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14. ABSTRACT:

Cognitive decline is a major non-motor dysfunction of Parkinson's disease (PD), still, little is known regarding the cellular mechanisms. Cognitive dysfunction is predominant in PD patients with GBA mutations who primarily develop Gaucher disease (GD). GBA is also the most common risk gene for Dementia with Lewy Bodies (DLB), a leading cause of dementia and cognitive disabilities. DLB shares α -synuclein aggregation pathology with that of PD. In this study, we used novel Gba mutant mice that develop GD to unravel the pathogenic mechanisms for cognitive decline in PD and DLB. We also generated double transgenic mice by crossing Gba mutants with SNCA transgenic mice model of PD to evaluate how α -synuclein aggregation might alter Gba/Gcase1 deficiency-mediated pathology and vice versa. Gba mutants did not show age-related PD-like motor deficits which were apparent in SNCA transgenic mice. However, Gba mutation in SNCA transgenic mice exacerbated the motor deficits. Gba mutants showed cognitive deficits, which, interestingly did not worsen with age. Gba-SNCA double transgenics showed similar cognitive deficits, whereas SNCA transgenics were cognitively normal. These results suggest that Gba/Gcase1 deficiency can independently impart cognitive decline, and when it co-occur with α -synuclein pathology as seen in Gba-SNCA double transgenics, it exacerbates the motor dysfunction. In the cognitive brain areas such as cortex and hippocampus, Gba mutants did not show pathological α -synuclein aggregation, however, an exacerbated α -synuclein pathology was seen in Gba-SNCA double transgenics. Unbiased proteomics of brains from these mice suggested a dysfunctional lipid metabolism in Gba mutants, which when co-existed with α -synuclein pathology as seen in double transgenics, leads to dysregulation in sirtuin signalling and reactive oxygen species production pathways. We indeed noted the accumulation of sphingolipid glucosylceramide (GlcCer) in the cognitive brain areas of Gba mutants, which was exacerbated in the brains of Gba-SNCA double transgenics. GlcCer was previously shown to promote pathological aggregation of α -synuclein. We began by examining synaptic proteins to understand how this lipid accumulation might lead to cognitive dysfunctions. Subset of synaptic proteins were upregulated/accumulated in Gba mutants akin to SNCA mutants, which was further exacerbated in Gba-SNCA double transgenics. Overall, our results so far suggest that Gba/Gcase1 deficiency-mediated lipid accumulation leads to cognitive decline, potentially through the dysregulation of synaptic proteins. We also show that this effect is exacerbated in the presence of α -synuclein pathology. We are now validating individual proteomic hits to identify potential modifiers of Gba/Gcase1 deficiency-mediated synaptic dysfunctions. We are testing if (AAV)-shRNA-mediated reduction in sphingolipids can restore synaptic health and reduce α -synuclein pathology in cognitive brain areas of Gba and Gba-SNCA transgenics, respectively. We will assess if this approach restores cognitive behavior. Thus, while identifying the modifiers of GBA mutation-induced risk to develop PD, DLB and associated cognitive deficits, this study unravels novel mechanisms that can be potential therapeutic targets for PD and DLB.

15. SUBJECT TERMS

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1. INTRODUCTION:

More than 80% of people with Parkinson's disease (PD) suffer from cognitive dysfunction, which is considered a major unmet need of people with PD and their caregivers. Still, little is known regarding the causative mechanisms. Cognitive dysfunction in PD exists on a broad spectrum from mild cognitive impairment (MCI) at an early stage to PD dementia (PDD) at advanced stages. Neocortical α -synuclein pathology has the strongest association with PDD and shares this feature with Dementia with Lewy Bodies (DLB), the second most common cognitive disorder. Another key pathological feature shared by PDD and DLB is their high occurrence in people carrying *GBA* mutations who primarily develop Gaucher disease (GD), a lysosomal lipid storage disorder. *GBA* encodes Gcase1, a lysosomal hydrolase that takes part in glycosphingolipid (GSL) metabolism. Most PD-causing *GBA* mutations are loss of function with a large fraction of them developing an early and worsened cognitive decline compared to sporadic PD cases. Post-mortem studies revealed reduced Gcase1 activity in several brain areas of PD patients with *GBA* mutations along with the accumulation of its substrate glucosylceramide (GlcCer) and other GSLs like glucosylsphingosine (GlcSph). Loss of Gcase1 activity and GSL accumulation is increasingly noted even in the brains of sporadic PD patients, suggesting its important role in overall PD pathogenesis. Decreased GCase activity and higher levels of GlcCer/GlcSph correlated with a higher burden of α -synuclein pathology in the cognitive brain areas of sporadic PD patients. This suggests an interplay between α -synuclein aggregation, GCase deficiency and GSL in causing cognitive dysfunction. Thus, studying the implications of *GBA* mutation-mediated GSL accumulation and its modulation in the presence of α -synuclein pathology provides a window into understanding the role of lipid dyshomeostasis in the cognitive dysfunction of PD and DLB. Thus, we used our well-established mice models of GD (L444P *Gba* mutation) and PD (A30P *Syn* transgenic), and their F1 crossbreds to decipher the role of lipid dyshomeostasis and its mechanisms leading to cognitive dysfunction of PD and DLB.

2. KEYWORDS:

Parkinson's disease, Dementia with Lewy Bodies, Cognitive dysfunctions, *GBA*, Lipid dyshomeostasis, Glycosphingolipids, Glucocerebrosidase 1, GCase1, Glucosylceramide, Glucosylsphingosine, ASAH1

3. ACCOMPLISHMENTS:

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: To characterize the cortical and hippocampal lipid profile in the brains of *Gba* KO, *SNCA tg* and *Gba/SNCA* mice as a function of age and correlate it with α -synuclein pathology. ($n=5$ /mice line/time point/experiment)

Major Task 1: Lipidomics for glycosphingolipids and phospholipids in *Gba* L444P KO, *SNCA tg* and *Gba/SNCA* mice at 3, 6, 9 and 12 months of age (1-36 months of project period)

Major Task 2: Immunohistochemistry in *Gba* L444P KO, *SNCA tg* and *Gba/SNCA* mice of 3, 6, 9 and 12 months of age (1-36 months)

Major Task 3: Western blotting (1-36 months)

Milestones achieved: Lipidomics and Western blotting for the symptomatic age of 12 months are completed. Completed the collection of 3 months old mice brain samples required for lipidomics and Western blotting. Perfusion to collect the mice's brain, cryosectioning, and immunohistochemistry are completed for early and late age groups (3 and 12 months, respectively). Accumulation of normal and pathological α -synuclein, neuronal numbers, lipid accumulation, synaptic health, and neuroinflammation are assessed by immunohistochemistry.

Specific Aim 2: *To assess the cognitive capabilities of Gba KO and SNCA tg mice and to evaluate if it worsens in Gba/SNCA mice (n=12/mice line/time point/experiment) (3-30 months)*

Milestones achieved: Completed longitudinal behavior assays (motor and cognitive) for WT (wild type), SNCA tg mice (PD mice), Gba L444P KO (n=12) and Gba/SNCA mice (n=13) (GD and GD/PD mice, respectively) at 3, 6, 9 & 12 months of age.

Specific Aim 3: *Evaluation of Long Term Potentiation (LTP) in Schaffer collateral synapses of hippocampal sections and to investigate if AAV-shRNA mediated inhibition of Asah1 and Ugcg ameliorate hippocampal pathology and cognitive deficits in Gba KO, SNCA tg and Gba/SNCA mice (18-36 months)*

Milestones achieved: Stereotaxic experiments are standardized. shRNA plasmids for Asah1 and Ugcg are tested for their transfection efficacy in N2a cells (mouse neuroblastoma cell lines). AAV-shRNA for Asah1 was prepared and ready to injected once we obtain mice of the right age.

LTP electrophysiology was originally planned in collaboration with a different institute. These experiments got delayed due to Covid-19-related restrictions. New mice are being bred to restart this experiment. To get additional mechanistic insights, we performed whole proteomic analyses on WT, Gba KO, SNCA tg and Gba/SNCA mice.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Specific Aim 1: *To characterize the cortical and hippocampal lipid profile in the brains of Gba KO, SNCA tg and Gba/SNCA mice as a function of age and correlate it with α -synuclein pathology. (n=5/mice line/time point/experiment)*

Milestones achieved:

GCase assay showed GD-like liver and brain pathology in Gba mutants: We performed GCase assay to confirm loss of GBA function in Gba L444P/KO mice, and Gba/SNCA double transgenic mice. *GBA1* mutation leads to decreased activity of the enzyme GCase1, which is encoded by *GBA1*. Liver is one of the organs which is severely affected in this mutation leading to GD. Thus, we measured GCase levels in the liver to validate the model. Both Gba L444P/KOs and Gba/SNCA double transgenics showed significant loss of GCase1 and GCase2 in the liver, with GCase3 showing similar trend when compared to WT and SNCA tg mice (Fig. 1A-C). Thus, total liver GCase levels were also significantly low in Gba L444P/KO and Gba/SNCA transgenic mice (Fig.

GCCase levels have been previously shown to be low in PD patients with GBA mutations, as well as in other familial and sporadic forms. Thus, we also looked in to GCCase level in the brain. Interestingly, the difference across the lines for all subtypes of GCCase and the total GCCase did not vary as significantly as we noted in the liver (Fig. 2E-H). GCCase1 showed trend toward a decrease in Gba L444P/KO, SNCA tg and Gba/SNCA mice (Fig. 2E). A decrease in GCCase1 level in SNCA tg mice is interesting, as this doesn't carry a Gba mutation, suggesting synuclein pathology has an effect on GCCase activity, corroborating findings in the sporadic PD cases. As expected, subtypes of GCCase, such as GCCase2, and GCCase3 were unchanged in the brain (Fig. 2F-G), which was also true in case of total GCCase (Fig. 2H). We are repeating our experiments on fresh brain samples, as the current GCCase assay were performed on those brains which were homogenized for lipidomics, and not optimized for GCCase assay, as we have done for liver samples.

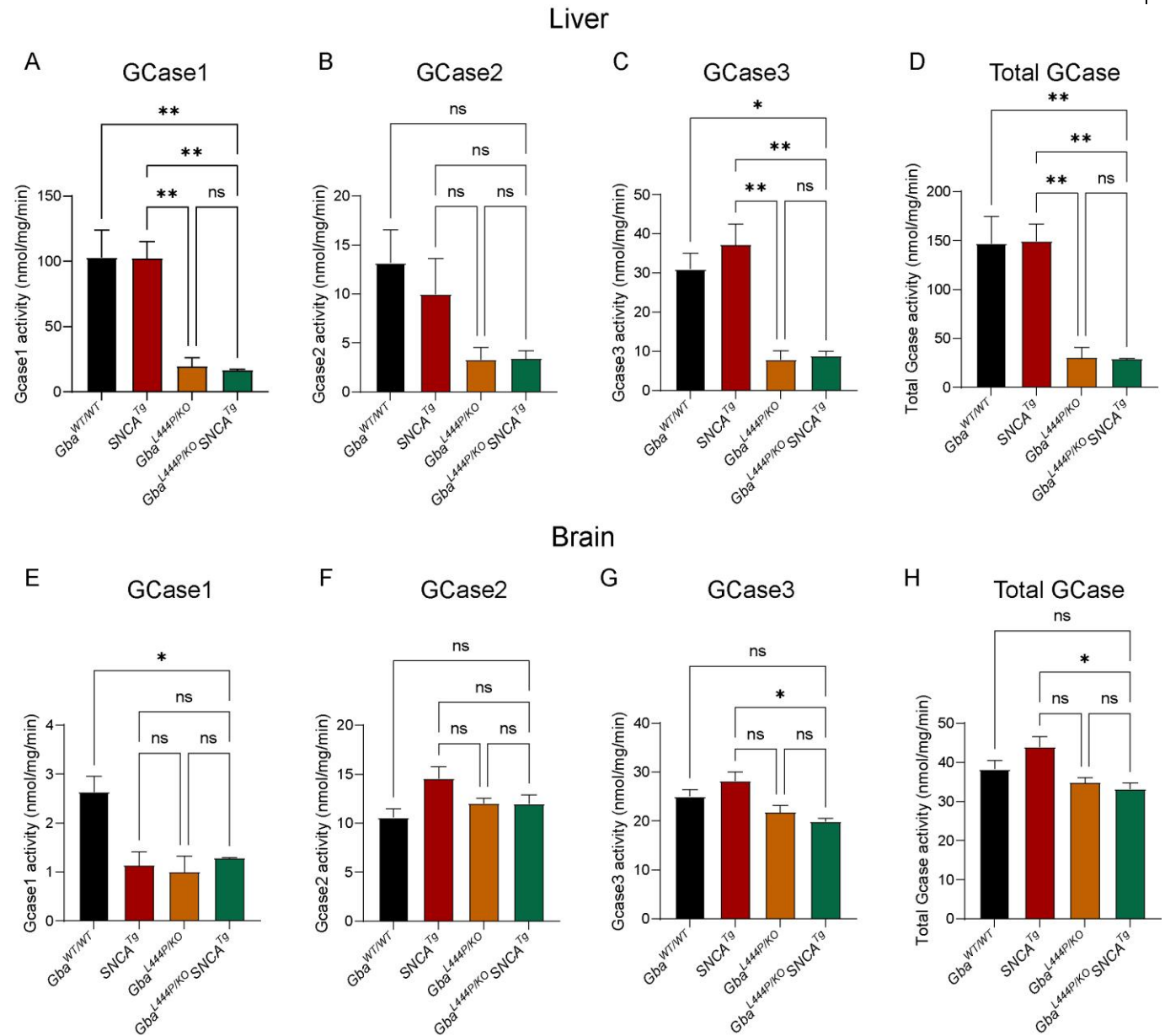


Fig 1: GCase1 expression was decreased in the liver of *Gba* mutants, and showed a trend towards differential expression in the brain. A-D: GCase1, GCase2, GCase3, and total GCase levels in the liver of WT, *L444P* KO, *SNCA* tg and *Gba*/*SNCA* mice. E-H: GCase1, GCase2, GCase3, and total GCase levels in the brains of WT, *L444P* KO, *SNCA* tg and *Gba*/*SNCA* mice.

Lipidomics confirmed GD phenotype in *Gba* mutants: Loss of function in the enzyme GCase1 due to *GBA* mutations results in accumulation of GSLs such as GlcCer and/or GlcSph in the liver leading to GD. In our mice model at 12 months of age, we did not see GlcCer accumulation in the liver of *Gba* L444P/KO or *SNCA* tg mice, which was also true for *Gba*/*SNCA* double transgenic mice (Fig. 2A-B). Interestingly, we saw a significant GlcSph accumulation both in *Gba* L444P/KO and *Gba*/*SNCA* double transgenic mice (Fig. 2C). These results suggest that, in mice carrying *Gba* L444P/KO mutation, accumulated GlcCer is broken down to more toxic GlcSph in the liver driving the GD pathology in our mice model. GlcCer levels did not change in the brain except for *Gba*/*SNCA* double transgenics which showed a trend towards GlcCer accumulation (Fig. 2D-E). GlcSph showed a mild increase in the brains of *Gba* L444P/KO and *Gba*/*SNCA* tg mice (Fig. 2F). These observations are in corroboration with patient data where liver pathology is severe in GD patients with *GBA* mutations, whereas the mild lipid accumulation seen in the brain increase the risk of developing neurodegenerative disorders like PD and DLB.

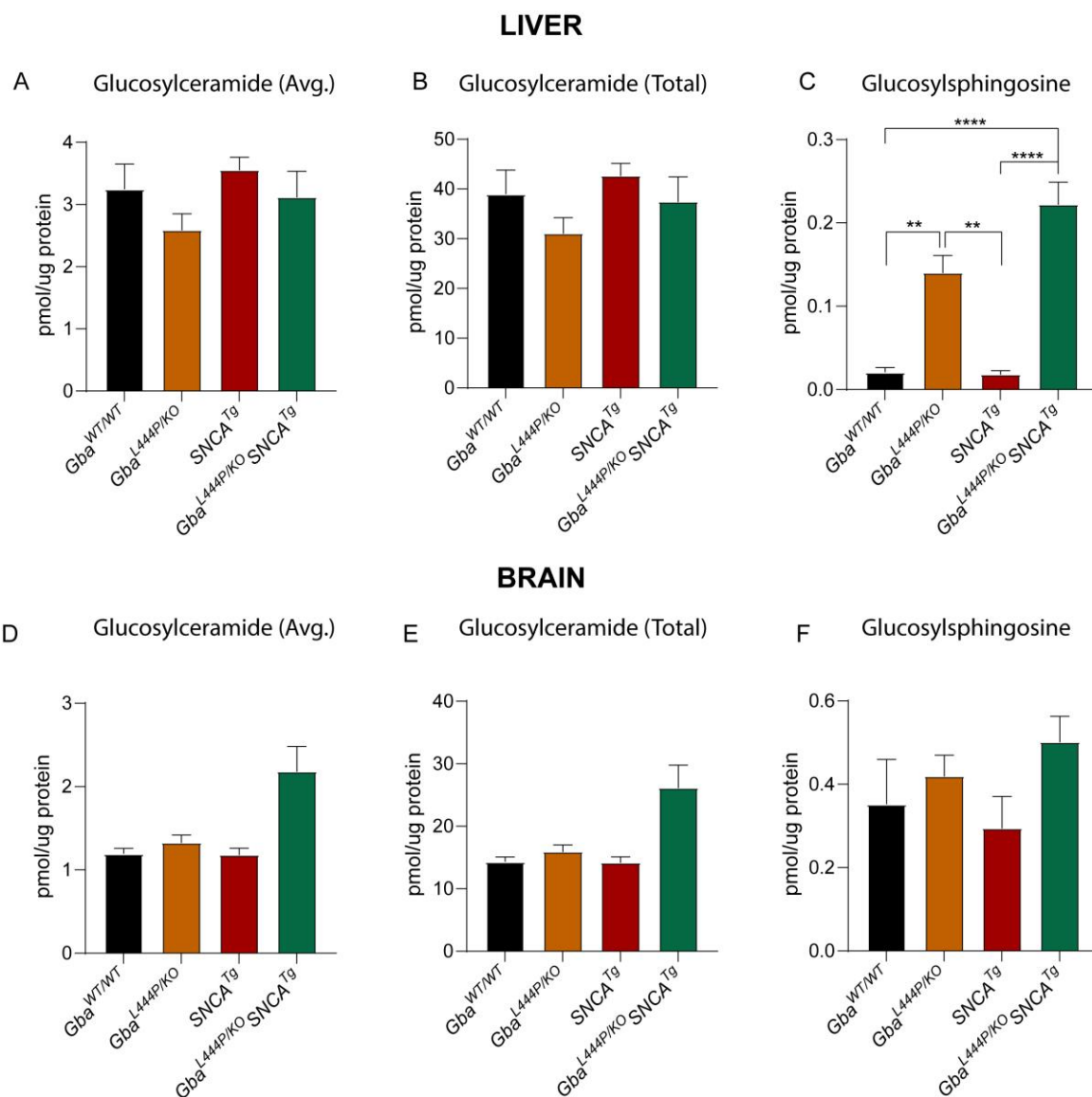


Fig. 2: Lipidomics in Gba mutants suggest severe and mild GlcSph accumulation in the liver and brain, respectively. A. Average levels of GlcCer in the liver. B. Sum of different GlcCer species in liver. C. GlcSph level in the liver. D & E. Average and sum of different GlcCer species in the brain, respectively. F. GlcSph levels in the brain.

Immunohistochemistry (IHC):

GlcCer accumulation was noted in the cortices of mice carrying Gba mutations: We performed these experiments at an early age of 3 months when the behavior deficits were mild/absent, and at the late age of 12 months when the behavior deficits were apparent (see Specific Aim 2 for the results). First, we tested if mice carrying Gba mutation such as Gba L444P/KO and Gba/SNCA tg mice show GSL accumulation in the cognitive brain areas such as cortex and hippocampus. We performed IHC for GlcCer, whereas IHC for GlcSph was not successful due to scarcity of good antibodies.

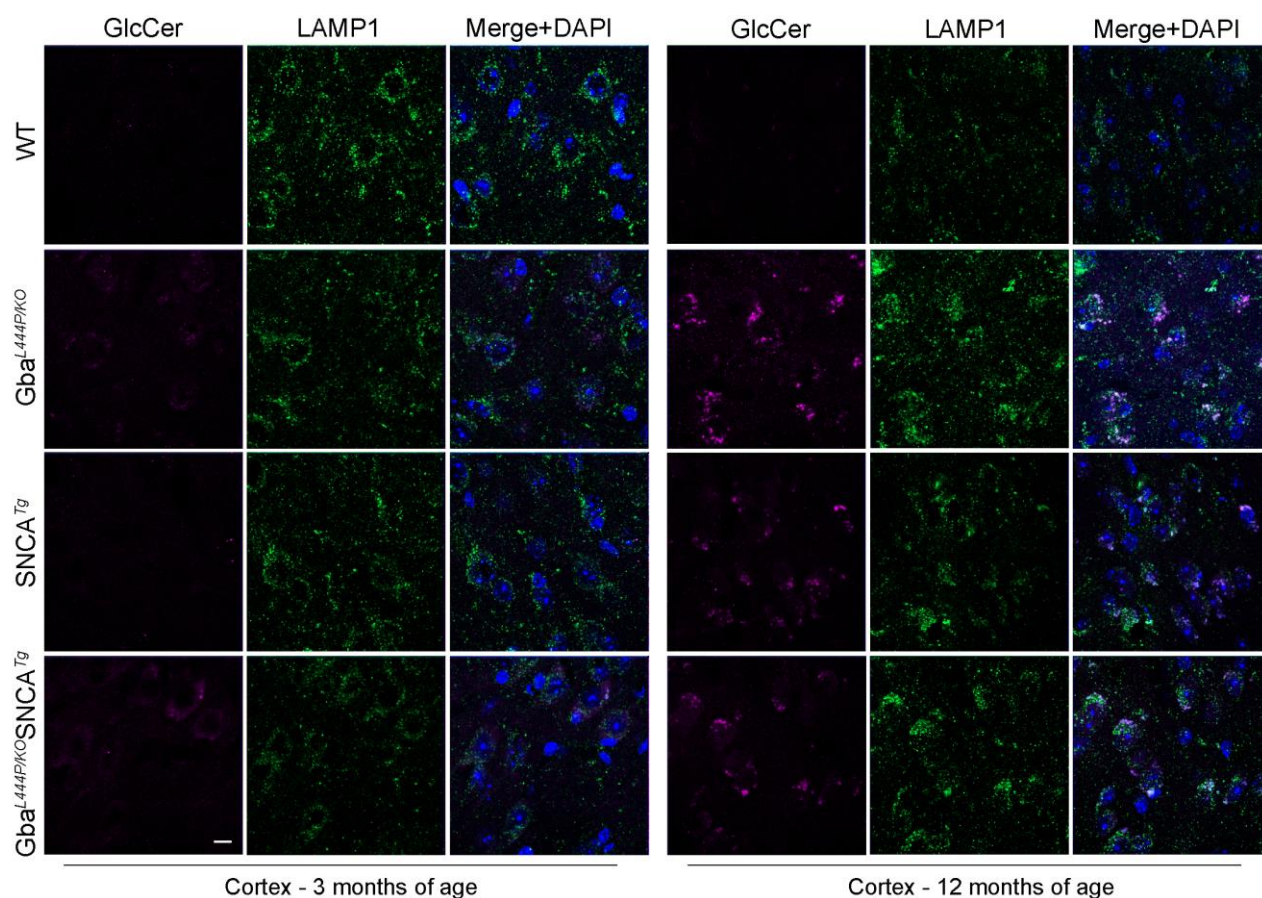


Fig. 3: Mice with Gba mutation or Gcase1 deficiency showed accumulation of GlcCer (Magenta) in the lysosomes (Green) in the cortex.

At 3 months of age, cortex of Gba L444P/KO mice showed only mild increase in GlcCer compared to WT, whereas Gba/SNCA double transgenic mice showed relatively higher GlcCer expression (Fig. 3, 3 months, magenta). SNCA tg showed no GlcCer expression at a younger age (Fig. 3, 3 months). GlcCer expression colocalized with that of lysosomal marker LAMP1 (Green) at 3 months, suggesting lipid accumulation only in the lysosome. At 12 months of age, we noted a severe GlcCer accumulation in the cortex of Gba L444P/KO and Gba/SNCA double transgenic mice (Fig. 3, 12 months, Magenta). Interestingly, SNCA tg mice which do not carry a Gba

mutation also showed a mild accumulation of GlcCer at 12 months of age (Fig. 3, 12 months, Magenta). This might be due to a mild loss of GCase1 in the brains of these mice (Fig. 1E). Most of GlcCer accumulation at 12 months of age seen in mutants co-localized with that of lysosomes (Fig. 3, 12 months, LAMP1, green), however, GlcCer accumulation was also seen outside lysosomes suggesting a widespread pathology at an older age. We also performed immunostaining to check hippocampal GlcCer accumulation, which is being quantitated.

Presence of Gba mutation in the SNCA tg PD mice exacerbates cortical α -synuclein pathology: To test if Gba mutation increase the risk of exacerbating the pre-existing synuclein pathology in SNCA transgenic mice, we performed IHC for normal α -synuclein and pathological phospho- α -synuclein (pSer-29) in the cortex of WT, Gba L444P/KO, SNCA tg, and Gba/SNCA double transgenic mice (Fig. 4). We also checked if there is cortical neuronal loss by using neuronal marker NeuN (Fig. 4). We noted that Gba L444P/KO mice did not show any α -synuclein

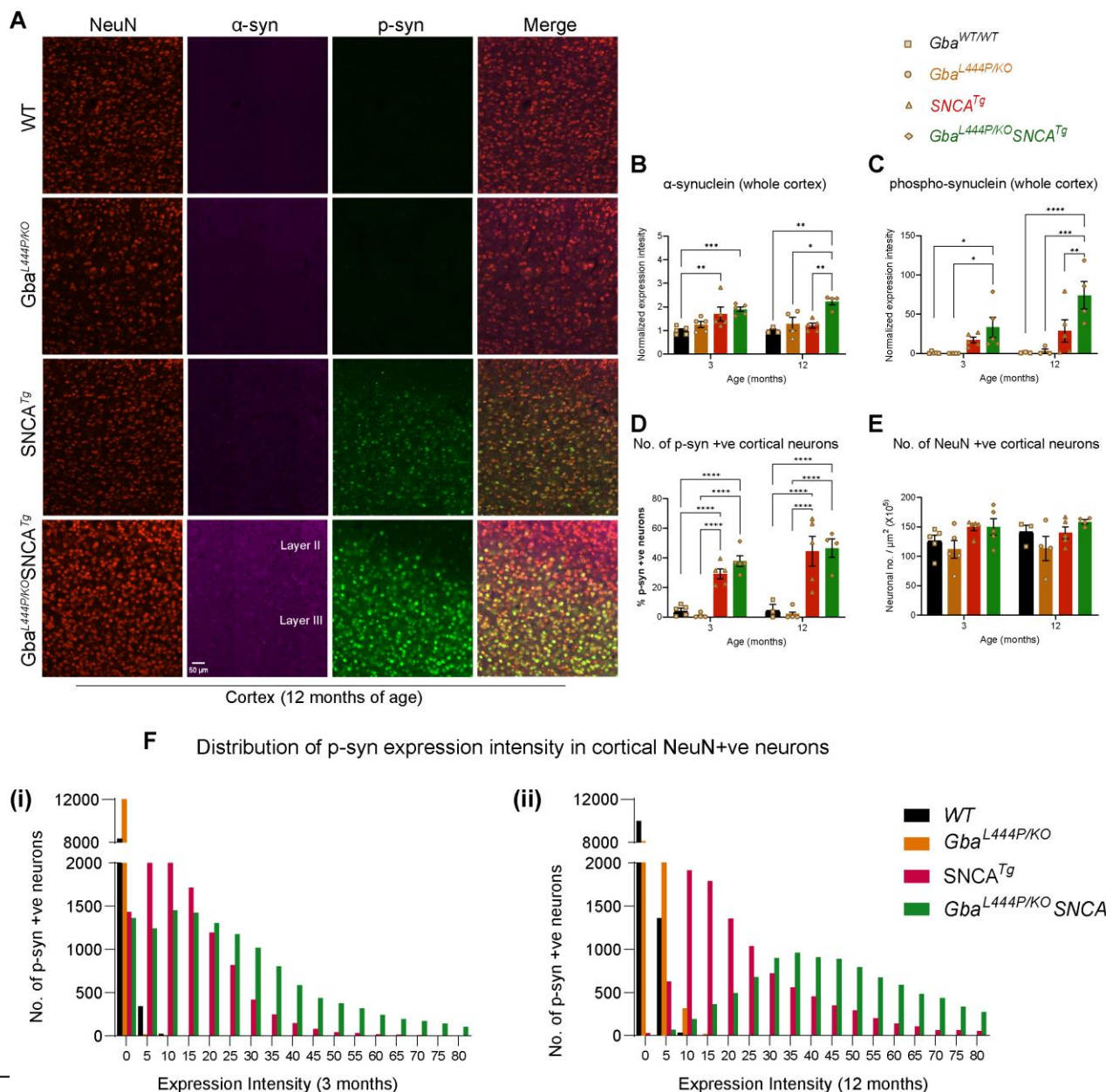


Fig. 4: Gba mutation exacerbates α -synuclein pathology in the cortices of SNCA tg mice. A. Representative photomicrographs of cortices of WT, Gba L444P/KO, SNCA tg, and Gba/SNCA tg mice immunostained for NeuN (red), α -synuclein (magenta) and phospho-synuclein (green). Not an exacerbated α -synuclein and phospho-synuclein expression in the Gba/SNCA double transgenics when compared to SNCA tg mice. B. Cortical α -synuclein expression at 3 and 12 months. C. Cortical phospho-synuclein expression at 3 and 12 months. D. Percentage of cortical neurons +ve for phospho-synuclein at 3 and 12 months. E. Cortical neuronal number at 3 and 12 months. F. Distribution of phospho-synuclein expression intensity in the cortical neurons at 3 (i) and 12 (ii) months of age.

overexpression or accumulation pathological phospho-synuclein in the cortex (Fig. 4: A-D). This suggest that the Gba L444P/KO mutation isn't potent enough to cause widespread α -synuclein pathology on its own, which is also true in majority of brains of GD patients. However, in the presence of a pre-existing α -synuclein pathology as seen in Gba/SNCA double transgenic mice, Gba L444P/KO mutation led to an overexpression of α -synuclein, as well as exacerbation of pathological phospho-synuclein accumulation (Fig. 4A-C). α -synuclein pathology was further exacerbated with age in these mice (Fig. 4B-C, compare 3 vs 12 months). Percentage of cortical neurons expressing phospho-synuclein was also increased in SNCA tg and Gba/SNCA double transgenic (Fig. 4D). When we looked into distribution of phospho-synuclein expression intensity in these neurons, we saw that Gba/SNCA double transgenic mice possess cortical neurons with higher burden of phospho-synuclein pathology (Fig. 4F). This burden of higher levels of phospho-synuclein worsened with age in Gba/SNCA double transgenics (Fig. 4F, i and ii, compared 3 vs 12 months). Though we saw a severe synuclein pathology, we did not see cortical neuronal loss in any of our mice akin to PD patients. Overall, our results suggest that Gba mutation can worsen pre-existing α -synuclein pathology in the cortex of our mice model. Similar pathogenic mechanisms might increase the risk of GD and PD patients with GBA mutation developing PDD and DLB.

Western blot experiment confirmed exacerbated α -synuclein and phospho-synuclein in Gba/SNCA double transgenics:

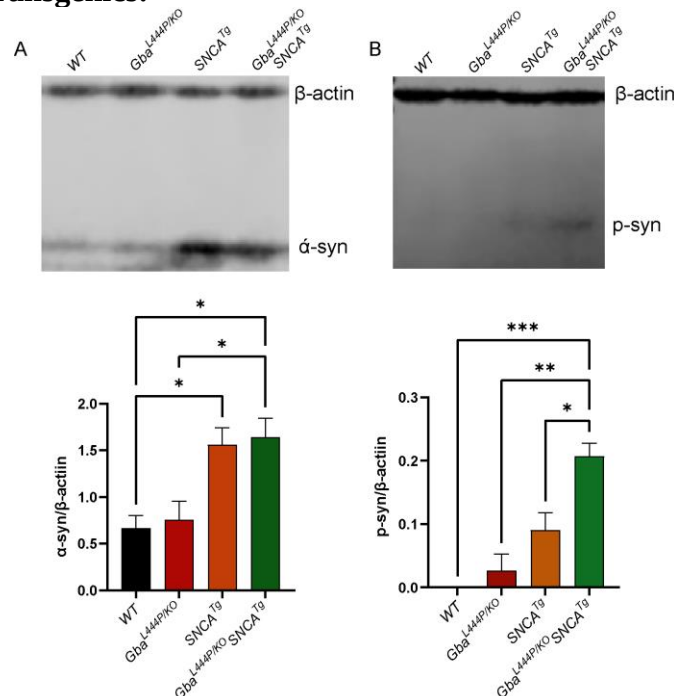


Fig. 5: Western blot showing α -synuclein (A) and phospho-synuclein (B) expression in the whole-brain homogenates of WT, Gba L444P/KO, SNCA tg, and Gba/SNCA tg mice.

Synaptic protein dyshomeostasis was exacerbated in the SNCA tg mice in the presence of Gba mutation:

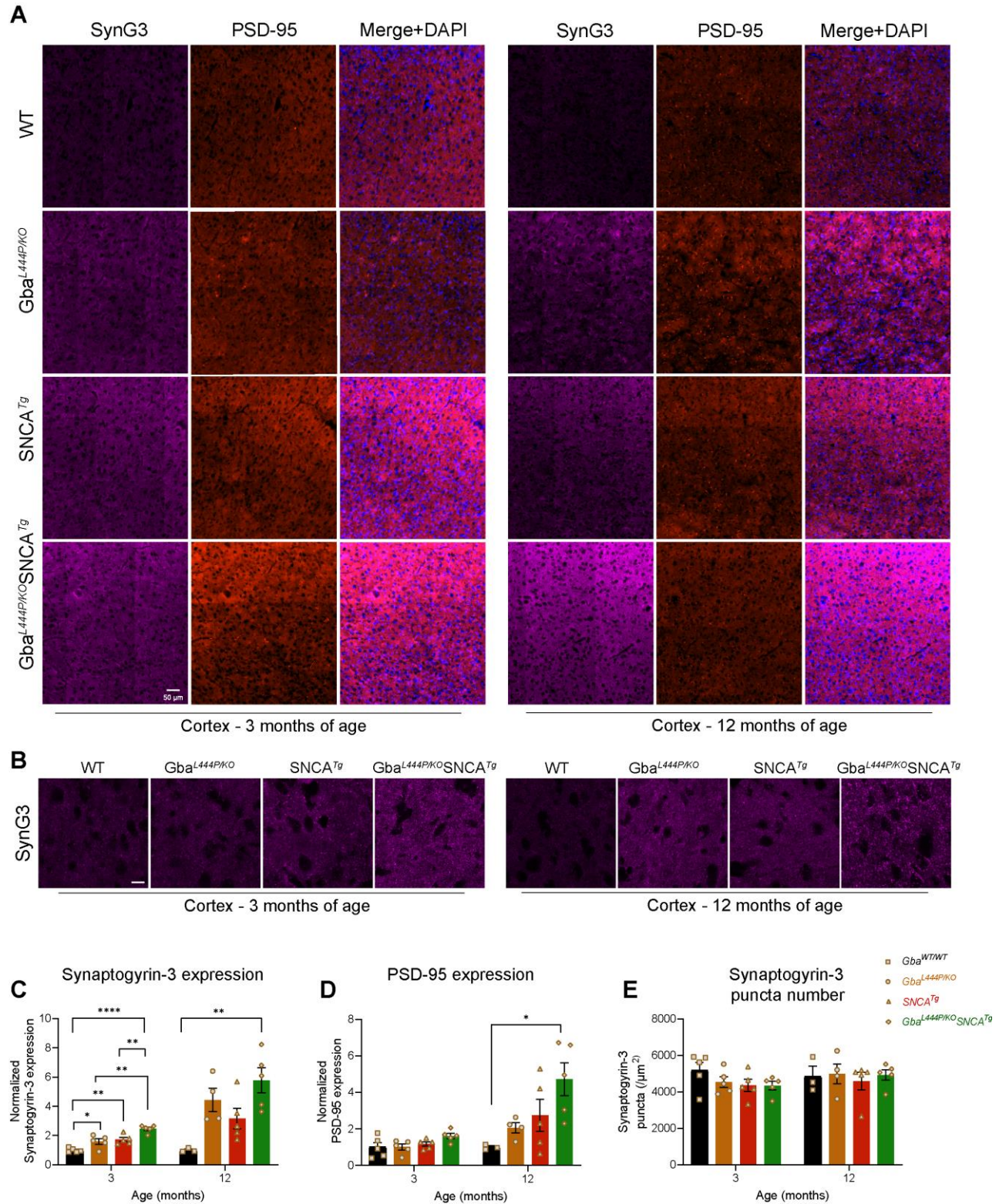


Fig. 6: Upregulation in synaptic proteins was seen in the cortex of Gba L444P/KO and SNCA tg, which was exacerbated in Gba/SNCA double transgenic mice. A. Representative photomicrographs of cortices immunostained for SynG3 (Magenta) and PSD-95 (Red) and their overlap with DAPI (Merge, blue). B. Representative high magnification (63X) images of SynG3 puncta in the cortex. C. SynG3 expression in the cortex. D. PSD-95 expression in the cortex, E. Cortical SynG3 puncta number.

Getting cues from the proteomics experiment (see Specific aim 3, Figure 10), we performed IHC for various pre- and post-synaptic proteins to check the synaptic health. We immunostained for presynaptic protein synaptogyrin-3 (SynG3, Magenta) and postsynaptic protein PSD-95 (Red) in the cortex and hippocampus. Interestingly, SynG3 expression in the cortex was significantly increased in Gba L444P/KO mice and SNCA tg mice. This overexpression was much more pronounced in Gba/SNCA double transgenics (Fig. 6A-B, 3 months). With age, the SynG3 expression was further increased in these mice (Figure 6A-B, 12 months). PSD-95 expression was not affected at 3 months of age across all the genotypes, however, at an old age of 12 months, Gba L444P/KO and SNCA tg mice showed a trend towards increase, with Gba/SNCA double transgenic showing a significant increase when compared to WT (Fig. 6A, C). To check if the increase in number is due to synaptogenesis, which has been recently seen in other cognitive disorders like Alzheimer's, we counted the SynG3 puncta representing presynaptic terminals (Fig. 6B). We did not see any change in presynaptic terminal number as marked by SynG3 puncta (Fig. 6E), suggesting upregulation in SynG3 is possibly due to overexpression of the protein in the synaptic terminals. We are performing post-synaptic terminal counting using homer as a marker, as PSD-95 immunostaining was more diffuse and unsuitable for puncta counting.

To check if a similar dysregulation in expression is seen in other presynaptic proteins in the cortex, we immunostained for synaptobrevin-2 (Fig. 7A). Though we saw a similar trend of increased protein expression at 12 months of age, it was not as severe as SynG3, suggesting dysregulation in only a subset of presynaptic proteins. In the hippocampus, we saw a significant upregulation in SynG3 in Gba L444P/KO, SNCA tg, and Gba/SNCA tg mice only at 3 months of age. This was also true for mild overexpression seen in PSD-95 expression in the hippocampus. These results suggest that only a subset of synaptic proteins are dysregulated, and the extent of dysregulation varies between cortex and hippocampus. These observations might hold key to cognitive dysfunctions seen in Gba L444P/KO and Gba/SNCA tg mice (see Specific Aim 2, Fig. X).

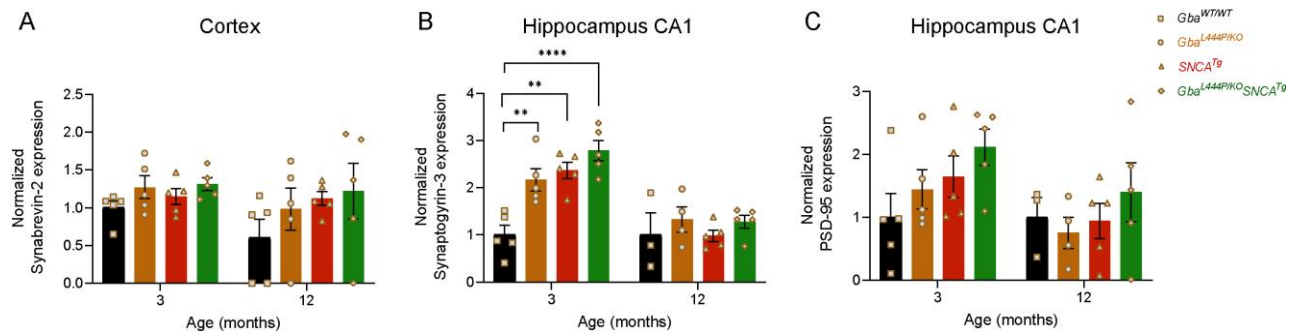


Fig 7: A. Cortical Synaptobrevin-2 expression did not change significantly. B. Hippocampus of Gba L444P/KO, SNCA tg, and Gba/SNCA tg mice showed an increase SynG3 expression at 3 months but not at 12 months of age. C. Hippocampal PSD-95 expression did not change significantly across the genotypes.

Specific Aim 2: To assess the cognitive capabilities of *Gba* KO and *SNCA* tg mice and to evaluate if it worsens in *Gba*/*SNCA* mice (n=12/mice line/time point/experiment) (3-30 months)

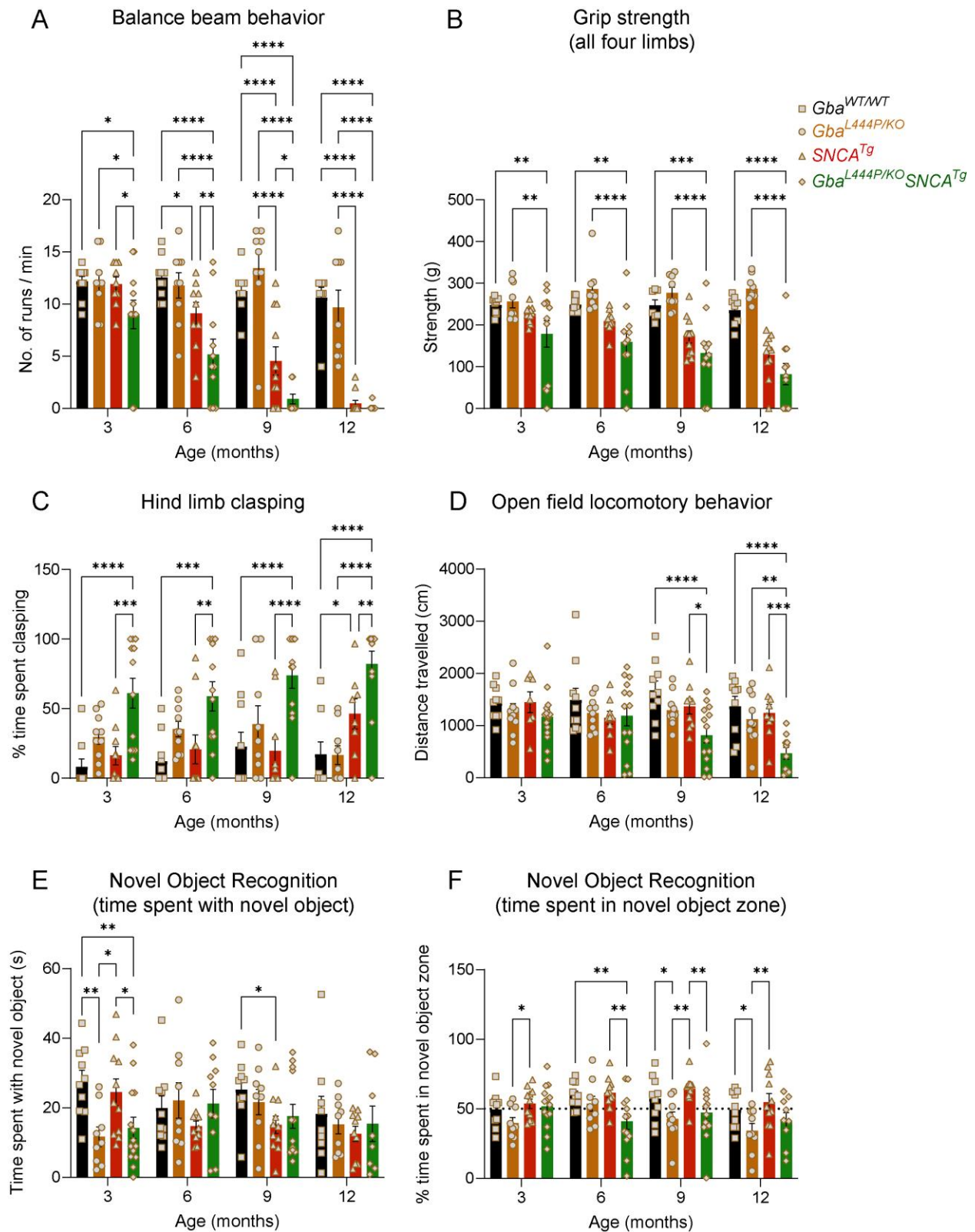


Fig. 8: Gba mutation leads to cognitive dysfunction in mice, and worsens motor deficits in SNCA tg mice. A. Balance beam behavior. B. Grip strength of all four limbs. C. Hind limb clasping behavior. D. Open field locomotory behavior. E. Novel object recognition test, time spent with novel object. F. Novel object recognition test, time spent in the novel object zone.

Gba mutation leads to cognitive dysfunction and exacerbates motor deficits in SNCA tg mice:

Test Longitudinal motor behavior evaluations such as balance beam, grip strength, hind limb clasping and open field locomotory behavior were performed to assess the age of onset of PD-like motor deficits, which was then compared with onset of cognitive behavior deficit as assessed by Novel Object Recognition test.

Balance beam: Balance beam test is used to assess the ability to walk straight on a narrow beam from brightly lit end towards a dark and safe platform/box (Fig. 8A). At 3 months of age, number of runs Gba L444P/KO and SNCA tg mice can perform in a minute were comparable to WTs. However, Gba/SNCA double transgenic mice showed balance beam behavior deficit as early as 3 months. SNCA tg mice developed balance beam deficits at 6 months and continued to worsen with age. The exacerbated balance beam deficit in Gba/SNCA tg seen at 3 months continued throughout, where by 9-12 months of age, they were severely affected and couldn't run on the balance beam. Gba L444P/KO mice did not develop any balance beam deficits across age groups.

Grip strength: Grip strength is a measure of force exerted by the animal in grasping specially designed pull bar assembly of a grip strength meter (Fig. 8B). Gba L444P/KO mice did not develop grip strength deficits, whereas SNCA tg mice developed age-related deficits in grip strength. Co-occurrence of Gba and SNCA mutations as in Gba/SNCA tg mice exacerbated grip strength deficits.

Hind limb clasping: Mice, when picked up by the tail and made to descend towards the ground, extend their limbs reflexly in anticipation of contact. Mice with certain neurological dysfunction display hind limb clasping instead of extension. We made mice to perform this maneuver for 30 seconds and noted the percentage of total time spent clasping (Fig. 8C). Gba L444P/KO mice showed a trend towards hind limb clasping throughout different ages. SNCA tg mice developed hindlimb clasping only at older age of 12 months. Gba/SNCA double transgenics showed an exacerbated hind limb clasping which worsened with progressing age.

Open field behavior: This assay was performed to understand overall locomotory behavior. Distance travelled exploring an open box (Fig. 8D) was comparable at 3 and 6 months among all the groups. At 9 months, a significant loss in exploratory/locomotory behavior was noted Gba/SNCA tg mice, which worsened further at 12 months of age when compared to other groups.

Novel object recognition (NOR): NOR test is used to assess the recognition memory. Here, mice were presented with two similar objects during the familiarization session, following which, after 18-20 hours, one of the two objects is replaced by a novel object. Mice being exploratory animals, spend more time with novel object when they are cognition is normal. Time spent with the novel object (Fig. 8E) and time spent in the novel object zone (Fig. 8F) were evaluated. Gba L444P/KO mice spent less time with the novel object at an early age of 3 months suggesting cognitive decline. Interestingly, at later ages, time spent with novel object was not significantly different for these mice from other groups. Time spent in novel object zone was also decreased in Gba/L444P KO mice at 3 months, which kept worsening with age. SNCA tg mice did not develop similar cognitive

decline, whereas cognitive decline seen in Gba/SNCA tg was comparable to that of Gba L444P/KO mice. Overall, this assay suggest that Gba mutation led to cognitive decline, and the cognitive decline seen in Gba/SNCA double transgenic mice is mainly driven by Gba mutation and not SNCA mutation.

