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Exogenous gangliosides increase the release of brain-derived neurotrophic factor

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Abstract

Gangliosides are lipophilic compounds found in cell plasma membranes throughout the brain that play a role in neuronal plasticity and regeneration. Indeed, absence or abnormal accumulation of gangliosides has been shown to lead to neurological disorders. Experimental data have shown that exogenous gangliosides exhibit properties similar to the neurotrophins, a family of neurotrophic factors that are important in the survival and maintenance of neurons and prevention of neurological diseases. Brain-derived neurotrophic factor (BDNF) is the most abundant of the neurotrophins. This work was done to reveal the neurotrophic mechanism of exogenous gangliosides. In particular, we examined whether gangliosides promote the release of BDNF. Rat hippocampal neurons or human neuroblastoma cells were transduced with a recombinant adenovirus expressing BDNF-flag to facilitate detection of BDNF. Release of BDNF was then determined by Western blot analysis and a two-site immunoassay of culture medium. The depolarizing agent KCl was used as a comparison. In hippocampal neurons, both GM1 ganglioside and KCl evoked within minutes the release of mature BDNF. In human cells, GM1 and other gangliosides released both mature BDNF and pro-BDNF. The effect of gangliosides was structure-dependent. In fact, GT1b preferentially released mature BDNF whereas GM1 released both mature and pro-BDNF. Ceramide and sphingosine did not modify the release of BDNF. This work provides additional experimental evidence that exogenous gangliosides can be used to enhance the neurotrophic factor environment and promote neuronal survival in neurological diseases.

Keywords

BDNF; pro-BDNF; GM1; GT1b; ceramide; NMDA receptor; recombinant adenovirus

1. Introduction

It is a pleasure to participate in this special issue of *Neuropharmacology* honoring the contributions of Dr. Erminio Costa to neuroscience. One of the authors (IM) was privileged to participate in a long discussion with Dr. Costa nearly 16 years ago in which Dr. Costa referred to the gangliosides as the new frontier in drug development for neurodegenerative

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diseases. Although explosive growth in the application of gangliosides in clinical trials has been hampered by the report of some side effects, this field of ganglioside neurobiology not only continued but has also accelerated into the characterization of novel mechanisms and signaling pathways and other manifestations of their biological activity. The data presented here are based in no small degree on Dr. Costa's pioneering achievements. Dr. Costa and colleagues would be gratified to know that their prediction of the neurotrophic activity of gangliosides has been revealed as true.

Gangliosides are sialic acid-containing glycosphingolipids (Fig. 1) that are abundant in the brain (Suzuki, 1965; Svennerholm, 1956) where they are synthesized by neuronal and glial cells (Derry and Wolfe, 1967). Gangliosides are crucial for brain development and plasticity. Indeed, accumulation of gangliosides leads to neurite outgrowth (Purpura and Suzuki, 1976), whereas lack of gangliosides inhibits nerve regeneration and induces axonal damage (Sparrow et al., 1984; Yamashita et al., 2005). Moreover, humans or mice that do not synthesize gangliosides suffer seizures (Simpson et al., 2004; Wu et al., 2005), and mice carrying a null mutation that abolishes synthesis of major gangliosides exhibit altered axon-glia interaction (Yamashita et al., 2005). Moreover, exogenously administered gangliosides have been shown to affect the survival of a number of central nervous system neurons, including glutamatergic (Favaron et al., 1988), dopaminergic (Hadjiconstantinou and Neff, 1988; Schneider et al., 1992), and cholinergic neurons (Sofroniew et al., 1986). However, the neurotrophic action of exogenous gangliosides is not completely understood.

