CHAPTER 8

Neuroprotection in Parkinson's Disease

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8.1 Neurotrophism and Neurotrophic Factors

Neurotrophic factors (NTFs) are small natural proteins necessary for the development and survival of nerve cells as well as for the maintenance of their morphological and functional phenotype. The 'neurotrophic hypothesis' enunciates that the establishment and maintenance of neuronal networks require the release at the target structures of NTFs, which are taken up by the nerve terminals and retrogradely transported to the soma of the projecting neurons. On reaching the nucleus, NTFs induce a gene programme that promotes neuronal survival and maintenance of phenotype.

Although the existence of 'chemotactic' influences between the growth cones of axons and their targets had already been postulated by Cajal's group,[1] the modern concept of neurotrophism is based on the work of Hamburguer and Levi-Montalcini[2] who reported that the phenomenon of naturally occurring

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cell death observed during development was dependent on the target where these dying neurons were projecting. Removing a prospective target early in development could dramatically increase neuronal loss. In the absence of target, the number of initially existing neurons did not change, indicating that the target influences survival and not the number of neurons generated. These seminal observations suggested that cells acting as a target of developing neurons produce limited amount of specific molecules which are required for their survival. The first molecule identified with these specific characteristics was the nerve growth factor (NGF).[3,4] As indicated above, the current concept of NTF applies not only to molecules regulating neuronal number during development but also to agents necessary for the maintenance of neuronal populations in adulthood.[5,6]

During the last decades, several proteins have been classified as NTFs because their effect on neuronal survival, differentiation (including synaptogenesis and neurite branching *in vitro*), maturation of electrophysiological properties, and plasticity. However, the role of these molecules as 'canonical' NTFs is not always well demonstrated. This is true even in the case of NGF (the 'prototypical NTF'), which is essential for the survival of sympathetic, sensory and central cholinergic neurons. Nevertheless, the dependence of these neuronal populations on NGF is not always conserved by the end of the developmental critical period. Indeed, some postnatal neurons (as it is the case of a subset of DRG neurons) can switch dependence from one (NGF) to another (glial cell line-derived neurotrophic factor) trophic factor.[7,8] Moreover, although deprivation of NGF in the adult compromises sympathetic neurons viability, it only affects gene expression in sensory neurons without decreasing their cell number.[9] However, it has not been definitely established whether, in the adult nervous system, neurons depend on one or several trophic factors with overlapping effects.

Because of their potent role in neuronal survival, NTFs have aroused clinical interest as potential neuroprotective agents that could prevent or retard the progression of neurodegenerative diseases. In this chapter we summarize current knowledge on the role of NTFs in the pathogenesis of Parkinson's disease (PD), emphasizing the data obtained from animal models of NTF deficiency. We also discuss the possible clinical applicability of NTFs as neuroprotective agents in PD.

8.2 Neuroprotection of Mesencephalic Dopaminergic Neurons: Role of GDNF

Parkinson's disease (PD), a neurodegenerative disorder that affects over one million Europeans,[10,11] is characterized by motor symptoms (tremor, bradykinesia, rigidity and alteration of gait).[12,13] The aetiology and pathogenesis of PD are essentially unknown, although several causative mechanisms have been proposed including alterations of protein folding/degradation, mitochondrial dysfunction and oxidative stress, neuroinflammation, and Ca^{2+} excitotoxicity.[14,15]

PD is caused by the progressive loss of specific sets of neurons both in the brainstem and in the peripheral nervous system. From a clinical standpoint, the most critical neuronal population affected corresponds to the mesencephalic dopaminergic (DA) neurons in the substantia nigra (SN) pars compacta projecting to the striatum (nigrostriatal neurons), thus leading to dysfunction of the neuronal circuits in the basal ganglia and alteration of motor control. DA neurons in the neighbouring ventral tegmental area (VTA) are less affected than SN neurons. Although the loss of nigrostriatal DA neurons is the most apparent pathological hallmark of PD, other cell types are affected even before SN cell death. Among those are noradrenergic neurons in the locus coeruleus (LC) and cells in the dorsal nucleus of the vagus or in the sympathetic ganglia. Peripheral sympathetic denervation (loss of cardiac or celiac fibres) has been proposed to be an early marker for PD.[16]