Specific Aim 3: Evaluation of LTP in Schaffer collateral synapses of hippocampal sections and to investigate if AAV-shRNA mediated inhibition of Asah1 and Ugcg ameliorate hippocampal pathology and cognitive deficits in Gba KO, SNCA tg and Gba/SNCA mice (16-36 months)

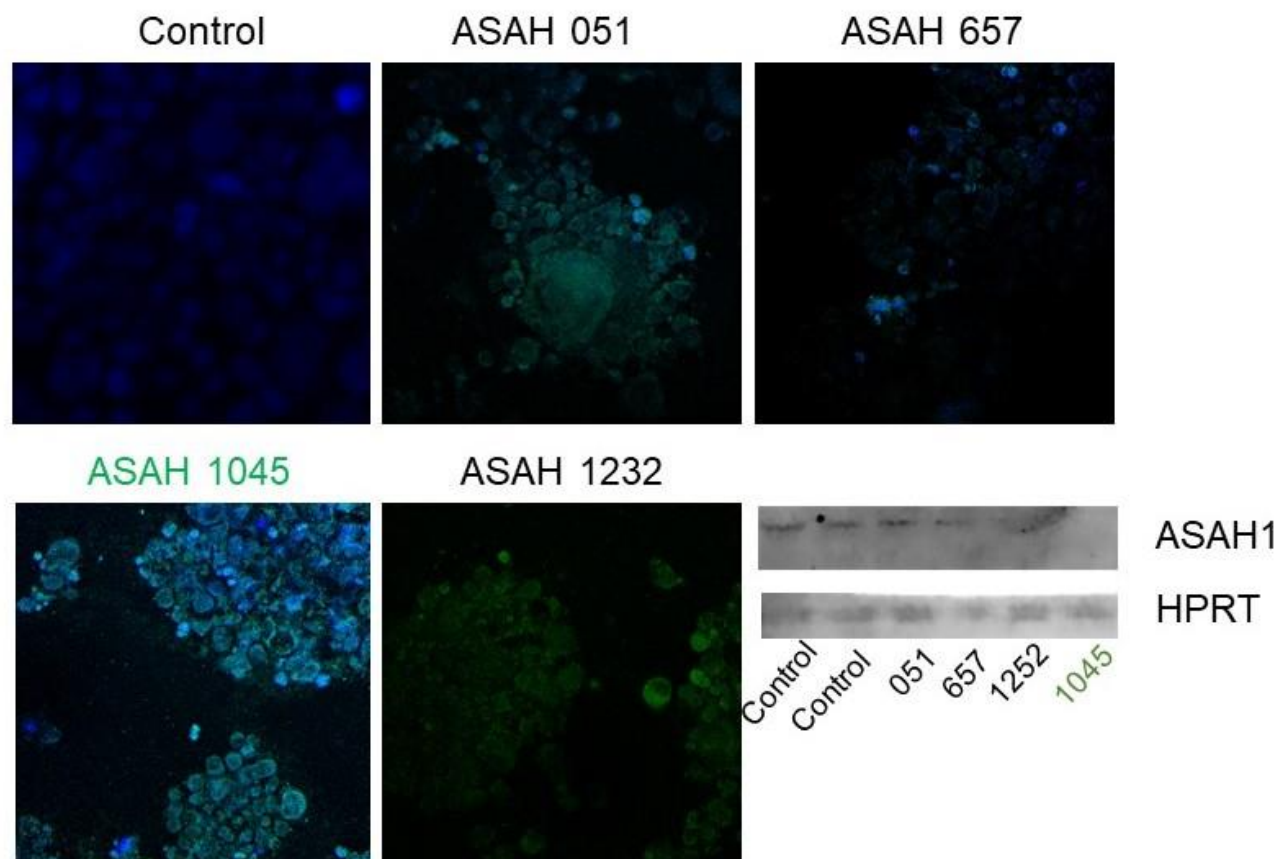


Fig. 9: Evaluating transfection efficacy of ASAHI1 shRNA plasmids in N2a cells

We prepared the shRNA plasmids for ASAHI1 (Fig. 9, green) and UGCG, and tested them for transfection efficacy in N2a cells (mouse neuroblastoma cell lines) (Fig. 10, blue). We then tested them for their efficiency to knockdown Asah1 and Ugcg protein using Western blotting. For ASAHI1, 4 different shRNA plasmids were tested for their transfection efficiency (Fig. 9, ASAHI1 051, ASAHI1 657, ASAHI1 1045, and ASAHI1 1232). ASAHI1 1045 showed the best transfection efficiency. It also showed good knockdown efficiency for asah1 protein as seen in Western blotting (Fig. 9, Western blots, 1045). We later prepared the AAV-shRNA for ASAHI1 1045 plasmid. We are now waiting for the right age mice to start stereotaxic injection of AAV-shRNA for ASAHI1 in our mice. Asah1 is the enzyme responsible for conversion of GlcCer to more toxic GlcSph. By decreasing the Asah1 levels through AAV-shRNA knock-down in the corticohippocampal area, we expect to rescue synaptic dysfunctions and cognitive decline noted in Gba L444P/KO mice and Gba/SNCA double transgenic mice.

Long Term Potentiation (LTP) is widely used to assess hippocampal functions that can be directly correlated to biological and behavioral phenotypes. We had planned these experiments to be performed under Aim 3 in collaboration with Dr. Pablo E. Castillo, Albert Einstein College of Medicine, New York. Covid effected this work due to logistic issues of transporting mice from one university to other, as well availability of enough mice numbers to do these experiments. We have now resumes breeding more mice, and we will restart the collaboration and complete these experiments.

To get more mechanistic insights, we used the time and resources that opened up due to the delay in LTP experiments to do proteomics. Brains from 12 month old WT, Gba L444P/KO mice, SNCA tg mice, and Gba/SNCA tg mice (n=3/genotype) were homogenized and subjected to Mass Spectroscopy (LFQ-MS). LFQ-MS was performed at Yale Mass Spectrometry & Proteomics Resource of the W.M. Keck Foundation Biotechnology Resource Laboratory. Samples were analysed in technical triplicates. The data was normalized to internal controls and total spectral counts. Proteins with two or more unique peptide counts listed using UniProt nomenclature were included for the analysis. To assess the effect of Gba mutation on the WT proteomics, WT and Gba L444P/KO mice were compared. To assess how Gba mutation might influence whole brain proteome of a SNCA tg mice, SNCA tg and Gba/SNCA tg mice were compared. A 1.5 fold change and a p-value difference of <0.05 between WT vs Gba L444P/KO mice, and SNCA tg vs Gba/SNCA double transgenic mice were considered significant. Volcano plots showing differentially expressed proteins were prepared using GraphPad Prism (Fig. 10A, B). Ingenuity Pathway Analysis (Qiagen) was used to determine the most significantly affected canonical pathways (Fig. 10C, D, i) as well as prediction of main cellular functions affected (Fig. 10C, D, ii). These results suggest that Gba mutation leads to abnormal lipid metabolism, and when it happens in the presence of SNCA mutation as seen in Gba/SNCA double transgenic mice, it leads to production of reactive oxygen species and sirtuin signalling pathway dysregulation. We validated these findings by immunohistochemistry. Differential expressed proteins obtained from the two different analyses were overlapped to obtain proteins that are affected by Gba mutation both in WT and Gba/SNCA tg mice (Fig. 10E, F). We are now performing further analysis of the significant hits and validating them by IHC and Western blotting to identify potential modifiers of Gba-mediated cognitive dysfunction.

In a related study, we also performed proteomics of olfactory bulb (OB) and olfactory cortex (OC) of WT and SNCA tg mice to get mechanistic insights into the previously seen olfactory deficits in SNCA tg mice (Martin-Lopez et al., 2022). These experiments were performed at 6-7 (n=3/genotype) and 12-14 (n=3/genotype) months. Proteomic data analysis was performed as described above with few modifications. Briefly, 1.25-fold change and a p-value difference of <0.05 between WT and SNCA Tg mice were considered as significant. Heat maps for significantly changed proteins were produced using Qlucore Omics Explorer. Volcano plots were produced using GraphPad Prism. Gene ontology (GO) enrichment analysis was used to determine significantly affected biological pathways. 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 SNCA tg mice (Fig. 11A, B). Protein kinase c-Src was the most significantly upregulated protein in the OB of SNCA tg mice (SRC, Fig. 11A, B, fold change of 2.88). Among other functions, protein kinase c-Src phosphorylates components of endocytic trafficking and notably, α -synuclein. GO enrichment analysis performed on the proteins that showed significant changes identified pathways involving vesicle-mediated transport, specifically endocytosis, which were significantly enriched (Fig. 11C). The vast majority of proteins from our dataset that were implicated in these pathways were downregulated in SNCA tg samples (Fig. 11A, B), suggesting a deficit in vesicle-mediated

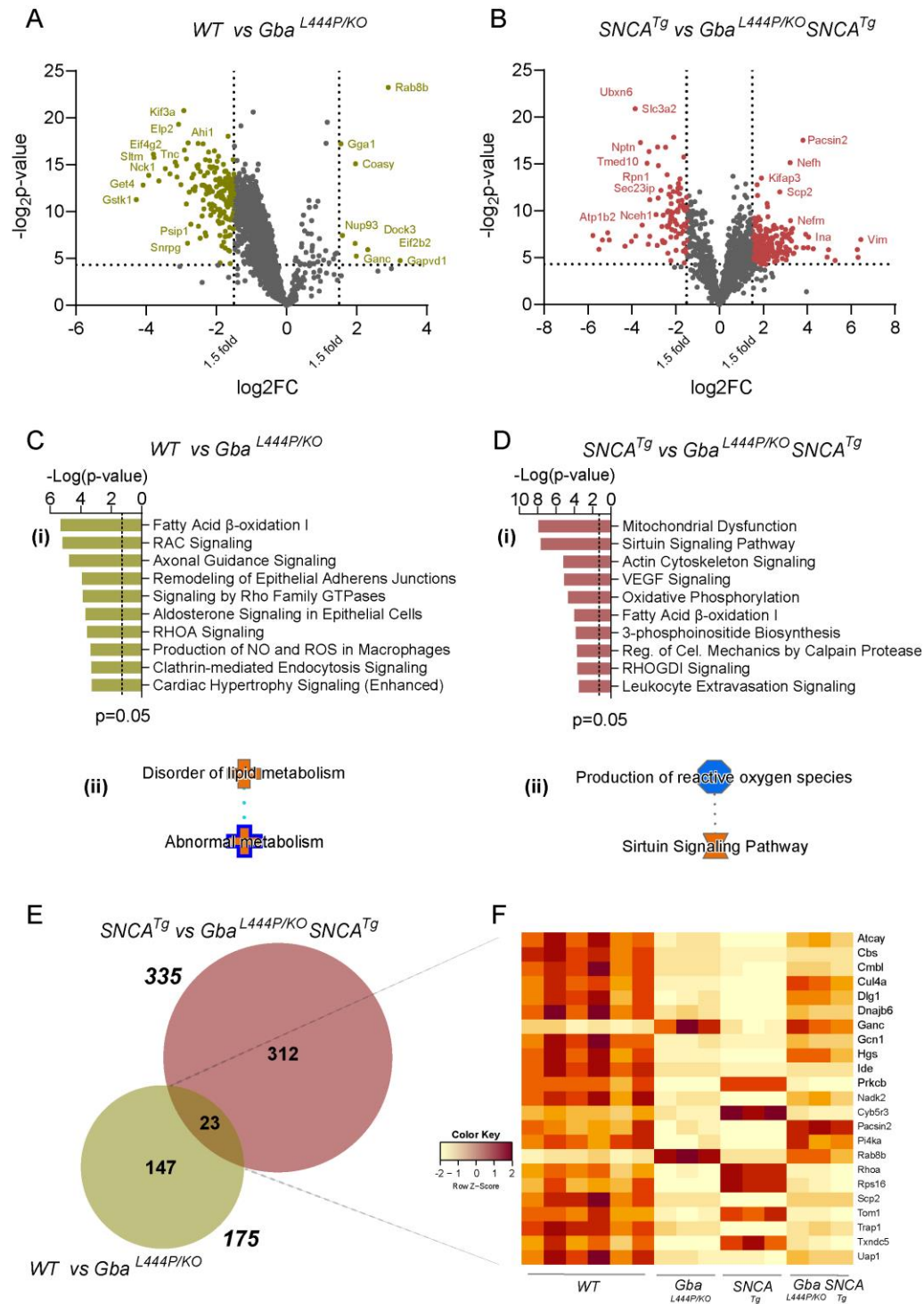


Fig. 10: Proteomics revealed lipid metabolism dysfunction in *Gba* mutations, which when co-existed with *SNCA* mutation, led to oxidative stress and dysfunction of sirtuin signaling pathway. A & B, Volcano plot depicting significantly upregulated (right) and downregulated (left) proteins in *Gba* L444P/KO mice in the WT background (WT vs *Gba* SNCA tg mice), and *SNCA* mutation background (*SNCA* tg vs *Gba*/SNCA tg mice), respectively. C & D, (i) Significantly dysregulated pathways, (ii) Top effected pathways. E. Overlap of significant proteins obtained from two different comparisons (WT vs *Gba* L444P/KO, and *Gba* L444P/KO vs *Gba*/SNCA tg mice). F. Heat map of proteins that were dysregulated in both analyses.

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 SNCA tg samples (Fig. 11D, E). 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 enriched (Fig. 8F). These pathways were driven by a majority of proteins that were downregulated in SNCA tg samples (Fig. 11D, 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. 11A-C), and provided additional evidence that, with age, these deficits became prominent in synaptic terminals of SNCA tg mice.

In the OC of 6-7 months mice, proteomic analysis identified a total of 2955 proteins, 11 of which were significantly differentially expressed in SNCA tg mice (Fig. 11G, H). As anticipated, we observed a significant increase in α -syn expression (SYUA, Fig. 11D, E). Interestingly, GO analysis largely revealed broader dysfunction in cellular metabolism (Fig. 11F) without enrichment of any vesicle mediated-transport pathways seen in the OB (Fig. 11C). In the OC of 12-14 months mice, proteomic analysis identified a total of 2596 proteins, 16 of which were significantly differentially expressed in SNCA tg samples (Fig. 11J, K). Similarly, GO analysis revealed only a subtle dysregulation in receptor clustering in SNCA tg samples, 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 OC, and that olfactory deficits seen at 12-14 months old SNCA tg mice might arise due to early age-related endocytic changes in the synaptic terminals of the OB circuitry.

Covid-19 pandemic delayed procuring required mice for this project, which opened time for the PI to get involved in another project to evaluate mechanisms for PD that arise due to presynaptic clathrin-mediated endocytosis dysfunction using auxilin (a clathrin uncoating protein) knockout mice. One paper from this study is published (Massaro Tieze et al., 2022) and another study is under revision (Vidyadhara et al., 2022, published in bioRxiv). Papers are attached in the ppendix.

What opportunities for training and professional development has the project provided?

Training: PI was trained in newer techniques such as proteomic data analysis, new behavioral experiments, subcellular fractionation to isolate synaptosomes and synaptic vesicles, *ex vivo* imaging of mice brain sections for dopamine transporters, Gcase/GBA enzyme assay, AAV-stereotaxic injections, sample collection for lipidomics, cortical and striatal dissection in mic brain, primary neuronal cultures, iPSCs differentiation, electron microscopy (EM) and EM image analysis. PI regularly interacted with Dr. Pramod Mistry who is a leader in GBA-related studies and his lab members through joint lab meetings. This helped him develop a clinical perspective on GBA-associated PD. Dr. Sreeganga Chandra, who is the mentor in this project interacted with PI one-on-one every week and helped him with the resources and provide suggestions on the techniques and concepts. With the help of Dr. Sreeganga Chandra, PI established successful collaborations with other labs such as Dr. David Sulzer and Dr. Dalibor Sames labs at Columbia University, Dr. Charles Greer and Dr. Michael Higley labs at Yale University, and Dr. Alexander Chubykin lab at Purdue University. Overall administrative process to get all the regulatory approvals, renewing the animal protocol, utilizing the grant money, and applying for no-cost extensions was also a learning experience for the PI, especially in these difficult times of Covid-19.

Professional development: PI had a detailed discussion with his mentor Dr. Chandra on his career interests and professional development plan in the beginning of every year since the inception of the project. Below are few of his accomplishments during the project period.

Courses/Conferences attended:

- a. MDS-PAS Neuroscience of Parkinson's Disease and Movement Disorders course, San Diego, 2022
- b. Society for Neuroscience Meeting 2022, San Diego
- c. Introduction to Biomedical Data Science and Health Informatics course, 2021
- d. Attended Yale innovation summit, 2020, 2021, 2022
- e. CSHL Neurodegenerative Diseases: Biology & Therapeutics conference, 2020
- f. CSHL meeting on Protein Homeostasis in Health and Disease, 2020
- g. International Congress of Parkinson's Disease and Movement Disorders, 2020
- h. Yale University Postdoc Symposium, 2020-2022
- i. Monsoon Brain Meeting 2020
- j. Neuroscience retreat Yale university 2020-2022
- k. Van Andel Institute Grand Challenges in Parkinson's Disease 2020, 2021

Teaching:

- a. Selected to teach Neurodegeneration at Yale in Modern Instructor Series (2021 and 2022), organized by Yale Postdoc Association (YPA) and Poorvu Center for Teaching and Learning
- b. Completed 90% of Certificate of College Teaching Preparation (CCTP) by Poorvu Center for Teaching and Learning
- c. Completed "An Introduction to Evidence-Based Undergraduate STEM Teaching" course by Cornell University, and the CIRTL network (online)

Mentoring:

- a. Mentored four Yale undergrads, including three of them for their "Senior Thesis".
- b. Mentored undergrads at Yale Biological & Biomed. Sciences Diversity & Inclusion Collective (YBDIC)
- c. Mentor at Annual Biomedical Research Conference for Minority Students (ABRCMS), USA
- d. Mentor at Women in STEM Research (WISR) India

Refereeing/Reviewing:

- a. Grant reviewer, National Science Centre, Poland
- b. Review Editor, Frontiers in Aging Neuroscience
- c. Topic Editor, Frontiers in Aging Neuroscience
- d. Book editor, Protein misfolding in Neurodegenerative Disorders, Elsevier
- e. Guest Editor, Journal of Visualized Experiments (JoVE)
- f. Judge at Yale Undergrad Research Symposium, USA
- g. Judge at ABRCMS, USA
- h. Judge at Intersections Science Fellows (ISFS) Symposium, USA
- i. Volunteered to give feedbacks to undergrads from NIH BP EDURE program
- j. Reviewed articles for Frontiers in Molecular Neuroscience, Frontiers in Neuroscience, Frontiers in Aging Neuroscience, Experimental Neurology, Translational Stroke Research, Journal of Neuroscience Research, Cell Transplantation, BMC Complementary and Alternative Medicine, J. of Chemical Neuroanatomy, BMC Complementary Medicine and Therapies, The FEBS Journal, Brain & Behavior

Talks:

- a. Society for Neuroscience Meeting, San Diego, USA 2022
- b. CUSAT Neuroscience Lecture Series, Cochin, India 2022

- c. CT Early Career Researcher Symposium, Cellular & Metabolic Pathways in Neurodeg. 2022
- d. Indian Academy of Neurosciences Society Meeting 2021
- e. Kasturba Medical College, Manipal University, Manipal, India 2021
- f. The Oxford College of Science, Bangalore University, India 2021
- g. Yale Neuroscience Research in Progress Seminars 2021
- h. Indian Academy of Neuroscience - Bangalore Chapter Symposium 2020
- i. Cold Spring Harbor Laboratory meeting on Protein Homeostasis in Health and Disease 2020
- j. International Congress of Parkinson's Disease and Movement Disorders 2020
- k. 3rd Annual Postdoc Symposium, Yale University 2020
- l. Monsoon Brain Meeting 2020

Posters:

- a. Society for Neuroscience meeting 2022, Award posters + virtual poster
- b. NeuroDay 2022, Yale University, New Haven, USA
- c. The 5th Annual Postdoctoral Symposium, Yale University, 2022
- d. Department of Neuroscience Retreat, Yale University, 2022
- e. Van Andel Institute Grand Challenges in Parkinson's Disease, 2021
- f. The 4th Annual Postdoctoral Symposium, Yale University, 2021
- g. Department of Neuroscience Retreat, Yale University, 2021
- h. Van Andel Institute Grand Challenges in Parkinson's Disease, 2020

Others:

- a. Founding member of Yale Neuroscience Postdoc Committee, 2020 - till date
- b. Member of Yale Neuroscience Committee for Diversity, Equity & Inclusion, 2020
- c. Member of Yale Neuroscience SYNAPSES committee, 2019-2020
- d. National Council for Behavioral Health, USA certified Mental Health First Aider, 2021-2024
- e. Member of Society for Neuroscience, USA 2022
- f. Member of International Parkinson and Movement Disorder Society 2021-2022
- g. Member of Aligning Science Across Parkinson's (ASAP), USA 2020 – till date
- h. Member of National Center for Faculty Development & Diversity, USA 2020 – till date

Awards:

- a. Society for Neuroscience Trainee Professional Development Award 2022
- b. Yale University Undergrad/Postgrad Mentoring Award 2022
- c. Parkinson Disease & Movement Disorders Society travel award 2022
- d. Best poster award, The 4th Annual Postdoctoral Symposium, Yale University 2021
- e. Top downloaded article in the Journal of Neurochemistry 2020

How were the results disseminated to communities of interest?

PI discussed the developments of the project with Dr. Pramod Mistry and his lab members, who are working extensively on Gaucher Disease and other *GBA*-related disorders. PI and the undergrad trainee who worked on the project presented the work in a few meetings (see above). A few results that came out of this grant are under peer review for publication. A review article and a methods article related to the topic of this grant are published in peer-reviewed journals (See Appendix).

What do you plan to do during the next reporting period to accomplish the goals?

“Nothing to report” as this is the final report. However, the evaluation of hippocampal LTP under Aim 3 will be completed with the collaborating institute once we get new mice. This experiment was delayed due to covid related restrictions.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

PD is diagnosed through motor abnormalities, and within several years, 80% of patients develop cognitive problems. Knowing this statistic provides a window for preventive measures and early treatment strategies for which understanding the mechanisms through which cognitive problems arise is crucial. This work provides major breakthroughs in this area and identifies shingolipids as important modifiers of α -synuclein pathology and cognitive dysfunction in PD and DLB. We show that the two-way interaction between lipid dyshomeostasis and α -synuclein pathology may lead to cognitive dysfunction through synaptic dysregulation. Future therapies may be developed targeting shingolipid dyshomeostasis to prevent synaptic dysregulation and cognitive dysfunction in PD and DLB patients, which is considered one of the main unmet needs. There are already several drugs commercially available that blocks accumulation of glycosphingolipids (GSLs) such as GlcSph in cancer. Drugs targeting GlcCer are under clinical trials for GD. Our results successfully establish the role of these two GSLs in cognitive dysfunction of PD, suggesting those drugs can be repurposed to treat PD and DLB patients. Our proteomic experiments might help us find new modifiers for *GBA*-mediated cognitive dysfunction (Martin-Lopez et al., 2022). This study will also provide evidence to support if *Ugcg* and *Asah1* which produce GSLs can be the potential targets to treat cognitive decline in PD.

Technically, this study establishes *Gba* L444P/KO mice as an ideal model to study *Gba*-mediated DLB, where the mice show classical peripheral features of GD and the cognitive dysfunctions seen in DLB without affecting the motor system. When this mouse is crossed with *SNCA* tg mouse to get *Gba*/*SNCA* double transgenic mice, it models PDD where the mice develop classical motor deficits as well as cognitive deficits of PD with α -synuclein pathology.

A review article on the “Role of the endolysosomal system in Parkinson’s disease” which came out of this grant is one the highly cited articles which proved to be valuable to PD research field (Vidyadhara et al., 2019). We also recently published a methods article through this grant titled “Subcellular Fractionation for the Isolation of Synaptic Components from the Murine Brain” (Massaro Tieze et al., 2022) which is well received and will be valuable to study synapses and its components in brain health and disease.

What was the impact on other disciplines?

This project is originally conceptualized to understand mechanisms of cognitive dysfunctions in PD. As we results started coming, as we now narrate the results, our study will also have a significant impact in understanding mechanisms for DLB, which is closely related to PD. We show that our Gba L444P/KO mice can be a good model of DLB, which can be used further to study DLB in detail. Thus, our work will broaden the therapeutic landscape for treatment of cognitive dysfunction also in other synucleinopathies such as DLB, along with unravelling the causative mechanisms. Through this study, we also provide a methods article to isolate various synaptic components which will be helpful broadly to any discipline under neuroscience.

What was the impact on technology transfer?

As described above, we propose a new mouse model to study DLB through this study. Our methods article to isolate synaptic components will also be helpful for others in the field. We have streamlined a collaborative effort to perform lipidomics, which could be used in other projects too. The complicated breeding experiments involved in this study allowed us to standardize various genotyping protocols which could be useful for other studies too. The stereotaxic apparatus set-up and the standardization of AAV injection methodology could also be used in other studies. Expertise in breeding methods enabled us to help others in the lab to generate mice for large-scale omics studies.

What was the impact on society beyond science and technology?

Even after more than 200 years of its first description, PD remains predominantly a movement disorder even in the public domain. It is important to sensitize the caregivers and the community about the immense cognitive and psychiatric disturbances the patients go through. We will communicate the intent and the results of our study to the public in lay words on all the available digital and print platforms.

Our results further support that a significant fraction of GD patients develop cognitive decline later in life. This information might guide patient behavior, practices, and may influence regulatory bodies to provide more resources to study and curb cognitive dysfunctions in patients with GD and other lysosomal storage disorders.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Effects of Covid-19: Restricted/limited access to the laboratory facilities was in place for a major part of this grant, which delayed our experiments significantly. PI also couldn't use the budget allotted for travel and attending conferences due to travel restrictions. We also had to cut down our mice colony during covid-19 lockdowns. It took time to bring back the colony. It was especially hard for this project because it involves crossing mice with different genetic backgrounds to ultimately get Gba L444P/KO mice, SNCA tg mice, and Gba/SNCA double transgenic mice with appropriate WT. An inadvertent error in breeding and genotyping to obtain Gba/SNCA double transgenic mice further delayed the process. PI used the time and resources that were made available due to these delays in writing a review article which is highly cited and made important contributions to PD research field (Vidyadhara et al., 2019). PI also got involved in another project to evaluate mechanisms for PD that arise due to presynaptic clathrin-mediated endocytosis dysfunction using auxilin (a clathrin uncoating protein) knockout mice. One paper from this study is published (Massaro Tieze et al., 2022) and another study is under revision (Vidyadhara et al., 2022, published in bioRxiv). PI also participated in various virtual career and professional development activities as in the "Researcher Development Plan".

PI took "no-cost extension" which helped complete the majority of the experiments. A small part of the experiment where mice had to be shipped to our collaborator's lab at an out-of-city institute (Dr. Pablo E. Castillo lab, Albert Einstein College of Medicine, NY) is pending, which will be complete soon. Alternatively, we performed proteomic experiments on the whole brain which gave us some important insights into possible modifiers of *GBA*-mediated PD and DLB. We also performed proteomics of olfactory bulb and olfactory cortex, which showed synaptic endocytic dysfunction in the olfactory bulb might be the key in initiating olfactory deficits in PD. Proteomics on olfactory system also helped us find out that olfactory deficits that occur in PD is mainly due to pathological changes in olfactory bulb, whereas olfactory cortex just has a participatory role (Martin-Lopez et al., 2022). Thus, proteomics experiments, which were not planned under this grant significantly added to the outcome of our project and helped in delineating molecular mechanisms for non-motor dysfunctions (including cognitive dysfunctions) of PD.

Changes that had a significant impact on expenditures

Shutdown or restricted entry to our laboratory due to Covid-19 pandemic for major part of this grant period along with limited access to the common facilities have significantly delayed our experiments. A no-cost extension for a year was helpful in utilizing the remaining grant to complete all the experiments.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- Publications, conference papers, and presentations

Journal publications.

1. **Vidyadhara DJ**, M. Somayaji, N Wade, J Ribaud, N Shashaank, H Zhao, B Yucel, J Gupta, T Lam, Dalibor Sames, Lois Greene, David Sulzer, and **Sreeganga S. Chandra**. 2022. Dopamine transporter and synaptic vesicle sorting defects initiate auxilin-linked Parkinson's disease. *BioRxiv*. <https://www.biorxiv.org/content/10.1101/2022.02.04.479203v1.full>, Under minor revision in *Cell Reports*, *Federal support acknowledged*
2. **Vidyadhara DJ**, JE Lee, SS Chandra, 2019. Role of the endolysosomal system in Parkinson's disease, Published in *Journal of neurochemistry* 150 (5), 487-506. <https://doi.org/10.1111/jnc.14820> (review), *Federal support acknowledged*
3. S. Massaro Tieze, **SS. Chandra, Vidyadhara DJ**, Subcellular Fractionation for the Isolation of Synaptic Components from the Murine Brain, Published in *J. of Visualized Experiments (JoVE)*, 2022, <https://dx.doi.org/10.3791/64574>, *Federal support acknowledged*
4. EM Lopez, **DJ Vidyadhara**, T Liberia, SJ. Meller, LE. Harmon, RM. Hsu, K Han, B Yücel, **SS Chandra**, CA Greer. α -Synuclein pathology and reduced neurogenesis in the olfactory system affect olfaction in a mouse model of Parkinson's disease. Under minor revision in *J. Neuroscience*, 2022, <https://doi.org/10.1101/2022.08.19.504562>, *Federal support acknowledged*

Books or other non-periodical, one-time publications.

1. Pathogenic mechanisms for cognitive dysfunctions of Parkinson's disease: insights from studies on GBA mutations, Risha Chakraborty, **Vidyadhara DJ**, David Backstrom, Jamie Park, Jiapeng Ruan, Pramod Mistry, **Sreeganga S. Chandra**., Abstract, Northeast Undergrad/Graduate Research Organisation for Neuroscience (NEURON) meeting, Quinnipiac University, Frank H. Netter School of Medicine on April 24, 2022, *Federal support acknowledged*
2. Pathogenic mechanisms for cognitive dysfunctions of Parkinson's disease: insights from studies on GBA mutations, Risha Chakraborty, **Vidyadhara DJ**, David Backstrom, Jamie Park, Jiapeng Ruan, Pramod Mistry, **Sreeganga S. Chandra**., Annual Biomedical Research Conference for Minoritized Scientists (ABRCMS), virtual, November 9-12, 2022, *Federal support acknowledged*
3. Presynaptic dopamine compartmentalization defects initiate auxilin-mediated parkinsonism, **Vidyadhara DJ**, M. Somayaji, N Wade, J Ribaud, N Shashaank, H Zhao, B Yucel, J Gupta, T Lam, Dalibor Sames, Lois Greene, David Sulzer, and **Sreeganga S. Chandra**. 2022, Abstract, Movement Disorders, 2022, Volume: 37 Page: S596, *Federal support acknowledged*
4. Understanding Endolysosomal Dysfunction in Parkinson's Disease Using Mutant Mouse Models, Lorenzo Arvanitis, Undergrad Senior Thesis 2021, Yale University, Mentored by **Sreeganga S. Chandra** and **Vidyadhara DJ**, *Federal support acknowledged*
5. Dopamine compartmentalization defects initiate auxilin-mediated parkinsonism, **Vidyadhara DJ**, M. Somayaji, N Wade, J Ribaud, N Shashaank, H Zhao, B Yucel, J Gupta, T Lam, Dalibor Sames, Lois Greene, David Sulzer, and **Sreeganga S. Chandra**. 2022, Abstract, Society for Neuroscience (SfN) meeting 2022, *Federal support acknowledged*

Other publications, conference papers and presentations.

1. Dopamine compartmentalization defects initiate auxilin-linked Parkinson's disease*, Presented in Society for Neuroscience Meeting 2022, San Diego, USA, Nov. 2022, *Federal support acknowledged*
2. Endolysosomal system dysfunction in Parkinson's disease*, Presented in CUSAT Neuroscience Lecture Series, Cochin, India, Aug. 2022, *Federal support acknowledged*
3. Dopamine transporter and synaptic vesicle sorting defects initiate auxilin-linked Parkinson's disease*, Presented in CT Early Career Researcher Symposium, Cellular & Metabolic Pathways in Neurodeg. March 2022, *Federal support acknowledged*
4. Endolysosomal system dysfunctions in Parkinson's disease* – evidence from recent studies, Presented by Indian Academy of Neurosciences Society Meeting 2021, *Federal support acknowledged*

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

We optimized subcellular fractionation technique to isolate synaptosomes and other synaptic components from murine brain through this study. This is published as methods article in audio-visual journal, Journal of Visualized Experiments (JoVE).

S. Massaro Tieze, **SS. Chandra, Vidyadhara DJ**, Subcellular Fractionation for the Isolation of Synaptic Components from the Murine Brain, Published in *J. of Visualized Experiments (JoVE)*, 2022, <https://dx.doi.org/10.3791/64574>, *Federal support acknowledged*

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

An audio-visual methods article is published as an outcome of this grant.

S. Massaro Tieze, **SS. Chandra, Vidyadhara DJ**, Subcellular Fractionation for the Isolation of Synaptic Components from the Murine Brain, Published in *J. of Visualized Experiments (JoVE)*, 2022, <https://dx.doi.org/10.3791/64574>, Federal support acknowledged

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Vidyadhara D J, PhD
Project Role: PI
Researcher Identifier (e.g. ORCID ID): <https://orcid.org/0000-0003-0974-0307>
Nearest person month worked: 24
Contribution to Project: Dr. Vidyadhara has obtained Yale IACUC and ACURO approvals, procured mice and set-up breeding colonies, all the experiments, data analysis, purchase of consumables and prepared necessary progress reports.

Name: Sreeganga S. Chandra, PhD
Project Role: Mentor
Researcher Identifier (e.g. ORCID ID): <https://orcid.org/0000-0001-9035-1733>
Nearest person month worked: 24
Contribution to Project: Dr. Chandra has obtained IACUC approval for her proposal, number 2018-11117 which is in congruent with this proposal. Dr. Chandra has also mentored PI at all the stages of experiments towards successful execution of the grant.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Dr. Pablo E. Castillo lab,
Dept. of Neuroscience,
Albert Einstein College of Medicine,
1410 Pelham Parkway South,
Kennedy Centre, Bronx, NY-10461.
USA

Contribution: This lab will help us complete Aim 3, in performing electrophysiological experiments to record hippocampal LTP in our mice models. These experiments were delayed due to Covid-19 restrictions.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable

QUAD CHARTS:

Submitted.

9. APPENDICES:

1. **Vidyadhara DJ**, M. Somayaji, N Wade, J Ribaud, N Shashaank, H Zhao, B Yucel, J Gupta, T Lam, Dalibor Sames, Lois Greene, David Sulzer, and **Sreeganga S. Chandra**. 2022. Dopamine transporter and synaptic vesicle sorting defects initiate auxilin-linked Parkinson's disease. *BioRxiv*. <https://www.biorxiv.org/content/10.1101/2022.02.04.479203v1.full>, Under minor revision in *Cell Reports*, *Federal support acknowledged* Page 32-85
 2. **Vidyadhara DJ**, JE Lee, SS Chandra, 2019. Role of the endolysosomal system in Parkinson's disease, Published in *Journal of neurochemistry* 150 (5), 487-506. <https://doi.org/10.1111/jnc.14820> (review), *Federal support acknowledged* Page 86-105
 3. S. Massaro Tieze, **SS. Chandra**, **Vidyadhara DJ**, Subcellular Fractionation for the Isolation of Synaptic Components from the Murine Brain, Published in *J. of Visualized Experiments (JoVE)*, 2022, <https://dx.doi.org/10.3791/64574>, *Federal support acknowledged* Page 106-121
 4. EM Lopez, **DJ Vidyadhara**, T Liberia, SJ. Meller, LE. Harmon, RM. Hsu, K Han, B Yücel, **SS Chandra**, CA Greer. α -Synuclein pathology and reduced neurogenesis in the olfactory system affect olfaction in a mouse model of Parkinson's disease. Under minor revision in *J. Neuroscience*, 2022, <https://doi.org/10.1101/2022.08.19.504562>, *Federal support acknowledged* Page 121-169
- Conference abstracts: Page 170-173

Dopamine transporter and synaptic vesicle sorting defects initiate auxilin-linked Parkinson's disease

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ABBREVIATIONS:

3-MT: 3-Methoxytyramine
 5-HIAA: 5-hydroxyindoleacetic acid
 ACSF: Artificial cerebrospinal fluid
 ALDH7A1: Aldehyde dehydrogenase 7A1
 BSA: Bovine serum albumin
 CCVs: Clathrin coated vesicles
 CME: Clathrin mediated endocytosis
 COMT: Catechol-o-methyltransferase
 DA: Dopaminergic
 DAT: Dopamine transporter
 DOPAC: 3,4-dihydroxyphenylacetic acid
 EM: Electron Microscopy
 FSCV: Fast scan cyclic voltammetry
 GAK: Cyclin G-associated kinase
 GFAP: Glial fibrillary acidic protein
 HPLC: High performance liquid chromatography
 HVA: Homovanillic acid
 Iba1: Ionized calcium-binding adapter molecule 1
 IPA: Ingenuity Pathway Analysis
 KO: Knockout
 RME-8: Receptor-mediated endocytosis 8
 SNG: Synaptogyrin
 SNpc: Substantia nigra pars compacta
 SEM: Standard error of the mean
 SV: Synaptic vesicle
 SV2: Synaptic vesicle glycoprotein 2
 SYP: Synaptophysin
 Syn: Synuclein
 SYT: Synaptotagmin I
 TH: Tyrosine hydroxylase
 VGAT: Vesicular GABA transporter
 VGLUT: Vesicular glutamate transporters
 VMAT2: Vesicular monoamine transporter-2
 VTA: Ventral tegmental area
 WT: Wildtype

SUMMARY:

Auxilin participates in the uncoating of clathrin-coated vesicles (CCVs), thereby facilitating synaptic vesicle (SV) regeneration at presynaptic sites. Auxilin (*DNAJC6/PARK19*) loss-of-function mutations cause early-onset Parkinson's disease (PD). Here, we utilized auxilin-knockout (KO) mice to elucidate the mechanisms through which auxilin deficiency and clathrin-uncoating deficits lead to PD. We demonstrate that auxilin KO mice display the cardinal features of PD, including progressive motor deficits, α -synuclein pathology, nigral dopaminergic loss, and neuroinflammation. Through unbiased proteomic and neurochemical analyses, we demonstrate that dopamine homeostasis is disrupted in auxilin KO brains, including via slower dopamine reuptake kinetics *in vivo*, an effect associated with dopamine transporter misrouting into axonal membrane deformities in the dorsal striatum. We also show that elevated macroautophagy and defective SV protein sorting contribute to ineffective dopamine sequestration and homeostasis, ultimately leading to neurodegeneration. This study advances our knowledge of how presynaptic endocytosis deficits lead to dopaminergic vulnerability and pathogenesis of PD.

KEYWORDS:

Clathrin mediated endocytosis, Dopamine, α -Synuclein, Lewy bodies, Axonal deformity, Synaptic autophagy, Clathrin coated vesicles, Endolysosomal system, Dorsal striatum, Substantia nigra

INTRODUCTION:

Presynaptic boutons of dopaminergic (DA) nigrostriatal neurons are sites of initiation for neurodegeneration in PD (Kordower et al., 2013). Nigrostriatal DA neurons possess long, hyperbranched axons as well as tonic firing properties that make them reliant on efficient synaptic vesicle (SV) recycling to maintain a steady state SV pool for neurotransmission (Vidyadhara et al., 2019). In presynaptic sites, SV recycling is supported by several endocytic pathways, including clathrin mediated endocytosis (CME), ultra-fast endocytosis, and bulk endocytosis (Chanaday et al., 2019). Clathrin coated vesicles (CCVs) are a common intermediate of these pathways. CCVs are uncoated by the coordinated action of auxilin (*DNAJC6*) or its ubiquitous homolog cyclin G-associated kinase (*GAK*), synaptojanin-1 (*SNJ1*), and endophilin-A1 (*ENDO1*) with the chaperone Hsc70. Interestingly, mutations in all four genes (*DNAJC6*, *GAK*, *SNJ1*, *ENDO1*) have

been identified as causal or risk alleles for PD/parkinsonism, suggesting a major role for altered clathrin uncoating in the initiation of DA presynaptic site degeneration and pathogenesis of both familial and sporadic PD (Vidyadhara et al., 2019). Animal models carrying these mutations also show clathrin uncoating and presynaptic endocytosis defects (Cao et al., 2017; Song et al., 2017; Yoshida et al., 2018), however, how these disturbances result in characteristics of PD is unclear.

Auxilin is a brain-specific heat shock protein 40 family co-chaperone that functions to uncoat CCVs to nascent SVs by recruiting Hsc70 (Fotin et al., 2004; Ungewickell et al., 1995). Unlike other co-chaperones, auxilin has a limited number of substrates (Roosen et al., 2021) and only one described function. Loss-of-function, autosomal recessive mutations of the auxilin gene (*PARK19*) cause juvenile, early-onset PD (Edvardson et al., 2012; Elsayed et al., 2016; Köroğlu et al., 2013; Mittal, 2020; Ng et al., 2020; Olgiati et al., 2016; Ray et al., 2021). A recent study also shows *PARK19* mutations in late-onset PD (Gialluisi et al., 2021). *In vivo* presynaptic dopamine transporter (DAT) imaging on a *PARK19* patient revealed DA terminal loss, which supports a parkinsonism diagnosis and suggests that clathrin uncoating deficits impact DA presynaptic sites (Ng et al., 2020). Furthermore, LRRK2 mutations, a common genetic cause for PD, may exert some of its pathological actions through auxilin. In LRRK2 patient induced pluripotent stem cell derived DA neurons, LRRK2 phosphorylation of auxilin led to decreased auxilin levels and clathrin binding, resulting in accumulation of oxidized-dopamine and α -synuclein overexpression (Nguyen and Krainc, 2018). Whether loss-of-function mutations in auxilin can also trigger PD through these mechanisms is unknown. Nonetheless, these newfound links between auxilin and LRRK2 implicate a role for auxilin in both familial and sporadic PD. The relevance of auxilin to all forms of PD is underscored by the finding that GAK (*DNAJC26*) is a risk allele for sporadic PD (Nalls et al., 2014).

Prior to the discovery of auxilin *PARK19* mutations, auxilin knockout (KO) mice were generated and characterized for CME deficits (Yim et al., 2010). Analysis of synapses in deep cerebellar nuclei of adult auxilin KO mice revealed accumulation of CCVs and empty clathrin cages (lacking SV membrane). Similar ultrastructural alterations were seen *in vitro* in primary cortical and hippocampal neurons and were accompanied by defective SV endocytosis (Yim et al., 2010). These findings confirmed that auxilin functions in clathrin uncoating *in vivo*. To determine how a primary deficit in clathrin uncoating that occurs in all neuronal types lead to selective vulnerability

of DA neurons in PD, we characterized auxilin KO mice for age-dependent nigrostriatal degenerative changes and investigated the underlying mechanisms. Here, we demonstrate that cytoplasmic dopamine accumulation, DAT mis-trafficking, SV sorting deficits and autophagic overload in dorsal striatal DA presynaptic sites of auxilin KO mice initiate behavioral and histochemical signatures of PD.

RESULTS:

Auxilin KO mice develop age-dependent PD-like behavioral abnormalities: We performed a battery of behavioral assays to evaluate if auxilin KO mice develop age-dependent motor behavior abnormalities akin to PD patients. We monitored cohorts of wildtype (WT, C57BL/6J) and auxilin KO (congenic B6.-*Dnajc6*^{tm1Legr}) mice longitudinally, assessing behavior at 3, 6, 9, 12, and 15 months of age. Locomotion and ambulatory behaviors were evaluated by the open field test. Auxilin KO mice behave like WT mice at 3 months of age but show a significant age-dependent decrease in overall distance travelled, starting at 9 months (Figure. 1a, b). Next, we tested the same cohorts on the balance beam test to evaluate motor coordination. We assessed the ability of mice to traverse a raised narrow beam by measuring the time taken to cross (Figure. 1c) and the number of runs performed in 1 minute (Figure. 1d). The performance of auxilin KOs was comparable to WT controls at 3 months (Figure. 1c, d), whereas it deteriorated in auxilin KOs at a later age with a significant deficit emerging at 9 months (Figure. 1c, d, Video 1). These results suggest that auxilin KOs appear to be normal at 3 months, whereas they become symptomatic by 9 months, exhibiting a progressive decrement in motor function at later ages. There was no difference in body weight between genotypes (Supplementary Figure. 1g). Furthermore, performance on the Rotarod and grip strength in auxilin KOs were comparable to that of WT (Supplementary Figure. 1a, e, f). In addition, auxilin KO mice do not exhibit anxiety-like behavior as evaluated by time spent in the inner and outer circle of an open field apparatus (Supplementary Figure. 1b, c), and by fecal pellet expulsion (Supplementary Figure. 1d). Together, these observations suggest that auxilin KO mice develop age-dependent, progressive motor deficits, consistent with *PARK19* and PD patients.

Aged auxilin KO mice faithfully replicate cardinal histopathological signatures of PD: Motor symptoms in PD manifest due to the degeneration of DA neurons in the substantia nigra pars compacta (SNpc) when DA loss reaches a threshold of 40-50% (Poewe et al., 2017). We performed stereological quantitation of SNpc DA neurons, that are immunoreactive to tyrosine hydroxylase

(TH), in WT and auxilin KO mice to understand the cellular basis for the motor symptoms we observed. No change in DA neuron numbers was seen in auxilin KO mice at 3 months (Figure. 1e, f). However, at the symptomatic age of 9 months, a significant loss of DA neurons was observed (~40%) (Figure. 1e, f), which did not increase further at 15 months (Supplementary Figure. 1h, i). Neuronal loss was distributed throughout the SNpc (Figure. 1e, arrows), as observed in models of α -synuclein overexpression (Chen et al., 2015) and vesicular dopamine storage deficits (Caudle et al., 2007), but unlike the ventrolateral loss seen in neurotoxic models (Vidyardhara et al., 2017). In addition, loss of DA neurons appears to be restricted to SNpc, as ventral tegmental area (VTA) TH expression and TH+ve neuron numbers were unchanged with age (Supplementary Figure. 2a-c), as in PD patients.

To assess if neurodegeneration was accompanied by neuroinflammation, we immunostained for glial fibrillary acidic protein (GFAP), an astroglial marker, and ionized calcium-binding adapter molecule 1 (Iba1), a microglial marker (Figure. 2a). At 3 months, the number of astroglia and microglia in the SNpc of auxilin KO mice were comparable to WT, whereas at 9 months, significant astrogliosis and microgliosis was seen in auxilin KO mice (Figure. 2a-c).

Next, we tested if auxilin KO brains exhibit α -synuclein pathology, a hallmark of PD (Poewe et al., 2017). Strikingly, auxilin KO brains showed age-dependent α -synuclein pathology. Phosphorylated and aggregated α -synuclein as determined by pSer129- α -synuclein immunostaining was seen in the TH+ve SNpc at 9 months, but not at 3 months of age in auxilin KO mice (Figure. 2d, e). Immunostaining also revealed a moderate decrease in TH expression at 3 months, with no significant change at 9 months of age in auxilin KO mice (Figure. 2d, f). The pSer129- α -synuclein pathology was also seen in the VTA at 9 months of age, but the expression did not reach significance (Supplementary Figure. 2a, c). These compelling data demonstrate that auxilin KO mice develop typical age-related parkinsonian pathology. Auxilin KO mice are thus a reliable and robust model for PD.

Proteomic analysis of auxilin KO mice brains implicate defective dopamine degradation: To gain unbiased insights into the consequences of auxilin loss-of-function, we performed proteomic analysis on whole brain and synaptosomes fractions from 3-month-old, WT and auxilin KO mice by label-free quantification mass spectrometry (LFQ-MS). WT and auxilin KO brains (n=3/genotype) were analyzed in technical triplicates. We detected a total of 2851 proteins in the

whole brain proteome, 22 of which were significantly changed in KO samples (Figure. 3a, b; Supplementary Table 1). We observed an expected decrease in auxilin levels and a compensatory increase in the auxilin homolog GAK, as previously published (Yim et al., 2010). Many of the prominent proteins whose levels are changed are linked to PD and neurodegeneration, including RAB3B, TBCD, ACAP2, HEBP1, WDFY1 and NNTM which are decreased, while CRYAB, PRIO, and NMRL1 are increased in auxilin KO brains (Figure. 3a, b; Supplementary Table 1). Notably, we did not identify any altered Golgi resident or trafficking proteins in auxilin KO brains. Ingenuity Pathway Analysis (IPA) revealed that the top pathways were highly overlapping and involve in the degradation of lysine and degradation of choline and monoaminergic neurotransmitters, including dopamine (Figure. 3c, d). Interestingly, a decrease in AL7A1 appears to drive the top canonical pathways (Figure. 3c). AL7A1 or aldehyde dehydrogenase 7A1 (ALDH7A1) is a multifunctional enzyme which plays crucial role in detoxification of reactive aldehydes and oxygen species that are generated during monoaminergic neurotransmitter metabolism (Brocker et al., 2011). Aldehydes that accumulate due to ALDH7A1 loss-of-function hinder dopamine synthesis (Clayton, 2020).

Proteomic analysis of synaptosomal fractions identified 3124 proteins, 24 of which were significantly dysregulated (Figure. 3e, f; Supplementary Table 2). Along with the expected downregulation of auxilin, KO mice showed decreased AL7A1, NNTM and WDFY1, and an upregulation in PURA2 and MTND, proteins which were also significantly changed in whole brain proteomic experiments (Figure. 3a, b). Three neurofilaments NFL, NFH, and AINX were upregulated and are candidate biomarkers for axonal damage in PD (Bäckström et al., 2020). A crucial dopamine metabolizing enzyme COMT (Myöhänen et al., 2010) (catechol-o-methyltransferase) was also significantly decreased in synaptosome preparations of auxilin KOs. HTRA1, PP2A, KCNJ4 and APC were a few PD-linked proteins that were also dysregulated (Figure. 3e, f; Supplementary table 2). In all, we find alterations in a high number of proteins linked to PD (13/23), including key dopamine metabolism enzymes. As the synaptosome preparations were purified from whole brain, these findings strongly suggests that loss-of-auxilin selectively impacts DA neurons. This is also evident from the IPA analysis, where the top affected pathways are related to dopamine degradation (Figure. 3g, h). A high fraction of the canonical pathways predicted for the whole brain analysis were replicated in IPA analysis for the

synaptosome samples (10/21, compare Figure. 3g, h with 3c, d), suggesting a major impact on the function of DA synapses upon loss-of-auxilin.