Experimental data have shown that gangliosides, and in particular, GM1 (monosialotetrahexosylganglioside), exhibit properties similar to the neurotrophins (Mocchetti, 2005), a family of neurotrophic factors that includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5. Neurotrophins, and in particular, BDNF, is believed to be necessary for the survival of specific neuronal populations (Zuccato and Cattaneo, 2009). The neurotrophic activity of GM1 appears to be derived from its ability to activate Trks, the high affinity neurotrophin receptors (Ferrari et al., 1995; Mutoh et al., 1995; Rabin and Mocchetti, 1995) and their associated signal transduction pathways (Duchemin et al., 2002). However, GM1 is more potent in activating TrkC than TrkB (Rabin et al., 2002). Moreover, the semisynthetic ganglioside LIGA20, which is more effective than GM1 *in vitro* (Manev et al., 1990) and *in vivo* (Schneider and DiStefano, 1993), promotes TrkB tyrosine phosphorylation more efficiently than TrkA (Bachis et al., 2002). LIGA20 differs from GM1 by the presence of a dichloroacetyl group which substitutes for the fatty acid tail of GM1 at the 2-amino position. How gangliosides activate different Trks is still debated. GM1 could bind to a Trk receptor and promote its dimerization (Farooqui et al., 1997). However, previous data have shown that GM1 induces TrkC tyrosine phosphorylation without binding to TrkC (Rabin et al., 2002). GM1 could induce the synthesis (Duchemin et al., 1997) and release (Rabin et al., 2002) of neurotrophins which, in turn, activate an autocrine loop. To examine this hypothesis, we utilized an approach similar to that used in previously published studies, namely, overexpressing BDNF by a viral vector (Heymach et al., 1996) in rat embryonic neuronal cultures and human neuroblastoma. Our results show that gangliosides regulate the release of BDNF.

2. Materials and Methods

2.1 Cell cultures

Rat hippocampal neurons were prepared from E18 Sprague Dawley rat embryos as previously described (Avdoshina et al., 2010). For biochemical experiments, neurons were plated on poly-D-lysine coated 12-well tissue culture plates (Sigma, St Louis, MO) at a density of 1×10^6 cells/ml and maintained in Neurobasal Medium with B27 supplement

(Invitrogen, Grand Island, NY). For immunocytochemistry, neurons were plated on cover slips coated with poly-L-lysine (at 8000 cells per ml). Cells were maintained in neurobasal medium containing 2% B27 supplement, and 500 μ M L-glutamine (Sigma). Cultures were treated with 5 μ M cytosine arabinoside to inhibit the proliferation of non-neuronal cells. Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The medium was changed every 3 to 4 days. At 8 days *in vitro*, neurons were infected with a recombinant adenovirus vector (ReAd) expressing BDNF (ReAd-BDNF) (see below). Two days after infection, neurons were washed in warm Hank's buffered salt solution (HBSS) and exposed to various stimuli (e.g. KCl, GM1) in HBSS for 10 min. The medium was then collected for the determination of BDNF by Western blot analysis or enzyme-linked immunosorbent assay (ELISA) (see below).

Human neuroblastoma cells H4 were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in high glucose Dulbecco's Modified Eagle Medium (MediaTech, Herdon, VA) supplemented with 10% fetal calf serum (Hyclone Lab, Logan, UT), 50 U/ml penicillin, 50 U/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂. Cultures were used when they reached ~75% confluence.

2.2. Generation of recombinant adenovirus expressing BDNF-flag

A recombinant adenovirus expressing BDNF-flag was generated by cloning the human BDNF cDNA with a C-terminal flag epitope into the multiple cloning site of the pAD-CMV vector (He et al., 1998). The vector expresses also enhanced green fluorescent protein (eGFP) under the independent CMV promoter (He et al., 1998), which allows a better visualization of synaptic spines. BDNF-flag cDNA was generated by polymerase chain reaction using BDNF primers 5'-CCCGCTAGCATGACCATCCTTTTCCTACT-3' and 5'-GGGGTCGACCTACTTATCGTCGTCATCCTTGTAATCTCTTCCCCTTTTAATGGTCAG-3'. The resultant product was purified, digested with NheI and HindIII, and then ligated into pVAX vector (Invitrogen) for expression. BDNF-flag was then removed from pVAX vector with NheI and XbaI and inserted into pAd-CMV vector. The orientation of BDNF-flag was checked by DNA sequencing. The pAd-CMV vector was then packaged into recombinant adenovirus to generate ReAd-BDNF. In brief, transfer vector was linearized with PmeI, and the PmeI-digested transfer vector and 1 μ l of pAdeasy-1 vector were cotransformed into BJ5183 competent bacterial cells. Cells were plated onto LB/kanamycin plates, and colonies, containing recombinant adenovirus plasmid, were screened. 4 μ g of linearized recombinant plasmid DNA were used to infect QBI-293A human embryonic kidney cells (Quantum Biotechnologies, Carlsbad, CA) by Lipofectamine 2000 (Invitrogen) in 200 μ l Opti-MEM (Invitrogen). Virus was harvested and amplified by repeating the infection of cells 4 times. The ReAd was concentrated by cesium chloride gradient.

