

Award Number: W81XWH-17-1-0564

TITLE: Mechanisms of Olfactory Deficits in Parkinson's Disease

PRINCIPAL INVESTIGATOR: Sreeganga S. Chandra

CONTRACTING ORGANIZATION: Yale University, New Haven, CT

REPORT DATE: December 2021

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE December 2021		2. REPORT TYPE Final		3. DATES COVERED 01Sep2017 - 31Aug2021	
4. TITLE AND SUBTITLE Mechanisms of Olfactory Deficits in Parkinson's Disease				5a. CONTRACT NUMBER W81XWH-17-1-0564	
				5b. GRANT NUMBER PD160079	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Sreeganga S. Chandra Charles Greer E-Mail: sreeganga.chandra@yale.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Yale University 15 York Street New Haven, CT 06520-8327				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Most Parkinson's disease patients lose their sense of smell, sometimes decades before they develop the symptoms typically associated with the disease, such as tremors and difficulty walking. Olfactory deficits, hyposmia or anosmia, is now recognized as a feature of early Parkinson's disease and an indication that the disease is progressing insidiously. In this FY16 PRP Focused Idea Award application, we will examine the underlying causes for olfactory deficits seen in Parkinson's patients. The focus of our application is directly in line with the Focus Area of ' <i>Identification and evaluation of mechanisms in early Parkinson's disease involving olfactory, microbiome, gastrointestinal, and/or autonomic nervous systems</i> '. In this grant, we will examine how a protein known as alpha-synuclein, that forms abnormal deposits that are a signature of Parkinson's disease, impacts nerve connections or synapses in the olfactory system. We suggest that in Parkinson's disease, due to the presence of these abnormal alpha-synuclein deposits, both the structure and function of olfactory nerve connections is not proper. We will examine this question through our complementary expertise in the olfactory system and alpha-synuclein biology, using high resolution microscopic methods on mouse models that express mutant forms of alpha-synuclein that are linked to the disease. When we have successfully finished this study, we anticipate having a detailed picture of the synaptic abnormalities in the olfactory system in an animal model of early Parkinson's disease. This will allow us to treat the loss of smell in early Parkinson's disease patients in 5-10 years. But more importantly, as the nose and the olfactory system are thought to be a conduit through which alpha-synuclein deposits spreads, eventually reaching the mid-brain, a greater understanding of this pathway will permit us to slow the progression or even prevent Parkinson's disease. This is the ultimate goal of all Parkinson's disease research.					
15. SUBJECT TERMS Hyposmia, Olfactory, Alpha-synuclein, Adult Neurogenesis, Parkinson's disease, Transgenic, Confocal, Electron Microscopy					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 79	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
Introduction	2
Keywords	2
Materials and Methods	4
Results	11
Discussion	23
Conclusions	27
Acknowledgments	28
Funding	28
References	28

Olfactory bulb is differentially susceptible to α -synuclein pathology leading to olfactory deficits in a mouse model of Parkinson's disease

Eduardo Martin-Lopez^{1,3}, D.J. Vidyadhara^{2,3}, Teresa Liberia^{1,3}, Sarah J. Meller^{1,3}, Leah E. Harmon^{2,3}, Ryan M. Hsu^{1,3}, Betül Yucel^{2,3}, Sreeganga S. Chandra^{2,3*} and Charles A. Greer^{1,3*}

¹Department of Neurosurgery, ²Department of Neurology, and ³Department of Neuroscience. Yale University School of Medicine, 310 Cedar Street, New Haven, CT 06510, USA

*Joint corresponding authors: Charles A. Greer: charles.greer@yale.edu and Sreeganga S. Chandra: sreeganga.chandra@yale.edu

Abstract

Parkinson's Disease (PD) is characterized by multiple symptoms including olfactory dysfunction. However, the mechanisms underlying olfactory dysfunction in PD remain unclear. Here, we explored pathological changes in the olfactory pathway of transgenic (Tg) mice expressing the human mutant A30P α -synuclein (α -syn) (α -syn-Tg mice) at 6-7 and 12-14 months of age, representing early and late-stage PD progression, respectively. α -Syn-Tg mice at late stages exhibited olfactory behavioral deficits, which correlated with severe α -syn pathology in all projection neurons throughout the olfactory pathway. The adult olfactory system has sustained neurogenesis in the subventricular zone where interneurons connected to the olfactory bulb (OB) are generated. Notably, in α -syn-Tg mice there was a reduction in neurogenesis at 6-7 months for granule cells, and at 12-14 months for periglomerular cells, both of which could contribute to olfactory dysfunction. Proteomic analyses showed a disruption in endo- and exocytic pathways in the OB during the early stages which appeared exacerbated at the synaptic terminals when the mice developed olfactory deficits. Our data suggest that (1) the α -syn-Tg mice recapitulates the olfactory functional deficits seen in PD; (2) olfactory structures exhibit spatiotemporal disparities for vulnerability to α -syn pathology; (3) α -syn pathology is restricted to projection neurons in the olfactory

pathway; (4) neurogenesis in adult α -syn-Tg mice is reduced; and (5) synaptic endo- and exocytosis defects in the OB may further explain olfactory deficits.

Keywords

α -synuclein, A30P, Parkinson's disease, olfactory system, neurogenesis; endocytosis; α -synuclein transgenic mice, presynaptic proteins, olfactory deficits

Introduction

Parkinson's Disease (PD) affects quality of life due to disparate motor and non-motor clinical symptoms. A common non-motor symptom in PD is hyposmia or anosmia which is seen in 75-90% of affected individuals (Constantinidis and de Ajuriaguerra, 1970; Ansari and Johnson, 1975; Haehner et al., 2009; Doty, 2012; Mahlknecht et al., 2016). Due to its high prevalence, anosmia is now included as a diagnostic criterion for PD by the Movement Disorder Society (Mahlknecht et al., 2016). Yet, the mechanisms underlying olfactory dysfunction in PD remain unclear.

PD is pathologically characterized by the neuronal accumulation of insoluble, cytoplasmic protein inclusions known as Lewy bodies (LBs) (Lewy, 1912; Braak et al., 1994; Del Tredici and Braak, 2013). The formation of LBs is associated with synaptic dysfunction and neurodegeneration (Mahul-Mellier et al., 2020) (Braak et al., 2003b). LBs consist mainly of α -synuclein (α -syn), a synaptic protein that regulates synaptic vesicle exo- and endocytosis (Spillantini et al., 1997; Vargas et al., 2014). α -Syn can assemble into multimers that form neurotoxic oligomers and fibrils (Shults, 2006; Wakabayashi et al., 2013). Pathological α -syn accumulation is accompanied by phosphorylation of the protein at serine 129 (pSer129- α -syn) (Okochi et al., 2000; Anderson et al., 2006) that increases with disease progression (Gorbatyuk et al., 2008; Walker et al., 2013). Indeed, ~90% of α -syn in LBs is phosphorylated at this position (Fujiwara et al., 2002; Samuel et al., 2016) and pSer129- α -syn antibodies are used to identify α -synuclein pathology in animal models of PD (Gentzel et al., 2021). In the olfactory system, increased levels of pSer129- α -syn are found in postmortem human brain tissues (Lue et al., 2012).

The events triggering α -syn pathology remain speculative, but there is evidence suggesting that α -syn misfolding and aggregation may originate in the olfactory epithelium and gastric system, after which the disease propagates to the brain via olfactory and enteric pathways (Braak et al., 2003a; Hawkes et al., 2007; Dunning et al., 2012; Svensson et al., 2015; Saito et al., 2016; Borghammer, 2018). Although environmental factors are reported to be involved in α -syn misfolding and PD pathogenesis (Kouli et al., 2018; Heinzel et al., 2021), other evidence point to mutations in genes including *PARKIN*, *PINK1*, *GBA*, *LRRK2* and *SNCA* as a cause of PD (Ferreira and Massano, 2017; Kouli et al., 2018; Simon et al., 2020). Among these, point mutations in the gene *SNCA* encoding for α -syn (A30P, A53T, E46K, G51D and H50Q) generate aggressive, fully penetrant, early-onset forms of PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004; Lesage et al., 2013; Proukakis et al., 2013). These and other mutations have been used to generate animal models to study familial forms of PD (van der Putten et al., 2000; Crabtree and Zhang, 2012).

In this study, we used a mouse model that overexpressed human A30P mutant α -syn (Chandra et al., 2005) (α -syn-Tg mice) to investigate the cellular and molecular mechanisms that lead to olfactory dysfunction. We followed disease progression by examining α -syn-Tg mice at 6-7 months of age and 12-14 months representing early and late stages of PD, respectively. α -Syn-Tg mice at late stages exhibited olfactory behavioral deficits, which correlated with severe α -syn pathology in all projection neurons throughout the olfactory pathway. Our analysis indicated that adult neurogenesis, which is characteristic of the adult olfactory system, was significantly reduced. To gain insights into the emergence of olfactory dysfunction in the α -syn-Tg mice, we performed a proteomic analysis which revealed decreased expression of proteins involved in exocytosis and endocytosis of synaptic vesicles in the olfactory bulb (OB) of both 6-7 months and 12-14 months α -syn-Tg mice. Our data suggest a preferential accumulation of α -syn in projection neurons throughout the olfactory pathway which, when coupled with decreased

neurogenesis and a decrease in synaptic exo and endocytic proteins, are likely to account for the olfactory dysfunction in the α -syn-Tg mice.

Materials and Methods

Mice

All experiments were performed with C57BL6/J congenic Thy-1 human A30P α -syn mice (α -syn-Tg) and their wild-type control littermates (WT) (Chandra et al., 2005). Analyses were carried out on either 6–7 months or 12–14 months using sex balanced cohorts of mice. Mice were raised and housed at Yale University vivarium on a 12 h light-dark cycle with access to standard chow *ad libitum*. All protocols were approved by Yale University Institutional Animal Care and Use Committee.

Buried food tests

To detect olfactory deficits and anosmia, we conducted two standardized buried food tests to optimize the detection of any anomalies in olfactory function in the early vs. late stages (Lehmkuhl et al., 2014; Machado et al., 2018; Marin-Pardo and Gimenez-Llort, 2021). Both tests are designed to determine the ability of mice to detect a food reward hidden in the bedding of clean housing cages.

Briefly, on day 1 (*habituation day*) 6-7 month old mice (WT: n=7 including 3 males and 4 females; α -syn-Tg: n=7 including 5 males and 2 females) were placed individually in Allentown PC7115 polycarbonate clean cages (Acecaging, USA) with a floor area of 451 cm² (69.90 in²) and filled with 3 cm-depth of 1/8" corncob bedding (Teklad, 7092). A piece of cheese flavored cookie (Better Cheddars, Nabisco) was left on the surface of the bedding and mice were allowed to habituate to the cookie smell for 5 min (*odor familiarization*). Before day 2, mice were partially deprived of food by providing them with 1.82 g of standard lab pellets (7 Kcal/mouse). On day 2 (*pre-test partially food deprivation test*), a piece of cheese cookie was hidden inside the bedding along the perimeter of the testing cage and the latency (in seconds) to find the cookie was recorded (trial 1). A latency longer than 300 sec. was

considered “failed” and mice were scored with 300 sec. Four identical trials were conducted with an interval of 1 h between trials. The whole bedding of each cage was changed between trials and the cookie was buried on different locations between days and trials. Mice were then fully deprived of food for 24 h, and on day 3, *score latency olfactory tests* were conducted identically to day 2. Weights were monitored daily due to food restrictions and performance between WT and α -syn-Tg males and females was compared to evaluate the influence of sex in the olfactory performance. The latency scores for each animal were calculated as the average between the trials and testing days. To exclude a bias produced by lack of motivation for food or because of motor deficits, a surface food test was also performed as previously described (Johnson et al., 2020). Briefly, a piece of the same cheese cookie was placed in a specific location of the surface of each cage with clean bedding, and mice were dropped in the opposite side of the cage. The latency to find and showing interest for the food was recorded for each individual animal.

Mice at 12-14 months (WT: n=6 including 4 males and 2 females; α -syn-Tg: n=8, all males) were tested using the equivalent protocol with a buried sweetened cereal. Tests were carried out during 3 consecutive days in Sealsafe Plus GM500 polysulfone clean cages (Techniplast) with a floor area of 501 cm² (77.66 in²) using a piece of sweetened cereal (Chocolate Cheerios, General Mills). On day 1 (*odor familiarization*), mice were exposed to a piece of cereal placed in each cage and the cereal was left for consumption overnight. Only those mice that consumed the cereal were selected for the analysis. On day 2 (*pre-test food deprivation*), mice were deprived of food for 24 h and weighed. On day 3 (*score latency olfactory test*), mice were weighed, and 3 consecutive trials were conducted as follows: (1) mice were habituated to the environment by placing them in a clean cage with 3 cm-depth of clean 1/8” corncob bedding (Teklad, 7092) for 60 min., after which mice were returned to home cage; (2) an olfactory test was carried out by burying inside the bedding a piece of cereal in a selected location in the cage, to then drop each animal in the opposite side of the burying site. Latencies to find the cereal were recorded and mice that took longer than 300 sec. to find the cereal were scored as 300 sec. (trial 1); (4) a second trial was performed identically by burying a second piece of cereal in a different corner of the cage (trial 2);

and (5) a third trial was conducted in an identical manner. The latency scores for each animal were the average between the 3 trials. For each animal, this test was repeated for 3 weeks, and the scores were averaged. A surface food test was also carried out to exclude bias due to lack of motivation for the food or motor deficits.

Tissue processing and immunohistochemistry

To obtain tissues for immunohistochemistry (IHC), mice were deeply anesthetized with an overdose of Euthasol (Virbac) and transcardially perfused first with 5 mL saline solution, followed by 1 mL/g of body weight of ice-cooled 4% paraformaldehyde (PFA) in PBS. Fixed brains were removed from skulls and postfixed for 24 h at 4°C. The brains were then transferred to 30% sucrose-PBS for cryoprotection and embedded in Tissue-Tek OCT compound (Fisher Scientific, Cat# 4585) for sectioning. Sections 25 µm thick were obtained using a Reichert Frigocut cryostat (E-2800) in the coronal plane, then air dried and stored at -80°C until use.

Before staining, sections were thawed in a slide warmer at 60°C for 10 min. and rehydrated in PBS for 10 min. An antigen unmasking step was conducted by incubating sections in 0.01M citrate buffer (pH 6.0) at 65°C for 35 min., followed by 5 min. incubation in ice-cooled citrate buffer to perform a heat-shock. After unmasking, slides were washed three times, 10 min. each with PBS + 0.1% Triton X-100 (Millipore-Sigma, Cat# x100) (PBST), and sections were incubated with blocking solution made of PBST supplemented with 5% normal goat serum (NGS) (Accurate Chemicals, Cat# CL1200) and 0.01% of protease-free bovine serum albumin (BSA) (Sigma-Aldrich, Cat# A3059) for 1 h at room temperature (RT). The blocking solution was removed, and sections were incubated overnight with primary antibodies (Table 1) diluted in 10% diluted blocking solution at 4°C. Sections were rinsed three times for 10 min. with PBST and incubated with secondary antibodies (Table 1) diluted in PBST, supplemented with 1 µg/mL of DAPI (Invitrogen, Cat# 1306) at RT for 2 h. Sections were washed three times with PBST, and slides mounted with Mowiol 4-88 (Sigma-Aldrich, Cat# 81381) prepared in 0.1M tris-glycerol. Imaging was done using a Zeiss LSM-800 confocal at 10/20/40X magnifications.

Electron microscopy

For electron microscopy (EM) analysis, we used 12-14 months WT and α -syn-Tg mice (n=3). Briefly, mice were administered an overdose of Euthasol (Virbac) and transcardially perfused with saline solution followed by 1 ml/g body weight of iced cooled 4% paraformaldehyde (PFA) + 0.2% glutaraldehyde in phosphate buffered saline (PBS) to fix the tissues. After overnight post-fixation at 4°C, 50 μ m-thick coronal sections were obtained in a vibratome (Pelco 101). Next, sections were treated with 4% osmium (Electron Microscopy Sciences, 19160), dehydrated with graded alcohols, and stained with uranyl acetate (Electron Microscopy Sciences, 22400) and lead citrate (Electron Microscopy Sciences). For micro-dissection, sections were embedded in EMbed 812 resin (Electron Microscopy Sciences, 14900), and both the OB and anterior piriform cortex (APC) were sectioned at 0.04 mm in an ultramicrotome (UltraCut-E). Microsections were mounted on formvar-coated slot grids (Electron Microscopy Sciences) and images were taken using a JEOL electron microscope (JEM-1200 EX II) at the level of the glomeruli in the OB, and layer I in APC with 12,000X magnification.

BrdU injections and detection

Neurogenesis in the OB was studied using the thymidine analog, 5-bromo-2'-deoxyuridine (BrdU) (Wojtowicz and Kee, 2006), prepared freshly before injections by diluting powdered BrdU (RPI, Cat# B74200) in sterile 1X-Dulbecco's PBS solution (Gibco, Cat# 14190-144) at 37°C for 5 min. to obtain a 5 mg/mL solution. Then, mice from the early (6-7 months, n=5/genotype) and late (12-14 months, n=3/genotype) experimental groups received two intraperitoneal injections of 50 mg/kg of BrdU, separated by 2 hours between 1 pm-3 pm to account for circadian variation.

At 25 days post-injection (DPI), mice were perfused, brains cryoprotected and tissues sectioned for IHC (see above). To detect BrdU, sections were thawed at 60°C for 10 min., rehydrated in PBS, and the antigen unmasking was made simultaneously to the DNA denaturalization by incubating sections in a 0.02M HCl at 65°C for 35 min. to break the DNA, followed by an incubation in the same solution in ice for 5 min. Next, acid was neutralized by incubating the sections in 0.1M boric acid-borax buffer (pH 8.6)

for 10 min. at RT. The subsequent steps including blocking solution and primary/secondary antibodies incubations were performed identically as described above.

Proteomics by LFQ-MS

Tissues were obtained from OB and APC of WT and α -syn-Tg mice at 6-7 months (n= 3/genotype) and 12-14 months (n=3/genotype). Briefly, mice were euthanized with an overdose of 5% isoflurane (Covetrus) and brains were rapidly extracted from skulls. Working on an ice-cooled petri dish, the left and right OBs and APCs were dissected from each animal using a scalpel and a 45° curved forceps (Dumont #5/45). Tissues were immediately frozen in liquid nitrogen and tissues from the left hemisphere were used for proteomic analysis. LFQ-MS was performed at the Yale Mass Spectrometry & Proteomics Resource of the W.M. Keck Foundation Biotechnology Resource Laboratory. Samples were analyzed as technical triplicates. The raw mass spectrometry data will be publicly available upon publication in the PRIDE depository. The data was normalized to internal controls and total spectral counts. Proteins with two or more unique peptide counts were listed using UniProt nomenclature and included for further analysis. A 1.25-fold change and a p-value difference of <0.05 between WT and α -syn-Tg mice are considered as significant. Heat maps for significantly changed proteins were produced using Qlucore Omics Explorer. Volcano plots were produced using GraphPad Prism 9.2 software. Gene ontology (GO) enrichment analysis (Ashburner et al., 2000; Mi et al., 2019; Carbon et al., 2021) was used to determine significantly affected biological pathways. Pathways with a false discovery rate of < 0.05 were considered as significantly enriched. Graphs depicting significantly affected pathways were produced using GraphPad Prism.

Western Blots

Western blots were performed using tissues from the right hemisphere obtained as described above. SDS-PAGE and western blots were performed using standard protocols, and images were collected using a LI-COR Odyssey imaging system. Densitometry values were collected using ImageJ. Western blot signal quantifications are represented as mean $\pm$ SEM of the endogenous/human α -synuclein, and

aggregated α -synuclein (Table 1) detected signal. As loading control, we labeled membranes against beta-actin (Table 1).

Quantifications and statistical analyses

Data from olfactory tests were first used to independently compare latency times between males and females in the WT and α -syn-Tg groups, and the data were analyzed using an unpaired non-parametric Mann-Whitney U test. Latency data between WT and α -syn-Tg groups were compared separately for the early (6-7 month) and late (12-14 month) groups using an unpaired non-parametric Mann-Whitney U test. For easier representation of the data in graphical form, latency time data were normalized between 0 (minimum latency time = 0 sec.) and 1 (maximum latency time = 300 sec.).

α -Syn pathology along the olfactory pathway was quantified by measuring the stained area for aggregated α -syn and pSer129 α -syn. Both markers were automatically quantified in the OB and APC layers by using Fiji (ImageJ) software with the MorphoLibJ integrated library and plugin (Legland et al., 2016). We first isolated the region of interest (ROI) on a 16-bit maximum projection of a 159.72 x 159.72 μ m confocal image of DAPI nuclear staining with a freehand drawing tool. We used DAPI channel to prevent bias when drawing the outline of the layers, and a mask of the drawn ROI was then applied to the corresponding channels stained with antibodies to quantify stained objects within this ROI. The uneven illumination was corrected by applying a “rolling ball” algorithm with a radius of 100 pixels. To address the noise of the aggregated α -syn and pSer129- α -syn channels, we applied the “remove outliers” function with a pixel radius of 2 and threshold of 50. To segment aggregated α -syn and pSer129- α -syn stained pixels from background, we used the Fiji automatic MaxEntropy threshold function. Due to the fainter staining within OB sections, we used the Fiji automatic “Triangle” threshold function in these sections instead. After making a mask of the images, the percent area occupied by aggregated α -syn and pSer129- α -syn segmented objects within the drawn ROI was quantified by applying the “Analyze Particles”

function with a minimum object area of 5 pixels. The stained area for both aggregated α -syn and pSer129- α -syn were compared using unpaired t-tests.

From the EM study, we quantified the numbers of mitochondria and synapses (total, asymmetric, symmetric, and reciprocal) from images taken in the two regions where most of the synapses between projection neurons occur: the glomeruli in the OB and layer I of the APC. Comparisons between WT and α -syn-Tg mice were made using unpaired t-tests.

Dopaminergic (DAergic) cells were quantified on sections stained for the enzyme tyrosine hydroxylase (TH) from 6-7 months mice. The number of TH-positive cells were counted in both the lateral and medial regions of three consecutive sections of the OB (n= 3) in the GL, and numbers averaged. Comparisons between WT and α -syn-Tg were made using an unpaired t-test.

