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Long-term striatal overexpression of GDNF selectively downregulates tyrosine hydroxylase in the intact nigrostriatal dopamine system

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Keywords: autoradiography, lentivirus, rat, striatum, substantia nigra

Abstract

Sustained neurotrophic factor treatment in neurodegenerative disorders such as Parkinson's disease is likely to affect both degenerating and intact neurons. To investigate the effect of long-term glial cell line-derived neurotrophic factor (GDNF) overexpression on intact nigrostriatal dopamine neurons, we injected a recombinant lentiviral vector encoding GDNF, or green fluorescent protein, in the right striatum of young adult rats. Thirteen months after viral injection GDNF levels were 4.5 ng/mg tissue in the striatum and 0.9 ng/mg in the substantia nigra as measured by ELISA, representing a 25–100-fold increase above control vector- or nontransduced tissue. GDNF overexpression significantly reduced tyrosine hydroxylase mRNA levels (by 39–72%) in the substantia nigra and ventral tegmental area neurons, and the optical density of tyrosine hydroxylase-immunoreactive innervation in the striatum was reduced by 25–52% with the most prominent reductions appearing caudally. No significant reduction was seen in striatal vesicular monoamine transporter 2-immunoreactivity or [³H]mazindole binding autoradiography to dopamine uptake sites, two other presynaptic markers in dopamine axon terminals. The striatal D1 and D2 receptor binding as determined by [³H]SCH23390 and [³H]spiperone binding, respectively, was unaltered relative to the intact side in both treatment groups. Preproenkephalin mRNA levels in postsynaptic striatal neurons, which increase upon removal of striatal dopamine, were also unaffected by the GDNF treatment. Taken together our findings indicate that sustained GDNF administration to intact nigrostriatal dopamine neurons selectively reduces tyrosine hydroxylase expression, without altering striatal dopamine transmission to the extent that compensatory changes in several other components related to dopamine storage and signalling occur.

Introduction

A growing body of evidence indicate that glial cell line-derived neurotrophic factor (GDNF) exert profound neurotrophic and pharmacological effects on injured nigrostriatal dopamine (DA) neurons and may therefore be useful to prevent the neurodegeneration underlying Parkinson's disease. Injections of GDNF protein have been shown to prevent the death of injured nigrostriatal dopamine neurons *in vivo* (Beck *et al.*, 1995; Sauer *et al.*, 1995; Tomac *et al.*, 1995; Kearns *et al.*, 1997; Tseng *et al.*, 1997), as well as the retrograde degeneration of DA axons induced by intrastriatal 6-hydroxydopamine (Kirik *et al.*, 2000a; Rosenblad *et al.*, 2000a). GDNF can also promote sprouting from axotomized DA neurons (Kirik *et al.*, 2000a, b; Rosenblad *et al.*, 2000b; Georgievska *et al.*, 2002). Preservation and/or regeneration of striatal DA axon terminals is pivotal for recovery of motor function (Bjorklund *et al.*, 2000) and is seen following GDNF administration to the DA axon terminals in the striatum, but not substantia nigra (SN) (Kirik *et al.*, 2000a, b). Moreover, functional recovery is best obtained with sustained GDNF treatment as indicated by recent studies in rodent and primate models of Parkinson's disease. Kirik *et al.* (2000b) showed that striatal but not nigral GDNF expression from a recombinant adeno-associated virus

vector could prevent degeneration as well as induce sprouting and functional recovery in the partial 6-hydroxydopamine lesion model, and Kordower *et al.* (2000) found that GDNF expression from a recombinant lentiviral (rLV) vector attenuated cell loss and functional impairments in a primate model of Parkinson's disease.

From a clinical perspective, it may be preferable if a protective therapy in patients with Parkinson's disease can be initiated early in the course of the disease to prevent further cell loss. Because not all DA neurons degenerate uniformly, this will mean that some intact DA neurons also receive sustained GDNF treatment. In the intact nigrostriatal system, exogenous GDNF increases DA levels and DA turnover on the treated side shortly after injection into the SN or striatum, and induced a turning response when animals were challenged with amphetamine (Hudson *et al.*, 1995; Beck *et al.*, 1996; Horger *et al.*, 1998). In line with these observations, electrophysiological recordings from cultures of early postnatal DA neurons have shown increased quantal release (Pothos *et al.*, 1998) and enhanced excitability (Yang *et al.*, 2001) following GDNF administration. However, the effects of continuous long-term GDNF administration (several months) to intact nigrostriatal DA neurons remain unknown. In the present study we addressed this question by overexpressing GDNF for 13 months in striatal cells of intact rats by means of an rLV-GDNF vector, followed by evaluation of pre- and postsynaptic components in the dopamine signalling pathway using immunohistochemistry, binding autoradiography and *in situ* hybridization techniques.

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Materials and methods

Viral vectors

VSV-G pseudotyped (rLV) vectors were produced as described previously (Zufferey *et al.*, 1997; Rosenblad *et al.*, 2000a). Briefly, the transfer plasmids pHR'CMV-W carrying the cDNA for green fluorescent protein (GFP) or GDNF was cotransfected with the helper plasmids pMD.G and pCMV Δ R8.91 into 293T cells. Virion-containing supernatants were collected on days 2 and 3 after transfection and concentrated at 116 000 g by ultracentrifugation. The titre of rLV-GFP vector stock was 1.1×10^8 TU/mL as determined by serial dilution of the concentrated supernatant on 293T cells. The viral particle titre was determined for rLV-GDNF and rLV-GFP virus stocks using an RNA slot blot technique as described previously (von Schwedler *et al.*, 1993) and from the ratio between transducing units (TU) and viral particle titre obtained for rLV-GFP, the titre of the rLV-GDNF vector was estimated to be 1.2×10^8 TU/mL.

Surgical procedures

All work involving experimental animals was conducted according to the guidelines set by the Ethical Committee for Use of Laboratory Animals at Lund University. Animals were housed in a 12:12 h light/darkness cycle with access to rat chow and water. In total, 24 female Sprague–Dawley rats (\approx 220 g by the time of surgery) were used. For stereotaxic surgery animals were anaesthetized using halothane and 2 μ L rLV-GFP ($n=8$) or rLV-GDNF ($n=15$) of a 1:2 diluted viral stock (i.e. 1.1 – 1.2×10^5 TU) was injected into two tracts in the right striatum at the following coordinates: (i) AP = +1.0 mm, ML = -2.6 mm, DV = -5.0 and -4.5 mm, Tb = 0.0; and (ii), AP = 0.0 mm, ML = -3.7 mm, DV = -5.0 and -4.5 mm, Tb = 0.0. Animals for ELISA ($n=4$) and autoradiography ($n=3$ and 6 for rLV-GFP and rLV-GDNF, respectively) were anaesthetized deeply with pentobarbital (70 mg/kg, Apoteksbolaget, Sweden), decapitated and the brains quickly dissected out. For ELISA, 2-mm-thick coronal slices were made through the brain and the entire striatum was dissected out and immediately froze on dry ice. For the SN, a 2 mm diameter punch was taken in the appropriate midbrain slice. Brains for autoradiography and *in situ* hybridization were immediately frozen on dry ice. Sections (14 μ m) were cut on a cryostat and thaw-mounted onto positively charged glass slides. Animals for immunohistochemical analysis ($n=5$ /group) were anaesthetized deeply with pentobarbital and transcardially perfused with 50 mL saline at room temperature, followed by 200 mL ice-cold phosphate-buffered 4% paraformaldehyde (pH 7.2–7.4). The brains were postfixed for 3–6 h in the same fixative, transferred to 30% sucrose for 24 h and cut into six series of 40- μ m-thick sections on a freezing microtome.

