

**Neuraminidases 3 and 4 direct neuronal development and function by reshaping the composition of brain gangliosides**

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By regulating recognition, signaling and physiology of neurons, gangliosides are essential to CNS function. The composition of gangliosides in the brain changes during development and aging being regulated by neuraminidases presumably, which cleave their terminal sialic acid residues. Two mammalian neuraminidases, Neu3 and Neu4 are major candidates for processing brain gangliosides, however, the corresponding knockout mice showed no changes in brain ganglioside metabolism or deficits in brain function.

To elucidate the physiological functions of Neu4 and Neu3 in the brain, we generated a gene-targeted mouse strain devoid of both enzymes. At the age of 10 months the mice showed deficits in spatial and short-term memory, whereas neuraminidase activity against gangliosides in their brain tissues was below detection levels.  $G_{M3}$  ganglioside was stored in lysosomes of neurons, microglia and vascular pericytes and  $G_{M1}$ -ganglioside decreased in the axons. The number of astrocytes and activated microglia was increased, consistent with neuroinflammation. Cultured hippocampal and cortical neurons also showed reduced levels of  $G_{M1}$  ganglioside in the axons and impaired neuritogenesis.

Our data provide evidence that Neu3 and Neu4 have unique roles in the regulation of brain ganglioside composition essential for CNS function: Neu4 generates the neuronal  $G_{M1}$  necessary for axon growth whereas Neu3 catabolizes  $G_{M3}$ .

## Introduction

Gangliosides are members of the larger glycosphingolipid family and consist of sialylated glycans linked to a ceramide lipid backbone. Their levels are approximately 10-fold higher in the brain as compared to other organs. Gangliosides are further enriched in the membranes of neurons usually as a part of distinct lipid patches or rafts, suggesting their important role in the development and function of the mammalian brain.

Numerous important functions have been attributed to gangliosides at the cell surface reviewed in<sup>1</sup>. In the *cis* mode, they associate laterally with membrane proteins, including receptors and ion channels, to modulate their activities. In the *trans* mode, ganglioside glycans, extending into the extracellular space, interact with glycan-binding proteins to mediate cell-protein and cell-cell interactions. Together in both *cis* and *trans* modes gangliosides carry out a number of functions essential for the central nervous system including myelination, neuritogenesis, synaptic plasticity, and transmission of nervous impulses reviewed in<sup>2</sup>. By modulating ion channel function and receptor signaling, gangliosides play an important role in neuronal excitability and synaptic transmission reviewed in<sup>3</sup>.

Ganglioside expression patterns in the brain are cell and organelle-specific, dynamic and are generated and maintained by highly coordinated processes of their biosynthesis, trafficking, processing and catabolism. General ganglioside content and composition of the mammalian brain also constantly changes over an individual lifetime. Early in development (such as E14 in the rat), the mammalian brain is dominated by G<sub>M3</sub> and G<sub>D3</sub><sup>4, 5</sup>. By the time of birth, G<sub>M3</sub> and G<sub>D3</sub> become minor components of the brain,

with G<sub>D1a</sub> and G<sub>T1b</sub> taking their place as the major sialoglycans. Finally, as the brain matures further to adulthood, G<sub>M1</sub> and G<sub>D1b</sub> content increases until the four species, G<sub>M1a</sub>, G<sub>D1a</sub>, G<sub>D1b</sub>, and G<sub>T1b</sub> are present at comparable levels together representing ~97% of brain gangliosides<sup>6</sup>.

Until now, it has been assumed that brain ganglioside composition is mainly regulated by changes in the rate of their biosynthesis in the Golgi apparatus by sialyltransferases that catalyze the transfer of Sia from CMP-Sia to an acceptor carbohydrate reviewed in<sup>2</sup>. Genetic deficiencies of individual sialyltransferases in human patients or in gene-targeted mouse models resulted in both absence of individual gangliosides and in severe neurological manifestations reviewed in<sup>2</sup>. However, transcriptional regulation of the sialyltransferase gene network alone cannot explain how the composition of brain gangliosides changes during development. Mice deficient in individual sialyltransferases lack the whole series of gangliosides: G<sub>D3</sub> and G<sub>M3</sub> in St8sia1 KO mice, G<sub>M1a</sub>, G<sub>D1a/b</sub> and G<sub>T1b</sub> gangliosides in B4galnt1 KO mice or G<sub>D1a</sub> and G<sub>T1b</sub> gangliosides in St3gal2/3-double KO mice<sup>7, 8</sup>. At the same time, no deficiency of any single sialyltransferase can specifically block production of G<sub>M1</sub> or G<sub>M3</sub> gangliosides in the mouse brain, suggesting that they are rather produced from more complex gangliosides by the action of neuraminidase(s) trimming the terminal Sia on the glycan chains.

Mammalians have 4 neuraminidases: Neu1, Neu2, Neu3 and Neu4, reviewed in<sup>9-11</sup>. Neu3 and Neu4 residing in lysosomal, endosomal and plasma membranes are dominantly expressed in the brain and are *in vitro*, primarily active against gangliosides reviewed in<sup>9</sup>. Neu3 and Neu4 have been previously implicated in neuronal differentiation, neuritogenesis, and axonal growth by *in vitro* and *in cellulo*

experiments<sup>12-14</sup>. However, no evidence of this has been obtained *in vivo*. Neu3 KO mice do not show signs of impaired brain function or brain ganglioside composition<sup>15</sup>. Neu4 KO mice have increased G<sub>D1a</sub> and decreased G<sub>M1</sub> ganglioside levels in the brain, but show normal behaviour and memory<sup>16</sup>. Considering the similar substrate specificity of Neu3 and Neu4<sup>17</sup> it is possible that the enzymes partially compensate each other's deficiency, which would explain the lack of neurological phenotypes in single KO models.

Here, we describe mice with a double deficiency of both ganglioside neuraminidases and demonstrate that they have a slowly-progressing lysosomal neurological disease causing gradual decline of short-term and spatial memory.

## RESULTS

### ***Neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice show progressive learning impairment.**

Mice with a combined deficiency of Neu3 and Neu4 were obtained by intercrossing of previously described *neu3<sup>-/-</sup>* and *neu4<sup>-/-</sup>* KO mouse lines, both in C57BL/6NCrl genetic backgrounds. Double heterozygous mice were crossed to obtain double homozygous *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* progeny, and their genotypes were confirmed by PCR (Figure S1 A). *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice were viable and born in the frequency expected from Mendelian inheritance (~12%) indicating that combined deficiency of both enzymes did not cause embryonic lethality.

*neu1* KO mouse was generated by microinjection in C57BL/6J blastocytes of the ES cells with targeted disruption of the Neu1 gene generated by the EUCOMM consortia. The targeted *neu1* allele contains *LacZ/BactPNeo* cassette flanked with FRT sites inserted into the intron 2 of the mouse *neu1* gene (Figure S1 B). This results in the expression of a fusion protein containing the mouse Neu1 amino acid sequence encoded by the exons 1-2 followed by bacterial  $\beta$ -galactosidase encoded by the *LacZ* gene under the control of the endogenous *neu1* promoter (Figure S1 B). Correct targeting event in the mice was confirmed by specific PCR that yielded expected band patterns for WT (*neu1<sup>+/+</sup>*), heterozygous (*neu1<sup>+/-</sup>*) or homozygous (*neu1<sup>-/-</sup>*) mice (Figure S1 C).

*neu1* mRNA levels in kidney (Figure S1 D) or brain (Figure S2) tissues of *neu1<sup>-/-</sup>* mice measured by RT-q-PCR were below detection limit. Levels of the *neu3* and *neu4* mRNA were not changed in the brain tissues of *neu1<sup>-/-</sup>* mice but significantly increased in the brain tissues of *neu4<sup>-/-</sup>* and *neu3<sup>-/-</sup>* single KO, respectively, as compared with those in

WT mice (Figure S2), suggesting the existence of mechanisms for compensatory regulation between these two genes.

