effects were attributable to the aSYN protein itself, luciferase (Luc) was used as a control protein. To investigate time-dependent effects, animals were consecutively sacrificed after 3 days, 1, 3, 6 and 9 weeks (Fig. 1b). By analyzing the first set of animals 3 days after viral injection, we confirmed that both vectors entered LC neurons equally (Fig. 1c, d), resulting in infection rates of 85.17 ± 2.53% for A53T-aSYN and 83.87 ± 3.31% for Luc (unpaired t-test, $p = 0.77$) (Fig. 1d). Double immunofluorescence stainings against TH and human aSYN or TH and Luc (Fig. 1e, f) revealed that both vectors induced protein expression already at this early time point with similar strength (A53T-aSYN 59.89 ± 2.95% and Luc 54.39 ± 3.57%, unpaired t-test, $p = 0.30$). Protein expression was mainly restricted to the LC covering the whole nucleus (Fig. 1g, h). In addition, a variable number of immuno-reactive cells were observed in the adjacent regions (ncl. parabrachialis, Barrington's nucleus, mesencephalic trigeminal nucleus and vestibular nuclei) (Fig. 1g). In LC neurons, cell bodies, as well as axons and dendrites were robustly labeled, indicating strong protein expression. Similar findings were observed for rAAV1/2-Luc injected animals. Notably, there was no aSYN or Luc signal in LC cells on the non-injected side at any time point (Fig. 1g). This allowed us to use the non-injected (left) side as an internal control.

A53T-aSYN overexpression causes LC neurodegeneration

In the first set of experiments, the extent of aSYN induced LC cell loss was assessed with unbiased stereological quantification of TH-positive LC cells 1, 3, 6 and 9 weeks after viral vector delivery. In the A53T-aSYN group, significant degeneration of TH-positive LC cells was measured already 3 weeks post-injection, with 15.86 ± 2.09% cell loss compared to control side. Neurodegeneration increased progressively reaching 34.84 ± 3.39% after 6 weeks and 56.25 ± 5.19% after 9 weeks (Fig. 2a, b). Cell loss was homogenously distributed over the complete rostro-caudal extent of the LC. No cellular pathology was observed in the Luc control group at any investigated time point, confirming that neither the viral vector nor overexpression of a cytoplasmic protein was able to induce neurodegeneration in our model (Fig. 2a, b).

Moreover, immunofluorescent TH-stainings and subsequent confocal imaging revealed that A53T-aSYN, but not Luc overexpression was accompanied by qualitative changes of neuronal morphology, including dystrophic axons and pyknotic perikarya (Fig. 2c).

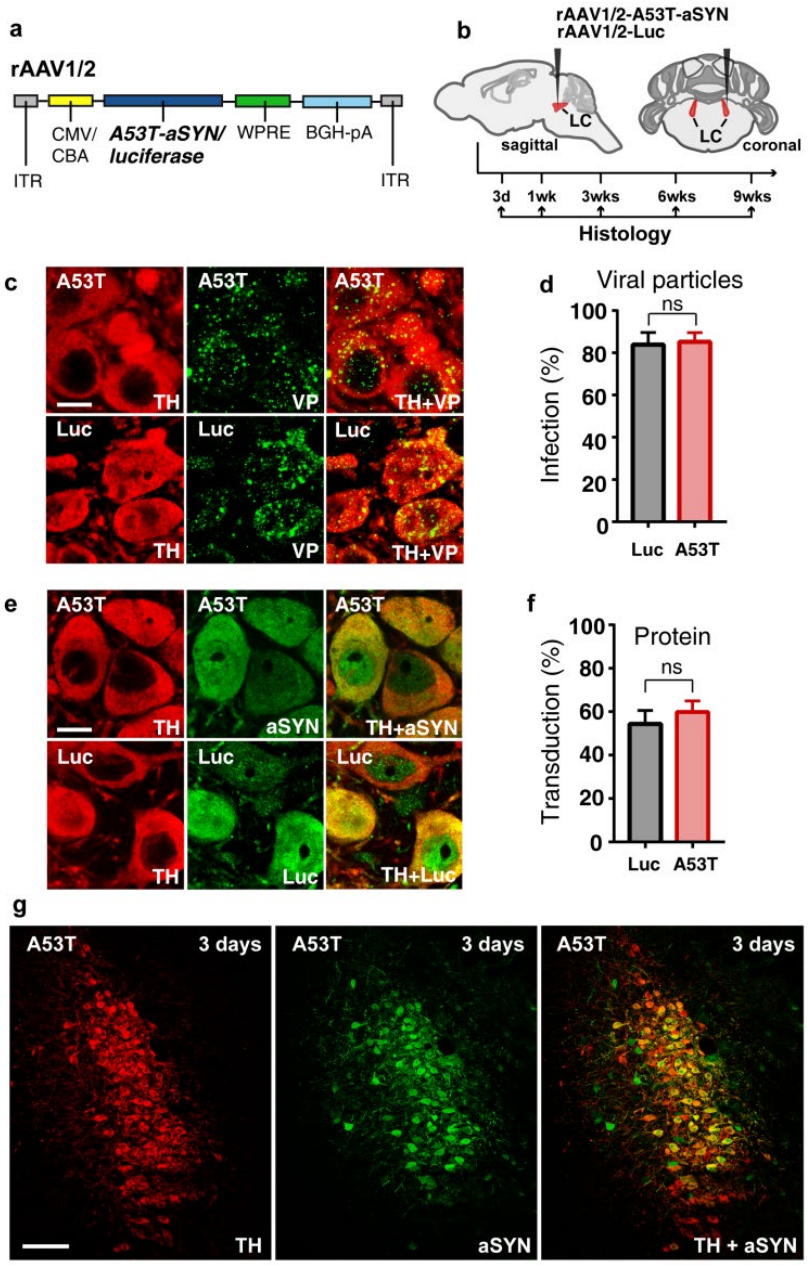

Figure 1 | Locally induced protein overexpression via injection of rAAV vectors in the LC region.

a rAAV1/2 vectors contain a chicken β-actin promoter hybridized with a CMV immediate early enhancer sequence (CMV/CBA) to drive expression of either A53T-aSYN or luciferase (control). ITR, inverted terminal repeat; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; BGH-pA, bovine growth hormone polyadenylation sequence. **b** Experimental design and schematic illustration of the injection site. Animals were consecutively sacrificed after 3 days, 1, 3, 6 and 9 weeks for immunohistochemical evaluation. **c-f** Analysis of the infection or transduction rates via double immunofluorescence staining for TH (red) and viral coating proteins (VP, green) (**c, d**) or TH (red) and human A53T-aSYN (green) or luciferase (green) (**e, f**), respectively. Co-localization of TH and VP indicates successful entry of viral particles, whereas co-localization of TH and A53T-aSYN/luciferase indicates successful protein expression. Student's t-test revealed no significant difference between the transduction rates of the two vectors ($p > 0.05$, $n = 3$ animals per protein) (**d, f**). Values (mean ± SEM) represent the percentage (%) of TH-positive neurons that were also positive for VP, aSYN or Luc. **g** Overview of the pontine brainstem (Bregma: -5.30 mm) stained against TH (red) and human aSYN (green) depicting the transduced area 3 days post-injection. Abbreviations: L, left; R, right; PB, parabrachial nucl.; SUV, superior vestibular nucl.; MV, medial vestibular nucl.; DTN, dorsal tegmental nucl.; LDT, laterodorsal tegmental nucl. h Higher magnification overview image of the TH-positive LC region (red) transduced with human A53T-aSYN (green). Scale bars 25 μm in **c**, **e**; 500 μm in **g** and 100 μm in **h**.

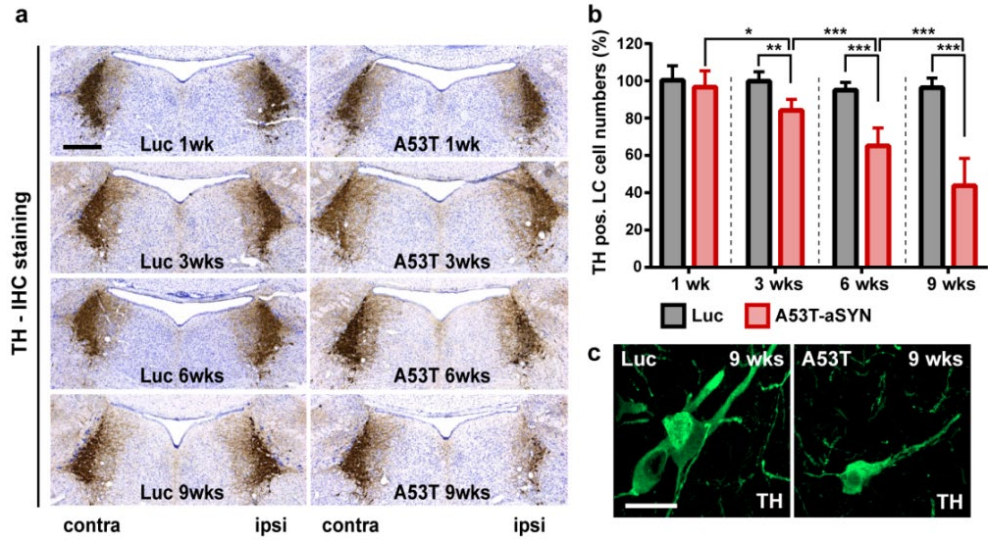

Figure 2 | Progressive loss of TH-immunoreactive LC cells after rAAV1/2-A53T-aSYN injection.

a, b Representative images (Bregma -5.40 mm) and unbiased stereology of TH-positive LC-neurons in A53T-aSYN (red bars, right column) or Luc (black bars, left column) overexpressing animals. Values (mean ± SEM) are expressed as cell numbers on the injected side compared to non-injected side (%). $n = 8$ per time point and group, two-way ANOVA analysis followed by Tukey's post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **c** Representative confocal images of neuronal morphology after 9 weeks of protein overexpression. Pyknotic cell bodies and dystrophic axons were observed in A53T-aSYN, but not in Luc overexpressing animals. Scale bars 250 μm in **a**, 50 μm in **c**.

Accumulation of phosphorylated-aSYN in the LC region

Phosphorylation of aSYN at amino acid serine 129 (p-aSYN) is a commonly observed phenomenon in human PD brain tissue and in animal models artificially overexpressing aSYN [39–43]. In these models, the S129 phosphorylation is frequently used as an indicator for aSYN aggregation. In our current study, we measured the signal intensity of p-aSYN systematically via double immunofluorescence stainings for TH and p-aSYN. Our data revealed that A53T-aSYN overexpression led to strong and progressive phosphorylation of aSYN in LC neurons (Fig. 3a, b). Accumulation of p-aSYN started early with positive cells being observable already 1 week post-injection, reaching highest levels at the latest time point. Generally, the p-aSYN signal was homogenously distributed in the cytoplasm of TH-positive LC cells. In addition, robust labeling of the nucleus was observed (Fig. 3d, e). To exclude the possibility of non-specific antibody labeling we analyzed rAAV-Luc injected animals, which showed no signal for p-aSYN at any time point (Fig. 3d, e). Next, we wanted to quantify if the degree of phosphorylation correlated with the degree of noradrenergic cell loss. Therefore, the p-aSYN signal intensity values were plotted and correlated with the percentage of LC cell loss (Fig. 3c). The strong correlation ($r = 0.67$, $p < 0.05$) indicates that the degree of aSYN phosphorylation can be used as a predictor of aSYN toxicity in our model.

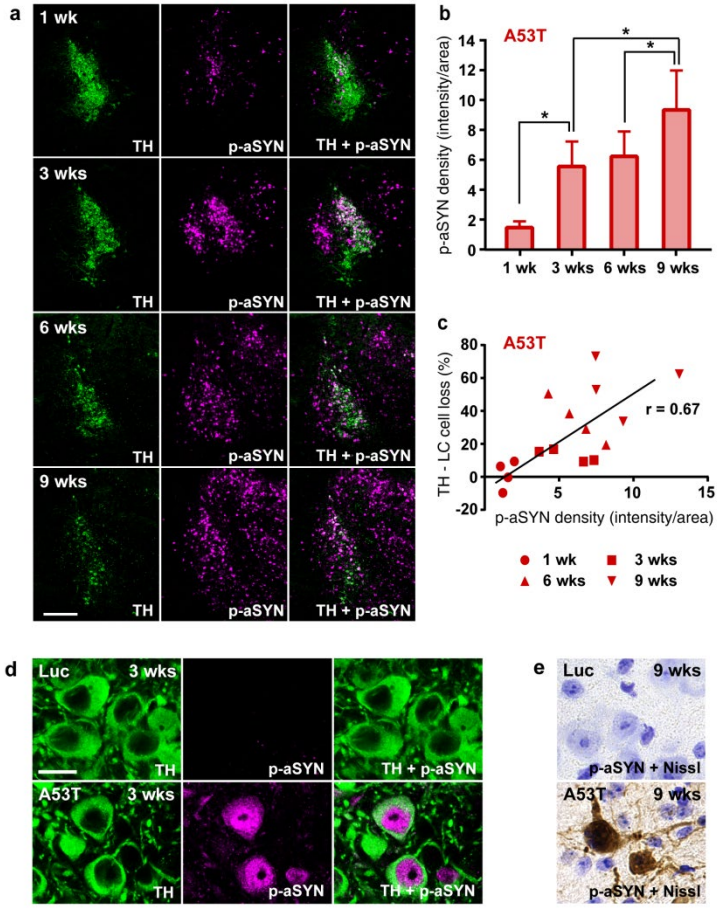

Figure 3 | Progressive accumulation of phosphorylated aSYN (p-aSYN) in the LC region.

a, b Representative images (Bregma -5.40 mm) and quantification of p-aSYN in the LC region via double immunofluorescence staining for TH (green) and p-aSYN (magenta). Values are presented as mean ± SEM, *n* = 4 per time point and group, one-way ANOVA analysis followed by Tukey's post-hoc test, * $p < 0.05$. **c** Robust correlation was observed between loss of TH-positive LC cells and accumulation of p-aSYN ($r = 0.67$, $p < 0.05$). **d** Confocal microscopy confirmed accumulation of p-aSYN (magenta) in TH-positive LC cells (green) 3 weeks post injection in A53T-aSYN (red) overexpressing animals (lower row). **e** p-aSYN immunoreactivity in the LC-region of A53T-aSYN overexpressing animals (lower row) 9 weeks post injection. No A53T-aSYN or p-aSYN immunoreactivity was observed in Luc overexpressing animals (upper row) at any time point. Scale bars 250 µm in **a**, 25 µm in **d-e**.

Formation of proteinase K (PK)-resistant, p62-, Ubi-1- and aSYN-positive aggregates

Lewy bodies in human PD brain tissue are characterized by immunoreactivity for insoluble (PK-resistant) aSYN, but also for a variety of other proteins, such as ubiquitin-1 (Ubi-1) and p62/SQSTM1/sequestosome-1 (p62) [44, 45]. Both of the latter proteins are implicated in the cellular clearance of aSYN. Occurrence of PK-resistant Ubi-1-positive aggregates indicates an overburdened proteasomal clearing system, while dysfunction of the lysosomal system can result in accumulation of p62-positive aggregates [46]. To test whether proteasomal and/or lysosomal clearance might be impaired in our model, we systematically screened A53T-aSYN and Luc overexpressing animals for p62- and Ubi-1-immunoreactivity. A53T-aSYN, but not Luc injected mice showed abundant p62- and Ubi-1-positive aggregates starting 3 weeks after viral vector delivery reaching highest numbers at the latest time point (Fig. 4a, b). Ubi-1-, as well as p62-positive inclusions appeared as small circular

objects surrounding the nuclei of the cells (Fig. 4b) and were restricted to the ipsilateral side of injection. As in the previous experiments most of the p-aSYN signal was seen in TH-positive neurons (Fig. 3a, d), we expected a high rate of co-localization for p62 and Ubi-1 with the LC marker TH. However, the majority of p62 and Ubi-1 immunoreactivity was located next to TH-positive LC cells, suggesting that other cells are involved in this process (Fig. 4a). To elucidate in which cell type the p62-positive aggregates were located, double immunofluorescence stainings for p62 with MAP2 (neuronal marker), Olig2 (oligodendroglial marker), GFAP (astrocytic marker) or IbA1 (microglial marker) were performed. While p62 did not co-localize with MAP2 (Fig. 5a), Olig2 (Fig. 5b) or GFAP (Fig. 5c), we observed clear co-localization with IbA1 (Fig. 5d), indicating that the p62-positive inclusions were located in microglial cells. Moreover, we further confirmed that Ubi-1-positive aggregates were also located in microglia (Fig. 5e). Double immunofluorescence stainings for IbA1 and aSYN (Syn211) (Fig. 5f, arrow) and GFAP and aSYN (Syn211) (Fig. 5g, arrow) revealed that microglia, as well as astroglia exhibited human aSYN after 3 weeks of aSYN overexpression.

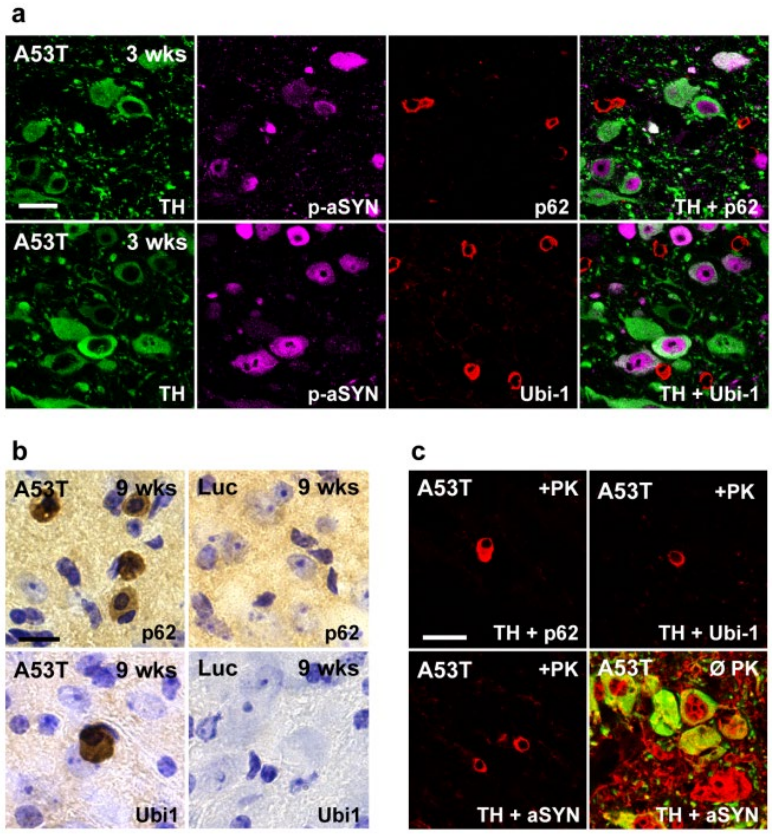

Figure 4 | Formation of insoluble protein aggregates in A53T-aSYN overexpressing animals.

a Staining for p62 (red, upper row) and Ubi-1 (red, lower row) revealed abundant p62- and Ubi-1-positive aggregates in the LC-region. These aggregates were found in close proximity to, but did not co-localize with TH-positive (green) LC cells, which were positive for p-aSYN (magenta). **b** Representative images of p62- (upper left) and Ubi-1 (lower left) positive aggregates in A53T-aSYN animals. No aggregates were observed in Luc overexpressing animals at any time point (right column). **c** p62-, Ubi-1 and aSYN stainings after proteinase K (PK) digestion or without digestion (Ø PK, lower right) confirmed that p62- (upper left), Ubi-1- (upper right) and human aSYN-positive (lower left) aggregates were insoluble. Scale bars 50 µm in **a** and **c**, 25 µm in **b**.

