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Is activation of GDNF/RET signalling the answer for successful treatment of Parkinson's disease?

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Is activation of GDNF/RET signalling the answer for successful treatment of Parkinson's disease? – A discussion of data from the culture dish to the clinic.

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Abstract

The neurotrophic signalling of glial cell line-derived neurotrophic factor (GDNF) with its canonical receptor, the receptor tyrosine kinase RET, coupled together with the GDNF family receptor alpha 1 (GFRα1) is important for dopaminergic neuron survival and physiology in cell culture experiments and animal models. This prompted the idea to try GDNF/RET signalling as a therapeutic approach to treat Parkinson's disease (PD) with the hallmark of dopaminergic cell death in the substantia nigra of the midbrain. Despite several clinical trials with GDNF in PD patients, which mainly focused on optimising the GDNF delivery technique, benefits were only seen in a few patients. In general, the endpoints did not show significant improvements. This suggests that it will be helpful to learn more about the basic biology of this fascinating but complicated GDNF/RET signalling system in the dopaminergic midbrain and about recent developments in the field to facilitate its use in the clinic. Here we will refer to the latest publications and point out important open questions in the field.

Introduction

The receptor tyrosine kinase rearranged during transfection (RET) can signal via several pathways to exert vital functions in neurons. The most widely studied ligand for RET is the glial cell line-derived neurotrophic factor (GDNF), which signals through binding the glycosylphosphatidylinositol (GPI)-linked GDNF family receptor alpha 1 (GFRα1) and to some extent also GDNF family receptor alpha 2 (GFRα2), which in turn binds RET to activate its intracellular tyrosine kinase activity (Figure 1). RET is responsible for activating downstream signalling cascades which can promote neurite growth, cell differentiation and survival, as well as synaptic plasticity (Enterria-Morales et al., 2020). Whilst GDNF-RET signalling has been widely studied for decades, as well as being the target of several clinical trials attempting to treat Parkinson's disease (PD), the full extent of GDNF-RET's physiological functions in the midbrain dopaminergic system, which is impaired in PD, remains unclear.

In vivo GDNF/RET signalling in the dopaminergic system and beyond

In a recent special issue on neurotrophic factors published in the journal Cell and Tissue Research, Conway *et al.* discuss the *in vivo* function of GDNF-RET signalling in midbrain dopaminergic neurons (Conway et al., 2020). The phenotypes of mice with GDNF and its canonical receptor RET knocked out are mentioned and data on the crosstalk of RET with genes known to be mutated in familial forms of PD, including *PARK1*/*SNCA* (α-synuclein), *PARK7/DJ1* (DJ-1), *PARK2*/*Parkin* (parkin) and *PARK6/PINK1* (PINK1*)* are summarised (Kramer et al., 2007; Kramer and Liss, 2015; Meka et al., 2015). RET deficient mice specifically lose dopaminergic neurons in the substantia nigra during ageing (Kramer et al., 2007), whilst more recent data support the notion that there is no or only a very mild defect on the maintenance of midbrain dopaminergic neurons in GDNF deficient mice (Kopra et al., 2015; Enterria-Morales et al., 2020). However, GDNF deficiency in mice might lead to a reduced amphetamine-induced locomotor response and striatal dopamine efflux (Kopra et al., 2017). GDNF seems therefore not essential for the development and maintenance of the midbrain dopaminergic system but is important for its normal physiological function, as well as remaining a viable therapeutic. This suggests that RET might additionally be activated in the dopaminergic system independently of GDNF by alternative signalling events, such as cross-activation by other

receptor tyrosine kinases (Volinsky and Kholodenko, 2013). Further research is needed to confirm this hypothesis. However, GDNF/RET signalling might be limited during mouse development, as a constitutively active RET mutation, MEN2B, and GDNF overexpression on its native locus increases the number of adult dopamine neurons in the substantia nigra pars compacta and the number of dopaminergic terminals in the dorsal striatum (Mijatovic et al., 2007; Kumar et al., 2015). More detailed analysis of when this phenotype appears and the mechanism at play is still required in these mice.

