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Parkinsonian GM2 Synthase Mice Lacking Mature Gangliosides Develop Urinary Dysfunction and Neurogenic Bladder

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Abstract

Parkinson's disease is a neurodegenerative disorder that reduces a patients' quality of life by the relentless progression of motor and non-motor symptoms. Among the non-motor symptoms is a condition called neurogenic bladder that is associated with detrusor muscle underactivity or overactivity occurring from neurologic damage. In Parkinson's disease, Lewy-body-like protein aggregation inside neurons typically contributes to pathology. This is associated with dopaminergic neuron loss in substantia nigra pars compacta (SNc) and in ventral tegmental area (VTA), both of which play a role in micturition. GM1 gangliosides are mature glycosphingolipids that enhance normal myelination and are reduced in Parkinson's brain. To explore the role of mature gangliosides in vivo, we obtained GM2 Synthase knockout (KO) mice, which develop parkinsonian pathology including a loss of SNc dopaminergic neurons, which we reconfirmed. However, bladder function and innervation have never been assessed in this model. We compared GM2 Synthase KO and wild type (WT) littermates' urination patterns from 9–19 months of age by counting small and large void spots produced during 1 hour tests. Because male and female mice had different patterns, we evaluated data by sex and genotype. Small void spots were significantly increased in 12–16 month GM2 Synthase KO females, consistent with overactive bladder. Similarly, at 9–12 month GM2 KO males tended to have more small void spots than WT males. As GM2 Synthase KO mice aged, both females and males had fewer small and large void spots, consistent with detrusor muscle underactivity. Ultrasounds confirmed bladder enlargement in GM2

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Author contributions

CAR and RGP conceived of the project and conducted pilot studies; CGT and GVM extended analyses with ISU, DM, GVH, SAC and SMM; CGT and DM performed immunohistochemistry; CGT and JVM did biochemical analyses; GVM did mouse breeding and genotyping with CGT, ISU, SAC and GVH; CGT and RGP analyzed data and wrote the paper with editorial assistance from GVM, CAR, and JVM.

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Competing interests

The authors declare no conflict of interest.

Synthase KO compared to WT mice. Tyrosine hydroxylase (TH) immunohistochemistry revealed significant dopaminergic loss in GM2 Synthase KO VTA and SNc, and a trend toward TH loss in GM2 KO periaqueductal gray (PAG) micturition center. Levels of the nerve growth factor precursor, proNGF, were significantly increased in GM2 Synthase KO bladders and transmission electron micrographs established atypical myelination of pelvic ganglion innervation in GM2 Synthase KO bladders. Cumulatively, our findings provide the first evidence that mature ganglioside loss affects micturition center TH neurons as well as proNGF dysregulation and innervation of the bladder. Thus, identifying therapies that will counteract these effects could be beneficial for those suffering from Parkinson's disease and related disorders.

1. Introduction

Parkinson's disease (PD) is an aging disorder that affects millions of people worldwide. The hallmark pathology of PD includes the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and pathological alpha-synuclein (aSyn) aggregation in Lewy bodies/Lewy neurites (Shulman et al., 2011). The substantia nigra pars compacta (SNc) dopaminergic neuron loss ultimately leads to movement problems, however, non-motor symptoms like urinary dysfunction are also common in PD (Winge et al., 2006). While not yet widely appreciated, there is growing evidence that the dopaminergic neurons of the ventral tegmental area (VTA) are also damaged in PD (Alberico et al., 2015; Harrison et al., 2016; Rinne et al., 1990). Up to 83% of those with idiopathic PD also exhibit urinary dysfunction with symptoms of nocturia, incontinence, urgency, and/or poor bladder emptying (Campos-Sousa et al., 2003). This type of dysfunction, also known as neurogenic bladder, results from damage to central, peripheral, and/or autonomic nervous system inputs to the bladder (Dubow, 2007; Ginsberg, 2013; Hall et al., 2012; Lemack et al., 2000). Neurogenic bladder has been associated with abnormal nerve growth factor (NGF) levels (Hamill et al., 2012), with NGF proposed as a neurogenic bladder biomarker (Seth et al., 2013). Interestingly, the precursor form of NGF, proNGF, is the main NGF found in adult human brain (Al-Shawi et al., 2007), and proNGF overexpression is associated with sympathetic and cholinergic system degeneration in animal models (Cuellar, 2012). ProNGF has also been shown to be implicated in bladder dysfunction (Ryu et al., 2018). Moreover, loss of modulatory inputs arising from SNc and VTA dopaminergic neurons can affect brain micturition centers in a manner to negatively impact bladder function (Kitta et al., 2008; Soler et al., 2011; Yoshimura et al., 2003). Together the data suggest that evaluating brain regions associated with micturition, bladder function, bladder volumes, as well as aSyn and NGF levels in bladder of GM2 Synthase deficient parkinsonian mice will be informative.

Among other major molecules that regulate neurodevelopment and neuronal function are the gangliosides, which are sialic acid-bearing glycosphingolipids that are highly expressed in brain (Schnaar, 2010). There are four major mature gangliosides that contribute to myelination and neurotransmission, GM1 (mono, with one sialic acid), GD1a and GD1b (di, with two sialic acids), and GT1b (tri, with three sialic acids) (Palmano et al., 2015; Posse de Chaves and Sipione, 2010; Sturgill et al., 2012). In fact, axon growth and stability utilize a lectin-glycan system composed of gangliosides that interact with myelin associated glycoprotein (MAG) (Schnaar, 2010). MAG, is one of the major sialic acid-binding lectin

molecules in brain that binds directly to GD1a and GT1b in a manner to optimize cell-cell recognition and structure, as well as neuronal signaling (Schnaar and Lopez, 2009). Moreover, deficiencies in GD1a and GT1b have been shown to contribute to neurodegeneration (Ohmi et al., 2014) and GM1 levels in substantia nigra dopaminergic neurons are lower than normal in PD brain (Hadaczek et al., 2015; Wu et al., 2012a). Recent data further confirm that levels of immature GM3 gangliosides are also elevated in PD serum (Chan et al., 2017). Treating PD patients with GM1 or the GM1-analogue, LIGA-20 can significantly reduce PD motor symptoms (Schneider et al., 1998; Schneider et al., 2010). These data imply that loss of GM1 gangliosides contributes significantly to PD pathogenesis, though the impact of GM1/GM2 ganglioside loss on bladder is unexplored.

Transgenic *B4galnt1* null mice entirely lack expression of GM2 synthase (EC 2.4.1.92), the enzyme that catalyzes the second step in ganglioside biosynthesis to convert immature GM3, GD3, and GT3 gangliosides to mature GM1, GD1, and GT1 gangliosides (Sheikh et al., 1999). The GM2 synthase KO mice have low MAG expression, demyelination, and develop age onset motor impairment (Chiavegatto et al., 2000; Schnaar and Lopez, 2009; Sheikh et al., 1999). More recently, motor impairment in GM2 Synthase KO (−/−) mice was shown to be associated with nigrostriatal dopaminergic aSyn-pathology (Wu et al., 2011) and model parkinsonism. Similar parkinsonian changes also occur in the heterozygous (+/−) GM2 Synthase mice (Wu et al., 2012a), making both models appropriate for PD research. Based on these findings we hypothesized that parkinsonian GM2 Synthase KO (−/−) mice would develop PD-like urinary dysfunction due to aSyn pathology and trophic factor dysregulation in the bladder.

