Interactive report

Towards a neuroprotective gene therapy for Parkinson’s disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model

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Abstract

During the last few years, recombinant viral vectors derived from adenovirus (Ad), adeno-associated virus (AAV) or lentivirus (LV) have been developed into highly effective vehicles for gene transfer to the adult central nervous system. In recent experiments, in the rat model of Parkinson’s disease, all three vector systems have been shown to be effective for long-term delivery of glial cell line-derived neurotrophic factor (GDNF) at biologically relevant levels in the nigrostriatal system. Injection of the GDNF encoding vectors into either striatum or substantia nigra thus makes it possible to obtain a regionally restricted over-expression of GDNF within the nigrostriatal system that is sufficient to block the toxin-induced degeneration of the nigral dopamine neurons. Injection of GDNF vectors in the striatum, in particular, is effective not only in rescuing the cell bodies in the substantia nigra, but also in preserving the nigrostriatal projection and a functional striatal dopamine innervation in the rat Parkinson model. Long-term experiments using AAV-GDNF and LV-GDNF vectors show, moreover, that sustained GDNF delivery over 3–6 months can promote regeneration and significant functional recovery in both 6-OHDA-lesioned rats and MPTP-lesioned monkeys. The impressive efficacy of the novel AAV and LV vectors in rodent and primate Parkinson models suggests that the time may now be ripe to explore these vector systems as tools for neuroprotective treatments in patients with Parkinson’s disease. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system

Topic: Degenerative disease: Parkinson’s

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1. Introduction

In Parkinson’s disease (PD) symptoms start to appear when about 70–80% of striatal dopamine is lost and about 50% of the dopamine neurons in the substantia nigra have degenerated. Autopsy data and neuroimaging, by 18F-fluorodopa PET (for dopamine synthesis and storage) or β-CIT SPECT (for dopamine uptake sites), indicate that there is a progressive loss of nigral dopamine neurons and a concomitant decline of striatal dopamine function at a rate of 5–10% per year [10,24]. This progressive nature of the disease offers opportunities for therapeutic interventions aimed at blocking or slowing down the ongoing degenerative process. Indeed, recent imaging data suggest that it may be possible to detect a decline in striatal dopamine function even before the onset of overt clinical symptoms, which would make it possible to initiate neuroprotective interventions in the very early stages of the disease, i.e. at the time when, or even before, the first symptoms appear (for a review, see Ref. [21]).

Neurotrophic factors are interesting candidates for neuroprotective therapies since they can interfere with both apoptotic and necrotic forms of cell death, and have been...
shown to rescue injured neurons after toxic, mechanical or ischemic damage in the adult nervous system. In PD, the affected dopamine neurons are likely to remain dysfunctional for long periods, perhaps years, before they are irreversibly lost. This suggests that neurotrophic factors may be able not only to prevent further cell loss, but also to restore function in dysfunctional or atrophic neurons in the degenerating nigrostriatal system.

Although a large number of growth factors can act as survival factors for nigral dopamine neurons, the members of the glial cell line-derived neurotrophic factor (GDNF) family are particularly interesting because of their potent in vivo effects in both rodent and primate models of PD. Studies using intracerebral injections of the recombinant protein have shown that GDNF can provide almost complete protection of nigral dopamine neurons against 6-hydroxydopamine (6-OHDA)- or MPTP-induced damage, promote axonal sprouting and regrowth of lesioned dopamine neurons, and stimulate dopamine turnover and function in neurons spared by the lesion [6,25]. Although these toxin-induced lesion models have a weakness in that they do not reproduce the same disease mechanism(s) and pathophysiology as seen in human PD, the results obtained in animal models are sufficiently impressive to suggest that GDNF, or its close relatives neurturin and artemin/neublastin, may be useful as therapeutic agents for neuroprotection in PD. However, given the chronic, progressive nature of PD it is likely that the factor should be administered continuously, over months or years, in order to sustain dopamine neuron survival and function long term. Moreover, since GDNF receptors are widely distributed in the nervous system, the factor may have to be delivered locally in order to avoid negative side effects.

For this reason, locally induced production of the neurotrophic factor by direct in vivo or indirect ex vivo delivery of the GDNF gene to the striatum and/or substantia nigra may offer distinct advantages. During the last few years, progress along these lines has been remarkable. Efficient long-term expression of GDNF in the nigrostriatal system has been achieved with three different vector systems, i.e. recombinant adenovirus (Ad), adeno-associated virus (AAV) and lentivirus (LV) vectors. Each of these vector systems holds great promise for gene transfer of therapeutic proteins to non-dividing cells of the adult central nervous system. In this review we will summarize the results obtained so far in rodent and primate models of PD, and discuss the steps that need to be taken in order to bring this gene transfer technology closer to the clinic.

2. Adenoviral (Ad) vectors

Ad vectors are advantageous in that they can accommodate large pieces of DNA (up to 8 kb), can be generated free of contaminant replication-competent virus at very high titers, and can infect both dividing and non-dividing cells. The transferred DNA remains as a non-integrated episome in the nucleus and is, therefore, most adequate for transient expression of transgenes in non-dividing cells.

The Ad vectors used thus far in GDNF transfer experiments have the disadvantage that the transduced cells express adenoviral proteins that may cause inflammation and trigger host immune reactions towards the infected cells (see Wood et al. [66] and Kajiwara et al. [30] for further discussion). This, in turn, may reduce transgene expression over time and contribute to the variable long-term expression of the transduced protein seen in several in vivo studies using first-generation Ad vectors [30,66]. The more recent, so-called helper-dependent, or gutless, Ad vectors may help to solve this problem and, with all wild-type Ad genes deleted, carries an enormous packaging capacity of around 35 kb [27,49,62].

