Downregulation of neurotrophic factors in the brain of a mouse model of Gaucher disease; implications for neuronal loss in Gaucher disease

Eun Young Kim[1], Young Bin Hong[1], Sang Hee Go[1], Beobyi Lee[2] and Sung-Chul Jung[3,4]

[1]Department of Biomedical Sciences
National Institute of Health
Seoul 122-701, Korea
[2]Department of Anatomy
College of Medicine, Konkuk University
Chungju 380-701, Korea
[3]Department of Biochemistry
College of Medicine, Ewha Womans University
Seoul 158-710, Korea
[4]Corresponding author: Tel, 82-2-2650-5725;
Fax, 82-2-2652-7842; E-mail, jungsc@ewha.ac.kr

Abstract

Gaucher disease is a glycosphingolipid storage disease caused by deficiency of glucocerebrosidase, resulting in the accumulation of glucosylceramide in lysosomes. The neuronopathic forms of this disease are associated with neuronal loss and neurodegeneration. However, the pathophysiological mechanisms leading to prenatal and neonatal death remain uncharacterized. To investigate brain dysfunction in Gaucher disease, we studied the effects of neurotrophic factors during development in a mouse model of Gaucher disease. The expression of brain-derived neurotrophic factor and nerve growth factor was reduced in the cerebral cortex, brainstem, and cerebellum of Gaucher mice, compared with that in wild-type mice. Extracellular signal-regulated kinase (ERK) 1/2 expression was downregulated in neurons from Gaucher mice and correlated with a decreased number of neurons. These results suggest that a reduction in neurotrophic factors could be involved in neuronal loss in Gaucher disease.

Introduction

Gaucher disease (OMIM 230800, 230900, 231000) is a lysosomal sphingolipid storage disorder caused by a deficiency of glucocerebrosidase (GC; glucosylceramidase, D-glucosyl-*N*-acylsphingosine glucohydrolase, EC 3.2.1.45) (Brady *et al.*, 1965). This disease is characterized by the accumulation of glucosylceramide in macrophages and macrophage-derived cells in various tissues (Brady *et al.*, 1966; Beutler and Grabowski, 2001). Patients with Gaucher disease are classified into three clinical phenotypes: non-neuronopathic (type I), acute neuronopathic (type II), and chronic neuronopathic (type III). Patients with Gaucher disease type I usually present with hepatosplenomegaly and bone disease, but do not have central nervous system symptoms. Patients with Gaucher disease type II or type III have additional neurological symptoms, which may lead to neurodegeneration in infancy or take an intermediate course (Conradi *et al.*, 1984; Brady *et al.*, 1993). The characteristics of the neuronopathic forms include extensive neuronal loss in the anterior horn, basal ganglia, pons, medulla, cerebellum, and hypothalamus, and an increased concentration of glucosylceramide and glucosylsphingosine in the cerebral cortex and cerebellum (Grafe *et al.*, 1988; Orvisky *et al.*, 2000; Wong *et al.*, 2004).

A mouse model of Gaucher disease has been created by targeted disruption of the GC gene (Tybulewicz *et al.*, 1992). These Gaucher mice are deficient in GC activity, show glucosylceramide accumulation in the bone marrow, liver, spleen, and brain, and diffuse storage of glucosylceramide in the microglial cells, brainstem neurons, and spinal cord neurons. They die within a day of birth, primarily from a skin defect (Tybulewicz *et al.*, 1992).

We recently showed that Bcl-2 is downregulated in the brains of fetal Gaucher mice, and we detected neural cell apoptosis (TUNEL-positive cells) in the brains of Gaucher mice (Hong *et al.*, 2004). However, the pathophysiological mechanisms that lead to the neurological symptoms of this disease have not been clearly defined. In this study, we

investigated the effects of neurotrophic factors in Gaucher disease. Neurotrophic factors play an important role in neuronal survival, differentiation, and plasticity (Dudek *et al.*, 1997; Miller *et al.*, 1997; Heese *et al.*, 2004). Previous studies have shown that deficiencies in neurotrophic factors, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), transforming growth factor β (TGF-β), and ciliary neurotrophic growth factor (CNTF), result in massive cell death and neuro-degeneration in the central nervous systems of mutant mice (Sendtner *et al.*, 1992; Crowley *et al.*, 1994; Ernfors *et al.*, 1994; Letterio, 2000). We compared the roles of neurotrophic factors through-out embryonic and postnatal development and analyzed the expression of neurotrophic factors in wild-type mice and the mouse model of Gaucher disease.