For the last decades, intrastriatal transplantation of dopamine-producing cells (most frequently foetal mesencephalic DA neurons) has been considered as an experimental therapeutic approach to advanced PD once pharmaceutical drugs have ceased to provide clinical benefit.[17–19] However, allogenic cell replacement therapies have recently been almost completely abandoned (for discussion, see refs. 20,21) due to the scarcity of tissue available for transplantation and because they do not always produce the expected beneficial effects (and in some cases can even induce the appearance of abnormal movements called dyskinesias[22,23]). Recently, intrastriatal delivery of NTFs, which could 'protect' nigrostriatal neurons and thus halt or retard PD progression, is being considered as an alternative therapeutic strategy to dopamine cell replacement.[24] The prototypical 'neuroprotective' agent used in most preclinical and clinical studies is glial cell line-derived neurotrophic factor (GDNF), which after isolation demonstrated a remarkable trophic effect on mesencephalic DA neurons *in vitro*.[25]

8.2.1 Biology of GDNF and Other Dopaminotrophic Factors

GDNF belongs to a family of ligands, which include artemin (ARTN), neurturin (NRTN) and persephin (PSPN), all being distantly related members of the transforming growth factor-beta superfamily. GDNF has attracted special attention due to its potent effect on dopaminergic and noradrenergic neuron survival[26,27] (see ref. 28 for a comprehensive review). Collectively, the members of the GDNF family of trophic factors are often grouped as 'dopaminotrophic' factors. GDNF is expressed in several regions of the adult brain (particularly in the striatum, anteroventral thalamus and septum)[29,30] and signals through an extracellular GPI-anchored receptor (GFRα1) that activates a tyrosine kinase transmembrane protein (c-ret).[26,31] A non-canonical form of GDNF signalling also exists in the adult rodent brain, which is independent of Ret and is mediated by neural cell adhesion molecule (NCAM).[32] Like other NTFs, GDNF is taken up by axon terminals of projecting neurons and transported to cell soma (for a review on retrograde transport, see ref. 33). Injection of

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^{125}I-GDNF in striatum results in labelled cells in the ipsilateral SN and VTA, thus suggesting a trophic role of GNDF in adult nigrostriatal neurons.[34]

GDNF has been shown to activate pathways associated with the promotion of antioxidant defence[35] and neuronal survival.[36] Among these pathways, the PI3K/AKT cascade[37] can protect neurons through several mechanisms including inactivation of apoptotic proteins.[38,39] GDNF overexpression (using lentiviral infection or engineered GDNF-producing cells) protects catecholaminergic neurons from toxic damage and induces fibre outgrowth *in vivo*.[26–27,40,41] However, the molecular mechanisms underlying these functional effects are still unknown. Recently, it has been reported that lentiviral GDNF delivered to the rat striatum induces gene expression in the SN, notably tyrosine hydroxylase (TH), GTP cyclohydrolase-I (which catalyses the synthesis of a cofactor of TH, tetrahydrobiopterin), GDNF receptors, and Dlk-1 (a factor involved in cell proliferation and differentiation). Since GDNF receptors are located in terminals of SN neurons, it is expected that these genes are upregulated in DA SN neurons in response to GDNF activation.[42] In any event, GDNF-dependent signalling pathways are as yet poorly studied and the role of GDNF in the maintenance of adult DA neurons remains essentially unknown.

8.2.2 Dopaminotrophic Factor-Mediated Neuronal Protection

As indicated above, GDNF has a well-recognized potent neurotrophic effect on DA neurons *in vitro*. Addition of GDNF to primary cultures of midbrain neurons favours the survival of the culture, and increases dopamine uptake, cell size and neurite length.[25] It has been demonstrated that GDNF exerts a protective role on DA neurons exposed to neurotoxic agents.[14] GDNF increases the survival of mesencephalic DA neurons treated with either 1-methyl-4-phenylpyridinium (MPP^+) or 6-hydroxydopamine (6-OHDA) and promotes the regrowth of damaged dopaminergic fibres.[43] A neuroprotective effect of GDNF against MPP^+ toxicity has been also shown in organotypic cultures of ventral mesencephalon, a preparation in which the integrity of DA neurons is better preserved than in enzymatically dispersed preparations.[44]

As it occurs with GDNF, the other trophic factors members of the GDNF family, NRTN, ARTN and PSPN, also increase the survival of DA neurons *in vitro* and show a neuroprotective effect on cells treated with 6-OHDA, although they seem to be less efficient than GDNF. The neuroprotective action of NRNT[45] and ARTN[46] is mediated by cross-reactive stimulation of the canonical GDNF receptor GFRα1-Ret that is expressed on DA neurons. Curiously, PSPN was believed to be unable to stimulate the GFRα1-Ret receptor[47] so the mechanism by which it exerted the protective action on dopaminergic neurons was uncertain. However, it has have been recently shown that PSPN can activate the GFRα1-Ret receptor as well,[48] explaining the protective effect that PSPN exerts on DA neurons.