Phosphorylated S6 ribosomal protein (pS6) intensity of staining was quantified in OB mitral cells of 12-14 months mice (n=4/genotype). Images were taken using an Olympus BX51 microscope, and intensity of the pixels was measured in two-line profiles per image, in two images per animal of both lateral and medial regions of the OB. Pixel intensity was measured using the Olympus cellSens Standard 3.1 software. Background line profiles were used to subtract the value from the stained profiles. Values obtained from line profiles per section and regions of the same animal were averaged to get the pS6 pixel intensity for each animal. Statistical comparisons were made using an unpaired t-test.

BrdU⁺ cells were quantified using an Olympus BX51 microscope. The analyzed sections were taken from midway through the olfactory bulb and were selected by finding the sections from each animal at the same coronal plane. The right OB sections were chosen arbitrarily, with left OB sections used at planes where the right section was torn or otherwise unusable. BrdU⁺ cells were manually counted via fluorescence microscopy at 20x magnification. The area of the glomerular layer (GL), granular cell layer (GCL) and bulbar rostral migratory stream (OB-RMS), were calculated by manual tracing of an image of the section taken at 4x magnification. Cell counts were divided by their respective areas to calculate average count per area, then averaged over the sections to find the average count per area in a select region,

over a given animal. Statistics for BrdU numbers on the average counts per area in the GL, GCL and the OB-RMS were compared separately between early (6-7 months) and late (12-14 months) stages using unpaired t-tests.

Western blot quantifications for endogenous/human α -syn and aggregated α -synu were normalized against the respective beta-actin quantifications. Comparisons between groups using this normalized data were made using unpaired t-tests.

All statistical analysis were carried out using the GraphPad Prism 9.2 software.

Results

Olfactory deficits found in late-stage mice expressing human A30P α -syn mutation

A progressive loss of olfaction is a well-known non-motor symptom in PD patients (Ansari and Johnson, 1975; Haehner et al., 2011). Here, we evaluated olfactory deficits in a mouse model of PD before and after the appearance of motor abnormalities, i.e. early (6-7 months) and late (12-14 months) stages, respectively (Chandra et al., 2005; Piltonen et al., 2013). Olfactory deficits were analyzed using standardized buried food tests as they are effective methods to evaluate the ability of trained mice to locate scented foods using olfactory cues (Dawson et al., 2005; Yang and Crawley, 2009; Lehmkuhl et al., 2014).

Our results from olfactory tests showed that α -syn-Tg mice displayed olfactory deficits only at late stages (12-14 months) compared to controls (WT) ($p=0.029$) but not at early stages (6-7 months) ($p=0.8048$) (Fig. 1A). These data suggested an olfactory impairment caused by α -syn-Tg overexpression in mice at advanced stages of the disease. In both α -syn-Tg and WT groups, data from males and females were merged after we ruled out the influence of sex on the results (comparisons between male and female's latencies were made at 6-7 months in WT: $p=0.2286$ and α -syn-Tg: $p=0.1905$; and at 12-14 months in WT: $p=0.1333$). Additionally, to exclude the influence of motor deficits or lack of motivation by food in mice, particularly at late stages, we performed a surface food test to find a cookie. Our results showed no differences between WT and α -syn-Tg at early ($p=0.9709$) and late ($p=0.1156$) stages (Fig. 1B),

indicating that these parameters did not influence olfactory performance, even though 12-14 months mice have motor deficits on more challenging motor tests (data not shown) In summary, we found that aged α -syn-Tg mice exhibit olfactory behavior deficits.

Architecture of the olfactory pathway of α -syn-Tg mice

Next, we sought to establish any α -syn pathology within the primary olfactory system to investigate the causes of olfactory deficits observed in 12-14 months mice. The olfactory pathway is organized in three fundamental processing stations interconnected by projection neurons that were the subject of our analysis (Fig. 2A).

(1) The olfactory epithelium (OE), which is the region of the nasal cavity where the olfactory receptor/sensory neurons (OSNs) are located. OSNs express olfactory receptors. Once these cells detect the odorants, the information is sent to the first olfactory processing center of the brain, the olfactory bulb (OB).

(2) The OB, which is formed by a variety of projection (PNs) and interneurons (INs) that are responsible for the initial identification of an odor. PNs are involved in receiving and processing the olfactory input received from OSNs, while INs modulate those signals. PNs include mitral and tufted cells (M/Tc), whose dendrites arborize in spherical structures called glomeruli (GL) where they form synapses with axon termini of OSNs, and send their axons to olfactory cortices, the third station studied in this work. Granule cells are the most abundant INs in the OB. The OB has a laminar organization with the GL, the external plexiform layer (EPL), mitral cell layer (MCL) and granule cell layer (GCL)

(3) The olfactory cortices (OCs), which are formed by the anterior olfactory nucleus (AON) and piriform cortex (PC), both responsible for the higher brain processing of an odor and memory formation (Shepherd et al., 2021).

We began by examining disruptions in the OE neuronal cytoarchitecture. OSNs in the OE are continuously renewed during the lifetime of an individual in a process that requires cell division of OE neural stem cells (horizontal and globose cells) and their differentiation to OSNs. The differentiation

stages of OSNs were identified using specific markers such as (1) olfactory marker protein (OMP) that is used to label mature OSNs, (2) adenylyl cyclase 3 (AC3) that is used to label differentiating OSNs, and (3) growth associated protein 43 (GAP43) that is used to label immature OSNs (Liberia et al., 2019). Our analysis showed no disrupted cytoarchitecture between WT controls and α -syn-Tg mice (Fig. 2B-G), which were also assessed for changes along the dorsal-ventral axis by staining with NADPH dehydrogenase [quinone] 1 (NQO1), a selective marker for the dorsal region (Fig. 2B, E). In addition, α -syn pathology was not detected in the OE, and there was no difference between genotypes in aggregated α -syn (Fig. 2B, E) and pSer129- α -syn (data not shown) staining. These antibodies detect two hallmarks of α -syn pathology, specifically, (1) the accumulation of α -syn aggregates (aggregated α -syn); and (2) phosphorylation of serine 129 of α -syn (pSer129- α -syn) (Narhi et al., 1999; Fujiwara et al., 2002). Our observations were consistent with data found in PD patients (Del Tredici and Braak, 2012).

Pathological α -synuclein accumulation is seen the olfactory bulb and cortices of α -syn-Tg mice

In the central olfactory regions, we first investigated which neurons express the human A30P α -syn transgene. Thy1 promoter is known to trigger protein expression predominantly in projection neurons (Feng et al., 2000), and so we sought to determine if our animal model showed a restricted expression of human α -syn in projection neurons of the olfactory pathway. To address this point, we performed triple IHC to detect endogenous mouse and human α -syn (endo- α -syn), in combination with aggregated α -syn and pSer129 α -syn, in the OBs and APC of mice at early (6-7 months) and late (12-14 months) stages of the disease progression. Our data showed an overexpression of endo- α -syn in both the OB and APC in α -syn-Tg mice in both age groups studied (Fig. 3). In the OB, the overexpression of endo- α -syn was strongly detected in mitral cells of the MCL (Compare Fig. 3a'', b'' with Fig. 3c'', d'') which is also true for the pyramidal neurons of MCL II in the APC (Compare Fig. 3e'', f'' with Fig. 3g'', h''). While there is increased α -syn expression in the transgenic, especially in the synaptic layers, the pattern of expression largely replicates endogenous α -syn expression. The overall increase of total endo- α -syn expression was

presumably the result of the overexpression of the hA30P α -syn, which was also confirmed by WB in the OB at both 6-7 and 12-14 months stages (Suppl. Fig. 1A). The MCL layer in the OB and APC contain projection neurons also exhibit both aggregated and pSer129 α -syn positive pathology in the OB (Compare Fig. 3A-a'', 3B-b'' with Fig. 3C-c'', D-d'', red and white arrowheads) and APC (Compare Fig. 3E-e'', F-f'' with Fig. 3G-g'', H-h'', red and white arrowheads). It is noteworthy that the pattern of expression is different between aggregated and pSer129 α -syn antibodies, with the latter largely staining the soma, consistent with published literature (Taguchi et al., 201).

To investigate α -syn pathology in the OB further, we determined and quantified α -syn pathology in the OB, in a layer-wise manner. We detected α -syn pathology in all OB layers, from the most superficial to the deepest as follows: (1) GL; (2) external plexiform layer (EPL); (3) mitral cell layer (MCL); (4) internal plexiform layer (IPL); and (5) granule cell layer (GCL). We quantified the expression of both aggregated α -syn and pSer129- α -syn along these layers in 12-14 months old mice (Fig. 4). In the GL, we did not find differences between WT and α -syn-Tg mice in either aggregated α -syn (Fig. 4a, b, g, and h) ($p=0.7187$, $t=0.3866$, $df=4$. Data not shown) or pSer129 α -syn ($p=0.4241$; $t=0.8893$, $df=4$. Data not shown), whose staining was scarcely detected in the periglomerular region. Similarly, the EPL showed minimal expression of aggregated α -syn in both WT and α -syn-Tg groups ($p=0.7187$; $t=0.3866$, $df=4$) (Fig. 4E). pSer129 α -syn was found to be expressed in both WT and α -syn-Tg with no statistical differences between experimental groups ($p=0.2336$; $t=1.402$, $df=4$) (Fig. 4K).

Interestingly, within the EPL layer of α -syn-Tg mice we found a strong expression of pSer129 α -syn in tufted cells, a subtype of OB projection neurons and therefore T-Box Brain 1, TBR1⁺ (Hevner et al., 2001) located in the outer EPL (oEPL) (Compare Fig. 5g, g' with Fig. 4h, h', white arrowheads). Both WT (Fig. 4g, g') and α -syn-Tg mice (Fig. 4h, h', green arrows) showed pSer129 α -syn in the apical and lateral dendrites of mitral cells. Lateral dendrites of mitral cells extend horizontally in the inner EPL (iEPL) and synapse with granule cell dendrites, while apical dendrites extend radially in the EPL to

innervate glomeruli. The restricted expression of pSer129- α -syn in the mitral cells-lateral dendrites of WT mice was unexpected, and we attributed its presence with a residual phosphorylation of α -syn previously reported to occur in healthy aged, adult brains representing ~4% of the total brain phosphorylated α -syn (Fujiwara et al., 2002). In the MCL, there was a strong trend to have higher levels of aggregated α -syn in α -syn-Tg mice compared to WT controls ($p=0.0553$; $t=2.679$, $df=4$) (Fig. 4C), while pSer129- α -syn expression level was significantly higher in the MCL of α -syn-Tg mice compared to WT controls ($p<0.01$; $t=5.204$, $df=4$), with a strong accumulation in TBR1⁺ mitral cell bodies (Fig. 4G-g'', H-h'', I). In the IPL, aggregated α -syn was significantly higher in α -syn-Tg than in WT ($p<0.01$; $t=4.934$, $df=4$) (Fig 4A-a'', B-b'', F), while pSer129- α -syn expression levels were unchanged between experimental groups ($p=0.1465$; $t=1.798$, $df=4$) (Fig 4G-g'', H-h'', F). This is consistent with the observation that pSer129- α -syn staining is mainly in cell bodies. In the GCL, the inner OB layer, we found aggregated α -syn expressed significantly higher in α -syn-Tg than in WT ($p<0.01$; $t=3.594$, $df=4$) (Fig. 4A-a'', B-b'', D), preferentially located in neuronal processes of the GCL parenchyma. On the other hand, and in contrast to a prior report in a similar animal model (Neuner et al., 2014), we observed a patchy accumulation of pSer129- α -syn in the GCL, slightly higher in α -syn-Tg than WT but without statistical differences ($p=0.3740$; $t=0.998$, $df=4$) (Fig. 4G-g'', H-h'', J), that likely corresponded to axonal bundles from M/Tc cells (Fig. 4G, H). An overall overexpression of pathological α -syn was confirmed by WB where there was significant increase in aggregated α -syn in OB homogenates of α -syn-Tg mice when compared to WT (Suppl. Fig. 1B). Taken together, our results indicate that the α -synuclein pathology in the OB of α -syn-Tg mice was restricted to M/Tc projection neurons.

We then examined the olfactory cortices formed by the anterior olfactory nucleus (AON) and piriform cortex (PC) for evidence of α -syn pathology. The AON is formed by a compact ring of cells, the *pars principalis*, surrounding the olfactory peduncle. This region is subdivided into two basic zones: an *outer plexiform layer*, and an *inner cell zone* containing pyramidal cells (Brunjes et al., 2005). We found

a trend for increased aggregated α -syn in the *inner cell zone* of α -syn-Tg mice (Fig. 5A-C). Interestingly, pSer129- α -syn was significantly increased in the projection neurons (TBR1⁺ cells) of the AON *inner cell zone* in α -syn-Tg mice with respect to controls (Fig. 5J-K) ($p < 0.01$; $t = 5.852$, $df = 4$), indicative of a severe pathology confined to these cells. These results showed compelling evidence of AON pathology as described in humans (Daniel and Hawkes, 1992; Pearce et al., 1995).

In the PC, we studied areas in the anterior piriform cortex (APC) and posterior piriform cortex (PPC) regions to cover the anatomical and functional differences along the rostral-caudal axis (Martin-Lopez et al., 2019; Shepherd et al., 2021). In the APC, we found an increase in aggregated α -syn in layers Ib and II (Fig. 5D-F, arrowheads), which are occupied predominantly by the apical dendrites and cell bodies of PC projection neurons (Martin-Lopez et al., 2019). This increase was not statistically significant probably due to the high variability among α -syn-Tg individuals ($p = 0.1489$; $t = 1.785$, $df = 4$), although a trend of higher aggregated α -syn was seen in α -syn-Tg (Fig. 5D-F). Results for aggregated α -syn in PPC were analogous (Fig. 5G-I) ($p = 0.3128$; $t = 1.154$, $df = 4$). Of interest was the accumulation of aggregated α -syn in cell bodies of some pyramidal neurons in layers II and III that was not seen in the OB (Fig. 5E, H, arrowheads), suggesting that PC neurons are more affected by α -syn pathology in α -syn-Tg mice. On the other hand, the expression of pSer129- α -syn highlighted an intense pathological profile in α -syn-Tg mice of both APC ($p < 0.01$; $t = 5.897$, $df = 4$) (Fig. 5M-O) and PPC ($p < 0.05$; $t = 3.383$, $df = 4$), Fig. 5P-R), where pSer129- α -syn staining appeared to be restricted to cell bodies and processes of projection neurons in layers II (a and b) and III (Fig. 5N and Q) of α -syn-Tg mice. Interestingly, there was a lack of labeling in layer IIa of APC compared to PPC (Fig. 5N, Q), which is the sublayer occupied by semilunar cells (SL) (Mazo et al., 2017). The reason for the differential effect in APC vs. PPC is a question requiring further analyses.

Collectively, these data showed substantial evidence of α -syn pathology throughout the central olfactory pathway where it was prominent in projection neuron cell bodies and processes, whose pathology seems likely to be linked to olfactory deficits at late stages of PD.

The olfactory bulb cytoarchitecture is unaffected by the expression of hA30P α -syn mutation

Next, we explored the anatomical architecture of the OB layers in mice at late stages of the disease (12-14 months). We first studied the GL, formed by the glomeruli and periglomerular neurons (PGNs) by staining against vesicular glutamate transporter 2 (VGLUT2) and OMP, both characteristic markers for OSNs axons. No evidence of anatomical disruptions was noticed in α -syn-Tg mice compared to WT controls (Suppl. Fig. 2A, B). In addition, we studied the dopaminergic (DAergic) local circuits established by PGNs expressing the enzyme tyrosine hydroxylase (TH⁺) (Kosaka and Kosaka, 2016), by quantifying TH⁺ cells around randomly selected glomeruli. Our results showed similar numbers of TH⁺ cells in WT (443 $\pm$ 43 cells/mm²) versus α -syn-Tg mice (535 $\pm$ 74 cells/mm²)(p=0.1358; t=1.864, df=4). The morphology of cells was also examined and found to be unaffected (Suppl. Fig. 2C, D). These data differ from those previously published showing a decrease in the number of TH⁺ cells, or an increase in the TH innervated area in transgenic mice expressing hA30P α -syn mutation (Lelan et al., 2011; Nuber et al., 2011; Neuner et al., 2014). Finally, we searched for changes in the cellular activity in mitral cells as the main projection neurons of the OB, by analyzing the expression levels of phosphorylated S6 ribosomal protein (Ser235/236) (pS6). Oscillations in pS6 levels have been correlated with the activation of neurons in response to stimuli, synaptic plasticity or pathological events such as traumatic injury or seizures (Biever et al., 2015). In the OB, pS6 expression levels are reported to be reduced in OB mitral cells after sensory deprivation (Galliano et al., 2021). Here, we quantified the intensity of pS6 staining along the MCL in both WT and α -syn-Tg mice showing a trend to lower intensity in α -syn-Tg mice compared to WT but with no statistically significant difference between the groups (Suppl. Fig. 2E, F, G). Furthermore, mitral cells expressing pSer129- α -syn in the α -syn-Tg mice also did not show any a change in pS6

expression compared to WT controls (Suppl. Fig. 2E-e''-F-f''). Overall, these results suggest no effect of α -syn pathology on the gross cytoarchitecture of the OB.

Ultrastructural analysis revealed unaltered synaptic and mitochondrial numbers in α -syn-Tg mice

We next asked if synaptic connectivity was impacted in α -syn-Tg mice at 12-14 months. We used EM to quantify synaptic number and features in the two primary regions in the olfactory pathway: (1) the OB glomerular neuropil (Gl), where M/Tc receive excitatory asymmetrical synapses from OSN axons and inhibitory symmetrical synapses with periglomerular interneurons (Kasowski et al., 1999); and (2) layer I of the APC, where axons from M/Tc make asymmetrical excitatory synapses with apical dendrites of PC pyramidal cells in layer Ia and local interneurons make symmetrical inhibitory synapses with the PC pyramidal apical dendrites in layer Ib (Sarma et al., 2011; Martin-Lopez et al., 2019). In both regions, we quantified the number synapses, differentiated among asymmetric (Fig. 6, red arrowheads), symmetric (Fig. 6, green arrowheads), and reciprocal (Fig. 6, yellow arrowheads), and also the number of mitochondria, whose dysfunction is reported to play a determining role in the progression of PD and can also be used as a proxy for neuronal activity (Trinh et al., 2021).

Total number of synapses in OB ($p=0.6819$; $t=0.4412$, $df=4$) and APC ($p=0.4953$; $t=0.7494$, $df=4$), asymmetric synapses in OB ($p=0.5449$; $t=0.6609$, $df=4$) and APC ($p=0.7396$; $t=0.3563$, $df=4$), symmetric synapses in OB ($p=0.2292$; $t=1.418$, $df=4$) and APC ($p=0.7096$; $t=0.400$, $df=4$), and reciprocal synapses in OB ($p=0.2301$; $t=1.414$, $df=4$) and APC ($p=0.1929$; $t=1.564$, $df=4$), were similar between WT and α -syn-Tg mice in both the Gl (Fig. 6A-C) or APC (Fig. 6E-G) parenchyma. The number of mitochondria in OB was also unaltered in α -syn-Tg mice ($p=0.2605$; $t=1.309$, $df=4$) and APC ($p=0.0685$; $t=2.475$, $df=4$). These data indicated that anomalies in number of mitochondria or synapses were unlikely to account for olfactory dysfunction. Interestingly, when the data is arranged by individual images from lower to highest number of elements (mitochondria or synapses), we found a consistent trend for fewer mitochondria and synapses in α -syn-Tg mice compared to WT controls (Fig. 6D, H). Collectively, our data did not show

evidence of morphological and ultrastructural changes that could explain the olfactory deficits observed in α -syn-Tg mice at late stages (12-14 months).

hA30P α -syn overexpression interferes with neurogenesis in the glomerular and granule cell layers of the olfactory bulb

Previous studies suggested that overexpression of human (normal or A30P mutant) α -syn in animal models of PD results in altered neurogenesis in different layers of the OB (Winner et al., 2004; Marxreiter et al., 2009; Neuner et al., 2014; Schreglmann et al., 2015). Neurogenesis of OB interneurons occurs throughout the lifetime of an individual and is crucial to the control and modulation of olfaction (Lazarini and Lledo, 2011). Newly generated interneurons proliferate from neural stem cells located in the subventricular zone (SVZ) of the lateral ventricles (LV), after which they migrate towards the OB in a migratory chain called the rostral migratory stream (RMS). New neuroblasts in this pathway take an average of 12-14 days to reach the OB in adult mice (Mobley et al., 2013) and approximately another week to integrate in the OB circuits (Whitman and Greer, 2007). Here, we sought to evaluate the effect of α -syn pathology on the neurogenesis in the OB at early (6-7 months) and late (12-14 months) stages of the disease, that is, prior to and after the onset of hyposmia. Newly formed neurons were identified by injecting BrdU, and tissues analyzed at 25 days post-injection (DPI). We quantified BrdU⁺ cells in three layers of the OB receiving interneurons from this route, namely, the GL, GCL and bulbar part of the RMS.

In the GL, we found no difference in the number of BrdU⁺ periglomerular neurons (PGNs) at 6-7 months by genotype ($p=0.7213$; $t=0.3695$, $df=8$) but showed a significant decrease at 12-14 months ($p=0.0491$; $t=2.460$, $df=6$), indicating that the hA30P α -syn mutation affected the neurogenesis at late stages (12-14 months) of the disease progression (Fig. 7A-D, a'-d', E). Since our quantifications on TH number of PGNs that were TH⁺ ve showed no differences between WT and α -syn-Tg mice (see above)

and considering that no BrdU⁺-PGNs expressed TH (Suppl. Fig. 3E, F), we concluded that the PGN population affected by the expression of hA30P α -syn mutation belonged to subpopulations of PGNs other than DAergic. In the GCL, we detected a significant decrease in the number of BrdU⁺ cells only at early (6-7 months) stages, ($p=0.0136$; $t=3.152$, $df=8$) highlighting that α -syn overexpression was affecting OB neurogenesis in the GCL prior to the onset of olfactory deficits (Fig. 7A-D, a-d, F). This is consistent with previous results from similar animal models expressing the hA30P α -syn mutation under the CaMK-tet regulated promoter (Marxreiter et al., 2009). At the 12-14 months late stage, there was a trend toward a decrease in BrdU⁺ in the GCL of α -syn-Tg mice compared to WT controls ($p=0.0955$; $t=1.976$, $df=6$) (Fig. 7F). Absence of statistical significance may be due to the global decrease in neurogenesis of cells targeting the OB during aging (Mobley et al., 2013; Mobley et al., 2014), which may have masked the effect of hA30P α -syn. Finally, there was no evidence of a change in the number of BrdU⁺ cells in the OB-RMS at either 6-7 months ($p=0.6048$; $t=0.5386$, $df=8$) or 12-14 months ($p=0.1076$; $t=1.890$, $df=6$) stages of the disease progression (Fig. 7A-D, a-d, G). Since BrdU injections at adult stages could only label OB interneurons generated in the SVZ and considering that α -syn pathology was only expressed in projection neurons of the olfactory pathway (Figs. 3-5), the effects observed in OB neurogenesis are presumably indirect. (Hoglinger et al., 2004; Taylor et al., 2014). Collectively, our data showed that hA30P α -syn mutation affected neurogenesis in the GCL of OB at early stage before the onset of olfactory deficits, whereas neurogenesis in the GL of OB is affected in the later stages when olfactory deficit was apparent in α -syn-Tg mice.

