GDNF Protection against 6-OHDA: Time Dependence and Requirement for Protein Synthesis

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Glial cell line-derived neurotrophic factor (GDNF) injected intranigrally protects midbrain dopamine neurons against 6-hydroxydopamine (6-OHDA) toxicity. The timing between GDNF administration and exposure to 6-OHDA is critical in achieving optimal protection. When injected 6 hr before an intranigral injection of 6-OHDA, GDNF provides complete protection as measured by the number of surviving neurons in the substantia nigra of adult rats. The surviving neuronal population decreases by ~50% with 12 and 24 hr separating GDNF and 6-OHDA administrations. In controls with 6-OHDA lesions, there is <10% survival of nigral dopamine neurons. No significant increase in survival is seen with either concurrent injections of GDNF and 6-OHDA or 1 hr GDNF pretreatment. Based on HPLC measurements, striatal and midbrain dopamine levels are at least twofold higher on the lesioned side in animals receiving GDNF 6 hr before a 6-OHDA lesion compared with vehicle recipients. Protein synthesis is necessary for GDNF-induced neuroprotective effects because cycloheximide pretreatment that inhibits protein synthesis also blocks neuroprotection.

Key words: GDNF; 6-OHDA; neuroprotection; substantia nigra; dopamine neurons; cycloheximide

Parkinson's disease is a progressive neurological disorder in which bradykinesia, balance and gait disturbances, muscular rigidity, and resting tremor predominate. The primary pathology of this disease is degeneration of the nigrostriatal system, resulting in significant loss of midbrain dopamine neurons. Glial cell line-derived neurotropic factor (GDNF) exerts significant trophic effects on midbrain dopamine neurons (Lin et al., 1993; Hoffer et al., 1994). GDNF is considered a distant member of the transforming growth factor β (TGF-β) superfamily and can be retrogradely transported from the striatum to dopamine neurons in the substantia nigra (Tomac et al., 1995b).

In characterizing the trophic actions of GDNF on dopamine neurons, several groups have reported both protective and regenerative effects *in vivo*. In adult rats, GDNF protects midbrain dopamine neurons against intranigral and intrastriatal injections of 6-hydroxydopamine (6-OHDA) (Kearns and Gash, 1995; Sauer et al., 1995; Choi-Lundberg et al., 1997), neurotoxic doses of methamphetamine (Cass, 1996), as well as axotomy-induced degeneration of the medial forebrain bundle (Beck et al., 1995). Protective effects have also been reported against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice (Tomac et al., 1995a). When administered after a medial forebrain bundle 6-OHDA lesion, GDNF normalizes nigral dopamine levels and increases the number of tyrosine hydroxylase immunoreactive (TH+) cells in the lesioned substantia nigra (Hoffer et al., 1994; Bowenkamp et al., 1995). In addition, GDNF has been shown to promote regeneration of dopamine neurons after MPTP-induced degeneration in mice and nonhuman primates (Tomac et al., 1995a; Gash et al., 1996).

To follow up on the initial reports on the neuroprotective properties of GDNF, we have conducted a series of experiments to better characterize the effects of GDNF on substantia nigra dopamine neurons exposed to 6-OHDA toxicity. In our initial study, pretreatment with GDNF 24 hr before an intranigral 6-OHDA lesion increased survival of nigral dopamine neurons to 47% of the population compared with 9% survival in vehicle-treated controls (Kearns and Gash, 1995). Therefore, the first experiment was designed to evaluate the effects of varying the time interval between GDNF administration and a 6-OHDA lesion on dopamine neuron survival. One purpose was to resolve clearly the issue of whether pretreatment was protective or regenerative. Neither our original study nor those by other groups have ruled out the possibility that the pretreatment effects of GDNF are restorative rather than neuroprotective. One possibility in the "protection" paradigms used was that the extent of the initial neurotoxic lesion was identical in both trophic factor and nontrophic factor recipients. However in GDNF recipients, sufficient GDNF then remained *in situ* to rapidly promote regeneration.

The second experiment evaluated preservation of neuronal dopamine after 6-OHDA toxicity in animals receiving GDNF pretreatment during the optimal period. HPLC was used to measure dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) levels in the striatum and midbrain of trophic factor recipients and controls. The final experiment in the current series was designed to determine whether protein synthesis is necessary for the neuroprotective effects of GDNF.

MATERIALS AND METHODS

Animals. Young adult male Fischer 344 rats weighing 200–250 gm at the start of each experiment were used. The animals were housed two per cage in a temperature-controlled room with a 12 hr light/dark cycle and were given food and water *ad libitum*. Animals were maintained according to the *NIH Guide for the Care and Use of Laboratory Animals.*