Materials and Methods

Mouse model of Gaucher disease

The GC-deficient model mouse (C57BL/6J-Gba^{tm1Nsb}) was obtained from the Jackson Laboratory (Bar Harbor, ME). In this homozygous mouse model of Gaucher disease, $< 4\%$ of normal GC activity is expressed, glucosylceramide is stored in the lyso-somes of cells, and the mice die within one day of birth (Tybulewicz *et al.*, 1992). All animals were housed at the Korea National Institute of Health, and were treated according to the National Institutes of Health guidelines for animal care.

Neuronal cultures

Neurons from Gaucher mice and their wild-type littermates were cultured. Briefly, the brains of wild-type and Gaucher mice at embryonic days 17.5 (E17.5) and 19.5 (E19.5) and postnatal day 1 (P1) were separated into cerebral cortex, brainstem, and cerebellar regions. The cells of the dissected regions were dissociated by trypsinization (0.25% w/v) for 15 min at 37°C, washed in Mg^{2+}/Ca^{2+}-free Hanks' balanced salt solution (Life Technologies, Grand Island, NY), and then further dissociated by repeated passages through a constricted Pasteur pipette. The cells were resuspended in Neurobasal^{TM} medium (Life Technologies) containing B27 supplements (Life Technologies) and plated at a density of 1×10^5 cells/well in six-well plates that had been pre-coated with poly-l-lysine (1 mg/ml). All cells were analyzed after 10-14 days *in vitro* (DIV). Glial proliferation was prevented by the addition to the medium of cytosine β-D-arabinoside (5 μM) on day 2 of culture.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the different brain regions (cerebral cortex, brainstem, and cerebellar regions) at E17.5, E19.5, and P1, and from the neuronal cells of wild-type and Gaucher mice collected at E17.5 and P1, using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). cDNA was transcribed using oligo dT primer and MMLV reverse trans-criptase (Promega, Madison, WI), according to the instructions of the manufacturer. Aliquots of the RT products were amplified by PCR using 20 cycles of 94°C for 45 s, 60°C for 30 s, and 72°C for 30 s. The PCRs were performed in reaction volumes of 25 μl, which included 2 μl of RT product as the template, 1 × reaction buffer, 1 U of Ex *Taq* DNA polymerase (TaKaRa, Japan), and 20 pmol of each primer. Primer sequences were as follows: β-actin, 5'-CCC-ACACTGTGCCCATCTAC-3' and 5'-AGTACTTGCG-CTCAGGAGGA-3'; BDNF, 5'-TGGCTGACACTTTT-GAGCAC-3' and 5'-TCAGTTGGCCTTTGGATACC-3'; and NGF, 5'-CTGTGGACCCCAGACTGTTT-3' and 5'-GCACCCACTCTCAACAGGAT-3'. The PCR products were analyzed on 1% agarose gels.

BDNF immunohistochemistry

Wild-type and Gaucher mice at P1 were perfused with 1.5 ml of saline, then with fixative containing 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS; pH 7.4). The brains were removed and stored in the same fixative for 4 h and in 30% sucrose cryoprotectant overnight. A series of 40-50-μm cryostat sections of whole brains were cut and collected in the wells of a tissue culture plate. Immunohistochemistry was performed using the conventional peroxidase-antiperoxidase (PAP) tech-nique of Sternberger and Sternberger (1986). Briefly, sections were incubated in 0.5% H$_2$O$_2$ in Tris-buffered saline (TBS) for 20 min, blocked in 10% normal goat serum for 1 h, and then incubated with rabbit anti-BDNF antibody (Chemicon, Temecula, CA) at a dilution of 1:500 for 48 h at 4°C. After the sections had been rinsed, they were incubated with goat anti-rabbit IgG (AP132; Chemicon) at a dilution of 1:100, and then with rabbit PAP (PAP18; Che-micon) at a dilution of 1:400. After the sections had been rinsed with TBS, they were reacted with the diaminobenzidine (DAB)- H$_2$O$_2$ kit (SK-4100; Vector, Burlingame, CA) for 3 min at room temperature.

