Parkin Cooperates with GDNF/Ret Signaling to Prevent Dopaminergic Neurodegeneration in Mice

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Declaration on oath

I hereby declare, an oath, that I have written the present dissertation by my own and have not used other than acknowledged resources and aids.

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To whom it may concern:

V.V. Durga Praveen Meka is submitting his doctoral dissertation in English. The title of his thesis is: **'Parkin Cooperates with GDNF/Ret signaling to Prevent Dopaminergic Neurodegeneration in Mice'**.

I hereby certify as a native speaker and molecular biologist that the English language used in this thesis is sufficiently correct for submission.

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To my parents and sisters

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic (DA) neurons in a specific region of the brain, namely the substantia nigra pars compacta (SNpc). The E3 ligase parkin and the receptor tyrosine kinase Ret are independently linked to the SNpc DA neuronal death in PD patients and animal models respectively. Parkin is shown to play a role in maintaining mitochondrial integrity and mitophagy of damaged mitochondria in cell culture. In contrast to its well-established roles *in vitro*, mice lacking parkin display no DA neuron loss in the SNpc. On the other hand, the neurotrophic factor glial cell-line derived neurotrophic factor (GDNF) and its receptor Ret were shown to be important for the survival and maintenance of the SNpc DA neurons in animal models. Mice without Ret in DA neurons display an age dependent and SNpc specific DA cell loss reminiscent to PD patients. Correspondingly, a mutation in the intracellular domain of the Ret receptor (MEN2B) renders it constitutively active, resulting in more DA neurons specifically in the SNpc region of the mice. However, association studies performed in humans did not find any polymorphism in the Ret gene that can be attributed to increased PD risk. These observations suggest that defects in Ret signaling could be a secondary, but not primary consequence in PD, where Ret might function along with other proteins that are linked to PD. Recently, a study unveiled the pro-survival function of DJ-1 in Ret deficient mice and a conserved interaction between DJ-1 and Ret mediated signaling in *Drosophila*. These findings encouraged us to investigate for functional interaction and downstream signaling of Ret and parkin.

In this study I used mouse genetics to investigate the functional cooperation and downstream signaling of Ret and parkin in SNpc DA neurons. To investigate the cell survival and maintenance function of parkin and Ret in the SNpc and its target region, the striatum (nigrostriatal DA system), we crossed conditional (dopaminergic specific) Ret deficient mice with complete parkin deficient mice. The double deficient mice thus obtained showed an enhanced and progressive age dependent DA cell loss (specifically in the SNpc) and striatal DA axon fiber loss compared to mice that only lack Ret, indicating cell survival and maintenance function of parkin together with Ret in ageing mice. Interestingly, the adult parkin and Ret double deficient mice showed significantly lowered total dopamine levels in the striatum and reduced total cellular ATP levels and decrease in mitochondrial complex I activity in the SN region. Taken together, these observations demonstrate that both Ret signaling and parkin activity are essential for energy production and mitochondrial function to maintain cellular physiology and morphology of the SNpc DA neurons in adult mice, which would otherwise lead to a progressive and age dependent degeneration.

In another approach, we wanted to investigate neuroprotective effects of parkin on the nigrostriatal system of ageing Ret deficient mice. In order to do this, we crossed transgenic human-parkin overexpressing mice with Ret deficient mice. Interestingly parkin overexpression in Ret deficient mice prevented SNpc DA cell body death and also protected striatal DA axonal fibers and total DA amounts. These findings not only suggest redundant functions of Ret and parkin, but also extend the knowledge about the neuroprotective properties of parkin, which is here demonstrated in neurons with trophic insufficiency.

Furthermore, to investigate the role of parkin in the increased DA neuron numbers in MEN2B mice, we crossed parkin deficient mice with MEN2B mice. Parkin deletion slightly but

significantly normalized the SNpc DA neuron numbers and striatal DA fiber innervation in the MEN2B mice. These findings demonstrate the functional interaction of constitutive active Ret signaling and parkin for the establishment of nigrostriatal DA system.

Altogether, our observations reveal an important functional crosstalk of parkin and Ret to maintain cellular ATP levels and proper mitochondrial function to promote the survival of SNpc DA neurons. Considering the cell survival role and redundant functions of Ret and parkin, striatal delivery of GDNF (or other Ret ligands) might be much more effective in PD patients with parkin mutations.

Zusammenfassung

Morbus Parkinson ist eine progressive neurodegenerative Krankheit, bei der dopaminerge Neuronen in einer spezifischen Gehirnregion, der Substantia nigra pars compacta (SNpc) absterben. Die E3-Ligase Parkin und die Rezeptortyrosinkinase Ret sind unabhängig voneinander mit dem dopaminergen Zellverlust in der SNpc bei Parkinson Patienten in Verbindung gebracht worden. Für Parkin konnte gezeigt werden, dass es in Zellkultur bei der Erhaltung der mitochondrialen Integrität und bei der Mitophagy in beschädigten Mitochondrien eine Rolle spielt. Trotz der wichtigen *in vitro* Funktionen von Parkin zeigen Mäuse, die mutant für dieses Protein sind, keinen dopaminergen Zellverlust in der SNpc. Andererseits wurde in Tiermodellen gezeigt, dass der neurotrophe Faktor glial cell-line derived neurotrophic factor (GDNF) und sein Rezeptor Ret wichtig für das Überleben und den Erhalt von SNpc dopaminergen Neuronen sind. Mäuse, die Ret nicht mehr in dopaminergen Neuronen produzieren zeigen einen Parkinson-ähnlichen, altersabhängigen und SNpc spezifischen Verlust von dopaminergen Neuronen. Übereinstimmend damit führt eine konstitutiv aktive Mutation in der intrazellulären Domäne des Ret Rezeptors (MEN2B) bei Mäusen zu einer erhöhten Anzahl von dopaminergen Neuronen in der SNpc Hirnregion. Dennoch konnten Assoziationsstudien im Menschen keine Polymorphismen im Ret Gen finden, die mit erhöhtem Risiko an Morbus Parkinson zu erkranken zusammenhängen könnten. Diese Beobachtungen weisen darauf hin, dass die Defekte in der Ret Signalweiterleitung eher sekundäre als primäre Konsequenzen der Parkinsonerkrankung sind, und Ret mit anderen Proteinen zusammenarbeitet, die mit Morbus Parkinson assoziiert werden. Vor kurzem konnte eine Studie zeigen, dass das Protein DJ-1 in Ret mutanten Mäusen eine überlebensfördernde Wirkung hat und dass die DJ-1 und Ret Signalweiterleitung in *Drosophila* miteinander interagiert. Diese Ergebnisse führten zu der Idee, eine funktionelle Interaktion in der Signalweiterleitung von Ret und Parkin zu untersuchen.

Für diese Arbeit haben wir Mausgenetik benutzt, um eine mögliche funktionelle Interaktion in der Signalweiterleitung von Ret und Parkin in der SNpc Gehirnregion und deren Zielregion, dem Striatum (nigrostriatales System) zu untersuchen. Um die Aufgaben von Ret und Parkin im Überleben von dopaminergen Neuronen und deren Erhalt genauer zu studieren, haben wir konditionelle (dopaminerg-spezifische) Ret knock-out Mäuse mit komplett knock-out Mäusen für Parkin gekreuzt. Diese Doppel-knock-out Mäuse zeigten im Vergleich zu Ret knock-out Mäusen einen verstärkten und mit dem Alter progredienten Zellverlust von dopaminergen Neuronen der SNpc Region, und einen verstärkten Verlust von striatalen Axonfasern. Dies deutet darauf hin, dass Ret und Parkin gemeinsame Funktionen im Überleben und im Erhalt von dopaminergen Neuronen in alternden Mäusen haben. Interessanterweise zeigten erwachsene Doppel-knock-out Mäuse signifikant reduzierte Mengen an Dopamin im Striatum, reduzierte zelluläre ATP-Mengen und reduzierte mitochondriale Komplex I Aktivität in der SN Region. Diese Beobachtungen zeigen, dass die essentiellen Funktionen von Ret und Parkin in der Energieproduktion und der mitochondrialen Funktion für die zelluläre Physiologie und Morphologie der SNpc dopaminergen Neuronen in erwachsenen Mäusen benötigt sind, und somit eine progressive und altersabhängige Degeneration verhindern.

In einem weiteren Ansatz untersuchten wir mögliche neuroprotektive Effekte von Parkin in alternden Ret mutanten Mäusen. Um dies zu erreichen, kreuzten wir Mäuse die, humanes Parkin bilden mit Ret mutanten Mäusen. Wir konnten zeigen, dass Überexpression von

Parkin in Ret mutanten Mäusen den dopaminergen Zelltod in der SNpc Gehirnregion verhindert, dass die striatalen dopaminergen Fasern geschützt waren und die Gesamtmenge an Dopamin unverändert war. Diese Ergebnisse weisen nicht nur auf redundante Funktionen von Ret und Parkin hin, sondern erweitern auch das Wissen über die neuroprotektiven Eigenschaften von Parkin, wie wir sie hier in Neuronen mit trophischer Insuffizienz zeigen konnten.

Zusätzlich haben wir die Rolle von Parkin in MEN2B Mäusen untersucht, die normalerweise eine erhöhte dopaminerge Neuronenzahl aufweisen. Parkin-MEN2B Mäuse zeigten eine milde, aber signifikante Normalisierung der dopaminergen Neuronen und der striatalen dopaminergen Fasern. Diese Ergebnisse demonstrieren die funktionale Interaktion von konstitutiv aktivem Ret und Parkin in der Etablierung des nigrostriatalen dopaminergen Systems.

Zusammenfassend zeigen unsere Daten einen wichtigen funktionellen Crosstalk zwischen Parkin und Ret bei der zellulären ATP Gesamtmenge und bei mitochondrialen Funktionen um das Überleben von dopaminergen SNpc Neuronen zu sichern. Wenn man die Zell-Überlebensfunktionen und die redundanten Funktionen von Ret und Parkin berücksichtigt, könnte man sich eine effektivere striatale Übermittlung von GDNF (oder anderen Ret Liganden) in Morbus Parkinson Patienten mit Parkin Mutationen vorstellen.

1. Introduction

A. Parkinson's Disease: Genetics and Pathogenesis

1.1 Neurodegeneration in Parkinson’s disease (PD)

Neurodegeneration is a slow and progressive process, which leads to the loss of structure and function of nerve cells, and in some cases the term neurodegeneration also refers to the death of the neurons itself. Based on the neuronal population that are affected, neurodegeneration can lead to specific pathological, behavioral and clinical phenotype as seen in Parkinson’s disease (PD), Alzheimer’s disease (AD), Huntington’s disease, Amyotrophic lateral sclerosis (ALS), Frontotemporal dementia (FTD) etc. Age of onset and rate of progression may vary from disease to disease and these might even differ from individual to individual with the same neurodegenerative disease. In most cases careful diagnosis is important to clinically distinguish these diseases for e.g. from Parkinsonism and PD, from AD and FTD. Genetic causes, protein misfolding and aggregation of toxic proteins, oxidative stress, mitochondrial dysfunction and energy failure, membrane damage, decreased axonal transport, and apoptosis are some of the common features shared by most of the neurodegenerative disorders (Bredesen *et al.*, 2006). Most of the neurodegenerative diseases seem to be tightly linked with ageing, since they are late-onset and are progressive with age (Rubinsztein, 2006); mitochondrial mutations and oxidative stress majorly contributes to the process of ageing (Lin and Beal, 2006).

PD is the second most common neurological disorder after AD, affecting approximately 1% of the population those who are over 60 years of age. PD is a progressive neurodegenerative disorder characterized by the loss of dopamine (DA) producing neurons specifically in the substantia nigra pars compacta (SNpc) region of the brain resulting in decreased dopamine (DA) levels in the striatum (illustrated in Figure 1.1 A and B). In PD, the DA neurons located in the ventrolateral and caudal portions of the SNpc are more affected, unlike during normal aging in which the dorsomedial part of SNpc is affected (Fearnley and Lees, 1991). In most cases of PD, the DA cell loss is associated with the presence of eosinophilic intra neuronal inclusions, called Lewy bodies in the DA soma and neurites. These Lewy bodies are composed of α-synuclein, neurofilaments and ubiquitin (Goldman *et al.*, 1983) (Figure 1.1 C). Symptoms of the disease only appear after the loss 50–60% of the SNpc neurons and depletion of 80–85% of the DA content in the striatum (Marsden, 1994). However, the pathological changes of PD may appear much earlier, up to three decades before the manifestation of any clinical signs. The cause of PD is probably multifactorial, with contributions from genetic predisposition, environmental toxins and ageing.

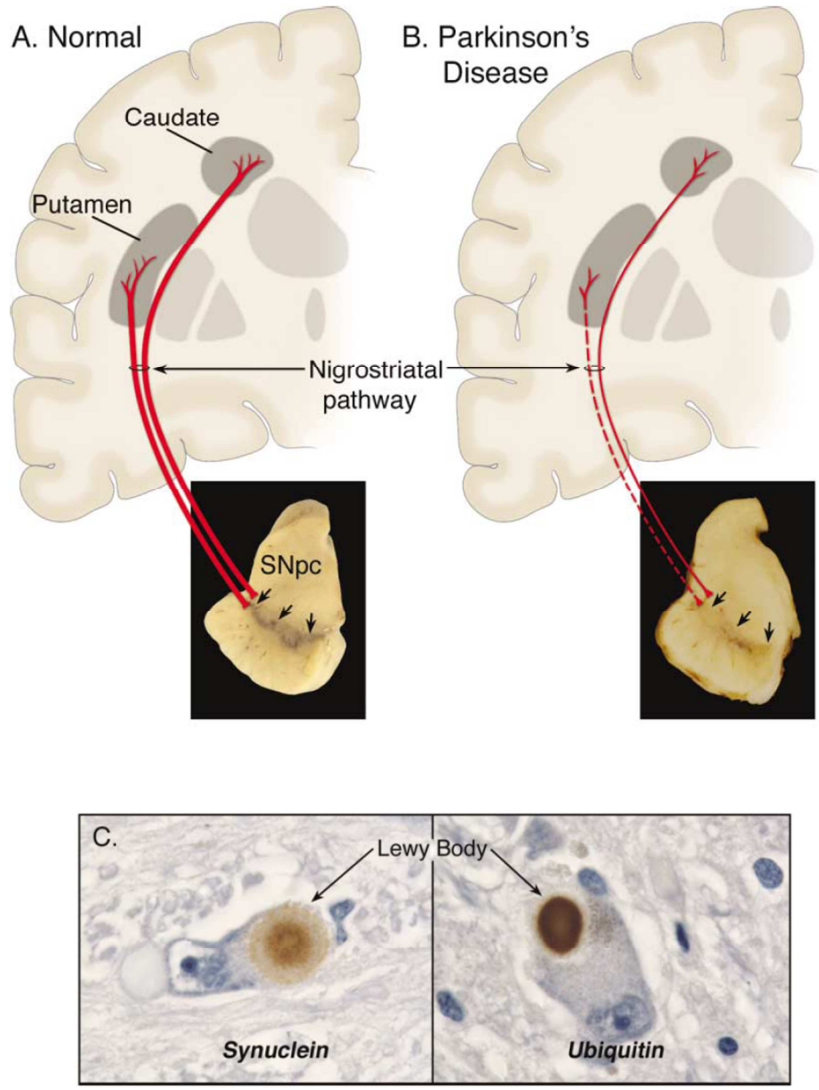

Figure 1.1 Neuropathology of Parkinson's disease (PD)

A. Schematic representation of the nigrostriatal pathway in normal individuals (shown in red). It is composed of DA neurons whose cell bodies are situated in the substantia nigra pars compacta (SNpc; shown in black arrows); these neurons project (thick solid red lines) to the basal ganglia and synapse in the striatum (i.e., putamen and caudate nucleus). The photograph shows normal pigmentation of the SNpc region due to the presence of neuromelanin within the DA neurons (this dark-brown pigment is not seen in rodents).

B. Schematic representation of the degenerated nigrostriatal pathway in PD patients (shown in red). There is a substantial loss of SNpc DA neurons (depigmentation or loss of neuromelanin can be seen in the area marked with black arrows) that project to the putamen (dashed red line) and a much more modest loss of those that project to the caudate (thin red solid line).

C. Immunohistochemical labeling of intraneuronal inclusions, termed Lewy bodies, in a SNpc dopaminergic neuron. Immunostaining with an antibody against α-synuclein reveals a Lewy body (black arrow) with an intensely immunoreactive central zone surrounded by a faintly stained peripheral zone (photograph on the left). Immunostaining with an antibody against ubiquitin yields more diffuse immunoreactivity within the Lewy body (photograph on the right). (Figure obtained from a review by Dauer and Przedborski, 2003).

1.2 Dopamine (DA) and its metabolism

Dopamine (DA) is a catecholamine, which functions as a neurotransmitter in the central nervous system (CNS) and as a hormone outside the CNS. In the brain, DA is synthesized and released by DA neurons. DA acts as a precursor in the synthesis of other neurotransmitters namely epinephrine and norepinephrine. DA is synthesized in the cytoplasm, by the action of tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC) (illustrated in Figure 1.2). After its synthesis DA is packaged in to presynaptic vesicles by VMAT2 (vesicular monoamine transporter 2) and in response to an action potential these DA filled vesicles fuse with the presynaptic membrane to release DA in to the synaptic cleft, which then binds to postsynaptic D1 and D2 receptors to exert its action. Unused synaptic DA is taken up into the presynaptic terminal via DA transporter (DAT) and re-stored in vesicles by VMAT2 (see Figure 1.3).

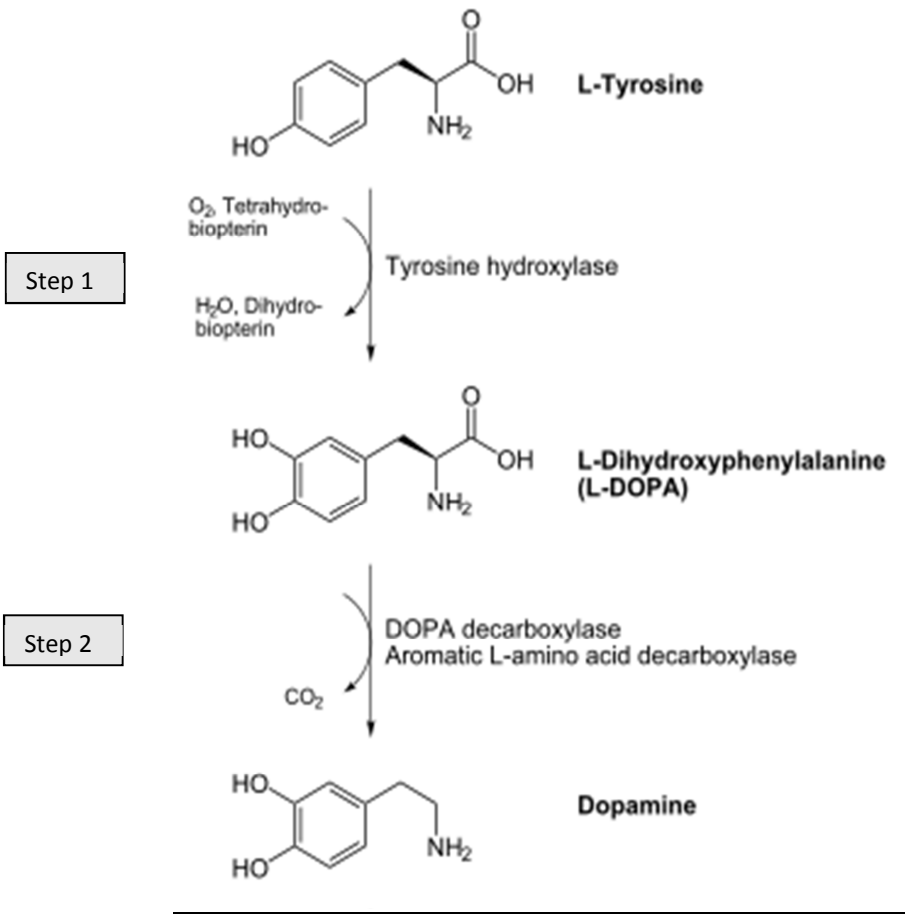

Figure 1.2 DA synthesis is a two-step process

Step1. L-Tyrosine is converted to L-Dihydroxyphenlyalanine (L-DOPA) by tyrosine hydroxylase (TH).
Step2. L-DOPA is then converted to DA by DOPA decarboxylase (which is an aromatic L-aminoacid decarboxylase)

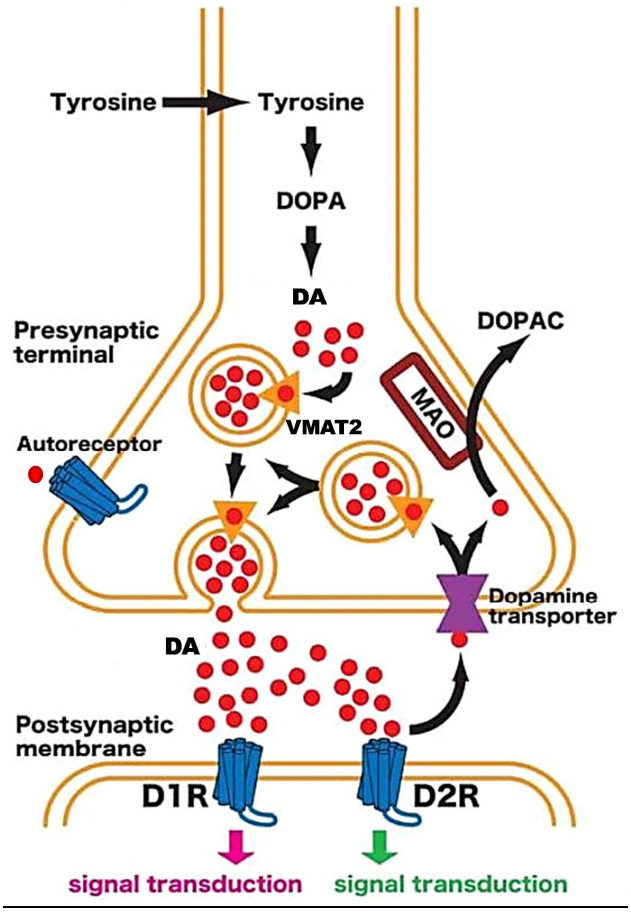

Figure 1.3 Schematic representation of DA release and uptake at the striatal DA synapse

DOPA = 3,4-dihydroxyphenylalanine; DA = Dopamine; VMAT2 = vesicular monoamine transporter 2; MAO = Monoamine oxidase; DOPAC = 3,4-dihydroxyphenylacetic acid; D1R = D1 receptor subtype; D2R = D2 receptor subtype (which is also autoreceptor on the presynaptic DA neuron). (Image source http://www.nibb.ac.jp/annual_report/2004/img/240-01.jpg)

Excess intracellular or synaptic DA should be degraded rapidly to prevent oxidation and other deleterious effects of this highly reactive compound. In case of excess intracellular DA levels, monoamine oxidase (MAO) metabolizes DA to corresponding aldehyde, which in turn is converted to a nontoxic and diffusible metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase (ALDH). Catechol-O-methyl-transferase (COMT) in the synapse converts the diffused DOPAC to homovanillic acid (HVA) - the final end product of DA metabolism. Small fraction of cytosolic DA, due to autoxidation can also form highly reactive DA quinones (DAQ), which can cause damage to the cells. Excess of DA in the synapse can be converted to 3-methoxytyramine (3-MT) by COMT. The 3-MT then can diffuse to the presynaptic neuron where it is metabolized by MAO to corresponding aldehyde and then by ALDH to HVA (depicted in Figure 1.4 A and B). Presynaptic DA neurons also possess auto receptors for DA to regulate its own synthesis, release, reuptake and degradation (shown in Figure 1.3).

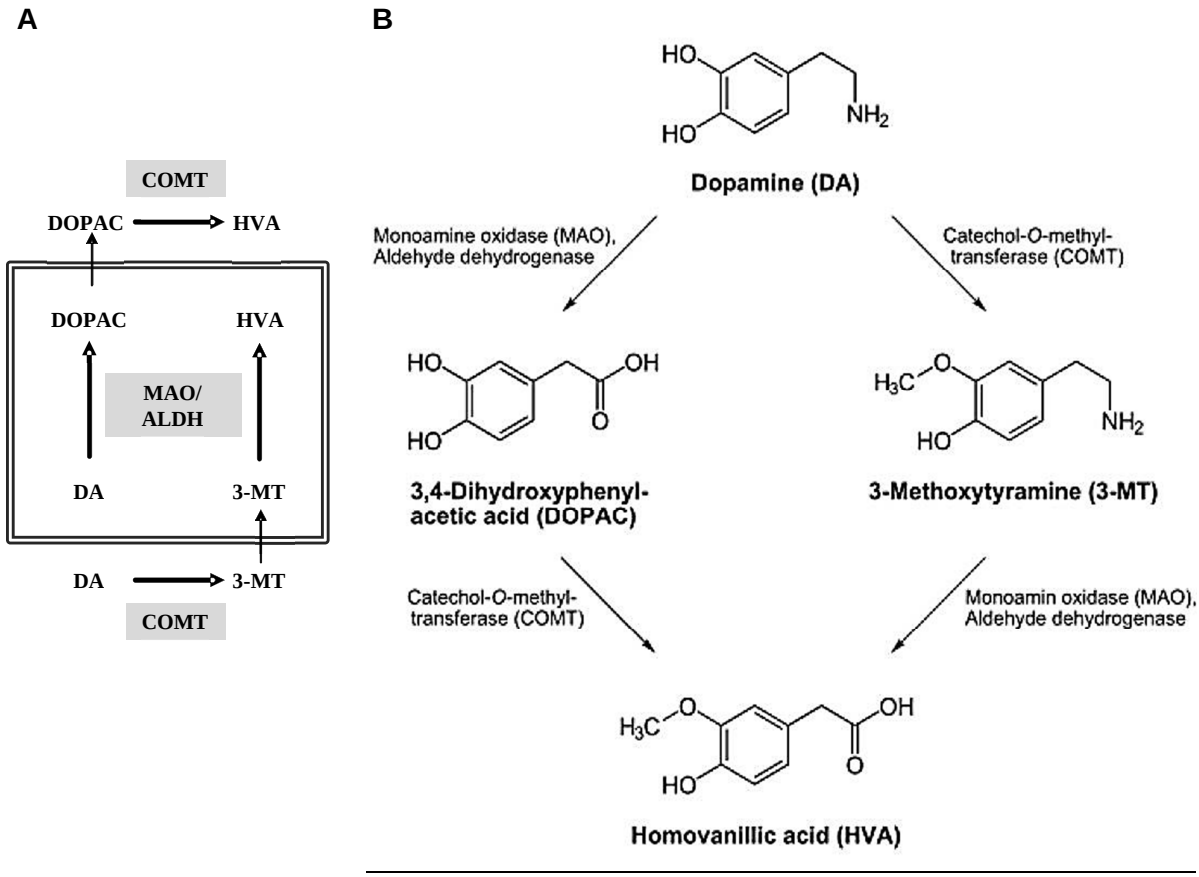

Figure 1.4 Enzymatic degradation of DA

A. Events of DA degradation occurring inside and outside the DA neuron are depicted. Monoamine oxidase (MAO), aldehyde dehydrogenase (ALDH) and Catechol-O-methyl-transferase (COMT) are the key enzymes that play a role in forming Homovanillic acid (HVA) - the end product of DA degradation pathway. Double lines represent cell membrane, thick arrows indicate enzymatic reactions and thin arrows indicate diffusion.
B. Degradation pathway shown with chemical structures

1.3 Dopaminergic neurons

DA neurons comprise less than 1% of all neurons in the brain. The DA neurons in the mammalian brain are classified into nine distinctive cell groups from A8 to A16, which was introduced by Dahlström and Fuxe in 1964 (Dahlström and Fuxe, 1964), these cell groups are distributed from the mesencephalon to the olfactory bulb (as illustrated schematically in sagittal view of the adult rat brain in Figure 1.5).

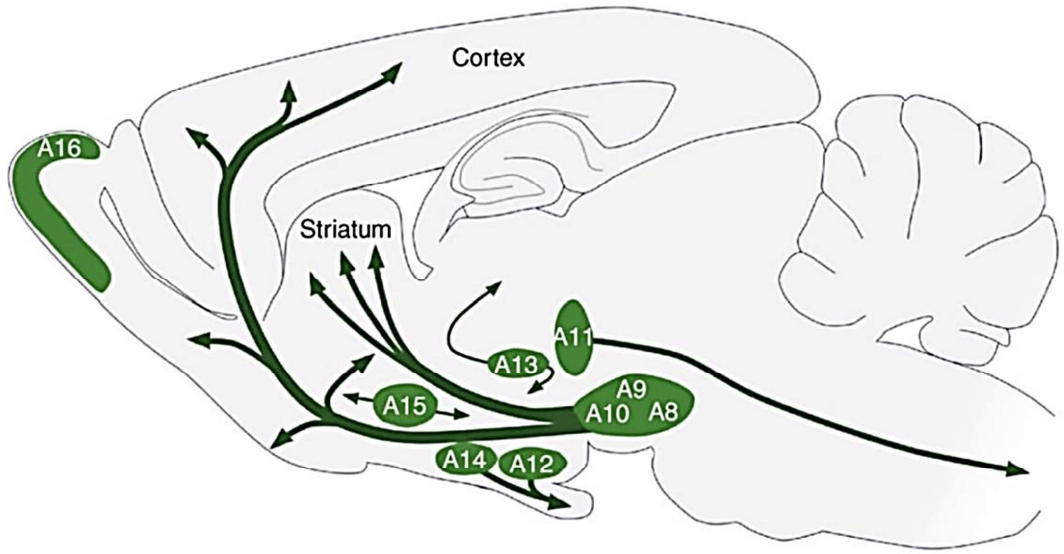

Figure 1.5 Distribution of DA neuron cell groups in the adult rodent brain

The DA neurons in the mammalian brain are localized in nine distinctive cell groups, distributed from the mesencephalon to the olfactory bulb, as illustrated schematically, in a sagittal view, in the adult rat brain. The principal projections of the DA cell groups, A8 to A16 are illustrated by arrows. (Figure obtained from Björklund and Dunnett, 2007). Midbrain comprises of three DA cell groups – A8 in the retrorubral field (RRF), A9 in the substantia nigra pars compacta (SNpc) and A10 in the ventral tegmental area (VTA); A11 cell group in the posterior thalamus projects to the spinal cord; the hypothalamic cell groups periventricular nucleus (A14) and arcuate nucleus (A12) sending projections to the pituitary gland; cells of the zona incerta belong to A13 cell group which diffusively innervates areas of the hypothalamus; cells in the pre-optic area and rostral hypothalamus comprises the A15 cell group; and A16 cell group are the periglomerular interneurons in the olfactory bulb.

1.3.1 Development of midbrain DA neurons

Midbrain DA neurons form roughly during the midpoint of embryonic development. These neurons originate from a single group of cells that arises at the mesencephalic-diencephalic junction. The multi-stage developmental process which leads to the formation of the adult midbrain DA neurons is marked by the identification and influence of distinct factors, both extrinsic and intrinsic. The early progenitor cells can be specified to become DA neurons under the influence of extracellular secretary signaling proteins like sonic hedgehog (Shh), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF8), Wnt1 and Wnt5a. The transcriptional factors which are identified to be important for the development of DA neurons include Nurr1, Lmx1a/Lmx1b, Pitx3, Otx2, EN1/En2, Pax2/Pax3/Pax5/Pax7/Pax8, Gli1/Gli2/Gli3, Gx2, Nkx etc., (Smidt and Burbach, 2007). All these factors have specific functional roles in the development of DA neurons, for e.g. Nurr1 for neurotransmitter synthesis (Zetterström *et al.*, 1997), Lmx1b for Pitx3 expression and Pitx3 is shown to be important for the development and survival of mesencephalic DA neurons (Nunes *et al.*, 2003). The DA neurons in the midbrain develop in two distinct regions, the SNpc and the VTA. In order to function properly, these neurons navigate and project their axons correctly

to their target areas with help of attractive and repulsive path finding cues like Netrin-DCC (deleted in colorectal cancer), Robo-Slit and semaphorin–plexin–neuropilin (Pasterkamp and Kolodkin, 2003; Smidt and Burbach, 2007) together with consecutive expression of certain heparin sulphate proteoglycans (HSPG) (Holt and Dickson, 2005). The DA neurons developed in the SNpc normally project to the neostriatum, whereas the VTA neurons form connections with striatal, cortical and limbic areas. Even though NTFs (GDNF, CDNF, MANF etc.,) are able to protect and repair lesioned DA system *in vivo*, their role in the development of DA neurons is not clear yet (reviewed by Andressoo and Saarma, 2008). After the differentiation into DA neurons and establishing their connections in their target striatal regions, the nigrostriatal pathway is further modified during two phases of naturally occurring programmed cell death (Burke, 2003). The first phase takes place shortly after birth and reaches its peak at postnatal day two. Whereas, the second phase of apoptosis occurs two weeks after birth, and lasts for about a week. Thus, a major fraction of SN neurons are eliminated during naturally occurring postnatal cell death and it is during this period that pro-survival or trophic signals secreted by the target area (the striatum) might actively regulate the shaping of nigrostriatal system with exact number of DA neurons. Several trophic factors have been shown to have neurotrophic effects on developing SN neurons, and might therefore be candidates for 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).

Identification of different factors involved in the DA neuronal formation not only improved our understanding of the pre- and post-mitotic development of these neurons, but also facilitated the development of novel approaches to derive DA neurons from stem cells (Wallén and Perlmann, 2003). Recent advancements have been made to generate stem cells from the fibroblasts of PD patients and these induced pluripotent stem cells (iPSCs) can be programmed to develop DA neurons with the help of different transcription factors (Chen *et al.*, 2011; Jung *et al.*, 2012). The patient iPSC derived DA neurons are valuable tools to understand the disease and to develop disease modifying therapies.

1.3.2 Distribution of midbrain DA neurons

The mesencephalon contains two major DA neuronal groups A9 (in the SNpc) and A10 (in the VTA) groups (shown in Figure 1.5). Neurons that belong to the A9 group are thought to project to the dorsal striatum (in rodents) through the nigrostriatal pathway, and the A10 group project to limbic and cortical areas to form the mesolimbic and mesocortical pathways respectively. This is now considered as an oversimplification, the SNpc neurons also have cortical and limbic connections in addition to their striatal innervation. The A10 neurons of the VTA also have connections to the ventral striatum and to the ventro-medial part of the head of the neostriatum in rodents. The A8 group neurons of the retrorubral field (which are located dorso-caudal to the A9 group) also connect to the striatal, cortical and limbic areas (Bentivoglio and Morelli, 2005).

The number of DA neurons in the midbrain varies greatly from rodents to primates and humans. Total number of neurons (bilaterally) expressing the DA neuronal marker tyrosine hydroxylase (TH) in the midbrain region of mice is nearly 25000, rats is about 45000, non-human primate (macaca monkey) is 165000 and humans (at 40-50 of age) is 600000 (German

& Manaye, 1993). More than 70% of the midbrain TH positive cells of non-human primates and humans are located in the SN, whereas this percentage is somewhat reduced (up to 60%) in rodents. However, the number of TH expressing neurons decreases in non-human primates and humans with age (Bogerts, Hantsch, & Herzer, 1983), these age-dependent changes are not observed in rodents, perhaps due to their short life span. The differences in the number of DA neurons from rodents to primates already explain the increasing anatomical and functional complexity of the brain in the latter. Unlike in rodents, the mesencephalic neurons of the primates innervate more to the cortical areas. In rodents, the cortical DA innervation is largely confined to areas of the frontal, cingulate and entorhinal cortex; whereas in primates, the mesencephalic DA neurons (the nigral neurons in particular) project to almost all cortical areas (Williams and Goldman-Rakic, 1998; Erickson *et al.*, 2000; Lewis *et al.*, 2002; Bentivoglio and Morelli, 2005).

The DA cell groups in the retrorubral field, SNpc and VTA regions can be divided in to dorsal and ventral tier based on their connectivity and morphology. The dorsal tier neurons innervate to the dorsal and ventral areas of the striatum, to cortical and limbic areas (Gerfen *et al.*, 1987a, 1987b; Lynd-Balta and Haber, 1994; Valente *et al.*, 2004b; Bentivoglio and Morelli, 2005). The dorsal tier neurons are round or fusiform in shape, express calbindin (a calcium binding protein) and relatively low levels of DAT (DA transporter) protein. The ventral tier neurons innervate to the striatum, dendrites of most of these cells extend ventrally to the neighboring SNpr region (Prensa *et al.*, 2009). The ventral tier neurons are angular in shape, in contrast to the dorsal tier neurons these cells do not express calbindin, express high levels of DAT protein and also express high levels of ion channel protein GIRK2. As described, the mesencephalic DA neurons exhibit complex innervating patterns; they differ in morphology as well as molecular markers they express (reviewed in Björklund and Dunnett, 2007). In patients with PD, the ventral tier DA neurons that express high levels of GIRK2 preferentially undergo degeneration (Yamada *et al.*, 1990; Liang *et al.*, 1996).

1.4 Genetics of PD

Identification of PD causing gene mutations is one of the major advances in PD research. Genetic causes contribute to 10% of all the PD cases, the remaining and vast majority are sporadic, without any family history and genetic defects. Understanding the function of monogenic forms of PD has provided valuable insights in studying the underlying molecular mechanisms that lead to the selective neurodegenerative process in PD. Monogenic PD-causing genes exhibits autosomal dominant (AD) or autosomal recessive (AR) forms of inheritance; mutations in α-synuclein and LRRK2 cause autosomal dominant PD, whereas parkin, DJ-1 and PINK1 are examples of autosomal recessive forms of the disease (more PD-linked genes are shown in the Table 1.1). Recent genome-wide association studies (GWAS) have confirmed that α-synuclein and leucine-rich repeat kinase 2 (LRRK2) causes PD and microtubule-associated protein tau (MAPT) gene loci as a risk factor to cause PD (Simón-Sánchez *et al.*, 2009). GWAS and exome approaches look promising in providing novel PD-associated loci in the near future. For example, it was demonstrated recently that common variants at the major histocompatibility complex class II, DR alpha (HLA-DRA) (Hamza *et al.*, 2010); bone marrow stromal cell antigen (BST1); and PARK16 loci as risk factors for PD

(Satake *et al.*, 2009; Simón-Sánchez *et al.*, 2009). Most cases of PD probably result from an intricate interaction of environmental and genetic factors; however, there is very limited information about gene-environment interactions. Future studies must be aimed at collecting information on both environmental exposures and genetic polymorphisms in relevant genes in large number of samples to enable the detection of these interactions.

Locus	Inheri-tance	Chromosome	Gene	Name of protein	Protein function	Pathology
PARK1	AD	4q21-23	*SNCA*	α-synuclein	Synaptic protein	LB+
PARK2	AR	6q25.2-q27	*PRKN*	Parkin	Ubiquitin-protein ligase	Pleomorphic (most LB-)
PARK3	AD	2q13	*SPR?*	Aldo-keto reductase?	Unknown	Unknown
PARK4	AD	4q21-23	*SNCA*	α-synuclein	Excess of α-synuclein protein	
PARK5	AD	4p14	*UCHL1*	UCHL-1	Hydrolyze small C-terminal adducts of ubiquitin	Unknown
PARK6	AR	1p36-p35	*PINK1*	PINK1	Mitochondrial kinase	Unknown
PARK7	AR	1p36	*DJ-1*	DJ-1	Oxidative stress protection	Unknown
PARK8	AD	12p11-q13	*LRRK2*	LRRK2	Muliple functions by several domains	Pleomorphic (LB+, tau+, ub+)
PARK9	AR	1p36	*ATP13A2*	ATPase type 13A2	Lysosomal protein	Unknown
PARK11	AD	2q37.1	*GIGYF2?*	GRB10 inte-racting GYF protein 2	Unknown	Unknown
PARK13	AD?	2p12	*OMI/ HTRA2*	HtrA serine peptidase 2	Serine protease+	Unknown
PARK14	AR	22q13.1	*PLA2G6*	A2 phospho-lipase	Phospholipid remodelling+	Unknown
PARK15	AR	22q12-q13	*FBXO7*	F-box protein 7	Phosphorylation-dependent ubiquitination	Unknown

Table 1.1 List of PD associated genes

PARK locus, mode of inheritance, chromosomal location, gene name, encoded protein, presumable function and associated pathology of each of the identified (in some cases suspected) genes are listed (Table obtained from web, source: http://dx.doi.org/10.5124/jkma.2011.54.1.70)
AD = Autosomal Dominant; AR =Autosomal Recessive; LB+ = lewybody positive; tau+= tau positive; ub+ = ubiquitin positive.

Five genes – α-synuclein, parkin, PINK1, DJ-1 and LRRK2 have been clearly linked to PD (discussed in this section) and a number of other genes or genetic linkages have been

identified that may cause PD (shown in the table 1.1). The identification of these genes has led to new insights and direction in understanding the molecular mechanisms underlying PD pathogenesis.

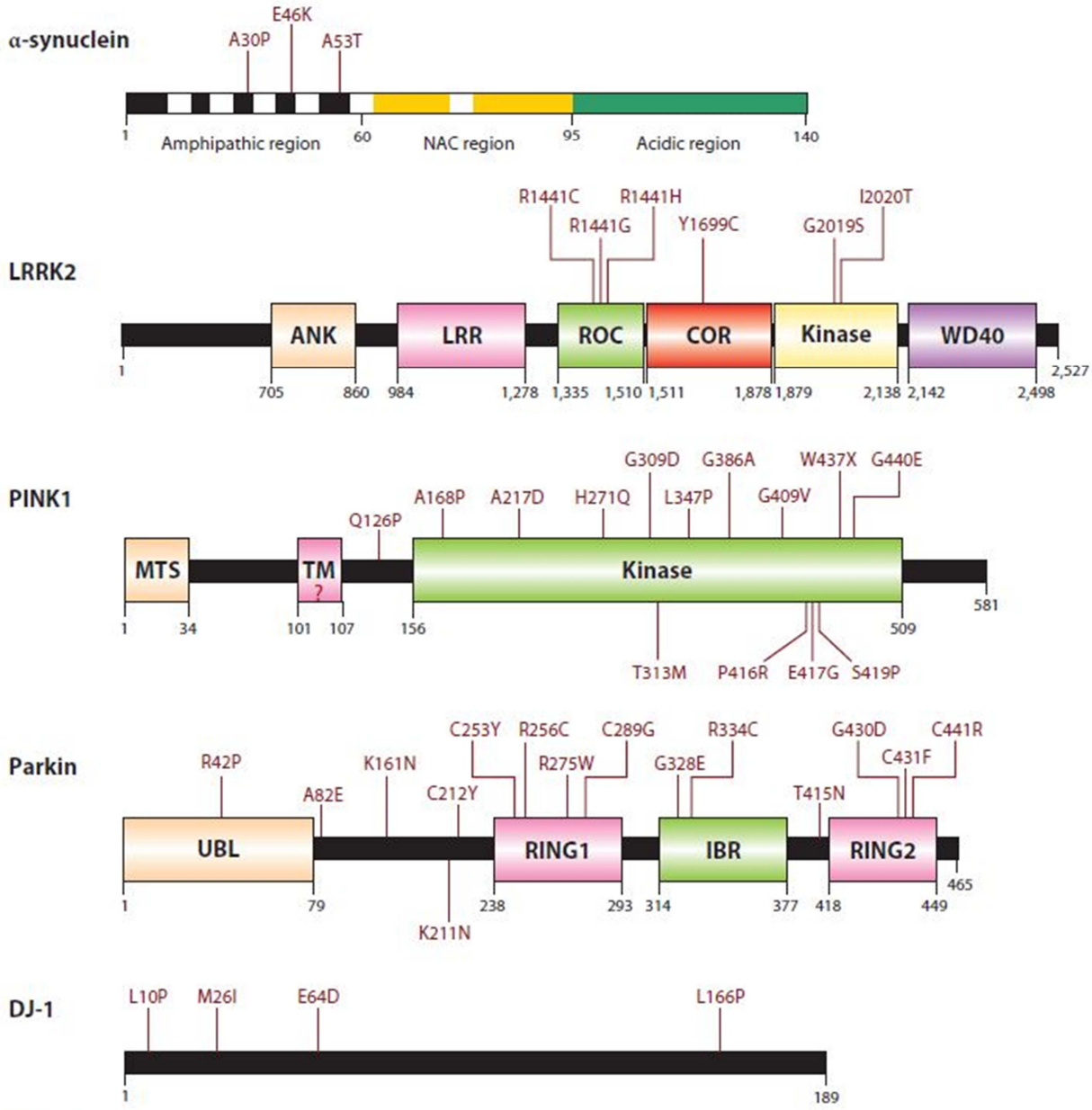

Figure 1.6 Important PD-related protein domains

Domains are always arranged from N-terminus to C-terminus. Numbers under the protein indicate domain boundaries. Mutations that segregate with PD are annotated at their approximate position (above) along the protein's length. For parkin, the recently identified RING0 domain is not shown here. (Figure obtained from Martin et al., 2011)

Genetic rodent models have made a significant contribution to the understanding of physiological functions of the major PD-associated proteins, namely α-synuclein, parkin,

PINK1, DJ1 and LRRK2 (Lee *et al.*, 2012). Details of these models are explained in the coming sections (1.4.1-1.4.5) with a particular emphasis on the parkin protein. Several research studies revealed that these PD-associated genes play important cellular roles, such as to support function of mitochondria, the ubiquitin-proteasomal system, the autophagy-lysosomal pathway and membrane trafficking (reviewed by Corti et al., 2011; Farrer, 2006; Martin et al., 2011; Shulman et al., 2011). However, the precise functions of these genes in health and disease remain poorly understood.

1.4.1 Alpha-synuclein (α-synuclein)

α-synuclein is a small protein with 140 AA (19 kDa) encoded by the SNCA gene. Point mutations (PARK1) or duplications or triplications (PARK4) of the SNCA gene are linked to familial PD and other synucleinopathies. α-synuclein was identified to be the principal component of Lewy bodies (cytoplasmic inclusions) observed in the surviving SNpc DA neurons of PD patients, even before its association with PD was known (Spillantini *et al.*, 1997). α-synuclein belongs to synuclein family members together with β and γ synucleins, identified in humans. All three synucleins are neuronal proteins preferentially localized in the presynaptic terminals under physiological conditions (George, 2001). The α-synuclein missense mutations so far identified in PD patients include Ala53Thr (A53T) (Polymeropoulos, 1997), Glu46Lys (E46K) (Zarranz *et al.*, 2004) and Ala30Pro (A30P) (Seidel *et al.*, 2010). The mutant patients' autopsies showed SNpc DA cell loss and accumulation of the α-synuclein protein in SN neurons (together with other neuronal populations), a phenotype showing a strong overlap with typical sporadic late-onset PD (Gasser, 2009) with some exceptions.

Structurally α-synuclein protein consists of three discrete regions (depicted in Figure 1.6): (1) an amino terminus (1-60 AA) – with apolipoprotein binding motifs, which confers the α-helical potential (2) a central hydrophobic region (61-95 AA) – the NAC (non-amyloid β component), conferring the β-sheet potential (3) a highly acidic carboxy terminus (see the Figure 1.8). The functional role of the two known shorter splice variants of the SNCA gene transcript have not been characterized well in physiological and pathological conditions (Maroteaux *et al.*, 1988; Maroteaux and Scheller, 1991; UEDA *et al.*, 1993). Deletion of a stretch in the NAC region (71-82 AA) prevents aggregation of α-synuclein overexpression, whereas overexpression of the NAC leads to aggregation and neurotoxicity *in vivo* (Periquet *et al.*, 2007). The missense A53T and A30P mutations promote aggregation of the protein (Dauer and Przedborski, 2003). Post translational modifications of α-synuclein, specifically phosphorylation and nitration, were shown to enhance aggregation (Giasson *et al.*, 2000; Fujiwara *et al.*, 2002)

1.4.2 LRRK2

Leucine-rich repeat kinase 2 (LRRK2) (also known as Dardarin) is a large protein (288 kDa) encoded by the PARK8 gene in humans, mutations in this gene is associated with PD and Crohn's disease. LRRK2 is an enzyme that belongs to the leucine-rich repeat kinase family. Structurally, LRRK2 protein contains several domains which include an ankyrin repeat region, a leucine-rich repeat (LRR) domain, a Roc (Ras of complex protein) domain that

shares homology with Ras-related GTPase superfamily, a COR (C-terminal of Roc) domain, a mitogen-activated protein kinase kinase kinase (MAPKKK) domain, and a C-terminal WD40 repeat domain (shown in Figure 1.6). The LRRK2 protein is localized in the Golgi apparatus, lysosomes, synaptic vesicles and on the outer mitochondrial memebrane (Biskup *et al.*, 2006; Galter *et al.*, 2006; Simón-Sánchez *et al.*, 2006; Taymans *et al.*, 2006; Higashi *et al.*, 2007). The neuropathological observations in PD patients with LRRK2 mutations are quite variable, most of them show LB pathology (as found in α-synuclein mutants), whereas tau and ubiquitin or LRRK2 positive inclusions were also observed, but less common (Zimprich *et al.*, 2004; Giasson *et al.*, 2006). The exact role of LRRK2 in cells remains largely unknown; however, recent studies suggest its potential function in mitogen-activated protein kinase (MAPK) pathways, protein translation control, programmed cell death pathways and activity in cytoskeleton dynamics (reviewed by Webber and West, 2009).

1.4.3 DJ-1

Deletion or missense mutations in DJ-1 gene are found to cause familial PD in Dutch patients (Bonifati *et al.*, 2003). Levels of DJ-1 are found to be increased in the frontal cortex of PD and AD patients compared to normal controls (Choi *et al.*, 2006). Pathological studies state that none of the Autosomal Recessive Juvenile PD (AR-JP) patients examined so far carries DJ-1 mutations. However, DJ-1 is rarely seen in the LBs of sporadic PD patients (Neumann *et al.*, 2004; Rizzu *et al.*, 2004). The DJ-1 gene (park7) encodes a protein with 189 AA, the sequences of DJ-1 are conserved from prokaryotes to eukaryotes, hence named as DJ-1 superfamily (Bandyopadhyay and Cookson, 2004). Structurally DJ-1 contains a catalytic site and α-helix at the C-terminal end (as shown in Figure 1.6). This catalytic site is very similar to the monomer subunit of intracellular cysteine protease of the bacteria *Pyrococcus horikoshii* (Du *et al.*, 2000). However, α-helix at the C-terminal blocks the catalytic site (Honbou *et al.*, 2003; Lee *et al.*, 2003; Tao and Tong, 2003).

DJ-1 functions as a dimer and is ubiquitously expressed in all cells and tissues of the body including brain (Nagakubo *et al.*, 1997). Expression of DJ-1 increases in astrocytes during oxidative stress and sporadic PD (Rizzu et al., 2004). During oxidative stress astrocytes mediate neuroprotective function, knockdown of DJ-1 in astrocytes results in loss of this protective function due to deregulation of mitochondrial complex I activity and inflammation (Waak *et al.*, 2009; Mullett and Hinkle, 2011). DJ-1 is localized in the cytoplasm, nucleus and in mitochondria. It is also found to be secreted by cells and tissues including cancer cells and astrocytes. DJ-1 is a multifunctional protein that functions in transcriptional regulation, mitochondrial regulation and preventing oxidative stress reaction. Functionally, DJ-1 has been shown to have chaperone and protease activity (reviewed in Ariga et al., 2013). Upon exposure to growth factors, DJ-1 is translocated from the cytoplasm to nucleus; this nuclear translocation is dependent on critical cysteine C106 oxidation (Kim *et al.*, 2012). Mutation or irreversible oxidation (SO_3H) of C106 results in loss of DJ-1's functions (Canet-Avilés *et al.*, 2004; Kinumi *et al.*, 2004). This C106 residue of DJ-1 is found to be phylogenetically conserved throughout the DJ-1 superfamily. DJ-1 is found to be excessively oxidized in the brains of patients with PD and AD (Choi *et al.*, 2006). In addition to oxidation, DJ-1 also undergoes sumoylation, S-nitrosylation and phosphorylation. Oxidative stress-dependent

acidic shift of DJ-1 results in excessive sumoylation of DJ-1 (Shinbo *et al.*, 2006). Nitrosative stress leads to S-nitrosylation of C46 and C53 residues, this prevents dimerization of DJ-1which is important for its function (Ito *et al.*, 2006). Proteomic identification of p53 dependent phosphorylation of DJ-1 was reported (Rahman-Roblick *et al.*, 2008), however biological function of this phosphorylation is not yet clear.