Cell survival study. Before surgery, 56 rats were randomly divided into

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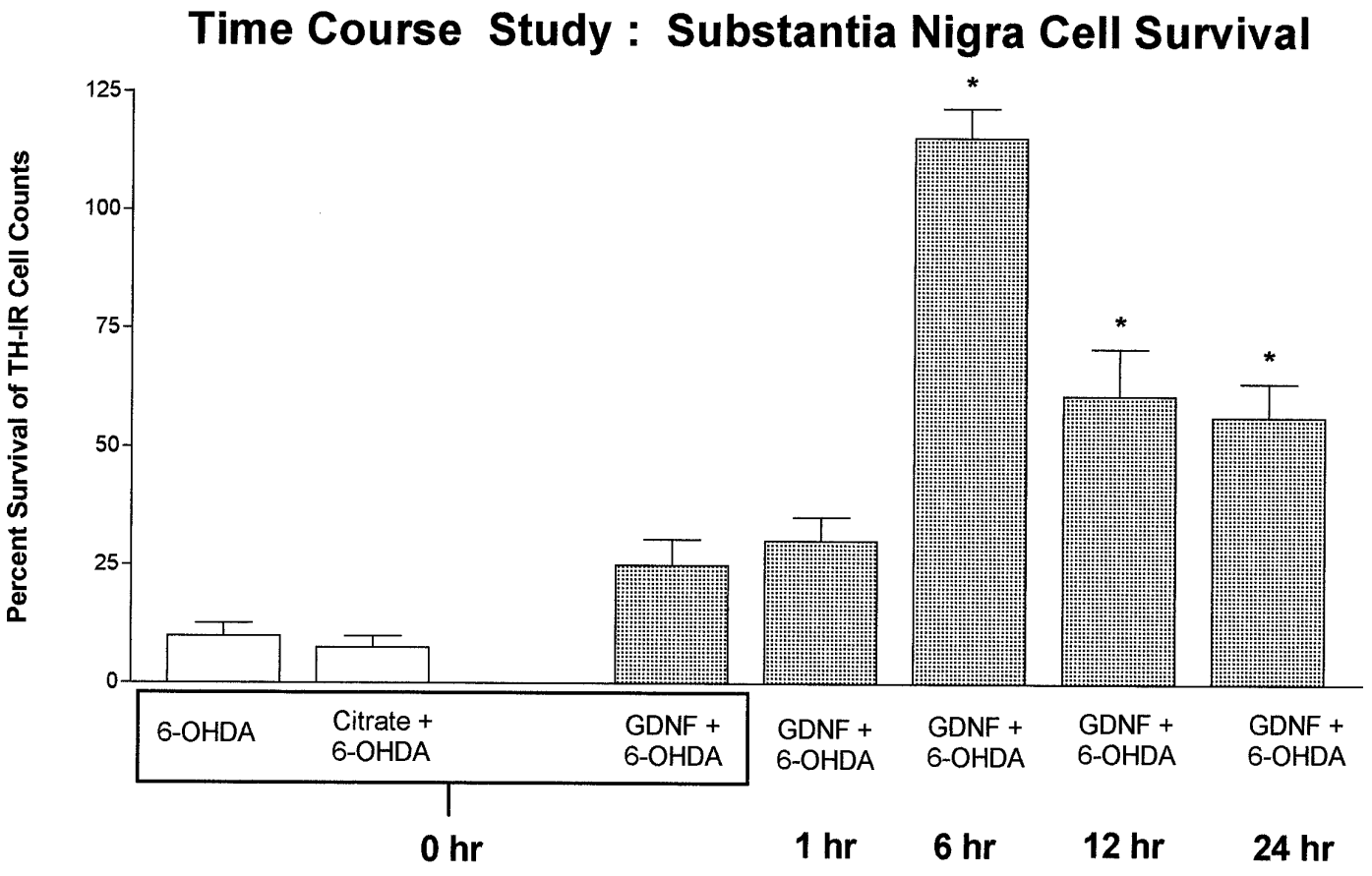

Figure 1. Percent survival of TH-IR neurons in the substantia nigra of the time course study. The *open bars* refer to the cell survival in non-GDNF-treated groups, whereas the *filled bars* correspond to the cell survival of the GDNF recipients. Values are represented as the mean ± SEM (*$p < 0.0001$, significantly different from the 6-OHDA group at the 0 hr time point).

seven groups with eight animals in each group. At the 0 hr time point, one group received 6-OHDA alone and served as the baseline. A second group at the 0 hr time point received citrate immediately followed by 6-OHDA. This second group tested whether the vehicle was protective in itself. The remaining five test groups received human recombinant GDNF (Amgen, Thousand Oaks, CA) dissolved in citrate buffer at five specified time points before a 6-OHDA challenge: 0, 1, 6, 12, and 24 hr. Three animals died during surgery because of anesthesia-related problems.

The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Butler, Columbus, OH) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) with the mouth bar set at −3.3 mm. The skull was exposed, and a burr hole was made using a high-speed dental drill.

Each injection of vehicle (10 mM citrate, pH 5.0), GDNF, or 6-OHDA (Sigma, St. Louis, MO) was administered directly into the right substantia nigra pars compacta using the following coordinates: anteroposterior, −5.4 mm; mediolateral, −2.2 mm; and dorsoventral, −8.5 mm from skull (Paxinos and Watson, 1986). Citrate-treated animals received 2 μl of citrate buffer directly into the substantia nigra. All trophic factor-treated animals received 10 μg of human recombinant GDNF in 2 μl of 10 mM citrate buffer delivered to the same site. The 6-OHDA alone group received only a single intranigral injection of this neurotoxin (8 μg in 2 μl of saline containing 0.02% ascorbic acid).

All injections were performed using a Hamilton 10 μl syringe with a 26 gauge tapered needle at a rate of 0.4 μl/min. At the completion of each injection, the needle was left in place for 5 min and then withdrawn at a rate of 2 mm/min. All surgeries were performed under aseptic conditions. Four weeks after surgery, the rats were anesthetized with an overdose of sodium pentobarbital and perfused with heparinized saline.

Cycloheximide study. First, the effects of cycloheximide on protein synthesis inhibition were determined. Eight rats were randomly divided into two groups with four rats each. One group received saline directly into the right substantia nigra, and the other group received 20 μg of cycloheximide dissolved in saline (Research Organics, Inc., Cleveland, OH) into the same site. The coordinates and surgical procedure were the same used in the cell survival study. One hour after saline or cycloheximide administration, [^{3}H]Leu (1 mCi/gm body weight; DuPont NEN, Wilmington, DE) was injected subcutaneously. One hour later, each animal was anesthetized with an overdose of sodium pentobarbital and killed by intracardiac perfusion with heparinized saline. Brains were removed, and 2 mm coronal sections were cut using a brain mold. Several tissue punches were taken from the substantia nigra, striatum, and cortex of the right side of the brain. A fluorescamine protein assay (Lorenzen and Kennedy, 1993) was used to determine micrograms per milliliter protein in each series of tissue punches. Each sample was then processed for liquid scintillation counting. Values were expressed as disintegrations per minute/(micrograms per millilter protein).

