European Journal of Neuroscience, Vol. 13, pp. 1085–1092, 2001

Immunophilin ligands can prevent progressive dopaminergic degeneration in animal models of Parkinson's disease

Lauren C. Costantini,[1] Douglas Cole,[2] Pravin Chaturvedi[2] and Ole Isacson[1]
[1]Neuroregeneration Laboratory, 115 Mill Street, Harvard Medical School/McLean Hospital, Belmont, MA 02178, USA and Department of Neurology and Psychiatry, Massachusetts General Hospital/McLean Hospital, Belmont, MA, USA
[2]Vertex Pharmaceuticals, Inc.; Cambridge, MA, USA

Keywords: animal models, FK-binding proteins, mouse, neuroprotection, neurotrophic factors, rat, substantia nigra

Abstract

Slowing or halting the progressive dopaminergic (DA) degeneration in Parkinson's disease (PD) would delay the onset and development of motor symptoms, prolong the efficacy of pharmacotherapies and decrease drug-induced side-effects. We tested the potential of two orally administered novel immunophilin ligands to protect against DA degeneration in two animal models of PD. First, in an MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model, we compared an immunophilin ligand (V-10,367) documented to bind the immunophilin FKBP12 with V-13,661, which does not bind FKBP12. Both molecules could prevent the loss of striatal DA innervation in a dose-dependent fashion during 10 days of oral administration. Second, to determine whether an immunophilin ligand can protect against progressive and slow DA degeneration typical of PD, an intrastriatal 6-hydroxydopamine-infusion rat model was utilized. Oral treatment with the FKBP12-binding immunophilin ligand began on the day of lesion and continued for 21 days. At this time point, *post mortem* analyses revealed that the treatment had prevented the progressive loss of DA innervation within the striatum and loss of DA neurons within the substantia nigra, related to functional outcome as measured by rotational behaviour. Notably, DA fibres extending into the area of striatal DA denervation were observed only in rats treated with the immunophilin ligand, indicating neuroprotection or sprouting of spared DA fibres. This is the first demonstration that immunophilin ligands can prevent a slow and progressive DA axonal degeneration and neuronal death *in vivo*. The effects of orally administered structurally related immunophilin ligands in acute and progressive models of DA degeneration are consistent with the idea that these compounds may have therapeutic value in PD.

Introduction

Parkinson's disease (PD) neuropathology is characterized by a selective degeneration of the nigrostriatal dopamine (DA) system that occurs over several years (reviewed in Calne & Eisen, 1990; Olanow & Tatton, 1999). The available pharmacological DA-substitution agents can alleviate PD signs during early stages of the disease, yet their efficacy diminishes with continued DA drug exposure, and side-effects eventually outweigh benefits (Oertel & Quinn, 1997; Chase, 1998). Intracranial surgery such as pallidotomy and subthalamic nucleus electrical stimulation can relieve subsets of PD signs (Benabid *et al*., 1996; Lozano & Lang, 1998; Shannon *et al*., 1998), and neuronal cell transplantation can replace a degenerated DA system by neuronal substitution rather than by drugs (Lindvall *et al*., 1989; Deacon *et al*., 1997; Lopez-Lozano *et al*., 1997; Wenning *et al*., 1997; Kordower *et al*., 1998). However, none of these approaches appear to alter the underlying progression and course of nigrostriatal degeneration.

Impressive neuroprotection of the DA system has been observed in animal experiments utilizing growth factors (Hagg, 1998; Dunnett & Bjorklund, 1999), antioxidants (Ebadi *et al*., 1996; Beal, 1999), glutamate antagonists (Rodriguez *et al*., 1998) and antiapoptotic agents (Koller, 1998; Olanow *et al*., 1998). The translation of these positive effects into the clinical setting has been cumbersome (or negative) due to difficulties in routes of administration and dosing parameters (Palfi, 1998; Shoulson, 1998). The discovery that the orally bioavailable immunosuppressive drug FK506 can enhance neurite outgrowth in cell lines and primary cultured neurons (Lyons *et al*., 1994; Steiner *et al*., 1997; Costantini *et al*., 1998), protect against acute glutamate toxicity and focal ischemia (Dawson *et al*., 1993; Sharkey & Butcher, 1994) and stimulate nerve regrowth after sciatic nerve injury (Gold *et al*., 1995) raised the possibility that FK506 could be a utilized as a neuroprotective agent in PD models. FK506 binds to an intracellular protein designated FKBP12 (FK506 binding protein, 12 kDa) (Steiner *et al*., 1992), and this FK506–FKBP12 complex inhibits a phosphatase called calcineurin (Liu *et al*., 1992). The concentration of FK506 required for neurotrophic activity is in the range that also causes inhibition of calcineurin's activity. However, because calcineurin inhibition also suppresses immune function, chronic administration of FK506 may not be desirable for patients with neurodegenerative disease.

Interestingly, there are now compounds available that bind FKBP12 but do not inhibit calcineurin, yet produce trophic effects both in culture and in models of nerve injury and neuronal degeneration (Gold *et al*., 1997; Steiner *et al*., 1997; Costantini

Correspondence: Dr O. Isacson, as above.
E-mail: isacson@helix.mgh.harvard.edu

Received 17 August 2000, revised 11 December 2000, accepted 8 January 2001

et al., 1998). We recently demonstrated that one of these 'neurophilin' ligands, V-10,367, has trophic activity and induces branching of neurites from primary fetal DA neurons in culture (Costantini *et al.*, 1998; Costantini & Isacson, 2000).

Here we demonstrate *in vivo* neuroprotective effects of two orally administered neurophilin ligands, V-10,367 and V-13,661. First in an *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse PD model, we tested V-10,367 at oral doses from 1 to 100 mg/kg/day and V-13,661 at oral doses from 0.15 to 15 mg/kg/day. Although structurally similar, only V-10,367 potently binds FKBP12 (Ki = 0.5 nM), whereas V-13,661 does not bind FKBP12 (Ki > 25 mM). These experiments therefore allowed us to examine the importance of FKBP12 binding in mediating the protection against MPTP-induced damage. We also tested the potential for V-10,367 to protect against a progressive DA denervation using an intrastriatal 6-hydroxydopamine (6-OHDA) rat model, evaluating effects at both the morphological and functional levels. In this model, in contrast to infusion of toxin directly into substantia nigra (SN), the infusion of toxin into the striatum causes a retrograde degeneration of the DA neurons within the SN over several weeks (Bjorklund *et al.*, 1997). The results demonstrate that orally administered neurophilin ligands are protective and/or regenerative.