Fluorescence-activated cell sorter (FACS) analysis

Cultured E17.5 and P1 neurons were fixed after 10-14 DIV with 10% ethanol, incubated in 2 N HCl, and blocked with 10% goat serum plus 0.01% Triton

X-100. The cells were incubated with primary antibodies (directed against MAP-2, BDNF, NGF, extracellular regulated kinase 1/2 [ERK 1/2], phospho-ERK 1/2, p38, c-Jun N-terminal kinase 1/2 [JNK 1/2], or phospho-JNK 1/2). After the cells had been washed, they were incubated with secondary antibody (FITC-conjugated anti-rabbit Ig; 1:200) for 2 h. Fluorescence-positive cells were analyzed by flow cytometry (FACS Vantage, Becton Dickinson, San Jose, CA).

Western blot analysis

P1 neuronal cells (cerebral cortex, brainstem, and cerebellar regions) of wild-type and Gaucher mice were cultured for 10-14 DIV and used to analyze the MAPK pathway. Western blots were performed as described previously (Seo *et al.*, 2004). Cells were homogenized with extraction buffer (10 mM PBS [pH 7.4], 0.5% sodium dodecyl sulfate [SDS], 10 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leu-
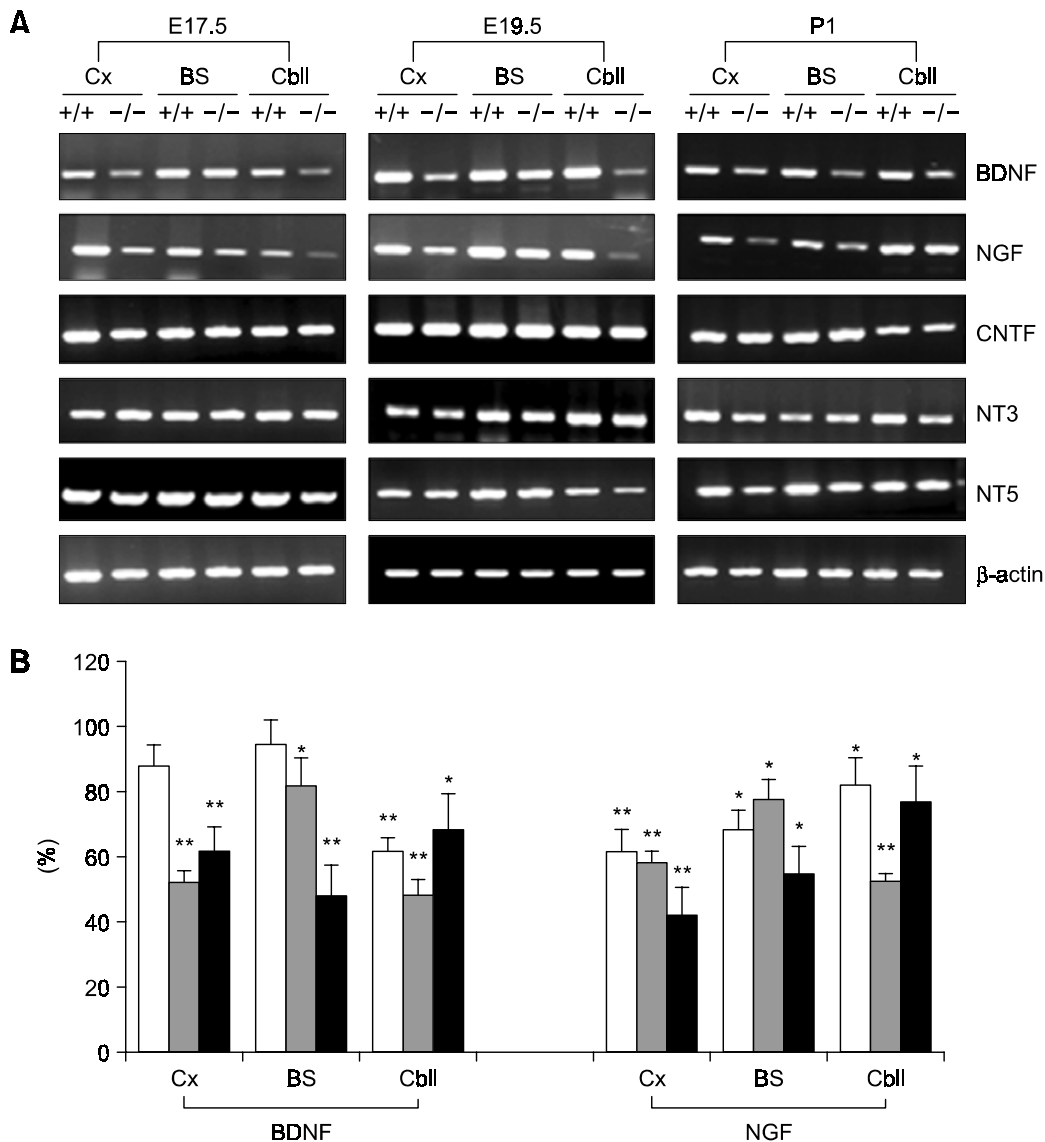

