Published in final edited form as:

Neurosci Lett. 2019 January 23; 692: 53–63. doi:10.1016/j.neulet.2018.10.038.

Lentiviral-mediated knock-down of GD3 synthase protects against MPTP-induced motor deficits and neurodegeneration

Anandh Dhanushkodi¹, Yi Xue¹, Emily E. Roguski³, Yun Ding⁴, Shannon G. Matta³, Detlef Heck², Guo-Huang Fan⁴, and Michael P. McDonald^{1,2}

¹Department of Neurology, University of Tennessee Health Science Center, Memphis, TN 38163

²Department of Anatomy & Neurobiology, University of Tennessee Health Science Center, Memphis, TN 38163

³Department of Pharmacology University of Tennessee Health Science Center, Memphis, TN 38163

⁴Department of Pharmacology & Toxicology, Virginia Commonwealth University, Richmond, VA 23284

Abstract

Converging evidence demonstrates an important role for gangliosides in brain function and neurodegenerative diseases. Exogenous GM1 is broadly neuroprotective, including in rodent, feline, and primate models of Parkinson's disease, and has shown positive effects in clinical trials. We and others have shown that inhibition of the ganglioside biosynthetic enzyme GD3 synthase (GD3S) increases endogenous levels GM1 ganglioside. We recently reported that targeted deletion of *St8sia1*, the gene that codes for GD3S, prevents motor impairments and significantly attenuates neurodegeneration induced by 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The current study investigated the effects of GD3S inhibition on the neurotoxicity and parkinsonism induced by MPTP. Mice were injected intrastrially with a lentiviral-vector-mediated shRNA construct targeting GD3S (shGD3S) or a scrambled-sequence control (scrRNA). An MPTP regimen of 18 mg/kg $\times$ 5 days reduced tyrosine-hydroxylase-positive neurons in the substantia nigra pars compacta of scrRNA-treated mice by nearly two-thirds. In mice treated with shGD3S the MPTP-induced lesion was approximately half that size. MPTP induced bradykinesia and deficits in fine motor skills in mice treated with scrRNA. These deficits were absent in shGD3S-treated mice. These results suggest that inhibition of GD3S protects against the nigrostriatal damage, bradykinesia, and fine-motor-skill deficits associated with MPTP administration.

Keywords

Parkinson's disease; genetic therapy; bradykinesia; MPTP; behavior; gangliosides

Address correspondence to: Mike McDonald, PhD, UTHSC Department of Neurology, 855 Monroe Ave., 415 Link Bldg., Memphis, TN 38163, (901) 448-4648, mike@tennessee.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Parkinson's disease is characterized by degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNc) and a progressive deterioration of motor function. This is typically preceded by a constellation of non-motor symptoms including gastric slowing and dysphagia, sleep disruption, anxiety and depression, and impaired executive function (reviewed in [57]). Unfortunately, treatment for Parkinson's patients typically does not begin until motor symptoms emerge, at which time nigro-striatal destruction is thought to exceed 70% [27]. Currently-available therapies for Parkinson's disease typically focus on enhancement of dopaminergic signaling but are not disease-modifying, i.e., they do not prevent ongoing neurodegeneration [53]. Of more than 50 drugs that were effective pre-clinically in MPTP and other toxin models, only two are considered to have disease-modifying potential in Parkinson's patients; those two are rasagiline and GM1 ganglioside [49].

Gangliosides are acidic glycosphingolipids that are highly enriched in lipid microdomains in the central nervous system. The distribution of gangliosides is altered in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [6,7,89]. However, the importance of these changes or their consequences is not fully understood. The investigation of gangliosides in neurodegenerative diseases has focused on GM1 because of its role in neuroprotection. GM1, one of the four major brain gangliosides (Fig. 1), has broad neuroprotective properties when used against insults including glutamate excitotoxicity, electrolytic lesions, and occlusive ischemia [32,42,54,70]. Ganglioside-based treatments show promise for Parkinson's disease. Dozens of studies have shown that GM1 ameliorates nigrostriatal lesions and behavioral impairments induced by MPTP in a number of species (e.g., [63,66,73]). Importantly, GM1 not only protects neurons from ongoing neurodegeneration, but also reverses dopamine D2 receptor supersensitivity following MPTP lesions [26,35,46,83]. D2 supersensitivity is thought to mediate dyskinesias induced by dopaminergic agonists such as levodopa [13,85]. Recent clinical trials have shown positive short- and long-term benefits of GM1 administration in Parkinson's patients [71,75]. However, patients in that study were required to self-inject GM1 twice daily for the duration of the study, which is not optimal for compliance [71].

Previous data from our lab suggest that inhibition of GD3S is neuroprotective and prevent behavioral and cognitive decline associated with neurodegenerative diseases [2,9]. For example, targeted deletion of *St8sia1*, the gene that codes for GD3 synthase (GD3S; Fig. 1), prevents cell death induced by A β 42 or folate deficiency in primary neurons and prevents oxidative stress and cognitive decline in a mouse model of Alzheimer's disease [9]. In another study we injected GD3S $^{-/-}$ and wild-type mice with MPTP and assessed balance, coordination, and fine motor skills. Knockout mice did not exhibit motor impairments, and the loss of tyrosine hydroxylase (TH) immunostaining was less than half that of MPTP-treated controls [2]. In the present study we used a lentiviral-mediated shRNA construct to knock down GD3S expression. Our hypothesis was that striatal injections of shGD3S would protect against nigro-striatal damage induced by MPTP.

Methods

Subjects.

Subjects were male wild-type C57BL/6N mice (#027, Charles River Laboratories, Wilmington, MA). Except during MPTP treatment and the 5 days following, mice were housed in an AAALAC-approved vivarium with a 12-hour light/dark cycle and free access to food and water throughout the study. During the 5 days of treatment with MPTP or saline and for the 5 days immediately afterward in which toxic metabolites may be excreted in the urine and feces, mice were housed in disposable cages under a fume hood. All procedures were approved by the Institutional Animal Care and Use Committee.

shRNA constructs.

Three shRNA constructs targeting mouse GD3S (*St8sia1*; Genbank number X84235) were created by *in vitro* transcription following the procedure described by Donze and Picard [25]. Briefly, desoxy-oligonucleotides were generated in which 20 nucleotides (nt) of sense or antisense sequence was flanked at 3' by the TATAGTGAGTCGTATTA sequence, complementary to the T7 promoter, and at 5' by an overhang of 2 A. The sense sequences were as follows: shGD3S-1 (at +29 nt from start codon), cgtccagaggggccatggcc; shGD3S-2 (at +129 nt), catcttcctgtctaccggc; and shGD3S-3 (at +333 nt), cttatactcgttcaccatcg. A BLAST search confirmed that the sequences hit only the corresponding *St8sia1* gene. The RNAs were transcribed *in vitro*, annealed, and used for transfection. Pilot studies were conducted to validate knock-down of GD3S in dissociated primary cortical/hippocampal neurons from 18-day-old mouse embryos as previously described [9,88]. Neurons were transfected with scrRNA or one of the three shGD3S constructs for 72 hours. Cells were then incubated with a goat polyclonal anti-GD3S antibody (#sc-46982, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min. followed by a Cy3-conjugated anti-goat IgG for 30 min. and visualized by fluorescent microscopy. Knock-down of GD3S was efficient and approximately equal using all three shGD3S constructs, by visual inspection (Fig. 2a). For the *in-vivo* studies we decided to use shGD3S-2, and quantified GD3S fluorescence at $74.4 \pm 3.8\%$ below scrRNA control levels.

Lentiviral vectors.

The shGD3S-2 and scrRNA constructs were cotransfected with a glycoprotein G vesicular stomatitis virus (VSV-G) packaging plasmid (Clontech, Mountain View, CA) to facilitate entry into the cells. The vectors were cotransfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 hours, the pseudovirus was harvested and diluted for use. To assess knock-down efficacy, pilot stereotactic injections of lenti.shGD3S or lenti.scrRNA were made bilaterally in the striatum of each mouse, 1.0 μ l per side, using the procedures described below. Mice were killed 2 weeks following the injections, perfused transcardially, and brain sections processed for fluorescent immunohistochemistry with an antibody targeting GD3S (#sc-46984, Santa Cruz Biotechnology). Additional sections were stained using antibodies targeting GD1b and GT1b (#G2004-90B & #G2006-90A, US Biologicals, Swampscott, MA).