To evaluate if the disruption in dopamine degradation predicted by the proteomic analysis of young auxilin KO brains (Figure. 3) leads to activation of downstream neurodegenerative pathways at an older age, we performed LFQ-MS on synaptosomes from 9-month-old auxilin KO mice. In this preparation, we still observed a compensatory increase in GAK in auxilin KO brains. NFL, AINX, and CADH2 were also upregulated as in the 3-month data set, reinforcing their potential as biofluid biomarkers of PD (Bäckström et al., 2020) (Supplementary Figure. 3a, b). Along with the expected downregulation of auxilin, mTOR, a key cell survival protein and an autophagy regulator which helps maintain striatal DA projections (Kosillo et al., 2019) and linked to PD (Querfurth and Lee, 2021), was decreased in auxilin KO mice. RGS6, a critical regulator of dopamine feedback signaling in nigrostriatal DA neurons and a modulator of PD pathology (Ahlers-Dannen et al., 2020) was also downregulated (Supplementary Figure. 3a, b; Supplementary table 4). IPA revealed highly overlapping autophagic pathways such as ILK, P13K/AKT, mTOR, and AMPK signaling, along with oxidative stress, DA signaling, and ubiquitination pathway as dysregulated in auxilin KOs at 9 months (Supplementary Figure. 3c), which are directly linked to neurodegeneration in PD (Poewe et al., 2017). Together, our proteomic analyses suggests that dysfunction of dopamine homeostasis in presynaptic sites is likely to be an early pathogenic event in auxilin-linked PD.

Disrupted striatal dopamine homeostasis in auxilin KO mice: To directly monitor dopamine homeostasis, we measured the levels of dopamine and its metabolites in dorsal striatum of WT and auxilin KO mice using high performance liquid chromatography (HPLC). Dopamine levels were moderately decreased (14.5%) at 3 months in auxilin KO mice compared to WT controls, whereas loss was more pronounced at 9 months of age (52%, Figure. 4a) when motor deficits are seen (Figure. 1). We also measured serotonin levels, which were unchanged in auxilin KOs (Supplementary Figure. 4a). Next, we evaluated the levels of dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), which are intra- and extra-cellular metabolites, respectively (Figure. 4e). Interestingly, DOPAC levels were significantly increased at 3 months (42%, Figure. 4b), even though dopamine levels are modestly decreased. DOPAC is a catabolite of cytosolic (non-vesicular) dopamine (Figure. 4e). Upregulation of DOPAC suggests cytosolic dopamine accumulation (Karoum et al., 1994), which is known to be

toxic, leading to oxidative stress and proteostasis deficits which culminate in neurodegeneration in both familial and sporadic models of PD (Masato et al., 2019). 3-Methoxytyramine (3-MT) is a dopamine metabolite, formed by direct catabolism of unused dopamine in the synaptic-cleft by COMT (Myöhänen et al., 2010) (Figure. 4e). 3-MT levels were significantly lower in auxilin KO mice (Figure. 4c) suggestive of a decrease in dopamine release (Waldmeier et al., 1981), and reflective of the downregulation of COMT seen in the proteomics data (Figure. 3e, f). Both DOPAC and 3-MT are metabolized further to HVA outside the DA termini (Figure. 4e), whose level did not change at 3 months in auxilin KO mice (Figure. 4d). This is possibly due to a balancing out of HVA levels attained by increased DOPAC and decreased 3-MT levels. At 9 months when motor abnormalities are apparent, both 3-MT and HVA levels were significantly decreased in auxilin KO mice (Figure. 4c, d), which is also the case in PD patients. Indeed, decreased HVA levels have also been observed in the cerebrospinal fluid of patients with auxilin mutations (Ng et al., 2020). DOPAC levels were unchanged at 9 months. Levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) did not change at 3 and 9 months (Supplementary Figure. 4b), suggesting dopamine-selective dysfunction. Overall, assessment of dopamine and its metabolites in dorsal striatum support the premise that dopamine homeostasis is altered in auxilin KO mice.

Dopamine reuptake is dysfunctional in young auxilin KO mice: Extracellular dopamine in the striatum is pumped back into DA axons by the dopamine transporter (DAT). Therefore, DAT controls the level of presynaptically available dopamine and is a key regulator of dopamine compartmentalization and homeostasis (Bu et al., 2021). DAT KO mice show a decreased striatal dopamine levels and release (Jones et al., 1998). We evaluated extracellular dopamine clearance on a sub-second timescale in the dorsal striatum of auxilin KO mice using fast scan cyclic voltammetry (FSCV) *in vivo*. The SNpc was stimulated using a 30 pulses of 50-Hz stimuli (0.6 sec) paradigm that drives burst firing by nigral DA neurons. This causes dopamine build-up in the extracellular space at levels sufficient to saturate DAT and to be detected by the carbon fiber electrode placed in the dorsal striatum (Somayaji et al., 2020) (Figure. 4f, Supplementary Figure 5a). Figure. 4g and Supplementary Figure. 5b depict the time course of evoked dopamine release and its clearance in the dorsal striatum, along with the characteristic background-subtracted voltammogram at the maximum oxidation peak for WT and auxilin KO mice. Surprisingly, evoked dopamine release was not significantly different between WT and auxilin KO mice (Figure. 4h). Interestingly, dopamine reuptake kinetics as measured by the time taken to clear 50% of the dopamine from its peak levels

($t_{1/2}$) was significantly delayed in auxilin KO mice (Figure. 4i), suggesting a deficit in DAT function.

To further analyze the difference in dopamine reuptake kinetics, we used a novel computational model of dopamine release derived from previous studies (Venton et al., 2003; Walters et al., 2014) to fit averaged FSCV traces from WT and auxilin KO mice. We found that the wider dopamine peak from auxilin KO mice can be closely fit by a ~60% reduction in DAT activity (parameter V_m) and a ~45% reduction in dopamine release per electrical pulse (parameter $[DA]_p$) compared to WT mice ($V_m = 3.3$ in auxilin KO vs $8.0 \mu\text{M/s}$ in WT, $[DA]_p = 0.333$ in auxilin KO vs $0.6 \mu\text{M/mA}$ in WT), while holding all other major parameters constant (Figure. 4j, Supplementary Figure. 5c). While DAT deficiency seen here was consistent with our FSCV recordings, the decrease in dopamine release in auxilin KOs in the computational model deviated from FSCV observations (Figure. 4h). However, decreased neurotransmitter release is expected in auxilin KOs as these mice have previously been shown to have SV recycling defects (Yim et al., 2010). Our neurochemical analyses show a decrease in the extracellular dopamine metabolite 3-MT (Figure. 4c) in the dorsal striatum of auxilin KO which also indicate dopamine release defects. Thus, it appears that a larger decrease in DAT activity is masking a decrease in dopamine release and can account for the minimal difference in total evoked dopamine release observed between WT and auxilin KO mice in the FSCV recordings.

Auxilin KO mice exhibit DAT deformities in the dorsolateral striatum: To visualize DAT in auxilin KO mice, we performed immunohistochemistry for DAT, co-labeling with the presynaptic SV protein synaptogyrin-3 in dorsal striatum (Figure. 5a). We found large DAT+ve structures ($6-8 \mu\text{m}$) in the dorsal striatum, but not in the ventral striatum of auxilin KO brains (Figure. 5a-c, Supplementary Figure. 6a), like structures that have been described in synaptojanin-1 knock-in mice (Cao et al., 2017). These structures were absent in WT, but ubiquitous in the dorsolateral striatum of auxilin KOs, localizing both with presynaptic sites (Figure. 5a, enlarged, arrowhead) and closer to the soma (as marked by DAPI) (Figure 5a, enlarged, arrows, Supplementary Figure. 6b). These DAT+ve structures were noted in auxilin KOs at both 3 and 9 months of age, though they were significantly higher (~40%) at the earlier time point (Figure. 5b). Synaptogyrin-3, which marks presynaptic termini and a known interactor of DAT, did not exhibit a change in distribution or expression level (Figure. 5a, Supplementary Figure. 7f). Glutamatergic and GABAergic termini,

which were imaged by staining for vesicular glutamate transporters (VGLUT1) and vesicular GABA transporter (VGAT), respectively, did not exhibit such structures (Supplementary Figure 7a-c). As DAT is typically localized to DA axonal projections (Block et al., 2015), these observations suggest that the large DAT+ve structures seen at the dorsal striatum of auxilin KO may be DA axonal membrane deformities.

To confirm that the DAT+ve structures are membrane-bound and surface accessible, we performed *ex vivo* imaging using the membrane DAT ligand dichloropane which binds to cell surface DAT preferentially from the extracellular side. Dichloropane was conjugated with rhodamine red-X, as described previously (Fiala et al., 2020) to obtain the dichloropane–rhodamine red-X probe (Fiala et al., 2020). Fresh striatal slices were incubated in artificial cerebrospinal fluid (ACSF) containing dichloropane probe (100 nM, 45 mins) and imaged for membrane-bound DAT. The number of small dichloropane-DAT+ve punctum that represent DA terminal varicosities in the dorsal striatum were not altered in auxilin KOs at 3 months of age (Figure. 5d, f). However, auxilin KOs revealed large dichloropane-DAT+ve structures (Figure. 5d, e, arrows) similar to those in the dorsal striatum of fixed brains, suggesting that the DAT structures are membrane accessible. Next, we performed an ultrastructure evaluation of dorsal striatum by electron microscopy (EM), which revealed multilayered axonal whirls in auxilin KO brains (Figure. 5g, Supplementary Figure. 8a, arrows). Additionally, there were early autophagic vacuole-like structures close to these axonal deformities (Figure. 5g, arrow heads). We performed immunogold labeling of DAT in the dorsal striatum, which revealed a uniform distribution of DAT-immunogold particles in WT, denoting DA axonal projections and presynaptic sites (Figure. 5h). In contrast, DAT-immunogold clusters were observed in auxilin KOs (Figure. 5h, arrows, Supplementary Figure. 8b, arrows), indicative of axonal membrane deformities of DA projections. Collectively, these observations suggest that DAT is mis-trafficked and trapped in large axonal deformities, which hinder DAT function in dopamine reuptake.

Increased presynaptic autophagy in auxilin KO mice clear CCVs: We evaluated the distribution pattern of endocytic partners of auxilin in the dorsal striatum by immunohistochemistry. Previous confocal imaging has shown that endocytic proteins have a clustered appearance in auxilin KO primary cortical neurons (Yim et al., 2010), mainly reflective of accumulation of CCVs and clathrin cages. We stained for clathrin, and unexpectedly saw that

neither the clathrin intensity nor the distribution pattern was altered in the dorsal striatum of young auxilin KO mice (Figure. 6a, b). Hsc70, the chaperone partner of auxilin, and endophilin-A1, another key endocytic protein required for uncoating also did not change significantly both in expression (Figure. 6a, d) or distribution (Figure. 6a, e) in auxilin KO mice striatum at 3 months. These observations remained true even at 9 months, except for endophilin-A1 which was significantly upregulated in auxilin KOs (Figure. 6a, c). We assessed the interaction of clathrin with Hsc70 and endophilin-A1 by their co-localization and determined it was not altered in auxilin KOs (Figure. 6a, f, g). Overall, there are no major changes in endocytic protein composition and distribution in young auxilin KO mice, in line with our synaptosomal proteomic experiments (Figure. 3e-h).

To confirm our immunohistochemistry findings, we performed EM of the dorsal striatum of 3-month-old WT and auxilin KO mice and quantitated the number of CCVs and SVs per synapse. We quantified these organelles in both asymmetric or Type I synapses which are predominantly glutamatergic (Figure. 6h), and symmetric or Type II synapses which are known to be DA or GABAergic in this brain region (Harris and Weinberg, 2012) (Figure. 6i). The number of CCVs in Type I synapses showed a moderate increase in auxilin KOs (~10%, Supplementary Figure. 9b). Increase in CCV numbers were more pronounced in Type II synapses of auxilin KO mice (~27%, Supplementary Figure. 9c). No notable difference between WT and auxilin KO were found in SVs number in both Type I and II synapses (Supplementary Figure. 9d, e). Cumulative effect of this was seen in CCV/SV ratio, which showed a modest but significant increase only in Type II synapses (Figure. 6k) but not in Type I synapses (Figure. 6j). It is worth noting that the distribution of CCVs and SVs was variable within the Type II synapses of auxilin KOs (Compare Figure. 6i and Supplementary Figure. 9a). Overall, these results are in consistent with our immunohistochemistry which did not show notable difference in clathrin distribution (Figure. 6a,b), and previous findings on cerebellar presynapses of auxilin KO mice (Yim et al., 2010).

The lack of a significant CCV accumulation in dorsal striatum was puzzling. To investigate whether CCVs and clathrin cages were being cleared by autophagy as suggested by several recent papers (Binotti et al., 2015; Yang et al., 2022), we examined the electron micrographs of WT and auxilin KO dorsal striatal presynapses for double membraned synaptic autophagosomes (Figure. 6l). The number of autophagosomes per synaptic site was significantly higher in auxilin KOs

compared to WT (Figure 6l, m). Both Type I and II synapses showed an enhanced number of autophagosomes, though Type II synapses revealed a relatively greater number (Supplementary Figure. 8f, g). Furthermore, we find several examples of autophagosomes, in the Type II synapses, containing CCVs and SVs as their cargo (Figure. 6l, arrowheads). This unexpected finding suggests that CCVs are cleared by presynaptic macroautophagy in auxilin KO mice. These observations also support our IPA analyses of the 9-month synaptosomal proteomics data which suggested activation of macroautophagy-related pathways (Supplementary Figure. 3c).

CCVs proteomics in auxilin KO mice suggest SV sorting defects: To understand the impact of loss of auxilin on SV sorting and composition, we performed EM and proteomic analysis of CCVs purified from brains of WT and auxilin KO mice (age: 3 months)(Blondeau et al., 2004). EM of the CCV preparations revealed that they contain both CCVs (arrows) and clathrin cages (arrowhead) but lack other organelles such as SVs (Figure. 7a) (Vargas et al., 2014). Auxilin KO mice displayed clathrin structures (CCVs + clathrin cages) which were significantly smaller in size compared to WT (Figure. 7a, b). This is in part because there was a larger proportion of clathrin cages in auxilin KOs (Figure. 7a, c), consistent with previously published findings (Yim et al., 2010).

LFQ-MS of CCVs revealed 891 proteins common to three independent experiments, 49 of which were significantly changed, with the majority being downregulated (38 down, 13 upregulated, Supplementary Table 3). Strikingly, all the proteins that exhibit decreased levels were SV transmembrane proteins (Takamori et al., 2006; Taoufiq et al., 2020), such as SNG 1 and 2, SYP, SYT 1 and 12, SV2-A and -B (Figure. 7d, e). VGLUT1 and VGLUT2, vesicular transporters for the excitatory neurotransmitter glutamate were decreased (Figure. 7d, e). Vesicular zinc transporters such as ZNT3 and TM163 were also decreased (Figure. 7d, e). By extension, this suggests that vesicular monoamine transporter-2 (VMAT2) may also be decreased, which was not detected by LFQ-MS, due to its low abundance (Taoufiq et al., 2020). IPA analysis of the CCV proteomics revealed dysregulation in the CME pathway in auxilin KO mice (Figure. 7f).

To rule out the possibility that the downregulation of certain SV transmembrane proteins seen in the auxilin KO CCV proteomics was due to the presence of clathrin cages, we compared our CCVs proteomics data with published SV proteomics data (Takamori et al., 2006). We find that the levels of synapsins, SCAMPs, syntaxins, SNAPs and several others which are categorized as SV

trafficking proteins were unchanged. Endocytic proteins such as dynamins, flotilins, RAB proteins, endophilin-A1, synaptojanin-1, that are peripherally associated with the SV membrane were also unchanged in auxilin KO CCVs compared to WT (data not shown). These observations indicate that the decrease in certain SV transmembrane proteins in auxilin KO mice is likely not an artifact due to the presence of empty clathrin cages. Overall, these results suggest SV sorting defects congruent with recently proposed roles for auxilin in endocytic proof reading (Chen et al., 2019) and indicate that uncoating of CCVs in auxilin KO by GAK or alternative ways would result in SVs with an improper protein stoichiometry.

DISCUSSION:

Recent advancements in PD genetics have led to the identification of mutations in proteins that play crucial roles in SV endocytosis (Gialluisi et al., 2021; Vidyadhara et al., 2019). Mutations in clathrin uncoating proteins auxilin (*PARK19*) and synaptojanin-1 (*PARK20*) were identified to be disease-causing; while, sequence variants in GAK, and endophilin-A1 that aids in the recruitment of synaptojanin-1 to CCVs, increase risk of developing PD. Mutations in receptor-mediated endocytosis 8 (RME-8; *PARK21*), which facilitates the formation of endosome-derived SVs through clathrin uncoating, is also linked to PD (Lopert and Patel, 2014). These genetic findings strongly point to disruptions in clathrin uncoating as important for the pathogenesis of PD.

Auxilin KO mice are the first endocytic mutants to faithfully replicate the cardinal features of PD. The only other murine model with an auxilin loss-of-function mutation (R927G) displayed moderate behavior deficits but was not accompanied by DA neurodegeneration (Roosen et al., 2021). Mice with a R258Q mutation in synaptojanin-1 also displayed motor behavior deficits with no loss of DA neurons or neuroinflammation (Cao et al., 2017). In *Drosophila* loss-of-function models for the GAK homolog, *auxilin*, and RME-8 mutations, PD phenotypes are only apparent with induced α -synuclein overexpression (Song et al., 2017; Yoshida et al., 2018). Thus, endocytic mutants, besides auxilin KOs, capture only a few aspects of PD. Here, we took advantage of the fact that auxilin has a defined function in clathrin uncoating, and auxilin KOs are a robust and faithful model of PD to elucidate the underlying mechanisms. We show that auxilin deficiency leads to neurodegeneration through three distinct, but overlapping mechanisms, in nigrostriatal DA termini: 1) Toxic accumulation of cytoplasmic dopamine due to imbalance in CCV/SV ratio and defective sorting of SVs, 2) Mis-trafficking of DAT that traps the protein in axonal membrane

whirls leading to defective dopamine reuptake, and 3) Synaptic autophagy overload. Collectively, these mechanisms lead to dopamine dys-homeostasis, a trigger of neurodegeneration in PD (Figure. 8).

Accumulation of cytoplasmic dopamine: Dopamine is typically sequestered into SVs via VMAT2 to avoid autooxidation. Dopamine that accumulates in the cytoplasm is oxidized predominantly to DOPAL and subsequently catabolized to DOPAC. Thus, the elevated DOPAC levels observed in the dorsal striatum of presymptomatic auxilin KO mice, is an indirect measure of cytoplasmic dopamine accumulation (Karoum et al., 1994) and increased conversion to DOPAL, a mediator of dopamine-related toxicity in PD (Masato et al., 2019). The accumulation of DOPAC is likely due to two factors- an imbalance in the CCV/SV ratio, and SVs with improper composition. As an outcome of slowed CME (Yim et al., 2010), auxilin KO neurons need to utilize alternative endocytic pathways to maintain SV pools, which are known to be less stringent in protein sorting, leading to SVs of variable protein composition (Wu et al., 2014). Proteomic analysis of CCVs from auxilin KO brains confirmed this tenet and revealed a decrease in copy number of integral SV membrane proteins. Thus, auxilin mutations are likely to lead to fewer functional SVs available for neurotransmitter filling and release. This is supported by our neurochemical analysis which showed a decrease in extracellular dopamine metabolite 3-MT which suggest dopamine release defects (Waldmeier et al., 1981). The FSCV based computational model also predicted dopamine release defects, suggesting defective SV sequestration of dopamine in auxilin KOs. Though our CCV analysis was not sufficiently sensitive to detect VMAT2, there is a possibility of its downregulation in auxilin KO CCVs considering the decrease of two other key vesicular neurotransmitter transporters, VGLUT1 and VGLUT2. Vesicular dopamine uptake is known to be decreased in patients with PD and other synucleinopathies (Goldstein et al., 2011). VMAT2 deficient mice develop PD phenotypes, similar to auxilin KO mice, via cytosolic dopamine accumulation (Caudle et al., 2007). Previous studies have shown that DOPAL-modified α -synuclein oligomers form pores in SVs causing increased DA leakage into the cytoplasm (Plotegher et al., 2017). While SV sorting deficits probably occur in all types of synapses leading to neurotransmitter compartmentalization defects, the properties of dopamine catabolites like DOPAL are likely to render DA synapses vulnerable (Figure. 8a-c).

Dopamine reuptake dysfunction and DAT mislocalization in membrane deformities:

Extracellular dopamine in the synaptic cleft is cleared by reuptake into the presynaptic sites through plasma membrane DATs and/or enzymatic degradation to 3-MT by COMT. The relative contribution of DAT and COMT to clear extracellular dopamine varies by brain region. In dorsal striatum, reuptake by DAT plays a major role in clearing extracellular dopamine, whereas COMT has negligible role (Myöhänen et al., 2010; Yavich et al., 2007). Nigrostriatal DA presynapses depend heavily on DAT-mediated dopamine reuptake to replenish their readily releasable neurotransmitter pool. A significant delay in clearing evoked dopamine from the dorsal striatum *in vivo* and *in silico* along with presence of large DAT+ve deformities both in fixed tissue and *ex vivo* clearly indicate DAT dysfunction in auxilin KO mice. This appears to be a defining feature of DA neurodegenerative phenotypes in auxilin KOs. Dopamine reuptake dysfunction for an extended period may lead to striatal dopamine loss as seen in DAT KOs (Jones et al., 1998), and exacerbate PD pathology in auxilin KO mice.

Live slice imaging of rhodamine-tagged dichloropane which binds to plasma membrane DAT, as well as the DAT-immunogold labelling in the dorsal striatum confirmed DAT-rich axonal membrane deformities in auxilin KOs. Similar DAT positive membrane whirls have been described for synaptobrevin-1 PD mutants (Cao et al., 2017), and may be a common feature of endocytic PD mutants. Other evidence of axonal damage comes from our proteomic findings as well, where neurofilament proteins that maintain axonal integrity were altered, including an increase in NF-L, a marker of neuroaxonal damage. In DA presynapses, DAT localization to the plasma membrane is dynamically regulated by endocytic trafficking and recycling. It remains to be determined whether auxilin and synaptobrevin-1 participate in endocytic recycling of DAT in the DA neurons and will be explored in the future. However, our observations suggest that the dopamine reuptake decrement seen in auxilin KOs occur principally due to DA axonal membrane deformities which trap DAT (Figure. 8d).

Synaptic autophagy overload: Owing to the higher turnover of synaptic proteins, vesicles, and mitochondria in presynaptic sites, autophagosome biogenesis occurs at a higher rate in the distal axons than in the soma (Maday and Holzbaur, 2014). Due to limited lysosomal activity, synaptic termini depend on retrograde microtubule-based axonal transport of autophagosomes towards the lysosome-rich soma for degradation. Tonic activity of DA neurons is likely to keep basal

autophagy rates high, and the requirement to transport autophagosomes long distances via extensive arborization make DA axons vulnerable to additional autophagic burden. Ultrastructural evaluation of dorsal striatum of auxilin KO mice revealed an increase in synaptic autophagosomes, which was more pronounced in Type II synapses. We also observed autophagosomes containing CCVs and SVs in auxilin KO synapses. We find evidence for increased mTOR signaling and activation of autophagic pathways in the synaptosomal proteomic data at symptomatic age, supporting elevated synaptic autophagy in auxilin KO mice. Rapamycin-induced enhancement of autophagy in DA presynapses of mice striatal slices have been shown to sequester SVs and decrease evoked dopamine release (Hernandez et al., 2012). A similar event in auxilin KO synapses might exacerbate cytosolic dopamine accumulation (Figure. 8a). Enhanced synaptic autophagy to clear missorted SVs and CCVs, as well as the products of toxic dopamine-oxidation could overload DA projections with autophagic vacuoles. Autophagosome accumulation, impaired retrograde transport as well as abnormal axonal deformities in DA axons have been previously noted in neurons from patients with PD and Alzheimer's disease (Hill and Colón-Ramos, 2020; Kouroupi et al., 2017; Nixon et al., 2005). EM revealed some of the autophagic vacuoles near the whirl-like axonal deformity in auxilin KO striatum. Though we presently do not understand the relationship between these two structures, DA axonal deformities observed in auxilin KOs may be a result of autophagic overload in DA termini.

In conclusion, our findings indicate that pathology of PD mediated by auxilin deficiency begin with a disruption of CME, which leads to fewer functional SVs for neurotransmitter filling. While these deficits occur at all synapses, it appears to have a particularly detrimental effect at nigrostriatal DA synapses due to the toxicity of cytosolic dopamine, and DAT reuptake alterations. Thus, investigating auxilin loss-of-function has also advanced our understanding of the mechanisms for DA vulnerability of PD.

STAR METHODS:

Mice: Auxilin KO mice have been previously described (Yim et al., 2010) and were bred to C57BL6/J mice to make them congenic. Auxilin homozygous KOs were compared to WT C57BL6/J from Jackson Laboratories, Maine. We have an IACUC approved protocol to maintain these mice.

Motor behavior evaluation: WT and auxilin KO cohorts were examined longitudinally at 3, 6, 9, 12, and 15 months of age (n=12-16 mice/genotype, sex-balanced) in motor behavioral assays. For evaluation of overall locomotory capabilities, mice were allowed to explore an open field arena, which was videotaped to assess the distance travelled in 5 mins using Noldus Ethovision CT software. The number of fecal pellets excreted during open field behavior test was evaluated as a measure of anxiety. The balance beam test was used to assess motor coordination by evaluating the ability to walk straight on a narrow beam from a brightly lit end towards a dark and safe box. Latency to traverse the beam and the number of times a mouse could perform this behavior in a minute were evaluated. The grip strength of the forelimbs and all the limbs was assessed by measuring the maximum force (g) exerted by the mouse in grasping specially designed pull bar assemblies attached to a grip strength meter (Columbus Instruments, Ohio, USA) in tension mode. A four-lane Rotarod was used to assess motor coordination and balance (Columbus Instruments, Ohio, USA). Mice were made to run for 300 secs on the rotating spindle of the Rotarod, which was accelerating from 4 to 40 rpm. Each mouse was subjected to three trials with a 30 min inter-trial recovery period. The average of the latency to fall and the rpm in these trials was used as a measure of motor performance. The procedure was repeated for four consecutive days in both WT and auxilin KO mice. We did not see a significant sex-based differences in all the behavior assays in auxilin KO mice and data from both sexes was collated.

Immunohistochemistry: WT and auxilin KO mice at 3 and 9 months of age (n=5-6/group; sex-balanced) were anaesthetized using isoflurane inhalation and perfused intracardially with 0.9 % heparinized saline followed by chilled 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were post-fixed in the same buffer for 48 hours and cryoprotected in increasing grades of buffered sucrose (15 and 30 %, prepared in 0.1 M PB), at 4 °C, and stored at -80 °C until sectioning. Serial sections of the brains (30 µm thick) were performed coronally using a cryostat (Leica CM1850, Germany), collected on gelatinized slides, and stored at -20 °C. Every sixth nigral section was subjected to immunoperoxidase staining and every 10th striatal section was used for immunofluorescence staining as per our earlier protocol(Vidyadhara et al., 2017). Briefly, for immunoperoxidase staining, endogenous peroxidase quenching was performed using 0.1 % H₂O₂ in 70 % methanol (30 mins), followed by blocking using 3 % bovine serum albumin (BSA) (2 hours) at room temperature (RT). Sections were then incubated at 4 °C with TH primary antibody (1:500, overnight) followed by biotin-conjugated secondary antibody at RT (1:200; 3-4

hours, Vector Laboratories, PK-6101). Tertiary labeling was performed with the avidin-biotin complex solution at RT (1:100; 3-4 hours, Vector Laboratories, PK-6101). Staining was visualized using 3,3'-diaminobenzidine (Fluka, 32750) as a chromogen in a solution of 0.1 M acetate imidazole buffer (pH 7.4) and H₂O₂ (0.1 %). For immunofluorescence staining, sections were incubated in 0.5 % Triton-X-100 (15 mins), followed by incubation in 0.3 M glycine (20 mins). Blocking was performed using 3% goat serum, followed by overnight incubation (4° C) in primary antibodies. Sections were then incubated in Alexa-conjugated secondaries (Thermo Fisher Scientific, USA) for 3-4 hours, followed by coverslip mounting using an antifade mounting medium with (H-1000, Vectashield) or without DAPI (H-1200, Vectashield). Coverslips were sealed using nail polish. 1X PBS with 0.1 % Triton-X-100 was used as both washing and dilution buffer for both immunoperoxidase and immunofluorescence staining, except for pSer129- α -Syn where 1X Tris buffer saline was used. Below is the list of antibodies used and their dilutions.

Antibody	Dilution	Manufacturer	RRID
Rabbit Anti-TH	1:500	Millipore (AB152)	RRID: AB_390204
Mouse Anti-TH	1:500	Synaptic Systems (213211)	RRID: AB_2636901
Rabbit Anti-Iba1	1:300	Wako Chemicals (019-19741)	RRID: AB_839504
Guinea Pig Anti-GFAP	1:400	Synaptic Systems (173004)	RRID: AB_10641162
Guinea Pig Anti-DAT	1:300	Synaptic Systems (284005)	RRID: AB_2620019
Rabbit Anti- α -synuclein (phospho S129)	1:800	Abcam (ab51253)	RRID: AB_869973
Mouse Anti-Clathrin light chain	1:200	Synaptic Systems (113011)	RRID: AB_887706
Rabbit Anti-Synaptogyrin 3	1:200	Synaptic Systems (103303)	RRID: AB_2619753
Rabbit Anti-VGAT	1:500	Synaptic Systems (131002)	RRID: AB_887871
Guinea Pig Anti-VGLUT2	1:500	Synaptic Systems (135418)	RRID: AB_2864786
Rat Anti-Hsc70	1:100	Enzo (ADI-SPA-815-D)	RRID: AB_2039279
Rabbit Anti-Endophilin-A1	1:200	Synaptic Systems (159002)	RRID: AB_887757

Unbiased stereology: The SNpc was delineated on every 6th TH+ve midbrain section(Fu et al., 2012) using a 10X objective of a brightfield microscope equipped with StereoInvestigator

(Software Version 8.1, Micro-brightfield Inc., Colchester, USA). The stereological quantification of TH+ve DA neurons was performed using the optical fractionator probe of the StereoInvestigator(Vidyadhara et al., 2017). The neurons were counted using 40X objective, with a regular grid interval of 22,500 μm^2 (x=150 μm , y=150 μm) and a counting frame size of 3600 μm^2 (x=60 μm , y=60 μm). The mounted thickness was identified to be around 22.5 μm , which was also determined at every fifth counting site. A guard zone of 3.5 μm was implied on either side, thus providing 15 μm of z-dimension to the optical dissector. The quantification began at the first anterior appearance of TH+ve neurons in SNpc to the caudal most part in each hemisphere(Fu et al., 2012) separately, which was later summed to derive total numbers.

Confocal microscopy and image analysis: Fluorescent images were acquired using a laser scanning confocal microscope (LSM 800, Zeiss) with a 20X or 40X objective for quantitation and 63X for representation using an appropriate Z-depth. All the images were blinded for genotype and age before subjecting to analysis using FIJI software from National Institute of Health (NIH). After performing sum intensity projection, the expression intensity was measured on an 8-bit image as the mean gray value on a scale of 0–255, where ‘0’ refers to minimum fluorescence and ‘255’ refers to maximum fluorescence. For counting Iba1+ve microglial cells, images were thresholded using the ‘otsu’ algorithm and the cells larger than 75-pixel units for a given image were counted using the ‘analyze particles’ function. GFAP+ve astroglial cells were counted manually using the ‘cell counter’ function. For counting DAT+ve structures, images were thresholded using ‘triangle’ algorithm, followed by ‘analyse particles’ function. All the structures of size 5 μm and above and the circulating between 0.3 to 1 were counted. SNpc, VTA and SNpr were demarcated as per Fu et al., 2012, colabeling with TH-immunostaining (Fu et al., 2012). Dorsal and ventral striata were demarcated as per Paxinos and Franklin, 2008 (Keith Franklin, 2008).

Proteomic analysis: Whole brain, synaptosomes and CCV samples were prepared from 3-month-old WT and auxilin KO mice. Brains from 3-month-old WT and auxilin KO mice (n=3/genotype) were homogenized in homogenization buffer (detergent-free 320 mM sucrose in 10 mM HEPES, pH 7.4 with protease and phosphatase inhibitors cocktail). Part of the homogenate was snap-frozen for whole brain proteomics. Rest of the homogenate was used to prepare synaptosomes as described previously (De Camilli et al., 1983). Synaptosomes integrity was confirmed by EM

before performing LFQ-MS. For CCVs sample preparation, brains from 14 pairs of WT and auxilin KO mice were pooled to obtain a single CCV fraction (Blondeau et al., 2004) as we have published previously (Vargas et al., 2014). Three independent purifications were performed and the resulting CCVs fractions were subjected to LFQ-MS. The purity of CCVs was confirmed by EM (Figure. 7a).

LFQ-MS was performed at Yale Mass Spectrometry & Proteomics Resource of the W.M. Keck Foundation Biotechnology Resource Laboratory. Samples were analyzed in 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 1.5-fold change and a p-value difference of <0.05 between WT and auxilin KO are considered as significant. Heat maps for significantly changed proteins were produced using Qlucore Omics Explorer. IPA (Qiagen) was used to determine the most significantly affected canonical pathways and their overlap.

High-performance liquid chromatography (HPLC): Sex balanced, auxilin KO mice at 3, 6, 9, 12, and 15 months of age with appropriate controls (n=8-12/genotype) were anesthetized using isoflurane inhalation. Mice were then sacrificed by cervical dislocation, and the brains were quickly removed and dissected for dorsal striatum. These samples were subjected to HPLC at Vanderbilt Neurochemistry Core Laboratory, Vanderbilt University. We did not notice a significant sex-based difference in HPLC results in auxilin KO mice.

Surgery and *in vivo* Fast Scanning Cyclic Voltammetry (FSCV): Surgeries and electrochemical recordings were conducted like our published procedure (Somayaji et al., 2020). Briefly, mice were anesthetized with isoflurane (SomnoSuite Small Animal Anesthesia System, Kent Scientific; induction 2.5%, maintenance 0.8–1.4% in O₂, 0.35 l/min) and head-fixed on a stereotaxic frame (Kopf Instruments, Tujunga, CA). Puralube vet ointment was applied on the eye to prevent cornea from drying out. Stereotactic drill (0.8 mm) was used to preform craniotomy (unilateral, right) to target the midbrain and dorsal striatum with the following coordinates (Keith Franklin, 2008) (values are in mm from Bregma); midbrain: anteroposterior = -2.9, mediolateral=+1.0, dorsoventral=+4; Dorsal Striatum: anteroposterior = +1.2, mediolateral = +1.3, dorsoventral = +3.1. An Ag/AgCl reference electrode via a saline bridge was placed under the skin. For electrical

stimulations, a 22G bipolar stimulating electrode (P1 Technologies, VA, USA) was lowered to target ventral midbrain (between 4-4.5mm). The exact depth was adjusted for maximal dopamine release. For recording the evoked dopamine release, a custom-built carbon fiber electrode (5 μ m diameter, cut to $\sim$ 150 μ m length, Hexcel Corporation, CT, USA) was lowered to reach dorsal striatum. Dil-coated carbon-fiber electrodes were used to identify the electrode position in the dorsal striatum and the electrode track in the brain tissue identified the position of the stimulation electrode (Supplementary Figure. 3c). The evoked dopamine release was measured using constant current (400 μ A), delivered using an Iso-Flex stimulus isolator triggered by a Master-9 pulse generator (AMPI, Jerusalem, Israel). A single burst stimulation consisted of 30 pulses at 50Hz (0.6s). Electrodes were calibrated using known concentration of dopamine in ACSF. Custom-written procedure in IGOR Pro was used for the data acquisition and analysis.

Computational model of dopamine reuptake and release: The model is comprised of a system of ordinary differential equations (ODEs), where equations (1) and (3) form a two-compartment model to simulate the release of dopamine (DA) from synaptic vesicles into the dorsal striatum and the diffusion of DA towards the carbon-fiber electrode through a “dead space” (i.e. an area of damaged tissue around the carbon fiber electrode) (Benoit-Marand et al., 2007):

$$\frac{d[DA]_S}{dt} = [DA]_P I f S + L[DA]_E - \frac{V_m[DA]_S}{[DA]_S + K_m} \quad (1)$$

$$S = \sum_i \theta(t - t_i) \theta\left(t_i + \frac{NP}{f} - t\right) \quad (2)$$

$$\frac{d[DA]_E}{dt} = L([DA]_S - [DA]_E) + \Gamma_{DA} \quad (3)$$

$$\frac{d\Gamma_{DA}}{dt} = k_{ads}[DA]_E - k_{des}\Gamma_{DA} \quad (4)$$

The first compartment $[DA]_S$ sums the concentration of DA released into the striatum, the concentration of DA that returns to the carbon-fiber electrode due to its reflective surface (Schmitz et al., 2001), and the concentration of DA removed through reuptake by DAT. $[DA]_P$ is the concentration of DA release per electrical stimulus pulse, while I and f are the stimulus current and stimulus frequency of the experimental protocol. The electrical pulse trains are modeled using the stimulation pattern S , where θ is the Heaviside theta function, t_i is the start time of the stimulus, and NP is the number of electrical pulses. DAT uptake is modeled using first-order Michaelis-

Menten kinetics (Michaelis et al., 2011; Wightman and Zimmerman, 1990), with V_m and K_m as the maximal velocity and affinity constant of DA. The second compartment $[DA]_E$ computes the difference between the DA that arrives from the striatum and the DA that bounces off the electrode, with a loss factor $L < 1$ used to account for the diffusion through the dead space. Equation (4) is used to model the electrochemical adsorption that occurs with carbon-fiber electrodes (Bath et al., 2000) and is included in the calculation of $[DA]_E$, and k_{ads} and k_{des} are the adsorption and desorption kinetic rate constants.

Ex vivo dichloropane-DAT imaging and quantitation: Dichloropane, a DAT ligand, was conjugated with rhodamine red-X as described by Fiala et al., 2020 (Fiala et al., 2020) to obtain dichloropane–rhodamine red-X probe (dichloropane probe). Mice (n=5/genotype) were sacrificed by cervical dislocation under isoflurane anesthesia and the brains were quickly dissected. Coronal slices (300 μ m) of striatum were cut (VT1200S, Leica) in ice-cold carbogenated solution containing: 100 mM choline chloride, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 7 mM MgCl₂, 0.5 mM CaCl₂, 15 mM glucose, 11.6 mM sodium ascorbate, and 3.1 mM sodium pyruvate. Striatal slices were incubated at 37 °C (30 mins) in carbogenated ACSF containing: 127 mM NaCl, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂ and 15 mM glucose. Slices were then warmed to room temperature in carbogenated ACSF and incubated in dichloropane probe (100 nM) for 45 mins at 37°C. Following this, slices were washed once in carbogenated ACSF and the dorsal striata maintained in ACSF were imaged under 25X water immersion objective at 561 nm excitation using a confocal microscope (LSM 900, Zeiss). Images were analyzed blind to the genotype for the presence of large DAT+ve structures and the number of DAT+ve puncta using FIJI software.

Electron microscopy: 3-month-old mice brains (n=2-3/genotype) were fixed by intracardial perfusion using 2% PFA and 2% glutaraldehyde prepared in 0.1M PB, followed by overnight immersion in 0.1 M cacodylate buffer with 2.5 % of glutaraldehyde and 2 % PFA. For DAT-immunogold labeling (15 nm gold particles), we used 3% PFA prepared in 1X PBS for intracardial perfusion, and 2% PFA and 0.15% glutaraldehyde prepared in 1X PBS for immersion fixation. Dorsal striatum was dissected, further processed at the Yale Center for Cellular and Molecular Imaging, Electron Microscopy Facility. EM imaging was performed using FEI Tecnai G2 Spirit BioTwin Electron Microscope. Images were analyzed blinded to the genotype using FIJI software

for synaptic autophagosomes, both in symmetric and asymmetric synapses. Similarly, synaptic CCVs and SVs per synapse were also counted, along with examining the images for axonal whirls and early autophagic vacuoles.

For EM of purified CCVs and clathrin cages, buffer containing CCVs was pipetted onto a parafilm containing glutaraldehyde and uranyl acetate to make a 18% glutaraldehyde and 73% uranyl acetate solution in 1X PBS. EM grids were floated on top of pipetted droplets and then dried for imaging, using Philips 301 Electron Microscope. Diameter of the CCVs and empty clathrin cages, as well as their numbers were measured using iTEM software (ResAlta Research Technologies, USA).

Statistics: For behavioral studies, two-way repeated measure ANOVA followed by Sidak's multiple comparison test was used. For all other experiments, Student's t-test with Welch's correction was used. Values are expressed as mean $\pm$ standard error of the mean (SEM) and *p* value of 0.05 or less was considered statistically significant. Student's t-test was also used to check if there are sex-based differences in the experimental results within auxilin KO mice, which was not significant.

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AUTHOR CONTRIBUTIONS: VDJ, DLS and SSC conceptualized the study. VDJ performed all behavior and histochemical experiments, imaging, quantitation and proteomic analyses. MS performed *in vivo* FSCV experiments. NW performed analyses of CCV proteomics and EM images. BY performed mice genotyping. HZ prepared CCVs. SN performed computational analysis. JR helped in immunofluorescence image analysis and illustrations. JG prepared *ex vivo* brain slices. TLL performed LFQ-MS. DS and LG provided reagents and founder mice colonies. VDJ and SSC wrote the manuscript. All authors have read and provided inputs to the manuscript.

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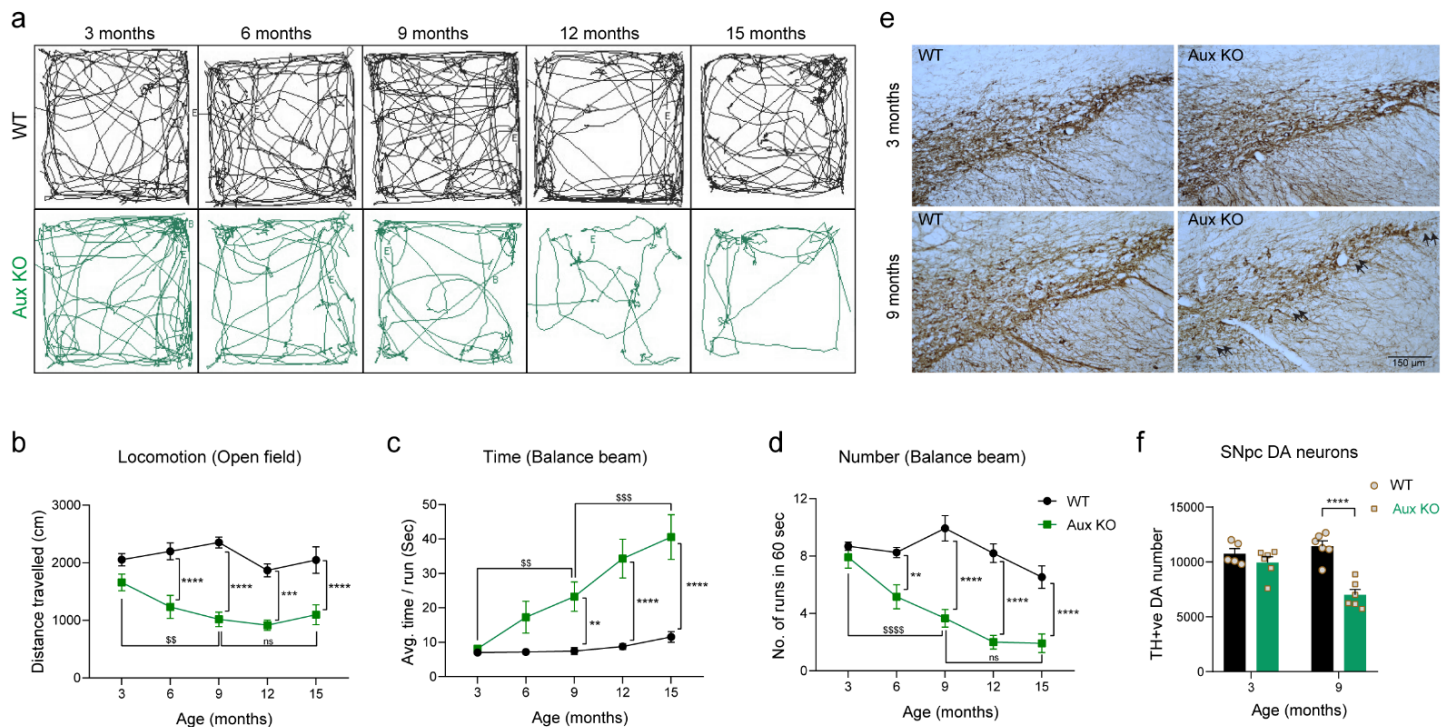


Figure. 1: Auxilin KO mice develop progressive motor behavior deficits that are accompanied by nigral DA neuronal loss. **a.** Longitudinal open field locomotory behavior tracings of WT and auxilin KO mice (Aux KO) from 3 to 15 months of age, performed at 3 months interval. **b.** Distance travelled in open field as a function of age. At 3 months, Aux KOs (n=12) were comparable to WT (n=16). A progressive diminishment in locomotion is seen in Aux KOs with age, which was significant beyond 9 months compared to their performance at 3 months. **c.** Time taken to traverse balance beam. Aux KOs take longer to cross the beam with age, with a significant difference after 9 months of age. **d.** Number of runs performed in a minute on a balance beam. **e.** Representative images showing TH+ve SNpc DA neurons in WT and Aux KO midbrain sections at 3 and 9 months of age. Fewer DA neurons (arrows) were present in the SNpc of Aux KO mice at 9 months. Scale bar: 150 μ m. **f.** Unbiased stereological counting of SNpc DA neurons. Note a significant (~40%) loss of DA neurons is seen in 9-month-old Aux KO mice (n=5-6/genotype). Statistics: For behavior, two-way repeated measure ANOVA followed by Sidak's multiple comparison test was used. For stereology, Student's t-test with Welch's correction was used. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, \$\$ $p < 0.01$, \$\$\$\$ $p < 0.0001$, \$\$ $p < 0.01$, \$\$\$\$ $p < 0.0001$, ns = not significant.

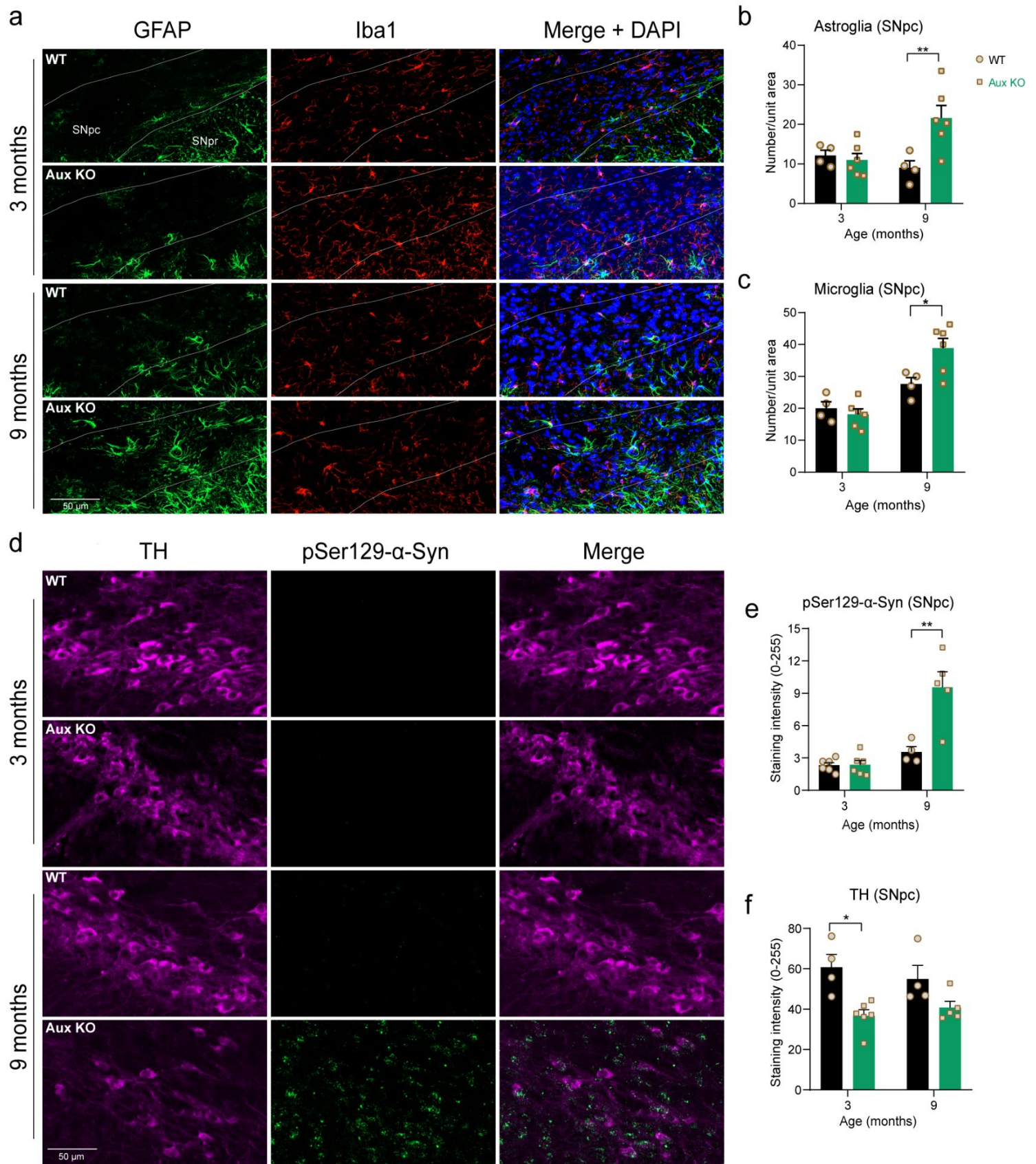


Figure. 2: Aged auxilin KO mice exhibit gliosis and α -synuclein pathology. **a.** Representative images of SNpc and SN pars reticulata (SNpr) of WT and Aux KO mice at 3 and 9 months (n=5-6/genotype) immunostained for the astroglial marker GFAP (green) and microglial marker Iba1 (red). Dashed line demarcates SNpc from SNpr. Scale bar: 50 μ m. **b.** Quantitation of GFAP+ve cells, show a significant astrogliosis at 9 months, but not at 3 months of age in the SNpc. **c.** Quantitation of Iba1+ve cells, show microgliosis in the SNpc of Aux KOs at 9 months. **d.** Representative images of the SNpc immunostained for pSer129- α -synuclein (green), a marker of α -synuclein pathology, co-stained with DA marker TH (magenta). Scale bar: 50 μ m. **e.** Quantitation of pSer129- α -synuclein +ve punctate aggregates in SNpc, which showed an increase at 9-month-old Aux KOs, but not at 3 months. **f.** Quantitation of TH staining intensity in SNpc, which showed a moderate decrease at 3 months in auxilin KO mice but not at 9 months, suggesting retention of TH phenotype in the surviving neurons. Statistics: Student's t-test with Welch's correction. * $p < 0.05$, ** $p < 0.01$.

