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Ethylenedioxy-PIP2 oxalate reduces ganglioside storage in juvenile Sandhoff disease mice

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Abstract

Sandhoff disease is an incurable neurodegenerative disorder caused by mutations in the lysosomal hydrolase β -hexosaminidase. Deficiency in this enzyme leads to excessive accumulation of ganglioside GM2 and its asialo derivative, GA2, in brain and visceral tissues. Small molecule inhibitors of ceramide-specific glucosyltransferase, the first committed step in ganglioside biosynthesis, reduce storage of GM2 and GA2. Limited brain access or adverse effects have hampered the therapeutic efficacy of the clinically approved substrate reduction molecules, eliglustat tartrate and the imino sugar NB-DNJ (Miglustat). The novel eliglustat tartrate analog, 2-(2,3-dihydro-1H-inden-2-yl)-N-((1R,2R)-1-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1-hydroxy-3-(pyrrolidin-1-yl)propan-2-yl)acetamide (EtDO-PIP2, CCG-203586 or “3h”), was recently reported to reduce glucosylceramide in murine brain. Here we assessed the therapeutic efficacy of 3h in juvenile Sandhoff (*Hexb* $-/-$) mice. Sandhoff mice received intraperitoneal injections of phosphate buffered saline (PBS) or 3h (60 mg/kg/day) from postnatal day 9 (p-9) to postnatal day 15 (p-15). Brain weight and brain water content was similar in 3h and PBS-treated mice. 3h significantly reduced total ganglioside sialic acid, GM2, and GA2 content in cerebrum, cerebellum and liver of Sandhoff mice. Data from the liver showed that 3h reduced the key upstream ganglioside precursor (glucosylceramide), providing direct evidence for an on target mechanism of action. No significant differences were seen in the distribution of cholesterol or of neutral and acidic phospholipids. These data suggest that 3h can be an effective alternative to existing substrate reduction molecules for ganglioside storage diseases.

Keywords

Glycosphingolipid; Ganglioside; Lysosomal Storage Disease; Neurodegeneration; Substrate Reduction Therapy; Sandhoff Disease

INTRODUCTION

Sandhoff disease is an incurable neurodegenerative lysosomal storage disease (LSD) caused by autosomal recessive mutations in the beta subunit of β -hexosaminidase (1). The deficiency of hexosaminidase A and B results in the storage of ganglioside GM2 and its asialo derivative (GA2) primarily in neurons. *Hexb*^{-/-} mice suffer from neurodegeneration, neuroinflammation, demyelination, progressive motor deterioration, and premature death by 16 weeks (2–6). Substrate reduction therapy (SRT) aims to prolong longevity in LSDs by reducing the production and load of stored lipid. Small molecule inhibitors of the ceramide-specific glucosyltransferase (glucosylceramide ceramide synthase) reduce downstream biosynthesis of gangliosides (7, 8). SRT has improved outcomes in murine models of Sandhoff disease, Tay-Sachs disease, GM1 gangliosidosis, Niemann Pick type C (NPC), Gaucher disease, and Fabry disease (9–20). Current clinically approved SRT molecules include imino sugar *N*-butyldeoxynojirimycin (*NB*-DNJ, Miglustat, Zavesca) and the D-threo-1-phenyl-2-decanoylamino-3-morpholino-propanol (PDMP) analog Genz-112638 (eliglustat tartrate). Both of these molecules have been administered to type 1 Gaucher disease patients (20–26). Due to its non-specificity, *NB*-DNJ has some adverse effects associated with prolonged usage, including weight loss, diarrhea, poor appetite, and tremor (20, 23). In addition, *NB*-DNJ binds directly to β -glucocerebrosidase where it acts as a molecular chaperone suggesting a more complicated mechanism of action on glucosylceramide metabolism (27, 28). Although eliglustat tartrate is therapeutically effective at lower doses (IC 50; 24 nM), therapeutic efficacy for neuronal LSDs is limited due to poor blood brain barrier (BBB) permeability (29, 30).

In contrast to eliglustat tartrate, its novel analog, *D*-threo-3',4'-ethylenedioxy-1-phenyl-2-indanylacetoamino-3-pyrrolidino-1-propanol-oxalate (EtDO-PIP2, or **3h**), enters the brain and reduces glucosylceramide in normal mice (Figure 1) (30). In this study, we have assessed the therapeutic effectiveness of **3h** in Sandhoff mice. Juvenile *Hexb*^{-/-} mice treated with **3h** showed significant reductions in total ganglioside and GM2 content in brain and liver. The ganglioside reductions in brain were similar to those reported previously in LSD mice treated with the imino sugars, *NB*-DNJ and *NB*-DGJ (9, 10, 13, 14). Our results suggest that **3h** could be a better substrate reduction therapy for prolonged treatment of peripheral and CNS LSDs.