The GDNF receptor GFRα1 has not yet been specifically deleted in dopaminergic neurons in the mouse and therefore the phenotype of adult GFRα1 deficient mice has not yet been reported, despite the creation of a floxed GFRα1 allele (Uesaka et al., 2007). Studies in adult GFRα1 global knockout mice are not possible, as these die shortly after birth due to renal agenesis, as do GDNF global knockout mice (Kramer and Liss, 2015). Only heterozygous GFRα1 knockout mice have been generated showing a mild age-related inflammation, reduction tyrosine hydroxylase-positive cells in the substantia nigra, reduced striatal dopamine and tyrosine hydroxylase staining and a reduced motor function also after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment (Boger et al., 2008; Zaman et al., 2008). Without the data from the conditional GFRα1 deficient mice in dopaminergic neurons, the picture of GDNF/RET/GFRα1 signalling in the dopaminergic system remains incomplete. Further important information might be gained from investigating GDNF/RET/GFRα1 signalling in human dopaminergic neurons, which so far remains a largely unexplored area of research.

Also, the role of alternative GDNF receptors such as syndecan-3, N-cadherin, integrins and NCAM in the dopaminergic system has not been carefully studied and requires more work (Figure 1). Despite recent data showing no effect of exogenous GDNF or RET agonists in RET deficient mice and cells from RET deficient mice, respectively (Drinkut et al., 2016; Mahato et al., 2020), this does not rule out a function of these alternative GDNF receptors in the dopaminergic system perhaps even without GDNF, as cell adhesion factors and in different signalling cascades.

The protein parkin appears to be an interesting crosstalk partner of GDNF/RET (Meka et al., 2015). Parkin is an E3 ubiquitin ligase that appears to mono- or poly-ubiquitinate proteins on the outer mitochondrial membrane as a result of cellular insults (Yoshii et al., 2011). In turn, this mediates the removal of damaged mitochondria by autophagy. Mice deficient for both parkin and RET exhibited an accelerated loss of both dopaminergic neurons and axons when compared with mice deficient for parkin or RET only, which showed no and moderate degeneration, respectively (Meka et al., 2015). Parkin overexpression provided a neuroprotective effect on the midbrain dopaminergic system of aged RET-deficient mice, which is consistent with a system of tight parkin/RET crosstalk (Meka et al., 2015). RET and parkin signalling proved to be important for mitochondrial integrity through activation of the pro-survival NF-κB pathway, which was mediated by RET through PI3K signalling (Meka et al., 2015). These data are encouraging, as GDNF/RET signalling may be able to target the often critically impaired mitochondrial function in both familial and sporadic forms of PD. RET has not been found to be a substrate of parkin, however in cell culture experiments other E3 ubiquitin ligases, including the E3 ligase Casitas B-lineage Lymphoma (CBL) and the neural precursor cell-expressed developmentally downregulated protein 4 (Nedd4) have been shown to target the long (RET51) and the short isoforms of RET (RET9), which differ only in their cytoplasmic domains, respectively (Ishiguro et al., 1999; Hyndman et al., 2017) (Figure 1). As a part of the Nedd4/GRB10/SHANK2 complex, Nedd4 localises to clathrin-coated pits and may play an important role in RET9 ubiquitination, endocytosis and degradation (Hyndman et al., 2017). Nedd4 may also ubiquitinate α-synuclein, resulting in its clearance via the endosome-lysosome pathway (Tofaris et al., 2011). Therefore, Nedd4 expression in midbrain dopaminergic neurons might be able to protect from α-synuclein toxicity and downregulate RET neurotrophic signalling simultaneously. It would be of great interest to study these possible functions of Nedd4 in more detail *in vivo* concerning stimulating RET and/or Nedd4 function in PD patients to protect midbrain dopaminergic neurons.