2.3. Transduction of hippocampal neurons and H4 cells

Hippocampal neurons and H4 cells were plated into 12-well plates containing 1 ml of Neurobasal medium and Dulbecco's Modified Eagle Medium, respectively. Cells were infected with ReAd-BDNF at multiplicity of infection (MOI) of 200. The plates were gently rocked every 15 min for 1 h. After 1 h exposure to virus, cells were washed with warmed phosphate buffer and the conditioned media was returned to wells. Transduced cells were exposed two days later to various experimental conditions (e.g. KCl, gangliosides). No cytotoxicity was observed at these MOIs.

2.4. Immunocytochemistry

Hippocampal neurons infected with ReAd-BDNF were maintained on glass coverslips for two days. Neurons were then fixed in 4% paraformaldehyde/phosphate buffer with 4% sucrose for 30 min at 4°C. Fixed cells were blocked and permeabilized in 4.5% nonfat dry

milk in TBS-T (150 mM NaCl, 20 mM Tris-base, pH 7.5, 0.1% Triton-X100) for 1 h at 4°C. Cells were incubated overnight at 4°C with mouse anti-flag monoclonal antibody M2 (1 µg/ml, Sigma). Coverslips were washed in blocking solution and fluorescence-conjugated secondary antibodies (AlexaFluor® 1:2000, Invitrogen) were applied for 1 to 2 h at room temperature. Coverslips were washed with phosphate buffer containing 0.05% Triton-X100, and mounted in Mowiol.

Human neuroblastoma cells H4 were seeded on poly-L-lysine pre-coated coverslips. Class III β -tubulin (1:1000, Covance, Emeryville, CA), anti-microtubule associated protein-2 (MAP2, 1:1000, Sigma), anti-Neuronal Nuclei (NeuN, 1:500, Millipore, Billerica, MA) and glial fibrillary acidic protein (GFAP, 1:2000, Wako, Japan) antibodies were used overnight at 4°C to visualize neurons and astrocytes, respectively. Coverslips were then incubated for 1 h at room temperature with corresponding secondary antibody (AlexaFluor®, 1:1000, Invitrogen) followed by 4',6'-diamidino-2-phenylindole (DAPI) (1:5000, Invitrogen) for 5 min, washed and mounted using ProLong® gold antifade reagent (Invitrogen). Images were captured on a Zeiss Axioskop microscope (Thornwood, NY) with a Photometrics Coolsnap-ix (Roper Scientific, Tucson, AZ) camera using Scanalytic's IPLab (Fairfax, VA) image capturing software.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Two days post-transduction with ReAd-BDNF, hippocampal neurons and H4 cells were washed twice with warmed HBSS and then exposed to GM1 or other stimuli. Medium was then collected and an aliquot (100 µl) used to measure BDNF levels by the E_{max} immunoassay systems (Promega, Madison, WI) as previously described (Bachis et al., 2002). Each experimental condition was performed in triplicate. Assay plates were analyzed on a Bio-Rad Microplate Reader Model 550 using Microplate ManagerIII software. Protein levels in the lysates were measured by the Bradford Coomassie blue colorimetric assay (Bio-Rad).

2.6. Western blot analysis

For analysis of BDNF immunoreactivity in the medium, cultures were washed twice with warmed HBSS and then exposed to KCl, GM1 and other stimuli. Medium was then collected and concentrated by Centricon® (Millipore) columns. Samples of equal volumes were mixed with a 2X reducing sample buffer, separated by 12% SDS-polyacrylamide gel (Mini-Protean III, BioRad, Hercules, CA), and transferred to nitrocellulose membranes (Millipore). Membranes were blocked in 5% nonfat dry milk TBS (150 mM NaCl, 20 mM Tris-base, pH 7.5) for 1 h at room temperature, and then probed with anti-flag antibody (Sigma) or rabbit pro-BDNF antibody (1 µg/ml, Millipore) overnight at 4°C. Blots were washed with TBS, and incubated in HRP-conjugated secondary antibody (Jackson ImmunoResearch, Inc, West Grove, PA) diluted in blocking buffer. To assure that each preparation has an equal amount of cells, protein levels were measured in cell lysates. In brief, cells were scraped in 50 mM Tris HCl, pH 7.0, and pelleted. Pellets were sonicated in the same buffer, centrifuged at 100,000 × g for 20 min, resuspended in 50 mM Tris HCl. Protein levels in the lysates were measured by the Bradford Coomassie blue colorimetric assay (Bio-Rad).

