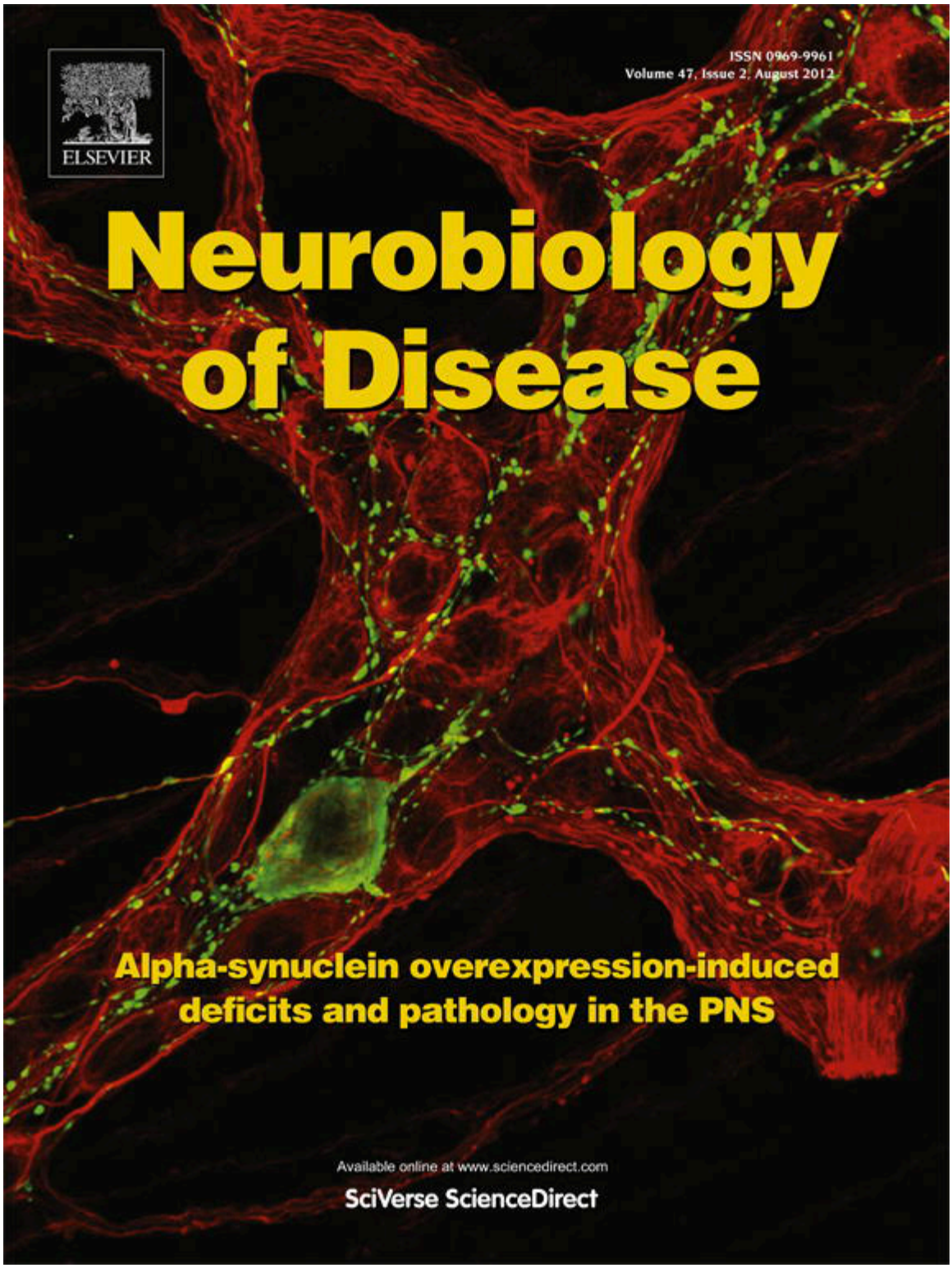
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Alpha-synuclein overexpressing transgenic mice show internal organ pathology and autonomic deficits

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ABSTRACT

While studying transgenic mice that overexpress human wildtype alpha-synuclein (Thy1-ASO, ASO) for typical brain alpha-synucleinopathy and central nervous system neuropathology, we observed progressive functional changes in the gastrointestinal and other peripheral organs. A more systematic study revealed that the gastrointestinal tract in ASO mice showed severe distension and blockage of the large intestine by 9–12 months of age. Functional assessments demonstrated a reduction in fecal water content and fecal pellet output, and increased whole gut transit time, in ASO mice compared to wildtype littermates, indicative of constipation, a symptom commonly reported by Parkinson's disease (PD) patients. Food intake was increased and body weight was decreased in 12 month old ASO mice, suggestive of metabolic abnormalities. Post-mortem histological analyses showed that human alpha-synuclein protein was robustly expressed in axonal fibers and in occasional cell bodies of the enteric nervous system, and in the heart of ASO mice. Accumulation of proteinase-K insoluble alpha-synuclein, reminiscent of neurodegenerative processes in PD was also observed. The functional and pathological changes we document here in ASO mice could relate to the autonomic deficits also seen in idiopathic and alpha-synuclein-mediated genetic forms of PD. These experimental data provide a foundation for therapeutic modeling of autonomic changes in PD and related alpha-synucleinopathies.

Introduction

The non-motor symptomatology of Parkinson's disease (PD) includes autonomic dysfunction, hyposmia, and sleep abnormalities (Chaudhuri et al., 2006). Such symptoms commonly experienced by PD patients are often detected before clinical motor symptoms, progressively become very disabling and significantly impact the quality of life for patients (Savica et al., 2010). Dysfunction of gastrointestinal and cardiovascular systems are significant autonomic features of PD, and post-mortem studies have identified PD neuropathology throughout the autonomic nervous system including the gastrointestinal tract, heart and sympathetic ganglia (Beach et al., 2010; Goldstein, 2010).

PD has multiple etiologies, including mitochondrial dysfunction and oxidative stress, and genes involved in such pathways have been identified (e.g. DJ-1, PINK1) (Cookson and Bandmann, 2010). Toxin-based and genetic approaches to induce mitochondrial dysfunction and oxidative stress in animals have been widely used to model central nervous system (CNS) dysfunction and the selective loss of midbrain dopamine neurons (Cannon and Greenamyre, 2010; Magen and Chesselet, 2010). These models have also indicated involvement of the peripheral nervous system (PNS). MPTP, a complex I inhibitor selective for the dopaminergic system, alters gastrointestinal dopaminergic transmission and related function, and reduces dopamine neurons in the enteric nervous system (Anderson et al., 2007; Chaumette et al., 2009; Natale et al., 2010; Tian et al., 2008). Systemic administration of rotenone, a general mitochondrial complex I inhibitor, in rats, induces clear gastrointestinal dysfunction and neuropathology in the enteric nervous system, similar to PD (Drolet et al., 2009; Greene et al., 2009). These toxin-based models demonstrate that mechanisms involved in CNS dysfunction in PD can also elicit functional deficits and pathology in the PNS. While such models depend on external agents that induce, for example, oxidative stress, a major genetic and pathologic component of PD is associated with the synaptic protein, alpha-synuclein. Mutations in, or multiplication of *SNCA* leads to early onset PD and diffuse Lewy body disease, and polymorphisms in the regulatory elements of *SNCA* can predispose to PD (Cookson and Bandmann, 2010). In both sporadic and familial PD, alpha-synuclein is a component of proteinacious Lewy body inclusions and neurites found throughout the CNS and also in the PNS. Alpha-synuclein is a highly expressed presynaptic protein,