Homozygous *neu1*<sup>-/-</sup> mice were viable and undistinguishable from WT and heterozygous littermates at birth, but showed slower development and at 2-3 month of age had a ~30% lower body weight than their heterozygous or WT littermates, resembling the previously described phenotype<sup>18</sup>.

Homozygous *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice showed normal growth, appearance and general behaviour indistinguishable from that of WT or single KO animals and could be bred to produce knockout litters. No visible signs of illness or decreased life span were observed while mice aged. Neurological assessment (gait, posture, avoidance response, righting reflex, horizontal bar test, inverted wire screen test) conducted on the group of ten *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> and ten WT mice every 2 months did not reveal any signs of neuromuscular pathology (data not shown).

However, progressive decline of memory and spatial learning capability in *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice was revealed by the spontaneous alternation in the Y-maze test. Learning progress of mutant mice at the age of 3 months was similar to that of the WT group (data not shown). In contrast, at the age of 6 months, Neu3/Neu4 double-deficient mice showed significantly lower alteration rate in Y-maze as compared to their WT counterparts (Fig. 1A), suggesting impaired spatial learning. At the age of 10 months the difference in the alternation rate between the WT and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice further increased (Fig. 1B) while *neu3*<sup>-/-</sup> and *neu4*<sup>-/-</sup> single KO still did not show a significant difference with the control. At 10 months *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice also displayed a deficit in

short-term memory measured as an ability to discriminate between a familiar and a novel object in a novel object recognition test (Fig. 1C).

**Neu3 and Neu4 are responsible for the majority of ganglioside sialidase activity in the mouse brain.**

Acidic neuraminidase activity in the mouse brain tissues was first assayed using a synthetic pan-neuraminidase 4MU-NaNa substrate. Residual activity was reduced to ~80% of the WT level in the brain tissues of the *neu1*<sup>-/-</sup> mice, to ~40% in the *neu3*<sup>-/-</sup> and the *neu4*<sup>-/-</sup> mice, and to ~20% in the *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mouse (Fig. 2A), reflecting the input of the three neuraminidases into the net brain acidic neuraminidase activity against 4MU-NaNa. Importantly, similarly to the previously described *neu1* KO strain (de Geest 2002) *neu1*<sup>-/-</sup> mice had almost complete deficiency of 4MU-NaNa neuraminidase activity in kidney, where Neu1 is the predominant neuraminidase (Figure S1E).

In contrast, the neuraminidase activity measured against purified porcine brain gangliosides (mainly GT<sub>1</sub>, GD<sub>1a</sub>, GD<sub>1b</sub> and GM<sub>1a</sub> gangliosides) was similar to the WT level in the brain tissues of *neu1*<sup>-/-</sup> mice, reduced by ~30% in the brain tissues of both *neu3*<sup>-/-</sup> and *neu4*<sup>-/-</sup> mice, and undetectable in brain tissues of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice (Fig. 2B). Neuraminidase activity measured against purified GM<sub>3</sub> ganglioside in the tissues of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice was also below the detection level (Fig. 2C). Altogether these data suggest that Neu3 and Neu4 are equally responsible for neuraminidase activity against gangliosides in the mouse brain without any significant contribution from Neu1.

**Progressive reduction of GM<sub>1</sub> and accumulation of GM<sub>3</sub> ganglioside in the brain of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice.**



The amount of protein-bound, lipid bound and total Sia measured by HPLC did not differ between WT and *neu3*<sup>-/-</sup> or *neu4*<sup>-/-</sup> mice, but was significantly increased in the tissues of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice (Fig. S3) indicating that a deficiency of neuraminidase activity in the brain of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice alters both protein and ganglioside sialome. The ganglioside composition of the brain was further analyzed by thin-layer chromatography (TLC) of total gangliosides extracted from the brain hemispheres of 2 and 10 month-old *neu3*<sup>-/-</sup>, *neu4*<sup>-/-</sup>, *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> and WT mice and 2 month-old *neu1*<sup>-/-</sup> mice (Fig. 3A,B). The ganglioside brain composition of *neu3*<sup>-/-</sup> mice at both ages as well as of 2 months-old *neu1*<sup>-/-</sup> mice did not differ from that of WT ones. In contrast, *neu4*<sup>-/-</sup> and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice showed ~2 fold decrease of G<sub>M1</sub> ganglioside at both ages. In addition, *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> animals showed progressive increase of G<sub>M3</sub> ganglioside, consistent with observed deficiency of G<sub>M3</sub> neuraminidase activity in the brain tissues (Fig. 3A,B).

The results of TLC experiments were further confirmed by HPLC analysis of derivatized gangliosides (Fig. 3C,D). The quantification of chromatograms demonstrated that difference in the brain ganglioside composition between the *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> and WT mice increased with mice age. At the age of 12 months G<sub>M3</sub> was increased 5 fold as compared with WT mice (p<0.001 in two-way ANOVA), G<sub>M1</sub> ganglioside decreased 2.3 fold (p<0.01) and G<sub>D1a</sub> ganglioside increased 1.4 fold (p<0.01). Mice with single Neu4 deficiency also showed similarly reduced G<sub>M1</sub> and increased G<sub>D1a</sub> levels. At the same time, G<sub>M3</sub> levels in the brains of *neu3*<sup>-/-</sup> and *neu4*<sup>-/-</sup> mice were similar to those in WT mice, confirming that both enzymes can convert this ganglioside, thus compensating each

other's deficiency. No significant changes in ganglioside profiles were detected in the brain tissues of *neu1*<sup>-/-</sup> mice.

Areas with accumulation of G<sub>M3</sub> ganglioside and decreased levels of G<sub>M1a</sub> ganglioside in brain tissues were studied by immunohistochemistry using monoclonal antibodies. G<sub>M3</sub> ganglioside staining showed a clear increase in double-KO *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice. G<sub>M3</sub> ganglioside storage was observed in most areas of the brain, including the CA1 level of hippocampus, but was particularly prominent in deep layers of the ventral cortex and along the vasculature (Fig. 4A). Dual-labelling studies showed that G<sub>M3</sub>-accumulating cells scattered along the cortex and hippocampus were different from NeuN-stained neurons and GFAP-stained astrocytes (Fig. S4), but were recognized by the isolectin B4, indicating that they represent microglia (Fig. 4B), while those associated with microvasculature were positive for the pericytes marker, NG2 (Fig. 4C). In both microglia and pericytes, G<sub>M3</sub> co-localized with the lysosomal marker LAMP2 showing that the ganglioside was stored in the endolysosomal compartment (Fig. 4D). Cholera toxin staining specific for G<sub>M1a</sub> ganglioside was reduced in myelinated axons of hippocampal and cortical neurons of Neu3/Neu4 double deficient mice as compared with those from the WT animals (Fig. 4E).

To verify if, in addition to G<sub>M3</sub>, Neu3 also desialylates G<sub>M2</sub> ganglioside *in vivo*, we crossed *neu3*<sup>-/-</sup> mice with those deficient in  $\beta$ -hexosaminidase A (*hexa*<sup>-/-</sup>).  $\beta$ -Hexosaminidase A catabolizes G<sub>M2</sub> ganglioside by converting it to G<sub>M3</sub> and the genetic deficiency of this enzyme causes G<sub>M2</sub> accumulation in Tay-Sachs disease. *hexa*<sup>-/-</sup> mice however, remain asymptomatic to 1 year of age, because G<sub>M2</sub> ganglioside is desialylated by a ganglioside neuraminidase into glycolipid G<sub>A2</sub> which is further processed by  $\beta$ -

hexosaminidase B to lactosyl-ceramide, thereby bypassing the  $\beta$ -hexosaminidase A defect<sup>23</sup>. Since this bypass is not effective in humans, infantile Tay-Sachs disease is fatal in the first years of life. The TLC analysis of the brain tissues of 2 month-old *hexa*<sup>-/-</sup> and double-knockout *hexa*<sup>-/-</sup>;*neu3*<sup>-/-</sup> mice demonstrated that Neu3 deficiency increases GM<sub>2</sub> accumulation at least 5 fold (Fig. S5), suggesting that Neu3 is the neuraminidase catabolizing GM<sub>2</sub> and responsible for the metabolic bypass in *hexa*<sup>-/-</sup> mice.

