Genetic analysis of dopaminergic neuron survival

GDNF/Ret signaling and the Parkinson's disease-associated gene DJ-1

Dissertation

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To my dear mother Mariana Aron (1954-1999) in loving memory

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(Unterschrift)

The work presented in this thesis was performed in the laboratory of Prof. Dr. Rüdiger Klein, Department of Molecular Neurobiology, Max-Planck Institute of Neurobiology, Martinsried, Germany.

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Abbreviations

AAV	Adeno-associated virus
AD	Alzheimer's disease
APAF-1	Apoptotic protease activating factor 1
AR-JP	Autosomal recessive juvenile Parkinsonism
ASK1	Apoptosis signal-regulating kinase 1
α-syn	Alpha-synuclein
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
ALS	Amyotrophic lateral sclerosis
cAMP	Cyclic adenosine monophosphate
CBD	Corticobasal degeneration
CNS	Central nervous system
CREB	cAMP response element binding protein
DA	Dopaminergic
DARPP-32	Dopamine and cAMP-regulated phosphoprotein, 32 kDa
DAT	Dopamine transporter
Df	(chromosomal) Deficiency
DLB	Dementia with Lewy Bodies
DTT	Dithiothreitol
e.g.	Example given
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
FGF	Fibroblast growth factor
FOXO	Forkhead box transcription factor, class O
FTD	Frontotemporal dementia
GABA	Gamma aminobutyric acid
GAB1/2	Grb2-associated binding protein
GDNF	Glial cell line-derived neurotrophic factor
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GFL	GDNF family ligand
GFRα	GDNF family receptor α
GIRK2	G protein-activated inward rectifier potassium channel 2
Glu	Glutamate
GMR	Glass multimer reporter
Gpe	*Globus pallidus*, external segment
Gpi	*Globus pallidus*, internal segment
Grb2	Growth factor receptor bound protein 2
GTP	Guanosine triphosphate
HD	Huntington's disease
HSCR	Hirschsprung's disease
Iba-1	Inonized calcium binding adaptor protein 1
i.e.	*Id est* (that is)
IRS-1	Insulin-receptor substrate 1
JNK	c-JUN N-terminal kinase
LB	Lewy Body
LBVAD	Lewy Body variant of Alzheimer's disease
L-DOPA	L-3,4-dihydroxyphenylalanine
LRRK2	Leucine-rich repeat kinase 2
MAPK	Mitogen-activated protein kinase
MEKK1	Mitogen-activated protein kinase (MAPK) kinase kinase 1
MEN2A/B	Multiple endocrine neoplasia type 2A/B

MF	Morphogenetic furrow
MFB	Medial forebrain bundle
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple system atrophy
MSN	Medium size spiny neuron
NCAM	Neural cell adhesion molecule
Nes	Nestin (promoter)
NeuN	Neuronal nuclei (marker)
NF	Neurotrophic factor
NFkB	Nuclear factor kB
NGF	Nerve growth factor
Nrf2	Nuclear Factor Erythroid 2-related Factor 2
NRTN	Neurturin
NS	Non significant (p>0.05, Student's t-test)
NT-4	Neurotrophin 4
P/O	Photoreceptors per ommatidium
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PDK1	Phosphatidylinositide-dependent protein kinase 1
PH	Pleckstrin homology (domain)
PI3K	Phosphoinositide-3 kinase
PINK1	(PTEN homolog-induced putative kinase
Pitx3	Paired-like homeodomain transcription factor 3
PKA	Protein kinase A
PSP	Progressive supranuclear palsy
PTB	Phosphotyrosine-binding (domain)
PTEN	Phosphatase and tensin homolog
R1-8	Photoreceptor neuron 1-8
Ret	Rearranged during transfection
RING	Really interesting new gene domain
Rl	Rolled
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-Polymerase chain reaction
RTK	Receptor tyrosine kinase
Sev	Sevenless (promoter)
sevRTK	Sevenless receptor tyrosine kinase
SH	Src-homology
SN	*Substantia nigra pars compacta*
SOS	Son of sevenless
STN	Subthalamic nucleus
STR	Striatum
SUMO	Small ubiquitin-like modifier
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
TM	Transmembrane (domain)
TRAF6	TNF receptor-associated factor 6
Trk	Tropomyosin-related kinase
AUS	Upstream activating sequence
UPS	Ubiquitin-proteasome system
UTR	Untranslated region
vs.	*Versus*
VTA	Ventral tegmental area
WT	Wild-type
6-OHDA	6-hydroxy-dopamine

Abstract

Pathological changes in the dopaminergic system account for a number of devastating illnesses including schizophrenia, psychosis, depression, addiction, obsessive compulsive disorder or the most well known Parkinson's disease (PD). The nigrostriatal pathway is an important component of the dopaminergic (DA) system mediating voluntary movement and originates in the ventral midbrain from where *substantia nigra pars compacta* (SN) neurons send their axons to the dorsal striatum. Massive loss of SN neurons as seen in PD leads to postural imbalance, rigidity, tremor and bradykinesia, however, the precise mechanisms involved in the maintenance and the demise of SN neurons are poorly understood.

Endogenous neurotrophic factors such as the Glial cell line-derived neurotrophic factor (GDNF; signaling via the Ret receptor tyrosine kinase) and Brain-derived neurotrophic factor (BDNF; signaling via the TrkB receptor tyrosine kinase) were reported to have protective and rescuing properties on DA neurons; however, their physiological roles in SN neurons remained unknown. Inactivation of the oxidative stress suppressor *DJ-1* causes PD; remarkably, mice lacking *DJ-1* function do not display overt SN degeneration, suggesting that additional *DJ-1* interactors compensate for loss of *DJ-1* function. To begin characterizing the cellular and molecular networks mediating SN neuron survival, I used mouse genetics to investigate the roles and the interaction between GDNF/BDNF-mediated trophic signaling and the *DJ-1*-mediated stress response in SN neurons.

While mice lacking *TrkB* function specifically in SN neurons display a normal complement of SN neurons up to 24-months, loss of *Ret* function in DA neurons causes adult-onset and progressive SN degeneration, suggesting that GDNF/Ret signaling is required for long-term maintenance of SN neurons. I then generated and aged mice lacking *Ret* and *DJ-1* and found remarkably that they display an enhanced SN degeneration relative to mice lacking *Ret*. Thus, *DJ-1* promotes survival of *Ret*-deprived SN neurons. Interestingly, the survival requirement for *Ret* and *DJ-1* is restricted to those SN neurons which express the ion channel GIRK2, project exclusively to the striatum and specifically degenerate in PD. This is the first *in vivo* evidence for a pro-survival role of *DJ-1*.

To understand how DJ-1 interacts molecularly with Ret signaling, I performed epistasis analysis in *Drosophila melanogaster*. Although *DJ-1* orthologs *DJ-1A* and *DJ-1B* are dispensable for fly development, the developmental defects induced by targeting constitutively active *Ret* to the retina were suppressed in a background of reduced *DJ-1A/B* function. Moreover, *DJ-1A/B* interacted genetically with Ras/ERK, but not PI3K/Akt signaling to regulate photoreceptor neuron development. Flies with reduced *ERK* activity and lacking *DJ-1B* function had more severe defects in photoreceptor neuron and wing development than flies with reduced ERK function. These observations establish, for the first time, a physiological role for *DJ-1B* in the intact *Drosophila*.

Our findings suggest that the triple interaction between aging, trophic insufficiency and cellular stress may cause Parkinsonism. Because *Ret* and *DJ-1* show convergence of their pro-survival activities, we predict that striatal delivery of GDNF might be most effective in PD patients carrying *DJ-1* mutations. A better understanding of the molecular connections between trophic signaling, cellular stress and aging will accelerate the process of drug development in PD.

Zusammenfassung

Pathologische Veränderungen im dopaminergen System sind für einige verheerende Krankheiten verantwortlich, unter anderem Schizophrenie, Psychosen, Depression, Drogenabhängigkeit, Zwangsneurosen und am besten bekannt, die Parkinson'sche Krankheit (PD). Die nigrostriatale Nervenbahn ist ein wichtiger Bestandteil des dopaminergen (DA) Systems und ist zuständig für bewusst ausgeführte Bewegungen. Sie entspringt im ventralen Mesencephalon, von wo die Axone der *substantia nigra pars compacta* (SN) Neurone ins dorsale Striatum verlaufen. Der massive Verlust von SN Neuronen, wie bei PD, führt zu den bekannten Symptomen Haltungsinstabilität, Muskelstarre, Zittern und verlangsamte Bewegungen. Allerdings sind die Mechanismen, die für die Erhaltung, bzw. den Verlust der SN Neurone verantwortlich sind, bisher nur schlecht verstanden.

Körpereigene neurotrophe Faktoren wie der *Glial cell line-derived neurotrophic factor* (GDNF, Signaltransduktion ueber die Ret Rezeptor Tyrosinkinase) und der *Brain-derived neurotrophic factor* (BDNF, Signaltransduktion ueber die TrkB Rezeptor Tyrosinkinase) wurden mit schützenden und erhaltenden Eigenschaften in DA Neuronen in Verbindung gebracht. Allerdings sind ihre physiologischen Funktionen noch weitgehend unbekannt. Inaktivierung von *DJ-1*, das vor oxidativem Stress schützt, führt bei Menschen zu PD. Erstaunlicherweise zeigen Mäuse, welchen *DJ-1* fehlt, keinen Verlust von SN Neuronen, was impliziert, dass weitere Interaktionspartner von *DJ-1* den Verlust der *DJ-1* Aktivität kompensieren können. Um die zellulären und molekularen Netzwerke zu charakterisieren, die für den Erhalt von SN Neuronen verantwortlich sind, verwendete ich genetische Methoden in der Maus. Hierbei war ich besonders an der Interaktion der neurotrophen Effekte von *GDNF/BDNF* und der *DJ-1* vermittelte Stressantwort in SN Neuronen interessiert.

Während Mäuse, welchen der *TrkB* Rezeptor spezifisch in SN Neuronen fehlt, bis zum Alter von 24 Monaten eine normale Anzahl und Morphologie von SN Neuronen zeigen, tritt bei Verlust der *Ret* Funktion in DA Neuronen in adulten Mäusen eine fortschreitende Degeneration von SN Neuronen auf. Dies deutet darauf hin, dass *GDNF/Ret* vermittelte Signale für das langfristige Überleben von SN Neuronen benötigt werden. Ich generierte und alterte daraufhin Mäuse, welchen *Ret* und *DJ-1* fehlte und konnte interessanterweise zeigen, dass bei diesen eine verstärkte SN Degeneration im Vergleich zu Mäusen auftrat, welchen nur der *Ret* Rezeptor fehlte. *DJ-1* unterstützt also das Überleben von Wachstumsfaktor-deprivierten SN Neuronen. Interessanterweise ist dieser Effekt beschränkt auf Zellen, die den Ionenkanal *GIRK2* exprimieren und ausschließlich Axone ins Striatum senden. Diese Zellen sterben auch speziell in der Parkinson'schen Krankheit ab. Diese Ergebnisse sind der erste *in-vivo* Beleg für eine positive Überlebensfunktion von *DJ-1*.

Um die Interaktion von *DJ-1* und *Ret*-vermittelter Signaltransduktion auf molekularer Ebene zu untersuchen, führte ich eine Epistaseanalyse in *Drosophila melanogaster* durch. Obwohl die *Drosophila DJ-1* Orthologe *DJ-1A* und *DJ-1B* für die Entwicklung von *Drosophila* erlässlich sind, konnte ein genetischer Hintergrund mit reduzierter *DJ-1A/B* Aktivität Augenentwicklungsdefekte unterdrücken, die durch Überexpression einer konstitutiv aktiven *Ret* Rezeptor in der Retina induziert wurden. Des weiteren interagierte *DJ-1A/B* genetisch mit dem *Ras/ERK* Signalweg, aber nicht mit dem *PI3K/Akt* Signalweg in der Regulation der Photorezeptorneuronenentwicklung. Fliegen mit reduzierter *ERK* Aktivität und fehlendem *DJ-1B* zeigten schwerwiegendere Defekte in der Photorezeptorneuron- und Flügelentwicklung als Fliegen mit reduzierter *ERK* Aktivität, aber normalem *DJ-1B* Level. Diese Beobachtungen zeigen zum ersten Mal eine physiologische Funktion für *DJ-1B* in *Drosophila*.

Unsere Ergebnisse legen nahe, dass die Interaktion der drei Faktoren Alterung, Mangel an trophischen Signalen und zellulärer Stress, Parkinson-ähnliche Symptome hervorrufen kann. Da *Ret* und *DJ-1* konvergente überlebensfördernde Aktivitäten zeigen, sagen wir voraus, dass

eine intrastriatale Behandlung mit *GDNF* in Parkinson-Patienten mit *DJ-1* Mutationen besonders wirksam sein könnte. Ein besseres Verständnis der molekularen Zusammenhänge zwischen trophischen Signalen, zellulärem Stress und Alterung wird den Prozess der Medikamentenentwicklung für PD deutlich beschleunigen.

Publications from the work presented in this thesis

Kramer ER, **Aron L**, Ramakers GM, Seitz S, Zhuang X, Beyer K, Smidt MP, Klein R (2007) Absence of Ret signaling in mice causes progressive and late degeneration of the nigrostriatal system. **PLoS Biology 5(3):e39**

Aron L, Klein P, Pham TT, Kramer ER, Wurst W, Klein R (2010) Pro-survival role for Parkinson's associated gene DJ-1 revealed in trophically impaired dopaminergic neurons. **PLoS Biology 8(4):e1000349**

I. INTRODUCTION

A. Genetics and biochemistry of Parkinson's disease

1.1 Neurodegenerative diseases: a challenge for modern neuroscience

The formation, activity and maintenance of nerve cells (neurons) are crucial for our capacity to perceive, analyze and react to the external world. Death of specific neuronal populations is seen in neurodegenerative diseases and has devastating consequences for affected individuals. Loss of memory and dementia are typical signs of Alzheimer's disease (AD) and mainly result from the depletion of acetylcholine-secreting cortical and hippocampal neurons; loss of voluntary movement, rigidity, tremor and imbalance are defining symptoms of Parkinson's disease (PD) and are mainly caused by degeneration of dopamine-secreting neurons in the *substantia nigra pars compacta*; uncontrolled movements (chorea) seen in Huntington's disease (HD) arise from loss of gamma-aminobutyric-acid-secreting inhibitory neurons in the striatum; finally, muscle weakness and progressive paralysis are typical signs of Amyotrophic lateral sclerosis (ALS) in which depletion of brain and spinal cord motoneurons leads to loss of acetylcholine-mediated muscle control (Figure 1).

Although manifesting with very different symptoms, neurodegenerative diseases share some common features, both at the clinical and cellular/molecular levels. These disorders typically affect individuals in their mid-life and the symptoms gradually worsen over time. Most of patients with neurodegenerative disease are sporadic, i.e. without any family history or known genetic defect. However, a minority of patients does inherit these disorders and it is the analysis of these families that provided essential clues about the pathophysiological processes that lead to nerve cell degeneration in these disorders. The identification of genes associated with familial neurodegenerative disease represented a breakthrough in neurodegeneration research as it allowed the generation of animal models that recapitulate these mutations and their detailed behavioral, anatomical and molecular analysis. It soon became clear that neurodegenerative disorders resemble each other in some aspects (Figure 1).

Dysfunction of the protein quality control and clearance machinery emerged as a common theme in neurodegeneration. Diseases as diverse as AD, PD, HD, ALS or Prion disease (caused by infectious Prion [PrP^{Sc}] particles accumulating in extracellular brain regions) display aggregation and deposition of misfolded proteins that lead to progressive CNS amyloidosis. Because of this distinctive property, neurodegenerative disorders are also called "protein clearance diseases". Accumulation of insoluble proteins impairs the function of the proteasome, the cellular machinery responsible for protein clearance. As a result, there is a general proteostasis defect, which has deleterious consequences for the cell. Another major recurring theme is the presence of mitochondrial dysfunction leading to enhanced production of reactive oxygen species (ROS). Increased levels of ROS (oxidative stress) negatively impact on cell viability, as ROS can covalently modify proteins, lipids and nucleic acids.

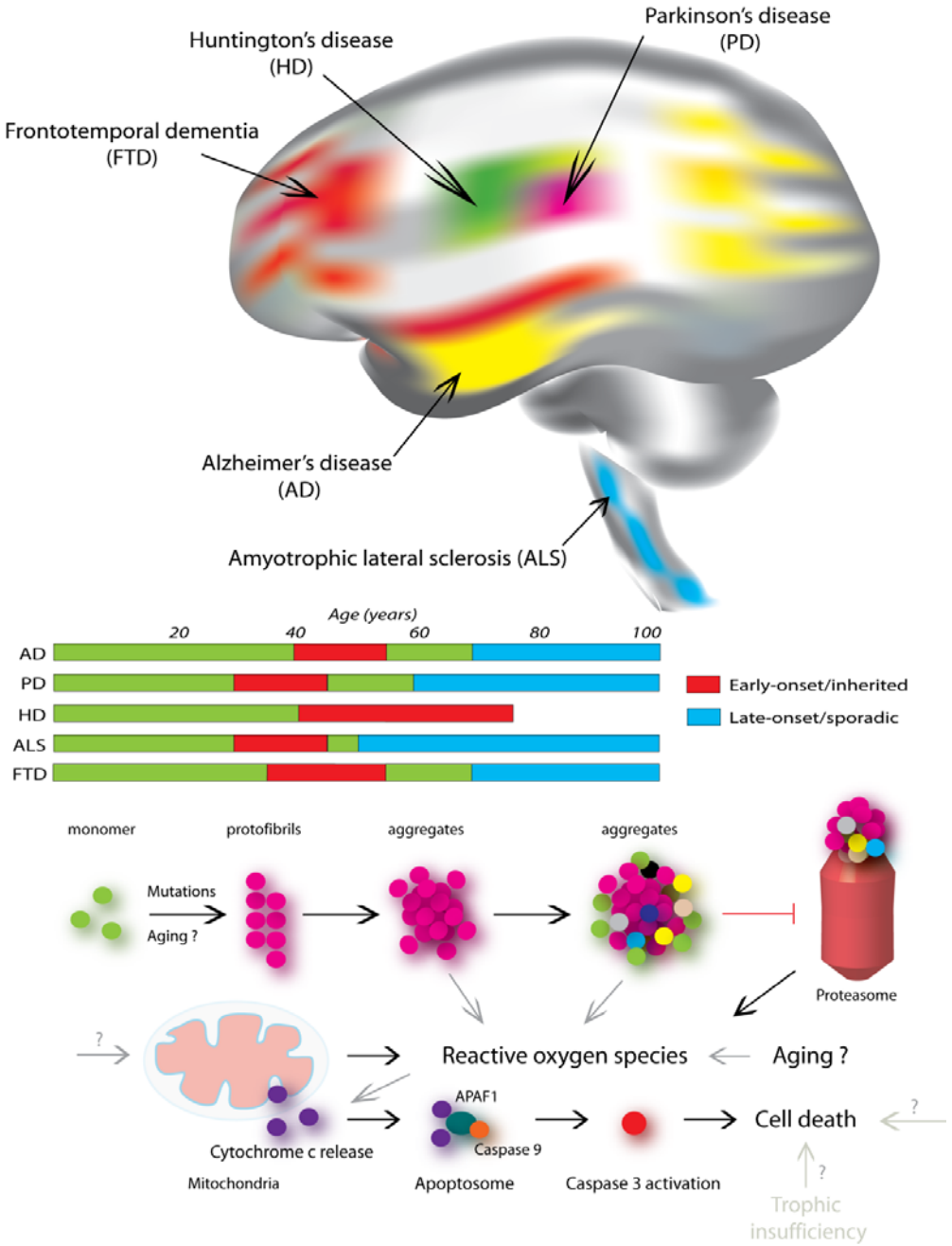


Figure 1. Anatomic and molecular aspects of neurodegenerative diseases. Each neurodegenerative disease shows relative specificity for a restricted brain region. AD affects mainly the temporal and parietal cortex and the hippocampus (yellow); Frontotemporal dementia (FTD) affects the frontal and temporal lobes of the cortex (red); Midbrain dopaminergic (DA) *substantia nigra pars compacta* (SN) neurons are lost in PD (purple); medium spiny striatal neurons are lost in HD (green); spinal cord (blue) and brain (not shown) motoneurons are lost in Amyotrophic lateral sclerosis (ALS). Shown in the middle is the age at disease onset; inherited forms (red) of disease start earlier, while sporadic forms (blue) display a slower progression. The lower part illustrates the common and most prominent pathological triggers of neurodegeneration. Abnormal protein aggregation generates oligomers and insoluble species (pink) that impair the proteasome and generate reactive oxygen species (ROS). Protein aggregates can further trap other functional proteins (other colors), leading to their inactivation. Mitochondrial dysfunction also generates ROS. The sum of all pathological insults initiates the apoptotic program that starts with cytochrome c release from mitochondria and culminates with the activation of caspases. It is currently unclear whether neurotrophic factor insufficiency has a disease-promoting effect or whether neurotrophic factor delivery into the diseased brain can halt neurodegeneration.

These and additional, yet unidentified pathological changes seem to converge on highly-conserved apoptotic pathways that culminate with the activation of caspases, which are highly specialized cysteine-aspartic proteases that execute the apoptotic program leading to cell death (Figure 1).

Although extensive research succeeded in identifying a number of risk factors and pathological mechanisms that might cause neurodegeneration, we currently do not know why only specific neuronal populations are lost in certain patients during aging. This lack of understanding of disease etiology is reflected in our poor therapeutic arsenal to treat neurodegeneration. No therapy to date is able to slow down or prevent neuronal cell death. The existing therapies are all symptomatic, have moderate efficacy and important side effects. Another major preoccupation is the increasing incidence of neurodegenerative disease in our aging society. There is high probability to develop AD after 85 years of age, and PD is most common in those above the age of 70 years. Amyotrophic lateral sclerosis (ALS), a disease in which motoneurons are specifically lost, rises sharply after the age of 40 years (Figure 1). The rising prevalence of neurodegenerative disease worldwide will have dramatic consequences for our economy and health care system if effective therapies are not soon developed.

Neurotrophic factors are molecules regulating neuronal survival and function and have been proposed to be potential therapeutic agents for these degenerative disorders. Current clinical trials dealing with neurotrophic factor delivery into the diseased brain have serious limitations and yield conflicting results, suggesting that improvements in both technology and understanding of neurotrophic factor biology are necessary.

The first part of the present work investigates the physiological relevance of two important neurotrophic factors, the Glial cell line-derived neurotrophic factor (GDNF) and the Brain-derived neurotrophic factor (BDNF) in the aging *substantia nigra pars compacta* (SN) neurons, the midbrain population that is lost in PD. Using mouse genetics, I provide evidence that GDNF signaling via the Ret receptor tyrosine kinase is required for maintenance of aging SN neurons. In the second part, I provide the first *in vivo* evidence for a pro-survival role of *DJ-1* in mouse SN neurons; moreover, using genetic analysis in *Drosophila melanogaster*, I show that *DJ-1* interacts genetically with *Ret*-associated signaling and uncover a novel physiological role for the *Drosophila* orthologue *DJ-1B* during retinal and wing development.

1.2. Two centuries of Parkinson's disease research

PD is a condition that has been known since ancient times. It is referred to in the ancient Indian medical system of *Ayurveda* under the name *Kampavata*, as far as 5000 BC. The physician Galen first described it in the Western literature in 175 AD, and named it 'shaking palsy'. However, it is only in 1817 that a detailed medical assay was published on PD. In his classical monograph "An essay on the shaking palsy", James Parkinson (1755-1824) described with amazing precision the core clinical features of PD. He described the disease as involving "involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported… [and] with a propensity to bend the trunk forwards". The term 'Parkinson's disease' was coined later by Jean-Martin Charcot. In 1912, the neurologist Frederic Lewy identified the presence of spherical cytoplasmic inclusions (Lewy bodies) in the brains of PD patients. The underlying biochemical changes in PD were identified largely thanks to the work of the Swedish pharmacologist Arvid Carlsson (born 1923). He demonstrated that dopamine was a neurotransmitter in the brain and not just a precursor of norepinephrine and developed methods to measure dopamine concentrations. In 1957, he performed a breakthrough experiment that led to the identification of dopamine as a critical regulator of movement. Carlsson treated animals with reserpine which depleted neurotransmitter stores which caused a strong decrease in dopamine levels and loss of movement control, similar to PD; he then administered the dopamine precursor L-DOPA (L-3,4-dihydroxyphenylalanine) to animals and succeeded in completely blocking the inhibitory effects of reserpine. L-DOPA was the first drug used to treat PD symptoms, and entered clinical practice in 1967 (Bjorklund and Dunnett, 2007). Subsequent studies established that the bulk of dopamine in the human brain was located in the striatum and that PD patients display a marked loss of striatal dopamine. Subsequent refinements of histological techniques allowed identification and mapping of midbrain *substantia nigra pars compacta* (SN) neurons, which densely innervate the striatum and which were found to be dramatically depleted in PD patients (Figure 2). In 1997 Maria Spillantini found that alpha-synuclein is the major component of Lewy Bodies (Spillantini et al., 1997). During the last 12 years, the study of familial PD led to the identification of PD-associated genes and the generation of animal models that recapitulate some features of PD, thus opening the molecular biology era of PD.

1.3. Loss of *substantia nigra* neurons causes Parkinson's disease

The demonstration by Carlsson that DA is a neurotransmitter in the brain was followed by the discovery that loss of *substantia nigra pars compacta* (SN) neurons leads to loss of striatal dopamine, which is responsible for the major symptoms of PD (Bjorklund and Dunnett, 2007). *Substantia nigra* is the largest structure in the midbrain, is located ventrally and plays an important role in movement, reward, addiction and learning. Its Latin name signifies "black substance" as parts of the human *substantia nigra* appear darker than neighboring areas due to the presence of the pigment melanin. Anatomical studies found that *substantia nigra* consists of 2 parts, with different connections and functions. The *substantia nigra pars reticulata* is the output of the basal ganglia and conveys signals to other brain regions involved in motor control (see below). The *substantia nigra pars compacta* (hereafter referred to as SN), with its melanine-containing DA cell bodies, is responsible for the darker appearance of this region (Figure 2A,C); loss of SN neurons as seen in PD leads to the disappearance of the

typical melanin pigment and the remaining SN neurons display intracytoplasmic protein aggregates containing alpha-synuclein (Lewy Bodies; Figure 2B,D,E).

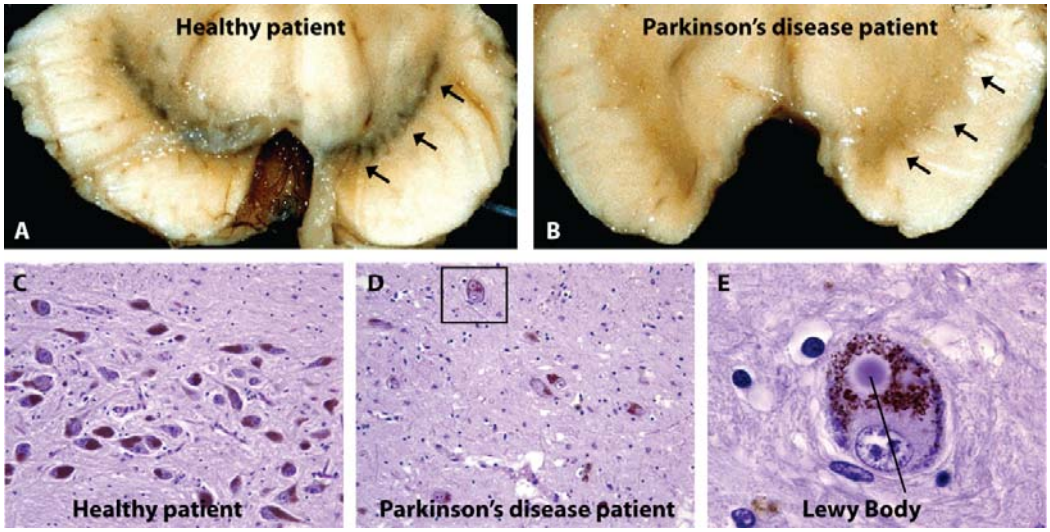


Figure 2. Loss of *substantia nigra* (SN) neurons causes Parkinson's disease. Pathological examination of a healthy patient (A) reveals typical pigmented DA neurons in the SN (arrows); in contrast, loss of SN neurons leads to pigment disappearance in the PD brain (B, arrows). Magnification of the SN area reveals a dense network of melanin-pigmented SN neurons in the healthy brain (C) while most of SN neurons are lost in PD (D). Some of the remaining neurons in PD contain insoluble cytoplasmic protein aggregates (Lewy Bodies, E) that are made of aggregated alpha-synuclein and other proteins. The melanin-containing granules have a red-brown hue and are distributed in the cytosol of all SN neurons (C-E). The picture in E is the higher magnification of the dark-boxed area in D. Adapted from Agamanolis, 2006.

SN serves mainly as input to the basal ganglia circuit, by supplying the striatum with dopamine (nigrostriatal pathway; Figure 3). Although the dorsal striatum is the major projection area for nigral DA neurons, SN neurons were recently found to project to other brain areas, including cortical and limbic areas (Bjorklund and Dunnett, 2007).

All DA neurons in the SN are positive for the DA marker tyrosine hydroxylase (TH), an enzyme involved in dopamine biosynthesis. However, two DA subpopulations were identified in the SN, based on the selective expression of additional markers and differential connectivity patterns. The majority (approx. 75 %) of TH-positive DA neurons in the SN also express the G protein-activated inward rectifier potassium channel 2 (GIRK2) and project exclusively to the striatum. A minority (approx. 25 %) of TH-positive SN DA neurons expresses the calcium-binding protein Calbindin; it is these neurons that project to limbic and cortical areas (Bjorklund and Dunnett, 2007; Korotkova et al., 2004).

1.3.1 Most substantia nigra neurons innervate the dorsal striatum

The development of the nigrostriatal system begins at the embryonic day E7.5 when the first progenitors located in the mesencephalon give rise to multiple cell types. In a second step, undifferentiated cells are progressively specified to become DA, by the orchestrated action of several extracellular modulators (Sonic hedgehog, Wnt1/5a, TGF-β, FGF-20) and intracellular transcription factors (Otx2, Gx2, En1/2, Lmx1a/b,

Pax2/3/5/7/8, Gli1/2/3, Nkx, Nurr1) (Smidt and Burbach, 2007). The midbrain precursor cells exit the cell cycle at E10-E11.5 and start expressing the first DA markers, including the tyrosine hydroxylase (TH) and other enzymes involved in the dopamine biosynthesis pathway (Abeliovich and Hammond, 2007). At E11.5 DA neurons engage in the process of axonal pathfinding and follow guidance cues provided by the Semaphorin family ligands, Slits, Netrins and Ephrins to reach their target area, the striatum (Abeliovich and Hammond, 2007). Upon completing target innervation (around E14.5), DA axons compete to establish synapses and survive; several target-derived neurotrophic factors have been proposed for DA neurons (Burke, 2003; Krieglstein, 2004; Smidt and Burbach, 2007), including GDNF and BDNF.

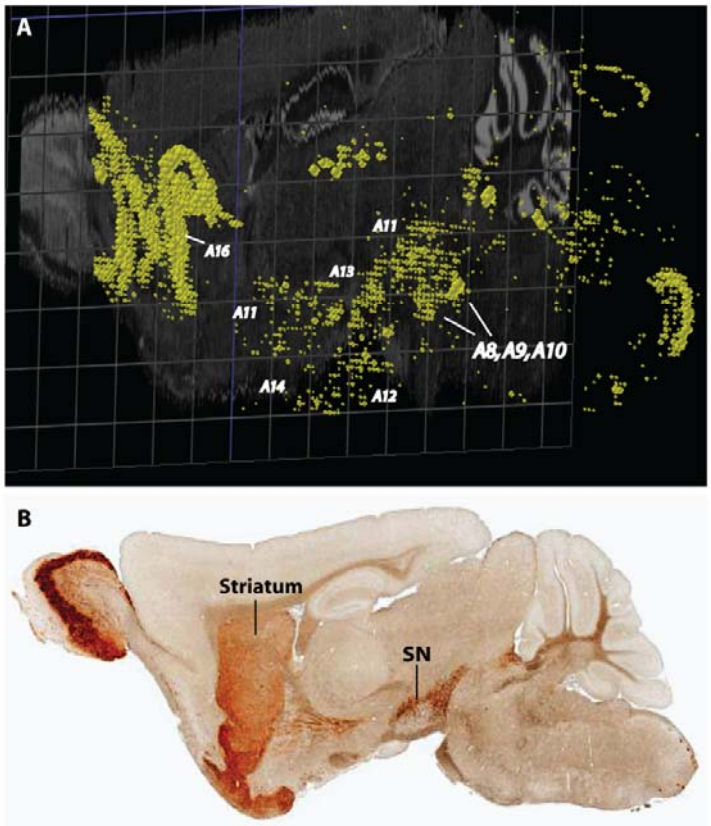


Figure 3. Dopaminergic cell groups and the nigrostriatal pathway. (A) Dopaminergic and non-DA (adrenergic and noradrenergic) neurons in the mouse brain expressing the tyrosine hydroxylase (TH) marker are visualized here in 3D (cell bodies are in yellow). Nine groups of DA neurons are found in the mouse brain (A8-A16, according to the classification by (Dahlstroem and Fuxe, 1964); three groups from these DA neurons are found in the midbrain : A8-retrorubral field, A9-*substantia nigra* (SN) and A10-ventral tegmental area (VTA). There are additional, non-DA neurons that also express the TH enzyme, which regulates synthesis of all dopamine, adrenaline and noradrenaline (groups A1-A7, localized caudal to the midbrain; not labeled). (B) The nigrostriatal pathway is shown in a sagital section of the mouse brain stained for TH. The SN cell bodies, located in the ventral midbrain send their axons (via the medial forebrain bundle) to the dorsal striatum. Note that the neighboring VTA neurons (also labeled by TH) innervate preferentially the ventral striatum. The picture in A was generated by the *Brain Explorer* software using the TH-expression data from the Allen Brain Atlas (www.brain-map.org).

Dopaminergic axons originating from the SN and innervating the dorsal striatum (Figure 3) travel through a major axon track (the *medial forebrain bundle,* MFB), that contains both ascending and descending fibers from different brain regions (Burke, 2003; Hattori, 1993).Transection of the MFB leads to massive degeneration of SN cell bodies and axons, and is a commonly used DA lesion in experimental studies investigating the survival requirements of SN cell bodies (Deumens et al., 2002). The dramatic degeneration of SN cell bodies after MFB axotomy also suggests that survival signals must travel retrogradely from the DA terminals in the striatum, along DA axons, to support survival of SN cell bodies. Finally, dopaminergic axons reaching the striatum elaborate distal terminations (axon terminals) that release dopamine to modulate motor control.

In contrast to the fast synaptic transmission mediated by glutamate or GABA (milliseconds) and involving opening of ion channels, dopamine mediates a slow synaptic transmission (hundreds of milliseconds to minutes) to impact its striatal target neurons. This slow synaptic transmission is mediated by dopamine binding to dopamine receptors. Five post-synaptic dopamine receptors have been identified, which either stimulate (D1, D5) or inhibit (D2-4) adenylyl cyclase activity and cAMP production. D1 and D2 receptors are abundant in the striatum and although they have opposite effects at the molecular level, they often have a synergistic action when more complex outputs are considered. Dopamine, acting via D1-5 receptors, exerts profound effects on postsynaptic neurons, ranging from short-term changes to long-term modulation of plasticity and synaptic efficacy (Greengard, 2001).

1.3.2. The basal ganglia modulate movement

Dopamine depletion due to loss of SN neurons as seen in PD leads to a severe impairment of voluntary movement and are responsible for the characteristic motor symptoms in PD: tremor at rest, postural imbalance, poverty of voluntary movements (akinesia), slowness and impaired scaling of movement (bradykinesia) and muscle rigidity (Dauer and Przedborski, 2003). These motor symptoms are the result of altered circuitry within the basal ganglia, a group of subcortical nuclei that are highly interconnected with the cerebral cortex, the thalamus and the brain stem (McHaffie et al., 2005). The basal ganglia consist of SN, the striatum globus pallidus (GP) and subthalamic nucleus (STN) (Figure 4) (Groenewegen, 2003). The striatum is the main input nucleus in the basal ganglia and receives excitatory (glutamatergic) input from the cortex and the thalamus and DA input from the SN. The DA input, transduced via dopamine D1 and D2 receptors has a modulatory role on striatal output activity. This information is processed by a striatal network comprising 10 % interneurons and 90 % GABAergic projection neurons, the so-called "medium spiny neurons" (MSNs). Striatal neurons then send inhibitory (GABAergic) connections to the GP external (GPe) and internal (GPi) segments and to the SN *pars reticulata*. The axis STR/GPi is known as the "*direct"* pathway and the STR/GPe/STN axis as the "*indirect"* pathway (Obeso et al., 2008). The output nuclei (GPi and SN *pars reticulata*) consist of GABAergic neurons that fire spontaneously at high frequency, continuously inhibiting neurons in target areas, such as the thalamus. Activation of striatal neurons by cortical inputs leads to inhibition of this output nuclei, via the *direct* pathway, and stimulates movement. The indirect pathway consists of two successive inhibitions and thus sustains the inhibitory action of the output nuclei, thus inhibiting movement (Figure 4). Loss of dopamine as seen in PD disrupts the cortico-striatal balance, leading to reduced

activity in the *direct* pathway and increased activity in the *indirect* pathway. As a consequence, there is hyperactivity in the STN and the STR and excessive inhibitory output in the GPi (Obeso et al., 2008), although the precise chain of events is incompletely understood (Nambu, 2008).

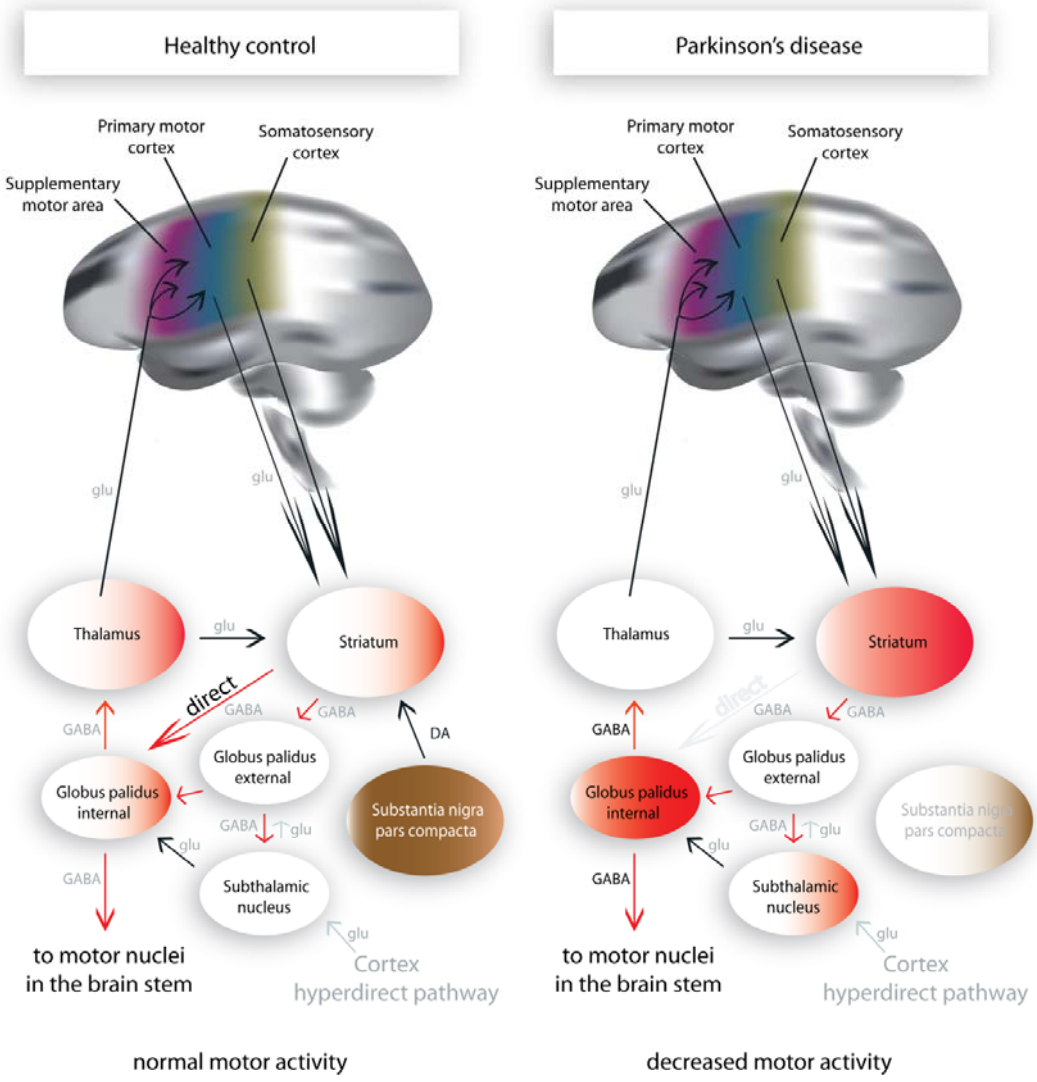


Figure 4. Basal ganglia and the control of movement. The striatum is the input structure of the basal ganglia. It receives excitatory input from the cortex and the thalamus and modulatory dopaminergic input from the *substantia nigra pars compacta* (SN). Two pathways originate in the striatum: the direct pathway (STR/GPi) that promotes movement and the indirect pathway (STR/GPe/STN/GPi) that inhibits movement. Loss of SN neurons leads to imbalance in the striatal output; the activity of the direct pathway is decreased and the indirect pathway is overactive. As a consequence, there is increased inhibitory activity in the GPi, which inhibits voluntary movement initiation (via the cortex) but also decreases movement execution (via inhibition of motor nuclei in the brain stem). GPe, *globus palidus* external segment; GPi, *globus palidus* internal segment; STN, subthalamic nucleus; STR, striatum. Excitatory connections use glutamate (glu) while inhibition is GABA-mediated (GABA). Adapted from (Groenewegen, 2003; Nambu, 2008).

1.3.3. The VTA neurons regulate emotional states and cognition

Besides SN neurons, another DA population is present in the ventral midbrain: the ventral tegmental area (VTA), the so-called A10 group (Figure 3) (Haber and Fudge, 1997). VTA neurons are TH-positive and the majority of them express the calcium-binding protein Calbindin, while a small fraction expresses the ion potassium channel GIRK2. VTA neurons play little, if any, role in movement control and mainly project to the limbic (including nucleus accumbens and the amygdala in the anteromedial and ventral striatum; and the septum; *the mesolimbic pathway*) and cortical (cingulated, prefrontal and perirhinal cortex; *mesocortical pathway*) areas (Fields et al., 2007). The mesolimbic and mesocortical pathways are involved in the regulation of emotional and cognitive states respectively; in addition, they are major regulators of reward behaviors and control drug and alcohol addiction (Bjorklund and Dunnett, 2007; Fields et al., 2007; Kauer, 2004).

1.3.4. Regional specificity and classification of Parkinsonian syndromes

PD has proven to be a heterogeneous condition. Although SN is dramatically affected in PD patients, neurodegeneration in PD extends beyond the SN, and affects (although with less severity) the neignboring DA VTA (A10) and retrorubral field (A8) regions, the noradrenergic (*locus coeruleus*), serotonergic (raphe) and the cholinergic (*nucleus basalis* of Meynert); in addition, the cerebral cortex, the olfactory bulb and the autonomic nervous systems are also affected and LB presence was equally documented in these brain areas (Dauer and Przedborski, 2003).This extended neurodegenerative pattern is likely underlying the non-motor symptoms observed in PD, including dementia and depression (Dauer and Przedborski, 2003).

Second, several partially distinct disorders manifest with PD symptoms. The term Parkinsonism has therefore been coined to encompass all syndromes that manifest with motor symptoms similar to PD. While nigrostriatal degeneration is the common pathological substrate of all Parkinsonism forms, additional changes might differentiate between PD and non-PD Parkinsonism (Figure 5). For example, in Multiple system atrophy (MSA; in which alpha-synuclein aggregates in glial cells), besides loss of SN neurons, striatal and basal ganglia output neurons (GPi and SN *pars reticulata*) are lost, in contrast to PD (Halliday, 2007). Similarly, some AD patients or patients with progressive supranuclear palsy (PSP) also manifest with Parkinsonism, although they display Tau, but not alpha-synuclein, aggregates (Forman et al., 2005); moreover, PSP patients also display loss of basal ganglia output neurons, similar to MSA patients (Halliday, 2007). The presence of LBs is also detected in dementia with LBs (DLB), making the distinction between PD and DLB difficult (Figure 5). These differences argue for a better understanding of the underlying molecular and cellular changes that generate this diversity of Parkinsonian phenotypes. In addition, the identification and development of specific biomarkers might improve the clinical diagnosis and therapeutic outcome for Parkinsonian disorders.

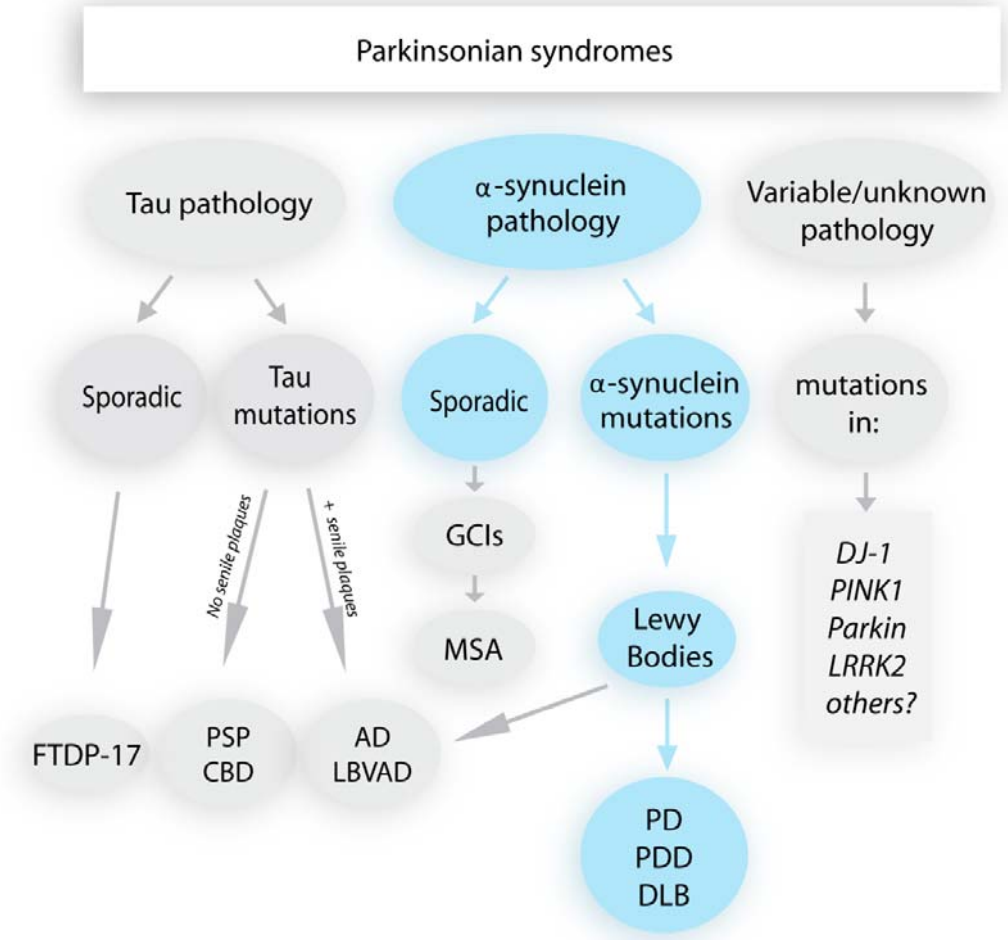


Figure 5. Classification of major diseases that manifest with Parkinsonism. Several molecularly distinct pathologies cause degeneration of SN neurons and manifest with PD-like symptoms. The presence of Lewy Bodies in SN neurons is by far the most common form of Parkinsonism. However, diseases that manifest with LBs in SN neurons and concurrent dementia (dementia with Lewy Bodies, DLB; Parkinson's disease dementia, PDD) are difficult to differentiate pathologically. Moreover, neuropathological examination of a subset of patients with Parkinsonism reveals that they do not display LBs in SN neurons; these patients display either alpha-synuclein inclusions in glial cells (Multiple system atrophy, MSA) or Tau inclusions in different brain areas. Tau mutations cause accumulation of aggregated Tau and concurrent frontotemporal dementia (FTD), while Tau pathology in sporadic cases without senile plaques is associated with either corticobasal degeneration (CBD) or progressive nuclear suprapalsy (PSP), in addition to Parkinsonism. The presence of senile plaques in sporadic patients is an indication of concurrent Alzheimer's disease (AD) or Lewy Body variant of Alzheimer's disease (LBVAD). In addition to motor impairments, all these concurrent disorders manifest with non-motor symptoms (e.g. dementia, depression, autonomic failure). Note that additional disorders might cause Parkinsonism in humans. Adapted from (Forman et al., 2005).

1.4. Genes associated with familial Parkinson's disease

One major advance in PD research was the identification of familial PD and the analysis of the underlying mutations in these families. The systematic analysis of extended pedigrees ultimately led to the discovery of PD-associated genes (Gasser, 2009b; Henchcliffe and Beal, 2008). About 10 % of all PD cases are genetic, the rest being sporadic (with no family history and no identified genetic defect). It is commonly believed that understanding the neurodegeneration in monogenic PD would lead to a better understanding of sporadic PD, which is likely caused by an interaction between a network of weakly-acting gene alterations and environmental factors. The genes linked to monogenic PD display either an autosomal-dominant or autosomal-recessive pattern of inheritance. Mutations in *alpha-synuclein* and *LRRK2* (also called *Dardarin*) cause autosomal dominant PD, while mutations in *Parkin*, *PINK1* or *DJ-1* cause autosomal recessive PD (Table 1).

Locus	Gene	Inheritance	Function
PARK1/4	*α-Synuclein*	AD	Involved in synaptic vesicle formation
PARK2	*Parkin*	AR	Ubiquitin E3 ligase
PARK6	*PINK1*	AR	Mitochondrial kinase
PARK7	*DJ-1*	AR	Oxidative stress suppressor
PARK8	*LRRK2*	AD	Multi-domain protein kinase
PARK9*	*ATP13A2*	AR	P-type ATPase
PARK13*	*OMI/HTRA2*	AD?	Serine protease

Table 1. The genes linked to familial Parkinson's disease. Mutations in alpha-synuclein or LRRK2 (also called Dardarin) cause autosomal dominant (AD) PD. Mutations in Parkin, PINK1 or DJ-1 cause autosomal recessive (AR) PD. * Mutations in two additional genes, ATP13A2 and OMI/HTRA2 have been tentatively associated with PD however, additional genetic evidence and replication are required. Note that additional loci were reported to be associated with PD or act as risk factors for PD development, but the evidence is currently weak (Gasser, 2009a; Hardy et al., 2009; Thomas and Beal, 2007).

1.4.1. The alpha-synuclein gene encodes a presynaptic terminal protein

Before its identification as a PD-linked gene, alpha-synuclein (*α-syn)* was found to be the major component of Lewy Bodies, the cytoplasmic insoluble aggregates often observed in the surviving SN neurons in PD (Spillantini et al., 1997). The identification of *α-syn* mutations in families with PD came therefore as a big surprise. The most common *α-syn* mutations are missense substitutions Ala53Thr (A53T) (Polymeropoulos et al., 1997) and Ala30Pro (A30P) (Kruger et al., 1998), although additional mutations were found (Gasser, 2009). These mutations induce a L-DOPA responsive Parkinsonism with a relatively early onset for A53T and somewhat later onset for A30P. Autopsy of patients with *α-syn* mutations showed cell loss in the SN and accumulation of α-syn not only in SN neurons but also in other neuronal populations, showing that dominant PD with *α-syn* mutations overlaps (but is not identical) with typical sporadic late-onset PD (Gasser, 2009). Remarkably, multiplications of the wild-type *α-syn* sequence (duplications and triplications) also cause Parkinsonism with α-syn inclusions (and with or without dementia) in some families (Ibanez et al., 2004; Singleton et al., 2003). This result is important as it shows

that mere increase in α-syn levels is toxic for neurons. Interestingly, further support for this hypothesis comes from the observation that triplications of *α-syn* induce an early onset (35 years) and high prevalence of dementia, while duplications have a later onset (Chartier-Harlin et al., 2004). As mentioned above the pathology of *α-syn* duplications was also detected in the cortex (with regions of spongiosis and gliosis) and α-syn inclusions were found both in cortical neurons (mostly in the lower cortical layers) and glial cells. These observations support the hypothesis that PD and DLB reflect the same pathogenic process (abnormal α-syn accumulation) with variable levels of severity.

