



## Review

## GDNF–Ret signaling in midbrain dopaminergic neurons and its implication for Parkinson disease

Edgar R. Kramer<sup>a,b,\*</sup>, Birgit Liss<sup>b</sup><sup>a</sup> Research Group Development and Maintenance of the Nervous System, Center for Molecular Neurobiology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany<sup>b</sup> Department of Applied Physiology, Ulm University, Ulm, Germany

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## ABSTRACT

**Glial cell line-derived neurotrophic factor (GDNF) and its canonical receptor Ret can signal together or independently to fulfill many important functions in the midbrain dopaminergic (DA) system. While Ret signaling clearly impacts on the development, maintenance and regeneration of the mesostriatal DA system, the physiological functions of GDNF for the DA system are still unclear. Nevertheless, GDNF is still considered to be an excellent candidate to protect and/or regenerate**

**Abbreviations:** 6-OHDA, 6-hydroxy-dopamine; Akt, PKB, protein kinase B, serine/threonine-specific protein kinase; BDNF, brain derived neurotrophic factor; CaMKII $\beta$ , calcium-calmodulin-dependent protein kinase II  $\beta$  isoform; cAMP, 3',5'-cyclic adenosine monophosphate; catecholamine, monoamine, neurotransmitter type including dopamine, epinephrine (adrenaline) and norepinephrine (noradrenaline); CCCP, carbonyl cyanide m-chlorophenyl hydrazone; Cdc42, cell division control protein 42 homolog, a plasma membrane-associated small GTPase of the Rho family; COMT, catechol-O-methyltransferase; CoREST, co-repressor for element-1-silencing transcription factor, a chromatin-modifying corepressor complex that acts with REST (repressor for element-1 silencing transcription factor) complex; GABA,  $\gamma$ -aminobutyric acid, inhibitory neurotransmitter in mammalian central nervous system; GTPase, cycles between an active GTP-bound and an inactive GDP-bound state; DA, dopaminergic; DAT, dopamine transporter; DJ-1, deglycase, oxidative stress sensor and redox-sensitive chaperone and protease; DOK1/4/5/6, docking proteins, have a PH and SH3 domain; EGFP, enhanced green fluorescent protein; EGR1, early growth response protein 1, Zif268 (zinc finger protein 225), NGFI-A (nerve growth factor-induced protein A), is a zinc finger transcription factor; Enigma, adaptor protein of the PDZ-LIM family; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinases, classical MAPKs; FosB/ $\Delta$ FosB, FBJ murine osteosarcoma viral oncogene homolog B, a transcription factor with a truncated  $\Delta$  form; FRS2, fibroblast growth factor receptor substrate 2, adaptor protein; GAP1/2, GTPase-activating proteins 1 and 2; GDNF, glia cell line-derived neurotrophic factor; GFLs, GDNF family of ligands; GFR $\alpha$ , GDNF family receptor  $\alpha$ ; GPI, glycosylphosphatidylinositol; GRB2/7/10, growth factor receptor-bound protein 2, 7 and 10, adaptor proteins; gsk3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; IRS1/2, insulin receptor substrate 1, adaptor protein, contains a PTB and PH domain; JNK, c-Jun N-terminal kinases, members of the MAPK family of proteins; Kv4.3 and KChip3, subunits of the Ca<sup>2+</sup>-sensitive, voltage gated A-type K<sup>+</sup> channel; LIM, (acronym combining the first letters of three proteins – Lin11, Isl-1 and Mec-3 – that have this common domain) protein interaction domain of two contiguous zinc finger domains, separated by a two-amino acid residue hydrophobic linker; MEN2B, multiple endocrine neoplasia 2 type B, mutation in the kinase domain of the Ret leading to constitutive active receptor; MAOA/MAOB, monoamine oxidases A and B; MAPK, mitogen-activated protein kinase, can phosphorylate serine, threonine, and tyrosine, e.g. p38MAPK; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NCAM, neuronal cell adhesion molecule; NF- $\kappa$ B, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells, transcription factor family with Rel homology domain (RHD); NGF, nerve growth factor; Nurr1, orphan nuclear receptor and transcription factor; PACE4, PCSK6, proprotein convertase subtilisin/kexin type 6; PC5A, PC5B, and PC7, proprotein convertases; PD, Parkinson disease; PDZ, (acronym combining the first letters of three proteins – post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) that have this common domain) protein interaction domain; PH, pleckstrin homology domain; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PINK1, PTEN-induced putative kinase 1; PKA, protein kinase A, a family of cAMP-dependent protein kinase; PLC $\gamma$ , phospholipase  $\gamma$  cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) ppp3R1/ppp3CB, calcineurin subunits; PTB, phosphotyrosine-binding domains; PI, phosphotyrosine-interaction domain; PTEN, phosphatase and tensin homolog; Rac1, Ras-related C3 botulinum toxin substrate 1 (Rho family), a small GTPase, like CDC42; Raf, (rapidly accelerated fibrosarcoma/rat fibrosarcoma) family of serine/threonine-protein kinase; Ras, (Rat sarcoma) family of small membrane-associated GTPase; Rho, (Ras homolog) family of small GTPases including Cdc42, Rac1, and RhoA; Ret, (rearranged during transfection) canonical GDNF receptor, a receptor tyrosine kinase; RRF, retro-rubal field; Ser/Thr, serine and threonine, which can be phosphorylated; Shank3, SH3 and multiple ankyrin repeat domains 3, proline-rich synapse-associated protein 2 (ProSAP2); SHC, SH2 domain containing transforming protein 1; SHP-2, cytoplasmic SH2 domain containing protein tyrosine phosphatase; SN, substantia nigra; SNP, single-nucleotide polymorphism analysis; SorLA, sorting protein-related receptor with A-type repeats, a member of the mammal Vps10p domain receptor; SOS, son of sevenless, family of guanine nucleotide exchange factors that act on Ras; Src, (sarcoma protein) membrane-associated tyrosine kinase with different Src homology (SH) domains characteristic for all 9 members of the Src family kinases; Tyr, tyrosines, which can be phosphorylated; TGF- $\beta$ , transforming growth factor  $\beta$  is a secreted protein that controls proliferation and cellular differentiation; TIEG, TGF- $\beta$ -inducible early-response gene, a zinc finger transcription factor Vav2, adaptor protein and guanine nucleotide exchange factor for the Rho family of Ras-related GTPases; VPS10P, vacuolar protein sorting 10 protein-domain receptors are type 1 transmembrane proteins; VTA, ventral tegmental area

\* Corresponding author at: Research Group Development and Maintenance of the Nervous System, Center for Molecular Neurobiology Hamburg (ZMNH), University Medical Center Hamburg-Eppendorf (UKE), Falkenried 94, 20251 Hamburg, Germany.

E-mail address: [edgar.kramer@uni-ulm.de](mailto:edgar.kramer@uni-ulm.de) (E.R. Kramer).