**Cultured hippocampal neurones from *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice show accumulation of GM<sub>3</sub> ganglioside, reduction of GM<sub>1</sub> ganglioside and severely impaired neurite outgrowth.**

To analyze if changes in ganglioside levels observed *in vivo* occur in cultured neurons, interfering with their growth and differentiation, hippocampal cultures were established from both WT and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mouse embryos at embryonic day 16. The cells were differentiated in culture for 21 days<sup>19</sup>. We first tested whether the hippocampal neurons in culture have increased level of GM<sub>3</sub> ganglioside. Staining of cultured cells from WT, *neu3*<sup>-/-</sup>, *neu4*<sup>-/-</sup> and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice with antibodies against LAMP2 and the neuronal marker Map2, detected an expanded lysosomal system in neurons, compatible with lysosomal storage (Fig. 5A). Further staining with antibodies against GM<sub>3</sub> ganglioside and neuronal marker NeuN (Fig. 5B) revealed that there is indeed an accumulation of GM<sub>3</sub> ganglioside in the hippocampal neurons from *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice. In contrast, GM<sub>1</sub> ganglioside, mainly associated with patches on the plasma membrane, was significantly reduced in both soma and neurites of the hippocampal cultured neurones from *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice (Fig. 5C).

Surprisingly, Map2 staining specific for neuronal dendrites revealed that dendrites were shorter and more scarce in neurons from *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice, showing on average a 25% reduction in dendrite length ( $p < 0.001$  in t-test) (Fig. 5D). Since these data suggested a potential defect in neuritogenesis we have compared the axon length of 2-day cultures of embryonic cortical neurons dissected from WT and the *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice. We found that axons (identified as the longest neurite in each neuron with a distinctive growing tip) were significantly shorter in the cultures of *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* as compared with WT mice (Fig. 5E). They also had lower amounts of G<sub>M1</sub> in the growing tip as determined by cholera toxin staining (Fig. 5F). Finally MAP-2 staining of the brain slices revealed reduced density of neuronal dendrites in the A1 level hippocampus (Fig. 5G) and other brain areas (Fig. S6) of *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice.

### Brain pathology

Brain cortex and hippocampus from WT, *neu3<sup>-/-</sup>*, *neu4<sup>-/-</sup>* and *neu3<sup>-/-</sup>; neu4<sup>-/-</sup>* mice were examined in 10-12 months-old animals, i.e. at the age when they demonstrate significant memory deficits. Microscopic examination at low magnification was performed on semi-thin (40  $\mu$ m) coronal and sagittal brain sections. The architecture of cortical and hippocampal neurons in the single and double KO as well as in the WT mice, showed a normal appearance (data not shown). However, immunochemical staining for NeuN, ILB4 and GFAP demonstrated that double KO mice had an increased number of astrocytes and activated microglia in all studied brain areas (Fig. 6A), consistent with brain inflammation such as observed in the mouse models of neurological lysosomal diseases<sup>20</sup>. Besides, brain tissues from double deficient mice had increased expression levels of inflammatory cytokines TNF-alpha and MIP-1-alpha also consistent with

activation of microglia (Fig. 6B). As described above, ILB4-positive microglia and microvasculature-associated cells (presumably pericytes) had increased storage of G<sub>M3</sub> ganglioside. To confirm that microglia and pericytes had a lysosomal storage phenotype, we performed a light microscopy examination of the brain cortex and hippocampus from single KO, double KO *neu3<sup>-/-</sup>,neu4<sup>-/-</sup>* mice and WT littermates carried out in Bouin's fixed sections stained with anti-lysosomal prosaposin (PSAP) polyclonal antibodies<sup>21</sup> which are highly specific for the lysosomes in professional phagocytes<sup>22</sup>. In the hippocampus of *neu3<sup>-/-</sup>* and *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice the PSAP antibody revealed foam microglial cells containing vacuoles typical of lysosomal storage (Fig.7A). This antibody also stained foam cells in the adventitial layer of vascular capillaries and post-capillary venules, most likely pericytes with storage materials (Fig. 7B). Both types of cells were absent in the hippocampus of *neu4<sup>-/-</sup>* and WT mice (Fig. 7A). On the other hand, no differences were observed in PSAP staining between cortical and hippocampal neurons from any KO and WT mice (Fig. 7A).

Further examination by transmission electron microscopy confirmed that, in mice with Neu3, Neu4 and Neu3/4 deficiency, the majority of cortical and hippocampal neurons were morphologically normal. However, a small number of cortical and hippocampal neurons in the Neu3, Neu4 and Neu3/4 KO mice had a few abnormal inclusions within the axons and cell bodies (Fig. 8A-D). The inclusions were membrane-bound structures containing whorls of membranes, typical of lysosomes with sphingolipid storage disorders<sup>23</sup>. The number of affected neurons was lower in the brain of *neu4<sup>-/-</sup>* mice ( $0.37 \pm 0.17$  SEM per frame) and higher in the brains of the *neu3<sup>-/-</sup>* ( $2.0 \pm$

0.28 SEM per frame) and *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice ( $2.46 \pm 0.29$  per frame). These inclusions were not found in either cortical or hippocampal neurons of WT brains.

Lipofuscin bodies, considered to be aged lysosomes and described as autofluorescent spherical or irregular structures of variable sizes with a homogenous electron dense content accompanied by the presence of small lipid droplets were present in the neuronal cell bodies of both neuraminidase-deficient and WT mice (Fig. 9A-D). However, in *neu3<sup>-/-</sup>* and *neu3<sup>-/-</sup>; neu4<sup>-/-</sup>* mice (Fig. 9C,D), lipofuscin bodies were heterogeneous in electron density, and contained multivesicular structures not observed in the lipofuscin bodies of the WT neurons or those from *neu4<sup>-/-</sup>* mice (Fig. 9A,B). This finding was consistent with increased size and number of autofluorescent inclusions in the brain sections of *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice (Fig. S7).

The most striking abnormality, observed in the brains of *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice at the ultrastructural level, was the presence of pericytes with lysosomal storage in the adventitia of brain capillaries and postcapillary venules. The affected cells were firmly attached to the surface of the venules and contained a plethora of lysosomal storage characterized by multivesicular bodies exhibiting a high level of electron density (Fig. 9 E-G). We have also identified microglial cells with lysosomal storage in Epon-embedded hippocampal sections of Neu3/4 deficient mice, both at the LM and EM levels (Figure 9H,K). At the EM level, vacuoles in the microglia resembled the abnormal lysosomes observed in pericytes, and contained multivesicular bodies surrounded by material with high electron density (Figure 9K).

## Discussion

The current study provides evidence that deficiency of ganglioside neuraminidases, Neu3 and Neu4 in mice causes lysosomal accumulation of G<sub>M3</sub> ganglioside, impairs production of G<sub>M1a</sub> ganglioside and leads to defects in neuritogenesis, neuronal death and memory loss. Hippocampal and cortical neurons from both Neu4 KO and double Neu3/Neu4 KO mice have low levels of G<sub>M1a</sub> ganglioside, suggesting that it is produced from G<sub>D1a</sub> by the action of Neu4. Besides, Neu4 deficiency severely impairs neuritogenesis in both types of cells. Previous data demonstrated that G<sub>M1a</sub> plays crucial role in neurite outgrowth by binding to laminin-1 and inducing its focal aggregation, enhancing the relocation of TrkA in lipid rafts and the subsequent activation of downstream signaling molecules including Lyn. Clustering of G<sub>M1a</sub> with laminin-1, along with laminin-1 self-assembly also promotes relocation and enrichment of  $\beta$ 1-integrin in lipid rafts and enhances combined laminin-integrin signaling to trigger neurite outgrowth<sup>24</sup>. Our current data show that, during neurite outgrowth, G<sub>M1a</sub> ganglioside is generated from G<sub>D1a</sub> directly at the growing tip by the action of Neu4. Importantly, Neu4 expression is undetectable at the embryonic stage, but rapidly increases at 3–14 days after birth. Its mRNA is present in a specific population of neurons scattered throughout the brain with a greater density in hippocampus and in the ventral cortex<sup>16, 23</sup>. Although a subject of experimental verification, it is tempting to speculate that these cells are neuroblasts that require generation of G<sub>M1a</sub> for axon and dendrite outgrowth. Due to a high expression of Neu4 during these first weeks of neonatal development, the level of G<sub>D1a</sub> ganglioside decreases and G<sub>M1a</sub> ganglioside increases until they reach equal levels characteristic for the adult brain.

