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Chronic isolation stress is associated with increased colonic and motor symptoms in the A53T mouse model of Parkinson's Disease

Running Title: Stress alters colonic and motor symptoms in PD

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Abstract

Background: Chronic stress exacerbates motor deficits and increases dopaminergic cell loss in several rodent models of Parkinson's Disease (PD). However, little is known about effects of stress on gastrointestinal (GI) dysfunction, a common non-motor symptom of PD. We aimed to determine if chronic stress exacerbates GI dysfunction in the A53T mouse model of PD and whether this relates to changes in α-synuclein distribution. **Methods:** Chronic isolation stress was induced by single-housing WT and homozygote A53T mice between 5 and 15 months of age. GI and motor function were compared with mice that had been group-housed. **Key Results:** Chronic isolation stress increased plasma corticosterone and exacerbated deficits in colonic propulsion and whole gut transit in A53T mice, and also increased motor deficits. However, our results indicated that the novel environment-induced defecation response, a common method used to evaluate colorectal function, was not a useful test to measure exacerbation of GI dysfunction, most likely because of the reported reduced level of anxiety in A53T mice. A53T mice had lower corticosterone levels than WT mice under both housing conditions, but single housing increased levels for both genotypes. Enteric neuropathy was observed in ageing A53T mice and A53T mice had a greater accumulation of alpha-synuclein (αsyn) in myenteric ganglia under both housing conditions. **Conclusions & Inferences:** Chronic isolation stress exacerbates PD-associated GI dysfunction, in addition to increasing motor deficits. However, these changes in GI symptoms are not directly related to corticosterone levels, worsened enteric neuropathy, or enteric αsyn accumulation.

Key words: Parkinson's disease, colonic propulsion, gut dysfunction, chronic isolation stress, alpha-synuclein, A53T

Introduction

Parkinson's Disease (PD) is a multi-system disorder that affects 1-2% of the population over 60 years of age [1]. In addition to the characteristic motor symptoms, which include rigidity, tremors, and bradykinesia [2], non-motor symptoms, such as gastrointestinal (GI) dysfunction, are a frequent occurrence. Five key features of GI dysfunction occur more frequently in PD patients compared to healthy individuals: these are excessive salivation, difficulty swallowing (dysphagia), nausea, slowed stomach emptying (gastroparesis), and severe chronic constipation [3, 4]. Chronic constipation can begin up to 20 years before motor symptom onset [5] and affects ~70-80% of PD patients [6-8]. The chronic constipation in PD is often unresponsive to conventional treatments (e.g. osmotic laxatives, changes in diet), and in many cases requires combination therapy. Moreover, many of the currently available treatments cause undesirable side-effects, including diarrhea, dehydration and severe abdominal cramps [4, 9]. Despite progress to alleviate motor symptoms caused by central nervous system (CNS) dysfunction, GI symptoms continue to contribute to the poor quality of life of patients with PD. In PD, constipation has been associated with slow gut motility, decreased fecal water content [10], and dopaminergic deficits in neurons of the gut [11]. Moreover, aggregation of alpha-synuclein (αsyn), a CNS hallmark of PD, has been found in the enteric nervous system (ENS) of PD patients and may be a cause of enteric neuron damage and subsequent GI dysfunction [12, 13].

Genetic and biochemical studies have revealed that missense mutations in the αsyn gene contribute to the onset of PD [14]. Mice overexpressing the A53T mutant human αsyn under the control of the mouse prion promoter develop and display severe motor impairments that are linked to the accumulation of αsyn in the brain, similar to patients with the A53T mutation [15, 16]. More recently, these mice have also been shown to suffer from GI dysfunction and have accumulation of αsyn in the gut [17, 18], making them a valuable model for studying both the motor and non-motor symptoms of the disease.

External factors such as chronic stress/anxiety have also been shown to contribute to the onset of PD [19, 20]. Chronic stress is known to exacerbate motor symptoms in several animal models of PD and it is thought that the effects are mediated by elevated glucocorticoid and corticosterone levels [19, 21]. Interestingly, patients with PD have been shown to have higher cortisol levels than healthy controls [22]. Glucocorticoids play an important role in regulating microglial activation and proinflammatory cytokine transcription factor expression and release [23]. Dysregulation of this system has been found to cause neuroinflammation, oxidative stress, and loss of dopamine producing neurons within the CNS [24, 25]. Although little is known with respect to

the effect of chronic stress on PD-associated constipation, stress is known to contribute to the onset and/or severity of GI dysfunction in the general population [26-28].

In the current study, we aimed to determine whether chronic isolation stress exacerbates or modifies the GI dysfunction associated with PD, using the A53T mouse model. A53T wildtype and A53T transgenic mice were single- or group-housed from 5 to 15 months of age, and GI function, which was assessed via fecal pellet output, colonic bead expulsion, and whole-gut transit, was correlated with deficits in motor function. In addition, enteric neuropathy and the accumulation of αsyn in the ENS was assessed. Our study showed that chronic stress in the form of social isolation further reduced colon motility and whole-gut transit, and exacerbated motor deficits in A53T mice, indicating that, in addition to CNS function, stress appears to exacerbate PD-associated GI dysfunction.

Experimental Procedures

Animals

All procedures involving mice conformed to the Australian National Health and Medical Research Council (NHMRC) code of practice for the care and use of animals for scientific purposes and were approved by the Institute Animal Ethics Committee (FINMH 16-110 and 16-029). Mice (B6;C3-Tg-Prnp/SNCA*A53T/83Vle/J) were originally obtained in breeding pairs from Jackson Laboratories (Bar Harbor, ME) to generate a stable breeding colony. A53T heterozygous breeders from this colony were used to produce both wildtype (WT) and homozygous A53T mice. All A53T mice used in this study were confirmed to have the transgene using Taqman probe real-time PCR. AS previously found, no significant differences in behaviour were found with respect to gender [18], therefore all results were combined for each genotype.

Chronic Isolation Stress

To investigate the effect of chronic isolation stress, mice (n=40 per cohort; 10 male and female WT mice and 10 male and female A53T mice) were either group housed or single-housed in a temperature- and humidity-controlled room under a 12-hr light/dark cycle. The number of mice in the study decreased as mice aged. This was due to humane killing of mice because of illness (tumors/wounds), or in the case of A53T mice, the onset of a severe motor phenotype. The number

of mice housed per cage in the group-housed cohort varied from 2 to 5 per cage due to fighting and subsequent separation (only males), or death. Food and water were available ad libitum. Mice were weighed weekly between 5-15 months of age.