Next, the effects of cycloheximide on the neuroprotective effects of GDNF were studied. Thirty-two animals were randomly distributed into the following four groups with eight rats each: (1) saline and citrate 1 hr later and 6-OHDA 6 hr later, (2) cycloheximide and citrate and 6-OHDA, (3) saline and GDNF and 6-OHDA, and (4) cycloheximide and GDNF and 6-OHDA. The timing of the citrate or GDNF and the 6-OHDA injections in groups 2–4 was identical to the timing of injections in group 1. Two rats died during surgery. All remaining animals received their injections directly into the right substantia nigra using the same coordinates and surgical procedure used in the cell survival study. Each animal was killed 5 d after surgery with an overdose of sodium pentobarbital and was perfused with heparinized saline.

Tissue preparation for immunocytochemistry. To evaluate cell survival, we removed the brains and post-fixed them for 24 hr in a 4% paraformaldehyde solution in 50 mM potassium phosphate-buffered saline (KPBS). The brains were then transferred to 30% sucrose in 50 mM KPBS at 4°C. Serial coronal frozen sections of 20 μm thickness were cut on a sliding microtome. Six sets of sections were collected in cryopro-

tectant solution (100 mM KPBS, 30% sucrose, polyvinylpyrrolidone (PVP-40; Sigma, St. Louis, MO), and 30% ethylene glycol) and stored at −20°C until immunocytochemical processing.

A series using every sixth section was stained for the primary antibody against TH (1:1000; Chemicon, Temecula, CA). Sections exposed to the primary anti-TH antibodies were incubated in biotinylated horse anti-mouse secondary antibody (1:500; Vector Laboratories, Burlingame, CA). Sections were then incubated in the avidin–biotin peroxidase complex using the Elite ABC Vectastain kit (Vector Laboratories). TH immunoreactivity (TH-IR) was visualized using 3,3′-diaminobenzidine as the chromagen with nickel enhancement (Date et al., 1990).

Cell counting. Unbiased stereological cell-counting procedures using the optical dissector method were used to count TH+ substantia nigra and ventral tegmental area neurons in all treated animals (West, 1993; Harding et al., 1994). Every sixth 20-μm-thick coronal tissue section through the substantia nigra was sampled for evaluation using the Bioquant image analysis system. The localization of the oculomotor nerve rootlets was the criteria for delineating the substantia nigra from the ventral tegmental area. The ventral tegmental area was considered to be within and medial to the rootlets, whereas the substantia nigra was considered to be located laterally. The extent of dopamine neuronal loss was estimated by the loss of TH+ substantia nigra neurons on the lesioned side with respect to the control side of the brain. Unbiased estimates of the number of mesencephalic dopamine neurons were produced because all regions of the system had an equal probability of being sampled. The total neuronal number was estimated based on the estimated volume of the structure being evaluated as well as on the neuronal density within the boundaries of the structure. Using an optical fractionator method for unbiased stereological cell counting, we subsequently estimated the number of TH-IR neurons in the substantia nigra and ventral tegmental area. On each section, a 200 × 200 μm grid was randomly superimposed with a 100 × 100 μm counting chamber placed on the image. A 15-μm-deep fraction of the counting chamber was determined by a stage encoder attached to the microscope to measure the *z*-axis. All neurons completely within the boundaries of the chamber or crossing the upper or right side of the chamber within the 15 μm depth were counted.

Neurochemistry study. Sixteen rats were randomly distributed into the following two groups of eight animals each: (1) citrate followed by 6-OHDA 6 hr later, and (2) GDNF followed by 6-OHDA. Two animals did not survive surgery because of complications from anesthesia. All animals underwent the same surgical procedure described previously for the 6 hr time point in the cell survival study. Animals were killed 3 weeks after surgery by decapitation under CO_2 anesthesia. The brains were rapidly removed, placed in ice-cold saline, and cut into 2-mm-thick coronal sections using a brain mold. The substantia nigra and striatum were dissected out, placed in preweighed vials, weighed, and frozen on dry ice. Levels of dopamine and DOPAC were determined by HPLC analysis with electrochemical detection using procedures described previously (Cass, 1996). Retention times of standards were used to identify peaks, and peak heights were used to calculate the recovery of internal standard as well as amounts of monoamines and metabolites. Levels of dopamine and DOPAC were expressed as nanograms per gram of wet weight of tissue.

RESULTS

Cell survival study

In controls receiving just 6-OHDA or citrate plus 6-OHDA at the 0 hr time point, there was <10% survival of TH+ dopamine neurons in the substantia nigra on the lesion side of the brain 4 weeks later (Fig. 1). In comparison, 25 and 30% survival rates of

