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Cellular/Molecular

Neuronal Expression of GalNAc Transferase Is Sufficient to Prevent the Age-Related Neurodegenerative Phenotype of Complex Ganglioside-Deficient Mice

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Gangliosides are widely expressed sialylated glyosphingolipids with multifunctional properties in different cell types and organs. In the nervous system, they are highly enriched in both glial and neuronal membranes. Mice lacking complex gangliosides attributable to targeted ablation of the B4galnt1 gene that encodes β-1,4-N-acetylgalactosaminyltransferase 1 (GalNAc-transferase; GalNAcT−/−) develop normally before exhibiting an age-dependent neurodegenerative phenotype characterized by marked behavioral abnormalities, central and peripheral axonal degeneration, reduced myelin volume, and loss of axo-glial junction integrity. The cell biological substrates underlying this neurodegeneration and the relative contribution of either glial or neuronal gangliosides to the process are unknown. To address this, we generated neuron-specific and glial-specific GalNAcT rescue mice crossed on the global GalNAcT−/− background [GalNAcT−/−/Tg(neuronal) and GalNAcT−/−/Tg(glial)] and analyzed their behavioral, morphological, and electrophysiological phenotype. Complex gangliosides, as assessed by thin-layer chromatography, mass spectrometry, GalNAcT enzyme activity, and anti-ganglioside antibody (AgAb) immunohistology, were restored in both neuronal and glial GalNAcT rescue mice. Behaviorally, GalNAcT−/−/Tg(neuronal) retained a normal “wild-type” (WT) phenotype throughout life, whereas GalNAcT−/−/Tg(glial) resembled GalNAcT−/− mice, exhibiting progressive tremor, weakness, and ataxia with aging. Quantitative electron microscopy demonstrated that GalNAcT−/− and GalNAcT−/−/Tg(glial) nerves had significantly increased rates of axon degeneration and reduced myelin volume, whereas GalNAcT−/−/Tg(neuronal) and WT appeared normal. The increased invasion of the paranode with juxtaparanodal Kv1.1, characteristically seen in GalNAcT−/− and attributed to a breakdown of the axo-glial junction, was normalized in GalNAcT−/−/Tg(neuronal) but remained present in GalNAcT−/−/Tg(glial) mice. These results indicate that neuronal rather than glial gangliosides are critical to the age-related maintenance of nervous system integrity.

Key words: ganglioside; glycosyltransferase; neurodegeneration; transgenic

Introduction

Gangliosides are sialylated glyosphingolipids widely expressed in vertebrate plasma membranes and intracellular compartments

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their roles. Mice generated with a disruption in the B4galnt1 gene are deficient in GalNAcT but are viable and appear grossly normal, indicating that complex ganglioside expression is not necessary for normal development (Takamiya et al., 1996) but later develop an age-dependent neurodegenerative phenotype characterized by weakness, ataxia, nerve degeneration and demyelination, and loss of nodal axo-glial junction adhesion and integrity (Sheikh et al., 1999; Chiavegatto et al., 2000; Susuki et al., 2007). Overexpression of the precursor simple gangliosides GM3, GD3, and 9-O-Ac(etyl)-GD3 in GalNAcT−/− mice may play a compensatory developmental role that limits the severity of the phenotype (Ngamukote et al., 2007; Furukawa et al., 2008).

Mice lacking b-series gangliosides (GD3s−/−) are grossly normal throughout life but repair peripheral nerve poorly (Kawai et al., 2001; Ōkada et al., 2002). Mice whose ganglioside repertoire is restricted to GM3 (GalNAcT−/− × GD3s−/− double knock-out) develop lethal audiogenic seizures (Kawai et al., 2001), age-dependent progressive motor and cognitive deficits (Tajima et al., 2009), and sensory loss (Inoue et al., 2002). Complete ganglioside ablation is not embryonic lethal; however, from 2 weeks of age, mice undergo progressive and severe neurodegeneration resulting in death at ~2 months (Yamashita et al., 2005). Together, these mouse data are suggestive of a more fundamental necessity for a-series gangliosides in age-related nervous system maintenance, although this is difficult to conclusively prove because a mouse with selective deficiency of a-series gangliosides has not been generated. Humans with inherited ganglioside deficiency also develop complex neurodevelopment and degenerative syndromes (Simpson et al., 2004; Boukhris et al., 2013).

