Gangliosides Activate Trk Receptors by Inducing the Release of Neurotrophins*

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We used NIH-3T3 fibroblasts expressing the different Trk receptors to examine whether GM1 ganglioside and its semisynthetic derivative LIGA20 activate various neurotrophin receptors. GM1 induced autophosphorylation of TrkC more potently than TrkA or TrkB receptors. In contrast, LIGA20 activated TrkB tyrosine phosphorylation only. Therefore, Scatchard analysis was performed to determine whether GM1 binds to TrkC. GM1 failed to displace neurotrophin-3 binding, suggesting that this ganglioside does not act as a ligand for Trk receptors. In addition, GM1 failed to induce autophosphorylation of a chimeric receptor consisting of the extracellular domain of the tumor necrosis factor receptor and the intracellular domain of TrkA, suggesting that GM1 does not affect the tyrosine kinase domain. We next determined whether GM1 induces the release of neurotrophins from fibroblast cells. GM1 induced a rapid and significant increase in the amount of neurotrophin-3, but not other neurotrophins. This effect was independent of the presence of Trk because K252a did not prevent GM1-mediated release of neurotrophin-3. Moreover, GM1-mediated TrkC autophosphorylation was blocked by TrkC-IgG (but not TrkB-IgG) receptor bodies, further suggesting that GM1 activates TrkC by inducing the release of neurotrophin-3. This hypothesis was also tested in cultured cerebellar granule cells. GM1 induced neurotrophin-3 (but not brain-derived neurotrophic factor or nerve growth factor) release. In contrast, LIGA20 increased the secretion of brain-derived neurotrophic factor. Our data show that gangliosides may activate different Trk receptors by differentially affecting the release of neurotrophins.

Gangliosides constitute a heterogeneous family of sialic acid-containing glycosphingolipids found in relative abundance in the nervous system (1, 2), where they influence the development and/or differentiation of neurons (3–5). Interest in these molecules has grown since the discovery that gangliosides displace neurotrophins from fibroblast cells (6–11). Indeed, the monosialotetrahexosylganglioside (GM1) prevents the dramatic loss of cholinergic neurons subsequent to hippocampal ablation or cortical lesion (6–9). GM1 also stimulates the regeneration of dopaminergic neurons (10), improves cell survival in injured substantia nigra (11), and ameliorates the abnormal motor responses in animals treated with the dopamine neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (12). Moreover, GM1 and other gangliosides have been shown to possess a neuroprotective action against excitatory amino acid toxicity in vitro (13–15) and after stroke or brain ischemia (16, 17) as well as after spinal cord injury (18). Despite these findings, the mechanisms of ganglioside trophic activity are still unclear.

Potential substrates GM1 that would explain its neurotrophic effects are the neurotrophins and their receptors. The neurotrophin family of neurotrophic factors includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, NT-4/5, and NT-6 (reviewed in Ref. 19). These trophic factors can promote the survival, growth, and differentiation of neurons through activation of the Trk transmembrane tyrosine kinase receptors (20, 21) and the p75 neurotrophin receptor (22, 23). NGF binds to TrkA; BDNF and NT-4/5 activate TrkB; and NT-3 primarily activates TrkC, but can also bind to TrkA and TrkB (reviewed in Ref. 24). p75 binds all members with relative equal affinity (25). Earlier studies have demonstrated that GM1 is able to activate the TrkA receptor (26–28), suggesting that GM1 may mimic NGF trophic activity. However, although the TrkA activation can justify the neurotrophic effects of GM1 on basal forebrain cholinergic neurons, it does not explain the effect of GM1 on other neuronal populations unresponsive to NGF. On the other hand, LIGA20, a semisynthetic derivative of GM1 (15, 29), induces TrkB tyrosine phosphorylation and shows responses similar to those of BDNF (30). We therefore tested the hypothesis that gangliosides activate various neurotrophin receptors. In addition, we have examined the molecular mechanism whereby GM1 and LIGA20 may activate TrkB by determining whether these gangliosides act as ligands for the TrkB receptors or induce the release of the neurotrophins. We provide evidence that GM1 induces the release of NT-3, whereas LIGA20 mediates the release of BDNF.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH-3T3 cells expressing each Trk receptor (kindly provided by Dr. D. Kaplan, Montreal Neurological Institute, Montreal, Canada) were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium supplemented with 10% donor calf serum (Invitrogen) and 200 μg/ml Geneticin in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. The medium was changed every 3 days, and confluent cells were used for the experiments. Incubation of cells with control medium (0.1% bovine serum albumin (BSA)), growth factors, and other compounds was carried out in serum-free medium.

