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Squalamine Restores the Function of the Enteric Nervous System in Mouse Models of Parkinson's Disease

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Running title: Squalamine restores ENS function in PD model mice

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Abstract and Keywords

BACKGROUND: Parkinson's disease (PD) is a progressive neurodegenerative disorder thought to be caused by accumulation of α -synuclein (α -syn) within the brain, autonomic nerves, and the enteric nervous system (ENS). Involvement of the ENS in PD often precedes the onset of the classic motor signs of PD by many years at a time when severe constipation represents a major morbidity. Studies conducted *in vitro* and *in vivo*, have shown that squalamine, a zwitterionic amphipathic aminosterol, originally isolated from the liver of the dogfish shark, effectively displaces membrane-bound α -syn.

OBJECTIVE: Here we explore the electrophysiological effect of squalamine on the gastrointestinal (GI) tract of mouse models of PD engineered to express the highly aggregating A53T human α -syn mutant.

METHODS: GI motility and *in vivo* response to oral squalamine in PD model mice and controls were assessed using an *in vitro* tissue motility protocol and via fecal pellet output. Vagal afferent response to squalamine was measured using extracellular mesenteric nerve recordings from the jejunum. Whole cell patch clamp was performed to measure response to squalamine in the myenteric plexus.

RESULTS: Squalamine effectively restores disordered colonic motility *in vivo* and within minutes of local application to the bowel. We show that topical squalamine exposure to intrinsic primary afferent neurons (IPANs) of the ENS rapidly restores excitability.

CONCLUSIONS: These observations may help to explain how squalamine may promote gut propulsive activity through local effects on IPANs in the ENS, and further support its possible utility in the treatment of constipation in patients with PD.

KEY WORDS:

Parkinson's disease, squalamine, intestinal motility, intrinsic primary afferent neuron, electrophysiology, gut-brain axis, vagus

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease associated with the accumulation of the protein α -synuclein (α -syn) within the peripheral and central nervous systems (CNS) [1]. Diagnosis of PD is primarily based on the presence of a combination of motor and non-motor symptoms [2]. The latter include severe neuropathic constipation which presents an important therapeutic challenge [3]. Lewy bodies containing α -syn are a pathognomonic sign of PD and are classically found in the CNS. However, they can also be detected within the enteric nervous system (ENS) of patients with sporadic disease [4, 5] and have been detected in the ENS before the clinical diagnosis was made [6]. In a majority of people with PD, constipation occurs many years before the onset of motor symptoms [7-9].

According to Braak's hypothesis PD may begin peripherally with the development of misfolded α -syn aggregates (Lewy bodies) in the olfactory nerves, submandibular gland and ENS [4, 5], then traffic to the CNS. In the case of the gut they move by vagal preganglionic fibres that arise from visceromotor projection neurons of the dorsal motor nucleus (DMV) [1]. This hypothesis has been supported recently by experiments in rodents. α -syn from PD patient brain lysate injected into the intestine progressed to the brainstem [10]. α -syn preformed fibrils injected into the duodenal and pyloric muscularis layer were observed in the DMV and then dispersed caudally in the hindbrain, eventually reaching the basolateral amygdala, dorsal raphe nucleus, and

substantia nigra pars compacta [11]. Truncal vagotomy prevented gut to brain spread of α -syn pathology [11]. In another study, α -syn preformed fibrils, injected into the duodenal wall of bacterial artificial chromosome (BAC) transgenic rats, propagated to the DMV as well as via a sympathetic route to the celiac ganglia and the intermediolateral nucleus of the spinal cord (IML) [12]. Additionally, low-dose, chronic administration of the pesticide rotenone in mice induced α -syn aggregation in the ENS, DMV, intermediolateral nucleus of the spinal cord, and the substantia nigra, reproducing PD pathological staging [13]. This progression was preventable by vagotomy [14]. Clinically Lewy bodies have been detected in the vagus of patients with PD [15] and a single large-scale retrospective epidemiological study of vagotomy in humans showed a reduced risk of development of PD in those patients who received truncal vagotomy [16]. However, some evidence suggests that ENS spread of α -syn pathology to the CNS after exposure may not be sufficiently sustained and may require other factors [17], although this may be influenced by differing experimental methodologies including type and quantity of α -syn and location of injection [11].

Ninety to 95% of the sensory neurites that innervate the intestinal epithelium originate from the ENS, with the rest arising from cell bodies outside the intestine [18, 19]. Intrinsic primary afferent neurons (IPANs) are ENS sensory neurons in the myenteric plexus that are essential for normal gut propulsive peristalsis and indeed life itself as their selective silencing in transgenic mice leads to death shortly after weaning [20]. We have shown recently that two thirds or more of intestinal vagal afferent signaling is relayed via IPANs through an IPANs-to-vagus intramural sensory synapse [21]. If myenteric IPANs' excitability is compromised in the hSNCA^{A53T} model used in the present report, constitutive afferent vagal firing rates could be reduced due to decreased activity at the intramural sensory synapse. To test this, we have

measured vagal afferent firing rates from the mesenteric nerve bundle of the small intestine of the hSNCA^{A53T} model mice and controls. Furthermore, we have examined these rates before and after adding squalamine to the gut lumen.

Squalamine is a natural zwitterionic amphipathic aminosterol with a net positive charge [22], produced in the liver of the dogfish shark and secreted along with bile salts into the proximal duodenum [23]. The synthetic squalamine salt (ENT-01), inhibits the formation of neurotoxic α -syn aggregates in a *C. elegans* model of PD, both *in vitro*, and *in vivo* [24]. We have recently demonstrated that acute intraluminal administration of squalamine reverses the reduced colonic propulsive motility and decreased constitutive vagus nerve firing observed in aged (≥ 2 y) compared to young (3 month) male mice [25]. The clinical prokinetic effect in gut motility appears to occur through local action of the compound on the ENS, since squalamine is not significantly absorbed into the systemic circulation [26].

In the present studies we explore the electrophysiologic effect of squalamine on the gastrointestinal (GI) tract of both normal mice and two mouse models of PD that express the highly aggregating A53T mutant form of human α -syn. The first model used was a mouse α -syn knock-out/knock in model that expressed four copies of human A53T α -syn (hSNCA^{A53T}) driven by the endogenous α -syn promoter but lacking the murine α -syn coding sequence (mSnca). The control is represented by a strain engineered to express two copies of the normal human α -syn protein (hSNCA) but also lacking the endogenous mouse α -syn coding region (mSnca) [27]. This model (dbl-PAC-Tg (SNCA^{A53T}), will be referred to as hSNCA^{A53T} in the subsequent text and in corresponding figures. The hSNCA^{A53T} strain exhibits a constipation phenotype that is more severe than that observed for the corresponding control strain [27]. These animals represent an early model of familial PD, as they display ENS dysfunction by 3 months, progressing in severity

to 6 months, in the absence of CNS pathologies such as olfactory dysfunction, dopaminergic deficits, Lewy body inclusions, and neurodegeneration [27, 28]. A recent study by the same group demonstrated that the translational inhibitor of α -syn, Posiphen, normalized colonic dysmotility in the hSNCA^{A53T} strain [28].

The second model, homozygotic A53T human α -syn overexpressing mice (7 months) [29] and their non-transgenic (non-Tg/WT) littermate controls (7 months), were compared to assess the effect of α -syn aggregation on colonic transit in another PD model. In this engineered mouse model, human A53T (hSNCA^{A53T}) expression is driven by a mouse prion promoter (PrP) resulting in the progressive accumulation of aggregates of A53T α -syn throughout the nervous system [29]. These mice are referred to as PrP-A53T and their controls as non-Tg in the following text and figures. When the homozygotes (PrP-A53T mice) reach an age of 7-8 months they begin to develop progressive impairment of motor function so severe that they are eventually unable to support themselves to feed and succumb at about 16 months [29]. Unlike the hSNCA^{A53T} model, PrP-driven human A53T α -syn expression results in accumulation of filamentous α -syn aggregates in the CNS leading to late-onset neurodegeneration (> 6 months) and mimicking human familial PD neuropathology [29].

We show here that colonic motility is impaired in both mouse models of PD, accompanied by reduced firing rate in vagal nerve fibres of the mesenteric nerve bundle and electrical behavior of IPANs in the hSNCA^{A53T} model. Application of squalamine rapidly restores the normal firing rate and electrical behavior together with promotion of propulsive activity.

Materials and Methods

All animal studies were approved by and performed in accordance with the Animal Research Ethics Board (AREB) of McMaster University (permit 16-08-30) and of the Florey Institute of Neuroscience and Mental Health (approval 16-029).

hSNCA^{A53T} mice and their controls (endogenous promotor) used for motility (Figure 1), mesenteric nerve recording (Figure 3), and electrophysiological recordings (Figure 4&5) were obtained from Jackson Laboratories (Maine, USA). 13-16 male PAC-Tg (SNCA^{WT}) (Stock No. 010710; Control) and (dbl-PAC-Tg (SNCA^{A53T}) (Stock No. 010799; PD mice) were aged 8-9 months prior to experiments. Researchers were blinded to animal groups.

7-month old male and female PrP-A53T human α -syn overexpressing transgenic mice (prion promotor) and their non-Tg littermate controls (25-35g) [29] were reared in a breeding colony at the Florey Institute. A total of 30 mice were used for *in vitro* colonic motility (Figure 2A). For the dose ranging *in vivo* fecal pellet output test, a total of 100 male mice, or 5 sets of non-Tg (N= 10) and PrP-A53T (N = 10) mice aged 7-months were used.

All mice were housed 3-5 per cage on a 12 h light/dark cycle with food and water provided *ad libitum* and allowed a 1-week acclimation period after arrival.

