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Protection by Synergistic Effects of Adenovirus-Mediated X-Chromosome-Linked Inhibitor of Apoptosis and Glial Cell Line-Derived Neurotrophic Factor Gene Transfer in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Model of Parkinson's Disease

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces clinical, biochemical, and neuropathological changes reminiscent of those occurring in idiopathic Parkinson's disease (PD). Here we show that a peptide caspase inhibitor, *N*-benzyloxycarbonyl-val-ala-asp-fluoromethyl ketone, or adenoviral gene transfer (AdV) of a protein caspase inhibitor, X-chromosome-linked inhibitor of apoptosis (XIAP), prevent cell death of dopaminergic substantia nigra pars compacta (SNpc) neurons induced by MPTP or its active metabolite 1-methyl-4-phenylpyridinium *in vitro* and *in vivo*. Because the MPTP-induced decrease in striatal concentrations of dopamine and its metabolites does not differ between AdV-XIAP- and control vector-treated mice, this protection is not associated with a preservation of nigrostriatal terminals. In contrast, the combination of adenoviral gene transfer of XIAP and of the glial cell line-derived neurotrophic factor to the striatum provides synergistic effects, rescuing dopaminergic SNpc neurons from cell death and maintaining their nigrostriatal terminals. These data suggest that a combination of a caspase inhibitor, which blocks death, and a neurotrophic factor, which promotes the specific function of the rescued neurons, may be a promising strategy for the treatment of PD.

Key words: Parkinson's disease; apoptosis; caspases; gene therapy; X-chromosome-linked inhibitor of apoptosis; glial cell line-derived neurotrophic factor

Pathologically, the hallmark of idiopathic Parkinson's disease (PD) is loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), leading to the major clinical and pharmacological abnormalities that characterize the disease. The cause of neuronal loss in the substantia nigra is not known. However, recent advances have been made in defining morphological and biochemical events in the pathogenesis of the disease. Inhibition of oxidative phosphorylation, excitotoxicity, and generation of reactive oxygen species are considered important mediators of neuronal death in PD (Beal, 1995). Evidence implicating apoptosis in PD has remained controversial. Some studies found evidence for apoptosis based on morphological criteria or *in situ* end labeling (Mochizuki et al., 1996; Anglade et al., 1997) of DNA strand breaks, whereas others did not (Wüllner et al., 1999).

Insights into the pathogenesis of PD have been achieved experimentally by using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP produces irreversible clinical, biochemical, and neuropathological effects that mimic those observed in idiopathic PD (Bloem et al., 1990). This meperidine analog is metabolized to 1-methyl-4-phenylpyridinium (MPP^+) by the enzyme monoamine oxidase B. MPP^+ is subsequently selectively taken up by dopaminergic terminals and concentrated in neuronal mitochondria in the substantia nigra. MPP^+ binds to and inhibits complex I of the electron transport chain (Tipton and Singer, 1993), thereby producing the same biochemical defects as those detected in SNpc of PD patients (Schulz and Beal, 1994).

Chronic administration of MPTP (daily over 5 d) induces apoptotic cell death in the SNpc of mice (Tatton and Kish, 1997), whereas no evidence of apoptosis was found in a more acute dosing regimen (Jackson-Lewis et al., 1995). The vulnerability for apoptosis is regulated by a number of proapoptotic and antiapoptotic factors. MPTP/MPP^+ toxicity involves the activation of caspases, key mediators in the apoptotic pathway, *in vitro* (Dodel et al., 1998) and *in vivo* (Yang et al., 1998). Caspases do not only play an essential role in initial signaling events but are also crucial components of the apoptotic machinery. If apoptosis is a major contributor to cell death in neurodegenerative diseases, inhibition of caspases may serve as a therapeutic opportunity (Schulz et al., 1999). Transgenic mice expressing a dominant negative inhibitor of caspase-1 are resistant to MPTP toxicity (Klevenyi et al., 1999). Furthermore, overexpression of Bcl-2 prevents activation of caspases and provides protection against MPTP toxicity (Yang et al., 1998). In addition, it was shown recently that the percentage of active caspase-3-positive neurons in SNpc was significantly higher in PD patients than in controls (Hartmann et al., 2000).

The inhibitor of apoptosis (IAP) family of proteins plays an evolutionary conserved role in regulating apoptosis in diverse species ranging from insects to humans (Deveraux and Reed, 1999). Human IAP (HIAP) family members [X-chromosome-linked inhibitor of apoptosis (XIAP), HIAP1, and HIAP2] are potent caspase inhibitors (Deveraux et al., 1997; Roy et al., 1997).

In this report, we investigate whether peptide and protein inhibitors of caspases provide protection from MPTP/MPP^+ toxicity. We demonstrate that, although peptide inhibitors of caspases block MPP^+-induced death of dopaminergic neurons, they do not rescue their terminals *in vitro*. Similarly, adenovirus-mediated transgene expression of XIAP blocks death of dopaminergic SNpc neurons in a chronic MPTP paradigm *in vivo* but does not prevent the decrease of dopaminergic terminal markers in the striatum. Contrary to the effects of XIAP, adenovirus-mediated expression of glial cell

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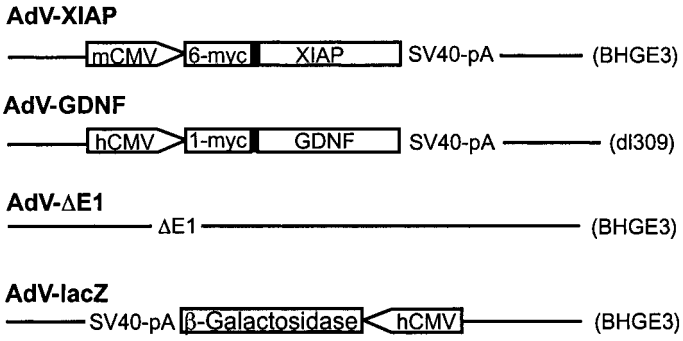

Figure 1. Schematical representation of adenoviral vectors used. The transgenic E1 regions are shown in detail. *MCMV*, Murine CMV; *hCMV*, human CMV; *SV40*, polyadenylation site; *myc*, c-Myc epitope; *BHGE3*, *dl309*, adenoviral backbones.

line-derived neurotrophic factor (GDNF) rescues the terminals of surviving dopaminergic neurons but does not block death.

MATERIALS AND METHODS

Adenoviral vector construction and virus purification. Replication-defective adenovirus vectors coding for rat GDNF and XIAP (Fig. 1) were constructed according to standard protocols (Gravel et al., 1997). They are derived from a mutant adenovirus type 5. AdV-dl390 or AdV-BHGE3 and are replication-deficient because of a deletion of the E1 (ΔE1) region. The transgenes are expressed under the control of a strong constitutive cytomegalovirus (CMV) promoter element. Control vectors express *Escherichia coli* β-galactosidase (AdV-LacZ) or do not carry a transgene (AdV-ΔE1). Determination of infectious titer was performed by plaque assay on HEK 293 cells. Titers obtained after concentration in two rounds of ultracentrifugation were 10^{11} pfu/ml. The particle ratio (plaque forming units per total adenovirus virions) were 1:52 for AdV-GDNF and 1:74 for AdV-XIAP. These ratios are considered reasonable (Mittereder et al., 1996).

Fetal mesencephalic cell cultures. Primary neuronal cultures were prepared from the ventral mesencephalon, which was dissected from embryonic day 14 rat embryos (Charles River Wiga, Sulzfeld, Germany) as described previously (Krieglstein et al., 1995). Tissue pieces were dissociated enzymatically in 0.25% trypsin (Life Technologies GmbH, Karlsruhe, Germany) and mechanically by trituration using fire-polished glass pipettes and then washed with DMEM/F12 in a 1:1 mixture (BioWhittaker, Walkersville, MD). Complete medium (DMEM/F12, containing 0.25% BSA, N1 supplements, penicillin–streptomycin, and 33 mM glucose) was used for single cell suspension. Cells were seeded at a density of $1.5 \times 10^5/cm^2$ on 10 mm glass coverslips coated with polyornithine and laminin.

At 4 days *in vitro* (DIV4) two-thirds (500 μl) of culture medium was replaced and *N*-benzyloxycarbonyl-val-ala-asp-fluoromethyl ketone (zVAD-fmk) (Bachem, Heidelberg, Germany), dissolved in 1% DMSO, was added to the culture medium at final concentrations ranging from 0 (vehicle alone) to 200 μM. MPP^+ dissolved in PBS was added at DIV4 after 2 hr of preincubation with zVAD-fmk, if applicable. At DIV5, culture medium was replaced by MPP^+-free medium. Respective concentrations of zVAD-fmk were maintained. Cultures were processed for immunocytochemistry at DIV6.

Cells were fixed with 4% paraformaldehyde (10 min, 20°C), permeabilized with acetone (10 min, −20°C), and blocked with H_2O_2 (10 min, 20°C) and 10% horse serum (10 min, 37°C). All steps were separated by washing three times with PBS. Incubation with mouse monoclonal antibody to rat tyrosine hydroxylase (TH) (1:200 with 5% horse serum, 1 hr at 37°C; Boehringer Mannheim, Mannheim, Germany) and biotinylated horse anti-mouse IgG antibody (1:200 with 5% horse serum, 15 min, 37°C; Vector Laboratories, Burlingame, CA) was followed by the staining procedure using the Vectastain ABC kit (15 min, 37°C; Vector Laboratories) in combination with diaminobenzidine reagents (5 min, 20°C). At 100× magnification, TH-immunoreactive neurons were counted across one diameter of the coverslip (an area comprising ~10% of the culture).