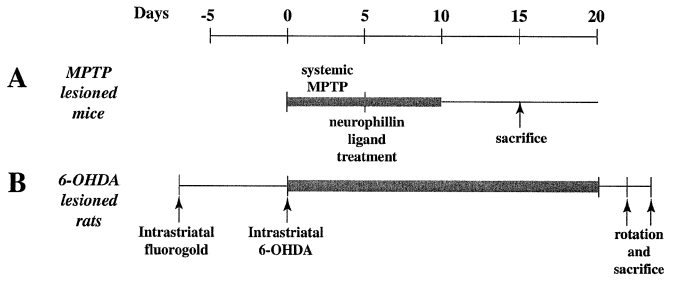


FIG. 1. Time line for the two experimental DA degenerative paradigms used. (A) The selective neurotoxin MPTP was infused i.p. in normal mice for 5 days. In parallel and for another 5 days (total 10 days), immunophilin ligands were orally administered (gavage). At day 15, all mice were killed for histological postmortem analysis. (B) To obtain information about immunophilin effects on long-term DA degeneration, a retrograde toxicity model was used. The striatum of rats was first injected bilaterally with fluorogold to prelabel SN DA neurons. One week later, 6-OHDA was infused unilaterally. Neuroimmunophilin treatment (V10,367) began thereafter and until death (day 21). Rats were also assessed from rotational asymmetry before killing.

Materials and methods

MPTP mouse model of Parkinson's disease

Male C57/BL6 mice (9-week-old; Charles River, Wilmington, MA, USA) were injected intraperitoneally (i.p.) with 20 mg/kg MPTP (Research Biochemicals International, Natick, MA, USA) twice per day for 2 days (at 12-h intervals), then once per day for the following 3 days (total MPTP dose, 140 mg/kg) (Fig. 1A). MPTP was made freshly each day as a 2.5-mg/mL solution in sterile saline. V-10,367, V-13,661 or vehicle (water/propylene glycol/ethanol at a ratio of 5 : 4 : 1, respectively) was administered orally by intubation once per day, 30 min prior to each MPTP injection, and for 5 more days after MPTP treatments (Fig. 1A). Mice were randomly distributed into four treatment groups: (i) V-10,367 at 1.0, 15.0, 50.0 and 100.0 mg/kg/day, $n = 8$ per dose; (ii) V-13,661 at 0.15, 1.5 and 15.0 mg/kg/day, $n = 10$–12 per dose; (iii) MPTP alone (MPTP/veh), $n = 16$; (iv) vehicle alone (veh/veh), $n = 15$.

Mice were anaesthetized with barbiturate and perfused on day 15 and analysed histologically for striatal tyrosine hydroxylase (TH; 1 : 500; Pel-Freez, Rogers, AK, USA) fibre density as previously described (Costantini *et al.*, 1998). Briefly, an equivalent field within the dorsolateral region of the striatum from four sections per mouse (120 μm apart, thus spanning the rostral–caudal 640-μm mouse striatum) was chosen by an investigator blinded to treatment, captured in NIH Image at 20×, and the optical density (OD) of TH^+ fibre staining was obtained. In addition, one field from the anterior thalamic nucleus (–1.3 mm from bregma) from each mouse was captured and an OD measurement obtained, which was subtracted from each striatal OD measurement to correct for assay background. The four final OD values were averaged for each mouse. All statistical comparisons were made using analysis of variance (ANOVA), and Wilcoxon rank-sum test to define significant differences between groups (in JMP Version 3.1, SAS Institute, NC, USA). All experiments were approved by the McLean Hospital Institutional Animal Care and Use committee.

6-OHDA rat model of Parkinson's disease

Male Sprague Dawley rats (Charles River) (≈ 300 g) received bilateral intrastriatal stereotaxic injections of 3% Fluorogold (FG; Molecular Probes, Eugene, OR, USA) in saline under ketamine/xylazine anaesthesia (60 mg/kg and 3 mg/kg, respectively) using a 2-μL Hamilton syringe fitted with a glass micropipette at the following striatal coordinate (0.2 μL/site) (calculated from Bregma): AP +1.0; L ±3.0; V –4.5 mm; incisor bar at –3.4 mm (3.4 mm below the ear bars). One week later (Fig. 1B), unilateral stereotaxic injections of 6-OHDA (Sigma, St. Louis, MO, USA) or vehicle (0.2% ascorbic acid/saline) were made (under ketamine/xylazine anaesthesia) into the right striatum using a 10-μL Hamilton syringe. A concentration of 3.0 μg/uL free base 6-OHDA dissolved in vehicle was injected into four striatal sites (2 μL/site, total dose 24 μg) at the following coordinates (calculated from bregma): site 1, AP +1.4, L –2.6, DV –5.0; site 2, AP +0.4, L –3.0, DV –5.0; site 3, AP –0.4, L –4.2, DV –5.0; site 4, AP –1.3, L –4.5, DV –5.0 mm. The tooth bar was set at 0 for all injections. Rate of injection was 1 μL/min, leaving the needle in place for a further 2 min before withdrawal. Rats were treated via oral intubation with V-10,367 (25 mg/kg) or vehicle (water/propylene glycol/ethanol at a ratio of 5 : 4 : 1, respectively) beginning the day of lesion and continuing once per day for 21 days.

Rats were randomly distributed in three treatment groups: (i) 6-OHDA + V-10,367, $n = 16$; (ii) 6-OHDA + vehicle, $n = 13$ (iii) 6-OHDA + no treatment, $n = 5$. These last two control groups were combined for 6-OHDA + vehicle, $n = 18$, because no differences between 6-OHDA + vehicle and 6-OHDA + no treatment were observed.

On day 21, rats were given an injection of 3.5 mg/kg D-amphetamine i.p. and placed (randomized) into automated rotometer bowls; full-body turns ipsilateral and contralateral to the lesion were monitored over a 90-min period via a computerized activity monitor system. Net rotational asymmetry scores were expressed as ipsilateral minus contralateral rotations. Rats were anaesthetized with barbiturate and perfused two days after behavioural testing (23 days postlesion) for histological analyses.

For DA neuron cell counts in the SN, three sections (200 μm apart) in which the medial region of the SN was clearly separated from the ventral tegmental area by the medial terminal nucleus of the accessory optic tract (level –5.3 mm in the atlas of Paxinos and Watson (1986) were selected for analysis, and only TH^+ cells lateral to the medial terminal nucleus were counted (10×, brightfield). For FG^+ cell counts, three sections adjacent to those utilized for TH^+ cell

