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Enhanced Glial Cell Line-Derived Neurotrophic Factor mRNA Expression Upon (−)-Deprenyl and Melatonin Treatments

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Glial cell line-derived neurotrophic factor (GDNF) has been shown to be a preferentially selective neurotrophic factor for dopamine (DA) neurons. In the present study, we have examined the distribution of GDNF mRNA expression in several major DA-containing cell body and terminal areas and the regulation of GDNF mRNA expression upon various pharmacological treatments. Results indicated that there is a relatively higher GDNF mRNA level in neurons of the nigrostriatal and mesolimbic dopaminergic pathways. Upon chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment (30 mg/kg, i.p., for 7 days), DA level was decreased, whereas GDNF mRNA expression was increased in the striatum, suggesting that more GDNF is synthesized and expressed to cope with the neurotoxin insult. Furthermore, among several DA neuron protective and/or therapeutic agents examined, both intrastriatal injections of (−)-deprenyl (1.25 μg and 2.5 μg) and melatonin (30 μg, 60 μg, and 120 μg) significantly enhanced GDNF mRNA expression in the striatum, whereas the same concentrations of (−)-deprenyl did not affect monoamine oxidase B (MAOB) activity, although it increased glutathione peroxidase (GPx) and/or superoxide dismutase (SOD) activities. Similarly, the same concentrations of melatonin did not alter SOD or GPx activities, except that the highest dose of melatonin (120 μg) increased lipid peroxidation in the striatum. Conversely, GM1 ganglioside injection (45 μg) lacked of an effect on GDNF mRNA expression. Together, these results suggest that both (−)-deprenyl and melatonin up-regulate GDNF gene expression at threshold doses lower than that needed for altering MAOB activity and/or the antioxidant enzyme systems, respectively. These results provide new information on the neuroprotective and therapeutic mechanisms of (−)-deprenyl and melatonin on DA neurons. J. Neurosci. Res. 53:593–604, 1998.

Key words: glial cell line-derived neurotrophic factor; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; (−)-deprenyl; GM1 ganglioside; melatonin; gene expression; striatum

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) is a growth factor that belongs to the transforming growth factor- superfamily, which shows preferential selectivity to dopamine (DA) neurons in the brain (Lin et al., 1993, 1994). Both in vitro and in vivo studies have shown that GDNF supports the survival of DA neurons and protects DA neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) toxicity (Kearns and Gash, 1995; Hou et al., 1996; Shults et al., 1996). When genetically engineered cells that contain GDNF are transplanted into 6-OHDA-lesioned rat brain, they survive and interact with tyrosine hydroxylase-positive neuronal fibers (Lindner et al., 1995). Furthermore, when GDNF is administered after MPTP administration, it restores DA levels and fiber densities in mice (Tomac et al., 1995a) and recovers dopaminergic function in monkeys (Gash et al., 1996). Although regional distribution studies have revealed the presence of GDNF mRNA throughout the central nervous system (Springer et al., 1994; Choi-Lundberg and Bohn, 1995), the relative distribution of GDNF mRNA in different DA-containing neurons in the brain is not known. The first part of the present study examined this distribution.

R(−)-deprenyl is known as a monoamine oxidase B (MAOB) inhibitor that binds to MAOB and irreversibly inhibits the enzyme (Youdim, 1978; Heinonen and Lammintausta, 1991), which consequently accumulates DA in

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neuronal tissues. Clinical trials have demonstrated that (−)-deprenyl delays the need for levodopa treatment in Parkinson's disease (Parkinson Study Group, 1993a,b). Animal studies with (−)-deprenyl have also shown that pretreatment with (−)-deprenyl protects DA neurons against the toxicity of MPTP. When it is administered after MPTP treatment, it also has a satisfactory therapeutic effect (Lee et al., 1994). More recently, in addition to its action as an MAOB inhibitor, deprenyl was found to suppress the hydroxyl radical formation and to exert antioxidant action in rescuing DA neurons (Wu et al., 1996; Thomas et al., 1997). Similarly, melatonin was suggested to be a free-radical scavenger and an antioxidant (for reviews, see Reiter 1995, 1996). Recent research indicates that prior administration of melatonin suppresses the increase in lipid peroxidation (LP) caused by MPTP and prevents MPTP-induced decrease in tyrosine hydroxylase immunoreactivity (Acuna-Castroviejo et al., 1997). On the other hand, glycosphingolipids are important constituents of vertebrate cell membrane and have been proposed to play a critical role in central nervous system growth and function (Ledeen, 1985). The monosialoganglioside GM1 is one of glycosphingolipids and has been shown to induce functional recovery upon nerve injury both behaviorally and neurochemically, particularly within the damaged dopaminergic system (Toffano et al., 1983; Sabel et al., 1985; Li et al., 1986; Skaper et al., 1989). With regard to the present study, GM1 ganglioside was found to promote the recovery of surviving DA neurons and enhanced dopaminergic innervation in MPTP-treated monkeys (Schneider et al., 1992; Herrero et al., 1993). Furthermore, when GM1 ganglioside was combined with basic fibroblast growth factor or epidermal growth factor (EGF), it produced a greater restoration of DA concentration in MPTP-treated mice (Schneider and DiStefano, 1995).

With the above-described neuron-rescuing and/or protective actions that have been observed with (−)-deprenyl, melatonin, and GM1 ganglioside, whether they also act through the GDNF signaling pathway is not known. The aim of the present study was to examine the effects of these compounds on GDNF mRNA expression in rat striatum. Meanwhile, their effects on the antioxidant enzyme systems, lipid peroxidation, and/or MAOB activity were also assayed.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (250–350 g) that were bred in the Institute of Biomedical Sciences, Academia Sinica (Taipei, Taiwan) were used in the present study. They were maintained on a 12/12 hr light/dark cycle (light on at 6:30 am) in a temperature-regulated room (23 ± 2°C) with food and water continuously available.