For further morphometric analysis, the total length of the cellular processes of 300 cells (randomly chosen on three different coverslips) was measured using an image analysis system (MCID-IV; Imaging Research, St. Catharines, Ontario, Canada).

For high-affinity uptake of [^{3}H]-labeled dopamine (Krieglstein and Unsicker, 1997), cells were seeded in 24-well plates. At DIV5, cells were washed three times with the incubation solution (5 mM glucose and 1 mM ascorbic acid in PBS, pH 7.4) and incubated (15 min, 37°C) in this solution, before adding 50 nM [^{3}H]dopamine (15 min, 37°C; Amersham Pharmacia Biotech, Braunschweig, Germany). Uptake was stopped by removal of the incubation mixture, followed by three rapid washes with ice-cold PBS. After removal of PBS, 300 μl of distilled water was added, cultures were frozen (2 hr, −80°C) and thawed, and cells were scraped twice with an additional volume of 200 μl of distilled water. Radioactivity-containing water was collected in vials, and extracted radioactivity was measured by liquid scintillation spectrometry after addition of 10 ml of scintillation cocktail per vial.

Striatal MPP^+ lesions. Male Sprague Dawley rats (Charles River Wiga) weighing 300–325 gm were anesthetized with methohexital (50 mg/kg, i.p.; Eli Lilly & Co., Bad Homburg, Germany) and placed in a stereotaxic instrument with the incisor bar set at 3.3 mm below the interaural line. A 1 mm burr hole was made in the skull over the left striatum, and a 1 mm guide cannula was affixed with dental acrylic vertically with the tip on top of the brain surface. MPP^+ (Research Biochemicals, Cologne, Germany) was dissolved in 0.1 M PBS, and zVAD-fmk (Bachem Biochemica GmbH, Heidelberg, Germany) was dissolved in 0.1 M PBS, containing 1% dimethyl sulfoxide (DMSO). After recovery for 48 hr, injections were made through the guide cannula into the left striatum (coordinates: bregma, 2.6 mm laterally, 4.5 mm below dura) in a volume of 1 μl (60 nmol of MPP^+, 1% DMSO or 1 μg of zVAD-fmk dissolved in 1% DMSO) using a blunt-tipped 26 gauge Hamilton syringe. All injections were made over 1 min. The needle was left in place for 1 min before being slowly withdrawn. The advantage of implanting a guide cannula for striatal injections were twofold: (1) drugs could be injected exactly into the same region at different time points, and (2) no anesthesia that might cause neuroprotective hypothermia was needed at the time of striatal injection.

Animals were decapitated at 7 d, and the brains were rapidly removed, placed in cold 0.9% saline for 10 min, and sectioned coronally into slices at 2 mm intervals. Slices were stained in 2% 2,3,5-triphenyl-tetrazolium chloride monohydrate (TTC) (Sigma, Deisenhofen, Germany) solution at room temperature in the dark for 30 min, followed by fixation in phosphate-buffered 4% paraformaldehyde. The lesioned area (noted by pale staining) was measured on the posterior surface of each section using an image processing system (MCID M4; Imaging Research) by an observer blinded to the experimental conditions. We previously verified the reliability of the TTC measurements in animals injected with malonate on adjacent sections stained with either TTC or Nissl stain (Schulz et al., 1995).

Adenovirus-mediated gene transfer to SNpc neurons and striatal injection of Fluorogold. For adenovirus-mediated gene transfer studies and MPTP experiments, we used 10- to 12-week-old male C57BL/6 mice. Mice were anesthetized with 420 mg/kg chloral hydrate, and 1×10^8 pfu units in a volume of 1 μl were stereotaxically introduced into the striatum (flat skull position, coordinates: bregma, 2.4 mm laterally, 3 mm below dura). Seven days later, animals were processed for detection of transgene expression. In parallel experiments, C57BL/6 mice ($n = 5$) were stereotaxically injected with 0.4 μl of 2% Fluorogold (Fluorochrome Inc., Denver, CO) at the same coordinates.

β-Galactosidase histochemistry. Mice were anesthetized with chloral hydrate (420 mg/kg) and perfused transcardially with 50 ml ice-cold saline (0.9% NaCl, 4°C), followed by 100 ml of fixative (4% paraformaldehyde and 0.2% glutaraldehyde in PBS, 4°C). The mouse brains were removed and post-fixed in the same fixative at 4°C overnight. Cryoprotection was performed by transferring the probes into PBS containing 30% sucrose and 0.4% paraformaldehyde at 4°C for 48 hr. Afterward, brains were shock-frozen on dry ice and stored at −80°C. Cryostat sections (16 μm) were cut from striatum and substantia nigra (coronal plane). For histochemistry, free-floating slices were washed in PBS (three times for 5 min each) and stained for β-galactosidase by using a β-galactosidase staining set (Boehringer Mannheim).

Immunohistochemistry for detection of active caspase-3 and DNA staining. Mice were killed each day (days 1–5) of MPTP treatment 6 hr after injection. Paraffin sections (10 μm) of SNpc (coronal plane) were mounted on coverslips, dewaxed, washed in PBS (three times for 5 min each), and blocked with normal goat serum (10% in PBS with 0.3% Triton X-100) for 10 min.

For labeling of active caspase-3, we used the CM1 rabbit polyclonal antibody, which recognizes the p20 processed band (Srinivasan et al., 1998) (diluted 1:1000 in PBS containing 0.3% Triton X-100 and 1% serum, overnight). After washing with PBS (three times for 5 min each), the sections were incubated at room temperature for 2 hr with secondary antibodies: carbocyanine 3 (CY3)-labeled goat anti-rabbit-IgG (1:200; Biotrend, Cologne, Germany). This was followed by a 5 min incubation step with Hoechst 33258 (Molecular Probes, Eugene, OR) for DNA staining. Sections were washed, mounted on coverslips, and then analyzed by confocal laser scanning microscopy (LSM 510; Carl Zeiss, Jena, Germany). The specificity of immune reactions was confirmed by substituting the primary antisera or monoclonal antibodies with nonimmune IgG.

Western blotting. AdV-GDNF (0.5×10^8 pfu) was injected into the left striatum of mice at the coordinates given above. Tissue from the left and right striatum and substantia nigra was dissected separately 1 week after the injection and frozen immediately. The following steps were essentially done as described previously (Schulz et al., 1996b). The tissue samples were lysed in lysis buffer containing leupeptin, aprotinin, and PMSF and subjected to mechanical homogenization. After centrifugation, Laemmli's buffer was added, and equal amounts of total protein (20 μg) were used for SDS-PAGE, followed by electroblotting to nitrocellulose membranes. A mouse monoclonal c-Myc antibody (9E10, 1:2000, incubation overnight; Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection of exogenous GDNF. Subsequently, blots were incubated for 1 hr with

anti-mouse-IgG-HRP antibody. Bound antibody was visualized using enhanced chemiluminescence. Blots were repeated at least three times for every condition.

Quantified immunodetection of GDNF. GDNF was quantified using the E_{max} ImmunoAssay system (Promega, Madison, WI) according to the protocol of the manufacturer.

MPTP mouse studies. One week after adenovirus delivery to the striatum, mice were treated either with normal saline or MPTP hydrochloride (Research Biochemicals). MPTP was administered in 0.1 ml of PBS at a dose of 30 mg/kg intraperitoneally at 24 hr intervals for five doses (Tatton and Kish, 1997). Ten animals were used in each group. Animals were killed 1 week after the last MPTP injection. The two striata were rapidly dissected, frozen, and stored at −80°C until analysis. On the day of the assay, tissue samples were sonicated in 20 μl of 0.1 M perchloric acid per milligram of striatal tissue. After centrifugation (15,000 × *g* for 10 min at 4°C), 20 μl of supernatant was injected onto a C18 reverse-phase HR-80 catecholamine column (ESA, Bedford, MA). Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were quantified by HPLC with electrochemical detection. The mobile phase (pH 2.9) consisted of 90% 75 mM sodium phosphate, 275 mg/l octane sulfonic acid solution, and 10% methanol. Flow rate was 1 ml/min. Peaks were detected by an ESA model Coulochem II with a 5010 detector (E1, 50 mV; E2, 400 mV). Data were collected and processed using the computer system Chromeleon (Gynkotek, Gemering, Germany).

