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GM1 Ganglioside Improves Dopaminergic Markers of Rat Mesencephalic Cultures Treated with MPP+

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Rat embryonic mesencephalic cultures were employed to evaluate the consequences of adding GM1 ganglioside to cultures lesioned with the selective neurotoxin 1-methyl-4-phenylpyridinium (MPP+). MPP+ reduced dopamine and DOPAC content, dopamine uptake, aromatic L-amino acid decarboxylase activity, and the number of tyrosine hydroxylase–immunopositive neurons. The immunopositive neurons that remained were aberrant. All of these parameters were partially restored by adding GM1 ganglioside to the cultures. The response to GM1 was not altered by prior treatment of the cultures with cytosine β-D-arabinofuranoside to reduce the number of glial cells. Dopamine uptake activity restored by GM1 was lost if GM1 was removed from the culture.

[Key words: GM1 ganglioside, mesencephalic cultures, dopaminergic neurons, neurotrophic activity, MPP+ (1-methyl-4-phenylpyridinium), Parkinson's disease]

There is now substantial evidence that GM1 ganglioside (II^3NeuAc-GgOse$_4$Cer) treatment improves the outcome of a variety of CNS insults when evaluated using *in vivo* or *in vitro* models (Ledeen, 1984). The mechanism(s) involved is unclear, though there is evidence that GM1 inserts into cell membranes (Toffano et al., 1980; Ghidoni et al., 1989) and may prevent Ca^{2+} influx from reaching toxic concentrations within cells (Vaccarino et al., 1987; Favaron et al., 1988; de Erausquin et al., 1990) and/or may support the activity of endogenous neurotrophic substances (Hakomori et al., 1990).

MPP+ (1-methyl-4-phenylpyridinium ion), formed in glial cells by monoamine oxidase B, is the neurotoxic product of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) metabolism in brain (Heikkila et al., 1984a,b, 1985; Nicklas et al., 1985). MPP+ selectively concentrates in dopaminergic neurons (Javitch et al., 1985), where it disrupts mitochondrial energy metabolism (Singer et al., 1988), resulting in neuronal death. MPTP has been used to induce model parkinsonism in animals. We have previously demonstrated that GM1 can facilitate the recovery of dopaminergic neurochemical, morphological, and behavioral markers in rodents treated with MPTP (Hadjiconstantinou et al., 1986, 1989a,b; Hadjiconstantinou and Neff, 1988, 1990; Weihmuller et al., 1988, 1989). These studies were recently extended to primates (Schneider et al., 1992). In this report, we show that GM1 can apparently rescue dopaminergic neurons in embryonic mesencephalic cultures treated with MPP+.

Materials and Methods

Preparation of mesencephalic cultures. Timed-pregnant Sprague–Dawley rats were purchased from Zivic-Miller Labs (Zelienople, PA). Mesencephalic cultures were prepared from embryonic day 15 (E15) embryos as described by Friedman and Mytilineou (1987). Pooled ventral mesencephalic tissues were mechanically dissociated and plated at a density of 1–1.5 × 10^6 cells in 35 mm plastic dishes that were precoated with poly-D-lysine. The number of viable cells added to a dish was determined from trypan blue exclusion and counting in a hemocytameter chamber. Culture medium consisted of Eagle's minimal essential medium supplemented with glucose, 30 mM; L-glutamine, 2 mM; fetal calf serum, 5%; horse serum, 5%; penicillin, 100 U/ml; and streptomycin, 100 μg/ml. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air with 100% relative humidity. The medium was changed every third day. For morphological studies cells were grown on 22 × 22 mm glass coverslips. The morphological appearance of the cells was followed with a phase-contrast microscope.

Treatments. Initial studies were performed to determine the concentration and duration of exposure to MPP+ that would reduce dopamine (DA) uptake in the cultures by about 50%. Based on these studies a concentration of 3 μM MPP+ was added on the fourth day in culture and removed 24 hr later. At this concentration of MPP+ there were no general toxic effects as evidenced by no detectable change of dish protein content and the morphological appearance of cells with a phase-contrast microscope.

GM1 ganglioside (generously supplied by Dr. Gino Toffano, FIDIA Research Laboratories) was added on day 5 in culture immediately after removing MPP+. Concentration–response and time–response studies were performed to establish the effectiveness of GM1. For most studies we treated the cultures for 7 d (day 12 in culture) with 0.5 μM GM1 unless otherwise indicated. GM1 had no discernable toxic effects on the cultures and did not alter the protein content of washed cultures. In addition, GM1 had no effect on DA uptake or aromatic L-amino acid decarboxylase (AAAD) activity when added to samples during an assay procedure.

For some studies monoclonal IgM anti-GM1 antibodies (generously provided by Dr. Norman Latov, Columbia University), diluted 1:100, or the B subunit of cholera toxin (5 μg/ml) (List Biological Laboratories, Campbell, CA), which selectively binds to GM1 (Fishman, 1986), was added to control and MPP+-treated cultures, either alone or together with GM1.

The influence of glial cells on the response of the dopaminergic neurons to GM1 was evaluated by treating the cultures with cytosine β-D-arabinofuranoside (Ara-C; Sigma, St Louis, MO), 1.25 μM on day 3 in culture for 24 hr. The cultures were washed on day 4 and MPP+ added for 24 hr as described above.

DA uptake estimation. Cultures were washed with 37°C phosphate-buffered saline (PBS) containing glucose, 5 mM; $CaCl_2$, 1 mM; $MgSO_4$, 1 mM; ascorbic acid, 0.1 mM; and pargyline, 0.1 mM. They were preincubated at 37°C for 5 min, and then ^{3}H-DA (dihydroxyphenylethylamine 3,4-[7-^{3}H]; New England Nuclear, Boston, MA), diluted with nonradiolabeled DA to a final concentration of 25 nM, was added and

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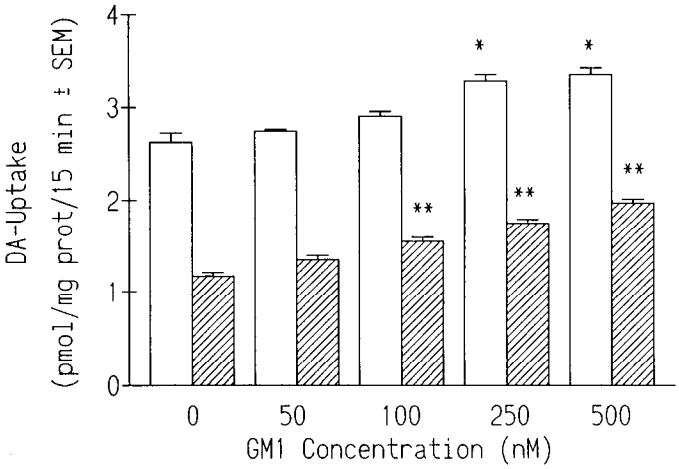

Figure 1. DA uptake by rat E15 mesencephalic cultures treated with MPP^+ and GM1 ganglioside. After 4 d in culture, MPP^+, 3 μM, was added for 24 hr. Then GM1 was added to the MPP^+-treated cultures (*hatched bars*) and to comparison normal cultures (*open bars*), and DA uptake was assayed on day 12 in culture. Data are presented as the mean ± SEM for 10–15 dishes. *, $p < 0.05$ compared with control untreated cultures. **, $p < 0.05$ compared with cultures treated with MPP^+ alone.

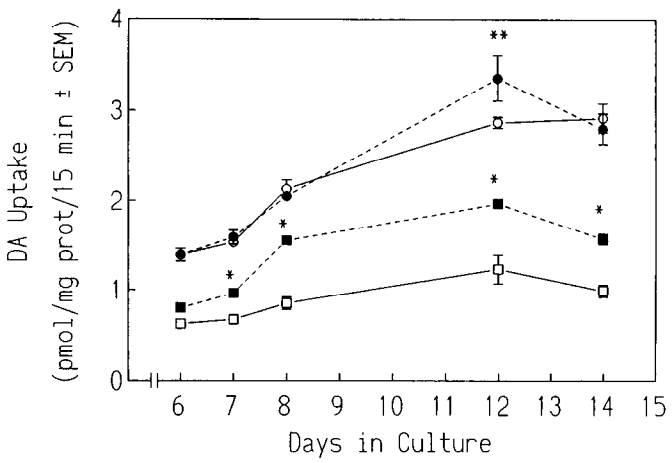

Figure 2. DA uptake by rat E15 mesencephalic cultures with time following MPP^+ or after MPP^+ plus GM1. Cultures were treated with MPP^+ as in Figure 1, and GM1, 0.5 μM, was added and the incubation continued. Data are presented as the mean ± SEM for control (*open circles*), control treated with GM1 (*solid circles*), MPP^+ (*open squares*), and MPP^+ treated with GM1 (*solid squares*). Data are presented as mean ± SEM for 5–12 dishes. *, $p < 0.05$ compared with MPP^+ alone. **, $p < 0.05$ compared with control.

the incubation continued for 15 min. Uptake was terminated by removing the medium and washing the cultures three times with ice-cold PBS solution described previously. ^{3}H-DA was extracted from the cultures by adding two separate 0.5 ml portions of a solution of 0.4 M $HClO_4$ and absolute ethanol (3:1 v/v) and scraping the dishes with a rubber policeman. The extract was sonicated and centrifuged, and a portion of the supernatant was counted for radioactivity. Nonspecific DA uptake was estimated in the presence of 5 μM benztropine. To exclude the possible interference of uptake by serotonergic or noradrenergic neurons fluoxetine, 1 μM, and desipramine, 5 μM, respectively, were included in the incubation medium (Prochiantz et al., 1979). Protein was estimated by the method of Lowry et al. (1951).

DA and DOPAC estimation. DA and DOPAC content in the cultures was established using HPLC with an electrochemical detector. In brief, the medium was removed and the dishes washed with the PBS solution described previously. Then the cells were removed from the dishes with 200 μl of $HClO_4$, 0.5 M, containing 0.002% ascorbic acid. The mixture was homogenized and centrifuged, and 100 μl of the supernatant was injected onto our HPLC system (Cohen et al., 1983).

AAAD estimation. After washing with the PBS solution, cells were scraped from the dishes and homogenized with 130 μl of ice-cold sucrose, 0.25 M. The homogenate, 30 μl, was incubated in 400 μl of a solution containing sodium phosphate buffer, 50 mM, pH 7.2; L-dopa, 0.5 mM (D-dopa for estimation of blank values); ascorbic acid, 0.17 mM; pyridoxal 5-phosphate, 0.01 mM; pargyline, 0.1 mM; 2-mercaptoethanol, 1 mM; and EDTA, 0.1 mM. Incubations were performed at 37°C for 20 min and the reaction was stopped by adding 80 μl of ice-cold $HClO_4$ 0.5 M, containing 10 pmol of 3,4-dihydroxybenzylamine as an internal standard. The mixture was transferred to a small conical polypropylene test tube containing about 20 mg of alumina, and catechol compounds were extracted and assayed by HPLC with electrochemical detection as described previously (Hadjiconstantinou et al., 1988).

Tyrosine hydroxylase immunohistochemistry. A method described by Beck et al. (1991) was used for the immunohistochemical studies. Briefly, cultures were washed with the PBS solution and fixed with formaldehyde, 4%, for 30 min. After washing again they were incubated for 24 hr with a monoclonal mouse anti-tyrosine hydroxylase (TH) antibody (LNC 1, generously provided by Dr. Gregory Kapatos, Wayne State University) diluted 1:1000 in PBS containing bovine serum albumin, 2%; Triton X-100, 0.2%; sodium azide, 0.02%; and goat serum, 1%. Following washing with PBS, cultures were incubated with goat anti-mouse IgG+A+M (H+L) diluted 1:100 (Zymed, San Francisco, CA) for 2 hr. After washing again, cultures were incubated for 2 hr with monoclonal mouse peroxidase–antiperoxidase complex (Zymed) diluted 1:100. Peroxidase was visualized by incubation with a solution of diaminobenzidine, 1 mg/ml, and 0.015% hydrogen peroxide in PBS. TH-immunopositive cells were visualized with a Leitz Orthoplan 2 microscope coupled to a Dage MTI CCD72 camera and projected onto a Sony Trinitron super fine pitch monitor PVN 1343MD. Images of the cultures were made with a Sony video printer UP 3000. The number of TH-immunopositive cells, the number of primary and secondary processes, the length of primary processes, and the soma perimeter were established with a computerized image analysis system.

Statistical analysis. Data were evaluated by analysis of variance followed by comparison of group differences with a Newman–Keuls test.

Results

Treating E15 mesencephalic cultures with the dopaminergic neurotoxin MPP^+, 3 μM, for 24 hr on day 4 in culture, decreased all of the dopaminergic markers evaluated by 40–60% (see figures and tables). The loss of dopaminergic markers appears to be rather selective for DA-containing neurons as there was no detectable loss of protein from the cultures and there were no striking changes of cell morphology except for the TH-immunopositive cells.

GM1 treatment enhanced DA uptake in MPP^+-lesioned cultures in a time- and concentration-dependent manner (Figs. 1, 2). The response appeared maximal at a concentration of about 0.5 μM GM1. Enhanced DA uptake was evident as early as 2 d after adding GM1 to the MPP^+-lesioned cultures and reached a maximum about 7 d later. When control mesencephalic cultures were incubated with GM1, there was a small but significant increase of DA uptake (Figs. 1, 2) when studied 7 d after initiating the treatment, day 12 in culture, but not at other days. The response of the MPP^+-treated cultures to GM1 was maintained as long as the compound was present in the medium. DA uptake decreased after removing GM1 to values found for untreated MPP^+-lesioned cultures (Fig. 3).

GM1 did not prevent the MPP^+-induced loss of DA uptake activity. When GM1 was added before or together with MPP^+ for 24 hr, there was no protection of DA uptake activity compared with cultures that were treated with MPP^+ alone (Table 1).

GM1 treatment elevated DA content in both control and

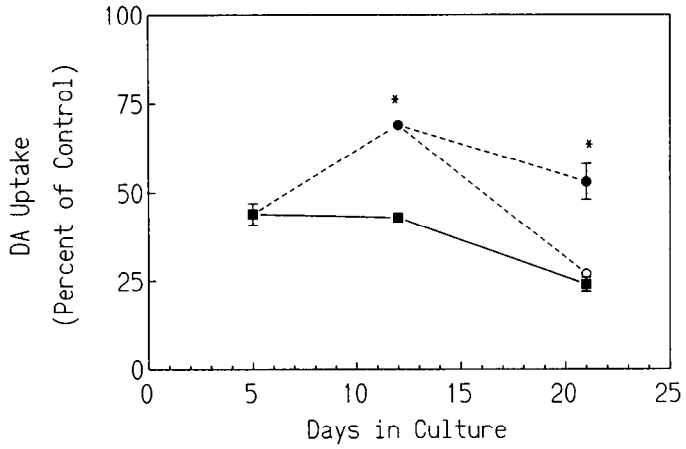

Figure 3. DA uptake by rat E15 mesencephalic cultures treated with MPP+ alone, MPP+ plus continuous treatment with GM1, or MPP+ plus limited treatment with GM1. Cultures were treated with MPP+, 3 μM, on day 4 in culture for 24 hr and then on day 5 MPP+ was removed and GM1, 0.5 μM, was added to two groups. The two groups were maintained with GM1 until day 12 in culture. Then GM1 was removed from one group (*open circle*) while GM1 was maintained in the other (*solid circles*). A comparison group consisted of MPP+ treatment alone (*solid squares*). Data are presented as the mean ± SEM for eight dishes. *, $p < 0.05$ compared with MPP+ alone.

MPP+-lesioned cultures. Only the lesioned cultures had elevated DOPAC content (Table 2).

We attempted to investigate TH activity in the cultures from the conversion of L-tyrosine to L-dopa with analysis by HPLC (Blank and Pike, 1976) and from the generation of ^3H_2O during the conversion of ^{3}H-tyrosine to dopa (Reinhard et al., 1986) but found both to be at the limits of sensitivity using the cultures and therefore inadequate for estimating enzyme activity. AAAD activity, however, was easily assayed in the cultures. Treatment with GM1 increased AAAD activity in control cultures (Table 2). Addition of MPP+ decreased AAAD in the cultures and treatment with GM1 enhanced enzyme activity.

Table 1. GM1 does not prevent MPP+-induced reduction of DA uptake in embryonic mesencephalic cultures

Conditions	DA uptake (pmol/mg protein/min ± SEM)
Contrast culture	
Medium	2.79 ± 0.12
MPP+	1.17 ± 0.04*
Pretreatment	
GM1	2.90 ± 0.06
GM1 plus MPP+	1.10 ± 0.06*
Cotreatment	
GM1	2.96 ± 0.05
GM1 plus MPP+	1.09 ± 0.04*

Mesencephalic cultures were prepared as follows: contrast culture, on day 4 in culture, MPP+, 3 μM, was added for 24 hr, the cultures were washed, and fresh medium was added for the remainder of the study; pretreatment, GM1, 0.5 μM, was added at plating and was present when MPP+ was added on day 4 for 24 hr; after washing fresh addition-free medium was added for the remainder of the study; cotreatment, on day 4 in culture both MPP+ and GM1 were added for 24 hr and removed, and fresh addition-free medium was added for the remainder of the study. DA uptake was assessed on day 12 in cultures. $N = 12$.

* $p < 0.05$ compared with culture medium alone or with GM1 treatment.

Table 2. GM1 enhances AAAD activity and DA and DOPAC content of mesencephalic cultures treated with MPP+

Conditions	AAAD activity (pmol/mg protein/ 20 min ± SEM)	DA (pmol/mg protein ± SEM)	DOPAC (pmol/mg protein ± SEM)
Medium	3.9 ± 0.2	6.8 ± 0.3	11.8 ± 0.5
MPP+	0.92 ± 0.08*	9.5 ± 0.6*	9.2 ± 0.8
GM1	4.4 ± 0.2*	2.7 ± 0.3*	4.2 ± 0.5*
MPP+ plus GM1	1.6 ± 0.1**	5.2 ± 0.5**	9.5 ± 0.2**

Mesencephalic cultures were treated on day 4 in culture with MPP+, 3 μM, for 24 hr. On day 5 fresh medium or GM1, 0.5 μM, was added where indicated. Fresh medium with GM1 was added every 3 d. On day 12 in culture AAAD activity and DA and DOPAC content were assayed. $N = 5$–10.

* $p < 0.05$ compared with Medium.

** $p < 0.05$ compared with MPP+.

About 1% of the cells in our cultures displayed TH-like immunoreactivity (Table 3). TH-immunopositive cells appeared to be bipolar or multipolar. Treatment with MPP+ decreased the number of TH-immunopositive cells by about 60% without significantly diminishing the total number of phase-bright cells. After MPP+, there were fewer TH-positive cells. They were smaller and had fewer primary processes or no processes, and the observed processes were shorter (Fig. 4, Table 4). Treatment of control cultures with GM1 did not change the number of TH-immunopositive cells (Table 3), and they appeared similar to untreated control cells (Fig. 4, Table 4). Addition of GM1 to the MPP+-treated cultures resulted in a two- to threefold increase of TH-immunopositive cells (Table 3) to values found for control cultures, and the cells appeared essentially normal (Fig. 4). This is evident from quantitative image analysis of soma perimeter, the number of primary and secondary processes, and the length of the primary processes (Table 4).

To study the specificity of the response of the cultures to GM1, either antibodies to GM1 or the B subunit of cholera toxin was added concomitantly with GM1 to control and MPP+-treated cultures and DA uptake assayed. Both treatments reduced DA uptake in control cultures and prevented the augmentation of transporter activity by added GM1 (Table 5). Both treatments also prevented the DA uptake augmentation that occurs following treatment of MPP+-lesioned cultures with GM1. Neither treatment, however, altered DA uptake in MPP+-lesioned cultures without GM1.

Cultures were treated with Ara-C to reduce the ratio of glial cells to dopaminergic neurons (Table 6), and then the cultures were evaluated for DA uptake. Ara-C reduced dish protein by about 60%, but DA uptake per dish was relatively unaffected; consequently, there was a rise of DA uptake per milligram of protein. Ara-C treatment had no discernable effect on the response of the cultures to GM1.

Discussion

MPTP administration results in the selective destruction of nigrostriatal dopaminergic neurons in humans and other species and therefore is used to induce experimental parkinsonism in animals. The biochemical, morphological, and behavioral deficits induced by MPTP *in vivo* can be reversed by administering GM1 ganglioside (Hadjiconstantinou et al., 1986, 1989a,b; Hadjiconstantinou and Neff, 1988, 1990; Janson et al., 1988; Weihmuller et al., 1988, 1989; Schneider et al., 1992). Improve-

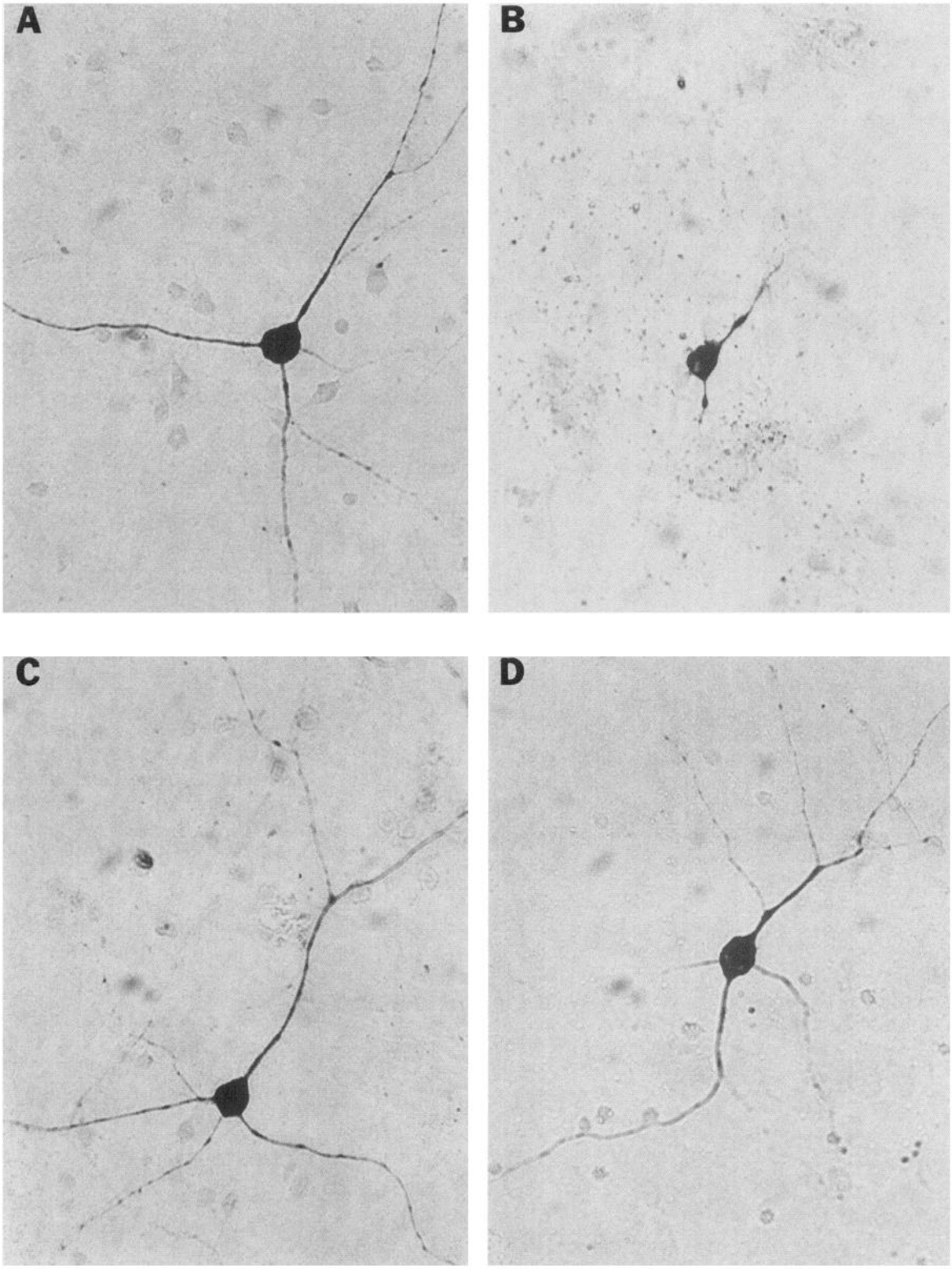

Figure 4. TH-immunopositive neurons in rat E15 ventral mesencephalic cultures after 12 d *in vitro*. *A*, Cells were maintained for 12 d in standard medium. *B*, After the fourth day in culture MPP^+, 3 μM, was added for 24 hr. The cultures were then maintained until day 12 in standard medium. *C*, Cultures were treated with MPP^+ as in *B*; however, GM1, 0.5 μM, was added to the cultures until assayed on day 12. *D*, Control cultures were maintained from day 5 to day 12 in the presence of GM1, 0.5 μM.

ment is also observed after administering some mitogenic peptide growth factors such as epidermal growth factor (Hadjiconstantinou et al., 1991) and basic or acidic fibroblast growth factor (Date et al., 1990; Otto and Unsicker, 1990). These findings suggest that parkinsonism might be treated by administering these substances early in the course of the disease.

MPP^+ at a concentration of 3 μM damaged dopaminergic mesencephalic neurons in culture when surveyed by biochem-

Table 3. GM1 restores the number of TH-immunopositive cells in mesencephalic cultures treated with MPP+

Conditions	Total cells ± SEM (1×10^4)	TH-positive cells ± SEM	Percentage of total cells
Medium	8.3 ± 0.1	819 ± 153	0.99
GM1	9.3 ± 0.3	1187 ± 92	1.27
MPP+	8.7 ± 0.3	335 ± 35*	0.39
MPP+ plus GM1	8.2 ± 0.6	828 ± 51**	1.00

Mesencephalic cells were grown on coverslips (22 × 22 mm) and treated as described in Table 2. On day 12 in culture the cells were fixed and the presence of TH was evaluated by immunohistochemistry. The number of cells on a coverslip was estimated by counting phase-bright cells in a superimposed counting frame and corrected for the size of the coverslip. $N = 4$–6 coverslips.

* $p < 0.05$ compared with Medium.

** $p < 0.05$ compared with MPP+.

ical and morphological criteria. There was about a 40–60% loss of DA uptake, DA and DOPAC content, AAAD activity, and TH-immunopositive cells when surveyed 7 d after MPP+ treatment. This observation is consistent with *in vivo* (Burns et al., 1983; Heikkila et al., 1984a) and *in vitro* (Mytilineou et al., 1985; Sanchez-Ramos et al., 1988) studies where there is evidence for selective destruction of dopaminergic neurons by MPP+. From an analysis of the TH-containing cells in the cultures, the progression of events appears to be loss of TH-immunopositive processes with shrinkage of the soma and eventual loss of the soma image. Loss of DA uptake activity is consistent with the loss of terminal processes and therefore it can be used as an indirect biochemical index to evaluate nerve terminal status.

GM1 added to normal or lesioned cultures increased DA uptake in a concentration-dependent manner. MPP+-lesioned cells show enhanced uptake as early as 2 d after adding GM1, and uptake enhancement is maintained whereas control cultures show enhanced uptake only on day 12 in culture. With the concentration of DA we used for studying DA uptake, the changes observed probably reflect an increased number of transporter sites. Indeed, Leon et al. (1988) found that GM1 treatment increases the V_{max} for DA uptake in embryonic mesencephalic cultures. We found that GM1 was able to enhance AAAD activity as well as DA and DOPAC content in both control and MPP+-treated cultures. Apparently the effect of GM1 on the biochemistry of dopaminergic neurons in culture is broad and not a selective effect on DA uptake.

GM1 does not protect dopaminergic neurons *in vivo* when administered before MPTP (Hadjiconstantinou and Neff, 1988) and it does not protect mesencephalic dopaminergic neurons from destruction by MPP+ *in vitro*. Our present finding that adding GM1 after a lesion has been initiated facilitates the recovery of all neurochemical parameters evaluated is consistent with the *in vivo* studies also (Hadjiconstantinou and Neff, 1988). *In vivo* early initiation of treatment is required for successful recovery as there appears to be a finite time after the insult when GM1 is effective. Moreover, GM1 treatment *in vivo* must be uninterrupted or recovery deteriorates (Hadjiconstantinou and Neff, 1988). Recovery of dopaminergic parameters also deteriorates in the mesencephalic cultures if GM1 is withdrawn.

Exogenous GM1 inserts into cell membranes (Toffano et al., 1980), and it induces differentiation and neurite outgrowth (Doherty et al., 1985; Skaper and Varon, 1985; Skaper et al., 1985; Mugnai and Culp, 1987). Antibodies to GM1 or the presence of the B subunit of cholera toxin modifies neurite outgrowth (Schwartz and Spirman, 1982; Spirman et al., 1982; Spoerri et al., 1988; Wu and Ledeen, 1991), demonstrating the importance of GM1 for this response. We found that DA uptake activity was decreased in control GM1-untreated cultures if antibody to GM1 or the B subunit of cholera toxin was added, implying that DA uptake activity, and perhaps other dopaminergic parameters, is dependent either directly or indirectly on GM1. The GM1 content of serum is low (Dacremont, 1972); thus, the response to antibody or the B subunit of cholera in the control untreated cultures is probably the consequence of their interaction with GM1 generated by the culture. Indeed, embryonic mesencephalic cultures can survive in a defined medium and added GM1 promotes DA uptake activity (Dal Toso et al., 1988). Both GM1 antibody and the B subunit prevented the GM1-induced increase of DA uptake in control and MPP+-treated cultures, implying that a contaminant in the GM1 preparation is not responsible for the enhanced dopaminergic parameters.

Treatment with GM1 restored the number of TH-immunopositive cells and their morphology to near normal in cultures lesioned with MPP+ yet had little effect on control cultures not lesioned with MPP+. This may represent rescue by GM1 of the MPP+-injured neurons from eventual destruction. Alternatively, it is possible that MPP+ reduces TH protein and thus neuron detectability by immunostaining and GM1 may upregulate TH expression restoring neuron detectability. This interpretation has been offered to explain the effect of NGF on the return of ChAT-immunopositive neurons following injury to cholinergic neurons (Varon et al., 1990). The fact that DA uptake does not

Table 4. GM1 restores the morphology of TH-immunopositive cells in mesencephalic cultures treated with MPP+

Conditions	Soma perimeter (μm ± SEM)	Primary process length (μm ± SEM)	Number of primary processes (mean ± SEM)	Number of secondary processes (mean ± SEM)
Medium	56 ± 3	164 ± 3	2.8 ± 0.17	5.8 ± 1
GM1	47 ± 4	180 ± 8	3.2 ± 0.5	5.8 ± 1
MPP+	37 ± 1*	65 ± 5*	1.8 ± 0.14*	2.1 ± 0.2*
MPP+ plus GM1	46 ± 1**	176 ± 5**	3.0 ± 0.2**	5.7 ± 0.4**

Mesencephalic cells were grown on coverslips (22 × 22 mm) and treated as described in Table 2. On day 12 in culture the cells were fixed and TH-containing cells were visualized by immunostaining. Morphometric analysis was performed with an image analyzer (Magiscan). Data are the average for four different studies where 50 cells were counted per treatment.

* $p < 0.05$ compared with Medium.