Drugs

MPTP hydrochloride, deprenyl, and GM1 ganglioside were purchased from Research Biochemical, Inc. (Wayland, MA). Melatonin was purchased from Sigma (St. Louis, MO). RNA isolation kit was purchased from Biotecx Laboratories, Inc. (Houston, TX). DNase, RNase inhibitor (RNasin), dNTP, avian myeloblastosis virus reverse transcriptase (AMVRT), and Taq polymerase were purchased from Promega (Madison, WI). Primer pairs for GDNF mRNA were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX).

Surgery and Drug Administration

For MPTP administration, one injection per day of MPTP (30 mg/kg, i.p.) was administered to rats continuously for 7 days. Animals were killed on the day 8, and the brains were removed. The striatal tissue was further punched out for GDNF mRNA determination, and the substantia nigra was punched out for DA assay. For other drug injections, rats were subjected to stereotaxic surgery under sodium pentobarbital anesthesia (40 mg/kg, i.p.). Twenty-three-gauge stainless-steel thin-wall cannulae (10 mm long) were implanted bilaterally into the striatum and affixed on the skull with dental cement. The coordinates for the striatum are: anteroposterior (AP) +1.0 mm from Bregma, mediolateral (ML) ±2.9 mm from the midline, and dorsoventral (DV) − 4.5 mm below the skull surface. The tooth bar was set at − 2.4 mm. Approximately 1 week after recovery from the surgery, deprenyl (1.25 μg, 2.5 μg, and 5.0 μg), GM1 ganglioside (45 μg), or melatonin (30 μg, 60 μg, and 120 μg) was infused directly into the striatum at a rate of 1.0 μl/min (1.2 μl for each side). Rats were decapitated 30 min after drug infusion; their brains were taken out, and the striatal tissue was dissected out for GDNF mRNA determination.

High-Performance Liquid Chromatography Analysis

Concentration of DA was estimated by high-performance liquid chromatography (HPLC) with fluorescence detection. The chromatographic system used was a 5-μm Ultrasphere ODS reverse-phase column (4.6 mm by 15 cm; Beckman, CA) with a Waters pump (model 501; Millipore Corp., Bedford, MA) and a Shimadzu RF 530 spectromonitor (Shimadzu, Japan): Excitation and emission wavelengths were set at 290 nm and 330 nm, respectively. The flow rate was maintained at 1.2 ml/min, and the sensitivity of the detector was set at 2 nA/V. The mobile phase consisted of 1.8 g/liter potassium-phosphate monobasic containing 0.66 g/liter of 1-heptanesul-

fonic acid sodium salt, pH 3.28, and 180 ml/liter of methanol. Output was recorded with a Shimadzu C-RIB integrator. DA was estimated according to the method of Peat and Gibb (1983) with some modifications. Briefly, tissue was weighed while it was still frozen and was homogenized in six volumes of 0.1 N perchloric acid containing 4 mM sodium metabisulfite. The homogenate was then centrifuged at 12,000 *g* for 15 min, and the clear supernatant (20 µl) was injected directly into the chromatographic system. DA was resolved around 5.2 min.

Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis

Because of the minute amount of GDNF mRNA level in the brain, the reverse transcription polymerase chain reaction (RT-PCR) method was adopted in the present study. Total RNA was prepared according to the method of Chomczynski and Sacchi (1987). Briefly, tissue was homogenized with 1 ml of RNA isolation reagent. The homogenate was then added with 0.2 ml of chloroform and centrifuged at 12,000 *g* for 15 min. The aqueous phase was removed and added with equal volume of isopropanol. The mixture was then stored on ice for 10 min and centrifuged again at 12,000 *g* for 15 min. The RNA was precipitated by centrifugation and washed twice with 75% ethanol. After that, total RNA was briefly dried under vacuum and then dissolved in diethylpyrocarbonate (DEPC)-treated H_2O.

To avoid DNA contamination, DNase treatment was applied. The 20-µl reaction solution contained 0.1 U/µl DNase, 0.2 U/µl RNasin, *Taq* buffer, and 20 µg total RNA. This mixture was incubated at 37°C for 30 min and then extracted by phenol/chloroform (3:1). The aqueous phase was recovered by centrifugation (12,000 *g* for 15 min) and was added with 1:10 volume of 2.5 mM sodium acetate, pH 5.01, and 2 volumes of 95% ethanol. The mixture was then placed in a − 20°C freezer overnight. Finally, total RNA was precipitated by centrifugation and washed with 75% ethanol. It was then dried and dissolved in DEPC-treated H_2O and quantified by using a spectrophotometer (U-2000; Hitachi, Japan) at 260 nm wavelength.

Variable amounts of total RNA were reverse transcribed by avain myeloblastosis virus reverse transcriptase (AMVRT; 8U) at 42°C with 0.5 µg oligo-dT as the primer in a 20-µl reaction buffer containing 50 mM Tris-HCl, pH 8.3; 50 mM KCl; 10 mM $MgCl_2$; 10 mM dithiothreitol; 0.5 mM spermidine; 1 mM dNTPs; and 1 U/µl of RNasin. After 1 hr, the AMVRT was heat inactivated at 95°C for 5 min. For PCR quantitation, the endogenously expressed mRNA for hypoxanthine phosphoribosyltransferase (HPRT; Jansen et al., 1992) was used as the internal control, which was coamplified along with the GDNF mRNA. The RT products were incubated with 0.4 µM of primer sets and 1 U of Taq polymerase in a 20-µl reaction mixture containing *Taq* buffer; 1.5 mM $MgCl_2$; 200 µM each of dATP, dTTP, and dGTP; 100 µM dCTP; and 5 µCi [^{35}S] dCTP. To increase the specificity of PCR amplification, a touch-down program was designed for the conditions of denaturing (95°C, 2 min), annealing (three cycles each at 54°C and 51°C in order and then 24 cycles at 48°C, 30 sec), and polymerization (72°C, 1 min). A final 10-min incubation at 72°C was carried out after these 30 cycles of PCR. Aliquots from PCR reaction were electrophoresed through an 8% polyacrylamide gel. The gel was dried and subjected to phosphorimager analysis (Molecular Dynamics, Eugene, OR). The gel was then exposed to x-ray film (Eastman Kodak, Rochester, NY). Pilot experiments were performed to determine the range of PCR cycles in which amplification efficiency remained constant and to demonstrate that the amount of amplified PCR product was also directly proportional to the amount of input RNA.

