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GDNF/RET signaling in dopamine neurons *in vivo*

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Abstract

The Glial cell-line derived neurotrophic factor (GDNF) and its canonical receptor Ret can signal both in tandem and separately to exert many vital functions in the midbrain dopamine system. It is known that Ret has effects on maintenance, physiology, protection and regeneration in the midbrain dopamine system, with the physiological functions of GDNF still somewhat unclear. Notwithstanding, Ret ligands such as GDNF are considered as a promising candidates for neuroprotection and/or regeneration in Parkinson disease (PD), although data from clinical trials is so far inconclusive. In this review, we discuss the current knowledge of GDNF/Ret signaling in the dopamine system *in vivo* as well as crosstalk with pathology-associated proteins and their signaling in mammals.

Introduction

Dopamine is a neurotransmitter produced by dopamine (DA) neurons in the midbrain. Dopamine is involved in a diverse range of functions throughout both the brain and body, including motor control, motivation, reward and emotional regulation (Bjorklund and Dunnett, 2007, Leknes and Tracey, 2008).

The DA system comprises of DA neuron cell bodies grouped in ventral midbrain in the retro-rubral field (RRF), ventral tegmental area (VTA) and substantia nigra (SN) (Fig. 1A,B). Axons from these areas project into the mesostriatal and mesocorticolimbic pathways (Bjorklund and Dunnett, 2007). As the name suggests, the mesostriatal pathway connects the SN and a portion of the VTA with the dorsal striatum (Fig. 1B,C). This pathway is particularly important in voluntary movement control. The mesocorticolimbic pathway is involved in emotional, cognitive and reward-based behaviors. It projects from the dorsal SN, VTA and RRF to the ventral striatum (putamen and caudate nucleus), cortex, hippocampus, habenula, septum, olfactory tubercle and nucleus accumbens (NAc). As the DA midbrain system is responsible for such a diverse range of functions, alterations can result in a number of neurological diseases. For example, degeneration of DA neurons in the SN results in a dopamine deficiency in the dorsal striatum, causing the motor pathology characteristic in Parkinson Disease (PD) (Fig. 1D) (Goedert et al., 2013, Obeso et al., 2001). As DA neurons are highly heterogeneous, a complex network of signaling pathways and events are involved in the development, maintenance and physiological functioning of the midbrain DA system. In recent years, neurotrophic factors, a multifarious group of polypeptides, have emerged as major players in the development and maintenance of the DA system (Lerner et al., 2015) (Airaksinen and Saarma, 2002). The neurotrophic factor Glial Cell Line-Derived Neurotrophic Factor (GDNF) is

the founding member of a group of GDNF Family Ligands (GFLs), also consisting of artemin, neurturin and persephin (Airaksinen and Saarma, 2002). GFLs generally signal by undergoing high affinity binding to a glycosylphosphatidylinositol (GPI)-linked GFRα members (1 to 4). GDNF, for example, binds to GFRα1, the only GFRα member expressed highly in DA neurons (Burazin and Gundlach, 1999, Yu et al., 1998, Sarabi et al., 2001). *GFRα2* mRNA but not GFRα2 protein was so far detected in non-DA cells in the mouse SN and striatum, unlike *GFRα3* mRNA, which was only found in the brain of mouse embryos (Golden et al., 1999, Yu et al., 1998, Trupp et al., 1998, Runeberg-Roos et al., 2016). These GFL-GFR complexes may subsequently activate either their canonical receptor, the receptor tyrosine kinase rearranged during transfection (Ret), or alternative receptors including neuronal cell adhesion molecule (NCAM), integrins, N-cadherin and syndecan-3 (Kramer and Liss, 2015) (Fig. 1D). There is not a one ligand one receptor relationship between GDNF and Ret, as both have alternative signaling partners also in the midbrain DA system (Kramer and Liss, 2015). In this review, we provide an update on the role of GDNF and Ret signaling in the midbrain DA system *in vivo* (Fig. 1E).

A) GDNF, GFRα1 and Ret expression in the midbrain dopamine system during development, adulthood, aging and PD

Since it was first isolated, GDNF has been considered to be a candidate target-derived neurotrophic factor involved in the development of SN dopamine neurons (Lin et al., 1993). Despite its name, GDNF is not expressed in the murine nervous system in glia cells but in neurons, especially in parvalbumin-positive (PV+) interneurons and cholinergic and somatostatin-positive interneurons in the striatum, as confirmed with mice carrying a lacZ gene in the GDNF locus (Gonzalez-Reyes et al., 2012, Pascual et al., 2008, Hidalgo-Figueroa et