MATERIALS AND METHODS

Mice

The SV/129 *Hexb*^{-/-} mice were obtained from Dr. Richard Proia (NIH). These mice were derived by the disruption of the murine *Hexb* gene and transferring this gene into the mouse genome via homologous recombination and embryonic stem cell technology as previously described (2). The genotypes of mice were determined by PCR as previously described. Mice were propagated in the Boston College Animal Facility and were housed in plastic cages with filter tops containing Sani-Chip bedding (P.J. Murphy Forest Products Corp.; Montville, NJ). Food and water were provided *ad libitum*. Nursing females were provided with cotton nesting pads for the duration of the experiment. All animal experiments were carried out with ethical committee approval in accordance with the National Institutes of

Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care Committee.

3h treatment and tissue collection

The **3h** molecule, D-*threo*-3',4'-ethylenedioxy-1-phenyl-2-indanylacetoamino-3-pyrrolidino-1-propanol-oxalate, was produced at the Vahlteich Medicinal Chemistry Core, University of Michigan, Ann Arbor, MI. **3h** was resuspended to a concentration of 1 mM in CHCl₃: CH₃OH 1:2 (v/v). The solution was dried under nitrogen, resuspended in 3.6 ml dH₂O, and then dissolved at 42°C in shaking water bath. The solution was neutralized by adding 400 µl of 10 × phosphate buffered saline (PBS), and was sterilized following passage through an Acrodisc 0.2 µm syringe filter (Sigma, St. Louis, MO). The final solution contained ~6.0 mg/ml of **3h**. Mice were weighed daily and received intraperitoneal (i.p.) injections equivalent to 60 mg/kg from postnatal day 9 (p-9) to postnatal day 15 (p-15). Control mice received i.p. injections of PBS. Injections were performed using a Hamilton syringe (26 gauge, point style 2, 0.5 inch needle) (Hamilton, Reno, NV), and volumes ranged from 50 µl to 100 µl/mouse. Mice were sacrificed 4 hrs after the final injection on p-15. Cerebrum, cerebellum, and liver were dissected and frozen on dry ice to determine wet weight. The tissues were then homogenized in 3.0 ml dH₂O, and 150 µl of each homogenate was set aside for analysis of protein and lysosomal enzyme activities. The remaining homogenate was frozen at -80°C, was lyophilized, and was then weighed before lipid extraction.

Enzyme assays

β-hexosaminidase and β-galactosidase specific activities were measured using either 1 mM 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich, St. Louis, MO) or 4-methylumbelliferyl-β-D-galactopyranoside (Sigma-Aldrich, St. Louis, MO) as the substrates, respectively (31). Tissue homogenates were centrifuged for 5 minutes at 2,000 × g. The collected supernatants were dispensed in duplicate to BD Falcon 96-well plates on ice. Increasing volumes of 40 µM 4-methylumbelliferone (Sigma-Aldrich, St. Louis, MO) in 0.9% NaCl were used as standards. Plates were incubated at 37°C for 30 minutes after the addition of substrate. The reaction was stopped by the addition of 0.5 M sodium carbonate (pH 10.7). A SpectraMax M5 micro-plate reader (Molecular Devices, Sunnyvale, CA) with excitation and emission set at 355 nm and 460 nm was used to estimate fluorescent emission of 4-methylumbelliferone. Total protein concentrations for each tissue were determined by mixing an aliquot of each sample with Bio-Rad Protein Dye Reagent (Bio-Rad, Hercules, CA) diluted 1:4 (v/v) in water. Increasing concentrations of bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) were used as standards. Plates were incubated at room temperature for 15 minutes, and read at 595 nm in the SpectraMax M5. Specific enzyme activity was expressed as nmol/mg protein/hr.

Lipid isolation, purification, and quantitation

Total Lipid Isolation and Purification—Lipid extraction of lyophilized tissue was performed overnight in 5 ml CHCl₃: CH₃OH 1:1 (v/v) according to our standard procedures (32). Samples were spun down at 2500 rpm for 20 min, and the supernatant was collected.