To ensure that all BrdU⁺ cells belonged to neuronal lineages and to exclude cell division by local glial cells, we characterized BrdU⁺ cell phenotypes with IHC using specific markers to detect neuron and glial cells. Neuroblasts were identified using doublecortin (DCX), a microtubule-associated protein required for the migration of cells. BrdU⁺ cells that were located either in the OB (Suppl. Fig. 3A, B) or the SVZ (Suppl. Fig 4A, B), did not colocalize with the DCX marker, suggesting that most BrdU⁺ cells

have completed migration and begun to differentiate into neurons at the time of the study, supporting the previously findings that OB neuroblasts require 3 weeks to migrate and differentiate into neurons after generation in the SVZ (Whitman and Greer, 2007; Mobley et al., 2013). Consequently, we looked for mature neurons using the neuronal nuclei marker NeuN. Unexpectedly, only a fraction of the total number of BrdU⁺ cells (representing approximately 40% of the total) colocalized this marker in the OB (Suppl. Fig. 3C, D), suggesting that the remaining ~60% of BrdU⁺ cells could be neurons in intermediate stages of differentiation in the OB that expressed a DCX⁻/NeuN⁻ phenotype. As mentioned above, we did not find any BrdU⁺ cells colocalizing with TH⁺ (Suppl. Fig. 3E, F), indicating that we did not isolate the DAergic OB subpopulation of PGNs during the duration of this study (25 days). Next, we searched for astrocytes and microglial cells using glial fibrillary acid protein (GFAP) and ionized calcium-binding adapter molecule 1 (IBA1), respectively. Neither GFAP in the OB (Suppl. Fig. 3G, H) and the SVZ (Suppl. Fig. 4C, D), nor IBA1 in the OB (Suppl. Fig. 3I, J) colocalized with BrdU⁺ cells, indicating they were not dividing glial cells. With respect to the BrdU⁺ cells that we found in the SVZ, the region where OB neuroblasts are produced but do not differentiate to neurons (Lim and Alvarez-Buylla, 2016), we determined that the BrdU⁺ cells belonged to the intermediate progenitor cells population known as transient amplifying cells (or “C” cells), which are characterized by the expression of Distal-Less Homeobox 2 (Dlx2) factor (Suppl. Fig. 4E, F) (Lim and Alvarez-Buylla, 2016). No differences in BrdU⁺ cell phenotypes were detected between in the SVZ of WT and α -syn-Tg mice (Suppl. Fig. 4).

Collectively, we found that the hA30P α -syn expression significantly reduced OB neurogenesis at early stages (6-7 months) in the GCL and at later stages (12-14 mo) in the GL, in a process that was likely independent of the α -syn pathology expressed in projection neurons of the olfactory system. Among the BrdU⁺ cells found in the OB, 40% of them were differentiated to neurons (DCX⁻/NeuN⁺), while the remaining 60% (DCX⁻/NeuN⁻) remained at intermediate stages of neuronal differentiation.

The olfactory bulb reveals age-related synaptic endocytic dysfunction in α -syn-Tg mice

To further understand development of olfactory system pathology in an unbiased manner, we performed proteomic analysis on OB and APC from 6-7 months and 12-14 months WT and α -syn-Tg mice (n=3/genotype) by label-free quantification mass spectrometry (LFQ-MS) as technical triplicates. Our proteomic analysis of the OBs from 6-7 months mice identified a total of 3328 proteins, 14 of which were significantly differentially expressed in α -syn-Tg mice (Fig. 8A, B, Suppl. table 1). Consistent with transgenic overexpression and immunohistochemical results, we observed a significant increase in α -syn expression in the OB of α -syn-Tg mice (SYUA, Fig. 8A, B). This was confirmed via Western Blot (Suppl. Fig. 1A). Protein kinase c-Src was the most significantly upregulated protein in the OB of α -syn-Tg mice (SRC, Fig. 8A, B, fold change of 2.88). Among other functions, protein kinase c-Src phosphorylates components of endocytic trafficking (Reinecke and Caplan, 2014) and α -syn (Ellis et al., 2001). Immunohistochemical results confirmed the upregulation of c-Src in 6-7 months α -syn-Tg mice and showed co-localization of c-Src and pSer129- α -syn in the OB (Suppl. Fig. 5). GO enrichment analysis performed on the proteins that showed significant change ($p > 0.05$) identified pathways involving vesicle-mediated transport, specifically endocytosis, which were significantly over-enriched (Fig. 8C). The vast majority of proteins from our dataset that were implicated in these pathways were downregulated in α -syn-Tg samples (Fig. 8A, B), suggesting a deficit in vesicle-mediated transport in the OB. Overall, proteomic analysis of the OB at early stages revealed dysfunction in endocytosis and vesicular transport.

In the OB of 12-14 months mice, proteomic analysis detected a total of 2712 proteins, 15 of which were significantly differentially expressed in α -syn-Tg samples (Fig. 8D, E, Suppl. Table 2). There was a trend in overexpression of α -syn (SYUA), which was confirmed via Western Blot (Suppl. Fig. 1A). Similar to what we found in 6-7 months mice, GO analysis identified pathways involving vesicle-mediated transport, specifically at the pre-synapses, as significantly over-enriched (Fig. 8F). These changes were driven by a majority of proteins that were downregulated in α -syn-Tg samples, including the vesicular transport proteins PICALM, UNC13A, SNAP29, and VPS45 (Fig. 8D, E). Thus, proteomic analysis at

late stages (12-14 months) reaffirms the endocytic and vesicular transport defects seen at 6-7 months in the OB (Fig. 8A-C), and provided additional evidence that, with age, these deficits became prominent in synaptic terminals of α -syn-Tg mice.

In the APC of 6-7 months mice, proteomic analysis identified a total of 2955 proteins, 11 of which were significantly differentially expressed in α -syn-Tg samples (Fig. 8G, H, Suppl. Table 3). As anticipated, we observed a significant increase in α -syn expression (SYUA, Fig. 8D, E). Interestingly, GO analysis largely revealed broader dysfunction in cellular metabolism (Fig. 8F) without over-enrichment of any vesicle mediated-transport pathways seen in the OB (Fig. 8C). In the APC of 12-14 months mice, proteomic analysis identified a total of 2596 proteins, 16 of which were significantly differentially expressed in α -syn-Tg samples (Fig. 8J, K, Suppl. Table 4). Similarly, GO analysis revealed only a subtle dysregulation in receptor clustering of α -syn-Tg mice, without any dysregulation of synaptic endo- and exocytosis as seen in the OB of these mice. Collectively, our proteomic analysis suggested that the OB is more vulnerable to α -syn pathology induced by the hA30P α -syn mutation expression than the APC, and that olfactory deficits seen at late stages might arise due to early age-related endocytic changes in the synaptic terminals of the OB circuitry.

Discussion

Olfactory deficits are proposed as prodromal for PD and have the potential to predict the risk and the severity of the disease (Doty et al., 1995; Haehner et al., 2007; Ross et al., 2008; Goetz, 2011; Postuma et al., 2015; Fullard et al., 2017; He et al., 2020; Zhou et al., 2020). However, the cellular and molecular mechanisms that affect olfaction in PD remain unknown. To study PD, animal models have been developed carrying mutated genes linked with familial forms of PD (Crabtree and Zhang, 2012; Konnova and Swanberg, 2018). Here, we used a mouse model of PD carrying the human A30P α -syn mutation expressed under the Thy1 promoter (Chandra et al., 2005) to study the cellular and molecular anomalies associated with olfactory deficits. We focused our analyses on two stages of the disease progression as

per the onset of olfactory dysfunction: (1) early-stage mice at 6-7 months, and (2) late-stage mice at 12-14 months. Our results showed that the hA30P α -syn mutation caused olfactory deficits at the late stages of the disease, correlating with strong α -syn pathology along the olfactory pathway projection neurons. α -Syn pathology was found at both early and late stages, but olfactory behavior deficits were not found until 12-14 months. Neurogenesis was affected at both stages but in different layers. Unbiased proteomics suggested that the OB was more vulnerable to the hA30P α -syn mutation than APC, causing perturbations in protein expression at both ages. Our data showed affected biological pathways involving synaptic transmission, specifically vesicle-mediated transport, suggesting a possible mechanism underlying olfactory dysfunction.

Spatiotemporal variabilities of α -synuclein pathology in the olfactory system of α -syn-Tg mice

Normal α -syn protein functions in the trafficking of synaptic vesicles (Lashuel et al., 2013; Vargas et al., 2017). However, in PD α -syn misfolds and produces insoluble oligomers and fibrils that, in conjunction with other proteins, aggregate in the cytoplasm of cells forming LB, a pathological hallmarks of PD (Shults, 2006). In the transition from oligomers to fibrils, α -syn is modified by phosphorylation of serine 129 (pSer129- α -syn), a process that is selective of α -syn pathology lesions (Fujiwara et al., 2002). The process of aggregation has been observed in animal models expressing the A30P α -syn mutation where pSer129- α -syn increases dramatically after the fifth month of age (Behere et al., 2021). We used this marker as well as aggregated α -syn to capture both phosphorylated and non-phosphorylated α -syn pathology, respectively.

We found accumulation of pathological α -syn throughout all projection neurons in the OB and APC (Figs. 3, 4 and 5), which is likely due to the hA30P α -syn mutation expression controlled by Thy1 promoter. Thy1 drives gene expression predominantly in projection neurons (Feng et al., 2000; Rockenstein et al., 2002) which accounts for our observation of selective accumulation of pathological α -syn in the projection neurons in olfactory system of α -syn-Tg mice. Comparable patterns of expression

are also seen with the A53T α -syn mutation under the Thy1 promotor (van der Putten et al., 2000). Synaptic transmission is regulated by α -syn through interactions with synaptic vesicle membranes and impacting on the presynaptic architecture (Vargas et al., 2017; Erustes et al., 2021; Liu et al., 2021). To explore this in the α -syn-Tg mice, we performed an ultrastructural analysis and quantified excitatory and inhibitory synapses and mitochondria in the presynapses of the OB and APC. These analyses did not show any statistically significant differences in any measures that might account for the behavioral deficits in α -syn-Tg mice (Fig. 6). Similarly, we did not find evidence of activity changes using the marker pS6 suggesting that at least basal levels of neuronal activity are present in the α -syn-Tg mice.

Beyond the spatiotemporal disparities of α -syn pathology in the olfactory system, what evidence did we find that may explain the late-stage olfactory deficits in α -syn-Tg mice? We propose two potential candidates: (1) decreased neurogenesis; and (2) perturbations of synaptic exo and endocytosis pathways proteins involved in the synaptic transmission.

The hA30P α -syn mutation reduces olfactory bulb neurogenesis at both early and late stages of PD

Neurogenesis in the OB occurs throughout the lifetime of an individual with a renewal of interneurons generated in subventricular zone (SVZ) in the lateral ventricle walls. From the SVZ, these newly formed cells join chains of migrating neuroblasts extending to the ependymal core of the OB via the rostral migratory stream (RMS) (Whitman and Greer, 2009). Once detached from the RMS, neuroblasts migrate radially to their final positions in either the GCL or GL. In PD, the evidence of aberrant neurogenesis is limited and controversial with some reports showing both decreases in neurogenesis in the SVZ (Hoglinger et al., 2004), or no effect (van den Berge et al., 2011). Here, using BrdU⁺ (5-bromo-2'-deoxyuridine) injections, we found a decrease in OB neurogenesis in α -syn-Tg mice (Fig. 7) that affected primarily the GCL at early stages (6-7 months) and the GL at later stages (12-14 months). A decrease in newly formed cells in these two layers was reported previously in similar animal models at earlier ages (Marxreiter et al., 2009; Zhang et al., 2019). The most likely explanation for

differences in onset of decreased neurogenesis is that it reflects the use of different promoters to trigger the expression of the α -syn containing the hA30P mutation. Both the expression levels as well as the temporal expression of proteins controlled by the Thy1 promoter are reported to influence adult neurogenesis in different degrees (Schreglmann et al., 2015), which could account for the differences seen in other PD models.

Although Zhang et al. (2019) reported cell death in the GCL of a PD mouse model, we did not find any evidence for the staining of cleaved Cas3 in the olfactory pathway (Suppl. Fig. 2C, D) that would account for the decrease in BrdU⁺ cells. The most plausible explanation for the observed decrease in BrdU⁺ cells is that SVZ proliferation was reduced, perhaps linked to an indirect effect of affected DAergic innervation into the SVZ. Thus, it is notable that reductions in SVZ neurogenesis were previously reported to accompany diminished DA levels in the SVZ (Hoglinger et al., 2004). Overall, it seems likely that the decrease in neurogenesis, seen as a decrease in BrdU labeling, contributed to the olfactory phenotype we report here. The delayed onset of a clear behavioral olfactory dysfunction suggests strongly that the effects of the α -syn pathology in the olfactory pathway must be cumulative.

Anomalies in OB and APC proteomics of synaptic trafficking in α -synuclein transgenics

We analyzed unbiased proteomics data in WT and α -syn-Tg mice in the OB and APC to determine cellular pathways that were affected by the overexpression of α -syn carrying the hA30P mutation. The hA30P α -syn mutation causes α -syn aggregation and phosphorylation of serine 129 leading to insoluble α -syn oligomerization and fibrillation (Lazaro et al., 2014; Behere et al., 2021). The pSer129- α -syn can accelerate the misfolding of normal α -syn leading to increased cytotoxicity, disruptions in vesicular trafficking, synaptic dysfunction, apoptosis, and formation of permeable annular pore-like protofibrils in the cell membrane that disrupt transmembrane trafficking (Fujiwara et al., 2002; Lashuel et al., 2002; Dalfo et al., 2004; Samuel et al., 2016; Schaser et al., 2019). How are these mechanisms affecting protein expression and dynamics of cells in olfactory regions remains to be investigated.

While 6-7 months mice did not exhibit olfactory deficits, the presence of α -syn pathology in olfactory areas suggested that the underlying mechanisms of olfactory deficits may arise early and could be cumulative leading to the deficits seen at older ages α -syn-Tg mice. Using proteomics at this early stage, we found perturbations of the proteome in both OB and APC brain areas, particularly in those proteins involved in the endocytic and vesicle-mediated transport pathways, which were significantly affected in the OB of α -syn-Tg mice. At later stages when olfactory deficits arose (12-14 months), these defects appear to be exacerbated in the synaptic terminals of the OB. This result was consistent with the fact that mutations in endocytic proteins cause familial PD (Vidyadhara et al., 2019) and that elevated α -syn levels and aggregation can cause inhibition of synaptic vesicle exo- and endocytosis (Nemani et al., 2010; Xu et al., 2016; Eguchi et al., 2017; Huang et al., 2019). Notably, we observed minimal disruption of biological pathways in the APC. These results suggest that the OB is more susceptible than APC to the α -syn pathology and may be a primary site in the olfactory pathway for olfactory behavior deficits seen in α -syn-Tg mice. Thus, together with defective neurogenesis, endocytic dysregulation in the synaptic terminals in the OB might initiate pathological processes leading to olfactory deficits in PD patients, especially those carrying α -syn mutations. Similar mechanisms might also exist in sporadic PD cases, as majority of them show LB formation and α -syn pathology.

Conclusions

Our work provides compelling evidence for the following: (1) mice overexpressing α -syn carrying the human A30P mutation exhibit olfactory deficits at a later age when PD-like motor deficits are visible (2) hA30P α -syn mutation causes α -syn pathology along the entire central olfactory pathway, restricted to projection neurons expressing TBR1; (3) α -syn pathology does not cause structural and morphological abnormalities in the olfactory system; (4) the overexpression of α -syn carrying hA30P causes a decrease in SVZ neurogenesis and interneurons targeted to the OB; and (5) hA30P α -syn mutation causes decreased expression of proteins involved in synaptic endocytosis and vesicle-mediated transport in the OB of early

and late α -syn-Tg mice. This, together with the alterations in neurogenesis, likely explain the olfactory deficits observed at late stages.

Acknowledgments

We thank Christine Kaliszewski for technical assistance with EM, and John E. Lee for α -syn-Tg and WT animal colonies maintenance and genotyping. We thank Phil Coish for reading the manuscript.

Funding

This work was funded by Department of Defense (81XWH-17-1-0564) to SSC and CAG, NIH (1RF1NS110354-01), Parkinson's Foundation Research 516 Center of Excellence (PF-RCE-1946) to SSC, Department of Defense (W81XWH1910264) to VDJ and NIH DC016851, DC017989 and DC013791 to CAG.

References

- Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, Barbour R, Huang J, Kling K, Lee M, Diep L, Keim PS, Shen X, Chataway T, Schlossmacher MG, Seubert P, Schenk D, Sinha S, Gai WP, Chilcote TJ (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.
- Ansari KA, Johnson A (1975) Olfactory function in patients with Parkinson's disease. *J Chronic Dis* 28:493-497.
- Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 25:25-29.

- Behere A, Thornqvist PO, Winberg S, Ingelsson M, Bergstrom J, Ekmark-Lewen S (2021) Visualization of early oligomeric alpha-synuclein pathology and its impact on the dopaminergic system in the (Thy-1)-h[A30P]alpha-syn transgenic mouse model. *J Neurosci Res* 99:2525-2539.
- Biever A, Valjent E, Puighermanal E (2015) Ribosomal Protein S6 Phosphorylation in the Nervous System: From Regulation to Function. *Front Mol Neurosci* 8:75.
- Borghammer P (2018) How does parkinson's disease begin? Perspectives on neuroanatomical pathways, prions, and histology. *Mov Disord* 33:48-57.
- Braak H, Rub U, Gai WP, Del Tredici K (2003a) Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *J Neural Transm (Vienna)* 110:517-536.
- Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E (2003b) Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* 24:197-211.
- Braak H, Braak E, Yilmazer D, de Vos RA, Jansen EN, Bohl J, Jellinger K (1994) Amygdala pathology in Parkinson's disease. *Acta Neuropathol* 88:493-500.
- Brunjes PC, Illig KR, Meyer EA (2005) A field guide to the anterior olfactory nucleus (cortex). *Brain Res Brain Res Rev* 50:305-335.
- Carbon S et al. (2021) The Gene Ontology resource: enriching a GOLD mine. *Nucleic Acids Research* 49:D325-D334.
- Chandra S, Gallardo G, Fernandez-Chacon R, Schluter OM, Sudhof TC (2005) Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. *Cell* 123:383-396.

- Constantinidis J, de Ajuriaguerra J (1970) [Familial syndrome with parkinsonian tremor and anosmia and its therapy with L-dopa associated with a decarboxylase inhibitor]. *Therapeutique* 46:263-269.
- Crabtree DM, Zhang J (2012) Genetically engineered mouse models of Parkinson's disease. *Brain Res Bull* 88:13-32.
- Dalfo E, Gomez-Isla T, Rosa JL, Nieto Bodelon M, Cuadrado Tejedor M, Barrachina M, Ambrosio S, Ferrer I (2004) Abnormal alpha-synuclein interactions with Rab proteins in alpha-synuclein A30P transgenic mice. *J Neuropathol Exp Neurol* 63:302-313.
- Daniel SE, Hawkes CH (1992) Preliminary diagnosis of Parkinson's disease by olfactory bulb pathology. *Lancet* 340:186.
- Dawson PA, Steane SE, Markovich D (2005) Impaired memory and olfactory performance in NaSi-1 sulphate transporter deficient mice. *Behav Brain Res* 159:15-20.
- Del Tredici K, Braak H (2012) Lewy pathology and neurodegeneration in premotor Parkinson's disease. *Mov Disord* 27:597-607.
- Del Tredici K, Braak H (2013) Dysfunction of the locus coeruleus-norepinephrine system and related circuitry in Parkinson's disease-related dementia. *J Neurol Neurosurg Psychiatry* 84:774-783.
- Doty RL (2012) Olfactory dysfunction in Parkinson disease. *Nat Rev Neurol* 8:329-339.
- Doty RL, Bromley SM, Stern MB (1995) Olfactory testing as an aid in the diagnosis of Parkinson's disease: development of optimal discrimination criteria. *Neurodegeneration* 4:93-97.
- Dunning CJ, Reyes JF, Steiner JA, Brundin P (2012) Can Parkinson's disease pathology be propagated from one neuron to another? *Prog Neurobiol* 97:205-219.

- Eguchi K, Taoufiq Z, Thorn-Seshold O, Trauner D, Hasegawa M, Takahashi T (2017) Wild-Type Monomeric alpha-Synuclein Can Impair Vesicle Endocytosis and Synaptic Fidelity via Tubulin Polymerization at the Calyx of Held. *J Neurosci* 37:6043-6052.
- Ellis CE, Schwartzberg PL, Grider TL, Fink DW, Nussbaum RL (2001) alpha-synuclein is phosphorylated by members of the Src family of protein-tyrosine kinases. *J Biol Chem* 276:3879-3884.
- Erustes AG, D'Eletto M, Guarache GC, Ureshino RP, Bincoletto C, da Silva Pereira GJ, Piacentini M, Smaili SS (2021) Overexpression of alpha-synuclein inhibits mitochondrial Ca(2+) trafficking between the endoplasmic reticulum and mitochondria through MAMs by altering the GRP75-IP3R interaction. *J Neurosci Res*.
- Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 28:41-51.
- Ferreira M, Massano J (2017) An updated review of Parkinson's disease genetics and clinicopathological correlations. *Acta Neurol Scand* 135:273-284.
- Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T (2002) alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol* 4:160-164.
- Fullard ME, Morley JF, Duda JE (2017) Olfactory Dysfunction as an Early Biomarker in Parkinson's Disease. *Neurosci Bull* 33:515-525.
- Galliano E, Hahn C, Browne LP, P RV, Tufo C, Crespo A, Grubb MS (2021) Brief Sensory Deprivation Triggers Cell Type-Specific Structural and Functional Plasticity in Olfactory Bulb Neurons. *J Neurosci* 41:2135-2151.