al., 2012). This corroborates three studies in rats which found GDNF protein only in patches in the postnatal striatum (Lopez-Martin et al., 1999) and *GDNF* mRNA only in neuronal cells of the adult striatum (Oo et al., 2005) such as 50-75% of choline acetyl-transferase (ChAT) positive interneurons and a minority of medium spiny GABAergic interneurons (Bizon et al., 1999). *GDNF* mRNA can be detected in the striatum and midbrain of rats and mice during embryonic development (Nosrat et al., 1997, Golden et al., 1999). High amounts of *GDNF* mRNA are found in the striatum of mice and rats during the early postnatal period (Stromberg et al., 1993, Schaar et al., 1993, Blum and Weickert, 1995, Choi-Lundberg and Bohn, 1995), which decreases during adulthood (Hidalgo-Figueroa et al., 2012, Nosrat et al., 1996, Trupp et al., 1997, Golden et al., 1999, Pochon et al., 1997). GDNF is expressed in human and rodent brains as a pre-pro-protein with two different splice isoforms: a full-length transcript (pre-α-pro-GDNF) and a shorter transcript (pre-β-pro-GDNF) that lacks 78 bp in the region encoding 26 amino acids of the pro-domain (Lin et al., 1993, Trupp et al., 1995, Lonka-Nevalaita et al., 2010). In humans, an additional 4 isoforms and 2 antisense RNAs have been described (Schaar et al., 1994) (Airavaara et al., 2011). The pre-region is cleaved off in the endoplasmic reticulum and the pro-domain mainly in secretory vesicles leading to the same secreted mature GDNF protein (Penttinen et al., 2018). The pre-α-pro-GDNF isoforms seems to be the most abundant isoform in the DA system (striatum and substania nigra)(Airavaara et al., 2011). In adult human brains there are very low amounts of *GDNF* mRNA under normal conditions but increased amounts have been reported during diseases such as PD, not only in neurons but also astrocytes, microglia and macrophages (Springer et al., 1994, Hunot et al., 1996, Nakagawa and Schwartz, 2004, Backman et al., 2006, Duarte Azevedo et al., 2020). Despite these advances in understanding as to which cells produce GDNF in the rodent striatum, the

nature of GDNF-producing striatal neurons in humans remains elusive and the actual role of PV+ cells on nigrostriatal protection is yet to be determined.

GFRα1 and Ret mRNA and protein have been found to be expressed in rodent midbrain DA neurons from early embryonic development through to adulthood (Marcos and Pachnis, 1996, Treanor et al., 1996, Nosrat et al., 1997, Trupp et al., 1997, Golden et al., 1999, Airaksinen and Saarma, 2002). While Ret seems to be exclusively expressed in the midbrain DA system of rodents by DA neurons, GFRα1 may additionally be expressed by cells in the striatum (Kramer et al., 2007, Cho et al., 2004). Although the expression of GFRα1 and Ret spliced isoforms have not been studied in detail for the midbrain DA system, there appears to be more *GFRα1a* than *GFRα1b* and more *Ret9* than *Ret 43* and *Ret51* mRNA expressed in the mouse brain (Yoong et al., 2005). In a rat PD model generated by 6-hydroxydopamine (6-OHDA), a transient increase has been reported for *GFRα1* and *Ret* mRNA levels in the SN followed by a decrease (Marco et al., 2002). In an mouse PD model generated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Ret protein levels were found to be reduced in the striatum (Hirata and Kiuchi, 2007). In these studies, the reduced GFRα1 and Ret expression correlates with a loss of tyrosine hydroxylase (TH) positive DA neurons in the midbrain and their innervation of the striatum (Hirata and Kiuchi, 2007, Blesa and Przedborski, 2014).

Most reports suggest no alteration in GFRα1 and Ret expression in aging rodents and monkeys (Kramer et al., 2007, Dass et al., 2006, Walker et al., 1998). Surprisingly, one publication claimed that in aging rats, a downregulation of the mRNA encoding the transcription factor Nurr1 in the SN, which is also needed for Ret expression but no *Ret* mRNA level changes in the SN and even a significant *Ret* mRNA increase in the striatum (Parkinson et al., 2015). In healthy human brains and *Ret* mRNA and protein levels in the midbrain and putamen seem to not be significantly altered with age (Backman et al., 2006, Alladi et al., 2010). However,

open discussion still remains as to how far Ret protein levels may be reduced in PD patients, if at all (Decressac et al., 2012, Hoffer and Harvey, 2011, Su et al., 2017, Chu et al., 2020). Comparing Ret protein expression in remaining substantia nigra DA neurons of PD patients with PD patients treated with a neurturin expressing virus and age-matched controls did not reveal an obvious difference (Chu et al., 2020).

B) GDNF, GFRα1 and Ret function in the midbrain DA during development, adulthood and aging

To determine the physiological function of GDNF, GFRα1 and Ret in the midbrain DA system, several rodent models were treated with GDNF and different knockout and transgenic mice were generated and analyzed.