Among the different NTFs tested *in vivo*, GDNF has shown the most potent and robust effect on animal (rodent and primate) models of parkinsonism.[28]

In the initial *in vivo* studies, the direct infusion of recombinant protein was used to deliver GDNF to the brain parenchyma. These experiments demonstrated that GDNF protects nigral DA neurons by activating their metabolism and dopamine turnover. GDNF also induced axonal sprouting and striatal reinnervation.[49]

As direct intrastriatal infusion of GDNF has potential complications derived from the chronically implanted infusion device or the diffusion of the trophic factor, an alternative approach for brain GDNF delivery is the administration of replication-deficient viral vectors. Three viral vector systems (adenovirus, adeno-associated virus and lentivirus) engineered to produce continuous expression of GDNF have been used with good experimental results.[40,50,51] The transfection based on adeno-associated virus and lentivirus is particularly interesting because it produces a long-term expression of GDNF without detectable cellular pathology or immune reaction (for a review, see ref. 28). However, the use of viral vectors in patients raises safety concerns because of their potential immunogenicity and the risk of mutagenesis by insertion into the genome of host cells. Another strategy to produce continuous intrastriatal GDNF delivery is the use of genetically modified cells that synthesize and release GDNF. In this regard, several groups have reported protective effects of GDNF-secreting genetically modified cells in animal models of PD.[52–55] The long-term survival of the engineered cells and the prevention of the immune reaction that they can trigger are two of the major hurdles that this technology must overcome before it can be considered for clinical application.[56]

Intrastriatal grafting of carotid body (CB) cells is a methodology developed in our laboratory that also seems to produce GDNF-mediated neuroprotection in PD.[57,58] The CB, a bilateral organ located in the bifurcation of the carotid artery, is a major arterial chemoreceptor organ responsible for the detection of changes in O_2 concentration in the blood. In conditions presenting hypoxemia, CB sensory cells release transmitters that activate afferent sensory fibres terminating in the respiratory centre to induce a compensatory hyperventilatory response.[59] CB sensory cells (called glomus cells) are of neural-crest origin and contain high levels of dopamine as well as GDNF (Figure 8.1)[60,61] and are thus well-suited to be used as donor tissue in transplantation studies in PD. Indeed, intrastriatal CB grafts can produce a significant histological and functional recovery in rodent and primate models of parkinsonism[57–58,60] (Figure 8.2A). It has been shown that the benefit induced by rat CB grafts is mainly due to a trophic effect on the nigrostriatal neurons rather than to the release of dopamine from the transplanted cells.[60] Once in the brain, grafted CB cells remain metabolically active during the entire animal lifespan and maintain their ability to produce GDNF (Figures 8.2B and 8.2C).[61] The reason for the long-lasting cell survival observed in CB transplants might be related to the fact that glomus cells are activated by hypoxia, an environmental condition presumed to be present inside the graft that is possibly deleterious for other cells types. Another advantage for the clinical application of CB cell therapy is that it allows autografts to be performed in PD patients, since unilateral CB surgical resection has no significant side effects in humans.[62]