Although these studies indicate that bodywide expression of simple gangliosides are sufficient to promote viability and complex gangliosides are required for nervous system maintenance, stability, and repair, it is unknown whether neuronal or glial ganglioside deficiency has the greater impact on the age-related phenotype and maintenance of the axon, myelin, and axo-glial junction. To assess the relative significance and necessity of complex ganglioside expression in neuronal and myelin-forming cells, we developed GalNAcT−/− rescue mice that selectively express gangliosides either neuronal [GalNAcT driven by the neurofilament-light (NFL) promoter; GalNAcT−/−-Tg(neuronal)] or in myelin [GalNAcT driven by the proteolipid protein (PLP) promoter;
GalNAcT<sup>−/−</sup>-Tg(glial)] and analyzed them for behavioral and structural abnormalities.

Materials and Methods

Generation of transgenic mice

The generation of GalNAcT<sup>−/−</sup> mice lacking complex gangliosides has been described previously (Takamiya et al., 1996) and backcrossed seven generations on a C57BL/6 background. Transgenic mice expressing the full-length cDNA encoding GalNAcT<sup>−/−</sup> under the control of the NFL or PLP promoter were generated by pronuclear injection to produce neuronal and glial expression, respectively (Fig. 1A). The activity of the NFL and PLP promoters are classically restricted to mature neurons and myelinating glia (oligodendrocytes and Schwann cells), respectively. GalNAcT DNA was cloned into the pGCHN-L vector (provided by J.-P. Julien, Lalaval University, Quebec, Quebec, Canada) for generating NFL–GalNAcT transgenic mice. Similarly, GalNAcT cDNA was cloned into PLP–SV40 (provided by Wendy Macklin, University of Colorado, Boulder, CO) for generating PLP–GalNAcT transgenic mice. Transgenic lines and germ-line transmitters were identified by PCR and backcrossed seven generations on a C57BL/6 background. NFL–GalNAcT and PLP–GalNAcT were then interbred with GalNAcT<sup>−/−</sup> mice to create GalNAcT<sup>−/−</sup>-Tg(neuronal) and GalNAcT<sup>−/−</sup>-Tg(glial) mice, respectively, that were used in all analyses. Evidence for GalNAcT enzyme activity restoration and complex ganglioside synthesis in neural tissues were confirmed by glycosyltransferase activity assays as described previously (Ruan and Lloyd, 1992; Ruan et al., 1995). Mice of either sex were killed by CO2 inhalation, and all experiments complied with United Kingdom Home Office guidelines.

Antibodies and reagents

Anti-ganglioside antibodies (AgAb) were generated as described previously (Bowes et al., 2002; Boffey et al., 2005); herein we used the monoclonal antibody MOG16 that reacts with complex gangliosides GD1b and GT1b, the latter being prominently restored in GalNAcT<sup>−/−</sup>-Tg(neuronal) and GalNAcT<sup>−/−</sup>-Tg(glial) mice (Fig. 1C,D). Immunostaining reagents were sourced as follows: e-bungarotoxin (BTX; Invitrogen); rabbit anti-Caspr (diluted 1:1000; gift from Elior Peles, Rehovot, Israel); rabbit anti-Kv1.1 (diluted 1:200; Alomone Labs); mouse anti-Caspr (diluted 1:500; NeuroMab); rabbit anti-Nav1.6 (diluted 1:100; Sigma); rabbit anti-neurofilament (diluted 1:750; Affinity Bioreagents); isotype-specific (IgG1, IgG2a, IgG2b, IgG3) Alexa Fluor 488- and 555-conjugated anti-mouse IgG antibodies; and Alexa Fluor 488- and 555-conjugated anti-rabbit secondary antibodies. Ringer’s solution (in mm: 116 NaCl, 4.5 KCl, 1 MgCl2, 2 CaCl2, 1 NaH2PO4, 23 NaHCO3, and 11 glucose, pH 7.4) was pregassed with 95% O2/5% CO2.

Lipid analysis and TLC

Gangliosides were extracted from brains removed and snap frozen from WT, GalNAcT<sup>−/−</sup>, GalNAcT<sup>−/−</sup>-Tg(neuronal), and GalNAcT<sup>−/−</sup>-Tg(glial) mice to label the neuromuscular junctions (NMJs). Spinal cord (Bowes et al., 2002), second segment of the cervical spinal cord, the optic nerve (OpN), and sciatic nerve (SN) were processed for resin embedding (Griffiths et al., 1981). Sections were cut for both light and electron microscopy. Tissue from 2- and 12-month-old mice (n = 4 per genotype, n = 3 WT) were studied for ultrastructural analysis. Electron micrographs from transverse sections of the OpN and the ventral columns of the spinal cord at 6700× and 2700× magnification, respectively, were taken on a Jeol CX-100 Electron microscope. Images from 1 μm semithin sections stained with methylene blue/azurII were captured of the SN at 100× magnification. For quantification, a minimum of 10 electron micrographs or six digital light microscopic images per animal were taken of randomly selected fields. All measurements were made on scanned or digitally captured images using NIH ImageJ software.