Burke *et al.* defined 2 postnatal cell death peaks in SN DA of rodents at postnatal day 2 and 14 and suggested by injecting GDNF and GDNF neutralizing antibodies that GDNF is limiting and protective during the first cell death period (Burke, 2004, Oo et al., 2003). Interestingly, mice transgenically expressing a monomeric form of GDNF in the forebrain exhibited double the amount of DA in the VTA but normal numbers of SN DA neurons at adulthood (Kholodilov et al., 2004). In these mice, the SN DA neuron number was only doubled after the first postnatal cell death period but was not maintained during adulthood (Kholodilov et al., 2004). Heterozygous hypermorphic GDNF mice encoding one copy of a more stable *GDNF* mRNA due to a modified 3' untranslated region (UTR) (Kumar et al., 2015) express the double amount of *GDNF* mRNA and protein in the striatum and *GDNF* mRNA was largely retained in PV+ expressing inhibitory neurons, as reported previously for wildtype mice (Gonzalez-Reyes et al., 2012, Pascual et al., 2008, Hidalgo-Figueroa et al., 2012). From postnatal day 7.5 through to adulthood, these mice were reported to have 15% more SN DA neurons and DA innervation

of the striatum as well as increased total DA levels, DA release and reuptake (Kumar et al., 2015). The heterozygous hypermorphic GDNF mice were also shown to have an increased response to amphetamine, are more sensitive to striatal 6-OHDA injection, but less susceptible to unilateral lactacystin injection proximally superior to the SN (Kumar et al., 2015). In a follow up study, amphetamine-induced conditional place preference was found not to be altered in the GDNF hypermorphic mice (Kopra et al., 2018). However, heterozygous hypermorphic mice seemed to perform better in motor function tests but did not exhibit psychiatric disease related phenotypes (Matlik et al., 2018).

GDNF knockout mice die shortly post-partum due to renal agenesis but with a normal DA system (Sanchez et al., 1996, Pichel et al., 1996, Moore et al., 1996). A group employing a heterozygous GDNF mouse model reported a 42% reduction in GDNF levels accompanied by a 20% loss of SN DA neurons in 12 month old mice but with no further neuron loss in 20 month old mice (Boger et al., 2006). Twelve-month-old GDNF heterozygous mice also exhibited a Parkinson–like spontaneous locomotion change in a three-dimensional rodent motion analysis (Karakostas et al., 2014). These heterozygous, GDNF-deficient mice were also more sensitive to methamphetamine (Boger et al., 2007). However, another group reported no alterations in response to amphetamine but a mild impairment of performance in the spatial Morris water maze (Gerlai et al., 2001). An enhanced reward value of sucrose was also described for heterozygous GDNF deficient mice (Griffin et al., 2006). While no changes in the response to cocaine was observed in heterozygous GDNF knockout mice, these mice seem to have more immediate early gene c-Fos positive neurons in the dorsal striatum and nucleus accumbens (Airavaara et al., 2004). More recently, the heterozygous GDNF deficient mice were also suggested to have an impaired latent inhibition to chronic stress and less active (c-Fos positive) neurons in the nucleus accumbens under stress (Buhusi et al., 2017).The first

conditional GDNF knockout mouse in the adult DA system was made using a tamoxifen Esr1-Cre system (Hayashi and McMahon, 2002) (0.2 mg tamoxifen per day and per gram body weight intraperitoneally for four consecutive days). Surprisingly, the authors claimed an absolute requirement of GDNF for adult catecholaminergic neuron survival (Pascual et al., 2008). In these mice they observed a 60% reduction of *GDNF* mRNA and GDNF protein in the striatum, leading to 60-70% degeneration of SN and VTA DA neurons 7 months after *GDNF* gene recombination (Pascual et al., 2008). These mice were reported to also have an almost 100% loss of noradrenergic neurons in the locus coeruleus, a brain region which has also been shown to exhibit neurodegeneration in PD (Pascual et al., 2008). However, another group has used the same Esr1-Cre mice (6–10 mg of tamoxifen was injected per mouse), as well as Nestin-Cre mice and an adeno-associated virus type 5 encoding Cre to recombine their floxed *GDNF* allele. They observed that in all 3 mouse models, GDNF was not required for catecholaminergic neuron survival *in vivo* (Kopra et al., 2015). Despite a stronger GDNF protein reduction, no loss of midbrain DA neurons in the SN and VTA or noradrenaline neurons of the locus coeruleus was observed in these 3 mouse models (Kopra et al., 2015). This very well controlled study strongly supports the idea of a dispensable function of GDNF for catecholaminergic neuron development and maintenance in the mouse (Kopra et al., 2015). Unfortunately, the comment by Pascual and Lopez-Barneo (Pascual and Lopez-Barneo, 2015) published in the same Nature Neuroscience issue as the Kopra *et al.* paper (Kopra et al., 2015), did not clarify why their results were so different. In a second approach the Lopez-Barneo laboratory tried without success to delete GDNF with a parvalbumin promoter-driven Cre line (Hippenmeyer et al., 2005) and partially successful with a ubiquitin C promoter-driven Cre recombinase-mutated esterogen receptor fusion protein (UBC-Cre-ERT2) (Ruzankina et al., 2007) treated again with a high amount of tamoxifen (0.18 mg tamoxifen per day and per