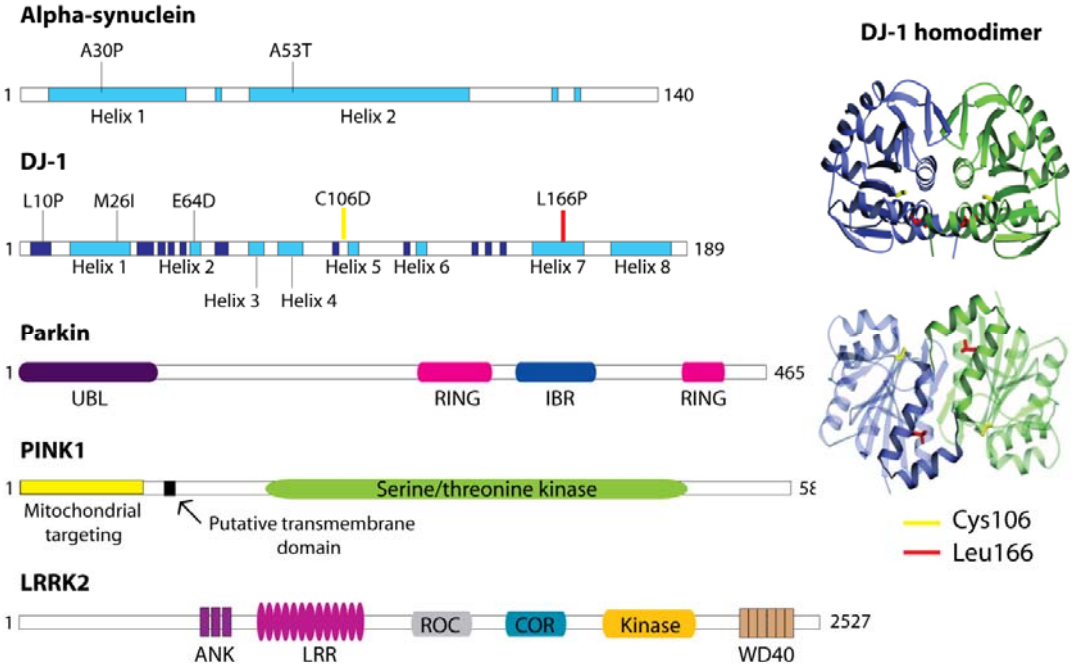


Figure 6. Structure of proteins linked to Parkinson's disease. Alpha-synuclein and DJ-1 are small proteins that lack conserved domains. Alpha-helixes are displayed in light blue and beta-sheets are in dark blue. The most common mutations are displayed only for alpha-synuclein and DJ-1. The 3D structure of the DJ-1 homodimer is displayed on the right. The second view of the dimer (below) is rotated by 90° relative to the top view. The oxidative-stress reactive Cysteine 106 is displayed in yellow, while the Leucine 166, mutated in PD, is displayed in red. Parkin contains an ubiquitin like domain (UBL) located N-terminally, a central linker region and a C-terminal domain comprising two RING (*really interesting new gene domain*) finger domains and a IBR (*in-between Ring domain*) domain. PINK1 contains an N-terminal mitochondrial targeting sequence, a putative transmembrane domain and a Serine/threonine kinase domain. LRRK2 is a large protein that comprises an ANK (*ankyrin repeat*) domain, a LRR (*leucine rich repeat*) domain, a ROC (Ras of complex proteins) domain, a COR (*C-terminal of ROC*), a WD40 (*Trp-Asp 40 repeats*) and a kinase domain. DJ-1 3D structure is from (Wilson et al., 2003).

The α-syn protein is a natively unfolded presynaptic protein, that is believed to regulate synaptic vesicle recycling and neurotransmitter storage and compartmentalization, and during these processes it associates with vesicular and membranous structures (Abeliovich et al., 2000; Yavich et al., 2006; Yavich et al., 2004). The protein consists of an N-terminal amphipathic region, a middle region containing a non-amyloid-beta component domain and a C-terminal acidic region (Figure 6). Removal of amino acids

71-82 prevents aggregation of overexpressed α-syn, while overexpression of the non-amyloid-beta domain alone led to increased aggregation and neurotoxicity *in vivo* (Periquet et al., 2007).The presence of the central hydrophobic non-amyloid-beta domain makes α-syn susceptible to aggregation. The A53T and A30P mutations increase the aggregation capacity of the protein (Dauer and Przedborski, 2003) and aggregated α-syn is the major component of LBs (Spillantini et al.,1997). Phosphorylation of α-syn at Ser129 was found to enhance its aggregation, and Ser129 phosphorylated α-syn is a major component of LBs (Anderson et al., 2006; Smith et al., 2005a). Truncated versions of α-syn have also been observed in LBs (Smith et al., 2005) and overexpression of C-terminally truncated α-syn in mouse SN neurons strongly increased aggregate formation and led to dopaminergic cell loss *in vivo* (Tofaris et al., 2006).

1.4.2. The LRRK2 gene encodes a multi-domain kinase

The second major locus for an autosomal dominant PD harbors the very large *LRRK2* (*Leucin-rich repeat kinase 2;* also called *Dardarin*) gene consisting of 51 exons and coding for a 2527 amino acid kinase (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Mutations in *LRRK2* cause typical L-DOPA responsive parkinsonism with relatively late-onset (late fifties) (Funayama et al., 2002) and remarkably, *LRRK2*-associated PD is much more prevalent than *α-syn*-associated PD; between 5-15% of dominant families carried mutations in *LRRK2*, as assessed across different populations (Berg et al., 2005) and a great majority of them carry the Gly2019Ser (G2019S) mutation (Gasser, 2009). The pathology of *LRRK2*-linked parkinsonism is unclear; post-mortem analysis of patients from one family found typical Lewy body disease, but also include variations like dementia with LBs (DLB, in which LBs are present in both the SN and the cortex), nigral degeneration without α-syn inclusions and rarely, PSP-like Tau aggregation (Ross et al., 2006; Zimprich et al., 2004). *LRRK2* mutations might thus represent an upstream event in the cascade leading to neurodegeneration with different pathologies (Gasser, 2009).

The *LRRK2* gene encodes a multidomain protein that possesses a Rho/Ras-like GTPase domain, a protein kinase domain of the MAPKKK family, as well as WD40-repeat and a leucine-rich repeat domains (Figure 6). Close to the GTPase domain, an additional domain termed COR (for carboxy-terminal of Ras) has an unknown function. The LRRK2 protein was found in the Golgi apparatus, in lysosomes and synaptic vesicles but also associated with the outer mitochondrial membrane (Biskup et al., 2006; Galter et al., 2006; Higashi et al., 2007; Simon-Sanchez et al., 2006; Taymans et al., 2006). The G2019S mutation is associated with increased kinase activity (Gloeckner et al., 2006; Smith et al., 2005b; West et al., 2007) and mutant G2019S *LRRK2* is found in LBs (Zhu et al., 2006). LRRK2 kinase activity is required for neurotoxicity *in vitro* (Greggio et al., 2006) and G2019S *LRRK2* induces a reduction in neurite growth and branching *in vitro* and causes aggregate formation and SN degeneration when virally delivered into the rat brain (MacLeod et al., 2006). Thus, inhibition of kinase activity is a potential therapeutic strategy for *LRRK2*-linked Parkinsonism.

The identification and genetic analysis of families with a recessive pattern of PD-inheritance ultimately led to the isolation of 3 additional genes, which cause PD when inactivated: *Parkin*, *Pink1* and *DJ-1*. Families carrying these autosomal recessive mutations experience an early onset of symptoms (second to fourth decade) and

clinically show L-DOPA responsive Parkinsonism. Because of this rapid progression, recessive Parkinsonism is also called autosomal-recessive juvenile Parkinsonism (AR-JP). Interestingly, although recessive Parkinsonism appears to progress rapidly, the development of dementia is slowed down in AR-JP patients compared to sporadic PD patients.

1.4.3. The Parkin gene encodes an Ubiquitin E3 ligase

The first gene linked to AR-JP to be identified was *Parkin* (Kitada et al., 1998) and *Parkin* mutations turned out to be a common cause of early onset AR-JP, estimated to account for 10 % of all cases (Periquet et al., 2003). Numerous mutations (missense mutations, nonsense mutations leading to premature termination of translation and exonic rearrangements [deletions, duplications, triplications]) were identified (Gasser, 2009). Except for the earlier onset, the clinical picture of most *Parkin*-linked AR-JP is quite similar to that of sporadic disease. The neuropathology of *Parkin*-linked AJ-RP is still a matter of debate; although degeneration of SN neurons and gliosis in SN and the *locus coeruleus* have been documented, many studies found no LBs in the remaining SN neurons, suggesting that *Parkin*-linked AR-JP might differ in some ways from the typical idiopathic (sporadic) PD (Hardy et al., 2009). It is therefore possible that some *Parkin* mutations cause SN degeneration in the absence of α-syn aggregation.

The *Parkin* gene encodes a 465 amino acid protein, containing an N-terminal ubiquitin like domain, a central linker domain and a C-terminal RING domain that consists of 2 RING motifs separated by an in between RING domain (Figure 6). The protein is an ubiquitin E3 ligase and participates in the targeting of misfolded proteins to the proteasome for degradation (Shimura et al., 2000; Zhang et al., 2000). Parkin appears to be a multipurpose protein that confers neuroprotection against a wide range on insults (Feany and Pallanck, 2003). It activates the NFkB signaling (Henn et al., 2007) and can promote PI3k/Akt signaling by interacting with ubiquitin interacting motifs (UIM) of Eps15. Eps15 is an adaptor protein of Epidermal growth factor receptor (EGFR) endocytosis and trafficking and its ubiquitination by Parkin interferes with its interaction with EGFR, therefore delaying EGFR degradation and prolonging PI3K/Akt survival signaling (Fallon et al., 2006). Besides loss of ubiquitin E3 ligase activity, aggregation of Parkin represents another *loss-of-function* mechanism, relevant for sporadic PD: oxidative damage alters Parkin solubility and phosphorylation by the cyclin-dependent kinase 5 (Cdk5) renders Parkin insoluble (Avraham et al., 2007). Finally, accumulating evidence suggests that Parkin regulates mitochondria function via several mechanisms, including regulation of fusion/fission (Van Laar and Berman, 2009), mitochondrial transport (Yang et al., 2005a) and remarkably, targeting of dysfunctional mitochondria for autophagic degradation (Narendra et al., 2008).

1.4.4. The PINK1 gene encodes a mitochondrial kinase

Inactivating mutations in the *PINK1* (*phosphatase and tensin (PTEN) homolog-induced putative kinase 1*) gene were identified in 2004 (Valente et al., 2004). The *PINK1*-linked AR-JP displays an early onset (32-48 years), a slow progression and is L-DOPA responsive (Valente et al., 2004). Patients with *PINK1* mutations appear to have a higher prevalence of psychiatric disturbances (anxiety and depression), which are only rarely observed in patients carrying *Parkin* mutations (Ephraty et al., 2007). Interestingly, a compound heterozygous *PINK1* mutation was found in a sporadic PD

patient with late onset (in the seventh decade and negative family history for movement disorders) and rapid progression (Gelmetti et al., 2008), suggesting that decreased *PINK1* function induces a slowly progressive SN degeneration that leads to overt symptoms only during aging. No post-mortem examination of PD patients carrying *PINK1* mutations has been reported so far.

The PINK1 protein has 581 amino acids and consists of an N-terminal mitochondrial targeting sequence and a highly conserved protein kinase domain similar to Serine/Threonine kinases of the Ca^{2+} Calmodulin family (Figure 6). Its localization is suggested to be mitochondrial (Gandhi et al., 2006) and its functions are only beginning to be elucidated. PD-associated *PINK1* mutations lead to altered kinase activity and mitochondrial dysfunction (Beilina et al., 2005; Leutenegger et al., 2006) and truncation of the C-terminal domain of PINK1 reduces its serine/threonine kinase activity (Sim et al., 2006). While *in vitro* overexpression of *PINK1* protects cells from staurosporine-induced cell death, overexpression of PD-associated *PINK1* mutants (Petit et al., 2005) or PINK1 knockdown (Deng et al., 2005) fail to confer protection. Evidence also accumulates for a role of PINK1 in mitochondria dynamics, including fusion/fission (Van Laar and Berman, 2009) or transport (Weihofen et al., 2009), although its relevance to SN neurodegeneration remains to be demonstrated.

1.5. DJ-1 is an oxidative stress suppressor

The third locus for AR-JP was found to be mutated in a Dutch family (van Duijn et al., 2001) and the gene was identified as the oncogene *DJ-1* (Bonifati et al., 2003; Nagakubo et al., 1997). Indeed, increased levels of DJ-1 were found in several cancers (Kim et al., 2005a). A number of *DJ-1 loss-of-function* mutations, including homozygous and heterozygous point mutations, truncations and exonic deletions, were identified in PD patients (Bonifati, 2007). The clinical features of *DJ-1*-linked AR-JP appear to be similar to that of *Parkin* and *PINK1*-cases, and thus similar to that of sporadic PD, but this is based on a rather small number of identified patients. Interestingly, in one recessive *DJ-1* family, early-onset Parkinsonism, dementia and ALS were found to co-exist, suggesting that although rare, *DJ-1* mutations might cause an extended clinical phenotype (Annesi et al., 2005). Remarkably, a family with AR-JP was identified and found to harbor heterozygous *loss-of-function* mutations in both *DJ-1* and *PINK1*, suggesting that interaction between hypomorphic alleles of *DJ-1* and *PINK1* cause Parkinsonism (Tang et al., 2006). To date, no pathological examination was reported for AR-JP patients carrying *DJ-1* mutations. Interestingly, while in sporadic PD patients DJ-1 is rarely present in Lewy bodies (Neumann et al., 2004; Rizzu et al., 2004), it was found to co-localize with α-syn-immunoreactive glial inclusions in MSA and with a subset of pathological Tau inclusions in a number of neurodegenerative tauopathies, including AD (Neumann et al., 2004; Rizzu et al., 2004). Remarkably, increased levels of DJ-1 were found in the frontal cortex of PD and AD patients compared to healthy controls (Choi et al., 2006).

DJ-1 shows a ubiquitous expression but is strongly expressed in testis, brain and kidney and has been implicated in processes as diverse as male fertility, oncogenesis and neuroprotection (Nagakubo et al., 1997; Olzmann et al., 2004). In the brain, *DJ-1* is expressed in both neurons and glia; moreover, its presence in areas involved in motor control like basal ganglia, thalamus, *substantia nigra*, red nucleus or the motor cortex (Bandopadhyay et al., 2005; Kotaria et al., 2005; Shang et al., 2004) suggests that loss

of *DJ-1* might underlie the motor dysfunction seen in PD. At the subcellular level, DJ-1 is mostly localized to the cytosol, but appears to translocate to mitochondria under oxidative stress conditions (Blackinton et al., 2005; Canet-Aviles et al., 2004) and can enter the nucleus following serum stimulation or after prolonged cellular stress (Nagakubo et al., 1997; Zhang et al., 2005). Several *in vitro* studies suggested that DJ-1 is a versatile co-transcriptional regulator; once in the nucleus DJ-1 co-transcriptionally promotes expression of genes involved in oxidative stress response, anti-apoptosis, or genes regulating male fertility (Kahle et al., 2009b). Remarkably, DJ-1 was also detected in the extracellular space and its levels in plasma of PD patients correlated with disease progression (Waragai et al., 2007), suggesting that DJ-1 could serve as a biomarker for PD.

1.5.1. Structural biology of DJ-1

The *DJ-1* gene encodes a 189 amino acid protein that comprises eleven β-strands (β1-β11) and eight alpha-helixes (αA-αH) (Honbou et al., 2003; Tao and Tong, 2003; Wilson et al., 2003) (Figure 6). The central β-sheet of the structure contains 7 strands (β1,2,5,6,7,10,11). Outside of this central β-sheet, strands β3 and β4 form a β-hairpin that is involved in DJ-1 dimerization and strands β8 and β9 form a β-α-β motif together with the helix αF. Most of the α-helices flank the two faces of the β-sheet, with the exception of helix αH, situated at the extreme C-terminus, which also mediates DJ-1 dimerization (Wilson et al., 2003). DJ-1 tertiary structure (Figure 6) has some resemblance with proteins from a superfamily that includes the archetypical bacterial ThiJ and PfpI proteases. However, in contrast to these proteins, DJ-1 appears to have no protease activity, because its catalytic triad is distorted and its putative active site is occluded by the additional C-terminal helix (αH) (Martinat et al., 2004; Wilson et al., 2003). The disease causing *DJ-1* mutant *Leu166Pro* disrupts the αG helix and leads to overall destabilization of DJ-1 and subsequent degradation by the ubiquitin-proteasome system (Gorner et al., 2004; Macedo et al., 2003; Miller et al., 2003; Moore et al., 2003; Olzmann et al., 2004).

1.5.2. DJ-1 controls the oxidative stress response

Research in the last 10 years identified several cellular functions for DJ-1 (Figure 7). Converging *in vitro* and *in vivo* evidence indicates that DJ-1 is a potent oxidative stress suppressor. In cultured cells exposed to oxidative stress conditions, the isoelectric point (pI) of DJ-1 shows an acidic mobility shift (Kahle et al., 2009b) and further studies revealed that the cysteine residue Cys106, which is conserved among all members of the DJ-1/ThiJ/PfpI superfamily, is the key target of oxidative modifications (Kinumi et al., 2004). Mild oxidation of this cysteine residue leads to a sulfinic acid derivative ($-SO_2^-$), while further oxidation leads to sulfonic acid ($-SO_3^-$) derivatives (Figure 7); remarkably, mild oxidation to sulfinic acid promotes chaperone activity of DJ-1 towards α-syn*,* both *in vitro* (Shendelman et al., 2004; Zhou and Freed, 2005; Zhou et al., 2006) and in animal models (Shendelman et al., 2004), while further oxidation impairs this activity (Zhou et al., 2006). It has thus been proposed that the sulfinic acid-containing DJ-1 is the active form of DJ-1 (Blackinton et al., 2009b; Canet-Aviles et al., 2004).

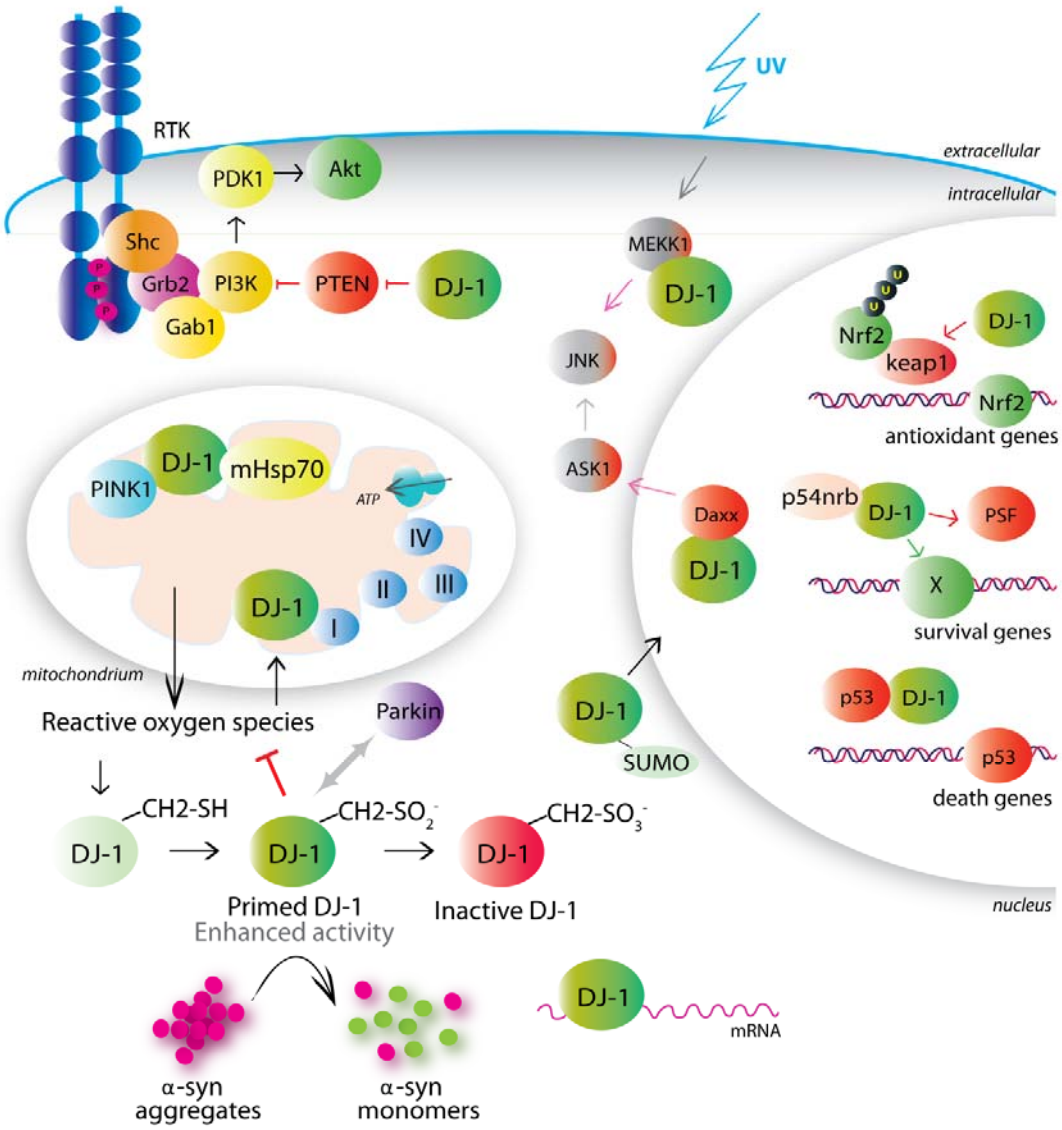


Figure 7. Putative functions for DJ-1. Converging *in vitro* and *in vivo* evidence suggests that DJ-1 is a potent oxidative stress suppressor. The protein can translocate to the mitochondria under conditions of oxidative stress; there, DJ-1 interacts with the resident chaperone mHsp70, and apparently with PINK1. Oxidative stress induces DJ-1 oxidation at the Cys106 residue, leading to a sulfinic acid derivative (DJ-1-$CH_2SO_2^-$ in green) that has enhanced chaperone activity towards misfolded α-syn; the Cys106 primed sulfinic derivative might also interact with Parkin. Further oxidation to a sulfonic derivative (DJ-1-CH_2-SO_3^- in red) inhibits DJ-1 function. Mutation of Cysteine106 inhibits the anti-oxidant and pro-survival activities of DJ-1 *in vivo*. Moreover, DJ-1 might inhibit the tumor suppressor PTEN, thereby promoting PI3K/Akt signaling. It can also negatively regulate JNK signaling, either by sequestering the ASK-1 activator Daxx in the nucleus, or by preventing MEKK1 from activating JNK, in UV-stressed cells. DJ-1 can also bind mRNA and influence translation of several targets. Finally, DJ-1 could act as co-transcriptional regulator to induce anti-oxidative genes (it promotes Nrf2 transcriptional activity by inhibiting its negative regulator Keap1) or survival genes (by promoting the activity of p54nrb or by inhibiting the pro-apoptotic transcription factor *pyrimidine tract-binding associated splicing factor*, PSF) or by binding to p53 to inhibit its pro-apoptotic targets. SUMOylation of DJ-1 is required for translocation to the nucleus. *In vivo* confirmation is required for most of the above proposed functions of DJ-1.

Interestingly, the amount of DJ-1 oxidized at Cys106 increases steadily with age both in humans and in mice (Meulener et al., 2006). Further *in vivo* support for a crucial role of Cys106 in DJ-1 anti-oxidative function comes from *Drosophila* studies; flies lacking *DJ-1B*, one of the two *Drosophila* homologues, are more sensitive to oxidative stress caused by PD-associated toxins like paraquat or rotenone or following H_2O_2 treatment; this sensitivity can be complemented by overexpression of wild-type fly *DJ-1B* and *DJ-1A*, suggesting that the two *Drosophila DJ-1* genes have a similar anti-oxidant function (Menzies et al., 2005; Meulener et al., 2006). Importantly, overexpression of *DJ-1B* in which Cys106 was mutated to alanine failed to rescue the increased lethality of *DJ-1B* null flies towards paraquat, providing direct *in vivo* evidence for a crucial role of Cys106 in DJ-1 function (Meulener et al., 2006). Besides Cys106 oxidation, DJ-1 was also found to be carbonylated and methionine-oxidized in the frontal cortex of PD and AD patients relative to controls (Choi et al., 2006), suggesting that DJ-1 may undergo extensive oxidative modifications in age-related neurodegenerative disorders. Another evidence for involvement of DJ-1 in oxidative stress control comes from *in vitro* studies where DJ-1 was identified as a co-transcriptional regulator that promotes expression of oxidative-stress responsive genes. DJ-1 was found to stabilize the Nuclear factor erythroid 2-related factor (Nrf2), a master regulator of antioxidant transcriptional responses by preventing it from interacting with Keap1. Keap1 negatively regulates Nrf2, by promoting its ubiquitination and subsequent degradation; stable Nrf2 activates numerous anti-oxidant genes, thereby exerting pro-survival effects (Clements et al., 2006).

1.5.3. Mitochondrial functions of DJ-1

Under conditions of oxidative stress, DJ-1 translocates to the mitochondria, and this event depends on an intact Cys106, suggesting that Cys106-oxidized DJ-1 might undergo mitochondrial re-localization (Canet-aviles et al., 2004). Moreover, wild-type DJ-1 protects cells against mitochondrial toxins *in vitro* and an engineered construct targeting DJ-1 to mitochondria led to significantly enhanced protection against H_2O_2 stress (Junn et al., 2009). Although *DJ-1* did not rescue the mitochondrial morphology alterations resulting from impaired *PINK1* and *Parkin* function *in vitro* (Exner et al., 2007; Yang et al., 2006) recent evidence suggests that DJ-1 influences mitochondrial morphology (Blackinton et al., 2009b), probably via a PINK1/Parkin-independent mechanism (Dodson and Guo, 2007).

1.5.4. DJ-1 and signal transduction

The human *DJ-1* gene was initially identified and cloned in the laboratory of Hiroyoshi Ariga (Nagakubo et al, 1997). *DJ-1* expression was induced by serum addition to starved HeLa cells, and the protein accumulated both in the cytoplasm and the nucleus. Moreover, DJ-1 displayed transforming activity when transfected into NIH3T3 cells; interestingly, when DJ-1 and c-myc or H-Ras were co-transfected, a synergistic effect was observed and the efficacy of DJ-1/H-ras was three times higher as the efficacy of H-Ras/c-myc (Nagakubo et al, 1997). These studies suggested that DJ-1 might display pro-mitogenic properties and might interact with Ras-signaling, at least in oncogenic conditions. A second study found that DJ-1 negatively regulates the PTEN phosphatase, the major negative regulator of the PI3K/Akt signaling. Increased DJ-1levels in breast cancer patients inversely correlated with PTEN immunoreactivity and *DJ-1* interacted genetically with *PTEN*, both in *Drosophila* and *in vitro* (Kim et al.,

2005a). Furthermore, DJ-1was found to modulate the activation status of the Akt kinase in CO7, A549 and NIH-3T3 cells (Kim et al., 2005), suggesting that altered PTEN activity might impact on the PI3K/Akt-dependent neuron survival in PD associated with DJ-1 mutations. Another possible DJ-1 interactor was suggested to be the tumor suppressor p53. *DJ-1* knockdown in zebrafish increased the levels of p53 and its target gene, the apoptotic mediator Bax (Bretaud et al., 2007). In addition, DJ-1 interacts physically with p53 in several cell types and inhibits its transcriptional activity; consequently, *DJ-1* overexpression leads to reduced Bax levels (Fan et al., 2008b).

DJ-1 was also found to inhibit JNK signaling *in vitro*. Via its nuclear interaction with the death protein Daxx in SH-SY5Y dopaminergic neuroblastoma cells, DJ-1 sequesters Daxx in the nucleus thus preventing it from gaining access to the cytoplasm. Cytoplasmic Daxx has pro-apoptotic effects - it binds to the intracellular domain of the Fas receptor and also directly activates the Apoptosis signal-regulating kinase 1 (ASK1), an upstream activator of the pro-apoptotic JNK signaling (Junn et al., 2005). In a second study, DJ-1 suppressed the ultraviolet-induced JNK activation in HEK293 cells by direct interaction the JNK upstream activator MEKK1 in the cytoplasm and inhibition of its nuclear translocation (Mo et al., 2008). Thus, DJ-1 might function as a negative regulator of JNK signaling.

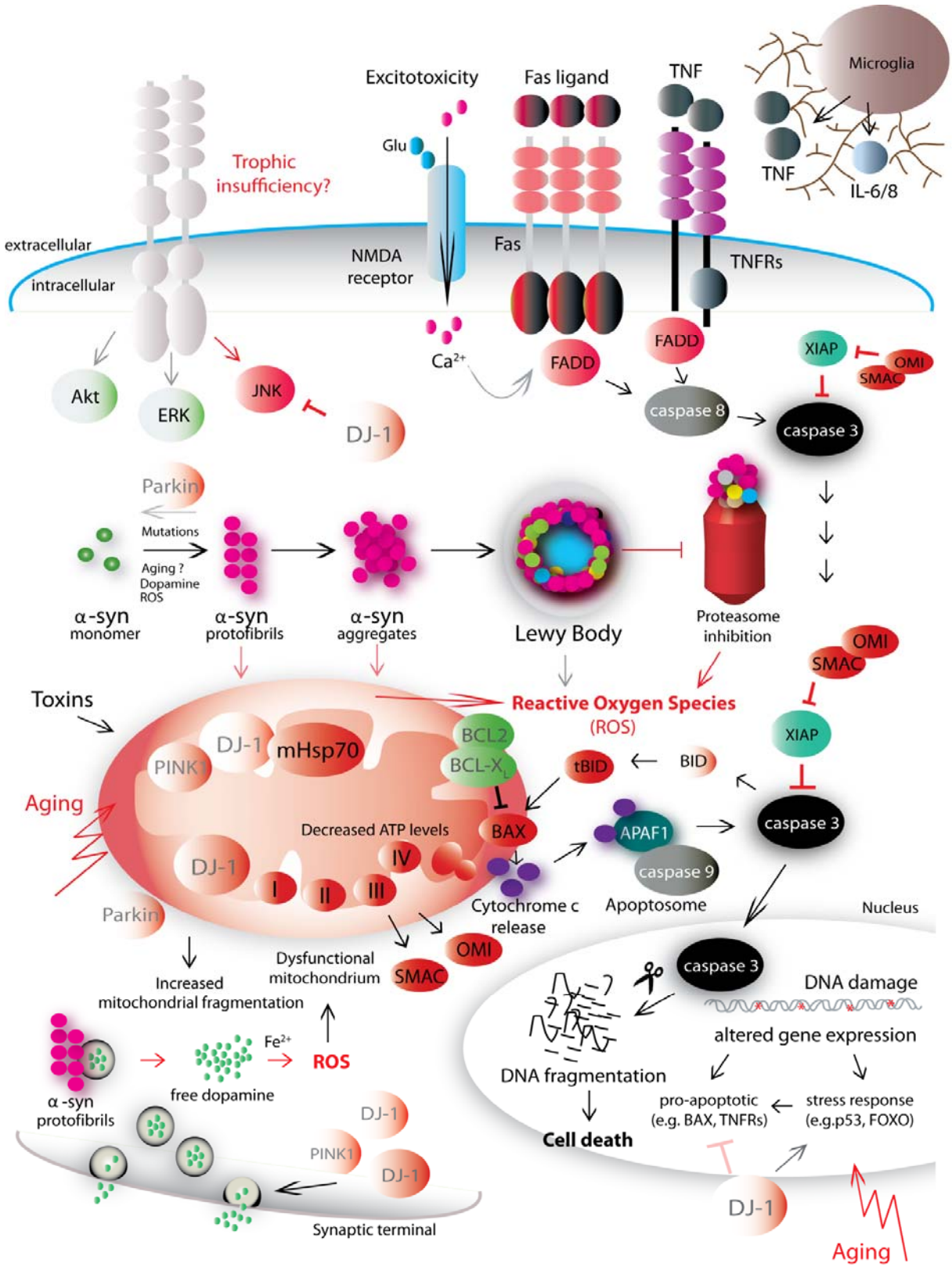
1.6. Mechanisms of *substantia nigra* cell death in Parkinson's disease

1.6.1. Animal models of Parkinson's disease

To understand the mechanisms underlying the demise of SN neurons and the occurrence of behavioral deficits in PD, the generation of animal models displaying select genetic and environmental alterations appears to be a necessary step. Following identification of familial PD-associated genes, numerous animal models have been developed in which individual or combined genetic and toxicologic manipulations have been performed. Ranging from *Caenorhabditis elegans* to non-human primates and with a strong focus on rodents, the current pre-clinical research in PD reached a considerable size. In the mouse, overexpression of *α-syn* using several promoters leads to protein accumulation in SN neurons and to behavioral alterations; similarly, deletion of the recessive genes *DJ-1*, *PINK1* or *Parkin-1* leads to mild physiological (altered dopamine dynamics) and behavioral (hypoactivity) impairments (Levine et al., 2004; Moore and Dawson, 2008). However, these models generally fail to display adult-onset and progressive DA degeneration, suggesting that they might be useful in modeling some aspects of PD (Moore and Dawson, 2008). In contrast, flies overexpressing mutant human *α-syn* (Feany and Bender, 2000) or lacking *PINK1* or Parkin (Clark et al., 2006; Park et al., 2006; Yang et al., 2006) function show loss of DA neurons and motor deficits and might be used to understand certain aspects of DA neuron function and maintenance. A second, independent approach was intoxication with DA-specific toxins, including 1–methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), rotenone or paraquat, following observations that exposure to these agents causes Parkinsonism in humans (Dauer and Przedborski, 2003). Interestingly, when *DJ-1*, *Pink1* or *Parkin* knockout mice and *Drosophila* mutants are exposed to these DA toxins, they display more severe DA deficits and generally display loss of SN neurons, suggesting that additional factors (environmental and/or genetic) might be involved in the development of recessive PD (Dauer and Przedborski, 2003; Moore and Dawson, 2008; Terzioglu and Galter, 2008; Whitworth

and Pallanck, 2008). These results raise the possibility that the phenotypic differences observed between patients carrying PD mutations and rodents might be due to the presence of additional (yet unidentified) modifiers, both genetic and environmental. Therefore, the search for these interactors in rodents might improve our understanding of the human nigral pathology. Integration of mechanistic information from cellular and animal models, *in vitro* studies and clinical studies (imaging, clinical trials, biopsies or post-mortem studies) revealed several common denominators that appear to be associated with DA neuron dysfunction and cell death (Figure 8).

Figure 8. Mechanisms of cell death in Parkinson's disease. Several pathogenic processes emerged as putative contributors to neurodegeneration in PD. *1. Alpha-synuclein and protein aggregation.* Aggregated alpha-synuclein (α-syn) forms oligomers (protofibrils) and insoluble aggregates that can impair the proteasome. In addition, insoluble α-syn aggregates can recruit other functional proteins leading to their inactivation. Aggregate formation and protofibrils impair the mitochondrial function leading to generation of ROS; *2. Recessive-PD genes and mitochondrial impairment.* Loss of DJ-1, PINK1 or Parkin activity decreases mitochondrial function and contributes to ROS formation. While DJ-1 translocates to mitochondria in response to oxidative stress, *PINK1* and *Parkin* act in a similar (yet poorly defined) pathway to regulate mitochondrial maintenance and possibly morphology. Loss of *DJ-1* function might decrease the activity of mitochondrial complexes I-IV and thereby lead to decreased ATP production; *3. Mitochondrial toxins.* Inhibition of mitochondrial activity by complex I-IV inhibitors (like rotenone or MPTP) or by oxidative stress generators (rotenone) is sufficient to cause Parkinsonism, further implicating the mitochondria in PD pathogenesis; *4. Altered dopamine release and storage.* Loss of *DJ-1*, *PINK1* or *Parkin* function decreases the amount of released dopamine, although it is not clear whether these proteins act directly at the pre-synaptic terminal or the decreased ATP production and mitochondrial impairment they generate are responsible for altered neurotransmission. Furthermore, α-syn protofibrils might directly act on synaptic vesicles to induce leakage of dopamine into the cytosol. Free dopamine has a high propensity to auto-oxidize, generating ROS; oxidized dopamine can covalently bind Parkin and inactivate its function in the ubiquitin-proteasome pathway; *5. Decreased pro-survival signaling* might accelerate cell dysfunction and cell death. Loss of trophic signaling might further accelerate cellular stress and dysfunction, however, the evidence so far is incomplete. Moreover, failure to induce oxidative-stress responsive genes might increase ROS levels and lead to oxidation of numerous proteins or to DNA oxidative damage. Chronic oxidative stress might lead to induction of pro-apoptotic proteins, including the mitochondrial pore-forming protein BAX or receptors for apoptotic ligands, like TNF; *6. Aging* is the major risk factor to develop PD and age-related changes in cellular signaling and function of mitochondria or maintenance of DNA function are believed to act as a substrate on which PD-linked mutations act to eventually mediate neuron demise. Therefore, it is possible that an interaction between age-related molecular changes and sub-lethal cellular alterations due to gene mutation and/or environmental stress leads to overt neurodegeneration; 7. *Activation of pro-apoptotic pathways* occurs through at least 2 pathways. Dysfunctional mitochondria lead to the opening of the mitochondrial permeability transition pore (mediated by BAX and BAK, and promoted by tBid - after priming by caspase-3) leading to cytochrome c release and formation of the apoptosome complex (intrinsic apoptotic pathway). Up-regulation of cell-death receptors (like TNFRs) and release of pro-apoptotic cytokines by microglia might activate the extrinsic apoptotic pathway, leading to caspase-8 and then caspase-3 activation. While active caspases can be held in check by IAP proteins (like XIAP), SMAC and OMI/HTRA2 released by the mitochondria might release this inhibition. Active effector caspase-3 and caspase-7 (not shown) use their cysteine-aspartyl protease activity to cleave at specific aspartic residues of numerous substrates, resulting in the apoptotic process. A third apoptotic pathway was proposed to originate downstream of ER stress (through activation of caspase-12), although its physiological and pathological roles are unclear yet (Szegezdi et al., 2003). Please note that the pathological relevance of several putative cell-death mechanisms presented here, issued from *in vitro* studies awaits confirmation in animal models. Adapted from (Abou-Sleiman et al., 2006; Bredesen et al., 2006; Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Mattson and Magnus, 2006; Moore et al., 2005a; Thomas and Beal, 2007; Vila and Przedborski, 2004).


Microglia
TNF
IL-6/8
Excitotoxicity
Fas ligand
TNF
Glu
Trophic insufficiency?
extracellular
intracellular
NMDA receptor
Fas
TNFRs
Ca2+
FADD
FADD
XIAP
OMI
SMAC
Akt
ERK
JNK
DJ-1
caspase 8
caspase 3
Parkin
Mutations
Aging ?
Dopamine
ROS
α-syn monomer
α-syn protofibrils
α-syn aggregates
Lewy Body
Proteasome inhibition
OMI
SMAC
Toxins
Reactive Oxygen Species (ROS)
XIAP
DJ-1
mHsp70
PINK1
BCL2
BCL-XL
tBID
BID
Aging
Decreased ATP levels
BAX
APAF1
caspase 3
DJ-1
IV
I
II
III
caspase 9
Parkin
Cytochrome c release
Apoptosome
Nucleus
OMI
SMAC
Dysfunctional mitochondrium
caspase 3
DNA damage
Increased mitochondrial fragmentation
altered gene expression
Fe2+
ROS
DNA fragmentation
α -syn protofibrils
free dopamine
pro-apoptotic (e.g. BAX, TNFRs)
stress response (e.g.p53, FOXO)
DJ-1
PINK1
DJ-1
Cell death
DJ-1
Aging
Synaptic terminal

Although the pathological mechanisms that lead to SN degeneration might be multiple (Figure 8), several pathways are considered to be central to DA dysfunction and cell death in a variety of systems: protein aggregation leading to proteasomal impairment (Figure 8.1), mitochondrial dysfunction (Figure 8.2, also caused by specific DA-toxins; Figure 8.3), altered dopamine release (Figure 8.4) that could all converge to generate oxidative stress. Aging (Figure 8.6) is known to impact on cell viability, but the mechanisms behind are unclear; decreased trophic signaling (Figure 8.5) might be a contributing factor, although its relevance is poorly understood. Finally, all these dysfunctions seem to converge on highly-conserved cell death pathways (Figure 8.7). Oxidative stress emerges as the major link between proteasomal impairment and mitochondrial dysfunction since inhibition of either the ubiquitin proteasome system (UPS) or mitochondrial respiration complexes generated ROS, a major determinant of DA dysfunction (Abou-Sleiman et al., 2006). Below, I will describe in more detail the protein aggregation stress/proteasomal impairment pathway and the mitochondrial dysfunction pathway, for which extensive evidence suggests a critical role in PD pathology.

1.6.2. Proteostasis defects impair neuron viability

The presence of α-syn in Lewy Bodies and the identification of point mutations and multiplications of the *α-syn* gene in PD argue for a critical role of protein aggregation in PD pathogenesis. In addition, Parkin is an ubiquitin E3 ligase that functions in the ubiquitin-proteasome system (UPS) to eliminate misfolded proteins (Shimura et al., 2000). Accumulation and aggregation of mutant α-syn has also been observed *in vitro* and in animal models (Dauer and Przedborski, 2004). The physiological function of α-syn has not been elucidated, but α-syn is associated with synaptic vesicles (Outeiro and Lindquist, 2003). Mutant α-syn has a high propensity to aggregate and forms oligomers termed protofibrils; several oligomers assemble into amyloid fibrils like those seen in LBs (Dauer and Przedborski, 2003). It is currently believed that α-syn protofibrils are actually the toxic species, and that fibrils might exert protective effects by sequestering toxic oligomers; in *Drosophila*, overexpression of the chaperone heat-shock protein 70 (HSP70) rescues DA degeneration caused by mutant *α-syn* overexpression, without reducing aggregate load (Auluck et al., 2002). In addition, α-syn aggregation might be a protective response to α-syn toxicity, at least in *Drosophila* (Chen and Feany, 2005) Protofibrils were also detected in brains from PD patients with triplication of the *α-syn* gene (Miller et al., 2004). Moreover, protofibrils were found to permeabilize dopamine-containing synaptic vesicles (Volles et al., 2001) freeing dopamine into the cytosol. Free dopamine is highly prone to auto-oxidation and generates reactive oxygen species (ROS); dopamine is also oxidized to dopamine quinones, which were shown to stabilize the α-syn protofibrils (Conway et al., 2001) and dopamine can covalently link and inactivate Parkin (LaVoie et al., 2005). Since Parkin functions as an ubiquitin E3 ligase, its inactivation might further impair the proteasome, and thus impair cell viability.

1.6.3. Dysfunctional mitochondria might promote neurodegeneration

Recent evidence that recessive PD-associated *DJ-1*, *PINK1* and *Parkin* have important roles in mitochondria identifies the mitochondria as important players in PD pathology. DJ-1 translocates to the mitochondria under stress conditions (Canet-aviles et al,

(Gandhi et al., 2006)2004), and PINK1 (Gandhi et al., 2006) possesses a N-terminal mitochondrial targeting sequence and localizes to mitochondria (Valente et al., 2004); within mitochondria, PINK1 might localize to the outer and inner mitochondrial membranes (Gandhi et al.,2006; Silvestri et al.,2005). Parkin localizes mainly in the cytosol and the ER; however, a pool of Parkin associates with the cytoplasmic surface of the outer mitochondrial membrane (Darios et al., 2003).

Perhaps the most solid indication that PINK1 and Parkin play important functions in mitochondria comes from *Drosophila* studies. *PINK1* and *Parkin* mutant flies display mitochondrial dysfunction, followed by loss of DA neurons and defects in flight muscles (Greene et al., 2003; Pesah et al., 2004); remarkably, *Parkin* overexpression rescued the *Pink1* mutant phenotypes, but not vice versa, providing the first *in vivo* evidence that the two genes function in the same pathway to regulate mitochondrial function (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Interestingly, although DJ-1 was found to interact physically with PINK1 (Tang et al., 2006) and, in conditions of oxidative stress with Parkin (Moore et al., 2005b), *DJ-1* overexpression did not rescue the *PINK1* and *Parkin Drosophila* phenotypes (Dodson and Guo, 2007), suggesting that DJ-1 might function in a different pathway. Moreover, PD patients with *PINK1* (Hoepken et al., 2007) or *Parkin* (Muftuoglu et al., 2004) mutations display reduced activities of mitochondrial respiratory chain complexes, further suggesting a crucial role of both PINK1 and Parkin in mitochondria. A possible common function might be the regulation of mitochondrial fission and fusion machineries or mitochondrial transport (Van Laar and Berman, 2009) although its relevance to SN neuron survival remains to be demonstrated. *DJ-1* deficient flies do not display loss of DA neurons and such severe mitochondrial defects but are selectively more sensitive to mitochondrial toxins like MPTP and rotenone (Menzies et al., 2005; Meulener et al., 2006).

There is evidence for a strong correlation between mitochondria and the UPS. Mitochondria generates ATP, required in UPS function; inhibition of complex I with rotenone led to 20 % ATP depletion and to increased ROS and to a marked reduction of proteasome activity (Shamoto-Nagai et al., 2003). Proteasomal inhibition renders neurons more sensitive to the mitochondrial inhibitor MPTP (Sullivan et al., 2004) and defects in mitochondrial complex I result in decreased proteasome activity (Hoglinger et al., 2003). Overexpression of aggregate-prone α-syn in mice leads to mitochondrial degeneration and to increased cellular sensitivity towards mitochondrial complex I inhibitors; conversely, loss of *α-syn* function promotes resistance to mitochondrial inhibitors (Abou-Sleiman et al., 2006; Martin et al., 2006). The best example of a tight connection between mitochondria and the UPS is perhaps the observation that chronic rotenone exposure in rats results in the formation of Lewy body-like aggregates in addition to Parkinsonism (Betarbet et al., 2000).

1.6.4. Current challenges in Parkinson's disease research

The analysis of the newly generated PD cellular and animal models allowed us to gain the first insights into the development of PD. While protein aggregation, mitochondrial dysfunction and the generation of ROS emerged as major cell-death promoters and provided some mechanistic answers, even more questions emerged. Why does neurodegeneration in PD affect SN neurons with relative specificity? Why do monogenic forms of PD manifest clinically only after several decades, although the

underlying mutations are inborn? What is the connection between sporadic and genetic forms of PD and what is the role of aging as modulator of neurodegeneration? In addition, are the genes linked to monogenic PD playing a prominent role in sporadic PD, and are they acting in a common pathogenic pathway? What are the environmental factors that accelerate or delay the onset of neurodegenerative disease? Can we identify specific biomarkers to follow disease progression and most importantly, will we be able to prevent, stop or cure neurodegenerative disease in the (near) future? These are the major challenges in neurodegeneration research for the next decades and we can only hope that some of these questions and challenges will receive appropriate answers and practical solutions.

B. Neurotrophic factors. BDNF/TrkB and GDNF/Ret signaling

1.7. Neurotrophic factors are potent survival agents for neurons

Neurotrophic factors (NFs) are secreted proteins that regulate development, maintenance, function and plasticity in the vertebrate nervous system. Although they were originally identified as neuronal survival factors, NFs control many other neuronal processes ranging from cell proliferation, differentiation to axon guidance, dendritic growth and modulation of synaptic formation and function (Chao, 2003; Davies, 2003). Several groups of closely related molecules make up the neurotrophic factors family: the neurotrophins, the GDNF family ligands and the neurotrophic cytokines (Table 2).

Family	Neurotrophic factor	Preferred receptor (s)
Neurotrophins	Nerve Growth factor (NGF)	TrkA, p75NTR
	Brain-derived neurotrophic factor (BDNF)	TrkB, p75NTR
	Neurotrophin-3 (NT-3)	TrkC, p75NTR
	Neurotrophin 4 (NT-4)	TrkB, p75NTR
GDNF family of ligands	Glial cell line-derived neurotrophic factor (GDNF)	GFRα1/Ret, GFRα1/NCAM
	Neurturin (NRTN)	GFRα2/Ret, GFRα2/NCAM
	Artemin (ARTN)	GFRα3/Ret
	Persephin (PSPN)	GFRα4/Ret
Neurotrophic cytokines	Ciliary neurotrophic factor (CNTF)	gp130, LIFRβ, CNTFRα
	Leukaemia-inhibitory factor (LIF)	gp130, LIFRβ
	Cardiotrophin-1 (CT-1)	gp130, LIFRβ
	Oncostatin-M (OSM)	gp130, OSMRβ
	Interleukin-6 (IL-6)	gp130, IL6Rα

Table 2. Families of neurotrophic factors. Neurotrophins constitute the first identified and best understood family of neurotrophic factors; all neurotrophins signal via Trk or p75NTR receptors. The GDNF family ligands (GFLs) use GFRα co-receptors and induce activation of the Ret RTK, as their common signaling receptor. Some GFLs can also signal independently of Ret, via GFRα/NCAM. Neurotrophic cytokines signal via gp130 but also have additional co-receptors. In contrast to Trk and Ret, the gp130 receptor has no intrinsic kinase activity and is tyrosine-phosphorylated by other cellular kinases.

The first family of neurotrophic factors to be identified were the neurotrophins, the best understood trophic factors in the nervous system to date (Figure 9). Pioneering work by Rita Levi-Montalcini and Victor Hamburger resulted in the identification of the first neurotrophic factor, the nerve growth factor (NGF). NGF was purified based on its ability to support survival of sympathetic and sensory spinal neurons in culture (Chao, 2009; Levi-Montalcini, 1987; Shooter, 2001). Subsequent studies demonstrated that NGF is synthesized and secreted by sympathetic and sensory target organs; once secreted, NGF is taken up by sympathetic and sensory nerve terminals via receptor-

mediated endocytosis and is transported through axons to neuronal cell bodies, where it mediates neuron survival and differentiation (Korsching, 1993). The second neurotrophin to be characterized, brain-derived neurotrophic factor (BDNF) was purified from pig brain as a survival factor for several neuronal populations not responsive to NGF (Barde et al., 1982). Two other neurotrophins NT-3 and NT-4 (also known as NT-5) were characterized in mammals (Figure 9). Neurotrophins are all derived from a common ancestral gene, are similar in sequence, and were therefore collectively named neurotrophins (Hallbook, 1999). Two additional neurotrophins, NT-6 and NT-7 are only found in fish and probably do not have mammalian orthologs (Huang and Reichardt, 2001).

1.7.1. *The neurotrophic factor hypothesis* and *extensions*

In the developing nervous system, natural cell death eliminates 50 % or more of neuronal populations. The central concept of the neurotrophic factor hypothesis is that limited amounts of secreted neurotrophic factors by the targets of innervation regulate the number of surviving neurons to match with the size of the target area (Purves et al., 1988; Thoenen et al., 1987). According to this hypothesis, developing neurons engage in a competition for trophic factors and neurons deprived of trophic support die by apoptosis. The foundations of this theory were the early observations that neurons are able to grow toward increasing concentrations of NGF (Gundersen and Barrett, 1979, 1980).While this model explains some aspects of neuronal development and the critical roles of NFs in regulation of neuron survival and function, the neurotrophic factor hypothesis cannot incorporate the diversity of functions and roles for NFs anymore. Most importantly, the diversity of functions regulated by neurotrophic factors (including neuron migration, neurite outgrowth, axon guidance, dendritic morphogenesis or synaptic plasticity) suggests that considerable revisions are required for the neurotrophic factor hypothesis to account for the extreme versatility (and beauty) of NF action.

1.8. Neurotrophins signal via Trk receptors and p75NTR.

The identification of the first neurotrophin NGF opened the era of cellular and molecular studies of neurotrophin action in the nervous system. NGF was shown to be internalized at nerve terminals and to be transported along axons in small vesicles by an energy- and microtubule dependent process; it was also found to exert both local and nuclear actions which regulate respectively growth cone motility and gene expression (Segal, 2003). However, these effects could not be explained molecularly, in the absence of an NGF receptor.

Initial efforts to identify a neurotrophin receptor resulted in the discovery of the low affinity receptor for NGF, p75NTR, which was subsequently shown to bind all neurotrophins (Rodriguez-Tebar et al., 1991). p75NTR is a distant member of the tumor necrosis factor receptor family and contains a “death” domain in its intracellular part (Chao, 1994). While for many years the roles of p75NTR remained unclear, the emerging picture of p75NTR is that of a versatile regulator of several crucial biological processes, including neuronal survival and apoptosis (see below).

An important advance in the neurotrophin field was facilitated by new discoveries from cancer research. Gene transfer assays from carcinoma cells led to the isolation of a

novel oncogene which represented a fusion between the nonmuscle tropomyosin gene and the transmembrane and cytoplasmic domains of a novel tyrosine kinase. The proto-oncogene was named tropomyosin-related kinase (*trk*) and is now commonly referred to as *trkA* (Barbacid et al., 1991; Huang and Reichardt, 2003); *trkB* and *trkC* genes were subsequently identified because of their high sequence homology with *trkA*. The breakthrough in neurotrophin biology came in 1991 with the identification of Trk receptors as specific binding partners for neurotrophins. TrkA was identified as a receptor for NGF (Kaplan et al., 1991; Klein et al., 1991a), TrkB was found to bind BDNF and NT-4 (Klein et al., 1991b) and TrkC binds NT-3 (Lamballe et al., 1991). NT-3 can also bind less efficiently to TrkA and TrkB (Huang and Reichardt, 2003). Endogenous signaling by Trk receptors was shown to mediate neuron survival and/or differentiation during development in all neuronal populations examined to date; in addition to neuronal survival, growth and differentiation, neurotrophin-induced Trk receptor activation was shown to mediate axonal and dendritic remodeling; assembly and remodeling of the cytoskeleton; membrane trafficking and fusion; and synapse formation, function and plasticity (Arancio and Chao, 2007; Chao, 2003; Reichardt, 2006; Zweifel et al., 2005).

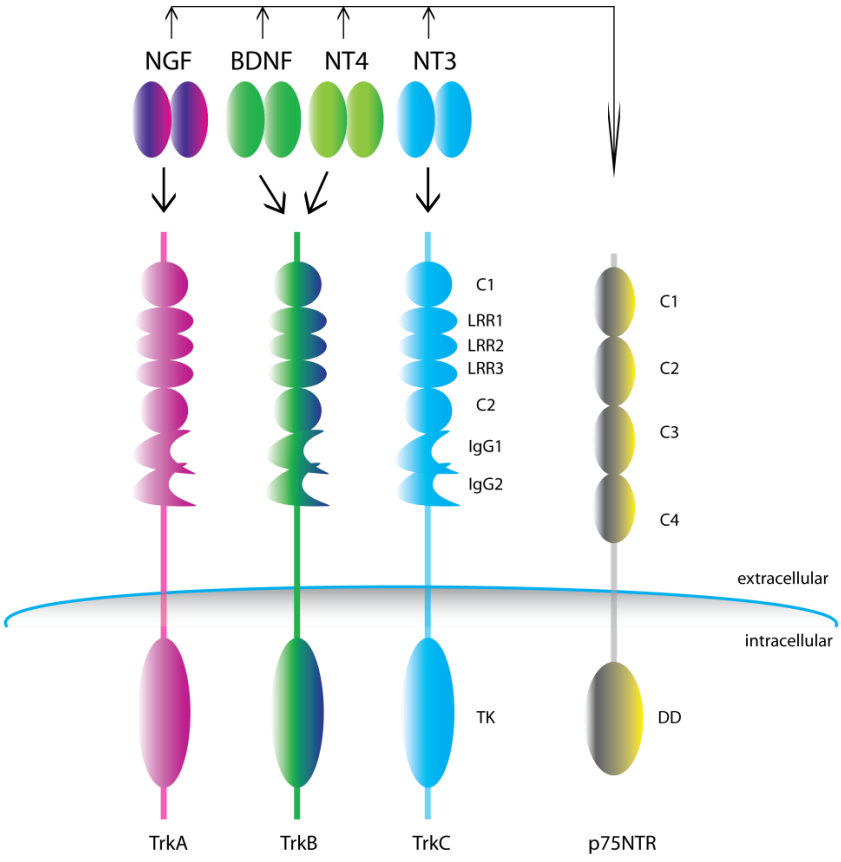


Figure 9. Neurotrophins signal via Trk receptors and p75NTR. NGF binds to TrkA, BDNF and NT4 to TrkB and NT3 binds TrkC. Neurotrophins also bind the low affinity receptor p75NTR (on the right). Trk receptors contain three leucine rich repeats (LRR1-3) in the extracellular part flanked by two cystein-rich domains (C1,C2) and two immunoglobulin G (IgG1, IgG2) domains for ligand binding; they also contain a catalytic tyrosine kinase (TK) domain in the intracellular part. p75NTR has four cysteine-rich repeats (C1-C4) in the extracellular part and a death domain (DD) in the intracellular part.

