

GDNF Is Predominantly Expressed in the PV + Neostriatal Interneuronal Ensemble in Normal Mouse and after Injury of the Nigrostriatal Pathway

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Glial cell line-derived neurotrophic factor (GDNF) is absolutely required for survival of dopaminergic (DA) nigrostriatal neurons and protect them from toxic insults. Hence, it is a promising, albeit experimental, therapy for Parkinson's disease (PD). However, the source of striatal GDNF is not well known. GDNF seems to be normally synthesized in neurons, but numerous reports suggest GDNF production in glial cells, particularly in the injured brain. We have studied in detail striatal GDNF production in normal mouse and after damage of DA neurons with MPTP. Striatal *GDNF* mRNA was present in neonates but markedly increased during the first 2–3 postnatal weeks. Cellular identification of GDNF by unequivocal histochemical methods demonstrated that in normal or injured adult animals GDNF is expressed by striatal neurons and is not synthesized in significant amounts by astrocytes or microglial cells. *GDNF* mRNA expression was not higher in reactive astrocytes than in normal ones. Approximately 95% of identified neostriatal GDNF-expressing cells in normal and injured animals are parvalbumin-positive (PV+) interneurons, which only represent ~0.7% of all striatal neurons. The remaining 5% of GDNF+ cells are cholinergic and somatostatin+ interneurons. Surprisingly, medium spiny projection neurons (MSNs), the vast majority of striatal neurons that receive a strong DA innervation, do not express GDNF. PV+ interneurons constitute an oscillatory functional ensemble of electrically connected cells that control MSNs' firing. Production of GDNF in the PV+ neurons might be advantageous to supply synchronous activity-dependent release of GDNF in broad areas of the striatum. Stimulation of the GDNF-producing striatal PV+ ensemble in PD patients could have therapeutic effects.

Introduction

The glial cell line-derived neurotrophic factor (GDNF) is a potent dopaminotrophic agent that promotes survival, differentiation, and phenotype of neurons. GDNF protects dopaminergic nigrostriatal neurons from neurotoxins in rodents and primates, and induces fiber outgrowth when administered into the brain parenchyma (Tomac et al., 1995; Gash et al., 1996; Kordower et al., 2000). GDNF has also a neuroprotective effect on noradrenergic neurons of the rodent locus ceruleus (Arenas et al., 1995; Pascual

et al., 2008). Based on these data, stimulation of endogenous GDNF production and/or exogenous GDNF administration are considered promising therapeutic strategies for Parkinson's disease (PD) (Kirik et al., 2004; Pascual et al., 2008). Indeed, the clinical effectiveness of intrastriatal infusion of recombinant GDNF has been evaluated in open (Gill et al., 2003) and double-blind (Lang et al., 2006) clinical trials. Intrastriatal transplantation of GDNF-producing cells has also been tested in parkinsonian animals (Akerud et al., 2001; Toledo-Aral et al., 2003) and humans (Arjona et al., 2003; Mínguez-Castellanos et al., 2007).

Clinical applicability of GDNF, still in an experimental stage, will surely benefit from refinement of the method of delivery in parallel with advances in the knowledge of GDNF function in the normal and injured brain (Check, 2007; Vastag, 2010). In this regard, the recent demonstration that adult nigrostriatal dopaminergic neuron survival is absolutely dependent on striatal GDNF production should stimulate future therapeutic developments in the field (Ibáñez, 2008; Pascual et al., 2008). However, the actual source of striatal GDNF is, surprisingly, not known in detail. GDNF is present in several parts of the brain (mainly striatum, thalamus, and septum) and is normally expressed in neurons (Pochon et al., 1997; Trupp et al., 1997), but the nature of striatal neurons responsible for GDNF synthesis is not fully characterized (Bizon et al., 1999). Moreover, *GDNF* mRNA is detected in cultured astrocytes and microglia, but whether the trophic factor is produced in relevant amounts *in vivo* by these cell types has not

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Table 1. GDNF expression in neonatal (P0) and adult (P55) mouse brain

| Structure | Postnatal day | |
|--------------------------|---------------|-------------|
| | Neonatal (P0) | Adult (P55) |
| Olfactory bulb | + | + |
| Septum | ++ | +++ |
| Striatum | + | +++ |
| Accumbens nucleus, core | +++ | +++ |
| Accumbens nucleus, shell | – | +++ |
| Striatal–pallidal border | +++ | ++ |
| Thalamus | – | +++ |
| Hypothalamus | ++ | + |
| Substantia nigra | – | – |
| Subcommissural organ | – | +++ |
| Interpeduncular nucleus | +++ | – |
| Retrobulbar field | ++ | ++ |
| Oculomotor nucleus | +++ | ++ |
| Motor trigeminal nucleus | +++ | – |
| Locus ceruleus | + | + |

The study is based on seven brains from *GDNF-LacZ* mice analyzed per age and structure. –, No X-gal-positive cell found; +, 1–10 GDNF-positive cells/analyzed structure; ++, 10–50 GDNF-positive cells/analyzed structure; +++, >50 GDNF-positive cells/analyzed structure.

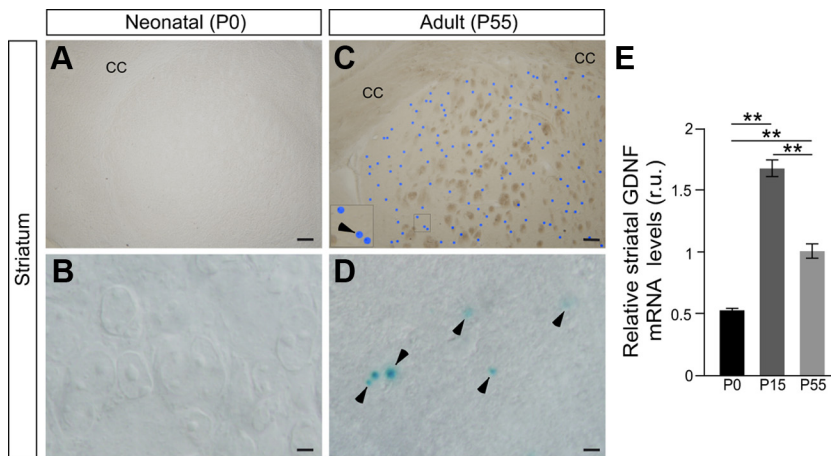


Figure 1. GDNF expression in neostriatum of neonatal and adult mice. **A–D**, GDNF promoter activity estimated by *in situ* β -galactosidase enzymatic detection from *GDNF-LacZ* mouse brain slices. Blue X-gal precipitate is indicated by arrows. Coronal sections through the neonatal (P0) (**A**, **B**) and adult (P55) (**C**, **D**) striatum are shown at low (**A**, **C**) and high (**B**, **D**) magnification. Scale bars: **A**, **C**, 100 μ m; **B**, **D**, 20 μ m. To make visible GDNF expression at low magnification (**C**) a blue dot was drawn over each X-gal-positive cell (see inset). **E**, Relative striatal GDNF mRNA levels estimated by qRT-PCR in neonatal (P0, black bar), juvenile (P15, dark gray bar), and adult (P55, light gray bar) mice. Data (relative to P55) are represented as mean \pm SEM ($n = 8$ for each age group). ** $p < 0.01$.