gram body weight intraperitoneally for five consecutive days) (Enterria-Morales et al., 2020). The authors reported that their tamoxifen treatment of the UBC-Cre-ERT2/GDNF conditional mice resulted in a mouse death rate of 11-28% (11% for heterozygous floxed GDNF mice, 14.3% in heterozygous whole body GDNF knockout mice and 28% in mice carrying the UBC-Cre-ERT2 construct in combination with homozygous whole body GDNF knockout), 60% loss of *GDNF* mRNA and protein in the striatum and a mild and moderate loss of a subpopulation of DA neurons of the SN and noradrenergic neurons in the locus coeruleus, respectively (Enterria-Morales et al., 2020). Papers on increased tamoxifen toxicity in cells lacking GDNF/Ret signaling (Plaza-Menacho et al., 2010) and in combination with CreER (Schmidt-Supprian and Rajewsky, 2007, Takebayashi et al., 2008) increase the possibility of a technical or methodological artefact in the two papers from the Lopez-Barneo laboratory (Pascual et al., 2008, Enterria-Morales et al., 2020). Further research is needed to resolve this issue. In a follow up study by Kopra *et al.,* conditional GDNF deficient mice were shown to have a reduced amphetamine induced locomotor response and increased dopamine transporter protein levels (Kopra et al., 2017). Taken together, GDNF may not be essential for development and maintenance of the midbrain DA system but could still be important for the normal physiology of these neurons and a possible therapeutic agent for PD patients.

Similar to GDNF knockout mice, GFRα1 knockout mice also die shortly after birth due to renal agenesis but with a normal DA system (Cacalano et al., 1998, Enomoto et al., 1998) (Tomac et al., 2000). It was reported that 18-month-old heterozygous GDNF mice have 30% fewer SN DA neurons and 24% fewer VTA DA neurons. Dopamine levels in the striatum and motor activity were lower and the stimulatory effects of the DA agonist were enhanced (Zaman et al., 2008). Twenty-six month old GFRα1 heterozygous mice exhibit reduced MPTP induced locomotion and greater expression of inflammatory markers (CD45 immunostaining and

phosphorylated p38 MAPK) in the SN (Boger et al., 2008). Although a floxed allele of *GFRα1* was generated a midbrain DA neuron knockout mouse was not produced or analyzed (Uesaka et al., 2007). More recently, it was reported in rats that GFRα1 expressing DA neurons in the SN seem to decrease with age and that physical exercise can increase GFRα1 expression as well as SN DA neuron activity (Arnold and Salvatore, 2016). So far, the precise function of the high affinity GDNF receptor GFRα1 in the postnatal midbrain DA system *in vivo* has not been resolved and more research is needed to do so.

Finally, Ret knockout mice also die shortly after birth without kidneys as GDNF and GFRα1 knockout mice do. In addition, no alterations in the DA system have been reported in these mice (Schuchardt et al., 1994, Marcos and Pachnis, 1996, Kramer et al., 2006). Adult heterozygous Ret knockout mice were only analyzed together with the conditional Ret deficient mice and did not reveal any phenotype in the midbrain DA system (Kramer et al., 2007).

Interestingly, mice homozygous for a constitutively active form of Ret with the Met918Thr mutation leading in humans to the dominantly inherited multiple endocrine neoplasia type 2B (MEN2B) exhibit 26% more DA neurons specifically in the SN combined with more DA innervation and more DA in the striatum, and more spontaneous and cocaine induced locomotor activity (Smith-Hicks et al., 2000, Mijatovic et al., 2007). In MEN2B mice, SN DA neurons may also be less sensitive to MPTP and 6-OHDA toxicity (Mijatovic et al., 2011) and demonstrate a greatly enhanced amphetamine-induced conditional place preference (Kopra et al., 2018). This suggests that Ret appears not only essential for maintenance of midbrain DA neurons but also that Ret signaling may be limiting during development.

Two laboratories independently deleted Ret specifically in DA neurons of mouse embryos using a dopamine transporter promoter driven Cre line (Zhuang et al., 2005) and consistently

found no histological alterations in the adult midbrain DA system (Jain et al., 2006, Kramer et al., 2007). Only at the age of one year did Ret deficiency cause a significant loss of 25% of midbrain DA neurons, specifically in the SN and not of the DA neurons in the nearby VTA (Kramer et al., 2007). This DA cell loss in the SN was progressive over time and reached 38% in two-year-old mice (Kramer et al., 2007). This DA cell degeneration in the SN was accompanied by a loss of DA innervation in the striatum, gliosis in the striatum and inflammation in the SN (Kramer et al., 2007). Deleting Ret in mice with a different dopamine transporter driven Cre line (Parlato et al., 2006, Turiault et al., 2007) lead to 15 to 21% DA cell loss in the SN in one and two year old mice, respectively (Meka et al., 2015). The DA cell loss in the SN but not in the VTA was also confirmed using a Nestin-Cre line to delete Ret in the nervous system (Kramer et al., 2007), which did not reveal an essential function for deleting GDNF in the DA system (Kopra et al., 2015). These data have clearly revealed an important function of Ret in maintaining some SN DA neurons in mice, although GDNF seems to not be the essential Ret ligand.