Axon morphology and quantification of axonal changes. All axons within or touching the top and left borders of an area of interest (AOI) were counted. The axon density, number of degenerating axons, or those containing accumulations of organelles within the AOI was counted. The volume occupied by compact myelin or axoplasm was quantified by a point counting method using an appropriately sized grid (Williams, 1977) and expressed as the ratio of the number of intercepts coinciding with the structure to the total number of intercepts.

Immunohistochemistry, imaging, and analysis

Ganglioside localization. Triangular sterni (TS) muscles (n = 3 per genotype, 6 months) were maintained in Ringer’s solution and incubated in 100 μg/ml Ag/Ab for 30 min at 4°C with 2 μg/ml FITC-conjugated BTX to label the neuromuscular junctions (NMJs). Spinal cord (n = 3 per genotype, 6 months) were snap frozen on removal, transversely cryosectioned at 10 μm onto 3-aminopropyltriethoxysilane (APES)-coated glass slides, and then incubated with 20 μg/ml Ag/Ab and anti-neurofilament antibody to identify axons (1:750) in PBS for 2 h at 4°C. All preparations were washed in Ringer’s solution or PBS before 20 min fixation in 4%
parafomaldehyde at room temperature, followed by 10 min washes with PBS, 0.1 % glycine, and PBS. Tissues were incubated with appropriate isotype-specific fluorescently labeled secondary antibodies at 2 μg/ml for 1 h at room temperature (cord) or 5 μg/ml with 1 % normal goat serum overnight at 4°C (TS), washed with PBS, and finally coveredslipped, or mounted and coverslipped in the case of TS. Images were captured on a Zeiss Axio Imager Z1 with Apo lume attachment.

Nodal integrity assessment. Fixed SN and OpN (n = 3 per genotype, 6 months) were teased into individual fibers or sectioned at 10 μm, respectively, on to APES-coated glass slides. Nerves were incubated with blocking solution (3 % normal goat serum plus 0.5 % Triton X-100 in PBS) for 30 min at 4°C before incubation overnight in the same solution plus primary antibodies (rabbit anti-Kv1.1 and mouse anti-Caspr). Nerves were washed in PBS and incubated for 3 h at room temperature with appropriate combination of fluorescently labeled secondary antibodies each at 2 μg/ml. Several features of nodal architecture were examined (and quantified in SN), including juxtaparanodal (JPN) Kv1.1 channel immunostaining invasion into paranodes, the distance between Kv1.1-positive domains, paranodal (PN) Caspr protrusions and the length of Caspr, and Nav1.6 staining. Nerves of Ranvier (NoR) were identified in phase contrast and by Kv1.1 channel staining. At least 20 nodes per mouse were imaged and assessed using NIH Image software.

Extracellular SN recordings
Nerves were mounted in a Perspex recording block across three chambers and sealed in with vacuum grease. SNs were maintained in Ringer’s solution, and recordings were performed at room temperature. To measure conduction velocity (CV) and rate of rise, nerves were stimulated at 1 Hz and supramaximal voltage (Grass S88 stimulator; Grass Instruments) for 30 min. Signals were amplified (CED 1902; Cambridge Electronic Design), digitized (NIDAQ-MX analog-to-digital converter; National Instruments), and analyzed using WinWCP version 4.5.2. Subsequently, paired-pulse recordings were performed on the same nerve to assess refractoriness; intervals between stimuli ranged from 3 to 30 ms. At termination, 5 μM tetrodotoxin was applied or nerve crush was performed to confirm that the recorded waveform originated from the opening of sodium channels. A minimum of 200 control waveforms were compared with the first was calculated for every interstimulus interval and plotted. Four to 6-month-old mice were used (n = 3 for WT; n = 4 for GalNAcT−/−-Tg(neuronal); n = 5 for GalNAcT−/− and GalNAcT−/−-Tg(glial)); both nerves were used for recordings per mouse. One-way ANOVA was used to compare CV and rate of rise among genotypes and two-way ANOVA to compare paired-pulse amplitudes with increasing interstimulus interval.

Statistics
Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software), and statistical significance was set at p < 0.05. Data in figures are presented as mean ± SEM. Behavioral data among genotypes was compared by two-way ANOVA, followed by Tukey’s post hoc tests for multiple comparisons; one-way ANOVA was applied to morphological studies and electrophysiological recordings, followed by Tukey’s post hoc analysis.

Results
GalNAcT enzyme activity and complex ganglioside expression is restored in neural tissue of GalNAcT−/−-Tg(neuronal) and GalNAcT−/−-Tg(glial) mice
Four neuronal and four glial transgenic lines were backcrossed onto GalNAcT−/− and screened for GalNAcT enzyme activity in whole-brain homogenate and for complex ganglioside expression in neural tissue by immunohistology. One line of each was then selected for additional study based on the highest enzyme activity. In the two selected lines, whole-brain GalNAcT enzyme activity (compared with WT, 100%) was present at 5.3% in GalNAcT−/−-Tg(neuronal) and 11.7% in GalNAcT−/−-Tg(glial) and undetectable in whole-brain homogenate from GalNAcT−/− mice.