Fecal pellet output and water content

Fecal pellet output testing was performed on mice every month between 5-15 months of age. Mice were placed individually in clean cages containing no bedding, food or water. The fecal pellets of each animal were collected in pre-weighed tubes every 15 minutes for 1 h after placement in the new environment. Water content in feces was measured by drying the pellets overnight at 65°C in an oven and reweighing the tubes. The % water content was calculated.

Colonic bead expulsion

Colonic propulsion was measured using the bead expulsion test, every 4 weeks between 5-15 months of age, immediately following fecal pellet output testing. Mice were lightly anesthetized with isoflurane to allow insertion of a bead (3 mm in diameter) into the distal colon 2 cm from the anus. Bead insertion was accomplished using a flexible plastic rod. Following bead insertion, mice were placed in individual cages to recover from anesthesia. The time taken from insertion of the bead to expulsion was recorded to the nearest second.

Whole gut transit

Whole gut transit time was performed in 12-15 month old WT and A53T mice as described in Vidal-Martinez et al. [17], with minor modifications. Briefly, transit time was assessed in mice after oral gavage of a 50% (v/v) cochineal solution prepared in drinking water (Queen Fine Foods, Alderley, QLD, Australia). Mice were placed in individual cages containing food, tissue for nesting, and had free access to water. Post gavage, the mice were observed for up to 9 h until the time of excretion of the first red stool, which was recorded for each mouse. Mice that had not passed a red stool by 9 h were scored as > 9 h.

Beam traversal test

Motor coordination and balance in 8-15 month old WT and A53T mice was tested using the ledged beam test, as previously described [29]. In brief, a beam, 1.5 m in total length, and

comprising of six (10 cm length) sections that decreased in width from 3.5 cm to 0.5 cm in 1 cm decrements 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 (4 trials each) followed by testing on the third day. During the testing phase, animals were recorded whilst traversing the beam, over 4 trials, with a 15-30 second inter-trial period. Videos were analyzed in slow-motion by an investigator blinded to the genotype of the animals. The total number of foot faults per section was averaged over the 4 trials.

Tissue collection and preparation

Prior to transcardial perfusion with 0.1M phosphate buffered saline (PBS), mice were anesthetized by intraperitoneal injection with a mix of ketamine/xylazine (100 mg/kg Ketamine and 10 mg/kg Xylazine). Gut tissue (colon and ileum) was flushed of fecal contents and tissues for immunohistochemistry were dissected along the mesenteric attachment, pinned flat onto balsa board with the lumen facing down, and fixed overnight in 2 % (v/v) formaldehyde plus 0.2% (v/v) picric acid in 0.1M sodium phosphate buffer, pH 7.2, at 4°C. Preparations were cleared of fixative by 3×10 min washes in dimethyl sulfoxide followed by 3×10 min washes in PBS. Fixed tissue was stored at 4°C in PBS containing sodium azide (0.1% w/v).

Immunohistochemistry of colon and ileum tissue

The mucosa and circular muscle were removed from the fixed tissue and wholemounts consisting of the myenteric plexus adhering to the longitudinal muscle were prepared. Wholemount preparations were incubated with either human anti-Hu (1:2000; a gift from Dr Miles Epstein [30]) and sheep anti-neuronal nitric oxide synthase (nNOS, 1:2000; a gift from Dr. Piers Emson [31]) or human anti-Hu, mouse anti-αsyn (1:200, Cell Signaling, QLD, Australia; for human αsyn) and rabbit anti-alpha-synuclein (1:1000, Abcam, VIC, Australia; for endogenous mouse αsyn) antibodies, overnight at 4°C. The wholemounts were then washed (3×10 min) in PBS before incubation with either donkey anti-human Alexa 594, donkey anti-sheep 488, donkey anti-rabbit 488, or donkey anti-mouse 594 secondary antibodies (Molecular Probes, Eugene, OR, USA), for 1 h at room temperature. Preparations were washed once with PBS, followed by 3×5-min washes in distilled water and incubated with Hoeschst 33258 solution (10 µg/ml Bisbenzimide-Blue in distilled water; Sigma-Aldrich, Sydney, NSW, Australia) for 5 min. Tissue was washed three times with distilled water for 10 min before being mounted on glass slides using fluorescence mounting

medium (Dako, Carpinteria, CA, USA). Images were captured using the Axio Imager.Z1 microscope (Carl Zeiss, Sydney, NSW, Australia) at 10× and 20× air objective. For Hu per ganglion studies, approximately 15-20 ganglion were counted per preparation. Approximately 100-200 neurons per preparation were counted for Hu translocation and nNOS quantitative studies. ImageJ (1.52i, http://imagej.nih.gov/ij)[32] was used to quantify the area of αsyn coverage in 10 ganglia per colon wholemount preparation. Approximately 100-150 neurons per colon preparation were counted to quantify the number of enteric neurons surrounded by αsyn particles. For all quantitative measurements, 5-6 wholemount preparations were used per cohort, which we have previously shown to be sufficient to reveal significant neuropathic changes [33].

Measurement of corticosterone levels in blood plasma

Terminal blood samples were collected from 15 months old mice via cardiac puncture. Corticosterone levels in blood plasma were measured in samples that were diluted 2-fold in calibrator diluent using the Parameter™ Corticosterone ELISA kit (R&D Systems, MN, USA) according to the manufacturer's specifications.

Statistical analysis

Data are expressed as the mean ± SEM. Comparisons between groups were performed using one- or two-way ANOVA with Sidak's or Tukey's multiple comparisons test. Combined αsyn data was analyzed by unpaired t-test. Analyses were performed using GraphPad Prism (GraphPad software Inc., San Diego, CA, USA). *P* values of less than 0.05 were considered statistically significant.

Results

Colonic bead expulsion and whole gut transit are modified by chronic social isolation stress.