Primer Pairs for PCR

The oligonucleotide primer pairs for PCR were designed by using the a computer program from Lowe et al. (1990). Murine gene sequences for GDNF and HPRT were obtained from GenBank (Genetic Computer Group). The primer pairs for GDNF and HPRT were designed to span introns. The sequences of primer pairs were as follows: 1) GDNF: 5′ primer, 5′-TGGGATGTCGTGGCTGTCTG-3′; 3′ primer, 5′-CCTCCTTGGTTTCGTAGCCC-3′; product size, 406 base pairs (bp); 2) HPRT: 5′ primer, 5′-CTCTGTGTGCTGAAGGGGGG-3′; 3′ primer, 5′-GGGACGCAGCAACTGACATT-3′; product size, 625 bp.

Measurement of Lipid Peroxidation

Malonaldehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE) which are indices of lipid peroxidation, were measured by using a colorimetric assay kit (LPO-586, CALBIOCHEM, San Diego, CA). Brain tissues were sonicated in an ice bath in 20 volumes (weight/volume) of 0.1 M phosphate buffer, pH 7.8. The homogenates were centrifuged at (1,000 *g* for 10 min. Aliquots of the supernatants were reacted with reagents, including N-methyl-2-phenylindole, methanol, and methanesulfonic acid, at 45°C for 40 min. This reaction was stopped by chilling samples on ice. These samples were then centrifuged at (12,000 *g* for 5 min. The resulting supernatants were measured at 586 nm, and concentrations of MDA and 4-HNE were calculated from a standard curve with known amounts of MDA or 4-HNE standards. The apparent molar extinction coefficient (ϵ) of the measured product is equal to the slope of standard curve. Furthermore, the ϵ values of MDA or 4-HNE are not significantly different from one another.

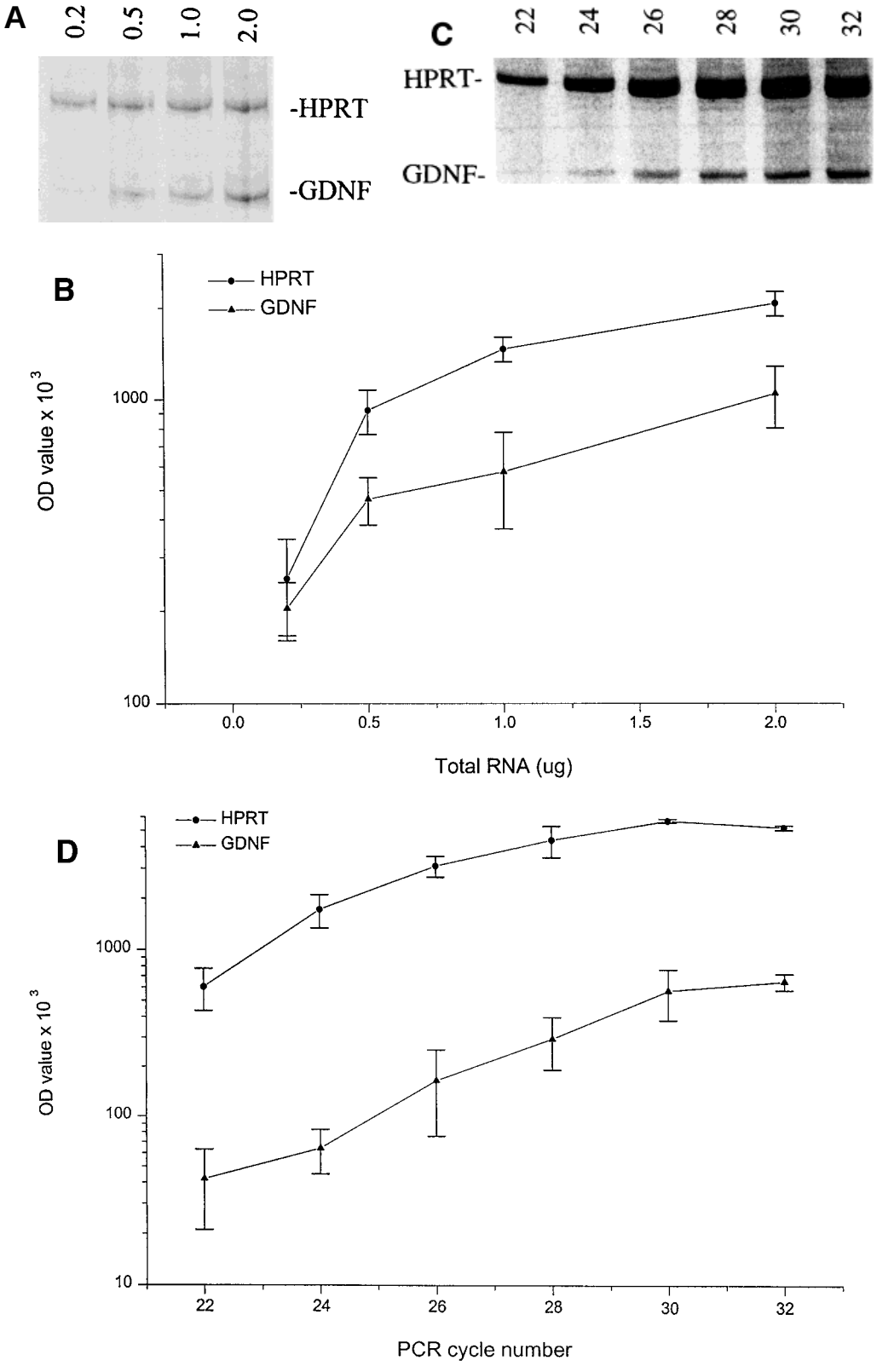
A
0.2
0.5
1.0
2.0
-HPRT
-GDNF
C
22
24
26
28
30
32
HPRT-
GDNF-
B
HPRT
GDNF
OD value x 10^3
1000
100
0.0
0.5
1.0
1.5
2.0
Total RNA (ug)
D
HPRT
GDNF
OD value x 10^3
1000
100
10
22
24
26
28
30
32
PCR cycle number