Binding autoradiography

Binding to D1- and D2-receptors was performed as described by Puschban *et al.* (2000). Briefly, sections were thawed at room temperature for 1 h, washed in Tris buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM CaCl₂, and 1 mM MgCl₂; pH 7.9) at 4 °C for 5 min, followed by incubation with 1.25 nM [³H]SCH23390 (specific activity 86 Ci/mmol; NEN Life Science Products) for 90 min. Unspecific binding was determined in adjacent sections in the presence of 1 μ M *cis*-flupenthixol (Sigma-Aldrich, St. Louis, MO, USA). Sections for D2 receptor binding were incubated in 1 nM [³H]spiperone (specific activity 15.7 Ci/mmol; NEN) + 100 nM ketanserin (to block binding to serotonin-2 receptors; Sigma-Aldrich) for 1 h. Nonspecific binding was determined in the presence of 1 μ M haloperidol (Sigma-Aldrich). Following two rinses in buffer and one in ddH₂O (2 min each) the sections were air dried and apposed to tritium-sensitive film

([³H]Hyperfilm, Amersham, Bucks, UK), and exposed for 21–28 days at 4 °C.

[³H]mazindole binding to dopamine transporter was determined as described by Henry *et al.* (1998). Sections were preincubated in Tris-Buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM KCl; pH 7.9) for 5 min at 4 °C, followed by 60 min incubation in the same buffer containing 10 nM [³H]mazindole (specific activity 24 Ci/mmol; NEN) and 50 nM desipramine (Sigma-Aldrich) to prevent binding to noradrenaline uptake sites. Nonspecific binding was determined in the presence of 100 μ M nomifensine (Sigma-Aldrich). Sections were then rinsed in buffer (2 \times) and ddH₂O, air dried, apposed to tritium-sensitive film ([³H]Hyperfilm, Amersham) and exposed for 14 days at 4 °C.

In situ hybridization

In situ hybridization was performed as described previously (Campbell *et al.*, 1992; Campbell & Bjorklund, 1995) using DNA oligonucleotide probes complementary to rat tyrosine hydroxylase (TH; nt 1441–1488) and rat preproenkephalin (PPE; nt 322–360). Probes were 3' end-labelled with [³⁵S]dATP (1250 Ci/mmol; NEN) using terminal deoxynucleotidyltransferase (New England Biolabs, USA) and purified using Chromaspin TE columns (Clontech, USA) to specific activities of $>10^9$ c.p.m./ μ g. Sections were thawed and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by three rinses in PBS (5 min each), and dehydration in 70% and 95% alcohol (2 min each). A total of 45 μ L of hybridization buffer containing 10^7 c.p.m. of probe/mL was applied to each section. The slide was then cover slipped and incubated in humidified chambers at 42 °C overnight. The cover slips were removed at 55 °C in 1 \times SSC (0.15 M NaCl/0.015 M sodium citrate) and washed 4 \times in the same buffer and finally cooled to room temperature and air dried before being apposed to autoradiographic film (β -Max, Amersham, UK) for 3–8 days at 20 °C. The slides were then dipped in liquid emulsion (Ilford, UK) and exposed for 3–4 weeks before being developed in Kodak D-19 (Kodak), fixed in AGFA 333 (AGFA) and counterstained with cresyl violet.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Rosenblad *et al.*, 2000a). Sections were quenched in 3% H₂O₂ for 5 min and preincubated in buffer (KPBS + 0.25% Triton X-100 + 2% serum) for 1 h, followed by incubation in buffer containing primary chicken anti-GFP (1:2000, Chemicon, USA), mouse anti-TH (1:2000; Chemicon), rabbit anti-vesicular monoamine transporter-2 (VMAT; 1:1000, Chemicon) or goat anti-GDNF (1:2000, R & D systems, USA) overnight at room temperature. Subsequently sections were rinsed three times, incubated for 2 h with biotinylated secondary antibody [rabbit anti-chicken (Promega, USA), horse anti-mouse (Vector Laboratories, USA), swine anti-rabbit (DAKO, Denmark), or horse anti-goat (Vector Laboratories), respectively], avidin–biotin–peroxidase (ABC kit; Vector Laboratories) according to manufacturers instructions, and finally visualized using 3'-3'-diaminobenzidine (Sigma-Aldrich) as a chromogen. The sections were mounted onto chrome-alum-coated slides and coverslipped with DPX.

Morphometric analysis

The density of D1, D2, and DA transporter binding, and TH- or VMAT-immunoreactive innervation in the striatum was assessed by measuring the optical density using the NIH 1.62 Image program as described previously (Kirik *et al.*, 1998). Black and white images for analysis were captured using a digital ProgRes camera adapted to an Olympus BX60 microscope and a Macintosh G3 PowerMac computer. Optical density was assessed by outlining the striatum at 2 or 3 rostrocaudal

levels (+1.0 mm, and -0.6 mm, or +1.0 mm, +0.2 mm and -0.6 mm in the atlas of Paxinos & Watson, 1998). Nonspecific optical density was measured from the striatum of autoradiography section for non-specific binding (*cis*-flupenthixol, halperidol or nomifensine), or in the corpus callosum in sections processed for immunohistochemistry.

Images for analysis of cellular levels of TH and PPE mRNA were grabbed using a Leica microscope, a digital video camera (Hamamatsu, Japan) and the Openlab 3.0 program run on a Power Macintosh G4 computer. At 20 \times magnification a black and white light microscopic picture of the SN, ventral tegmental area (VTA), or striatum was captured and a second picture of the exact same area was then taken after switching to dark-field. Images from the SN and VTA was captured in two sections corresponding to levels -5.0 and -5.3 mm in the atlas of Paxinos & Watson (1998); sections for analysis of PPE mRNA was taken at level +0.5 mm. In the light microscopy picture (where also the silver grains could be seen), all cresyl violet-stained cells with a neuronal shape and weakly stained cytoplasm was selected and in the corresponding dark-field image the density of silver grains located over the cell was then measured using the ScionImage program run on a Compac computer by placing circle of constant diameter over the cell. Unspecific signal was measured in an adjacent region devoid of DA cells. A minimum of 75 and 35 cells were measured on each side in the SN and VTA, respectively. The levels of PPE mRNA in the striatum was assessed by sampling two equal size regions in the head of the striatum on each side where the density of grains were measured.

The number of TH-immunoreactive (IR) and VMAT-IR nigral neurons was assessed by counting all immunoreactive neurons lateral to the medial terminal nucleus of the accessory optic tract in three consecutive sections through the SN, as described previously (Sauer & Oertel, 1994).