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involved in SNARE protein assembly (Burre et al., 2010), and with an important role in neurotransmitter release (Nemani et al., 2010). Overexpression of either wildtype or mutant alpha-synuclein, or knockdown of alpha-synuclein together with beta and gamma synucleins, causes neuronal dysfunction and degeneration (Burre et al., 2010; Chung et al., 2009; Greten-Harrison et al., 2010; Nemani et al., 2010; Ulusoy et al., 2010). A variety of transgenic mouse models of alpha-synucleinopathy exist and although CNS deficits have been the focus of studies in such models (Chesselet, 2008; Chesselet et al., 2008; Dawson et al., 2010; Fleming et al., 2004, 2008; McLean et al., 2012; Rockenstein et al., 2002; Song et al., 2004; Watson et al., 2009), more recently, functional deficits in cardiac and gastrointestinal systems have also been described (Fleming et al., 2007, 2009; Kuo et al., 2010; Wang et al., 2008).

We first noticed evidence for autonomic dysfunction in transgenic mice that overexpress human wildtype alpha-synuclein on the Thy1 promoter (ASO, Thy1-ASO) by their extreme sensitivity to the alpha2-adrenergic agonist, xylazine, used as a routine anesthetic agent (Hallett et al., unpublished data). We also observed at post-mortem examination, clear gut and bladder distension in older (>12 months) ASO mice. Based on these observations we investigated gastrointestinal functional deficits, and neuropathology of the gastrointestinal system in ASO mice.

Materials and methods

Animals

All animal procedures were performed in accordance with the guidelines of the National Institute of Health and were approved by the Institutional Animal Care and Use Committee (IACUC) at McLean Hospital, Harvard Medical School. Animals were housed according to standard conditions, in a dark/light cycle of 12 h, with ad libetum access to food and water. Transgenic mice overexpressing human wildtype alpha-synuclein under the Thy1 promoter have been previously described (Rockenstein et al., 2002). Mice hemizygous for alpha-synuclein overexpression were maintained on a mixed C57BL/6-DBA/2 background by breeding female hemizygous mice with male BDF1 hybrids (Charles River, Wilmington, USA). Genotypes were verified using polymerase chain reaction amplification of tail DNA. For all experimentation only male ASO and wildtype littermate mice were used.

Beam traversal test

Motor coordination and balance in 12–15 month old ASO and WT littermate mice was tested using a challenging beam traversal test, as previously described (Fleming et al., 2004). In brief, a plexiglass beam (Plastics Zone, Woodland Hills, CA), 1 m length total, and comprising of four (25 cm length) sections that gradually decreased in diameter from 3.5 cm to 0.5 cm in 1 cm increments was used. Animals were trained to traverse the beam (from widest to narrowest) directly into the animal's home cage. Each mouse received two days of training (5 trials each) followed by testing on the third day. During the testing phase, a wire mesh grid (1 cm^2) of corresponding beam width was placed over the beam. Animals were videotaped while traversing the beam, over 5 trials. Videotaped were analyzed at slow-motion, by an investigator blinded to the genotype of the animals and the time taken to traverse the beam, and number of footslips off the beam were determined over the 5 trials and averaged.

Assessment of fecal water content

Fecal pellet output and water content was assessed in 2–4 and 9–12 month old ASO and WT littermate mice as previously described (Anderson et al., 2007; Kuo et al., 2010; Taylor et al., 2009) (n = 10–15 per group). Fecal water content provides an indication of constipation (reduced fecal water content resulting from reduced gastrointestinal motility and subsequent increased reabsorption of water), diarrhea (increased fecal water content) and malabsorption. Mice were placed individually in a novel environment (clean cage) and observed for 60 min. Testing was performed at the same time each day (2–3 PM). Fecal pellets from individual mice were collected immediately after expulsion in a pre-weighed sealed 2 mL microcentrifuge tube. The number of pellets expulsed by each mouse was recorded. Tubes were weighed to obtain the wet weight of the stool. Stool was then dried overnight at 60 °C and reweighed to obtain the dry weight. The difference in wet and dry weight was expressed over the wet stool weight to calculate fecal water content.

Whole gut transit time

Whole gut transit time (WGTT) was assessed in 2–4 and 12 month old ASO and WT littermate mice as previously described (Kuo et al., 2010) (n = 7–14 per group). Animals were placed in individual cages and food deprived for 1 h prior to receiving 0.3 mL 6% (w/v) carmine solution in 0.5% methylcellulose by oral gavage. The time taken for each mouse to produce a red fecal pellet after the administration of carmine dye was recorded. Animals which did not produce a pellet containing red dye after 12 h were recorded as >12 h. WGTT was calculated as time (min) per 20 g animal bodyweight.

Food and water intake

Food and water intake were measured over 3 consecutive days in 2 and 12 month old ASO and WT littermate mice. Individually housed mice were provided with a preweighed amount of rodent chow, placed in a heavy glass jar in the homecage. Remaining chow was weighed at the same time each day, over the next 3 days. Similarly, mice were provided with a premeasured volume of water in the drinking bottle, and the volume of water consumed was determined by calculating the remaining volume at the same time each day over 3 consecutive days. Food and water intake were determined as total consumption over 3 days, and as average consumption per 20 g animal bodyweight.

Immunohistochemistry

12–15 month old mice (n = 5 WT, n = 6 ASO) were terminally anesthetized by interaperitoneal injection of sodium pentobarbital (130 mg/kg) and intracardially perfused with heparinized saline followed by 4% paraformaldehyde in PBS. The brain, heart and gastrointestinal tissues were quickly dissected (gastrointestinal tissue were flushed with saline) and post-fixed overnight in 4% paraformaldehyde at 4 °C for 8 h, before cryoprotection in 30% sucrose/PBS until equilibration. Brains were sectioned at 40 μm on a freezing microtome, and serially collected in PBS. Gastrointestinal tract and heart were cryosectioned at 30 μm and collected directly onto slides in 4 series. Whole mount sections of the myenteric plexus were also prepared from PFA-fixed gastrointestinal tissues by manual separation of the myenteric plexus attached to longitudinal muscle layers, from the submucosal and mucosal layers.

Free-floating brain sections, whole-mount and slide-mounted tissue sections of heart and gastrointestinal tract were permeabilized in 0.1 or 0.5% Triton X-100/PBS. Non-specific labeling was blocked by incubating sections in 10% donkey or goat serum in 0.1 or 0.5% Triton X-100/PBS for 1 h at room temperature. Sections were then incubated with primary antibodies for 12 h at 4 °C, washed in PBS and incubated for 1 h at room temperature with secondary antibody. For immunofluroescent staining, anti-rabbit, anti-rat, anti-sheep and anti-goat AlexaFluor 488, 568 and 680-conjugated fluorescent secondary antibodies were used (1:500, Molecular Probes), followed by DAPI

staining to visualize nuclei. For peroxidase-based detection sections were incubated in 3% in hydrogen peroxide prior to the serum blocking step. Biotinylated anti-rabbit, anti-rat, anti-sheep and anti-goat secondary antibodies (1:300, Vector Labs) were used followed by ABC complexes and DAB substrate (Vector Labs). The following commercially available primary antibodies were used for both fluorescence and peroxidase immunostanings: sheep anti-alpha-synuclein (Millipore, AB5336P) at 1:1500; rat anti-alpha-synuclein 15G7 (Axxora, ALX-804-258) at 1:10; mouse anti-alpha-synuclein Syn211 (Millipore, 36-008); sheep or rabbit anti-tyrosine hydroxylase (PelFreez, P60101 or P40101 respectively), 1:300; rabbit anti-vasoactive intestinal peptide (AbCam, AB22736), 1:300; rabbit anti-peripherin (Millipore, AB1530), 1:500; goat anti-vesicular acetylcholine transporter (VAChT) (Millipore, AB1578), 1:750; rabbit neuronal nitric oxide synthase (nNOS) (Abcam, ab76067), 1:100). NeuroTrace™ red fluorescent Nissl stain (Invitrogen) (1:500) was used for labeling neurons in brain tissue.

Proteinase-K digestion

For assessing accumulation of insoluble alpha-synuclein, a proteinase-K digestion step was included prior to immunostaining with alpha-synuclein and DAB visualization. Proteinase-K digestion was performed as previously described with slight modifications (Fernagut et al., 2007). Briefly, free-floating sections of tissue, or tissue sections mounted onto slides, were incubated with Proteinase-K (Promega) at 10 μg/mL in PBS for 15 min at room temperature. Sections were then washed in PBS and processed as described above.

Imaging

Confocal analysis of immunofluorescent immunostainings was performed using a Zeiss LSM510/Meta station. Light microscopy was visualized using a Zeiss Axiovert microscope coupled to an Optronics Microfire digital camera. For quantification of neuronal number in the dorsal motor nucleus of vagus, the total number of Nissl-stained neurons in the dorsal motor nucleus of vagus in every 6th section containing this nucleus were counted. For quantification of peripherin-immunoreactive neurons in the myenteric plexus, the average number of neurons in 15–20 myenteric ganglia per animal was quantified.

Statistical analyses

A Student's *t*-test was used to compare two groups on a single dependent measure. 1-way ANOVA was used to compare more than 2 groups on the same dependent measure followed by a Bonferroni post-hoc test. All analyses were conducted using GraphPad Prism (Version 4.0) (GraphPad Software, Inc). All tests were considered significant at $p<0.05$.

Results

Assessment of CNS functional deficits and pathology in ASO mice

To verify involvement of the CNS in ASO mice, behavioral analyses of sensorimotor function, using the challenging beam traversal test, and post-mortem analysis to examine proteinase-K resistant alpha-synuclein immunostaining in the brain, was performed in a cohort of 12–15 month old ASO and WT littermate mice, as previously described (Fernagut et al., 2007; Fleming et al., 2004). In the beam traversal test (Figs. 1A, B), ASO mice had a significantly increased number of footslips off the beam, compared to WT mice ($p<0.01$, unpaired *T*-test). Post-mortem analyses were performed in the same cohort of animals. A proteinase-K pretreatment of brain tissue sections followed by immunostaining with a pan-alpha-synuclein antibody (detecting both mouse and human forms of the protein), was used to reveal proteinase-K resistant accumulations of alpha-synuclein, as previously described (Fernagut et al., 2007) (Figs. 1C–E). Following a proteinase-K digestion, no alpha-synuclein immunostaining was observed in the substantia nigra pars reticulata of WT mice (Fig. 1C), whereas robust accumulations of alpha-synuclein were detected in ASO mice in the SNr (Fig. 1D) and throughout the brain. At higher magnification, structures that morphologically resembled swollen neurites were observed in ASO mice (Fig. 1E).

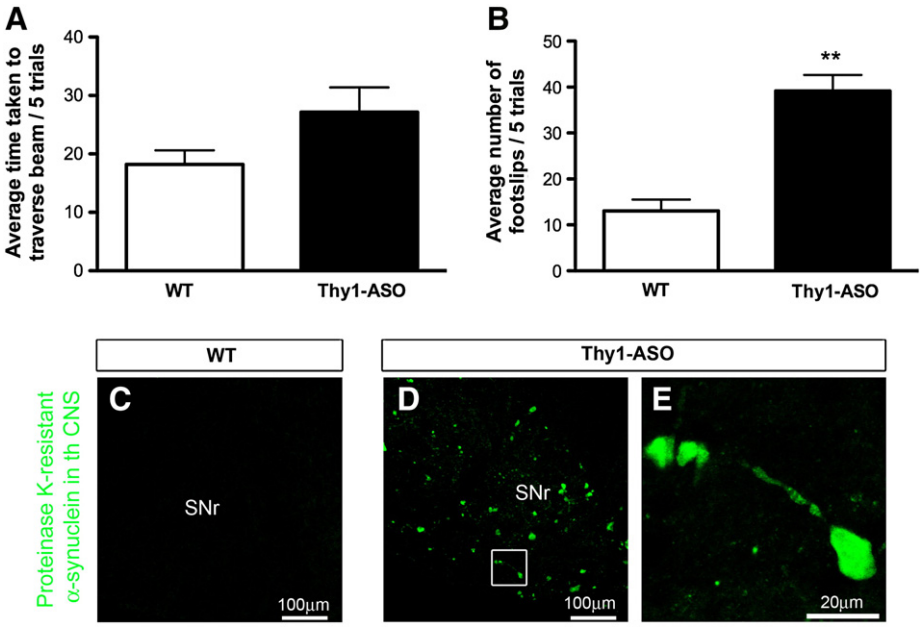

Fig. 1. Functional motor deficits and CNS alpha-synuclein pathology in ASO mice. (A and B) Functional testing of motor performance in 12–15 month old ASO and wildtype (WT) mice on the challenging beam traversal test, shows that the average number of foot slips was significantly increased in ASO mice (B). The average time taken to traverse the beam was not significantly increased in this group of animals (A). ** $p<0.01$, unpaired *T*-test. (C–E) Proteinase-K-resistant alpha-synuclein immunostaining in the substantia nigra pars reticulata of 12–15 month old WT and ASO mice, using an antibody that detects both human and endogenous mouse alpha-synuclein, reveals robust staining of proteinase-K insoluble alpha-synuclein in ASO mice (D and E), but not in WT mice (C). Panel E is a high magnification of the boxed area in panel D, and shows a structure that morphologically resembles a swollen neurite. SNr = substantia nigra pars reticulata. Scale bar = 100 μm (C and D) and 20 μm (E).

Assessment of gastrointestinal gross pathology and function in ASO mice

Initial observations of gross pathology of freshly dissected WT and ASO mice revealed clear abnormalities (Fig. 2). On inspection, the intestinal tract was of different pallor and size, and showed distinct areas of enlargement in ASO mice (Fig. 2A). These changes were already apparent in 2 month old mice, and in older (15 month old) mice, severe distension and blockage was observed. The urinary bladder was also noticeably enlarged in some, but not all, 15 month old ASO mice, but was never enlarged in WT littermates (Fig. 2B). We also observed urinary bladder enlargement in 9 month old ASO mice (data not shown). Of note, it was apparent during dissections that the urinary bladder was frequently expelled in WT mice, or if full, was no larger than ~0.75 cm wide (see Fig. 2B). In comparison, the urinary bladder was rarely empty in ASO mice, and on occasion, as described above, was noticeably enlarged compared to WT mice (see Fig. 2B).

Based on our observations of gross pathology, we next examined more quantitative measures of gastrointestinal function. The total number of fecal pellets expelled in individual mice over a one hour period was determined (Figs. 2C, D) and was reduced in older (9–12 months) ASO mice compared to WT littermates, whereas there was no significant different in younger (2–4) month old mice. Fecal water content was determined by calculating the water content in fecal pellets collected over a one hour period. Between 2 and 4 months of age, the fecal water content (Fig. 2E) was not significantly different in ASO and WT mice. However, in older mice at 9–12 months of age, fecal water content was significantly reduced in ASO mice compared to WT mice (Fig. 2F). We also measured the total wet weight of a 3.5 cm sample of small intestine isolated from 2–4 and 9–12 month old mice and found that this was significantly increased in 2–4 month ASO mice (66.6 ± 7.2 mg WT vs. 113.7 ± 5.0 mg ASO, $p < 0.001$, unpaired *T*-test), suggesting early signs of intestinal dysfunction. The intestinal weight was not significantly different between ASO and WT mice at 9–12 months of age (132.1 ± 6.5 mg WT vs. 150.3 ± 10.5 mg ASO, $p > 0.05$, unpaired *T*-test), suggesting an overall slowing of gastrointestinal function or motility in WT mice as they age. Interestingly, when examining comparable dissected samples of small intestine in ASO and WT littermate mice at both age-groups, it was noticeable that distinct localized areas of fecal matter were observed in ASO mice, which differed to a more homogenous distribution of fecal matter throughout the small intestine in WT mice. In addition, during observations of older mice (9+ months of age) during collections of fecal pellets, and during dissections of the gastrointestinal tract, it was apparent that the fecal pellets in ASO mice were abnormally hard (data not shown).

We also measured whole-gut transit time (WGTT) in WT and ASO mice as a marker of gastrointestinal motility (Figs. 2G, H). A red carmine dye was administered directly into the stomach using oral gavage, and the time until excretion was monitored. Although there was no overall significant different in WGTT between ASO and WT mice at 2–4 months of age, 10 out of 14 ASO mice showed a longer WGTT than the average WGTT measured in WT mice. At 12 months of age, WGTT in ASO mice was significantly increased compared to WT mice, and 6 out of 7 mice showed a longer WGTT than the average WGTT in age-matched WT mice.

In order to determine whether gastrointestinal functional deficits in ASO mice such as reduced fecal water content and increased WGTT, were a result of reduced food consumption, we measured daily food and water consumption over a total of 3 days in 2 and 12 month old ASO and WT mice, as well as determining animal weight (Figs. 2I–N). As has been previously described (Fleming et al., 2004), ASO mice have a significantly reduced body weight compared to age-matched WT littermates at both 2 and 12 months of age (Figs. 2 I, L). In ASO mice at 12 months of age, both the average food intake over 3 days (expressed per 20 g bodyweight), and total food intake over 3 days, was significantly increased compared to WT mice (Figs. 2M–N). Food intake was not altered in younger (2 month old) animals (Figs. 2J–K). Water intake was not significantly changed in ASO mice although average water intake showed a tendency to increase in 12 month old ASO mice (Figs. 2J–K, M–N).

Histological assessment of the gastrointestinal system in ASO mice

Our observations on abnormal gastrointestinal pathology and function, together with previously published work in ASO mice supporting our functional findings (Wang et al., 2008), led us to further investigate whether neuronal pathology at the histological level could be observed in ASO mice. We examined whole mounts and also cryosectioned PFA-fixed tissue from the gastrointestinal tract (ileum and distal colon) of 15 month old WT and ASO mice. We first confirmed expression of the human alpha-synuclein protein in whole mount sections of colon, using a monoclonal rat antibody specific for the human form of the protein (directed against amino acids 116–131 of human alpha-synuclein) (Neumann et al., 2000) (Fig. 3A). We observed structures resembling axonal swellings that were immunoreactive for human alpha-synuclein in ASO mice, and therefore next determined whether there was evidence for accumulation of insoluble alpha-synuclein. A proteinase-K digestion was performed followed by immunolabeling with a polyclonal sheep antibody that detects both mouse and human forms of alpha-synuclein (directed against amino acids 108–120 of human alpha-synuclein). In mice at 15 months of age, small accumulations of insoluble alpha-synuclein were observed in the myenteric plexus of wildtype mice, however, these appeared larger and more frequent in ASO mice (Figs. 3B, C). Overexpression of alpha-synuclein in peripheral tissues (heart and ileum) in ASO mice, was also confirmed using western blot analysis of total alpha-synuclein protein (Supplementary Fig. 1).

Next, we examined further the expression of human alpha-synuclein protein in whole mount sections of colon, (Fig. 4). Alpha-synuclein-immunoreactive fibers and occasional cell bodies, that were colocalized with the pan-neuronal (intermediate filament) marker peripherin (Fig. 4B), were localized in the myenteric plexus. Using cryosections of ileum, we also observed human alpha-synuclein immunoreactive fibers in the submucosal plexus and in the villi (data not shown). There was no positive immunolabeling for human alpha-synuclein in WT mice (Fig. 4A). Coimmunolabeling for human alpha-synuclein with neuronal markers of extrinsic and intrinsic enteric neuron innervation (Figs. 4D–K), shows little colocalization of human alpha-synuclein with noradrenergic (TH-immunoreactive), cholinergic (VAChT-immunoreactive) and VIP-immunoreactive neurons in ASO mice. Although human alpha-synuclein was not colocalized also with nNOS immunoreactive neurons, the human alpha-synuclein puncta were localized around nNOS cell bodies, suggesting innervation of this population cells. Quantification of peripherin-immunoreactive neurons in myenteric ganglia showed no significant change in neuronal number in 15 month old ASO mice compared to age-matched wildtype littermate control mice (1951 ± 226 neurons/mm^2, WT mice; 2399 ± 87 neurons/mm^2, ASO mice; $p > 0.05$, unpaired *T*-test).

The dorsal motor nucleus of vagus located in the brainstem provides parasympathetic preganglionic extrinsic innervation to enteric neurons, and alpha-synuclein pathology and neuronal degeneration has been reported in this nucleus in PD patients (Braak et al., 2001; Gai et al., 1992). Immunofluorescence labeling with a human-specific alpha-synuclein antibody combined with a fluorescent Nissl stain to label all neurons, was used to examine the dorsal motor nucleus of vagus in 2 and 12–15 month old ASO and wildtype littermate mice. There was an absence of human alpha-synuclein labeling in this region in ASO mice at both ages studied, despite intense labeling for human alpha-synuclein in the adjacent hypoglossal nucleus (Supplementary Figure 2). Quantification of neuronal number in the dorsal

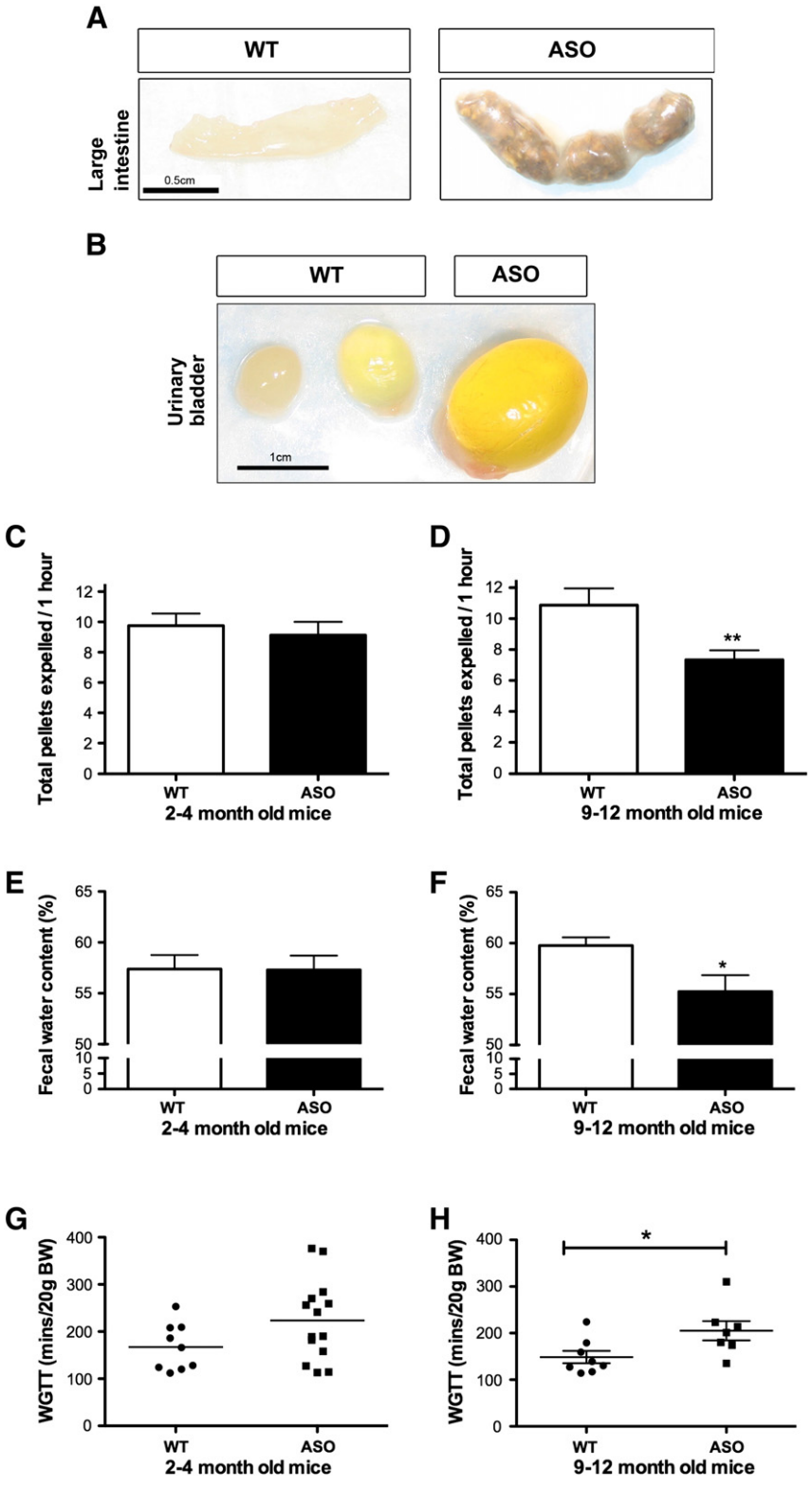

Fig. 2. Gastrointestinal and urinary bladder pathology in ASO mice. Macroscopic assessment of the large intestine (A) and urinary bladder (B) in wildtype (WT) and ASO mice. Comparable regions of the large intestine were dissected from 15 month old ASO and WT littermate mice and photographed. Urinary bladders were photographed immediately prior to dissection of the gastrointestinal tract. Note the pronounced distension of the large intestine and urinary bladder in ASO mice. Scale bar = 0.5 cm (A) and = 1 cm (B). (C and D) The total number of fecal pellets expelled in 2–4 month (C) and 9–12 month (D) WT and ASO mice over a 1 h period after mice was individually placed in a clean cage. (E and F) Fecal water content was then determined in the collected fecal pellets expelled over 1 h. Water content was significantly reduced in 9–12 month old ASO mice compared to WT littermates. (G and H) Whole gut transit time (WGTT), defined as the time taken for animals to produce a red pellet after receiving a red carmine dye by oral gavage, was determined in 2–4 and 9–12 month old WT and ASO mice. WGTT was significantly increased in 9–12 month ASO mice compared to WT littermates * $p<0.05$, unpaired *T*-test. (I–N) Animal weight and assessment of food and water intake in 2 and 12 month old WT and ASO mice. ASO mice have a significantly lower body weight than age-matched WT littermate mice at both 2 and 12 months old age (I and L). Food and water intake was measured over 3 days in 2 and 12 month old animals. The average food intake over 3 days, expressed over body weight (J and M), and total food intake over 3 days (K and N), is significantly increased in 12 month old ASO mice compared to WT mice. * $p<0.05$, ** $p<0.01$, unpaired *T*-test. Error bars represent standard error mean.

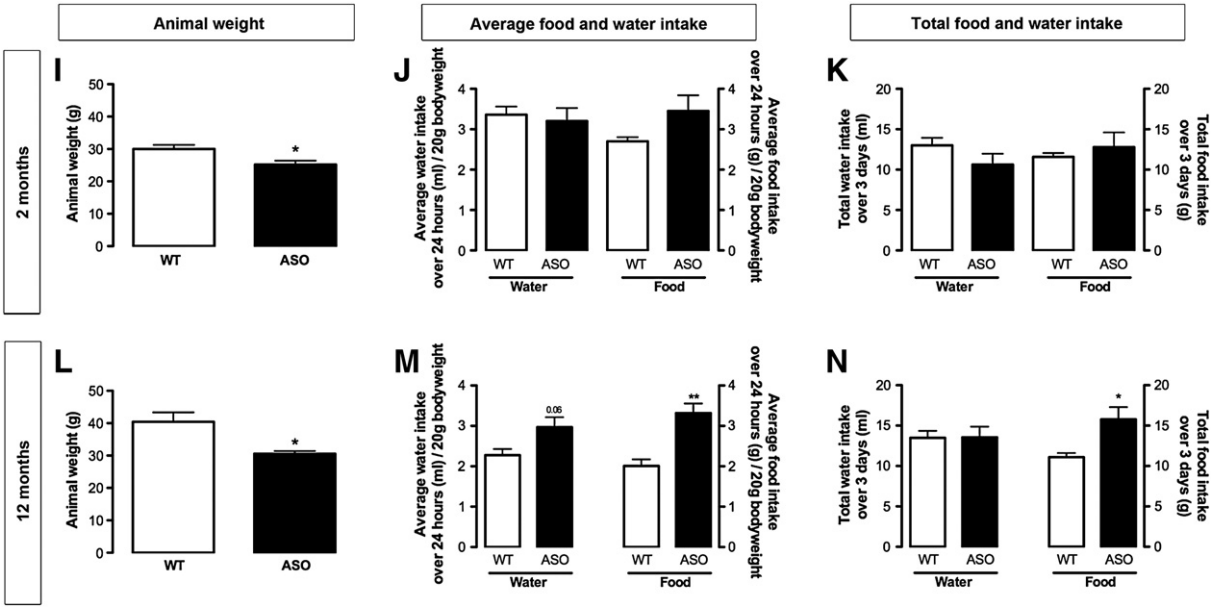

Fig. 2 (*continued*).

motor nucleus of vagus in 12 month old animals showed no difference between ASO and WT mice. We have also examined alpha-synuclein overexpression in ASO mice in additional CNS regions relevant in autonomic control, and find human alpha-synuclein overexpression in the hypothalamus and brainstem reticular formation in ASO mice (data not shown).

Histological assessment of the cardiac system in ASO mice

In order to determine whether alpha-synuclein induced pathological changes could be observed in other peripheral organs in addition to the gastrointestinal tract, we also examined proteinase-K resistant alpha-synuclein immunostaining in cryosections of heart tissue from ASO and WT mice at 15 months of age (Figs. 4L–M). Proteinase-K resistant accumulations of alpha-synuclein resembling axonal swellings were observed in the ventricle and atrial walls of ASO mice, which were not observed in WT littermate mice (Fig. 4L). Overexpression of alpha-synuclein was confirmed in heart tissue lysates in ASO mice using western blot analysis (Fig. 4M), and immunostaining with an antibody specific for the human form of alpha-synuclein showed expression of the human alpha-synuclein protein in noradrenergic neuronal fibers.

During our analyses of human alpha-synuclein expression in peripheral organs of ASO mice, we also noticed that human alpha-synuclein is also expressed in the skin of these animals. Lewy body pathology has been reported in the skin of patients with PD, and cutaneous autonomic innervation, including that to sweat glands is decreased in PD patients (Dabby et al., 2006; Jellinger, 2011). Further analysis of potential PD-like pathology in cutaneous nerves in ASO mouse skin, and functional assessments of cutaneous innervation in this model, are warranted.

Discussion

In summary, we show that the Thy1-ASO transgenic model of alpha-synuclein overexpression displays pathological changes in peripheral organ systems, including the gastrointestinal and cardiac systems, and functional autonomic deficits, similar to the pathology and debilitating clinical symptoms observed in patients with genetic and sporadic forms of PD. Specifically, the severely distended large intestine, decreased fecal water content and increased whole gut transit time in ASO mice are indicative of constipation, a symptom commonly reported by PD patients. Axonal swellings and insoluble alpha-synuclein accumulations in heart and gut tissues, as revealed using

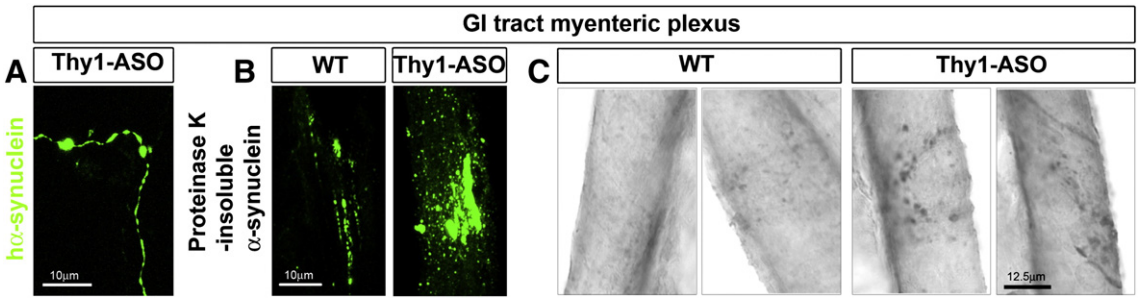

Fig. 3. Accumulation of alpha-synuclein in the myenteric plexus of ASO mice. (A) Immunostaining for alpha-synuclein in whole mount sections of colon myenteric plexus from 15 month old ASO mice, using an antibody specific for the human form of the protein, reveals neuritic swellings. (B and C) Proteinase-K-resistant alpha-synuclein immunostaining in cryosections of ileum myenteric plexus from 15 month old ASO and WT mice, reveals accumulation of insoluble alpha-synuclein in mice overexpressing wildtype alpha-synuclein. Panel B shows staining after immunofluorescence detection, and panel C shows staining after peroxidase-based detection.

proteinase-K resistant immunostaining for alpha-synuclein, are indicative of neurodegenerative processes.

Pathological and functional assessment of the gastrointestinal system in ASO mice and PD

Gastrointestinal symptoms in PD include constipation, increased gastrointestinal transit time and difficulty with defecation (Pfeiffer, 2011), and can significantly impact quality of life for PD patients (Gallagher et al., 2010; Pfeiffer, 2010). Generally, constipation is associated with a higher risk of PD and incidental Lewy body disease (Abbott et al., 2001, 2003; Savica et al., 2009), and severe gastrointestinal symptoms and other non-motor PD symptoms can lead to patient morbidity (Zesiewicz et al., 2010). Our macroscopic pathological assessment of the gastrointestinal tract in WT and ASO mice at different ages, demonstrated that older (15 month) ASO mice showed severe distension and blockage of the large intestine. Our data show deficits in ASO mice as early as 2 months of age, with increased retention of fecal matter throughout the gastrointestinal tract, and increased intestinal weight compared to WT littermates. Our functional analyses show that older ASO mice model constipation, by reduced fecal water content and increased whole gut transit time (WGTT). Interestingly, although we found no overall significant difference in WGTT between ASO and WT mice at 2–4 months of age, 71% of ASO mice showed a longer WGTT than the average WGTT observed in the WT littermate mice. At 12 months of age, WGTT in ASO mice was significantly longer than in WT mice, with 86% mice exhibiting a longer WGTT than the average WGTT in WT mice. Clinically, between 20 and 89% PD patients report decreased bowel movement frequency, and greater disease severity appears to be associated with increased severity of constipation (Pfeiffer, 2011). Indeed our data on fecal water content and WGTT in ASO transgenic mice also indicates that gastrointestinal deficits increase in severity with increasing age. In order to ascertain that the gastrointestinal deficits in ASO mice were not a result of a reduced food intake, we measured food and water intake in 2 and 12 month old mice. Interestingly we found

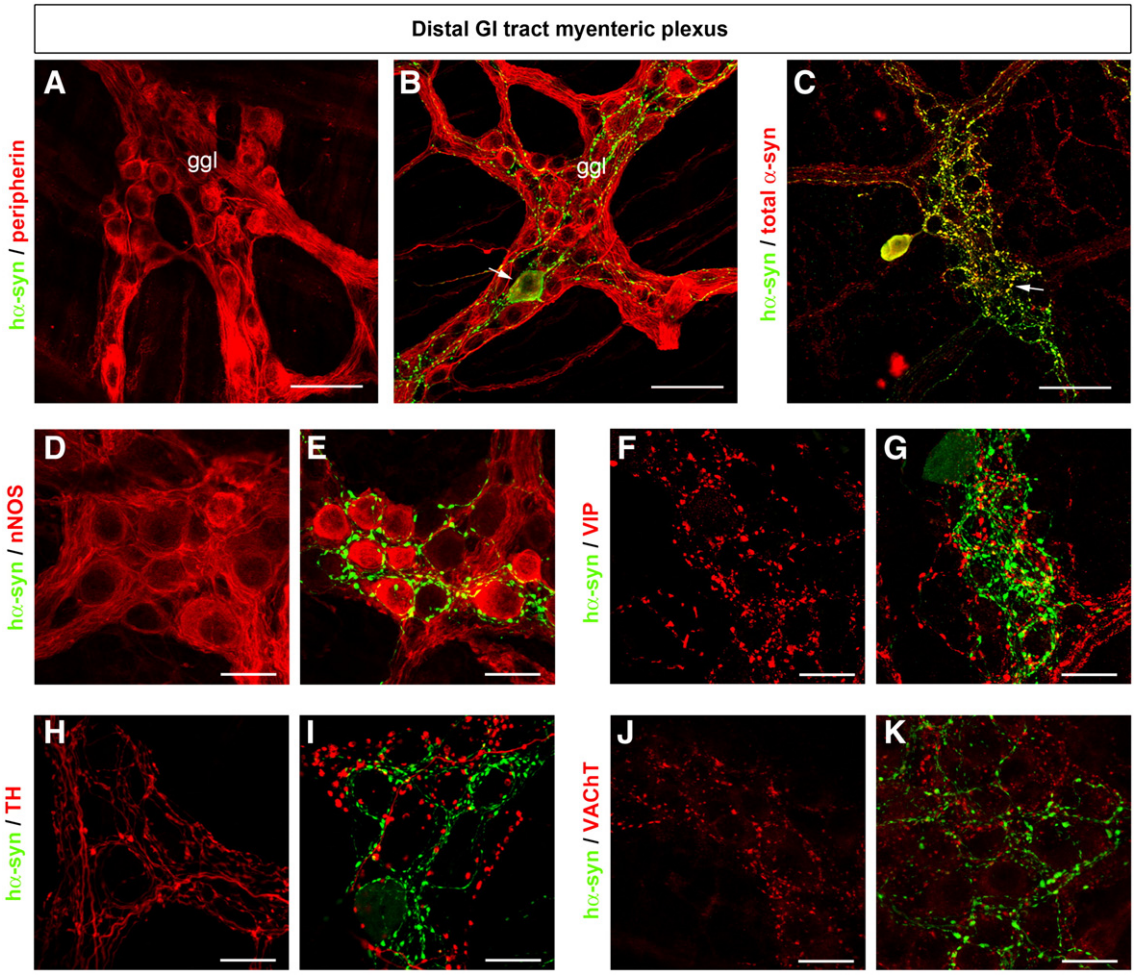

Fig. 4. Robust neuronal expression of human alpha-synuclein protein in the gastrointestinal myenteric plexus and in the heart of ASO mice. (A and B) Immunostaining for human alpha-synuclein (green) using a specific antibody for the human form of the protein, and the pan-neuronal (intermediate filament) marker peripherin (red) in whole mounts of colonic myenteric plexi, shows a neuronal expression of human alpha-synuclein in neuronal fibers and in occasional cell bodies in ASO mice (B) but no expression of human alpha-synuclein in WT mice. (C) Coimmunostaining for alpha-synuclein using a human specific antibody (green) and an antibody that detects both mouse and human alpha-synuclein (red) demonstrates a punctate localization of human alpha-synuclein, and shows that human alpha-synuclein is not overexpressed in all alpha-synuclein immunoreactive fibers. (D–K) Coexpression patterns of human alpha-synuclein with neuronal nitric oxide synthase (nNOS) (D and E), vasoactive intestinal peptide (VIP) (F and G), tyrosine hydroxylase (TH) (H and I) and vesicular acetylcholine transporter (VAChT) (J and K). Human alpha-synuclein puncta appear to surround and innervate nNOS immunoreactive neurons, and show little colocalization with VIP, TH and VAChT immunoreactive neurons. Scale bar = 50 μm (A, B), 20 μm (C–K). (L) Accumulation of insoluble alpha-synuclein in structures resembling axonal swellings is observed in the heart wall of 15 month-old ASO mice but not WT littermates. (M) Co-immunofluorescence labeling using an antibody that recognizes human alpha-synuclein (green) and tyrosine hydroxylase (TH) (red) shows a punctate expression of human alpha-synuclein protein in many but not all TH-immunoreactive fibers, suggesting that in ASO mice, human alpha-synuclein is expressed in the sympathetic nervous system. Insets show higher magnification images of boxed areas.

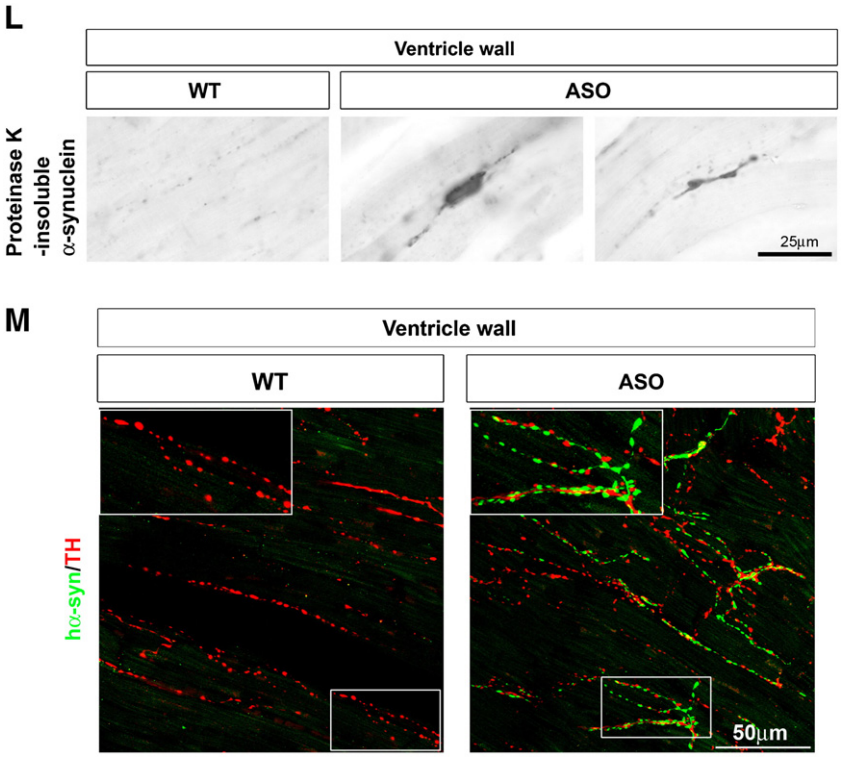

Fig. 4 (*continued*).

that food consumption was significantly increased in the older ASO mice. Given that the average weight of ASO mice is significantly reduced compared to WT mice, we hypothesize that basal metabolism may be increased in ASO mice. Our gastrointestinal functional data are complementary to and consistent with those published by Wang et al., 2008, where abnormal stool frequency was observed in 11–12 month old Thy-1 ASO mice — after exposure to a novel environment, stool frequency was increased, and after environmental habituation, stool frequency was reduced (Wang et al., 2008). Overall, the dramatic macroscopic pathology and clear deficits in gastrointestinal function observed in ASO mice are similar to the symptoms reported in patients with Parkinson's disease and related disorders (Lebouvier et al., 2009).

Alpha-synuclein histopathology in the gastrointestinal system of ASO mice and PD

The gastrointestinal tract is controlled by intrinsic and extrinsic neuronal innervation; extrinsic innervation connects the gut to the CNS and is provided by the sympathetic and parasympathetic nervous systems. Parasympathetic preganglionic motor pathways innervating intrinsic gut neurons originate from the dorsal motor nucleus of vagus (cranial nerve X; CNX) in the medulla, and sympathetic post-ganglionic neurons originate from sympathetic chain vertebral ganglia. Extrinsic innervation to the gut is responsible for modulating gut motility, secretion and absorption. Alpha-synuclein Lewy body pathology and neuronal degeneration in the dorsal nucleus of vagus has been reported in human PD patients (Braak et al., 2001; Gai et al., 1992), suggesting that PD pathology affecting extrinsic innervation to the gut may contribute to gastrointestinal dysfunction in PD. We examined CNX in ASO and WT mice at 2 and 13 months of age. Strikingly, despite intense labeling for human alpha-synuclein throughout the brainstem, including the adjacent CNXII nucleus, cholinergic neurons in CNX were devoid of human alpha-synuclein expression. In addition, there was no evidence for neuronal degeneration in CNX in ASO mice. These results suggest that gastrointestinal dysfunction in ASO mice is independent of cell loss in CNX and/or alpha-synuclein overexpression.