Western blot analysis was used to determine levels of NMDA receptor subunit 2A as previously described (Brandoli et al., 1998). In brief, control and GM1-treated hippocampal neurons were harvested in 50 mM Tris HCl, pH 7.0, and pelleted. Pellets were homogenized in the same buffer, centrifuged at 100,000 × g for 20 min, resuspended in 1 ml of Tris HCl. After removal of cellular debris by centrifugation, protein levels in the lysates were measured by the Bradford Coomassie blue colorimetric assay (Bio-Rad). Equal amounts of

proteins were loaded onto an 8.5% SDS-polyacrylamide gel. Proteins were transferred on a nitrocellulose membrane and blocked as above. Membranes were incubated with rabbit affinity-purified polyclonal antibodies anti-NR2A (1:2500, Millipore). Blots were exposed to chemiluminescence substrates (Thermo Fisher Scientific, Rockford, IL). Detection of chemiluminescence was captured on Hyperfilm (GE Healthcare Biosciences, Pittsburgh, PA).

2.7. Reagents

D-erythro-Sphingosine and *N-stearoyl-D-erythro*-Sphingosine (C18 ceramide) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). GT1b was obtained from Sigma. GM1 was a gift from FIDIA s.p.a., Abano Terme, Italy.

3. Results

3.1. Adenovirus BDNF localizes in neurite processes

In neurons BDNF is packaged into secretory granules, and sorted in synaptic spines (Danzon and McNamara, 2004; Kohara et al., 2001). To determine whether C-terminal flag-tagged BDNF generated from ReAd-BDNF localizes in secretory granules similarly to endogenous BDNF, we examined BDNF-flag immunoreactivity after adenovirus-mediated gene transfer in hippocampal neurons. In transduced neurons, BDNF-flag was detected as discrete immunoreactive puncta within the cell soma and neurite processes (Fig. 2), suggesting that BDNF-flag is packaged into secretory granules. Moreover, BDNF-flag puncta were distributed within dendritic spines, which were visualized by eGFP (Fig. 2). These data indicate that viral-produced BDNF is sorted in synaptic spines. In addition, these results show that the distribution of BDNF-flag is similar to that of wild-type BDNF, suggesting that the flag tag did not disrupt the natural distribution of BDNF.

3.2 GM1 and KCl promote the release of mature BDNF

We have previously shown that GM1 induces, within 10–15 min, the release of NT-3 from neuronal cells (Rabin et al., 2002). To examine whether GM1 increases the release of BDNF, hippocampal neurons were exposed to GM1 at a concentration (60 μ M) previously shown to promote the release of NT-3 (Rabin et al., 2002). Moreover, neurons were exposed to KCl (55 mM), a depolarizing agent that evokes a release of BDNF from neurons (Aloyz et al., 1999). A sensitive ELISA was used to determine the amount of BDNF released after GM1 and KCl. In neurons exposed to either KCl or GM1 for 10 min we observed a statistically significant accumulation of BDNF in the medium, suggesting that these stimuli increase the release of BDNF (Fig. 3). GM1 was less potent than KCl (Fig. 3), which is consistent with the inability of GM1 to change membrane depolarization (de Erausquin et al., 1990).

Once in secretory granules, BDNF can be released by depolarization as mature BDNF or as the glycosylated precursor pro-BDNF (Yang et al., 2009). To determine which BDNF species is released, we examined BDNF immunoreactivity in the culture medium by Western blot analysis. The conditioned medium contained only a single BDNF species that migrated at an apparent molecular weight of ~15,000 (Fig. 4), which corresponds to mature BDNF. The relative amount of BDNF immunoreactivity was greater in neurons exposed to KCl or GM1 for 10 min than controls (Fig. 4), suggesting that GM1, similarly to KCl, promotes the release of mature BDNF.