Intracerebral delivery of GDNF by Ad-GDNF vectors has been explored in rats with 6-OHDA lesions of the nigrostriatal dopamine system [5,12,13,15,38]. Choi-Lundberg et al. [12,13] have shown that Ad-GDNF injected either close to the substantia nigra or into the striatum, given 1 week before an intrastriatal 6-OHDA lesion, can afford significant protection of the nigral dopamine neurons against the toxic insult: after intranigral Ad-GDNF injection, 79% of the nigral dopamine neurons survived, compared to 31% in the controls (given a similar injection of Ad-lacZ); and after intrastriatal injection, 64% of the neurons survived, compared to 36% in the controls, as determined 6 weeks after the 6-OHDA lesion. The size of the striatal lesion, as assessed by immunohistochemistry for the tyrosine hydroxylase (TH) enzyme, was unaffected by the Ad-GDNF treatment. Thus, the intensity of TH-positive fiber staining in the striatum was reduced equally, by about 40%, in all treatment groups, suggesting that GDNF over-expression did not modify the extent of the acute toxic damage to the dopamine terminals in the striatum.

In these studies, transduction efficiency was measured in two ways: first, by counting β-galactosidase (β-gal)-positive cells in the Ad-lacZ-injected animals and, secondly, by measurements of tissue GDNF protein levels by ELISA, and tissue GDNF DNA and RNA levels by PCR and rtPCR in the Ad-GDNF-injected animals. Both parameters indicated down-regulation over time: the number of β-gal-positive cells in the mesencephalon declined from 39 000 at 1 week to 7100 at 7 weeks in the intranigraly injected animals [12]. In the striatum, the intensity of β-gal staining decreased between 1 and 7 weeks, although the total number of β-gal-positive cells was relatively unchanged (27 000 and 24 000 cells at 1 and 7 weeks, respectively [13]). Tissue GDNF levels declined from 13 to 4.7 ng (per nigral sample) between 1 and 4 weeks in the substantia nigra, and from 14 to 11 ng (per striatal sample) between 1 and 7 weeks in the striatum (see Table 1). GDNF DNA and RNA levels determined by PCR and rtPCR indicated a similar extent of down-regulation over the first 4–7 weeks.
Table 1

Levels of GDNF expression in the basal ganglia obtained by three viral vector systems

<table>
<thead>
<tr>
<th>Vector</th>
<th>Host [Ref.]</th>
<th>Injection volume (μl)</th>
<th>Site of injection</th>
<th>GDNF content in striatum (ng/mg tissue)</th>
<th>GDNF content in substantia nigra (ng/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>Rat [13]</td>
<td>2</td>
<td>str (one site)</td>
<td>1–2*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Monkey [9]</td>
<td>30</td>
<td>str (one site)</td>
<td>0.09*</td>
<td>ND</td>
</tr>
<tr>
<td>rAAV</td>
<td>Rat [34]</td>
<td>9</td>
<td>str (three sites)</td>
<td>0.22</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Rat [34]</td>
<td>2</td>
<td>sn (two sites)</td>
<td>1.58</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>Rat [45]</td>
<td>2</td>
<td>sn (two sites)</td>
<td>ND</td>
<td>0.2–0.6*</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Rat</td>
<td>3</td>
<td>str (three sites)</td>
<td>2.3–6.3</td>
<td>0.2–0.9</td>
</tr>
<tr>
<td></td>
<td>Mouse [4]</td>
<td>1</td>
<td>sn (one site)</td>
<td>ND</td>
<td>0.5–0.6*</td>
</tr>
<tr>
<td></td>
<td>Monkey [37]</td>
<td>45</td>
<td>str (five sites)</td>
<td>0.22–0.35*</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Approximate levels of GDNF from the indicated anatomical areas and species are presented for purposes of a rough comparison rather than a precise quantitative analysis. For example, the adenovirus injections were made in both anatomical structures as were the lentivirus injections in the monkey, but the rAAV injections were made in the individual structures. Different vector particle concentrations were used in each study and are not taken into account in this comparison because of the difficulty in comparing this parameter between different vectors. Vector titers can be found in the cited references. In addition, the survival times varied widely between the studies. Some of the values are estimated as detailed below. An asterisk (*) indicates that the ng/mg tissue was calculated from a reported ng per punch value by a rough estimate of the mg per punch. The dagger (²) indicates that the reported value’s units were originally ng/mg protein. Therefore, we estimated that protein constitutes approximately 10% of the tissue weight and calculated the values in the table accordingly (ND, not done; str, striatum; sn, substantia nigra). The rat LV data are from our own unpublished study (Georgievska et al., to be published).