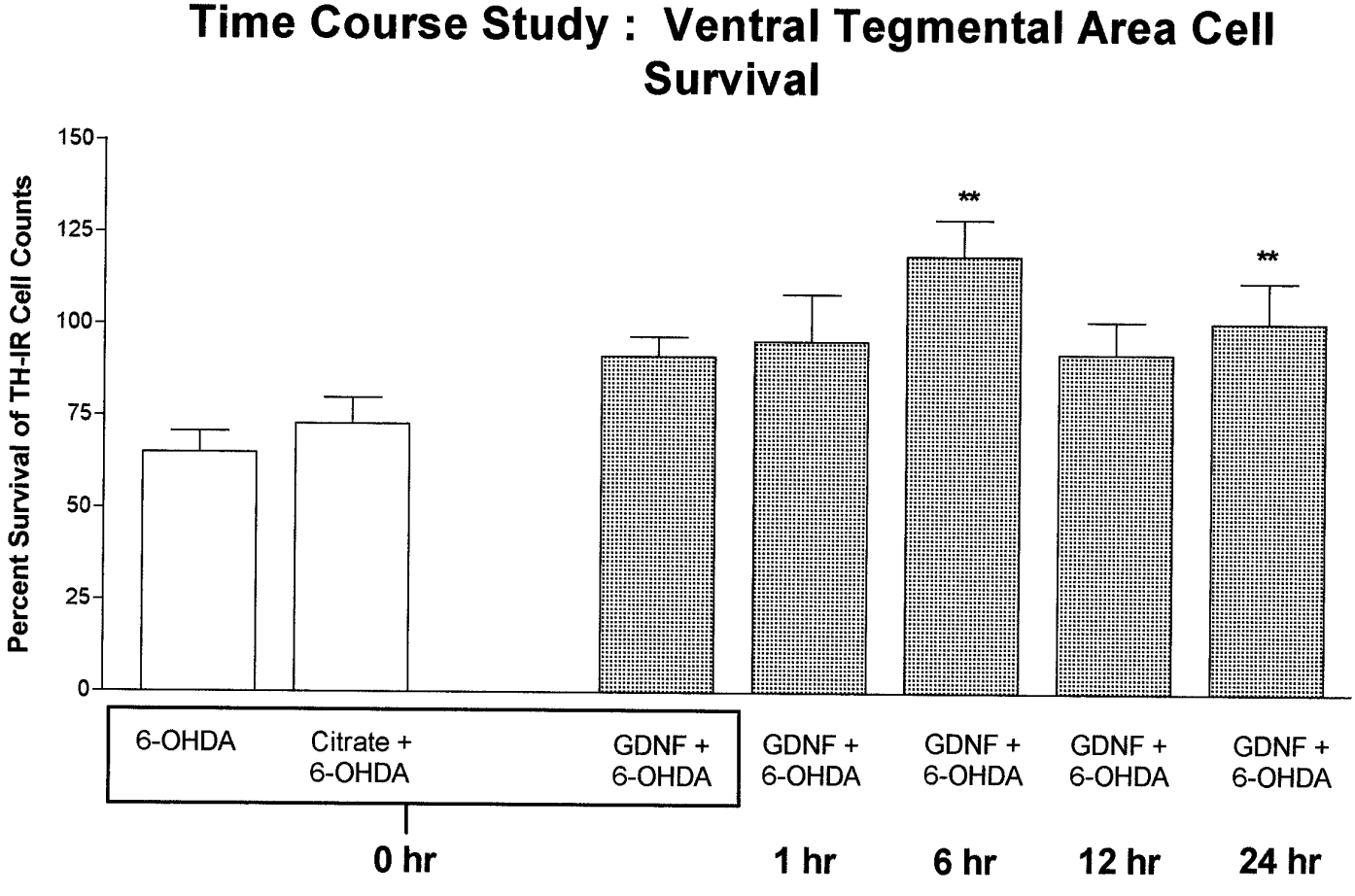

Figure 2. Percent survival of TH-IR neurons in the ventral tegmental area. Cell survival of the non-GDNF recipients is shown in the *open bars*, whereas the *filled bars* demonstrate the cell survival in GDNF-treated groups. Values are expressed as the mean ± SEM (**$p < 0.05$, significantly different from the 6-OHDA group at the 0 hr time point).