TH immunohistochemistry was performed on 10 μm paraffin midbrain sections. The sections were mounted on glass slides and processed for immunostaining as described above (adenoviral gene transfer to SNpc neurons). For detection of TH-immunopositive structures, a monoclonal mouse antibody was used (1:1000; Diasorin, Stillwater, MN). The primary antibody was visualized by a secondary CY3-labeled goat anti-rabbit-IgG (Biotrend). TH-positive cells were quantified in two ways. (1) The areas of nigral TH-positive structures, cell bodies, and processes were bilaterally measured by the LSM 510 image processing software on at least five TH-immunostained mesencephalic sections at the widest dimension of the SNpc at anteroposterior −3.16 (Franklin and Paxinos, 1996) lateral to the roots of the third cranial nerve separating medial and lateral SNpc by observers blinded to the treatment schedule. To prevent double counting of TH-positive cells, every third section was analyzed. In a first step, the mean of background staining of the slice was determined. Next, the area of SNpc was coarsely outlined by hand. Inside this selected area, the area of TH-positive structures was calculated after background correction. (2) Counts of TH-positive cells were also performed manually. Nucleated, process-bearing TH-positive SNpc cells were counted unilaterally on at least five TH-immunostained mesencephalic sections from the same location as described above. Results are expressed as TH-positive cell counts per section.

Statistical analysis. Data are expressed as means ± SEM values. Tests of variance homogeneity, normality, and distribution were performed to ensure that the assumptions required for standard parametric ANOVA were satisfied. Statistical analysis was performed by ANOVA, followed by Tukey's *post hoc* test to compare group means. To study whether the simultaneous treatment with AdV-XIAP and AdV-GDNF has not only additive but synergistic effects, we used the fractional product method (Greco et al., 1995). In the fractional product method, the effect of two independently acting agents is defined as the product of the unaffected fractions after treatment with either drug alone: $fu(1,2) = fu(1) \times fu(2)$. This formula allows to calculate the predicted effect of cotreatment, based on the assumption that two agents do not interact or cooperate in inducing their effect. If the unaffected fraction, that is, the remaining reduction of striatal catecholamine concentrations or TH-positive cells is below the calculated product fu(1,2) after cotreatment, and then the two agents show synergy.

Animal guidelines. Studies were done in accordance with the European Convention for Animal Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

RESULTS

Peptide inhibitors of caspases protect against MPP^+-induced death of cultured fetal dopaminergic mesencephalic neurons but not against the loss of their neurites

Treatment of mesencephalic cultures with MPP^+ resulted in a concentration-dependent decrease in the number of TH-positive neurons when cell counts were assessed at 48 hr after treatment (Fig. 2*A*). Confirming previously published results (Dodel et al., 1998), coincubation with 200 μM of the panspecific caspase inhibitor zVAD-fmk significantly reduced the extent of MPP^+-induced cell death of TH-positive neurons. Administration of zVAD-fmk alone had no effect on the survival of dopaminergic neurons (Fig. 2*B*). In contrast, the same concentration of zVAD-fmk had no effect on MPP^+-induced reduction of [^{3}H]dopamine uptake (Fig. 2*C*), suggesting that, although the somata of TH-immunoreactive neurons were rescued from cell death, these cells lost their capability to take up dopamine. This observation is in accordance with the morphological finding of remaining TH-positive neuronal cell bodies with vastly devastated cell processes after treatment with MPP^+ (Fig. 3*A*,*B*), quantified as a marked decrease of the mean length of cell processes (Fig. 3*D*). Cultures simultaneously treated

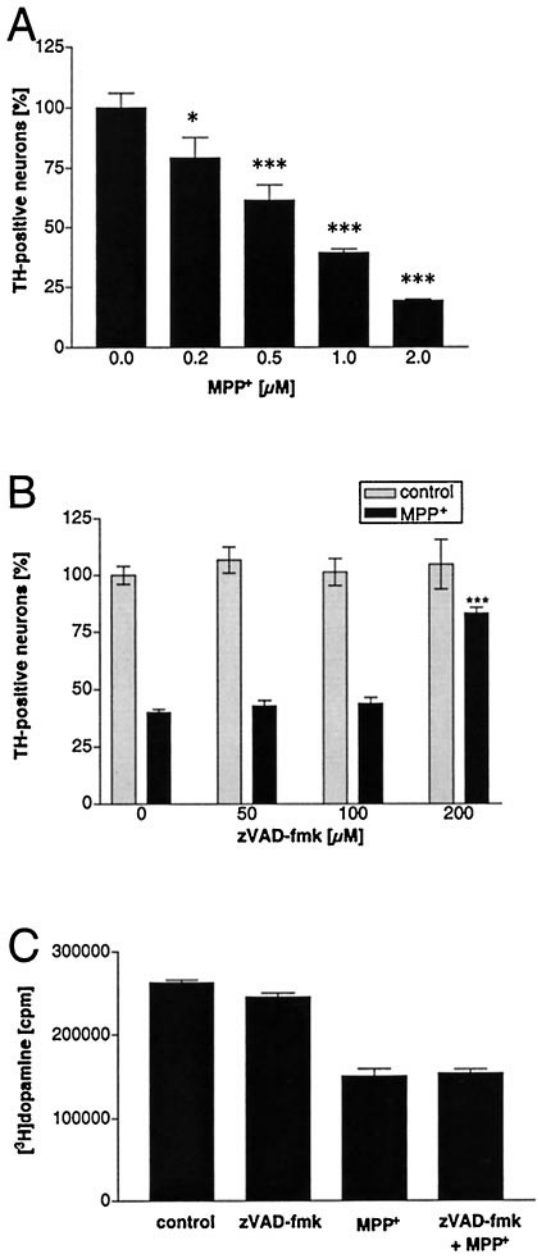

Figure 2. zVAD-fmk inhibits the MPP^+-induced loss of TH-positive somata but does not protect against the reduction of [^{3}H]dopamine uptake in cultured fetal mesencephalic dopaminergic neurons. *A*, MPP^+ concentration-dependently induces cell death of TH-positive neurons. Cultures of mesencephalic neurons were treated with MPP^+ at various concentrations for a period of 24 hr beginning at DIV4. After fixation at DIV6 and subsequent immunocytochemical staining, TH-positive cells were counted. Results are mean ± SEM ($n = 5$), expressed as percentages of control. *B*, zVAD-fmk inhibits MPP^+-induced cell death of TH-positive neurons. On DIV4, zVAD-fmk was added to the culture medium at concentrations ranging from 0 (vehicle alone) to 200 μM. Starting 2 hr later, cultures were treated with 1 μM MPP^+ or vehicle (*control*) for a period of 24 hr. After fixation on DIV6 and immunocytochemical staining, TH-positive cells were counted. Results are mean ± SEM ($n = 5$), expressed as percentages of control. *C*, zVAD-fmk has no effect on MPP^+-induced decrease of [^{3}H]dopamine uptake of cultured mesencephalic dopaminergic neurons. Mesencephalic cultures were exposed to MPP^+ (0.2 μM) after 2 hr of preincubation with zVAD-fmk (200 μM). After 24 hr, uptake of [^{3}H]dopamine was assessed. Results are mean ± SEM ($n = 4$).

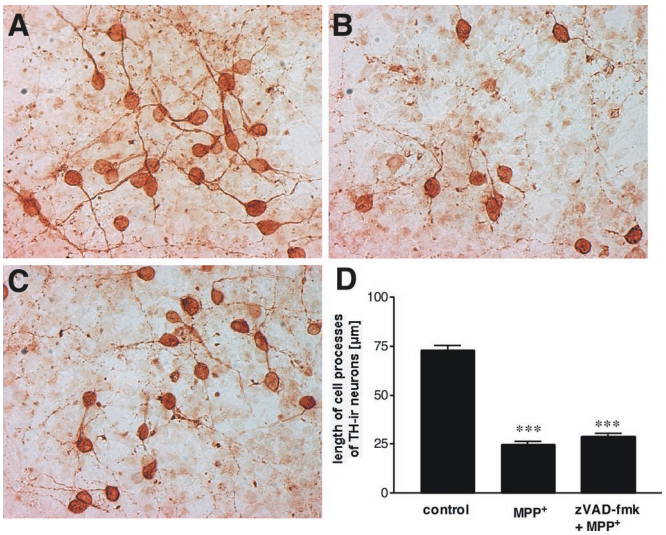

Figure 3. zVAD-fmk does not protect cultured fetal mesencephalic dopaminergic neurons from MPP^+-induced loss of their neurites. Cultures of mesencephalic neurons were treated with vehicle (*A*, *B*) or 200 μM zVAD-fmk (*C*). Starting 2 hr later, cultures were exposed to vehicle (*A*) or 1 μM MPP^+ (*B*, *C*) for a period of 24 hr. At DIV6, TH-positive neurons were visualized by immunocytochemical staining and further analyzed at 250× magnification. The length of the cell processes of TH-positive neurons was determined by morphometric analysis (*D*). Results are mean ± SEM (n = 4). ***$p < 0.001$ compared with controls and no statistical significance after exposure to MPP^+ between treatment with vehicle and zVAD-fmk (ANOVA followed by Tukey's *post hoc* test).

with 200 μM zVAD-fmk showed similarly damaged cell processes despite increased survival of TH-positive neurons (Fig. 3*C*,*D*).

MPTP induces caspase activation and apoptosis in dopaminergic SNpc neurons and leads to cell death

It has been suggested by morphological criteria that chronic MPTP treatment of mice leads to apoptosis of dopaminergic SNpc neurons (Tatton and Kish, 1997), whereas no evidence of apoptosis was found in a more acute dosing regimen (Jackson-Lewis et al., 1995). Using an antibody generated against active caspase-3 (Srinivasan et al., 1998), we therefore asked whether MPTP activates caspase-3 in dopaminergic SNpc neurons in a chronic dosing paradigm. We found the peak of immunopositive cells at 6 hr after the second injection of 30 mg/kg MPTP (Fig. 4). At this time point, the cytosol of the majority of TH-positive neurons was labeled by the antibody recognizing active caspase-3 (Fig. 4*A*,*B*). Several nuclei of the same neurons showed chromatin condensation, a typical morphological feature of apoptosis, visualized by DNA staining with Hoechst 33258 (Fig. 4*C*,*D*).