In addition, the review by Conway *et al.* comprehensively discusses *in vivo* mRNA and protein expression data for GDNF, GFRα1 and RET in humans and rodents during development, adulthood and ageing (Conway et al., 2020). Within the midbrain, only dopaminergic neurons appear to express

RET and GFRα1 (Kramer and Liss, 2015), with cells in the striatum only expressing GFRα1 (Kramer et al., 2007). The consensus in the literature is that there is no alteration in GFRα1 and RET expression in ageing mammals (Kramer et al., 2007). Regarding PD patients with an accumulation of α-synuclein, an open discussion remains as to whether RET protein level may be reduced. Viral overexpression of α-synuclein in the rat substantia nigra has been found to lead to neurodegeneration of dopaminergic neurons that cannot be prevented by GDNF treatment (Lo Bianco et al., 2004; Decressac et al., 2011), which is thought to be mediated through transcriptionally and translationally downregulated of the transcription factor nuclear receptor related 1 (Nurr1) and its target gene RET (Wallen et al., 2001; Decressac et al., 2012). It seems that high viral overexpression of α-synuclein levels can cause an interruption of GDNF/RET signalling in rats which might not be of physiological relevance nor recapitulate the situation in humans, where a triplication of the α-synuclein locus leads to only double the amount of soluble α-synuclein but still an early-onset form of PD (Olgiati et al., 2015; Albert et al., 2017; Duffy et al., 2018; Polinski et al., 2018). α-synuclein mRNA expression seems to not be upregulated in PD patients (Su et al., 2017) and can even be downregulated (Kingsbury et al., 2004), with no alteration of Nurr1 or growth factor receptors observed in PD patients either (Su et al., 2017). It has also been shown that no (Backman et al., 2006; Su et al., 2017) or very little (Decressac et al., 2012) reduction in RET is observed in the dopaminergic system of PD patients. It remains an open question how much RET is needed to mediate GDNF's beneficial effect on dopaminergic neurons. Recently it has been reported that GDNF overexpression may protect from α-synuclein triggered dopamine neuron death in cell culture and mice (Chmielarz et al., 2020). Further research is needed to clarify the crosstalk of α-synuclein and GDNF/RET signalling in the midbrain dopaminergic system.

It has become more widely accepted of recent that PD starts outside of the brain most likely in the gut, where the first α-synuclein aggregates are found in the enteric nervous system and neurons seem to die which correlates with the early PD non-motor symptoms such as constipation (Siddiqui et al., 2002; Braak et al., 2006; Pedrosa Carrasco et al., 2018). This α-synuclein pathology seems to spread along the gut-brain axis through the dorsal motor nucleus of the vagus nerve into the

brainstem leading to rapid eye movement (REM) sleep behaviour disorder, depression and finally leading to the typical PD motor symptoms via the death of midbrain dopaminergic neurons of the substantia nigra (Greene, 2014; Braak and Del Tredici, 2017; Schaeffer et al., 2020). Interestingly, the neurons of the gut-brain axis also express RET (Nosrat et al., 1997; Wallen et al., 2001; Barlow et al., 2003). The RET receptor mediates the development of enteric nervous system neurons but RET's physiological function in the adult enteric nervous system is not well understood (Taraviras et al., 1999). It remains an interesting question what the physiological function of GDNF/RET signalling could be in these different neurons along the gut-brain axis and how far crosstalk of α-synuclein and GDNF/RET signalling takes place here, too.