Pellets were washed in 2 ml CHCl_3 : CH_3OH 1:1 (v/v), spun down again, and the total 7 ml of supernatant was brought up to a final volume of 19.6 ml at a ratio of 30:60:8 CHCl_3 : CH_3OH : dH_2O (v/v/v). Neutral lipids and cholesterol were separated from acidic lipids and gangliosides by ion exchange chromatography as we described (13, 32, 33). The column was washed twice with 20 mL solvent A and the entire neutral lipid fraction, consisting of the initial eluent plus washes, was collected. This fraction contained cholesterol, phosphatidylcholine, phosphatidylethanolamine and plasmalogens, sphingomyelin, and neutral GSLs, including cerebroside and asialo-GM2 (GA2). Next, acidic lipids were eluted from the column with 35 mL CHCl_3 : CH_3OH : 0.8 M Na acetate, 30:60:8 by volume. Gangliosides were separated from acidic phospholipids by Folch partitioning, base treated, and desalted as previously described (13, 33). Neutral lipids were dried by rotary evaporation and resuspended in 10 mL CHCl_3 : CH_3OH (2:1; v/v). A 4 mL aliquot was evaporated under nitrogen, base treated with 1 N NaOH, and Folch partitioned. The Folch lower phase containing GA2 and GlcCer was evaporated under nitrogen and resuspended in 4 mL CHCl_3 : CH_3OH (2:1; v/v).

Ganglioside Sialic Acid Quantification—Total ganglioside content was quantified before and after desalting using the resorcinol assay as we previously described (33). Sialic acid values were fit to a standard curve using N-acetylneuraminic acid as a standard.

High Performance Thin Layer Chromatography—All lipids were analyzed qualitatively by high-performance thin-layer chromatography (HPTLC) according to previously described methods (32, 33). The amount of lipid per lane was equivalent to 1.5 μg of total sialic acid for gangliosides, and 70 μg , 200 μg , and 300 μg of tissue dry weight for neutral lipids, acidic lipids, and GA2, respectively. For GlcCer analysis in liver, a total of 5.0 mg of tissue dry weight were spotted on the plate. HPTLC plates were developed by a single 90 min ascending run with CHCl_3 : CH_3OH : dH_2O (55:45:10; v/v/v for gangliosides; 65:35:8, v/v/v for GA2) containing 0.02% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The plates were sprayed with either the resorcinol-HCl reagent or the orcinol- H_2SO_4 reagent and heated at 95°C for 10 min to visualize gangliosides or neutral glycosphingolipids (GA2 and glucosylceramide, GlcCer), respectively. The HPTLC distribution of neutral and acidic lipids was as previously described (10, 32). The bands were visualized by charring with 3% cupric acetate in 8% phosphoric acid solution (34). For GlcCer analysis in liver, a total of 5.0 mg of tissue dry weight were spotted on the HPTLC and developed by a single 30 min ascending run with CHCl_3 : CH_3OH : dH_2O (65:35:8, v/v/v).

Quantitation of Individual Lipids—The total brain ganglioside distribution was normalized to 100%, and the percentage distribution values were used to calculate sialic acid concentration (micrograms of sialic acid per 100 mg dry weight) of individual gangliosides. The density values for neutral lipids, acidic lipids, GA2 and GlcCer were fit to either a standard curve or known standard and were used to calculate individual concentrations expressed as milligrams per 100 mg dry weight. Oleyl alcohol was also run on the HPTLC plates as an internal standard for quantitation of cholesterol, and the neutral and acid phospholipids (10, 34).

Results

Our objective was to determine the influence of **3h** on brain development and to assess whether **3h** might be an effective substrate reduction therapy for juvenile Sandhoff disease (*Hexb* $-/-$) mice. The motor behavior of the **3h**-treated mice with respect to ambulation was not noticeably different from that of the PBS control mice. Brain water content, β -hexosaminidase specific activity, and β -galactosidase specific activity were similar in the control and *Hexb* $-/-$ mice treated with **3h** at 60 mg/kg/day from p-9 to p-15 (Table 1). Water content is a sensitive indicator of brain development (35, 36). Body weight was slightly higher in the **3h**-treated *Hexb* $-/-$ mice than in the control *Hexb* $-/-$ mice and was similar to that seen in the normal *Hexb* $+/-$ mice. Total sialic content in **3h**-treated mice was significantly lower in the **3h**-treated *Hexb* $-/-$ mice than in the control *Hexb* $-/-$ mice in cerebrum (11%), cerebellum (14%), and in liver (38%) (Table 2).