In the second study, Choi-Lundberg et al. [13] monitored changes in nigrostriatal function in tests of drug-induced rotation and spontaneous forelimb use. However, with the type of lesion used (16 μg of 6-OHDA in a single striatal deposit) the extent of striatal denervation (about 40%) is insufficient to induce any consistent functional effects that are stable over time (see Ref. [32] for further discussion). Thus, although the Ad-GDNF vector-injected animals displayed less motor asymmetry acutely after the lesion (at 4–12 days after a unilateral 6-OHDA injection), these differences were not maintained at longer survival times, which at least in part was due to spontaneous recovery in the control groups. In a follow-up study, Connor et al. [15] used the same lesion parameters, but this time in aged rats. In 20-month-old rats, the lesion-induced deficits, albeit small, were more stable over time. Significant improvement in side bias andamphetamine-induced rotation was observed when the Ad-GDNF vector was injected into the striatum. Vector injection into the substantia nigra, by contrast, had a detrimental, rather than somewhat complicated by the toxicity induced by the large amount of vector, 1.5×10⁸ pfu, was four to five times higher than those used in the Choi-Lundberg et al. [12,13] experiments (3.2–3.9×10⁷ pfu). The level of GDNF expression in the Ad-GDNF-injected animals was not determined in the Bilang-Bleuel et al. [5] study, but the multiple injection protocol used suggests that GDNF may have been expressed more widely and at higher levels throughout the striatum. This would explain why Bilang-Bleuel et al. [5] observed not only partial protection of the TH-positive nigral cell bodies (to a mean of 62% of normal) but also partial sparing of the striatal TH-positive innervation, which was semi-quantitatively estimated to be in the range of 75%. In line with these morphological data, amphetamine-induced rotation was reduced by 80–90% in the Ad-GDNF-injected animals at 1–3 weeks after 6-OHDA treatment. Although no observations on spontaneous (i.e. non-drug-induced) motor behaviors were made in this study, the data suggest partial sparing of a functional nigrostriatal pathway in the Ad-GDNF-injected animals. The interpretation of the results, however, is somewhat complicated by the toxicity induced by the large amounts of Ad vectors used (1.5×10⁸ pfu in 9 μl). Both Ad vectors, containing GDNF or lacZ, caused both inflammation and atrophy of the injected striatum. The control Ad-lacZ vector, in addition, induced a non-specific 37% loss of dopamine neurons in the ipsilateral substantia nigra (i.e. in the absence of any 6-OHDA lesion).

Clearly, with the current E1, E3 deleted Ad vectors used so far, the magnitude of the inflammatory response and the toxicity increases with increasing titers of the injected vector. These host responses, which are likely to be triggered by the expression of viral proteins on the surface of the transduced cells, may also suppress the expression...
of the transgene or even kill the transduced cells [66]. This is particularly well illustrated in the experiments of Bohn et al. [8] and Lawrence et al. [40], using injections of Ad-lacZ vector in the caudate nucleus of intact monkeys. In a group of 10 monkeys, all given the Ad-lacZ vector in the same dose, the number of β-gal-expressing cells in the caudate varied from 0 to 600,000. Staining with inflammatory markers revealed an inverse correlation between transgene expression and extent of the inflammatory response, including demyelination [40]. Injection of varying titers of the vector (from 5×10^4 to 2×10^9 pfu) showed that the intensity of the host's immune response increased with increasing titers. At 1 month after vector injection, no β-gal expression was seen in the animal given the highest titer [8]. These data clearly support the view that the host’s immune response is a major limitation for high-level, stable transgene expression in the brain from the currently used Ad vectors, and is probably a major reason why GDNF expression from intracerebrally injected Ad vectors declines and becomes more variable over time. In the recent study of Connor et al. [15], for example, only 10 of 14 animals showed GDNF expression at 6 weeks after Ad-GDNF injection into striatum or nigra of aged rats, as revealed by immunohistochemistry. Ad-GDNF expression at survival times longer than 6–7 weeks has so far not been reported in the rat PD model.

3. Adeno-associated viral (AAV) vectors

The recombinant AAV vectors have 96% of the viral genome removed, leaving only the two short inverted terminal repeats (ITRs) which are sufficient for packaging and integration. The advantage of these vectors is that they can integrate and stably express their transgene product in non-diving cells, including neurons, and that the absence of viral genes minimizes the expression of foreign proteins and hence the risk of triggering host immune responses [50]. The disadvantage is that the rAAV DNA packaging capacity is small, less than 5 kb, which limits the size of the gene constructs that can be delivered with the rAAV system. Another limiting factor is that the transgene is expressed with a delay of several days, and increases gradually over the first 2–3 weeks, probably due to the fact that a second strand of DNA needs to be synthesized in the transduced cells before the transgene can be expressed (for recent reviews, see Refs. [48,54,61]).

Studies using β-gal or GFP as reporter genes have shown that the rAAV vector is efficient in transducing non-dividing cells, mainly neurons, in the adult CNS [3,31,35,43,55]. However, not all types of neurons are equally good targets and the transduction efficiency varies greatly between different brain regions. This is, at least in part, likely to be due to the fact that AAV requires binding to heparan sulfate proteoglycans, as well as to co-receptors (such as the FGF receptor 1). Thus, the presence or absence of such receptor molecules on the cell surface may determine the efficiency by which the rAAV vectors are internalized into different types of neurons [3,54,61].

In the nigrostriatal system, rAAV vectors have been shown to be effective in transducing neurons in both substantia nigra, globus pallidus and striatum, and high levels of transgene expression have been observed over at least 6 months after vector injection [34,35,41,42] (Figs. 1 and 2). Over 90% of the transduced cells can, by morphological criteria, be classified as neurons. The high affinity of the rAAV vector for the pars compacta of the substantia nigra makes it possible to express the transgene in a high proportion of the nigral dopamine neurons with a single 1–2 μl injection of the vector (Fig. 1J–L). The transduction efficiency is, by comparison, lower in the striatum. In our recent study [34], using a single 3 μl injection of the rAAV-GFP vector in the striatum, we observed GFP-positive cells around the injection site within a radius of about 0.3 mm, compared to a radius of about 1.5 mm in the substantia nigra after a 1 μl injection. To reach larger areas of the striatum, therefore, we have had to use multiple injections of the vector (3×3 μl), spaced with a distance of about 1 mm between the injection sites.