It has been questioned whether, similar to cholinergic neurons of the medial septum after target ablation (Sofroniew et al., 1993), dopaminergic SNpc neurons may not die after MPTP treatment but remain in a metabolically inactive, atrophic state that can be rescued by certain therapies, e.g., treatment with growth factors. Therefore, we retrogradely labeled dopaminergic SNpc neurons by injecting 0.4 μl of 2% Fluorogold into the striatum at 7 d before treatment with 30 mg/kg MPTP for 5 consecutive days. The animals were killed at day 7 after initiation of MPTP treatment, and Fluorogold-positive cells in the SNpc were quantified (Fig. 4*E*,*F*). Approximately 90% of the TH-positive neurons were labeled with this method. MPTP treatment led to a 45% decrease of Fluorogold-labeled cells in the SNpc (24.4 ± 0.8 vs 43.3 ± 4.6 cells per section; n = 4 and 5 sections per animal; $p < 0.05$).

Peptide inhibitors of caspases attenuate MPP^+-induced striatal lesion volume in rats

In addition to inducing death of dopaminergic SNpc neurons, MPP^+ leads to a substantial striatal lesion when injected stereotaxically into the striatum (Storey et al., 1992; Schulz et al., 1996a). To test whether peptide inhibitors of caspases block MPP^+-induced cell death *in vivo* as well, we examined the effects of zVAD-fmk on MPP^+-induced striatal lesion volumes in rats. Intrastriatal injection of 1 μg of zVAD-fmk (dissolved in PBS, containing 1% DMSO) 1 hr before and 6 hr after intrastriatal injection of 60 nmol of MPP^+, via chronically implanted guide cannulas, significantly attenuated the lesion volume compared with vehicle-treated controls (Fig. 5).

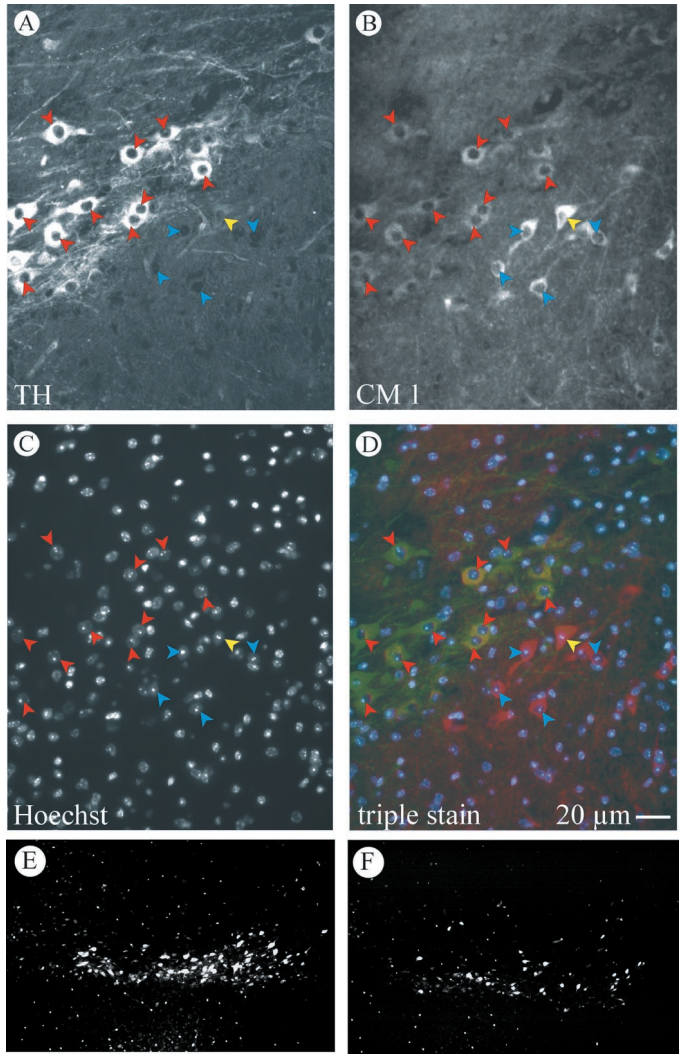

Figure 4. MPTP induces caspase-3 activation in SNpc neurons and leads to cell death. Immunohistochemistry for TH (*A*) and activated caspase-3 (CM1 antibody) (*B*) was performed at 6 hr after the second injection of 30 mg/kg MPTP. SNpc neurons with a strong signal for CM1 show reduced staining for TH (*blue arrowheads*), whereas neurons with a weak CM1 signal still show strong TH-positivity (*red arrowheads*). In *C*, the same section was labeled with Hoechst dye. In *D*, the data presented in *A–C* are presented as a fused micrograph. Thus, *yellow* staining indicates TH, *red* staining indicates activated caspase-3 reactivity, and *light blue* staining indicates chromatin. Neurons intensively stained for CM1 show condensed and fragmented nuclei as detected by Hoechst staining. Note the condensed and fragmented nucleus of the intensively CM1-labeled neuron (*yellow arrowhead*). *E*, *F*, Sections from the substantia nigra of mice injected with Fluorogold into the striatum, followed by subcutaneous treatment with saline (*E*) or $5 \times 30\ mg \cdot kg^{-1} \cdot d^{-1}$ MPTP (*F*).

Adenovirus-mediated gene expression in SNpc dopaminergic neurons after gene delivery to the striatum in mice

We have shown previously that our adenoviral vector system effectively transduces primary neurons and yields significant levels of transgene expression (Simons et al., 1999). To test whether the vector system is suitable for gene delivery to the nigrostriatal

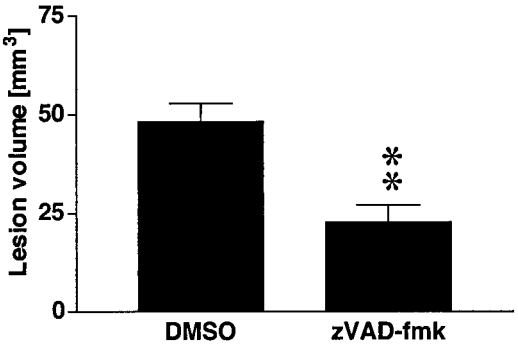

Figure 5. zVAD-fmk attenuates striatal lesions produced by MPP^+ in rats. zVAD-fmk (1 μg) dissolved in 1 μl of DMSO or 1 μl of DMSO alone (vehicle-treated controls) were injected into the striatum 1 hr before and 3 hr after striatal injection of 60 nmol of MPP^+. Lesion volumes were analyzed after 7 d by TTC staining (mean ± SEM; $n = 7$; $^{**}p < 0.01$; two-tailed Student's t test).

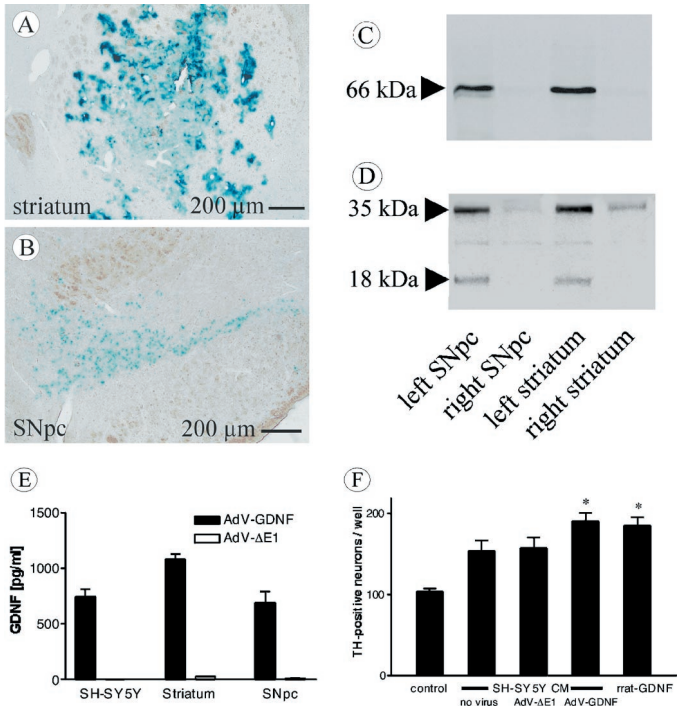

Figure 6. Transgene expression in striatum and substantia nigra at 7 d after adenoviral-mediated gene transfer into the striatum. β-Galactosidase activity staining in frontal section through the striatum close to the injection site of AdV-LacZ (*A*) and coronal section through the substantia nigra (*B*). Retrogradely transduced neurons are visible as *blue dots* in SNpc. In the striatum, β-galactosidase is mainly expressed by astroglia but also by some neuronal cells. No staining was observed in the contralateral hemisphere. At 7 d after stereotaxic AdV-XIAP and AdV-GDNF injection into the left striatum, XIAP (*C*) and GDNF (*D*) expression in the left striatum and SNpc was detected by immunoblot analysis for their c-Myc moiety. The molecular weight of XIAP detected (66 kDa) corresponds to the molecular weight of XIAP (55 kDa) fused with six copies of the Myc tag. GDNF is fused to one copy of a Myc tag only containing 10 amino acids. The bands represent GDNF (~18 kDa) and a nonreduced, disulfide-bonded dimer (~35 kDa). *E*, Immunodetection of GDNF by ELISA in the conditioned medium of 10^6 SH-SY5Y cells at 48 hr and in 10 μg of protein of mouse striatum and SNpc at 7 d after AdV-GDNF or AdV-ΔE1 transfection (some values are too small to be shown). *F*, Biological activity of GDNF secreted from SH-SY5Ycells without (no virus) or after transfection with AdV-ΔE1 or AdV-XIAP on mesencephalic embryonic cultures. $^*p < 0.001$ compared with controls; $p < 0.05$ compared with conditioned medium of AdV-ΔE1-transfected SH-SY5Y cells.