1.4.4 PINK1

PINK1 (phosphatase and tensin (PTEN)-induced putative kinase 1) is a mitochondrial ser/thr protein kinase (with 581 AA) encoded by the PINK1 gene (park6). Mutations in the PINK1 gene cause L-DOPA responsive PD with an early onset (Valente et al., 2004). However, PINK1 mutations were so far not seen in post-mortem PD brains. PINK1 is ubiquitously expressed in all tissues of the body; high expression of it is seen in skeletal muscle and testis (Unoki and Nakamura, 2001; Nakajima *et al.*, 2003). PINK1 is expressed throughout the brain (Taymans *et al.*, 2006), its levels seems not to be altered due to PD pathogenesis (Blackinton *et al.*, 2007). Intracellularly, PINK1 is localized in mitochondria and also in the cytoplasm. Structurally, PINK1 consists of a mitochondrial targeting sequence (34 AA in length) and a highly conserved protein kinase domain which shows a high degree of homology to the ser/thr kinases of the calcium/calmodulin family (Valente *et al.*, 2004a) (shown in Figure 1.6). There are two PINK1 isoforms found in human brain extracts with molecular mass of 66 and 55 kDa. The smaller one (55 kDa) being the mature form derived from the larger isoform (66 kDa) (Beilina *et al.*, 2005). However the ratio of 66:55 kDa PINK1 isoforms seems to be important for normal cellular functions. Reduced 66:55 kDa ratio were detected in the SN and cerebellar brain regions of PD patients and in cellular systems with disease causing PINK1 mutants overexpression (Weihofen *et al.*, 2008). PINK1 is involved in mitochondrial response to cellular and oxidative stress (Valente *et al.*, 2004b). PINK1 together with parkin (another PD-linked protein) plays an important role in regulating mitochondrial morphology and function in response to mitochondrial stressors by promoting mitophagy (discussed in 1.7.5.3) (Exner *et al.*, 2007; Deng *et al.*, 2008; Poole *et al.*, 2008; Parachoniak and Park, 2009; Weihofen *et al.*, 2009).

1.4.5 Parkin

Parkin is an E3 ubiquitin ligase encoded by the park2 gene. Deletion or missense mutations can lead to loss of function of parkin and causes AR-JP (Kitada *et al.*, 1998; Matsumine *et al.*, 1998). Parkin has also been shown to play an important role in sporadic PD (Klein and Schlossmacher, 2007; Klein and Lohmann, 2009). Parkin can be inactivated due to nitrosative or dopaminergic or oxidative stresses (Reviewed in (Dawson and Dawson, 2010), which are commonly seen in sporadic PD. Mutations in the parkin gene can lead to changes in parkin function that result in decreased catalytic activity, unusual ubiquitination, decreased solubility or increased proteasomal degradation (Winklhofer *et al.*, 2003; Wang *et al.*, 2005; Hampe *et al.*, 2006; Matsuda *et al.*, 2006). Neuropathological studies of parkin mutant PD patients show selective DA neuronal loss accompanied by gliosis in the SNpc and locus coeruleus (Ishikawa and Takahashi, 1998). Few parkin mutated PD patients show α-synuclein containing Lewy body inclusions (Farrer *et al.*, 2001; Sasaki *et al.*, 2004; Pramstaller *et al.*, 2005); a hallmark

of PD pathology and others lack these inclusions (Ishikawa and Takahashi, 1998). There are also cases reported with Tau-neurofibrillary tangles (Mori *et al.*, 1998; van de Warrenburg *et al.*, 2001).

Parkin is a cytosolic protein with 465 AA, consists of an N-terminal ubiquitin-like (UBL) domain (which resembles an ubiquitin monomer). The UBL domain has been implicated in proteasome binding, substrate recognition, and regulation of parkin stability and two RING finger domains (RING1 and RING2) - which show zinc binding activity - at the c-terminal, these RING domains are separated by an in between ring (IBR) domain forming the RING-IBR-RING (RBR) motif and this motif is responsible for its E3 ligase activity and is found to be highly conserved in eukaryotes (Beasley *et al.*, 2007) (structure shown in Figure 1.6). An additional RING finger domain (RING0) has been identified between the UBL and RBR motifs, which also show zinc-binding activity (Hristova *et al.*, 2009) (not shown in the Figure 1.6). Binding to zinc ions confer proper 3-D conformation to parkin, mutations in the zinc binding domain causes misfolding of parkin protein (Cookson *et al.*, 2003; Gu *et al.*, 2003; Sriram *et al.*, 2005; Exner *et al.*, 2012). The RBR motif is cysteine rich, and is prone to oxidative stress induced inactivation (Winklhofer *et al.*, 2003; LaVoie *et al.*, 2007; Wong *et al.*, 2007; Schlehe *et al.*, 2008).

1.4.5.1 Ubiquitin ligase activity of parkin

The domain architecture of parkin resembles that of any E3 protein ligase which can catalyze the covalent linkage of ubiquitin to lysine residues within its substrate proteins in a process called ubiquitination (Tanaka *et al.*, 2001; Trempe *et al.*, 2013). The process of ubiquitination is a multi-step process, where a ubiquitin monomer from the active E1 enzyme is transferred to a conjugating E2 enzyme, from which the ubiquitin is finally transferred to a substrate protein by the E3 ligase. The E3 enzymes typically act in a rather substrate-specific manner. Being an E3 ligase, parkin can mediate different modes of ubiquitination, either poly- (K48 or K63 or K27 linked) or monoubiquitination (reviewed by Kulathu and Komander, 2012). The fate of substrate protein is determined by the mode of its ubiquitination. K48-linked polyubiquitination (ubiquitin linkage via Lys48) of the substrate protein results in its proteasomal degradation, whereas K63- or K27-linked polyubiquitination or monoubiquitination of the substrate protein alters its functional properties to play a wide range of regulatory functions implicated in signal transduction, DNA repair, endocytosis, and autophagy. Thus, parkin is a multifunctional E3-ligase, which has the capability of performing a variety of ubiquitin linkages and cellular functions (reviewed by Kuang et al., 2013; Kulathu and Komander, 2012).

1.4.5.2 Neuroprotective functions of parkin

Parkin is shown to be protective when overexpressed in a variety of stress induced-cellular systems and in animal models of PD. Parkin protects cells against ceramide induced mitochondrial swelling (Darios *et al.*, 2003), kainic acid excitotoxicity (Staropoli *et al.*, 2003), manganese induced cell death (Higashi *et al.*, 2004) and dopaminergic toxicity (Jiang *et al.*, 2004). Parkin overexpression reduced α-synuclein toxicity in rat, drosophila, and other cellular models (Petrucelli *et al.*, 2002; Yang *et al.*, 2003; Yamada *et al.*, 2005). Parkin

overexpression also reduced mutant LRRK2-mediated DA neuron toxicity in *Drosophila* (Ng *et al.*, 2009). Overexpression of parkin also protected SN DA neurons in mice against chronic MPTP toxicity (Yasuda *et al.*, 2011; Sheng *et al.*, 2012). Whereas, parkin-deficient mice did not show increased sensitivity to mutant α-synuclein toxicity (von Coelln *et al.*, 2006) or MPTP or 6-OHDA intoxication (Perez *et al.*, 2005; Thomas *et al.*, 2007). A recent study also uncovers protective role of parkin overexpression in AD mouse model, parkin overexpression restored activity-dependent synaptic plasticity and rescued behavioral abnormalities in APP/PS1 transgenic mice; parkin overexpression also downregulates APP protein expression, decreases β-amyloid load and reduces inflammation (Hong *et al.*, 2013).

All these findings show that overexpression of parkin may provide a nonphysiologic protection to a variety of stressors, but endogenous levels of parkin do not participate in neuronal survival to these various stressors (Reviewed in Dawson and Dawson, 2010). However, the exact molecular mechanisms through which parkin overexpression exerts protective functions are not known.

1.4.5.3 Role of parkin and PINK1 in mitochondrial integrity and mitophagy

The functional interaction of parkin and PINK1 was first observed in *Drosophila*. Parkin and PINK1 deficient flies show indistinguishable phenotypes with reduced life span, apoptotic flight muscle degeneration and subsequent motor deficits. Interestingly, parkin overexpression restored PINK1 deficient phenotype, but PINK1 overexpression could not compensate for parkin loss, indicating that parkin and PINK1 act in a common pathway with PINK1 acting upstream of parkin (Greene *et al.*, 2003; Clark *et al.*, 2006; Park *et al.*, 2006; Yang *et al.*, 2006). Similar results were observed in cultured human cells: parkin overexpression could prevent mitochondrial alterations induced by PINK1 deficiency (Exner *et al.*, 2007; Lutz *et al.*, 2009). In cultured cells, parkin and PINK1 knockdown show similar mitochondrial defects including mitochondrial fragmentation, decreased mitochondrial membrane potential and reduced ATP production (Exner *et al.*, 2007; Dagda *et al.*, 2009; Lutz *et al.*, 2009; Sandebring *et al.*, 2009). Direct interaction between parkin and PINK1 has been observed in SH-SY5Y cell lysates (Geisler *et al.*, 2010b), lysates from striatum and SN of rats (Um *et al.*, 2009), and human brain lysates (Sha *et al.*, 2010). It has been shown that parkin marks PARIS for proteasomal degradation to increase the expression of PGC-1α, which is essential for mitochondrial biogenesis (Shin *et al.*, 2011); so far, PINK1 is not implicated in this pathway. Parkin can also prevent proapoptotic BH3 domain-mediated cytochrome c release, whereas PINK1 cannot (Berger *et al.*, 2009). All these additional roles of parkin argue for PINK1 independent protective functions of parkin.

Autophagy is a process in which cytoplasmic material including organelles are sequestered in an autophagosome, which later fuses with the lysosome for eventual degradation. The process of autophagy can be selective or nonselective. Selective autophagy requires labeling of the organelles (eg., via ubiquitination) and binding of adaptor proteins that are required for the initiation of autophagy. Dysfunction of autophagic mechanisms has been reported in several neurodegenerative disorders (reviewed in (Levine and Kroemer, 2008; Chu, 2010; García-Arencibia *et al.*, 2010; Komatsu and Mizushima, 2011). In cell culture models of PD, dysfunction of selective autophagy of mitochondria (‘mitophagy’) has been reported

(Narendra *et al.*, 2008). Upon acute treatment of exogenous parkin-expressing HeLa cells with mitochondrial uncoupler - CCCP (carbonyl cyanide 3-chlorophenylhydrazone), Narendra et al 2008, have observed a robust recruitment of parkin to the uncoupled mitochondria. However, prolonged CCCP treatment resulted in the clearance of the damaged mitochondria (mitophagy) in parkin-expressing cells compared to normal parkin non-expressing HeLa cells. A recent study demonstrated that ER and impaired mitochondria contact regions serve as initiation platforms for omegasome generation and subsequent bit-by-bit, parkin-mediated mitophagy in parkin overexpressing HeLa and COS7 cells (Yang and Yang, 2013).

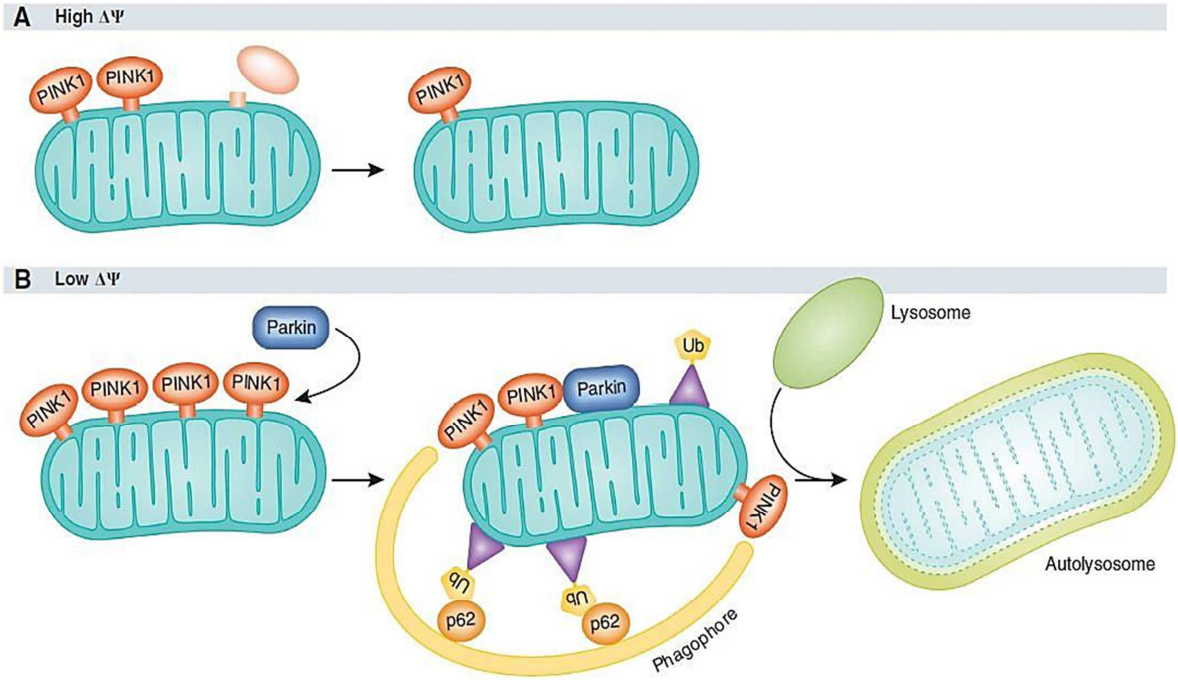

Figure 1.7 PINK1 and parkin induced mitophagy

(A) Under basal conditions (high membrane potential), PINK1 is imported into the mitochondria where it is proteolytically processed and rapidly degraded.

(B) Under conditions of low membrane potential (for eg. after CCCP treatment), the full length PINK1 gets accumulated on the damaged mitochondria and recruits parkin. Then, parkin ubiquitinates outer mitochondrial membrane proteins (like VDACs), allowing adaptor proteins like p62 to link the ubiquitinated cargo to autophagic machinery. Thus damaged mitochondria are surrounded by autophagosomes, which then fuse with lysosomes to form autolyosomes, ultimately resulting in the degradation of their content. (Figure obtained from Exner et al., 2012).

Several studies have demonstrated the essential role of full-length PINK1 in CCCP-induced mitochondrial recruitment of parkin and subsequent mitophagy (demonstrated in Figure 1.7). In PINK1-silenced cells, PINK1 ko mouse fibroblasts and 'kinase dead' PINK1 mutant-expressing cells, CCCP treatment does not result in parkin being recruited to the damaged mitochondria (reviewed by Pilsl and Winklhofer, 2012). PINK1-dependent mitochondrial recruitment of parkin might also require other proteins. Upon PINK1-dependent recruitment

of parkin to the damaged mitochondria, parkin ubiquitinates mitochondrial outer membrane proteins such as VDAC1 (Geisler *et al.*, 2010a) and mitofusin 1 and 2 (Gegg *et al.*, 2010; Glauser *et al.*, 2011) and the type of ubiquitination can be either degradative or non-degradative. Degradative ubiquitination (K48-linked) of mitofusin 1 and 2 prevents mitochondrial fusion, leading to isolation of the damaged mitochondria to promote their mitophagy. The non-degradative ubiquitination, in case of VDAC1 serves as a signal for the autophagic machinery, for the recruitment of adaptor proteins like p62 which links the ubiquitinated mitochondria to the autophagic machinery to form phagophores, which later mature to autophagosome and fuse with lysosome to form autolyosome, which eventually leads to degradation of their content (reviewed in Exner et al., 2012). However, degradation of mitofusin1 or 2 seems not be a pre-requisite for mitophagy as cells derived from MFN1/2 double ko mice show parkin mediated mitophagy (Tanaka, 2010). It was also shown that VDACs (1, 2 and 3) can function redundantly, in the absence of all the three VDACs the recruitment of parkin and subsequent mitophagy is impaired in parkin overexpressing HEK 293 cells after CCCP treatment (Sun *et al.*, 2012).

Additionally, PINK1 and Parkin can also regulate mitochondrial trafficking and quarantine damaged mitochondria by detaching them from the microtubule network. In cells with healthy mitochondria, Miro and its binding partners connect mitochondria to microtubules, facilitating trafficking of the organelles to various cellular locations. Upon mitochondrial damage, PINK1 is stabilized on the outer membrane, resulting in the recruitment of Parkin forming the PINK1/parkin/Miro complex, leading to subsequent phosphorylation and ubiquitination of Miro for its proteasomal degradation; damaged mitochondria thus segregated undergo mitophagy (Youle and Kane, 2011).

1.4.5.4 Mitophagy is dispensable for mitochondrial maintenance function of parkin

A recent study (Müller-Rischart *et al.*, 2013) revealed that parkin can perform its stress-protective function in the absence of mitophagy by depending on NEMO (NF-κB essential modulator), the core regulatory component of the IκB kinase complex essential for classical NF-κB signaling (Makris *et al.*, 2000; Schmidt-Supprian *et al.*, 2000)Parkin can ubiquitinate (K-63 linked) NEMO, leading to an increased expression of OPA1, a mitochondrial GTPase that plays a key role in mitochondrial inner membrane fusion and preventing apoptosis (Olichon *et al.*, 2003; Frezza *et al.*, 2006). Thus, parkin not only plays an important role in maintaining mitochondrial network by promoting fusion mechanisms, but also has a prosurvival role. Consistent with this notion, OPA1 overexpression could prevent parkin silencing induced mitochondrial fragmentation in cultured cells (Lutz *et al.*, 2009). Supporting the existence of two mutually exclusive mitochondria related protective functions of parkin; in NEMO- or OPA1-deficient cells, parkin-induced mitophagy is not impaired (Müller-Rischart *et al.*, 2013).

Altogether, parkin is thought to act in different ways to handle cellular stress. Under moderate stress conditions, parkin can activate NF-κB signaling pathway to deal with mild mitochondrial defects. However, in severe stress conditions when mitochondria are irreversibly damaged, parkin eliminates damaged mitochondria via mitophagy.

1.4.5.5 Putative parkin substrates

In addition to the above discussed parkin substrates, several studies have identified putative substrates of parkin to provide more insights to its E3 ligase function. The first identified putative parkin substrate *in vitro* is CDCre-1, which belong to a family of GTPases called septins that are highly expressed in the nervous system (Zhang *et al.*, 2000). However there is no *in vivo* evidence for CDCre-1 being a substrate for parkin (Periquet *et al.*, 2005). Pael-R (Parkin-associated endothelin receptor-like receptor) is also considered as a putative parkin substrate. Pael-R is a G protein coupled transmembrane protein that is highly expressed in oligodendrocytes and DA neurons. Overexpression of human Pael-R makes it insoluble and is shown to induce DA neurodegeneration in *Drosophila* (Yang *et al.*, 2003). Parkin with its E3 ligase function is thought to mark the Pael-R for degradation to protect DA neurons; however, there is no *in vivo* evidence for this functional relationship. Parkin is shown to attach K-63 linked polyubiquitin chains to another protein called syniphilin-1 (interacting partner of α-synuclein) (Lim *et al.*, 2005). Upon overexpression in cell culture, parkin, syniphilin-1 and α-synuclein forms protein aggregates (Chung *et al.*, 2001)which are then cleared by autophagic mechanisms.

FBP-1 (far up stream element binding protein 1) and AIMP2 (Aminoacyl-tRNA synthetase-interacting multifunctional protein type 2) are shown to accumulated in patients with AR-PD due to parkin mutations and also in sporadic PD. FBP1 functions as an ATP-dependent DNA helicase, whereas AIMP2 is reported to be present in Lewy bodies These proteins FBP-1 and AIMP2 which are substrates of parkin undergo K-48 linked polyubiquitination for proteasomal degradation and are reported to accumulate not only in parkin null mice and also in MPTP treated mice (in which parkin is inactivated due to S-nitrosylation) (Ko *et al.*, 2005, 2006). These *in vivo* reports suggest that FBP-1 and AIMP2 are likely to be true parkin substrates (reviewed in Dawson and Dawson, 2010)

Parkin is thought to play a role in microtubule stabilization by ubiquitinating its substrates, the α/β tubulin heterodimers and microtubules. Synaptotagmin XI and SEPT5_v2/CDCrel-2 (another member of the septin family) were also reported as parkin substrates, also found to be accumulated in diseased brains (Choi *et al.*, 2003; Yang *et al.*, 2005). *In vitro* studies report that parkin can modulate the function of cyclin E (Staropoli *et al.*, 2003) and RanBP2 (Um and Chung, 2006) via its ubiquitination function. DJ-1 is also reported as a putative parkin substrate, misfolded DJ-1 is attached to K-63 linked polyubiquitin chains by parkin which then forms aggregosome by binding to HDAC6 to facilitate autophagy (Olzmann *et al.*, 2007). Though the unmodified form of α-synuclein doesn't interact with parkin, a rare O-glycosylated form of α-synuclein (αSp22) has been identified that interacts with and is ubiquitinated by parkin (Shimura *et al.*, 2001)

Monoubiquitination substrates of parkin include HSP70, PICK1 (a PDZ protein) and Eps15. However, physiological function of this parkin-mediated modification of HSP70 is not clear (Moore *et al.*, 2008); whereas, monoubiquitination of PICK1 regulates the activity of acid-sensing ion channels (Joch *et al.*, 2007). Interestingly, parkin is reported to regulate EGF trafficking and promote PI3K signaling via its interaction and subsequent monoubiquitination of the UIM protein, Eps15 (Fallon *et al.*, 2006). Even though many *in vivo* and *in vitro*

substrates of parkin have been identified, there is generally not sufficient data to demonstrate a role for many of these putative substrates in parkin-mediated PD.

1.4.5.6 Parkin regulates EGF (Epidermal Growth Factor) receptor turnover and signaling

Recently, it has been shown that parkin regulates the trafficking and signaling of the epidermal growth factor receptor (EGFR) by monoubiquitinating Eps15 (Fallon *et al.*, 2006). They propose that by monoubiquitinating Eps15, parkin interferes with the ability of the Eps15 UIMs (ubiquitin interacting motifs) to bind ubiquitinated EGFR, thereby delaying its internalization and degradation, and thus promoting PI3K–Akt signaling. However, there is no data so far demonstrating the involvement of EGFR in the development and/or maintenance of the DA system and therefore the question arises on the specificity of the parkin-Eps15 regulation for EGFR due its irrelevance in PD pathogenesis (Husnjak and Dikic, 2006). Functional interaction of parkin and Eps15 with other receptors which are having critical functions in the DA system for example, the GDNF receptor Ret, needs to be investigated, since all the receptor tyrosine kinases use the same fundamental endocytotic machinery (Sorkin and von Zastrow, 2009).

1.4.5.7 Distinct properties of C- and N-terminally truncated parkin mutants

Parkin mutants with pathogenic C-terminal truncations cannot associate with cellular membranes, and thus are rapidly misfolded and aggregated. In cell culture, after transient expression larger fraction of the C-terminal parkin deletion mutants (W453Stop, E409Stop or Q311Stop) was found in the detergent-insoluble fraction, in contrast to the wild-type parkin, which was found mostly in the detergent soluble fraction (Henn *et al.*, 2005). By indirect immunofluorescence experiments it appeared that the C-terminal mutants (W453Stop, E409Stop or Q311Stop) were like scattered aggregates in contrast to the wildtype parkin which is homogenously distributed throughout the cytosol (Henn et al 2005). Cell culture studies also show that, transient expression of N-terminal missense mutations (R33Q, R42P, K48A and V56E), located within the ubiquitin-like domain (UBL), decrease the stability of parkin; as a consequence, these mutants are rapidly degraded by the proteasome. Moreover, there were reports about a smaller parkin isoform (of 42 kDa) which can be found in human brain lysates and cultured cells, which originates from an internal translation start site and this isoform lacks the UBL domain (Henn *et al.*, 2005).

1.4.5.8 Parkin animal models

As described in previous sections, parkin-deficient *Drosophila* shows flight muscle phenotype and mitochondrial abnormalities, but not DA neurodegeneration. Deletion of parkin in mice (like for the PINK1 and DJ-1 ko deletion in mice) also does not lead to DA neurodegeneration (shown in the Table 1.2). However, decreased DAT protein levels and increased Glutathione (GSH) levels were observed in the striatum of parkin ko mice, suggesting a compensatory mechanism that may protect SNpc DA neuronal loss in these mice (Itier *et al.*, 2003). Another parkin ko mice, generated by deleting exon 7 showed cell loss in LC but not in SNpc (Von Coelln *et al.*, 2004). Increase in extracellular DA concentration was observed in the striatum

of parkin ko mice, after quantitative *in vivo* microdialysis (Goldberg *et al.*, 2003). Parkin ko mice also exhibit behavioral abnormalities, including impaired exploration (thigmotaxis behavior in the open field) and abnormal anxiety-related behavior (Zhu *et al.*, 2007). However, the exon 2 deleted parkin ko mice did not show any behavior alterations (Perez and Palmiter, 2005). Amperometric recordings in acute striatal sections of parkin ko mice showed a decrease in evoked dopamine release (Kitada *et al.*, 2009). Comparison between different complete parkin ko mice generated is shown in the Table 1.2.

	Itier et al., 2003	Goldberg et al., 2003	von Coelln et al., 2004	Perez and Palmiter, 2005
Mutation	Δ exon 3	Δ exon 3	Δ exon 7	Δ exon 2
Histology	No inclusions No nigral cell loss (15)	No inclusions No nigral cell loss (12,18,24)	Locus ceruleus cell loss (2,12,18) No nigral cell loss (2,12,18) No inclusions	No inclusions
Behavior	Reduced locomotor activity (5–6) Reduced locomotor response to amphetamine (6) Reduced alternance in T-maze (5)	More slips traversing a beam (2–4,7,18) Somatosensory deficits (2–4,7)	Reduced acoustic startle response (9)	No robust differences in 25 behavioral tests (18–22)
Neurochemistry	Increased DA and DOPAC in limbic system (11)	Increased extracellular DA in striatum (8,9)	Reduced NE in olfactory bulb and spinal cord (18)	No changes observed in NE, DA, DOPAC, HVA or 3-MT
Electrophysiology	Deficits in glutamate neuro-transmission in hippocampus (13)	Decreased synaptic excitability of striatal neurons (6–9)	Not examined	Not examined
Biochemistry	Reduced DAT and VMAT2 in striatum Decreased DA uptake in cultured neurons Increased levels of reduced glutathione in striatum and cultured neurons	Normal abundance of PARKIN substrates Reduced levels of mitochon-drial and antioxidant proteins in ventral midbrain	Not examined	Not examined

Abbreviations: DA, dopamine; DAT, DA transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; NE, norepinephrine; VMAT2, vesicular monoamine transporter.

Table 1.2 List of parkin-deficient mouse models published by different groups which have been analyzed for different parameters - with age of analysis indicated in parentheses (in months)

(this modified table belongs to the book of 'Parkinson's Disease Genetics and Pathogenesis' edited by Ted M. Dawson from the chapter Mouse Models of Recessive Parkinsonism written by Matthew S. Goldberg and Jie Shen)

However, overexpression of wildtype human parkin protected DA neurons from MPTP (Yasuda *et al.*, 2011; Sheng *et al.*, 2012) and amyloid toxicity (Hong *et al.*, 2013), whereas overexpression of mutant human parkin in both *Drosophila* and mice led to progressive DA neuron degeneration (Sang *et al.*, 2007; Wang *et al.*, 2007; Lu *et al.*, 2009). Therefore, studies on transgenic human parkin-overexpressing mice might be an attractive alternative to the parkin-deficient models to demonstrate the physiological functions of parkin *in vivo* and to develop therapeutic strategies.

1.4.5.9 Mitochondrial abnormalities in parkin-deficient mice

Mitochondria isolated from parkin ko mice, showed altered respiratory chain activity (Palacino et al 2004; Stichel et al 2007). Reduced antioxidant capacity and increased oxidative damage was also observed in aged parkin ko mice (Palacino et al 2004). In one study immuno-electron microscopic examinations did not reveal any mitochondrial damage in the SN and striatal regions of aged parkin ko mice (Stichel et al 2007). In apparent contradiction, another study reports severe mitochondrial damage in astrocytes, oligodendrocytes, microglia and neurons of the mesencephalon in adult and aged parkin ko mice (Schmidt et al 2011). It is important to note that the later study did not perform immuno EM to specifically label DA neurons; hence they might have also looked at non-DA neurons which led to discrepant outcomes.

1.5 Mitochondrial complex I inhibition and oxidative stress in PD

Mitochondrial dysfunction, decrease in Complex I activity and other respiratory chain defects have been reported in brain, skeletal muscle and platelets of PD patients (Mizuno *et al.*, 1989; Parker Jr. *et al.*, 1989; Bindoff *et al.*, 1991). Similar observations have been reported in several genetic and toxin-induced animal models of PD (already discussed in previous sections). It was also known that the generic complex I inhibitors, such as MPTP or rotenone can induce Parkinsonism in humans and animals (mainly primates and rodents). Supporting this hypothesis, expression of a yeast single-subunit NADH dehydrogenase - Ndi1p (that is insensitive to MPTP and rotenone) protects DA neurons from MPTP and rotenone toxicity *in vitro* and *in vivo* (Exner *et al.*, 2012). However, deletion of a Complex I subunit protein Ndufs4 (which is important for the assembly and functionality of complex I) did not cause DA neuron-specific degeneration in mice (Kruse *et al.*, 2008; Quintana *et al.*, 2010; Choi *et al.*, 2011). Even more interestingly, the primary midbrain neurons derived from the Ndufs4 ko mice are still sensitive to the complex I inhibitors, MPP^+ or rotenone (Choi *et al.*, 2008). This argues for complex I-independent actions of these toxins, rotenone has also been shown to cause microtubule disassembly (Ren *et al.*, 2005; Choi *et al.*, 2011). More recently, reasons for these discrepant findings have been reported: the Ndufs4 ko mice forms respiratory super complexes with the stabilizing effect of complex III and this leads to the sensitivity to complex I inhibitors (Calvaruso *et al.*, 2012; Sterky *et al.*, 2012).

As a consequence of complex I inhibition, mitochondrial ATP production is decreased which could then lead to an increase in ROS (reactive oxygen species) formation, which affects mtDNA, ETC (electron transport chain) and other mitochondrial proteins (reviewed in Abou-Sleiman et al., 2006; Henchcliffe and Beal, 2008; Lin and Beal, 2006). This hypothesis has been used to explain the selective degeneration of the midbrain DA neurons (which are at a high risk of oxidative damage with low anti-oxidant environment) in PD patients and animal models. However, experimental evidence demonstrating a direct link between mitochondrial defects and oxidative stress is rather weak. Indeed, several mouse models with respiratory chain defects resulted in apoptotic cell death without any indications of ROS generation (reviewed in Exner *et al*., 2012).

1.6 Neuroinflammation in PD

The three predominant types of glial cells found in the central nervous system (CNS) are the astrocytes, oligodendrocytes and microglia. The major function of these non-neuronal cells in the CNS is to physically support the neurons and to regulate local environment. However, in PD pathogenesis only astrocytes and microglia have been so far implicated (Teismann *et al.*, 2003; Teismann and Schulz, 2004; Hirsch *et al.*, 2005). Astrocytes are known to regulate potassium concentration in the extracellular compartment to regulate synaptic activity of the neurons (Filosa *et al.*, 2009). Upon neuronal dysfunction or degeneration, astrocytes get activated and increase the expression of Glial fibrillary acidic protein (GFAP), and there will be enlargement of their cell body and extension of the processes into the injured area (Eddleston and Mucke, 1993). Compared to astrocytes, the role of microglial cells in the brain is less well understood (Teismann and Schulz, 2004). Microglia arises from macrophages outside the nervous system and they are distinct from other cells of the nervous system. During seizures, damage or neuronal dysfunction, they undergo proliferation, migration and eventually acquire macrophage-like properties to actively participate in the clearance of dying cells to prevent harmful effects of cell debris and thereby protect the local environment (Banati *et al.*, 1993). Compared to other brain regions, SN is relatively rich in resting microglia (Kim *et al.*, 2000) leading to further assumptions and implications about their role in increasing the vulnerability of SNpc DA neurons in PD (Teismann *et al.*, 2003; Andressoo and Saarma, 2008). Post-mortem PD brains showed indications of increased inflammation in the SN and striatum (Teismann and Schulz, 2004; Hirsch *et al.*, 2005). However, observations indicate increased density of microglia in the SN region, rather than in the striatum (McGeer *et al.*, 1988). In contrast, the increase in astrocyte density was relatively mild, both in the SN and the striatum (Mirza *et al.*, 2000). It is rather unclear whether activated glial cells are protective or harmful for neurons and the CNS. On the one hand, activated glial cells might secrete neurotrophic factors like GDNF or BDNF to support the dying or damaged neurons (Batchelor *et al.*, 1999; Hirsch, 2000). In contrast, they were also shown to secrete and release a variety of toxic and pro-apoptotic molecules, 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 accelerate the neurodegenerative process (Hunot *et al.*, 1999). More studies need to be aimed at understanding the role of these glial cells in disease pathology.

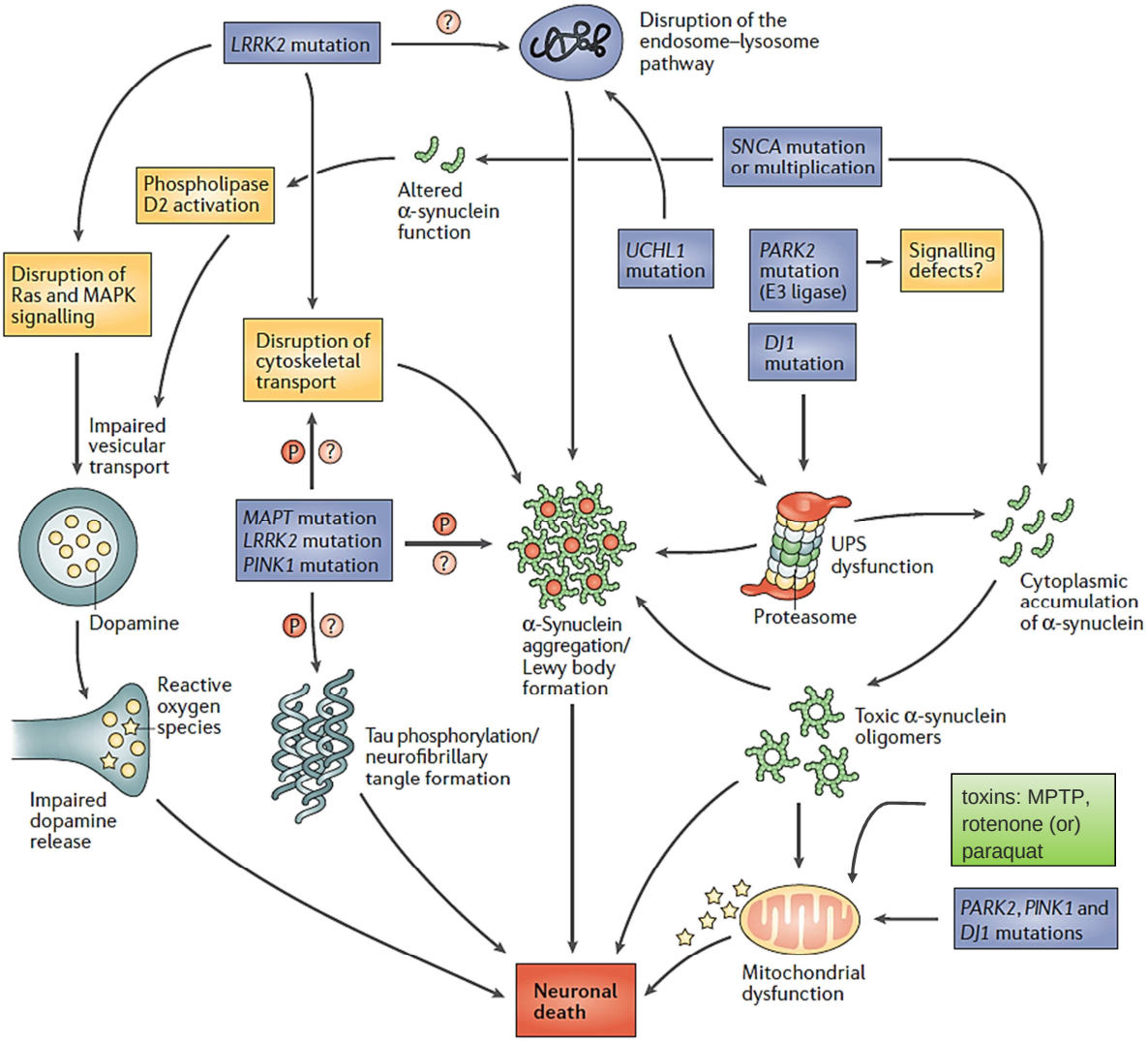

Figure 1.8 Genetics and etiology of Parkinson's disease (PD)

Several pathogenic intracellular processes have emerged as putative contributors to neurodegeneration in PD. Some of them are listed below.

i) Dominant PD genes: Mutations or multiplication of α-synuclein genes forms aggregated α-synuclein protein. The aggregated protein forms oligomers (protofibrils) and insoluble aggregates that can impair the proteasome. In addition, insoluble α-synuclein aggregates can recruit other functional proteins leading to their inactivation. Aggregate formation and protofibrils impair permeabilization of vesicles and altered DA storage leading to generation of oxidative stress. Aggregated α-synuclein is a principal component of Lewy bodies. However, controversy exists regarding whether LBs promote toxicity or protect a cell from harmful effects or misfolded proteins by sequestering them in an insoluble compartment away from cellular elements. Furthermore, α- synuclein protofibrils might directly act on synaptic vesicles to induce leakage of dopamine into the cytosol. Free dopamine is prone to auto-oxidize, generating oxidative stress; oxidized dopamine can covalently bind parkin and inactivate its function in the ubiquitin-proteasome pathway. The role of another PD-linked gene product LRRK2 remains unclear; however, recently it is found to have potential function in mitogen-activated protein kinase (MAPK) pathways, protein translation control, programmed cell death pathways and activity in cytoskeleton dynamics. Another dominant gene, UCH-L1 (Ubiquitin carboxy-terminal hydrolase L1) is a de-ubiquitinating enzyme; mutations in this gene might lead to proteasomal dysfunction and disruption in endosome-lysosome pathway.

ii) Recessive PD genes: Loss of DJ-1, PINK1 or Parkin activity decreases mitochondrial function and contributes to oxidative stress. While DJ-1 translocates to mitochondria in response to oxidative stress, PINK1 and Parkin cooperate and act in a similar (yet poorly defined) pathway to regulate mitochondrial maintenance and possibly morphology. Loss of function mutations of some of these PD-linked genes might decrease the activity of mitochondrial complexes I and thereby lead to decreased ATP production; Mutations in E3 ligase parkin can lead to accumulation of its protein substrates namely CDCre-1, Pael-R etc., contributing to DA neuron degeneration. Mutations in microtubule-associated protein tau (MAPT) gene is associated with PD (revealed recently by genome wide association studies-GWAS) might contribute to α-synuclein aggregation (and Lewy body pathology), formation of Tau tangles and disruption of cytoskeletal transport ultimately leading to DA neuron degeneration.

iii). Mitochondrial toxins and oxidative stress: Inhibition of mitochondrial activity by complex I inhibitors (like rotenone, paraquat or MPTP) or by oxidative stress generators (rotenone) is sufficient to cause Parkinsonism, further implicating the mitochondria in PD pathogenesis.

(Figure modified from a review by Farrer, 2006)

B. GDNF/Ret Signaling in Midbrain Dopaminergic Neurons

1.7 Neurotrophic factors (NTFs)

Neurotrophic factors (NTFs) are a family of proteins that play a very important role in the development (growth and survival) and maintenance of neurons both in the central and peripheral nervous system. NTFs are often secreted by cells of the target tissue. They bind to specific receptors at the nerve terminals of the projecting neurons and are typically transported retrogradely to the soma to induce intracellular signaling. In some cases they are also transported themselves to the nucleus to induce expression of certain genes. In either case, the resulting signaling effects enable proper growth and innervation of the projecting neurons. Hamburger and Levi-Montalcini (1949) first demonstrated the influence of certain substances in the target regions that promote the survival of projecting neurons, and removal of a prospective target can lead to drastic loss of neurons that should have been projecting to that target region (reviewed in Cowan, 2001).

NTFs belong to one of the three families; (1). neurotrophins, (2). glial cell-line derived neurotrophic factor family ligands (GFLs), and (3). neuropoietic cytokines (reviewed in Boyd and Gordon, 2003). Ligands from each of these families have their own distinct signaling mechanisms with different functions; however their cellular responses overlap. These proteins have been shown to have neuroprotective and neuroregenerative functions against neurodegenerative diseases, neurotoxins and axonal injuries.

Among the NTFs known, the glial cell line derived neurotrophic factor (GDNF) which belongs to GFLs has been shown to be a promising candidate to treat PD, due to its potent trophic action on cultured DA neurons (Lin *et al.*, 1993). GDNF is produced by neurons in the striatum, a target region of the SNpc DA projecting neurons which is shown to influence the development and maintenance of the nigrostriatal DA system. GDNF that is expressed in the striatum is largely (upto 95%) by the parvalbumin fast-spiking interneurons and to a much lower extent (upto 5%) by cholinergic and somatostatin positive interneurons. Other cell populations like the medium spiny projection neurons (MSNs) - which are the vast majority of striatal neurons that receive DA innervation, the astrocytes and microglial cells do not express GDNF (Pascual *et al.*, 2012). It was also shown that DA neurons utilize Shh (sonic hedgehog) to regulate the expression of Acetylcholine and GDNF in striatal cholinergic and fast-spiking interneurons. Conversely, Shh expression by DA neurons is repressed by signals that arrive from cholinergic neurons and involves the GDNF receptor Ret on DA neurons (Blesa *et al.*, 2012).

Infusion of exogenous GDNF into respective brain regions can prevent neurotoxin-induced damage of midbrain DA neurons in PD animal models (Tomac *et al.*, 1995a, 1995b). Subsequently, GDNF was found to be a potent survival factor for motor neurons (Henderson *et al.*, 1994; Arenas *et al.*, 1995) and central noradrenergic neurons (Arenas *et al.*, 1995). The positive effects of GDNF in animal models of PD encouraged the initiation of several clinical

trials. In the phase I and II trials, GDNF and its family members were directly infused into the striatum or expressed by viral vectors in PD patients with the hope to slow down or inhibit disease progression or even to cure the symptoms. Unfortunately, to date the efficacy of these treatments could not be reproducibly demonstrated, perhaps due to differences in the study design (Lang *et al.*, 2006; Manfredsson *et al.*, 2009). GDNF has been defined as an important player in these processes, but our knowledge about its signaling mechanisms in DA neurons is very limited. In addition to its pro-survival role 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).

1.8 Glial cell-line derived neurotrophic factor family ligands (GFLs)

Glial Cell-line Derived Neurotrophic Factor Family Ligands (GFLs) are distantly related to TGF-β (transforming growth factor-β) superfamily, with seven cysteine residues with similar relative spacing as other members of this family (Lin *et al.*, 1993). GFLs together with TGF-β belongs to the cysteine knot protein family and function as homodimers. GFLs are secreted and activated by a variety of tissues and can bind to receptors on projecting neurons and play an important role in their development, supporting their survival and maintenance in adult animals (Airaksinen *et al.*, 1999). Glial cell line-derived neurotrophic factor (GDNF) and Neurturin (NTRN) were the first identified and biochemically purified GFL members, based on their biological activity. The two other GFLs Artemin (ARTN) and Persephin (PSPN) were identified by a database search and homology cloning (Baloh *et al.*, 2000).

Like any other secreted proteins, GFLs are also secreted in a precursor form, prepro-GFL. Upon secretion, the signal sequence is cleaved to form pro-GFL. For complete maturation, the pro-GFL might probably undergo proteolytic cleavage. In order to raise GFLs concentrations at the sites of release and to prevent diffusion, extra cellular proteoglycans bind to the GFLs with their heparin sulphate side chains (Hamilton *et al.*, 2001). However, very little is known about the secretory and precursor activation mechanism of GFLs. Some studies also report biological activity for pro-NTFs (Lee *et al.*, 2001).

1.9 GDNF family ligands (GFLs) signals via the Ret receptor tyrosine kinase

Unlike other ligand-RTK (receptor tyrosine kinase) signaling, GFLs first have to bind to specific GFRα co-receptors to interact and activate Ret, which was originally discovered as a proto-oncogene (reviewed in Takahashi, 2001). The Ret receptor can only be activated if the GFL is first bound to a novel class of proteins, known as GDNF family receptor-α (GFRα) receptors, which are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. GFLs, acting as homo-dimers bring two molecules of GFRα co-receptor together to form a high-affinity complex. Subsequently, the GFL-GFRα complex (with two molecules each) recruits two molecules of Ret and transphosphorylates the intracellular tyrosine kinase domains of each Ret molecule (reviewed in Sariola and Saarma, 2003). There are four

different GFRα receptors (GFRα1–4), which determine the ligand specificity of the GFRα–Ret complex. GDNF binds to GFRα1, and then forms a complex with Ret. Similarly, before binding to Ret, NRTN binds to GFRα2, ARTN to GFRα3, and PSPN to GFRα4 (shown in Figure 1.9). Additionally, NRTN and ARTN might crosstalk with GFRα1, and GDNF might also signal via GFRα2 and GFRα3 co-receptors (Airaksinen *et al.*, 1999; Baloh *et al.*, 2000; Lindahl *et al.*, 2001; Takahashi, 2001).

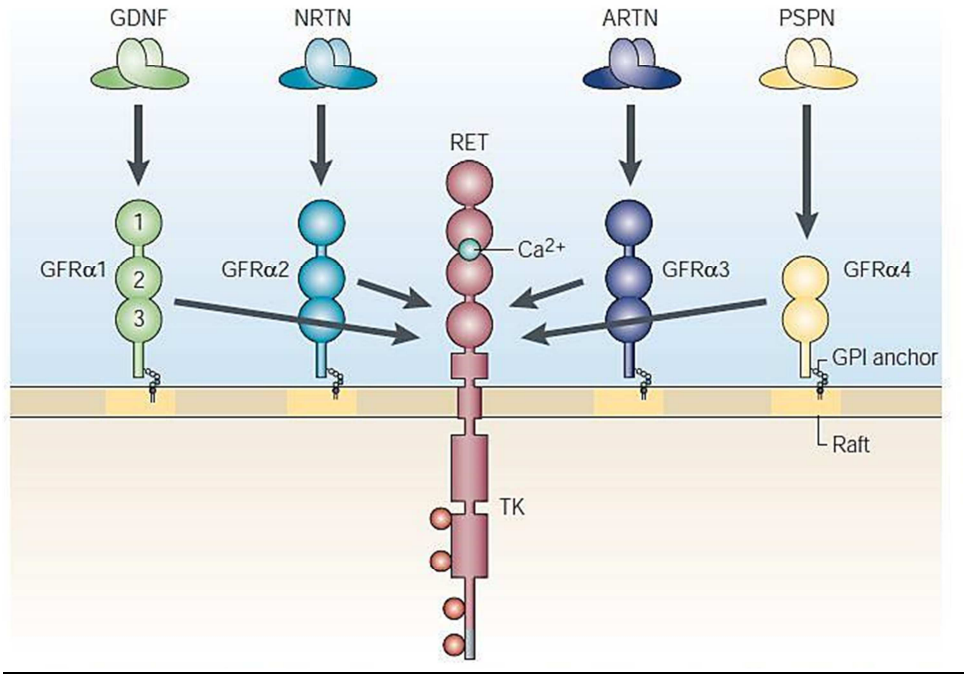

Figure 1.9 GDNF-family ligands and Ret receptor interactions

Glial cell line-derived neurotrophic factor (GDNF)-family ligands (GFLs) forms homodimers to activate Ret tyrosine kinase (TK) by first binding their cognate GDNF-family receptor-α (GFRα) receptors. Most preferred ligand–receptor interactions that are known to occur physiologically *in vivo* are shown by black arrows. GFRα proteins are attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor. All the GFRα proteins are predicted to have three cysteine-rich globular domains (1, 2 and 3), except for GFRα4, which has only two. Four tyrosine residues in the Ret intracellular part (Tyr905, Tyr1015, Tyr1062 and Tyr1096; red balls) serve as docking sites for different adaptors. One of them (Tyr1096) is in the carboxy-terminal end of the long isoform of Ret (grey). Membrane rafts are shown in yellow. ARTN, artemin; NRTN, neurturin; PSPN, persephin. (Figure obtained from Airaksinen and Saarma, 2002)

Ret was initially identified as an unusual rearranged transforming gene in NIH3T3 cells transfected with human lymphoma DNA (Takahashi *et al.*, 1985). This transforming gene resulted is formed due to the fusion of 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 (Takahashi *et al.*, 1988; Iwamoto *et al.*,

1993). Ret homologues have been identified in higher and lower vertebrates, including *Drosophila* (Hahn and Bishop, 2001). The human Ret gene is localized to the chromosome 10 (10q11.2), with 21 exons in total. Loss-of-function mutations and point mutations in the Ret gene can cause several diseases. For example, loss of Ret function causes severe enteric denervation leading to congenital aganglionic megacolon (Hirschsprung's disease) and constitutive activation mutations in Ret are responsible for dominantly hereditary endocrine cancer syndromes (Kodama *et al.*, 2005). Based on the clinical symptoms, the multiple endocrine neoplasia type 2 (MEN2) is divided into three groups: multiple endocrine neoplasia type 2A (MEN2A), 2B (MEN2B) and familial medullary thyroid carcinoma (FMTC) (Hansford and Mulligan, 2000). In MEN2A, the disease has been linked to point mutations, which are mostly located to cysteine residues in the extracellular cysteine-rich domain of Ret. These point mutations introduce abnormal intermolecular cysteine bridges, and cause an auto-activation of Ret-MEN2A by dimerization. In the case of Ret-MEN2B, the point mutations are located in the intracellular domain of Ret (M918T), where they cause an auto-activation of Ret-MEN2B monomers. In FMTC patients, point mutations have been found both in the extracellular and the intracellular domains of Ret (Hansford and Mulligan, 2000).

1.9.1 Structure of the Ret protein

Ret is a single transmembrane protein with three structurally distinct domains: an N-terminal extracellular domain which contains four cadherin-like repeats with a calcium binding domain and a cysteine-rich region; a hydrophobic transmembrane domain and a typical cytoplasmic tyrosine kinase domain at the C-terminal (see Figure 1.10). Considering their homology with cadherins, the cadherin-like domains are thought to play a role in cell adhesion, however their function is not very well defined (Anders *et al.*, 2001). The calcium binding site, which might be present between the second and third cadherin-like domains, is required for proper folding, secretion and activation of the Ret receptor (Nozaki *et al.*, 1998; van Weering *et al.*, 1998; Anders *et al.*, 2001). The 16-cysteine residues of the cysteine-rich region of the extracellular domain are important for proper folding of the receptor and for its binding to the GFRα co-receptor (Runeberg-Roos and Saarma, 2007). Mutations in this region can either cause receptor misfolding, which could result in protein degradation leading to Hirschsprung's disease, or constitutive activation, which could result in ligand independent activation of the Ret receptor leading to endocrine neoplasia type 2A (MEN2A) tumors (Dvorakova *et al.*, 2005). The hydrophobic transmembrane domain, which was originally thought just to be a link for the extra and intracellular domains, was recently shown to be important for dimerization of the Ret molecules to facilitate the formation of GFL/GFRα/Ret signal transduction complex (Runeberg-Roos and Saarma, 2007). Transmembrane domain mutations were found in some patients with MEN2 tumors (Kjaer *et al.*, 2006). The intracellular tyrosine kinase domain (TK) split by the insertion of 27 amino acids. This domain of Ret is homologous to that of other RTKs such as FGFR-1 and VGFR-2 (Tuccinardi *et al.*, 2007). GFL-induced Ret dimerization juxtaposes the two catalytic domains, allowing the trans-phosphorylation of tyrosines which then act as docking sites for several intracellular signaling effectors.

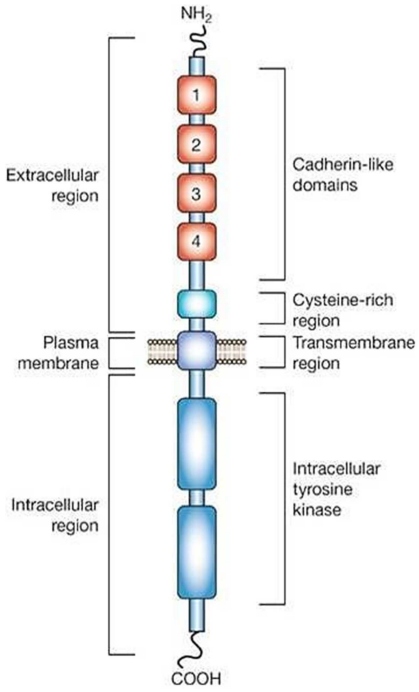

Figure 1.10 Monomeric structure of the receptor tyrosine kinase Ret (Obtained from a review by Drosten and Pützer, 2006)

1.9.2 Ret receptor isoforms

The natural alternative splicing of the Ret gene results in 3 different isoforms of the Ret protein. The Ret51, Ret43 and Ret9 isoforms contain 51, 43 and 9 amino acids in their C-terminal tail respectively (Myers *et al.*, 1995). Ret51 and Ret9 are the most studied Ret isoforms; they are conserved over a broad range of species and consist of 1114 and 1072 amino acids, respectively (Kodama *et al.*, 2005). The cytoplasmic tyrosine kinase domain of Ret9 contains 16 tyrosines, whereas the Ret51 isoform contains 18 tyrosines. Tyr1090 and Tyr1096 are present only in the Ret51 isoform due its longer C-terminal tail (Arighi *et al.*, 2005). The N-terminal extracellular domain of Ret contains nine N-glycosylation sites. The fully glycosylated Ret protein is reported to have a molecular weight of 170 kDa although it is not clear to which isoform this molecular weight relates (Takahashi *et al.*, 1993).