Immunocytochemistry of brain slices revealed accumulation of G<sub>M3</sub> only in the brains of mice double deficient in Neu3 and Neu4 and only in the lysosomes of the brain's phagocytic cells, microglia and pericytes which gain a foamy appearance and contain multivesicular bodies typical of lysosomal ganglioside storage disorders such as G<sub>M1</sub> and G<sub>M2</sub> gangliosidoses<sup>23, 25, 26</sup>. This phenotype is different from the secondary storage of gangliosides occurring in many lysosomal disorders, mostly in neurons and mainly in the vesicles free of the lysosomal markers<sup>20</sup>. We speculate that the accumulation of G<sub>M3</sub> ganglioside in pericytes and microglia arises due to the high phagocytic activity of these cells causing an extracellular substrate burden. We recently identified vascular pericytes as one of the sites for lysosomal storage of GAGs in the MPS IIIC mouse model<sup>20</sup>, but to our knowledge the current report provides the first description of the lysosomal accumulation of gangliosides in pericytes. Ganglioside storage is most likely responsible for activation of microglia cells, secretion of cytokines, brain inflammation, micro/astrogliosis detected in *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice and eventually triggers neurodegeneration also observed in other neurological lysosomal disorders<sup>20</sup>.

Further analysis of cultured neurons from *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice have shown that they also accumulated G<sub>M3</sub> ganglioside. In addition, the EM analysis of mouse brain sections have demonstrated that a fraction of cortical neurons in *neu3*<sup>-/-</sup> and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice as well as hippocampal neurons in *neu3*<sup>-/-</sup>; *neu4*<sup>-/-</sup> mice had abnormal lysosomal structures containing whorls of membranes both in the neuronal bodies and axons consistent with ganglioside accumulation. Neurons of the *neu3*<sup>-/-</sup> and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice also contained heterogeneous lipofuscin bodies comprising multivesicular structures not observed in the WT neurons. In the aging human brain lipofuscin structures composed of



tightly packed protein and lipoprotein aggregates are the result of incomplete lysosomal degradation of damaged organelles reviewed<sup>27</sup>. However, a growing body of evidence also suggests that lipofuscin is not benign since it may impair the function of other cellular systems, including the ubiquitin/proteasome pathway<sup>27</sup>. In particular, lipofuscin bodies are observed at the late disease stage in the brains of humans and mice affected with neuropathic lysosomal diseases and are attributed to progressive mitochondrial damage and autophagy block<sup>20</sup>. We show that lipofuscin bodies are also triggered by the accumulation of gangliosides and co-appear with (and most probably contribute to) memory deficits observed in the late-age *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice.

Our data do not provide evidence for the participation of the ubiquitously expressed lysosomal neuraminidase Neu1 in the conversion of brain gangliosides. Neu1 has much lower *in vitro* activity against gangliosides as compared with Neu3 and Neu4, but its low specific activity could be compensated by 10–20 time higher expression level<sup>28</sup>. The genetic deficiency of Neu1 in human sialidosis patients and KO mice causes major block in the catabolism of sialylated glycoproteins and oligosaccharides reviewed in<sup>29</sup>, but the brain gangliosides in the Neu1 KO mouse were never analyzed and increased ganglioside levels in brain autopsies reported for human sialidosis patients could be caused by the secondary storage of gangliosides. Our current data show that Neu1 deficiency in the mouse brain does not alter composition of gangliosides at least until the age of 2 months, but we acknowledge that at a later age, lysosomal storage of sialylated peptides and oligosaccharides can possibly cause an autophagy block and accumulation of the secondary storage materials, including gangliosides.

Taken together, our results suggest a novel concept for the biogenesis of mammalian brain gangliosides and provide the first *in vivo* evidence that ganglioside composition is modulated by the interplay of sialyltransferases and neuraminidases in response to external or internal stimuli. The sialyltransferases generate essential (mainly complex) gangliosides in early Golgi, while neuraminidases **modulate** gangliosides at the required time/site by “trimming” Sia residues. It is also possible that some of gangliosides at the cell surface can undergo rapid resialylation by plasma membrane associated sialyltransferases as occurs for the glycan chains of glycoproteins<sup>30</sup>. In addition, our data identify lysosomes of microglia and pericytes as the major places for ganglioside catabolism in the brain, with Neu3 and Neu4 playing partially redundant roles in desialylation of G<sub>M3</sub> ganglioside and Neu3 playing preferential role in desialylation of G<sub>M2</sub> ganglioside stored in the mouse model of Tay-Sachs disease.

## METHODS

**Animals.** Mice with targeted disruption of the *neu3* (*neu3*<sup>-/-</sup>) and *neu4* (*neu4*<sup>-/-</sup>) genes, all in C57BL/6NCrl genetic background have been previously described<sup>16</sup>. Mice with a combined deficiency of Neu4 and Neu3 were obtained by intercrossing *neu4* and *neu3* knockout (KO) mouse strains. Doubly homozygous *neu4*<sup>-/-</sup>;*neu3*<sup>-/-</sup> progeny were viable and their genotypes were confirmed by PCR of tail DNA. The absence of *Neu4* transcripts in total mRNA extracted from the brain of *neu4*<sup>-/-</sup>;*neu3*<sup>-/-</sup> mice was confirmed by RT-PCR (Figure S1). Mice were housed in an enriched environment with continuous access to food and water, under constant temperature and humidity, on a 12 h light:dark cycle. Approval for the animal care and the use in the experiments was granted by the Animal Care and Use Committee of the Ste-Justine Hospital Research Center.

**Neurological and behavioural examination of mice.** The motor performance of mice was evaluated using a simplified neurological examination as previously described<sup>31</sup>.

The 3, 6, and 9-month-old mice (ten per age per genotype) were subjected to the spontaneous alternation in the Y-maze test performed as previously described (Yadav et al., 2013). A custom-made Y-maze with three identical white Plexiglas arms (40 × 10 × 20 cm, 120° apart) was placed at the centre of a room under dim lighting conditions. The walls of each arm had a distinct design to provide visual cues. At the beginning of the test, each mouse was placed at the end of one arm facing the centre and allowed to explore the maze for 8 min. All experiments were started at the same time every day and performed by the same investigator (XP). Sessions were video-recorded and arm entries

scored by a trained observer, unaware of the treatment group. Successful alternation was defined as consecutive entries into a new arm before returning to the two previously visited arms.

Alternation was calculated as:  $[\text{number of alternations}/(\text{total number of arm entries}-2)] \times 100$

Novel object recognition test was used for assessing short-term recognition memory<sup>32, 33</sup>. Mice were placed individually in a 50 × 40 × 20 cm (length x width x height) testing chamber with white Plexiglas walls for a 10 min habituation period and returned to home cage. Next day mice were placed in the testing chamber for 3 min with two identical objects (acquisition session). Mice were returned to home cages and one hour later placed back into the testing chamber in the presence of one of the original objects and one novel object (recognition session) for 3 minutes. The original objects consisted of two plastic red cubes 4 cm high × 4 cm diameter (base). The novel object consisted of a blue, 7 cm high × 5 cm diameter (base) round plastic pyramid. The acquisition and recognition sessions were video recorded and an observer who was blinded for the mouse genotype scored the time spent exploring the objects. The chambers and objects were cleaned with ethanol between trials. Exploratory behaviour was defined as sniffing, touching and directing attention to the object. In preliminary studies, naive mice exhibited no significant preference for the red cylinder or the blue pyramid. Exploration time was expressed as the mean ± the standard error of the mean (S.E.M.). For the acquisition session, the recognition index (RI) was calculated as the ratio of the time exploring one of the objects to the time spent exploring both objects × 100. For the recognition session, the RI was calculated as the ratio of the time spent

exploring the novel object to the time spent exploring both the familiar and novel objects  $\times 100^{34}$ .