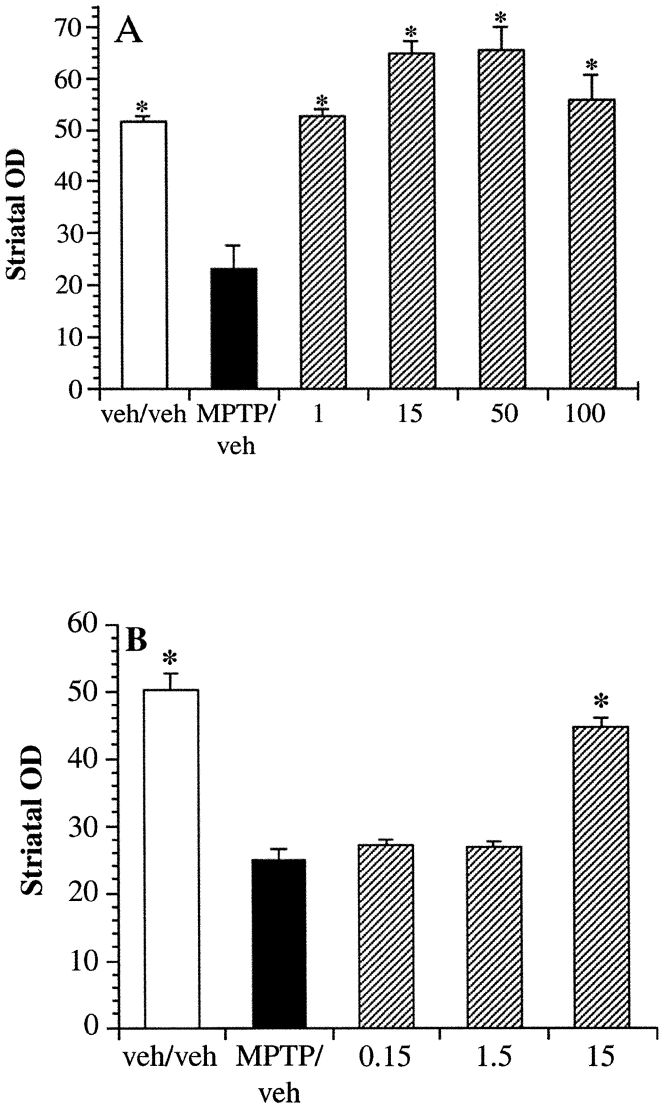


FIG. 2. MPTP mouse lesion model of Parkinson's disease. (A) All doses of V-10,367 (hatched bars) produced significantly higher striatal TH^+ fibre density when compared with lesioned mice treated with vehicle (black bar). *Post hoc* analyses showed that 15.0 and 50.0 mg/kg/day were significantly more effective than 1.0 mg/kg/day. (B) of the three doses of V-13,661 tested (hatched bars), significantly higher striatal TH^+ fibre density was observed with 15 mg/kg/day when compared with lesioned mice treated with vehicle (black bar). $*P < 0.05$ vs. MPTP/veh.

counts were sampled (10×, UV fluorescence). Two distinct populations of FG^+ cells were observed (as previously reported by Sauer & Oertel, 1994); FG^+ cells were counted when they were brightly white/yellow fluorescent and exhibited at least one long dendrite (average cell soma diameter of 31 μm). Smaller cells (average cell soma diameter of 9.1 μm) with fine reticular cell processes reminiscent of phagocytic cells were excluded (Sauer & Oertel, 1994). These cells were labelled secondary to phagocytosis of labelled cellular debris. Cell numbers (TH^+ and FG^+) were obtained from lesioned and unlesioned sides, and data are appropriately expressed as relative '% of unlesioned' calculated from these three sections per rat.

Quantification of striatal area of lesion and TH innervation was obtained using a video capture microscope image and image analysis (NIH Image). The area of lesion was outlined and measured (in mm) in four sections per rat (200 μm apart). OD measurements of TH^+ innervation were taken from the lesioned and unlesioned cross-section of the entire striatum (corresponding to Paxinos and Watson +1.0, +0.5 and –0.3 mm from Bregma, excluding the nucleus accumbens) in three sections per rat. These measurements were averaged per rat and expressed as '% unlesioned' of intact contralateral side. All comparisons of the treatment vs. the vehicle groups were made by Wilcoxon rank-sum test (nonparametric comparison). Overall, volume and area of the anatomical structures or cells analysed did not differ in any of the lesion or treatment groups and thus all sampling and comparisons were made appropriately according to morphometric methods previously published (Frim *et al*., 1994; Sauer *et al*., 1995; Lee *et al*., 1996; Costantini *et al*., 1998; Hagg, 1998; Horger *et al*., 1998).

Results

Neurophilin ligands protect against MPTP-induced degeneration in the mouse

In a pilot study, utilizing the 5-day regimen of MPTP injections in mouse (Fig. 1A; total dose = 140 mg/kg), degeneration of the DA system was predominantly observed at the striatal level, with no significant loss of TH^+ neurons within the SN (Costantini *et al*., 1998). Therefore, this MPTP paradigm in mouse and possibly others is most appropriate for analysis of loss of TH^+ fibre density in the striatum, but not actual cell loss in the SN. Quantification by densitometric analysis showed that mice receiving MPTP exhibited a significant decrease (≈ 50%) in striatal TH^+ fibre density (Fig. 2A, black bar, 'MPTP/veh') when compared to unlesioned mice (Fig. 2A, white bar, 'veh/veh'). When MPTP-lesioned mice were treated orally with V-10,367 at doses ranging from 1.0 to 100 mg/kg/day (Fig. 2A, hatched bars), a significantly higher striatal TH^+ fibre density was observed when compared to MPTP-lesioned mice receiving vehicle (Fig. 2A, black bar, 'MPTP/veh'). TH^+ fibre density in mice treated with 15 mg/kg/day and 50 mg/kg/day doses was significantly greater than in mice treated with the 1 mg/kg/day dose; the dose of 15 mg/kg/day showed higher TH^+ density than vehicle-treated mice.

In a preliminary experiment, V-13,661 prevented MPTP-induced loss of striatal TH fibre density when administered orally at 15 mg/kg per day (data not shown). To determine whether lower doses can also prevent this degeneration, follow-up experiments utilized V-13,661 in this paradigm over a dose range of 0.15–15.0 mg/kg per day (Fig. 2B). TH^+ fibre density was significantly greater in MPTP-lesioned mice receiving 15 mg/kg/day of V-13,661 (Fig. 2B, hatched bar, '15') than in MPTP-lesioned mice receiving vehicle (Fig. 2B, black bar, 'MPTP/veh'). The lower doses of V-13,661 had no effect on TH^+ fibre density.

Neurophilin ligand protects against progressive 6-OHDA-induced degeneration in the rat

Adult rats were injected with FG (bilaterally into striatum) to prelabel DA neurons within the SN, then injected with 6-OHDA (unilaterally into striatum) one week later (Fig. 1B). A progressive degeneration of the DA nigrostriatal system occurs after axon terminal damage induced by this intrastriatal injection of 6-OHDA (Bjorklund *et al*., 1997). Rats receiving unilateral injections of 6-OHDA into the striatum show a marked depletion of striatal TH^+ fibres at 3 weeks postlesion (Fig. 3B) when compared with intact side (Fig. 3A), whilst intrinsic striatal neurons remain relatively intact (data not shown). In addition to this striatal TH loss, there was a loss of TH^+ neurons within the SN (Fig. 3D, 62% decrease at 23 days after lesion) when compared to the intact side (Fig. 3C).