The signaling cascades downstream of GDNF/Ret have been mainly investigated in cell culture and were summarized in recent reviews (Airaksinen and Saarma, 2002, Kramer and Liss, 2015). Little is known about the specific pathways used in DA neurons *in vivo* (Figure 1E). Also, the exact pro-survival mechanism of GDNF is not known, activation of Ret can initiate several signaling cascades, of which the mitogen activated protein (MAP) kinase and phosphoinositositide-3-kinase (PI3K) pathways have been suggested to play a role in the survival promoting actions (Penttinen et al., 2018, Airaksinen and Saarma, 2002). Phosphorylated tyrosine residues in the cytosolic domain of Ret are binding sites for several adaptor proteins. Binding of SH2 domain containing transforming protein 1 (SHC) to Ret can for example stimulate the recruitment of GTPase-activating proteins 1 and 2 (Gab1/2) and

subsequent activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), AKT and the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) signaling cascade stimulating mitochondrial activity and cell survival (Meka et al., 2015). The association of the adaptor protein fibroblast growth factor receptor substrate 2 (FRS2) to Ret can lead to the binding of growth factor receptor-bound protein 2 (GRB2) and son of sevenless (SOS) stimulating the Rat sarcoma family of small membrane-associated GTPase (RAS)/MAPK pathway also important for cell survival (Aron et al., 2010). But Ret has also been suggested to activate sarcoma protein membrane-associated tyrosine kinase (SRC) and phospholipase γ (PLCγ) (Kramer and Liss, 2015). Further details about the involved signaling pathways are given in chapter D) where we discuss the crosstalk of GDNF/Ret with PD-associated genes.

This raises several important questions:

- Why do only some aging DA neurons of the SN rely on Ret receptor signaling for survival and not other SN DA neurons or VTA DA neurons?
- How is Ret activated in the midbrain DA system if GDNF is not the essential ligand? Is GFRα1 essential for the development and maintenance of the midbrain DA system and is it required for Ret activation?
- Does an adult deletion of Ret in midbrain DA neurons lead to a similar or different phenotype to the one reported embryonic for Ret deletion?
- At which stage(s) during development do MEN2B mice show an increase in SN DA neurons?
- Which downstream signaling pathways do GDNF/Ret use *in vivo* to exert their different functions?

More research is needed to address and satisfactorily answer these questions.

C) Protective and regenerative functions of GDNF/Ret in the midbrain DA system of pharmacological and genetic PD models

GDNF has been shown to be protective and regenerative in the midbrain DA system of rodents and monkeys against many pharmaceutical challenges such as MPTP (Tomac et al., 1995, Gash et al., 2005, Su et al., 2009) and 6-OHDA (Bilang-Bleuel et al., 1997, Choi-Lundberg et al., 1997, Bjorklund et al., 1997, Kirik et al., 2001, Yang et al., 2009, Lindgren et al., 2012). Both GDNF isoforms (pre-α-pro-GDNF and (pre-β-pro-GDNF) have been shown to be neuroprotective against 6-OHDA in rats (Penttinen et al., 2018). DA neurons in Ret deficient mice are not more sensitive to MPTP toxicity but show an impairment regarding the regeneration of DA axons in the striatum, which can be triggered by endogenous GDNF (Kowsky et al., 2007). In addition, in MPTP treated Ret deficient mice striatal overexpression of GDNF did not lead to neuroprotective and regenerative effects of GDNF usually observed in the midbrain DA system of wildtype mice (Drinkut et al., 2016, Drinkut et al., 2018). Ret signaling is therefore absolutely required for GDNF to exert its neuroprotective and regenerative effects to ameliorate neurodegeneration in the DA system. Ret activation should therefore be considered the primary target of GDNF therapy to treat PD. GDNF is unable to cross the blood-brain barrier and seems to work best in the midbrain DA system if provided to the striatum via GDNF expressing viruses, or cells or small tube injections (Aron and Klein, 2011).

In some genetic mouse models of PD, GDNF has also been shown to protect DA neurons and their striatal innervation such as in the MitoPark mouse, where mitochondrial function is disrupted in DA neurons by selective deletion of the mitochondrial transcription factor Tfam (Chen et al., 2018).

GDNF/Ret signaling has therefore shown to be a promising candidate for PD treatment when tested in PD animal models. A number of human trials have been undertaken to utilize the beneficial effect of GDNF/Ret for the midbrain DA system of PD patients, with so far still an overall inconclusive outcome (Barker et al., 2020). It became obvious that the therapy can only be successful in PD patients with sufficient midbrain DA neurons remaining which are the only cells in the midbrain expressing the GDNF receptor Ret (Kramer and Liss, 2015). A second important issue is that we still do not understand the full extent as to how GDNF/Ret signaling leads do neuroprotection and regeneration.