The ability of the colon in conscious mice to propel solid contents was evaluated using the bead expulsion test. There was no effect of housing condition on WT mice with respect to bead expulsion time (Figure 1A), however, housing conditions had a greater effect on expulsion time in A53T mice (Figure 1B). A significantly greater slowing of expulsion time at 13 ($P<0.05$), 14

($P<0.05$), and 15 months of age ($P<0.001$) in single-housed A53T mice was observed when compared with group-housed A53T mice, indicating an exacerbation of the phenotype (Figure 1B). At 15 months, the expulsion time in A53T mice was doubled in single versus group housed mice. In both group- and single-housed mice, bead expulsion time was greater for A53T mice when compared with WT mice at 15 months of age, an indication of constipation in A53T mice. In group-housed A53T mice, bead expulsion time at 15 months of age was 279±46 seconds compared with 196±27s in WT mice (Figure 1C; $P<0.02$). A similar result was observed when comparing single-housed A53T mice (456±1451 seconds) with single-housed WT mice (164±45 seconds; Figure 1D; $P<0.0001$). Significant changes in colonic bead expulsion were observed earlier in single-housed A53T mice, with a significant delay in bead expulsion time occurring at both 13 ($P<0.05$) and 14 months of age ($P<0.001$), compared to WT mice (Figure 1D).

Similar to bead expulsion, housing conditions had a minimal effect on the whole-gut transit time of WT mice (Figure E), however, chronic isolation stress resulted in changes to transit time occurring earlier, indicating exacerbation of the phenotype. A significant delay in transit time was observed at 13 ($P<0.05$) and 14 ($P<0.02$) months in single-housed A53T mice when compared with group-housed A53T mice (Figure 1F). At 15 months of age, A53T mice from both housing conditions had similar transit times (A53T group: 382±26 min; A53T single: 423±27 min) (Figure 1F). Whole gut transit time was significantly slower in group-housed A53T mice (382±26 min) when compared with WT mice (287±11 min) at 15 months of age ($P<0.02$; Figure 1G). However, significant differences in transit time in single-housed A53T mice were observed as early as 13 months ($P<0.0001$), and this delay in gut transit was maintained at 14 ($P<0.01$) and 15 ($P<0.05$) months of age when compared with single-housed WT mice (Figure 1H).

Group and single housed mice differ in their defecation responses to a novel environment and chronic isolation stress alters stool water content.

The differences in relative responses of A53T vs. WT mice related to the substantial difference in responses of group-housed vs. single-housed WT mice (Figure 2A). Between 9-15 months of age, single-housed WT mice produced approximately double the number of pellets produced by group-housed WT mice when they were moved to a novel environment. Interestingly, housing conditions had no consistent effect on responses of A53T mice (Figure 2B). For group-housed A53T mice, total fecal pellet output (FPO) in response to being moved to a novel environment was greater than WT mice over the 1-hour collection period, particularly between 10-

15 months of age (Figure 2C). In addition, there was a trend towards increased stool wet weight and dry weight in A53T mice when compared with WT mice (data not shown). In contrast, single-housed A53T mice exhibited lower FPO in response to a novel environment when compared with single-housed WT mice between 9-15 months of age (Figure 2D).

Housing conditions had no consistent effect on water content in WT mice (Figure 2E), however, a significant reduction was observed in single-housed A53T mice when compared to group-housed A53T mice between 13-15 months of age ($P<0.05$; Figure 2F). Group-housed mice displayed no significant change in stool water content when comparing WT and A53T mice between 5-15 months of age (Figure 2G). Interestingly, stool water content in single-housed mice was significantly reduced for A53T mice when compared with WT mice between 12-15 months of age ($P<0.05$; Figure 2F), however the reduction in water content was minimal (~3-5%).

A53T mice differ in body weight and housing conditions affect weight gain in WT mice only.

Overall, the body weights of A53T mice were lower compared with WT mice from the same housing condition, and the percentage body weight change did not differ between WT and A53T mice under the same housing conditions (data not shown). However, WT mice that were single housed did not gain as much weight over the experimental period when compared with group-housed WT mice (Figure 3A), with a significant reduction in percentage body weight observed following the first week of single housing. Housing conditions had no significant effect on percentage body weight change in A53T mice (Figure 3B).

Hind limb function is impaired in A53T mice and chronic isolation stress exacerbates the motor deficit.

The beam traversal test was used to detect subtle motor deficits in hind limb function. Under both housing conditions, A53T mice developed progressively worse motor coordination at 8 (Figure 4A and D), 10 (Figure 4B and E) and 15 months (Figure 4C and F) of age when compared with WT mice ($P<0.05$). When comparing within genotype, the motor deficit became worse with age under both housing conditions, particularly for A53T mice, indicating the progressive nature of the disease.

Interestingly, when comparing between housing conditions, group-housed WT mice produced a significantly greater number of foot faults compared to single-housed WT mice at both

8 (P<0.05; Figure 4G) and 10 months of age (P<0.05; Figure 4H). This result was not as prominent in A53T mice. In contrast, at 15 months of age hind limb motor deficits were significantly exacerbated in single-housed A53T mice at all sections of the beam, excluding the narrowest section, when compared to group-housed A53T mice (P<0.05; Figure 4I). Group-housed WT mice performed similar to single-housed WT mice at 15 months.

The A53T mutation induces ENS neuropathy and alters the distribution of α-synuclein

To further understand the GI dysfunction observed *in vivo*, alterations at the cellular level were assessed using immunohistochemistry. ENS neuropathy was initially assessed by staining for the RNA binding protein Hu, which is expressed in all enteric neurons. There were no changes in the number of Hu-positive neurons per ganglion in the myenteric plexus in the distal ileum between A53T and WT mice under either housing condition (Figure 5A). However, there was a significant decrease in the number of Hu-positive neurons per ganglion in the distal colon when comparing single-housed A53T mice (24.0±1.4) with single-housed WT mice (34.8±5.9; P<0.05; Figure 5B-D). This result was not observed for group-housed A53T mice (27.1±1.6) when compared with group-housed WT mice (31.2±2.4), although there was a trend towards a decrease.

Enteric neuron damage was also assessed by measuring the extent of Hu translocation from the cytoplasm to the nucleus. There was no significant change in Hu translocation when comparing genotype or housing condition in either the distal ileum (Figure 5E) or distal colon (Figure 5F), however, there was a trend for an increase in the number of neurons observed to have nuclear translocation of Hu in the distal ileum when compared with single-housed mice with that of group housed mice.

Because nNOS is an important regulator of intestinal motility, we next investigated nNOS expression in the distal ileum and colon. nNOS-expressing neurons are abundant in the myenteric plexus compared with the submucosal plexus [34], therefore, we focused on the myenteric plexus to monitor ENS neuropathy. There was a significant decrease in the proportion of nNOS neurons in the distal ileum of A53T mice (group: 23.8±0.7; single: 21.5±1.8) when compared to WT mice (group: 29.5±0.5; single: 28.2±1.8) under both housing conditions (P<0.05; Figure 6A). In contrast, there was no change in the proportion of nNOS-positive neurons in the distal colon (Figure 6B).