Recent improvements in rAAV vector production has resulted in 100–1000-fold higher titers, a higher proportion of infectious particles (relative to empty ones), as well as completely Ad helper virus free vector preparations [67,68]. The new vector we are exploring now (provided by the University of Florida vector core) includes, in addition, a modified promoter construct, i.e. a hybrid CMV/chicken β-actin (CBA) promoter with the woodchuck hepatitis virus posttranscriptional element (WPRE) [18,69], instead of the MD promoter [53] used in our earlier studies [34,43,45]. The new vector has resulted in a substantial increase in transduction efficiency in both striatum and substantia nigra, both in terms of the number of GFP-expressing cells and the level of GFP per cell. The transduced protein is transported intra-axonally, from the nigra along the nigrostriatal pathway to the terminals in the striatum (Fig. 1), and from the striatum along the striatonigral pathway to the terminals in the globus pallidus, entopeduncular nucleus and pars reticulata of the substantia nigra (Fig. 2). Indeed, with the new vector construct, the level of expression is such that the axonal and dendritic projections of the transduced nigral and striatal neurons appear to be completely filled by the transduced GFP.

Intracerebral delivery of GDNF by means of AAV-GDNF vectors has so far been explored in the intrastral 6-OHDA lesion model in three studies [34,44,45], using a rAAV vector of high titer (1.0×10^12 viral particles per ml) and the MD promoter (a CMV immediate-early promoter with an intervening β-globin intron) which has been shown to support sustained, long-term expression in the rat brain [43]. In the first two studies [44,45] the AAV-GDNF vector
Fig. 1. Distribution of the GFP protein in an intact animal receiving an injection of the AAV-GFP vector into the substantia nigra (SN) (2 μl, 5 weeks survival, CBA promoter). The transgene is highly expressed within the nigral dopamine neurons (J–L) and transported anterogradely in the axons to fill out virtually the entire nigrostriatal pathway and its axonal branches in the entopeduncular nucleus (EP; G–I), globus pallidus (GP; D–F) and the striatum (STR; A–C). Also, the dendrites in the substantia nigra, pars reticulata contain high levels of the transduced GFP protein (L).

was injected over the nigra, either 3 weeks before or immediately after the intrastriatal 6-OHDA injection. In the first case, the time interval between vector injection and lesion was sufficient to allow GDNF to become fully expressed at the time of the 6-OHDA injection. In the second case, GDNF was expressed with a delay of a few
days, with full expression at about 10 days after the initial 6-OHDA-induced axon terminal damage. This means that the onset of GDNF production coincided with the onset of degeneration of the nigral dopamine cell bodies, which starts 5–7 days after the 6-OHDA injection [63]. Both injection paradigms were equally efficient in rescuing the dopamine neuron cell bodies: 92–94% survival in the AAV-GDNF-injected groups, compared to 45–51% surviv-

Fig. 2. Injection of the AAV-GFP vector in the striatum (3×3 µL, 5 weeks survival, CBA promoter) will transduce a large number of cells (almost exclusively neurons) in both striatum and globus pallidus (B,C,E,F). The transduced protein is effectively transported anterogradely along the striatogniral pathway to the globus pallidus (GP, D–F), entopeduncular nucleus (EP, G–I) and substantia nigra (SN, J–L).
al in the AAV-lacZ-injected groups. The extent of denervation of the TH-positive fibers in the striatum, however, was unaffected by the AAV-GDNF treatment.

The level of GDNF expression, as determined in 2-mm diameter punches from the transduced substantia nigra, remained fairly stable over the 10-week observation period, at a level of 0.7–1.2 ng/punch (approximately equivalent to 0.3–0.6 ng/mg tissue; see Table 1). In the Choi-Lundberg et al. [12] study, using Ad-GDNF injections into the substantia nigra, the protection of the nigral cell bodies was incomplete (79%) despite several-fold higher GDNF tissue levels (see Table 1). This difference may be explained by the fact that the Ad vector is expressed mainly outside the dopamine neurons. Thus, over-expression of GDNF within the dopamine neurons themselves, as obtained with the AAV-GDNF vector, may be particularly efficient for rescue of the axotomised nigral cell bodies. With the new generation of AAV vector now available (see above) the level of GDNF obtained after injection in either nigra or striatum is 7–50-fold higher that that achieved with the previous vector (Kirik et al., unpublished data).

In the Kirik et al. [34] study, the AAV-GDNF vector was injected at multiple sites in the striatum, in the nigra, or in both striatum and nigra, 4 weeks before the 6-OHDA injection. Expression of GDNF, as observed by immunohistochemistry, was maintained at high levels in both sites throughout the 6-month experimental period. In animals receiving vector injection into the striatum, the GDNF protein was widely distributed throughout the striatum and transported along the striatoniigral pathway to the globus pallidus (Fig. 3D–F), the entopeduncular nucleus (Fig. 3G–I) and the substantia nigra (Fig. 3J–L). In this experiment, a four-site intrastriatal 6-OHDA lesion was used (4×7 μg) which gives a substantial, 80–90%, denervation of the striatum, sufficient to induce marked motor impairments that are stable over time [32,34]. Vector injection into the nigra was more efficient in protecting the nigral dopamine cell bodies: 91% cell survival in the nigral injection group, compared to 57% in the striatal injection group and 12% in the lesion-only controls. However, only the rats receiving AAV-GDNF injections into the striatum showed behavioral recovery, and this was accompanied by partial sparing of the nigrostriatal projection and reinnervation of the lesioned striatum (see Fig. 5C). As illustrated in Fig. 4, the recovery developed gradually over 4–5 months after the lesion and was observed in both drug-induced rotation (Fig. 4A) and in tests of spontaneous motor behavior, i.e. forelimb use in the so-called cylinder and staircase tests (Fig. 4B–D). No functional sparing or recovery was seen in the animals receiving AAV-GDNF injections into the substantia nigra, despite near-complete protection of the cell bodies. In these animals, the nigrostriatal projection and striatal TH-positive innervation was damaged to the same extent as in the lesion-only animals that received injections of the control vector (Fig. 5B,D).