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**the mesostriatal DA system in Parkinson disease (PD). Clinical trials with GDNF on PD patients are, however, so far inconclusive. Here, we review the current knowledge of GDNF and Ret signaling and function in the midbrain DA system, and their crosstalk with proteins and signaling pathways associated with PD.**

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## 1. Introduction

The neurotransmitter dopamine is produced by dopaminergic (DA) neurons and modulates diverse functions in the brain and throughout the body, including movement, memory, motivation and emotions [1,2]. The cell bodies of DA neurons are grouped in the ventral midbrain in the substantia nigra (SN), the ventral tegmental area (VTA) and the retro-rubal field (RRF). Axonal projections of midbrain DA neurons are split into the mesostriatal and the mesocorticolimbic pathways [1]. The complex projections, functions and interactions of distinct types of midbrain DA neurons have recently been further dissected [3–10]. To briefly summarize, the mesostriatal pathway connects the SN and some VTA DA neurons with the dorsal striatum and is important for the control of voluntary movement. The mesocorticolimbic pathway projects from the VTA, the dorsal tier of the SN and the RRF to the ventral striatum (caudate nucleus and putamen), nucleus accumbens, olfactory tubercle, septum, amygdala, habenula, hippocampus and cortex and is involved in cognitive, rewarding/aversive and emotion-based behavior. According to its diverse projections and functions, alterations of the midbrain DA system can lead to a variety of neurological diseases. For example, the progressive loss of SN DA neurons in particular and the related dopamine deficit within the dorsal striatum cause the classical motor-function related symptoms in Parkinson disease (PD) [11,12]. Characterizing the rare familial cases of PD with mutations in specific genes has shed light onto the etiology of PD and facilitated the discovery of common pathological alterations, such as mitochondrial dysfunction, metabolic and oxidative stress, axonal transport defects, and abnormal protein degradation and aggregation [13,14]. The heterogeneity of midbrain DA neurons suggests that a multitude of signaling events are required during development and maintenance to ensure proper functioning including different neurotrophic support [9,15–17]. The midbrain DA system is largely conserved between humans and rodents and studies in transgenic mice have identified the basic requirements for generation and maintenance of the DA system [1,18,19] (Fig. 1). Here we review the emerging roles of neurotrophic factors for the midbrain DA system during physiological and pathophysiological conditions such as PD, with a focus on GDNF (glial cell line-derived neurotrophic factor) and Ret (rearranged during transfection) signaling.

## 2. Role of neurotrophic factors in the midbrain DA system

Neurotrophic factors are a diverse group of polypeptides that function as growth and survival factors during development, adulthood and aging [20,21]. According to the neurotrophic factor hypothesis originally postulated by Rita Levi-Montalcini and Victor Hamburger, more neurons are born during embryogenesis than later survive, and target-derived neurotrophic factors are one limiting factor determining which neurons survive or die during pre- and postnatal development [22]. They can also stimulate axon outgrowth and guidance [23–25]. Neurotrophic factors also

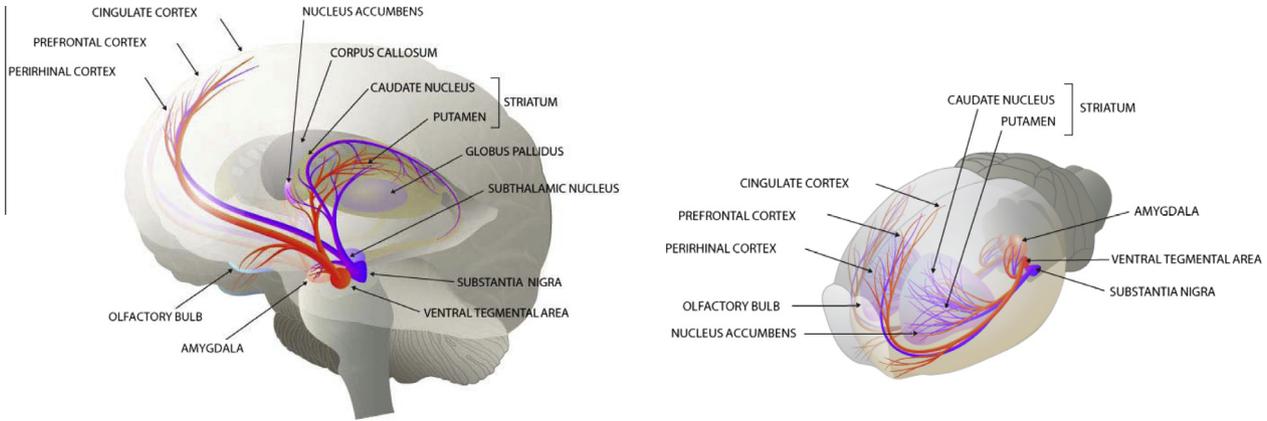
prevent degeneration associated with neurodegenerative diseases, stimulate differentiation and synaptogenesis, and are essential for maintaining normal physiological functions in the nervous system, including adult synaptic plasticity and behavior [21,26–30]. In general, neurotrophic factors may be secreted into the extracellular space from both neurons and glia. They can diffuse and are actively transported over long distances in antero- and retrograde directions [31,32]. Neurotrophic autocrine loops have been suggested to support midbrain DA neuron survival in culture [33]. DA neurons require specific neurotrophic factors and their cell surface receptors for proper *in vivo* differentiation and maintenance, which have not yet been fully characterized [17,34]. Neurotrophic factors of the DA system include the neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), as well as the four GDNF family ligands (GFLs) GDNF, neurturin, artemin and persephin, which are distantly related members of the transforming growth factor- $\beta$  superfamily and the focus of this review [17,35,36].

## 3. Function of neurotrophic factor GDNF in the midbrain DA system

In general, GFLs mediate their actions by utilizing a complex signaling network consisting of several different binding and signaling partners [37]. As summarized in Fig. 2, each GFL binds with high affinity to one of the glycosylphosphatidylinositol (GPI)-linked GDNF family receptor  $\alpha$  (GFR $\alpha$ ) members 1–4 [17]. GDNF binds with high affinity to GFR $\alpha$ 1, which is the only GFR $\alpha$  receptor expressed at high mRNA and protein levels in midbrain DA neurons [38,39]. GFR $\alpha$ 1 is alternatively spliced and both isoforms are highly expressed in the SN [40–42]. The long a form including the exon 5 encoded sequence was found to bind GDNF less efficiently than the short b form lacking the exon 5 encoded sequence; the long a form also promotes axon outgrowth through MAPK, Rac1 and Cdc42 signaling, in contrast to the short b form [41,42]. GFR $\alpha$ 2 is also expressed in the ventral midbrain, but in non-DA neurons [43–45]. GFR $\alpha$ 3 and GFR $\alpha$ 4 seem not to be expressed in the ventral midbrain [46,47]. The GDNF/GFR $\alpha$ 1 signaling complex can recruit transmembrane receptors such as the canonical GDNF receptor Ret, a receptor tyrosine kinase [17,48–50], or the neuronal cell adhesion molecule (NCAM) [51–53], to trigger downstream signaling events in midbrain DA neurons (Fig. 2).

GDNF seems to be the most prominent neurotrophic factor within the midbrain DA system and a promising therapeutic candidate for neuroprotective and regenerative interventions in PD patients [21,54,55]. GDNF was described more than 20 years ago as a survival factor for rat embryonic DA neurons of the midbrain in culture [23]. Later, this positive *in vitro* survival effect of GDNF was extended to other neuronal cell types such as motor neurons, adrenergic neurons, parasympathetic neurons, enteric neurons, and somatic sensory neurons [17,56–59].