Mice lacking the Ret9 isoform specifically show kidney abnormalities and enteric aganglionosis, which are similar and almost as severe as in Ret-null mice; however, mice which lack Ret51 showed no obvious developmental problems (Schuchardt *et al.*, 1994). Consistent with these reports, Ret9 expression could rescue the Ret loss phenotype (Srinivas *et al.*, 1999; de Graaff *et al.*, 2001). These studies argue for the crucial role of Ret9 during development, but not Ret51. The Ret 51 isoform seems to be more important for inner-medullary collecting duct cell survival in mice (Lee *et al.*, 2002) and in oncogenic transformation (Iwashita *et al.*, 1999). Taken together, it appears that the Ret51 and Ret9 isoforms have distinct functions.

1.9.3 Ret RTK intracellular signaling

Like the majority of receptor tyrosine kinases studied, signaling pathways initiated by the Ret receptor include the Ras-MAPK, PI3K-AKT, and phospholipase Cγ (PLCγ) pathways. On activation, Ret undergoes autophosphorylation of intracellular tyrosine residues, which serve as docking sites for downstream signaling effectors either with Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains. Upon activation, at least 14 of the 18 tyrosine residues in the intracellular domain of Ret can become phosphorylated (Liu *et al.*, 1996; Kawamoto *et al.*, 2004; Knowles *et al.*, 2006).

Autophosphorylation of the key residue Tyr1062 is required for activation of Ras-MAPK and PI3K-AKT pathways (Besset *et al.*, 2000; Hayashi *et al.*, 2000; Segouffin-Cariou and Billaud, 2000; Coulpier *et al.*, 2002). This residue appears to be critical for Ret function, and mice with a point mutation in Tyr1062 show a severe loss-of-function phenotype (Jijiwa *et al.*, 2004; Wong *et al.*, 2005; Jain *et al.*, 2006a). Both FRS2 and Shc (Src-homologous and collagen-like protein) adaptor proteins can bind to phosphorylated Tyr1062 of the activated Ret receptor. Activated Ret has been shown to interact with FRS2 in lipid rafts, but with Shc outside lipid rafts (Paratcha *et al.*, 2001), leading to differential downstream signaling of Ret. It has been shown that binding of Ret to Shc, but not FRS2, is responsible for cell survival effects of Ret in neuroblastoma cells (Lundgren *et al.*, 2006). Ret stimulation by GPI-anchored GFRα in rafts promotes the binding of lipid-anchored adaptors such as FRS2 and the activation of Src. Binding of activated Ret to FRS2 results in subsequent recruitment of GRB2 and SOS to activate the RAS-MAPK (ERK) pathway. Ret stimulation by GFL bound to soluble GFRα outside the rafts initially activates signaling pathways that are mediated by soluble adaptors, such as Shc, to recruit adaptor proteins GRB2 and GAB2 for the activation of the PI3K-AKT pathway (reviewed in Sariola and Saarma, 2003). A recent study has established a biochemical function and physiological role for the phosphorylation of Tyr687 in the juxtamembrane region of the Ret intracellular domain (Perrinjaquet *et al.*, 2010). SHP2 (a phosphotyrosine phosphatase) through its interaction with Tyr687 and association with components of the Tyr1062 signaling complex is recruited to phosphorylated Ret to activate the PI3K-AKT pathway and promote survival and neurite outgrowth in primary neurons (Perrinjaquet *et al.*, 2010). Phosphorylation of Tyr1096, which is present only in the long Ret51 isoform, can also contribute to these pathways. The Grb2/Gab2 complex can also assemble directly onto phosphorylated Tyr1096, offering an alternative route to PI3K activation by GDNF ligands. Three other adaptor proteins: DOK4/5 (downstream of tyrosine kinase 4/5), IRS1/2 (insulin receptor substrate 1/2) and enigma, also bind to the same Tyr1062 site. However, binding of enigma to Ret is phosphorylation-independent (reviewed in Sariola and Saarma, 2003).

Regarding the remaining autophosphorylation sites (Iba, 2013), it has been found that phosphorylation of Tyr1015 leads to activation of PLCγ (Borrello *et al.*, 1996). Phosphorylation of Tyr900 and Tyr905 (which are present in the kinase activation loop) are known to contribute to full kinase activation (Knowles *et al.*, 2006). A recent yeast-two-hybrid screen identified a GTPase-activating protein (GAP) for Rap1, Rap1GAP, as a novel Ret-binding protein (Jiao *et al.*, 2011). Like Src, Rap1GAP was also found to require

phosphorylation of Tyr981 for Ret binding and suppressed GDNF-induced activation of ERK and neurite outgrowth. In addition to tyrosine autophosphorylation, Ret has been found to undergo serine phosphorylation at Ser696 by protein kinase A (PKA). Mutation of Ser696 affected the ability of Ret to activate the small GTPase Rac1 and stimulate formation of cell lamellipodia (Fukuda *et al.*, 2002).

Ret-mediated activation of PI3K-AKT and RAS-MAPK signaling might promote survival by modulating several downstream targets, including transcription factors: p53, cAMP response element binding protein (CREB) and forkhead box O (FOXO), transcription factor inhibitors: the inhibitor IκB of NF-κB and pro-apoptotic proteins: caspases, BAD and glycogen synthase kinase-3 (GSK3); however, these targets need to be validated in DA neurons. Whereas the downstream signaling of other proteins (IRS1/2; Dok1/4/5; STAT3 or Enigma) that are recruited to activated Ret is poorly understood. It remains to be determined whether all or a subset of these putative downstream targets promote survival of DA neurons (Reviewed by Aron and Klein, 2011).

1.10 Ret-independent signaling of GDNF

The expression patterns of GFRα receptors and Ret are not totally overlapping (Trupp *et al.*, 1997). This observation raised questions about alternate GDNF receptors. GDNF, in absence of Ret has been shown to activate signaling pathways/molecules like ERK/MAPK, PLCγ, CREB, and Src-family kinases (Poteryaev *et al.*, 1999; Trupp *et al.*, 1999); these findings strengthened the notion of the existence of alternate GDNF receptors. Another RTK, Met, which is a receptor for hepatocyte growth factor (HGF), has been shown to be activated by GDNF together with syndecans to trigger Src activation, in the absence of Ret (Popsueva *et al.*, 2003) (Figure 1.11A).

Another heavily discussed alternate GDNF receptor is the p140 isoform of Neural Cell Adhesion Molecule (NCAM), which belongs to the immunoglobulin superfamily. GDNF has low affinity for the p140 isoform of NCAM, but this affinity is enhanced the presence of GFRα1. Upon activation, NCAM acts on downstream signaling molecules like cytoplasmic protein tyrosine kinases Fyn and FAK (Paratcha *et al.*, 2003) (depicted in Figure 1.11B). GDNF/GFRα1/NCAM signaling has been shown to regulate neuronal morphology, cell migration and synapse formation (reviewed in Ibáñez, 2010). Genetic ablation of NCAM in mice resulted in a mild loss of DA neurons in the midbrain (SNpc and VTA), with impaired DA signaling (Xiao *et al.*, 2009). Moreover, use of NCAM-blocking antibody inhibited the *in vivo* and *in vitro* effects of GDNF in SNpc and VTA DA neurons (Chao *et al.*, 2003) suggesting a functional role of NCAM together with GDNF/GFRα1 in DA neurons. Recently, cell adhesion molecules integrin β1 and N-cadherin have been shown to be involved in Ret-independent GDNF signaling (Cao *et al.*, 2008b, 2010) (Figure 1.11C). It has also been proposed that GDNF-Ret signaling can be modulated by heparan sulphate proteoglycans (HSPG). These extracellular structures immobilize GDNF, raise its local concentration, and thus enhance Ret activation (Hamilton *et al.*, 2001; Barnett *et al.*, 2002). The GDNF, immobilized at the extracellular matrix (ECM), can also signal independent of Ret via HSPG syndecan-3 (Bespalov *et al.*, 2011) (Figure 1.11C).

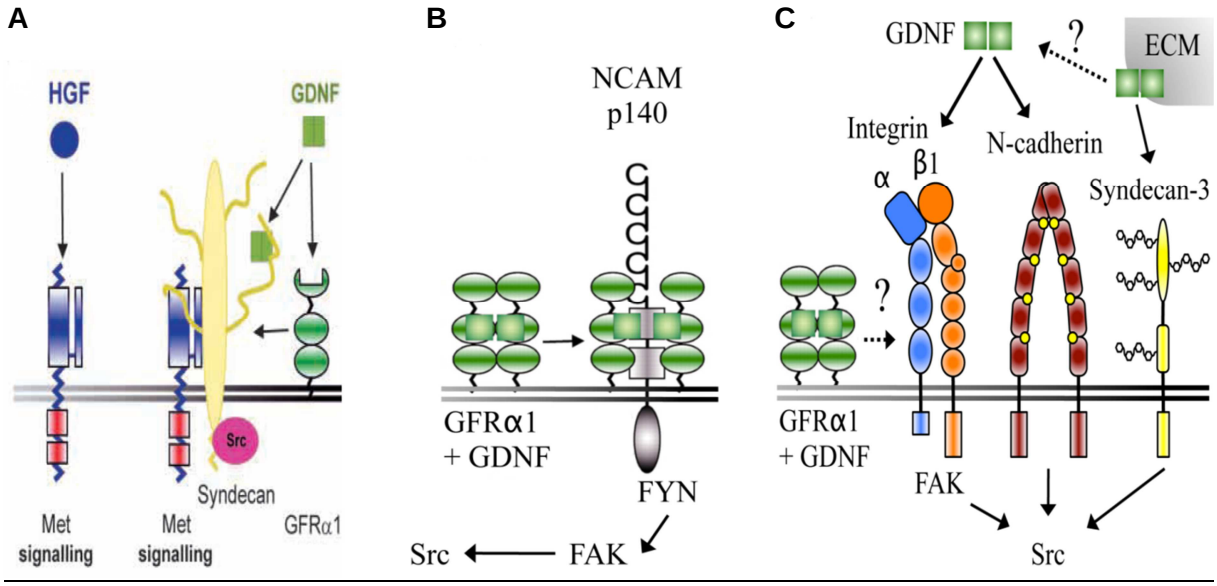

Figure 1.11 Ret-independent signaling of GDNF

A. Met together with Syndecans functions as GDNF receptor: Met RTK, which is a receptor for hepatocyte growth factor (HGF), can function as GDNF receptor together with Syndecans to activate Src in a Ret independent fashion. (Figure obtained from Sariola and Saarma, 2003).
B. NCAM, an alternate GDNF receptor: Similar to Ret, the NCAM p140 isoform also requires the co-receptor GFRα1 for binding to GDNF. After activation, NCAM triggers downstream signaling via the FYN and FAK kinases. (Figure kindly provided by Karsten Tillack).
C. Integrin β1, N-cadherin and Syndecan-3 as putative Ret-independent GDNF receptors: The molecular mechanisms underlying these ligand receptor interactions, requirement of GFRα1 co-receptor for the function of these putative receptors is not known. (Figure kindly provided by Karsten Tillack).

1.11 GFL-independent signaling of Ret

For embryonic sympathetic superior cervical ganglion (SCG) neurons, Nerve growth factor (NGF)/tyrosine kinase receptor type 1 (TrkA) signaling is essential for survival, however, NGF/TrkA is dispensable for postnatal SCGs, wherein NGF via its ligand TrkA promotes the activation of the Ret51 isoform in a GFL-independent manner thus inducing Ret-mediated growth, metabolism and gene expression (Tsui-Pierchala *et al.*, 2002). TrkA and Ret do not interact with each other, suggesting that TrkA indirectly mediates phosphorylation of Ret, although the exact mechanisms underlying functional interaction of TrkA and Ret are not clear (reviewed in Sariola and Saarma, 2003).

GDNF ligand-independent signaling of Ret can also occur in the dominantly inherited cancer syndromes MEN2A, MEN2B and FMTC, which arise due to point mutations in the Ret receptor (Santoro *et al.*, 2002). The MEN2A disease has been linked to point mutations that occur in the extracellular cysteine-rich domain of Ret. These point mutations cause abnormal intermolecular cysteine bridges leading to dimerization and subsequent autoactivation of the Ret receptor independent of GDNF. Ret-MEN2A mediates activation of AKT and MAPK

pathways, leading to the survival of PC12 cells in the absence of growth factors (De Vita *et al.*, 2000). In the case of MEN2B syndrome, the point mutation (Met918Thr) is located in the intracellular domain of Ret, which leaves the receptor auto active even in its monomeric form, without any interaction with GDNF/GFRα1. The Ret MEN2B variant has been shown to be active already during its synthesis in the ER, it was also demonstrated that the oncogenic precursor of the receptor has the capacity to activate AKT, ERK and signal transducer and activator of transcription 3 (STAT3) pathways from the ER (Runeberg-Roos and Saarma, 2007). In FMTC syndrome, point mutations have been found both in the extracellular and the intracellular domain of Ret (Hansford and Mulligan, 2000). The Ret-FMTC mutants Y791F and S891A have been shown to activate a Src/JAK/STAT3 pathway, independent of GDNF (Plaza Menacho *et al.*, 2005).

1.12 Genetic animal models for understanding GDNF/GFRα1/Ret signaling

Several genetic mouse models have been generated to study the essential role of GDNF/GFRα/Ret signaling, *in vivo*. The first model was GDNF complete knock out (GDNF -/-) mice. These mice die after birth due to renal agenesis and absence of the enteric plexus (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sánchez *et al.*, 1996). Surprisingly, the GDNF -/- mice have been shown to develop a normal number and organization of mesencephalic DA neurons. Experiments on embryonic (E14) slice cultures have shown the importance of GDNF in neurite outgrowth and DA neuronal survival *in vitro* (af Bjerkén *et al.*, 2007). These observations suggest that the trophic dependence of nigrostriatal neurons on GDNF after exogenous administration of the trophic factor might be acquired during postnatal maturation. In an *in vivo* study, GDNF function blocking antibodies enhanced cell death during the first period of naturally occurring apoptosis in the SN (Oo *et al.*, 2003). Striatal GDNF expression has been shown to transiently increase the SN cell number (Kholodilov *et al.*, 2004). Heterozygous GDNF mice (GDNF +/-) are viable and show normal development with few behavioral alterations observed during their first postnatal weeks; however, the nigrostriatal DA system remains unaltered (Gerlai *et al.*, 2001). Inadequate GDNF levels have been shown to increase the adverse phenotypes caused by chronic administration of drugs of abuse. These interesting findings led to the proposal of GDNF as a potential target to treat addiction (reviewed in Carnicella and Ron, 2009). However, loss of SNpc DA neurons have been reported in aged GDNF +/- mice (Table 1.3; Boger et al., 2006). Similar results have been observed in aged GFRα1 heterozygous ko (GFRα1 +/-) mice (Table 1.3; Boger et al., 2008; Zaman et al., 2008). To address the physiological functions of GDNF in catecholaminergic neurons, Pascual et al. have developed a conditional GDNF -/- mouse where GDNF expression can be selectively decreased during adulthood, to avoid developmental compensation, if any (Pascual *et al.*, 2008). The conditional GDNF -/- mice showed a selective and extensive catecholaminergic neuronal loss, specifically the SNpc, VTA and LC; these alterations correspond to behavioral changes in these mice (Table 1.3; Pascual et al., 2008).

Conditional Ret deletion resulted in a significant decrease of SNpc DA neurons and striatal fiber density of aged mice (12 and 24 months) accompanied by inflammation and gliosis in

the nigrostriatal system. In these mice viability of neurons in the VTA and LC are not compromised (Table 1.3; Kramer et al., 2007). The findings of Kramer et al., did not go in line with the observations by another group (Table 1.3; Jain et al., 2006), which reported no differences in the adult (6–12 months of age) nigrostriatal DA neuronal numbers after conditional ablation of Ret. The variable results on conditional Ret-deficient mice from these two groups might be due to the fact that Jain et al 2006 pooled animals of 6-12 months of age for analysis. Inclusion of significantly younger animals could obscure effects with 12 month-old-mice which were selectively analyzed in Kramer et al., 2007. Supporting the role of Ret signaling in the DA system, MEN2B knock-in mice have been shown to develop more DA neurons specifically in the SNpc region (Mijatovic *et al.*, 2007). A mouse model generated by Tillack et al., (unpublished data), which expresses Tet-system transactivator protein under control of the mouse TH promoter for enabling Ret ablation specifically in DA neurons during adulthood revealed no increase in DA neurodegeneration compared to the originally published conditional Ret-deficient mice by Kramer et al., 2007. These results rule out the argument of early embryonic compensation to explain the different levels of neurodegeneration observed with the Ret receptor-deficient mice and the conditional GDNF-deficient mice.

Reference	**Animal model**	**TH+ cells SN**	**TH+ cells VTA**	**TH+ fibers** (striatum)
Boger *et al.* (2006)	*GDNF*$^{+/-}$	4 m: n.s.d. 8 m: n.s.d. 12 m: 81† 20 m: 85†	n.a.	12 m: 58†
Zaman *et al.* (2008)[(1)] Boger *et al.* (2008)[(2)]	*GFRα1*$^{+/-}$	8 m: n.s.d.[(1)] 18 m: 70*[(1)] 26 m: 83*[(2)]	n.a.	8 m: n.s.d.[(1)] 18 m: n.s.d.[(1)] 26 m: 60*[(2)]
Jain *et al.* (2006)	*Ret*$^{F/-}$; *Dat-Cre*	8–12 m: n.s.d.	8–12 m: n.s.d.	8–12 m: n.s.d.
Kramer *et al.* (2007)	*Ret*$^{F/F}$; *Dat-Cre*	3 m: n.s.d. 12 m: 75† 24 m: 62†	12 m: n.s.d.	12 m: 60† (dSt) 72† (vSt)
Pascual *et al.* (2008)	*GDNF*$^{F/-}$; *Cre-EsR1*	1 m$^{\$}$: n.s.d. 7 m$^{\$}$: 42†	1 m$^{\$}$: n.s.d. 7 m$^{\$}$: 34†	7 m$^{\$}$: 60† (dSt) 72† (vSt)

Table 1.3 List of mouse models with defective GDNF or GFRα1 or Ret signaling

+/- indicates heterozygous knockout mice; F/F indicates mice with both the alleles of desired gene floxed; F/- indicates mice with only one allele of desired gene, which is floxed. Numbers indicate percentage with respect to control animals. X m = age of the mice used for analysis (months). X m$ = months after GDNF depletion. (dSt) dorsal striatum; (vSt) ventral striatum. n.a., = not analyzed; n.s.d., = no significant differences. *P≤0.05; †P≤0.01. (Table obtained from Pascual et al., 2011)

Taken together, these mouse lines suggest an important function of GDNF/GFRα1/Ret signaling in aged mice. These results also argue that GDNF can signal through 'non-canonical' NCAM or other alternate receptors (Chao *et al.*, 2003; Paratcha *et al.*, 2003; Cao *et al.*, 2008a). These receptors may compensate for the absence of Ret, and therefore provide an

explanation for why the DA system loss is milder in the conditional Ret-deficient mice relative to the conditional GDNF-deficient mice.

Even though several of the *in vivo* and *in vitro* studies emphasize the importance of GDNF/Ret signaling in the survival and maintenance of DA neurons (reviewed by Aron and Klein, 2011), there is very limited information about the downstream signaling mechanisms of Ret signaling through which it exerts its functions.

1.12.1 Mice deficient of Ret signaling in midbrain DA neurons - a preclinical animal model for PD

The conditional Ret-deficient mice generated by Kramer et al 2007 (DAT-Ret ko) display several pathologic features of pre-symptomatic PD. The conditional Ret-deficient mice exhibit - an adult onset, specific and progressive degeneration of the SNpc DA neurons with no alterations in VTA DA neurons; degeneration of DA nerve terminals in the striatum; the presence of substantial neuroinflammation and gliosis in the degenerated nigrostriatal system; and reduced levels of evoked dopamine release in striatum. Their observations reveal that Ret signaling is crucial for long-term maintenance of SN axons and cell bodies. The Ret-deficient mice showed significant degeneration of DA fiber innervation at 9 months of age, which then continued to progress until 24 months, indicating a clear progressive pattern of axonal fiber degeneration. However, the cell bodies started to degenerate later: significant cell body loss was only observed at 12 months, which was found to also be progressive with ageing until 24 months. The aged Ret-deficient mice displayed up to 60 % loss of DA axons innervating the striatum, while the loss of SN cell bodies was 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, experimental evidence suggests that neurotrophic factors exert different actions at nerve terminals and in the cell body (Zweifel *et al.*, 2005). The aged Ret-deficient mice (at 24 months) also showed neuroinflammation at the site of degeneration similar to that was observed in post mortem brains of PD patients (Teismann *et al.*, 2003; Teismann and Schulz, 2004; Hirsch *et al.*, 2005). They displayed an enhanced astrocyte recruitment to the striatum and microglial recruitment to the SNpc regions only at 24 months, not at 12 months suggesting that these neuroinflammatory changes are rather post neurodegenerative effects but not the primary causes of degeneration. However, these mice don't show other hallmark symptoms of PD - as they exhibit no cytoplasmic α-synuclein containing inclusions; no behavioral alterations; and no changes in striatal total DA levels despite of striatal DA innervation loss (Kramer *et al.*, 2007). In addition, mice without Ret signaling are not more sensitive to MPTP toxicity, but they failed to show regeneration of damaged dopaminergic axon terminals after MPTP toxicity (Kowsky *et al.*, 2007).

Since the Ret receptor is well demonstrated for its cell survival signaling, it may not contribute directly to α-synuclein accumulation. Moreover, one should also consider the changes in total DAT protein levels that were observed in the DA specific Cre expressing mice (DAT-Cre) which were crossed with floxed Ret ($Ret^{lx/lx}$) mice to generate the Ret-deficient (DAT-Ret ko) mice (Kramer *et al.*, 2007). In the DAT-Cre line, the Cre transgene insertion in to the 5'-UTR of one of the endogenous DAT locus resulted in only one

functional copy of DAT gene, which led to an age-dependent decrease in DAT protein levels along with increased striatal DA levels and hyperactive behavior in the mice (Parlato *et al.*, 2006; Kramer *et al.*, 2007; Turiault *et al.*, 2007). This could be the reason why Ret-deficient (DAT-Ret ko) mice did not show decreased striatal DA levels and behavioral changes despite the age-dependent degeneration of nigrostriatal DA system. Accordingly, it might be worthwhile to generate an improved conditional Ret-deficient mouse line to overcome problems related to DA metabolism thereby generate an even better model for understanding PD pathogenesis.

1.12.2 Constitutive active Ret signaling causes nigrostriatal DA system enlargement in mice

Another interesting study revealed the importance of Ret signaling for shaping the brain DA system. A study published by Mijatovic et al (in 2007) used knock-in mice with the Ret-MEN2B mutation to study the effects of constitutive Ret activity on the brain dopaminergic system. They found robustly increased concentrations of dopamine (DA) and its metabolites in the striatum and other brain regions (cortex and hypothalamus). Moreover, in the SNpc of homozygous MEN2B mice, they reported a 26% increase in the number of TH-positive cells (but not in the VTA). These mice also showed more DA innervation in the striatum. These findings clearly suggest that Ret activity can have a direct biological function that actively changes and shapes the brain dopaminergic system in mice (Mijatovic *et al.*, 2007). The most likely explanation for the increased number of SNpc TH-positive cells in the homozygous MEN2B mice is the role of GDNF-dependent signaling in the postnatal development of the nigrostriatal DA system. It is known that the nigral DA neurons undergo apoptosis during the first three postnatal weeks and in mice overexpression of GDNF in the DA targets promoted postnatal survival of nigrostriatal DA neurons during the first phase of natural cell death by suppressing apoptosis (Oo and Burke, 1997; Burke *et al.*, 1998; Oo *et al.*, 2003; Kholodilov *et al.*, 2004). Thus, the increased number of TH-positive SNpc neurons in MEN2B mice could be because of the GDNF-independent signaling of constitutively active Ret, resulting in more DA neurons. In a follow-up study Mijatovic et al further showed that the DA neuronal cell bodies of the MEN2B mice were shown to be more resistant (but not the striatal DA axons) to the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA); suggesting a protective effect of constitutive active Ret signaling on the DA cell bodies of the SNpc, but not on the DA axons in the striatum (Mijatovic *et al.*, 2011). Considering the significant role of constitutively active Ret signaling on the development of DA system, MEN2B mice can be a perfect animal model to evaluate the function of other candidate proteins that may also contribute to the establishment of nigrostriatal DA system.

Despite the fact that Ret signaling has been shown to be important during postnatal development and also for the maintenance of the nigrostriatal DA system in aged mice, association studies performed in humans did not find any polymorphism in the Ret gene that can be attributed to increased PD risk (Wirdefeldt *et al.*, 2003; Lücking *et al.*, 2010). These observations suggests that defects in Ret signaling are not a primary cause of PD, but a secondary consequence in PD pathogenesis in which Ret is one of the many proteins that are

altered. To get more insights into Ret signaling and its role within the altered protein network of PD, a study by Aron et al., 2010 investigated the functional interaction of Ret and the PD-related protein DJ-1. In that study they crossed the conditional Ret-deficient mice (Kramer *et al.*, 2007) with DJ-1 complete knock-out mice (Pham *et al.*, 2010), and showed that mice lacking both DJ-1 and Ret in the DA system upon ageing display an accelerated loss of SNpc DA neurons without any enhanced striatal innervation loss compared to the single Ret-deficient mice. DJ-I/Ret double loss-of-function experiments revealed interaction of DJ-1 with ERK signaling to control eye and wing development in *Drosophila*. This study uncovers the *in vivo* cell survival function for DJ-1 in mice and a conserved interaction between DJ-1 and Ret-mediated signaling in *Drosophila* (Aron *et al.*, 2010). These findings open up new areas of research and highlight the current need to investigate the function of Ret together with other key players which are implicated in PD. This should enable the design of new therapies that could potentially include customized therapies for PD patients with specific gene mutations to increase the efficacy of compounds like GDNF and its family members, which are currently under clinical trials.

2. The Thesis Project

Background information

Parkin and the receptor tyrosine kinase Ret are independently linked to the neurodegenerative processes leading to the selective cell death of substantia nigra (SN) dopaminergic (DA) neurons in Parkinson's disease (PD) patients and animal models respectively. Recessive mutations of parkin, an E3 ubiquitin protein ligase causes PD, but mouse models lacking parkin show no SNpc DA neuron loss (Itier *et al*., 2003; Goldberg *et al*., 2003), suggesting additional embryonic compensatory mechanisms in the parkin deficient mice. However, parkin overexpression protects the nigrostriatal DA system in several toxin-induced models of PD. Furthermore, cell culture studies have shown diverse functions of parkin related to ubiquitin proteasomal system, mitochondrial integrity and mitophagy (Reviewed in Exner *et al*., 2012). On the other hand, mice lacking the GDNF receptor Ret in DA neurons develop an age dependent and SNpc specific DA cell loss reminiscent to PD patients (Kramer *et al*., 2007), but so far no mutations in Ret are linked to PD. Moreover, a mutation in the intracellular domain of the Ret receptor (MEN2B) renders it constitutively active, resulting in more DA neurons specifically in the SNpc region of the mice (Mijatovic *et al* 2007). However, association studies performed in humans did not find any polymorphism in the Ret gene that can be attributed to increased PD risk (Wirdefeldt *et al*., 2003; Lucking *et al*., 2008). These observations suggest that defects in Ret signaling could be a secondary, but not primary consequence in PD, where Ret might function along with other proteins that are linked to PD. Consistent with this notion, a study performed by Aron *et al*., in 2010 showed genetic interaction of Ret receptor with a PD-linked protein, DJ-1. In that study they could unveil the pro-survival function of DJ-1 in Ret deficient mice, and a conserved interaction between DJ-1 and Ret mediated signaling in *Drosophila* (Aron *et al*., 2010).

Recently, parkin (another PD-linked protein) has been shown to enhance the signaling of a receptor tyrosine kinase EGFR to promote cell-survival by activating PI3K-AKT pathway (Fallon *et al* 2006). However, there is no data so far demonstrating the involvement of EGFR in the development and/or maintenance of the DA system, this arises the question on specificity of parkin mediated regulation of EGFR. Since all the receptor tyrosine kinases use the same fundamental cell survival mechanisms it is important to extend our understanding on the functional interaction of parkin with other receptors which are having critical functions in the DA system for example the GDNF receptor, Ret. Moreover, findings from different studies suggest that both GDNF/Ret signaling (Hayashi *et al*., 2000) and parkin (Reviewed in Exner *et al*., 2012) can act on the NF-kB pathway to meditate cell survival functions, so far there is no information about their functional cooperation in this context. Considering these different possible converging points of Ret signaling and Parkin activity, in the current study I want to investigate the downstream effects of parkin and Ret signaling and their possible genetic and functional interaction if any, to promote the survival of DA neurons using mouse as a model organism.

The main objectives of my project

1. To examine the long-term survival and maintenance function of parkin and Ret signaling in mouse SNpc DA neurons.

2. To investigate the *in vivo* neuroprotective effect of human parkin over expression on trophically impaired Ret deficient DA neurons.

3. To study the influence of parkin in the establishment of SNpc DA neurons with constitutive active Ret signaling.

4. To understand the common downstream signaling cross points of Ret and parkin in DA neurons and to study the effect of Ret signaling together with parkin on mitochondrial integrity, the heavily discussed intracellular target in PD.

My approaches

To accomplish my objectives, I used different transgenic mouse models in which the function of Ret and parkin are modulated.

Primarily, I generated new conditional Ret deficient mice (DCB-Ret ko; Figure 4.1 A) which is slightly different from the already published Ret deficient (DAT-Ret ko) mice (Kramer *et al*., 2007). The newly generated DCB-Ret ko mice carries a bacmid with an extra DAT promoter upstream to Cre expressing gene to compensate for the undesirable age dependent total DAT protein changes observed in the DAT-Ret ko (Kramer *et al*., 2007), which is due to the knock-in of Cre expressing gene in to the endogenous DAT locus for DA neuron specific conditional ablation of Ret.

1. To examine the long-term survival and maintenance function of parkin and Ret signaling in mouse SNpc DA neurons, I generated mice that lack both Ret and parkin (DCB-Ret/parkin ko; Figure 4.1 B) and investigated them in their adulthood and during ageing.

2. In order to investigate the *in vivo* neuroprotective effect of human parkin over expression on trophically impaired Ret deficient DA neurons, I generated two mouse lines that over express different forms of human parkin in Ret deficient mice (DCB-Ret ko/wt h-parkin & DCB-Ret ko/Δ h-parkin; Figures 4.16 A and B) and analyzed them during ageing.

3. To study the influence of parkin in the establishment of SNpc DA neurons with constitutive active Ret signaling, I generated parkin deficient mice with constitutive active Ret mutation, MEN2B (MEN2B/parkin ko; Figure 4.20) and analyzed them in their adulthood.

4. To understand the common downstream signaling cross points of Ret and parkin in DA neurons, I looked for signaling and physiological changes in the nigrostriatal DA system of Ret/parkin double ko mice. Whereas to study the effect of Ret signaling together with parkin on mitochondrial integrity, I analyzed for energy production and mitochondrial activity in the SN DA neurons of Ret/parkin double ko mice.

Outcome

Here, for the first time I demonstrate biochemical and mild behavioral changes in the newly developed conditional Ret deficient mice (DCB-Ret ko) in addition to the age dependent nigrostriatal DA system degeneration as described in the previously reported DAT-Ret ko mice (Kramer *et al*., 2007). More importantly, I provide genetic evidence that Ret and parkin functionally interact to promote SNpc DA neurons survival in mice. Aging mice lacking Ret and parkin show enhanced degeneration of DA neurons in the SN and their axons in the striatum. Interestingly, human parkin overexpression could protect the degenerating DA system of the Ret deficient mice suggesting redundant functions of Ret and parkin. Moreover, parkin deletion partly normalized the enlarged DA system in mice with MEN2B mutation suggesting the functional cooperation of Ret and parkin for the development of nigrostriatal DA system. I also provide the first *in vivo* evidence for converging functions of Ret signaling and parkin activity to maintain the proper cellular ATP levels and mitochondrial function in the SNpc DA neurons. All together, these observations reveal an important crosstalk of Ret and parkin to ensure proper mitochondrial function to prevent degeneration of nigrostriatal DA system. The knowledge about functional interaction and redundant functions of parkin and Ret might help to develop novel strategies to prevent, to slow down or to treat PD; particularly in PD patients with parkin mutations, GDNF and other Ret activating ligands might be more effective.

3. Materials and Methods

A. Materials

Table 3.1 List of chemicals and reagents

Substance	Company
2-Mercaptoethanol	Invitrogen
3,4-Dihydroxybenzylamine	Aldrich
Acrylamide	Roth
Agarose	BIOzym
Ammonium Persulfate (APS)	Appli Chem
Bovine serum albumin (BSA)	PAA
Bromophenol blue	Carl Roth
Celvol® 205	Celanese chem
Protease Inhibitor Cocktail Tablets	Roche
Coumaric acid	Carl Roth
Di amino benzidine (DAB)	Sigma-Aldrich
Disodium hydrogen phosphate (Na_2HPO_4)	Carl Roth
DMEM	Invitrogen
DMSO	Carl Roth
dNTP Set, 100mM Solutions	Fermentas
Ethidiumbromide	Carl Roth
Ethylene diamine tetra acetic acid (EDTA)	Carl Roth
Ethylene glycol	Sigma-Aldrich
Flouromount G	Southern biotech
Ficoll Type 400	Sigma-Aldrich
Glutaraldehyde	Fluka
Glycerol	Carl Roth
Hydrochloric acid (HCl)	Carl Roth
Hydrogen peroxide (H_2O_2)	Merck

Ketamine	UKE Apotheke
Luminol	Carl Roth
Magnesium chloride ($MgCl_2$)	Carl Roth
Osmium tetroxide (OsO_4)	Sigma-Aldrich
Paraformaldehyde (PFA)	Appli Chem
Pepsin	Appli Chem
perchloric acid	Appli Chem
Ponceau S	Sigma-Aldrich
Potassium chloride (KCl)	Carl Roth
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Rompun	UKE Apotheke
sodium cacodylate	Sigma-Aldrich
Sodium chloride (NaCl)	Carl Roth
Sodium dodecyl sulfate	Carl Roth
Sodium hydroxide (NaOH)	Carl Roth
Sucrose	Sigma-Aldrich
TEMED	Carl Roth
Tris(hydroxymethyl)aminomethane	Merck
Triton X-100	Carl Roth
Tween20	Carl Roth
Xylene cyanol dye solution	Sigma-Aldrich

Table 3.2 List of buffers and solutions

Buffer name	**Constituents**
1X Phosphate buffer saline (PBS)	10 mM Na_2HPO_4; 2 mM KH_2PO_4; 0.137 M NaCl; 2.7 mM KCl
1X Tris buffer saline (TBS)	50 mM Tris pH 7.4; 150 mM NaCl
Tris buffer saline with Tween (TBST)	0.1 % Tween20 in 1 X TBS

Tail lysis buffers	
buffer A	5 M NaOH; 0.5 M EDTA pH 8.0
buffer B	1 M Tris HCl pH 5.0
10X TAQ buffer	1 M KCl; 1 M Tris HCl pH 9.0; 1 M $MgCl_2$
Loading dye (for DNA)	3 % (w/v) Ficoll Type 400; 0.05 % (w/v) bromophenol blue; 0.05 % (w/v) xylene cyanol F
50X Tris Acetate-EDTA (TAE) buffer (1L)	2 M Tris pH 7.8; 0.5 M NaOAc; 50 mM EDTA
Anaesthetic solution	120 µL ketamine 100mg/mL; 80 µL rompun 20mg/mL
4% PFA	1L PBS (warm up to 60°C); 40 g PFA; 400 µL 5M NaOH + 150 µL 37% HCl pH 7.5; Cool down to 4°C before use
Sucrose solution	30 % (for IHC) and 80 % (for immuno-EM) of Sucrose in 100mL PBS
Brain embedding solution	mix egg yelow and sucrose 10 : 1 (g/g); use cold mix for embedding (4 ^{0}C) to polymerize, add 0.5 mL glyceraldehyde 25 % to 10 mL egg mix; mix well and allow 45 min for polymerization at room temperature, then move the embedded brains to dry ice after 30mins store the moulds until used at -80^{0}C
Celvol® mounting medium	Celvol®205 125 g/L; glycerol 25 % (v/v); in 0.1 M PBS pH 7.4
Cryoprotection solution (1L)	30 % glycerol (v/v); 30 % ethylene glycol (v/v) in 0.1 M PBS pH 7.4
Blocking solution (for IHC)	5 % BSA; 0.3 % Triton X-100 in TBS
Antibody carrier solution (for IHC)	2 % BSA; 0.1 % Triton X-100 in TBS
DAB solution (for IHC)	5 mL of H_2O; Each tablet of DAB and H_2O_2
Tissue homogenization buffer (for Western blotting)	50 mM Tris HCL pH 7.5; 150 mM NaCl; 0.5 % Triton X 100; 1 tablet Protease Inhibitor cocktail EDTA free (for 50 mL of buffer); 1 tablet of Phostop phosphatase inhibitor cocktail (for 10 mL of buffer)
6X Loading dye (for proteins)	125 mM Tris pH 6.8; 4 % SDS; 20 % v/v Glycerol 100%; 200 mM 1M DTT; 0.02 % bromo phenol blue
APS (10%)	1 g of APS in 10 mL ddH_2O
Blotting buffer (1X) (for Western blotting)	20 mM Tris; 150 mM glycerine; 0.1 % (w/v) SDS; 20 % (v/v) methanol
Blocking solution and antibody carrier solution (for Western blotting)	5 % BSA; 0.1 % Tween20 in TBS

ECL (10mL) **(for Western blotting)**	0.1 M Tris pH 8.5; 3 µL of 30 % H_2O_2; 50 µL of 250 mM luminol(DMSO); 25 µL 90 mM coumaric acid(DMSO)
Blocking solution **(for immuno-EM)**	0.3 % BSA; 10 % horse serum in 1 X PBS pH-7.4 **Note**: No detergent
Antibody carrier solution **(for immuno-EM)**	0.2% BSA; 1% horse serum; in 1 X PBS pH-7.4 **Note**: No detergent
Osmium (for immuno-EM)	1 % Osmium tetroxide in 0.1 M Cacodylate buffer
HPLC lysis buffer	0.1 M perchloric acid ($HClO_4$); 0.5 mM disodium EDTA; 100 ng/mL of 3,4 dihydroxybenzylamine

Table 3.3 List of kits

Kit name (order number)	company	purpose
Vectastain ABC (peroxidase) Standard (PK-4000)	Linaris	antibody signal enhancer
Pierce™ BCA protein assay kit (Thermo Scientific #23225)	Thermo Scientific	estimation of protein concentration
OxyBlot Protein Oxidation Detection (S7150)	Millipore	detection of protein carbonylation
Complex I Enzyme Activity Microplate assay (ab109721)	Abcam	complex I activity measurements
ATP (Colorimetric/Fluorometric) Assay (ab83355)	Abcam	total cellular ATP measurements

B. Methods

3.1 Transgenic mouse lines

3.1.1 Animal housing

All animal (mouse) experiments were performed in accordance with the German Animal Welfare Act and with the approval of local authorities of the city state Hamburg and the animal care committee of the University Medical Center Hamburg-Eppendorf (UKE). Mice were housed in the mouse facility of the Center for Molecular Neurobiology Hamburg (ZMNH) under constant conditions temperature (at 22 °C) and humidity (at 40-50%) in a 12 h light-dark cycle with unrestricted access to food and water. All routine mouse work, like changing the cages and bedding material, water and food supply, breeding, weaning, collection of biopsies and marking mice for identification was done by the animal care takers of the UKE mouse facility.

3.1.2 Transgenic mouse lines

All transgenic mouse lines used in this study (Table 3. 4) were maintained on a C57BL/6J inbred strain background.

Table 3.4 List of transgenic mouse lines

Name (abbreviation)	**Type**	**Description**	**Reference**
DAT-Cre BAC (DCB)	transgenic	Cre recombinase expression by exogenous DAT locus in the bacmid	Parlato et al., 2006
Ret floxed ($Ret^{lx/lx}$)	knock-in	loxP conditional alleles flanking exon 12 of Ret gene	Kramer et al., 2006
Parkin ko	knock out	exon 3 deletion resulting	Itier et al., 2003
wt human parkin (wt h-parkin)	transgenic	human parkin (full length) overexpression driven by mouse prion promoter	Unpublished
Δ human parkin (Δ h-parkin)	transgenic	human parkin (with 13 AA C-terminal truncation) overexpression driven by mouse prion promoter	Unpublished
MEN2B	Knock-in	constitutive active form of Ret expression driven by endogenous Ret promoter	Smith-Hicks et al., 2000

3.1.2.1 The Cre/loxP system was used to specifically ablate Ret in the mouse midbrain DA neurons

The Cre/loxP system allows site-specific DNA recombination (excision and consequently inactivation of the target gene) in defined cells or tissues. Cre Recombinase is a Type I

topoisomerase from bacteriophage P1 that catalyzes the site-specific recombination of DNA between loxP (locus of X-over P1, a 34-bp site normally found in the bacteriophage P1) sites. Two mouse lines are required to achieve conditional gene deletion. One is a conventional transgenic mouse line with Cre specifically expressed in a defined tissue or cell type (we used DCB) and the second is a mouse strain in which the target gene (here Ret) is flanked by two loxP sites in a direct orientation ("floxed allele"). Recombination occurs only in the Cre recombinase expressing cells leaving the target gene active in the remaining cells and tissues. In this study, we crossed DAT-Cre BAC mouse line (DCB; a transgenic line that carries a bacmid which expresses Cre recombinase under the control of midbrain DA neurons specific promoter, DAT) with a second mouse line that carries floxed alleles of Ret ($Ret^{lx/lx}$; loxP conditional alleles flanking exon 12 of Ret gene) to obtain midbrain DA neurons specific conditional Ret knockout mice (referred as DAT-Cre BAC/ $Ret^{lx/lx}$ or DCB-Ret ko; Figure 3.1).

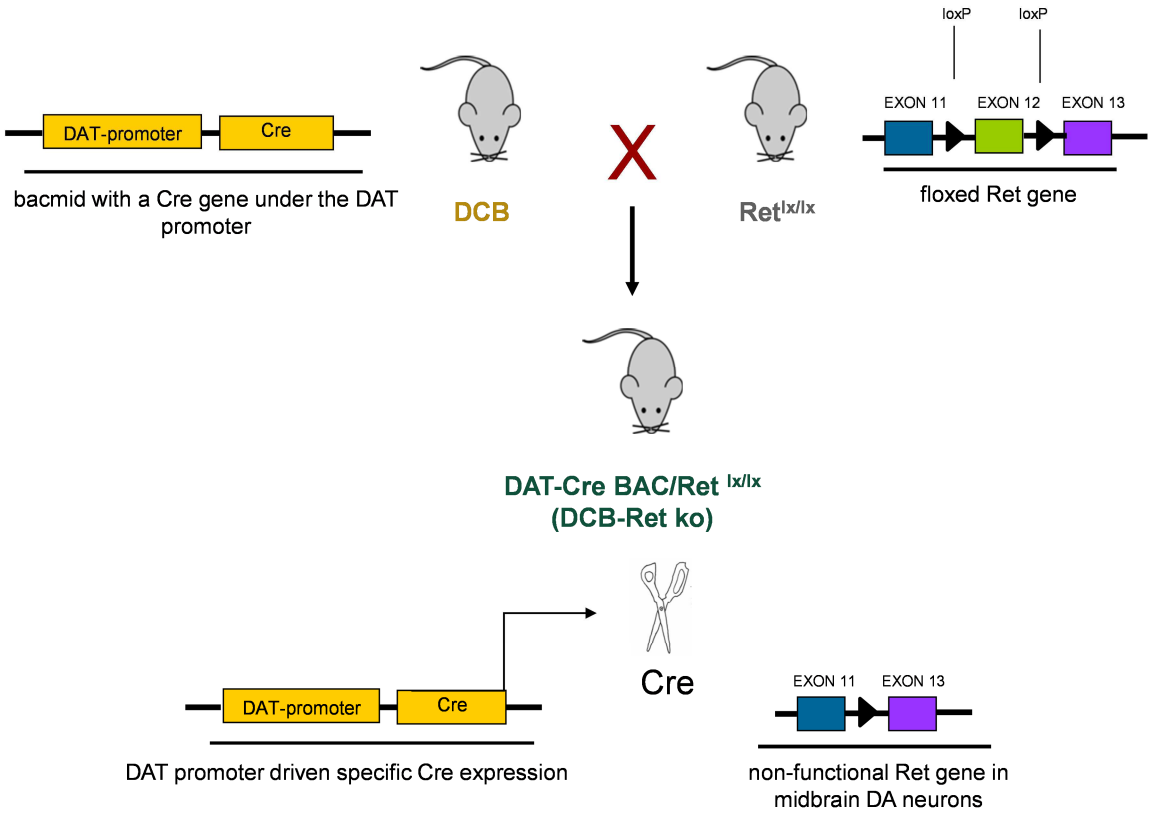

Figure 3.1 Illustration of Cre/loxP system used for the conditional ablation of Ret from midbrain DA neurons

3.2 Mouse genotyping (DNA extraction and PCR amplification)

DNA was extracted from mouse tails after incubation of each tail biopsy with 100 µL tail lysis buffer A and heated for 20 min at 95 °C in a thermocycler and subsequent neutralization with 100 µL tail lysis buffer B. The resulting DNA solution was kept at +4 °C for immediate use or stored at -20 °C for later use. A PCR mastermix (50 µL) was prepared using homemade Taq DNA polymerase enzyme and 2 µl of genomic DNA per reaction.

The PCR master mix composition (for 50 μL):

1 μL each of the reverse and forward primers (30 μM), (see Table 3.5)

5 μL of 10X TAQ buffer (1X)

5 μL of dNTPs mix (10 μM),

1 μL Taq DNA polymerase,

36 μL distilled water and

2 μL from the DNA solution

Sequence specific forward and reverse primers and PCR T_m conditions and expected band(s) sizes for each transgene are listed in Table 3.5. The Ret gene conditional alleles were tested for germline recombination events using an additional PCR, Ret^{rec}. Amplified DNA was then analyzed by agarose gel electrophoresis.

Table 3.5 Genotyping primers list

PCR – primers	Primer sequence (5'-3')	band(s) size	Tm
DCB			
forward	TCC CTG TGG ATG CCA CCT CTG ATG	500 bp	67 °C
reverse	GCA GAA GGG GCA GCC ACA CCA TTC		
Ret^{lx}			
forward	CCA ACA GTA GCC TCT GTG TAA CCC C	300bp (Ret wt)	62 °C
reverse	GCA GTC TCT CCA TGG ACA TGG TAG	350bp (Ret^{lx})	
Ret^{rec}			
forward	CGA GTA GAG AAT GGA CTG CCA TCT CCC	600 bp	72 °C
reverse	ATG AGC CTA TGG GGG GGT GGG CAC		
parkin wt			
forward	CTC CTG GAC TCC CAT ATG GAG CCC	300 bp	65 °C
reverse	CCT TCT GTT GCT CCA CTG GCA GAG		
parkin ko			
forward	GAA CGA GAT CAG CAG CCT CTG TTC C	350 bp	65 °C
reverse	CCT TCT GTT GCT CCA CTG GCA GAG		
MEN2B			
forward	CCT CTC ACA CAC CAC AAC C	300 bp (Ret wt)	60 °C
reverse	GCT CAG TCT GAG ATG CTG GG	350bp (MEN2B)	
(wt/Δ) h-parkin			
forward	ACC TGC AGG CAG GCA ACG CTC AC	361 bp	64 °C
reverse	GCA GGG AGT AGC CAA GTT GAG GG		

3.3 Histology and Immunohistochemistry

For immunohistochemistry mice were perfused with PBS and 4% paraformaldehyde. Subsequently, brains were removed from the skull, post fixed overnight in the same fixative

and cyroprotected by incubating them in 30% sucrose solution. 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. The 30 µm thick coronal sections were cut on a cryostat, collected free floating, and then directly used for staining or stored in a cryoprotection solution at -20˚C until utilized. For DA fiber staining, sections were premounted; for all other staining procedures free-floating sections were used. Free floating or mounted sections were blocked for 1 h in blocking solution (5% BSA, 0.3% Triton X-100 in TBS) at room temperature, and incubated with the first antibody diluted in carrier solution (2% BSA, 0.1%Triton X-100 in TBS) at 4˚C overnight (see Table 3.6).

For TH/GIRK2 cell body and astrocyte/microglia staining, the sections were washed three times in TBS for 5 min and incubated at 4˚C overnight with a species-specific biotin-coupled secondary antibody (dilution 1:200 in antibody incubation buffer) from Vectastain ABC kits (Linaris). 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 h at room temperature). Finally, after three washes in TBS, a substrate of HRP, the diaminobenzidine (DAB) which is diluted in ddH_2O was added. Sections were incubated until a brown precipitate was formed that allowed specific visualization of neurons or glial cells. The DAB exposure was optimized to allow a high signal-to-noise ratio, usually not more than 20 min. The sections were then washed once with ddH_2O for 15 min and mounted on glass slides, then after air drying, they are finally mounted with the Celvol® mounting medium.

For TH/DAT fiber immunofluorescence staining, the sections were washed three times in TBS for 5 min after overnight primary antibody incubation and then the sections were incubated in biotinylated secondary antibody (1:200 anti-mouse or anti-rat, Linaris) diluted in carrier solution for 2 h at room temperature, again washed as described above, and treated with streptavidin-Cy3 (1:500; Sigma) diluted in carrier solution, for 2 h at room temperature. For other immunofluorescence staining procedures, the sections were washed three times in TBS for 5 min after overnight primary antibody incubation and then sections were incubated in respective secondary antibodies that are coupled to either Alexa488 (Invitrogen) or Cy3 (Invitrogen) at a dilution of 1:500 in carrier solution at 4˚C overnight. After another three washing steps, all the immunofluorescence stained sections were mounted in aqueous mounting medium with anti-fading reagent (Fluoromount G, Southern biotech).

Table 3.6 List of primary antibodies for immunohistochemistry

antibody (α-)	Host	clonality	working dilution	company/source
Ret	Rabbit	monoclonal	1:250	lab made
Parkin	Goat	polyclonal	1:500	AbD serotec
TH	Mouse	monoclonal	1:1000	Diasorin
DAT	Rat	monoclonal	1:500	Chemicon
GIRK2	Rabbit	polyclonal	1:500	Almone labs
GFAP	Rabbit	polyclonal	1:500	Dako
Iba1	Rabbit	polyclonal	1:500	Wako

3.3.1 Quantification of cell populations

For stereological analysis, unbiased counting relative to genotype and condition was performed using the optical fractionator work flow of the StereoInvestigator software program (MicroBrightField, Williston, Vermont, United States) on 30 µm thick coronal serial sections with a section interval of six for the midbrain (SNpc and VTA) using an oil immersion 63x objective, a counting frame of 50 x 50 µm, and a grid size of 100 x 100 µm.

3.3.2 Quantification of soma size

TH immunostained coronal sections were analyzed using a bright field microscope with a 63x objective. Random cells were selected using optical fractionator work flow and soma size was determined using the 'nucleator' probe in the StereoInvestigator software (MicroBrightField, Williston, Vermont, United States). For each mouse 100 to 150 cells were analyzed, experimenters were blinded to the genotype of each animal.

3.3.3 Fiber density measurement

Striatal fiber density measurements were performed 30 µm thick coronal striatal sections as described by Kowsky and others (Kowsky *et al.*, 2007). Images of TH or DAT immunofluorescent stainings were taken using an epifluorescent upright microscope Axio Imager.M1 (Zeiss, Goettingen, Germany) equipped a Hamamatsu camera C8484. Five coronal striatal sections with a section interval of six, distributed between bregma +1.10 and −0.10 mm were analyzed. For every section, three pictures in the dorsal and two pictures in the ventral striatum were acquired using an oil immersion 63x objective. 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).

3.3.4 Astrocyte and microglial density quantifications

Every sixth section (30 µm thick coronal) from the striatum and the midbrain was used to determine the density of astrocytes (immunostained for GFAP) or microglial cells (immunostained for Iba1) in the dorsal striatum and SN respectively. For each section, the area containing labeled cells was delineated and 3 images of dorsal striatum and 2 images of SN were acquired using the upright microscope Axio Imager.M1 (Zeiss, Goettingen, Germany). The number of cells was later determined from the images obtained from 5 sections per animal by using the cell counter plugin in the ImageJ program (NIH).

