CHARACTERIZATION OF MJFF SNCA KO AND HUMANIZED A53T SNCA KI RATS

Phenotyping Data Results

STUDY OVERVIEW

MODEL OVERVIEW

- The goal of the study was to perform in depth characterization of the MJFF-generated aSyn KO and Humanized aSyn A53T KI rats that are available through Inotiv (formerly Horizon/Envigo):
- **aSyn A53T KI Model Description**
  - This model contains a knockin of the A53T-mutated SNCA gene deeming the rat SNCA gene non-functional. The knockin contains humanized amino acids for the region spanning amino acids 53-122. The resulting model expresses a humanized A53T alpha-synuclein protein without endogenous rat alpha-synuclein. This model was generated using CRISPR/Cas9 genome targeting strategies.
  - This model is available at Inotiv (https://www.inotivco.com/model/hsdsage-sd-sncaem1-snca-a53t-sage).
- **aSyn KO Model Description:**
  - This model contains a deletion of the endogenous rat SNCA gene, encoding the alpha-synuclein protein. This model was generated using the CRISPR/Cas9 genome targeting strategy.
  - This model is available at Inotiv (https://www.inotivco.com/model/hsdsage-sd-scnaem1sage).

MODEL OVERVIEW

» The goal of the study was to perform in depth characterization of the MJFF-generated aSyn KO and Humanized aSyn A53T KI rats that are available through Inotiv (formerly Horizon/Envigo).

– **Evaluation Plan:** Homozygous rats and wildtype littermate controls were evaluated for the following outcome measures at the following ages. Groups were gender-matched to enable evaluation of sex differences in phenotypes.

aSyn A53T KI Rats

4mo → 8mo → 12mo → 18mo

	Structures	Outcome Measures	N
Behavioral Tests	N/A	Open field test (4, 8, 12, 18 mo)	10 male + 10 female
		GI Motility (4, 8, 12, 18 mo)	10 male + 10 female
		Beam Walk (4, 8, 12, 18 mo)	10 male + 10 female
		Fine Motor Kinematics (4, 8, 12, 18 mo)	10 male + 10 female
Biochem	STR, CTX, HPC, SNpc, CRBL	Branched DNA for Rat and Human SNCA mRNA (4, 8, 12, 18 mo)	5 male + 5 female
	CTX, SNpc	Western blot for total, soluble, insoluble aSyn (4, 18 mo)	5 male + 5 female
Neurochem	STR	HPLC for DA and DA metabolites (18 mo)	5 male + 5 female
Peripheral Collections	N/A	PBMC Banking (4, 8, 12, 18 mo)	10 male + 10 female
Histology	STR, CTX, HPC, SNpc, CRBL	TH Stain and Stereology (4, 8, 12, 18 mo)	5 male + 5 female
		pS129 aSyn Stain and Images (18 mo)	5 male + 5 female
		Total aSyn Stain and Images (12, 18 mo)	5 male + 5 female
		pTau (AT8) Stain and Images (4, 8, 12, 18 mo)	5 male + 5 female
		GFAP Stain and Images (4, 8, 12, 18 mo)	5 male + 5 female
		Iba-1 Fluorescent Stain and Images (12, 18 mo)	5 male + 5 female
	Colon, Duodenum	pS129 aSyn Stain (18 mo)	10 male + 10 female
		Total aSyn Stain (4, 8, 12, 18 mo)	10 male + 10 female

aSyn KO Rats

6mo → 12mo

	Structures	Outcome Measures	N
Biochem	STR, CTX, HPC, SNpc, CRBL	Branched DNA for Rat SNCA mRNA (6, 12 mo)	10 male + 10 female
	CTX, SNpc	Western blot for total aSyn (6, 12 mo)	10 male + 10 female
Neurochem	STR	HPLC for DA and DA metabolites (12 mo)	10 male + 10 female
Peripheral Collections	N/A	PBMC Banking (6, 12 mo)	10 male + 10 female

Abbreviations: STR = Striatum, CTX = Cortex, HPC = Hippocampus, SNpc = Substantia Nigra pars compacta, CRBL = Cerebellum

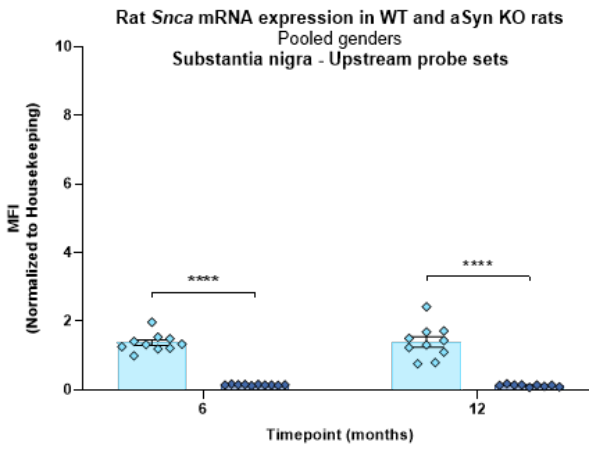
ASYN KNOCKOUT RAT PHENOTYPES

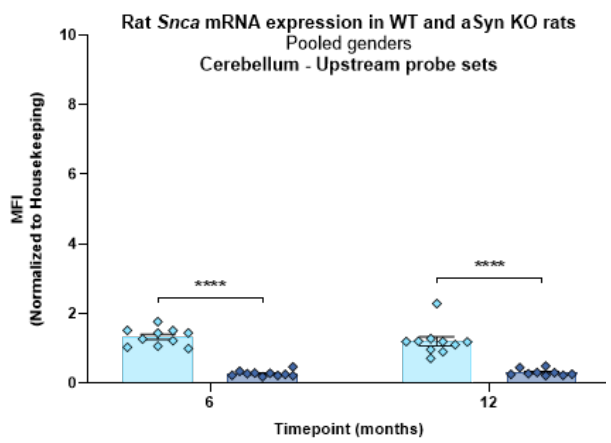
aSyn KO Rat Phenotype Summary

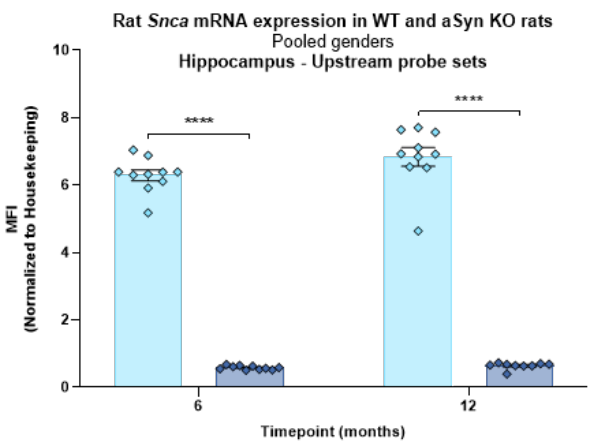
	Structures	Outcome Measures	N	Results
Biochem	STR, CTX, HPC, SNpc, CRBL	Branched DNA for Rat SNCA mRNA (6, 12 mo)	10 male + 10 female	Rat SNCA levels absent.
	CTX, SNpc	Western blot for total aSyn (6, 12 mo)	10 male + 10 female	aSyn absent
Neurochem	STR	HPLC for DA and DA metabolites (12 mo)	10 male + 10 female	No genotype-related changes.
Peripheral Collections	N/A	PBMC Banking (6, 12 mo)	10 male + 10 female	N/A

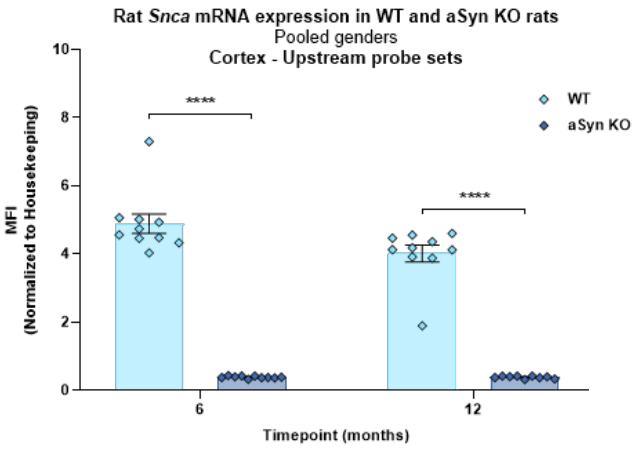
Abbreviations: STR = Striatum, CTX = Cortex, HPC = Hippocampus, SNpc = Substantia Nigra pars compacta, CRBL = Cerebellum, PBMC = peripheral blood mononuclear cells, aSyn = alpha-synuclein; DA = dopamine, mo = months of age

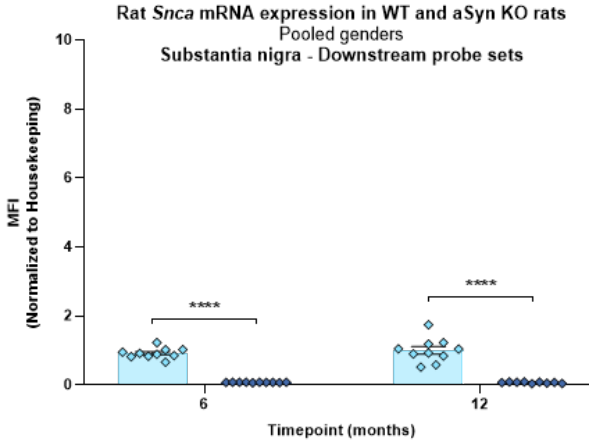
aSyn KO Rat Phenotyping – Rat Snca

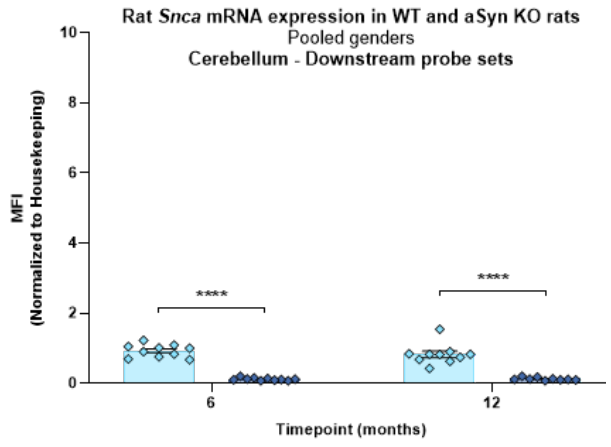


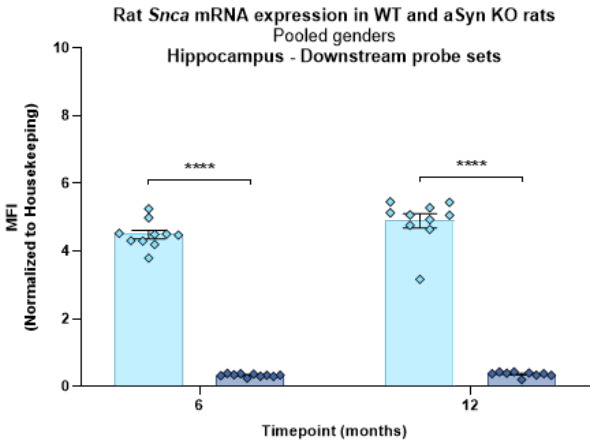


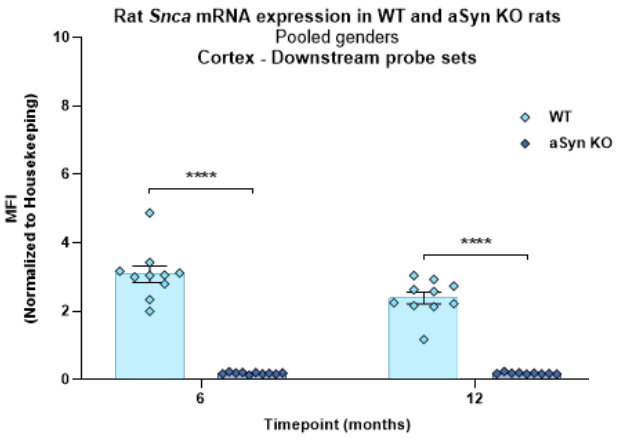








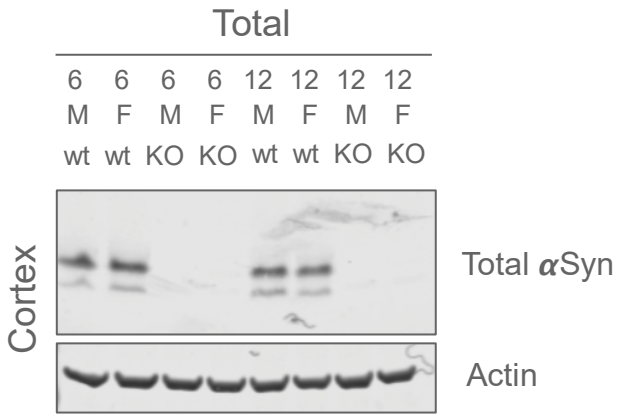




No rat Snca mRNA expressed. All animals shown – no sex differences.

