Development and Survival of Neurons in Dissociated Fetal Mesencephalic Serum-Free Cell Cultures: II. Modulatory Effects of Gangliosides

A. Leon, R. Dal Toso, D. Presti, D. Benvegnù, L. Facci, G. Kirschner, G. Tettamanti,¹ and G. Toffano

Fidia Neurobiological Research Laboratories, Abano Terme, and ¹Institute of Biological Chemistry, University of Milano, Milano, Italy

This paper analyzes the effects of exogenously supplied GM1 on the development, i.e., specific neurotransmitter uptake capability and survival, of the dopaminergic neurons present in fetal mouse-dissociated mesencephalic cells. Exogenous GM1, but not asialo-GM1, sialic acid, or the oligosaccharide chain of GM1, enhances in a time- and concentration-dependent manner the specific ³H-dopamine uptake (increase of the apparent V_{max} and decrease of the apparent K_m value) and the long-term survival of the dopaminergic neurons. The GM1 effects on the behavior of the dopaminergic neurons require the presence of cell-derived neuronotrophic influences present within the culture system and are associated with an increase in the response of the cells to the trophic influences. GM1 effects are not limited to dopaminergic neurons, and depend on the stable association of the ganglioside molecule with the cells. It is suggested that GM1 is not a trophic agent per se, but rather potentiates neuronotrophic activities and/or exerts independent influences to which neurons respond only if appropriately supported.

It is becoming increasingly evident that not only the development but also the repair of the nervous system is controlled by extracellular signals whose physiological effects most probably depend on their interaction with molecules expressed on the cell surface (Frazier and Glaser, 1979). Although detailed studies of glycolipid expression and function on the neuronal cell surface are still in their infancy, gangliosides appear to play a prominent role in regulating both neuronal development and repair. During development, brain ganglioside patterns undergo marked changes, both quantitatively and qualitatively (Willinger and Schachner, 1980; Koulakoff et al., 1983). In regenerating adult axons, cell synthesis and axonal transport of gangliosides increase (Sbaschnig-Agler et al., 1984). While antibodies to gangliosides inhibit nerve regeneration (Sparrow et al., 1984), ganglioside administration *in vivo* has been reported to facilitate nerve regeneration and functional recovery of the PNS following

injury (Ceccarelli et al., 1976; Gorio et al., 1980, 1983; Sparrow and Graftstein, 1982; Robb and Keynes, 1984). Furthermore, the administration of gangliosides, in particular monosialoganglioside GM1 (nomenclature according to Svennerholm, 1963), has been reported to ameliorate the outcome following injury to the CNS (Wojcik et al., 1982; Agnati et al., 1983; Karpiak, 1983; Toffano et al., 1983, 1984; Carmignoto et al., 1984; Fass and Ramirez, 1984; Kojima et al., 1984; Jonsson et al., 1984; Oderfeld-Nowak et al., 1984; Sabel et al., 1984, 1985; Casamenti et al., 1985; Fujito et al., 1985; Commissiong and Toffano, 1986; Cuello et al., 1986; Fusco et al., 1986; Gradkowska et al., 1986; Hadjiconstantinou and Neff, 1986; Hadjiconstantinou et al., 1986; Sofroniew et al., 1986).

In an attempt to understand the role of gangliosides in regulating neuronal development and repair, the effects of exogenous GM1 addition to neuronal cells in culture have been evaluated in a variety of studies. Neurons, both primary and clonal, respond to the ganglioside with pronounced morphological changes characteristic of cell differentiation (Leon et al., 1982, 1984; Byrne et al., 1983; Ferrari et al., 1983; Rybak et al., 1983; Facci et al., 1984; Katoh-Semba et al., 1984; Doherty et al., 1985; Skaper and Varon, 1985; Skaper et al., 1985; Matta et al., 1986). In this paper we report the effects of GM1 addition on the behavior of dopaminergic neurons in dissociated embryonic mouse mesencephalic cells cultured in a serum-free, hormone-supplemented medium. In these cultures, more than 98% of the mesencephalic cells can be classified as neurons on the basis of immunocytochemical criteria (Dal Toso et al., 1988). Survival and expression of specific high-affinity neurotransmitter uptake of the dopaminergic neurons appear to be dependent on adequate levels of trophic influences, either produced in culture or exogenously supplied. GM1 ganglioside enhances both dopaminergic cell survival and maturation only in the presence of adequate trophic influences. This effect is correlated with stable cell association of the gangliosides, and suggests that exogenously supplemented gangliosides may affect neuronal behaviors *in vitro*, and perhaps also *in vivo*, by facilitating membrane-mediated cellular responses to endogenously occurring neuronotrophic signals.