3.3.5 Fluorescence intensity measurements and co-localization studies

To correlate protein expression levels in tissue with fluorescence intensities (Figure 4.24 A and B) and for co-localization studies (Figure 4.14), immunofluorescent staining was performed on 30 µm coronal sections (of the regions of interest) from 3 different animals per genotype. Multicolor images of RGB samples were obtained with a Leica TCS SP2 confocal

microscope system and 10x (0.3 NA) and 63x (oil 1.32 NA) objectives. Image stacks were maximally projected and contrast and intensity levels were uniformly adjusted using the ImageJ program (NIH). For fluorescence intensity measurements, intensity values of at least 150 cells from an animal was averaged represented as mean pixel intensities using Image Studio Lite (from LI-COR). For co-localization studies, at least 150 cells were counted from each animal using the cell counter plugin in the imageJ program (NIH).

3.3.7 Immunoelectron microscopy

After perfusing mice with 0.9% saline and fixative (4% paraformaldehyde with 0.1% glutaraldehyde), brains were removed from the skulls and postfixed overnight in 4% paraformaldehyde at 4˚C. The brains were then cut with a vibratome in to 100 µm thick sections and incubated in 2.3 M sucrose (in PBS, pH 7.4) overnight at 4˚C. Sections were then permeabilized by a process called cracking, in which the sections were alternatively incubated for 3 min in 2- methyl-butane (cooled below -100°C in liquid N_2) and 2.3 M sucrose (at room temperature) for three times and then washed three times with PBS, each wash for 5 min and incubated in 0.3% H2O2 and washed with PBS. Sections were blocked with 0.3% BSA and 10% horse serum in 1X PBS for 30 min and then incubated in mouse anti-TH antibody (1:1000, Diasorin), diluted in carrier solution (1X PBS with 0.2% BSA and 1% horse serum) for 48 h at 4˚C. After primary antibody incubation, sections were washed with PBS and incubated in HRP coupled anti-mouse secondary antibody (1:500, Vectastain) diluted in carrier solution for 2 h at room temperature and then washed with PBS before incubating the sections in Vectastain ABC solution (Vector Laboratories, Burlingame, California, United States) for 2 h at room temperature. For brown staining, sections were washed with PBS and incubated with diaminobenzidine (DAB) for 5 min following three PBS washes. The stained sections were then rinsed three times in 0.1 M sodium cacodylate buffer (pH 7.2–7.4) and osmicated using 1% osmium tetroxide (Science Services, München, Germany) in cacodylate buffer. Following osmication, the sections were dehydrated using ascending ethyl alcohol concentration steps, followed by two rinses in propylene oxide. Infiltration of the embedding medium was performed by immersing the sections in a 1:1 mixture of propylene oxide and Epon and finally in neat Epon and hardened at 60 °C. Ultrathin sections (60 nm) were examined in EM902 (Zeiss, Germany). Pictures were taken with a MegaViewIII digital camera (A. Tröndle, Moorenweis, Germany).

3.3.7.1 Immunoelectron microscopy data analysis

Total number of mitochondria and percentage of damaged mitochondria per 100 μm^2 of cytosolic area was analyzed from 1500±250 μm^2 TH immunolabelled cytosolic area of SNpc neurons obtained from two to three vibrotome sections for each animal. Mitochondria were considered as damaged when the cristae are distorted or disrupted, when the outer membrane is detached or when the outer membrane has protrusions (Stichel *et al.*, 2007). For estimating the length of mitochondria, a minimum of 500 mitochondria were counted from 20–28 TH immunolabeled SNpc neurons obtained from two to three vibratome sections per animal. We classified the mitochondria into two groups based on their length, smaller than 0.5 µm and larger than 0.5 µm, slightly different from what was described previously (Gautier *et al.*,

2008). The analysis was done independently by two examiners blinded to the genotype using the ImageJ program (NIH).

3.4 Biochemical analysis

3.4.1 Tissue preparation for biochemical analysis

Mice euthanized through cervical dislocation, brains were removed from the skull, quickly frozen on dry ice and stored at -80˚C until used. The frozen brains were cut in to 1 mm thick (for SN) and 2 mm thick (for striatum) coronal sections (2 mm rostral or caudal to the interaural line); and tissue was punched out with 2mm diameter sample corers (Fine Science Tools, Heidelberg, Germany). The dissected SN and striatum samples were used for different experiments as described.

3.4.2 Sample preparation for Western blotting

For mouse tissue, dissected SN and striatum samples from control and mutant mice were homogenized in pre-cooled lysis buffer (50 mM Tris HCl pH-7.5, 150 mM NaCl, 0.5% triton x-100) with appropriate amounts of protease and phosphatase inhibitor cocktail (Roche) using Teflon Dounce. The homogenate was centrifuged at 20000g for 15 min at 4˚C. Supernatant obtained was used for western blotting proteins in the soluble fraction and for determination of protein concentration using BCA (Pierce); whereas, for detergent insoluble proteins the pellets obtained after centrifugation were boiled in buffer containing 25 mM Tris pH 6.8, 1% SDS, 4% glycerol and 5% β-mercaptoethanol for 10 min at 95˚C. For mammalian cell lines, cell pellets were lysed in 0.1% Triton X-100 in PBS with appropriate amounts of protease and phosphatase inhibitor cocktails (Roche) by pipetting up and down then centrifuged at 20000g for 15 min, supernatants were used for determining protein concentration using BCA (as described in 3.4.2.1) and Western blotting (as described in 3.4.3).

3.4.2.1 Estimation of protein concentration

Protein concentrations of brain tissue lysates and SH-SY5Y cell lysates were determined using the Pierce™ BCA protein assay kit (Thermo Scientific) according to the manufacturer´s instructions. Along with test samples, BSA standards with 5 different concentrations in the range of 0-10 µg/µL were measured. All samples and standards were measured in triplicates.

3.4.3 Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their molecular weight and the resolved proteins were then transferred on to PVDF membranes for immunodetection. A Hoefer SE 600/400 system with 1.5 mm spacers and 10 well Teflon combs were used for SDS-PAGE. For resolving proteins of my interest I casted mini gels until 3/4th of the total volume between the glass plates with 10 % (w/v) resolving gels, after polymerization of the resolving gel, the remaining 1/4th of the volume was filled with 4 % stacking gels (composition of resolving and stacking gels are shown below). After adding the stacking gel 10 well Teflon combs were placed, which were removed after polymerization to create sample wells.

10% (w/v) resolving gel: (10mL)	4.05 mL of ddH2O 3.3 mL of 30% acrylamide 2.6 mL of 1.5 M Tris pH 8.8 with 0.4 % SDS 50 µL of 10 % APS 5 µL of TEMED
4% (w/v) stacking gel: (5mL)	3.05 mL of ddH2O 0.65 mL of 30% acrylamide 1.3 mL of 0.5 M Tris pH 8.8 with 0.4 % SDS 50 µL of 10 % APS 5 µL of TEMED

Striatal and SN protein lysates (with 3-5 µg/µL) concentrations were mixed with equal volumes of protein loading buffer and heated for 5 min at 95 °C before loading into the sample wells of the gel. One well in each gel was loaded with prestained protein molecular weight standard to identify molecular weights (in kDa). Electrophoresis run was performed at 100 V until bromophenol blue (which was used in the loading buffer) dye front reaches the bottom of the separating gel. The SDS-PAGE gel is freed from the glass plates and incubated in blotting buffer. To start the blotting procedure, PVDF membranes were activated by incubation in methanol for 3-5 min and transferred to blotting buffer together with six Whatmann blotting papers. For transferring the resolved protein from the gel to the membrane, 3 sheets of Whatmann blotting papers were placed on a semi dry transfer cell, on top of the blotting papers PVDF membrane was placed on which the SDS-PAGE gel is placed on which 3 sheets of blotting paper were placed. The lid of the transfer apparatus is carefully placed on the stack and connected to a power source. Transfer was carried out with 1 mA/cm^2 (for 1 blot: ~0.12 A) for 2 h and after which the efficiency was checked by staining the PVDF membrane with a temporary Ponceau S staining. Membranes were then blocked in appropriate amounts of blocking solution (5% BSA powder in TBS-T) for 1 h at room temperature while gently rocking on a horizontal shaker. Next, membranes were incubated with appropriate amounts of primary antibody diluted (Table 3.7) in carrier solution and placed on a rolling mixer at 4 °C over night. After incubation in primary antibody, the membrane was washed 3 times with TBS-T (5 min each wash) with constant agitation on a horizontal shaker. Membranes were then incubated with respective Horse radish peroxidase (HRP)-conjugated secondary antibody (Jackson Immunoresearch) diluted in carrier solution for 1 h at room temperature, the membrane was washed 3 times in TBS-T (5 min each wash) again followed by one time in TBS. Immunodetection was achieved by incubating the membrane for 3 min in ECL solution. Finally, chemiluminescent signals were detected under the Image Reader LAS-4000 system. The chemiluminescent signals were saved as pictures by the image reader at pre-determined time points and later quantified (in some cases) using the Image Studio Lite (from LI-COR).

Table 3.7 List of primary antibodies for Western blotting

antibody (α-)	host	clonality	working dilution	company/source
Ret	rabbit	monoclonal	1:1000	lab made
Parkin	mouse	monoclonal	1:500	Santa Cruz Biotechnology
TH	mouse	monoclonal	1:20000	Diasorin
β-actin	mouse	monoclonal	1:20000	Sigma Aldrich
phospho-AKT (Ser 473)	mouse	monoclonal	1:1000	Cell Signaling Technology
phospho-S6 (S235/236)	rabbit	monoclonal	1:1000	Cell Signaling Technology
phospho-ERK1/2 (Thr202/Tyr204)	rabbit	monoclonal	1:1000	Cell Signaling Technology
AKT (pan)	rabbit	monoclonal	1:1000	Cell Signaling Technology
S6	mouse	monoclonal	1:1000	Cell Signaling Technology
ERK1/2	rabbit	monoclonal	1:1000	Cell Signaling Technology
DAT	rat	monoclonal	1:500	Chemicon
NDUFA10	rabbit	polyclonal	1:1000	Santa Cruz Biotechnology
Histone 3 (H3)	rabbit	monoclonal	1:1000	Cell Signaling Technology
Opa1	mouse	monoclonal	1:1000	BD Transduction Laboratories
Drp1	mouse	monoclonal	1:1000	BD Transduction Laboratories
Mfn1	mouse	monoclonal	1:1000	Abcam
Mfn2	rabbit	monoclonal	1:1000	Sigma-Aldrich
COXII	rabbit	monoclonal	1:1000	Epitomics
COXIV	rabbit	polyclonal	1:500	Cell Signaling Technology

3.4.4 Detection of protein carbonylation

For detecting the carbonylation of proteins in control and mutant mice (24 months old), SN and striatum tissue samples were prepared (as described in 3.4.1). After protein concentration determination using BCA (as described in 3.4.2.1), 15 µg of protein per sample was used to detect protein carbonylation using Oxyblot kit (Millipore) and rest of the procedure was carried out as per the kit manufacturer's instructions.

3.4.5 Striatal total dopamine measurements using HPLC-ECD system

Dissected striatal tissue from control and mutant mice was homogenized in 0.1 M perchloric acid containing 0.5 mM disodium EDTA and 100 ng/mL, 3,4-dihydroxybenzylamine (internal standard) and then centrifuged at 50,000 g for 30 min. Pellets thus obtained were resuspended in 200 µL neutralizing buffer (lysis buffer used for Western blotting) for protein determination using BCA (as described in 3.4.2.1); whereas, the supernatants after filtering through a 0.22 µM PVDF membrane were subjected to HPLC electrochemical detection system analysis as described previously (Yang and Beal 2011) with the following modifications, 3,4 dihydrobenzylamine (100 ng/mL lysate buffer) was used as internal standard; flow rate of the mobile phase was 1.2 mL/min; the sample injection volume was 20µL. The applied electrochemical potentials were: Conditioning cell = +10 mV; analytical

cell: E1 = + 50 mV; E2 = 360 mV. The retention times of the measured metabolites were as follows: DOPAC (3.80 min), 3,4-dihydroxybenzylamine (4.25 min), dopamine (6.88 min) and HVA (10.30 min). The peak areas of Dopamine and its metabolites were normalized with the peak areas of internal standard to obtain the total amount DA and its metabolites present in the injected sample volume and the final values were represented as nanogram per milligram of striatal protein.

3.4.6 Measurement of Cellular ATP Levels in mouse brain tissue

Dissected SN tissue samples from control and mutant mice were homogenized in 200 µL of pre-cooled ATP assay buffer provided in the kit (ATP Assay Kit - Abcam) using Teflon Dounce. Small volume of the homogenate was used for protein concentration determination using BCA (as described in 3.4.2.1), whereas, rest of the homogenate was centrifuged at 14000g for 15 min and 50 µL of the supernatant was used to perform fluorometric ATP assay according to the kit manufacturer's instructions.

3.4.7 Cell cultures and RNA interference

SH-SY5Y (human neuroblastoma cells; ATCC® CRL-2266™) cells were cultivated in DMEM supplemented with 15% FBS (Sigma) and maintained at 37˚C, 5% CO_2. For RNAi intereference, cells were reversely transfected with the following stealth siRNA oligos (Invitrogen): human Ret HSS109181; human Parkin HSS107594 using Lipofectamine RNAiMAX (Invitrogen). After 4 h medium was changed and cells were harvested 48 h post-transfection using cell scraper, after a brief centrifugation for 5 min at 1000 rpm in 4 °C the cell pellets are immediately flash frozen in liquid N_2 and then stored at -80 °C until used.

3.4.8 Mitochondrial enrichment and Complex I activity (from SN tissue and SH-SY5Y cell samples)

Dissected SN tissue samples were homogenized in 1 ml and siRNA transfected cell pellets (obtained from one 6 well plate) were lysed in 0.5ml of pre-cooled homogenization buffer containing 320 mM Sucrose, 5 mM Tris pH-7.4, 2 mM EGTA along with appropriate amounts of protease inhibitor cocktail (Roche). After ten strokes with Teflon dounce (for tissue) or pipetting the pellet up and down for 30 times in the lysis buffer (for SH-SY5Y cells), the lysates were centrifuged for 3 min at 2000g to remove nuclei and other cell particles. Supernatants were collected and centrifuged for 10 min at 12,000g to pellet mitochondria and synaptosomes. The crude pellet was resuspended in 1 ml (for tissue) and 0.5ml (for SH-SY5Y cells) of homogenization buffer containing 0.02% w/v of digitonin to disrupt synaptosomal membranes and release trapped mitochondria (Palacino *et al.*, 2004). The resuspended samples were centrifuged for 10 min at 12,000g to pellet mitochondria, which were again resuspended in 100 µl of the homogenization buffer, and protein content was determined by BCA assay (as described in 3.4.2.1). Complex I enzyme activity assay was performed using the complex I enzyme activity microplate assay kit (Abcam) according to the manufacturer's instruction with 30 µg protein (for tissue) and 50 µg protein (for SH-SY5Y cells) from the enriched mitochondrial preparations.

3.5 Behavioral Experiments

Aged male mice (12 and 24 month old) were housed individually with free access to water and food in a room with 12 h /12 h reversed day-night cycle. All behavioral experiments were conducted during the dark period in a quiet room with 12 lux light intensity.

3.5.1 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; the mice that were tested at 12 months were aged and tested again at 24 months. Each mouse was placed into a 50 x 50 cm arena enclosed by 40 cm high walls for 10 min and their horizontal activity was monitored by an observer and by using the EthoVision software (Noldus, Sterling, USA). The results represent locomotory and thigmotactic behavior per 10 min.

3.5.2 Elevated plus maze

Mice (24 month old) were placed in an elevated plus maze with four 30 cm long and 5 cm wide arms, connected by a 5 x 5 cm center. Two opposing arms were bordered by 15 cm high walls (closed arms), whereas the other two arms (open arms) were bordered by a 2 mm rim. The maze was elevated 75 cm from the floor and illuminated with 3 lux to allow video recording. The mouse was placed into the center facing one open arm and observed for 5 min. The following parameters were obtained from the pre-recorded video with the software The Observer (Noldus); entries and duration into the open and closed arms (calculated when all four paws were on an arm), entries into the end of the open arms (calculated when the mouse reaches with its snout the end of an open arm) and head dipping events in the open arms (Trullas and Skolnick, 1993).

4. Results

A. Parkin deletion enhances nigrostriatal degeneration in the aged Ret-deficient mice

4.1 Generation and characterization of Ret/parkin double-deficient mice

In the process of generating Ret/parkin double-deficient mice, we first had to generate conditional Ret-deficient mice, since complete ablation of Ret would lead to embryonic lethality. Using a conditional approach we ablated Ret specifically from the midbrain DA neurons during development, this is achieved by crossing mice carrying a floxed allele of Ret (the $Ret^{lx/lx}$ mice; Kramer et al., 2006) with transgenic mice carrying a bacmid that expresses Cre recombinase (as early as E13.5) under the dopamine transporter (DAT) promoter (the DCB mice; Parlato et al., 2006; Turiault et al., 2007) to generate the DCB-$Ret^{lx/lx}$ (referred to as DCB-Ret ko) mice (Figure 4.1 A). The DCB Cre expressing mice used in this study has several advantages over the previously known DAT-Cre line (Kramer et al., 2007). In the DAT-Cre line, the Cre transgene insertion in to the 5'-UTR of one of the endogenous DAT locus resulted in only one functional copy of DAT gene, which led to an age dependent decrease in DAT protein levels along with increased striatal DA levels and hyperactive behavior in the mice (Kowsky et al., 2007; Parlato et al., 2006; Turiault et al., 2007). With the use of BAC transgenesis approach, we could express Cre under the exogenous DAT promoter that is engineered in to the bacmid to ablate Ret specifically in the DA neurons leaving the endogenous DAT locus remained intact and functional without any age dependent changes in DAT protein levels or alterations in striatal DA levels or behavior changes. The DCB-Ret ko mice thus generated were then crossed with general parkin-deficient mice (parkin ko) (Itier et al., 2003) to obtain Ret/parkin double-deficient mice (referred to as DCB-Ret/parkin ko) (Figure 4.1 B).

The DCB-Ret/parkin ko mice were viable and fertile. Double and single mutant mice of Ret and parkin together with DCB or $Ret^{lx/lx}$ control mice were allowed to age and used for analyzing changes in the nigrostriatal DA system at different time points during the aging process (at 3-6 months, 12 months and 24 months). Ret and parkin which are highly expressed otherwise were efficiently removed in the midbrain DA neurons of the DAT-Ret/parkin ko mice as shown by immunohistochemical co-stainings for Ret and parkin with the DA cell marker tyrosine hydroxylase (TH) in SNpc brain sections (Figures 4.2 A and B) and western blotting of striatal brain lysates (Figure 4.2 C), whereas the levels of DA neuron specific proteins, TH and DAT did not change in the adult Ret/parkin double ko and Ret, parkin single ko mice when compared with DCB controls (Figure 4.2 C).

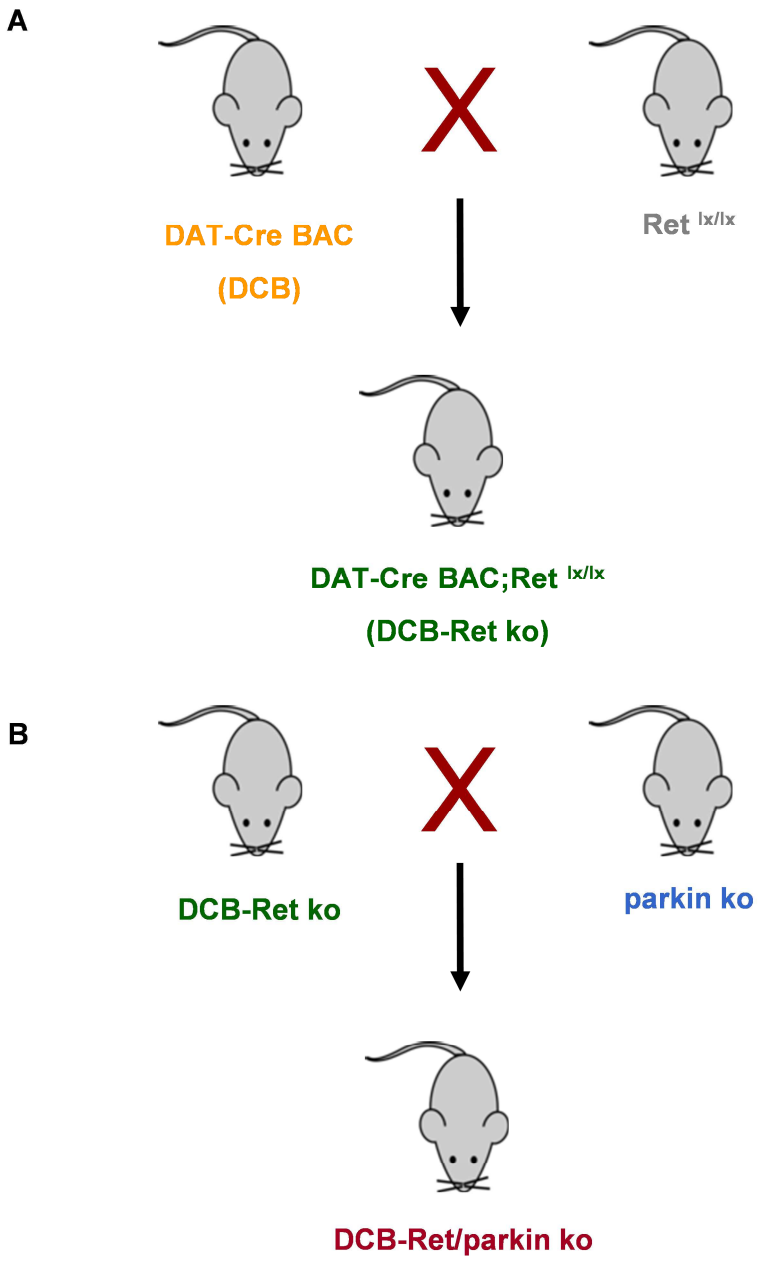

Figure 4.1 Schematic representations of the crosses made to obtain Ret/parkin double-deficient mice

(A) Generation of conditional Ret-deficient mouse: DAT-Cre BAC mice, obtained from Günther Schütz (Parlato et al., 2006) were crossed with Floxed Ret ($Ret^{lx/lx}$) mice (Kramer et al., 2007) to create DAT-Cre BAC/$Ret^{lx/lx}$ (DCB-Ret ko) mice which specifically lack Ret in their DA neurons.
(B) Generation of Ret/parkin double-deficient mice: The conditional Ret-deficient mice were crossed with parkin-deficient mice obtained from Alexis Brice (Itier et al., 2003) to create double KO mice (DCB-Ret/parkin ko) which are completely deficient for parkin and lack Ret specifically in the DA neurons. The Ret/parkin ko mice were analyzed at adult (3-6 months), middle (12 months) and late (24 months) ages.

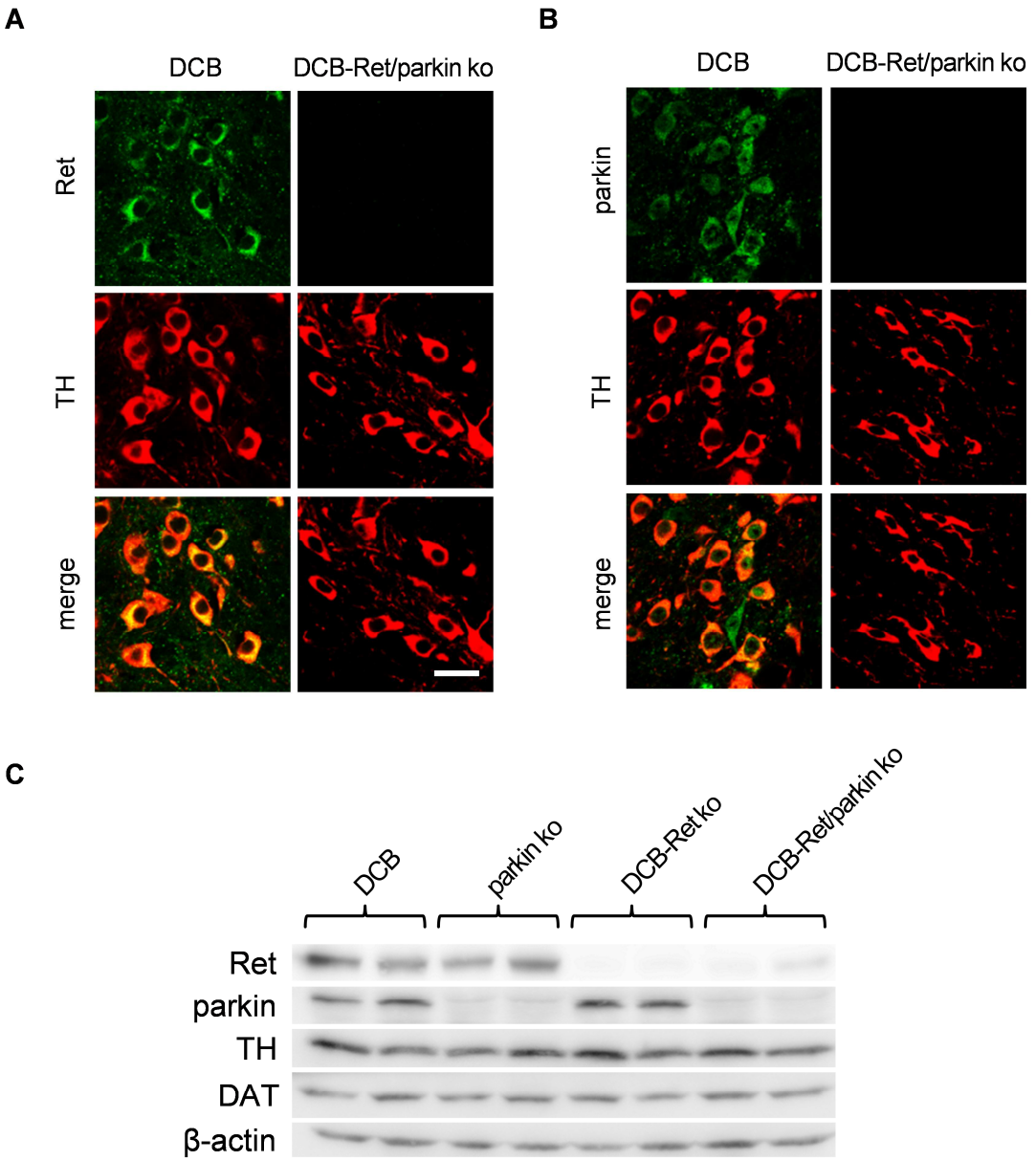

Figure 4.2 Characterization of Ret and parkin deletion in SNpc DA (TH) neurons of Ret/parkin double ko mice

(A-B) Representative images of coronal sections from 3 month old control (DCB) and DCB-Ret/parkin ko showing loss of (A) Ret and (B) parkin expression in TH stained DA neurons of the SNpc in the DCB-Ret/parkin ko mice but not in the DCB control. Scale bar = 25 µm.
(C) Western blot analysis of Ret and parkin expression in striatal tissue lysates from 3 month old mice of the indicated genotypes confirms the efficient deletion of parkin and Ret proteins in their respective knockouts. TH and DAT expression seems not change across the genotypes when normalized with β-actin (loading control), 2 mice per genotype are shown.

4.2 Parkin deletion enhances the age dependent progressive DA neuronal loss in the SNpc of conditional Ret-deficient mice

To study the cell survival function of parkin in Ret-deficient mice, with decreased trophic support. First, I investigated the number of DA neurons in adult (3-6 month old) DCB-Ret/parkin ko mice by stereological quantification of TH antibody stained brain sections. As reported previously for the single Ret ko (Kramer et al., 2007) and parkin ko mice (Goldberg et al., 2003; Itier et al., 2003), the DCB-Ret/parkin ko mice also showed normal number of DA neurons in the SNpc compared to age-matched control mice (DCB) (Figure 4.3 B). To study the effect of ageing in these mice, I analyzed the number of DA neurons in DCB-Ret/parkin ko mice at 12 and 24 months of age by stereological quantification of TH antibody stained brain sections, by which I confirmed the original finding that the number of DA neurons in the SNpc does not change in 12 and 24 month old parkin ko mice (Goldberg et al., 2003; Itier et al., 2003) (Figures 4.3 A, C and D). Whereas, I observed a progressive SNpc DA neuron loss in the new DCB-Ret ko mouse model from 15% in 12 months old mice (Figure 4.3 C) to 21% in 24 month old animals compared to the DCB control mice (Figures 4.3 A and D) similar to what has been reported for the original DAT-Cre/$Ret^{lx/lx}$ mice (Kramer et al., 2007). However, the DCB-Ret/parkin ko mice show an enhanced degeneration of the SNpc DA neurons losing 19% at one year to 28% at two years (Figures 4.3 A, C and D). As reported for the single Ret ko mice (Kramer et al., 2007) the cell survival function of Ret and parkin together seems also to be specific for the SNpc DA neurons; whereas, DA neurons in the adjacent ventral tegmental area (VTA) region remains unaltered in aged mice (Figure 4.3 E). These findings for the first time show an essential cell survival function of parkin in trophically impaired DA neurons of the SNpc.

To address the survival function of Ret and parkin specifically in the subgroup of SNpc DA neurons that were reported to preferentially die in PD patients; I stereologically quantified the amount of high G-protein-activated inward rectifier potassium channel (GIRK2) expressing DA neurons. I observed significant cell body loss in the 24 month old mice specifically in the SNpc region but not in the VTA, this loss was up to 20% in DCB-Ret ko and 27% in DCB-Ret/parkin ko mice (Figures 4.3 F, G and H), which is very much similar to that of my TH neuron quantifications of the 24 month old mice (Figure 4.3 D). The DA neurons loss in the Ret/parkin double-deficient mice and single Ret was not specific for the GIRK2 subgroup.

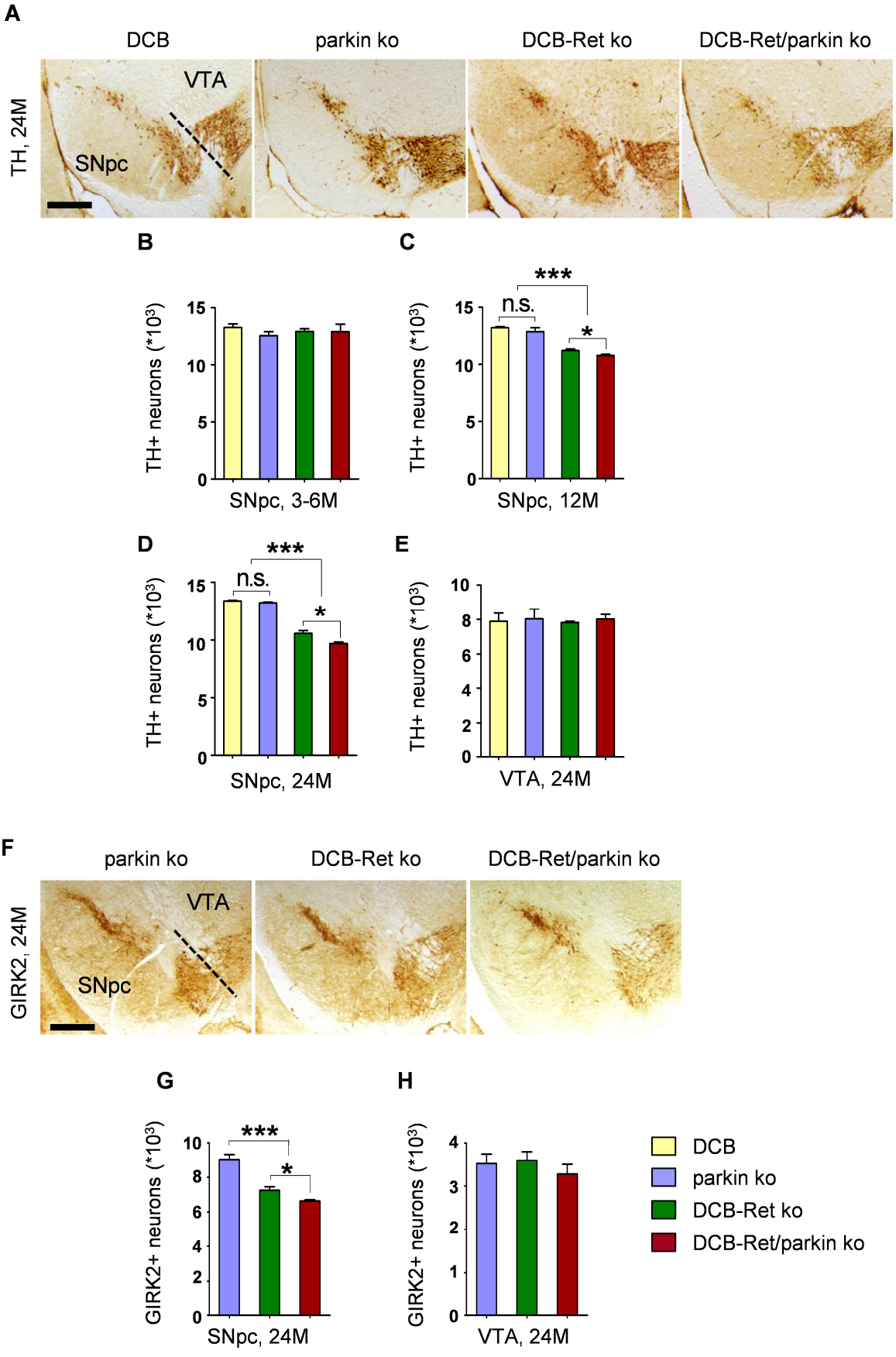
A
DCB
parkin ko
DCB-Ret ko
DCB-Ret/parkin ko
TH, 24M
VTA
SNpc
B
C
TH+ neurons (*10³)
SNpc, 3-6M
SNpc, 12M
n.s.
***
*
D
E
SNpc, 24M
VTA, 24M
F
GIRK2, 24M
G
H
GIRK2+ neurons (*10³)
DCB
parkin ko
DCB-Ret ko
DCB-Ret/parkin ko

Figure 4.3 Parkin deletion enhances the age dependent SNpc specific progressive DA neuronal loss in the conditional Ret-deficient mice

(A) Representative images of coronal sections from 24 months old DCB, parkin ko, DCB-Ret ko and DCB-Ret/parkin ko mice showing dopaminergic neurons in the SNpc and VTA stained with TH antibody. Scale bar = 250 µm.
(B-E) Stereological quantifications of the TH positive neurons of the indicated genotypes in the (B) SNpc of 3-6 months old mice, n = 3 mice per genotype; (C) SNpc of 12 months, n = 3-4 mice per genotype; (D) SNpc and (E) VTA of 24 months old mice, n = 3-4 mice per genotype.
(F) Representative images of coronal sections from 24 months old parkin ko, DCB-Ret ko and DCB-Ret/parkin ko mice showing GIRK2 positive neurons in the SNpc and VTA. Scale bar = 250 µm.
(G-H) Stereological quantifications of the GIRK2 positive neurons in the (G) SNpc and (H) VTA of 24 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, *** $p \leq 0.001$, Student's t-test.

4.3 Parkin together with Ret is important for the maintenance of DA innervation in aged mice

To understand the possible maintenance function of Ret together with parkin in the nigrostriatal DA system innervation; I labeled DA fibers in the striatum using TH antibody and compared the fiber density in adult (3-6 months) and aged (12 months and 24 months) mutants with their respective controls (Figures 4.4 A-F). Quantifications in the dorsal and ventral striatum of adult mice performed together with Kumar Ponna, did not reveal any significant alterations in the single parkin or Ret ko mice as reported previously (Itier et al., 2003; Kramer et al., 2007) or in the newly generated Ret/parkin double ko mice (Figures 4.4 B and E). I then quantified DA innervation in 12 and 24 months old mice. Parkin ko mice did not exhibit any target innervation phenotype as reported previously (Itier et al., 2003); whereas in the DAT-Ret ko mice, progressive DA fiber loss was observed in the dorsal striatum, which is 33% at 12 months and 48% at 24 months when compared to the DCB control mice similar to what has been reported previously for the DAT-Ret ko mice (Kramer et al., 2007) (Figures 4.4 A, C and D). In the Ret/parkin double-deficient mice this innervation loss was even stronger and reached 51% in 12 months and 56% in 24 month old mice compared to the DCB control mice (Figures 4.4 A, C and D). The reduction of TH innervations in the ventral striatum corresponded to the changes observed in the dorsal striatum at 12 and 24 months (Figures 4.4 F and G). The enhanced innervation loss observed in the 12 and 24 month old Ret/parkin double ko mice was statistically significant when compared to the single Ret ko mice. I then used dopamine transporter (DAT) as a second, independent DA fiber marker to make sure that the previously observed effects were not due to downregulation of TH expression but due to the loss of DA fibers itself (Figures 4.4 H and I). Parkin ko mice were used as controls, since they did not show any DA fiber loss phenotype (Figures 4.4 A-G), (Itier et al., 2003). Both DCB-Ret ko and DCB-Ret/parkin ko 24-month-old mutants displayed a 35% and 49% reduction in DAT-immunoreactive fiber density relative to age-matched parkin ko control mice respectively. Taken together data from the TH and DAT fiber immunostaining demonstrate the importance of parkin activity for the long-term maintenance of DA axons deprived of Ret mediated trophic support.

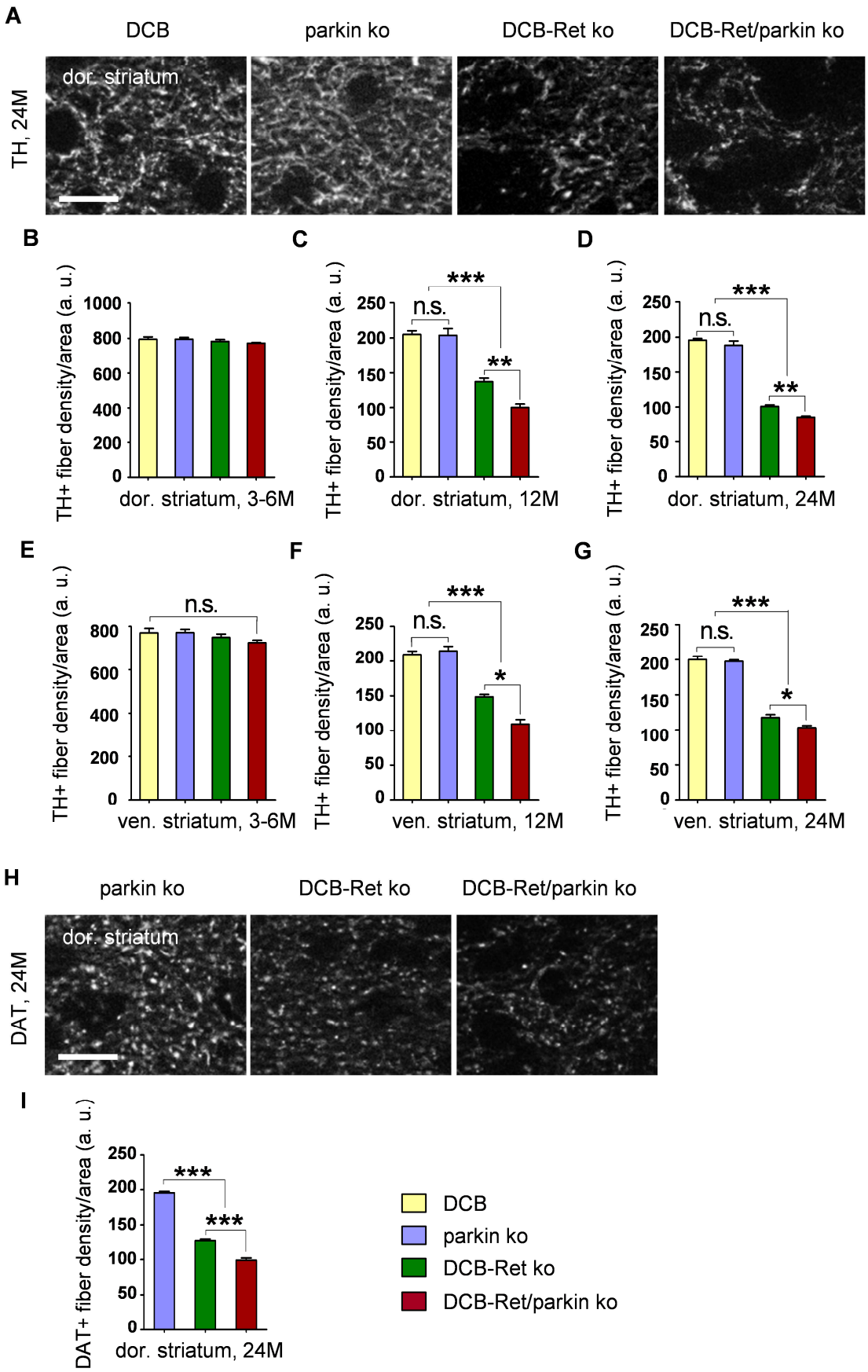

Figure 4.4 Parkin along with Ret is required for the maintenance of striatal dopaminergic innervation in ageing mice.

(A) Representative images of coronal sections from 24 months old DCB, parkin ko, DCB-Ret ko and DCB-Ret/parkin ko mice showing dopaminergic fiber innervation in the dorsal striatum immunofluorescently labeled with TH antibody. Scale bar = 10 µm.
(B-D) Quantifications of the TH fiber density in dorsal striatum of the indicated genotypes in (B) 3-6 months old mice, n = 3 mice per genotype (C) 12 months old mice, n = 4 mice per genotype and (D) 24 months old mice, n = 4 mice per genotype.
(E-G) Quantifications of the TH fiber density in ventral striatum of the indicated genotypes (E) 3-6 months old mice, n = 3 mice per genotype (F) 12 months old mice, n = 4 mice per genotype and (G) 24 months old mice, n = 4 mice per genotype.
(H) Representative images of coronal sections from 24 months old parkin ko, DCB-Ret ko and DCB-Ret/parkin ko mice showing DA fiber innervation in the dorsal striatum immunofluorescently labeled with DAT antibody. Scale bar, 10 µm.
(E) Quantifications of the DAT fiber density in dorsal striatum of 24 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test.

4.4 Ret and parkin double deletion leads to decrease in the levels of DA and its metabolite DOPAC in the striatum of aged mice

In order to investigate the effect of nigrostriatal DA system degeneration on striatal total DA amounts, with the help of Barbara Finckh, I measured the levels of total DA and its metabolites (DOPAC and HVA) in the striatal lysates by using HPLC-ECD system. As reported already (Goldberg et al., 2003; Itier et al., 2003), aged parkin ko mice did not show any changes in the levels of DA and its metabolites in the striatum (Figures 4.5 A-F). Consistent with the strong DA innervation loss in the striatum, I also detected around 19% less total dopamine in 12 months and 30% less in 24 month old Ret single and Ret/parkin knockout mice compared to control mice ($Ret^{lx/lx}$ or DCB) (Figures 4.5 A and B). Also the dopamine degradation product 3,4-Dihydroxyphenylacetic acid (DOPAC) was significantly reduced in the 12 month and 24 month old Ret/parkin double-deficient mice compared to their age matched control mice (Figures 4.5 C and D). However, no differences were observed in the levels of the final end product of DA, homovanillic acid (HVA) at 12 or 24 months across the genotypes (Figures 4.5 E and F). Importantly, I did not observe any significant differences in the total levels of DA, and its metabolites DOPAC and HVA of DCB when compared to $Ret^{lx/lx}$ mice at 24 months (Figures 4.5 B, D and F). Moreover, the Cre expressing DCB control mice (when compared to $Ret^{lx/lx}$ mice at 24 months) showed unaltered DA metabolism, unlike the DAT-Cre mice (Aron et al., 2010; Kramer et al., 2007); which gave us the possibility to visualize changes in total DA levels in the striatum of Ret-deficient mice for the first time, very well corresponding with the DA cell and fiber loss phenotypes (Figures 4.3 and 4.4). These observations suggest an important role for Ret in maintaining the total levels of striatal DA, whereas parkin and Ret together are playing a role in the metabolism of striatal DA in aged mice.

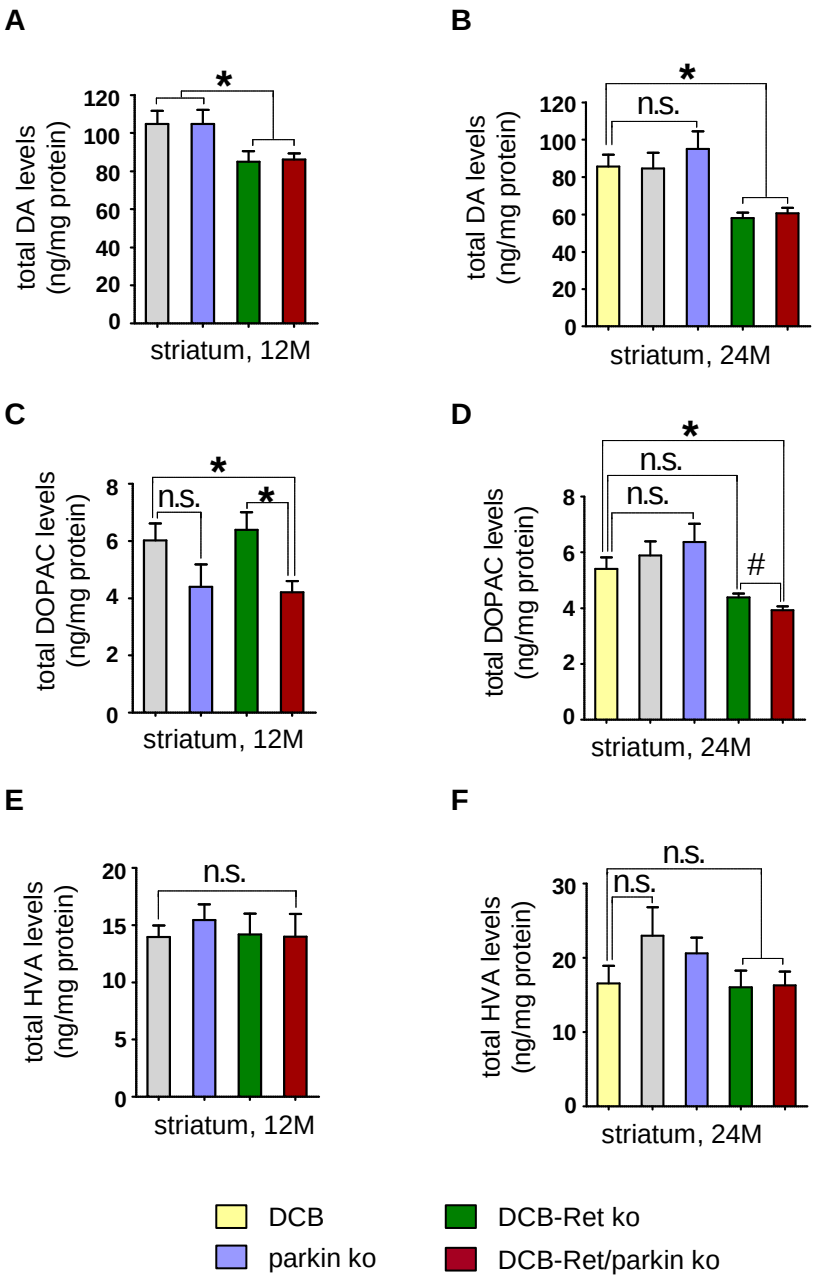

Figure 4.5 Ret and parkin double deletion leads to decrease in the levels of DA and its metabolite DOPAC in the striatum of aged mice

(A-B) HPLC measurements of total DA levels of the indicated genotypes in (A) 12 months old mice, n = 6-7 mice per genotype and (B) 24 months old mice, n = 4-7 mice per genotype
(C-D) HPLC measurements of total DOPAC levels of the indicated genotypes in (C) 12 months old mice, n = 6-7 mice per genotype and (D) 24 months old mice, n = 4-7 mice per genotype
(E-F) HPLC measurements of total HVA levels of the indicated genotypes in (E) 12 months old mice, n = 6-7 mice per genotype and (F) 24 months old mice, n = 4-7 mice per genotype
Data are represented as mean +/- SEM; n.s., not significant, # $p = 0.057$, * $p \leq 0.05$, Student's t-test.

4.5 Parkin deletion in Ret-deficient mice did not enhance gliosis and inflammation in the single Ret-deficient mice

To support the idea that this is a progressive neurodegenerative process which is just too slow to detect dying neurons, I went ahead (with help of Anil Annamneedi) to visualize gliosis and inflammation in the nigrostriatal system of aged mice which is thought to be an intrinsic part of the degeneration process. I stained striatal and SN sections for glial fibrillary acidic protein (GFAP) positive astrocytes (Figures 4.6 A and B) and ionized binding calcium adaptor molecule (Iba1) positive microglia (Figures 4.7 A and B).

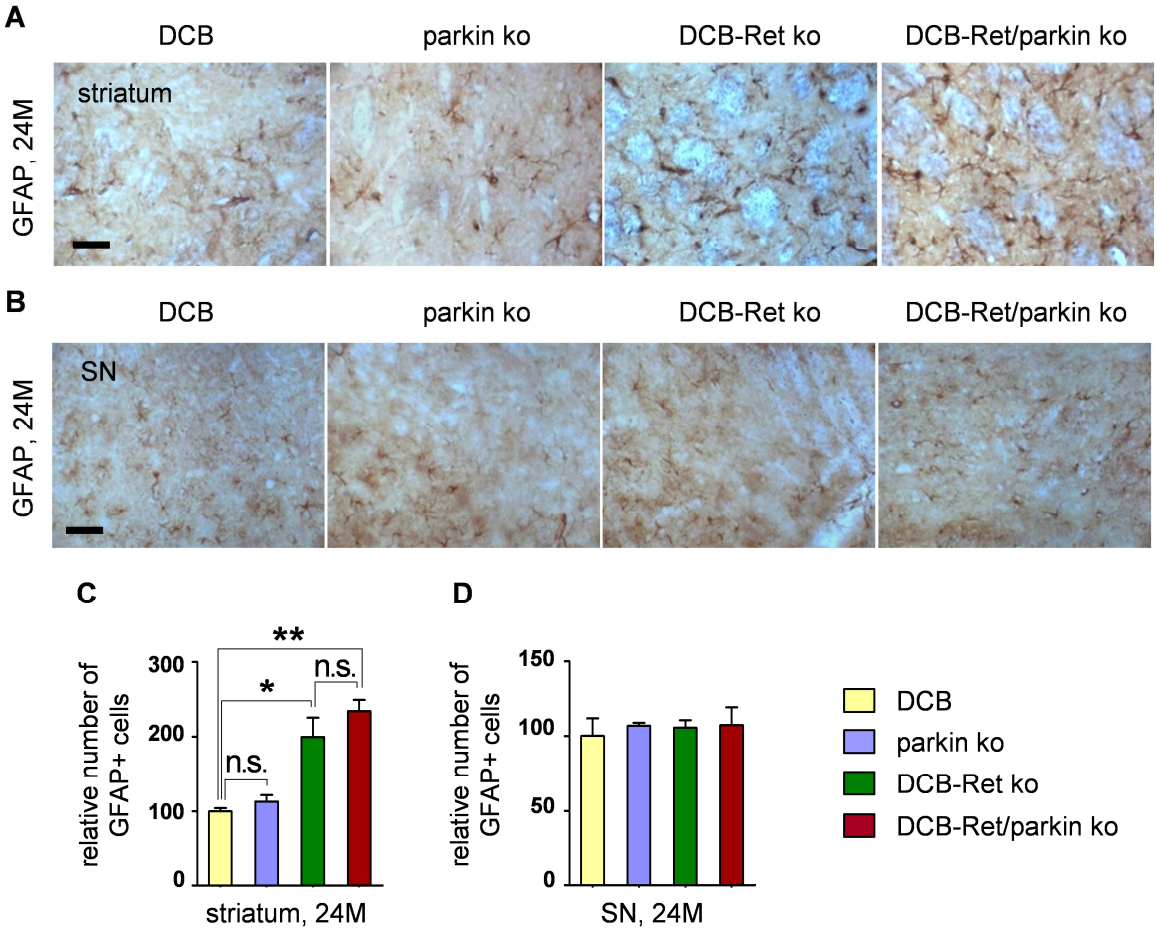

Figure 4.6 Enhanced gliosis in the striatum of aged Ret KO and Ret/parkin double ko mice

(A-B) Representative images of coronal sections from 24 months old DCB, parkin ko, DCB-Ret ko and DCB-Ret/parkin ko mice stained with GFAP antibody showing astrocytes in the (A) striatum and (B) SN. Scale bar = 250 µm.
(C-D) Quantifications of GFAP positive cells in the (C) striatum and (D) SN of 24 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, Student's t-test.

As reported previously for the single Ret ko mice (Aron et al., 2010; Kramer et al., 2007), I observed enhanced gliosis in the striatum (Figure 4.6 C) and inflammation in the SN (Figure 4.7 D) of Ret single and Ret/parkin double-deficient mice compared to DCB controls and

parkin ko mice. However, these changes in the Ret/parkin double ko mice are further not enhanced when compared with Ret single ko mice. The increased number of astrocytes and microglia observed in the striatum and SN respectively (Figures 4.6 and 4.7) of both the Ret/parkin double ko mice and single Ret ko mice directly corresponded to the nigrostriatal degenerative process as these changes were not observed in the parkin ko and DCB control mice which showed no nigrostriatal degeneration (Figures 4.3-4.5).