Figure 1. (A) RT-PCR analysis of the expression of the genes encoding neurotrophin family in the brains of wild-type mice and Gaucher mice at three developmental stages (E17.5, E19.5, and P1). Brains were dissected from wild-type mice and Gaucher mice and the cerebral cortex, brainstem, and cerebellar regions were separated. β-actin was used as the internal control. +/+, wild-type mice; -/-, Gaucher mice; Cx, cerebral cortex; BS, brainstem; Cbll, cerebellum. (B) Expression levels of BDNF and NGF proteins in the brains of wild-type and Gaucher mice at E17.5 and P1 were analyzed by FACS. white bar, E17.5; gray bar, E19.5; black bar, P1 mice. Data are expressed as means ± SD (*n* = 3). *, *P* < 0.01 and **, *P* < 0.001 versus corresponding wild-type mice using Student's *t* test.

peptin). The proteins were boiled for 5 min, and 50 μg protein samples were separated by 9% SDS-PAGE and blotted onto Hybond membrane (Amersham, Buckinghamshire, UK). The blots were probed with specific antibodies and then with the corresponding horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The proteins were visualized using enhanced chemiluminescence (ECL; Amersham, DE).

Measurement of cell viability

Live and dead cells were distinguished using 2 μM calcein acetoxymethyl ester (calcein-AM) and 4 μM ethidium homodimer 1 (EthD-1), respectively, as described in the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes Inc., Eugene, OR). We compared survival under specific culture conditions at 1 DIV with survival under the same culture conditions at 10-14 DIV. Live cells were analyzed by flow cytometry (FACS Vantage).

Statistical analysis

Statistical analyses were performed using Student's t test. Data are presented as means ± SD.

Results

Expression of neurotrophic factors in the brains of Gaucher mice

To examine the roles of neurotrophic factors in Gaucher disease, we first investigated the expression of the genes for neurotrophic factors and their receptors in Gaucher mice. Expression of the mRNAs of the neurotrophin family (BDNF, NGF, neurotrophin 3 [NT3], and NT4/5), and of CNTF, GDNF, TGF-β, NGFR, and the tyrosine kinase receptor (Trk) family (TrkA, TrkB, and TrkC) was analyzed in the brains of wild-type and Gaucher mice at three developmental stages (E17.5, E19.5, and P1). Of the neurotrophic factors and the corresponding receptors that were analyzed, the expression of the genes encoding BDNF and NGF was reduced in Gaucher mice at all developmental stages (E17.5, E19.5, and P1) (Figure 1A). In the brains of Gaucher mice, low levels of BDNF expression were observed relative to those observed in wild-type mice in the cerebral cortex (88 ± 4%, 52 ± 4%, and 62 ± 7%; at E17.5, E19.5, and P1, respectively), brainstem (95 ± 2%, 82 ± 8%, and 48 ± 9%, respectively), and cerebellum (62 ± 5%, 48 ± 5%, and 68 ± 11%, respectively). The expression of NGF was also lower in Gaucher mice
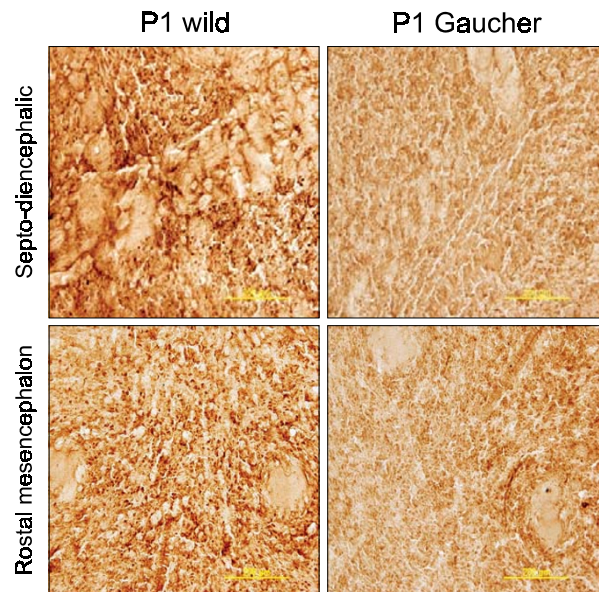

