Characterization of Humanized A53T Alpha-synuclein (aSyn A53T KI) and Alpha-synuclein KO (aSyn KO) Rat Models

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1 BACKGROUND

Mutations and multiplications in the SNCA gene encoding the alpha-synuclein protein are linked to an autosomal dominant form of Parkinson's disease (PD). One such mutation is the alanine-to-threonine substitution at amino acid 53, leading to early onset PD, possibly through an increased propensity for the A53T alpha-synuclein to aggregate. To better understand the biology of this mutation and develop a preclinical *in vivo* model for PD, we have generated a humanized A53T alpha-synuclein rat model with a non-functional rat *SNCA* gene (aSyn A53T KI). The model was created by applying CRISPR/Cas9 genome targeting strategy where humanized amino acids were inserted for the region spanning amino acids 53-122 in the rat *SNCA* gene. In addition, a rat *SNCA* KO model (aSyn KO) was developed using CRISPR/Cas9 through the insertion of a single base pair to read a premature stop codon.

The aim of this collaborative rat phenotyping effort is to extensively characterize the aSyn A53T KI rat at 4, 8, 12, and 18 months of age as well as assess the biochemical characteristics of aSyn KO rat at 6 and 12 months of age. During the course of this project, aSyn A53T KI rats and their wildtype littermates (mixed gender) are subjected to a battery of behavior tests, including fine motor kinematic analysis, home cage motor activity, open field, tapered beam balance, and GI motility. After the behavioral assessment, various tissue samples are collected from each age cohort for subsequent histological and biochemical analyses. *Ex vivo* outcome measures include expression of *SNCA* gene in different brain regions, immunohistochemical analysis of pS129 aSyn and total aSyn in the gut, and immunohistochemical analysis of the brains for proteinase K resistant aSyn, GFAP, Iba-1, AT8 pS202/pT205 Tau, pS129 aSyn, total aSyn, and tyrosine hydroxylase. In addition, striatal dopamine levels as well as total and soluble/insoluable aSyn will be analyzed. Here, we report interim results for the aSyn A53T KI rats up to 8-12 months. The testing and data analysis for the 12 and 18-month cohorts are still ongoing. Tissue sample collection of 6 and 12-month old aSyn KO rats has been completed and results from the *SNCA* gene expression assay are being presented.

2 METHODS

Fine Motor Kinematic Analysis

Rats were analyzed in the MotoRater test (TSE) using walking mode. The movement data was captured using a high-speed camera. 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).

Open Field

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. Rats were placed in the center of the chamber and their behavior was recorded for 30 minutes.

Beam Walk

Sensorimotor functions of forelimbs and hindlimbs were tested using tapered/ledged beam. 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 rats' performance was videotaped and analyzed by calculating the slip ratio (the number of slips/number of total steps).

Home Cage Motor Activity

Rats were first subjected RF chip implantations. Implanted chips provide an identifier of an animal in a cage in home cage setting where receiver plate (Actual Analytics, UK) is placed underneath the plastic home cage. Transmitting RF chips and receiver plates allow analysis of home cage behavior of rats. Recordings for rats was performed over 72 h period.

GI Motility

One-hour stool frequency was measured. The stools were weighed to provide a wet weight, then dried overnight at 65°C and weighed again to provide a dry weight. Bead expulsion time was measured. Briefly, a single 3 mm colored plastic bead is inserted into the distal colon (3 cm past the anus) with a plastic rod, while each animal is under brief isoflurane anesthesia. Animals were observed for 4 hours to measure time until bead expulsion.

TH+ Cell Counts Using Unbiased Stereology

Fixed, cryoprotected and frozen midbrain samples were sectioned as 20 µm coronal sections at 200 µm intervals through substantia nigra (SN) and ventral tegmental area (VTA) and mounted on slides. Sectioning was started at -4.6 mm from bregma and continued to -6.2 mm from bregma in AP axis. The sections were first thawed and air dried. Anti-TH immunohistochemistry as performed with a standard IHC protocol at CRL DRS. Nissl staining was used as a counterstain. Numbers of TH-positive neurons were determined by counting immunopositive cells through the SNpc.

QuantiGene Multiplex Gene Expression Analyses

Various brain regions (cerebellum, cerebral cortex, hippocampus, striatum and substantia nigra) were collected and preserved with RNAlater, and stored at -80°C until analyzed using the QuantiGene multiplex gene expression assay platform (Thermo Fisher Scientific). Briefly: tissues were lysed using the QuantiGene Sample Processing kits, and the supernatants were used to determine the mRNA expression of the native rat *SNCA* gene (aSyn KO model) or the native rat *SNCA* gene vs. the partial human *SNCA* (aSyn A53T KI model) with custom-prepared QuantiGene Plex sets. The *SNCA* mRNA expression levels were normalized to the geometric mean of 3 different housekeeping genes. Representative data are shown for substantia nigra (aSyn KO) and cerebral cortex (aSyn A53T KI), respectively.