Given the high structural similarity of neurotrophins and their receptors, it does not come as a surprise that the signaling pathways they initiate share many common features. In the following, I will describe the signaling mediated by BDNF.

1.8.1. BDNF signaling via TrkB

Since neurotrophins were initially believed to act specifically in the nervous system, it came as a surprise that their receptors TrkA-C also possess tyrosine kinase activity, similar to the receptors for mitogenic growth factors like plateled derived growth factor (PDGF) or epidermal growth factor (EGF). Neurotrophins function generally as non-covalently associated homodimers (Figure 9). The most important site at which Trk receptors interact with neurotrophins has been localized to the most proximal immunoglobulin (IgG2 in Figure 9) domain of each receptor (Ultsch et al., 1999). Ligand engagement of Trk receptors results in phosphorylation of their cytoplasmic tyrosine residues. 10 conserved tyrosine residues are contained within the cytoplasmic part of Trk receptors, while three residues (Y670, Y674, and Y675) are found in the auto-regulatory loop of the kinase domain that regulates kinase activation. Phosphorylation of these 3 tyrosines further activates the receptor (Inagaki et al., 1995). Phosphorylation of the other tyrosine residues creates docking sites for adapter proteins containing phosphotyrosine-binding (PTB) and Src-homology-2 (SH-2) motifs (Pawson and Nash, 2000).

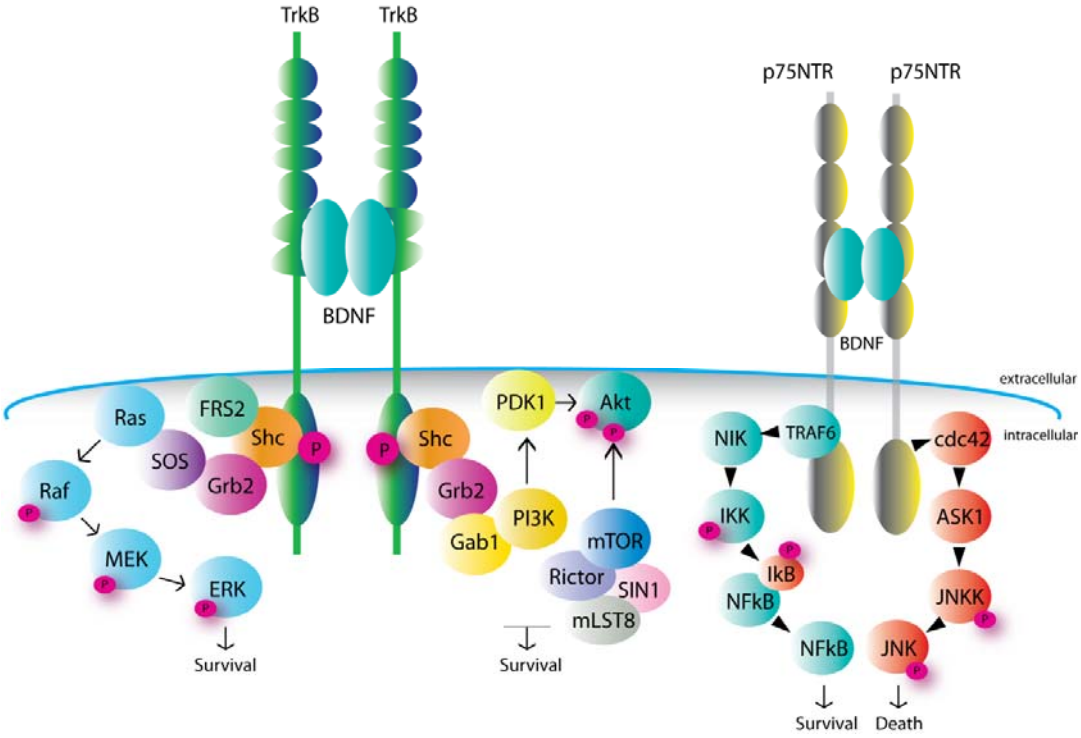


Figure 10. BDNF signaling via TrkB and p75NTR. BDNF can promote survival of different neuronal population by engaging TrkB and/or p75NTR receptors. Binding of BDNF homodimer to TrkB induces TrkB dimerization and trans-autophosphorylation on conserved tyrosine residues in the cytoplasmic part of the receptor. Phosphorylated tyrosines serve as docking sites for the Shc adaptor that recruits further adaptors to activate the Ras/ERK signaling and PI3K/Akt signaling pathways. BDNF binding to p75NTR leads to recruitment of TRAF6 and activation of NFkB pro-survival pathway; in contrast, activation of JNK signaling mediates pro-apoptotic effects. It appears that p75NTR binding to mature neurotrophins promote survival, while binding to proneurotrophins induces apoptosis (Lu et al., 2005).

These adapter proteins couple Trk receptors to intracellular signaling pathways, which include the Ras/ERK (extracellular signal–regulated kinase) protein kinase pathway, the phosphatidylinositol-3-kinase (PI-3 kinase)/Akt kinase pathway, and phospholipase C (PLC)-γ 1 (Chao, 2003; Kaplan and Miller, 2000). The PI3K/Akt and Ras/ERK signaling pathways promote survival in a broad variety of neuronal cell types (see below).

1.8.2. BDNF signaling via p75NTR

p75NTR emerged as a versatile regulator of neurotrophin signals. The traditional view that p75NTR is a pro-survival molecule was shattered in 2001 when p75NTR was found to bind with high affinity to proneurotrophins and to mediate apoptosis (Lee et al., 2001). Proneurotrophins are the uncleaved precursors of neurotrophins, and are processes by extracellular proteases (like matrix metalloproteinase 7, MMP7) to release the mature neurotrophins. proBDNF was subsequently shown to induce apoptosis by activating p75NTR (Teng et al., 2005) and mounting evidence suggests that binding of p75NTR to mature neurotrophins promotes cell survival, while binding to proneurotrophins promotes apoptosis (Lu et al., 2005). p75NTR does not contain any catalytic domain in its intracellular side (Figure 10) but can recruit several adaptors to signal; thus, its pro-survival activities are mediated by the association with TRAF6 and subsequent activation of NFkB signaling, while its pro-apoptotic activities require cdc42-activation of ASK1, the initiator of JNK signaling (Figure 10). p75NTR also has modulatory roles on neurotrophin signaling (Bibel et al., 1999), and forms the signal-transmitting subunit of the Nogo receptor complex (NogoR, Lingo, p75NTR) that inhibits axon growth downstream of inhibitory glycoproteins like Nogo (Lu et al., 2005; Reichardt, 2006).

1.9. GDNF family ligands signal via the Ret receptor tyrosine kinase

The Glial cell line-derived neurotrophic factor (GDNF) is the prototypical member of the GDNF family ligands (GFLs) that also includes Neurturin (NRTN), Artemin (ARTN) and Persephin (PSPN). GFLs play important roles during the development and function of the nervous system. GDNF was isolated in 1993 as a growth factor promoting survival of embryonic midbrain dopaminergic neurons (Lin et al., 1993) (Lin et al, 1993). This discovery raised hopes that GDNF might be used as therapeutic agent to treat PD. Subsequently, GDNF was found to be a potent survival factor for motoneurons (Henderson et al., 1994) (Arenas et al., 1995) and central noradrenergic neurons (Arenas et al., 1995). In addition to its pro-survival roles in neurons, GDNF and the related GFLs have additional functions in neuronal proliferation, migration, differentiation and synapse formation (Paratcha and Ledda, 2008) and also play crucial roles as morphogenetic factors in kidney and spermatogonia development (Airaksinen and Saarma, 2002).

Unlike other ligands for receptor tyrosine kinases, GDNF and the related GFLs require a glycosylphosphatidylinositol (GPI)-anchored cell surface protein as a ligand binding component (Figure 11). This co-receptor was termed GDNF family receptor (GFRα); four GFRα co-receptors for GFLs exist: GFRα1 (binds GDNF), GFRα2 (binds NRTN), GFRα3 (binds ARTN) and GFRα4 (binds PSPN). GFLs, acting as homo-dimers bind the GFRα co-receptor to form a high-affinity complex. Subsequently, the GFL- GFRα complex recruits the Ret receptor tyrosine kinase, the signaling receptor of the GDNF

family ligands. Thus, Ret RTK is the common signal transducer of all GDNF family ligands. To date, Ret is the only known RTK that does not bind directly to its ligands but requires co-receptors for activation.

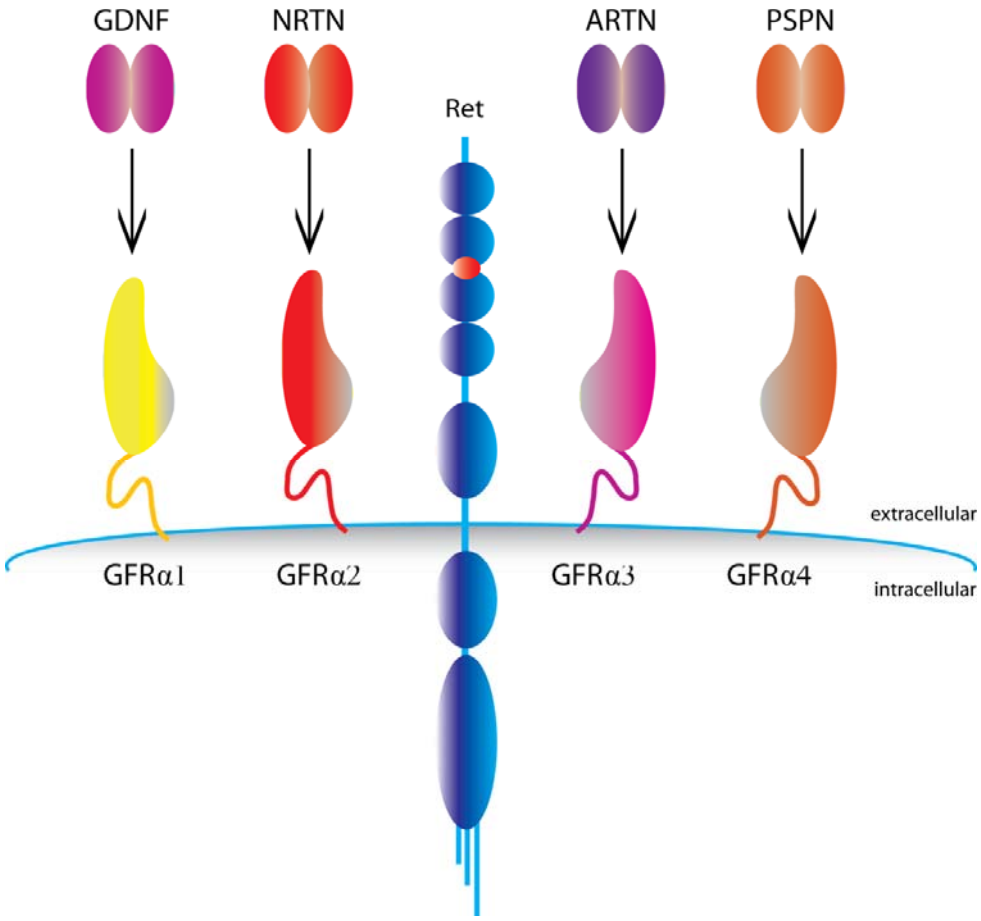


Figure 11. GDNF family ligands (GFLs) signal via the Ret receptor tyrosine kinase. Soluble GFLs, acting as homodimers, recruit one of the GFRα1-4 co-receptors. Each GFL has a high affinity for its corresponding GFRα (as indicated), while weaker binding is known to occur between GDNF and GFRα2, ARTN and GFRα1 or NRTN and GFRα4 (not shown). The GFL/GFRα complex then recruits the Ret receptor tyrosine kinase and induces its dimerization and trans-autophosphorylation, leading to activation of the kinase activity of Ret.

Ret was initially identified as a novel rearranged transforming gene in NIH3T3 cells transfected with human lymphoma DNA (Takahashi et al., 1985). This transforming gene resulted from the fusion between two unlinked DNA fragments which occurred during the transfection process. The resulting chimeric gene encodes a fusion protein comprising an N-terminal domain with a dimerizing motif fused to a carboxy-terminal tyrosine kinase domain (Takahashi and Cooper, 1987). Subsequently, the name *Ret* (***Re****arranged during* ***t****ransfection*) was retained to designate the gene encoding the receptor tyrosine kinase that contains the kinase domain of the fused oncogene (Iwamoto et al., 1993; Takahashi et al., 1988). The human *Ret* gene lies on the chromosome band 10q11.2 and comprises 21 exons. Homologues of *Ret* have been identified in higher and lower vertebrates, including the fruit fly *Drosophila melanogaster* (Hahn and Bishop, 2001). To date, more than 100 mutations were described in the Ret gene; mutations leading to constitutive activation of *Ret* cause

cancer, while loss of Ret function causes severe enteric denervation leading to megacolon (Hirschsprung's disease [HSCR]). Germline mutations leading to constitutive activity of Ret have been identified in three related dominantly inherited cancer syndromes: *multiple endocrine neoplasia* type 2A (MEN2A), 2B (MEN2B) and familial medullary thyroid carcinoma (FMTC) (Santoro et al., 2004). Substitution of cysteine Cys634 (located in the cysteine-rich domain at the boundary with the trans-membrane domain) with other amino acids accounts for 85 % of all MEN2A cases and leads to the formation of a covalent link between two mutated Ret^{MEN2A} receptors (thus generating a dimer that displays constitutive kinase activity) (Figure 12). Substitution of methionine 918 from the kinase domain of Ret with threonine (M918T) accounts for 95 % of all MEN2B cases and leads to a constitutively active, monomeric, Ret^{MEN2B} receptor (Santoro et al., 2004) (Figure 12).

1.9.1. Structural aspects of Ret signaling

According to the classical RTK paradigm, GFLs –acting as homo-dimers– recruit 2 molecules of GFRα and subsequently 2 molecules of Ret receptor (Figure 12).

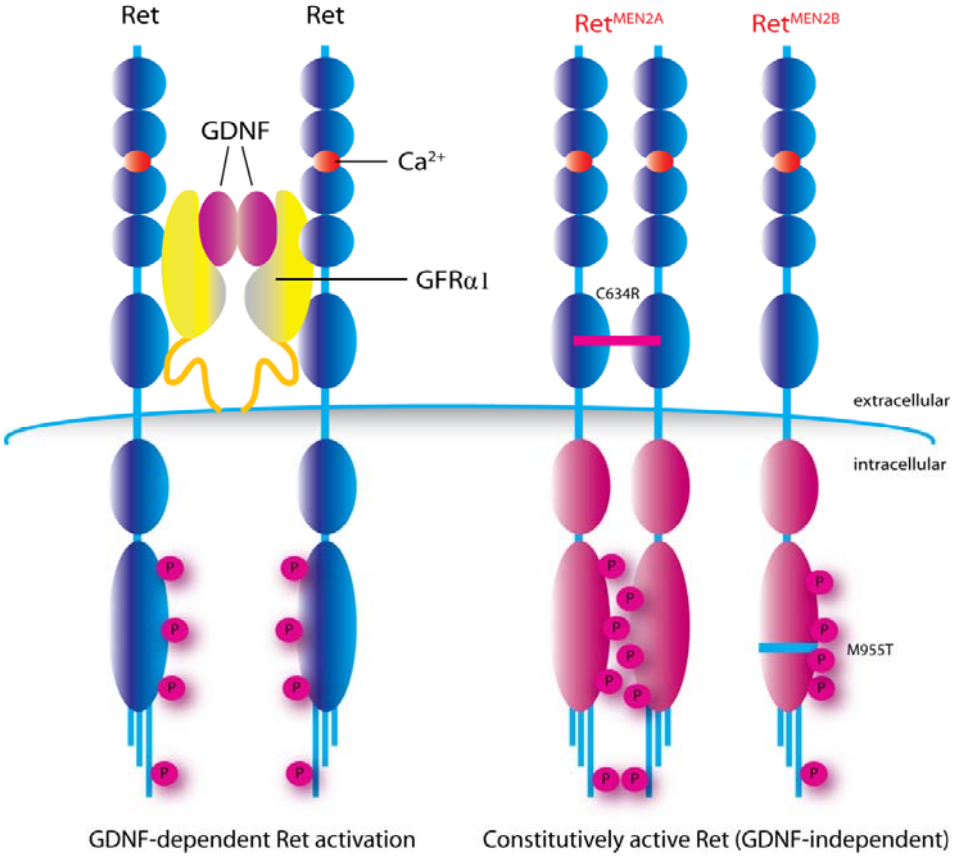


Figure 12. Activation of Ret signaling by GDNF and constitutively active Ret forms. The formation of the GDNF/GFRα1 complex recruits two molecules of Ret receptor and promotes Ret autophoshorylation on conserved tyrosine residues. A similar situation is seen when the other GFLs recruit their GFRα2-4 co-receptors to activate Ret (not shown). Mutations in the extracellular cysteine-rich domain (C634R) lead to constitutive-active dimeric Ret receptor. Mutations in the tyrosine kinase domain (M955T) also have the same effect, but the receptor remains monomeric. The C634R and M955T mutations are the most common *Ret* mutations, which cause *multiple endocrine neoplasia* (MEN type 2A and 2B, respectively) in humans.

Formation of the GDNF/GFRα/Ret tripartite complex promotes RET dimerization and receptor *trans*-autophosphorylation within the RET intracellular kinase domain leading to recruitement of phospho-tyrosine-binding adaptors that lead to activation of downstream signaling cascades (Figure 12).

Ret is a single-pass transmembrane protein (Figure 13). In its extracellular part, Ret contains four cadherin-like repeats, a calcium-binding site and a cysteine-rich domain. The intracellular portion features a typical tyrosine kinase domain. Based on their homology to cadherins, the cadherin-like domains might mediate cell adhesion; however, their function is currently ill defined (Anders et al., 2001). The calcium binding site, located between the second and third cadherin-like domain, is needed for the folding, secretion and the signal transduction capacity of Ret (Nozaki et al., 1998; van Weering et al., 1998). The cysteine-rich domain contains 16 cysteine residues and was suggested to play a role in receptor folding and binding to the GFRα co-receptor (Runeberg-Roos and Saarma, 2007). Mutations in this domain can lead to protein misfolding; intriguingly, mutation of the same cysteine residues can lead to either protein degradation (*loss-of-function*) or constitutive activation (*gain-of-function*) and families with both endocrine neoplasia type 2 (MEN2) tumors and HSCR have been identified (Dvorakova et al., 2005).

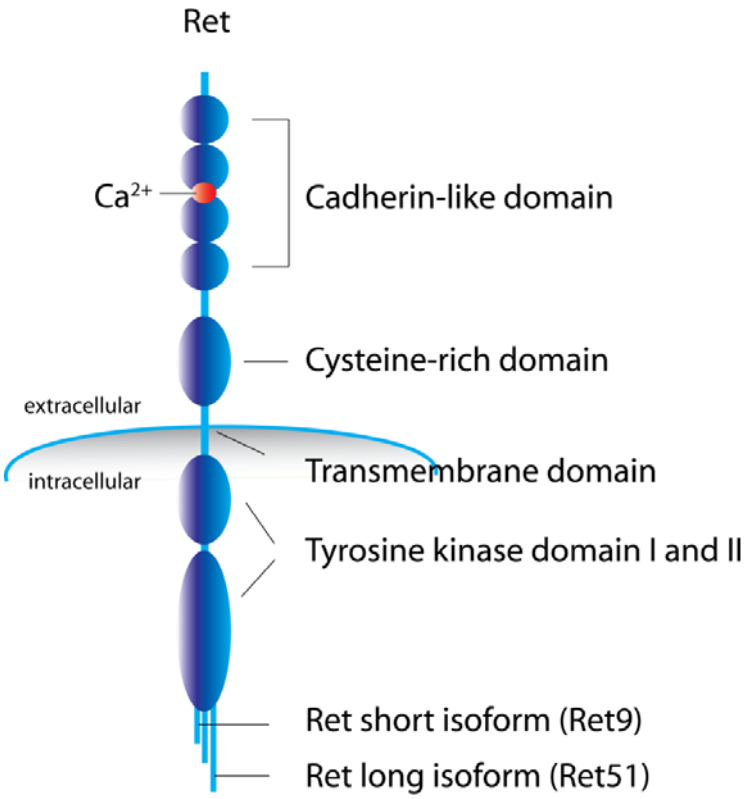


Figure 13. Structural organization of the Ret receptor tyrosine kinase. In the extracellular part, four cadherin-like domains and a Ca^{2+} binding site might regulate the adhesive properties of Ret. A cysteine-rich domain is responsible for receptor folding and binding to the ligand/co-receptor complex. In the intracellular part, the tyrosine kinase domain, consisting of two parts, mediates auto-phosphorylation following receptor activation. Three Ret isoforms are known to exist, which differ in their C-terminal sequence, however only two (Ret9 and Ret51) are conserved over a broad range of species.

The transmembrane (TM) domain of Ret is no longer seen as a passive link between the extracellular ligand-sensing part and the intracellular signal-transducing part and recent evidence suggests that it plays an active role during the activation of Ret. The TM domain may drive self-association of two Ret molecules, which may facilitate complex formation during GFL/GFRα/Ret signal transduction (Runeberg-Roos and Saarma, 2007); consequently, mutations in three residues within the TM domain were found in patients with MEN2 tumors (Kjaer et al., 2006). Below the TM domain, the juxtamembrane domain of Ret might be involved in the control of cell migration via cAMP/PKA-dependent phosphorylation of its serine 696 (Asai et al., 2006; Fukuda et al., 2002).

A central function of the Ret RTK is carried out by its intracellular kinase domain (KD). The KD of Ret is homologous to that of other tyrosine kinases (e.g. FGFR-1, VGFR-2) (Tuccinardi et al., 2007). Ligand-induced Ret dimerization juxtaposes the two catalytic domains, thereby allowing mutual trans-phosphorylation; phosphotyrosines propagate the signal by recruiting intracellular proteins. Studies using phosphopeptide mapping and mass spectrometry identified 12 autophosphorylation sites in the Ret RTK (Kawamoto et al., 2004; Liu et al., 1996) but only five tyrosines have been found phosphorylated in intact cells so far. Three of these tyrosine are found within the KD (Y905, Y981, Y1015), and two in the C-terminal tail (Y1062, Y1096); of note, the Y1096 residue is only found in the Ret51 isoform (Coulpier et al., 2002; Salvatore et al., 2001). Tyrosine 905 is the autocatalytic residue, whose phosphorylation generates a conformational change that allows kinase activation; the other four phosphorylated tyrosines serve as docking sites for adaptor proteins, with tyrosine 1062 playing the most prominent role in activation of Ret-downstream signaling. Indeed, mutation of Y1062 in mice induces severe defects in the development of the enteric nervous system and nephrogenesis, which are seen in *Ret* null mice (Jijiwa et al., 2004); in addition Y1062 is required for the transforming activity of oncogenic Ret (Degl'Innocenti et al., 2004).

Three C-terminal Ret splicing variants are generated by alternative splicing of the 3' region: Ret9, Ret43 and Ret51. Ret9 and Ret51 are conserved over a broad range of species and consist of 1072 and 1114 amino acids, respectively (Kodama et al., 2005). Ret9 and Ret51 differ in the amino acid sequences immediately downstream from Y1062: following glycine 1063, Ret9 has 9 extra residues, while Ret51 has 51 amino acids, which include the phosphorylation site Y1096. Therefore, the two Ret isoforms were suspected to display differences in signaling capacities; indeed, Vassilis Pachnis and colleagues generated mice that only expressed Ret9 or Ret51. Mice lacking *Ret9* had a phenotype similar to *Ret* null mice, albeit milder, while lack of *Ret51* did not significantly affect development (Schuchardt et al., 1994). Moreover, only *Ret9* is able to rescue the phenotype of *Ret*-null mice (de Graaff et al., 2001; Srinivas et al., 1999). Thus, *Ret9* but not *Ret51* appears to be crucial for normal development. Additional differences between the two isoforms were also noticed in the regulation of mouse inner-medullary collecting duct cell survival, which is promoted by *Ret51* but not *Ret9* (Lee et al., 2002) and in oncogenic transformation where *Ret51* mutants are more active than the corresponding *Ret9* mutants (Iwashita et al., 1999). Therefore, it appears that Ret51 and Ret9 isoforms fulfill different functions.

1.9.2. Dynamic aspects of Ret signaling

Binding of adaptors to phosphorylated Y1062 triggers activation of several signaling pathways; binding of Shc and FRS2 proteins mediates recruitment of the Grb2-SOS complex that leads to Ras/ERK stimulation, while binding of the Grb2-GAB1/2 complex leads to stimulation of the PI3K/Akt pathway. Binding of DOK4 and DOK5 adaptors is implicated in ERK stimulation, while binding of ShcC and IRS is required to trigger PI3K/Akt activation (Santoro et al., 2004). Moreover, binding of the DOK1 adaptor to phosphorylated Y1062 leads to c-Jun N-terminal kinase (JNK) activation via recruitment of the NCK adaptor (Manie et al., 2001). The extraordinary versatility of Y1062, which can recruit this diversity of adaptors, suggests a model whereby competition between various signaling intermediates for binding to the phosphorylated Y1062 docking site allows switching between different biological responses to Ret-dependent signaling. Several factors have been suggested to contribute to substrate selection including the subcellular localization of Ret, its turn-over rate, the proportion of activated Ret in the cell or the genetic background (Runeberg-Roos and Saarma, 2007). Ret signaling activates two major signaling cascades that promote cell survival in diverse neuronal and non-neuronal populations and which are described in more detail below: the Ras/ERK (MAPK) and the PI3K/Akt pathways (Figure 14).

1.9.2.1. Ret signaling via the Ras/ERK pathway

The Mitogen-activated protein kinase (MAPK) pathway is an evolutionary conserved pathway that regulates diverse cellular programs including embryogenesis, proliferation, cell size, differentiation or survival, based on cues derived from the cell surface and the metabolic state of the cell (Dhillon et al., 2007). Tyrosine-phosphorylated Ret recruits the PTB-containing adaptor protein Shc; after Shc phosphorylation, a complex containing the SH2/SH3-containing adaptor protein Grb2 and the SH3-binding Ras exchange factor SOS is recruited (Figure 14). SOS is a guanine nucleotide exchange factor (GEF) for the small GTP-ase protein Ras (Figure 14). Membrane associated Ras (via C-terminal farnesylation) interacts with SOS, which facilitates exchange of GDP with GTP and thereby maintains Ras in an active state. GTP-bound Ras has an increased affinity for several substrates, including PI3K (see below) or the kinase Raf. Binding of active Ras to the Ser/Thr kinase Raf (also termed mitogen activated protein kinase kinase kinase [MAPKKK]) allows Raf activation by other proteins (following repeated cycles of phosphorylation and de-phosphorylation and protein-protein interactions that are incompletely understood) (McKay and Morrison, 2007). Once activated, the Raf kinase phosphorylates and activates the MEK1/2 (MAPKK) kinase. Activated MEK1/2 then phosphorylates the extracellular signal-regulated kinases (ERKs, also called MAPKs) on both serine and tyrosine residues in their activation loop. Once phosphorylated, ERK1/2 (also known as $p44^{MAPK}$ and $p42^{MAPK}$) use their Ser/Thr activity to phosphorylate several cytoplasmic (e.g. the RSK protein) or nuclear targets. Thus, translocation of activated ERK1/2 to the nucleus leads to activation of several transcription factors, including c-Fos and c-Myc, p53, SMAD1-4, Sap-1a, SP1 and Elk-1 to control cell motility, proliferation, differentiation or survival downstream of Ras (Dhillon et al., 2007; Raman et al., 2007; Turjanski et al., 2007). A prominent pro-survival effect of ERK1/2 is via phosphorylation and activation of RSK (p90 ribosomal S6 kinase) kinases, which then phsophorylate the transcription factor CREB (cAMP responsive

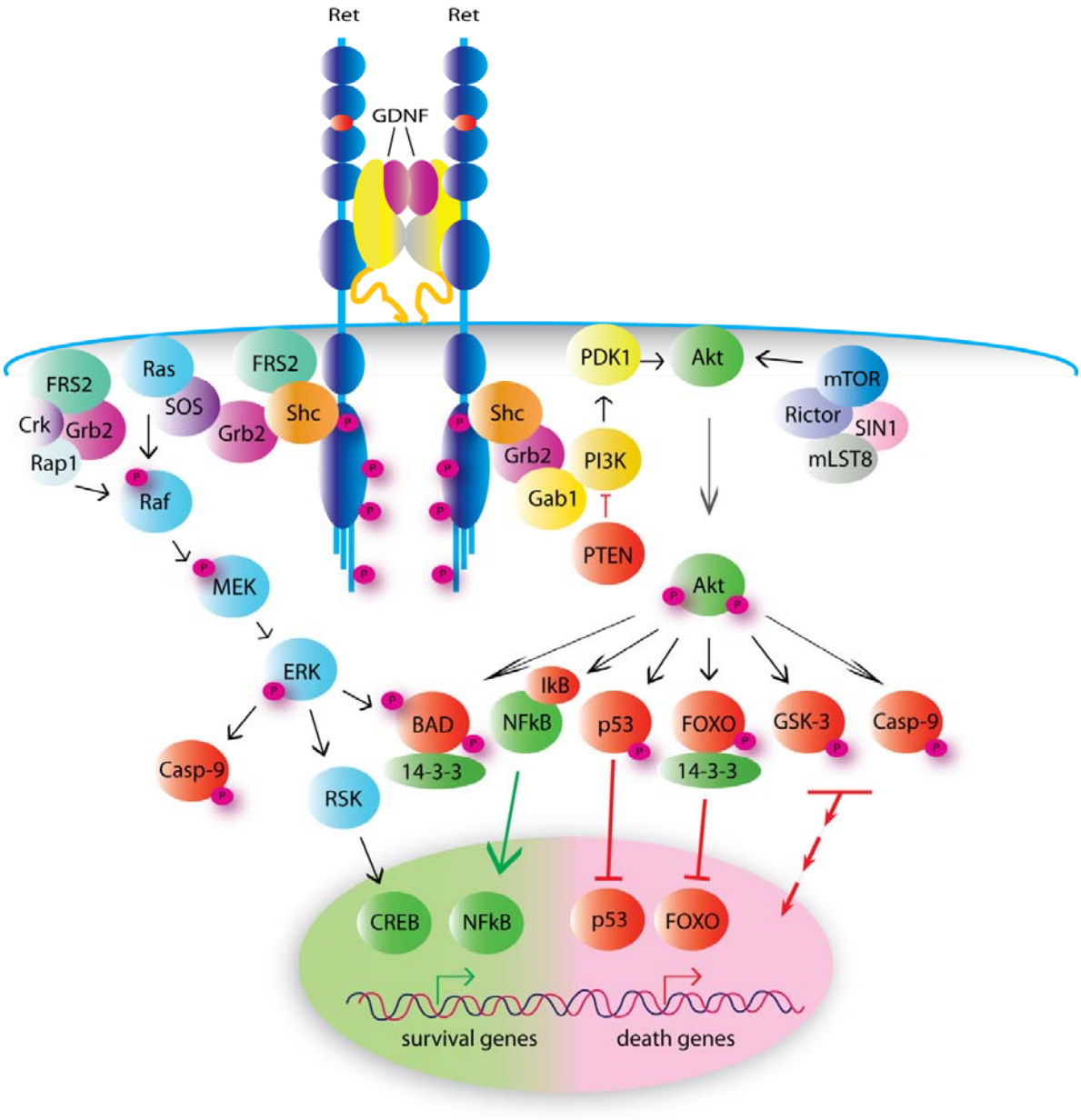


Figure 14. Ret signaling via the Ras/MAPK and PI3K/Akt pathways. Activation of Ret by the GDNF/GFRα1 complex (or by the other GFLs/GFRα 2-4 complexes) leads to recruitment of molecular adaptors that bind to phosphorylated tyrosine residues. These adaptors recruit additional transducers; Shc recruits Grb2 and then SOS which induces Ras activation; GTP-bound Ras promotes activation of Raf and signaling via the ERK/MAPK pathway. In contrast to this transient mode of Ras activation, signaling via FRS2/Grb2/Crk/Rap1 provides a more sustained activation of MAPK. Phosphorylated ERK translocates to the nucleus where it phosphorylates additional targets (not shown), but can also phosphorylate RSK1 to activate CREB; in addition, it phosphorylates the pro-caspase 9 thereby inhibiting caspase-3 activation. Binding of the Shc/Grb2/Gab1 complex to phosphorylated tyrosine residues induces recruitment and activation of the PI3K, which phosphorylates phosphoinositides from the plasma membrane and provides a docking site for the protein kinase B (Akt). Membrane-associated Akt is then phosphorylated by PDK1 and the mTOR/Rictor/SIN1/mLST8 complex. Activated Akt promotes survival by modifying several downstream targets, via both non-transcriptional and transcriptional effects (see text for details). Note that Ras can also directly bind and activate the PI3K (not shown).

element binding protein) to induce activation of pro-survival genes (Reichardt, 2006). ERK were also shown to directly phosphorylate pro-caspase 9, thereby blocking caspase-3 activation (Allan et al., 2003). Besides ERK1/2, three other MAPKs (ERK3, ERK5 and ERK7) have a distinct regulation and are less well understood (Raman et al, 2007). Increasing evidence suggests a tight temporal and spatial dynamics of Ras/ERK signaling. Thus, while transiently activated Ras at the nerve terminal mediates growth cone motility or exocytosis, retrograde transport of the ligand/receptor complex via endosomes (i.e. signaling endosomes) in the cell soma induces a prolonged activation of ERK signaling, that is required for neuronal survival or differentiation (Huang and Reichardt, 2003; Reichardt, 2006). Moreover, while transient Ras activation requires the Shc/Grb2/SOS adaptors, prolonged Ras/ERK activation appears to require a different set of adaptors. The FRS2 adaptor is phosphorylated by active RTK receptors and recruits the adaptor proteins Grb2 and Crk and the tyrosine kinase Src; Crk then binds the Rap1 exchange factor C3G, which then activates the Raf kinase and thus ERK signaling (Huang and Reichardt, 2003; Reichardt, 2006; Figure 14).

1.9.2.2. Ret signaling via the PI3K/Akt pathway

The phosphoinositide-3-kinase (PI3K) is a major regulator of cell survival in diverse cellular populations (Manning and Cantley, 2007). Three families of PI3K are known to date, the predominant form of PI3K existing as a heterodimer of a catalytic subunit (molecular weight 110 kDa) and a regulatory subunit (molecular weight 85 kDa (Fruman et al., 1998). Two major modes for PI3K activation are known; PI3K can be directly activated by GTP-bound Ras or it can be recruited via the formation of the Grb2/Gab1/2 complex (Figure 14). Once activated, PI3K phosphorylates the D-3 position of the inositol ring of phosphoinositides (PI) in the membrane and generates $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (PI di-and tri-phosphates); phosphoinositides generated by PI3K participate in the activation of the protein kinase B (also called Akt), which has emerged as a critical regulator of signaling and one of the most versatile and important protein in physiology and disease (Manning and Cantley, 2007).

Phosphatidylinositides generated following PI3K action recruit both Akt and the phosphatidylinositide-dependent protein kinase (PDK-1) at the plasma membrane. Both Akt and PDK-1 possess *pleckstrin homology* (PH) domains, which exhibit high affinity for $PI(3,4)P_2$ and $PI(3,4,5)P_3$. Membrane-associated Akt is then phosphorylated by PDK1 at Threonine 308 (Kim and Chung, 2002); following this initial phosphorylation, a second kinase phosphorylates Akt at Serine 473; this unknown enzyme, long termed PDK2 is now identified as mTOR kinase complex 2 (mTORC2) (Guertin and Sabatini, 2007; Manning and Cantley, 2007). Phosphorylation at both Thr308 and Ser473 are required to fully activate the Akt kinase. PTEN is the major negative regulator of the PI3K (Salmena et al., 2008). It is best knwon for its lipid-phosphatase activity and $PI(3,4,5)P_3$ is one of its substrates; $PI(3,4,5)P_3$ de-phosphorylation reduces the activation of the PI3K: Besides its lipid-phosphatase activity, it also posesses a protein-phosphatase activity (its substrates known so far are phosphorylated IRS-1(Weng et al., 2001), Shc (Gu et al., 1999) and can mediate protein-protein interactions to control genome maintenance, cell cycle progression or apoptosis, independently of PI3K/Akt (Salmena et al., 2008).

Activated Akt phosphorylates numerous downstream targets (Figure 14). Most relevant for survival are its effects on pro-apoptotic proteins, both at transcriptional and non-transcriptional level. Thus, Akt can directly phosphorylate Bad, a Bcl-2 family member that promotes apoptosis through sequestration of Bcl-X_L, which otherwise would inhibit Bax, a pro-apoptotic protein. Following Bad phosphorylation by Akt, 14-3-3 proteins bind to phosphorylated Bad, thereby preventing Bad from promoting apoptosis (Kim and Chung, 2002). A second target of Akt is the pro-caspase 9; during apoptosis, release of cytochrome c from mitochondria promotes the formation of the apoptosome (cytochrome c and APAF-1) followed by activation of procaspase-9 and subsequent activation of effector caspases (3 and 7; (Bredesen et al., 2006). Direct phosphorylation of pro-caspase 9 by Akt renders it resistant to processing and activation (Kim and Chung, 2002). Elevated levels of GSK-3β promote apoptosis, and GSK-3β phosphorylation by Akt inhibits its pro-apoptotic action. Akt also promotes NFkB signaling by phosphorylating the inhibitory binding partner of NFkB, IkB (Brunet et al., 2001); NFkB is released from the inhibition and translocates to the nucleus to promote transcription of pro-survival genes (Bcl-2 family members and IAPs). The Forkhead family of transcription factors (FKHRLs) induce expression of several pro-apoptotic genes, and FOXO (the Forkhead box transcription factor, class O) has prominent pro-apoptotic roles (Brunet et al., 2001); following direct phosphorylation by Akt, FOXO associate with 14-3-3 proteins which promote its export from the nucleus and sequestration in the cytoplasm (Brunet et al., 2001).

1.9.3. GDNF and Ret can signal independently of each other

1.9.3.1. Ret-independent GDNF signaling

The expression of GFRα proteins in several regions devoid of Ret and the ability of GDNF to trigger downstream signaling in Ret-deficient cell lines and primary neurons suggested the existence of a Ret-independent GDNF/GFRα1 signaling. Carlos Ibanez and collaborators identified the Neural Cell Adhesion Molecule (NCAM) as a novel GDNF receptor in neurons (Paratcha et al., 2003). The association between GFRα1 and NCAM generated a high-affinity receptor for GDNF and following ligand-induced NCAM activation, the cytoplasmic Src-like kinase Fyn and the focal adhesion kinase (FAK) were activated (Figure 15); remarkably while presence of GDNF promoted cell adhesion via Fyn and FAK, GFRα1 inhibited NCAM-mediated cell-adhesion in the absence of GDNF (Paratcha et al., 2003). GDNF binding to NCAM stimulates Schwann cell migration and axonal growth in hippocampal and cortical neurons in a Ret-independent manner. Mice that lack GFRα1 only in cells that do not express Ret (but still express GFRα1 in cells expressing Ret) were recently found to display loss of parvalbumin-immunoreactive cortical interneurons, further confirming the in vivo relevance of Ret-independent GDNF signaling (Canty et al., 2009). In a recent study, Carlos Ibanez and collaborators found a novel function for GDNF/GFRα1complex, working independently of Ret but requiring NCAM function, as mediators of hippocampal and cortical synapse formation. While all cell adhesion systems studied so far involve the direct interaction between membrane-bound partners in trans, GFRα1-mediated cell adhesion is dependent on the presence of its ligand, GDNF; GDNF promotes the cell-adhesion capacity of GFRα1 during trans-homophilic cell adhesion that occurs during synapse formation (Ledda et al., 2007) providing the first example of a ligand-induced cell adhesion system. Finally, GDNF/GFRα1signaling promotes differentiation and migration of cortical GABAergic neurons, in a Ret- and NCAM-

independent manner (Pozas and Ibanez, 2005), suggesting that existence of additional transmembrane receptors for GDNF.

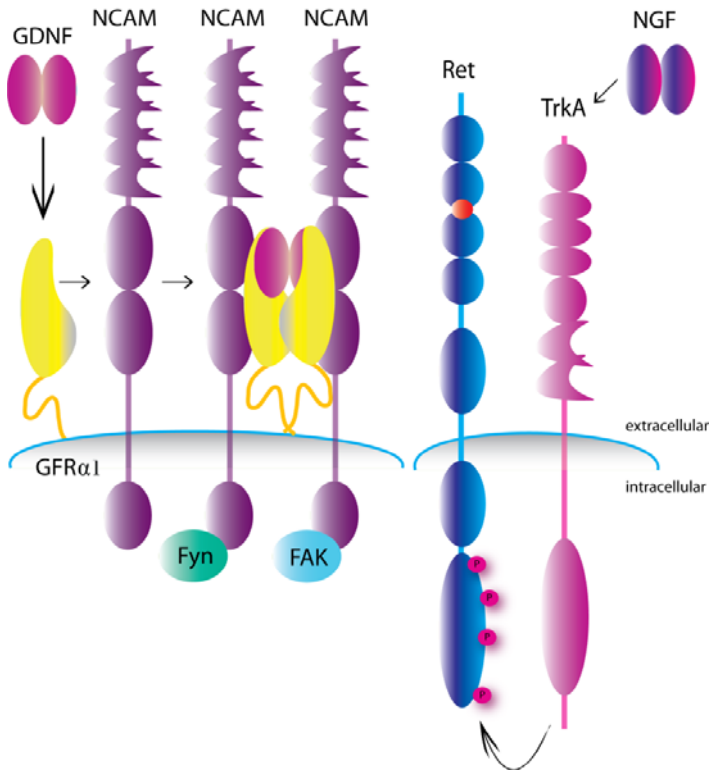


Figure 15. GDNF and Ret can signal independently of each other. Left: In cells lacking Ret but expressing GFRα1-2, GDNF (or ARTN, not shown) can recruit the neural cell adhesion molecule (NCAM) and induce activation of Fyn and FAK kinases. Note that the existence of alternative GDNF receptors has been postulated. Right: In superior cervical ganglion (SCG) cells, activation of TrkA by NGF leads to phosphorylation and activation of Ret signaling, probably in an indirect manner (e.g. by recruitment of a downstream kinase).

1.9.3.2. GFL-independent Ret signaling

Although embryonic sympathetic superior cervical ganglion (SCG) neurons require NGF/TrkA signaling for survival, postnatal SCGs do not depend on NGF/TrkA survival signals anymore. In postnatal SCGs, NGF-acting via TrkA- promotes phosphorylation of the Ret RTK in a GFL-independent manner thus inducing enhanced Ret-mediated growth, metabolism and gene expression (Tsui-Pierchala et al., 2002). Although the exact mechanism of activation is unclear, TrkA does not directly associate with Ret, suggesting that TrkA indirectly promotes Ret phosphorylation.

1.9.4. Study of Ret signaling in Drosophila melanogaster

The *Drosophila* compound eye is composed of an orderly array of about 800 identical units called ommatidia. Each ommatidium contains eight photoreceptors neurons called R1-R8, four non-neuronal lens secreting cone cells, seven pigment cells and a sensory bristle (Figure 16). Each photoreceptor extends a single rhabdomere, the light sensing organelle containing the visual pigment Rhodopsin. R1-R6 photoreceptors have larger

rhabdomeres and are called “outer photorecetors”, as they surround the smaller “inner” rhabdomeres of R7 and R8 cells.

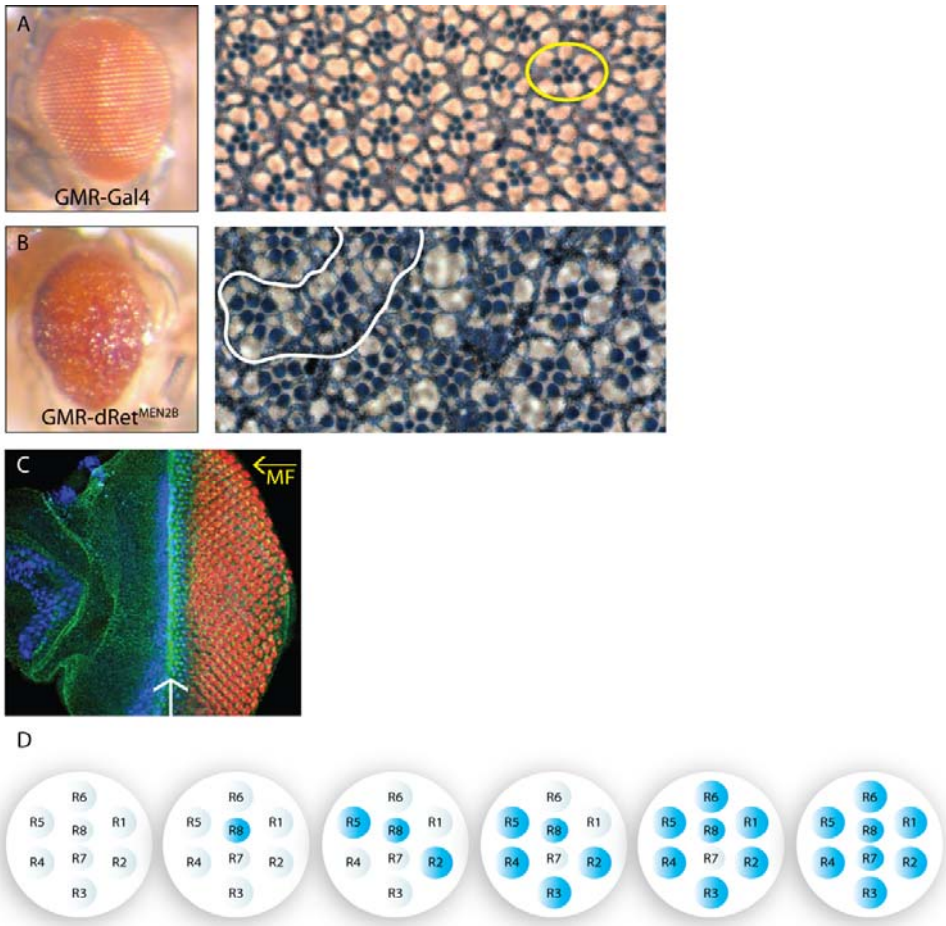


Figure 16. *Drosophila* eye development and constitutively active Ret signaling. (A) Picture of a control *Drosophila* eye, showing the regular organization of ommatidia. On the right side, histological analysis of retinal organization reveals the presence of an organized array of photoreceptor neurons, within each ommatidium (yellow circle). Each ommatidium contains 8 photoreceptors, however, only 7 photoreceptors are in the same plane. Each photoreceptor contains a cell body (white) and a light-sensing organelle (rhabdomere, in blue). (B) Expression of a constitutively active version of Ret during development (targeted specifically to photoreceptor neurons using a photoreceptor-specific driver GMR; i.e. *GMR-dRet*^{MEN2B}) leads to severe defects in eye development. The eye has a rough appearance, is disorganized and has a reduced size. Histologically, the ommatidia are also affected; the size of individual photoreceptors is increased and the photoreceptors display positioning defects. In addition, non-neuronal cells display defects in development, and ommatidia are often fused together (white line) and abnormally positioned with respect to each other. (C) During eye development, the formation of the morphogenetic furrow (MF, white arrow) initiates the cell differentiation process in the embryonic retina. The MF proceeds from right to left (yellow arrow) and leaves behind differentiated cells. In red, Elav labeling reveals photoreceptor neurons; in blue, atonal staining reveals non-differentiated cells, while the green signal marks the armadillo-labeled cells that stopped proliferating within the MF. (D) After passage of the MF, the first cell to differentiate is the photoreceptor 8 (R8), followed by R2 and R5, then by R3 and R4, followed by R1 and R6. The last photoreceptor to differentiate is R7. After differentiation of R cells, non-neuronal cells are recruited in each ommatidium and at the end, all excess non-differentiated cells are eliminated by apoptosis.

During embryonic development, the eye forms from a monolayer epithelium called eye imaginal disc. In early stages, all undifferentiated cells divide synchronously. The formation of an apical constriction in the disc epithelium (called morphogenetic furrow, MF; Figure 16C) marks the beginning of the differentiation process (in the middle of the third instar larval stage). The MF generated a wave of differentiation signals that traverses the entire antennal disc, from posterior to anterior (Figure 16C). While anterior to MF, all cells divide synchronously, in MF division stops; after MF passage, cells undergo two further synchronous cell divisions (Heberlein and Moses, 1995). The first photoreceptor neuron to be specified after MF passage is R8. Every newly specified R8 cell behaves as a founder cell for one ommatidium by initiating the sequential recruitment of other cells (Figure 16D) (Frankfort and Mardon, 2002). Shortly after, R2 and R5 are added, followed by R3 and R4 to form a five-cell pre-cluster. At this stage, all remaining undifferentiated cells undergo a coordinated S-phase and then undergo mitosis in a process called "second mitotic wave", resulting in R1 and R6 recruitment. R7 is the last photoreceptor neuron to be specified (Voas and Rebay, 2004). After all photoreceptors have been specified, non-neuronal cells join the ommatidium. First, four lens-secreting cone cells, then primary, secondary and tertiary pigment cells (1', 2', 3' PCs); finally, unused cells are eliminated by apoptosis, at which point the fly has completed about half of the pupal development (Voas and Rebay, 2004).

Recruitment of cells in the developing retina involves a stereotyped sequence of inductive interactions between extensive networks of intercellular signaling events. Thus, a complex interplay between RTK (Sevenless and EGF), Notch, Hedgehog, Wingless and Decapentaplegic (Dpp/TGF-beta) pathways is required for many aspects of retinal development, including eye fate specification, regulation of proliferative vs. differentiative choice points and establishment of distinct cell fates (Baker, 2007; Hsiung and Moses, 2002).

The recruitment of the last R7 photoreceptor is dependent on the function of the *sevenless* (*sev*) gene, which encodes the sevenless receptor tyrosine kinase (sevRTK). The sevRTK binds to its specific ligand, *bride-of-sevenless* (*Boss*). Out of five equal-potential cells that express *sev*, only one finds itself in direct contact with the *Boss*-expressing R8 cell and goes on to become the R7 cell. The other four cells of this equivalence group, which do not contact the R8 cell, instead adopt the fate of non-neuronal lens-secreting cone cells. Ectopic expression of Boss or expression of activated forms of SevRTK and its downstream effectors leads to transformation of cone cells into R7 cells; activation of the Ras/Raf/MEK/ERK signaling cascade downstream of sevRTK leads to very similar phenotypes, suggesting that the Ras/Raf/MEK/ERK signaling cassette mediates the differentiation of the R7 cell induced by the Boss-sevRTK interaction (Karim et al., 1996).

The extremely stereotyped control of *Drosophila* eye development and the evolutionary conservation of the genes controlling retinal development made Drosophila an organism of choice in the study of signal transduction. Numerous major player in RTK signaling have been initially identified and characterized in the fly, including the members of the Ras/MAPK signaling. Although *Ret* is expressed in *Drosophila* in tissues that roughly parallel their mammalian counterparts (Hahn and Bishop, 2001) its expression in the developing eye is weak (Read et al., 2005) and its ligand in *Drosophila* is currently unknown. To gain insight into the molecular interactions

involved in Ret signaling, the laboratory of Ross Cagan at the Mount Sinai School of Medicine in New York generated flies carrying MEN2 versions of Ret. In these flies, expression of constitutively active Ret (Ret^{MEN2A} or Ret^{MEN2B}) is targeted to the developing photoreceptors (using the photoreceptor specific driver *glass multimer reporter*, *GMR*). Ret^{MEN2} flies, in contrast to controls, display a rough eye phenotype due to excessive cell proliferation, differentiation defects, defective patterning of ommatidia and inter-ommatidial spaces and excessive apoptosis (Figure 16B). The use of Ret^{MEN2} flies allows the identification of specific suppressors (mutations that rescue the Ret^{MEN2} eye phenotype and restore eye development) or enhancers (mutations that further worsen the Ret^{MEN2} phenotype). In the first screen using Ret^{MEN2} flies, Ross Cagan and colleagues found that most of the identified interactors are known interactors of mammalian WT Ret; in addition, they performed a drug screen and discovered a potent Ret^{MEN2} suppressor (ZD6474) that is now in clinical development (phase III) and holds promise as the first chemotherapeutic approved for MEN2 (Vidal et al., 2005). Thus, the use of Ret^{MEN2} flies allows identification of novel Ret interactors.

1.10. Functions of BDNF and GDNF in *substantia nigra* neurons

Following differentiation into DA neurons and establishment of connections with the target area, the striatum, the nigrostriatal pathway is thought to be further refined during two waves of naturally occurring cell death (Burke, 2003). The first wave takes place shortly after birth and achieves its peak two days after; the second wave of apoptosis takes place two weeks after birth and lasts approx. one week. Thus, an important fraction of SN neurons are eliminated during the naturally occurring postnatal cell death and it is during this period that pro-survival molecules secreted by the target area, the striatum, might actively control survival of SN neurons. Several trophic factors have been shown to have neurotrophic effects on developing SN neurons, and might therefore be candidates as target-derived neurotrophic factors for SN neurons: BDNF; GDNF and the related NRTN; NT-4 and fibroblast growth factor 2 (FGF-2) (Krieglstein, 2004; Smidt and Burbach, 2007). I will discuss below the evidence for BDNF and GDNF as candidate neurotrophic factors for SN neurons.

