A Functional Role for Complex Gangliosides: Motor Deficits in GM2/GD2 Synthase Knockout Mice

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Although gangliosides are abundant molecular determinants on all vertebrate nerve cells (comprising ≈1.5% of brain dry weight) their functions have remained obscure. We report that mice engineered to lack a key enzyme in complex ganglioside biosynthesis (GM2/GD2 synthase), and which express only the simple ganglioside molecular species GM3 and GD3, develop significant and progressive behavioral neuropathies, including deficits in reflexes, strength, coordination, and balance. Quantitative indices of motor abilities, applied at 8 and 12 months of age, also revealed progressive gait disorders in complex ganglioside knockout mice compared to controls, including reduced stride length, stride width, and increased hindpaw print length as well as a marked reduction in rearing. Compared to controls, null mutant mice tended to walk in small labored movements. Twelve-month-old complex ganglioside knockout mice also displayed significant incidence of tremor and catalepsy. These comprehensive neurobehavioral studies establish an essential role for complex gangliosides in the maintenance of normal neural physiology in mice, consistent with a role in maintaining axons and myelin (Sheikh, K. A., J. Sun, Y. Liu, H. Kawai, T. O. Crawford, R. L. Proia, J. W. Griffin, and R. L. Schnaar. 1999. Mice lacking complex gangliosides develop Wallerian degeneration and myelination defects. Proc. Natl. Acad. Sci. USA 96: 7532-7537), and may provide insights into the mechanisms underlying certain neural degenerative diseases.

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system axon degeneration and dysmyelination consistent with a role for complex gangliosides in axon–myelin interactions (28). As these animals aged, behavioral deficits consistent with neural degeneration appeared in GalNAcT\(^{2/2}\) mice. Comprehensive neurobehavioral studies, reported here, establish an essential role for complex gangliosides in the maintenance of normal neural physiology in mice and may provide insights into certain neural degenerative diseases.

**MATERIALS AND METHODS**

**Animals**

Thirty-six male mice including those with a disrupted gene for GM2/GD2 synthase GalNAcT\(^{-/-}\), heterozygotes GalNAcT\(^{+/--}\), and wild-type littersmates GalNAcT\(^{+/+}\) were used (19). Mice were tested at 7–9 months old (8-month-old group; 12 mice each genotype), and at 11–13 months old (12-month-old group; 11 knockout, 9 heterozygote, 7 wild type). Mice were individually housed for 15 days prior to the experiments and maintained in a light-dark (14:10) photoperiod (lights on at 0700 h) in a temperature- and humidity-controlled room. Food was placed on the floor of the cage and the water bottle nozzle was also located close to the floor. All behavioral tests were conducted between 1300–1800 h with randomly chosen mice by two investigators who were uninformed of their genotypes. Mice were allowed to acclimate in the testing room 1 h prior to experiments. All mice were tested on the same day for each behavioral parameter. The sequence of behavioral tests was standardized (as follows) and used for both 8- and 12-month-old groups.

Care and handling procedures were in accordance with institutional and government guidelines.

**Neurological Assessment**

Visual placing response. Each mouse was lifted vertically midtail ∼15 cm above the edge of a table and slowly lowered to elicit the visual placing response, characterized by an extension of fore- and hindlimbs before contact. A positive score of 1 was recorded if the animal extended its forepaws before touching the table. The mean score was recorded in three consecutive trials.

Negative geotaxis. Each mouse was placed, facing downward, at the center of a wire mesh screen tilted at 45° and 75 cm above a table. The latency to turn to face upward was recorded up to 120 s in three consecutive trials.

Beam balance. Each mouse was placed at the center and perpendicular to a rounded wooden beam (60-cm length, 2-cm diameter) suspended 60 cm above a foam pillow. The mean latency to fall (up to 120 s) was registered in three consecutive trials.