Squalamine dilactate

Squalamine dilactate was donated by Dr Michael Zasloff, Georgetown University (Washington, DC, United States). Squalamine dilactate powder was dissolved in 90% ethanol to make a stock solution, then aliquoted and stored at -20°C until use. Stock solution was diluted in Krebs buffer to a working concentration of 30 μ M for *in vitro* experiments, as determined in a

dose-ranging pilot experiment [30]. Squalamine was diluted to 20, 40, 80, or 120 mg/kg for oral gavage (2% ethanol, included in the control gavage), in order to study *in vivo* fecal pellet output.

***In vitro* colon motility**

The *in vitro* colonic segment experiments were performed with only minor differences in equipment/protocol and as described previously [31-35]. The colon was excised and placed within an organ-bath perfusion chamber filled with warmed, oxygenated Krebs buffer or physiological saline (35°C, 95% O₂, 5% CO₂). The colon was flushed and cannulated at the oral and anal ends to a manifold and syringe to allow inflow of oxygenated Krebs buffer (or physiological saline) or Krebs and squalamine and to maintain intraluminal pressure. Recordings in the first portion of the study (data in Figure 1) were measured at a mechanical threshold causing a pressure differential of 2 hPa. The height of the inflow tube at baseline measurements (data in Figure 2A) was parallel to the height of the colon in the organ bath (1.1 cm). Mechanical threshold was defined as an inflow pressure great enough to generate a contraction in under 30 sec (1.8 cm). Motor patterns were recorded using a Microsoft LifeCam 3000 web camera (Figure 1) positioned 7-8 cm above the tissue. Videos were recorded during a 20-minute Krebs control and a 20-minute Krebs plus squalamine period in which solutions were added to the inflow syringe.

***In vivo* fecal pellet output (FPO)**

PrP-A53T and non-Tg controls were subjected to the FPO test 1 day prior to the start of dosing (day 0). Mice were fasted for one hour and then given access to food one hour before testing. Baseline values were obtained on day 0, following oral gavage with vehicle (sterile water). On days 1-5 mice were fasted for one hour prior to oral gavage with vehicle or 20, 40, 80, or 120 mg/kg squalamine. Oral gavage occurred between 10:00 to 11:00 am daily. On day 5,

the FPO test was performed 1 hour after the final dose was administered. Total number of stool pellets produced in the first 15 min and over a 60 min period was measured in each group.

Mesenteric nerve recording

Jejunal mesenteric nerve recordings were performed as described previously [21]. A segment of hSNCA^{A53T} mouse jejunum (2-3 cm) was mounted on an agar-coated petri dish filled with oxygenated Krebs buffer and nicardipine (3 μ M) and the attached mesentery was pinned out. The luminal contents of the tissue were flushed with Krebs and the oral and anal ends of the tissue were cannulated with silicon tubing. The mesenteric nerve bundle was exposed using fine-tipped forceps by gently removing excess mesentery around the mesenteric arteries. The petri dish nerve preparation was then placed on an inverted microscope stage and perfused with fresh oxygenated Krebs in the serosal compartment using a pump. The nerve bundle was sucked onto a glass micropipette attached to a microelectrode to record multi-unit electrical activity recorded using a Multi-Clamp 700B amplifier and Digidata 1440A signal converter. Baseline afferent firing was recorded for 20-30 min during luminal Krebs perfusion. Intraluminal squalamine (30 μ M) was perfused following the control (Krebs) for a duration of 30 min. Single-unit activity was sorted from multi-unit recordings using principal component analysis (PCA) and spike waveform analysis for spike shape, size and duration in the Dataview program [36]. Vagal fibres were identified by response to cholecystokinin (CCK) applied to the serosa 10 min following the cessation of treatment [37, 38]. The mean interspike interval between vagal afferent spike firing (the inverse of firing frequency) was measured during Krebs and squalamine treatment periods for control and hSNCA^{A53T} mice.

Whole-cell patch clamping

Whole-cell patch clamp was performed on a myenteric plexus preparation as previously described [39]. The mucosa, submucosa, and circular muscle were removed to expose the myenteric plexus over about half the length of the preparation while the adjacent epithelium remained intact. The preparation was dissected in a carbogen-bubbled recording dish filled with Krebs buffer. A single myenteric ganglion was cleaned for patching by application of 0.02% protease type XI (Sigma-Aldrich, Oakville, Canada) dissolved in Krebs for 10-15 min followed by washout with perfused Krebs for 5 min. Only whole-cell recordings greater than 4 G Ω were used. IPANs' excitability parameters were measured as described previously first with perfusion with Krebs buffer for 20 min followed by perfusion with Krebs buffer containing squalamine for 15-30 min. The patched cells were confirmed to be IPANs at the end of the recording by injection of Neurobiotin and confirming the morphology of the cell was that of a Dogiel type II neuron [40]. The neurons were ionophoretically filled with Neurobiotin, from the intracellular patch pipette saline and then fixed overnight at 4°C in Zamboni's fixative (2% vol/vol picric acid, 4% paraformaldehyde in 0.1 M Na₂HPO₄/NaH₂PO₄ buffer, pH =7.0) [41]. Neurons were subsequently washed using three 10-min washes of DMSO and then PBS and then visualized with fluorescence epi-illumination after exposure to streptavidin-Texas Red (Vector, <http://www.vectorlabs.com>), diluted 1:50, revealing Neurobiotin [41] [42].

Statistical Analysis

Effects of squalamine on IPANs' excitability in control and PD model mice were assessed in paired experiments following Krebs control and subsequent squalamine exposure. Percent difference was calculated by (treatment-control)/control. Data are presented as mean $\pm$ S.E.M. *In vitro* statistical comparisons were performed using paired or unpaired, two-tailed t-tests or 1 or 2-

way ANOVA using Graphpad Prism software (Version 7.0). *In vivo* studies were analysed using 1-way and 2-way ANOVA. Statistical significance was determined when $P < 0.05$.

Results

Squalamine increased colonic motor activity in hSNCA^{A53T} mice, *in vitro*

Colonic segments from the homozygotic A53T human α -syn overexpressing mice (hSNCA^{A53T}) (8-9 months) and their controls (8-9 months) were compared to assess the effect of α -syn aggregation on colonic motility. The velocity of propagating contractile clusters (PCCs) was not significantly different in colonic segments from control mice compared with hSNCA^{A53T} mice (N = 12-13 mice/group) ($P = 0.0946$) (Figure 1A). According to the 2-way ANOVA, there would be 9.5% chance of observing the present effect if the animal model had no effect at all, therefore the observed effect of the animal model was not quite significant. Addition of intraluminal squalamine (30 μ M) increased PCC velocity by 20% in the control ($P = 0.044$) and by 38% in hSNCA^{A53T} mice ($P < 0.0001$), compared to Krebs. In contrast neither the frequency nor force (amplitude) of the PCCs differed significantly between control and hSNCA^{A53T} intestinal preparations, nor did squalamine increase either parameter in control or hSNCA^{A53T} intestinal segments (Figure 1B&C). In addition, while the colonic segment from the control animals exhibited a regular period pattern of peristaltic waves, the colonic segments from the hSNCA^{A53T} model exhibited an irregular peristaltic wave pattern characterized by discontinuous contractions, as shown in the spatiotemporal maps (Figure 1D&E, top). Addition of intraluminal squalamine stimulated regular peristaltic waves that travelled the full length of the colonic segment from the oral to anal direction (Figure 1D&E, bottom).

Colonic motor activity is reduced in a second mouse model (PrP-A53T), *in vitro*

We evaluated the total number of contractions in non-Tg and PrP-A53T mice at baseline and during pressure-induced distention (threshold) (Figure 2A). We also evaluated the effect of intraluminal squalamine on PCCs when colon segments were not distended (baseline) and during pressure induced distension (Figure 2B). The total number of contractions was significantly reduced between PrP-A53T and non-Tg mice both at baseline (1.3 ± 0.4 mm/s compared to 4.7 ± 0.9 mm/s, $P < 0.01$) and at threshold (5.5 ± 1.6 mm/s compared to 13.4 ± 1.4 mm/s, $P < 0.01$) (Figure 2A). Threshold significantly increased the total number of contractions compared to baseline in both groups ($P < 0.0001$ in non-Tg and $P < 0.05$ in PrP-A53T). There was no difference in PCC velocity for segments from PrP-A53T (prion promotor) mice compared to non-Tg controls ($N = 6-12$ mice/group) at both baseline (1.2 ± 0.2 mm/s compared to 1.7 ± 0.3 mm/s) and upon distention (1.6 ± 0.3 mm/s compared to 3.0 ± 0.7 mm/s) (Figure 2B). Intraluminal squalamine ($30 \mu\text{M}$) increased baseline PCC velocity from 1.2 ± 0.2 mm/s to 2.3 ± 0.4 mm/s for PrP-A53T mice ($P < 0.05$) and from 1.7 ± 0.3 mm/s to 2.8 ± 0.4 mm/s in non-Tg mice ($P < 0.05$) (Figure 2B). Upon colonic distension, squalamine had no effect on PCC velocity in PrP-A53T mice (from 1.6 ± 0.3 to 2.1 ± 0.3 mm/s) or non-Tg mice (from 3.0 ± 0.7 to 2.4 ± 0.3 mm/s) ($P > 0.05$). These experiments demonstrate that colonic motility was impaired *in vitro* in both hSNCA^{A53T} and PrP-A53T models and that squalamine increased PCC velocity for both control and hSNCA^{A53T} and PrP-A53T mice.