- Gentzel RC, Toolan D, Jinn S, Schachter JB, Ma L, Kahle PJ, Smith SM, Marcus JN (2021) Intracranial administration of alpha-synuclein fibrils in A30P-synuclein transgenic mice causes robust synucleinopathy and microglial induction. *Neurobiol Aging* 106:12-25.
- Goetz CG (2011) The history of Parkinson's disease: early clinical descriptions and neurological therapies. *Cold Spring Harb Perspect Med* 1:a008862.
- Gorbatyuk OS, Li S, Sullivan LF, Chen W, Kondrikova G, Manfredsson FP, Mandel RJ, Muzyczka N (2008) The phosphorylation state of Ser-129 in human alpha-synuclein determines neurodegeneration in a rat model of Parkinson disease. *Proc Natl Acad Sci U S A* 105:763-768.
- Haehner A, Hummel T, Reichmann H (2011) Olfactory loss in Parkinson's disease. *Parkinsons Dis* 2011:450939.
- Haehner A, Hummel T, Hummel C, Sommer U, Junghanns S, Reichmann H (2007) Olfactory loss may be a first sign of idiopathic Parkinson's disease. *Mov Disord* 22:839-842.
- Haehner A, Boesveldt S, Berendse HW, Mackay-Sim A, Fleischmann J, Silburn PA, Johnston AN, Mellick GD, Herting B, Reichmann H, Hummel T (2009) Prevalence of smell loss in Parkinson's disease--a multicenter study. *Parkinsonism Relat Disord* 15:490-494.
- Hawkes CH, Del Tredici K, Braak H (2007) Parkinson's disease: a dual-hit hypothesis. *Neuropathol Appl Neurobiol* 33:599-614.
- He R, Zhao Y, He Y, Zhou Y, Yang J, Zhou X, Zhu L, Zhou X, Liu Z, Xu Q, Sun Q, Tan J, Yan X, Tang B, Guo J (2020) Olfactory Dysfunction Predicts Disease Progression in Parkinson's Disease: A Longitudinal Study. *Front Neurosci* 14:569777.

- Heinzel S, Aho VTE, Suenkel U, von Thaler AK, Schulte C, Deuschle C, Paulin L, Hantunen S, Brockmann K, Eschweiler GW, Maetzler W, Berg D, Auvinen P, Scheperjans F (2021) Gut Microbiome Signatures of Risk and Prodromal Markers of Parkinson Disease. *Ann Neurol* 90:E1-E12.
- Hevner RF, Shi L, Justice N, Hsueh Y, Sheng M, Smiga S, Bulfone A, Goffinet AM, Campagnoni AT, Rubenstein JL (2001) *Tbr1* regulates differentiation of the preplate and layer 6. *Neuron* 29:353-366.
- Hoglinger GU, Rizk P, Muriel MP, Duyckaerts C, Oertel WH, Caille I, Hirsch EC (2004) Dopamine depletion impairs precursor cell proliferation in Parkinson disease. *Nat Neurosci* 7:726-735.
- Huang M, Wang B, Li X, Fu C, Wang C, Kang X (2019) alpha-Synuclein: A Multifunctional Player in Exocytosis, Endocytosis, and Vesicle Recycling. *Front Neurosci* 13:28.
- Johnson ME, Bergkvist L, Mercado G, Stetzk L, Meyerdirk L, Wolfrum E, Madaj Z, Brundin P, Wesson DW (2020) Deficits in olfactory sensitivity in a mouse model of Parkinson's disease revealed by plethysmography of odor-evoked sniffing. *Sci Rep* 10:9242.
- Kasowski HJ, Kim H, Greer CA (1999) Compartmental organization of the olfactory bulb glomerulus. *J Comp Neurol* 407:261-274.
- Konnova EA, Swanberg M (2018) Animal Models of Parkinson's Disease. In: *Parkinson's Disease: Pathogenesis and Clinical Aspects* (Stoker TB, Greenland JC, eds). Brisbane (AU).
- Kosaka T, Kosaka K (2016) Neuronal organization of the main olfactory bulb revisited. *Anat Sci Int* 91:115-127.

- Kouli A, Torsney KM, Kuan WL (2018) Parkinson's Disease: Etiology, Neuropathology, and Pathogenesis. In: Parkinson's Disease: Pathogenesis and Clinical Aspects (Stoker TB, Greenland JC, eds). Brisbane (AU).
- Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat Genet* 18:106-108.
- Lashuel HA, Overk CR, Oueslati A, Masliah E (2013) The many faces of alpha-synuclein: from structure and toxicity to therapeutic target. *Nat Rev Neurosci* 14:38-48.
- Lashuel HA, Petre BM, Wall J, Simon M, Nowak RJ, Walz T, Lansbury PT, Jr. (2002) Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. *J Mol Biol* 322:1089-1102.
- Lazarini F, Lledo PM (2011) Is adult neurogenesis essential for olfaction? *Trends Neurosci* 34:20-30.
- Lazaro DF, Rodrigues EF, Langohr R, Shahpasandzadeh H, Ribeiro T, Guerreiro P, Gerhardt E, Krohnert K, Klucken J, Pereira MD, Popova B, Kruse N, Mollenhauer B, Rizzoli SO, Braus GH, Danzer KM, Outeiro TF (2014) Systematic comparison of the effects of alpha-synuclein mutations on its oligomerization and aggregation. *PLoS Genet* 10:e1004741.
- Legland D, Arganda-Carreras I, Andrey P (2016) MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ. *Bioinformatics* 32:3532-3534.
- Lehmkuhl AM, Dirr ER, Fleming SM (2014) Olfactory assays for mouse models of neurodegenerative disease. *J Vis Exp*:e51804.

- Lelan F, Boyer C, Thinard R, Remy S, Usal C, Tesson L, Anegon I, Neveu I, Damier P, Naveilhan P, Lescaudron L (2011) Effects of Human Alpha-Synuclein A53T-A30P Mutations on SVZ and Local Olfactory Bulb Cell Proliferation in a Transgenic Rat Model of Parkinson Disease. *Parkinsons Dis* 2011:987084.
- Lesage S, Anheim M, Letournel F, Bousset L, Honore A, Rozas N, Pieri L, Madiona K, Durr A, Melki R, Verny C, Brice A, French Parkinson's Disease Genetics Study G (2013) G51D alpha-synuclein mutation causes a novel parkinsonian-pyramidal syndrome. *Ann Neurol* 73:459-471.
- Lewy F (1912) Paralysis agitans. I. Pathologische Anatomie. Lewandowsky's Handbuch der Neurologie, Band 3: Spezielle Neurologie II. In, pp 920-933: Berlin: Springer.
- Liberia T, Martin-Lopez E, Meller SJ, Greer CA (2019) Sequential Maturation of Olfactory Sensory Neurons in the Mature Olfactory Epithelium. *eNeuro* 6.
- Lim DA, Alvarez-Buylla A (2016) The Adult Ventricular-Subventricular Zone (V-SVZ) and Olfactory Bulb (OB) Neurogenesis. *Cold Spring Harb Perspect Biol* 8.
- Liu C, Zhao Y, Xi H, Jiang J, Yu Y, Dong W (2021) The Membrane Interaction of Alpha-Synuclein. *Front Cell Neurosci* 15:633727.
- Lue LF, Walker DG, Adler CH, Shill H, Tran H, Akiyama H, Sue LI, Caviness J, Sabbagh MN, Beach TG (2012) Biochemical increase in phosphorylated alpha-synuclein precedes histopathology of Lewy-type synucleinopathies. *Brain Pathol* 22:745-756.
- Machado CF, Reis-Silva TM, Lyra CS, Felicio LF, Malnic B (2018) Buried Food-seeking Test for the Assessment of Olfactory Detection in Mice. *Bio Protoc* 8:e2897.

- Mahlknecht P et al. (2016) Optimizing odor identification testing as quick and accurate diagnostic tool for Parkinson's disease. *Mov Disord* 31:1408-1413.
- Mahul-Mellier AL, Burtscher J, Maharjan N, Weerens L, Croisier M, Kuttler F, Leleu M, Knott GW, Lashuel HA (2020) The process of Lewy body formation, rather than simply alpha-synuclein fibrillization, is one of the major drivers of neurodegeneration. *Proc Natl Acad Sci U S A* 117:4971-4982.
- Marin-Pardo D, Gimenez-Llort L (2021) Olfactory Signatures in the Food Finding Test in Mice With Normal and Alzheimer's Disease-Pathological Aging With Special Concerns on the Effects of Social Isolation. *Front Neurosci* 15:733984.
- Martin-Lopez E, Ishiguro K, Greer CA (2019) The Laminar Organization of Piriform Cortex Follows a Selective Developmental and Migratory Program Established by Cell Lineage. *Cereb Cortex* 29:1-16.
- Marxreiter F, Nuber S, Kandasamy M, Klucken J, Aigner R, Burgmayer R, Couillard-Despres S, Riess O, Winkler J, Winner B (2009) Changes in adult olfactory bulb neurogenesis in mice expressing the A30P mutant form of alpha-synuclein. *Eur J Neurosci* 29:879-890.
- Mazo C, Grimaud J, Shima Y, Murthy VN, Lau CG (2017) Distinct projection patterns of different classes of layer 2 principal neurons in the olfactory cortex. *Sci Rep* 7:8282.
- Mi H, Muruganujan A, Ebert D, Huang X, Thomas PD (2019) PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res* 47:D419-D426.
- Mobley AS, Rodriguez-Gil DJ, Imamura F, Greer CA (2014) Aging in the olfactory system. *Trends Neurosci* 37:77-84.

- Mobley AS, Bryant AK, Richard MB, Brann JH, Firestein SJ, Greer CA (2013) Age-dependent regional changes in the rostral migratory stream. *Neurobiol Aging* 34:1873-1881.
- Narhi L, Wood SJ, Steavenson S, Jiang Y, Wu GM, Anafi D, Kaufman SA, Martin F, Sitney K, Denis P, Louis JC, Wypych J, Biere AL, Citron M (1999) Both familial Parkinson's disease mutations accelerate alpha-synuclein aggregation. *J Biol Chem* 274:9843-9846.
- Nemani VM, Lu W, Berge V, Nakamura K, Onoa B, Lee MK, Chaudhry FA, Nicoll RA, Edwards RH (2010) Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis. *Neuron* 65:66-79.
- Neuner J, Filser S, Michalakis S, Biel M, Herms J (2014) A30P alpha-Synuclein interferes with the stable integration of adult-born neurons into the olfactory network. *Sci Rep* 4:3931.
- Nuber S, Petrasch-Parwez E, Arias-Carrion O, Koch L, Kohl Z, Schneider J, Calaminus C, Dermietzel R, Samarina A, Boy J, Nguyen HP, Teismann P, Velavan TP, Kahle PJ, von Horsten S, Fendt M, Kruger R, Riess O (2011) Olfactory neuron-specific expression of A30P alpha-synuclein exacerbates dopamine deficiency and hyperactivity in a novel conditional model of early Parkinson's disease stages. *Neurobiol Dis* 44:192-204.
- Okochi M, Walter J, Koyama A, Nakajo S, Baba M, Iwatsubo T, Meijer L, Kahle PJ, Haass C (2000) Constitutive phosphorylation of the Parkinson's disease associated alpha-synuclein. *J Biol Chem* 275:390-397.
- Pearce RK, Hawkes CH, Daniel SE (1995) The anterior olfactory nucleus in Parkinson's disease. *Mov Disord* 10:283-287.

- Piltonen M, Savolainen M, Patrikainen S, Baekelandt V, Myohanen TT, Mannisto PT (2013) Comparison of motor performance, brain biochemistry and histology of two A30P alpha-synuclein transgenic mouse strains. *Neuroscience* 231:157-168.
- Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045-2047.
- Postuma RB, Berg D, Stern M, Poewe W, Olanow CW, Oertel W, Obeso J, Marek K, Litvan I, Lang AE, Halliday G, Goetz CG, Gasser T, Dubois B, Chan P, Bloem BR, Adler CH, Deuschl G (2015) MDS clinical diagnostic criteria for Parkinson's disease. *Mov Disord* 30:1591-1601.
- Proukakis C, Dudzik CG, Brier T, MacKay DS, Cooper JM, Millhauser GL, Houlden H, Schapira AH (2013) A novel alpha-synuclein missense mutation in Parkinson disease. *Neurology* 80:1062-1064.
- Reinecke J, Caplan S (2014) Endocytosis and the Src family of non-receptor tyrosine kinases. *Biomol Concepts* 5:143-155.
- Rockenstein E, Mallory M, Hashimoto M, Song D, Shults CW, Lang I, Masliah E (2002) Differential neuropathological alterations in transgenic mice expressing alpha-synuclein from the platelet-derived growth factor and Thy-1 promoters. *J Neurosci Res* 68:568-578.
- Ross GW, Petrovitch H, Abbott RD, Tanner CM, Popper J, Masaki K, Launer L, White LR (2008) Association of olfactory dysfunction with risk for future Parkinson's disease. *Ann Neurol* 63:167-173.

- Saito Y, Shioya A, Sano T, Sumikura H, Murata M, Murayama S (2016) Lewy body pathology involves the olfactory cells in Parkinson's disease and related disorders. *Mov Disord* 31:135-138.
- Samuel F, Flavin WP, Iqbal S, Pacelli C, Sri Renganathan SD, Trudeau LE, Campbell EM, Fraser PE, Tandon A (2016) Effects of Serine 129 Phosphorylation on alpha-Synuclein Aggregation, Membrane Association, and Internalization. *J Biol Chem* 291:4374-4385.
- Sarma AA, Richard MB, Greer CA (2011) Developmental dynamics of piriform cortex. *Cereb Cortex* 21:1231-1245.
- Schaser AJ, Osterberg VR, Dent SE, Stackhouse TL, Wakeham CM, Boutros SW, Weston LJ, Owen N, Weissman TA, Luna E, Raber J, Luk KC, McCullough AK, Woltjer RL, Unni VK (2019) Alpha-synuclein is a DNA binding protein that modulates DNA repair with implications for Lewy body disorders. *Sci Rep* 9:10919.
- Schreglmann SR, Regensburger M, Rockenstein E, Masliah E, Xiang W, Winkler J, Winner B (2015) The temporal expression pattern of alpha-synuclein modulates olfactory neurogenesis in transgenic mice. *PLoS One* 10:e0126261.
- Shepherd GM, Rowe TB, Greer CA (2021) An Evolutionary Microcircuit Approach to the Neural Basis of High Dimensional Sensory Processing in Olfaction. *Front Cell Neurosci* 15:658480.
- Shults CW (2006) Lewy bodies. *Proc Natl Acad Sci U S A* 103:1661-1668.
- Simon DK, Tanner CM, Brundin P (2020) Parkinson Disease Epidemiology, Pathology, Genetics, and Pathophysiology. *Clin Geriatr Med* 36:1-12.
- Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. *Nature* 388:839-840.

- Svensson E, Horvath-Puho E, Thomsen RW, Djurhuus JC, Pedersen L, Borghammer P, Sorensen HT (2015) Vagotomy and subsequent risk of Parkinson's disease. *Ann Neurol* 78:522-529.
- Taylor TN, Potgieter D, Anwar S, Senior SL, Janezic S, Threlfell S, Ryan B, Parkkinen L, Deltheil T, Cioroch M, Livieratos A, Oliver PL, Jennings KA, Davies KE, Ansorge O, Bannerman DM, Cragg SJ, Wade-Martins R (2014) Region-specific deficits in dopamine, but not norepinephrine, signaling in a novel A30P alpha-synuclein BAC transgenic mouse. *Neurobiol Dis* 62:193-207.
- Trinh D, Israwi AR, Arathoon LR, Gleave JA, Nash JE (2021) The multi-faceted role of mitochondria in the pathology of Parkinson's disease. *J Neurochem* 156:715-752.
- van den Berge SA, van Strien ME, Korecka JA, Dijkstra AA, Sluijs JA, Kooijman L, Eggers R, De Filippis L, Vescovi AL, Verhaagen J, van de Berg WD, Hol EM (2011) The proliferative capacity of the subventricular zone is maintained in the parkinsonian brain. *Brain* 134:3249-3263.
- van der Putten H, Wiederhold KH, Probst A, Barbieri S, Mistl C, Danner S, Kauffmann S, Hofele K, Spooren WP, Ruegg MA, Lin S, Caroni P, Sommer B, Tolnay M, Bilbe G (2000) Neuropathology in mice expressing human alpha-synuclein. *J Neurosci* 20:6021-6029.
- Vargas KJ, Makani S, Davis T, Westphal CH, Castillo PE, Chandra SS (2014) Synucleins regulate the kinetics of synaptic vesicle endocytosis. *J Neurosci* 34:9364-9376.
- Vargas KJ, Schrod N, Davis T, Fernandez-Busnadiego R, Taguchi YV, Laugks U, Lucic V, Chandra SS (2017) Synucleins Have Multiple Effects on Presynaptic Architecture. *Cell Rep* 18:161-173.
- Vidyadhara DJ, Lee JE, Chandra SS (2019) Role of the endolysosomal system in Parkinson's disease. *J Neurochem* 150:487-506.

- Wakabayashi K, Tanji K, Odagiri S, Miki Y, Mori F, Takahashi H (2013) The Lewy body in Parkinson's disease and related neurodegenerative disorders. *Mol Neurobiol* 47:495-508.
- Walker DG, Lue LF, Adler CH, Shill HA, Caviness JN, Sabbagh MN, Akiyama H, Serrano GE, Sue LI, Beach TG, Arizona Parkinson Disease C (2013) Changes in properties of serine 129 phosphorylated alpha-synuclein with progression of Lewy-type histopathology in human brains. *Exp Neurol* 240:190-204.
- Whitman MC, Greer CA (2007) Synaptic integration of adult-generated olfactory bulb granule cells: basal axodendritic centrifugal input precedes apical dendrodendritic local circuits. *J Neurosci* 27:9951-9961.
- Whitman MC, Greer CA (2009) Adult neurogenesis and the olfactory system. *Prog Neurobiol* 89:162-175.
- Winner B, Lie DC, Rockenstein E, Aigner R, Aigner L, Masliah E, Kuhn HG, Winkler J (2004) Human wild-type alpha-synuclein impairs neurogenesis. *J Neuropathol Exp Neurol* 63:1155-1166.
- Wojtowicz JM, Kee N (2006) BrdU assay for neurogenesis in rodents. *Nat Protoc* 1:1399-1405.
- Xu J, Wu XS, Sheng J, Zhang Z, Yue HY, Sun L, Sgobio C, Lin X, Peng S, Jin Y, Gan L, Cai H, Wu LG (2016) alpha-Synuclein Mutation Inhibits Endocytosis at Mammalian Central Nerve Terminals. *J Neurosci* 36:4408-4414.
- Yang M, Crawley JN (2009) Simple behavioral assessment of mouse olfaction. *Curr Protoc Neurosci* Chapter 8:Unit 8 24.
- Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B, Llorens V, Gomez Tortosa E, del Ser T, Munoz DG, de Yebenes JG (2004) The new

mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann Neurol* 55:164-173.

Zhang XM, Anwar S, Kim Y, Brown J, Comte I, Cai H, Cai NN, Wade-Martins R, Szele FG (2019) The A30P alpha-synuclein mutation decreases subventricular zone proliferation. *Hum Mol Genet* 28:2283-2294.

Zhou Y, He R, Zhao Y, He Y, Hu Y, Sun Q, Xu Q, Tan J, Yan X, Tang B, Guo J (2020) Olfactory Dysfunction and Its Relationship With Clinical Features of Parkinson's Disease. *Front Neurol* 11:526615.

Tables

Table 1. Primary and secondary antibodies

Antigen	Primary Ab	Source (Cat. #)	Dilution	Secondary Ab	Source	Dilution
AC3	Rabbit Poly.	ThermoFisher (PA5-35382)	1:300	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
α -synuclein endo(M, H)	Mouse IgG1	BD Bioscience (610786)	1:500	Goat anti-Ms IgG1 Alexa 555 (IHC)	Invitrogen	1:1000
			(IHC)	Goat anti-Ms IgG1 IRDye 800	LICOR	1:10000
			1:1000 (WB)			
α -synuclein, aggregated	Mouse IgG1	BioLegend (847801)	1:500	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
α -synuclein, aggregated	Mouse IgG1k	EMD Millipore (MABN389)	1:300	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
α -synuclein, aggregated	Rat IgG2a, k	BioLegend (864901)	1:300	Goat anti-Rat IgG Alexa 647	Invitrogen	1:1000
α -synuclein pSer129	Mouse IgG2a	BioLegend (825702)	1:1000	Goat anti-Ms IgG2a Alexa 488	Invitrogen	1:1000
α -synuclein pSer129	Rabbit IgG	Abcam (ab51253)	1:500	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000
β -actin	Mouse IgG1	GeneTex (GT5512)	1:1000	Goat anti-Ms IgG1 IRDye 800	LICOR	1:10000
BrdU	Rat IgG	Abcam (ab6326)	1:300	Goat anti-Rat IgG Alexa 488	Invitrogen	1:1000
Caspase 3 cleavage	Rabbit Poly.	Cell Signaling (9661L)	1:500	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
c-Src/Src	Mouse IgG2a	Santa Cruz (sc-5266)	1:250	Goat anti-Ms IgG2a Alexa 555	Invitrogen	1:1000
Dlx2	Rabbit Poly.	EMD Millipore (AB5726)	1:200	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
Doublecortin (DCX)	Guinea Pig	EMD Millipore (AB2253)	1:500	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
GAP43	Mouse IgG2a	ThermoFisher (33-5000)	1:100	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000
GFAP	Rabbit Poly.	BioLegend (840001)	1:1000	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
Iba1	Rabbit Poly.	Wako (016-20001)	1:200	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
NQO1	Goat IgG	Abcam (ab2346)	1:100	Donkey anti-Goat Alexa 555	Invitrogen	1:1000
OMP	Goat Poly.	Wako (019-22291)	1:500	Donkey anti-Goat Alexa 546	Invitrogen	1:1000
Phospho-S6 (pS6)	Rabbit IgG	Cell Signaling (4858T)	1:400	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
Tbr1	Rabbit Poly.	Abcam (ab31940)	1:500	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
TH (clone LNC1)	Mouse IgG1	EMD Millipore (MAB318)	1:500	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
VGLUT2	Rabbit Poly.	SySy (135-403)	1:200	Donkey anti-Rb IgG Alexa 488	Invitrogen	1:1000

Figure Legends

Figure 1. α -syn-Tg mice show olfactory deficits at the age of 12-14 months. (A) Wild type (WT) and α -syn-Tg (overexpressing α -syn carrying the hA30P mutation) mice are evaluated for olfactory deficits using standard buried food tests. Mice at early stages of the disease (6-7 months) do not exhibit olfactory dysfunction measured as the latency to find a hidden food. At late stages (12-14 months), α -syn-Tg mice show olfactory deficits indicative of dysregulated olfactory pathway. (B) Surface food location test did not show any difference between WT and α -syn-Tg mice, performed to exclude bias due to motor deficits or lack of motivation for food. Data are normalized between 0 as the minimum latency time (0 sec), and 1 as the maximum latency time (300 sec) (* = $p < 0.05$). Statistics: Unpaired non-parametric Mann-Whitney U test.