**Neuronal cultures.** Embryos were extracted from pregnant female mice at embryonic day 14-15 (hippocampal neurons) or 15-17 (cortical neurons). The hippocampi and cortex were dissected out and kept in cold Hanks Balanced Salt Solution (HBSS) containing antibiotics. The tissue was incubated with 2.5% trypsin solution (Gibco) at 37°C for 15 minutes, rinsed 3 times with HBSS, and the cells were dissociated by consecutive pipetting using glass Pasteur pipettes with 3 different opening sizes (3, 2 and 1 mm). The cells were counted using a hemocytometer, and re-suspended in Neurobasal medium with antibiotics, B27, N2, and glutamine supplements. The hippocampal cells were plated in 12-well plates at 60,000 cells per well on Poly-L-Lysine coated coverslips, left to differentiate for 21 days while changing half of the medium on days 3, 10 and 17, fixed on day 21 using a 4% PFA in 4% sucrose solution for 20 minutes and then stored in phosphate-buffered saline (PBS), pH 7.4 at 4°C. The cortical neurons were plated at a density of 50,000 cells per well.

**Tissue processing for morphological studies.** To prepare tissues for morphological studies, animals were deeply anesthetised with sodium pentobarbital and perfused via intracardiac catheter with PBS followed by 4% paraformaldehyde in PBS for preparation of OTC-embedded brain samples or by Bouin's solution for preparation of paraffin-embedded brain samples. Brains and visceral organs (liver, spleen, heart, lungs, kidney and urinary bladder) were removed and immersed in 4% paraformaldehyde in PBS overnight. Tissues for light microscopy were trimmed, dehydrated with an ethanol series followed by acetone, acetone-xylene mixture and xylene and then embedded in

paraffin. Tissues for fluorescent confocal microscopy were treated sequentially in 10%, 20% and 30% sucrose in PBS overnight at 4° C and embedded in OCT compound before freezing at -80° C.

**Histopathology and immunohistochemistry.** Five µm-thick sections of paraffin-embedded tissues were deparaffinized in xylene and rehydrated with isopropyl alcohol, followed by 96%, 70%, and 60% ethanol. The sections were treated with 1% NaN<sub>3</sub> and 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min to inactivate endogenous peroxidase, blocked with 5% bovine fetal serum in PBS for 30 min (both at room temperature), and incubated with primary anti-prosaposin (PSAP; 1:100) diluted in 5% bovine serum albumin in PBS overnight at 4°C. Detection of the primary antibody was performed using Dako EnVision System-HRP Rabbit kit (Dako). Finally, the sections were counter stained in 1% methylene blue and viewed and photographed using a Nikon epifluorescent light microscope (E800) equipped with Olympus digital camera (DP70).

**Fluorescent confocal microscopy.** Forty µm-thick sagittal sections were cut from OTC-embedded frozen brains using CM3050 S Microtome (Leica). The sections were treated with 1% Triton X-100, blocked with 10% goat serum in PBS and incubated overnight at 4°C with primary antibodies in 3% goat serum, 0.1% Triton X-100 in PBS. The following antibodies were used: mouse anti-mouse NeuN (Millipore MAB377, 1:400); rat anti-mouse LAMP2 (DHSB ABL-93-s, 1:100); rabbit anti-mouse GFAP (Abcam, 1:100-600); mouse humanized anti-G<sub>M2</sub> (KM966, 1:400); mouse anti-G<sub>M3</sub> (Cosmo Bio M2590, 1:50-300); chicken polyclonal anti-MAP2 (EnCor Biotechnology, 1:2000); rabbit anti-mouse NG2 (Abcam AB129051, 1:200); mouse anti-mouse/human MAG (Abcam AB89780, 1:200) and rabbit polyclonal anti mouse/rat CD68 (Abcam

AB125212, 1:200). The slides were further stained with Alexa Fluor 555-conjugated Goat anti-mouse IgG, Alexa Fluor 488, 555 and 647-conjugated Goat anti-rat IgG (all Life Technologies); cholera toxin subunit B AlexaFluor 555 conjugate for G<sub>M1</sub> (1:10,000); Phalloidin-Tetramethyl-rhodamine B isothiocyanate for actin; isolectin GS-IB4 Alexa Fluor 568 conjugate (Invitrogen, 1:20); and DyLight 488-conjugated Affinipure Goat anti-human IgG (Jackson immunoresearch laboratories). The slides were mounted with Vectashield mounting medium and analyzed using a Leica DM 5500 Q upright confocal microscope (63x oil objective, N.A. 1.4). Images were processed and quantified using the ImageJ 1.50i software and Photoshop (Adobe).

**Electron Microscopy of mouse tissues.** *Neu3*<sup>-/-</sup> (n=3), *neu4*<sup>-/-</sup> (n=3), and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> (n=3) mice and their WT littermates (n=3) at 12 to 14 months of age were anesthetised with sodium pentobarbital and fixed by intracardiac perfusion with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0). After perfusion, the brains were carefully removed and post-fixed by immersion in the same fixative for 48 hours at 4°C. The brains were sliced at the level of the hippocampus by a coronal section. The slices were approximately 2 mm thick and were further cut near the medial site by a sagittal incision. The resulting blocks containing an area of the cortex and the hippocampus were washed in 0.1 M buffer phosphate (pH 7.0) and immersed for 2 hours in ferrocyanide reduced osmium tetroxide. The blocks were dehydrated in ethanol and propylene oxide and embedded in Epon. Semithin sections (1 µm thick) were cut, mounted on glass slides, stained with toluidine blue, and visualized with a Leica DMS light microscope. The use of light microscopy (LM) allowed us to select cortical and hippocampal areas for electron microscopy (EM).

Ultrathin sections (120 nm thick) were cut with a diamond knife and mounted on 200 mesh copper grids. Staining of the grids was done with uranyl acetate for 5 min, followed by lead citrate for 2 min. The grids were viewed on a FEI Tecnai 12 electron microscope at the McGill University Facility for Electron Microcopy Research (FEMR).

**Quantitative EM analysis.** EM pictures were taken at random from hippocampal regions of *neu3*<sup>-/-</sup> (n=3), *neu4*<sup>-/-</sup> (n=3), *neu3*<sup>-/-</sup>; *neu4*<sup>-/-</sup> (n=3) and WT (n=3) mice at a magnification of x2,900. Each picture/frame represented an area of 816  $\mu\text{m}^2$ . The number of vacuoles containing whorls of membrane typical of sphingolipid storage disorders was counted by an observer blind to the mouse genotype.

**Enzyme assays in mouse tissues.** At the age of 12 weeks mice were sacrificed using CO<sub>2</sub> chamber and their brains extracted, snap-frozen with liquid nitrogen and kept at -80 °C. A hundred mg of frozen brain tissue was homogenized in 300  $\mu\text{L}$  of water in the 1.5 mL Eppendorf tubes using Kontes Pellet motorized pestle. Protein concentration in the homogenate was measured by Bradford method using the Bio-Rad reagent.