The influence of **3h** on the qualitative and quantitative distribution of brain gangliosides is shown in Figure 2 and Table 3. Gangliosides that were undetectable or represented less than 1% of the total distribution (such as GD3 in cerebrum) were omitted from the analysis. Ganglioside GM2 comprised ~5% of ganglioside sialic acid content in p-15 *Hexb* $-/-$ mouse cerebrum and cerebellum (Figure 2 and Table 3). **3h** reduced GM2 content by 52% and 53%, respectively, in cerebrum and cerebellum of *Hexb* $-/-$ mice. GM1, GD1a, GD1b, and GT1b content were also significantly reduced in cerebrum of *Hexb* $-/-$ mice treated with **3h** (Table 3). GD3, GD1b, and GT1b were significantly reduced in cerebellum of *Hexb* $-/-$ mice treated with **3h** (Table 3). In normal and Sandhoff mouse liver, GM2 containing N-glycolylneuraminic acid (NGNA) is the primary ganglioside (Figure 2 and Supplementary Figure S1). NGNA-GM2 content was significantly reduced by 45% in *Hexb* $-/-$ mice treated with **3h** compared to PBS-treated *Hexb* $-/-$ controls (Table 2).

The influence of **3h** on storage of asialo GM2 (GA2) is shown in Figure 3. GA2 is undetectable in normal mouse brain and liver (Figure 3, Table 4). GA2 in liver resolved as a doublet, similar to NGNA-GM2 (Figure 3, Figure 2, Supplementary Figure S1). **3h** reduced GA2 in *Hexb* $-/-$ liver, but this difference was not significant ($p < 0.06$). There were no differences in neutral and acidic lipid content for Sandhoff mice treated with PBS or **3h** (Figure 4, Figure 5, Table 4). Notably, cerebellar cerebroside, consisting of largely of galactosylceramide, were unchanged in *Hexb* $-/-$ mice receiving **3h** (Table 4). The content of GlcCer was evaluated only in liver, as liver did not contain detectable galactosylceramide, which would obscure low amounts of GlcCer. The content of GlcCer ($\mu\text{g}/100$ mg dry weight) in two independent liver samples from the control (PBS)-treated SD mice was 4.38 μg and 6.84 μg , whereas the GlcCer content in two independent liver samples from the **3h**-treated SD mice was 2.91 μg and 3.62 μg . The average reduction in liver GlcCer content (41%) was comparable to the reduction of liver GM2 content (Table 2). These findings indicate that **3h** targets the glucosyltransferase as shown in Fig. 1.

Discussion

Substrate reduction therapies using *N*-butyldeoxynojirimycin (*NB*-DNJ, Miglustat, Zavesca) or the glucose analog of *NB*-DGJ (*N*-butyldeoxygalactonojirimycin), are effective in

reducing ganglioside storage and pathogenesis in mouse models of Tay–Sachs disease, Sandhoff disease, and GM1 gangliosidosis (4, 10–14, 17, 37, 38). Recently, we showed that intraperitoneal administration of the novel eliglustat tartrate analog, **3h**, to normal adult C57BL/6 mice for three days caused a significant dose dependent decrease in the content of brain glucosylceramide, the upstream precursor for ganglioside biosynthesis (Figure 1) (30). It was not determined from this study, however, whether the reduction of glucosylceramide was associated with reduction of downstream synthetic products (gangliosides).

In this study we show that the daily injection of **3h** from postnatal day 9 to 15 significantly reduced the total content of brain and liver gangliosides in SD (*Hexb* $-/-$) mice. Despite the absence of hexosaminidase β subunit, the degree of GM2 storage is considerably less in the SD mouse brain than in the infantile human SD brain (32). This might result in part from the activity of the hexosaminidase α subunit towards the GM2 substrate (2). **3h** significantly reduced the accumulation of GM2 and GA2 in cerebrum, cerebellum, and in liver indicating a systemic therapeutic response. The reduction of ganglioside in liver was likely linked to the reduction observed for GlcCer, the up-stream metabolic precursor.