Figure 2. Organization of mouse olfactory system, and anatomy of the olfactory epithelium (OE) in WT and α -syn-Tg mice. (A) Diagram of the mouse olfactory system summarizing the different station relays used by the olfactory information to travel from the OE to the brain. (B, E) Immunohistochemistry against aggregated α -syn (green) showing an absence of labeling in the OE. Staining against NAD(P)H dehydrogenase [quinone] 1 (NQO1) (red) is used to label dorsal OE. Differences in NQO1 staining correspond to slight variations in the section plane along the rostral-caudal axis between the different mice. (C, F) Differentiating and mature OSNs labeled with AC3 (red) and OMP (blue) respectively. (D, G) Immature and mature olfactory sensory neurons (OSNs) labeled with growth associated protein-43 (GAP43) (green) and olfactory marker protein (OMP) (blue) respectively. No differences in the anatomy and morphology of the OE are seen with any of these markers between WT and α -syn-Tg mice at 12-14 months of age. Staining using mouse anti- aggregated α -syn (EMD Millipore, Table 1). Abbreviations: LP: lamina propia; SP: septum. Scale bars of 500 μ m in A, B; 50 μ m in C, D, E, F.

Figure 3. Validation of the α -syn-Tg mice model by detecting endogenous α -synuclein in the OB and APC. Pathological α -syn is detected by the expression of pSer129- α -syn (green) and aggregated α -syn (cyan), while endogenous (endo-) α -syn, which detect normal mouse and human α -syn, is shown in red. Nuclei counterstained in blue. (A-a'', B-b'') Staining in the OB and APC layer II of WT mice at 6-7 months showing residual expression of pSer129- α -syn in MCL and iEPL (a), and endo- α -syn in some mitral cells (a'', arrowhead), with no staining in APC. (C-c'', D-d'') Staining in α -syn-Tg mice showing overexpression of pSer129- α -syn and aggregated α -syn in the MCL/IPL layers of the OB (c, c'), with strong accumulation in mitral cells apical dendrites (green and cyan arrowheads), and in neurons of APC layer II (d, d'). Endo- α -syn is overexpressed in OB mitral cells with pathology (C, white and c'' red arrowheads, respectively) and some APC layer II pyramidal neurons (D, white and d'' red arrowheads, respectively). In APC, some neurons expressing pSer129- α -syn do not colocalized with endo- α -syn (D, green arrowheads). (E-e'', F-f'') Representative images of pSer129- α -syn staining in the OB and APC of WT mice at 12-14 months showing residual expression in the OB-iEPL (e), and endo- α -syn in some mitral cells (e'', red arrowheads). (G-g'', H-h'') pSer129- α -syn staining in α -syn-Tg mice at 12-14 months. Overexpression of endo- and pathologic α -syn is observed in some mitral cells (G, white arrowheads) and pyramidal neurons of APC layer II (H, white arrowheads). Other neurons only overexpress pSer129- α -syn pathology (G-g, H-h, green arrowheads) or only endo- α -syn (G-g'', H-h'', red arrowheads). At 12-14 months endo- α -syn seems to be localized more dispersedly in the cellular parenchyma than that of 6-7 months old mice. Staining is a triple using mouse anti- endo- α -syn (BD Biosciences), rabbit anti- pSer129- α -syn (Abcam) and rat anti- aggregated α -syn (BioLegend) (Table 1). Abbreviations: APC: anterior piriform cortex; iEPL: inner external plexiform layer; IPL: internal plexiform layer; MCL: mitral cell layer; OB: olfactory bulb. Scales of 25 μ m.

Figure 4. α -synuclein pathology in the olfactory bulb (OB) of mice at 12-14 months. (A-a'', B-b'') Representative images of OB coronal sections from WT and α -syn-Tg mice stained against aggregated α -

syn (green) and TBR1 (red) as a marker for projection neurons (a'', b'', g'', h''). A significant accumulation of aggregated α -syn is observed in α -syn-Tg mice in the GCL (D) and IPL (D), with a strong trend in MCL (C). In the EPL there is almost no accumulation of aggregated α -syn between WT and α -syn-Tg mice (E). (G-g'', H-h'') Representative images of OB coronal sections from WT and α -syn-Tg mice stained against pSer129- α -syn (green) and TBR1 (red). (I) pSer129- α -syn is significantly increased in the MCL of α -syn-Tg mice compared to controls. (K) no statistical differences are observed on pSer129- α -syn expression between WT and α -syn-Tg mice, but an evident accumulation in the inner EPL of WT (g, g') and α -syn-Tg (h, h') mice is observed. Remarkably, a strong accumulation of pSer129- α -syn is seen in apical dendrites of mitral cells of α -syn-Tg mice (h, h', green arrowheads). Tufted cells also overexpress pSer129- α -syn (h, h', arrowheads). (J, L) No differences are detected in the GCL and IPL between WT and α -syn-Tg mice, although a characteristic patchy accumulation is seen in GCL that likely belong to M/Tc axons. Nuclei counterstained in blue. Staining using mouse anti aggregated α -syn (BioLegend) and mouse anti- pSer129- α -syn (BioLegend) (Table 1). Abbreviations: EPL: external plexiform layer; GCL: granule cell layer; IPL: internal plexiform layer; M/Tc: mitral and tufted cells; TBR1: T-Box Brain Transcription Factor-1. Scale bars of 500 μ m in A, B, G, H of; 100 μ m in a'a'', b-b'', g-g'', h'-h''. *= p<0.05; **= p< 0.01. Statistics: Unpaired t-test.

Figure 5. α -synuclein pathology in anterior olfactory nucleus (AON) and piriform cortex (PC) of mice at 12-14 months. (A, B) Representative images of AON coronal sections from WT and α -syn-Tg mice stained against aggregated α -syn (green) and TBR1 (red) as a marker for projection neurons. (C) Percentage of stained area in the AON showing higher levels of aggregated α -syn in α -syn-Tg vs WT mice with no statistical differences due to high variability. (D, E, G, H) Representative images of aggregated α -syn staining from the APC and PPC showing α -syn aggregation in layer II of α -syn-Tg mice, with accumulation in some neuronal cell bodies (arrowheads). (F, I) Quantification of aggregated α -syn

stained area in APC and PPC showing no statistical differences between WT and α -syn-Tg mice, although there is a trend to be higher in α -syn-Tg mice. (J, K) Representative images of pSer129- α -syn staining (green) in AON, showing a strong accumulation of α -syn pathology in cell bodies of projection neurons co-labeled with TBR1 (red). (L) The comparison of stained area for pSer129- α -syn in AON shows statistically significant higher accumulation in α -syn-Tg mice. (M, N, P, Q) Examples of images taken in APC and PPC showing an intense labeling for pSer129- α -syn in neuronal cell bodies of layers IIa and IIb in APC, and IIb in PPC (dotted line). (O, R) Quantification of pSer129- α -syn stained area in APC and PPC showing statistically significant differences between WT and α -syn-Tg mice. Nuclei counterstained in blue. Staining using mouse anti- aggregated α -syn (BioLegend) and mouse anti- pSer129- α -syn (BioLegend) combined with anti-TBR1 (Table 1). Abbreviations: APC: anterior PC; PPC: posterior PC; TBR1: T-Box Brain Transcription Factor-1. Scale bars of 200 μ m in A, B, J, K; 50 μ m in D, E, G, H, M, N, P, Q. * = $p < 0.05$; ** = $p < 0.01$. Statistics: Unpaired t-test.

Figure 6. No ultrastructural alterations were seen in the OB and layer I in APC of α -syn-Tg mice.

(A, B) Representative images of electron microscopy in the glomeruli of OBs from WT and α -syn-Tg mice. Number of mitochondria (Mito) and different types of synapses are quantified and highlighted as: asymmetric (Asym. Red arrowheads), symmetric (Sym. Green arrowheads) and reciprocal (Rec. Yellow arrowheads). (C) Quantification of Mito. and synapses in the glomeruli parenchyma show no statistical differences between WT and α -syn-Tg mice. (D) Representation of the number of Mito. and total synapses counted per image and arranged from the smallest to the greatest number of objects, showing a strong trend of α -syn-Tg mice to have less Mito. although no statistical differences are found. (E, F) Representative images of APC layer I parenchyma at EM. In these images it is shown Asym. (red arrowheads) and Sym. (green arrowheads) synapses. (G) Quantification of Mito. and synapses in APC showing no statistical differences between WT and α -syn-Tg mice. (H) Representation of the distribution

of Mito. and synapses on each image arranged from lowest to highest number of objects showing a similar trend to that observed in OB. Scale bars of 0.5 μm . Statistics: Unpaired t-test.

Figure 7. Perturbations in neurogenesis was seen in the OB of α -syn-Tg mice. Neurogenesis is studied by counting newly generated cells identified by detecting the thymidine analog BrdU incorporated during cell division. BrdU staining is shown in green while immature neurons (neuroblasts) are identified with doublecortin (DCX) staining (red). Nuclei counterstained in blue. (A to d') Representative images of OB coronal sections in WT and α -syn-Tg mice at 6-7 months (A-a', B-b') and 12-14 months (C-c', D-d') stages. Insets are high magnification images of BrdU and DCX labeled cells located in the RMS + GCL (a-d) or the GL (a'-d'). (E) Quantification of BrdU cells in the GL shows a statistically significant decrease of cells in α -syn-Tg mice at late stages 12-14 months, and an overall increase in the total number of BrdU⁺ cells in this layer between 6-7 to 12-14 months mice. (F) Analysis of BrdU cells in the GCL shows a statistically significant decrease of labeled cells in α -syn-Tg mice exclusively at early stages of the disease (6-7 months) (G) Quantification of BrdU⁺ cells in the RMS showing no statistical differences between WT and α -syn-Tg mice. Abbreviations: EPL: external plexiform layer; GL: glomerular layer; Gl: individual glomeruli; GCL: granule cell layer; MCL: mitral cell layer; RMS: bulbar portion of the rostral migratory stream. Scale bars of 500 μm in A-D; 100 μm in a-d; 50 μm in a'-d'. *= $p < 0.05$. Statistics: Unpaired t-test.

Figure 8. Proteomics of OB and APC revealed defects in synaptic endo- and exocytosis pathways in the OB, but minimum alterations in APC of α -syn-Tg mice. (A) Volcano plot of fold change in the OB proteome of 6-7 months α -syn-Tg compared to control wild type (WT) mice (n=3 mice/genotype). Proteins that changed 1.25-fold (vertical dotted lines) with a p-value of < 0.05 (horizontal dotted line) are considered to be significantly differentially expressed. Among the 14 significant proteins, 8 are upregulated (red) and 6 are downregulated (blue). (B) Heat map of significantly differentially expressed proteins in OB of α -syn-Tg compared to WT mice at early stage (6-7 months) for each technical replicate

(3 technical replicates/mouse). Red indicates increased expression (+2), and blue indicates decreased expression (-2). (C) Biological pathways that are significantly ($FDR < 0.05$) affected in the OB of 6-7 months α -syn-Tg mice, determined by GO enrichment analysis. (D) Volcano plot of fold change in the OB proteome of 12-14 months α -syn-Tg compared to WT mice ($n=3$ mice/genotype). Among the 15 significantly differentially expressed proteins (1.25-fold, $p < 0.05$), 7 are upregulated (red) and 8 are downregulated (blue). (E) Heat map of significantly differentially expressed proteins in OB of late stage (12-14 months) α -syn-Tg mice compared to their WT counterparts for each technical replicate (3 technical replicates/mouse). (F) Biological pathways that are significantly ($FDR < 0.05$) affected in the OB of 12-14 months α -syn-Tg mice, determined by GO enrichment analysis. (G) Volcano plot of fold change in the APC proteome. α -syn-Tg compared to WT mice ($n=3$ mice/genotype) at 6-7 months All the 11 significantly differentially expressed proteins (1.25-fold, $p < 0.05$) are upregulated in α -syn-Tg mice. (H) Heat map of significantly differentially expressed proteins in APC of α -syn-Tg compared to WT mice at 6-7 months for each technical replicate (3 technical replicates/mouse). (I) Biological pathways that are significantly ($FDR < 0.05$) affected in the APC of 6-7 months α -syn-Tg mice, determined by GO enrichment analysis. (J) Volcano plot of fold change in the APC proteome of α -syn-Tg compared to WT mice ($n=3$ mice/genotype) at 12-14 months Among the 16 significantly differentially expressed proteins (1.25-fold, $p < 0.05$), 12 are upregulated (red) and 4 are downregulated (blue). (H) Heat map of significantly differentially expressed proteins in APC of α -syn-Tg mice compared to WT controls for each technical replicate (3 technical replicates/mouse) at 12-14 months (I) Biological pathways that are significantly ($FDR < 0.05$) affected in the APC of 12-14 months α -syn-Tg mice, determined by GO enrichment analysis.

Supplementary Figure 1. Quantification of endogenous/human and aggregated α -synuclein by western blot (WB). (A) WB detecting total (endogenous and human) α -syn in WT controls and α -syn-Tg mice at both studied stages (6-7 and 12-14 months mice). The amount of protein quantified by densitometry showed a significant increase of total α -syn between WT vs α -syn-Tg mice at both 6-7 and 12-14 months in the OB (B) Detection of aggregated α -syn (from EMD Millipore, Table 1) by WB showed an increase in the amount of protein in the OB of α -syn-Tg mice at 12-14 months β -actin is used as a loading control. * = $p < 0.05$, ** = $p < 0.01$. Statistics: Unpaired t-test.

Supplementary Figure 2. Structure of the OB and general cell activity of mitral cells in 12-14 months mice. (A, B) OSNs axons identified by labeling with VGLUT2 (green) and OMP (red) show no differences in the glomerular structure between WT and α -syn-Tg mice. (C, D) Staining of dopaminergic neurons with TH in the GL (green) shows no disruptions in the morphology or cellular processes in α -syn-Tg mice. Quantifications of TH cell numbers is similar between WT ($443 \pm 43 \text{ mm}^2$) and α -syn-Tg ($535 \pm 74 \text{ mm}^2$) mice (data not represented). Apoptosis is detected using cleaved caspase-3 (red) and no differences are evident between WT and α -syn-Tg mice, indicative of an increased cell death caused by the hA30P α -syn mutation. (E-e', F-f') Mitral cells pathology detected with pSer129- α -syn from BioLegend (Table 1) (green), and cellular activity detected using pS6 (red). Residual pSer129- α -syn is seen in WT (E-e) that is overexpressed in α -syn-Tg mice (F-f). pS6 staining reveals similar activity in mitral cells of both WT and α -syn-Tg mice (e', f'). (G) Quantification of pS6 expression levels by analyzing the pixel intensity in mitral cells shows no significant differences between WT and α -syn-Tg mice indicative of decreased activity caused by the hA30P α -syn mutation. Abbreviation: OMP: olfactory marker protein; OSNs: olfactory sensory neurons; pS6: phosphorylated S6 Ribosomal Protein (Ser235/236); TH: tyrosine hydroxylase; VGLUT2: vesicular glutamate transporter 2. Scale bars of 200 μm in A, B; 100 μm in C, D; 50 μm in E-e', F-f'. Statistics: Unpaired t-test.

Supplementary Figure 3. Analysis of cell phenotypes of BrdU-expressing cells in OB of WT and α -syn-Tg mice at 6-7 months. Newly generated cells incorporate BrdU are shown in green in WT controls and α -syn-Tg mice; markers to identify different cell types are shown in red. Nuclei counterstained in blue. (A, B) no BrdU⁺ cell expresses the neuroblast marker DCX in either the GCL or RMS. (C, D) Mature neurons are labeled with NeuN in the GCL and roughly 43% and 30% of BrdU cells co-express this marker in WT and α -syn-Tg mice, respectively. (E, F) Analysis of cells in the periglomerular region showing that no BrdU⁺ cell express the dopaminergic marker TH. (G, H) GFAP marker is used to identify astrocytes showing no colocalization with BrdU. (I, J) Microglial cells are identified with IBA1 which are not part of the pool of BrdU⁺ cells either. Abbreviations: DCX: doublecortin; GCL: granule cell layer; GFAP: glial fibrillary acidic protein; IBA1: ionized calcium-binding adapter molecule-1; NeuN: neuronal nuclei marker; RMS: rostral migratory stream; TH: tyrosine hydroxylase. Scale bars of 50 μ m.

Supplementary Figure 4. Characterization of BrdU-expressing cells in the subventricular zone (SVZ) of mice at early stages of the disease (6-7 months) BrdU⁺ cells are shown in green. In red, we show staining for the different subtype of cells located within the SVZ: neuroblasts that express DCX (a.k.a. “A” cells), radial glia neural stem cells expressing GFAP (a.k.a. “B” cells) and transient amplifying cells expressing Distal-Less Homeobox 2 (Dlx2) (a.k.a. “C” cells). Nuclei counterstained in blue. (A, B) DCX staining do not colocalizes with any BrdU⁺ cell indicating that BrdU⁺ are not part of the “A” pool. (C, D) Similarly, any BrdU⁺ expressed GFAP as marker for “B” cells. (E, F) However, marker to identify “C” cells (Dlx2) co-localized with BrdU⁺ cells located in this region, indicating they were part of the “C” cells pool of the SVZ in these mice. (G) greyscale image of the analyzed region showing location of the SVZ next to the ventricular zone (VZ) and the lateral ventricle (LV). Scale bars of 400 μ m in G; 50 μ m in A, B; 25 μ m in C, D, E, F.

Supplementary Figure 5. Expression of the membrane-associated tyrosine protein kinase (c-Src) in cells with α -synuclein pathology in the olfactory bulb (OB). α -Synuclein pathology is shown by staining with pSer129- α -syn (green), while c-Src is shown in red. Nuclei counterstained in blue. c-Src is reported to phosphorylate α -syn (Ellis et al., 2001) and its overexpression may indicate a role in α -syn phosphorylation under pathology. (A-a', C-c') Labeling of WT mice at 6-7 and 12-14 months, respectively showing residual expression of pSer129- α -syn (a, c) and c-Src (a', c') in the MCL and EPL. (B-b', D-d') Expression in α -syn-Tg mice showing the overexpression of α -synuclein pathology (b, d), and c-Src (b', d') colocalizing in mitral cells (arrowheads). c-Src overexpression detected by immunohistochemistry confirms the results seen in proteomics. Staining using rabbit anti- pSer129- α -syn (Abcam) (Table 1). Scale bars of 50 μ m.

No.	Accession	Gene name	Protein name	Fold change
1	C10	Grc10	Protein C10	1.28436951
2	SYUA	Snc1	Alpha-synuclein	2.295515511
3	H2A1B	H2ac4	Histone H2A type 1-B	1.643448378
4	MTSS2	Mtss2	Protein MTSS 2	1.262605161
5	YLPM1	Ylpm1	YLP motif-containing protein 1	1.292921147
6	UBAC1	Uba1	Ubiquitin-associated domain-containing protein 1	1.410921697
7	SRC	Src	Neuronal proto-oncogene tyrosine-protein kinase Src	2.876813941
8	RELL2	Rell2	RELT-like protein 2	1.34090359
9	CCD22	Ccdc22	Coiled-coil domain-containing protein 22	0.571394901
10	CPT1A	Cpt1a	Carnitine O-palmitoyltransferase 1, liver isoform	0.759995746

11	TIAM1	Tiam1	Rho guanine nucleotide exchange factor TIAM1	0.719080008
12	FXR2	Fxr2	Fragile X mental retardation syndrome-related protein 2	0.749066485
13	HMGA1	Hmga1	High mobility group protein HMG-I/HMG-Y	0.793204904
14	DIRA2	Diras2	GTP-binding protein Di-Ras2	0.770665441

Supplementary Table 1: Proteins that are significantly changed in the proteomic analysis of olfactory bulb of α -syn-Tg mice in comparison to WT at 6-7 months of age.

No.	Accession	Gene name	Protein name	Fold change
1	SYTC	Tars1	Threonine--tRNA ligase 1, cytoplasmic	0.795958
2	COX1	Mtco1	Cytochrome c oxidase subunit 1	0.698021
3	VPS45	Vps4b	Vacuolar protein sorting-associated protein 4B	0.793934
4	PICAL	Picalm	Phosphatidylinositol-binding clathrin assembly protein	0.754455
5	ENOPH	Enoph1	Enolase-phosphatase E1	0.683989
6	UN13A	Unc13a	Protein unc-13 homolog A	0.671959
7	NEO1	Neo1	Neogenin	0.71851
8	CLPT1	Clptm1	Cleft lip and palate transmembrane protein 1	0.778314
9	SNP29	Snap29	Synaptosomal-associated protein 29	0.777816
10	CA198	N/A	Uncharacterized protein C1orf198	1.346692
11	SMCE1	Smarce1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	1.507282
12	P66B	Gatad2b	Transcriptional repressor p66-beta	1.515755
13	ZYX	Zyx	Zyxin	1.26207
14	AT8A1	Atp8a1	Phospholipid-transporting ATPase 1A	1.260929
15	NACA	Naca	Nascent polypeptide-associated complex subunit alpha	1.307384

Supplementary Table 2: Proteins that are significantly changed in the proteomic analysis of olfactory bulb of α -syn-Tg mice in comparison to WT at 12-14 months of age.

No.	Accession	Gene name	Protein name	Fold change
1	SMCA2	Smarca2	Probable global transcription activator SNF2L2	1.279780958
2	ARAF	Araf	Serine/threonine-protein kinase A-Raf	1.268139535
3	ZY11B	Zyg11b	Protein zyg-11 homolog B	1.268384587
4	SUGP2	Sugp2	SURP and G-patch domain-containing protein 2	1.405654737
5	F177A	Fam177a1	Protein FAM177A1	1.27607181
6	ATE1	Ate1	Arginyl-tRNA--protein transferase 1	1.252608747
7	AASD1	Aarsd1	Alanyl-tRNA editing protein Aarsd1	1.335151511
8	PTEN	Pten	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	1.720804806
9	PABP1	Pabpc1	Polyadenylate-binding protein 1, PABP-1, Poly(A)-binding protein 1	1.364366716
10	MON2	Mon2	Protein MON2 homolog	1.766115672
11	SYUA	Snca	Alpha-synuclein	4.090515865

Supplementary Table 3: Proteins that are significantly changed in the proteomic analysis of anterior piriform cortex of α -syn-Tg mice in comparison to WT at 6-7 months of age.