To characterize complex ganglioside profiles, ganglioside fractions were extracted from brains for resorcinol staining of TLC and accurate molecular mass identification using liquid chromatography mass spectrometry (Fig. 1C,D). TLC of brain extracts confirmed the presence of complex gangliosides in expected profiles (Fig. 1C). WT brain contains a full complement of simple and complex gangliosides, with greater expression of the complex gangliosides GD1a, GT1b, GM1, and GD1b, as also indicated by liquid chromatography mass spectrometry (Fig. 1D). In GalNAcT−/− brain, complex gangliosides are absent; instead, GM3, GD3, and 9-O-Ac(etyl)-GD3 that are upstream of GalNAcT in the biosynthetic pathway are highly enriched, as reported previously (Takamiya et al., 1996; Furukawa et al., 2008). Enrichment of these simple gangliosides is maintained in GalNAcT−/−-Tg(neuronal) and GalNAcT−/−-Tg(glial) brain; additionally, the complex gangliosides GD1a, GT1b, GM1, and GD1b are also present at approximately equal levels in each strain and at lower levels than in WT brain. Based on the normalized intensities from the extracted ion chromatogram of the D18:1/18:0 species for each complex ganglioside, restoration levels are 25–40 % of WT whole-brain values. The lower levels of complex gangliosides seen in both GalNAcT−/−-Tg(neuronal) and GalNAcT−/−-Tg(glial) shown by TLC and enzyme activity assays compared with WT may be in part attributable to a dilution effect, because analyses were conducted on whole brain. The enrichment of simple gangliosides in the rescue strains is likely attributable to restricted cell specificity of the transgenic GalNAcT enzyme, with consequent buildup in the un-rescued tissue compared with the global expression of GalNAcT in WT brain.

Immunostaining of PNS and CNS tissue was performed using AgAbs to delineate cell specificity of the transgenically reinstated gangliosides (Fig. 2). Monoclonal antibodies raised against complex gangliosides were applied to spinal cord sections and ex vivo whole-mount TS nerve–muscle preparations to demonstrate appropriate expression in CNS and PNS among genotypes and compare fluorescence levels as an indication of expression levels in the target membranes. Despite lower levels of whole-brain ganglioside as determined by TLC above, AgAb immunostaining at similar levels was evident in the distal axon of the NMJ and also on spinal cord ventral column fibers in WT and GalNAcT−/−-Tg(neuronal) mice (Fig. 2). Complex ganglioside expression was absent on axons of GalNAcT−/− mice as expected. In GalNAcT−/−-Tg(glial) mice, pronounced immunostaining was observed at sites corresponding to perisynaptic Schwann cells overlying the nerve terminal (in which the PLP promoter is active; Michalski et al., 2011) and surrounding neurofilament immunoreactivity in ventral spinal cord. It is not possible to establish whether the latter immunostaining is present on the axonal membrane or the adaxonal myelin membrane at this level of resolution, because the two membranes are very closely apposed. With some monoclonal antibodies, we observed perisynaptic Schwann cell labeling at the NMJ of GalNAcT−/−-Tg(neuronal) mice, consistent with activity of the NFL promoter in non-myelinating Schwann cells (Haynes et al., 1999; data not shown). However, primary cultures of Schwann cells and oligodendrocytes from GalNAcT−/−-Tg(neuronal) mice were negative when immunostained with AgAbs to complex gangliosides compared with positive WT glial cultures (data not shown).

Neuronal but not glial expression of GalNAcT attenuates the behavioral deficits seen in GalNAcT−/− mice
Because GalNAcT−/− mice exhibit age-dependent locomotor deficits, we performed behavioral tests to assess the impact of neuronal and glial complex ganglioside rescue on motor coordina-
tion, strength, gait, and balance. Mice of all genotypes maintained a normal lifespan; however, \textit{GalNAcT}^{+/+}/\textit{H11022}/\textit{H11002} and \textit{GalNAcT}^{+/+}/\textit{H11022}/\textit{H11002} -Tg(glial) mice developed a severe locomotor deficit with age, manifested most obviously by hindpaw clasping and impaired extension (Fig. 3A) and a markedly ataxic gait with pronounced tremor. Interestingly, old (>18 months) \textit{GalNAcT}^{+/+}/\textit{H11002} -Tg(glial) mice exhibited excessive facial scratching with excoriation and hair loss, not seen in other genotypes, including \textit{GalNAcT}^{+/+}/\textit{H11002} (data not shown). \textit{GalNAcT}^{+/+}/\textit{H11002} -Tg(neuronal) mice appeared normal throughout life, indistinguishable from WT.