The distribution of αsyn in myenteric ganglia from the distal colon was assessed to determine if αsyn may contribute to the reduction in neurons per ganglion and the significant

difference in bead expulsion time between A53T grouped and single housed mice. Immunostaining against endogenous mouse αsyn revealed a punctate pattern in both WT and A53T ganglia (Figure 7A and B); however, the number and size of particles in A53T mice from both housing conditions, as measured by the area of αsyn coverage per area of ganglion, was markedly greater when compared with WT mice (Figure 7C). This increase in αsyn levels in A53T mice was further highlighted when the area of αsyn coverage for each genotype from the different housing conditions was combined (Figure 7D; $P<0.05$). Within ganglion, punctate staining around neurons was also observed. These neurons surrounded by αsyn were also observed in WT mice, but not to the same extent as A53T mice (Figure 7E-H). Although not significant, there was an increasing trend towards the number of myenteric neurons surrounded by punctate αsyn particles in A53T mice under both housing conditions when compared with WT mice (Figure 7G). This increase in A53T mice was confirmed when combining housing data (Figure 7H; $P<0.05$). Interestingly, human αsyn distribution did not appear to differ between group- and single-housed A53T mice, with abundant staining being observed in the neurites of the tertiary plexus (Figure 7I and J).

Corticosterone levels in blood plasma ~~are altered with chronic isolation stress~~

To assess the effect of chronic isolation on stress hormones in WT and A53T mice, corticosterone levels were determined using terminal blood plasma. The corticosterone levels in WT mice were significantly higher when compared with A53T mice regardless of housing condition ($P<0.002$). Housing conditions had a significant effect on corticosterone levels for both genotypes, with single-housed WT mice having higher levels than group-housed WT mice (102.7 ± 4.7 vs. 63.0 ±5.7 ng/ml, respectively) and single-housed A53T mice having higher levels than group-housed A53T mice (73.0 ± 6.8 vs. 33.5 ± 3.0 ng/ml, respectively) (Figure 8; $P<0.0001$). These results indicate that chronic isolation elevates plasma levels of corticosterone.

Discussion

Chronic stress is known to exacerbate neurodegeneration and the progression of several neurodegenerative diseases [35-37]. A key symptom of PD is severe GI dysfunction, and the current study aimed to determine if chronic stress, induced via long-term individual housing, could exacerbate GI dysfunction in the A53T mouse model of PD. We demonstrated that A53T mice

develop progressive motor deficits and GI dysfunction, with respect to colonic bead expulsion time and whole gut transit, and that chronic stress exacerbated these PD-induced deficits.

Long term single housing is a well-established method for inducing chronic stress in rodents [38-40]. In the current study, our results confirmed that long-term individual housing of both WT and A53T mice induced a significant increase in plasma levels of corticosterone, a critical mediator of the physiological stress response, compared with group housed mice. Interestingly, in both housing conditions, WT mice had greater levels of corticosterone than A53T mice, indicating a genotype specific difference in stress hormone levels. In addition, housing conditions had more of an effect on body weight in WT mice, with group-housed WT mice gaining more weight over time than single-housed WT mice. This difference in percentage body weight was not observed in A53T mice. These differences observed between WT and A53T mice in response to single housing may be related to the reduced anxiety-like behaviour that A53T mice display when compared with WT mice [41, 42]. The mechanisms underlying the hypoanxiety-like behaviour in A53T mice remain unclear, but it has been suggested that this behavioural phenotype may be caused by early αsyn-mediated effects on central dopaminergic activity [41].

Investigation of fecal output in responses to a novel environmental stress revealed a greater difference between single and group housing in WT compared to A53T mice. Furthermore, reduced weight gain occurred in WT, but not A53T mice that were single housed, and A53T mice had lower corticosterone levels. These differences support the conclusion that anxiety-like behaviour is reduced in A53T mice [41, 42].

Although a genotype specific difference in corticosterone levels was observed, single-housed A53T mice had higher levels of the stress hormone when compared with group-housed A53T mice. This increase in corticosterone levels in combination with the A53T mutation may contribute to the exacerbated gut and motor PD phenotype. It is possible that an increase in stress hormones, in particular corticosterone, can affect the normal activity of the dopamine system, such as the mesolimbic and mescocortical pathway [43, 44]. Previous reports have demonstrated that stress leads to decreased dopamine levels in the striatum [45], accelerated dopaminergic neuron loss in the nigrostriatum [46, 47], and promotes aggregation and accumulation of αsyn [48]. Administration of cortiscosterone has been shown to impair motor performance in rodents [49]. Therefore, with respect to our study, the increase in corticosterone levels may have contributed to the acceleration of motor dysfunction in A53T mice. However, it should be noted that corticosterone levels were measured in terminal blood plasma samples, which could enhance stress-related differences.

Chronic stress has been shown to alter gut motility and intestinal permeability [50-53]. Elevated levels of corticosterone have been shown to decrease motility and depress myenteric neuron function in the small intestine by reducing electrical stimulation-induced Ca^{2+} responses, which are important for enteric neuron transmission and gut motility [53]. In our study, single-housed A53T mice had slower transit time and reduced colon motility when compared with group-housed A53T mice, which could be contributed to by increased corticosterone levels, if corticosterone has inhibitory effects in the colon that are similar to those in the small intestine. Clearly, elevation in stress hormone levels alone are not the sole cause of GI dysfunction as WT mice under both housing conditions had higher levels of corticosterone, but less GI dysfunction when compared with A53T mice.

The propulsive activity of the colorectum in response to a pellet or other hard mass in its lumen is dependent on intrinsic reflexes of the ENS and remains intact when all connections with the CNS are removed [54, 55]. Thus, bead expulsion is predicted to be less sensitive to emotional state, compared to defecation, which is triggered by a behavioural stimulus such as the stress of moving to a novel environment. A clear deficit in colorectal bead expulsion time was observed in A53T mice when compared to the WT mice, which indicated that A53T mice did indeed have compromised ability to propel contents of the colorectum. There was a gradual increase in expulsion time in both group- and single-housed A53T mice, indicating the progressive nature of colorectal dysfunction, and this deficit was significantly exacerbated by chronic isolation stress.

