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Down-regulation of Bcl-2 in the fetal brain of the Gaucher disease mouse model: a possible role in the neuronal loss

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Abstract Gaucher disease is a lysosomal storage disorder resulting from an inborn deficiency of glucocerebrosidase. To investigate the genes responsible for the neuronal symptoms of Gaucher disease, gene expression profiles were analyzed in brains of the Gaucher disease mouse model using a cDNA microarray, and it was found that the *bcl-2* gene is down-regulated. Immunoblotting and apoptosis assay were performed to study the relationship between the decreased expression of Bcl-2 and neuronal death on the brains of Gaucher mice fetuses at embryonic day 17.5 (E17.5) and E19.5. Decreased expression of Bcl-2 was observed in the brain stem and cerebellum but not in cortex by immunoblotting. In situ labeling of DNA fragmentation using terminal transferase-mediated dUTP nick-end-labeling (TUNEL) assay confirmed that apoptosis occurred in the brain stem and cerebellum. More apoptotic cells were detected in the brains of Gaucher mice fetuses at E19.5 than at E17.5. These results suggest that the accumulation of either glucocerebroside or glucosylsphingosine, as a result of glucocerebrosidase deficiency, affects gene expression and could be responsible for neuronal cell death.

Keywords Gaucher disease · Glucocerebrosidase · Mouse · Fetus · Brain · DNA microarray · Bcl-2 · Neuronal loss

Introduction

Gaucher disease (MIM 230800, 230900, 231000) is a lysosomal storage disorder in which deficient

glucocerebrosidase (glucosylceramidase, D-glucosyl-N-acylsphingosine glucohydrolase, EC 3.2.1.45) activity allows glucocerebroside to accumulate in cells (Brady et al. 1966; Beutler and Grabowski 2001). Clinically, patients can be divided into three major phenotypes based on severity and age of disease onset: chronic nonneuronopathic (type I), acute neuronopathic (type II), and subacute neuronopathic (type III). Type I, which is the most prevalent, is characterized by the accumulation of glucosylceramide, primarily in Kupffer cells, osteoclasts, and splenic macrophages. Types II and III are characterized by an earlier age of onset and significantly more severe systemic clinical symptoms. Oculomotor abnormalities, hypertonia of the neck muscles with extreme arching of the neck, bulbar signs, limb rigidity, seizures, and sometimes choreoathetoid movements are common neuronopathic symptoms of this disease, and neuronal loss is also observed (Beutler and Grabowski 2001). However, the pathophysiological mechanism leading to the neurologic symptoms of this disorder remains unknown. In this study, we investigated the genes involved in the manifestation of neurological symptoms using gene expression analysis of the Gaucher disease mouse model.

Materials and methods

Mouse and cell culture

The glucocerebrosidase-deficiency mouse (C57BL/6J-Gba^{tm1Nsb}) was originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA). This glucocerebrosidase gene knockout-homozygote mouse expresses <4% of normal GC activity, stores glucocerebroside in the lysosomes of cells of the reticuloendothelial system, and dies within a day of birth (Tybulewicz et al. 1992). All the animals were housed in the Korean Food and Drug Administration, which is accredited by the American Association for the Accreditation of Laboratory Animal Care, and were treated

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according to the Korean Food and Drug Administration and National Institutes of Health guideline for animal care. To induce a cell model of Gaucher disease, mouse neuroblastoma Neuro-2a and microglial BV-2 cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum with or without 200- μ M conduritol B epoxide (CBE, Sigma, St Louis, MO, USA), a specific glucocerebrosidase inhibitor (Pelled et al. 2000) at 37°C in a 5% CO₂ incubator for 8 days.