2. Materials and Methods

2.1 Animals.

Heterozygous *B4galnt1* (+/−) breeders were generously provided by Drs. Ledeen and Wu of Rutgers School of Medicine (Newark, NJ, USA), to generate our mouse colony. Genotyping was performed by PCR as previously described (Wu et al., 2001). For this study, we evaluated a cohort of male and female WT (+/+) mice that express two copies of GM2 Synthase, and GM2 Synthase KO mice that totally lack GM2 Synthase (−/−) expression, from 9–23 months of age. Animals were maintained on 12-hr light/dark cycles with access to food and water *ad libitum* except during 1 hour urinary pattern tests as described below. All experiments were conducted using protocols approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee (IACUC) in accordance with AALAC and National Institutes of Health guidelines.

2.2 Behavioral Tests and Other Measures.

Open Field. (N = 20, 9 WT and 11 KO). Locomotor activity was assessed using a TruScan™ open field activity monitor (Coulbourn Instruments, Whitehall, PA, USA). Mice were acclimated to the test room for 15 min prior to being placed individually in the center of the arena with total movement monitored for 15 minutes. Total movement was measured as successive coordinate movements made across the floor plane while mice were continuously active. Mice were tested in random order on two independent occasions essentially as

previously described (Farrell et al., 2014). *Urination Patterns*. (N = 26, 15 WT and 11 KO). Food and water were removed during tests. Five independent tests were conducted per mouse per group at each time point. Each mouse was individually placed in a clean cage for 1 hour (10:00–11:00 am) with the cage bottom covered with a fitted clean white filter paper (Bio-Rad, Hercules, CA, USA, cat# 1650962) as shown in Figure 1a. Filter papers were collected, labeled, and allowed to dry. Urine spots illuminated with UV light allowed spots to be categorized as small ($< 0.2 \text{ cm}^2$) or large ($> 0.2 \text{ cm}^2$) and counted by individuals blinded to genotypes, using established methods (Birder et al., 2002; Bjorling et al., 2015; Hamill et al., 2012; Hodges et al., 2008; Studeny et al., 2008; Yu et al., 2014). If overlapping urine spots were detected, they were not included in the counts. An image of a UV illuminated filter shows large and small urine spots that can be easily differentiated (Figure 1b). *Water Intake*. (N = 10, 5 WT and 5 KO). Mice were individually housed in cages with water delivered from a 50 mL conical tube sealed with a #7 single hole rubber stopper containing a double-ball water sipper. Tubes were weighed before testing and the next morning in order to calculate water intake. *Bladder Volumes*. (N = 15, 8 WT and 7 KO). Mice were anesthetized with isoflurane and abdomens were shaved with electric clippers. Post void bladder volumes were measured by ultrasonography using a moveable Real Time 4D Volume probe with data collected by associated imaging software (Voluson® VE8/VE8 Expert General Electric® Health Care; Wauwatosa, Wisconsin, USA). Briefly, the transducer was coated with ultrasound gel (McKesson Ultrasound Gel PINK; McKesson Medical–Surgical, Richmond, VA, USA), placed at a 45° degree angle on the abdomen and moved until the bladder image appeared on the monitor. Bladder volumes were calculated by measuring bladder length and depth according to established methods (Al-Izki et al., 2009).

2.3 Tissue Collection and Sample Preparation.

Mice were euthanized by CO₂ inhalation followed by decapitation. Bladder and brain tissues were dissected and prepared as described below. *Immunohistochemistry*. Brains (N = 6 WT, 6 KO) were collected and post-fixed in 4% formaldehyde/sucrose overnight. Tissues were immunostained free-floating using antibodies for aSyn (C20, sc-7011-R, Santa Cruz, CA, 1:100) and tyrosine hydroxylase (TH; chicken anti-TH, Aves Labs, Tigard, OR, 1:200–1:250) followed by secondary antibodies, then evaluated by confocal microscopy using the Olympus FluoView 1000 with quantification as previously described (Alerte et al., 2008; Farrell et al., 2014; Vidal-Martinez et al., 2016) or with the EVOS Cell Imaging System (Thermo-Fisher, USA) and ImageJ Software (Rueden et al., 2017). *Transmission electron microscopy*. (N = 2 WT, 2 KO). We followed traditional protocols for fixing and embedding tissues. Briefly, bladders with the bladder base and major pelvic ganglia intact, were fixed in 4% paraformaldehyde overnight at 4°C then washed with 10 mL of 0.1 M imidazole-HCl buffer at pH 7.2 and fixed in 2.5% glutaraldehyde in 0.1 M imidazole buffer at room temperature. Epoxy resin embedded-tissue samples were trimmed and regions of interest were surveyed in semi-thin sections stained with a methylene blue dye mixture (Richardson et al., 1960) on glass microscope slides with a bright field optical microscope. Thin sections of tissue regions, including selected bundles of nerve fibers and nerve cells, were cut with a diamond knife and mounted on 100 mesh copper grids, followed by staining with 2% uranyl acetate solution and lead citrate. Grids were examined at 80 kV accelerating voltage on a model H-7650 transmission electron microscope (Hitachi High Technologies, Dallas, TX)

with digital images collected using a mid-mount CCD camera system and acquisition software (Advanced Microscopy Techniques, Woburn, MA). *Immunoblots*. For standard immunoblots, tissues were homogenized in 8 volumes (w/v) ice-cold buffer containing 1 mM AEBSF, 5 μ M aprotinin and 5 μ M leupeptin using a Bullet Blender (Next Advance, Inc., Averill Park, NY, USA). Samples were then sonicated (Sonic Dismembrator Model 100, Thermo-Fisher, USA) and spun at 14,000 \times g, 10 min at 4°C to remove particulates. *Sequential protein extraction* to separate soluble from insoluble proteins uses a series of buffers and pellet re-extractions performed with ultracentrifugation as previously described (Vidal-Martinez et al., 2016; Waxman and Giasson, 2008; Wu et al., 2012b). (N = 7, 1 A53T, 3 WT, and 3 KO). Protein concentrations were determined by bicinchoninic acid assay (Thermo-Fisher, USA). Gels, loaded with 50 μ g total protein per sample, were transferred to nitrocellulose, blocked in 5% non-fat milk and incubated in primary antibodies overnight at 4°C. Immunoblot antibodies included aSyn (C20, sc-7011-R, Santa Cruz, CA), proNGF (H-20, sc-548, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β -actin (4970S, Cells Signaling Technology, Danvers, MA, USA). Secondary antibodies IRDye 800CW (green) or 680RD (red) produced infrared fluorescence for capture using the Li-Cor Odyssey system (Lincoln, NE, USA).

2.4 Statistical Analysis.

Data from WT and GM2 Synthase KO mice were analyzed by independent sample *t*-tests or two way ANOVA, as appropriate to the data, using Prism 6 or InStat (GraphPad Software Inc., San Diego, CA, USA), with significance levels set to $p < 0.05$ and variability shown as the standard error of the mean.

3. Results

3.1 Total movement and water intake are similar in GM2 Synthase KO and WT mice.

As previously shown by others (Chiavegatto et al., 2000; Sheikh et al., 1999), we also noted that GM2 Synthase KO mice developed coordination and balance problems as they aged (not shown). Though GM2 Synthase KO mice became uncoordinated, they still efficiently moved around the cage during urinary pattern tests. This was confirmed by measuring total movement in an open field for WT and GM2 Synthase KO mice at 9, 12, 16, and 19 months of age. We noted that though GM2 Synthase KO mice tended to move slightly less than WT mice, all mice had similar total movement at all time points (Figure 2). We also assessed overnight water intake for GM2 Synthase KO and WT mice and confirmed equivalent hydration of the mice for our study (WT, 4.08 ± 0.3 mL; KO, 4.07 ± 0.4 mL; $p = 0.52$). These data show that the GM2 Synthase KO and WT mice had similar baselines for measuring potential differences in urinary patterns using the void spot test.

3.2 GM2 Synthase KO mice initially show urinary frequency that diminishes with age.

We noted that male mice of both genotypes consistently produced more large and small void spots than female mice. Thus, we compared mouse urination patterns according to genotype and sex. Regarding small spots at 9–12 months, GM2 Synthase KO males tended to have more small spots than WT males, however, at 16 months the KO males tended to have fewer small spots than WT males. Yet, small spot differences were not statistically significant for

males (Figure 3a). With regard to large spots, KO males initially showed a tendency to produce more large spots, but at 16–19 months KO males had significantly fewer large spots than WT males (Figure 3b). Regarding female small spots, KO females had significantly more small spots at 12–16 months than WT females (Figure 3c), as can be better appreciated in the inset graph. With regard to large spots, KO females tended to have more large spots than WT females at 9–12 months, but then tended to have fewer large spots produced at 16–19 months though not significantly (Figure 3d). The finding that all KO mice initially had more void spots that reduced with age suggested that KO mice initially had bladder hyperreflexia and then developed hyporeflexia. This led us to suspect that GM2 Synthase KO mice developed urine retention that can manifest as bladder enlargement, which we tested by doing ultrasounds.

3.3 GM2 Synthase KO mice exhibit significant bladder enlargement.

We noted urine retention and bladder enlargement in a GM2 Synthase KO mouse that had to be euthanized (Figure 4a). We later obtained ultrasound equipment which allowed us to evaluate bladders of live mice. Using ultrasonography, we measured bladder volumes of 19–20 month old GM2 Synthase KO and WT male and female mice after all urination pattern testing was complete. Bladders (volume–cm³) of WT mice were smaller than those of GM2 Synthase KO mice. In GM2 Synthase KO males, bladders were two times larger (0.18 ± 0.03 cm³) than were the bladders of age matched WT males (0.09 ± 0.01 cm³) (Figure 4b). For KO females, their bladders were ~ 6 times larger (0.61 ± 0.16 cm³) than the bladders of age matched WT females (0.09 ± 0.03 cm³) (Figure 4c). This strongly suggested that the reduction in void spots seen for GM2 Synthase KO mice paralleled an increase in urine retention that had produced bladder enlargement.

3.4 Tyrosine hydroxylase differences in WT and GM2 Synthase KO brain micturition centers.

In addition to the PAG micturition center, dopamine signaling related to VTA significantly modulates micturition (Hashimoto et al., 2003). Moreover, accumulating data show that VTA dopaminergic cell loss is relatively common in patients with PD (Alberico et al., 2015; Dragicevic et al., 2015; Harrison et al., 2016; Rinne et al., 1990) as are problems with urinary bladder function (Dubow, 2007; Ginsberg, 2013; Hall et al., 2012; Lemack et al., 2000). Thus, we assessed tyrosine hydroxylase (TH) immunohistochemistry in PAG and VTA of WT and GM2 Synthase KO mice. There is a significant loss of TH in the VTA of GM2 KO mice compared to WT mice (Figure 5a) and a trend toward reduced TH in GM2 KO PAG compared to WT littermates (Figure 5b). This provides new evidence that dopaminergic damage in brain micturition centers can impact voiding in association with a loss in mature gangliosides. Moreover, SNc dopaminergic neurons also contribute to micturition based on data from 6-hydroxydopamine lesioned rats (Soler et al., 2011; Yoshimura et al., 2003). Thus, we also evaluated TH immunohistochemistry which reconfirmed SNc dopaminergic loss in our GM2 Synthase KO mice (Figure 5c). We also noted that aSyn levels were somewhat increased in the GM2 Synthase KO brain.

3.5 Assessment of aSyn in WT and GM2 Synthase KO bladders.

As GM2 Synthase KO mice had significant micturition problems, we measured insoluble aSyn in bladder using an established sequential protein extraction and immunoblot method as before (Lou et al., 2010; Vidal-Martinez et al., 2016; Waxman et al., 2008; Wu et al., 2012b) (Figure 6). With this method, soluble proteins are released by the first three buffers used for sequential extraction of tissue: high salt (HS), high salt triton (HST), and the radio immunoprecipitation assay buffer (RIPA). Insoluble, *aggregated*, proteins are extracted in the final SDS-Urea buffer. As a positive control we used a bladder from an A53T aSyn transgenic mouse that develop age-onset bladder dysfunction (Hamill et al., 2012). Monomeric 17–19 kDa aSyn was present in soluble extracts of A53T Tg, WT, and GM2 KO bladders, however, insoluble high molecular weight (HMW) aSyn was found only in A53T bladder (at arrow, Figure 6, left side) but not in WT or GM2 Synthase KO bladders (at arrows, Figure 6, right side). These findings revealed that aSyn did not aggregate in GM2 Synthase KO bladders. We then evaluated other possible mechanisms known to be associated with bladder dysfunction.

3.6 ProNGF levels are elevated in GM2 Synthase KO bladder.

As bladder NGF is a proposed neurogenic biomarker, we evaluated NGF levels in whole bladders from WT and GM2 Synthase KO mice (N = 4 WT, 5 KO). We noted that there was no mature NGF in the bladders of either WT or GM2 KO mice (not shown). However, proNGF levels were significantly increased in GM2 KO as compared to WT bladders (Figure 7a). Quantification of bladder proNGF levels in 12–23 month old GM2 KO and WT mice confirmed a significant increase in proNGF protein levels only in GM2 KO bladders (Figure 7b, WT 155.9 ± 28.23 ; KO 555.4 ± 183.9 , *t* test, $p = 0.049$). Others recently confirmed that bladder proNGF is detrimental to normal bladder function (Ryu et al., 2018). Because trophic factors can also modulate innervation, we next evaluated bladder innervation by transmission electron microscopy.

3.7 GM2 Synthase KO mice have abnormal myelination in the bladder innervation.

Transmission electron micrographs revealed differences in bladder innervation in the major pelvic ganglion of 17 month old WT and KO Synthase GM2 mice. Large and small nerve fibers, as well as neurons in and around the ganglia were examined with cross-sections of nerve fibers from GM2 Synthase KO and WT mice compared. In WT samples, nerve fibers appeared uniformly filled with cytoplasm containing profiles of neurotubules and neurofilaments along with profiles of mitochondria and small vesicles, surrounded by myelin sheaths of uniform thickness and electron-density (Figure 8a). In contrast, the nerve fibers of the GM2 Synthase KO samples included large irregular vacuoles at the interface between the axolemma and the myelin sheath, which also contained many smaller electron-lucent pockets (Figure 8b, at red arrows). Moreover, the distribution of fibers (collagen) in the extracellular space of the nerve fiber bundles in WT mice were more uniform (Figure 8a) than in corresponding areas from GM2 Synthase KO mice, where large spaces between nerve fibers appeared to be devoid of visible extracellular material (Figure 8b).

The discovery of abnormal bladder innervation in GM2 Synthase KO mice parallels the myelination abnormalities previously described in the optic and sciatic nerves in this mouse

model (Chiavegatto et al., 2000; Schnaar and Lopez, 2009; Sheikh et al., 1999). Moreover, our findings provide new insight into the urinary dysfunction found in aging GM2 Synthase KO mice. Collectively, the data suggest that in male and female GM2 Synthase KO mice the loss of mature gangliosides reduced dopaminergic function in brain micturition centers and increased bladder proNGF levels in a manner to impair bladder innervation and detrusor function without stimulating bladder aSyn aggregation.

4. Discussion

4.1 Role of mature gangliosides in humans with PD and in PD mouse models.

PD patients have lower GM1 ganglioside levels in brain (Hadaczek et al., 2015; Wu et al., 2012a) and GM1 administration to parkinsonian rodents induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or 6-hydroxydopamine, enhances nigral regeneration and reduces their motor impairment (Mocchetti, 2005). PD patients also respond favorably to GM1 treatment by showing significant improvement in motor function (Schneider, 1998; Schneider et al., 2013; Schneider et al., 1995; Schneider et al., 2010). GM1 is able to stimulate signaling through the NGF, BDNF, and GDNF receptors which may underlie improvements seen in response to GM1 (Hadaczek et al., 2015; Mocchetti, 2005; Mocchetti et al., 2007; Posse de Chaves and Sipione, 2010) or a GM1 analog, LIGA-20 (Wu et al., 2011). Treating diabetic rats with mixed brain gangliosides containing 20% GM1 reversed their urinary dysfunction (Paro et al., 1994), establishing a role for mature gangliosides in normal function. While the above findings provide evidence for a role for GM1 in normal motor and urinary function, as we did not treat our GM2 Synthase KO mice with GM1, it is possible that a complete loss of GM1, GM2 or other mature gangliosides may have produced the changes found in this model.

4.2 Urinary dysfunction and aSyn pathology in humans and in PD mouse models.

PD patients often present with age-onset urinary dysfunction (Araki et al., 2000; Martignoni et al., 1995; VanderHorst et al., 2015) which has been shown to correlate with aSyn accumulation in neurons of the spinal cord, paraspinal sympathetic ganglia, and vagal nerve (Braak et al., 2003). Bladder aSyn pathology has been reported only in one mouse model of multiple system atrophy and in one patient with Lewy Body dementia (Beach et al., 2010; Boudes et al., 2013). aSyn pathology was also identified in a region of human PAG that regulates arousal and wakefulness in patients with Lewy body dementia and multiple system atrophy (Benarroch et al., 2009).

4.3 GM2 Synthase KO mice as a parkinsonian neurogenic bladder model.

The above findings suggested that the GM2 Synthase KO mice, which completely lack all mature gangliosides, would develop age-onset autonomic problems in addition to parkinsonian abnormalities related to motor pathways as previously reported for both the GM2 Synthase KO mice and GM2 Synthase Heterozygous mice (Wu et al., 2011; Wu et al., 2012a). While original reports on the KO mice showed Wallerian degeneration, demyelination of the optic and sciatic nerves, and development of movement problems (Chiavegatto et al., 2000; Sheikh et al., 1999), more recent studies suggest that KO mice nicely model parkinsonism (Forsayeth and Hadaczek, 2018; Hadaczek et al., 2015). Yet, it is

worth noting that humans that entirely lack GM2 Synthase expression do not develop parkinsonism but rather spastic paraplegia (Harlalka et al., 2013). Still the data suggest that loss of mature gangliosides in the GM2 Synthase KO mice may have impacted their urinary function.

To test for this, we evaluated GM2 KO mice for PD-like urinary problems as well as their bladder biochemistry and innervation. By measuring void spot patterns from 9–19 months in GM2 Synthase KO and WT mice we obtained data suggesting that our hypothesis was at least partially correct: overall GM2 Synthase KO mice had more void spots than WT mice, especially at younger ages. This suggested that GM2 Synthase KO mice initially suffered bladder hyperreflexia of the detrusor muscle causing them to have urinary frequency at 9–12 months in males and 9–16 months in females.

As they aged more, both male and female GM2 Synthase KO mice showed a decrease in the numbers of void spots produced in urinary pattern tests. By 19–20 months, GM2 Synthase KO mice also developed bladder enlargement that paralleled the decrease in void spots, which is consistent with hyporeflexia and/or urinary retention due to detrusor underactivity (Cockayne et al., 2000) a dysfunction that is common in patients with PD (Kadow et al., 2015). We had anticipated that such dysfunction may have resulted from PD-like aggregation of aSyn and Lewy body/Lewy neurite formation in neurons in the brain micturition centers or in bladder. As a positive control we used bladders of A53T aSyn Tg mouse, which had abundant bladder aSyn aggregation consistent with the known urinary problems in this model (Hamill et al., 2012). However, GM2 Synthase KO mice did not accumulate insoluble aSyn in bladder, suggesting that their urinary problems were not due to aSyn pathology in bladder tissue. Yet, the finding of TH loss in both VTA and SNc of GM2 Synthase KO mice revealed a loss of dopaminergic function that appears to have contributed to the dysregulation of micturition in these mice.

Denervation of the bladder can produce voiding abnormalities ranging from overactivity to urinary retention. Neurological bladder damage can also cause a loss of detrusor movement, or areflexia, that results in incomplete bladder emptying (Yoshimura and Chancellor, 2004). In the rat, the major pelvic ganglion supplies efferent neurons to regulate bladder function (Birder et al., 2010). Using electron microscopic analyses, we assessed myelinated axons running within the pelvic nerve and major pelvic ganglion of KO and WT mice, and detected atypical myelination and dysmorphic axonal structures only in KO mice. Interestingly, unmyelinated axons in the base of the bladder appeared to have larger diameters in KO compared to WT GM2 Synthase mice, consistent with prior findings for unmyelinated axons in the KO optic nerve (Sheikh et al., 1999). It has been shown that impaired bladder innervation can affect bladder trophic factor levels.

4.4 Contribution of trophic factors to bladder dysfunction.

Denervation can increase NGF levels in bladder smooth muscle (Steers and Tuttle, 2006; Tanner et al., 2000), and elevated levels of NGF have been associated with bladder dysfunction in A53T aSyn Tg parkinsonian mice and other rodents (Hamill et al., 2012; Vizzard, 2000). However, we did not see changes in bladder NGF in the GM2 Synthase KO mice, perhaps because NGF levels are typically so low in the adult nervous system (Al-

Shawi et al., 2007). We did however find high levels of bladder proNGF, the precursor form of NGF, in GM2 Synthase KO mouse bladder. ProNGF has been implicated in bladder dysfunction in rodents after spinal cord injury (Ryu et al., 2018) and has also been implicated in neurodegeneration of peripheral and central nervous system neurons in adult humans and rodents, as well as in cell models (Al-Shawi et al., 2008; Fahnstock et al., 2001; Ioannou and Fahnstock, 2017; Sobottka et al., 2008).

5. Conclusions

Cumulatively, the findings suggest that loss of mature gangliosides in the GM2 Synthase KO mice is associated with dopaminergic cell loss in brain micturition centers, increased proNGF expression in bladder, and abnormal myelination associated with the major pelvic ganglion leading to neurogenic bladder symptoms. Studying animals like the GM2 Synthase KO mice, which model motor and non-motor PD symptoms, may help assess emerging PD therapies. Also, as urinary dysfunction can manifest before motor symptoms in PD (Sakakibara et al., 2012; Takahashi et al., 2014), careful evaluation of patients above 45 years of age may help identify those who could benefit most from neuroprotective therapies that are in development as therapeutics for PD and related disorders. In addition, if such therapies also reverse urinary problems they may benefit not only parkinsonian patients but anyone with urinary dysfunction, regardless of the cause.

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Abbreviations used

aSyn	alpha-synuclein
A53T	alanine to threonine mutant human aSyn
BDNF	brain derived neurotrophic factor
GDNF	glial cell line derived neurotrophic factor
HMW	high molecular weight
HS	high salt buffer
HST	high salt triton-X 100 buffer

KO	knockout
MAG	myelin associated glycoprotein
NGF	nerve growth factor
PAG	periaqueductal gray
PD	Parkinson's disease
proNGF	pro nerve growth factor
RIPA	radio immunoprecipitation assay buffer
SNe	substantia nigra pars compacta
Tg	transgenic
TH	tyrosine hydroxylase
VTA	ventral tegmental area
WT	wild type

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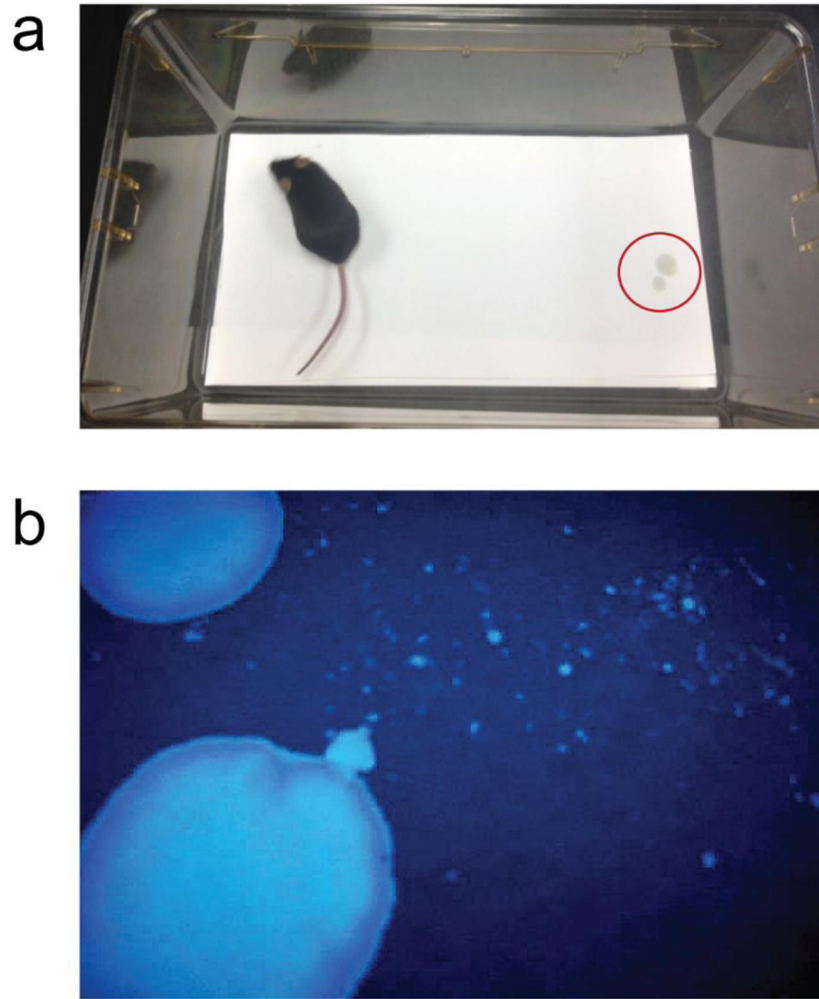


Figure 1. Void spot method to assess urinary patterns in GM2 Synthase KO and WT mice. (a) A GM2 Synthase KO mouse is shown in a cage with a filter paper covering the entire bottom surface. Damp urine spots, circled in red, can be seen on the filter. (b) A representative image of two large and many small void spots on a dried filter illuminated by UV light, on which spot sizes were easily discernable for measurement and counting using established methods. (N = 26, 15 WT and 11 KO).

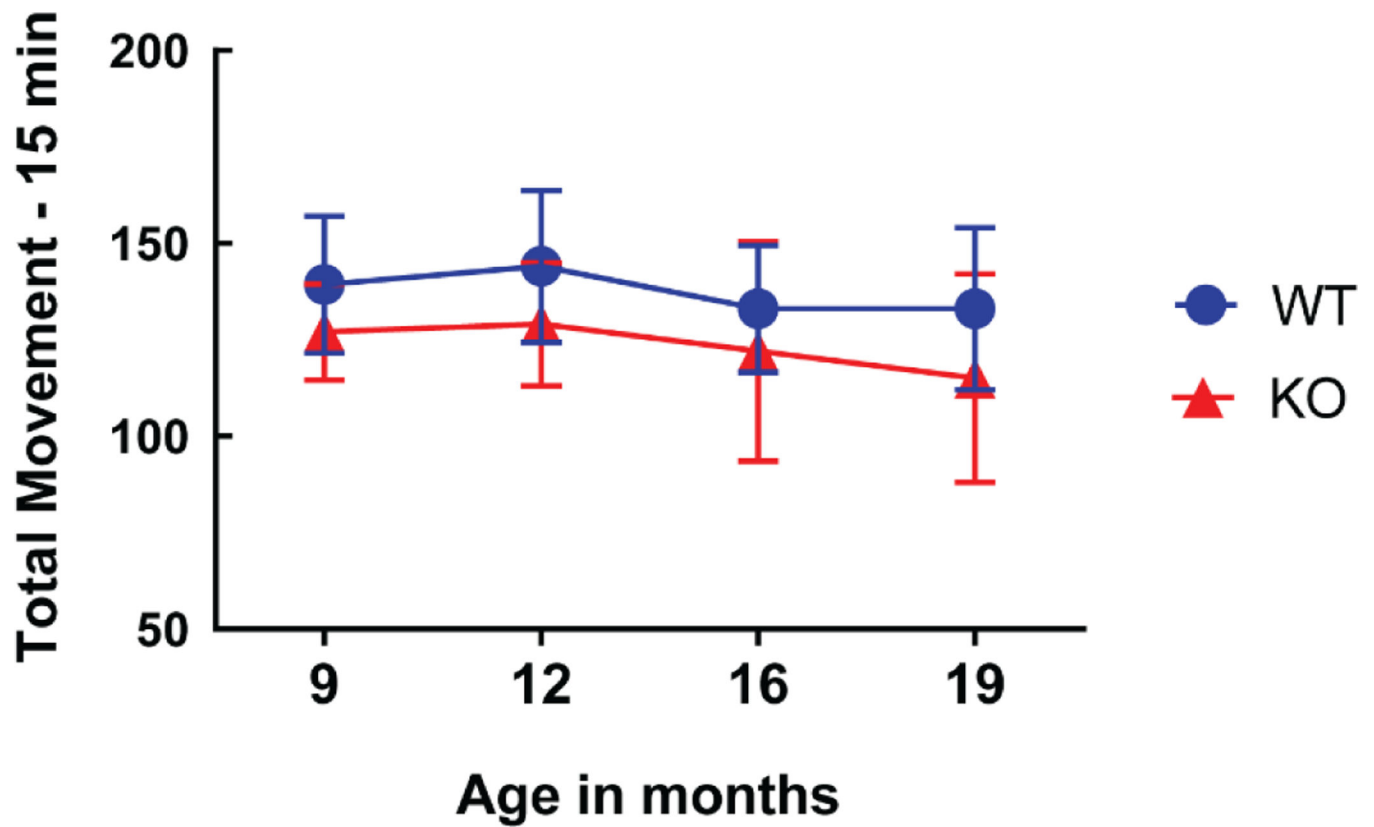


Figure 2. Total movements are similar in WT and GM2 Synthase KO mice.

The movement of WT and KO mice across the floor plane in an open field during 15 minute trials was evaluated at 9–19 months. Both KO and WT mice have similar total movement at all ages. (N = 20, 9 WT and 11 KO).

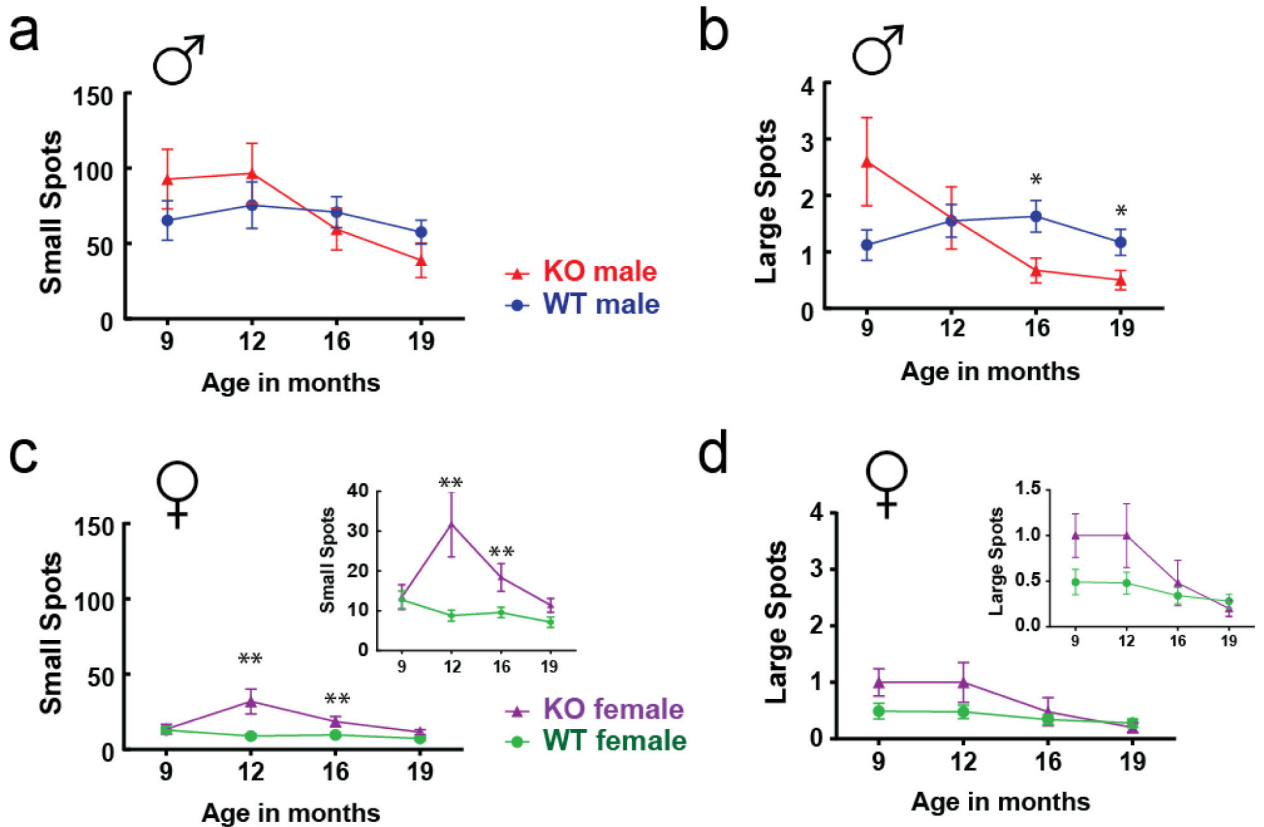


Figure 3. Urination patterns of GM2 Synthase KO and WT mice from 9–19 months.

Due to sex differences data for male (σ) and female (ϱ) mice are plotted separately. (a) KO males tend to have more small spots than WT males at 9–12 months, but fewer small spots at 16–19 months, though differences are not significant. (b) KO males tend to have more large spots than WT males at 9 months, though not significantly different. However, by 16–19 months, KO males had significantly fewer large spots than WT males. (c) At 9 months WT and KO females had similar numbers of small spots. From 12–16 months, KO females had significantly more small spots than WT females, as is better appreciated in the inset graph with a different Y axis. Though not significantly different, KO females show a trend toward more large spots at 9–12 months that decrease at 16–19 months, (N = 26, 15 WT and 11 KO), * $p < 0.05$; ** $p < 0.01$.

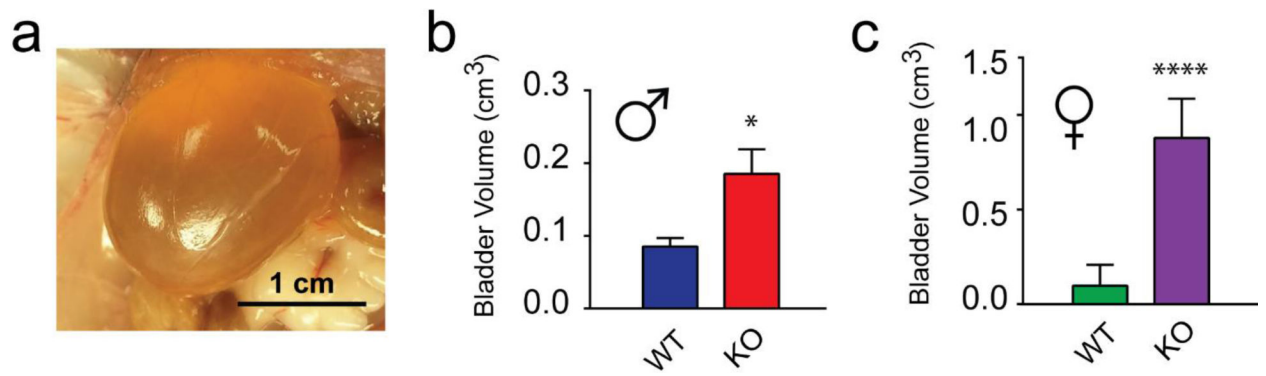


Figure 4. Bladder enlargement in aging GM2 Synthase KO mice.

Due to sex differences, quantitative data for male (♂) and female (♀) mice are plotted separately. (a) A representative enlarged bladder from a 12 month old mouse collected before initiating ultrasound analyses. Data in (b) and (c) are from bladder ultrasounds of 20 month old mice. (b) Male KO bladders (volume–cm³) are significantly enlarged compared to bladders of WT males. (c) Female KO bladders are also significantly enlarged compared to WT females. These increases paralleled a decrease in the number of void spots produced by both sexes of KO mice as they aged. (N = 15, 8 WT and 7 KO), * p < 0.05; **** p < 0.0001.

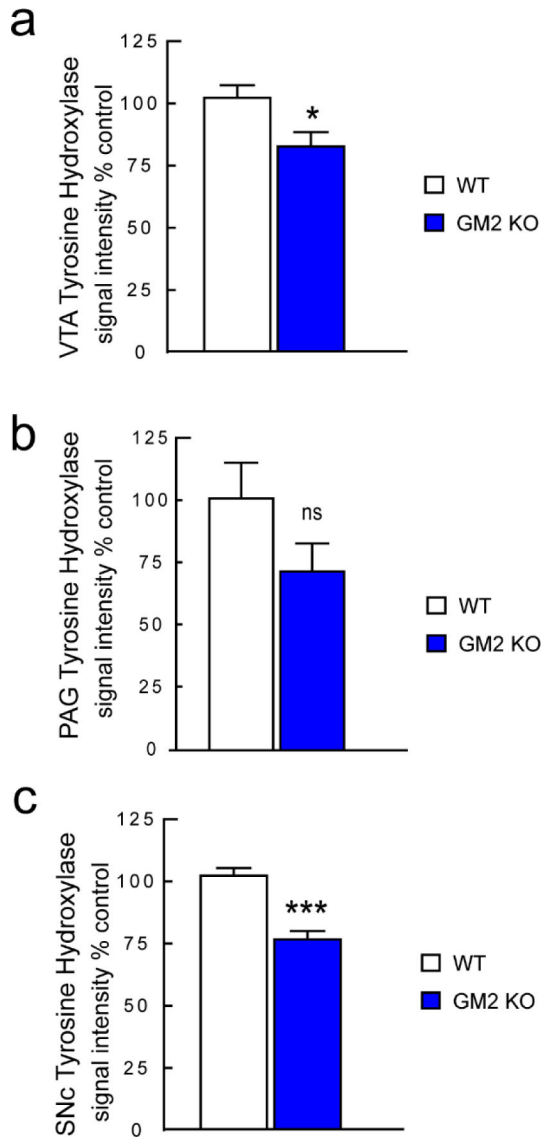


Figure 5. Ventral tegmental area, periaqueductal gray, and nigral micturition centers have diminished tyrosine hydroxylase staining in 19 month old GM2 Synthase KO brain.

(a) VTA dopaminergic TH signal intensity reveals a significant loss in KO VTA compared to WT VTA. (b) Similar analysis of the PAG micturition center shows a trend toward a loss in dopaminergic TH signal intensity in KO as compared to WT mice, but that difference is not statistically significant. (c) SNc dopaminergic TH signal intensity reveals a significant loss in KO compared to WT SNc. (N = 12, 6 WT and 6 KO), * p < 0.05, *** p < 0.005, ns = not significant.

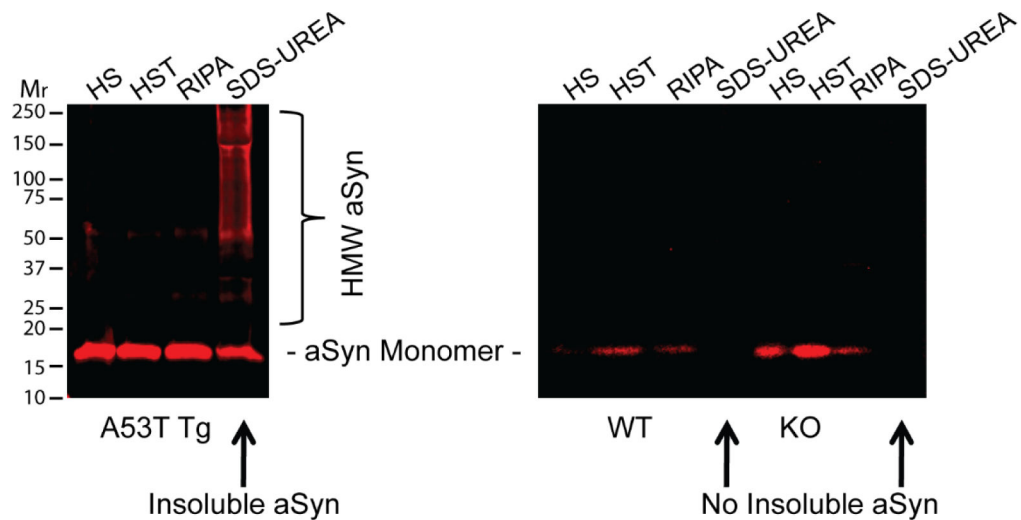


Figure 6. A positive control bladder from an A53T aSyn transgenic (Tg) mouse shows robust insoluble aSyn in the SDS-Urea sample in contrast to SDS-Urea samples from WT and GM2 Synthase KO mice that lack insoluble aSyn.

Bladders were assessed using a method to isolate soluble from insoluble proteins in tissue as detailed in Methods. Soluble aSyn appears as a single band of 17–19 kDa in samples extracted with High Salt (HS) buffer, High Salt Triton (HST) buffer, and RIPA buffer from bladders. Insoluble high molecular weight (HMW) aSyn is seen only in the A53T Tg SDS-Urea sample that was used as a positive control, and not in SDSUrea samples from 20–23 month WT or GM2 Synthase KO bladders. (N = 7, 1 A53T, 3 WT, and 3 KO). (Red signal was produced using C20, Cat # sc-7011-R antibody, Santa Cruz, CA, 1:100; IRDye 680 anti-Rabbit Secondary antibody, 1:10,000 using the LiCor Odyssey system).