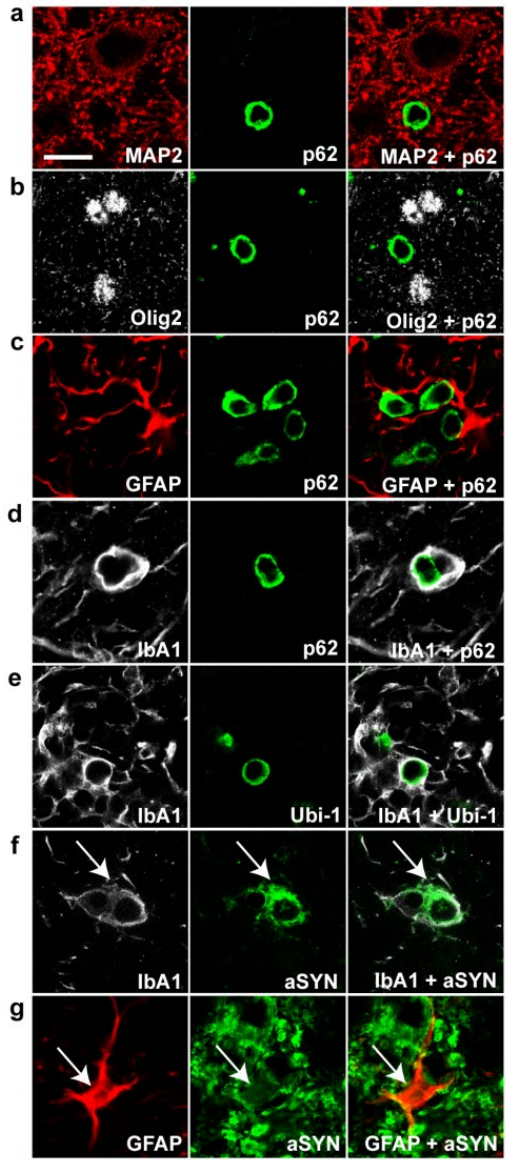

Figure 5 | p62- and Ubi-1 positive aggregates co-localize with IbA1-positive microglia.

a-c Representative confocal microscopy images of p62 (green, *second column*) and MAP2 (**a,** red, *first row*), Olig2 (**b,** gray, *second row*), or GFAP (**c,** red, *third row*) show no co-localization of p62 and the different cellular markers. In contrast, co-staining p62 (green) and IbA1 (**d,** gray, *fourth row*) or Ubi-1 (green) and IbA1 (**e,** gray, *fifth row*) revealed clear co-localization. IbA1 positive microglial cells (**f,** gray, *sixth row*) and GFAP-positive astroglia (**g,** red, *seventh row*) also co-stained for aSYN (*arrows* in **f, g**). Scale bar for **a-g** 25 µm.

Next, we aimed to investigate if the observed p62- and Ubi-1-positive inclusions indeed consisted of insoluble aggregated proteins. Since PK resistance is accepted as a valid marker for the formation of insoluble aggregates in human PD samples and animal models [33, 36, 47], we digested tissue samples of A53T-aSYN and Luc injected mice of all time points with PK. As a result, numerous PK-resistant insoluble aggregates positive for p62, Ubi-1 and aSYN were found in A53T-aSYN injected mice (Fig. 4c). Notably, PK-resistant aSYN aggregates had the same shape and size as Ubi-1- and p62-inclusions. Further, all three kinds of aggregates started to appear 3 weeks after initiation of A53T-aSYN overexpression and were restricted to the site of viral injection. PK digestion and subsequent analysis of rAAV-Luc injected animals revealed no signal for aSYN, p62, Ubi-1 or Luc in any analyzed section.

Targeted α-synucleinopathy induces reactive micro- and astrogliosis in the LC region

Microglia activation and reactive astrocytes have been observed by respective PET imaging in human prodromal and manifest PD patients [48, 49], post-mortem PD brain samples [50, 51] and aSYN animal models [52–54]. Most of the studies using animal models focused on the impact of microglia activation following nigrostriatal degeneration. In the current study, we aimed to investigate whether a focally induced α-synucleinopathy in the LC region would lead to reactive micro- and astrogliosis. Therefore, a triple immunofluorescence staining for IbA1 (microglial marker), GFAP (astroglial marker) and TH was carried out and the intensity of fluorescence signal was quantified (Fig. 6a-c). Already 3 weeks of A53T-aSYN overexpression were sufficient to induce a 3.5-fold increase of astroglial signal intensity in the injected LC region compared to Luc control. The astrogliosis further progressed up to a 6-fold increase after 9 weeks (Fig. 6b). Simultaneously, a 3-fold signal increase for microglia was measured after 3 weeks of A53T-aSYN overexpression and a 5-fold increase after 9 weeks, compared to Luc (Fig. 6c). 3D reconstructed high magnification confocal images revealed a dense glial network in A53T-aSYN overexpressing animals, in which the remaining TH-positive LC neurons were embedded already 3 weeks after viral vector delivery (Fig. 6d). Abundant direct physical contacts between TH-positive LC neurons and astro- and microglia could be resolved. In addition, numerous LC cells appeared to be nearly completely engulfed by microglial processes (Fig. 6d, arrows). In contrast, overexpression of Luc did not lead to any significant increase of astro- or microglia intensity values (Fig. 6a-d). Besides the interaction of astro- and microglia with LC neurons, we also observed direct physical contacts between astrocytes and microglial cells (Fig. 6e, arrow).

To underline our hypothesis that the degree of aSYN-induced pathology is closely associated with the degree of microgliosis, we correlated the microglial intensity values with the percentage of LC neurodegeneration (Fig. 6f, g). This revealed a correlation coefficient of $r = 0.80$ ($p < 0.05$) for A53T-aSYN, whereas for the Luc overexpressing animals no significant correlation was found ($r = 0.09$, $p > 0.05$).

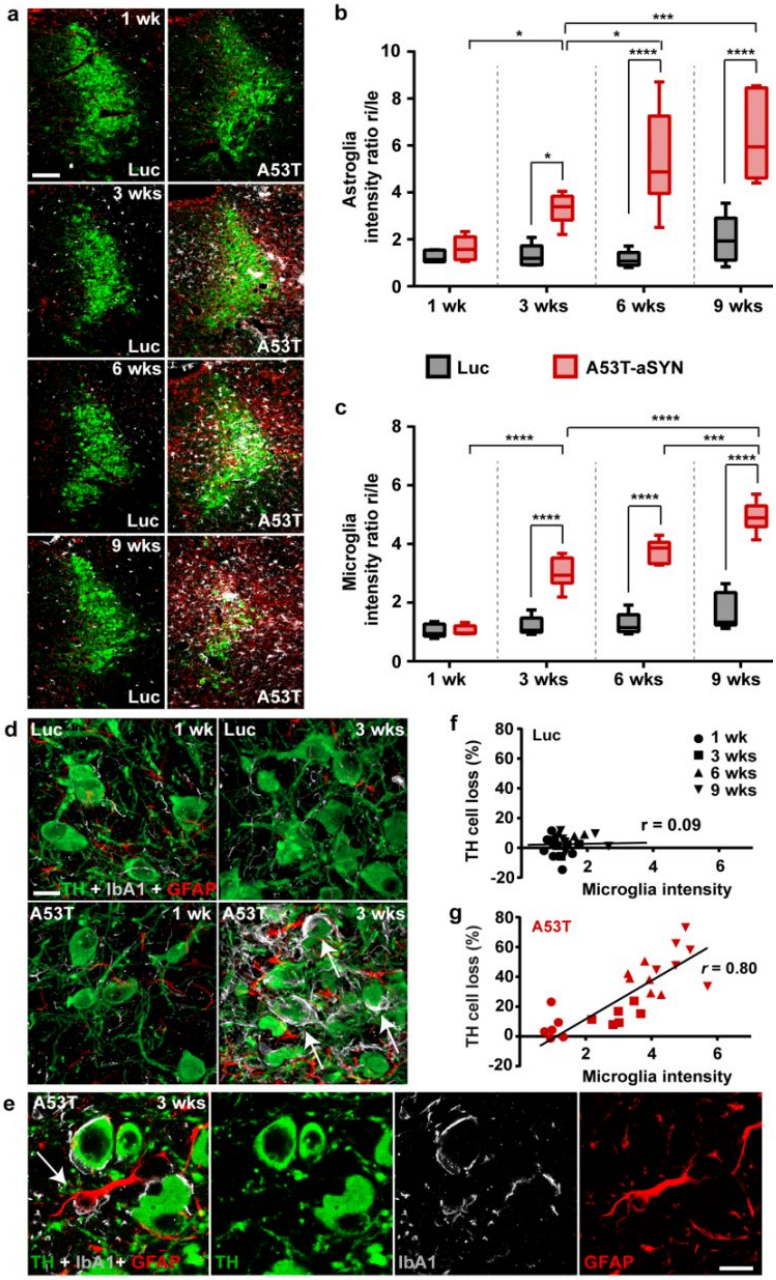

Figure 6 | A53T-aSYN overexpression leads to a pronounced reactive micro- and astrogliosis in the LC-region.

a Representative images of the LC region of Luc (left column) or A53T-aSYN (right column) injected animals stained for TH (green), IbA1 (gray) and GFAP (red) display a marked increase of micro- and astroglia over time in A53T-aSYN overexpressing mice. Quantification of GFAP (**b**) and IbA1 (**c**) signal intensity revealed a progressive increase of astro- and microglia signal in A53T-aSYN injected animals (red boxes) compared to Luc control (black boxes). Values (mean ± min/max) are expressed as the signal intensity ratio of the injected side compared to the non-injected side. n = 6 animals per time point and group. Two-way ANOVA analysis followed by Tukey's post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **d** Reconstructed high magnification confocal images of the LC region showing physical contacts between TH-positive (green) LC cells and IbA1-positive micro- (gray) and GFAP-positive astroglia (red) after 3 weeks of A53T-aSYN overexpression (lower right). Engulfment (arrow) of TH-positive neurons by glial cells was only observed in A53T-aSYN expressing animals and not in Luc control mice (upper row). **e** Direct physical contacts were also observed between micro- and astroglia (arrow). **f, g** Correlating TH cell loss with the microglia intensity values indicates a strong association between increase of microglia and severity of TH cell loss in A53T-aSYN overexpressing animals ($r = 0.80$, $p < 0.05$), whereas there was no correlation in Luc expressing animals ($r = 0.09$, $p > 0.05$). Pearson's correlation coefficient with 95% confidence interval. Scale bars 100 µm in **a**, 25 µm in **d** and **e**.

Extensive transport of human A53T-aSYN to efferent brain regions

After investigating the local effects of A53T-aSYN overexpression, we addressed the question whether the aSYN pathology can propagate to anatomically connected brain regions. A variety of studies using overexpression of rAAV-aSYN or injection of preformed aSYN fibrils (PFF's) have described transport or spread of aSYN to anatomically connected brain regions [41, 55–59]. To investigate the propagation of human A53T-aSYN after inducing the α-synucleinopathy in LC neuronal somata, we stained predetermined brain sections against human aSYN (Syn211) or Luc and rated the occurrence of aSYN- or Luc-positive axons or cell bodies (Table 2). While overexpression of Luc resulted in a staining pattern, which was limited to the injection site and absent in distant brain regions, we observed aSYN signal in a high number of brain regions in A53T-aSYN injected mice (Fig. 7). One week after injection of rAAV-A53T-aSYN in the right LC region, abundant aSYN-positive axons were observed in various brain regions which are known output regions of LC neurons [60]. The human aSYN signal was solely axonal and no aSYN-positive cell bodies were detected. Regions showing the strongest aSYN signal included the main olfactory bulb, lateral septal nucleus, diagonal band nucleus, bed nuclei of the stria terminalis, central amygdalar nucleus, periaqueductal gray, midbrain reticular nucleus, substantia nigra (SN) pars compacta and the ventral tegmental area (Table 2). We counted 36 brain regions, which contained human aSYN-positive axons after one week, indicating that human A53T-aSYN was transported rapidly along the axons towards the synaptic terminals in an anterograde direction. Despite the increase of axonal aSYN signal, no aSYN-positive cell bodies were detected outside of the LC region at any investigated time point, arguing against the hypothesis that human A53T-aSYN is released in LC output regions and taken up by synaptically connected cells in the short time frame of 9 weeks. This is highlighted by the finding that staining against p-aSYN revealed no signs of phosphorylation or aggregation of endogenous aSYN in distant brain regions after 9 weeks whereas the axons containing human (non-phosphorylated) A53T-aSYN stained positive for TH (Fig. 8c).

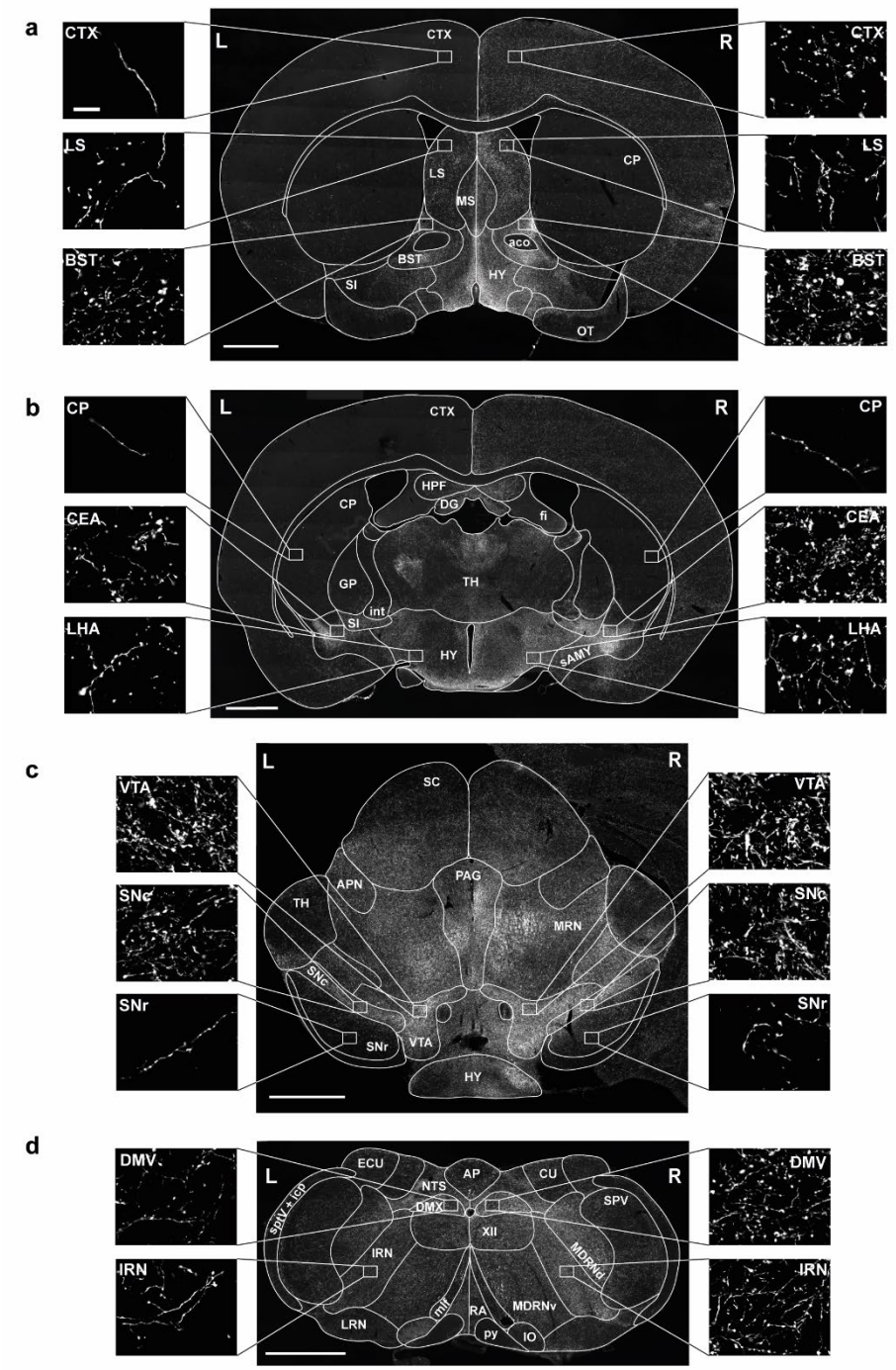

Figure 7 | Widespread transport of human A53T-aSYN to interconnected brain regions.

a-d Representative images of analyzed brain sections stained against human aSYN (Syn211) (Bregma +0.50 mm a, Bregma -0.94 mm b, Bregma -3.16 mm c, Bregma -7.56 mm d). Scale bars 1 mm in **a-d**, 25 µm in all high magnification images. Abbreviations: CTX, cortex; CP, caudoputamen; LS, lateral septal nucleus; MS, medial septal nucleus; aco, anterior commissure; BST, bed nuclei of stria terminalis; HY, hypothalamus; SI, substantia innominata; OT, olfactory tubercle; HPF, hippocampal formation; DG, dentate gyrus; fi, fimbria hippocampi; int, internal capsule; TH, thalamus; GP, globus pallidus; sAMY, striatum-like amygdalar nuclei; LHA, lateral hypothalamic area; CEA, central amygdalar nucleus; SC, superior colliculus; APN, anterior pretectal nucleus; PAG, periaqueductal gray; MRN, midbrain reticular nucleus; VTA, ventral tegmental area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; AP, area postrema; NTS, nucleus of the solitary tract; CU, cuneate nucleus; ECU, external cuneate nucleus; DMX, dorsal motor nucleus of the vagus nerve; XII, hypoglossal nucleus; SPV, spinal nucleus of the trigeminal; MDRNd, medullary reticular nucleus, dorsal part; MDRNv, medullary reticular nucleus, ventral part; IRN, intermediate reticular nucleus; IO, inferior olivary complex; py, pyramid; RA, raphe nuclei; mlf, medial longitudinal fascicle; LRN, lateral reticular nucleus; sptV, spinal tract of the trigeminal nerve; icp, inferior cerebellar peduncle; L, left (contralateral); R, right (ipsilateral).