Materials and Methods

Materials. Commercial chemicals were of analytical grade, or of the highest purity available. Solvents were distilled before use. The water routinely employed was freshly taken from a Milli-Q reagent water system (Millipore, Molsheim, France). BSA, bovine pancreatic insulin, human transferrin, putrescine, L-3,5,3'-triiodothyronine (T_3), progesterone, sodium selenite, L-2,4-diaminobutyric acid (DABA), amino-

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Correspondence should be addressed to Dr. Alberta Leon, Fidia Research Laboratories, Via Ponte della Fabbrica 3/A, 35031 Abano Terme, Italy.

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Table 1. Effects of GM1 on BZT-sensitive ^3H -DA uptake by mesencephalic cell cultures

	GM1 concentrations (M)			
	0	10^{-8}	5×10^{-8}	10^{-7}
DNA content ($\mu\text{g}/\text{dish}$)	7.5 ± 0.9	7.6 ± 0.9	7.1 ± 1.2	7.2 ± 0.7
BZT-sensitive ^3H -DA uptake ^a (fmol/ μg DNA per 15 min)	11.03 ± 0.8	20.25 ± 0.4^b	23.3 ± 2.2^b	29.5 ± 3.5^b
Kinetic parameter				
K_m (10^{-7} M)	1.045	—	—	0.354
$V_{\max}$ (fmol/ μg DNA per 15 min)	55	—	—	174

Dissociated cells were suspended in control and GM1-containing (serum-free) media, seeded at 1×10^6 per plate/2 ml, and cultured for 4 d. See Materials and Methods for assay of DNA content and BZT-sensitive ^3H -DA uptake. Values are mean $\pm$ SEM of triplicate analyses. For kinetic analysis ^3H -DA uptake was measured using 30, 50, 100, 200, and 400 nM DA and apparent K_m and $V_{\max}$ values were calculated from Hofstee plots (triplicate points for each concentration).

^a Assessed using 5×10^{-8} M ^3H -DA.

^b $p < 0.01$; Student's t test (vs control value).

oxyacetic acid, pargyline chlorhydrate, ascorbic acid, and calf thymus type I DNA were purchased from Sigma (St. Louis, MO); ^3H -dopamine (DA; sp act, 22–33 Ci/mmol) and GABA (sp act, 225 mCi/mmol) from New England Nuclear (NEN, Dreieich, FRG); 3,5-diaminobenzoic acid from Serva (Heidelberg, FRG); bztropine mesylate (BZT) from Merck, Sharp & Dohme (Quebec, Canada); Eagle's basal medium from Gibco (New York); Ham's F12 nutrient mixture from Flow Laboratories (McLean, VA); bovine skin collagen (Vitrogen) from Collagen Corp. (Palo Alto, CA); the preblended liquid-scintillation solution, Instagel II, from Packard (Downers Grove, IL). Mouse monoclonal antibody to rat neurofilament RT97 (anti-NF) (Anderton et al., 1982) and mouse glial fibrillary acidic protein (GFAP) antiserum were a gift of Dr. F. S. Walsh (Institute of Neurology, The National Hospital, London, UK).

Cell culture preparation and composition. Dissociated mesencephalic and striatal cell cultures were prepared as previously described (Prochiantz et al., 1979; Di Porzio et al., 1980). Briefly, rostral mesencephalic tegmentum and corpus striatum from brains of 13- and 15-d-old mouse embryos, respectively, were dissected under the microscope in sterile conditions and dissociated mechanically. The cells were centrifuged at $45 \times g$ for 4 min and resuspended in a serum-free medium consisting of equal volumes of Eagle's basal medium and Ham's F12, supplemented with components as reported in the preceding paper by Dal Toso et al. (1988). Two milliliters of cell suspension containing the desired amounts of cells were added to collagen-coated 35-mm-diameter tissue culture dishes (Falcon). The cultures were incubated at 37°C in a water-saturated (95% air; 5% CO_2) atmosphere. As previously reported (Dal Toso et al., 1988), more than 98% of the cells plated at 0.5 – 2×10^6 /dish and cultured for 4 or 8 d were identified as neurons by their immunoreactivity to anti-NF, and less than 1% of them stained positively with GFAP antiserum.

^3H -dopamine and GABA uptake. Prior to uptake studies, culture medium was removed, cells washed extensively with PBS containing glucose (6 gm/liter), Ca^{2+} (1 mM), and Mg^{2+} (1 mM). Assay of ^3H -dopamine (DA) uptake was conducted as previously described (Prochiantz et al., 1981; Dal Toso et al., 1988). With the exception of kinetic studies, 5×10^{-8} M ^3H -DA was routinely employed. GABA was assayed as described by Prochiantz et al. (1981). In brief, uptake of GABA was routinely measured by addition of $0.1 \mu\text{M}$ ^{14}C -GABA for 15 min at 37°C . Amino-oxyacetic acid ($10 \mu\text{M}$) was used to prevent GABA catabolism. GABA uptake inhibitors were added when necessary. Washing and extraction procedures were as described for ^3H -DA uptake studies. GABA identification was performed by thin-layer chromatography (Lasher, 1974). More than 90% of the radioactivity was associated with a spot comigrating with authentic GABA.