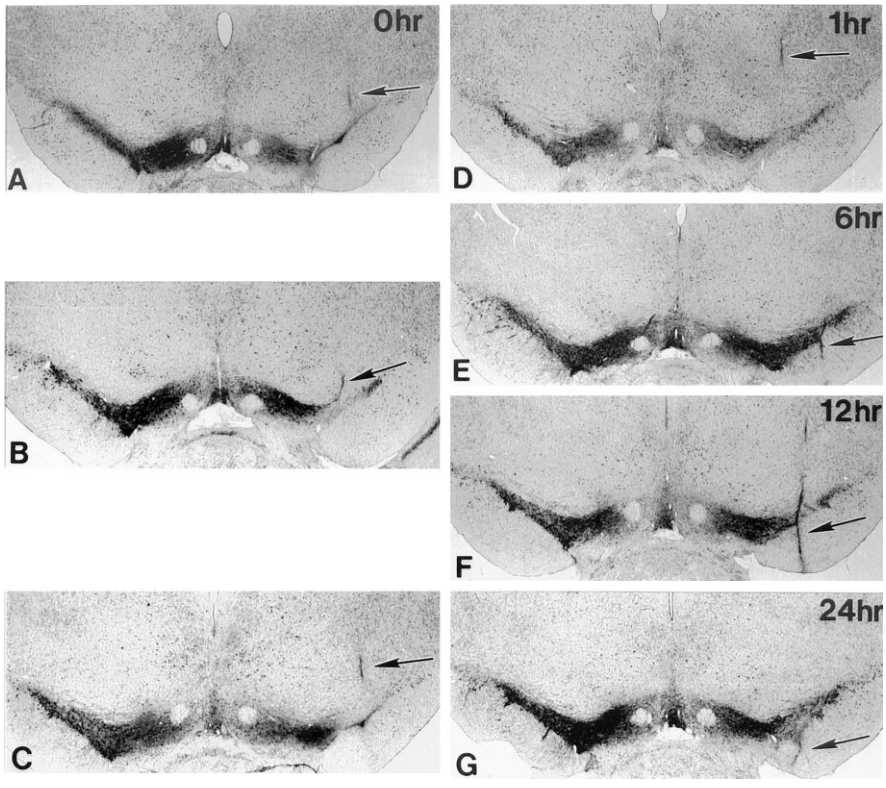

Figure 3. Representative sections are shown through the midbrain of animals in all treatment groups processed for TH immunocytochemistry. *A–C*, The 0 hr time point. The 6-OHDA alone (*A*), citrate and 6-OHDA (*B*), and GDNF and 6-OHDA (*C*) treatment groups all show extensive loss of midbrain dopamine neurons. *D*, GDNF treatment 1 hr before 6-OHDA neurotoxicity also results in a severe loss of dopamine neurons in the substantia nigra. *E*, In contrast, at the 6 hr time point of GDNF administration, there is complete sparing of dopamine neurons in the substantia nigra. *F*, *G*, There is also significant sparing of dopamine neurons in the substantia nigra of animals treated with GDNF at the 12 and 24 hr time points, respectively, but this sparing does not seem to be as complete as the sparing observed at the 6 hr time point. A needle track (*arrows*) can be identified either above or entering the substantia nigra in all photomicrographs.

TH+ neurons were found in animals treated with GDNF at the 0 and 1 hr time points, respectively. The protective effect of GDNF became most evident at the 6 hr time point, at which the number of TH+ neurons on the lesioned side at least equaled the number of neurons in the contralateral substantia nigra. This protection declined to 61% at the 12 hr time point and 57% at the 24 hr time point. Dopamine neurons were less affected by the lesion in the ventral tegmental area (Fig. 2); a significant number of neurons survived in the 6-OHDA-treated (65%) and citrate and 6-OHDA-treated (73%) controls. Survival rates were even >90% in the ventral tegmental area of the GDNF-treated groups.

The goal of the statistical analysis was to compare each of the six treatment group means with the 6-OHDA control mean. Therefore, one-way ANOVA and Dunnett's many-to-one *t* test procedure were used. The ANOVA *F* statistic, based on 6 and 46 df, was significant ($p < 0.0001$ for the substantia nigra and $p < 0.0045$ for the ventral tegmental area). Dunnett's procedure revealed that TH+ neuron numbers in the substantia nigra of the 6, 12, and 24 hr GDNF treatment groups were significantly different ($p < 0.0001$) from the control mean. However, only the 6 and 24 hr GDNF treatment groups were significantly different from the control group in the ventral tegmental area ($p < 0.05$). Two-tailed tests of significance were used at the 0.05 level. Although cell numbers at the 6 hr time point seemed to be higher on the lesioned side (6527 ± 283) than in the contralateral nigra (5781 ± 399), this difference was not statistically significant ($p < 0.15$). This same observation was made in the ventral tegmental area in which the ipsilateral side showed higher cell counts (4971 ± 339) compared with the control side (4408 ± 468). However, this increase was not statistically significant ($p < 0.35$).

The photomicrographs in Figure 3 further document the results from cell counting. They demonstrate extensive loss of TH+ neurons in the substantia nigra after 6-OHDA lesions without trophic factor pretreatment. In animals pretreated with GDNF at the 0 and 1 hr time points, although TH-IR cell loss was pronounced, it seems less severe. In animals administered GDNF 6 hr before 6-OHDA, there was complete sparing of TH+ neurons in the substantia nigra. However, when the time between GDNF administration and 6-OHDA lesion was extended to 12 and 24 hr, damage to substantia nigra neurons is again evident, although less extensive than the damage seen at the 1 hr time point. At 4 weeks after 6-OHDA lesioning, mild macrophage infiltration was evident around all injection sites.

Neurochemical analysis

In GDNF recipients, dopamine levels in the lesioned striatum were 80% of the levels in the unlesioned side of the brain (Table 1). In contrast, dopamine levels decreased to 36% in the lesioned striatum compared with the contralateral side in the citrate-treated group. In the substantia nigra of animals treated with GDNF, dopamine levels increased to 128% of the levels in the noninjected side, whereas levels in the lesioned nigra were only 43% of the levels in the contralateral nigra in the vehicle recipients. Although dopamine levels in the GDNF-treated animals seemed higher on the lesioned side of the brain compared with the nonlesioned side, this difference was not statistically significant (two-tailed *t* test). Further analysis of this experiment compared the striatum and substantia nigra of the GDNF-treated animals with that of the citrate-treated animals. The results were statistically significant in both the striatum and substantia nigra ($p < 0.01$) as determined by a two-tailed *t* test at the 0.05 level (Table 1).

Levels of DOPAC, the primary metabolite of dopamine in rats, were also measured. In the lesioned striatum, DOPAC levels were reduced to 95% of the contralateral levels in GDNF-treated animals, whereas in the citrate-treated animals, the levels fell to 49% of contralateral values. In the lesioned substantia nigra, DOPAC was decreased to 96% of that in the noninjected side in the

Table 1. Dopamine and DOPAC levels

	Region	Dopamine	DOPAC
Substantia nigra (SN)			
Citrate	Left SN	1179 ± 162	382 ± 34
GDNF		995 ± 150	648 ± 123
Citrate	Right SN	509 ± 199	175 ± 58
GDNF		1276 ± 204*	619 ± 98**
Striatum (Str)			
Citrate	Left Str	9749 ± 918	2298 ± 380
GDNF		10412 ± 1011	2747 ± 320
Citrate	Right Str	3505 ± 1602	1137 ± 442
GDNF		8323 ± 1139*	2609 ± 335**

Values are expressed as nanograms per gram of wet weight of tissue.
Significantly different from the citrate controls, same side *$p < 0.01$, **$p < 0.05$.