Figure 2. BDNF immunohistochemical staining of the septo-diencephalic region (upper panel) and rostral mesencephalon (lower panel) at P1 in the brains of wild-type mice (left panel) and Gaucher mice (right panel). Cryosections (40-50 μm) of whole brains were mounted on slides. Scale bar, 200 μm.

relative to that in wild-type mice in the cerebral cortex (61 ± 8%, 42 ± 9%, and 58 ± 3%; at E17.5, E19.5, and P1, respectively), brainstem (68 ± 5%, 55 ± 8%, and 78 ± 6%, respectively), and cerebellum (82 ± 6%, 77 ± 11%, and 52 ± 3%, respectively) (Figure 1B). In contrast, the expression levels of NT3, NT4/5, CNTF, GDNF, TGF-β, NGFR, and the Trk family were similar to those of wild-type mice at all stages of development. We then used immunohistochemistry to assess whether the decrease in BDNF mRNA correlated with a reduction in the expression of BDNF protein. BDNF protein was reduced in the septo-diencephalic region and the rostral mesencephalon in Gaucher mice at P1 (Figure 2).

Expression levels of neurotrophic factors in cultured neurons from Gaucher mice

Primary neurons from E17.5 and P1 mice were cultured for 10-14 DIV. Cultured neurons from Gaucher mice exhibited neuronal morphologies (axonal growth and branching, at P1, about 80% MAP-2 positive) similar to those of wild-type neurons (data not shown). Consistent with the results for brain tissue, RT-PCR indicated that the expression of BDNF and NGF transcripts was reduced in neuronal cultures (E17.5 and P1) (Figure 3A and B). The expression of the neurotrophic factors was
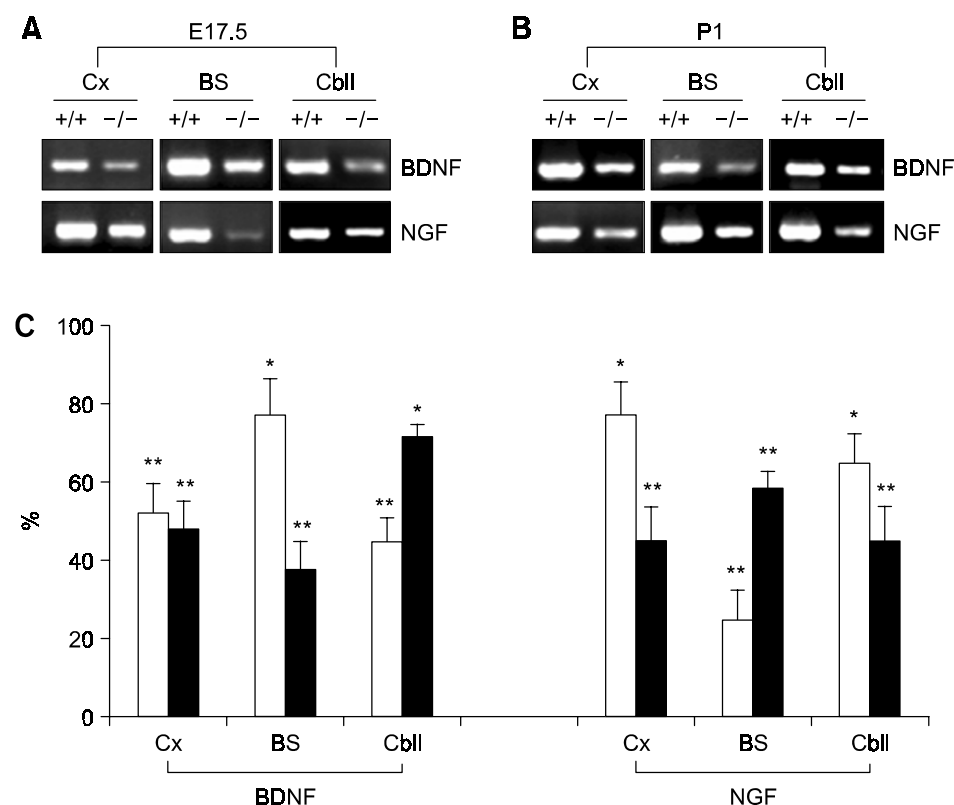
A