1.10.1. BDNF as a candidate target-derived neurotrophic factor

BDNF meets all the important criteria to be considered as a candidate neurotrophic factor for SN neurons. Thus, BDNF is expressed in the striatum, the SN target area (Friedman et al., 1991; Hofer et al., 1990) and was shown to be retrogradely transported by DA neurons when injected into the striatum (Mufson et al., 1994). In addition BDNF (and the related neurotrophins, except NGF) promotes survival of cultured midbrain DA neurons (Hyman et al., 1991) and stimulates their activity (Knusel et al., 1991). The *in vivo* evidence for BDNF acting as a target-derived neurotrophic factor is incomplete. Thus, mice lacking *BDNF* or the *TrkB* receptor in all cells die early after birth due to several sensory and motor defects (Klein, 1994) and their DA system was therefore not evaluated.

To understand the in vivo relevance of BDNF/TrkB signaling for developing neurons, mice heterozygous for *TrkB* null alleles have been used. These animals display a moderate loss of SN neurons (26 %) specifically during aging and interestingly, removal of both *TrkB* and *TrkC* increased the loss of SN neurons to 34 % (von Bohlen

und Halbach et al., 2005). Another group generated hypomorphic *TrkB* mice (in which TrkB levels were reduced to 25 % of wild-type levels) and found a 40 % loss of SN neurons, but during development, together with increased levels of striatal dopamine (Zaman et al., 2004). Most BDNF constitutive knockout mice die after 2 weeks, while some survive up to 4 weeks. At postnatal day 14, absence of BDNF function did not affect survival of SN DA neurons (Jones et al., 1994). Interestingly, conditional removal of BDNF function from the midbrain-hindbrain (using the Wnt1-Cre line) area led to a 23 % loss of SN neurons at postnatal day 21 (Baquet et al., 2005). These two studies seem therefore to suggest that BDNF is dispensable for the initial establishment and survival (up to P15) of SN neurons, but might be required between P15 and P21 for their survival.

A major problem with the above-mentioned studies is that the function of *TrkB* was not specifically removed from SN neurons, and thus additional non-cell autonomous effects might be involved in the regulation of SN neuron survival. Moreover, removal of BDNF from several neuronal populations might also generate non-cell autonomous effects; finally, the findings that *TrkB* might regulate maintenance during aging (von Bohlen und Halbach et al., 2005) or initial SN development (Zaman et al., 2004), while BDNF rather regulates early maintenance (Baquet et al., 2005) are partially conflicting and additional evidence is required to establish whether BDNF is a target-derived neurotrophic factor for developing neurons.

1.10.1. GDNF as a candidate target-derived neurotrophic factor

GDNF was initially identified as a potent survival factor for cultured embryonic DA neurons (Airaksinen and Saarma, 2002; Lin et al., 1993) and numerous subsequent studies confirmed its pro-survival effects *in vitro* (Burke, 2003). GDNF is expressed throughout the CNS and high levels are detected in the striatum (Choi-Lundberg and Bohn, 1995; Springer et al., 1994), specifically in the dopaminoceptive medium size striatal neurons, (Burke, 2004). Moreover, its developmental pattern of expression also correlates with that of striatal innervation and naturally occurring cell death of SN neurons (Burke, 2004) and GDNF undergoes retrograde transport in SN neurons (Tomac et al., 1995).

The *in vivo* evidence for GDNF acting as a target-derived neurotrophic factor for SN neurons is currently missing. Considering the known *in vitro* pro-survival effects of GDNF/Ret on DA and motor neurons, it came as a surprise that the CNS development, including DA neurons, in mice lacking GDNF, GFRα1 or Ret was mostly unaffected (Paratcha and Ledda, 2008). These results suggest that GDNF/Ret signaling is dispensable for the initial establishment of the nigrostriatal system; however because these mice die before the complete establishment and maturation of the nigrostriatal system, the role of GDNF/Ret signaling as a target derived neurotrophic factor for SN neurons remains unclear.

1.11. BDNF and GDNF as a therapy for Parkinson's disease?

Besides potentially regulating SN neuron function and survival *in vivo*, both BDNF and GDNF showed potent rescuing effects *in vitro* and in animal models of PD, in which adult or aging neurons were challenged; however, GDNF consistently showed more promising effects in these studies (Kordower, 2003). Several delivery modes into the brain were tested pre-clinically for GDNF, including: 1) Direct injections or chronic pump perfusions in the striatum 2) gene therapy, using adeno-associated viruses (AAV) or lentiviruses 3) encapsulated cell technology, in which cells engineered to secrete GDNF are implanted in the striatum. Lentiviral delivery is perhaps the most promising strategy to be applied clinically in the near future. However, safety issues including a regulatable promoter to control gene expression *in vivo* and the exact injection site of the viral vector need to be addressed before considering human clinical trials (Kirik et al., 2004; Kordower, 2003; Kordower and Olanow, 2008).

Three clinical trials on GDNF delivery have been performed so far. Two delivered GDNF directly into the striatum of five (Gill et al., 2003) or ten (Slevin et al., 2005) PD patients; the third study, was placebo-controlled and injected either GDNF or a placebo in the striatum of 34 PD patients (Lang et al., 2006). The first two studies reported positive effects on motor function after 18 and 6 months, respectively, while the third study failed to detect any improvement after 6 months. Several reasons might explain the conflicting results in these clinical trials. First, the major problem is that these trials have reduced statistical power, given the reduced number of patients that were tested. Second, the age of the PD-patients was relatively atypical in the third study (their mean age was 46 while the mean age at PD onset is considered to be 68).Third, the delivery of GDNF was not enough standardized in all three studies. Fourth, the patients were followed for only a short period, precluding the analysis of later-onset positive effects (Barker, 2006). These results suggest that additional technical (Kordower and Olanow, 2008) and non-technical improvements are required in order to properly evaluate the potential therapeutic effects of GDNF and other neurotrophic factors in PD. One such necessary improvement was suggested to be a better understanding of the biological roles played by neurotrophic factors in the aging dopaminergic system.

1.12. The thesis project

The present work deals with the survival requirements of SN neurons, the neuronal population that degenerates in PD. To begin characterizing the cellular and molecular networks mediating SN survival, we characterized genetically the roles and interaction between two candidate survival pathways: neurotrophic support and oxidative stress suppression. GDNF and BDNF neurotrophic factors were proposed to exert pro-survival effects in SN neurons, however, their physiological roles in the establishment and maintenance of the nigrostriatal system remained unknown. Inactivation of the oxidative stress suppressor *DJ-1* leads to Parkinson's disease but animal models lacking *DJ-1* show no DA neurodegeneration, suggesting that additional factors interact with loss of *DJ-1* function to cause nigral degeneration. The major aims of my work were:

1. To determine whether *Ret* and *TrkB*, the signaling receptors for GDNF and BDNF respectively, are required for long-term maintenance of SN neurons.

2. To determine whether *Ret* interacts genetically with *DJ-1* in the aging mouse dopaminergic system.

3. To evaluate whether mouse dopaminergic neurons lacking *Ret*-mediated support are sensitive to cellular stress caused by misfolded alpha-synuclein.

4. To determine whether mice lacking *Ret* and *DJ-1* function recapitulate pathological features of PD.

5. To assess whether *DJ-1* interacts genetically with constitutively active *Ret* signaling in *Drosophila melanogaster*.

6. To investigate the molecular mechanisms underlying the genetic interaction between *DJ-1* and *Ret* in *Drosophila melanogaster*.

Here, I provide genetic evidence that GDNF/Ret, but not BDNF/TrkB signaling is required for long-term maintenance of SN neurons. I also provide the first *in vivo* evidence for a pro-survival role of the PD-associated gene and oxidative stress suppressor *DJ-1*. Aging mice lacking *Ret* and *DJ-1* function loose 51 % of SN neurons projecting to the striatum, suggesting that the triple interaction between aging, trophic insufficiency and cellular stress might cause Parkinsonism. Interestingly, *Ret*-deprived SN neurons are not sensitive to alpha-synuclein aggregation *in vivo*, suggesting some degree of specificity for the interaction between *Ret* and *DJ-1*. Furthermore, using genetic analysis in *Drosophila melanogaster*, I show that *DJ-1* interacts genetically with *Ret* and associated Ras/ERK signaling and uncover a novel physiological role for the *Drosophila* orthologue *DJ-1B* during retinal and wing development.

II. RESULTS

A. *Ret* signaling maintains a fraction of aging *substantia nigra* neurons

To circumvent the early lethality of *TrkB* and *Ret* null mice, a conditional approach was taken in our laboratory and conditional knockout mice lacking *Ret* or *TrkB* function in defined tissues were generated. Mice carrying floxed alleles of *Ret* (Ret^{lx}) or *TrkB* ($TrkB^{lx}$) were crossed to mice in which the expression of *Cre* recombinase was driven either in the whole nervous system (using the Nestin-Cre mouse line) or in the dopaminergic (DA) system only (using the dopamine transporter [*DAT*]-*Cre* line). After several crosses, *DAT-Cre;*$Ret^{lx/-}$ (referred to as *DAT-Ret*) , *DAT-Cre;*$TrkB^{lx/-}$ (*DAT-TrkB*), *DAT-Cre;* $Ret^{lx/-}$*;*$TrkB^{lx/-}$ (*DAT-Ret/TrkB*), *Nes-Cre;*$Ret^{lx/-}$ (*Nes-Ret*) and *Nes-Cre;*$TrkB^{lx/-}$ (*Nes-TrkB*) mice and corresponding controls ($Ret^{lx/lx}$, $Ret^{lx/-}$, $TrkB^{lx/lx}$, $TrkB^{lx/-}$, *Dat-Cre*, *Nes-Cre*) were generated. *Nes-Cre* mice display robust *Cre* expression in the whole nervous system, starting during embryonic (E10.5) development (Kramer et al., 2006; Tronche et al., 1999). *Cre* expression is induced at embryonic day 15 in *DAT-Cre* mice when almost all (95 %) TH-positive cells in the SN and VTA areas of the ventral midbrain express the Cre protein. Weak expression was detected in DA neurons in the olfactory bulb and hypothalamus and no *Cre* expression was seen in the striatum (Zhuang X, 2005). *Ret* is highly expressed in the SN and VTA areas, and Ret expression is efficiently removed from these areas in *DAT-Ret* mice (Kramer et al., 2007). *TrkB* is expressed in nigral DA neurons but also in other neuronal populations in the ventral midbrain. Single-cell PCR analysis revealed a 65 % reduction of TrkB mRNA-positive DA neurons in the SN of *DAT-TrkB* mice compared to controls (Kramer et al., 2007).

DAT-Ret, *DAT-TrkB* and *DAT-Ret/TrkB* mice develop normally without any obvious morphological defects, while *Nes-Ret* mice develop with stiff hind limbs, reflecting a critical requirement for Ret signaling during motoneuron axon guidance (Kramer et al., 2005). *Nes-TrkB* mice are born but display an increased lethality after 3 months of age and were not used in this study. Mutant and control mice were allowed to age and animals were analyzed at different time points during the aging process for the integrity of the nigrostriatal system.

2.1. *Ret* is required for long-term maintenance of *substantia nigra* neurons

To determine whether Ret or TrkB signaling is required for the establishment and the maintenance of the DA system, we performed stereological quantifications of DA neurons in mutant and control animals at diverse time points during adulthood and aging. Using several DA independent markers (tyrosine hydroxylase, Dopa-decarboxylase, Pitx3) Edgar Kramer detected a progressive loss of DA neurons in the SN, but not VTA area in *DAT-Ret, DAT-Ret/TrkB* and *Nes-Ret* but not *DAT-TrkB* aging mice (12 to 24 months); out of approx. 13 000 DA neurons in SN, about 30 % were lost in aging *DAT-Ret* mice. Remarkably, no loss of DA neurons was observed in 3-month-old mutant and control animals, suggesting that Ret and TrkB signaling are dispensable for the development of SN and VTA neurons (Kramer et al, 2007). These observations raised the possibility that in the absence of Ret signaling, a fraction of SN DA neurons died in aging conditions.

To provide more definitive evidence of SN neuron cell death, I used the general neuronal marker NeuN, which labels almost all post-mitotic neurons in the nervous system and investigated whether aging *DAT-Ret* mice have altered numbers of NeuN-immunoreactive neurons in the SN. To allow delineating the SN area, I co-stained the NeuN labeled sections with TH (Figure 17A,B). I found that aging (12 and 24-months) control mice display approx. 19 000 NeuN-immunoreactive neurons in the SN while the absence of Ret signaling in DA neurons leads to loss of approx. 3 500 neurons, starting after 12 months (Figure 17C; n=4-5 mice/group * $p < 0.001$ and ** $p < 0.0001$ for 12- and 24-months-old *DAT-Ret* vs. control mice, respectively). These independent quantifications correlate very well with the differences seen by Edgar Kramer when using dopaminergic specific markers and suggest that DA neurons are the only affected neuronal population in the SN of aging *DAT-Ret* mice. In addition, these results rule out the possibility of decreased expression of DA markers in aging *DAT-Ret* mutants and indicate that a fraction of Ret-deficient DA neurons are actually lost during aging. Thus, absence of Ret signaling in the mouse dopaminergic system leads to a progressive loss of SN neurons.

To determine whether DA neuron degeneration in the SN has any influence on the neighboring glial cells, I used Nissl staining to label all neurons and glia cells in the ventral midbrain (Figure 17D). To allow delineating the SN area in the ventral midbrain, I performed tyrosine hydroxylase (TH) immunohistochemistry (Figure 17D,E) and quantified the number of Nissl-stained cells in the SN of aging (12 months) *DAT-Ret* and control mice. I found that both *DAT-Ret* and control mice display about 75 000 neurons and glial cells in the SN (Figure 17F). This suggests that the overall tissue integrity and composition is grossly maintained in aging *DAT-Ret* mutants and the only affected population are the DA neurons (n = 4 mice/group and *DAT-Ret* vs. CTRL p=0.17, t-test). Thus, absence of Ret signaling in aging DA neurons causes cell-autonomous DA neurodegeneration in a subpopulation of aging SN neurons suggesting that Ret, but not TrkB signaling is required for long-term survival of SN DA neurons.

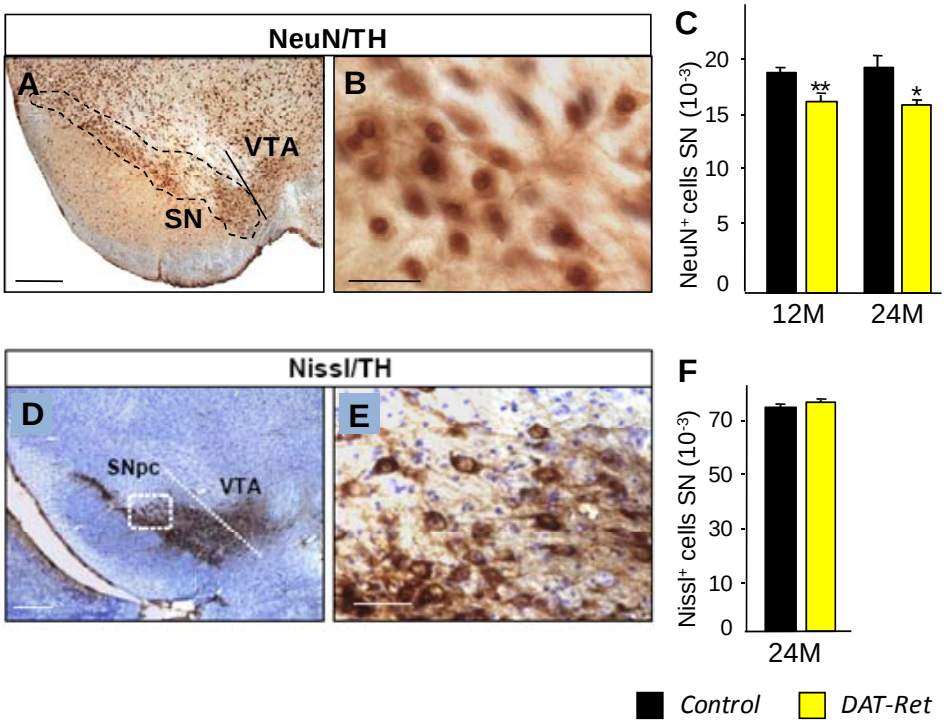


Figure 17. Loss of *substantia nigra* neurons in aging *DAT-Ret* mice. (A-C) Aging *DAT-Ret* mice display loss of neurons in the substantia nigra (SN). (A) Double immunostaining for NeuN and TH (very weak staining to outline the SN [stippled area]). (B) higher magnification view of the stippled area, showing cytoplasmic TH and nuclear NeuN labeling. (C) Stereological quantification of NeuN-positive neurons in the SN of 12- and 24-month-old control and *DAT-Ret* mice (n = 5 [at 12 months] and n = 4 [at 24 months] mice/group; * $p < 0.001$ and ** $p < 0.0001$ for 12- and 24-months-old *DAT-Ret* vs. control mice, respectively).(D-F) Similar numbers of Nissl-positive cells in 12-month-old *DAT-Ret* and control mice. (D) Coronal section of a 12-month-old control mouse showing DA neurons in the ventral midbrain labeled for both Nissl (blue) and TH (brown) (E) Higher magnification view of the boxed area of (D) showing the presence of numerous cells labeled only by Nissl and not by TH (F) Stereological quantification of Nissl-stained cells in the SN of 12-month-old control and *DAT-Ret* mice (n = 4 mice/group, p = 0.17). Scale bars indicate 200 μm.

2.2. *Ret* is required for long-term maintenance of striatal dopaminergic fibers

Dopaminergic neurons in the SN send axonal projections that densely innervate the dorsal striatum. To evaluate whether GDNF/Ret and BDNF/TrkB signaling are required for the establishment and maintenance of SN DA target innervations, I immunolabeled the DA fibers innervating the striatum using the DA-specific marker TH. TH labeling of brain coronal sections revealed the presence of a dense network of DA fibers that innervates the striatum in control animals (Figure 18C,D). Remarkably, the DA fiber network of aging *DAT-Ret* mice was visibly altered (Figure 18D,E). Using a semi-automated method I then performed extensive quantifications of DA fiber density in *Ret* and *TrkB* mutant and control animals (Figure 18G,I). At 12 months, the DA fiber density was markedly reduced (40 %) in the dorsal striatum of *DAT-Ret*, *DAT-Ret/TrkB* and *Nes-Ret*, but not *DAT-TrkB* mice, as compared to control animals (Figure 18G-dorsal). The same situation was observed in the ventral striatum, although the degree of fiber loss was less pronounced, consistent with the innervation preference of SN neurons for the dorsal striatum (Greengard et al., 1999) (Figure 18G-ventral).

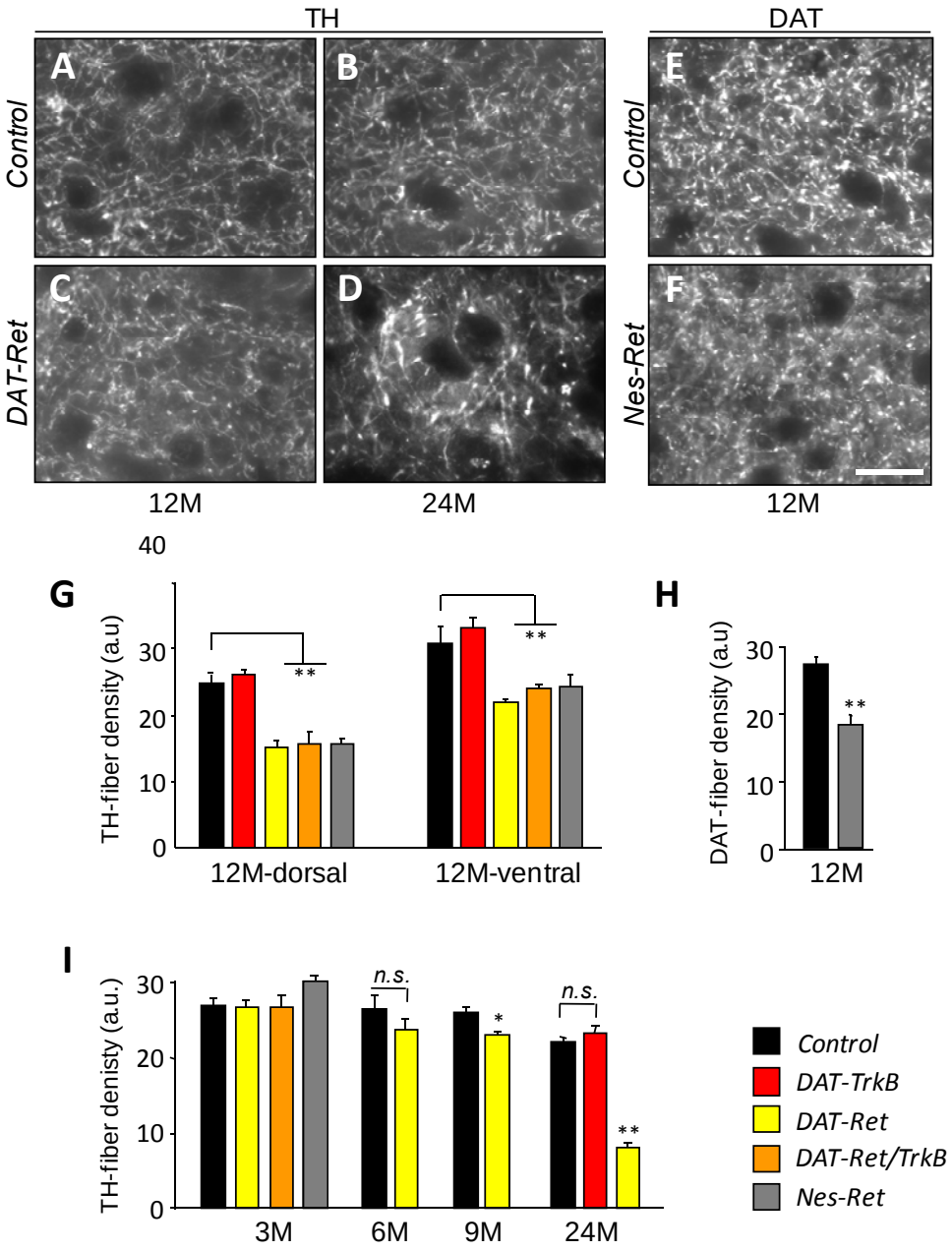


Figure 18 *Ret* is required for long-term maintenance of *substantia nigra* axons. (A–D, E, F) Photomicrographs showing dopaminergic fibers labeled by immunofluorescence using antibodies against TH (A–D) and DAT (E and F) in control (A, B, E), *DAT-Ret* (C, D) and *Nes-Ret* (F) mutants at 12 (A, C, E, F) and 24 months of age (B and D). (G) Quantification of TH-immunoreactive fiber density in dorsal versus ventral striatum of 12-month-old controls ($n = 16$), *DAT-TrkB* ($n = 4$), *DAT-Ret* ($n = 6$), double *DAT-Ret/TrkB* ($n = 5$), and *Nes-Ret* mutants ($n = 7$). Significant reaductions in TH fiber density were observed for *DAT-Ret,* double *DAT-Ret/TrkB,* and *Nes-Ret* mutants in dorsal ($p < 0.001$) and ventral striatum ($p < 0.01$). (H) The density of DAT-immunoreactive fibers was quantified in 12-month-old *Nes-Ret* mutants and age-matched controls ($n = 4$ per genotype; $p < 0.001$, *t*-test). (I) Time course of TH-positive fiber loss from 3 to 24 months of age. The loss of TH fibers begins after 6-9 months ($p = 0.09$ and $p < 0.05$ at 6 months and 9 months, respectively) and is very pronounced at 24 months ($p < 0.0001$). *DAT-TrkB* mutant mice do not show any signs of fiber loss even at 24 months of age ($p = 0.13$). *, $p < 0.05$; **, $p < 0.01$ *t*-test. Scale bar indicates 25 µm. *n.s.-non significant*.

A similar degree of fiber loss was observed in dopaminergic-specific *DAT-Ret* and whole nervous system-specific *Nes-Ret* mutant mice, suggesting that the loss of DA fibers in aging *Ret* mutants is cell autonomous. Moreover, the degree of fiber loss was similar in *DAT-Ret* and *DAT-Ret/TrkB* suggesting that Ret does not cooperate with TrkB signaling to promote maintenance of DA fibers innervating the striatum. These results indicate that Ret, but not TrkB signaling is required for maintenance of striatal innervation in aging mice.

I next determined whether the initial growth and striatal targeting of DA axons is altered in mice lacking Ret and TrkB-mediated trophic support. I used sections from 3-month-old *Ret* and *TrkB* mutants and control animals. Intriguingly, the density of DA fibers in the striatum was similar in all mutant and control animals, suggesting that both Ret and TrkB signaling are dispensable for the initial development, striatal targeting and maintenance of DA fibers (Figure 18I). These results also correlate with the previous stereological results obtained by Edgar Kramer, which suggested a normal development of SN cell bodies in *Ret* and *TrkB* mutants.

To determine the onset of DA fiber degeneration in dopaminergic- and whole nervous system-specific *Ret* mutant mice, I performed a time-course analysis of DA innervation density in *Ret*-deficient and control animals. *DAT-Ret* mice display the first significant loss (approx. 10 %) of DA fibers after 9 months (Figure 18I). Remarkably, the loss of fibers is dramatically increased (60 %) in 24-month-old animals, as compared to controls and to *DAT-TrkB* mice (no fiber loss). To rule out that the observed fiber loss is due to an age-dependent downregulation of TH expression, I used an independent marker, the dopamine transporter (DAT) to label the dopaminergic fibers (Figure 18E,F). A dense network of DAT-immunoreactive striatal fibers was observed in control animals (Figure 18E). Because *DAT-Ret* mice have altered levels of DAT protein (due to the insertion of the Cre coding sequence in the 5' UTR of the DAT gene; data not shown), we used *Nes-Ret* mice for this analysis. I found, similarly, that the DA fiber network is visibly altered in 12-month-old *Nes-Ret* mutants (Figure 18F) and the DA fiber density is reduced by 35 % relative to age-matched controls (Figure 18H). Taken together, these results indicate that Ret, but not TrkB signaling is required for maintenance of DA fibers innervating the striatum, specifically during aging.

2.3. Post-synaptic dysfunction in aging *DAT-Ret* mice

Dopaminergic input to post-synaptic striatal neurons is necessary for proper movement control. Given the significant loss of DA neuron cell bodies and fibers, I wondered whether these anatomical changes in the DA pre-synapse have any impact on the post-synaptic striatal neurons (Figure 19). To evaluate the overall impact of DA innervation (and thus presynaptic input) loss on striatal neurons, I used the general neuronal marker NeuN to label all striatal neurons, including interneurons. Analysis of 24-month-old *DAT-Ret* and control mice revealed that *Ret* mutant mice display an overall weaker NeuN labeling in the striatum (Figure 19A,B). Quantification of NeuN-immunoreactive neuron density in the dorsal striatum revealed a small but significant reduction of NeuN-labelled neurons (8 %; n=5 mice/group, p< 0.05 Student's t-test) in the striatum of 24-month-old *DAT-Ret* mice compared to age-matched control animals (Figure 19C).

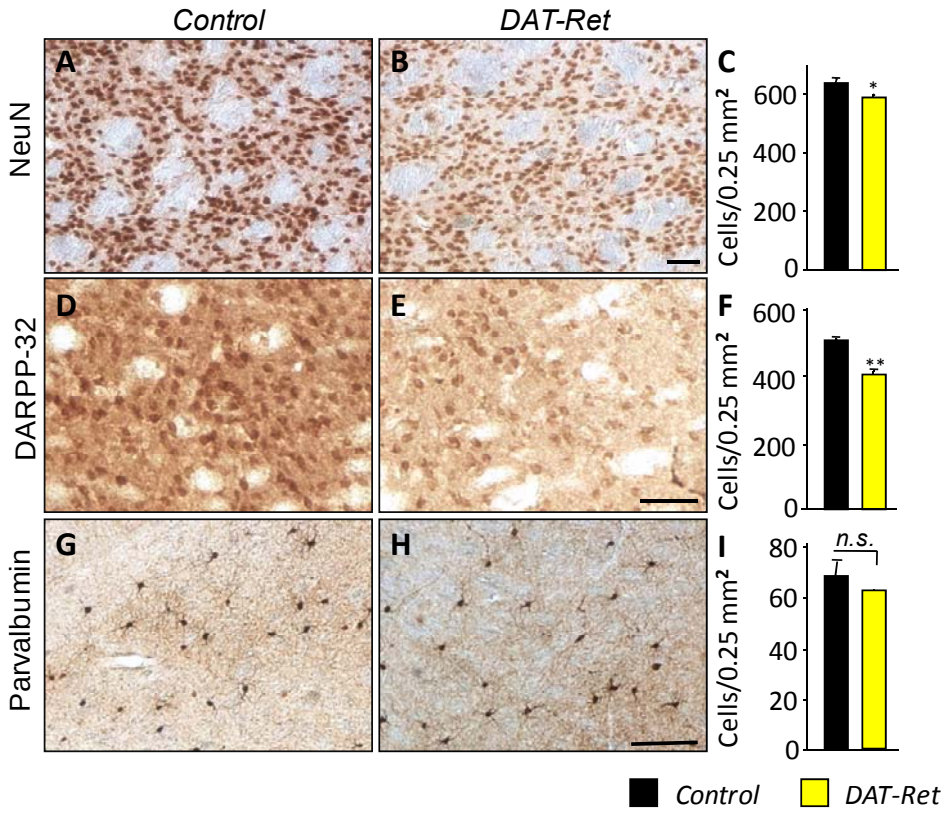


Figure 19. Postsynaptic dysfunction in aging DAT-*Ret* mice. Immunolabelings revealing the expression of NeuN (A,B), DARPP-32 (D,E) and parvalbumin (G, H) in the dorsal striatum of 24-month-old control (A, D, G) and *DAT-Ret* mutants (B, E, H). Histograms showing the density of NeuN-positive (C), DARPP-32–positive (F), and parvalbumin-positive cells (I) in *DAT-Ret* mutants and age-matched controls (n = 3–5 animals/group). Note the reduced NeuN and DARPP-32 staining intensities in *DAT-Ret* compared to control mice. *, $p < 0.05$; **, $p < 0.01$ (Student t-test). Scale bars indicate 50 µm. *n.s.- non significant.*

To investigate more specifically the postsynaptic dopaminoceptive neurons in the striatum, I used a more specific marker for this neuronal population. The protein DARPP-32 (dopamine and cAMP-regulated phosphoprotein, 32 kDa) is expressed by nearly all dopaminoceptive striatal projection neurons (Greengard et al., 1999) and plays an important role in dopamine-mediated signaling. Dysfunctional striatal projection neurons were found to have reduced DARPP-32 expression (Alves et al., 2008; Canals et al., 2004; Regulier et al., 2002). Remarkably, when analyzing the density of DARPP-32-immunoreactive striatal projection neurons, I found a 20 % decrease in density in 24-month-old *DAT-Ret* mutant relative to control mice (Figure 19D-F; n=3 mice/group, $p<0.01$, Student's t-test). It is likely that abnormalities in dopamine signaling have a direct effect on the expression and activation status of dopamine downstream transducers, including DARPP-32. Therefore, dysfunction in the pre-synaptic DA compartment in aging *DAT-Ret* mice induces post-synaptic dysfunction and possibly loss of striatal dopaminoceptive neurons.

To determine whether this observed striatal dysfunction in *DAT-Ret* mice is specific to dopaminoceptive neurons, I analyzed a striatal interneuron population which expresses the calcium-binding protein parvalbumin (Parent et al., 1996). Labeling of parvalbumin-immunoreactive striatal interneurons revealed a similar staining intensity in both *DAT-Ret* and control mice (Figure 19G,H). In addition, the density of parvalbumin-immunoreactive striatal interneurons was similar in both *DAT-Ret* and control mice (Figure 19I), further confirming that the loss of DA fibers in *DAT-Ret* mice affect specifically the post-synaptic striatal projection neurons. Taken together, these results suggest that loss of striatal innervation in aging *DAT-Ret* mice exerts an indirect (non cell-autonomous) effect on the function of dopaminoceptive striatal neurons.

2.4. Enhanced reactive gliosis in the striatum of aging *DAT-Ret* mice

Axonal dysfunction and degeneration was previously associated with recruitment of reactive astrocytes (Butt et al., 2004) and an increased density of reactive astrocytes was detected in the nigrostriatal system of PD patients (Hunot and Hirsch, 2003). We asked whether degenerating SN somata, loss of DA axons and post-synaptic dysfunction in *DAT-Ret* mice would cause gliosis by recruitment of reactive astrocytes.

I used the astrocytic marker glial fibrillary acidic protein (GFAP) to detect alterations in astrocyte number in the brains of *Ret* and *TrkB* mutant and control mice. GFAP-labeled astrocytes displayed their typical star-shape morphology (Figure 20) and were found in several brain regions, including the cortex, the striatum or the SN (data not shown). The measurement of GFAP-immunoreactive astrocyte density in the dorsal striatum revealed a similar (low) astrocyte density in both 12-month-old *DAT-Ret* and control mice (Figure 20A-C; n=3 mice/group, $p=0.9$, Student's t-test). Remarkably, at 24 months, a massive striatal astrogliosis was detected in *DAT-Ret* mice as compared to control and *DAT-TrkB* mice (Figure 20D-F; n=4-5 mice/group, *DAT-Ret* vs. CTRL or *DAT-TrkB* $p<0.001$, Student's t-test). This result raises the possibility that degenerating DA fibers or post-synaptic dysfunction in *DAT-Ret* mice causes a late recruitment of reactive astrocytes to the striatum. Interestingly, no increased astrogliosis was detected in the SN of 24-month-old *DAT-Ret* relative to age-matched

control mice, despite the marked degeneration of DA neuron cell bodies in this area (Figure 20G-I; n=3 mice/group, p=0.24, Student's t-test). Thus, degenerating DA fibers and/or dysfunctional post-synaptic MSNs in *DAT-Ret* mice trigger a late recruitment of reactive astrocytes to the striatum, while degenerating SN cell bodies do not appear to be a strong recruiting signal for astrocytes. Because *Ret* is not genetically ablated in astrocytes, these results suggest that the astrogliosis in the striatum of aging *DAT-Ret* mice is caused by DA fiber degeneration, in a non-cell autonomous manner.

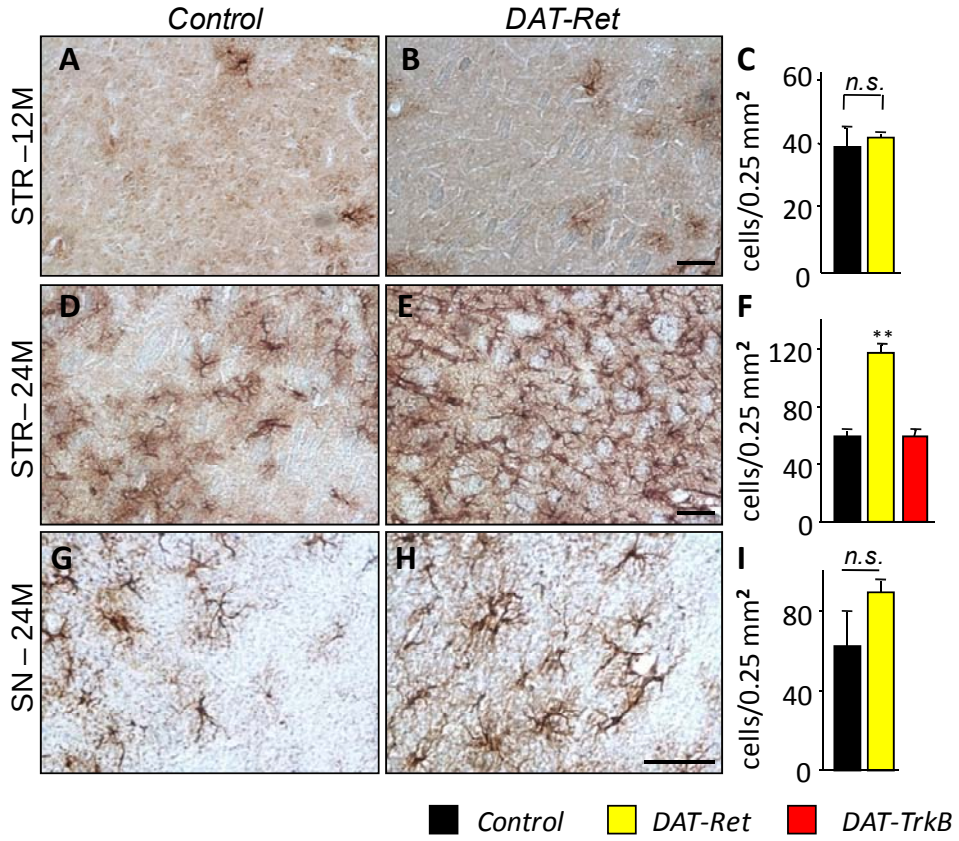


Figure 20. Enhanced reactive gliosis in the striatum of aging *DAT-Ret* mice. (A, B, D, E, G, H) Bright-field photomicrographs of dorsal striatum (STR; A, B, D, E) and substantia nigra (SN; G, H) of 12-month-old (A, B) and 24-month-old (D, E, G, H) control (A, D, G) and *DAT-Ret* mutants (B, E, H) stained for the astrocytic marker GFAP. (C, F, I) Histograms showing the density of GFAP-positive reactive astrocytes (n = 3–5 mice/group). There is a 2-fold increase in the number of reactive astrocytes in the striatum of 24-month-old *DAT-Ret* mutants relative to wild-type controls and *DAT-TrkB* mutants (F) ($p < 0.0001$, Student's t-test), whereas no difference is seen in 12-month-old *DAT-Ret* mutants compared to controls (C) ($p = 0.9$, Student's t-test). No significant increase in the number of reactive astrocytes is seen in the SN of 24-month-old *DAT-Ret* mutants compared to controls (I) ($p = 0.24$). **, $p < 0.01$ Student's t-test. Scale bars indicate 50 µm. *n.s.*–non significant.

2.5. Enhanced microglial recruitment in aging *DAT-Ret* mice

Inflammatory processes are associated with a wide variety of neuronal pathologies and post-mortem analysis of PD patients revealed massive neuro-inflammation in the substantia nigra (Hirsch et al., 2003; Hunot and Hirsch, 2003; Teismann and Schulz,

2004). Microglial cells recruited in areas of neurodegeneration are able to change morphology and acquire phagocytic properties, thus helping eliminate neuronal corpses that underwent apoptosis (Hunot and Hirsch, 2003). We asked whether dying SN cell bodies and axons in aging *DAT-Ret* mice would cause recruitment of microglial cells in the SN or the striatum.

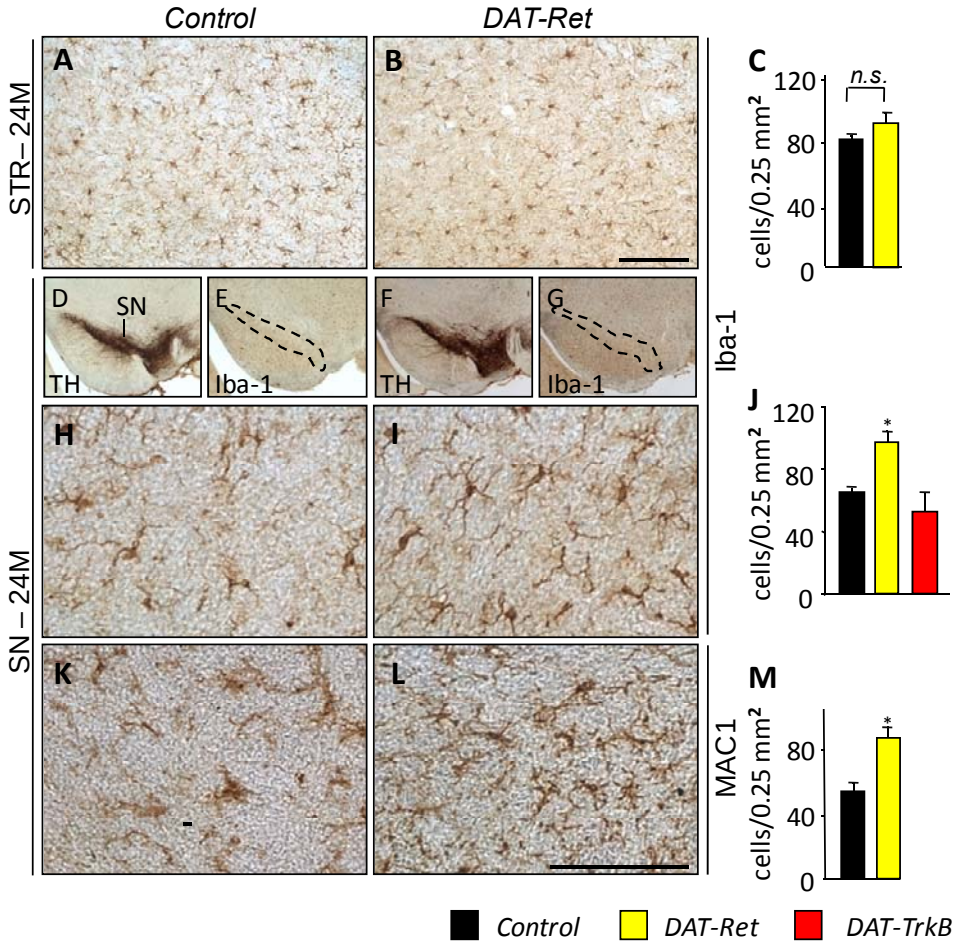


Figure 21. Enhanced inflammation in the substantia nigra of aging *DAT-Ret* mice. (A, B, D–I, K, L) Immunohistochemical stainings of dorsal striatum (STR; A,B) and substantia nigra (SN; D–I, K, L) of 24-month-old control (A, D, E, H, and K) and *DAT-Ret* mice (B, F, G, I, and L) for Iba-1 (A, B, E, G, H, I), TH (D, F), and MAC1/CD11b (K, L). To localize microglial cells in SN, adjacent sections were stained for TH to define the SN area which was copied to the adjacent section stained for microglial cells. (C, J, M) Histograms showing the density of Iba-1–positive (C, J) and MAC1-positive (M) cells in the striatum (C) and SN (J, M) of 24-month-old (C, J) *DAT-Ret* mice and controls. Similar densities of Iba-1–positive cells were detected in the striatum of 24-month-old mutants and controls (C; $n = 4$, $p = 0.065$, Student's t-test). In the SN however, there was a significant increase in the density of Iba-1–positive cells in 24-month-old *DAT-Ret* mice compared to controls (J; $n = 5$, $p < 0.05$). Using MAC1 as a second, independent marker for microglia, a similar result was observed (M) ($n = 3$, $p < 0.05$, Student's t-test). * $p < 0.05$ Student's t-test. Scale bars indicate 100 µm. *n.s.*-non significant.

I used immunohistochemistry for the Ionized binding calcium adapter molecule (Iba)-1 to specifically label microglia in brains of *DAT-Ret* and control mice. Iba-1 labeling revealed the presence of microglial cells both in the striatum (Figure 21A,B) and the SN (Figure 21H,I) of mutant and control mice. I first used Iba-1 stained sections form

the striatum of mutant and control mice and determined the density of microglial cells in this area. The density of microglial cells in the striatum is similar in 24-month-old *DAT-Ret* and control mice (Figure 21A-C; n=4 mice/group, p=0.065, Student's t-test), suggesting that degenerating DA fibers and post-synaptic dysfunction in *DAT-Ret* mice are not a strong recruiting signal for microglial cells.

I then set up to determine the density of microglial cells in the SN area. For each coronal section stained for Iba-1, I used the consecutive section stained for TH, to allow precise delineation of the SN area (Figure 21D-G). I found that 24-month-old *DAT-Ret* mice display a significantly increased density (45 %) of Iba-1 positive microglial cells in the SN as compared to age-matched controls and *DAT-TrkB* mice (Figure 21H-J; n=5 mice for *DAT-Ret* and controls and n=3 for *DAT-TrkB* mice; $p<0.05$ Student's t-test). I then used a second, independent marker to label microglial cells, the macrophage antigen alpha (MAC1, CD11b, or CR3; Figure 21K,L). I found, similarly, a significant increase in the number of MAC1-positive microglial cells in the SN of 24-month-old *DAT-Ret* mice compared to controls (Figure 21M; n=3 mice/group, $p<0.05$, Student's t-test). Interestingly, when analyzing the density of microglial cells in the SN of 12-month-old *DAT-Ret* and control mice, no difference in the microglial cell density was detected (data not shown). These results raise the possibility that degenerating DA cell bodies, in contrast to axons, are a strong recruiting signal for microglial cells. Moreover, the recruitment of microglial cells is delayed compared to the onset of SN neuron degeneration, suggesting that microglial cells do not play a causative role in the neurodegeneration of SN cell bodies. Similar to reactive astrocytes, Ret expression was not ablated in microglial cells of DAT-*Ret* mice, suggesting that the enhanced microglial recruitment in the SN of aging *DAT-Ret* mice occurred as a result of DA neuron cell death.

2.6. No major behavioral alterations in aging *DAT-Ret* mice

Release of dopamine in the striatum is an essential event required for the proper control of movement initiation. Dramatic depletion of DA as seen in PD leads to the typical signs of the disease that include the inability to initiate movement, tremor, postural instability and rigidity. We wanted to determine whether any behavioral and physiological changes accompanied the loss of a fraction of DA neurons in aging *DAT-Ret* mice.

I performed a series of behavioral tests that are routinely used to detect motor alterations in rodents (Figure 22). To evaluate the balance and coordination abilities of mutant and control mice, the rotarod test and the swimming test were used; the open-field and forced swimming test allowed the analysis of both general and horizontal activity. Because in *DAT-Ret* mice the levels of DAT protein are altered (due to the insertion of the *Cre* transgene in the 5'UTR of the *DAT* gene), the control groups also included *DAT-Cre* mice. When recording the spontaneous general activity of mutant and control mice in the open-field arena, no major differences between *DAT-Ret* and control animals were observed (data not shown). When analyzing the general and horizontal activity in the forced swimming test, no difference was observed between the different groups of mice (Figure 22B). Similarly, the coordination and balance abilities as assessed in the rotarod and swimming tests were similar in aging *DAT-Ret* and control mice (Figure 22A,C, n>15, *p=NS*, Student's t-test).

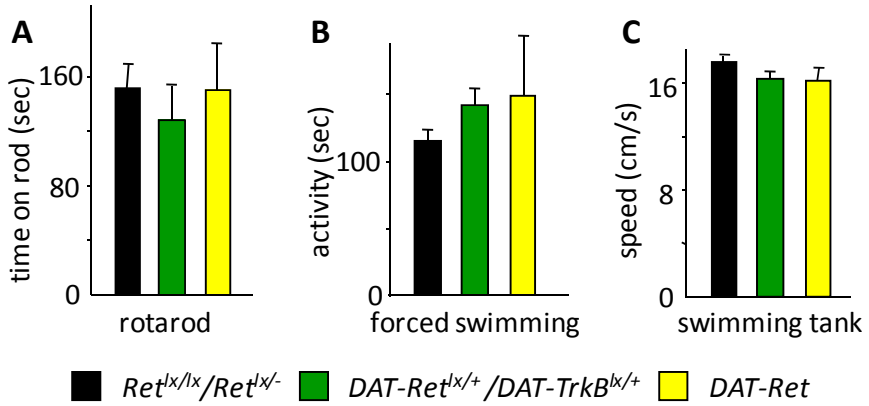


Figure 22. No major behavioral alterations in aging *DAT-Ret* mice. General activity (B) and motor performance (A, C) were tested in 18-24 month-old mice. Motor coordination was tested in the rotarod test (A) and in a swimming tank (C) while the forced swimming test allowed the measurement of horizontal activity (B). No major behavioral alterations were detected in *DAT-Ret* mice as compared to controls carrying floxed alleles of Ret or the *DAT-Cre* construct ($n > 15$, p=NS student's test).

Moreover, measurements performed by Edgar Kramer also revealed that the total levels of striatal dopamine were not significantly altered in aging *DAT-Ret* and *DAT-TrkB* mice compared to controls (data not shown). Thus, although at the anatomical level aging *DAT-Ret* mice display a moderate loss of DA neurons and a marked loss of DA fibers, the nigrostriatal system compensates physiologically by maintaining an optimal dopamine output, suggesting that aging *DAT-Ret* mice are in a pre-symptomatic state of nigral pathology. Indeed, studies of PD patients revealed that motor deficits only become apparent after a "threshold" for SN cell body and axon loss (situated at 50-60 % loss of SN cell bodies and approx. 80 % for DA terminals) is reached (Dauer and Przedborski, 2003).

In summary, the results presented so far suggest that Ret, but not TrkB signaling is necessary for maintenance of aging DA neuron cell bodies and axons. In the absence of Ret signaling and during aging, additional histological changes occur indirectly as consequences of DA dysfunction and include postsynaptic dysfunction, increased astrogliosis and neuroinflammation. Despite these anatomical changes, aging *DAT-Ret* mice are in a pre-symptomatic state of nigral pathology. The moderate anatomical phenotype and the absence of behavioral alterations in *DAT-Ret* mice suggest that additional factors are required for maintenance and functioning of aging DA neurons.

B. *DJ-1* promotes survival of *Ret*-deficient *substantia nigra* neurons

We searched for additional *Ret*-interactors in the process of DA neuron maintenance. We chose to investigate *DJ-1* as a potential *Ret* interactor, for several reasons. First, loss of *DJ-1* function in humans causes Parkinson's disease, suggesting that *DJ-1* is required for survival of human DA neurons. Surprisingly, mice lacking *DJ-1* function do not display loss of SN neurons and DA fibers during aging (Chandran et al., 2008; Chen et al., 2005; Goldberg et al., 2005; Kim et al., 2005b; Yamaguchi and Shen, 2007) suggesting the existence of additional factors that compensate for loss of *DJ-1* function in the mouse. Second, *DJ-1* is expressed in SN and the nearby ventral tegmental area (Bader et al., 2005) of the mouse brain, similar to *Ret* (Kramer et al., 2007). Third, DJ-1 is an oncogene and was suggested to interact with RTK-related signaling pathways including PI3K/Akt (Gorner et al., 2007; Kim et al., 2005a; Yang et al., 2005b) and p53 (Fan et al., 2008a; Fan et al., 2008b; Giaime et al., 2009).

To address a possible pro-survival role for *DJ-1* in neurons lacking Ret-mediated trophic support, I generated animals lacking *Ret* in the dopaminergic system and *DJ-1* in all cells (*DAT-Cre*; $Ret^{lx/lx}$; $DJ\text{-}1^{-/-}$ in short *DAT-Ret/DJ-1*) and corresponding controls. To determine whether Ret and DJ-1 show convergent pro-survival activities in DA neurons, I analyzed adult and aging mutant and control mice for the integrity of the nigrostriatal system.

2.7. Enhanced loss of *substantia nigra* neurons in aging *DAT-Ret/DJ-1* mice

We hypothesized that loss of *Ret* and *DJ-1* function would accelerate the neurodegenerative process in the DA system of aging *DAT-Ret* mice. To test our hypothesis, I first performed stereological quantifications to determine the number of SN DA neurons in adult and aging *DAT-Ret/DJ-1*, *DAT-Ret*, $DJ\text{-}1^{-/-}$ and control mice.

Using brain coronal sections immunostained for the DA marker TH (Figure 23A-C), I determined the number of TH-positive DA neurons in the SN of 3-month-old mutant and control animals. The same number of DA neurons was found in *DAT-Ret;DJ-1* double mutant and in control animals, indicating that the nigrostriatal system developed normally in the absence of *Ret* and *DJ-1* function (Fig. 23G). I then evaluated the number of aging (18- and 24-month-old) SN neurons in mutant and control mice (Figure 23).