In contrast to NB-DNJ, which is known to reduce mouse body weight (16, 38), no adverse effects of **3h** were detected on body weight over the treatment period. The reduction of total brain gangliosides and of GM2 and GA2 content in the *Hexb* $-/-$ mice was obtained with a **3h** dosage of 60 mg/kg/day. Although the reductions in mouse brain ganglioside content seen using **3h** were similar to those seen previously using the NB-DNJ or NB-DGJ imino sugars, the therapeutic dosages used in the studies with the imino sugars ranged from 400–1200 mg/kg body weight (10, 11, 13, 14, 38). The influence of **3h** on brain ganglioside content that we observed in this study and in our previous studies with the NB-DNJ or NB-DGJ imino sugars are markedly different from the findings reported recently for the Genzyme drug Genz-529468 (39). Although Genz-529468 is also an imino sugar, it increases rather than decreases GlcCer and GM2 content in the *Hexb* $-/-$ mice (39). Both NB-DNJ and NB-DGJ significantly reduce GlcCer content in mouse embryos and in proliferating cells (7, 40, 41). The **3h**-induced reduction of GlcCer we observed in liver and the **3h**-induced inhibition of glucosyltransferase seen previously (30), indicates that these imino sugars and **3h** target the same synthetic pathway. **3h** may offer improved outcome over Genz-529468, as **3h** targets GlcCer synthase and decreases rather than increases the deleterious storage materials. The concentration of therapeutic efficacy also appears to be better for **3h** (60 mg/kg) than for the imino sugars (10, 38).

We previously showed that the therapeutic efficacy of NB-DNJ in targeting ganglioside storage could be enhanced when administered with a calorie restricted ketogenic diet (38). Although the mechanism remains unclear, the restricted ketogenic diet appears to enhance delivery of NB-DNJ through the blood brain barrier. Further studies will be needed to determine whether the ketogenic diet can enhance delivery of **3h** to the brain. Long term survival studies and behavioral assessments will determine if **3h** (CCG-203586) is a more efficient SRT than are the imino sugars for glycosphingolipid storage diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

GSL	glycosphingolipid
GlcCer	glucosylceramide
LSD	lysosomal storage disease
SD	Sandhoff disease
3h	EtDO-PIP2-oxalate, ethylenedioxy-PIP2-oxalate, CCG-203586, 2-(2,3-dihydro-1H-inden-2-yl)-N-((1R,2R)-1-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1-hydroxy-3-(pyrrolidin-1-yl)propan-2-yl)acetamide oxalate NGNA, N-glycolylneuraminic acid
NB-DNJ	N-butyldeoxynojirimycin
NB-DGJ	N-butyldeoxygalactonojirimycin.

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Figure 1. Ethylenedioxy PIP2 oxalate, an inhibitor of ceramide glucosyltransferase.
The compound was synthesized and reported by Larsen et al. (30).

Figure 2. HPTLC of gangliosides from p-15 Sandhoff mice brain and liver.

Gangliosides from PBS-treated normal mice (+/-), PBS-treated Sandhoff mice (-/-), and Sandhoff mice treated with **3h** at 60 mg/kg/day from p-9 to p-15 (**3h**) were separated in a single 90 min ascending run in CHCl_3 : CH_3OH : dH₂O, 55: 45: 10 (v/v/v) with 0.02% CaCl_2 . The equivalent of 1.5 μg of sialic acid was spotted for each sample. The plate was sprayed with the resorcinol-HCl reagent and then charred at 95°C to visualize the ganglioside bands. A summary of the densitometric analyses of the HPTLC plates is shown in Table 2.

Figure 3. HPTLC of GA2 from p-15 Sandhoff mice brain and liver.

Asialo The HPTCL was developed in a single 90 min ascending run in CHCl₃: CH₃OH: dH₂O, 65: 35: 8 (v/v/v) with 0.02% CaCl₂. The amount of neutral lipid spotted per lane was equivalent to 300 µg tissue dry weight. The equivalent of 4 µg GA2 is shown in the standard (STD) lane. The bands were visualized by spraying the plate with orcinol-H₂SO₄ and charring at 95°C. Other conditions were as described in Figure 2. A summary of the densitometric analyses is shown in Table 3.

Figure 4. HPTLC of neutral lipids from p-15 Sandhoff mice brain and liver.