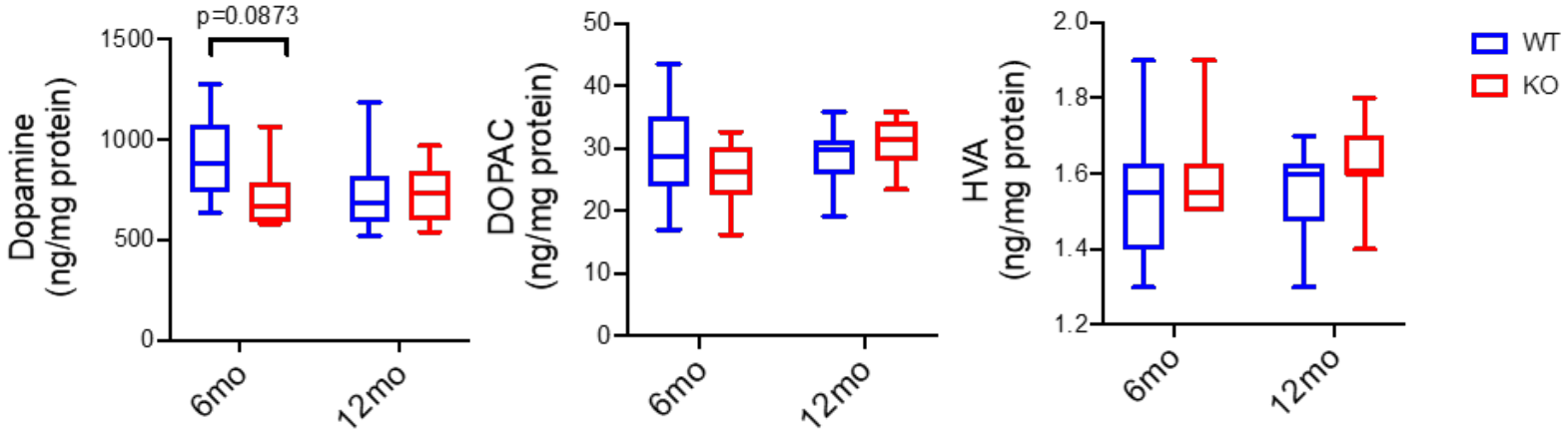
aSyn KO Rat Phenotyping -

aSyn Protein



No rat aSyn protein expressed

Neurochemistry



No alterations in striatal dopamine or dopamine metabolites.
All animals shown – no sex differences.

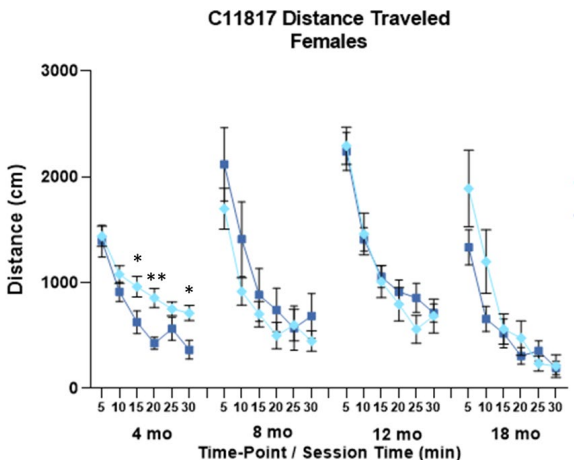
HUMANIZED ASYN A53T KNOCKIN RAT PHENOTYPES

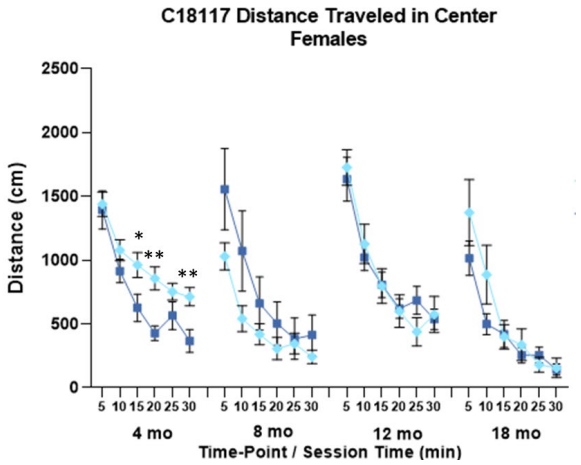
Humanized aSyn A53T KI Rat Phenotype Summary

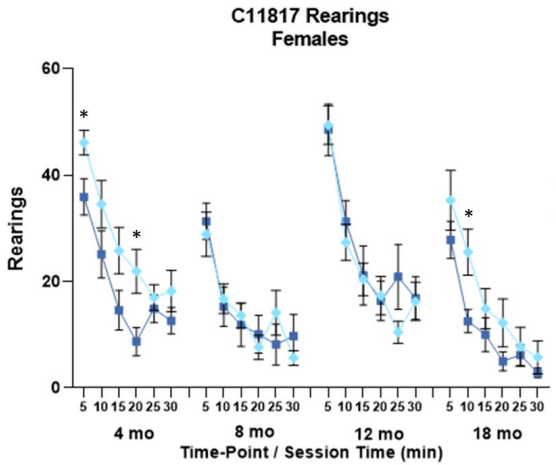
	Structures	Outcome Measures	N	Results
Behavioral Tests	N/A	Open field test (4, 8, 12, 18 mo)	10 male + 10 female	Female phenotypes at 4mo Male phenotypes at 18mo
		GI Motility (4, 8, 12, 18 mo)	10 male + 10 female	No compelling deficits
		Beam Walk (4, 8, 12, 18 mo)	10 male + 10 female	No compelling deficits
		Fine Motor Kinematics (4, 8, 12, 18 mo)	10 male + 10 female	Female phenotypes at 4mo Male phenotypes at 18mo
Biochem	STR, CTX, HPC, SNpc, CRBL	Branched DNA for Rat and Human SNCA mRNA (4, 8, 12, 18 mo)	5 male + 5 female	Rat SNCA absent. Human SNCA present.
	CTX, SNpc	Western blot for total, soluble, insoluble aSyn (4, 18 mo)	5 male + 5 female	No apparent differences
Neurochem	STR	HPLC for DA and DA metabolites (18 mo)	5 male + 5 female	No genotype-related changes.
Peripheral Collections	N/A	PBMC Banking (4, 8, 12, 18 mo)	10 male + 10 female	N/A
Histology	STR, CTX, HPC, SNpc, CRBL	TH Stain and Stereology (4, 8, 12, 18 mo)	5 male + 5 female	No cell loss
		pS129 aSyn Stain and Images (18 mo)	5 male + 5 female	No pS129 aSyn pathology
		Total aSyn Stain and Images (12, 18 mo)	5 male + 5 female	No apparent change in total aSyn
		pTau (AT8) Stain and Images (4, 8, 12, 18 mo)	5 male + 5 female	No pTau pathology
		GFAP Stain and Images (4, 8, 12, 18 mo)	5 male + 5 female	No GFAP pathology
		Iba-1 Fluorescent Stain and Images (12, 18 mo)	5 male + 5 female	No Iba-1 pathology
	Colon, Duodenum	pS129 aSyn Stain (18 mo)	10 male + 10 female	No pS129 aSyn pathology
		Total aSyn Stain (4, 8, 12, 18 mo)	10 male + 10 female	No apparent change in total aSyn

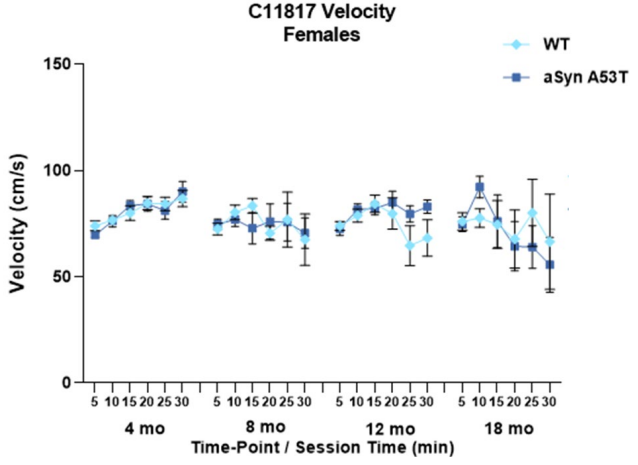
Abbreviations: STR = Striatum, CTX = Cortex, HPC = Hippocampus, SNpc = Substantia Nigra pars compacta, CRBL = Cerebellum, PBMC = peripheral blood mononuclear cells, aSyn = alpha-synuclein; DA = dopamine, mo = months of age

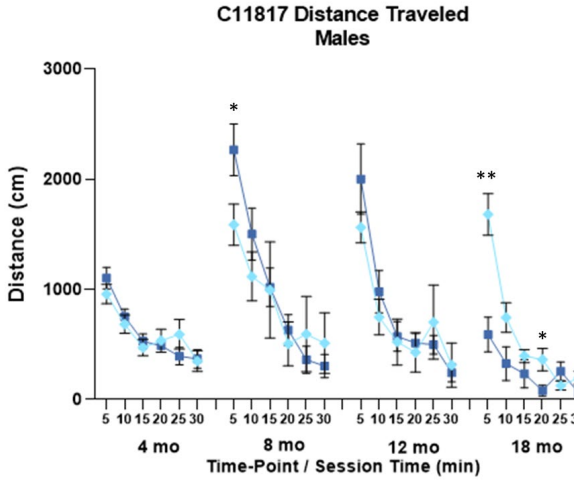
aSyn A53T KI Rat Phenotyping – Open Field