Stereotactic surgery.

Mice were anesthetized using a ketamine (92.3 mg/kg) + xylazine (13.82 mg/kg) cocktail, and an incision made at the midline. Burr holes were drilled targeting the striatum, using coordinates for C57BL/6J mice from Hof et al. [43] and adapted in pilot surgeries for the C57BL/6N strain (AP +0.2; ML $\pm$ 2.1; DV -3.6). We administered the vectors in the striatum because it is a larger target and there are a number of papers showing rapid and robust transduction to the SNc when administered in the striatum [18,65,82]. A 31-ga. injection cannula was lowered to -3.6 mm ventrally into the striatum. Each side was injected individually with 2.0 μ l of shGD3S-2 or scrRNA. The cannula was left in place for 10 min. following each injection to allow dispersion of the injectate. We targeted the striatum because it is a large structure and a more reliable approach to retrogradely transducing neurons in the SNc with a single injection per hemisphere [1,16,18,65,82,84].

1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

MPTP hydrochloride was purchased from Sigma (#M0896, lot 128K1549, St. Louis, MO) and dissolved in physiological (0.9%) saline at a concentration of 1.8 mg (2.106 mg salt) per ml. Mice were injected intraperitoneally in a volume of 10 ml/kg of body weight to achieve a dose of 18 mg/kg. Control mice were injected with an equivalent volume of physiological saline. A sub-chronic injection regimen was used, in which a single injection was given each day for 5 days, beginning 1 month following the intracranial injections. We have previously shown that this dose induces a robust SNc lesion with little or no mortality [23]. Mice were left undisturbed in their cages for the 5 days of injections and 5 days afterwards, except during the injection procedure. Because of the presence of putatively toxic urine and feces, all mice within a cage were assigned to the same lesion group (saline or MPTP). Use of MPTP and disposal of hazardous waste was done in strict accordance with safety standards [64], and its use was approved by the Institutional Biosafety Committee.

Sensorimotor function.

Starting on the sixth day following the last injection of MPTP or saline, mice completed a battery of behavioral tasks to measure sensorimotor function. Mice were first placed in commercially-available activity monitors (MED-Associates, Inc., Georgia, VT) for a 60-min. session as previously described [2,24,36,37,38]. The activity monitors measured 27 $\times$ 27 cm, with 16 infrared photocell beams equally spaced in the x and y axes of the horizontal plane, 1 cm from the floor of the monitor. An additional vector of 16 photobeams was situated 4 cm above the floor to track rearing. Gait, balance, coordination, and fine motor skills were assessed using the horizontal beam, inverted screen, sticker removal, vertical grid, and painted paw gait tasks as previously described [24,52,68,69]. The horizontal beam task required the mouse to traverse a 0.64-cm diam., 81-cm long painted wooden beam elevated 40 cm above a soft blanket. Mice were motivated by a 25-watt white light bulb at the starting platform, and reinforced with entry into a dark box on the other side of the beam. After training with increasingly longer distances, mice were placed on the 5-cm² starting platform, and latency to traverse the beam was recorded. Each subject was given three trials, and the fastest latency to cross was taken as that subject's score. The inverted screen task tested the mouse's ability to hang upside down from a screen of 1-cm² hardware cloth in a

wooden frame. The screen was waved gently in the air and then turned upside down for up to 60 s, and the latency to fall was recorded. Mice not falling within 60 s were given a score of 60. Three trials were given and the longest latency for the three trials used as the mouse's score. The sticker-removal test is the classic demonstration of MPTP-induced loss of fine-motor skills. On this test, the mouse was gently scruffed and a 0.6-cm-diam. adhesive sticker placed on the top of its snout. The mouse was then placed in a clean tub cage and observed. The latency to the first attempt to remove the sticker, and the latency to its final removal were recorded. Gait was assessed using the painted paw test. Mice were trained to traverse an 87.5-cm runway to enter a dark box, motivated by a 25-watt bulb at the start of the runway. When they reliably ran into the dark box, a length of paper was placed on the floor of the runway. The subject's paws were painted with red (forepaws) and black (hind paws) non-toxic tempera paint. The mouse was then immediately placed at the start of the runway, and allowed to run into the dark box.

Licking behavior.

To assess licking behavior, mice were placed individually in standard housing cages outfitted with contact lickometers as previously described [11,15,39,40]. A metal wire mesh was placed on the bottom of the cage, and a lick was recorded in the form of a junction potential when the animal's tongue touched the water spout and completed the circuit between the mesh and the lickometer. The cages were maintained within a quiet area of the vivarium, and food and water were freely available at all times. Water was the only fluid available, and all water was dispensed through the lickometer for the duration of the assessment. Mice were kept in the lickometer cages for 3 consecutive days following surgery but before MPTP lesioning, and for 3 consecutive days following MPTP injections. The sessions were conducted from Friday to Sunday to minimize disruption from animal care staff. For each set of 3-day measurements, the last 2 days were combined for data analysis. Variables of interest included the number of licks, visits to the lickometer, bursts of licking, and inter-lick interval (ILI). Measures of variability, such as coefficient of variation (σ/μ) and Fano factor (σ^2/μ), were also tracked. A visit to the lickometer was defined as at least one burst of licking, preceded by a period of at least 60 s without a lick. A burst of licks was defined as at least two licks preceded and followed by a lick-free interval longer than 180 msec but shorter than 500 msec. An ILI was defined as the interval between two licks separated by at least 40 msec but less than 180 msec.

High-performance liquid chromatography (HPLC) with electrochemical detection.

Mice were sacrificed 12 weeks following the first MPTP injection. Mice used for HPLC were killed by cervical dislocation without anesthesia. Brains were rapidly removed and a section extending from 0.0 to 1.0 mm rostral to bregma was removed for dissection using a brain matrix, as previously described [2,23,55,77,78]. A 1-mm punch was taken from the left dorsolateral striatum and rapidly frozen on dry ice. The frozen striatal punches were suspended in 400 μ l aCSF and 600 μ l 0.2 mM perchloric acid, homogenized for 30 sec., and then centrifuged at 10,000 RPM for 15 min at 4°C. The supernatant was filtered through a nylon syringe filter (0.2 μ m) and frozen at -80° C. Frozen samples (20 μ l; 1:1 dilution with polished HPLC grade water) were automatically injected by a refrigerated autosampler (ESA, Inc., Sunnyvale, CA) onto a 150 $\times$ 2 mm ODS C18 column connected to an ESA

model 580 HPLC pump. The mobile phase, containing 80 mM sodium dihydrogen phosphate monohydrate, 2.0 mM 1-octanesulfonic acid sodium salt, 100 μ l/l triethylamine, 5 nM EDTA, and 10% acetonitrile, pH 3.0, was perfused at 0.25 ml/min. Dopamine levels were determined using an ESA 5041 high-sensitivity analytical cell, an ESA 5020 guard cell and an ESA Coulochem II 5200A electrochemical detector at a potential of 220 mV with the current gain at 10 nA and the guard cell at +350mV. Under these conditions, the limit of detection for dopamine is 100 fg per sample.

Histology and immunohistochemistry.

Mice used for stereology and immunohistochemistry were sacrificed under brief isoflurane anesthesia and perfused transcardially, first with saline and then with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 min. Brains were removed and fixed overnight in the same fixative, and 40- μ m coronal sections were taken throughout the extent of the substantia nigra pars compacta (SNc) for histological analysis. Free-floating sections were treated first with phosphate buffered saline (PBS) containing 20% methanol and 3% hydrogen peroxide for 30 min., and rinsed thoroughly in PBS. Sections were then incubated in 10% normal horse serum in PBS containing 0.1% Triton X-100 for 30 min. and incubated overnight at 4° C in the primary antibody (dilution 1:200) targeting tyrosine hydroxylase (TH; #AB152, Millipore), GD3S (#sc-99093, Santa Cruz), GD1b (#G2004-90D, US Biologicals), GM1 (GM1-1, Developmental Studies Hybridoma Bank, Iowa City, IA), or GT1b (#MAB5608, Millipore). Following incubation sections were rinsed three times in PBS, incubated for an hour in the appropriate secondary antibody. Sections used for stereology were then washed thrice, and treated with avidin–biotin complex (Vectastain Elite ABC kit, Vector Labs) reagent for an hour, and visualized using diaminobenzidine.