Statistical analysis

Comparison of striatal densitometry measurements was made using two-way factorial analysis of variance (ANOVA; group \times level) followed when significant by *post hoc* Newman-Keuls test. Comparisons of tissue levels of GDNF, nigral cell counts and cellular TH and PPE

mRNA density was made using an unpaired *t*-test. For all comparisons $P < 0.05$ was considered significant.

Results

Long-term transgene expression in vivo

Thirteen months after rLV injections, many GFP-IR cells were found centrally in the striatum of rLV-GFP-transduced animals (Fig. 1C). Both neurons and glia were transduced but the majority of GFP-IR cells had the morphology of medium-sized spiny neurons. In animals injected with rLV-GDNF strong GDNF staining was seen in the striatum (Fig. 1A) and in the SN pars reticulata (data not shown) on the ipsilateral side. The pattern of GDNF-IR was diffuse, which is consistent with GDNF being released into the extracellular space.

Consistent with the prominent GDNF immunoreactivity on the ipsilateral side, tissue levels of GDNF as determined by ELISA were increased significantly both in the striatum (4.59 ± 0.81 ng/mg tissue; $P < 0.001$) and SN (0.83 ± 0.25 ng/mg tissue; $P < 0.001$) as compared with the untreated contralateral side (0.04 ± 0.01 ng/mg tissue and 0.03 ± 0.01 ng/mg tissue in the striatum and SN, respectively). The increased GDNF levels in the SN primarily resulted from anterograde transport and release from transduced striatonigral projection neurons (Georgievska *et al.*, 2002).

GDNF-induced changes in TH

In sections through the midbrain processed for TH by *in situ* hybridization, a prominent signal was detected in the SN and VTA (Fig. 2A and B). The level of TH mRNA was similar on the intact and treated side of rLV-GFP injected animals (Fig. 2A), whereas the signal was clearly reduced on the treated side in rLV-GDNF animals (Fig. 2B). Inspection of emulsion-dipped sections showed that silver grains were located on top of large cresyl violet-stained perikarya in the SN pars compacta and VTA (Fig. 2C, and data not shown). Compared with the contralateral intact side (Fig. 2C and E) the amount of grains/cell was clearly reduced ipsilaterally in rLV-GDNF-treated animals (Fig. 2D and F). No such side difference was observed in rLV-GFP-injected animals (data not shown). Quantification showed that the amount of

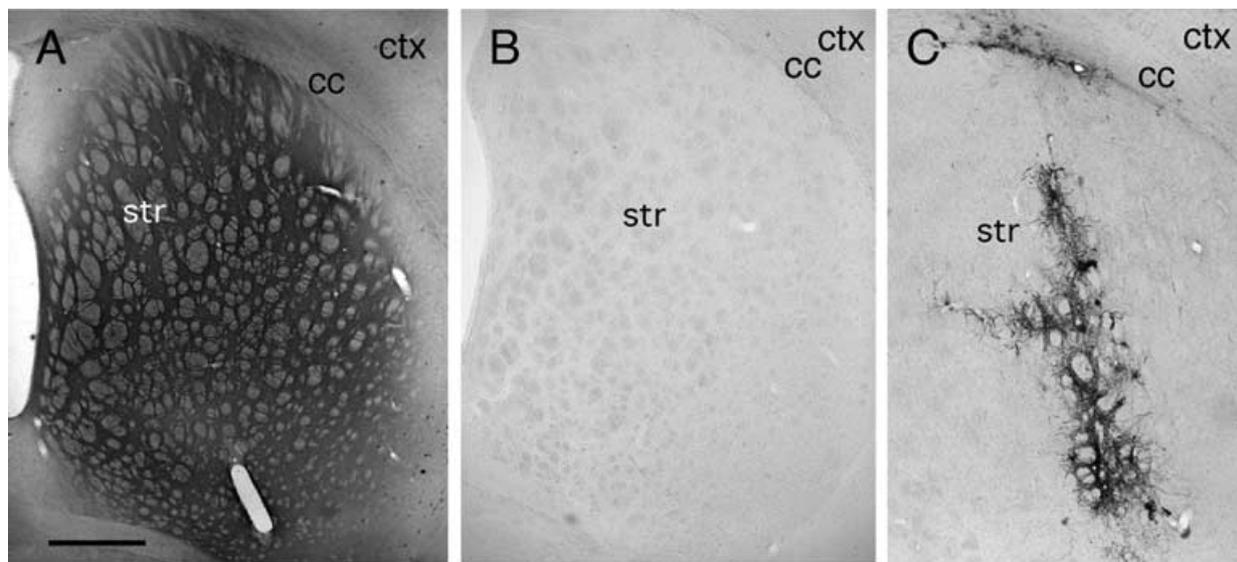


FIG. 1. Transgene expression from rLV-GFP and rLV-GDNF vectors in the striatum at 13 months post-transduction. Striatal sections processed for GDNF immunohistochemistry show a diffuse staining throughout the striatum rLV-GDNF (A), but not in rLV-GFP (B) animals, consistent with a secretion of GDNF from transduced striatal cells. (C) GFP-IR cells, predominantly of neuronal morphology, are seen in a 0.5-mm-wide and 2–3 mm deep area surrounding the injection tract. cc, corpus callosum; ctx, cortex; str, striatum. Scale bar, 600 μ m.