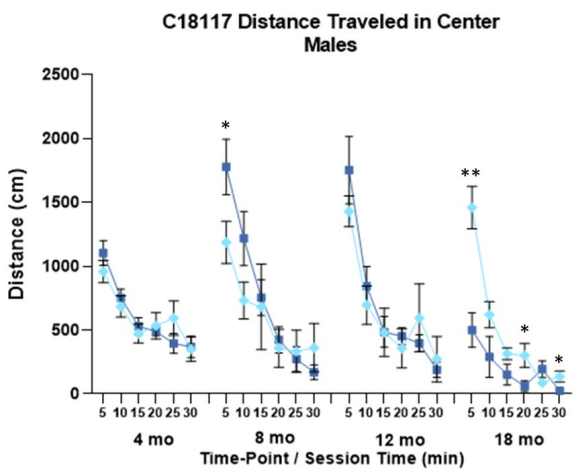


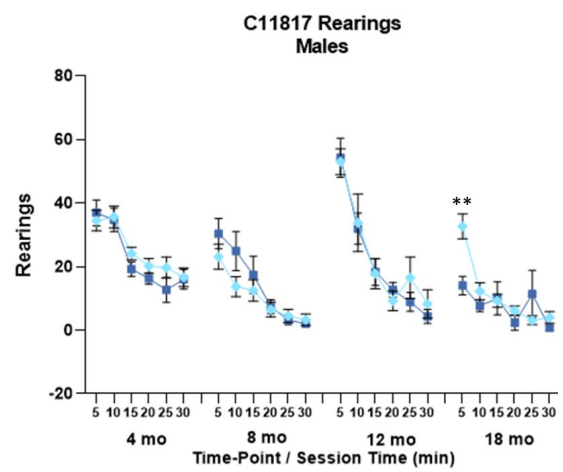


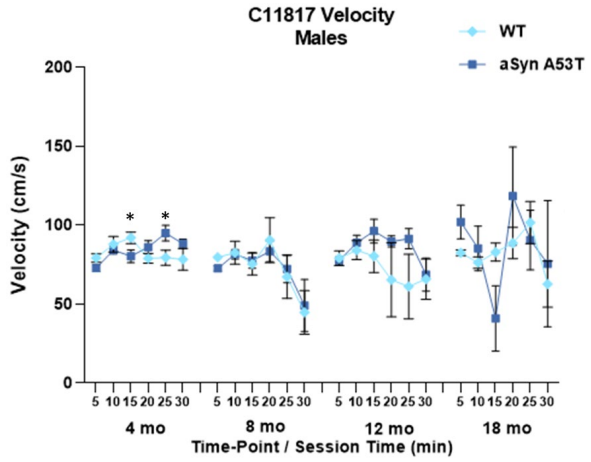






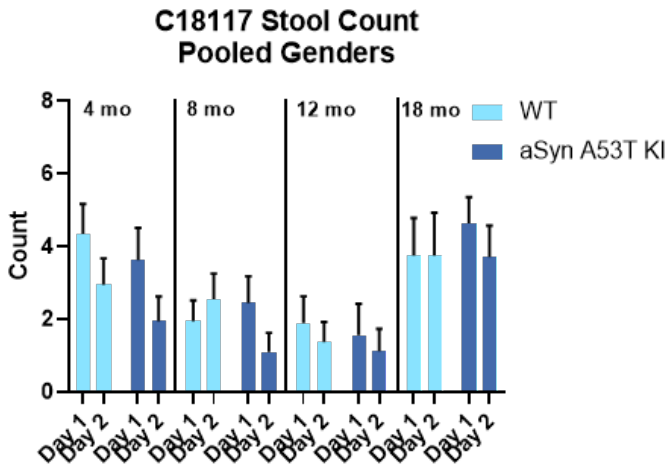


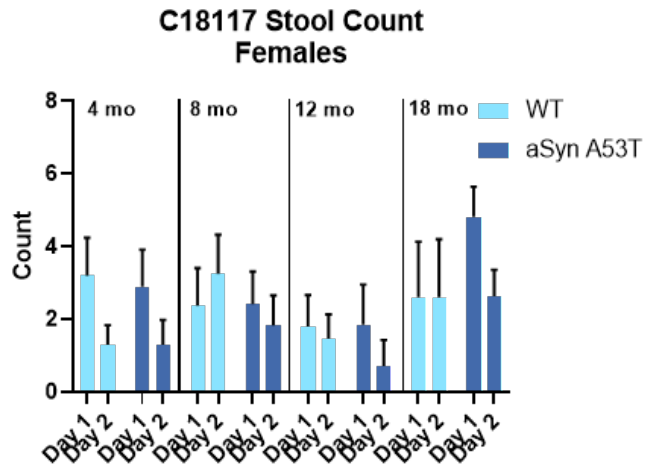


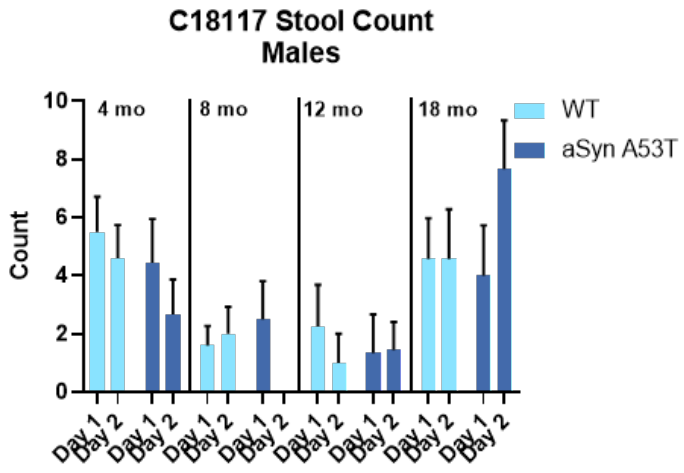


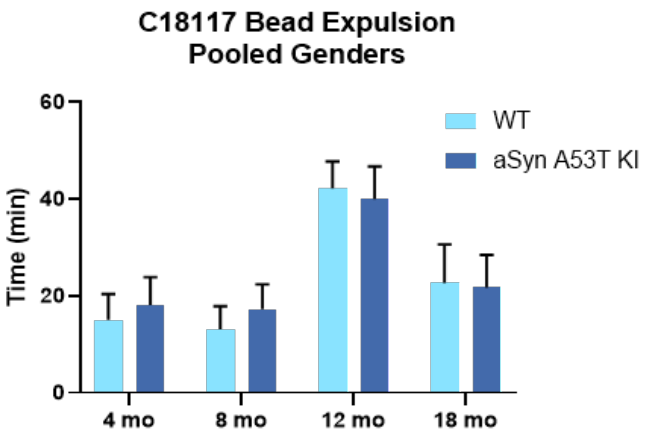
When sexes were pooled, differences were observed in total distance travelled at 8 and 18 mo, distance travelled in center at 4, 8, and 18 mo, number of rearings at 4 and 18 mo, and average velocity at 12 mo. In female rats, differences were observed in total distance travelled at 4 mo, distance travelled in center at 4 mo, and in number of rearings at 4 and 18 mo. In male rats, statistical differences were observed in total distance travelled at 8 and 18 mo, distance travelled in center at 18 mo, and number of rearings at 18 mo.

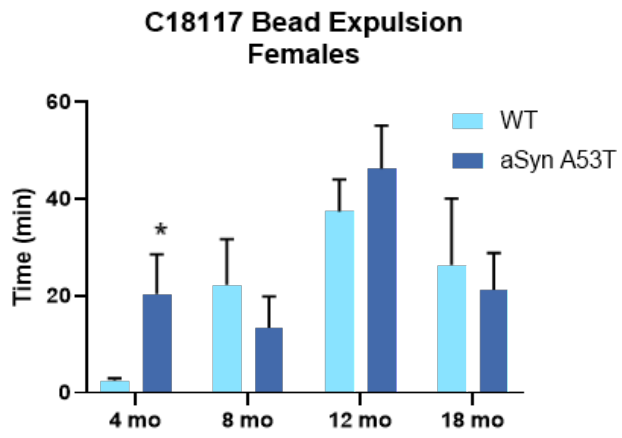
aSyn A53T KI Rat Phenotyping – GI Motility

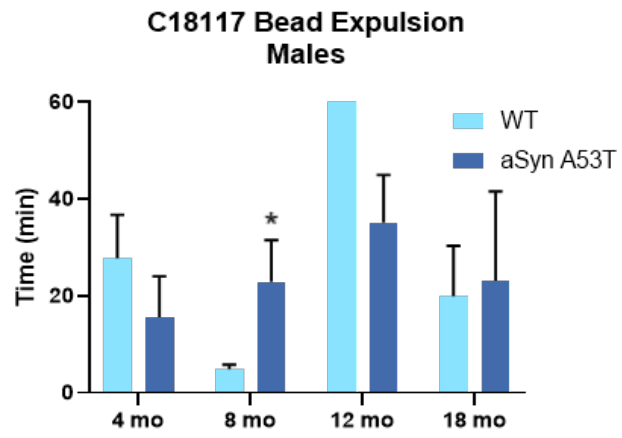






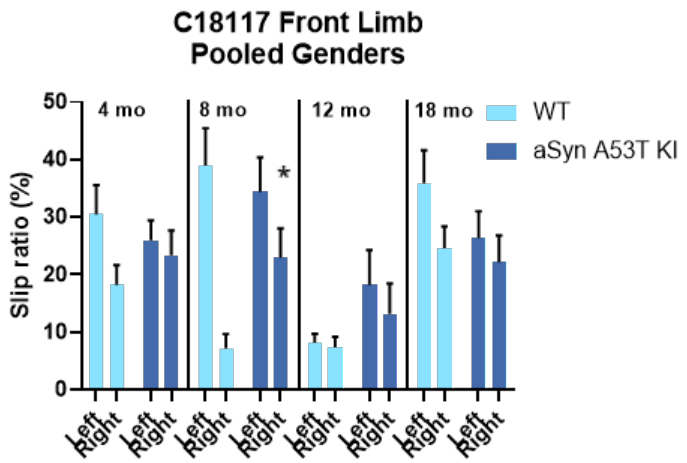


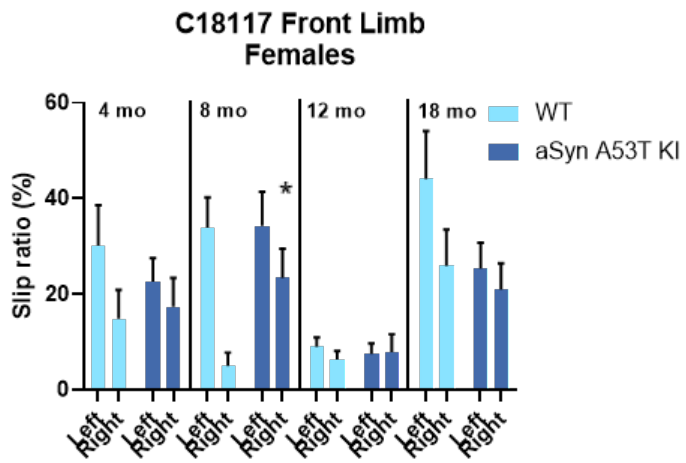


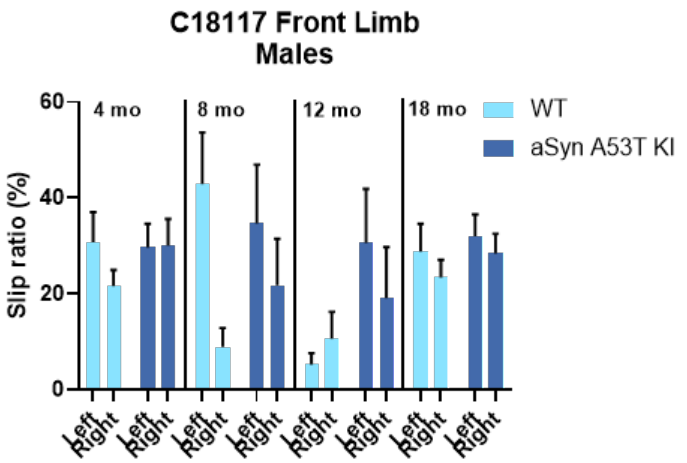


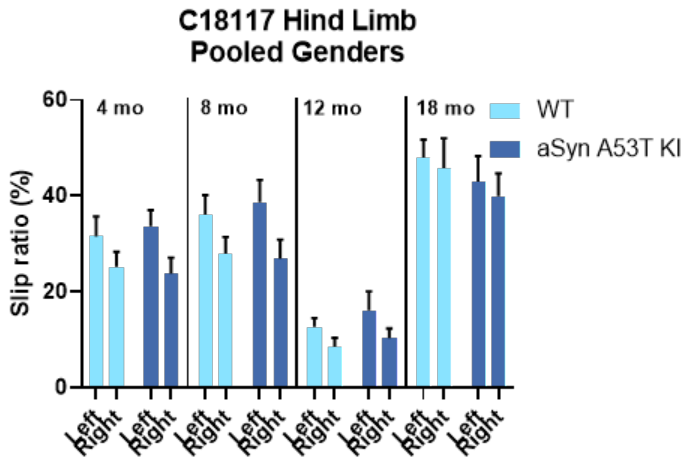
There were no statistical differences in stool count between genotypes. In bead expulsion, female KI rats took more time to egest the bead at 4 mo and male KI rats took more time to egest the bead at 8 mo when compared to WT.

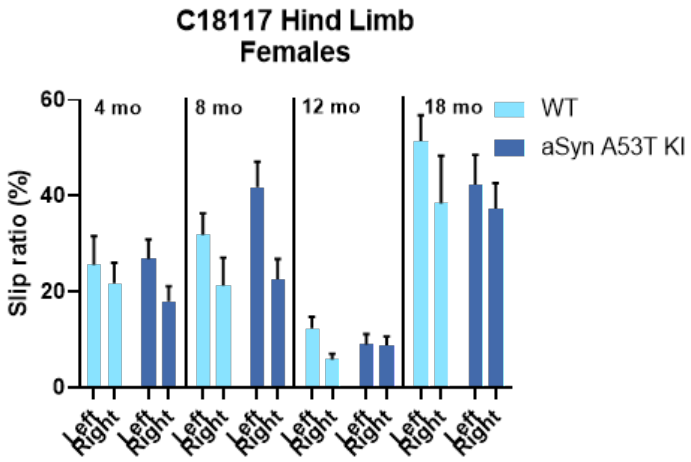
aSyn A53T KI Rat Phenotyping – Beam Walk