The HPTLC was developed to a height of 4.5 cm with chloroform: methanol: acetic acid: formic acid: water (35:15:6:2:1; v/v/v/v/v), then run to the top with hexane: diisopropyl ether: acetic acid (65:35:2; v/v/v). The amount of neutral lipid spotted per lane was equivalent to 70 µg tissue dry weight. The bands were visualized by charring with 3% cupric acetate in 8% phosphoric acid solution. Other conditions were as described in Figure 2. CE – Cholesterol Esters; TG – Triglycerides; IS – Internal Standard (Oleyl Alcohol); Chol – Cholesterol; Cer – Ceramide; CB – Cerebrosides (doublet); PE-phosphatidylethanolamine; PC – Phosphatidylcholine; SM – Sphingomyelin.

Figure 5. HPTLC of acidic lipids from p-15 Sandhoff mice brain and liver.

The HPTLC was developed to a height of 6.0 cm with chloroform: methanol: acetic acid: formic acid: water (35:15:6:2:1; v/v/v/v/v), then run to the top with hexane: diisopropyl ether: acetic acid (65:35:2; v/v/v). The amount of acidic lipid spotted per lane was equivalent to 200 µg tissue dry weight. The bands were visualized by charring with 3% cupric acetate in 8% phosphoric acid solution. Other conditions were as described in Figure 2. FA – Fatty Acids; IS – Internal Standard (Oleyl alcohol) CL – Cardiolipin; PA – Phosphatidic Acid; Sulf – Sulfatides (doublet); PS - Phosphatidylserine; PI – Phosphatidylinositol.

Table 1
Body weight, brain water content, and enzymatic activity in p-15 Sandhoff mice treated with **3h**.

<i>Hxb</i> genotype	Treatment	<i>n</i> ^a	Bodyweight (g)	Wet weight		Water content		β -Hexosaminidase specific activity		β -Galactosidase specific activity	
				Cerebrum (mg)	Cerebellum (mg)	Cerebrum (%)	Cerebellum (%)	Cerebrum (nmol/mg protein g/h)	Cerebellum (nmol/mg protein g/h)	Cerebrum (nmol/mg protein g/h)	Cerebellum (nmol/mg protein g/h)
+/-	PBS ^b	4	8.7 $\pm$ 0.2	287.6 $\pm$ 2.5	94.1 $\pm$ 6.6	84.45 $\pm$ 0.01	83.72 $\pm$ 0.01	293.8 $\pm$ 3.5	107.5 $\pm$ 4.4	42.0 $\pm$ 1.6	33.2 $\pm$ 1.7
-/-	PBS ^b	4	7.8 $\pm$ 0.1	264.4 $\pm$ 6.8	102.0 $\pm$ 4.0	84.59 $\pm$ 0.01	84.09 $\pm$ 0.01	3.9 $\pm$ 0.1	3.0 $\pm$ 0.2	62.5 $\pm$ 3.4	61.1 $\pm$ 1.7
-/-	3h ^c	3	9.0 $\pm$ 0.5	271.0 $\pm$ 2.3	87.7 $\pm$ 7.7	84.45 $\pm$ 0.01	83.88 $\pm$ 0.01	4.5 $\pm$ 0.0	2.8 $\pm$ 0.2	69.4 $\pm$ 6.0	54.8 $\pm$ 7.6

Values are expressed as the mean $\pm$ standard error of the mean (SEM)

^a *n*, the number of independent samples per group

^b Mice were injected daily from p-9 to p-15 with 1 $\times$ phosphate buffer saline

^c Mice were injected daily from p-9 to p-15 with **3h** (EtDO-PIP2 oxalate) at 60 mg/kg/day

Table 2

Effect of **3h** on total ganglioside content in cerebrum, cerebellum and liver in p-15 Sandhoff mice

Genotype	Treatment	^a <i>n</i>	Ganglioside sialic acid (mg/100 mg dry weight) ^b
<i>Cerebrum</i>			
+/-	PBS ^c	4	487 ± 11
-/-	PBS ^c	4	500 ± 4
-/-	3h ^d	3	432 ± 11 *
<i>Cerebellum</i>			
+/-	PBS ^c	4	373 ± 6
-/-	PBS ^c	4	391 ± 17
-/-	3h ^d	3	318 ± 15 *
<i>Liver</i>			
+/-	PBS ^c	4	57 ± 5
-/-	PBS ^c	4	113 ± 6
-/-	3h ^d	3	70 ± 5 **

Values are expressed as the mean ± standard error of the mean (SEM).

* Significantly different from the PBS-treated -/- group a $P < 0.05$ using the student's *t* test.

** Significantly different from the PBS-treated -/- group a $P < 0.01$ using the student's *t* test.

^a *n*, the number of independent samples per group.