Animals with gastrointestinal slowing consistent with constipation, develop αsyn aggregates in enteric neurons that innervate the gut [56]. Previous studies have shown that αsyn accumulates in enteric neurons in A53T transgenic mice as early as 4 months of age [17, 57] and that ageing A53T mice have greater amounts of aggregated αsyn in myenteric neurons of the small intestines [17]. This aggregation of αsyn may result in enteric neuron damage and/or loss via mitochondrial dysfunction and subsequent oxidative stress [58]. In the present study, we observed an increase in the number of large αsyn particles in ganglion and surrounding enteric neurons in A53T mice. However, housing conditions had no additional effect on αsyn distribution or localization, suggesting that a change in αsyn accumulation is not driving the difference between the groups.

We investigated the numbers of nNOS neurons and the translocation of Hu protein to the nucleus as indices of stress to enteric neurons. Neurons containing nNOS are reported to be the most sensitive to stress, probably because of the reactivity of the free radical, NO, which is produced by these neurons [59]. Translocation of Hu protein to the nucleus is also observed when

enteric neurons are challenged [60, 61]. We saw fewer nNOS neurons in myenteric ganglia of the ileum in A53T compared to WT mice, but there was no difference in the proportion of nNOS-positive neurons in the distal colon. However, we did see significantly fewer Hu-positive nerve neurons per ganglion in the colon of single housed A53T mice when compared with single-housed WT mice. This change in total neuron number may be related to the greater reduction in colon motility seen in single-housed A53T mice when compared with group-housed A53T mice. Although there was a tendency for a greater occurrence of nuclear Hu in A53T, compared to WT mice, and in single housed compared to group housed mice, these differences were not statistically significant.

In conclusion, our results clearly show that chronic stress increases both the GI dysfunction and motor deficits in A53T mice. In addition, we provide further evidence for the reduced anxiety-like behaviour that was previously observed in A53T mice. Because of the confounding effect of the difference in anxiety-like behavior in A53T compared with WT mice, provoking defecation by stress (the novel environment test) is not recommended for characterizing GI dysfunction in this PD model. On the other hand, the bead expulsion test provides a clear indication of the exacerbated GI phenotype.

Authorship: SD, JPB, and JF designed the research study, SD, RC, OA, RMM, XC, MTR, VAL, and LJE conducted the experiments, SD, RC, MD, EL, and LJF analyzed the data, SD, JF, and DF wrote the manuscript.

Conflict of interest Statement: Takeda Pharmaceuticals International, Inc., was Joel P. Berger's employer at the time the research was conducted.

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Figure Legends

Figure 1. Colonic bead expulsion and whole-gut transit time are increased in A53T mice when compared with WT mice and chronic isolation stress exacerbates the phenotype in A53T mice. A: Housing conditions did not affect colonic bead expulsion time in WT mice that were group or single housed. B: Deficits in colonic bead expulsion time were exacerbated in single-housed A53T mice when compared with group-housed A53T mice between 13-15 months of age. C: For group housed mice, colonic bead expulsion time was significantly slower in A53T mice when compared with WT mice at 15 months of age. D: In single-housed mice, colonic bead expulsion was

significantly slower in A53T mice when compared with WT mice at 13-15 months of age. E: Housing conditions did not affect whole-gut transit time in WT mice that were group or single housed. F: A significant delay in whole gut transit was observed at 13 and 14 months of age in single-housed A53T mice when compared with group-housed A53T mice. G: Whole gut transit time was significantly delayed in group-housed A53T mice when compared with WT mice at 15 months of age. H: In single-housed mice, whole-gut transit was significantly delayed in A53T mice when compared with WT mice at 13 months of age, and this delay continued at 14, and 15 months of age. F: Data were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test. Data represent the mean ± SEM; $^{*}P<0.05$; n=15-20/group.

Figure 2. Housing conditions change the relationships of fecal pellet output in the novel environment challenge between WT and A53T mice and chronic isolation stress affects stool water content in A53T mice.

A: Fecal pellet output (FPO) was markedly different in WT mice in response to housing conditions, with single-housed mice producing approximately double the number of pellets when compared to group-housed WT mice between 9-15 months. B: No consistent effect of housing on FPO was observed for A53T mice. C: Total FPO in response to being moved to a novel environment was increased in group-housed A53T mice when compared to group-housed WT mice between 10-15 months of age. D: Single-housed A53T mice exhibited lower FPO in response to a novel environment when compared with single-housed WT mice between 9-15 months of age. E: Housing conditions had minimal effect on water content in WT mice. F: A significant reduction in water content was observed in single-housed A53T mice when compared to group-housed A53T mice between 13-15 months of age. G: There was no change in water content when comparing group-housed WT mice with group-housed A53T mice between 5-15 months of age. H: Stool water content in single-housed mice was significantly reduced for A53T mice when compared with WT mice between 12-15 months of age. Data were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test. Data represent the mean ± SEM; $^{*}P<0.05$; n=15-20/group.

Figure 3. Housing conditions affect the rate of body weight gain in WT mice, but not A53T mice. A: The percentage body weight gained over time for single-housed WT mice was markedly less when compared with group-housed WT mice. B: Chronic isolation stress did not affect the percentage body weight gained in A53T mice when compared with group-housed A53T mice. Data

were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test. Data represent the mean ± SEM; *P<0.05; n=15-20/group.

Figure 4. Hind limb motor function is impaired in A53T mice under both housing conditions, deficits in motor function are exacerbated at 15 months of age in single-housed A53T mice. A and D: Under both housing conditions, the number of foot faults made by A53T mice at 8 months of age was significantly greater than WT mice at the indicated section of beam. B and E: The number of foot faults made by A53T mice at 10 months of age was significantly greater than WT mice at the indicated section of beam. C and F: Similar to 8 and 10 months, the number of foot faults made by A53T mice at 15 months of age was significantly greater than WT mice at the indicated section of beam. At 15 months of age, the number of foot faults were significantly greater for single housed than group housed A53T mice. G: Group housing produced a greater number of foot faults in both WT and A53T mice at 8 months of age when compared with single-housed mice. H: Group housing consistently produced a greater number of foot faults in WT mice at 10 months of age when compared with single-housed WT mice. I: At 15 months of age, single-housed A53T mice produced a significantly greater number of foot faults when compared with group-housed A53T mice. Data were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test. Data represent the mean ± SEM; *P<0.05; n=15-20/group. For G-I, *P<0.05 for A53T group vs. A53T single and #P<0.05 for WT group vs. WT single mice; n=15-20/group.