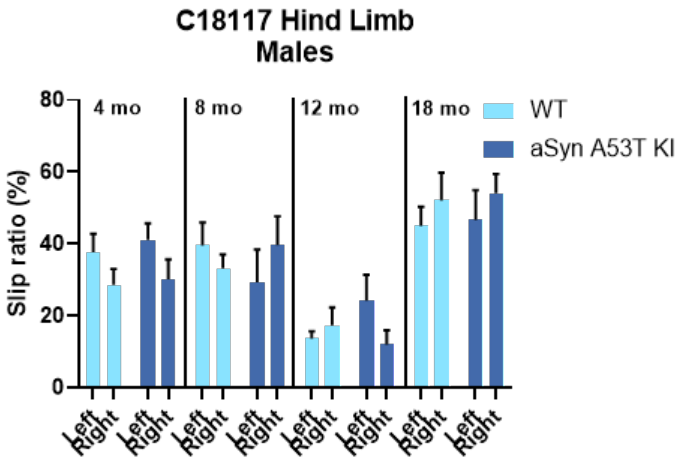






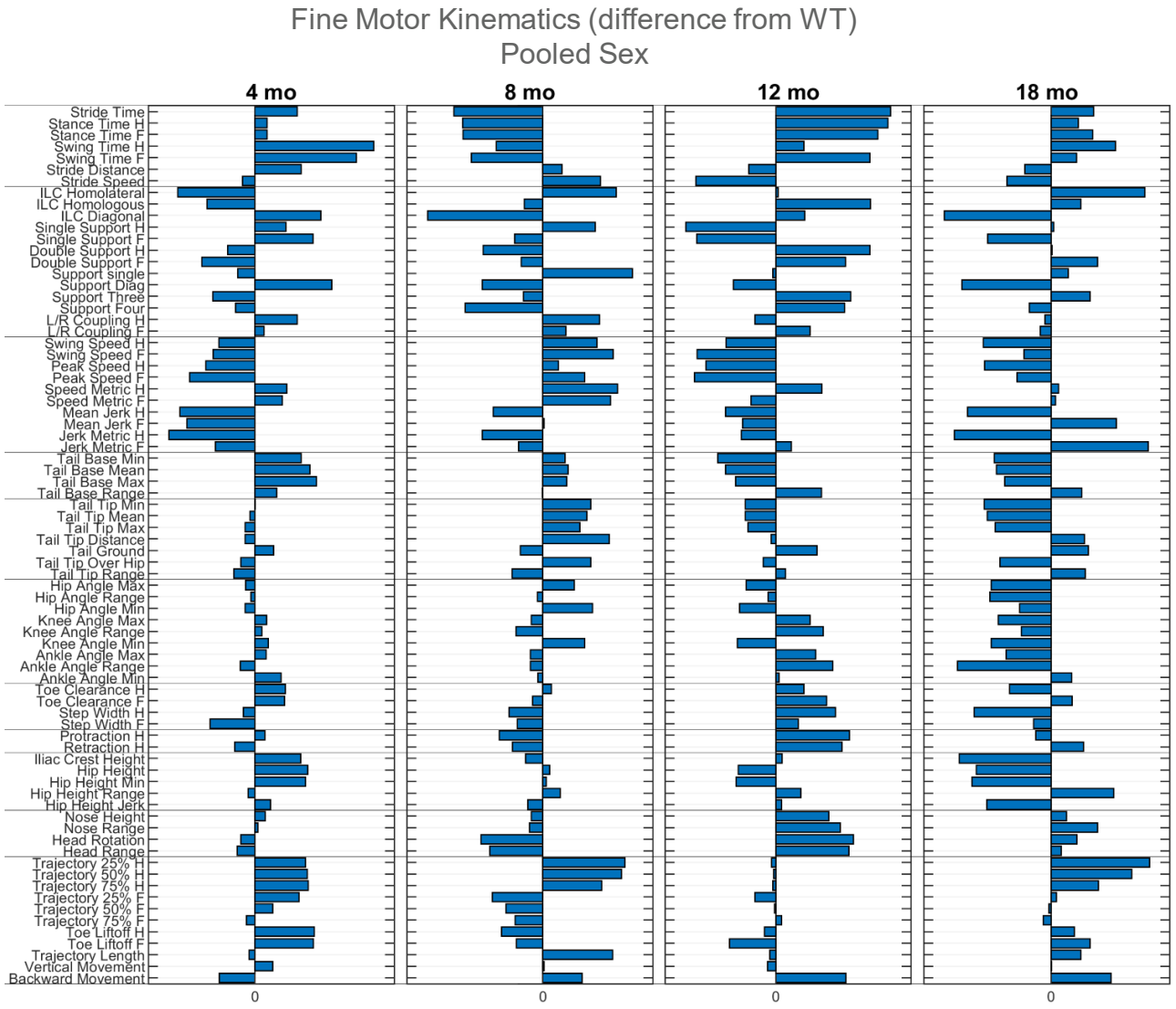


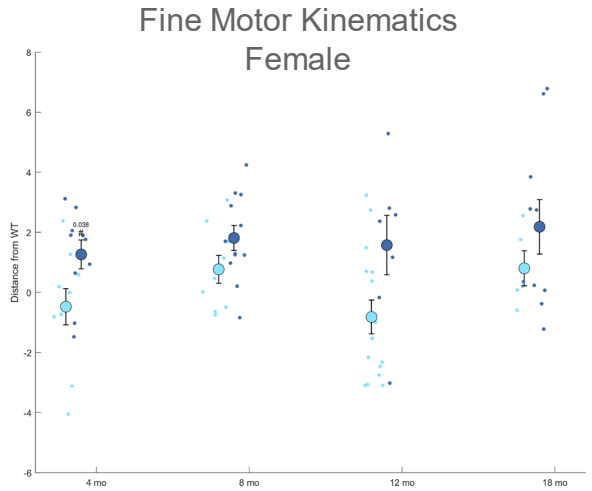


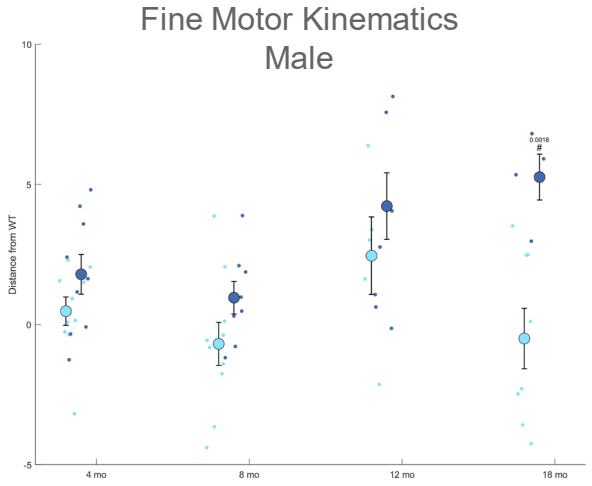


Female KI rats had statistically more slips on the right side front limb steps when compared to WT. This is also seen in the pooled genders. There were no other statistically significant differences when the males were compared.

aSyn A53T KI Rat Phenotyping – Fine Motor



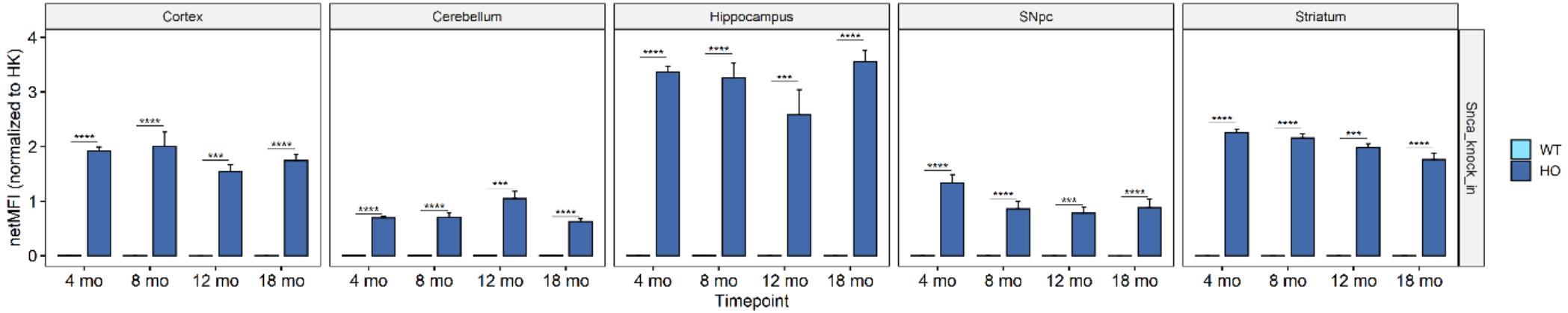




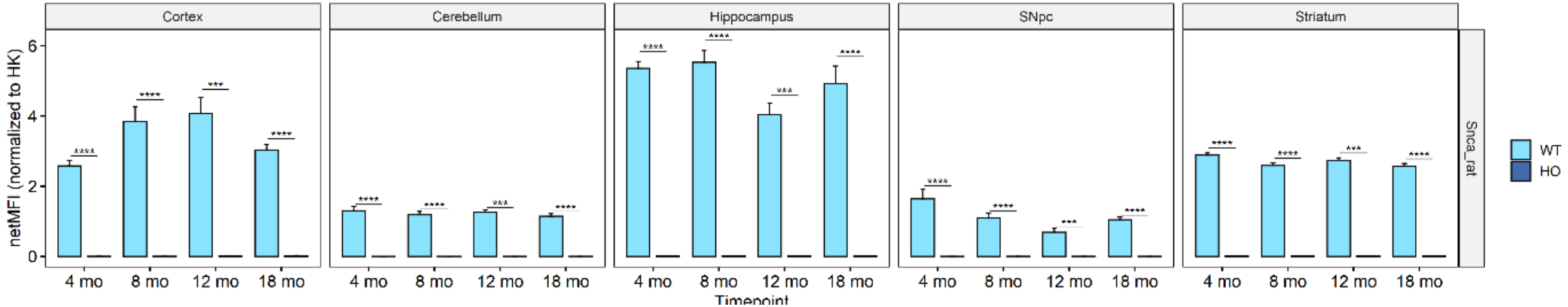
When the sexes were pooled the KI rats were statistically different compared to WT in all age groups. When sexes were separated, female KI rats were statistically different from WT at 4 months of age and male KI rats were statistically different from WT 18 months of age.

aSyn A53T KI Rat Phenotyping -

Human *SNCA* mRNA



Rat *Snca* mRNA

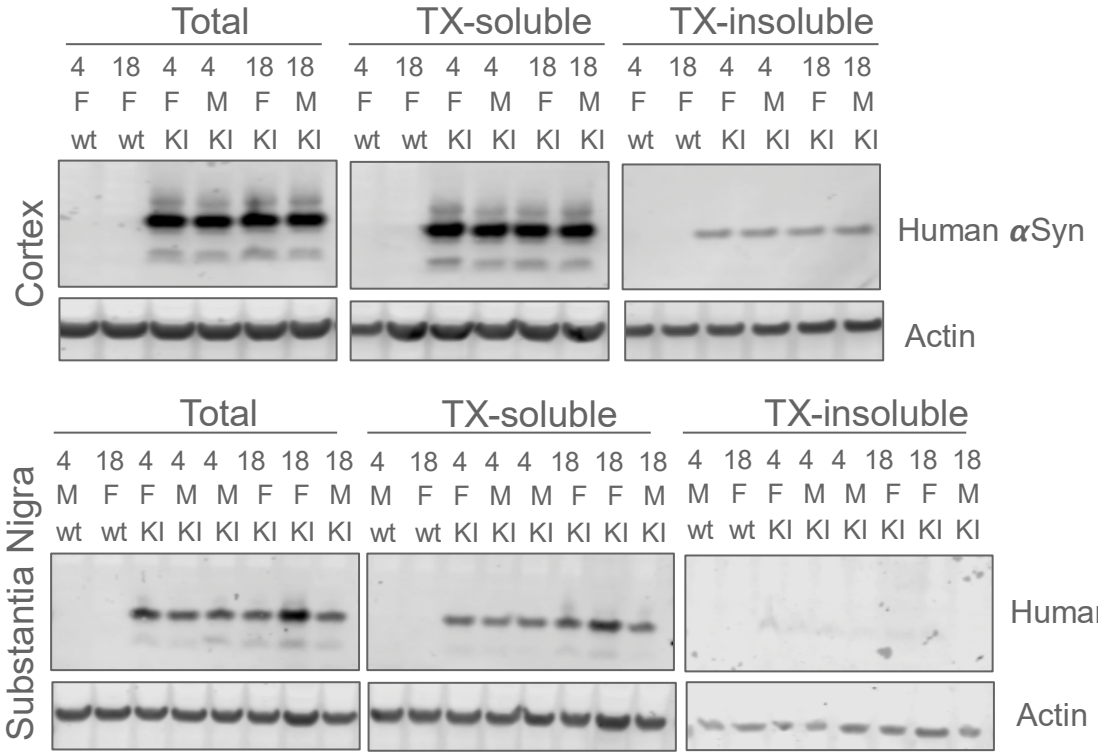


KI rats display high expression of human *SNCA* mRNA with absence of rat *Snca* mRNA.

All animals shown – no sex differences.