been precisely evaluated (Saavedra et al., 2008). It has been reported that GDNF expression increases under brain injury due to recruitment of GDNF-synthesizing glial cells (astrocytes and microglia), which may eventually become the predominant source of the trophic agent (Hughes et al., 1999; Bresnahan and Antauer, 2000). Nonetheless, this concept is under debate and the actual role of reactive glia in GDNF production *in vivo* is unsettled (Saavedra et al., 2008).

The objective of this work was to investigate brain GDNF expression in mice, with special emphasis in the identification of the striatal cell (neuron or glia) population producing GDNF. We have studied several genetically modified mouse models to precisely identify the sites of GDNF synthesis. These mice were used in combination with MPTP-treated animals to evaluate *in vivo* GDNF production by reactive glia. Our data show an unexpected pattern of striatal GDNF expression, linked to the organization and function of the GABAergic synchronous interneuron ensemble. The findings suggest that

stimulation of endogenous GDNF-secreting cells could be a feasible methodology of potential clinical relevance.

Materials and Methods

Mice (C57BL/6N strain) were housed under temperature-controlled conditions (22°C) in a 12 h light/dark cycle with access *ad libitum* to food and water. All experiments were performed according to institutional guidelines approved by the ethics committee of the Hospital Universitario Virgen del Rocío and the European Community (Council Directive 86/609/EEC). Heterozygous *GDNF-LacZ* mice of either sex and outcrossed to C57BL/6N background for >10 generations (Sánchez et al., 1996) were used for analysis of the pattern of GDNF expression in neonates (P0; i.e., up to 24 h after birth), and at P8, P17, and adulthood (2 months old). GDNF promoter activity was estimated by β -galactosidase enzymatic detection in brains from heterozygous *GDNF-LacZ* mice (Sánchez et al., 1996; Villadiego et al., 2005). Transgenic *GDNF-EGFP* mice of either sex were obtained from GENSAT on a mixed background (Gong et al., 2003). Animals were killed either by administration of chloral hydrate anesthesia (490 mg/kg) (adult mice) or by decapitation (neonatal animals).

Histological analyses. Mouse brain was removed from anesthetized animals and immediately fixed overnight (15 h; adult mice) or 4 h (neonatal mice) at 4°C with 4% paraformaldehyde in PBS. Brains included in gelatin were cut in 50- μ m-thick coronal sections using a vibratome (Leica). For the histological detection of GDNF expression, we used heterozygous *GDNF-LacZ* mice (Sánchez et al., 1996) and the 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal)-staining, or *GDNF-EGFP* mice (Gong et al., 2003) and immunohistochemistry for GFP. In either case, a wild-type animal was used as a negative control in all the experiments. For colocalization studies, immunohistochemistry was performed with the appropriate antibody after X-gal staining or GFP immunohistochemistry. The Envision+ kit (Dako) was used for chromatic immunohistochemistry according to the recommended manufacturer's protocol. Primary antibodies used were as follows: anti-NeuN (1:200; Millipore Bioscience Research Reagents); anti-choline acetyltransferase (ChAT) (1:1000; Millipore Bioscience Research Reagents); anti-SS (1:800; Millipore); anti-GFP (monoclonal, 1:100; Roche); anti-IBA1 (1:500; Wako); anti-tyrosine hydroxylase (TH) (1:5000; Novus Biologicals); anti-PV28 (1:6000; Swant); anti-gial fibrillary acid protein (1:500; Dako); anti-GFP (polyclonal, 1:1000; Invitrogen); anti-DARPP32 (1:200, Millipore; and 1:50, Santa Cruz Biotechnology); anti-calbindin (CB) (1:10,000; Swant); and anti-CR (1:8000; Swant). For GFP detection in colocalization studies, we used either mouse (monoclonal) or rabbit (polyclonal) anti-GFP antibodies. In general, the latter provided a stronger signal. Secondary antibodies anti-rabbit or anti-mouse were added in a second step and the reaction was developed with 3,3'-diaminobenzidine (Dako). For the immunofluorescent studies, we used secondary antibodies anti-mouse IgG or anti-rabbit conjugated with Alexa Fluor 488 or Alexa Fluor 568 (1:800; Invitrogen).

Stereological cell counts. Quantification of GDNF expression in brains of MPTP-treated and control heterozygous *GDNF-LacZ* mice was done by counting the total number of cells containing X-gal dots. We counted parvalbumin (PV)-, somatostatin (SS)-, and ChAT-immunopositive neurons in 50- μ m-thick coronal sections with a vibratome using the C.A.S.T. (Computer Assisted Stereological Toolbox) system (Olympus). Cells positive for both markers (immunohistochemistry and X-gal) were estimated in the caudate–putamen nucleus following Franklin and Paxinos (2008), using 205–273 ran-

dom 14,260 μm^2 dissectors. Values are given as mean \pm SEM and relative to the total proportion of each cell type in the striatum (Tepper and Bolam, 2004).

Acute MPTP treatment. Heterozygous, 2-month-old, wild-type *GDNF-LacZ* or *GFAP-EGFP* mice were rendered parkinsonian by a single subcutaneous administration (40 mg/kg) of MPTP (Sigma). Mice were killed 7, 14, or 21 d after the lesion, and their brains were processed for immunohistochemistry, cell sorting, or real-time PCR analysis.