Figure 1.

Measurement of GPx Activity

Selenium-dependent peroxidase activity was measured by using a coupled enzyme procedure with glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH; Flohe and Gunzler, 1984). Tissue homogenates were centrifuged at (12,000 *g* for 15 min. Aliquots of the supernatants were added with 0.1 M phosphate buffer, pH 7.8, containing 1 mM NaN_3, 1 mM glutathion, 0.2 mM NADPH, and 0.24 U GSH reductase. Reaction was started by adding 100 (l of 2.5 mM H_2O_2 (total reaction volume was 1 ml) and was measured at 340 nm for 5 min. Units of enzyme activity were calculated by using the (value of 6.22 $mM^{-1}cm^{-1}$ for NADPH. One unit of GPx activity is defined as 1 (mol NADPH consumed/min.

Measurement of SOD Activity

SOD activity was assayed according to the method of Misra and Fridovich (1972). Tissue homogenates were centrifuged at (1,000 *g* for 10 min. Aliquots of the supernatants were added with 0.1 M phosphate buffer, pH 7.8, containing 0.2 mM xanthine and 0.3 mM epinephrine. Xanthine oxidase was diluted appropriately, so that an assay mixture without SOD source increased OD value 0.03 per min at 320 nm. Reaction was started by the addition of xanthine oxidase (total reaction volume was 1 ml), and absorbance was measured at 320 nm continuously for 3 min. Fifty percent inhibition of epinephrine oxidation is defined as 1 unit of enzyme activity.

Measurement of MAOB Activity

The activity of MAOB was assayed radioenzymatically, as described by Kindt et al. (1988), with minor modifications. Briefly, [^{14}C] benzylamine was used as the substrate for MAOB. Enzyme activity was expressed as nanomole substrate oxidized/hr/mg protein. Blank values were obtained by adding to the incubating mixture 10 μM (−)-deprenyl to inhibit MAOB. The striatal tissue was sonicated in an ice bath in 10 volume (weight/volume) of 0.3 M ice-cold sucrose containing 10 mM phosphate buffer, pH 7.4. The homogenate was centrifuged at 1,000 *g* for 10 min. Duplicate aliquots containing 100–200 μg of protein were incubated with 0.1 mM benzylamine (containing 0.25 μCi [^{14}C] benzylamine, 59 mCi/mmol) in 0.05 M potassium phosphate buffer, pH 7.4, at a final volume of 0.5 ml for 10 min at 37°C. The reaction was terminated by adding 5 ml of toluene:ethylacetate (1:1, volume/volume) and vortexing for 30 sec. The samples were centrifuged at (400 *g* for 5 min to separate the organic and aqueous phases. Then, 3 ml from the organic layer were removed, added to 5 ml of Beckman Ready Safe Liquescent (Palo Alto, CA), and analyzed by using liquid scintillation spectrometry. The protein content in the brain tissue was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Protein Determination

Protein concentration was determined by using the method of Bradford (1976) with bovine serum albumin as standard.

Statistics

The results were analyzed with a Student's t-test or with a one-way analysis of variance followed by Dunnett's t-test for comparisons between the experimental group and a common control group.

RESULTS

Quantitative RT-PCR in Determining GDNF and HPRT mRNA Levels

The quantitative RT-PCR analyses yielded a linear relationship between the OD value and the amount of total RNA ranging from 0.2 μg to 2.0 μg for both primer sets (Fig. 1A–D). Similarly, there was a linear relationship between the OD value and the PCR cycle number ranging from 22 to 32. Based on these results, 0.5 μg total RNA and a PCR cycle number of 30 were used for further experiments.

Distribution of GDNF mRNA Level in Different Brain Regions

The preferential, selective effect of GDNF on DA neurons made us interested in examining the GDNF mRNA level in different DA-containing areas. Figure 2 shows that statistical analysis revealed an overall significant effect in GDNF mRNA regional distribution ($F = 4.30$; $P < 0.01$). Further analyses indicated that the GDNF mRNA level is higher in general in neurons along

Fig. 1. Calibration and quantification of glial cell line-derived neurotrophic factor (GDNF) mRNA for the reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Total RNA was isolated from the hippocampus of rat and was reverse-transcribed and amplified by PCR. **A:** Representative x-ray film of reaction using primer pairs of GDNF and hypoxanthine phosphoribosyltransferase (HPRT) for coamplification with different amounts (μg) of total RNA. **B:** Quantitative results of A from two independent experiments. Data are mean (SEM. **C:** Representative x-ray film of reaction also with GDNF and HPRT primer pairs but with different PCR cycle numbers. **D:** Quantitative results of C also from two independent experiments. Data are mean (SEM.