On quantitative testing, hindlimb grip strength is similar in the four genotypes at 2–3 months and then significantly and progressively deteriorates (two-way ANOVA, \(p < 0.001\); Fig. 3B) in \textit{GalNAcT}^{+/+} and \textit{GalNAcT}^{+/+}/\textit{Tg(glial)} mice from 6 months of age, as revealed by \textit{post hoc} tests. \textit{GalNAcT}^{+/+} and \textit{GalNAcT}^{+/+}/\textit{Tg(glial)} mice also exhibit equally poor motor performance and coordination that progresses with age as determined by a reduced latency to fall from the rotarod (two-way ANOVA, \(p < 0.001\); Fig. 3C) and an increase in the number of foot falls on grid walking (two-way ANOVA, \(p < 0.001\); Fig. 3D). There is no significant difference in task performance between WT \textit{GalNAcT}^{+/+} -Tg(neuronal) mice.

**Morphological appearances as a function of complex ganglioside expression in neurons and glia**

Ultrastructural abnormalities in \textit{GalNAcT}^{+/+} mice principally include an age-dependent increase in degenerate axon number, abnormal myelination (CNS dysmyelination and PNS demyelination), loss of innermost PN transverse bands, and axolemmal protrusions at the NoR (Sheikh et al., 1999; Sugiura et al., 2005; Susuki et al., 2007). Therefore, we assessed these parameters in groups of normal, \textit{GalNAcT}^{+/+}, \textit{GalNAcT}^{+/+}/\textit{Tg(neuronal)}, and \textit{GalNAcT}^{+/+}/\textit{Tg(glial)} mice at young (2 months) and aged (12 months) time points to determine whether neuronal or glial ganglioside expression was critical to the development of these pathological abnormalities and that correlated with behavioral
Figure 3. Neuronal but not glial expression of complex gangliosides in GalNAcT−/− mice rescues behavioral deficiencies. A, Hindpaw extension on lifting by the tail occurs in WT mice; this is replicated in GalNAcT−/−/Tg(neuronal) mice, but abnormal clasping of the hindpaws occurs in GalNAcT−/− and GalNAcT−/−/Tg(glial) mice. Grip strength (B), latency to fall from the rotarod (C), and number of foot slips (D) are comparable among genotypes at 2 months. There is significant deterioration with age in GalNAcT−/− and GalNAcT−/−/Tg(glial) mice (2-way ANOVA, p < 0.001) compared with WT and GalNAcT−/−/Tg(neuronal), *** indicates significance compared with WT; ### signifies significance compared with neuronal rescue. *p < 0.05; *** and ###, p < 0.001.

Restoration of nodal architecture by neuronal expression of complex gangliosides

The aberrant distribution of nodal ion channels (Kv1.1 and Nav1.6) described previously in GalNAcT−/− mice (Susuki et al., 2007) indicates a role for complex gangliosides in the maintenance of nodal domains. In both SN (PNS) and OpN (CNS), rectification of nodal ion channel localization to discrete domains was observed after neuronal, but not glial, expression of complex gangliosides (Fig. 5). In the SN of GalNAcT−/− and GalNAcT−/−/Tg(glial) mice, immunohistology reveals significant invasion of the PN with JPN potassium channels (Kv1.1) compared with WT mice (Fig. 5 A, B, indicated by orange arrows; one-way ANOVA, p < 0.05). In GalNAcT−/−/Tg(neuronal) mice, Kv1.1 localization is restored to normal. A consequence of the Kv1.1 PN invasion in GalNAcT−/− and GalNAcT−/−/Tg(glial) mice is a reduction in the distance between JPN Kv1.1-positive domains compared with WT, whereas Caspr staining lengths remain similar (Fig. 5A–C; one-way ANOVA, p < 0.001), thereby effectively indicating an overlap in Kv1.1 and Caspr staining at the PN/JPN border. In contrast, in GalNAcT−/−/Tg(neuronal) mice, the inter-Kv1.1 distance and Caspr staining domains are significantly longer than in GalNAcT−/− and GalNAcT−/−/Tg(glial) mice (Figure 5A–C; one-way ANOVA, p < 0.01). The number of PN protrusions filled with Caspr immunoreactivity were significantly greater in GalNAcT−/− and GalNAcT−/−/Tg(glial) mice compared with both WT and GalNAcT−/−/Tg(neuronal) mice (Fig. 5A–C; one-way ANOVA, p < 0.05, indicated by white arrows). These protrusions do not contain neurofilament immunoreactivity, suggesting that they...
are attributable to discrete local disturbances in the membrane rather than alterations in neurofilament ultrastructure (data not shown). The lateral extent of Nav1.6 immunostaining increased with loss of complex gangliosides compared with WT and remained increased in both GalNAcT<sup>−/−</sup>-Tg(glial) and GalNAcT<sup>−/−</sup>-Tg(neuronal) mice (Fig. 5 B, C). These results suggest a role for complementary complex ganglioside expression in closely apposed membranes and the reliance on these lipid interactions for the fine-tuning of domain organization.