Stereological quantification of cells in SNc.

Unbiased stereological quantification of TH-positive cells was conducted in 3-5 mice per group as previously described [2,23,28,55], by an experimenter blind to the treatment conditions. Every fourth section through the rostro-caudal extent of the SNc was measured using the optical fractionator counting method and Stereo Investigator software (MicroBrightField), starting with a random section for each mouse. The contour of the SNc was first delineated using Stereo Investigator's anatomical mapping tool at low power. TH-positive cells within the SNc were counted in from 10 to 20 frames measuring 25 $\times$ 25 μ m in each of the selected sections, generated using Stereo Investigator's random-sampling grid. The frames were selected using the systematic random sampling scheme, which provides an unbiased and efficient sampling technique. In every counting-frame location, the top of the section was identified, after which the plane of the focus was moved 4 μ m deeper through the section (guard zone) to prevent counting inaccuracies due to uneven section surfaces. The resulting focal plane served as the first point of the counting process. All TH-positive cells that came into focus in the next 8- μ m segment (dissector height) were counted if they were entirely within the counting frame or touching the upper or right side of the counting frame. Based on these parameters and counts, the total number of TH-positive cells per selected region were counted using the optical fractionator formula $N = 1/ssf \times 1/asf \times 1/hsf \times Q$, where ssf is the section sampling fraction, asf is the area sampling fraction, hsf is the height sampling fraction (dissector height divided by the section thickness after shrinkage),

and Q denotes the total count of particles sampled for each region. After completion of stereological counts, sections were counter-stained with cresyl violet (CV) and the number of SNc neurons positive for CV but negative for TH were counted in a similar fashion.

Statistical analyses.

Most behavioral data were analyzed using one- or two-way factorial analyses of variance (ANOVA), with lesion (Saline or MPTP) and treatment groups (scrRNA or shGD3S) as between-subjects factors. Follow-up comparisons were made using orthogonal paired t-tests or Fishers protected LSD as appropriate. Conceptually-related dependent measures were analyzed together using multivariate ANOVA (MANOVA) fitted to an identity matrix. Time-series data were analyzed using hierarchical linear modeling, with time as a balanced continuous repeated measure and subject as a random nominal factor nested within treatment and lesion. Categorical data from the inverted-screen and sticker-removal tests were analyzed using a one-way Kruskal-Wallis test followed by Mann-Whitney paired comparisons. The nearest integer to the population mean minus 1.0 SEM was used as the cutoff for categorizing proficient vs. impaired behavior on the sticker-removal test. Thus 14 s was used as a cutoff for first attempt to remove the sticker, and a further 5 s to actually remove it. To protect against spurious Type I errors, follow-up analyses were conducted only after a significant omnibus effect, except with comparisons having specific *a priori* hypotheses. All comparisons were two-tailed with α set at 0.05.

Results

To validate the efficacy of the construct *in vivo*, mice were injected intrastrially and sacrificed 2 weeks later. Figure 2b shows that GD3S was largely absent from striatal neurons in these mice. We further verified that shGD3S reduced expression of the major brain b-series gangliosides GD1b and GT1b (Fig. 2c).

In the activity monitors there were no main effects of treatment or lesion, and no Treatment X Lesion interaction effects on any measure related to locomotor activity, rearing, velocity, or repetitive beam breaks [F 's < 2.9, p 's > .097; data not shown]. Non-parametric analysis of sticker-removal data showed no difference in the proportion of mice making a first attempt to remove the sticker within 14 s [$X^2(3) = 1.1$, $p = .789$], but a significant group difference in the proportion of mice removing the sticker within 5 s once the initial attempt was made [Fig. 3a; $X^2(3) = 11.8$, $p = .0083$]. Follow-up tests showed that MPTP significantly slowed sticker-removal time in scrRNA-treated mice [$p = .0109$]. The median removal latencies in saline- and MPTP-lesioned mice were 1.0 and 6.5 s, respectively, in scrRNA-treated mice. There was no effect of MPTP on sticker-removal latencies in shGD3S-treated mice [$p = 1.0$]. there was a significant effect of lesion on the horizontal beam crossing latency [Fig. 3b; $F(1,31) = 7.7$, $p = .0093$]. The effects of treatment and the Treatment X Lesion interaction were not significant [F 's < 0.9, p 's > .357]. Follow-up tests showed that MPTP slowed crossing latency in scrRNA-treated [$p = .0172$] but not shGD3S-treated [$p = .184$] mice. There were no significant group differences in the number of foot-slips on the horizontal beam [F 's < 1.0, p 's > .325; data not shown]. There were also no group differences on the inverted screen [$X^2(3) = 4.3$, $p = .236$; data not shown] or vertical grid [F 's < 1.1, p 's > .320;

data not shown] tests, and on the gait assessment there were no differences in left or right stride, uniformity of stride, or hind-paw base [F 's < 1.4; p 's > .246; data not shown].

Mice were pre-exposed to the lickometers 3 days before the first MPTP injection, to habituate them to the apparatus and ensure stable licking rates. During this time there were no group differences on any measure of licking [F 's < 3.8, p 's > .072; data not shown]. In contrast to pre-MPTP recordings, there were significant main and interaction effects following MPTP injections. Follow-up tests showed that there was no effect of MPTP on any measure of licking in mice treated with shGD3S [F 's < 3.8, p 's > .101]. In contrast, MPTP increased the mean ILI [Fig. 3c; $F(1,12) = 21.3$, $p = .0006$] and the total number of licks [Fig. 3d; $F(1,12) = 5.8$, $p = .0327$] in the scrRNA control mice. The mode and median ILI showed the same pattern as the mean, suggesting a shift of the distribution of licks as opposed to a change in shape of the curve (Table 1). Additional results are summarized in Table 1. Within the scrRNA-treated mice, MPTP did not affect the number of visits to the lickometer [$F(1,6) = 0.03$, $p = .873$], but the number of bursts, licks per burst, and licks per visit were all significantly increased [F 's > 7.0, p 's < .038]. MPTP also decreased variability of the ILI in scrRNA-treated mice, but not in their shGD3S-treated counterparts, as measured by coefficient of variation and Fano factor [$\lambda = 0.18$, $F(6,22) = 5.0$, $p = .0024$].

At the completion of the behavioral experiments and 12 weeks following the last MPTP injection, mice were sacrificed for histology and assessment of nigrostriatal integrity. Figure 4a shows that the shGD3S vector transduced to the SNc where it reduced GD3S expression, consistent with other reports showing retrograde transduction from the striatum to the SNc [18,41,60]. The reduced GD3S resulted in elevated GM1 levels as constitutively high levels of ganglioside were being synthesized but converted to a-series rather than b-series gangliosides (Fig. 4b), consistent with the pattern observed in the GD3S^{-/-} mouse [9,47,48]. There were significant effects of lesion [$F(1,12) = 42.9$, $p < .0001$] and treatment [$F(1,12) = 6.6$, $p = .0250$] on TH+ cell counts (Fig. 5a,b). The Lesion X Treatment interaction was not significant [$p = .107$]. Follow-up tests showed that 18 mg/kg MPTP induced a 64.3% loss of TH-positive neurons in the SNc in scrRNA control mice [$p = .0004$]. MPTP also induced a significant lesion in subjects treated with shGD3S [$p = .0181$], although the extent of neuronal loss was much smaller (35.1%). There were no main or interaction effects on SNc neurons that were positive for CV but negative for TH [F 's < 0.6; p 's > .458; data not shown]. There were no significant effects of lesion, treatment, or their interaction on dopamine content in the dorsolateral striatum [Fig. 5c; F 's < 3.1; p 's > .103]. Follow-up comparisons by treatment showed that dopamine was reduced 30.6% in MPTP-lesioned mice pre-treated with scrRNA [$F(1,9) = 5.8$, $p = .0427$], but unchanged in shGD3S-treated mice [$p = .630$]. Dopamine levels in the two MPTP-lesioned groups did not differ significantly [$p = .085$]. There were no significant main or interaction effects on striatal DOPAC or the DOPAC/DA ratio [F 's < 2.5; p 's > .142; data not shown].