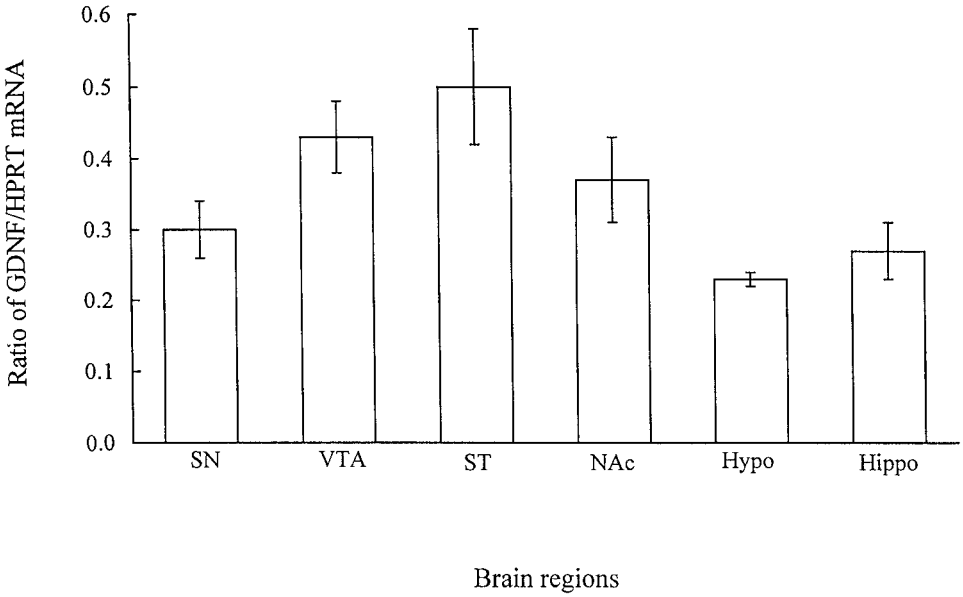

Fig. 2. Quantitative analyses of GDNF mRNA expression in several dopamine (DA)-containing cell body and terminal regions. Five independent assays were performed in each area. Data are expressed as mean ± SEM. SN, substantia nigra; VTA, ventral tegmental area; ST, striatum; NAc, nucleus accumbens; Hypo, hypothalamus; Hippo, hippocampus.

the nigrostriatal and mesolimbic dopaminergic pathways. The order from the most abundant to the least expressed area is striatum > ventral tegmental area > nucleus accumbens > substantia nigra > hippocampus > hypothalamus.

Effects of Chronic MPTP Injection on GDNF mRNA Expression

Figure 3A shows the autoradiographic results of GDNF mRNA expression in the striatum upon chronic MPTP injection. Figure 3B shows that MPTP significantly increased GDNF mRNA level ($t = 2.33$; $P < 0.05$). Meanwhile, MPTP markedly decreased DA concentration in the corresponding cell body area, the substantia nigra ($t = 1.70$; $P = 0.05$).

Effects of (−)-Deprenyl, GM1 Ganglioside, and Melatonin on GDNF mRNA Expression

Figure 4A illustrates the representative autoradiographic results of GDNF mRNA expression in the striatum upon various doses of (−)-deprenyl, GM1 ganglioside, and melatonin injections into this area. Figure 4B shows that statistical analysis indicated an overall significant effect of drug treatment ($F = 6.27$; $P < 0.01$). Further analyses revealed that (−)-deprenyl both at 1.25 μg and at 2.5 μg significantly increased GDNF mRNA level in the striatum ($tD = 3.24$, $P < 0.01$ and $tD = 3.61$, $P < 0.01$, respectively). However, (−)-deprenyl at a higher dose (5 μg) was without such an effect ($tD = 0.27$; $P < 0.05$). Similarly, melatonin both at 60 μg and at 120 μg markedly elevated GDNF mRNA level in the striatum ($tD = 3.78$, $P < 0.01$ and $tD = 4.73$, $P < 0.01$, respectively). Melatonin at 30 μg was without such an effect ($tD = 0.68$; $P > 0.05$). On the other hand, GM1 at 45 μg did not affect GDNF mRNA expression in the striatum ($tD = 1.36$; $P > 0.05$).

Effects of (−)-Deprenyl and Melatonin on SOD, GPx Activities, Lipid Peroxidation, and/or MAOB Activity in the Striatum

We have examined the antioxidant enzyme activities, and lipid peroxidation, and/or MAOB activity upon various doses of (−)-deprenyl and melatonin treatments. Table I shows that separate sets of one-way analysis of variance revealed that there was not an overall significant effect of drug treatment on SOD activity ($F = 1.71$; $P > 0.05$). Further analyses indicated that (−)-deprenyl at 5.0 μg markedly elevated SOD activity in the striatum ($tD = 2.86$; $P < 0.05$). On the other hand, there was an overall significant drug effect on GPx activity ($F = 4.40$; $P < 0.01$). Further analyses revealed that this was due to (−)-deprenyl treatment, because (−)-deprenyl at 2.5 μg significantly elevated GPx activity in the striatum ($tD = 4.77$; $P < 0.01$). Moreover, there was an overall significant effect on lipid peroxidation measure ($F = 4.52$; $P < 0.01$). Further comparisons indicated that this was due to melatonin treatment at 120 μg, because this dose mark-