Oral administration of squalamine increased colonic transit in the PrP-A53T mice strain *in vivo*

Fecal pellet output following oral gavage with squalamine was measured in the PrP-A53T mice to assess repeated oral administration of squalamine in a dose ranging study on defecation, *in vivo* (Figure 2C). Pellet output within the first 15 minutes following oral gavage provided an estimate of colonic motility, since gastric feeding stimulates colonic peristalsis, resulting in the discharge of fecal pellets. Both PrP-A53T and non-Tg mice (N = 10 mice/group/dose) were administered vehicle only on Day 0 to establish each animal's baseline pellet output. From Day 1 to Day 5 the animals received vehicle only or squalamine orally by gavage at a range of daily doses from 0 (vehicle only), 20, 40, 80 and 120 mg/kg. Pellet output within the first 15 minutes at Day 5 were compared to baseline values measured at Day 0. We could not detect a significant difference in the fecal pellet output between non-Tg and PrP-A53T mice in the groups receiving only vehicle ($P > 0.05$), possibly due to the poor sensitivity of this assay when compared to measuring PCC velocity and frequency, *in vitro*. However, following gavage of squalamine on Day 5, a treatment associated increase was observed in non-Tg mice dosed with 40 and 120 mg/kg ($P < 0.01$ and $P < 0.05$, respectively) as well as in PrP-A53T mice dosed with 20, 40, and 80 mg/kg ($P < 0.005$, $P < 0.0001$, and $P < 0.01$, respectively). These experiments demonstrated that oral administration of squalamine stimulated colonic motility in both non-Tg and PrP-A53T mice. Effect of squalamine on wet weight, dry weight, % water content of stool, and % change in stool output/body weight are presented in Supplementary Figure 1.

Squalamine significantly increased vagal afferent firing frequency in hSNCA^{A53T} mice *in vitro*

Vagal afferent firing rates were measured by extracellular action potential (multiunit) recording from a mesenteric nerve bundle attached to segments of the jejunum excised from hSNCA^{A53T} mice and controls. The interspike interval of vagal single units were measured and these represent the reciprocal of their firing rate. Mean afferent vagal firing rate was significantly reduced in the hSNCA^{A53T} mice compared to the control group ($p = 0.0375$). The mean interspike interval between vagal spikes was shorter in control mice compared to hSNCA^{A53T} mice, indicating a lower firing rate in the latter (2389 ± 230 ms compared to 3088 ± 392 ms), (Figure 3A). Squalamine had strong effects on vagal firing rates in both groups of mice (Figure 3B&C). Intraluminal addition of squalamine ($30\mu\text{M}$) decreased the interspike interval between vagal afferent spikes in the control mice by 63% to 874 ± 60 ms ($p < 0.0001$). Acute treatment with squalamine also decreased the interspike interval between vagal afferent spikes in the hSNCA^{A53T} model mouse by 47% to 1646 ± 168 ms ($p < 0.0001$). These results demonstrate that the vagal afferent firing rate is reduced in the hSNCA^{A53T} mice and is potently stimulated by the addition of intraluminal squalamine in both the control and hSNCA^{A53T} and PrP-A53T model mice.

hSNCA^{A53T} model mice have reduced myenteric intrinsic primary afferent neuron (IPANs) excitability

To help elucidate the mechanism by which squalamine stimulated intestinal motility we conducted electrophysiological studies on single neurons within the intact myenteric plexus of the hSNCA^{A53T} mice [27] and corresponding control animals using published methods [18]. IPANs [40] in the myenteric plexus have neurites that project to the epithelial layer where they

respond to molecules present in the gut lumen and then conduct action potentials to the myenteric plexus [39, 43, 44]. IPANs excitation generates PCCs that move luminal contents in the oral to anal direction [44]. We investigated whether IPANs' excitability was reduced in the hSNCA^{A53T} mice compared to the control mice and whether squalamine administration to intestinal segments taken from hSNCA^{A53T} mice could facilitate IPANs' excitability.

Using whole-cell patch pipette recordings from IPANs, we measured the threshold for action potential generation in response to intracellular injection of square depolarising current pulses (AP threshold), the number of action potentials (AP) generated in response to current injection of 2x threshold intensity (No. AP 2x threshold), the area under the curve for the slow after-hyperpolarisation generated by 3 action potentials (sAHP AUC), and the resting membrane potential. In this experiment, 14 IPANs from 14 hSNCA^{A53T} mice and 9 IPANs from 9 control mice were successfully studied (Figure 4). The AP threshold (mean $\pm$ SEM) was 32.2 ± 20.0 pA for the control compared to 59.2 ± 46.1 pA for the hSNCA^{A53T} IPANs ($P = 0.036$) (Figure 4E). The number of action potentials produced by a current 2x the threshold intensity was 3.9 ± 5.1 for the control versus 1.6 ± 0.6 for the hSNCA^{A53T} IPANs ($P = 0.06$) (Figure 4F). The area under of the curve for the sAHP was -49.5 ± 63.7 mV s for the control versus -85.5 ± 78.2 mV s for the hSNCA^{A53T} cells ($P = 0.17$) (Figure 4G). The resting membrane potential was -56 ± 10 mV for control IPANs versus -62 ± 6 mV for those from the hSNCA^{A53T} model ($P = 0.034$) (Figure 4H). Thus, IPANs from the hSNCA^{A53T} mouse strain exhibited a reduced excitability compared to those from the control animals.

Squalamine excites IPANs

We tested for the effect of squalamine on the excitability of an isolated colonic segment from the hSNCA^{A53T} (endogenous promoter) mouse using preparations in which myenteric neurons are exposed on one half of a small intestinal segment [39]. In this experiment we asked whether squalamine influenced the activity of the IPANs through direct interaction with the neuron or indirectly, by stimulating release of epithelial mediators that influenced IPANs' behaviour. Addition of squalamine (30 μ M) to the Krebs buffer in either the epithelial or the myenteric plexus compartments of the preparation increased IPANs' excitability (Figure 5). Adding squalamine to the luminal side of the hSNCA^{A53T} mouse colon (N = 15 cells) decreased the mean AP threshold from 63.7 ± 50.4 to 35.7 ± 22.3 pA ($P=0.037$) (Figure 5C) and increased the number of APs produced by a current 2x the threshold intensity from 1.6 ± 0.6 to 3.1 ± 0.7 ($P=0.0016$) (Figure 5A, 5C). Addition of squalamine decreased the area under the curve of the sAHP from 86.8 ± 88.2 to 20.3 ± 25.3 mV s ($P=0.0035$) (Figure 5C) and depolarised the resting membrane potential from -62 ± 7 to -54 ± 6 mV ($P< 0.001$) (Figure 5C). Adding squalamine directly to the myenteric plexus of the hSNCA^{A53T} mouse colon (N = 5 cells) (Figures 5D, 5E) did not significantly decrease mean AP threshold from 46.0 ± 31.3 to 29.0 ± 10.1 pA ($P=0.058$) and increase the number of APs produced by a current 2x the threshold intensity from 1.4 ± 0.5 to 4.4 ± 2.8 ($P=0.095$). Squalamine significantly depolarised the resting membrane potential from -63 ± 4 to -55 ± 6 mV ($P= 0.0067$), but did not significantly decrease the area under the curve of the sAHP (-71.9 ± 60.1 to -9.6 ± 15.1 mV s ($P=0.099$)), when added to the myenteric plexus of the hSNCA^{A53T} mouse. These experiments demonstrated that squalamine can augment the reduced excitability of the IPANs in tissue taken from hSNCA^{A53T} mice and that squalamine can act directly on the IPANs, rather than indirectly through release of a chemical such as a neurotransmitter from a luminal epithelial cell, like an enteroendocrine cell.

Discussion

The purpose of the present study was to investigate the ability of squalamine, a zwitterionic amphipathic aminosterol that has previously been shown to improve age-related gut dysmotility [25, 30] and inhibit the formation of α -syn aggregates [24], to improve colonic motility and constipation in two mouse models of Parkinson's disease (PD). We demonstrate in this report that squalamine can restore gastrointestinal motility in mouse models of PD.

Constipation in PD presents a significant challenge in the management of the disease and often precedes the onset of motor symptoms by years or decades [9]. It has been recently reported that α -syn expression is induced in the ENS in response to viral, bacterial and fungal infections [45] and that excessive intraneuronal accumulation of α -syn promotes formation of toxic aggregates [24]. Because of the normal trafficking of α -syn aggregates from the ENS to the central nervous system (CNS) via the vagus [10, 16], neurotoxic aggregates accumulate progressively within the brainstem and more rostral structures. Additionally, there is potential for bidirectional spread of α -syn between the ENS and CNS via efferent vagal fibres, as suggested by Di Monte [46]. α -syn overexpression in the DMV and vagal ganglia was efficiently trafficked via vagal afferents and efferents to the stomach wall [46]. It would be logical to suggest therefore that targeting α -syn aggregation in the ENS may be beneficial in the treatment of PD constipation [47].

The two models of PD used in this particular study both expressed the human A53T α -syn autosomal dominant mutation, one being driven by a prion promoter (PrP-A53T) [29], the other by the endogenous mouse sequence (hSNCA^{A53T}) [27] and both exhibit GI dysmotility.

Overexpression of normal human α -syn in the ENS of the control strain was not associated with apparent gastrointestinal dysfunction, nor were α -syn aggregates seen on pathology, in contrast to the strain engineered to overexpress A53T (hSNCA^{A53T}) [27]. Addition of squalamine increased colonic PCC velocity in both strains and FPO in the PrP-A53T strain. It is notable however, that squalamine also produced prokinetic effects on motility in control strains in the absence of α -syn pathology, which likely occurs via stimulation of the ENS. These observations support the hypothesis that the beneficial effect of squalamine in PD model mice with, respect to motility, may in part involve displacement of membrane disruptive aggregates within the enteric neurons.

