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 glycosphingolipids 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 neuronal-specific and glial-specific GalNAcT rescue mice crossed on the global GalNAcT+/− background [GalNAcT+/−-Tg(neuronal) and GalNAcT+/−-Tg(gial)] 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(gial) resembled GalNAcT−/− mice, exhibiting progressive tremor, weakness, and ataxia with aging. Quantitative electron microscopy demonstrated that GalNAcT+/− and GalNAcT−/−-Tg(gial) 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(gial) 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 glycosphingolipids widely expressed in vertebrate plasma membranes and intracellular compartments

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Ledeen and Wu, 2011; Yu et al., 2011; Sandhoff and Harzer, 2013). They are particularly enriched in nervous system gray and white matter, in which they have roles in development and maintenance, cell–cell recognition, signal transduction, growth, and motility. They are key components of lipid raft membrane domains (Sonnino et al., 2007; Ohmi et al., 2012) and function as receptors for microbial pathogens and toxins (Fewou et al., 2013; Ravindran et al., 2013) and neuropathy-associated auto-antibodies (Willison and Yuki, 2002; Kusunoki et al., 2008). Gangliosides are synthesized by stepwise addition of monosaccharides to ceramide by glycosyltransferases (Hakomori, 1981; Yu et al., 2004; Fig. 1B). The predominant gangliosides found in neural tissue are the complex gangliosides GM1, GD1a, GD1b, and GT1b, likely because of high activity of their synthesizing enzyme, β-1,4-N-acetylgalactosaminyltransferase 1 (GalNAc–transferase; GalNAcT, encoded by the B4galnt1 gene) in the nervous system (Dicsere and Dain, 1971). Transgenic mice with disruptions in specific glycosyltransferase genes, and thus deficient in downstream ganglioside biosynthesis, have been critical tools in advancing understanding of
Figure 1. Expression of GalNAcT and complex gangliosides in rescue mice. (A), Constructs were generated to drive GalNAcT expression in the neurons and glia of GalNAcT−/− mice by promoting the GalNAcT gene under the control of the hNFL and mPLP promoters, respectively. (B), Ganglioside biosynthetic pathway. The GalNAcT enzyme is necessary for generation of complex gangliosides (surrounded by the green box). (C), TLC of extracts from brains of WT, GalNAcT−/−, GalNAcT−/−-Tg(neuronal), and GalNAcT−/−-Tg(glia) mice, sprayed with resorcinol to label gangliosides. Standards for the complex gangliosides GM1, GD1a, GD1b, and GT1b are labeled in the right lane. WT extracts contain all complex gangliosides, which are absent from GalNAcT−/− extract and restored in GalNAcT−/−-Tg(neuronal) and GalNAcT−/−-Tg(glia) mice. GalNAcT−/− extracts are enriched with simple gangliosides. GalNAcT−/− extracts are also enriched with simple gangliosides and additionally express the complex gangliosides but at lower levels than WT. (D), Chromatograms of extracted major gangliosides in mouse brains of all genotypes confirm TLC findings. All major gangliosides can be putatively detected. The complex gangliosides GT1b, GD1b, GD1a, and GM1 are the most abundant gangliosides found in WT extracts. These are also present in rescue mice at lower levels. GD3, 9-O-Ac(etyl)-GD3, and GM3 are highly abundant in GalNAcT−/− extracts and remain at high levels in both rescue mice.

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; Okada 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 is 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 neurally (GalNAcT driven by the neurofilament-light (NFL) promoter; GalNAcT−/−-Tg(neuronal)) or in myelin (GalNAcT driven by the proteolipid protein (PLP) promoter;
GalNAcT{\textsuperscript{−/−}}-Tg(glial) and analyzed them for behavioral and structural abnormalities.

Materials and Methods

Generation of transgenic mice

The generation of GalNAcT{\textsuperscript{−/−}}-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 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 cDNA was cloned into the pGCHNF-L vector (provided by J.-P. Julien, Laval 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{Tg(neuronal)} mice to create GalNAcT{Tg(neuronal)} and GalNAcT{Tg(glial)} mice, respectively, that were used in all analyses. Evidence for GalNAcT enzyme activity restoration and complex gangliosides 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 Tg(neuronal), the latter being prominently restored in.Ptr

Lipid analysis

Extraction and TLC. Gangliosides were extracted from brains removed and snap frozen from WT, GalNAcT{\textsuperscript{−/−}}, GalNAcT{Tg(neuronal)}, and GalNAcT{Tg(glial)} mice (Fig. 1C,D). Immuno-reacting 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, 2 NaHCO₃, 10 glucose, pH 7.4) was pregassed with 95% O₂/5% CO₂.