Figure 5. The number of Hu-positive neurons per ganglion is reduced in the colon of single-housed A53T mice, but there is no change in Hu translocation. A: There was no change in the number of Hu-positive neurons per ganglion in the myenteric plexus of the distal ileum between WT and A53T mice under both housing conditions. B: A significant decrease in the number of Hu-positive neurons per ganglion in the distal colon between single-housed A53T and WT mice was observed (*P<0.05). C and D: Representative photomicrographs of Hu staining in distal colon tissue from single-housed WT mice and group-housed WT mice. Scale bar = 50μm; * = neurons with Hu translocation. E: In the distal ileum, there was no significant change in the number of neurons exhibiting Hu translocation to the nucleus, however there was a trend for an increase in single-housed mice. F: Similar to the distal ileum, genotype and/or housing had no effect on the number of neurons exhibiting Hu translocation. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. For Hu per ganglion, data represent the mean ± SEM (n= 15-20 ganglia

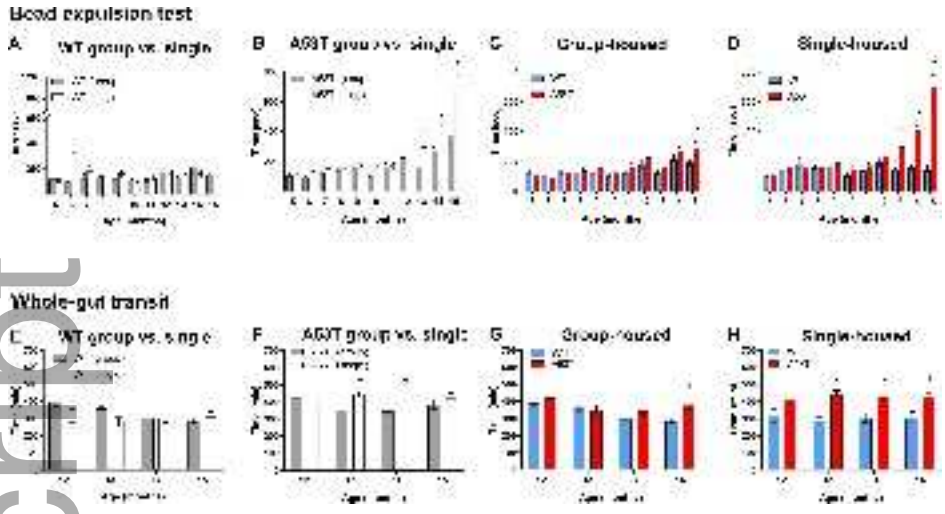
from 5 mice per group). For Hu translocation, data represent the mean ± SEM (n= 100-200 neurons from 5 mice per group).

Figure 6. The proportion of nNOS-positive neurons is decreased in A53T mice when compared with WT mice under both housing conditions. A: There was a significant decrease in the proportion of nNOS-positive neurons in the distal ileum between A53T and WT mice under both housing conditions ($P<0.05$). Representative photomicrographs show a decrease in the proportion of nNOS-positive neurons in A53T mice compared with WT. Scale bar = 50 µm. B: There was no change in the proportion of nNOS-positive neurons in the distal colon between WT and A53T mice under both housing conditions. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Data represent the mean ± SEM (n= 100-200 neurons from 5 mice per group).

Figure 7. Endogenous αsyn distribution and the number of enteric neurons surrounded by αsyn particles are greater in A53T mice when compared to WT mice. A: Staining against endogenous αsyn revealed a punctate staining pattern in WT and B: A53T mice. C: The size and number of αsyn particles, as measured by the area of αsyn coverage per area of ganglion, trended towards an increase in A53T mice under both housing conditions when compared with WT mice. D: The increase in αsyn coverage in A53T mice was confirmed by combining the data for each housing condition and genotype. E and F: αsyn particles were observed to surround some neurons within ganglion from WT mice, but a greater number of larger particles were observed to surround neurons in A53T mice. G: There was an increasing trend towards the number of myenteric neurons surrounded by punctate αsyn particles in A53T mice under both housing conditions when compared with WT mice. H: The increase in the number of neurons surrounded by αsyn was confirmed by combining the data for each housing condition and genotype. I and J: There was no observable change in human αsyn expression/distribution in group-housed A53T mice when compared with single-housed A53T mice. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test or unpaired t-test. Data represent the mean ± SEM (n= 100-200 neurons from 6 mice per group). Scale bar in A, B, I, and J = 25 µm. Scale bar in E and F = 20 µm.

Figure 8. Housing conditions affect terminal plasma corticosterone levels. Under both housing conditions, WT mice had higher levels of corticosterone when compared with A53T mice (*$P<0.002$). Single-housed mice had significantly higher corticosterone levels when compared with

group-housed mice when comparing within genotype (**P<0.0001). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Data represent the mean ± SEM (n=10).



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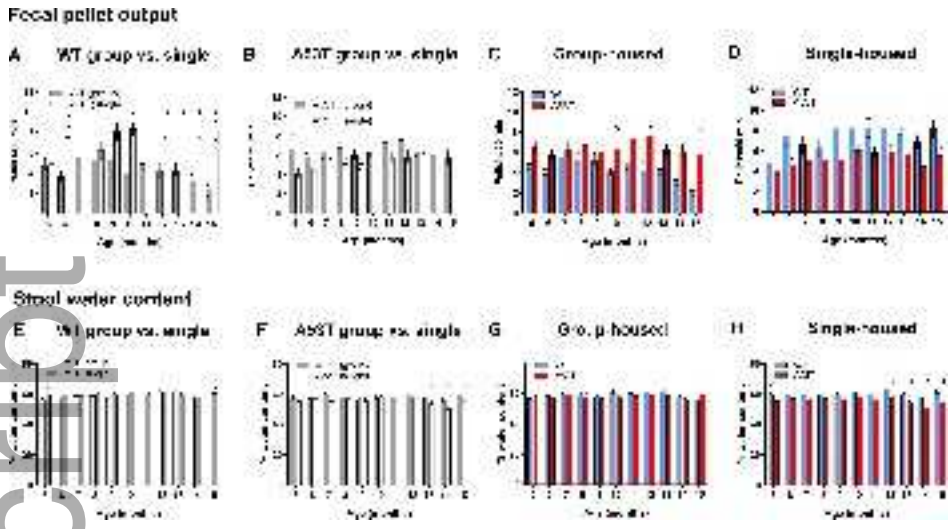