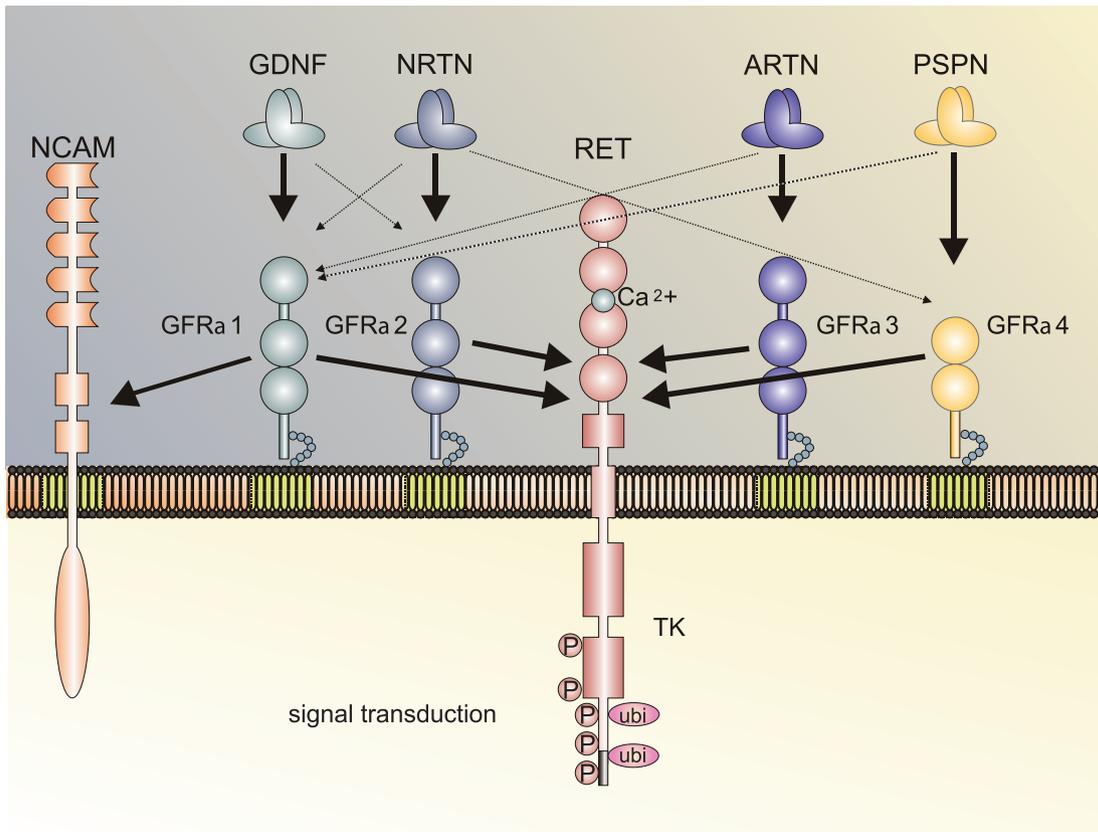
Mature GDNF is a homo-dimeric glycoprotein [23]. GDNF is expressed as a pre-pro-domain containing precursor protein with



**Fig. 1.** The midbrain dopaminergic system is conserved in humans and mice. The cell bodies of the midbrain dopaminergic (DA) neurons that are preferentially dying in Parkinson disease (PD) patients are located in the substantia nigra (SN) pars compacta (cell bodies in the midbrain labeled in purple). They innervate with their axons mainly the dorsal striatum (a subcortical telencephalon region labeled in gray). Addiction affects DA neurons of the ventral tegmental area (VTA) (cell bodies in the midbrain labeled in red) mainly innervate the ventral striatum, cortex, amygdala and olfactory tubercle compacta.

two splice variations in the pro-domain leading to a long  $\alpha$  and a short  $\beta$  pro-protein detected in and outside the DA system [60–63]. The pre-domain of GDNF is cleaved upon secretion and the pro-domain can be removed for activation by several proteases in the extracellular space, such as furin endoprotease, PACE4, and proprotein convertases PC5A, PC5B, and PC7 [17,63]. Potassium-stimulated secretion of long  $\alpha$  GDNF protein was enhanced by interaction with the sorting protein-related receptor with A-type repeats (SorLA), a member of the mammal Vps10p

domain receptor family, but the secretion-defective short  $\beta$  pro-protein did not efficiently bind to SorLA [64,65]. Interestingly, SorLA was also found to act as a sorting receptor for the internalization of the GDNF/GFR $\alpha$ 1/Ret complex, leading to GDNF degradation in lysosomes and recycling of the receptors GFR $\alpha$ 1 and Ret back to the cell membrane [66]. Secreted GDNF can bind to polysaccharides such as heparin sulfate (HS) proteoglycans on syndecan 3 [67] or polysialic acid (PSA) on NCAM [51]. This binding might reduce GDNF diffusion and allow for concentration of GDNF



**Fig. 2.** GDNF family of ligands and their receptors. The four members of the glial cell line-derived neurotrophic factor (GDNF) family of ligands, which include GDNF, neurturin, artemin, and persephin, are homodimers which bind with high affinity to one of the four members of the GDNF receptor  $\alpha$  family (GFR $\alpha$ 1–4). These receptor-ligand complexes can interact with and activate the canonical GDNF receptor Ret, a receptor tyrosine kinase. GDNF can also activate alternative GDNF receptors, such as the neuronal cell adhesion molecule (NCAM). The intracellular domain of Ret can be phosphorylated and ubiquitinated.

at specific sites. These data illustrate the complex multiple step maturation process of GDNF, with many possibilities for fine-tuning its expression and tissue availability also in the DA system.

GDNF mRNA is expressed in the adult DA system in the striatum, the nucleus accumbens, thalamus, hippocampus and cerebellum [68–70], which are known to be brain areas innervated by axonal projections of midbrain DA neurons [1,71–73]. Despite its name, and expression in cultured astrocytes, microglia and oligodendrocytes [68,74–76], GDNF seems to be absent in glia cells of the mouse striatum even under 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced DA degeneration conditions [77]. In the adult striatum, GDNF mRNA was only detected in neuronal cells [78]. In mice carrying the lacZ gene in the GDNF locus [79], GDNF was also found in the DA innervated striatum, thalamus, septum and subcommissural organ [80]. In the adult striatum of these mice, GDNF was found to be expressed in 80% of fast spiking, parvalbumin-positive GABAergic interneurons (which representing 0.7% of all striatal neurons and 95% of GDNF-positive cells). In the striatum, 5% of GDNF positive neurons appear to be somatostatinergic or cholinergic interneurons. GDNF was not expressed in medium spiny neurons (MSN) [55,77]. The lack of GDNF detection in postnatal and adult midbrain DA neurons suggests that the existence of an autocrine loop of GDNF stimulation of DA neurons is unlikely [77]. However, midbrain DA neurons express sonic hedgehog (Shh) and require Shh for long-term maintenance [81]. Shh is released from DA neuron axons and inhibits the muscarinic autoreceptor in cholinergic interneurons. Shh also downregulates GDNF expression in cholinergic and GABAergic interneurons of the striatum. Conversely, GDNF in the striatum activates the Ret receptor on midbrain DA neurons and in turn inhibits their Shh expression [81]. This inhibitory feedback loop might be important for maintaining the homeostasis between midbrain DA neurons and striatal neuronal activity.

Constitutive GDNF knockout mice were shown to die soon after birth without developing kidneys, but with a normal DA system, proving GDNF not essential during development of the prenatal DA system in the mouse [79,82,83]. In mice, GFR $\alpha$ 1 was also found to be dispensable for the embryonic DA system development [84–86]. Antibodies against the rate limiting enzyme for dopamine synthesis, tyrosine hydroxylase (TH), on wildtype mouse midbrain tissue revealed a naturally occurring DA cell death already during late embryogenesis. Half of the DA neurons remaining at birth are eliminated by two postnatal apoptotic processes which peak at day 2 and 14 after birth [87,88]. Intrastriatal injection of GDNF-blocking antibody in wild-type mice can augment cell death, and expression of GDNF in the striatum has been found to inhibit the natural apoptosis at postnatal day 2 [88,89]. However, GDNF expression only transiently increased the midbrain DA cell number, which returned to normal within a few weeks during adulthood, suggesting a rather minor role for GDNF in normal postnatal development of the midbrain DA system [88,89].