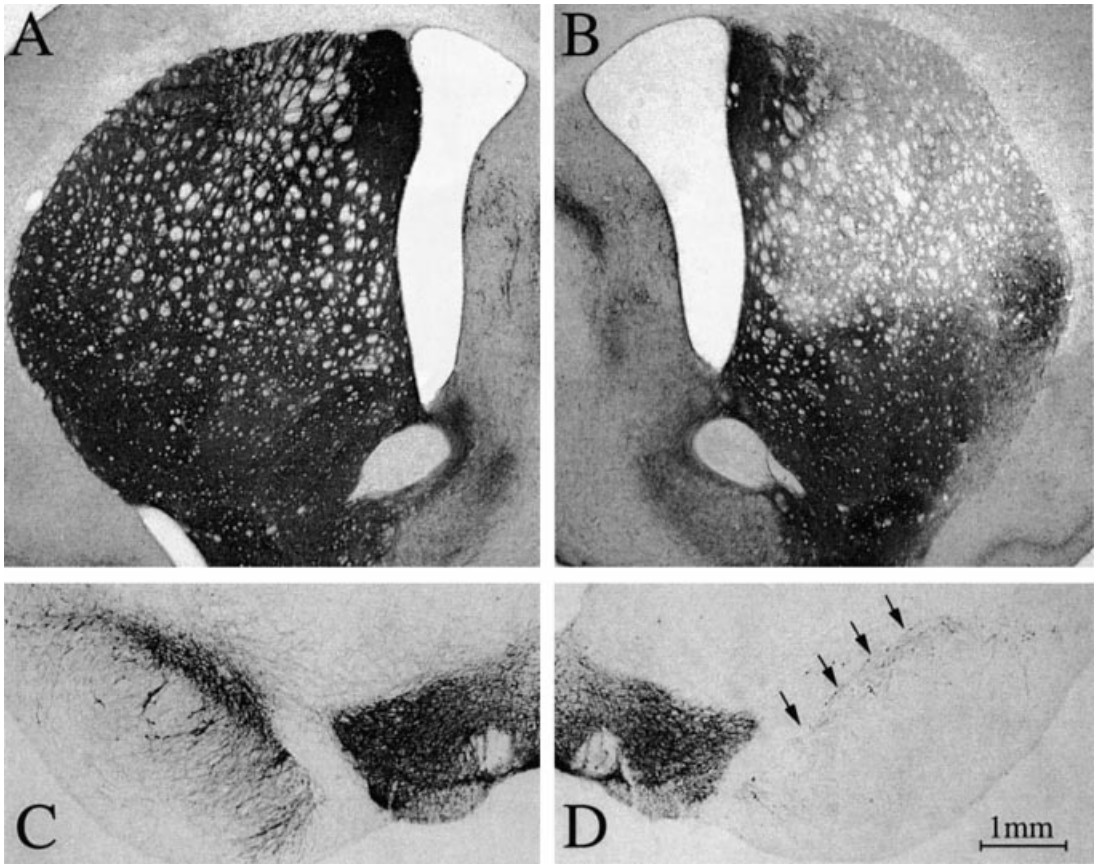


FIG. 3. Morphology of progressive 6-hydroxydopamine lesion rat model of Parkinson's disease. (A) Representative section from one of the three levels through the striatum (intact side) utilized for quantification of TH^+ striatal fibre density. (B) Intrastriatal injection of 6-OHDA induced clear loss of TH^+ fibre density within the dorsolateral region of the striatum (23 days after 6-OHDA). (C) Representative section from one of the three levels through the SN (intact side) utilized for quantification of TH^+ neuron number. (D) Intrastriatal injection of 6-OHDA produced a retrograde degeneration of TH^+ neurons within the SN (arrows; 23 days after lesion). Scale bar, 1 mm (A–D).

Quantification within the striatum showed that a large area of the striatum was depleted of TH^+ fibres in lesioned rats treated with vehicle (Fig. 4A, white bar). By contrast, the depleted area was significantly smaller in similarly lesioned rats treated orally with V-10,367 (Fig. 4A, hatched bar). Furthermore, quantification of total striatal TH^+ fibre density showed that 6-OHDA-lesioned rats treated orally with V-10,367 showed significantly higher (24%) striatal TH^+ fibre density when compared with lesioned rats treated with vehicle (Fig. 4B).

Intrastriatal 6-OHDA markedly decreased the number of DA neurons in the SN ipsilateral to the lesion (Fig. 4C, white bar). Lesioned rats treated orally with V-10,367 showed significantly higher numbers of TH^+ neurons (Fig. 4C, hatched bar) than vehicle-treated rats. Intrastriatal 6-OHDA also induced a loss of a subset of FG^+ cells within the SN. Rats treated with V-10,367 showed more FG^+ cells (27.7% $\pm$ 4.48 SEM of unlesioned FG^+ cells) than rats treated with vehicle (22.52% $\pm$ 2.82 of unlesioned FG^+ cells) although this difference was not statistically significant ($P = 0.493$). FG prior to lesion was injected at one site within the striatum, whereas 6-OHDA was subsequently administered to four sites within the striatum. Therefore, FG-labelled cells probably represented only a subset of the neurons exposed to 6-OHDA.

Interestingly, detailed microscopic analysis of the striatum in 6-OHDA-lesioned rats treated with neurophilin ligand clearly demonstrated the presence of TH^+ fibres extending from the border of the denervated area of striatum towards the centre of this area of total TH^+ loss (Fig. 5A). Such fibre patterns were never observed in striatum of vehicle-treated rats (Fig. 5B).

Rats receiving direct 6-OHDA infusions into the neostriatum (unilateral) have highly variable rotations (ipsilateral to the lesion) when exposed to DA-releasing drugs such as amphetamine (Dunnett *et al*., 1983; Dunnett *et al*., 1987). This limits the use of this model. However, in the present study, we observed amphetamine-induced rotation 21 days after the progressive 6-OHDA lesion (Fig. 4D), and many rats treated orally with V-10,367 displayed a tendency for less motor asymmetry than did vehicle-treated lesioned rats, although these differences did not reach statistical significance as a group ($P = 0.208$).

Discussion

Experimental strategies to slow or halt neuronal cell death and stimulate axonal regeneration in the CNS include several neurotrophic factors, antioxidants and antiapoptotic agents. Protective effects in animal models of PD have been obtained with various molecules including GDNF (reviewed in Bjorklund *et al*., 1997; Rosenblad *et al*., 1999), neurturin (Horger *et al*., 1998; Tseng *et al*., 1998; Akerud *et al*., 1999; Rosenblad *et al*., 1999), BDNF (Altar *et al*., 1994; Frim *et al*., 1994; Galpern *et al*., 1996), TGF-B (Kreiglstein *et al*., 1995; Unsicker *et al*., 1996) and bFGF (Date *et al*.,