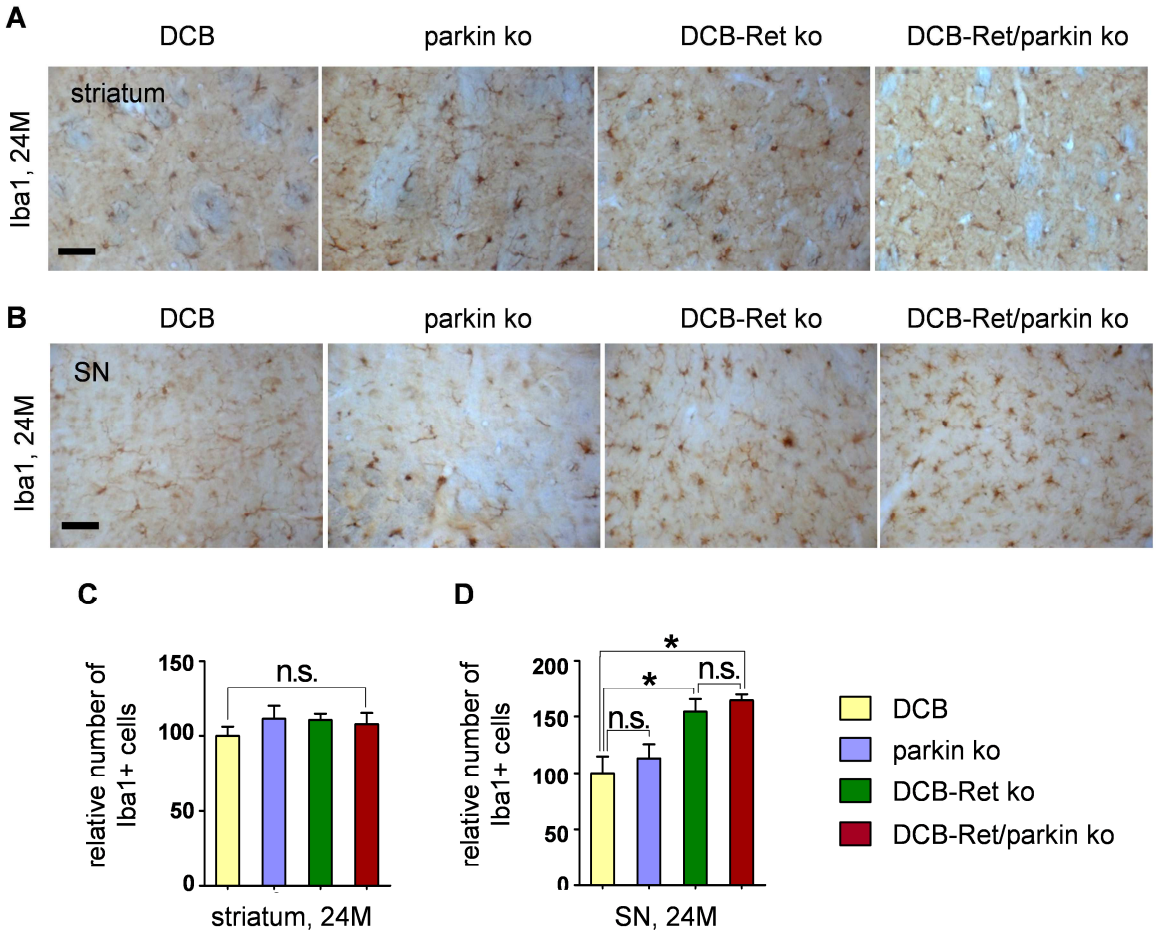

Figure 4.7 Enhanced inflammation in the SN of aged Ret KO and Ret/parkin double ko mice

(A-B) Representative images of coronal sections from 24 months old DCB, parkin ko, DCB-Ret ko and DCB-Ret/parkin ko mice stained with Iba1 antibody showing microglia in the (A) striatum and (B) SN. Scale bar = 250 µm.
(C-D) Quantifications of Iba1 positive cells in the (C) striatum and (D) SN of 24 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, Student's t-test.

4.6 Parkin and Ret loss leads to behavioral abnormalities in aged mice

Having observed cellular and physiological changes in the nigrostriatal system of the aged Ret single ko mice and Ret/parkin double mutant mice, next I wanted to investigate behavioral alterations in Ret ko and Ret/parkin double ko mice relative to controls and parkin ko mice at

12 and 24 months. To measure alterations in general activity and anxiety related behavioral changes, I performed open-field test (in 12 and 24 months old mice) and elevated plus maze (EPM) test (in 24 months old mice) respectively. In the open-field test, I recorded the horizontal activity of each mouse (from control and mutant groups) in an open-field arena (50x50 cm arena with 40cm high walls on all the sides) for 10 minutes. I then used the Ethovision (Noldus) software to analyze movement and thigmotactic behavior (the tendency to remain close to vertical surface, in this case in the border zone of the arena) of each mouse from the 10 minutes video recording. At both 12 and 24 months of age, parkin ko mice has significantly moved less distance compared to DCB controls (Figures 4.8 A and C), as previously reported by others (Zhu et al., 2007). I have also observed similar alterations in DCB-Ret ko and Ret/parkin double ko mice at 12 and 24 months. Both the single Ret ko and Ret/parkin double ko mice also moved less distance compared to DCB controls (Figures 4.8 A and C). The discrepancy in the behavior of DCB-Ret ko and DAT-Cre-Ret ko mice which was published earlier (Aron et al., 2010; Kramer et al., 2007) could be due to the use of different Cre lines. In the current study we used an improved Cre driver line to generate conditional Ret ko mice without disturbing the endogenous DAT locus, resulting in unaltered DAT protein levels, striatal total DA levels and no hyperactivity in our DCB control mice. Interestingly, the Ret/parkin double ko mice showed increased thigmotactic behavior, these mice spent more time in the border zone during the trial when compared to single parkin ko, Ret ko mice and DCB controls. However, this thigmotactic behavior was only significantly changed in 12 months Ret/parkin double ko mice but showed only a tendency in 24 months old double ko mice (Figures 4.8 B and D), which could be due to less number of animals used to perform the test at 24 months (n = 6-17 mice per genotype).

In the EPM test, I recorded the behavior of each mouse in an elevated plus maze with four 30 cm long and 5 cm wide arms, connected by a 5 x 5 cm center. Two opposing arms were bordered by 15 cm high walls (closed arms), whereas the other two arms (open arms) were bordered by a 2 mm rim and the whole maze was elevated 75 cm from the floor. The mouse was placed into the center facing one open arm and 5 min observation was recorded. Different parameters were analyzed from the recorded video with the software The Observer (Noldus); entries into the open arms (calculated when all four paws were on an open arm), entries into the end of the open arms (calculated when the mouse reaches with its snout the end of an open arm) and head dipping events (calculated when the mouse dips its head in the open arm) as previously described (Trullas and Skolnick, 1993). Less open arm entries and decreased exploration in the open arm indicates anxiety. The 24 month old parkin ko and Ret/parkin double ko mice show less open arm entries (Figure 4.8 E) and these mice rarely reached the end of the open arms (Figure 4.8 F) compared to the DCB controls; whereas, these differences were not seen in the DCB-Ret ko mice (Figures 4.8 E and F). In addition, parkin ko, DCB-Ret ko and Ret/parkin double ko mice show less open arm head dipping events compared to DCB controls (Figure 4.8 G). An abnormal anxiety-related behavior was already observed in a previous study in parkin ko mice (Zhu et al., 2007). However, in all the different parameters analyzed, Ret/parkin double ko mice seems to show stronger behavior alterations when compared to the Ret ko mice (which is statistically significant or close to significance with a p-value < 0.05) and a trend in enhancement of behavior alterations (with no statistical significance) when compared to the parkin ko mice (Figures 4.8 E, F and G).

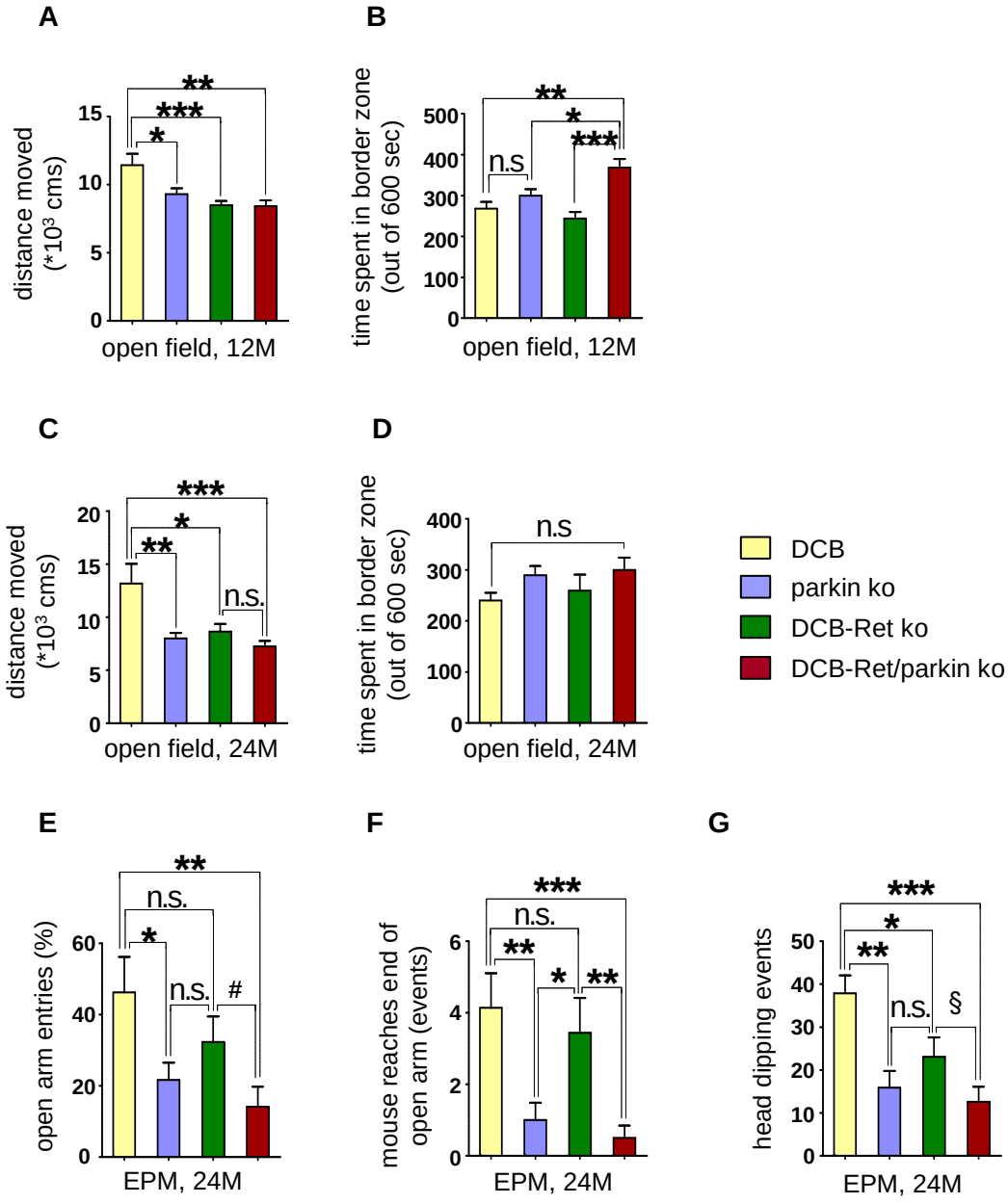

Figure 4.8 Parkin and Ret deletion leads to behavior abnormalities in aged mice

(A-D) Quantifications showing the horizontal activity of aged mice of the indicated genotypes in the open field test. Distance moved (in 10 min) by (A) 12 months and (C) 24 months mice in 50*50 cm arena is shown. Time spent in the border zone of the arena (out of 600 sec) by (B) 12 months and (D) 24 months mice are shown; n =11-24 per genotype for 12months, n = 6-17 per genotype for 24 months

(E-F) Quantifications showing the behavior during 5min on an elevated plus maze of 24 months old mice of the indicated genotypes. (E) open arm entries (F) number of times mouse reached the end of an open arm, (G) head dipping events, is shown. n = 7-11 mice per genotype.

Data are represented as mean +/- SEM; n.s., not significant, § p = 0.07, # p = 0.057, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, test

Taken together, the parkin, Ret single and Ret/parkin double ko mice showed locomotory and anxiety related behavior alterations. However, the anxiety related phenotype observed on the EPM seems to be more parkin-dependent which is slightly (and insignificantly) enhanced due to Ret ablation in the Ret/parkin double ko mice.

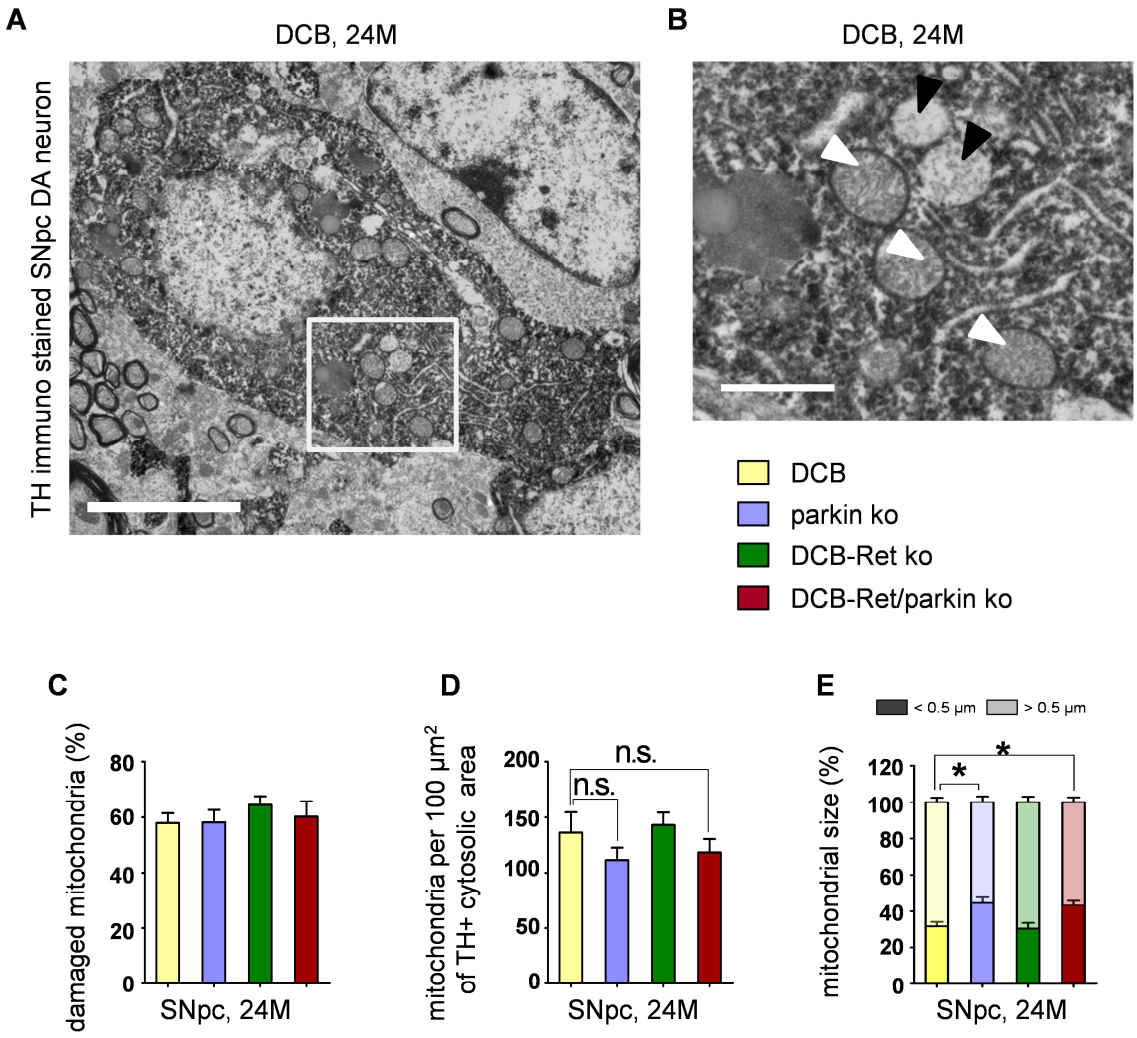

Figure 4.9 Parkin deletion results in smaller mitochondria in the SNpc DA neurons of aged mice

(A) Representative TEM photograph of TH immuno-labelled DA neuron (darkly stained due to DAB reaction) from the SNpc of 24 month old DAT-Cre BAC control mouse. Scale bar = 5 µm.

(B) Higher magnification of the boxed region of (A) depicting normal (marked with white triangles) and damaged (marked with black triangles) mitochondria. Scale bar = 1 µm.

(C-E) Quantifications of (C) total number of mitochondria per 100 μm^2 TH+ cytosolic area, (D) percentage of damaged mitochondria and (E) percentage of larger mitochondria (> 0.5 µm, shown as light shaded bars) and smaller mitochondria (< 0.5 µm, shown as dark shaded bars) of 24 months old mice of the indicated genotypes, n = 3-4 mice per genotype.

Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, Student's t-test.

4.7 Parkin deletion leads to increase in smaller mitochondria in mouse SNpc DA neurons

To understand the underlying causes of nigrostriatal degeneration in Ret single and Ret/parkin double ko mice, I wanted to investigate the ultrastructural changes of SNpc DA neuronal mitochondria in the 24 month old mice.

To achieve that, I immunolabeled midbrain coronal sections from 24 month old DCB, parkin ko, DCB-Ret ko and DCB-Ret/parkin ko mice with TH antibody for an electron microscopic study performed with help of the ZMNH imaging facility (headed by Michaela Schweizer). I used the high-magnification EM pictures (Figures 4.9 A and B) to examine morphological changes in mitochondria between the different groups of mice. I did not observe any alterations in the percentage of damaged mitochondria per 100 μm^2 area cytoplasm (Figure 4.9 C) or total number of mitochondria per 100 μm^2 area cytoplasm (Figure 4.9 D) in parkin ko (Stichel et al., 2007), Ret ko and Ret/parkin double ko mice compared to DCB controls. However, the percentage of smaller mitochondria (size smaller than 0.5µm) is increased in groups which lack parkin, the parkin ko and the Ret/parkin double ko mice but not in the Ret single ko mice when compared to DCB control mice (Figure 4.9 E).

4.8 No signs of oxidative stress or signaling abnormalities in the Ret/parkin double ko mice

To investigate the role of oxidative stress in the nigrostriatal degeneration in Ret single and Ret/parkin double ko mice, I further detected the amount of protein oxidation in the SN (Figure 4.10 A) and striatum (Figure 4.10 B) lysates of 24 months old single and double mutants using OxyBlot Protein Oxidation Detection Kit (from Millipore).

To detect carbonylated proteins, I incubated the protein lysates with DNPH (2,4-dinitrophenylhydrazine), which then is converted to DNP (2,4-dinitrophenylhydrazone) upon binding to carbonylated proteins. The derivatized lysates were subjected to western blot and probed with anti-DNP antibody to detect protein carbonylation (Figures 4.10 A and B). Contradicting the previous finding from Palacino et al. (Palacino et al., 2004), I did not observe any increase in protein carbonylation in the SN and striatum lysates obtained from parkin ko mice. I also did not observe any increase in the protein carbonylation in the DCB-Ret ko and Ret/parkin double ko tissue lysates compared to DCB controls (Figures 4.10 A and B).

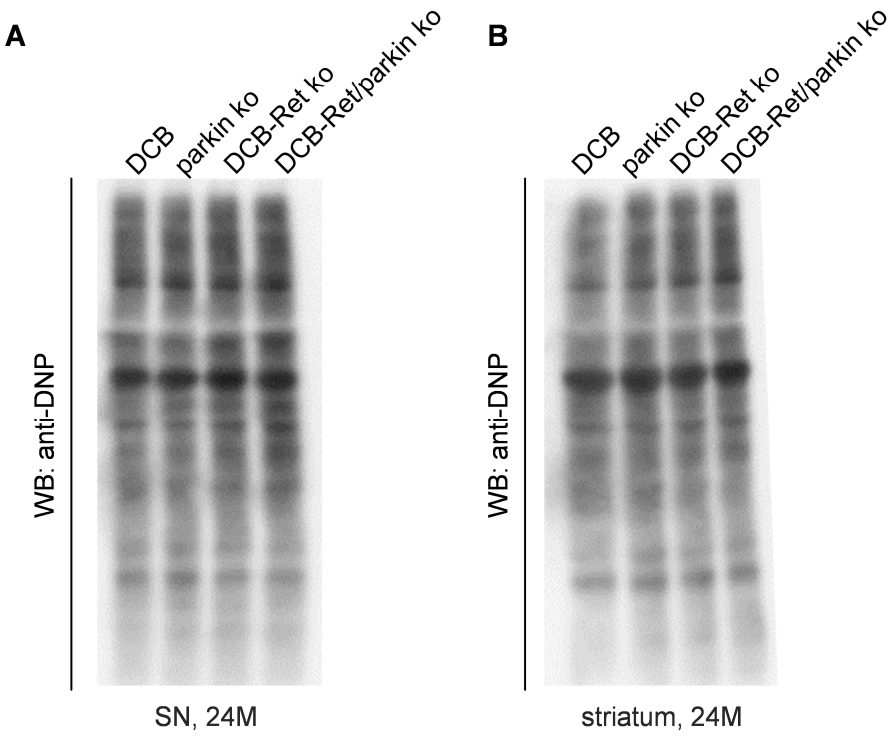

Figure 4.10 No indications of oxidative stress in the SN and striatum of aged Ret/Parkin double ko mice

(A-B) Western blot images depicting carbonylated proteins detected by anti-DNP (2,4-dinitrophenylhydrazone) antibody following derivatization of the (A) SN and (B) striatum protein lysates with DNPH (2,4-dinitrophenylhydrazine) provided in the OxyBlot Protein Oxidation Detection Kit (from Millipore); 24 months old mice of indicated genotypes were used for the assay (n = 1 per genotype is shown)

To look for signaling changes due to Ret and parkin single and double deletion, I performed western blot analysis with the SN (Figure 4.11 A) and striatum (Figure 4.11 B) protein lysates of 24 month old mice. I observed no alterations in the activation and amount of signaling molecules namely AKT (protein kinase B), the ribosomal protein S6 (a component of the 40S ribosomal subunit that might regulate translation) and mitogen-activated protein kinase (ERK kinase 1/2) during the slow progressive *in vivo* nigrostriatal degeneration process in the single Ret and Ret/parkin double ko mice (Figures 4.11 A and B). I also did not observe any changes in the levels of DA neuronal marker proteins, TH (in both SN and striatum) or DAT (in the striatum) in these 24 month old mice (Figures 4.11 A and B). The protein levels of mitochondrial NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10 (NDUFA10), a component of the complex I of the electron transport chain of mitochondria did not change in the SN region (Figure 4.11 A); β-actin protein levels served as loading control for all the proteins mentioned above. It is possible that in my Western blot probes, for which I carefully punched out the SN/VTA region, other neuronal and glia cells in the SN and striatum regions masked the mild signaling changes, which otherwise could have been detected.

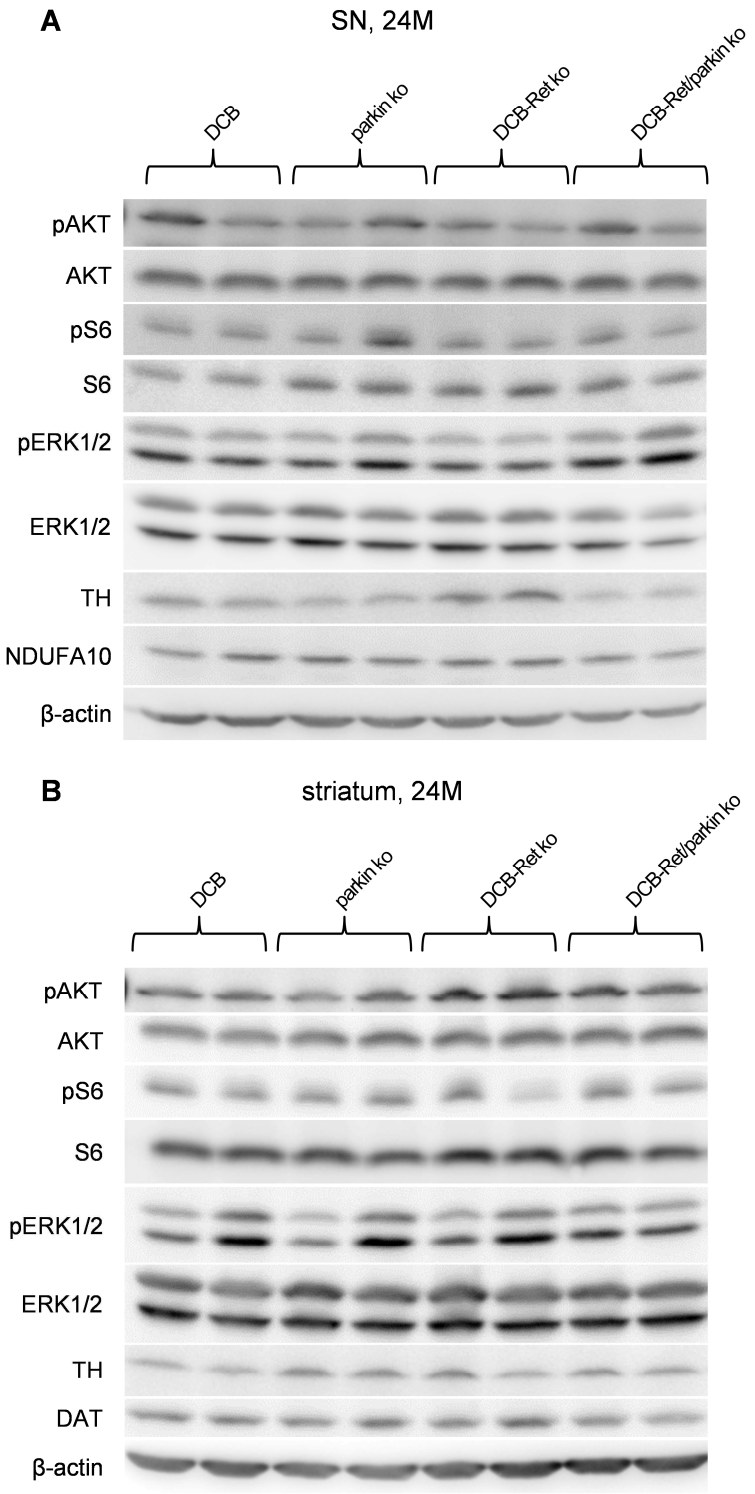

Figure 4.11 Ret and Parkin deletion does not lead to any detectable changes in the activation or amount of key signaling proteins in the SN and striatum of aged mice

(A-B) Western blot images depicting the expression of different signaling proteins (as indicated always on the left) in the (A) SN and (B) striatum protein lysates of 24 months old mice of indicated genotypes (n = 2 per genotype are shown)

4.9 Morphological and physiological changes in the nigrostriatal system of adult Ret/parkin double-deficient mice

To investigate the role of parkin in maintaining the size of DA neuronal soma, together with Kumar Ponna, I measured the TH immunostained DA neuronal soma size the SNpc of 24 months old mice using the nucleator probe of StereoInvestigator software (from MBF bioscience). As reported earlier (Goldberg et al., 2003), we did not find any decrease in the TH soma size for parkin ko mice compared to DCB controls (Figure 4.12 A). We could also reproduce the previous findings of reduced soma size in single Ret ko mice which was originally reported for GIRK2 positive neurons in the SNpc (Aron et al., 2010), and in this study we extend their findings to TH positive neuronal soma. Since the GIRK2 positive neurons in the SNpc are a subgroup of TH positive cells, it could be that the cell size phenotype is more general but not GIRK2 specific which was overlooked in the previous study (Aron et al., 2010). This reduced TH soma size phenotype observed in the Ret single ko mice did not enhance in the Ret/parkin double ko mice (Figure 4.12 A).

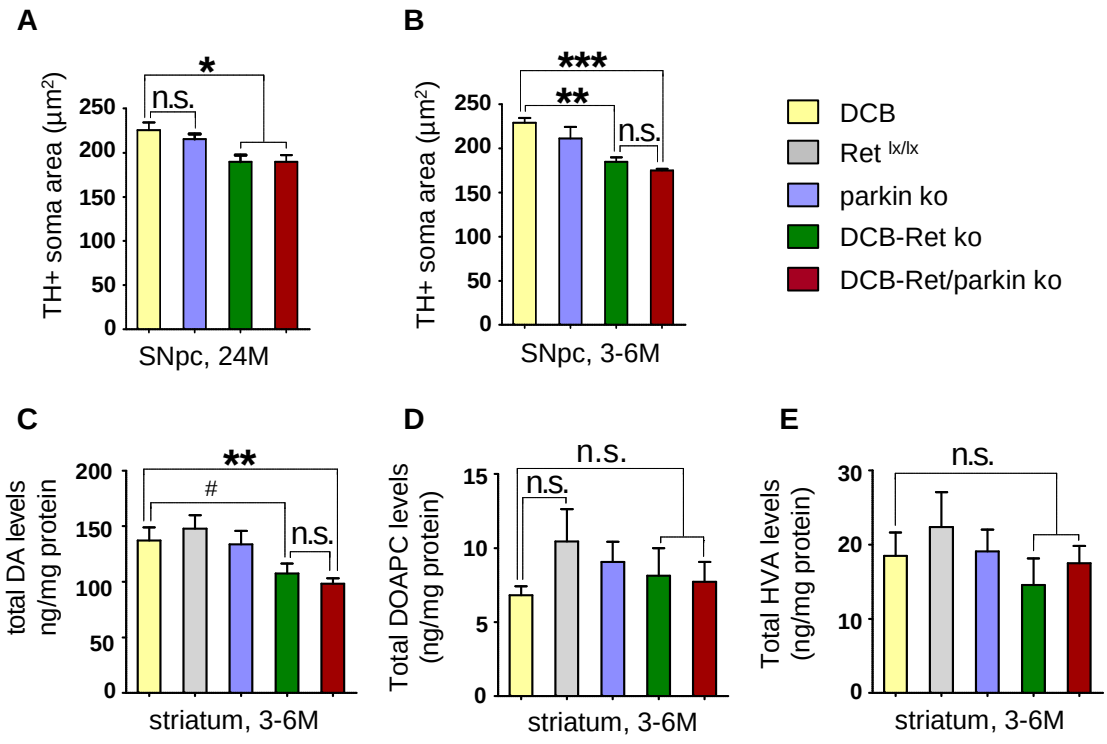

Figure 4.12 Parkin and Ret double deficiency causes early morphological and physiological changes in the nigrostriatal DA system of adult mice, before any indications of neurodegeneration

(A-B) Quantifications of soma area measurements of TH positive neurons in the SNpc of (A) 24 months old mice and (B) 3-6 months old mice of the indicated genotypes, n = 3 mice per genotype.
(C-E) HPLC measurements of (C) total DA levels (D) total DOPAC levels and (E) total HVA levels in the striatum of 3-6 months old mice of the indicated genotypes, n = 5-7 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, # $p = 0.09$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test.

In order to investigate the correlation between ageing and soma size reduction, I investigated with the help of Kumar Ponna the TH stained DA neuron soma size in the SNpc of adult (3-6 months) mice as mentioned above for the 24 months. As expected (and reported for the 24 months mice), parkin ko did not show any alterations in TH positive soma size, compared to DCB controls (Figure 4.12 B). However, the single Ret ko and Ret/parkin double ko mice when compared to DCB controls, already show a decrease in SNpc TH positive soma size in their adulthood (Figure 4.12 B) before any signs of axonal or neuronal degeneration (Figures 4.3 and 4.4). As observed for the 24 months, the adult Ret/parkin double ko mice did not show any additional soma size reduction phenotype compared to that of the age matched Ret single ko mice. The reduced cell soma size is so far the earliest significant alteration found in SNpc DA neurons of Ret-deficient mice.

These findings prompted me to look for further changes in the nigrostriatal DA system of the adult single and double ko mice. By using HPLC-ECD system, I measured the total levels of DA and its metabolites in the striatal lysates of adult (3-6 months) single parkin ko, Ret ko mice and double Ret/parkin ko mice along with controls ($Ret^{lx/lx}$ and DCB mice). In the parkin ko mice (as reported by Goldberg et al., 2003; Itier et al., 2003) and DCB-Ret ko mice, I did not observe any significant differences in the total levels of DA and its metabolites (DOPAC and HVA). However, there is a trend of less total DA levels in the DCB-Ret ko mice (p value = 0.09) when compared to DCB controls (Figure 4.12 C). The reduction of total DA levels is statistically significant in the Ret/parkin double ko mice compared to DCB controls (Figure 4.12 C). The adult Ret/parkin double ko mice did not show any alterations in the total levels of DOPAC or HVA (Figures 4.12 D and E). These early detectable changes in the striatal DA levels of Ret/parkin double ko mice argues for a physiological role of Ret and parkin in regulating dopamine synthesis or turn over.

4.10 Reduced total cellular ATP levels and decreased mitochondrial complex I activity is in the SN of adult Ret/parkin double ko mice

The reduced cell size and DA levels observed in Ret/parkin double-deficient mice could be due to decreased energy supply. Since parkin has been shown to be a master regulator of mitochondrial integrity (Corti and Brice, 2013; Pilsl and Winklhofer, 2012), I had a closer look at the mitochondria function in all the mouse mutants at adulthood. Indeed, when I measured the total ATP levels in SN tissue lysates, I observed a 20% reduction in the Ret/parkin double but not in single parkin or Ret ko mice compared to DCB control mice (Figure 4.13 A). Next, I wanted to know if the reduced ATP levels in the double-deficient mice and cells was due to a reduced respiratory chain activity as suggested for parkin-deficient mice (Palacino et al., 2004; Stichel et al., 2007). To investigate this, I performed mitochondrial complex I activity assay in the enriched mitochondrial fractions from the SN of the single and double mouse mutants. Only the mitochondrial enriched fraction obtained from the SN of Ret/parkin double-deficient mice showed a highly significant 20% reduction of complex I activity but not the SN mitochondrial preparations of the single parkin or Ret-deficient mice when compared to DCB control mice (Figure 4.13 B).

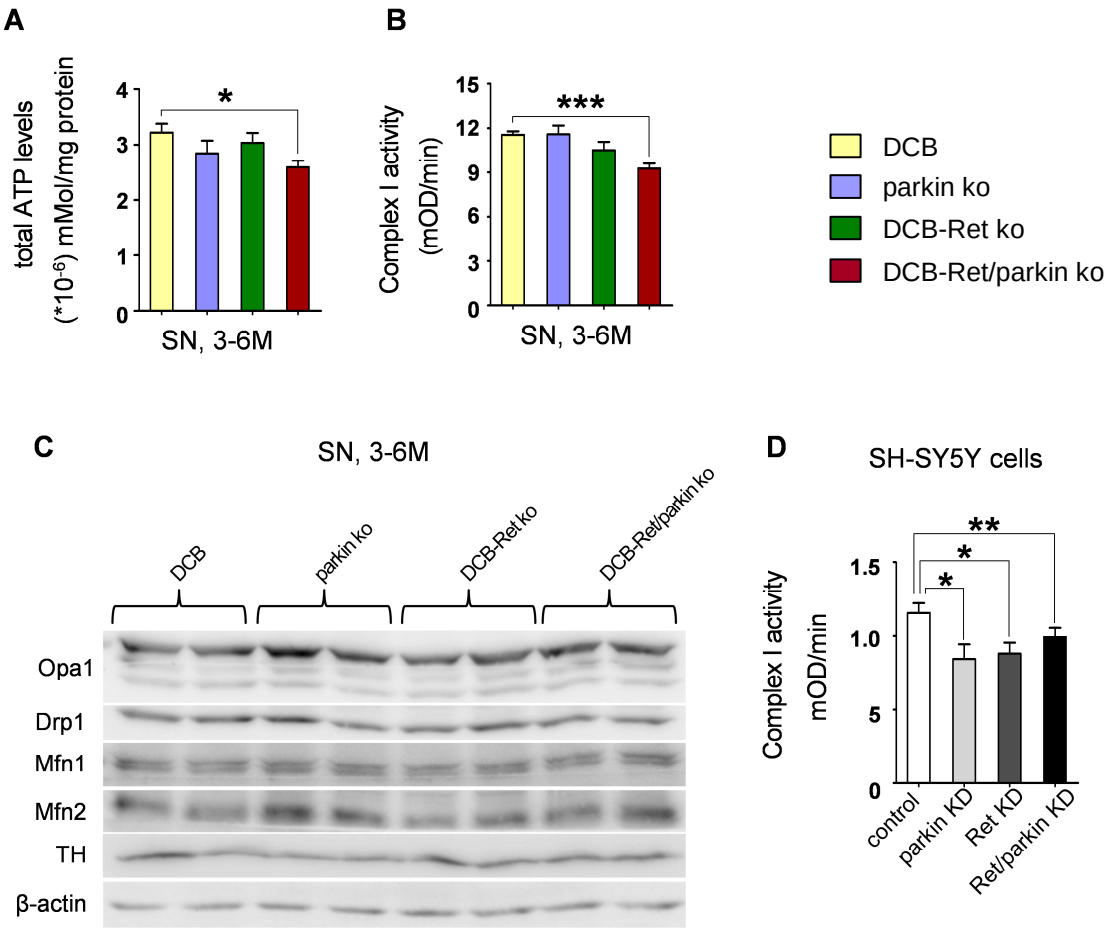

Figure 4.13 Parkin together with Ret deficiency leads to decrease in mitochondrial complex I activity and total ATP levels in the SN of adult mice with no detectable changes in mitochondrial fission and fusion protein levels

(A) Quantifications of mitochondrial complex I enzyme activity measurements in the enriched mitochondrial preparations obtained from the SN of 3-6 months old mice of the indicated genotypes, n = 6-9 mice per genotype.
(B) Quantifications of total cellular ATP levels in the SN of 3-6 months old mice of the indicated genotypes, n = 5 mice per genotype.
(C) Western blot images depicting the expression of different mitochondrial proteins (as indicated always on the left) in the SN protein lysates of 3-6 months old mice of indicated genotypes; TH and β-actin were used as loading controls (n = 2 per genotype are shown).
(D) Quantifications of mitochondrial complex I enzyme activity measurements in the enriched mitochondrial preparations obtained from the SH-SY5Y cell lysates treated with control siRNA, parkin siRNA, Ret siRNA and Ret/parkin siRNA as indicated, lysates from 5 independent experiments were used for the assay.
Data are represented as mean +/- SEM; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test

However, the protein levels of key molecules involved in mitochondrial fission (Drp1) /fusion mechanisms (Mfn1, Mfn2 and Opa1) did not change in the single or double mutant mice; TH and β-actin were used as loading controls (Figure 4.13 C). To confirm these findings in a well-controlled cellular system with the help of Merit Wildung, I performed mitochondrial complex I activity assay in the enriched mitochondrial preparations obtained from SH-SY5Y

cells treated with siRNAs to knockdown the expression of parkin, Ret or both. I also observed around 20% reduction of complex I activity in the mitochondrial preparations of parkin, Ret single KD cells and Ret/parkin double KD cells compared to the preparations from the cells treated with control siRNA (Figure 4.14 D).

These observations all together suggests for the first time - as previously reported for PINK1 and parkin (Gautier et al., 2008; Gispert et al., 2009; Palacino et al., 2004) - an important role of Ret alone and in cooperation with parkin in regulating mitochondrial complex I activity and ATP production in mice and SH-SY5Y cells without altering the levels of proteins involved in fission/fusion mechanisms or protein levels of NDUFA10, a critical subunit of complex I (Figure 4.11 A).

B. Parkin overexpression prevents nigrostriatal degeneration in the aged Ret-deficient mice

After revealing the cell survival function of parkin in the tropically impaired DA system of Ret-deficient mice by knocking out the parkin gene, I wanted to investigate the neuroprotective function of parkin in the degenerating nigrostriatal DA system of Ret-deficient mice by overexpressing parkin. To answer this question, our lab obtained two unpublished transgenic human parkin overexpressing mouse lines (from the lab of Konstanze Winklhofer, LMU, Münich). These transgenic mice express either full-length wildtype human parkin (wt h-parkin) or C-terminal truncated human parkin (Δ h-parkin) under the mouse prion promoter.

4.11 Characterization of transgenic human parkin overexpressing mice

First we had to answer the question, if the parkin transgenic mice express the human parkin transgene under the control of the mouse prion promoter in the DA neurons of these mice. Since our parkin antibodies which work in IHC detect human and mouse parkin protein and cannot distinguish between them. Thus, to allow unbiased determination of transgenic human parkin expression in DA neurons we had to cross the parkin transgenic mice (wt h-parkin and Δ h-parkin) into the parkin-deficient background. With the help of Kumar Ponna, I immunohistochemically co-stained the coronal midbrain sections from parkin ko/wt h-parkin and parkin ko/Δ h-parkin mice (with parkin ko and wildtype as controls) using a general parkin and TH antibody (Figure 4.14 A). I then estimated the percentage of TH and parkin double positive cells. I found that almost all DA neurons of the ventral midbrain (SNpc and VTA together) express wt h-parkin (93% of TH positive neurons) and Δ h-parkin (94% of TH positive neurons) (Figure 4.14 B).

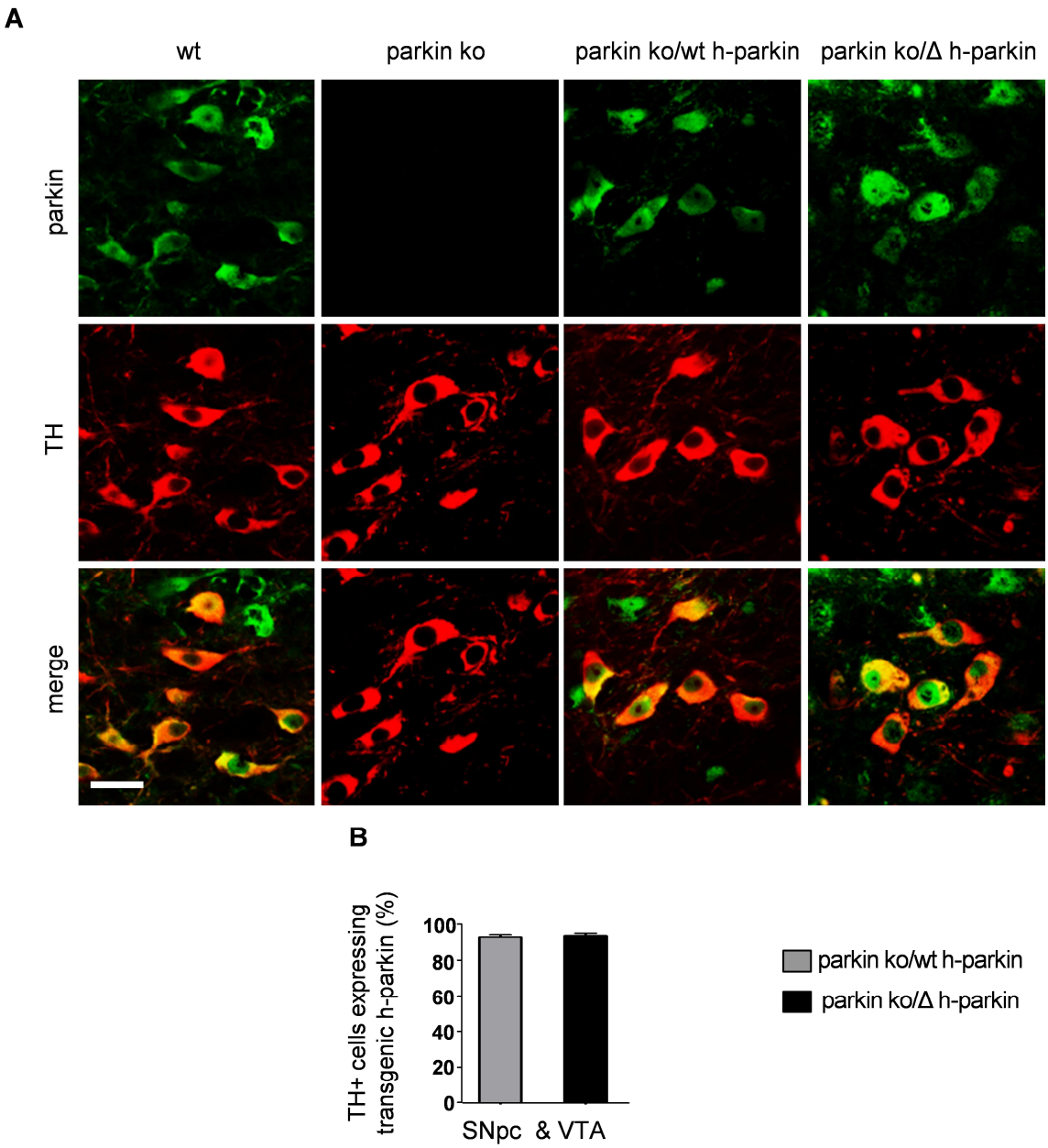

Figure 4.14 Characterization of transgenic human parkin overexpression in midbrain DA neurons of transgenic human parkin overexpressing parkin ko mice

(A) Expression of wt h-parkin and Δ h-parkin in DA (TH) neurons of the SNpc in transgenic mice crossed with parkin ko mice. Wildtype and transgenic mice but not parkin ko mice show parkin expression. Scale bar = 25 µm.
(B) Quantification of TH positive cells expressing transgenic wt h-parkin and Δ h-parkin (n = 3 mice per genotype, 150-200 cells analyzed/mouse.

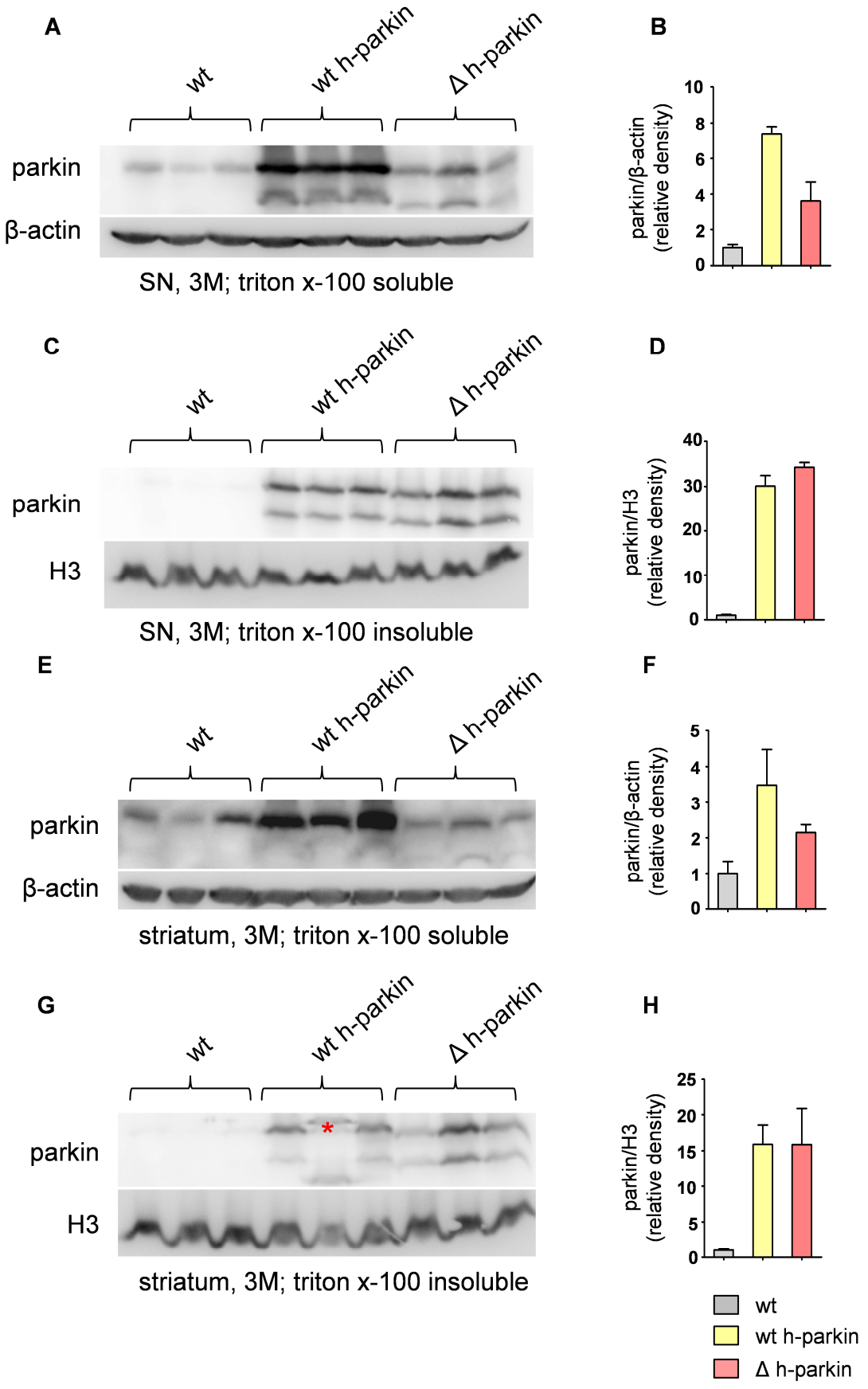

Figure 4.15 Characterization of transgenic wildtype (wt) and truncated (Δ) human parkin protein levels in the striatum and SN protein lysates

(A-B) Western blot images in (A) showing the amount of parkin protein in triton x-100 soluble fraction of the SN protein lysates from wt, wt h-parkin and Δ h-parkin overexpressing mice; β-actin was used as loading control. Respective densitometric quantifications were shown in (B), n = 3 mice per genotype.
(C-D) Western blot images in (C) showing the amount of parkin protein in triton x-100 in-soluble fraction of the SN protein lysates from wt, wt h-parkin and Δ h-parkin overexpressing mice; Histone 3 (H3) was used as loading control. Respective densitometric quantifications were shown in (D), n = 3 mice per genotype.
(E-F) Western blot images in (E) showing the amount of parkin protein in triton x-100 soluble fraction of the striatal protein lysates from wt, wt h-parkin and Δ h-parkin overexpressing mice; β-actin was used as loading control. Respective densitometric quantifications were shown in (F), n = 3 mice per genotype.
(G-H) Western blot images in (G) showing amount of parkin protein in triton x-100 in-soluble fraction of the striatal protein lysates from wt, wt h-parkin and Δ h-parkin overexpressing mice; H3 was used as loading control. Red asterisk indicates degraded sample. Respective densitometric quantifications were shown in (H), n = 3 or 4 mice per genotype.
Note: In some cases (in the wt h-parkin and Δ h-parkin lanes) an extra band observed below the normal parkin protein band, which originates from an internal translation start site in the human parkin transcript and this isoform lacks the UBL domain (Henn et al., 2005).

Next, I wanted to estimate the levels of transgenic human parkin in adult (3 months old) mice. Therefore, I performed western blot analysis on Triton X-100 soluble and insoluble protein lysates of SN and striatum from wildtype (wt), wt h-parkin and Δ h-parkin mice. In the Triton X-100 soluble fraction of the SN, I observed nearly 6-fold more parkin protein levels in wt h-parkin and 2.5-fold more in Δ h-parkin compared to wildtype mouse parkin (Figures 4.15 A and B). In the Triton X-100 soluble fractions of the striatum, I observed 2.5-fold more of wt h-parkin and one-fold of Δ h-parkin compared to wildtype mouse parkin protein levels (Figures 4.15 E and F). Concerning the Triton X-100 insoluble protein fractions of both SN (Figures 4.15 C and D) and striatum (Figures 4.15 G and H), I did not find any major differences in the levels of wt h-parkin and Δ h-parkin; whereas wildtype mouse parkin was completely absent in these fractions (Figures 4.15 C and D; G and H). Taken together my results show that the SN and striatal lysates from wt h-parkin transgenic mice contains more soluble form of parkin protein, when compared to the Δ h-parkin transgenic mice.

To test the *in vivo* neuroprotective potential of parkin against the trophic insufficiency induced neurodegeneration, we mated the newly characterized transgenic parkin overexpressing mice wt h-parkin and Δ h-parkin with DCB-Ret ko mice to obtain the DCB-Ret ko/ wt h-parkin and DCB-Ret ko/Δ h-parkin mice respectively (Figures 4.16 A and B). The DCB-Ret ko/wt h-parkin and DCB-Ret ko/Δ h-parkin mice were viable and fertile. These mice were then aged (until 12 months) to study the influence of human parkin overexpression on degenerating DA system of Ret-deficient mice.