In adult mice, conditional ubiquitous removal of GDNF triggered by tamoxifen in Esr1-Cre mice [90] led to a ~40–80% reduction of striatal GDNF protein levels and reportedly caused an approximate 60% degeneration of SN DA and VTA DA neurons [80]. Behavioral analysis of these mice in the open field test revealed a hypokinetic syndrome with reduced distance travelled, less rearing events and diminished accumulated rearing time. Surprisingly, these mice also lost almost 100% of noradrenergic neurons from locus coeruleus, another brain region that displays high vulnerability to degeneration in PD [80]. However, the opposite result was found when another group used the same Esr1-Cre mice together with their own floxed GDNF mice; no loss of midbrain DA neurons or locus coeruleus noradrenergic neurons was observed and no alterations in the open field motor activity test

[91]. This discrepancy might be due to different genetic backgrounds of the mice used in these two studies starting already with different embryonic stem cells utilized to generate the mice (R1 ES cells derived from mouse strain 129X1/SvJ x 129S1/Sv)F1-Kitl<+> [80]; IB10/E14IB10 ES cells derived from mouse line 129P2/OlaHsd [91]). In addition, this discrepancy emphasized the need to characterize alternative GDNF-deficient mouse models. Likewise, Kopra et al. reported no catecholaminergic cell loss in the brain of adult Nestin-Cre/GDNF mice when GDNF was deleted in neurons and glia cells during embryogenesis, or by injection of AAV5-Cre virus during adulthood [91]. Heterozygous GDNF knockout mice showed about 35–65% decrease in GDNF protein levels, a slight age-dependent loss of SN DA neurons (14–20% in 20 and 12 month old mice, respectively), and an aged-dependent motor impairment in the open field and accelerated rotarod test [92]. In addition, adult heterozygous GDNF knockout mice have increased extracellular dopamine levels and FosB/ $\Delta$ FosB levels in the striatum and an impaired water maze learning performance [93,94]. Also heterozygous GFR $\alpha$ 1 knockout mice were investigated and showed at the age of 18 month 24% loss of DA neurons in the SN but not in the VTA, reduced dopamine levels in the striatum and reduced locomotor activity [95,96]. Data on the midbrain DA system of conditional GFR $\alpha$ 1 knockout mice have not yet been reported but might help to shed light on the importance of GDNF/GFR $\alpha$ 1 signaling in the midbrain DA system. Taken together, the discrepancy between data from current GDNF and GFR $\alpha$ 1 mouse models suggest that the *in vivo* GDNF and GFR $\alpha$ 1 functions for midbrain DA neuron survival during postnatal development and aging of mice remain controversially discussed and further experiments are needed to clarify this issue [80,91,97].

Besides GDNF's effect on DA cell survival, GDNF has also been reported to influence the physiology of midbrain DA neurons. This important aspect of GDNF is often been neglected and is therefore discussed here in more detail. In adult and aged rats, intranigral injection of 10  $\mu$ g GDNF increased dopamine levels in the striatum and also augmented motor behavior [98,99]. Treatment of primary midbrain DA culture from newborn rats with 10 ng/ml GDNF enlarged the number of dopamine molecules released per quantum by 380% compared to controls [100]. GDNF treatment could also acutely and reversibly potentiate the excitability of rat VTA DA neuron cultures and increase the number of dopamine vesicles [101]. GDNF potentiates the excitability of rat midbrain slices and DA neuron cultures by an MAPK-dependent inhibition of Ca<sup>2+</sup>-sensitive, voltage gated A-type K<sup>+</sup> channels [102] composed of Kv4.3 and KChip3 subunits [103], which has similarly been described for  $\alpha$ -synuclein in adult mice [104]. Adult rats injected intrastrially with 10  $\mu$ g GDNF after one week show an increase of dopamine release and reuptake with two 20-min infusions of K(+) (70 mM), as measured by microdialysis [105]. Chronic infusions of 7.5  $\mu$ g of GDNF per day for 2 months into the right lateral ventricle in 21–27 year old monkeys increased stimulus-evoked release of dopamine significantly in the SN and striatum and also increased hand movement speed up to 40% [106]. GDNF (3–30 ng/ml) increased dopamine release from K(+) stimulated synaptosomes (20 mM, 2 min) and from electrically stimulated rat striatal slices (2 Hz, 2 min), an effect dependent upon tonic adenosine A(2A) receptor activation [107]. Single bilateral injections of 10 mg of GDNF (5 mg each side) into the striatum of postnatal day 2 (P2) rats produced forelimb hyperflexure, clawed toes of all limbs, a kinked tail, increased midbrain dopamine levels, and enhanced TH activity [108]. GDNF/Ret signaling was shown using the human neuroblastoma cell line TGW to increase mRNA and protein levels of TH [109]. Also striatal GDNF injection (100  $\mu$ g) in young and 2 year old rats enhanced expression and phosphorylation dependent activation of TH in the SN and striatum and increased both amphetamine- and potassium-evoked dopamine

release [110]. Acute GDNF stimulation of midbrain DA neuron cultures increased the expression of the transcription factors EGR1 and TIEG, and ERK or PKA activation. ERK or PKA activation increased expression of caveolin1 and calcineurin subunits ppp3R1 and ppp3CB, while calcium-calmodulin-dependent protein kinase II beta isoform (CaMKIIbeta) and the glycogen synthase kinase 3-beta (gsk3beta) expression, involved in neuronal apoptosis, was decreased [111]. GDNF might reduce dopamine reuptake by stimulating the internalization of the protein complex containing the GDNF receptor Ret, the adaptor protein and Rho-family guanine nucleotide exchange factor Vav2, and the dopamine transporter (DAT) [112]. The influence of GDNF on dopamine and on the electrophysiological properties of midbrain DA neurons is important because modulation of activity pattern has emerged as a crucial factor for differential midbrain DA neuron function and vulnerability to degeneration in PD [113–115]. Postsynaptic dopamine-mediated responses of GABAergic medium spiny neurons in the striatum or other midbrain DA projections areas are critically dependent not only on the binding to respective G-protein coupled stimulatory (D1, D5) or inhibitory (D2, D3, D4) dopamine receptors [116], the presynaptic activity of the dopamine transporter (DAT), the monoamine oxidases MAOA/MAOB and the catechol-O-methyltransferase (COMT) [117], but also depend on the amount of dopamine released from presynaptic dopamine vesicles in response to their activity pattern [118]. Thus, due to its stimulation of dopamine release, GDNF alters complex dopamine-dependent behavior, e.g. voluntary movement, reward/reinforcement/aversive behavior, cognition, and stress-responses [1,2].

In this light, it is interesting to note that epigenetics (specially histone modifications and DNA methylation of the *Gdnf* gene promoter in the ventral striatum) modulates the susceptibility and adaptation to chronic stress of adult wild-type mice, and that GDNF expression can normalize behavior in these chronically stressed mice [119]. GDNF infusion (2.5–10 µg/day) into the VTA of adult mice has also been reported to specifically block biochemical and behavioral adaptations to chronic cocaine, morphine and alcohol exposure [120,121].

In neurotoxic PD rodent and monkey models generated by administering drugs such as MPTP or 6-hydroxy-dopamine (6-OHDA) [122,123], GDNF protects SN DA neurons from degeneration, if provided before this neurotoxic lesion [124–126]. On previously lesioned rodents and monkeys, GDNF provided a neurorestorative function on midbrain DA neurons, in particular of the mesostriatal projection, by resprouting of remaining axonal branches and formation of new synapses [124–127]. While it is clear that GDNF is beneficial in animal models with DA system alterations, GDNF treatment of PD patients was found in clinical phase II trials to be safe but did not show efficacy. This is most likely due to technical problems and the selection of advanced PD patients with only few remaining DA neurons that would be able to respond [21,54,128,129]. The same holds true for clinical trials with neurturin. The neurturin receptor GFR $\alpha$ 2 is not expressed on DA neurons, but neurturin can also bind to GFR $\alpha$ 1 with less affinity. This may allow neurturin to activate DA neurons directly, despite the lack of its high-affinity receptor. Alternatively, a non-cell autonomous function for neurturin would need to be proposed to explain this result [45,54,129]. The clinical trials will not be discussed here in detail as they have recently been summarized in several excellent reviews [21,54,128,129].

In summary, there is clear evidence that GDNF supports the normal physiology of midbrain DA neurons and that GDNF is beneficial in animal models of DA dysfunction. However, it is still uncertain whether GDNF is crucial for development and/or maintenance of midbrain DA neurons, and if GDNF offers a neuroprotective therapeutic strategy for SN DA neurons in human PD patients.

#### 4. GDNF/GFR $\alpha$ 1 can mediate its actions through distinct transmembrane receptors in midbrain DA neurons

In DA neurons in culture, GDNF can activate different signaling cascades and can utilize a complex signaling network consisting of several binding and signaling partners [37]. As discussed above and illustrated in Fig. 2, GDNF binds to GFR $\alpha$ 1 and this complex then recruits other receptors, in particular Ret [48,49,50], but also NCAM [51–53], integrins (e.g. integrin  $\alpha$ V and  $\beta$ 1) [52,130], syndecan-3 [67], or N-cadherin [131]. In accordance with the variety of possible downstream interaction partners of GDNF/GFR $\alpha$ 1, GFR $\alpha$ 1 mRNA is strongly expressed in many adult brain regions, while Ret mRNA expression is mainly restricted to the midbrain, cerebellum, pons and thalamus [50,70,132–135]. In the midbrain DA system Ret protein is only expressed in DA neurons [136–139] while all other GDNF receptors are additionally found in postsynaptic neurons and use a variety of different downstream signaling components [17,140]. It is still not understood which functions of GDNF in DA neurons are mediated by which GDNF receptor. And it is also unknown how important these GDNF receptors are in the DA system in vivo as cell adhesion molecules or cell recognition receptors independent of their GDNF receptor function [140]. The functions of GDNF/GFR $\alpha$ 1/Ret signaling are best understood in midbrain DA neurons, and will be summarized in the following section.

#### 5. Function of the receptor tyrosine kinase Ret in the midbrain DA system

As highlighted above, the GDNF receptor Ret is expressed in all DA neurons during development and maintenance, as well as in motor neurons, somatic sensory neurons, enteric neurons, and sympathetic and parasympathetic neurons [17]. Accordingly, Ret signaling is involved in a variety of different functions, including cell survival, differentiation, proliferation, migration, chemotaxis, branching, morphogenesis, neurite outgrowth, axon guidance and synaptic plasticity [17,141].

Ret is alternatively spliced and there are at least three isoforms, with Ret9 (1072 amino acids) and Ret51 (1114 amino acids) being more abundant than Ret43 (1106 amino acids). The three isoforms are identical until Tyr 1062 and differ only at the C-terminus [142–145]. The last amino acids at the C-terminus of Ret9 are encoded by intron 19, for Ret51 in exon 20 and for Ret43 in exon 21 [144]. Interestingly, the Ret knockout lethality and kidney agenesis can be rescued by a Ret9 transgene, but not a Ret51 transgene [146]. Mice engineered to express only Ret51 die as neonates or young adults that exhibit severe growth retardation and kidney abnormalities. Mice expressing only Ret9 are viable and appear to be normal [146].

Binding of GDNF/GFR $\alpha$ 1 to Ret leads to homo-dimerization of Ret and subsequent autophosphorylation of certain tyrosine residues within its intracellular domain. Ret9 and Ret51 isoforms are phosphorylated with different preference on at least 12 tyrosines: Tyr687, Tyr752, Tyr806, Tyr809, Tyr826, Tyr900, Tyr905, Tyr928, Tyr981, Tyr1015, and Tyr1062. Ret51 is also phosphorylated on Tyr1096 [147–151]. The phosphotyrosines of Ret are docking sites for adapter proteins that activate different intracellular signaling cascades [17,21,141,151,152] (Fig. 2 and 3). For example, phospho-Tyr905 can bind Grb7/10; phospho-Tyr981 binds to Src (Raf/Ras and PI3K/Akt signaling); phospho-Tyr1015 binds to PLC $\gamma$  (Ca<sup>2+</sup> release, PKC activation, neurotransmission); phospho-Tyr1062 recruits Enigma, IRS1/2, DOK1/2/4/5/6, Shank3, FRS2 and Shc1/GRB2/GAP1/2 (which activates PI3K/Akt/NF- $\kappa$ B for mediating cell survival, proliferation and neurotransmission, PI3K/Rac induces differentiation, SOS/Ras/MAPK stimulates cell survival, growth,

differentiation and neurogenesis, JNK, p38MAPK and ERK5 signaling), FRS2/GRB2/SHP-2 (RAS/MAPK signaling); and phospho-Tyr1096 can bind GRB2 (RAS/MAPK and PI3K/Akt signaling) [17,153,154]. Ser696 might be phosphorylated by cAMP-dependent PKA and prevents phosphorylation of Tyr 687 that blocks Rac mediated cytoskeletal rearrangements [153]. The differing signaling properties of Ret isoforms were also demonstrated in mice; kidney defects were observed when Tyr1015 of Ret9 or Ret51 and Tyr1062 of Ret9 were mutated to the non-phosphorylatable phenylalanine, but were not observed in mice with a Ret51 Tyr1062 mutation [154]. These mice were already used to analyze Ret signaling events for sympathetic neuron survival [155] and are an excellent tool to address the open question which downstream signaling cascades of Ret are required *in vivo* for DA cell survival and function. It is also largely unknown which of the above mentioned adaptor proteins of the Ret receptor are really important *in vivo* in DA neurons.

The GPI anchor localizes GFR $\alpha$ 1 to lipid rafts in the plasma membrane, which leads to the recruitment of Ret into lipid rafts after GDNF binding [156,157]. Phospholipase or protease cleavage can produce soluble GFR $\alpha$ 1 that can bind GDNF and activate Ret outside lipid rafts [17]. In addition, Ret activated by soluble GFR $\alpha$ s can be recruited to lipid rafts through Tyr1062 phosphorylation [158]. Ret can signal inside lipid rafts for example through the membrane-tethered Src (Src-homologous and collagen-like protein) protein and the lipid-anchored adaptor protein FRS2 (fibroblast growth factor receptor substrate 2), leading to Ras/MAPK activation. Ret outside lipid rafts can bind the adaptor protein SHC and can prolong activation of the PI3K/Akt signaling cascade [158]. For full MAPK (but not Akt) activation, internalization of the Ret receptor through clathrin-coated pits is required in addition to lipid raft recruitment [159]. The protein stability of Ret9 and Ret51 has been reported to be different, but further experiments are needed to clarify this issue in DA neurons [159–162]. Members of the Cbl family of E3 ubiquitin ligases can ubiquitinate Ret at Lys 1060 and Lys 1107 [160,163,164] and thereby mark Ret for lysosomal [160,162] and/or proteasomal degradation [161,165].

Not much is known currently about the precise signaling properties and events of Ret in DA neurons. In the DA-like cell line SH-SY5Y, Ret9 seems to be expressed at higher levels than Ret51, to accumulate in the Golgi apparatus and after stimulation and internalization, to be mainly degraded in lysosomes. Ret51 is efficiently delivered to the plasma membrane and partially recycled back to the plasma membrane from the endosomal compartment [159,162]. Despite the fact that Ret is expressed in mice from embryonic day 10 and is an early embryonic marker for postmitotic DA neurons [34,166], constitutive Ret knockout mice which die shortly after birth without kidneys show normal numbers of midbrain DA neurons [132,167,168]. This suggests that Ret, like GDNF and GFR $\alpha$ 1, is dispensable in DA neurons during embryonic development.

To study Ret function in the adult midbrain DA system *in vivo*, two laboratories generated conditional DA neuron-specific Ret knockout mice by deleting floxed Ret in the DATCre knock-in mouse background [169]. Both laboratories found no significant loss of midbrain DA neurons in these mice till the age of 9 months [24,137]. The early postnatal development of the DA system at P4 and P16 has not yet been systematically investigated in Ret- or GDNF-deficient mice, but if there are any alterations, they seem to be compensated during adulthood [24,91,137,139]. One laboratory aged the conditional DA neuron-specific Ret mice to two years and detected a significant and progressive loss of DA neurons specifically in the SN but not in nearby VTA from age one to two years [24]. SN DA neurons were reduced by 25% in one-year-old mice and 38% in two-year-old mice and this was accompanied by an inflammation in the SN. Furthermore, in the striatum of these

mice, reduced axonal DA innervation was first detected at the age of 9 months, followed by additional postsynaptic neuron loss and reduced dopamine release at 12 months. Ultimately, a gliosis and inflammation was present in two-year-old mice [24]. In PD, a corresponding dying back neurodegeneration process has been postulated to start with the loss of DA innervation in the striatum, followed by SN DA cell body loss accompanied by inflammation and gliosis within the SN [13,170,171]. As the DA neuron-specific Ret knockout mice show a similar mesostriatal dying back process, they might provide a pre-symptomatic model of PD. A similar selective SN DA neuron degeneration phenotype was found in Nestin-Cre/Ret mice lacking Ret in almost the complete nervous system [24] in contrast to the Nestin-Cre/GDNF mice which showed no DA phenotype [91]. Recently the mild but important maintenance function of Ret for aging DA neurons of the SN was confirmed in a third mouse model [139] using DATCreBAC mice which express the Cre recombinase from a DAT locus on a bacmid construct (BAC) [172,173]. In these mice also the mean SN DA soma size was reduced to about 84% in adult mice [139]. Furthermore, besides striatal dopamine levels and motor activity, mitochondrial complex I activity was reduced. The reduced complex I activity is an important link to selective SN DA degeneration, as pharmacological complex I inhibition (e.g. by MPTP, see above) directly induces Parkinsonism in mice and humans [122,139]. Experiments in SH-SY5Y cells suggest that Ret supports mitochondrial integrity and ATP production through the PI3K/NF- $\kappa$ B pathway [139]. It cannot yet be ruled out that the mild Ret conditional knockout mouse phenotype that has been described might be due to early embryonic compensation. The DAT and Nestin promoters drive Cre expression and thus Ret deletion already during the early prenatal development [174]. A mouse model with an inducible DA neuron specific postnatal deletion of Ret is needed to address this issue.

An important role for Ret in the DA system is further supported by investigating mice expressing a constitutively active form of Ret with Met918 mutated to threonine in the Ret kinase domain, called MEN2B mutation, integrated into the endogenous genomic Ret locus [175]. This mutation is found in Multiple endocrine neoplasia 2 type B (MEN2B) patients with medullary thyroid carcinoma, pheochromocytoma, and hyperparathyroidism [176]. MEN2B signaling was reported to be similar to the wildtype Ret protein but the MEN2B protein seems to be activated by autophosphorylation in the endoplasmic reticulum (ER) before even reaching the cell surface [176–178]. Because GDNF can hyperactivate MEN2B [178,179], endogenous GDNF might additionally stimulate MEN2B activity in mice. Homozygous MEN2B mice respond to cocaine with an increased locomotor activity and longer travelled distances compared to wild-type mice [180], suggesting a supersensitive midbrain DA system. In line with this, and consistent with a midbrain DA development and/or maintenance promoting function of GDNF/Ret signaling, homozygous MEN2B mice were found to establish about 26% more midbrain DA neurons until adulthood, specifically in the SN but not in the VTA region [180]. The MEN2B mice also show more striatal TH and DAT protein levels, an elevated dopamine synthesis rate, more striatal dopamine and dopamine metabolites, a larger releasable dopamine pool in the striatum, and an increased activity-dependent dopamine release capacity in SN DA neurons [180,181]. It remains an open question if reduced cell death of DA neurons or increased proliferation and altered differentiation of DA progenitor cells lead to the enlarged DA system in MEN2B mice. This *in vivo* MEN2B phenotype supports the view that Ret activation is the primary target of GDNF to trigger DA cell survival. The SN specific DA neuron phenotype of MEN2B mice and Ret-deficient mice suggest that Ret activity is limiting for the establishment and maintenance of SN DA neurons in mice and that tight regulation of Ret signaling is required

to maintain the DA system in a normal state. Ret signaling seems a critical modulator for development and maintenance of SN DA neurons, which are highly vulnerable to degeneration in PD. Thus manipulation of Ret signaling might offer a molecular tool for a pharmacological neuroprotective strategy for PD patients.

In this context it is interesting to mention, that in the sub-chronic MPTP-model of PD [122], young adult Ret-deficient mice not yet showing a DA neurodegeneration phenotype did not display an increased DA cell vulnerability to the toxin. However, the axonal resprouting within the striatum was completely abolished [182]. In contrast, in the 6-OHDA model of PD, young adult MEN2B mice challenged with 6-OHDA in the striatum and analyzed after 3 weeks were shown to have more surviving SN DA neurons but the striatal dopamine loss was similar to 6-OHDA treated wildtype mice [183]. The striatal DA innervation at 3 weeks and the possible resprouting effect occurring over several months in the 6-OHDA model was not assessed in MEN2B mice, but was shown to be enhanced in wild-type mice with viral GDNF expression [184]. Further experiments are needed to better understand the neuroprotective and regenerative effects of MEN2B and Ret, respectively, and to determine whether GDNF can still provide its neuroprotective and regenerative effects in these mouse models.