Indeed, squalamine has been shown to have two beneficial properties of relevance to PD. By displacement of monomeric α -syn from a membrane, squalamine reduces the surface concentration of the protein, reducing the probability that neurotoxic aggregates will form. In addition, squalamine can also displace aggregates that have already formed on the membrane, restoring membrane function, and releasing the aggregate into the cytoplasm for subsequent digestion. Upon entry into a cell, or potentially a primary afferent neuron [48], squalamine is electrostatically attracted to the negatively charged phospholipid head groups that comprise the cytoplasmic surface of the plasma membrane thereby neutralizing its negative electrostatic surface potential [48, 49]. In a *C. elegans* model of PD, in which the animal has been engineered to express human α -syn in its muscles, orally administered squalamine inhibits formation of membrane disruptive intracellular aggregates and prevents the onset of disordered motility and paralysis [24]. In addition, *in vitro*, squalamine prevents the binding of membrane disruptive α -syn aggregates to the surface of neuronal cells, protecting them from loss of viability [24]. We believe a similar effect occurs when squalamine enters the enteric neuron of the hSNCA^{A53T} mouse. Our studies demonstrate, as well, that the enteric nerves in the hSNCA^{A53T} mouse models

are not irreversibly damaged, since squalamine restored the disordered GI motility in both models of PD. Orally administered squalamine also increased FPO in A53T mice at the mid-range doses of 20, 40, and 80 mg/kg and at the doses of 40 and 120 mg/kg in the non-Tg controls, which may represent a bell-shaped dose response curve for squalamine, with an optimal range of 40 mg/kg. Additionally, chronic administration may produce significant effects on α -syn aggregates by day 5. Indeed, in a recent Phase 2a clinical study of 50 PD patients with severe constipation, orally administered ENT-01 reversed constipation dose dependently in most patients some suffering for 60 years [26].

As predicted in the introduction, constitutive vagal afferent firing rates were decreased (increased interspike intervals) for hSNCA^{A53T} model animals compared to controls (Figure 3). Squalamine (30 μ M) potently increased vagal fibre firing rates when it was added to the Krebs buffer perfusing the lumen for both controls and hSNCA^{A53T} animals. Given the important role of enteric IPANs in gut to brain signaling [21], the vulnerability of the ENS to misfolded α -syn in terms of reduced function or IPANs excitability would have a significant impact on the amount and quality of information reaching the brain from the gut. If behavioural or mood-related Parkinsonian symptoms are mediated, in part, by reduced afferent vagal discharge, the excitatory action of squalamine on IPANs and the vagus may serve to reverse the symptoms.

We have shown here that IPANs from animals in which A53T is overexpressed (hSNCA^{A53T}) exhibit reduced excitatory activity compared with IPANs from controls. Reduced excitability was characterized by a more hyperpolarized resting membrane potential in the hSNCA^{A53T} mouse, requiring a larger threshold current for action potential generation. The number of action potentials produced by a current 2x the threshold intensity was much lower for the hSNCA^{A53T} mouse, which correlates with the larger area under the curve of the slow after-

hyperpolarization (sAHP). These results demonstrate that the IPANs from the hSNCA^{A53T} mouse requires a greater stimulus to fire an action potential and takes longer to repolarize before it can fire a subsequent action potential (AP) than the control mouse and is thus less excitable.

Subsequent squalamine exposure restores normal excitatory responses of the colonic IPANs within minutes of local application to preparations from hSNCA^{A53T} animals; the resting membrane potential is more depolarized. In this model less current is required to reach threshold and generate an AP, and the sAHP area under the curve (AUC) is smaller, indicating faster repolarization and the ability to respond and produce a subsequent AP sooner. It is possible that the effects on PD model mice are not α -syn specific, given the prokinetic effects of squalamine in young and old wild-type mice [25] and in the controls. In the setting of the GI tract, we hypothesize that squalamine can be readily transported across the intestinal epithelium and, as described in this report, stimulate the activity of local IPANs and, in turn, GI motility in a healthy animal. It is also possible that IPANs are stimulated via a sensory transduction cascade mediated by intestinal epithelial cells but given that squalamine increases IPAN excitability when applied luminally or directly to the myenteric plexus, we suggest the former. Interestingly, squalamine does not seem to enter the systemic circulation so that the effects of oral administration are focused in the gut [26].

Locally applied squalamine can stimulate the peristaltic activity of both WT and A53T mice minutes after exposure of the colonic lumen to the compound. Similarly, squalamine restores normal excitatory responses of the colonic IPAN within minutes of local application to preparations from both control and A53T animals. These observations are consistent with a mechanism in which the squalamine ion increases the excitatory status of the IPAN, regardless of the presence of functionally disruptive α -syn aggregates. A reduction in the negative surface

charge on intracellular membranes could both influence the electrical behavior of the neuron as well as displace membrane associated aggregates of α -syn. Because squalamine can displace proteins that are bound electrostatically to intracellular membranes, it rapidly activates the AMPA glutamate receptor in primary cortical neurons by displacing the inhibitory TARP regulatory protein from the cytoplasmic face of the plasma membrane [50]; similarly the squalamine ion inhibits the sodium hydrogen exchanger (type 3) by displacing its positively charged carboxyl-terminus from the cytoplasmic face of the plasma membrane [51]. The mechanism by which squalamine alters the electrical excitability of the enteric neuron, independent of the presence of α -syn, is under investigation.

The action of squalamine on IPANs to increase their excitability is entirely consistent with its prokinetic effects since propulsive motility throughout the small and large intestines is critically dependent on normal IPANs function and excitability [20, 52]. Silencing of IPANs by inhibition of protein kinase A activity produces lethal pseudo-obstruction in the murine intestine [20], and the pharmacological inhibition of IPANs excitability by application of 5,6-Dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one to the Krebs buffer superfusing *ex vivo* segments of mouse intestine reduces and then abolishes all propulsive PCCs [52]. Manipulation of the current underlying the AHP by calcium-activated, intermediate-conductance potassium channels (IK or IKCa) block or activation has similar effects in the small intestine [53]. Because of their role in generating the propulsive peristaltic reflex, action at myenteric IPANs provides a cellular explanation for the increased propulsive motility and reduced constipation in the hSNCA^{A53T} and PrP-A53T animals caused by squalamine. We found evidence for the direct action of squalamine on the IPANs as demonstrated by the increased excitability of the neuron when squalamine was applied directly to the myenteric plexus.

In conclusion, we have shown that squalamine, locally administered to the lumen of the GI tract of hSNCA^{A53T} mice, can restore intestinal motility and enteric sensory neuron function, suggesting that the ENS is not irreversibly damaged, at least in murine models of PD.

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Conflict of Interest

M.Z. and D.B. are employees of Enterin Inc. J.B. and W.A.K. are members of the scientific advisory board of Enterin Inc. The authors have no additional conflicts of interest.