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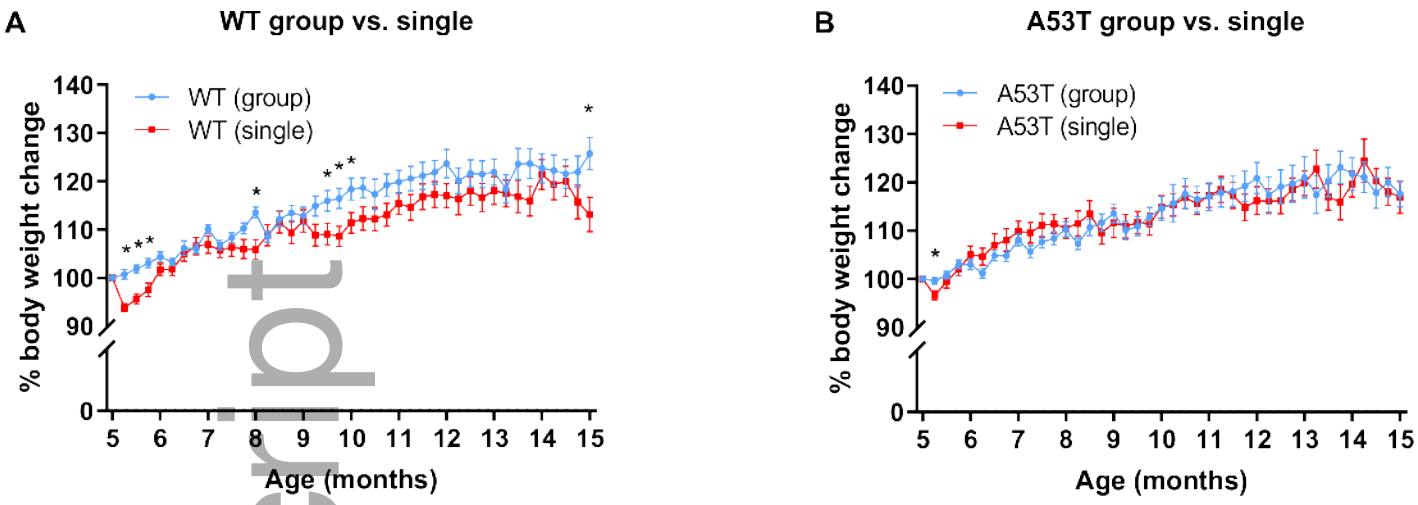

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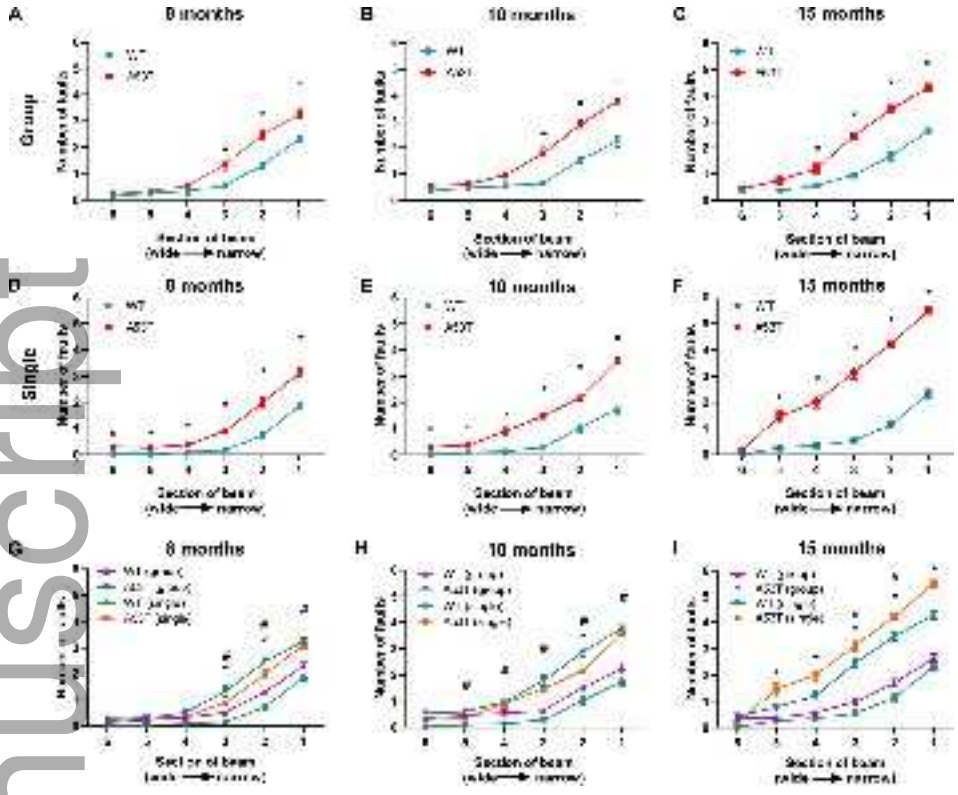

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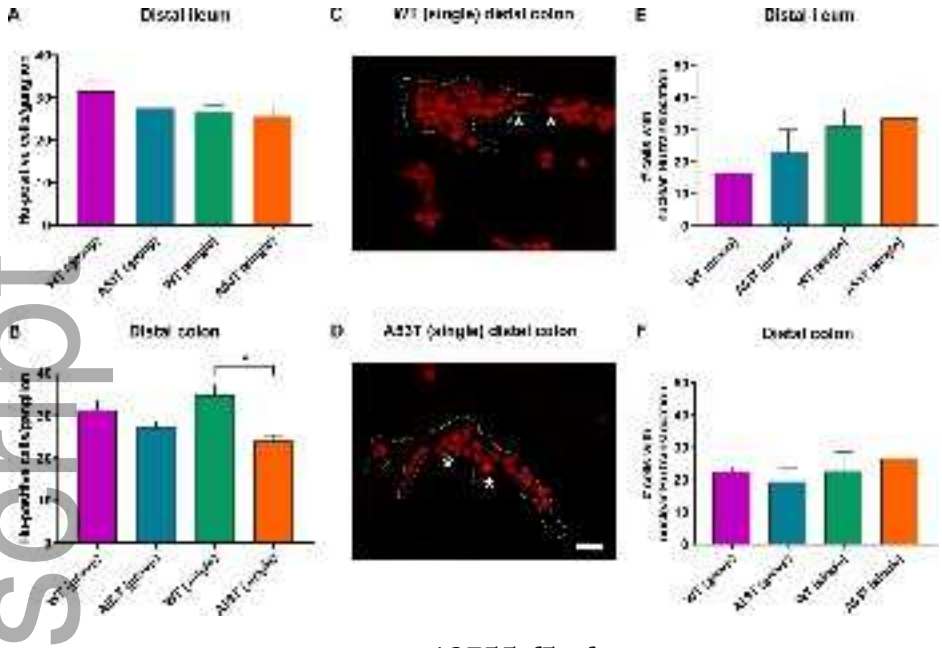