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‡ The abbreviations used are: GM1, monosialotetrahexosylganglioside; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; BSA, bovine serum albumin; TNF, tumor necrosis factor; MTT, mitochondrial 3(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide; NIH, NIH-3T3 cells expressing TrkA; NIH, NIH-3T3 cells expressing TrkB; NHIC, NIH-3T3 cells expressing TrkC; C6Trk, C6 glioma cells expressing TrkA.

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Cerebellar granule cells were prepared from postnatal day 8 Sprague-Dawley rat pups (Taconic Farms Inc., Germantown, NY) as previously described (31, 32). In brief, neurons were plated onto 1% poly-L-lysine-pretreated 100-mm plastic dishes at a density of 2.5 × 10^5 cells/ml and grown in basal Eagle's medium (Invitrogen) containing glutamine (2 mM), fetal calf serum (10%), KCl, HEPES (25 mM), gentamicin (100 μg/ml), and penicillin/streptomycin (10,000 units/ml). Cells were maintained at 37°C in 5% CO2 and 95% air. Cytosine arabinoside (10 μM) was added 24 h after cell plating to inhibit non-neuronal cell proliferation. Cultures were used on day 8 in vitro for all experiments.

**Tyrosine Phosphorylation**—Trk tyrosine phosphorylation was carried out as described (28). In brief, cells were exposed to neurotrophins (Promega, Madison, WI), gangliosides (Fidia S.p.A., Abano Terme, Italy), or Trk-IgG receptor bodies (Regeneron Pharmaceuticals, Tarrytown, NY) for the appropriate time points and lysed in lysis buffer (1% Nonidet P-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 1% glycerol, and 0.25% bromphenol blue) for electrophoresis on 7.5% SDS-polyacrylamide gel. Gels were transferred to nitrocellulose and probed overnight at 4°C with anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) as previously described (28). Blots were analyzed using an enhanced chemiluminescence system (Amersham Biosciences) and then stripped and reprobed with anti-pan Trk antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Binding Studies**—Cell membranes for receptor binding were prepared according to the method of Hempstead et al. (33) with some modifications. Briefly, cells were harvested in a buffer solution containing 1 mM Tris-Cl (pH 8.0) and 1 mM EDTA and lysed using a Tekmar Tissumizer. Nuclei were pelleted by centrifugation at 1000 g for 5 min. Cell membranes were isolated by centrifugation of the supernatant for 30 min at 15,000 g. The membrane pellet was resuspended in the same buffer and stored at −70°C. Binding studies were performed with 30 μg of protein suspended in a binding solution of culture medium containing 5 mM mg/ml BSA and 20 mM HEPES (pH 7.2). 125I-Labeled NT-3 and 125I-labeled NGF (Bachem, San Carlos, CA) were added to the mixture (0.21-ml final volume), followed by incubation for 2 h at 4°C. The bound complexes were isolated by filtration according to an established method (34). 125I-Labeled neurotrophins were added to the mixture (0.21-ml final volume), followed by incubation for 2 h at 4°C. The bound complexes were isolated by filtration through GF/C filters and washed using the binding solution; the filters were then counted using a γ-counter with a counting efficiency of 80%. Nonspecific binding was determined using a 200-fold excess of unlabeled neurotrophins in the binding solution. Saturation and Scatchard analyses were performed using the LIGAND program.