These results are consistent with previous studies using injections or infusions of recombinant GDNF protein, which have shown that survival of the nigral dopamine neurons in the absence of a functional nigrostriatal projection is insufficient for functional sparing or functional recovery in the intrastriatal 6-OHDA lesion model [33,59,65].

The animals receiving AAV-GDNF injections into the striatum were as impaired as the control lesion rats acutely after the lesion (Fig. 4A,C), indicating that the over-expression of GDNF, at the levels obtained, was not sufficient to protect the striatal dopamine terminals against the toxic insult. However, the TH-positive axons along the nigrostriatal pathway were partly preserved and sprouting fibers were abundant in the globus pallidus and in the caudal and ventral parts of the striatum (Fig. 5C). The efficient striatal reinnervation seen in these animals thus appeared to be caused by a combination of protection of lesioned nigrostriatal axons followed by regeneration towards and into the region of high GDNF expression. This sequence of axonal damage followed by a protracted remodelling of the nigrostriatal projection is consistent with the slow and progressive functional recovery that developed during the first months after the lesion.

It is notable that over-expression of GDNF in the nigra failed to preserve the TH-positive axons along the nigrostriatal pathway. In these animals, there was massive axonal sprouting in and around the medial forebrain bundle, close to the rescued cell bodies. These sprouting fibers were seen to extend up to the border of the globus pallidus, but not further rostrally (Fig. 5D). Indeed, the area containing dense sprouting fibers coincided with the area of high GDNF-immunoreactivity, as seen in sections stained with the GDNF antibody.

These data are in agreement with results obtained with direct intracerebral injections of recombinant GDNF protein [33,60]. As illustrated in Fig. 7, GDNF injected into the striatum either 6 h before or 1 day after the intrastriatal 6-OHDA injection is efficient in preserving the cell bodies and the axons of the nigrostriatal pathway, as well as inducing axonal sprouting in the globus pallidus and regeneration of TH-positive fibers in the striatum, while GDNF over the substantia nigra induces sprouting locally around the injection site. In both cases, sprouting of TH-positive fibers occurs in the area reached by high concentrations of the GDNF protein. Consistent with these morphological data, significant sparing or recovery of motor functions was obtained only in the animals receiving GDNF injection into the striatum, i.e. in those animals which had significant sparing of the nigrostriatal projection in combination with GDNF-induced sprouting of TH-positive axons in the lesioned striatum [33].

Additionally, in the Kirik et al. [34] study, it is important to point out that no significant reinnervation of the striatum or functional recovery was seen in the animals with combined AAV-GDNF injections in both nigra and...
Fig. 3. Immunohistochemical visualization of the GDNF protein in an animal receiving injections of the AAV-GDNF vector (3×3 μl, MD promoter) in the striatum, 6 months survival. The transduced GDNF is widely distributed not only throughout the striatum (STR; A–C) but also along the axons of the striatal projection neurons to the globus pallidus (GP; D–F), entopeduncular nucleus (EP; G–I) and substantia nigra (SN; J–L). The efficient intra-axonal transport of the expressed protein suggests that it may be able to exert effects throughout the nigrostriatal system even when the vector is injected in the striatum only.
Fig. 4. Functional recovery in drug-induced rotation (A), and forelimb use in the paw-reaching (B) and cylinder tests (C and D) in 6-OHDA lesioned rats that had received AAV-GDNF injections into either striatum (STR), substantia nigra (SN) or both striatum and nigra (STR/SN). The vector injection was made 3 weeks before the intrastriatal 6-OHDA injection. Note that functional recovery is seen only in animals with intrastriatal vector injections (modified after Kirik et al. [34]).

striatum. We believe this is attributable to the intense local sprouting close to the substantia nigra seen in these animals, which may prevent regrowth of the lesioned axons towards the striatal source of GDNF (see Section 5).

Expression of GDNF in the nigral dopamine neurons themselves, therefore, may be detrimental, rather than positive, for the recovery of function in 6-OHDA lesioned rats.
Fig. 5. Over-expression of GDNF in the striatum by intrastriatal injections of the AAV-GDNF vector can block dopamine neuron degeneration induced by the intrastriatal 6-OHDA lesion, as illustrated in B and C. Importantly, the axons along the nigrostriatal pathway are partially preserved. These rescued axons provide the substrate for sprouting and regrowth into the area of high GDNF expression (indicated by green color in C), which in turn is accompanied by a gradual functional recovery (as illustrated in Fig. 4). Injection of the vector into the substantia nigra (D), by contrast, protects the nigral cell bodies but is unable to preserve the axonal projection to the striatum. Instead, there is extensive local sprouting of TH-positive fibers in regions close to the rescued cell bodies, i.e. into the area of high GDNF expression (green color in D). (Based on data from Ref [34].)