Histochemical visualization. Histochemical visualization of catecholaminergic neurons in culture was performed following noradrenergic uptake using the glyoxylic acid-induced fluorescence (GIF) technique, as previously described (Dal Toso et al., 1988). The GIF⁺ neurons were visualized with a Zeiss III photomicroscope equipped with catecholamine epifluorescence and phase-contrast optics. Using prefixed coordinates, GIF⁺ neurons were counted over at least 3% of the total surface area.

GM1 characterization and preparation of GM1-containing media. GM1 ganglioside, extracted from calf brain according to Tettamanti et al.

(1973), was purified and chemically characterized as described by Sonnino et al. (1978). Chromatographic analysis indicated that the purity of GM1 was over 99%. No amino acid peaks were detected by automated amino acid analysis after acid hydrolysis of 100 mg GM1. Desired amounts of purified GM1 were dissolved in chloroform-methanol (1:1 by volume), dried again and redissolved in an appropriate volume of incubation medium in order to reach the final ganglioside concentration (from 10^{-8} to 10^{-7} M). The solution was maintained at 37°C for at least 1 hr, with intermittent swirling. The recovery of GM1 by this procedure, as measured using ^3H -GM1 labeled on the terminal galactose moiety (Facci et al., 1984), exceeded 90%. The control medium was treated in the same way except for the omission of GM1. Solvents were distilled before use.

When needed, appropriate amounts of GM3, asialo-GM1, and GD1b were substituted for GM1. These were a gift from Professor G. Tettamanti (Institute of Biological Chemistry, University of Milano, Milano, Italy) and their purity exceeded 99%.

Determination of cell-associated GM1 radioactivity. Association of GM1 to mesencephalic cells in culture was essentially conducted as described by Facci et al. (1984). ^3H -GM1 labeled on the terminal galactose moiety was used. The specific radioactivity was 1.5 Ci/mmol and its specific radiochemical purity was over 99%. In brief, collagen-coated coverslips without and with mesencephalic cells were incubated at 37°C for different times, with ^3H -GM1 (10^{-8} – 10^{-7} M) in serum-free medium. After incubation, coverslips were washed twice with PBS, once with the culture medium supplemented with 10% fetal calf serum (30 min at 37°C), and 3 more times with PBS. The radioactivity of each coverslip was extracted twice with 5 ml aliquots of tetrahydrofuran (THF), as described by Tettamanti et al. (1973). The THF extracts were brought to dryness under a nitrogen stream and resuspended with 1 ml distilled water prior to the addition of 10 ml of emulsifier (Instagel II). Samples were then counted by liquid scintillation with a Packard Tricarb (model 460 C) counter. Residual radioactivity due to GM1 association to collagen was subtracted. TLC identification of radioactivity was conducted as reported previously (Facci et al., 1984). Dried coverslips were then processed for DNA assay (Erwin et al., 1981). THF treatment did neither interfere with the DNA assay nor modify the DNA content of the coverslips.

Results

GM1 effects on specific ^3H -DA uptake

The high-affinity ^3H -DA uptake of the fetal mouse-dissociated mesencephalic neurons, specifically inhibited by BZT, was used as an index to determine the effect of exogenously supplied GM1 on dopaminergic cells (Dal Toso et al., 1988). Addition of GM1 (from 10^{-8} to 10^{-7} M) to dissociated mesencephalic cells at plating time did not modify the neuronal (RT97⁺) to astroglial (GFAP⁺) cell ratio nor the total DNA content per dish when assessed at day 4 (Table 1). GM1 produced a concentration-dependent increase of the BZT-sensitive ^3H -DA uptake without changes in the BZT-insensitive levels. Kinetic analysis indicated

Figure 1. BZT-sensitive DA uptake (A) and DABA-sensitive GABA uptake (B) as a function of cell density. Effect of GM1. Dissociated mesencephalic cells were seeded at various cell densities in serum-free culture medium without (○) or with (●) 10^{-7} M GM1. Uptakes were assayed at day 4 *in vitro*, as reported in Materials and Methods. Each value is the mean $\pm$ SEM of triplicate analyses. See text for further details.

that the GM1 effect was associated with a 3-fold increase of the apparent $V_{\max}$ and 3-fold reduction of the apparent K_m value. The GM1-induced increase was evident after 2 d (control = 25.15 ± 5.84 fmol/plate; GM1, 10^{-7} M, = 41.17 ± 1.34 fmol/plate), and persisted up to at least 8 d (Table 2). Transient exposure of cell cultures to GM1 for up to 2 hr before or during the ^3H -DA uptake assay was totally ineffective.