		Injected (right) hemisphere Time of A53T-aSYN overexpression				Non-injected (left) hemisphere Time of A53T-aSYN overexpression			
		1 wk	3 wks	6 wks	9 wks	1 wk	3 wks	6 wks	9 wks
Bregma	Main olf. bulb, gr. layer	+	++	++(+)	++	(+)	+	+	(+)
+4.28 mm	Inner plexiform layer	–	+(+)	+(+)	+	–	(+)	(+)	(+)
	Outer plexiform layer	(+)	+	+	+	–	(+)	(+)	(+)
Bregma	Cortex	+	++	++(+)	++	(+)	+	+	(+)
+2.96 mm	Main olf. bulb	+	++	++(+)	++	(+)	(+)	+	(+)
	Ant. olf. nucleus	+	+(+)	+(+)	+	–	(+)	(+)	(+)
	Lateral olf. tract	(+)	+(+)	+	+	–	–	–	–
Bregma	Cortex	+	++(+)	++(+)	++(+)	–	+	+	+
+1.18 mm	Taenia tecta, dors. part	+	++	++	+(+)	–	+	+	(+)
	Corpus callosum	(+)	+(+)	+	+	–	(+)	(+)	(+)
	Lateral septal nucleus	+(+)	++(+)	+++	++(+)	(+)	+	+(+)	+(+)
	Diagonal band nucleus	+(+)	++(+)	+++	++(+)	(+)	+	+(+)	+(+)
	Nucleus accumbens	+	+	+	+	–	(+)	(+)	(+)
	Caudoputamen	(+)	+	+	(+)	–	(+)	(+)	(+)
Bregma	Medial septal nucleus	+	+(+)	++	+(+)	(+)	+	+(+)	+
+0.38 mm	Cortex	(+)	++(+)	++(+)	++	–	+	+	+
	Bed ncl. of stria term.	++	+++	+++(+)	+++	(+)	+	++	++
	Magnocellular ncl.	(+)	+	++	++	–	(+)	+	+
	Hypothalamus	+	++(+)	++(+)	+++	–	+(+)	+(+)	++
	Lateral septal nucleus	(+)	++	++(+)	++(+)	–	+	+(+)	+(+)
	Caudoputamen	(+)	+	+	(+)	–	(+)	(+)	(+)
	Subst. innominata	+	+	+(+)	+(+)	(+)	(+)	+	+
Bregma	Central amygdalar ncl.	+	++	+++	+++(+)	(+)	+(+)	++	++
–0.58 mm	Subst. innominata	+(+)	++	++(+)	+++	+	+(+)	++	++
	Globus pallidus	(+)	+	+	+	–	(+)	(+)	(+)
	Hypothalamus	+	++	++(+)	++	(+)	+	+(+)	+(+)
	Lat. hypothalamic area	+(+)	++	+++	++(+)	(+)	+	++	++
	Fimbria	(+)	+	(+)	(+)	(+)	+	(+)	(+)
	Stria terminalis	+	+(+)	+(+)	+(+)	–	(+)	+	+
	Corpus callosum	–	+	+	+	–	–	(+)	(+)
	Cortex	(+)	++(+)	++(+)	++	–	+	+	+
Bregma	Cortex	–	+(+)	+(+)	+	–	(+)	(+)	(+)
–3.16 mm	Sup. colliculus, sens.	–	+(+)	+(+)	+	–	+	+	+
	Sup. colliculus, motor	(+)	+(+)	++	++	(+)	+(+)	++	++
	Periaqueductal gray	+(+)	++	++(+)	++(+)	(+)	+(+)	++	++
	Midbrain reticular ncl.	+(+)	++	++	++(+)	+	+(+)	++	++
	Red nucleus	(+)	+	+(+)	+	+	++	++(+)	++
	Ventral tegmental area	+	+(+)	++	++(+)	+(+)	++	++(+)	++(+)
	Subst. nigra, pars compacta	+(+)	+(+)	++	++	(+)	+	+(+)	+(+)
	Subst. nigra, pars reticulata	(+)	+	+	(+)	(+)	(+)	(+)	(+)
	Hippocampus	–	+	+(+)	+	–	(+)	+	+
	Interpeduncular ncl.	(+)	+(+)	++	++(+)	(+)	+(+)	++(+)	++(+)
	Thalamus	(+)	+(+)	++	++	(+)	+	+(+)	+(+)
Bregma	Dorsal motor ncl. of n. X	–	+	+(+)	+(+)	–	(+)	+	+
–7.56 mm	Hypoglossal ncl.	–	+	+	+	(+)	(+)	+	(+)
	Parvicell. reticular ncl.	+(+)	+(+)	+(+)	+(+)	(+)	(+)	+	+
	Intermediate ret. ncl.	+	+	++	+(+)	–	(+)	+	+
	Medullary reticular ncl.	+	+(+)	+(+)	+(+)	–	+	+	+
	Lateral ret. ncl.	–	(+)	+	(+)	–	(+)	(+)	(+)
	Ncl. of the solitary tract	–	(+)	+	+(+)	–	(+)	+	+
	Spinal ncl. of the n. V	–	(+)	(+)	(+)	–	–	(+)	(+)
	Area postrema	(+)	(+)	+	+	(+)	(+)	+	+
	Arbor vitae	–	+	+	+	–	+	+	+
	Gr. layer (cerebellum)	+	+(+)	+(+)	+(+)	(+)	+	+	+
	Mol. layer (cerebellum)	–	+	+	+	–	+	+	+

Table 2 | Semiquantitative analysis of human aSYN-pathology in distant brain regions.

Occurrence of human aSYN-positive axons was graded out of seven coronal brain sections as follows: – no positive axons; + sparse (few positive axons); ++ mild (more positive axons); +++ moderate (many positive axons, covering almost the complete brain region) and ++++ severe pathology (large number of positive axons densely covering the complete brain region); (+) describes an intermediate state between two categories to allow a more accurate description. n = 6 per time point. Abundance of aSYN-positive axons increased over time and was more prominent in the injected (right) hemisphere. The signal for human aSYN was solely axonal and no aSYN-positive cell bodies were detected.

No substantia nigra (SN) cell loss after 9 weeks of human A53T-aSYN overexpression in LC neurons

Already after 1 week of A53T-aSYN overexpression in LC neurons, human aSYN positive axons passing by DA SN neurons could be detected. After 9 weeks, SN neurons were densely surrounded by aSYN containing axons (Fig. 8a) but no human aSYN signal was observed in the somata of SN cells. Stereological quantification of TH-positive SN neurons (Fig. 8b) revealed no significant difference of TH-immunoreactive neurons between A53T-aSYN compared to Luc overexpressing mice neither for the left nor for the right SN (One-way ANOVA; $p > 0.05$). This result points out that LC degeneration, in combination with profound local axonal aSYN accumulation was not sufficient to induce degeneration of DA SN neurons within the relatively short period of 9 weeks.

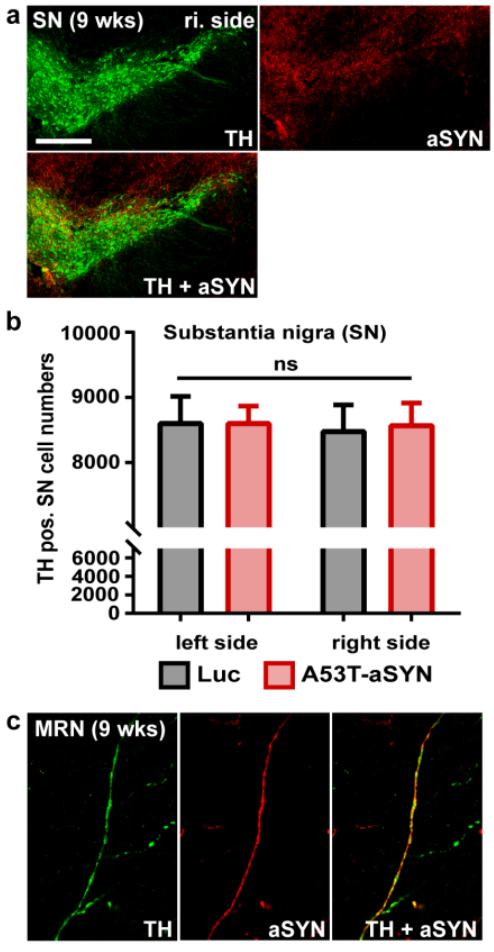

Figure 8 | No SN cell loss after 9 weeks of A53T-aSYN overexpression in LC region.

a Abundant human aSYN-positive (red, *upper right*) axons were observed in the TH-positive SN region (green, *upper left*) after 9 weeks of A53T-aSYN overexpression in the LC. In contrast, no aSYN-positive cell bodies could be detected. **b** Quantification of TH-positive SN neurons 9 weeks post injection revealed no significant difference between A53T-aSYN group (*red bars*) and Luc control group (*black bars*) for either side. Values are presented as mean ± SEM, $n = 8$ per group and side. One-way ANOVA analysis ($p > 0.05$). **c** Representative image of TH and human aSYN axonal co-localization in distant brain regions, exemplified for midbrain reticular nucleus (MRN). The majority of aSYN positive axons co-stained for TH indicating that they origin from the LC. Scale bars 250 µm in **a,** 25 µm in **c**.

Discussion

Degeneration of the LC noradrenergic system is a key event during PD pathogenesis in the prodromal phase of the disease. In this study, we present the first targeted LC α-synucleinopathy mouse model, which replicated cardinal features of human PD pathology. We have designed our rAAV vector-based overexpression model to generate robust and rapid induction of aSYN pathology, including phosphorylation and aggregation of aSYN, noradrenergic neurodegeneration, development of dystrophic axon morphology, signs of proteasomal and lysosomal dysfunction and prominent neuron-glial interactions. Furthermore, the herein characterized aSYN transport pattern allows investigating the effects of aSYN-induced LC neurodegeneration on anatomically connected LC output structures.

Progressive S129 phosphorylation and formation of PK-resistant aSYN-positive aggregates

Phosphorylation of aSYN at amino acid S129 is a dominant pathological modification of aSYN [40] since approximately 90% of aSYN in human Lewy bodies is phosphorylated at this position, whereas only 4% of soluble aSYN exhibits this posttranslational modification [39]. In PD animal models, phosphorylation at S129 is used as a key marker to investigate an induced α-synucleinopathy and its occurrence has often been interpreted as formation of aSYN aggregates [41, 42, 54, 61, 62]. In our current study, we show abundant and over time increasing S129-phosphorylation of aSYN in the cytoplasm and nucleus of LC cells (Fig. 3a, d). Previous studies have pointed out that aSYN has different cellular localizations. Beside the presynaptic and cytoplasmic localization, a nuclear occurrence of aSYN is known [63]. Nuclear p-aSYN has been observed in previous studies where aSYN was overexpressed [64, 65] and it could be shown that nuclear aSYN interacts with histone molecules. It was even suggested that the S129-phosphorylation may play an important role for the nuclear translocation of aSYN [66]. To confirm that phosphorylation of aSYN was accompanied by formation of high molecular weight aSYN aggregates we performed PK digestion experiments that revealed small circular aSYN positive inclusion bodies restricted to the injection site (Fig. 4c). Our model thereby reproduces a key feature of the LC pathology observed in human PD patients. Importantly, since PK digestion led to the destruction of all soluble proteins it did not allow us to investigate if the developing aSYN-positive inclusions are located in neurons or glial cells. The observed discrepancy between a high amount of p-aSYN-positive cells and a relatively limited number of aSYN-positive PK-resistant inclusions raises the question whether aSYN S129-phosphorylation can solely be used as a sufficient marker for aSYN aggregation. Our data indicate that S129-phosphorylation is an important indicator for aSYN pathology, but immunohistochemistry for other aggregation markers should be added to confirm the occurrence of aSYN aggregates [67, 68].

Formation of p62- and Ubi-1-positive proteinaceous inclusions in microglia

Additional markers, which are also commonly accepted to investigate protein aggregation and simultaneously serve as indicators for dysfunction of the proteasomal or lysosomal protein degradation system include Ubi-1 and p62 [44, 45]. Based on previous reports, which showed close co-localization of p-aSYN and Ubi-1 or p62 [56, 69], we expected to find overlap of these markers in our model. But notably, all p62 and Ubi-1 aggregates were located next to p-aSYN positive LC cells (Fig. 4a). Co-staining p62 and Ubi-1 with different glial and neuronal markers revealed that the p62 and Ubi-1 inclusions were located in IbA1-positive microglial cells (Fig. 5d, e). Further, we also show that microglia exhibited human aSYN, probably as a result of local aSYN uptake or phagocytosis of aSYN containing cellular debris. (Fig. 5f). The possibility that the microglial cells were transduced by rAAV1/2-A53T seems to be unlikely, since the used rAAV1/2 vector possesses a high neuronal tropism [32] and triple stainings for IbA1 (microglia), GFAP (astroglia) and aSYN revealed no aSYN-immunoreactivity 3 days after viral vector delivery within micro- or astroglial cells. Despite this, we cannot completely exclude the possibility of microglia transduction by the initial injection of rAAV1/2-A53T. Deposition of internalized aSYN aggregates in microglia has already been observed in vitro [70], but our study represents (to our knowledge) the first in vivo evidence of inclusion formation in microglial cells. We hypothesize that these p62- und Ubi-1-positive aggregates develop de novo in microglial cells possibly because of massive human aSYN uptake, which exceeds the lysosomal degradation capacities and leads to protein aggregation.

Currently we can only speculate why p62 and Ubi-1 reactivity was observed in microglia but absent in LC neurons. Previous studies have shown that p62-positive inclusions co-localize with Ubi-1 not only in neuronal but also in glial cells in neurodegenerative diseases including Alzheimer's disease, dementia with Lewy bodies and PD [44]. Furthermore, it has been shown that microglia rapidly internalize aSYN thereby representing the most efficient scavengers of neuronal released aSYN [71, 72]. By clearing aSYN, microglia might actively delay accumulation of aSYN and maturation of aSYN aggregates in LC neurons. One could hypothesize that in our model aSYN is rapidly released from LC neurons and taken up by microglia, which in turn leads to microglial but not neuronal accumulation of p62 und Ubi-1 aggregates. A longer duration of the experiment may clarify the question, whether Ubi-1- and p62-positive inclusions might also develop in LC neurons.

Reactive astro- and microglia and their implication in aSYN-induced LC pathology

Another key aspect in several animal models in which aSYN was injected or overexpressed [52, 54, 73] is the profound involvement of reactive astro- and microgliosis during the development of the aSYN pathology. It has been shown that activated microglial cells, besides their implication in clearing aSYN, are able to trigger the release of inflammatory cytokines and accelerate the production of reactive oxygen species, thereby likely contributing to the process of neurodegeneration [73–75]. In our model, LC cells were surrounded by a massive network of astro- and microglia already after 3

weeks of aSYN overexpression, with many microglial cells almost completely engulfing the surviving LC neurons (Fig. 6d, arrows). This early induction of microgliosis (Fig. 6c) is in line with previous findings where microgliosis even preceded the onset of neurodegeneration [53, 76]. Furthermore, we observed a strong correlation between the increase of microglial signal and LC cell loss (Fig. 6g), implying the conclusion that reactive microglial cells are important modulators of aSYN-induced toxicity not only in the dopaminergic SN but also in the noradrenergic LC. Microgliosis was accompanied by severe and progressively increasing astrogliosis. Reactive astrocytes surrounded and partially engulfed LC neurons. Furthermore, they formed direct physical contacts with reactive microglia (Fig. 6e, arrow) and exhibited clear signal for human aSYN (Fig. 5g). Importantly, reactive astrocytes can also take part in clearing aSYN by endocytosis and degradation in their lysosomal system [77, 78]. Furthermore, they interact closely with microglia and release pro- and anti-inflammatory molecules [79, 80]. Our LC model exemplifies this close interdependency between neurons, micro- and astroglia. We show that glial cells are highly involved in the process of aSYN degradation and that glial dysfunction or failure could be a factor of PD progression. However, it should also be considered that the LC itself plays a central role in decreasing neuroinflammation [81]. Noradrenaline is able to suppress the expression of pro-inflammatory cytokines in glial cells while simultaneously elevating the expression of anti-inflammatory markers [82, 83]. Hence, it is reasonable to assume that loss of LC neurons additionally increases the neuroinflammatory response and contributes to the progressive increase of micro- and astroglial activity seen in our model.

Anterograde axonal transport of aSYN to LC output regions

To slow or prevent the progression of PD, it is essential to investigate if and how the α-synucleinopathy propagates within the brain. Recent evidence [41, 56, 58, 84, 85] suggests that toxic aSYN species formed in a small number of cells can spread trans-synaptically to distant but anatomically connected brain regions where they act as seeds to trigger the formation of insoluble aSYN aggregates [56, 86]. Furthermore, cell culture experiments have demonstrated that aSYN can be taken up by cells and transported in both the retrograde and anterograde direction [87–89]. The noradrenergic LC has a broad input-output connectome [60, 90] making this brain region suitable to investigate trans-neuronal spread. Moreover, Iba and colleagues [91] have demonstrated in a tauopathy model that injections of synthetic tau fibrils were able to induce tau pathology in LC neurons which then propagated to LC afferents and efferents. To investigate if this also translates into our LC aSYN overexpression model we systematically analyzed and scored the aSYN pathology after 1, 3, 6 and 9 weeks (Fig. 7, Table 2). Our results indicate that the overexpressed human A53T-aSYN, once produced in the cytoplasm of LC neurons, is only transported in the anterograde direction towards the synaptic terminals, as abundant aSYN-positive axons and terminals in efferent LC regions co-stained for TH (Fig. 7, Fig. 8c, Table 2). The broad LC output connectome [60] likely explains this high amount of aSYN-positive axons in distant brain regions of the ipsilateral but also contralateral hemisphere. The mild aSYN pathology of the contralateral (non-injected) side can be

explained by LC projections crossing the midline and innervating brain structures of the contralateral hemisphere [92]. In contrast to the profound axonal aSYN immunoreactivity, we found no aSYN-positive cell bodies outside of the LC region, arguing against trans-neuronal spread of aSYN in the relatively short time frame of 9 weeks. The absence of Luc-positive axons in LC output regions might be explained on one hand by protein size (Luc 62 kDa vs. aSYN 15 kDa) and on the other hand by the naturally presynaptic localization of aSYN [93, 94]. We therefore conclude that the aggregation prone aSYN species created by overexpression of human A53T-aSYN in the LC region are not transferred to other neuronal populations within the investigated 9 weeks. A longer time period and subsequently higher aSYN burden in the LC system may be necessary to enable such a transfer at later time points. This is in line with the finding that despite the severe degree of axonal aSYN accumulation in the SN region after 9 weeks of A53T-aSYN overexpression in the LC region, no statistically significant SN neurodegeneration was observed (Fig. 8).