Acidic  $\alpha$ -neuraminidase,  $\beta$ -galactosidase, and  $\beta$ -hexosaminidase activities in cellular and tissue homogenates were assayed at pH 4.75 using the corresponding fluorogenic 4-methylumbelliferyl glycoside substrates as previously described<sup>16</sup>. Protein concentration was measured using a Bio-Rad Bradford kit. Neuraminidase activity against gangliosides mixed porcine brain gangliosides, (Avanti chemicals) was measured as described<sup>35</sup>. The concentration of released sialic acid was measured by the thiobarbituric method<sup>36</sup>. The reaction mixture for neuraminidase assays against G<sub>M3</sub> contained 5  $\mu\text{mol}$  of sodium acetate buffer (pH 4.5), 10 nmol G<sub>M3</sub> 50  $\mu\text{g}$  Triton X-100 and 50  $\mu\text{L}$  of the brain homogenate, and was incubated for 30 min at 37°C. The released



Sia were measured by fluorometric high-performance liquid chromatography (HPLC) with malononitrile<sup>37</sup>.

**Analysis of brain gangliosides by TLC.** Briefly, frozen brain tissues were homogenized in water (10% v/w) using a FastPrep-24 MP homogenizer. Lipids were extracted by addition of 2 volumes of methanol and 1 volume of chloroform to one volume of the homogenate. After 10 min centrifugation at 1000 g the organic phase was collected, and used to analyse gangliosides by phase separation as described<sup>16</sup>. The upper phase containing gangliosides was isolated and passed through a Supelclean LC-18 column (Supelco). Gangliosides were eluted first using methanol and then the chloroform/methanol mixture. After evaporation under a stream of nitrogen, the residue was resuspended in 0.1 ml of the chloroform/methanol mixture and applied to a silica gel thin-layer chromatography (TLC) plate that was developed using chloroform/methanol/0.22% CaCl<sub>2</sub> (55:45:10, by volume). After staining with orcinol, gangliosides were identified by comparing their R<sub>f</sub> to those of authentic porcine brain ganglioside standards (Avanti Polar Lipids). Lipids present in the lower phase were separated by TLC using chloroform/methanol/ammonia/water (70:30:2:3, by volume).

**Analysis of brain glycosphingolipids by HPLC.** Glycosphingolipids (GSLs) were isolated and analysed essentially as described in<sup>38</sup>. Mouse brain hemispheres were homogenised in water using an Ultraturax T25 probe homogeniser (IKA, Germany). Protein concentrations in the homogenates were determined using bicinchoninic acid (BCA) assay (Sigma, UK). Lipids from the homogenates were then extracted with chloroform/methanol and GSLs were further purified using solid phase C18 columns (Telos, Kinesis, UK). Eluted lipid fraction GSLs were dried under nitrogen and digested

overnight with ceramide glycanase, prepared in-house from medicinal leeches (*Hirudo medicinalis*). The released glycans were then fluorescently-labelled with anthranilic acid (2AA) prior to purification using DPA-6S SPE amide columns (Supelco, PA, USA). Purified, 2AA-labelled glycans were then separated and quantified by normal-phase high-performance liquid chromatography (NP-HPLC) as described previously<sup>38</sup>. In order to calculate GSL molar quantities, 2.5 pmol of a 2-AA-labelled Chitotriose standard (Ludger, UK) was also included in the HPLC sample group.

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## REFERENCES

1. Hakomori, S. The glycosynapse. *Proc Natl Acad Sci U S A* **99**, 225-232 (2002).
2. Schnaar, R.L., Gerardy-Schahn, R. & Hildebrandt, H. Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. *Physiol Rev* **94**, 461-518 (2014).
3. Palmano, K., Rowan, A. *et al.* The role of gangliosides in neurodevelopment. *Nutrients* **7**, 3891-3913 (2015).
4. Yu, R.K., Macala, L.J. *et al.* Developmental changes in ganglioside composition and synthesis in embryonic rat brain. *J Neurochem* **50**, 1825-1829 (1988).
5. Yu, R.K., Tsai, Y.T. & Ariga, T. Functional roles of gangliosides in neurodevelopment: an overview of recent advances. *Neurochem Res* **37**, 1230-1244 (2012).
6. Tettamanti, G., Bonali, F., Marchesini, S. & Zambotti, V. A new procedure for the extraction, purification and fractionation of brain gangliosides. *Biochim Biophys Acta* **296**, 160-170 (1973).
7. Kawai, H., Allende, M.L. *et al.* Mice expressing only monosialoganglioside GM3 exhibit lethal audiogenic seizures. *J Biol Chem* **276**, 6885-6888 (2001).
8. Sturgill, E.R., Aoki, K. *et al.* Biosynthesis of the major brain gangliosides GD1a and GT1b. *Glycobiology* **22**, 1289-1301 (2012).
9. Monti, E. & Miyagi, T. Structure and Function of Mammalian Sialidases. *Top Curr Chem* **366**, 183-208 (2015).
10. Miyagi, T. & Yamaguchi, K. Mammalian sialidases: physiological and pathological roles in cellular functions. *Glycobiology* **22**, 880-896 (2012).
11. Pshezhetsky, A.V. & Ashmarina, L.I. Desialylation of surface receptors as a new dimension in cell signaling. *Biochemistry (Mosc)* **78**, 736-745 (2013).
12. Rodriguez, J.A., Piddini, E. *et al.* Plasma membrane ganglioside sialidase regulates axonal growth and regeneration in hippocampal neurons in culture. *J Neurosci* **21**, 8387-8395 (2001).
13. Da Silva, J.S., Hasegawa, T. *et al.* Asymmetric membrane ganglioside sialidase activity specifies axonal fate. *Nat Neurosci* **8**, 606-615 (2005).
14. Takahashi, K., Mitoma, J. *et al.* Sialidase NEU4 hydrolyzes polysialic acids of neural cell adhesion molecules and negatively regulates neurite formation by hippocampal neurons. *J Biol Chem* **287**, 14816-14826 (2012).
15. Yamaguchi, K., Shiozaki, K. *et al.* Reduced susceptibility to colitis-associated colon carcinogenesis in mice lacking plasma membrane-associated sialidase. *PLoS One* **7**, e41132 (2012).
16. Seyrantepe, V., Canuel, M. *et al.* Mice deficient in Neu4 sialidase exhibit abnormal ganglioside catabolism and lysosomal storage. *Hum Mol Genet* **17**, 1556-1568 (2008).
17. Smutova, V., Albohy, A. *et al.* Structural Basis for Substrate Specificity of Mammalian Neuraminidases. *PloSOne* (2014).
18. de Geest, N., Bonten, E. *et al.* Systemic and neurologic abnormalities distinguish the lysosomal disorders sialidosis and galactosialidosis in mice. *Hum Mol Genet* **11**, 1455-1464 (2002).