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 AgAb for 5 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 AgAb 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%
Furukawa et al., 2008

GalNAcT enzyme activity and complex ganglioside expression was calculated for every interstimulus interval and plotted. Four- to 6-month-old mice were used. 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 and Alexa Fluor 647 goat anti-rabbit (1:1000) and Alexa Fluor 488 goat anti-mouse (1:500) antibodies. Nerves were imaged and assessed using NIH ImageJ software. GalNAcT enzyme activity is shown by TLC and enzyme activity assays 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<sup>−/−</sup>-Tg(neuronal) and GalNAcT<sup>−/−</sup>-Tg(gial) mice

Four neuronal and four glial transgenic lines were backcrossed onto GalNAcT<sup>−/−</sup> 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<sup>−/−</sup>-Tg(neuronal) and 11.7% in GalNAcT<sup>−/−</sup>-Tg(gial) and undetectable in whole-brain homogenate from GalNAcT<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup>-Tg(neuronal) and GalNAcT<sup>−/−</sup>-Tg(gial) 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<sup>−/−</sup>-Tg(neuronal) and GalNAcT<sup>−/−</sup>-Tg(gial) 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<sup>−/−</sup>-Tg(neuronal) mice (Fig. 2). Complex ganglioside expression was absent on axons of GalNAcT<sup>−/−</sup> mice as expected. In GalNAcT<sup>−/−</sup>-Tg(gial) 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<sup>−/−</sup>-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<sup>−/−</sup>-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<sup>−/−</sup> mice

Because GalNAcT<sup>−/−</sup> 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, GalNAcT/H11002/H11002 and GalNAcT/H11002/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) GalNAcT/H11002/H11002 mice exhibited excessive facial scratching with excoriation and hair loss, not seen in other genotypes, including GalNAcT/H11002/H11002 (data not shown). GalNAcT/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 GalNAcT/H11002 and GalNAcT/H11002-Tg(glial) mice from 6 months of age, as revealed by post hoc tests. GalNAcT/H11002 and GalNAcT/H11002-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 GalNAcT/H11002-Tg(neuronal) mice.

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

Ultrastructural abnormalities in GalNAcT/H11002 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, GalNAcT/H11002, GalNAcT/H11002-Tg(neuronal), and GalNAcT/H11002-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 2. Restoration of complex ganglioside expression in PNS and CNS tissue identified by anti-ganglioside mAb immunostaining. Complex ganglioside at the NMJ (PNS) and in the ventral spinal cord (CNS) are labeled by mouse monoclonal AgAb, followed by detection with a fluorescently labeled anti-mouse IgG antibody (green in merged images). In nerve–muscle preparations, the NMJs are identified by fluorescently conjugated BTx, which binds the AChR on the postsynaptic membrane. The axons of ventral columns in spinal cord sections are identified by anti-neurofilament antibody (red). Complex gangliosides are present on nerve fiber axolemma in WT and GalNAcT/H11002-Tg(neuronal) mice in both preparations and absent in the GalNAcT/H11002 mice. Perisynaptic Schwann cells at the motor nerve terminal of the GalNAcT/H11002-Tg(glial) mice are positive for AgAb, and AgAb immunoreactivity also surrounds the neurofilament marker in the ventral spinal cord. Scale bars, 10 μm.
performance. None of the parameters measured were significantly different among the four genotypes at 2 months (data not shown); however, by 12 months, significant pathological deviations in genotypes were seen (Fig. 4), confirming the age-dependent nature of neurodegeneration and corresponding to the timeframe of the development of behavioral deficiencies.

At 12 months of age, the density (cord) or number (OpN and SN) of degenerate axons is significantly greater in both GalNAcT\(^{-/-}\) and GalNAcT\(^{-/-}\)-Tg(neuronal) SN and in OpN for GalNAcT\(^{-/-}\) and ventral spinal cord for GalNAcT\(^{-/-}\)-Tg(glial) compared with WT and GalNAcT\(^{-/-}\)-Tg(neuronal) mice (one-way ANOVA, \(p < 0.05\); Fig. 4A). In contrast GalNAcT\(^{-/-}\)-Tg(neuronal) mice have no significant axon degeneration in any of the three sites assessed compared with WT. Degenerate axons are indicated by red arrowheads in representative images in Figure 4C. Average myelin volume varies significantly among genotypes (one-way ANOVA, \(p < 0.05\)) and also varies between neural tissues (Fig. 4B). GalNAcT\(^{-/-}\) and GalNAcT\(^{-/-}\)-Tg(glial) myelin volume is significantly reduced compared with WT mice in all three sites. GalNAcT\(^{-/-}\)-Tg(neuronal) myelin volumes does not significantly differ from WT in any site. Together, these findings indicate that GalNAcT\(^{-/-}\)-Tg(glial) mouse morphology (degenerate axon number and myelin volume) is not significantly different from GalNAcT\(^{-/-}\), whereas neuronal expression of gangliosides in GalNAcT\(^{-/-}\)-Tg(neuronal) is sufficient to attenuate age-related axonal degeneration and myelin volume changes.