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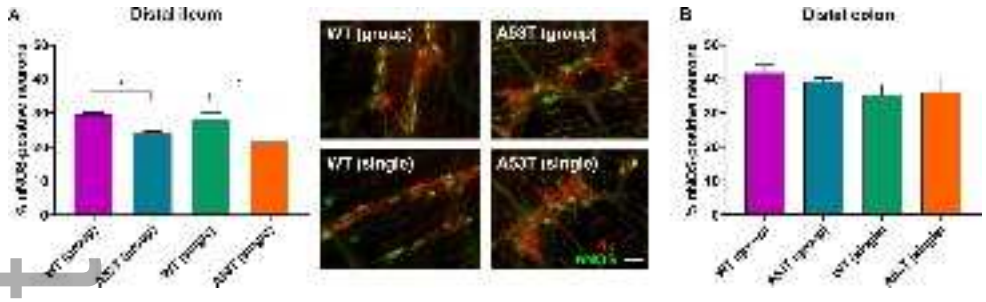

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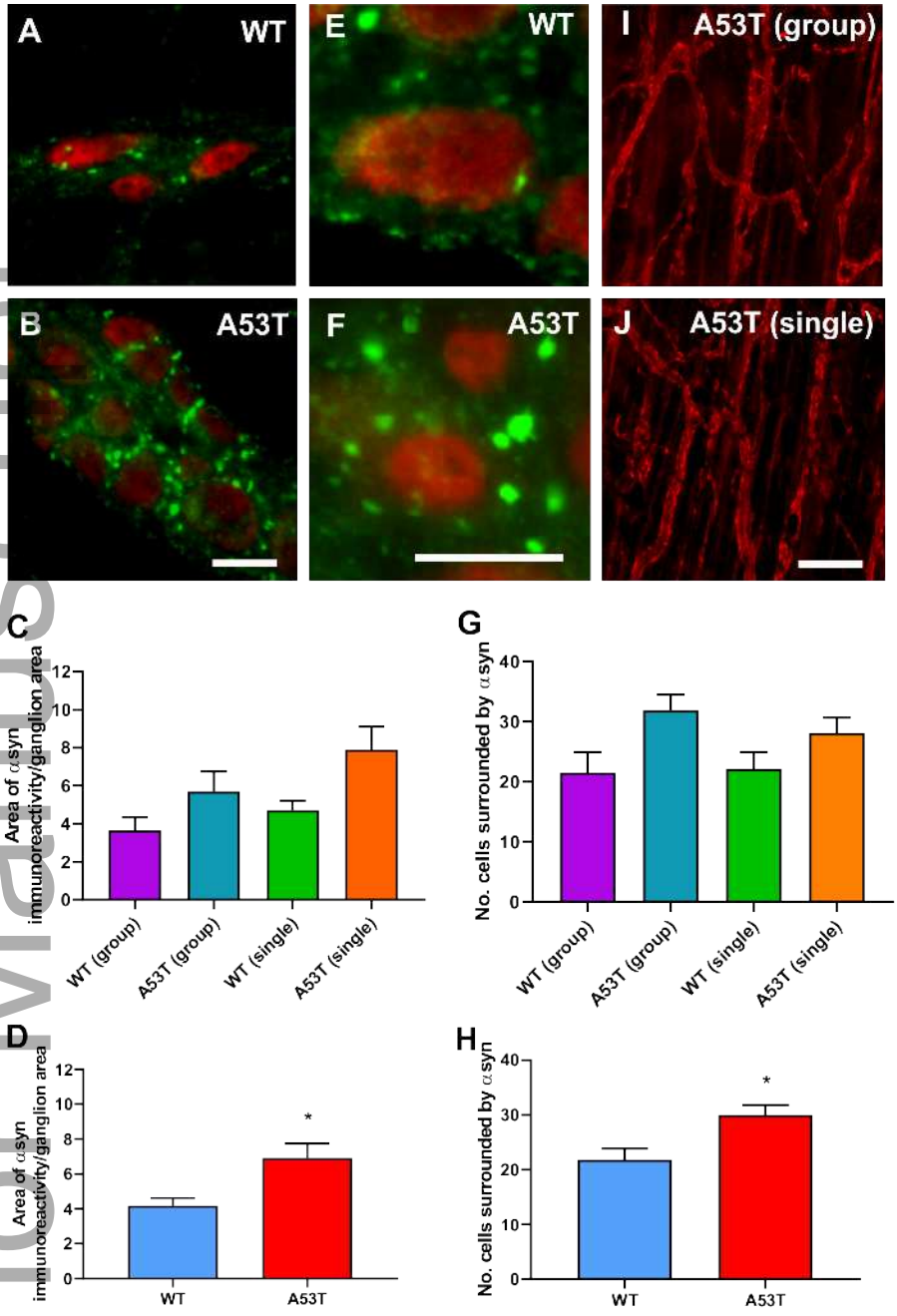

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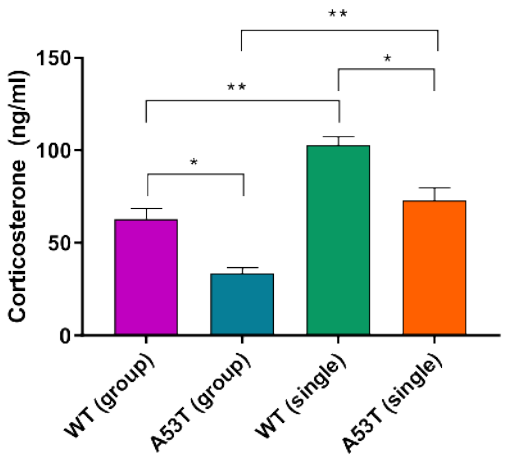

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