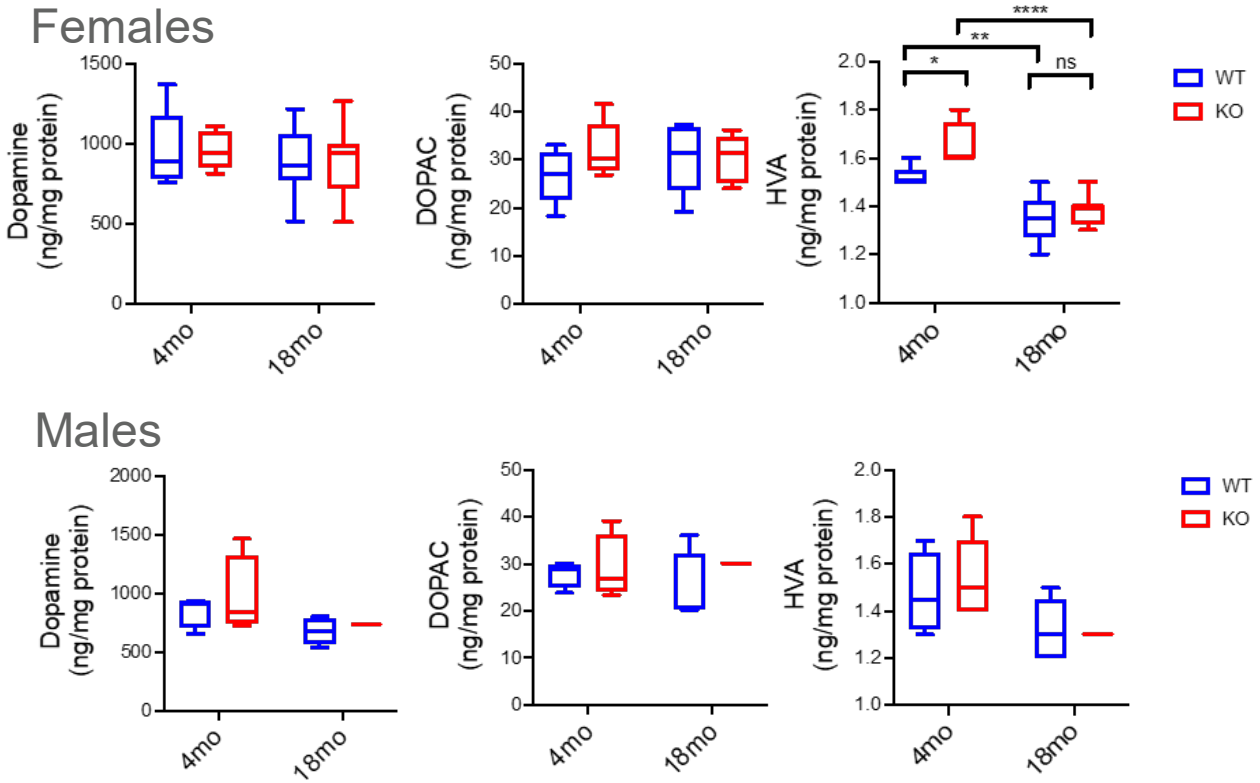
aSyn A53T KI Rat Phenotyping -

aSyn Protein



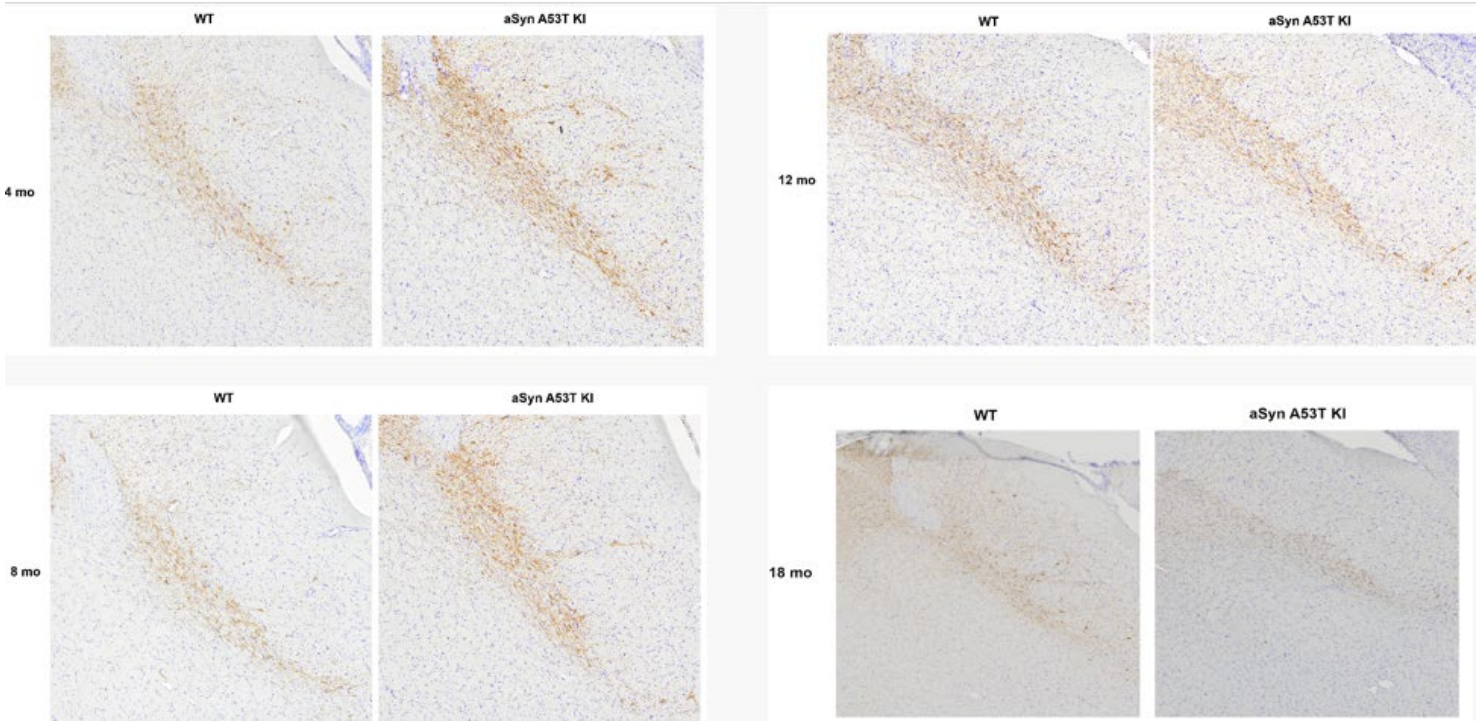
Expression of human aSyn in KI rats.
Insoluble aSyn detected in the cortex.

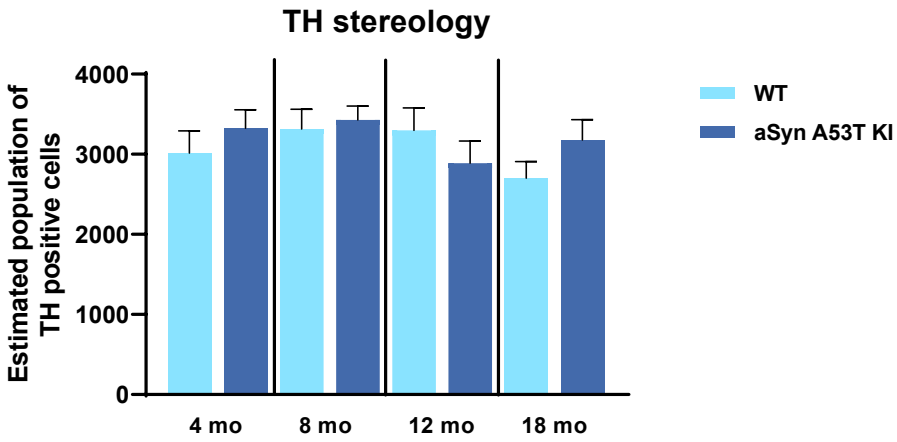
Neurochemistry



No alterations in striatal dopamine. Slight increase in HVA in females only at 4mo.

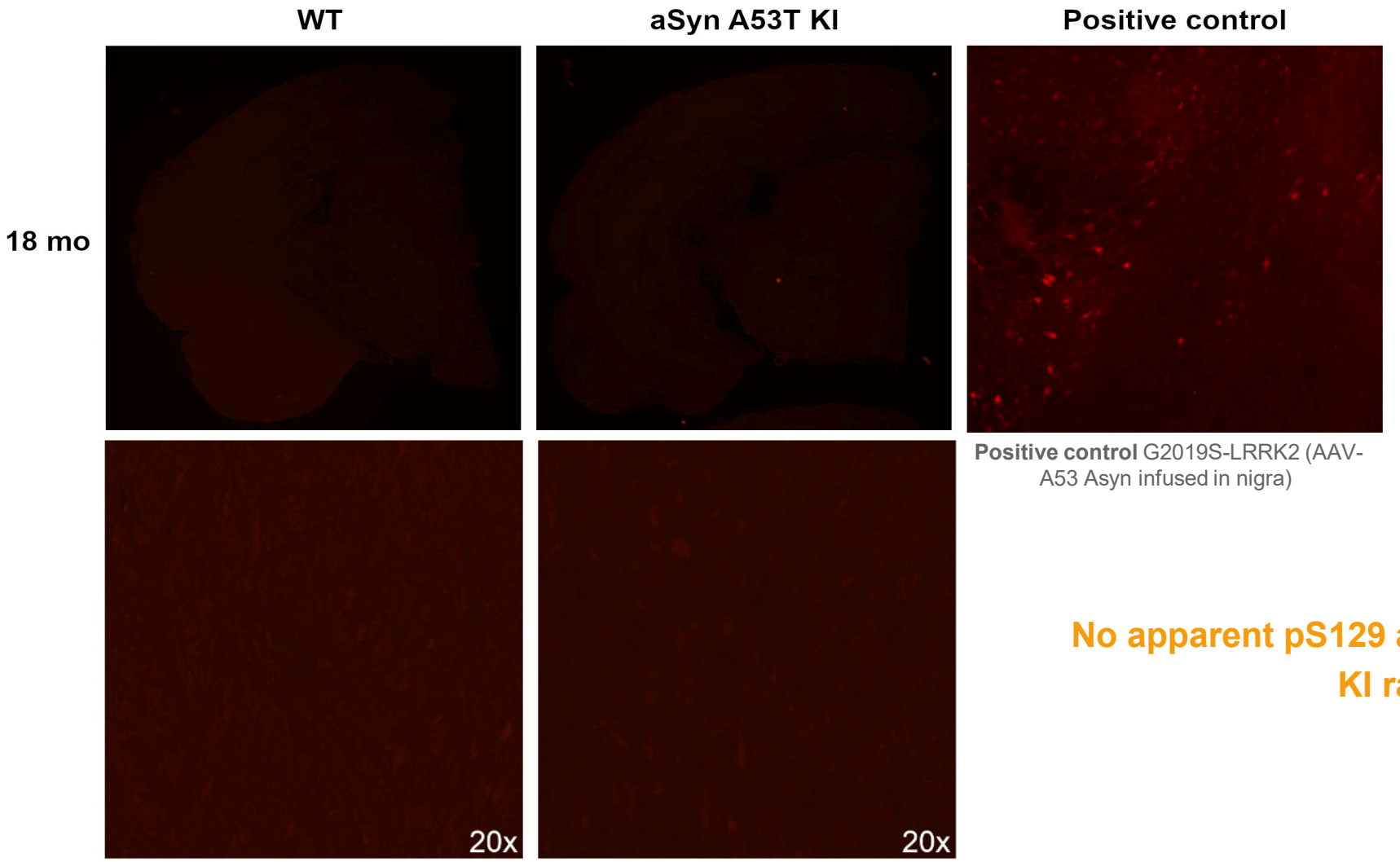
aSyn A53T KI Rat Phenotyping – TH Stereology





No TH cell loss in the SNpc

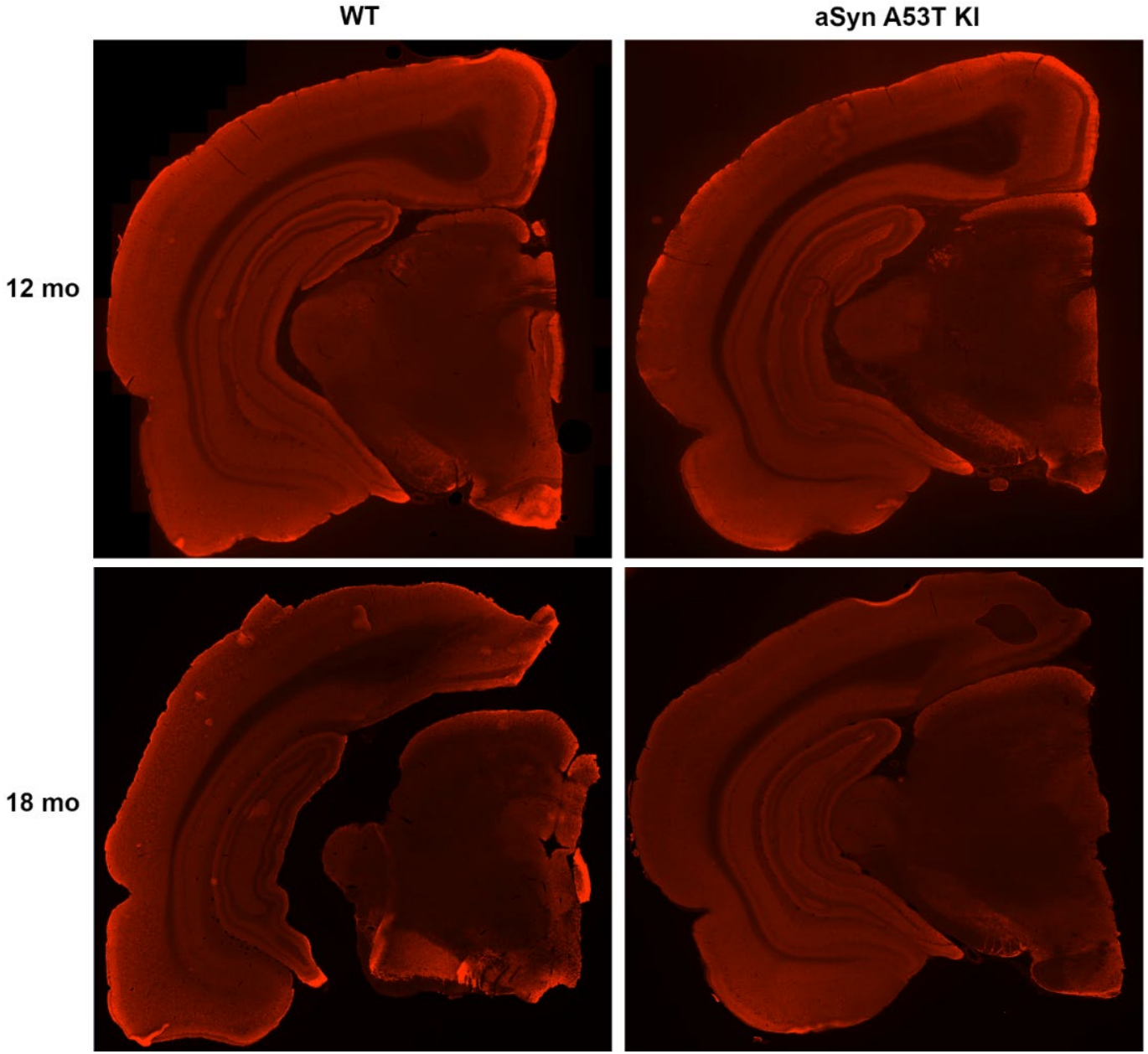
aSyn A53T KI Rat Phenotyping – pS129 aSyn Stain



Positive control G2019S-LRRK2 (AAV-A53 Asyn infused in nigra)

No apparent pS129 aSyn in the SNpc of KI rats.

aSyn A53T KI Rat Phenotyping – Total aSyn Stain



Total aSyn staining present in WT and KI rats.
Does not appear to be increased in KI vs WT.