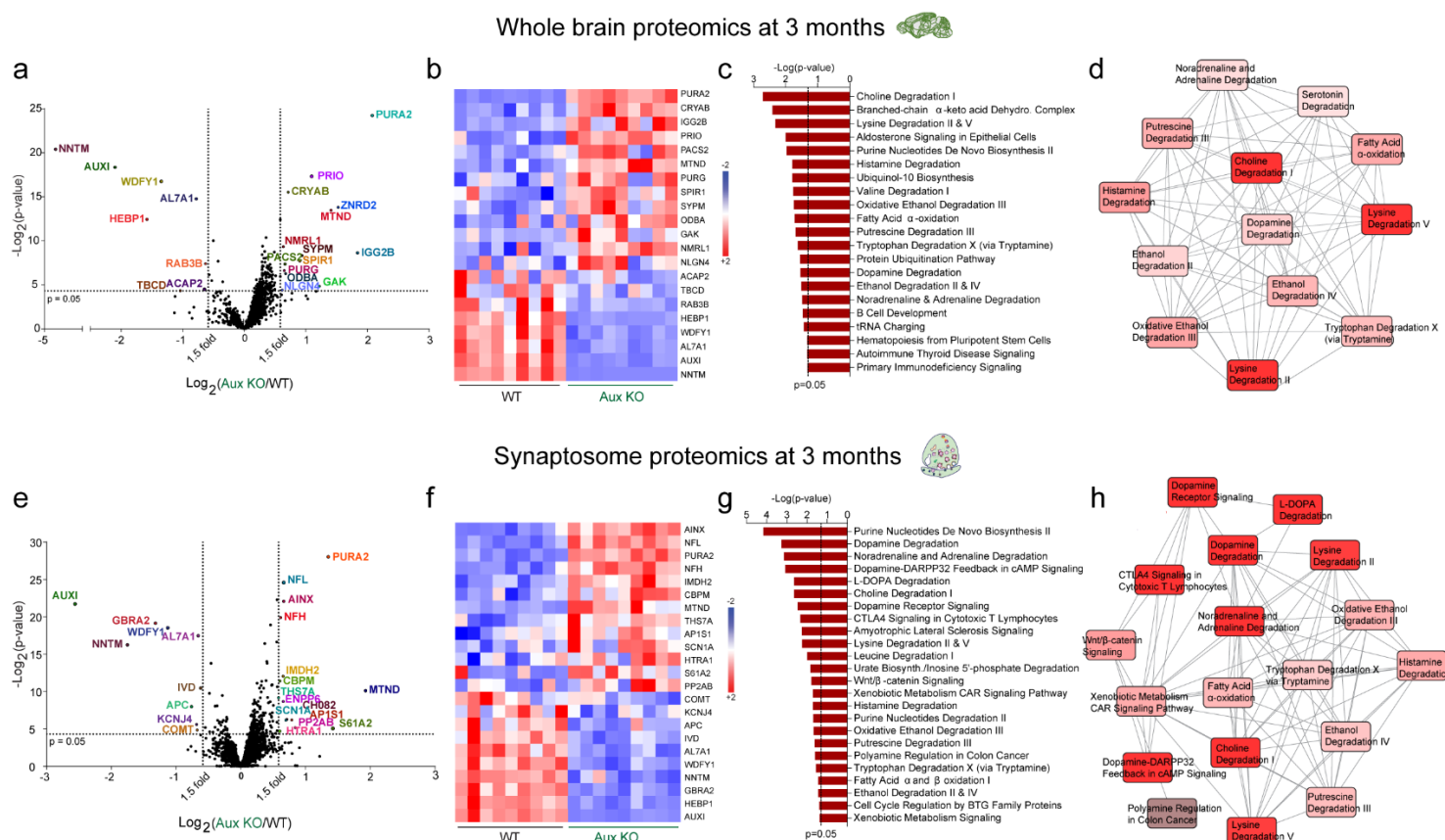


Figure 3: Whole brain and synaptosome proteomics reveal dopamine degradation dysfunction in young auxilin KOs. **a.** Volcano plot of whole brain proteome of Aux KOs compared to WT (age=3 months, n=3 mice/genotype). Proteins that exhibit a 1.5-fold change (vertical dotted lines) and a p-value of 0.05 (Student's t-test) or lesser (horizontal dotted line) were considered as significantly changed. Among the 22 proteins that significantly changed, 8 were decreased (left quadrant) and 14 were increased (right quadrant). **b.** Heat map of significantly changed proteins in whole brain homogenates of WT and Aux KOs for all 9 technical replicates (3 technical replicates/mouse). Red indicates an increase (+2) and blue indicates decreased levels (-2). **c.** Pathways that are significantly ($p < 0.05$) affected in whole brain of Aux KOs as determined by IPA. **d.** Diagram showing the overlap of significantly affected pathways, where intense red depicts most affected and light red depicts moderately affected pathways. **e.** Volcano plot of synaptosome proteome of Aux KOs compared to WT (age=3 months, n=3 mice/genotype). Out of 24 proteins that were significantly changed (1.5-fold, $p < 0.05$, Student's t-test), 9 were decreased (left quadrant) and 15 were increased (right quadrant). **f.** Heat map of significantly changed proteins in synaptosome preparations from Aux KOs in comparison to WT for each technical replicate. Red indicates an increase (+2) and blue indicates decreased levels (-2). **g.** Significantly affected pathways due to synaptosome proteomic changes as determined by IPA. **h.** Overlap of significantly affected pathways showing highly affected (intense red) and moderately affected (light red) pathways.

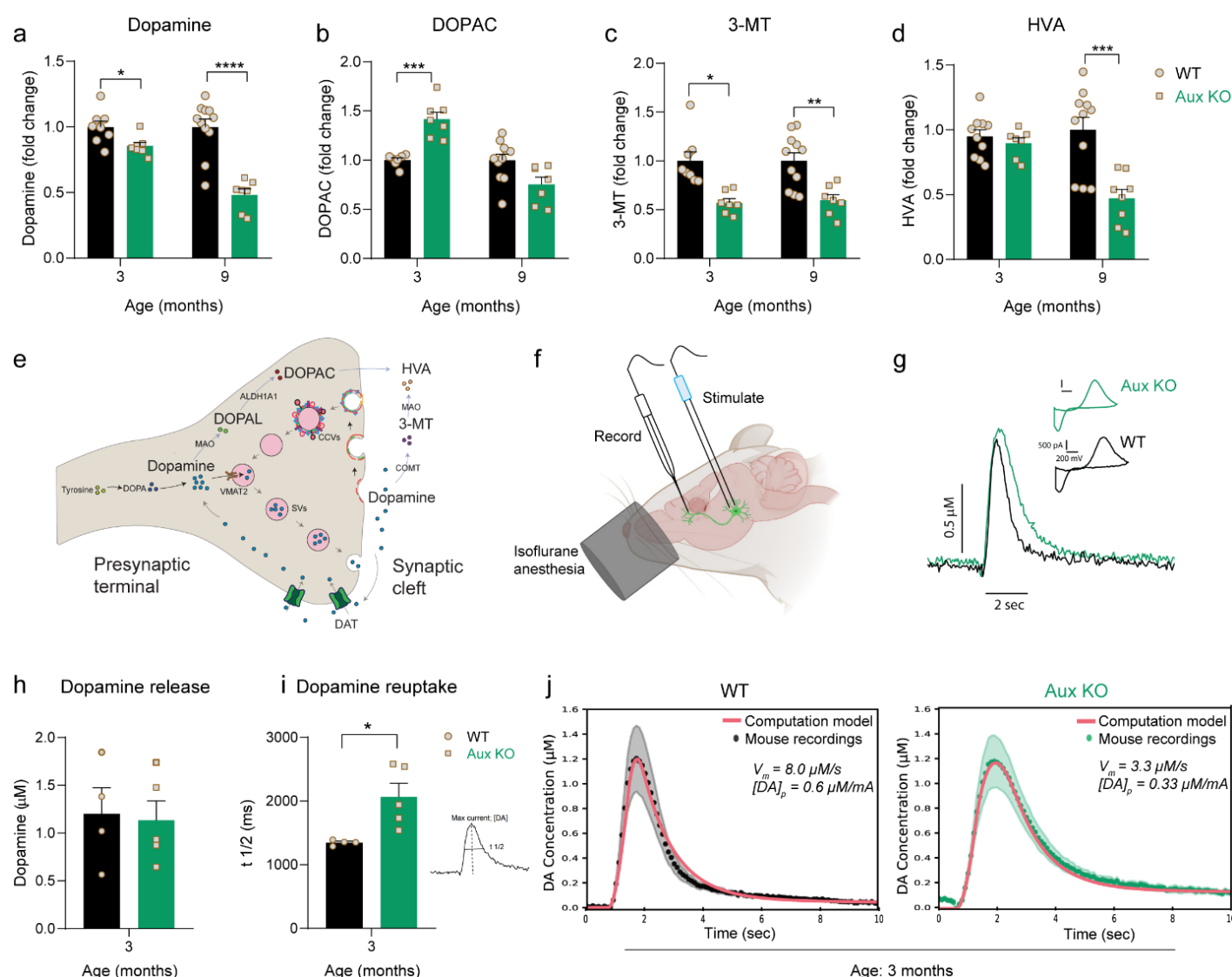


Figure 4: Dopamine catabolism and dopamine reuptake deficits in young auxilin KO mice.

a. Dopamine levels in the dorsal striatum of WT and Aux KO mice. Note a modest decrease in dopamine at 3 months, but a larger decrease at 9 months of age (n=7-11 mice/genotype). **b.** DOPAC levels in the dorsal striatum. At 3 months, a significant increase in the intracellular dopamine catabolite DOPAC is seen in Aux KOs, whereas no change is observed at 9 months. **c.** 3-MT levels in the dorsal striatum. An extra-synaptic dopamine catabolite 3-MT was decrease both at 3 and 9 months of age in Aux KOs. **d.** HVA levels in the dorsal striatum. HVA, another extra-synaptic dopamine catabolite did not change at 3 months but decreased significantly at 9 months in Aux KO mice. **e.** Schematic showing compartmentalization of dopamine and its catabolites in intra- and extra-synaptic space. **f.** Schematic showing the location of FSCV recording electrode in the dorsal striatum and the bipolar stimulating electrode in the ventral midbrain of mice under isoflurane anesthesia. **g.** Example trace of evoked dopamine release following stimulation of midbrain DA neurons by 30 pulses at a constant 50-Hz frequency in 3-month-old Aux KO and WT mice (scale: y axis, 0.5 μ M dopamine; x axis, 2 sec). **h.** Dopamine release in the dorsal striatum. No significant change in dopamine release was seen in Aux KO mice when compared to WT (n=4-5/genotype). **i.** Dopamine reuptake in the dorsal striatum. Reuptake kinetics measured

by time taken to clear half the dopamine from its peak levels ($t_{1/2}$) was significantly delayed in Aux KO mice. Statistics: Student's t-test with Welch's correction. **j.** Best-fits of computational model of dopamine (DA) release (red lines) to averaged FSCV recordings in the dorsal striatum of WT (Black dots; $R^2 = 0.98$, $n = 4$) and Aux KO (Green dots; $R^2 = 0.99$, $n = 5$) mice. Black/green ribbons report SEM. Dopamine release from Aux KO mice is closely fit by a ~60% reduction in DAT activity (parameter V_m) and a ~45% reduction in dopamine release per electrical pulse (parameter $[DA]_P$). $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$.

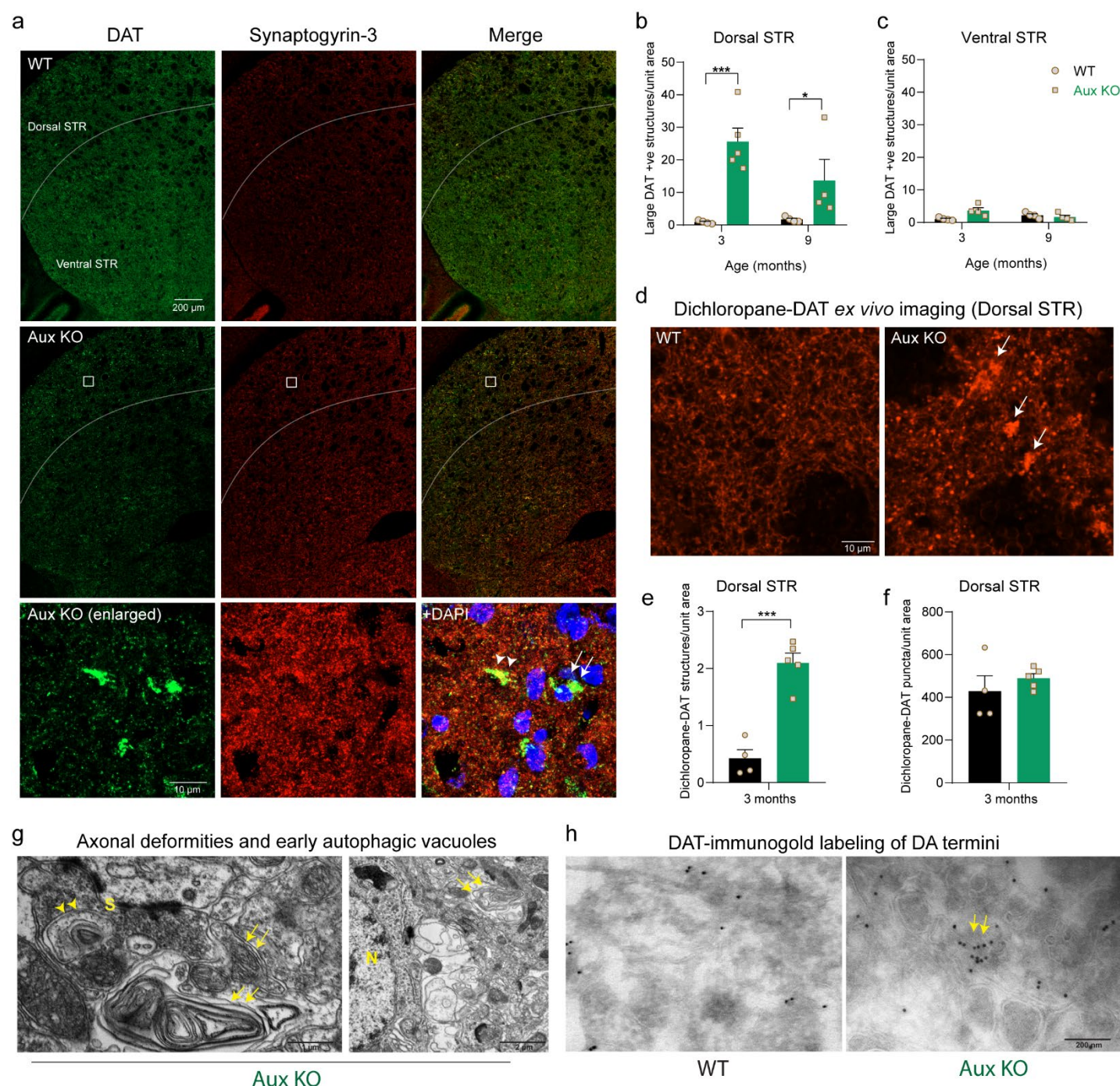


Figure 5: DAT+ve axonal deformities in the dorsal striatum of auxilin KO. **a.** Representative images of dorsal and the ventral striatum (STR) in WT and Aux KO mice, immunostained for DAT and synaptogyrin-3. Note large DAT+ve structures in the dorsal STR of Aux KOs, that are absent in the ventral STR. Scale bar: 200 μ m. These DAT+ve structures were juxtaposed to presynaptic sites as seen by colocalization with synaptogyrin-3 (Aux KO, enlarged, arrowhead), as well as in the soma marked by DAPI staining (Aux KO, enlarged, arrow). Scale bar: 10 μ m. **b.** Number of DAT+ve structures/unit area in the dorsal striatum. Aux KO showed a significantly higher DAT+ve structures both at 3 and 9 months. **c.** Number of DAT+ve structures in the ventral

striatum. DAT+ve structures were not observed in ventral striatum in Aux KOs. **d.** Representative images of *ex vivo* staining of dichloropane–rhodamine red-X in the dorsal striatum of WT and Aux KO mice. DA axonal projections and presynaptic sites appear as small puncta, whereas axonal deformities appear as large dichloropane-DAT+ve structures (arrows). Scale bar: 10 μ m **e.** Number of large dichloropane-bound DAT+ve structures/unit area in the dorsal striatum, were significantly higher in Aux KOs. **f.** Number of small dichloropane-bound DAT+ve puncta, was not altered in Aux KO dorsal striatum in comparison to WT. **g.** EM of dorsal striatum of Aux KO mice showing large axonal whirl like deformities (arrows), which were present ubiquitously, closer to both synaptic terminals (S) and soma (N: nuclei). Early autophagic vacuole like structures were also seen in dorsal striatum (arrow heads), closer to axonal whirls. Scale bar: 1 μ m and 2 μ m. **h.** DAT-immunogold labeling of dorsal striatum that mark only DA axonal termini showed dispersed labeling in WT. In Aux KOs, DAT-immunogold clusters were seen in the dorsal striatum (arrows). Scale bar: 200 nm. Statistics: Student’s t-test with Welch’s correction. *** $p < 0.001$.

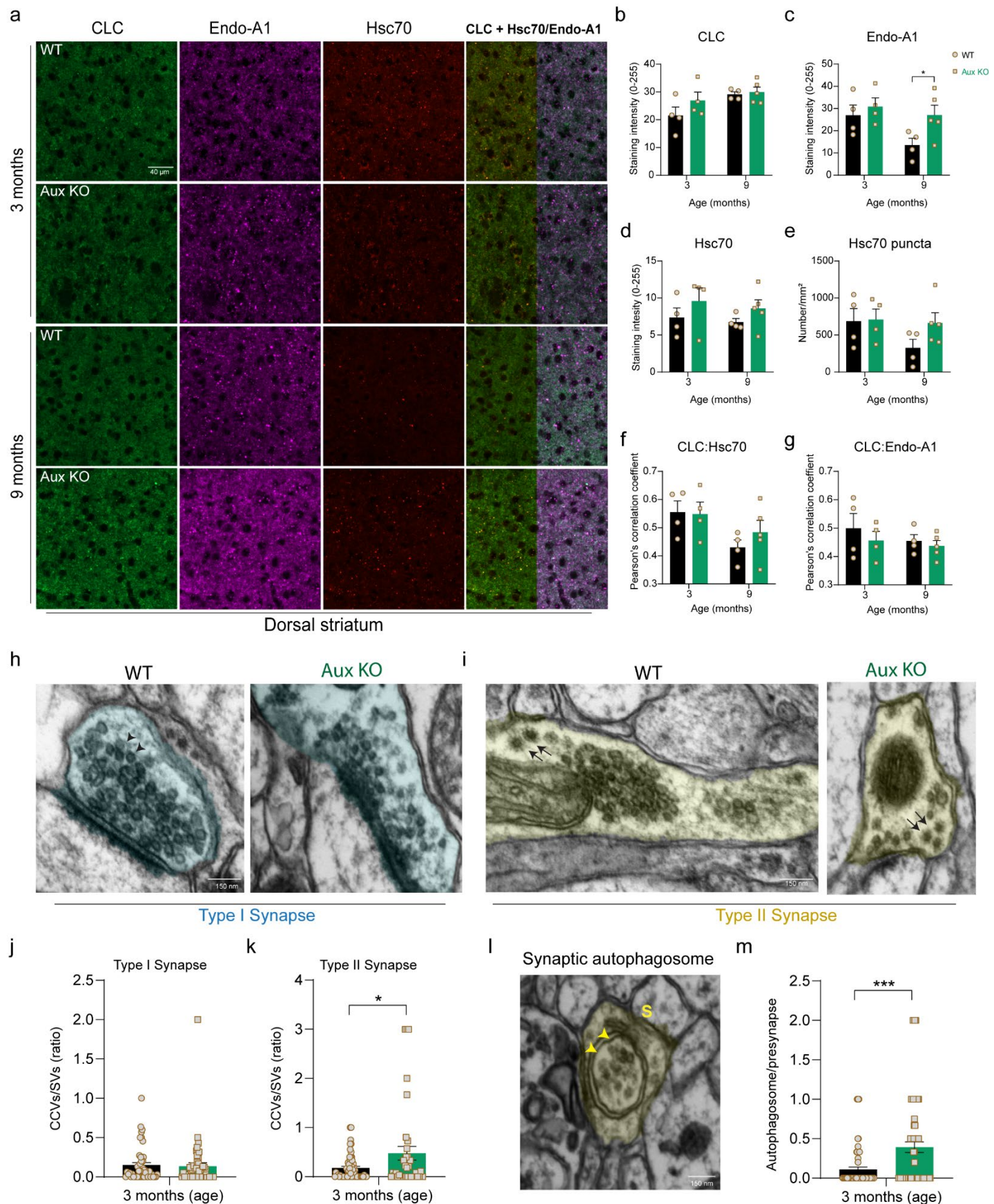


Figure. 6: CCVs are cleared by synaptic autophagy in Auxilin KOs. **a.** Representative images of dorsal striatum immunostained for clathrin light chain (CLC), endophilin-A1 (Endo-A1) and Hsc70, in WT and Aux KO mice at 3 and 9 months of age. Scale bar: 40 μ m. **b.** CLC expression the dorsal striatum, was not altered in Aux KOs. **c.** Endo-A1 expression in the dorsal striatum, was not changed at 3 months but was increased at 9 months in Aux KOs. **d.** Hsc70 expression in the dorsal striatum, which was also not altered. **e.** Number of Hsc70+ve puncta in the dorsal striatum is unaltered. **f.** Colocalization of Hsc70 with CLC in WT and Aux KOs. **g.** Endo-A1 colocalization with CLC. **h.** Representative EM image of a Type I excitatory presynapse with SVs (arrow heads) from dorsal striatum of WT and Aux KO. Scale bar: 150 μ m. **i.** Representative EM image of a Type II inhibitory presynapse in the dorsal striatum of WT and Aux KO mice with SVs and CCVs (arrows). Note Aux KO presynapse showing a decrease accumulation of CCVs, accompanied by a decrease in SVs. Scale bar: 150 μ m. **j.** The CCV to SV ratio in Type I synapses of dorsal striatum (Age: 3 months). This ratio was not altered in Aux KOs. **k.** The CCV to SV ratio in Type II synapses of dorsal striatum, which was significantly increased in Aux KOs in comparison to WT. **l.** EM image of a Type I synaptic terminal (S) in the dorsal striatum of Aux KOs showing double membraned autophagosomes containing CCVs (arrows). **m.** Number of autophagosomes per presynaptic terminal in the dorsal striatum, which was significantly increased in Aux KOs compared to WT (Age: 3 months). Statistics: Student's t-test with Welch's correction. $*p<0.05$, $***p<0.001$.

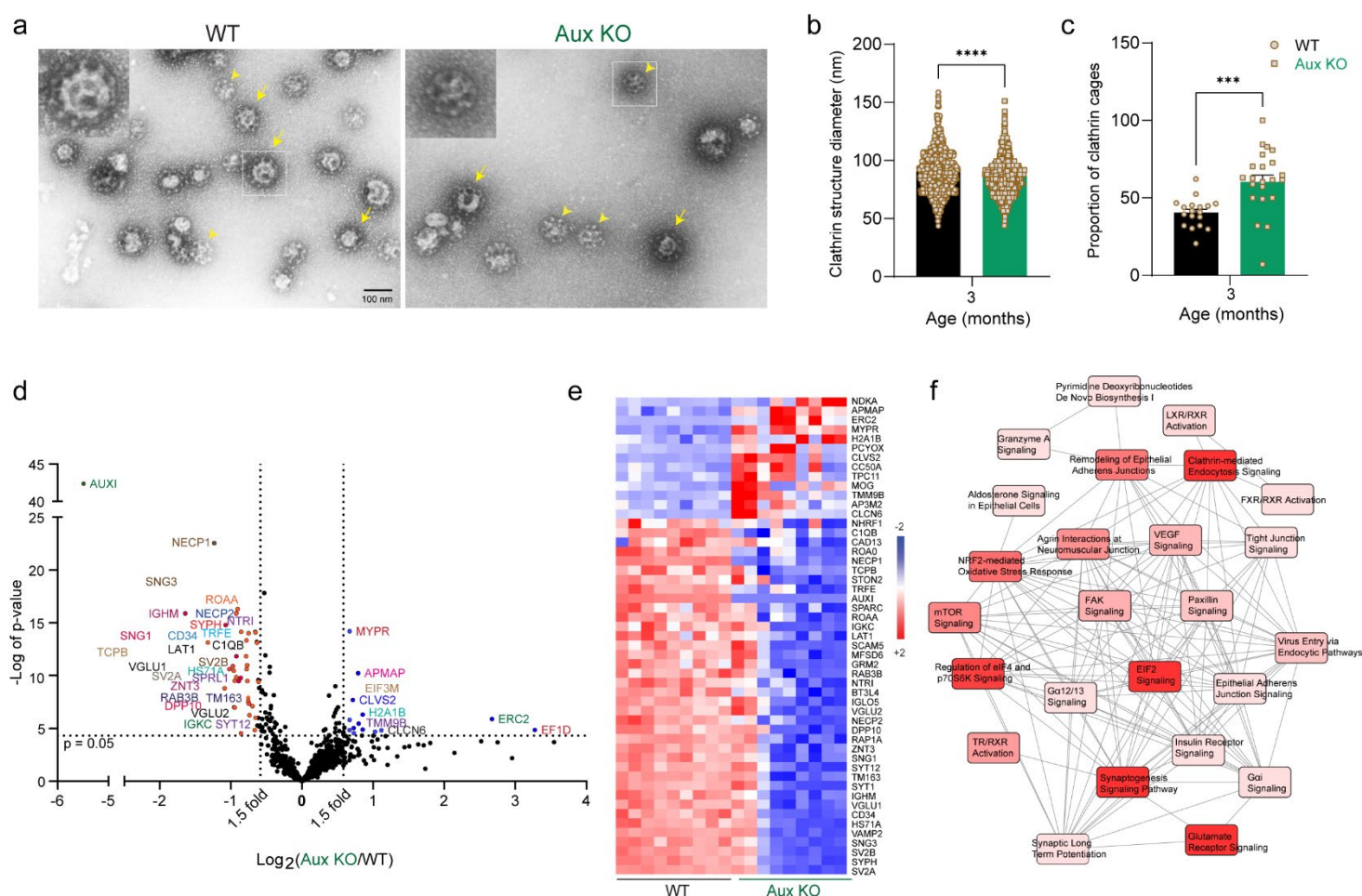


Figure 7: EM and proteomics of CCVs reveals SVs with variable membrane composition in auxilin KOs. **a.** Representative EM images of CCV preparation showing CCVs (arrows) and empty clathrin cages (arrow heads) in WT and Aux KO mice. Scale bar: 100 nm. **b.** Diameter of clathrin structures (CCVs + clathrin cages), which was significantly smaller in Aux KO samples compared to WT. **c.** Proportion of clathrin in WT and Aux KO mice. Note, Aux KOs show significantly larger proportion of clathrin cages. **d.** Volcano plot of CCV proteome of Aux KOs in comparison to WT (n=14 mice/experiment, N=3 experiments/genotype). Proteins that were changed 1.5-fold (vertical dotted lines) with a p-value of 0.05 (Student's t-test) or lower (horizontal dotted line) were considered as significantly changed. Among the 49 proteins that were significantly changed, 37 were decreased and 12 were increased. **e.** Heat map of significantly changed proteins in Aux KOs in comparison to WT for each experiment (3 technical replicates per experiment). Red indicates increased expression (+2), and blue indicates a decrease (-2). **f.** Pathways that are significantly affected ($p < 0.05$) in Aux KO mice due to CCV proteome changes, and their overlap. Pathways depicted in intense red are highly affected, whereas the light red are moderately affected. Statistics: Student's t-test with Welch's correction. *** $p < 0.001$, **** $p < 0.0001$.

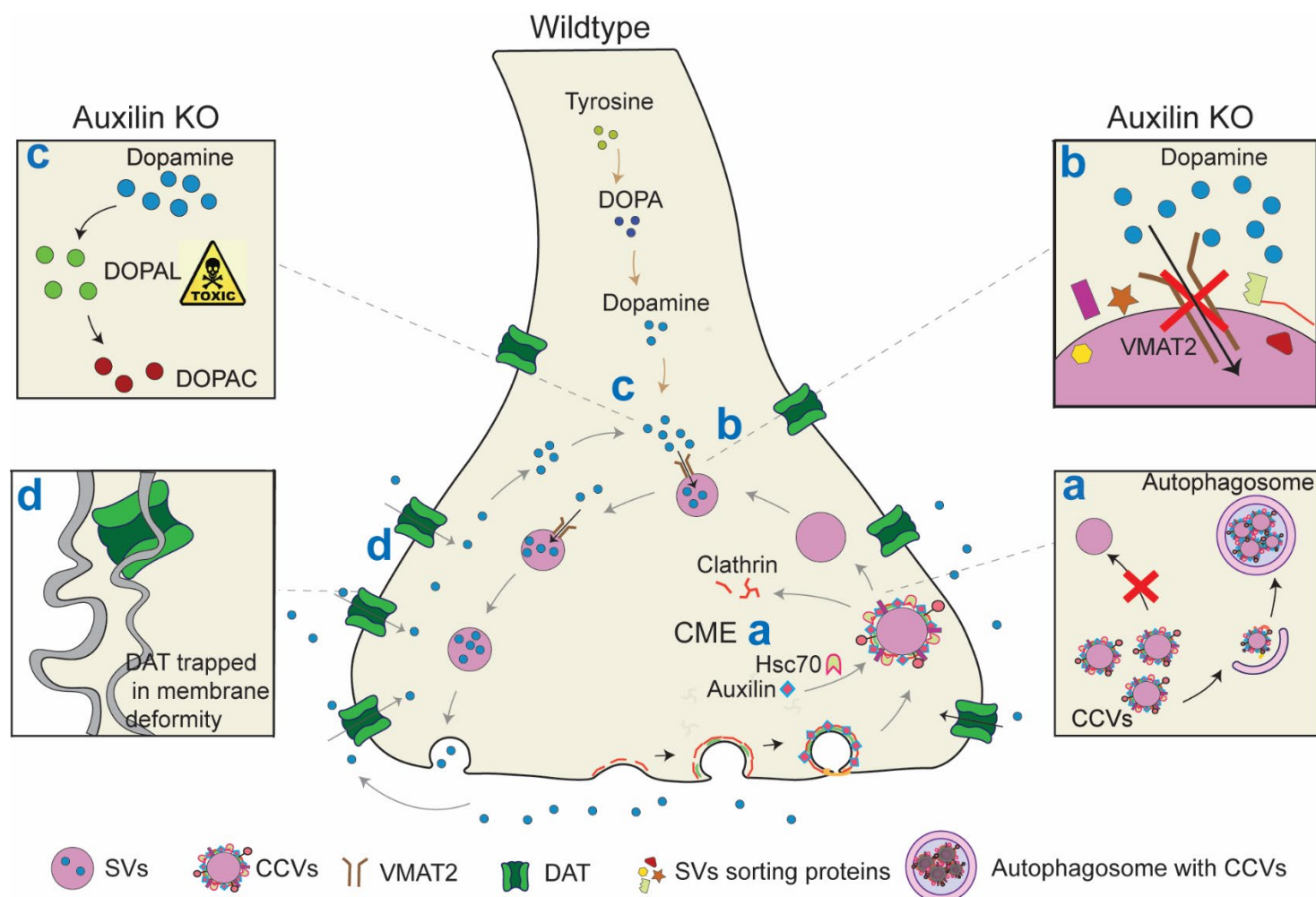
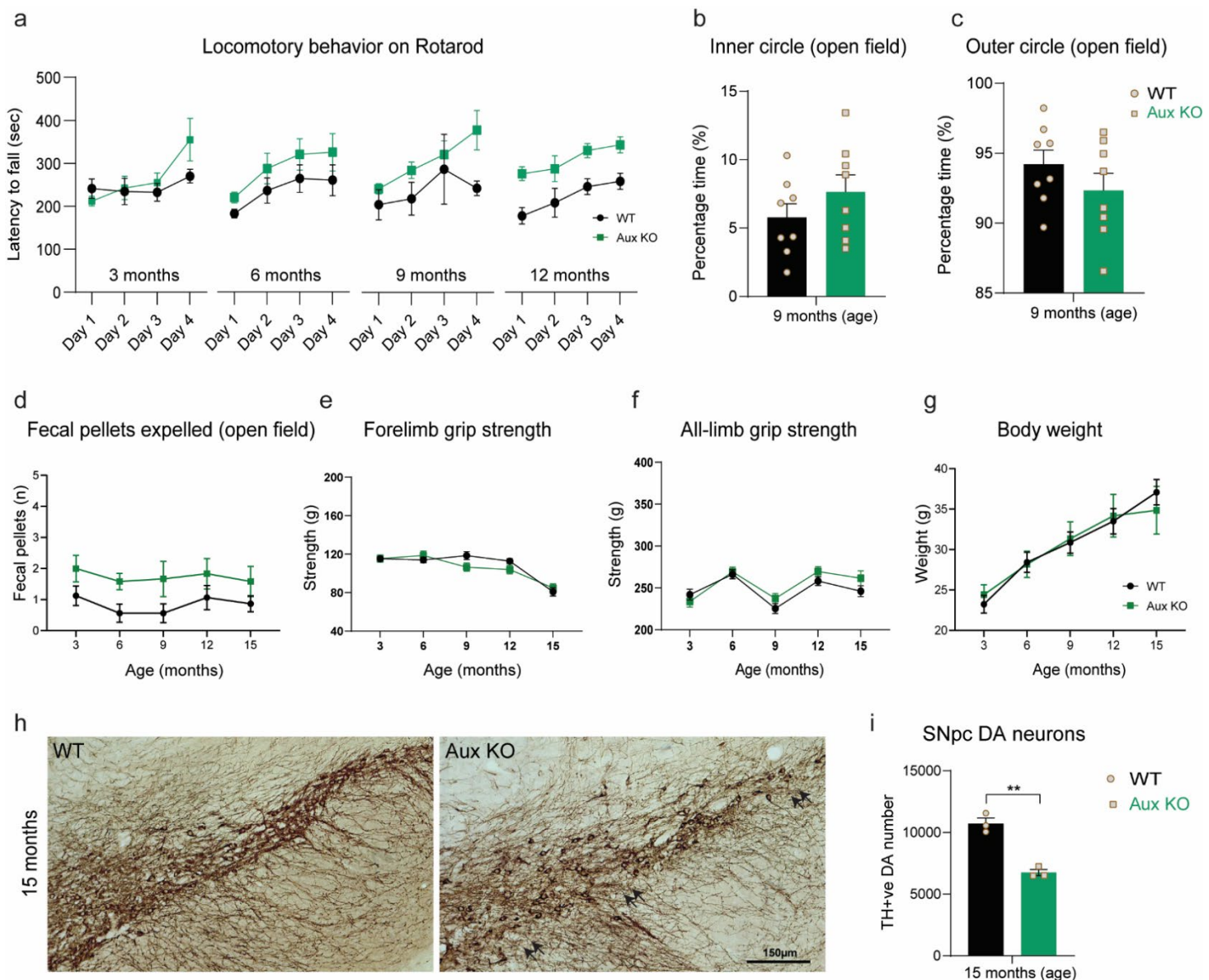
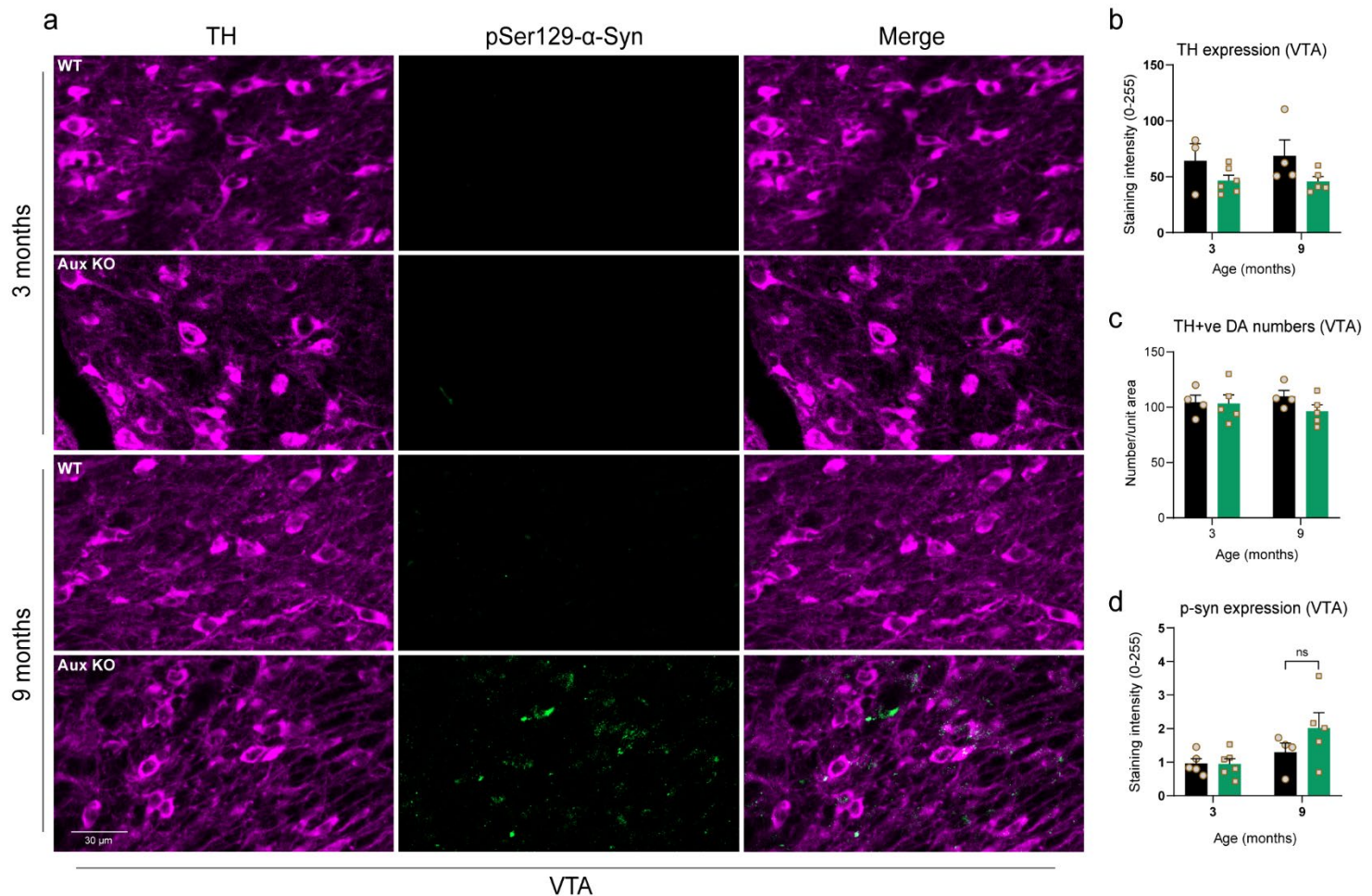


Figure 8: Schematic showing dopamine compartmentalization defects in a DA presynapse of auxilin KO mice. **a.** In a WT DA presynapse, after release of dopamine, SVs are recycled principally through CME. Clathrin forms a coat on the nascent SV membrane to form CCVs with the aid of adaptors. Auxilin recruits Hsc70 to CCVs and functions in its uncoating to generate SVs. In Aux KO mice, CCV uncoating is affected, leading to their accumulation and their subsequent clearance by an enhanced synaptic autophagy. Consequently, there is an imbalance in CCV to SV ratio. **b.** In WT DA synapses, dopamine once synthesized is immediately sequestered into the SVs by the vesicular transporter VMAT2. Aux KO mice have SVs with varied protein stoichiometry and a decrease in the copy number of vesicular transporters, hindering dopamine sequestration to SVs. **c.** In WT, there is minimal cytosolic dopamine present. In Aux KO, due to aforementioned events, there is an elevation in cytosolic dopamine, which is oxidized to its toxic intermediates such as DOPAL and DOPAC. **d.** Normally, DAT plays a pivotal role in dopamine reuptake and replenishing presynaptic vesicular dopamine for future release. In Aux KOs, DAT is misrouted and trapped in the axonal membrane whirls/deformities in the DA projections, compromising dopamine reuptake.

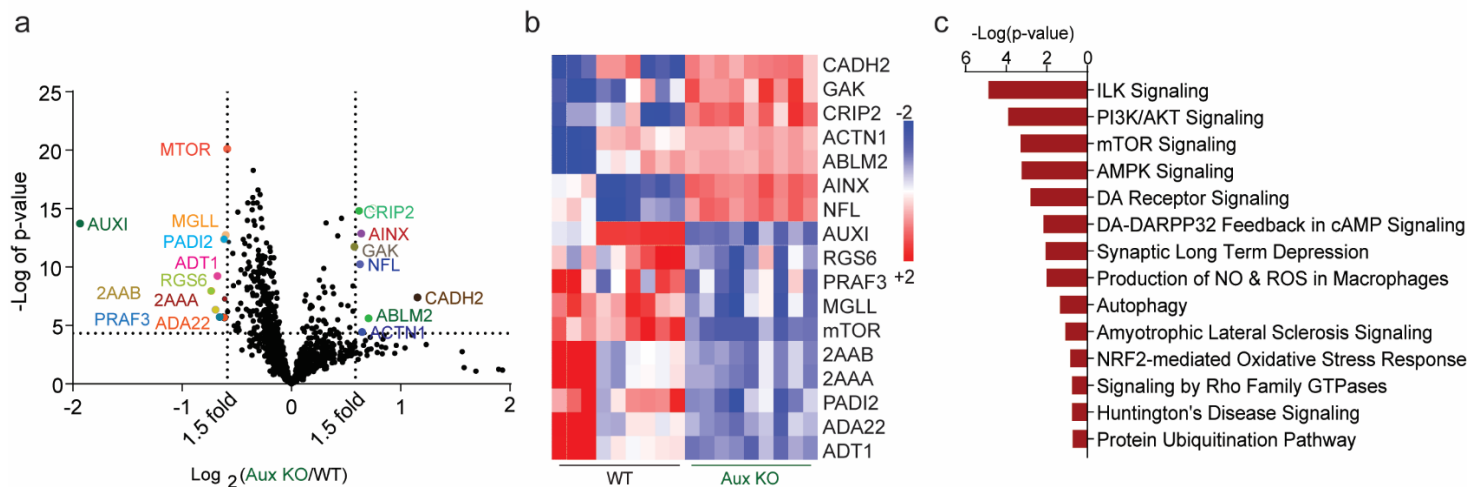


Supplementary Figure. 1: Auxilin KO mice show selective behavioral deficits **a.** Motor coordination on Rotarod measured as latency to fall. Aux KOs did not show significant alteration in this behavioral test when compared to WT. **b.** Percentage time spent in inner circle of the open field. This is a measure of anxiety and was not different from WT at 9 months in Aux KOs, even though motor deficits were apparent. **c.** Percentage time spent in outer circle of the open field. **d.** Number of fecal pellets expelled during open field behavior, which did not change significantly between WT and Aux KOs across age. **e.** Forelimb grip strength measured as a function of age in WT and Aux KOs. **f.** All-limb grip strength was also not affected. **g.** Body weight of WT and Aux KOs as a function of age. **h.** Representative images of TH+ve DA neurons in SNpc of WT and Aux KO mice at 15 months of age. Note a loss of DA neurons in the SNpc of Aux KO mice (arrows). Scale bar: 150 μ m. **i.** Stereological counting of SNpc DA neurons, which revealed a significant loss of DA neurons in Aux KO mice at 15 months of age. Statistics: For age-related behavior, two-way repeated measure ANOVA followed by Sidak's multiple comparison test was used. For others, Student's t-test with Welch's correction was used. ****** $p < 0.01$.

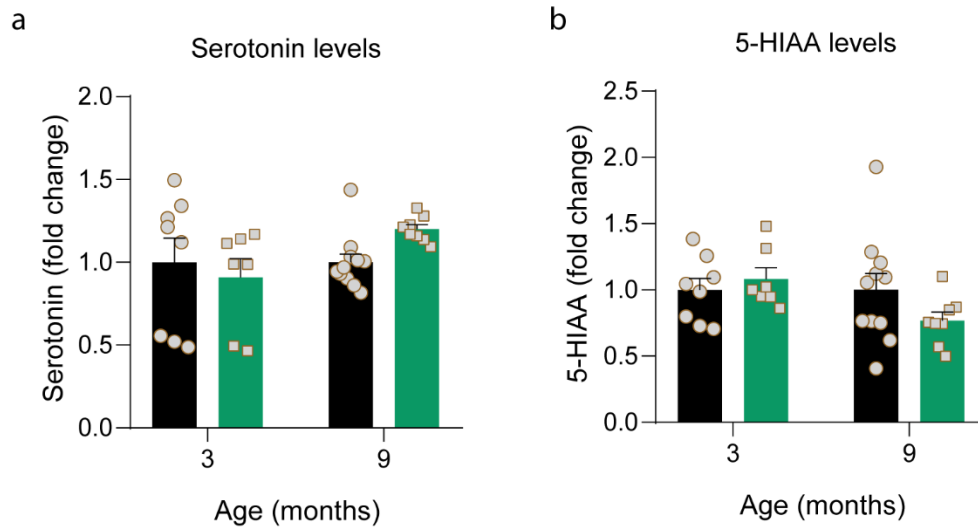


Supplementary Figure. 2: VTA was relatively preserved in auxilin KOs. **a.** Representative images VTA immunostained for α -synuclein aggregation marker pSer129- α -synuclein (green) co-labelled with DA marker TH, at 3 and 9 months in WT and Aux KOs. Scale bar: 30 μ m. **b.** TH expression in VTA, which did not change with age in Aux KOs. **c.** Number of TH+ve DA neurons in VTA, which was not altered in Aux KO mice. **d.** p-Ser 129- α -synuclein expression in VTA, which showed a trend of higher expression at 9 months but did not reach significance in Aux KOs. Statistics: Student's t-test with Welch's correction.