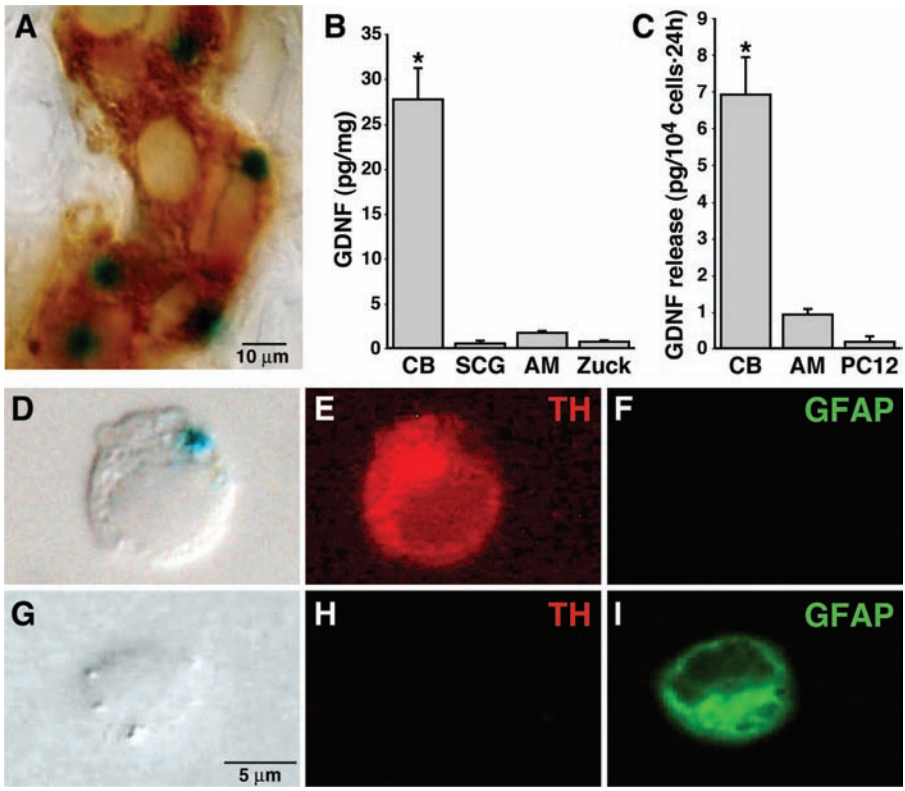

Figure 8.1 Synthesis and release of GDNF in dopaminergic carotid body glomus cells. (**A**) Histological section of mouse carotid body tissue showing the expression of GDNF (green colour aggregasomes, X-gal staining) in tyrosine hydroxylase (TH) positive glomus cells (brown colour). (**B**) and (**C**) Measurement of GDNF content (**B**) and release to the medium (**C**) in cultures of rat carotid body (CB), adrenal medulla (AM), organ of Zuckerkandl (Zuck) and PC12 cells. (**D**–**I**) Immunohistochemical analysis showing that the GDNF promoter is active in mouse type I carotid body cells (TH positive and glial fibrillary acidic protein (GFAP) negative cells). Modified from ref. 61. Animals GDNF/LacZ were used to study activity of the GFAP promoter (see refs. 60,71).

Two phase I/II clinical trials have shown that CB autotransplantation induces clinical benefits in PD patients, particularly in those who are not in an advanced stage of the disease.[63,64] The CB is small organ and therefore a limitation of CB-based cell therapy is the scarcity of tissue available for transplantation. To overcome this obstacle we have started a programme to expand CB cells *in vitro*. It is well-known that, in conditions of chronic hypoxemia (*i.e.* in high altitude residents or in patients with chronic obstructive pulmonary diseases), the CB undergoes a compensatory hypertrophy.[65–67] This led us to hypothesize that adult mammalian CB could contain latent neural progenitors activated by low O_2 tension. In accord with this proposal, we have

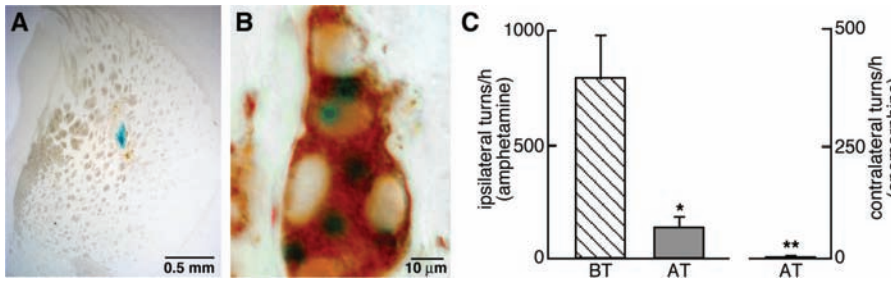

Figure 8.2 Transplantation of carotid body cell aggregates in rodent models of Parkinson's disease. (**A**) Histological section at the level of the mouse striatum showing the expression of GDNF (green colour) in a carotid body transplant from a GDNF/LacZ mouse (see ref. 71). (**B**) Histological section of a striatum containing a carotid body transplant. Cells in the graft show the expression of GDNF (green colour aggregasomes, X-gal staining) in tyrosine hydroxylase (TH) positive glomus cells (brown colour). (**C**) Rotational behavior of a hemiparkinsonian rat before (BT) and after (AT) intrastriatal transplantation of carotid body tissue. Modified from refs. 60,61.