system *in vivo,* we compared the efficacy of stereotaxic gene delivery to the striatum (Fig. 6*A*,*B*) with gene delivery directly above (dorsal to) the substantia nigra (data not shown). We found the transgene expression in SNpc neurons to be higher and better reproducible after gene delivery to the striatum than to or slightly above the substantia nigra. Using β-galactosidase activity staining (Fig. 6*A*,*B*) or immunohistochemistry (data not shown) for the detection of transgene expression, injection of AdV-LacZ into the striatum led to the expression of β-galactosidase in neurons and glial cells but also of almost all dopaminergic cells in SNpc, presumably as a result of retrograde axonal transport. Gene delivery to the striatum was therefore chosen for all further experiments.

The adenovirus constructs for XIAP and GDNF contained a 6-Myc and 1-Myc tag, respectively, that allowed to control for the transgene expression encoded by the XIAP-Myc-tagged and GDNF-Myc-tagged constructs by immunoblot analysis (see Materials and Methods). Seven days after stereotaxic injection of the AdV-XIAP vector into the left striatum, a protein with the molecular weight expected for XIAP fused with a Myc tag (66 kDa) is expressed in the left but not in the right striatum and SNpc (Fig. 6*C*). Similarly, the GDNF-Myc-tagged protein is expressed with a molecular weight of ~18 and ~35 kDa (Fig. 6*D*). The latter is likely to represent a nonreduced, disulfide-bonded dimer. This nonreduced form was also detected at low amounts in the contralateral striatum.

To show *in vitro* that the Myc-tagged GDNF secreted by infected cells is biologically active, we infected SH-SY5Y neuroblastoma cells with 50 pfu/cell of AdV-GDNF or AdV-ΔE1. Three days later, 48 hr conditioned medium was analyzed by ELISA (Fig. 6*E*). GDNF (742 pg) was secreted per 10^6 infected cells per day compared with 0.2 pg of GDNF in AdV-ΔE1-infected cells. Seven days after stereotaxic injection of 0.5×10^8 pfu AdV-GDNF into the striatum, there was a substantial increase in the concentration of GDNF in the striatum and substantia nigra (Fig. 6*E*). Bioactivity was further confirmed with embryonic mesencephalic cultures (Fig. 6*F*). After seeding, mesencephalic cultures were maintained on 90% of DMEM/F12 medium and 10% of conditioned serum-free medium from SH-SY5Y cells. Seven days later, the cultures were stained for TH. Treatment with conditioned medium from SH-SY5Y cells increased the survival of TH-positive neurons. Maintaining mesencephalic cultures in conditioned medium from SH-SY5Y cells infected with AdV-GDNF but not with AdV-ΔE1 provided additive effects. The survival of TH-positive neurons maintained in conditioned medium from AdV-GDNF-treated SH-SY5Y cells was as good as the survival after treatment of mesencephalic cultures with 10 ng/ml recombinant rat GDNF. These results confirmed that bioactive GDNF was produced and secreted by cells infected with GDNF.

AdV-XIAP gene transfer promotes survival of dopaminergic SNpc neurons but does not protect against the MPTP-induced loss of striatal catecholamine concentrations in mice

When analyzed at 7 d after the last administration, treatment with 30 mg/kg MPTP intraperitoneally at 24 hr intervals for five doses significantly reduced the number of TH-positive neurons in SNpc (Fig. 7*A*,*B*). Treatment with a control vector had no effect on survival (Fig. 7*C*). Gene transfer-mediated expression of XIAP almost completely protected TH-positive cells in the SNpc against MPTP toxicity (Fig. 7*D–F*). The protective effects were restricted to the side of adenovirus injection and transgene expression and did not extend to the contralateral side (Fig. 7*E*,*F*). In contrast, XIAP transgene expression had no effect on the ipsilateral striatal concentrations of dopamine, DOPAC, or HVA in the same animals (Fig. 8).

Synergistic effects of AdV-XIAP and AdV-GDNF against MPTP toxicity

Because treatment with AdV-XIAP alone did not rescue nigrostriatal terminals, we studied whether administration of AdV-GDNF promoted the function of rescued neurons. In contrast to AdV-XIAP, striatal administration of AdV-GDNF alone did not promote survival of TH-positive SNpc neurons (Fig. 9*B*,*E*,*F*). However, striatal administration of both AdV-XIAP and AdV-GDNF

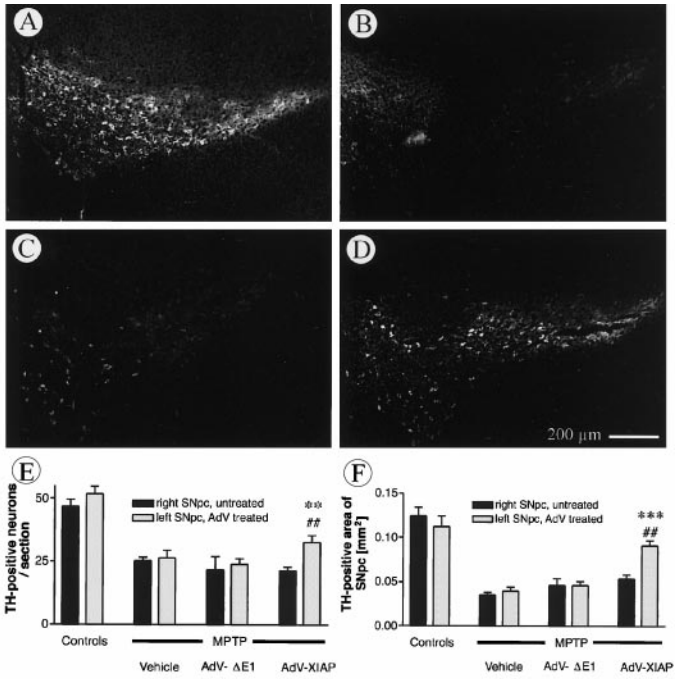

Figure 7. TH-positive neurons are rescued by AdV-XIAP adenoviral gene transfer. MPTP treatment severely depletes TH-positive neurons in SNpc (*B*) compared with untreated mice (*A*). Adenoviral vectors were injected into the left striatum (*C*, *D*) 7 d before MPTP treatment. Treatment with AdV-XIAP (*D*) but not treatment with the control vector AdV-ΔE1 (*C*) rescued TH-positive neurons ipsilateral to gene delivery. In all cases, AdV treatment had no effect on the contralateral SNpc (data not shown). TH-positive cells were counted (*E*), and TH-positive structures were quantified by image analysis (*F*). Mean ± SEM; $n = 8$–10 mice per group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with AdV-ΔE1-treated mice (ANOVA followed by Tukey's *post hoc* test); $^{\#\#}p < 0.01$ compared with untreated contralateral side (two-tailed *t* test for matched pairs).

provided almost complete protection against MPTP-induced cell death (Fig. 9*D–F*). Again, the protective effects did not extend to the contralateral SNpc (Fig. 9*C*,*E*,*F*).

In contrast to AdV-XIAP treatment, delivery of the GDNF-expressing vector into the striatum 1 week before treatment with MPTP protected against MPTP-induced depletion of catecholamines in the striatum when analyzed at 7 d after the last MPTP injection (Fig. 9*G*). The combined treatment with AdV-XIAP and AdV-GDNF provided complete protection against MPTP-induced depletion of dopamine (30.2 compared with 33.1 nmol/mg striatal tissue of untreated controls), DOPAC (8.1 compared with 7.8 nmol/mg), and HVA (10.5 compared with 6.2 nmol/mg). The simultaneous treatment with AdV-XIAP and AdV-GDNF did not only protect against the MPTP-induced loss of striatal catecholamine concentrations compared with vehicle- or control vector-treated controls or with the contralateral untreated striatum, but also provided significantly better protection than either treatment alone ($p < 0.01$). To investigate that the effects of AdV-XIAP and AdV-GDNF were not only additive but also synergistic, we used the fractional product method (Greco et al., 1995). The combination of GDNF- and XIAP-expressing vectors showed synergistic effects (Fig. 9*G*).

