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FTY720 Improves Behavior, Increases Brain Derived Neurotrophic Factor and Reduces α-Synuclein Pathology in Parkinsonian GM2+/− Mice

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Abstract

Parkinson's disease (PD) is a progressive aging disorder that affects millions worldwide, thus, disease-modifying-therapies are urgently needed. PD pathology includes α-synuclein (aSyn) accumulation as synucleinopathy. Loss of GM1 gangliosides occurs in PD brain, which is modeled in GM2 synthase transgenic mice. GM2+/− mice have low, not absent GM1 and develop age-onset motor deficits, making them an excellent PD drug testing model. FTY720 (fingolimod) reduces synucleinopathy in A53T aSyn mice and motor dysfunction in 6-OHDA and rotenone PD models, but no one has tested FTY720 in mice that develop age-onset PD-like motor problems. We confirmed that GM2+/−mice had equivalent rotarod, hindlimb reflexes, and adhesive removal functions at 9 mo. From 11 mo, GM2+/− mice received oral FTY720 or vehicle 3x/week to 16 mo. As bladder problems occur in PD, we also assessed GM2+/− bladder function. This allowed us to demonstrate improved motor and bladder function in GM2+/− mice treated with FTY720. By immunoblot, FTY720 reduced levels of proNGF, a biomarker of bladder dysfunction. In humans with PD, arm swing becomes abnormal, and brachial plexus modulates arm swing. Ultrastructure of brachial plexus in wild type and GM2 transgenic mice confirmed abnormal myelination and axons in GM2 transgenics. FTY720 treated GM2+/− brachial plexus sustained myelin associated protein levels and reduced aggregated aSyn and PSer129 aSyn levels. FTY720 increases brain derived neurotrophic factor (BDNF) and we noted increased BDNF in GM2+/− brachial plexus and cerebellum, which contribute to rotarod performance. These findings provide further support for testing low dose FTY720 in patients with PD.

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Keywords

Brachial plexus synucleinopathy; Fingolimod; Improved bladder function; Low dose drug; Motor improvement; Myelination biomarker increases; Neuroprotection; Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is an age-onset neurodegenerative disorder that affects ~ 1% the population over 50 and ~4% over 85 yr of age (Bekris *et al.* 2010). Males are 1.5 times more likely than females to be diagnosed with PD (Wooten *et al.* 2004). Early onset PD (21–49 years old) and juvenile onset PD (before 21 years old) do occur, but most PD is age-related and considered to be sporadic (Bonifati 2012), with familial PD accounting for only ~ 5% of all cases.

Most PD brains at autopsy exhibit a loss of midbrain substantia nigra pars compacta (SNc) dopaminergic neurons, which is apparent as a loss of neuromelanin-pigment. SNc supplies dopaminergic input to striatum that helps control normal body movement (Turner & Desmurget 2010) in association with cerebellar function (Bostan & Strick 2010; Bostan *et al.* 2010). The dying back of SNc axons that innervate striatum is part of the progressive pathology that subsequently leads to PD motor symptoms (Alexander 2004; Cheng *et al.* 2010).

Surviving midbrain SNc neurons often contain Lewy Bodies, inclusions with abundant aSyn, a presynaptic protein that broadly contributes to dopamine regulation (Perez *et al.* 2002; Benskey *et al.* 2016; Porras & Perez 2014; Mor & Ischiropoulos 2018; Perez & Hastings 2004; Peng *et al.* 2005; Tehranian *et al.* 2006). Phosphorylation of aSyn PSer129 not only affects normal aSyn function but also increases its aggregation potential (Lou *et al.* 2010; Oueslati 2016). aSyn aggregation in Lewy bodies/neurites is common in both sporadic and familial PD. Furthermore, though under-appreciated, it is known that cerebellum is affected in patients with PD (Wu & Hallett 2013) in part by interconnecting circuitry with the striatum (Bostan & Strick 2010; Bostan *et al.* 2010). Moreover, PSer129 aSyn has been found in adrenal gland, retina, salivary gland, enteric nervous system, and pancreas of PD patients; confirming extensive synucleinopathy in PD (Stoddard 1994; Bencsik *et al.* 2014; Antunes *et al.* 2016; Martinez-Valbuena *et al.* 2018; Beach *et al.* 2014; Ortuno-Lizaran *et al.* 2018; Fumimura *et al.* 2007; Beach *et al.* 2010; Jain 2011), further establishing that PD is more than just a brain disorder. Thus, protective therapies that reduce synucleinopathy are urgently needed.

Among key biological molecules that regulate optimal neuronal function are gangliosides, sialic acid-bearing glycosphingolipids that are highly expressed in the brain (Schnaar 2010). The most abundant mature gangliosides are GM1 (mono, with one sialic acid), GD1a/GD1b (di, with two sialic acids), and GT1b (tri, with three sialic acids) (Palmano *et al.* 2015; Sturgill *et al.* 2012; Posse de Chaves & Sipione 2010). GD1a and GT1b deficiencies contribute to neurodegeneration (Ohmi *et al.* 2014) and GM1 levels are significantly lower, but not entirely absent in SNc of PD brain (Wu *et al.* 2012a; Hadaczek *et al.* 2015). Gangliosides are also ligands that enhance myelin stability (Vyas & Schnaar 2001) and

recent data from our lab confirmed bladder innervation myelination abnormalities in GM2−/− KO mice entirely lacking GM2/GD2 Synthase, the enzyme for mature ganglioside synthesis (Gil-Tommee *et al.* 2019). GM2−/− KO mice have only immature GM3 gangliosides a species of gangliosides that reportedly are elevated in sera of PD patients (Chan *et al.* 2017). GM1 has been shown to reduce aSyn aggregation *in vitro* (Martinez *et al.* 2007), suggesting that normal GM1 levels act to keep aSyn soluble. Prior data also confirm that ganglioside decreases in PD can be alleviated by treating with GM1 or with the GM1-analogue LIGA-20, both of which reduce PD motor symptoms (Schneider *et al.* 2010; Schneider *et al.* 1998). These data imply that mature GM1 gangliosides contribute significantly to brain function and that mice with reduced GM1 levels and progressive motor dysfunction make an excellent PD model (Wu *et al.* 2012a; Hadaczek *et al.* 2015) supporting their use for testing candidate PD therapies.