Quantitative real-time PCR. Total striatal levels of GDNF mRNA were determined by quantitative real-time PCR (qRT-PCR) in controls (P0, P15, and adult mice) and MPTP-injured mice (7, 14, and 21 d after lesion). Striatal RNA was extracted using 300 μl of TRIzol reagent (Invitrogen) in a homogenizer (Omni TH). RNA samples (0.5 μg) were treated with RNase-free DNase (GE Healthcare) and copied to cDNA using SuperScriptII reverse transcriptase (Invitrogen) in a final volume of 20 μl . Real-time PCR was performed in an Applied Biosystems Prism 7500 Sequence Detection System using TaqMan Gene Expression Master Mix (Applied Biosystems). In the case of mice with MPTP treatment, *Actb* and *Gapdh* RNA levels were estimated to normalize for RNA input amounts. A Taqman probe for *hydroxymethylbilane synthase (HMBS)* gene was used to normalize RNA levels in P0, P15, and adult mice. This gene has been shown to be a bona fide unvaried transcript for quantitative PCR studies of brain genes with low expression levels (Sieber et al., 2010). Two different TaqMan probes were used for estimation of *GDNF* mRNA levels (exon 1–2 and exon 2–3), and the values obtained were pooled together. To normalize mRNA levels in treated/aged mice to control samples, we calculated an average cycle threshold of the control samples and processed all the samples in the experiment relative to this average cycle threshold. Primers and Taqman sequences are available upon request.

Isolation of GFAP+ striatal cells. Briefly, we dissected the striatum from the brain of *GFAP-EGFP* mice (Zhuo et al., 1997) without lesion and 7, 14, or 21 d after MPTP administration. The dispersed striatal cells were resuspended in medium containing (for 50 ml): 44 ml L15 medium (Invitrogen), 0.5 ml of penicillin/streptomycin (Cambrex), 0.1 g of BSA (Sigma), 0.05 ml of HEPES (Invitrogen), and 5 ml of distilled and deionized water. Dead cells (7-AAD+) were eliminated from sorts and analyses. GFP-positive and -negative striatal subpopulations were isolated in a MoFlo three-laser flow cytometer (Dako and Cytomation) and processed for qRT-PCR analysis.

Statistics. Data are presented as the mean \pm SEM and were analyzed with one-way ANOVA followed by Tukey's test. A p value <0.05 was considered statistically significant.

Results

Postnatal changes of GDNF expression in mouse brain

We evaluated the level of GDNF expression in neonatal (P0) and adult (P55) animals using a semiquantitative methodology, as determined by the number of X-gal-positive deposits in different brain areas of the *GDNF-LacZ* mouse. The activity of the *GDNF* promoter was estimated using β -galactosidase as reporter (Sánchez et al., 1996; Toledo-Aral et al., 2003).

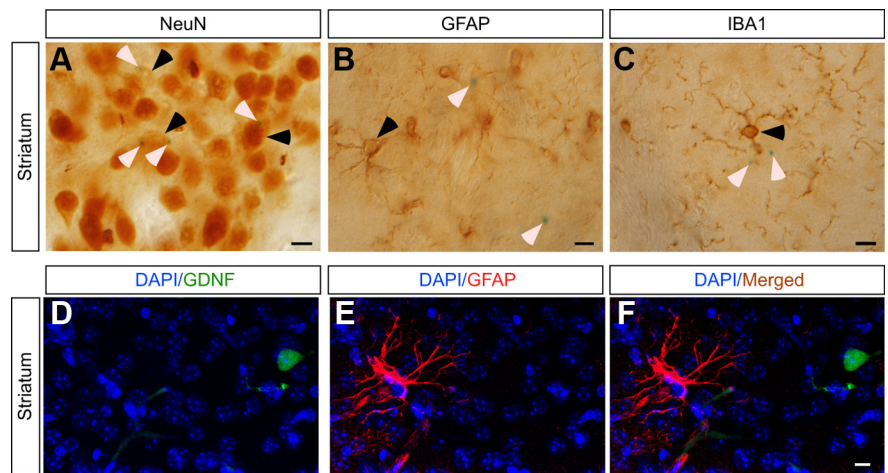


Figure 2. Selective neuronal GDNF expression in adult mouse striatum. **A–C**, Striatal coronal sections from *GDNF-LacZ* mouse showing GDNF expression (X-gal staining, blue dots indicated by white arrowheads) and neurons (**A**, NeuN+), astrocytes (**B**, GFAP+), and microglia (**C**, IBA1+) cell types (black arrowheads). **D–F**, Confocal microscopy Z-stacks of striatal coronal sections from *GDNF-EGFP* mouse. GDNF expression is revealed with an antibody against GFP (**D**, green), and astrocytes are detected by GFAP immunofluorescence (**E**, red). Nuclei are shown in blue (DAPI staining), and a merged image is shown in **F**. Scale bars, 20 μm .

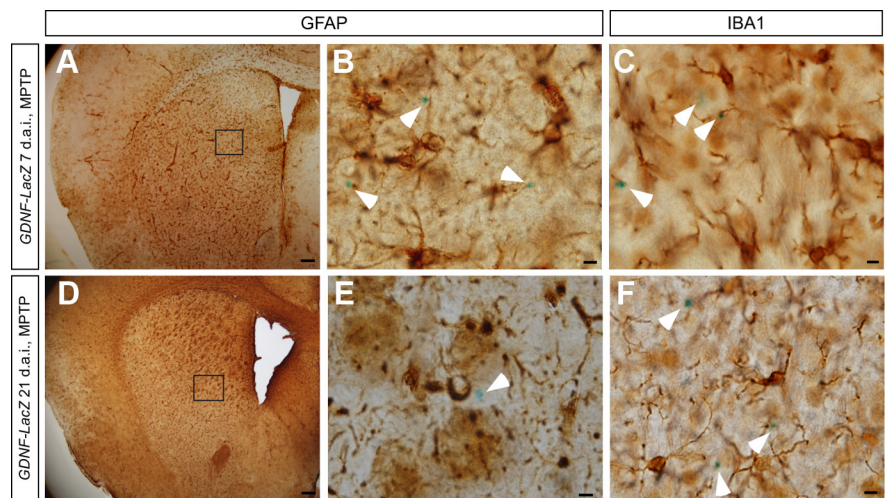


Figure 3. GDNF expression after MPTP treatment is restricted to striatal neurons. **A, D**, Low magnification of coronal brain sections showing a strong striatal astrogliosis in MPTP-treated animals 7 (**A**) and 21 (**D**) d after injury (d.a.i.). **B, E**, Striatal areas inside the squares in **A** and **D** are shown at higher magnification to demonstrate that the X-gal+ cells (blue dots indicated by arrowheads) are not GFAP+ astrocytes. **C, F**, Striatal IBA1-positive cells do not express GDNF (X-gal staining, blue dots indicated by arrowheads) in MPTP-lesioned (7 and 21 d.a.i.) animals. LV, Lateral ventricle. Scale bars: **A, D**, 200 μm ; **B, C, E, F**, 10 μm .