GDNF-treated animals, with levels reduced to 46% compared with animals that received vehicle. Based on two-tailed *t* tests at the 0.05 level, these values were also statistically significant ($p < 0.05$).

Cycloheximide study

This third study was divided into two parts to examine the effects of protein synthesis inhibition on GDNF neuroprotection of substantia nigra dopamine neurons challenged with 6-OHDA. The first step was to verify that the cycloheximide dose used inhibited protein synthesis in the midbrain. One group received saline and the other received cycloheximide directly injected into the substantia nigra. The rate of protein synthesis was measured 6 hr later because protein turnover within neurons occurs over a 6 hr period (Clemens, 1980).

In saline-treated animals, [^{3}H]Leu incorporation was similar in the substantia nigra, striatum, and cortex [12–14 dpm/(μg/ml protein)]. However, when cycloheximide was administered directly into the substantia nigra, [^{3}H]Leu incorporation declined to 2 dpm/(μg/ml protein). These results were statistically significant ($p < 0.05$) in all three brain regions as determined by paired two-tailed *t* tests based on 3 df (data not shown). The fact that protein synthesis was inhibited not only in the substantia nigra but also in the striatum and cortex may be a result of the diffusion of cycloheximide through the brain parenchyma. Nonetheless, because protein synthesis in the substantia nigra was inhibited and the animals appeared healthy, the next study was performed.

The optimal 6 hr interval between GDNF and 6-OHDA administration identified in the cell survival study was used, and animals were distributed into the following groups: (1) saline, citrate, and 6-OHDA; (2) cycloheximide, citrate, and 6-OHDA; (3) saline, GDNF, and 6-OHDA; and (4) cycloheximide, GDNF, and 6-OHDA.

The results of cell counting in the two citrate-treated groups showed only a 6% survival of dopamine neurons in the substantia nigra pretreated with saline and an 18% survival in animals receiving cycloheximide (Fig. 4). When animals received saline followed by GDNF and 6-OHDA, there was complete preservation of TH+ neurons in the substantia nigra (139% survival) similar to that observed in the cell survival study. Although the cell numbers in this group seemed to be higher on the lesioned side than in the contralateral nigra, again the difference was not statistically significant (two-tailed *t* test). When animals were treated with cycloheximide before GDNF, the protective effect of GDNF was primarily lost (28% survival). All groups that showed significant TH+ cell loss in the substantia nigra displayed neuron survival levels between 60 and 70% in the ventral tegmental area. In animals receiving saline followed by GDNF, cell survival in the ventral tegmental area approached 100%. The goal of the statistical analysis for this study was to compare each of the four group means with each other. Two-sample *t* statistics were used for each pairwise (substantia nigra and ventral tegmental area) comparison. Satterthwaite's approximation was used to determine the df (between 8 and 26) for the *t* statistic. Bonferroni's correction factor was applied to each comparison to protect against the inflation of the type I error rate caused by multiple comparisons among the means. These results show that the substantia nigra and ventral tegmental area of animals that received saline followed by GDNF and 6-OHDA were significantly protected ($p < 0.05$), whereas these same brain regions in animals treated with cycloheximide followed by GDNF and 6-OHDA were not statistically different from the regions in the citrate-treated groups (Fig. 4).

The photomicrographs in Figure 5 show minimal survival of TH+ neurons in the substantia nigra of both citrate-treated groups. This result is particularly informative, because it suggests that cycloheximide is not protective in itself. It has been hypothesized that cycloheximide may be able to inhibit the synthesis of "killer proteins" (Goto et al., 1990). However in this animal model, this is not the case. In group 3 (Fig. 5*C*), the animals that received saline before GDNF showed complete protection of the substantia nigra as described previously in the cell survival study, whereas in group 4 (Fig. 5*D*), there was extensive loss of TH-IR neurons in the substantia nigra when the protective effect of GDNF was challenged with cycloheximide. Some macrophage infiltration was evident around all injection sites.

DISCUSSION

The present study demonstrates that GDNF rapidly induces changes in midbrain dopamine neurons that provide protection against 6-OHDA neurotoxicity. Significantly increased survival of TH+ neurons in the substantia nigra was found in animals receiving a single injection of GDNF at either 6, 12, or 24 hr before an intranigral 6-OHDA lesion. The effect peaked with pretreatment 6 hr before the neurotoxic challenge. At this time point, virtually all nigral neurons survived the subsequent 6-OHDA lesion. Because injections of GDNF either 1 hr before or concurrent with the 6-OHDA challenge did not significantly increase cell survival, protection rather than restoration of dopamine neurons was involved. If restoration was occurring, then survival would have increased with the shortest intervals between GDNF treatment and the 6-OHDA lesion.

The decline in percent survival at the 12 and 24 hr time points to 61 and 57%, respectively, reflects the narrow window of opportunity for completely protecting dopamine neurons against the levels of 6-OHDA used in this study. Intranigral injections of 6-OHDA produce an acute lesion resulting in cell death within 10 min (Ungerstedt and Arbuthnott, 1970). Therefore, the cellular changes providing protection against neurotoxicity (such as protein synthesis of free radical-scavenging enzymes or calcium-binding proteins) must be in place before the 6-OHDA challenge. Once GDNF and its receptor are internalized by the dopamine neuron, a series of intracellular events occurs by way of a signal transduction pathway based on tyrosine kinase activity (Ullrich and Schlessinger, 1990). The results indicate that the molecular events leading to neuroprotection after GDNF administration peak during the 6 hr time interval and then quickly attenuate.