Ultrastructural examination of longitudinal sections through WT SN NoR demonstrates individual PN loops aligning with the axon, connected by transverse bands (Fig. 5 D). In all three complex ganglioside-deficient genotypes, the PN loops appear disorganized and aberrantly stacked, particularly at the nodal/PN border of GalNAcT<sup>−/−</sup>-mice, we observed that they were present here both in GalNAcT<sup>−/−</sup>-Tg(glial) and GalNAcT<sup>−/−</sup>-Tg(neuronal) mice (Fig. 6, enlargements shown in insets). The neurofilament appears normal in all genotypes in this region.

Electrophysiological examination of SN
In view of the ion channel and nodal architecture abnormalities, degenerate axons, and myelin volume loss present in ganglioside null and rescue mice, we performed ex vivo extracellular recordings on SN from the different genotypes. Subtle reductions in CV were seen in all ganglioside-deficient mice compared with WT, but this did not reach significance (Fig. 7A). There is a significant difference in rate of rise of the compound nerve action potential in all genotypes compared with WT mice (Fig. 7B, C; one-way ANOVA, * p < 0.01). Paired pulse recordings show that refractoriness is not altered in GalNAcT<sup>−/−</sup>, GalNAcT<sup>−/−</sup>-Tg(glial), or GalNAcT<sup>−/−</sup>-Tg(neuronal) mice compared with WT (Fig. 7D). These results suggest that the behavioral phenotype is not accounted for by SN conduction slowing because recovery of the behavioral phenotype in neuronal rescue mice is not mirrored by an improvement in conduction. It is likely that changes to the refractory period were not detected

**Figure 4.** Prevention of morphological abnormalities in neuronal but not glial rescue mice. A, Degenerate axon density or number is greater in 12-month-old GalNAcT<sup>−/−</sup> mice and reaches significance in OpN and SN compared with age-matched WT mice. Neuronal expression of complex gangliosides significantly attenuates this degeneration, whereas degenerate axon density and number in glial rescue mice remains significantly greater in spinal cord and SN, respectively. B, Myelin volume is significantly decreased in all tissues studied for GalNAcT<sup>−/−</sup> and GalNAcT<sup>−/−</sup>-Tg(glial) mice compared with WT and also compared with GalNAcT<sup>−/−</sup>-Tg(neuronal) in SN. C, Representative EM and light microscopic images from transverse sections of spinal cord, OpN, and SN for all genotypes show normalization of axon and myelin in neuronal rescue mice and increased degenerate axons (indicated by red arrowheads), myelin thinning, and poorer ultrastructure in GalNAcT<sup>−/−</sup> and GalNAcT<sup>−/−</sup>-Tg(glial) mice. Organellle-filled axons and redundant myelin occurred frequently in GalNAcT<sup>−/−</sup>-Tg(glial) mice OpN and are indicated by red asterisks and arrows, respectively. One-way ANOVA, * p < 0.05, ** p < 0.01, *** p < 0.001. Scale bars: Cord, 2 μm; OpN, 1 μm; SN, 25 μm.
Figure 5. Restoration of normal nodal architecture by expression of complex gangliosides in neurons of GalNAcT−/− mice. A. Representative illustrative images per genotype of Caspr (green) and Kv1.1 (magenta) immunoreactivity at SN and OpN NoR in 6-month-old mice. In SN Kv1.1, invasion into the PN is indicated by orange arrows (SN and OpN) and Caspr protrusions (SN only) by white arrows. B. Invasion of the PN (identified by Caspr) with JPN marker Kv1.1 staining significantly increased in GalNAcT−/− and GalNAcT−/−-Tg(glial) mice compared with WT and GalNAcT−/−-Tg(neuronal) mice SN. Consequently, the distance between Kv1.1-positive domains significantly decreased for both genotypes. This distance was lengthened in GalNAcT−/−-Tg(neuronal) mice compared with WT. The number of PN Caspr staining protrusions significantly increased for GalNAcT−/− and GalNAcT−/−-Tg(glial) SN compared with WT and GalNAcT−/−-Tg(neuronal) levels, which were comparable. Compared with WT nerve, Caspr staining was significantly lengthened in GalNAcT−/−-Tg(neuronal) nerve. Conversely, Caspr staining length was significantly shorter in GalNAcT−/− and GalNAcT−/−-Tg(glial) SN compared with GalNAcT−/−-Tg(neuronal). The length of Nav1.6 immunostaining significantly increased in GalNAcT−/− and both rescue mice compared with WT. C. To scale, schematic representing the length of staining in each domain per genotype. One-way ANOVA, p < 0.05. * signifies significance compared with WT; # signifies significance compared with GalNAcT−/−-Tg(neuronal). ** and ###, p < 0.01; *** and ####, p < 0.001. Scale bar, 10 μm.
because of very subtle invasion of the paranodes by Kv1.1 channels.