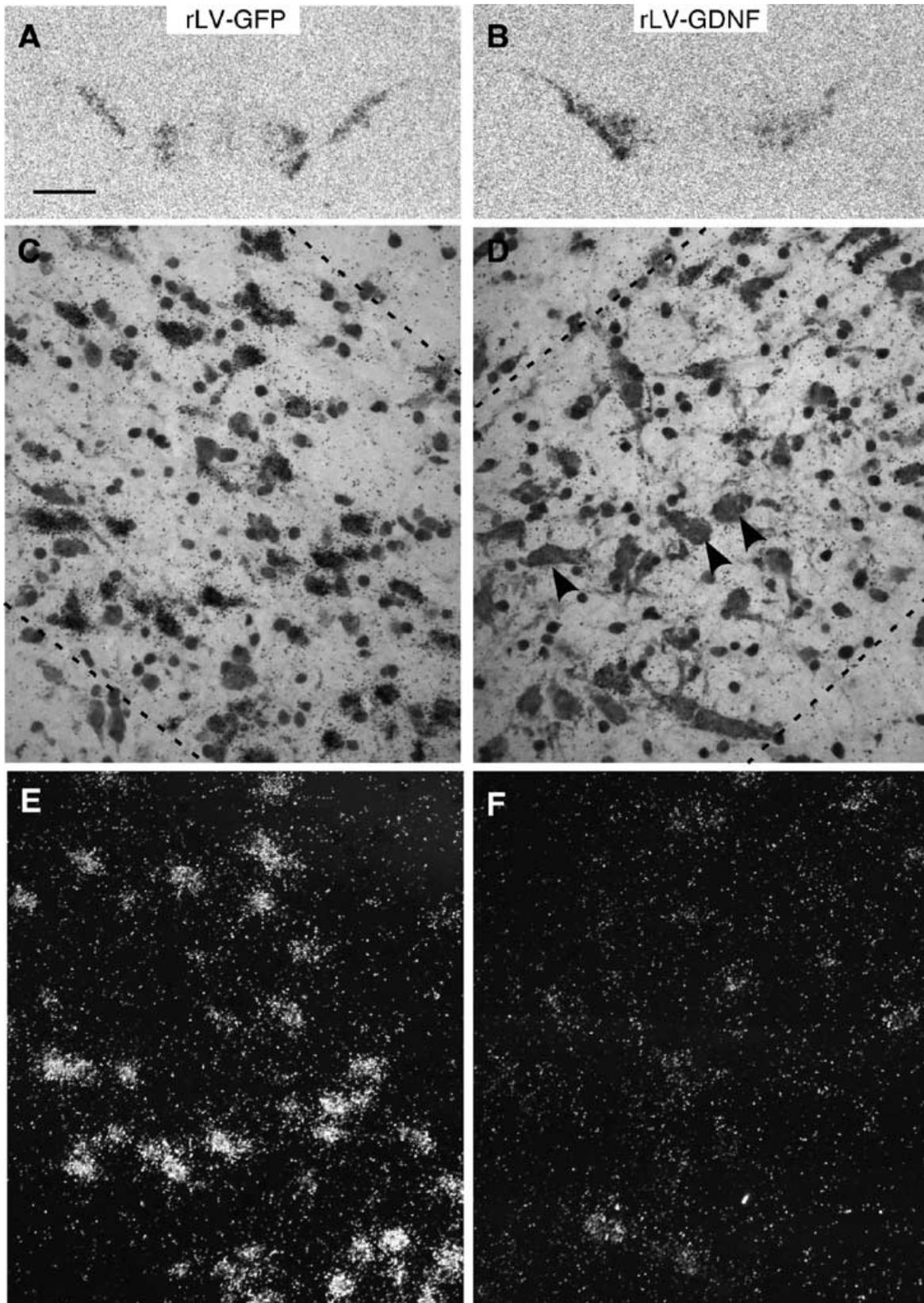


FIG. 2. Tyrosine hydroxylase mRNA expression in SN and VTA neurons. (A and B) Photomicrographs from the autoradiographic film illustrating the markedly reduced levels of TH mRNA on the treated (right) side of rLV-GDNF treated animals (B) as compared to rLV-GFP treated (A). Bright-field (C and D) and dark-field (E and F) microscopic pictures of emulsion dipped sections through the substantia nigra (delineated by the dotted lines in C and E) show a marked reduction in the number of silver grains overlying large faintly cresyl violet-stained perikarya on the GDNF treated side (D and F) as compared with the intact side (C and E). Arrowheads in (D) indicate large presumably dopaminergic neurons on the GDNF-treated side that have markedly reduced expression of TH. Scale bar, 800 μ m (A and B); 40 μ m (C–F).

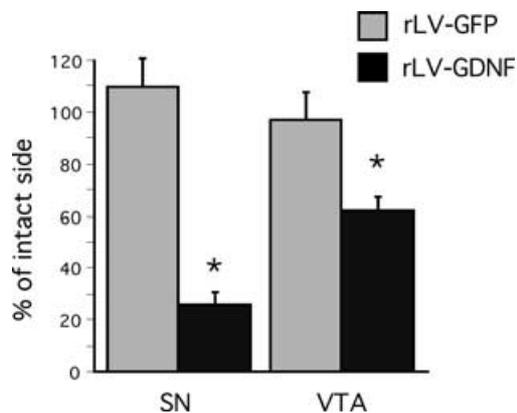


FIG. 3. Quantification of the density of grains/cell showed a significant reduction in both the SN and VTA of GDNF treated animals. * $P < 0.001$ compared with the rLV-GFP-treated group (*t*-test).

TH mRNA/cell on the treated side in rLV-GFP animals was indistinguishable from that on the untreated side ($108 \pm 10\%$ and $100 \pm 8\%$ in the SN and VTA, respectively; Fig. 3). By contrast, the TH mRNA/cell was reduced significantly in the ipsilateral SN and VTA of rLV-GDNF-injected animals (to $28 \pm 4\%$ and $61 \pm 3\%$ of that on the intact side, respectively; $P < 0.01$; Fig. 2G).

Next we determined if the reduction in TH mRNA in the DA neurons of the SN and VTA translated into a change in the amount of TH protein as determined by TH immunoreactivity. In the striatum, the TH-IR innervation on the intact and rLV-GFP-treated side was similar at all rostro-caudal levels examined (Fig. 4A–B). By contrast, in the rLV-GDNF-treated group a weaker intensity of TH staining was seen on the treated side as compared with the intact. The reduced intensity had a patchy distribution with the most prominent reductions in dorso-lateral and caudal aspects of the striatum (Fig. 4C). No signs of aberrant sprouting of TH-IR fibres were found along the nigrostriatal projection. Optical density measurements of TH-IR innervation at three rostro-caudal levels through the striatum confirmed this impression. In the rLV-GDNF-treated group the density of TH immunoreactivity on the ipsilateral as compared with the contralateral side, was reduced to $75 \pm 5.1\%$, $66 \pm 3.8\%$, $48 \pm 6.1\%$ in the rostral, middle and caudal striatum, respectively ($P < 0.01$ for all levels; Fig. 5A). No reduction compared with the intact side was found in GFP-treated animals at any of the three levels examined ($111 \pm 3.6\%$, $94 \pm 2.4\%$ and $121 \pm 17\%$, in the rostral, middle and caudal striatum, respectively; Fig. 5A).

At the level of the cell bodies in the SN pars compacta, many TH-IR neurons extending dendritic processes into the SN pars reticulata were seen on the intact side as well as ipsilaterally in rLV-GFP-treated animals (Fig. 4G and H), whereas in rLV-GDNF-injected animals the SN pars compacta seemed to contain fewer and more faintly stained TH-IR neurons (Fig. 4I). Quantification showed a significant reduction in the number of TH-IR nigral neurons on the treated side of rLV-GDNF animals ($85 \pm 4.9\%$) as compared with the intact side, or the rLV-GFP-treated side ($108 \pm 4.7\%$; $P < 0.05$ for both comparisons; Fig. 5B).

VMAT immunoreactivity

To further investigate if the reduction in TH mRNA and TH protein in the SN and striatum was because of a loss of DA terminals in the striatum and cell bodies in the SN or downregulation of TH protein, we processed serial sections for immunohistochemistry with an antibody directed against VMAT, another marker for nigrostriatal DA neurons

(Miller *et al.*, 1999). In contrast to TH, the striatal VMAT-IR innervation was equally dense on both sides of either treatment group (Fig. 4D–F). This was confirmed by optical density measurements, which did not differ significantly between the treated and intact side in either the rLV-GFP ($99 \pm 3.1\%$) or rLV-GDNF ($106 \pm 4.9\%$) group ($P > 0.05$ for all comparisons). Similarly, the number of VMAT-IR cells in the SN of both rLV-GFP- and rLV-GDNF-treated animals was identical to that on the intact side ($P > 0.05$; Fig. 5B).