Synaptosome proteomics at 9 months

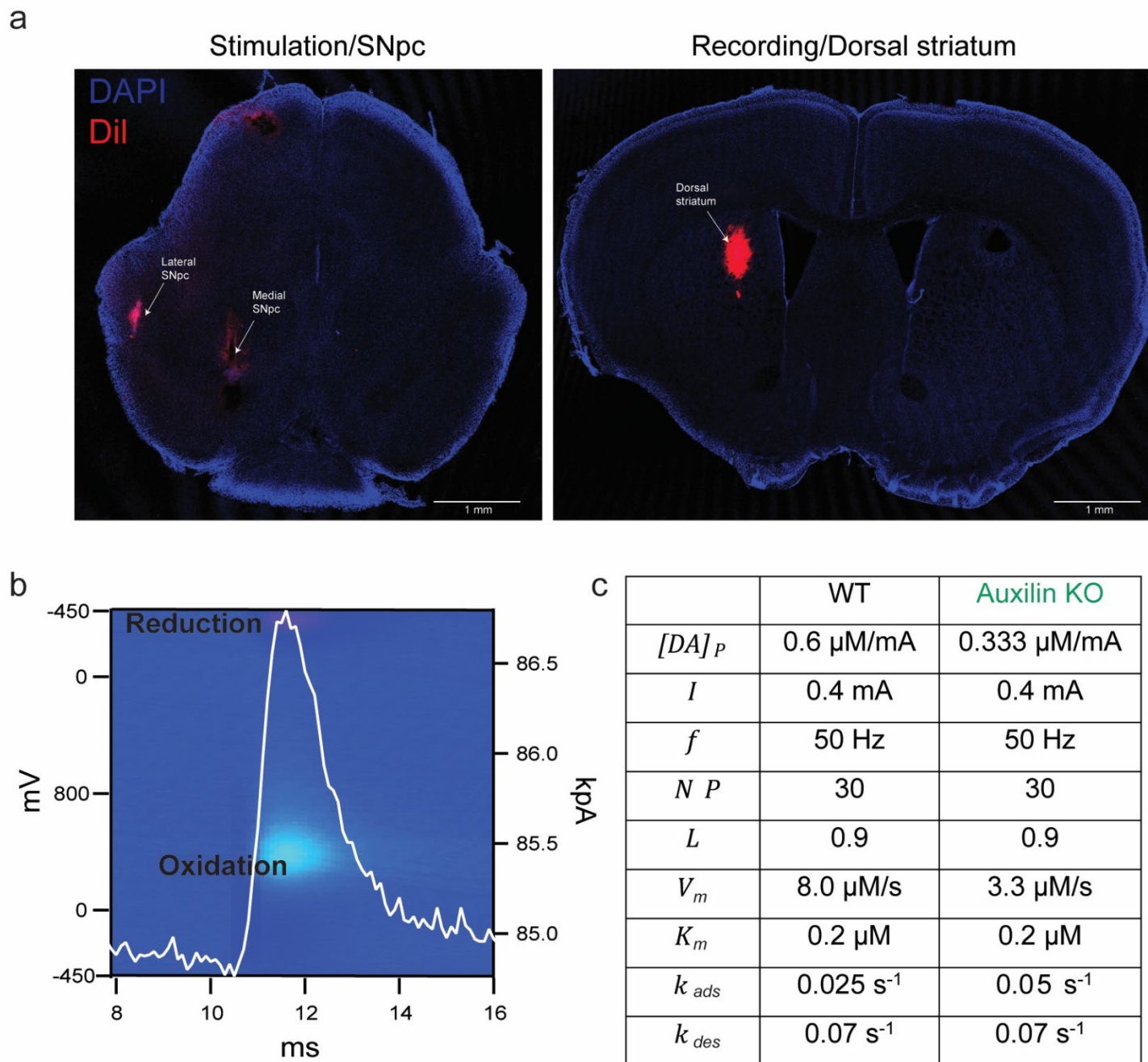


Supplementary Figure. 3: Synaptosome proteomics at 9 months revealed several PD-linked proteins and pathways to be altered. **a.** Volcano plot of synaptosome proteome of 9-month-old Aux KOs compared to WT (n=3 mice/genotype). Proteins that were altered greater than 1.5-fold (vertical dotted lines) with a p-value of 0.05 (Student's t-test) or lesser (horizontal dotted line) were considered as significantly changed. Among 17 proteins that significantly changed, 10 were decreased (left) and 7 were increased (right). **b.** Heat map of significantly changed proteins in synaptosomes of Aux KOs in comparison to WT depicted for each technical replicate (3 technical replicates/mouse). Red indicates an increase (+2) and blue indicates decreased levels (-2). **c.** Pathways that are significantly ($p < 0.05$) affected in whole brain synaptosomes of Aux KOs as determined by IPA.

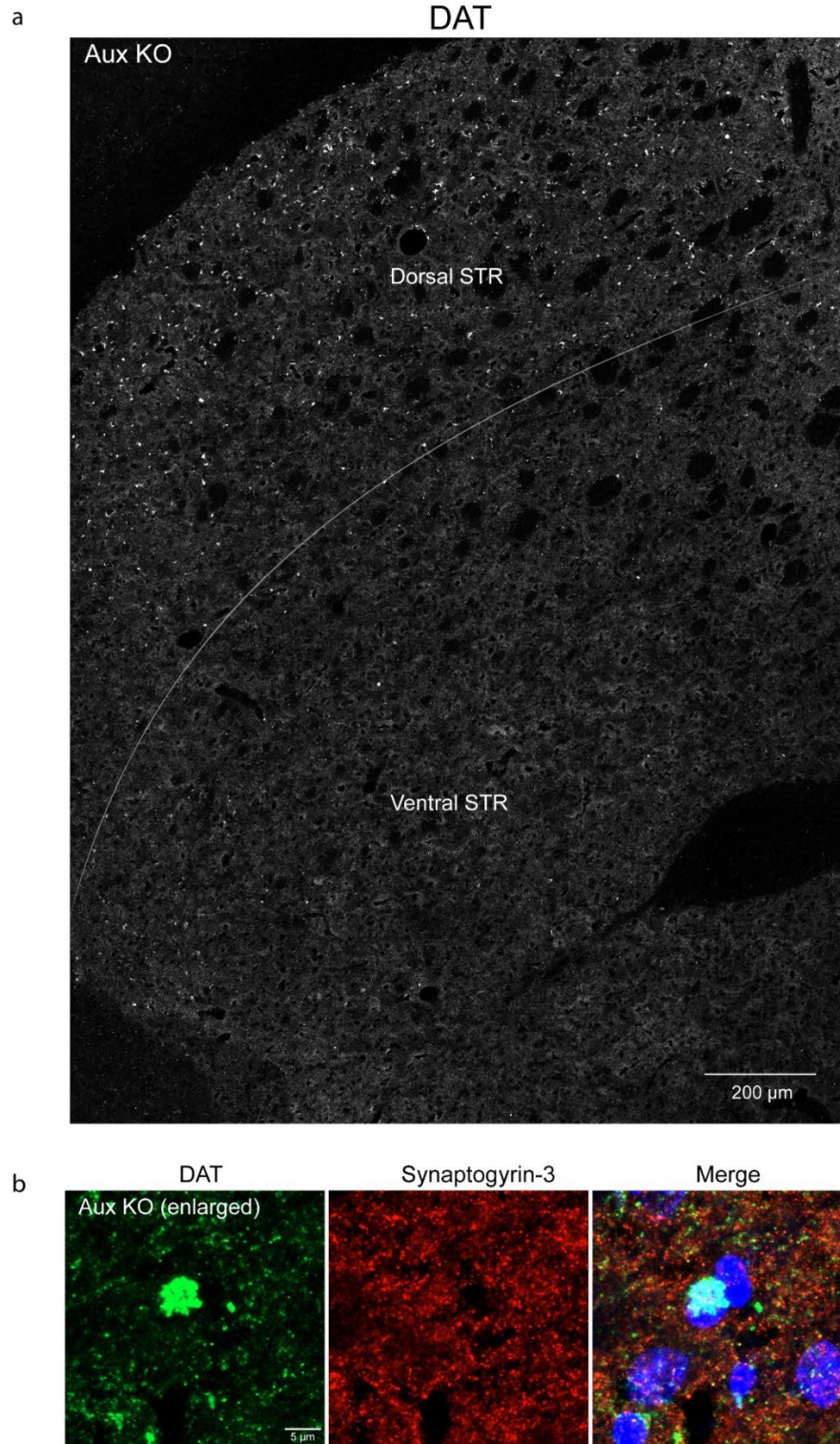


Supplementary Figure. 4: Serotonin and its metabolites were unaltered in auxilin KO brains.

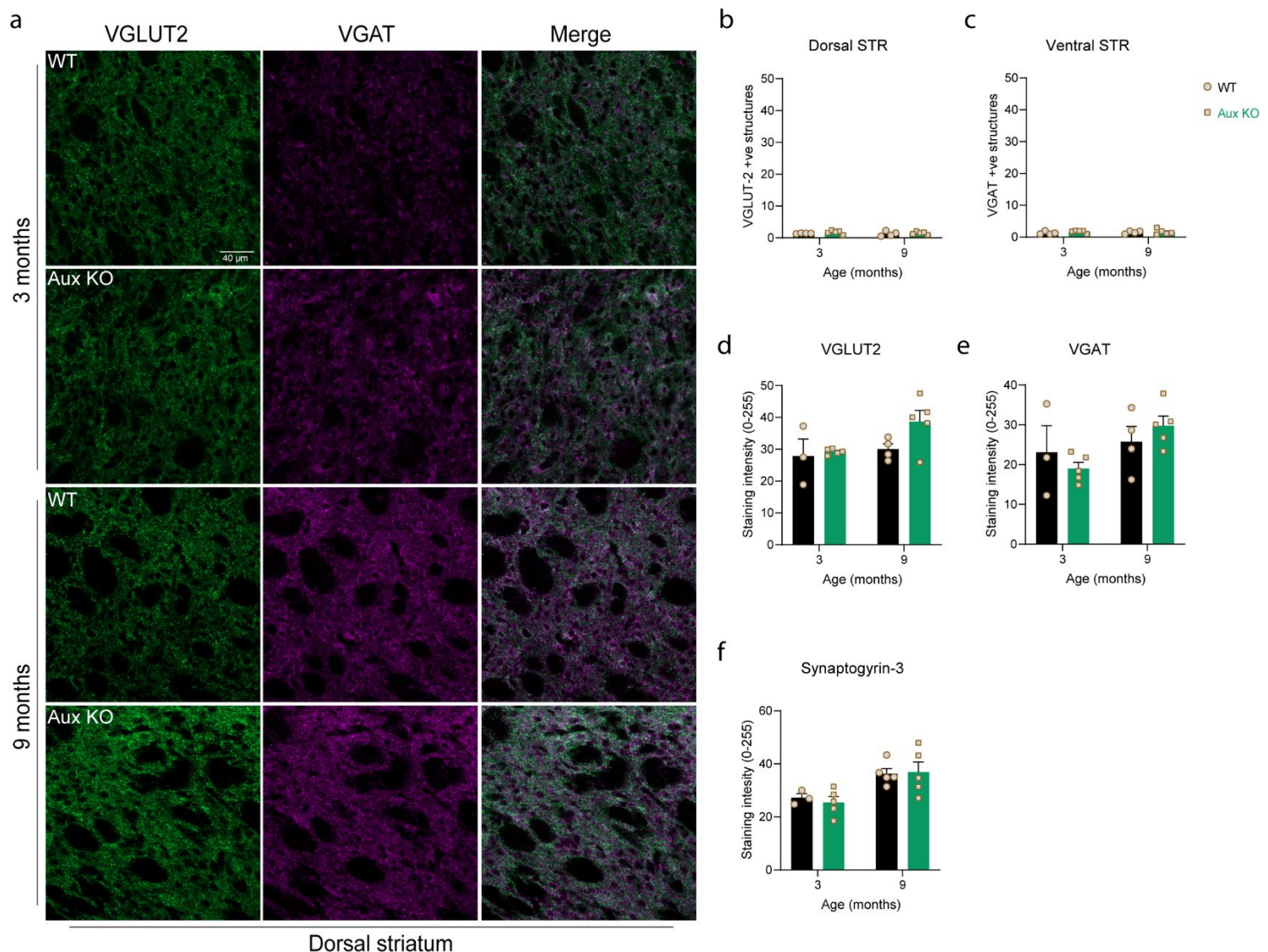
a. Serotonin levels in the dorsal striatum of WT and Aux KOs at 3 and 9 months, as measured by HPLC, which was not altered in Aux KO mice. **b.** Levels of 5-HIAA, a serotonin metabolite, was also unaltered. Statistics: Student's t-test with Welch's correction.



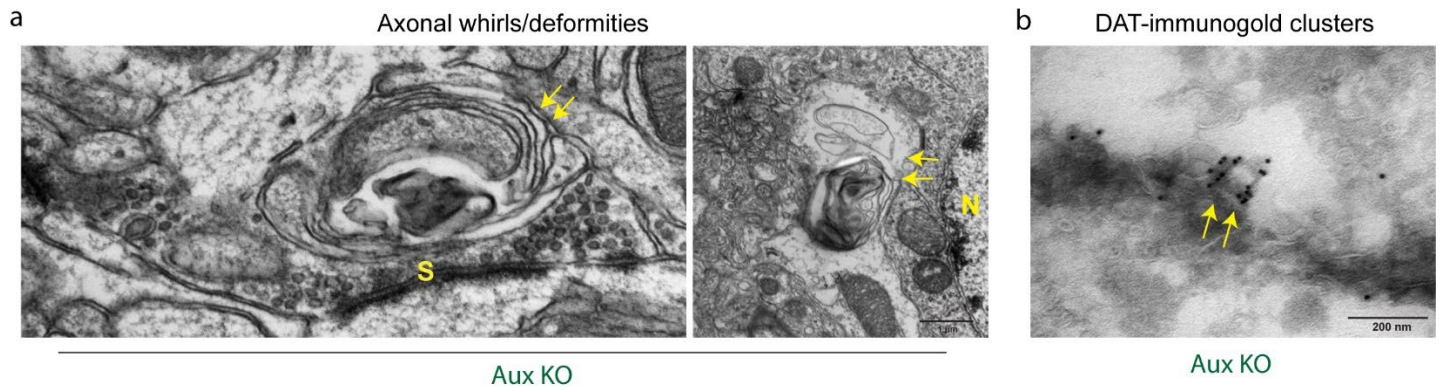
Supplementary Figure. 5: FSCV recording. **a.** Representative images of coronal mouse brain sections showing the location of the bipolar stimulating electrode in the SNpc and the FSCV recording electrode in the dorsal striatum (STR), as marked by DiI staining (DiI: red, DAPI: blue). Scale bar: 1mm **b.** The 3-dimensional pseudocolor plot showing oxidation (cyan) and reduction (red) of dopamine. **c.** Best fit parameters of the dopamine computational model to fit FSCV recordings.



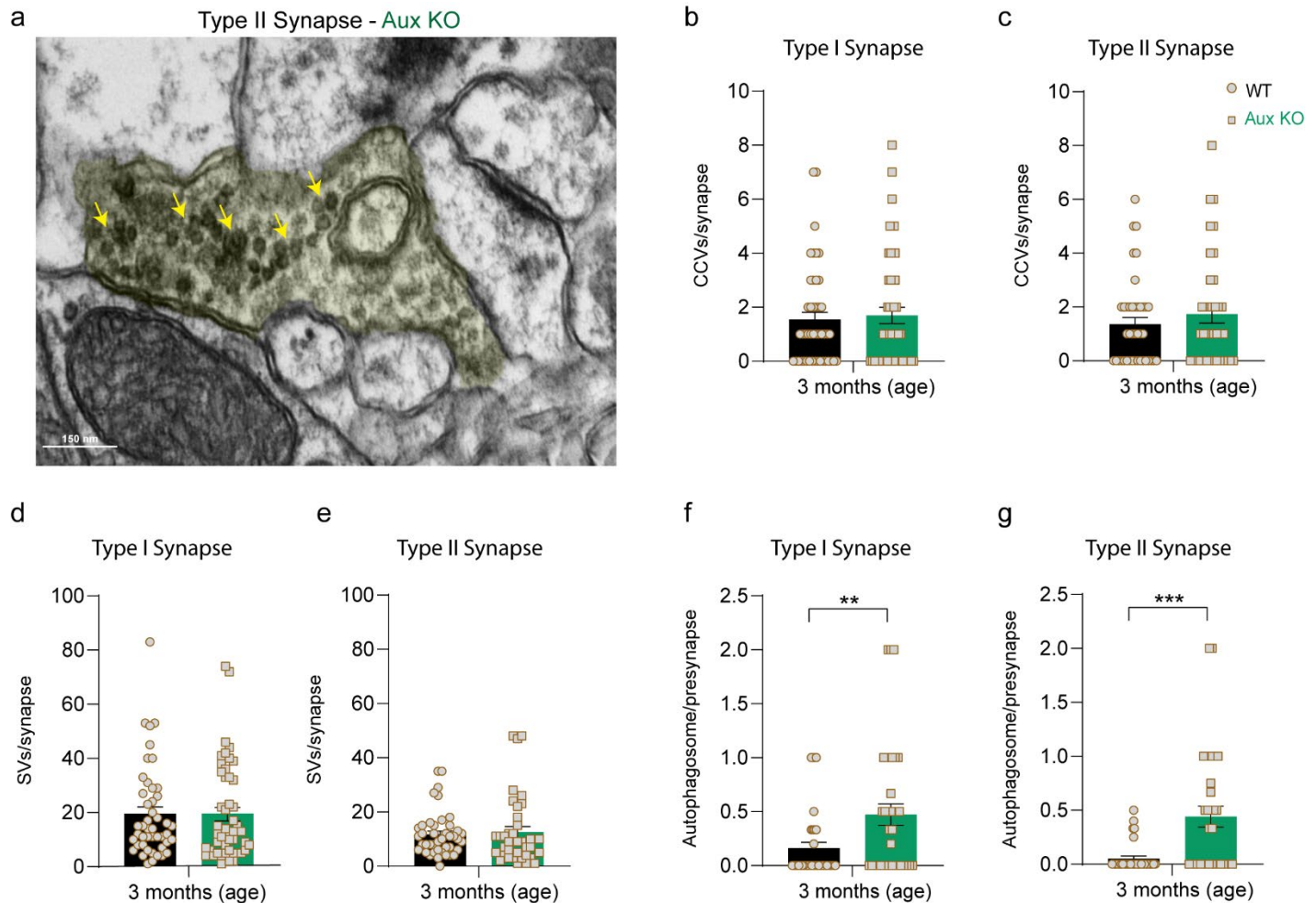
Supplementary Figure. 6: Large DAT+ structures in the dorsal striatum of auxilin KOs. a. Representative grayscale image of striatum of Aux KOs immunostained for DAT, showing large DAT+ structures are enriched in the dorsolateral striatum (STR), but not in the ventral STR. Scale bar: 200 μ m. **b.** Enlarged image of DAT+ve structures (green) in the dorsal striatum, co-immunostained with synaptogyrin-3 (red). Scale bar: 5 μ m.



Supplementary Figure. 7: Axonal deformities were not seen in glutamatergic and GABAergic termini. **a.** Representative images showing dorsal striatum immunostained for glutamatergic marker VGLUT2 and GABAergic marker VGAT, at 3 and 9 months of age, in WT and Aux KO mice. Scale bar: 40 μ m. **b.** Quantitation for VGLUT2+ve large structures/whirls in dorsal striatum of WT and Aux KO mice. We did not observe any differences between the two genotypes. **c.** Quantitation for VGAT+ve large structures in dorsal striatum of Aux KO mice which revealed no alterations. **d.** Expression of VGLUT2 in the dorsal striatum of WT and Aux KO mice at 3 and 9 months. **e.** Expression of VGAT in the dorsal striatum of WT and Aux KO mice at 3 and 9 months, which did not alter in Aux KOs. **f.** Synaptogyrin-3 expression in the dorsal striatum of WT and Aux KO mice at 3 and 9 months, which was unaltered in Aux KOs (See Figure. 4 for representative images). Statistics: Student's t-test with Welch's correction.



Supplementary Figure. 8: Auxilin KO mice show large axonal whirls/deformities in the dorsal striatum. **a.** Ultrastructure of axonal whirls/deformities in the dorsal striatum of Aux KO mice (arrows). These structures were present both close to synaptic termini (S) and soma (as identified by nucleus, N). Scale bar: 1 μ m. **b.** DAT-immunogold clusters in the dorsal striatum of Aux KO mice (arrows). Scale bar: 200 nm.



Supplementary Figure. 9: Synaptic autophagy clears accumulated CCVs. **a.** Representative EM image of Aux KO Type I synapse (shaded in yellow) showing CCVs or clathrin cage accumulation, as well as SV clusters (arrows). Scale bar: 150 μ m. **b.** CCVs number in Type I synapses, which showed a minor increase in Aux KOs. **c.** CCVs number in Type II synapses, which showed a minor increase in Aux KO mice. **d.** SVs number in Type I synapses, which was not altered. **e.** SVs number in Type II synapses, which also did not change significantly in Aux KOs. **f.** Autophagosomes per presynaptic terminal in Type I synapses of dorsal striatum in WT and Aux KOs, which were significantly higher in Aux KOs. **g.** Autophagosomes per presynaptic terminal in Type II synapses, which were also increased significantly in Aux KO mice. Student's t-test with Welch's correction. ** $p < 0.01$, *** $p < 0.001$

Supplementary Table 1: Proteins that are significantly changed in the proteomic analysis of brains of auxilin KO mice, in comparison to WT (age: 3 months).

No.	Accession ID	Gene Name	Protein Name	fold change
1	NNTM	Nnt	NAD(P)	-29.7108
2	AUXI	Dnajc6	Putative tyrosine protein phosphatase auxilin	-4.43618
3	HEBP1	Hebp1	Heme-binding protein 1	-3.09322
4	WDFY1	Wdfy1	WD repeat and FYVE domain-containing protein 1	-2.63747
5	TBCD	Tbcd	Tubulin-specific chaperone D	-2.05534
6	AL7A1	Aldh7a1	Alpha-aminoacidic semialdehyde dehydrogenase	-1.77566
7	ACAP2	Acap2	Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2	-1.55571
8	RAB3B	Rab3b	Ras-related protein Rab-3B	-1.5479
9	NLGN4	Nlgn4l	Neurologin 4-like	1.531757
10	NMRL1	Nmral1	NmrA-like family domain-containing protein 1	1.551981
11	ODBA	Bckdha	2-oxoisovalerate dehydrogenase subunit alpha	1.56953
12	PACS2	Pacs2	Phosphofurin acidic cluster sorting protein 2	1.575139
13	PURG	Purg	Purine-rich element-binding protein gamma	1.591686
14	CRYAB	Cryab	Alpha-crystallin B chain	1.592596
15	SPIR1	Spire1	Protein spire homolog 1	1.804854
16	SYPM	Pars2	Probable proline--tRNA ligase	1.916042
17	PRIO	Prnp	Major prion protein	2.101816
18	GAK	Gak	Cyclin-G-associated kinase	2.335293
19	ZNRD2	Znrd2	Protein ZNRD2	2.57939
20	MTND	Adi1	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	2.843007
21	IGG2B	Igh-3	Ig gamma-2B chain C region	3.601283
22	PURA2	Adss2	Adenylosuccinate synthetase isozyme 2	4.116065

Supplementary Table 2: Proteins that are significantly changed in the proteomic analysis of synaptosomes prepared from the brains of auxilin KO mice, in comparison to WT (age: 3 months).

No.	Accession	Gene Name	Protein Name	fold change
1	AUXI	Dnajc6	Putative tyrosine-protein phosphatase auxilin	-5.93229
2	NNTM	Nnt	NAD(P) transhydrogenase	-3.37721
3	HEBP1	Hebp1	Heme-binding protein 1	-3.2288
4	GBRA2	Gabra2	Gamma-aminobutyric acid receptor subunit alpha-2	-2.5017
5	WDFY1	Wdfy1	WD repeat and FYVE domain-containing protein 1	-2.19771
6	APC	Apc	Adenomatous polyposis coli protein	-1.70123
7	KCNJ4	Kcnj4	Inward rectifier potassium channel 4	-1.60661
8	COMT	Comt	Catechol O-methyltransferase	-1.59569
9	AL7A1	Aldh7a1	Alpha-aminoacidic semialdehyde dehydrogenase	-1.56755
10	IVD	Ivd	Isovaleryl-CoA dehydrogenase Ivd, mitochondrial	-1.53054
11	HTRA1	Htra1	Serine protease HTRA1	1.518369
12	CBPM	Cpm	Carboxypeptidase M	1.520221
13	NFH	Nefh	Neurofilament heavy polypeptide	1.533002
14	IMDH2	Impdh2	Inosine-5'-monophosphate dehydrogenase 2	1.573405
15	THS7A	Thsd7a	Thrombospondin type-1 domain-containing protein 7A	1.573839
16	AINX	Ina	Alpha-internexin	1.57441
17	NFL	Nefl	Neurofilament light polypeptide	1.59067
18	AP1S1	Ap1s1	AP-1 complex subunit sigma-1A	1.618179
19	SCN1A	Scn1a	Sodium channel protein type 1	1.636414
20	CH082	N/A	UPF0598 protein C8orf82 homolog	1.726623
21	PP2AB	Ppp2cb	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	1.789953
22	PURA2	Adss2	Adenylosuccinate synthetase isozyme 2	2.577984
23	S61A2	Sec61a2	Protein transport protein Sec61 subunit alpha isoform 2	2.644442
24	MTND	Adi1	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	4.038564

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Supplementary Table 3: Proteins that are significantly changed in the proteomic analysis of CCVs prepared from the brains of auxilin KO mice, in comparison to WT, at 3 months of age.

No.	Accession	Gene Name	Protein Name	fold change
1	AUXI	Dnajc6	Putative tyrosine-protein phosphatase auxilin	-44.906
2	IGHM	IGHM	Ig mu chain C region	-3.12027
3	CD34	Cd34	Hematopoietic progenitor cell antigen CD34	-2.50216
4	NECP1	Necap1	Adaptin ear-binding coat-associated protein 1	-2.35292
5	SNG3	Syng3	Synaptogyrin-3	-2.10263
6	HS71A	Hspa1a	Heat shock 70 kDa protein 1A	-2.02752
7	SV2B	Sv2b	Synaptic vesicle glycoprotein 2B	-1.9614
8	SPRL1	Sparcl1	SPARC-like protein 1	-1.94395

9	GRM2	Grm2	Metabotropic glutamate receptor 2	-1.9365
10	IGKC	Igkc	Ig kappa chain C region	-1.92748
11	TM163	Tmem163	Transmembrane protein 163	-1.92239
12	TCPB	Cct2	T-complex protein 1 subunit beta	-1.8972
13	SNG1	Syng1	Synaptogyrin-1	-1.8946
14	NECP2	Necap2	Adaptin ear-binding coat-associated protein 2	-1.89401
15	ROAA	Hnrnpab	Heterogeneous nuclear ribonucleoprotein A/B	-1.86664
16	RAB3B	Rab3b	Ras-related protein Rab-3B	-1.85144
17	VGLU1	Slc17a7	Vesicular glutamate transporter 1	-1.8164
18	TRFE	Tf	Serotransferrin	-1.80762
19	LAT1	Slc7a5	Large neutral amino acids transporter small subunit 1	-1.72256
20	SYPH	Syp	Synaptophysin	-1.71627
21	SV2A	Sv2a	Synaptic vesicle glycoprotein 2A	-1.71423
22	ROA0	Hnrnpa0	Heterogeneous nuclear ribonucleoprotein A0	-1.70418
23	ZNT3	Slc30a3	Zinc transporter 3	-1.70291
24	DPP10	Dpp10	Inactive dipeptidyl peptidase 10	-1.68897
25	NTRI	Ntm	Neurotrimin	-1.68091
26	SYT12	Syt12	Synaptotagmin-12	-1.67682
27	IGLO5	Iglon5	IgLON family member 5	-1.668
28	VGLU2	Slc17a6	Vesicular glutamate transporter 2	-1.66765
29	SYT1	Syt1	Synaptotagmin-1	-1.61913
30	CAD13	Cdh13	Cadherin-13	-1.58329
31	BT3L4	Btf3l4	Transcription factor BTF3 homolog 4	-1.57599
32	SCAM5	Scamp5	Secretory carrier-associated membrane protein 5	-1.57105
33	C1QB	C1qb	Complement C1q subcomponent subunit B	-1.55463
34	STON2	Ston2	Stonin-2	-1.5433
35	VAMP2	Vamp2	Vesicle-associated membrane protein 2	-1.52957
36	MFSD6	Mfsd6	Major facilitator superfamily domain-containing protein 6	-1.5266
37	RAP1A	Rap1a	Ras-related protein Rap-1A	-1.51838
38	NHRF1	Slc9a3r1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	-1.51157
39	CC50A	Tmem30a	Cell cycle control protein 50A	1.511254
40	TPC11	Trappc11	Trafficking protein particle complex subunit 11	1.588902
41	MYPR	Plp1	Myelin proteolipid protein	1.59174
42	CLVS2	Clvs2	Clavesin-2 OS=Mus musculus	1.642113
43	MOG	Mog	Myelin-oligodendrocyte glycoprotein	1.655013
44	AP3M2	Ap3m2	AP-3 complex subunit mu-2	1.657988
45	APMAP	Apmmap	Adipocyte plasma membrane-associated protein	1.727314
46	TMM9B	Tmem9b	Transmembrane protein 9B	1.738029
47	H2A1B	H2ac4	Histone H2A type 1-B	1.807098
48	NDKA	Nme1	Nucleoside diphosphate kinase A	1.808866
49	PCYOX	Pcyox1	Prenylcysteine oxidase	2.039561
50	CLCN6	Clcn6	Chloride transport protein 6	2.169131
51	ERC2	Erc2	ERC protein 2	6.374011

1163 **Supplementary Table 4:** Proteins that are significantly changed in the proteomic analysis of
 1164 synaptosomes prepared from the brains of auxilin KO mice, in comparison to WT, at symptomatic
 1165 age of 9 months.

No.	Accession	Gene Name	Protein Name	fold change
1	AUXI	Dnajc6	Putative tyrosine-protein phosphatase auxilin	-3.82296
2	RGS6	Rgs6	Regulator of G-protein signaling 6	-1.66333
3	ADA22	Adam22	Disintegrin and metalloproteinase domain-containing protein 22	-1.61876
4	ADT1	Slc25a4	ADP/ATP translocase 1	-1.59798
5	2AAB	Ppp2r1b	Serine/threonine-protein phosphatase 2A	-1.57516
6	2AAA	Ppp2r1a	Serine/threonine-protein phosphatase 2A	-1.54334
7	PADI2	Padi2	Protein-arginine deiminase type-2	-1.53356
8	PRAF3	Arl6ip5	PRA1 family protein 3	-1.52679
9	MGLL	Mgll	Monoglyceride lipase	-1.51729
10	MTOR	Mtor	Serine/threonine-protein kinase mTOR	-1.50276
11	CRIP2	Crip2	Cysteine-rich protein 2	1.543321
12	NFL	Nefl	Neurofilament light polypeptide	1.545559
13	AINX	Ina	Alpha-internexin	1.557636
14	CADH2	Cdh2	Cadherin-2	2.225312
15	ABLM2	Ablim2	Actin-binding LIM protein 2	1.631285
16	ACTN1	Actn1	Alpha-actinin-1	1.5648206
17	GAK	GAK	Cyclin-G-associated kinase	1.4910263

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REVIEW

Role of the endolysosomal system in Parkinson's disease

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Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative disorders, affecting 1–1.5% of the total population. While progress has been made in understanding the neurodegenerative mechanisms that lead to cell death in late stages of PD, mechanisms for early, causal pathogenic events are still elusive. Recent developments in PD genetics increasingly point at endolysosomal (E-L) system dysfunction as the early pathomechanism and key pathway affected in PD. Clathrin-mediated synaptic endocytosis, an integral part of the neuronal E-L system, is probably the main early target as evident in auxilin, RME-8, and synaptojanin-1 mutations that cause PD. Autophagy, another important pathway in the E-L system, is crucial in maintaining proteostasis and a healthy mitochondrial pool, especially in neurons considering their inability to divide and requirement to function an entire life-time. *PINK1* and *Parkin* mutations severely

perturb autophagy of dysfunctional mitochondria (mitophagy), both in the cell body and synaptic terminals of dopaminergic neurons, leading to PD. Endolysosomal sorting and trafficking is also crucial, which is complex in multi-compartmentalized neurons. *VPS35* and *VPS13C* mutations noted in PD target these mechanisms. Mutations in *GBA* comprise the most common risk factor for PD and initiate pathology by compromising lysosomal function. This is also the case for *ATP13A2* mutations. Interestingly, α -synuclein and LRRK2, key proteins involved in PD, function in different steps of the E-L pathway and target their components to induce disease pathogenesis. In this review, we discuss these E-L system genes that are linked to PD and how their dysfunction results in PD pathogenesis.

Keywords: Autophagy, Clathrin-mediated endocytosis, LRRK2, Lysosomes, Retromer complex, α -Synuclein.

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Parkinson's disease (PD) is the second most common neurodegenerative disorder, clinically identified by motor symptoms, with an increasing recognition of non-motor manifestations (Hoehn and Yahr 1967; Chaudhuri and Schapira 2009). Loss of dopaminergic neurons of the substantia nigra pars compacta (SNpc) (40–50%) and the presence of α -synuclein pathology in surviving neurons are the neuropathological hallmarks of PD. At the onset of motor symptoms, dopamine levels have decreased 60–70% in the dorsal striatum, where most SNpc dopaminergic neurons form synaptic connections (Fearnley and Lees 1991; Ma *et al.* 1997; Dickson *et al.* 2009; Kordower *et al.* 2013). Under physiological conditions, α -synuclein is predominantly present in

Abbreviations used: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATP13A2, ATPase cation transporting 13A2; CCVs, clathrin coated vesicles; CME, clathrin-mediated endocytosis; DCVs, dense core vesicles; E-L, endolysosomal; ESCRT, endosomal sorting complex required for transport; GAK, cyclin G associated kinase; GBA, Glucosidase Beta Acid; Gcase1, glucocerebrosidase 1; GD, Gaucher disease; GlcCer, glucosylceramide; GTPase, guanosine triphosphatase; GWAS, genome wide association study; Hsc70, heat shock cognate 70; HSP40, heat shock protein 40; HSPGs, heparan sulfate proteoglycans; ILVs, interluminal vesicles; iPSCs, induced pluripotent stem cells; KO, knockout; LAMP2a, lysosome-associated membrane glycoprotein 2; L-dopa, Levodopa; LRRK2, leucine-rich repeat kinase 2; M6P, mannose-6-phosphate; MAPK/ERK, mitogen-activated protein kinase/ extracellular signal-regulated kinase; MSNs, medium spiny neurons; MVBs, multivesicular bodies; PARK, Parkinson's disease gene; PD, Parkinson's disease; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PINK1, PTEN Induced putative Kinase 1; PIPs, phosphoinositide phosphates; PRKN, parkin gene; RME-8, receptor mediated endocytosis 8; siRNA, small interfering ribonucleic acid; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; SNCA, alpha-synuclein gene; SNpc, substantia nigra pars compacta; SVs, synaptic vesicles; SYNJ1/ SynJ1, synaptojanin-1; TGN, trans-golgi network; USP19, ubiquitin-specific protease 19; VPS, vacuolar protein sorting.

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pre-synaptic terminals, aiding neurotransmitter release, whereas in PD, α -synuclein tends to aggregate and form Lewy bodies (the defining pathology of PD) (Spillantini *et al.* 1997; Spillantini *et al.* 1998). Aggregation of α -synuclein, perturbed proteostasis, mitochondrial dysfunction, oxidative stress, and impaired calcium homeostasis are the major cell-autonomous mechanisms of PD pathogenesis. Non-cell-autonomous mechanisms include cell-to-cell transmission of pathological α -synuclein and neuroinflammation (Jyothi *et al.* 2015; Poewe *et al.* 2017). So far, incomplete knowledge on causal mechanisms has limited treatment strategies to symptomatic relief. Recent studies targeting the endolysosomal (E-L) system reveal that its dysfunction can lead to the mentioned disease mechanisms, suggesting the E-L system as a key pathway in PD pathogenesis. In this review, we discuss important E-L system components, their roles in neuronal survival, and how their dysfunction causes PD.

Endolysosomal system in neurons: Custom-made for neuronal function

The E-L system comprises of complex, highly dynamic membrane-enclosed tubular-vesicular structures with overlapping properties and functions (Klumperman and Raposo 2014). They facilitate nutritional intake from a cell's microenvironment through endocytosis, neutralize pathogenic materials via phagocytosis, promote cellular proteostasis through autophagy, and maintain overall cellular homeostasis through endosomal sorting and trafficking of various proteins/enzymes across organelles (Repnik *et al.* 2013; Klumperman and Raposo 2014). E-L system functions vary greatly depending on cell type and functional state. Neurons are unique in their highly polarized intracellular compartmentalization of cell body/soma and processes including axons, axon terminals, and dendrites. This organization enables them to form complicated networks that mediate specialized functions (Fig. 1). This complexity comes at a price: burdensome demands on the E-L system, which have been increasingly implicated in neurological diseases including PD (Kett and Dauer 2016; Wang *et al.* 2018).

In neurons, synthesis of E-L components occurs primarily in the cell body/soma (Fig. 1a). These components are trafficked through the secretory pathway to their subcellular location. A prime example of this is lysosomal hydrolases trafficking. Lysosomal hydrolases are produced in the endoplasmic reticulum, then undergo glycosylation and other post-translational modifications at the Golgi apparatus [e.g. addition of mannose-6-phosphate (M6P) groups] (Nixon and Cataldo 1995). This modification directs hydrolases to lysosomes. More specifically, hydrolases form complexes with transmembrane M6P receptors, which are sequestered into clathrin-coated vesicle (CCVs) budding from the trans-Golgi networks (TGN) to form endosomes/early endosomes (Nixon and Cataldo 1995) (Fig. 1a). Inside early endosomes,

M6P receptors detach from hydrolases and are recycled back to the TGN for their next round of transport (Saftig and Klumperman 2009), while hydrolases are delivered to lysosomes (discussed below).

Simultaneously, early endosomes are also derived from the cell surface through the endocytic pathway, an integral part of the E-L system. Many of the nutrients required for neuronal function and surface receptors designated for recycling are internalized through clathrin-mediated endocytosis (CME) at the plasma membrane and delivered to early endosomes (Fig. 1a). Early endosomes derived from both the TGN and the plasma membrane mature into late endosomes, where Rab5 and Rab7 play a prominent role (Saftig and Klumperman 2009). This process occurs with the help of the endosomal sorting complex required for transport (Fig. 1a), which facilitates ubiquitin-dependent sorting of cargo/hydrolases from the outer membrane of early endosomes into interluminal vesicles (ILVs) (Wollert *et al.* 2009). Early endosomes derived from CME, if not sorted, follow a recycling pathway to the plasma membrane (Fig. 1a) (Saftig and Klumperman 2009). ILVs are enveloped inside multivesicular bodies (MVBs), which mature into late endosomes. These late endosomes deliver their luminal contents either by forming tubular extensions or by fusing with pre-existing lysosomes to form endolysosomes (Luzio *et al.* 2007). These may be considered the final compartment of the endocytic pathway, where hydrolases degrade endocytic cargo and the membranes of ILVs for reuse (Saftig and Klumperman 2009; Klumperman and Raposo 2014). In addition, there is retrograde transport of receptors (such as M6P, LAMP2a, etc) from endosomes to the TGN for reuse through the retromer complex, comprised mainly of VPS35, VPS26 and VPS29 (Saftig and Klumperman 2009) (Fig. 1a). Proteins that are meant for secretion follow a secretory pathway from the TGN (Saftig and Klumperman 2009; Klumperman and Raposo 2014). Selective capture and elimination of a cell's damaged organelles and misfolded proteins happens through a process called macroautophagy (termed hereafter as autophagy) (Ohsumi 2014), an integral part of the E-L system. Double membrane vesicles called autophagosomes encapsulate unwanted/toxic cytosolic constituents including aggregated proteins and deliver them to the lysosome for degradation (Fig. 1a) (Mizushima 2007; Kawamata *et al.* 2008). Non-selective autophagy also occurs during starvation, through which cells can recycle proteins and reuse amino acids for the synthesis of basic proteins essential for cell survival (Ohsumi 2014). Therefore, autophagy plays a pivotal role in house-keeping and maintenance of neuronal homeostasis.

Customization of the E-L system for neurons mainly happens in axons (Fig. 1b) and synapses (Fig. 1c). At pre-synaptic terminals, vesicle precursors are delivered from the cell body and undergo vesicle cycling to form functional synaptic vesicles (SVs). However, delivery of new vesicle precursors cannot match the number of SVs undergoing

exocytosis to achieve neurotransmission at an active synapse (Saheki and De Camilli 2012). This deficit is solved by highly responsive CME, recapturing fused vesicular membranes from the pre-synaptic membrane to form CCVs, which are in turn converted back to SVs through clathrin uncoating (Fig. 1c) (Heuser and Reese 1973; Morgan *et al.* 2000; Saheki and De Camilli 2012). Less understood clathrin-independent bulk endocytosis (Holt *et al.* 2003; Wu and Wu 2007) and the “kiss and run” mechanism (Fesce *et al.* 1994) are also parts of the synaptic endocytic repertoire. In addition, there are dense-core vesicles derived from the TGN that are transported down the axons. Unlike SVs, dense core vesicles do not participate in SV recycling and are primarily used to secrete long-acting neuropeptides (Nurrish 2014). While these processes are mostly confined to the pre-synaptic compartment, the E-L system's postsynaptic compartment contributes to membrane trafficking pathways primarily aimed at receptor, transporter, and ion-channel recycling (Rosendale *et al.* 2017).

Although this complex E-L system allows a neuron to function far away from its site of protein synthesis, the neuron is beholden to anterograde transport of proteins and membranes synthesized and modified in the soma for maintenance of axons and the synapses (Maday *et al.* 2014). Furthermore, constant synaptic activity, especially in continuously firing neurons like SNpc dopaminergic neurons may result in considerable build-up of damaged organelles, membranes, and misfolded proteins. These need to undergo encapsulation by an autophagosome or endosome (Fig. 1C) and retrograde transport to the soma for lysosomal degradation (Fig. 1B) (Yang *et al.* 2013). Here, long-distance axonal microtubule networks provide significant support to the neuronal E-L system (Yang *et al.* 2013). These spatial challenges to address a unique structural geometry, high metabolic demand, enhanced responsiveness to chemical/electrical stimulation, and the requirement for longevity because of their postmitotic nature, place a tremendous burden on the E-L system. Nigrostriatal dopaminergic neurons have an additional burden in terms of long and highly branched axons, larger surface area, selective transporters for external toxins, large fluctuations in free Ca^{2+} , less buffering capacity, and higher oxidative stress, reducing their threshold for degeneration (Vidyadhara *et al.* 2016; Surmeier *et al.* 2017; Vidyadhara *et al.* 2017; Vidyadhara *et al.* 2019). These vulnerabilities are further exacerbated with aging. Faced with environmental and genetic risk factors, disease pathogenesis abounds.

E-L system dysfunction: the holy grail of PD pathophysiology?

In 1997, identification of dominant mutations in the α -synuclein gene (*SNCA*, *PARK1*) which encodes α -synuclein as a cause of familial PD (Polymeropoulos *et al.* 1997),

brought a conceptual shift toward the importance of genetics in disease pathogenesis. Subsequent findings revealed various other genetic mutations, with at least 10–15% of PD patients having familial PD of an absolute genetic origin. Leucine-rich repeat kinase 2 (*LRRK2*, *PARK8*) and Parkin (*PRKN*, *PARK2*) mutations are the most common genetic variants of dominant and recessively inherited PD respectively (Deng *et al.* 2018). Mutations and gene multiplications of *SNCA* (*PARK1* and *PARK4*) (Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004) and Glucosidase Beta Acid (*GBA*) are also highly prevalent, whereas PD pathology was also noted with abnormal forms of Parkinsonism associated with deglycase or DJ1 (*PARK7*), and PTEN induced putative kinase 1 (*PINK1*, *PARK6*) (Deng *et al.* 2018). There is also compelling evidence for a genetic component to sporadic PD. In fact, a rigid distinction between the genetic and environmental basis of PD pathogenesis may not exist considering that cases are phenotypically indistinguishable (except for the onset) (Baba *et al.* 2006), implying genetic and sporadic cases share common underlying mechanisms. Even after considerable studies on these familial mutations and their interaction with environment, the core pathomechanisms of PD are still unclear, limiting treatment strategies. Over the past 10 years, advancements in high-throughput sequencing, the application of genome wide association studies (GWAS), and meta-analysis, have identified new genetic mutations (refer to Deng *et al.* 2018 for a list of all PARK genes and risk alleles). Intriguingly, most of these identified genes have links to the E-L system (refer Table 1). Mechanistic studies also suggest that these proteins are the downstream targets of previously identified broad spectrum dysfunction in α -synuclein and *LRRK2* mutations. It is now becoming clear that most PD-linked genes (Table 1) and known pathomechanisms are, in one way or another, associated with the E-L system, strongly suggesting this pathway as the key master regulator of PD pathogenesis. Recent studies suggest that neurodegeneration is initiated at the dopaminergic pre-synaptic terminals of striatum, years before the clinical diagnosis (Imbriani *et al.* 2018). Evidence that most of the E-L system genes associated with PD function at this terminal further establishes their fundamental role in PD pathogenesis. Below, we discuss our understanding of the functions and dysfunctions of these PD-linked E-L system genes. We also discuss how other key PD-associated proteins target E-L pathways.

Clathrin-mediated synaptic vesicle endocytosis: A prime target?

SV endocytosis is predominantly mediated through CME (Fig. 1c). Following exocytosis, the SV membrane is endocytosed primarily through CME, which involves formation of a clathrin coat, composed of an outer clathrin layer, and an inner layer of adaptors (Saheki and De Camilli 2012). The most common adaptors, such as AP-2 and AP180, bind

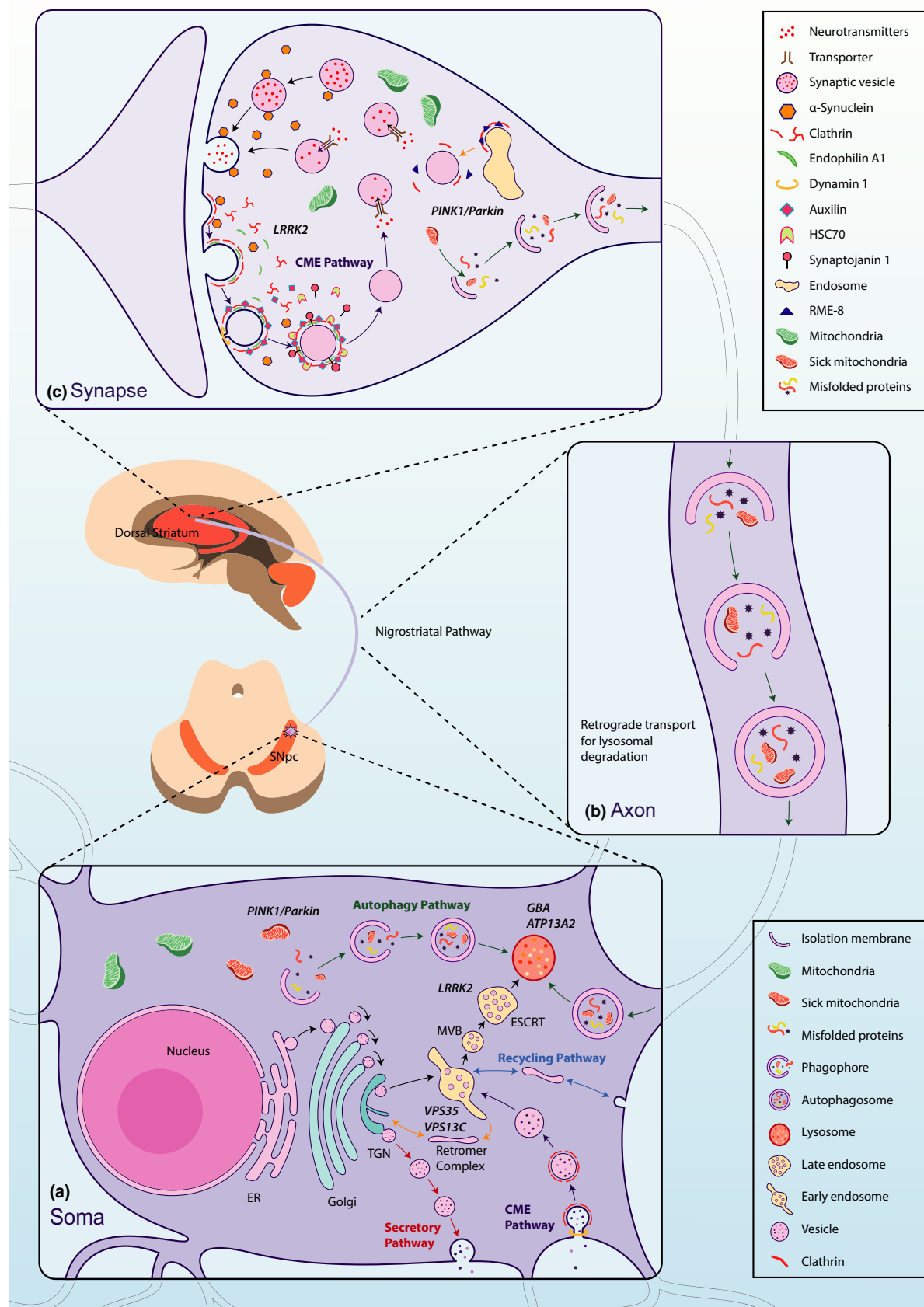


Fig. 1 E-L system in the compartments of a nigrostriatal dopaminergic neuron, with the normal functional locations of E-L system proteins linked to PD. (a) Soma: E-L system proteins that form structural and functional components are synthesized in the neuronal soma. Following production in the endoplasmic reticulum (ER) and post-translational modifications in the Golgi apparatus, proteins (e.g. lysosomal hydrolases) are packed into vesicles and delivered to the trans-Golgi network (TGN). In the TGN, based on the nature of post-translational modification, the vesicles are diverted either toward lysosomes (black arrows) or to the secretory pathway (red arrows). Vesicles that are destined for lysosomes bud off from the TGN to form early endosomes. Simultaneously, early endosomes carrying nutrients and surface receptors for recycling are also derived from the plasma membrane through clathrin mediated endocytosis (CME, purple arrows). These early endosomes, with the help of the endosomal sorting complex required for transport (ESCRT), pass through highly dynamic tubulovesicular structures like multivesicular bodies (MVB) and/or late endosomes before fusing with lysosomes. Leucine-rich repeat kinase 2 (LRRK2) plays a significant role in this sorting, whereas Glucocerebrosidase 1 (GBA/Gcase 1) and ATPase cation transporting 13A2 (ATP13A2) are crucial for normal lysosomal function. Early endosomes from the cell surface that are not sorted follow the recycling pathway back to the plasma membrane (blue arrows). Retrograde transport of receptors that are used for sorting from early endosomes to TGN occurs through the retromer complex where Vacuolar Protein Sorting 35 (VPS35) and VPS13C take part (yellow arrows). An expanding membrane sac called a phagophore sequesters malfunctioning organelles (e.g. sick mitochondria) and misfolded proteins to form a double-membraned autophagosomes. These later

fuse with lysosomes and release contents for degradation and recycling of components; the complete process is called autophagy (green arrows). PTEN Induced Putative Kinase 1 (PINK1) and Parkin are important in identifying sick mitochondria and diverting them to a specialized form of autophagy called mitophagy. (b) Axon: Axons form a crucial bridge for retrograde transport of endosomes/autophagosomes from pre-synaptic terminals to soma for lysosomal degradation. A gradual development of phagophore to full-fledged autophagosome happens within the axon (green arrows) as the encapsulated cargo arrives towards the proximal end. (c) Synapse: Neurotransmitter release in the pre-synaptic terminal is regulated principally by the synaptic vesicle (SV) cycle, an integral part of the neuronal E-L system. Neurotransmitters are loaded into these SVs through specialized transporters. Upon arrival of an action potential in the synaptic terminal, these SVs fuse with the plasma membrane at active sites and release neurotransmitters via exocytosis (black arrows). Empty SVs are then recycled through CME (Purple arrows). α -Synuclein and endophilin A play roles here in membrane bending and SV curvature formation. Clathrin forms a layer on these invaginations with the help of adaptors, and in conjunction with dynamin leads to membrane fission forming clathrin coated vesicles (CCV). Clathrin uncoating is a prerequisite for SV recycling, which is performed by coordinated action of auxilin, Hsc70, endophilin A1, and synaptojanin 1. Phosphorylation by LRRK2 is necessary for activation of these proteins. Endosomes derived from the plasma membrane also form SVs in the nerve terminal where RME-8 plays a role in clathrin sorting (yellow arrow). Synaptic autophagy and mitophagy are also crucial in maintaining the health of the pre-synaptic terminal (green arrows), where PINK1/Parkin are crucial.