Correlation with cell density

Since DA uptake capacity of the mesencephalic dopaminergic neurons is dependent on the cell density (Dal Toso et al., 1988), we investigated the effects of GM1 at increasing plating cell densities (Fig. 1A). As previously reported, in the absence of GM1 the BZT-sensitive DA uptake was constant up to an apparent threshold of approximately $3 \mu\text{g}$ DNA at day 4 (about 0.5×10^6 plated cells) and subsequently increased linearly with increasing cell density (or proportional to the square of the cell density on a per-plate basis). In GM1-treated cultures, the same pattern was observed, but the slope of the cell density-induced increase was approximately 1.5–2-fold greater. Although slopes of the amplitude of the cell-derived influences differed among different cell batches, the relative effect of GM1 was similar.

Interestingly, the cell density effect on specific DA uptake was only kinetically correlated with an increase in the apparent $V_{\max}$ ($V_{\max} = 72.8$ or 230.0 fmol/ μg DNA in 15 min when using 1×10^6 or 2×10^6 cells plated/dish, respectively). No significant modifications in the apparent K_m values were observed ($K_m = 0.127$ or $0.110 \mu\text{M}$, respectively, using 1×10^6 or 2×10^6 plated cells). However the addition of GM1, as reported above, both increased the apparent $V_{\max}$ values and decreased the apparent K_m values.

Correlation with number and viability of dopaminergic neurons

Table 2 shows data relative to the time course of the number of dopaminergic, i.e., GTF⁺ cells seeded at a density of 1 or 2×10^6 cells. In GM1-free cultures, as previously reported (Dal Toso et al., 1988), doubling of the 1×10^6 plating cell density did not significantly alter the proportion of dopaminergic cells surviving at 4 d (about 0.15% of the total seed), but reduced by half the dopaminergic cell losses between 4 and 8 d (from 75% to about 35%). The higher plating cell density further caused an approximately 2-fold increase of the BZT-sensitive DA uptake per dopaminergic neuron, the latter uptake also increasing with

Table 2. Correlation between BZT-sensitive DA uptake and number of dopaminergic neurons in mesencephalic cell cultures: effects of GM1

Parameters	Cells plated	Days	Control	GM1 (10^{-7} M)
DA cells/plate	1×10^6	4	1451 ± 152	1632 ± 186
	1×10^6	8	350 ± 157	368 ± 148
	2×10^6	4	3340 ± 234	3592 ± 301
	2×10^6	8	2159 ± 171	3030 ± 250^a
DA uptake (dpm)/plate	1×10^6	4	2852 ± 210	4613 ± 511^a
	1×10^6	8	1396 ± 116	2681 ± 215^a
	2×10^6	4	$14,940 \pm 1035$	$21,740 \pm 1957^a$
	2×10^6	8	$13,192 \pm 1123$	$19,546 \pm 1467^a$
DA uptake (dpm)/DA cell	1×10^6	4	1.96	2.83
	1×10^6	8	3.99	7.28
	2×10^6	4	4.47	6.06
	2×10^6	8	6.11	6.45

Dissociated mesencephalic cells (1 and 2×10^6 plated cells) were grown for 4 or 8 d *in vitro* in the presence of serum-free culture media with or without 10^{-7} M GM1. At appropriate times, the BZT-sensitive DA uptake and number of GTF⁺ neurons were assessed in sister cultures, as reported in Materials and Methods. Values are mean $\pm$ SEM of triplicate analyses for each assay.

^a $p < 0.05$; Student's *t* test (vs control value).

Table 3. Specificity of the GM1 molecule

Addition (10^{-7} M)	BZT-sensitive ^3H -DA uptake ^a (%)	DABA-sensitive ^{14}C -GABA uptake ^a (%)
—	100 ± 15	100 ± 28
GM1	154 ± 9	557 ± 20
GM3	142 ± 5	579 ± 47
GD _{1b}	159 ± 10	645 ± 30
Asialo-GM1	93 ± 15	118 ± 31
Sialic acid	88 ± 14	96 ± 34
Oligosaccharide ^b	98 ± 17	102 ± 41

Mesencephalic cells (1×10^6 /plate) were grown for 4 d in serum-free medium with and without the above components. All components were added to a concentration of 10^{-7} M. Uptake assays were conducted as reported in Materials and Methods. Values are referred to specific uptake per plate with respect to control values (87.1 fmol ^3H -DA/plate and 0.68 pmol ^{14}C -GABA/plate) and are the mean ± SEM of 2 independent experiments conducted in triplicate with each assay. See Materials and Methods for preparation and purity of compounds.