Open questions and limitations

In this study, we have decided to overexpress human mutant A53T-aSYN by injection of a previously well-established rAAV vector. The vector used in this study has proven effective in inducing progressive neurodegeneration of SN neurons by several groups in several PD animal models [32, 33, 95–97]. In this context, it would be of relevance to investigate whether overexpression of wild-type aSYN in the LC would have led to a different histopathological phenotype. Considering the lower rate of β-sheet and fibril formation of wild-type aSYN compared to the A53T-aSYN variant [98, 99], one could hypothesize that overexpression of wild-type aSYN might lead to milder histopathological alterations, but this has to be demonstrated in the LC model in a further study. For this initial study we have focused on a relatively short time frame of up to 9 weeks which allowed us to characterize the initial, local and time-dependent histopathological alterations of LC neurons caused by A53T-aSYN. Since 9 weeks is likely too short to observe the full neuropathology, a future study containing longer observation times of up to 52 weeks or even longer would be suggested. This would allow to further investigate whether trans-synaptic spread of aSYN and subsequent degeneration of dopaminergic SN neurons occur at a later time-point. As our study primarily aims to address the histopathological consequences of A53T-aSYN overexpression in LC neurons, we have not carried out a behavioral assessment. Nevertheless, the model would benefit from a thoroughly carried out behavioral characterization, including sleep recordings covering the possible occurrence of any non-motor or subtle motor symptoms.

Conclusions

In a time, in which on one hand the clinical research focus shifts away from the neurodegeneration of the dopaminergic nigrostriatal pathway towards the prodromal stages of PD and on the other hand the first potentially disease modifying therapies enter clinical testing [100], animal models mimicking the prodromal phase of PD are needed. In this study, we have reproduced cardinal histopathological features of the human LC PD-pathology, delineated the time-course of noradrenergic neurodegeneration and characterized robust histological markers, which are sufficient to assess the pathological changes in a quantitative and qualitative way. Taken together, this animal model may contribute to the research on the pathophysiology of the prodromal stage of PD. Further studies with longer observation times and additional characterization (e.g. behavioral assessment, biochemical analyses) are required to determine whether the herein presented model will prove helpful in the development and testing of disease-modifying therapy.

List of abbreviations

α-synuclein – aSYN; dopaminergic – DA; locus coeruleus – LC; luciferase – Luc; paraformaldehyde – PFA; Parkinson's disease – PD; phosphate buffer – PB; phosphate buffered saline – PBS; phosphorylated α-synuclein – p-aSYN; proteinase K – PK; recombinant adeno-associated viral – rAAV; REM sleep behavior disorder – RBD; substantia nigra – SN; tyrosine hydroxylase – TH

Declarations

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Authors' contributions

WHO, LAM and WHC designed the study. MTH, FFG and BL performed experiments, conducted immunohistochemical analysis and analyzed data. WHO, LAM and ND supervised the project. MTH, FFG, LAM and WHO wrote the manuscript. ND, LT, JBK and JMB critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All animal experiments were approved by the local authorities (Regierungspräsidium Giessen, Germany V54-19 c 20 15 h 01 MR 20/15 Nr. 66/2015).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Competing interests

The authors declare no competing financial interests. JBK and JMB have equity stakes in, and have received consultancy fees from, Atuka Inc., outside of the submitted work. WHO received personal fees for educational talks and/or consultancy, outside of the submitted work, from Abbvie, Adamas, Bristol-Myer-Squibb, Desitin, Mundipharma, Neuropore, Novartis, Roche and UCB Pharma and grants from the Deutsche Forschungsgemeinschaft, the International Parkinson-Fonds The Netherlands, the Michael J. Fox Foundation, USA, the National Research Fond Luxembourg and from Novartis Pharma, Germany.

Consent for publication

Not applicable.

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8.2. Publication 2

The locus coeruleus – another vulnerability target in Parkinson's disease

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Introduction

The α-synucleinopathy Parkinson's disease (PD) affects, in addition to the dopaminergic substantia nigra pars compacta (SNc), other vulnerable neurotransmitter systems in the CNS, for example the noradrenergic locus coeruleus (LC) or cholinergic neurons in the vagal nuclear complex. The histopathological distribution of α-synuclein (aSYN) aggregates containing Lewy-bodies and neurites, the pattern of neurodegeneration, various imaging studies, and the broad symptomatology indicate that PD neuropathology does not develop simultaneously in all vulnerable brain regions, but rather in a sequential way.[1] In REM sleep behavior disorder (RBD), a specific prodromal stage of PD, both clinical (imaging studies) and neuropathological evidence indicates that the region of the noradrenergic LC system is involved early in the topographical sequence of pathological changes, years before the dopaminergic SNc is affected and motor symptoms become apparent.[2–4] The resulting central deficiency of noradrenergic neurotransmission contributes to both the non-motor and motor symptomatology of PD.[5,6] Furthermore, experimental evidence from PD animal models suggests that deficient LC-noradrenergic neurotransmission enhances the nigral toxicity of several neurotoxins (e.g. MPTP or 3,4-Methylendioxy-N-methylamphetamin),[7–9] whereas an increase of noradrenergic neurotransmission may exert a neuroprotective effect on the SNc.[10] This introduces an intriguing possibility for disease-modification.

This viewpoint article argues that the early involvement of the LC in the progression of PD and its contribution to the pathophysiology and symptomatology places the LC in a unique position. Future research on the LC-noradrenergic system should include: 1) testing of symptomatic therapy for alleviating noradrenergic deficiency; 2) the development of disease-modifying therapeutic approaches at the LC level based on the reported potential neuroprotective effect of the LC on the dopaminergic SNc; and 3) the search for LC-related disease progression biomarkers.

The noradrenergic LC – a structure to be rediscovered for PD research

The human noradrenergic LC, a small nucleus in the pontine brainstem, contains only around 35 000 neurons per hemisphere[11] while representing the major source of noradrenaline (NA) for vast parts of the human brain. LC cells are involved in several highly preserved brain functions including, but not limited to, generation of arousal, facilitation of behavioral adaptions following new sensory information, memory consolidation, learning, modulation of motor control, and regulation of local blood flow. In PD, Lewy-body formation, axonal loss of noradrenergic projections, and altered synaptic morphology of LC cells are features of the early phase of the disease.[12,13] Several lines of evidence indicate that accumulation of pathological aSYN in LC cells occurs not just in early disease stages, but it also exceeds the observed Lewy pathology in the SNc.[1,14] The resulting decrease of NA in the neocortex, thalamus, hypothalamus, and cerebellum contributes to several non-motor symptoms of PD, including cognitive impairment, affective symptoms such as depression, anxiety, apathy, fatigue, and REM sleep behavior disorder.[15–18] Furthermore, dysfunctional noradrenergic

neurotransmission is also implicated in impaired motor control and freezing of gait.[5,6] Notably, despite the early and profound burden of Lewy pathology and loss of noradrenergic axons in output projection targets, the majority of LC neurons can survive the pathological process for many years, thereby even outliving the loss of SNc neurons.[14,19] Although unbiased stereological quantifications are lacking, reported LC cell loss ranges from 21-93% and is commonly observed in advanced PD stages.[20] The time lag between early alterations of LC neurons and the final cell loss during advanced disease stages leaves the LC for many years in a dysfunctional state.[1,14,19] It is therefore tempting to speculate that LC neurons may have so far unidentified intrinsic properties which render them partially resilient to the disease process. This could explain the significantly longer duration between cellular pathological changes (Lewy body formation) and neuronal cell death in the noradrenergic LC compared to the dopaminergic SNc.

Determinants of LC vulnerability in PD

Noradrenergic LC cells share common morphological, electrophysiological, and metabolic features with other neuronal cell groups known to degenerate in PD. These intrinsic cellular factors are thought to render certain neuronal populations particularly vulnerable to the disease process. For the LC system, these include an extensive axonal arborization with multiple synaptic and paracrine neurotransmitter release sites that lead to high energetic demand, the electrophysiological phenotype of a pacemaker neuron continuously generating slow tonic spiking, the burden to generate and metabolize a highly reactive neurotransmitter, high amounts of intracellular neuromelanin and heavy metals, and its location directly next to the 4th ventricle (Fig. 1).[12,21] All of these features combined set the LC in a critical at-risk position regarding energetic failure, metabolic burden, and possible exposure to toxins or inflammatory cytokines.[12,21] The studies conducted so far on LC vulnerability and degeneration in PD animal models (Table 1) further reveal that neurotoxins which are commonly used to lesion the nigrostriatal system, e.g. MPTP or the pesticide rotenone, also cause degeneration of the LC. Furthermore, aSYN-overexpression models demonstrate that LC cells are susceptible to artificially increased intracellular aSYN levels.[22] Compared to the wealth of studies conducted on the SNc, research on the LC in the preclinical as well as in the clinical setting is sparse. This situation offers the unique opportunity to transfer the existing expertise on catecholaminergic neurons and the fast growing body of knowledge on the nigrostriatal system to research on the LC-noradrenergic system.

LC pathology in prodromal and manifest PD – opportunities for improved symptomatic treatment and neuroprotection

The increasing knowledge of noradrenergic deficiency in PD has resulted in several promising attempts to restore noradrenergic neurotransmission for improved control of certain non-motor and motor manifestations (Table 2). The studies conducted so far employed mainly three distinct

pharmacological strategies: 1) direct increase of NA by administration of NA precursor substances (droxidopa/L-threo-DOPS); 2) increasing the available concentration of NA in the synaptic cleft by synaptic reuptake inhibitors selective for NA alone (atomoxetine, reboxetine) or NA and serotonin (duloxetine, venlafaxine) or NA and dopamine (methylphenidate); or 3) enhanced synaptic release of NA by presynaptic α_2-adrenoreceptor antagonists (idazoxan, fipamezole). The results obtained indicate that enhancement of noradrenergic neurotransmission can alleviate several non-motor and motor manifestations of PD while simultaneously increasing patients' quality of life. Notably, all of the trials listed were conducted in de novo or manifest PD patients. We therefore argue that efforts should be increased and noradrenergic replacement therapy should be carried forward in prodromal PD patient groups as manifestations of noradrenergic shortage, such as depression, anxiety, or cognitive impairment are already evident.[23,24]

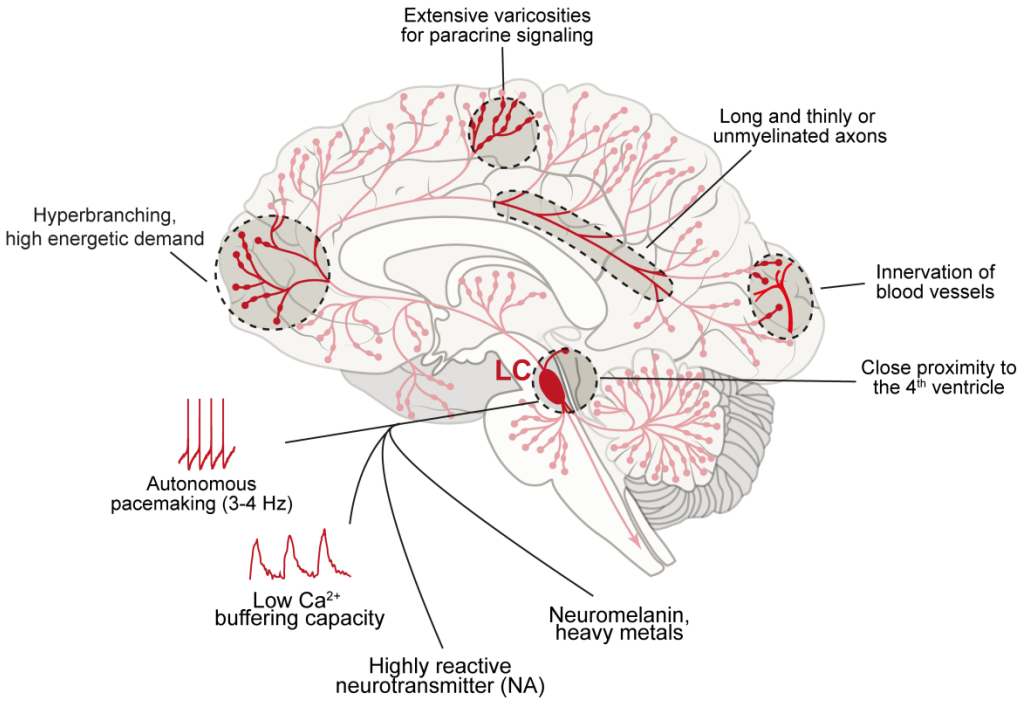

Figure 1 | Vulnerability factors of noradrenergic LC neurons.

			Species	Reference	LB/LN -like struc-tures	Loss of neurons in the SNc (%)	Loss of neurons in the LC (%)	Loss of neurons in other regions (%)	Biochemical / electrophysiolo-gical alterations
Toxin-based models	MPTP i.p.		NHP	Forno et al. 1986[41]	In LC/SNc	Moderate to severe degeneration	Focal lesions, degree comparable to SNc	–	–
			NHP	Mitchell et al. 1985[42]	–	Considerable damage	Obvious damage in 1/3 monkeys	Considerable damage to the cells of the VTA	50% ↓ NA in CTX, and ACB >90% ↓ DA in vSTR and dSTR
			Swiss Webster mice	German et al. 2000[43]	–	47	None	–	48% ↓ NA in CTX 68% ↓ DA in STR
			C57Bl/6J mice	Seniuk et al. 1990[44]	–	Dose-dependent degeneration	Dose-dependent degeneration	Dose-dependent degeneration of VTA, A13	↓ NA in CTX ↓ DA in STR
			C57Bl/6 mice	Gupta et al. 1986[45]	–	86	64	66 (VTA)	–
			C57Bl/6J mice	Fornai et al. 2004[46]	In SNc/LC	~90	~60	–	Marked decline of DA, DOPAC, HVA in STR Up to 80% ↓ NA in forebrain
	Rotenone	s.c.	Wistar rats	Lin et al. 2008[47]	SNc	~10	~60	–	
		i.v.	Lewis rats	Betarbet et al. 2000[48]	SNc	Mild to severe	Mild to moderate	VTA relatively spared	Striatal dopaminergic denervation from partial to complete
	Paraquat i.p.		Mice	Fernagut et al. 2007[49]	None	26.7	26.2	–	–
	6-OHDA intraventricular		Sprague-Dawley rats	Chiodo et al. 1983[50]	–	–	–	–	increase in firing frequency of LC neurons 85-90% ↓ NA in HC and CBX No change of NA in LC
			Sprague-Dawley rats	Descarries et al. 1972[51]	–	–	20-85 (time-dependent)	–	–
Genetic models	Parkin$^{-/-}$		Mice	Von Coelln et al. 2004[52]	–	None	~20	–	No change in striatal DA, DOPAC, HVA No change of NA in CTX, BS, HC, CBX ~30% ↓ NA in OB ~20% ↓ NA in spinal cord
			Mice	Key et al. 2019[53]	–	–	–	–	Acceleration of the spontaneous pacemaker frequency of LC neurons
	PINK1$^{-/-}$		Rats	Grant et al. 2015[54]	In LC/SNc / PAG/AMB	None	41	–	–
	Thy1-WT-aSYN		Mice	Fernagut et al. 2007[49]	In SNc/LC	None	None	–	–
	PrP-A53T-aSYN		Mice	Sotiriou et al. 2010[55]	–	None	None	–	↓ NA (-33% STR, -40% OB, -31% spinal cord) No change of NA in CTX No change of DA, DOPAC, HVA in STR
Over-expression models	CMV-A53T-aSYN in the LC		C57Bl/6N mice	Henrich et al. 2018[22]	In LC	None	Up to 56.25	–	–
	Syn1-WT-aSYN in vagal nerve		Sprague-Dawley rats	Rusconi et al. 2017[56]	–	–	Up to 15	Up to 30 in DMV No cell loss in AMG	–

Table 1 | Involvement of the LC-noradrenergic system in animal models of PD

Abbreviations: ACB, nucleus accumbens; AMG, amygdala; CBX, cerebellar cortex; CTX, cortex; DA, dopamine; DMV, dorsal motor nucleus of the vagal nerve; DOPAC, 3,4-Dihydroxyphenylacetic acid; HC, hippocampus; HVA, homovanillic acid; LB, Lewy body; LN, Lewy neurite; OB, olfactory bulb; STR, striatum; vSTR, ventral striatum; VTA, ventral tegmental area.

Another aspect to be considered is the growing body of experimental evidence that suggests the LC exerts neuroprotective effects on the nigrostriatal system. Early research on the LC in MPTP-based animal models indicated a neuroprotective role of NA neurotransmission on SNc survival. Ablation of LC neurons by systemic N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) or local 6-OHDA injection prior to systemic MPTP treatment resulted in enhanced neurodegeneration of DA SNc cells.[7–9] In contrast, noradrenergic hyperinnervation reduced the MPTP-induced nigral toxicity.[10] While the NA neuroprotection hypothesis has not been followed up initially, a series of more recent experiments has put this idea on firmer footing. Administration of reboxetine decreased the magnitude of SNc degeneration in a progressive parkinsonian mouse model[25] in which nigral neurodegeneration was induced by inhibition of rRNA synthesis due to genetic ablation of the transcription initiation factor IA (TIF-IA). In addition, the β_2-agonists salmeterol and clenbuterol facilitated a reduction of MPTP-induced SNc cell loss.[26,27] Taken together, these studies suggest that augmentation of noradrenergic neurotransmission might not only alleviate symptoms manifesting as a result of NA deficiency, but may even have disease-modifying efficacy by exerting neuroprotective effects on the SNc. Finally, ongoing trials and studies being planned of potentially disease modifying compounds that target aSYN in the oligomeric or aggregate form should assess LC-related outcome measures, since systemic treatment against α-synucleinopathy might not only improve the function of the dopaminergic, but also of the noradrenergic system.

Kinetics of LC neurodegeneration – Potential for novel LC progression biomarkers?