chez et al., 1996; Toledo-Aral et al., 2003). In this animal model, previously used by our group to characterize brain regions with strong GDNF mRNA and protein expression (Pascual et al., 2008), β -galactosidase activity is only found in the sites of GDNF expression in adult brain, as described by using radioactive *in situ* hybridization (Trupp et al., 1997). We observed a clear change of brain GDNF expression pattern with postnatal maturation (Table 1), thus indicating that in addition to its role in organ and PNS development, GDNF acquires new neurotrophic functions in adult life. Interestingly, GDNF expression was practically absent from neonatal caudate-putamen (Fig. 1*A, B, E*), although a significant increase of *GDNF* mRNA level was observed soon (P15) during postnatal maturation (Fig. 1*E*). In young adults (P55), cells expressing GDNF were sparsely, but rather uniformly, distributed in the neostriatum (Fig. 1*C, D*). These observations further demonstrate that GDNF is highly expressed in the adult

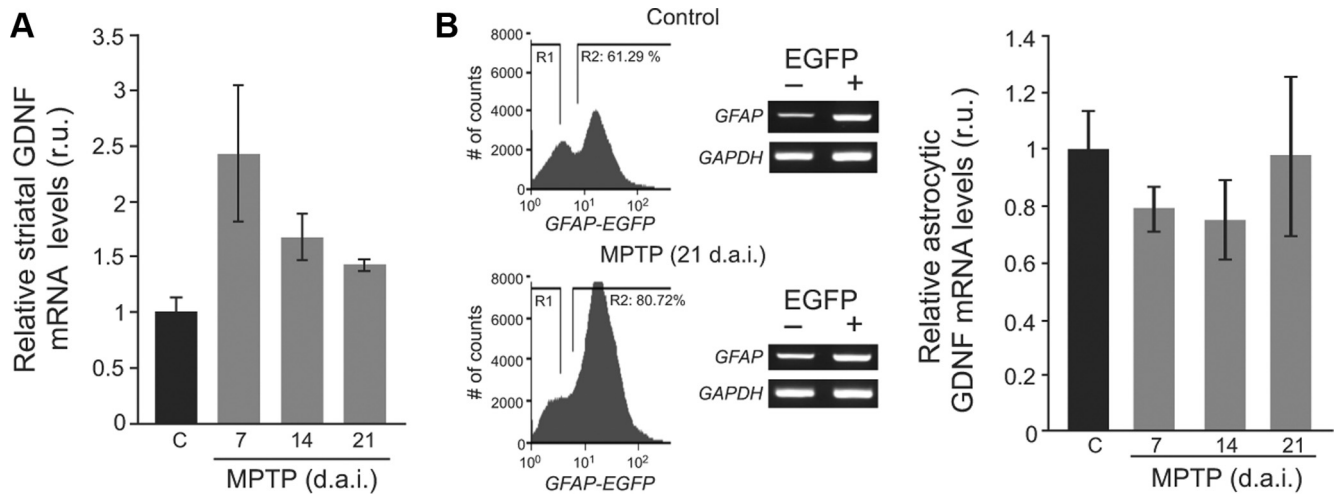


Figure 4. Striatal astrocytic *GDNF* mRNA expression does not increase after MPTP treatment. **A**, Striatal *GDNF* mRNA levels estimated by qRT-PCR from total striatum of control (black bar) and MPTP-treated wild-type mice [gray bars; 7, 14, and 21 d after injury (d.a.i.)]. No significant differences were observed ($p = 0.051$, one-way ANOVA, Tukey's test). **B**, Left, Flow cytometry diagrams showing astrocyte separation by cell sorting from striata of untreated (control) and MPTP-treated (21 d.a.i.) *GFAP-EGFP* mice. The diagrams show the increase of *GFAP-EGFP*-positive cells in the striatum after MPTP treatment (R2 increased from 61.29 to 80.72%). Center, *GFAP* mRNA is enriched in the isolated population (EGFP+) as shown by RT-PCR. The *GAPDH* gene was used as a loading control. Right, Astrocytic *GDNF* mRNA levels estimated by qRT-PCR from isolated EGFP+ cells of untreated (black bar) and MPTP-treated (gray bars; 7, 14 and 21 d.a.i.) *GFAP-EGFP* mice. Data [relative units (r.u.) to untreated group] are represented as mean \pm SEM; $n = 4-8$.

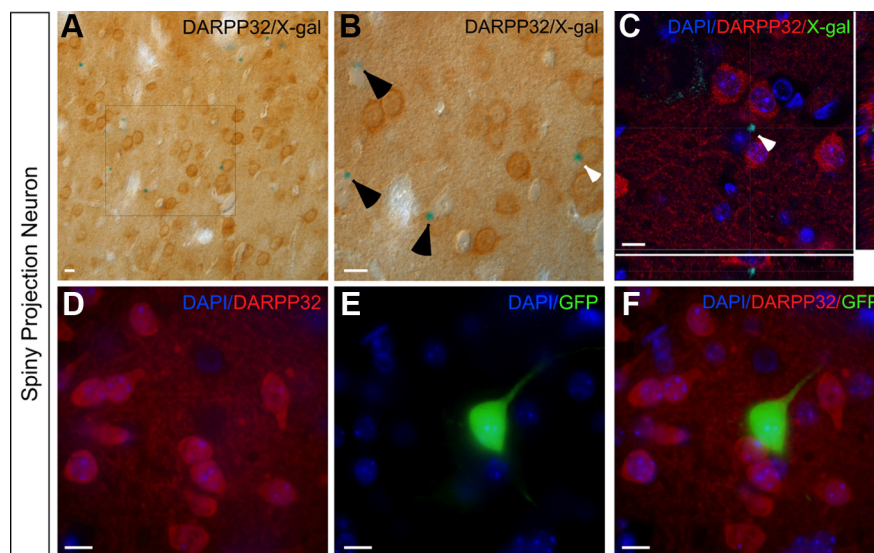


Figure 5. GDNF is not expressed in striatal medium spiny projection neurons. **A**, Striatal coronal sections from *GDNF-LacZ* mouse showing absence of GDNF expression (X-gal staining, blue dots) in MSNs (DARPP32+). **B**, High magnification of **A**. The white arrowhead points to a blue dot in close apposition to a DARPP32+ neuron. **C**, Confocal microscopy Z-stacks showing that DARPP32+ MSNs (red) were not X-gal+ (green). **D-F**, Striatal slices from *GDNF-EGFP* mouse stained with fluorescent antibodies against DARPP32 and GFP (polyclonal). Note the lack of overlapping between the two signals. The planes of **C** and **D** were slightly adjusted to make the cells appear into focus.