Forelimb grip strength. Each mouse was suspended by its forepaws on a rounded metal bar (diameter 3 mm) positioned 60 cm above a foam pillow. The forelimb grip strength was assessed by measuring the mean time (up to 30 s) the mice hung from the bar in three consecutive trials.

Hindlimb reflex extension (2). Each mouse was suspended by its forelimbs on a rounded metal bar (diameter 3 mm) positioned 60 cm above a foam pillow. The latency of hindlimbs to extend was scored as follows: 0, one or both hindlimbs paralyzed; 1, loss of reflex and hindlimbs and paws held close to the body with clasp- ing toes; 2, loss of reflex with flexion of hindlimbs; 3, hindlimbs extended to form ∼90° angle; 4, hindlimbs extended to form >90° angle.

Platform. Each mouse was placed at the center of a wooden dowel bridge (60-cm length, 2-cm diameter, suspended 60 cm above a foam pillow) facing one of the platforms on either end of the bridge (15 × 15 cm). The mean latency to reach either platform with four paws (up to 60 s) was registered in three consecutive trials. In each trial the mouse faced alternate platforms and a score of 60 s was given to the animal that failed to reach the platform or fell during the test.

Motor coordination and balance (rotarod). A rotarod apparatus (Economex, Columbus Instruments, Columbus, OH) was used in both a fixed speed and accelerating speed modes. Fixed speed: Each mouse was placed in a separate lane of the apparatus on a rotating cylinder (5 cm diameter) at 5 rpm. Accelerating speed: The mouse was placed on a stopped rod. Rotation was initiated at 2.5 rpm and increased in increments of 0.2 rpm every second. The performance

**FIG. 1.** Biosynthesis of major brain gangliosides. The relationships between major brain gangliosides and their precursors are shown schematically, along with the ganglioside nomenclature of Svennerholm (31). The block in ganglioside biosynthesis due to disruption of the GalNAcT gene (19) is indicated by a vertical double line.
was recorded as the mean latency to fall from the rod (up to 300 s) in 3 consecutive trials with a resting period between each trial. These two tests were conducted on different days.

Open field activity. Each mouse was placed in the center of an acrylic open arena (1 m$^2$) with the floor marked off in 42 squares. The frequency of locomotion (number of squares entered with all four paws), rearing (number of times the mouse stood on its hindlimbs), and grooming (face or body grooming) were recorded during 300 s. The latency to leave the central area toward the squares along the walls (border of 10 cm) was also registered. The floor of the arena was washed with a 5% alcohol solution between experimental trials. Each trial was videotaped and scored by two observers uninformed of the genotype.

Walking pattern (9). Each mouse was placed in a confined runway (5.5 cm wide, 60 cm long, and 15 cm high) illuminated by a bright light with a dark chamber at the end. After two or three trials, when the animals walked reproducibly to the dark compartment, each mouse was briefly suspended by the tail and a nontoxic ink was painted onto its hindpaws with a small paintbrush. It was then allowed to walk over a strip of paper placed in the floor of the corridor. A series of at least six prints of each foot, recorded in two tests, were used in obtaining the measurements. Stride length (largest distance between two consecutive hindpaw prints on each side), stride width (largest distance perpendicular to parallel left and right paw prints), and print length (the longest print) were determined.

Tremor. Each mouse was scored for the presence or absence of whole body tremor (at resting or during movement) in an observational plastic cage with acrylic cover in three consecutive observations of 10 s interspersed with an interval of 10 s. The scoring began 1 min after being placed in the test cage.

Catalepsy. Each mouse was placed with its hindlimbs on a flat surface and its forelimbs on a horizontal metal bar suspended 5 cm above the surface. The immobility state (time that the animal spent motionless hanging from the bar) was recorded in three consecutive trials. If an animal fell or climbed the bar, it was placed immediately on the bar again; the test was terminated after a maximum of three such escapes.