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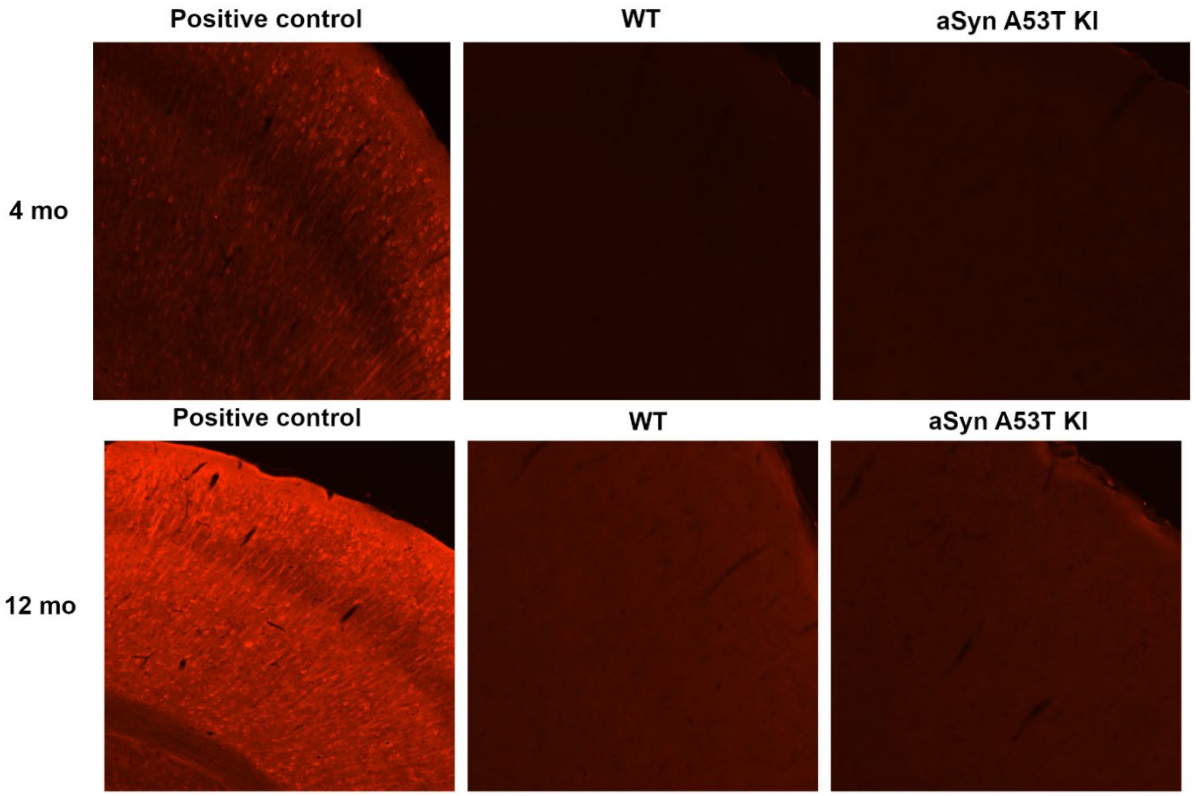
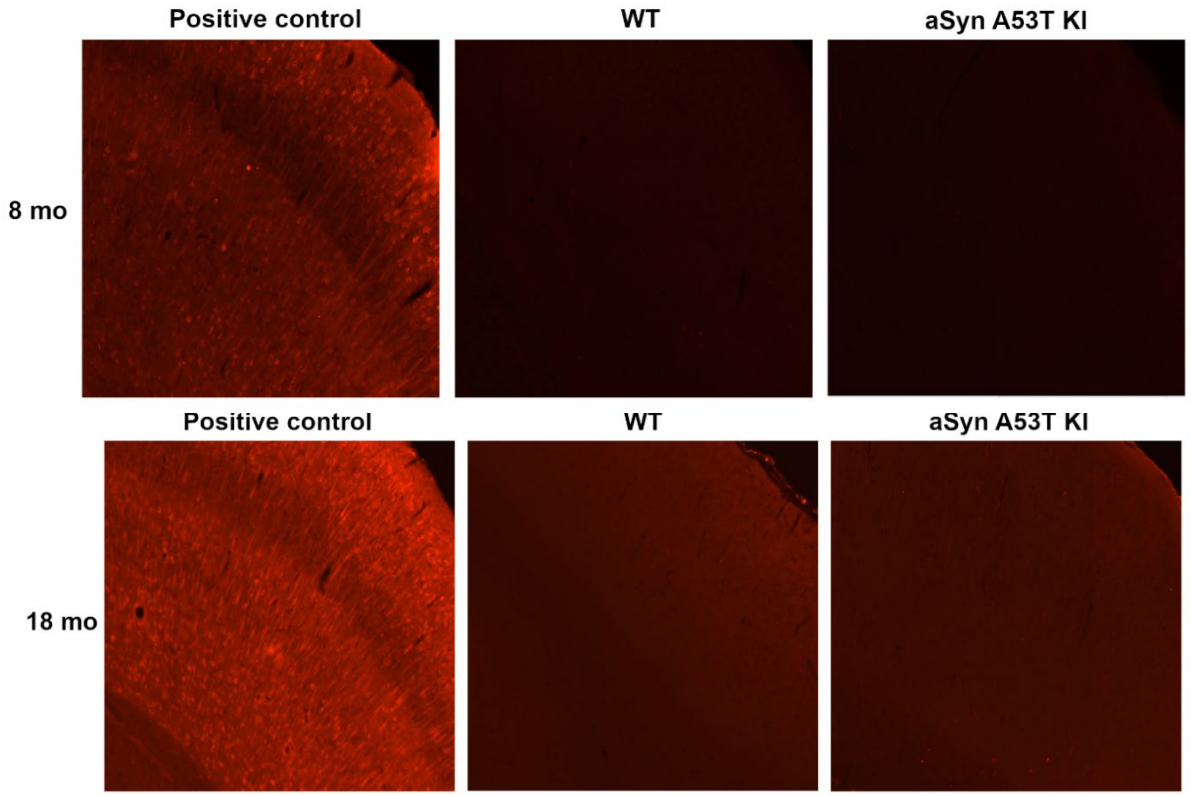
aSyn A53T KI Rat Phenotyping – Phospho-Tau Staining

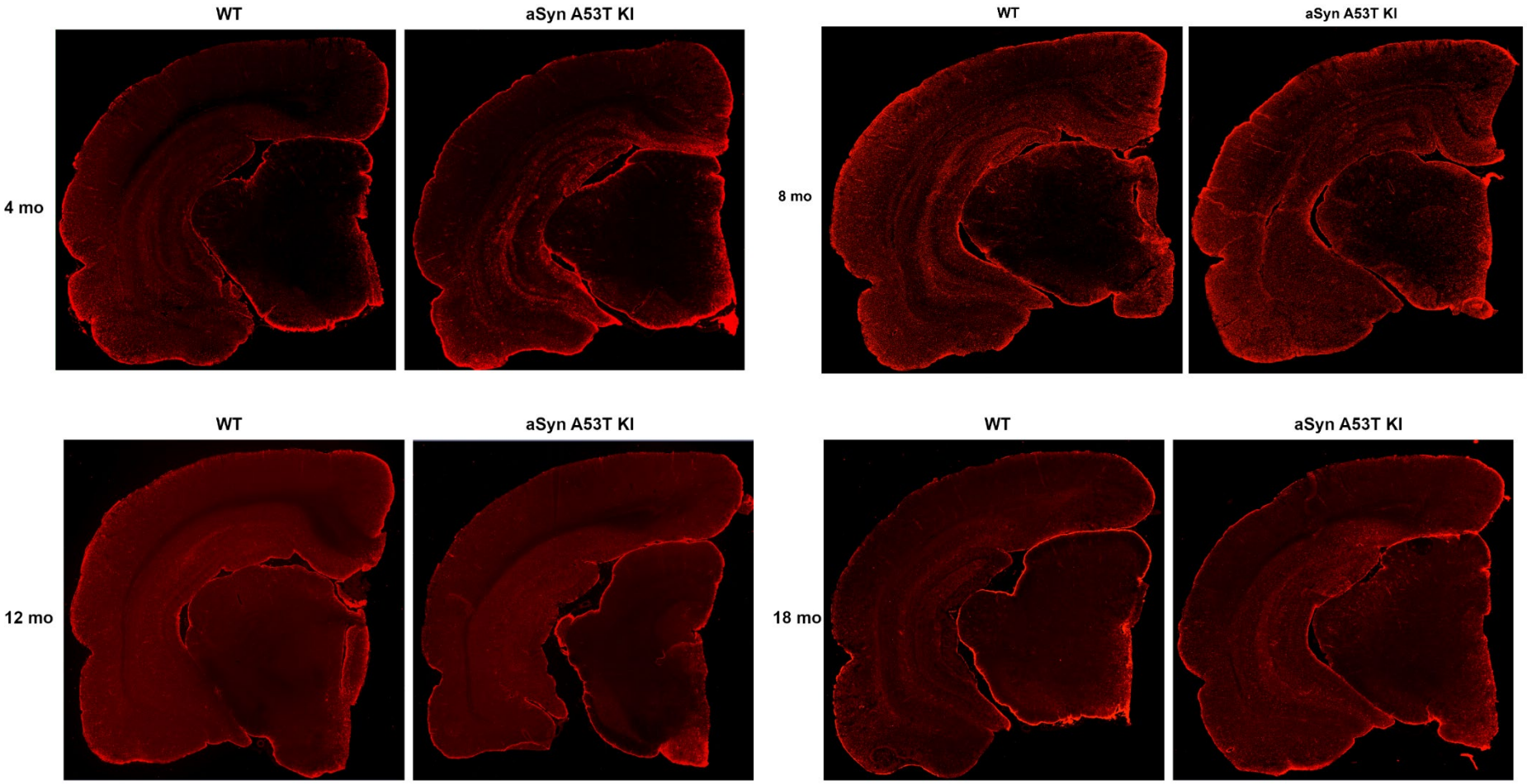




Positive control
p301S Tau
Transgenic

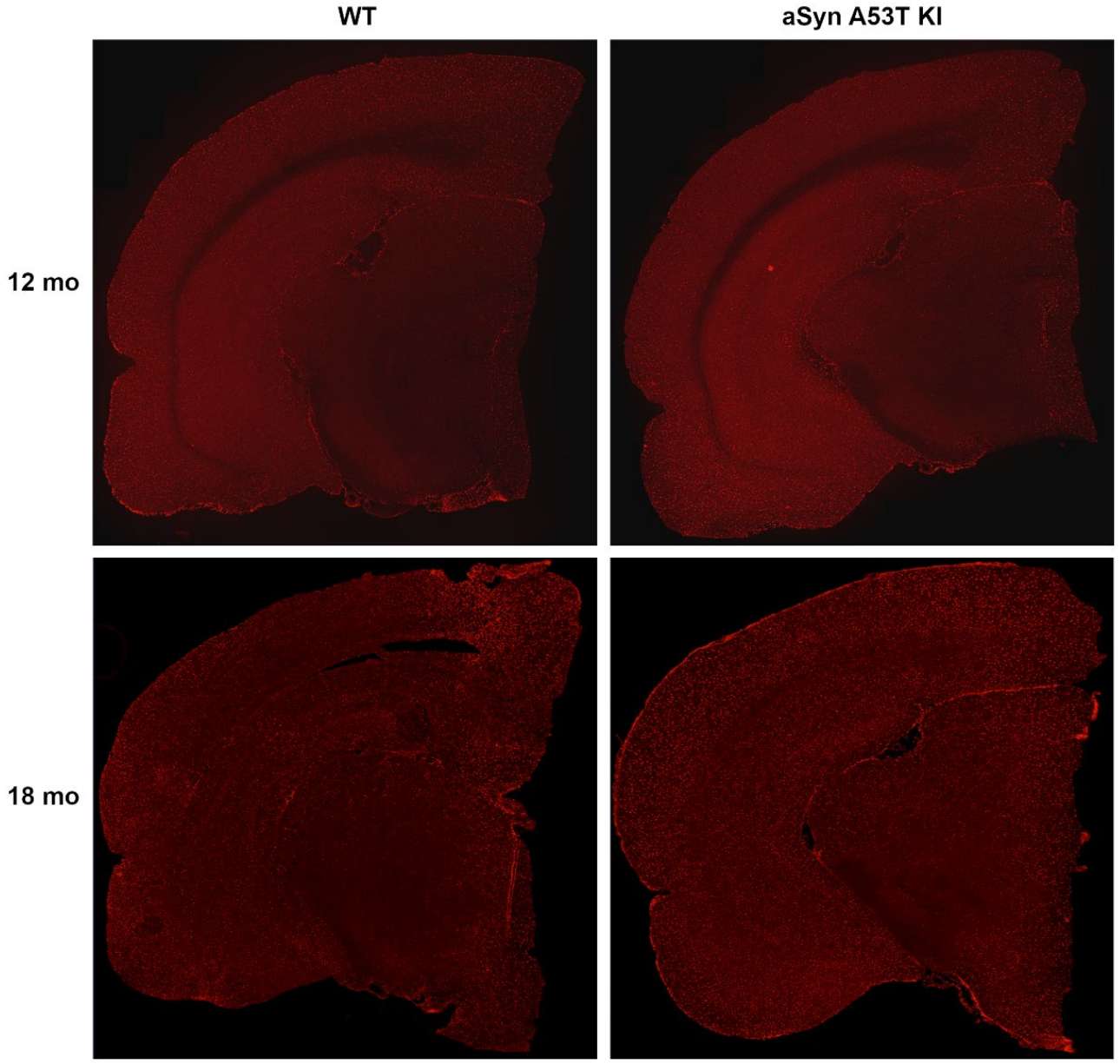
No difference in pTau staining between KI and WT rats

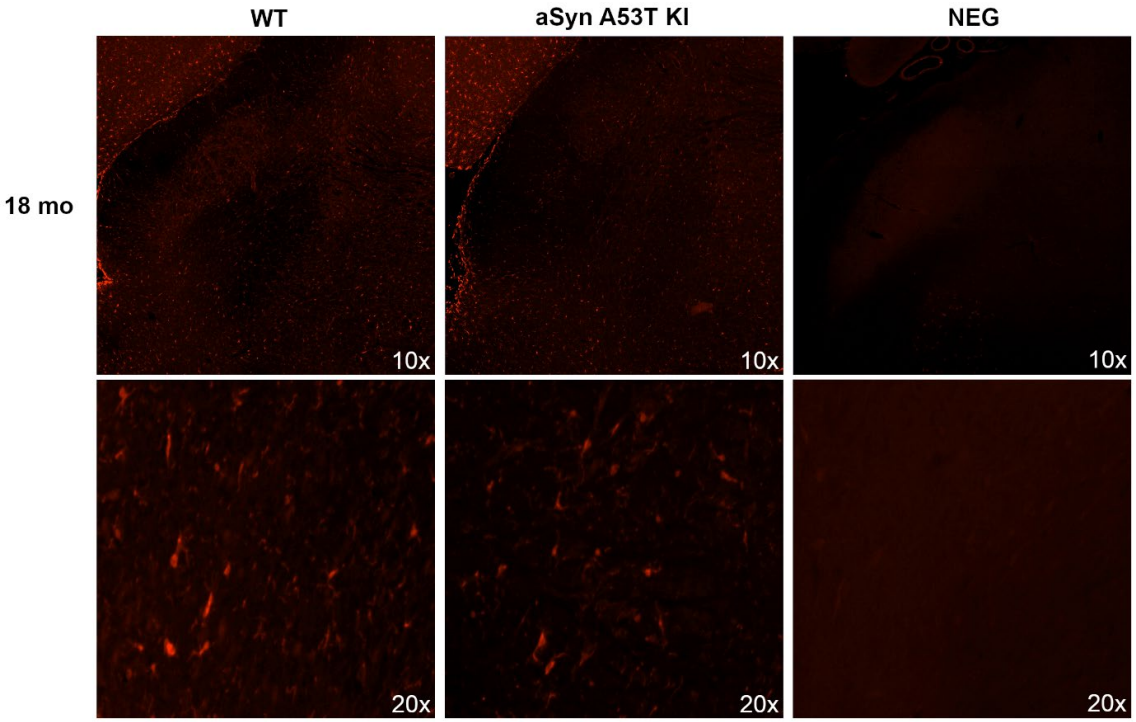
aSyn A53T KI Rat Phenotyping – GFAP Staining



No difference in GFAP staining between KI and WT rats

aSyn A53T KI Rat Phenotyping – Iba-1 Staining





No difference in Iba-1 staining between KI and WT rats.