480 **References**

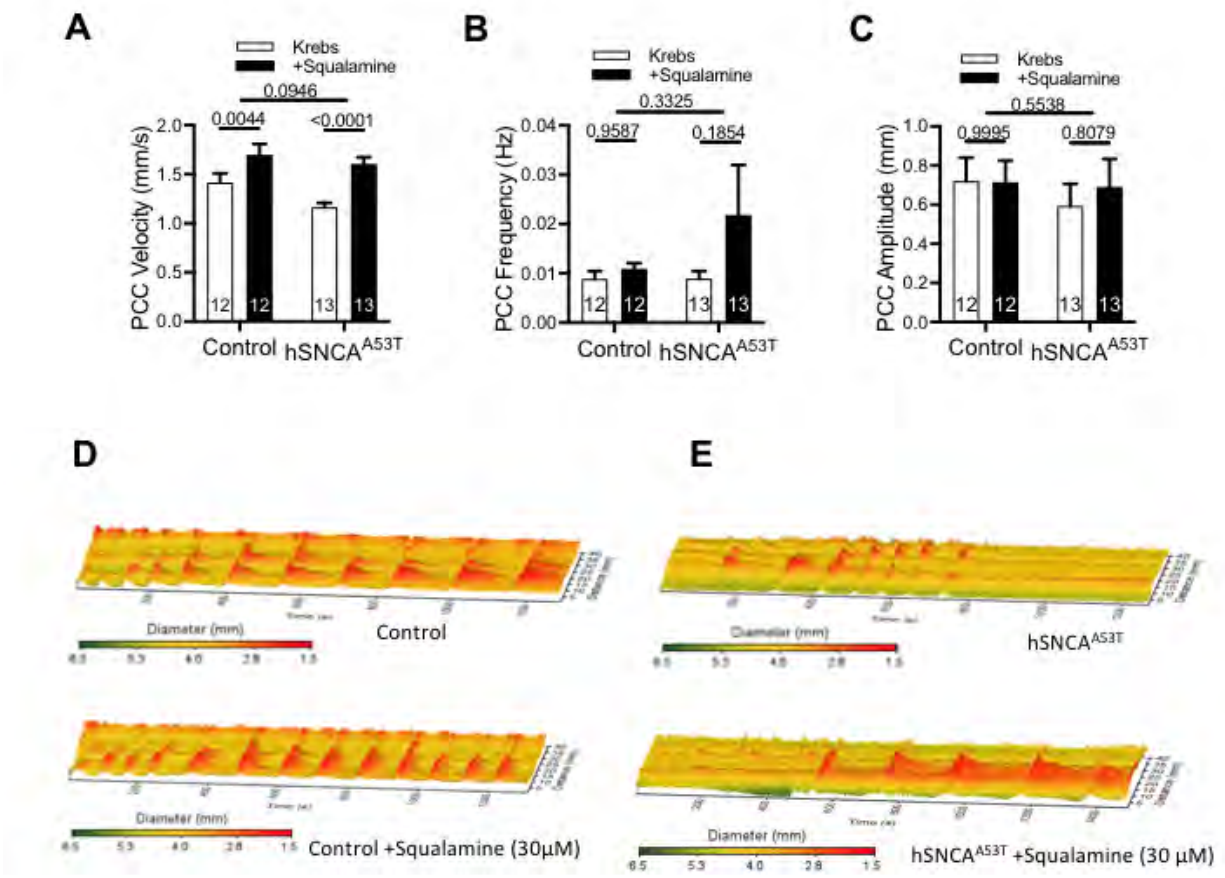
- 481 [1] Braak H, Rub U, Gai WP, Del Tredici K (2003) Idiopathic Parkinson's disease: possible
482 routes by which vulnerable neuronal types may be subject to neuroinvasion by an
483 unknown pathogen. *J Neural Transm (Vienna)* **110**, 517-536.
- 484 [2] Hughes AJ, Daniel SE, Kilford L, Lees AJ (1992) Accuracy of clinical diagnosis of
485 idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J Neurol*
486 *Neurosurg Psychiatry* **55**, 181-184.
- 487 [3] Fasano A, Visanji NP, Liu LWC, Lang AE, Pfeiffer RF (2015) Gastrointestinal
488 dysfunction in Parkinson's disease. *The Lancet Neurology* **14**, 625-639.
- 489 [4] Braak H, de Vos RAI, Bohl Jr, Del Tredici K (2006) Gastric α -synuclein
490 immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for
491 Parkinson's disease-related brain pathology. *Neuroscience Letters* **396**, 67-72.
- 492 [5] Rietdijk CD, Perez-Pardo P, Garssen J, van Wezel RJ, Kraneveld AD (2017) Exploring
493 Braak's Hypothesis of Parkinson's Disease. *Front Neurol* **8**, 37.
- 494 [6] Shannon KM, Keshavarzian A, Mutlu E, Dodiya HB, Daian D, Jaglin JA, Kordower JH
495 (2012) Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease. *Mov*
496 *Disord* **27**, 709-715.
- 497 [7] Cersosimo MG, Raina GB, Pecci C, Pellene A, Calandra CR, Gutierrez C, Micheli FE,
498 Benarroch EE (2013) Gastrointestinal manifestations in Parkinson's disease: prevalence
499 and occurrence before motor symptoms. *J Neurol* **260**, 1332-1338.
- 500 [8] Lin CH, Lin JW, Liu YC, Chang CH, Wu RM (2014) Risk of Parkinson's disease
501 following severe constipation: a nationwide population-based cohort study. *Parkinsonism*
502 *Relat Disord* **20**, 1371-1375.
- 503 [9] Klingelhoefer L, Reichmann H (2015) Pathogenesis of Parkinson disease-the gut-brain
504 axis and environmental factors. *Nat Rev Neurol* **11**, 625-636.
- 505 [10] Holmqvist S, Chutna O, Bousset L, Aldrin-Kirk P, Li W, Bjorklund T, Wang ZY, Roybon
506 L, Melki R, Li JY (2014) Direct evidence of Parkinson pathology spread from the
507 gastrointestinal tract to the brain in rats. *Acta Neuropathol*.
- 508 [11] Kim S, Kwon SH, Kam TI, Panicker N, Karuppagounder SS, Lee S, Lee JH, Kim WR,
509 Kook M, Foss CA, Shen C, Lee H, Kulkarni S, Pasricha PJ, Lee G, Pomper MG, Dawson
510 VL, Dawson TM, Ko HS (2019) Transneuronal Propagation of Pathologic alpha-
511 Synuclein from the Gut to the Brain Models Parkinson's Disease. *Neuron* **103**, 627-641
512 e627.
- 513 [12] Van Den Berge N, Ferreira N, Gram H, Mikkelsen TW, Alstrup AKO, Casadei N, Tsung-
514 Pin P, Riess O, Nyengaard JR, Tamguney G, Jensen PH, Borghammer P (2019) Evidence
515 for bidirectional and trans-synaptic parasympathetic and sympathetic propagation of
516 alpha-synuclein in rats. *Acta Neuropathol* **138**, 535-550.
- 517 [13] Pan-Montojo F, Anichtchik O, Dening Y, Knels L, Pursche S, Jung R, Jackson S, Gille G,
518 Spillantini M, Reichmann H, Funk R (2010) Progression of Parkinson's disease pathology
519 is reproduced by intragastric administration of rotenone in mice. *PLoS One* **5**, e8762.
- 520 [14] Pan-Montojo F, Schwarz M, Winkler C, Arnhold M, O'Sullivan G, Pal A, Said J, Marsico
521 G, Verbavatz J, Rodrigo-Angulo M, Gille G, Funk R, Reichmann H (2012)
522 Environmental toxins trigger PD-like progression via increased alpha-synuclein release
523 from enteric neurons in mice. *Sci Rep* **2**, 898.

- [15] Del Tredici K, Braak H (2008) A not entirely benign procedure: progression of Parkinson's disease. *Acta Neuropathol* **115**, 379-384.
- [16] Svensson E, Horvath-Puho E, Thomsen RW, Djurhuus JC, Pedersen L, Borghammer P, Sorensen HT (2015) Vagotomy and subsequent risk of Parkinson's disease. *Ann Neurol* **78**, 522-529.
- [17] Manfredsson FP, Luk KC, Benskey MJ, Gezer A, Garcia J, Kuhn NC, Sandoval IM, Patterson JR, O'Mara A, Yonkers R, Kordower JH (2018) Induction of alpha-synuclein pathology in the enteric nervous system of the rat and non-human primate results in gastrointestinal dysmotility and transient CNS pathology. *Neurobiol Dis* **112**, 106-118.
- [18] Keast J, Furness J, Costa M (1987) Distribution of peptide-containing neurons and endocrine cells in the rabbit gastrointestinal tract, with particular reference to the mucosa. *Cell Tissue Res* **248**, 565-577.
- [19] Ekblad E, Winther C, Ekman R, Hakanson R, Sundler F (1987) Projections of peptide-containing neurons in rat small intestine. *Neuroscience* **20**, 169-188.
- [20] Howe D, Clarke C, Yan H, Willis B, Schneider D, McKnight G, Kapur R (2006) Inhibition of protein kinase A in murine enteric neurons causes lethal intestinal pseudo-obstruction. *Journal of Neurobiology* **66**, 256-272.
- [21] Perez-Burgos A, Mao Y, Bienenstock J, Kunze W (2014) The gut-brain axis rewired: adding a functional vagal nicotinic "sensory synapse". *FASEB J* **28**, 3064-3074.
- [22] Wehrli SL, Moore KS, Roder H, Durell S, Zasloff M (1993) Structure of the novel steroidal antibiotic squalamine determined by two-dimensional NMR spectroscopy. *Steroids* **58**, 370-378.
- [23] Moore KS, Wehrli S, Roder H, Rogers M, Forrest JN, Jr., McCrimmon D, Zasloff M (1993) Squalamine: an aminosterol antibiotic from the shark. *Proc Natl Acad Sci U S A* **90**, 1354-1358.
- [24] Perni M, Galvagnion C, Maltsev A, Meisl G, Muller MB, Challa PK, Kirkegaard JB, Flagmeier P, Cohen SI, Cascella R, Chen SW, Limbocker R, Sormanni P, Heller GT, Aprile FA, Cremades N, Cecchi C, Chiti F, Nollen EA, Knowles TP, Vendruscolo M, Bax A, Zasloff M, Dobson CM (2017) A natural product inhibits the initiation of alpha-synuclein aggregation and suppresses its toxicity. *Proc Natl Acad Sci U S A* **114**, E1009-E1017.
- [25] West CL, Amin JY, Farhin S, Stanis AM, Mao Y-K, Kunze WA (2019) Colonic Motility and Jejunal Vagal Afferent Firing Rates Are Decreased in Aged Adult Male Mice and Can Be Restored by an Aminosterol. *Frontiers in Neuroscience* **13**.
- [26] Hauser RA, Sutherland D, Madrid JA, Rol MA, Frucht S, Isaacson S, Pagan F, Maddux BN, Li G, Tse W, Walter BL, Kumar R, Kremens D, Lew MF, Ellenbogen A, Oguh O, Vasquez A, Kinney W, Lowery M, Resnick M, Huff N, Posner J, Ballman KV, Harvey BE, Camilleri M, Zasloff M, Barbut D (2019) Targeting neurons in the gastrointestinal tract to treat Parkinson's disease. *Clinical Parkinsonism & Related Disorders* **1**, 2-7.
- [27] Kuo YM, Li Z, Jiao Y, Gaborit N, Pani AK, Orrison BM, Bruneau BG, Giasson BI, Smeyne RJ, Gershon MD, Nussbaum RL (2010) Extensive enteric nervous system abnormalities in mice transgenic for artificial chromosomes containing Parkinson disease-associated alpha-synuclein gene mutations precede central nervous system changes. *Hum Mol Genet* **19**, 1633-1650.
- [28] Kuo YM, Nwankwo EI, Nussbaum RL, Rogers J, Maccacchini ML (2019) Translational inhibition of α -synuclein by Posiphen normalizes distal colon motility in

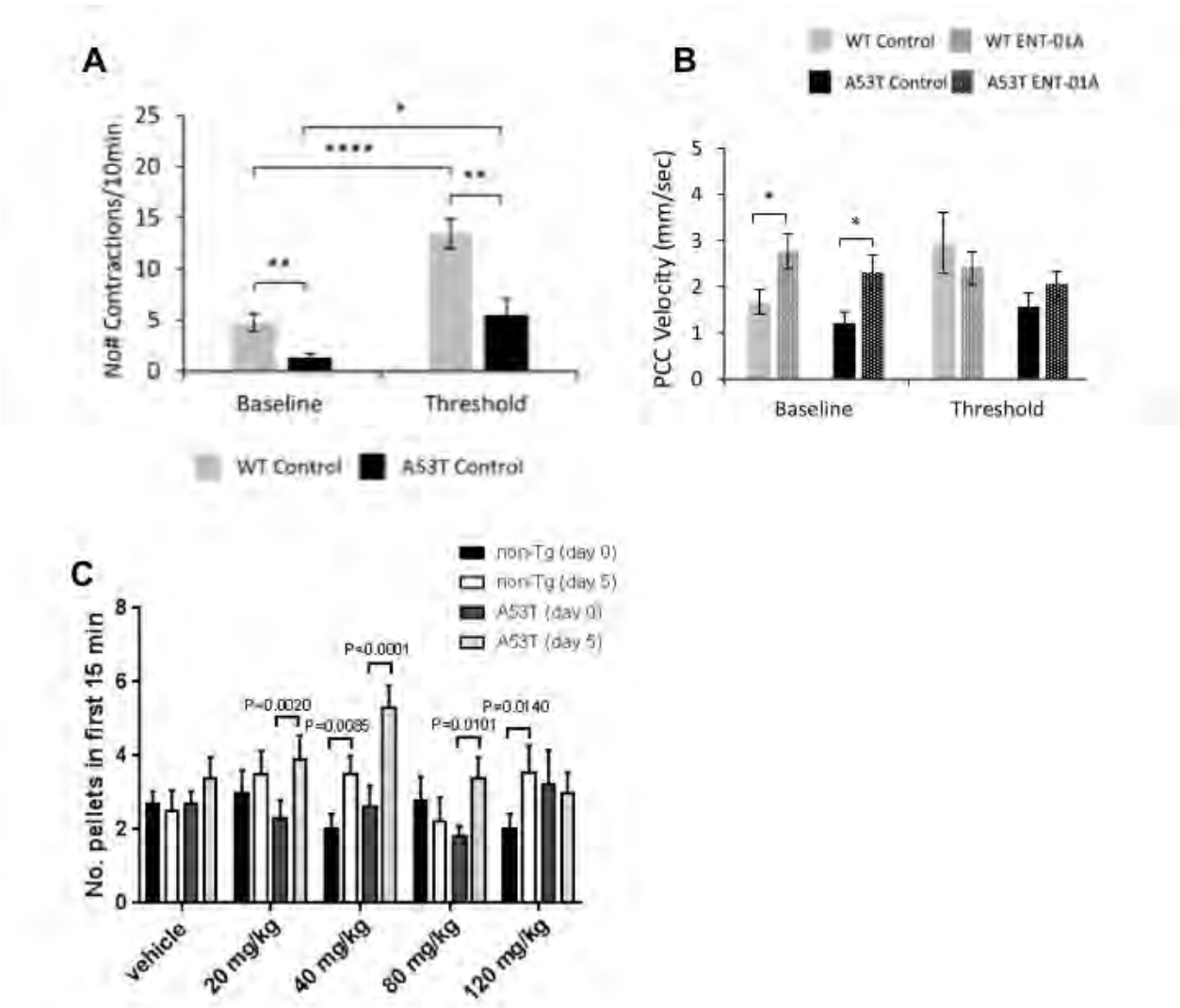
- transgenic Parkinson mice. *Am J Neurodegener Dis* **8**, 1-15.
- [29] Giasson BI, Duda JE, Quinn SM, Zhang B, Trojanowski JQ, Lee VM (2002) Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron* **34**, 521-533.
- [30] Kunze W, Yan RM, Min KK, Pasyk M, Stanis A, Zasloff M (2014) in *Gastroenterology* Elsevier, Chicago, p. S356.
- [31] Delungahawatta T, Amin JY, Stanis AM, Bienenstock J, Forsythe P, Kunze WA (2017) Antibiotic Driven Changes in Gut Motility Suggest Direct Modulation of Enteric Nervous System. *Front Neurosci* **11**, 588.
- [32] Wafai L, Taher M, Jovanovska V, Bornstein JC, Dass CR, Nurgali K (2013) Effects of oxaliplatin on mouse myenteric neurons and colonic motility. *Front Neurosci* **7**, 30.
- [33] West C, Stanis AM, Wong A, Kunze WA (2016) Effects of *Saccharomyces cerevisiae* or *boulardii* yeasts on acute stress induced intestinal dysmotility. *World J Gastroenterol* **22**, 10532-10544.
- [34] West C, Wu RY, Wong A, Stanis AM, Yan R, Min KK, Pasyk M, McVey Neufeld KA, Karamat MI, Foster JA, Bienenstock J, Forsythe P, Kunze WA (2017) *Lactobacillus rhamnosus* strain JB-1 reverses restraint stress-induced gut dysmotility. *Neurogastroenterol Motil* **29**, 1-11.
- [35] Wu RY, Pasyk M, Wang B, Forsythe P, Bienenstock J, Mao YK, Sharma P, Stanis AM, Kunze WA (2013) Spatiotemporal maps reveal regional differences in the effects on gut motility for *Lactobacillus reuteri* and *rhamnosus* strains. *Neurogastroenterol Motil* **25**, e205-214.
- [36] Heitler WJ (2007) DataView: A Tutorial Tool for Data Analysis. Template-based Spike Sorting and Frequency Analysis. *Journal of Undergraduate Neuroscience Education* **6**, A1-A7.
- [37] Hillsley K, Grundy D (1998) Serotonin and cholecystokinin activate different populations of rat mesenteric vagal afferents. *Neurosci Lett* **255**, 63-66.
- [38] Richards W, Hillsley K, Eastwood C, Grundy D (1996) Sensitivity of vagal mucosal afferents to cholecystokinin and its role in afferent signal transduction in the rat. *J Physiol* **497 (Pt 2)**, 473-481.
- [39] Mao YK, Kasper DL, Wang B, Forsythe P, Bienenstock J, Kunze WA (2013) *Bacteroides fragilis* polysaccharide A is necessary and sufficient for acute activation of intestinal sensory neurons. *Nat Commun* **4**, 1465.
- [40] Furness JB, Kunze WA, Bertrand PP, Clerc N, Bornstein JC (1998) Intrinsic primary afferent neurons of the intestine. *Prog Neurobiol* **54(1)**, 1-18.
- [41] Mao Y, Wang B, Kunze W (2006) Characterization of myenteric sensory neurons in the mouse small intestine. *J Neurophysiol* **96**, 998-1010.
- [42] Kunze WA, Mao YK, Wang B, Huizinga JD, Ma X, Forsythe P, Bienenstock J (2009) *Lactobacillus reuteri* enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. *J Cell Mol Med* **13**, 2261-2270.
- [43] Bertrand P (2009) The cornucopia of intestinal chemosensory transduction. *Front Neurosci* **3**, 48.
- [44] Thomas EA, Sjoval H, Bornstein JC (2004) Computational model of the migrating motor complex of the small intestine. *Am J Physiol Gastrointest Liver Physiol* **286**, G564-572.
- [45] Stolzenberg E, Berry D, Yang D, Lee EY, Kroemer A, Kaufman S, Wong GCL, Oppenheim JJ, Sen S, Fishbein T, Bax A, Harris B, Barbut D, Zasloff MA (2017) A Role for Neuronal Alpha-Synuclein in Gastrointestinal Immunity. *J Innate Immun*.

- [46] Ulusoy A, Phillips RJ, Helwig M, Klinkenberg M, Powley TL, Di Monte DA (2017) Brain-to-stomach transfer of alpha-synuclein via vagal preganglionic projections. *Acta Neuropathol* **133**, 381-393.
- [47] Barbut D, Stolzenberg E, Zasloff M (2019) Gastrointestinal Immunity and Alpha-Synuclein. *J Parkinsons Dis* **9**, S313-S322.
- [48] Sumioka A, Yan D, Tomita S (2010) TARP phosphorylation regulates synaptic AMPA receptors through lipid bilayers. *Neuron* **66**, 755-767.
- [49] Yeung T, Gilbert G, Shi J, Silvius J, Kapus A, Grinstein S (2008) Membrane phosphatidylserine regulates surface charge and protein localization. *Science* **319**, 210-213.
- [50] Sumioka A, Yan D, Tomita S (2009) TARP phosphorylation regulates synaptic AMPA receptors through lipid bilayers. *Neuron* **66**, 755-767.
- [51] Alexander RT, Jaumouille V, Yeung T, Furuya W, Peltekova I, Boucher A, Zasloff M, Orlowski J, Grinstein S (2011) Membrane surface charge dictates the structure and function of the epithelial Na⁺/H⁺ exchanger. *EMBO J* **30**, 679-691.
- [52] Wang B, Mao YK, Diorio C, Pasyk M, Wu RY, Bienenstock J, Kunze WA (2010) Luminal administration ex vivo of a live *Lactobacillus* species moderates mouse jejunal motility within minutes. *FASEB J* **24**, 4078-4088.
- [53] Ferens D, Baell J, Lessene G, Smith JE, Furness JB (2007) Effects of modulators of Ca(2+)-activated, intermediate-conductance potassium channels on motility of the rat small intestine, in vivo. *Neurogastroenterol Motil* **19**, 383-389.

FIGURE 1

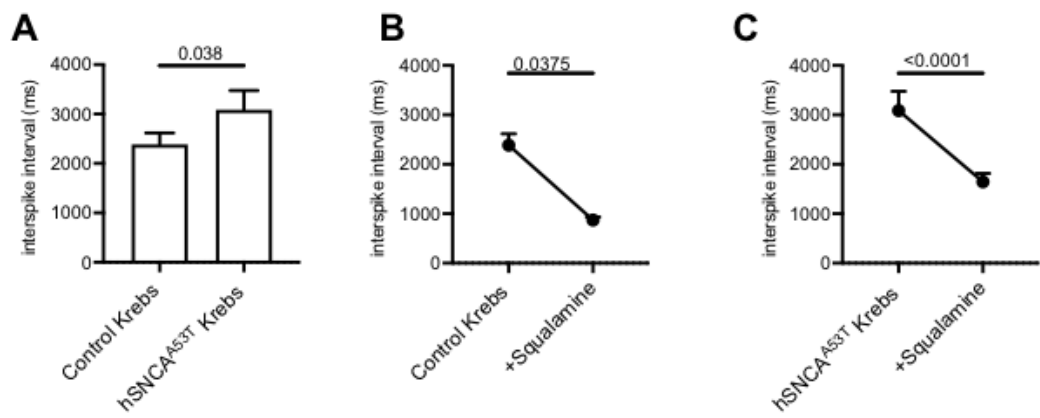


642 **FIGURE 2**



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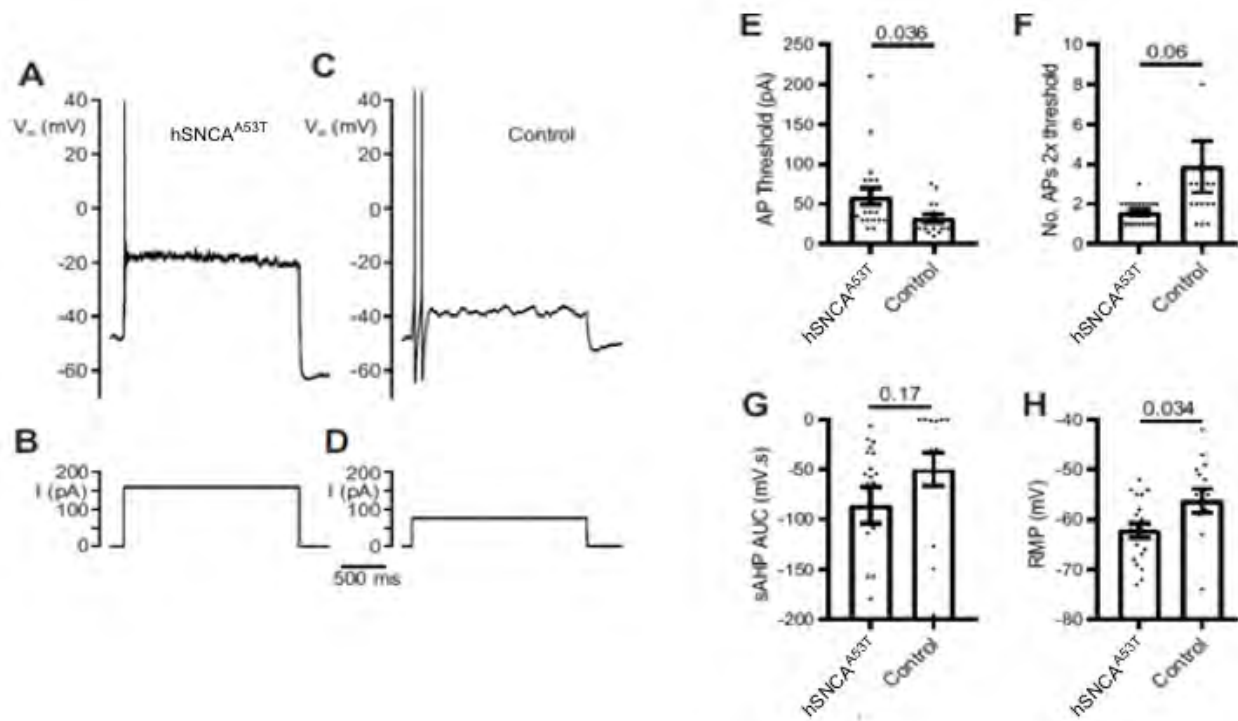
644 **FIGURE 3**



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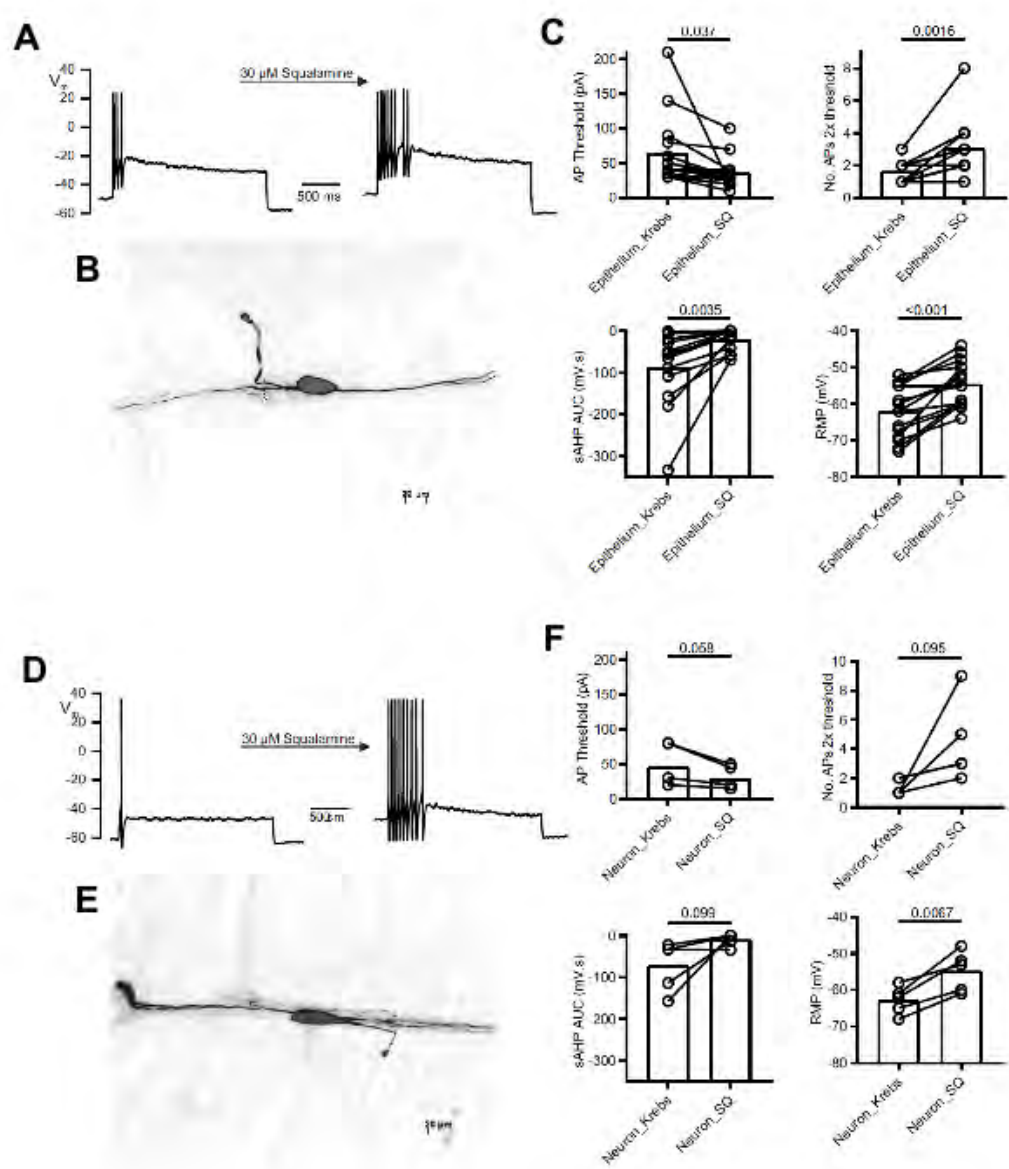


FIGURE LEGENDS

Figure 1. Colonic motor activity was increased with intraluminal squalamine in both hSNCA^{A53T} (endogenous promoter) mice and control mice (A) hSNCA^{A53T} mice did not have reduced PCC velocity compared to control mice ($P = 0.0946$). Intraluminal squalamine (30 μ M) significantly increased colonic PCC velocity in control and hSNCA^{A53T} mice ($N = 12-13$ mice/group, 2-way ANOVA with Sidak's multiple comparisons test). (B) hSNCA^{A53T} mice did not have reduced colonic frequency compared to control, nor did squalamine produce an effect on colonic frequency ($N = 12-13$ mice/group, 2-way ANOVA with Sidak's multiple comparisons test). (C) hSNCA^{A53T} mice did not have reduced colonic amplitude compared to control, nor did squalamine produce an effect on colonic amplitude ($N = 12-13$ mice/group, 2-way ANOVA with Sidak's multiple comparisons test). (D) Control mice had more uniform and consistent colonic contractions during Krebs control compared to (E) hSNCA^{A53T} mice who had infrequent shorter contractions as represented in the 3D heat maps. Intraluminal squalamine increased colonic contractions, particularly in hSNCA^{A53T} mice. Contractions (red) and relaxation (yellow-green) are depicted across time and distance along the colon. All data represented as mean $\pm$ S.E.M, * $P < 0.05$.

Figure 2. A53T (prion promoter) mice had reduced colonic motor activity compared to non-Tg control mice but improved fecal pellet output with intraluminal squalamine. (A) PrP-A53T mice (black) had a significantly reduced total number of contractions compared to non-Tg (gray) at baseline and threshold. Threshold significantly increased the total number of contractions in both non-Tg and PrP-A53T mice ($N = 6-12$ mice/group, 1-way ANOVA). (B) There was no difference in PCC velocity between PrP-A53T mice (black) compared to non-Tg

(gray) at baseline and threshold. Intraluminal squalamine (30 μ M) significantly increased colonic PCC velocity in non-Tg (gray patterned) and A53T (black patterned) at baseline (N= 6-12 mice/group, 1-way ANOVA). (C) Feeding of squalamine for 5 days increased fecal pellet output in non-Tg and A53T mice at several doses. (N = 10 mice/group/dose, 2-way ANOVA). All data represented as mean $\pm$ S.E.M, * P < 0.05.