GDNF clinical trials

Despite these encouraging preclinical data, the results from GDNF clinical trials on PD patients are still inconclusive (Barker et al., 2020; Manfredsson et al., 2020; Sidorova and Saarma, 2020). The first clinical trial saw intraventricular GDNF administration failing to improve motor symptoms in PD patients due to its inability to cross cell barriers, thus not reaching target neurons. Patients in this trial also reported suffering adverse effects including nausea, paraesthesia, weight loss and anorexia (Nutt et al., 2003). Following an improvement of the knowledge of GDNF's biodistribution features, two small, phase I, open-label trials were embarked upon. These trials employed an intraputaminal delivery of GDNF, which increased ^{18}F-DOPA) uptake on PET scans by 19%, with an absence of major side effects (Gill et al., 2003; Slevin et al., 2005). These effects persisted for several months after the end of GDNF treatment (Slevin et al., 2007). A phase II, placebo-controlled trial followed this in an attempt to replicate the earlier observations but it did not meet its primary endpoints (Lang et al., 2006). Another more recent phase II trial also failed to reach its primary endpoints, however, all patients in the GDNF-treated group had significantly increased ^{18}F-DOPA uptake on PET scans; when *post-hoc* analyses were performed, 43% of these patients had at least a 10 point Unified Parkinson's Disease Rating Scale (UPDRS) increase, with no increase in the control group (Whone et al., 2019a; Whone et al., 2019b). Ninety-five percent of these patients had at least one clinically significant

outcome measure 80 weeks after administration ended (Whone et al., 2019a; Whone et al., 2019b). In a recent workshop by many working on these clinical trials, it was agreed that throughout these trials that a statistical significance is shown in some but not all patients, resulting in the overall trials not meeting their primary endpoint (Barker et al., 2020). The results of the GDNF clinical trials and the influence of *placebo and nocebo* effect on these studies has also recently been reviewed (Gash et al., 2020). Restoration of motor function was often observed in patients receiving both GDNF and *placebo* treatments (Gill et al., 2003). One of the factors responsible for this effect may be that many patients are subject to long-term distress as a result of PD. When these patients take positive action by agreeing to participate in a clinical trial, with a positive outcome expected, a more positive outlook develops with time, thus creating a strong *placebo* effect. This *placebo* effect might be mediated by increased release of the neurotransmitter dopamine in the brain, which is reduced in PD. The *nocebo* effect is a term describing here the worsening of symptoms and clinical features in patients with progressive and currently incurable PD due to their own negative perceptions and expectations.

There was great promise for the GDNF clinical trials from the beginning, however, technical problems with the production and delivery of GDNF, as well as the selection of the patients still require optimisation (Gill et al., 2003). One issue is that GDNF therapy is only viable for PD patients with sufficient midbrain dopaminergic neurons remaining, which are the only cells in the midbrain expressing the GDNF receptor RET (Kramer and Liss, 2015; Quintino et al., 2019). Therefore, the ideal candidate for GDNF therapy is a younger, early-stage, sporadic PD patient with many dopaminergic neurons left (Barker et al., 2020). This is however a patient that might benefit still for many years from a symptomatic treatment. Another issue is that we are still not fully certain as to how GDNF/RET signalling results in maintenance, neuroprotection and regeneration of midbrain dopaminergic neurons. A difficult issue remains the delivery of GDNF and its close family member neurturin since they do not pass the blood-brain barrier and there is very little or no transport to the substantia nigra if injected in the striatum (Bartus et al., 2015). Intracranial surgery for delivery causes a great burden on PD patients and there have been patients in multiple trials who have