GDNF expression in normal and injured adult striatum is confined to neurons and is practically absent from glial cells

To ascertain the cell types expressing GDNF in the normal brain, we stained brain sections from *GDNF-LacZ* animals with antibodies against neuronal (NeuN), astroglial (GFAP), and microglial (IBA1) markers. Of >150 striatal X-gal deposits analyzed (14 sections from three animals), all were found in neurons. In striatal sections stained with antibodies against either GFAP (33 sections from 4 animals) or IBA1 (26 sections from 6 animals), we have never detected any X-gal deposit in these types of glial cells. This typical distribution was found in the striatum (Fig. 2A–C) as well as in other brain areas (thalamus and septum) expressing GDNF (data not shown). The lack of GDNF in normal neostriatal astrocytes was further confirmed using brain sections from *GDNF-EGFP* mice (Gong et al., 2003) costained with antibodies against GFAP (Fig. 2D–F). These data indicated that in normal brain, GDNF is mostly confined to neurons and is absent from or expressed at low levels in glial cells.

striatum (Trupp et al., 1997; Bizon et al., 1999; Pascual et al., 2008), and support its neurotrophic function on the dopaminergic nigrostriatal and mesolimbic pathways. During embryonic development, GDNF is not necessary for maintenance of mesencephalic dopaminergic neurons, as these cells are unaffected in GDNF-null animals (Moore et al., 1996; Sánchez et al., 1996). The neurotrophic role of GDNF seems to be acquired later in life, probably once GDNF-producing cells are phenotypically matured in parallel with the postnatal development of the dopaminergic nigrostriatal system (Burke, 2003) (see Identification of GDNF-expressing striatal neurons, below).

As it has been proposed that GDNF production increases upon brain damage (Hanbury et al., 2003), mainly due to up-regulation of the *GDNF* gene in glial cells (Hughes et al., 1999; Bresjanac and Antauer, 2000; Saavedra et al., 2008), we studied GDNF expression in *GDNF-LacZ* animals treated with a single MPTP dose to induce partial or complete destruction of dopaminergic nigrostriatal neurons (Mejías et al., 2006). These animals, examined 7–21 d after MPTP injection, exhibited a characteristic strong striatal astroglial reaction (Figure 3A,D). However, the

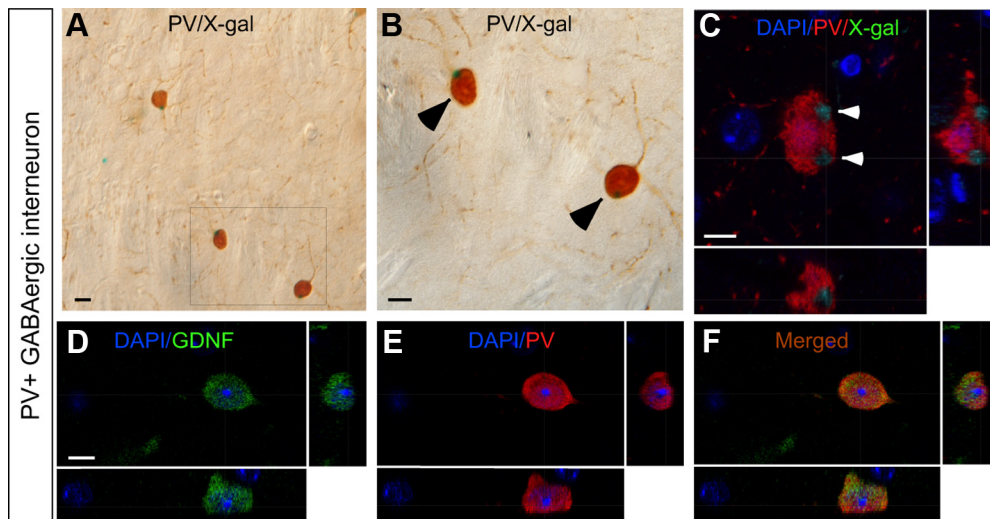


Figure 6. Expression of GDNF in PV+ interneurons of adult striatum. **A, B**, Striatal coronal sections from *GDNF-LacZ* mouse showing GDNF expression (X-gal staining, blue dots) in PV+ interneurons. The square in **A** is shown at high magnification in **B**. Black arrowheads indicate neurons positive for X-gal and PV. **C**, Confocal microscopy Z-stacks showing a PV+ interneuron (red) that is X-gal+ (green). **D–F**, Confocal microscopy Z-stacks of striatal slices from *GDNF-EGFP* mouse. *GDNF-EGFP*+ neurons (**D**, green) also express PV marker (**E**, red). A merged image is shown in **F**. Scale bars, 10 μ m. In **D–F**, we used a mouse (monoclonal) anti-GFP antibody. Highly visible GFP expression was also seen with the GFP polyclonal antibody (data not shown).

pattern of GDNF expression remained unchanged with respect to the one in normal animals. None of the X-gal deposits examined ($n = 2946$ in three animals) appeared in either astrocytes (GFAP+) (Fig. 3*B,E*) or microglial (IBA1+) cells (Fig. 3*C,F*). *GDNF* mRNA levels were also measured in the striatum removed from MPTP-injured animals by qRT-PCR. In accord with the data obtained with the X-gal technique, lesioned animals showed an increase of *GDNF* mRNA expression that reached a peak and then declined over the weeks after MPTP injection (Fig. 4*A*). Three weeks after the lesion, we still observed a nonsignificant 1.5-fold increase of striatal *GDNF* mRNA with respect to controls ($p = 0.051$, one-way ANOVA). To further investigate whether brain damage specifically induces GDNF in astrocytes, we performed experiments in MPTP-treated *GFAP-EGFP* mice (Zhuo et al., 1997) in which striatal astrocytes (GFAP-EGFP+) can be efficiently separated by flow cytometry. As illustrated in Figure 4*B*, MPTP treatment induced a relative $\sim 30\%$ increase of the GFAP+ cell population (R2 gate) as a consequence of the reactive astrocytosis shown before (Fig. 3). However, in this enriched astrocyte population *GDNF* mRNA levels were similar to those measured in unlesioned animals (Fig. 4*B*). Altogether, these findings strongly suggest that striatal GDNF production increases moderately in response to damage of the dopaminergic nigrostriatal neurons. However, glial cells, particularly astrocytes whose number is strongly increased in lesioned animals, do not seem to be a significant source of GDNF. Therefore, the data support the view that striatal neurons, and not glial cells, are the predominant sites of GDNF production in normal and injured animals.