^a Evaluated at day 4 (1×10^6 cells/plate) *in vitro*.

^b Oligosaccharide component is referred to the oligosaccharide chain containing sialic acid of the GM1 molecule and was obtained from Professor G. Tettamanti (Institute of Biological Chemistry, University of Milano, Milano, Italy). For ganglioside nomenclature, see Svennerholm (1963).

time in culture. GM1 treatment did not significantly affect the number of dopaminergic cells at all plating densities or culture times, except that in the higher-density cultures it reduced the 8 d cell loss to only about 15% of the 4 d number. At the lower plating density, GM1 also increased by 50% the DA uptake per dopaminergic cell at both 4 and 8 d, and at the higher plating density, only at 4 d, perhaps because the uptake capacity was already maximal in the higher-density 8 d cultures. The protective effect of GM1 on the late dopaminergic cell losses was not restricted only to dopaminergic neurons, since the DNA content per plate also decreased less in the presence than in the absence of GM1 (15% decrease instead of 52%).

Neuronal specificity

The high-affinity ^{14}C -GABA uptake, abolished by 1 mM DABA (an inhibitor of neuronal GABA uptake), is considered specific to GABAergic neurons and is used as a parameter of GABAergic activity (Prochiantz et al., 1981). In the mesencephalic cultures, the DABA-sensitive ^{14}C -GABA uptake was linear up to at least 30 min of incubation at 37°C and reached its maximum value after 4 d in culture. Addition of 1 mM β -alanine, a selective inhibitor of glial GABA uptake, did not produce any further inhibition. This is in accord with the presence of very few glial elements in our culture system.

As already reported, in cultures of increasing cell density the DABA-sensitive ^{14}C -GABA uptake, when referred to per 10^6 plated cells, increased, if at all, only at relatively high cell densities (Dal Toso et al., 1988). As shown in Figure 1B, GM1 (10^{-7} M) markedly enhanced the DABA-sensitive ^{14}C -GABA uptake in 4-d-old cultures at all cell densities. Moreover, at 1×10^6 cells/plate, the GM1-induced increase of specific GABA uptake was concentration-dependent (97 and 156% increase with 10^{-8} and 10^{-7} M GM1, respectively). The kinetic analysis, conducted in the presence of 10^{-7} M GM1, indicated that, also in this case, there was an increase in the apparent $V_{\max}$ (control = 2.47 ± 0.06 ; GM1 = 3.22 ± 0.03 pmol/ μg DNA per 15 min) and a reduction in the apparent K_m value (from 1.67 ± 0.10 to 0.78 ± 0.09 M). In addition, the GM1 effect on specific GABA uptake was not restricted to mesencephalic cells. GM1 stimulated the

Table 4. Serum-stable association of ^3H -Ga1-GM1 to cultured mesencephalic cells as a function of ligand concentration or time

Ligand concentration (M)	Serum-stable-associated ^3H -GM1 (pmol/ μg DNA)
1×10^{-8}	0.201 ± 0.019
5×10^{-8}	1.373 ± 0.080
1×10^{-1}	2.960 ± 0.130
Time of exposure (hr)	
0.5	0.941 ± 0.087
1.0	1.193 ± 0.012
2.0	1.373 ± 0.153
24.0	4.597 ± 0.341

Mesencephalic cells (1×10^6 /dish) were cultured for 1 d, then exposed for 2 hr to serum-free medium containing ^3H -Ga1-GM1 at the concentrations indicated, or exposed to 5×10^{-8} M ^3H -Ga1-GM1 for the indicated times. Ligand exposures were at 37°C in a water-saturated 95% air, 5% CO_2 incubator. At all times, the extracted radioactivity comigrated with authentic GM1 on TLC plates. Values are means ($\pm$ SEM) from triplicate samples in each of 2 independent experiments.

DABA-sensitive ^{14}C -GABA uptake in 4-d-old dissociated fetal striatal cell cultures in serum-free conditions (from a control value of 1.12 ± 0.16 to 1.80 ± 0.09 pmol/ μg DNA per 15 min, with 10^{-7} M GM1 added to 1×10^6 plated cells). There too, no GM1 effect was observed on striatal cell viability at 4 d, as evaluated on the basis of phase-contrast microscopy and DNA content per dish.

Molecular specificity

Other gangliosides, possessing a chemical structure similar to GM1, were tested for their effects on specific DA and GABA uptake by cultured mesencephalic cells. Table 3 shows that similar increases were produced by monosialogangliosides such as GM3, which lacks the 2 terminal sugar residues, and disialogangliosides, such as GD_{1b}. In contrast, asialo-GM1, which lacks the *N*-acetylneuraminic acid, was totally ineffective. Free sialic acid or the oligosaccharide chain of GM1 also had no activity.