In the last 10 years, increasing efforts have been undertaken to identify new biomarkers that correlate with PD progression and thereby allow for monitoring ongoing neurodegenerative alterations and the therapeutic efficacy of a given neuroprotective compound in clinical trials.[28] Ideally, such a marker should reflect the prodromal phase of the disease as well as the advanced stages, and its changes should be linked directly to the progressing neuropathology. In 2019, three groups of prodromal PD patients are, in principle, available for clinical research for identification and characterization of PD progression markers: the asymptomatic carriers of one of the numerous mutations of the 1) leucine-rich repeat kinase 2 (LRRK2), or 2) glucocerebrosidase (GBA) genes, and 3) patients suffering from RBD. We argue that involvement of the noradrenergic LC, most likely in RBD, fulfills several key requirements for research on new disease progression markers. These consist of: 1) involvement in the prodromal phase of PD; 2) the long time lag between initial Lewy-pathology and neurodegeneration in advanced PD stages; 3) contribution to several early non-motor symptoms; and 4) LC pathology in not only idiopathic PD but also in PD caused by genetic alterations (e.g. SNCA

duplication/triplication/point mutation carriers, LRRK2 and GBA mutation carriers)[29] and RBD patients.[2,3]

Mechanism of action	Drug	Reference	Target	Outcome	Study design
NA precursor	Droxidopa / L-DOPS	Biaggioni et al., 2017[57]	Orthostatic hypotension	Improved symptoms Increase in standing systolic BP	DB, OL, PC
		Kaufmann et al., 2014[58]	Orthostatic hypotension	Improved symptoms Increase in standing systolic BP	R, PC
		Fukada et al., 2012[59]	FOG	Improvement FOG only when co-administered with entacapone	R, OL
SNRI	Atomoxetine	Weintraub et al., 2010[60]	Depression	No significant change in depression	R, PC, DB
			Global cognition	Improvement in global cognition	
			Daytime sleepiness	Improvement in daytime sleepiness	
		Kehagia et al., 2014[61]	Impulsivity	Improvement in distinct behavioral tasks	DB, R, PC
		Marsh et al., 2009[62]	Executive dysfunction	Improvement in executive dysfunction	OL
	Reboxetine	Pintor et al., 2006[63]	Depression	Improvement of depressive symptoms	OL
		Lemke et al., 2002[64]	Depression	Improvement of depressive symptoms	OL
SSNRI	Duloxetine	Djaldetti et al., 2007[65]	Central pain	Subjective pain relief Quantitative pain threshold did not change	OL
	Venlafaxine	Takahashi et al., 2019[66]	Depression	Improvement in depression	R, OL
			Apathy	No change in apathy scores	
			FOG	Improvement of FOG	
		Richard et al., 2012[67]	Depression	Improvement of depressive symptoms	R, DB, PC
DNRI	Methylphenidate	Devos et al., 2006[70]	Gait disorders	Improvement of gait and motor symptoms	RM
		Moreau et al., 2012[71]	Gait disorders, FOG	Improvement of gait and FOG	R, DB, PC
		Espay et al., 2011[68]	Gait impairment	No improvement of gait Worsening of motor function, sleepiness and QoL	R, PC, DB
		Mendonça et al., 2007[69]	Fatigue	Significantly lower fatigue scores No change in motor function	R, DB, PC
α_2-agonist	Clonidine	Riekkinen et al., 1999[72]	Spatial working memory	Improvement in spatial working memory	OL
			Attentional set shifting	No effect on attentional set shifting	
α_2-antagonist	Idazoxan	Rascol et al., 2001[73]	LID	Improvement of LIDs No worsening of parkinsonism	R, PC
	Fipamezole	LeWitt et al., 2012[74]	LID	Improvement of LIDs No worsening of parkinsonism	DB, R, PC

Table 2. Selected clinical trials with noradrenergic agents in PD

Abbreviations: CO, crossover design; DB, double-blinded; DNRI, dopamine and noradrenaline reuptake inhibitor; FOG, freezing of gait; L-DOPS, L-threo-3,4-dihydroxyphenylserine; LID, levodopa induced dyskinesia; OL, open label; PC, placebo-controlled; R, randomized; SNRI, selective noradrenaline reuptake inhibitor; SSNRI, selective serotonin and noradrenaline reuptake inhibitors; QoL, quality of life.

In the following, we will summarize three possible avenues for further potential disease progression biomarker development.

Neuromelanin-sensitive MR imaging (NM-MRI) visualizes neuromelanin, a dark colored pigment found in high concentrations in the catecholaminergic neurons of the SNc and LC as T1 hyperintense regions.[30,31] Studies of LC dysfunction and degeneration in manifest PD patients report significant bilateral reduction of NM-MRI signal intensity in the LC region, suggesting a loss of pigmented noradrenergic neurons.[32,33] Application of the NM-MRI technique in RBD cohorts has revealed similar results, thus indicating early involvement of neuromelanized LC neurons in the disease process.[4,34] However, no data are available regarding LRRK2 or GBA prodromal patients, and follow-up studies in the RBD cohorts have not been reported. Therefore, a conclusion of whether NM-MRI imaging can be used as a progression marker of LC degeneration awaits further research.

PET imaging to monitor noradrenergic LC function during the course of PD is now possible due to optimized monoaminergic radiotracers and general technological progress. The studies conducted to date in manifest PD patients showed an increase of tracer binding in the LC region in early motor PD, indicating a compensatory up-regulation of noradrenergic function in early disease stages.[35,36] Interestingly, a recent 3-year follow-up study reported that, after the initial increase, an annual decline of 7.8% of ^{18}F-DOPA uptake in the LC region takes place, suggesting progressive degeneration of LC cells in manifest PD.[36] Furthermore, the use of a newly developed noradrenergic-specific radiotracer on manifest PD patients[37] revealed a significant decrease of tracer binding in known LC output regions, such as the red nucleus and the thalamus, likely reflecting noradrenergic denervation in those structures.[37] In light of a lack of data in RBD, LRRK2 and GBA prodromal PD patients, it is essential to carry noradrenergic PET imaging forward into prodromal patient cohorts.

Attentional set shifting refers to the ability of switching the focus of attention between different perceptual dimensions. When combined with pupillometry the Wisconsin Card Sorting Test (WCST) or the Intra-/Extra-Dimensional Attentional Set-Shifting Task (IED) can be used to investigate LC function in humans. According to rodent studies, performance in attentional set shifting is highly dependent on prefrontal cortical noradrenergic activity originating in the LC.[38] Moreover, it is already known that PD patients perform worse in shifting their focus of attention during the WCST.[39,40] However, there is no pupillometric data available during attentional set shifting in manifest, not to speak of prodromal PD patient groups.

Conclusion

In this viewpoint article we argue that the LC represents a suitable structure for identification of prodromal disease progression markers in PD in order to monitor ongoing neurodegeneration during the prodromal phase of the disease. The wealth of information available on the physiology and pathophysiology of the dopaminergic nigrostriatal neurons is abundant, and a clinical phenotype

hallmarking a lesion at level of the LC, i.e. RBD, is available. Thus, by combining both assets, it should be possible to reach a level of knowledge on the LC which will have an impact on the discovery of prodromal PD progression markers and development of symptomatic or disease-modifying treatments.

Authors' roles

(1) Research Project: A. Conception, B. Organization, C. Execution. (2) Manuscript Preparation: A. Writing of First Draft, B. Review and Critique, C. Design of Figures, D. Final Editing.

W.H.O.: 1A, 1B, 1C, 2A, 2D.

M.T.H.: 1A, 1B, 1C, 2B, 2D.

A.J.: 1A, 2B.

F.F.G.: 1A, 1B, 1C, 2B, 2C, 2D.

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8.3. Publication 3

Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease

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Abstract

Neurodegeneration of the nigrostriatal dopaminergic system and concurrent dopamine (DA) deficiency in the basal ganglia represent core features of Parkinson's disease (PD). Despite the central role of DA in the pathogenesis of PD, dopaminergic systems outside of the midbrain have not been systematically investigated for Lewy body pathology or neurodegeneration. Dopaminergic neurons show a surprisingly rich neurobiological diversity suggesting that there is not one general type of dopaminergic neuron but rather a spectrum of different dopaminergic phenotypes. This heterogeneity on the cellular level could account for the observed differences in susceptibility of the dopaminergic systems to the PD disease process.

In this review we will summarize the long history from the first description of PD to the rationally derived DA replacement therapy, describe the basal neuroanatomical and neuropathological features of the different dopaminergic systems in health and PD, explore how neuroimaging techniques broadened our view of the dysfunctional dopaminergic systems in PD, and discuss how dopaminergic replacement therapy ameliorates the classical motor symptoms but simultaneously induces a new set of hyperdopaminergic symptoms.

Keywords

Parkinson disease; dopamine; dopaminergic systems; motor symptoms; dopaminergic therapy; L-DOPA

List of abbreviations

123I-IBZM, 123I-iodobenzamide; 18F-FDG, 18F-fluorodeoxyglucose; AADC, aromatic L-amino acid decarboxylase; DA, dopamine; DAT, dopamine transporter; ICD, impulse control disorder; LB, Lewy body; LID, L-DOPA induced dyskinesia; LN, Lewy neurite; MCI, mild cognitive impairment; MSA, mutiple system atrophy; PD, Parkinson's disease; PDD, Parkinson disease dementia; PSP, progressive supranuclear palsy; RBD, REM sleep behavior disorder; RRF, retrorubral field; SN, substantia nigra; STR, striatum; TH, tyrosine hydroxylase; UPDRS, Unified Parkinson's Disease Rating Scale; VMAT2, vesicular monoamine transporter 2; VTA, ventral tegmental area.

Introduction

The basic clinical symptomatology of Parkinson's disease (PD) is well-known for 200 years and has been expanded ever since. But how can we identify the neuropathological correlates of these symptoms? How can we link symptoms to certain brain circuit dysfunction, or vice versa, how can we predict the clinical manifestation of a dysfunctional system? The comprehension of the association of neuronal systems and physiological functions/dysfunctions is crucial for the rational development of therapy to alleviate disabling symptoms. To investigate the link between symptomatology and neuropathology in humans we own the following toolkit: (i) post-mortem neuropathological studies to explore neurodegeneration, distribution of Lewy bodies/neurites (LB, LN) and biochemical alterations; (ii) neuroimaging studies in combination with radiotracers to examine dysfunctional neurotransmission; and (iii) clinical studies investigating the potential of certain medications to alleviate, worsen or even provoke certain symptoms.

In this review, we will briefly summarize the long road to the discovery of a dysfunctional dopaminergic system in PD laying the ground for the still up-to-date gold standard of therapy and then focus on the emerging evidence of the dysfunctional dopaminergic systems of the brain in PD.

A long road to go

The first medical description of PD dates back to 1817 when James Parkinson published his monograph entitled 'An Essay on the Shaking Palsy' based on the depiction of the clinical picture of six patients (Fig. 1) (Parkinson 1817). Fifty-five years later, in 1872, Jean-Martin Charcot identified bradykinesia as a defining feature of PD and suggested that tremor is not an obligate symptom of the disease. He therefore proposed the term "Parkinson's disease" thereby arguing against the term 'shaking palsy'. At this time, neuropathological correlate(s) of the diverse symptoms had not been resolved and PD remained a highly debilitating disorder without effective treatment.

Almost one hundred years after the first description, neuropathological studies brought a first breakthrough in PD linking degeneration of the substantia nigra (SN) to the characteristic parkinsonian motor symptoms. In 1893, Georges Marinesco and Paul Blocq were the first to suggest that a lesion of the midbrain could contribute to the motor symptoms seen in PD. Their hypothesis was based on an autopsy of a patient with unilateral parkinsonism, which revealed a tuberculous nodule confined to the right cerebral peduncle. Two years later, Edouard Brissaud hypothesized that the SN might be the major pathological site of PD. This hypothesis was validated by the pioneering work of Constantin Trétiakoff in 1919 who demonstrated substantial loss of pigmented nigral cells in post-mortem PD brains and inclusion bodies in the remaining neurons which he called 'corps de Lewy' (Lewy body, LB), in honor of their first describer Friederich H. Lewy (Trétiakoff 1919). The neurochemical consequences of SN degeneration, that is, dopamine (DA) deficiency in the basal ganglia of PD patients however remained unknown until 1960.

Thus, almost forty years later, in 1957, Arvid Carlsson demonstrated in a pioneering work that administration of reserpine lead to depletion of brain DA levels and onset of motor deficits in animals mimicking the symptomatology of parkinsonism. He also proved that application of L-DOPA, a blood-brain barrier-passing precursor of DA and noradrenaline could alleviate these symptoms by restoring the brain DA to normal levels (Carlsson 1959, Carlsson et al. 1957). This work built the basis for the DA era of PD and was later honored with the Nobel Prize of medicine. Soon after this, Oleh Hornykiewicz and Herbert Ehringer demonstrated that DA is depleted in the putamen, caudate nucleus and SN of post-mortem brains of Parkinson patients (Hornykiewicz 1963, Ehringer and Hornykiewicz 1960). Subsequently, they intravenously administered L-DOPA to volunteering patients. The effect of this therapy was the complete abolishment of the akinesia (Birkmayer and Hornykiewicz 1961). Thus, they introduced L-DOPA to the field of neurology as the first rationally developed therapy of PD. In 1970, based on the elaborative work of George C. Cotzias (Cotzias et al. 1969), the US Food and Drug Administration (FDA) finally approved L-DOPA as the first drug to treat PD.

Although we know for a long time that the dopaminergic system of the brain is neither among the first regions affected in the course of the disease, nor is it solely accountable for the wide spectrum of symptoms, the gold standard of therapy is still based on the restoration of dopaminergic neurotransmission by means of administration of L-DOPA or DA receptor agonists (Oertel 2017).

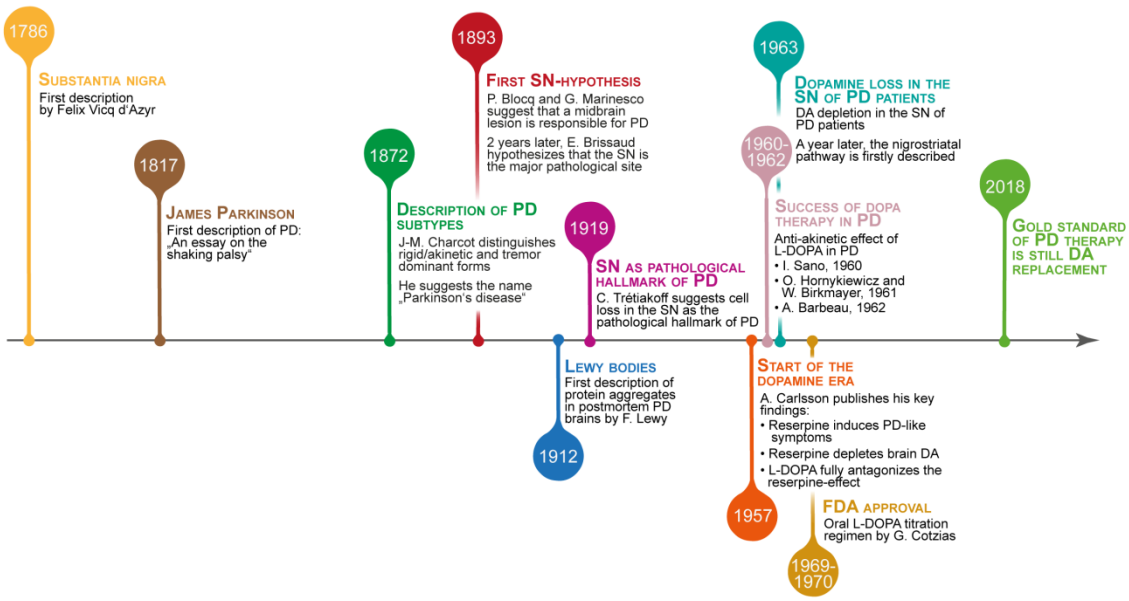

Figure 1 | Milestones of PD pathogenesis and therapy

Parkinsonism as the core feature of PD

PD is a clinical diagnosis based on the occurrence of the characteristic parkinsonian motor abnormalities plus at least two supportive criteria and the complete absence of absolute exclusion criteria and red flags (Table 1) (Postuma et al. 2015). The three cardinal motor features of PD are bradykinesia/hypokinesia, tremor, and rigidity. Typically, PD patients initially present with unilateral motor signs, most commonly with akinesia in combination with resting tremor affecting one of the upper extremities (Pallone 2007). The motor symptoms then gradually spread to the contralateral and lower limbs, but the initial asymmetry remains (Weintraub et al. 2008, Rodriguez-Oroz et al. 2009). Bradykinesia means slowness of movement, whereas hypo-/akinesia is defined as reduced or diminished amplitude and frequency of spontaneous movements (Rodriguez-Oroz et al. 2009). Patients often describe this as 'weakening of the limb', but upon examination, the muscle strength is not altered. Bradykinesia usually presents as a slowness in everyday routine activities and reduced unilaterally arm swing during walking (Lewek et al. 2010, Jankovic 2008). Other signs of this symptom can be a decreased blinking rate (Biousse et al. 2004, Karson 1983), reduced facial expressions (hypomimia) and gesturing, micrographia and a monotone, soft speech (hypophonia) (Ho et al. 1999). Resting tremor (4-6 Hz), representing the most obvious and therefore stigmatizing symptom of PD, is defined as an involuntary rhythmical movement of a body part, which affects usually one of the upper extremities in the early phase of the disease (Jankovic 2008). Tremor is commonly one of the first motor signs to appear, and starts generally in the fingers or the thumb, resulting in the typical "pill-rolling tremor" (Kalia and Lang 2015). It becomes apparent during resting state, weakens or even disappears during voluntary movement of the limb and worsens when the patient is stressed or anxious. Rigidity refers to an increased muscle tone in both the agonist- and antagonist muscles resulting in stiffness of the limb. Upon clinical examination, a resistance to passive movement in the extremity can be observed. The resistance may be either smooth (lead-pipe phenomenon) or fluctuating (cogwheel rigidity) (Weintraub et al. 2008), the latter rather representing a mixture of tremor and rigidity.

STEP 1. DIAGNOSIS OF PARKINSONISM Bradykinesia/hypokinesia + one of the following: • Resting tremor • Rigidity
STEP 2. SUPPORTIVE CRITERIA At least 2 out of 4 • Clear and dramatic response (>30% in UPDRS III score) to dopaminergic therapy • L-DOPA-induced dyskinesia • Resting tremor of a limb • Positive test of either olfactory dysfunction or cardiac sympathetic denervation (scintigraphy)
STEP 3. ABSENCE OF ABSOLUTE EXCLUSION CRITERIA
STEP 4. ABSENCE OF RED FLAGS

Table 1 | Diagnostic criteria for PD

As the disease advances, postural instability becomes progressively apparent, representing the most common cause of falls and significantly decreasing the quality of life (Williams et al. 2006, Koller et al. 1989, Michalowska et al. 2005). Although postural instability usually develops during the course of the disease, it is mostly not present in early PD and an early occurrence therefore suggests an alternative diagnosis (Jankovic 2008, Postuma et al. 2015). The combination of cardinal motor symptoms, impairment of balance and an anterior shift of the mean center of gravity position finally results in the fully evolved late-stage parkinsonian posture and gait: the patient is bending forward into a flexed truncal position and the stride length and walking pace substantially decrease. The patient begins to shuffle and may scrape the foot on the floor with reduced or absent arm swing (Morris et al. 1994, Jankovic 2008, Ebersbach et al. 2013, Błaszczyk et al. 2007).