3 RESULTS

Body Weight and Behavior

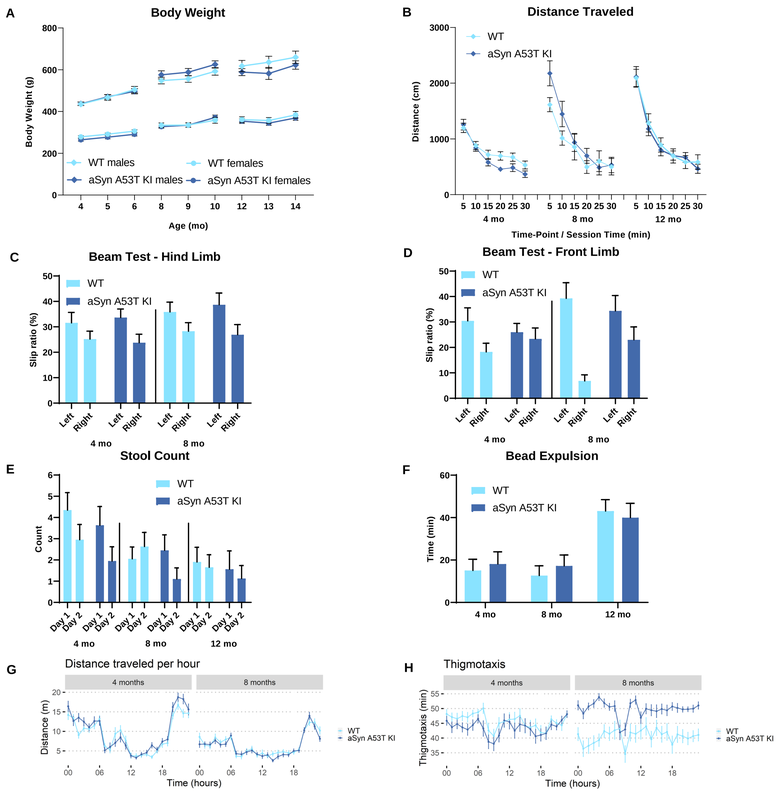

Fig. 1. (A) No differences in the body weight between aSyn A53T KI and WT rats until 14 months of age (n=5-10); (B) No differences in the spontaneous activity between aSyn A53T KI and WT rats until 12 months of age (n=19-20); (C) and (D) No differences in sensorimotor functions between aSyn A53T KI and WT rats until 8 month of age. (n=19-20); (E) and (F) No differences in GI tract motility between aSyn A53T KI and WT rats until 12 months of age (n=19-20); (G) No differences in circadian total activity between aSyn A53T KI and WT rats until 8 months of age; (H) Increased time spent close to cage walls at 8 timepoint in aSyn A53T KI (n=19-20). All data are presented as mean +/- SEM.

Fine Motor Kinematic Gait Analysis

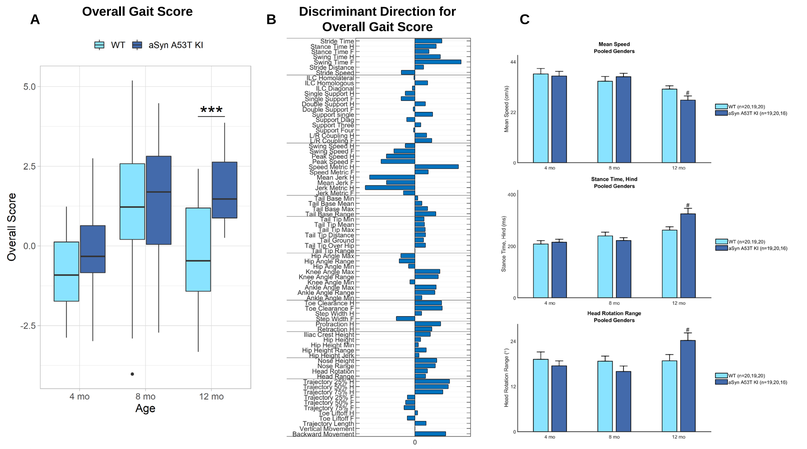

Fig. 2. Fine motor kinematics of aSyn A53T KI and WT rats. The overall gait score (A) is based on discriminant direction vector (B) which emphasizes gait changes associated to aSyn A53T rats in all timepoints: the aSyn A53T rats express slower overall speed, slight changes in paw trajectories and head movements (seen in toe clearance, protraction, trajectory parameters, and in nose and head positions). Separate graphs for mean speed, hind limb stance time, and head rotation range, are shown in (C). Data presented as boxplot (A) or mean +/- SEM (C), n=16-20.