3.3. GM1 causes a decrease in N-methyl-D-aspartate receptor subunit 2A

We have previously shown that recombinant BDNF reduces the expression of N-methyl-D-aspartate (NMDA) receptor subunits 2A (NR2A) in cerebellar granule cells in cultures

(Brandoli et al., 1998). This subunit is one of the four known isoforms of the NR2 subunit involved in the functional assembly of the NMDA receptor channel. The decrease of NR2A occurs within 6 h of exposure to BDNF (Brandoli et al., 1998), and it is prevented by K252a (Brandoli et al., 1998), an inhibitor of Trk tyrosine kinase activity that has been used to block neurotrophin neurotrophic activities (Berg et al., 1992) that occur through Trk activation. To determine whether the GM1-evoked release of BDNF has a biological activity similar to the recombinant BDNF, we examined whether GM1 could reduce the levels of NR2A. Hippocampal neurons were exposed to GM1 for 6 h in the presence or absence of K252a. NR2A levels were then determined by Western blot analysis. GM1 elicited a significant decrease in NR2A levels (Fig. 5). K252a, which did not change NR2a levels *per se*, abolished the GM1-mediated decrease of NR2A subunit (Fig. 5), suggesting that the effect of GM1 involves the activation of Trk tyrosine kinase activity, most likely through a BDNF autocrine loop.

3.4. GM1 promotes the release of BDNF in human neuroblastoma cells

We next examined whether GM1 affects the release of BDNF from human cells by using the neuroblastoma cell line H4 transduced with ReAd-BDNF. We first characterized which cell type was present in this cell line by staining this culture with antibodies that recognize an astrocytic marker such as GFAP, and neuronal markers such as β III-tubulin, MAP-2 and NeuN. GFAP was undetected, whereas cultures were mostly comprised of β III-tubulin positive (Fig. 6) and but MAP-2 and NeuN negative cells (data not shown). Because MAP-2 and NeuN are considered markers for mature neurons (Magavi et al., 2000), these cultures appear to represent transformed derivatives of neuronal progenitor cells.

Cells were then exposed to KCl and two concentrations of GM1 for 10 min, the medium collected and BDNF levels determined by ELISA. Moreover, to assure that H4 cells contain functional neurotransmitter receptors, cells were also exposed to glutamate or NMDA, two stimuli that have been shown to produce a rapid increase of BDNF release from neurons (Marini et al., 1998). More BDNF immunoreactivity was observed in cells exposed to GM1, KCl, glutamate or NMDA (Fig. 7). The effect of GM1 was weaker than the other stimuli, supporting the data that membrane depolarization is a stronger stimulus than GM1 for inducing BDNF release.

Western blot analysis was then carried out to reveal which BDNF species is released from H4 cells. The conditioned medium of control cells contained a ~36KDa and a ~15KDa BDNF immunoreactive band (Fig. 8A) indicating pro-BDNF and mature BDNF, respectively. This result was confirmed by probing the blot with an antibody that recognizes pro-BDNF only (Fig. 8B). Pro-BDNF appeared to be more abundant than the mature form (Fig. 8). In cells exposed to KCl the increase was more evident for the mature form whereas GM1 was more potent in increasing the pro-BDNF form (Fig. 8). Thus, GM1 may promote the release of pro-BDNF depending upon the cellular environment.

3.5. The effect of gangliosides on BDNF release is structure-dependent

The typical structure of gangliosides consists of a lipid component, ceramide, which is attached to an oligosaccharide chain (Ledeen and Yu, 1982). The length of the lipid chain and the inclusion of sialic acid residue are important determinants of the degree of neurotrophic activity (Manev et al., 1990). To determine which portion of gangliosides is required for the neurotrophic effect, we exposed infected H4 cells to GM1, GT1b, sphingosine and ceramide. BDNF immunoreactivity was then determined 15 min later in the medium. GT1b was stronger than GM1 in inducing the release of mature BDNF (Fig. 9), supporting previous data that the sialic portion of gangliosides is crucial for their neuroprotective effect (Manev et al., 1990). Moreover, we observed that ceramide and

sphingosine did not increase BDNF immunoreactivity (Fig. 9), suggesting that the lipid portion of gangliosides alone does not promote the release of BDNF.