In qualitative observations, abnormal organelle-filled axons and redundant myelin (Fig. 4C, indicated by red asterisks and arrows, respectively) were frequently observed in OpN axons from GalNAcT\(^{-/-}\) and GalNAcT\(^{-/-}\)-Tg(glial) mice. Whereas myelin volume measurements were quantitatively normal in GalNAcT\(^{-/-}\)-Tg(neuronal) mice, the myelin appeared looser than in WT, suggesting that subtle defects in compaction were not ameliorated in either this or the GalNAcT\(^{-/-}\)-Tg(glial) mice.

**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. 5A,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 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 WT, suggesting that subtle defects in compaction were not ameliorated in either this or the GalNAcT\(^{-/-}\)-Tg(glial) mice.
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\(^{-/-}\)-Tg(glial) and GalNAcT\(^{-/-}\)-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. 6D). In all three complex ganglioside-deficient genotypes, the PN loops appear disorganized and aberrantly stacked, particularly at the nodal/PN region in GalNAcT\(^{-/-}\)-Tg(glial) and GalNAcT\(^{-/-}\)-Tg(neuronal) mice. These focal areas of disorganization may correspond to the sites of Caspr-filled protrusions observed by immunostaining. Whereas transverse bands between axons and PN loops are absent at the PN/JPN border of GalNAcT\(^{-/-}\) mice, we observed that they were present here both in GalNAcT\(^{-/-}\)-Tg(glial) and GalNAcT\(^{-/-}\)-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\(^{-/-}\), GalNAcT\(^{-/-}\)-Tg(glial), or GalNAcT\(^{-/-}\)-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 in all tissues studied for GalNAcT\(^{-/-}\) and GalNAcT\(^{-/-}\)-Tg(glial) mice compared with WT and also compared with GalNAcT\(^{-/-}\)-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\(^{-/-}\) and GalNAcT\(^{-/-}\)-Tg(glial) mice. Organelle-filled axons and redundant myelin occurred frequently in GalNAcT\(^{-/-}\)-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 \(\mu m\); OpN, 1 \(\mu m\); SN, 25 \(\mu m\).
Figure 5. Restoration of normal nodal architecture by expression of complex gangliosides in neurons of GalNAcT<sup>−/−</sup> 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<sup>−/−</sup> and GalNAcT<sup>−/−</sup>-Tg(glial) mice compared with WT and GalNAcT<sup>−/−</sup>-Tg(neuronal) mice SN. Consequently, the distance between Kv1.1-positive domains significantly decreased for both genotypes. This distance was lengthened in GalNAcT<sup>−/−</sup>-Tg(neuronal) mice compared with WT. The number of PN Caspr staining protrusions significantly increased for GalNAcT<sup>−/−</sup> and GalNAcT<sup>−/−</sup>-Tg(glial) SN compared with WT and GalNAcT<sup>−/−</sup>-Tg(neuronal) levels, which were comparable. Compared with WT nerve, Caspr staining length was significantly increased in GalNAcT<sup>−/−</sup> and GalNAcT<sup>−/−</sup>-Tg(glial) SN compared with GalNAcT<sup>−/−</sup>-Tg(neuronal). The length of Nav1.6 immunostaining significantly increased in GalNAcT<sup>−/−</sup> and both rescue mice compared with WT. C, To scale, schematic representing the length of staining in each domain per genotype. One-way ANOVA, <i>p</i> < 0.05. * signifies significance compared with WT; # signifies significance compared with GalNAcT<sup>−/−</sup>-Tg(neuronal). * and #, <i>p</i> < 0.05; ** and ##, <i>p</i> < 0.01; *** and ###, <i>p</i> < 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; Sugiuira 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 GalNAC" deficiency states. These findings clearly demonstrate the importance of neuronally expressed GalNAC in maintaining nervous system integrity throughout the lifespan.