E17.5

B

P1

C

Figure 3. RT-PCR and FACS analysis of BDNF and NGF expression in cultured neurons. Brains of wild-type mice and Gaucher mice at E17.5 (A) and P1 (B) were separated into cerebral cortex, brainstem, and cerebellar regions, and the cells were then dissociated. RNA was extracted from neurons that had been maintained in Neurobasal™ medium and analyzed at 10-14 DIV. (C) Expression levels of BDNF and NGF proteins in cultured neurons at E17.5 (open bar) and P1 (closed bar) were analyzed by FACS. +/+, wild-type mouse neurons; -/-, Gaucher mouse neurons; Cx, cerebral cortex; BS, brainstem; Cbll, cerebellum. Data are expressed as means ± SD (n = 3). *, $P < 0.01$, and **, $P < 0.001$ versus corresponding wild-type neurons using Student's t test.

quantified by FACS analysis (Figure 3C). BDNF and NGF expression was significantly reduced in cultured Gaucher mice neurons collected at P1 from the cerebral cortex (48 ± 8% and 45 ± 7% of the levels observed in cultured wild-type neurons, respectively), brainstem (38 ± 7% and 58 ± 5% of the levels observed in cultured wild-type neurons, respectively), and cerebellum (72 ± 8% and 45 ± 9% of the levels observed in cultured wild type neurons, respectively). In Gaucher mice at E17.5, the expression of BDNF and NGF was reduced in the cerebral cortex, brainstem, and cerebellum. The mean value of BDNF expression was 1.7-fold higher in neurons from wild-type mice (100%) than that in neurons from Gaucher mice (58.3 ± 8%), and the level of NGF in neurons from wild-type mice was 1.8-fold higher than the level of NGF in neurons from Gaucher mice (54.6 ± 6%).

Analysis of the MAPK pathway in neurons from Gaucher mice

We then investigated whether this reduction in BDNF and NGF expression involved the MAPK pathway (ERK 1/2, p38, and JNK 1/2). Using immunoblotting (Figure 4A) and FACS analysis (Figure 4B), we found that ERK 1/2 phosphorylation was lower in the neurons of Gaucher mice than in those of wild-type mice. The expression of p38 and JNK was not significantly altered in Gaucher mice compared with that of wild-type mice (Figure 4C-F). Phosphorylation of ERK 1/2 in the cerebral cortex, brainstem, and cerebellum of Gaucher mice at P1 was reduced to 65 ± 8%, 80 ± 11%, and 45 ± 7% of wild-type values, respectively. There was no difference in total ERK protein in cultured wild-type and Gaucher mouse neurons.