Identification of GDNF-expressing striatal neurons

Medium spiny projecting neurons (MSNs) represent 90–95% of the rodent striatal neurons, being the remaining cells in a mixed population of interneurons (Tepper and Bolam, 2004). Among these, a small percentage ($\sim 0.3\%$ of all striatal neurons) comprises cholinergic interneurons, and the rest are GABAergic interneurons. GABAergic interneurons are further divided into several neurochemically distinct classes. Approximately 0.7% of all striatal neurons are interneurons that express the calcium

binding protein PV (Tepper and Bolam, 2004). The other populations of GABAergic interneurons express CB, calretinin (CR), or SS (Gerfen et al., 1985; Kawaguchi, 1993; Rymar et al., 2004). Using selective markers to identify the various neuronal subtypes indicated above, we analyzed by immunohistochemistry striata of *GDNF-LacZ* animals. It was surprising that projection neurons (DARPP32+), the most abundant in the striatum that receive a strong innervation from nigrostriatal dopaminergic cells (Tepper et al., 2010), were never seen to contain X-gal aggregates (Fig. 5*A,B*). Apparent colocalization of X-gal deposits with DARPP32+ cells observed in some cases, was not confirmed by confocal analyses (Fig. 5*B,C*). This finding was also supported by analyzing the *GDNF-EGFP* mouse, in which DARPP32+ cells (examined in nine striatal sections from three animals) never colocalized with GFP+ neurons (Figure 5*D–F*).

Among the population of striatal interneurons, X-gal expression was seen in 83% of PV+ GABAergic interneurons. Qualitatively, GDNF expression was demonstrated both in *GDNF-LacZ* animals (X-gal deposits) (Fig. 6*A–C*) and in *GDNF-EGFP* mice, as well as with the two different GFP antibodies used (Fig. 6*D–F*). Although in smaller proportion (6% and 3%, respectively), GDNF expression was also observed in cholinergic interneurons (ChAT+), a population of large aspiny cells sparsely but evenly distributed throughout striatum (Fig. 7*A,B*) as well as in SS+ (Fig. 7*C,D*) interneurons. In contrast, GDNF signals were absent in CB+ or CR+ cells (Fig. 7*E,F*). The quantitative summary of neostriatal GDNF expression (Fig. 8*A*) indicates that among the population of identified GDNF+ (X-gal+) cells, $\sim 95\%$ were PV+. The remaining GDNF-expressing cells were either ChAT+ (3%) or SS+ (4%). This distribution of GDNF-synthesizing interneurons remained essentially unaltered in striatum from MPTP-treated animals studied 7 d after injury (Fig. 8*A*). Therefore, the data indicate that both in normal conditions and after toxic damage of dopaminergic nigrostriatal neurons in adult mouse, PV+ GABAergic interneurons are the main neostriatal source of the GDNF necessary for maintenance and survival of the nigrostriatal system. Appearance of striatal GDNF (delayed to the initial P10–P15) (Fig. 1) parallels the ontogenic maturation of PV neurons, which starts in the lateral striatum at approxi-

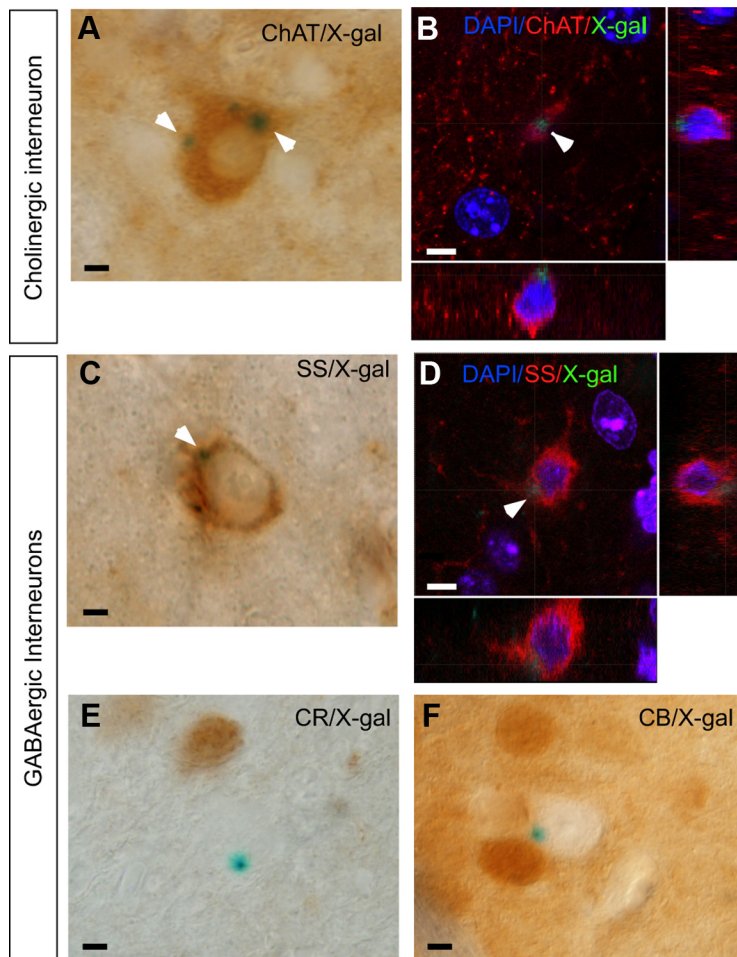


Figure 7. Selective expression of GDNF in ChAT- and SS-positive GABAergic interneurons of adult striatum. **A, B,** Striatal coronal sections from *GDNF-LacZ* mouse showing GDNF expression (X-gal, blue dots indicated by arrowheads) in ChAT+ interneurons. **B,** Confocal microscopy Z-stacks analysis of ChAT+ interneurons. Arrowhead indicates the X-gal precipitate. **C–F,** Striatal coronal sections from *GDNF-LacZ* mouse showing GDNF expression (X-gal, blue dots) in SS+ (**C, D**) but not in CR+ (**E**) or CB+ (**F**) GABAergic interneurons. **D,** Confocal microscopy Z-stacks analysis of SS+ interneurons. Arrowheads indicate the X-gal precipitate. Scale bars, 10 μ m.

mately P9 in rodents (Schlösser et al., 1999). At this stage (Fig. 8B, top), GDNF+ neurons are observed in the region where PV expression initiates but overcome in number the PV+ cells. This suggests that GDNF synthesis could precede PV expression during interneuron postnatal maturation. Nonetheless, at P18 the number of PV+ cells expressing GDNF was similar to that found in adult striatum (Fig. 8B, bottom).