Certain technical caveats may affect the interpretation of these results. The targeting strategy we adopted used NFL and PLP promoters to drive GalNAC 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 mRNA has been described in non-myelinating Schwann cells (Fabrizi et al., 1997; Sotelo-Silveira et al., 2000), thereby providing one explanation for the presence of complex ganglioside immunoreactive perisynaptic Schwann cells we observed at the GalNAC"-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 GalNAC 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 Heffer-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 GalNAC"-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. GalNAC" mice have increased expression of the simple gangliosides (Takamiya et al., 1996; Furukawa et al., 2008). Both GalNAC"-Tg(neuronal) and GalNAC"-Tg(glia) retained high levels of these simple gangliosides, although we do not know whether these were present in GalNAC"-deficient cells or were still accumulated in GalNAC"-replete cells containing rate-limiting levels of enzyme activity. Although it has been proposed previously that neurodegeneration in GalNAC" 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 GalNAC"-Tg(neuronal) mice and was absent in both compartments in GalNAC" mice. GalNAC"-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 GalNAC" 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 GalNAC" mice (Chiavegatto et al., 2000) are normalized in GalNAC"-Tg(neuronal) mice. Additionally, the characteristic tremor and ataxia observed in the GalNAC"-deficient mice that has been likened to parkinsonism (Wu et al., 2011) is also completely attenuated in GalNAC"-Tg(neuronal) mice. The precise source(s) of the gross behavioral abnormalities in GalNAC" 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;
A prominent suggestion is that complex ganglioside deficiency leads to impairment of axon–myelin stability and consequent axonal degeneration (Sugiura 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 their reversal in at least some sites in GalNAcT−/−-Tg(neuronal) mice, we were not able to confirm peripheral CV slowing in GalNAcT−/−-Tg(glial) mice, indicating that peripheral nerve CV defects cannot be responsible for the clinical phenotype, although the situation may differ in the CNS, which is the more likely source of the phenotype.

Sodium channel dispersion and myelin sheath disruption lead to conduction slowing (Gutiérrez et al., 1995; Ichimura et al., 2005). The necessary spatial separation of sodium and potassium channels at the NoR is provided by PN loop contact with the axolemma and axo-glial transverse band formation (Rasband et al., 2003; Rosenbluth et al., 2003). Our immunohistochemical data demonstrate lengthened Nav1.6 clusters in the PNS for all ganglioside-deficient genotypes compared with WT, most apparent in GalNAcT−/−-Tg(glial) mice. This correlates with our CV data. Additionally, PN loops only form orderly attachments on the axon in WT mice and instead have stacked PN loops in all mutants, primarily seen at the nodal border. This suggests that the abnormal Nav1.6 distribution may be attributable to abnormal PN loop formation. Lengthening of sodium channel domains reported here and previously in GalNAcT−/− mice (Susuki et al., 2007) and prominently in GalNAcT−/−-Tg(glial) mice 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(glial) 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.

Our principal finding that function is restored by neuronal rather than glial GalNAcT expression indicates the requirement for an axonally expressed ligand in fine tuning the interacting molecules at axo-glial junctions that are required for maintaining myelin, axonal, and nodal integrity. The complex gangliosides GD1a and GT1b have been described as axonal ligands for myelin-associated glycoprotein (MAG) localized on the innermost wrap of the myelin sheath (Collins et al., 1997). The age-related degenerative phenotype, functional, and morphological deficits displayed by GalNAcT−/− mice parallel those found in

**Figure 7.** Peripheral nerve functional recovery in rescue mice. Analysis of extracellular recordings from 4- to 6-month-old GalNAcT−/−, GalNAcT−/−-Tg(neuronal), and Tg(glial) SN show a reduction in CV that does not reach significance (A). B, Rate of rise of the compound nerve action potential is significantly reduced compared with WT 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 < 0.01, ***p < 0.001. Individual mice data are plotted to demonstrate the variance in the recordings among animals.
MAG null mice and mice created from interbreeding the two genotypes (Pan et al., 2005). Restoration of MAG–ganglioside interaction in GalNAcT−/−-Tg(neuronal) mice might thus account for at least some components of the preserved phenotype. Other abundant gangliosides, GM1 and GD1b, are likely to also have MAG-independent neuroprotective roles. Complex gangliosides, particularly GM1, are an integral member of membrane-specific microdomains, lipid rafts (Simons and Ikonen, 1997), that serve many signaling and trafficking functions that could be disrupted by ganglioside loss, such as complement regulator dysfunction (Ohmi et al., 2009). Lipid rafts are also involved in the recruitment and stabilization of the glial adhesion molecule neurofascin 155 at the NoR (Schafer et al., 2004). Indeed, GalNAcT−/− mice have mislocalized neurofascin at the NoR (Susuki et al., 2007), and mice lacking neurofascin fail to develop normal NoR (Sherman et al., 2005), demonstrating the importance of such an interaction.

The global loss of complex gangliosides results in an obvious behavioral phenotype whose specific origin is unknown but likely attributable to multiple impairments. Here we show that the wide-ranging degenerative features and consequent behavioral phenotype can be overcome by the expression of complex gangliosides limited to neuronal membranes. Strikingly, glial expression of complex gangliosides does not have the same effect. This demonstrates the necessity for site-specific expression of complex gangliosides in maintenance of the nervous system and opens up experimental avenues for more precisely defining the role of complex gangliosides in nervous system maintenance. The discovery of human disorders of ganglioside biosynthesis (Simpson et al., 2004; Boukhris et al., 2013) and autoimmune disorders in which gangliosides are targets (Willson and Plomp, 2008) adds important clinical dimensions to this area.

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