No.	Accession	Gene name	Protein name	Fold change
1	CHM2A	Chmp2a	Charged multivesicular body protein 2a	0.418897
2	CRYAB	Cryab	Alpha-crystallin B chain	0.70911
3	KPCB	Prkcb	Protein kinase C beta type	0.715298
4	TIPRL	Tiprl	TIP41-like protein	0.701951
5	ADDG	Add3	Gamma-adducin	1.290529
6	PP6R3	Ppp6r3	Serine/threonine-protein phosphatase 6 regulatory subunit 3	1.552139
7	ASTRB	Gramd1b	Protein Aster-B	1.266189
8	RLGPB	Ralgapb	Ral GTPase-activating protein subunit beta	1.260604
9	SC23A	Sec23a	Protein transport protein Sec23A	1.338548
10	ENAH	Enah	Protein enabled homolog	1.293051
11	AASD1	Aarsd1	Alanyl-tRNA editing protein Aarsd1	1.583143
12	MIC26	Apoo	MICOS complex subunit Mic26	1.260649
13	OPLA	Oplah	5-oxoprolinase	1.597227
14	MARC2	Mtarc2	Mitochondrial amidoxime reducing component 2	1.274637
15	FA81A	Fam81a	Protein FAM81A	1.695311
16	DLGP1	Dlgap1	Disks large-associated protein 1	1.309585

Supplementary Table 4: Proteins that are significantly changed in the proteomic analysis of anterior piriform cortex of α -syn-Tg mice in comparison to WT at 12-14 months of age.

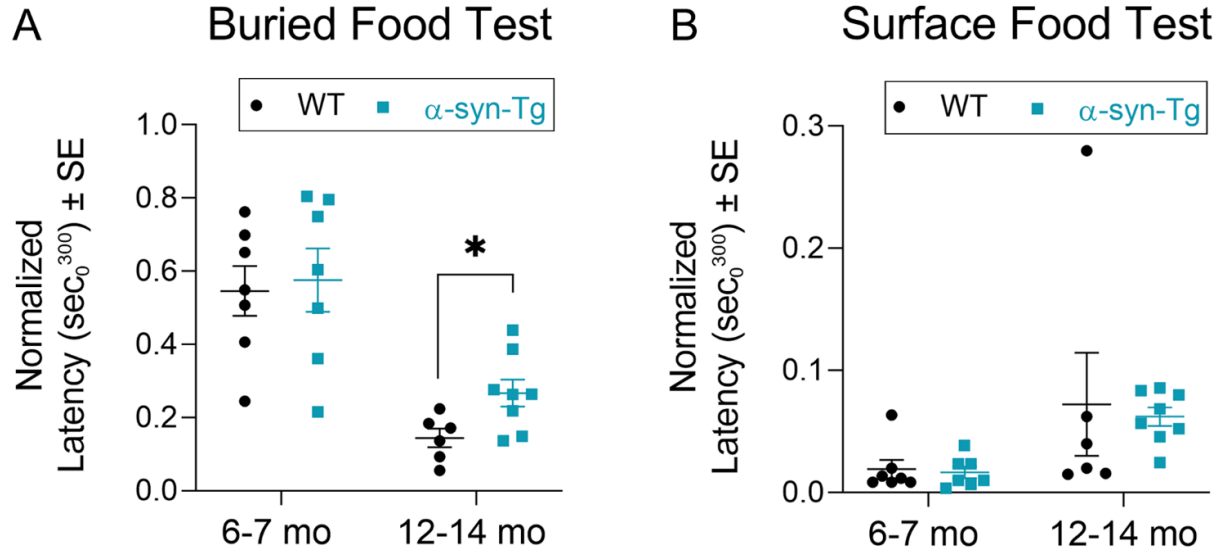


Figure 1. α -Syn-Tg mice show olfactory deficits at an older age of 12-14 months. (A) WT and α -syn-Tg (overexpressing α -syn carrying the hA30P mutation) mice are evaluated for olfactory deficits using standard buried food tests. Animals at early stages of the disease (6-7 mo.) do not exhibit olfactory dysfunction measured as the latency to find a hidden food. At late stages (12-14 mo.), α -syn-Tg mice show olfactory deficits indicative of dysregulated olfactory pathway. (B) Surface food location test did not show any difference between WT and α -syn-Tg mice, performed to exclude bias due to motor deficits or lack of motivation for food. Data are normalized between 0 as the minimum latency time (0 sec), and 1 as the maximum latency time (300 sec) (* = $p < 0.05$). Statistics: Unpaired non-parametric Mann-Whitney U test.

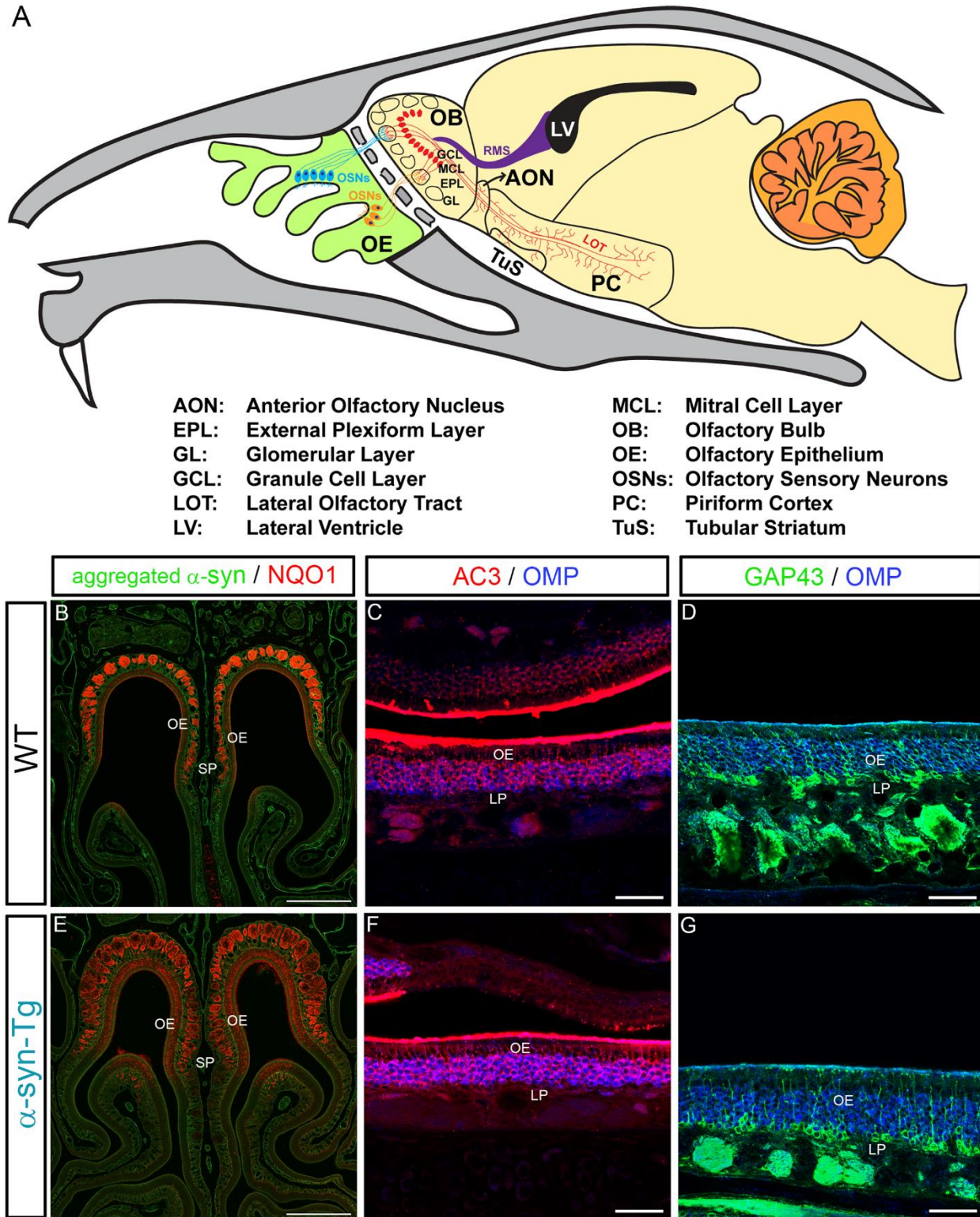


Figure 2. Organization of mouse olfactory system, and anatomy of the olfactory epithelium (OE) in WT and α -syn-Tg mice at 12-14 mo. (A) Diagram of the mouse olfactory system summarizing the different station relays used by the olfactory information to travel from the OE to the brain. (B, E) IHC against aggregated α -syn (green) showing an absence of labeling in the

OE. Staining against NQO1 (red) is used to label dorsal OE. Differences in NQO1 staining correspond to slight variations in the section plane along the rostral-caudal axis between the different animals. (C, F) Differentiating and mature olfactory sensory neurons OSNs labeled with AC3 (red) and OMP (blue), respectively. (D, G) Immature and mature OSNs labeled with growth GAP43 (green) and OMP (blue), respectively. No differences in the anatomy and morphology of the OE are seen with any of these markers between WT and α -syn-Tg mice. Staining using mouse anti- aggregated α -syn (EMD Millipore, Table 1). Abbreviations: LP: lamina propia; SP: septum. Scale bars of 500 μ m in A, B; 50 μ m in C, D, E, F.

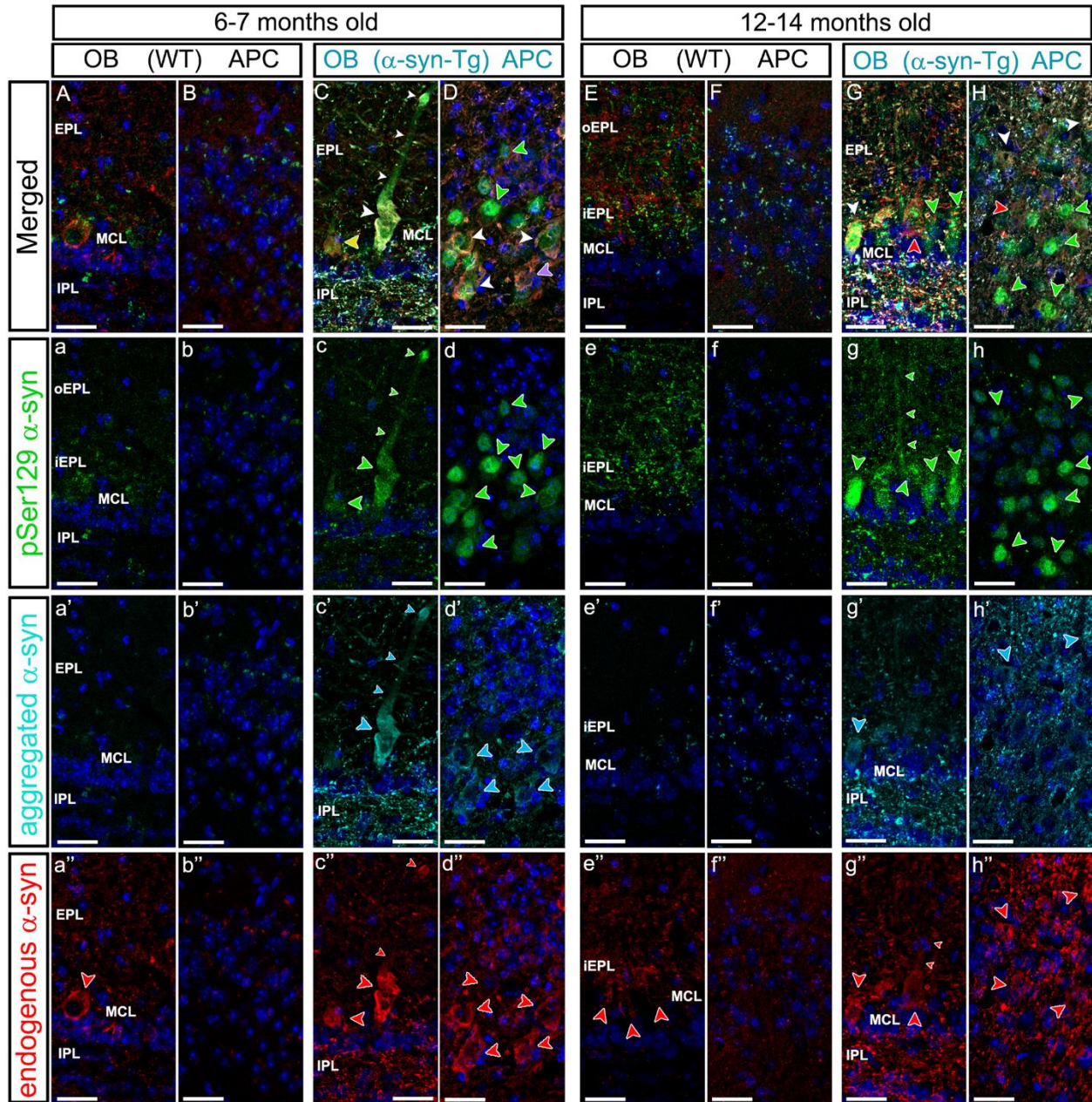


Figure 3. Validation of the α -syn-Tg mice model by detecting endogenous α -synuclein in the OB and APC. Pathological α -syn is detected by the expression of pSer129- α -syn (green) and aggregated α -syn (cyan), while endogenous (endo-) α -syn, which detect normal mouse and human α -syn, is shown in red. Nuclei counterstained in blue. (A-a'', B-b'') Staining in the OB and APC layer II of WT animals at 6-7 mo. showing residual expression of pSer129- α -syn in MCL and iEPL (a), and endo- α -syn in some mitral cells (a'', arrowhead), with no staining in APC. (C-c'', D-d'') Staining in α -syn-Tg mice showing overexpression of pSer129- α -syn and aggregated α -syn in the MCL/IPL layers of the OB (c, c'), with strong accumulation in mitral cells apical dendrites (green and cyan arrowheads), and in neurons of APC layer II (d, d'). Endo- α -syn is overexpressed in OB mitral cells with pathology (C, white and c'' red arrowheads, respectively) and some APC layer II pyramidal neurons (D, white and d'' red arrowheads, respectively). In APC,

some neurons expressing pSer129- α -syn do not colocalized with endo- α -syn (D, green arrowheads). (E-e'', F-f''). Representative images of pSer129- α -syn staining in the OB and APC of WT mice at 12-14 mo. showing residual expression in the OB-iEPL (e), and endo- α -syn in some mitral cells (e'', red arrowheads). (G-g'', H-h'') pSer129- α -syn staining in α -syn-Tg mice at 12-14 mo. Overexpression of endo- and pathologic α -syn is observed in some mitral cells (G, white arrowheads) and pyramidal neurons of APC layer II (H, white arrowheads). Other neurons only overexpress pSer129- α -syn pathology (G-g, H-h, green arrowheads) or only endo- α -syn (G-g'', H-h'', red arrowheads). At 12-14 mo. endo- α -syn seems to be localized more dispersedly in the cellular parenchyma than that of 6-7 mo. old mice. Staining consists of a triple IHC using mouse anti- endo- α -syn (BD Biosciences), rabbit anti- pSer129- α -syn (Abcam) and rat anti-aggregated α -syn (BioLegend) (Table 1). Abbreviations: APC: anterior piriform cortex; iEPL: inner external plexiform layer; IPL: internal plexiform layer; MCL: mitral cell layer; OB: olfactory bulb. Scales of 25 μ m.

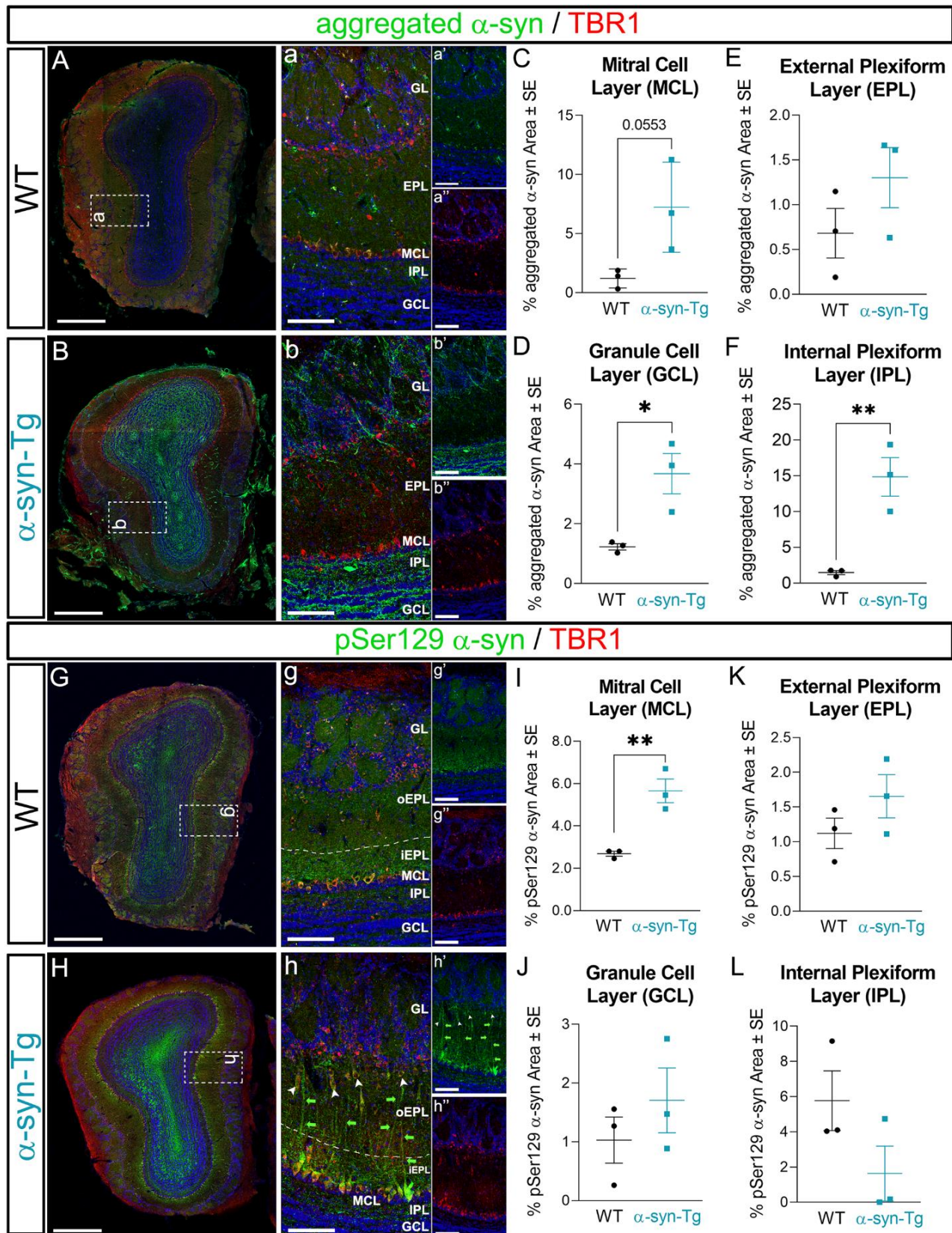


Figure 4. α -synuclein pathology in the OB of mice at late stage of 12-14 mo. (A-a'', B-b'') Representative images of OB coronal sections from WT and α -syn-Tg mice stained against aggregated α -syn (green) and TBR1 (red) as a marker for projection neurons (a'', b'', g'', h''). A significant accumulation of aggregated α -syn is observed in α -syn-Tg mice in the GCL (D) and IPL (D), with a strong trend in MCL (C). In the EPL there is almost no accumulation of aggregated α -syn between WT and α -syn-Tg mice (E). (G-g'', H-h'') Representative images of OB coronal sections from WT and α -syn-Tg mice stained against pSer129- α -syn (green) and TBR1 (red). (I) pSer129- α -syn is significantly increased in the MCL of α -syn-Tg mice compared to controls. (K) no statistical differences are observed on pSer129- α -syn expression between WT and α -syn-Tg mice, but an evident accumulation in the inner EPL of WT (g, g') and α -syn-Tg (h, h') mice is observed. Remarkably, a strong accumulation of pSer129- α -syn is seen in apical dendrites of mitral cells of α -syn-Tg mice (h, h', green arrowheads). Tufted cells also overexpress pSer129- α -syn (h, h', arrowheads). (J, L) No differences are detected in the GCL and IPL between WT and α -syn-Tg mice, although a characteristic patchy accumulation is seen in GCL that likely belong to M/Tc axons. Nuclei counterstained in blue. Staining using mouse anti aggregated α -syn (BioLegend) and mouse anti- pSer129- α -syn (BioLegend) (Table 1). Abbreviations: EPL: external plexiform layer; GCL: granule cell layer; IPL: internal plexiform layer; M/Tc: mitral and tufted cells; TBR1: T-Box Brain Transcription Factor-1. Scale bars of 500 μ m in A, B, G, H of; 100 μ m in a'a'', b-b'', g-g'', h'-h''. * = $p < 0.05$; ** = $p < 0.01$. Statistics: Unpaired t-test.