Table 1 PD genes linked to E-L system with their PARK nomenclature, proteins they encode, and their primary function and original reference of their discovery.

PD genes that are linked to E-L system				
PARK	Gene	Protein	Function	Reference
PARK1	<i>SNCA</i>	α -Synuclein	Synaptic vesicle cycling	Polymeropoulos <i>et al.</i> (1997)
PARK2	<i>PRKN</i>	Parkin	E3 Ubiquitin ligase	Mori <i>et al.</i> (1998) Kitada <i>et al.</i> , (1998)
PARK4	<i>SNCA</i>	α -Synuclein	Synaptic vesicle cycling	Singleton <i>et al.</i> , (2003) Chartier-Harlin <i>et al.</i> , (2004)
PARK6	<i>PINK1</i>	PINK1	Serine/Threonine kinase	Valente <i>et al.</i> , (2004)
PARK8	<i>LRRK2</i>	LRRK2	Kinase/GTPase	Paisán-Ruiz <i>et al.</i> , (2004); Zimprich <i>et al.</i> , (2004)
PARK9	<i>ATP13A2</i>	ATPase type 13A2	ATPase	Ramirez <i>et al.</i> , (2006); Di Fonzo <i>et al.</i> , (2007)
PARK16	<i>RAB29</i>	Ras-Related Protein Rab-7L1	Rab GTPase	Satake <i>et al.</i> (2009)
PARK17	<i>VPS35</i>	VPS35	Retromer	Wider <i>et al.</i> , (2008) Vilariño-Güell <i>et al.</i> , (2011)
PARK19	<i>DNAJC6</i>	Auxilin	Co-chaperone	Edvardson <i>et al.</i> , (2012) Köroğlu <i>et al.</i> , 2013
PARK20	<i>SYNJ1</i>	Synaptojanin-1	Polyinositol phosphatase	Krebs <i>et al.</i> , (2013) Quadri <i>et al.</i> , (2013)
PARK21	<i>DNAJC13</i>	RME-8	Co-chaperone	Vilariño-Güell <i>et al.</i> , (2014)
PARK23	<i>VPS13C</i>	VPS13C	Retromer, Lipid transporter	Lesange <i>et al.</i> , (2014), Schormair <i>et al.</i> , (2018)
Risk Genes	<i>GBA</i>	Glucocerebrosidase 1	Glucosidase	Sidransky <i>et al.</i> , (2009)
	<i>GAK</i>	Cyclin-G Associated Kinase	Co-chaperone	Nalls <i>et al.</i> , (2014) Chang <i>et al.</i> , (2017)
	<i>SH3GL2</i>	Endophilin A1	Membrane bending protein	Nalls <i>et al.</i> , (2014) Chang <i>et al.</i> , (2017)

to endocytic motifs on the SV membrane, as well as phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] present in the plasma membrane. Endophilin A1 and α -synuclein drive membrane bending and curvature formation of the developing clathrin coated pit (Bai *et al.* 2010; Westphal and

Chandra 2013). Following this, the clathrin coated pit in conjunction with dynamin and other accessory proteins undergoes membrane fission to form a CCV (Saheki and De Camilli 2012). Regeneration of SVs from CCVs and recycling of clathrin and related coat components for further

endocytosis requires un-coating of clathrin from CCVs (Fig. 1c). The clathrin uncoating step is carried out by the coordinated action of the PI phosphatase synaptojanin-1 and the co-chaperone auxilin with Hsc70. Endophilin A1 and cyclin G Associated Kinase (GAK) also regulate clathrin uncoating to facilitate CME at the pre-synaptic terminal (Fig. 1c) (Saheki and De Camilli 2012). Mutations of these E-L system genes in familial PD suggest synaptic endocytosis dysfunction as a prime trigger for PD pathogenesis.

The DNAJ family of heat shock proteins in the pre-synaptic terminal and beyond

Auxilin

DNAJC6 gene encodes auxilin, the major pre-synaptic endocytic protein linked to PD. Auxilin is a brain specific co-chaperone belonging to the DNAJC family (Table 1), a subclass of heat shock proteins (HSP40). Auxilin plays an important role in clathrin un-coating, recruiting ATP-activated Hsc70 to CCVs, which in turn, disassembles clathrin from vesicles facilitating their recycling (Lemmon 2001; Gorenberg and Chandra 2017) (Fig. 1c). Loss-of-function autosomal recessive mutations of auxilin (*PARK19*) have been shown to cause juvenile/early onset PD, first observed in two teenage brothers of a Palestinian family (Edvardson *et al.* 2012). The study noted abnormal mRNA splicing and a decrease in auxilin mRNA levels. Other variants of auxilin mutations were later noted in a Brazilian family with similar mis-splicing of mRNA, whereas mutations affecting the J-domain (p.Q791*, p.Q846* and p.R927G) were observed in a few other consanguineous families (Koroglu *et al.* 2013; Olgiati *et al.* 2016). Along with typical PD symptoms, patients with nonsense mutations have cognitive impairment, one of the cardinal non-motor symptoms of PD. Epilepsy and mental retardation have also been noted for some PD patients with auxilin mutations (Koroglu *et al.* 2013). These human genetic studies suggest widespread neuronal dysfunction besides nigrostriatal dopaminergic neurodegeneration. Hence, future studies on post-mortem brains documenting PD-typical Lewy body pathology and dopaminergic neuronal loss are necessary to concretely categorize the pathology as PD or parkinsonism.

One of the few preclinical studies characterizing *DNAJC6* reveals that auxilin down-regulation leads to progressive dopaminergic neuronal loss and motor disabilities in *Drosophila*. Furthermore, loss of auxilin exacerbates α -synuclein over-expression and paraquat (an environmental toxin)-mediated dopaminergic loss, thus aggravating previously established phenotypes in fly models of PD (Song *et al.* 2017). Interestingly, the brains (deep cerebellar nuclei) of adult homozygous auxilin knockout (KO) mice revealed an increased number of CCVs and empty cages at the synapse. Similar defects were noted in primary cortical and hippocampal neurons accompanied by defective SV endocytosis

(Yim *et al.* 2010). The impairment was not immediately detrimental to the neurons, which may explain the post-developmental onset of PD symptoms and slow disease progression. Evaluating whether similar disruptions occur in nigrostriatal dopaminergic neurons and how it mediates pathogenesis will prove crucial in understanding the role of auxilin and the E-L system in PD. An initial step toward this suggests that LRRK2 phosphorylates auxilin, attenuating auxilin's normal function in human iPSC-derived dopaminergic neurons. The study further revealed that phosphorylation of auxilin by over-expressing LRRK2 leads to accumulation of oxidized dopamine and α -synuclein over-expression, pathological hallmarks of PD (Nguyen and Krainc 2018). Whether loss-of-function mutations in auxilin independently exert pathology through the same mechanisms is unknown. However, this newfound link between LRRK2 and auxilin points to the crucial and widespread involvement of E-L system in PD pathogenesis.

GAK

GAK is another DNAJC family protein (*DNAJC26*/Auxilin 2), homologous (~50%) to auxilin but expressed throughout the body (Lemmon 2001). In a meta-analysis of PD GWAS across 13,708 patients and 95,282 controls (Nalls *et al.* 2014), GAK was identified as a risk gene for PD which was confirmed in another recent largescale study (Chang *et al.* 2017) (Table 1). However, GAK's role in PD is still contentious because of presence of mutations in *TMEM175* in a nearby independent locus, which shares the same promoter as GAK. What adds credence is that, like auxilin, GAK functions in clathrin un-coating by recruiting Hsc70 to CCVs (Lemmon 2001) (Fig. 1C). Results show that GAK over-expression can partially compensate for loss of auxilin, and is also noted to up-regulate naturally in auxilin-KO mice (Yim *et al.* 2010). Another similarity between auxilin and GAK has been noted in their interaction with LRRK2 (Jinn *et al.* 2017; Nguyen and Krainc 2018). Brain-specific loss of GAK and ubiquitous KO of GAK are both lethal (postnatal and embryonic respectively) (Lee *et al.* 2008a). The inability of auxilin to compensate for GAK in these mice suggests another unique role for GAK in neurons, perhaps a significant one in dopaminergic neurons. Further *TMEM175* deficiency is noted to cause PD-like α -synuclein aggregation, along with impairment of lysosomal and mitochondrial function (Jinn *et al.* 2017). Clarity on the role of GAK will prove to be crucial not only in establishing its involvement in PD but also in consolidating CME as the prime target in PD pathogenesis.

RME-8

Receptor mediated endocytosis 8 (RME-8) or *DNAJC13* is another DNAJC family co-chaperone and principally functions in regulating retromer and clathrin un-coating dynamics on endosomes (Gorenberg and Chandra 2017) (Fig. 1C).

DNAJC13 mutations (p.N855S) in autosomal-dominant PD (*PARK21*) noted first in a Dutch-German-Russian family of Mennonite origin, with multiple incidences (Table 1) (Vilarino-Guell *et al.* 2014; Gustavsson *et al.* 2015). Post-mortem brains of PD patients with *DNAJC13* mutations revealed strong immunoreactivity for Lewy bodies in the substantia nigra, along with brain areas implicated in non-motor symptoms of PD. Nigral Lewy bodies were also found to be co-localized with RME-8 (Vilarino-Guell *et al.* 2014). Unlike other DNAJC proteins implicated in PD, *in vitro* studies have revealed that the *DNAJC13* mutation confers toxic gain-of-function, leading to E-L cargo trafficking deficits (Norris *et al.* 2017). The *DNAJC13* mutation is also predicted to affect overall endosomal trafficking, even in the soma and postsynaptic compartment (Vilarino-Guell *et al.* 2014), unlike the synapse-specific dysfunction predicted for auxilin. Recently, the link between *DNAJC13* and PD has been muddled by an independent analysis of the same Mennonite family which did not find *DNAJC13* mutations. Instead, all the affected individuals were identified to carry mutations in *TMEM230* (Deng *et al.* 2016), which also functions in E-L system, perhaps at synapses. Hence, further studies are warranted to confirm the genetic link between *DNAJC13* and PD. Still, evaluating the RME-8/*DNAJC13* mediated E-L system dysfunction might provide valuable insights in understanding PD.

Synaptojanin-1 and endophilin A: Partners in crime?

Two other major synaptic endocytic proteins besides the DNAJ family that are implicated in PD are synaptojanin-1 (SYNJ1) and endophilin A. SYNJ1 is a Sac domain-containing protein with two different phosphatase domains; 5-phosphatase and Sac1, which target different phosphoinositide phosphates (PIPs) (McPherson *et al.* 1996). 5-phosphatase regulates SV endocytosis by dephosphorylating PI(4,5)P₂ to facilitate uncoating of CCVs in cooperation with Hsc70 and auxilin (Fig. 1c), whereas Sac1 domain has gained importance because of its link to PD (*PARK20*, Table 1) (Cremona *et al.* 1999; Mani *et al.* 2007; Saheki and De Camilli 2012). The p.R258Q missense mutations in the Sac domain were identified independently in two consanguineous families of Italian and Iranian origin. Homozygous missense mutations of *SYNJ1* Sac domain are noted to cause autosomal recessive PD with extrapyramidal features identical to patients with auxilin mutations (Krebs *et al.* 2013; Quadri *et al.* 2013; Olgiati *et al.* 2014). Interestingly, they responded poorly to L-dopa treatment, indicating the need for preclinical studies to understand the exact pathomechanisms. Recent observations by Cao *et al.*, suggest that the Sac domain plays a previously unappreciated role in clathrin uncoating. Mice with *SynJ1* Sac mutations (knock-in) developed PD-like characteristics and featured synaptic endocytosis defects, including accumulating of CCVs. Furthermore, elevated

auxilin and Parkin levels were noted in these mice, suggesting the profound effect of Sac mutations on other proteins linked to PD (Cao *et al.* 2017). Studies on fibroblasts from patients with *SYNJ1* mutations suggested defective endocytic trafficking (Quadri *et al.* 2013). Other studies have shed light on the involvement of SynJ1 in regulating synaptic autophagy. Mutations in the Sac1 domain of *SynJ1* in both *Drosophila* neurons and *PARK20* patient-derived iPSCs severely affected synaptic autophagosome formation but not endocytosis (Vanhouwaert *et al.* 2017). This discrepancy and the precise relationship between SV endocytosis and synaptic autophagy remains to be clarified. However, the *Drosophila* model revealed loss of dopaminergic neurons, providing evidence for the contribution of synaptic autophagy defects in pathogenesis of PD (Vanhouwaert *et al.* 2017). Considering its dual enzymatic activity, SynJ1 is also believed to play a role in fast endophilin-mediated, clathrin-independent endocytosis which is an alternative to CME (Watanabe *et al.* 2018) and several other processes. It remains to be determined if SYNJ1 exert its effects on PD pathogenesis through any of the alternative endocytic pathways.

SYNJ1 recruitment to endocytic membrane is facilitated by endophilin A (Fig. 1c), a protein drawing attention in PD pathogenesis, despite its locus variation only being identified as a risk factor (Table 1) (Chang *et al.* 2017). This is because of endophilin A's close association with SYNJ1 (Song and Zinsmaier 2003; Cao *et al.* 2017) and its link to most important proteins involved in PD. One of our earlier studies indicated that over-expression of α -synuclein decreases endophilin A levels, while deletion of synucleins had the opposite effect (Westphal and Chandra 2013). As noted in *SynJ1* knock-ins, endophilin KO also leads to synaptic vesicle endocytosis defects, accumulation of CCVs, elevation of Parkin levels, and other parkinsonian features (Milosevic *et al.* 2011; Cao *et al.* 2014). Gain-of-function mutations in LRRK2 hyper-phosphorylate endophilin A, the fly homolog, leading to synaptic endocytosis dysfunction (Matta *et al.* 2012). LRRK2-mediated phosphorylation of endophilin A is also noted to regulate synaptic autophagy (Soukup *et al.* 2016). Owing to the relatively higher frequency of LRRK2 mutations and its involvement in sporadic PD, endophilin A may prove to be another crucial protein involved in PD.

Autophagy in PD; cause and effect, hand in hand

Neurons need to constantly maintain homeostasis by preserving healthy organelles and functional proteins, while selectively capturing and eliminating damaged organelles and misfolded proteins. Autophagy, an integral component of the E-L system, is an intracellular proteostasis pathway that performs this housekeeping function (Mizushima *et al.* 1998;

Nixon 2013; Suresh *et al.* 2018b) (Fig. 1A, 1). It was initially identified in cell lines to function in maintaining energy homeostasis during critical periods of development and in response to nutrient depletion (Mizushima 2007). Intriguingly, nutrient depletion does not induce autophagy in neurons, but a complete lack of autophagy can cause lethal neurodegeneration, emphasizing its important role in survival (Hara *et al.* 2006; Komatsu *et al.* 2006). Since neurons are post-mitotic and long-lived, they are particularly vulnerable to accumulation of toxic proteins (Nixon 2013). In addition, greater dependency of neurons on mitochondria for energy instead of glycolytic pathways demands constant mitochondrial turnover (Kann and Kovacs 2007), leading to many malfunctioning mitochondria. Both these factors play major roles in the pathogenesis of PD.

PINK1 and Parkin: Role in mitophagy and beyond

Under physiological conditions, autophagy plays a crucial role in clearing unwanted proteins, as well as damaged mitochondria through a targeted mechanism called mitophagy (Nguyen *et al.* 2016). Loss-of-function mutations in *PINK1* and *Parkin* (*PARK2* and *PARK6*), result in recessively inherited early onset PD and significantly, both genes were discovered to be essential for mitophagy (Kitada *et al.* 1998; Mori *et al.* 1998; Valente *et al.* 2004). *PINK1* is a serine/threonine protein kinase that acts as a surveillance molecule for mitochondrial health and accumulates on the outer membrane of abnormal mitochondria (Nguyen *et al.* 2016). *PINK1* attracts recruitment of *Parkin* to damaged mitochondria, which through its E3 ubiquitin ligase activity (Narendra *et al.* 2010; Kane *et al.* 2014), coats the mitochondria with ubiquitin triggering autophagosomes to engulf the mitochondria (Narendra *et al.* 2008), culminating in lysosomal degradation (Fig. 1a1). Discoveries of *PINK1* and *Parkin* mutations were the first to shed light on the direct involvement of autophagy and E-L system in PD pathogenesis (Pickrell and Youle 2015). These observations also helped in validating previous neurotoxic studies (Przedborski *et al.* 2004) which identified mitochondrial dysfunction as one of the primary causes of neurodegeneration. Though the symptoms are indistinguishable from sporadic cases, several studies on brains of PD patients with *Parkin* mutations do not show α -synuclein aggregation and Lewy body pathology (Doherty and Hardy 2013). Only one report suggests *PINK1*-linked parkinsonism to be associated with Lewy pathology (Samaranch *et al.* 2010). This prompts us to speculate if there are alternative neuropathological cascades in PD that are α -synuclein independent but centered around the E-L system.

In addition to their well-defined roles in mitophagy, *Parkin* was identified to modulate E-L system through Rab7 ubiquitination (Song *et al.* 2016), whereas *PINK1* phosphorylates some Rab proteins (Lai *et al.* 2015). It is also worth noting that several of these Rab proteins (for example Rab7) regulate mitochondrial dynamics and quality control independent of

Parkin/PINK1 (Wang *et al.*, 2018). *Parkin* mutations are also known to directly affect retromer recruitment (Williams *et al.* 2018) and interact strongly with VPS35 by ubiquitinating it (Malik *et al.* 2015; Martinez *et al.* 2017). *Parkin* binds to endophilin A and functions in its ubiquitination (Cao *et al.* 2014). *Parkin* also ubiquitinates EPS15 to regulate epidermal growth factor receptors and phosphoinositide 3-kinase (PI(3)K)-Akt signaling that plays a role in neuronal survival (Fallon *et al.* 2006). These developments suggest mitophagy-independent routes these proteins may take toward PD. Further investigations of *Parkin* and *PINK1* in this direction might provide crucial insights in understanding neurodegenerative mechanisms that are Lewy-body independent.

Synaptic autophagy and axonal transport: a road less travelled in PD research

α -Synuclein aggregates initially in pre-synaptic termini, where the protein is enriched (Kramer and Schulz-Schaeffer 2007). α -Synuclein aggregates are a major target for autophagy-mediated degradation (Xilouri *et al.* 2008). In both genetic and sporadic PD, aggregated α -synuclein is likely to enhance autophagosome formation to meet the higher demand for its degradation (Wong and Holzbaur 2015). Once threshold reaches beyond compensatory limits, mutant/over-expressed α -synuclein starts to accumulate, turning detrimental to cellular homeostasis. In fact, A53T and A30P α -synuclein mutants blocked their own uptake and other substrates by lysosomes for degradation through the chaperone-mediated autophagy pathway *in vitro* (Cuervo *et al.* 2004). Dopamine-modified α -synuclein is identified to further exacerbate this process, which may explain the selective vulnerability of dopaminergic neurons in PD (Martinez-Vicente *et al.* 2008). A recent study on mammalian cells and transgenic mice shows that α -synuclein over-expression mediated autophagy defects are exerted through Rab1a inhibition (Winslow *et al.* 2010). Further understanding of the mechanisms for α -synuclein-autophagy crosstalk in PD might have crucial therapeutic applications. Backing this notion, both *in vitro* and *in vivo* studies show that enhancing autophagy and autophagosome-lysosome fusion could ameliorate PD-like features by clearing aggregated α -synuclein (Suresh *et al.* 2017; Suresh *et al.* 2018a).

Several other PD causing genes associated with the E-L system exert their pathological effects through impairing synaptic autophagy. The R258Q SynJ1 mutation directly affects autophagosome biogenesis and maturation at synaptic terminals of flies and neurites of human iPSC-derived neurons. The observation of dopaminergic neuronal loss in these flies suggests autophagosome generation defects as one of the possible mechanisms through which SynJ1 mutations lead to dopaminergic neurodegeneration and PD (Vanhouwaert *et al.* 2017). Pre-synaptic endocytic membrane bending protein endophilin A also has functions in providing docking sites for autophagy proteins and facilitating autophagosome expansion.

This is achieved by phosphorylation of the endophilin A-BAR domain by LRRK2, as noted in a fly model (Soukup and Verstreken 2017). Congruent to these observations, mice lacking endophilin A revealed deficiency in autophagosome formation and developed a parkinsonian phenotype (Murdoch *et al.* 2016). These studies, along with recent finding of clathrin involvement in autophagy regulation (Rong *et al.* 2012), suggest a cooperative cross-talk between SV endocytosis and autophagy.

Macromolecules and organelles in the pre-synaptic terminal travel a long distance from the soma, making them old and vulnerable. Rapid turnover of proteins, membranes, receptors, and vesicles in the pre-synapse assigns autophagy a major role in maintaining synaptic homeostasis. This tasks synaptic autophagy with quality control responsibilities. Though there is compelling evidence that compromised autophagy contributes to PD, these studies primarily looked at the soma from a proteinopathy or mitophagy perspective (Lynch-Day *et al.* 2012). Selective inactivation of autophagy by conditional knockout of autophagy protein atg7 in dopaminergic neurons leads to enhanced dopamine release and rapid pre-synaptic recovery with abnormal axonal profiles (Hernandez *et al.* 2012). Conversely, autophagy enhancement through mTOR inhibition slows dopamine release, reduces the number of synaptic vesicles, and increases autophagosome density in the axons (Hernandez *et al.* 2012). These results emphasize the importance of synaptic autophagy in normal dopamine transmission and further evaluation is required to understand if additional synaptic autophagy defects exist, which may lead to PD pathogenesis.

Another unique and stressful situation for neurons is that autophagosomes loaded with unwanted materials from an active pre-synaptic terminal, need to be transported a long distance to the soma for lysosomal degradation (Fig. 1b). Microtubule and scaffolding proteins aid in this retrograde axonal transport of autophagosomes and are crucial for the normal functioning of E-L system (Birdsall and Waites 2019). During their transport, autophagosomes mature into autolysosomes by fusing with late-endosomes/lysosomes towards the proximal end of axon (Nikolietopoulou and Tavernarakis 2018). There are recent studies suggesting lysosomal degradation, especially of mitophagosome mediated by Parkin-PINK1, occurs at the distal end. This is predicted to provide rapid neuroprotection against oxidative stress by damaged mitochondria, without requirement for retrograde transport (Ashrafi *et al.* 2014; Ashrafi and Schwarz 2015). Axonal transport aids this function through anterograde transport of E-L system components required for degradation from soma towards the distal end. Any defect in this to-and-fro transport machinery can completely clog the E-L system and other processes. Considering its functional significance in neuronal survival, axonal transport might hold clues for understanding pathological mechanisms. Abnormal

axonal swelling with lysosomal accumulation has been noted in Alzheimer's disease, blocking the retrograde transport of autophagosomes (Nixon *et al.* 2005; Gowrishankar *et al.* 2015). Similar understanding on the health of axonal transport machinery and its dynamics in PD is crucial toward finding commonalities amongst neurodegenerative diseases.

Sometimes, there are traffic issues: E-L system trafficking in PD

The multi-compartmentalized nature of neurons puts tremendous pressure on the endosomal sorting and trafficking machinery, which is also true in case of nigrostriatal neurons. The vacuolar protein sorting (VPS) group of proteins that form retromer complex comprising of VPS35, VPS26, and VPS29 are crucial in retrograde trafficking and sorting of protein cargos from endosomes to the TGN, facilitating their recycle and reuse (Fig. 1a) (Seaman *et al.* 1998; Haft *et al.* 2000). The retromer complex also functions in sorting proteins/cargo to regulate endosomal and lysosomal maturation (Vagnozzi and Pratico 2018). Pedigrees with single allelic mutation in *VPS35* revealed autosomal dominant, late-onset, L-dopa-responsive PD, with striking similarities to idiopathic PD (Wider *et al.* 2008; Vilarino-Guell *et al.* 2011). Broadly, it is proposed that the mutant *VPS35* fails to associate with other components of the retromer complex, resulting in impaired retrograde trafficking (Williams *et al.* 2017). Induced loss-of-function mutations of *VPS35* *in vitro* resulted in enlargement of late-endosomes and aggregation of α -synuclein oligomers (Follett *et al.* 2016). Another study on flies revealed ceramide accumulation because of *VPS35* deficiency, suggesting lysosomal dysfunction (Lin *et al.* 2018). α -Synuclein accumulation in nigral dopaminergic neurons, loss of dopaminergic neurons, reduced dopamine transporter levels in the striatum, and locomotory deficits were noted in *VPS35*-deficient mice (Tang *et al.* 2015a; Ishizu *et al.* 2016). Further *in vitro* studies of *VPS35* (D620N) mutant cells revealed dysfunction in endosome-to-Golgi retrieval of lysosomal membrane proteins (LAMP2a) and accelerated lysosomal degradation of LAMP2a (Tang *et al.* 2015a). LAMP2a is considered important in directing misfolded α -synuclein to lysosomes, suggesting a mechanism for α -synuclein accumulation in *VPS35* deficiency (Tang *et al.* 2015a). *VPS35* mutations also affect autophagy and dendritic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking (Zavodszky *et al.* 2014; Munsie *et al.* 2015). *VPS35* mutations bring severe abnormality in mitochondrial dynamics, as seen in mouse dopaminergic neuronal cultures and in PD patient derived fibroblasts (Tang *et al.* 2015b). Oxidative stress implicated in sporadic PD might mediate pathogenesis through *VPS35*-driven mitochondrial defects (Wang *et al.* 2016). *VPS35* also has a role in biogenesis of mitochondria-derived vesicles,

further suggesting that its dysfunction causes mitochondrial pathology and neurodegeneration (Sugiura *et al.* 2014). VPS35 is functionally linked to LRRK2, boosting its kinase activity (Mir *et al.* 2018). In fact, decreased VPS35 levels were noted in the SNpc of PD patients with *LRRK2* mutations (Zhao *et al.* 2018). Interestingly, over-expression of VPS35 ameliorated locomotor deficits and enhanced lifespan in *LRRK2* mutant models (MacLeod *et al.* 2013; Linhart *et al.* 2014), which was also seen in *Parkin* mutant models (Malik *et al.* 2015). Neuroprotection was also noted against MPP⁺ induced toxicity in dopaminergic neurons (Bi *et al.* 2013). Along with its E-L sorting functions, these links between VPS35 and α -synuclein, LRRK2, Parkin, lysosomal dysfunction, autophagy, mitochondrial perturbations, and oxidative stress strongly suggest VPS35 as a good therapeutic target.

Another VPS family member associated with PD is VPS13C. Five allelic variations in the *VPS13C* gene can lead to autosomal recessive, early-onset PD, primarily because of premature termination of VPS13C (Lesage *et al.* 2016; Schormair *et al.* 2018). Patients were noted with cardinal PD symptoms and extrapyramidal signs. Post-mortem studies revealed α -synuclein/ubiquitin positive-Lewy bodies in the brainstem, limbic system, hippocampus, and cortical associative areas (Lesage *et al.* 2016). A few studies reveal that VPS13 functions in the TGN-endosomal cycle (De *et al.* 2017), whereas recent discoveries have also located it on the outer mitochondrial membrane (Lesage *et al.* 2016). In fact, siRNA-mediated silencing of VPS13C resulted in lower mitochondrial membrane potential, mitochondrial fragmentation, increased respiratory rates, exacerbated Parkin-dependent mitophagy, and up-regulation of Parkin in response to mitochondrial damage (Lesage *et al.* 2016). A new study has revealed VPS13C's role in forming membrane contact sites and facilitating lipid transfer between the ER and mitochondria (Kumar *et al.* 2018). This is noteworthy, considering lipid transfer's increasing recognition in disease pathogenesis.

Lysosomes: PD may also begin at the end of the tunnel

Since lysosomes are the destination for most E-L system vesicles carrying a variety of cargos for degradation and recycling (Fig. 1a), dysfunction in any of its components results in lysosomal storage disorders, severely perturbing cellular homeostasis. A growing body of evidence suggests lysosomal storage disorders increase risk for PD pathogenesis (Robak *et al.* 2017). Among them, Gaucher disease (GD) confers the greatest risk, where 5–15% of GD patients or carriers develop PD later in life, to an extent where causal *GBA* mutations are the most common genetic risk factor for PD (Table 1) (Aharon-Peretz *et al.* 2004; Simon-Sanchez *et al.* 2009; Bultron *et al.* 2010). *GBA* encodes glucocerebrosidase 1 (Gcase1), which facilitates breakdown of glucosylceramide (GlcCer) into

glucose and ceramide. In its absence, GlcCer accumulates and undergoes partial enzymatic deacylation to glucosylsphingosine and other more complex glycosphingolipids, which mediate disease pathogenesis. *GBA* is a relatively well-studied E-L system gene with regards to PD, in terms of post-mortem brains, clinical investigations, and basic research. Reduced Gcase1 activity in substantia nigra, putamen, and other brain areas of PD patients with *GBA* mutations have been noted along with GlcCer and glycosphingolipids accumulation (Gegg *et al.* 2012; Alcalay *et al.* 2015). Interestingly, similar observations were made even in sporadic PD patients (Alcalay *et al.* 2015; Rocha *et al.* 2015), suggesting an even broader role for *GBA* than just mutations and potentially involve epigenetic and environmental modifiers of *GBA*. Gcase1 levels decrease with aging, the major risk factor for PD (Rocha *et al.* 2015; Hallett *et al.* 2018), indicating *GBA*'s possible role in lowering the nigral neurons' threshold to develop PD. Cognitive areas such as hippocampus, frontal cortex, and amygdala also showed relative decrease in Gcase1 activity, along with a significant elevation of hippocampal glucosylsphingosine in sporadic cases (Gegg *et al.* 2012). This correlates with a higher Lewy body burden identified in the hippocampus and medial temporal regions, as well as in a diffused cortical Lewy body pathology in *GBA*-linked PD (Watson and Leverenz 2010; Hallett *et al.* 2018). These pathological observations strengthen clinical findings that cognitive deterioration is severe in PD patients with *GBA* mutations compared to that of sporadic cases (Liu *et al.* 2016; Mata *et al.* 2016). These features indicate that mouse models of PD with *GBA* mutations are important in studying lipid dyshomeostasis and mechanisms of cognitive dysfunction in PD.

A recent study by Taguchi *et al.*, provides evidence for an association between loss of Gcase1 activity, glycosphingolipids accumulation, and α -synuclein aggregation, both *in vitro* and *in vivo* models of *Gba* mutation (Taguchi *et al.* 2017). Glucosylsphingosine which promoted pathological α -synuclein aggregates, capable for further self-templating, was shown to accumulate in the brains of young homozygous *Gba* mutant and KO mice. This strongly implicates glucosylsphingosine in PD pathogenesis. Even in heterozygous *Gba* mutations, the Gcase1 activity decreased with age to an extent where mice reached threshold for haploinsufficiency, mimicking the human condition. Furthermore, the *Gba* mutant mice, when crossed with well-characterized α -synuclein transgenic PD mice, revealed accelerated PD progression including α -synuclein aggregation and motor deficits (Taguchi *et al.* 2017). α -Synuclein aggregation has also been seen in human iPSCs-derived neurons from GD and PD patients that are exposed to Gcase1 inhibitors, and in those carrying *GBA* mutations (Zunke *et al.* 2018). Heterozygous mutations in *GBA* also cause misprocessing of Gcase1 in the ER, leading to ER retention of Gcase1. This produces ER stress and auto-lysosomal perturbations causing an abnormal lipid profile and increased α -synuclein

accumulation and secretion (Ron and Horowitz 2005; Fernandes *et al.* 2016). This study along with a few other observations suggest dopaminergic dysfunction because of gain-of-function mutations in *Gba* (Cullen *et al.* 2011; Sidransky and Lopez 2012; Fernandes *et al.* 2016). However, therapeutic strategies based on loss-of-function observations have been successful, arguing against a gain-of-function model. Activation of residual Gcase1 by small molecules and chaperones and ameliorating glycosphingolipid accumulation by inhibiting GlcCer synthase lowered α -synuclein pathology and reinstated lysosomal health (Richter *et al.* 2014; Sardi *et al.* 2017; Charvin *et al.* 2018). These strategies are already being tested in clinics (Richter *et al.* 2014).

ATPase cation transporting 13A2 (ATP13A2), another lysosomal protein which localizes on its membrane (Fig. 1a) with unknown function, has been implicated in PD (*PARK9*, Table 1). Autosomal recessive, early-onset parkinsonism because of *ATP13A2* mutations first noted in Kufor-Rakeb Syndrome, followed by recent studies in other forms of parkinsonism (Ramirez *et al.* 2006; Di Fonzo *et al.* 2007; Park *et al.* 2011; Eiberg *et al.* 2012). Fibroblasts from these patients showed impaired lysosomal acidification and proteostasis, leading to inefficiency in substrate degradation and autophagosome clearance. These observations were replicated in *ATP13A2* knockdown dopaminergic cell lines, where its restoration re-established lysosomal homeostasis and ameliorated cell death (Dehay *et al.* 2012). Down-regulation of *ATP13A2* was also seen in nigral neurons of sporadic PD patients, along with *ATP13A2* traces in Lewy bodies (Dehay *et al.* 2012). A loss-of-function *ATP13A2* mutant mouse model revealed age-related lysosomal dysfunction, protein aggregation, and gliosis leading to motor abnormalities. Interestingly, the levels of total and oligomeric α -synuclein was normal, despite ubiquitinated protein aggregation, cathepsin-D dysfunction, and autophagy defects (Kett *et al.* 2015). These observations suggest that *ATP13A2* mutations may lead to PD through an α -synuclein independent mechanism.

E-L system; a key target of key PD-associated proteins?

α -Synuclein

Misfolded α -synuclein can trigger PD pathogenesis in the pre-synaptic terminals of nigrostriatal neurons (Kramer and Schulz-Schaeffer 2007; Spinelli *et al.* 2014; Imbriani *et al.* 2018). This may well be one of the earliest pathogenic events in PD, where α -synuclein aggregates target SV endocytosis, SV trafficking, and autophagy components of E-L system to initiate or exacerbate dopaminergic neuronal dysfunction.

α -Synuclein and other family members function in pre-synaptic terminals, where they modulate SV distribution and neurotransmission (Greten-Harrison *et al.* 2010; Burre 2015;

Vargas *et al.* 2017) (Fig. 1c). The biophysical properties of synucleins reveal their ability to sense and modify membrane curvature (Middleton and Rhoades 2010; Shen *et al.*, 2012; Westphal and Chandra 2013). These functions suggest that synucleins regulate SV exocytosis and endocytosis (Greten-Harrison *et al.* 2010; Vargas *et al.* 2014). Studies in KO mice in fact show that synucleins normally function post SV exocytosis, in fusion pore expansion and/or control the kinetics of SV endocytosis by facilitating membrane curvature (Vargas *et al.* 2014; Lautenschlager *et al.* 2017; Logan *et al.* 2017). α -Synuclein also appears to control SV availability for release by regulating SV clustering (Diao *et al.* 2013; Vargas *et al.* 2017).

On a pathological level, several studies show that over-expression of α -synuclein inhibits neurotransmission. The locus of this consistent effect is not clear. Studies have shown that elevated α -synuclein levels and oligomeric α -synuclein inhibit SV exocytosis in a SNARE-dependent manner or by affecting SV clustering (Nemani *et al.* 2010; Huang *et al.* 2019). On the other hand, considerable evidence suggests that aggregated α -synuclein primarily impacts SV endocytosis, which is of relevance to E-L system genes linked to PD. In lamprey giant synapses, injection of aggregated α -synuclein inhibit clathrin uncoating as evidenced by the accumulation of CCVs when SV recycling was evoked by high-frequency stimulation (Busch *et al.* 2014). A recent study also suggests that extra synuclein might sequester Hsc70, thus affecting clathrin uncoating and subsequently causing SV recycling defects (Banks *et al.*, 2019). A similar observation was made in mammalian Calyx of Held synapse, where acute elevation of monomeric and mutant α -synuclein levels decreased SV endocytosis with a frequency-dependent effect on SV exocytosis. This was also seen upon transgenic expression of α -synuclein (Xu *et al.* 2016; Eguchi *et al.* 2017). Over-expression of mutant α -synuclein also affects dopamine transporter trafficking on the pre-synaptic membrane, leading to dopamine dyshomeostasis (Kisos *et al.* 2014). The observations that over-expression and deletion of synucleins have similar effects on SV exo- and endocytosis (Lautenschlager *et al.* 2017) suggest that SV cycling is exquisitely sensitive to α -synuclein levels. Further understanding of the precise mechanisms for interactions of α -synuclein and synaptic E-L system proteins involved in PD will provide crucial insights on early causal pathogenic events in PD.

At the soma, aggregated α -synuclein mainly targets E-L system trafficking, especially the retromer pathway. Recent studies propose that aggregated α -synuclein disrupts the retromer complex by interacting through VPS26 or VPS29, and thus preventing the recruitment of adaptors like Snx3 and SNX-BAR dimers (Chung *et al.* 2017; Patel and Witt 2018). Deficiency of VPS35 in dopaminergic neurons impairs endosome-to-Golgi retrieval of receptors like LAMP2a,

enhancing its lysosomal degradation. LAMP2a is a critical receptor for chaperone-mediated autophagy of misfolded α -synuclein, so its deficiency leads to a vicious cycle of α -synuclein accumulation (Tang *et al.* 2015a), which might be the case in patients with VPS35 mutations. Over-expression of α -synuclein also directly compromises macroautophagy through inhibition of Rab1a (Winslow *et al.* 2010). It is also pertinent to note that aggregated α -synuclein in the soma is a key target of treatment strategies in development through induction of macroautophagy (Suresh *et al.* 2017; Suresh *et al.* 2018a).

Recent developments suggest that α -synuclein pathology spreads in a prion-like manner through synaptically connected regions in a manner consistent with Braak staging (Braak *et al.* 2003; Luk *et al.* 2012). The E-L system plays a prominent role in cell-to-cell transmission of preformed α -synuclein filaments, leading to the spread of phospho- α -synuclein pathology. Internalization of fibrillar α -synuclein occurs via endocytosis (as well as tunnelling nanotubules) and follows the neuronal endosomal pathway to lysosomal degradation (Rodriguez *et al.* 2018). Heparan sulfate proteoglycans, lymphocyte-activation gene 3, $\alpha 3$ isoform of Na^+/K^+ ATPase, and a few endocytic vesicle receptors are predicted to act as receptors for α -synuclein preformed fibrils (Holmes *et al.* 2013; Shrivastava *et al.* 2015; Mao *et al.* 2016; Ihse *et al.* 2017). *In vitro* studies suggest that internalized α -synuclein fibrils are localized in both early endosomes and late endolysosomes (Lee *et al.* 2008b; Karpowicz *et al.* 2017). The lipid bilayer of endosomes restricts cytoplasmic spillage, so most endocytosed α -synuclein is degraded by lysosomes. How a fraction of α -synuclein preformed fibrils escapes this barrier and goes on to initiate seeding still remains unclear (Bieri *et al.* 2018). Studies of α -synuclein axonal transport predict that retrograde transport of α -synuclein preformed fibrils is mediated by endocytic vesicles and is twice as fast and more efficient than anterograde transport (Brahic *et al.* 2016). This strengthens the notion that PD pathology spreads retrogradely from the synaptic terminal to soma. The E-L system is also involved in exocytosis of fibrillized α -synuclein, facilitating its prion-like spread. An ER-bound enzyme with chaperone activity USP19 (ubiquitin-specific protease 19) has been shown to deliver misfolded α -synuclein to late endosomes, which then fuses with the plasma membrane through a unique process called misfolding-associated protein secretion (Lee *et al.* 2016). Another recent study suggests that the DNAJ/Hsc70 complex could facilitate α -synuclein exocytosis (Fontaine *et al.* 2016). Over-expression of *ATP13A2* and *GBA* have been shown to increase exocytosis of α -synuclein (Tsunemi *et al.* 2014; Fernandes *et al.* 2016). Together, these observations identify the E-L system as the primary conduit for the spread of α -synuclein-mediated pathology in PD.

LRRK2

Leucine-Rich Repeat Kinase 2 (LRRK2) is a multidomain large protein with both GTPase and kinase activity along with other domains for protein-protein interactions (Kett and Dauer 2012). Epidemiological, histopathological, and clinical investigations have all established *LRRK2* gene mutations as the most common genetic cause of PD (*PARK8*, Table 1) (Paisan-Ruiz *et al.* 2004; Zimprich *et al.* 2004), with a considerable contribution to sporadic cases (Kett and Dauer 2012). Over eight identified *LRRK2* mutations cause PD. The mutation at G2019S is the most common and is known to increase LRRK2 kinase activity (West *et al.* 2005; Smith *et al.* 2006; Kett and Dauer 2012). LRRK2 functions have been explored through PD-related studies, which have revealed its close association with the E-L system. LRRK2's role in vesicular trafficking, lysosomal homeostasis, and autophagy are well-accepted. Phosphorylation by LRRK2 is essential for a subset of RAB GTPases' function of E-L vesicular sorting and trafficking (Steger *et al.*, 2016; Steger *et al.*, 2017). In an over-expression system, RAB7L1 was noted to interact strongly with LRRK2 at the switch-II site (Steger *et al.*, 2017), unlike as seen in *in vitro* kinase assays (Steger *et al.*, 2016). Several *LRRK2* mutations significantly affect RAB pathways, where variations in Rab7L1's interactions with LRRK2 have been identified to increase the risk of sporadic PD. In fact, recent GWAS identify *RAB29* encoding RAB7L1 (*PARK16*, Table 1) as an independent risk gene for PD (Satake *et al.* 2009). Rab7L1 up-regulation could attenuate LRRK2-induced toxicity in dopaminergic neurons (MacLeod *et al.* 2013). This is predicted to be functioning through VPS35 and VPS26 (Linhart *et al.* 2014). VPS35 also cooperates with LRRK2 to regulate SV recycling and dopaminergic synaptic release (Inoshita *et al.* 2017), thus suggesting a prominent role for the retromer complex in LRRK2-mediated PD pathogenesis.

LRRK2 regulates lysosomal protein trafficking and morphology (Kuwahara *et al.* 2016; Eguchi *et al.* 2018). In *LRRK2* mutants, perinuclear lysosomal clustering was noted, mediated by increased phosphorylation of RAB7 (Hockey *et al.* 2015). Two-pore channels are the calcium channels present on acidic vesicles of the E-L system and help in regulating their trafficking and fusion of lysosomes with autophagosomes/endosomes (Calcraft *et al.* 2009). Abnormal lysosomal morphology and density seen in fibroblasts from patients with LRRK2 mutations could be reversed by inhibiting these two-pore channels (Hockey *et al.* 2015). Thus, these channels might be one potential mediator of E-L system dysfunction in LRRK2-associated PD. Lysosome-related proteins involved in PD such as Gcase1, LAMP2A, and ATP13A2 are also down-regulated in PD patients with *LRRK2* mutations (Zhao *et al.* 2018), suggesting that lysosomes are one of LRRK2's prime targets.

Mechanisms for LRRK2-mediated autophagy defects are still elusive. The ability of LRRK2 kinase inhibitors to reverse autophagy dysfunction suggest that kinase activity may be significant (Manzoni *et al.* 2013; Saez-Atienzar *et al.* 2014). Mutant *LRRK2* is also a substrate for chaperone-mediated autophagy, impairment of which could exacerbate pathological implications (Orenstein *et al.* 2013). Accumulation of MVBs and malformed autophagosomes were noted in *LRRK2* R1441C mutations (Alegre-Abarrategui *et al.* 2009). Intra-axonal autophagic vacuole defects in nigral dopaminergic projections were seen in mice expressing human *LRRK2* (Li *et al.* 2009; Tagliaferro *et al.* 2015). *LRRK2* also regulates synaptic autophagy through phosphorylation of endophilin A (Soukup *et al.* 2016). G2019S mutations are known to induce autophagy through MAPK/ERK kinases (Bravo-San Pedro *et al.* 2012; Bravo-San Pedro *et al.* 2013), and some studies suggest that *LRRK2*-mediated autophagy regulation is largely independent of mTOR (Manzoni *et al.* 2013). Since RAB-mediated trafficking is crucial for autophagy, which is also affected in *LRRK2* mutations, it is important to evaluate if autophagy defects are the consequences of RAB dysregulation or a direct effect of *LRRK2* mutations.

LRRK2 regulates the SV cycle by interacting with E-L system proteins that are also implicated in PD: auxilin, SYNJ1, and endophilin A. *LRRK2* overactivity phosphorylates auxilin at its clathrin binding domain, making their interaction weaker. This leads to disruption of SV endocytosis (Nguyen and Krainc 2018). This was seen in patient iPSC-derived dopaminergic neurons, along with accumulation of oxidized dopamine, increased α -synuclein levels, and decreased Gcase1 activity (Nguyen and Krainc 2018). Hyperphosphorylation of endophilin A and synJ1 by *LRRK2* significantly altered synaptic endocytosis (Matta *et al.* 2012; Islam *et al.* 2016; Pan *et al.* 2017). A recent study showed that *LRRK2* can directly bind to clathrin-light chains through its GTPase domain, affecting Rac1-mediated endosomal dynamics (Schreij *et al.* 2015). *LRRK2* also perturbs synaptic exocytosis through phosphorylation of N-ethylmaleimide-Sensitive Factor which up-regulates SNARE disassembly (Belluzzi *et al.* 2016). Furthermore, *LRRK2* is highly expressed in dorsal striatal medium spiny neurons (MSNs), the post-synaptic compartment of nigrostriatal pathway. G2019S mutation of *LRRK2* leads to abnormally high activity of MSNs and morphological perturbations during development (Matikainen-Ankney *et al.* 2016). Abnormal synaptogenesis and dopamine receptor activation in the developing MSNs mediated by dysfunctional protein kinase A was seen in *LRRK2* R1441C mutation (Parisiadou *et al.* 2014). These defects can permanently modify the circuit structure and function, predisposing the individuals to both motor and non-motor dysfunctions of PD. *LRRK2* mutation-mediated endocytosis defects were noted predominantly in these MSNs, along with nigral dopaminergic

neurons and not in cortical or hippocampal neurons (Pan *et al.* 2017; Maas *et al.* 2017). This suggests a heightened vulnerability in the E-L system of nigrostriatal circuit for *LRRK2* mutations.

Increased *LRRK2* kinase activity mediated elevation of exocytosis may also be relevant for cell-to-cell transfer of misfolded α -synuclein (Lin *et al.* 2009; Bae *et al.* 2018; Schapansky *et al.* 2018). *LRRK2* can be selectively recruited to overloaded lysosomes and divert undegraded materials out of the kidney cells, along with secretion of α -synuclein from endosomes (Eguchi *et al.* 2018). Studies also demonstrate heightened uptake of α -synuclein fibrils through endocytosis in the presence of pathogenic *LRRK2* (Bae *et al.* 2018). However, *LRRK2*'s role in prion-like spreading of α -synuclein remains speculative, until confirmation that similar mechanisms exist in neurons.

Future directions

Clinical description and diagnosis of PD has changed little since James Parkinson's characterization of the disease 200 years ago (Parkinson 2002). In the 1960s, identification of dopamine deficiency as the cause of motor abnormalities led to symptomatic relief for patients (Carlsson *et al.* 1957). Although some success has been noted with deep brain stimulation (Limousin *et al.* 1998), we are still relying on symptomatic relief. The last three decades of basic research have enriched our understanding of PD's cellular and molecular mechanisms, bringing tangible hope for disease modifying treatment strategies. Identification of risk genes and conceptual shifts in our understanding through genetic models point to E-L system dysfunction as the key mediator of PD pathogenesis. Further strengthening this concept, PD pathogenesis appears to begin at synaptic terminals where endo- and exocytotic machinery is most active and where α -synuclein and *LRRK2* exert their effects through the E-L system and vice-versa. Studies so far suggest that altered flux through the E-L system in nigral dopaminergic neurons that have lesser threshold for degeneration is the key trigger for neurodegeneration. Moving forward, critical elucidation of the mechanisms behind sorting, trafficking, and recycling defects demands detailed studies of auxilin and other DNAJ family proteins, along with SYNJ1, endophilin A, and the retromer complex. Studying these proteins/genes will also provide crucial insights into less explored roles of synaptic autophagy, axonal transport, and clathrin-independent endocytosis in PD. Recent surveys of PD patients note preservation of cognitive functioning as a major unmet need. Further investigation of *GBA* mutations implicated in PD is a promising avenue considering *GBA*'s known involvement in cognitive disorders like Dementia with Lewy Bodies. Along with *GBA*, studying *ATP13A2* and other lysosomal genes will enrich our understanding of lysosomal dysfunction, potentially bringing us closer to repurposing drugs used

in lysosomal storage disorders for PD. Affirming ongoing clinical trials targeting autophagy, α -synuclein, and LRRK2, an enhanced understanding of E-L system-mediated pathogenesis will prove crucial in developing novel treatment strategies and diagnostic tools for PD.