^b Total sialic acid content was determined by the resorcinol assay.

^c Mice were injected daily from p-9 to p-15 with 1 × phosphate buffered saline.

^d Mice were injected daily from p-9 to p-15 with **3h** (EtDO-PIP2 oxalate) at 60 mg/kg/day.

Table 3
Effect of **3h** on cerebrum, cerebellum, and liver ganglioside distribution in p-15 Sandhoff mice.

<i>Hexb</i> Genotype	Treatment	<i>b</i> <i>n</i>	Concentration (µg sialic acid/100 mg dry weight) ^a							
			GM2		GM1		GD3		GD1a	
			Percent	Concentration	Percent	Concentration	Percent	Concentration	Percent	Concentration
<i>Cerebrum</i>										
+/-	PBS ^c	4	ND	ND	7.9 ± 0.1	38.6 ± 1.3	ND	ND	44.5 ± 0.5	217.0 ± 5.6
-/-	PBS ^c	4	5.1 ± 0.3	25.6 ± 1.4	7.0 ± 0.4	34.8 ± 1.5	ND	ND	43.0 ± 0.4	215.1 ± 3.1
-/-	3h ^d	3	2.8 ± 0.1 [*]	12.3 ± 0.3 ^{**}	6.1 ± 0.2	26.4 ± 1.6 [*]	ND	ND	43.4 ± 0.3	187.4 ± 5.2 [*]
<i>Cerebellum</i>										
+/-	PBS ^c	4	ND	ND	6.5 ± 0.3	24.2 ± 1.4	5.3 ± 0.5	19.8 ± 1.6	22.8 ± 0.7	84.9 ± 3.6
-/-	PBS ^c	4	5.3 ± 0.2	20.8 ± 1.6	5.8 ± 0.2	22.9 ± 1.9	4.9 ± 0.3	19.2 ± 1.1	22.7 ± 0.2	88.7 ± 4.5
-/-	3h ^d	3	3.1 ± 0.1 [*]	9.8 ± 1.2 ^{**}	5.5 ± 0.4	17.4 ± 1.4	4.3 ± 0.2	13.6 ± 0.7 ^{**}	24.1 ± 0.3 [*]	76.6 ± 3.7
<i>Liver</i>										
+/-	PBS ^c	4	59.5 ± 1.4	33.6 ± 2.5	25.3 ± 0.7	14.5 ± 1.6	ND	ND	15.2 ± 1.2	8.6 ± 1.1
-/-	PBS ^c	4	68.7 ± 2.8	77.1 ± 4.8	18.0 ± 1.3	20.3 ± 1.9	ND	ND	13.3 ± 1.6	15.1 ± 2.2
-/-	3h ^d	3	60.5 ± 1.3	42.1 ± 2.6 ^{**}	21.9 ± 1.5	15.4 ± 2.1	ND	ND	17.6 ± 1.1	12.2 ± 0.3
<i>Hexb</i> Genotype										
			Concentration (µg sialic acid/100 mg dry weight) ^a		GD1b		GT1b		GQ1b	
			GT1a/LD1	Percent	Concentration	Percent	Concentration	Percent	Concentration	Percent
<i>Cerebrum</i>										
+/-	PBS ^c	4	4.0 ± 0.4	19.4 ± 1.4	9.6 ± 0.2	46.9 ± 1.3	27.0 ± 0.3	137.7 ± 4.3	6.9 ± 0.1	33.8 ± 0.9
-/-	PBS ^c	4	3.8 ± 0.1	18.9 ± 0.2	8.4 ± 0.1	42.2 ± 0.9	26.2 ± 0.5	131.3 ± 3.2	6.5 ± 0.1	32.4 ± 0.5
-/-	3h ^d	3	4.8 ± 0.3	20.8 ± 0.7 [*]	7.3 ± 0.1 [*]	31.7 ± 0.5 ^{**}	27.8 ± 0.1 [*]	119.8 ± 3.3	7.7 ± 0.1 [*]	33.3 ± 0.7
<i>Cerebellum</i>										
+/-	PBS ^c	4	5.6 ± 0.4	21.0 ± 1.7	17.1 ± 0.4	63.7 ± 2.3	30.8 ± 1.0	114.6 ± 3.5	12.0 ± 0.6	44.7 ± 2.1