4.12 Transgenic overexpression of human parkin prevents DA cell body loss in the SNpc of aged Ret-deficient mice

To study the effect of transgenic human parkin overexpression on DA cell bodies in wildtype and Ret-deficient background, I stereologically quantified together with Kumar Ponna the number of DA neurons in the TH antibody stained coronal midbrain sections of 12 month old control ($Ret^{lx/lx}$), single transgenic parkin mice (wt h-parkin and Δ h-parkin), single Ret ko mice and human parkin overexpressing Ret ko mice (DCB-Ret ko/ wt h-parkin and DCB-Ret ko/Δ h-parkin) (Figures 4.17 A-C). We found that the number of DA neurons in the SNpc and in the VTA of mice with transgenic overexpression of wildtype or truncated human parkin (wt h-parkin and Δ h-parkin) was similar to the $Ret^{lx/lx}$ control mice (Figures 4.17 B and C). As expected, in the aged (12 month old) Ret-deficient mice we observed a significant 18% loss of TH positive neurons in the SNpc region compared to $Ret^{lx/lx}$ controls (Figure 4.17 B), whereas the number of TH positive neurons in the adjacent VTA region (Figure 4.17 C) was unchanged, which is consistent with my own results (Figure 4.3) and also others (Aron et al., 2010; Kramer et al., 2007). To study the effect of transgenic parkin overexpression in aged Ret-deficient mice, we extended our stereological analysis of midbrain TH positive neurons to DCB-Ret ko/wt h-parkin and DCB-Ret ko/Δ h-parkin mice. Compared to the DCB-Ret ko mice, both DCB-Ret ko/wt h-parkin and DCB-Ret ko/Δ h-parkin mice at 12 months showed significantly higher number of SNpc TH positive neurons similar to what has been observed in the wt h-parkin, Δ h-parkin and $Ret^{lx/lx}$ controls (Figure 4.17 B).

Transgenic overexpression of wt h-parkin or Δ h-parkin in Ret-deficient background did not alter VTA DA neuron numbers (Figure 4.17 C). We also confirmed these observations by stereological quantification of GIRK2 subgroup of DA neurons in the coronal midbrain sections of 12 month old DCB-Ret ko, DCB-Ret ko/wt h-parkin and DCB-Ret ko/Δ h-parkin mice immunohistochemically stained with GIRK2 antibody (Figures 4.17 D and E). These results altogether suggest that human parkin overexpression (either wt h-parkin or Δ h-parkin) can prevent DA neuronal cell body loss in the aged Ret-deficient mice.

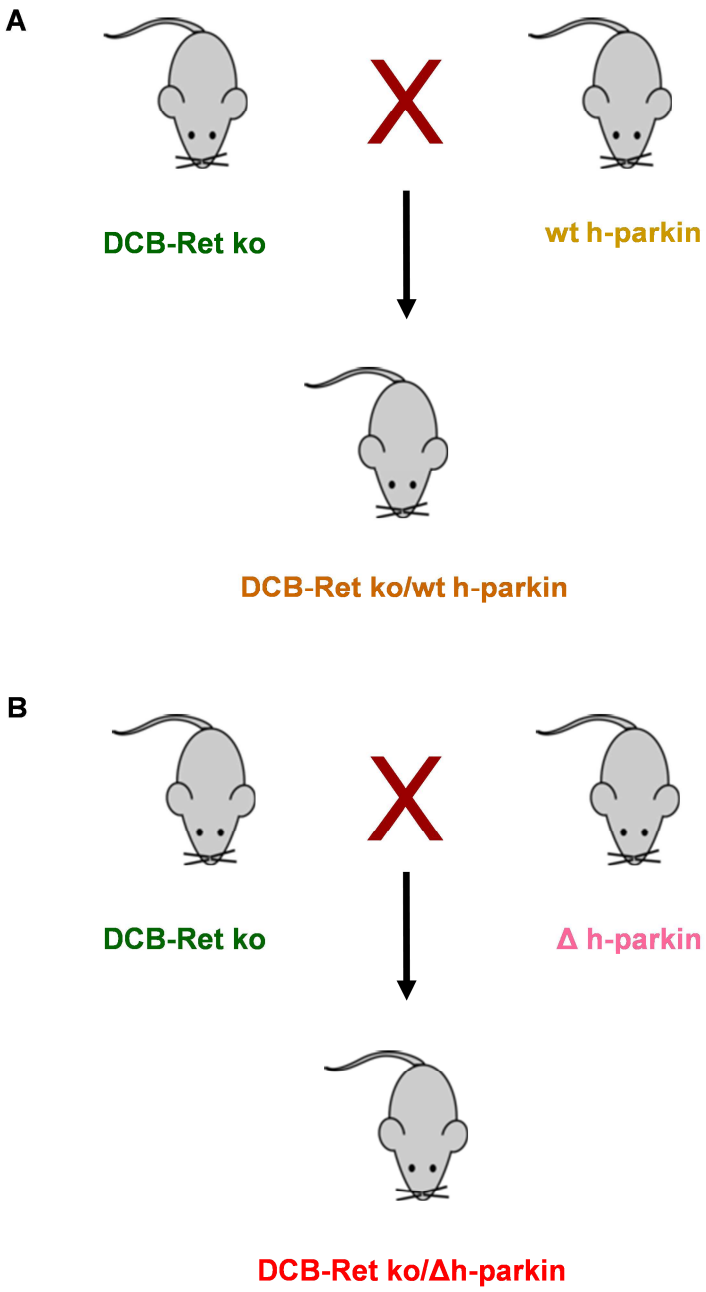

Figure 4.16 Schematic representations of the crosses made for the generation of transgenic human parkin over expressing Ret-deficient mice

(A) Generation of wildtype human parkin overexpressing Ret-deficient (DCB-Ret ko/wt h-parkin) mice: The conditional Ret-deficient mice (described in Figure 4.1 A) were crossed with wt h-parkin overexpressing mice (obtained from Konstanze Winklhofer) to obtain DCB-Ret ko/wt h-parkin mice.
(B) Generation of C-terminal truncated human parkin overexpressing Ret-deficient (DCB-Ret ko/Δ h-parkin) mice: The conditional Ret-deficient mice (described in Figure 4.1 A) were crossed with Δ h-parkin overexpressing mice (obtained from Konstanze Winklhofer) to obtain the DCB-Ret ko/Δ h-parkin mice.

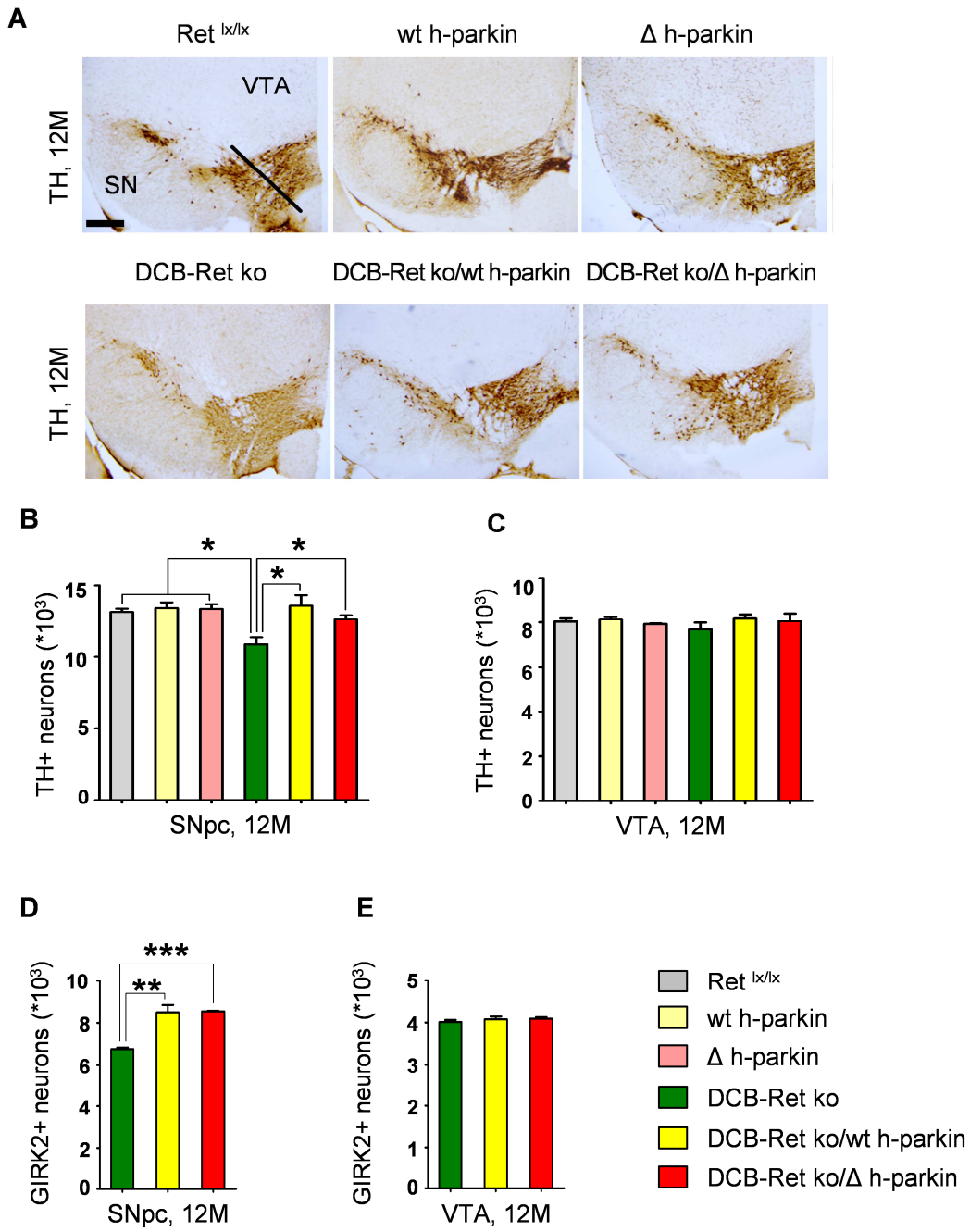

Figure 4.17 Transgenic overexpression of human parkin prevents DA neuronal loss in aged Ret-deficient mice

(A) Representative images of coronal midbrain sections showing DA neurons in the SNpc and VTA stained with TH antibody from 12 months old mice of indicated genotypes. Scale bar = 250 µm.
(B-C) Stereological quantifications of TH positive neurons in the (B) SNpc and (C) VTA of 12 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
(D-E) Stereological quantifications of GIRK2 positive neurons in the (D) SNpc and (E) VTA of 12 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test

4.13 Transgenic overexpression of human wildtype parkin prevents DA innervation loss and maintains total DA levels in the striatum of aged Ret-deficient mice

Next, we wanted to investigate if parkin overexpression can also protect the striatal DA innervation in the aged Ret-deficient mice. With the help of Anil Annamneedi and Kumar Ponna, I immunofluorescently labeled and quantified DA fiber innervation in the coronal striatal sections with antibodies against two independent DA markers TH and DAT in 12 month old mice overexpressing human parkin (wt h-parkin or Δ h-parkin) in wildtype and Ret-deficient background (Figure 4.18). Human parkin overexpression (wt h-parkin or Δ h-parkin) did not influence DA (TH and DAT positive) in the dorsal and ventral striatum when compared to the $Ret^{lx/lx}$ mice. As reported earlier, the DCB-Ret ko mice showed significant DA (TH and DAT) innervation loss relative to $Ret^{lx/lx}$ controls, both in dorsal and ventral striatum (Figures 4.18 A-E) (Aron et al., 2010; Kramer et al., 2007). When compared to the DA innervation (stained with TH and DAT) loss in the 12 month DCB-Ret ko mice, it was only the full-length human parkin overexpressing Ret-deficient mice (DCB-Ret ko/wt h-parkin) that showed significantly more TH and DAT positive innervation but not the C-terminal truncated human parkin overexpressing Ret-deficient mice (DCB-Ret ko/Δ h-parkin), both in the dorsal and ventral striatum (Figures 4.18 A-E).

To investigate the effect of parkin overexpression on total striatal DA levels of the Ret-deficient mice, I measured the total DA levels and its metabolites in the striatum lysates of 12 month old $Ret^{lx/lx}$ control mice, parkin overexpressing mice (wt h-parkin , Δ h-parkin), Ret-deficient mice (DCB-Ret ko) and parkin overexpressing Ret-deficient mice (DCB-Ret ko/wt h-parkin, DCB-Ret ko/Δ h-parkin) using HPLC-ECD system. To reduce the amount of animals needed for the experiment I used the data from 12 month old DCB-Ret ko and $Ret^{lx/lx}$ mice shown in the (Figures 4.5 A, C and E) as controls again; the preparation and HPLC measurements for all the samples described here and in (Figures 4.5 A, C and E) were done at the same time.

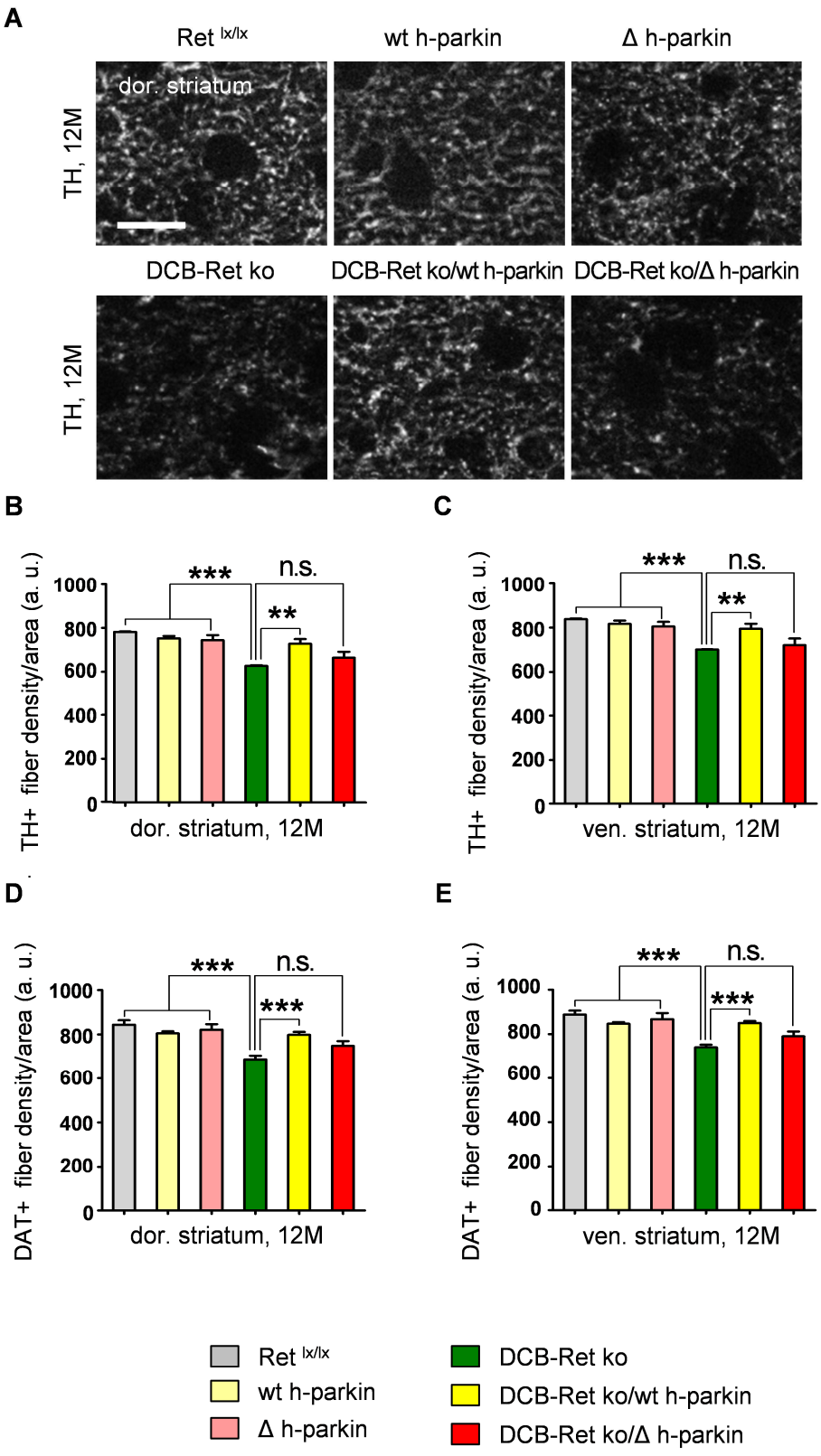

Figure 4.18 Transgenic overexpression of wt but not truncated human parkin prevents DA innervation loss in aged Ret-deficient mice

(A) Representative images of coronal midbrain sections showing DA innervation in the dorsal stained with TH antibody from 12 months old mice of indicated genotypes. Scale bar = 10 µm.

(B-C) Quantifications of TH positive fibers in the (B) dorsal striatum and (C) ventral striatum of 12 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
(D-E) Quantifications of DAT positive fibers in the (D) dorsal striatum and (E) ventral striatum of 12 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test.

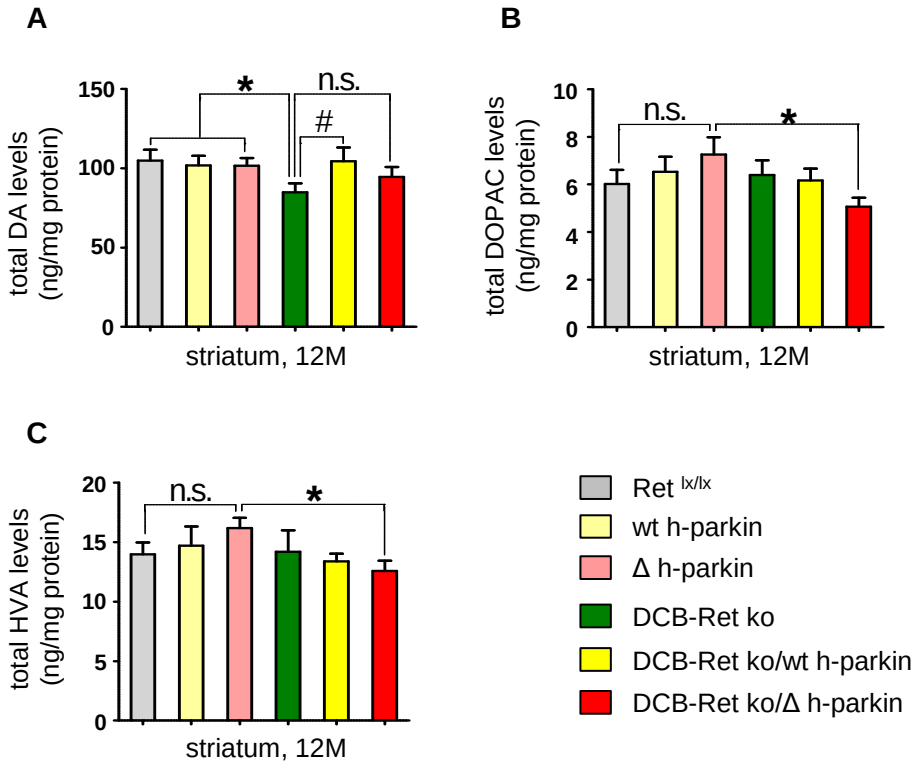

Figure 4.19 Transgenic overexpression of wt but not truncated human parkin preserves total DA levels in aged Ret-deficient mice

(A-C) HPLC measurements of (A) total DA levels, (B) total DOPAC levels and (C) total HVA levels in the striatum of 12 months old mice of the indicated genotypes, n = 4-7 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, # $p = 0.07$, * $p \leq 0.05$, Student's t-test.

The total DA levels in the striatum of parkin over expressing mice, wt h-parkin and Δ h-parkin did not alter when compared to $Ret^{lx/lx}$ mice. As described previously, there was nearly 20% total DA loss in the striatum of DCB-Ret ko mice and this DA loss was prevented only in the DCB-Ret ko/wt h-parkin (with p value = 0.07), but not in the DCB-Ret ko/Δ h-parkin mice. Whereas the total levels of DOPAC and HVA showed an increase in trend in the Δ h-parkin mice when compared to $Ret^{lx/lx}$ mice (statistically not significant), this increase was significantly normalized and reached control ($Ret^{lx/lx}$) levels in the DCB-Ret ko/Δ h-parkin mice. No major differences were observed in the levels of DOPAC and HVA in mice with other genotypes. It was only DCB-Ret ko/wt h-parkin mice, but not the DCB-Ret ko/Δ h-parkin mice that showed significant protection of striatal DA innervation and DA levels in comparison to DCB-Ret ko mice. This could be explained by relatively low amount of soluble parkin protein the Δ h-parkin transgenic mice, particularly in the striatum with only one-fold higher expression (Figures 4.15. E-H); whereas, in the SN there was nearly 2.5-fold higher

amounts of parkin protein in the Δ h-parkin transgene compared to wildtype mouse parkin and this is probably enough to show its protective effect in the SNpc DA neurons. These observations all together suggests that overexpression of parkin seems to be neuroprotective in trophically impaired Ret-deficient mice and can maintain striatal total DA levels, prevent age dependent DA cell body and innervation loss.

C. Parkin deletion partially normalizes the enlarged nigrostriatal system in the adult MEN2B mice

Using two different approaches, we could show that parkin is important for the maintenance of nigrostriatal DA system in trophically impaired Ret-deficient mice. In a third approach, I wanted to investigate if loss of parkin can normalize the DA system enlargement visible in adult mice carrying a constitutive active form of the Ret receptor with a mutation in the intracellular domain (MEN2B mutation) (Mijatovic et al., 2007). To answer this question, we crossed parkin ko mice (Itier et al., 2003) with MEN2B mice (Smith-Hicks et al., 2000) to generate MEN2B/parkin ko mice (Figure 4.20).

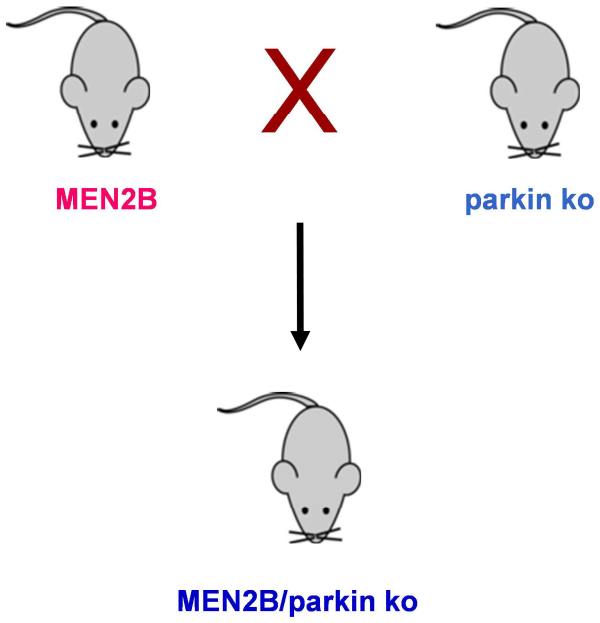

Figure 4.20 Schematic representations of the crosses to generate parkin-deficient MEN2B mice

Mice with a constitutive active mutant of Ret (MEN2B) were obtained from Frank Costantini (Smith-Hicks et al., 2000) and crossed with parkin-deficient mice (parkin ko) obtained from Alexis Brice (Itier et al., 2003) to create parkin-deficient MEN2B mice (MEN2B/parkin ko).

The MEN2B/parkin ko mice were viable, but the male homozygous MEN2B/parkin ko mice where infertile. I analyzed DA system in these mice in their adulthood (3-6 months), as I wanted to study the early developmental role of parkin in the enlarged DA system of MEN2B

mice. Throughout these experiments (unless mentioned), I considered parkin ko mice as control group, since previous studies (Goldberg et al., 2003; Itier et al., 2003) and the present study (Figures 4.3-4.5) did not observe any significant alterations in the DA system of these mice.

4.14 Parkin deletion normalizes the increased number of SNpc DA neurons in the adult MEN2B mice

To study the effect of parkin deletion on increased number of DA cell bodies in the MEN2B mice, I stereologically quantified the number of DA neurons in the coronal midbrain sections of 3-6 month old parkin ko, MEN2B and MEN2B/parkin ko mice stained with antibodies against two different DA markers TH and GIRK2 (Figures 4.21 A and D).

As described previously (Mijatovic et al., 2007), I confirmed that adult homozygous MEN2B mice show more DA neurons specifically in the SNpc (21% more TH positive cells; 31% more GIRK2 positive cells) compared to parkin ko mice (Figures 4.21 A, B, D and E). However, in the MEN2B/parkin ko double mutant mice the number of DA neurons are normalized (a reduction of 12% TH positive and 15% GIRK2 positive cells) compared to MEN2B mice (Figures 4.21 A, B, D and E). As reported previously, the number of VTA DA neurons is not increased in MEN2B mice and also stays unaltered in MEN2B/parkin ko mice when compared to parkin ko mice (Figures 4.21 C and F).

4.15 Parkin deletion slightly normalizes the increased DA innervation and increases DA turnover in the striatum of adult MEN2B mice

To study the effect of parkin deletion on increased DA innervation of the MEN2B mice, I labeled the DA innervation in the coronal striatal sections of 3-6 month old parkin ko, MEN2B and MEN2B/parkin ko mice with antibodies against two independent DA markers TH and DAT. As reported previously (Mijatovic et al., 2007), I confirmed that adult homozygous MEN2B mice show more DA innervation (44% more TH positive fibers and 56% more DAT positive fibers in the dorsal striatum) compared to parkin ko mice (Figures 4.22 A, B, C and D). The increased striatal DA innervations observed in MEN2B mice is slightly but significantly reduced by 10 and 11% in the dorsal striatum of MEN2B/parkin ko mice when stained with TH and DAT antibodies, respectively (Figures 4.22 A, B, C and D).

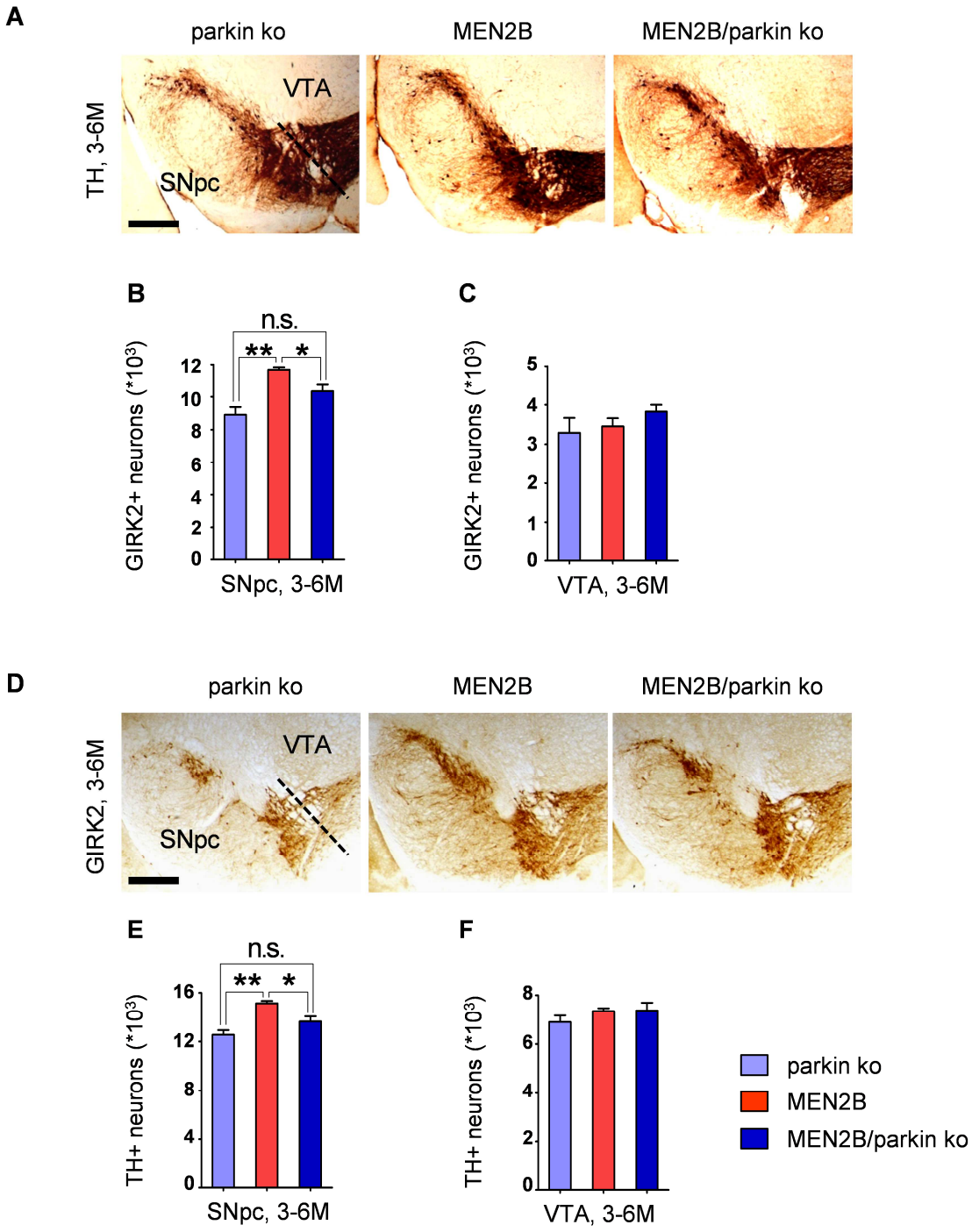

Figure 4.21 Parkin deletion partly normalizes the increased SNpc DA neuron numbers in the MEN2B mice

(A) Representative photomicrographs of coronal sections from 3-6 months old parkin ko, MEN2B and MEN2B/parkin ko mice showing TH positive neurons in the SNpc and VTA. Scale bar = 250 µm.
(B-C) Stereological quantifications of the TH positive neurons in the (B) SNpc and (C) VTA of 3-6 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
(D) Representative photomicrographs of coronal sections from 3-6 months old parkin ko, MEN2B and MEN2B/parkin ko mice showing GIRK2 expressing neurons in the SNpc and VTA. Scale bar = 250 µm.
(E-F) Stereological quantifications of the GIRK2 positive neurons in the (E) SNpc and (F) VTA of 3-6 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, Student's t-test.

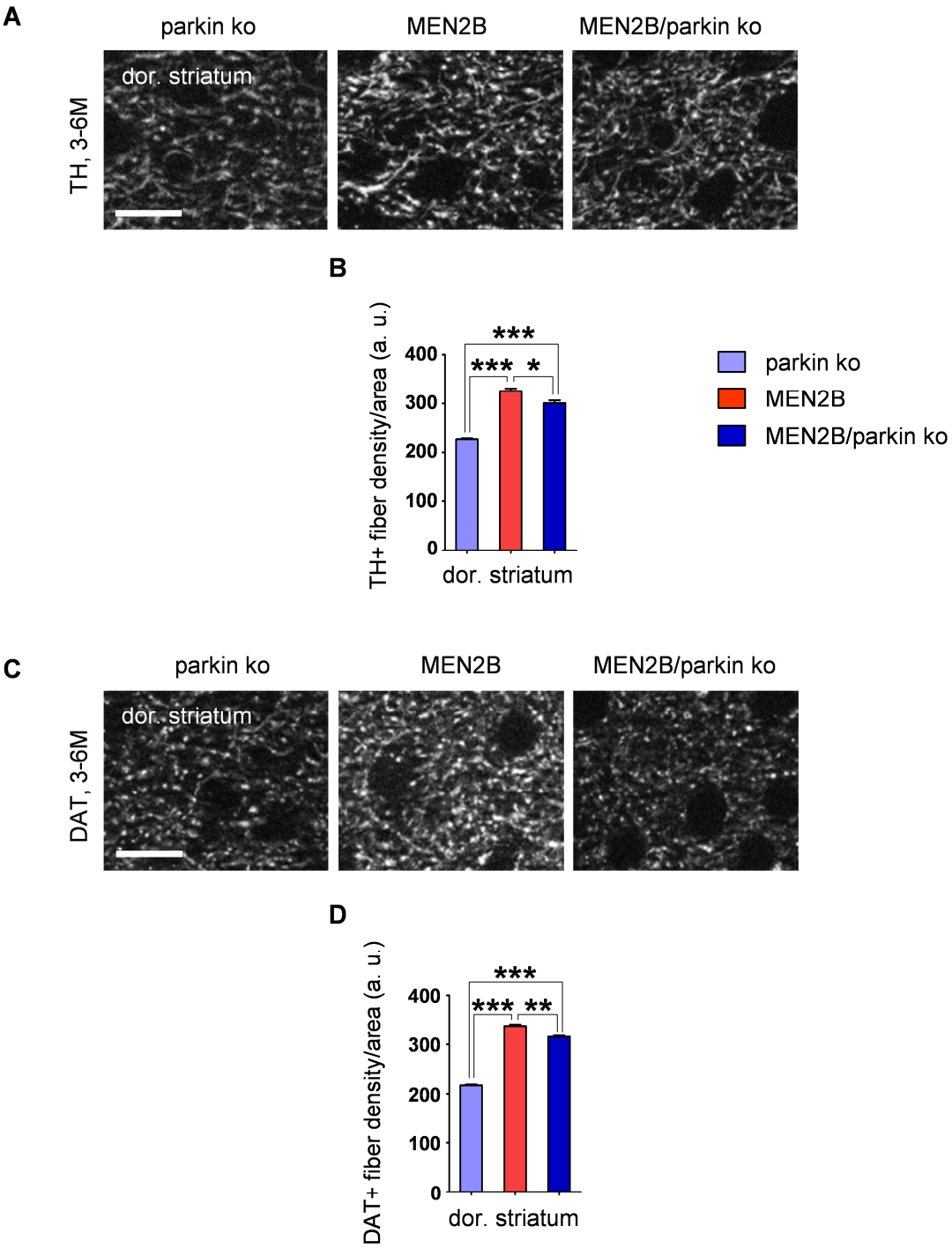

Figure 4.22 Parkin deletion slightly normalizes the increased striatal DA innervation in the MEN2B mice

(A) Representative photomicrographs of coronal sections from 3-6 months old parkin ko, MEN2B and MEN2B/parkin ko mice showing TH positive fibers in the dorsal striatum. Scale bar = 10 µm.
(B) Quantifications of the TH positive fiber density in the dorsal striatum of 3-6 months old mice of the indicated genotypes, n = 3-4 mice per genotype.
(C) Representative photomicrographs of coronal sections from 3-6 months old parkin ko, MEN2B and MEN2B/parkin ko mice showing DAT positive fibers in the dorsal striatum. Scale bar = 10 µm.
(D) Quantifications of the DAT positive fiber density in the dorsal striatum of 3-6 months old mice of the indicated genotypes, n = 3-4 mice per genotype. Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, ** $p \leq 0.001$, Student's t-test.

In order to investigate the effect of parkin deletion in MEN2B mice on the striatal total DA levels, I measured the total levels of DA and its metabolites in the striatum lysates of MEN2B/parkin ko mice together with MEN2B, parkin ko and wildtype mice using HPLC-ECD system. As already reported for the MEN2B mice (Mijatovic et al., 2007), I observed very high levels of DA (Figure 4.23 A) and its metabolites DOPAC (Figure 4.23 B) and HVA (Figure 4.23 C) in the striatum compared to parkin ko or wildtype mice. Whereas, the MEN2B/parkin ko mice show only a very slight and insignificant reduction of total DA levels (Figure 4.23 A) and increase in the levels of DOPAC (Figure 4.23 B) and HVA (Figure 4.23 C) compared to MEN2B mice. However, the ratio of the DA degradation product HVA to DA (HVA/DA) is significantly recovered in the MEN2B/parkin ko mice compared to MEN2B mice (Figure 4.23 D).

Taken together all these observations show that loss of parkin helps to normalize the enlarged DA system of MEN2B mice suggesting an important genetic cross talk of Ret and parkin already during development and adulthood to establish correct number of DA cell and their innervation.

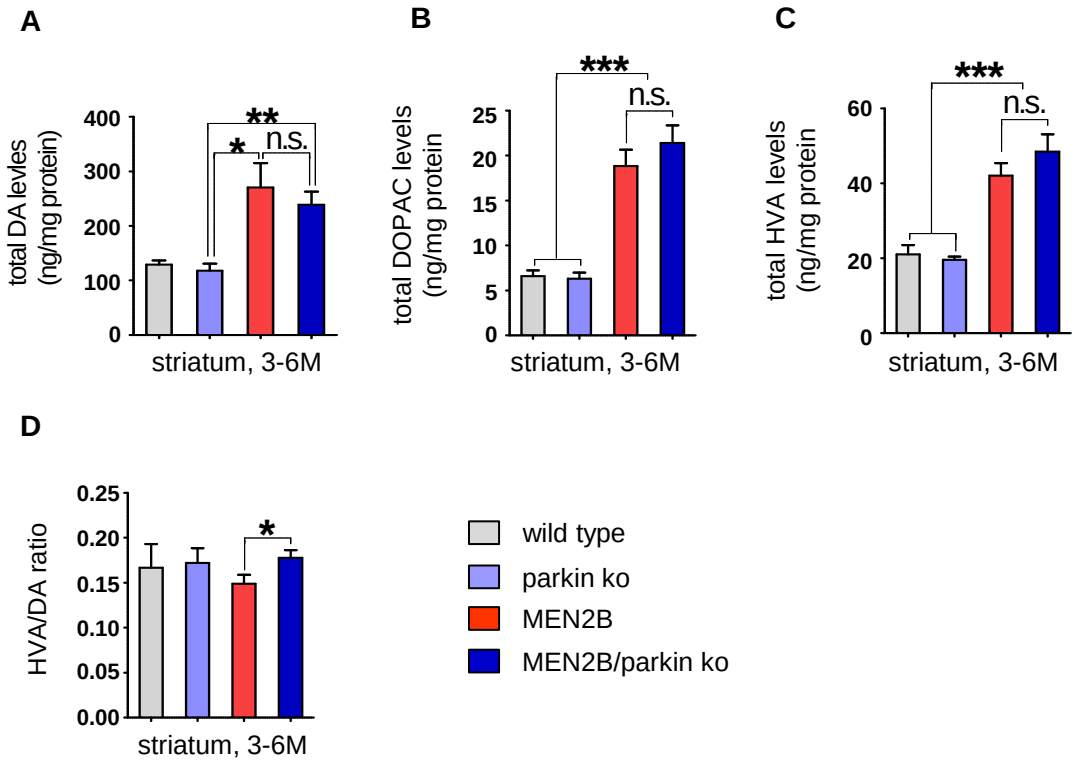

Figure 4.23 Parkin deletion in MEN2B mice normalizes DA turnover but cannot influence total DA or its metabolites

(A-D) HPLC measurements of (A) total DA levels, (B) total DOPAC levels and (C) total HVA levels and (D) ratio of HVA to DA levels in the striatum of 3-6 months old mice of the indicated genotypes, n = 4-8 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Student's t-test.

4.16 MEN2B mutation in mice enhances Ret protein levels both in the midbrain and striatum, and also leads to increased soma size of midbrain DA neurons

To investigate the levels of Ret protein in MEN2B and MEN2B/parkin ko mice, I performed immunohistochemical staining and western blot analysis on the SN sections and striatum lysates respectively and probed with antibody against Ret. Interestingly, for the first time I could show that MEN2B mutation in mice does not only lead to enlarged DA system but also to more Ret protein that is detected in the midbrain DA neurons (Figures 4.24 A and B) and also in the striatum (Figures 4.24 C and D) compared to wildtype mice. However, parkin deletion in MEN2B mice has a slight but insignificant effect on the increased Ret protein levels both in midbrain (Figures 4.24 A and B) and striatum (Figures 4.24 C and D).

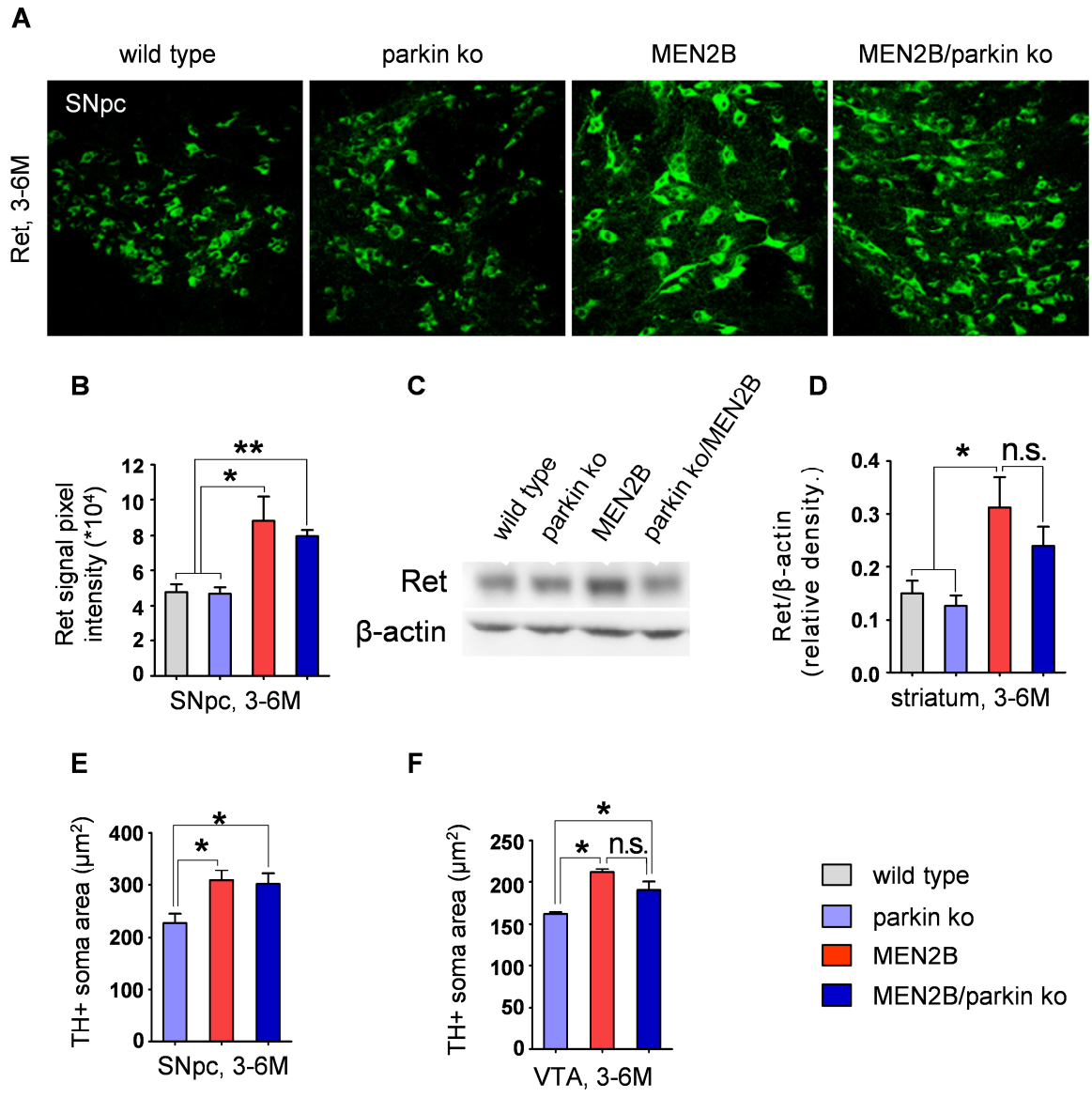

Figure 4.24 Ret-MEN2B mutation leads to an increase in Ret protein levels in the nigrostriatal system and also increases neuronal soma size of the midbrain DA neurons in mice

(A) Representative images of coronal sections from 3-6 months old parkin ko, MEN2B and MEN2B/parkin ko mice showing Ret expressing neurons in the SNpc region.
(B) Quantifications of Ret signal pixel intensity in the SNpc of 3-6 months old mice of the indicated genotypes, n = 3 mice per genotype.
(C) Western blot images from 3-6 months old wildtype, parkin ko, MEN2B and MEN2B/parkin ko mice showing Ret expression in striatal protein lysates, β-actin was used as loading control. One mouse per genotype is shown.
(D) Western blot quantifications of Ret expression (normalized with β-actin) in the striatal protein lysates is shown, n = 4 mice per genotype
(E-F) Quantifications of TH+ soma area of 3-6months old mice of the indicated genotypes in the (E) SNpc and (F) VTA regions is shown, n = 3 mice per genotype.
Data are represented as mean +/- SEM; n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, Student's t-test.

Having observed a decrease in DA soma size in the SN of DA neuron specific Ret-deficient mice (Figure 4.12 A and B); I wanted to investigate the effect of MEN2B mutation on DA soma size, together with the role of parkin. To answer this question I measured soma size of TH labeled DA neurons in the midbrain coronal sections (using the nucleator probe of the StereoInvestigator program) from adult parkin ko, MEN2B and MEN2B/parkin ko mice. As I have not observed any changes in the soma size of adult and aged parkin ko mice (Figures 4.12 A and B), I considered parkin ko mice as my control group. My results for the first time revealed an increase in the soma size of DA neurons in the SNpc (Figure 4.24 E) and VTA (Figure 4.24 F) of MEN2B and MEN2B/parkin ko mice when compared with parkin ko control mice. However, the MEN2B/parkin ko mice did not show any significant decrease in the size of DA neuronal soma when compared with MEN2B mice (Figures 4.24 E and F). These observations reveal an important function of neurotrophic signaling mediated by Ret in establishing and maintaining the soma size of DA neurons in mice (Figures 4.12 A and B; 4.24 E); whereas, parkin deletion could not modulate the Ret mediated function on DA cell size.

5. Discussion

In the present study, I demonstrate biochemical and mild behavioral changes in the newly developed conditional Ret-deficient mice (DCB-Ret ko, Figure 4.1 A) along with nigrostriatal degeneration upon ageing (Figures 4.1-4.8), which was described in the previously reported Ret ko mice (DAT-Ret ko mice) (Kramer *et al.*, 2007). These findings make the DCB-Ret ko mice an even exciting preclinical genetic mouse model to understand etiologies and pathogenesis of PD. More importantly, I report that the survival and maintenance function of Ret is tightly linked with parkin, a PD-related protein, in the SNpc DA neurons. Deletion of parkin in Ret receptor deficient mice leads to enhanced neurodegeneration phenotype with increased SNpc DA cell body and innervation loss, when compared to mice that only lack the Ret receptor (Figure 4.3 and 4.4). Interestingly, transgenic overexpression of parkin prevented the DA neurodegeneration phenotype of Ret-deficient mice (Figures 4.17 and 4.18). Parkin deficiency also normalized the enlarged DA system of MEN2B mice (Figures 4.21 and 4.22). It was also shown that both, Ret signaling and parkin activity is essential for maintaining total cellular ATP levels and complex I activity of the mitochondria contributing to appropriate cellular morphology and physiology of the SNpc DA neurons (Figures 4.12 and 4.13). Altogether, these data demonstrates that parkin cooperates with GDNF/Ret signaling to prevent degeneration of SNpc DA neurons in mice by maintaining proper mitochondrial function. Supporting my findings, cell culture experiments performed (on SH-SY5Y cells) in collaboration with the lab of Konstanze Winklhofer, LMU, Münich for the first time demonstrated the role of Ret in maintaining mitochondrial integrity (Meka *et al*., unpublished data), which is somewhat similar to what has been previously reported for parkin (reviewed in Exner *et al*., 2012). The findings in SH-SY5Y cells also confirms the requirement of Ret signaling and parkin activity for mitochondrial complex I activity and total cellular ATP levels (Figures 4.13 D and 5.2). Moreover, it was shown that overexpression of parkin or activation of the GDNF/Ret signaling can rescue the mitochondrial loss of function phenotype of Ret or parkin (Figures 5.3 and 5.4). The newly discovered mitochondrial maintenance function of GDNF/Ret signaling in SH-SY5Y cells has been shown to signal via the PI3K pathway to activate the classical NF-κB pathway (Meka *et al*., unpublished data), which is also a downstream target of parkin signaling for maintaining mitochondrial integrity (Henn *et al*., 2007; Müller-Rischart *et al*., 2013; Figure 5.5).

5.1 Ret deficiency in mice also causes biochemical and behavioral alterations along with age-dependent nigrostriatal DA neurodegeneration

Previously, DAT-Cre mice were crossed with floxed Ret mice to generate midbrain DA neuronal specific conditional Ret-deficient mice, DAT-Ret ko (Kramer *et al.*, 2007). In the DAT-Cre line, the Cre transgene insertion in to the 5'-UTR of one of the endogenous DAT locus resulted in only one functional copy of DAT gene, which led to an age-dependent decrease in DAT protein levels along with increased striatal DA levels and hyperactive

behavior in the mice (Parlato *et al.*, 2006; Kowsky *et al.*, 2007; Turiault *et al.*, 2007). It could be the reason why the previously generated Ret-deficient (DAT-Ret ko) mice did not show decreased striatal DA levels and behavioral changes despite the age-dependent degeneration of nigrostriatal DA system. To overcome this problem we used new Cre expressing mice - the DAT-Cre BAC (DCB) mice, for generating midbrain DA neuron specific conditional Ret-deficient mice, DCB-Ret ko (Figure 4.1 A). The DCB mice contain a bacmid that is engineered to expresses Cre recombinase under an exogenous dopamine transporter (DAT) promoter (Parlato *et al.*, 2006; Turiault *et al.*, 2007) which gave the possibility to drive the expression of Cre under the DA neuron specific promoter, DAT without altering the activity of endogenous DAT promoters and therefore the DCB mice contained normal DAT protein levels; hence abnormal DA metabolism and subsequent behavioral changes were prevented (Figures 4.2 C; 4.5 A-D; 4.8 and 4.11 B).

The conditional Ret-deficient (DCB-Ret ko) thus generated showed an age-dependent decrease in striatal total DA levels (19% at 12 months and 30% at 24 months; Figure 4.5 A and B) and displayed locomotory behavior alterations in open field (at 12 and 24 months; Figures 4.8 A and C) and mild anxiety-related behavior on the elevated plus maze (Figure 4.8 G) which were previously not described. The newly discovered biochemical and behavioral alterations observed in the Ret single deficient mice corresponded very much with the progressive DA nigrostriatal degeneration phenotype signifying the importance of trophic signaling for normal physiology and behavior of the mice. The DCB-Ret ko mice showed a 15% DA neuronal loss in 12 months old (Figure 4.3 C) and 21% loss in 24 month old animals specifically in the SNpc but not in the adjacent VTA region when compared to the DCB control mice (Figures 4.3 D and E). This is consistent with the already published DAT-Ret ko mice (Kramer *et al.*, 2007), which also demonstrated that Ret signaling is dispensable for VTA DA neuronal survival. When compared to SNpc DA neurons, the VTA neurons are shown to be less sensitivity to 6-OHDA treatment (Barroso-Chinea *et al.*, 2005) or α-syn overexpression (Maingay *et al.*, 2006). Moreover the presence of a functional K-ATP channel Kir6.2 was shown to promote cell death of SN, but not VTA DA neurons in two mechanistically distinct mouse models of dopaminergic degeneration (Liss and Roeper, 2001). Remarkably, significant loss of VTA DA neurons was observed in a conditional GDNF adult knockout mouse (Pascual *et al.*, 2008), raising the possibility that Ret-independent GDNF signaling (via NCAM or other alternate receptors) is required for survival of VTA neurons. It is possible that GDNF can signal through different receptors localized to specific neuronal population to exert its functions. Moreover, I detected indistinguishable cell loss percentage differences both in TH and G-protein coupled potassium inwardly rectifying channel (GIRK2) stained SNpc neurons in the DCB-Ret ko mice (Figure 4.3 F-H), suggesting no specific requirement of Ret signaling for GIRK2-positive DA neuronal subtype in the SNpc which are more prone to degeneration in PD patients (Yamada *et al.*, 1990; Liang *et al.*, 1996). These findings exclude the possibility of Ret signaling dependence specifically in the GIRK2 DA neuronal subtype in the SNpc, at the same time confirmed my results obtained from the TH staining (Figures 4.3 D and E).

Corresponding to the cellular changes, the striatal DA innervation is significantly decreased in the aged mice upto 33% at 12months and 48% in the 24months old mice when compared to

the DCB controls (Figures 4.4 A, C and D). The fiber loss phenotype in the DCB-Ret ko mice has been confirmed with an independent DA marker, DAT in 24 months old mice (Figures 4.4 H and I). This striatal TH fiber degeneration phenotype observed DCB-Ret ko mice is rather uniform throughout the striatum, as the percentage of fiber loss observed in the dorsal side corresponded very much to that of ventral side of the striatum in these genotypes (Figure 4.4 F and G). These observations argue for differential survival or maintenance functions of Ret in the DA axonal terminal of the striatum and in the cell bodies of the SNpc. Moreover, previous study on Ret-deficient mice showed that the loss of DA fibers starts at 9 months, with no indications of cell body loss; the first significant changes with respect to DA cell bodies have been observed at 12 months (Kramer *et al.*, 2007). This increased or advanced vulnerability of DA fiber innervation relative to cell body loss observed in Ret-deficient mice could be due to several reasons. It is possible that both DA fiber terminals and cell bodies require Ret signaling for maintenance, could it be that the DA fiber terminals are more trophic signaling dependent than the DA cell bodies. Another plausible justification is the very well-known dying back hypothesis of degeneration in which the axonal compartment is firstly vulnerable, which then retrogradely proceeds to cell bodies. This situation can be explained form the post-mortem histological observations of PD-brains (Bernheimer *et al.*, 1973; Lach *et al.*, 1992). Typically at the onset of PD symptoms, nearly 50-60 % SN cell bodies are lost while the loss of striatal DA levels exceeds 80 % (Dauer and Przedborski, 2003). Experiments performed on MPTP animal models also support the dying back model, monkeys when treated with MPTP showed striatal terminal loss that precedes SNpc DA cell bodies loss (Herkenham *et al.*, 1991); moreover in rats, protection of striatal axon terminals prevented loss of SNpc cell bodies after MPTP-treatment (Wu *et al.*, 2003). To solve this puzzle, retrograde tracing experiments must be performed to determine whether some DA cell bodies in Ret-deficient mice are deprived of terminal innervation, before the onset of SNpc cell body degeneration.

