Gangliosides potentiate in vivo and in vitro effects of nerve growth factor on central cholinergic neurons

(trophic factors/neural plasticity/neuronal degeneration/septal cell culture/nucleus basalis)

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ABSTRACT The effects of nerve growth factor β (β-NGF) and ganglioside GM1 on forebrain cholinergic neurons were examined in vivo and in vitro. Following unilateral decortication of rats, GM1 (5 mg/kg per day) administered intracerebroventriculally could protect forebrain cholinergic neurons of the nucleus basalis magnocellularis from retrograde degeneration in a manner comparable to β-NGF. Administered in combination with β-NGF, GM1 produced a significant increase in choline acetyltransferase activity in the nucleus basalis magnocellularis and remaining cortex ipsilateral to the lesion. Concentrations of GM1 that were ineffective when administered alone in this lesion model, when given with β-NGF, potentiated β-NGF effects in both of the above brain areas. In dissociated septal cells in vitro, an increase in choline acetyltransferase activity was noted at β-NGF concentrations as low as 0.1 pM and reached a plateau at 1 nM. A moderate (up to 35%) stimulation of choline acetyltransferase activity was observed with 10 μM GM1. The application of β-NGF in combination with 10 μM GM1 or 0.1 μM GM1, a concentration that is ineffective in these cultures, produced a much greater increase in choline acetyltransferase activity than did β-NGF alone. These observations support the idea that exogously applied gangliosides can elicit trophic responses in cholinergic neurons of the central nervous system. That GM1 increases and even potentiates β-NGF effects suggests that some of the trophic actions of this compound may be mediated through endogenous trophic factors.

Nerve growth factor β (β-NGF) can be considered the prototype for substances exerting in vivo and in vitro trophic effects on defined cells of the nervous system (1). In addition to its peripheral actions, β-NGF acts on subsets of centrally located neurons (2, 3). Central forebrain cholinergic neurons contain β-NGF binding sites, and their terminal targets produce the trophic peptide, which can be transported retrogradely to cell bodies of these cholinergic neurons (4). In the adult, these cholinergic neurons respond to exogenous β-NGF after partial or total damage of the septal-hippocampal connections (5–7). In addition, β-NGF has been found to affect forebrain cholinergic neurons in vitro (8, 9). Sialogangliosides, in particular GM1, exert trophic-like activity, both in vivo and in vitro, and resemble “bona fide” trophic factors in many ways (for review, see ref. 10). When applied in vivo, they promote the anterograde regeneration of acetylcholinesterase-reactive fibers in the hippocampus after partial fimbria transections (11). Administration of GM1 also prevents the retrograde cell shrinkage of cholinergic neurons of the nucleus basalis magnocellularis (NBM) that follows cortical infarction (12), as well as cell death in the medial septum after unilateral hippocampal ablation (13). These findings have prompted us to investigate the possible in vivo and in vitro interactions between a selective and a specific trophic factor (β-NGF) and a biological substance (GM1) whose apparent trophic activity is less well defined. Our preliminary observations in the in vivo model (14) and the results presented here strongly suggest that the ganglioside GM1 has an enabling or potentiating effect on β-NGF-mediated responses in central cholinergic neurons.

MATERIAL AND METHODS

Cortical Lesions and Drug Treatment. Male Wistar rats (Charles River Breeding Laboratories, 300–350 g) were used in these experiments and were subjected to a unilateral left-side cortical devascularizing lesion as described (15). The surgical procedure produced a limited, well-defined infarction of the neocortex without affecting underlying brain structures (15).

A group of lesioned rats (n = 5) received GM1 (0.5 or 5 mg/kg per day) intracerebroventricularly (i.c.v.) for 1 week through a stainless steel cannula (23-gauge) permanently implanted into the right lateral ventricle (coordinates from Bregma (16): AP, −0.8; L, 1.4; V, 3.5). The cannula was connected by flexible polyethylene tubing to a subcutaneously implanted osmotic minipump (Alzet 2001, Alza). Another lesioned group (n = 5) received β-NGF (12 μg per day) alone or in combination with GM1 (0.5 or 5 mg/kg per day) in the same manner. A group of lesioned rats (n = 6) received physiological saline (0.9% NaCl containing 0.1% bovine serum albumin; Sigma), and rats that had not undergone surgery (n = 6) served as controls. Animals were decapitated 30 days after the surgical procedure (i.e., 23 days after treatment with β-NGF and/or GM1 ceased). Discrete brain areas were microdissected from fresh tissue slices as described (15).

Biochemical Analysis of Microdissected Tissue. Microdissected tissues were kept frozen at −80°C until required. Choline acetyltransferase (ChAT) activity was determined by a radiometric assay described by Fonnum (17), and protein content was determined by the Bradford method (18).

Immunohistochemical Analysis of Brain Tissue. Three additional rats from each group were anesthetized and perfused as described (19). Sections (50 μm) of the entire NBM were obtained from frozen brain tissue blocks by using a sliding microtome (Reichert). Sections were then processed free-floating for ChAT immunocytochemistry (19).

Cell Culture. The septal area was dissected from brain of day-17 embryonic rat fetus (Sprague-Dawley) as described by Dunnett et al. (20). Dissociated septal cells were prepared by a modification of a published method (21). Viable cell yield was determined by the trypan blue-exclusion test. The dissociated cells were resuspended in culture medium to a

Abbreviations: ANOVA, analysis of variance; ChAT, choline acetyltransferase; NBM, nucleus basalis magnocellularis; β-NGF, nerve growth factor β, i.e.v., intracerebroventricularly.

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final cell density of $0.6 \times 10^6$ viable cells per ml and seeded (1 ml of cell suspension per well) on poly(l-lysine) (Sigma)-coated culture wells (16 mm diameter) in 24-well plates (Falcon). One rat embryo yielded on average $0.5-0.75 \times 10^6$ viable septal cells. Exposure of the cells to either $\beta$-NGF or GM1 began immediately after seeding. Cultures were maintained at $37^\circ C$ in a humidified incubator in a 95% air/5% CO$_2$ atmosphere. The cells were inspected regularly and medium was replenished every 3 days. Data were analyzed by analysis of variance (ANOVA) and/or two-tailed $t$ test.

Biochemical Determinations of Tissue Culture Preparations. The cells in culture were washed free of medium with Ca$^{2+}$/Mg$^{2+}$-free phosphate-buffered saline (PBS, pH 7.4), collected in a total volume of 100 $\mu$l of homogenization buffer (10 mM EDTA/0.5% Triton X-100, pH 7.4), and homogenized on ice in a glass homogenizer. Aliquots of the homogenate were taken for determination of ChAT activity (17) and protein concentration (Bio-Rad protein assay).

Immunocytochemistry of Tissue Culture Preparations. Cultures were washed free of medium with Ca$^{2+}$/Mg$^{2+}$-free PBS, fixed for 20 min at room temperature in 4% paraformaldehyde, and then washed four or five times with PBS/0.02% Triton X-100 before incubation with the primary antibodies [anti-glia fibrillary acidic protein, anti-neurofilament, anti-ChAT, anti-NGF receptor (22, 36)]. Immunocytochemical staining was obtained by adapting previously described methods (22) to our tissue culture conditions.

RESULTS

In Vivo Studies. These studies confirm that ChAT activity decreases significantly in the ipsilateral NBM of mature rats 30 days following a unilateral devascularizing lesion of the neocortex. The previously described (23) retrograde cell shrinkage and loss of neurites of these forebrain cholinergic neurons were clearly apparent in NBM sections from lesioned rats (compare Fig. 1 a and b). As previously reported (15), when compared with the control group, no significant changes in ChAT activity were found in any other microdissected brain areas (data not shown).

The i.c.v. administration of $\beta$-NGF (12 $\mu$g per day), for 7 days from the onset of the lesion, prevented a decrease in ChAT activity in the NBM after partial cortical infarction. The magnitude of this protective effect was shown to be comparable to that obtained with the i.c.v. administration of GM1 alone (5 mg/kg per day) (Table 1, experiment A). The combined administration of $\beta$-NGF and GM1 in the partially decorticated animals increased ChAT activity in the NBM, ipsilateral to the lesion, above control levels. Immunocytochemical analysis revealed not only full protection of the cholinergic neurons from retrograde cell shrinkage and loss of dendritic extensions but also an apparent increase in the number of ChAT-immunoreactive processes in the neuropil (Fig. 1c).

In this series of experiments ChAT activity in the remaining cortex of the lesioned animals was found to be similar to that of the unlesioned side. Furthermore, treatment with $\beta$-NGF or GM1 alone increased ChAT activity of the lesioned side over that of control. A more noticeable cooperativity between these two factors was observed in the remaining neocortex ipsilateral to the lesion, where the combined treatment brought ChAT activity to 237% of control values (Table 1).

In cortically lesioned animals treated with low doses of GM1 (0.5 mg/kg per day, i.c.v., 7 days), a significant decrease (32% of control values) in ChAT activity was observed in the NBM ipsilateral to the lesion (Table 1, experiment B). However, if these subthreshold amounts of ganglioside were administered concurrently with effective doses of $\beta$-NGF to lesioned animals, a significant increase (21%) in ChAT activity was observed in the area of the affected forebrain cholinergic neurons. Even more remarkable were the changes observed in ChAT activity in the ipsilateral cortex of the lesioned animals after simultaneous i.c.v. administration of subthreshold amounts of GM1 and effective doses of $\beta$-NGF. While this low dosage of GM1 per se did not alter ChAT activity in the remaining ipsilateral cortex, in combination with $\beta$-NGF it produced a marked increase of the ChAT enzymatic activity.

In Vitro Studies. Dissociated septal cells in culture represent a mixed neuronal-glial cell population as determined immunocytochemically with the use of antisera to glial fibrillary acidic protein and antiserum to neurofilament pro-
Table 1. Effect of β-NGF administered for 7 days in combination with an effective (5 mg/kg per day; experiment A) or an ineffective (0.5 mg/kg per day; experiment B) dose of GM1 on ChAT activity in the NBM and cortex of mature rats, 30 days after unilateral decortication

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ChAT activity</th>
<th>% control</th>
<th>ChAT activity</th>
<th>% control</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>57.67 ± 3.86</td>
<td>—</td>
<td>35.81 ± 2.39</td>
<td>—</td>
</tr>
<tr>
<td>Lesion plus vehicle</td>
<td>6</td>
<td>31.16 ± 3.17</td>
<td>54*</td>
<td>35.85 ± 1.74</td>
<td>100</td>
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<tr>
<td>Lesion plus GM1</td>
<td>5</td>
<td>61.94 ± 6.55</td>
<td>107</td>
<td>50.70 ± 2.44</td>
<td>142*</td>
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<tr>
<td>Lesion plus β-NGF</td>
<td>5</td>
<td>50.94 ± 3.75</td>
<td>88</td>
<td>47.63 ± 3.12</td>
<td>132*</td>
</tr>
<tr>
<td>Lesion plus GM1 plus β-NGF</td>
<td>5</td>
<td>69.41 ± 1.06</td>
<td>120*</td>
<td>84.82 ± 10.42</td>
<td>237*</td>
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</table>

Experiment B

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ChAT activity</th>
<th>% control</th>
<th>ChAT activity</th>
<th>% control</th>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>69.06 ± 6.67</td>
<td>—</td>
<td>39.20 ± 2.77</td>
<td>—</td>
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<td>Lesion plus vehicle</td>
<td>6</td>
<td>44.87 ± 6.60</td>
<td>65*</td>
<td>38.20 ± 4.69</td>
<td>97</td>
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<tr>
<td>Lesion plus GM1</td>
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<td>46.92 ± 2.80</td>
<td>68*</td>
<td>36.93 ± 2.80</td>
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<tr>
<td>Lesion plus β-NGF</td>
<td>5</td>
<td>73.07 ± 3.30</td>
<td>109</td>
<td>59.06 ± 2.90</td>
<td>151*</td>
</tr>
<tr>
<td>Lesion plus GM1 plus β-NGF</td>
<td>5</td>
<td>83.87 ± 6.56</td>
<td>121*</td>
<td>72.98 ± 4.08</td>
<td>186*</td>
</tr>
</tbody>
</table>

Tissues were obtained 30 days after lesion (i.e., 23 days after treatment with GM1 and/or β-NGF ceased). Values for ChAT activity are means ± SEM and are expressed as nmol per mg of protein per hr; n indicates number of rats.

*Significantly different from control at P < 0.01 (ANOVA followed by a post-hoc Dunnett’s test).

ChAT enzymatic activity that was significantly greater than that obtained with β-NGF alone. This potentiating effect of GM1 was more dramatic when GM1 was applied in combination with 0.1 pM β-NGF. We found that even 0.1 μM GM1, which was ineffective when applied alone, potentiated the β-NGF-induced increase in ChAT activity (Figs. 3 and 4).

**DISCUSSION**

The trophic effects of β-NGF on central and peripheral neurons are in all likelihood receptor-mediated. Although there is still no definite information on the cellular and molecular events that take place following the interaction of β-NGF with its receptor, several mechanisms have been postulated (for review, see ref. 24). The interaction of the ganglioside with endogenous trophic factors, and with β-NGF in particular, could take place at any level. Nevertheless, it is likely that this interaction occurs at the level of the cell membrane where GM1 is incorporated (25). In this regard, it is interesting that immobilized GM1 is capable of binding β-NGF with low affinity (26). Thus, gangliosides could provide additional binding sites for growth factors or, on the other hand, may modify the growth factor receptor state, as is the case for the ganglioside GD2 and the vitronectin receptor (27).

![Image](a.png)  
**Fig. 2.** Detection of ChAT (α)- and NGF receptor (β)-immunoreactive cells in septal cell culture. Note the similarity in incidence and in morphology of ChAT- and NGF receptor-immunoreactive cells. Single arrows indicate immunoreactive cell bodies, and paired arrows indicate neurites. (Interference contrast optics; bar = 20 μm.)
Certain conditions are required for gangliosides to show a trophic effect in vivo or in vitro. These have been referred to as "permissive conditions" for the in vivo effects (19) or a "window of opportunity" for the in vitro effects. It is conceivable that under certain conditions the availability of endogenous trophic factors is affected, and consequently the ability of cells to respond to these factors is influenced. In the in vivo experimental model, the early initiation of ganglioside treatment is essential (19). This is in agreement with the evidence that, in response to injury, the brain produces low amounts of endogenous trophic factors immediately after the insult (e.g., ref. 28). Therefore, in instances of extensive neural lesions, a situation of extreme vulnerability might arise. In such a case, irreversible anterograde and retrograde cellular damage would occur. In the case of central cholinergic neurons, retrograde degenerative changes can be in part reversed by the timely administration of β-NGF (5–7). In view of the above, it can be proposed that in the present in vivo situation the administration of gangliosides prevents anterograde and retrograde neuronal degeneration by potentiating the action of the limited quantity of endogenous trophic factors produced in the first few days subsequent to the lesion. The difficulties encountered in rescuing cholinergic neurons by administration of exogenous gangliosides in aged rats (19) could be explained in the same way, since aging is accompanied by an apparent loss of β-NGF receptors.


and a diminished production of endogenous factors after injury (30).

The idea that gangliosides act cooperatively with β-NGF in the present in vivo model is reinforced by the observations that the expression of β-NGF is increased in the target areas of the basal forebrain cholinergic neurons following mechanical lesions (for review, see ref. 30). Although β-NGF apparently does not act as a trophic agent for dopaminergic neurons, the reported actions of GM1 over the nigrostriatal system (31) might be similarly explained. For this, the identification of a specific trophic factor(s) that acts on this subset of central neurons would be necessary.

The effects of β-NGF and/or GM1 in the cortex suggest that their administration may provoke an important reorganization of the cholinergic fibers of the remaining neocortex. Whether this is due to an increased production of the biosynthetic enzyme or due to sprouting of cholinergic terminals is not known.

The concept that gangliosides potentiate β-NGF-mediated effects on cholinergic neurons is supported by in vitro studies, in which the exogenous concentrations of the two factors can be accurately controlled. In other cell culture systems, the trophic actions of gangliosides are dependent upon the presence of β-NGF (32). Although GM1 does exert some trophic action when administered alone in our in vitro model, we have established that there is also an important interaction between specific (β-NGF) and non-specific (GM1) factors on cholinergic markers. It is interesting that both effective and subthreshold concentrations of the ganglioside, when applied in combination with β-NGF, produced supra- and over maximal responses in ChAT activity, consistent with the in vivo observations in the cerebral cortex. Although Hefti et al.
reported that gangliosides did not potentiate the effects of \(\beta\)-NGF on septal cells in culture, their experiments differed from ours, as they employed a mixture of gangliosides rather than pure GM1 and a single, high concentration of \(\beta\)-NGF. We have observed that the synergism between GM1 and \(\beta\)-NGF occurs at submaximal concentrations of \(\beta\)-NGF. However, the molecular mechanisms underlying these interactions have not yet been properly examined. Nevertheless, it is possible that the contribution of glial cells is important both \textit{in vivo} and \textit{in vitro}. There is evidence that \(\beta\)-NGF is produced by glial cells (34). \textit{In vivo}, neural damage results in reactive gliosis, a phenomenon that may contribute to increased availability of endogenous trophic factors.

Validation of the ganglioside–trophic factor cooperativity hypothesis will require further investigations of the molecular mechanisms underlying their interactions in the central and peripheral nervous systems. There is already evidence of the cooperativity of \(\beta\)-NGF and gangliosides in the peripheral nervous system (35). The investigation of the interaction of gangliosides with \(\beta\)-NGF and other trophic factors may provide valuable insight for the establishment of therapeutic regimes in neurodegenerative diseases.

We dedicate this paper to the memory of Mr. Manuel Madanes. We thank Drs. R. Levi-Montalcini, L. Aloe, and W. Mushynski for valuable advice and materials. We thank J. Seguin for secretarial assistance and A. Foster for photography. This work was supported by the Research Council (Canada) and, in part, by FIDIA Laboratories (Italy). L.G. received a Fonds de la Recherche Scientifique (Quebec) studentship, and R.L.K., a postdoctoral fellowship (Medicorp, Canada).