Statistical analyses. A two-way repeated measures ANOVA (genotype $\times$ age) was used to compare the groups at 8 and 12 months old. Pairwise comparisons were conducted using Tukey test. Mean differences were considered statistically significant when $P < 0.05$. In a few instances, the omnibus F test was not significant ($P > 0.05$) but the statistical analyses proceeded with a priori planned comparisons. This statistical approach is consistent with recommendations for analyses of planned comparisons (16). For tremor and catalepsy tests, Kruskal–Wallis followed by Dunn's method were employed.

RESULTS

Balance, Coordination, Strength, and Reflexes

Mice lacking complex gangliosides displayed highly significant progressive behavioral deficits consistent with ongoing neural degeneration. The most striking deficits were in the areas of balance, coordination, muscle strength, and reflexes. GalNAcT $^{1/-}$ mice were clearly distinguished from wild-type and heterozygote littermates based on hindlimb reflexes (Figs. 2a and 2b). Normally, mice lifted by the tail extend their hindlimbs outward at an angle of 90–120°. Whereas this behavior was seen with wild-type and heterozygote mice, GalNAcT $^{1/-}$ mice displayed impaired hindlimb extension, with most animals retracting the hindlimbs, which were held close to their bodies with an inversion and flexion of the paws (Fig. 2a). The severity of this profile was equally evident at 8 and 12 months of age (Fig. 2b). Forelimb grip strength showed progressive impairment in GalNAcT $^{1/-}$ mice, with statistically significant reduction at 12 months of age in GalNAcT $^{2/-}$ mice (Fig. 2c).

Balance was also significantly impaired in GalNAcT $^{1/-}$ mice (Fig. 3). Wild-type and heterozygote mice routinely remained on the beam for the maximum allowed time (120 s), whereas GalNAcT $^{1/-}$ mice showed a deteriorating ability to do so with age (Fig. 3b). The rotarod test, the time an animal remains on a rotating rod, is an objective measure of motor balance, coordination, and control (17, 24). Compared to wild-type littermates, GalNAcT $^{1/-}$ mice displayed a marked impairment in their performance on the rotarod at a fixed speed (Fig. 3c) and at an accelerating speed (not shown). Heterozygote mice displayed less severe deficits, which were significantly different from wild type littermates at 8 but not 12 months of age.

The time required for mice to reach a platform along a balance beam was significantly increased in both GalNAcT $^{1/-}$ and GalNAcT $^{2/-}$ mice at both ages tested (Fig. 3d). Instead of walking and maintaining a stable upright posture, knockout mice at both ages displayed ventral recumbence, with their entire body flattened against the surface of the beam, and their hind- and forelimbs wrapped laterally around the beam (see Fig. 3a). While some of the GalNAcT $^{1/-}$ mice failed to maintain their balance and fell, others used their forelimbs to drag themselves along the beam; their hindlimbs were generally unutilized.

Visual acuity and spatial perception, measured as visual placing (15), did not differ as a function of genotype at 8 months of age, but a marked impairment in
the percentage of mice demonstrating the appropriate placing response was observed in 12-month-old GalNAcT (2/2) mice (30 ± 6.9%, P > 0.05). Visual ability of 12-month-old mice was further tested by moving an object (cotton swab) toward each mouse and recording their reactions in three consecutive trials. All three genotypes had the maximum score (avoiding or trying to investigate the object), indicating similar visual capability (data not shown). The poor performance in the visual placing task suggests impairment in extending the forelimbs to reach the edge of the table (motor deficit), a slow reaction time (decreased reflex), or possibly a deficit in positional placing (spatial deficit). The negative geotaxis test is indicative of spatial placing because normal mice avoid the downward position by turning upward. No significant differences were seen between mutant and wild-type mice in this task (data not shown), indicating that GalNAcT (2/-) perceive spatial orientation. The poor performance in visual placing and forelimb grip tests at 12 months of age (Fig. 2c) points to a progressive functional impairment in the forelimbs of GalNAcT (2/-) mice.