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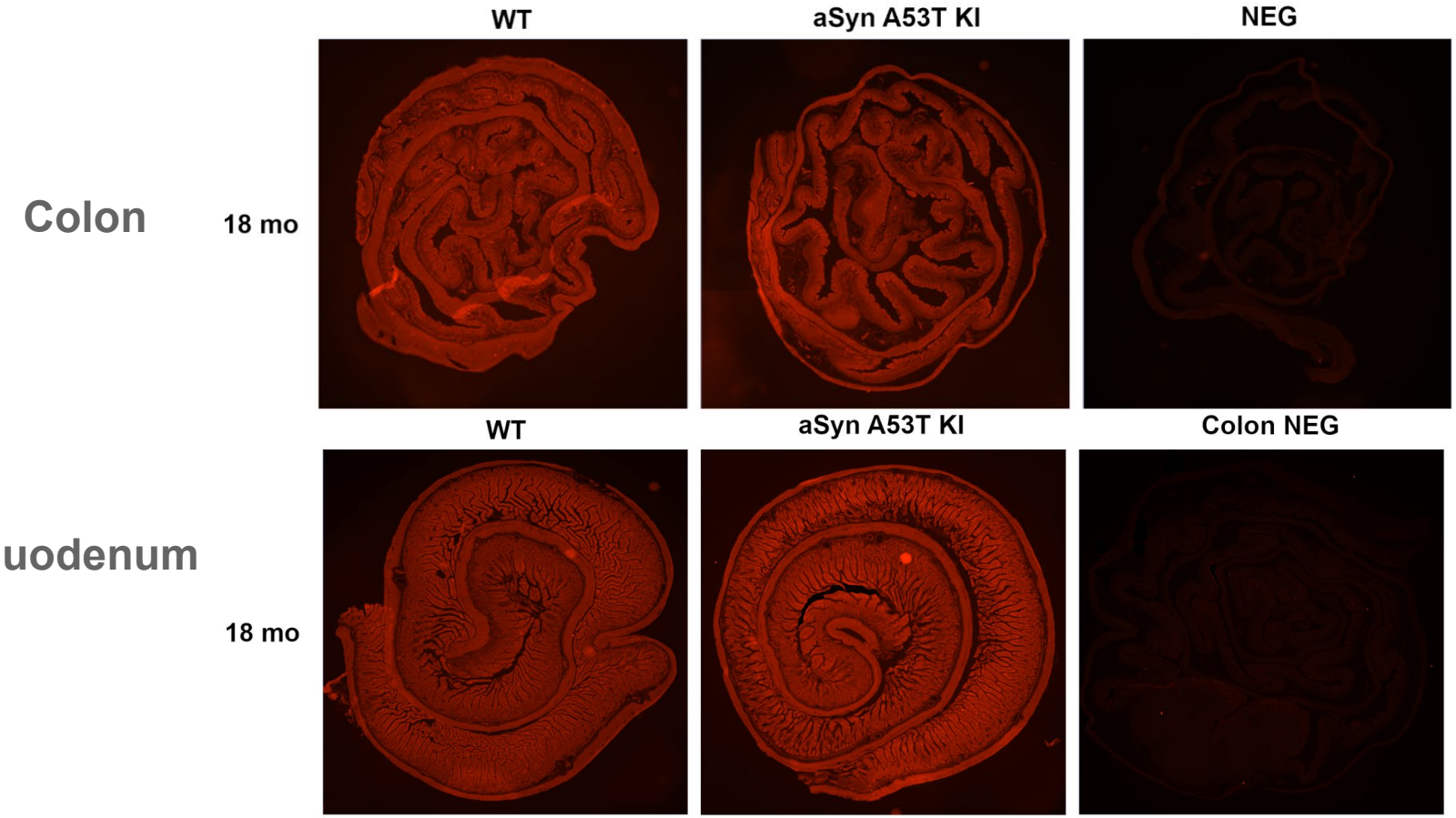
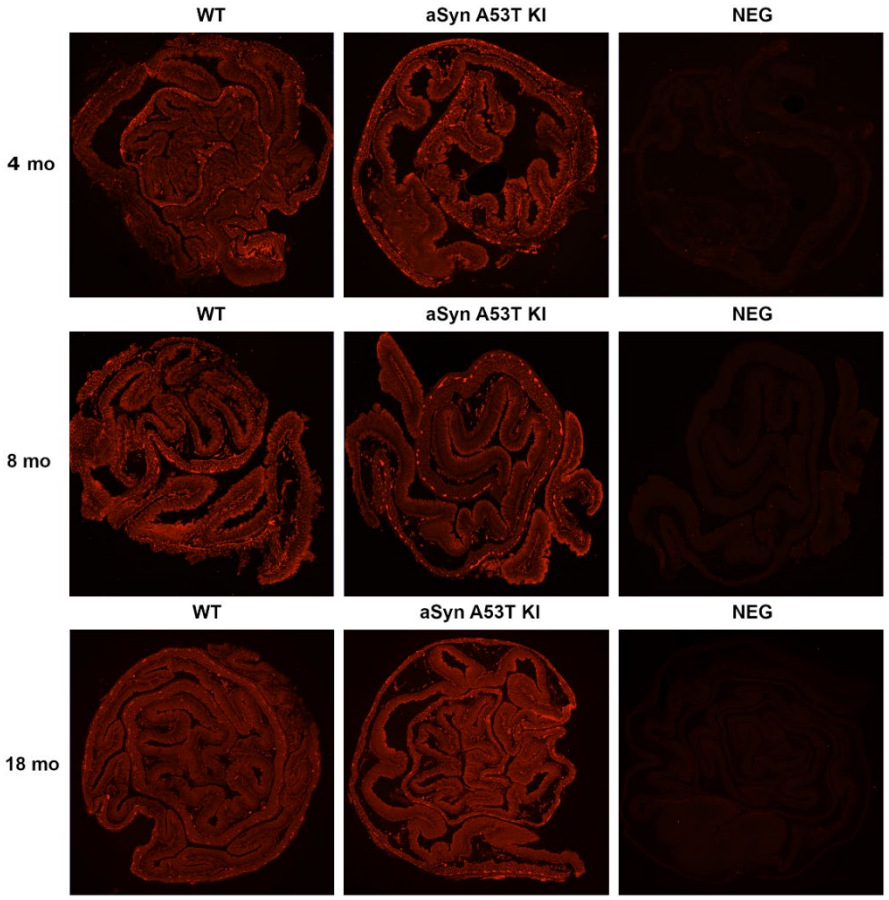
aSyn A53T KI Rat Phenotyping – Gut pS129 aSyn Stain



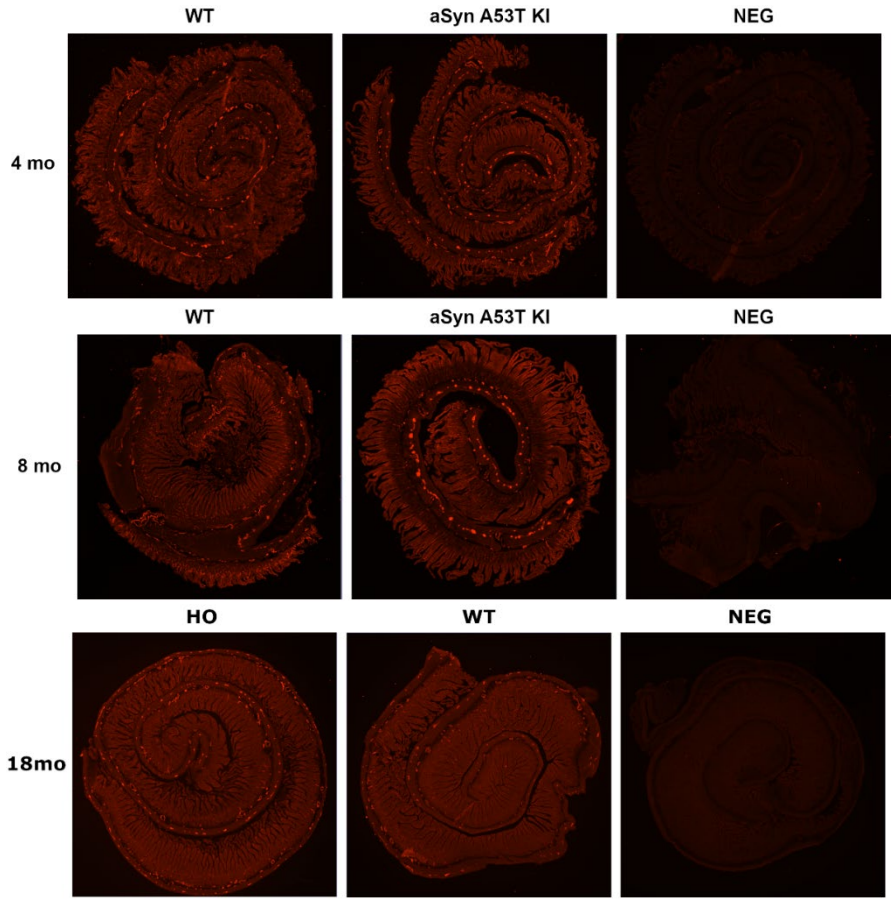
Autofluorescence at 18 months. No apparent difference in pS129 aSyn staining between KI and WT rats.

aSyn A53T KI Rat Phenotyping – Gut Total aSyn Stain

Colon



Duodenum



No difference in total aSyn staining between KI and WT rats

METHODS

METHOD OVERVIEW - BEHAVIOR

Outcome Measures	Method Description
Open Field Test	Exploratory activity was studied in an open field test. Activity chambers (Med Associates Inc, St Albans, VT; 43.2 x 43.2 x 40 cm) were equipped with infrared (IR) beams. Animals were tested at low-stress conditions where the light is lowered to approximately 10-30 lux of red light. Rats were placed in the center of the chamber and their behavior was recorded for 30 minutes in 5-minute intervals. Quantitative analysis was performed on the following measures: distance moved, rearing frequency, time spent in center area, and velocity.
GI Motility	One-hour stool frequency was measured. Testing was repeated for two days in a row. Each animal was removed from its home cage and placed in a clean, clear plastic cage without food or water for one hour. Stools were collected immediately after expulsion and placed in sealed tubes. The total stools were weighed to provide a wet weight, then dried overnight at 65°C and weighed again to provide a dry weight. In addition, number of stools was measured. Bead expulsion time was measured. Animals were fasted for 12 hours before the measurements. Briefly, a single 3 mm colored plastic bead was inserted into the distal colon (3 cm past the anus) with a plastic rod, while each animal was under brief isoflurane anesthesia. Animals were observed for 1 hour to measure time until bead expulsion.
Beam Walk	Sensorimotor functions of forelimbs and hindlimbs were tested using tapered/ledged beam (Zhao et al., Behav. Brain Res. 156 (2005) 85–94). The rats were pre-trained for 3 days to traverse the beam prior to testing day. The beam-walking apparatus consisted of a horizontal 160 cm tapered (square) beam with underhanging ledges on each side to permit foot faults without falling. The end of the beam was connected to a black box (20.5 cm × 25 cm × 25 cm) with a platform at the starting point. A bright light was placed above the start point to motivate the rats to traverse the beam. The rats' performance was videotaped and later analyzed by calculating the slip ratio (the number of slips/number of total steps). Steps onto the ledge were scored as a slip. The mean of three trials was used for statistical analyses.
Fine Motor Kinematics	Rats were analyzed in the MotoRater test using walking mode. On the day of testing, the rats were marked in appropriate points of body, such as joints of limbs and parts of tail to ease the data analysis process. The movement data was captured using a high speed camera (300 frames / second) from three different dimensions, from below and both sides. The captured videos of each rats were converted to SimiMotion software to track the marked points of body to have the raw data i.e. the movement of the different body points in coordinates in relation to the ground, and each of the three dimensions were correlated. Different gait patterns and movements were analyzed using a custom made automated analysis system. The analyzed parameters included: 1) general gait pattern parameters (stride time and speed, step width, stance and swing time during a stride, interlimb coordination), 2) body posture and balance (toe clearance, iliac crest and hip height, hind limb protraction and retraction, tail position and movement), and 3) fine motor skills (swing speed during a stride, jerk metric during swing phase, angle ranges and deviations of different joints, vertical and horizontal head movement).