4. Lentiviral (LV) vectors

LV vectors are derived from a group of highly pathogenic retroviruses, which includes the HIV viruses. They share the useful properties of the commonly used oncoretroviral vectors, with the additional advantage that the LV vectors can integrate also into non-dividing cells. They have a large cloning capacity, at least 9 kb, and are stably integrated into the genome of the target cells, i.e. properties that are highly favorable for long-term expression of transgenes in the nervous system (for recent reviews, see Refs. [11,64]). Most of the efforts so far have been focused on the development of efficient vector systems based on the HIV-1 virus. This raises a number of important safety issues that have to be solved before these vectors can be considered for clinical use. In this respect, the alternative vector systems based on equine or feline lentiviruses, which appear to be non-pathogenic for humans, may be particularly attractive candidates for clinical use [17,28,46,56]. In the current versions of the HIV-1-based LV vector, up to 60% of the viral genome has been eliminated and only three or four of the nine genes of HIV-1 are retained [20,71]. The viral particles are generated by transient transfection of 293T cells with three or four different plasmids, which further reduces the risk of such recombination events that may lead to the generation of an infectious, replication-competent retrovirus [20,51,52,64,71]. The introduction of the so-called self-inactivating (SIN) version of the vector has further increased the biosafety of this vector system [19].

In the current version of LV vectors, the particles are pseudotyped with the G envelope protein of the vesicular stomatitis virus (VSV-G). This gives the vector the capacity to infect a broad range of tissues, including nervous tissues, and is probably responsible for their high affinity for fully differentiated neurons within the CNS [7,14,47,51,52]. The level of expression in the brain is further increased by the introduction of the woodchuck regulatory element (WPRE) into the vector construct [19,70].

The VSV-G pseudotyped LV vectors are highly efficient in transducing cells in both striatum and substantia nigra, and in both sites the majority of the transduced cells are neurons [7,19,20,36,52]. Deglon et al. [19] and Bensadoun et al. [4] reported a total of 21 800 and 38 000 cells expressing β-gal after injection of 1–2 µl of a high titer LV-lacZ vector in the substantia nigra in rat and mouse, respectively. Up to 50% of the TH-positive neurons were labelled when the most efficient SIN vector with the WPRE was used. In the monkey, using the same efficient vector, Kordower et al. [36] reported an average of 187 000 β-gal-positive cells in the nigra and up to 1.5 million positive cells in the striatum at 3 months post-injection. In the striatum, stable expression of reporter genes, β-gal or GFP has been observed for up to 6 months without any signs of toxicity or adverse inflammatory reaction in the host tissue ([7], Kirik et al., unpublished data).

Expression of GDNF in the nigrostriatal system with LV-GDNF vectors has so far been reported in four pub-
lished studies [4,19,37,58]. In the Deglon et al. [19] study, 2 μl of the LV-GDNF vector was injected unilaterally above the substantia nigra, followed 1 week later by a knife transection of the ipsilateral medial forebrain bundle (MFB). At 1 week after the lesion, 56% of the TH-positive neurons remained in the LV-GDNF-injected animals, compared to 24% in the LV-LacZ-injected lesion controls. In the Bensadoun et al. [4] study, which was carried out in mice, 1 μl of the LV-GDNF vector was injected over the nigra, followed 2 weeks later by an intrastriatal injection of 6-OHDA on the same side. At 4 weeks after the lesion, 70% of the nigral TH-positive neurons survived compared to 33% in the controls. Striatal dopamine levels were reduced by 90% in both groups, indicating that the striatal dopamine innervation was not spared in the LV-GDNF-injected animals. The level of GDNF, as determined by ELISA in dissected pieces containing the whole nigral region, was 2.6 and 1.95 ng at 1–6 weeks post-injection in the two studies. Assuming that the weight of the dissected piece was in the range of 4–6 mg, this is equivalent to 0.4–0.6 ng/mg, i.e. similar to the tissue levels obtained in the rat substantia nigra with the old version of the AAV.GDNF vector [34,44,45] (see Table 1).

In our own study [58], the LV-GDNF vector was injected both over the nigra (1 μl) and in the striatum (2×1 μl). One week later, the rats received a single injection of 20 μg 6-OHDA into the ipsilateral striatum. To help in the assessment of cell survival, the nigrostriatal neurons were labeled retrogradely by an injection of fluoro-gold in the striatum 2 weeks before the vector injection. With this combined vector injection there was almost complete rescue of the nigrostriatal neurons: 87% of the fluoro-gold labeled cells and 81% of the TH-positive cell bodies, compared to 17–24% survival in the LV-GFP-injected lesion controls. In addition, there was a significant partial preservation of the TH-positive innervation in the striatum, suggesting that over-expression of GDNF in the striatum had afforded partial protection of the nigrostriatal dopamine terminals against the toxic insult. Interestingly, a similar level of neuroprotection and sparing of the striatal TH-positive innervation was obtained with a vector expressing artemin/neublastin, suggesting that this newly discovered member of the GDNF family has prominent neurotrophic actions on the nigrostriatal dopamine neurons [58].

In a subsequent experiment (Georgievska et al., unpublished data) we have obtained a similar degree of protection following injection of LV-GDNF in the striatum only, i.e. 80% survival of nigral TH-positive neurons compared to 20% in the controls at 4 weeks after the intrastriatal 6-OHDA lesion (6 weeks after vector injection). In these animals the level of GDNF expression in the striatum (by ELISA) was 2–6 ng/mg tissue, i.e. 5–10-fold higher than that seen after vector injection in the substantia nigra (see Table 1). Immunohistochemical staining showed widespread distribution of GDNF throughout the striatum, and transport in the striatal projection neurons down to the globus pallidus, entopeduncular nucleus and substantia nigra (Fig. 6). Indeed, the GDNF level measured in the nigra in the striatum-injected animals was quite high, 0.4 ng/mg tissue, which is similar to that obtained by Deglon et al. [19] in the nigra after intranigral injection of the LV-GDNF vector.