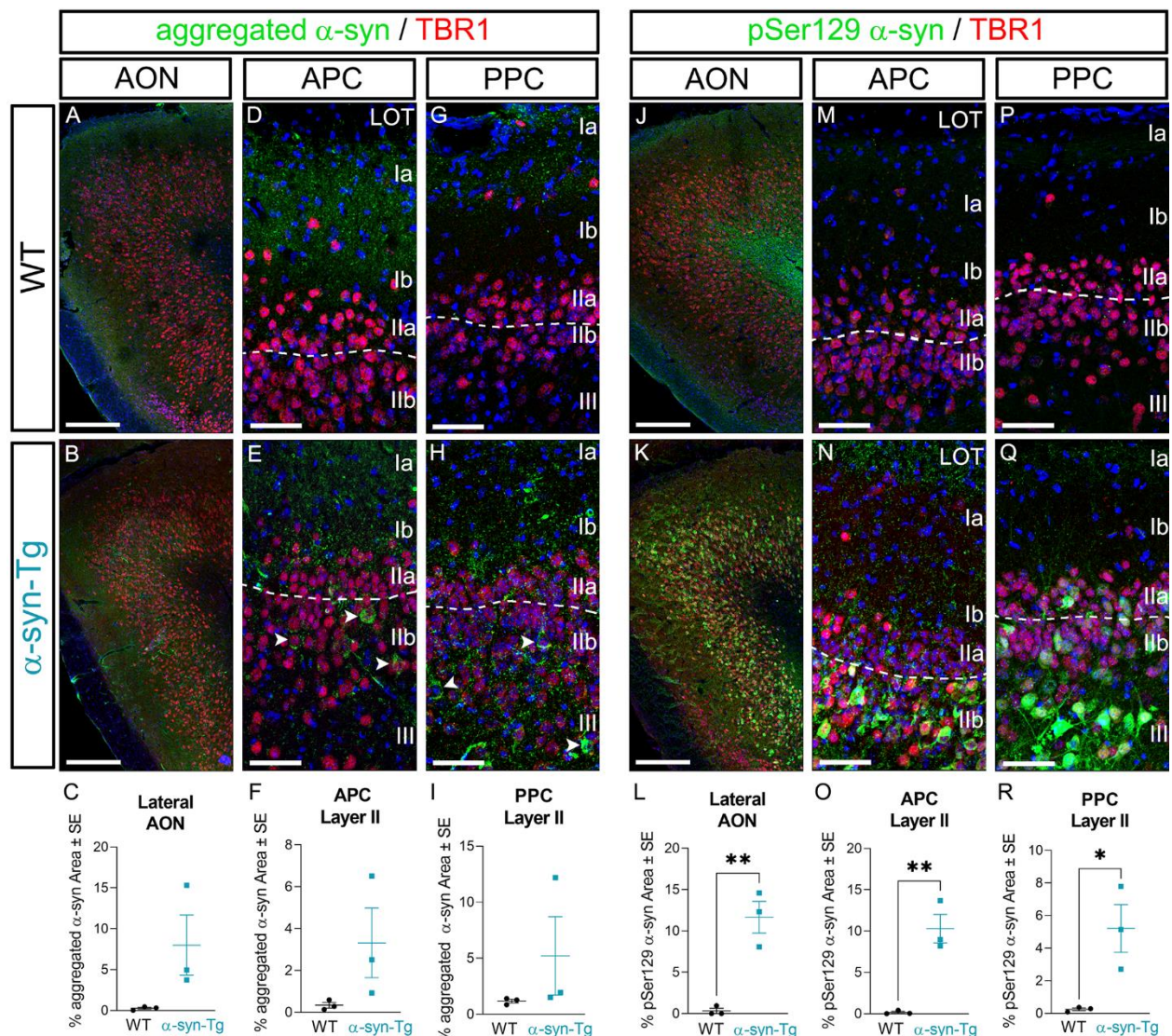


Figure 5. α -Synuclein pathology in anterior olfactory nucleus (AON) and piriform cortex (APC) of mice at late stages of 12-14 mo. (A, B) Representative images of AON coronal sections from WT and α -syn-Tg mice stained against aggregated α -syn (green) and TBR1 (red) as a marker for projection neurons. (C) Percentage of stained area in the AON showing higher levels of aggregated α -syn in α -syn-Tg vs WT mice with no statistical differences due to high variability. (D, E, G, H) Representative images of aggregated α -syn staining from the APC and PPC showing α -syn aggregation in layer II of α -syn-Tg mice, with accumulation in some neuronal cell bodies (arrowheads). (F, I) Quantification of aggregated α -syn stained area in APC and PPC showing no statistical differences between WT and α -syn-Tg mice, although there is a trend to be higher in α -syn-Tg mice. (J, K) Representative images of pSer129- α -syn staining (green) in AON, showing a strong accumulation of α -syn pathology in cell bodies of projection neurons co-labeled with TBR1 (red). (L) The comparison of stained area for pSer129- α -syn in AON shows statistically significant higher accumulation in α -syn-Tg mice. (M, N, P, Q) Examples of images taken in APC and PPC showing an intense labeling for pSer129- α -syn in neuronal cell bodies of layers IIa and IIb in APC, and IIb in PPC (dotted line). (O, R) Quantification of pSer129- α -syn stained area in APC and PPC

showing statistically significant differences between WT and α -syn-Tg mice. Nuclei counterstained in blue. Staining using mouse anti- aggregated α -syn (BioLegend) and mouse anti-pSer129- α -syn (BioLegend) combined with anti-TBR1 (Table 1). Abbreviations: APC: anterior PC; PPC: posterior PC; TBR1: T-Box Brain Transcription Factor-1. Scale bars of 200 μ m in A, B, J, K; 50 μ m in D, E, G, H, M, N, P, Q. *= $p < 0.05$; **= $p < 0.01$. Statistics: Unpaired t-test.

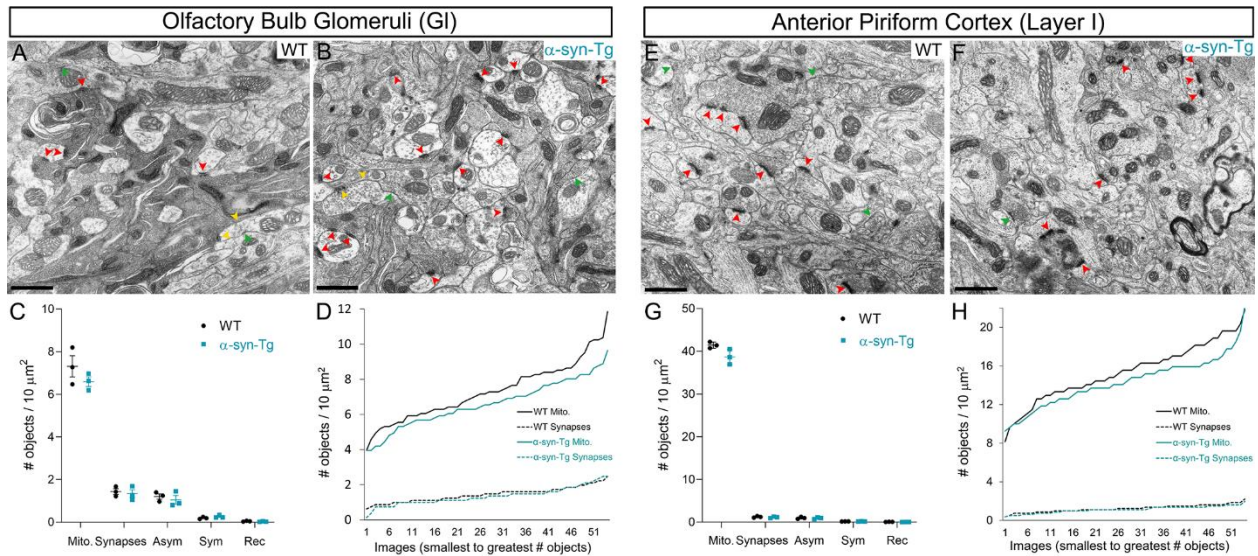
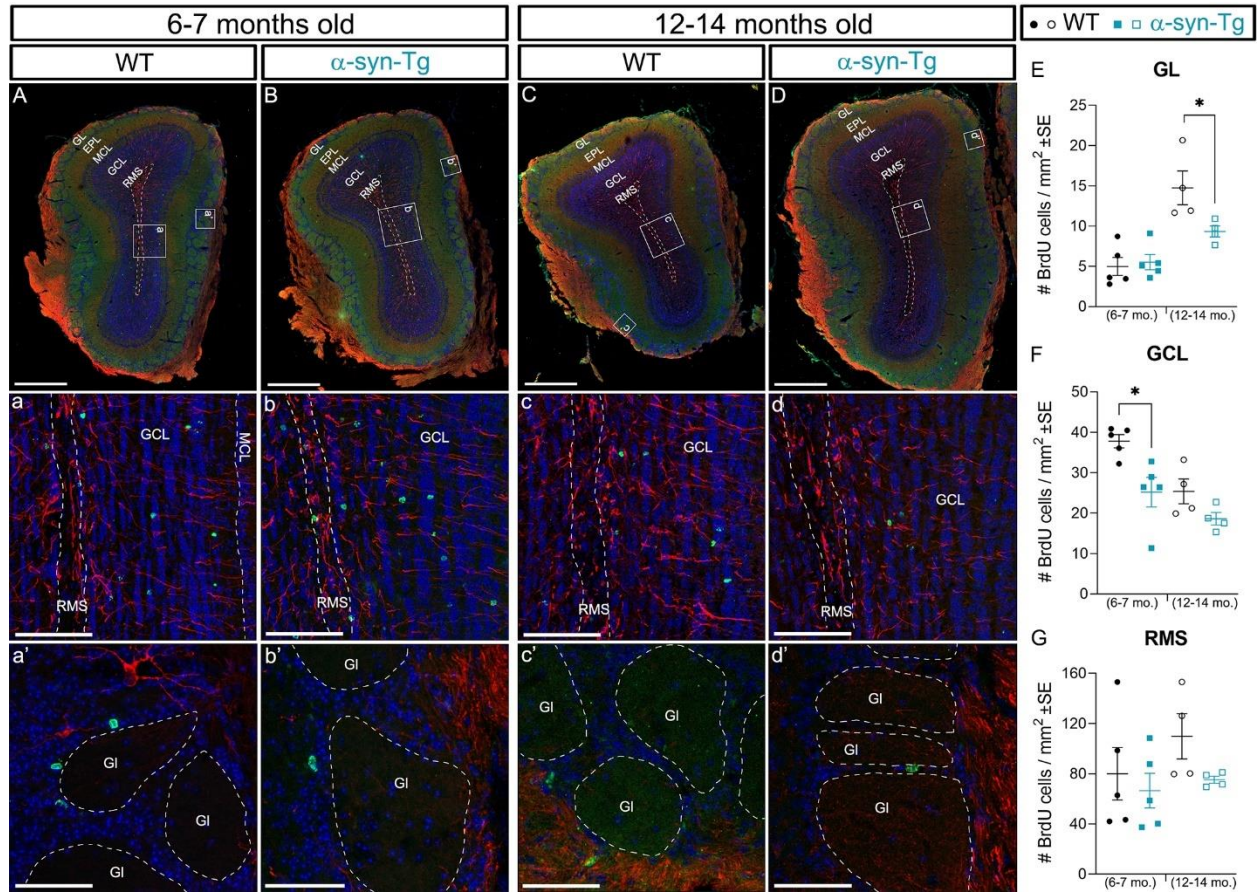


Figure 6. No ultrastructural alterations were seen in the OB, and layer I in the APC of α -syn-Tg mice. (A, B) Representative images of electron microscopy in the glomeruli of OBs from WT and α -syn-Tg mice. Number of mitochondria (Mito) and different types of synapses are quantified and highlighted as: asymmetric (Asym. Red arrowheads), symmetric (Sym. Green arrowheads) and reciprocal (Rec. Yellow arrowheads). (C) Quantification of Mito. and synapses in the glomeruli parenchyma show no statistical differences between WT and α -syn-Tg mice. (D) Representation of the number of Mito. and total synapses counted per image and arranged from the smallest to the greatest number of objects, showing a strong trend of α -syn-Tg mice to have less Mito. although no statistical differences are found. (E, F) Representative images of APC layer I parenchyma at EM. In these images it is shown Asym. (red arrowheads) and Sym. (green arrowheads) synapses. (G) Quantification of Mito. and synapses in APC showing no statistical differences between WT and α -syn-Tg mice. (H) Representation of the distribution of Mito. and synapses on each image arranged from lowest to highest number of objects showing a similar trend to that observed in OB. Scale bars of 0.5 μ m. Statistics: Unpaired t-test.



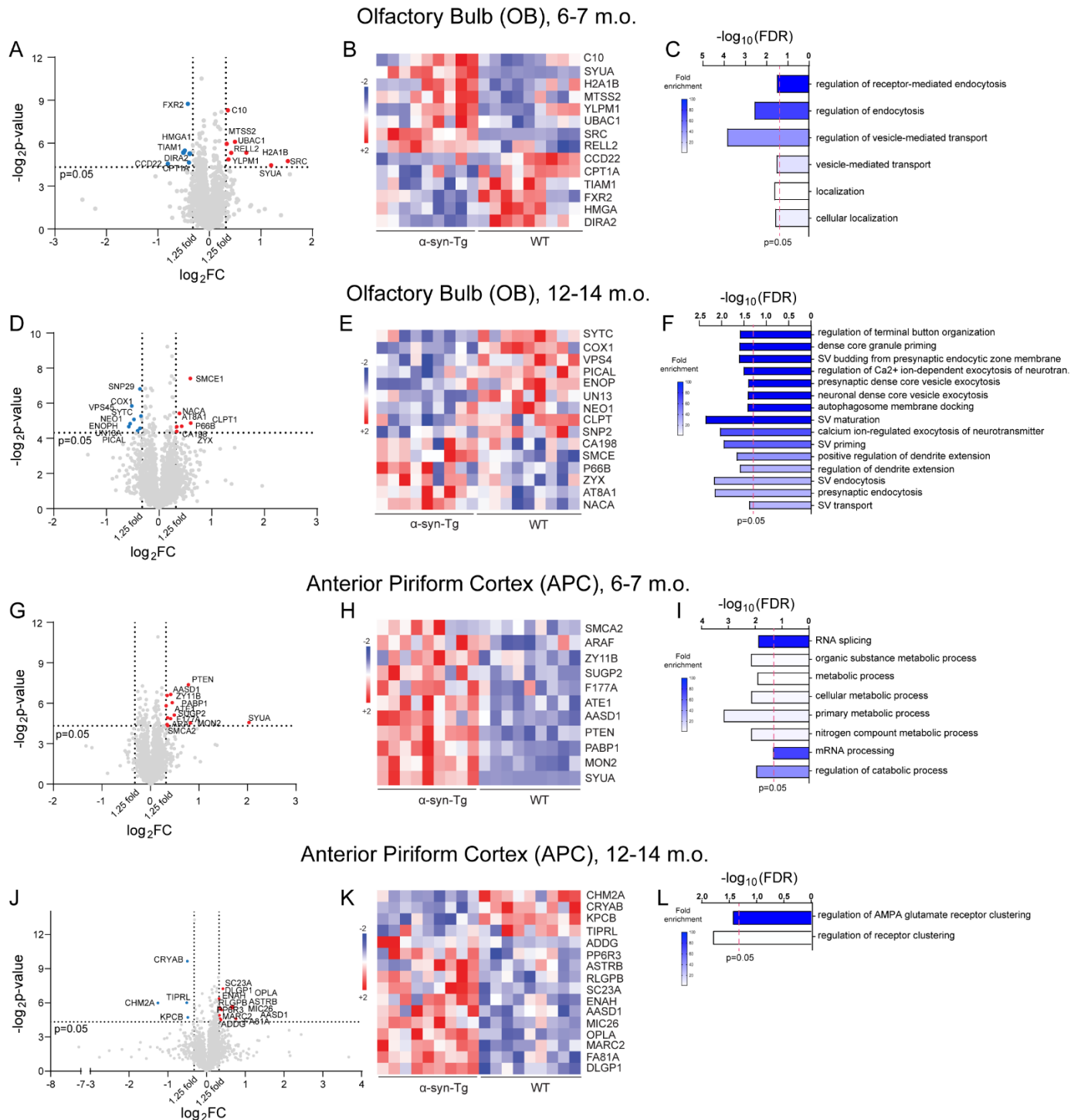
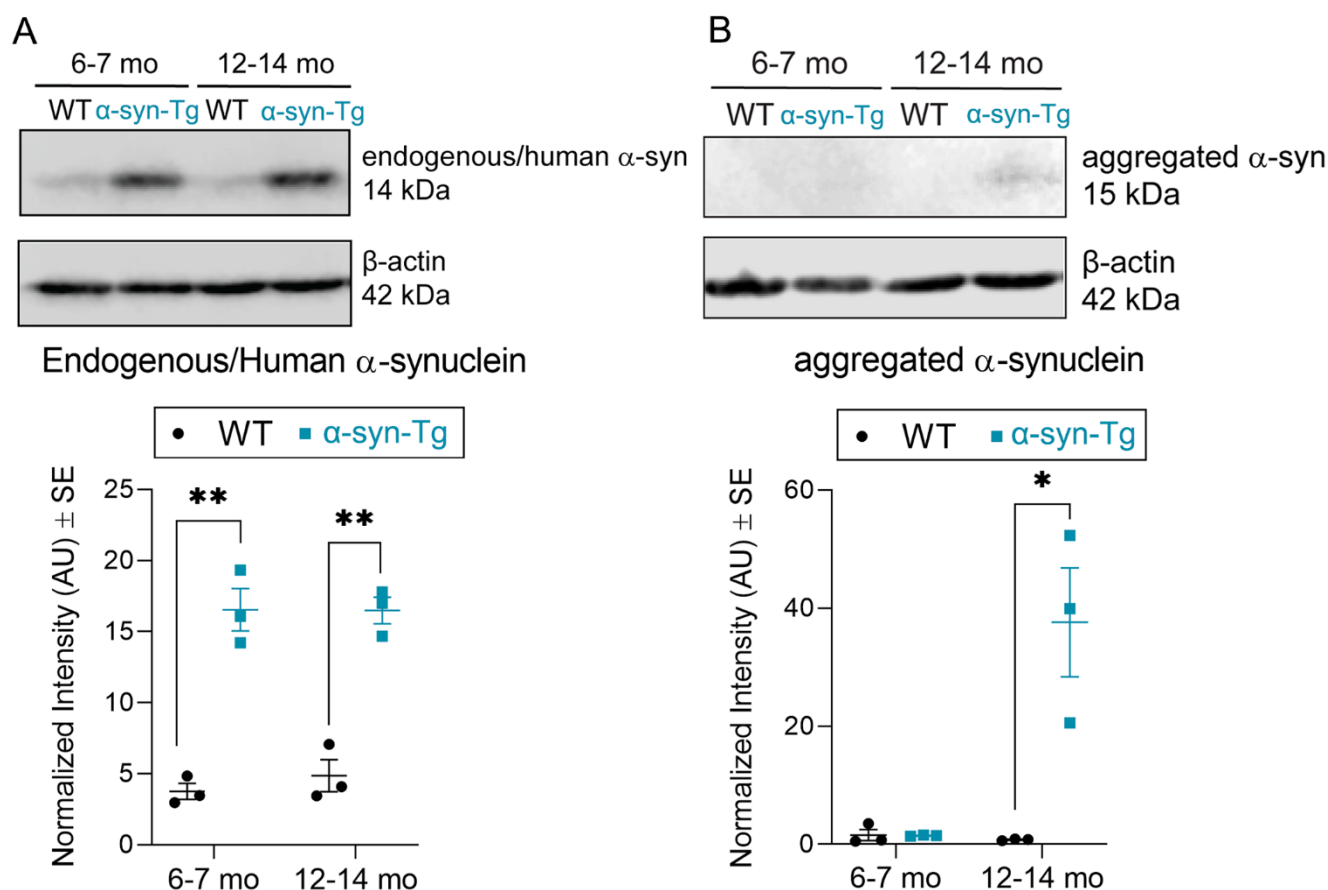
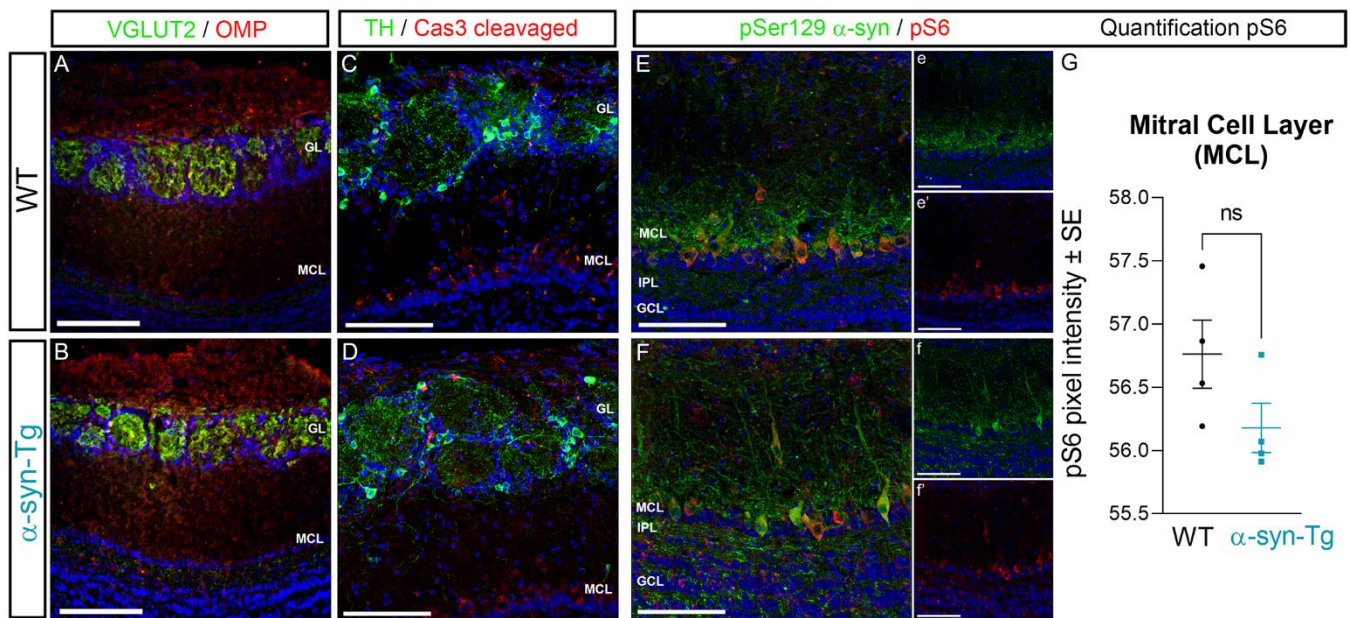


Figure 8. Proteomics of OB and APC revealed defects in synaptic endo- and exocytosis pathways in the OB, but minimum alterations in APC of α -syn-Tg mice. (A) Volcano plot of fold change in the OB proteome of 6-7 mo. α -syn-Tg compared to control WT mice (n=3 mice/genotype). Proteins that changed 1.25-fold (vertical dotted lines) with a p-value of <0.05 (horizontal dotted line) are considered to be significantly differentially expressed. Among the 14 significant proteins, 8 are upregulated (red) and 6 are downregulated (blue). (B) Heat map of significantly differentially expressed proteins in OB of α -syn-Tg compared to WT mice at early