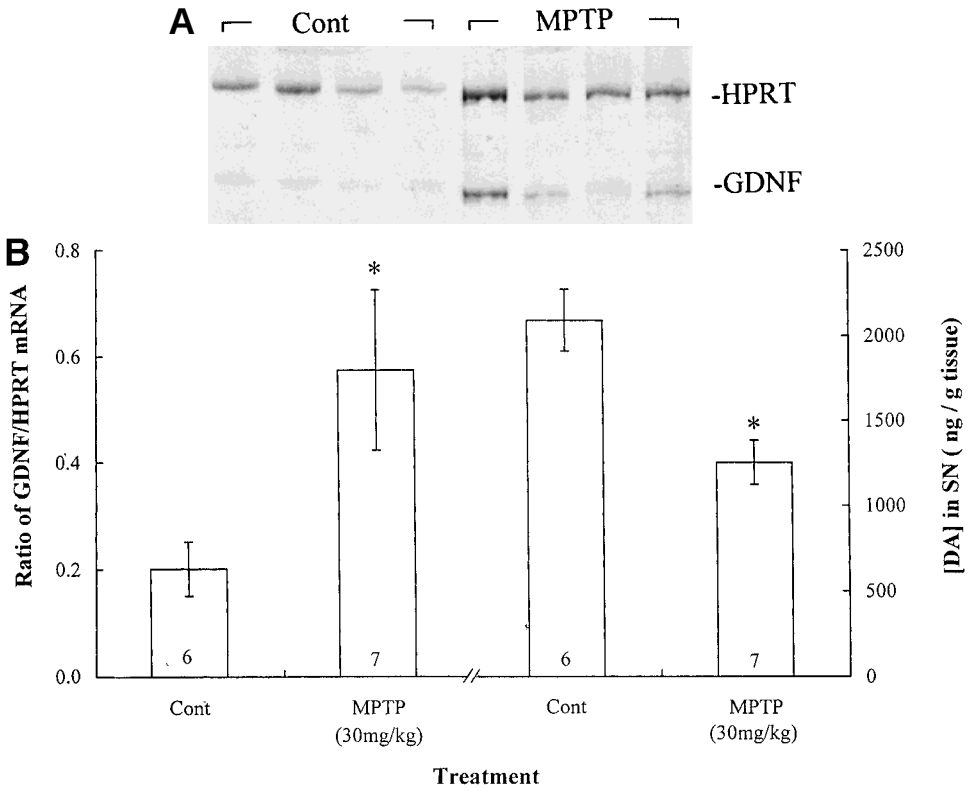

Fig. 3. Alterations in GDNF mRNA expression in the striatum following subchronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment. The amount of GDNF mRNA was expressed relative to that of HPRT. **A:** Representative x-ray film. **B:** Quantitative results of A are shown on the left. The right shows a decreased DA level in the substantia nigra upon MPTP treatment. Data are expressed as mean ± SEM. Asterisk indicates $P < 0.05$ compared with the corresponding control group. The number in each column indicates animal number.

edly increased lipid peroxidation in the striatum (tD = 3.68; $P < 0.01$). For MAOB activity measure, no treatment at any dose affected MAOB activity in the striatum ($F = 0.97$; $P > 0.05$).

DISCUSSION

Results of the present study demonstrated that GDNF mRNA expression is present in various DA-containing nuclei and terminal areas in the brain, with the nigrostriatal and mesolimbic dopaminergic neurons showing relatively higher distributions. These results are consistent with many reports that have suggested the role of GDNF as a selective neurotrophic factor for DA neurons (Lin et al., 1993, 1994). Furthermore, we have found that the striatal astrocyte culture expressed little GDNF mRNA (unpublished observation). This finding is consistent with the observation that the GDNF mRNA is expressed mainly in neurons (Pochon et al., 1997). The regional distribution results also revealed that the ventral tegmental area expressed more GDNF mRNA than the substantia nigra. Similar results were found with brain-derived neurotrophic factor (BDNF), another dopaminergic neurotrophic factor in which expression is also higher in the ventral tegmental area than in the substantia nigra (approximately 1.7 fold; Hung and Lee, 1996). These results suggest that DA neurons in the ventral tegmental area are better protected than DA neurons in the substantia nigra. This suggestion may also explain partially the differential vulnerability of the nigrostriatal and mesolimbic dopaminergic pathways upon MPTP toxicity and in Parkinson's disease (Jacobowitz et al., 1984; Schneider et al., 1987; Hirsch et al., 1988). However, at present, we do not know the origin of GDNF mRNA in the nondopaminergic neurons. GDNF may be synthesized locally in the striatum and nucleus accumbens and may be transported retrogradely to the substantia nigra and ventral tegmental area to support DA neurons (Tomac et al., 1995b).

We have also found that chronic MPTP injections decreased DA concentration in the substantia nigra, whereas it significantly increased GDNF mRNA expression in the striatum. These results suggest that, upon dopaminergic damage, more GDNF is synthesized and