Discussion

Our data show that inhibition of GD3S is neuroprotective against MPTP lesions. This is the first demonstration that knock-down of GD3S *in vivo* is neuroprotective. In addition, we show that MPTP induces bradykinesia and impairs fine motor skills at a relatively low dose

of MPTP administered sub-chronically. When treated with shGD3S, motor impairments were not evident. Taken together, these results suggest that inhibition of GD3S may be able to prevent ongoing nigrostriatal degeneration and associated behavioral impairments.

MPTP lesions induced subtle but significant motor deficits in scrRNA-treated control mice. The inability to remove the sticker quickly is the classic test of loss of fine-motor skills associated with MPTP lesioning [10,29]. The slower crossing latencies observed in the horizontal-beam task is indicative of the bradykinesia characteristic of Parkinson's disease, a slowness that was absent in mice treated with shGD3S. MPTP also disrupted licking rhythms in scrRNA-treated mice. Like the beam-crossing, MPTP slowed licking rate, reflected in an increased inter-lick interval (ILI). Although the difference in mean ILI between saline and MPTP-lesioned groups was less than 6 msec, the normal licking rhythm is tightly regulated in mice and small differences can be highly significant [11,12,40]. The median and modal ILIs were similarly slowed by MPTP, suggesting a shift in the entire distribution of ILIs as opposed to idiosyncratic changes in a few mice or under certain circumstances, e.g., modulating lick frequency only at the beginning or end of bursts. Lingual bradykinesia is a symptom of Parkinson's disease that gets worse over time [86]. In a retrospective study of motor symptoms, [18F]fluorodopa-positron emission tomography, and clinical outcomes, Gallagher et al. [30] found that tongue protrusion force and movement time on a reaction-time task 4 years earlier better-differentiated Parkinson's patients from normal controls than any other measures. Lingual bradykinesia associated with reduced tongue strength is thought to contribute to the slower oropharyngeal transit times characteristic of Parkinson's disease [86]. Although MPTP-induced changes in licking behavior have not previously been reported in the literature, several studies have shown similar reductions in lick frequency in rats lesioned unilaterally with 6-hydroxydopamine (6-OHDA) when striatal dopamine depletion exceeds 75% of the contralateral (unlesioned) side [19,20,59,61,80]. In addition to slower licking, 6-OHDA-lesioned rats exerted significantly less tongue force on the lickometer in all five studies. Although we did not specifically measure tongue force in the present study, these findings are consistent with our results. We observed that the number of licks per visit was increased more than 50% in the MPTP-lesioned mice treated with scrRNA, with no significant difference in the number of visits per day. Although this may suggest increased water intake, perhaps secondary to dehydration, such an effect of MPTP or 6-OHDA has never been reported in the literature. Indeed, bilateral striatal dopamine depletion following MPTP or 6-OHDA administration has only been shown to reduce water intake, and only with very large bilateral lesions [17,21,51,56,67,87]. Thus, rather than reflecting an increase in water intake, it is more plausible that the increased number of licks in the scrRNA-treated MPTP mice in the present study reflects a compensatory response to a less-efficient lick motor pattern, e.g., due to reduced tongue force resulting in less water per lick.

The mechanism of neurodegeneration is not known in most cases of Parkinson's disease, but is thought to involve hydrolysis of sphingomyelin by sphingomyelinase to produce ceramide, which generates *de novo* GD3 ganglioside synthesis by GD3S [3,4,6,14,58]. GD3 translocates to the mitochondrial membrane where it accumulates in lipid microdomains, a necessary step in this apoptotic cascade [22,31,50,81]. Thus knock-down of GD3S may exert its neuroprotective effect through reduction of GD3 or the resulting increase in

neuroprotective GM1, or both [2,9,24]. The increase in GM1 occurs simultaneously with GD3S inhibition as constitutively high levels of ganglioside are converted to a-series rather than b-series gangliosides (Figs. 1,4b; [5,8,9,47,48]). All known manipulations that alter GD3 *in vivo* necessarily affect other brain gangliosides, so it's impossible to determine definitively whether one or both of the mechanisms is responsible for the neuroprotective effect of shGD3S in the present study.

Although GD3 cannot be altered *in vivo* without affecting GM1 levels, many studies have examined the neuroprotective effects of exogenous GM1 independent of changes in GD3. Exogenous GM1 has been shown to protect against insults as varied as excitotoxicity, ischemia, ethanol intoxication, and X-irradiation [33,42,44,45,54]. GM1 also protects against MPTP-induced nigro-striatal damage in mice, cats, rats, and monkeys [34,46,62,63,73,74,76,79]. In double-blind, placebo-controlled studies Schneider et al. ([71,72]) showed that GM1 is safe and prevents deterioration of motor function and activities of daily living in patients with mild-to-moderate Parkinson's disease, over periods up to 24 weeks. In an open-label study they showed continued positive effects over a 5-year period [75]. The clinical trials required patients to self-inject exogenous GM1 twice daily for the duration of the study, which is not ideal for medication compliance. In addition, GM1 is expensive and only poorly crosses the blood-brain barrier. Thus the investigation of approaches that increase endogenous GM1 are warranted. In our hands, all efforts to increase endogenous GM1 have been neuroprotective. In addition to the results of the present paper and those with the GD3S^{-/-} mice [2,9], we have shown *in vivo* neuroprotective effects of purified sialidase (VCS) from the *Vibrio cholerae* bacterium. VCS increases GM1 and reduces b-series gangliosides, by cleaving the α 2-8 and external α 2-3 sialic acid linkages on gangliosides (see Fig. 1). However, while GD3S inhibition results in an increase in GD1a, VCS hydrolyzes it; thus GD1a, GD1b, and GT1b are all converted to GM1, creating an even greater elevation of endogenous GM1 than is achievable with deletion of GD3S [5,9,24,47,90]. When infused in the dorsal third ventricle, VCS protects against degeneration of CA3 pyramidal neurons following kainite-induced *status epilepticus* [24]. Intraventricular VCS is also partially protects against MPTP-induced nigro-striatal damage [76]. Thus three different approaches to increasing endogenous GM1 are neuroprotective in several *in vivo* and *in vitro* models.

Despite multiple possible neuroprotective mechanisms associated with GD3S inhibition, the neuroprotection was not complete in the present study. The MPTP-induced SNc cell loss in the shGD3S-treated mice was just under half that of scrRNA controls. However, this may be more a function of the lesion model than the intervention. MPTP is a "sledge-hammer" that recapitulates in 5 days what occurs over many years in humans. GD3S knock-down may afford full protection in a model that recapitulates the slower neurodegeneration of Parkinson's disease. Nevertheless, the protective effect of shGD3S in the present study was sufficient to prevent the expression of motor symptoms observed in MPTP-lesioned mice treated with scrRNA. Typically, it is thought that SNc neuronal loss must approach two-thirds before motor symptoms appear. If this is the case then partial protection may be sufficient to delay the exacerbation of symptoms for many years in Parkinson's patients. Thus inhibition of GD3S may warrant further study as a potential therapeutic for Parkinson's disease.

Acknowledgements.

This work was supported by the National Institutes of Health [grant number NS065063]; and American Health Assistance Foundation/Alzheimer's Disease Research. Image production and stereological analyses were conducted at the Neuroscience Institute's Imaging Center at UTHSC (cns.uthmem.edu/imaging-center).