**Walking Pattern** (9) and Open Field Behavior

Although heterozygote and wild-type mice showed similar walking patterns at both ages tested, the GalNAcT (2/-) mice exhibited significantly impaired walking performance (Fig. 4). The gait of GalNAcT (2/-) mice was slower and they tended to walk in small labored movements as compared to wild-type littermates. Stride length was significantly reduced in GalNAcT (2/-) mice compared to wild-type mice (Fig. 4a) with a narrowing in the base of support (stride width, Fig. 4b). There was an increase in the hindpaw print length in the GalNAcT (2/-) mice at both ages (Fig. 4c). Print length is short in normal animals, which walk on their toes, and is increased in animals with nerve damage, which place the entire foot on the ground (9).

To assess motor and exploratory behavior, each mouse was placed in the center of an open field. Latency to leave the central area, locomotor activity, rearing, and grooming were determined in a 5-min test. Although no statistical difference in locomotor activity was detected among 8-month-old mice, significantly reduced locomotor activity, measured as the number of squares traversed in the open field in 5 min, was observed in 12-month-old GalNAcT (2/-) and GalNAcT (1/-) mice (Fig. 5a). This deficit was completely attributable to a hesitation in initiating movement rather than a deficit in locomotor velocity. GalNAcT (2/-) mice (and to a lesser extent GalNAcT (1/-) littermates) took significantly more time to leave the center square of the open field (Fig. 5b). To account for increased latency, locomotion velocity after initiation was calculated as the number of squares traversed by the mice after exiting the center square, as a function
of time remaining in the test. This revealed equivalent locomotor velocity after initiation for all genotypes and age groups tested (data not shown), indicating decreased motivation to explore the new environment rather than a locomotor deficit per se.

Rearing behavior was severely affected in GalNAcT (−/−) mice at both ages tested. Although the aging process per se significantly reduced rearing behavior in wild-type mice (between 8 and 12 months), aging resulted in even more notable rearing impairment in GalNAcT (−/−) mice (Fig. 5c). GalNAcT (+/−) mice displayed an intermediate rearing behavior. Grooming behavior was not significantly different among the mice (data not shown).

Tremor and Catalepsy

A marked whole body tremor was apparent in GalNAcT (−/−) mice at 12 months of age. When quantified as percentage of incidence of tremor in repeated tests, wild-type mice were generally free of tremor (14 ± 10%), whereas all GalNAcT (−/−) mice showed tremors in most of the trials (90 ± 0.5%, P < 0.05 relative to wild type) and some GalNAcT (+/−) mice had some episodes of tremor as well (52 ± 11%, P > 0.05). During the forelimb grip test at 12 months of age, GalNAcT (−/−) mice displayed rigidity and a static position. When catalepsy was quantified, GalNAcT (−/−) mice spent significantly longer hanging motionless on the bar in a vertical position (27.3 ± 6.1 s, P < 0.05) as compared to wild-type littermates (6.7 ± 2.7 s), whereas GalNAcT (1/2) mice displayed intermediate behavior (18.2 ± 7.8 s, P > 0.05).

At 8 and 12 months of age, GalNAcT (−/−) mice were distinguished from wild-type littermates by a modest (9–11%), but statistically significant (P < 0.01) reduction in body weight, which was measured prior to the first set of behavioral experiments at each age. However, the percentage weight gain over the 4 months between tests was similar (9–10%). The modest difference in weight would not be expected, per se, to impair behavioral performance.

FIG. 3. Motor coordination and balance. (a) 8-Month-old mice show different strategies to move along a round wooden beam. Whereas the GalNAcT (+/+) mouse (upper panel) walks in a stable upright posture, the GalNAcT (−/−) mouse (lower panel) displays a ventral recumbence, with the body flattened against the beam, typically keeping hind- and forelimbs wrapped laterally and using them to drag itself along the beam. (b) Balance, evaluated as the time each mouse remained on a 2-cm-diameter round beam (up to 120 s). The GalNAcT (−/−) mice at 8 and 12 months of age had impaired performance compared to wild-type mice (P < 0.001 at both ages) with an age-dependent impairment in this genotype (P < 0.042). (c) Motor coordination was evaluated using a rotarod test at a fixed speed (5 rpm, 5 cm diameter) by measuring the latency to fall from the rod. The GalNAcT (−/−) mice displayed a marked impairment at 8 (P < 0.001) and 12 (P = 0.016) months of age compared to wild-type littermates. Heterozygote mice also had impaired performance at 8 months of age (P = 0.007). (d) Balance and coordination were measured as the time required for each mouse to reach a platform along a balance beam. This time was significantly increased in GalNAcT (−/−) mice (P = 0.036 at 8 months and P = 0.014 at 12 months of age) and in GalNAcT (+/−) mice (P = 0.033 at 8 months and P = 0.029 at 12 months of age) in comparison with wild-type littermates of the same age. Data are expressed as mean ± SE, and groups were compared by two-way, repeated measures analysis of variance (ANOVA), *P < 0.05, **P < 0.01 relative to wild-type littermates at the same age, post hoc Tukey tests. Tests were performed at 8 months of age (black bars) and 12 months of age (gray bars).
Gangliosides, although found in all vertebrate tissues, are remarkably abundant in brain, comprising ≈1.5% of the brain dry weight (33, 35). As prominent cell surface molecules on all nerve cells, they have been proposed to serve a multitude of functions from neuronal pathfinding to synaptic transmission (7, 30), and have been implicated in the pathology of neurodegenerative diseases from multiple sclerosis to Alzheimer’s disease (25, 32). Genetic mouse models with altered ganglioside expression provide an opportunity to test these proposals in vivo.

GalNAcT (−/−) mice, lacking all complex brain gangliosides, develop to reproductive age with grossly normal nervous system morphology and physiology (34), and their survival (up to 14 months) was not reduced compared to wild-type mice. However, the current results indicate a role for complex gangliosides in long-term neuronal stability and function, consistent with axonal degeneration and dysmyelination we reported in earlier neuroanatomical studies (28).

Total brain ganglioside and sialic acid expression are equivalent in GalNAcT (−/−) and wild-type mice (data not shown), indicating that the behavioral changes reported here are due to qualitative and/or quantitative

**FIG. 4.** Walking patterns. Nontoxic ink was applied to the hindpaws of each mouse, and the walking pattern recorded on a strip of paper placed on the floor of a runway. (a) Stride length was reduced in GalNAcT (−/−) mice at 8 (P = 0.048) and at 12 months of age (P = 0.01). (b) The stride width (base of support) was reduced in the null mutants at 8 (P < 0.001) and at 12 months of age (P = 0.031). (c) The hindpaw print length was increased at 8 (P = 0.007) and at 12 (P = 0.001) months of age in GalNAcT (−/−) mice, and an aging effect was significant (P < 0.001). Data are expressed as mean ± SE; groups were compared by two-way, repeated measures analysis of variance (ANOVA). *P < 0.05, **P ≤ 0.01 relative to wild-type littermates at the same age, post hoc Tukey test or t test in a priori planned comparisons. Tests were performed at 8 months of age (black bars) and 12 months of age (gray bars).

**FIG. 5.** Open field exploratory activity. (a) At 12 months of age both GalNAcT (+/−) (P = 0.04) and GalNAcT (−/−) mice (P = 0.001) traversed fewer squares compared to wild-type littermates. (b) The latency to exit the central area (starting point) towards the quadrants along the walls (border of 10 cm) was markedly increased in GalNAcT (−/−) mice at 8 (P = 0.039) and 12 (P = 0.015) months of age. (c) Rearing behavior was reduced in GalNAcT (−/−) mice at both ages (P = 0.014 at 8 and P = 0.02 at 12 months of age). An aging effect was detected in wild-type (P = 0.031) as well as in the null mutant (P = 0.039). Data are expressed as mean ± SE; groups were compared by two-way, repeated measures analysis of variance (ANOVA). *P < 0.05, **P ≤ 0.01 relative to wild-type littermates at the same age, post hoc Tukey test or t test in a priori planned comparisons. Tests were performed at 8 months of age (black bars) and 12 months of age (gray bars).
changes in the expression of specific ganglioside species. A potential molecular basis for neural degeneration is the absence of complex ganglioside ligands for myelin-associated glycoprotein (3, 4, 38), leading to a decrease in myelin-axon stability with accompanying nerve degeneration. GalNAcT (−/−) mice also have increased GD3, which is reported to be proapoptotic (8). However, neuronal cell bodies (motorneurons, dorsal root ganglion cells, and retinal ganglion cells) are normal in GalNAcT (−/−) mice compared to control mice, indicating a primary neuropathic process leading to axon degeneration and dysmyelination (28). Although in most behavioral tests heterozygotes were statistically indistinguishable from wild-type mice, they often showed behavioral trends which were intermediate between wild type and knockout, and these trends reached statistical significance for rotarod performance, platform acquisition, and locomotion. Ganglioside quantification in GalNAcT (+/−) mice (data not shown) indicates modestly reduced total ganglioside expression and increased GD3 expression, suggesting that qualitative changes in ganglioside expression may subtly affect behavior.

A deficit in the same enzyme targeted in the current animal model, GM2/GD2 synthase, was reported in a human patient (10, 20). The affected child had increased brain GM3 and GD3, reduced GM2/GD2 synthase levels, and lacked complex gangliosides. Unlike the animal model, the patient had an 80% reduction in total brain gangliosides and displayed severe deficits, including poor physical and motor development, seizures, and death in infancy. The more severe deficits may have been due to reduced total ganglioside expression, which is not recapitulated in the animal model. The genetic basis for the human disorder was not determined.

In addition to their accumulation in lysosomal storage diseases (e.g., Tay Sachs disease) (36), gangliosides are implicated as autoimmune targets in certain neurodegenerative disorders, including Guillain-Barré syndrome and multiple sclerosis (25, 27). There is a high correlation between circulating anti-GD1a antibodies and a form of Guillain-Barré syndrome marked by immunological attack on the axolemma (acute motor axonal neuropathy) (14). Although the autoimmune disorders may have a different molecular basis than the disorder quantified here, it is of note that the absence of these same target gangliosides results in behavioral deficits associated with axonal neuropathy.

As well as symptoms of peripheral axonal neuropathy, older complex ganglioside knockout mice display deficits consistent with central nervous system (CNS) dysfunction, including tremor, catalepsy, and latency to initiate movement. These deficits are consistent with our prior anatomical observations of neural degeneration in the CNS of these mutants (28). Although our data do not, per se, demonstrate selective susceptibility of any particular neural pathways, the behavioral deficits observed in older mutant mice warrant further exploration of dopaminergic systems. Catalepsy has been extensively used in rodents to characterize striatal dopamine dysfunction (6, 11). An observed increase in immobility when animals are placed on a hanging bar is obtained by neuroleptics and dopamine-blocking agents (5, 23, 39). The behavioral effects of dopamine dysfunction or block in rats and mice also include akinesia (18) and decreases in locomotion, exploration, rearing (1), and rotarod performance (24), all of which were seen in the complex ganglioside knockout mice.

Our data strongly implicate complex ganglioside structures in long-term nervous system maintenance and are consistent with their role in axon-myelin stabilization. Other potential neuroanatomical correlates in this model, as well as the molecular mechanisms by which ganglioside expression controls neural cell physiology, are issues for future investigation.

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