## 6. Possible role of GDNF/Ret in PD pathology and therapy

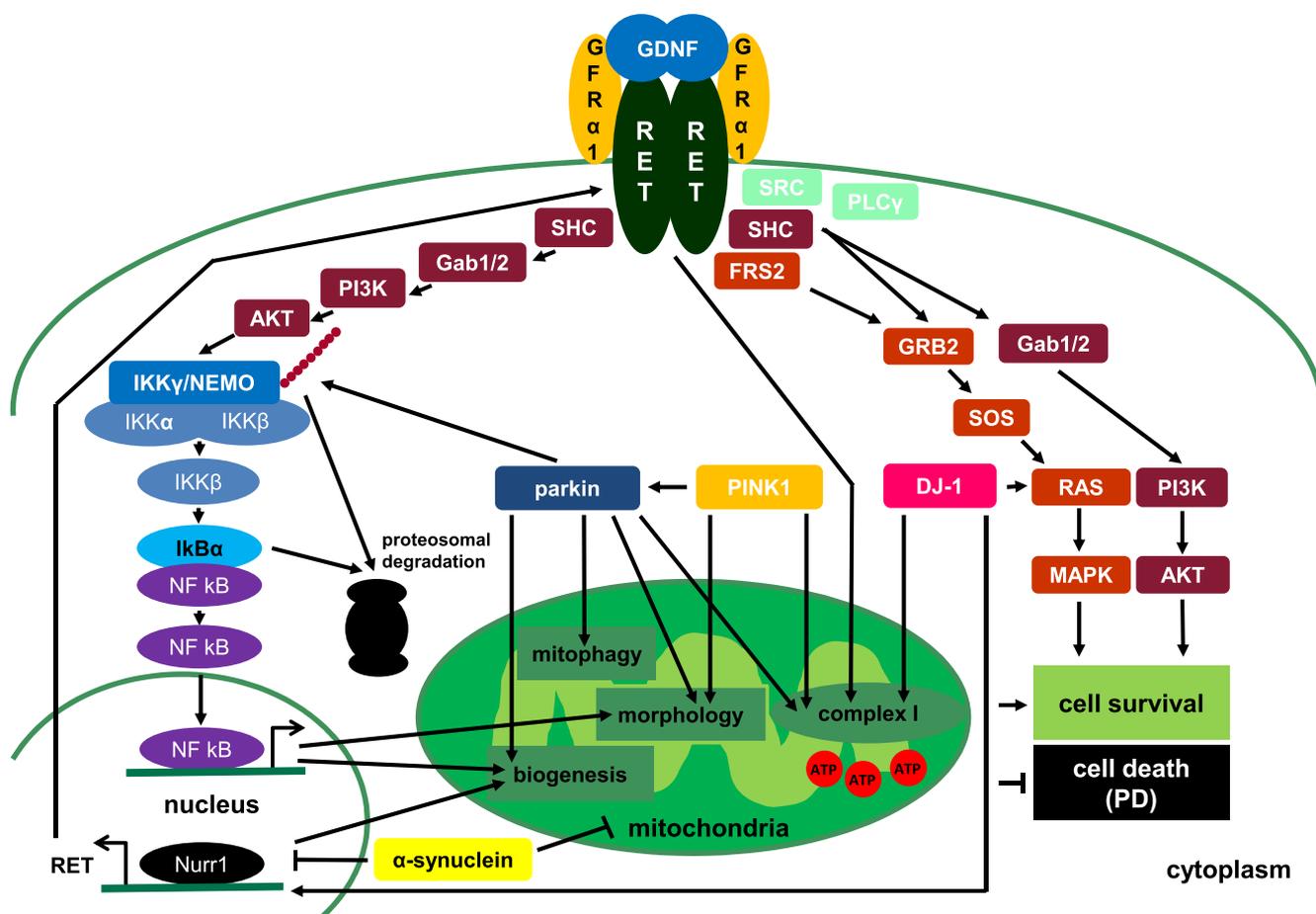
Despite the Ret knockout and MEN2B mouse phenotypes in the SN DA neurons, so far no Mendelian mutations in the genes encoding GDNF, GFR $\alpha$ 1, or Ret have been found in PD patients. This is most likely due to the essential function of GDNF/Ret signaling during mammalian development outside the midbrain DA system (e.g. kidney development). Most homozygotes loss-of-function mutations of GDNF, GFR $\alpha$ 1, and Ret hitting the whole body might therefore be lethal. Some loss- and gain-of-function mutations of Ret have been well demonstrated to be viable in humans but lead to severe diseases. These Ret mutations will be mentioned here, although their brains have not yet been analyzed for the above mentioned alterations. It has not yet been investigated whether the brain is affected and might show an enlarged midbrain DA system in MEN2B patients (autosomal dominant inherited or *de novo* mutation in the sperm from the father) or if this makes them less prone to develop PD; in the past, a patient's life expectation was short, which may have hindered this analysis [185]. Likewise, the short life of Hirschsprung disease patients, 50% of whom also carry a Ret loss-of-function mutation (autosomal dominant inherited or a *de novo* mutation), might have prevented analysis of aged brains and their susceptibility to develop PD [186]. One single-nucleotide polymorphism (SNP) analysis revealed a linkage of Ret with PD [187], while other studies found no linkage of GDNF and Ret SNPs with PD [188,189]. In addition, one compound heterozygous PD patient was described who carried a mutation in Ret and in the neuroprotective immunophilin FKBP52 (also known as FKPB4). These mutations are sufficient to disrupt the RET51/FKBP52 complex formation normally triggered by GDNF stimulation in cultured cells [190].

Nevertheless, as illustrated in Fig. 3 and detailed below, increasing evidence suggests that the protein network regulated by GDNF/Ret signaling is overlapping or even identical with the protein network that is altered in PD patients. The first reported crosstalk between these pathway components was of Ret with a protein mutated in some rare familial forms of PD, the chaperon DJ-1 (PARK7, autosomal recessive loss of function mutation). DJ-1 was found to be neuroprotective against oxidative stress and environmental toxins and localized to mitochondria [14,191,192]. Ret and DJ-1 double-deficient mice showed an accelerated degeneration of SN DA neurons compared to the mild loss observed in

Ret-deficient mice and the absence of histological alteration in DJ-1 knockout mice [193]. Loss of DJ-1 in the Ret-deficient background did not, however, worsen the striatal DA innervation loss of Ret-deficient mice. Using the *Drosophila* eye as a genetic screening tool, DJ-1 and Ret were found to genetically interact and to specifically activate the Ras/MAPK, but not the PI3K/Akt signaling cascade [193]. Also Ret and DJ-1 proteins are not known to physically interact and bind to each other their signaling converges and overlaps in activating the Ras/MAPK pathway. In addition, inducible loss of DJ-1 in SH-SY5Y cells was shown to trigger hypoxia and the production of reactive oxygen species that stabilize the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ). HIF-1 $\alpha$  expression was correlated with down-regulation of Ret protein levels, while overexpression of DJ-1 increased Ret protein levels [194].

Recently GDNF/Ret signaling was also linked to  $\alpha$ -synuclein (PARK1 and PARK4, autosomal dominant mutation), a ubiquitously expressed protein of the brain and the main component of Lewy bodies and neurites (protein aggregates frequently found in PD patients).  $\alpha$ -Synuclein gain-of-function mutations or genomic  $\alpha$ -synuclein gene (SNCA) duplications can lead to PD [14,195]. While the physiological function of  $\alpha$ -synuclein is still not entirely clear, it is known to ensure synapse function by associating with DA vesicles. Misfolded and aggregated  $\alpha$ -synuclein seems to be toxic for mitochondria, general protein expression, degradation of organelles and neighboring cells [196,197]. In a rat PD model, viral overexpression of GDNF in the SN or in the striatum was not protective against viral overexpression of human wild-type  $\alpha$ -synuclein in the SN [198–200]. In this model, the orphan nuclear receptor Nurr1 and Ret, its downstream target, were found to be transcriptionally down-regulated by  $\alpha$ -synuclein accumulation. Reduced Ret protein levels might have prevented the GDNF-induced survival response in midbrain DA neurons [199]. This study also linked GDNF/Ret signaling to Nurr1 (autosomal recessive loss of function mutation), another protein found to be mutated in a rare familial form of PD [201]. Together with the CoREST repressor complex, sumoylated Nurr1 was previously shown to repress NF- $\kappa$ B induced inflammatory gene expression in astrocytes and microglia in lipopolysaccharide or  $\alpha$ -synuclein overexpression models of PD, and thereby to prevent SN DA degeneration [202,203]. Nurr1 is required for maintenance of DA neurons and is proposed to stimulate mitochondrial gene and Ret expression [199,204–206]. The negative effect of  $\alpha$ -synuclein overexpression on Nurr1 and Ret expression was thought to be a possible explanation for the observation that GDNF has shown no beneficial effect in clinical phase II trials on PD patients, who might have high  $\alpha$ -synuclein protein levels [54,129,207]. However, so far no [207] or only mild [199] reductions of Ret mRNA and protein levels were found in the SN of PD patients, making this hypothesis less likely.