References

- [1]. Ahmed MR, Berthet A, Bychkov E, Porras G, Li Q, Bioulac BH, Carl YT, Bloch B, Kook S, Aubert I, Dovero S, Doudnikoff E, Gurevich VV, Gurevich EV, Bezard E, Lentiviral overexpression of GRK6 alleviates L-dopa-induced dyskinesia in experimental Parkinson's disease, *Science translational medicine* 2 (2010) 28ra28.
- [2]. Akkhwattanakul Y, Maiti P, Xue Y, Aryal D, Wetsel WC, Hamilton D, Fowler SC, McDonald MP, Targeted deletion of GD3 synthase protects against MPTP-induced neurodegeneration, *Genes, brain, and behavior* 16 (2017) 522–536.
- [3]. Anglade P, Vyas S, Javoy-Agid F, Herrero MT, Michel PP, Marquez J, Mouatt-Prigent A, Ruberg M, Hirsch EC, Agid Y, Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease, *Histol. Histopathol* 12 (1997) 25–31. [PubMed: 9046040]
- [4]. Arboleda G, Cardenas Y, Rodriguez Y, Morales LC, Matheus L, Arboleda H, Differential regulation of AKT, MAPK and GSK3 β during C2-ceramide-induced neuronal death, *Neurotoxicology* 31 (2010) 687–693. [PubMed: 20696185]
- [5]. Ariga T, Itokazu Y, McDonald MP, Hirabayashi Y, Ando S, Yu RK, Brain gangliosides of a transgenic mouse model of Alzheimer's disease with deficiency in GD3-synthase: expression of elevated levels of a cholinergic-specific ganglioside, GT1a α , *ASN Neuro* 5 (2013) 141–148.
- [6]. Ariga T, Jarvis WD, Yu RK, Role of sphingolipid-mediated cell death in neurodegenerative diseases, *J. Lipid Res* 39 (1998) 1–16. [PubMed: 9469581]
- [7]. Ariga T, McDonald MP, Yu RK, Role of ganglioside metabolism in the pathogenesis of Alzheimer's disease, *J. Lipid Res* 49 (2008) 1157–1175. [PubMed: 18334715]
- [8]. Ariga T, Yanagisawa M, Wakade C, Ando S, Buccafusco JJ, McDonald MP, Yu RK, Ganglioside metabolism in a transgenic mouse model of Alzheimer's disease: expression of Chol-1a antigens in the brain, *ASN Neuro* 2 (2010).
- [9]. Bernardo A, Harrison FE, McCord M, Zhao J, Bruchey A, Davies SS, Jackson Roberts L, 2nd, Mathews PM, Matsuoka Y, Ariga T, Yu RK, Thompson R, McDonald MP, Elimination of GD3 synthase improves memory and reduces amyloid- β plaque load in transgenic mice, *Neurobiol. Aging* 30 (2009) 1777–1791. [PubMed: 18258340]
- [10]. Bouet V, Boulouard M, Toutain J, Divoux D, Bernaudin M, Schumann-Bard P, Freret T, The adhesive removal test: a sensitive method to assess sensorimotor deficits in mice, *Nat Protoc* 4 (2009) 1560–1564. [PubMed: 19798088]
- [11]. Boughter JD, Jr., Baird JP, Bryant J, St John SJ, Heck D, C57BL/6J and DBA/2J mice vary in lick rate and ingestive microstructure, *Genes, brain, and behavior* 6 (2007) 619–627.
- [12]. Boughter JD, Jr., Mulligan MK, St John SJ, Tokita K, Lu L, Heck DH, Williams RW, Genetic control of a central pattern generator: rhythmic oromotor movement in mice is controlled by a major locus near Atp1a2, *PLoS One* 7 (2012) e38169. [PubMed: 22675444]
- [13]. Brooks DJ, Piccini P, Turjanski N, Samuel M, Neuroimaging of dyskinesia, *Ann. Neurol* 47 (2000) S154–158; discussion S158–159. [PubMed: 10762143]
- [14]. Brugg B, Michel PP, Agid Y, Ruberg M, Ceramide induces apoptosis in cultured mesencephalic neurons, *J. Neurochem* 66 (1996) 733–739. [PubMed: 8592146]
- [15]. Bryant JL, Boughter JD, Gong S, LeDoux MS, Heck DH, Cerebellar cortical output encodes temporal aspects of rhythmic licking movements and is necessary for normal licking frequency, *Eur. J. Neurosci* 32 (2010) 41–52. [PubMed: 20597972]
- [16]. Carpentier DC, Vevis K, Tralbalza A, Georgiadis C, Ellison SM, Asfahani RI, Mazarakis ND, Enhanced pseudotyping efficiency of HIV-1 lentiviral vectors by a rabies/vesicular stomatitis virus chimeric envelope glycoprotein, *Gene Ther.* 19 (2012) 761–774. [PubMed: 21900965]

- [17]. Casas M, Prat G, Robledo P, Barbanoj M, Kulisevsky J, Jane F, Methylxanthines reverse the adipsic and aphagic syndrome induced by bilateral 6-hydroxydopamine lesions of the nigrostriatal pathway in rats, *Pharmacol. Biochem. Behav* 66 (2000) 257–263. [PubMed: 10880677]
- [18]. Cearley CN, Wolfe JH, A single injection of an adeno-associated virus vector into nuclei with divergent connections results in widespread vector distribution in the brain and global correction of a neurogenetic disease, *J. Neurosci* 27 (2007) 9928–9940. [PubMed: 17855607]
- [19]. Ciucci MR, Russell JA, Schaser AJ, Doll EJ, Vinney LM, Connor NP, Tongue force and timing deficits in a rat model of Parkinson disease, *Behav. Brain Res* 222 (2011) 315–320. [PubMed: 21459116]
- [20]. Ciucci MR, Schaser AJ, Russell JA, Exercise-induced rescue of tongue function without striatal dopamine sparing in a rat neurotoxin model of Parkinson disease, *Behav. Brain Res* 252 (2013) 239–245. [PubMed: 23756136]
- [21]. Davis BJ, Blair ML, Sladek JR, Jr., Sladek CD, Effects of lesions of hypothalamic catecholamines on blood pressure, fluid balance, vasopressin and renin in the rat, *Brain Res.* 405 (1987) 1–15. [PubMed: 3552113]
- [22]. De Maria R, Lenti L, Malisan F, d'Agostino F, Tomassini B, Zeuner A, Rippo MR, Testi R, Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis, *Science* 277 (1997) 1652–1655. [PubMed: 9287216]
- [23]. Dhanushkodi A, Akano EO, Roguski EE, Xue Y, Rao SK, Matta SG, Rex TS, McDonald MP, A single intramuscular injection of rAAV-mediated mutant erythropoietin protects against MPTP-induced parkinsonism, *Genes, brain, and behavior* 12 (2013) 224–233.
- [24]. Dhanushkodi A, McDonald MP, Intracranial V cholerae sialidase protects against excitotoxic neurodegeneration, *PLoS One* 6 (2011) e29285. [PubMed: 22195039]
- [25]. Donze O, Picard D, RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase, *Nucleic Acids Res* 30 (2002) e46. [PubMed: 12000851]
- [26]. Fazzini E, Durso R, Davoudi H, Szabo GK, Albert ML, GM1 gangliosides alter acute MPTP-induced behavioral and neurochemical toxicity in mice, *J. Neurol. Sci* 99 (1990) 59–68. [PubMed: 2250172]
- [27]. Fearnley JM, Lees AJ, Ageing and Parkinson's disease: substantia nigra regional selectivity, *Brain* 114 (Pt 5) (1991) 2283–2301. [PubMed: 1933245]
- [28]. Flanigan TJ, Xue Y, Kishan Rao S, Dhanushkodi A, McDonald MP, Abnormal vibrissa-related behavior and loss of barrel field inhibitory neurons in 5xFAD transgenics, *Genes, brain, and behavior* 13 (2014) 488–500.
- [29]. Fleming SM, Schallert T, Ciucci MR, Cranial and related sensorimotor impairments in rodent models of Parkinson's disease, *Behav. Brain Res* 231 (2012) 317–322. [PubMed: 22394540]
- [30]. Gallagher CL, Johnson SC, Bendlin BB, Chung MK, Holden JE, Oakes TR, Brooks BR, Konopacki RA, Dogan S, Abbs JH, Xu G, Nickles RJ, Pyzalski RW, DeJesus OT, Brown WD, A longitudinal study of motor performance and striatal [18F]fluorodopa uptake in Parkinson's disease, *Brain Imaging Behav* 5 (2011) 203–211. [PubMed: 21556744]
- [31]. Garofalo T, Giammarioli AM, Misasi R, Tinari A, Manganelli V, Gambardella L, Pavan A, Malorni W, Sorice M, Lipid microdomains contribute to apoptosis-associated modifications of mitochondria in T cells, *Cell Death Differ.* 12 (2005) 1378–1389. [PubMed: 15947792]
- [32]. Glasier MM, Janis LS, Goncalves MI, Stein DG, GM1 produces attenuation of short-term memory deficits in Hebb-Williams maze performance after unilateral entorhinal cortex lesions, *Physiol. Behav* 66 (1999) 441–446. [PubMed: 10357433]
- [33]. Guelman LR, Zorrilla Zubilete MA, Rios H, Dopico AM, Zieher LM, GM1 ganglioside treatment protects against long-term neurotoxic effects of neonatal X-irradiation on cerebellar cortex cytoarchitecture and motor function, *Brain Res.* 858 (2000) 303–311. [PubMed: 10708681]
- [34]. Hadjiconstantinou M, Rossetti ZL, Paxton RC, Neff NH, Administration of GM1 ganglioside restores the dopamine content in striatum after chronic treatment with MPTP, *Neuropharmacology* 25 (1986) 1075–1077. [PubMed: 3490632]