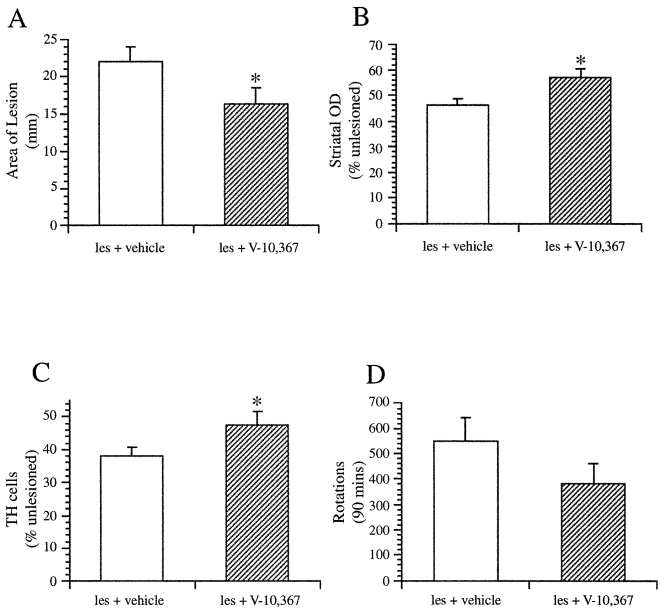
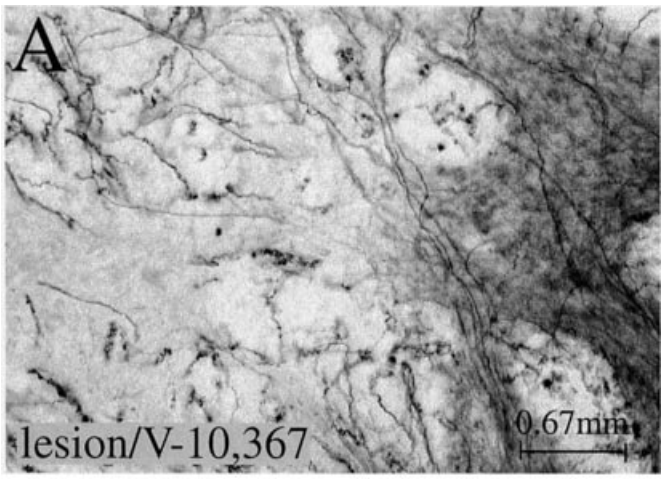


FIG. 4. Quantification of progressive 6-hydroxydopamine lesion model. (A) Rats treated with V-10,367 showed a significantly smaller area of 6-OHDA-induced loss of striatal TH^+ fibres when compared with vehicle-treated rats ($^*P < 0.05$). (B) Quantification of striatal TH^+ fibre density after intrastriatal injection of 6-OHDA revealed significant decreases in fibre density in rats treated with vehicle, whilst lesioned rats treated with V-10,367 maintained significantly higher (57% of unlesioned) TH^+ fibre density ($^*P < 0.05$). (C) Quantification of TH^+ cells within the SN revealed a significantly higher number of TH^+ cells in lesioned rats treated with V-10,367 when compared with lesioned rats treated with vehicle ($^*P < 0.05$). (D) When tested for amphetamine-induced rotation 21 days after progressive 6-OHDA lesion, there was a trend towards a decrease in rotation in V-10,367-treated rats ($P = 0.208$).



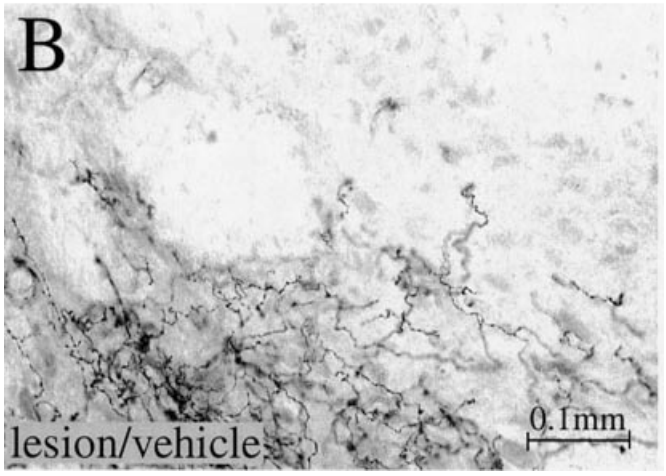


FIG. 5. Morphological analyses of striatal 6-OHDA lesion. Upon microscopic analyses of the striatum in 6-OHDA-lesioned rats, TH^+ fibres were observed within the core of the lesion only in (A) neurophilin ligand-treated rats when compared with (B) the core of vehicle-treated rats 23 days after lesion.

1993). However, clinical application has been limited because of difficulties in delivery and side-effects. These peptides do not readily diffuse across the blood–brain barrier or ventricular lining and have limited or unstable bioavailability and some toxicity (Kordower *et al*., 1999). The present study shows significant protection of the nigrostriatal system with orally administered nonpeptidergic factors in animal models of PD.

Orally administered neurophilin ligands are protective in rodent models of PD

We previously demonstrated (at higher doses than those presented here) that the neurophilin ligand V-10,367 (which binds and inhibits the immunophilin FKBP12) can induce branching of neurites from primary DA neurons in culture (Costantini and Isacson, 2000), and completely protect against MPTP-induced loss of striatal DA innervation after oral administration (Costantini *et al*., 1998). Other studies have observed similar effects in an MPTP mouse model after subcutaneous delivery of an FKBP12-binding compound (Steiner *et al*., 1997). Here, we show that the effects of neurophilin ligands V-10,367 and V-13,661 correlate with oral dose and can confer complete protection against MPTP-induced toxicity. These effects do not appear to require interaction with FKBP12, because V-13,661 does not bind to FKBP12. Although one dose of V-10,367 (15 mg/kg/day) produced supranormal levels of TH^+ staining (Fig. 2A), the difference in TH density is modest and may reflect TH protein levels (induction) rather than axonal density increases (Costantini *et al*., 1998). Whether such TH induction results in increased DA release must be experimentally determined. Most probably, the dual controls of DA release and reuptake will be more important for DA concentration at the synapses than an increased activity of L-dopa-synthesizing enzymes (such as TH). This positive effect is relevant in a clinical sense only by reference to trophic support of as yet undamaged remaining striatal innervation in PD patients, because they present with signs after ≈80% loss of striatal DA.

In a second part of this investigation, we utilized an intrastriatal 6-OHDA lesion model which is more analogous to the progressive DA degeneration seen in PD. In this model, atrophy of striatal DA terminals begins within one week after lesion and is followed by rapid, yet partial, cell death of DA cells within the SN (Bjorklund *et al*., 1997). There is long-term atrophy of SN DA neurons and TH downregulation 1–4 weeks after lesion. The incomplete loss of DA neurons and partial remaining striatal TH^+ fibres resemble the early

stages of PD, leaving part of the nigrostriatal system intact as a potential substrate for sprouting and functional recovery after administration of growth-promoting factors (Lee *et al*., 1996). Notably, within the striatum of neurophilin ligand-treated rats, TH^+ fibre innervation was maintained to almost 60% of unlesioned levels, suggesting that the neurophilin ligand protected DA fibres, induced regeneration or sprouting from spared DA fibres, or prevented the lesion-induced decreases in TH levels (prevention of the down-regulation of TH would still serve to enhance the biochemical functionality of the injured nigrostriatal system).

In addition, we found TH^+ fibres extending into the area of TH^+ loss within the striatum (Fig. 5), suggesting that sprouting of spared fibres in the area of lesion may have occurred. Alternatively, but less likely, would be a protection of a subpopulation of striatal fibres around the core of the lesion. An analogous sprouting effect of TH^+ axons has been observed within the globus pallidus when GDNF was administered (injected into striatum or ventricle) every third day for 4 weeks beginning the day after progressive 6-OHDA lesion, though no sprouting or regrowth of DA axon terminal were seen within the striatum (Rosenblad *et al*., 1999). In the SN, the protection of TH^+ neurons in neurophilin-treated rats after 6-OHDA may reflect a protection of the acute-phase cell death of SN neurons after lesion.