The crosstalk of GDNF/Ret signaling with the protein network encoded by genes found to be mutated in familial forms of PD or gene variants being risk factors for PD appears to be important in this context (Kramer and Liss, 2015, Blauwendraat et al., 2020).

D) GDNF/Ret signalling crosstalk with PD-associated genes

The products of PD-related genes are specifically implicated in distinct mechanisms including proteostasis, mitochondrial functionality, the autophagy-lysosome system and calcium homeostasis reviewed in (Michel et al., 2016). Therefore, they contribute to maintenance of DA cell viability. Neuroprotective and regenerative mechanisms converge with the pathways PD-related genes serve a function in, so that also the crosstalk between GDNF/Ret signaling with the protein network altered in PD is a very fruitful area to study.

The first crosstalk of GDNF/Ret described with a PD linked gene was with DJ-1 (Parkinsonism associated deglycase) encoded by the *PARK7* gene (Aron et al., 2010). DJ-1 is cleaved in a redox-dependent manner, inactivated by oxidation and functions as a chaperone, protease, and as a transcriptional and mitochondrial regulator to protect midbrain DA neurons against oxidative stress-induced apoptosis (Chen et al., 2010, Ariga et al., 2013). DJ-1 deficient mice

exhibit only 7% fewer DA neurons in the VTA and a normal numbers of SN DA neurons but are more sensitive to MPTP (Kim et al., 2005, Pham et al., 2010). Interestingly, aging mice lacking DJ-1 and Ret display an accelerated loss of G-protein-regulated inward-rectifier potassium channel 2 (Girk2) positive SN DA neurons innervating the striatum, but no change in the number of axons, compared to mice deficient of Ret only (Aron et al., 2010). In combination with fly data, it seems likely that DJ-1 and Ret also converge in mammals by activating the Ras/MAPK pathway to support midbrain DA neurons (Aron et al., 2010). Data from SH-SY5Y cells with some DA cell features suggest that DJ-1 is important to down-regulate the hypoxia-inducible factor-1α which negatively effects Ret protein levels (Foti et al., 2010). This suggests that DJ-1 and Ret crosstalk on two levels, they share the Ras/MAPK signaling pathway to maintain midbrain DA neurons and DJ-1 increases Ret protein levels which might also be important for DA neuron maintenance.

A GDNF/Ret crosstalk with the PTEN-induced kinase (PINK1) encoded by the *PARK6* gene has been found in flies and in SH-SY5Y cells and may also occur in mammals (Klein et al., 2014). PINK1 is a Ser/Thr kinase that accumulates on the outer mitochondrial membrane after sufficient loss of membrane potential, which allows for the recruitment and phosphorylation of parkin and ubiquitin (Pickrell and Youle, 2015). PINK1, together with parkin, initiates the degradation of damaged mitochondria by autophagy (mitophagy) but is also involved in controlling mitochondrial fission and motility (Pickrell and Youle, 2015). It has been shown that GDNF/Ret signaling can rescue PINK1 knockdown induced cell morphology and bioenergetics defects by improving electron transport chain complex I activity (Klein et al., 2014). This suggests an important link as to how GDNF/Ret signaling and PINK1 can act in tandem to positively influence mitochondrial integrity. PINK1 deficient mice do not show DA cell or innervation loss and only a mild impairment of DA release and synaptic plasticity in the

striatum and an increased sensitivity to MPTP, which can be rescued by parkin and DJ-1 overexpression (Haque et al., 2012, Kitada et al., 2007) (Gispert et al., 2009). It could be revealing to investigate in detail the crosstalk of PINK1 and Ret in PINK1 and Ret double deficient mice.

GDNF/Ret seems to also crosstalk with the protein parkin (Meka et al., 2015). Parkin is an E3 ubiquitin protein ligase encoded by the *PARK2* gene and seems to mono- or polyubiquitinate proteins on the outer membrane of mitochondria upon cellular insults and mediates the clearance of damaged mitochondria via mitophagy (Seirafi et al., 2015, Pickrell and Youle, 2015). Disappointingly, all parkin deficient mice do not show a clear DA cell loss phenotype but link PINK1 deficient mice reduced dopamine release and synaptic plasticity (Itier et al., 2003, Kitada et al., 2009, Perez and Palmiter, 2005). In contrast to PINK1 deficient mice, Parkin deficient mice seem not to be more sensitive to MPTP and 6-OHDA (Perez et al., 2005). Mice deficient for parkin and Ret exhibit an accelerated DA neuron and axonal loss compared with parkin-deficient mice, which showed none, and Ret-deficient mice, which showed moderate degeneration (Meka et al., 2015). Consistent with a tight parkin and Ret crosstalk, parkin overexpression protected the midbrain DA system from degeneration in aged Ret deficient mice (Meka et al., 2015). Ret and parkin signaling was shown to be important for mitochondrial integrity by activating the pro-survival NF-κB pathway which was mediated by Ret through the phosphoinositie-3-kinase pathway (Kramer, 2015a, Kramer, 2015b, Meka et al., 2015). These data are encouraging that GDNF/Ret signalling might be able to target the frequently critically impaired mitochondrial function in sporadic and familial forms of PD.