- [35]. Hadjiconstantinou M, Weihmuller F, Neff NH, Treatment with GM1 ganglioside reverses dopamine D-2 receptor supersensitivity induced by the neurotoxin MPTP, *Eur. J. Pharmacol* 168 (1989) 261–264. [PubMed: 2532603]
- [36]. Harrison FE, Allard J, Bixler R, Usoh C, Li L, May JM, McDonald MP, Antioxidants and cognitive training interact to affect oxidative stress and memory in APP/PSEN1 mice, *Nutritional neuroscience* 12 (2009) 203–218. [PubMed: 19761651]
- [37]. Harrison FE, May JM, McDonald MP, Vitamin C deficiency increases basal exploratory activity but decreases scopolamine-induced activity in APP/PSEN1 transgenic mice, *Pharmacol. Biochem. Behav* 94 (2010) 543–552. [PubMed: 19941887]
- [38]. Harrison FE, Yu SS, Van Den Bossche KL, Li L, May JM, McDonald MP, Elevated oxidative stress and sensorimotor deficits but normal cognition in mice that cannot synthesize ascorbic acid, *J. Neurochem* 106 (2008) 1198–1208. [PubMed: 18466336]
- [39]. Hayer A, Bryant JL, Boughter JD, Heck DH, A low-cost solution to measure mouse licking in an electrophysiological setup with a standard analog-to-digital converter, *J. Neurosci. Methods* 153 (2006) 203–207. [PubMed: 16364450]
- [40]. Heck DH, Gu W, Cao Y, Qi S, Lacaria M, Lupski JR, Opposing phenotypes in mice with Smith-Magenis deletion and Potocki-Lupski duplication syndromes suggest gene dosage effects on fluid consumption behavior, *Am J Med Genet A* 158A (2012) 2807–2814. [PubMed: 22991245]
- [41]. Herzog CD, Dass B, Holden JE, Stansell J, 3rd, Gasmi M, Tuszynski MH, Bartus RT, Kordower JH, Striatal delivery of CERE-120, an AAV2 vector encoding human neurturin, enhances activity of the dopaminergic nigrostriatal system in aged monkeys, *Mov. Disord* 22 (2007) 1124–1132. [PubMed: 17443702]
- [42]. Hicks D, Heidinger V, Mohand-Said S, Sahel J, Dreyfus H, Growth factors and gangliosides as neuroprotective agents in excitotoxicity and ischemia, *Gen. Pharmacol* 30 (1998) 265–273. [PubMed: 9510073]
- [43]. Hof PR, Young WG, Bloom FE, Belichenko PV, Celio MR, *Comparative Cytoarchitectonic Atlas of the C57BL6 and 129Sv Mouse Brains*, Elsevier, New York, 2000.
- [44]. Hungund BL, Ross DC, Gokhale VS, Ganglioside GM1 reduces fetal alcohol effects in rat pups exposed to ethanol in utero, *Alcohol. Clin. Exp. Res* 18 (1994) 1248–1251. [PubMed: 7847614]
- [45]. Hungund BL, Zheng Z, Lin L, Barkai AI, Ganglioside GM1 reduces ethanol induced phospholipase A2 activity in synaptosomal preparations from mice, *Neurochem. Int* 25 (1994) 321–325. [PubMed: 7820065]
- [46]. Kastner A, Herrero MT, Hirsch EC, Guillen J, Luquin MR, Javoy-Agid F, Obeso JA, Agid Y, Decreased tyrosine hydroxylase content in the dopaminergic neurons of MPTP-intoxicated monkeys: effect of levodopa and GM1 ganglioside therapy, *Ann. Neurol* 36 (1994) 206–214. [PubMed: 7914399]
- [47]. Kawai H, Allende ML, Wada R, Kono M, Sango K, Deng C, Miyakawa T, Crawley JN, Werth N, Bierfreund U, Sandhoff K, Proia RL, Mice expressing only monosialoganglioside GM3 exhibit lethal audiogenic seizures, *J. Biol. Chem* 276 (2001) 6885–6888. [PubMed: 11133999]
- [48]. Kawai H, Sango K, Mullin KA, Proia RL, Embryonic stem cells with a disrupted GD3 synthase gene undergo neuronal differentiation in the absence of b-series gangliosides, *J. Biol. Chem* 273 (1998) 19634–19638. [PubMed: 9677390]
- [49]. Koprach JB, Kalia LV, Brotchie JM, Animal models of alpha-synucleinopathy for Parkinson disease drug development, *Nature reviews. Neuroscience* 18 (2017) 515–529. [PubMed: 28747776]
- [50]. Kristal BS, Brown AM, Apoptogenic ganglioside GD3 directly induces the mitochondrial permeability transition, *J. Biol. Chem* 274 (1999) 23169–23175. [PubMed: 10438487]
- [51]. Kryzhanovskii GN, Krupina NA, Kucherianu VG, [A new model of an experimental depressive syndrome in rats induced by the systemic administration to the animals of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine], *Zh. Vyssh. Nerv. Deiat. Im. I. P. Pavlova* 45 (1995) 377–387. [PubMed: 7597834]
- [52]. Liu Y, Hoffmann A, Grinberg A, Westphal H, McDonald MP, Miller KM, Crawley JN, Sandhoff K, Suzuki K, Proia RL, Mouse model of GM2 activator deficiency manifests cerebellar