D1- and D2 receptor and DA transporter binding autoradiography

Pre-terminal DA uptake sites are likely to participate in termination of DA signalling by removal of extracellular DA, and to determine if the number of binding sites were altered by GDNF overexpression, we performed binding autoradiography with [3 H]mazindole on striatal sections. Inspection of the autoradiographic films showed a strong binding in the striatum and nucleus accumbens (Fig. 6A) and the binding was symmetrical on the treated and untreated sides in either rLV-GFP- or rLV-GDNF-injected animals. This was confirmed by densitometry measurements in the rostral and caudal striatum where no significant difference was found between the rLV-GFP and rLV-GDNF treated groups at either rostro-caudal level ($P > 0.05$; two-way ANOVA; Fig. 6D). The density on the treated compared to the untreated side was $103 \pm 9.9\%$ in the rostral striatum, and $85 \pm 10\%$ in the caudal in animals receiving rLV-GFP. The corresponding percentages in rLV-GDNF-injected animals were $94 \pm 7.5\%$ and $82 \pm 5.5\%$ in the rostral and caudal sections, respectively.

Changes in postsynaptic D1 and D2 receptor binding has been reported following changes in DA transmission in the striatum and [3 H]SCH23390 and [3 H]spiperone binding autoradiography was therefore used to investigate if GDNF treatment induced compensatory changes in the number of D1 and D2 receptors, respectively. Prominent [3 H]SCH23390 binding was seen in the striatum and nucleus accumbens areas (Fig. 6B), as well as in the SN pars reticulata (data not shown). Comparison of density of D1 receptor binding on the ipsilateral side of rLV-GFP and rLV-GDNF treated animals did not show any significant difference in either the rostral or caudal striatum ($104 \pm 3.2\%$ vs. $91 \pm 3.6\%$, and $70 \pm 9.1\%$ vs. $93 \pm 5.5\%$, respectively; $P > 0.05$, two-way ANOVA; Fig. 6E). Furthermore, no significant difference in binding density was observed between the treated and nontreated side in either the GFP or the GDNF group ($P > 0.05$).

Similarly to the D1 receptor, a prominent binding of the D2 ligand [3 H]spiperone was found throughout the striatal complex as well as in the SN (Fig. 6C and data not shown). No significant difference in striatal [3 H]spiperone binding was found neither between the ipsilateral and contralateral sides of rLV-GFP- or rLV-GDNF-treated animals (Fig. 6F), nor between the treated sides of rLV-GFP- and rLV-GDNF-transduced animals, either rostrally ($86 \pm 7.2\%$ and $91 \pm 2.4\%$, respectively) or caudally ($73 \pm 11\%$ and $87 \pm 11\%$; $P > 0.05$ for all comparisons).

Preproenkephalin mRNA

Preproenkephalin mRNA levels have been found to increase in striatal projection neurons following removal of the DA input to the striatum (Gerfen *et al.*, 1991). To investigate whether reduced TH levels in the nigrostriatal DA neurons was perceived as a loss of DA tone by the postsynaptic neurons we performed *in situ* hybridization for PPE mRNA. Cells containing PPE mRNA were seen throughout the striatum (Fig. 7A–D). The distribution of silver grains was similar on the intact and treated side in both rLV-GFP- (Fig. 7A and B) and rLV-GDNF- (Fig. 7C and D) treated animals. This was confirmed by optical density readings of silver grain density from darkfield images