DNA microarray

Gene expression profiles were analyzed by DNA microarray in wild-type ($n=3$) and Gaucher mice brains ($n=3$) at E19.5 (embryo at 19.5 days after conception) and for CBE-treated Neuro-2a and BV-2 cells and their controls. RNA was isolated from mice brains and cultured cells using an RNeasy minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Fluorescently labeled cDNA copies of the total RNA pool were prepared by direct incorporation of fluorescent nucleotide analogs during synthesis of the first-strand cDNA by reverse transcription (RT). Each 40 μ l labeling reaction consisted of 100 μ g of total RNA, 2 nmol of random primers, 400 U of reverse transcriptase (Superscript II, Stratagene, La Jolla, CA, USA) in 1 \times reaction buffer containing dNTP, and 2 nmol of either Cy-3-dUTP or Cy-5-dUTP (Amersham Pharmacia, Amersham, UK). RNA and primers were preheated to 70°C for 5 min and cooled in ice water before the remaining reaction components were added. RT reactions were incubated for 2 h at 42°C. Reaction products were purified by QIAquick PCR Purification Kit (Qiagen). The two cDNA pools to be compared were mixed and applied to the DNA microarray (TwinChip mouse 7.4K, Digital Genomics, Seoul, South Korea) in a hybridization mixture containing 10 μ g yeast tRNA. Hybridization took place under a glass coverslip in a humidified slide chamber in an incubator at 58°C for 20 h. DNA chips were scanned using ScanArray Lite (Perkin Elmer Life Sciences, Billerica, MA, USA). Scanned images were analyzed with GenePix 3.0 software (Axon Instruments, Union City, CA, USA) to obtain gene expression ratios. Logged gene expression ratios were normalized by LOWESS regression (Yang et al. 2002). Fluorescent intensity of each spot was calculated by local median background subtraction. We used the robust scatter-plot smoother LOWESS function to perform intensity-dependent normalization for gene expression. Significance analysis of microarray (SAM) was performed for the selection of genes with significant gene expression changes (Tusher et al. 2001). Statistical significance of the differential expression of any gene was assessed by computing a q value (the lowest false discovery rate at which the gene is called significant) for each gene. Software for clustering was

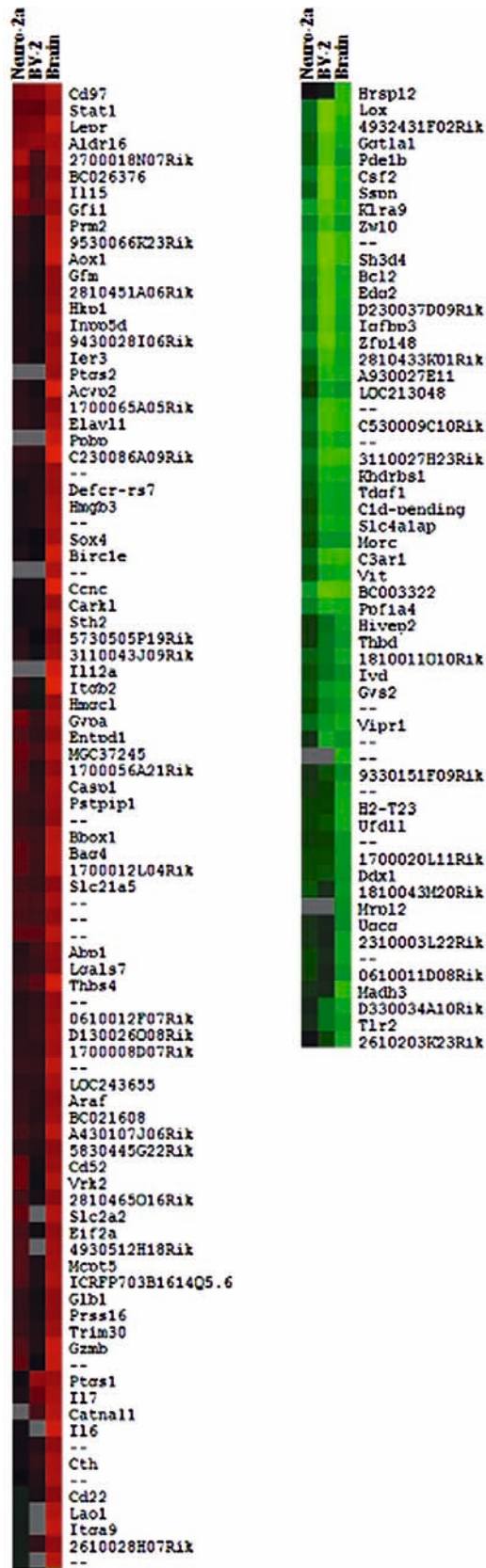
downloaded from Eisen Lab Web site (<http://www.rana.lbl.gov/EisenSoftware.htm>).

Semiquantitative RT-PCR and quantitative real-time PCR

Fetal brains of wild-type and Gaucher mice at E17.5 and E19.5 were separated into cerebral cortex, brain stem, and cerebellar regions. RNAs were purified as previously described, and RT reactions were performed with 2 μ g of total RNA using 50 μ g/ml of oligo(dT) primer at 42°C for 1 h in the presence 20 U of MMLV reverse transcriptase (Promega, Madison, WI, USA). An aliquot of the RT product was amplified by polymerase chain reaction (PCR; 20 cycles of 94°C for 45 s, 60°C for 30 s, 72°C for 30 s). PCRs were performed in a reaction volume of 25 μ l including 2 μ l RT products as a template, 1 \times reaction buffer, 1 U of Ex Taq DNA polymerase (TaKaRa, Japan) and 20 pmol of each primer. Primer sequences were as follows: β -actin (forward: 5'-CCCACACTGTGCCCATCTAC-3', reverse: 5'-AGTACTTGCGCTCAGGAGGA-3'), *bcl-2* (forward: 5'-AAGCTGTACACAGAGGGGCTA-3', reverse: 5'-CAGATGCCGGTTCAGGTTACT-3') and *bax* (forward: 5'-TGCAGAGGATGATTGCTGAC-3', reverse: 5'-GATCAGCTCGGGCACTTTAG-3'). PCR products were analyzed on a 1% agarose gel, and the images were analyzed with densitometric software (Tina v.2.09; Raytest, Courbevoie, France). Real-time PCRs were performed in a reaction volume of 20 μ l including 5 μ l RT products as a template, 10 μ l SYBR Green PCR master mix (Applied Biosystems, Warrington, UK), and 20 pmol of each primer described above, and were performed with 40 cycles of 95°C for 60 s and 60°C for 60 s using ABI 7700 (Applied Biosystems). Average C_t values of *bcl-2* and *bax* from triplicate PCR reactions were normalized from average C_t values of β -actin. Ratios of expression levels of *bcl-2* and *bax* between wild-type and Gaucher mice were calculated as $2^{-(\text{mean}\Delta\Delta C_t)}$.

Immunoblotting

For the preparation of total protein extracts, the cerebral cortex, brain stem, and cerebellar regions were washed with cold phosphate-buffered saline (PBS) and homogenized with extraction buffer [10 mM PBS (pH 7.4), 0.5% sodium dodecyl sulfate (SDS), 10 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml leupeptin]. Homogenates were centrifuged at 14,000g for 20 min at 4°C, and the supernatant was used for immunoblotting. Proteins were boiled for 5 min, and 50 μ g of protein samples were separated using 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto Hybond membranes (Amersham, Buckinghamshire, UK). Blots were then probed with anti- β -Actin, anti-Bcl-2, and anti-Bax (Pharmingen) then with the corresponding horseradish-peroxidase-conjugated secondary



antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Blots were developed using chemiluminescent reagent (Amersham, DE, USA).



Fig. 1 Expression levels of 148 genes increased or decreased more than fourfold. Gene expression profiles were analyzed using fetal brains of wild-type ($n=3$) and Gaucher mice ($n=3$) at E19.5 and CBE-treated Neuro-2a and BV-2 cells together with their controls. Three separated reactions were performed for each mouse model and cell model. Expression levels are depicted by color coding with green representing down-regulation and red representing up-regulation. Black and gray represent zero and missing, respectively

TUNEL assay

In situ labeling of DNA fragmentation using terminal transferase-mediated dUTP nick-end-labeling (TUNEL) was performed on brain sections from wild-type and Gaucher mice with an In situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Frozen 10 μm coronal sections were cut using a cryostat and mounted on glass slides. Sections were fixed in 4% neutral-buffered paraformaldehyde for 20 min at room temperature then washed for 30 min with PBS. Sections were then incubated in 1% Triton X-100 at room temperature for 1 h. Sections were then incubated with TUNEL reaction mixture contained in the kit for 1 h at 37°C and counter-stained with propidium iodide (PI).

Results and discussion

To investigate the genes responsible for the neuronal death typical of Gaucher disease, gene expression profiles were analyzed using fetal brains of wild-type ($n=3$) and Gaucher mice ($n=3$) at E19.5 and CBE-treated Neuro-2a and BV-2 cells together with their controls. Of the 7,744 genes on the DNA microarray, the expression of 58 genes decreased more than fourfold and of 90 genes increased more than fourfold in the Gaucher mouse brain (Fig. 1). Of the 148 genes that either decreased or increased in expression, we chose to examine the *bcl-2* gene because it is one of the genes related to cell survival or death and especially to apoptosis. The *bcl-2* was demonstrated to have log ratios [logarithm of expression score (CBE-treated cells/untreated control cells or Gaucher mouse brain/wild-type mouse brain)] of -1.81 , -3.41 , and -2.38 in CBE-treated Neuro-2a and BV-2 cells and Gaucher mouse brain, respectively.

To confirm the decreased expression of the *bcl-2* gene in brains of Gaucher mice, semiquantitative RT-PCR and quantitative real-time PCR were performed to detect *bcl-2*, *bax*, and β -actin mRNAs (Fig. 2). Mean C_t values of *bcl-2* and *bax* were calibrated with β -actin C_t values, and variations in *bcl-2* and *bax* between wild-type and Gaucher mice were calculated. Low levels of *bcl-2* mRNA were observed in the brain stem (57% of wild-type mice) and cerebellum (46%) of the Gaucher mouse at E19.5, whereas *bax* mRNA was 104 and 115% of wild-type mice, respectively. In contrast, there was no decrease in *bcl-2* mRNA level in the brain stem and cerebellum of the Gaucher mouse at E17.5. In the

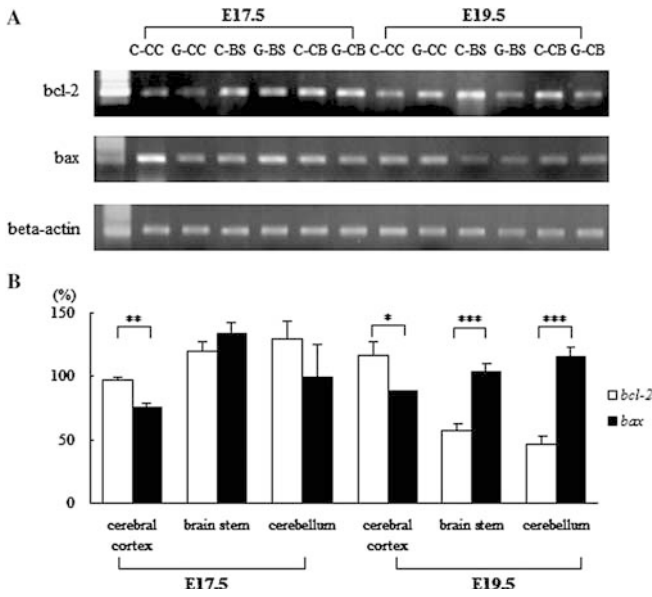


Fig. 2 **a** Semiquantitative RT-PCR analysis of *bcl-2*, *bax* expression in the mouse brain at E17.5 and E19.5. Brains were dissected from wild-type and Gaucher mice ($n=3$). Cerebral cortex, brain stem, and cerebellar regions were separated, and mRNAs were purified. After RT reactions with 2 μ g of total RNA, PCR was performed with β -actin primers to calibrate the concentration of cDNAs. Adjusted volumes of template were used for PCR of *bcl-2* and *bax*. C-CC cerebral cortex from the wild-type mouse, G-CC cerebral cortex from the Gaucher mouse, C-BS brain stem from the wild-type mouse, G-BS brain stem from the Gaucher mouse, C-CB cerebellum from the wild-type mouse, G-CB cerebellum from the Gaucher mouse. **b** Expression levels of *bcl-2* and *bax* in the mouse brain at E17.5 and E19.5 analyzed by quantitative real-time PCR. Data are expressed as means \pm SD of three observations. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus corresponding control using Student's *t* test

cerebral cortex, *bcl-2* mRNA was slightly down-regulated at E17.5 and slightly up-regulated at E19.5 in the Gaucher mouse compared with the wild-type mouse. These results suggest that a deficiency of glucocerebrosidase affects the expression of the *bcl-2* gene in the brain stem and cerebellum.

Immunoblotting was used to determine whether the down-regulation of *bcl-2* expression causes reduced expression of Bcl-2 protein (Fig. 3). Variations in band intensities for Bcl-2 and Bax between wild-type and Gaucher mice were calculated by the same method used in RT-PCR. Expression levels of Bcl-2 and Bax are shown in Fig. 3c. Bcl-2 levels in the cerebral cortex, brain stem, and cerebellum were 77, 110, and 81% of wild-type values, respectively, in the E17.5 Gaucher mouse (Fig. 3a). In contrast, Bcl-2 levels were significantly reduced in the brain stem (32% of wild type) and cerebellum (55% of wild type) in the Gaucher mouse at E19.5, whereas Bax expression was 71% in the brain stem and 95% in the cerebellum at this stage relative to the expression detected in wild-type mice (Fig. 3b). In the cerebral cortex of the E17.5 Gaucher mouse, Bcl-2 expression was 77% and Bax expression was 90% of the wild-type value, whereas at E19.5, Bcl-2 was 100% and

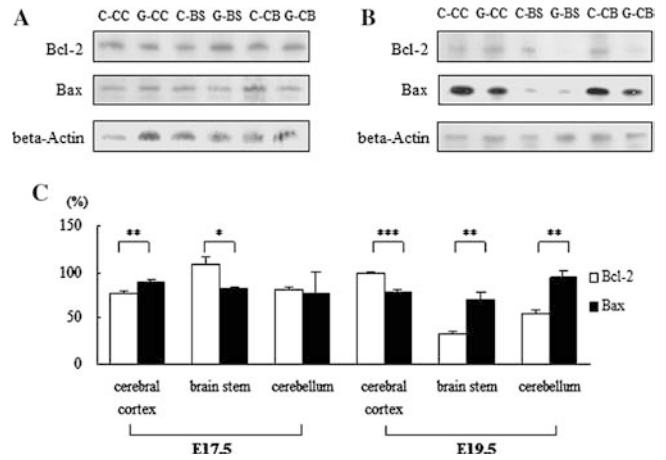


Fig. 3 Immunoblotting for Bcl-2 and Bax from the mouse brain at **a** E17.5 and **b** E19.5. Cerebral cortex, brain stem, and cerebellar regions ($n=3$ for each mouse strain) were separated, and proteins were extracted. Total proteins (50 μ g) were separated by 12.5% SDS-PAGE then transferred onto PVDF membrane. Membranes were cut into three parts according to the molecular weights of Bcl-2, Bax, and β -Actin and probed with primary antibodies as described in the text. C-CC cerebral cortex from the wild-type mouse, G-CC cerebral cortex from the Gaucher mouse, C-BS brain stem from the wild-type mouse, G-BS brain stem from the Gaucher mouse, C-CB cerebellum from the wild-type mouse, G-CB cerebellum from the Gaucher mouse. **c** Relative expression of Bcl-2 and Bax was evaluated as described in the text. Data are expressed as means \pm SD of three observations. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus corresponding control using Student's *t* test

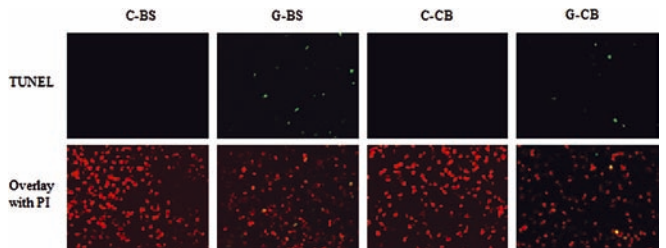


Fig. 4 Neuronal apoptosis was detected with a DNA fragmentation [terminal transferase-mediated dUTP nick-end-labeling (TUNEL)] assay. Cryosections (10 μ m) of whole brains were mounted on slides. Slides were viewed with a Zeiss Axiovert confocal microscope at 20 \times magnification. The upper panel is TUNEL stained with fluorescein and in the lower panel, the fluorescein staining is overlaid with PI stain. C-BS brain stem from the wild-type mouse, G-BS brain stem from the Gaucher mouse, C-CB cerebellum from the wild-type mouse, G-CB cerebellum from the Gaucher mouse

Bax was 78% of the wild-type value. Although there were some variations between mRNA levels and protein expression, Bcl-2 in the brain stem and cerebellum at E19.5 displayed the lowest expression in terms of both mRNA and protein.

Since Bcl-2 was down-regulated in the brain stem and cerebellum at E19.5, we investigated neuronal apoptosis in the brains of Gaucher mice at that age using a TUNEL assay. Apoptotic cells were observed with fluorescence microscopy. There were more apoptotic cells in the brain stem and cerebellum of the Gaucher mouse than in those of the wild-type mouse at E19.5 (Fig. 4).

However, there were no apoptotic cells in the cerebral cortex of either (data not shown). In the E17.5 mouse brain, some apoptotic cells were also detected in the brain stem and cerebellum (data not shown), but there were fewer than observed in the E19.5 mouse brain.

Although the mechanism by which Bcl-2 delays or prevents the onset of cell death in the central nervous system is not completely understood, Bcl-2 is designated a repressor of apoptosis and programmed cell death. Down-regulation of Bcl-2 causes neuronal cell death (Raghupathi et al. 2002), and inactivation of Bcl-2 results in progressive degeneration of motoneurons and sympathetic and sensor neurons (Michaelidis et al. 1996). On the other hand, overexpression of Bcl-2 can protect against neuronal cell death (Farlie et al. 1995).

In this study, we found that Bcl-2 was down-regulated in the brain stem and cerebellum of the Gaucher mouse and that this is related to neuronal cell death in these regions. In contrast, there was no marked decrease in Bcl-2 levels and no apoptosis in the cerebral cortex. These results are consistent with previous studies that reported severe neuronal loss in the pons and medulla and active apoptosis in the midbrain and thalamus in patients with type II Gaucher disease but less neuronal loss in the cerebral cortex (Finn et al. 2000; Kaye et al. 1986).

Gaucher disease is caused mainly by glucocerebroside, a metabolic intermediate in the synthesis and degradation of complex glycosphingolipids. Accumulation of glucocerebroside in the liver, spleen, and bone marrow is believed to contribute to the massive enlargement of these organs. Recently, the deacylated analog of glucocerebroside, glucosylsphingosine, was reported to accumulate in the cerebral and cerebellar cortices of patients with type II or type III Gaucher disease (Orvisky et al. 2002) and in the brains of Gaucher mice (Orvisky et al. 2000). Glucosylsphingosine has been suggested to be a candidate neurotoxin (Lloyd-Evans et al. 2003; Schueler et al. 2003). It is a potent inhibitor of mitochondrial cytochrome-c-oxidase (Igisu et al. 1988) and could affect cellular signal transduction and differentiation (Hannun and Bell 1987; Shayman et al. 1991).

Although sphingosine decreases the expression of Bcl-2 in HK-60 cells (Sakakura et al. 1996), direct experimental evidence that the down-regulation of *bcl-2* is the consequence of the accumulation of either glucocerebroside or glucosylsphingosine is poor. However, there could be some relationship between the progressive incremental accumulation of glucocerebroside and glucosylsphingosine in utero (Orvisky et al. 2000) and the progressive decrease of expression of *bcl-2* gene throughout gestation. The *bcl-2* gene down-expression could affect neuronal loss in fetuses with Gaucher disease. Further investigation of the relationship between the regulation of neuronal gene expression and the accumulation of glucocerebroside substrates should clarify the role of the neuronal death in Gaucher disease.

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