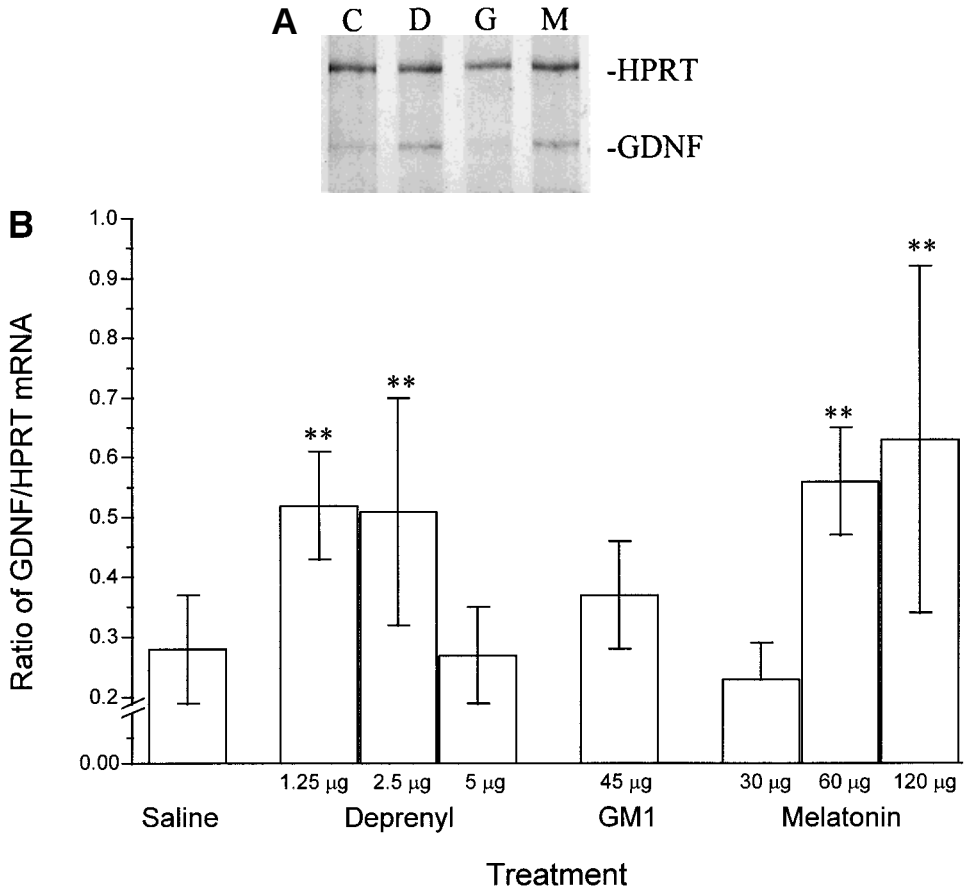

Fig. 4. Alterations in GDNF mRNA expression in the striatum upon (−)-deprenyl, GM1 ganglioside, and melatonin injections into this area. The amount of GDNF mRNA was expressed relative to that of HPRT. **A:** Representative x-ray film. C, control; D, (−)-deprenyl 2.5 μg; G, GM1 ganglioside 45 μg; M, melatonin 60 μg.**B:** Quantitative results of A. Data are expressed as described in Figure 3B. Asterisks indicate $P < 0.01$ compared with the control group.

expressed in the striatum: It is then transported retrogradely to the substantia nigra (Tomac et al., 1995b) to cope with the environmental insult. Similar results were found with BDNF, in which expression is also enhanced upon MPTP and MPP^+ treatments (Hung and Lee, 1996). However, at present, we do not know which signals were sent to those neurons and/or the to surviving DA neurons to express more GDNF and BDNF mRNAs upon DA neuron damage. This awaits clarification.

(−)-Deprenyl has been reported previously to inhibit MAOB activity and to reduce free-radical formation. Upon neurotoxin insults, such as MPTP, it protects and rescues the dying DA neurons, possibly through the mechanisms described above (for review, see Magyar et al., 1996). However, in the present study, we found that direct injections of (−)-deprenyl into the striatum at concentrations that did not significantly affect MAOB activity in this area (1.25 μg and 2.5 μg doses) markedly enhanced GDNF mRNA expression almost twofold. These results suggest a new pharmacological action of (−)-deprenyl in addition to its action as an MAO inhibitor. Indeed, we have found that the same concentrations of (−)-deprenyl also increased BDNF mRNA level in the striatum (unpublished observation). In another study, Seniuk et al. (1994) reported similarly that (−)-deprenyl augmented ciliary neurotrophic factor (CNTF) gene expression in injured astrocytes. Moreover, Ansari et al. (1993) reported that (−)-deprenyl rescued axotomized rat facial motoneurons independent of MAO inhibition. However, in this study, we also found that the highest dose of (−)-deprenyl examined (5 μg) lacked of an effect on GDNF mRNA expression. The reason for this is not known yet. It is possible that, at higher concentrations, the pharmacological action of (−)-deprenyl switches to other mechanisms. This speculation is supported by the examination of the effects of (−)-deprenyl on antioxidant

TABLE I. Effects of (−)-Deprenyl and Melatonin on Superoxide Dismutase and Gluthathione Peroxidase Activities, Lipid Peroxidation, and Monoamine Oxydase B Activity in Rat Striatum (n = 5–12 in each group)

Treatment	SOD (U/mg protein)[a]	GPx (U/mg protein)	LP (nmol/mg protein)	MAOB (nmol/mg protein/min)
Saline (1.2 µl)	5.29 ± 0.61	1.26 ± 0.30	1.17 ± 0.02	1.55 ± 0.07
(−)-Deprenyl (1.25 µg)	6.16 ± 0.15	1.78 ± 0.07	0.75 ± 0.03	1.34 ± 0.12
(−)-Deprenyl (2.5 µg)	5.40 ± 0.20	2.73 ± 0.03**	1.13 ± 0.03	1.56 ± 0.13
(−)-Deprenyl (5 µg)	6.63 ± 1.47*	1.40 ± 0.08	1.28 ± 0.38	1.35 ± 0.16
Melatonin (30 µg)	6.17 ± 1.27	1.31 ± 0.07	1.63 ± 0.14	
Melatonin (60 µg)	5.99 ± 0.60	1.36 ± 1.16	1.22 ± 0.08	
Melatonin (120 µg)	6.07 ± 1.40	1.67 ± 0.30	2.06 ± 0.92**	

[a]SOD, superoxide dismutase; GPx, glutathione peroxidase; LP, lipid peroxidase; MAOB, monoamine oxydase B. Data are expressed as mean ± SEM. Statistical significance was evaluated by analysis of variance followed by Dunnett's t-test. U is defined as one unit of the amount that reduces the absorbance change by 50% per minute in SOD assay. U is defined as 1 µmole nicotinamide adenine dinucleotide phosphate oxidized per minute in GPx assay.
*$P < 0.05$.
**$P < 0.01$.

enzyme systems, because (−)-deprenyl at 2.5 µg and 5.0 µg, but not 1.25 µg, markedly increased GPx and/or SOD activity (Table I). These results are consistent with the finding that chronic (−)-deprenyl treatment increases SOD activity (Knoll, 1989; Clow et al., 1991). These results are also in line with the report that (−)-deprenyl reduces hydroxyl radical formation produced by DA autoxidation (Wu et al., 1996). These results suggest that (−)-deprenyl can readily remove the superoxide molecule in the cell to prevent the possible occurrence of Fenton reaction. However, this observation is not consistent with that of Carrillo et al. (1991), who reported that (−)-deprenyl altered SOD activity but not GPx activity in rat striatum. At present, we do not have an explanation for this discrepancy; however, in their study, Carrillo et al. adopted a chronic injection regimen, whereas we used the acute injection paradigm. Nonetheless, the present results demonstrate that, other than functioning as an MAO inhibitor and a free-radical scavenger, (−)-deprenyl also enhanced GDNF mRNA expression to protect DA neurons, and the concentration of (−)-deprenyl that was needed to enhance GDNF mRNA expression was lower than that needed for its antioxidant action and for MAO inhibition.