Neuronal viability *in vitro*

We measured the percentage of viable neurons present in each culture period. Most P1 wild-type neurons from the cerebral cortex, brainstem, and cerebellum remained viable after 1 DIV (94 ± 5%, 83 ± 7%, and 76 ± 9%, respectively). However, the proportions of viable cells from the same brain regions of Gaucher mice were slightly reduced (71 ± 4%, 67 ± 5%, and 59 ± 8%, respectively). Cell viability was almost unchanged after 10-14 DIV in both wild-type mice (mean, 79 ± 5%) and Gaucher mice (mean, 61 ± 8%) compared with the level of viability observed after one day in culture for wild-type mice (mean, 84 ± 7%) and Gaucher mice (mean, 63 ± 6%).
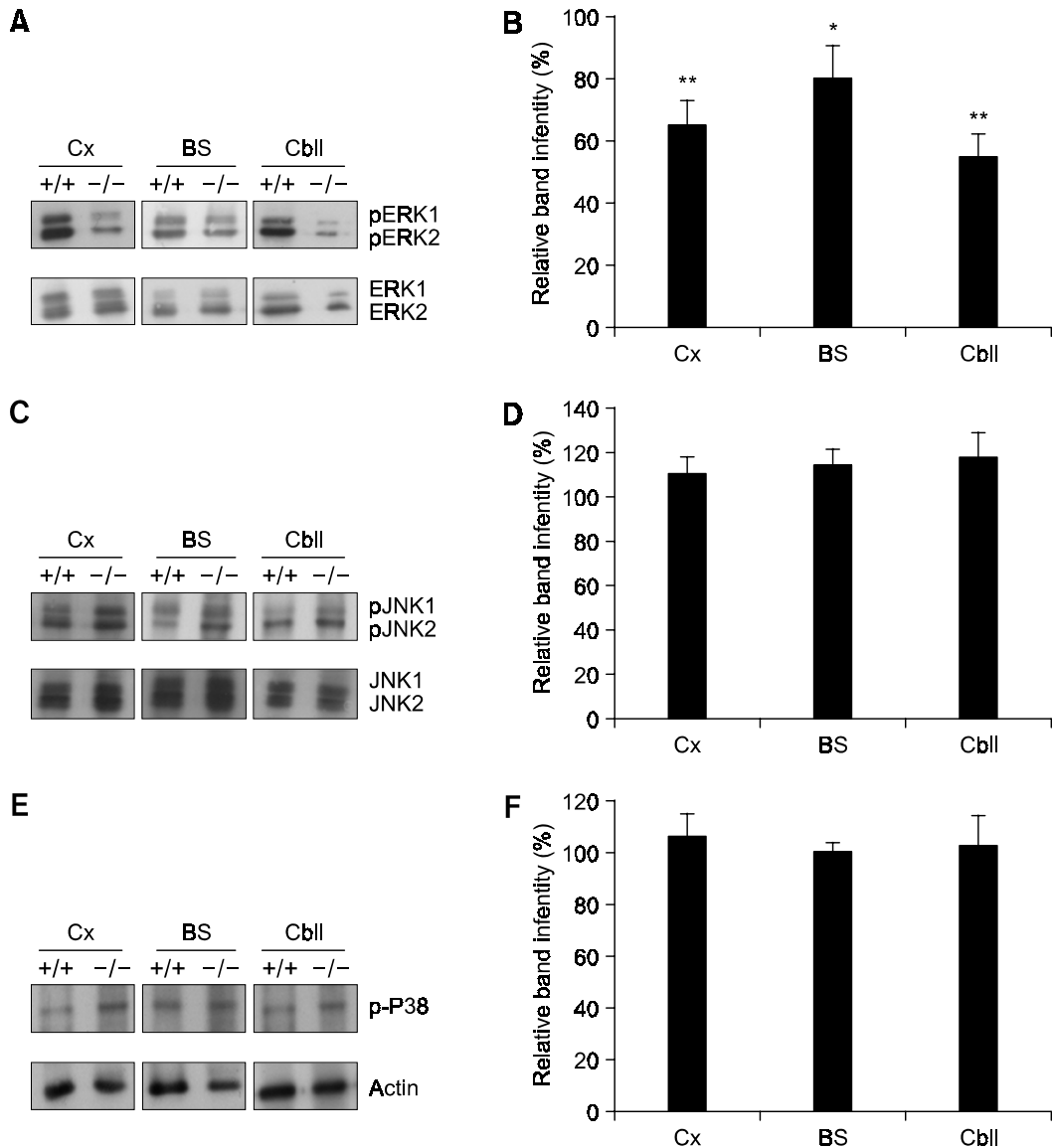
Figure 4. Western blot analysis of the MAPK pathway. Brains of wild-type mice and Gaucher mice at P1 were separated into cerebral cortex, brainstem, and cerebellar regions, and the cells were dissociated. Cell lysates from the P1 neuronal cells of wild-type and Gaucher mice from 10-14 DIV were immunoblotted with anti-phospho-ERK 1/2 and anti-ERK 1/2 antibodies (A), with anti- phospho-JNK 1/2 and anti-JNK 1/2 antibodies (C), and with anti-phospho-P38 antibody (E). Levels of phospho-ERK 1/2 (B), phospho-JNK 1/2 (D), and phospho-P38 (F) were quantified by FACS. +/+, wild-type mouse neurons; -/-, Gaucher mouse neurons; Cx, cerebral cortex; BS, brainstem; Cbll, cerebellum. Values shown are means $\pm$ SD (n = 3). *, $P < 0.01$, and **, $P < 0.001$ versus corresponding wild-type neurons using Student's t test.

Discussion

The brain pathology of neuronopathic forms of Gaucher disease is attributable to a deficiency in GC that leads to neuronal dysfunction (Brady *et al.*, 1993). Type II patients show extensive damage in the brain, including gliosis in the dorsal brainstem and cochlear nuclei (Grafe *et al.*, 1988; Kaga *et al.*, 1998). It has recently been reported that the accu-

mulation of glucosylceramide in cultured neurons can cause the release of Ca^{2+} from intracellular stores. These neurons are sensitive to neurotoxic agents that induce neuronal cell death and the accumulation of glucosylsphingosine, which contributes to neuronal destruction (Korkotian *et al.*, 1999; Lloyd-Evans *et al.*, 2003; Pelled *et al.*, 2005). However, many questions remain unanswered regarding the pathogenesis of brain dysfunction in Gaucher
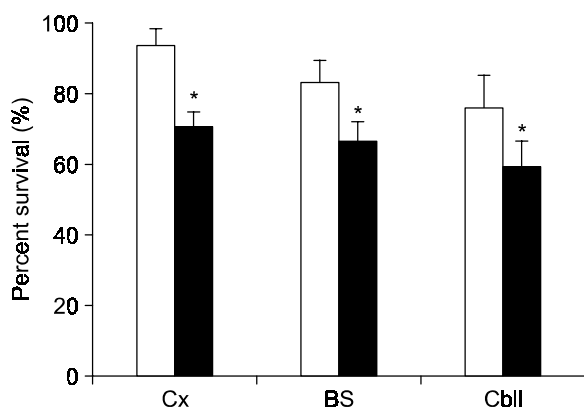
Figure 5. Cell viability assay on cultured neurons. P1 neurons from wild type and Gaucher mice were cultured in neurobasal medium for 14 days. Cells that survived were quantified using the Live/Dead Viability/Cytotoxicity assay. Open bar, wild type neuron; closed bar, Gaucher neuron; Cx, cerebral cortex; BS, brainstem; Cbll, cerebellum. Data are expressed as means ± SD (*n* = 3). *, *P* < 0.01 versus corresponding wild type neurons using Student's *t* test.

disease.

In this study, we found that the expression of genes for the neurotrophic factors BDNF and NGF is reduced in the cerebral cortex, brainstem, and cerebellum of the Gaucher mouse compared with that of the wild-type mouse at various developmental stages. Both neurotrophic factors were gradually downregulated by developmental stages, except in cerebellum. Among the regions in the brain of Gaucher mouse, there were no significant differences of the expression levels of neurotrophic factors. Similarly, a deficiency of neurotrophic factors was observed in cultured neurons (E17.5 and P1) from Gaucher mice. These results suggest that the accumulation of glucosylceramide or glucosylsphingosine affects the expression of the genes for BDNF and NGF in the brain. These results are consistent with those of previous studies, which found that BDNF and NGF can protect cultured cerebellar granule neurons against apoptosis (D'Arcangelo and Halegoua, 1993; Lindholm *et al.*, 1993) and that trophic factor deprivation induces apoptosis in neurons (Dugan *et al.*, 1995).

BDNF and NGF protect neurons, and these neurotrophic factors activate the MAPK pathway (Crowley *et al.*, 1994; Nonomura *et al.*, 1996; Dudek *et al.*, 1997). The MAPK pathway comprises a family of signaling molecules that transduce extracellular stimuli into intracellular responses under a wide variety of circumstances. Neurotrophic factors activate the MAPK pathway in cerebellar granule cells and this activation mediates cell survival and cell growth (Zirrgiebel *et al.*, 1995). The MAPK family

includes ERK 1/2, p38, JNK, and ERK 5 (Widmann *et al.*, 1999). The role of ERK in neuronal degeneration is unclear. However, the activation of ERK is typically associated with cell survival, cell proliferation, and cell differentiation, given that ERK is activated by mitogens and some cell survival factors (Xia *et al.*, 1995; Li *et al.*, 1998). Therefore, the reduced phosphorylation ratio of ERK 1/2 that was observed in this study could have a negative effect on the survival of neurons in Gaucher mice. These results indicate that reductions in BDNF and NGF may be involved in causing neuronopathic forms of Gaucher disease, because neurotrophic factors are associated with neuronal survival during development.

However, it is unclear whether neurotrophic factors protect neurons or whether they are beneficial to neurons in Gaucher mice. In this study, the levels of the pro-neurotrophins, pro-BDNF and pro-NGF, were not quantified. Pro-neurotrophins induce apoptosis in neurons and glia (Lee *et al.*, 2001; Beattie *et al.*, 2002). The effects of reduced levels of BDNF and NGF on the expression of pro-neurotrophins in the brain of the Gaucher mouse should be clarified because apoptosis is a programmed event, possibly resulting from cellular dysfunction in another area. *In vivo* approaches to understanding the pathophysiology of Gaucher disease and the development of new therapeutic strategies to address it have previously been hindered by the lack of a viable animal model (Tybulewicz *et al.*, 1992; Liu *et al.*, 1998). However, the generation of viable mouse models of Gaucher disease has recently been reported (Xu *et al.*, 2003; Sun *et al.*, 2005), and these mouse models will be useful in elucidating the pathogenesis of Gaucher disease.

Further investigation of the relationship between the regulation of neurotrophic factors and the accumulations of GC substrates should clarify the role of neuronal death in Gaucher disease.

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