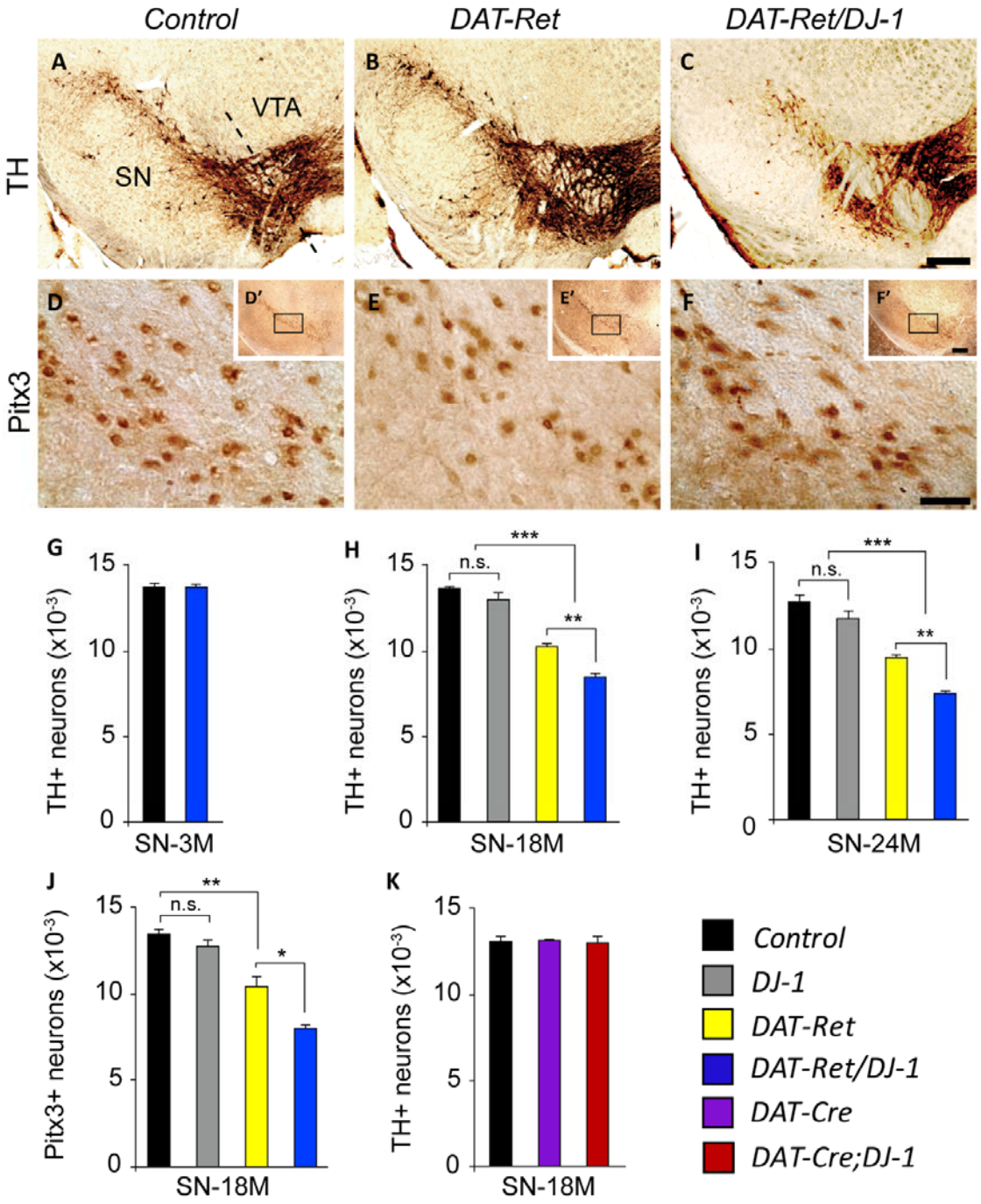


Figure 23. *DJ-1* promotes survival of aging *Ret*-deprived *substantia nigra* neurons. (A-F) Photomicrographs of 18-month-old control (A,D), *DAT-Ret* (B,E) and *DAT-Ret/DJ-1* (C,F) double mutant coronal brain sections showing DA neurons in the substantia nigra (SN) and the ventral tegmental area (VTA) stained for the DA markers TH (A-C) and Pitx3 (D-F). The images in panels D-F are higher magnification views of the stippled area in the insets (D'-F') of each panel. (G-K) Stereological quantifications of TH- and Pitx3-immunoreactive DA neurons in the SN of 3-month-old (G), 18-month-old (H,J,K) and 24-month-old (I) mice of the indicated genotypes (n=5 mice per genotype) * $p<0.05$, ** $p<0.01$,*** $p<0.001$, Student's t-test. Scale bars: (C,F') 250 µm, (F) 50 µm. *n.s.- not significant.*

I found that 18-month-old mice lacking *DJ-1* function have a normal complement of SN neurons, consistent with the previously mentioned studies (Figure 23H; *DJ-1*$^{-/-}$ vs. CTRL p= *NS*, Student's t-test). *DAT-Ret* mice display a moderate loss of DA neurons (25 % loss), in agreement with our previous observations (Figure 23H; n=5 mice/group, *DAT-Ret* vs. CTRL p<0.01 Student's t-test). Remarkably, when analyzing 18-month-old *DAT-Ret/DJ-1*aging mice, I found a significant decrease in the number of SN neurons (37 % loss) compared to *DAT-Ret* mice and *DJ-1*$^{-/-}$ and controls mice (Figure 23H; n=5 mice/group; *DAT-Ret/DJ-1* vs. CTRL p<0.001, *DAT-Ret/DJ-1* vs. *DJ-1*$^{-/-}$ p<0.001 and *DAT-Ret/DJ-1* vs. *DAT-Ret* p<0.01, Student's t-test). At 24-months, *DAT-Ret/DJ-*1 mice lost 41 % of their SN neurons, in contrast to *DAT-Ret* (25 % loss), *DJ-1*$^{-/-}$ and control mice (no loss of SN neurons; Figure 23I; n=5 mice/group; *DAT-Ret/DJ-1* vs. CTRL p<0.001, *DAT-Ret/DJ-1* vs. *DJ-1*$^{-/-}$ p<0.001 and *DAT-Ret/DJ-1* vs. *DAT-Ret* p<0.01, Student's t-test). The difference between *DAT-Ret/DJ-1* double and *DAT-Ret* single mutant mice was statistically significant and was not additive, suggesting that *Ret* interacts genetically with *DJ-1* to promote survival of aging SN DA neurons.

I then used as a second, independent DA marker to rule out that the previously observed effects were due to downregulation of TH expression; I used an antibody against Pitx-3 (Figure 23D-F), which is expressed by all differentiated DA (Smidt et al., 2004). I found, similarly, that 18-month-old *DJ-1*$^{-/-}$ mice have a normal complement of SN neurons and *DAT-Ret* mice display a moderate loss of SN neurons (25 %; Figure 23J; *DJ-1*$^{-/-}$ vs. CTRL p=NS; *DAT-Ret* vs. CTRL p<0.01 Student's t-test). 18-month-old *DAT-Ret;DJ-1* double mutant mice lost 41 % of their SN neurons, significantly more than age-matched *DAT-Ret, DJ-1*$^{-/-}$ and control mice (Figure 23J; n=5 mice/group; *DAT-Ret/DJ-1* vs. CTRL p<0.001, *DAT-Ret/DJ-1* vs. *DJ-1*$^{-/-}$ p<0.001 and *DAT-Ret/DJ-1* vs. *DAT-Ret* p<0.01, Student's t-test). Thus, combined deletion of *Ret* and *DJ-1* causes loss of neurons rather than reduced TH expression in a subpopulation of the SN. Furthermore, to exclude that the *DAT-Cre* transgene and the mutant *DJ-1* allele somehow genetically interacted, I compared the numbers of TH-positive neurons in 18-month-old *DAT-Cre;DJ-1*$^{-/-}$ mice, *DAT-Cre* transgenics and littermate controls; in all these three mutant lines, a similar number of SN neurons was found (Fig. 23K). In addition, quantifications performed by Pontus Klein revealed no loss of VTA neurons in 18-month-old *DAT-Ret/DJ-1* mice as compared to controls, further suggesting that the survival dependency on *Ret* and *DJ-1* signaling is specific for SN neurons (data not shown). Taken together, these results indicate that *DJ-1* is required for survival of aging SN neurons that were impaired in receiving Ret-mediated trophic signals.

2.8. *Ret* and *DJ-1* maintain *substantia nigra* neurons that express GIRK2

Two subpopulations make up the SN: the predominant population of neurons is located ventrally, expresses the G-protein gated, inwardly rectifying potassium channel GIRK2 and projects to the dorsal striatum; the second subpopulation is located dorsally, expresses the calcium-binding protein Calbindin and projects to different areas including limbic and neocortical regions (Bjorklund and Dunnett, 2007). It is the GIRK2 neurons that are preferentially lost in PD (Liang et al., 1996; Yamada et al., 1990). We asked whether both subpopulations require *Ret* and *DJ-1* activity for survival during aging (Figure 24).

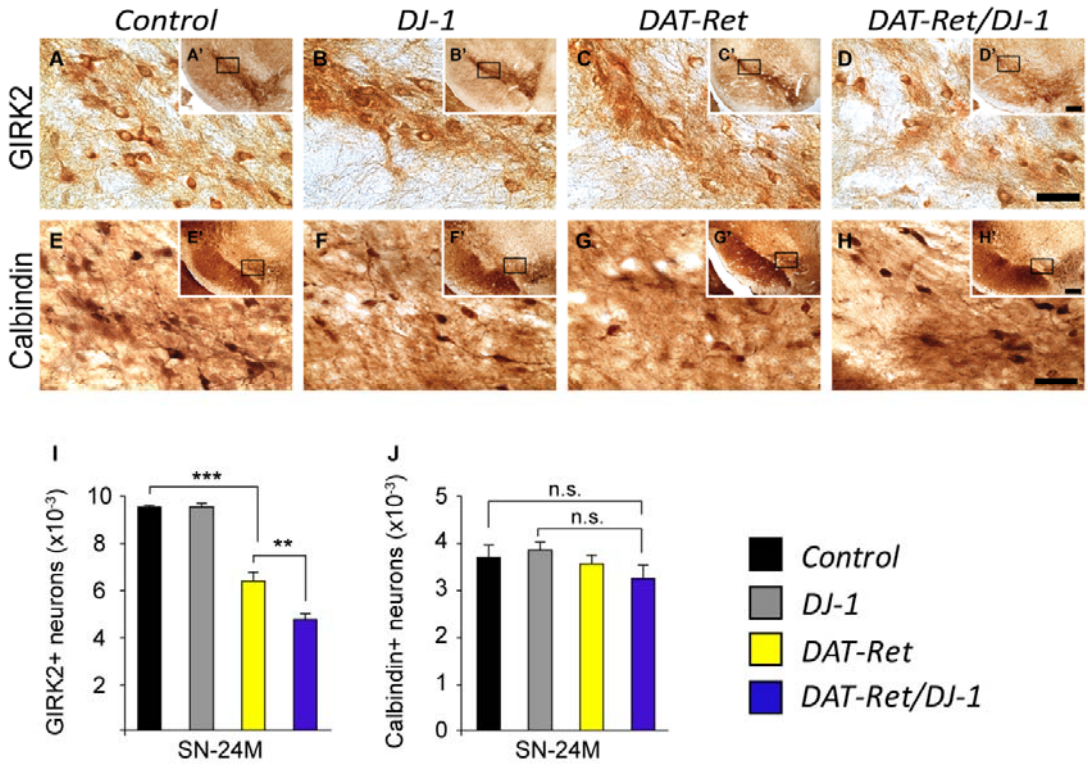


Figure 24. *Ret* and *DJ-1* maintain GIRK-2- positive *substantia nigra* neurons. (A-H) Photomicrographs of 24-month-old control (A,E), *DJ-1*single (B,F), *DAT-Ret* single (C,G), and *DAT-Ret/DJ-1* double mutant (D,H) coronal brain sections stained for GIRK2 (A-D) and Calbindin (E-H). All images are higher magnification views of the stippled area in the insets of each panel. (I,J) Stereological quantification of GIRK-2- (I) and Calbindin-immunoreactive neurons (J) in the SN of 24-month-old mice of the indicated genotypes (n=5 mice per genotype; n.s., not significant;* $p<0.05$, ** $p<0.01$, *** $p<0.001$, t-test). Scale bars: (d,h) 50 µm, (d',h') 250 µm.

I stained coronal brain sections from mutant and control mice for GIRK2 (Figure 24A-D) and performed stereological quantifications to determine the number of GIRK2-positive SN neurons in 24-month-old animals. Removal of *DJ-1* had no effect on the number of GIRK2-positive neurons (Figure 24A,B,I). Remarkably, while removal of *Ret* function alone caused a partial reduction of GIRK2-positive neurons (33% loss), combined removal of *Ret* and *DJ-1* had the strongest effect (51% loss; $p<0.001$ *DAT-Ret/DJ-1* double vs. *CTRL*; $p<0.01$ *DAT-Ret/DJ-1* double vs. *DAT-Ret* single mutants, Student's t-test; Figure 24C,D,I). Therefore, Ret and DJ-1 activity is required to maintain half of the GIRK2-immunoreactive DA neurons in the SN during aging.

The second DA population in the SN was labeled using a Calbindin-specific antibody. Interestingly, the Calbindin-immunoreactive subpopulation in the SN was unaffected in all groups (Figure 24E-H,J). Thus, the neuronal population that is most critically dependent on Ret and DJ-1 function is the GIRK2-positive subpopulation, while the other midbrain dopaminergic cells are grossly unaffected in the mutant mice. Furthermore, given that the SN sub-population most dependent on Ret and DJ-1 activity contains the GIRK2-imunoreactive SN neurons which project exclusively to the striatum, these results raise the possibility that the target of DA neurons is also a

factor of neuronal vulnerability during aging. Therefore, aging GIRK2-, but not Calbindin-positive neurons require *DJ-1* and *Ret* function for survival.

2.9. *DJ-1* is dispensable for long-term maintenance of *Ret*-deficient axons

We next evaluated the possibility that *Ret* and *DJ-1* cooperate in maintaining target innervation of nigral DA neurons. I labeled DA fibers in the striatum using TH as a marker and compared the fiber density in 18- and 24-months mutant and control mice (Figure 25A-D). Quantification of TH-positive fiber density confirmed a marked decrease in the dorsal striatum of 18- and 24-month-old *DAT-Ret* single mutants compared to age-matched controls (Figure 25C,H,I; n= 5 mice/group; *DAT-Ret* vs. *CTRL* $p<0{,}001$, Student's t-test). In contrast, no significant reductions in the density of DA fibers were observed in the striatum of *DJ-1* single mutant mice (Figure 25B,H,I). Interestingly, *DAT-Ret/DJ-1* double mutants displayed reductions of TH-positive fibers that were in the same range as *DAT-Ret* single mutants (46 % at 18-months and 52 % at 24-months, *DAT-Ret/DJ-1* vs. *DAT-Ret* $p=NS$, Student's t-test; Figure 25D,H,I).

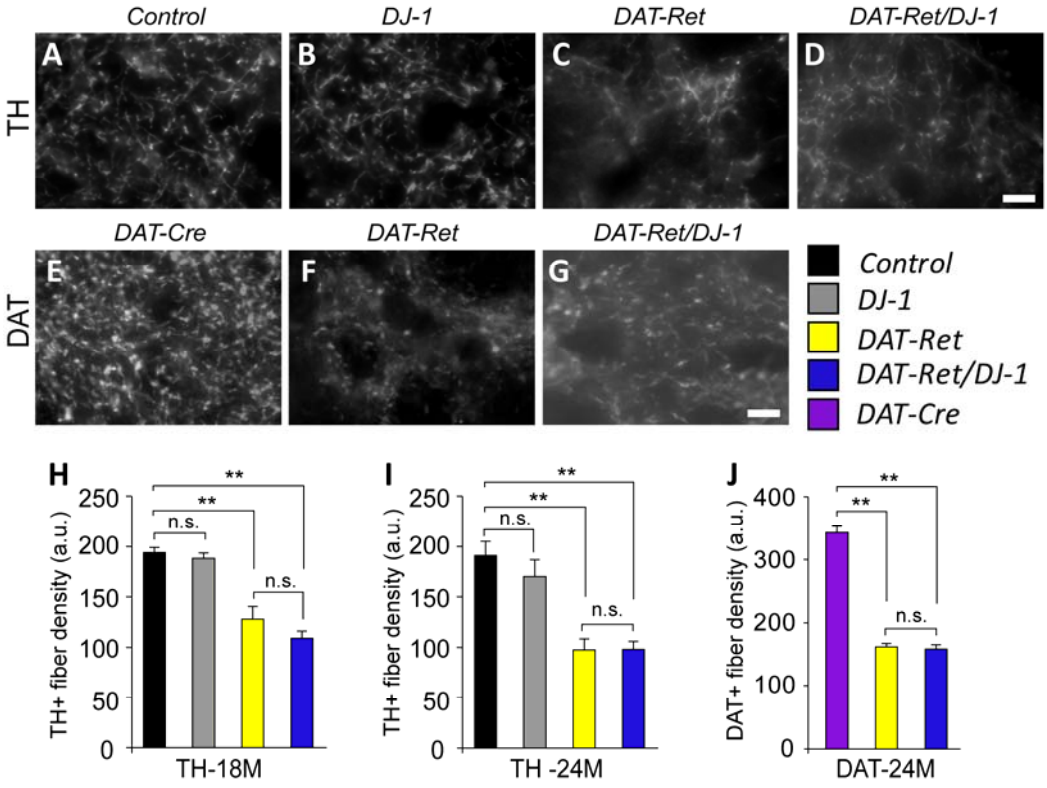


Figure 25. *DJ-1* is dispensable for maintenance of *Ret*-deficient dopaminergic fibers. (A-G) Photomicrographs of DA fibers innervating the dorsal striatum in 24-month-old control (A,E), *DJ-1* single (B), *DAT-Ret* single (C,F) and *DAT-Ret/DJ-1* double (D,G) mutant mice labeled by immunofluorescence using the DA markers TH (A-D) and DAT (DAT; E-G). (H,I) Quantification of TH-fiber density in the dorsal striatum of 18-month-old (H) and 24-month-old (I) mice of the indicated genotypes (n=5-7 mice per genotype; $p<0.01$, Student's t-test). (J) Quantification of DAT-fiber density in the dorsal striatum of 24-month-old mice of the indicated genotypes (n=5-7 mice per genotype; ** $p<0.001$, t-test). Scale bars: (D,G) 10µm. *n.s.-not significant.*

I then used the dopamine transporter (DAT) as a second, independent marker for DA axons (Figure 25E-G). *DAT-Cre* knock-in mice were used as controls, since they have reduced levels of DAT protein (the *Cre* transgene being inserted in the 5'-UTR of the *DAT* gene). Both *DAT-Ret* and *DAT-Ret/DJ-1* 24-month-old mutants displayed a 54 % reduction in DAT-immunoreactive fiber density relative to age-matched *DAT-Cre* control mice (n =4-5 mice/group; *DAT-Ret* or *DAT-Ret/DJ-1* vs. *DAT-Cre* $p< 0{,}001$ and *DAT-Ret* vs. *DAT-Ret/DJ-1* $p=NS$, Student's t-test; Figure 25E-G,J;). Thus, *DJ-1* activity is dispensable for long-term maintenance of DA fibers deprived of Ret-mediated trophic support.

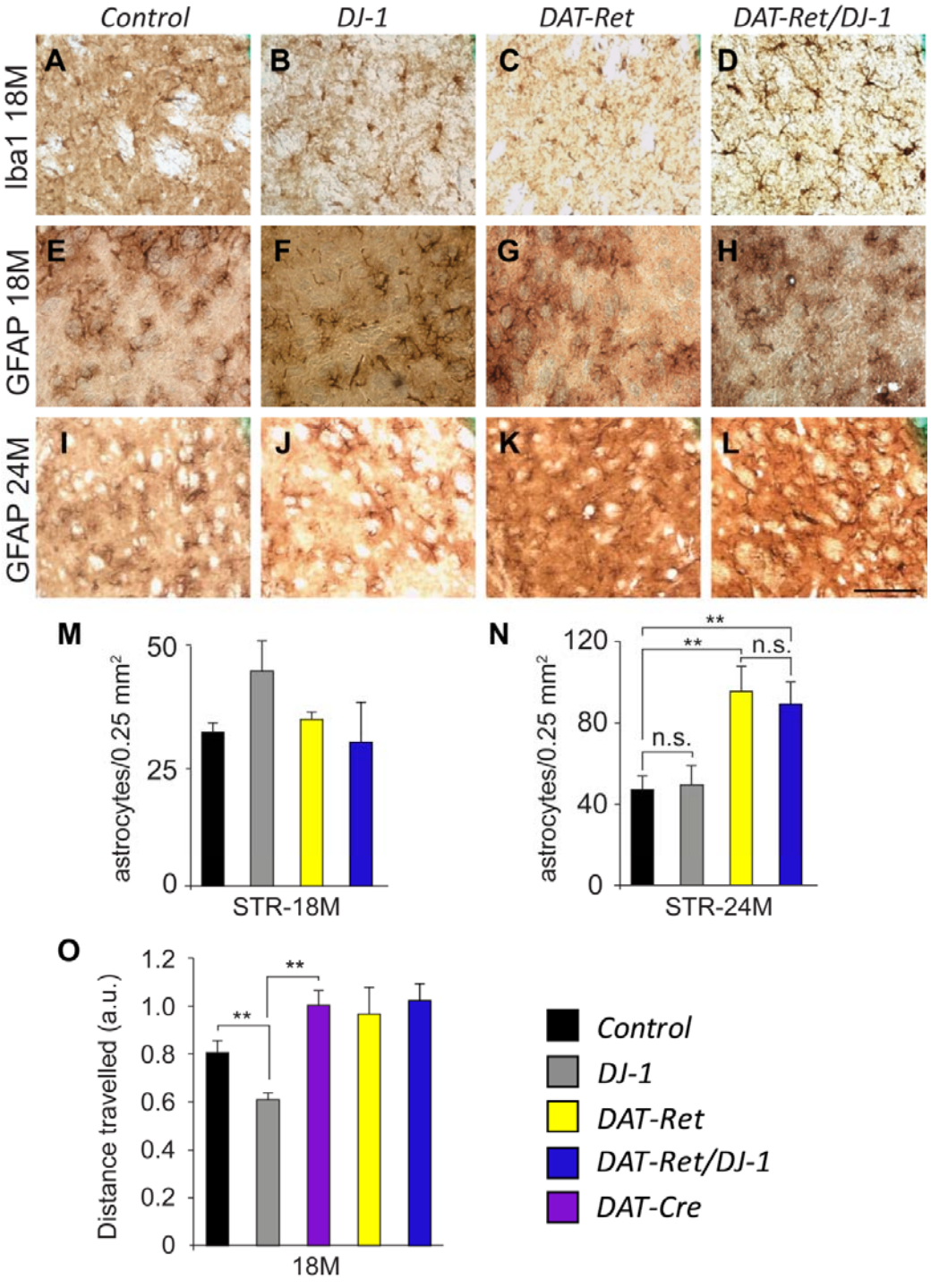
2.10. No enhanced striatal pathology in aging *DAT-Ret/DJ-1* mice

Aging *DAT-Ret* mice were found to have post-synaptic defects (Figure 19), enhanced glial cell recruitment in the dorsal striatum (Figure 20) but no significant behavioral deficits (Figure 22). To determine whether the increased degeneration of SN DA cell bodies in *DAT-Ret/DJ-1* mice has any impact on neuroinflammatory processes in the striatum, I labeled microglial cells and astrocytes in the dorsal striatum of 18- and 24-months old control, *DAT-Ret* and *DJ-1*$^{-/-}$ single and *DAT-Ret/DJ-1* double mutant mice (Figure 26).

No visible differences in the density of Iba-1-immunoreactive microglial cells in the striatum were observed among the aforementioned groups of animals (Figure 26A-D). This situation parallels the previous finding that 24-month-old *DAT-Ret* and control mice have similar microglial cell densities in the striatum (Figure 21A-C). I then used an anti-GFAP antibody to label astrocytes in 18- and 24-months animals. At 18-months, the density of GFAP-labeled astrocytes was similar in all mutant and control groups (Figure 26E-H,M). At 24 months however, the astrocyte density was visibly increased in both *DAT-Ret* and *DAT-Ret/DJ-1* mice, relative to control and *DJ-1* deficient mice (Figure 26I-L), consistent with the previously observed increase in 24-month-old *DAT-Ret* vs. control mice (Figure 20D-F). Quantification of astrocyte density revealed a 2-fold increase in 24-month-old *DAT-Ret* and *DAT-Ret/DJ-1* mice relative to control and *DJ-1* mutant mice, but no significant difference between *DAT-Ret* single and *DAT-Ret/DJ-1* double mutants (n=4-5 mice/group; *DAT-Ret* or *DAT-Ret/DJ-1* vs. *CTRL* $p< 0{,}01$ and *DAT-Ret* vs. *DAT-Ret/DJ-1* p=NS, Student's t-test; Figure 26N). Therefore, combined deletion of *Ret* and *DJ-1* does not accelerate the striatal neuroinflammatory processes observed in aging *DAT-Ret* deficient mice.

To understand whether absence of Ret and DJ-1 function affects the DA neurotransmission in the striatum we performed, together with Pontus Klein, open-field analysis on 18-month-old mutant and control mice (Figure 26O). Mice were tested, without previous training, in an open-field arena and their horizontal activity was measured during two consecutive sessions.

Figure 26. No enhanced neuroinflammation and behavioral deficits in aging *DAT-Ret/DJ-1* mice. (A-L) Photomicrographs of 18-month-old (A-H) and 24-month-old (I-L) control (A,E,I), *DJ-1* (B,F,J), *DAT-Ret* (C,G,K) and *DAT-Ret/DJ-1* (D,H,L) mouse striatal sections stained for the microglial marker Iba-1 (A-D) and the astrocytic marker GFAP (E-L). (M,N) Quantification of astrocyte density in the dorsal striatum (STR) at 18 and 24 months. At 24 months, *DAT-Ret* and *DAT-Ret/DJ-1* mice display a similar increase in astrocyte density (n=4-5 mice/group, ** $p<0.01$, t-test). (O) Open field behavioral assessment of 18-month-old animals of indicated genotypes. *DJ-1* mice were hypoactive, while *DAT-Cre*, *DAT-Ret* and *DAT-Ret/DJ-1* mice had similar horizontal activities (n=7-16, ** $p<0.01$, t-test). Scale bar indicates 200 μm. *n.s.-non significant.*


Control
DJ-1
DAT-Ret
DAT-Ret/DJ-1
Iba1 18M
GFAP 18M
GFAP 24M
A
B
C
D
E
F
G
H
I
J
K
L
M
astrocytes/0.25 mm²
50
25
0
STR-18M
N
astrocytes/0.25 mm²
120
80
40
0
**
**
n.s.
n.s.
STR-24M
O
Distance travelled (a.u.)
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
18M
Control
DJ-1
DAT-Ret
DAT-Ret/DJ-1
DAT-Cre

Consistent with previous reports (Chen et al., 2005; Goldberg et al., 2005) we found that *DJ-1* deficient mice were moderately hypoactive as compared to controls carrying floxed alleles of *Ret* or heterozygous *Ret*-mutants (n=7-16 mice/group and *DJ-1*$^{-/-}$ vs. *CTRL* $p<0{,}01$ Student's t-test; Figure 26O). In contrast, mice carrying the *DAT-Cre* transgene were found to be hyperactive (*CTRL* vs. *DAT-Cre* $p<0.01$, Student's t-test; Figure 26O); this is likely due to reduced levels of dopamine transporter, caused by the insertion of the *Cre* transgene in the DAT 5'-UTR (data not shown). Reduced levels of dopamine transporter allow more dopamine to accumulate at the synapse, as a result of decreased reuptake by DA presynaptic terminals; indeed, mice that partially or completely lack the dopamine transporter are hyperactive and display more dopamine in the striatum (Perona et al., 2008). When analyzing the horizontal activity of *DAT-Ret* and *DAT-Ret/DJ-1* mice, we found that these animals remained hyperactive compared to controls that have normal DAT levels ($Ret^{lx/+}$, $Ret^{lx/lx}$ and $Ret^{lx/-}$) while their horizontal activity did not significantly differ from that of *DAT-Cre* mice (n= 7-16 mice/group; *DAT-Ret* or *DAT-Ret/DJ-1vs. CTRL* $p<0{,}05$; *DAT-Ret* or *DAT-Ret/DJ-1vs. DAT-Cre* $p=NS$, Student's t-test; Figure 26O). Measurement of total levels of striatal dopamine (performed by Pontus Klein) revealed normal dopamine levels in *DJ-1* mutant mice as compared to controls with normal DAT levels and increased dopamine levels in *DAT-Cre*, *DAT-Ret* and *DAT-Ret/DJ-1* mice (these 3 mutant groups had similar dopamine levels; data not shown). Taken together, these results suggest that combined deletion of *Ret* and *DJ-1* does not enhance the DA neurotransmission and striatal pathology observed in aging *DAT-Ret* mutants.

2.11. *Ret*-deprived nigral neurons are not sensitive to α-syn aggregation stress

Mice lacking *Ret* and *DJ-1* have an enhanced nigral degeneration compared to mice that only lack *Ret*, suggesting that absence of *Ret* signaling renders SN neurons more vulnerable to loss of protective *DJ-1* function. Are *Ret*-deficient SN neurons more vulnerable to any kind of cellular stress?

To evaluate this possibility, *DAT-Ret* mutant mice were crossed to mice overexpressing an aggregation-prone mutant of *α-syn* in SN neurons. Transgenic mice overexpressing a PD-causing mutant (Ala30Pro) version of human *α-syn* under the control of the TH promoter (*TH-α-synA30P*) were previously shown to have very mild DA deficits and displayed no loss of DA neurons, up to 12-months of age (Rathke-Hartlieb et al., 2001).

I first confirmed that mutant *α-synA30P* shows abnormal accumulation in SN cell bodies. Using an antibody that recognizes both the endogenous and the human transgenic α-syn, I found that endogenous α-syn is located at axon terminals in control animals (Figure 27C), and no protein is detected in SN cell bodies (Figure 27A). In contrast, transgenic *α-syn Ala30Pro* localizes at axon terminals (Figure 27, compare D with C) but also accumulates in the SN cell body (Figure 27B).

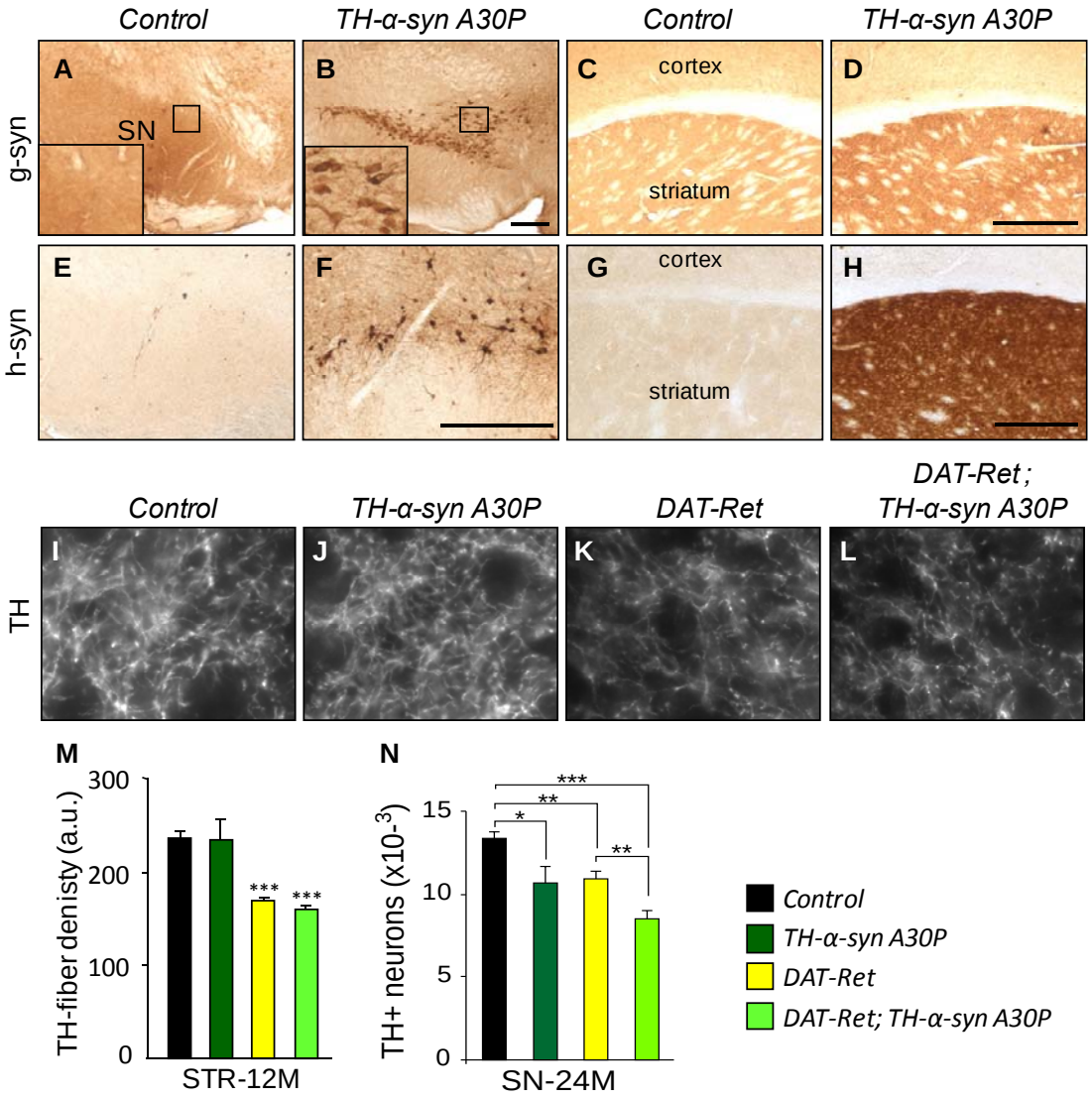


Figure 27. No synergistic effects of Ret deficiency and α-syn aggregation in nigral neurons. (A-H) Photomicrographs of 3-month-old control (A,C,E,G) and *TH-α-synA30P* (B, D, F, H) brain coronal sections from the substantia nigra (SN; A,B,E,F) or the striatum (STR; C,D,G,H) stained for total (endogenous and transgenic; A-D) and human transgenic (E-H) alpha-synuclein (α-syn). Antibodies against α-syn recognized both mouse and human (g-syn) or only the human (h-syn) protein. Note the abnormal accumulation of human α-syn in the SN (B,F) of *TH-α-synA30P* mice. (I-L) TH labeling of striatal DA fibers in 12-month-old control (I), *TH-α-synA30P* (J), *DAT-Ret* (K) and *DAT-Ret;TH-α-synA30P* (L) mice. (M) Quantification of DA fiber density for the genotype groups mentioned above (n=4-5 mice/group; ***p<0.001, t-test). (N) Stereological quantification of TH-positive SN neurons in 24-month-old control, T*H-α-synA30P*, *DAT-Ret* and *DAT-Ret;TH-α–synA30P* mice. While *TH-α-synA30P* mice lose 20 % and *DAT-Ret* mice lose 18 % SN neurons, the loss in *DAT-Ret;TH-α –synA30P* mice is additive (36 % loss) and not synergistic (n=4-5 mice/group; * p<0.05,** p<0.01, ***p<0.001, Student's t-test). Scale bars indicate 250 µm.

The same conclusion was reached using a human specific α-syn antibody; the transgenic protein localizes both to the striatum (Figure 27H) and SN cell body (Figure 27F), while the endogenous α-syn is not detected by the human-specific antibody (Figure 27E,G). Thus, overexpressed mutant *Ala30Pro α-syn* shows abnormal accumulation in the SN cell body and is a potential source of cellular stress.

I then determined whether the presence of α-syn aggregates in SN DA fibers renders them more vulnerable to loss of Ret-mediated trophic support. I analyzed the DA fiber density in the striatum of 12-month-old control, *TH-α-synA30P*, *DAT-Ret* and *DAT-Ret* ;*TH-α-synA30P* animals using TH as a marker for DA axons (Figure 27I-L). I found that *DAT-Ret* mice display a moderate loss of DA fibers innervating the striatum (30 %; n=5 mice/group; *DAT-Ret* vs. *CTRL* $p<0,01$, Student's t-test; Figure 27M), as previously mentioned (Figure 18). Interestingly, the degree of DA fiber loss in 12-month-old *DAT-Ret;TH-α-synA30P* mice was not different from that of age-matched *DAT-Ret* mice (n=5 mice/group; *p=NS*, Student's t-test; Figure 27M), suggesting that DA axons deprived of Ret-mediated trophic support are not more vulnerable to the presence of misfolded alpha-synuclein.

Second, to investigate whether SN cell bodies deprived of Ret function are more vulnerable to aggregation of α-syn, I determined the number of SN cell bodies in 24-month-old mutant and control animals. 24-month-old animals were chosen since the interaction between *Ret* and *DJ-1* in controlling survival of SN cell bodies is maximal at this time point. I found surprisingly that 24-month-old *TH-α-synA30P* transgenic and *DAT-Ret* single mutant mice lose about 20 % of TH-positive DA neurons in the SN, relative to controls (Figure 27N). This suggests that the presence of aggregated *α-synA30P* leads to the degeneration of a fraction of aging SN neurons. Furthermore, *DAT-Ret;TH-α-synA30P* mice lose 36 % of TH-positive DA neurons in the SN, as compared to controls (n=5 mice/group; *DAT-Ret;TH-α-synA30P* vs. CTRL $p<0.001$ and *DAT-Ret;TH-α-synA30P* vs. *DAT-Ret* or *TH-α-synA30P* $p<0.01$, Student's t-test; Figure 27N). Comparison of neuronal loss in *TH-α-synA30P*, *DAT-Ret* single and *DAT-Ret;TH-α-synA30P* mutants suggests that SN neuron loss in *DAT-Ret;TH-α-synA30P* mice is additive and is not caused by a genetic interaction between trophic insufficiency (*Ret* loss) and increased cellular stress due to protein aggregation (*α-syn* overexpression); moreover, this result further suggests that distinct neurons are affected by loss of *Ret* function and α-syn aggregation. Therefore, aging SN cell bodies lacking *Ret* signaling are not more sensitive to the presence of aggregated *α-syn.*

Taken together, these two lines of evidence suggest that Ret deficiency does not render SN cell bodies and axons more vulnerable to any kind of cellular stress, but rather to defined types of dysfunction (e.g. absence of *DJ-1* function) that amplify the effects of trophic deprivation in aging conditions.

C. Genetic interaction between *Ret* and *DJ-1* in *Drosophila melanogaster*

The convergent pro-survival activities of *Ret* and *DJ-1* in aging SN neurons raised the possibility that these two proteins might function in similar pathway(s) or influence common downstream targets. To understand how Ret and DJ-1 activities mutually interact, we decided to use the fruit fly *Drosophila melanogaster* as a starting point for more detailed signal transduction studies. *Drosophila* has a long and successful history in signal transduction studies and allowed the identification and characterization of many critical regulators of cellular signaling. The ability to manipulate lethal mutations in non-essential fly tissues (e.g. retina, wings), to spatially and temporally control transgene expression and to specifically label diverse cellular populations has greatly accelerated cell signaling research. In addition, a vast majority of cell signaling regulators in *Drosophila* are conserved throughout evolution, further validating studies in this simpler organism. Signaling via receptor tyrosine kinases (RTKs) controls many aspects of *Drosophila* development and function (Voas and Rebay, 2004); for example, *Drosophila* eye development is very sensitive to dosage changes in RTKs like *sevRTK* or *EGFR* and downstream components of the PI3K/Akt and Ras/MAPK pathways (Duffy and Perrimon, 1994). Genetic alterations in these pathways lead to defects in eye development and adult eye morphology that can be easily analyzed and thus allow identification of novel interactors.

Drosophila has two *DJ-1* genes, named *DJ-1A* and *DJ-1B*. While *Drosophila DJ-1B* is ubiquitously expressed, *DJ-1A* appears to be enriched in certain tissues such as testes (Menzies et al., 2005; Meulener et al., 2005). Using a DJ-1B specific antibody, I confirmed that endogenous *DJ-1B* is expressed in *Drosophila* heads at postnatal day 5; in addition, *DJ-1B* expression is lost in *DJ-1A*$^{-/-}$*;DJ-1B*$^{-/-}$ flies (Figure 28A). Similarly, Pontus Klein used RT-PCR to show that the *DJ-1A* transcript is present in fly heads (data not shown). I then tested whether overexpression of constitutively active versions of Ret, Raf, ERK/rolled or wild-type Akt1 in the *Drosophila* eye system modulates the levels of endogenous DJ-1B protein. I used the photoreceptor neuron specific *glass multimer reporter* (*GMR*) which allows transgene overexpression via the GAL4/UAS system in post-mitotic photoreceptor neurons in the developing retina, starting in late larval stages (Read et al., 2005). I found that increased levels of constitutively active Ret, Raf, ERK or increased levels of WT Akt1 did not modify endogenous DJ-1B levels (Figure 28A). This suggests that the expression of *DJ-1B* is not modified following activation of Ret, Ras/ERK or Akt signaling. Moreover, I found that flies homozygous for *DJ-1A* and/or *DJ-1B* null alleles and flies overexpressing *DJ-1A* or *DJ-1B* in the eye (using the *GMR* driver) displayed normal development and eye ultrastructure (Fig. 18B and data not shown). Therefore, *DJ-1A/B* activity is dispensable for normal fly development.

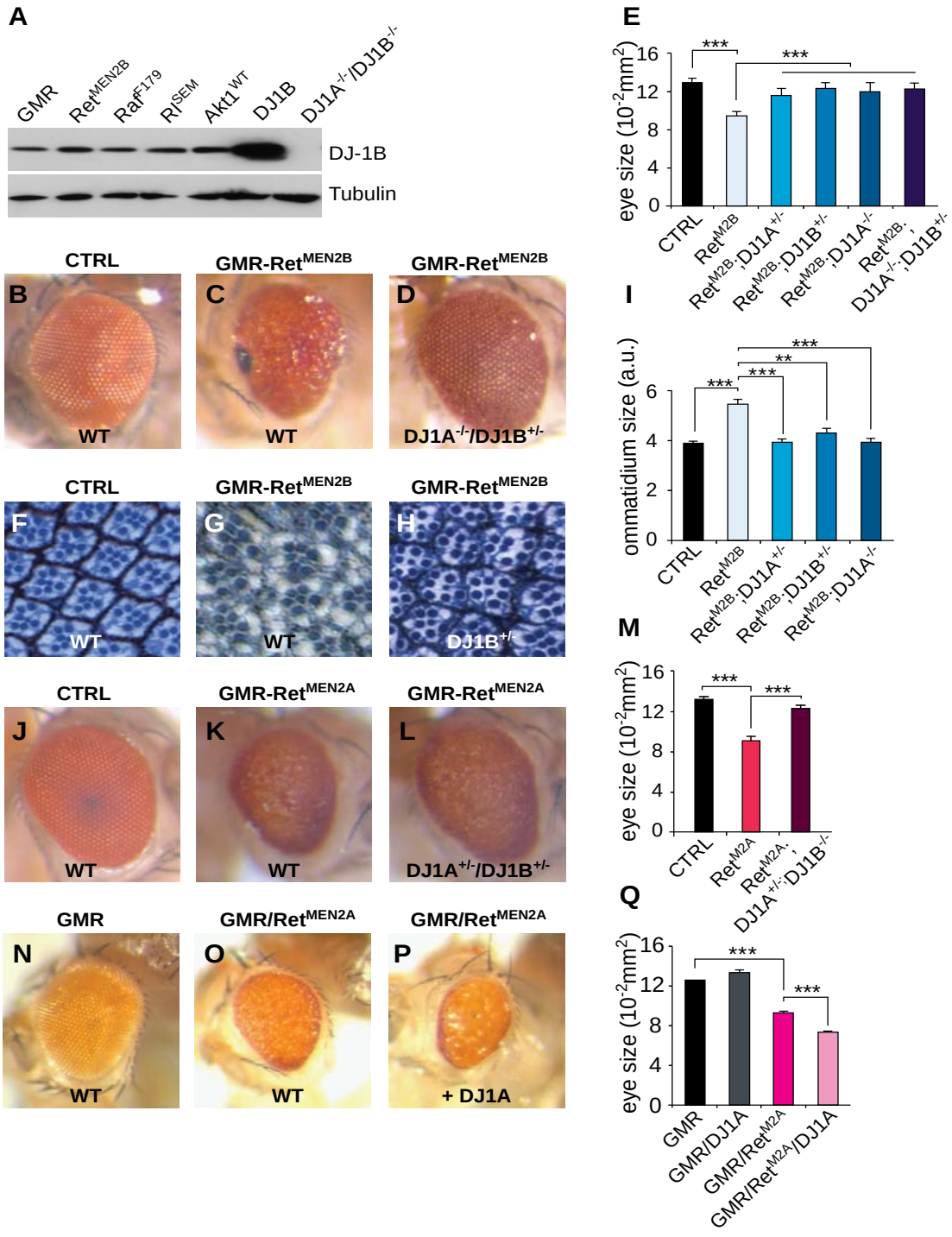
Our mouse experiments suggested that while DJ-1 function is dispensable for development and long-term maintenance of SN neurons, DJ-1 activity is required for survival of a subset of aging SN neurons deprived of Ret-mediated trophic support. We therefore addressed the possibility that the roles of *DJ-1* in *Drosophila* might become important in situations of increased or decreased Ret and RTK signaling. We

started by testing whether *DJ-1A/B* interacts with constitutively active Ret and its downstream signaling during eye development.

2.12. *Ret* interacts genetically with *DJ-1A/B* in the *Drosophila* eye system

The roles of *Ret* signaling in *Drosophila* are poorly understood. Although *Ret* expression in *Drosophila* roughly parallels that in mammals, the ligand responsible for its activation remains unknown (Abrescia et al., 2005; Hahn and Bishop, 2001). In contrast, the use of constitutively active versions of Ret circumvents this regulation step and allows expression of ligand-independent active Ret protein in tissues like the developing retina. Flies carrying constitutively active versions of *Ret* fused to the GMR promoter (*GMR-dRet*^{MEN2}) were generated by the laboratory of Ross Cagan at the Mount Sinai School of Medicine in New York and shown to interact with many already-identified WT Ret interactors (Read et al., 2005). *GMR-dRet*^{MEN2B} flies develop with adult eyes of reduced size and rough morphology; this phenotype results from excessive proliferation, patterning defects and increased apoptosis triggered by overactive Ret (Read et al., 2005). I confirmed that *GMR-dRet*^{MEN2B} flies develop with retinal defects and then complemented this observation with a quantitative evaluation of the *GMR-dRet*^{MEN2B} phenotype. The overall eye size in *GMR-dRet*^{MEN2B} flies was decreased by 30 % compared to controls (Figure 28B,C,E). I then sectioned the fly eyes and used a contrasting agent (toluidine blue) to highlight retinal organization. I found, as previously reported, that numerous ommatidia are often fused together and display abnormal polarity and poorly patterned inter-ommatidial spaces (Figure 28G). In addition, I found that the average ommatidium size was increased by 35% in *GMR-dRet*^{MEN2B} mutants relative to controls (that carried the *GMR-Gal4* construct; Figure 28G,I); a similar increase in photoreceptor cell size is seen in mutants overexpressing constitutively active Sevenless/Ras or PI3K/Akt components (Rubin et al., 1997; Vanhaesebroeck et al., 1997).

Figure 28. Genetic interaction between Ret and *DJ-1A/B* in the *Drosophila* eye. (A) Western blot analysis showing normal levels of endogenous DJ-1B after overexpression of constitutively active Ret, Raf, ERK/rl or wild-type Akt1and DJ-1B, using the GMR promoter. No *DJ-1B* was detected in *DJ-1A*$^{-/-}$*DJ1B*$^{-/-}$ heads. (B-D) Transgenic *GMR-dRet*^{MEN2B} flies have smaller and rougher eyes (C) compared to wild-type controls (B); this phenotype is suppressed in a background of reduced *DJ-1A/B* activity (D). (E) Quantification of eye sizes in the indicated mutant and control flies (n=17-25 eyes/genotype; *** p<0.001, Student's t-test).(F-H) Photomicrographs of ultrathin eye sections stained with toluidine blue showing the normal size and pattern of individual ommatidia of an adult control fly (F), the larger ommatidia of a *GMR-dRet*^{MEN2B} fly (G), and the near normal ommatidia of a *GMR- dRet*^{MEN2B}*;DJ-*1B$^{+/-}$ fly (H). (I) Quantification of ommatidia sizes in the indicated mutant and control flies (n=4 eyes/genotype and n>150 ommatidia analyzed per animal; ** p<0.01, *** p<0.001, Student's t-test). (J-L) Flies carrying a *GMR-dRet*^{MEN2A} construct display smaller and rougher eyes (K) as compared to controls (J); this phenotype is rescued in a background of reduced *DJ-1A/B* activity (L). (M) Quantification of eye sizes in the indicated mutant and control flies (n >10 eyes/genotype; *** p<0.001, t-test). (N-P) Overexpression of dRet^{MEN2A} using the GMR promoter (*GMR/UAS-dRet*^{MEN2A}) leads to a moderate rough eye phenotype (O) compared to control (N), which can be further enhanced by DJ-1A co-overexpression (P). *GMR/UAS-dRet*^{MEN2A}*/UAS-DJ1A* eyes are rougher, have a glassy appearance (P), and a reduced size compared to control and *GMR/UAS-dRet*^{MEN2A} *flies* (Q; quantification of n>10 eyes per genotype, ***p<0.001, Student's t-test).



To determine whether *DJ-1* is a *Ret* interactor, I crossed *GMR-dRet*^{MEN2B} flies with flies carrying *DJ-1A* and/or *DJ-1B* microdeletions that specifically inactivate *DJ-1A* or *DJ-1B* function (here named "knockout alleles") (Meulener et al., 2005). Remarkably, reducing *DJ-1A/B* activity lead to a strong suppression of *GMR-dRet*^{MEN2B} phenotype (Figure 28D,E), the resulting eyes having an improved overall organization. At the histological level, the ommatidial organization of *GMR-dRet*^{MEN2B} flies heterozygous for *DJ-1A* (*GMR-dRet*^{MEN2B}*;DJ-1A*$^{+/-}$) or *DJ-1B* (*GMR-*

dRet^{MEN2B}*;DJ-1B*$^{+/-}$), or lacking completely *DJ-1A* (*GMR-dRet*^{MEN2B}*; DJ-1A*$^{-/-}$) was significantly restored and the size of ommatidial units was almost completely rescued (Figure 28I). Thus, *DJ-1A/B* activity is required for full manifestation of *Ret*^{MEN2B}-induced eye phenotype.

We next asked whether reducing *DJ-1A/B* activity ameliorates the retinal defects induced by another constitutively active *Ret* form (*dRet*^{MEN2A}). *GMR-dRet*^{MEN2A} flies also display a rough eye phenotype and a reduction in total eye size (Figure 28K,M). Remarkably, when *DJ-1A/B* function is reduced (*GMR-dRet*^{MEN2A}*;DJ-1A*$^{+/-}$*;DJ-1B*$^{-/-}$ flies) the phenotype caused by *Ret*^{MEN2A} overexpression is strongly suppressed (Figure 28L,M). Therefore, *DJ-1A/B* activity is required for full manifestation of *Ret*^{MENA}-induced eye phenotype.

I then evaluated whether increasing *DJ-1* activity is sufficient to further impair eye development in flies overexpressing constitutively active *Ret*^{MEN2}. For this, I used the moderate phenotype caused by *Ret*^{MEN2A} overexpression in photoreceptor neurons using the GAL4/UAS system (*GMR-Gal4/UAS-dRet*^{MEN2A}; Figure 28O,Q). When *DJ-1A* was co-expressed with *Ret*^{MEN2A} in developing photoreceptor neurons (*GMR-Gal4/UAS-dRet*^{MEN2A}*/UAS-DJ1A*^{WT}) the eye phenotype was further enhanced, and the resulting eyes became even smaller (Figure 28P,Q). Therefore, *DJ-1A/B* activity is both necessary and sufficient to allow manifestation or to modulate *Ret*^{MEN2}-eye phenotypes in *Drosophila*, suggesting that *DJ-1A/B* interact genetically with *Ret*^{MEN2} in the developing fly retina.

To further confirm that the modulatory effects on *Ret*^{MEN2}-eye development can be attributed specifically to *DJ-1A/B*, I used independent *DJ-1A/B* loss-of-function alleles. P-element insertions that disrupt both *DJ-1A* (Ins[2R]^{k04204}) and *DJ-1B* (Ins[3R]^{16805}) genes have been previously generated and are predicted to function as either hypomorphic (reduced protein function) or null (complete loss of protein function) alleles. Similarly, chromosomal deficiencies that remove several genes including DJ-1A (Df[2R]^{CX1}) or DJ-1B (Df[3R]^{7917}) are null alleles for *DJ-1A* or *DJ-1B*, respectively. I generated *GMR-dRet*^{MEN2B} flies that carried these *loss-of-function* alleles and characterized their retinal organization and ultrastructure. I found remarkably, that the defects in eye and organization and size induced by overactive *Ret*^{MEN2B} were strongly suppressed in these backgrounds of reduced DJ-1A/B activity; the resulting eyes displayed an improved retinal organization and restored eye size (Figure 29C-F,G). Similarly, the abnormal increase in ommatidia size and abnormal ommatidial fusion induced by overactive *Ret*^{MEN2B} were significantly suppressed in a *DJ-1A* heterozygous background (*GMR-dRet*^{MEN2B}*/* Df(2R)^{CX1}; Figure 29H-K). Thus, the *GMR-dRet*^{MEN2B}-mediated eye phenotype is partially suppressed in backgrounds of reduced *DJ-1A/B* activity.

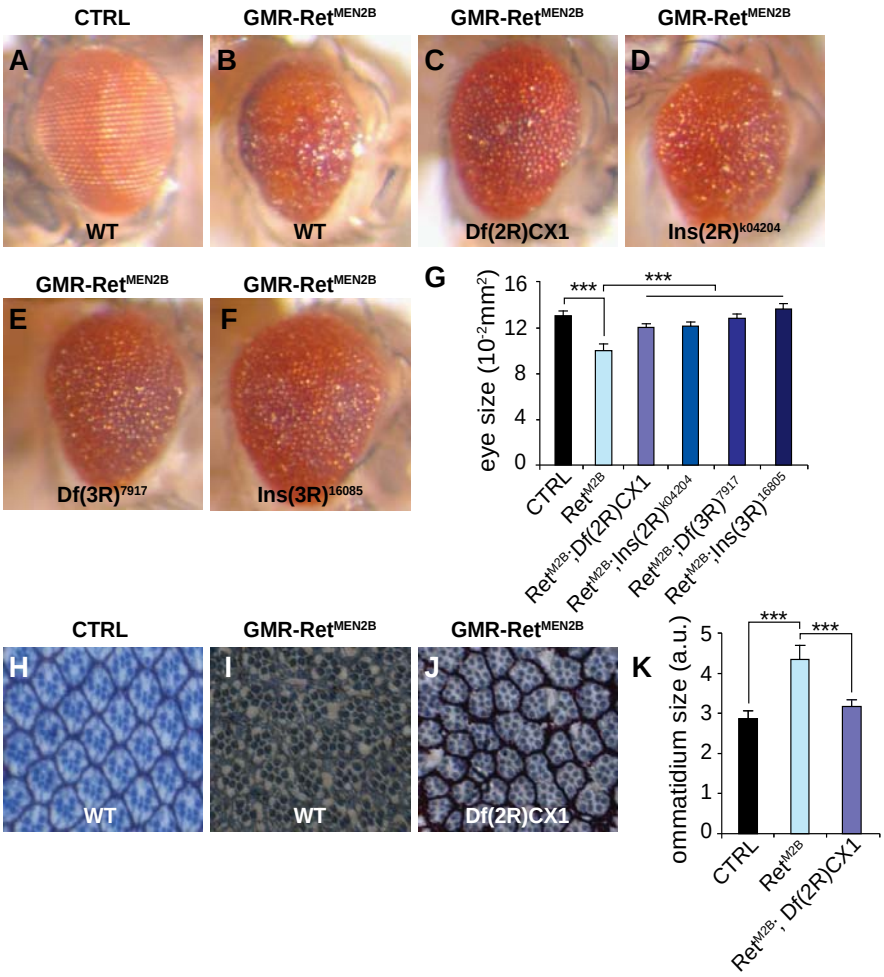


Figure 29. Active *Ret* interacts with independent *DJ-1A/B* loss-of-function alleles. (A-F) Overexpression of constitutively active Ret in the developing retina (*GMR-dRet*^{MEN2B}) leads to a rough eye phenotype (B) which can be rescued in backgrounds of reduced *DJ-1A* or *DJ-1B* activity (C-F); deficiencies that remove *DJ-1A* (C) or *DJ-1B* (E) genes or insertions in the *DJ-1A* (D) or *DJ-1B* (F) genes were used. (G) Quantification of eye sizes in the indicated mutant and control flies (n>15 eyes/group; *** p<0.001, Student's t-test). (H-J) Photomicrographs of ultrathin eye sections stained with toluidine blue showing enlarged ommatidia in *GMR-dRet*^{MEN2B} flies (I) as compared to controls (H) and near normal ommatidia in *GMR-dRet*^{MEN2B} flies carrying a *DJ-1A* deficiency (J). (K) Quantification of ommatidia sizes in the indicated mutant and control flies (n=4 eyes/genotype and n>150 ommatidia analyzed per animal; *** p<0.001, Student's t-test).