Previously, subcutaneous delivery of a FKBP12-binding compound (GPI-1046) was shown to increase striatal TH^+ fibre density to 36–43% of unlesioned levels when administered after an acute lesion of the rat nigrostriatal system (intranigral 6-OHDA), and to decrease amphetamine-induced rotations; however, analysis of surviving SN DA cell number was not presented (Steiner *et al*., 1997). In a separate study, utilizing the same FKBP12-binding compound in the acute intranigral 6-OHDA model, Harper and colleagues observed no sparing effect of GPI-1046 on striatal TH^+ fibres or SN DA neurons after subcutaneous delivery; no reduction in amphetamine-induced rotations was observed in animals treated with this compound, though the duration of circling behaviour was reduced (Harper *et al*., 1999). Delayed treatment with GPI-1046, starting 14 days after lesion, showed no behavioural or morphological effects. Notably, this study reported that GPI-1046 is rapidly cleaved after systemic administration, generating an apparently biologically inactive free acid. Further studies will be required to resolve these observations.

How do orally administered neurophilin ligands compare with intracranially injected peptidergic factors in rodent models of PD?

Protective and regenerative effects similar in magnitude to the present findings with neurophilin ligands in the progressive 6-OHDA lesion model have been obtained with trophic peptide factors, though their optimal route of administration has yet to be determined. For example, repeated intranigral injections of GDNF beginning 5 or 7 days after lesion (when the terminal degeneration is ongoing but prior to DA cell death) produced protection of TH^+ neurons, though less complete protection of FG^+ cells (Sauer *et al*., 1995; Winkler *et al*., 1996). In contrast to our observations with V-10,367, Winkler *et al*. (1996) did not observe protection of striatal DA innervation or sprouting into the lesioned area, and no effects on amphetamine-induced rotation or forelimb akinesia were detected (Winkler *et al*., 1996). Intranigral injection of GDNF one day prior to lesion spared a proportion of SN cells, yet only half of the spared neurons were TH^+, suggesting that GDNF does not affect TH levels (Kearns & Gash, 1995). Similarly, repeated neurturin (a peptidergic factor related to GDNF) or GDNF injected into SN beginning 3 days after lesion spared FG^+ cells but not TH^+ cells (Horger *et al*., 1998), consistent with GDNF effects on cell survival but not TH expression. This SN population may contain protected neurons (FG^+ but TH^-) that are unable to produce sufficient levels of TH to have functional capacity. In contrast, the present data demonstrate significant protection of TH^+ cells after treatment with V-10,367, consistent with a trend in the same direction for FG^+ neurons. Studies of intranigral infusion of GDNF after medial forebrain-bundle lesion of the nigrostriatal pathway, either prior to lesion (Lu & Hagg, 1997) or 2 weeks after lesion (Bowenkamp *et al*., 1995), demonstrated sparing of TH^+ cells but no effect on striatal innervation or lesion-induced decreases in TH levels.

Improved effects following GDNF administration were obtained in studies utilizing intrastriatal administration after 6-OHDA lesion, perhaps because GDNF injected into striatum may more effectively accumulate in the SN through retrograde transport (Tomac *et al*., 1995). Intrastriatal GDNF injected at the time of lesion protected TH^+ cells, partially spared striatal TH^+ innervation (5–15% of unlesioned) in the area around the injection site, and produced a 50% reduction in amphetamine-induced rotation (Shults *et al*., 1996). Repeated injections of GDNF into striatum or ventricle (ICV) beginning one day after lesion protected TH^+ (and FG^+) neurons, but had no effect on striatal DA innervation or amphetamine-induced rotation (Rosenblad *et al*., 1999). However, intrastriatal injection of GDNF beginning 4 weeks after 6-OHDA restored striatal DA innervation to 70–95% of unlesioned levels, increased numbers of surviving TH^+ cells by 20% and normalized forelimb stepping deficits (Rosenblad *et al*., 1998). Neurturin partially protected TH^+ neurons only after intrastriatal administration, but showed no effect after ICV injection (Rosenblad *et al*., 1999). This illustrates the problematic diffusion kinetics of peptidergic factors after intracranial administration, resulting in limited diffusion and hence incomplete protection of damaged areas. The lack of protection observed with ICV neurturin is presumably due to its poor solubility and diffusion kinetics (Rosenblad *et al*., 1999). Systemic-delivery methods for neuropeptides have been developed and tested with success in animal PD models, such as conjugation of the trophic factor to a transferrin receptor antibody (Backman *et al*., 1996). In contrast, as described here, neurophilin ligands exert growth-promoting effects on both spared and damaged axons as well as TH^+ neurons in the lesioned adult nigrostriatal DA system after oral administration.

Potential relationship between morphological and behavioural effects

In our current experiments, the trend towards normalization of rotational behaviour in the 6-OHDA-lesioned rats receiving V-10,367 suggests that the observed morphological effects at the nigral and striatal levels were functionally relevant. Although protection was not complete, the maintenance of almost 60% of striatal DA innervation and almost 50% of DA neurons could potentially overcome the threshold for Parkinsonian symptoms (Dunnett & Bjorklund, 1999). Depending on the lesion and behavioural paradigm, studies have shown a close correlation between degree of striatal DA levels (or DA uptake sites) and drug induced amphetamine rotation (Przedborski *et al*., 1995; Lee *et al*., 1996). However, in protective/regenerative paradigms (GDNF) in the model utilized in our study, improvement in forelimb use can occur in the absence of significant effects on amphetamine-induced rotation (Winkler *et al*., 1996; Rosenblad *et al*., 1998; Rosenblad *et al*., 1999). What could explain these apparent differences in recovery in the striatal DA toxicity model? The intrastriatal delivery of 6-OHDA could cause additional striatal neuronal cell dysfunction, even after a mild lesion, though no apparent damage was observed in the present study. In addition, the time course of trophic factor treatment may play a role in the effects

observed; analysis in the present study was performed one day after the last oral administration of neurophilin ligand, at 3 weeks after lesion. It is possible that significant functional recovery may develop over a longer time period. Future studies are needed to evaluate these possibilities.

In summary, we have demonstrated protection by neurophilin ligands of the DA system in one acute and one progressive degeneration model of PD. The neurotrophic effects of neurophilin ligands after oral administration were similar to those obtained with intracerebrally delivered neurotrophic factors in such animal models.

Acknowledgements

We are grateful for research funds provided to McLean Hospital/Harvard Medical School through a sponsored research agreement with Vertex Pharmaceuticals, Inc., by the Century Foundation of Sarasota Memorial Hospital, and by an NIH/NINDS award (P50-NS39793) Morris K. Udall Research Center of Excellence for Parkinson's disease. We also thank Dr Stephan Ogenstad for statistical advice and analysis and Ms Anna Moore for assistance with the figures and drawings.

Abbreviations

6-OHDA, 6-hydroxydopamine; DA, dopamine; FG, Fluorogold; FKBP12, FK506 binding protein, 12 kDa; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; OD, optical density; PD, Parkinson's disease; SN, substantia nigra; TH, tyrosine hydroxylase.

References

Akerud, P., Alberch, J., Eketjall, S., Wagner, J. & Arenas, E. (1999) Differential effects of glial cell line-derived neurotrophic factor and neurturin on developing and adult substantia nigra dopaminergic neurons. *J. Neurochem.*, **73**, 70–78.