METHOD OVERVIEW – TISSUE COLLECTION

Outcome Measures	Method Description
Perfusion	All rats were transcardially perfused with saline.
Brain Tissue Collection and Fixation for Histology	Group A (n=5/sex): The left hemisphere was collected in toto and post-fixed with 4% PFA for 24 hours (immersed), then cryoprotected by 30% sucrose in solution for 72 hours (until sunk), after which the brain samples were frozen in liquid nitrogen and used for immunohistochemistry.
Brain Tissue Sectioning	The fixed, cryoprotected and frozen left hemisphere samples were sectioned in the coronal plane at an interval of 200 µm. In each cohort, the brains were cut into 6-8 full series of 20-µm thick cryosections each containing representative sections of cortex, striatum, hippocampus, substantia nigra pars compacta and cerebellum. Sections were collected on Superfrost glass slides. The sections were collected at the following coordinates: **Slide A**: 0.4 mm to -0.4 mm from bregma (striatum and cortex; 5 sections), **Slide B**: -3.2 mm to -4.0 mm from bregma (hippocampus; 5 sections), **Slides C and D**: -4.6 mm to -6.2 mm from bregma (SNpc; altogether 9 sections) and **Slide E**: -9.2 mm to -10.0 mm from bregma (cerebellum; 5 sections)
Gut Tissue Collection and Fixation for Histology	Group A (n=5/sex): The gut was also dissected and processed into duodenum and colon. The small intestine and large intestine samples from the gut were collected from the rats as follows. Specifically: one 3-cm sample from the duodenum (6 cm anal to the pyloric sphincter) and one 3-cm piece from the proximal colon (the first 6 cm distal to the cecum) were collected. After removing the 3-cm gut samples, they were cut open longitudinally (sample was like a sheet) and carefully washed with PBS (intestine contents washed away). These longitudinally cut, PBS-washed intestine were then rolled into a package. In rolling, the opposite way of the intestinal structure was followed (= against the longitudinal axis) to form a roll-like structure. The package was tied with threads prior to post-fixing. Post-fixation step involved immersion of the fixed sample in 4% PFA in 0.1 M PB for a minimum of 24 h. The samples were then transferred to a 30% sucrose solution at +4°C for 48-72 h. After post-fixation, the threads were removed and the samples were frozen in liquid nitrogen and placed in foil on dry ice in order to maintain the original organ structure. The samples were stored at -80°C until sectioning. Duodenum and colon from the same rat were placed in a separate Peel-A-Way® Disposable Embedding Mold with OCT Tissue Tek compound.
Gut Tissue Sectioning	All the collected gut blocks prepared as rolls were sectioned coronally (against the axis of the roll). The duodenum and colon were sectioned separately into 20-µm thick cryosections. All together three series at an interval of 200 µm were collected, with 4 sections per series. One series was used for total aSyn staining, one for phosphorylated aSyn staining and one was reserved for piloting and negative controls.
Brain Tissue Collection for mRNA Analysis	Group A (n=5/sex): The right hemisphere of half the rats was dissected into cortex, striatum, hippocampus, substantia nigra pars compacta and cerebellum for gene expression analysis: These samples were collected as 5-10 mg punches, immersed in 5-10x volume of RNAlater, and stored at +4°C overnight before removing the RNAlater and storing the samples at -80°C until used in the bDNA (QuantiGene) assays.
Brain Tissue Collection for Protein Analysis	Group B (n=5/sex): For the remaining half of the rats, the left hemisphere was dissected into cortex, striatum, hippocampus, substantia nigra pars compacta and cerebellum. All the tissues were weighed, fresh frozen in liquid nitrogen and stored at -80°C until used for WB.
Brain Tissue Collection for Protein Analysis	Group B (n=5/sex): The right hemisphere striatum was dissected, frozen in liquid nitrogen and stored at -80°C until used for HPLC.

METHOD OVERVIEW - HISTOLOGY

<table>
<tr><th>Outcome Measures</th><th>Method Description</th></tr>
<tr><td>Immunostaining</td><td>The used primary antibodies and performed stainings are listed in the below table. In short, the stainings were performed according to Charles River SOPs. All immunohistochemical stainings were piloted prior to performing the actual stainings. All stained sections were scanned with Olympus VS120 slide scanner.
<table>
<tr><th>Stain</th><th>Antibody</th></tr>
<tr><td>Rabbit anti-Glial Fibrillary Acidic Protein (GFAP)</td><td>DAKO Z0334</td></tr>
<tr><td>Rabbit anti-Iba-1</td><td>WAKO 019-19741A</td></tr>
<tr><td>Mouse anti-Phospho-Tau (S202, T205) (AT8)</td><td>Thermo Scientific MN1020</td></tr>
<tr><td>Rabbit anti-Tyrosine Hydroxylase</td><td>Millipore AB152</td></tr>
<tr><td>Cresyl Fast Violet</td><td>Sigma C5042</td></tr>
<tr><td>Rabbit anti-human pS129 alpha-synuclein</td><td>Abcam Ab51253</td></tr>
<tr><td>Rabbit anti-alpha-synuclein</td><td>Cell Signaling 2628S</td></tr>
<tr><td>Goat anti-Rabbit AF568</td><td>Molecular Probes A11036</td></tr>
<tr><td>Goat anti-Mouse AF568 (IgG gamma 1)</td><td>Invitrogen A21124</td></tr>
<tr><td>Biotinylated Goat anti-Rabbit</td><td>Vector BA-1000</td></tr>
</table></td></tr>
<tr><td>TH Stereology</td><td>The optical fractionator method (Gundersen, 1986) was used to estimate the total numbers of TH-positive cells in SNpc. The cells of interest were counted manually with Stereo Investigator software (MicroBrightField, VT, USA). A total of 5 sections from stained series for each animal were used for stereological analysis.</td></tr>
</table>

METHOD OVERVIEW – BIOCHEMISTRY

<table>
<tr><th>Outcome Measures</th><th>Method Description</th></tr>
<tr><td>Branched DNA for Rat and Human SNCA mRNA</td><td>The RNAlater preserved tissue pieces from the 5 brain regions were thawed, cleared of any residual preservative and homogenized using the QuantiGene Sample Processing Kit (Thermo Fisher Scientific) with the TissueLyser II bead homogenizer. The homogenization volumes were adjusted to tissue weight (300 µl of the homogenizing solution + 3 µl of proteinase K per every 5 mg of tissue). The RNA-containing supernatants were then collected according to the manufacturer's instructions for use in the QuantiGene Plex assays. The tissue supernatants were frozen as 2 aliquots at -80°C, where possible, to avoid repeated freeze-thaw cycles.

The target expression levels were determined using custom-prepared QuantiGene Plex sets (see below table) and QuantiGene Plex Assay Kits. The assay was performed according to the instructions provided by the manufacturer (Invitrogen). Small pilots were run before the actual assays to determine optimal sample input for the different tissues. All tissues/samples were run with the original homogenate diluted 1:6 or 1:8, giving all targets in the preferred fluorescence range (above background and below 20,000 FI). The expression levels of the SNCA transcripts were normalized using the geometrical mean of the housekeeping genes Hmbs, Gapdh and Ppib.
<table>
<tr><th>Target symbol</th><th>Species</th><th>Target name</th><th>Accession</th><th>Probe set region</th></tr>
<tr><td>human SNCA</td><td>human</td><td>Humanized A53T SNCA (Snca_knock_in)</td><td>GS03265</td><td>95-548*</td></tr>
<tr><td>rat Snca (upstream)</td><td>rat</td><td>Rat α-synuclein</td><td>NM_019169</td><td>3-309</td></tr>
<tr><td>Rat Snca (downstream)</td><td>rat</td><td>Rat α-synuclein</td><td>NM_019169</td><td>564-1082#</td></tr>
<tr><td>Hmbs</td><td>rat</td><td>Hydroxymethylbilane synthase</td><td>NM_013168</td><td></td></tr>
<tr><td>Gapdh</td><td>rat</td><td>Glyceraldehyde-3-phosphate dehydrogenase</td><td>NM_017008</td><td></td></tr>
<tr><td>Ppib</td><td>rat</td><td>Peptidylprolyl isomerase B</td><td>NM_022536</td><td></td></tr>
</table>
*SNCA knock-in: NM_019169 bases 1-327 + NM_000345 (424-648); designed to produce signal only if the knock-in is present (captured human portion only, but amplified anywhere)
#Outside of the KI region in the last exons</td></tr>
<tr><td>Western Blot</td><td>For tissue homogenization, fractionation, and western blot analysis, we applied a slightly modified version of previously described protocol (Weihofen at al, 2019, Neurobiology of Disease). Tissue was homogenized using FastPrep®-24 device in 8 volumes (v/w) 50 mM HEPES-KOH pH 7.6, 1% Triton X-100, 750 mM NaCl, 5 mM EDTA supplemented with Complete Mini protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche Diagnostics). For fractionation the protein content normalized homogenates were centrifuged at 16,100 g for 45 min at 4 °C. The supernatant was collected (soluble fraction) and the pellet was resuspended in 4 volumes 50 mM HEPES-NaOH 7.6, 1% SDS (insoluble fractionation). Lysates normalized for total protein concentration were then subjected to SDS-PAGE on Bis-Tris NuPAGE 4-12% gradient gels under reducing conditions and transferred to polyvinylidene difluoride membranes. Membranes were fixed with fresh 0.4% paraformaldehyde in PBS for 30 min. Membranes were incubated with 4B12 for human α-syn (Thermo Fisher, MA-90346), syn-1 (BD Bioscience Clone 42) for total α-syn or C4 (Millipore MAB1501) for actin. Bound antibodies were detected and quantified by LICOR infrared imaging system and ImageStudio Lite 5.2.5 software, respectively.</td></tr>
</table>

METHOD OVERVIEW – NEUROCHEMISTRY

Outcome Measures	Method Description
HPLC for DA and DA Metabolites	Samples were received in an aqueous buffer follow homogenization and protein normalization. Samples were derivatized using a slightly modified version of a previously reported method (Song et al., 2012 Analytical Chem 84: 412-419). Briefly, 20ul of diluted homogenate was added to a round bottom 96 well plate. 10 ul of 100mM Sodium Carbonate was added to each sample and agitated by pipetting up and down for 5 seconds. Then, 10 ul of 2% Benzoyl Chloride was added to each sample and again pipetted up and down for 5 seconds. Finally, 10 ul of an internal standard mixture (containing 13C-benzoyl chloride labeled dopamine, HVA, DOPAC) was added to the sample and pipetted up and down. Samples were then loaded onto a Shimadzu Sil-20AC autosampler which injected samples onto a Waters BEH C18 reverse phase column (2mm ID x 5cm long). Samples were separated and eluted over 10 minutes and an AB Sciex 6500 triple quadrupole mass spectrometer was used to detect benzoylated dopamine (466 -> 105), benzoylated HVA (304 -> 105), and benzoylated DOPAC (394 -> 105). Concentrations were calculated using a standard curve and the light:heavy internal standard ratio.

METHOD OVERVIEW – DATA ANALYSIS

Outcome Measures	Method Description
Behavioral Data	Behavioral data was analyzed for aSyn A53T KI vs. WT difference by Welch t-test and Two-Way ANOVA for 4, 8, 12 and 18 time points separately.
Immunohistochemistry	Data from immunohistochemical stainings was analyzed for aSyn A53T KI vs. WT differences by Welch t-test for 4, 8, 12 and 18 time points separately.
Branched DNA mRNA	The non-normalized fluorescence (median) intensity values (MFI) obtained from the Bio-Plex 200 reader, and representing the expression/quantity of each gene, were converted to normalized gene expression values as detailed below. For assay quality control: The limit of detection (LOD) in the assays was considered the signal above the background (average MFI of the assay background control wells plus 3 standard deviations), calculated for each assay plate. Here, signals remaining below the LOD were not removed from analyses, since such signals were in groups that were expected to be low, and exclusion would have decreased the effective n. Thus all values were used for statistical analyses, even though the exact values that remain close to background may not be fully quantitative (e.g. the humanized *SNCA* in the WT animals). Additionally, after removing potential (technical) outliers, the MFI values were corrected to the baseline (each analyte separately) by subtracting the average MFI in the background wells. These net MFI values were averaged, and then normalized by dividing each target gene signal (net MFI) by the geometric mean of the housekeeping genes (averaged and with background subtracted). The statistical significances for the endogenous rat *Snca* and the humanized *SNCA* (Snca_knock_in) as well as the housekeeping genes were performed as pairwise comparisons at each timepoint by the Mann-Whitney U-test. Each tissue was analyzed separately. The significances are shown in the data sets such that the genders are either pooled or the genders are analyzed separately.

METHOD OVERVIEW – PBMC COLLECTION

Outcome Measures	Method Description
PBMC Banking	The whole blood collected upon animal euthanization was kept in the collection tubes at RT until the isolation procedure (max. 3 hours). All the reagents used were taken to RT the previous day to temper them properly. During isolation, each blood sample (~ 2 ml) was divided into 4 SepMate tubes not to exceed their capacity. The isolation was performed according to the manufacturer's instructions. Sepmate is a tube that facilitates the isolation of PBMCs by density gradient centrifugation using density gradient medium. Briefly: 4.5 ml of the density gradient medium was added to the SepMate™ tube by carefully pipetting it through the central hole of the SepMate™ insert. Each sample was diluted with an equal volume of DPBS + 2% FBS and mixed gently. The diluted sample was added by pipetting it down the side of the tube, with the sample mixing with the density gradient medium above the insert. The tubes were centrifuged at 1200 x g at RT for 15 minutes with the brake on. After this, the top layer, which contained the enriched MNCs, was transferred into a new tube. The PBMCs were then washed twice with 1000 µl of sterile DPBS-2% FBS, centrifuging each time at 300 g for 10 min at RT (brake on for pellet formation). After removing the supernatant from the last wash, the remaining pellet was resuspended gently into 500 µl of DPBS-2% FBS to get a clear suspension (the tube was put on ice). For cell counting, a sample of 10 µl was taken and 89 µl HBSS was added. Right before measurement, 1 µl of propidium iodide was added, and the cell number was determined by the MACSquant 10 flow cytometer. After this, the cells were centrifuged at 300 g for 10 min +4°C. After removing the supernatant carefully, the PBMC pellet was stored at -80°C.