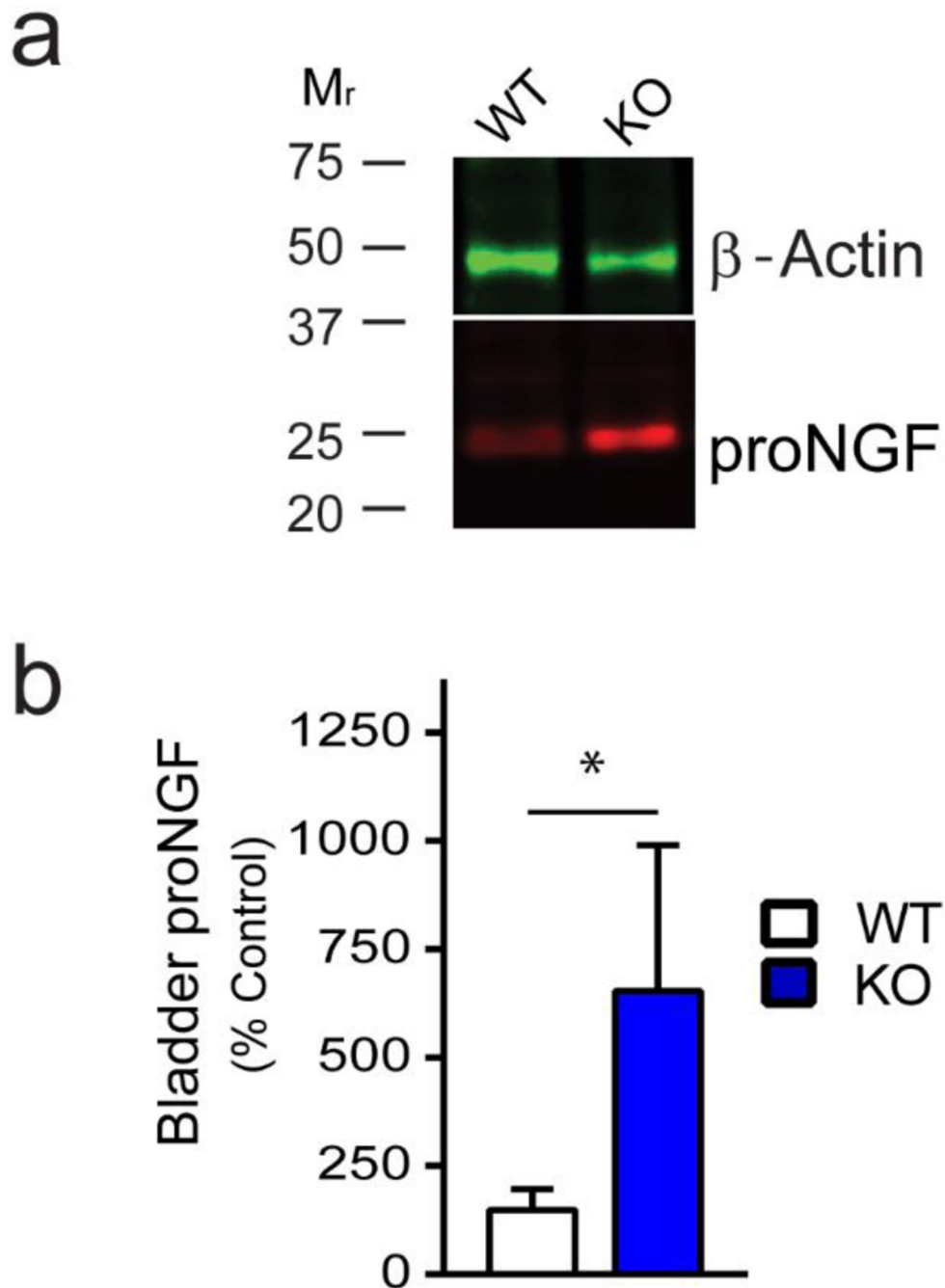


Figure 7. Levels of the precursor form of Nerve Growth Factor, proNGF, are elevated in GM2 Synthase KO bladder as compared to WT bladder.

(a) Representative immunoblot from 20 month old GM2 Synthase KO and WT mice shows much more proNGF in KO than in WT bladder, probed with antibody for NGF (H-20, Cat # sc-548, Santa Cruz Biotechnology, Santa Cruz, CA, USA), with β -actin (4970S, Cell Signaling Technology, Danvers, MA, USA) used as an internal control. (b) Quantification of proNGF normalized to β -actin from WT and KO bladders (N = 9, 4 WT and 5 KO) confirms significant increase in bladder proNGF in GM2 KO bladders. * $p < 0.05$.

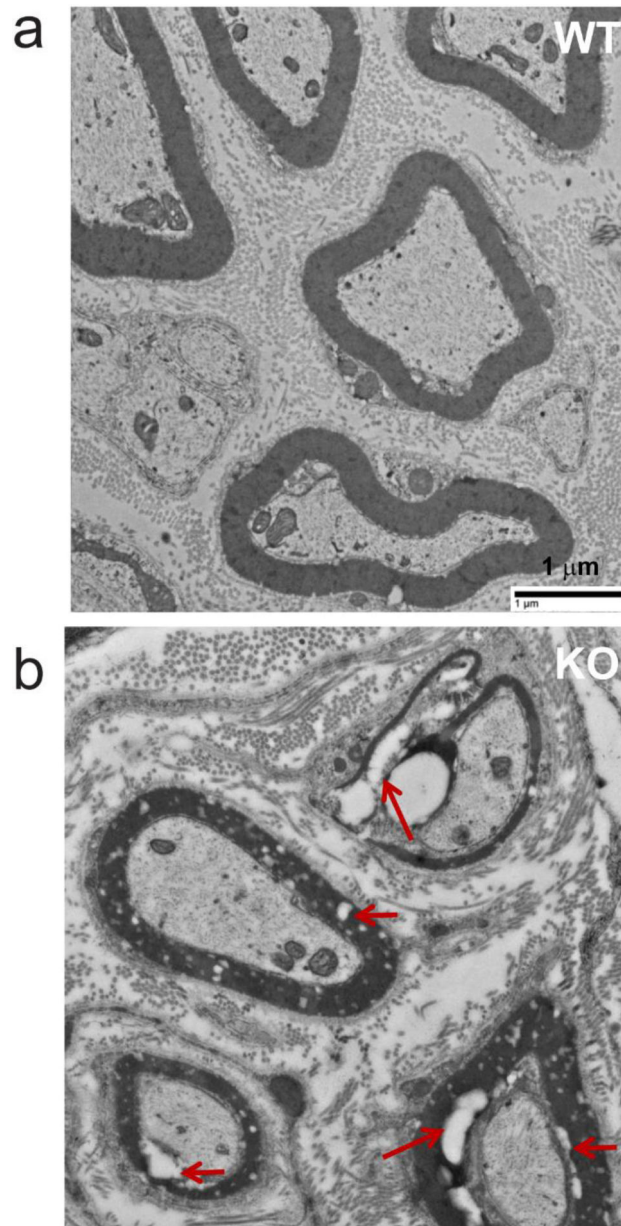


Figure 8. Ultrastructural differences in axons within the major pelvic ganglion at the base of the bladder of 17 month old WT and GM2 Synthase KO mice.

(a) Representative transmission electron micrograph of myelinated fibers found coursing through the major pelvic ganglion of a WT mouse shows uniform myelin sheaths surrounding axons with uniform morphologies. (b) Transmission electron micrograph of myelinated fibers coursing through the major pelvic ganglion of GM2 Synthase KO mouse shows large and small vacuoles in the myelin sheaths (at red arrows) surrounding axons of variable sizes and morphologies. (N = 4, 2 WT and 2 KO).