Altar, C.A., Boylan, C.B., Fritsche, M., Jones, B.E., Jackson, C., Weigand, S.J., Lindsay, R.M. & Hyman, C. (1994) Efficacy of brain-derived neurotrophic factor and neurotrophin-3 on neurochemical and behavioral deficits associated with partial nigrostriatal dopamine lesions. *J. Neurochem.*, **63**, 1021–1032.

Backman, C., Rose, G.M., Hoffer, B.J., Henry, M.A., Bartus, R.T., Friden, P. & Granholm, A.C. (1996) Systemic administration of nerve growth factor conjugate reverses age-related cognitive dysfunction and prevents cholinergic neuron atrophy. *J. Neurosci.*, **16**, 5437–5442.

Beal, M. (1999) Coenzyme Q10 administration and its potential for treatment of neurodegenerative diseases. *Biofactors*, **9**, 261–266.

Benabid, A.L., Pollak, P., Gao, D., Hoffman, D., Limousin, P. & Gay, E. (1996) Chronic electrical stimulation of the ventralis intermedius nucleus of the thalamus as a treatment of movement disorders. *J. Neurosurg.*, **84**, 203–214.

Bjorklund, A., Rosenblad, C., Winkler, C. & Kirik, D. (1997) Studies on neuroprotective and regenerative effects of GDNF in a partial lesion model of Parkinson's disease. *Neurobiol. Dis.*, **4**, 186–200.

Bowenkamp, K., Hoffman, A., Gerhardt, G., Henry, M., Granholm, A., Hoffer, B. & Mahalik, T. (1995) Glial cell line derived neurotrophic factor supports survival of injured midbrain dopaminergic neurons. *J. Comp. Neurol.*, **355**, 479–489.

Calne, D. & Eisen, A. (1990) Parkinson's disease, motoneuron disease and Alzheimer's disease: origins and interrelationship. *Adv. Neurol.*, **53**, 355–360.

Chase, T.N. (1998) Levodopa therapy: consequences of the nonphysiologic replacement of dopamine. *Neurology*, **50**, S17–S25.

Costantini, L., Chaturvedi, P., Armistead, D., McCaffrey, P., Deacon, T. & Isacson, O. (1998) A novel immunophilin ligand: distinct branching effects on dopaminergic neurons in culture and neurotrophic actions after oral administration in an animal model of Parkinson's disease. *Neurobiol. Dis.*, **5**, 97–106.

Costantini, L.C. & Isacson, O. (2000) Immuophilin ligands and GDNF enhance neurite branching or elongation from developing dopamine neurons. *Exp. Neurol.*, **164**, 60–70.

Date, I., Yoshimoto, Y., Imaoko, T., Miyoshi, Y., Gohda, Y., Furuta, T., Asari, S. & Ohmoto, T. (1993) Enhanced recovery of the nigrostriatal dopaminergic system in MPTP-treated mice following intrastriatal injection of basic fibroblast growth factor in relation to aging. *Brain Res.*, **621**, 150–154.

Dawson, T.M., Steiner, J.P., Dawson, V.L., Dinerman, J.L., Uhl, G.R. & Snyder, S.H. (1993) Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate toxicity. *Proc. Natl. Acad. Sci. USA*, **90**, 9808–9812.

Deacon, T., Schumacher, J., Dinsmore, J., Thomas, C., Palmer, P., Kott, S., Edge, A., Penney, D., Kassissieh, S., Dempsey, P. & Isacson, O. (1997) Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. *Nature Med.*, **130**, 350–353.

Dunnett, S. & Bjorklund, A. (1999) Prospects for new restorative and neuroprotective treatments in Parkinson's disease. *Nature*, **399** (Suppl.), A32–A39.

Dunnett, S., Bjorklund, A., Schmidt, R., Stenevi, U. & Iverson, S. (1983) Intracerebral grafting of neuronal cell suspensions. IV. Behavioral recovery in rats with unilateral 6-OHDA lesions in different brain sites. *Acta Physiol. Scand.*, **522**, 29–37.

Dunnett, S., Wishaw, I., Rogers, D. & Jones, G. (1987) Dopamine-rich grafts ameliorate whole body motor assymmetry and sensory neglect but not independent limb use in rats with 6-OHDA lesions. *Brain Res.*, **75**, 63–78.

Ebadi, M., Srinivasan, S. & Baxi, M. (1996) Oxidative stress and antioxidant therapy in Parkinson's disease. *Prog. Neurobiol.*, **48**, 1–19.

Frim, D.M., Uhler, T.A., Galpern, W.R., Beal, M.F., Breakefield, X.O. & Isacson, O. (1994) Implanted fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevent 1-methyl-4-phenylpyridinium toxicity to dopaminergic neurons in rat. *Proc. Natl. Acad. Sci. USA*, **91**, 5104–5108.

Galpern, W.R., Frim, D.M., Tatter, S.B., Altar, C.A., Beal, M.F. & Isacson, O. (1996) Cell-mediated delivery of brain-derived neurotrophic factor enhances dopamine levels in an MPP+ rat model of substantia nigra degeneration. *Cell Transplant.*, **5**, 225–232.

Gold, B.G., Katoh, K. & Storm-Dickerson, T. (1995) The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. *J. Neurosci.*, **15**, 7509–7516.

Gold, B.G., Zeleny-Pooley, M., Wang, M.S., Chaturvedi, P. & Armistead, D.M. (1997) A non-immunosuppressive FKBP-12 ligand increases nerve regeneration. *Exp. Neurol.*, **147**, 269–278.

Hagg, T. (1998) Neurotrophins prevent death and differentially affect tyrosine hydroxylase of adult rat nigrostriatal neurons in vivo. *Exp Neurol.*, **149**, 183–192.

Harper, S., Bilsland, J., Young, L., Bristow, L., Boyce, S., Mason, G., Rigby, M., Hewson, L., Smith, D., O'Donnell, R., O'Connor, D., Hill, R., Evans, D., Swain, C., Williams, B. & Hefti, F. (1999) Analysis of the neurotrophic effects of GPI-1046 on neuron survival and regeneration in culture and in vivo. *Neuroscience*, **88**, 257–267.

Horger, B., Nishimura, M., Armanini, M., Wang, L., Poulsen, K., Rosenblad, C., Kirik, D., Moffat, B., Simmons, L., Johnson, E., Milbrandt, J., Rosenthal, A., Bjorklund, A., Vandlen, R., Hynes, M. & Phillips, H. (1998) Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. *J. Neurosci.*, **18**, 4929–4937.

Kearns, C.M. & Gash, D.M. (1995) GDNF protects nigral dopamine neurons against 6-hydroxydopamine in vivo. *Brain Res.*, **672**, 104–111.

Koller, W. (1998) Neuroprotection for Parkinson's disease. *Ann. Neurol.*, **44** (Suppl.), S155–S159.

Kordower, J., Freeman, T., Chen, E., Mufson, E., Sanberg, P., Hauser, R., Snow, B. & Olanow, C. (1998) Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. *Mov. Disord.*, **13**, 383–393.