The results of the cell survival experiment complement and

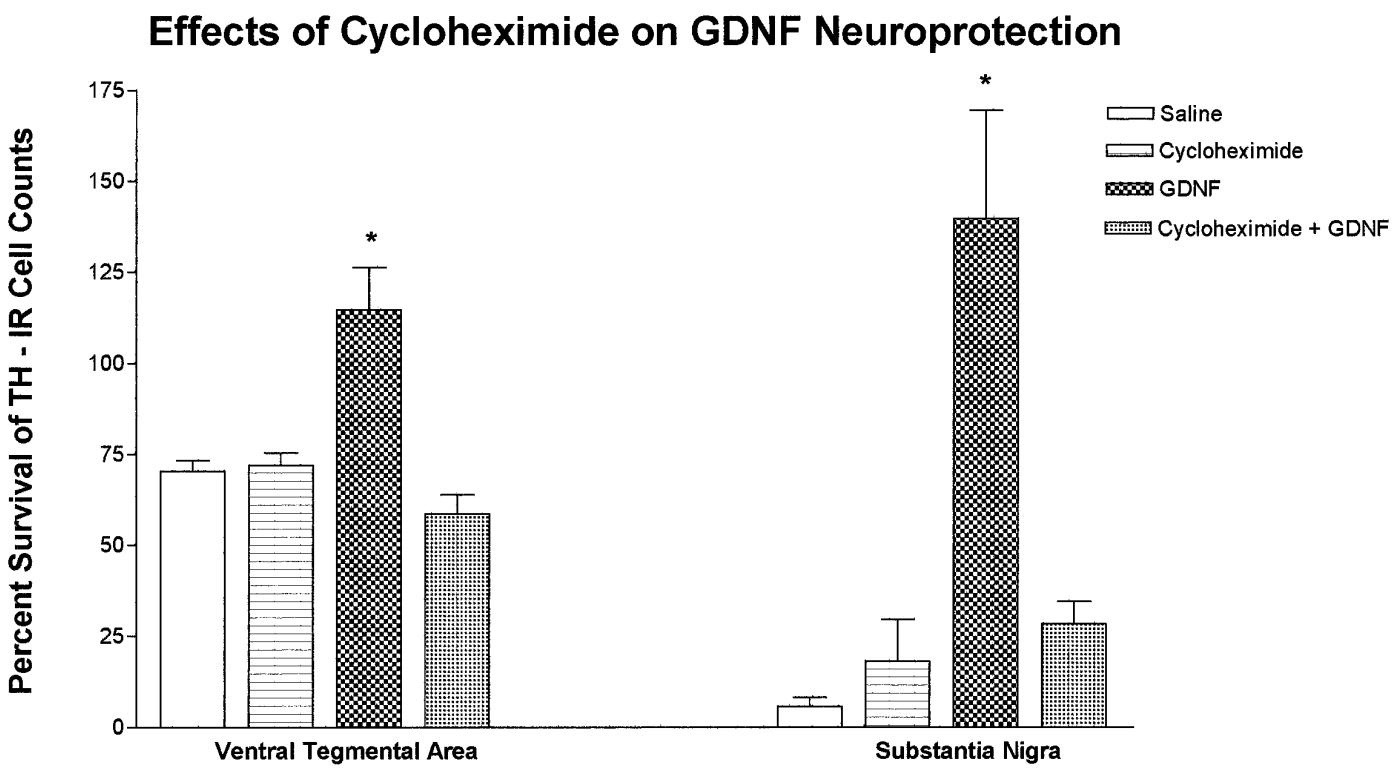

Figure 4. Effects of cycloheximide on GDNF administration in the substantia nigra and ventral tegmental area. Values are expressed as the mean percent survival of TH-IR neurons ± SEM (*$p < 0.05$, statistically different from the saline plus citrate-treated group).

expand the findings from other studies evaluating GDNF administration before a lesion. Both intranigral and intrastriatal injections of 10 μg of GDNF in mice, 24 hr before an MPTP challenge, partially preserved nigral dopamine levels in animals examined 7 d after the lesion (Tomac et al., 1995a). The numbers of TH+ dopamine neurons in the midbrain and of TH+ fibers in the striatum of MPTP-lesioned mice were also partially maintained by GDNF pretreatment. Recently, Tseng et al. (1997) implanted in rats capsules baby hamster kidney cells transfected with cDNA for rat GDNF that released ~5 ng of GDNF/d rostral to the substantia nigra 1 week before a medial forebrain bundle lesion. One week after the lesion, nearly 65% of the nigral dopamine neurons were found to have survived in animals receiving GDNF-producing capsules versus 27% survival in animals with control capsule implants. Gene therapy has also been used to deliver GDNF to dopamine neurons. Approximately 75% of the nigral TH+ neuronal population examined was preserved in rats injected with an adenoviral vector that transfected cells with GDNF DNA in the midbrain 7 d before a chronic 6-OHDA lesion (Choi-Lundberg et al., 1997). In comparison, dopamine neuronal survival ranged from 25 to 35% in animals receiving either no virus or adenovirus that did not encode biologically active GDNF. Thus, in at least two species and under different experimental approaches, GDNF pretreatment has been demonstrated to mediate consistently the survival of dopamine neurons from injuries induced by axotomy and neurotoxicity.

The window for the protective effects of GDNF may be wider for the 6-OHDA striatal lesion model. A striatal injection of 6-OHDA induces progressive axonal degeneration and loss of midbrain dopamine neurons over a period of weeks to months as opposed to death within minutes from a nigral injection (Sauer et al., 1995). Administration of GDNF in striatal 6-OHDA lesions from 1 d before to 7 d after the lesion has been demonstrated to partially preserve nigral dopamine neuronal number (Kearns and Gash, 1995; Sauer et al., 1995; Winkler et al., 1996). The problem with post-GDNF administration in the striatal lesion model is separating neuroprotective from neurorestorative effects.