DISCUSSION

Adenovirus-mediated gene transfer is a promising tool for the treatment of various clinical disorders, including neurodegenerative diseases. Recombinant adenoviral vectors effectively target gene expression to the brain and offer long-term expression of foreign proteins without disturbing survival, electrophysiological function, or cytoarchitecture of neuronal cells (Le Gal La Salle et al., 1993; Slack et al., 1996). Here we present evidence that dopaminergic SNpc neurons can effectively be infected and express a

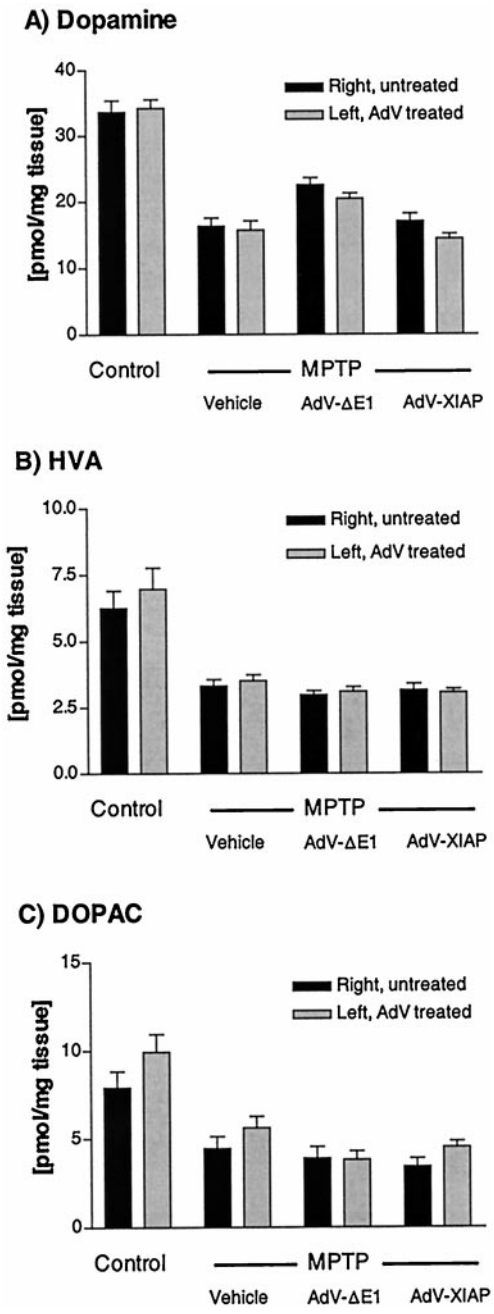

Figure 8. Gene transfer of AdV-XIAP does not protect against the MPTP-induced decrease of striatal catecholamine concentrations. Mice received injections of vehicle or adenoviral vectors into the left striatum. Seven days later, mice were treated with 5 × 30 mg/kg MPTP intraperitoneally at 24 hr intervals or were left untreated (*Control*) and, after another 7 d, they were killed, and concentrations of dopamine (*DA*; *A*), HVA (*B*), or DOPAC (*C*) in the striatum were measured. Mean ± SEM; $n = 8$–10 mice per group.

transgene after delivery of the vector to the terminals in the striatum (Fig. 6). Because XIAP and LacZ are not known to be secreted, their transgene expression in the substantia is likely to result from retrograde axonal virus transport, whereas the detection of GDNF in the substantia nigra may result from retrograde transport of GDNF (Tomac et al., 1995b), the virus, or both. Interestingly, we detected some GDNF in the contralateral striatum but no expression of LacZ or XIAP (Fig. 6), suggesting that this may be a result of GDNF diffusion. In contrast to the findings of retrograde axonal transport in motoneurons in which a lesion of the muscle fibers is required for significant transport to occur (Ghadge et al., 1995), the nigrostriatal system appears to be retrogradely transducible with high and reproducible efficacy.

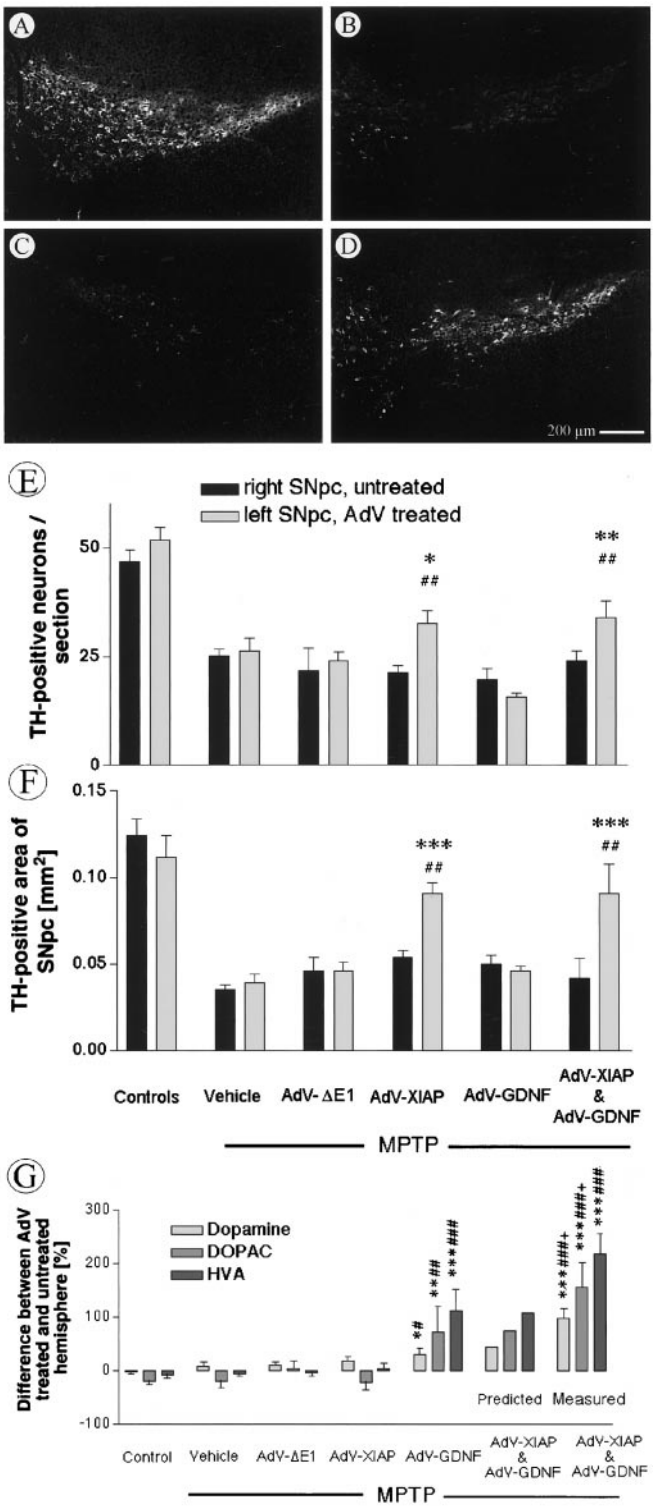

Figure 9. Gene transfer of AdV-GDNF and AdV-XIAP has synergistic effects. Mice received injections of vehicle or adenoviral vectors into the left striatum. Seven days later, mice were treated with 5 × 30 mg/kg MPTP intraperitoneally at 24 hr intervals or were left untreated (*Control*) and, after another 7 d, they were killed. MPTP treatment led to a severe depletion of TH-positive neurons in SNpc (see Fig. 6*B*) compared with untreated mice (*A*). AdV-GDNF treatment had no effects on neuronal survival (*B*). Combination of AdV-XIAP and AdV-GDNF protected TH-positive cells from MPTP-induced cell death ipsilateral to the side of treatment (*D*) but not on the contralateral side (*C*). TH-positive cells were counted (*E*), and TH-positive structures were quantified by image analysis (*F*). ANOVA for repeated measures; $F_{(1,5,27)} = 10.5$; $p < 0.001$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with AdV-ΔE1-treated mice (Tukey's *post hoc* test); $^{\#\#}p < 0.01$ compared with right untreated side (two-tailed *t* test for matched pairs). Mean ± SEM; n = 8–10 mice per group. *G*, Concentrations of dopamine, HVA, or DOPAC were quantified in the same animals. Because the right side remained untreated, it served as a control (100% of each condition). The observed effects of combined treatment with AdV-XIAP and AdV-GDNF are better than the calculated values for independent effects (see Materials and Methods). The statistics were calculated using the original raw data. ANOVA for repeated measures; $F_{(1,5,51)} = 18.7$; $p < 0.001$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{**}p < 0.001$ compared with AdV-ΔE1-treated mice (Tukey's *post hoc* test). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with untreated right side (two-tailed *t* test for matched pairs). Mean ± SEM; n = 8–10 mice per group. The combined treatment with AdV-XIAP and AdV-GDNF is significantly better than either treatment alone ($p < 0.01$).

We show here that virally mediated overexpression of XIAP protects dopaminergic SNpc neurons from cell death in a chronic MPTP treatment paradigm (Fig. 7). Because caspase peptide inhibitors block MPP^+ toxicity *in vitro* (Fig. 2) and *in vivo* (Fig. 5), the protection against MPTP toxicity is likely attributable to inhibition of caspases and not related to an interference with MPTP metabolism, e.g., the inhibition of monoamine oxidase B in the striatum. Using retrograde Fluorogold labeling, we provide evidence that a decrease of the number of TH-positive cells at 7 d after completion of MPTP treatment corresponds well with death of dopaminergic neurons (Fig. 4). Therefore, protection against the loss of TH-positive cells by XIAP is likely to reflect neuroprotection.