Kordower, J., Palfi, S., Chen, E., Ma, S., Sendera, T., Cochran, E., Mufson, E., Penn, R., Goetz, C. & Comella, C. (1999) Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. *Ann. Neurol.*, **46**, 419–424.

Kreiglstein, K., Suter-Crazzolara, C., Hotten, G., Pohl, J. & Unsicker, K. (1995) Trophic and protective effects of growth/differentiation factor 5, a member of the transforming growth factor-beta superfamily, on midbrain dopaminergic neurons. *J. Neurosci. Res.*, **42**, 724–732.

Lee, C., Sauer, H. & Bjorklund, A. (1996) Dopaminergic neuronal degeneration and motor impairments following axon terminal lesion by intrastriatal 6-hydroxydopamine in the rat. *Neuroscience*, **72**, 641–653.

Lindvall, O., Rehncrona, S., Brundin, P., Gustavi, B., Astedt, B. & Widner, H. (1989) Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease: a detailed account of methodology and a 6-month follow-up. *Arch. Neurol.*, **46**, 615–631.

Liu, J.M.W.A., Wandless, T.J., Alberg, D.G., Belshaw, P.J., Cohen, P., Mackintosh, C., Klee, C.B. & Schreiber, S.L. (1992) Inhibition of T cell signalling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. *Biochemistry*, **31**, 3896–3901.

Lopez-Lozano, J., Bravo, G., Brera, B., Millan, I., Salmean, J., Uria, J. & Insausti, J. (1997) Long-term improvement in patients with severe Parkinson's disease after implantation of fetal ventral mesencephalic tissue in a cavity of the caudate nucleus: 5-year follow up in 10 patients. *J. Neurosurg.*, **86**, 931–942.

Lozano, A. & Lang, A. (1998) Pallidotomy for Parkinson's disease. *Neurosurg. Clin. N Am.*, **9**, 325–336.

Lu, X. & Hagg, T. (1997) Glial cell line-derived neurotrophic factor prevents death, but not reductions in tyrosine hydroxylase, of injured nigrostriatal neurons in adult rats. *J. Comp. Neurol.*, **388**, 484–494.

Lyons, W.E., George, E.B., Dawson, T.M., Steiner, J.P. & Snyder, S.H. (1994) Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC12 cells and sensory ganglia. *Proc. Natl. Acad. Sci. USA*, **91**, 3191–3195.

Oertel, W. & Quinn, N. (1997) Parkinsons disease: drug therapy. *Baillieres Clin. Neurol.*, **6**, 89–108.

Olanow, W., Jenner, P. & Brooks, D. (1998) Dopamine agonists and neuroprotection in Parkinson's disease. *Ann. Neurol.*, **44** (Suppl.), S167–S174.

Olanow, C. & Tatton, W. (1999) Etiology and pathogenesis of Parkinson's disease. *Annu. Rev. Neurosci*, **22**, 123–1244.

Palfi, S. (1998) Clinical and pathological evaluation of patient with Parkinson's disease (PD) following intracerebroventricular (icv) GDNF. *Soc. Neurosci. Abstr.*, **24**, 42.

Paxinos, G. & Watson, C. (1986) *The Rat Brain in Stereotaxic Coordinates.* Acadmic Press, San Diego.

Przedborski, S., Levivier, M., Jiang, H., Ferreira, M., Jackson-Lewis, V., Donaldson, D. & Togasaki, D. (1995) Dose-dependent lesions of the dopaminergic nigrostriatal pathway induced by intrastriatal injection of 6-hydroxydopamine. *Neuroscience*, **67**, 631–647.

Rodriguez, M., Obeso, J. & Olanow, W. (1998) Subthalamic nucleus-mediated excitotoxicity in Parkinson's disease: a target for neuroprotection. *Ann. Neurol.*, **44** (Suppl.), S175–S188.

Rosenblad, C., Kirik, D., Devaux, B., Moffat, B., Phillips, H. & Bjorklund, A. (1999) Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or lateral ventricle. *Eur. J. Neurosci.*, **11**, 1554–1566.

Rosenblad, C., Martinez-Serrano, A. & Bjorklund, A. (1998) Intrastriatal glial cell line-derived neurotrophic factor promotes sprouting of spared nigrostriatal dopaminergic afferents and induces recovery of function in a rat model of Parkinson's disease. *Neuroscience*, **82**, 129–137.

Sauer, H. & Oertel, W. (1994) Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in rat. *Neuroscience*, **59**, 401–415.

Sauer, H., Rosenblad, C. & Bjorklund, A. (1995) Glial cell-line derived neurotrophic factor but not transforming growth factor B3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. *Proc. Natl. Acad. Sci. USA*, **92**, 8935–8939.

Shannon, K., Penn, R., Kroin, J., Adler, C., Janko, K., York, M. & Cox, S. (1998) Stereotactic pallidotomy for the treatment of Parkinson's disease. Efficacy and adverse effects at 6 months in 26 patients. *Neurology*, **50**, 434–438.

Sharkey, J. & Butcher, S.P. (1994) Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia. *Nature*, **371**, 336–339.

Shoulson, I. (1998) Mortality in DATATOP: a multicenter trial in early Parkinson's disease. *Ann. Neurol.*, **43**, 318–325.

Shults, C., Kimber, T. & Martin, D. (1996) Intrastriatal injection of GDNF attenuates the effects of 6-hydroxydopamine. *Neuroreport*, **7**, 627–631.

Steiner, J.P., Connelly, M.A., Valentine, H.L., Hamilton, G.S., Dawson, T.M., Hester, L. & Snyder, S.H. (1997) Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin, and cyclosporin A. *Nature Med.*, **3**, 421–428.

Steiner, J.P., Dawson, T.M., Fotuhi, M., Glatt, C.E., Snowman, A.M., Cohen, N. & Snyder, S.H. (1992) High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature*, **358**, 584–586.

Tomac, A., Lindquist, E., Lin, L.F., Ogren, S.O., Young, D., Hoffer, B.J. & Olson, L. (1995) Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature*, **373**, 335–339.

Tseng, J., Bruhn, S., Zurn, A. & Aebische, P. (1998) Neurturin protects dopaminergic neurons following medial forebrain bundle axotomy. *Neuroreport*, **9**, 1817–1822.

Unsicker, K., Suter-Crazzolara, C. & Krieglstein, K. (1996) Growth factor function in the development and maintenance of midbrain dopaminergic neurons: concepts, facts and prospects for TGF-β. *Ciba Found. Symp.*, **196**, 70–80.

Wenning, G., Odin, P., Morrish, P., Rehncrona, S., Widner, H., Brundin, P., Rothwell, J., Brown, R., Gustavii, B., Hagel, P., Jahanshahi, M., Sawle, G., Bjorklund, A., Brooks, D., Marsden, C., Quinn, N. & Lindvall, O. (1997) Short- and long-term survival and function of unilateral intrastriatal dopaminergic grafts in Parkinson's disease. *Ann. Neurol.*, **42**, 95–107.

Winkler, C., Sauer, H., Lee, C. & Bjorklund, A. (1996) Short-term GDNF treatment provides long-term rescue of lesioned nigral DA neurons in a rat model of Parkinson's disease. *J. Neurosci*, **16**, 7206–7215.