4. Discussion

Neurotrophins could be an ideal therapy for neurological disorders because they combine a positive effect on regeneration of axotomized neurons with the ability of preventing neuronal apoptosis (Mocchetti and Brown, 2008). In addition, neurotrophins promote neurogenesis (Barnabe-Heider and Miller, 2003), which is essential for specific cognitive functions that decline in some neurological disorders and in aging. A major challenge in a neurotrophic factor therapy is represented by the molecular structure that impairs the ability of neurotrophic factor to cross the blood brain barrier. Therefore, compounds that mimic the neurotrophins but that cross the blood brain barrier have an enormous potential to slow down neurodegenerative disorders. Previous studies have shown that GM1 ganglioside evokes the release of two neurotrophins, NT-3 and NGF (Rabin et al., 2002). In this work, we have provided evidence that GM1 promotes also the release of another neurotrophin, BDNF.

Our data show that hippocampal neurons infected with ReAd-BDNF exhibited BDNF immunoreactivity in synaptic spines, indicating that recombinant BDNF is stored in the same synaptic compartment of endogenous BDNF. BDNF is synthesized as a precursor, or pro-BDNF, which is then converted to mature BDNF in the trans-Golgi by members of the subtilisin-kexin family of endoproteases such as furin, or in the immature secretory granules by proprotein convertases (Greenberg et al., 2009). It is currently believed that this process occurs through an extracellular pathway upon the activity-dependent release of pro-BDNF from neurons. Pro-BDNF activates p75 (Woo et al., 2005), the low affinity neurotrophin receptor that has been implicated in cell death (Casaccia-Bonnet et al., 1996). Mature BDNF instead, plays a role in synaptic plasticity. Thus, it was important to ascertain which BDNF species is released upon GM1 treatment. Hippocampal neurons failed to release pro-BDNF even under depolarizing conditions, thus supporting the notion that neurons are able to rapidly convert pro-BDNF into BDNF (Matsumoto et al., 2008). In these neurons GM1 increased the release of mature BDNF only with a time course similar to that of KCl. Thus, GM1 appears to promote the release of mature BDNF from neurons without interfering with the processing of pro-BDNF.

Unlike hippocampal neurons, the conditioned media of H4 cells contained more pro-BDNF than mature BDNF. The amount of pro-BDNF was further increased by KCl or gangliosides, suggesting increased release. This was not surprising, because recent studies have shown that pro-BDNF can be released by depolarizing conditions (Yang et al., 2009). However, the fact that H4 cells do not efficiently convert pro-BDNF into mature BDNF is puzzling considering that these cells were infected with the same ReAd-BDNF used for hippocampal neurons. Recent studies have shown that non-neuronal cells are able to secrete and uptake pro-BDNF (Bergami et al., 2008; Mowla et al., 2001). We have noticed that these cultures do not express markers of differentiated neurons such as MAP-2 and NeuN, but are β III tubulin positive. Thus, it appears that these cultures are transformed derivatives of neuronal progenitor cells. Perhaps progenitor cells do not effectively process pro-BDNF into mature BDNF. In fact, little or no pro-BDNF has been detected in the rodent adult central nervous system (Yang et al., 2009). Nevertheless, it remains to be established whether the production of pro-BDNF is associated with the unique cellular environment of H4 cells.

The mechanism(s) underlying the ability of gangliosides to induce the release of the neurotrophins is still under investigation. Neurotrophins have been shown to regulate their own release. In particular, BDNF release can be triggered by NGF in PC12 cells (Canossa et

al., 1997; Kruttgen et al., 1998). This event is TrkA mediated (Canossa et al., 1997). Thus, GM1 could promote BDNF release by binding and activating Trks. However, this does not appear to be the case because previous studies have shown that the ability of GM1 to release NT-3 or NGF does not depend upon binding to TrkC (Rabin et al., 2002) or TrkA (Rabin and Mocchetti, 1995). Moreover, K252a, which inhibits Trk activation, did not prevent the ability of GM1 to induce the release of neurotrophins (Rabin et al., 2002). In addition, the GM1 effect on neurotrophin release can be seen in cells that do not express Trk (Rabin and Mocchetti, 1995). Lastly, previous data have shown that neutralization of neurotrophins by Trk receptor bodies abolishes the ability of gangliosides to activate Trk tyrosine phosphorylation but not the release of neurotrophins (Rabin et al., 2002). An attractive possibility to explain the release of BDNF by GM1 is suggested by experimental data showing that gangliosides increase intracellular Ca^{2+} levels in neurons *via* Ca^{2+} channels (Ando et al., 1998; Fang et al., 2002; Quattrini et al., 2001). Indeed, intracellular Ca^{2+} has been shown to modulate the presynaptic release of BDNF (Canossa et al., 2001; Hartmann et al., 2001; Heymach et al., 1996). On the other hand, gangliosides, like other glycosphingolipids, participate in the formation of membrane microdomains such as glycosphingolipid-based membrane rafts or bind to VIP21-caveolin within the plasma membrane (Fra et al., 1995). Caveolin has been shown to exert an important function as an anchoring protein for signaling molecules including various kinases, tyrosine kinase receptors, Trk and p75 within the plasma membrane (Bilderback et al., 1999; Huang et al., 1999; Okamoto et al., 1998). By interacting with caveolin, gangliosides may enhance or inhibit the activity of these molecules, which, in turn, may promote the release of neurotrophins. Alternatively, gangliosides, through their lipid moiety, may interact with synaptophysin or other proteins that are essential for synaptic vesicles (Thiele et al., 2000) and modulate both the fusion of vesicles with membranes and the release of their content. This may explain why gangliosides promote the release of pro-BDNF in H4 cells more than do depolarization conditions. Therefore, it is possible that multiple mechanisms control ganglioside-mediated release of neurotrophins.