The most compelling data on the efficacy of the LV-GDNF vector have been obtained by Kordower et al. [37] in their recent study in aged and MPTP-treated monkeys. In their experiment, LV-GDNF was injected into both caudate nucleus (10±5 μl), putamen (10±10±5 μl), and substantia nigra (5 μl) 1 week after an intracarotid MPTP injection. As in our AAV-GDNF experiment in the rat (Kirik et al. [34], see above) the acute behavioral impairments were as severe in the LV-GDNF-treated animals as in the controls. However, over the subsequent 3 months, the motor deficits seen in the Parkinson rating scale and an operant hand-reaching task were reversed in the LV-GDNF-treated animals, in three of the four animals near normal levels. The loss of TH-positive neurons in the nigra, which was –89% in the LV-βGal-treated lesion controls, was completely prevented, and the TH-positive innervation in the striatum was significantly preserved (70–80% of normal, compared to about 25% in the LV-βGal controls). Consistent with this, striatal 18F-fluorodopa uptake (as assessed by PET prior to sacrifice) was increased by 300% in the LV-GDNF-treated striatum. Interestingly, sparing of striatal TH-positive fibers, and increase in 18F-fluorodopa uptake, was best in those monkeys showing the most pronounced recovery in the behavioral tests, suggesting that a functional nigrostriatal projection had been preserved in the LV-GDNF-treated animals.

In a second experiment, the LV-GDNF vector was injected in striatum and nigra in aged (25 years old) monkeys [37]. Three months later the LV-GDNF-treated animals displayed an increase in the number of TH-positive neurons in the ipsilateral substantia nigra, as well as an increase in the size and the expression of TH-mRNA in individual nigral TH-positive neurons. Striatal TH-immunoreactivity was increased by about 40%, striatal tissue levels of dopamine and HVA by 47–207%, and striatal 18F-fluorodopa uptake by 27%. Since aged monkeys show a decline in these parameters with increasing age [23], the likely interpretation of these data is that over-expression of GDNF is able to reverse the age-dependent functional decline by up-regulation of dopamine synthesis and turnover in impaired, but still surviving, nigrostriatal neurons. Sustained, high-level expression of GDNF in both striatum and substantia nigra was demonstrated over 3–8 months by both immunohistochemistry and ELISA. At 8 months after vector injection the GDNF level in tissue punches from the caudate and putamen was 2.25±3.5 ng/mg protein (approx. equivalent to 0.23–0.35 ng/mg tissue), which is close to the expression level obtained with the same vector by Bensadoun et al. [4] in the mouse substantia nigra (see
Fig. 6. Immunohistochemical visualization of the GDNF protein in an animal receiving injections of the LV-GDNF vector (3×1 μl) in the striatum, 10 weeks survival. Similar to the results obtained with the AAV-GDNF vector (Fig. 3), the transduced GDNF is widely distributed throughout the striatum (STR; A–C) and along the axons of the striatal projection neurons to the globus pallidus (GP, D–F), entopeduncular nucleus (EP; G–I) and substantia nigra (SN; J–L). The level of GDNF, as measured by ELISA, was 4–6 ng/mg tissue in the striatum and 1–2 ng/mg in the substantia nigra (see Table 1), showing that the factor is efficiently distributed throughout the nigrostriatal system (Georgievska et al., unpublished data).
Table 1). Consistent with the rodent data, efficient anterograde transport of the transduced GDNF was observed from the striatum to both globus pallidus and substantia nigra in the LV-GDNF-injected monkeys.

5. How should GDNF be applied for optimal therapeutic effect?

The results obtained in the rat PD model show that the ability of GDNF to preserve or restore nigrostriatal function depends on the site of administration of the trophic factor (or the vector). Thus, in the intrastriatal 6-OHDA lesion model where the initial insult is at the level of the axon terminals in the striatum, administration of GDNF into the striatum — but not into substantia nigra — is efficient in preserving a functional striatal dopamine innervation. And, only intrastriatal GDNF administration is efficient in promoting regeneration or sprouting into the striatal target (Fig. 7). However, not only the site of injection but also the timing, i.e. the degenerative state of the lesioned nigrostriatal dopamine neurons, is important: regardless of how GDNF is administered — as recombinant protein or via viral vectors — a substantial level of functional sparing or recovery is obtained only when both the cell bodies and the axons along the nigrostriatal pathway remain intact up into, or very close to, the striatum. If administered early in the degenerative process, GDNF can prevent further die-back of the lesioned axons and induce regrowth and sprouting into the area of high GDNF expression. Clearly, GDNF has to be delivered at the level of the axonal stumps in order to elicit the

A. Degeneration induced by intrastriatal 6-OHDA

![Image of degeneration process]

B. Rescue and sprouting induced by GDNF in striatum

![Image of rescue and sprouting process]

Fig. 7. The ability of GDNF to preserve a functional nigrostriatal projection in the rat PD model depends both on the site of administration and the timing relative to the toxin injection, as revealed in experiments using injections or infusion of the recombinant protein. GDNF is most effective when given prior to or shortly after the toxic insult. (Based on data from Refs. [33,60] and Kirik et al., unpublished data.)
sprouting in the striatum; administration of GDNF at the level of the cell bodies does not induce any sprouting in the striatum (Fig. 7C). When the degenerative process has progressed further, intrastriatal GDNF is no longer capable of rescuing either axons or cell bodies of lesioned nigral neurons, as indicated in Fig. 7B. In this case, sprouting in the striatum is much less pronounced. This suggests that GDNF is able to induce sprouting primarily from lesioned axons or axon terminals. Intact axons, either the ones spared by a 6-OHDA lesion or those in non-lesioned animals, appear to respond primarily by a functional up-regulation of the transmitter machinery, i.e. by increased dopamine synthesis or turnover [39].