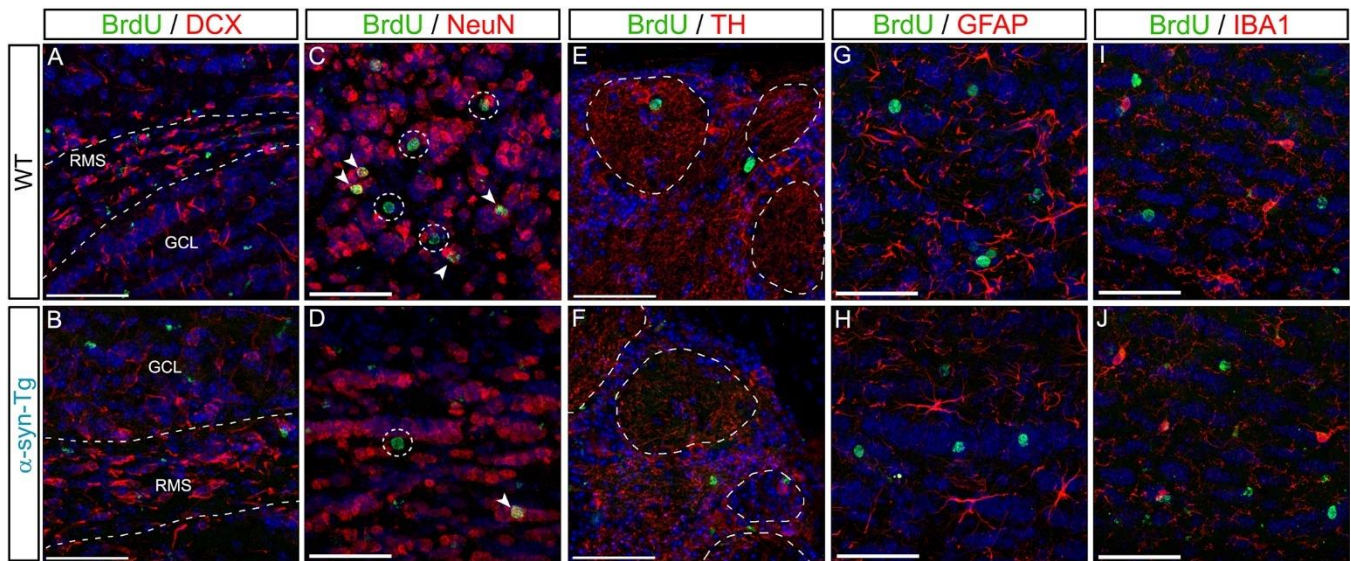
stage (6-7 mo.) for each technical replicate (3 technical replicates/mouse). Red indicates increased expression (+2), and blue indicates decreased expression (-2). (C) Biological pathways that are significantly ($FDR < 0.05$) affected in the OB of 6-7 mo. α -syn-Tg mice, determined by GO enrichment analysis. (D) Volcano plot of fold change in the OB proteome of 12-14 mo. α -syn-Tg compared to WT mice ($n=3$ mice/genotype). Among the 15 significantly differentially expressed proteins (1.25-fold, $p < 0.05$), 7 are upregulated (red) and 8 are downregulated (blue). (E) Heat map of significantly differentially expressed proteins in OB of late stage (12-14 mo.) α -syn-Tg mice compared to their WT counterparts for each technical replicate (3 technical replicates/mouse). (F) Biological pathways that are significantly ($FDR < 0.05$) affected in the OB of 12-14 mo. α -syn-Tg mice, determined by GO enrichment analysis. (G) Volcano plot of fold change in the APC proteome. α -syn-Tg compared to WT mice ($n=3$ mice/genotype) at 6-7 mo. All the 11 significantly differentially expressed proteins (1.25-fold, $p < 0.05$) are upregulated in α -syn-Tg mice. (H) Heat map of significantly differentially expressed proteins in APC of α -syn-Tg compared to WT mice at 6-7 mo. for each technical replicate (3 technical replicates/mouse). (I) Biological pathways that are significantly ($FDR < 0.05$) affected in the APC of 6-7 mo. α -syn-Tg mice, determined by GO enrichment analysis. (J) Volcano plot of fold change in the APC proteome of α -syn-Tg compared to WT mice ($n=3$ mice/genotype) at 12-14 mo. Among the 16 significantly differentially expressed proteins (1.25-fold, $p < 0.05$), 12 are upregulated (red) and 4 are downregulated (blue). (H) Heat map of significantly differentially expressed proteins in APC of α -syn-Tg mice compared to WT controls for each technical replicate (3 technical replicates/mouse) at 12-14 mo. (I) Biological pathways that are significantly ($FDR < 0.05$) affected in the APC of 12-14 mo. α -syn-Tg mice, determined by GO enrichment analysis.



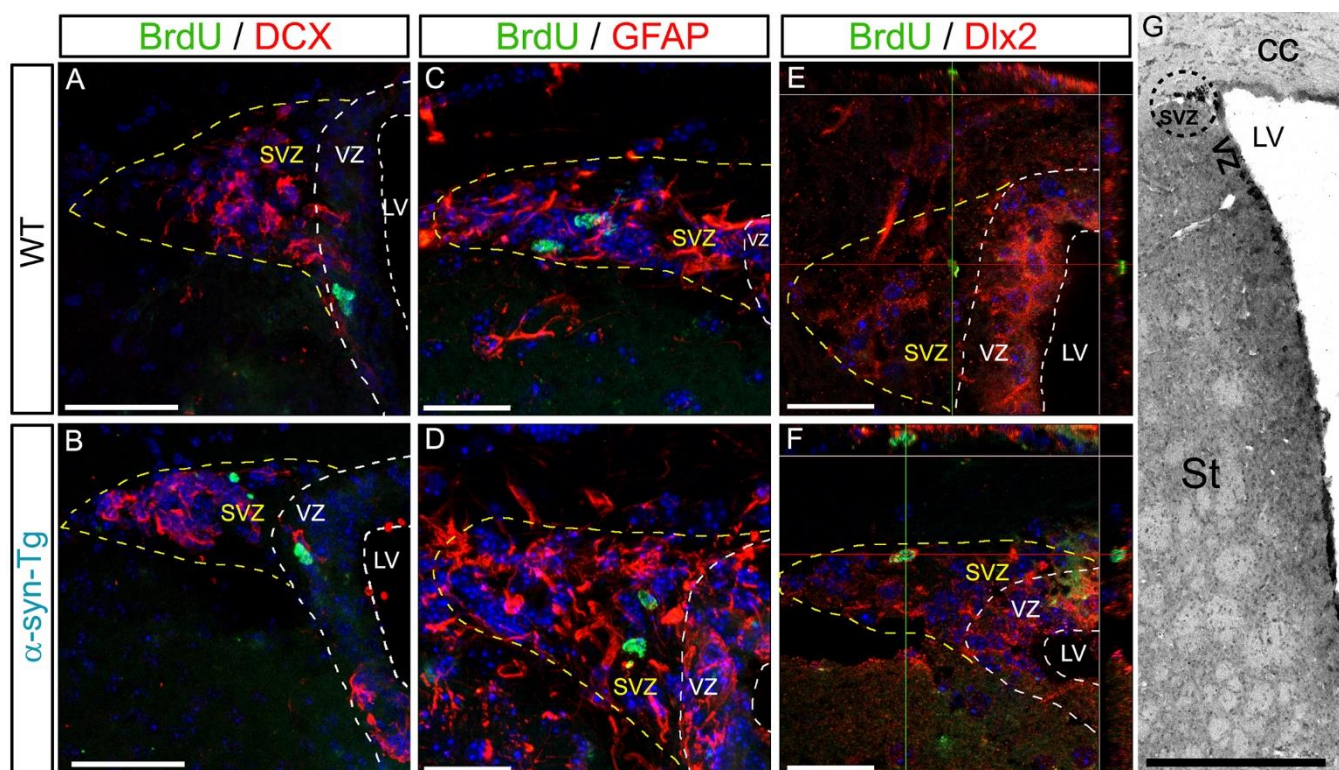
Supplementary Figure 1. Quantification of endogenous/human and aggregated α -synuclein by Western blot (WB). (A) WB detecting total (endogenous and human) α -syn in WT controls and α -syn-Tg mice at both studied stages (6-7 and 12-14 mo. mice). The amount of protein quantified by densitometry showed a significant increase of total α -syn between WT vs α -syn-Tg mice at both 6-7 and 12-14 mo. in the OB. (B) Detection of aggregated α -syn (from EMD Millipore, Table 1) by WB showed an increase in the amount of protein in the OB of α -syn-Tg mice at 12-14 mo. β -actin is used as a loading control. * = $p < 0.05$, ** = $p < 0.01$. Statistics: Unpaired t-test.



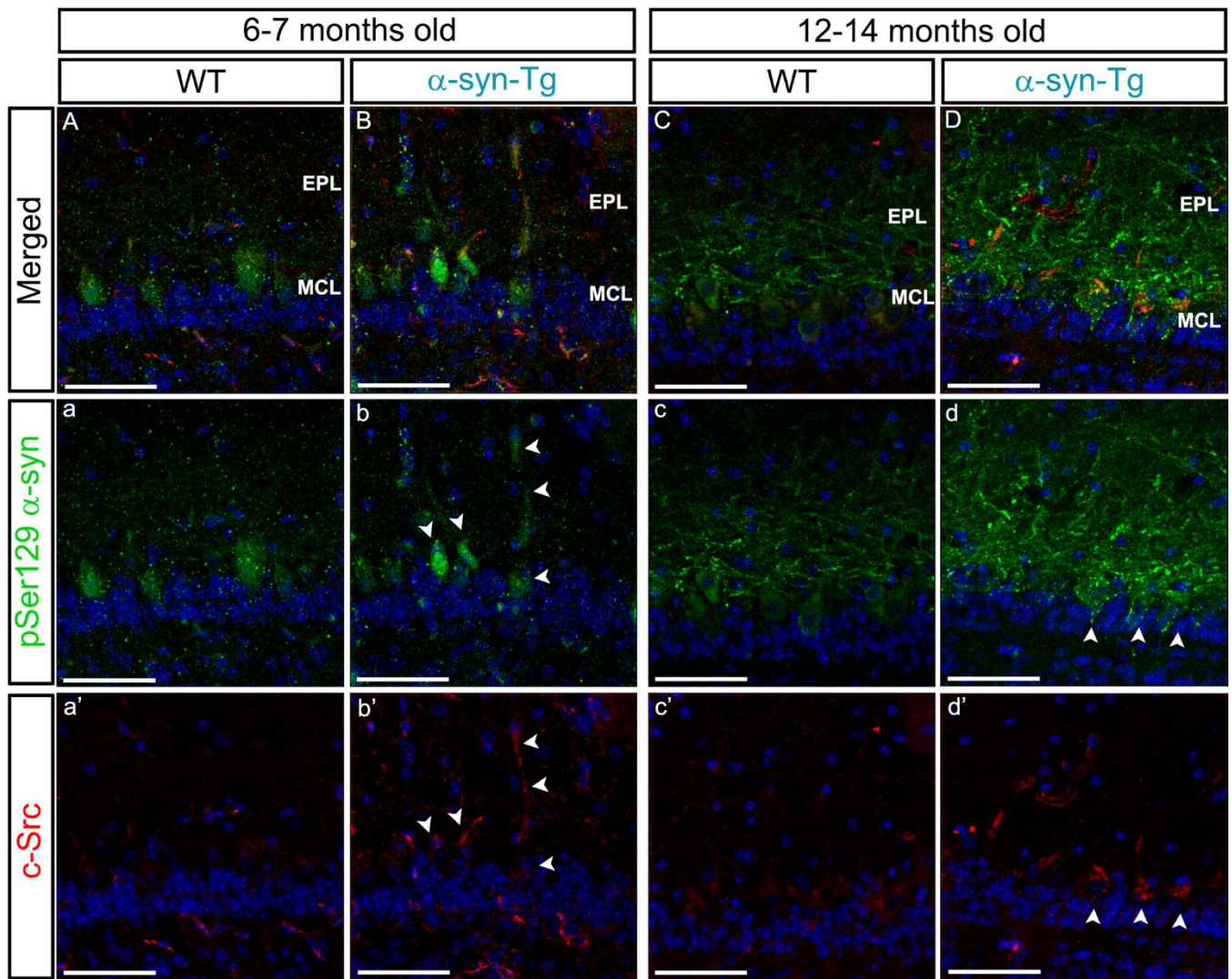
Supplementary Figure 2. Structure of the OB and general cell activity of mitral cells in 12-14 mo. mice. (A, B) OSNs axons identified by labeling with VGLUT2 (green) and OMP (red) show no differences in the glomerular structure between WT and α -syn-Tg mice. (C, D) Staining of dopaminergic neurons with TH in the GL (green) shows no disruptions in the morphology or cellular processes in α -syn-Tg mice. Quantifications of TH cell numbers is similar between WT ($443 \pm 43 \text{ mm}^2$) and α -syn-Tg ($535 \pm 74 \text{ mm}^2$) mice (data not represented). Apoptosis is detected using cleaved caspase-3 (red) and no differences are evident between WT and α -syn-Tg mice, indicative of an increased cell death caused by the hA30P α -syn mutation. (E-e', F-f') Mitral cells pathology detected with pSer129- α -syn from BioLegend (Table 1) (green), and cellular activity detected using pS6 (red). Residual pSer129- α -syn is seen in WT (E-e) that is overexpressed in α -syn-Tg mice (F-f). pS6 staining reveals similar activity in mitral cells of both WT and α -syn-Tg mice (e', f'). (G) Quantification of pS6 expression levels by analyzing the pixel intensity in mitral cells shows no significant differences between WT and α -syn-Tg mice indicative of decreased activity caused by the hA30P α -syn mutation. Abbreviation: OMP: olfactory marker protein; OSNs: olfactory sensory neurons; pS6: phosphorylated S6 Ribosomal Protein (Ser235/236); TH: tyrosine hydroxylase; VGLUT2: vesicular glutamate transporter 2. Scale bars of 200 μm in A, B; 100 μm in C, D; 50 μm in E-e', F-f'. Statistics: Unpaired t-test.



Supplementary Figure 3. Analysis of cell phenotypes of BrdU-expressing cells in the OB of mice at early stages of the disease (6-7 mo.). Newly generated cells incorporate BrdU are shown in green in WT controls and α -syn-Tg mice; markers to identify different cell types are shown in red. Nuclei counterstained in blue. (A, B) no BrdU⁺ cell expresses the neuroblast marker DCX in either the GCL or RMS. (C, D) Mature neurons are labeled with NeuN in the GCL and roughly 43% and 30% of BrdU cells co-express this marker in WT and α -syn-Tg mice, respectively. (E, F) Analysis of cells in the periglomerular region showing that no BrdU⁺ cell express the dopaminergic marker TH. (G, H) GFAP marker is used to identify astrocytes showing no colocalization with BrdU. (I, J) Microglial cells are identified with IBA1 which are not part of the pool of BrdU⁺ cells either. Abbreviations: DCX: doublecortin; GCL: granule cell layer; GFAP: glial fibrillary acidic protein; IBA1: ionized calcium-binding adapter molecule-1; NeuN: neuronal nuclei marker; RMS: rostral migratory stream; TH: tyrosine hydroxylase. Scale bars of 50 μ m.



Supplementary Figure 4. Characterization of BrdU-expressing cells in the SVZ of mice at early stages of the disease (6-7 mo.) BrdU⁺ cells are shown in green. In red, we show staining for the different subtype of cells located within the SVZ: neuroblasts that express DCX (a.k.a. “A” cells), radial glia neural stem cells expressing GFAP (a.k.a. “B” cells) and transient amplifying cells expressing Distal-Less Homeobox 2 (Dlx2) (a.k.a. “C” cells). Nuclei counterstained in blue. (A, B) DCX staining do not colocalizes with any BrdU⁺ cell indicating that BrdU⁺ are not part of the “A” pool. (C, D) Similarly, any BrdU⁺ expressed GFAP as marker for “B” cells. (E, F) However, marker to identify “C” cells (Dlx2) co-localized with BrdU⁺ cells located in this region, indicating they were part of the “C” cells pool of the SVZ in these animals. (G) greyscale image of the analyzed region showing location of the SVZ next to the ventricular zone (VZ) and the lateral ventricle (LV). Scale bars of 400 μm in G; 50 μm in A, B; 25 μm in C, D, E, F.



Supplementary Figure 5. Expression of the membrane-associated tyrosine protein kinase (c-Src) in cells with α -synuclein pathology in the OB. α -Synuclein pathology is shown by staining with pSer129- α -syn (green), while c-Src is shown in red. Nuclei counterstained in blue. c-Src is reported to phosphorylate α -syn (Ellis et al., 2001) and its overexpression may indicate a role in α -syn phosphorylation under pathology. (A-a', C-c') Labeling of WT animals at 6-7 and 12-14 mo., respectively showing residual expression of pSer129- α -syn (a, c) and c-Src (a', c') in the MCL and EPL. (B-b', D-d') Expression in α -syn-Tg mice showing the overexpression of α -synuclein pathology (b, d), and c-Src (b', d') colocalizing in mitral cells (arrowheads). c-Src overexpression detected by immunohistochemistry confirms the results seen in proteomics. Staining using rabbit anti- pSer129- α -syn (Abcam) (Table 1). Scale bars of 50 μ m.

Supplementary Table 1: Proteins that are significantly changed in the proteomic analysis of olfactory bulb of α -syn-Tg mice in comparison to WT at 6-7 months of age.

No.	Accession	Gene name	Protein name	Fold change
1	C10	Grccl0	Protein C10	1.28436951
2	SYUA	Snca	Alpha-synuclein	2.295515511
3	H2A1B	H2ac4	Histone H2A type 1-B	1.643448378
4	MTSS2	Mtss2	Protein MTSS 2	1.262605161
5	YLPM1	Ylpm1	YLP motif-containing protein 1	1.292921147
6	UBAC1	Ubac1	Ubiquitin-associated domain-containing protein 1	1.410921697
7	SRC	Src	Neuronal proto-oncogene tyrosine-protein kinase Src	2.876813941
8	RELL2	Rell2	RELT-like protein 2	1.34090359
9	CCD22	Ccdc22	Coiled-coil domain-containing protein 22	0.571394901
10	CPT1A	Cpt1a	Carnitine O-palmitoyltransferase 1, liver isoform	0.759995746
11	TIAM1	Tiam1	Rho guanine nucleotide exchange factor TIAM1	0.719080008
12	FXR2	Fxr2	Fragile X mental retardation syndrome-related protein 2	0.749066485
13	HMGA1	Hmga1	High mobility group protein HMG-I/HMG-Y	0.793204904
14	DIRA2	Diras2	GTP-binding protein Di-Ras2	0.770665441

\

Supplementary Table 2: Proteins that are significantly changed in the proteomic analysis of olfactory bulb of α -syn-Tg mice in comparison to WT at 12-14 months of age.

No.	Accession	Gene name	Protein name	Fold change
1	SYTC	Tars1	Threonine--tRNA ligase 1, cytoplasmic	0.795958
2	COX1	Mtco1	Cytochrome c oxidase subunit 1	0.698021
3	VPS45	Vps4b	Vacuolar protein sorting-associated protein 4B	0.793934
4	PICAL	Picalm	Phosphatidylinositol-binding clathrin assembly protein	0.754455
5	ENOPH	Enoph1	Enolase-phosphatase E1	0.683989
6	UN13A	Unc13a	Protein unc-13 homolog A	0.671959
7	NEO1	Neo1	Neogenin	0.71851
8	CLPT1	Clptm1	Cleft lip and palate transmembrane protein 1	0.778314
9	SNP29	Snap29	Synaptosomal-associated protein 29	0.777816
10	CA198	N/A	Uncharacterized protein C1orf198	1.346692
11	SMCE1	Smarce1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	1.507282
12	P66B	Gatad2b	Transcriptional repressor p66-beta	1.515755
13	ZYX	Zyx	Zyxin	1.26207
14	AT8A1	Atp8a1	Phospholipid-transporting ATPase 1A	1.260929
15	NACA	Naca	Nascent polypeptide-associated complex subunit alpha	1.307384

Supplementary Table 3: Proteins that are significantly changed in the proteomic analysis of anterior piriform cortex of α -syn-Tg mice in comparison to WT at 6-7 months of age.

No.	Accession	Gene name	Protein name	Fold change
1	SMCA2	Smarca2	Probable global transcription activator SNF2L2	1.279780958
2	ARAF	Araf	Serine/threonine-protein kinase A-Raf	1.268139535
3	ZY11B	Zyg11b	Protein zyg-11 homolog B	1.268384587
4	SUGP2	Sugp2	SURP and G-patch domain-containing protein 2	1.405654737
5	F177A	Fam177a1	Protein FAM177A1	1.27607181
6	ATE1	Ate1	Arginyl-tRNA--protein transferase 1	1.252608747
7	AASD1	Aarsd1	Alanyl-tRNA editing protein Aarsd1	1.335151511
8	PTEN	Pten	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	1.720804806
9	PABP1	Pabpc1	Polyadenylate-binding protein 1, PABP-1, Poly(A)-binding protein 1	1.364366716
10	MON2	Mon2	Protein MON2 homolog	1.766115672
11	SYUA	Snca	Alpha-synuclein	4.090515865

Supplementary Table 4: Proteins that are significantly changed in the proteomic analysis of anterior piriform cortex of α -syn-Tg mice in comparison to WT at 12-14 months of age.

No.	Accession	Gene name	Protein name	Fold change
1	CHM2A	Chmp2a	Charged multivesicular body protein 2a	0.418897
2	CRYAB	Cryab	Alpha-crystallin B chain	0.70911
3	KPCB	Prkcb	Protein kinase C beta type	0.715298
4	TIPRL	Tiprl	TIP41-like protein	0.701951
5	ADDG	Add3	Gamma-adducin	1.290529
6	PP6R3	Ppp6r3	Serine/threonine-protein phosphatase 6 regulatory subunit 3	1.552139
7	ASTRB	Gramd1b	Protein Aster-B	1.266189
8	RLGPB	Ralgapb	Ral GTPase-activating protein subunit beta	1.260604
9	SC23A	Sec23a	Protein transport protein Sec23A	1.338548
10	ENAH	Enah	Protein enabled homolog	1.293051
11	AASD1	Aarsd1	Alanyl-tRNA editing protein Aarsd1	1.583143
12	MIC26	Apoo	MICOS complex subunit Mic26	1.260649
13	OPLA	Oplah	5-oxoprolinase	1.597227
14	MARC2	Mtarc2	Mitochondrial amidoxime reducing component 2	1.274637
15	FA81A	Fam81a	Protein FAM81A	1.695311
16	DLGP1	Dlgap1	Disks large-associated protein 1	1.309585