developed issues with their cannulae but the Renishaw and Clearpoint systems seems to improve the intracranial delivery with each trial. Other delivery methods including intranasal administration and transient disruption of the blood-brain barrier are also being tested and may hold promise, however more study is required here (Hernando et al., 2018; Li et al., 2018). In early trials it is thought that low dose or biological activity of particular GDNF batches which were not sufficient to provide clinical benefit may have caused a variation in results (Kirkeby and Barker, 2019). Interestingly, neurotrophic factors often require only a short term, pulsatile interaction with their receptors to activate the signalling required for neuronal survival, which can persist for days or months afterwards (Sidorova and Saarma, 2020). Overactivation of neurotrophic receptors due to prolonged interaction with their ligand may be detrimental to the neurons due to some negative feedback loops which serve to limit uncontrolled propagation or multiplication of these signals (Lake et al., 2016). Additionally, very high concentrations of growth factors, which produce biphasic response curves in clinical trials, may inhibit the formation of oligomeric signalling complexes required for function because each monomer of the receptor is bound to a ligand (Schlee et al., 2006). For example, GDNF overexpression in rats was shown to downregulate the rate-limiting enzyme for dopamine synthesis, tyrosine hydroxylase, which is indicative of reduced dopamine syntheses and dopaminergic function (Georgievska et al., 2004). Therefore, the beneficial amount of GDNF to be delivered to patients may be in a narrow range, too much or too little might result in a negative outcome. It was however recently shown in aged mice that a two –fold increase in endogenous GDNF levels enhances dopaminergic function and appears to be safe (Turconi et al., 2020). Despite this, it seems worth exploring alternative delivery methods such as small RET agonistic drugs or gene therapy approaches.

Small molecule RET agonists and altered RET ligands – A new hope?

Since GDNF does not cross the blood-brain barrier and needs to be supplied directly into the brain when used as a PD drug, there are great efforts to develop small RET/GFRα1 activator molecules or GDNF mimetics that do pass the blood-brain barrier (Mahato and Sidorova, 2020). As only very small amounts of neurotrophic factors are required, small quantities of well-designed molecules might

yield clinically significant results, providing that they activate the correct downstream signalling cascades. Examples of such drugs are DNSP-11, XIB4035, Q525, compound 8, BT13 or BT44, which are currently being tested in different *in vivo* and *in vitro* models of PD (Tokugawa et al., 2003; Stenslik et al., 2015; Ivanova et al., 2018; Jmaeff et al., 2020b; Jmaeff et al., 2020a; Mahato and Sidorova, 2020; Viisanen et al., 2020).

DNSP-11 has been shown to work *in vitro*, however, it was only neuroactive but not neuroprotective on dopaminergic neurons *in vivo* (Fuqua et al., 2014; Stenslik et al., 2015). XIB4035 has been shown shown to be an allosteric regulator of GDNF increasing GDNF activity (Tokugawa et al., 2003; Hedstrom et al., 2014). Q525 and compound 8 are defined as RET agonists (Jmaeff et al., 2020b; Jmaeff et al., 2020a). The last three molecules are yet to be tested in dopaminergic neurons, so it is not yet clear whether this could translate well into patients.

Two RET agonists, BT13 and BT44, have been shown to stimulate RET both *in vitro* and *in vivo* (Sidorova et al., 2017; Viisanen et al., 2020). It is speculated that BT13 mimics soluble GDNF/GFRα1, rather than GDNF itself, since both proteins may signal in a soluble way. BT13 does not require GFRα co-receptors to elicit its effects and can bind either directly to RET or allosterically modulate the receptor complex to GDNF (Sidorova et al., 2017). The consensus is that RET must homodimerise to become activated. BT13 either induces RET homodimerisation by conformational changes or by stabilising it in its active conformation if the signalling complex is already formed (Bespalov and Saarma, 2007). Striatal delivery of BT13 has been shown to stimulate neuronal signalling and dopamine release similarly to GDNF (Mahato et al., 2020). In contrast to BT13, the third-generation selective RET agonist BT44 has some selectivity to the GFRα1/RET complex when compared to RET alone (Viisanen et al., 2020). This may be beneficial when attempting to ameliorate potential side effects (Viisanen et al., 2020). BT44 is more potent than BT13 but less potent than the GDNF molecule it is mimicking (Sidorova et al., 2017; Mahato and Sidorova, 2020; Viisanen et al., 2020). It was demonstrated that BT44 promotes the survival of primary dopaminergic neurons from wild-type but not RET knockout mice indicating an *in vivo* selectivity of BT44 for RET (Renko et al., 2021). In a 6-hydroxydopamine PD rat model, BT44 protected dopaminergic fibres in the striatum as well as