identified in the CB a population of neural crest-derived stem cells that in response to hypoxia can proliferate and differentiate in new dopaminergic and GDNF-producing glomus cells.[68] We are currently investigating whether these newly identified progenitors are active in humans and if they can be used to expand CB tissue before transplantation.

8.3 Genetic Models of NTF Depletion: Conditional GDNF Knock-out Mice

Most of the knowledge available on the physiological function of GDNF has come from the analysis of genetically modified mice models. It is more than 15 years since three groups independently showed that ablation of the GDNF gene (*Gdnf* $^{-/-}$ mice) results in animal death after birth due to renal agenesis and the absence of then enteric plexus.[69–71] Unexpectedly, the *Gdnf* $^{-/-}$ mice had an apparently normal number and organization of mesencephalic DA neurons. These observations suggested that the trophic dependence of nigrostriatal neurons on GDNF, supported by the pharmacological experiments (exogenous administration of the trophic factor), must be acquired during postnatal maturation. Heterozygous *Gdnf* mice are fertile and develop normally, although they manifest an accelerated decline in spontaneous motor activity and coordination with age.[72] Nevertheless, this embryonic GDNF deficit seems to have little impact on the adult, since at 20 months of age, the mice show only a 15% decrease of TH-positive SN neurons and no difference in striatal TH^+ fibre density with respect to controls.[72] *Gdnf* $^{+/-}$ mice, however, seemed to show a higher susceptibility to neurotoxin-induced long-term degeneration of monoaminergic neurons than wild-type littermates.[73]

Region-specific genetic deletion (driven by the promoter of the dopamine transporter gene) of Ret (the canonical GDNF receptor) in DA neurons has provided conflicting results regarding the role of this pathway in the maintenance of adult neurons. No differences in adult DA nigrostriatal neurons have been shown in Ret-null mice *versus* controls, as determined by comparative morphometric and biochemical analysis.[31] However, another group has reported that embryonic deletion of Ret in catecholaminergic neurons results in a significant decrease of TH^+ SN neurons and striatal nerve terminals of aged mice although, unexpectedly, neurons in the VTA and LC remain unaffected.[74] The variable results obtained with the regional Ret-null mouse might be related to the fact that, besides the Grfa-1/Ret pathway, GDNF can signal through 'non canonical' N-CAM receptors,[32] that may compensate for the absence of Ret.

We have recently reported the generation of a conditional GDNF-null mouse in which GDNF expression was markedly reduced in adulthood.[29] This model avoids the establishment of developmental compensatory modifications, which could mask the true physiologic action of GDNF in the adult nervous system. The conditional GDNF deficient mice show selective and extensive catecholaminergic neuronal death, most notably in the LC, SN and VTA (Figure 8.3). GABAergic and cholinergic pathways appear to be unaffected. The neurochemical and histological alterations in GDNF-deprived mice induce the appearance of behavioural motor disturbances characterized by a progressive akinetic syndrome (Figure 8.4). These data have demonstrated that endogenous GDNF is absolutely required for trophic maintenance of mesencephalic