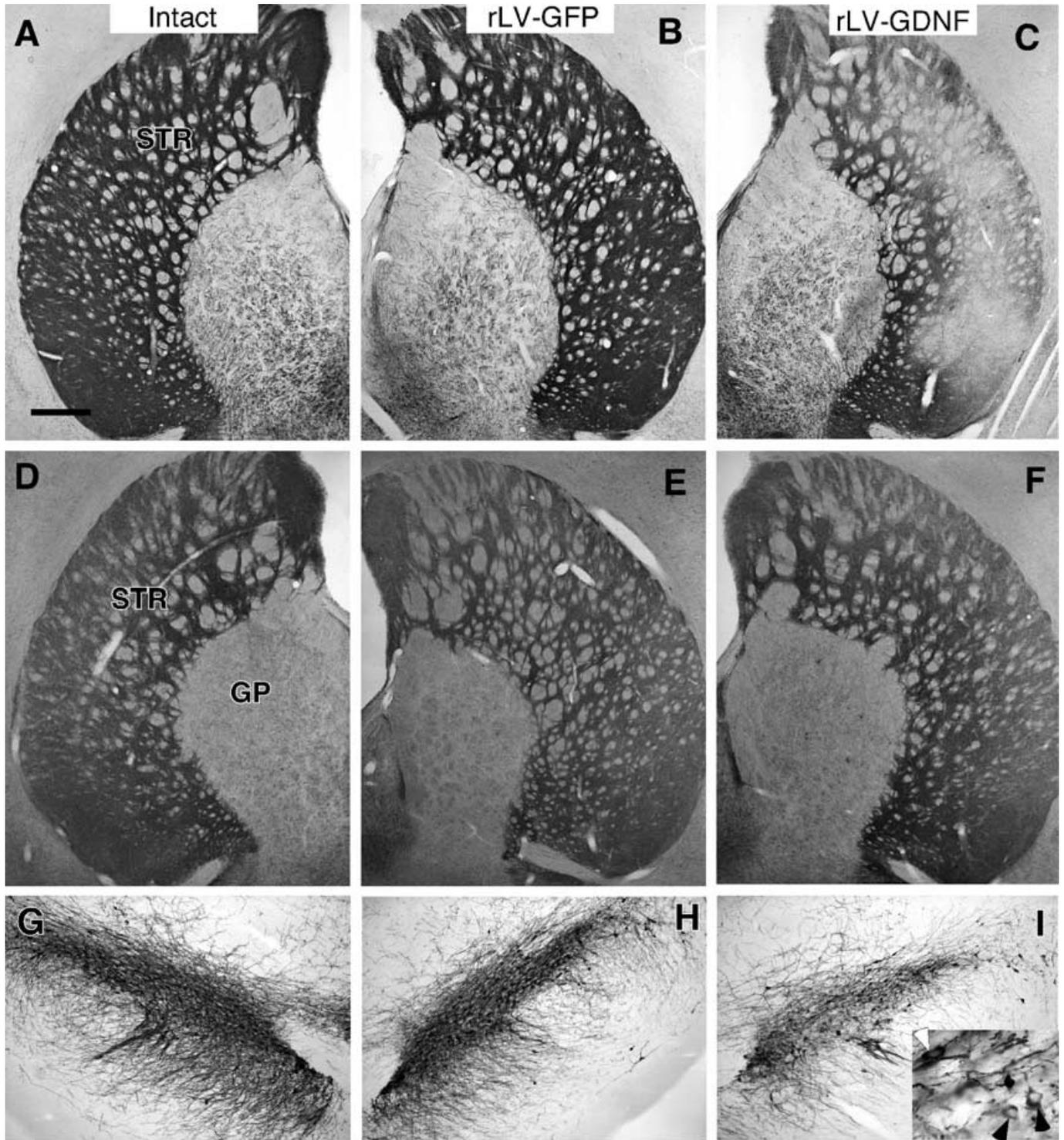


FIG. 4. Coronal sections through the striatum and substantia nigra of the intact (A, D and G), rLV-GFP-treated (B, E and H) and rLV-GDNF-treated (C, F and I) sides illustrating the extent of TH-IR and VMAT-IR innervation in the striatum (A–F) and cell bodies in the SN (G–I). Following GDNF treatment the striatal TH-IR innervation was markedly reduced (C) as compared with the intact (A), or control vector-injected (B) sides. The degree of downregulation varied within the structure and was most prominent in the caudal and lateral aspects. By contrast, the VMAT-IR innervation in adjacent sections was similar in intact (D), rLV-GFP- (E) and rLV-GDNF-injected animals (F). At low power the SN pars compacta of GDNF-treated animals appeared to contain fewer TH-IR neurons and dendrites extending into the SN pars reticulata (I) compared with the intact (G) or rLV-GFP-treated (E) side. At higher magnification (insert in I), the central portion of the SN pars compacta of GDNF-treated animals contain some neurons with reduced TH expression (filled arrowheads) whereas others retain a staining intensity similar to that seen on the intact side (open arrowhead). Scale bar, 600 μ m (A–F); 300 μ m (G–I). STR, striatum; GP, globus pallidum.

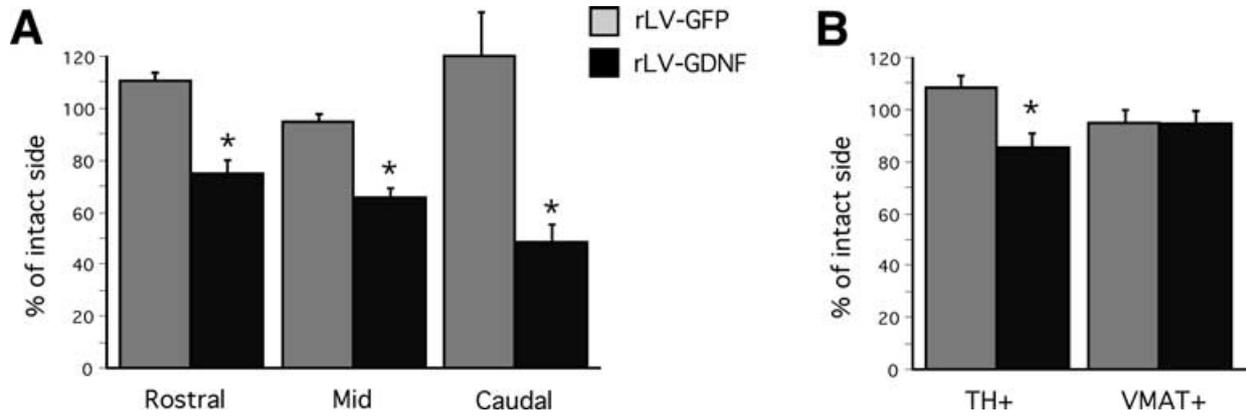


FIG. 5. (A) Quantification of the optical density of TH-IR innervation in the striatum. GDNF-treated animals showed a significant reduction at all three rostro-caudal levels examined (see Material and methods for details) but most prominently in the caudal aspects ($P < 0.01$, two-way ANOVA followed by *post hoc* Newman–Keuls; $n = 5$ /group). (B) Cell counts revealed a significant reduction in the number of TH-IR (TH+) neurons, but not VMAT-IR (VMAT+), neurons in the SN pars compacta of GDNF-treated animals as compared to rLV-GFP injected group ($P < 0.05$, Student's *t*-test; $n = 6$ /group). This indicates that the TH levels were reduced below the detection limit in a population of SN pars compacta cells. *Denotes significantly different from GFP-treated animals.

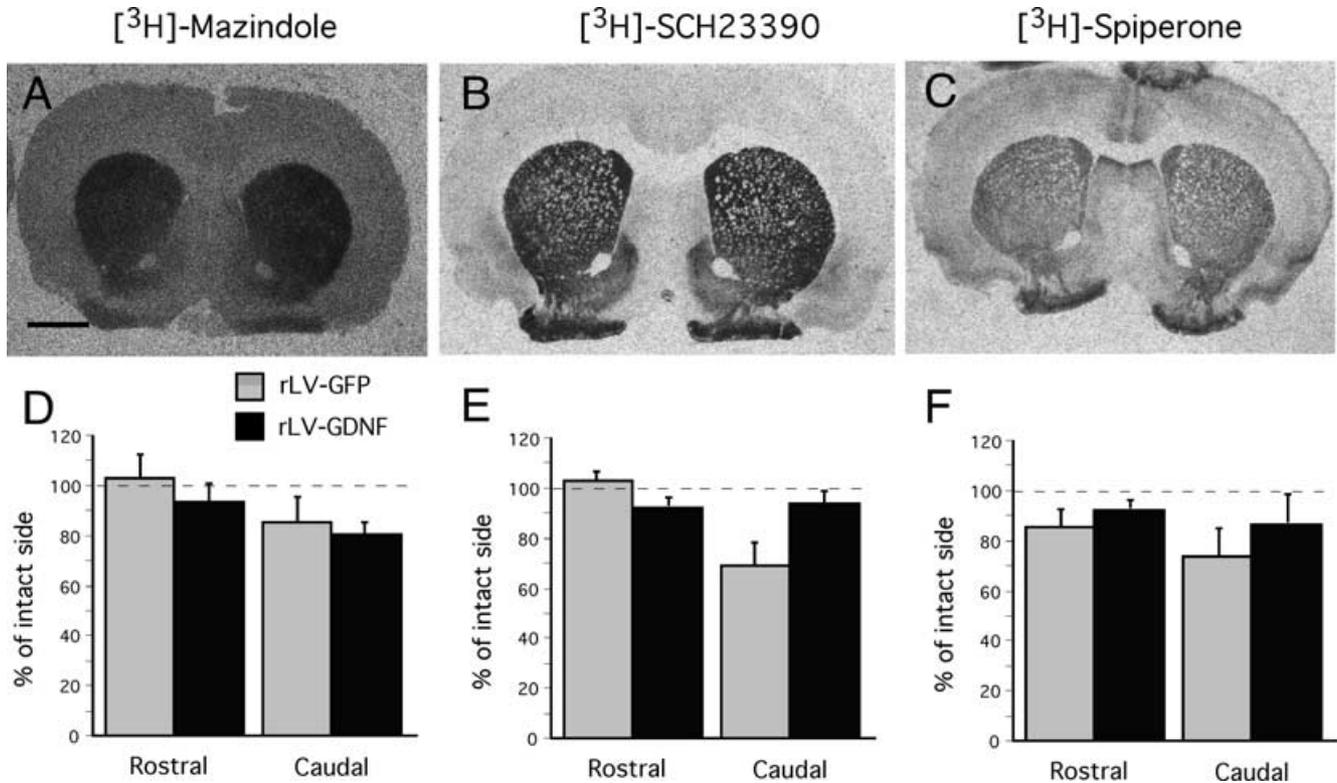


FIG. 6. Representative photomicrographs from autoradiographic films show binding of [³H]mazindole to presynaptic DA uptake sites (A), [³H]SCH23390 to postsynaptic D1 receptors (B) and [³H]spiperone to D2 receptors (C) in coronal sections through the striatum. Prominent specific binding was seen throughout the striatal complex in all animals, and no side difference was seen in either the rostral or caudal striatum following GDNF overexpression as determined by optical density measurements ([³H]mazindole, D; [³H]SCH23390, E; [³H]spiperone, F; $P > 0.05$, two-way ANOVA). Scale bar, 1500 μ m.

of emulsion dipped sections, which did not differ between and rLV-GFP- ($91 \pm 2\%$) and rLV-GDNF- ($96 \pm 1\%$) treated animals, respectively (Fig. 7E).