Apart from these motor symptoms, several non-motor features occur in PD, with a substantial impact on the quality of life of patients (Schrag 2000). The most prevalent non-motor features are the following: reduced gastric and bowel motility resulting in constipation, olfactory dysfunction, sleep disturbances (e.g. REM sleep behavior disorder (RBD)), forgetfulness (cognitive decline), depression, apathy, and symptoms of autonomic dysfunction (e.g. urinary urgency, dysfunctional thermoregulation, sweating, orthostatic hypotension, erectile dysfunction) (Martinez-Martin et al. 2007). Importantly, non-motor features often precede the motor symptoms and therefore the diagnosis of PD by decades (Siderowf and Lang 2012).

In contrast to the well-known symptomatology of PD, it has been increasingly difficult to identify the neurobiological correlates underlying the parkinsonian symptoms and integrate them in a pathophysiological model that explains the origin of brady-/akinesia, tremor and rigidity. While striatal DA deficiency could be clearly linked to the onset of motor dysfunction, the wide spectrum of symptoms and compensatory mechanisms in PD cannot be attributed solely to the loss of DA.

The dopaminergic systems of the brain

To understand the link between symptoms and a dysfunctional neuronal brain circuit, it is essential to explore the neurotransmitter system and its physiological functions. Therefore, we will briefly summarize the dopaminergic systems of the brain and their implications in distinct physiological functions. The dopaminergic neurons of the mammalian central nervous system are distributed along ten distinct neuronal populations located in the ventral mesencephalon (A8-A10), diencephalon (A11-A15), olfactory bulb (A16) and retina (A17) (Fig. 2) (Björklund and Hökfelt, Björklund and Dunnett 2007, Dahlstroem and Fuxe 1964). All of these different subsystems are engaged in several biological functions such as motor, sensory and autonomic control, reward mechanisms, and cognition (Smeets and González 2000, Montague et al. 2004).

The neurons of the ventral mesencephalic dopaminergic complex (A8-A10) are morphologically indistinguishable and rather form a continuum without clear anatomical boundaries. The A8 cell group is primarily located in the retrorubral field (RRF), whereas A9 neurons are found in the SN

pars compacta, and A10 refers to dopaminergic neurons within the ventral tegmental area (VTA) (Vogt Weisenhorn et al. 2016, Yetnikoff et al. 2014). All of them form one extensive mesotelencephalic dopaminergic projection system comprising three major pathways: (i) a ventral mesostriatal or mesolimbic system which is involved in motivated behaviors predominantly originating in the VTA (A10); (ii) a mesolimbocortical or mesocortical system responsible for memory and learning, mainly originating in the VTA (A10); and (iii) a dorsal mesostriatal or nigrostriatal pathway which is engaged in voluntary motor control mainly originating in the SN pars compacta (A9) (Fig. 3) (Björklund and Dunnett 2007, Zeiss 2005, Flückiger et al. 1985). The diencephalic dopaminergic system (A11-A15) contains five distinct cell groups. The neurons of A11 are located in the periventricular gray of the caudal hypothalamus and thalamus and project mainly to the dorsal horn of the spinal cord giving rise to the diencephalospinal pathway (Flückiger et al. 1985, Watson et al. 2012). It was suggested that these neurons contribute to anti-nociception, motor and autonomic reflexes (Clemens and Hochman 2004, Lindvall et al. 1983, Fleetwood-Walker et al. 1988). The tuberoinfundibular dopaminergic neurons of the arcuate nucleus (A12) and the dopaminergic neurons of the preoptic area (A14) are engaged in neuroendocrine functions by secreting DA to the hypophyseal portal blood system thereby regulating prolactin (PRL) and growth hormone (GH) secretion (Ben-Jonathan and Hnasko 2001, Turiault et al. 2007). The A13 dopaminergic cell group is located within the medial part of the zona incerta and projects locally into the hypothalamus forming the incertohypothalamic pathway (Flückiger et al. 1985). This subsystem is engaged in the regulation of gonadotropin-releasing hormone (GnRH) secretion (Turiault et al. 2007). The neurons of the A15 cell group are located in the rostral hypothalamic periventricular area. Their function is not yet fully understood but they seem to be involved in the regulation of GnRH as well (Brown et al. 2015, Clarkson and Herbison 2011).

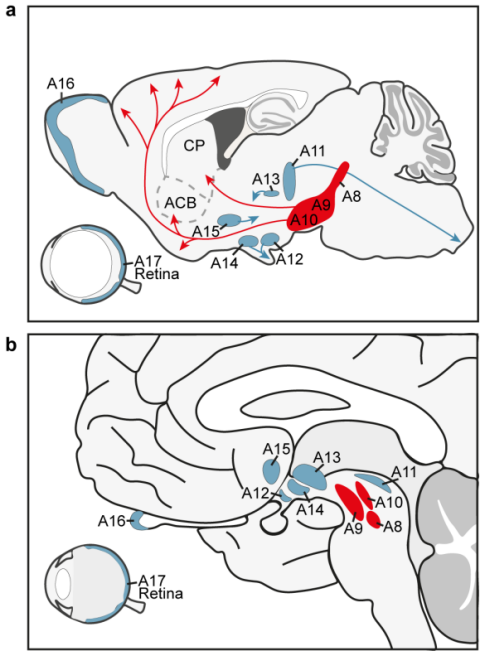

Figure 2 | Localization and PD-pathology of the dopaminergic systems in mice (a) and humans (b).
The color of the different nuclei refers to the presence or absence of pathology seen in PD. green – not affected, red – affected, blue – not sufficient data available. (**a**) modified from (Björklund and Dunnett 2007)

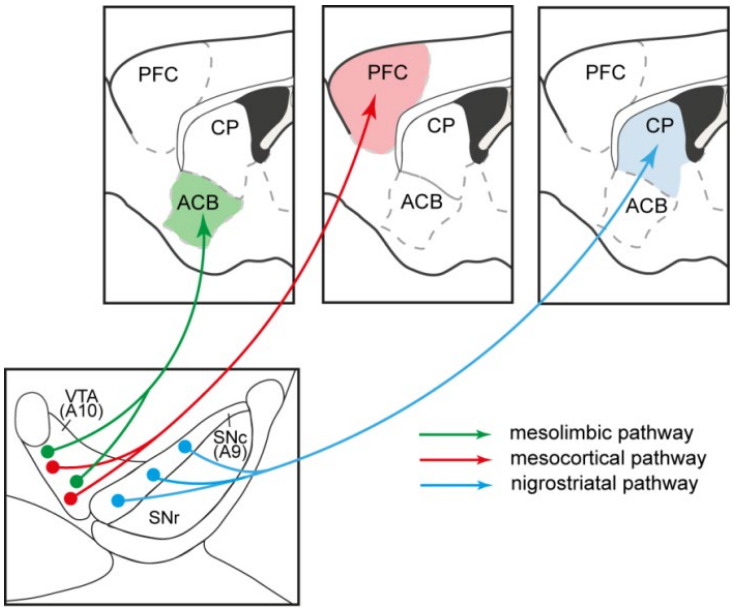

Figure 3 | Mesotelencephalic pathways. The A8-A10 dopaminergic cell groups are found in the ventral midbrain.

They form one extensive mesotelencephalic pathway comprising three major pathways: (1) mesolimbic pathway projecting mainly from the VTA to the ventral STR; (2) mesocortical pathway mainly originating in the VTA and projecting to the prefrontal cortex (PFC); and (3) nigrostriatal pathway originating in the substantia nigra and projecting to the dorsal STR. Abbreviations: ACB – nucleus accumbens, CP – caudoputamen; VTA – ventral tegmental area, SNr – substantia nigra pars reticulata; SNc – substantia nigra pars compacta

The A16 dopaminergic cells of the olfactory bulb are interneurons found in the periglomerular layer (Halász et al. 1981) and play a pivotal role in odor discrimination and odor processing (Wilson and Sullivan 1995, Tillerson et al. 2006, Taylor et al. 2009). The retinal dopaminergic cells (A17) are neurons of the amacrine subtype found in the inner nuclear and inner plexiform layers of the retina (Archibald et al. 2009). Retinal dopaminergic neurotransmission plays a central role for contrast sensitivity, visual acuity and retinal light adaption by induction of the transition from the rod-circuit (dark-adapted state) to the cone-circuit (light-adapted state) (Archibald et al. 2009, Jackson et al. 2012, Korshunov et al. 2017, Ribelayga et al. 2008).

What are the prerequisite features a neuron has to possess to be considered dopaminergic? The classical dopaminergic neuron is defined by the presence of: (i) DA, (ii) DA synthetizing enzymes (i.e. tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC)), (iii) DA degrading enzymes (i.e. monoamino oxidases), (iv) DA transporters (i.e. vesicular monoamine transporter 2 (VMAT2), DA transporter (DAT)), and (v) autoreceptors (i.e. D_2-receptor) (Vernier et al. 2004). Simultaneously, dopaminergic neurons lack dopamine-β-hydoxylase and phenylethyl-N-methyl transferase, the two enzymes required for the conversion of DA into noradrenaline and subsequently adrenaline (Vernier et al. 2004). Importantly, not all of the above mentioned neuronal populations (A8-A17) contain the complete set of proteins involved in dopaminergic neurotransmission, that is, some cell groups only partially fulfill all of the criteria of a traditional dopaminergic phenotype. For example, in the non-human primate, the A11 neurons contain TH, but at the same time lack detectable levels of AADC or DAT, suggesting that these neurons are L-DOPAergic, rather than

dopaminergic (Barraud et al. 2010). Among all dopaminergic cell groups, the A8 (RRF), A9 (SN pars compacta) and A10 (VTA) neurons exhibit the most complete dopaminergic phenotype (Vernier et al. 2004). The above introduced traditional map of the brain's dopaminergic system was generated based on detecting DA complemented with visualizing the distribution of TH immunoreactivity (Björklund and Dunnett 2007, Björklund and Hökfelt, Dahlstroem and Fuxe 1964). As a consequence, the map does not reflect the diversity of the different dopaminergic subsystems. The heterogeneity furthermore suggests that there is not one general type of dopaminergic neurons but rather a spectrum of different dopaminergic phenotypes.

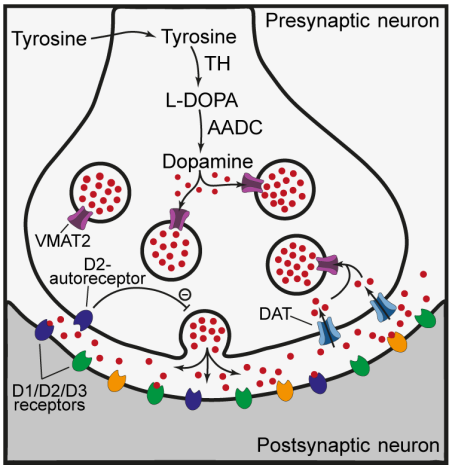

Figure 4 | A traditional dopaminergic neuron.
Abbreviations: TH – tyrosine hydroxylase; AADC – aromatic acid decarboxylase; VMAT2 – vesicular monoamine transporter 2; DAT – dopamine transporter

Neuropathological alterations of the dopaminergic systems in PD

Neuropathological studies, given their cross-sectional nature, allow the investigation of the spatial pattern of pathology, i.e. the aspect of the localization of LB pathology, neurodegeneration and consequent biochemical alterations. The advantages of neuropathological studies are: (i) high resolution in space which enables the detection of pathology at single-cell level, and (ii) detection of LB/LN pathology distribution, which is currently not possible with in vivo neuroimaging studies due to the lack of an α-synuclein radiotracer.

Although it is commonly accepted that a dysfunctional dopaminergic neurotransmission is one of the core features of PD, and that the dopaminergic systems of the brain are heterogeneous and therefore may have different susceptibility to neurodegenerative processes, the dopaminergic neuronal populations outside the midbrain have not been systematically investigated in PD.

Since the discovery of DA depletion in the SN and striatum (STR) of Parkinson patients (Hornykiewicz 1963), several neuropathological studies were conducted to estimate dopaminergic neurodegeneration of the SN pars compacta. The average loss of pigmented nigral neurons compared to age-matched healthy controls ranges between 41% and 79% across studies, on average 67% (Javoy-Agid et al. 1984, Bogerts et al. 1983, Waters et al. 1988, Hirsch et al. 1988, German et al. 1989, Alberico et al. 2015, Zarow et al. 2003, Damier et al. 1999, Gibb and Lees 1991, Kempster et al. 1989, Halliday et al. 1996). Interestingly, the neuropathological process does not homogeneously affect the

full extent of the dopaminergic SN. A characteristic topology of neurodegeneration can be observed: neurons of the ventrolateral and caudal subregion, called ventral tier are primarily affected (around 70-90% cell loss), whereas neurons in the dorsal tier are relatively resistant to the degenerative process (25-70% cell loss) (Damier et al. 1999, Fearnley and Lees 1991, Halliday et al. 1996, Hirsch et al. 1997). In consent with these findings is the uneven pattern of DA depletion in the STR. The putamen, mostly receiving input from the ventral tier of the SN, shows almost complete DA depletion (<1% of DA remaining), whereas the caudate nucleus has still substantial levels of DA (~40% of DA remaining) (Fig. 6b) (Kish et al. 1988, Waters et al. 1988, Fahn et al. 1971). One study showed that the cell loss of pigmented, neuromelanin-containing SN neurons is less than the loss of TH-positive cells at all studied timepoints, indicating that prior to cell death, dopaminergic neurons become dysfunctional and decrease their dopaminergic phenotypic expression (ghost cells) (Kordower et al. 2013). This suggests that at the time of the manifestation of the cardinal motor symptoms, symptoms most likely occur due to nigrostriatal dysfunction rather than frank neurodegeneration (Kordower et al. 2013). Besides neurodegeneration, the SN pars compacta also exhibits severe LB and LN pathology (Braak et al. 2003, Gibb and Lees 1989, Seidel et al. 2015). In fact, a combination of nigral Lewy pathology and neurodegeneration of the dopaminergic SN is highly specific for PD and even a prerequisite for the definite neuropathological diagnosis (Gelb et al. 1999, Dickson et al. 2009).

The A8 dopaminergic neurons of the RRF show minor or no degenerative changes in PD (McRitchie et al. 1997), whereas the dopaminergic neurons of the VTA (A10) show abundant LB/LN pathology (Seidel et al. 2015) and substantial neurodegeneration in PD. The reported cell loss of neuromelanin-pigmented VTA neurons in PD ranges between 40% and 77%, on average 53% (Hirsch et al. 1988, German et al. 1989, Alberico et al. 2015, Javoy-Agid et al. 1984, Bogerts et al. 1983, Uhl et al. 1985, Waters et al. 1988, Damier et al. 1999, McRitchie et al. 1997, Javoy-Agid and Agid 1980). A direct comparison of the VTA and SN cell counts is – due to the different samples and statistical methods – difficult. Only a few studies directly compared nigral and ventral tegmental neuromelanized cell counts by investigating the same midbrain tissue samples. According to their results, the degree of neurodegeneration in the SN usually exceeds that of the VTA by 20% on average (Damier et al. 1999, German et al. 1989, Hirsch et al. 1988). This suggests that, although these two cell populations have a lot of common traits, certain factors partially decrease the susceptibility of VTA (A10) neurons to neurodegeneration, and/or increase the vulnerability of SN (A9) neurons.

The diencephalic dopaminergic neuronal populations (A11-A15) have not raised much attention in PD yet, although their possible involvement in the disease process might contribute to certain autonomic and neuroendocrine dysfunctions seen in PD patients (Politis et al. 2008, Chaudhuri and Schapira 2009). It is reported that virtually all nuclei of the hypothalamus exhibit LB pathology to some extent after a certain disease duration (Langston and Forno 1978). The most severely affected hypothalamic regions are the tuberomammillary nucleus and the lateral and posterior hypothalamic nuclei, regions that do not contain dopaminergic cell groups (Langston and Forno 1978, Braak et al. 2003, Braak et al. 2004). Interestingly, the tuberoinfundibular region which exhibits the highest

density of hypothalamic dopaminergic neurons (A12) is relatively spared of LB pathology (Langston and Forno 1978). Nevertheless, to date no study exists which investigated LB formation specifically in hypothalamic dopaminergic cells. In addition, studies on hypothalamic dopaminergic neurodegeneration are also sparse. Only one study aimed to quantify pigmented neuronal cell counts in hypothalamic nuclei of PD patients. Interestingly, no significant cell loss was detected (Matzuk and Saper 1985). Taken together, studies of the hypothalamic dopaminergic system in PD are sparse and their results are controversial.

The olfactory bulb is one of the first brain regions affected during PD (Braak et al. 2003) and hyposmia is present in up to 90% of PD patients (Doty et al. 1988, Haehner et al. 2011), often preceding the classical motor symptoms by more than a decade (Kalia and Lang 2015). Several studies detected dense accumulation of LBs in granule-, mitral- and tufted cells and the anterior olfactory nucleus (Ubeda-Bañon et al. 2010, Sengoku et al. 2008, Braak et al. 2004). Interestingly, the periglomerular layer, in which the dopaminergic A16 neurons are localized, is relatively spared of the α-synucleinopathy and LBs only occasionally co-localize with TH immunoreactivity (Ubeda-Bañon et al. 2010, Sengoku et al. 2008, Cave et al. 2016). Studies estimating the number of bulbar dopaminergic neurons are contentious. Two independent studies reported that TH-positive neuronal count was doubled in the olfactory bulbs of PD patients compared to healthy controls (Huisman et al. 2004, Mundiñano et al. 2011), while other studies revealed no significant difference between PD and healthy controls (Cave et al. 2016, Ubeda-Bañon et al. 2010, Huisman et al. 2008). Taken together, dopaminergic cells of the olfactory bulb are spared of the α-synucleinopathy. However, whether their neuronal numbers increase during the disease duration and whether this change arises as a consequence of the disease process or DA replacement therapy needs to be further investigated.

Data on the retinal dopaminergic system (A17) in PD are sparse. A very recently published study was the first one to examine and describe phosphorylated α-synuclein-positive, LB- and LN-like inclusions in the retina of PD patients (Ortuño-Lizarán et al. 2018). Morphological changes were exclusively found in the ganglion cell layer and exclusively co-localized with ganglionic cell markers thereby excluding the possibility of LB-formation in dopaminergic amacrine cells. However, despite the lack of α-synucleinopathy in retinal dopaminergic cells, neurochemical evidence of a dysfunctional retinal DA neurotransmission exists. Retinal dopaminergic cells of PD patients show decreased TH-immunoreactivity (Nguyen-Legros 1988), and simultaneously significantly lower levels of retinal DA were measured (Harnois and Di Paolo 1990).