In addition to gangliosides, myelin associated proteins contribute to neuron function by supportive effects. For example, myelin basic protein (MBP) enhances neuronal signaling while mutations or absence of MBP cause impaired motor function *in vivo* (Nave 2010; Harauz & Boggs 2013). Similarly, the oligodendrocyte-specific-protein 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP), contributes to axonal integrity (Lappe-Siefke *et al.* 2003). Moreover, oligodendrocytes and other glial cells produce neurotrophic factors that are protective of brain neurons, and GM1 itself functions as a trophic factor (Mocchetti 2005; Mocchetti & Bachis 2004).

PD has no cure and existing treatments can only modify symptoms and do not slow disease progression. Levodopa, a dopamine precursor that crosses the blood brain barrier then is converted to dopamine, acts to reduce PD rigidity and bradykinesia. Another PD treatment, deep brain stimulation (DBS), is more often used in those who respond poorly to PD medications. However, DBS surgery is invasive so it is not a first line PD therapy. Even so, a recent report showed less tremor progression in patients after DBS during a relatively early stage of the disease (Hacker *et al.* 2018). PD patients also can benefit from exercise/physical therapy programs, which improve muscle tone, balance, and quality of life in PD patients (Oertel 2017).

FTY720/fingolimod is an approved oral treatment for relapsing/remitting multiple sclerosis (MS) (Sanford 2014; Brinkmann 2009; Brinkmann *et al.* 2010). The drug is protective by increasing BDNF expression (Vargas-Medrano *et al.* 2014; Vidal-Martinez *et al.* 2016; Deogracias *et al.* 2012) and also by improving myelination in MS patients as well as in MS models (Gurevich *et al.* 2018; Yazdi *et al.* 2015).

In association with PD, BDNF has been shown to be a major neurotrophin for SNc dopaminergic neurons (Dluzen *et al.* 1999), thus using a drug that can stimulate BDNF expression has considerable potential as a PD therapeutic. In addition to its role in PD pathology, we discovered that aSyn normally interacts with and modulates protein phosphatase 2A (PP2A) enhancing its activity (Peng *et al.* 2005; Lou *et al.* 2010; Farrell *et al.* 2014; Wang *et al.* 2009). We also found that in PD brain, PP2A activity is reduced in tissue with robust aSyn aggregation (Wu *et al.* 2012b). This suggests that a loss of aSyn function toward PP2A as aSyn accumulates in Lewy bodies/neurites may require

enhancement. We and others have shown that FTY720 also stimulates PP2A activity (Vargas-Medrano *et al.* 2014; Lek *et al.* 2017; Perrotti & Neviani 2008). Here we show the protective benefits of low dose FTY720 in parkinsonian GM2+/− mice. Our findings suggest that FTY720 has the potential to counteract PD pathology, reverse dysfunction and slow disease progression. As the drug is already Food and Drug Administration (FDA) approved, it could be repurposed in short order for PD clinical trials.

EXPERIMENTAL PROCEDURES

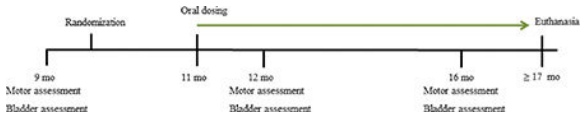
Mice and Genotyping

A heterozygous (+/−) breeding pair of GM2/GD2 synthase/*B4galnt1* mice (Sheikh *et al.* 1999) was a gift from Drs. Ledeen and Wu of Rutgers New Jersey Medical School and used to produce most mice for this study. Experiments were conducted on protocols approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee (IACUC #11025) in accordance with AALAC and National Institutes of Health guidelines. Mice were housed under 12-hr light/dark cycles with access to food and water *ad libitum*. Genotyping was performed using DNA purified from 3 mm tail snips with QIAamp DNA Mini Kit (QIAGEN.com). PCR primers were 5′-TAC CAG GCC AAC ACA GCA-3′ and 5′-CAG GTC CAG GGG CGT CTT-3′. PCR products stained with ethidium bromide were photographed under UV light to identify bands of 2.9 Kb and 2.5 Kb in GM2+/− mice as previously described (Wu *et al.* 2001).

FTY720 Preparation and Dosing

FTY720 was dissolved in dimethyl sulfoxide (DMSO) to create a 5 mg/mL stock solution that was stored at −20°C. Animal weights were obtained the day before dosing to calculate the amount of FTY720 needed for 0.5 mg/kg/mouse dosing. Beginning at 11 mo of age, mice received oral 0.5 mg/kg FTY720 or an equivalent amount of DMSO, as vehicle 3x/week. Doses were prepared and given in a blinded manner according to mouse ear punch patterns. For drug delivery, mice were held by the scruff of the neck, in a semi-supine position, with solutions delivered using a 10 μl pipette with the pipette tip gently placed in the mouth.

As previously confirmed by others, GM2+/− mice can begin showing motor deficits by 7 mo (Wu *et al.* 2012a), thus we set 9 mo as our baseline and randomly selected mice after confirming equivalent motor and bladder function. For behavioral tests, mice were placed in clean quiet rooms 15 min prior to evaluation in order to acclimate to the environment. Tests were performed between 1:00 – 3:00 PM on each test day for 9, 12 and 16 mo old GM2+/− mice (as shown in the time line below).



Behavioral Assessments

Rotarod. Balance and coordination were measured using rotarod (Colburn Instruments, PA) by experimenters blinded to the treatment condition for this and all behavioral tests. Mice were pre-trained in two independent sessions at a constant rate of 4 rpm and then at 8 rpm. On the day of testing, each mouse received an accelerating warm-up trial. Three trials were then performed with the rotarod set to accelerate from 4 – 40 rpm over 5 min time, with rotation increasing in increments of 0.12 rpm/sec. Between trials, mice were allowed a resting period of 5 min. Accelerating rotarod tests for each time point were done over two consecutive days. *Adhesive removal sensorimotor tests*. Adhesive removal allows determining motor response to a sensory stimulus (Fleming *et al.* 2004). Each mouse was restrained and a small adhesive sticker was placed on their forehead using forceps. The mouse was placed in a cage and a timer was set to record how long it took each mouse to remove the sticker, typically with their forepaws. The maximum amount of time each mouse was tested was 2 min (120 sec), and if a mouse failed to remove the sticker it scored 120. This testing was performed over 5 trials with mice having a short break between trials, over 2 consecutive days. *Hindlimb reflex tests*. To measure motor nerve function we evaluated hindlimb reflexes. Each mouse was suspended by the tail for 5 sec during which the position of the hindlimbs was scored as previously described (Chiavegatto *et al.* 2000). Data were collected over 3 trials performed on 2 different days, with short breaks between trials. Scores range from impaired to normal as follows: 0 = one or both hindlimbs paralyzed, 1 = hindlimbs and paws close to the body with clasping toes, 2 = loss of flexion of hindlimbs, 3 = hindlimbs extended < 90°angle, and 4 = hindlimbs extended > 90°angle. *Urinary Void Spot Analysis*. Food and water were removed during 1 hr tests performed on 5 different days, with each mouse placed individually into a clean cage (between 10:00–11:00 am) with cage bottom covered with filter paper (Bio–Rad, Hercules, CA, USA, cat# 1650962) as before (Gil-Tommee *et al.* 2019). Filters were collected, labeled, and allowed to dry. Void spots were illuminated with a UV light and counted as small (≤ 0.2 cm^2) or large (> 0.2 cm^2) by individuals blinded to treatments using established methods (Bjorling *et al.* 2015; Gil-Tommee *et al.* 2019; Hodges *et al.* 2008; Studeny *et al.* 2008; Yu *et al.* 2014; Birder *et al.* 2002; Hamill *et al.* 2012). Pooled data for each mouse at 12 and 16 mo time points provided the final values.

Euthanasia and Tissue Collection

Animals were euthanized by CO_2 inhalation followed by decapitation. Tissues were rapidly dissected, placed in labeled tubes and stored at −80°C until analysis. We collected brachial plexus, brain, and urinary bladders at necropsy. For electron microscopy, tissue was handled as described below.

Transmission Electron Microscopy

Dissected brachial plexus was placed in 4% PFA overnight then rinsed and transferred to 10 mL pH 7.2 containing 2.5% glutaraldehyde-0.1 M imidazole buffer. Tissues were then immersed in 2 mL 2% osmium tetroxide in imidazole buffer for 4 hr and washed in distilled water followed by dehydration in a graded series of ethanol (50%, 80%, absolute). Sequentially, tissue sections were immersed in a mixture of 1:1 absolute ethanol and

propylene oxide and then in propylene oxide in sealed vials on a rotating mixer for 15 min. Finally, tissue was infiltrated with a 1:1 mixture of propylene oxide and epoxy resin overnight in open vials then embedded with epoxy resin in molds at 60°C for 48 hr. Embedded tissue was first 'thick' sectioned at 0.25 micrometers with glass knives and stained with epoxy tissue stain (Electron Microscopy Sciences, Hatfield, PA). Selected areas were then thin sectioned at 70 nm with diamond knives and mounted on Formvar-Carbon coated grids and stained with 2% uranyl acetate and lead citrate solution. Digital images were collected with a mid-mount XR611 camera (AMT, Inc., Woburn, MA) in a model H-7650 electron microscope (Hitachi High-Technologies, Dallas, TX).

Gene Expression

Total mRNA extracted from cerebellum used a miRNeasy mini kit (Qiagen, catalog no. 217004) and RNase-free DNase kit (Qiagen, catalog no. 79254) according to the manufacturer. RNA concentration and purity were assessed using NanoDrop 2000 spectrophotometry (Thermo Scientific). RNA quality was assessed by measuring 28S/18S band ratios in RNA "bleach" gels exactly as described (Aranda *et al.* 2012; Segura-Ulate *et al.* 2017). Reverse transcription of mRNAs was performed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, catalog no. 4387406) as per manufacturer instructions. Amplification was measured by real-time quantitative PCR (qPCR) with Taqman probes in a RealPlex Mastercycler 2 (Eppendorf Inc., Westbury, NY). Relative expression of mRNAs was measured using Taqman probe assays (Life Technologies, Inc.) for BDNF (catalog no. Mm04230607_s1), glial cell line derived neurotrophic factor (GDNF) (Mm00599849_m1), nerve growth factor (NGF) (Mm00443039_m1), and ciliary neurotrophic factor (CNTF) (Mm00446373_m1) with GAPDH (catalog no. Mm99999915_g1) and eukaryotic 18S ribosomal RNA (catalog no. Hs99999901_s1) as internal expression controls. Molecular assays were evaluated in 2–3 independent experiments in duplicate or triplicate.

Protein Isolation and Immunoblots

Soluble and insoluble protein was isolated as before (Waxman & Giasson 2008; Gil-Tommee *et al.* 2019; Wu *et al.* 2012b; Vidal-Martinez *et al.* 2016). Standard immunoblot tissue was homogenized in 6 volumes (w/v) of ice-cold buffer containing 1 mM AEBSF, 5 μM aprotinin and 1 mM benzamidine using a Bullet Blender (Next Advance, Inc., Averill Park, NY, USA). Samples were sonicated and spun at 14,000 × g for 10 min at 4°C to remove particulates. Protein concentrations were determined by bicinchoninic acid assay (Thermo-Fisher, USA). Gels, loaded with 10 – 50 μg protein per sample were transferred to nitrocellulose, blocked in buffer containing 5% nonfat milk, and incubated in primary antibodies overnight at 4°C. Antibodies included aSyn (C20, sc-7011-R, Santa Cruz Biotechnology, Santa Cruz, CA, USA), aSyn PSer129 (11A5, gift of Dr. J. Anderson of Elan Pharmaceuticals), β-actin (4970S, Cells Signaling, Danvers, MA, USA), CNP (D83E10, Cell Signaling, Danvers, MA, USA), MBP (ab40390, Abcam), proNGF (H-20, sc-548, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and BDNF (ab203573, Abcam). Secondary antibodies were IRDye 800CW (green) or 680RD (red) to generate signals that were quantified in Image Studio (v2) by LiCor Odyssey (Lincoln, NE, USA). Biochemical and molecular evaluations were from multiple independent experiments.