Inhibition of caspases may provide promising opportunities for the treatment of acute or chronic neurodegenerative disorders (Schulz et al., 1999). IAPs inhibit the group II caspases-3 and -7 but not caspases-1, -6, -8, or -10. In addition, XIAP may also block active caspase-9, an initiator caspase that is activated by the apoptotic protease activating factor-1 in combination with cytochrome c released from mitochondria and dATP (Deveraux et al., 1998, 1999). Our results provide the first evidence that XIAP, a group II and III inhibitor of caspases, is effective in an *in vivo* model of PD. Further indication for the importance of caspases in the death of dopaminergic neurons comes from studies showing that caspase inhibitors increase survival of dopaminergic neurons grafted to hemiparkinsonian rats and thereby substantially improve functional recovery (Schierle et al., 1999).

Surprisingly, the protective effects of XIAP did not extend to the dopaminergic terminal markers in the striatum (Fig. 8). The concentrations of dopamine and its metabolites were similarly decreased in control vector (AdV-ΔE1)- and AdV-XIAP-treated mice. Because MPTP is metabolized by monoamine oxidase B to MPP^+, which then is selectively taken up by dopaminergic terminals, dopaminergic nerve terminals may be the primary target of MPTP neurotoxicity, followed by a slower and secondary death of the SNpc dopaminergic cell bodies mediated by caspase activation and apoptosis. The likely explanation for the dissociation between the rescue of TH-positive substantia nigra neurons and the failure to maintain biochemical parameters of dopaminergic function in the striatum with XIAP gene transfer is the loss of dopaminergic synaptic terminals. Although an important role for synaptic caspase activation and apoptosis has been proposed (Mattson and Duan, 1999), axonal degeneration after withdrawal of trophic support occurs without the activation of caspases in contrast to the cell death of the soma (Finn et al., 2000). We observed the same dissociation of protective effects using peptide caspase inhibitors against MPP^+ toxicity *in vitro* (Fig. 2). These findings suggest that, although dopaminergic cell somata are protected from MPTP toxicity, they may be functionally impaired. Similar observations of functional impairment have been made in NGF-deprived sympathetic neurons rescued by peptide caspase inhibitors, which showed smaller somata and no dendrites, and maintained only basal levels of protein synthesis (Deshmukh et al., 1996). However, even after a longer period of time, readdition of NGF restored growth and metabolism.

GDNF is the major neurotrophic factor for dopaminergic mesencephalic neurons. It promotes survival of cultured mesencephalic neurons and is expressed in the developing striatum. GDNF promotes recovery of the injured nigrostriatal dopamine system and improves motor functions in both rodent and nonhuman primate

models of Parkinson's disease (Beck et al., 1995; Tomac et al., 1995a; Gash et al., 1996). Adenoviral gene delivery of GDNF to the striatum or substantia nigra prevents neuronal degeneration and restores dopaminergic and motor function in the 6-hydroxydopamine lesion model of PD (Bilang-Bleuel et al., 1997; Choi-Lundberg et al., 1997, 1998; Kirik et al., 2000). In summary, GDNF provides neuroprotective and neurorestorative effects against 6-hydroxydopamine toxicity in the rat (Gash et al., 1998).

Although the 6-hydroxydopamine model is suitable to study symptomatic effects of drug treatments and neurorestorative effects of therapies, the MPTP model more faithfully recapitulates many of the features of sporadic PD when it comes to the molecular mechanisms of dopaminergic cell death (Dawson, 2000). MPTP elicits many of the biochemical, neuropathological, and clinical features of PD in humans, nonhuman primates, and rodents. Although 6-hydroxydopamine and MPTP induce selective death of dopaminergic neurons, their mechanisms are different, and protective effects of a given therapy in one model may not apply to the other.

GDNF has pronounced neurorestorative effects on dopaminergic markers in mice (Tomac et al., 1995a; Date et al., 1998) or monkeys (Gash et al., 1996; Zhang et al., 1997) when administered after MPTP and shows neuroprotective effects on the concentrations of dopamine and its metabolites when administered before or during MPTP administration (Kojima et al., 1997; Cheng et al., 1998). There is one report of protective effects against MPTP-induced death of dopaminergic SNpc neurons (Tomac et al., 1995a), showing enhanced survival of ipsilateral and contralateral dopaminergic neurons when GDNF was stereotaxically injected above the substantia nigra of one side. Because the nigrostriatal dopamine system is uncrossed, these effects were interpreted as attributable to diffusion of GNDF across the midline after mesencephalic injections. In contrast, we did not observe protective effects against the MPTP-induced loss of TH-positive neurons, although GDNF was expressed in the striatum and the SNpc after adenoviral gene delivery as detected by Western blot and ELISA, and this GDNF showed biological activity on dopaminergic mesencephalic cultures *in vitro* and on terminal markers of dopamine *in vivo*. For GDNF, we only observed protective–restorative effects on the concentrations of dopamine and its metabolites in the striatum. Our results also show that most of the GDNF expression is restricted to the ipsilateral striatum and substantia nigra.

The discrepancy between the protective effects reported by Tomac et al. (1995) and the findings presented here may result from the different models used. In the same strain of mice, Tomac et al. used two subcutaneous injections of 40 mg/kg MPTP, whereas we treated chronically for 5 d with 30 $mg \cdot kg^{-1} \cdot d^{-1}$. The chronic exposure to MPTP may result in a failure of protection compared with acute treatment. This hypothesis is supported by a study by Cheng and colleagues (1998) who showed that repeated intrastriatal administration of GDNF provides only small protective effects against the loss of dopaminergic markers in the striatum induced by chronic MPTP treatment for 7 consecutive days compared with the complete protection reported by Tomac and colleagues (1995). In mesencephalic cultures, GDNF did not prevent acute toxicity to dopaminergic neurons by MPP^+ but only protected dopaminergic neurons from continuous cell death after termination of the exposure to MPP^+ and stimulated the regrowth of dopaminergic fibers damaged (Hou et al., 1996).

A clinicopathological analysis of the first patient from a multicenter clinical trial who had come to autopsy after multiple injections of GDNF into the right lateral ventricle (Kordower et al., 1999) showed no significant regeneration of nigrostriatal neurons. Furthermore, this treatment did not improve clinical parkinsonism and did not prevent deterioration of clinical symptoms. Because no GDNF immunoreactivity was observed in the patient's brain at 3 weeks after the final GDNF injection and the diffusion of GDNF was extremely limited in three primates treated with chronic intraventricular GDNF infusion, it was concluded that the intracerebroventricular route of delivery is unsuitable in primates and that gene therapy approaches may have the potential to overcome these limitations.

We show here that AdV-GDNF delivery to the striatum has no protective effects against the loss of TH-positive neurons in SNpc in a chronic systemic MPTP paradigm (Fig. 9), although the infection efficacy is high and dopaminergic markers in the striatum are preserved. Because virally mediated expression of XIAP rescues dopaminergic somata and acts in synergy with GDNF gene delivery to restore dopaminergic synaptic markers, the combination of this neuroprotective (XIAP) and neurorestorative (GDNF) strategy may provide a promising opportunity to counteract progressive cell loss and functional impairment in PD.

REFERENCES

Anglade P, Vyas S, Javoy-Agid F, Herrero MT, Michel PP, Marquez J, Mouatt-Prigent A, Ruberg M, Hirsch EC, Agid Y (1997) Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. Histol Histopathol 12:25–31.

Beal MF (1995) Aging, energy and oxidative stress in neurodegenerative diseases. Ann Neurol 38:357–366.

Beck KD, Valverde J, Alexi T, Poulsen K, Moffat B, Vandlen RA, Rosenthal A, Hefti F (1995) Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. Nature 373:339–341.

Bilang-Bleuel A, Revah F, Colin P, Locquet I, Robert JJ, Mallet J, Horellou P (1997) Intrastriatal injection of an adenoviral vector expressing glial-cell-line-derived neurotrophic factor prevents dopaminergic neuron degeneration and behavioral impairment in a rat model of Parkinson disease. Proc Natl Acad Sci USA 94:8818–8823.

Bloem BR, Irwin I, Buruma OJS, Haan J, Roos RAC, Tetrud JW, Langston JW (1990) The MPTP model: versatile contributions to the treatment. J Neurol Sci 97:273–293.

Cheng FC, Ni DR, Wu MC, Kuo JS, Chia LG (1998) Glial cell line-derived neurotrophic factor protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in C57BL/6 mice. Neurosci Lett 252:87–90.

Choi-Lundberg DL, Lin Q, Chang YN, Chiang YL, Hay CM, Mohajeri H, Davidson BL, Bohn MC (1997) Dopaminergic neurons protected from degeneration by GDNF gene therapy. Science 275:838–841.

Choi-Lundberg DL, Lin Q, Schallert T, Crippens D, Davidson BL, Chang YN, Chiang YL, Qian J, Bardwaj L, Bohn MC (1998) Behavioral and cellular protection of rat dopaminergic neurons by an adenoviral vector encoding glial cell line-derived neurotrophic factor. Exp Neurol 154:261–275.