19. Grabrucker, A., Vaida, B., Bockmann, J. & Boeckers, T.M. Synaptogenesis of hippocampal neurons in primary cell culture. *Cell Tissue Res* **338**, 333-341 (2009).
20. Martins, C., Hulkova, H. *et al.* Neuroinflammation, mitochondrial defects and neurodegeneration in mucopolysaccharidosis III type C mouse model. *Brain* **138**, 336-355 (2015).
21. Zhao, Q. & Morales, C.R. Identification of a novel sequence involved in lysosomal sorting of the sphingolipid activator protein prosaposin. *J Biol Chem* **275**, 24829-24839 (2000).
22. Carvelli, L., Libin, Y. & Morales, C.R. Prosaposin: a protein with differential sorting and multiple functions. *Histol Histopathol* **30**, 647-660 (2015).
23. Seyrantepe, V., Lema, P. *et al.* Mice doubly-deficient in lysosomal hexosaminidase A and neuraminidase 4 show epileptic crises and rapid neuronal loss. *PLoS Genet* **6** (2010).
24. Ichikawa, N., Iwabuchi, K. *et al.* Binding of laminin-1 to monosialoganglioside GM1 in lipid rafts is crucial for neurite outgrowth. *J Cell Sci* **122**, 289-299 (2009).
25. Yamanaka, S., Johnson, M.D. *et al.* Targeted disruption of the Hexa gene results in mice with biochemical and pathologic features of Tay-Sachs disease. *Proc Natl Acad Sci U S A* **91**, 9975-9979 (1994).
26. Yamano, T., Shimada, M. *et al.* Ultrastructural study on nervous system of fetus with GM1-gangliosidosis type 1. *Acta Neuropathol* **61**, 15-20 (1983).
27. Gray, D.A. & Woulfe, J. Lipofuscin and aging: a matter of toxic waste. *Sci Aging Knowledge Environ* **2005**, re1 (2005).
28. Yamaguchi, K., Hata, K. *et al.* Evidence for mitochondrial localization of a novel human sialidase (NEU4). *Biochem J* **390**, 85-93 (2005).
29. Pshezhetsky, A.V. & Ashmarina, M. Lysosomal multienzyme complex: biochemistry, genetics, and molecular pathophysiology. *Prog Nucleic Acid Res Mol Biol* **69**, 81-114 (2001).
30. Kreisel, W., Hanski, C. *et al.* Remodeling of a rat hepatocyte plasma membrane glycoprotein. De- and reglycosylation of dipeptidyl peptidase IV. *J Biol Chem* **263**, 11736-11742 (1988).
31. Lema, P.P., Girard, C. & Vachon, P. Evaluation of dexamethasone for the treatment of intracerebral hemorrhage using a collagenase-induced intracerebral hematoma model in rats. *J Vet Pharmacol Ther* **27**, 321-328 (2004).
32. Antunes, M. & Biala, G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn Process* **13**, 93-110 (2012).
33. Akkerman, S., Blokland, A. *et al.* Object recognition testing: methodological considerations on exploration and discrimination measures. *Behav Brain Res* **232**, 335-347 (2012).
34. Ennaceur, A. & Delacour, J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behav Brain Res* **31**, 47-59 (1988).
35. Schneider-Jakob, H.R. & Cantz, M. Lysosomal and plasma membrane ganglioside GM3 sialidases of cultured human fibroblasts. Differentiation by detergents and inhibitors. *Biol Chem Hoppe Seyler* **372**, 443-450 (1991).
36. Manzi, A.E. *Preparation and Analysis of Glycoconjugates*. in *Current Protocols in Molecular Biology* (ed. F.M. Ausubel, *et al.*) 17.18.18-17.18.10 (John Wiley & Sons, Inc., New York, USA, 2003).

37. Hata, K., Tochigi, T. *et al.* Increased sialidase activity in serum of cancer patients: Identification of sialidase and inhibitor activities in human serum. *Cancer Sci* **106**, 383-389 (2015).
38. Neville, D.C., Coquard, V. *et al.* Analysis of fluorescently labeled glycosphingolipid-derived oligosaccharides following ceramide glycanase digestion and anthranilic acid labeling. *Anal Biochem* **331**, 275-282 (2004).

## Figure Legends

### Figure 1. *Neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice show learning impairment at the age of 10 months.

(A-B) *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice show a trend for impaired performance at the age of 6 months (A) and impaired performance at the age of 10 months (B) as compared to WT mice in Y-MAZE test for the spatial memory. \* - significantly different from WT ( $P < 0.05$ ) as calculated by one-way ANOVA. Ten naïve mice were studied per age/per sex/per genotype.

(C) Ten month old *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice showed impaired performance in novel object recognition test. Ten naïve mice were studied for each sex/age/genotype. \*\* - significantly different from WT ( $P < 0.01$ ) as calculated by one-way ANOVA.

**Figure. 2. Neuraminidase activity in mouse brain tissues.** The activity was measured at pH-optima in the homogenates of whole brain tissues of WT C57Bl6 mice (WT), gene-targeted C57Bl6 mice deficient in Neu1 (*neu1<sup>-/-</sup>*), Neu3 (*neu3<sup>-/-</sup>*), Neu4 (*neu4<sup>-/-</sup>*) and double-knockout *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice against MufNaNa, mixed porcine brain gangliosides or purified G<sub>M3</sub> ganglioside. Values are shown as means ( $\pm$ S.E). N-value for each genotype is as follows: for WT n=6, and for *neu3<sup>-/-</sup>*, *neu4<sup>-/-</sup>* and *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* n=4, for *neu1<sup>-/-</sup>* n=8. \*, \*\* and \*\*\* - significantly different from WT ( $P < 0.05$ , 0.01 and 0.001, respectively) by repeated measurements ANOVA.

### Figure 3. Alteration of ganglioside profiles in the brain tissues of *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice.

(A) Representative TLC images of orcinol-stained gangliosides from brain of the WT, *neu1<sup>-/-</sup>*, *neu3<sup>-/-</sup>*, *neu4<sup>-/-</sup>* and *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice at 2 months of age. The positions of the ganglioside standards are indicated on the left.

**(B)** Levels of G<sub>M1a</sub> and G<sub>M3</sub> gangliosides normalized to the level of G<sub>T1b</sub> ganglioside in the brains of WT, Neu1, Neu3, Neu4 and double Neu3/Neu4 deficient mice at 2 and 10 months of age were measured by quantification of stained TLC plates using ImageJ software. Values represent means  $\pm$  S.D. of duplicate measurements. \*\*  $p < 0.01$  as compared with the WT animals by one-way ANOVA.

**(C)** Representative HPLC chromatograms of glycan chains of mouse brain GSLs labelled with anthranilic acid and quantification of GSLs in the brains of WT, Neu1, Neu3, Neu4 and double Neu3/Neu4 deficient mice at 2, 6 and 10 months of age.

**(D)** Levels of gangliosides measured by quantification of HPLC chromatograms. Values represent means  $\pm$  S.D. of duplicate measurements. \*\*\*  $p < 0.001$  as compared with the WT animals by one-way ANOVA.

**Figure 4. Changes in G<sub>M1</sub> and G<sub>M3</sub> gangliosides in the brain cells of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice**

**(A-D)** Accumulation of G<sub>M3</sub> ganglioside in microglia and perivascular cells of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice. Forty  $\mu$ m-thick sagittal brain sections of 10 month-old WT and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice were stained with anti-G<sub>M3</sub> antibodies (**A-C**, green) and either microglial marker, isolectin B4 (**B**, red), or antibodies against the pericytes marker NG2 (**C**, red) or lysosomal marker LAMP-2 (**D**, red). The nuclei were stained with DRAQ5 (**B,C,D**). Slides were studied on a Axioscanner (**A**) or Leica DM 5500 Q upright confocal microscope (63x oil objective, N.A. 1.4) (**B,C,D**). Panels show representative images of at least 30 studied for 3 *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice and 3 WT mice. Bars represent 100  $\mu$ m (**A**), 25  $\mu$ m, 10  $\mu$ m or 5  $\mu$ m (**B,C,D**). Bar graph shows average numbers of G<sub>M3</sub>-positive cells per 100  $\mu$ m<sup>2</sup>.

(E) Reduction of G<sub>M1</sub> ganglioside in neurons of *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice. Forty µm-thick sagittal brain sections of 10 month-old WT and *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice were stained for G<sub>M1</sub> ganglioside with fluorescently-labeled cholera toxin (red) and antibodies against neuronal myelinated axon marker MAG (green). The nuclei were stained with DRAQ5 (blue). Bar graph shows average areas of cholera-toxin stained puncta per 100 µm<sup>2</sup>. All panels show representative images of at least 30 studied for 3 *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice and 3 WT mice. Quantifications were performed using ImageJ software by an observer blinded to mouse genotype, on 3 different panels from 3 different experiments. Values are shown as means (±S.E). \*, \*\* and \*\*\* -significantly different from WT (P<0.05, 0.01 and 0.001, respectively) by one-way ANOVA.

**Figure 5. Neurones from *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice show accumulation of G<sub>M3</sub> ganglioside, reduction of G<sub>M1</sub> ganglioside and severely impaired neurite outgrowth.**

(A) Increased Lamp2 immunostaining (green) compatible with lysosomal storage in 21 DIV cultured hippocampal neurons from *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice. Dendrites are stained with anti-MAP2 antibodies (red) and nuclei, with DRAQ5<sup>TM</sup> (blue).