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Subcellular Fractionation for the Isolation of Synaptic Components from the Murine Brain

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Abstract

Synaptic terminals are the primary sites of neuronal communication. Synaptic dysfunction is a hallmark of many neuropsychiatric and neurological disorders. The characterization of synaptic sub-compartments by biochemical isolation is, therefore, a powerful method to elucidate the molecular bases of synaptic processes, both in health and disease. This protocol describes the isolation of synaptic terminals and synaptic sub-compartments from mouse brains by subcellular fractionation. First, sealed synaptic terminal structures, known as synaptosomes, are isolated following brain tissue homogenization. Synaptosomes are neuronal pre- and post-synaptic compartments with pinched-off and sealed membranes. These structures retain a metabolically active state and are valuable for studying synaptic structure and function. The synaptosomes are then subjected to hypotonic lysis and ultracentrifugation to obtain synaptic sub-compartments enriched for synaptic vesicles, synaptic cytosol, and synaptic plasma membrane. Fraction purity is confirmed by electron microscopy and biochemical enrichment analysis for proteins specific to sub-synaptic compartments. The presented method is a straightforward and valuable tool for studying the structural and functional characteristics of the synapse and the molecular etiology of various brain disorders.

Introduction

Synapses are the basic computational units of the brain through which neurons communicate and exert diverse and exquisitely complex functions. Synapses are, thus, fundamental to the health of the brain¹; synaptic dysfunction is implicated as a source or result of many disorders². Synapses are constituted by pre- and post-synaptic terminals, extensions of two different neurons that are closely apposed

and separated by a synaptic cleft traversed by synaptic adhesion molecules. Information flows from the pre- to post-synaptic compartment in the form of chemical messengers called neurotransmitters¹. The molecular processes involved in neurotransmission are active areas of research^{3,4,5}. Understanding the pathogenic processes within synaptic terminals and the response of synapses to pathology

in other neuronal sub-compartments are crucial steps to addressing disorders of the brain^{1,2}. Several methodological advancements, predominantly applied to murine models, have advanced this pursuit⁶. The isolation of synaptic fractions by differential centrifugation is one such paradigm-shifting method that has enabled the detailed evaluation of synaptic processes in health and disease.

The adult human brain consists of 80-90 billion neurons^{7,8}. Among murine species, the rat brain contains approximately ~200 million neurons, while mice have ~70 million^{9,10}. Each neuron forms thousands of specific synaptic connections with a network of highly polarized neurons intermingled with glial cells and dense vasculature. In such complex and heterogeneous tissue, it was once unthinkable to isolate and study synapses as an independent system. In the 1960s, Victor Whittaker, Catherine Hebb, and others made this possible by isolating intact synaptic terminals using subcellular fractionation^{11,12,13,14}. In an attempt to isolate synaptic vesicles (SVs), they homogenized brains through liquid shear force in iso-osmotic (0.32 M) sucrose followed by ultracentrifugation. They obtained pinched-off, plasma membrane-enclosed, intact nerve terminals or varicosities, which they called nerve-ending particles (NEPs)^{11,13}. As the structural and functional characteristics of the synapse were preserved in these structures, NEPs were later termed "synaptosomes" for congruence with other subcellular organelles^{13,15}. It is worth noting that the work of Eduardo de Robertis and colleagues, who coined the term "synaptic vesicle", overlapped with that of Whittaker and colleagues and contributed to the validation of "synaptosome" isolation and characterization^{16,17,18}.

Synaptosomes are physiologically active structures that contain all the cellular and molecular properties required for

the storage, release, and reuptake of neurotransmitters^{13,18}. The preservation of key synaptic characteristics *in vitro* and freedom from non-synaptic components also contribute to the utility of this isolation method. Synaptosomes have contributed immensely to the understanding of the chemical and physiological properties of neurotransmission and are now being used to study synaptic molecular processes and their alterations in disease^{19,20,21,22,23}. Synaptosomes are also the initial source material for isolating synaptic components such as SVs, clathrin-coated vesicles (CCVs), synaptic cytosol, synaptic plasma membrane, synaptic mitochondria, synaptic adhesion molecules, and other components of interest, which can facilitate the understanding of the molecular mechanisms of synaptic function^{18,19,20,24,25,26,27,28}. These sub-synaptic components can be obtained by the osmotic lysis of synaptosomes and sucrose density gradient ultracentrifugation^{15,29}. Although the original subcellular fractionation method by Whittaker's research group is known to be efficient in isolating quality synaptosomes and SVs^{13,30}, recent optimizations enhance the purity of the subcellular fractions^{22,23,31,32}. This article provides a highly detailed and accessible version of a classic protocol for the subcellular fractionation of murine brain tissue to isolate synaptosomes, SVs, and other sub-synaptic components.

Protocol

All experiments with mice were approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University (Protocol 2021-11117) and performed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Animal care and housing complied with the Guide for the Care and Use of Laboratory Animals³³ and were provided by the Yale Animal

Resource Center (YARC). Animals were maintained in a 12 h light/dark cycle with *ad libitum* access to food and water. Five to eight mice or two to four rats per genotype or condition are required for the following protocol. Fewer rats are necessary due to their larger brain volumes. Similarly, the age of the experimental animals may affect fraction yield; additional mice may be required for ages less than 2 months. Otherwise, the outlined procedures apply to both murine species and healthy adult animals of any age. The representative data presented in this study utilized wild-type (C57BL/6J) mice

(age = 2 months; four males and four females per replicate) obtained from a commercial source (see **Table of Materials**).

1. Experimental preparation

NOTE: This protocol requires ~11 h for a single researcher to complete. It is highly recommended to complete benchtop setup (**Figure 1**), buffer preparation (**Table 1**), the precooling of centrifuges and rotors to 4 °C, and the collection and labeling of necessary materials and equipment (see **Table of Materials**) the day prior to protocol execution, where applicable.



Figure 1: Benchtop setup. Prior to brain dissections, (A) Dounce glass homogenizers and (B) all buffers were chilled on ice. (C) Protease inhibitor stock solutions were thawed on ice. A second container of wet ice for centrifuge tubes, a Dewar of liquid nitrogen (not shown), and (D) a container of dry ice for short-term storage of the samples flash-frozen in liquid nitrogen were obtained. (E) Microcentrifuge tubes were pre-labeled for all samples, as four aliquots of each subcellular fraction sample per genotype or condition were collected during this procedure (time-saving tip: thoroughly label all the tubes the day before the experiment is performed). (F) An appropriate biohazard waste container, (G) 70% ethanol, (H) surgical tools, and (I) an absorbent surface pad. The required centrifuge tubes and disposables were set aside for efficient access during protocol implementation (not shown). [Please click here to view a larger version of this figure.](#)

1. Prepare the benchtop for surgery and collect the scissors and forceps required for brain excision (see **Table of**

Materials). Pre-label 1.5 mL microcentrifuge tubes for

mouse tail biopsies and four tubes per collected fraction, as outlined in **Figure 2**.

- Obtain two containers of wet ice, one container of dry ice, and a benchtop liquid nitrogen Dewar flask.
- Thaw phenylmethylsulfonyl fluoride (PMSF), pepstatin A, aprotinin, and leupeptin stock solutions on ice (see **Table of Materials**). Prepare the necessary buffers (**Table 1**).

NOTE: Sucrose solutions can be prepared in advance and stored at 4 °C. However, protease inhibitors (thawed stocks and tablets) must be added fresh to all the buffers at the start of the experiment due to the instability of

these reagents in aqueous solutions. Further, all the buffers must be prepared with detergent-free glassware and detergent-free water to enable the collection of intact synaptosomes.

- Chill all the buffers and glass Dounce homogenizers (see **Table of Materials**) on ice. Set the centrifuges to 4 °C and chill the rotors to 4 °C.
- Add 14 mL of Buffer A (**Table 1**) to a Dounce homogenizer on ice.

Table 1: Composition of the subcellular fractionation buffers. [Please click here to download this Table.](#)

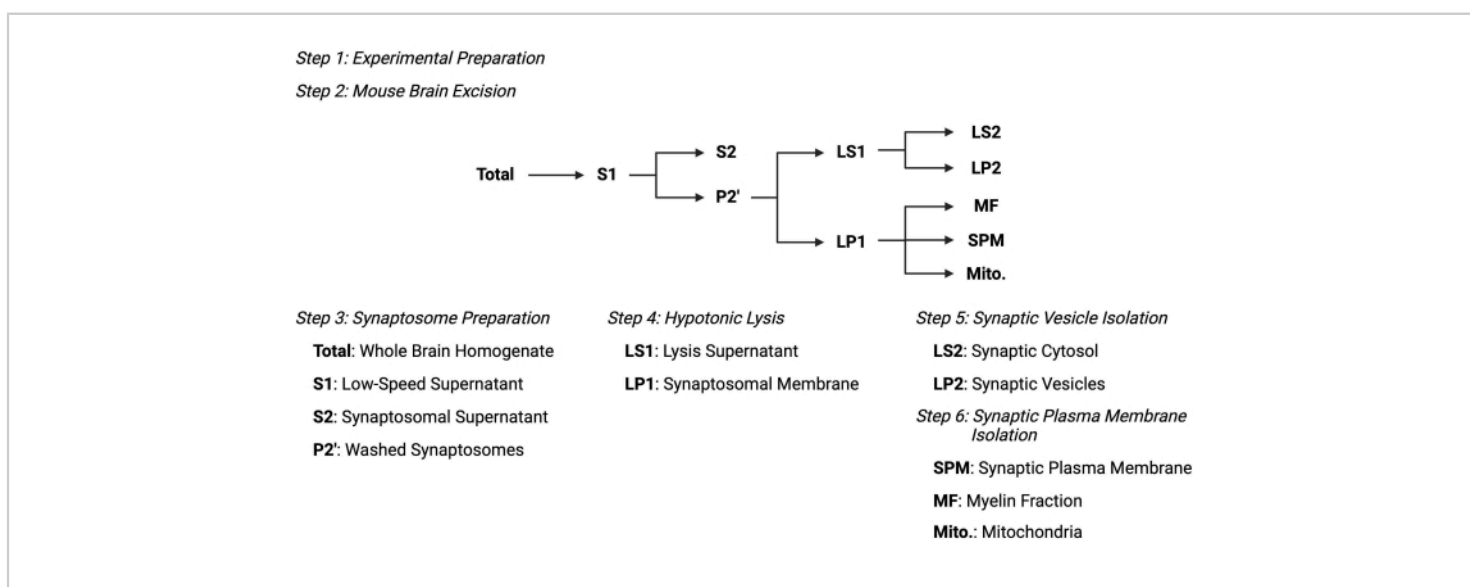


Figure 2: Overview of the subcellular fractionation protocol. Summary schematic of the subcellular fractionation steps and collected samples. [Please click here to view a larger version of this figure.](#)

2. Mouse brain excision

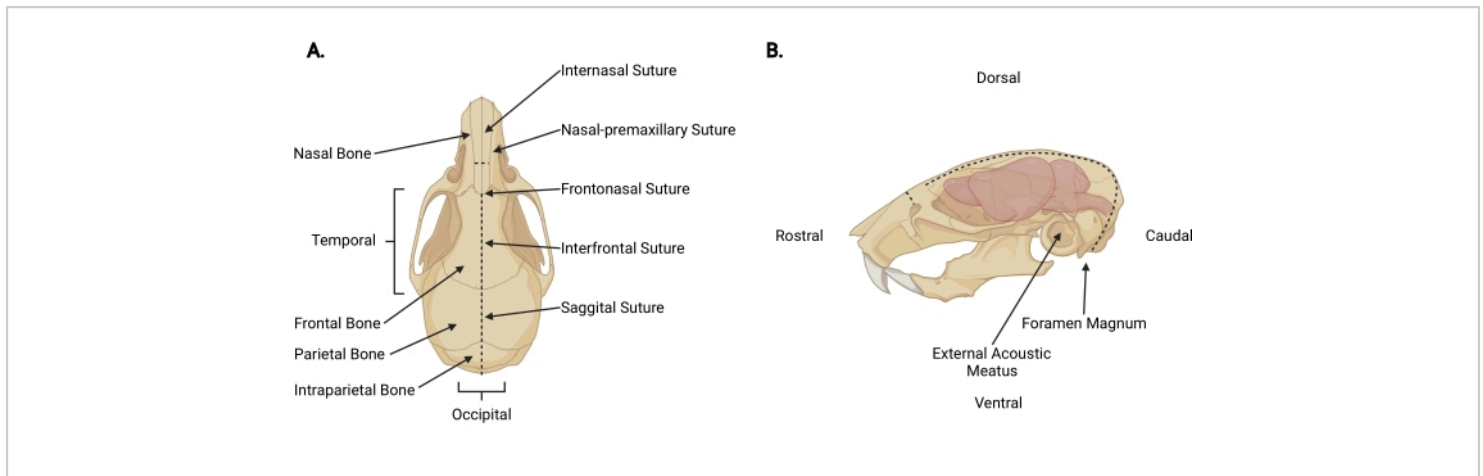


Figure 3: Craniofacial anatomy. (A) Dorsal view of a mouse skull with relevant cranial structures indicated. (B) Left lateral view of a mouse skull and brain with relevant cranial structures and anatomical directions indicated. The dashed lines represent the locations where incisions should be made. [Please click here to view a larger version of this figure.](#)

1. Deeply anesthetize each mouse with 100% isoflurane in an anesthesia chamber located in a fume hood or biosafety cabinet using an open drop method³⁴. Sacrifice each mouse by cervical spine dislocation followed swiftly by decapitation. Alternate between genotypes or experimental groups for each sacrifice and dissection¹². Obtain tail biopsies after euthanasia by excising 2 mm of the distal tail tip with fine scissors. Store the tissue for genotyping.
 2. Spray the decapitated head with 70% ethanol to prevent hair from adhering to the tissue and surgical instruments during dissection.
 3. Insert fine scissors under the skin at the decapitation incision to a pericranial depth and make a midsagittal incision up to the internasal suture (**Figure 3A**) to retract the scalp from the skull.
 4. Working from the occipital area toward each temporal aspect, trim the fascia and muscle to expose the external surface of the skull beyond each external acoustic meatus (**Figure 3B**).
 5. Secure the scalp and rostral aspect of the skull with the non-dominant hand. With the other, insert fine scissors 2 mm into the caudal side of the foramen magnum, where the spinal cord is visible exiting. Make a midline incision until the scissors reach the internal surface of the intraparietal bone (**Figure 3**; dashed lines).
- NOTE:** During the initial incision, the scissors must be parallel to the spinal cord with pressure applied toward the internal surface of the skull to prevent damage to the brainstem and cerebellum.
6. Change the angle of the scissors so the blades run parallel with the dorsal surface of the skull. Continue advancing the midsagittal incision rostrally through the parietal and frontal bones, using the sagittal and interfrontal sutures as a guide. Use constant upward pressure to avoid damage to the cortex. Terminate the incision just beyond the internasal suture (**Figure 3A**).

- Make a small perpendicular incision (~3 mm) to the nasal bone, rostral to the internasal suture, by placing the scissors perpendicular to the skull with each blade positioned at a nasal-premaxillary suture and making one even cut (**Figure 3**; dashed lines).

NOTE: This step will increase the ease of retracting the skull and will be critical for collecting the olfactory bulb if this area is of interest.

- While securing the rostral aspect, use one side of a pair of textured forceps to gently lift the skull up from the brain, then laterally and ventrally. Repeat along the midline as

needed, then for the other hemisphere until the entire brain surface is exposed.

- Using curved forceps or a fine spatula, gently lift the rostral side of the brain. Cut the optic and cranial nerves to complete the excision from the skull.
- For each condition, collect five to eight mouse brains together into the chilled glass Dounce homogenizer containing 14 mL of Buffer A (**Table 1**).

3. Synaptosome preparation

- NOTE:** The schematics of this procedure are shown in **Figure 4**.

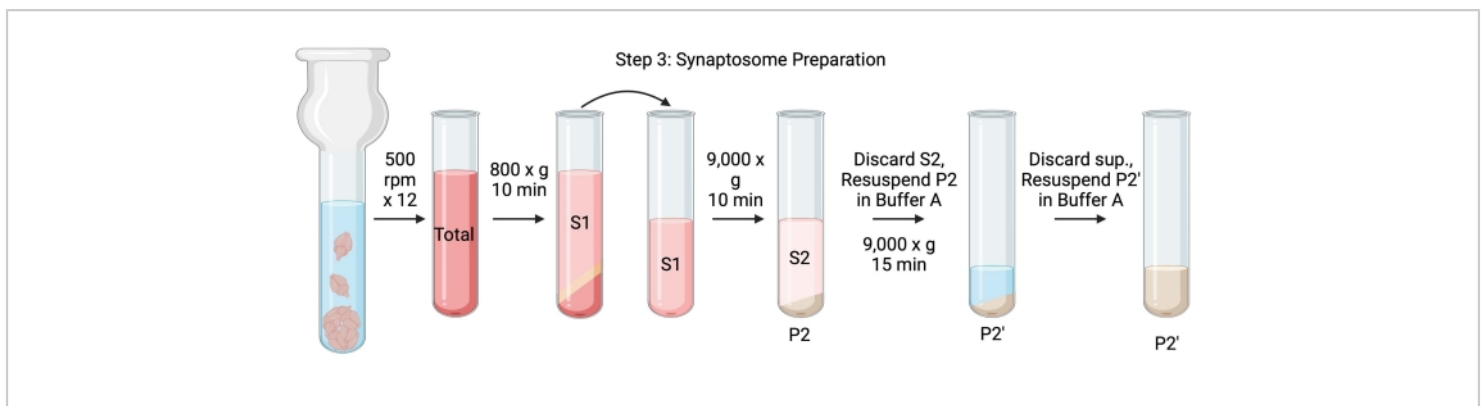


Figure 4: Synaptosome preparation. Schematic of step 3, the generation of synaptosomes (P2'). [Please click here to view a larger version of this figure.](#)

- Homogenize the brains using a glass Dounce homogenizer in 12 up-down passes at 500 rpm (total). Pause briefly at each downstroke to ensure thorough homogenization of the tissue. Homogenize preferentially in an ice bath to avoid warming and protein denaturation. Take 5 μ L aliquots for protein concentration determination by the bicinchoninic acid assay (BCA, see **Table of Materials**). Take 100 μ L of whole brain lysate aliquots for western blot (WB). For this and all

subsequent samples (**Figure 2**), take two aliquots for BCA and two aliquots for WB. Flash-freeze all the collected aliquots in liquid nitrogen and store them at -80°C .

- Spin the total brain homogenate in a high-speed round bottom centrifuge tube (14 mL) (see **Table of Materials**) at $800 \times g$ for 10 min at 4°C to obtain the supernatant (S1). Transfer S1 to a new centrifuge tube, leaving the pellet behind (P1), which contains intact cells and nuclei.

- Avoid pipetting up the fluffy, white, loose, superficial pellet. Take 2 x 5 μL of S1 for BCA and 2 x 100 μL of S1 for WB.
- Spin S1 at 9,000 x g for 15 min at 4 °C to obtain the synaptosomal supernatant (S2) and crude synaptosome pellet (P2). Take 2 x 10 μL of S2 for BCA and 2 x 500 μL of S2 for WB. Discard the supernatant after obtaining aliquots and proceed to the next step with the pellet.
 - Resuspend P2 in 3 mL of ice-cold Buffer A with protease inhibitors and centrifuge at 9,000 x g for 15 min at 4 °C to obtain the supernatant (S2') and washed synaptosomes (P2'). Discard the supernatant and keep the pellet.
 - Resuspend P2' in 3 mL of Buffer A. Avoid resuspending the dark red portion at the bottom of the pellet, which mainly contains mitochondria. Take 2 x 20 μL of P2' for BCA and 2 x 100 μL of P2' for WB.
- NOTE:** This can be achieved by gently pipette-mixing the edges and surface of the pellet to resuspend the white washed synaptosomes while directing the pipette tip away from the red center of the pellet.

4. Hypotonic lysis

NOTE: The schematics of this procedure are shown in **Figure 5**.

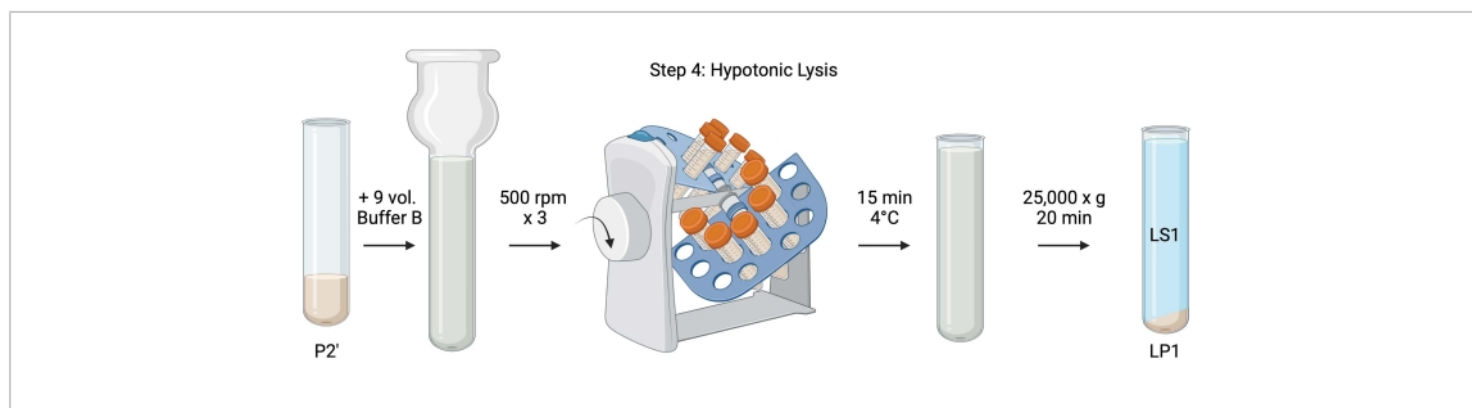


Figure 5: Hypotonic lysis. Schematic of step 4, the hypotonic lysis of synaptosomes to generate the lysis supernatant (LS1) and synaptosomal membrane fractions (LP1). [Please click here to view a larger version of this figure.](#)

- For the hypotonic lysis of washed synaptosomes, add 9 volumes of chilled Buffer B (**Table 1**) to resuspended P2' (~27 mL). Homogenize the synaptosomes in a glass Dounce homogenizer (three up-down passes at 500 rpm).
- Transfer the samples to 50 mL capped conical centrifuge tubes. Rotate them on a tube revolver in a 4 °C cold room for 15 min.
- Centrifuge lysed P2' at 25,000 x g for 20 min at 4 °C to obtain the lysis supernatant (LS1) and the lysis pellet containing synaptosomal membranes (LP1). Take 2 x 50 μL of LS1 for BCA and 2 x 400 μL of LS1 for WB. Transfer LS1 into a capped centrifuge tube for ultracentrifugation (see **Table of Materials**).

5. Synaptic vesicle isolation

NOTE: The schematics of this procedure are shown in **Figure**

6.

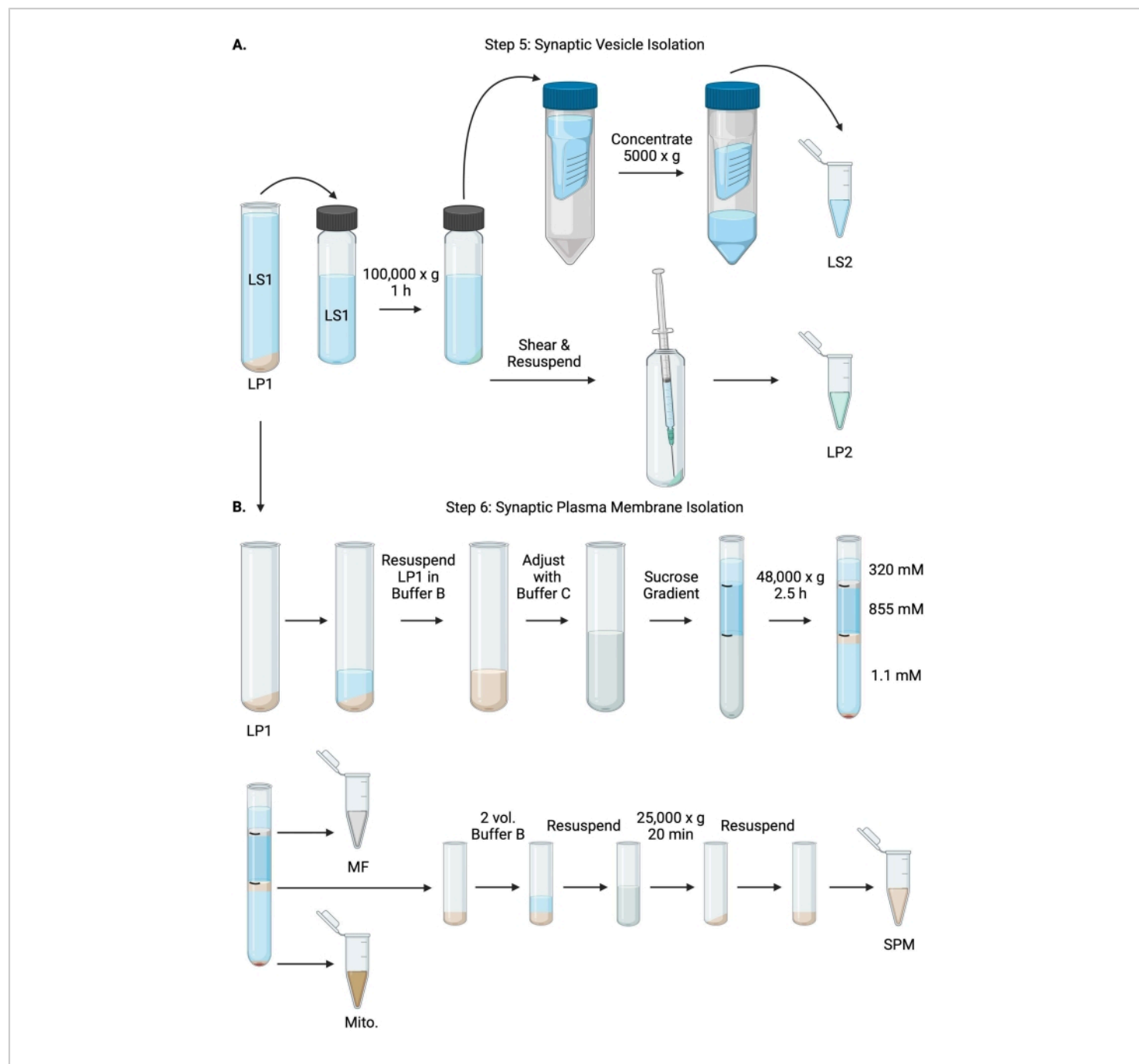


Figure 6: Synaptic vesicle isolation and synaptic plasma membrane isolation. (A) Schematic of step 5, the isolation of synaptic cytosol (LS2) and synaptic vesicle (LP2) fractions, and **(B)** step 6, the generation of myelin (MF), synaptic plasma

membrane (SPM), and mitochondrial (Mito.) fractions following the ultracentrifugation of sucrose gradients. [Please click here to view a larger version of this figure.](#)

1. Centrifuge LS1 in a fixed angle ultracentrifuge rotor (see **Table of Materials**) at 100,000 x g for 60 min at 4 °C to obtain synaptic cytosol supernatant (LS2) and synaptic vesicle pellet (LP2). LP2 will be small, translucent, and strongly adhered to the side of the centrifuge tube.
2. Resuspend LP2 in 500 µL of Buffer A. Using a 23 G needle and a 1 mL syringe, shear LP2 with gentle trituration. Take 2 x 10 µL of LP2 for BCA and 2 x 250 µL of LP2 for WB.
3. Transfer LS2 (~30 mL) to centrifugal filter units with a 10 kDa cutoff (see **Table of Materials**).
NOTE: If proteins smaller than 10 kDa are of interest, 4 kDa cutoff centrifugal filter units are available but will result in longer spin times.
4. Concentrate LS2 to approximately 0.5 mL by spinning at 5000 x g for up to 1 h at 4 °C. Take 2 x 10 µL of concentrated LS2 for BCA and 2 x 250 µL of concentrated LS2 for WB. After starting the spin, proceed directly to step 6.1.

6. Synaptic plasma membrane isolation

1. Resuspend LP1 (step 4.3) in 1 mL of Buffer B (**Table 1**). Take 2 x 10 µL of LP1 for BCA and 2 x 50 µL of LP1 for WB. Adjust the remaining LP1 to a final volume of 7.5 mL and a final sucrose concentration of 1.1 M with Buffer B and Buffer C (**Table 1**).
2. Transfer 7.5 mL of resuspended LP1 into a 14 mL ultracentrifuge tube (see **Table of Materials**). Carefully overlay LP1 with 3.75 mL of Buffer D (**Table 1**), and then overlay with 1.25 mL of Buffer A (or a larger volume to fill just below the top of the centrifuge tube). Avoid pipetting down the side of the tube, which will disrupt the sucrose gradient interfaces. After overlaying each sucrose fraction, mark the top of the solution with a pen. Balance the tubes for ultracentrifugation by weight, not volume, with the dropwise addition of Buffer A to within 10 mg. Centrifuge at 48,000 x g for 2.5 h at 4 °C in a swinging bucket ultracentrifuge rotor (see **Table of Materials**).
3. Acquire images of the intact gradients following ultracentrifugation to document the distinctness of each sucrose interface and the success of fractionation.
4. Carefully remove the superficial layer of 320 mM sucrose (Buffer A). Recover the myelin fraction (MF) at the 320 mM/855 mM sucrose interface in an 800 µL volume. Recover the synaptic plasma membrane (SPM) fraction at the 855 mM/1.1 M sucrose interface in a 1,000 µL volume. Pipette each fraction up from the wall of the tube in a circular manner to ensure the complete fraction is collected. Carefully aspirate off the remaining sucrose and recover the mitochondrial pellet (Mito.) by resuspending in 200 µL of Buffer B. Take 2 x 100 µL of MF for BCA and 2 x 10 µL of Mito. for BCA; divide the remainder of MF and Mito. samples in half for WB.
5. Dilute the SPM fraction with 2 volumes of Buffer B (~2 mL), and then centrifuge in a fixed angle rotor in a 3.5 mL centrifuge tube (see **Table of Materials**) at 25,000 x g for 20 min at 4 °C. Discard the supernatant and resuspend the SPM pellet in Buffer A for a final volume of 250 µL. Take 2 x 5 µL of SPM for BCA and divide the remaining SPM in half for WB.
6. Perform a BCA to determine the protein concentration of each sample, accounting for variable aliquot volume.

NOTE: For WB analysis, the suggested working protein concentration for all subcellular fractions is 2 µg/µL (or as high as achievable for LS1 and MF).

Representative Results

The presented method results in 11 brain subcellular fractions that can be subjected to further purification and various forms of downstream analysis^{35,36}. The gold standard method to assess the quality of synaptosomes, SVs²³, and other components is electron microscopy (EM) (**Figure 7**). Quantitative immunoblotting for proteins that are present in specific subcellular fractions can also be performed to assess markers of fraction purity (**Figure 8**). For example, immunoblot analysis of fractions reveals the enrichment of N-cadherin (CDH2, UniProt name) in the synaptic plasma membrane fraction (SPM), α-synuclein (SYUA) in the synaptic cytosol (LS2), synaptophysin (SYPH) in the synaptic

vesicle fraction (LP2), and myelin basic protein (MBP) in the myelin fraction (MF) when compared to protein levels in the initial whole brain homogenate (Total) (**Figure 8**). Once fraction purity has been established (for example, note the absence of CDH2 in the LS2 fraction or the many-fold increase in SYPH in the LP2 fraction), quantitative immunoblotting can be used to determine the localization of proteins of interest or query differences in protein distribution between genotypes or treatments. Understanding the subcellular localization of synaptic proteins can enable the dissection of previously undescribed protein functions. Further, this method may elucidate trafficking defects or synaptic dysfunction in disease states, especially when paired with functional assays. For example, our team has used this method to identify a pool of enzymatically active palmitoyl protein thioesterase 1 that is enriched in the synaptic cytosol¹⁹.

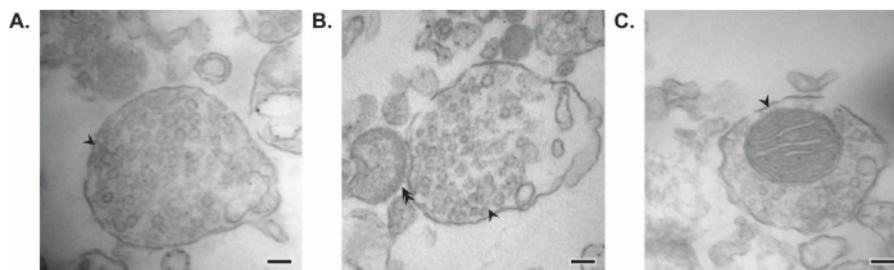


Figure 7: Electron microscopy (EM) of synaptosomes. (A) Representative EM image of a synaptosome containing synaptic vesicles (arrow). (B) Representative EM image of a synaptosome with both pre- (arrow) and post-synaptic components (double arrow). (C) Representative EM image of a synaptosome containing synaptic vesicles and a mitochondrion (arrow) (scale bars = 100 nm). [Please click here to view a larger version of this figure.](#)

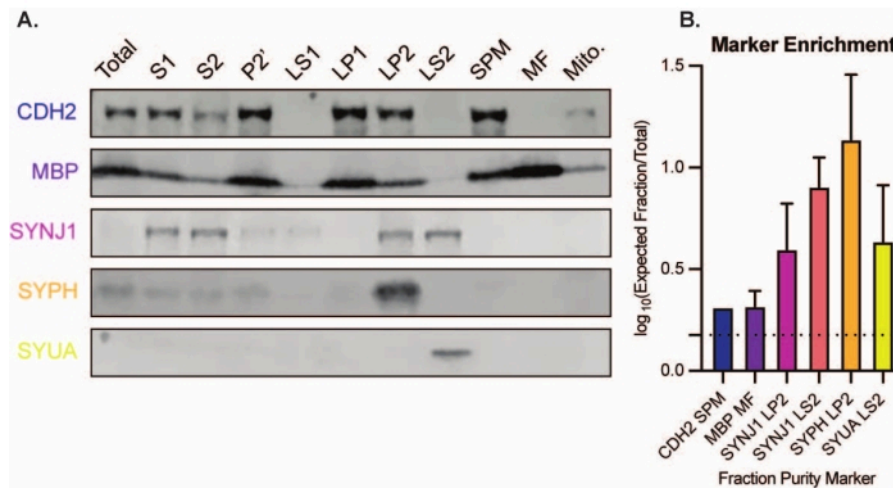


Figure 8: Immunoblot analysis of subcellular fractions. (A) Markers of subcellular fraction purity (indicated with UniProt nomenclature) are appropriately localized compared to the whole brain homogenate (total): N-cadherin (CDH2) in the synaptic plasma membrane fraction (SPM), synaptophysin 1 (SYPH) and synaptotagmin 1 (SYNJ1) in the synaptic vesicle enriched fraction (LP2), α -synuclein (SYUA) in the synaptic cytosol (LS2), and myelin basic protein (MBP) in the myelin fraction (MF). (B) Immunoblot quantification analysis reveals the enrichment (fold-change from total) of fraction purity markers. Data are represented as mean $\pm$ standard deviation on a log₁₀ scale. The dotted line indicates a 1.5-fold change ($y = 0.176$) ($n = 3$ replicate experiments with 8 wild-type mice; age = 2 months; $n = 4-5$ blots for SYPH, SYUA, MBP, with $n = 3$ plotted values previously published by Gorenberg et al.¹⁹; $n = 5$ for SYNJ1; $n = 1$ for CDH2). [Please click here to view a larger version of this figure.](#)

Discussion

In their seminal studies, Whittaker and colleagues used four morphological criteria to identify synaptosomes: (1) the structures have a sealed plasma membrane; (2) the structures contain SVs resembling those in nerve terminals and varicosities *in situ* in size and number; (3) the structures possess one or more small mitochondria; and (4) the presynaptic membrane is frequently adhered to a post-synaptic component^{11,12,13}. Though the first two criteria generally apply to every isolation method, in the most recent protocols described in this article, not all resulting synaptosomes will have mitochondria and attached post-

synaptic terminals. Approximately 60% of the synaptosomes will have mitochondria, and only up to 15% are estimated to have attached post-synaptic terminals³⁷. If post-synaptic components are of particular interest, the use of an isotonic Krebs-like homogenization buffer and pressure filtration for enrichment are known to yield high concentrations of synaptosomes with post-synaptic terminals (also termed synaptoneuroosomes)^{22,38}.

The method of sacrificing the animal can impact the quality of synaptosomes and synaptic subfractions. Adult animals sacrificed using a euthanasia method that does not require

anesthesia will result in the best fraction quality. Further, the brains should be freshly dissected, not frozen, and homogenized using a 1:10 ratio of homogenization buffer (weight/volume) for the most viable synaptic fractions²². The brain has a heterogeneous population of synapses that can be differentiated by the type of neurotransmitters they carry. Synaptosome formation is generally unaffected by synapse type or neurotransmitter content¹³. An exception is mossy fibers in the cerebellum, which are known to be disrupted in optimal conditions for obtaining synaptosomes from the rest of the brain^{39,40}. Thus, removal of the cerebellum prior to brain homogenization is recommended if the exclusion of this region does not affect the experimental goal. If interested in isolating synaptosomes of a particular neurotransmitter character, areas of the brain that are enriched for neurons containing the neurotransmitter of interest can first be isolated. However, this approach will impose limitations on the final fraction yield, depending on the size of the region of interest (the age of animals is also, therefore, a consideration). There are immunochemical methods for the isolation of neurotransmitter-specific synaptosomes, but the viability and yield will be significantly compromised²². If assessing synaptosome metabolic viability is important, the measurement of neurotransmitter release^{41,42} or certain enzymatic assays⁴³ can be employed.

Common contaminants in synaptosome preparations include microsomes, free mitochondria, SVs, and neuronal and glial membranes. Contamination can be reduced by increasing the number of washes at the P1 and P2 fractions²² and avoiding resuspension of the red mitochondrial pellet in subsequent steps. In experiments where metabolic viability and time are crucial, reducing the number of washes and using Ficoll or Percoll gradients over sucrose gradients will be helpful^{44,45,46}. These methods also reduce contamination

significantly. Whittaker's original protocol yielded high-quality SVs. Further optimization by Nagy et al.²³, included in this method, produces SVs with remarkable homogeneity and purity without compromising significantly on yield³⁶. If specific SV subtypes are of interest, such as glutamatergic (VGLUT-1-containing) or GABAergic (VGAT-1-containing) SVs, immunoisolation using specific antibodies can be performed^{47,48}. Alternative methods are also available for isolating CCVs from synaptosomes, which, due to differential density, may not be present at the same interface as SVs obtained with this method^{20,49,50}.

Overall, the present protocol to isolate synaptic components can be further optimized to obtain fractions with improved homogeneity and viability based on the quality and quantity of the source brain tissue and the experimental goals. For further troubleshooting details, one should refer to book chapters by Dunkley and Robinson²² and Ganzella et al.³⁶.

Disclosures

The authors have nothing to disclose.

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α -Synuclein pathology and reduced neurogenesis in the olfactory system affect olfaction in a mouse model of Parkinson's disease

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Abstract

Parkinson's Disease (PD) is characterized by multiple symptoms including olfactory dysfunction, whose underlying mechanisms remain unclear. Here, we explored pathological changes in the olfactory pathway of transgenic (Tg) mice expressing the human A30P mutant α -synuclein (α -syn) (α -syn-Tg mice) at 6-7 and 12-14 months of age, representing early and late-stages of motor progression, respectively. α -Syn-Tg mice at late stages exhibited olfactory behavioral deficits, which correlated with severe α -syn pathology in projection neurons of the olfactory pathway. In parallel, olfactory bulb (OB) neurogenesis in α -syn-Tg mice was reduced in the OB granule cells at 6-7 months, and OB periglomerular cells at 12-14 months, respectively, 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 at 12-14 months. Our data suggest that, 1) the α -syn-Tg mice recapitulate 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 in the OB; and 5) synaptic endo- and exocytosis defects in the OB may further explain olfactory deficits.

Keywords

α -Synuclein, A30P, Parkinson's disease, olfaction, neurogenesis, endocytosis, presynaptic proteins

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 (1-5). Due to its high prevalence, anosmia is now included as a diagnostic criterion for PD by the Movement Disorder Society (6). Yet, the mechanisms underlying olfactory dysfunction in PD remain unclear and not fully investigated.

PD is pathologically characterized by the neuronal accumulation of insoluble, cytoplasmic protein inclusions known as Lewy bodies (LBs) and Lewy neurites (7-9). LBs consist mainly of α -synuclein (α -syn), a synaptic protein that regulates synaptic vesicle exo- and endocytosis (10, 11). Thus, the formation of LBs is associated with synaptic dysfunction and neurodegeneration (12, 13). In PD, pathological α -syn can assemble into multimers that form neurotoxic oligomers and fibrils (14, 15), whose accumulation is accompanied by phosphorylation of the protein at serine 129 (pSer129- α -syn) (16, 17) that increases with disease progression (18, 19). Indeed, ~90% of α -syn in LBs is phosphorylated at this position (20, 21) and pSer129- α -syn antibodies are used to identify α -syn pathology in animal models of PD (22). Furthermore, increased levels of pSer129- α -syn are found in postmortem human olfactory bulbs (23).

The events triggering LB pathology in the brain 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 (24-29). Although environmental factors are reported to be involved in α -syn misfolding and PD pathogenesis (30, 31), other evidence points to genetic factors including mutations in *GBA*, *LRRK2* and *SNCA* genes as a cause of PD and associated LB pathology (30, 32, 33). Among these, point mutations in the gene *SNCA* encoding for α -syn (e.g., A30P, A53T, E46K, G51D and H50Q), generate aggressive, fully penetrant, early-onset forms of PD (34-38). These and other mutations have been used to generate animal models to study familial forms of PD (39, 40).

As noted above, anosmia is among the criteria used to diagnose PD. In a comparable vein, analyses of mouse models with *SNCA* mutations suggested the development of olfactory deficits (41-44). To understand more fully the mechanisms that may account for any olfactory deficits associated with *SNCA* mutations, in this study we used a mouse model that overexpressed human A30P mutant α -syn (45) (α -syn-Tg mice) to investigate the olfactory system. Our findings indicated a preferential accumulation of α -syn pathology in projection neurons throughout the olfactory pathway and a decreased OB neurogenesis. In addition, we detected a decrease in synaptic exo- and endocytic protein expression, events that were likely to account for the olfactory dysfunction in the α -syn-Tg mice.

Materials and Methods

Animals

All experiments were performed in C57BL6/J congenic Thy-1 human A30P mutant α -syn mice (α -syn-Tg) and their wild-type (WT) control littermates (45). Analyses were carried out on either 6–7 months (early stage) or 12–14 months (late stage) 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 behavioral olfactory deficits, we conducted two standardized buried food tests to optimize the detection of any anomalies in olfactory function in the early vs. late stages (46-48). Both tests determine the ability of mice to detect a food reward hidden in the bedding of clean housing cages.

On 6-7 months mice (WT: n=7, 3 males and 4 females; α -syn-Tg: n=7, 5 males and 2 females) testing took place over 3 consecutive days(49). On day 1 (*habituation day*) mice were placed individually in clean polycarbonate cages (Acecaging, USA) filled with 3 cm 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, food consumption was restricted by providing them with only 1.82 g of standard lab pellets (7 Kcal/mouse). On day 2 (*pre-test partial 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 bedding of each cage was changed between trials and the cookie was buried at different locations between days and trials. At the end of day 2 mice were fully deprived of food for 24 h, and on day 3, *score latency olfactory tests* were conducted as described for 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 (50). 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, 4 males and 2 females; α -syn-Tg: n=8 males) were tested using an equivalent protocol (51). Tests were carried out over 3 consecutive days in clean polysulfone cages (Techniplast) using a piece of sweetened cereal. As described for the 6-7 months mice, on day 1 (*odor familiarization*), the 12-14 months mice were exposed to a piece of cereal (Chocolate Cheerios, General Mills) placed in each cage. The cereal was left for consumption overnight and only those mice that consumed the cereal were selected for the further analysis. On day 2 mice were deprived of food for 24 h. On day 3 mice were habituated to the testing environment by placing them in a clean cage with 3 cm of clean 1/8" corncob bedding (Teklad, 7092) for 60 min., after which mice were returned to home cage. Test trials (*score latency olfactory test*) were as follows: during Trial 1 a piece of cereal was buried in a selected location in the test cage and the mouse then returned to the test cage at a location opposite the site of the buried cereal. The latency to find the cereal was recorded; mice that took longer than 300 sec. to find the cereal were scored as 300 sec. Trials 2 and 3 were performed identically but the pieces of cereal were placed in a different corner of the cage. The latency scores for each animal were the average for the 3 trials. For each animal, this test was repeated for 3 weeks, and the scores were averaged. As for the 6-7 months mice, body weights were monitored to ensure the mice were in good health and a surface food test as described above 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 μ m 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. Then, 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 µm-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) (52), 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 1-3 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 (n= 3/genotype) and 12-14 (n=3/genotype) months, respectively. 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 were 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 (53-55) 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 months) and late (12-14 months) 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 layers, the *inner cell zone* in the AON and in PC layer 2 by using Fiji (ImageJ) software with the MorphoLibJ integrated library and plugin (56). 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 1 of APC. Comparisons between WT and α -syn-Tg mice were made using unpaired t-tests.

Dopaminergic 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 OB 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 human/mouse endogenous α -syn and aggregated α -syn 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 α -syn-Tg mice

A progressive loss of olfaction is a well-known non-motor symptom in PD patients (4, 57). 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 (45, 58). 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 (48, 49, 51).

Our results from olfactory tests showed that α -syn-Tg mice displayed olfactory deficits only at late stages (12-14 months) compared to WT controls ($p=0.029$) 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. 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 in which the latency to find the food was recorded as a measure of mobility. There were no differences between WT and α -syn-Tg at early ($p=0.9709$) and late ($p=0.1156$) stages (Fig. 1B), indicating that motor agility did not influence olfactory performance, even though 12-14 months mice had motor deficits on

more challenging motor tests (data not shown; (45)). In summary, we found that aged α -syn-Tg mice exhibited olfactory behavior deficits.

Cytoarchitecture 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 (Fig. 2A):

- I. Olfactory sensory neurons (OSNs) expressing odor receptors are located in the olfactory epithelium (OE) lining the nasal cavity. When odorants bind, the information is sent to the first olfactory processing center of the brain, the olfactory bulb (OB).
- II. The OB has a laminar organization formed by 6 layers which go from the most superficial to the deepest as follows: (1) olfactory nerve layer; (2) glomerular layer (GL); (3) external plexiform layer (EPL); (4) mitral cell layer (MCL); (5) internal plexiform layer (IPL); and (6) granule cell layer (GCL). These layers are formed by a variety of projection neurons (PNs), involved in receiving and processing the olfactory signals received from OSNs, and interneurons (INs) that modulate those signals. PNs include mitral and tufted cells (M/Tc), whose cell bodies locate in the MCL and outer EPL, respectively. PNs extend both primary apical dendrites that arborize in spherical structures called glomeruli (Gl) where they form synapses with axon termini of OSNs, and lateral secondary dendrites that extend laterally within the EPL, where olfactory information is highly modulated. Both type of PNs send their axons to higher brain structures, predominantly the tubular striatum (TuS) and the olfactory cortex (OC), the last the subject of our analysis.
- III. The OC is the third station studied in this work and is divided in the anterior olfactory nucleus (AON) and piriform cortex (PC), both responsible for the higher brain processing of an odor and memory formation (59).

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) to label mature OSNs, (2) adenylyl cyclase 3 (AC3) to label differentiating OSNs, and (3) growth associated protein 43 (GAP43) to label immature OSNs (60). Our analysis showed no disrupted OE cytoarchitecture between WT controls (Fig. 2B, D) and α -syn-Tg mice at 12-14 months of age (Fig. 2C, E). We tested if the unaffected morphology was due to the absence of the disease in the OE by detecting 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) (20, 61). The data showed that α -syn pathology was not detected in the OE, and there was no difference between genotypes in aggregated α -syn (Fig. 2F, G) and pSer129- α -syn (Fig. 2H, I) staining. We also assessed for changes along the dorsal-ventral axis by staining with NADPH dehydrogenase [quinone] 1 (NQO1), a selective marker for the dorsal region, and we did not find any difference between the groups (Fig. 2F-I). Our observations were consistent with data found from PD patients (62).

Next, we explored the anatomy of the OB layers and general activity of OB projection neurons in 12-14 months mice. 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 found in α -syn-Tg mice compared to WT controls (Fig. 2J, K). In addition, we studied the dopaminergic local circuits established by PGNs expressing the enzyme tyrosine hydroxylase (TH⁺) (63), by quantifying TH⁺ cells around randomly selected glomeruli. Our results showed similar morphologies (Fig. 2L, M) and no statistical differences ($p=0.1358$; $t=1.864$, $df=4$) in the number of TH⁺ cells in WT (443 ± 43 cells/mm²) versus α -syn-Tg mice (535 ± 74 cells/mm²). 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 mutant α -syn (44, 64, 65). 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 (66). In the OB, pS6 expression levels are reported to be reduced in OB mitral cells after sensory deprivation (67). Here, we quantified the intensity of pS6 staining along the MCL in both WT and α -syn-Tg mice, showing no statistically significant difference between the groups (Fig. 2N-P). Overall, these results suggested no effect of α -syn pathology on the gross cytoarchitecture and mitral cell activity of the OB in aged α -syn-Tg mice.