Because GDNF can promote the survival of dopamine neuronal perikarya after their projections to the striatum have been lost (Beck et al., 1995; Winkler et al., 1996; Tseng et al., 1997), it is important to determine whether function has been maintained in the various experimental models used to study neuroprotection. In the present study, the neurochemical measures indicate that striatal function was preserved. GDNF recipients displayed significantly higher levels of dopamine and DOPAC in the striatum as well as in the substantia nigra 3 weeks after lesion than lesioned controls displayed. In rats pretreated with an intrastriatal injection of 10 μg of GDNF 24 hr before methamphetamine-induced injury to the nigrostriatal dopamine system, Cass (1996) found that reduction in striatal dopaminergic function, measured by potassium-evoked dopamine overflow, was prevented. In addition, Hebert et al. (1996) have shown that potassium-evoked dopamine release in the rat striatum is significantly increased 3 weeks after an intranigral injection of 10 μg of GDNF. Therefore, in addition to maintaining dopamine perikarya where striatal projections have been lesioned, GDNF can preserve and upregulate the function of residual dopaminergic processes in the striatum.

Dopamine levels in the substantia nigra of the GDNF-treated animals seemed higher than levels on the nonlesioned side. In both experiments in the present study in which cell numbers were counted, the number of TH+ neurons in the GDNF-pretreated, lesioned nigra also seemed to be higher than the number in the nonlesioned contralateral substantia nigra. This trend that has

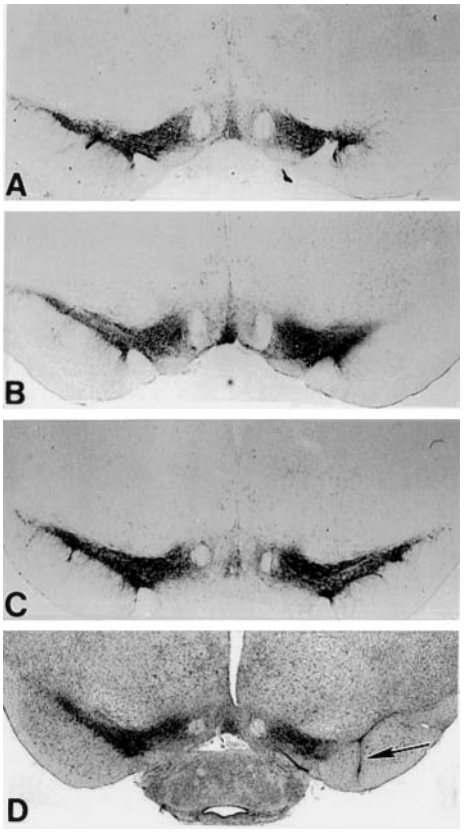

Figure 5. Representative sections processed for TH immunocytochemistry of all treated midbrains of the cycloheximide study. *A*, Animals that received intranigral injections of saline and citrate and 6-OHDA show extensive loss of midbrain dopamine neurons. *B*, Similarly, there is also a severe depletion of dopamine neurons in the substantia nigra of animals treated intranigrally with cycloheximide and citrate and 6-OHDA. *C*, In contrast, animals treated with intranigral injections of saline and GDNF and 6-OHDA show complete sparing of nigral dopamine neurons. *D*, However, when the rat midbrain is treated with cycloheximide and GDNF and 6-OHDA, there is a significant depletion of nigral dopamine neurons reflecting the inhibition of GDNF neuroprotection by cycloheximide. The needle track is shown (*arrow*) in *D* passing through the substantia nigra.

been reported before (see Kearns and Gash, 1995; Tseng et al., 1997) likely reflects on the ability of GDNF to increase both TH+ immunoreactive staining and dopamine levels in nigral dopamine neurons (Hudson et al., 1995; Gash et al., 1995). With regard to dopamine metabolism, DOPAC levels were considerably increased in the substantia nigra of GDNF-treated rats on both the lesioned (162%) and nonlesioned (170%) sides of the brain compared with the control side of the vehicle-treated animals. This result suggests that GDNF may have the potential to upregulate dopamine turnover in both ipsilateral and contralateral sides of the brain.

GDNF-induced neuroprotection against 6-OHDA toxicity requires protein synthesis. In animals receiving cycloheximide, the neuroprotective effects from GDNF pretreatment were greatly reduced. Cycloheximide inhibits protein synthesis by interfering with the formation of the peptide chain during translation (Tornheim et al., 1969). When 6-OHDA enters a dopamine neuron, it undergoes auto-oxidation, yielding compounds such as the superoxide radical, hydroxyl radical, and hydrogen peroxide (Cohen and Heikkila, 1974). These highly reactive oxygen species initiate cell destruction by lipid peroxidation and nucleic acid and protein degradation. One possible mechanism by which GDNF may elicit its neuroprotective effect is by enhancing the production of antioxidant enzymes (such as superoxide dismutase and glutathione peroxidase) that reduce the levels of these free radicals. Another possibility is that GDNF may be targeting the mechanisms involved in the modulation of internal cellular calcium levels such as calcium-binding proteins, specific ionic pumps, or calcium transporters. It has been postulated that abnormalities in internal calcium level homeostasis may be responsible for the selective neuronal degeneration of dopamine neurons in Parkinson's disease (De Erausquin et al., 1994).

In summary, GDNF induces significant changes in substantia nigra dopamine neurons within 6 hr of administration that protects them from 6-OHDA neurotoxicity. Based on the neurochemical measurements, levels of dopamine and DOPAC were also protected. Finally, the demonstration that protein synthesis is needed provides a preliminary basis for understanding the mechanisms underlying GDNF-induced neuroprotection in this experimental model of Parkinson's disease.

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