I also detected neuroinflammation in the nigrostriatal system of 24 month old Ret-deficient mice. I noticed an increased number of reactive astrocytes (by immunostaining with GFAP antibody), specifically in the striatum but not in the SNpc region (Figure 4.6). The increase of astrocytes in the striatum could be due to degenerated DA fiber or dysfunctional post-synaptic MSNs. Since astrocyte recruitment was only observed in 24 month but not 12 month old mice, it can speculated that the recruitment of astrocytes is not the primary cause of DA fiber degeneration which was already observed in 9 month old Ret-deficient mice (reported in Kramer *et al*., 2007). It could be that the signal which drives the recruitment of astrocytes comes from the striatal DA axonal fibers or other post synaptic cells, but not from the SNpc DA neuronal soma. Moreover, I observed microglial recruitment (by immunostaining with Iba1 antibody) in the SNpc but not in the striatum of 24 month old DCB-Ret ko (Figure 4.7), which is in contrast with astrocytes recruitment. Previously, Kramer *et al*., in 2007 reported similar findings in 24 month old mice but not in the 12 month old DAT-Ret ko mice, implying that microglial recruitment is also not the primary cause of SNpc DA neuron loss which is already observed in 12 month old mice. Moreover, microglial recruitment is required to clear the apoptotic SN DA neurons to prevent the accumulation of cellular debris and consequent harmful effects on the neighboring healthy DA neurons and other cells. The microglial recruitment in the aged Ret-deficient mice is consistent with the observations made

in the SNpc of PD patients' brains (Teismann *et al.*, 2003). However, the exact role of astrocyte and microglial recruitment to the site of neurodegeneration is largely unknown and it requires further studies to understand their contribution before, during and after the occurrence of degenerative process.

Taken together, these observations supports the idea that selective age-dependent nigrostriatal DA system is accompanied by inflammation and decreased total DA levels, behavioral alterations in the Ret-deficient mice making them an exciting preclinical genetic model to study the neurodegeneration in PD

5.2 Parkin cooperates with GDNF/Ret signaling to prevent DA neurodegeneration

In order to investigate the *in vivo* functional interaction of parkin and Ret, we generated three different mouse model systems, in which we modulated the normal physiological state of the proteins, Ret and parkin.

The three mouse models generated and analyzed in the current study are:

1. Ret/parkin double-deficient mice: The conditional Ret-deficient mice (DCB-Ret ko) which are pre-disposed to PD-like DA neurodegeneration were crossed with complete parkin-deficient mice mice which showed no histological alterations (Itier *et al.*, 2003) to obtain the Ret/parkin double-deficient mice (DAT-Ret/parkin ko; Figure 4.1 and 4.2). These mice were analyzed at early (3-6 months) and late ages (at 12 and 24 months) to study the effect of Ret/parkin double deletion on the nigrostriatal DA system.

2. Ret-deficient/human parkin overexpressing mice: The conditional Ret-deficient mice which are pre-disposed to PD-like DA neurodegeneration (Figures 4-1-4-8; Kramer *et al*., 2007; Aron *et al*., 2010) were crossed with transgenic mice over human parkin either the wildtype (wt h-parkin) or C-terminal truncated (Δ h-parkin) form under the mouse prion promoter to obtain two variants Ret-deficient/human parkin overexpressing mice, one with wt h-parkin (DCB-Ret ko/ wt h-parkin) and other with Δ h-parkin (DCB-Ret ko/ Δ h-parkin) (Figure 4.16 A and B). These mice were analyzed at 12 months in order to study the effect of parkin overexpression on the nigrostriatal DA system of the Ret-deficient mice which shows significant signs of degeneration already at that age.

3. MEN2B/parkin-deficient mice mice: The MEN2B mutant (with a constitutive active mutation in the intracellular domain of the Ret receptor) mice which show enlarged nigrostriatal DA system (Mijatovic *et al*., 2007) were crossed with complete parkin-deficient mice, which showed no histological alterations (Itier *et al*., 2003) to generate MEN2B mice with parkin deficiency (MEN2B/parkin ko; Figure 4.20). These mice were analyzed during their adulthood (at 3-6 months) in order to investigate the influence of parkin deletion on the enlarged nigrostriatal DA system of the MEN2B mice.

5.2.1 Parkin deletion exacerbates the nigrostriatal dopaminergic neurodegeneration process in the aged Ret-deficient mice

To investigate the functional interaction of Ret and parkin, we generated Ret/parkin double-deficient mice, by crossing the conditional Ret-deficient mice with complete parkin knockouts. The Ret/parkin double-deficient mice thus generated are viable, developed normally and are fertile. The Ret/parkin double deletion did not show any histological alterations in the SNpc DA neurons in adult (3-6 months old) mice (Figure 4.3 B). However, I observed an enhanced loss of SNpc DA neurons in the aged Ret/parkin double-deficient mice compared to mice that lack Ret alone. The DCB-Ret/parkin ko mice show an enhanced degeneration of the SNpc DA neurons losing 19% at 12 months to 28% at 24 months (Figure 4.3A, C and D) which is slightly but significantly enhanced when compared to the cell loss observed in single Ret-deficient mice with 15% loss in 12 months old (Figure 4.3 B) to 21% in 24 month old animals compared to the DCB control mice (Figure 4.3 A and D). Whereas, the number of VTA DA neurons remain unchanged in 24 month old Ret/parkin double ko mice as observed for the single Ret ko mice. Moreover, deletion of parkin along with Ret did not increase the susceptibility of GIRK2 expressing SNpc DA neurons to age-dependent neurodegeneration, as I have not observed any additional loss of GIRK2 stained neurons in the SNpc relative to the percentage of TH positive cell loss (Figure 4.3 F-H). Taken together these results suggest a mild enhancement effect of parkin deficiency on the SNpc DA neurons of the Ret-deficient mice without GIRK2 subtype specificity.

Remarkably, parkin deletion showed a much stronger impact on the striatal fiber innervation of the aged Ret-deficient mice. Whereas, parkin deletion alone did not cause any DA fiber degeneration in adult and aged mice as reported (Itier *et al*., 2003). In the Ret/parkin double-deficient mice the TH innervation loss in the dorsal striatum reached upto 51% in 12 months and 56% in 24 months old mice which is considerably severe when compared to the fiber loss in DCB-Ret ko mice which was upto 33% at 12 months and 48% at 24 months when compared to the DCB control mice (Figures 4.4 A, C and D; similar to the observations from Kramer *et al.*, 2007). The enhanced fiber loss phenotype in the Ret/parkin double ko mice compared to DCB-Ret ko mice has been confirmed with an independent DA marker, DAT in 24 month old mice. The fiber degeneration phenotype observed is rather uniform throughout the striatum, as the percentage of fiber loss observed in the dorsal side corresponds very much to that of ventral side of the striatum in all the genotypes (Figure 4.4 E-G). In the 24 month old Ret/parkin double-deficient mice the nigrostriatal degeneration is also accompanied by increased astrocyte recruitment in the striatum and microglial migration in the SN which is similar to what has been observed for the age matched single Ret-deficient mice with no further significant enhancement (Figures 4.6 and 4.7; Kramer *et al*., 2007; Aron *et al*., 2010). It can be speculated that the mild enhancement in the DA degeneration phenotype observed in the Ret/parkin double-deficient mice compared to the single Ret-deficient mice might not be enough to enhance the astrocyte and microglial recruitment to striatum and SNpc respectively.

As reported already (Itier *et al*., 2003), aged parkin ko mice did not show any changes in the levels of DA and its catabolites (HVA and DOPAC) in the striatum (Figures 4.5 A-F). Despite the enhanced striatal fiber degeneration phenotype both at 12 and 24 months, Ret/parkin

double-deficient mice (upto 19% in 12 month and 30% in 24 month old mice) did not show any further decrease in the striatal total DA levels when compared to Ret single deficient mice (Figures 4.5 A and B). However, I did not observe any age related alterations in the total levels of TH or DAT protein levels in the SN or striatum of single parkin, Ret and Ret/parkin double ko mice (Figures 4.11 A and B). Altogether, suggesting an additional compensatory mechanism in the Ret/parkin double ko mice to maintain the striatal total DA levels despite of DA neurodegeneration. However, in ageing mice (both at 12 and 24 months) the total levels of DOPAC a metabolite and intermediate of DA degradation product which can only be metabolized in the DA neuron terminals, was found to be significantly lowered only in the Ret/parkin double ko mice but not in the single parkin or Ret ko mice when compared to the control (DCB) mice (Figures 4.5 C and D). This can be explained by the enhanced loss of DA innervation observed in the striatum of Ret/parkin double ko mice reaching a critical threshold resulting in a decreased reuptake of DA and subsequent decline in the total levels of DOPAC.

Interestingly, Ret and parkin single deletion led to significant behavioral abnormalities with respect to their horizontal activity in the open field test performed on ageing mice, this behavioral phenotype is already described for parkin ko (Zhu *et al*., 2007) and first described for the Ret-deficient mice (Figures 4.8 A and C). However in the Ret/parkin double-deficient mice, thigmotactic behavioral changes (anxiety related behavior) have also been observed at 12 months (Figure 4.8 C) but not in 24months (Figure 4.8 D), which could be due to less number of animals used for performing the test (n = 6-17 per group). Nevertheless, after performing the most stringent anxiety related behavioral assessment on the elevated plus maze (EPM), I observed increased anxiety related behavior in the 24 month Ret/parkin double ko mice, with preferentially less exploration on the open arms (Figure 4.8 E-G). However, the single parkin ko mice which showed a trend (but statistically insignificant) for thigmotactic behavior in the open field test showed a significant anxiety related behavior on the EPM with less exploration on the open arms, consistent with earlier reports. Whereas the single Ret ko mice did not show any significant changes on the EPM compared to controls except for decreased number of head dipping events on the open arm indicating slight changes in anxiety related behavior (Figure 4.8 G). These behavioral changes observed on the EPM of Ret/parkin double ko mice is significantly greater (close to the p value = 0.05) when compared to Ret single ko mice; and with an increasing trend (statistical insignificant) when compared to the parkin single ko mice, which could be again due to number of animals used for performing the test (n = 7-11 per group) (Figure 4.8 E-G). However, the anxiety related phenotype observed on the EPM seems to be more parkin dependent which is slightly (but insignificantly) enhanced due to Ret ablation in the Ret/parkin double ko mice. The observed changes in DA metabolism and behavioral changes in Ret/parkin double ko mice signifies the functional interaction between parkin and Ret, having influence not only at the cellular and physiological levels but also on the overall behavior of the mice.

This parkin dependent nigrostriatal DA degeneration phenotype in the aged Ret-deficient mice strongly signifies the functional cooperation between parkin and Ret for the maintenance of the nigrostriatal DA system in ageing mice, in contrast to another PD-linked protein DJ-1, which has significant role only in the maintenance of DA cell bodies but not innervation of the Ret-deficient mice (Aron *et al*., 2010). Under trophic deprivation the functional role of

DJ-1 seems to be more restricted for cell body maintenance, whereas parkin has a more general function both in the maintenance of DA cell bodies and the striatal axonal terminals, which can be explained by differential functional properties of DJ-1 and parkin in mitochondrial maintenance and activation of survival signaling (reviewed in Exner *et al* 2012). These results also suggest that the nigrostriatal DA system of parkin-deficient mice mice is more sensitive to trophic insufficiency but not for the previously described mutant α-synuclein toxicity (von Coelln *et al.*, 2006) or MPTP or 6-OHDA intoxication (Perez *et al.*, 2005; Thomas *et al.*, 2007).

5.2.2 Parkin overexpression can complement for Ret loss in the nigrostriatal DA system of mice

In order to understand the direct influence of parkin on Ret signaling, we wanted to investigate the effect of parkin overexpression on degenerating nigrostriatal system of the aged Ret-deficient mice. To achieve this, we obtained two mouse lines from the lab of Konstanze Winklhofer at the LMU, Münich; which transgenically overexpressed either wildtype (wt h-parkin) or truncated human parkin (Δ h-parkin) under the mouse prion promoter (Structures of wt and Δ h-parkin are shown in the Figure 5.1). After characterizing the overexpression of human parkin (both wt and Δ h-parkin) in the midbrain DA neurons (Figure 4.14 and 4.15), we crossed the transgenic mice with conditional Ret-deficient mice (schematically shown in Figure 4.16 A and B). The human parkin overexpressing Ret-deficient mouse lines, DCB-Ret ko/wt h-parkin and DCB-Ret ko/Δ h-parkin are viable, showed normal development and are fertile. In order to investigate the function of parkin on the degenerating nigrostriatal system of Ret-deficient mice, we aged the mice until 12 months and then euthanized for DA system analysis, at this age Ret-deficient mice shows significant nigrostriatal degeneration (Figures 4.3-4.5; Kramer *et al.*, 2007).

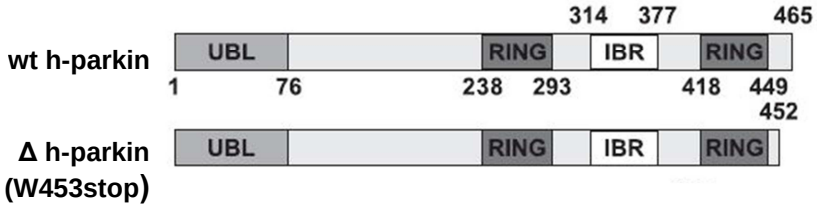

Figure 5.1 Structural differences in wt h-parkin and Δ h-parkin (with a W453stop mutation)

wt h-parkin is full-length form of human parkin, whereas the Δ h-parkin is a C-terminal truncation mutant which is 13 amino acids shorter than its wildtype counterpart.

At 12 months, the human parkin overexpressing mice (wt h-parkin and Δ h-parkin) showed normal number of midbrain TH positive (DA) neurons, whereas the Ret-deficient mice showed an 18% loss in the SNpc TH positive neurons (Figure 4.17 A and B) as observed earlier in this study (Figure 4.3 C) and by others (Kramer *et al.*, 2007). Interestingly, this loss has been prevented when wt h-parkin or Δ h-parkin is overexpressed in mice with Ret-deficient background, indicating neuroprotective function of parkin on the degenerating nigrostriatal DA system due to trophic insufficiency. Moreover these observations have been

confirmed after analyzing the GIRK2 positive subgroup of DA neurons (Figure 4.17 D and E).

Corresponding to the normal number of DA neurons in the midbrain region, the 12 month old human parkin overexpressing mice (wt h-parkin and Δ h-parkin) also showed no DA innervation loss, when immunofluorescently stained with TH and DAT antibodies (Figure 4.18). However, the DA innervation loss observed in the striatum of Ret-deficient mice (Figures 4.3-4.5; Kramer *et al*., 2007), has been significantly prevented when wt h-parkin but not Δ h-parkin is overexpressed in mice with Ret-deficient background (Figure 4.18). In accordance with these findings, the total striatal DA levels measured using HPLC ECD system also signifies normal striatal total DA levels when wt or Δ h-parkin is overexpressed in wildtype Ret background. However, when overexpressed in the Ret-deficient background it was only the wt h-parkin overexpression mice but not its Δ h-parkin counterpart which showed a nearly normal total DA levels (with p value = 0.07) (Figure 4.19 A). These discrepancies in the neuroprotective effect of wt and Δ h-parkin with respect to the DA innervation and total DA content in the striatum can be explained by the differential amounts of transgenic parkin protein detected in the two transgenic parkin lines (wt and truncated) (Figure 4.15). Even after C-terminal truncation (W453stop) all the functional domains of the parkin protein will be still intact (shown in the Figure 5.1); but, this truncation mutation was earlier shown to affect the solubility and localization of the protein (Winklhofer *et al*., 2003; Henn *et al*., 2005). Interestingly, truncated human parkin overexpression does not seem to be toxic to the cells as we did not observe any loss of SNpc DA neurons or striatal innervation in the Δ h-parkin mice compared to controls (Figures 4.17 and 4.18).

When I performed western blot on SN and striatum tissue lysates, I observed differences in the amount of parkin protein across the two transgenic mouse lines in the Triton X-100 soluble fractions, but not in the insoluble fractions (Figure 4.15). In the SN Triton X-100 soluble fraction, compared to endogenous mouse parkin, the wt h-parkin is nearly 6-fold and Δ h-parkin is 2.5-fold more which seemed be adequate to exert neuroprotective function in the DA cell bodies that lack Ret (Figures 4.15 A and B). In the striatal Triton X-100 soluble fraction wt h-parkin is 2.5-fold highly expressed, which appears to be sufficient for its neuroprotective effect unlike for Δ h-parkin which is just one-fold higher when compared to endogenous mouse parkin (Figures 4.5 E and F), and is insufficient to protect the Ret loss dependent DA fiber degeneration and total DA loss. Taken together, these observations reveal the presence of higher amounts of soluble parkin protein in the wt h-parkin overexpressing mice both in the SN and striatal regions when compared to the Δ h-parkin overexpressing mice.

Altogether these findings support the previous analogy of neuroprotective function of parkin overexpression which was demonstrated in several cellular and *in vivo* models. Parkin overexpression protected cells against ceramide induced mitochondrial swelling (Darios *et al*., 2003), kianic acid excitotoxicity (Staropoli *et al*., 2003), manganese induced cell death (Higashi *et al*., 2004) and dopaminergic toxicity (Jiang *et al*., 2004). Parkin overexpression also reduced α-synuclein toxicity *in vivo* in rat, Drosophila, and other cellular models (Petrucelli *et al.*, 2002; Yang *et al.*, 2003; Yamada *et al.*, 2005). Parkin overexpression also

reduced mutant LRRK2 mediated DA neuron toxicity in Drosophila (Ng *et al.*, 2009). In addition to its protective function against a variety of environmental/misfolded protein stressors, our findings show that parkin overexpression can also compensate for trophic insufficiency in SNpc DA neurons.

5.2.3 Parkin deletion can partly normalize the enlarged nigrostriatal DA system of MEN2B mice

After observing the synergistic effect of Ret and parkin on the nigrostriatal system of Ret/parkin double ko mice and complementary function of parkin when overexpressed in Ret-deficient mice. We further wanted to investigate the role of parkin in the establishment of enlarged DA system of the adult MEN2B mice. A constitutive active mutation in the intracellular domain of the Ret receptor (MEN2B) resulted in more number of DA neurons specifically in the SNpc region of the mouse midbrain, which most likely arouse due to post natal developmental role of the GDNF independent function of the Ret-MEN2B mutation (Mijatovic *et al*., 2007). The mutant Ret receptor in the nigrostriatal system of MEN2B mice also lead to an increase in the DA innervation and subsequent increase in the striatal levels of DA and its catabolites (DOPAC and HVA). Here, in this study we crossed the complete parkin ko mice (Itier *et al*., 2003) with MEN2B mice (Smith-Hicks *et al.*, 2000; Mijatovic *et al.*, 2007) to obtain the MEN2B/parkin ko double mutant mice (Figure 4.20) to investigate the effect of parkin deletion on the enlarged DA system. The MEN2B/parkin ko mice are viable and developed normally, whereas the male homozygous mutants are infertile for unknown reasons.

Compared to parkin-deficient mice mice (which showed normal number of DA neurons in the SNpc and VTA midbrain regions; Figure 4.3), the MEN2B mice showed a significant 21% increase in TH stained neurons specifically in the SNpc region but not in the VTA as observed previously (Mijatovic *et al.*, 2007). However in the MEN2B/parkin ko mice the TH positive numbers in the SNpc was significantly less by 12% when compared to MEN2B, whereas the VTA TH neuronal numbers remained unaltered across the genotypes. These observations were furthermore confirmed by analyzing GIRK2 positive neurons in the midbrain region (SNpc and VTA) of the parkin ko, MEN2B and MEN2B/parkin ko mice (Figure 4.21). Corresponding with the neuronal numbers in the SNpc, the DA fiber innervation in the dorsal striatum (stained with TH and DAT) was also significantly higher in the MEN2B mice (44% higher with TH and 55% higher with DAT) when compared with parkin ko, which showed normal fiber density (Figure 4.4). However in the MEN2B/parkin ko mice the DA innervation is slightly but significantly decreased (upto 10% less with TH and 11% less with DAT) when compared to MEN2B mice. We further investigated the consequences of these changes observed in the DA innervation on the striatal total DA levels and its catabolites. MEN2B mice showed a massive 150% increase in the total DA levels and along with a significant increase in its catabolites, HVA and DOPAC (Figure 4.23 A-C). However, parkin deletion could not alter the increased levels of DA and its catabolites in the MEN2B/parkin ko, but showed a significant recovery in the ratio of HVA/DA levels when compared to MEN2B mice (Figure 4.23 D). Interestingly, the MEN2B mutation which originally was reported to enhance the activity of Ret receptor, also led to increase in the

overall amount of Ret protein that can be detected immunofluorescently in the SNpc (Figure 4.24 A and B) and by western blot analysis in the striatum tissues (Figure 4.24 C and D). However, parkin deletion had no effect on the increased Ret protein levels (Figure 4.24 A-D).

These observations in the nigrostriatal DA system of the MEN2B/parkin ko mice suggest a specific role of parkin in the establishment of Ret-MEN2B mutation dependent nigrostriatal DA system enlargement, predominantly in the SNpc DA neuronal cell bodies. Parkin is considered to be neuroprotective and anti-apoptotic perhaps due to its influence on mitochondria and the p53/caspase activation pathway (reviewed in Exner *et al*., 2012). This might be the reason why MEN2B mice lose their excess number of DA neurons in the absence of parkin.

5.2.4 Role of Ret signaling and parkin activity in the neuronal and mitochondrial morphology of SNpc DA neurons

In addition to the physiological, behavioral and nigrostriatal DA neuronal maintenance function, we also demonstrate the influence of Ret signaling on the soma size of SNpc DA neurons. The GIRK2 subgroup of DA neurons in the SNpc of aged Ret-deficient mice was earlier shown to have significantly smaller soma size (Aron *et al*., 2010). However, in the current study we extend our findings to all TH expressing neurons as we observe a reduced neuronal soma size of the TH positive cells in the SNpc of aged and also in the adult Ret-deficient mice and Ret/parkin double-deficient mice (Figures 4.12 A and B). Supporting this notion we also observed larger soma size for the midbrain TH positive neurons in adult MEN2B mice (and also in MEN2B/parkin ko mice) with constitutive active mutation in the Ret receptor (Figures 4.24 E and F). Thus we demonstrate an important and rather generalized function of Ret signaling in DA neuronal development to attain normal soma size. As reported by others we did not observe any effect of parkin deletion on the soma size of DA neurons in adult and ageing mice (Goldberg *et al*., 2003; Figures 4.12 A and B).

To gain insights into the causes of neuronal death, we investigated mitochondrial morphology specifically in the SNpc DA neurons of aged Ret, parkin single and Ret/parkin double-deficient mice using immuno electron microscopy. Parkin, Ret single and double deficiency upon ageing did not lead to increased mitochondrial damage or change in the total mitochondrial number in the SNpc DA neurons (Figures 4.9 A-D). However, we discovered parkin dependent changes in the mitochondrial morphology, smaller mitochondrial populations (with less than 0.5 µm in size) are significantly higher in aged parkin single and Ret/parkin double-deficient mice compared to age matched control and Ret-deficient mice (Figure 4.9 E), a phenotype so far not reported for parkin (Palacino *et al.*, 2004; Stichel *et al.*, 2007; Schmidt *et al.*, 2011), but well documented for DJ-1 deficient mice (Irrcher *et al*., 2010). The smaller mitochondria in parkin knockout mice might be due to a reduced fusion or increased fission rate both tightly control by parkin (reviewed in Pilsl and Winklhofer, 2012; Corti and Brice, 2013). This is consistent with results from cultured cells and primary neurons with parkin knock down leading to robust increase in mitochondrial fragmentation (Pilsl and Winklhofer, 2012). Apart from the changes in mitochondrial morphology, I did not observe any increase in protein carbonylation (indication of oxidative stress; Figure 4.10) or changes

in the activation or total amount of key signaling molecules in the SN and striatal regions of aged (24 month old) parkin, Ret single and double ko mice (Figure 4.11). Moreover, neither in the adult single ko (parkin or Ret) nor in the double-deficient mice we found altered protein levels of the pro-fission proteins Drp1 or pro-fusion proteins Mfn1, Mfn2, or OPA1 (Figure 4.13 C). Though parkin was reported to promote the ubiquitin-dependent degradation of Drp1, Mfn1, and Mfn2 proteins and regulate OPA1 expression through ubiquitinating NF-κB essential modulator (NEMO) (Pilsl and Winklhofer, 2012; Corti and Brice, 2013; Müller-Rischart *et al.*, 2013). In addition the high number of non-dopaminergic neurons and other cells in our tissue lysates from the SN used for Western blotting might have masked small differences, if any. Alternatively, these observations raised a possibility that the most important intracellular events that could lead to neurodegeneration might be detectable during adulthood before the start of neurodegenerative process; hence we looked for early biochemical and physiological changes in adult mice that lack parkin and Ret.

5.2.5 Ret signaling and parkin activity is essential for the maintenance of physiological striatal DA levels, for cellular ATP levels and mitochondrial complex I activity in the SN of adult mice

Interestingly, Ret and parkin deletion together led to a significant decrease in the total striatal DA levels in mice already during adulthood (at 3-6 months) before any indications of nigrostriatal neurodegeneration. These changes can be seen exclusively in the Ret/parkin double ko mice but not in the single parkin or Ret-deficient mice (Figure 4.12 C). However, at this age (3-6 months) there were no alterations in the total levels of DA catabolites (DOPAC and HVA; Figure 4.12 D and E). Moreover the total protein levels of TH and DAT in the adult mice did not change, which otherwise could have explained the decreased total DA levels (Figure 4.2 C). The Ret/parkin double deletion (as reported by some studies for parkin; Palacino *et al.*, 2004; Stichel *et al.*, 2007) might alter energy production which could influence the activity of TH or other enzymes that can directly or indirectly influence striatal DA levels.

When investigated for changes in cellular energy levels and mitochondrial activity in the adult mice, I have observed a significant 20% reduction in cellular ATP levels (Figure 4.13 A), corresponding with a decrease in mitochondrial complex I activity by 20% specifically in the adult Ret/parkin double-deficient mice (Figure 4.13 B), but not in the age matched Ret and parkin single ko mice. However, no changes in the expression of mitochondrial NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10 (NUFA10), a component of the complex I of the electron transport chain of mitochondria were seen (Figure 4.12A). The changes observed with respect to total cellular ATP levels and complex I activity observed in mice have also been confirmed by cell culture experiments performed together with the lab of Konstanze Winklhofer at the LMU, Münich. The TH producing SH-SY5Y cells lacking Ret and parkin showed a decrease in total cellular ATP levels (Figure 5.2) and corresponding decrease in complex I activity (Figure 4.13 D) in the SH-SY5Y cells.

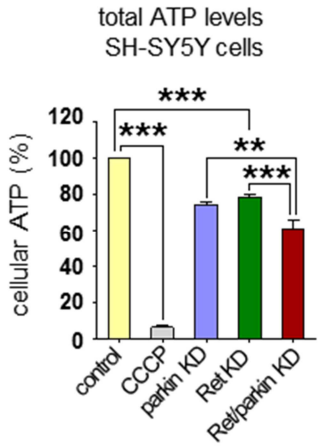

Figure 5.2 Ret signaling and parkin activity is essential for maintaining cellular ATP levels in SH-SY5Y cells (data from the lab of Konstanze Winklhofer; Meka *et al* unpublished data)

Graph represents the quantifications of cellular ATP levels (%) in the SH-SY5Y cells treated with control siRNA or mitochondrial uncoupler, CCCp or siRNAs to knock down parkin, Ret or both Ret and parkin as indicated. Data are represented as mean +/- SEM of four independent experiments; ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test.

However, these changes in total ATP levels and complex I activity were already reported for the single parkin-deficient mice cells (Pilsl and Winklhofer, 2012) and in this study we extend these findings also to cells that are only deficient for Ret. Furthermore, there was an enhanced reduction in the total cellular ATP levels in the Ret/parkin double deficient cells compared to cells that only lack Ret or parkin (Figure 5.2). The decreased cellular ATP levels and reduced complex I activity observed in the Ret-deficient SH-SY5Y cells and Ret/parkin double-deficient mice, for the first time reveal an important functional relevance of Ret with respect to cellular energy metabolism and mitochondrial function which could ultimately lead to age-dependent degeneration of nigrostriatal DA system.

Even though basic cellular model systems that lack parkin showed decreased mitochondrial membrane potential and ATP production along with reduced mitochondrial complex I activity (Pilsl and Winklhofer, 2012; Matsui *et al.*, 2013), similar to what has been observed in the SHSY5Y cells that lack parkin; in animal models these findings are rather discrepant, unlike in this study some earlier reports suggest decreased complex I activity already in single parkin-deficient mice mice (Palacino *et al.*, 2004; Stichel *et al.*, 2007). Moreover Palacino et al. (Palacino *et al.*, 2004) also showed increased protein carbonylation in single parkin ko mice, which was not seen in our parkin-deficient mice mouse lines (parkin ko and Ret/parkin double ko). However a recent study performed in medaka fish (Oryzias latipes) model, showed that parkin/PINK1 double deletion could only lead to mitochondrial complex I activity loss, but not parkin or PINK1 single deletion (Matsui *et al.*, 2013). These recent *in vivo* findings in medaka fish supports the requisite of an additional component deletion that is involved in the functional network to visualize mitochondrial abnormalities in parkin-deficient mice animals, similar to what has been observed in our Ret/parkin double-deficient mice (Figure 4.13 A and B). These *in vivo* and *in vitro* findings, for the first time reveal an important functional cooperation of Ret signaling and parkin activity in maintaining total

cellular ATP levels by preserving the complex I activity and thus might play a role in maintaining physiological striatal DA levels in adult mice and ultimately preventing age-dependent degeneration of nigrostriatal DA system observed in the Ret/parkin double-deficient mice. Decreased complex I activity observed in our animal and cellular models that lack Ret and parkin fits very well with the existing literature obtained from several toxin-induced (MPTP, rotenone) and genetic models (DJ-1 deficient mice) of PD and also with the data obtained from PD patients' brains (Mizuno *et al.*, 1989; Parker Jr. *et al.*, 1989; Bindoff *et al.*, 1991), supporting the notion that Ret could be one of those proteins whose function might be altered in PD.

5.3. Convergent signaling pathways of Ret signaling and parkin activity that determine mitochondrial morphology

5.3.1 GDNF/Ret signaling can prevent mitochondrial fragmentation phenotype in parkin-deficient mice SH-SY5Y cells

Cell culture experiments performed on SH-SY5Y cells in collaboration with the lab of Konstanze Winklhofer (at the LMU, Münich) strongly supports the functional interaction of Ret and parkin observed in our mouse models. As reported by other studies parkin knock down led to mitochondrial fragmentation in SH-SY5Y cells (shown in Figure 5.3 A and B). However, the fragmentation phenotype in parkin-deficient mice cells can be completely prevented by treating the cells with GDNF and soluble GFRα1 – which possibly signals via the Ret receptor (Figure 5.3 B and C). Moreover It was also shown that GDNF/GFRα1 treatment has no effect on parkin expression per se, suggesting that the beneficial effects of GDNF/GFRα1 are explicitly due to signaling changes, but not because of transcriptional activation of parkin expression (Figure 5.3 D).

Using specific inhibitors for PI3K, MEK1 and MEK1/2 signaling pathways it was shown that the GDNF/GFRα1/Ret signals via the PI3K pathway but not the MEK (or Ras-Raf-MEK-ERK) pathway (Figure 5.3 B and C). Furthermore, experimental evidences also suggest that the protective effect of GDNF/Ret signaling can be reverted by inhibiting the classical NF-κB signaling pathway with IκB super-repressor (IκBΔN) co-expression (Figure 5.3 E). Additionally it was also shown that the classical NF-κB signaling pathway activation by overexpressing IKKβ (NF-κB pathway activator) alone is enough to rescue the parkin dependent mitochondrial fragmentation phenotype (Figure 5.3 E).

The GDNF/Ret signaling particularly involving the soluble GFRα1 can activate PI3K signaling pathway but not the MEK (or Ras-Raf-MEK-ERK) pathway (reviewed in Airaksinen and Saarma 2002). It was also shown that GDNF/Ret can activate the NF-κB pathway in neuroblastoma cells through binding of the adaptor protein SHC on the phosphorylated Ret tyrosine 1062 recruiting GAB1 and activating PI3K-AKT pathway (Hayashi *et al.*, 2000). Consistent with all these previous findings, the GDNF/soluble GFRα1/Ret signals via the PI3K signaling to activate the classical NF-κB pathway in preventing the parkin deficiency dependent mitochondrial network alterations.

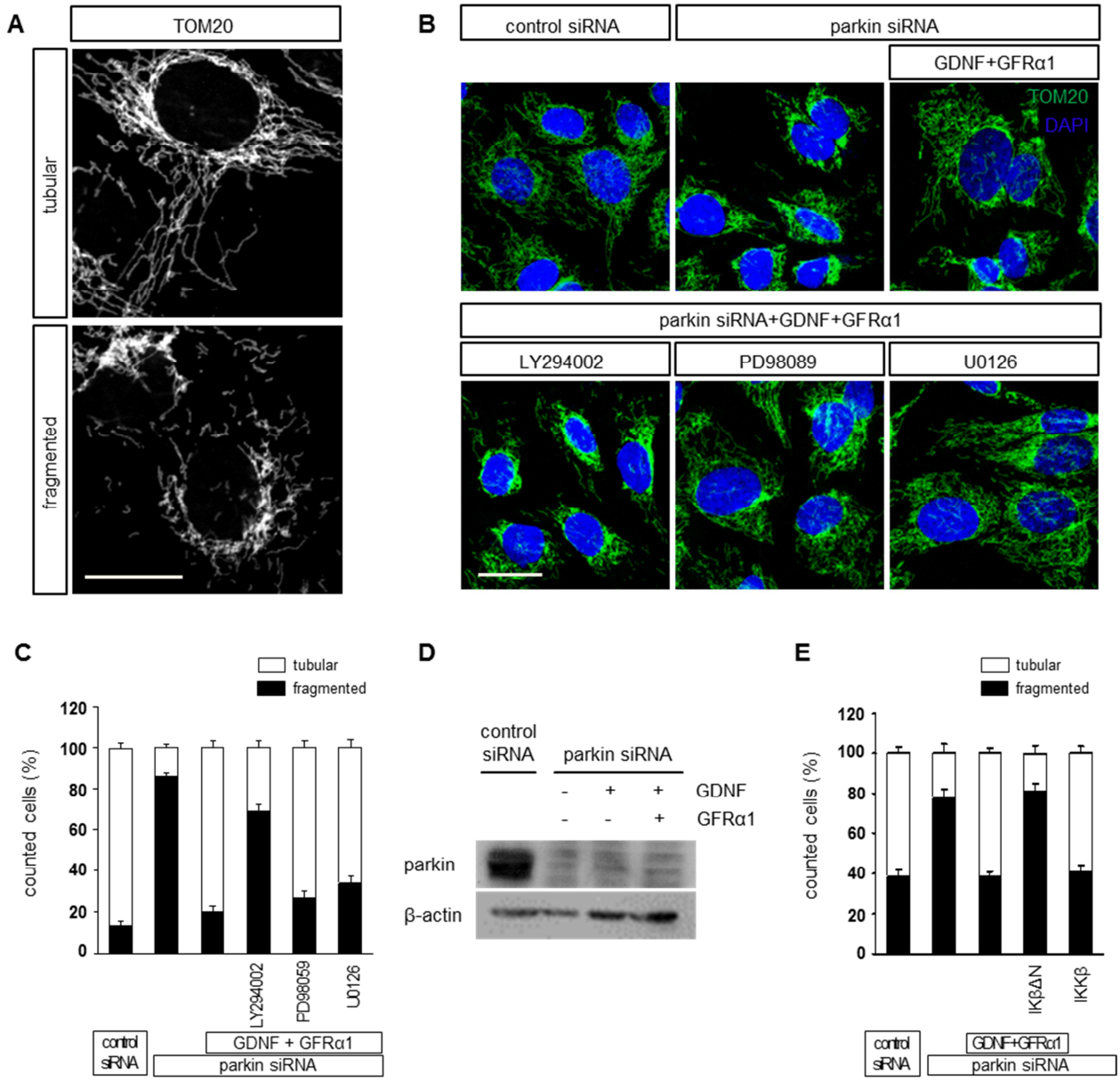

Figure 5.3 GDNF/GFRα1/Ret signaling can prevent parkin deficiency induced mitochondrial fragmentation (data from the lab of Konstanze Winklhofer; Meka *et al* unpublished data)

(A) Photomicrographs representing tubular (normal) and fragmented (damaged) mitochondrial network in SH-SY5Y cells immunofluorescently stained with TOM20 antibody. Scale bar = 10 µm.

(B) Representative photomicrographs of mitochondrial network immunofluorescently labeled with TOM20 antibody (green) and nuclei stained with DAPI (blue) in SH-SY5Y cells after treatment with control siRNA , parkin siRNA , parkin siRNA in combination with GDNF+GFRα1 alone and together with one of the compounds LY294002 (PI3K inhibitor), PD98059 (MEK1 inhibitor) and U0126 (MEK 1+2 inhibitor). Scale bar = 10 µm.

(C) Quantifications showing the effect of control siRNA, parkin siRNA, and parkin siRNA in combination with GDNF+GFRα1 alone and together with one of the compounds LY294002 (PI3K inhibitor) PD98059 (MEK1 inhibitor) and U0126 (MEK 1+2 inhibitor) on mitochondrial network; white bars represent SH-SY5Y cells with

tubular mitochondrial network whereas black bars represent cells with fragmented mitochondrial network. Data are represented as mean +/- SEM of at least four independent experiments.
(D) Western blot image showing the efficient knock down of parkin protein after parkin siRNA treatment alone or together with GDNF and GDNF+ GFRα1, but not in control siRNA treated SH-SY5Y cells; β-actin is used as a loading control.
(E) Quantifications showing the effect of control siRNA and parkin siRNA alone or together with GDNF+GFRα1 (with or without overexpressing IKβΔN, inhibitor of NF-κB signaling pathway) and IKKβ (constitutive activator of NF-κB signaling pathway) on mitochondrial network; white bars represent SH-SY5Y cells with tubular mitochondrial network whereas black bars represent cells with fragmented mitochondrial network. Data are represented as mean +/- SEM of at least four independent experiments.

5.3.2 Ret knock down induces mitochondrial network alterations in SH-SY5Y cells, which can be prevented by parkin overexpression

In addition to decrease in cellular ATP levels and reduced mitochondrial complex I activity, SH-SY5Y cells that lack Ret also exhibit mitochondrial network alterations. Ret knockdown resulted in 56% of cells with fragmented mitochondria and 20% of cells with atypically condensed mitochondria (Figure 5.3 C). The mitochondrial fragmentation phenotype seen in majority of the Ret-deficient cells is very much similar to what has been described for parkin, whereas the condensation phenotype was first time observed, in which the mitochondrial tubules are thinner, highly curly and concentrated around the nucleus (Figure 5.3 A and B). It could be that the condensed state is an intermediate form of mitochondria before they are fragmented or a completely different phenotype.

The Ret deficiency dependent mitochondrial phenotypes (both fragmentation and condensation) can be prevented by overexpressing parkin as well as Ret but not by the kinase dead form of Ret (Ret KiD). These findings for the first time reveal an important role of Ret signaling in the maintenance of mitochondrial structure and function. The redundant functions of Ret and parkin shown here are in agreement with the findings obtained from our parkin overexpressing Ret-deficient mice (Figure 5.3 C), where our results demonstrated the neuroprotective function of parkin on the degenerating nigrostriatal system of aged Ret-deficient mice.

Inhibiting the classical NF-κB pathway (by overexpressing IkβΔN) did not alter the Ret deficiency dependent mitochondrial phenotypes, implying that Ret signaling is upstream to the NF-κB signaling pathway. However, wt PI3K overexpression significantly reduced the severity of Ret loss dependent mitochondrial network alterations which again are reverted by blocking the NF-κB pathway (by co-expressing IkβΔN). Overexpression of constitutive active PI3K (PI3K myr) had a better protective effect on the Ret loss dependent mitochondrial phenotype than wt PI3K overexpression. However, the effect of PI3K myr overexpression was not further enhanced due to the addition of GDNF/GFRα1, implying that PI3K signaling is the predominant downstream target of GDNF/GFRα1/Ret signaling, which then activates the classical NF-κB signaling to mediate the mitochondrial integrity. Furthermore, overexpression of a constutive active mutant of the Ret receptor (MEN2A) which can signal independent of GDNF also reduced the severity of Ret loss dependent mitochondrial network alterations, with a mild beneficial effect when GNDF/GFRα1 is additionally provided (Figure 5.4 E).

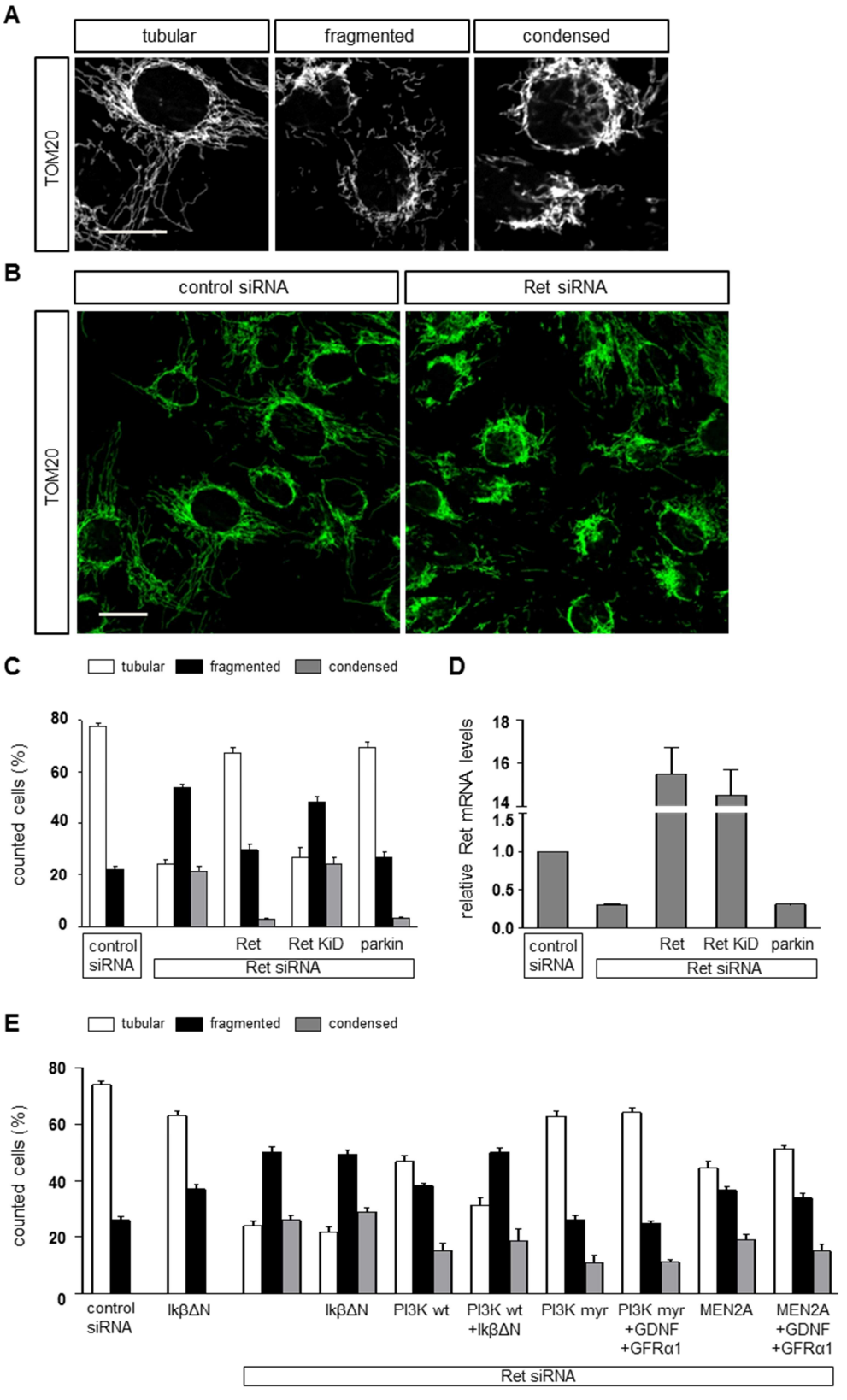

Figure 5.4 Ret knock down induces mitochondrial network alterations in SH-SY5Y cells
(data from the lab of Konstanze Winklhofer; Meka *et al* unpublished data)

(A) Photomicrographs representing tubular (normal), fragmented (damaged) and condensed (damaged) mitochondrial network in SH-SY5Y cells immunofluorescently stained with TOM20 antibody. Scale bar = 10 µm.

(B) Representative photomicrographs of mitochondrial network in SH-SY5Y cells immunofluorescently labeled with TOM20 after treatment with control and Ret siRNA. Scale bar = 10 µm.
(C) Quantifications showing the effect of control siRNA and Ret siRNA in combination with constructs overexpressing Ret, Ret kinase dead domain (Ret KiD) and parkin on mitochondrial network; white bars represent SH-SY5Y cells with tubular mitochondrial network whereas black and grey bars represent cells with fragmented and condensed mitochondrial network respectively. Data are represented as mean +/- SEM of three independent experiments.
(D) Relative Ret mRNA levels quantified by RT-PCR for the samples used in Figure 5.4 C.
(E) Quantifications showing the effect of overexpression of different constructs (as indicated) on mitochondrial network alterations induced due to Ret knock down in SH-SY5Y cells; white bars represent SH-SY5Y cells with tubular mitochondrial network whereas grey and black bars represent cells with condensed and fragmented mitochondrial network respectively. Data are represented as mean +/- SEM of three independent experiments.

Altogether, these findings suggest a direct role of Ret signaling on mitochondrial network integrity and function in the TH expressing SH-SY5Y cells, signaled via the PI3K/NF-κB pathway.

5.3.3 Activation of NF-κB pathway, the common downstream effect of GDNF/Ret signaling and parkin activity to promote mitochondrial integrity and thus cell survival

Recently, it was described that GDNF/Ret can activate the NF-κB pathway in neuroblastoma cells through binding of the adaptor protein SHC on the phosphorylated Ret tyrosine 1062 recruiting GAB1 and activating PI3K-AKT signaling (Hayashi *et al.*, 2000). The PI3K/NF-κB pathway and the preservation of mitochondrial function is an important additional pathway besides activating the transcription factor CREB by the Ras/ERK pathway. For CREB activation, SHC is also recruited to the phosphorylated Ret tyrosine 1062 but binds to Grb2 and SOS (Hayashi *et al.*, 2000). Moreover, Ret tyrosine 1062 signaling though the NF-κB pathway is also considered essential to ensure the development and maintenance of the enteric nervous system (ENS) and the inner ear neurons in mammals, therefore this pathway is also essential to prevent Hirschsprung's disease (megacolon disease) and congenital hearing loss (Ohgami *et al.*, 2012). Constitutive active Ret mutations, such as the MEN2B mutation, account for most of the inherited medullary thyroid cancer incidents (Wells *et al.*, 2013) and NF-κB signaling might contribute to the neoplastic transformation and anti-apoptotic function of these cells (Pacifico and Leonardi, 2010), It seems reasonable to speculate that carcinogenic Ret enhances also in these cells the mitochondrial activity and thereby supports the malignancy of these cancer cells. In this context it was suggested that oncogenic Ret stimulates the NF-κB pathway not via the PI3K pathway but by the Ras/Raf/MEK1 pathway (Ludwig *et al.*, 2001). Wildtype and mutant Ret might use preferentially different signaling pathways to alter mitochondria function which might allow on one hand to develop new drugs to specifically interfere with disease-causing Ret signaling events or on the other hand stimulate healthy Ret signaling pathways.

On the other hand, previous studies from the lab of Konstanze Winklhofer have identified a stress-protective pathway regulated by parkin that links NF-κB signaling and mitochondrial integrity (Henn *et al.*, 2007; Müller-Rischart *et al.*, 2013). Inhibition of NF-κB pathway activation by an IκB super-repressor (IκBΔN) or a kinase-inactive IKKβ interferes with the

neuroprotective activity of parkin (Henn *et al.*, 2007). Moreover, pathogenic parkin mutants with an impaired neuroprotective capacity show a reduced ability to stimulate NF-κB-dependent transcription (Henn *et al.*, 2007). They have demonstrated parkin mediated linear (k63 linked) ubiquitination of NF-κB essential modulator (NEMO), which is essential for canonical NF-κB signaling. Subsequently, the mitochondrial guanosine triphosphatase OPA1 is transcriptionally upregulated via NF-κB-responsive promoter elements for maintenance of mitochondrial integrity and protection from stress-induced cell death. However, parkin-dependent stress protection is lost in the absence of either NEMO or OPA1, but not in cells defective for the mitophagy pathway. Notably, in parkin-deficient cells linear ubiquitination of NEMO, activation of NF-κB, and upregulation of OPA1 are significantly reduced in response to TNF-α stimulation, supporting the physiological relevance of parkin in regulating the NF-κB pathway for mitochondrial integrity and cell survival.

The newly discovered mitochondrial maintenance function of GDNF/Ret signaling in SH-SY5Y cells has been also shown to signal via the PI3K pathway to finally activate the classical NF-κB pathway (Meka *et al*., unpublished data), which is also a downstream target of parkin signaling for maintaining mitochondrial integrity (Henn *et al*., 2007; 2013) (Figure 5.5).

Taken together, the results from cell culture experiments complement my observations from different mouse lines that indicate *in vivo* functional cell survival functions of Ret and parkin. Since knockdown of Ret in cells alone already changes the mitochondrial morphology, complex I activity and ATP levels significantly like parkin or PINK1, suggesting an essential function of Ret to prevent mitochondrial inactivation. Thus, Ret deletion seems to have similar effects on mitochondria like the ionophore CCCP, uncoupling the proton gradient generated by the electron transport chain or the complex I inhibitors rotenone, paraquate, 6-hydroxy-dopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the last two also causing selectively DA neuron death with a PD like syndrome in animals and humans. This supports the idea that the selective age-dependent degeneration of DA neurons of the SNpc accompanied by decreased striatal fiber innervation and total DA levels, behavioral alterations in the Ret-deficient mice makes them an exciting genetic model to study the etiologies and pathogenesis of PD and especially defects in bioenergetics and mitochondrial quality control, but also oxidative stress and protein misfolding and aggregation.

The synergistic effect of Ret and parkin double deletion on the nigrostriatal DA system of aged mice, can be explained by the mitochondrial maintenance function of Ret and parkin (shown in mice and cell culture) which might be mediated by the NF-κB pathway (shown in cell culture). These observations also demonstrate that Ret signaling and parkin activity together are essential for proper energy metabolism and to maintain normal mitochondrial function contributing to appropriate cellular physiology and morphology of the SNpc DA neurons in adult mice, which would otherwise lead to a progressive and age-dependent degeneration. Our results here might be explained by a moderate level of cellular stress in our mice leading to the slow progressive neurodegeneration phenotype during aging and unlike the chronic stress paradigms which are normally employed in cell culture experiment which

results in high stress conditions in almost all the cells at the same time leading to a non-biological situation (Pilsl and Winklhofer, 2012; Corti and Brice, 2013). Data from the parkin overexpressing Ret-deficient mice uncovers redundant functions of Ret and parkin in the maintenance of nigrostriatal DA system, which fits very well with the cell culture data showing the complementary functions of Ret and parkin in maintaining mitochondrial integrity involving the NF-κB pathway. These findings also extend the neuroprotective properties of parkin in a new direction, which is further demonstrated in neurons with trophic insufficiency. Finally, the parkin-deficient mice MEN2B mice strengthens the possible functional interaction of Ret and parkin is observed also during the establishment of nigrostriatal DA system, in which parkin deficiency normalized the enlarged nigrostriatal DA system of MEN2B mice. Taking the facts from cell culture experiments, it can be inferred that the signaling effects of the constitutive active Ret receptor in the MEN2B mice (which resulted in more DA neurons) can be moderately diminished by parkin deletion in the MEN2B/parkin ko mice which ultimately resulted in the DA system normalization.