In this work we have examined the potential mechanism by which GM1 may exert a neuroprotective action by looking at its BDNF releasing properties. BDNF has been shown to activate an autocrine loop in neurons of both the peripheral (Acheson et al., 1995) and central nervous system (Marini et al., 1998). This autocrine loop is crucial for neuronal survival. To ascertain whether GM1 activates a BDNF autocrine loop, we examined the ability of this ganglioside to reduce the amount of NR2A, one of the subunits of NMDA receptor that has been shown to be down-regulated by BDNF through TrkB (Brandoli et al., 1998). We found that GM1 decreases NR2A levels with a time course similar to BDNF. Most importantly, the effect of GM1 was blocked by K252a, a tyrosine kinase inhibitor that has been used to block BDNF effects by hampering Trk signaling. These data support the notion that GM1 evokes a BDNF autocrine loop which involves the release of BDNF and activation of TrkB (Mutoh et al., 1995). In addition, our data help explain why exogenous gangliosides protect neurons from excitatory amino acid-induced neuronal apoptosis (Bachis et al., 2002; Favaron et al., 1988; Manev et al., 1990; Wu et al., 2004). Since excitotoxicity, such as that obtained after acute stroke, is believed to be caused by an overactivation of the NMDA channel and a sustained rise in cytosolic Ca^{2+} concentration (Choi, 2005), reducing the amount of NMDA subunit may prevent the intracellular Ca^{2+} from reaching lethal concentrations.

GT1b is a trisialoganglioside that has the lipophilic tail similar to that of GM1 but a sugar portion that contains three sialic acid residues. Sphingosine and ceramide, instead, compose the lipid tail of gangliosides. In this work we show that the lipid portion of gangliosides does not influence the release of BDNF. In fact, ceramide or sphingosine failed to change the release of BDNF. This data might explain why these sphingolipids do not prevent glutamate

toxicity (Manev et al., 1990). Moreover, GM1 was less potent than GT1b in inducing the release of BDNF. Thus, the combination of oligosaccharide portion and sialic acid in the chemical structure of gangliosides may be crucial for the observed effect on neurotrophins. It remains to be established whether the sugar portion and sialic acid alone are able to induce the release of the neurotrophins. Nevertheless, this result supports previous suggestions that the oligosaccharide portion requires the presence of the sialic acid to be fully biologically active, because asialoGM1 does not exhibit neurotrophic activity (Manev et al., 1990). Intriguingly, we found that in H4 cells GT1b preferentially activates the release of mature BDNF over pro-BDNF. Thus, by modifying ganglioside structure we might be able to obtain a compound that induces the release of mature BDNF only. This is important because mature BDNF and pro-BDNF exhibit distinct actions on neuronal survival.

In summary, our data provide a plausible link between the neuroprotective properties of gangliosides and their ability to induce the release of neurotrophins. Because released neurotrophins, in turn, activate Trk tyrosine kinase and associated target molecules such as extracellular signal-regulated kinase (Rabin and Mocchetti, 1995) and phosphatidylinositol 3-kinase (Duchemin et al., 2008), we suggest that exogenous gangliosides can be considered indirect Trk agonists.