Statistics

Student t tests were performed using Prism 6 Software (GraphPad Inc., San Diego, CA, USA). Group effects (GM2+/− Vehicle vs FTY720), time effects, and group × time interactions were evaluated by parametric repeated measures ANOVA (RM-ANOVA) with post hoc Bonferroni tests using SPSS (v20, IBM, New York, NY). Expression of trophic factor mRNAs were calculated by comparative Ct method ($2^{-\Delta\Delta Ct}$) using the Relative Expression Software Tool (REST) (Pfaffl *et al.* 2002) (http://www.REST.de.com). Data represent mean ± standard error (SEM), except for mRNA whisker box plots created with REST 2009, demonstrating median (dotted line inside the box), interquartile ranges 1 and 3 (upper and lower edges of the box), and maximum and minimum expression values (top and bottom whiskers). Significance was set to $p < 0.05$ for all assessments.

RESULTS

FTY720 improves movement and sensorimotor function in aging GM2+/− parkinsonian mice

Rotarod measures balance and coordination as well as physical endurance. At 9 mo, in baseline tests, all GM2+/− mice performed similarly. Dosing stared at 11 mo, and at 12 mo, after 4 weeks of FTY720 or vehicle, GM2+/− mice treated with FTY720 showed no decline in performance while vehicle treated littermates showed a trend toward a decline in performance. However, by 16 mo, after 5 mo of FTY720 or vehicle, the FTY720 treated GM2+/− mice performed significantly better than their vehicle treated littermates, and even better than their own performance at 9 and 12 mo (Fig. 1A).

We also evaluated sensorimotor function by timing the ability to remove an adhesive sticker placed on the forehead of GM2+/− mice. As can be appreciated in Figure 1B, at 9 mo, all GM2+/− mice removed the sticker within approximately 10 sec. At 12 mo, all GM2+/− mice took much longer to remove the sticker. By 16 mo, after 5 mo treatment with FTY720 or vehicle, the FTY720 GM2+/− mice had improved timing (~ 25 seconds) while vehicle treated GM2+/− performance continued to worsen (~57 seconds) (Fig. 1B).

As a test of motor nerve function, we also measured hindlimb reflexes of GM2+/− mice. At 9 mo all GM2+/− mice had scores that were within a relatively normal range (3.25). At 12 mo, after 4 weeks of FTY720 or vehicle, all GM2+/− mice still had similar scores regardless of treatment (3.25). However, by 16 mo, after 5 mo of FTY720 or vehicle, the FTY720 treated GM2+/− mice had hindlimb reflexes similar to their 9 and 12 mo scores, while vehicle treated littermates had scores consistent with a significant decline (~2.6). These findings demonstrate that FTY720 sustained hindlimb reflexes (Fig. 1C), while vehicle treated GM2+/− mice showed parkinsonian progression akin to what occurs over time in PD.

Bladder function is also improved by FTY720 in GM2+/− mice

Abnormal bladder function is common in patients with PD (Araki *et al.* 2000), and we confirmed bladder dysfunction in the related GM2−/− KO parkinsonian model (Gil-Tommee *et al.* 2019). Those findings prompted us to measure bladder function in GM2+/− mice treated with vehicle or FTY720. Mice with bladder hyperreflexia produce more small spots

and fewer large spots as measured using a urinary void spot test (described in Experimental Procedures). We noted that aging GM2+/− mice treated with vehicle had significantly more small spots (Fig. 2A) and significantly fewer large spots (Fig. 2B) than littermate GM2+/− mice treated with FTY720. This suggested that bladder function was significantly improved by FTY720. As an additional measure of bladder health we biochemically evaluated proNGF protein levels in bladders of vehicle and FTY720 treated GM2+/− mice, as proNGF is known to increase along with bladder dysfunction (Ryu *et al.* 2018). Evaluation of bladder proNGF by immunoblot revealed that FTY720 significantly reduced bladder proNGF, as can be appreciated in Figure 2C ($p < 0.05$).

FTY720 reduces pathological aSyn and increases BDNF and myelin associated protein levels in brachial plexus of aging GM2+/− mice

Optic and sciatic nerves of GM2−/− KO mice have abnormal myelination/degeneration (Sheikh *et al.* 1999) and aSyn has been shown to increase in aging GM2−/− KO and GM2+/− parkinsonian mouse brain (Wu *et al.* 2012a; Wu *et al.* 2011). However, no one has evaluated the brachial plexus of GM2+/− mice for synucleinopathy or protection in response to a therapeutic. We chose to do so because brachial plexus is the main innervation of the forelimb that is associated with rotarod and adhesive removal tests, and also contributes to arm swing in humans (Mirelman et al. 2016). We collected brachial plexus, brain, and urinary bladders at necropsy. We assessed aSyn aggregation in GM2+/− brachial plexus by immunoblot and saw abundant aggregated aSyn in vehicle treated GM2+/− mice (Fig. 3A, lane 1). In contrast, brachial plexus of FTY720 treated GM2+/− mice had monomeric aSyn with no high molecular weight bands (Fig. 3A, lane 2). These findings suggest that FTY720 has potent anti-synucleinopathy properties, as previously seen in A53T aSyn mice treated with FTY720 (Vidal-Martinez *et al.* 2016). Evaluation of multiple brachial plexus samples by dot blot allowed us to quantify total aSyn levels, which was reduced in GM2+/− mice by FTY720 (Fig. 3B, left; Vehicle = 1.01 ± 0.02; FTY720 = 0.25 ± 0.08; $p < .0001$). As Lewy body aggregates are enriched in PSer129 aSyn we also quantified PSer129 aSyn on dot blots and saw that FTY720 significantly reduced aSyn PSer129 levels in GM2+/− brachial plexus (Fig. 3B, right side; Vehicle = 1.01 ± 0.22; FTY720 = 0.25 ± 0.08; $p < .001$). We then measured brachial plexus BDNF by immunoblot, and found that FTY720 increased proBDNF and preproBDNF levels in GM2+/− brachial plexus compared to vehicle treated littermates (Fig. 3C). We also measured markers of myelin and axonal integrity, MBP and CNP on brachial plexus by immunoblot. It is known that MBP and CNP proteins decrease in association with aging and/or pathology (Ansari & Loch 1975; Xie *et al.* 2013; Kuhlmann *et al.* 2008; Wang *et al.* 2004). After equal protein loading, we compared MBP and CNP levels in 9 mo GM2+/− brachial plexus as a positive control, to MBP and CNP protein in brachial plexus of vehicle treated GM2+/− mice, which was nearly absent (Fig. 3D). In contrast, FTY720 treated GM2+/− brachial plexus had CNP and MBP levels similar to those seen at 9 mo (Fig. 3D), a time when GM2+/− motor function was relatively normal (Fig. 1A – 1C).

Cerebellar BDNF mRNA is also increased in response to FTY720 in GM2+/− mice

As FTY720 improved movement of GM2+/− mice (Fig. 1A – 1C) we measured neurotrophic factor expression in cerebellum, a brain region that contributes significantly to balance and coordination. Similar to our earlier findings in A53T aSyn transgenic mice

treated with FTY720 (Vidal-Martinez *et al.* 2016), we saw significantly increased BDNF mRNA levels as measured using qPCR. However, GDNF, NGF, and CNTF levels were not changed after oral FTY720 in GM2+/− mice (Fig. 4). This BDNF increase in cerebellum parallels the BDNF increases noted in the brachial plexus of GM2+/− mice (Fig. 3C).

Abnormal brachial plexus myelin and axons in untreated GM2 transgenic mice

We previously demonstrated atypical myelination and axons of GM2−/− KO bladder innervation by transmission electron microscopy (TEM) (Gil-Tommee *et al.* 2019). As demonstrated above, vehicle treated GM2+/− mice showed a loss of MBP and CNP proteins in brachial plexus (Fig. 3D). Unfortunately, brachial plexus from treated mice was frozen and thus could not be evaluated by TEM. However, no one has ever assessed the brachial plexus of any GM2 mice by TEM. Thus, we obtained GM2 mice from Dr. Mariko Saito (Nathan Kline Institute, Orangeburg, NY) and in collaboration with Dr. Peter Cooke (New Mexico State University, Las Cruces, NM) performed brachial plexus TEM (Fig. 5). This demonstrated that brachial plexus axons and myelin sheaths in WT GM2 mouse are normal (Fig. 5A), in GM2+/− mice are abnormal (Fig. 5B), and even more abnormal in GM2−/− KO mice (Fig. 5C). Although we did not evaluate FTY720 treated GM2+/− brachial plexus by TEM, it is tempting to speculate that FTY720 may have improved its ultrastructure as we did see BDNF, MBP, and CNP levels increase in the nerve.

DISCUSSION

The World Health Organization lists neurological disorders as a great risk factor facing human health due to both aging and the exponential rise in the world's population (https://www.who.int/news-room/fact-sheets/detail/ageing-and-health). As the main risk factor for PD is aging, its incidence is approaching epidemic levels. This awareness has prompted a concerted effort by the research community to identify early PD biomarkers and to develop protective therapies that can slow or halt PD progression. With this in mind, we began preclinically evaluating the anti-parkinsonian potential of the FDA approved MS drug, FTY720. Importantly, FTY720 has a long half-life (Kovarik *et al.* 2004; Meno-Tetang *et al.* 2006), allowing us to give the drug just 3 times/week, which was well-tolerated and protective. Our findings in this parkinsonian mouse model, GM2+/− mice, along with data from other parkinsonian models support FTY720 repurposing for PD (Ren *et al.* 2017; Zhao *et al.* 2017; Vidal-Martinez *et al.* 2016).

Movement abnormalities are typically the main symptom at PD diagnosis. However, few parkinsonian animal models replicate the age-onset movement problems seen in PD (Dawson *et al.* 2010; Farrell *et al.* 2014; Chesselet & Richter 2011; Potashkin *et al.* 2010). Fortunately, GM2+/− mice develop such motor deficits over time (Wu *et al.* 2012a; Wu *et al.* 2011), making it an excellent model for testing prolonged drug treatment. As it is also important to test drugs after symptoms have manifested, we aged our GM2+/− mice then treated them with FTY720 or vehicle from 11 – 16 mo. This allowed us to show that 5 mo of oral FTY720 significantly improved movement as measured by rotarod, adhesive removal and hindlimb reflex tests (Fig. 1). Bladder muscle can also be affected in PD, as previously shown in GM2−/− KO mice, which develop age-onset bladder dysfunction (Gil-Tommee *et*

al. 2019). We measured void patterns of GM2+/− mice and saw that mice treated with FTY720 had more normal void patterns compared to vehicle treated littermates with hyperreflexia (Fig. 2A, 2B). In GM2−/− KO bladder, we found abundant proNGF, a biomarker of bladder dysfunction (Ryu *et al.* 2018; Gil-Tommee *et al.* 2019). We therefore measured bladder proNGF by immunoblot in FTY720 and vehicle treated mice, revealing significantly lower levels of proNGF in FTY720 treated GM2+/− mice (Fig. 2C).

Another common motor problem in PD is decreased arm swing, which may serve as a PD diagnostic marker (Mirelman *et al.* 2016). Brachial plexus modulates arm swing in humans (Souza *et al.* 2016; Pontzer *et al.* 2009; Collins *et al.* 2009), thus we evaluated brachial plexus of aging GM2+/− mice. By immunoblot we saw abundant Lewy-like aSyn aggregation in vehicle treated brachial plexus that was reversed by FTY720 (Fig. 3A). We also found abundant pathological PSer129 aSyn in the brachial plexus of vehicle treated mice that was reversed by FTY720 (Fig. 3B). As myelination problems can affect movement, we measured levels of key myelin associated proteins in GM2+/− brachial plexus, which revealed that FTY720 maintained MBP and CNP at young levels compared to a loss of MBP and CNP in vehicle treated GM2+/− brachial plexus by immunoblot (Fig. 3D).

FTY720 improves function at least in part by increasing BDNF expression *in vivo* and *in vitro* (Deogracias *et al.* 2012; Doi *et al.* 2013; Smith *et al.* 2015; Vidal-Martinez *et al.* 2016; Ren *et al.* 2017; Segura-Ulate *et al.* 2017). As brachial plexus showed changes in myelin associated proteins we also measured brachial plexus BDNF on immunoblots. This revealed increased levels of proBDNF and preproBDNF proteins in GM2+/− mice treated with FT720 (Fig. 3C). As cerebellum is a brain region that regulates balance and coordination, we also measured expression of BDNF and other trophic factors in GM2+/− cerebellum by qPCR. While a significant increase in BDNF mRNA was seen in cerebellum of FTY720 treated mice, the levels of GDNF, NGF, and CNTF were not increased after 3x/week FTY720 (Fig. 4). Remarkably, cerebellum also contributes to urinary function (Chou *et al.* 2013; Sugiyama *et al.* 2009).

We and others have shown abnormal myelination in nerves in GM2−/− KO mice (Gil-Tommee *et al.* 2019; Sheikh *et al.* 1999; Chiavegatto *et al.* 2000), but no one has assessed any nerves in GM2+/− mice. We saw increased levels of MBP and CNP myelin associated proteins in GM2 +/− brachial plexus in response to FTY720 (Fig. 3D). And though we could not perform TEM from aging studies with FTY720, we obtained GM2 mice in which to assess brachial plexus ultrastructure by TEM. This allowed us to show that WT brachial plexus had normal myelin surrounding healthy axons (Gil-Tommee *et al.* 2019), while GM2+/− and GM2−/− KO myelination and axons were abnormal (Fig. 5). These findings paralleling findings in brachial plexus for aSyn, MBP, and CNP (Fig. 3), which likely contributed to improved function of GM2+/− mice treated with FTY720.

In summary, low dose oral FTY720 given 3x/week was well-tolerated and increased BDNF expression in parkinsonian GM2+/− mice. FTY720 also reduced pathological Lewy-like aSyn aggregation in brachial plexus. Regarding urinary bladder, FTY720 improved voiding and reduced levels of the bladder dysfunction marker, proNGF. Taken together these

preclinical findings in GM2+/− parkinsonian mice provide compelling support for testing FTY720 in patients with PD in hopes of slowing or delaying PD progression.

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HIGHLIGHTS

- GM2+/− mice, with reduced GM1 levels, develop age-onset parkinsonian symptoms
- Reduced GM1 levels increase GM2+/− synucleinopathy and loss of myelin markers
- Low dose FTY720 reduces synucleinopathy in the GM2+/− nervous system
- Low dose FTY720 increases BDNF and myelin markers in GM2+/− mice
- Movement and bladder function are improved by low dose FTY720 in GM2+/− mice

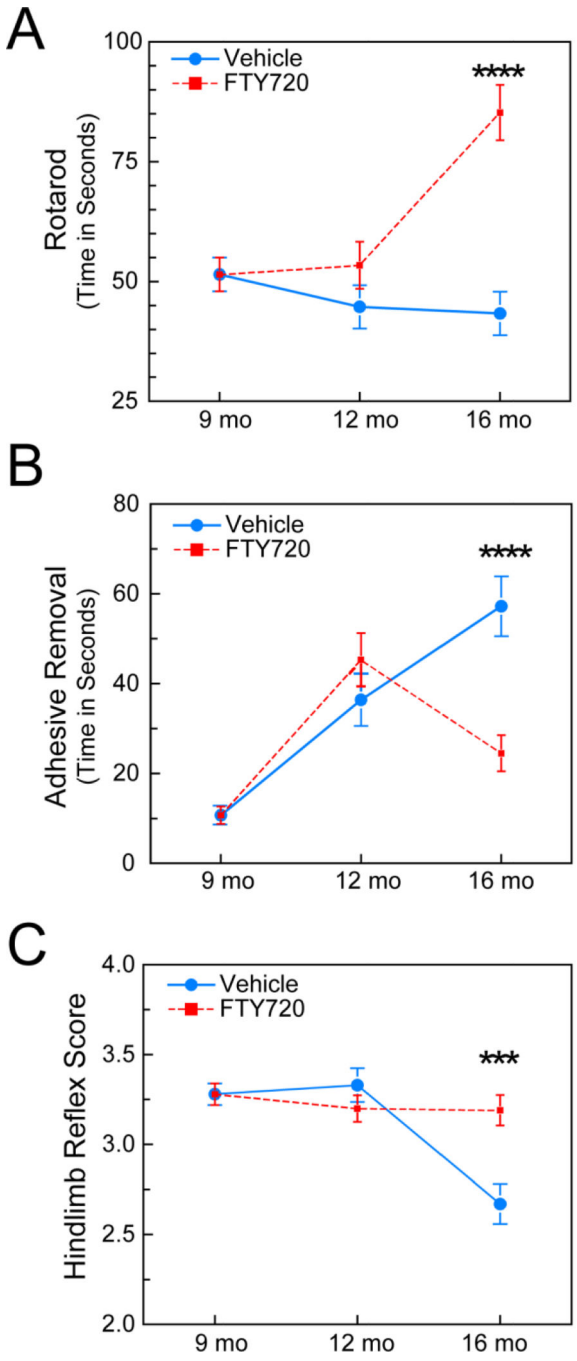

Figure 1. GM2+/− movement is improved or sustained in response to FTY720.

All motor tasks were evaluated at baseline (9 mo) and again at 12 mo and 16 mo for GM2+/− mice treated with vehicle or FTY720. (**A**) FTY720 treated GM2+/− mice significantly improved their time on the rotarod at 16 mo, while vehicle treated littermates rotarod performance declined. (**B**) Adhesive removal timing is better when riming is shorter. GM2+/− mice treated with FTY720 performed significantly better than vehicle treated GM2+/− littermates at 16 mo. (**C**) The hindlimb reflexes of GM2+/− mice treated with FTY720 were

sustained at relatively normal levels, while vehicle treated GM2+/− mice developed poorer hindlimb reflexes by 16 mo. RM-ANOVA, ***, $p < .001$; ****, $p < .0001$.

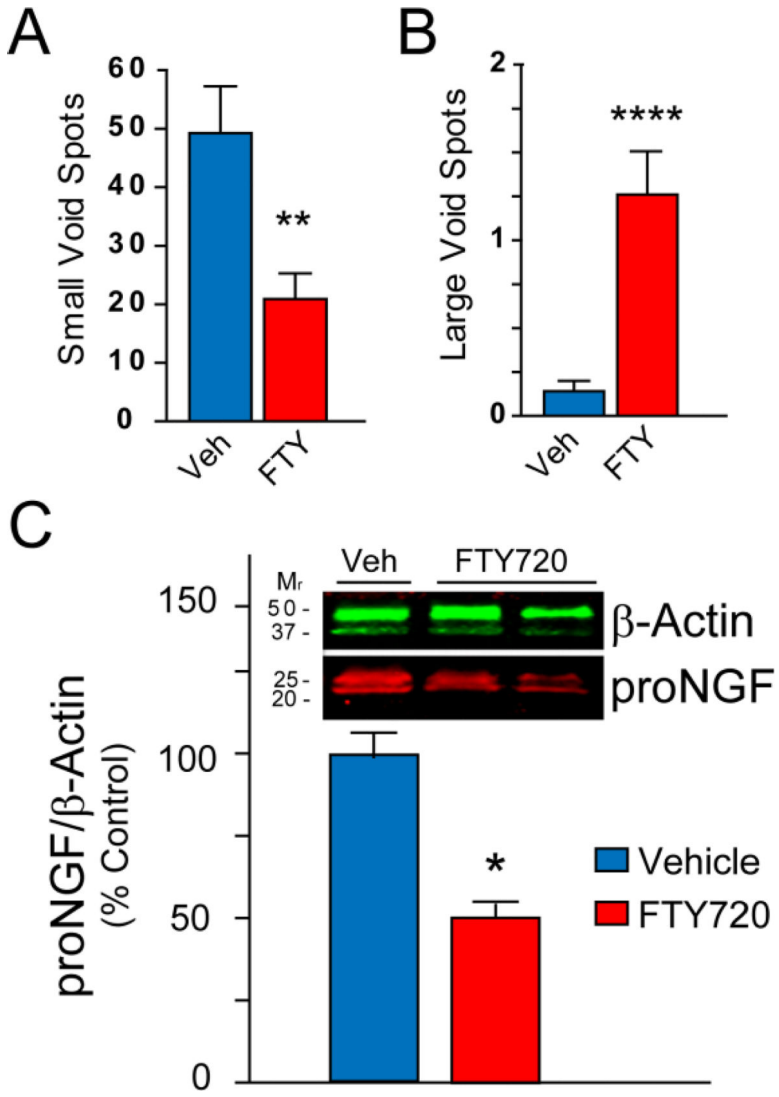

Figure 2. FTY720 improves voiding function and reduces proNGF levels in GM2+/− bladder.

The void spot test is a measurement tool in which fewer small spots and more large spots indicate normal bladder function. Levels of proNGF increase with bladder dysfunction. (**A**) FTY720 treated GM2+/− mice have significantly fewer void spots at 16 mo. (**B**) FTY720 treated GM2+/− mice have significantly more large void spots at 16 mo. (**C**) Representative immunoblots from vehicle and FTY720 treated GM2+/− bladder, confirm reduced proNGF levels in GM2+/− mice treated with FTY720. Student's t test, *, $p < .05$, **, $p < 0.01$; ****, $p < .0001$.

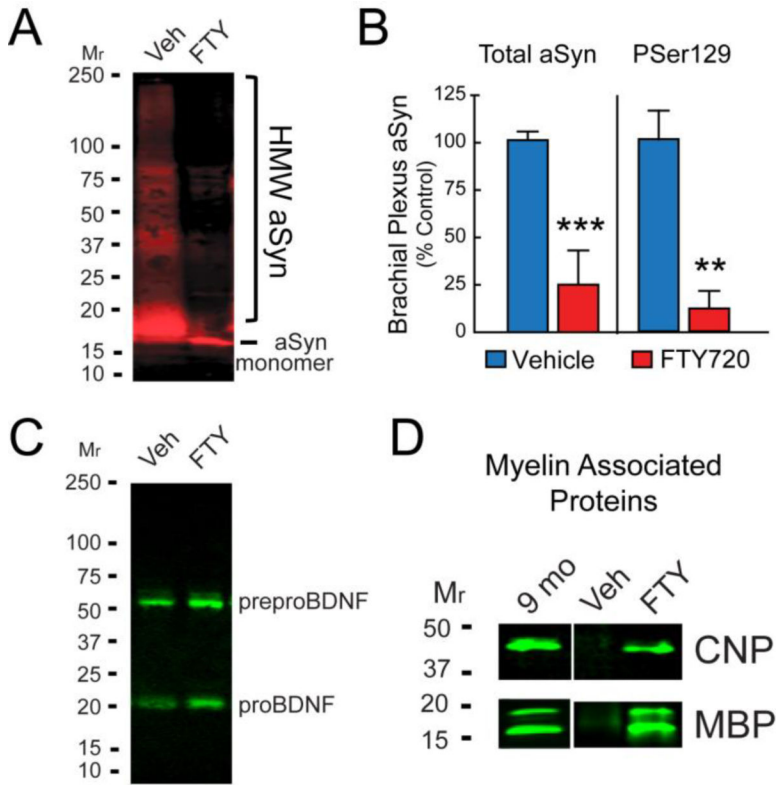

Figure 3. FTY720 reduces synucleinopathy, increases BDNF protein, and sustains MBP and CNP at younger levels in aging GM2+/− brachial plexus.