Discussion

Many neurological abnormalities associated with global complex ganglioside deficiency achieved through targeted gene knock-out have been described previously (Takamiya et al., 1996; Sheikh et al., 1999; Chiavegatto et al., 2000; Sugiiura et al., 2005; Susuki et al., 2007). However, the precise cellular contribution to these phenotypes has never been investigated. Herein, through selective reintroduction of glycosyltransferase activity in a site-specific manner, we find that neuronal, and not glial, rescue of complex gangliosides is both necessary and sufficient to prevent the age-dependent neurodegenerative phenotype seen in global GalNAcT−/− deficiency states. These findings clearly demonstrate the importance of neurally expressed GalNAcT in maintaining nervous system integrity throughout the lifespan.

Certain technical caveats may affect the interpretation of these results. The targeting strategy we adopted usedNFL and PLP promoters to drive GalNAcT expression in a cell-restricted manner to neurons and myelin-forming glia, respectively. Although both vectors have been used widely to achieve this, it is recognized that, at different stages of mouse development and in different neuronal and glial subtypes, these promoter activities may be more promiscuously active than generally stated (Haynes et al., 1999; Michalski et al., 2011). For example, transient expression of neurofilament mRNAs has been described in non-myelinating Schwann cells (Fabrizi et al., 1997; Sotoelo-Silveira et al., 2000), thereby providing one explanation for the presence of complex ganglioside immunoreactive perisynaptic Schwann cells we observed at the GalNAcT−/−-Tg(neuronal) mouse NMI. However, the absence of complex gangliosides in cultured Schwann cells and oligodendrocytes provides overall confidence in the validity of using the NFL promoter for this model. Furthermore, the activity of the natural GalNAcT promoter and of the enzyme itself, which is highly regulated in developmental and spatial patterns, is not recapitulated in these transgenic mice, especially when considering that the regional patterns of brain ganglioside composition will change continuously throughout life (Segler-Stahl et al., 1983; Ikarashi et al., 2011). It is also known that gangliosides can transfer between membranes by shedding and uptake (Olsheski and Ladisch, 1996; Lauc and Hefer-Lauc, 2006). Indeed, substantial levels of different gangliosides circulate in the plasma and may be derived from dietary intake or acquired transplacentally from heterozygous dams used in our breeding programs (McJarrow et al., 2009; Mitchell et al., 2012). Therefore, it is possible that glial membranes might acquire complex gangliosides from neuronal membranes in GalNAcT−/−-Tg(neuronal) mice and vice versa, regardless of their primary site of biosynthesis.

Notwithstanding these caveats, our analyses demonstrate that site-specific reintroduction of complex gangliosides has been achieved. GalNAcT−/− mice have increased expression of the simple gangliosides (Takamiya et al., 1996; Furukawa et al., 2008). Both GalNAcT−/−-Tg(neuronal) and GalNAcT−/−-Tg(glia) retained high levels of these simple gangliosides, although we do not know whether these were present in GalNAcT-deficient cells or were still accumulated in GalNAcT-replete cells containing rate-limiting levels of enzyme activity. Although it has been proposed previously that neurodegeneration in GalNAcT−/− mice might in part be attributable to a toxic gain of function, our data showing retention of very high levels of these simple gangliosides would argue that deficiency of complex gangliosides appear to be the major factor.

Complex gangliosides are present in both axons and glia (Ogawa-Goto et al., 1992; Svennerholm, 1994; Ogawa-Goto and Abe, 1998), with greater enrichment of GM1 and GD1a in axons (Ogawa-Goto and Abe, 1998) and immunostaining (Gong et al., 2002). Our immunostaining for complex gangliosides was most apparent on the axons rather than myelin in WT and GalNAcT−/−-Tg(neuronal) mice and was absent in both compartments in GalNAcT−/− mice. GalNAcT−/−-Tg(glia) perisynaptic Schwann cells at motor nerve terminals were labeled by AgAbs, and ventral column fibers were also immunopositive. If indeed complex gangliosides are more prevalent in the axonal compartment in WT mice, this could explain why a rescue of neuronal complex gangliosides restores the GalNAcT−/− phenotype to near normality, whereas recovery of gangliosides in the glial compartment has no effect.