Relationship between association of GM1 to cells and uptake parameters

As shown in Table 4, stable association of GM1 to mesencephalic cells increased with increasing concentrations and time of incubation, as already reported for N₂A neuroblastoma cells (Facci et al., 1984). Exposure of mesencephalic cells to 10^{-7} M GM1 only for the first 24 hr in culture affected the specific uptake of both DA and GABA (Table 5). The effect was, however, smaller than that in cultures in which GM1 was present throughout the 4 d without changes of the culture medium. The GM1 effects after 24 hr of incubation were accompanied only by a decrease in the apparent K_m values: from 9.1 to 4.05×10^{-8} M for the DA uptake and from 1.8 to 0.87×10^{-6} M for the GABA uptake. No significant changes were observed in the apparent $V_{\max}$ values. Apparently, the affinity constants of the uptake systems are more readily sensitive to GM1 addition, while modifications in the apparent $V_{\max}$ value seem to necessitate longer exposure to GM1. It should be mentioned that the change of the incubation medium to interrupt the effect of GM1 may result in modification not only of the extent of GM1 association to the cells, compared to the 4 d incubation, but also in cell density effects. Whether the former or the latter factor is of greater importance is at present unclear.

Table 5. Effect of the addition, at various times, of GM1 on specific ³H-DA uptake in mesencephalic cells in culture

Treatment time	BZT-sensitive ³ H-DA uptake (fmol/plate for 15 min)	DABA-sensitive ¹⁴ C-GABA uptake (pmol/plate for 15 min)
0–24 hr		
Control	8.56 ± 1.04	0.11 ± 0.01
GM1	14.98 ± 0.70	0.30 ± 0.07
0–96 hr		
Control	15.22 ± 3.55	0.15 ± 0.03
GM1	41.41 ± 1.70	0.58 ± 0.12

Mesencephalic cells (1×10^6) were cultured in serum-free medium with or without 10^{-7} M GM1 either for only 1 d or for 4 consecutive d. In the former case, medium was removed from control and GM1-treated cultures and substituted with fresh culture medium containing no GM1. All cultures were incubated for another 3 d and uptakes were assessed at d 4. Values are the mean ± SEM of triplicate analyses.

Discussion

The data reported here show that exogenously supplied GM1, but not asialo-GM1, sialic acid, or the oligosaccharide chain of GM1, enhances DA uptake and long-term survival of dopaminergic neurons in fetal mouse-dissociated mesencephalic cells cultured in serum-free conditions. GM1 also increases specific ¹⁴C-GABA uptake in mesencephalic and striatal cultures and long-term survival of the bulk population of mesencephalic neurons (as judged by DNA content of the culture). This multiplicity of GM1 effects seems to reflect a capacity to affect development of neurotransmitter uptake activity and survival of various CNS neuronal cell types *in vitro* without any regional specificity. This is in accord with previous reports concerning facilitation by gangliosides of neuronal differentiation (i.e., neurite outgrowth, synaptogenesis, and/or tubulin mRNA induction) of a variety of clonal and primary neuronal cells in culture (Obata et al., 1977; Morgan and Seifert, 1979; Dimpfel et al., 1981; Hauw et al., 1981; Roisen et al., 1981; Leon et al., 1982; Byrne et al., 1983; Ferrari et al., 1983; Rybak et al., 1983; Spoerri, 1983; Tsuji et al., 1983; Dreyfus et al., 1984; Katoh-Semba et al., 1984; Doherty et al., 1985; Hefti et al., 1985b; Skaper and Varon, 1985; Skaper et al., 1985; Matta et al., 1986).

Relationship between GM1 effects and trophic influences in vitro

The GM1 effects on the dopaminergic neurons in the mesencephalic cell cultures appear to require the presence of cell-derived influences supporting their development, i.e., in terms of specific neurotransmitter uptake capability, and survival. GM1 is ineffective in low-density cultures and effective in higher-density cultures. Thus, GM1 seems to be unable to substitute for the cell-derived influences, but rather to potentiate the action of the latter or, in any case, to exert effects to which neurons respond only if appropriately supported. A similar relationship has been observed between GM1 and NGF in pheochromocytoma cells (PC12) and in fetal chicken dorsal root (DRG) and sympathetic ganglionic cells in culture (Ferrari et al., 1983; Katoh-Semba et al., 1984; Leon et al., 1984; Doherty et al., 1985; Skaper and Varon, 1985; Skaper et al., 1985; Matta et al., 1986). In addition, GM1 has been shown to have a permissive effect on the response of fetal chicken ciliary ganglia neurons to the chicken eye ciliary neuronotrophic factor (CNTF) (Skaper et al., 1985). Together these data suggest that the GM1 molecule does

not possess per se trophic activity, but is capable of affecting the efficacy of different trophic agents acting on specific neuronal types. Nonetheless, on comparison in the mesencephalic cell cultures between dopaminergic and GABAergic neurons, it appears that the GM1 effects and their relationship to trophic support may vary among neuronal cell types *in vitro*. In particular, the amplitude of the GM1-induced potentiation of the cell-derived influences on DA uptake in the mesencephalic cells was constant (approximately 1.7-fold) at all cell densities beyond the threshold. Furthermore, the effects on DA uptake of GM1 plus fetal mouse-dissociated striatal cells, known to enhance specific DA uptake per dopaminergic cell (Prochiantz et al., 1979; Di Porzio et al., 1980), were additive (data not reported). This indicates that GM1 and both the mesencephalic and striatal cell-derived influences affect dopaminergic neurons via different mechanisms. In contrast, the amplitude of the GM1-induced potentiation of GABA uptake increased with increasing cell density (at least up to 1.5×10^6 plated cells), suggesting that GM1 may have increased the sensitivity of the GABAergic cells to subthreshold concentrations of the cell-derived influences. Hence, the mechanisms underlying the GM1 potentiation of trophic activity on neuronal cells *in vitro* may not be unique, and may vary with the cell type and its developmental stage. In addition, it may depend on the type of trophic signal (e.g., survival versus cell–cell interactions) operative at a particular developmental stage of the neurons. Support for this latter view derives from evidence indicating that cell surface gangliosides play a modulatory role not only in signal-mediated cell growth and differentiation but also in cell adhesion and cell–cell recognition (Hakomori, 1981; Ando, 1983; Bremer et al., 1984, 1986; Blackburn et al., 1986; Thompson et al., 1986).

It is noteworthy that ganglioside addition to embryonic rat-dissociated septal cells in culture has recently been reported to enhance choline acetyltransferase (ChAT) activity but not NGF-mediated increase of ChAT activity (Hefti et al., 1985b). Furthermore, NGF does not increase survival or fiber outgrowth of the cultured fetal septal cholinergic neurons (Hefti et al., 1985a). The functional significance of the NGF-stimulated ChAT activity in these cultures and its relationship to its well-known neuronotrophic effect in PNS neurons requires further clarification. In this study, NGF addition to the mesencephalic cell cultures was ineffective both on uptake parameters and cell survival.

Types of GM1 effects in vitro

Two neuronal behaviors are affected by the cell density and GM1. One of these is neuronal survival. Endogenous agents provided by 1×10^6 cells/dish are adequate to support neuronal survival, but only for 4 d (during which time GM1 addition yields no numerical improvement). Longer survival (e.g., 8 d) requires exogenous supplementation from additional CNS cells or CNS tissue extracts (Dal Toso et al., 1988). The present study shows that GM1 enhances the effects of cell supplementation. In addition, similar results were obtained in experiments conducted using GM1 and a bovine striatal extract (data not shown). Hence, GM1 enhances the benefits of both supplements. The other neuronal behavior affected by GM1 and cell density is DA (or GABA) uptake. In principle, an increase of neurotransmitter uptake at nonsaturating substrate concentrations may be due to decrease of apparent K_m , presumably a reflection of an increased efficiency of the transport mechanism. This is not the case for the increase of V_{max} . In fact, the increase of V_{max} reflects

not only the number of specific cells but also their membrane surface (e.g., neurite extension) and/or the number of uptake sites per unit surface. While the apparent K_m is decreased by GM1 but not by an increase of the cell density, V_{max} is increased by increase of cell density as well as by GM1. The question arises as to the relationship between this differential behavior and the molecular mechanisms underlying the cell density and the GM1 effects.

One may visualize the occurrence of GM1 effects on multiple-membrane constituents (see below). The first, reflected by an increase in number of uptake sites, is apparently related to the potentiation by GM1 of the neuronotrophic, cell-derived influences, and may be due to ganglioside potentiation of transmembrane signaling of the trophic influences. This is probably related to ganglioside stimulation of the trophic-dependent neurite outgrowth. In fact, although neurite outgrowth has not been evaluated in this study, GM1 has been reported to facilitate neurite outgrowth in cells both with and without an absolute neuronotrophic requirement for survival and/or neurite extension (Byrne et al., 1983; Ferrari et al., 1983; Facci et al., 1984; Katoh-Semba et al., 1984; Leon et al., 1984; Skaper and Varon, 1985; Skaper et al., 1985; Matta et al., 1986). Interestingly, in chicken DRG cells *in vitro*, gangliosides potentiate NGF-induced neurofilament expression and maintenance (Doherty et al., 1985) while antibodies to GM1 block in a dose-dependent manner the NGF-induced neurite outgrowth (Schwartz and Spirman, 1982). In SB2IB2 cells, there occurs during ganglioside-induced differentiation a significant increase in the expression of mRNA for tubulin (Rybak et al., 1983). Besides the above, gangliosides are known to modify a number of membrane functions, presumably depending on the nature of the membrane constituent with which they interact. In fact, gangliosides are known to cause changes of membrane properties including enzyme activity (Partington and Daly, 1979; Davis and Daly, 1980; Leon et al., 1981; Goldenring et al., 1985), receptor binding (Hollmann and Seifert, 1986; Montecucco, 1986), receptor-mediated signal transduction (Bremer et al., 1984, 1986; Berry-Kravis and Dawson, 1985; Bar-Sinai et al., 1986), and ion permeability (Vyskocil et al., 1985). Likely, the GM1-induced increased efficiency of the uptake system is a reflection of GM1 interactions with the neurotransmitter carrier system.

Mechanisms of GM1 effects: relationship to cell association of GM1

The question now arises with regard to the mechanisms underlying the multiple ganglioside effects. Although the answer is largely obscure, the results reported here have shown that the portion of stably associated GM1 is probably involved in the effects observed on the dopaminergic and GABAergic neurons. Incubation of various cells in the presence of gangliosides, including GM1, results in a time- and temperature-dependent cell accumulation of the added glycolipid, even in the absence of endocytosis (Moss et al., 1976; Radsak et al., 1982; Schwarzmann et al., 1983; Facci et al., 1984). Although some of the gangliosides can be released by treatment of the cells with serum or trypsin, it appears that a residual portion (trypsin-insensitive) is tightly associated with the outer leaflet of the plasma membrane. At the concentration used in this study, the cell-associated GM1 is mainly trypsin-resistant (Facci et al., 1984). Spin-labeled ganglioside analogs indicate that the trypsin-insensitive form of cell membrane-associated glycolipid displays characteristic anisotropic ESR spectra (Schwarzmann et al., 1983),

which closely resemble those of spin-labeled analogs at low dilution in artificial vesicles. The stably associated gangliosides are both functionally and metabolically active (Moss et al., 1976; Fishman et al., 1983; Sonderfeld et al., 1985). Likely, the surface packing of gangliosides and/or the ganglioside interactions with membrane proteins and phospholipids in defined microdomains of the bilayer play a strategic role in the supramolecular organization and functional properties of the membrane components (Spiegel et al., 1985; Tettamanti et al., 1985). Another possibility is that the stably cell-associated exogenous gangliosides undergo internalization and recycling via direct glycosylation, resulting in *de novo* synthesis of more complex gangliosides (Ghidoni et al., 1983; Sonderfeld et al., 1985). These cause modification of the cell surface ganglioside pattern, which, in turn, may affect the membrane properties.

Relationship between ganglioside effects in vitro and in vivo

The question has been raised as to whether the findings obtained *in vitro* are relevant as models for the ganglioside-mediated effects *in vivo*. Hefti et al. (1985b) have concluded that the ganglioside effects on maintenance and/or recovery of function following CNS injury *in vivo* are related to the ganglioside-induced attenuation of astrocyte proliferation, rather than to their effects on neuronal cells *in vitro*. The argument has been that the concentrations at which gangliosides act on neuronal cells *in vitro* (0.05–0.75 mg/ml of medium) are higher than those obtained during *in vivo* administration (10–50 mg/kg of body weight). While, in the above-mentioned study, high concentrations of gangliosides *in vitro* were employed to evidence their effects on astrocyte morphology and proliferation, others have shown that similar concentrations of individual gangliosides may be toxic for astroglial cells in culture (Skaper et al., 1986). In the present study, the effect of gangliosides is obtained at concentrations of 15–150 ng/ml, compatible with the ganglioside concentrations obtainable in the brain after its systemic administration *in vivo* (Ghidoni et al., 1986). We therefore favor the view that the GM1 effects *in vivo* on neuronal repair processes are related to its capability to enhance neuronal cell responsiveness to endogenously occurring neuronotrophic factors.

Neuronotrophic factors are known to be present in the adult brain and to increase following brain injury. Although their physiological relevance still remains to be established, recent experiments conducted with NGF (Hefti, 1986; Williams et al., 1986) suggest that they presumably play a primary role in the maintenance of cell survival following CNS injury. Interestingly, GM1 treatment has been reported to facilitate neuronal cell survival not only of dopaminergic and noradrenergic neurons, but also of cholinergic neurons known to be NGF-responsive (Agnati et al., 1983; Toffano et al., 1984; Commissiong and Toffano, 1986; Cuello et al., 1986; Sofroniew et al., 1986).

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