- pathology and motor impairment, *Proc. Natl. Acad. Sci. U. S. A* 94 (1997) 8138–8143. [PubMed: 9223328]
- [53]. Ma L, Wei L, Wu F, Hu Z, Liu Z, Yuan W, Advances with microRNAs in Parkinson's disease research, *Drug design, development and therapy* 7 (2013) 1103–1113.
- [54]. Mahadik SP, Hungund BL, Gokhale VS, Ortiz A, Makar TK, Karpiak SE, Monosialoganglioside (GM1) restores membrane fatty acid levels in ischemic tissue after cortical focal ischemia in rat, *Neurochem. Int* 23 (1993) 163–172. [PubMed: 8369740]
- [55]. Maiti P, Gregg LC, McDonald MP, MPTP-induced executive dysfunction is associated with altered prefrontal serotonergic function, *Behav. Brain Res* 298 (2016) 192–201. [PubMed: 26393431]
- [56]. Marshall JF, Levitan D, Stricker EM, Activation-induced restoration of sensorimotor functions in rats with dopamine-depleting brain lesions, *J. Comp. Physiol. Psychol* 90 (1976) 536–546. [PubMed: 8470]
- [57]. McDonald MP, Methods and models of the non-motor symptoms of Parkinson disease In: LeDoux MS (Ed.), *Movement Disorders: Genetics and Models*, Elsevier Academic Press, Amsterdam, 2015.
- [58]. Mochizuki H, Goto K, Mori H, Mizuno Y, Histochemical detection of apoptosis in Parkinson's disease, *J. Neurol. Sci* 137 (1996) 120–123. [PubMed: 8782165]
- [59]. Nuckolls AL, Worley C, Leto C, Zhang H, Morris JK, Stanford JA, Tongue force and tongue motility are differently affected by unilateral vs bilateral nigrostriatal dopamine depletion in rats, *Behav. Brain Res* 234 (2012) 343–348. [PubMed: 22796604]
- [60]. Paterna JC, Feldon J, Bueler H, Transduction profiles of recombinant adeno-associated virus vectors derived from serotypes 2 and 5 in the nigrostriatal system of rats, *J. Virol* 78 (2004) 6808–6817. [PubMed: 15194756]
- [61]. Plowman EK, Maling N, Rivera BJ, Larson K, Thomas NJ, Fowler SC, Manfredsson FP, Shrivastav R, Kleim JA, Differential sensitivity of cranial and limb motor function to nigrostriatal dopamine depletion, *Behav. Brain Res* 237 (2013) 157–163. [PubMed: 23018122]
- [62]. Pope-Coleman A, Schneider JS, Effects of Chronic GM1 Ganglioside Treatment on Cognitive and Motor Deficits in a Slowly Progressing Model of Parkinsonism in Non-Human Primates, *Restor Neurol Neurosci* 12 (1998) 255–266. [PubMed: 12671296]
- [63]. Pope-Coleman A, Tinker JP, Schneider JS, Effects of GM1 ganglioside treatment on pre- and postsynaptic dopaminergic markers in the striatum of parkinsonian monkeys, *Synapse* 36 (2000) 120–128. [PubMed: 10767059]
- [64]. Przedborski S, Jackson-Lewis V, Naini AB, Jakowec M, Petzinger G, Miller R, Akram M, The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety, *J. Neurochem* 76 (2001) 1265–1274. [PubMed: 11238711]
- [65]. Reimsnyder S, Manfredsson FP, Muzyczka N, Mandel RJ, Time course of transgene expression after intrastratial pseudotyped rAAV2/1, rAAV2/2, rAAV2/5, and rAAV2/8 transduction in the rat, *Mol Ther* 15 (2007) 1504–1511. [PubMed: 17565350]
- [66]. Rothblat DS, Schneider JS, Effects of GM1 ganglioside treatment on dopamine innervation of the striatum of MPTP-treated mice, *Ann. N. Y. Acad. Sci* 845 (1998) 274–277. [PubMed: 9668361]
- [67]. Sakai K, Gash DM, Effect of bilateral 6-OHDA lesions of the substantia nigra on locomotor activity in the rat, *Brain Res.* 633 (1994) 144–150. [PubMed: 7907930]
- [68]. Sango K, McDonald MP, Crawley JN, Mack ML, Tifft CJ, Skop E, Starr CM, Hoffmann A, Sandhoff K, Suzuki K, Proia RL, Mice lacking both subunits of lysosomal β -hexosaminidase display gangliosidosis and mucopolysaccharidosis, *Nat. Genet* 14 (1996) 348–352. [PubMed: 8896570]
- [69]. Sango K, Yamanaka S, Hoffmann A, Okuda Y, Grinberg A, Westphal H, McDonald MP, Crawley JN, Sandhoff K, Suzuki K, Proia RL, Mouse models of Tay-Sachs and Sandhoff diseases differ in neurologic phenotype and ganglioside metabolism, *Nat. Genet* 11 (1995) 170–176. [PubMed: 7550345]
- [70]. Santucci AC, Gluck R, Kanof PD, Haroutunian V, Induction of memory and cortical cholinergic neurochemical recovery with combine fetal transplantation and GM1 treatments in rats with lesions of the NBM, *Dementia* 4 (1993) 273–281. [PubMed: 8261024]

- [71]. Schneider JS, Gollomp SM, Sendek S, Colcher A, Cambi F, Du W, A randomized, controlled, delayed start trial of GM1 ganglioside in treated Parkinson's disease patients, *J. Neurol. Sci* 324 (2013) 140–148. [PubMed: 23199590]
- [72]. Schneider JS, Roeltgen DP, Mancall EL, Chapas-Crilly J, Rothblat DS, Tatarian GT, Parkinson's disease: improved function with GM1 ganglioside treatment in a randomized placebo-controlled study, *Neurology* 50 (1998) 1630–1636. [PubMed: 9633704]
- [73]. Schneider JS, Rothblat DS, Differences in release and clearance of extracellular dopamine in the striatum after spontaneous or GM1-ganglioside-stimulated recovery from experimental Parkinsonism, *Restor Neurol Neurosci* 16 (2000) 97–104. [PubMed: 12671212]
- [74]. Schneider JS, Schroeder JA, Rothblat DS, Differential recovery of sensorimotor function in GM1 ganglioside-treated vs. spontaneously recovered MPTP-treated cats: partial striatal dopaminergic reinnervation vs. neurochemical compensation, *Brain Res.* 813 (1998) 82–87. [PubMed: 9824674]
- [75]. Schneider JS, Sendek S, Daskalakis C, Cambi F, GM1 ganglioside in Parkinson's disease: Results of a five year open study, *J. Neurol. Sci* 292 (2010) 45–51. [PubMed: 20206941]
- [76]. Schneider JS, Seyfried TN, Choi HS, Kidd SK, Intraventricular Sialidase Administration Enhances GM1 Ganglioside Expression and Is Partially Neuroprotective in a Mouse Model of Parkinson's Disease, *PLoS One* 10 (2015) e0143351. [PubMed: 26629687]
- [77]. Siesser WB, Cheng SY, McDonald MP, Hyperactivity, impaired learning on a vigilance task, and a differential response to methylphenidate in the TR β PV knock-in mouse, *Psychopharmacology (Berl)*. 181 (2005) 653–663. [PubMed: 15983791]
- [78]. Siesser WB, Zhao J, Miller LR, Cheng SY, McDonald MP, Transgenic mice expressing a human mutant β 1 thyroid receptor are hyperactive, impulsive, and inattentive, *Genes, brain, and behavior* 5 (2006) 282–297.
- [79]. Skaper SD, Facci L, Schiavo N, Vantini G, Moroni F, Dal Toso R, Leon A, Characterization of 2,4,5-trihydroxyphenylalanine neurotoxicity in vitro and protective effects of ganglioside GM1: implications for Parkinson's disease, *J. Pharmacol. Exp. Ther* 263 (1992) 1440–1446. [PubMed: 1361575]
- [80]. Skitek EB, Fowler SC, Tessel RE, Effects of unilateral striatal dopamine depletion on tongue force and rhythm during licking in rats, *Behav. Neurosci* 113 (1999) 567–573. [PubMed: 10443783]
- [81]. Sorice M, Garofalo T, Misasi R, Manganelli V, Vona R, Malorni W, Ganglioside GD3 as a raft component in cell death regulation, *Anti-cancer agents in medicinal chemistry* 12 (2012) 376–382. [PubMed: 21554197]
- [82]. Taymans JM, Vandenberghe LH, Haute CV, Thiry I, Deroose CM, Mortelmans L, Wilson JM, Debyser Z, Baekelandt V, Comparative analysis of adeno-associated viral vector serotypes 1, 2, 5, 7, and 8 in mouse brain, *Hum. Gene Ther* 18 (2007) 195–206. [PubMed: 17343566]
- [83]. Toffano G, Agnati LF, Fuxe K, Aldinio C, Consolazione A, Valenti G, Savoini G, Effect of GM1 ganglioside treatment on the recovery of dopaminergic nigro-striatal neurons after different types of lesion, *Acta Physiol. Scand* 122 (1984) 313–321. [PubMed: 6151339]
- [84]. Trbalza A, Georgiadis C, Eleftheriadou I, Hislop JN, Ellison SM, Karavassilis ME, Mazarakis ND, Venezuelan equine encephalitis virus glycoprotein pseudotyping confers neurotropism to lentiviral vectors, *Gene Ther.* 20 (2013) 723–732. [PubMed: 23171919]
- [85]. Turjanski N, Lees AJ, Brooks DJ, In vivo studies on striatal dopamine D1 and D2 site binding in L-dopa-treated Parkinson's disease patients with and without dyskinesias, *Neurology* 49 (1997) 717–723. [PubMed: 9305330]
- [86]. Umemoto G, Tsuboi Y, Kitashima A, Furuya H, Kikuta T, Impaired food transportation in Parkinson's disease related to lingual bradykinesia, *Dysphagia* 26 (2011) 250–255. [PubMed: 20803220]
- [87]. Ungerstedt U, Adipsia and aphagia after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system, *Acta Physiol. Scand. Suppl* 367 (1971) 95–122. [PubMed: 4332694]
- [88]. Wang B, Liu Y, Huang L, Chen J, Li JJ, Wang R, Kim E, Chen Y, Justicia C, Sakata K, Chen H, Planas A, Ostrom RS, Li W, Yang G, McDonald MP, Chen R, Heck DH, Liao FF, A CNS-permeable Hsp90 inhibitor rescues synaptic dysfunction and memory loss in APP-overexpressing

Alzheimer's mouse model via an HSF1-mediated mechanism, *Mol. Psychiatry* 22 (2017) 990–1001. [PubMed: 27457810]

- [89]. Wu G, Lu ZH, Kulkarni N, Ledeen RW, Deficiency of ganglioside GM1 correlates with Parkinson's disease in mice and humans, *J. Neurosci. Res* 90 (2012) 1997–2008. [PubMed: 22714832]
- [90]. Yang LJ, Lorenzini I, Vajn K, Mountney A, Schramm LP, Schnaar RL, Sialidase enhances spinal axon outgrowth in vivo, *Proc. Natl. Acad. Sci. U. S. A* 103 (2006) 11057–11062. [PubMed: 16847268]

Highlights:

- A low dose of MPTP induced a moderate nigrostriatal lesion and mild parkinsonism
- Lentiviral-mediated shRNA knockdown of GD3S (shGD3S) prevented MPTP-induced parkinsonism
- Nigrostriatal lesion size in shGD3S -treated mice was half that of controls

Figure 1. Synthesis and degradation of brain gangliosides.

Gangliosides are acidic glycosphingolipids synthesized through addition of carbohydrate moieties to UDP-galactose-glucosylceramide (Gal-Glc-Cer). The four major brain gangliosides are shaded pink. GD3 synthase (GD3S; EC 2.4.99.8) converts GM3 to GD3, from which all the b-series gangliosides are synthesized. Central nervous system knock-down of GD3S reduces synthesis of GD1b and GT1b, resulting in greater levels of GD1a and GM1 as constitutively high levels of ganglioside are converted to a-series instead of b-series gangliosides [7,9]. β-hexosaminidase (βHex; EC 3.2.1.52) cleaves the GalNac residue on GM2 to generate GM3. β-galactosidase (βGal; EC 3.2.1.23) hydrolyzes the β-glycosidic linkage between Gal and GalNac to convert GM1 into GM2. Abbreviations: Cer, ceramide; Glc, glucose; Gal, galactose; GalNac, N-acetylgalactosamine; NeuAc, N-acetylneuraminic

acid (sialic acid); GNT, GalNac transferase (EC 2.4.1.92); $\alpha 3$, $\alpha 2$ -3 sialic acid bond; $\alpha 8$, $\alpha 2$ -8 sialic acid bond.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. shGD3S reduces intrastriatal GD3S and b-series gangliosides.

(a) Primary neurons were treated with one of three shRNA vectors targeting GD3S in pilot studies to validate knock-down. After 72 hours cells were fixed and treated with antibodies against MAP2 or GD3S. All three vectors knocked down GD3S approximately equally, by visual inspection. (b) Mice were injected intrastratially with 1.0 μ l of shGD3S or scrambled-sequence control (scrRNA), and sacrificed 2 weeks later. High magnification images (lower panels) show that GD3S immunoreactivity present in striatal neurons of uninjected mice and those injected with scrRNA, but was almost completely absent in mice treated with the active shRNA vector. (c) Loss of GD1b and GT1b immunoreactivity in shGD3S-treated mice confirms the knock-down of GD3S (see Fig. 1). Scale bars = 200 μ m (b&c, upper panels), 100 μ m (c, middle panels), 50 μ m (b&c, lower panels).

Figure 3. GD3S knock-down protects against MPTP-induced behavioral impairments.

Mice were assessed on a battery of behavioral tasks to assess motor function. (a) On the sticker-removal test, scrRNA-treated mice had much greater difficulty removing the sticker when injected with MPTP. shGD3S-treated mice performed as well as saline controls. (b) Mice were trained to cross an elevated horizontal beam to enter a dark box. The scrRNA-treated mice lesioned with MPTP were slower to cross the 81-cm beam than their saline-treated counterparts. In contrast, shGD3S-treated mice performed equally well after saline or MPTP. (c,d) In scrRNA-treated mice, MPTP induced a significant slowing of the lick rate (c) and increase in the number of licks (d). shGD3S treatment prevented these deficits. * $p < .05$, *** $p < .001$ compared to respective saline control group.

Figure 4. GD3S knock-down reduces GD3S and increases GM1 in the SNc.

(a) When injected into the striatum, shGD3S transduces retrogradely to the SNc and reduces GD3S expression. **(b)** Silencing GD3S increases GM1 ganglioside, as constitutively high levels of ganglioside are converted to a-series rather than b-series gangliosides (see Fig. 1).

Figure 5. GD3S knock-down protects against nigrostriatal damage.

(a,b) Stereological analysis showed that MPTP destroyed 64.3% of the TH-positive neurons in the SNc, in mice treated with the scrambled-construct control. In contrast, lesion size was approximately half that in shGD3S-treated mice. (c) MPTP induced a significant loss of dopamine in the dorsolateral striatum in scrRNA-treated but not shGD3S-treated mice. * $p < .05$, *** $p < .001$ compared to respective saline control group. Scale bars = 100 μm .

Table 1.

Measures of licking behavior following MPTP or saline injections. All measures are presented as mean $\pm$ SEM unless otherwise stated. Abbreviations: ILI, inter-lick interval; IVI, inter-visit interval

Measure	ScrRNA		shGD3S	
	Saline	MPTP	Saline	MPTP
ILI Coefficient of variation	0.24 $\pm$ 0.01	0.18 $\pm$ 0.02 [*]	0.26 $\pm$ 0.01	0.24 $\pm$ 0.01
ILI Fano factor	7.47 $\pm$ 0.61	4.67 $\pm$ 0.87 [*]	8.58 $\pm$ 0.41	7.79 $\pm$ 0.66
Median ILI	122.20 $\pm$ 0.73	128.60 $\pm$ 0.57 ^{***}	122.60 $\pm$ 1.80	123.75 $\pm$ 0.93
Modal ILI	119.50 $\pm$ 2.96	128.25 $\pm$ 1.03 [*]	123.00 $\pm$ 2.27	122.00 $\pm$ 1.68
ILIs (thousands)	5.90 $\pm$ 0.62	8.09 $\pm$ 0.22 [*]	4.00 $\pm$ 0.49	4.76 $\pm$ 0.74
Bursts	418.00 $\pm$ 9.67	559.75 $\pm$ 26.63 ^{**}	464.25 $\pm$ 99.63	357.75 $\pm$ 54.34
Visits	114.50 $\pm$ 7.84	117.00 $\pm$ 12.71	126.75 $\pm$ 29.23	106.25 $\pm$ 14.35
Licks per visit	49.02 $\pm$ 9.85	76.05 $\pm$ 2.58 [*]	37.77 $\pm$ 7.31	52.43 $\pm$ 2.08
Max. licks per visit	203.00 $\pm$ 8.12	229.75 $\pm$ 17.26	173.75 $\pm$ 34.46	181.25 $\pm$ 20.11
Licks per burst	13.87 $\pm$ 2.46	16.71 $\pm$ 0.34	11.05 $\pm$ 2.48	15.34 $\pm$ 1.32
Max. licks per burst	110.50 $\pm$ 2.06	162.00 $\pm$ 6.56 ^{***}	105.00 $\pm$ 15.43	93.75 $\pm$ 1.70
Bursts per visit	3.37 $\pm$ 0.28	4.48 $\pm$ 0.10 [*]	3.41 $\pm$ 0.61	3.41 $\pm$ 0.34
Max. bursts per visit	16.50 $\pm$ 1.26	18.25 $\pm$ 1.84	12.25 $\pm$ 2.39	16.50 $\pm$ 2.96

^{*} p < .05;

^{**} p < .01;

^{***} p < .001