Behavioral features associated with motor coordination and balance that are impaired in GalNAcT−/− mice (Chiavegatto et al., 2000) are normalized in GalNAcT−/−-Tg(neuronal) mice. Additionally, the characteristic tremor and ataxia observed in the GalNAcT-deficient mice that has been likened to parkinsonism (Wu et al., 2011) is also completely attenuated in GalNAcT−/−-Tg(neuronal) mice. The precise source(s) of the gross behavioral abnormalities in GalNAcT−/− mice has never been established because of the remarkably preserved gross brain architecture (Takamiya et al., 1996) combined with the multitude of cell biological functions modulated by gangliosides (Wu et al., 2005;
Ohmi et al., 2009). A prominent suggestion is that complex ganglioside deficiency leads to impairment of axon–myelin stability and consequent axonal degeneration (Sugiuira et al., 2005; Schnaar, 2010). The myelin structural abnormalities seen in GalNAcT-/- mice and their reversal in at least some sites in GalNAcT-/- Tg(neuronal) mice point toward an impairment in myelination and axo-glial junction formation as at least partially responsible for the behavioral phenotype rather than being directly attributable to the rather low frequency of degenerate axons. Electrophysiologically, we only examined peripheral myelinated axons; although confirming peripheral CV slowing in GalNAcT-/- mice and axo-glial invasion occurred in GalNAcT-/- Tg(neuronal) mice, here Kv1.1 mislocalization in ganglioside deficiency states is insufficient to directly cause lengthened Nav1.6 clusters in the PNS for all other genotypes (one-way ANOVA, p < 0.05). C. Representative trace for each genotype from which analysis was performed. D. The amplitude of the second pulse per interstimulus interval in paired pulse recordings were plotted per genotype; there were no significant changes to refractory period (two-way ANOVA, p > 0.05). ** and *** signifies significance compared with WT. ***p < 0.001. Individual mice data are plotted to demonstrate the variance in the recordings among animals.

Our principal finding that function is restored by neuronal rather than glial GalNAcT expression indicates a breakdown in the nodal/PN border that may be influenced by complex ganglioside interactions with ligands in both the glial and axonal membranes. Indeed, a breakdown in the axo-glial junction and subsequent lengthening of sodium channel domains and PN invasion by Kv channels is also seen in the sulfatide-deficient mouse (Ishibashi et al., 2002). Unexpectedly, despite the dogma that potassium channels mislocalize to the paranodes attributable to loss of transverse bands, here Kv1.1 invasion occurred in GalNAcT-/- Tg(gliarial) mice although we observed intact transverse bands ultrastructurally, albeit in qualitative rather than quantitative observations. It is possible that the physical barrier provided by transverse bands is only one component required for Kv1.1 localization, additionally requiring specialized lipid raft associated anchoring domains that involve gangliosides (Gu and Gu, 2011). Normal refractory periods were recorded in all mice, suggesting that the Kv1.1 channel mislocalization in ganglioside deficiency states is insufficient to directly result in major functional disruption.
Huntington’s disease (HD) is an inherited neurodegenerative disorder characterized by motor, cognitive, and psychiatric symptoms. It is caused by the expansion of a CAG trinucleotide repeat in the Huntington’s disease (HD) gene, which results in the production of a toxic polyglutamine tract in the huntingtin protein (Htt). The polyglutamine tract leads to the aggregation of Htt, which disrupts cellular functions and results in neuronal death. The exact mechanism of how the polyglutamine tract causes neurodegeneration is still not fully understood, but it is thought to involve impaired protein homeostasis, oxidative stress, and synaptic dysfunction.

Another key factor in HD is the role of gangliosides, which are complex glycosphingolipids that are abundant in the central nervous system (CNS). Gangliosides have been implicated in the regulation of membrane trafficking, signal transduction, and cell adhesion. In HD, the expression of certain gangliosides is altered, which may contribute to the pathology of the disease.

The role of gangliosides in HD is primarily studied using animal models. For example, mice expressing the human Htt with a polyglutamine tract develop HD-like symptoms, including motor and cognitive impairments. These models have been used to investigate the role of gangliosides in HD. For instance, mice deficient in the enzyme that synthesizes a specific ganglioside, GM3, show improved survival and reduced motor symptoms compared to control mice. This suggests that gangliosides may be a therapeutic target for HD.

In summary, gangliosides play a crucial role in the regulation of cellular functions, and their dysregulation may contribute to the pathogenesis of Huntington’s disease. Further research is needed to fully understand the role of gangliosides in HD and to develop targeted therapies for this disease.

References:


