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ORIGINAL ARTICLE

Non-motor parkinsonian pathology in aging A53T α-Synuclein mice is associated with progressive synucleinopathy and altered enzymatic function

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Abstract

Aging, the main risk factor for Parkinson's disease (PD), is associated with increased α–synuclein levels in substantia nigra pars compacta (SNc). Excess α-synuclein spurs Lewy-like pathology and dysregulates the activity of protein phosphatase 2A (PP2A). PP2A dephosphorylates many neuroproteins, including the catecholamine rate-limiting enzyme, tyrosine hydroxylase (TH). A loss of nigral dopaminergic neurons induces PD movement problems, but before those abnormalities occur, behaviors such as olfactory loss, anxiety, and constipation often manifest. Identifying mouse models with early PD behavioral changes could provide a model in which to test emerging therapeutic compounds. To this end, we evaluated mice expressing A53T mutant human (A53T) α–synuclein for behavior and α–synuclein pathology in olfactory bulb, adrenal gland, and gut. Aging A53T mice exhibited olfactory loss and anxiety that paralleled olfactory and adrenal α-synuclein aggregation. PP2A activity was also diminished in olfactory and adrenal tissues harboring insoluble α-synuclein. Low adrenal PP2A activity co-occurred with TH hyperactivity, making this the first study to link adrenal synucleinopathy to anxiety and catecholamine dysregulation. Aggregated A53T α–synuclein recombinant protein also had impaired stimulatory effects on soluble recombinant PP2A. Collectively, the data identify an excellent model in which to screen compounds for their ability to block the spread of α-synuclein pathology associated with pre-motor stages of PD.

Keywords: enzymatic dysregulation, loss of a-Syn function, synucleinopathy.

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α-Synuclein (a-Syn) is a widely expressed pre-synaptic protein (Maroteaux *et al.* 1988). a-Syn gene mutations and multiplications cause early onset Parkinson's disease (PD) (Polymeropoulos *et al.* 1997; Kruger *et al.* 1998; Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004; Zarranz *et al.* 2004), however, most PD is sporadic and linked to aging. Increases in a-Syn protein are noted in aging human Substantia nigra pars compacta (SNc) (Li *et al.* 2004; Chu and Kordower 2007), which can stimulate neuroinflammation and microglial activation

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Abbreviations used: A53T, mice expressing A53T mutant human a-Syn; a-Syn, α-Synuclein; LMMP, longitudinal muscle of the myenteric plexus; Non-Tg, non-transgenic littermate mice expressing mouse a-Syn; OB, olfactory bulb; PD, Parkinson's disease; PK, proteinase K; PP2A, protein phosphatase 2A; PSer129, phospho-serine 129 on a-Syn; PSer19, phospho-serine 19 on TH; PSer40, phospho-serine 40 on TH; PTyr307, phospho-tyrosine-307 on PP2A; Thio-S, thioflavin-S; TH, tyrosine hydroxylase.

(Croisier *et al.* 2005), leading to Lewy body formation. Families expressing A53T mutant a-Syn (A53T) develop early onset PD and Lewy bodies with highly phosphorylated a-Syn (Anderson *et al.* 2006). Cumulatively, these data suggest that having too much a-Syn protein in neurons is problematic.

PD motor symptoms emerge after extensive loss of SNc dopaminergic neurons (Bernheimer *et al.* 1973). Yet, non-motor symptoms precede motor onset by years, during a pre-motor phase of PD. Constipation is associated with low gut motility (Ashraf *et al.* 1997), dopaminergic defects (Singaram *et al.* 1995), and a-Syn accumulation in colonic neurons (Shannon *et al.* 2012). An impaired sense of smell affects many PD patients (Bohnen *et al.* 2007) and anosmia and hyposmia are common initial symptoms of pre-motor PD (Haehner *et al.* 2011). Olfactory impairment correlates with olfactory bulb (OB) Lewy body pathology (Beach *et al.* 2009), which occurs early in the course of PD (Braak *et al.* 2004). These findings suggest that gastrointestinal or OB assessment for biomarkers, coupled with behavioral tests could identify PD at a time when SNc remains intact (Doty *et al.* 1995; Savica *et al.* 2009).

Anxiety and depression (Dooneief *et al.* 1992) can also precede PD motor symptoms (Lauterbach and Duvoisin 1991; Shiba *et al.* 2000; Weisskopf *et al.* 2003), suggesting a neurochemical basis. Indeed, anxiety is associated with elevated adrenal catecholamines (Kvetnansky and Mikulaj 1970), as well as hyperactivity of tyrosine hydroxylase (TH; EC 1.14.16.2) in adrenal gland (Chobotska *et al.* 1998). Measuring behavior in combination with sensitive bioassays (Bidinosti *et al.* 2012) may help identify pre-motor PD cases.

Although a-Syn is implicated in PD neuropathology, it also contributes to normal physiology by interacting with key regulatory proteins in a chaperone-like manner (Perez and Hastings 2004; Sidhu *et al.* 2004; Geng *et al.* 2011). The catalytic subunit of protein phosphatase 2A (PP2A; EC 3.1.3.16) interacts with and is stimulated by soluble a-Syn *in vitro* and *in vivo* (Peng *et al.* 2005; Lou *et al.* 2010). Another enzyme that a-Syn modulates is TH which is inhibited (Perez *et al.* 2002; Peng *et al.* 2005; Lou *et al.* 2010). Too much or too little soluble a-Syn (Lou *et al.* 2010) or loss of soluble a-Syn by its aggregation, contributes to dysregulated TH and PP2A activity in brain (Alerte *et al.* 2008; Wu *et al.* 2012). However, whether a-Syn aggregation might affect TH or PP2A in the PNS is unknown.

In the current studies we assessed movement, olfaction, anxiety, gut pathology, and synucleinopathy in aging A53T homozygous mice and their non-transgenic (Non-Tg) littermates. We also measured phosphorylation of a-Syn serine 129 (PSer129), a modification abundant in Lewy bodies/Lewy neurites (Fujiwara *et al.* 2002); PP2A tyrosine 307 phosphorylation (PTyr307), a marker of low PP2A activity (Chen *et al.* 1992); and TH serine 40 (PSer40) phosphorylation, a marker of high TH activity by immunoblot and immunohistochemistry. In addition, we measured PP2A and TH activity in olfactory and adrenal homogenates to compare to behavioral data. Collectively, the results identify an excellent model for screening therapeutic compounds for efficacy for PD.

Methods

Mice

A53T a-Syn (B6; C3-Tg-Prnp/SNCA*A53T/83Vle/J) heterozygous breeders (Jackson Laboratories, Bar Harbor, ME, USA), produced our cohort of A53T homozygous (n = 7) and Non-Tg littermates (n = 5). Genotyped mice (SeqWright-DNA-Technology, Houston, TX, USA) were housed in temperature and humidity-controlled rooms on 12-h light–dark cycles, with food and water *ad libitum*, except during olfactory testing (see below). Ethical treatment of animals followed AALAC, DLAR, ARRIVE, and NIH Animal Care Guidelines on IACUC approved protocols at the University of Pittsburgh.

Behavioral assessment

All tests were performed in clean quiet test rooms at the University of Pittsburgh BST Rodent Behavioral Core.

Movement

Open field tests (12–14 month old mice) utilized TruScan™ activity monitors (Coulbourn Instruments, Whitehall, PA, USA). Mice acclimated 5–15 min in test rooms prior to placement in the center, then floor plane velocity (cm/s) and distance (cm) were monitored for 15 min using established methods (Roy *et al.* 2006; Hunt *et al.* 2013). *Anxiety Test 1* - The open field arena is 26.67 cm^2, with the margin being the area within 3.81 cm from the wall, and the center > 3.81 cm. Anxious mice have longer margin times (Crawley 1985), as demonstrated by margin/center time ratios (Pinna *et al.* 1997). Mice were tested in random order on three independent occasions.

Food pre-training for olfactory testing

Mice (12–14 month of age) were trained individually to eat the food pellets (~ 250 mg Cap'n Crunch Cereal; Quaker Oats Co., Chicago, IL, USA) placed on top of the bedding in the home cage. Training continued until all mice ate pellets in < 30 s. *Anxiety Test 2* - Mice receiving familiar palatable food in a novel environment explore it extensively before eating, a behavior termed *hyponeophagia* (Merali *et al.* 2003). Mice, placed individually into clean cages for 5 min, had familiar palatable food placed on top of bedding while latency to approach, sniff, and begin eating the pellet was recorded in seconds.

Olfactory test

Once mice ate food in a novel environment in < 30 s, buried pellet testing commenced (Nathan *et al.* 2004; Fleming *et al.* 2008; Lu *et al.* 2008). Testing began when mice were 13–15 months old (Session 1). Mice were retested at 14–16 months of age (Session 2), and again at 15–17 months (Session 3). To ensure motivation, food was removed 14–18 h prior to testing (Fleming *et al.* 2008), but weight loss never exceeded 10%. Room lighting was identical for each test, and mice were placed individually in clean holding cages for 5 min, then transferred individually to test cages for 2 min acclimation, then returned to the holding cage while a pellet was

buried ~ 0.5 cm below the bedding in a random location to eliminate a learning component. After food placement, each mouse was placed in the center of the test cage and given 5 min to find the pellet while latency to sniff, dig up, and begin eating food was recorded using LimeLight™ (Coulbourn Instruments, Whitehall, PA, USA). Test cages were cleaned and fresh bedding applied before additional mice were tested.

Tissue collection/handling

Mice were killed by CO2 inhalation and decapitation. ***Brains -*** Brains with OBs were rapidly extracted and bisected longitudinally, freezing half for biochemistry and post-fixing half for immunohistochemistry. ***Adrenal -*** Adrenal glands were collected as previously described (Kolski-Andreaco *et al.* 2007), flash frozen, and stored at −80°C until analyzed. Tissues were homogenized in buffer that allowed measuring TH and PP2A activity from each sample. This buffer contained 52.5 mM HEPES, 1.05 mM AEBSF, 5.25 μg/mL aprotinin, 2.25 μg/mL leupeptin, 1.05 mM benzamidine, 10.5% glycerol. Adrenals were homogenized using Bullet Blender® (Next Advance Inc., Averill Park, NY) at speed 9, 4 min, 4°C. After homogenization, supernatants were split for TH and PP2A assays. Pellets were re-extracted in 50 mM Tris, 150 mM NaCl, 0.1% Triton-X 100, 0.2% sodium dodecyl sulfate for immunoblots. ***Gut -*** A dissecting microscope was used to prepare small intestine longitudinal muscle with myenteric plexus whole mounts as previously described (Drolet *et al.* 2009). Segments (5 cm) were isolated, bisected longitudinally and pinned flat in sylgard-coated dishes with mucosa facing down. Similar regions from each mouse were post-fixed overnight in 4% formaldehyde/sucrose.

Immunohistochemistry

Brains

Unperfused brains were sectioned sagittally (30 μm) and handled as previously described (Alerte *et al.* 2008; Lou *et al.* 2010).

Antibodies/Thioflavin

TH (Aves Labs; MAB318, Millipore, Billerica, MA, USA), phospho-serine 19 on TH (AB5425, Chemicon), a-Syn (AB5334P, Chemicon; sc-7011-R, Santa Cruz, Santa Cruz, CA, USA), PP2A (sc-6110, Santa Cruz), PTyr307 PP2A (sc-12615, Santa Cruz). a-Syn aggregation was assessed with 1% Thioflavin-S (Thio-S, T1829, Sigma-Aldrich) on sections first immunolabeled for a-Syn and TH. As Thio-S signal is green, secondary antibodies for double labeling a-Syn and TH were Cy-5 and Cy-3, respectively. a-Syn Cy-5 signal was pseudocolored red on Thio-S stained sections to permit demonstrating a yellow co-localization signal.

Gut

Longitudinal muscle with myenteric plexus tissues were immunolabeled as previously described (Drolet *et al.* 2009). ***Quantification of Gut a-Syn -*** Optical densities of 10 fields/sample/mouse were generated using ImageQuant, (GE Healthcare, Waukesha, WI, USA). Quantitative analyses used baseline correction to normalize data by measuring the lightest-stained 15 nm^2 region of each tissue, set as background. That value was subtracted from the signal for all other regions.

Immunoblots

Proteins (10–20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated in primary antibody overnight, 4°C. a-Syn antibodies were sc-7011-R (Santa Cruz); Syn-1 (610787, BD Biosciences, San Jose, CA, USA), and a-Syn PSer129 (11A5, from John Anderson, Elan Pharmaceuticals). PP2Ac antibodies were 1D6 (05-421, Millipore/Upstate, EMD Millipore, Billerica, MA, USA) and sc-6110 (Santa Cruz), with β-actin (A5441, Sigma-Aldrich) used as a loading control. Blots were imaged using LiCor Odyssey (Lincoln, NE, USA).

PP2A assay

Brain

Tissues were homogenized in imidazole buffer [20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA plus protease inhibitors], 4°C, centrifuged to remove particulates followed by free phosphate removal with MicrospinTM-G-25 columns (GE Healthcare). Supernatants were incubated in 4-Nitrophenyl-phosphate (pNPP) buffer [50 mM Tris-HCl, pH 7.0, 0.1 mM $CaCl_2$] with KRpTIRR substrate, 10 min, 30°C. Samples were assayed at 650 nm relative to fresh standards by malachite green assay (17-127; Millipore/Upstate). Inhibitors for specificity included: 50 nM protein-phosphatase-inhibitor 2 (PP1-specific), 3 nM fostriecin (PP2A/PP4-specific), 50 nM cantharidin (PP4-specific), and 10 nM okadaic acid as previously described (Peng *et al.* 2005; Lou *et al.* 2010; Wu *et al.* 2012).

Adrenal gland

Tissues were homogenized in simple buffer as described above with samples supplemented to [0.1 mM CaCl2].

Recombinant protein

a-Syn (gift of Dr. John Rosenberg, University of Pittsburgh) was incubated with PP2A (Cayman Chemicals, Ann Arbor, MI, USA; or Millipore), 4°C for 30 min in pNPP buffer then with substrate, 30°C, 10 min. Duplicate Samples were assayed in three independent experiments.

Tyrosine hydroxylase assay

TH activity utilized a colorimetric assay (Daubner and Fitzpatrick 1993). Adrenal tissue prepared in simple buffer was supplemented to [25 mM EDTA]. Samples were evaluated as previously described (Wang *et al.* 2009; Lou *et al.* 2010) spectrophotometrically at 490 nm (UV-1800, Shimadzu, Columbia, MD).

Recombinant A53T a-Syn aggregation

Protein was aggregated using established protocols (Giasson *et al.* 1999) which produces insoluble a-Syn pellets (P) and soluble a-Syn supernatants (S) exactly as previously described (Wu *et al.* 2012).

Statistics

Data were transformed to log scale to induce normality (for distance, velocity, and latency to find food). Group effects (Non-Tg and A53T), time effects, and group x time interaction effects were evaluated by parametric repeated measures analysis of variance

(RM-ANOVA) with post hoc Bonferroni analysis. Data were also evaluated by non-parametric Friedman tests. Both RM-ANOVA and Friedman tests utilized SPSS (v20, IBM, New York, NY), *t*-test and one-way ANOVA utilized InStat (Graph Pad, San Diego, CA, USA). Data represent the mean ± SEM, with significance set to $p < 0.05$.

Results

Aging A53T mice exhibit hyperactivity and anxiety

Homozygous A53T mice are a good model of synucleinopathy, but do not develop SNc-associated motor complications (Giasson *et al.* 2002; Lee *et al.* 2002; Tanji *et al.* 2010; Tsika *et al.* 2010). As movement is important for olfactory testing of mice, we confirmed their ability to move in an open field. The average distance moved in 15 min was 2323.9 ± 158.1 cm for A53T and 2016.5 ± 209.2 cm for wild type Non-Tg mice; demonstrating that A53T mice moved quite well. Data were log transformed to induce normality, adjusted for time × interaction effects and assessed by repeated measures analysis of variance (RM-ANOVA). This revealed that A53T mice moved greater distances than Non-Tg littermates (A53T, 6.29 ± 1.53; Non-Tg, 5.74 ± 2.03; $p = 0.009$; RM-ANOVA). The movement velocity of A53T mice was 7.6 ± 0.53 cm/s and for Non-Tg mice 6.6 ± 0.69 cm/s; when evaluated by RM-ANOVA of log transformed data revealed significant differences (A53T, 0.55 ± 1.53; Non-Tg, 0.22 ± 1.79; $p = 0.031$). Thus, A53T mice were hyperactive. Hyperactivity has been reported for another strain of A53T a-Syn mice (Unger *et al.* 2006), raising the possibility that A53T mutant a-Syn expression may cause anxiety.

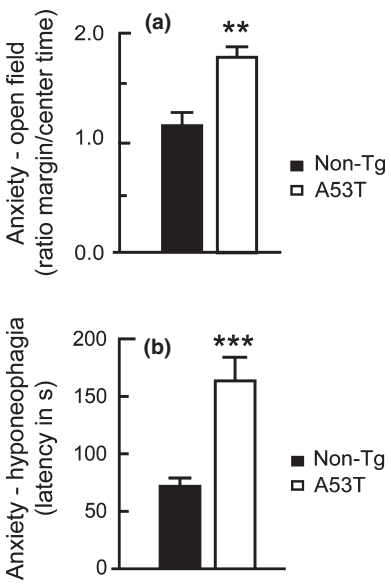

Fig. 1 Anxiety is present in mice expressing A53T mutant human a-Syn (A53T). (a) A53T mice spent significantly more time in the margin than in the center of an open field. (b) Hyponeophagia (see Methods for details) was significantly greater in A53T mice compared to non-transgenic littermate mice expressing only mouse a-Syn (Non-Tg) littermates. ***$p < 0.001$, **$p < 0.03$.

To assess this we used established methods in which the amount of time mice spent in the margin versus center of an open field during 15 min trials was recorded. A53T a-Syn mice spent more time in the margin than in the center (Fig. 1a). Anxiety was also assessed by comparing the latency to approach and begin eating a familiar palatable food, placed on top of the bedding in a novel environment, a measure of anxiety called hyponeophagia. Mice quickly ate the familiar palatable in their home cages, but A53T mice were significantly slower to approach and taste the food in a new cage, further demonstrating anxiety (Fig. 1b, A53T, 137.1 ± 23.4 s; Non-Tg, 55.2 ± 14.8 sec; $p = 0.03$, *t*-test).

Anxiety is associated with catecholamine synthesis (Leduc 1961; Kvetnansky and Mikulaj 1970; Anisman and Zacharko 1986), and stress increases adrenal catecholamine levels (LeBlanc and Ducharme 2007). Furthermore, Lewy bodies occur in PD adrenal medulla (Fumimura *et al.* 2007; Wakabayashi *et al.* 2010). Therefore, we next assessed adrenal a–Syn solubility, and TH and PP2A activity as described below.

Aging A53T adrenal glands have synucleinopathy and dysregulated enzymatic activity

Soluble a-Syn normally regulates TH and PP2A activity in opposite directions (Perez *et al.* 2002; Peng *et al.* 2005), while loss of soluble a-Syn by its aggregation, impairs PP2A activity in human brain (Wu *et al.* 2012). However, no one has evaluated the impact of a-Syn aggregation in adrenal gland in any model. We measured a-Syn levels in soluble adrenal extracts and found low amounts of monomeric a-Syn in Non-Tg mice, and as expected, higher levels of a-Syn monomers in A53T adrenal tissue (data not shown). When we re-extracted pellets, we noted high and low molecular weight insoluble a-Syn species in A53T adrenal but little signal in Non-Tg adrenal homogenates (Fig. 2a). a-Syn oligomers quantified by densitometry, were significantly elevated in A53T mice compared to Non-Tg littermates, demonstrating synucleinopathy in aging A53T mouse adrenal gland (Fig. 2b; $p < 0.001$, *t*-test).

Phosphorylated TH is the active form of the protein (Kumer and Vrana 1996) and adrenal catecholamine levels are significantly elevated in anxious animals (Kvetnansky and Mikulaj 1970). We therefore measured adrenal TH activity first indirectly by quantifying serine 40 phosphorylation (PSer40) levels by immunoblot (Bobrovskaya *et al.* 2007), and then directly using established methods (Lou *et al.* 2010). Aging A53T adrenal gland had 27% higher TH PSer40 levels compared to Non-Tg adrenal (Fig. 2c, $p < 0.001$, *t*-test), a finding that paralleled significantly higher TH activity in A53T adrenal homogenates (Fig. 2d; $p < 0.01$, *t*-test).

Dephosphorylation of TH PSer40 is mediated by PP2A (Haavik *et al.* 1989; Kumer and Vrana 1996), therefore, we

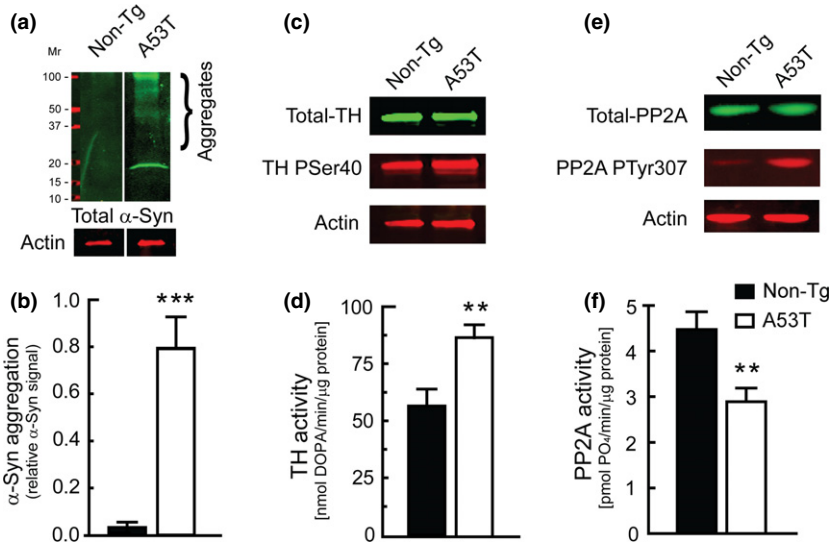

Fig. 2 Adrenal gland a-Syn aggregates contribute to TH and PP2A dysregulation in mice expressing A53T mutant human a-Syn (A53T). Adrenal homogenates from non-transgenic littermate mice expressing mouse a-Syn (Non-Tg) and A53T a-Syn homozygous littermates were evaluated by immunoblot and enzymatic assays. (a) a-Syn oligomers are evident in A53T adrenal, while Non-Tg adrenal contains little a-Syn signal in the pellet fraction. (b) Histogram demonstrating total a-Syn in Non-Tg and A53T adrenal immunoblots. (c) Total TH signal was similar in Non-Tg and A53T mice; however, PSer40 TH signal was stronger in A53T mice, consistent with elevated TH activity. (d) TH activity was significantly greater in A53T compared to Non-Tg mouse adrenal. (e) Total PP2A levels were similar in Non-Tg and A53T immunoblots, but PP2A PTyr307 signal, which correlates with low PP2A activity, was increased only in A53T adrenal. (f) PP2A activity was significantly lower in A53T adrenal gland. Actin was used as a loading control. Data represent duplicate samples from three independent experiments. ***$p < 0.001$, **$p < 0.01$.

also measured PP2A activity, first indirectly by quantifying PP2A PTyr307, a marker of less active PP2A by immunoblot; and then directly by PP2A assay (Peng *et al.* 2005; Lou *et al.* 2010). A53T adrenal PP2A had 24% more PTyr307 than Non-Tg adrenal (Fig. 2e, $p < 0.001$, *t*-test). This paralleled significantly less PP2A activity in A53T compared to Non-Tg adrenal (Fig. 2f; $p < 0.01$, *t*-test), further suggesting that a-Syn-mediated-stimulation of PP2A activity becomes impaired when a-Syn aggregates. This is the first demonstration that adrenal a-Syn pathology dysregulates TH and PP2A in a manner to induce anxiety in aging A53T mice.

Aging A53T mouse myenteric neurons accumulate aggregated a-Syn

Constipation is a common complaint occurring long before motor onset in PD. Animals with gastrointestinal slowing

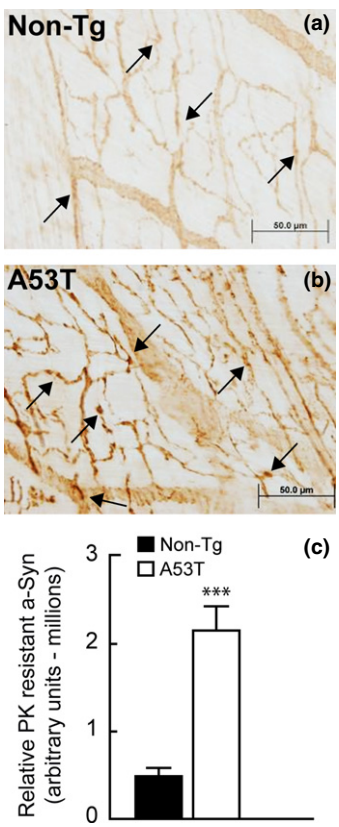

Fig. 3 a-Syn aggregation is abundant in the small intestine of aging mice expressing A53T mutant human a-Syn (A53T). Proteinase K (PK) treated longitudinal muscle with myenteric plexus (LMMP) gut tissue from non-transgenic littermate mice expressing mouse a-Syn (Non-Tg) and A53T mice reveal aggregated a-Syn. (a) Myenteric neurons of Non-Tg mice contain little PK-resistant a-Syn (arrows). (b) Myenteric neurons in A53T mice contain abundant darkly stained PK-resistant a-Syn (arrows). (c) Quantification confirms significantly more a-Syn aggregation in myenteric neurons of A53T mice compared to their Non-Tg littermates. Size bars = 50.0 μm. ***$p < 0.001$.

consistent with constipation, develop a-Syn aggregates in enteric neurons that innervate the gut (Drolet *et al.* 2009). Although gut motility was not evaluated in our mice, we used established methods to assess a-Syn aggregation in their small intestines. Proteinase K (PK) treatment removes soluble, while leaving insoluble proteins. When PK treated gut tissues were evaluated immunohistochemically, only low levels of PK resistant a-Syn were present in Non-Tg myenteric neurons (Fig. 3a, at arrows), in stark contrast to high level PK resistant a-Syn in A53T myenteric neurons (Fig. 3b). Quantitative analyses, with baseline correction, revealed significantly more PK resistant a-Syn in aging A53T mouse myenteric neurons compared to Non-Tg mice (Fig. 3c; A53T, 2.15 ± 0.15 M; Non-Tg, 0.49 ± 0.14 M; $p < 0.001$, *t*-test), nearly identical to data from rotenone treated rats with low gut motility (Drolet *et al.* 2009).

Aging A53T mice develop progressive olfactory loss

Olfaction was assessed using a buried pellet test (Fleming *et al.* 2008), performed during three sessions over 8 weeks. Results, evaluated by repeated measures analyses on log transformed data (Fig. 4), confirmed interaction effects between groups and sessions. The group effect was examined at each session using unpaired *t*-tests as well as by Wilcoxon rank sum tests after Bonferroni correction. In Session 1, when mice were 13–15 months old, both groups found the buried pellet with similar latencies (A53T, 4.45 ± 0.65; Non-Tg, 4.01 ± 0.78; $p = 0.412$, Wilcoxon rank sum test). By Session 2, when mice were 14–16 months old, A53T a-Syn mice were significantly slower at finding food (A53T, 5.10 ± 0.44; Non-Tg, 3.74 ± 0.74; $p = 0.024$, Wilcoxon rank sum test). In Session 3 when mice were 15–17 months old, olfaction remained significantly impaired in A53T mice (A53T, 5.17 ± 0.42, Non-Tg, 4.08 ± 0.63; $p = 0.012$, Wilcoxon rank sum test). Prior studies, using mice that express wild-type human a-Syn, showed OB a-Syn aggregates with a parallel loss of olfaction (Fleming *et al.* 2008), but the impact of synucleinopathy on OB PP2A has never been assessed.

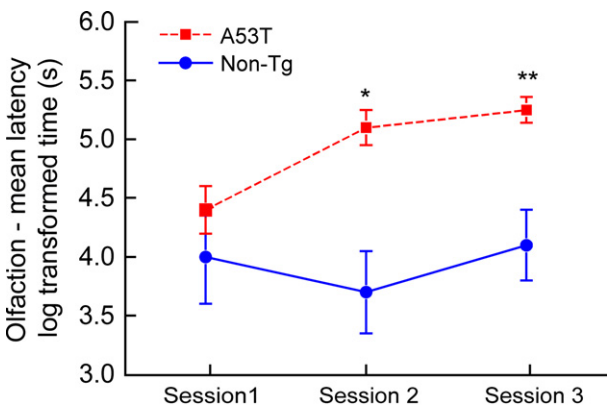

Fig. 4 Mice expressing A53T mutant human a-Syn (A53T) develop progressive olfactory impairment. A53T homozygous a-Syn mice showed age onset olfactory loss. For each test, experimenters placed familiar palatable food in a random location ~ 0.5 cm under the bedding, before putting the food-restricted mouse in the center of the cage. Latency to find, dig up, and begin eating the food was recorded in 5 min trials for each mouse independently. Homozygous A53T a-Syn mice had loss of olfaction over time, while non-transgenic littermate mice expressing mouse a-Syn (Non-Tg) littermates were unaffected. *$p < 0.05$, **$p = 0.012$.

Aging A53T olfactory bulb with a-Syn aggregation has impaired PP2A activity

Highly phosphorylated a-Syn Ser129 is present in Lewy bodies (Saito *et al.* 2003; Anderson *et al.* 2006), and Lewy bodies are common in OB of individuals with PD (Braak *et al.* 2004). To determine if our aging A53T mice that developed progressive olfactory loss, had Lewy-like pathology in OB, we used Thio-S staining and a-Syn immunohistochemistry. Non-specific Thio-S signal was noted in blood vessels of our unperfused mice, similar to what was reported by others using unperfused animals (Sun *et al.* 2002). The non-specific labeling appeared as linear groupings of less intense Thio-S signal in both A53T and Non-Tg OB and likely represents staining of red blood cells. In contrast, large irregularly shaped Thio-S stained aggregates were found in A53T OB (Fig. 5a, bottom left panel), while Non-Tg Thio-S signal appeared at background levels (Fig. 5a, top left panel). In A53T OB, Thio-S and a-Syn signals overlapped (Fig. 5a, bottom middle panel), as can be appreciated in the yellow merged image (Fig. 5a, bottom right) confirming Lewy-like a-Syn aggregation in aging A53T OB. Non-Tg OB had uniform a-Syn signal (Fig. 5a, top middle panel) that did not overlap Thio-S (Fig. 5a, top right panel). Lewy-like phosphorylated Ser129 (PSer129) a-Syn immunoreactivity was also strong in a-Syn stained profiles in A53T OB (Fig. 5b, bottom panels). When quantified in FluoView (Olympus America Inc., Center Valley, PA, USA), PSer129 signal intensity was significantly greater in A53T compared to Non-Tg littermate OB ($p < 0.0001$, *t*-test). Similar total PP2A immunoreactivity was apparent in A53T a-Syn and Non-Tg OB (Fig. 5c, left panels), yet PP2A phosphorylated on Tyr307 which labels less active PP2A, was significantly greater in A53T OB (Fig. 5c, middle panels), as confirmed by quantitation (Fig. 5c right panels; $p < 0.001$; *t*-test). Despite equal amounts of PP2A catalytic subunit on immunoblots (Fig. 5d), PP2A activity was significantly lower in A53T OB (Fig. 5e), further demonstrating that aggregated A53T a-Syn is a poor stimulator of PP2A activity. To directly assess this, we next compared PP2A activation in response to soluble and insoluble recombinant A53T a-Syn.

Aggregated A53T a-Syn has less PP2A stimulating capacity

Soluble a-Syn interacts with PP2A catalytic subunit and stimulates its activity *in vitro* and *in vivo* (Peng *et al.* 2005;

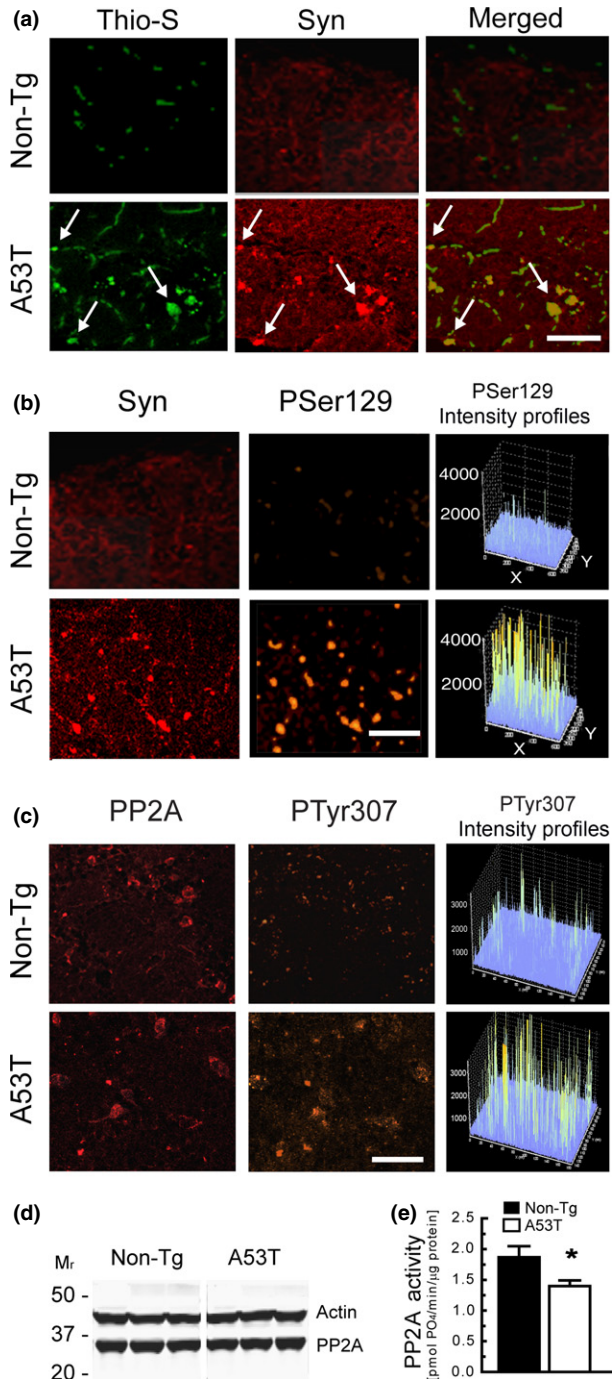

Fig. 5 Mice expressing A53T mutant human a-Syn (A53T) mice develop Lewy-like a-Syn aggregates and impaired protein phosphatase 2A (PP2A) activity in olfactory bulb. Representative olfactory bulb (OB) immunohistochemistry from aging A53T a–Syn and non-transgenic littermate mice expressing mouse a-Syn (Non-Tg) littermates. (a) a-Syn aggregation was assessed with Thioflavin-S (Thio-S) staining and a-Syn immunohistochemistry, with a-Syn Cy-5 signal pseudocolored red. Non-Tg olfactory bulb had non-specific staining of blood vessels (green Thio-S signal, upper left panel) and no evidence of a–Syn aggregation (red signal, upper middle panel), which when merged do not overlap. In contrast A53T olfactory bulbs have large Thio-S profiles (arrows, lower left panel) that colocalize with a–Syn signal (arrows, lower middle panel) and appear yellow in the merged image (arrows, lower right panel). (b) A53T OB has aggregated a-Syn (lower left panel) with highly phosphorylated Ser129 (center lower panel), as demonstrated quantitatively in intensity profiles (lower right panel). (c) OB from age-matched Non-Tg and A53T a-Syn mice contain similar total PP2A (left panels), however, A53T mice have more phosphorylated Tyr307 (lower middle panel) as demonstrated quantitatively in signal intensity profiles (lower right panel). (d) Immunoblots of Non-Tg and A53T OB tissues have equal total PP2A, with actin serving as a loading control. (e) PP2A activity is significantly reduced in A53T OB that has widespread a-Syn aggregation. Size bars = 50 µm. $*p < 0.05$.

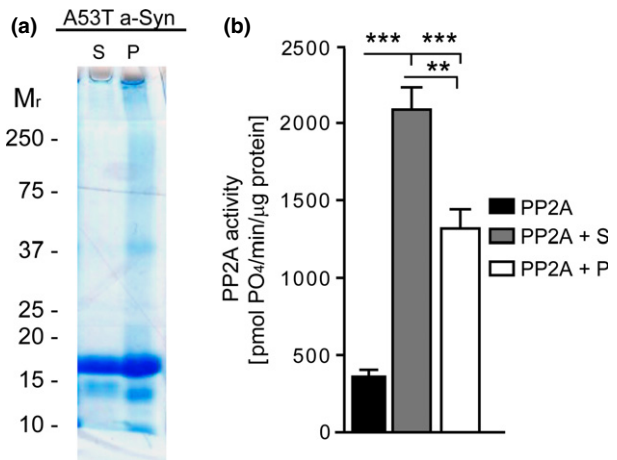

Fig. 6 Insoluble recombinant mutant human a-Syn (A53T) is less effective at stimulating PP2A activity. (a) Representative Coomassie stained gel showing recombinant mutant human A53T a-Syn in soluble (S) and insoluble pellet (P) fractions. (b) Aggregated A53T a-Syn was significantly less able to stimulate PP2A activity than soluble A53T a-Syn. Data are from duplicate samples in three independent experiments. $**p < 0.01$, $***p < 0.001$.

Lou *et al.* 2010). In contrast, aggregated a-Syn is less able to stimulate PP2A activity in human brain and *in vitro* (Wu *et al.* 2012). Here, we compared soluble and aggregated mutant human A53T recombinant a-Syn for its ability to stimulate the activity of soluble recombinant PP2A. A 19 kDa a-Syn band on Coomassie stained gels, was insoluble when present in the pellet (Fig. 6a), a fraction that contained 68% ± 3% of total A53T a-Syn protein after the aggregation step. When we assayed PP2A activity, recombinant PP2A efficiently cleaved PO_4 from the substrate at baseline, and soluble A53T a-Syn strongly stimulated PP2A. In contrast, aggregated A53T a-Syn was much less able to stimulate PP2A activity (Fig. 6b), further demonstrating that a-Syn

aggregation causes a loss of function of A53T a-Syn for PP2A stimulation, as also noted for wild type a-Syn (Wu *et al.* 2012).

Discussion

Aging is the major risk factor for PD (Collier *et al.* 2011) and is highly correlated with increasing a-Syn levels in human brain (Li *et al.* 2004; Chu and Kordower 2007). Such age-dependent increases in a-Syn likely contribute to Lewy body formation and eventually to PD motor symptoms. Before motor onset, however, constipation, olfactory impairment, and anxiety are behaviors common to the pre-motor, prodromal phase of PD (Shiba *et al.* 2000; Weisskopf *et al.* 2003; Pellicano *et al.* 2007). Anxiety also manifests after PD onset, however, that may occur primarily in response to fluctuations associated with levodopa therapy (Maricle *et al.* 1995). In this study, our data reveal a strong association between accumulation of insoluble a-Syn and pre-motor behavioral markers of PD.

Regarding mice, the original A53T M83 mice developed a motor phenotype by ~ 8 month and had no a-Syn pathology in OB (Giasson *et al.* 2002). Their rapid demise may have prevented OB a–Syn accumulation. A53T M83 mice that are now commercially available remain healthy as they age (Oaks *et al.* 2013). Using this same model, we evaluated homozygous A53T a-Syn mice for synucleinopathy and pre-motor PD behaviors with aging. Like most a-Syn transgenic mice, these mice do not model nigrostriatal damage (Blesa *et al.* 2012) and as expected, their movement was also unimpaired (Fig. 1). However, they developed age onset abnormalities in enzymatic activity that could underlie early behavioral and physiological changes of PD. These mice could thus be useful for pre-clinical evaluation of novel therapeutics for PD.

This is important because levodopa, the current major therapy for PD, does nothing to slow progression and may even contribute to pathogenesis (Simuni and Stern 1999). With society aging worldwide, there is an urgent need to find therapies that will halt PD progression. One strategy for accomplishing this is to elucidate the normal function(s) of proteins implicated in PD, especially those that affect dopaminergic/catecholaminergic physiology. For example, a-Syn contributes to dopamine handling at many levels. We discovered that soluble a-Syn controls dopamine synthesis by binding to and regulating dopamine regulatory proteins including the rate limiting enzyme, TH, the next biosynthetic enzyme, aromatic amino acid decarboxylase, and the catalytic subunit of PP2A. PP2A is the main phosphatase that dephosphorylates and inhibits TH and aromatic amino acid decarboxylase (Perez *et al.* 2002; Peng *et al.* 2005; Tehranian *et al.* 2006; Lou *et al.* 2010). Soluble a-Syn differentially regulates PP2A (activates) and TH (inhibits) under normal conditions. Furthermore, soluble a-Syn also controls dopamine release and reuptake (Sidhu *et al.* 2004; Sulzer 2010). This implies that therapies that sustain soluble a-Syn at low levels will protect against dopamine mishandling and its toxic consequences (Caudle *et al.* 2007).

In humans, the a-Syn A53T mutation induces Lewy body/Lewy neurite pathology and early onset PD (Polymeropoulos *et al.* 1997). In our current study, we assessed the impact of the A53T a-Syn mutation on pre-motor PD-like symptoms in mice. Evaluating A53T a-Syn mice over time allowed us to correlate progressive synucleinopathy with behavioral markers of early PD. The A53T mice had good mobility but displayed anxiety (Fig. 1). In exploring the source of anxiety we found that a-Syn aggregation contributes to abnormal TH and PP2A activity in the adrenal gland (Fig. 2), which may provide novel biomarkers for early PD detection. We further established that aging A53T mice develop gut pathology associated with a-Syn over-expression (Fig. 3), as well as olfactory loss (Fig. 4) and Lewy-body-like pathology in OB (Fig. 5a, b). OB synucleinopathy was paralleled by impaired PP2A activity (Fig. 5e). These data suggest that with aggregation, insoluble a–Syn loses normal functionality toward both PP2A and TH, producing effects exactly opposite to the effects of soluble a–Syn. This was confirmed *in vitro* using recombinant A53T a-Syn and PP2A (Fig. 6).

The etiology of anxiety in PD is complex and data from mouse models vary, which may be because of differences in the ages of the mice evaluated and the levels of a-Syn expression. For instance, animals expressing only mouse a-Syn or entirely lacking a-Syn have no anxiety (Peña-Oliver *et al.* 2010). Homozygous A53T a-Syn mice at 2 month of age are less anxious than controls (George *et al.* 2008), while A53T heterozygous mice are less anxious than controls at 12 month (Graham and Sidhu 2010). We, however, evaluating older homozygous A53T a-Syn mice. Some have noted that a loss of olfaction can produce anxiety in mice (Glinka *et al.* 2012), though our mice had normal olfaction (Fig. 4) when anxiety was first observed (Fig. 1), implying an a-Syn effect. We noted a–Syn aggregation comparable to PD (Fumimura *et al.* 2007) in A53T adrenal glands with altered enzymatic activity (Fig. 2), making ours the first report to link adrenal synucleinopathy with catecholamine dysregulation and anxiety.

In conclusion, PP2A appears to be a rational therapeutic target for PD. While there is no evidence directly linking PP2A to PD, a large PD-linkage-region on human chromosome 5 encompasses genes for both the PP2A catalytic subunit (5q31.1) and a PP2A B subunit (5q32) (Scott *et al.* 2001; Lill *et al.* 2012), suggesting that further evaluation is warranted. Furthermore, a-Syn mice (Fleming *et al.* 2008) respond well to treatments aimed at stimulating PP2A activity (Lee *et al.* 2011). Because aging is a major contributing factor to PD, our aging A53T model should be particularly useful for testing medications aimed at slowing synucleinopathy. The model will also be appropriate for evaluating

a-Syn imaging agents that are currently in development. As the diagnosis of pre-motor PD improves and novel PD therapies are developed, "cures" for this devastating neurodegenerative disorder may be on the near horizon.

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References

Alerte T. N., Akinfolarin A. A., Friedrich E. E., Mader S. A., Hong C. S. and Perez R. G. (2008) Alpha-synuclein aggregation alters tyrosine hydroxylase phosphorylation and immunoreactivity: lessons from viral transduction of knockout mice. *Neurosci. Lett.* **435**, 24–29.

Anderson J. P., Walker D. E., Goldstein J. M. *et al.* (2006) Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J. Biol. Chem.* **281**, 29739–29752.

Anisman H. and Zacharko R. M. (1986) Behavioral and neurochemical consequences associated with stressors. *Ann. N. Y. Acad. Sci.* **467**, 205–225.

Ashraf W., Pfeiffer R. F., Park F., Lof J. and Quigley E. M. (1997) Constipation in Parkinson's disease: objective assessment and response to psyllium. *Mov. Dis.* **12**, 946–951.

Beach T. G., White C. L., 3rd, Hladik C. L. *et al.* (2009) Olfactory bulb alpha-synucleinopathy has high specificity and sensitivity for Lewy body disorders. *Acta Neuropathol.* **117**, 169–174.

Bernheimer H., Birkmayer W., Hornykiewicz O., Jellinger K. and Seitelberger F. (1973) Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J. Neurol. Sci.* **20**, 415–455.

Bidinosti M., Shimshek D. R., Mollenhauer B., Marcellin D., Schweizer T., Lotz G. P., Schlossmacher M. G. and Weiss A. (2012) Novel one-step immunoassays to quantify alpha-synuclein: applications for biomarker development and high-throughput screening. *J. Biol. Chem.* **287**, 33691–33705.

Blesa J., Phani S., Jackson-Lewis V. and Przedborski S. (2012) Classic and new animal models of Parkinson's disease. *J. Biomed. Biotechnol.* **2012**, 845618.

Bobrovskaya L., Gilligan C., Bolster E. K., Flaherty J. J., Dickson P. W. and Dunkley P. R. (2007) Sustained phosphorylation of tyrosine hydroxylase at serine 40: a novel mechanism for maintenance of catecholamine synthesis. *J. Neurochem.* **100**, 479–489.

Bohnen N. I., Gedela S., Kuwabara H., Constantine G. M., Mathis C. A., Studenski S. A. and Moore R. Y. (2007) Selective hyposmia and nigrostriatal dopaminergic denervation in Parkinson's disease. *J. Neurol.* **254**, 84–90.

Braak H., Ghebremedhin E., Rub U., Bratzke H. and Del Tredici K. (2004) Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res.* **318**, 121–134.

Caudle W. M., Richardson J. R., Wang M. Z. *et al.* (2007) Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. *J. Neurosci.* **27**, 8138–8148.

Chartier-Harlin M.-C., Kachergus J., Roumier C. *et al.* (2004) alpha-Synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* **364**, 1167–1169.

Chen J., Martin B. L. and Brautigan D. L. (1992) Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science* **257**, 1261–1264.

Chobotska K., Arnold M., Werner P. and Pliska V. (1998) A rapid assay for tyrosine hydroxylase activity, an indicator of chronic stress in laboratory and domestic animals. *Biol. Chem.* **379**, 59–63.

Chu Y. and Kordower J. H. (2007) Age-associated increases of alpha-synuclein in monkeys and humans are associated with nigrostriatal dopamine depletion: Is this the target for Parkinson's disease? *Neurobiol. Dis.* **25**, 134–149.

Collier T. J., Kanaan N. M. and Kordower J. H. (2011) Ageing as a primary risk factor for Parkinson's disease: evidence from studies of non-human primates. *Nat. Rev. Neurosci.* **12**, 359–366.

Crawley J. N. (1985) Exploratory behavior models of anxiety in mice. *Neurosci. Biobehav. Rev.* **9**, 37–44.

Croisier E., Moran L. B., Dexter D. T., Pearce R. K. and Graeber M. B. (2005) Microglial inflammation in the parkinsonian substantia nigra: relationship to alpha-synuclein deposition. *J. Neuroinflammation* **2**, 14.

Daubner S. C. and Fitzpatrick P. F. (1993) Alleviation of catecholamine inhibition of tyrosine hydroxylase by phosphorylation at serine40. *Adv. Exp. Med. Biol.* **338**, 87–92.

Dooneief G., Mirabello E., Bell K., Marder K., Stern Y. and Mayeux R. (1992) An estimate of the incidence of depression in idiopathic Parkinson's disease. *Arch. Neurol.* **49**, 305–307.

Doty R. L., Bromley S. M. and Stern M. B. (1995) Olfactory testing as an aid in the diagnosis of Parkinson's disease: development of optimal discrimination criteria. *Neurodegeneration* **4**, 93–97.

Drolet R. E., Cannon J. R., Montero L. and Greenamyre J. T. (2009) Chronic rotenone exposure reproduces Parkinson's disease gastrointestinal neuropathology. *Neurobiol. Dis.* **36**, 96–102.

Fleming S. M., Tetreault N. A., Mulligan C. K., Hutson C. B., Masliah E. and Chesselet M. F. (2008) Olfactory deficits in mice overexpressing human wildtype alpha-synuclein. *Eur. J. Neurosci.* **28**, 247–256.

Fujiwara H., Hasegawa M., Dohmae N., Kawashima A., Masliah E., Goldberg M. S., Shen J., Takio K. and Iwatsubo T. (2002) alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat. Cell Biol.* **4**, 160–164.

Fumimura Y., Ikemura M., Saito Y. *et al.* (2007) Analysis of the adrenal gland is useful for evaluating pathology of the peripheral autonomic nervous system in Lewy body disease. *J. Neuropathol. Exp. Neurol.* **66**, 354–362.

Geng X., Lou H., Wang J. *et al.* (2011) Alpha-Synuclein binds the K (ATP) channel at insulin-secretory granules and inhibits insulin secretion. *Am. J. Physiol. Endocrinol. Metab.* **300**, E276–E286.

George S., van den Buuse M., San Mok S., Masters C. L., Li Q. X. and Culvenor J. G. (2008) Alpha-synuclein transgenic mice exhibit reduced anxiety-like behaviour. *Exp. Neurol.* **210**, 788–792.

Giasson B. I., Uryu K., Trojanowski J. Q. and Lee V. M. (1999) Mutant and wild type human alpha-synucleins assemble into elongated filaments with distinct morphologies in vitro. *J. Biol. Chem.* **274**, 7619–7622.

Giasson B. I., Duda J. E., Quinn S. M., Zhang B., Trojanowski J. Q. and Lee V. M. (2002) Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron* **34**, 521–533.

Glinka M. E., Samuels B. A., Diodato A., Teillon J., Feng Mei D., Shykind B. M., Hen R. and Fleischmann A. (2012) Olfactory deficits cause anxiety-like behaviors in mice. *J. Neurosci.* **32**, 6718–6725.

Graham D. R. and Sidhu A. (2010) Mice expressing the A53T mutant form of human alpha-synuclein exhibit hyperactivity and reduced anxiety-like behavior. *J. Neurosci. Res.* **88**, 1777–1783.

Haavik J., Schelling D. L., Campbell D. G., Andersson K. K., Flatmark T. and Cohen P. (1989) Identification of protein phosphatase 2A as the major tyrosine hydroxylase phosphatase in adrenal medulla and corpus striatum: evidence from the effects of okadaic acid. *FEBS Lett.* **251**, 36–42.

Haehner A., Hummel T. and Reichmann H. (2011) Olfactory loss in Parkinson's disease. *Parkinsons Dis.* **2011**, 450939.

Hunt R. F., Girskis K. M., Rubenstein J. L., Alvarez-Buylla A. and Baraban S. C. (2013) GABA progenitors grafted into the adult epileptic brain control seizures and abnormal behavior. *Nat. Neurosci.* **16**, 692–695.

Kolski-Andreaco A., Cai H., Currle D. S., Chandy K. G. and Chow R. H. (2007) Mouse adrenal chromaffin cell isolation. *J. Vis. Exp.* **5**, 129.

Kruger R., Kuhn W., Muller T. *et al.* (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat. Genet.* **18**, 106–108.

Kumer S. C. and Vrana K. E. (1996) Intricate regulation of tyrosine hydroxylase activity and gene expression. *J. Neurochem.* **67**, 443–462.

Kvetnansky R. and Mikulaj L. (1970) Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. *Endocrinology* **87**, 738–743.

Lauterbach E. C. and Duvoisin R. C. (1991) Anxiety disorders in familial parkinsonism. *Am. J. Psychiatry* **148**, 274.

LeBlanc J. and Ducharme M. B. (2007) Plasma dopamine and noradrenaline variations in response to stress. *Physiol. Behav.* **91**, 208–211.

Leduc J. (1961) Catecholamine production and release in exposure and acclimation to cold. *Acta Physiol. Scand. Suppl.* **183**, 1–101.

Lee M. K., Stirling W., Xu Y. *et al.* (2002) Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 –> Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. *Proc. Natl Acad. Sci. USA* **99**, 8968–8973.

Lee K. W., Chen W., Junn E. *et al.* (2011) Enhanced phosphatase activity attenuates alpha-synucleinopathy in a mouse model. *J. Neurosci.* **31**, 6963–6971.

Li W., Lesuisse C., Xu Y., Troncoso J. C., Price D. L. and Lee M. K. (2004) Stabilization of alpha-synuclein protein with aging and familial parkinson's disease-linked A53T mutation. *J. Neurosci.* **24**, 7400–7409.

Lill C. M., Roehr J. T., McQueen M. B. *et al.* (2012) Comprehensive research synopsis and systematic meta-analyses in Parkinson's disease genetics: The PDGene database. *PLoS Genet.* **8**, e1002548.

Lou H., Montoya S. E., Alerte T. N. *et al.* (2010) Serine 129 phosphorylation reduces the ability of alpha-synuclein to regulate tyrosine hydroxylase and protein phosphatase 2A in vitro and in vivo. *J. Biol. Chem.* **285**, 17648–17661.

Lu D. C., Zhang H., Zador Z. and Verkman A. S. (2008) Impaired olfaction in mice lacking aquaporin-4 water channels. *FASEB J.* **22**, 3216–3223.

Maricle R. A., Nutt J. G. and Carter J. H. (1995) Mood and anxiety fluctuation in Parkinson's disease associated with levodopa infusion: preliminary findings. *Mov. Dis.* **10**, 329–332.

Maroteaux L., Campanelli J. T. and Scheller R. H. (1988) Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. *J. Neurosci.* **8**, 2804–2815.

Merali Z., Levac C. and Anisman H. (2003) Validation of a simple, ethologically relevant paradigm for assessing anxiety in mice. *Biol. Psychiatry* **54**, 552–565.

Nathan B. P., Yost J., Litherland M. T., Struble R. G. and Switzer P. V. (2004) Olfactory function in apoE knockout mice. *Behav. Brain Res.* **150**, 1–7.

Oaks A. W., Frankfurt M., Finkelstein D. I. and Sidhu A. (2013) Age-dependent effects of A53T alpha-synuclein on behavior and dopaminergic function. *PLoS ONE* **8**, e60378.

Pellicano C., Benincasa D., Pisani V., Buttarelli F. R., Giovannelli M. and Pontieri F. E. (2007) Prodromal non-motor symptoms of Parkinson's disease. *Neuropsychiatr. Dis. Treat.* **3**, 145–152.

Peña-Oliver Y., Buchman V. L. and Stephens D. N. (2010) Lack of involvement of alpha-synuclein in unconditioned anxiety in mice. *Behav. Brain Res.* **209**, 234–240.

Peng X., Tehranian R., Dietrich P., Stefanis L. and Perez R. G. (2005) Alpha-synuclein activation of protein phosphatase 2A reduces tyrosine hydroxylase phosphorylation in dopaminergic cells. *J. Cell Sci.* **118**, 3523–3530.

Perez R. G. and Hastings T. G. (2004) Could a loss of alpha-synuclein function put dopaminergic neurons at risk? *J. Neurochem.* **89**, 1318–1324.

Perez R. G., Waymire J. C., Lin E., Liu J. J., Guo F. and Zigmond M. J. (2002) A role for alpha-synuclein in the regulation of dopamine biosynthesis. *J. Neurosci.* **22**, 3090–3099.

Pinna G., Galici R., Schneider H. H., Stephens D. N. and Turski L. (1997) Alprazolam dependence prevented by substituting with the beta-carboline abecarnil. *Proc. Natl Acad. Sci. USA* **94**, 2719–2723.

Polymeropoulos M. H., Lavedan C., Leroy E. *et al.* (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* **276**, 2045–2047.

Roy M., David N. K., Danao J. V., Baribault H., Tian H. and Giorgetti M. (2006) Genetic inactivation of melanin-concentrating hormone receptor subtype 1 (MCHR1) in mice exerts anxiolytic-like behavioral effects. *Neuropsychopharmacology: official publication of the American College of. Neuropsychopharmacology* **31**, 112–120.

Saito Y., Kawashima A., Ruberu N. N. *et al.* (2003) Accumulation of phosphorylated alpha-synuclein in aging human brain. *J. Neuropathol. Exp. Neurol.* **62**, 644–654.

Savica R., Carlin J. M., Grossardt B. R., Bower J. H., Ahlskog J. E., Maraganore D. M., Bharucha A. E. and Rocca W. A. (2009) Medical records documentation of constipation preceding Parkinson disease: A case-control study. *Neurology* **73**, 1752–1758.

Scott W. K., Nance M. A., Watts R. L. *et al.* (2001) Complete genomic screen in Parkinson disease: evidence for multiple genes. *JAMA* **286**, 2239–2244.

Shannon K. M., Keshavarzian A., Dodiya H. B., Jakate S. and Kordower J. H. (2012) Is alpha-synuclein in the colon a biomarker for premotor Parkinson's disease? Evidence from 3 cases. *Mov. Dis.* **27**, 716–719.

Shiba M., Bower J. H., Maraganore D. M., McDonnell S. K., Peterson B. J., Ahlskog J. E., Schaid D. J. and Rocca W. A. (2000) Anxiety disorders and depressive disorders preceding Parkinson's disease: a case-control study. *Mov. Dis.* **15**, 669–677.

Sidhu A., Wersinger C. and Vernier P. (2004) Does alpha-synuclein modulate dopaminergic synaptic content and tone at the synapse? *FASEB J.* **18**, 637–647.

Simuni T. and Stern M. B. (1999) Does levodopa accelerate Parkinson's disease? *Drugs Aging* **14**, 399–408.

Singaram C., Ashraf W., Gaumnitz E. A., Torbey C., Sengupta A., Pfeiffer R. and Quigley E. M. (1995) Dopaminergic defect of

enteric nervous system in Parkinson's disease patients with chronic constipation. *Lancet* **346**, 861–864.

Singleton A. B., Farrer M., Johnson J. *et al.* (2003) alpha-Synuclein locus triplication causes Parkinson's disease. *Science* **302**, 841.

Sulzer D. (2010) Clues to how alpha-synuclein damages neurons in Parkinson's disease. *Mov. Dis.* **25**(Suppl 1), S27–S31.

Sun A., Nguyen X. V. and Bing G. (2002) Comparative Analysis of an Improved Thioflavin-S Stain, Gallyas Silver Stain, and Immunohistochemistry for Neurofibrillary Tangle Demonstration on the Same Sections. *J. Histochem. Cytochem.* **50**, 463–472.

Tanji K., Mori F., Mimura J., Itoh K., Kakita A., Takahashi H. and Wakabayashi K. (2010) Proteinase K-resistant alpha-synuclein is deposited in presynapses in human Lewy body disease and A53T alpha-synuclein transgenic mice. *Acta Neuropathol.* **120**, 145–154.

Tehranian R., Montoya S. E., Van Laar A. D., Hastings T. G. and Perez R. G. (2006) Alpha-synuclein inhibits aromatic amino acid decarboxylase activity in dopaminergic cells. *J. Neurochem.* **99**, 1188–1196.

Tsika E., Moysidou M., Guo J. *et al.* (2010) Distinct region-specific alpha-synuclein oligomers in A53T transgenic mice: implications for neurodegeneration. *J. Neurosci.* **30**, 3409–3418.

Unger E. L., Eve D. J., Perez X. A., Reichenbach D. K., Xu Y., Lee M. K. and Andrews A. M. (2006) Locomotor hyperactivity and alterations in dopamine neurotransmission are associated with overexpression of A53T mutant human alpha-synuclein in mice. *Neurobiol. Dis.* **21**, 431–443.

Wakabayashi K., Mori F., Tanji K., Orimo S. and Takahashi H. (2010) Involvement of the peripheral nervous system in synucleinopathies, tauopathies and other neurodegenerative proteinopathies of the brain. *Acta Neuropathol.* **120**, 1–12.

Wang J., Lou H., Pedersen C. J., Smith A. D. and Perez R. G. (2009) 14-3-3zeta contributes to tyrosine hydroxylase activity in MN9D cells: localization of dopamine regulatory proteins to mitochondria. *J. Biol. Chem.* **284**, 14011–14019.

Weisskopf M. G., Chen H., Schwarzschild M. A., Kawachi I. and Ascherio A. (2003) Prospective study of phobic anxiety and risk of Parkinson's disease. *Mov. Dis.* **18**, 646–651.

Wu J., Lou H., Alerte T. N., Stachowski E. K., Chen J., Singleton A. B., Hamilton R. L. and Perez R. G. (2012) Lewy-like aggregation of alpha-synuclein reduces protein phosphatase 2A activity in vitro and in vivo. *Neuroscience* **207**, 288–297.

Zarranz J., Alegre J., Gomez-Esteban J. *et al.* (2004) The new mutation, E46K, of alpha-synuclein causes parkinson and Lewy body dementia. *Ann. Neurol.* **55**, 164–173.