Abbreviations

BDNF	brain-derived neurotrophic factor
DAPI	diamidino-2-phenylindole
ELISA	enzyme-linked immunosorbent assay
eGFP	enhanced green fluorescent protein
GFAP	glial fibrillary acidic protein
GM1	monosialotetrahexosylganglioside
HBSS	Hank's buffered salt solution
MAP-2	microtubule associated protein-2
NGF	nerve growth factor
NT-3	neurotrophin-3
NeuN	Neuronal Nuclei
NMDA	N-methyl-D-aspartate
NR2A	NMDA receptor subunit 2A
ReAd	recombinant adenovirus

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Figure 1.

Structure of exogenous gangliosides and sphingolipids used in this study. Gangliosides have a lipid tail (ceramide) linked by a glycosidic bond to an oligosaccharide chain. The prototype GM1 has four carbohydrates, one glucose, two galactoses and one N-acetylgalactosamine, and N-acetylneuraminic acid known also as sialic acid. GT1b has three sialic acid residues.

Figure 2.

Localization of recombinant BDNF in hippocampal neurons. Hippocampal neurons were transduced with ReAd-BDNF as described in Materials and Methods. This vector co-expresses eGFP (to better visualize neurite processes and dendritic spines) and flag-tagged BDNF under the two independent CMV promoters. Two days later, neurons were fixed and stained with an antibody against flag tag. Lower panels, higher magnification (40X) of upper panels. Scale bar: 10 μ m

Figure 3.

GM1 increases BDNF levels in the medium of hippocampal neurons. ReAd-BDNF infected hippocampal neurons were exposed to medium alone or containing KCl or GM1 for 10 min. Medium was then collected and 100 μ l used to measure BDNF immunoreactivity by ELISA as described in Materials and Methods. Data are expressed as mean $\pm$ SEM of 6 separate samples. * p <0.05, ** p <0.01 vs control (ANOVA and Holm-Sidak test).

Figure 4.

Western blot analysis of released BDNF from hippocampal neurons. Two days after ReAd-BDNF infection, hippocampal neurons were exposed to medium alone (Ctrl=control) KCl or GM1 for 10 min. Medium was collected and fractionated on a SDS-polyacrylamide gel as described in Materials and Methods. Blots were probed with M2 flag antibody. The blots are from two independent neuronal preparations.

Figure 5.

GM1 decreases NR2A subunit levels. Neurons were exposed to GM1 alone or in the presence of K252a (100nM) for 6 h. Lysates were prepared, and Western blot analysis was performed using a NR2A antibody as described in Materials and Methods. A.

Representative blot showing NR2A immunoreactivity. B. Semiquantitative analysis of NR2A immunoreactivity. Data are expressed in arbitrary units (densitometric values of NR2A immunoreactivity/total protein content) are the mean $\pm$ SEM of 6 separate samples.

*p<0.05 vs control, **p<0.05 vs GM1 (ANOVA and Dunnett's test).

Figure 6. Immunohistochemical analysis of H4 cells. H4 cells were plated on poly-L-lysine coated coverslips. Two days after plating, cells were fixed and stained for β III-tubulin, and GFAP as described in Materials and Methods. DAPI was used as a counterstaining. Bar=10 μ m.

Figure 7.

GM1 and depolarization induce the release of BDNF from human H4 cells. BDNF levels were determined by ELISA in an aliquot of the medium of H4 cells exposed for 10 min to the indicated stimuli. Data are the mean $\pm$ SEM of three independent experiments ($n=3$ each group, each experiment). * $p<0.05$, ** $p<0.01$, # $p<0.001$ vs control (ANOVA and Holm-Sidak test).

Figure 8.

pro-BDNF is released by GM1 from human H4 cells. H4 cells were exposed to the indicated stimuli for 10 min. The medium was collected, concentrated and fractionated on 12% SDS-polyacrylamide gel. A. Blot was probed with M2 flag antibody. B. The same blot shown in A was striped and reprobed with a pro-BDNF antibody.

Figure 9.

Influence of sphingolipids on BDNF release. H4 cells were infected with ReAd-BDNF and 48 h later were stimulated with GM1, GT1b, sphingosine (SPH), and ceramide (CER) for 10 min (all at 60 μ M). The medium was collected, concentrated and fractionated on 12% SDS-polyacrylamide gel. The blot was probed with M2 anti-flag antibody. The experiments were repeated twice with comparable results.