Intrinsic innervation is provided by the enteric nervous system, and has been described as a “second brain”. The enteric nervous system contains 80–100 million neurons, with autonomous function and showing broad chemical and functional diversity (Lebouvier et al., 2009). Neurochemicals identified in the enteric nervous system include dopamine, acetylcholine, norepinephrine, serotonin, GABA and glutamate, and a large number of other neurotransmitters and hormones including, vasoactive intestinal peptide (VIP), nitric oxide and substance P. The neuronal network comprising the enteric nervous system is organized into the myenteric (Auerbach's) and submucosal (Meissner's) plexi, which, through local reflexes, control gut motility and secretion respectively. With respect to PD, Lewy body and alpha-synuclein pathology have been found in myenteric and submucosal plexi of PD patients throughout the entire digestive tract (Beach et al., 2010; Braak et al., 2006; Lebouvier et al., 2010; Wakabayashi et al., 1988). We examined the morphology of alpha-synuclein expressing neurons and fibers in the ileum and observed swollen axons indicative of neuronal pathology and proteinase-K resistant accumulations of alpha-synuclein in ASO mice. Smaller and fewer accumulations of proteinase-K insoluble alpha-synuclein were also observed in WT mice, however, this is in accord with previous literature demonstrating alpha-synuclein accumulation in the enteric nervous system in aged rodents, and also in control patients (Beach et al., 2010; Braak et al., 2006; Lebouvier et al., 2010; Phillips et al., 2009). We confirmed that human alpha-synuclein protein was expressed in the enteric nervous system of ASO mice where it showed a neuronal localization. Given the local expression of human alpha-synuclein in the gastrointestinal tract, we hypothesize that gastrointestinal dysfunction in ASO mice could be a direct consequence of neuronal dysfunction from alpha-synuclein overexpression. Although there was no robust colocalization with the extrinsic (cholinergic and noradrenergic) or intrinsic (vasoactive intestinal peptide, nNOS)

gastrointestinal neuronal populations examined, human alpha-synuclein was localized around nNOS-immunoreactive neurons, suggesting that human alpha-synuclein-overexpressing fibers innervate this neuronal population. By inference we hypothesize that human alpha-synuclein overexpression in ASO mice is localized in other enteric neuron subpopulations, for example calbindin, somatostatin, tachykinin or serotonergic neurons; this warrants further investigation in future studies. Alternatively, overexpression of human alpha-synuclein may induce a loss of neurotransmitter staining, as has been previously reported following axotomy (Lams et al., 1988). Our quantification of peripherin immunoreactive neurons in myenteric ganglia shows no significant change in neuronal number in 12 month old ASO mice compared to age-matched controls. Given our knowledge of the extensive CNS pathology and behavioral abnormalities in ASO mice in the absence of gross neuronal loss in the brain, our finding that myenteric ganglia neuronal number is not changed is consistent with the idea that alpha-synuclein overexpression can cause neuronal dysfunction and neuronal pathology prior to, or in the absence of neuronal loss. We have previously shown that AAV-mediated overexpression of A53T mutant alpha-synuclein in nigral dopamine neurons in rats causes a progressive and protracted degeneration of dopamine neurons, that is preceded by several predegenerative changes including axonal dystrophy, disrupted neurotransmission, and changes in levels of proteins involved in synaptic transmission and cellular transport (Chung et al., 2009).

Histopathological analysis of the heart in ASO mice as a model of PD

Clinical symptoms of cardiovascular autonomic dysfunction in Parkinson's disease patients include orthostatic hypotension and decreased heart rate variability, and can result in serious complications (Goldstein, 2010; Ziemssen and Reichmann, 2010). Cardiac imaging studies using scintigraphy and PET to assess sympathetic noradrenergic transmission, have shown cardiac sympathetic denervation (Braune et al., 1998; Goldstein et al., 2000, 2009; Mitsui et al., 2006; Orimo et al., 2007). In support of this are histological assessments of post-mortem tissue, which show neuronal loss in sympathetic ganglia, reduced tyrosine hydroxylase innervation to the heart (Ghebremedhin et al., 2009; Mitsui et al., 2006; Orimo et al., 2008a, 2008b), and alpha-synuclein immunoreactive Lewy body and neurite pathology in cardiac tissue (Fujishiro et al., 2008; Orimo et al., 2008b). It has previously been reported that heart weight in WT and ASO mice is unchanged (Fleming et al., 2007), suggesting that there is no major heart tissue atrophy or hypertrophy. We also found no obvious gross pathology of the heart in ASO or WT mice for the ages studied (2–15 months). However, our analyses of alpha-synuclein-induced pathological changes in the heart of 15 month old ASO mice showed the presence of swollen alpha-synuclein-immunoreactive fibers, indicative of axonal pathology, which were not present in WT mice. Human alpha-synuclein protein was expressed locally in the ventricular and atrial walls of ASO mice in a punctate pattern, where it was localized within TH-immunoreactive (noradrenergic) fibers. The alpha-synuclein histopathological changes we have shown in heart tissue in ASO mice are consistent with functional cardiac abnormalities previously described in these mice, including altered baroreceptor function and reduced heart rate variability (Fleming et al., 2007, 2009) and also seen in PD patients (Goldstein, 2010). In addition we have observed that ASO mice show an extreme sensitivity to anesthesia with xylazine (an alpha2 adrenergic agonist), which is suggestive of general autonomic dysfunction.

General autonomic dysfunction associated with models of alpha-synuclein pathology

During the course of this work, a parallel study described autonomic dysfunction combined with alpha-synuclein pathology in alpha-synuclein PAC transgenic mice that have an insertion of the entire human SNCA gene on a null background for mouse alpha-synuclein (Kuo et al., 2010). In that study, gastrointestinal deficits (using similar assays to those performed in the current study) were detected in mice overexpressing A53T and A30P mutant alpha-synuclein, as early as 3 months of age, but were not detected at any time point in mice overexpressing WT alpha-synuclein. We believe that the autonomic nervous system pathology and gastrointestinal functional deficits described in the current study in ASO mice overexpressing WT human alpha-synuclein, and the lack of deficits/pathology in PAC transgenic mice overexpressing WT human alpha-synuclein, could be due to the differences in expression of the alpha-synuclein gene. Using the Thy1 promoter, alpha-synuclein expression could be considered to be “spear-headed” within neuronal systems, as compared to a more generalized expression using the artificial chromosome (PAC).

Conclusion

These results emphasize that deficits and pathology in peripheral neuronal systems caused by the overexpression of human wildtype alpha-synuclein are similar to those that occur both in age-related sporadic PD and alpha-synuclein-mediated genetic forms of the disease. In addition, our work demonstrates that overexpression of wildtype alpha-synuclein on the Thy1 promoter is sufficient to cause autonomic deficits and systemic pathology outside of the CNS. ASO mice offer a useful platform for the testing of mechanisms and novel therapies for autonomic dysfunction in PD-related disorders.

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