Genetic crosstalk has also been reported between Ret and another PD-associated protein, the PTEN-induced putative kinase 1 (PINK1) (PARK6, autosomal recessive loss of function mutation), a mitochondria-associated Ser/Thr kinase involved in initiating mitophagy for removal of damaged mitochondria [14,192,208,209]. PINK1 has been shown to phosphorylate ubiquitin and parkin and thereby activate and recruit parkin to mitochondria [210–212]. Parkin (PARK2, autosomal recessive loss of function mutation), an E3 ubiquitin ligase that insures mitochondrial integrity by stimulating mitogenesis and regulating mitochondrial fission and fusion, is also a PD-associated protein. Together with PINK1, parkin promotes autophagy of damaged and depolarized mitochondria [14,192,209,213]. Co-expressing *Drosophila* Ret with the MEN2B mutation (Met918Thr) in the *Drosophila* indirect flight muscle rescued the mitochondrial morphology and muscle degeneration phenotype in PINK1 loss-of-function mutant flies [214]. Expressing the *Drosophila* MEN2B



**Fig. 3.** Signaling network of GDNF/Ret with proteins encoded by genes mutated in some familial forms of Parkinson disease. DJ-1 (PARK7) is involved in GDNF/Ret cell survival signaling through the RAS/MAPK pathway and stimulates Ret expression.  $\alpha$ -Synuclein (PARK1 and 4) inhibits Nurr1 and Ret expression. PINK1 (PARK6) and GDNF/Ret together control mitochondrial morphology and complex I activity in thorax extracts from PINK1 deficient *Drosophila* [214]. Surprisingly, fly MEN2B did not rescue the muscle degeneration, mitochondrial morphological alterations in muscle and DA cells, or the ATP generation and complex I activity reduction in the thorax of parkin-deficient flies. Neither carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) induced parkin recruitment to mitochondria, nor mitophagy in parkin overexpressing SH-SY5Y cells, was prevented by MEN2B overexpression or GDNF/GFR $\alpha$ 1 stimulation [214]. The work described above clearly showed beneficial effects of GDNF/Ret signaling in PINK1 loss-of-function conditions, but it left open the question of whether GDNF/Ret signaling intersects with that of parkin, which has now been addressed in a publication this year.

protein in flies also prevented the mitochondrial morphological alterations in *Drosophila* PINK1-deficient DA neurons and in mammalian SH-SY5Y cells. In addition, MEN2B expression rescued the oxygen consumption in PINK1 knock-down SH-SY5Y cells and also the reduced ATP levels and complex I activity in thorax extracts from PINK1 deficient *Drosophila* [214]. Surprisingly, fly MEN2B did not rescue the muscle degeneration, mitochondrial morphological alterations in muscle and DA cells, or the ATP generation and complex I activity reduction in the thorax of parkin-deficient flies. Neither carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) induced parkin recruitment to mitochondria, nor mitophagy in parkin overexpressing SH-SY5Y cells, was prevented by MEN2B overexpression or GDNF/GFR $\alpha$ 1 stimulation [214]. The work described above clearly showed beneficial effects of GDNF/Ret signaling in PINK1 loss-of-function conditions, but it left open the question of whether GDNF/Ret signaling intersects with that of parkin, which has now been addressed in a publication this year.

Mice that were double-deficient for parkin and Ret in midbrain DA neurons showed an accelerated SN DA cell and striatal DA innervation loss compared to the moderate degeneration in Ret-deficient mice and absence of alterations in parkin-deficient mice [139]. The down-regulation of parkin and Ret simultaneously led to impaired mitochondrial integrity in mice and SH-SY5Y cells. On the other hand, elevated expression of parkin protected the DA system of aged Ret-deficient mice [139]. Furthermore, in SH-SY5Y cells, parkin and GDNF/Ret could substitute for each other

to ensure proper mitochondrial function and morphology by converging signaling cascades that activate NF- $\kappa$ B. Ret is known to activate NF- $\kappa$ B through the PI3K pathway [139]. Taken together, this work revealed an essential *in vivo* survival function linking parkin and the GDNF/Ret signaling cascade to maintain both the highly vulnerable SN DA neurons and, in contrast to GDNF/Ret and DJ-1 crosstalk [193], also the DA innervation in the striatum [139,215].

In summary, GDNF/Ret seems to signal together with proteins encoded by PD-linked genes such as DJ-1, PINK1, parkin,  $\alpha$ -synuclein and Nurr1 in flies and mice. These studies emphasize the influence of GDNF/Ret and PD-associated proteins on mitochondria function, which is frequently altered in PD patients [216–218]. The intersection of GDNF/Ret with proteins found to be mutated in some familiar forms of PD are most likely just the tip of the iceberg and exciting undiscovered interactions can be found in the future.

## 7. Summary and future prospects

GDNF/GFR $\alpha$ 1/Ret signaling seems to be an important modulator of DA midbrain neuron development and maintenance under both physiological and pathophysiological conditions such as PD. GDNF and Ret can signal together as well as independently of each other. GDNF and Ret signaling shows a tight intersection with the network of proteins encoded by genes found to be mutated in familial

forms of PD to ensure mitochondrial integrity. Despite the great progress made in recent years, detailed functions and underlying molecular mechanisms of GDNF/GFR $\alpha$ 1/Ret signaling still need to be resolved.

When it was thought that GDNF was absolutely required for catecholaminergic neuron maintenance, and that Ret only promoted maintenance of a subpopulation of SN DA neurons, the analysis of compensatory mechanisms and alternative GDNF receptors seemed to be an urgent question. Considering that GDNF might be dispensable for midbrain DA system development and maintenance, an equally important question now seems to be which Ret ligand (if not GDNF) stimulates Ret to ensure survival of SN DA neurons during aging and in PD, and which additional neurotrophic factors might be essential for the midbrain DA system development and maintenance.

The findings summarized here support the on-going clinical phase I and II trials with GDNF and other GFR members, such as neurturin, for therapy for PD patients. As summarized in several recent reviews, GDNF and Ret hold still great promise for the treatment of PD, if the remaining technical problems might be overcome to show their full potential [21,54,128,129]. The critical task here is still to prove that the beneficial effects of GFLs described in many animal models of PD can be reproduced in human PD patients. In this context, identifying which GDNF receptors and downstream signaling cascades are involved in mediating the beneficial effects of exogenously provided GFLs might help to develop new and more efficient strategies to enhance neurotrophic signaling for DA system maintenance and regeneration.

In conclusion, the complex GDNF and Ret signaling events in DA neurons remain an exciting and promising field for basic and applied research with important therapeutic implications and many surprising findings are anticipated.

### Conflict of interest

The authors have declared that no conflict of interest exists.

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