Date I, Aoi M, Tomita S, Collins F, Ohmoto T (1998) GDNF administration induces recovery of the nigrostriatal dopaminergic system both in young and aged parkinsonian mice. NeuroReport 9:2365–2369.

Dawson TM (2000) New animal models for Parkinson's disease. Cell 101:115–118.

Deshmukh M, Vasilakos J, Deckwerth TL, Lampe PA, Johnson EM (1996) Genetic and metabolic status of NGF deprived sympathetic neurons saved by an inhibitor of ICE family proteases. J Cell Biol 135:1341–1354.

Deveraux QL, Reed JC (1999) IAP family proteins—suppressors of apoptosis. Genes Dev 13:239–252.

Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388:300–304.

Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 17:2215–2223.

Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC (1999) Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J 18:5242–5251.

Dodel RC, Du Y, Bales KR, Ling ZD, Carvey PM, Paul SM (1998) Peptide inhibitors of caspase-3-like proteases attenuate 1-methyl-4-phenylpyridinum-induced toxicity of cultured fetal rat mesencephalic dopamine neurons. Neuroscience 86:701–707.

Finn JT, Weil M, Archer F, Siman R, Srinivasan A, Raff MC (2000) Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. J Neurosci 20:1333–1341.

Franklin KBJ, Paxinos G (1996) The mouse brain in stereotaxic coordinates. New York: Academic.

Gash DM, Zhang Z, Ovadia A, Cass WA, Yi A, Simmerman L, Russell D, Martin D, Lapchak PA, Collins F, Hoffer BJ, Gerhardt GA (1996) Functional recovery in parkinsonian monkeys treated with GDNF. Nature 380:252–255.

Gash DM, Zhang Z, Gerhardt G (1998) Neuroprotective and neurorestorative properties of GDNF. Ann Neurol 44:S121–S125.

Ghadge GD, Roos RP, Kang UJ, Wollmann R, Fishman PS, Kalynych AM, Barr E, Leiden JM (1995) CNS gene delivery by retrograde transport of recombinant replication-defective adenoviruses. Gene Ther 2:132–137.

Gravel C, Gotz R, Lorrain A, Sendtner M (1997) Adenoviral gene transfer of ciliary neurotrophic factor and brain-derived neurotrophic factor

leads to long-term survival of axotomized motor neurons. Nat Med 3:765–770.
Greco RW, Bravo G, Parsons JC (1995) The search for synergy: a critical review from a response surface perspective. Pharmacol Rev 47:331–385.
Hartmann A, Hunot S, Michel PP, Muriel MP, Vyas S, Faucheux BA, Mouatt-Prigent A, Turmel H, Srinivasan A, Ruberg M, Evan GI, Agid Y, Hirsch EC (2000) Caspase-3: a vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. Proc Natl Acad Sci USA 97:2875–2880.
Hou JG, Lin LF, Mytilineou C (1996) Glial cell line-derived neurotrophic factor exerts neurotrophic effects on dopaminergic neurons in vitro and promotes their survival and regrowth after damage by 1-methyl-4-phenylpyridinium. J Neurochem 66:74–82.
Jackson-Lewis V, Jakowec M, Burke RE, Przedborski S (1995) Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurodegeneration 4:257–269.
Kirik D, Rosenblad C, Bjorklund A, Mandel RJ (2000) Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. J Neurosci 20:4686–4700.
Klevenyi P, Andreassen O, Ferrante RJ, Schleicher Jr JR, Friedlander RM, Beal MF (1999) Transgenic mice expressing a dominant negative mutant interleukin-1beta converting enzyme show resistance to MPTP neurotoxicity. NeuroReport 10:635–638.
Kojima H, Abiru Y, Sakajiri K, Watabe K, Ohishi N, Takamori M, Hatanaka H, Yagi K (1997) Adenovirus-mediated transduction with human glial cell line-derived neurotrophic factor gene prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopamine depletion in striatum of mouse brain. Biochem Biophys Res Commun 238:569–573.
Kordower JH, Palfi S, Chen EY, Ma SY, Sendera T, Cochran EJ, Mufson EJ, Penn R, Goetz CG, Comella CD (1999) Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. Ann Neurol 46:419–424.
Krieglstein K, Unsicker K (1997) Protein from chromaffin granules promotes survival of mesencephalic dopaminergic neurons by an EGF-receptor ligand-mediated mechanism. J Neurosci Res 48:18–30.
Krieglstein K, Suter-Crazzolara C, Fischer WH, Unsicker K (1995) TGF-beta superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. EMBO J 14:736–742.
Le Gal La Salle G, Robert JJ, Berrard S, Ridoux V, Stratford-Perricaudet LD, Perricaudet M, Mallet J (1993) An adenovirus vector for gene transfer into neurons and glia in the brain. Science 259:988–990.
Mattson MP, Duan W (1999) "Apoptotic" biochemical cascades in synaptic compartments: roles in adaptive plasticity and neurodegenerative disorders. J Neurosci Res 58:152–166.
Mittereder N, March KL, Trapnell BC (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J Virol 70:7498–7509.
Mochizuki H, Goto K, Mori H, Mizuno Y (1996) Histochemical detection of apoptosis in Parkinson's disease. J Neurol Sci 137:120–123.
Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J 16:6914–6925.
Schierle GS, Hansson O, Leist M, Nicotera P, Widner H, Brundin P (1999) Caspase inhibition reduces apoptosis and increases survival of nigral transplants. Nat Med 5:97–100.
Schulz JB, Beal MF (1994) Mitochondrial dysfunction in movement disorders. Curr Opin Neurol 7:333–339.
Schulz JB, Henshaw DR, Siwek D, Jenkins BG, Ferrante RJ, Cipolloni PB, Kowall NW, Rosen BR, Beal MF (1995) Involvement of free radicals in excitotoxicity *in vivo*. J Neurochem 64:2239–2247.
Schulz JB, Matthews RT, Henshaw DR, Beal MF (1996a) Neuroprotective strategies for treatment of lesions produced by mitochondrial toxins: implications for neurodegenerative diseases. Neuroscience 71:1043–1048.
Schulz JB, Weller M, Klockgether T (1996b) Potassium deprivation-induced apoptosis of cerebellar granule neurons: a sequential requirement for new mRNA and protein synthesis, ICE-like protease activity, and reactive oxygen species. J Neurosci 16:4696–4706.
Schulz JB, Weller M, Moskowitz MA (1999) Caspases as treatment targets in stroke and neurodegenerative diseases. Ann Neurol 45:421–429.
Simons M, Beinroth S, Gleichmann M, Liston P, Korneluk RG, MacKenzie AE, Bähr M, Klockgether T, Robertson GS, Weller M, Schulz JB (1999) Adenovirus-mediated gene transfer of IAPs delays apoptosis of cerebellar granule neurons. J Neurochem 72:292–301.
Slack RS, Belliveau DJ, Rosenberg M, Atwal J, Lochmüller H, Aloyz R, Haghighi A, Lach B, Seth P, Cooper E, Miller FD (1996) Adenovirus-mediated gene transfer of the tumor suppressor, p53, induces apoptosis in postmitotic neurons. J Cell Biol 135:1085–1096.
Sofroniew MV, Cooper JD, Svendsen CN, Crossman P, Ip NY, Lindsay RM, Zafra F, Lindholm D (1993) Atrophy but not death of adult septal cholinergic neurons after ablation of target capacity to produce mRNAs for NGF, BDNF, and NT3. J Neurosci 13:5263–5276.
Srinivasan A, Roth KA, Sayers RO, Shindler KS, Wong AM, Fritz LC, Tomaselli KJ (1998) *In situ* immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. Cell Death Differ 5:1004–1016.
Storey E, Hyman BT, Jenkins B, Brouillet E, Miller JM, Rosen BR, Beal MF (1992) 1-Methyl-4-phenylpyridinium produces excitotoxic lesions in rat striatum as a result of impairment of oxidative metabolism. J Neurochem 58:1975–1978.
Tatton NA, Kish SJ (1997) *In situ* detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. Neuroscience 77:1037–1048.
Tipton KF, Singer TP (1993) Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. J Neurochem 61:1191–1206.
Tomac A, Lindqvist E, Lin LF, Ogren SO, Young D, Hoffer BJ, Olson L (1995a) Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. Nature 373:335–339.
Tomac A, Widenfalk J, Lin LF, Kohno T, Ebendal T, Hoffer BJ, Olson L (1995b) Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. Proc Natl Acad Sci USA 92:8274–8278.
Wüllner U, Kornhuber J, Weller M, Schulz JB, Löschmann P-A, Riederer P, Klockgether T (1999) Cell death and apoptosis regulating proteins in Parkinson's disease: a cautionary note. Acta Neuropathol 97:408–412.
Yang L, Matthews RT, Schulz JB, Klockgether T, Liao AW, Martinou J-C, Penney Jr JB, Hyman BT, Beal MF (1998) MPTP neurotoxicity is attenuated in mice overexpressing Bcl-2. J Neurosci 18:8145–8152.
Zhang Z, Miyoshi Y, Lapchak PA, Collins F, Hilt D, Lebel C, Kryscio R, Gash DM (1997) Dose response to intraventricular glial cell line-derived neurotrophic factor administration in parkinsonian monkeys. J Pharmacol Exp Ther 282:1396–1401.