In the rat PD model, where the primary insult is in the striatum, intrastriatal delivery is clearly the preferred route of administration of GDNF. In this acute lesion model administration of GDNF in the nigra induces extensive sprouting locally in the midbrain. Such aberrant sprouting appears to impair rather than promote functional recovery. Whether these observations are relevant for PD may depend on the nature of the degenerative process in the human disease: if nigrostriatal degeneration in PD starts in the terminals and progresses towards the cell bodies, then intrastriatal application is the obvious choice. If degeneration starts at the cell body level, on the other hand, the trophic factor may have to be administered in the nigra to have any protective effect. Interestingly, in their LV-GDNF study, Kordower et al. [37] obtained substantial protection and functional recovery in MPTP-treated monkeys after injection of LV-GDNF in both striatum (caudate nucleus and putamen) and substantia nigra. This is at variance with our results in the rat model where combined AAV-GDNF injection in nigra and striatum was less effective than injection in striatum alone [34]. This suggests that the effect of nigral transduction is different in the monkey model where degeneration of the nigrostriatal pathway is likely to be more protracted in time. In rats given intrastriatal 6-OHDA, axonal degeneration in the striatum occurs within the first day, and the die-back of axons along the nigrostriatal pathway is complete within a week. In MPTP-treated monkeys axonal degeneration is delayed and proceeds over several weeks [22]. The slow axonal degeneration in the monkey model may thus permit the transduced GDNF to preserve the axons while they still remain within or close to the striatum. The 6-OHDA lesioned rat, by contrast, probably represents a more acute lesion in which the time-window for axonal rescue is shorter. With this type of lesion, administration of GDNF in the nigra will act on neurons whose axons terminate very close to the cell bodies. In this case the axons are clearly unable to regenerate back to the striatum.

The clinical implication of these experimental data is that GDNF should be applied in the early stages of the disease process, while a significant portion of the nigrostriatal pathway still remains. In more advanced stages of the disease, cell bodies may still survive in the substantia nigra but the dopaminergic innervation in the striatum is largely gone, particularly in the putamen where less than 10% of the innervation may remain in severe cases.

### 6. Clinical perspective

The techniques for direct intracerebral gene delivery using recombinant viral vectors are still highly experimental. However, the efficiency and safety of the vector systems have during the last few years been improved to such an extent that they are now seriously considered for clinical application in conditions of CNS disorders. PD is likely to be one of the neurodegenerative diseases in which this technique will be first tested. The reason for this is that the underlying neuropathology is well known, and that there is a well-defined and anatomically restricted target for intervention, i.e. the slowly degenerating nigrostriatal dopamine system. With all vector systems discussed here, localized deposits of microliter amounts of vector are sufficient for efficient transduction of the substantia nigra or striatum not only in rodents, as demonstrated for both Ad, AAV and LV vectors, but also in non-human primates as shown for the LV vector [36,37]. Although the experimental data on GDNF gene transfer in the animal models demonstrate quite convincingly the ability of transduced GDNF to protect the nigrostriatal dopamine neurons against the 6-OHDA- or MPTP-induced toxic insult, it is unclear whether results obtained in these models can help us to predict the effects in patients with PD. The animal models used to date display Parkinson-like damage to the nigrostriatal dopamine system, as well as relevant long-lasting motor impairments, but they do not model the specific disease process characteristic for the human disease. It is unclear, therefore, whether the cellular mechanisms involved in neurotoxic damage, induced by, for example, 6-OHDA in the rat or MPTP in the monkey, are representative of the neurodegenerative process in the diseased human brain. Eventually, only carefully monitored trials in patients will help to resolve this issue.

The first gene transfer procedure tested clinically in the CNS is the encapsulation technique. In the trials initiated so far, cells engineered to secrete a neurotrophic factor, CNTF, have been enclosed within a semi-permeable membrane and implanted either in the intrathecal space (in ALS patients [1,2]) or in the lateral cerebral ventricle (in patients with Huntington’s disease; M. Peschanski, personal communication). In this procedure, the engineered cells remain confined within the capsule, and can thus be removed if necessary, which adds an extra level of safety. For direct gene delivery to the CNS the safety issues are more complex. Thus, for direct injection of viral vectors the potential risk of contaminating helper virus, and unforeseen possible recombination with wild-type virus, need to be carefully assessed. The possibility of insertional mutagenesis, or activation of cellular proto-oncogenes, in
the cases where the vector is integrated into the genome, is also a concern that needs to be taken seriously.

There are also important issues related to the level and site of the expression of the transgene, in this case GDNF, as well as the possible negative or adverse side effects that might be induced by sustained, high-level expression of this biologically active molecule in the human brain. Since we do not yet have any relevant human data to guide us on these important safety issues, it appears essential to develop vector constructs that are regulatable, most importantly so that the GDNF production can be substantially reduced, or completely switched-off, if necessary. Regulatable promoters, such as the tetracycline-based systems, are currently being explored in both the Ad, AAV and LV vector systems [16,26,29,57].

Of the three vector systems discussed here, the AAV and LV vectors seem to be the most promising ones for long-term high-level transgene expression in the CNS. From available data, it seems that these vector systems are essentially non-toxic, even when injected in high titers, and that they induce, at most, limited inflammation or cellular immune responses in the brain. The AAV-GDNF and LV-GDNF vectors are thus interesting candidates for intracerebral gene delivery in clinical trials in PD patients. However, before going to the clinic, it will be essential to demonstrate not only safety but also efficacy, both anatomically and functionally, in long-term experiments in the monkey MPTP model; preferably with vectors that allow external regulation of GDNF transgene expression. The recent study of Kordower et al. [37], demonstrating efficacy of the LV-GDNF vector in MPTP-treated monkeys, is an important first step in this direction.

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