(**A**) aSyn immunoblot from representative vehicle (Veh) treated GM2+/− mice shows abundant high molecular weight (HMW) aSyn, which is eliminated in brachial plexus of age matched FTY720 treated GM2+/− mice. (**B**) Quantification of total aSyn and pathological PSer129 aSyn from multiple brachial plexus samples confirms a significant reduction in synucleinopathy in FTY720 treated GM2+/− mice compared to vehicle treated GM2+/− mice. (**C**) Reprobing the immunoblot shown in A for BDNF, confirms FTY720 increased preproBDNF and proBDNF levels in brachial plexus, as compared to vehicle treated GM2+/− mice. (**D**) Myelin associated proteins MBP and CNP are abundant in brachial plexus of untreated 9 mo mouse, absent in 17 mo vehicle treated GM2+/mouse, and sustained in 17 mo GM2+/− mouse treated with FTY720. Student's t test, **, $p < 0.01$; ***, $p < .001$.

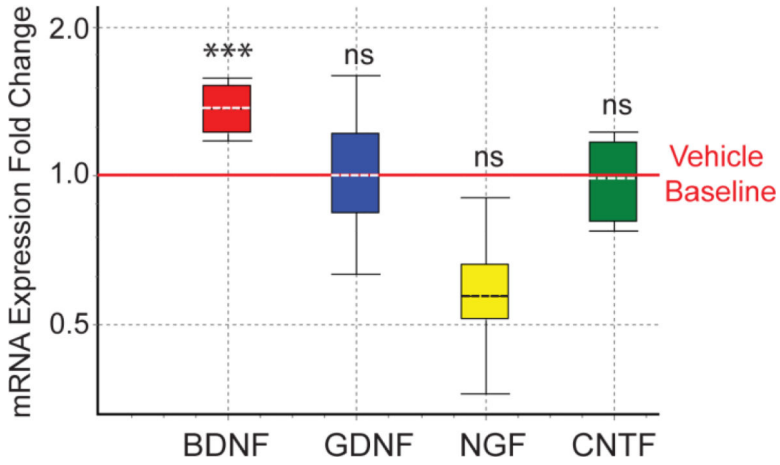

Figure 4. FTY720 increases BDNF mRNA in GM2+/− cerebellum.

Cerebellar qPCR from vehicle treated and FTY720 treated littermates confirm a significant increase in BDNF but not in GDNF, NGF, or CNTF neurotrophic factors. ***, $p < .001$, ns, not significant.

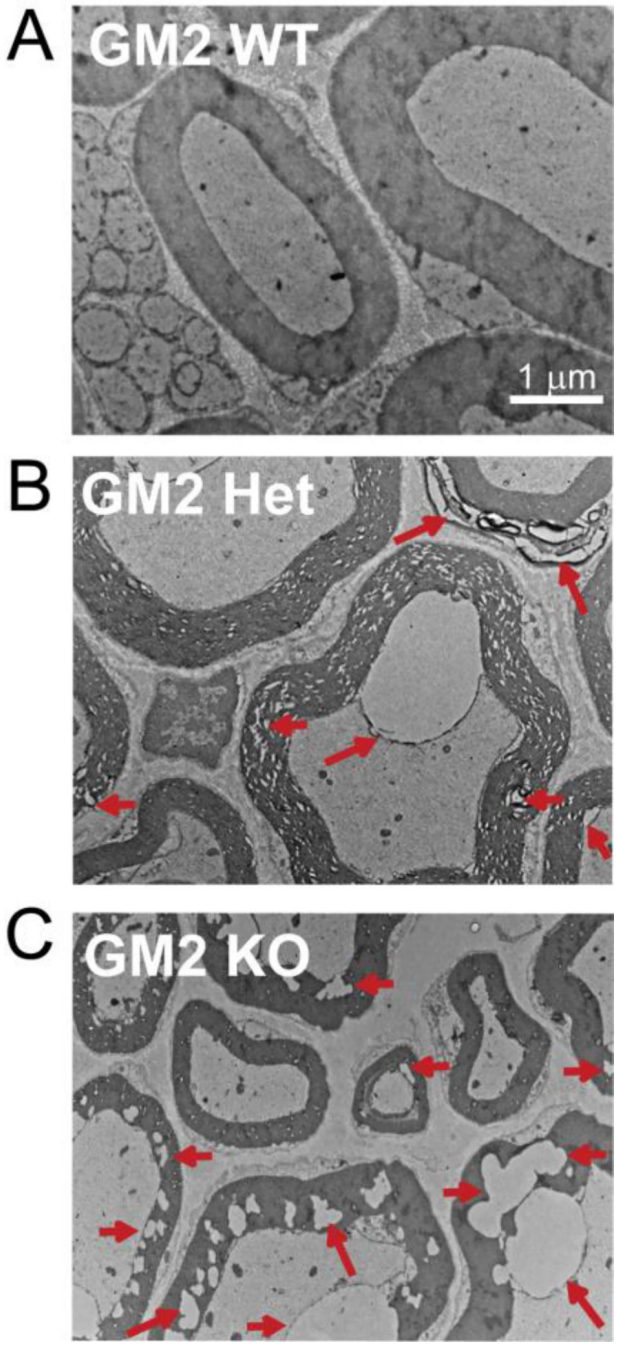

Figure 5. Brachial plexus myelination and axonal abnormalities in GM2 transgenic mice.

(**A**) Myelin sheaths are uniform and surround normal axons in the brachial plexus of a wild type (WT) GM2 mouse. (**B**) Arrows point to areas of abnormal myelin sheaths as well as to atypical axonal changes in the brachial plexus of a GM2+/− mouse. (**C**) Arrows point to areas of abnormal myelin sheaths and axonal changes in the brachial plexus of a GM2 synthase KO mouse. Scale bar = 1 µm.