Taken together, these independent lines of evidence suggest that *DJ-1A/B* interact genetically with *Ret*^{MEN2} in the developing fly retina.

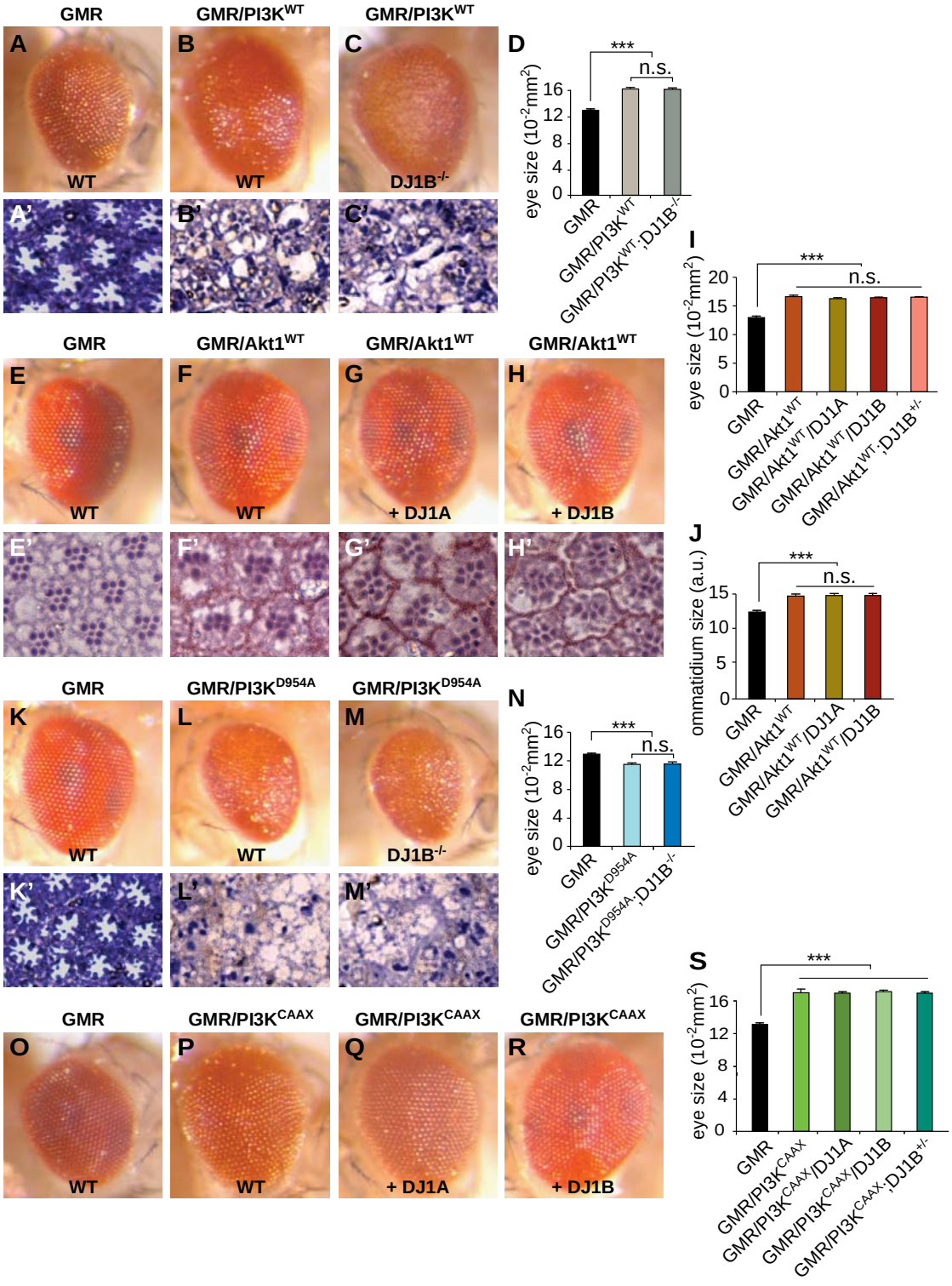
2.13. No interaction between PI3K/Akt signaling and *DJ-1A/B*

Activation of the Ret RTK leads to the activation of two major signaling pathways: PI3K/Akt and MAPK signaling. To understand whether the interaction between *Ret* and *DJ-1* involves these Ret-mediated signaling pathways, I investigated the capacity of fly *DJ-1A/B* to genetically modify the phenotypes associated with altered activity in the PI3K/Akt and MAPK signaling.

In the developing eye system of *Drosophila*, PI3K/Akt signaling controls photoreceptor patterning, proliferation, differentiation and cell size (Vanhaesebroeck et al., 1997). Consequently, upon strong overexpression of wild-type *PI3K* using the photoreceptor specific driver *GMR* (*GMR/PI3K^{WT}* at 30°C), I observed a 25% increase in eye size and a disorganized retina compared to controls (*GMR-Gal4*) (Figure 30A,B,A',B',D). This phenotype is likely due to excessive cell proliferation, increase in cell size and patterning defects. To test whether the absence of *DJ-1B* function can suppress this phenotype, I generated flies that overexpress *GMR*-driven *PI3K^{WT}* in a *DJ-1B$^{-/-}$* background (GMR/*PI3K^{WT}; DJ-1B$^{-/-}$*). The increase in eye size and retinal defects were not suppressed in these flies (Figure 30C,C',D), suggesting that *DJ-1B* activity does not play a role in the *PI3K^{WT}* overexpression phenotype.

I next tested whether overexpression of *DJ-1A* or *DJ-1B* is sufficient to enhance the phenotype of increased PI3K/Akt signaling. For this, I used the moderate eye phenotype induced by wild-type Akt1 overexpression (*GMR/Akt1^{WT}* at 25ºC), which causes a 25 % increase in eye size without creating other visible morphological defects (Figure 30E,F,I). At the histological level, *GMR/Akt1^{WT}* retinas display a 20% increase in ommatidia sizes, consistent with the role of PI3K/Akt signaling in cell size regulation (Leevers et al., 1996) (Figure 30E',F',J). When *DJ-1A* or *DJ-1B* were co-overexpressed with *Akt1^{WT}*, no additional increase in eye size was observed nor did the resulting eyes become disorganized (Figure 30G,H,I). Histologically, *GMR/Akt1^{WT}/DJ1A* and *GMR/Akt1^{WT}/DJ1B* ommatidia displayed the same size increase as those in *GMR/Akt1^{WT}* flies (Figure 30 G',H',J). Similar results were obtained in flies expressing a constitutively active version of PI3K (*PI3K^{CAAX}*) which is membrane-bound PI3K; the increase in eye size and mild rough eye phenotype (Figure 30O,P,S) were not further modulated after altering the levels of *DJ-1A* or *DJ-1B* (Figure 30Q,R,S). Thus, *DJ-1A/B* fails to interact with overactive PI3K/Akt signaling.

Figure 30. No interaction between *PI3K/Akt* and *DJ-1A/B* in the *Drosophila* eye. (A-C) Overexpression of *PI3K^{WT}* in photoreceptor neurons (*GMR-Gal4; UAS-PI3K^{WT}*) leads to a rough eye of increased size (B), as compared to control (A); in a *DJ-1B$^{-/-}$* background, this phenotype is not modified (C). Flies were raised at 30ºC to enhance the phenotype; similar results were obtained at 25ºC (data not shown). (D) Quantification of eye sizes in the indicated mutant and control flies (n>25 eyes/group; *** p<0.001, Student's t-test). (A'-C') Photomicrographs of ultrathin eye sections stained with toluidine blue showing the normal size and pattern of individual ommatidia of an adult control fly (A'), and the disorganized eyes of both GMR/*PI3K^{WT}* (B') and a GMR/*PI3K^{WT}; DJ-1B$^{-/-}$* fly (C'). (E-H) No enhancement of Akt1^{WT} overexpression (*GMR/Akt1^{WT}*) phenotype by *DJ-1A* or *DJ-1B* co-overexpression or in a heterozygous *DJ-1B* knockout background. The increased eye size compared to controls (E) after *Akt1^{WT}* overexpression (F) is not further modified after *DJ-1A* (G) or *DJ-1B* (H) overexpression or in a *DJ-1B* heterozygous background (I). Similarly, the increased ommatidial size induced by Akt1^{WT} overexpression (F', J) is not modified by *DJ-1A* (G',J) or *DJ-1B* (H',J) overexpression. (I,J) Quantifications of eye (n>25 animals) and ommatidia sizes (n=4 eyes/genotype and n>150 ommatidia analyzed per animal; *** p<0.001, Student's t-test). (K-M) No further enhancement of a dominant-negative PI3K overexpression phenotype in a *DJ-1B* null background. The decreased eye size after *PI3K^{D954A}* overexpression (at 30ºC; L, N) is not modified in a *DJ-1B* null background (M, N). Similarly, the retinal defects induced by *PI3K^{D954A}* (L) are not enhanced by *DJ-1B* loss-of-function (M). (N) Quantification of eye sizes (n>25 animals per group, *** p<0.001, Student's t-test). (O-R) *DJ-1A* and *DJ-1B* do not modulate the eye phenotype induced by overexpression of constitutive active *PI3K*. The increased eye size and eye roughness after *PI3K^{CAAX}* overexpression (P) are not modified after *DJ-1A* (Q, S) or *DJ-1B* (R, S) co-overexpression or in a *DJ-1B* heterozygous background (S). (S) Quantification of eye sizes (n>25 animals per group, *** p<0.001, Student's t-test).



I then considered the possibility that DJ-1 might interact with decreased levels of PI3K/Akt signaling. I used a dominant-negative version of the PI3K (*PI3K*^{D954A}) which carries a mutation in the catalytic domain leading to reduced kinase activity (Leevers et al., 1996). When *PI3K*^{D954A} is targeted to developing photoreceptors (*GMR/PI3K*^{D954A}), the development of the eye is severely impaired; although total eye size is only moderately reduced (10-20%; Figure 30K,L,N), many photoreceptor

neurons are lost, suggesting that apoptosis in the retina occurs relatively late during development (Figure 30K',L'). When overexpressing *PI3K*^{D954A} in a *DJ-1B* null background (*GMR/PI3K*^{D954A}*;DJ-1B*$^{-/-}$), no enhancement of retinal phenotype relative to *GMR/PI3K*^{D954A} flies was observed; the total eye size (Figure 30M,N) and retinal morphology (Figure 30M') remained similar. Therefore, absence of *DJ-1B* activity does not further exacerbate the loss of *PI3K* activity in the developing fly retina.

Taken together, these independent experiments results suggest that *DJ-1A/B* do not interact with the PI3K/Akt pathway in controlling eye development in *Drosophila*.

2.14. *DJ-1A/*B activity is sufficient to suppress the *PTEN*-mediated eye phenotype

A previous report suggested that Drosophila *DJ-1A* is both necessary and sufficient to interact with the tumor suppressor PTEN during the development of the retina and in mammalian cell culture (Kim et al., 2005a). Moreover, *DJ-1* also modulated the activation status of the Akt kinase *in vitro* (Gorner et al., 2007; Kim et al., 2005a). Our findings that *DJ-1A/B* do not interact with PI3K/Akt signaling are in apparent contrast to these published reports. To determine whether *DJ-1A/B* interact genetically with *PTEN* in the *Drosophila* eye system, I used the eye specific promoter *eyeless*, which induces transgene expression already in proliferating cells. As previously reported (Kim et al., 2005a), such a strong overexpression of *PTEN* (*ey/PTEN*) leads to an adult eye of severely reduced size (Figure 31A,B,K). At the histological level, the resulting ommatidia are smaller, consistent with the role of PTEN as negative regulator of the PI3K/Akt signaling (Figure 31A',B',L). Interestingly, this *PTEN*-mediated phenotype can be partially suppressed when *WT Ret*, *DJ-1A* or *DJ-1B* activity is increased (Figure 31C-E,K); the resulting ommatidia have a partially restored size (Figure 31A'-E',L). These results confirm the previous observations that *DJ-1A* is a novel *PTEN* regulator, and also identify *DJ-1B* as a potential *PTEN* interactor.

To determine whether *DJ-1A* or *DJ-1B* activity is required to suppress the *PTEN*-induced eye phenotype, I used flies in which *PTEN* was mildly overexpressed to allow testing for enhancement. For this, I used the *GMR* driver which is active in postmitotic cells only, in contrast to *eyeless* is activated in actively proliferating cells. In contrast to the more affected ey/PTEN flies, which display reduced cell proliferation and cell size, *GMR/PTEN* flies display only a (moderately) reduced cell and retina size (Figure 31F,G). Surprisingly, *GMR/PTEN* flies with reduced levels of *DJ-1A/B* do not display more severe eye phenotypes as compared to flies carrying *GMR/PTEN* in a wild-type background (Figure 31H,I). In addition, *GMR/PTEN* flies lacking completely *DJ-1B* function (*GMR/PTEN;DJ-1B*$^{-/-}$) were similarly not more affected than *GMR/PTEN* flies (Figure 31J).

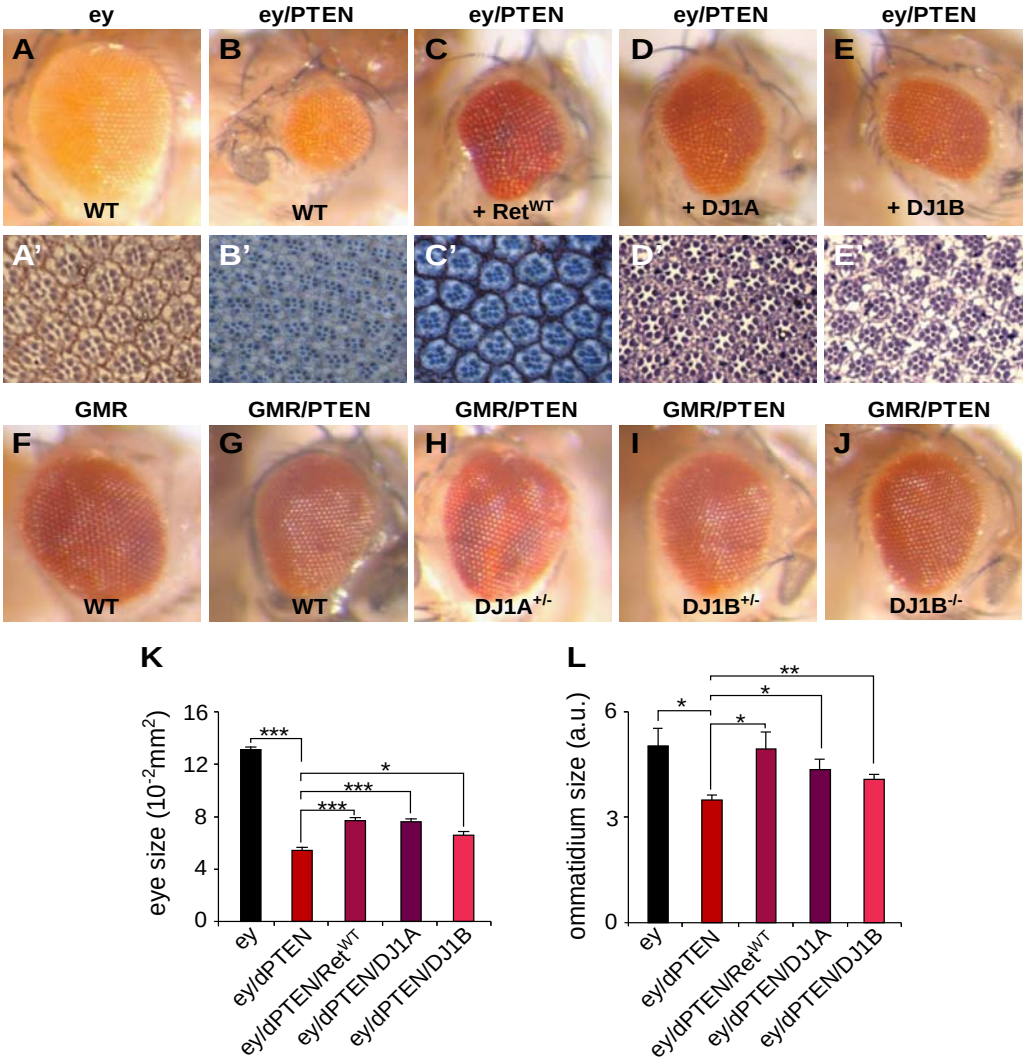


Figure 31. DJ-1 activity is sufficient to suppress the PTEN-mediated eye phenotype. (A-E) Images of the normal eye of an adult control fly (A), the smaller and rough eye of a fly overexpressing *PTEN* controlled by the eyeless promoter (B; *ey-Gal4; UAS-PTEN*), and rescued eyes of *ey-PTEN* flies that co-overexpress Ret^{WT} (C) *DJ-1A* (D) or *DJ-1B* (E). (A'-E') Photomicrographs of ultrathin eye sections stained with toluidine blue showing the normal size and pattern of individual ommatidia of an adult control fly (A'), the smaller ommatidia of an *ey-PTEN* fly (B'), and the rescue of ommatidial sizes in *ey-PTEN* flies that co-overexpress Ret^{WT} (C'), *DJ-1A* (D') or *DJ-1B* (E'). (K) Quantification of eye sizes in the indicated mutant and control flies (n>25 eyes per genotype; *** $p<0.001$, t-test). (L) Quantification of ommatidia sizes in the indicated mutant and control flies (n=4 eyes/group and n>150 ommatidia analyzed/eye; * $p<0.05$ and ** $p<0.01$, t-test). (F-J) Little effect of *DJ-1A/B* inactivation on the phenotype induced by PTEN overexpression. Images of an adult control eye (F), the slightly reduced eye size of a fly overexpressing *PTEN* controlled by the GMR promoter (Q; *GMR-Gal4; UAS-PTEN*), and eye of similar size in *GMR/PTEN* flies heterozygous knockout for *DJ-1A* (H) or *DJ-1B* (I) or homozygous knockout for *DJ-1B* (J).

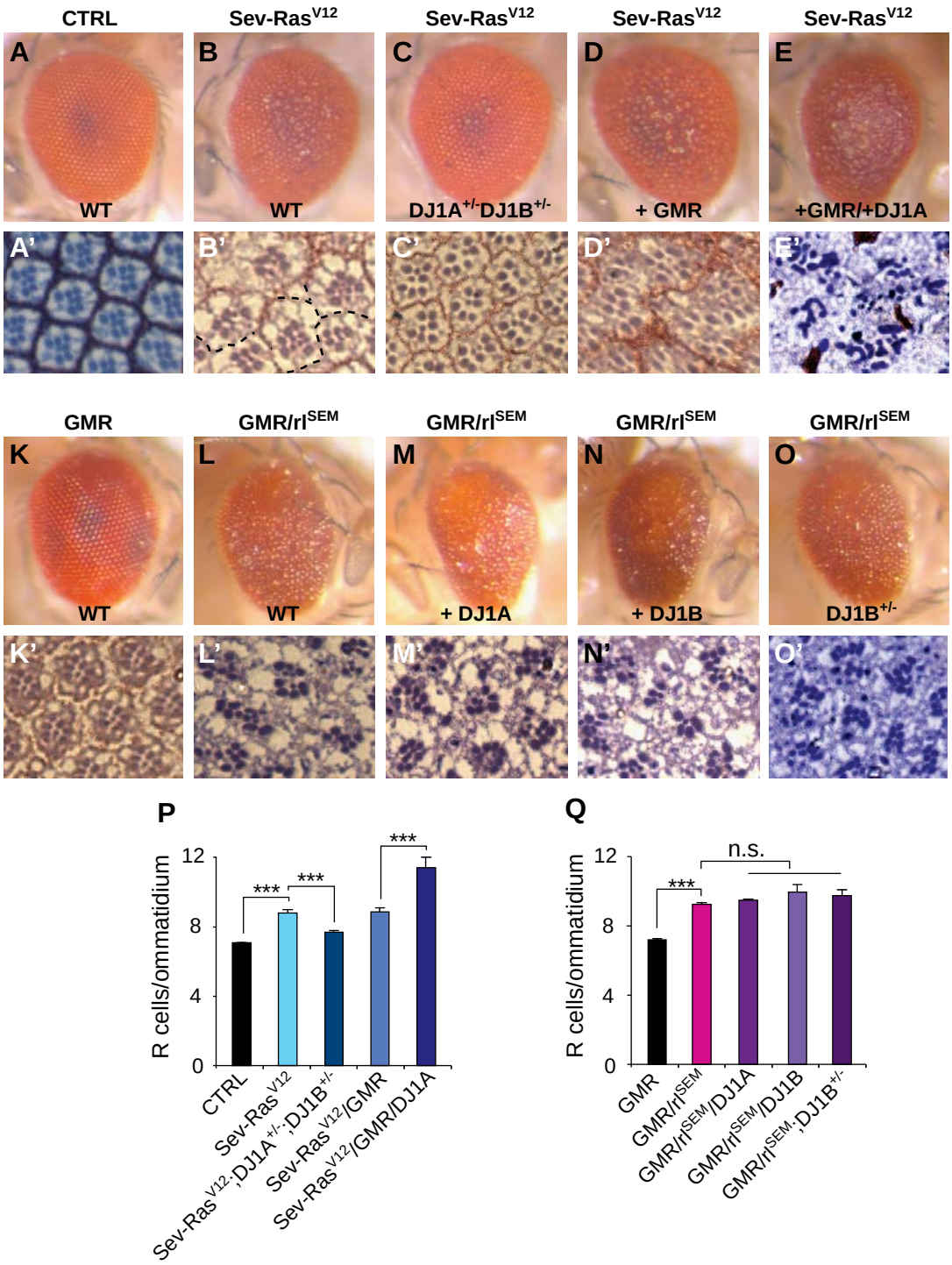
Thus, while *DJ-1* overexpression is sufficient to inhibit the effects of PTEN-overexpression, *DJ-1A/B* is not required to mediate this inhibiting effect. Therefore, DJ-1 might interact with PTEN only in defined situations (e.g. in oncogenic conditions), but in a PI3K-Akt independent manner (see Discussion).

2.15. *DJ-1* interacts genetically with overactive Ras/ERK signaling

Activation of Ras/MAPK signaling in *Drosophila* controls cell differentiation, proliferation and size (Friedman and Perrimon, 2006; Prober and Edgar, 2000). Flies with increased Ras/Raf/ERK activity display patterning and proliferation defects and exhibit an increase in the number of photoreceptors per ommatidium, while reducing the activity in this pathway leads to failure of non-neuronal cells to differentiate into neurons (Biggs et al., 1994). Moreover, a tight regulation of Ras/MAPK signaling is required during the embryonic development of numerous structures, including eye and wing (Friedman and Perrimon, 2006; Johnson Hamlet and Perkins, 2001).

To investigate whether DJ-1 interacts with the Ras/ERK pathway, I used constitutively active versions of *Ras* and the MAPK ERK/*rolled* (*rl*), which impair eye development by promoting excessive proliferation and altered cell differentiation (Brunner et al., 1994). Point mutations in the *Ras* oncogene are found in 30 % of all cancers. The most common mutation is the substitution of glycine 12 with valine (Ras^{G12V}, also called Ras^{V12}), that leads constitutive association with GTP; Ras is "locked" in this GTP-bound state and is therefore constitutively active (Dhillon et al., 2007). Ras/ERK signaling was found to be particularly important for the development of the seventh photoreceptor neuron (R7) contained in each ommatidium. Overexpression of consitutively active Ras^{V12} in R7 photoreceptor neurons driven by the R7-neuron specific promoter *sevenless* (that is fused to the coding sequence for Ras^{V12}; *Sev-*Ras^{V12}) is sufficient to induce abnormal differentiation of additional cells into R7 photoreceptors; as a result, multiple R7 photoreceptors are induced in each ommatidium (Karim et al., 1996). To test whether *DJ-1A/B* interacts genetically with activated *Ras,* I generated flies expressing *Sev-*Ras^{V12} in either a wild-type background or a background of reduced *DJ-1A/B* activity (Figure 32). As expected, flies expressing *Sev-*Ras^{V12} in a wild-type background developed with rough eyes (Figure 32B); histological examination of their retina revealed that the total number of photoreceptor neurons in each ommatidium increased to an average of 8,5 photoreceptors /ommatidium (P/O; Figure 32B',P).

Figure 32. *DJ-1A/B* interact genetically with Ras signaling in *Drosophila*. (A-E, P) *DJ-1A/B* interact genetically with constitutively active *Ras* in the fly retina. (A-E) eye images showing a normal control eye (A), the rough eye phenotype of flies carrying a *Sev-*Ras^{V12} construct (B) and the rescue of the rough eye in a *DJ-1A/DJ-1B* heterozygous knockout background (C). *GMR*-mediated *DJ-1A* overexpression further enhances the Ras-induced phenotype (*Sev-*Ras^{V12}*/GMR/DJ1A*, E) as compared to control (*Sev-*Ras^{V12}*/GMR*, D). (A'-E') Photomicrographs of ultrathin eye sections showing normal control ommatidia (A'), the *Sev-*Ras^{V12}-induced increase in photoreceptors and fused ommatidia (B', stippled lines indicate missing separations due to abnormal fusions of ommatidia) and the rescue after reducing *DJ-1A/B* function (C'). (E') Further increase of the number of photoreceptors/ommatidium and worsening of retinal ultrastructural defects after *DJ-1A* overexpression as compared to control (D'). (P) Quantification of the numbers of photoreceptors/ommatidium (n=4 eyes/group and n>150 ommatidia analyzed per eye; ** $p<0.01$ and *** $p<0.001$, t-test). (K-O,Q) No further modulation of constitutively active *rolled*-induced eye phenotype by *DJ-1A* or *DJ-1B*. (K-O) eye images showing a control eye (K), the rough eye phenotype induced by rl^{SEM} overexpression (L; *GMR-Gal4; UAS-*rl^{SEM}), and the lack of modulation after co-overexpression of *DJ-1A* (M) or *DJ-1B* (N) or after decreasing *DJ-1B* function (O). (K'-O') Photomicrographs of ultrathin eye sections showing normal control ommatidia (K'), the *GMR/*rl^{SEM}-mediated increase in photoreceptor number/ommatidium (L') and the lack of modulation after co-overexpression of *DJ-1A* (M'), *DJ-1B* (N') or after decreasing DJ-1B levels (O'). (Q) Quantification of the numbers of photoreceptors/ommatidium (n=4 eyes/group and n>150 ommatidia analyzed per eye; *** $p<0.001$, t-test).



When reducing the *DJ-1A/B* levels (*Sev-Ras^{V12};DJ-1A$^{+/-}$;DJ-1B$^{+/-}$*), this phenotype was significantly rescued (Figure 32C) the *Sev-Ras^{V12};DJ-1A$^{+/-}$;DJ-1B$^{+/-}$*flies displaying an improved retinal morphology while the number of P/O was restored (Figure 32C',P). In addition, while in *Sev-Ras^{V12}* flies 67 % of ommatidia were abnormally fused with their neighbors, only 5 % abnormally fused ommatidia were detected in the rescued *Sev-Ras^{V12};DJ-1A$^{+/-}$;DJ-1B$^{+/-}$* flies (Figure 32 C'and data not shown). Thus, *DJ-1A/B* activity is required for the full manifestation of the *Ras^{V12}*-induced eye phenotype.

To test whether *DJ-1* is sufficient to modulate the Ras^{V12}-mediated eye phenotype, I co-overexpressed Ras^{V12} in R7 neurons (*Sev-*Ras^{V12}) and *DJ-1A* in all photoreceptor neurons (using the *GMR* driver). While *Sev-*$Ras^{V12}$and *Sev-*Ras^{V12}*/GMR-Gal4* flies displayed the same moderate eye phenotype (Figure 32D,D',P), *Sev-*Ras^{V12}*/GMR/DJ-1A* eyes were severely affected and very rough (Figure 32E). At the histological level, *Sev-*Ras^{V12}*/GMR/DJ-1A* eyes displayed a strong increase in the number of P/O (11 on average) compared to *Sev-RasV12 and Sev-*Ras^{V12}*/GMR-Gal4* retinas (Figure 32E',P). Thus, *DJ-1* activity appears sufficient to modulate the Ras^{V12}-mediated eye development.

The use of a constitutively active version of *Ras* also allows us to determine whether the novel *Ras* interactor *DJ-1* functions upstream, downstream or in parallel to Ras in controlling photoreceptor neuron development. Constitutively active Ras^{V12} cannot be further regulated by any upstream interactor that normally regulates the wild-type Ras. In contrast, the phenotype mediated by Ras^{V12} could still be modulated by interactors acting downstream of Ras or in a parallel pathway. Since modulation of DJ-1A/B levels caused a modification of the Ras^{V12}-mediated eye phenotype, DJ-1A/B functions either downstream of Ras or in a parallel pathway that cooperates with Ras signaling to control *Drosophila* eye development.

I then assessed the modulation of constitutively active *rolled(Erk)* signaling by *DJ-1*. A substitution of a single conserved amino acid in the kinase domain of *rolled* (called *sevenmaker*) is sufficient to constitutively activate its Ser/Tyr kinase activity; the mutation was named *sevenmaker* (*SEM*) because its targetting to the developing retina leads to the formation of multiple R7 photoreceoptor neurons in each ommatidium (Brunner et al., 1994). I overexpressed rl^{SEM} in all photoreceptor neurons using the *GMR* driver. The resulting *GMR/*rl^{SEM} eyes were rough, slightly reduced in size and relatively disorganized compared to control GMR-Gal4 eyes (Figure 32L). When analyzing the retinal ultrastructure, I found an increase in the number of P/O (9,5 P/O on average; Figure 32Q). I then generated flies that co-overexpress rl^{SEM} and *DJ-1A* (*GMR/*rl^{SEM}*/DJ-1A*) or *DJ-1B* (*GMR/*rl^{SEM}*/DJ-1B*); I also generated flies overexpressing rl^{SEM} in a *DJ-1B* heterozygous knockout background (*GMR/*rl^{SEM}*;DJ-*$1B^{+/-}$). The rough eye phenotype induced by rl^{SEM} overexpression was not further enhanced after *DJ-1A/B* overexpression (Figure 32M,N) nor did a decrease in DJ-1B activity exert a suppressing effect on the rl^{SEM}-mediated phenotype (Figure 32O); at the histological level, the increase in the number of P/O caused by rl^{SEM} overexpression was not further modulated after modifying the levels of DJ-1A/B (Figure 32M'-O',Q). Because *DJ-1A/B* activity failed to further modulate the constitutively active *rolled* signaling, *DJ-1A/B* function either upstream of Rolled/ERK or in parallel to the Rolled/ERK signaling pathway to control cell differentiation and proliferation in the retina.

Taken together, these results indicate that *DJ-1A/B* interacts with Ras-mediated signaling and *DJ-1A/B* function either between Ras and ERK or in parallel to Ras/ERK signaling to control eye development induced by overactive Ras/ERK signaling.

2.16. *DJ-1* and *ERK* interaction controls *Drosophila* eye and wing development

The interaction between endogenous DJ-1A/B and the ectopically expressed overactive Ras/ERK prompted us to investigate whether DJ-1A/B and the endogenous Ras/ERK interact during the normal development of the fly. To test whether DJ-1A/B interacts with endogenous ERK signaling in *Drosophila*, I performed double *loss-of-function* experiments. I investigated this potential interaction in two places where *ERK/Rolled* is known to play a crucial role during development: the development of photoreceptor neurons and wing venation (Friedman and Perrimon, 2006).

I used a *rolled*(*rl*)/ERK hypomorphic allele (rl^1) that leads to a moderate loss of rl/ERK activity. Flies carrying two rl^1 alleles (rl^1/rl^1) are viable and display moderate rl-associated phenotypes. Consistent with a role for *rl/ERK* in proper photoreceptor neuron development, rl^1/rl^1 eyes were slightly smaller and displayed a mild reduction in the number of P/O (6.64; Figure 33C,C',I,J,K). Remarkably, while control and *DJ-1B*$^{-/-}$ flies had a normal appearance and a normal complement of 7 P/O (Figure 33A,A',B,B',I,J,K), eyes with reduced *rl/ERK* function and lacking *DJ-1B* (rl^1/rl^1;*DJ-1B*$^{-/-}$) flies were significantly smaller, rough and displayed on average only 5.34 P/O (Figure 33D,D',I,J,K; $p<0.05$ CTRL vs. rl^1/rl^1; $p<0.001$ rl^1/rl^1 *vs.* rl^1/rl^1;*DJ-1B*$^{-/-}$, Student's t-test). In addition, while a fraction of rl^1/rl^1 ommatidia contained 6 P/O, many ommatidia in rl^1/rl^1;*DJ-1B*$^{-/-}$ flies have 5, 4 or 3 P/O (Figure 33J). This suggests that *DJ-1B* is required as a *rolled* interactor to control photoreceptor neuron development.

I then investigated the development of the wing and found that rl^1/rl^1 flies had a very mild defect in wing venation, the vein L4 being sometimes thinner (in about 20 % of the animals; Figure 33F,F',L) (Brunner et al., 1994). While in control and *DJ-1B*$^{-/-}$ flies the L4 vein developed normally, in wings with reduced *rl/ERK* activity and lacking *DJ-1B* (rl^1/rl^1;*DJ-1B*$^{-/-}$) the thinning of the L4 vein was either short (in 33 % of animals), long (37 % of all cases) or the L4 vein was interrupted (in 25 % of animals; Figure 33G,G',H,H',L). Such an enhanced phenotype was previously seen in flies with a very strong reduction in rl/ERK function – these flies carried a combination of rl^1 and the stronger allele rl^{10} (a deficiency; Brunner et al., 1994). *DJ-1B* is thus required, as a *rolled* interactor, to control the development of the wing.

These results uncovered, for the first time, a role for *DJ-1B* in the unchallenged fly and suggest that DJ-1B cooperates with Ras/MAPK signaling during photoreceptor neuron and wing imaginal disc development.

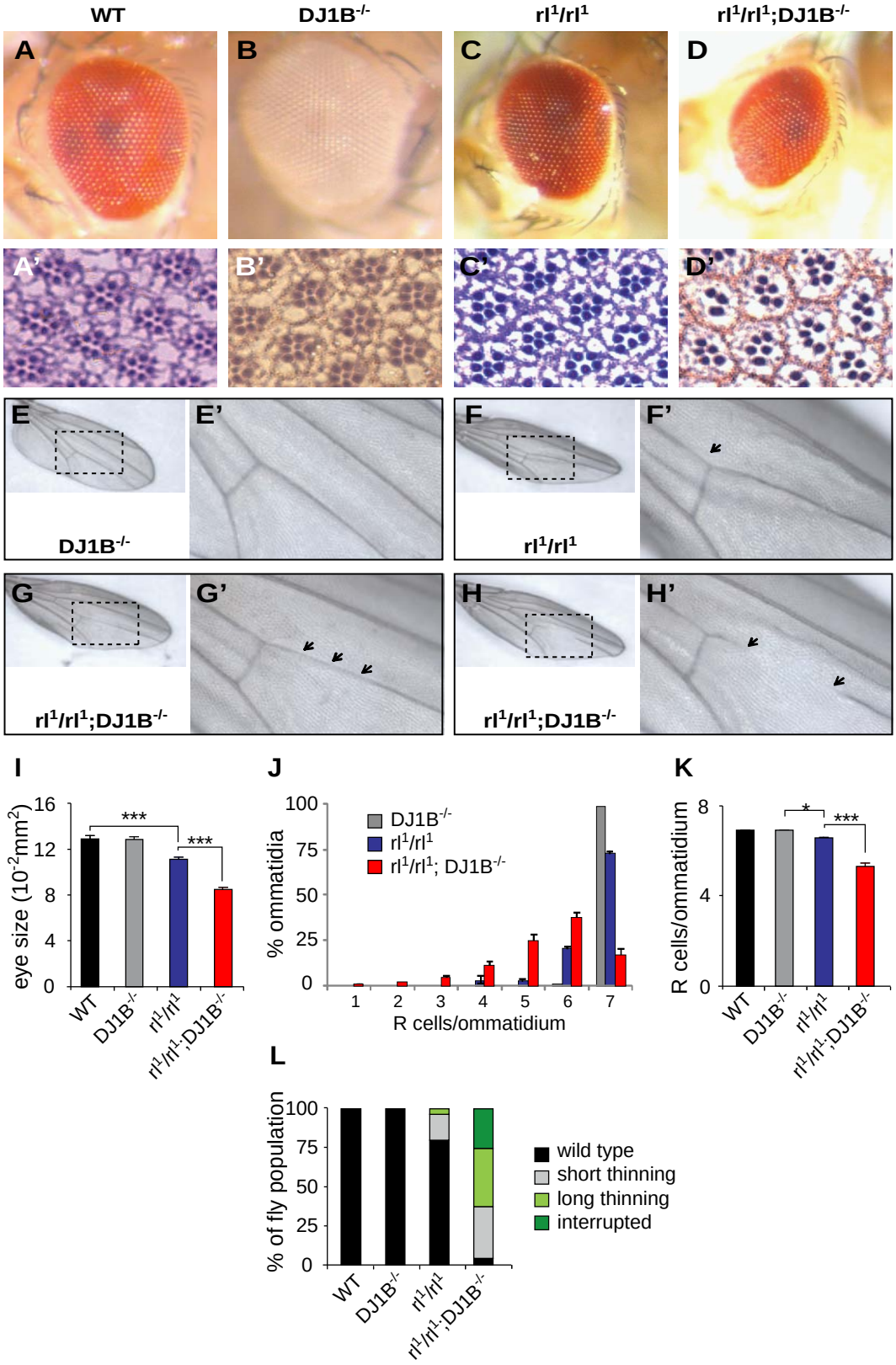

WT
DJ1B-/-
rl1/rl1
rl1/rl1;DJ1B-/-
A
B
C
D
A'
B'
C'
D'
E
E'
DJ1B-/-
F
F'
rl1/rl1
G
G'
rl1/rl1;DJ1B-/-
H
H'
rl1/rl1;DJ1B-/-
I
eye size (10-2mm2)
16
12
8
4
0
***
***
WT
DJ1B-/-
rl1/rl1
rl1/rl1;DJ1B-/-
J
% ommatidia
100
75
50
25
0
DJ1B-/-
rl1/rl1
rl1/rl1; DJ1B-/-
1
2
3
4
5
6
7
R cells/ommatidium
K
R cells/ommatidium
8
4
0
*
***
WT
DJ1B-/-
rl1/rl1
rl1/rl1;DJ1B-/-
L
% of fly population
100
75
50
25
0
wild type
short thinning
long thinning
interrupted
WT
DJ1B-/-
rl1/rl1
rl1/rl1;DJ1B-/-

Figure 33. *DJ-1B* cooperates with endogenous *Ras/ERK* signaling to control eye and wing development. (A-D,I-K) Interaction between *DJ-1B* and *rolled/ERK* controls photoreceptor neuron development. (A-D) Images of normal eyes of control (A) and *DJ-1B*$^{-/-}$ (B) flies, of a smaller *rl*^{1}/*rl*^{1} eye (C; *rl*^{1} is a hypomorphic *rolled* allele) and a very small and rough eye in a fly with reduced *rl*^{1} activity and completely lacking DJ-1B function (*rl*^{1}/*rl*^{1}; *DJ1B*$^{-/-}$; D). (I) Quantification of eye sizes (n>50 eyes per group, *** p<0.001 t-test). (A'-D') Photomicrographs of ultrathin eye sections showing the normal retina of control (E) and *DJ-1B*$^{-/-}$ flies (F), the mild reduction in photoreceptors/ommatidium (P/O) in the *rl*^{1}/*rl*^{1} retina (G) and the stronger reduction seen in the *rl*^{1}/*rl*^{1}; *DJ1B*$^{-/-}$ retina (H). (J) Histogram showing the distribution of photoreceptor neurons/ommatidium in *DJ-1B*$^{-/-}$, *rl*^{1}/*rl*^{1} and *rl*^{1}/*rl*^{1};*DJ-1B*$^{-/-}$ flies. All ommatidia in *DJ-1B*$^{-/-}$ flies carry 7 photoreceptor neurons in one plane (identical to wild-type control flies; not shown). While some *rl*^{1}/*rl*^{1} ommatidia have 6 P/O, many *rl*^{1}/*rl*^{1};*DJ-1B*$^{-/-}$ ommatidia develop with less than 6 P/O. (K) Quantification of the average number of photoreceptors/ommatidium (n=4 eyes/group and n>150 ommatidia analyzed per eye, * p<0.05 and *** p<0.001, t-test). (E-H,L) Interaction between *DJ-1B* and *rolled/ERK* controls wing vein development. (E-H) images of full-size wings and (E'-H') magnifications showing the central portion of the L4 vein (arrows). *DJ-1B*$^{-/-}$ flies develop with normal wings (E,E') while in *rl*^{1}/*rl*^{1} flies the L4 vein has short stretches of thinner diameter (F, F'). In contrast, *rl*^{1}/*rl*^{1} *DJ1B*$^{-/-}$ flies often develop with the L4 vein having long stretches of thinner diameter (G,G') or with an interrupted L4 vein (H,H'). (L) Quantification of the L4 vein phenotype categorized as wild-type, with short stretches of thinner diameter, with long stretches of thinner diameter, or interrupted. The observed frequencies are shown for the different genotypes (n>115 wings analyzed per animal).

2.17. *DJ-1* controls oxidative stress response in *Drosophila*

Oxidative stress was proposed to one of the major pathological events that lead to dopaminergic neurodegeneration (Abou-Sleiman et al., 2006). The control of oxidative stress might play a pivotal role in therapies for PD. Flies lacking *DJ-1B* were previously shown to be deficient in control of oxidative stress response. When challenged with toxins associated with PD (paraquat, rotenone) and with other oxidative stress inducers (H_2O_2), *DJ-1B* mutants were significantly more sensitive and displayed an enhanced lethality ; they were not, however, more sensitive to any toxic stress, as these mutants were not more sensitive to other toxins like DTT (reducing agent and protein denaturant) or MG132 (proteasome inhibitor). This lethality was rescued by overexpression of either Drosophila *DJ-1B* or *DJ-1A* genes (Menzies et al., 2005; Meulener et al., 2005).

I administered either paraquat or H_2O_2 to WT and *DJ-1A* or *DJ-1B* single and DJ-1A/DJ-1B double knockout flies and tested their long-term survival. Absence of *DJ-1B* function (in *DJ-1B* single and *DJ-1A/DJ-1B* double KO) rendered the flies more sensitive to these toxins, consistent with previous observations (Figure 34A). In contrast, flies lacking *DJ-1A* function were only mildly affected. These results suggest that *DJ-1B* controls the oxidative stress response to PD-associated toxins. My preliminary results also suggest that overexpression of human *DJ-1* is able to partially rescue the increased sensitivity due to loss of fly DJ-1B, suggesting that the anti-oxidant activity of *DJ-1* is evolutionary conserved (data not shown). I am now determining whether flies with reduced *rl* and *DJ-1B* function show increased toxin-sensitivity compared to *rl*^{1} and *DJ-1B* deficient flies and whether activation of rl signaling (using a *rl*^{SEM} allele that is expressed in all cells) can complement the toxin-sensitivity of *DJ-1B* null flies.

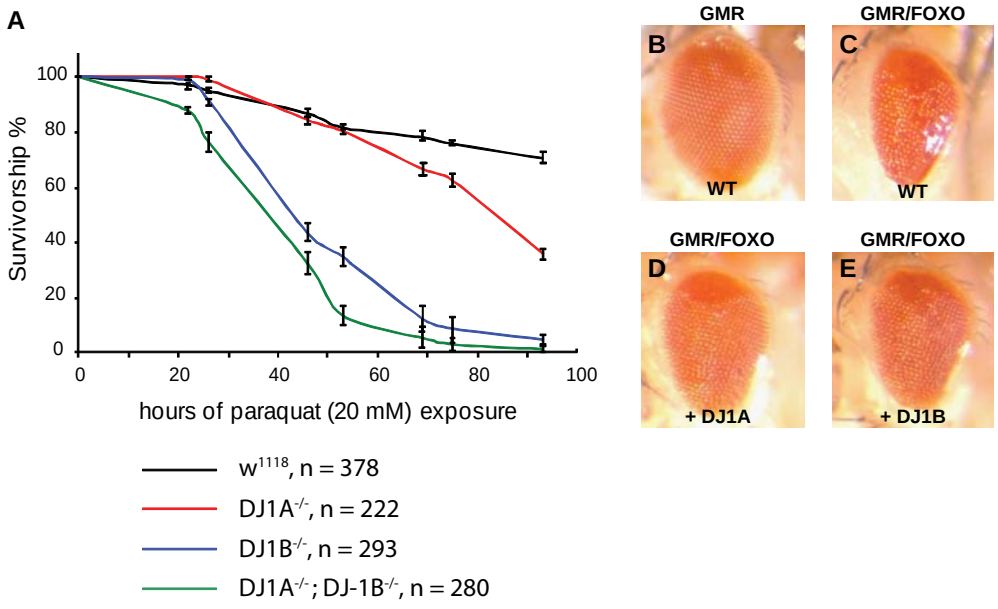


Figure 34. *DJ-1B* controls oxidative stress response and interacts with *FOXO* in *Drosophila*. (A) Survival of wild-type (black line), *DJ-1A*$^{-/-}$ (red line), *DJ-1B*$^{-/-}$ (blue line) and *DJ-1A*$^{-/-}$*; DJ-1B*$^{-/-}$ double mutant (green line) adult flies exposed to 20 mM paraquat. Flies lacking *DJ-1B* function show an enhanced sensitivity to paraquat, as compared to controls, while lack of *DJ-1A* function only partially affects fly survival. (B-E) *DJ-1A/B* interact genetically with *FOXO* in the *Drosophila* eye system. Overexpression of *FOXO* in photoreceptor neurons (*GMR-Gal4; UAS-FOXO*) leads to a smaller, rough eye (C) compared to control (B). Co-overexpression of *DJ-1A* (D) or *DJ-1B* (E) partially rescues the *GMR/FOXO* eye phenotype, the resulting rescued eyes displaying an improved overall organization.

A major regulator of oxidative stress response, longevity and apoptosis is the Forkhead family member FOXO; *FOXO* is required to protect against oxidative stress, while increased levels of FOXO during chronic stress can induce apoptosis (Giannakou et al., 2004; Junger et al., 2003). Remarkably, the extension of longevity mediated by Sirtuins (Sir2) in several organisms involve the deacetylation of FOXO, which switches the function of FOXO from pro-apoptotic to pro-survival (Giannakou and Partridge, 2004). I tested whether *DJ-1* can interact with FOXO signaling in the developing retina. In this system, overexpression of *FOXO* induces excessive apoptosis and generates a rough eye of reduced eye size (Figure 34C). Remarkably, when *DJ-1A* or *DJ-1B* are co-overexpressed with *FOXO* in developing photoreceptor neurons (using the *GMR* driver), the FOXO-induced phenotype is partially suppressed, the resulting retina displaying less irregularities (Figure 34D,E). These initial results suggest that *DJ-1* interacts genetically with the transcription factor FOXO, and raise the possibility that DJ-1 might control stress response by modulating this master transcriptional regulator.

III. DISCUSSION

The mechanisms underlying the selective death of SN neurons in PD are incompletely understood. Although several symptomatic therapies exist, none of them is able to halt PD progression, suggesting that a better understanding of the molecular mechanisms regulating maintenance and degeneration of SN neurons is needed in order to successfully fight against PD. Although neurotrophic factors like GDNF and BDNF are considered to be rescuing agents for dysfunctional SN neurons, the results from clinical trials where they were administered to PD patients are conflicting. To understand the physiological relevance of GDNF and BDNF signaling for the aging DA system, we deleted their respective signaling receptors *Ret* and *TrkB* in dopaminergic neurons specifically.

The present work identifies the GDNF signaling receptor Ret as a critical regulator of SN long-term survival. Mice lacking *Ret*, but not the *TrkB* receptor, show progressive degeneration of SN neurons and their axons, postsynaptic dysfunction in the SN target area, the striatum, and enhanced neuroinflammation.

The identification of genes causing familial PD has raised hopes for a better understanding of the molecular pathology underlying nigral degeneration. These initial expectations were tempered by the lack of SN degeneration in mice genetically engineered to carry PD-causing mutations. This raises the possibility that several independent pathological defects of reduced severity add up to cause SN cell death. To unravel additional pro-survival regulators of reduced power in the aging DA system, we analyzed the survival requirements of trophically deprived DA neurons.

Remarkably, we found that the PD-associated gene *DJ-1*, although dispensable for survival of wild-type SN neurons, is required for survival of Ret-deprived DA neurons during aging. *Ret* and *DJ-1* double knockout mice show an enhanced loss of SN neurons, specifically in the GIRK2 subpopulation, which projects exclusively to the striatum and is specifically affected in PD patients. Thus, the triple interaction between aging, trophic insufficiency and cellular stress due to loss of *DJ-1* function causes degeneration of 51 % SN neurons that innervate the striatum. Using *Drosophila* genetics, we found that *Ret* and *DJ-1* interact genetically to control eye development; moreover, *DJ-1* interacts with Ras/ERK, but not PI3K/Akt pathway to promote photoreceptor neuron and wing development.

Taken together, these results indicate that aging SN DA neurons rely on a pro-survival network that includes *Ret* and *DJ-1*. Therefore, mice lacking *Ret* and *DJ-1* could serve as models to further investigate the pathological mechanisms underlying PD and associated disorders. Moreover, we suggest that activation of Ret signaling (including for example, the striatal delivery of GDNF) might be employed as a therapeutic strategy to mitigate SN neurodegeneration in PD patients carrying *DJ-1* mutations.

3.1. *Ret* and *TrkB* are dispensable for the establishment of the nigrostriatal system

Neurotrophic factors are potent mediators of neuronal development. The development of DA neurons is initiated at the embryonic day E7.5 when the first progenitors engage in the differentiation process to eventually become dopaminergic. After birth, DA neurons undergo two rounds of programmed cell death (at postnatal days 2 and 14) which are thought to eliminate an important fraction of DA neurons. This situation is seen *in vivo* for the majority of developing neurons and it is estimated that 20-80 % of neurons undergo cell death shortly after their axons reach their targets (Davies, 2003; Oppenheim, 1991). During this postnatal period, DA neurons might require trophic support for survival.

GDNF was initially identified as a potent survival factor for cultured embryonic midbrain dopaminergic neurons (Lin et al., 1993). Similarly, BDNF was found to promote survival of cultured embryonic DA neurons (Hyman et al., 1991). Our finding that Ret and TrkB signaling are dispensable for the initial maintenance of the DA system (Figures 18 and 23; see also Kramer et al., 2007) was unexpected and suggests several possibilities. First, the removal of both *Ret* and *TrkB* function was defined by the activity of the Nestin or DAT promoters (which induced the expression of the Cre recombinase); the Nestin promoter induces Cre expression after E10.5 (Kramer et al., 2006; Tronche et al., 1999) while the DAT promoter is activated at E15 (Zhuang et al., 2005). Although unlikely, it remains possible that the initial expression of *Ret* or *TrkB* before onset of Nestin or DAT activity was sufficient to provide trophic support for the developing embryonic DA neurons. A second, more plausible explanation is that Ret and TrkB signaling are indeed dispensable for survival of embryonic DA neurons, and that GDNF-Ret independent and BDNF-TrkB independent signaling might promote survival of DA neurons; the analysis of DA neuron development in *NCAM* and *p75NTR* deficient mice might clarify this possibility. A third possibility is that embryonic neurons deprived of GDNF or BDNF trophic support gain access to alternative trophic pathways; because *DAT-Ret/TrkB* mice also show a normal development, we can rule out that *Ret*-deficient DA neurons use TrkB signaling for survival, and vice versa. To test if other survival pathways are upregulated in *DAT-Ret* and *DAT-TrkB* knockout mice, the activation status of alternative survival pathways could be evaluated.

3.2. *TrkB* is dispensable for long-term maintenance of the nigrostriatal system

Previous studies suggested that BDNF/TrkB might be required for survival of SN neurons *in vivo*, starting from P15 (Baquet et al., 2005; von Bohlen und Halbach et al., 2005). Our finding that removal of TrkB signaling did not affect the survival of aging SN neurons (Figure 18 and Kramer et al., 2007) was rather unexpected. The efficiency of Cre-mediated recombination in *DAT-TrkB* mice was estimated by Edgar Kramer to be at least 60 % (using single-cell PCR analysis; Kramer et al., 2007). It is therefore possible that some DA neurons still have access to TrkB-mediated trophic support during aging. Another source of differences between the previous studies and ours relates to the regional specificity of *BDNF* or *TrkB* inactivation. Thus, while previous studies deleted *TrkB* or *BDNF* expression in either all cells (von Bohlen und Halbach et al., 2005) or in the entire midbrain-hindbrain region (Baquet et al., 2005),

we deleted *TrkB* specifically in DA neurons. Therefore, the *TrkB* activity in SN neurons might indeed be dispensable for cell survival, in which case the effects observed in mice with decreased TrkB levels in all cells must be non cell-autonomous, meaning that they are not caused by removal of *TrkB* function from DA neurons. Loss of *TrkB* in non-DA neurons might have indirectly impacted on the long-term survival of SN DA neurons in these mice. Similarly, mice lacking BDNF in the midbrain-hindbrain region might have suffered other non-DA alterations that exerted an indirect effect on SN neuron function and survival. Additional studies, in which *TrkB* is deleted using independent *TrkB* floxed alleles and DA-specific Cre lines will address this possibility. Moreover, the analysis of mice in which *TrkB* is deleted in non-DA territories will reveal whether aging DA neurons engage in specific cell-cell interactions that promote their function and survival during aging.

3.3. Ret signaling mediates survival of a fraction of aging *substantia nigra* neurons

In contrast to TrkB, we found that Ret signaling is crucial for long-term maintenance of SN axons and cell bodies (Figures 17, 18, 23, 24). We observed that aging mice lacking Ret signaling in DA neurons display a marked loss of DA axons innervating the striatum (up to 60 % in 24-month-old mice), while the loss of SN cell bodies was relatively moderate (about 30 % in 24-month-old mice). This suggests that SN DA axons are more dependent on Ret signaling than the SN cell bodies. Indeed, mounting evidence suggests that neurotrophic factors exert different actions at nerve terminals and in the cell body (Zweifel et al., 2005). Although the exact temporal, spatial and molecular dynamics for GDNF/Ret signaling in DA neurons is unknown, results from studies on neurotrophin signaling showed the existence of local and long-distance signaling (Ibanez, 2007). While signaling at the axon terminal is more transient in nature and controls exocytosis or cytoskeleton rearrangement, signaling in the cell body is more sustained and mediates neuronal survival (Reichardt, 2006).

Three possibilities might explain the increased vulnerability of axons relative to cell somata following loss of *Ret* function. First, both DA axon terminals and somata require Ret signaling for maintenance, although DA terminals depend more on Ret signaling than DA cell bodies. In this scenario, Ret signaling at DA terminals might control processes crucial for terminal maintenance, while its functions in the soma are less critical for survival. Second, Ret signaling might only be required for survival of DA cell bodies, but not axons. Loss of about 30 % DA cell bodies should lead to a corresponding decrease in terminal density (30 %), unless a fraction of DA neurons have exceedingly large terminal arbours compared to the remaining neurons. However, since single-cell labeling of individual DA neurons revealed a relative uniformity of DA neuron terminal fields (Finkelstein et al., 2000), we find this possibility unlikely. A third more likely explanation is that the axonal compartment is the first affected by the degenerative process, followed retrogradely by SN cell bodies (i.e. ‘dying back’ process). This situation was also observed in post-mortem histological analysis of PD-brains (Bernheimer et al., 1973; Lach et al., 1992). At the onset of PD symptoms, about 50-60 % SN cell bodies are lost while the loss of dopamine in the striatum exceeds 80 % (Dauer and Przedborski., 2003). Experimental evidence for the ‘dying back’ model also comes from studies on MPTP-treated monkeys in which loss of striatal terminal precedes the loss of SN cell bodies (Herkenham et al., 1991) while protection of axon terminals prevents loss of SN

somata in MPTP-treated rats (Wu et al., 2003). To test whether the axon terminal compartment is indeed the first affected by the degenerative insult, the time-course analysis on *DAT-Ret* and control mice could be extended to include additional time points. Thus, while the loss of DA fibers starts at 9 months (Figure 18), no information about the first degeneration signs in the soma compartment is available, the first measurement being performed at 12 months (Kramer et al., 2007). Furthermore, there is compelling evidence that loss of axon terminals in neurodegenerative disease is preceded by micro-structural deficits like synaptic loss (Conforti et al., 2007). Therefore, it would be interesting to determine whether synaptic loss precedes the relatively delayed loss of axons in *DAT-Ret* mice. Finally, retrograde tracings might be used to determine whether some DA cell bodies in *DAT-Ret* mice are deprived of terminal arbor, before the onset of SN soma degeneration.

If GDNF is a survival factor for aging DA neurons, why is there only partial neurodegeneration in *DAT-Ret* mice? The loss of a fraction of SN DA neurons in aging *DAT-Ret* mice suggests that additional survival factors are active in aging DA neurons. A recent study found that chronic removal of GDNF function from the brain (at 2 months of age, using tamoxifen-induced Cre-mediated recombination of a floxed *GDNF* allele in all cells) leads to a dramatic degeneration of SN neurons (60 % neurons lost after 5 months); in addition, the neighbouring VTA was also affected (70 % loss after 5 months) and the noradrenergic *locus coeruleus* was virtually absent in GDNF knockout mice (Pascual et al., 2008). These results suggest that *GDNF* has a more important role in regulating DA neuron survival than *Ret*. One possibility is that GDNF controls DA neuron survival via a Ret-independent signaling pathway. The alternative receptor for GDNF, NCAM, is a good candidate for such a pro-survival effect. NCAM is expressed in adult DA neurons and interestingly, application of anti-NCAM blocking antibodies to cultured DA neurons strongly decreased the neurite outgrowth promoting effects of GDNF while delivery of NCAM blocking antibodies into the SN of rats inhibited the effects of GDNF on locomotor activity (Chao et al., 2003). A second possibility is that GDNF might use a yet non-identified receptor to mediate survival of aging SN neurons, and the existence of additional GDNF receptor(s) was suggested (Pozas and Ibanez, 2005). A third possibility that explains the mild DA phenotype in *DAT-Ret* vs. adult GDNF KO mice is the existence of trophic compensation in Ret-deficient SN neurons, via up-regulation of alternative trophic pathways. CDNF was identified as a novel putative neurotrophic factor for DA neurons and was shown to completely rescue the effects of 6-hydroxydopamine when injected in the mouse striatum (Lindholm et al., 2007). Therefore, the analysis of aging *NCAM* deficient mice and investigation of the activation status of alternative neurotrophic factor pathways in *DAT-Ret* mice should better define the role of GDNF signaling in the dopaminergic system.

DAT-Ret mice display a normal complement of SN neurons at 3 months (Figure 23 and Kramer et al., 2007) while degeneration in the SN starts during aging (12-24 months; Figures 18 and 23). Why do aging, but not adult DA neurons require Ret-mediated signaling for survival?

While during embryonic development neurons are ‘competent to die’ and require neurotrophic input to survive, it appears that adult neurons switch to become ‘competent to live’ and require active pro-apoptotic processes to undergo apoptosis (Benn and Woolf, 2004). Factors that might actively induce apoptosis in mature

neurons are for example ROS and other kinds of cellular stress, as those seen in neurodegenerative disease. Therefore, it remains possible that adult SN neurons do not require trophic signaling (or at least GDNF/Ret or BDNF/TrkB) for survival.

Aging SN neurons, in contrast to adult neurons, might re-become vulnerable to a series of age-related changes and might re-activate trophic signaling pathways to actively promote neuronal survival. It is possible that aging SN neurons start using several survival pathways, which would explain the moderate DA neurodegeneration in our aging *DAT-Ret* mice. Hence, a combination of age-related adaptive changes and trophic support might be necessary for maintenance of SN neurons. In this scenario, age-related deleterious sub-threshold changes that do not directly impact on cell survival might become critically amplified in the absence of neurotrophic support and might lead to neurodegeneration. Extensive research into the mechanisms of aging identified several classes of proteins that regulate longevity, including the Sirtuins, FOXO and p53 transcription factors, heat shock proteins or components of the insulin signaling pathway (Curtis et al., 2005; Vijg and Campisi, 2008). These pathways promote longevity by adapting the cell to different kinds of cellular stressors, including ROS, genotoxic stress and other consequences of cellular senescence. It is interesting to note that some of these pathways regulating aging are also regulated by RTKs, raising the possibility that RTK activity might regulate some aspects of the aging process in neurons. It remains therefore possible that removal of trophic factors generates additional cellular stress in DA neurons or deprives the cell of some critical anti-aging signals. A better understanding of the molecular pathways that regulate organism and tissue-aging and the connection between aging regulators and RTK signaling might uncover common regulators that could be employed as therapeutic targets for age-related diseases caused by decreased trophic signaling.

3.4. *DJ-1* promotes survival of trophically impaired *substantia nigra* neurons

To extend our understanding of the survival networks used by midbrain DA neurons, we searched for additional interactors of Ret in the process of DA neuron maintenance. We focused our attention on the Parkinson's disease-associated gene *DJ-1*. Loss of *DJ-1* in humans is sufficient to cause PD (Bonifati et al., 2003), while mice lacking *DJ-1* show no overt neurodegeneration in the SN (Gasser, 2009), suggesting additional compensatory mechanisms in *DJ-1* mice; in addition, experiments in mammalian cell cultures and *Drosophila* suggested that DJ-1 interacts with RTK-related pathways, like the PI3K/Akt (Gorner et al., 2007; Kim et al., 2005a; Yang et al., 2005b). We therefore generated mice lacking *Ret* in DA neurons and *DJ-1* in all cells (*DAT-Ret/DJ-1* mice). While these animals developed normally and showed no overt behavioral alterations, we found surprisingly that combined loss of *Ret* and *DJ-1* leads to enhanced loss of midbrain DA neurons as compared to mice lacking Ret alone. About 40 % SN cell bodies were lost in 24-month-old *Ret/DJ-1* mice, compared to only 25 % in mice lacking Ret function (Figure 23). The interaction between *Ret* and *DJ-1* is restricted to SN neurons (the VTA neurons are not affected) and is age-dependent, as adult (3-month-old) *DAT-Ret/DJ-1* mice do not display DA neurodegeneration. The enhanced degeneration seen in mice lacking *Ret* and *DJ-1* indicates that *DJ-1* promotes survival of SN dopaminergic neurons deprived of Ret-mediated trophic support, specifically during aging.

Our results that *DJ-1* promotes cell survival only under conditions of environmental (trophic) stress are in agreement with previous published observations. *DJ-1* inactivation in mice and flies does not cause cell loss in the unchallenged animal, but increases its sensitivity PD-related toxins (Andres-Mateos et al., 2007; Chen et al., 2005; Goldberg et al., 2005; Kim et al., 2005b; Menzies et al., 2005; Meulener et al., 2005; Yamaguchi and Shen, 2007). The reported photoreceptor degeneration after Drosophila *DJ-1A* RNAi knock down (Yang et al., 2005b) cannot be reproduced by us and is not observed in *DJ-1A/B* genetic mutants (data not shown) suggesting either the existence of some compensatory mechanisms in the *DJ-1A/B* null mutants or unspecific off-target effects in the *DJ-1A* RNAi knock down flies. In vitro, *DJ-1* inactivation in neuronal cells does not lead to increased cell death, but renders these neurons more sensitive to oxidative stress (Martinat et al., 2004; Taira et al., 2004). One of the advantages of the present study is that it provides evidence for DJ-1 promoting cell survival in the unchallenged or uninjured mouse brain. The genetic changes in the DAT-Ret/DJ-1 mutant mice are well defined and allow the further analysis of the underlying mechanisms.

Does absence of DJ-1 function renders cells more sensitive to any kind of cellular stress? A remarkable recent finding is that combined deletion of all three PD-recessive genes *DJ-1*, *PINK1* and *Parkin* does not cause degeneration of SN neurons, even in 27-month-old mice (Kitada et al., 2009), suggesting that the pro-survival activity of *DJ-1* becomes critical only in specific situations. It appears therefore that increased cellular stress and mitochondrial dysfunction are not sufficient to trigger SN cell death, as long as SN neurons receive trophic signals like the ones mediated by GDNF/Ret. Additional studies evaluating the long-term maintenance of SN neurons deprived of *Ret* and *Parkin* or *PINK1* function will reveal if other PD-linked genes are essential for survival of trophically impaired SN neurons.

In *DAT-Ret* mice the degree of fiber loss (50%) exceeds the degree of SN cell body loss (25 %); this suggests that a fraction of aging SN neurons deprived of Ret-mediated signaling might have lost their target innervation. When analyzing the density of DA fibers in *DAT-Ret/DJ-1* mice, we found interestingly that it matches exactly the degree of fiber loss in *DAT-Ret* mice (50 % in both groups, Figure 25). The simplest explanation is that additional *DJ-1* removal caused degeneration of *Ret*-deficient SN cell bodies that lost their target innervation (the target deprived pool), while *Ret*-deficient SN cell bodies with functional connections were largely unaffected (Figure 35). Alternatively, removal of *DJ-1* could affect both the target-deprived cells and the cells properly connected to the striatum; however, this would imply that some SN neurons would have to re-sprout during aging to compensate for the enhanced fiber loss. Work in Jörg Schulz and our own laboratory indicates, however that DA neurons lacking *Ret* signaling cannot re-sprout to re-establish a functional DA terminal arbor following toxic (MPTP) lesions, in contrast to wild-type DA neurons, further suggesting that Ret signaling is required for the re-sprouting of DA terminals (Kowsky et al., 2007). Therefore, we favor the model in which *DJ-1* deficiency causes degeneration of the target-deprived pool of Ret-deficient DA neurons (Figure 35). To test the validity of this model, we are currently performing tracing experiments in which the retrograde tracer *Fluoro-gold* is injected in the striatum. We will determine the percentage of SN cell bodies that send projections to the striatum in control, *DAT-Ret* and *DAT-Ret/DJ-1* mice. To validate our model, we expect the percentage of functionally connected cell bodies in *DAT-Ret* to be

decreased relative to control or *DAT-Ret/DJ-1* mice, in spite of *DAT-Ret/DJ-1* mice having less DA cell bodies (i.e. all remaining cell bodies in *DAT-Ret/DJ-1* mice are functionally connected with their target area).

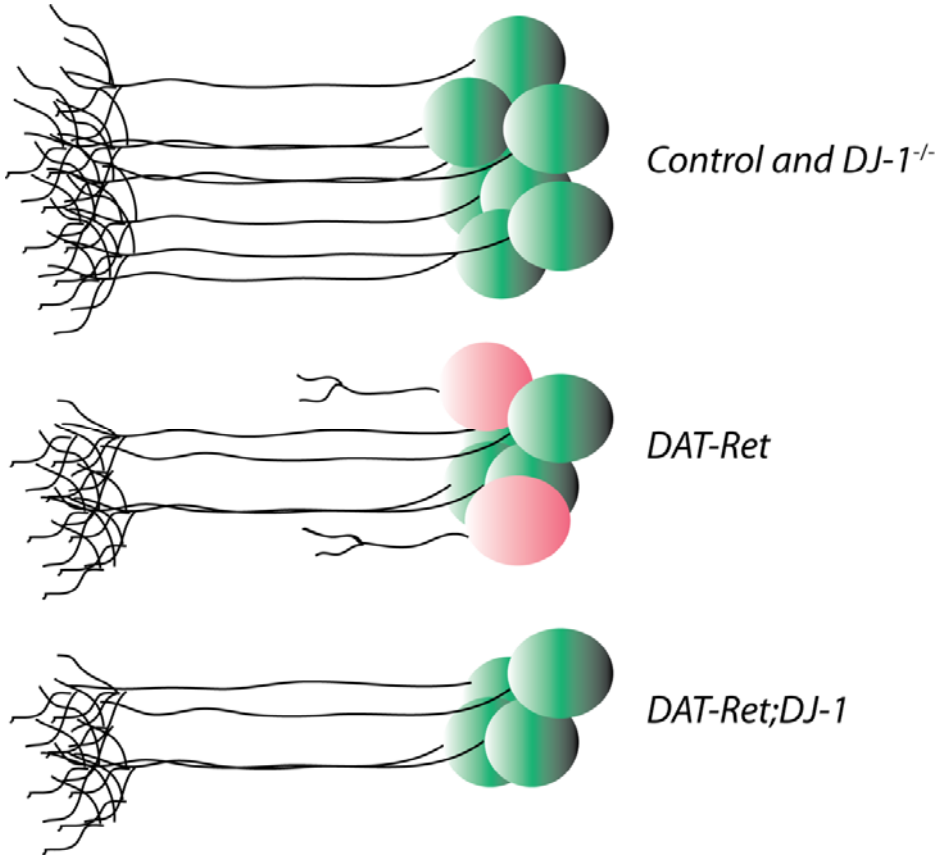


Figure 35. Enhanced loss of *substantia nigra* cell bodies in *DAT-Ret/DJ-1* mice. Removal of DJ-1 function does not affect the survival of SN neurons (upper part), which send axonal projections to the striatum to participate in the control of movement. Ret-deprived SN neurons loose a fraction (25 %) of SN cell bodies while the loss of DA fibers innervating the striatum (51%) exceeds that of cell body. Therefore, we hypothesize the existence of a DA population of neurons in the SN of *DAT-Ret* mice which are devoid of target innervation (in pink). It is this target-deprived subpopulation of neurons that might be primarily lost in *DAT-Ret;DJ-1* mice, in which the density of target innervation in the striatum matches exactly that of DAT-Ret mice, although the degree of SN cell body loss (41 %) is enhanced. In addition, both Ret and DJ-1 deficiency affect primarily the survival of GIRK2-positive neurons (shown here) that project exclusively to the dorsal striatum.

Our finding that *DJ-1* promotes survival of *Ret*-deficient SN cell bodies but not axons suggests that the SN cell body might be the primary site of *DJ-1* pro-survival activity. Neurotrophic factor receptors, such as Ret, are transported from distal sites to the cell body, where they signal to promote survival. Components of the signaling machinery including activated Ras are also transported to the cell body in signaling vesicles (Howe et al., 2001). The signaling mediated by RTKs at axon terminals vs. the soma appears to have at least some distinct aspects; for example, while activation of

Ras/ERK via Shc/Grb2/SOS interactions promote a transient activation at the axon terminal and activates mainly the ERK1/2 MAPKs, in the cell body Ras/ERK is activated via FRS2/Crk binding to receptor-containing endosomes to provide a sustained pro-survival signal (Huang and Reichardt, 2003; Reichardt, 2006). It is therefore possible that DJ-1 interacts with the Ras/ERK machinery that is activated in SN cell bodies to promote DA neuron survival.

The differential effects we observed in the maintenance of SN axons vs. somata in *DAT-Ret* mice raise the possibility that the axonal and cell body compartments might have different survival regulations and requirements. Indeed, increasing evidence suggests that these two compartments might be at least partially uncoupled with respect to their survival requirements. Specific molecules were found that primarily regulate maintenance of cell bodies, but not axons - including Bax, Bcl2 and JNK (Conforti et al., 2007; Ries et al., 2008); conversely, molecules like the Wallerian degeneration slow protein Wld^s promote axonal maintenance but not soma survival in 6-hydroxydopamine (Sajadi et al., 2004) or MPTP (Hasbani and O'Malley, 2006) challenged SN neurons. Remarkably, a very recent paper by Marc Tessier-Lavigne and colleagues suggested that trophically-deprived neurons activate two distinct capsases to mediate loss of axons and somata. Thus, during trophic (NGF, BDNF, NT3) deprivation, the shedding of surface β-amyloid precursor protein (APP) by the β-secretase (BACE) generates a pro-apoptotic shedded product that interacts with the transmembrane death receptor 6 (DR6) protein. DR6 mediates apoptosis by activating caspase-3 in cell soma and caspase-6 in axons (Nikolaev et al., 2009). Another very recent interesting observation is that DJ-1 is proteolytically cleaved by caspase-6 in vitro to generate a C-terminal fragment that exhibits anti-apoptotic activity by negatively regulating p53-dependent transcription (Giaime et al., 2009). A better understanding of the molecular mechanisms regulating axon and soma maintenance and the role of DJ-1 in these processes is required and *DAT-Ret/DJ-1* mice could be valuable tools for this purpose. Understanding the differential vulnerability of the axonal and cell-body compartments to aging and degenerative insults might improve our understanding of neurodegeneration and open new therapeutic avenues.

3.5. Neuroinflammatory changes and postsynaptic dysfunction in *Ret*-deficient mice

Although the family of glial cells has three prominent members (astrocytes, oligodendrocytes and microglia), only astrocytes and microglia have been implicated in the pathogenesis of PD so far (Hunot and Hirsch, 2003; Teismann and Schulz, 2004; Teismann et al., 2003). Astrocytes regulate extracellular potassium concentration and regulate synaptic activity by uptake of synaptic transmitters like glutamate (Filosa et al., 2009) thereby critically regulating neuron function. Following neuronal dysfunction, astrocytes become activated and increase the expression of the Glial fibrillary acidic protein (GFAP), followed by the enlargement of the cell body and extension of processes into the injured area (Eddleston and Mucke, 1993). In contrast to astrocytes, the role of microglial cells in the brain is less well understood (Teismann and Schulz, 2004). Microglia arise from macrophages outside of the nervous system and they are not related to the other cells in the nervous system; however, they might become activated during seizures, injury or neuronal dysfunction, and they undergo proliferation, migration and eventually acquire macrophage-like properties to actively participate in the clearance of dying cells

(Banati et al., 1993). Interestingly, SN is relatively rich in resting microglia in comparison to other brain region (Kim et al., 2000) leading to the proposal that the increased microglia density might also represent a vulnerability factor in PD (Andressoo and Saarma, 2008; Teismann et al., 2003).

Post-mortem examination of patients with PD revealed massive neuroinflammation in the SN and the striatum (Hunot and Hirsch, 2003; Teismann and Schulz, 2004). Interestingly, the degree of microglial activation was found to be more robust than that of astrocyte recruitment and activation, and the microglial activation was found in the SN rather than in the striatum (McGeer et al., 1988). The microglial activation observed in the SN was dramatic (Banati et al., 1998; Mirza et al., 2000) and numerous microglial cells were found in close proximity to DA neurons, onto which they sometimes agglomerated to produce an image of "neuronophagia" (McGeer et al., 1988). In addition, the density of CD8 cytotoxic T cells was also increased in the SN of PD patients (McGeer et al., 1987). In contrast, the increase in astrocyte density was found to be relatively mild, both in the SN and the striatum (Forno et al., 1992; Mirza et al., 2000). Activated glial cells might exert both protective and deleterious effects on neurons; thus, they can secrete neurotrophic factors (GDNF or BDNF) and are able to scavenge toxic compounds released by dysfunctional or dying neurons (Batchelor et al., 1999; Hirsch et al., 1999). On the other hand, activated glial cells also secrete a variety of toxic and pro-apoptotic molecules including noxious compounds (diverse types of ROS) and pro-inflammatory and pro-apoptotic cytokines (including tumor necrosis factor alpha [TNF-α], interleukins [IL-1β/2/4/6], interferon-γ [IFN-γ]) that might activate pro-apoptotic pathways and might thus accelerate neurodegeneration (Hunot et al., 1999). It is therefore unclear whether activated gilal cells are protective or deleterious for neurons, or both, and research in animal models could clarify their mode of action.

We detected an increased density of reactive astrocytes in *DAT-Ret* mice, specifically during aging (24 months) and in the striatum (Figures 20, 26). Two cellular entities are altered in the striatum of 24-month-old *DAT-Ret* mice as compared to age-matched controls: the DA terminals projecting from the SN are markedly depleted (Figures 18 and 25) and the post-synaptic medium spiny neurons (MSNs) downregulated the neuronal marker NeuN and the post-synaptic DA marker DARPP-32, thereby suggesting cellular dysfunction (Figure 19). This suggests that loss of pre-synaptic DA input leads to post-synaptic alterations in dopaminoceptive cells. The increase of astroglial recruitment in this area therefore raises the possibility that dysfunctional DA axons or dysfunctional post-synaptic MSNs, or both, represent a strong recruiting signal for reactive astrocytes. This astroglial recruitment displays a delayed kinetics, as *DAT-Ret* mice did not display increased astrogliosis at 12 months relative to control animals, although they underwent DA neurodegeneration and lost a fraction of their DA terminals in the striatum (Figure 18). Therefore, reactive astrocytes might not be directly involved in the DA fiber degeneration process, but are recruited after the onset of nigrostriatal degeneration. Moreover, since no increased density of astrocytes was detected in the SN (where DA neurodegeneration was ongoing; Figure 20) it appears that dysfunctional DA axons and/or MSN postsynaptic neurons, rather than SN cell bodies, generate recruiting signals for reactive astrocytes.

In contrast to the astrogliosis in the striatum of aging *DAT-Ret* mice, the microglial response we detected was restricted to the substantia nigra (Figure 21). Similar to

astrocytes, microglial cells were also recruited after the degeneration occurred (at 24 but not 12 months). The fact that dysfunctional and dying SN cell bodies induce a local recruitment of microglial cells in aging *DAT-Ret* mice is not surprising, given the known roles of microglia in maintaining tissue homeostasis and their robust association with SN degeneration in post-mortem PD patients (Hunot and Hirsch, 2003; Teismann et al., 2003). Microglial cells might thus clear apoptotic SN neurons and might prevent the accumulation of cellular debris, which might be deleterious for the neighboring unaffected cells. Chronic activation of microglia might on the other hand be deleterious for SN neurons, and microglial cells might accelerate neurodegeneration in its late stages in PD. Further studies that investigate the properties of both astrocytes and microglia at diverse steps in the DA neurodegeneration process might provide new clues about the dual role played by glial cells in nigral disease.

3.6. Regional specificity of neurodegeneration in mice lacking *Ret* and *DJ-1*

The major symptoms in PD are mainly caused by loss of SN neurons and their input to the striatum. Although additional brain areas are affected, the degeneration in the SN is the most consistent alteration detected in the brains of patients with Parkinsonism, including PD patients. The reasons for this relative specificity are currently unknown, although it is believed that a combination of intracellular metabolic and ionic age-related changes in conjunction with extracellular factors (presence of microglia, connectivity, trophic support, toxins) might influence DA neuron survival in a unique way (Abou-Sleiman et al., 2006; Andressoo and Saarma, 2008; Dauer and Przedborski, 2003; Lotharius and Brundin, 2002). For example, SN neurons synthesize the neurotransmitter dopamine, which has a high propensity to auto-oxidize and generate ROS if it abnormally accumulates in the cytosol; therefore, it has been suggested that altered dopamine release and clearance might promote neuronal cell death (Lotharius and Brundin., 2002) and dopamine can covalently bind and inactivate Parkin, thereby potentially contributing to PD pathogenesis (LaVoie et al., 2005). However, dopamine is produced by other neuronal populations (including the VTA) which are less affected in PD, suggesting that other or additional factors might underlie the relative specificity of DA neurodegeneration in PD.

Our finding that VTA DA neurons do not require Ret and DJ-1 signaling for survival is therefore interesting (Kramer et al., 2007). VTA neurons were previously found to be less sensitive than SN neurons to 6-OHDA treatment (Barroso-Chinea et al., 2005) or *α-syn* overexpression (Maingay et al., 2006) and the presence of a functional K-ATP channel Kir6.2 promotes cell death of SN, but not VTA neurons in two chronic mouse models of DA degeneration (Liss et al., 2005). It is therefore possible that VTA neurons do not require Ret signaling for survival, in contrast to SN neurons. Interestingly, a dramatic loss of VTA DA neurons was observed in the above-mentioned GDNF adult knockout mouse (Pascual et al., 2008), raising the possibility that Ret-independent GDNF signaling (via NCAM or via a yet unidentified receptor) is required for survival of VTA neurons. The analysis of VTA neuron survival in aging *NCAM* deficient mice might help solve this issue.

Perhaps more interesting is our finding that loss of Ret and DJ-1 signaling affects a subpopulation of SN neurons that project exclusively to the striatum. We found a specific requirement for Ret and DJ-1 activity in the GIRK2-positive subpopulation,

while the other Calbindin-positive subpopulation did not require Ret/DJ-1signaling for survival (Figure 24). Interestingly, Calbindin-positive neurons in the SN were found to be specifically spared in PD (Yamada et al., 1990) and in mice treated with the PD-causing toxin MPTP (Liang et al., 1996). Conversely, in primary ventral mesencephalic cultures treated with MPTP, GIRK2-postive SN neurons showed enhanced sensitivity (Chung et al., 2005). It is thus interesting that the GIRK2-positive SN neurons, which are more prone to degeneration, are also the ones depending on pro-survival signals mediated by Ret and DJ-1. It appears that elevated levels of the G-protein coupled potassium inwardly rectifying channel GIRK2 is a cause of vulnerability, since elevating the levels of GIRK2 further sensitizes these neurons (Chung et al., 2005). Remarkably, GDNF was found to acutely modulate the excitability of midbrain dopaminergic neurons by inhibiting A-type potassium channels, a function that involves specifically the MAPK pathway (Yang et al., 2001). It is tempting to speculate that Ret and DJ-1 affect SN intracellular potassium concentration by modulating the ERK1/2 signaling and that loss of input from Ret and DJ-1 might further exacerbate the increase in intracellular potassium concentration induced by the presence of GIRK2. Further studies focusing specifically on the GIRK2 subpopulation will better define the exact biochemical processes involved in their survival and their interplay with other age-related cellular changes.

3.7. Ret-deprived nigral neurons are not sensitive to alpha-synuclein aggregation stress

The enhanced neurodegeneration of *Ret*-deprived SN neurons when the function of the oxidative stress suppressor *DJ-1* was removed raised the possibility that *Ret*-deficient DA neurons are generally more sensitive to any deleterious agent. To test this hypothesis genetically, we used transgenic mice that overexpress an aggregation-prone version of human alpha-synuclein specifically in dopaminergic neurons. Overexpression of Ala30Pro human α-syn in DA neurons was shown to be insufficient to trigger overt SN neurodegeneration in these mice, up to 12 months of age, despite the presence of aggregated alpha-synuclein in the SN cell body (Rathke-Hartlieb et al., 2001). When aging *DAT-Ret;TH-α-synA30P* mice were generated and aged, we found surprisingly that they display an accelerated loss of SN cell bodies, relative to *DAT-Ret* mice (Figure 27). We also found, however that overexpression of mutant *α-syn* alone leads to a mild SN neurodegeneration (20 % loss of cell bodies) in 24-month-old animals. Thus, the increased DA neurodegeneration in *DAT-Ret;TH-α-synA30P* mice is due to an additive, but not synergistic effect of Ret and DJ-1 deletion on SN neuron survival. We reached a similar conclusion when analyzing the maintenance of DA fibers in *DAT-Ret;TH-α-synA30P* mice (Figure 27); the degeneration of DA fibers caused by loss of Ret was not further enhanced when mutant α-syn was overexpressed in SN neurons. These results suggest that Ret-deprived DA neurons are not more sensitive to the cellular stress caused by misfolded α-syn. Additional support for this conclusion comes from the study performed in our laboratory in collaboration with Jörg Schulz (University of Göttingen) and in which the sensitivity of adult *Ret*-deficient DA neurons towards the PD toxin MPTP was evaluated. DA neurons lacking Ret were found to display the same sensitivity towards MPTP as wild-type neurons (Kowsky et al., 2007). Therefore, we suggest that *DAT-Ret* mice are not generally more sensitive to any cellular stressor, but rather to defined types of neuronal dysfunctions (e.g. loss of *DJ-1* function) that amplify the negative effects of trophic deprivation during aging.

3.8. *DJ-1* interacts genetically with constitutively active *Ret* in *Drosophila melanogaster*

The enhanced degeneration of SN neurons in mice that lack *Ret* and *DJ-1* signaling suggests that Ret and DJ-1 act in parallel pathways to promote neuron survival. To gain insight into the molecular mechanisms used by Ret and DJ-1 to regulate maintenance of DA neurons, we employed *Drosophila melanogaster*. Drosophila has a long and successful history in signal transduction studies and allowed the discovery and characterization of important regulators of RTK signaling. In addition, flies that lack the PD-associated recessive genes show similar or even aggravated dopaminergic phenotypes compared to mice (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Given that GDNF does not bind Drosophila Ret, we used constitutively active versions of Ret, in order to overcome this ligand-receptor regulatory step. Two constitutively associated versions of Ret (Ret^{MEN2A} and Ret^{MEN2B}) cause *multiple endocrine neoplasia* in humans, a cancer of neural crest origin (Read et al., 2005). Targeted expression of these active Ret versions in the developing photoreceptor neurons of the fly disrupts the normal development of photoreceptors by promoting excessive cell proliferation, abnormal differentiation and by increasing their size (Figures 28, 29); these effects are followed by a secondary apoptotic wave that leads to adult flies of reduced size and rough eye morphology (Read et al., 2005). When DJ-1A/B function is decreased, these retinal defects are significantly rescued and the resulting rescued eyes have a completely restored size. Conversely, DJ-1A overexpression is sufficient to enhance the Ret^{MEN2A} phenotype (Figures 28, 29). Therefore, DJ-1A/B represent novel interactors of Ret signaling in *Drosophila*. Our results also suggest that selective inhibition of DJ-1 activity might represent a novel therapeutic strategy for patients with Ret^{MEN2} tumors.

We then set out to determine which of the Ret-induced cellular pathways interact genetically with *DJ-1A/B*. We found, surprisingly, that *DJ-1* does not modulate the phenotypes caused by alterations in the PI3K/Akt signaling (Figure 30). However, *DJ-1A/B* interacted genetically with *PTEN*, the negative regulator of the PI3K/Akt signaling, but only when overexpressed (Figure 31). Previous studies reported that *DJ-1A* interacts genetically with *PTEN* in *Drosophila* and *in vitro* and furthermore, DJ-1 modulated the activation status of the Akt kinase (Gorner et al., 2007; Kim et al., 2005a; Yang et al., 2005b). We could not reproduce the previously reported (Yang et al., 2005b) interaction with Akt and the modulation of Akt activation by DJ-1, both in *Drosophila* and in mammalian cell culture systems. Although *DJ-1* was sufficient (but not necessary) to inhibit the effects of overexpressed *PTEN*, the negative regulator of PI3K activation, we believe that *DJ-1* may interact with *PTEN* only in defined pathophysiological situations (e.g. oncogenic). In agreement with this suggestion, a recent study showed that *DJ-1* interacts with *PTEN* in hypoxia conditions, similar to those seen in cancer (Vasseur et al., 2009). We predict that the interaction between *DJ-1* and *PTEN* in these circumstances is independent of PI3K/Akt signaling. Indeed, emerging evidence suggests lipid phosphatase-independent roles of PTEN (Gao et al., 2000) the best studied being a protein-phosphatase-dependent inhibition of Ras/MAPK signaling (Kerr et al., 2006; Nayeem et al., 2007), modulation of JNK signaling (Vivanco et al., 2007) and several nuclear functions that involve protein-protein interactions, to control cell cycle progression maintenance of genomic stability or apoptosis (Yin and Shen, 2008).

Activation of MAPK signaling by Ras is required for proper photoreceptor proliferation and differentiation in *Drosophila* (Fortini et al., 1992) and constitutively active Ras/ERK signaling severely impairs eye development (Figure 32). We found surprisingly that *DJ-1* modulated the eye phenotype of constitutively active Ras^{V12} (Figure 32), suggesting that DJ-1 functions downstream of Ras activation or in parallel to Ras-mediated signaling to mediate photoreceptor neuron development. To map the interaction site between DJ-1 and Ras/MAPK signaling, we used a constitutively active version of ERK/rolled, which also induces defects in eye development when targeted to developing photoreceptor neurons. The abnormal proliferation and eye roughness induced by constitutively active rolled were not modulated by DJ-1A/B (Figure 32), suggesting that DJ-1 functions either upstream or in parallel to ERK activation. Preliminary evidence suggests that *DJ-1* does not modulate the phenotype of constitutively active Raf, further restricting the interaction site between DJ-1 and the Ras/Raf/MEK/ERK pathway to Ras/Raf. Therefore, we suggest that DJ-1A/B act either between Ras and Raf or in a parallel pathway to control eye development in *Drosophila* (see below).

The roles of endogenous Ret signaling in *Drosophila* are currently unknown; Ret expression in the fly parallels the expression in mammals (Hahn and Bishop, 2001) and *Drosophila* Ret elicits neurotrophic activities in cultured mammalian cells (Abrescia et al., 2005), suggesting that Ret might mediate trophic signals in *Drosophila*. In this context, it is interesting to note that the relevance of neurotrophic factors to *Drosophila* development is poorly understood (Hidalgo et al., 2006). Although neurotrophins and their receptors are not present in *Drosophila*, it is becoming increasingly clear that neurotrophic activities are important for a variety of processes; thus, during eye development EGFR RTK exerts neurotrophic activities in photoreceptor neurons (Baker, 2001). The identification of the *Drosophila* ligand for Ret might better define the role of Ret signaling during evolution.

3.9. *DJ-1* interacts with *ERK* signaling to control eye and wing development in the fly

The interaction between DJ-1A/B and ectopic Ras signaling in *Drosophila* prompted us to investigate whether DJ-1 plays any role during the development of the fly. The only described functions for DJ-1 in *Drosophila* are regulation of PTEN and Akt signaling (mentioned above) and the control of oxidative stress response (Lavara-Culebras and Paricio, 2007; Menzies et al., 2005; Meulener et al., 2005; Meulener et al., 2006; Moore et al., 2006; Park et al., 2005).

To test whether *DJ-1* controls fly development as a *Ras/ERK* interactor, we focused on two tissues where Ras/ERK signaling is known to play a major role: the development of the eye (Bergmann et al., 1998; Biggs et al., 1994; Fortini et al., 1992; Kim et al., 2006) and wing (Friedman and Perrimon, 2006; Li et al., 2000). We found that, while *DJ-1B* function is dispensable for eye development, lack of *DJ-1B* function severely impairs the development of photoreceptors with reduced ERK (rl) levels. While rl^1/rl^1 flies display about 6.64 photoreceptors/ommatidium (P/O), $rl^1/rl^1;DJ\text{-}1B^{-/-}$ flies have only 5.34 P/O (Figure 33). In addition, consistent with previous observations (Biggs et al., 1994), additional photoreceptor neurons other than R7 failed to differentiate, further implicating the *DJ-1/rolled* axis in the control

of proper photoreceptor neuron differentiation in *Drosophila*. Therefore, *DJ-1B* is required - as a *rolled* interactor- to control photoreceptor neuron development. This is the first evidence for a role of *DJ-1B* in the intact (unchallenged) fly.

We searched for additional roles for *DJ-1B* in the development of the fly. Previous studies revealed an important role for *rolled* signaling in the proper induction of wing veins, therefore contributing to the establishment of the wing. Flies with reduced Ras/ERK signaling all have vein differentiation defects in the wing, although with varying degrees of severity, reflecting the amount of *Ras*, *Raf* or *rolled* function that was removed (Brunner et al., 1994; Li et al., 2000; Lim et al., 1999).Therefore, wing vein development is sensitive to dosages in the Ras/ERK pathway, similar to retinal photoreceptor neurons. We hypothesized that reducing the levels of *rolled* and *DJ-1B* concomitantly would exacerbate the phenotype caused by reducing only ERK levels; to test this hypothesis, we analyzed the wing development in control, *DJ-1B* null, rl^1/rl^1 and $rl^1/rl^1;DJ\text{-}1B^{-/-}$ flies. We found, as expected that removal of *DJ-1B* function does not impact on vein development, while reducing ERK/rolled functio in rl^1/rl^1 flies causes a moderate defect in vein differentiation. When $rl^1/rl^1;DJ\text{-}1B^{-/-}$ wings were analyzed, more severe defects that in rl^1/rl^1 flies were observed; the L4 vein was often interrupted or poorly differentiated (Figure 33), further suggesting an important role for DJ-1B as a *rolled* interactor in *Drosophila* development.

As previously mentioned, endogenous Ret plays probably no major role in photoreceptor neuron and wing development in *Drosophila* (Hahn and Bishop, 2001; Read et al., 2005) the major regulators of fly photoreceptor neuron development being sevRTK and EGRF RTK receptors, while wing development is mainly under the control of *EGFR* (*DER*) RTK (Baonza et al., 2000). Therefore, DJ-1 might act in parallel to sevRTK and/or EGFR RTK in *Drosophila*. We are currently testing whether *DJ-1* interacts with *EGFR* to control eye and wing development in *Drosophila*. Interestingly, the EGFR is also expressed in aging mouse DA neurons and EGF was suggested to be a candidate neurotrophic factor for aging and dysfunctional SN neurons (von Bohlen und Halbach and Unsicker, 2009) and showed rescuing effects when delivered into the brain of rats in which the medial forebrain bundle (MFB) was transected (Pezzoli et al., 1991; Ventrella, 1993) or treated with 6-OHDA (Iwakura et al., 2005). EGF also regulates TH expression in postnatal rats and elevates dopamine turnover in the striatum (Futamura et al., 2003) and decreased levels of EGF are found in the striatum of PD patients (Iwakura et al., 2005). Therefore, it is possible that additional RTKs besides Ret control DA neuron function and survival in the mouse and these observations argue for additional investigations in *Drosophila melanogaster*, as a complement to mammalian studies.

3.10. What are the molecular functions of DJ-1 in *substantia nigra* neurons?

Several functions for DJ-1 emerged from studies in mammalian cell culture and in animal models. Besides the above-mentioned regulation of PI3K/Akt, DJ-1 might inhibit JNK signaling (Junn et al., 2005; Kahle et al., 2009a; Mo et al., 2008) and promotes expression of anti-apoptotic (Fan et al., 2008b; Xu et al., 2005) and anti-oxidant genes (Clements et al., 2006). In addition, DJ-1 might be activated following exposure to moderate levels of ROS and might exert crucial survival promoting functions in mitochondria or as a redox-dependent molecular chaperone (Shendelman et al., 2004). DJ-1 was also found to bind mRNA (Blackinton et al., 2009a) and to

modulate dopamine release (Goldberg et al., 2005). However, the lack of a strong phenotype in *DJ-1* null mutants has prevented the analysis of *DJ-1* function *in viv*o and the above-mentioned putative functions for DJ-1 remain to be validated in physiological and pathophysiological contexts relevant to PD.

How is the expression of *DJ-1* regulated in mouse DA neurons and in *Drosophila*? We first tested whether *DJ-1* expression is modified by *Ret*. Removal of *Ret* function (in the mouse) did not modify DJ-1 protein levels; similarly, in *Drosophila*, targeted expression of constitutively active versions of Ret (Ret^{MEN2}) or the related Akt, Raf, ERK in the developing retina did not modify DJ-1A/B levels, suggesting that *DJ-1* is not regulated via *Ret* and its related PI3K/Akt or Ras/MAPK. Other RTKs might induce DJ-1 expression or alternatively, its expression can be regulated by other transcription factors in response to e.g. oxidative stress (in aging DA neurons) or other developmental cues (in *Drosophila*). Indeed, the levels of DJ-1 were found to be elevated in the brains of PD and AD patients (Choi et al., 2006) and the levels of DJ-1 in the plasma of sporadic PD patients correlated with disease progression (Waragai et al., 2007) suggesting that DJ-1 expression can be upregulated following cellular stress. Failure to upregulate DJ-1 expression in conditions of cellular dysfunction (e.g. PD patients with *DJ-1* mutations) might leads to DA neurodegeneration. This possibility is also supported by research in cellular and animal models of PD, in which removal of DJ-1 function sensitized DA neurons and other non-DA cells to toxic exposure (Kahle et al., 2009a; Menzies et al., 2005; Meulener et al., 2005).

Our genetic analysis provides the first evidence for a crucial pro-survival role of *DJ-1* in aging DA neurons. We found that *DJ-1* promotes survival of dopaminergic neurons only in conditions of aging and trophic insufficiency suggesting that the function(s) of *DJ-1* might only be uncovered in specific circumstances.

It is interesting to note that our genetic analysis in the mouse uncovered the same interaction pattern between DJ-1and RTK-related signaling as in *Drosophila*. Thus, in both systems DJ-1 is dispensable for cell survival or development; moreover, reduced Ret (in the mouse) or RTK (in the fly) signaling leads to moderate effects on cell survival or development. If DJ-1 would act in the same pathway as Ret or related RTKs, mice and flies lacking DJ-1 should display the same moderate defects as seen in Ret and RTK-deficient animals. However, since loss of RTK and DJ-1 function leads to a synergistic effect, DJ-1 acts in parallel to RTK-signaling pathways to exert its functions.

When using *Drosophila* to further localize the interaction point between DJ-1 and Ras/ERK signaling, we obtained evidence that DJ-1 might function either between Ras and Raf or in parallel to the Ras/ERK pathway to control fly development (Figure 36). Since DJ-1 modulated the phenotypes induced by constitutively active Ras^{V12}, DJ-1 does not appear to regulate Ras activation. Instead, we propose that DJ-1 might control the activation of the Raf kinase, or its subcellular localization.

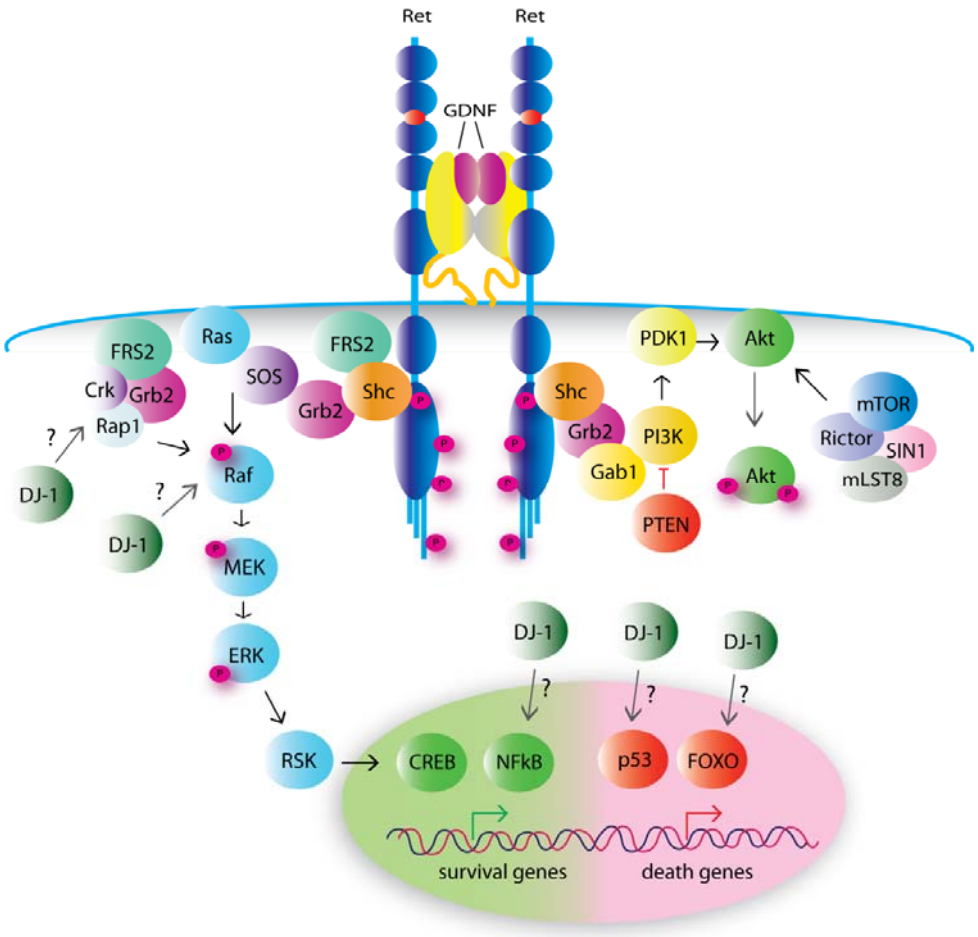


Figure 36. Interaction between *DJ-1* and Ras/ERK signaling. Genetic evidence from aging mouse DA neurons suggests that DJ-1 acts in parallel to a Ret-associated pathway to control survival. Complementary studies in Drosophila suggest that DJ-1 interacts preferentially with the Ras/ERK pathway. DJ-1 connects to the Ras/ERK pathway either between Ras and Raf, or acts completely independent of Ras/ERK. Thus, DJ-1 might regulate the activation status of Raf by modulating the activity of Raf-activating factors; DJ-1 could also regulate Raf localisation and thereby the activation of downstream targets. DJ-1 could also act in a parallel pathway to reinforce the pro-survival activity of the Ras/ERK pathway. For example, DJ-1 might regulate the transcriptional activity of NFkB, p53 or FOXO, but might also have another target not presented here.

The serine/threonine kinase Raf is activated following its recruitment by activated Ras-GTP; while association between Raf and a 14-3-3 dimer stabilizes Raf in the inactive form, binding to Ras-GTP interferes with 14-3-3 binding and promotes Raf phosphorylation at several serine and tyrosine resides (Leicht et al., 2007). It is believed that Raf activation involves several rounds of phosphorylation and dephosphorylation events and multiple protein-protein interactions. The major phosphorylating factors for Raf identified so far are the p21 Rac-activated kinase family (PAK) and the Src-family of tyrosine kinases (Leicht et al., 2007). In addition, Raf associates with chaperones (HSP-90, HSC-70) and with scaffold proteins (KSR, CNK, RKIP) that supposedly promote its activation (Kolch, 2000, 2005; Kyriakis, 2007), although much remains to be learned about its regulation. Since Raf is a mitogen activated protein kinase kinase kinase (MAPKKK), it is interesting to note that the previously reported interaction between DJ-1 and JNK signaling in cells

stressed with UV light involved the specific association of DJ-1 with MEKK1, the MAPKKK upstream of MEK and JNK (Mo et al., 2008). DJ-1 sequesters MEKK1in the cytoplasm, thereby preventing it from entering the nucleus and activating apoptosis (Mo et al., 2008). Whether DJ-1 also binds Raf remains to be elucidated and additional studies are required to address the regulation of Raf by DJ-1.

An alternative route for DJ-1 to interact with Ras/ERK signaling is by acting in an independent pathway that cooperates with Ras/ERK signaling during development and perhaps in DA neurons. DJ-1 might thus act on targets like p53 (Fan et al., 2008b) or FOXO (Figure 34), or might regulate NFkB (Figure 36). It is interesting to note that the survival extension mediated by Sirtuins (Sir2) in several organisms requires FOXO activity and Sirtuins deacetylate FOXO, thereby shifting its pro-apoptotic activity into survival-promoting (Giannakou and Partridge, 2004). Both p53 and FOXO possess this dual death/survival switch and it is possible that stress-response proteins like DJ-1act to switch the balance towards survival, while absence of DJ-1 is pro-apoptotic. Additional studies will elucidate the interaction between DJ-1 and these transcription factors.

Does DJ-1 also control Ras/ERK signaling in aging DA neurons? Mechanistic studies in the Ret/DJ-1 mouse model are difficult to pursue, because of the late-onset and slowly progressing phenotype. Interestingly, mice over-expressing activated Ras (Ras^{V12}) in the nervous system have larger neurons and embryonic mesencephalic neurons derived from these mice are resistant to MPTP or 6-hydroxydopamine - induced degeneration, suggesting that Ras signaling promotes survival of SN neurons (Heumann et al., 2000). More recently, the analysis of Ret knockin mice revealed a critical role for Ras/B-Raf/IKK signaling, but not for PI3K and ERKs, in the survival of sympathetic neurons (Encinas et al., 2008). It remains to be shown whether similar mechanisms operate in the survival of dopaminergic neurons, but it is interesting that this signaling pattern shares resemblance with the interaction we found between *Ret* and *DJ-1* in *Drosophila*.

3.11. Interaction between aging, trophic insufficiency and cellular stress as cause for Parkinson's disease

3.11.1. DAT-Ret/DJ-1 mice as a model for Parkinsonism

Are mice lacking Ret and DJ-1 useful for understanding PD? *DAT-Ret/DJ-1* mice recapitulate some critical PD characteristics, which were difficult to obtain in previous models. First, lack of *Ret* and *DJ-1* causes adult-onset and progressive loss of SN neurons similar to PD; moreover, the degeneration shows specificity for the GIRK2 subpopulation of SN neurons, and does not affect the neighboring VTA neurons. While GIRK2 neurons are indeed preferentially lost in PD (Yamada et al., 1990), VTA neurons also degenerate in PD (although much less than SN neurons) suggesting that additional pathological triggers add-up to cause multi-network dysfunction. Second, *DAT-Ret/DJ-1* mice also show degeneration of SN axons, similar to PD. The loss of axons precedes the loss of SN cell bodies, at least in *DAT-Ret* mice, consistent with a "dying back" model suggested for the neurodegeneration in PD (Dauer and Przedborski, 2003). Moreover, because *DAT-Ret/DJ-1* mice show increased degeneration of SN cell bodies but not axons relative to *DAT-Ret* mice, it is possible that two independent pathological alterations act in different sub-neuronal

compartments to negatively impact on neuronal viability. Third, *DAT-Ret/DJ-1* mice show enhanced neuroinflammatory responses (astrogliosis and microgilal recruitment) relative to control mice and excessive neuroinflammatory responses were also observed in PD patients. Therefore, we conclude that the chain of cellular and network changes induced by neurotrophic factor insufficiency and cellular stress in DA neurons shows some striking similarities to human DA neurodegeneration, that argue for the usefulness of *DAT-Ret/DJ-1* mice as PD models. However, *DAT-Ret/DJ-1* mice do not reproduce all pathological alterations seen in PD. Thus, we detected no accumulation of aggregated alpha-synuclein in aging *DAT-Ret* or *DAT-Ret/DJ-1* mice and these animals are not behaviorally impaired. Therefore, the mechanisms regulating protein homeostasis in DA neurons do not require trophic input and appear to be independent on the chaperone activity of DJ-1.

The absence of overt behavioral alterations in aging *DAT-Ret/DJ-1* mice also parallels observations made in human PD studies. The symptoms of PD only become apparent when a critical threshold of nigrostriatal degeneration is reached. It is estimated that 50-60% SN neurons and 80-90 % of their terminals in the striatum must be lost in order to trigger motor deficits (Dauer and Przedborski, 2003). The phase preceding behavioral alteration, called presymptomatic, might involve several compensatory mechanisms that ensure a proper SN output in the basal ganglia or a proper output of the basal ganglia when DA neurodegeneration is significant (Bezard et al., 2003). Among these physiological compensations, up-regulation of DA signaling – via increased synthesis, reduced re-uptake or increased activity of post-synaptic dopamine receptors – is believed to compensate for moderate loss of SN neurons; alternatively, loss of SN cell bodies might induce re-sprouting of striatal terminals to maintain a normal terminal arborisation, despite cell body degeneration (Bezard et al., 2003). Interestingly, the process of striatal terminal resprouting in the intact striatum (Hudson et al., 1995) or following striatal lesions (Bjorklund et al., 1997) or after MPTP treatment (Kowsky et al., 2007) requires GDNF/Ret signaling; remarkably, although the current clinical trials dealing with GDNF delivery have reduced power to predict the therapeutic outcome of GDNF, post-mortem examination of one PD-patient treated with GDNF showed extensive resprouting in the striatum (Love et al., 2005), suggesting that GDNF promotes terminal resprouting in humans and is supposedly involved in some aspects of presymptomatic compensation in PD. Additional presymptomatic compensations were suggested to take place within the basal ganglia (the subthalamic nucleus [STN] and the globus pallidus [GPi] increase their activity) or even outside of the basal ganglia (changes in thalamo-cortical activity) but they are poorly understood (Bezard et al., 2003). *DAT-Ret/DJ-1* mice could therefore be used to better understand these presymptomatic compensations; in addition, the development of therapeutic strategies that target this compensatory period might delay onset of PD in humans.

3.11.2. The multiple hit hypothesis of Parkinson's disease

The low penetrance of PD and the variability of symptoms in family members who inherit PD-associated mutations have raised the possibility that several risk factors interact to promote SN neuronal demise (the multiple hit hypothesis of PD (Sulzer, 2007). Common risk factors for PD are aging, environmental toxins, different mutations of low frequency but also developmental and disease-induced abnormalities (e.g. neuroinflammation). At the cellular level, distinct molecular alterations could

synergize to cause neuronal dysfunction and cell death. The lack of appropriate disease models makes the study of multiple hit interactions difficult. David Sulzer and colleagues recently reported that a combination of high cytoplasmic calcium, elevated levels of free cytoplasmic dopamine and the presence of alpha-synuclein induces selective death of cultured DA neurons at postnatal day 0-2 and interference with any of these individual hits alleviated neuronal cell death (Mosharov et al., 2009). We report here a chronic and slowly progressive genetic mouse model in which the interplay between three factors (aging, trophic insufficiency and increased cellular stress due to DJ-1 inactivation) synergize and cause the loss of approx. 50 % of GIRK2 DA neurons in the SN. Aging is the critical component of this triple interaction network, as young and adult mice lacking *Ret* and *DJ-1* do not display SN degeneration. The second risk factor is loss of Ret-mediated trophic support, which causes degeneration of a subset of SN neurons during aging. Finally, lack of *DJ-1* function only becomes critical in aging SN neurons deprived of Ret-mediated trophic support. This triple-interaction model likely reflects the synergism generated by several independent pathological triggers, acting at sub-threshold levels, and which together induce degeneration of SN neurons.

We also tested the interaction between aging, loss of Ret-mediated trophic support and defective proteostasis. *Alpha-synuclein* (*α-syn*) mutations that render the protein insoluble cause PD and aggregated α-syn is found in Lewy Bodies, both in monogenic and sporadic PD. We found that *DAT-Ret* mice which overexpress aggregation-prone α-syn lose indeed more SN neurons than *DAT-Ret* single mutants or mice that only overexpress α-syn, but the loss is additive and not synergistic. This suggests that some pathological changes (loss of *Ret* and α-syn aggregation), acting in different neurons to cause cell death, add up to enhance tissue loss, while others (loss of *Ret* and *DJ-1*) synergize within the same cells to cause their dysfunction and demise. The distinction between additive and synergistic effects of pathological changes is important for the choice of therapy, as additive effects require independent management of individual causes, whereas synergistic pathological changes might be prevented in one step. The fact that mice that lack *Ret* and overexpress mutant α-syn in DA neurons do not show a synergistic loss of neurons also suggests that *Ret* mutants are not generally more sensitive to any deleterious agent, but appear to be specifically sensitive to changes that amplify the effects of trophic deprivation. Thus, trophic insufficiency and aging predispose neurons more to loss of *DJ-1* function rather than to abnormal aggregation of mutant α-syn. Further investigation of interactions between different pathological changes will lead to a better understanding of the cellular and molecular mechanisms that are critical for the function and survival of SN neurons.

3. 12. Perspectives

3.12.1. Toward integrative models of Parkinson's disease

Two major approaches have been used to mimic PD in an animal model. The first is based on the administration of specific dopaminergic toxins, and the second is using transgenic animals. These approaches were successful in reconstituting some of the major PD characteristics. First, delivery of toxins like 6-OHDA and MPTP induce dopaminergic neurodegeneration by inhibiting the mitochondrial complex I of the electron transport chain and lead to dramatic behavioral alterations (Dauer and Przedborski, 2003), but the initial acute behavioral changes in mice are reversible,

suggesting neuronal recovery. Although these two models show a clear SN cell loss and some behavioral phenotypes, their major disadvantage is that they do not - or only sporadically- lead to the occurrence of intracellular inclusions. Moreover, although some sub-chronic protocols can be applied, they do not resemble the progressive course of the disorder. Second, models based on PD-associated genes are successful in reproducing defects in proteostasis (e.g. *α-syn* overexpression) and display moderate behavioral phenotypes (Moore et al., 2008); however, they generally fail in reproducing SN neurodegeneration.

Therefore, integrated strategies, combining both neurotoxin-based and transgenic animals should be used in order to dissect the complex interactions leading to SN dysfunction and death. In addition, I propose that the storage, integration and analysis of experimental data collected from all PD-models might be amenable to system analysis (Figure 37) and might accelerate the pre-clinical and drug development in PD. PD is a complex disorder with a strong environmental (90 %) contribution, supporting the idea that SN cell death is a multifactorial process (Dauer and Przedborski, 2003). This complex biological system can be defined as a highly interconnected map of signaling pathways involved in multiple biological processes (Figure 37).

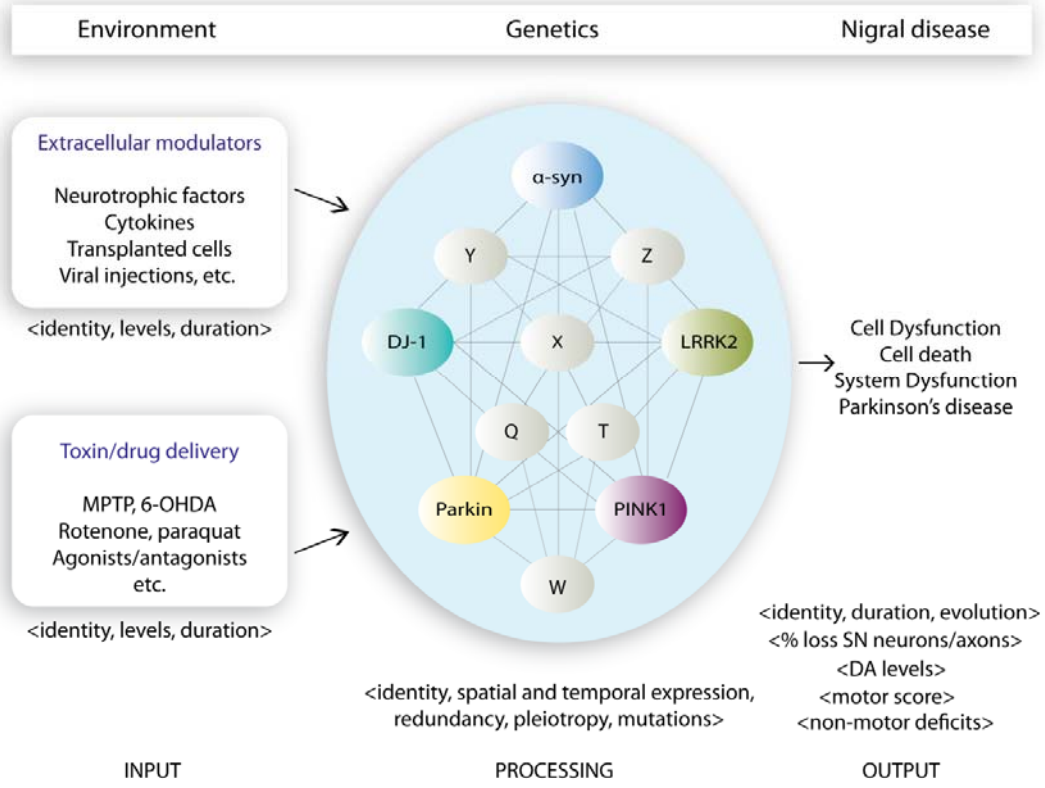


Figure 37. Toward integrative models of Parkinson's disease. Extracellular factors (e.g. neurotrophic factors, toxins and other modulators [endogenous or experimentally induced]) interact with intracellular factors inside the SN (genetically controlled and subjected to multiple interactions) to control their fate. Some pathways inside SN neurons might be preferential, and some factors more important than others. Dysfunction in some pathways might lead to specific outcomes. Choosing the best PD model for a given application means selecting the combination of factors that generates an output best fitting to pre-established criteria. Standardized information about all parameters involved (genetic, toxicologic, histologic, behavioral, others) should facilitate the establishment and development of integrative models for PD.

The input of this system is represented by all extracellular factors acting on SN neurons (e.g. cytokines, trophic factors). The processing of all the input signals is performed at several sites within SN neurons. The output is represented by the SN neuron behavior, that can vary from no or slight dysfunction to cell death. When a certain threshold of cell death is reached, behavioral alterations will be visible at the organism level. Several requirements are necessary to achieve this purpose: 1. Creation of an easily-accessible common resource; this might represent a website where all relevant information (concerning the type of model; the type of experimental alteration; histological, behavioral and molecular analysis; other parameters) is structured and compared in a useful way; 2. Standardization of all protocols used during these experiments, or a clear description of them, to allow analysis and reproducibility; 3. Development of *in silico* tools to allow data analysis and most importantly, to allow prediction of experimental outcomes; development of *in silico* tools to interactively adjust experimental predictions based on available new data; 4. Establishment of a policy concerning the eventual conflicts of interest, data sharing and other legal rights.

Because it appears that distinct sets of pathological changes interact in specific ways to generate distinct outcomes, it is logical to assume that integrative models of PD might require both genetic and non-genetic manipulations. The use of multiple experimental hits might lead to more overt degeneration signs, and provided they are properly controlled, they might be used for drug development. For example, delivery of a specific MPTP dose to a mouse lacking *DJ-1* function and overexpressing alpha-syn in SN neurons might be used to screen for compounds that are more effective for *DJ-1* linked PD than compound testing on *LRRK2* mice treated with MPTP and overexpressing alpha-syn. Moreover, *DJ-1* deficient mice challenged with MPTP and overexpressing alpha-synuclein might also receive defined doses of GDNF in the striatum, and the outcome might be compared to other similar studies to allow further refinements. The use of a common resource might allow sharing important information about associations of pathological changes and their prevalent behavioral outcomes. Prediction models based on known pathological associations and behavioral correlates could be developed and further refined in this interactive common resource.

3.12.2. The future of neurotrophic factor-based therapies for Parkinson's disease

Although the already existing clinical trials met with several technical problems, neurotrophic factors still hold promise as rescuing agents for neurodegenerative disease. It is currently acknowledged that serious improvements concerning the delivery mode of neurotrophic factors, the ideal time-window for delivery but also safety issues are needed before we can critically assess their therapeutic power (Barker, 2006; Kordower and Olanow, 2008; Ramaswamy et al., 2009). New therapeutic avenues are also provided by the development of small molecule agonists that specifically activate neurotrophic factor signaling and several such molecules were identified for GDNF. One of them is a non-peptidyl small molecule that binds GFRα1 and activates the GFRα1-Ret complex (Tokugawa et al., 2003) and several patents for small molecule Ret-modulators exist already (Jurvansuu and Goldman, 2008). Our current results raise the possibility that activation of Ret signaling via these agonists might be beneficial for nigral disease and future research will evaluate the effects of activating Ret signaling in Parkinson's disease patients.

IV. MATERIALS AND METHODS

A. BUFFERS, SOLUTIONS AND MEDIA

PBS	10 mM Na_2HPO_4 pH 7.4 2 mM KH_2PO_4 0,137 M NaCl 2,7 mM KCl
TBS	50 mM Tris pH 7.4 150 mM NaCl
PFA 4 %	40 g PFA in 1 L warm PBS (60 ^{0}C) add 400 µL NaOH 5M to help dissolving then neutralize with 150 µL HCl 37 % for perfusions, use cold (4 ^{0}C) PFA 4 % solution
Sucrose solutions	15 % and 30 % sucrose in PBS used for brain immersion
Cryoprotection solution (1L)	300 mL distilled water 300 mL glycerol 300 mL Ethyleneglycol 100 mL PBS used to store mouse brain sections at -20 ^{0}C
Egg embedding mix	mix egg yelow and sucrose 10 : 1 (g/g) use cold mix for embedding (4 ^{0}C) to polymeraze, add 1 mL glyceraldehyde 25 % to 20 mL egg mix; mix well and allow 45 min for after polymerization at r.t., store at -80^{0}C
Fly food (1L)	15 g yeast 11.7 g agar 80 g molasses 60 g corn flower 6.3 mL propionic acid 2.4 g methylparaben yeast paste (yeast granules and fly water) was added to bottles to enhance egg laying
Fly food with paraquat *for fly toxicity tests*	paraquat was directly dissolved in boiled food food was dispensed in vials and allowed to dry
H_2O_2 solutions *for fly toxicity tests*	5 % sucrose in distilled water 2 % H_2O_2
Fly water	0.8 % acetic acid in distilled water

PCR buffer A	25 mM NaOH 0.2 mM EDTA
PCR buffer B	40 mM Tris HCl pH 5.0
Blocking buffer	5 % bovine serum albumin (BSA) in TBS 0.3 % Triton X-100
Antibody incubation buffer	2 % BSA in TBS 0.1 % Triton X-100
Modified RIPA buffer	20 mM HEPES 100 mM NaCl 10 mM NaF 1.5 mM sodium vanadate 5 mM EDTA 2 % Triton X-100 5 mM sodium pyrophosphate 1mM sodium molybdate 0,1 % SDS 1 tablet *Complete* protease inhibitors
Chloral hydrate	8 % chloral hydrate (Roth, Germany) in PBS

Unless otherwise mentioned, chemicals were purchased from Sigma-Aldrich and Merck (Germany).Fly food components were from ProBio and Primavera (Germany).

B. MOUSE GENETICS, HISTOLOGY AND BEHAVIOR

4.1. Transgenic animals

The *Ret* floxed allele was generated by flanking the exon 12 of the *Ret* gene, which encodes the ATP binding site of the tyrosine kinase domain, with loxP sites (Kramer et al., 2006). The second exon of the *TrkB* gene, encoding a part of the tyrosine kinase domain, was flanked by loxP sites to generate the *TrkB* floxed allele (Minichiello et al., 1999). *Nestin-Cre* mice express Cre from a transgene (Tronche et al., 1999), while *DAT-Cre* mice were generated by knocking *Cre* into the 5' UTR region of the endogenous mouse *DAT* locus (Zhuang et al., 2005). The *DJ-1* gene trap mice were generated by Thu-Trang Pham and Daniela Vogt-Weisenhorn (Helmholtz Research Center, Germany) using the XE726 ES cell clone (Bay Genomics, USA) in which the pGT1Lxf vector (containing a splice acceptor, LacZ and neomycin genes) was inserted between the exons 6 and 7 of the DJ-1 gene; this generates a *DJ-1* null allele, as the resulting DJ-1 protein is unstable (Pham et al., submitted). Mice overexpressing a mutant version of human *alpha-synuclein* (Ala30Pro) under the control of the TH promoter (TH-α-synA30P) were from Philipp Kahle and Christian Haass (LMU University, Munich, Germany) (Rathke-Hartlieb et al., 2001). The mice used in this

study were kept on a C57Bl6/J genetic background with contributions of 129/sv from the embryonic stem cell culture and the different Cre mouse lines. Because both *DAT*-Cre and *Nes*-Cre lines show significant recombination in germ cells, the floxed allele derived from the parent that carries the Cre recombinase is often constitutively recombined; therefore, the homozygous *Ret* mutants carry one *Ret* allele recombined in a regionally specific manner and one *Ret* allele recombined constitutively (i.e. exact genotypes are *DAT-Cre*; $Ret^{lx/-}$ and *Nes-Cre*;$Ret^{lx/-}$). The control mice for all experiments carried floxed alleles of *Ret* ($Ret^{lx/lx}$, $Ret^{lx/+}$), *TrkB* ($TrkB^{lx/lx}$, $TrkB^{lx/+}$), or one copy of Cre (*DAT-Cre* or *Nestin-Cre*), unless otherwise mentioned. The following abbreviations are used throughout this thesis: *DAT-Ret* for *DAT-Cre;*$Ret^{lx/-}$, *DAT-TrkB* for *DAT-Cre;*$TrkB^{lx/-}$, *DAT-Ret/TrkB* for *DAT-Cre;*$Ret^{lx/-}$;$TrkB^{lx/-}$, *Nes-Ret* for *Nes-Cre;*$Ret^{lx/-}$, *DAT-Ret/DJ-1* for *DAT-Cre;*$Ret^{lx/-}$; *DJ-*$1^{-/-}$, *TH*-αsynA30P for *TH-α-syn*(Ala30Pro).

4.2. Generation of conditional *Ret* and *TrkB* mutants: the *Cre/loxP* system

To ablate *Ret* or *TrkB* expression in defined tissues (dopaminergic system or the whole nervous system), the *Cre/loxP* system was used (Figure 38).

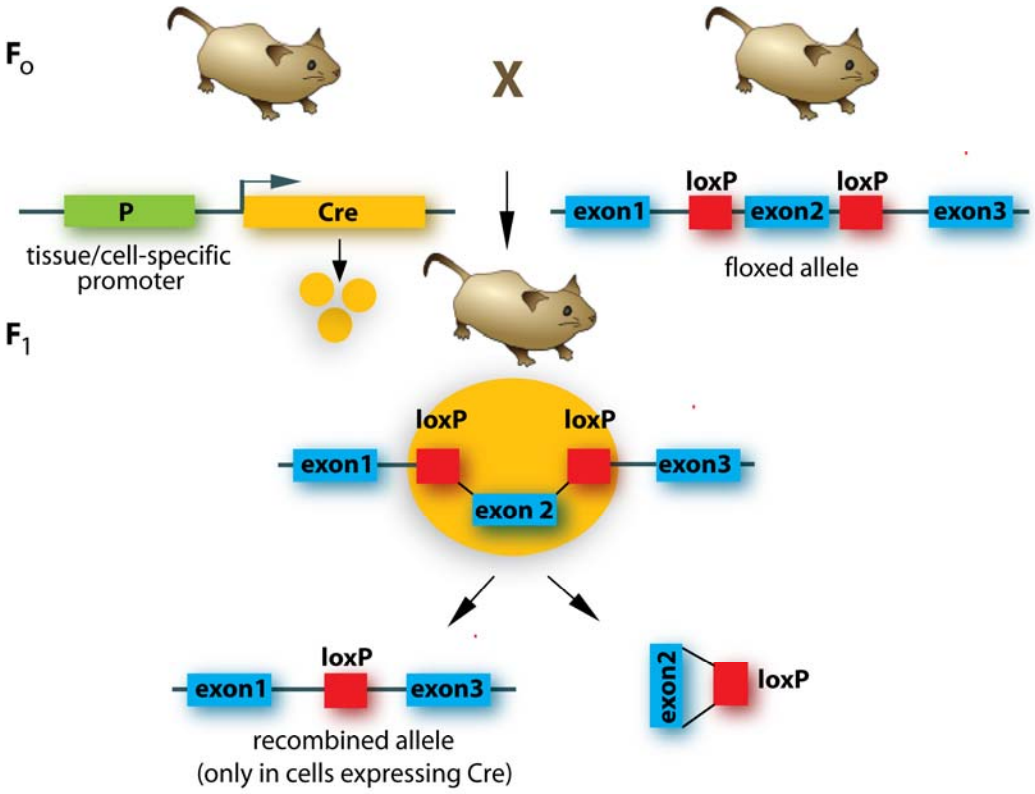


Figure 38. The Cre/LoxP system. To generate region-specific knockout mice, two lines are required: a line carrying the *Cre* recombinase under the control of a tissue/cell specific promoter (P); a second line in which the gene of interest has been engineered to contain loxP sites. Upon crossing these two mouse lines, the resulting progeny will express *Cre* in cells/tissue in which the promoter P is active. In these cells/tissue *Cre* will mediate site-specific DNA recombination and will remove the DNA piece contained between the engineered loxP sites. Therefore, the resulting progeny will carry the new mutation only in the cells/tissue in which the promoter P was active (conditional knockout mice).

The *Cre/loxP* system allows site-specific DNA recombination in defined cells or tissues. The site-specific DNA recombinase Cre catalyses recombination between two engineered sites, loxP (locus of X-over P1, a 34-bp site found in the bacteriophage P1). Two mouse lines are required to achieve conditional gene deletion. One is a conventional transgenic mouse line with Cre targeted to a specific tissue or cell type, and the second is a mouse strain in which the target gene (here *Ret* or *TrkB*) is flanked by two loxP sites in a direct orientation ("floxed allele"). Recombination (excision and consequently inactivation of the target gene) occurs only in those cells expressing *Cre* recombinase. Thus, the target gene remains active in all cells and tissues which do not express *Cre* (Figure 38). For example, mice that carry floxed alleles of *Ret* ($Ret^{lx/lx}$) and express the *Cre* recombinase under the control of a dopaminergic specific (e.g. dopamine transporter-DAT) promoter (*DAT-Cre;* $Ret^{lx/lx}$) will therefore lack Ret expression only in dopaminergic neurons.

4.3. Mouse genotyping

DNA extraction. DNA was extracted from mouse tails after incubation with 100 µL PCR buffer A (45 minutes at 95°C) and subsequent neutralization with 100 µL PCR buffer B. The resulting DNA solution was kept at +4 °C (for immediate use) or at -20 °C (for long-term storage).

*PCR amplification***.** The PCR mix (50 µL) contained 1 µL from the reverse and forward primers (30 µM), 5 µL ThermoPol PCR buffer (New England Biolabs), 5 µL of dNTPs mix (10 µM), 1 µL Taq DNA polymerase (New England Biolabs), 36 µL distilled water and 1 µL from the DNA solution. The following primers were used:

Primer name	**Sequence (5'-3')**
DJ-1 Forward	AGG CAG TGG AGA AGT CCA TC
DJ-1 Reverse WT	AAC ATA CAG ACC CGG GAT GA
DJ-1 Reverse mutant	CGG TAC CAG ACT CTC CCA TC
Annealing at 58 °C	$DJ\text{-}1^{+}$ (475 bp) and $DJ\text{-}1^{-}$ (240 bp) products
Ret^{lx} Forward (geno 5)	CCA ACA GTA GCC TCT GTG TAA CCC C
Ret^{lx} Reverse (geno 7)	GCA GTC TCT CCA TGG ACA TGG TAG
Annealing at 62 °C	Ret^{+} (300 bp) and Ret^{lx} (350 bp) products
Cre Forward (A)	GCC TGC ATT ACC GGT CGA TGC AAC GA
Cre Reverse (B)	GTG GCA GAT GGC GCG GCA ACA CCA TT
Annealing at 64°C	500 bp product
Ret^{rec} Forward (geno6)	CGA GTA GAG AAT GGA CTG CCA TCT CCC
Ret^{rec} Reverse (3E)	ATG AGC CTA TGG GGG GGT GGG CAC
Annealing at 72 °C	600 bp product

For each reaction, two primers (indicated above) were used, except for DJ-1 genotyping where 3 primers were used. The *DJ-1* genotyping was also performed as

two separate PCR reactions, to detect either the WT (475 bp product) or the mutant (240 bp product) *DJ-1* allele. To detect the floxed vs. WT allele of *Ret*, Ret^{lx} forward and reverse primers were used and generated a 300 bp product (WT allele) or a 350 bp product (floxed allele). The presence of the *Cre* construct was tested in the Cre PCR, while the presence of the recombined *Ret* allele was detected in a Ret^{rec} PCR reaction (the product sizes are indicated above). The PCR conditions were: an initial denaturation step at 94°C (5 minutes) was followed by 35 cycles that comprised denaturation (94°C; 30 seconds), primer annealing (at annealing temperature indicated above; 30 seconds) and elongation (at 72°C; 30 seconds); finally, a final elongation step at 72 °C (10 minutes) completed the PCR amplification. The PCR products were analyzed on 1 % or 2 % agarose gels.

4.4. Histology, immunohistochemistry and Nissl staining

Perfusion and tissue processing. Mice were given a lethal dose (0.5 ml) of 8 % chloral hydrate solution. Mice were perfused transcardially with PBS and 4 % PFA (20 minutes each). Subsequently, brains were removed from the scull, post-fixed overnight in the same fixative, and cyroprotected by incubation in 15 % and 30 % sucrose solutions. Left and right brain halves were embedded separately in egg yolk with 10 % sucrose and 5 % glutaraldehyde, and kept frozen at −80°C until analyzed. 30 µm–thick coronal sections were cut on a cryostat, collected free floating, and then directly used for stainings or stored in a cryoprotection solution at −20 °C until utilized. For fluorescent immunohistochemical and Nissl stainings, sections were premounted; for all other stainings, free-floating sections were used.

Primary antibodies. The following primary antibodies were diluted in antibody incubation buffer:

Antibody	**Species**	**Dilution**	**Source/donor**
Tyrosine hydroxylase	mouse	1: 2000	Immunostar, USA
Dopamine transporter	rat	1: 500	Chemicon, Germany
Pitx3	rabbit	1: 1000	M.P. Smidt, Netherlands
NeuN	mouse	1: 200	Chemicon, Germany
GFAP	rabbit	1: 500	DakoCytomation, Denmark
DARPP-32	rabbit	1: 50	US Biological, USA
Parvalbumin	mouse	1: 10000	Swant, Switzerland
Iba-1	rabbit	1: 1000	Wako, Germany
MAC-1/CD11b	rat	1: 200	Serotec, UK
GIRK2	rabbit	1: 80	Alomone labs, Israel
Calbindin	mouse	1: 500	Chemicon, Germany
Alpha-synuclein	sheep	1: 500	Chemicon, Germany
Alpha-synuclein	rat	1: 500	Philipp Kahle, Germany

Free floating sections were first incubated 1 hour in blocking buffer at room temperature (r.t.). The solution containing the primary antibody in antibody incubation buffer was then added, and sections were incubated over-night at 4°C.

After three washes in TBS (5 minutes each), the sections were incubated 1 hour at r.t. with a species-specific biotin-coupled secondary antibody (dilution 1:200 in antibody incubation buffer) from Vectastain ABC kits (Vector Laboratories, Burlingame, California, United States). After another three washes in TBS, sections were incubated with a complex of avidin-biotin (in which a part of biotin-binding sites are vacant) coupled to horseradish peroxisade (HRP; dilution 1:200 in TBS buffer; incubation 1 hour at r.t.). Finally, after three washes in TBS, a substrate of HRP - the diaminobenzidine (DAB; Sigma-Aldrich, Germany) - diluted in tap water was added. Sections were incubated until a brown precipitate was formed that allowed specific visualization of neurons or glial cells. The exposure times to DAB (typically less than 20 minutes) were optimized to allow a high signal-to-noise ratio. For NeuN/TH double labeling, sections were first stained for NeuN as described above, followed by a weak TH staining with more-diluted primary (1:20,000) and secondary antibodies (1:2,000) and avidin-HRP/biotin complexes (1:2,000).

To fluorescently label DA fibers, sections were first-premounted, allowed to dry then incubated in blocking buffer (1 hour at r.t.). Then, the primary antibody (TH or DAT) diluted in antibody incubation buffer was added and the sections were incubated over-night at 4°C. After three washes in TBS, the secondary biotin-coupled antibody (Vectastain ABC kit; Vectorlabs, USA) diluted 1:500 in antibody incubation buffer was added (2 hours of incubation at r.t.). After another three washes, Cy3-coupled Streptavidin (Sigma-Aldrich, Germany) diluted 1:1,000 in TBS was added (incubation 1 hour at r.t.). The sections were again washed three times in TBS buffer and then mounted in an aqueous mounting medium with anti-fading reagent (Biomedia, Foster City, California, United States).

Nissl staining was performed according to standard procedures. Briefly, mounted sections were successively dehydrated with 50 % and 100 % ethanol, and then incubated in xylene (5 minutes) to remove fat. Sections were then re-hydrated by incubation in ethanol solutions of decreasing concentrations (100 %, 70 %, 50 % and then in water). Sections were then incubated in a 0.5 % cresyl violet solution for 8 minutes, followed by a quick wash in water and destaining in 70 % ethanol until an optimal contrast is achieved (microscope examination). Finally, after incubation in 96 % ethanol (5 minutes), butanol (3 minutes) and xylene (10 minutes), sections were allowed to dry and were mounted with a non-aqueous mounting medium (DPX).

4.5. Stereological quantification of *substantia nigra* neurons

Stereological counts were performed with the StereoInvestigator program (MicroBrightField, Williston, Vermont, United States) on every third or sixth section spanning the SN. After immunolabeling of SN neurons, the exact order of sections spanning the SN area was established. On each individual section, the area of the SN was delineated and an automated method allowed selection of smaller SN sub-territories (each territory was a 50 x 50 µm square) that were uniformly distributed (distance between two consecutive sub-territories was 75 µm on the x-axis and 100 µm on the y-axis). Inside each selected sub-territory, neurons found within 10 µm depth (z-axis) were counted. After all sections were processed, the program used the determined neuronal densities to estimate the total number of neurons found within the selected SN volume. 3-5 animals were tested per group.

4.6. Determination of dopaminergic fiber density in the striatum

Pictures of fluorescently labeled DA fibers in the striatum were acquired using a fluorescence microscope (Axioplan; Zeiss, Göttingen, Germany) at 63x magnification. For every section, three pictures in the dorsal striatum and two pictures in the ventral striatum were acquired. DA fiber density in the striatum was assessed on every third section spanning the striatum (between Bregma +1.10 mm and −0.10 mm). In order to automatically delineate the fibers and to increase the signal-to-noise ratio, the images were first thresholded and subsequently quantified with an automatic counting-grid macro implemented in the Metamorph software (Molecular Devices, Sunnyvale, California, United States) (Kramer et al., 2007).

4.7. Determination of neuron, astrocyte and microglial densities

Every sixth section from the dorsal striatum or the SN was used to determine the density of astrocytes or microglial cells. For each section, the area containing labeled cells was delineated. The number of cells was then determined and the density of cells in the selected area was calculated. 6-8 sections were analyzed /animal and at least 4 animals were analyzed per group.

4.8. Behavioral tests

Experimental protocols were approved by the government of Oberbayern, Germany. 18- and 24-month-old mice were housed individually with free access to water and food in a room with 12h/12h reversed day-night cycle. All experiments were conducted during the night period in a quiet room with 12 lx light intensity.

Open field. To test the general activity of aging control and mutant mice, animals were subjected to open field behavioral assessment. Mice received no prior training. They were placed into a 59 cm x 59 cm large arena for 20 minutes and their horizontal movement was monitored by an observer and by using the EthoVision software (Noldus, Sterling, USA). The experiment was repeated on the consecutive day and the average of the two trials per mouse was taken for further analysis (Valenti et al., 2001). The results represent the average travelled distance (cm)/20 minutes.

Swimming tank. To monitor leg movements, mice were swimming 60 cm in a water filled glass-tank (100 cm long and 6 cm wide, filled with 23ºC water to a depth of 20 cm) to a visible black escape platform (6 cm long, 20.5 cm high) 0.5 cm above the water level (Carter et al., 1999). Before testing, mice were given three training sessions per day for three consecutive days in which they were allowed to swim 4 times per session. During the tests, mice were tested 6 times per day (in two separate test sessions each comprising 3 consecutive runs) during two consecutive days. The tests were videotaped and the time mice needed to reach the platform was determined.

Forced swimming test. To test spontaneous activity, mice (without any previous training) were placed in a plexiglas cylinder (20 cm high and 14 cm in diameter) filled with water to a height of 10 cm. A camera was used to observe and record the animal's behavior. Within the 6 minutes of the trial, the first 2 minutes the animal was

allowed to adjust to the new conditions. In the next 4 minutes, the duration of two types of motor activity, struggling and floating status, was recorded. The results are expressed as activity (sec)/240sec.

Rotarod. To monitor limb motor coordination and balance, a computerized rotarod apparatus was used (TSE, Bad Homburg, Germany). Mice were trained at several constant speeds (0, 4, 10, 25 rpm) and increasing speeds (4-10, 10-20, 20-30 rpm) three times five minutes each and were placed back on the rod every time they fell. After the mice attained a steady baseline level of performance, they were tested 4 times five minutes at 25 rpm (in separate trials, during one day) and 4 times five minutes at increasing speed 4-25 rpm (separate trials, the next day). The latency to fall off the rotarod during the 300 sec test was recorded (Valenti et al., 2001).

C. DROSOPHILA GENETICS, HISTOLOGY AND TOXICITY TESTS

4.9. *Drosophila* lines

The following lines were used during this study:

***Drosophila* stocks**	**Source/donor**
WT	Bloomington stock center (No. 1)
w^{1118}	Bloomington stock center (No. 5905)
w^{1118}	Bloomington stock center (No. 6326)
DJ-1A$^{-/-}$ *(Δ72)*	Nanci Bonini (USA)
DJ-1B$^{-/-}$ *(Δ93)*	Nanci Bonini (USA)
DJ-1A$^{-/-}$*; DJ-1B*$^{-/-}$	Nanci Bonini (USA)
Df(2R)CX1	Bloomington stock center (No. 442)
Ins(2R)^{k04204}	Bloomington stock center (No. 102296)
Df(3R)^{7917}	Bloomington stock center (No. 7917)
Ins(3R)^{16085}	Bloomington stock center (No. 16085)
rl^{SEM}	Kyoto stock Center (No. 108365)
rl^{1}	Bloomington stock center (No. 386)
GMR-dRet^{MENA}	Ross Cagan (USA)
GMR-dRet^{MEN2B}	Ross Cagan (USA)
Sev-Ras^{V12}	Marc Therrien (canada)
GMR-Gal4 (II)	Takashi Suzuki (Germany)
GMR-Gal4 (III)	Bloomington stock center (No. 8121)
Ey-gal4	Takashi Suzuki (Germany)
Sev-Gal4	Bloomington stock center (No.5793)
Da-Gal4	Bloomington stock center (No. 5460)
Tubulin-gal4	Gaia Tavosanis (Germany)
Actin-Gal4	Gaia Tavosanis (Germany)
UAS-dRet^{MEN2A}	Ross Cagan (USA)
UAS-dRet^{MEN2B}	Ross Cagan (USA)
UAS-DJ1A	Nanci Bonini (USA)

UAS-DJ1B	Nanci Bonini (USA)
UAS-hDJ1	Nanci Bonini (USA)
UAS-PI3K^{WT}	Sally Leevers (UK)
UAS-PI3K^{D954A}	Sally Leevers (UK)
UAS-PI3K^{CAAX}	Sally Leevers (UK)
UAS-Akt1^{WT}	Bloomington stock center (No. 8191)
UAS-rl^{SEM}	Jongkyeong Chung (Korea)
UAS-Ras^{V12}	Bloomington stock center (No. 4847)
UAS-Raf^{F179}	Bloomington stock center (No. 2033)
UAS-dPTEN	Tak Mak (Canada)
UAS-dFOXO	Bloomington stock center (No. 9575)

The following *Drosophila* stocks were generated during this study:

w^{1}; BL/Cyo; DJ-1B^{-}/TM2
w^{1}; BL/Cyo; UAS-DJ-1A/TM2
w^{1}; BL/Cyo; UAS-DJ-1B/TM2
w^{1}; BL/Cyo; UAS-dRet^{MEN2B} :: DJ-1B^{-}/TM2
w^{1}; BL/Cyo; da-Gal4 :: DJ-1B^{-}/TM2
w^{1}; GMR-Gal4/Cyo; TM2/TM6
w^{1}; GMR-Gal4/Cyo; DJ-1B^{-}/TM6
w^{1}; GMR-Gal4/Cyo; UAS-DJ-1A/TM6
w^{1}; GMR-Gal4/Cyo; UAS-DJ-1B/TM6
w^{1}; GMR-dRet^{MEN2B}/Cyo; TM2/TM6
w^{1}; GMR-dRet^{MEN2B}/Cyo; DJ-1B^{-}/TM6
w^{1}; GMR-dRet^{MEN2B} :: DJ-1A^{-}; DJ-1B^{-}/TM2
w^{1}; GMR-dRet^{MEN2A}/Cyo; TM2/TM6
w^{1}; GMR-dRet^{MEN2A}/Cyo; DJ-1B^{-}/TM6
w^{1}; GMR-Gal4/Cyo; UAS-DJ-1A RNAi/TM2
w^{1}; Sev-Gal4/Cyo; DJ-1B^{-}/TM2
w^{1}; Sev-Gal4/Cyo; UAS-DJ-1A/TM2
w^{1}; Sev-Gal4/Cyo; UAS-DJ-1B/TM2
w^{1}; Elav-Gal4/Cyo; DJ-1B^{-}/TM6
w^{1}; DJ-A$^{-/-}$; GMR-Gal4/TM6
w^{1}; UAS-dRet^{MEN2A}/Cyo; DJ-1B^{-}/TM2
w^{1}; UAS-dRet^{MEN2A}/Cyo; UAS-DJ-1A/TM2
w^{1}; UAS-dRet^{MEN2A}/Cyo; UAS-DJ-1B/TM2
w^{1}; UAS-PI3K^{WT}/Cyo; TM2/TM6
w^{1}; UAS-PI3K^{D954A}/Cyo; TM2/TM6
w^{1}; UAS-PI3K^{WT}/Cyo; DJ-1B^{-}/TM6

w^1; UAS-PI3K^{D954A}/Cyo; DJ-1B$^-$/TM6
w^1; UAS-dPTEN/Cyo; TM2/TM6
w^1; UAS-dPTEN/Cyo; UAS-DJ-1B$^-$/TM6
w^1; UAS-dPTEN/Cyo; UAS-DJ-1A/TM6
w^1; UAS-dPTEN/Cyo; UAS-DJ-1B/TM6
w^1; DJ-1A$^-$/Cyo; UAS-dRaf^{F179}/TM2
w^1; DJ-1A$^-$/Cyo; UAS-rl^{SEM}/TM2
w^1; UAS-hDJ-1/Cyo; DJ-1B$^-$/TM6
w^1; UAS-dFOXO/Cyo; DJ-1B$^-$/TM6
rl^1/Cyo; DJ-1B$^-$/TM2
rl^{SEM}/Cyo; DJ-1B$^-$/TM2

Unless otherwise stated, flies were raised at 25ºC with 70 % relative humidity on standard cornmeal medium. Embryos and larvae were hydrated using fly water. Fly stocks were kept at 18ºC on cornmeal medium and were flipped regularly.

4.10. Genotype abbreviations

A fusion between a promoter and a cDNA is indicated by a hyphen (-). For example, GMR-dRet^{MEN2A}, GMR-dRet^{MEN2B} and Sev-Ras^{V12} flies carry fusions between the photoreceptor neuron specific promoters *glass multimer reporter* (GMR) or *sevenless* (Sev) and mutated cDNAs of Ret and Ras genes.

Flies that carry promoter-Gal4 constructs and one or several UAS constructs (i.e. targeted expression using the GAL4-UAS system) are labeled as in the following examples: GMR/DJ-1A (instead of GMR-Gal4; UAS-DJ-1A), GMR/PI3K^{WT}/DJ-1A (instead of GMR-Gal4; UAS-PI3K^{WT}; UAS-DJ-1A).

4.11. Assembly of two mutations on the same chromosome (recombination)

During meiosis, *Drosophila* females, in contrast to males, undergo recombination between homologous chromosomes at high rates. This property can be used to generate fly lines that carry two distinct mutations on the same chromosome. Two stocks each carrying one mutation are crossed together and virgin females (carrying both mutations but each on a homologous chromosome) are collected. During meiosis, recombination occurs and hybrid chromosomes are eventually produced. To screen for flies carrying recombined chromosomes, virgin females are crossed to males carrying balancer chromosomes (that do not undergo recombination and whose presence in the progeny causes an easily identifiable phenotype); thus, some of the resulting progeny will carry a new hybrid chromosome that has both mutations (Figure 39). To screen for flies carrying the hybrid chromosome, males issued from this cross (and thus potential carriers of the hybrid chromosome) are first allowed to mate with virgin females carrying balancer chromosomes (to insure the chromosome is maintained in a population); after several days, each individual male is re-isolated. To determine which males carry the hybrid chromosome, several strategies can be used. DNA from each male can be isolated and tested for the presence of both mutations (by PCR). Males can also be genotyped directly, by visual inspection, when

the presence of both mutations leads to a visible and unambiguous phenotype. Alternatively, males can be crossed to defined virgin females and the progeny can be screened by western blotting for expected proteomic changes.

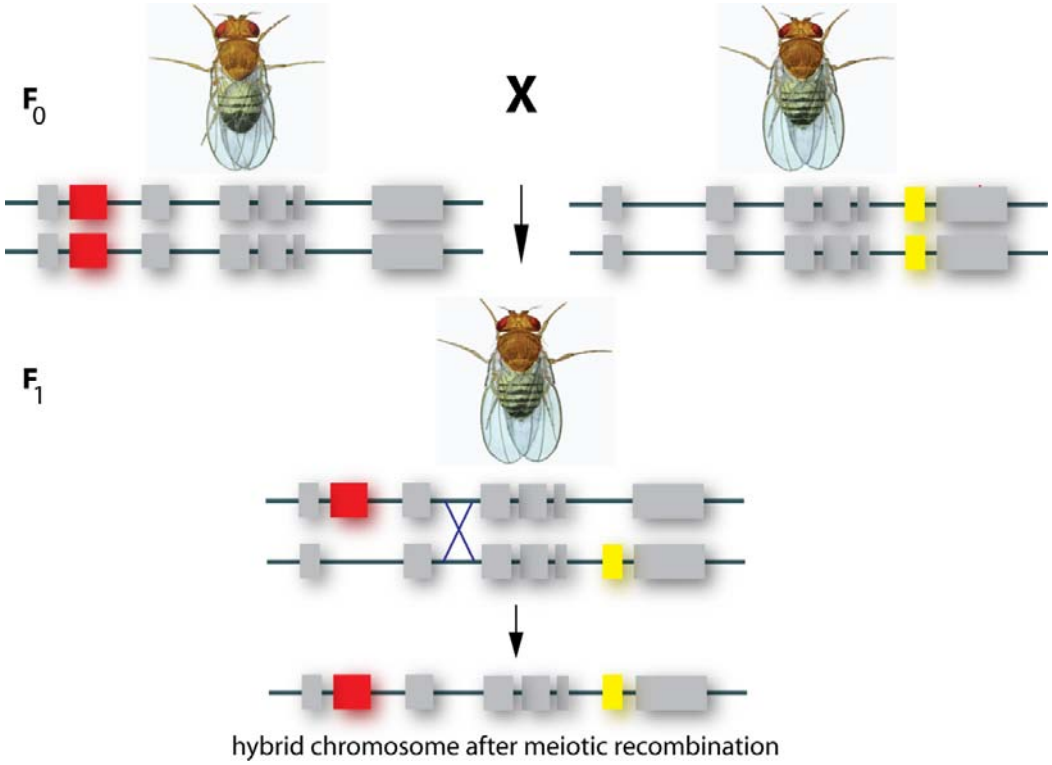


Figure 39. Performing recombinations in *Drosophila melanogaster*. To assemble two mutations (red and yellow) on the same chromosome, flies carrying each mutation are crossed; the resulting virgin females will undergo recombination between homologue chromosomes at high rates; the frequency of recombinants containing both mutations (red and yellow) will depend on the chromosomal distance between the two mutations. Virgin females are then crossed to defined males and the presence of the recombined chromosomes is detected using several methods (see text for details).

4.12. The GAL4-UAS system

The GAL4-UAS binary system allows the expression of a given transgene under the indirect control of a given promoter (Figure 40). (1) The gene (*X*) to be expressed is positioned downstream of an upstream activating sequence (UAS) found in yeast; flies carrying the UAS-X construct are generated (line 1). Conventional lines exist in which the Gal4 transactivating yeast gene is placed under the control of a cell/tissue specific promoter (P; line 2), allowing expression of the Gal4 protein in cells/tissue where the promoter P is active. When the two lines 1 and 2 are crossed, the Gal4 protein generated in defined cells/tissue binds specifically the UAS sequence and induces the expression of the X gene in the same cells/tissue. If, for example, P is *GMR* and X is *DJ-1A*, expression of *DJ-1A* will be specifically induced in post-mitotic photoreceptor neurons, where *GMR* is active.

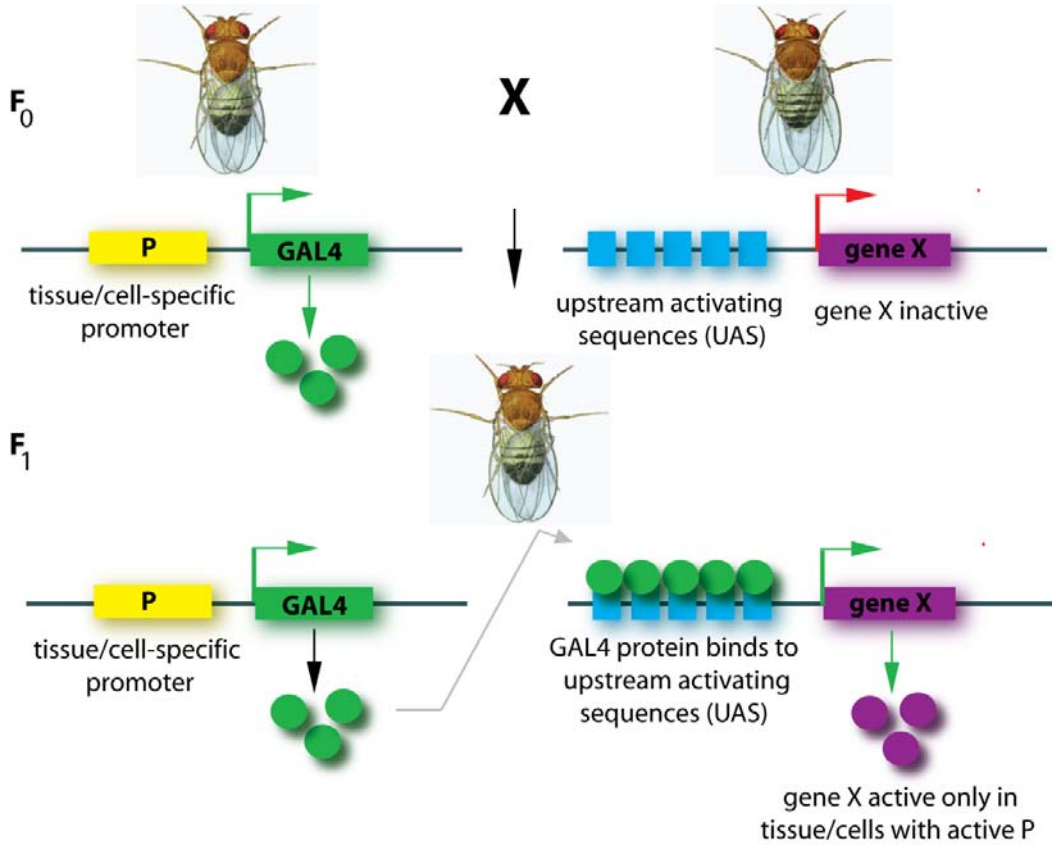


Figure 40. Targeted gene expression using the GAL4-UAS system. The GAL4-UAS system allows the expression of a given gene (*X*) in defined tissue/cells, using a tissue/cell specific promoter (P). One fly line carries a transgene in which the coding sequence of the yeast transactivator GAL4 is placed downstream of promoter P. Alternatively, the coding sequence of GAL4 can be knocked in downstream of an endogenous promoter. A second fly line carries a transgene in which the gene of interest (X) is placed downstream of an upstream activating sequence (UAS). Upon crossing these two fly lines, the resulting progeny will express the GAL4 transactivator in the cells/tissue defined by the promoter P; in these cells, GAL4 binds the UAS sequence and leads to activation of transcription downstream of UAS. The gene *X* is therefore active only in P-positive cells, while it remains inactive in all other cells/tissues.

4.13. Imaging of fly eyes and wings

Pictures of postnatal day P1-P5 eyes and wings were acquired using a Leica MZ 9.5 stereomicroscope equipped with a Leica DFC320 digital camera (LeicaMicrosystems, Wetzlar, Germany). Total eye area was determined using the ImageJ software (NIH, USA).

4.14. Toluidine blue staining and analysis

To highlight the cellular anatomy of the fly retina and to visualize the sub-cellular morphology of photoreceptor neurons, toluidine blue was used as contrasting agent. Heads from postnatal day P1-P5 flies were dissected and post-fixed in 2.5% glutaraldehyde. After washing with PBS, heads were incubated in a 1% osmium

tertaoxide solution (Science Services, Munich, Germany), then dehydrated in ethanol solutions of increasing concentrations (25-100 %), followed by a 10 minute incubation in propylene oxide. Heads were then incubated overnight in a solution containing 50 % propylene oxide and 50 % durcupan epoxy resin, which contained 48 % component A/M, 40 % hardener B, 2.25 % accelerator C and 9 % plasticizer D (Sigma-Aldrich). Then, heads were incubated overnight in 100 % durcupan epoxy resin. The next day, heads and fresh durcupan resin were transferred to molds; heads were oriented tangentially and then cooked overnight at 60ºC. The polymerized resin containing the specimens was then removed from molds and cut using a 2088 ultrotome (LKB, Bromma, Sweden). 3 µm-thick sections were collected, mounted and then stained using a pre warmed toluidine blue solution, that contained 0.1% toluidine blue (Serva Electrophoresis, Heidelberg, Germany) and 2.5% sodium carbonate. After a quick wash in water, sections were allowed to dry and were then covered with paraffin oil. Pictures at different retinal depths were acquired for each head (at 40x magnification). To determine ommatidium size and the number of photoreceptor neurons/ommatidium at least 150 ommatidia/animal from at least 4 animals were analyzed.

4.15. Toxicity tests

Postnatal day P1-P5 fly progeny was first starved in empty vials for 6 hours. Flies were then placed in new vials containing either 1) fly food with paraquat (20 mM) or 2) 2 % H_2O_2 in 5 % sucrose solution (dispensed on a Whatmann paper at the bottom of an empty vial). The number of surviving flies was scored periodically (every 8-12 hours) and the food-containing vials were exchanged regularly.

4.16. Immunoblotting

Postnatal day P1-P5 fly heads from at least 50 animals were collected and snap frozen in liquid nitrogen, then stored at -80ºC. The lysis and detection were performed as previously described (Meulener et al., 2005) using an anti-DJ1A/B antibody (rabbit polyclonal, 1:500, kind gift from Leo Pallanck, USA). Briefly, fly heads were lysed in a RIPA modified buffer, followed by two centrifugations at 13 000 rpm. The supernatant was used for immunoblotting using the anti-DJ-1B antibody and anti-tubulin antibody (mouse monoclonal, 1:1000, Sigma-Aldrich, Germany).

4.17. Statistics

Unpaired Student's t-test was used to compare mean values and to evaluate significance. Differences were considered statistically significant for *p*-values inferior to 0.05.

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Education

1998 – 2004	Master of Engineering and Engineering degree in Biochemistry and Biosciences at the *National Institute of Applied Sciences (INSA de Lyon),* Dept. of Biosciences, Lyon-France. Bachelor studies at *EURINSA* (the European department of *INSA de Lyon*, 1998-2000) and the department of BioSciences (2000-2004).
Oct. 2003-Aug.2004	M.Sc. in biochemistry from the *Claude Bernard* University-Lyon and *INSA de Lyon*-France. Research training at the *National Institute of Health and Medical Research (INSERM)*, Laboratory of Experimental Neurobiology and Physiopathology U 433 (Dr. Marie-Françoise Belin) Lyon-France.
1998	Baccalaureate in sciences, Bucharest-Romania (score 9,77/10).

Pre-doctoral research

Oct. 2002-July 2003	Pre-doctoral research training at the *Karolinska Institute*, Dept. of Molecular Medicine, Neurogenetics unit (Prof. Dr. Martin Schalling) Stockholm-Sweden.

Doctoral research

Oct. 2004 – present	Ph.D. thesis at the *Max Planck Institute of Neurobiology* Dept. of Molecular Neurobiology (Prof. Dr. Rüdiger Klein) Munich-Germany.

Courses and workshops

Jan.-May 2003	Neuroscience research course and Magnetic Resonance Imaging (MRI) course at *Karolinska Institute*-Stockholm, Sweden.
Oct.2004-Oct.2007	Took part at 7 workshops organized by the *Nervous System Repair* (*NSR*) research training network: Lausanne, Lund, Stockholm, Cambridge, Turin, Marseille and Munich. Each workshop (duration 4-7 days) consisted of lectures, practicals and company visits.
Apr. 2006	*Cambridge Spring school* on Neurodegeneration, Cambridge University, UK. Presented one poster.

Meetings and conferences

2004	*NSR* meeting, Prague-Czech Republic, project presentation.
2005, 2008	Poster and talk at Klein lab retreat, Ringberg-Germany.
2005-2009	Gave oral presentations at meetings of the SFB596 network *(Mechanisms of neurodegeneration)* in Munich-Germany.
2006	Meeting of the APOPIS -*Abnormal proteins in the pathogenesis of neurodegenerative disorers*- in Madrid-Spain; gave talk
2006	Midterm *NSR* meeting in Munich; co-organizer and gave talk.
2007	International Parkinson's meeting, Salzburg-Austria, poster
2007	Final meeting *NSR* network, Marseille-France, gave talk.
2007	PhD retreat of the SFB596 network, Chiemsee, gave talk.
2008	International Symposium of the SFB596 on Parkinson's disease and prion disease, Ringberg-Germany, gave talk.
2008	SFB 596 evaluation, Munich-Germany, presented poster.
2008	3rd annual meeting of the *Neuroscience Network of Excellence* (*NeuroNE*), Lisbon-Portugal, gave talk.
2008	Member of the Organization Committee of the *Drosophila Regional Meeting in Munich-Germany,* poster.
2009	Fly retreat, Chiemsee, gave talk

Awards, fellowships, honors

2004-2007	Fellow of the *Marie Curie* Research Training Network *Nervous System Repair (NSR*)- official EU research program
2004	Label *Euforia* of European Engineering (*INSA de Lyon*)
1998-2000	Scholarship awarded by the *INSA de Lyon* and the Rhône-Alpes region during my training in the European Department of *INSA de Lyon (EURINSA).*
1998	Prize *Summa cum laudae* awarded by the *Decebal* high school in Deva-Romania, for contributions to its prestige.
1998	Selected in the *Romanian Chemistry Olympic Team* and took courses at Iasi and Bucharest Universities (2 months).
1994-1998	Received two first prizes, two second and a third prize at the *National Chemistry Olympiads* of Romania, and other prizes at regional chemistry competitions**.**

Previous publications

Partonen T, Treutlein J, Alpman A, Frank J, Johansson C, Depner M, **Aron L**, Rietschel M, Wellek S, Soronen P, Paunio T, Koch A, Chen P, Lathrop M, Adolfsson R, Persson ML, Kasper S, Schalling M, Peltonen L, Schumann G. Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression (2007) *Ann. Med*. 39(3):229-38

Johansson C, Willeit M, **Aron L**, Smedh C, Ekholm J, Paunio T, Kieseppa T, Lichtermann D, Praschak-Rieder N, Neumeister A, Kasper S, Peltonen L, Adolfsson R, Partonen T, Schalling M. Seasonal affective disorder and the G-protein beta-3-subunit C825T polymorphism (2004). *Biol. Psychiatry* 1;55(3):317-9.