It has been hypothesized for a long time that a dysfunctional DA homeostasis might contribute to the selective vulnerability of catecholaminergic neurons in PD (Lotharius 2002, Lohr et al. 2014, Pifl et al. 2014, Uhl 1998, Caudle et al. 2007, Post et al. 2018, Segura-Aguilar et al. 2014, Gandhi et al. 2012, Bayersdorfer et al. 2010, Surmeier 2018, Surmeier et al. 2017). Moreover, DA seems to promote the formation and secretion of SDS-resistant α-synuclein oligomers thereby eventually contributing to the initiation and progression of the disease (Lee et al. 2011). Despite this central role of DA, extramesencephalic dopaminergic systems have not been systematically investigated for α-

synucleinopathy and/or neurodegeneration. Substantial literature exists on the ventral mesencephalic dopaminergic (A8-A10) nuclei considering their involvement in the disease process. These comparative data allow to clearly recognize a spectrum of susceptibility, in which the nigral dopaminergic cells of the ventral tier (A9) are the most vulnerable, followed by the VTA (A10), the dorsal tier of the SN (A9), and the RRF (A8). Identifying the factors which render certain neurons particularly vulnerable or resistant to the disease process remains a key challenge. It has been suggested that the different protein expression pattern, and thus the interaction of various proteins influences the susceptibility of these neuronal populations (Double et al. 2010). Specific proteins, and protein expression patterns (proteomes) which could account for the observed spectrum of vulnerability within the mesencephalic dopaminergic cell populations have been found. These are of interest for cellular metabolism but also for the electrophysiological firing patterns of these cell groups. Whereas the pacemaking of the less vulnerable VTA neurons relies on voltage-dependent Na^+ channels (Puopolo et al. 2007), adult nigral neurons use L-type voltage gated Ca^{2+} channels of the Cav1.3 subtype to maintain autonomous pacemaking leading to sustained Ca^{2+} influx to the cytosol (Chan et al. 2007, Nedergaard et al. 1993). In the most vulnerable ventral tier of the SN, latter is combined with a substantially lower intracellular Ca^{2+} buffering capacity due to the absence of calbindin and significantly lower expression levels of parvalbumin and calretinin compared to the dorsal tier of the SN or the VTA, respectively (Chung et al. 2005, Yamada et al. 1990, Parent et al. 1996, McRitchie et al. 1996). As a consequence, these neurons have a high intracellular Ca^{2+}-burden leading to high energy demands due to ATP-dependent Ca^{2+} extrusion mechanisms (Surmeier et al. 2011, Chan et al. 2010). Furthermore, metabolic studies have shown that nigral neurons have an almost 3-fold higher basal oxidative phosphorylation rate than VTA neurons and thus a substantially elevated basal oxidative stress level and a significantly lower reserve respiratory capacity (Pacelli et al. 2015). This means that nigral neurons are less capable of increasing their ATP production when higher energy demands occur. For thourough reviews on additional potential vulnerability factors see (Double et al. 2010) and (Brichta and Greengard 2014).

What we can learn from neuroimaging studies

Neuroimaging studies become increasingly valuable tools to link pathological alterations with motor and non-motor symptoms, to investigate etiology and pathomechanisms, to monitor disease progression, to support differential diagnosis of parkinsonism and to assess the outcome of therapeutic approaches (Politis 2014). In this review we will focus on three major applications which are relevant regarding dopaminergic dysfunction in PD: (1) the variety of imaging agents allows us to investigate the changes in the dopaminergic systems and metabolic activity caused by PD and thereby broadens our understanding of the molecular and cellular disease pathogenesis and progression (Weingarten et al. 2015, Politis 2014). (2) PET, SPECT and MRI based imaging can be used in the clinical setting to assist differential diagnosis of idiopathic PD vs. atypical parkinsonian syndromes or other causes of parkinsonism (Kägi et al. 2010, Scherfler et al. 2007). (3) Neuroimaging studies can

be used to detect subclinical levels of dopaminergic dysfunction and thus facilitate the identification and risk stratification of prodromal PD patients (Meles et al. 2017, Heller et al. 2017). Apart from these indications, functional neuroimaging has various other applicabilities, such as assessing the therapeutic effect of deep brain stimulation or embryonic cell transplantation (Weingarten et al. 2015, Natale et al. 2018).

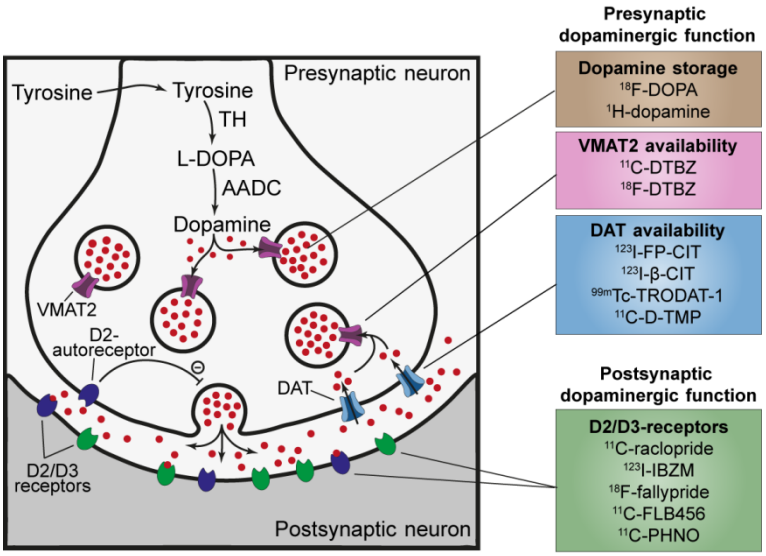

Figure 5 | Available radiotracers for in vivo imaging of the dopaminergic systems.
Radiotracers can be used to image the presynaptic dopaminergic activity (DA storage, VMAT2 and DAT availability) or the postsynaptic dopaminergic function (D_2/D_3-receptors) with PET and SPECT approaches.

A general advantage of functional neuroimaging studies is their potential to assess in vivo dysfunction of neuronal circuits, i.e. how the affected neurons behave in their neuronal network once they have reached a dysfunctional state. Additionally, they enable the analysis of the spatio-temporal pattern of neuropathology, that is, the progression of cellular and regional dysfunction in space and time. Dopaminergic dysfunction has been one of the major interests over the past 30 years of imaging in PD. The development of different imaging agents and tracers enabled the assessment of presynaptic dopaminergic dysfunction and postsynaptic DA receptor changes (Fig. 5). As expected, the ventral midbrain (A8-A10) of PD patients exhibits a reduction of presynaptic dopaminergic tracer uptake indicating dopaminergic degeneration (Joutsa et al. 2015, Goldstein et al. 2008, Ito et al. 2002, Hsiao et al. 2014). As a consequence, brain regions receiving dopaminergic input from A8-A10, namely the putamen, caudate nucleus and ventral STR (nucleus accumbens and olfactory tubercle) show reduced tracer binding reflecting dopaminergic denervation (Pavese et al. 2011, Joutsa et al. 2015, Lewis et al. 2012, Hsiao et al. 2014). It could be shown that the loss of tracer binding is uneven between the subregions of the STR: the dorsal putamen displays the most severe reduction, followed by the caudate nucleus and the ventral STR (Bohnen et al. 2011, Lewis et al. 2012, Hsiao et al. 2014). This is in accordance with the neuropathological studies describing a stereotypical pattern of ventral mesencephalic dopaminergic neurodegeneration resulting in uneven dopaminergic denervation of the STR (Fig. 6b) (Damier et al. 1999, Fearnley and Lees 1991, Waters et al. 1988, Halliday et al. 1996).

The decrease in striatal tracer binding significantly correlates with the degree of locomotor disability, particularly with bradykinesia and rigidity (Vingerhoets et al. 1997, Holthoff-Detto et al. 1997, Rinne et al. 2000, Otsuka et al. 1996). Interestingly, it does not correlate with the degree of rest tremor, suggesting that the neural substrate of this motor symptom might be distinct from the nigrostriatal pathway (Otsuka et al. 1996, Vingerhoets et al. 1997). Longitudinal follow-up PET tracer studies have estimated the progression of mesencephalic dopaminergic dysfunction over time and found that presynaptic dopaminergic function declines exponentially indicating that the progression of the disease tends to be faster at the early phases (Nandhagopal et al. 2009, Hilker et al. 2005). This finding is of potential importance in therapeutic trials testing compounds with disease modifying potential, if DAT SPECT is chosen as a surrogate marker for progression of PD. It would mean that such clinical trials should be preferentially performed in de novo PD patients or even in prodromal stages of PD with phenoconversion to manifest motor PD as a clinical endpoint (see also RBD – below).

Distinct behavioral and pharmacological triggers can be used to detect dysfunctional patterns of brain activity and neurotransmitter release. Several studies have been conducted to measure changes of striatal DA release triggered by L-DOPA challenge and correlated this with different symptoms of PD. They have consistently found that L-DOPA induces striatal DA release and that the degree of L-DOPA-induced DA outflow strongly correlated with disease duration (La Fuente-Fernández et al. 2004), Hoehn-Yahr stage (Pavese et al. 2006), and motor disability measured by the Unified Parkinson's Disease Rating Scale (UPDRS) (Tedroff et al. 1996). This means, that patients who had a longer disease duration, higher Hoehn-Yahr stages or higher UPDRS scores have larger putamenal DA release upon L-DOPA administration. Furthermore, the amplitude of striatal DA release positively correlated with dyskinesia scores indicating that a failure in the regulation of DA release contributes to the development of L-DOPA-induced dyskinesias (Pavese et al. 2006, La Fuente-Fernández et al. 2004), an adverse effect of L-DOPA affecting up to 90% of patients after 10 years of DA replacement therapy (Lopez et al. 2010, Hauser et al. 2007).

Reduction of the hypothalamic ^{18}F-DOPA uptake indicating monoaminergic dysfunction and reduced AADC activity has been reported in PD patients (Pavese et al. 2010, Moore et al. 2008, Pavese et al. 2011). However, since the hypothalamus, apart from its intrinsic dopaminergic neurons (A11-A15), receives dense monoaminergic innervation originating from the serotonergic median and dorsal raphe nuclei and noradrenergic A1 and A6 (locus coeruleus) cell groups (Palkovits et al. 1980, van de Kar and Lorens 1979), changes in ^{18}F-DOPA PET reflect the net alterations of all these systems (Pavese et al. 2011). Consequently, direct conclusions on the hypothalamic dopaminergic system (A11-A15) cannot be drawn from ^{18}F-DOPA results. Nevertheless, significant reduction of postsynaptic D_2 and D_3 receptors has been observed in a ^{11}C-raclopride study indicating dopaminergic dysfunction in the hypothalamus of PD patients (Politis et al. 2008).

Currently, the diagnosis of clinical PD is exclusively based on the presenting symptomatology (Table 1) and neuroimaging techniques like SPECT, PET or conventional MRI are not recommended as a first-line diagnostic approach. However, under certain conditions (e.g. atypical symptomatology or

unclear response to dopaminergic treatment) imaging techniques can be of use to better differentiate idiopathic PD from atypical parkinsonian syndromes like multiple system atrophy (MSA) or progressive supranuclear palsy (PSP) and from secondary causes of parkinsonism (e.g. vascular parkinsonism, neoplasms or drug-induced parkinsonism). DAT SPECT using the ^{123}I-Ioflupane ligand (DATScan) can be used to measure the strength of dopaminergic innervation of the STR (striatal DAT levels) and is thereby able to differentiate neurodegenerative forms of parkinsonism (e.g. PD, MSA, PSP) which normally show reduced striatal DAT levels, from essential tremor and healthy controls (normal striatal DAT levels) (Kägi et al. 2010, Scherfler et al. 2007). However, this technique does not allow to further distinguish idiopathic PD from the atypical parkinsonian syndromes (MSA, PSP). In this case one could perform a D_2/D_3 receptor SPECT with the ligand ^{123}I-iodobenzamide (^{123}I-IBZM). PD patients generally show normal postsynaptic D_2/D_3 signal intensities, while MSA and PSP patients are commonly characterized by decreased postsynaptic D_2/D_3 receptor availability. However, a normal D_2/D_3 signal does not exclude MSA or PSP (Vlaar et al. 2007). Other possibilities to differentiate idiopathic PD from atypical parkinsonian syndromes are metabolic PET imaging with ^{18}F-FDG (Hellwig et al. 2012, Juh et al. 2004), which relies on disease specific alterations of brain glucose metabolism, or MRI based approaches like T2 weighted structural MRI, voxel-based morphometry and diffusion tensor imaging (Price et al. 2004, Paviour et al. 2005, Ota et al. 2013).

At the onset of motor symptoms and, consequently, at the time of diagnosis 30% of nigral cells have already been lost and only 50-60% of normal TH-immunoreactivity is present in the STR (Fearnley and Lees 1991, Greffard et al. 2006, Cheng et al. 2010, Kordower et al. 2013). The time period, in which the pathological process of PD has already started but the motor symptoms are not yet present is the so-called premotor or prodromal phase of PD (Kalia and Lang 2015). During this period, functional neuroimaging of the nigrostriatal system is able to detect subclinical levels of dopaminergic dysfunction and thus facilitates the identification and risk stratification of patients with prodromal PD (Bauckneht et al. 2018, Heller et al. 2017, Meles et al. 2017, Iranzo et al. 2010, Stiasny-Kolster et al. 2005, Piccini et al. 1999). Patients suffering from idiopathic RBD, a parasomnia characterized by the absence of atonia during REM sleep in combination with abnormal nocturnal behavior, represent a specific prodromal risk population for developing PD (Iranzo et al. 2013). Several studies found that RBD manifestation precedes the onset of PD, dementia with Lewy bodies and MSA, thereby representing an early and specific symptom of these neurodegenerative α-synucleinopathies (Postuma et al. 2012, Schenck et al. 2013). Applying DAT SPECT imaging with ^{123}I-Ioflupane on RBD patients revealed a progressive decrease of presynaptic striatal DAT availability from "mild" or "subclinical" RBD to manifest RBD to PD (Heller et al. 2017). Moreover, 18F-FDG-PET imaging showed that a subgroup of RBD patients already possessed the same altered brain glucose metabolism pattern which is related to PD patients (Meles et al. 2017). Taken together, neuroimaging techniques can help to differentiate RBD patients from healthy controls, to monitor RBD disease progression, to stratify

the risk of phenoconversion to PD, and thereby identify and characterize eligible patients for neuroprotective trials (Heller et al. 2017, Baumeister et al. 2018).

Symptomatology 'off'/'on' dopaminergic medication – conclusions of clinical studies

Clinical drug studies investigating the potential of dopaminergic medication to improve, but also to worsen or even induce some of the PD symptoms are valuable tools to identify distinct symptoms which are linked to the dysfunctional dopaminergic neurotransmission.

Physiological dopaminergic functions, such as voluntary motor control, require optimal DA levels in dopaminergic output regions. Both a hypodopaminergic and hyperdopaminergic state result in neural network dysfunction, and eventually in clinical symptoms. At the time of PD motor symptoms onset around 30% of dopaminergic nigral neurons are lost and 50-60% of their axon terminals show a marked decrease of TH immunoreactivity (Fearnley and Lees 1991, Greffard et al. 2006, Cheng et al. 2010, Kordower et al. 2013). This indicates that even when the disease process started and moderate DA shortage is present in the STR, intrinsic mechanisms compensate the DA deficit retaining normal physiological functions and thereby an asymptomatic state (Fig. 6a) (Perez et al. 2008, Zigmond et al. 1990, Garris et al. 1997, Bergstrom and Garris 2003, Obeso et al. 2004). Interestingly, patients with incidental LB disease, i.e. healthy individuals without apparent parkinsonism or dementia with LB/LN pathology upon autopsy, show a 27% cell loss of pigmented nigral neurons (Fearnley and Lees 1991) and a 33-50% decrease of striatal TH immunoreactivity (DelleDonne et al. 2008, Dickson et al. 2008, Beach et al. 2008). As PD progresses and the severity of hypodopaminergism increases, the compensatory mechanisms fail and symptoms of a hypoactive dopaminergic system manifest. Replenishment of DA neurotransmission in these hypodopaminergic regions via DA replacement therapy (e.g. L-DOPA, DA agonists) will ameliorate the manifestation of DA shortage (Fig. 6a, b) (Birkmayer and Hornykiewicz 1961, Cotzias et al. 1969). Concurrently, given the uneven nature of neurodegeneration across the dopaminergic systems of the brain in PD, doses of L-DOPA which are necessary to restore dopaminergic neurotransmission in the most severely depleted nigrostriatal system simultaneously 'overdose' the better preserved mesolimbic and mesocortical brain networks. Thus, dopaminergic treatment with the focus on, and the primary clinical aim of ameliorating the motor symptoms leads to overactivation of the mesolimbic and mesocortical systems thereby resulting in symptoms of hyperdopaminergism (Fig. 6b) (Gotham et al. 1988, Swainson et al. 2000, Voon et al. 2017, Vriend et al. 2014, Joutsa et al. 2015, Vaillancourt et al. 2013). Therefore, symptoms of a dysfunctional dopaminergic neurotransmission represent a continuum in which hypodopaminergic states develop as a consequence of disease progression, whereas hyperdopaminergic states emerge as side effects of DA replacement therapy. In the following section, we will briefly give an insight into the dopaminergic symptoms of PD.

The pioneering work of A. Carlsson showed that DA deficiency in the brain of rabbits resulted in parkinsonian symptoms which could be alleviated by administration of L-DOPA, a blood-brain barrier crossing precursor of DA (Carlsson 1959, Carlsson et al. 1957). Since then, both neuropathological and neuroimaging studies of PD patients showed that the degree of DA deficiency in the dorsal STR significantly correlates with the Hoehn-Yahr stage and UPDRS motor disability, especially with bradykinesia and rigidity scores (Hornykiewicz 1963, Morrish et al. 1995, Broussolle et al. 1999, Seibyl et al. 1995, Hsiao et al. 2014, Pavese et al. 2011, Nandhagopal et al. 2009, Price et al. 1978). Consequently, administration of L-DOPA significantly improves the two latter motor symptoms of PD (Birkmayer and Hornykiewicz 1961, Cotzias et al. 1969). Although dopaminergic replacement therapy is the most effective symptomatic treatment of PD, longterm L-DOPA administration leads to motor side effects, the so called L-DOPA induced dyskinesias (LIDs). LIDs are among the most common adverse effects of L-DOPA therapy and affect up to 80% of patients after 5 years, and up to 90% after 10 years of treatment (Hauser et al. 2007, Ahlskog and Muenter 2001, Rajput et al. 1984, Jong et al. 1987). The term LIDs refers to a variety of motor side effects which can be classified based on the clinical movement pattern and the temporal correlation between the occurrence of the dyskinesia and the administration of dopaminergic medication (Luquin et al. 1992, Pandey and Srivanitchapoom 2017, Bastide et al. 2015). Interestingly, severe nigrostriatal damage seems to be a prerequisite of LIDs when L-DOPA is administered in pharmacologically relevant doses. Neither healthy controls, nor non-human primates with only moderate DA deficiency develop LIDs as a result of longterm L-DOPA treatment (Boyce et al. 1990, Schneider 1989, Hagenah et al. 1999, Di Monte et al. 2000, Jenner 2008). This indicates that nigrostriatal hyperdopaminergism per se is not sufficient to induce dyskinesias, but other factors have to be involved additionally. It is hypothesized that a 'dysregulated DA release' is responsible for the development of LIDs (see above), which originates in the compensatory mechanisms and changes due to dopaminergic denervation of the STR. It was shown that sprouting of serotonergic axon terminals takes place in the STR of PD patients (Rylander et al. 2010). Serotonergic neurons, due to their partially overlapping protein expression with dopaminergic cells (e.g. AADC, VMAT2), are able to take up L-DOPA, convert it to DA and store it in synaptic vesicles (Tison et al. 1991, Arai et al. 1995, Arai et al. 1994, Butcher et al. 1970). As a consequence, these neurons, although normally not relying on DA as a neurotransmitter, synthetize and release DA upon administration of L-DOPA (Carta et al. 2007). However, since they neither express D_2 autoreceptors mediating the natural feedback of DA release, nor DAT to clear DA from the synaptic cleft, the dopaminergic neurotransmission becomes dysregulated resulting in swings of synaptic DA levels manifesting as LIDs (Mosharov et al. 2015, Carta and Bezard 2011).