<i>Hexb</i> Genotype	Treatment	^b <i>n</i>	Concentration (µg sialic acid/100 mg dry weight) ^a	GM2		GM1		GD3		GD1a	
			Percent	Concentration	Percent	Concentration	Percent	Concentration	Percent	Concentration	Percent
−/−	PBS ^c	4	6.5 ± 0.1	25.3 ± 1.2	15.2 ± 0.2	59.3 ± 2.9	29.1 ± 0.1	113.7 ± 5.3	10.6 ± 0.5	41.1 ± 1.1	
−/−	3h ^d	3	7.2 ± 0.1 [*]	22.9 ± 1.0	13.2 ± 0.1 [*]	42.1 ± 2.0 ^{**}	29.7 ± 0.8	94.4 ± 3.9 [*]	12.9 ± 0.1	41.3 ± 3.8	
<i>Liver</i>											
+/-	PBS ^c	4	ND	ND	ND	ND	ND	ND	ND	ND	ND
−/−	PBS ^c	4	ND	ND	ND	ND	ND	ND	ND	ND	ND
−/−	3h ^d	3	ND	ND	ND	ND	ND	ND	ND	ND	ND

Values are expressed as the mean ± standard error of the mean (SEM).

^{*} Significantly different from the PBS-treated −/− group at $P < 0.05$ using the student's *t* test.

^{**} Significantly different from the PBS-treated −/− group at $P < 0.01$ using the student's *t* test. ND, not detectable; **3h**, ethylenedioxy-PIP2 oxalate.

^a Percent distribution and concentration of individual gangliosides were determined by densitometric scanning of HPTLC plates.

^b *n*, the number of independent samples per group.

^c Mice were injected daily from p-9 to p-15 with 1 × phosphate buffered saline.

^d Mice were injected daily from p-9 to p-15 with 3h (EtDO-PIP2 oxalate) at 60 mg/kg/day.

Table 4

Neutral and acidic lipid content in p-15 Sandhoff mice treated with 3h.

	Cerebrum		Cerebellum		Liver	
	+/-	-/-	+/-	-/-	+/-	-/-
Neutral lipid ^a						
TG	ND	ND	ND	ND	1.34 ± 0.28	0.06 ± 0.03
Choi	5.35 ± 0.09	5.22 ± 0.11	5.13 ± 0.11	4.00 ± 0.28	1.43 ± 0.33	1.37 ± 0.04
Cer	0.59 ± 0.01	0.56 ± 0.02	0.59 ± 0.05	2.23 ± 0.49	1.54 ± 0.29	4.49 ± 0.08
CB	Trace	Trace	Trace	2.11 ± 0.08	1.61 ± 0.04	ND
PE	9.95 ± 0.35	9.77 ± 0.65	9.11 ± 0.58	3.34 ± 0.63	3.12 ± 0.94	5.22 ± 0.16
PC	5.55 ± 0.33	5.91 ± 0.74	5.02 ± 0.91	3.70 ± 0.30	3.68 ± 0.34	2.27 ± 0.09
SM	0.94 ± 0.07	0.87 ± 0.07	0.83 ± 0.09	0.34 ± 0.03	0.43 ± 0.04	1.20 ± 0.02
GA2	ND	0.70 ± 0.03	0.51 ± 0.01 ^{**}	ND	0.55 ± 0.01 [*]	1.31 ± 0.07
Acidic lipids						
CL	1.63 ± 0.03	1.70 ± 0.07	1.62 ± 0.11	4.45 ± 0.15	3.63 ± 0.24	2.02 ± 0.26
Sulf	0.50 ± 0.02	0.38 ± 0.02	0.44 ± 0.06	2.33 ± 0.29	1.97 ± 0.19	1.81 ± 0.56
PS	4.21 ± 0.22	4.38 ± 0.30	4.08 ± 0.14	5.91 ± 0.16	5.37 ± 0.35	0.90 ± 0.05
PI	2.20 ± 0.06	2.22 ± 0.12	2.14 ± 0.12	1.75 ± 0.04	1.57 ± 0.11	2.46 ± 0.12

Values are expressed as mg/100 mg dry weight. N= 3–4 mice per group.

^{*} Significantly different from the PBS-treated -/- group at $P < 0.05$ using the student's *t*-test.

^{**} Significantly different from the PBS-treated -/- group at $P < 0.01$ using the student's *t*-test. ND, not detectable; **3h**, EtDO-PIP2. Lipid abbreviations are described in Figs. 4 and 5.

^a Concentrations of individual lipids were determined by densitometric scanning of HPTLC plates.