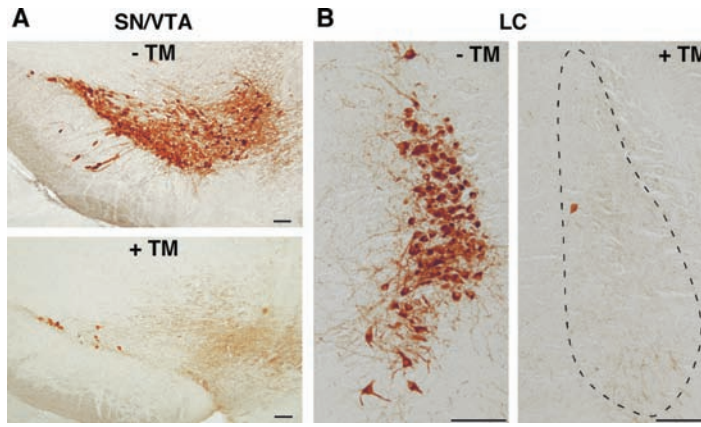

Figure 8.3 Mesencephalic catecholaminergic neuronal death in the adult conditional GDNF knout out mouse. (**A**) Mesencephalic dopaminergic neurons in substantia nigra (SN) and ventral tegmental area (VTA) on a conditional GDNF-null animal before (−TM) and seven months after (+TM) deletion of the GDNF gene. (**B**) Same experiment showing the disappearance of cells in the locus coeruleus (LC) after GDNF depletion. Modified from ref. 29.