Figure 3. Intraluminal squalamine significantly increased vagal afferent firing (reduced interspike interval) in both control and hSNCA^{A53T} mice. (A) The interspike interval was significantly longer in hSNCA^{A53T} mice than control mice (N= 13-14 mice/group). (B) Intraluminal squalamine (30 μ M) significantly reduced the vagal afferent interspike interval for control mice (N = 13 mice). (C) Intraluminal squalamine (30 μ M) significantly reduced the vagal afferent interspike interval for hSNCA^{A53T} mice (N = 14 mice). 1-way ANOVA, Holm-Sidak's multiple comparisons test. All data represented as mean $\pm$ S.E.M, * P < 0.05.

Figure 4. hSNCA^{A53T} (endogenous promoter) mice had decreased intrinsic excitability of myenteric intrinsic primary afferent neurons compared to control mice. (A) Representative action potential firing response to injected depolarizing square wave current stimulus (B) of 2x threshold intensity (hSNCA^{A53T}). (C) Representative action potential firing response to current stimulus (D) of 2x threshold intensity (control). (E-H) Probabilities under the null hypothesis of no difference given above dot plots, mean values given by open bars, error bars represent SEM. (E) The threshold intracellular current (AP threshold) required to evoke a single action potential (AP) was larger for hSNCA^{A53T} (N = 20 recordings) than for control mice (N = 16 recordings) (t = 2.2, t-test unpaired 2-tailed). (F) The number of action potentials evoked at 2 times threshold current intensity (No. APs 2x threshold) was greater for control (N = 19 recordings) than for hSNCA^{A53T} mice (N = 16 recordings) (t = 1.9, t-test unpaired 2-tailed). (G) The slow action

potential after hyperpolarisation area under the curve (sAHP AUC) showed little difference between hSNCA^{A53T} (N = 19) and control mice (N = 14) (t = 1.4, t-test unpaired 2-tailed). **(H)**

The resting membrane potential was more hyperpolarised for hSNCA^{A53T} (N = 20) than for control mice (N = 16 recordings) (t = 2.2, t-test unpaired 2-tailed).

Figure 5. Application of squalamine onto the intestinal epithelium or directly onto the exposed myenteric plexus increased excitability of intrinsic primary afferent neurons

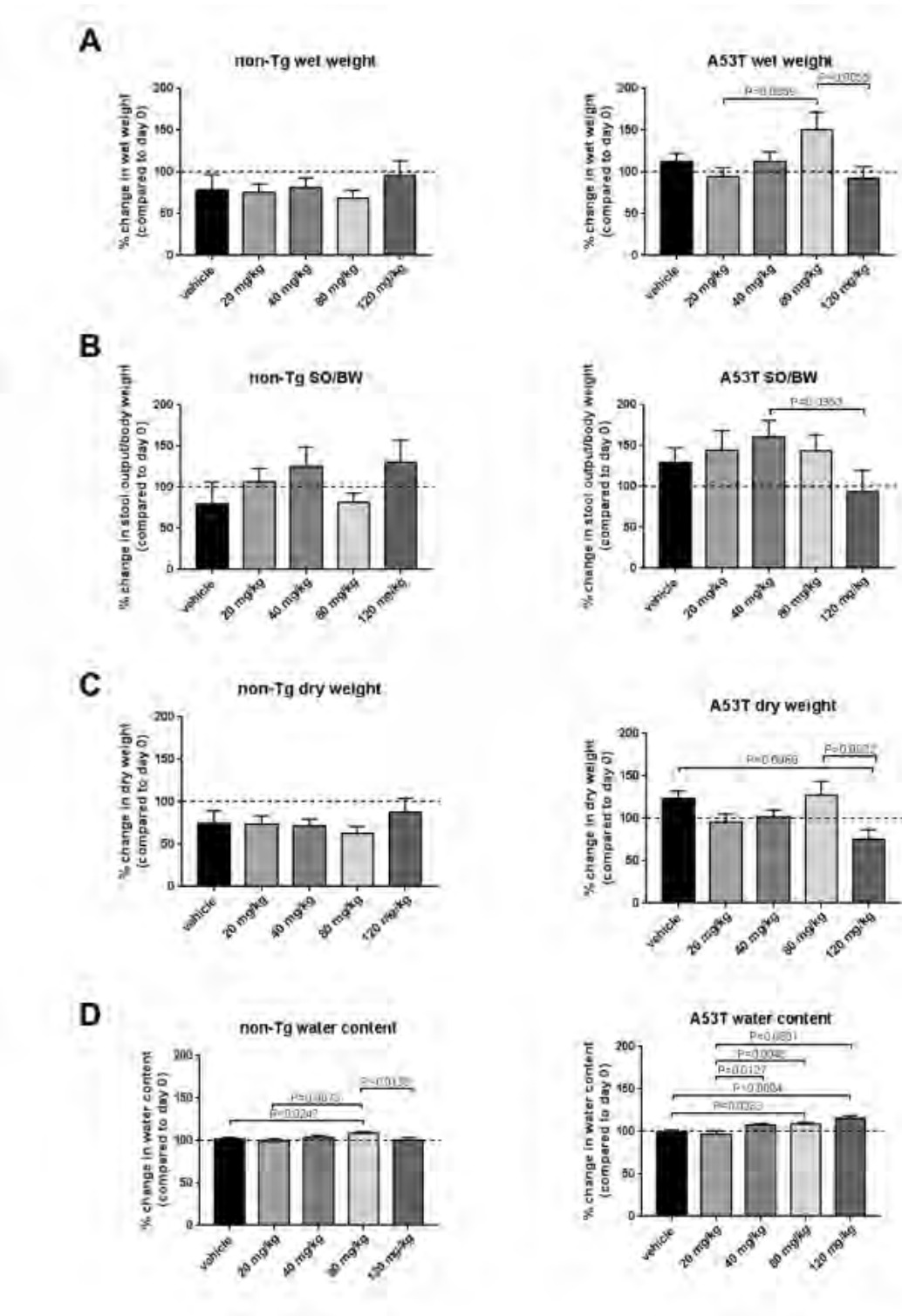
(IPANs) in hSNCA^{A53T} mice. **(A)** Representative action potential firing increase to injected square wave current stimulus after acute application of 30 μ M squalamine onto the *intestinal epithelium* using the divided hemidissection preparation. **(B)** Texas Red fluorescence image of neuron recorded from in **(A)** after tissue fixation revealing flattened oval soma and circumferentially directed neurites (Dogiel type II morphology) characteristic of chemosensitive myenteric intrinsic primary afferent neurons. **(C)** Addition of squalamine to the epithelial layer decreased the action potential firing threshold (AP Threshold) (t = 2.3, t-test paired 2-tailed), increased the number of action potentials discharged (No. AP 2x threshold) (N = 15 recordings, t = 4, t-test paired 2-tailed), decreased the area under the curve for the post action potential slow after hyperpolarisation (sAHP AUC) (N = 14 recordings, t = 3.6, t-test paired 2-tailed) and depolarised the neuron membrane potential (N = 15 recordings, t = 5.9, t-test paired 2-tailed). **(D)** A representative action potential firing increase in response to an injected square wave current stimulus after application of 30 μ M squalamine onto the myenteric plexus. **(E)** Texas Red fluorescence image of neuron recorded from in **(D)** reveals Dogiel type II morphology. **(F)** Addition of squalamine to the myenteric plexus decreased AP threshold (N = 5 recordings, t = 2.6, t-test paired 2-tailed), increased No. AP 2x threshold (N = 5 recordings, t = 2.2, t-test paired 2-tailed) decreased the post action potential sAHP AUC (N = 5 recordings, t = 2.1, t-test paired 2-

tailed), and depolarised resting membrane potential (N = 5 recordings, $t = 5.2$, t-test paired 2-tailed). (C & F) Probabilities under the null hypothesis of no difference given above individual value barbell plots mean values given by open bars, error bars represent SEM.

SUPPLEMENTARY FIGURES

Figure S1. Effect of increasing doses of squalamine (ENT-01) on stool wet and dry weight in non-Tg and PrP-A53T mice. (A) Squalamine did not affect stool wet weight in the non-Tg or A53T mice when the % change in wet weight between day 5 and 0 was compared to vehicle-treated mice. High dose squalamine (80 mg/kg) increased wet weight compared to 20 mg/kg and decreased between 80 and 120 mg/kg in A53T mice, but not compared to vehicle. (B) Squalamine had no effect on dry stool weight in non-Tg mice when the % change in dry weight between day 5 and 0 was compared to the vehicle-treated mice. Dry stool weight was decreased at 120 mg/kg in the A53T mice compared to vehicle-treated and 80 mg/kg treated mice. (C) Squalamine increased % water content between day 5 and 0 at 80mg/kg in non-Tg mice when compared to vehicle-treated mice, but this effect was not seen at 120mg/kg. Squalamine increased % water content at 80 and 120 mg/kg in A53T mice when compared to vehicle-treated mice. (D) Squalamine had no effect on the % change in stool output/body weight between day 5 and 0 in non-Tg or A53T mice compared to vehicle-treated mice. (N = 10 mice/group/dose, 1-way ANOVA). All data represented as mean $\pm$ S.E.M, $*P < 0.05$.

741 **FIGURE S1**



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