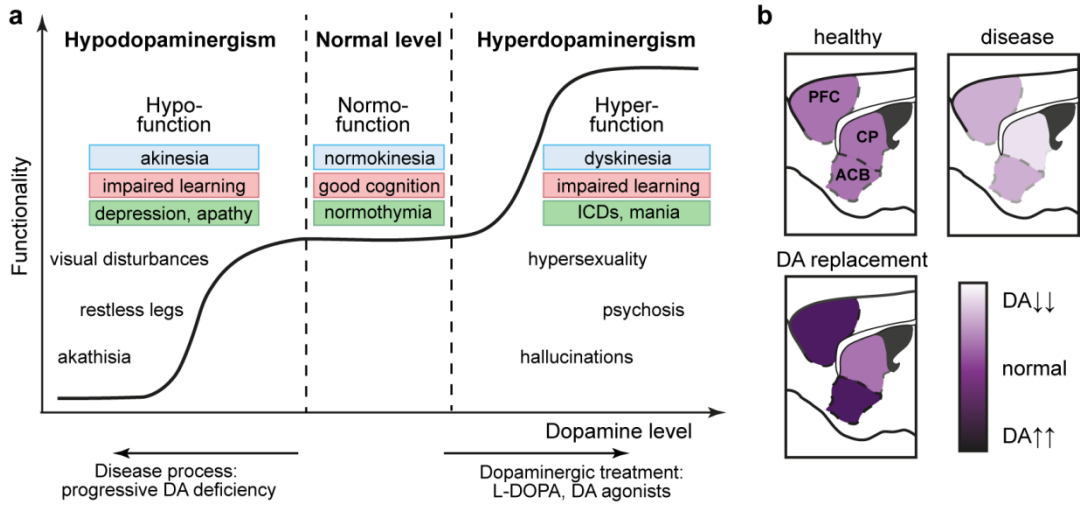

Figure 6 | The relationship between DA levels and physiological functions/dysfunctions.

a As a consequence of diverse compensatory mechanisms, mild to moderate changes of DA levels remain asymptomatic (plateau). When the severity of hypo- or hyperdopaminergism increases, compensatory mechanisms fail to retain physiological functions leading to dysfunctional neural circuits manifesting as clinical symptoms. Hypodopaminergic states develop as a consequence of disease progression, whereas hyperdopaminergic states emerge as side effects of DA replacement therapy. **b** Ventral mesencephalic dopaminergic (A8-A10) neurodegeneration shows a stereotypical pattern resulting in severe hypodopaminergism in the caudoputamen (CP, nigrostriatal pathway), and mild to moderate hypodopaminergism in the ventral STR (ACB – nucleus accumbens, mesolimbic pathway) and prefrontal cortex (PFC, mesocortical pathway). As a consequence, doses of DA replacement therapy which are necessary to remedy the nigrostriatal pathway simultaneously overdose the mesocortical and mesolimbic pathways.

Cognitive deficits ranging from mild cognitive impairment (MCI) not yet qualifying as dementia to manifest dementia are common non-motor symptoms in PD with significant impact on the quality of life (Chaudhuri and Schapira 2009). MCI can be observed in prodromal and manifest PD affecting around 20% of patients at the time of diagnosis (Muslimovic et al. 2005, Aarsland et al. 2009a) and displays a major risk factor for the progression to PD dementia (PDD) (Hoogland et al. 2017, Hobson and Meara 2015, Pedersen et al. 2017). Cognitive impairment most commonly affects executive functions resulting in a 'dysexecutive syndrome' resembling to that seen in patients with frontal lobe damage (Owen et al. 1995, Taylor et al. 1986, Owen et al. 1993, Muslimovic et al. 2005, Rowe et al. 2002). The pathophysiology of MCI and dementia in PD is heterogeneous and involves a combination and synergism of distinct pathological changes. These include cortical LB pathology (Mattila et al. 2000, Apaydin et al. 2002, Hurtig et al. 2000, Irwin et al. 2012, Compta et al. 2011), cortical cholinergic deficiency due to the degeneration of the nucleus basalis of Meynert (Mattila et al. 2001, Perry et al. 1991), noradrenergic loss as a consequence of locus coeruleus degeneration, cortical and limbic reduction of DA due to the degeneration of the mesolimbic and mesocortical dopaminergic systems (Rinne et al. 1989, Paulus and Jellinger 1991, Zweig et al. 1993, Ito et al. 2002, Scatton et al. 1983), potential occurrence of Alzheimer's disease co-pathology (Boller et al. 1980, Paulus and Jellinger 1991, Irwin et al. 2012, Compta et al. 2011), and others – for a thourough review see (Halliday et al. 2014). This means that a DA deficit in certain brain regions contributes to the cognitive deficit seen in nondemented PD patients with MCI and in PDD patients (Rinne et al. 1989,

Paulus and Jellinger 1991, Zweig et al. 1993, Ito et al. 2002, Scatton et al. 1983). However, DA deficiency per se is not considered to be sufficient for the development of the full range of cognitive deficits (Bosboom et al. 2004, Caballol et al. 2007). Interestingly, studies examining the effects of L-DOPA in PD patients with dysexecutive syndrome report beneficial, neutral, and detrimental effects (Gotham et al. 1988, Swainson et al. 2000, Kulisevsky 2000, Downes et al. 1989, Bowen et al. 1975, Kulisevsky et al. 1996, Lange et al. 1992, Pillon et al. 1989). This is due to the fact that executive functions can be split into different components, such as working memory, inhibition, attentional set-shifting, and planning, whose neurobiological correlates are distinct (Smith 1999, Rabinovici et al. 2015). During an executive task, depending on the subdomain required, different neural networks are active including the prefrontal and parietal cortices, the basal ganglia, the thalamus and the cerebellum (Collette et al. 2005, Monchi et al. 2001, Monchi et al. 2006, Wager et al. 2004, Wager and Smith 2003, Cools et al. 2004). As a consequence, cognitive tasks which rely on DA-depleted brain regions (dorsal STR) will be ameliorated by DA replacement therapy, whereas cognitive functions associated with relatively intact or less affected, DA-dependent brain regions (ventral STR and prefrontal cortex) will be impaired due to a relative 'overactivation' of these systems (Gotham et al. 1988, Cools et al. 2001). This explains why studies investigating the effect of DA replacement therapy on cognitive function report both detrimental and beneficial effects: depending on the cognitive task, distinct subdomains of executive function are examined which all have a different grade of hypodopaminergism.

Neuropsychiatric syndromes ranging from major depression to psychosis and impulse control disorders (ICDs) are highly prevalent in PD affecting the vast majority of PD patients during the course of the disease (Aarsland et al. 2009b). Major depression occurs in approximately 17% of PD patients (Reijnders et al. 2008), apathy is present in up to 60% (Yamanishi et al. 2013, Pedersen et al. 2009), whereas the prevalence of anxiety in cross-sectional studies ranges between 20 and 49% (Chen et al. 2010, Dissanayaka et al. 2010, Kulisevsky et al. 2008, Nègre-Pagès et al. 2010, Nuti et al. 2004). All three disorders are suggested to be – even if partly – associated with a deficient mesolimbic dopaminergic neurotransmission, i.e. with a mesolimbic hypodopaminergic state (Remy et al. 2005, Voon et al. 2011, Weintraub et al. 2005). This notion is further supported by clinical trials investigating the efficacy of DA agonists in depressive syndromes showing significant improvement of these symptoms (Reichmann et al. 2003, Reichmann et al. 2002, Barone et al. 2010, Lemke et al. 2005, Pahwa et al. 2007, Bodkin and Amsterdam 2002, Thobois et al. 2013). A wide range of ICDs, such as pathological gambling, compulsive sexual behavior, and binge eating are associated with dopaminergic treatment affecting around 13.6% of PD patients on DA replacement therapy compared to 1.7% of patients neither receiving DA agonists nor L-DOPA (Weintraub et al. 2010). ICDs are suggested to develop as a consequence of a dopaminergic hyperactivity in ventral striatal reward circuitry (mesolimbic system) resulting in an increased drive to perform a certain behavior, and to be maintained by an impaired learning from negative consequences due to prefrontal cortical hyperdopaminergism (mesocortical system) (Fig. 6b) (Weintraub 2008, Evans et al. 2006, O'Sullivan

et al. 2011, Steeves et al. 2009, Cilia et al. 2008). Interestingly, the largest multicenter study (DOMINION) investigating the occurrence of ICDs in 3090 PD patients found that the frequency of developing an ICD was 2-fold higher in patients receiving DA agonists compared to patients taking L-DOPA (14.0% vs. 7.2%) (Weintraub et al. 2010). This can be explained by a significantly higher affinity of DA agonists to D_3 receptors compared to D_1 and D_2 receptors (Gerlach et al. 2003). While D_1 and D_2 receptors are more abundant in the dorsal STR (nigrostriatal pathway) mediating voluntary motor control, D_3 receptors are predominantly found in the ventral STR (mesolimbic pathway) playing an important role in reward mechanisms (Sokoloff et al. 1990, Gurevich 1999). As a consequence, doses of DA agonists required to improve motor symptoms may overactivate the mesolimbic system resulting in ICDs (Weintraub 2008, Voon et al. 2017). The association between developing an ICD and a hyperdopaminergic state in the mesocortical and mesolimbic systems is further supported by longitudinal studies showing that DA agonist dose reduction or discontinuation, even in combination with an increased L-DOPA dose, significantly improves ICD symptoms (Mamikonyan et al. 2008).

Concluding remarks

Neuropathological analysis, neuroimaging studies and clinical trials have enabled us to better understand dopaminergic dysfunction in PD. It has become evident that, although being one of the core features of PD, nigrostriatal degeneration cannot be solely accountable for the wide range of PD symptoms. Despite the central role of DA in PD, extramesencephalic, i.e. diencephalic, olfactory bulbar and retinal dopaminergic systems have not been systematically investigated yet – not to speak of the dopaminergic system related to the gastrointestinal tract. The distinct dopaminergic systems have a surprisingly high neurobiological diversity suggesting that there is not one general type of dopaminergic neuron but rather a spectrum of different dopaminergic phenotypes. This heterogeneity on the cellular level could account for the observed differences in susceptibility of the dopaminergic systems to the disease process. To finally understand which factors render neurons particularly vulnerable, we first ought to investigate which neuronal populations are affected in the course of PD, with emphasis on neuronal cell groups sharing common traits, like the synthetic machinery, the metabolism and the overall reliance on DA as a neurotransmitter.

Arvid Carlsson and his fundamental discovery of DA deficiency in PD paved the way for the still ongoing era of dopaminergic replacement therapy and fueled 50-years of research on the dopaminergic systems in PD. Within the last two decades, the research focus slowly shifted towards other important areas such as neuropathological research on other neurotransmitter systems involved in PD, identification of genetic mutations or environmental risk factors. A major focus of the basic research field has been set on unravelling the pathogenesis and progression of PD including research on α-synuclein aggregation and interneuronal trafficking on one side and the identification of cell-

autonomous factors rendering certain cell groups more vulnerable to the disease process, like mitochondrial dysfunction or electrophysiological cell properties on the other side.

Conflict of interest

The authors declare no conflict of interest.

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8.4. Abstract 1

Poster as first author at the Meeting of the German Neuroscience Society, Göttingen, Germany (March 2017).

Targeted overexpression of A53T-α-synuclein induces progressive neurodegeneration and electrophysiological changes of noradrenergic locus coeruleus neurons – a preclinical model of Parkinson's disease

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Introduction

Neurodegeneration of Locus coeruleus (LC) neurons is a common feature of the early prodromal phase of Parkinson`s disease (PD) and occurs at Braak's stage 2, actually years before the substantia nigra is affected. However, the mechanisms underlying α-synuclein accumulation and neurodegeneration in LC neurons are still unclear. In our present study we have developed a new mouse model to study the time dependent effects of cellular A53T-α-synuclein overexpression in the LC, regarding α-synuclein aggregation, changes in electrophysiological properties and noradrenergic cell loss.

Methods

Serotype 1/2 recombinant AAV vectors carrying the genetic information of A53T-α-synuclein or luciferase were unilaterally injected in the right LC of C57Bl/6 wild-type mice to induce continuous overexpression of A53T-α-synuclein in LC neurons for 1, 3, 6 or 9 weeks. At each time point eight animals per group were sacrificed for immunohistochemical analysis, whereas four animals per group and time point were used for patch-clamp recordings of LC neurons in acute brainstem slices.

Results

We found that targeted overexpression of A53T-α-synuclein in the LC of wild-type mice caused progressive α-synuclein aggregation and significant loss of noradrenergic LC neurons in a time dependent manner, starting three weeks post-injection. Accumulation of α-synuclein in the LC was accompanied by transport of α-synuclein to various interconnected brain regions observed even after one week of A53T-α-synuclein overexpression. In our model, neurodegeneration of the LC was associated with significant changes in the electrophysiological properties of the neurons. Time dependently, A53T-α-synuclein overexpression induced alterations in action potential shape and increase in the pacemaking frequency.

Conclusions

Our data indicate that overexpressed A53T-α-synuclein accumulates steadily in LC neurons while simultaneously inducing major changes in electrophysiological properties of these noradrenergic cells which might ultimately result in cell death.

BL is a DAAD fellow. WHC and LAM have received a grant by the intramural research fund of the Rhön-AG. WHO is supported by the Charitable Hertie Foundation, Frankfurt/Main, Germany.

8.5. Abstract 2

Poster as first author at the 21st International Congress of Parkinson's Disease and Movement Disorders, Vancouver, Canada (June 2017).

Targeted overexpression of A53T-α-synuclein induces progressive neurodegeneration and electrophysiological changes of noradrenergic locus coeruleus neurons – a preclinical model of Parkinson's disease

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Introduction

Dysfunction of the noradrenergic locus coeruleus (LC) is an early hallmark of Parkinson's disease (PD). Neurodegeneration of LC neurons occurs in Braak's stage 2, years before the substantia nigra is affected. The extensive loss of noradrenergic LC neurons in PD is responsible for a large amount of non-motor symptoms that occur in early stages of the disease. However, the mechanisms that render LC neurons prone to α-synuclein accumulation and neurodegeneration are still unclear. In our present study, we developed a new mouse model to study the time dependent effects of cellular A53T-α-synuclein overexpression in the LC, regarding the toxicity caused by α-synuclein accumulation, the alteration in electrophysiological properties and noradrenergic cell loss.

Methods

Serotype 1/2 recombinant adeno-associated viral vectors (rAAV) carrying the genome for A53T-α-synuclein or luciferase were unilaterally injected in the right LC of C57Bl/6 wildtype mice to induce continuous protein overexpression. At 1, 3, 6 and 9 weeks post injection, eight animals overexpressing either A53T or luciferase were sacrificed for immunohistochemical analysis. In addition, four animals per group and timepoint were used to study the biophysical characteristics of LC neurons by patch-clamp recordings in acute brainstem slices.

Results

We show, that targeted overexpression of A53T-α-synuclein in the LC of wildtype mice caused progressive α-synuclein accumulation and significant loss of noradrenergic LC neurons in the injected side in a time dependent manner, starting 3 weeks post-injection. Aggregated forms of α-synuclein were confirmed by Proteinase K resistance and Ser129 phosphorylation. Furthermore, overexpression of α-synuclein led to a progressive increase of astro- and microglia density in the injected LC region. In our model, neurodegeneration of LC cells was associated with significant changes of their electrophysiological properties. Time dependently, A53T-α-synuclein overexpression induced alterations in action potential shape and an acceleration of the pacemaking frequency.

Conclusions

Our data indicate that overexpressed A53T-α-synuclein accumulates steadily in LC neurons while simultaneously induces neuroinflammation and major changes in electrophysiological properties, which might be responsible for the observed cell death of LC neurons.

Disclaimer: L.A. Matschke and WH. Chiu have received a grant by the intramural research fund of the Rhön-AG. B. Lee is a DAAD fellow. W.H. Oertel is supported by the Charitable Hertie Foundation, Frankfurt/Main, Germany.

8.6. Abstract 3

Poster as shared first author at the α-synuclein Meeting, Athens, Greece (September 2017).

Targeted overexpression of human-A53T-α-synuclein in locus coeruleus neurons causes widespread transport of α-synuclein to interconnected brain regions

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Dysfunction of the noradrenergic locus coeruleus (LC) is an early hallmark of Parkinson's disease (PD) and contributes to a variety of non-motor symptoms. LC cells exhibit a common at-risk phenotype compared to other neurons that undergo neurodegeneration in PD, like autonomous pacemaking and cytosolic Ca^{2+} oscillations. In our present study, we aimed to elucidate, whether a locally induced α-synucleinopathy can cause transport of α-synuclein to brain regions distant from side of injection.

AAV1/2 carrying the gene for either human-A53T-α-synuclein or luciferase were unilaterally injected into the LC-region of wild-type mice to induce continuous protein overexpression. After 1, 3, 6 and 9 weeks, the whole brain of four animals per group was immunohistochemically analyzed for deposits of human-α-synuclein or luciferase.

Targeted overexpression of human-A53T-α-synuclein causes progressive α-synuclein accumulation at the side of injection. Aggregated forms of α-synuclein were confirmed by Proteinase K resistance and Ser129 phosphorylation. Furthermore, α-synucleinopathy in the LC-region leads to widespread

axonal transport of human-A53T-α-synuclein as early as 1 week post injection to interconnected brain areas, as the olfactory bulb, central amygdala and lateral septal nucleus. At early time-points, deposits of human-A53T-α-synuclein are mainly seen in axons but start to appear over time also in cell bodies in certain brain regions like the bed nuclei of the stria terminalis or the central amygdala. No protein transport was seen in luciferase injected animals.

Our results show that overexpressed A53T-α-synuclein is transported anterogradely over long distances to anatomically connected brain regions. Occurrence of α-synuclein positive cell bodies in a subset of the affected brain regions indicates additional retrograde axonal transport of α-synuclein.

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8.7. Verzeichnis der akademischen Lehrer/-innen

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