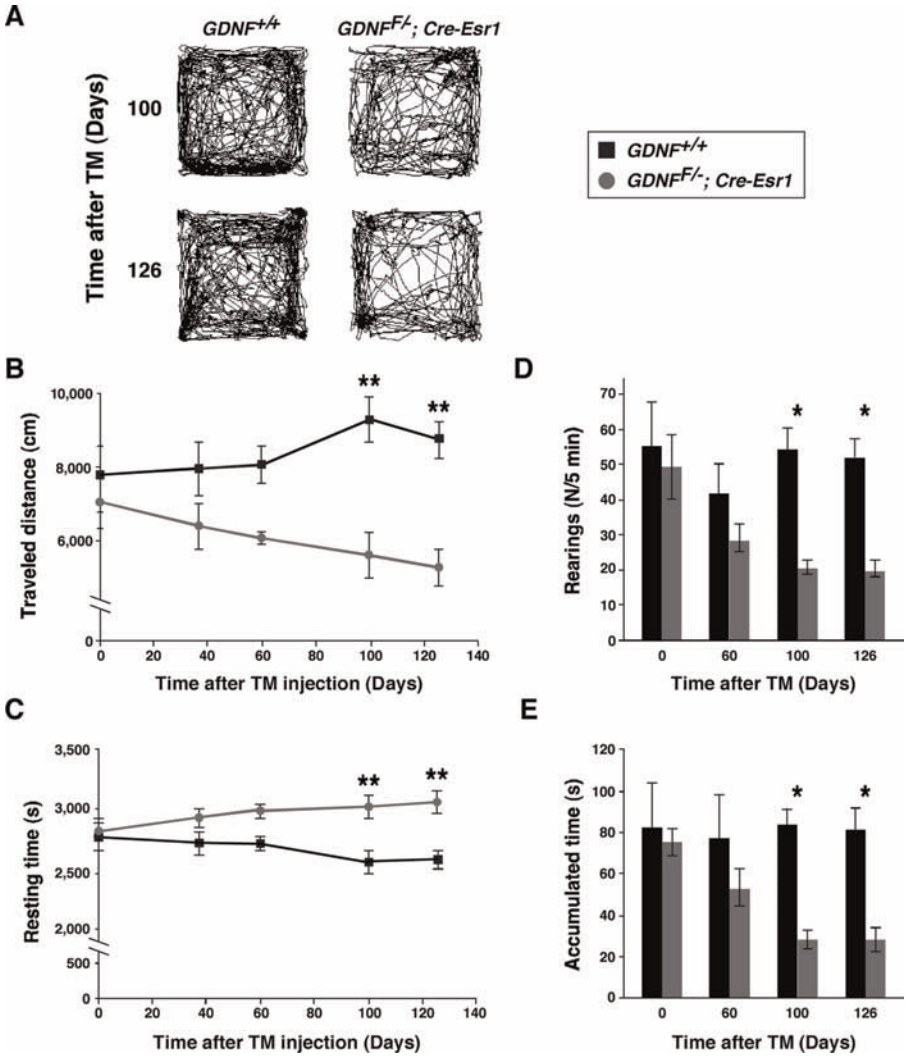

Figure 8.4 Motor abnormalities in the adult conditional GDNF knock-out mouse parkinsonian model. Spontaneous activity (animals were recorded during 60 min in an open field chamber) measured in wild-type (black bars: $GDNF^{+/+}$; +/+ mice treated with TM; $n = 13$) and GDNF depleted animals (grey bars: $GDNF^{F/-}$; *Cre-Esr1* mice treated with TM; $n = 7$) three days before and 37, 60, 100, and 126 days after TM injection. (**A**) Four traces from wild-type and GDNF-depleted animals are shown as representative of the time points 100 and 126 days after TM injection. Activity trace during minutes 16–30 is presented. (**B**) Travelled distance (cm) was calculated by following the centre of gravity of the subject. (**C**) Resting time (s) was the time spent in resting state (with reference to the default velocity threshold of 2.57 cm/s). (**D**,**E**) Vertical movements quantified in three periods of five minutes from each animal and time point. Averaged readings (**D**) and the accumulated time spent with both forepaws without contacting the floor (**E**) are plotted. Each individual point represents 3–8 animals. One-way ANOVA followed by the Tukey test. Means $\pm$ S.E.M.; *, $p < 0.05$; **, $p < 0.01$. Modified from ref. 29.

dopaminergic and noradrenergic neurons. The GDNF-deficient mouse is a well-defined model in which to study neuroprotection in experimental PD. It remains to be investigated in the future which essential GDNF-controlled targets are required for mammalian SN, VTA and LC neuronal survival.

8.4 Clinical Effects of GDNF

The good results obtained in the preclinical studies on the neuroprotective role of GDNF have stimulated the development of clinical studies designed to test the therapeutic effects of GDNF in advanced PD patients. Besides the clinical studies with CB tissue referred to above, several clinical trials have been performed using direct intracerebral infusion of GDNF.

In a controlled clinical trial, monthly intraventricular GDNF injection failed to provide clinical benefit in advanced PD patients and instead resulted in frequent adverse events.[75] A postmortem examination in one patient suggested that GDNF did not reach the target cells *via* this route. However, encouraging clinical and neurochemical results were observed with continuous intraputaminal GDNF infusion on PD patients in two independent open-label clinical trials. One of the trials performed on five PD patients reported encouraging clinical outcomes at one year, while [^{18}F]-dopa PET studies showed an increase in putaminal uptake around the tip of each catheter.[76] The second study on 10 patients using a different delivery protocol also reported positive results at six months.[77] However, a randomized placebo-controlled trial involving 34 PD patients showed no significant clinical differences between groups at six months despite increased [^{18}F]-dopa uptake in the recombinant GDNF-treated group.[78] The open-label extension of this study was halted due to safety concerns: three patients developed neutralizing antibodies, which could potentially cross-react with endogenous GDNF, while in a parallel toxicology study some monkeys developed cerebellar damage. Besides GDNF, other members of the same protein family (particularly neurturin) are also being assayed in pilot clinical trials with yet inconclusive results.

8.5 Conclusions and Perspectives

NTFs exert a potent effect on the survival and maintenance of phenotype in adult neurons. Therefore intracerebral administration of these factors is a promising therapeutic strategy in neurodegenerative disorders, such as PD, presenting progressive neuronal death. There is a vast scientific literature supporting the neuroprotective role of exogenous GDNF on the nigrostriatal pathway. However, most of the clinical trials performed to test the efficacy of NTF-based therapies in advanced PD patients have been quite discouraging. The generation of the conditional GDNF-null mouse model has recently allowed us to show the absolute requirement of GDNF for the survival of dopaminergic and noradrenergic mesencephalic neurons in adult brain.

These data unequivocally demonstrate a major physiological neuroprotective effect of GDNF and thus it should revive interest in GDNF-based therapies.

Clinical application of NTFs is confronted with several technological and scientific challenges that should be addressed in future preclinical and clinical research. Before new clinical trials are performed, a safe and efficacious route of GDNF delivery (produced in cells, purified, or encoded in viral vectors) must be clearly established. In this regard, diffusion of GDNF in the brain parenchyma and the appropriate concentration of GDNF delivered to cells are critical issues that might determine the clinical outcome. It must be also investigated whether the administration of appropriate cocktails of several trophic factors offer advantages over the use of GDNF alone. Besides these technologically oriented studies, much research should be done to unravel the actual physiological role of NTFs and their molecular mechanism of action on adult neurons. This work might eventually lead to the identification of new signalling pathways that will provide targets accessible to small molecules amenable for their use as pharmaceutical drugs. The molecular physiology and pharmacology of neuroprotection are still in their infancy. Therefore, it can be presumed that the development of these fields will surely offer new opportunities for a more effective fight against PD and other neurodegenerative diseases.

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