Discussion

In the last decade, GDNF-based therapies have become a promise for the treatment of PD because of the potent trophic effect of this agent on dopaminergic neurons both *in vitro* (Lin et al., 1993) and *in vivo* (Tomac et al., 1995). It has also recently been demonstrated that maintenance of the mouse dopaminergic adult nigrostriatal system absolutely depends on neostriatal GDNF production (Pascual et al., 2008), although the source of GDNF is not well known. The design of new GDNF-based therapeutic approaches for PD would surely benefit from a more precise knowledge of endogenous GDNF function in the normal and injured brain. In this article, we show that GDNF production in mouse striatum develops in early postnatal life and is essentially confined to neurons. We have not detected large amounts of GDNF in glial cells either in normal animals or after damage of

the nigrostriatal pathway. In both circumstances, GDNF is absent from the densely innervated striatal MSNs and appears predominantly expressed in PV+ GABAergic interneurons.

As other neurotrophic factors, GDNF is normally expressed in low quantities, and its identification by means of standard immunohistochemical methods is hampered by the lack of highly specific antibodies and the low level of expression of the protein (Villadiego et al., 2005; Lonka-Nevalaita et al., 2010). We have overcome this limitation using the *GDNF-LacZ* mouse (Sánchez et al., 1996), which permits unequivocal identification of GDNF-expressing cells. We have shown before in several cell types a close relationship between *GDNF* promoter activity (as evidenced by X-gal histochemistry) and GDNF synthesis and release (Villadiego et al., 2005). Critical observations on GDNF expression were also confirmed using the *GDNF-EGFP* mice (Gong et al., 2003) or by qRT-PCR of highly enriched glial cell populations obtained by cell sorting from striata of *GFAP-EGFP* transgenic animals. Therefore, the main conclusions in this article are based on data obtained with various animal models and complementary methodologies.

In accord with previous reports (Trupp et al., 1997; Pascual et al., 2008), we have further confirmed that the thalamus, septum, nucleus accumbens, and neostriatum show the highest levels of GDNF expression in the adult brain (Table 1). Striatal GDNF is practically absent in neonates, but appears during the second to third postnatal weeks, and its production remains during adulthood (Fig. 1; Table 1). This temporal pattern coincides with postnatal maturation of the nigrostriatal pathway (Antonopoulos et al., 2002; Burke, 2003) and striatal PV+ neurons (Schlösser et al., 1999; Chesselet et al., 2007), the ones that show the highest expression of GDNF in adult mouse (see below). Neonatal mesencephalic dopaminergic neurons are normal in the GDNF-null animals (Moore et al., 1996; Sánchez et al., 1996); therefore, it seems that their trophic dependence on this factor develops early after birth in parallel with the maturation and phenotypic specification of dopaminergic nigrostriatal axon terminals and their targets in the striatum. If the same process occurs in humans, one could speculate that subtle developmental alterations produced during this critical time period could eventually result in higher susceptibility to parkinsonism in adulthood.

In normal brain, GDNF was found in significant quantities in striatal neurons rather than in astrocytes or microglia (Fig. 2). In glial cells, *GDNF* mRNA was amplified by RT-PCR, but the presence of the trophic factor was not observable using either the *GDNF-LacZ* or the *GDNF-EGFP* mice (Figs. 2–4). Although there is an abundant literature on the glial expression of GDNF, our findings are in accord with reports suggesting that in central and peripheral neuronal tissues GDNF is normally confined to

neurons (Pochon et al., 1997; Bizon et al., 1999). In this regard, it is worth mentioning that, similar to the brain, GDNF expression in the adult carotid body, a neuronal crest-derived organ of the peripheral nervous system that contains high levels of this trophic factor (Toledo-Aral et al., 2003; Pardo et al., 2007), appears in neuron-like glomus cells rather than in the glia-like sustentacular type II cells (Villadiego et al., 2005). As reported before (Hanbury et al., 2003; Saavedra et al., 2008), striatal GDNF expression was upregulated in response to toxic injury of dopaminergic nigrostriatal neurons, but, surprisingly, this appeared to be a consequence of increased GDNF production in neurons rather than in glial cells. Although in MPTP-lesioned animals we observed a clear increase in the number of striatal astrocytes and microglia, evidence of *GDNF-LacZ* transcriptional activity was seen only in neurons. Furthermore, qRT-PCR analyses of enriched astrocyte populations did not indicate an increase of GDNF expression in reactive glia (studied 7–21 d after lesion) in comparison with controls. These findings clearly contradict a common view, albeit not accepted by all authors, that GDNF production switches from neurons to glial cells in the damaged brain (Saavedra et al., 2008). They could also question, at least in terms of quantitative significance, the putative neuroprotective action of activated glia. Nonetheless, the current study focuses on the MPTP-injured nigrostriatal system, and, therefore, its conclusions cannot be extended to other models in which astrocytic GDNF production has been proposed to have a neuroprotective role (Saavedra et al., 2008).

Our data indicate that the most abundant sources of neostriatal GDNF, either in normal or MPTP-lesioned animals, are GABAergic PV+ interneurons that represent ~0.7% of neostriatal neurons but ~95% of all GDNF-expressing cells identified in this structure. The remaining GDNF-expressing cells were SS+ and ChAT+ interneurons (Figs. 5–8). MSNs, the most abundant population in the striatum that receive the majority of the nigral dopaminergic afferent fibers were never seen to show X-gal marks or GFP expression indicative of *GDNF* promoter activity. The fact that MSNs do not seem to contribute to the retrograde neurotrophic support of the nigrostriatal system is an unexpected finding that suggests adaptive specializations within the striatal connectome (Fig. 8C, scheme). PV+ interneurons are sparsely distributed over the striatum, and its distribution obeys a ventral-to-dorsal, medial-to-lateral, and caudal-to-rostral gradient of increasing density. This striatal topology coincides with the area innervated by dopaminergic terminals from the substantia nigra (Björklund and Dunnett, 2007). PV+ interneurons receive dopaminergic inputs from the substantia nigra and glutamatergic synapses

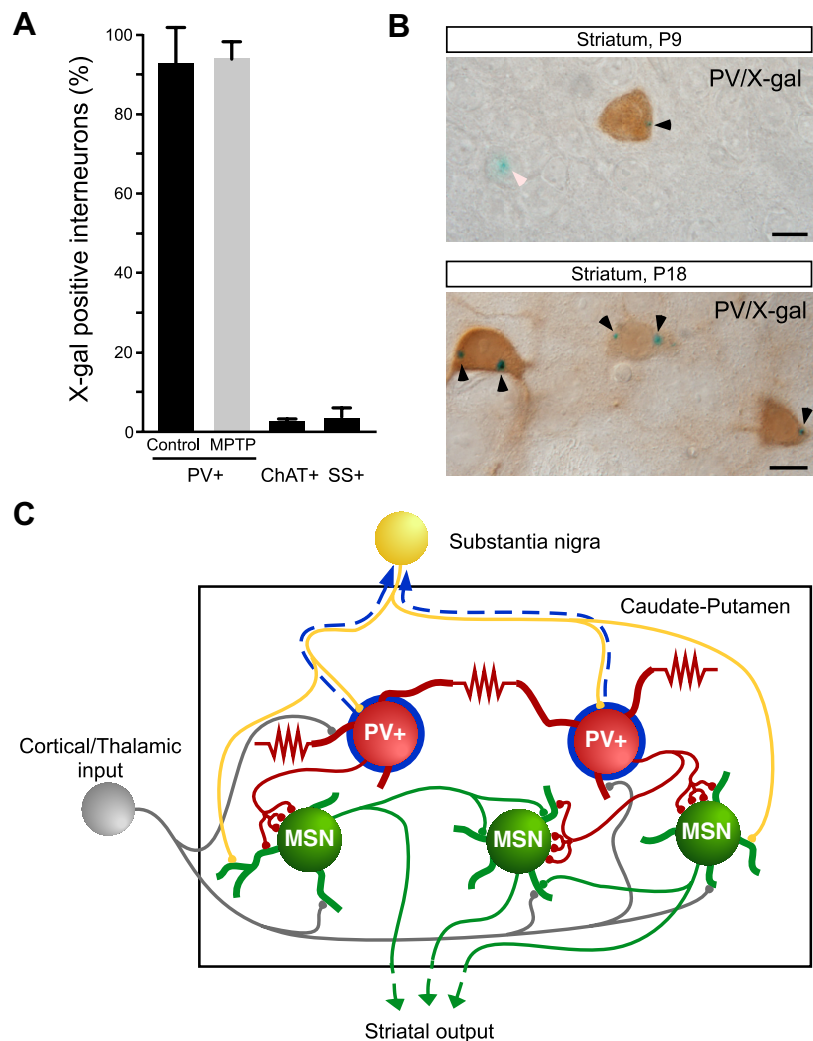


Figure 8. *A*, Quantitative summary of neostriatal GDNF-expressing interneurons. Percentage of identified X-gal-positive cells in striatum from normal animals (black bars) and from MPTP-treated mice 7 d after injection (gray bar). *B*, Parallel appearance of GDNF and PV in striatal interneurons during postnatal maturation. Representative striatal coronal sections from P9 (top) and P18 (bottom) *GDNF-LacZ* mice showing GDNF (X-gal staining) and PV (brown color) expression. Arrowheads indicate X-gal blue dots. *C*, Simplified scheme of the striatal connectome. Axons of MSNs (green) send collaterals to neighboring MSNs. These cells are under control of parvalbumin + GABAergic interneurons (red), a cell ensemble synchronized through multiple dendrodendritic electrical synapses (resistances in the scheme). Glutamatergic cortical and thalamic inputs to PVs and MSNs are indicated in gray color. Dopaminergic nigrostriatal fibers (yellow) innervate both PVs and MSNs. Retrograde GDNF signaling supporting trophic maintenance of dopaminergic neurons is represented by blue discontinuous lines and the halo surrounding the GDNF-producing PV+ interneurons.

from thalamus and cortex. Their axons generate powerful proximal inhibitory synapses onto MSNs. As in other parts of the CNS (i.e., cortex or hippocampus), striatal PV+ neurons are “fast-spiking” cells capable of sustaining high-frequency (>400 Hz) action potential firing with little afterhyperpolarization or spike frequency adaptation. PV+ cells constitute a unique network interconnected by electrical synapses due to dendrodendritic gap junctions, which enable them to fire almost synchronously. Striatal fast-spiking PV cells can resonate at the gamma frequency (Sharott et al., 2009) and are considered to be the major component of a powerful feedforward inhibition that controls spike timing in MSNs, thereby regulating striatal output (Gittis et al., 2010; Tepper et al., 2010; Chuhma et al., 2011). GDNF production in PV+ neurons could be advantageous if the trophic factor is released during periods of activity of the cell functional ensemble (Lonka-Nevalaita et al., 2010), thus interacting with axon

terminals of practically the entire nigrostriatal pathway. Additional advantages that PV+ interneurons could offer as the source of neurotrophic support for the nigrostriatal pathway result from their presumable high resistance to excitotoxicity, due to the cytosolic content of parvalbumin (a powerful Ca^{2+} buffering protein) and because repetitive electrical firing depends on Na^+ rather than on Ca^{2+} currents (Tepper et al., 2010). Metabolic demands in “fast-spiking” interneurons appear to be coped with through the expression of particularly high levels of oxidative phosphorylation enzymes (Gulyás et al., 2006). In addition to PV+ cells, the other GDNF-producing interneurons might also have a role in the maintenance of projection or local striatal circuits. SS+ and ChAT+ interneurons, although less numerous than PV+ cells, could contribute to nigrostriatal trophic support since they also receive dopaminergic innervation. On the other hand, exogenous GDNF administration seems to protect MSNs in models of Huntington’s disease (Alberch et al., 2002). Thus, it could be that in normal conditions GDNF dependent trophic support is provided by cholinergic interneurons, which receive axon collaterals of MSNs (Tepper et al., 2010).

Identification of the ensemble of PV+ interneurons as the main source of striatal GDNF raises numerous questions of potential clinical relevance. Neuroprotection in PD has been attempted so far by continuous administration of exogenous GDNF released locally in the striatum. If endogenous GDNF secretion is actually associated with the synchronized electrical activity of striatal PV cells, it would imply that, in physiologic conditions, the trophic factor is simultaneously released in broad areas of the striatum and with a pulsating or discontinuous time course, circumstances that probably help optimize its neurotrophic actions. In this sense, it is worthy to remark that the number of neostriatal interneurons in primates is higher than in rodents (Tepper and Bolam, 2004). Therefore, stimulation in PD patients of the striatal PV+ cell population (e.g., by pharmacological, electric, or magnetic means) to synthesize and release GDNF under controlled conditions could have therapeutic effects.

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