Discussion

To investigate the effects of long-term continuous GDNF administration on intact nigrostriatal DA neurons, we injected rLV-GDNF, or

rLV-GFP control vector, into the striatum of young adult rats. At 13 months post-transduction significantly elevated levels of GDNF (25- to 100-fold compared with the contralateral nontransduced side) were found both in the ipsilateral striatum, and SN (because of anterograde axonal transport and release from transduced striato-nigral projection neurons; Georgievska *et al.*, 2002) of rLV-GDNF-treated animals. As a result of the GDNF overexpression, cellular TH mRNA levels were reduced significantly, on average by 72% and 39% in the

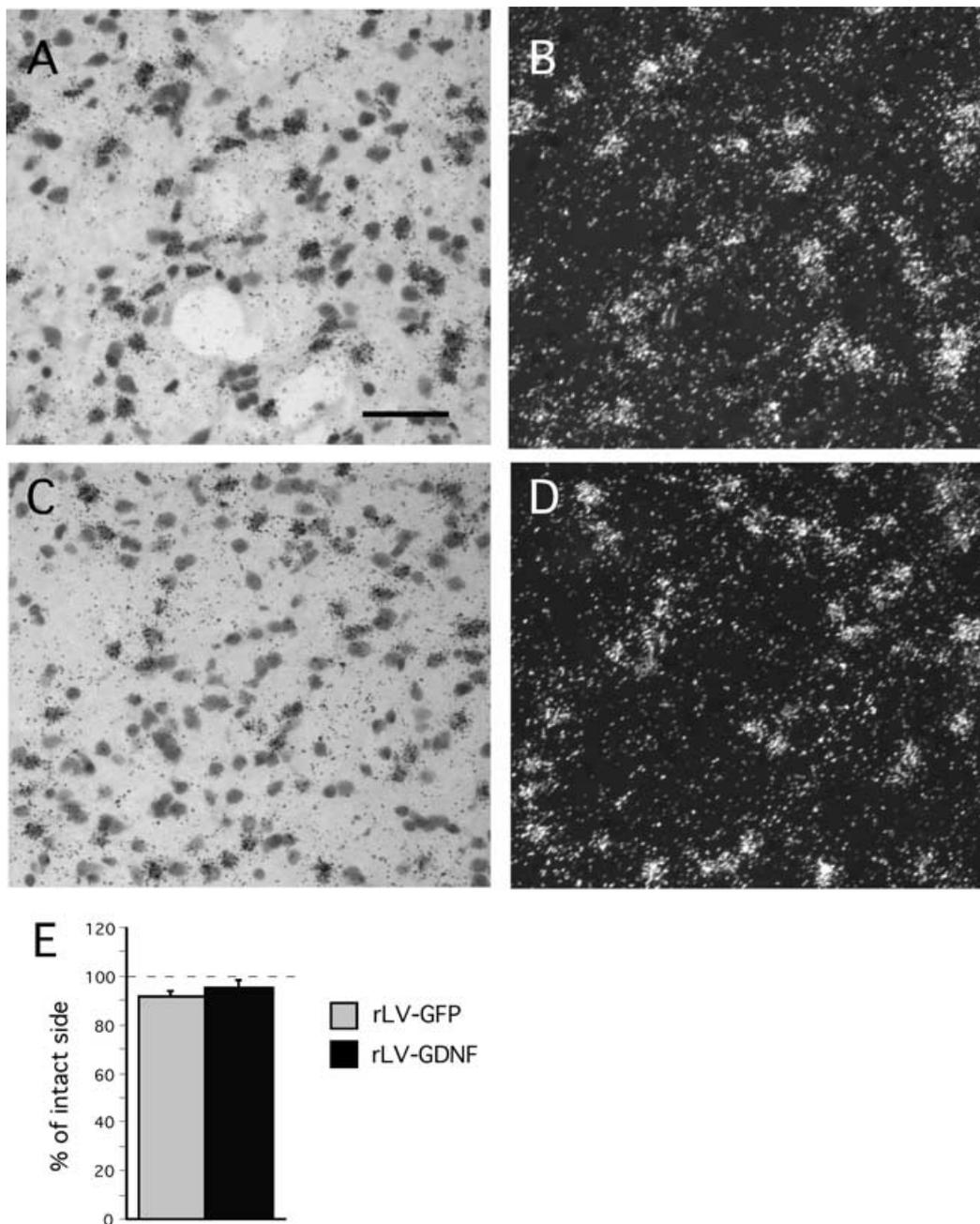


FIG. 7. *In situ* hybridization on coronal sections through the striatum using a probe specific for PPE mRNA (A–D). Bright-field photomicrographs show location of silver grains on top of neuronal profiles in the striatum consistent with expression in medium sized spiny projection neurons. Dark-field photomicrographs show a similar number of grains/cell on the rLV-GFP- and rLV-GDNF-treated sides. (E). The cellular PPE mRNA levels as measured by optical density of silver grains in dark-field images did not differ significantly from the intact side in either rLV-GDNF- or rLV-GFP-treated animals ($P > 0.05$, Student's *t*-test; $n = 5$ /group). Scale bar in (A), 50 μ m.

SN pars compacta and VTA neurons, respectively, and on the ipsilateral side the TH-IR were reduced both in the axon terminals in the striatum (–5% to –52% with the most prominent reductions appearing in the caudal aspects of the striatum) as well as in the TH-IR cell bodies in the SN (–15% TH-IR neurons). The changes in TH mRNA and TH-IR were not accompanied by any compensatory changes in a number of other DA transmission-associated markers such as VMAT-IR axon terminal density or cell number, [3 H]mazindole binding to striatal DA uptake sites, D1 and D2 receptor binding sites, or the levels of PPE mRNA in the striatum.