Different crosstalk pathways have been postulated for GDNF/Ret signaling and α-synuclein. Multiple copies of the gene *SNCA* (*PARK*1 und 4) encoding α-synuclein or mutations such as A30P or A53T have been found to lead to PD (Goedert et al., 2013). In two rat models

overexpressing α-synuclein intranigrally via a lentiviral vector encoding a human A30P mutant from of α-synuclein (Lo Bianco et al., 2004) or via a recombinant AAV-encoding human wild type α-synuclein (Decressac et al., 2011), GDNF exerted no positive effect on either DA neuron survival or motor function. It was hypothesized by the authors that α-synuclein caused repression of nuclear receptor related 1 (Nurr1), an upstream transcription factor of Ret, thus reducing Ret translation and limiting downstream signaling (Decressac et al., 2012)(Figure 1.). In PD patients showing frequently α-synuclein accumulation in Lewy bodies and Lewy neurites would also have downregulation of Nurr1 and Ret protein levels this could explain why GDNF has not been shown to be beneficial in clinical trials on PD patients (Barker et al., 2020). As mentioned previously, without the presence of Ret on midbrain DA neurons GDNF cannot elucidate its beneficial function (Drinkut et al., 2016). Decressac *et al*. presented data from SN sections of PD patient stained with anti-Ret antibodies and suggested a strong downregulation of Ret protein levels in PD midbrain DA neurons (Decressac et al., 2012). However other independent studies conducted on patients with Lewy bodies rodent did not find a reduction of *Ret* mRNA or protein levels (Walker et al., 1998, Backman et al., 2006, Su et al., 2017). Su et al. also used transgenic mice overexpressing either wild-type or doubly mutated A30P and A53T α-synuclein under regulation of the TH promoter, as well as AAV-mediated α-synuclein transgenic rats and did not find alterations in Nurr1 and Ret mRNA and protein levels (Su et al., 2017). As suggested by Hoffer and Harvey (Hoffer and Harvey, 2011), it is possible that the α-synuclein concentration in the rat substantia nigra produced by the viral vectors was much greater than the levels observed in PD patients, making this rodent model not translatable to human pathology. This emphasizes the need for physiological and pathophysiological relevant α-synuclein animal models which recapitulate the PD pathology reliably. Since Ret seems to still bedetectable in remaining midbrain DA neurons in PD

patients, there must be other factors to explain the non-significant improvements of GDNF in clinical trials on PD patients. More research is needed to clarify the precise crosstalk of α-synuclein and GDNF/Ret signaling.

Concluding Remarks

GDNF/Ret signaling appears to be an influential modulator of development and maintenance in midbrain DA neurons under both physiological and pathophysiological conditions including PD and Dementia with Lewy bodies (DLB). GDNF/Ret signaling has been shown to crosstalk with proteins encoded by familial PD-related genes. Although great progress has been made over recent decades to elucidate at least some mechanisms of GDNF/Ret signaling and their crosstalk with DJ-1, parkin and alpha-synuclein, work is still required to determine the crosstalk to other PD-related proteins such as LRRK2 and to study the detailed molecular mechanisms of the different crosstalks. As outlined above, GDNF and Ret can signal both together and independently of one another. Further investigation is encouraged for both of these signaling mechanisms, as differentiation as to which signaling mechanism is responsible for which process will be useful when developing and applying therapeutics against PD using Ret and/or GDNF signaling.

The main task in this field is to reproduce findings where GDNF family members have shown to be beneficial in *in vivo* animal models, to show the same in human patients with diseases including PD and DLB. GDNF/Ret signaling remains a promising avenue for research, particularly in the context of neurodegenerative diseases affective the midbrain DA system, including PD and DLB. In tandem with early diagnosis, GDNF stimulation to induce neuroprotection in dopamine neurons may hold promise as a potential preventative therapy.

For PD, signaling of Ret appears to be essential for midbrain DA neuron protection, maintenance and regeneration and GDNF may be only one of several Ret ligands in the midbrain DA system. Endogenous or artificial Ret activators could be exciting tools to be explored further in this context.