Other than its well-known role in reproductive and circadian physiology, in recent years, melatonin was also found to act as a free-radical scavenger and an antioxidant (for reviews, see Reiter et al., 1995; Reiter 1996). In a cell-free in vitro system, Poeggeler et al. (1994) have demonstrated that, by adding exogenous melatonin to the system, it reacts with the hydroxyl radical to form a much less reactive indolyl cation radical: It therefore reduces the toxic environment of a cell. Furthermore, Tan et al. (1993) have reported that melatonin was also able to inhibit DNA damage caused by the chemical carcinogen safrole, which is believed to exert its toxicity through free-radical production in animals. More related to the present study, melatonin was found to decrease lipid peroxidation produced by MPTP and to protect DA neurons against MPTP toxicity, and these effects are probably due to the antioxidant action of melatonin (Acuna-Castroviejo et al., 1997). However, in the present study, we found that direct injections of melatonin into rat striatum enhanced GDNF mRNA expression in a dose-dependent manner in this area. Most of these injections did not affect SOD, GPx, or lipid peroxidation, except that the highest dose of melatonin (120 µg) increased lipid peroxidation in the striatum. The mechanism for this action is not known at present, although it is possible that, at such a high dose, some nonspecific, physiological effects may occur that may cause cell damage indirectly. Together, the above results suggest that melatonin has multiple protective mechanisms on DA neurons and that the threshold dose for melatonin to increase GDNF mRNA expression is lower than that for functioning as a free radical scavenger. These results also provide new information on the therapeutic aspect of melatonin for disorders associated with DA neuron degeneration.

The monosialoganglioside, GM1 ganglioside, was shown to modulate neuronal differentiation, neuronal development, and synaptic plasticity (Nagai and Tsuji, 1988). More related to the present study, GM1 ganglioside was shown to rescue the injured DA neurons and to recover the dopaminergic neuronal function in monkeys (Schneider et al., 1992; Herrero et al., 1993). Furthermore, when GM1 ganglioside was coadministered with neurotrophic factors, such as BDNF (Fadda et al., 1993) and EGF (Schneider and DiStefano, 1995), it protected DA neurons to a greater extent. These results prompted our interest in examining the effect of GM1 ganglioside

on GDNF gene expression, because GDNF shows preferential selectivity to DA neurons. However, recently, we have found that direct injection of GM1 ganglioside into the rat striatum did not affect GDNF mRNA expression, and it did not alter BDNF mRNA expression either (unpublished observation). These results suggest that GM1 ganglioside probably does not act through enhancing neurotrophic factor gene expression to protect DA neurons. However, these results do not rule out the possibility that an interaction between GM1 ganglioside and neurotrophic factor does occur. For example, GM1 ganglioside may interact with the neurotrophic factor signaling pathways to potentiate their effects. This explanation is supported by the finding that GM1 ganglioside increased tyrosine kinase nerve growth factor receptor autophosphorylation (Ferrari and Greene, 1996). Moreover, in most studies, GM1 treatment has been administered as a chronic regimen, whereas, in the present study, we adopted the acute-injection paradigm. The relatively long treatment duration that is required for GM1 ganglioside to be effective may explain partially the lack of an effect of GM1 ganglioside on GDNF and BDNF mRNA expression in the present study. Nevertheless, the cellular and molecular mechanisms underlying the pharmacological actions of GM1 ganglioside need to be elucidated.

In summary, in the present study, we have found that GDNF mRNA expression is present in the major DA-containing cell body and terminal areas and, along with the ventral tegmental area, has a higher expression level than the substantia nigra. Chronic MPTP treatment decreased the DA level in the substantia nigra, whereas it significantly increased GDNF mRNA expression in the striatum. These results suggest that neurons in the striatum may synthesize and express more GDNF so that the surviving DA neurons can cope with the environmental insult. Investigations of the dopaminergic protective agents indicate that intrastriatal injections of (−)-deprenyl and melatonin both significantly up-regulated GDNF mRNA expression, whereas GM1 ganglioside was without such an effect. Furthermore, at the effective concentrations in enhancing GDNF expression, (−)-deprenyl did not alter MAOB activity but increased GPx and/or SOD activity at higher doses. Similarly, at the effective doses of melatonin for enhancing GDNF expression, it did not alter SOD and GPx activities or lipid peroxidation, except at the highest dose examined. These results suggest a new protective mechanism of (−)-deprenyl and melatonin other than their roles as MAO inhibitors and/or antioxidants. In addition to the neuroprotective action of GDNF on DA neurons, accumulative evidence has further implicated the therapeutic potential for GDNF in treating disorders associated with DA neuron dysfunction, such as Parkinson's disease (for reviews, see Lapchak et al., 1996, 1997). Although the GDNF-RET receptor signaling pathway has been reported (Mason, 1996), and there is evidence indicating that concurrent activation of the cAMP-dependent signaling pathway is needed for GDNF to manifest its function (Engele and Franke, 1996), the exact cellular and molecular mechanisms underlying the pharmacological actions of GDNF are not well known yet. Further investigations are ongoing in this laboratory to elucidate these mechanisms.

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