To rule out the possibility that the reduction in TH mRNA and TH-IR was because of a loss of nigrostriatal terminals we investigated the density of VMAT-IR and [3 H]mazindole binding in striatal sections. Both the VMAT and the DA transporter are located preterminally on DA axons in the striatum (Hersch *et al.*, 1997; Miller *et al.*, 1999), and have been shown to disappear progressively as the DA innervation is lost in patients with Parkinson's disease (Miller *et al.*, 1997; Miller *et al.*, 1999). The absence of changes in striatal VMAT-IR and [3 H]mazindole binding following GDNF treatment therefore strongly suggest that TH had been selectively downregulated in DA neurons

whereas the number of DA neurons in the SN and their axon projections to the striatum remained intact. Importantly, the normal appearance of VMAT and [³H]mazindole binding also support the impression from TH-stained sections through the nigrostriatal pathway that aberrant sprouting, which has been observed following GDNF treatment of damaged neurons (Kirik *et al.*, 2000a; Kirik *et al.*, 2000b; Georgievska *et al.*, 2002), does not occur in the intact DA system, at least not with the GDNF doses and mode of treatment used here. In line with our findings that GDNF can downregulate TH, Lu & Hagg (1997) reported that continuous unilateral infusion of 3 µg/day over the SN for 14 days reduced the number of TH-IR neurons in the SN to 70% of that on the intact side, whereas no side difference was detectable in the number of retrogradely DiI-labelled neurons (independent of TH), and Messer *et al.* (1999) found that injection of 10 µg GDNF into the SN/VTA area of mice significantly reduced the activity of the TH promoter and subsequent TH mRNA expression by 40–65% at 7 days after injection. Contrary to these findings, a recent study in aged monkeys (Palfi *et al.*, 2002) reports an increased number of striatal TH-IR neurons following rLV-GDNF treatment, indicating a GDNF-induced upregulation of TH. However, in this study the increase in TH-IR was paralleled by an increased number of DA transporter-IR neurons. This could in fact reflect induction of a DA phenotype in striatal neurons and GDNF might, rather than directly enhancing TH (and DA transporter) expression, activate a programme in the striatal neurons that make them responsive to DA phenotype inductive cues present in the striatum. This property could be unique to a subset of intrastriatal DA neurons and it might therefore be difficult to extrapolate these findings to the situation in intact nigrostriatal neurons. Moreover, in this study (Palfi *et al.*, 2002) an increase in TH-IR cell number was seen also following removal of nigrostriatal DA innervation by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and was further enhanced by rLV-GDNF treatment, corroborating the notion that this phenomenon is not directly dependent upon, but might be facilitated by, GDNF administration.

The mechanism behind the GDNF-induced TH downregulation remains unclear but our findings are consistent with a model where GDNF enhances the activity of nigrostriatal DA neurons, which in turn leads to a compensatory downregulation in TH in order to return the dopamine activity in these neurons back to normal levels. The level of TH mRNA and protein are regulated very intricately at several points from transcription of the TH gene to protein phosphorylation (Kumer & Vrana, 1996) and negative feedback regulation following stimulation of DA receptors has been reported. For example, decreased levels of TH mRNA and TH protein was found after sustained, but not intermittent, pharmacological overstimulation of the DA receptors by administration of the DA receptor agonist apomorphine (Iwata *et al.*, 2000). Therefore, it is intriguing that there is strong electrophysiological, biochemical and functional evidence that GDNF facilitates DA transmission shortly after GDNF administration in intact DA neurons. Recordings from DA neurons have shown that the spontaneous firing rate and the quantal size of terminal DA release is increased following GDNF exposure (Pothos *et al.*, 1998; Yang *et al.*, 2001). Second, the levels of DA in the striatum and/or SN are transiently increased for 1–3 weeks following a single GDNF injection (Hudson *et al.*, 1995; Beck *et al.*, 1996; Martin *et al.*, 1996). Increased *in vivo* TH activity (as measured by L-dopa accumulation) and DA turnover have also been found when GDNF was overexpressed in the nigrostriatal system using a recombinant adeno-associated virus vector (Kirik *et al.*, 2000b). Finally, functional studies show that following unilateral GDNF administration to the intact nigrostriatal DA system animals develop a turning bias toward the contralateral side in response to amphetamine, consistent with an overactivity in the dopamine system on the

GDNF-treated side (Hudson *et al.*, 1995; Kirik *et al.*, 2000b; Georgievska *et al.*, 2002). It is thus possible that GDNF administration acutely increases the DA release from DA terminals, leading to overactivation of pre- and postsynaptic DA receptors, which in turn activates a negative feedback regulation of TH in the nigrostriatal neurons. If the change in TH levels is an adaptive response to GDNF induced over-activity in the DA system, the tissue levels of DA should remain similar on the treated and nontreated sides in animals with sustained unilateral GDNF treatment. Indeed, in a parallel study (Georgievska *et al.*, unpublished data) it was found that sustained striatal GDNF overexpression from an rLV vector similar to the present study led to a time-dependent downregulation of TH protein levels and TH activity over 6 weeks, whereas striatal DA levels quickly return to normal and are maintained over the following months. This could suggest that storage and/or turnover of DA have been enhanced by the GDNF so that less TH is required to maintain normal DA levels in the striatum.

Similar to the observations of presynaptic axon terminal density as measured by VMAT immunoreactivity and [³H]mazindole binding, the levels of postsynaptic striatal D1 and D2 receptor binding and PPE mRNA were unchanged following GDNF treatment. In animals models of Parkinson's disease where striatal DA signalling has been disrupted by removal of DA axon terminals through 6-hydroxydopamine lesions, or pharmacologically by application of DA receptor antagonists, enkephalin protein and mRNA levels (Tang *et al.*, 1983; Gerfen *et al.*, 1991) as well as D2 receptor binding (Creese *et al.*, 1977) have been reported to increase. Similar denervation-induced changes have also been seen at post mortem analysis of brains from patients with Parkinson's disease (Piggott *et al.*, 1999; Calon *et al.*, 2002). Although an increased DA release from the remaining axon terminals might compensate for those lost in a partially denervated striatum (Chritin *et al.*, 1996), the normal innervation density, as shown by striatal VMAT immunoreactivity and [³H]mazindole binding, and the unchanged levels of postsynaptic components of DA signalling in the present study are consistent with the notion that GDNF-induced reductions in TH occur without impaired striatal DA signalling. However, we can not from the present data rule out the possibility that by increasing the GDNF levels further, one would be able to achieve more dramatic reductions in TH levels, which could in turn lead to changes in DA receptor binding or PPE mRNA.

Conclusions

The current study provides evidence for prominent downregulation of TH in nigrostriatal DA neurons as a result of long-term overexpression of GDNF in the striatum. As new therapeutic strategies based on sustained GDNF treatment progress towards evaluation in patients with Parkinson's disease (Bjorklund & Lindvall, 2000; Bjorklund *et al.*, 2000), it becomes increasingly important to further address the functional and morphological consequences of this mode of treatment on the intact neurons. In the present study the level of GDNF expressed from the rLV vector was 4.5 ng/mg tissue, which is more than 20-fold higher than the 0.2 ng/mg tissue that has been considered the threshold for neuroprotection in the nigrostriatal DA system (Bjorklund *et al.*, 2000). It is therefore possible that one can attenuate the TH reduction by regulating the amount of GDNF administered. One should also note that the present data do not allow us to discriminate between the relative contribution of GDNF in the striatum and SN to these findings as GDNF produced by the transduced striatal neurons is also transported to the SN (Georgievska *et al.*, 2002). Future studies will have to address the influence of dose and site of administration for the GDNF-induced TH downregulation.

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Abbreviations

DA, dopamine(rgic); GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; IR, immunoreactive; PBS, phosphate buffered saline; PPE, preproenkephalin; rLV, recombinant lentiviral vector; SN, substantia nigra; TH, tyrosine hydroxylase; VMAT, vesicular monoamine transporter-2; VTA, ventral tegmental area.

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