** $p < 0.05$ compared with MPP+.

Table 5. Cholera toxin B subunit or antibodies to GM1 prevent the GM1-induced increase of DA uptake in mesencephalic cultures treated with MPP+

Conditions	DA uptake (pmol/mg protein/15 min ± SEM) Control	MPP+
Medium	3.0 ± 0.09	1.7 ± 0.07
GM1	3.5 ± 0.2*	2.6 ± 0.2*
Ab-GM1	2.3 ± 0.2	1.7 ± 0.2
CT-B	2.0 ± 0.2	1.7 ± 0.06
GM1 plus Ab-GM1	2.0 ± 0.2	1.5 ± 0.2
GM1 plus B CT	2.4 ± 0.1	1.6 ± 0.2

Mesencephalic cultures were treated on day 4 in culture with MPP+, 3 μM, for 24 hr. On day 5 the medium was changed and the cultures treated for the remainder of the study as indicated: medium alone, GM1 0.5 μM, antibody to GM1 diluted 1:100, cholera toxin B subunit 5 μg/ml, GM1 plus antibody to GM1, GM1 plus cholera toxin B subunit. DA uptake was assayed on day 12 in culture. $N = 12$.

* $p < 0.05$ compared with all Control or MPP+ groups, respectively. All MPP+-treated cultures were significantly different from Control cultures, $p < 0.05$ compared with other groups in the column.

Table 6. Enhanced DA uptake induced by GM1 in MPP+-treated mesencephalic cultures is not affected by prior treatment with Ara-C

	DA uptake (pmol/dish)	DA uptake (pmol/mg protein)	Protein (μg/dish)
Mixed cultures			
Control	1.5 ± 0.1	4.06 ± 0.16	346 ± 20
MPP+	0.72 ± 0.12*	2.11 ± 0.14*	331 ± 12
GM1	1.76 ± 0.05	4.4 ± 0.2	372 ± 12
MPP+ plus GM1	1.24 ± 0.05**	3.6 ± 0.4**	335 ± 13
Neuronal cultures			
Control	1.29 ± 0.07	8.6 ± 0.9	124 ± 22
MPP+	0.67 ± 0.09*	5.5 ± 1.0*	147 ± 15
GM1	1.39 ± 0.05	9.1 ± 1.3	157 ± 8
MPP+ plus GM1	1.82 ± 0.05**	10.9 ± 1.4**	153 ± 14

Mesencephalic cultures were treated with Ara-C, 1.25 μM, for 24 hr on day 3 in culture. On day 4 in culture they were washed and MPP+, 3 μM, added for 24 hr. On day 5 in culture GM1 was added, indicated in the table, and maintained for the remainder of the study. DA uptake was assayed on day 12 in culture. Data are presented as the mean ± SEM for three or four dishes.

* $p < 0.05$ compared with control.

** $p < 0.05$ compared with MPP+.

recover in the MPP+-treated cultures with time suggests that dopaminergic neurons are eventually lost. Therefore, we postulate that GM1 rescues moribund neurons, resulting in the return of dopaminergic parameters along with TH immunoreactivity.

Although the mechanism(s) for recovery of function after an injury is unknown, there is evidence that GM1 may have anti-neurotoxic and/or pro-neurotrophic actions. Neuronal insults are associated with excessive prolonged Ca^{2+} influx that is detrimental for neuronal survival (Rothman and Olney, 1987). In glutamate-induced neurotoxicity GM1 prevents the translocation of protein kinase C in injured neurons and may thereby limit intracellular Ca^{2+} increases (Vaccarino et al., 1987; Favaron et al., 1988; de Erausquin et al., 1990), thus protecting the neuron from delayed death (Manev et al., 1990). MPP+ increases glutamate in brain (Carboni et al., 1990) and NMDA receptor antagonists can prevent MPP+ neurotoxicity (Turski et al., 1991). Pretreatment or cotreatment with GM1 together with MPP+ or MPTP does not appear to prevent the toxic effects on dopaminergic neurons in culture or *in vivo*. However, early and continuous treatment with GM1 after an MPTP or MPP+ lesion is required for a response. The early administration of GM1 after an insult may rescue neurons from delayed degeneration and death associated with elevated glutamate release. Brain-derived neurotrophic factor (BDNF) has been shown to prevent the MPP+ neurotoxicity in mesencephalic cultures by inducing glutathione synthetase activity (Spina et al., 1992).

In addition to possible anti-neurotoxic properties, GM1 may have restorative effects by modulating endogenous neurotrophic factor activity. Gangliosides modulate tyrosine kinase activity of trophic factor receptors, such as epidermal growth factor (Bremer et al., 1986), insulin growth factor-I, and platelet-derived growth factor (Hakomori et al., 1990). A tyrosine kinase receptor appears important for BDNF activity (Klein et al., 1991). Epidermal growth factor (Hadjiconstantinou et al., 1991) and BDNF promote recovery of mesencephalic neurons in culture (Hyman et al., 1991). Trophic factors for dopaminergic neurons are present in brain (Prochiantz et al., 1979, 1981; di Porzio et al., 1980), and added GM1 may facilitate their action on the injured neurons by modulating their synthesis, release, and/or receptor characteristics. For example, GM1 has a synergistic effect with NGF on brain cholinergic neurons (Cuello et al., 1989).

Enhanced DA uptake induced by epidermal growth factor is abolished by prior treatment of cultures with Ara-C, implying that the presence of glial cells is necessary for the response (Knusel et al., 1990). In contrast, Ara-C treatment did not alter the ability of GM1 to enhance DA uptake in cultures lesioned with MPP+, negating a role for glial cells in this response. Moreover, embryonic mesencephalic neurons in a defined culture medium show enhanced DA uptake when GM1 is included (Leon et al., 1988).

Based on reports in the literature and our studies with MPTP, neurotrophic peptides, and GM1, we postulate that the following events occur in the cultures lesioned with MPTP and treated with GM1. MPP+ is transported into the mesencephalic dopaminergic neurons and inhibits mitochondrial energy metabolism and all dependent biochemical events. Phenotypic expression is lost and the neurons begin degenerating. Added GM1 inserts into membranes, limiting excessive Ca^{2+} influx and facilitating the action of neurotrophic factors present in the cultures, hence rescuing the cells and stimulating phenotypic expression, regeneration, and repair. Apparently, the rescued dopaminergic neurons are dependent on added GM1 as they lose their ability to take up DA if GM1 is removed. This observation is consistent with *in vivo* studies where dopaminergic parameters were found to deteriorate in MPTP-lesioned animals if GM1 treatment was terminated (Hadjiconstantinou and Neff, 1988).

In conclusion, MPP+ induces selective destruction of dopaminergic neurons in rat embryonic mesencephalic cultures. There is loss of DA and DOPAC, AAAD activity, DA uptake, and TH-immunopositive cells. All of these parameters are partially restored by adding GM1 ganglioside to the cultures.