**p70G'** Chimera—Transient transfection of NIH-3T3 cells with the pcDM8 expression vector (Invitrogen) was carried out as previously described (35) using LipofectAMINE reagent (Invitrogen). Transfection included 8 μg of pcDM8 or pcDM containing a chimeric receptor consisting of the extracellular domain of the tumor necrosis factor (TNF) receptor and the intracellular domain of Trkα (36), a gift from Dr. Eric M. Shooter (Stanford University, Stanford CA), and 0.2 μg of pRSV-β-galactosidase reporter vector as an internal control. After 6 h, cells were washed twice and incubated with growth medium. Cells were allowed to express this receptor for 2 days before harvesting. After exposure to GM1, NGF, or TNF for 5 min, cells were lysed as described above. The lysates were incubated with an anti-Trk antibody, and Trk tyrosine phosphorylation was determined as described above.

**Enzyme Immunoassay**—Neurotrophin levels were determined by a two-site immunoassay as previously described (37, 38). In brief, cells were allowed to confluence and medium was then changed and replaced with unconditioned serum-free medium containing 0.1% BSA or gangliosides. Incubation was allowed to proceed for 5, 15, or 30 min or additional times. The medium was then removed and concentrated using Centricon YM-10 concentrators (Millipore Corp., Bedford, MA), and neurotrophin levels were determined using the NGF/NT-3/BDNF Emax™ immunoassay system (Promega) according to the manufacturer’s instructions.

**Neuronal Survival**—Cerebellar granule cell survival was determined by mitochondrial dehydrogenases 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (30, 32) and according to the manufacturer’s specifications (MTT Kit I, Roche Molecular Biochemicals). Briefly, neurons were cultured on 96-well plates; 10 μl of the 5 mg/ml MTT labeling reagent was added to each well containing neurons; and plates were incubated for 4 h in a humidified atmosphere. After the incubation, 100 μl of the solubilization solution was added to each well for 18 h. The absorbance of the samples was measured at wavelengths of 570 and 700 nm.

**RESULTS**

**Gangliosides Demonstrate Differential Specificity upon Trk Autophosphorylation**—GM1 activates TrkA (28) more than TrkB (30), suggesting that this ganglioside may affect different Trk receptors. To examine the relative potency of GM1 in Trk activation, we used NIH-3T3 fibroblasts transfected with cDNAs for TrkA (NIHA), TrkB (NIHB), or TrkC (NIHC). Cells were exposed to GM1 (60 μM), and Tyrosine phosphorylation was determined at various time points. Cells were also exposed to NGF, BDNF, or NT-3 (100 ng/ml each) as a positive control and to ceramide, the lipophilic portion of GM1. In NIHA (Fig. 1A) and NIHC (Fig. 1C) cells, GM1 increased TrkA and TrkC tyrosine phosphorylation, respectively, within 10 min, although its potency was weaker than that of NGF or NT-3. The effect of GM1 on TrkC phosphorylation lasted up to 30 min and was over by 3 h (Fig. 2A). Surprisingly, exposure of NIH cells to GM1 for 10, 30, or 180 min did not result in any significant increase in TrkB tyrosine phosphorylation (Figs. 1B and 2A). Therefore, the GM1-mediated increase in TrkC tyrosine phosphorylation was quantitatively stronger than that of other Trk receptors (Fig. 2A). Ceramide failed to change the basal levels of Trk tyrosine phosphorylation in any cell line examined (Fig. 1).

The inability of GM1 to evoke a significant activation of TrkB...
prompted us to examine whether NIHB cells respond to gangliosides. Cells were then exposed to LIGA20, which we have previously shown to activate TrkB in cerebellar granule cells more potently than GM1 (30). In contrast to GM1, LIGA20 elicited a significant increase in TrkB tyrosine phosphorylation within 10 min (Fig. 2B), suggesting that the weaker effect of GM1 on TrkB is not attributable to lower expression of these receptors or to unresponsiveness of NIHB cells to gangliosides.

**GM1 and Neurotrophin Binding**—The relative potency of GM1 in activating TrkC more than other Trk receptors could be due to a quantitatively different number of Trk receptors expressed in NIH-3T3 cells. To test this hypothesis, equilibrium binding and Scatchard plot analyses were carried out. Crude membranes were prepared from NIHC and NIHA cells and assayed for binding to 125I-labeled NT-3 or 125I-labeled NGF, respectively. In NIHC cells, a specific and saturable NT-3 binding was observed with one site in the high affinity range (KD = 9 ± 1.1 pM) (Fig. 3A). Similarly, NIHA cells exhibited a saturable and high affinity binding site for NGF (KD = 12 ± 2.5 pM) (data not shown). The single binding site in both cell lines most likely results from the fact that these cells do not express the low affinity component of the neurotrophin receptor p75 (21). Moreover, both cell lines expressed a comparable amount of receptors (NIHC, Bmax = 700 ± 88 fmol/mg of protein; and NIHA, Bmax = 778 ± 79 fmol/mg of protein). Therefore, the stronger activation of TrkC versus TrkA by GM1 is not the result of differential expression of Trk receptors in these cells.

GM1 may act as a ligand for TrkC. To test this hypothesis, we examined the ability of GM1 to displace 125I-labeled NT-3. Unlabeled NT-3 and NGF were used as positive and negative controls, respectively. NT-3 was able to elicit a concentration-dependent decrease in 125I-labeled NT-3 binding starting at 10 pM (Fig. 3B). In contrast, neither GM1 nor NGF displaced 125I-labeled NT-3 binding even when used at 10 μM (Fig. 3B). These data indicate that GM1 does not interact with TrkC at the NT-3-binding site. However, GM1 may interact at an allosteric site of the TrkC receptors. Thus, we examined whether GM1 changes the affinity of NT-3 for its receptor. The binding assay was performed with membranes incubated with 125I-labeled NT-3 (10 pM) in the absence or presence of various concentrations of GM1 (up to 100 μM) (data not shown), and binding data were analyzed by Scatchard plot. GM1 failed to significantly change the affinity of 125I-labeled NT-3 for TrkC (NT-3, KD = 10 ± 1.9 pM; and NT-3 + GM1, KD = 11 ± 1.5 pM), suggesting that GM1 does not act at an allosteric site of the TrkC receptor.

**GM1 Does Not Activate the p70trk Chimeric Receptor**—Activation of TrkA by inducing receptor dimerization (40). To test whether either one of these mechanisms may account for the ability of GM1 to induce TrkA tyrosine phosphorylation, NIH-3T3 cells were transfected with an expression vector containing p70trk, a chimeric receptor consisting of the extracellular domain of the TNF receptor and the transmembrane and intracellular domains of TrkA (36). p70trk has been shown to induce neuronal differentiation of PC12 cells in response to TNF, similar to NGF (36). Cells were then exposed to GM1 (60 μM) or NGF or TNF (100 ng/ml each) for 10 min. TNF promoted a strong autophosphorylation of p70trk (Fig. 4). The tyrosine kinase inhibitor K252a (100 nM) reduced this activation. In contrast, neither GM1 nor NGF increased the tyrosine phosho-
rlation of p70(S6K). GM1 does not induce tyrosine phosphorylation of p70(S6K), NIH-3T3 cells were transiently transfected with pCDM870-Trk or the empty vector (mock). Two days later, cells were exposed to control medium alone (lane 1) or containing TNF (lane 2), TNF + K252a (lane 3), GM1 (lane 4), or NGF (lane 5) for 10 min. Mock-transfected cells were exposed to TNF (lane 6). Cells were then lysed, and the lysates were immunoprecipitated with anti-pan Trk antibody, separated on a gel, and immunoblotted with anti-phosphoserylserine antibody (Ph870trk). The blot was stripped and reblotted with anti-pan Trk antibody.

**Fig. 4.** GM1 does not induce tyrosine phosphorylation of p70(S6K). NIH-3T3 cells were transiently transfected with pCDM870-Trk or the empty vector (mock). Two days later, cells were exposed to control medium alone (lane 1) or containing TNF (lane 2), TNF + K252a (lane 3), GM1 (lane 4), or NGF (lane 5) for 10 min. Mock-transfected cells were exposed to TNF (lane 6). Cells were then lysed, and the lysates were immunoprecipitated with anti-pan Trk antibody, separated on a gel, and immunoblotted with anti-phosphoserylserine antibody (Ph870trk). The blot was stripped and reblotted with anti-pan Trk antibody.

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**Table I**

| Treatment     | NIH-3T3 | NIHHC |
|---------------|---------|-------|
| Control       | 8.5 ± 1.7 | 8.2 ± 4.2 |
| GM1           | 22.1 ± 4.7* | 16.7 ± 2.8* |
| K252a         | 7.8 ± 2.0 | 9.1 ± 3.3 |
| GM1 + K252a   | 21.2 ± 3.2* | 17.1 ± 2.7* |

* p < 0.001 versus control (analysis of variance and Dunnett’s test).

**Fig. 5.** GM1 increases the release of NT-3 from NIH and NIHHC cells. Cells were grown in medium containing serum until reaching 85% confluence. The medium was then removed and replaced with serum-free unconditioned medium containing 0.1% BSA (control) or GM1 (60 μM) for 10 min. The medium was concentrated and assayed by a two-site immunoassay for different neurotrophins. Data are expressed as percent of control wells and are the means ± S.E. of at least two independent experiments (n = 6 for each group). *p < 0.05 versus control.

**Fig. 6.** TrkC-IgG inhibits GM1-mediated TrkC autophosphorylation. NIHHC cells were exposed to TrkC-IgG or TrkB-IgG receptor bodies (10 μg/ml each) for 15 min prior to the addition of NT-3 (100 ng/ml) or GM1 (60 μM). Cells were then incubated for an additional 10 min. Lysates were prepared and processed for TrkC tyrosine phosphorylation as described in the legend to Fig. 1. The blot was stripped and reprobed with anti-pan Trk antibody.
Gangliosides and Release of Neurotrophins

Fig. 7. GM1 induces the release of NT-3 from C6Trk cells. Confluent cells were exposed to unconditioned medium containing 0.1% BSA (control) or GM1 for the indicated times. NT-3 levels were measured in the media. Data are expressed as percent of control cells (NT-3 levels in control cells, 47.4 ± 5.2 pg/mg of protein) and are the means ± S.E. of at least three independent experiments (n = 8 for each group). The medium (from control or treated cells) did not contain detectable amounts of NGF immunoreactivity. *, p < 0.05 versus control.

1 h, C6Trk cells were exposed for 5 min and up to 3 h to concentrations of GM1 previously shown to either increase (80 μM) or to fail to affect (40 μM) TrkA tyrosine phosphorylation in these cells (28). GM1 elicited an increase in NT-3 release starting at 1 h and only when used at the higher concentration (Fig. 7). C6Trk cells did not secrete detectable amounts of NGF either before or after exposure to GM1 for up to 3 h (data not shown), supporting the suggestion that NT-3 release underlies the ability of GM1 to activate Trk receptors.

Gangliosides Increase the Release of Neurotrophins in Cerebellar Granule Cells—Up to this point, our data have shown that GM1 induces the release of NT-3 in transformed cells expressing Trk receptors. However, due to their phenotype, these cells may not represent a "physiologically" relevant model. On the other hand, it is difficult to measure NT-3 release in vivo. Therefore, the release hypothesis was examined in cultured cerebellar granule cells, in which gangliosides exert neuroprotective activity by activation of the TrkB receptor (30).

Cerebellar granule cells at day 8 in vitro were exposed to GM1 for 5 or 15 min in serum-free medium. The medium was collected; concentrated into small aliquots; and analyzed for BDNF, NGF, or NT-3 immunoreactivity. LIGA20, which is more potent than GM1 in inducing TrkB autophosphorylation (30), was used as a positive control. GM1 elicited a rapid (within 5 min) accumulation of NT-3 in the medium, but failed to affect BDNF or NGF release (Table II). The increased release of NT-3 was observed up to 15 min (Table II). LIGA20 instead evoked a significant increase in BDNF release only (Table II). Overall, these data confirm that the release phenomenon is not an artifact of tumor cell lines.

TrkB-IgG Blocks LIGA20-mediated Neuronal Protection—To further support and strengthen the release hypothesis, we tested whether TrkB-IgG, which is known to block the neuroprotective property of BDNF released in cerebellar granule cells (37), also blocks LIGA20-mediated neuroprotection. Cerebellar granule cells were exposed to TrkB-IgG (10 μg/ml) immediately prior to LIGA20 (5 μM); then 3 h later, a toxic concentration of glutamate (300 μM) was added. Neurons were also exposed to BDNF (100 ng/ml) as a positive control. Cell death was measured by MTT assay 14 h later. As expected, the neurotoxic effect of glutamate was blocked by LIGA20 and BDNF (Fig. 8). Both compounds failed to prevent glutamate toxicity when added together with TrkB-IgG. In contrast, TrkC-IgG did not block the neuroprotective effect of LIGA20 or BDNF (Fig. 8), further suggesting that LIGA20 activates a BDNF autocrine loop.

DISCUSSION

In this work, we have explored the interaction of gangliosides with the neurotrophin receptors TrkA, TrkB, and TrkC. We have found that GM1 activates TrkC more strongly than TrkA or TrkB and that LIGA20 activates predominantly TrkB. Moreover, we have shown that this phenomenon is a direct result of the ability of gangliosides to increase the release of NT-3 or BDNF. Several evidences confirmed this novel mechanism of action of gangliosides. First, NT-3 and BDNF concentrations were increased within minutes in the medium of cells exposed to GM1 or LIGA20, respectively. Second, TrkC receptor bodies reduced the GM1-mediated activation of TrkC, whereas TrkB-IgG prevented LIGA20-mediated neuroprotection. Third, GM1 failed to displace [125I]-labeled NT-3 binding and to induce autophosphorylation of the p75[trk] chimeric receptor, suggesting that GM1 does not act as a Trk ligand. Moreover, we have demonstrated that the presence of Trk receptors is not a prerequisite for GM1-mediated NT-3 release. Therefore, our findings suggest a new and important physiological property of gangliosides because they indicate that these compounds exert trophic activity by inducing a rapid release of the neurotrophins that, in turn, activate Trk receptors by an autocrine loop.

TrkC is not the only receptor activated by GM1. In fact, this ganglioside has been shown to induce autophosphorylation of TrkA in various cell lines (26–28). However, GM1 did not induce a significant accumulation of NGF in all cell lines tested in this study. Instead, GM1 increased NT-3 release in both NIHIC and NIHA cells as well as C6Trk cells. Because NT-3 enhanced TrkA autophosphorylation in NIHIC and C6Trk cells, whereas NGF failed to produce an appreciable induction of TrkC tyrosine phosphorylation in NIHIC and C6Trk cells, our suggestion that TrkA is activated by an NT-3 autocrine loop supports our hypothesis that NT-3 is the main target of GM1 and strengthens the suggestion that gangliosides facilitate the activation of Trk receptors by enhancing a constitutive and/or regulated secretion of neurotrophins (51–55).

In C6Trk cells, the effect of GM1 on TrkA autophosphorylation occurs with a time delay of at least 1 h (28) compared with NIHIC and PC12 cells. A similar time delay was observed in the ability of GM1 to selectively induce NT-3 release in C6Trk cells. At present, it is unclear whether the tardy effect of GM1 in C6Trk cells might be due to the different composition of membranes of these cells. Nevertheless, the temporal correlation, together with the lower NGF synthesis in C6Trk cells (38), supports our suggestion that TrkA is activated by an NT-3 autocrine loop. Moreover, GM1 caused a weaker activation of TrkA in these cells compared with NIHIC cells. This difference can be ascribed to the presence of p75 in C6Trk cells (49), which reduces the ability of NT-3 to activate TrkA. In fact, p75 has been shown to have a significant effect on the interaction between TrkA and neurotrophins. In particular, high concentrations of p75 increase the affinity of TrkA for NGF and reduce that of NT-3 for TrkA (41, 42). Thus, depending upon the amount of p75 in Trk-expressing cells, GM1 may be more potent than GM1 in activating TrkB in...
Gangliosides increase neurotrophin release in cerebellar granule cells

Table II

| Treatment         | BDNF (pg/mg protein) | NT-3 (pg/mg protein) | NGF (pg/mg protein) |
|-------------------|----------------------|----------------------|---------------------|
| Control (5 min)   | 100.8 ± 9.1          | 58.6 ± 13.7          | 14.6 ± 2.6          |
| GM1 (5 min)       | 113.4 ± 12.3         | 91.1 ± 11.1          | 16.8 ± 1.1          |
| LIGA20 (5 min)    | 191.7 ± 3.4^a        | 73.7 ± 11.1          | 17.7 ± 2.1          |
| Control (15 min)  | 139.4 ± 12.8         | 67.4 ± 9.1           | 17.4 ± 2.3          |
| GM1 (15 min)      | 155.1 ± 6.6          | 109.1 ± 13.4^a       | 20.6 ± 2.4          |
| LIGA20 (15 min)   | 234.8 ± 31.7^a       | 81.4 ± 7.7           | 18.5 ± 2.1          |

* p < 0.01 versus control (analysis of variance and Dunnett’s test).

the central and peripheral nervous system (56–58). Still, it appears that the effect of ceramide on TrkA dimerization might be an event secondary to the neurotrophin release.

The molecular mechanisms whereby gangliosides induce the release of neurotrophins from neuronal and non-neuronal cells are unclear. Trk receptors have been shown to regulate the release of neurotrophins (52), suggesting that GM1 could induce the release of NT-3 by interacting with Trk. We can rule out this hypothesis because GM1 induced the release of NT-3 in wild-type NIH-3T3 cells, which do not express Trk receptors, as well as in NIH3T3 cells in the presence of the tyrosine kinase inhibitor K252a. On the other hand, neurotrophins are released from neurons in an activity-dependent manner and from non-neuronal cells in an activity-independent manner (37, 51–55). Previous studies have suggested that both sodium and intracellular calcium are intimately involved in the NGF release process in hippocampal neurons (51). GM1 has been shown to modulate intracellular calcium levels in neurons (63–66). Thus, it may be possible that gangliosides may induce the release of NT-3 and BDNF from neurons in a calcium-dependent manner. However, in non-neuronal cells, gangliosides could regulate the release of neurotrophin in a calcium-independent manner, perhaps through an interaction with VIP21-caveolin within the plasma membrane (67). Caveolin is a membrane protein distinct from the coated pit and localized in caveolae (68), plasma membrane invaginations rich in cholesterol and sphingolipids and specialized in the cellular transport of molecules. Caveolin has been shown to exert an important function as an anchoring protein for signaling molecules, including various kinases and tyrosine kinase receptors within the plasma membrane (69, 70). Moreover, by interacting with caveolin, gangliosides may enhance or inhibit the activity of these molecules, which, in turn, may promote the release of NT-3. Therefore, it is possible that multiple mechanisms control ganglioside-mediated release of neurotrophins. Such complexity might be critical for generating the remarkable diversity and specificity with which gangliosides regulate neuronal function.

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