reducing motor imbalance in these animals (Renko et al., 2021). Taking all of these findings together, BT13 and BT44 might have the potential to be developed into further treatments. Unlike GDNF, their production and administration are simpler and may cause fewer side effects, as they seem selective to RET and their signalling might be influenced by the presence of GFRα1 (Jmaeff et al., 2020b; Jmaeff et al., 2020a). This may provide hope to use neurotrophic signalling as a therapeutic approach for PD patients, subverting the requirement for the highly invasive neurosurgery currently required for successful striatal GDNF delivery (Gill et al., 2003; Whone et al., 2019a; Whone et al., 2019b).

Another promising direction to stimulate RET in PD patients might be the use of modified versions of GDNF and neurturin which might diffuse better in the tissue and perhaps even penetrate the blood-brain barrier (Runeberg-Roos et al., 2016; Runeberg-Roos and Penn, 2020). Care must be taken if these proteins are routinely tested preclinically in rhesus monkeys, which have a putamen five times smaller than humans, making it difficult to extrapolate their distribution behaviour in PD patients (Yin et al., 2009). For example, an N-terminally truncated GDNF variant was created which exhibited a two-fold increase in distribution when administered to rats and non-human primates, whilst retaining its ability to activate the RET receptor complex (Smith et al., 2015; Grondin et al., 2019). These proteins could also be delivered in viral vectors with specific promoters and inducible systems limiting the expression to particular cell types and time points, thus reducing the potential for off-target effects and deleterious overexpression (Chtarto et al., 2016; Kordower, 2016; Axelsen and Woldbye, 2018; Chen et al., 2020; Runeberg-Roos and Penn, 2020). In a phase I clinical trial, treatment with AAV2-GDNF was well tolerated by patients with an enhanced putaminal uptake of ^{18}F-DOPA suggestive of increased neurotrophic signalling in dopaminergic neurons (Heiss et al., 2019).

Concluding Remarks

Activating GDNF/RET signalling has yet to show its full beneficial potential in PD patients. Important parameters to consider for further clinical trials are the strata of PD patients, the kind, dose, place, time and application technique of RET activators. It seems sensible to consider alternative RET

stimulators such as small drugs, peptides and modified GDNF with high RET receptor selectivity, improved tissue distribution properties and the ability to penetrate the blood-brain barrier. Small molecules may additionally be able to treat non-motor symptoms of PD and could be a better option. More preclinical research is needed to carefully evaluate the properties of different RET activators and optimizing the parameters for clinical application. Stimulating RET signalling in the gut-brain axis and midbrain dopaminergic system is most likely just one of several approaches which might help to slow down, stop or reverse this devastating neurodegenerative disease. We envision that the majority of PD patients could benefit best from receiving a combination of different treatments and that stimulating RET could be one of them.

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Conflict of interest

We declare no conflict of interest that relate to this paper.

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Figure 1 | GDNF family of ligands and the different GDNF receptors. In the dopaminergic system only two out of the four GDNF-family members are of interest, the glial cell line-derived neurotrophic factor (GDNF) and neurturin. These ligands form homodimers that undergo high-affinity binding with one of the four GDNF family receptor α members (GFRα1-4) with only GFRα1 being expressed in the dopaminergic system. The receptor-ligand complexes may then interact with RET, GDNF's canonical receptor, or also activate alternative GDNF receptors including syndecan-3, N-cadherin, integrin α and β1 and the neural cell adhesion molecule (NCAM). RET's intracellular domain can be subject to post-translational modifications, including phosphorylation at a number of tyrosine and serine domains and ubiquitination. RET51 (long) and RET9 (short) isoforms, which differ only in their cytoplasmic domains, may be subject to different post-translational modifications. The phosphorylation sites on Ret provide docking sites for adaptor proteins, resulting in differing downstream signalling cascades.

Figure