References

Beck KD, Knusel B, Pasinetti G, Michel PP, Zawadzka H, Goldstein M, Hefti F (1991) Tyrosine hydroxylase mRNA expression by dopaminergic neurons in culture: effect of 1-methyl-4-phenylpyridinium treatment. J Neurochem 57:527–532

Blank CL, Pike R (1976) A novel, inexpensive, and sensitive method for analysis of tyrosine hydroxylase activity in tissue samples. Life Sci 18:859–866.

Bremer EG, Schlessinger J, Hakomori SI (1986) Ganglioside-mediated modulation of cell growth. Specific effects of GM3 on tyrosine phosphorylation of the epidermal growth factor receptor. J Biol Chem 261: 2434–2440.

Burns SP, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin IJ (1983) A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by *N*-methyl-1,2,3,6-tetrahydropyridine. Proc Natl Acad Sci USA 80: 4546–4550.

Carboni S, Melis F, Pani L, Hadjiconstantinou M, Rossetti ZL (1990) The non-competitive NMDA-receptor antagonist MK-801 prevents the massive release of glutamate and aspartate from rat striatum induced by 1-methyl-4-phenylpyridinium (MPP^+). Neurosci Lett 117: 129–133.

Cohen J, Hadjiconstantinou M, Neff NH (1983) Activation of dopamine-containing amacrine cells of retina: light-induced increase of acidic dopamine metabolites. Brain Res 260:125–127.

Cuello AC, Garofalo L, Kenigsberg RL, Maysinger D (1989) Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. Proc Natl Acad Sci USA 86:2056–2060.

Dacremont G (1972) Ganglioside concentration in human plasma. Clin Chim Acta 37:449–454.

Dal Toso R, Giorgi O, Soranzo C, Kirschner G, Ferrari G, Favaron M, Benvegnu D, Presti D, Vicini S, Toffano G, Azzone GF, Leon A (1988) Development and survival of neurons in dissociated fetal mesencephalic serum-free cell cultures. I. Effects of cell density and of an adult mammalian striatal-derived neuronotrophic factor. J Neurosci 8:733–745.

Date I, Notter MFD, Felten SY, Felten DL (1990) MPTP-treated young mice but not aging mice show partial recovery of the nigrostriatal dopaminergic system by stereotaxic injection of acidic fibroblast growth factor (aFGF). Brain Res 526:156–160.

de Erausquin GA, Manev H, Guidotti A, Costa E, Brooker G (1990) Gangliosides normalize distorted single-cell intracellular free Ca^{2+} dynamics after toxic doses of glutamate in cerebellar granule cells. Proc Natl Acad Sci USA 87:8017–8021.

di Porzio U, Daguet M-C, Glowinski J, Prochiantz A (1980) Effect of striatal cells on *in vitro* maturation of mesencephalic dopaminergic neurons grown in serum-free conditions. Nature 288:370–373.

Doherty P, Dickson JG, Flanigan TP, Leon A, Toffano G, Walsh FS (1985) Molecular specificity of ganglioside effects on neurite regeneration of sensory neurons *in vitro*. Neurosci Lett 62:193–198.

Favaron M, Manev H, Alho H, Bertolino M, Ferret B, Guidotti A, Costa E (1988) Gangliosides prevent glutamate and kainate neurotoxicity in primary neuronal cultures of neonatal rat cerebellum and cortex. Proc Natl Acad Sci USA 85:7351–7355.

Fishman PH (1986) Recent advances in identifying the functions of gangliosides. Chem Phys Lipids 42:137–151.

Friedman L, Mytilineou C (1987) The toxicity of MPTP to dopamine neurons in culture is reduced at high concentrations. Neurosci Lett 79:65–72.

Ghidoni R, Fiorilli A, Trinchera M, Venerando B, Chigorno V, Tettamanti G (1989) Uptake, cell penetration and metabolic processing of exogenous administered GM1 ganglioside in rat brain. Neurochem Int 15:455–465.

Hadjiconstantinou M, Neff NH (1988) Treatment with GM1 ganglioside restores striatal dopamine in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mouse. J Neurochem 51:1190–1196.

Hadjiconstantinou M, Neff NH (1990) Differential recovery of dopamine synthetic enzymes following MPTP and the consequences of GM1 ganglioside treatment. Eur J Pharmacol 181:137–139.

Hadjiconstantinou M, Rossetti ZL, Paxton RC, Neff NH (1986) Administration of GM1 ganglioside restores the dopamine content in striatum after chronic treatment with MPTP. Neuropharmacology 25:1075–1077.

Hadjiconstantinou M, Rossetti ZL, Silvia C, Krajnc D, Neff NH (1988) Aromatic L-amino acid decarboxylase activity of the rat retina is modulated *in vivo* by environmental light. J Neurochem 51:1560–1564.

Hadjiconstantinou M, Mariani AP, Neff NH (1989a) GM1 ganglioside-induced recovery of nigrostriatal dopaminergic neurons after MPTP: an immunohistochemical study. Brain Res 484:297–303.

Hadjiconstantinou M, Weihmuller FB, Neff NH (1989b) Treatment with GM1 ganglioside reverses dopamine D-2 receptor supersensitivity induced by the neurotoxin MPTP. Eur J Pharmacol 168:261–264.

Hadjiconstantinou M, Fitkin JG, Dalia A, Neff NH (1991) Epidermal growth factor enhances striatal dopaminergic parameters in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mouse. J Neurochem 57:479–482.

Hakomori S, Igarashi Y, Nojiri H, Bremer E, Hanai N, Nores GA (1990) Bioactive gangliosides modulating transmembrane signaling. In: Trophic factors and the nervous system (Horrocks LA, Neff NH, Yates AJ, Hadjiconstantinou M, eds), pp 135–153. New York: Raven.

Heikkila RE, Hess A, Duvoisin RC (1984a) Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. Science 224:1451–1453.

Heikkila RE, Manzino L, Cabbat FS, Duvoisin RC (1984b) Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors. Nature 311: 467–469.

Heikkila RE, Manzino L, Cabbat FS, Duvoisin RC (1985) Studies on the oxidation of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase B. J Neurochem 45:1049–1054.

Hyman C, Hofer M, Barde YA, Juhasz M, Yancopolos GD, Squinto SP, Lindsay RM (1991) BDNF in a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350:230–232.

Janson AM, Agnati LF, Fuxe K, Cintra A, Sundstrom E, Zini I, Toffano G, Goldstein M (1988) GM1 ganglioside protects against the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced degeneration of nigrostriatal dopamine neurons in the black mouse. Acta Physiol Scand 132:587–588.

Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH (1985) Parkinsonism-inducing neurotoxin, *N*-methyl-4-phenyl-1,2,3,6 tetrahydropyridine: uptake of the metabolite *N*-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proc Natl Acad Sci USA 82:2173–2177.

Klein R, Nanduri V, Jing SA, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991) The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66:395–403.

Knusel B, Michel PP, Schwaber JS, Hefti F (1990) Selective and non-selective stimulation of central cholinergic and dopaminergic development *in vitro* by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin, and the insulin-like growth factors I and II. J Neurosci 10:558–570.

Ledeen RW (1984) Biology of gangliosides: neuritogenic and neuronotrophic properties. J Neurosci Res 12:147–159.

Leon A, Dal Toso R, Presti D, Benvegnu D, Facci L, Kirschner G, Tettamanti G, Toffano G (1988) Development and survival of neurons in dissociated fetal mesencephalic serum-free cell cultures. II. Modulatory effects of gangliosides. J Neurosci 8:746–753.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Manev H, Costa E, Wroblewski JT, Guidotti A (1990) Abusive stimulation of excitatory amino acid receptors: a strategy to limit neurotoxicity. FASEB J 4:2789–2797.

Mugnai G, Culp LA (1987) Cooperativity of ganglioside-dependent with protein-dependent substratum adhesion and neurite extension of human neuroblastoma cells. Exp Cell Res 169:328–344.

Mytilineou C, Cohen G, Heikkila RE (1985) 1-Methyl-4-phenylpyridine (MPP^+) is toxic to mesencephalic dopamine neurons in culture. Neurosci Lett 57:19–24.

Nicklas WJ, Vyas I, Heikkila RE (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci 36:2503–2508.

Otto D, Unsicker K (1990) Basic FGF reverses chemical and morphological deficits in the nigrostriatal system of MPTP-treated mice. J Neurosci 10:1912–1921.

Prochiantz A, di Porzio U, Kato A, Berger B, Glowinski J (1979) *In vitro* maturation of mesencephalic dopaminergic neurons from mouse embryos is enhanced in presence of their striatal target cells. Proc Natl Acad Sci USA 76:5387–5391.

Prochiantz A, Daguet M-C, Herbet A, Glowinski J (1981) Specific

stimulation of *in vitro* maturation of mesencephalic dopaminergic neurons by striatal membranes. Nature 293:570–572.

Reinhard JF Jr, Smith GK, Nichol CA (1986) A rapid and sensitive assay for tyrosine-3-monooxygenase based upon the release of ^3H_2O and the adsorption of ^{3}[H]-tyrosine by charcoal. Life Sci 39:2185–2189.

Rothman SM, Olney JW (1987) Excitotoxicity and the NMDA receptor. Trends Neurosci 7:299–302.

Sanchez-Ramos JR, Michel P, Weiner WJ, Hefti F (1988) Selective destruction of cultured dopaminergic neurons from fetal rat mesencephalon by 1-methyl-4-phenylpyridinium: cytochemical and morphological evidence. J Neurochem 50:1934–1944.

Schneider JS, Pope A, Simpson K, Taggart J, Smith MG, DiStefano L (1992) Recovery from experimental parkinsonism in primates with GM1 ganglioside treatment. Science 256:843–846.

Schwartz M, Spirman N (1982) Sprouting from chicken embryo dorsal root ganglia induced by nerve growth factor is specifically inhibited by affinity-purified anti-ganglioside antibodies. Proc Natl Acad Sci USA 79:6080–6083.

Singer TP, Ramsay RR, McKeown K, Trevor A, Castagnoli NE Jr (1988) Mechanism of the neurotoxicity of 1-methyl-4-phenylpyridinium (MPP^+), the toxic bioactivation product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Toxicology 49:17–23.

Skaper SD, Varon S (1985) Ganglioside GM1 overcomes serum inhibition of neurite outgrowth. Int J Dev Neurosci 3:187–198.

Skaper SD, Katoh-Semba R, Varon S (1985) GM1 ganglioside accelerates neurite outgrowth from primary peripheral and central neurons under selected culture conditions. Brain Res 355:19–26.

Spina MB, Squinto SP, Miller J, Lindsay RM, Hyman C (1992) Brain-derived neurotrophic factor protects dopamine neurons against 6-hydroxydopamine and *N*-methyl-4-phenylpyridinium ion toxicity: involvement of the glutathione system. J Neurochem 59:99–106.

Spirman N, Sela B-A, Schwartz M (1982) Antiganglioside antibodies inhibit neuritic outgrowth from regenerating goldfish retinal explants. J Neurochem 39:874–877.

Spoerri PE, Rapport MM, Mahadik SP, Roisen FJ (1988) Inhibition of conditioned media-mediated neuritogenesis of sensory ganglia by monoclonal antibodies to GM1 ganglioside. Dev Brain Res 41:71–77.

Toffano G, Benvegnu D, Bonetti AC, Facci L, Leon A, Orlando P, Ghidoni R, Tettamanti G (1980) Interaction of GM1 ganglioside with crude rat brain neuronal membranes. J Neurochem 35:861–866.

Turski L, Bressler K, Rettig KJ, Loschmann PA, Wachtel H (1991) Protection of substantia nigra from MPP^+ neurotoxicity by *N*-methyl-D-aspartate antagonists [see comments]. Nature 349:414–418.

Vaccarino F, Guidotti A, Costa E (1987) Ganglioside inhibition of glutamate-mediated protein kinase C translocation in primary cultures of cerebellar neurons. Proc Natl Acad Sci USA 84:8707–8711.

Varon S, Hagg T, Conner J, Fass B, Vahlsing HL, Manthorpe M (1990) Consequences to CNS cholinergic neurons of *in vivo* nerve growth factor administration. In: Trophic factors and the nervous system (Horrocks LA, Neff NH, Yates AJ, Hadjiconstantinou M, eds), pp 219–228. New York: Raven.

Weihmuller FB, Hadjiconstantinou M, Bruno JP, Neff NH (1988) Administration of GM1 ganglioside eliminates neuroleptic-induced sensorimotor deficits in MPTP-treated mice. Neurosci Lett 92:207–212.

Weihmuller FB, Hadjiconstantinou M, Bruno JP, Neff NH (1989) Continued administration of GM1 ganglioside is required to maintain recovery from neuroleptic-induced sensorimotor deficits in MPTP-treated mice. Life Sci 45:2495–2502.

Wu G, Ledeen RW (1991) Stimulation of neurite outgrowth in neuroblastoma cells by neuraminidase: putative role of GM1 ganglioside in differentiation. J Neurochem 56:95–104.