Immunohistochemistry

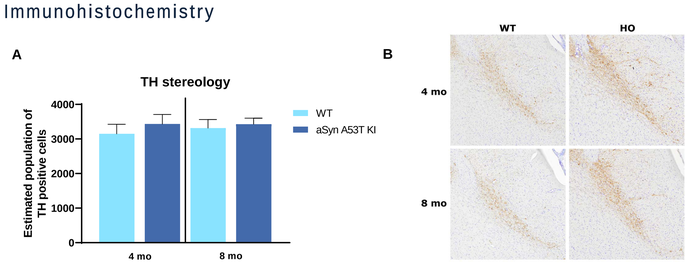

Fig. 3. (A) Estimated TH positive cells in substantia nigra of aSyn A53T KI and WT rats at the ages of 4 and 8 months. No statistical significances between the genotypes (Student's t-test). Data presented as mean +/- SEM, n=8-9; (B) A representative photomicrograph of TH-positive cells in SNpc of the aSyn A53T KI and WT rats at 4 and 8 months, with CFV counterstaining. No clear differences were observed between the genotypes at these timepoints.

Gene Expression Assay (QuantiGene)

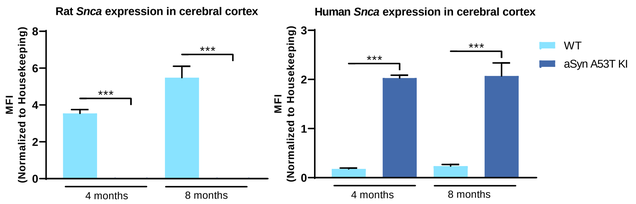

Fig. 4. Phenotype of the aSyn A53T KI rat having a partial human cDNA insertion. The wildtype animals have a robust native rat *SNCA* mRNA expression, whereas the KI animals give a positive signal for the humanized transcript. Data represent means with SEM (pooled genders) in the cerebral cortex (n = 19-20 in each age cohort).

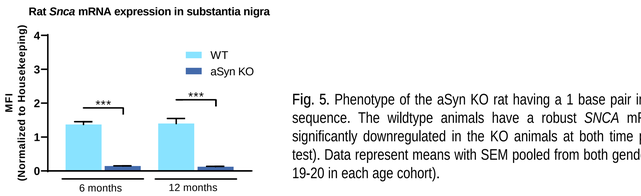

Fig. 5. Phenotype of the aSyn KO rat having a 1 base pair insertion in the SNCA gene sequence. The wildtype animals have a robust *SNCA* mRNA expression, which is significantly downregulated in the KO animals at both time points checked (Welch's t-test). Data represent means with SEM pooled from both genders in substantia nigra (n = 19-20 in each age cohort).

4 CONCLUSIONS

- Overall, the behavioral phenotype and body weight of the aSyn A53T KI rats seem to be very similar to their WT littermates through the timepoints tested. However, according to the highly sensitive fine motor kinematic gait analysis, the aSyn A53T KI rats has significantly altered overall gait score compared to the WT rats at 12-month timepoint. In addition, aSyn A53T KI rats spent more time closer to cage walls at the 8-month time point which could potentially be an indicator of an increased anxiety.
- aSyn A53T KI rats gave a clear signal for the inserted partial human *SNCA* at the mRNA level, whereas they essentially lacked the native rat signal. Conversely, the WT rats had a robust expression of the native rat *SNCA*, although they also produced some residual signal with the human probes, most likely due to partial sequence homology. These data indicate that genotype is reflected in the phenotype as expected.
- There were no differences in the number of TH-positive cells analyzed in the aSyn A53T KI and WT rats either in the 4 or 8-month cohorts. No a-Syn immunoreactivity was observed in the 4-mo cohort (data not shown), and therefore, GFAP, Iba1, proteinase K resistant α-synuclein, AT8 pTau, and pS129 α-synuclein/Total α-synuclein will be reported for the upcoming 12-month cohort.
- The aSyn KO rats exhibited a clear phenotype with significant downregulation of the native rat *SNCA* gene expression at the mRNA level, indicating that transcription and/or processing of the gene product are altered.