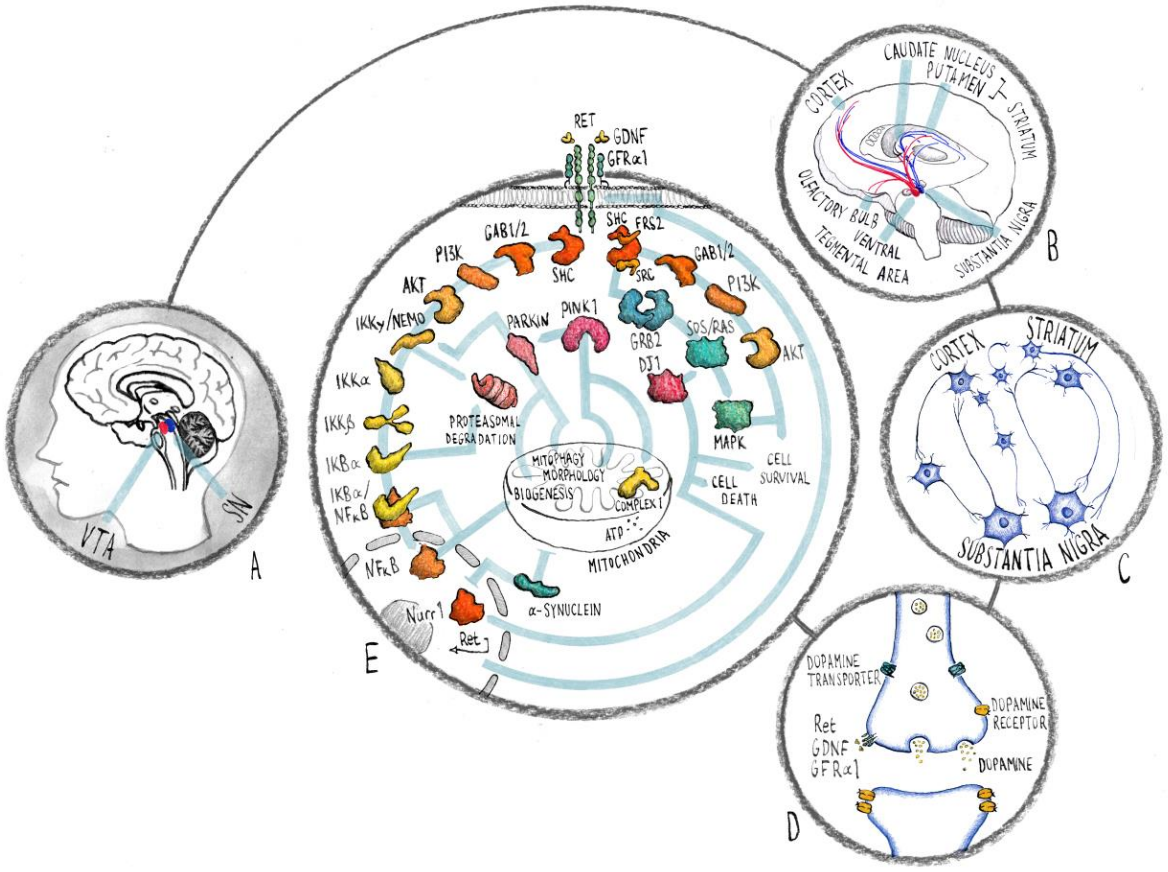

Figure legend

Figure 1. GDNF-Ret signaling in the midbrain dopamine system. A. A sagittal cross section of a human head with the brain visible. The cell bodies of the midbrain dopamine neurons are placed in the substantia nigra (SN) and the ventral tegmental area (VTA) **B.** The dopamine system within the brain. Red indicates dopamine neurons with cell bodies originating from

the ventral tegmental area (VTA). These neurons are affected in addiction and predominantly innervate the ventral striatum (telencephalic region shaded in gray), amygdala, cortex, and olfactory tubercle compacta. Purple indicates dopamine neurons with cell bodies originating in the substantia nigra (SN). These SN dopaminegric neurons preferentially die in Parkinson disease. Their axons predominantly innervate the dorsal striatum. **C.** A network of dopamine neurons of the SN innervating the striatum and cortex with feedback loops back onto dopamine neurons. **D.** A simplified dopamine synapse. It is visible that dopamine receptors are expressed both pre- and post-synaptically outside the synaptic cleft, with Ret and the dopamine transporters only expressed presynaptically. **E.** A summary of GDNF/Ret intracellular signaling cascades. Some proteins shown are encoded for by genes which are mutated in familial forms of Parkinson disease. For example, DJ-1 (PARK7) has involvement in GDNF/Ret signaling via the Ras/MAPK pathway to stimulate expression of Ret. α-synuclein (PARK1 and 4) has been proposed to have an inhibitory effect on the Ret transcription factor Nurr1, therefore reducing Ret expression. PINK1 (PARK6) in tandem with GDNF/Ret signaling, controls mitochondrial complex I regulation and morphology. PARKIN (PARK2) and GDNF/Ret also influence mitochondria by both stimulation of complex I activity and preserving mitochondrial integrity through the NF-κB pathway.

Compliance with Ethical Statements

Conflict of interest

The authors declare that research and writing was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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