Bars represent 15 µm. Bar graph shows average total areas of LAMP2 staining per neuron.

(B) Increased G<sub>M3</sub> ganglioside immunostaining compatible with its storage (green) in 21 DIV cultured hippocampal neurons from *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice. Neurons are stained with antibodies against NeuN (red) and nuclei, with DRAQ5 (blue). Bars represent 10 µm. Bar graph shows % of NeuN-positive and NeuN-negative cells positive for G<sub>M3</sub>.

(C) Decreased cholera-toxin staining (red) compatible with reduced G<sub>M1</sub> ganglioside level in 21 DIV cultured hippocampal neurons from *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice. Neurons are



stained with antibodies against NeuN (green) and nuclei, with DRAQ5 (blue). Bars represent 10  $\mu\text{m}$ . Bar graph shows average total areas of G<sub>M1</sub> ganglioside staining per neuron.

**(D)** Decreased dendrite length in 21 DIV cultured hippocampal neurons from *neu3<sup>-/-</sup>*; *neu4<sup>-/-</sup>* mice. Dendrites are stained with antibodies against MAP2 (red) and nuclei, with DRAQ5 (blue). Bar represents 75  $\mu\text{m}$ . Bar graph shows average dendrite length.

**(E)** Decreased axon length in 2 DIV cultured cortical neurons from *neu3<sup>-/-</sup>*; *neu4<sup>-/-</sup>* mice. Neurites are stained with tetramethyl-rhodamine B conjugated falloidin (red) and nuclei, with DRAQ5 (blue). Axons (marked with arrows) were identified as the longest neurite in each neuron with a distinctive growing tip. Bar represents 10  $\mu\text{m}$ . Bar graph shows average axon length.

**(F)** Decreased G<sub>M1</sub> level in 2 DIV cultured cortical neurons from *neu3<sup>-/-</sup>*; *neu4<sup>-/-</sup>* mice. G<sub>M1</sub> ganglioside is labeled with cholera toxin (red) and nuclei, with anti-NeuN antibodies (green). Bar represents 75  $\mu\text{m}$ . Bar graph shows average areas of cholera-toxin stained puncta per 100  $\mu\text{m}^2$ .

**(G)** Decreased dendrite length and G<sub>M1</sub> level in hippocampal neurons from 10 month-old *neu3<sup>-/-</sup>*; *neu4<sup>-/-</sup>* mice. Dendrites are stained with antibodies against MAP2 (green), G<sub>M1</sub> ganglioside, with cholera toxin (red) and nuclei, with DRAQ5 (blue). Bar represents 10  $\mu\text{m}$ . Bar graph shows average dendrite length and average areas of cholera-toxin stained puncta per 100  $\mu\text{m}^2$ .

All panels show representative images of at least 10 studied for 3 *neu3<sup>-/-</sup>*; *neu4<sup>-/-</sup>* mice and 3 WT mice. Quantifications were performed using ImageJ software by an observer blinded to mouse genotype, on 3 different panels from 3 different experiments. Values

are shown as means ( $\pm$ S.E). \*, \*\* and \*\*\* -significantly different from WT ( $P < 0.05$ , 0.01 and 0.001, respectively) by one-way ANOVA.

**Figure 6. CNS inflammation in *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice**

(A) Increased numbers of GFAP-positive astrocytes and C68-positive microglia are detected in the somatosensory cortex (layer V, upper panel) and hippocampus (CA1 layer, lower panel) of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice. Forty  $\mu$ m-thick sagittal brain sections of 10-month-old *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mouse and WT littermate control were stained with antibodies against GFAP (green) and CD68 (red). The nuclei were stained with DRAQ5 (blue). Bars represent 100  $\mu$ m.

Bar graphs show average number of astrocytes and activated microglial cells counted for nine adjacent 0.25 mm<sup>2</sup> sections of somatosensory cortex and hippocampus. Data show mean values ( $\pm$ SD). Two mice were analyzed for each sex and genotype. \*\*  $P < 0.001$  in unpaired two-tailed t-test.

(B) Total brain tissues of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice show progressively increased expression of the inflammation marker, MIP1 $\alpha$  (CCL3). Total RNA was isolated from whole mouse brain, reverse-transcribed to cDNA and quantification of cytokines was performed by RT-qPCR. The data show ratios of the cytokine levels in *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice and those in WT controls (both normalized for the content of *RLP32* mRNA). Data show mean values ( $\pm$ SD). At least three mice were analyzed for each age, sex and genotype. Two-way ANOVA was used to test differences between the mouse groups: significant differences between the mean values in Bonferroni post-test (\* $P < 0.05$ , \*\*  $P < 0.001$ ) are shown.

**Figure 7. Lysosomal storage in the brain cells of *neu3*<sup>-/-</sup> and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice**

(A, B) Hippocampal sections of *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mouse brain showing PSAP-positive neurons (arrowheads with N) and microglia (arrows). The microglia have fine processes and foamy cytoplasm. (C) *neu3<sup>-/-</sup>* brain exhibits a pattern similar to the *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* brain but the number of microglia in the hippocampal region is lower. PSAP-positive microglia are absent in the hippocampus of *neu4<sup>-/-</sup>* (D) and WT (E) animals. (F) unstained control. The bar is equal to 30  $\mu$ m and applies to all sections.

**(G-K) Lysosomal storage in vascular pericytes.** (G) Hippocampus of a *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mouse showing a PSAP-positive pericytes in the adventitial layer of a postcapillary venule (arrow). (H) Pericytes of *neu3<sup>-/-</sup>* exhibit a staining pattern similar to that of *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* brains. PSAP-positive microglia are encircled. (K) Pericytes are unreactive and microglial cells are seemingly absent in the hippocampus of WT mouse. The bar is equal to 30  $\mu$ m and applies to all sections.

**Figure 8. Abnormal vacuoles containing whorls of membranes in the neurons of *neu3<sup>-/-</sup>* and *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* mice**

(A) *neu3<sup>-/-</sup>* brain, cross section of axons at the level of hippocampus. The axon with an abnormal vacuole containing whorls of membranes is marked by an arrow. (B) *neu4<sup>-/-</sup>* brain, section of brain cortex. Small vacuoles containing whorls of membranes are present in the bodies of cortical neurons (arrow). (C) *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* brain, cross section of axons at the level of hippocampus. Abnormal vacuoles containing whorls of membranes are frequently observed within the axons (arrow). (D) *neu3<sup>-/-</sup>;neu4<sup>-/-</sup>* brain, section of brain cortex. Vacuoles containing whorls of membranes are observed within the body of hippocampal and cortical neurons (arrows).

**Figure 9. Ultrastructural pathology in the brain of neuraminidase-deficient mice**

**(A-D). Lipofuscin bodies in *neu3*<sup>-/-</sup> and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> mice are heterogeneous in electron density and contain multi-vesicular structures.** (A) Section of WT brain. The micrograph illustrates a typical lipofuscin vacuole found in the cell body of hippocampal and cortical neurons (arrow and inset). (B) Section of *neu4*<sup>-/-</sup> brain. Cortical and hippocampal neurons contain lipofuscin bodies similar to the ones observed in WT neurons. (C, D) Section of *neu3*<sup>-/-</sup> and *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> brains, respectively. Cortical and hippocampal neurons contain lipofuscin bodies, which are heterogeneous in size and electron densities, and contain multi-vesicular structures.

**(E-G) Lysosomal storage in pericytes of postcapillary venules.** Adventitial cells, possibly pericytes in and around the choroid plexus of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> brains have large heterogonous lysosomes containing multivesicular structures and material of varying electron densities.

**(H,K) Lysosomal storage in hippocampal microglia.** (H) LM of the Epon section of hippocampus of *neu3*<sup>-/-</sup>;*neu4*<sup>-/-</sup> brain showing small cells containing vacuoles (square frame and inset). (K) EM shows that the same cells have large heterogeneous lysosomes containing multi-vesicular structures and material of varying electron density similar to those found in pericytes.