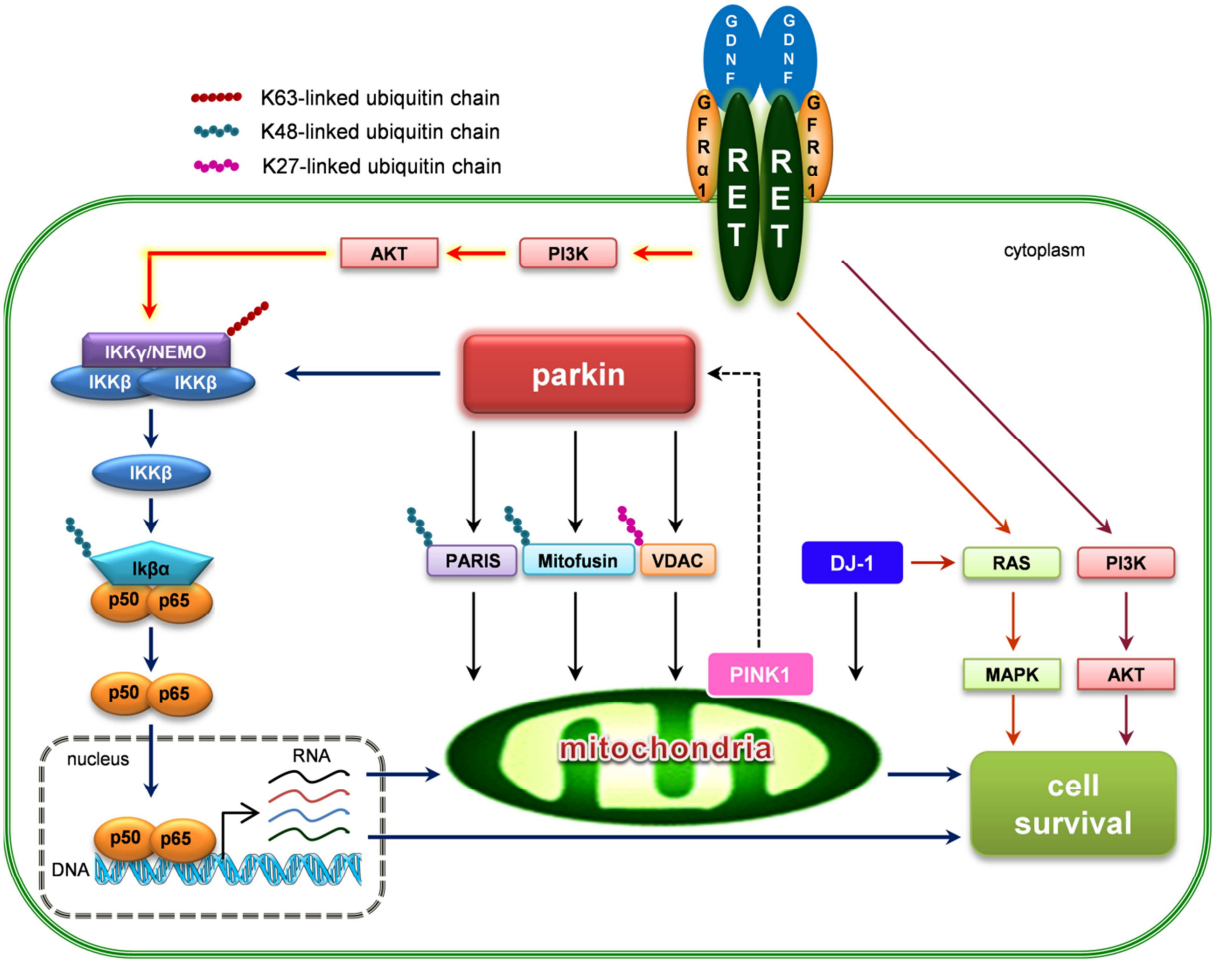

Figure 5.5 Downstream signaling events of Ret and parkin in SNpc DA neurons (only major ones are outlined)

i) Cell survival pathways of PD-linked proteins parkin, PINK1 and DJ-1

Under mitochondrial depolarization (a severe stress condition), PINK1 which is stabilized on the mitochondrial membrane recruits parkin to the damaged mitochondria by unclear mechanism. Upon recruitment parkin prevents the fusion of damaged mitochondrial tubules by marking mitofusins - Mfn1 and Mfn2 (through attaching K48 linked polyubiquitin chains) for proteasomal degradation (Gegg *et al.*, 2010; Glauser *et al.*, 2011); subsequently, parkin attaches K27 linked polyubiquitin chains to VDACs (VDAC 1 or 3) to the isolated damaged mitochondrial tubules (Geisler *et al.*, 2010; Sun *et al.*, 2012). The K27 linked polyubiquitin chains attached to the VDACs serves as signals for the binding of autosomal adaptor proteins like p62, which initiates the formation of phagophore around the damaged mitochondrial tubules to activate mitophagy (mitochondrial autophagy) (reviewed in Exner *et al*., 2012). In a PINK1 independent manner, parkin targets the mitochondrial biogenesis repressor protein PARIS for proteasomal degradation (by attaching K48 linked polyubiquitin chains) and thus initiates mitochondrial biogenesis to regulate mitochondrial homeostasis (Shin *et al.*, 2011). Under mild cellular stress conditions, parkin can also activate the classical NF-κB signaling. Parkin can attach K63 linked polyubiquitin chains to NEMO, which then lead to the activation of IKKβ which in turn marks Ikβα for proteasomal degradation, finally releasing the p50/p65 complex which can translocate to the nucleus to initiate transcription of genes (like OPA1) to maintain mitochondrial integrity and to initiate anti-apoptotic signaling pathways (Müller-Rischart *et al.*, 2013). Another protein DJ-1 can also protect mitochondrial integrity by a parkin/PINK1 independent mechanism (reviewed in Exner *et al*., 2012).

ii) GDNF/Ret cell survival signaling together with DJ-1 and parkin

After the formation of the GDNF/GFRα1/Ret hetero multimeric complex, the critical tyrosine residues in the intracellular domain of the Ret receptor are phosphorylated, which subsequently activates the downstream signaling pathways such as the PI3K-AKT or the RAS-MAPK pathways, which ultimately lead to cell survival (reviewed in Airaksinen and Saarma, 2002). The PD-linked protein, DJ-1 has been shown to functional interact with GDNF/Ret signaling to activate the RAS-MAPK pathway to mediate cell survival (Aron *et al*., 2011). The current study, taking the support of cell culture data (from the lab of Konstanze Winklhofer) suggests that GDNF/Ret signaling and parkin together role in maintaining mitochondrial integrity. Upon activation, GDNF/Ret signaling acts via the PI3K-AKT signaling to activate the downstream NF-κB pathway, which is also a target of parkin activity to induce several genes that are important for mitochondrial structure and function (Meka *et al*., unpublished data).

5.4 Functional cooperation of Ret with PD-linked proteins, namely parkin and DJ-1 to prevent DA neurodegeneration in mice

PD is a sporadically occurring neurodegenerative disorder; the potential risk factors associated with the onset of the disease include genetic predisposition, environmental factors and ageing. Remarkable progress has been made in understanding the disease after the identification of PD-linked genes. Monogenic PD causing genes exhibits autosomal dominant (AD) or autosomal recessive (AR) form of inheritance; mutations in α-synuclein and LRRK2 causes autosomal dominant PD, whereas parkin, DJ-1 and PINK1 are examples of autosomal recessive forms of the disease (more putative PD-linked genes are known). Recent genome-wide association studies (GWAS) have confirmed that α-synuclein and leucine-rich repeat kinase 2 (LRRK2) are linked to PD and microtubule-associated protein tau (MAPT) gene loci was identified as risk a factor (Simón-Sánchez *et al.*, 2009). However, the penetrance of these PD causing genes is often incomplete; stating that not everyone who carries the PD-associated gene mutation develop the disease. This genetic variation suggests a possible interplay of genetic, epigenetic or environmental factors that can ultimately leads to the disease development (reviewed by Farrer, 2006). Genetic rodent models have made a significant contribution to the understanding of physiological function of PD associated proteins namely

α-synuclein, parkin, PINK1, DJ1 and LRRK2. However, none of these models could recapitulate the typical characteristics of PD pathology. But *in vivo* and *in vitro* studies revealed that these PD-associated genes play important roles in cellular functions, such as mitochondrial functions, ubiquitin-proteasomal system, autophagy-lysosomal pathway and membrane trafficking (reviewed by Corti *et al*., 2011; Dawson *et al*., 2010; Farrer, 2006; Martin *et al*., 2011; Shulman *et al*., 2011).

On the other hand, a recent study discovered that mice which have genetically lost the expression of the canonical receptor for glial cell line-derived neurotrophic receptor (GDNF), the receptor tyrosine kinase Ret, showed an age- and cell type-specific loss of SNpc DA neurons reminiscent to the histological alterations observed in PD patients (Kramer *et al.*, 2007). Data from GDNF deficient mice support the essential function of GDNF/Ret signaling for maintaining the DA system (Pascual *et al*., 2008). In addition, mice carrying the constitutive active MEN2B mutation in their Ret gene were shown to maintain more DA neurons in the SNpc and DA innervations in the striatum than wildtype mice (Mijatovic *et al.*, 2007). These findings are surprising considering the fact that there are no familiar PD cases reported so far with mutations in neurotrophic factors or receptors (Wirdefeldt *et al.*, 2003; Lücking *et al.*, 2010). However, reports suggests that infusion of exogenous GDNF into respective brain regions can prevent neurotoxin-induced damage of midbrain DA neurons in PD animal models (Tomac *et al.*, 1995a, 1995b). The positive effects of GDNF in animal models of PD encouraged the initiation of several clinical trials, though the reasons for inconsistent outcome is still debated (Lang *et al.*, 2006; Manfredsson *et al.*, 2009). These observations suggests that defects in GDNF/Ret signaling are not a primary cause of PD, but a secondary consequence in the PD pathogenesis in which Ret is one of the many proteins that are altered. To get more insights on Ret signaling and to investigate its role in altered protein network of PD, a study by Aron *et al*., 2010 investigated the functional interaction of Ret and a PD-related protein DJ-1. In that study they showed that mice lacking both DJ-1 and Ret in the DA system upon ageing display an accelerated loss of SNpc DA neurons without any enhanced striatal innervation loss compared to the single Ret-deficient mice. DJ-I/Ret double loss-of-function experiments revealed interaction of DJ-1 with ERK signaling to control eye and wing development in Drosophila (Aron *et al.*, 2010). Furthermore, in the current study I report a functional interaction between Ret and parkin shown in three different genetic mouse models. Moreover cell culture experiments performed in collaboration with the lab of Konstanze Winklhofer provided more mechanistic insights about the Ret/parkin functional interaction and their contribution to energy metabolism, mitochondrial function and morphology, which is shown to be mediated by the NF-κB pathway (Meka *et al* unpublished data).

The *in vivo* cell survival functions of parkin and DJ-1 is only revealed in the absence of the neurotrophic GDNF/Ret signaling. This might be explained by compensatory mechanisms in the classical knockout mice that keep up the survival signal in DA neurons. Upon deletion of Parkin or DJ-1 in addition to the Ret receptor, the signaling intensity of survival pathways like PI3K/NF-κB and Ras/ERK are lowered below a critical threshold which eventually leads to enhanced neurodegeneration. DJ-1 and parkin are both considered to be neuroprotective and anti-apoptotic perhaps due to their influence on mitochondria and the p53/caspase activation

pathway (reviewed in Exner *et al*., 2012).This might also be the reason why the MEN2B mice in the absence of parkin lose their excess number of SNpc DA neurons.

Interestingly, in the Ret-deficient background DJ-1 seems only to be essential for cell body survival (Aron *et al.*, 2010), while we found here that parkin is important for cell body and axonal maintenance. Proper mitochondrial function is essential for the neuronal cell body and the axon to provide enough energy for their proper functioning. Both, parkin and DJ-1 have been shown to protect mitochondria from toxic insults and ensure proper mitochondrial morphology and membrane potentiation although by parallel signaling pathways (Exner *et al*., 2012). Interestingly, the mitochondrial fragmentation and depolarization in DJ-1 deficient cells can be rescued by parkin and PINK1 overexpression, but DJ-1 cannot prevent the parkin or PINK1 deficient phenotype (reviewed in Exner *et al*., 2012). Parkin, but not DJ-1, was shown to promote mitochondrial biogenesis by facilitating the degradation of the transcriptional repressor PARIS (parkin interacting substrate), which inhibits the expression of the peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), involved in the upstream control of mitochondrial biogenesis (Shin *et al.*, 2011). Perhaps most important, parkin and PINK1, but not DJ-1, have been recently implicated in autophagic clearance of mitochondria with poor membrane potential and axonal transport of mitochondria by regulating the degradation of the outer mitochondrial membrane Rho-like GTPase Miro, leading to the release of mitochondria from the adaptor protein Milton connecting them via kinesin heavy chain to microtubules (reviewed in Schwarz, 2013). The function of parkin and PINK1 to control kinesin-dependent anterograde and dynein-dependent retrograde movements of mitochondria might be important to ensure a healthy pool of mitochondria in the axons essential for axonal maintenance. Under physiological conditions the mitochondrial transport in parkin-deficient mice seems not altered but under conditions of mitochondrial damage the parkin-controlled mitochondrial transport mechanisms become essential (reviewed in Schwarz, 2013). Under neurotrophic depletion in Ret-deficient mice the observed changes of mitochondria might trigger the switch to the parkin-dependent mitochondrial transport and allows revealing its essential function for axonal maintenance.

The enhanced DA cell and fiber loss and mitochondrial dysfunction in the Ret/parkin double-deficient mice has most likely a wide spread effect on mouse brain physiology and behavior. The reduced amount of energy and DA terminals can explain the lower level of dopamine in the striatum (Figure 2) and dopamine release and re-uptake. High ATP is required in DA neurons for example for the vesicular monoamine transporter VMAT2 responsible for the dopamine uptake into presynaptic vesicles to decrease the toxic oxidation-prone cytosolic dopamine and for removing the toxic amounts of calcium from the cytosol into the endoplasmic recticulum by high-affinity ATP-dependant transporters. The calcium is getting into the cell by the sustained pacemaking activity of the L-type Cav1.3 calcium channels in adult mice (Pilsl and Winklhofer, 2012). Therefore, reduced ATP levels might alter the total DA network which control many behaviors such as movement, memory, motivation and emotions (Björklund and Dunnett, 2007; Leknes and Tracey, 2008). Interestingly we could detect first changes in motor and emotional behavior in our DCB-Ret ko and DCB-Ret/parkin double ko mice consistent with the neurodegeneration phenotype (Aron *et al*., 2010; Kramer *et al*., 2007).

There is still the need for clinically relevant animal models showing significant degeneration and a related Parkinsonian phenotype including motor and non-motor deficits (Meissner *et al.*, 2011). Having shown the neuroprotective effect of overexpressed parkin on the DA neuron degeneration in Ret knockout mice this is a good indication that the Ret-deficient mice are a reasonable model to test new protective strategies against PD. However, the Ret/parkin double knockout mouse model might be preferred to investigate the molecular mechanisms involved in neurodegeneration and protection because of the enhanced neurodegeneration phenotype and the observed behavioral changes which can be used as an additional readout.

The presented data raises the question if Ret also cross talks with other genes found to be mutated in familiar forms of PD. Answering this question to some extent, in an ongoing study in our lab, Karsten Tillack is investigating the consequences of toxic α-synuclein overexpression in midbrain DA neurons with trophic insufficiency in the double mutant mice (Ret ko/synA53T), which is generated by crossing the conditional synA53T mice (which specifically overexpress human mutant A53T α-synuclein in midbrain DA neurons) with conditional Ret ko mice. However there were no obvious morphologic differences or increase in the α-synuclein accumulation in the Ret ko/synA53T mice at 12 months compared to single mutant mice. However, all mutant and double mutant mice showed comparably reduced levels of DA in the striatum similar to what has been described in previous studies of Ret single deficient mice and α-synuclein overexpressing mice (Tofaris *et al.*, 2006; Kramer *et al.*, 2007; Daher *et al.*, 2009; Lin *et al.*, 2012). Moreover, there was an additional increase of DA metabolites in the striatum of synA53T and Ret ko/synA53T mice, which suggests an unknown role of α-synuclein in DA turnover. There was a clear tendency of DA fiber loss and cell loss in synA53T expressing mice which was even more significant in Ret ko/synA53T double mutant mice. Interestingly, the DA neurons in the VTA were also more severely affected in the Ret ko/synA53T double mutant mice compared to the synA53T single mutant mice, although VTA neurons do not degenerate in PD and have been found to be insensitive to α-synuclein toxicity in previous animal models (Maingay *et al.*, 2006). An even more drastic change in the 24 month Ret ko/synA53T mice can be expected when compared to the existing data from the 12 month old mice, since ageing contributes to progressive phenotype. However, none of the previous studies performed by crossing α-synuclein mutant mice and knockout mice of other PD-related proteins, like parkin (von Coelln *et al.*, 2006; Stichel *et al.*, 2007) and DJ-1 (Ramsey *et al.*, 2010) resulted in a pronounced loss of DA neurons. Altogether, these results suggest a rather general susceptibility of Ret-deficient midbrain DA neurons to α-synuclein toxicity. It is also important to note that some additional signaling events in the VTA DA neurons that are triggered due to α-synuclein toxicity makes them more vulnerable to degeneration in the absence of Ret, which suggests a novel neuroprotective function of the Ret receptor in the midbrain DA neurons against α-synuclein proteotoxicity. However, it requires further investigation to understand the underlying mechanisms causing the enhanced midbrain DA neuronal death in the Ret ko/synA53T mice, and whether it is the PI3K/NF-κB pathway or the RAS-MAPK pathway that is involved in this context. Furthermore, investigating the downstream signaling effects of NCAM and integrins (alternate GDNF receptors) on mitochondrial integrity and cell survival pathways would uncover new therapeutic possibilities or even lead to combinatorial approaches to enhance DA cell survival.

Although in a different context, it is worthwhile investigating the biochemical interaction between parkin, Ret and Eps15 keeping in view a previous study that is published explaining the role of parkin in regulating EGF receptor internalization together with Eps15 (Fallon *et al.*, 2006). Even though it was shown that the presence of parkin can enhance the intracellular survival signaling of EGFR by delaying its internalization, there is not much known about the importance of EGFR signaling in the development and maintenance of the DA system. Considering the facts that all receptor tyrosine kinases use the same cellular machinery for signaling and internalization and having known about the essential functions of Ret signaling in DA system maintenance (Kramer *et al.*, 2007) it is important to investigate the role of parkin in Ret receptor internalization and subsequent downstream signaling together with alternate GDNF receptors like NCAM and integrins.

In further studies with other PD-related proteins, what can we expect for example from Ret/PINK1 double-deficient mice? Since PINK1 is considered upstream of parkin concerning mitochondrial fission and fusion dynamics, transport, and mitophagy - we can expect a similar phenotype in Ret/PINK1 double-deficient mice as reported here for the Ret/parkin mice. But since there are a few functions of parkin not found for PINK1, such as preventing cytochome c release induced by proapoptotic BH3 domains (Pilsl and Winklhofer, 2012), these mice are still worthwhile to be generated and analyzed to investigate parkin independent functions of PINK1 which might show up under trophic insufficiency.

In conclusion, all these findings suggest that, the Ret signaling pathway is an essential part of the protein network altered in PD patients, functionally in close vicinity to parkin and DJ-1 and also has the capacity to modulate toxic effects of mutant α-synuclein. Therefore Ret can be suggested as one of the possible targets of the multiple hit hypotheses leading to PD. The tight cross talk of Ret with parkin to prevent mitochondrial defects in DA neurons provides new information how the neurotrophic signaling of Ret mediates maintenance of cell bodies and axons. Because of the close link of Ret to many diseases the Ret/PI3K/NF-κB/mitochondria link might shed new light on the molecular mechanisms leading not only to DA system related diseases such as PD, but also cancer, Hirschsprung's disease and hearing loss. Altogether, these findings opened up new areas of research and the present need to investigate the function of Ret together with other key proteins which are implicated in PD to design new therapies as well as to provide customized therapies for PD patients with specific gene mutations to increase the efficacy of compounds like GDNF and its family members, which are currently under clinical trials.

Supplementary Information

S.1 Introduction

S.A. Parkinson's Disease: Genetics and Pathogenesis

S.1.1 History of PD

Parkinson's disease (PD) was described in Ayurveda, the ancient Indian medical system under the name Kampavata, as early as 5000 BC. In Western medical literature, PD was illustrated by the famous physician Galen as "shaking palsy" in 175 AD. However a detailed medical essay was published on the disease by a London doctor James Parkinson in 1817 entitled "An Essay on the Shaking Palsy". This essay established ‘shaking palsy’ as a recognized medical condition. Jean Martin Charcot, a French neurologist was the first to truly identify the importance of Parkinson's work and named the disease after him. Spherical cytoplasmic inclusions in PD patients’ brains were first identified by Frederic Lewy in 1912 which are later named as Lewy bodies. Despite a lot of progress in understanding the disease, much of it remains a mystery. The chemical differences in the brains of PD patients were identified in the 1960s. A Swedish pharmacologist, Arvid Carlsson for the first time described DA itself as a neurotransmitter, which was until then thought just as a precursor for norepinephrine (NE). Further extending his findings, Carlsson (in 1957) could further elucidate the regulatory role of DA in motor behavior. In his candid experiment, Carlson treated the animals with reserpine, which potentially decreases the DA levels in the brain and thus leads to motor impairments. By subsequent administration of L-DOPA (L-3,4-dihydroxyphenylalanine), a DA precursor, he could completely restore the impaired motor behavior in the reserpine injected animals. Subsequently, DA deficiency is considered the cause of PD symptoms; but the underlying mechanisms causing the death of the DA producing neurons in specific brain regions remained largely unknown. Discovery of DA insufficiency in PD patients lead to the first effective medical treatment of the disease with L-DOPA. L-DOPA (or) Levodopa a precursor of DA which can cross the blood brain barrier. L-DOPA was first administered in 1967 to treat the disease symptoms; barring its side effects, this drug still remains as the "gold standard" in PD medication. In the ancient times, the Mucuna pruriens plant extract was used to treat PD symptoms, and was later learned to contain L-DOPA (Katzenschlager *et al.*, 2004).

S.1.2 Symptoms of PD

PD symptoms can be broadly classified as motor and non-motor symptoms. The type of symptoms and the rate of disease progression may differ significantly among the patients.

S.1.2.1 Motor Symptoms

These are the "cardinal" symptoms of PD, which are visible from outside. They include: bradykinesia (slowness of movement), rigidity, resting tremor, postural instability and other physical symptoms may include gait problems and reduced facial expression resulting in a mask like face. These symptoms are mainly due to the loss of SNpc DA neurons and subsequent reduction of striatal DA levels. They can be controlled or reduced by administering DA and its agonists.

S.1.2.2 Non-motor Symptoms

These symptoms are DA-non-responsive and can have a drastic impact on patients. They include: cognitive impairment, depression and anxiety, sleep difficulties, hyposmia (loss of sense of smell), constipation, speech and swallowing problems. These symptoms are related to the degeneration of non-DA neurons including the serotoninergic neurons of the raphe nucleus, noradrenergic neurons of the locus cerulus (LC), or cholinergic neurons of the nucleus basalis of Meynert.

S.1.3 Neuropathological staging (or Braak staging) of PD

Braak, with his coworkers proposed a neuropathological staging hypothesis for PD (Braak *et al.*, 2003). The classification of disease stages was based on the presence of lewy body pathology. According to this staging hypothesis, the standard PD nigral pathology occurs in the midway (see Figure S.1.1). Whereas, the Lewy bodies seem to appear first in the olfactory bulb, medulla oblongata and pontine tegmentum, individuals in any of these stages are clinically asymptomatic. With disease progression, Lewy bodies start to appear in the SN, then in the midbrain areas and in the basal forebrain before they finally appear in the neocortex. The pathological stages for PD, described by Braak share similarities to that found in LBD described by Kosaka (reviewed in Kosaka & Iseki, 1996). According to Braak stages, Lewy body pathology precedes neuronal loss by at least one or more stages.

If Braak stages are to be believed then, non-motor and non-DA symptoms should appear prior to the ‘cardinal’ motor symptoms of PD. Due to the early involvement of olfactory bulb and brain stem one would expect olfactory dysfunction, sleep disorders and depression. Patients in the end stage may suffer from cognitive and psychiatric problems similar to those found in LBD due to the involvement of cortex (Aarsland *et al.*, 2001). Several studies which suggest that autonomic symptoms, anxiety and depression may precede clinical PD (Shiba *et al.*, 2000; Abbott *et al.*, 2005), support this staging system. The Lewy body pathology did not correlate with neuronal loss in the affected brain areas in many PD cases; hence this hypothesis needs further validation.

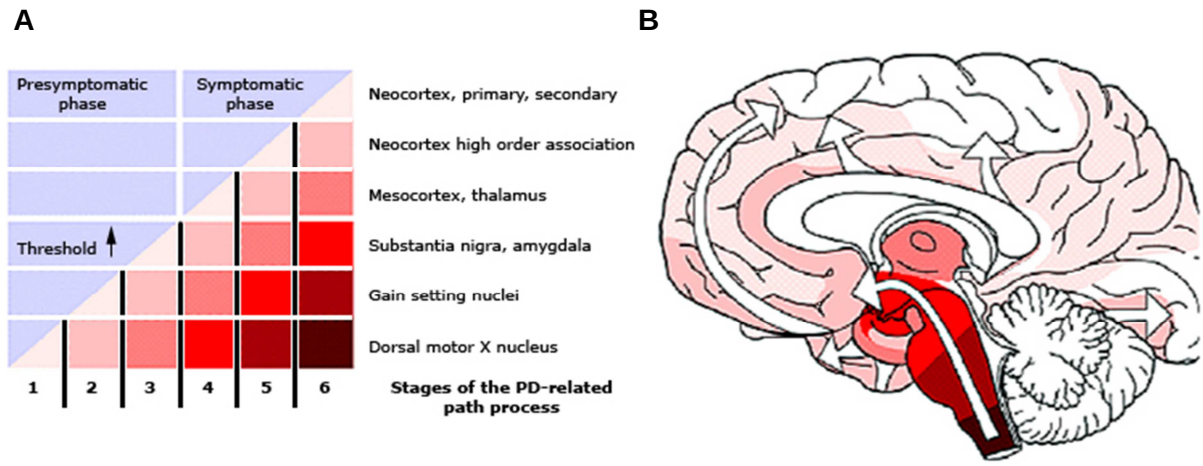

Figure S.1.1 Braak stages of PD

A) The presymptomatic (stage 1-3) and symptomatic phases (stage 4-6) are shown. The presymptomatic phase is marked when the Lewy bodies or neurites appear in persons without any symptoms. The asymptomatic phase is followed by the symptomatic phase when the neuropathological threshold in the affected individual is exceeded (black arrow), in this phase the PD-related symptoms starts to appear. The increasing slope and intensity of the colored squares (below the diagonal) is an indication of increasing pathology in the vulnerable brain regions (mentioned on the right hand side)

B) Diagram depicting the ascending pathological process (white arrows). The shading intensity of the colored areas refers to panel A. (Figures adapted from Braak et al., 2004)

S.1.4 Parkinsonism vs. Parkinson's disease (PD)

Parkinsonism (Parkinsonian syndrome) is any condition that causes a combination of movement abnormalities seen in PD, resulting from the loss or damage of DA producing neurons. PD is the main neurodegenerative cause of Parkinsonism. Not everyone who has Parkinsonism symptoms has PD, other causes of Parkinsonism can be due to the result of: medications to treat psychosis and other major psychiatric disorders, repeated head trauma, stroke, neurodegenerative disorders: such as multiple system atrophy (MSA), progressive supranuclear palsy (PSP) and Lewy body dementia (LBD).

S.1.5 Basal ganglia (or Basal nuclei)

The basal ganglia are a group of nuclei from different areas of brain that forms an interconnected uni-functional unit. Anatomically, these nuclei are located at the base of the forebrain which receives inputs from cortical layer, and sends information to thalamus and other brain stem regions (McHaffie *et al.*, 2005). The basal ganglia are involved in regulating involuntary movements, procedural learning related behaviors (like eye movements, bruxism -gnashing of teeth etc.,), cognitive and emotional functions (Mink and Thach, 1993). The components of human basal ganglia system (illustrated in Figure S.1.2) include the neostriatum (caudate and putamen) and the paleostriatum - globus pallidus external (GPe) and globus pallidus internal (GPi) segments, the subthalamic nucleus, the substantia nigra pars compacta (SNpc) and substantia nigra pars reticulata (SNpr) (Groenewegen, 2003). Although

GPi and SNpr are anatomically apart they are functionally identical with same kind of pre- and postsynaptic connections.

The basal ganglia nuclei, neostriatum receives major stimulatory input from almost all areas of the layer 5 glutamatergic neurons of the cortex via the corticostriatal pathway. Whereas the neostriatum receives another major source of input from the SNpc forming the nigrostriatal DA system, these DA projections can either be excitatory or inhibitory, depending on the DA receptor subtype expressed on the postsynaptic neurons. The neostriatum also receive inputs from the striatal cholinergic interneurons; these neurons release acetylcholine. The cholinergic input from the interneurons functions exactly opposite to the DA inputs that come from the SNpc. The coordinated action of all these different input mechanisms to the basal ganglia is important for the initiation of movements

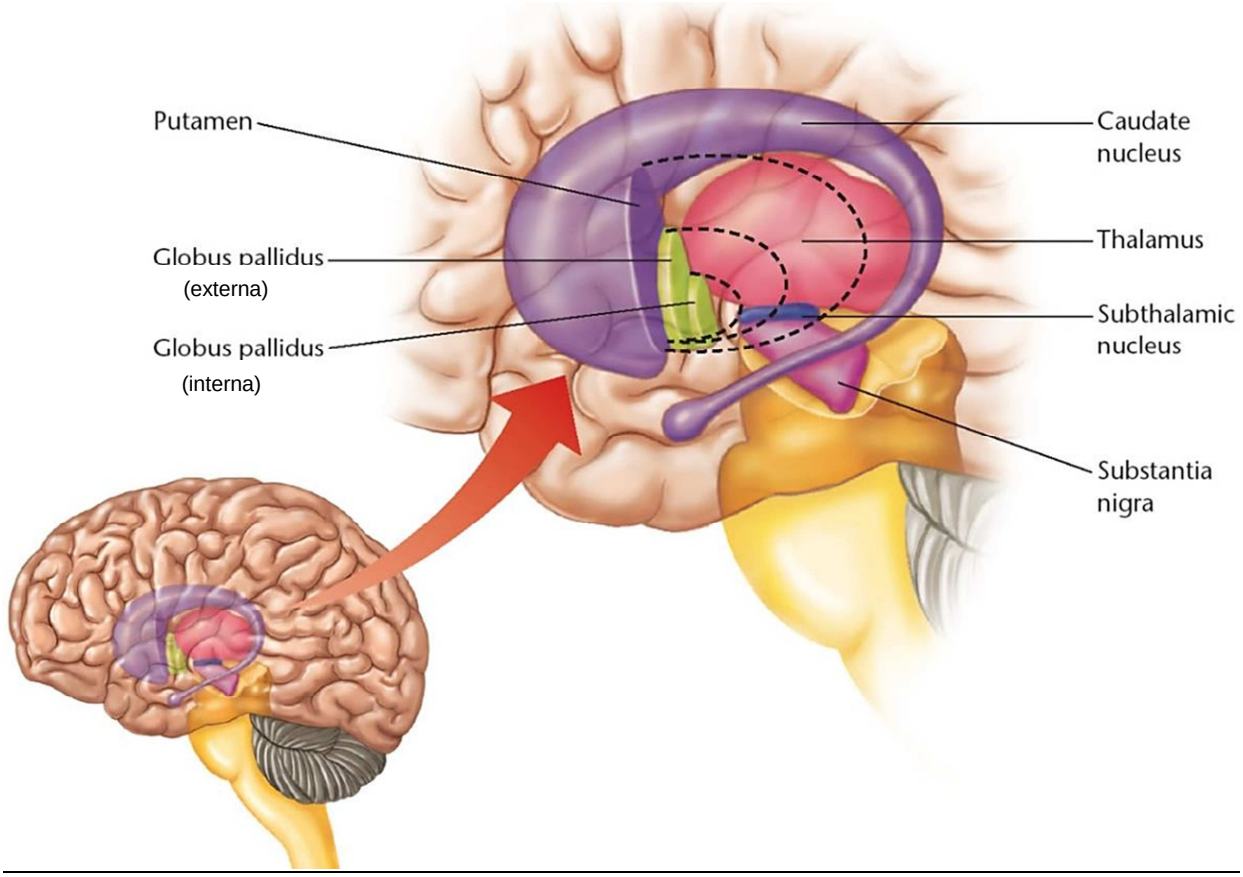

Figure S.1.2 Components of the human basal ganglia system

The components of human basal ganglia system shown here includes: caudate and putamen (together called as neostriatum); the external and internal segments of globus pallidus (together called as paleostriatum); the subthalamic nucleus; and the substantia nigra. Thalamus which receives output from the basal ganglia is also shown (Figure obtained from suicidionuncamais.wordpress.com)

The neostriatum largely contains medium spiny neurons (MSNs), which make up to 95% of the total striatal neurons. These MSNs are divided into two groups based on their direct or indirect connections with the efferent nuclei of the BG (GPi/SNpr). One group of MSNs directly innervate to the GPi/SNpr forming the **direct pathway** and other group of MSNs

which possesses short axons, makes connections with the neurons in the closely located GPe region and these neurons in the GPe forms connections with the neurons in the STN, which are connected with the GPi/SNpr regions to complete the **indirect pathway** of the BG (Obeso *et al.*, 2008) (illustrated in Figure S.1.3 A).

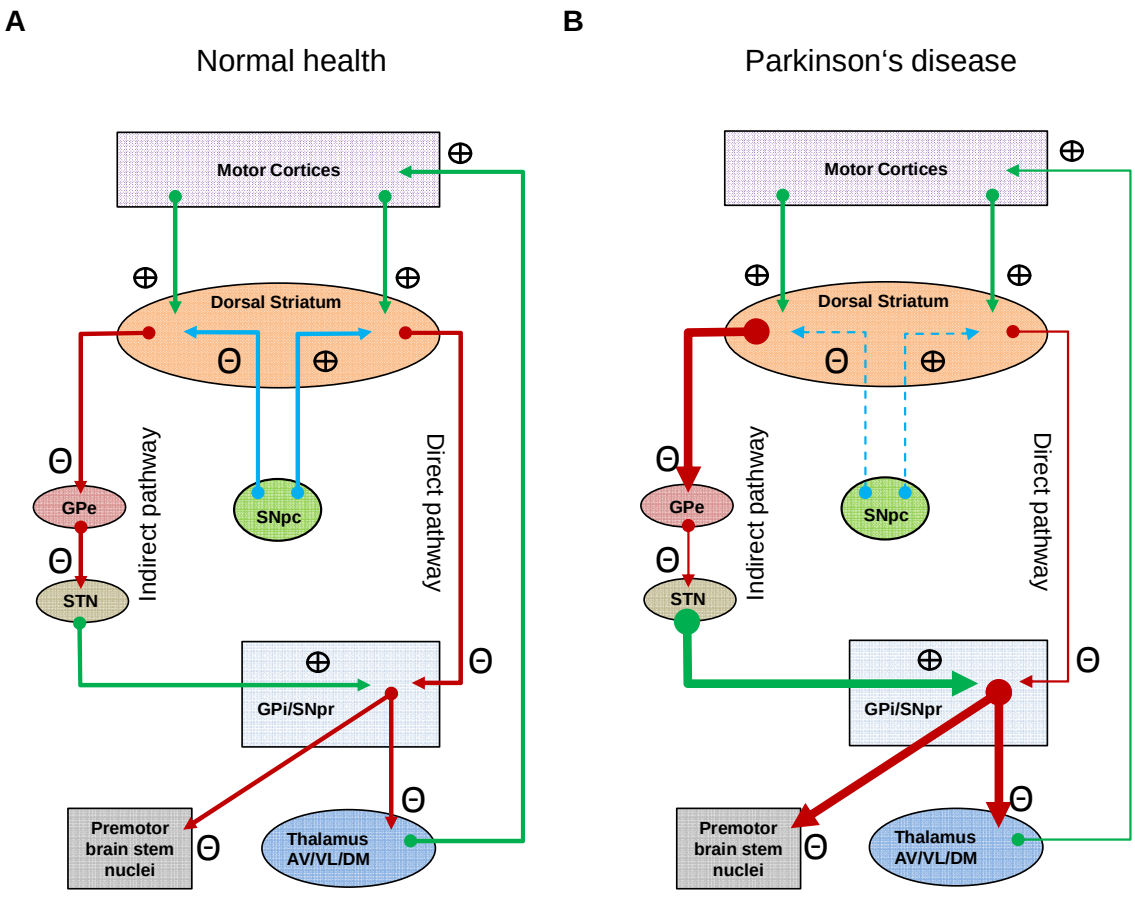

Figure S.1.3 Schematic outline of basal ganglia pathways in (A) normal health and (B) Parkinson's disease (PD)

SNpc = substantia nigra pars compacta; SNpr = substantia nigra pars reticulacta; GPe = globus pallidus externa; GPi = globus pallidus interna; STN = subthalamic nucleus; AV = anteroventral nucleus of the thalamus; VL = ventral lateral nucleus of the thalamus; DM = dorsomedial nucleus of the thalamus
⊕ indicates excitatory projections; Θ indicates inhibitory projections. Green arrows indicate glutamatergic connections; red arrows indicate GABAergic connections, whereas the blue arrows indicate DA connections. In (B) dotted blue lines indicate degenerated DA neuronal projections. The thickness of arrows corresponds to activity - in (A) & (B) arrows with intermediate thickness indicates normal activity of the respective projections; in (B) thinner arrow lines indicate decreased activity and thicker arrow lines indicate increased activity of the respective connections. (Figure adapted from Cenci, 2007)

The direct and indirect striatal projection neurons are segregated mainly by the DA receptor subtype (D1 or D2 subtype) they express (Gerfen *et al.*, 1990). The D1 receptor mRNA is restricted to those neurons which are directly connected to the output nuclei of the basal ganglia, these D1 receptor containing GABAergic neurons selectively express substance P

and dynorphin. Contrarily, the MSNs that project to the GPe contain D2 receptor mRNA, these GABAergic neurons express enkephalin. There is also a small proportion of MSNs which express both D1 and D2 receptors (Thibault *et al.*, 2013).

S.1.5.1 Direct pathway

The DA inputs from the SNpc to the D1R expressing MSNs are excitatory because this subtype of DA receptors are coupled to the stimulatory G-protein (G_s); on the other hand cortical (glutamatergic) inputs to these MSNs are also excitatory. Upon excitation these neurons release GABA to reduce the strong action of tonically active GABAergic output nuclei (GPi/SNpr) on the thalamus, and some regions of the brain stem. Upon decreased inhibition, the thalamic nuclei release more glutamate to stimulate certain areas of the motor cortex to initiate movements.

S.1.5.2 Indirect pathway

The DA inputs that come from the SNpc to these MSNs are inhibitory due to the presence of the inhibitory G-protein (G_i) coupled D2 receptors, whereas the cortical (glutamatergic) inputs are excitatory. Upon the action of DA, these MSNs become depolarized and release less GABA to the GPe, this results in the release of more GABA from the GPe neurons to the STN. The glutamatergic STN neurons undergo depolarization, which results in decreased stimulation of the tonically active GABAergic output nuclei (GPi/SNpr). Upon decreased stimulation of the output nuclei (GPi/SNpr) less GABA is released to the thalamus, and some regions of the brain stem. This finally leads to increased release of glutamate in the cortical areas via the thalamocortical fibers to stimulate certain areas of the motor cortex to make movements. The final action of the indirect pathway depends on the availability of glutamate and DA in the neostriatum.

S.1.5.3 Basal ganglia in PD

In PD, the degenerated nigrostriatal system results in reduced DA levels in the striatum. Decreased DA levels have different effects on the direct and indirect pathways (illustrated in Figure S.1.3 B). Due to reduced DA levels the direct pathway becomes less inhibitory to the GPi/SNpr output nuclei. This result in increased firing of the GABAergic projections those come from the GPi/SNpr to the thalamic and some brain stem regions. This decreases the stimulatory action of thalamus on the motor cortical areas.

Decreased DA levels leads to decreased inhibitory effect of the D2 receptor containing MSNs on the GPe neurons, this leads to decreased release of GABA from of the GPe neurons on the STN glutamatergic neurons, which results in the release of more glutamate from the STN to the GPi/SNpr output nuclei (Figure S.1.3 B). The hyperactive output nuclei release more GABA to the thalamus and certain and brain stem areas, which in turn leads to less stimulation of the motor cortical areas. Thus, decreased DA levels results in the slowing of movements.

S.1.6 Epidemiology of PD

In the general population, the prevalence of PD is thought to be around 0.3%, whereas, in aged population (>60 years), this rises to around 1%. There are approximately five million PD sufferers across the world. Many epidemiologic studies suggest that men are at a higher risk to develop PD than women. This could be due to neuroprotective effects of estrogens in women, a higher rate of occupational toxin exposure as well as minor head trauma in men, or recessive susceptibility genes on the X chromosome (Elbaz *et al.*, 2002; Wooten *et al.*, 2004). A study on rats suggests that down regulation of sex determining gene – SRY gene (present on the male specific - Y chromosome) specifically decreased the expression of TH in the SNpc DA neurons leading to motor deficits (Dewing *et al.*, 2006). This observation provides further evidence for male susceptibility in PD.

PD is largely sporadic, whereas the potential risk factors associated with the onset of this disease include genetic predisposition, environmental factors and ageing; PD may perhaps be multifactorial, due to a combination of two or more of these factors (reviewed by Farrer, 2006). In the last decade, there has been a remarkable progress in the identification of genes that are responsible for causing PD and related disorders. Only 10% of the PD cases are due to genetic causes, but studying the function of the disease-linked genes and gene products has helped the researchers to understand the molecular mechanisms underlying the disease. However, the penetrance of these PD causing genes is often incomplete; stating that not everyone who carries the PD-associated gene mutation develop the disease. This genetic variation suggests a possible interplay of genetic, epigenetic or environmental factors that can ultimately leads to disease development.

S.1.7 Role of environmental factors in PD

Several studies associate a number of environmental factors with increased risk of PD, including occupational exposure to pesticides, heavy metals, organic solvents, magnetic fields, dietary intake of dairy products etc.; however, their causal relationship have not been well established. Among the association studies, reports on exposure to pesticides and increasing risk of PD seem to be consistent. In 1980s, discovery of MPTP (1-methyl-4-phenyl-1, 2, 3, 6,-tetrahydropyridine) induced SNpc DA neuronal degeneration and a resultant Parkinsonism in humans strengthened the relationship between pesticides and PD. Other pesticides namely, rotenone and paraquat were also implicated in PD (reviewed in Wirdefeldt et al., 2011)). Based on these epidemiological studies different toxin-induced PD animal models have been developed to study SNpc DA neurodegeneration. Moreover, blood urate levels, use of NSAID (Non-steroidal anti-inflammatory drug), adiposity, and brain injury have limited or conflicting evidence of relationship to PD. On the other hand, cigarette smoking, caffeine consumption, alcohol consumption, dietary intake of Vitamin E (or other anti-oxidants) and physical activity were reported to reduce the risk of PD, but the physiologic mechanisms for these relations are poorly understood (Wirdefeldt *et al.*, 2011). To make further progress in understanding the role of environmental factors in PD, larger studies with more precise quantifications should be performed.

S.1.8 Genetic animal models of PD

S.1.8.1 α-synuclein animal models

Overexpression of α-synuclein in *Drosophila* and *C. elegans* also leads to loss of DA neurons. Lewy body-like inclusions with progressive DA neuronal loss and L-DOPA responsive motor defects were observed in α-synuclein overexpressing *Drosophila* (Feany and Bender, 2000); whereas, Lewy body inclusions and progressive DA neuronal loss was not observed in α-synuclein expressing *C. elegans* (Lakso *et al.*, 2003; Kuwahara *et al.*, 2006). Since *Drosophila* and *C. elegans* do not contain endogenous α-synuclein, these models cannot be considered as relevant systems to study the in vivo function of α-synuclein. Several mouse models have been developed to study the function of α-synuclein in vivo. However, knocking out α-synuclein in mice did not result in any significant effect on the development or maintenance of DA system (Abeliovich *et al.*, 2000; Chandra *et al.*, 2004). Several researchers have developed transgenic α-synuclein-overexpressing mice under different promoters. The phenotypic outcome in these mice varied heavily and is mostly promoter dependent (Chesselet, 2008). Nevertheless, some of these mouse models serve as excellent models of α-synuclein-induced neurodegeneration. One such model is the mouse prion promoter (mPrP) driven A53T mutant α-synuclein overexpressing mice, these mice exhibit α-synuclein pathology that is very similar to what is observed in humans, which include α-synuclein aggregation, fibrils formation and truncation, and post translation modification of α-synuclein like phosphorylation and ubiquitination, ultimately leading to progressive age-dependent neurodegeneration (Giasson *et al.*, 2002; Lee *et al.*, 2002; Chesselet, 2008).

The toxicity of α-synuclein seems to occur due to mitochondrial dysfunction and proteasomal and lysosomal impairments and disruption of ER-Golgi trafficking (Tanaka *et al.*, 2001; Cuervo *et al.*, 2004; Cooper *et al.*, 2006; Martin *et al.*, 2006). Mouse DA neuronal toxicity induced by mitochondrial toxins seems to be dependent on the expressions levels of α-synuclein. Mice deficient of α-synuclein are more resistant to MPTP and other mitochondrial toxins (Dauer and Przedborski, 2003; Klivenyi *et al.*, 2006) compared to wildtype, whereas mice overexpressing α-synuclein are more sensitive to paraquat and other mitochondrial toxins (Norris *et al.*, 2007). Transgenic human A53T α-synuclein overexpressing mice exhibit mitochondrial defects (Martin *et al.*, 2006). Consistent with these findings, SN and striatum of PD patients with α-synuclein aggregates also exhibit decreased mitochondrial complex I activity (Devi *et al.*, 2008). All these studies provide a direct link between α-synuclein toxicity and mitochondrial dysfunction.

In the context of functional interaction between α-synuclein and parkin (another PD-related gene product), it has been shown that wildtype but not mutant parkin could protect the DA neurons from the proteasome inhibition mediated neurotoxicity of mutant α-synuclein. α-synuclein has been shown to interact with synphilin-1 (Engelender *et al.*, 1999) and syniphilin-1 is a known *in vitro* substrate of parkin, an E3 ubiquitin ligase (Chung *et al.*, 2001). All these finding argue for a coordinated function of different proteins that are linked to PD.

S.1.8.2 LRRK2 animal models

As observed for α-synuclein, overexpression of LRRK2 in *Drosophila* and *C. elegans* also leads to DA neuron degeneration (Liu *et al.*, 2008; Ng *et al.*, 2009; Saha *et al.*, 2009; Venderova *et al.*, 2009); whereas, no Lewy body-like inclusions were found in these models as they do not express α-synuclein. In mouse, LRRK2 deficiency doesn't seem to be important for the development or maintenance of the nervous system, in contrast to what was observed in *Drosophila* (Lee *et al.*, 2007; Sakaguchi-Nakashima *et al.*, 2007; Imai *et al.*, 2008; Wang *et al.*, 2008; Andres-Mateos *et al.*, 2009). Transgenic expression of LRRK2WT, LRRK2 R1441C, LRRK2 G2019S or conditional expression of LRRK2WT, LRRK2 G2019S or knock-in of LRRK2 R1441C in mice doesn't lead to DA neurodegeneration (reviewed in Dawson et al., 2010). The exact reason for these discrepancies in mice and humans with respect to LRRK2 mediated DA neuron loss is unclear.

S.1.8.3 DJ-1 animal models

Several *Drosophila* models have been generated to study the physiological functions of DJ-1. *Drosophila* mutants that lack both the orthologs of DJ-1 (DJ-1a and DJ-1b) are sensitive to oxidative stress and displayed motor deficits and reduced lifespan without showing any DA neurodegeneration (Park *et al.*, 2005; Lavara-Culebras and Paricio, 2007) However, transgenic RNAi dependent knock down of DJ-1 resulted in age dependent loss of DA neurons (Yang *et al.*, 2005). In mice, DJ-1 deficiency has no impact on the SNpc DA neurons and total DA levels in the striatum; however, some studies report decreased mitochondrial dysfunction in DJ-1 ko mice (Goldberg *et al.*, 2005; Andres-Mateos *et al.*, 2007; Pham *et al.*, 2010). DJ-1 mutant mice and flies have decreased mitochondrial complex I activity (Hao *et al.*, 2010; Giaime *et al.*, 2012) it was also shown that DJ-1 binds to complex I subunits to ensure proper mitochondrial function (Hayashi *et al.*, 2009).

S.1.8.4 PINK1 animal models

Drosophila PINK1 deficiency causes mitochondrial abnormalities leading to apoptosis of flight muscle cells and behavioral deficits (Clark *et al.*, 2006; Park *et al.*, 2006). The flies lacking PINK1 contain swollen mitochondria and produce less ATP. Whereas, PINK1 ko mice did not show any histological or physiological alterations in the DA system except for mild changes in striatal DA transmission (Gautier *et al.*, 2008; Gispert *et al.*, 2009). In drosophila and mouse models of PINK1, complex I activity is found to be decreased (Gautier *et al.*, 2008; Morais *et al.*, 2009). This PINK1 dependent mitochondrial phenotype can be rescued by expressing Ndi1p (a yeast complex I equivalent) (Haddad *et al.*, 2012). Vitamin K2 (which serves as an electron carrier in bacteria) was also reported to rescue the mitochondrial morphology and maintenance of ATP production in the PINK1 fly model (De Strooper *et al.*, 2012).

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Appendices

Abbreviations

AA	Amino acid
AADC	L-amino acid decarboxylase
AD	Alzheimer's disease
ALDH	Aldehyde dehydrogenase
AR-JP	Autosomal recessive juvenile Parkinsonism
ARTN	Artemin
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
bp	Base pair
BSA	Bovine serum albumin
CDNF	Cerebral dopamine neurotrophic factor
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
DA	Dopamine
DAB	Di-amino-benzidine
DAT	Dopamine transporter
ddH_2O	Double-distilled water
DNA	Deoxy ribose nucleic acid
DOPAC	3,4-Dihydroxyphenylacetic acid
dNTP	Deoxyribonucleotide triphosphate
e.g.	Example given
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinases
Eps15	Epidermal growth factor receptor pathway substrate 15
et al.,	et alii
FGF8	Fibroblast growth factor 8
FMTC	Familial medullary thyroid carcinoma
FTD	Frontotemporal dementia
GABA	Gamma aminobutyric acid
GAB1/2	Grb2-associated binding protein
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFLs	Glial cell derived neurotrophic factor family ligands
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
GPI	Glycosyl phosphatidylinositol
Grb2	Growth factor receptor bound protein 2
GTP	Guanosine triphosphate
h	Hour(s)

h-parkin	Human parkin
HPLC-ECD	High-performance liquid chromatography - electrochemical detection
HRP	Horse radish peroxidase
HVA	Homovanillic acid
Iba1	Inonized calcium binding adaptor protein 1
IBR	In between RING domain
IL	Interleukin
INF	Interferon
JAK-STAT	Janus-activated kinase-signal transducer, activator of transcription
JNK	c-JUN N-terminal kinase
Kb	Kilobase(s)
KDa	Kilodalton(s)
ko	Knockout
L	Liter
LB	Lewy Body
L-DOPA	L-3,4-dihydroxyphenylalanine
LC	Locus Coeruleus
LRRK2	Leucine-rich repeat kinase 2
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MEKK1	Mitogen-activated protein kinase (MAPK) kinase kinase 1
MEN2	Multiple endocrine neoplasia 2
min	Minute(s)
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
MSN	Medium size spiny neuron
3-MT	3-methoxytyramine
NCAM	Neural cell adhesion molecule
NE	Norepinephrine
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NRTN	Neurturin
NSE	Neuron-specific enolase
n.s.	Non-significant ($p>0.05$, Student's t-test)
NT	Neurotrophin
PARIS	Parkin interacting substrate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson’s disease
PFA	Paraformaldehyde
pH	Potential hydrogen
PI3K	Phosphoinositide-3 kinase
PINK1	PTEN homolog-induced putative kinase 1
Pitx3	Paired-like homeodomain transcription factor 3
PKA	Protein kinase A
PSPN	Persephin
p75NTR	p75 neurotrophin receptor
PTB	Phosphotyrosine-binding (domain)

PTEN	Phosphatase and tensin homolog
Ret	Rearranged during transfection
RING	Really interesting new gene domain
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SH	Src-homology
Shh	Sonic hedgehog
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TAE buffer	Tris/Acetic acid/EDTA buffer
TBS	Tris buffered saline
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
T_m	Melting temperature
TNF	Tumor necrosis factor
Trk	Tropomyosin-related kinase
UBL	Ubiquitin like
UCH	Ubiquitin carboxyl-terminal hydrolase
UPS	Ubiquitin-proteasome pathway
UTR	Untranslated region
vs.	Versus
VDAC	Voltage-dependent anion channel
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area
wt	Wild type
6-OHDA	6-hydroxydopamine
ºC	Degree centigrade
µ	Micro
%	Percent
Δ	Truncated

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S.1 Supplementary Information (Introduction)

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