Accumulation of α -synuclein pathology in α -syn-Tg mice

The α -syn-Tg mouse model of PD expresses the human A30P mutant α -syn under the Thy1 promoter, which is known to trigger protein expression predominantly in projection neurons (68). To validate how this model was affecting the olfactory system, we first investigated where human α -syn (h α -syn) was expressed. We performed triple IHC to detect h α -syn in combination with pSer129- α -syn and aggregated α -syn, as hallmarks of α -syn pathology, in α -syn-Tg mice at both 6-7 and 12-14 months (Fig.

3). We performed the study in two main areas of the olfactory system, the OB and the anterior region of piriform cortex (APC) which is the area that receives the most innervation from the OB (69).

As expected, h α -syn was not detected in WT animals (Fig. 3A-a, B-b, E-e, F-f), although we observed some residual expression of pSer129- α -syn in the OB-MCL (Fig. 3a', e') that did not occur in APC (Fig. 3b', f'). In transgenic mice, the h α -syn expression pattern was synaptic and appeared distributed predominantly in neuronal fibers in the GCL and IPL of the OB at 6-7 months (Fig. 3c, white arrowheads). However, the h α -syn staining pattern became more somatic in the MCL at 12-14 months (Fig. 3g, white arrowheads). In APC, the h α -syn expression pattern was found predominantly in, 1) the densely packed cell layers 2a+b and 3 formed by superficial and deep pyramidal neurons, respectively (Fig. 3d, h), with higher expression at 12-14 months (compare Fig. 3d staining with 3h). In layers 2 and 3 a more pronounced somatic expression was observed at 12-14 months (Fig. 3d, h, white arrowheads); 2) in layer 1 stronger staining was evident at 12-14 months, suggesting an increase of h α -syn expression with age (Fig. 3D-d, H-h). Sublayer 1a contains the primary synapses from the OB projection neurons onto the apical dendrites of the pyramidal neurons in layers 2 and 3 while sublayer 1b includes the associational synapses with cortico-cortical axons coming from other OC regions (69). α -Syn pathology was detected in both OB and APC tissues from mice at 6-7 (Fig. 3C-c'', D-d'') and 12-14 (Fig. 3G-g'', H-h'') months, colocalizing predominantly with cell bodies and processes expressing h α -syn (white arrowheads). The overall increase of total α -syn (both human and mouse endogenous) and aggregated α -syn in α -syn-Tg mice was confirmed by WB in OB homogenates of α -syn-Tg mice when compared to WT at both 6-7- and 12-14-months (Fig. 4A, B). The data were not significant at 6-7 months probably because of the insensitivity of the antibody in western blotting (Fig. 4B).

To further investigate which neuronal types were targeted by the overexpression of hA30P mutant α -syn, we performed a triple IHC analysis along the olfactory pathway of 12-14 months mice, to detect h α -syn together with aggregated α -syn as a marker for pathology and either TBR1 (T-Box Brain Protein-1) to identify excitatory/glutamatergic projection neurons (70), or GAD65/67 (Glutamic Acid Decarboxylase isoform 65 and 67) to identify GABA inhibitory neurons (71) (Fig. 5). Our results with TBR1 showed that all cells expressing h α -syn colocalized with TBR1 in the OB-MCL, AONl and APC (Fig. 5B-b', C', c', D', d', white arrowheads) but not in the OB-GL region (Fig. 5A-a'). However, not all TBR1 cells expressed h α -syn (Fig. 5A-D, red arrowheads, and red cells). α -Syn pathology studied with aggregated α -syn colocalized predominately with TBR1 cells that also expressed h α -syn (Fig. 5a''-d'', white arrowheads). There were instances of GAD65/67 overlapping with h α -syn or aggregated α -syn (Fig. 5E-h'', red arrowheads), but it was not as robust as seen in projection neurons and was less somatic.

Collectively, we concluded that the α -syn pathology in the α -syn-Tg mouse model was found throughout the olfactory pathway, most prominently in projection/excitatory neurons.

α -Synuclein pathology in the central olfactory pathway of α -syn-Tg mice

We next investigated α -syn pathology in the three main stations of the central olfactory pathway: the OB, the AON, and PC of mice at 12-14 months. In the OB, we quantified the expression of both aggregated α -syn and pSer129- α -syn in each of the OB layers and found that α -syn pathology was laminar specific (Fig. 6). There was no difference in GL expression between WT and α -syn-Tg mice in either aggregated α -syn (Fig. 6C; $p=0.7187$, $t=0.3866$, $df=4$) or pSer129- α -syn (Fig. 6J; $p=0.4241$; $t=0.8893$, $df=6$). In the EPL, minimal expression of aggregated α -syn was seen in both WT and α -syn-Tg groups (Fig. 6A-a'', B-b'', D; $p=0.7187$; $t=0.3866$, $df=4$). In WT and α -syn-Tg mice pSer129- α -syn staining was equivalent in the EPL (Fig 6H-h'', I-i'', K; $p=0.2336$; $t=1.402$, $df=4$). A subpopulation of OB projection neuron somata expressing TBR1⁺ in the outer EPL (oEPL), tufted cells, were pSer129- α -syn labeled in α -syn-Tg mice (Fig. 6h, h').

The 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. Both dendrites were stained with pSer129- α -syn in WT (Fig. 6h, h') and α -syn-Tg mice (Fig. 6i, i', green arrows), but the distribution was different between the experimental groups: the expression was limited to the lateral dendrites in the WT mice while both the lateral and apical dendrites showed expression in the α -syn-Tg mice (Fig. 6h, i). As mentioned above, we attributed its presence in the WT mice 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 (20). In the MCL, there was a strong trend of higher levels of aggregated α -syn in α -syn-Tg mice (Fig. 6E; $p=0.0553$; $t=2.679$, $df=4$), while pSer129- α -syn expression was significantly higher in α -syn-Tg mice compared to WT (Fig. 6L; $p<0.01$; $t=5.204$, $df=4$). Labeling of pSer129- α -syn which was most evident in the cell bodies of mitral cells of both WT (Fig. 6H-h'') and α -syn-Tg (Fig. 6I-i'') mice, colocalized with TBR1. These data together with our previous observations using h α -syn (Fig. 5A-d''), were consistent with the notion that α -syn pathology, detected by phosphorylation of α -syn in Ser129, is a process restricted to projection/non-GABAergic neurons, as previously reported (72). Deeper in the IPL, aggregated α -syn was significantly higher in α -syn-Tg (Fig. 6b, b') than in WT controls (Fig. 6F; $p<0.01$; $t=4.934$, $df=4$), while pSer129- α -syn expression levels were unchanged between experimental groups (Fig. 6M; $p=0.1465$; $t=1.798$, $df=4$). This is consistent with the observation that pSer129- α -syn staining is mainly concentrated in cell bodies and thicker neurites (73). In

the GCL aggregated α -syn (Fig. 6A-a'', B-b'') expression was significantly higher in α -syn-Tg than in WT (Fig. 6G; $p < 0.01$; $t = 3.594$, $df = 4$) and appeared preferentially localized to neurites interdigitated with the granule cell islets. The staining for pSer129- α -syn within the GCL (Fig. 6H-h'', I-i'') showed a pattern characteristic of the mitral/tufted cell (M/Tc) axons as they coalesce and subsequently form the lateral olfactory tract (LOT). There was only a trend toward a higher expression of pSer129- α -syn in the GCL (Fig. 6N; $p = 0.3740$; $t = 0.998$, $df = 4$). Together, our results indicated that α -synuclein pathology accumulation was enhanced in the OB projection M/Tc neurons of α -syn-Tg mice.

We next 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 (opl)*, and an *inner cell zone (icz)* containing pyramidal cells (74). We found a trend for increased aggregated α -syn in the *inner cell zone* of α -syn-Tg mice (Fig. 7A-C; $p = 0.1033$; $t = 2.103$, $df = 4$). Interestingly, pSer129- α -syn was significantly increased in projection neurons (TBR1⁺ cells colocalizing with pSer129- α -syn) of the AON *inner cell zone* in α -syn-Tg mice with respect to controls (Fig. 7J-K; $p < 0.01$; $t = 5.852$, $df = 4$), consistent with the pathology restricted to these cells. These results showed compelling evidence of AON pathology as described in humans (75, 76).

In PC, we studied areas in the anterior- (APC) and posterior- (PPC) piriform cortex to cover the anatomical and functional differences along the rostral-caudal axis (59, 69). In the APC, we found an increase in aggregated α -syn in layers 1b, occupied predominantly by the apical dendrites of PC projection neurons (69) (Fig. 7D, E; compare similar staining on Fig. 3d'', h''), and layer 2b, occupied predominantly by pyramidal neuron cell bodies some of which were strongly labeled for aggregated α -syn (Fig. 7E, arrowheads). Quantification of the stained area showed a trend of higher aggregated α -syn in α -syn-Tg mice, although this increase was not statistically significant most likely due to the high variability among individual mice (Fig. 7F; $p = 0.1489$; $t = 1.785$, $df = 4$). Results for aggregated α -syn in PPC were analogous (Fig. 7G-I; $p = 0.3128$; $t = 1.154$, $df = 4$). In contrast, the expression levels of pSer129- α -syn highlighted an intense pathological profile in α -syn-Tg mice in both APC (Fig. 7M-O; $p < 0.01$; $t = 5.897$, $df = 4$) and PPC (Fig. 7P-R; $p < 0.05$; $t = 3.383$, $df = 4$) compared to WT controls. In these cases, pSer129- α -syn staining was restricted to cell bodies and processes of projection neurons in layers 2a and b, and layer 3 (Fig. 7N and Q) of α -syn-Tg mice. Interestingly, there was a lack of pSer129- α -syn labeling in layer 2a of APC compared to PPC (Fig. 5N, Q). Sublayer 2a is occupied by semilunar cells (SL) (77), and the reasons for the differential expression in APC vs. PPC requires further analyses.

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

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 number and type of synapses found in the two primary regions in the olfactory pathway where projection neurons make synaptic contacts: (1) the OB glomerular neuropil (Gl), where M/Tc receive excitatory asymmetrical synapses from OSN axons and inhibitory symmetrical synapses with periglomerular interneurons (78); and (2) layer 1 of the APC, where axons from M/Tc make asymmetrical excitatory synapses with apical dendrites of PC pyramidal cells in layer 1a and local interneurons make symmetrical inhibitory synapses with the PC pyramidal apical dendrites in layer 1b (69, 79). In both regions, we quantified asymmetric (Fig. 8, red arrowheads), symmetric (Fig. 8, green arrowheads), and reciprocal (Fig. 8, yellow arrowheads) synapses. We also quantified 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 (80).

Total numbers of synapses and mitochondria between WT and α -syn-Tg mice in both the Gl (Fig. 8A-C) or APC (Fig. 8E-G) parenchyma showed no statistical differences: 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 ($p=0.7096$; $t=0.400$, $df=4$), reciprocal synapses in OB ($p=0.2301$; $t=1.414$, $df=4$) and APC ($p=0.1929$; $t=1.564$, $df=4$), and mitochondria in OB ($p=0.2605$; $t=1.309$, $df=4$) 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. 8D, 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).

Adult neurogenesis in the olfactory system of α -syn-Tg mice

Previous studies suggested that overexpression of human WT or hA30P mutant α -syn in animal models of PD results in altered neurogenesis in different layers of the OB (44, 81-83). Neurogenesis of OB interneurons occurs throughout the lifetime of an individual and is crucial to the control and modulation of olfaction (84, 85). 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) (86). New neuroblasts in this pathway take an average of 12-14 days to reach the OB in adult mice (87) and approximately another week to integrate in the OB circuits (88). Here, we sought to evaluate the effect of α -syn pathology on the neurogenesis of interneurons targeted to 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 (Fig. 9E; $p=0.7213$; $t=0.3695$, $df=8$) but showed a significant decrease at 12-14 months (Fig. 9E; $p=0.0491$; $t=2.460$, $df=6$), indicating that the hA30P mutant α -syn affected the neurogenesis at late stages of the disease progression (Fig. 9A-D, a'-d', E). Since our quantification of PGNs that were TH⁺ showed no differences between WT and α -syn-Tg mice (see above) and considering that no BrdU⁺-PGNs expressed TH (Fig. 10E, F), we concluded that the PGN population affected by the expression of hA30P mutant α -syn at 12-14 months did not include dopaminergic PGNs. In the GCL, we detected a significant decrease in the number of BrdU⁺ cells only at early (6-7 months) stages (Fig. 9F; 6-7 months: $p=0.0136$; $t=3.152$, $df=8$; vs 12-14 months: $p=0.095$; $t=1.976$, $df=6$), highlighting that hA30P mutant α -syn overexpression was affecting OB neurogenesis in the GCL prior to the onset of olfactory deficits (Fig. 9A-D, a-d, F). This is consistent with results from similar animal models expressing the hA30P α -syn mutation under the CaMK-tet regulated promoter (82). Absence of statistical significance at late stages (12-14 months) may be due to the global decrease in neurogenesis of cells targeting the OB during aging (87, 89), which may have masked the effect of hA30P mutant α -syn. Finally, there was no evidence of a change in the number of BrdU⁺ cells in the OB-RMS at either 6-7 (Fig. 9G; $p=0.6048$; $t=0.5386$, $df=8$) or 12-14 months (Fig. 9G; $p=0.1076$; $t=1.890$, $df=6$) (Fig. 9A-D, a-d). 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. 3,5-7), the effects observed in OB neurogenesis are presumably indirect, for instance via an alteration in the dopamine release into the SVZ (90, 91). Collectively, our data showed that hA30P mutant α -syn 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 neuroblasts,

mature neurons and glial cells in the two regions we found BrdU labeled cells: the OB and the SVZ. First, we looked for neuroblasts by using doublecortin (DCX), a microtubule-associated protein required for the migration of cells. BrdU⁺ cells that were located in the OB did not colocalize with the DCX marker (Fig. 10A, B), suggesting that most BrdU⁺ cells have completed migration and begun to differentiate into neurons at the time of the study. This is consistent with our prior studies showing that OB neuroblasts require 3 weeks to migrate and differentiate into neurons after generation in the SVZ (87, 88). Mature neurons in the OB were identified 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 (Fig. 10C, D), suggesting that the remaining ~60% of BrdU⁺ cells could be neurons in intermediate stages of differentiation with a DCX⁻/NeuN⁻ phenotype. We also looked for an effect in the TH subpopulation within the GL, a group of mature PGNs reported to be affected in PD patients (92) and in similar animal models (44, 65). Our data demonstrated that none of the BrdU⁺-cells in the GL colocalized with the TH marker (Fig. 10E, F). To ensure that the DCX⁻/NeuN⁻ phenotypes did not belong to dividing glial cells, we searched for astrocytes and microglial cells using glial fibrillary acid protein (GFAP) and ionized calcium-binding adapter molecule 1 (IBA1), respectively. Neither GFAP (Fig. 10G, H) nor IBA1 (Fig. 10I, J) colocalized with BrdU⁺ cells, indicating they were not dividing glial cells. In the SVZ, these markers identify characteristic subpopulations of cells that represent the three differentiation stages of OB interneurons: “A” cells or neuroblasts (DCX⁺); “B” cells or neural stem cells, which share a phenotype with radial glial cells (GFAP⁺); and “C” cells or intermediate transient amplifying cells (TACs) (Dlx2⁺ and Tbr2⁺ for dorsal TACs) (86). Our results showed that BrdU⁺ cells found in the SVZ were negative for DCX (Fig 10K, L), GFAP (Fig. 10M, N) and Tbr2 (T-Box Brain Protein-2) (Fig. 10O, P), indicating they were no “A”, “B” or dorsal “C” cells. However, they expressed Distal-Less Homeobox 2 factor (Dlx2), characteristic of all “C” cells (86), indicating that some “C” (TAC) cells remained in the SVZ 25 days after the BrdU injection. These cellular classifications were uniform for both WT and α -syn-Tg mice.

Collectively, we found that the hA30P mutant α -syn expression significantly reduced OB neurogenesis in the GCL at early stages (6-7 months) and in the GL at later stages (12-14 months), respectively. 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. In addition, the scarce number of BrdU⁺ cells found in the SVZ were transient amplifying cells or “C” cells.

Age-related changes in synaptic endocytic proteins 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 with technical triplicates) by label-free quantification mass spectrometry (LFQ-MS). 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. 11A, B. Table 2). 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 Blotting (Fig. 4A). Protein kinase c-Src was the most significantly upregulated protein in the OB of α -syn-Tg mice (SRC, Fig. 11A, B, fold change of 2.88). Among other functions, protein kinase c-Src phosphorylates components of endocytic trafficking (93) and notably, α -syn (94). GO enrichment analysis performed on the proteins that showed significant changes ($p > 0.05$) identified pathways involving vesicle-mediated transport, specifically endocytosis, which were significantly enriched (Fig. 11C). The vast majority of proteins from our dataset that were implicated in these pathways were downregulated in α -syn-Tg samples (Fig. 11A, 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. 11D, E. Table 3). There was a trend in overexpression of α -syn (SYUA), which was confirmed via Western Blot (Fig. 4A). 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 enriched (Fig. 8F). These pathways were driven by a majority of proteins that were downregulated in α -syn-Tg samples, including the vesicular trafficking proteins PICALM, UNC13A, SNAP29, and VPS45 (Fig. 11D, 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. 11A-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. 11G, H. Table 4). As anticipated, we observed a significant increase in α -syn expression (SYUA, Fig. 11D, E). Interestingly, GO analysis largely revealed broader dysfunction in cellular metabolism (Fig. 11F) without enrichment of any vesicle mediated-transport pathways seen in the OB (Fig. 11C). 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. 11J, K. Table 5). Similarly, GO analysis revealed only a subtle dysregulation in receptor clustering in α -syn-Tg samples, 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 (6, 95-101). 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 (39, 102). Here, we used a mouse model of PD carrying the human A30P mutant α -syn expressed under the Thy1 promoter or α -syn-Tg (45) 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.

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

We screened for h α -syn expression and pathology throughout the olfactory pathway examining the different stations (Fig. 3 and 5). We find broad expression of h α -syn throughout the olfactory pathway with prominent expression in projection neurons (68, 103). Interestingly, pSer129 α -syn pathology was found mainly in projection neurons, suggesting that these cells drive the olfactory dysfunction seen in these transgenic mice.

The presence of α -syn pathology in the olfactory system of transgenic mice at 6-7 months was unexpected because a behavioral deficit was not detected at the early stage. At advanced stages of the disease progression (12-14 months), the α -syn-Tg mice exhibited significant olfactory deficits in parallel with the α -syn pathology. Why α -syn pathology did not affect the olfactory behavior at early stages remain unknown, but one possibility suggested by our proteomic data was the appearance of changes in the synaptic vesicle trafficking proteome in older mice that were not found in the younger mice. As noted above, the delayed onset of olfactory deficits could be attributed to regulated protein expression controlled by genetic modifiers (104), including those that could act on the gene promoter employed here, Thy1. Some of these modifiers have been reported to interact with the expression of the hA30P mutant α -syn to modify the toxicity of this protein (105).

To establish a mechanism that explained the temporal susceptibility of olfactory neurons to the hA30P mutant α -syn, we investigated the anomalies in synaptic architecture and intracellular signaling pathways in 12-14 months mice. Synaptic transmission is regulated by α -syn through interactions with synaptic vesicle membranes and impacting on the presynaptic architecture (106-108). To explore this in the α -syn-Tg mice, we performed an ultrastructural analysis and quantified excitatory and inhibitory synapses and mitochondria in the projection neurons synaptic regions of the OB and APC, the glomeruli and layer 1, respectively. Surprisingly, these analyses did not show any differences in ultrastructural features that might account for the behavioral deficits in α -syn-Tg mice (Fig. 8). Similarly, although mitochondria function deficits have been reported in PD (Barbuti et al., 2021) we did not find evidence of alterations in the number/density of mitochondria.

We identify two plausible mechanisms to explain the late-stage olfactory deficits in α -syn-Tg mice: (1) decreased neurogenesis; and (2) perturbations of synaptic exo- and endocytosis pathways proteins involved in the synaptic transmission.

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

Neurogenesis in the olfactory system occurs throughout the lifetime of an individual, where newly formed neuroblasts join chains of migrating neuroblasts extending to the ependymal core of the OB via the rostral migratory stream (RMS) (85). Once detached from the RMS, neuroblasts migrate radially to their final positions in either the GCL or GL of the OB. In PD, the evidence of aberrant neurogenesis is limited and controversial with some reports showing either decreases in neurogenesis in the SVZ (90), or no effect (109). Here, using BrdU⁺ injections, we found a decrease in OB neurogenesis in α -syn-Tg mice (Fig. 9) that affected primarily the GCL at early (6-7 months) and the GL at later (12-14 months) stages, respectively. A decrease in OB neurogenesis in these two layers was reported previously in similar animal models but at earlier ages (82, 110), so that it is reasonable to speculate on an effect of hA30P mutant α -syn in OB neurogenesis.

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 (Fig. 2L, M) 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 dopaminergic innervation into the SVZ, as reported before (90). 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.

Alterations 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 hA30P mutant α -syn. The hA30P α -syn mutation causes α -syn pathology (111, 112) and is implicated in trafficking mechanism including those associated with both endo- and exocytosis (113). The hA30P α -syn mutation was previously associated with disruptions in vesicular trafficking, synaptic dysfunction, apoptosis, and formation of permeable annular pore-like protofibrils in the cell membrane that disrupt transmembrane trafficking (20, 21, 114-116). 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 (117) and that elevated α -syn levels and aggregation can cause inhibition of synaptic vesicle exo- and endocytosis (118-121). 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 contribute to 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 carrying the human A30P mutant α -syn exhibit olfactory deficits at a later stages of the disease progression, when PD-like motor deficits are apparent; (2) transgenic expression causes α -syn pathology along the entire central olfactory pathway, affecting predominantly projection neurons (TBR1⁺); (3) α -syn pathology does not cause structural and morphological abnormalities in the olfactory system; (4) the overexpression of hA30P mutant α -syn causes a decrease in SVZ neurogenesis and interneurons targeted to the OB in the GL and GCL at late and early stages of the disease, respectively; and (5) hA30P mutant α -syn 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.

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Figures

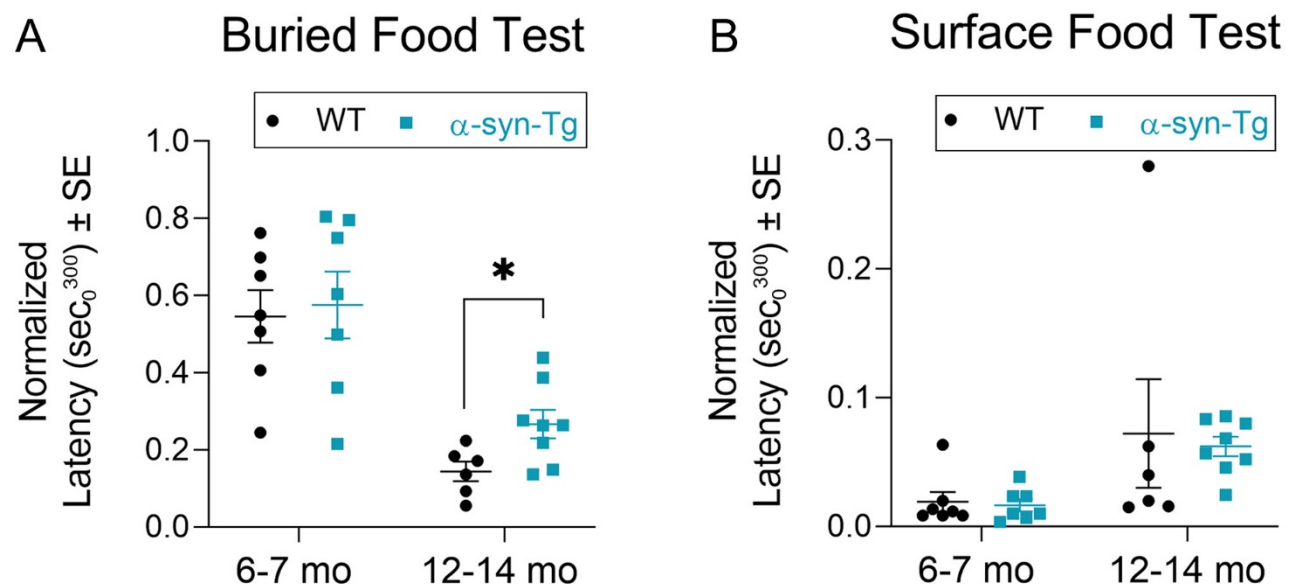
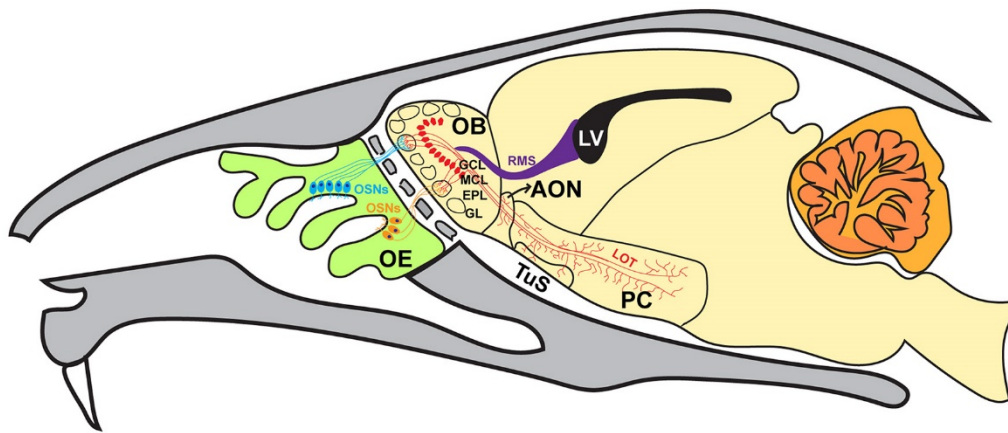
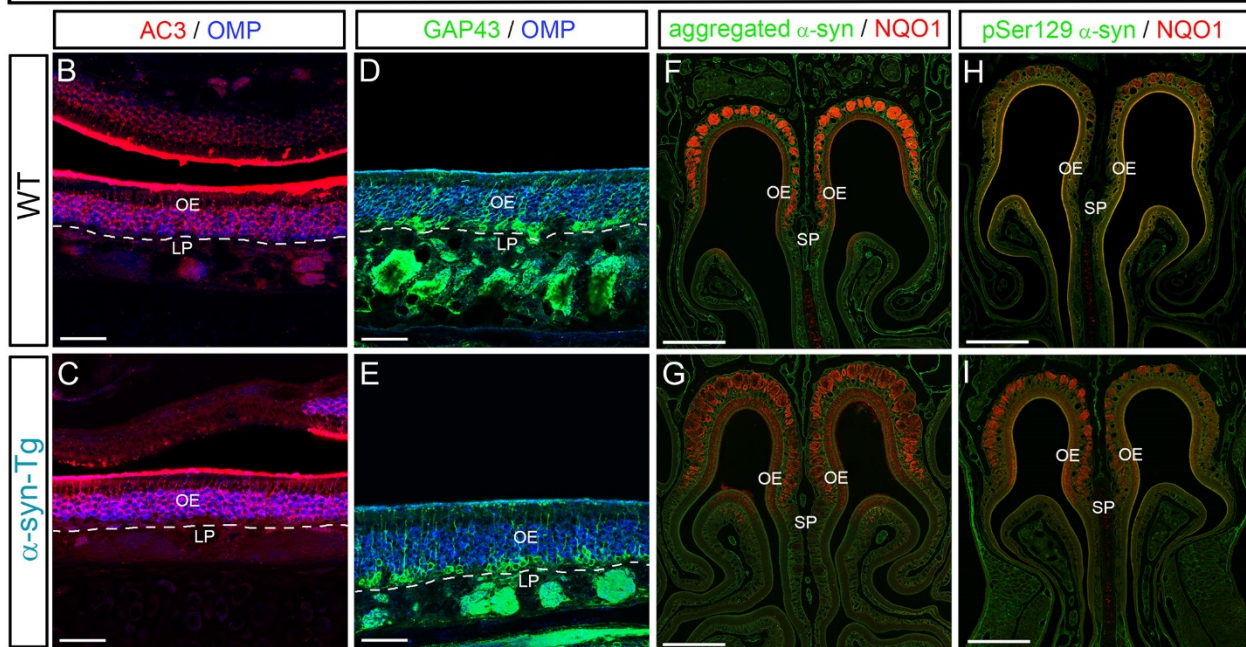


Figure 1. α -syn-Tg mice show olfactory deficits at 12-14 months. (A) Wild type (WT) and α -syn-Tg (carrying the hA30P mutant α -syn) mice are evaluated for olfactory deficits using standard buried food tests. Mice at early stages of the disease (6-7 months) do not exhibit olfactory dysfunction measured 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.

A



OLFACTORY EPITHELIUM CYTOARCHITECTURE



OLFACTORY BULB CYTOARCHITECTURE AND MITRAL CELLS ACTIVITY

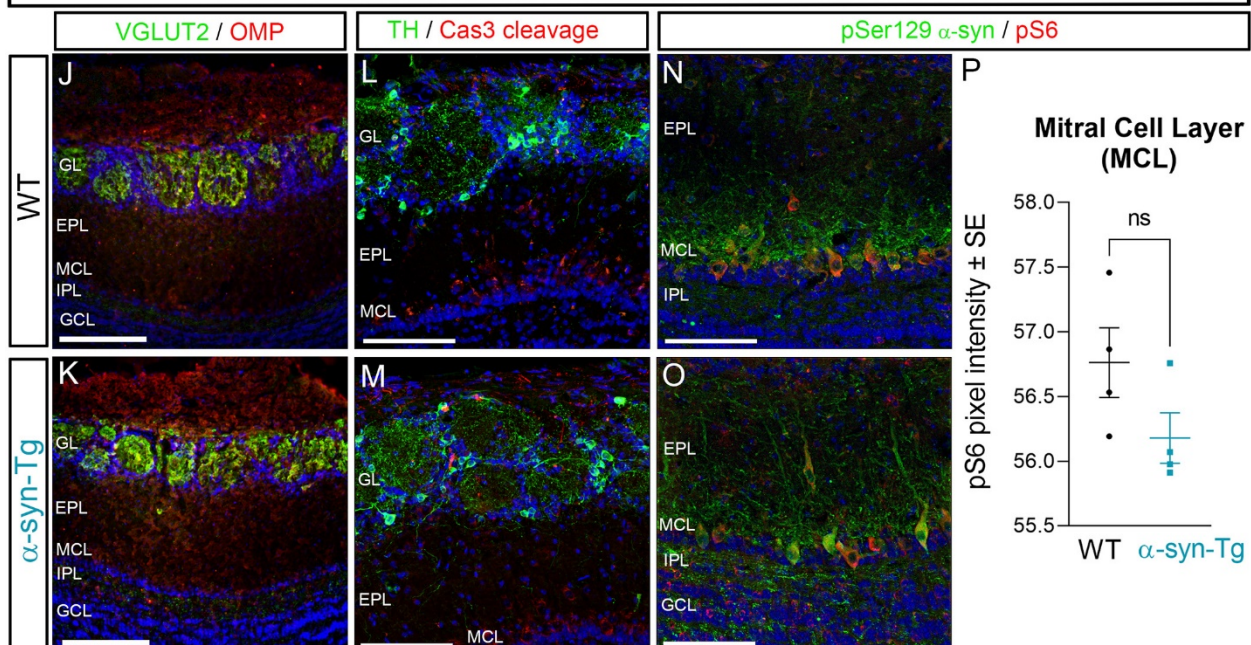


Figure 2. Organization of mouse olfactory system, and cytoarchitecture of the olfactory epithelium (OE) and bulb (OB) in α -syn-Tg mice at 12-14 months. (A) Diagram of the mouse olfactory system summarizing the different station relays used by the olfactory information to travel from the OE to the brain. (B, C) Immunohistochemistry against AC3 (red) and OMP (blue) to label intermediate differentiating and mature OSNs, respectively. (D, E) Staining for GAP43 (green) and OMP (blue) showing immature and mature OSNs, respectively. No alterations in the OE cytoarchitecture are seen in α -syn-Tg mice. (F, G) Low magnification images of the OE stained for α -syn pathology using aggregated α -syn (green; Mouse EMD Millipore, Table 1) and NQO1 (red), specific for dorsal OE. No evidence of α -syn aggregation is seen in the OE. (H, I) Low magnification images of the OE stained for α -syn pathology using pSer129- α -syn (green, Rabbit Abcam, Table 1) and NQO1 (red). No evidence of α -syn pathological phosphorylation is seen in the OE. Differences in NQO1 staining correspond to slight variations in the section plane along the rostral-caudal axis between the different mice. All these staining show an unaltered cytoarchitecture in the OE of α -syn-Tg mice with lack of α -syn pathology. (J, K) OSNs axons labeled with VGLUT2 (green) and OMP (red) in the OB show no differences in the glomerular structure between WT and α -syn-Tg mice. (L, M) Staining of dopaminergic neurons with TH in the OB-GL (green) and signs of apoptosis using Caspase 3 cleavage (red), showing no disruptions in the morphology or cellular processes or increased cell death in α -syn-Tg mice. (N, O) Staining for α -syn pathology in the OB-MCL using pSer129 α -syn (green; Mouse BioLegend, Table 1) and cellular activity detected using pS6 (red) in mitral cells. (P) Quantification of pS6 expression levels shows no significant differences between WT and α -syn-Tg mice. Nuclei counterstained with Dapi (blue). Abbreviations: AC3: adenylyl cyclase type 3; AON: anterior olfactory nucleus; EPL: external plexiform layer; GAP43: growth associated protein-43; GL: glomerular layer; GCL: granule cell layer; LOT: lateral olfactory tract; LP: lamina propia; LV: lateral ventricle; MCL: mitral cell layer; NQO1: NAD(P)H dehydrogenase [quinone] 1; OB: olfactory bulb; OE: olfactory epithelium; OMP: olfactory marker protein; OSNs: olfactory sensory neurons; PC: piriform cortex; pS6: phosphorylated S6 Ribosomal Protein (Ser235/236); RMS: rostral migratory stream; SP: septum; TuS: tubular striatum; TH: tyrosine hydroxylase; VGLUT2: vesicular glutamate transporter 2. Scale bars of 500 μ m in F-I; 200 μ m in J, K; 100 μ m in L, M; 50 μ m in B-E, N, O. Statistics: Unpaired t-test.

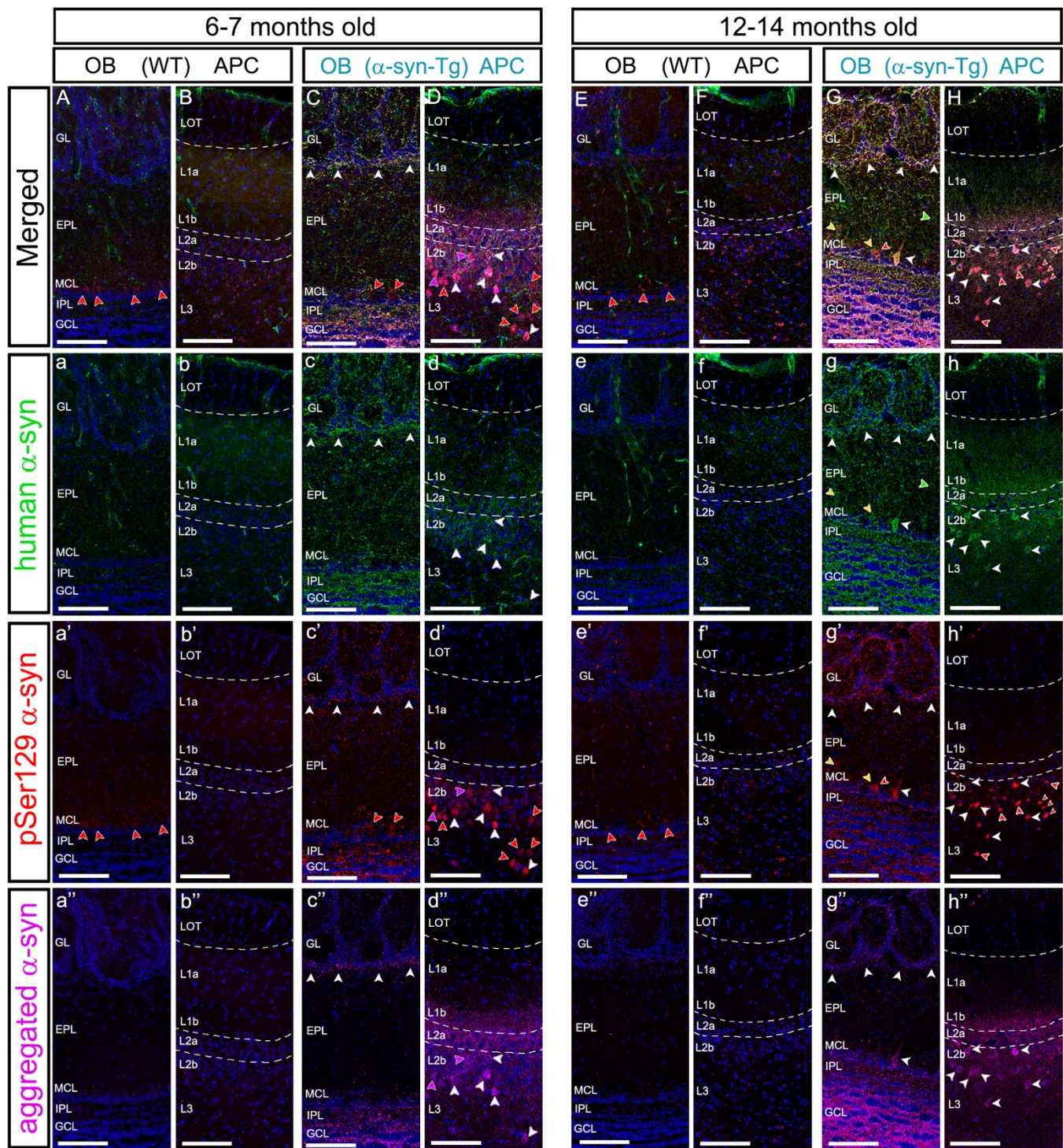


Figure 3. Detection of transgenic human α -synuclein expression and associated α -syn pathology in α -syn-Tg mice. A human specific anti- α -syn antibody (h α -syn) is used to detect the expression of the transgene in the α -syn-Tg mice (green). α -Syn pathology is detected using pSer129- α -syn (red; Rabbit Abcam, Table 1; largely somatic) and aggregated α -syn (magenta; Rat BioLegend, Table 1; largely synaptic). (A-a''; B-b'') Staining in the OB and APC of WT control mice at 6-7 months. Only a residual expression of pSer129- α -syn is seen in OB mitral cells (A,a', red arrowheads). (C-c''; D-d'') Staining in the OB and APC of α -syn-Tg mice at 6-7 months. Transgene expression in the OB is seen in neuronal processes of the GL, EPL, IPL and GCL layers. (c). In APC, h α -syn stains neuronal processes of layers 1, 2 and 3 and it appears somatic in some neurons of 2b (d). Most of these processes and neuronal bodies

in APC co-expressed α -syn pathology (white arrowheads) ($h\alpha$ -syn⁺; red arrowheads in C, c', D, d'; magenta arrowheads in D, d', d''). Strong expression of aggregated α -syn is detected in layers 1b and 2a of APC (d''). (E-e''; F'f'') Staining in the OB and APC of WT control mice at 12-14 months. A residual expression of pSer129- α -syn remains in the OB mitral cells at this age (E, e', red arrowheads). (G-g''; H-h'') Staining in the OB and APC of α -syn-Tg mice at 12-14 months. Compared with 6-7 months, at 12-14 months there is a stronger accumulation of the transgene ($h\alpha$ -syn⁺, green) along all layers of OB (g), and layers 1b, 2a and 2b in APC (h). $h\alpha$ -syn staining is somatic in some mitral cells (g). In APC, most neurons expressing $h\alpha$ -syn also show pathology (H-h''). In APC no $h\alpha$ -syn or α -syn pathology is seen in layer 2a (H-h''). Nuclei counterstained with Dapi (blue). Abbreviations: APC: anterior piriform cortex; EPL: external plexiform layer; GCL: granule cell layer; GL: glomerular layer; IPL: internal plexiform layer; LOT: lateral olfactory tract; MCL: mitral cell layer; OB: olfactory bulb. Scales of 100 μ m.

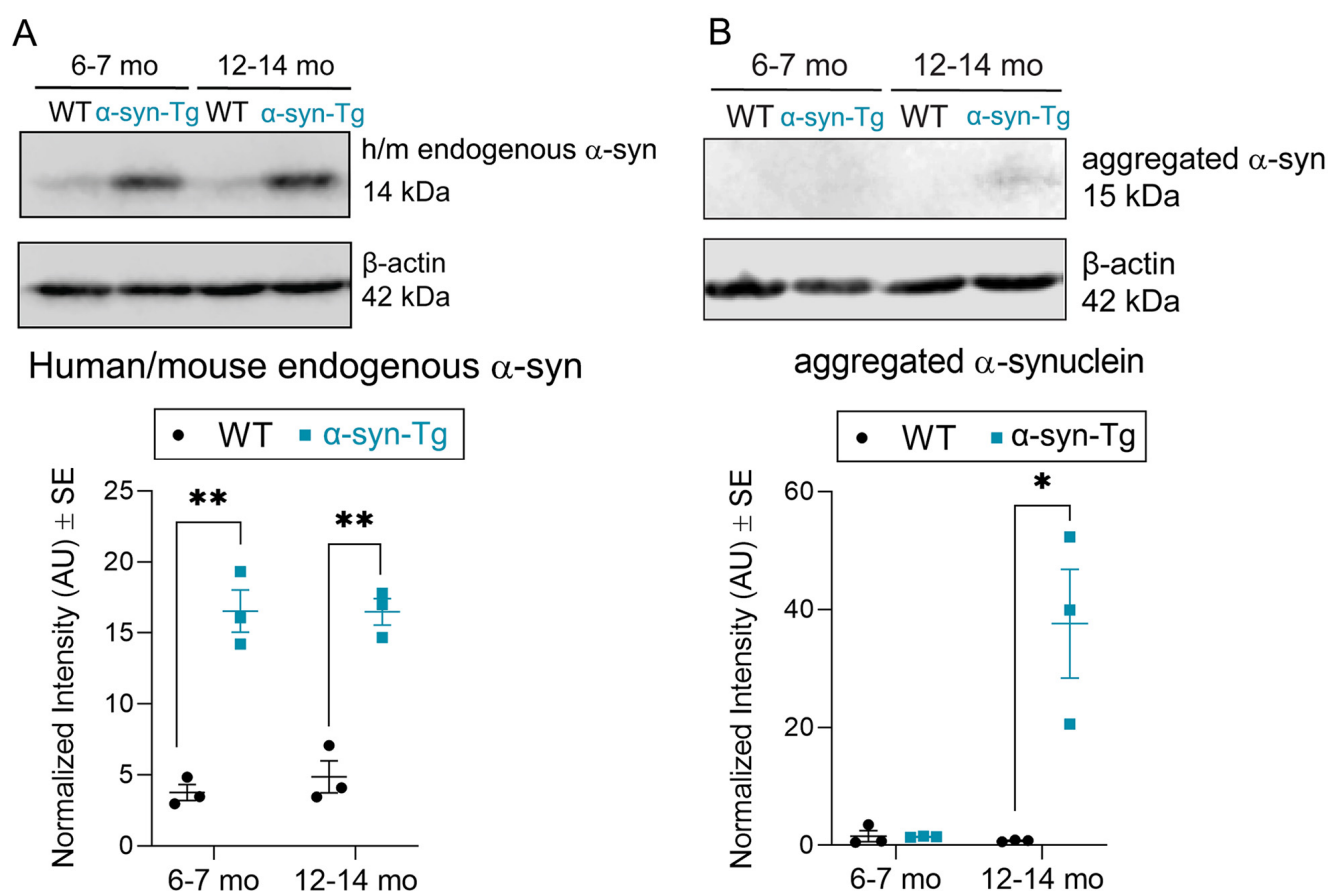


Figure 4. Quantification of human/endothelial and aggregated α -synuclein by western blot (WB). (A) WB detecting total (endogenous and human) α -syn in WT controls and α -syn-Tg mice at both studied stages (6-7- and 12-14-months mice). The amount of protein quantified by densitometry showed a significant 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 (Mouse 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.

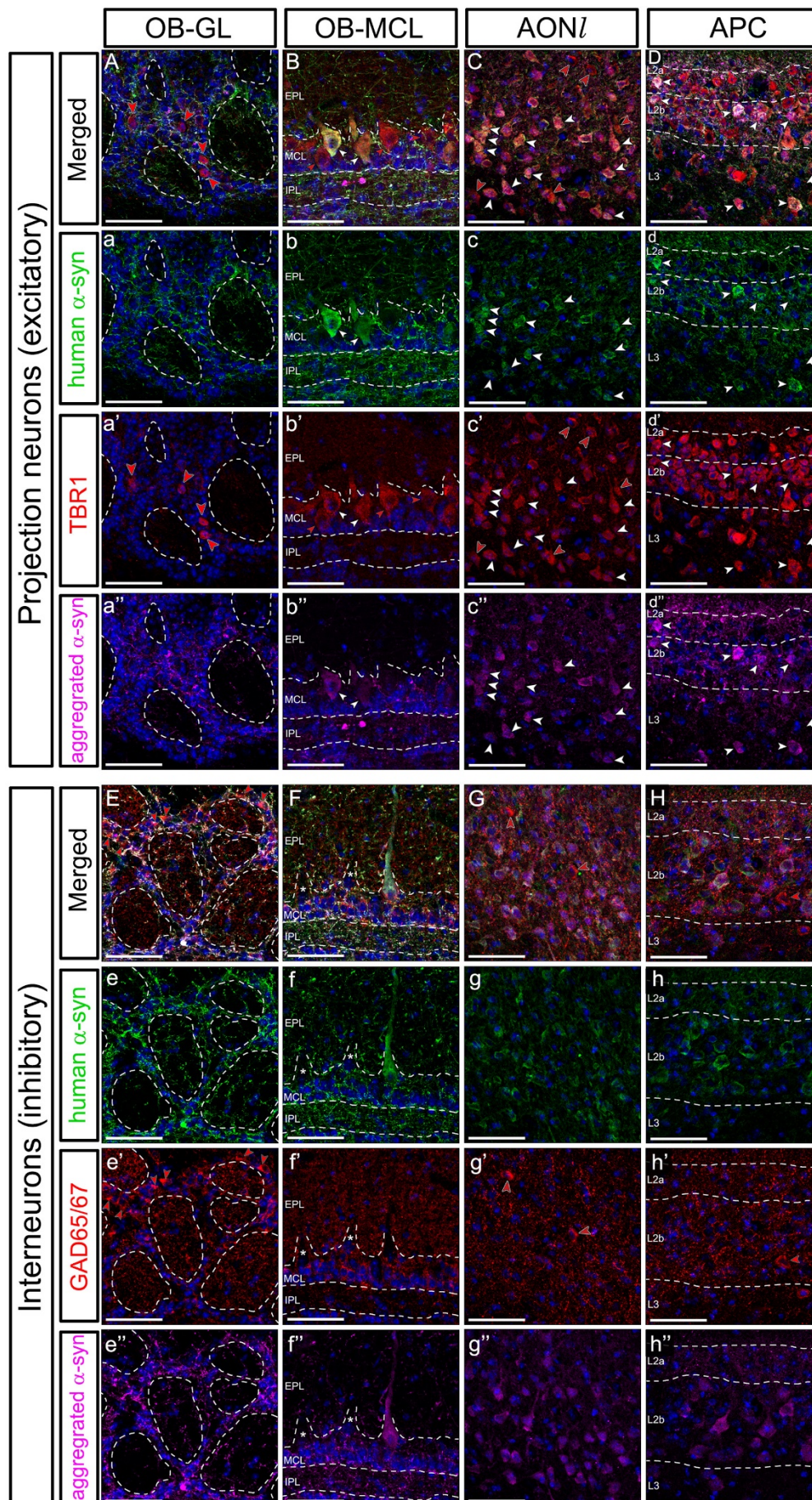


Figure 5. Detection of the transgenic human α -synuclein expression in excitatory projection and inhibitory neurons at 12-14 months. (A-a'' to D-d'') Staining for human specific α -syn ($h\alpha$ -syn; green) with the projection neurons marker TBR1 (red) and aggregated α -syn (magenta, Rat BioLegend, Table 1) in the OB: GL and MCL layers, the lateral AON and APC. No colocalization is seen in TBR1⁺ neurons in the OB-GL with $h\alpha$ -syn or aggregated α -syn (A-a', red arrowheads). In the OB-MCL, AONl and APC, some neurons express the transgene ($h\alpha$ -syn⁺) which always colocalized with TBR1 and α -syn pathology (B-b'', C-c'', D-d'', white arrowheads). (E-e'' to H-h'') Staining for human specific α -syn ($h\alpha$ -syn; green) with the inhibitory neurons marker GAD65/67 (red) and aggregated α -syn (magenta, rat BioLegend, Table 1) in the same olfactory regions. GAD65/67⁺ neuron colocalizes with $h\alpha$ -syn or aggregated α -syn. The IPL, which is occupied predominantly by OB projection neuron axons is labeled for $h\alpha$ -syn or aggregated α -syn but not for GAD65/67, indicating that the transgene is prominent in projection excitatory neurons. Nuclei counterstained with Dapi (blue). Abbreviations: AONl: lateral anterior olfactory nucleus; APC: anterior piriform cortex; EPL: external plexiform layer; GAD65/67: glutamate decarboxylase isoforms 65 and 67; IPL: internal plexiform layer; MCL: mitral cell layer; OB: olfactory bulb; TBR1: T-Box Brain Transcription Factor-1. Scales of 50 μ m.

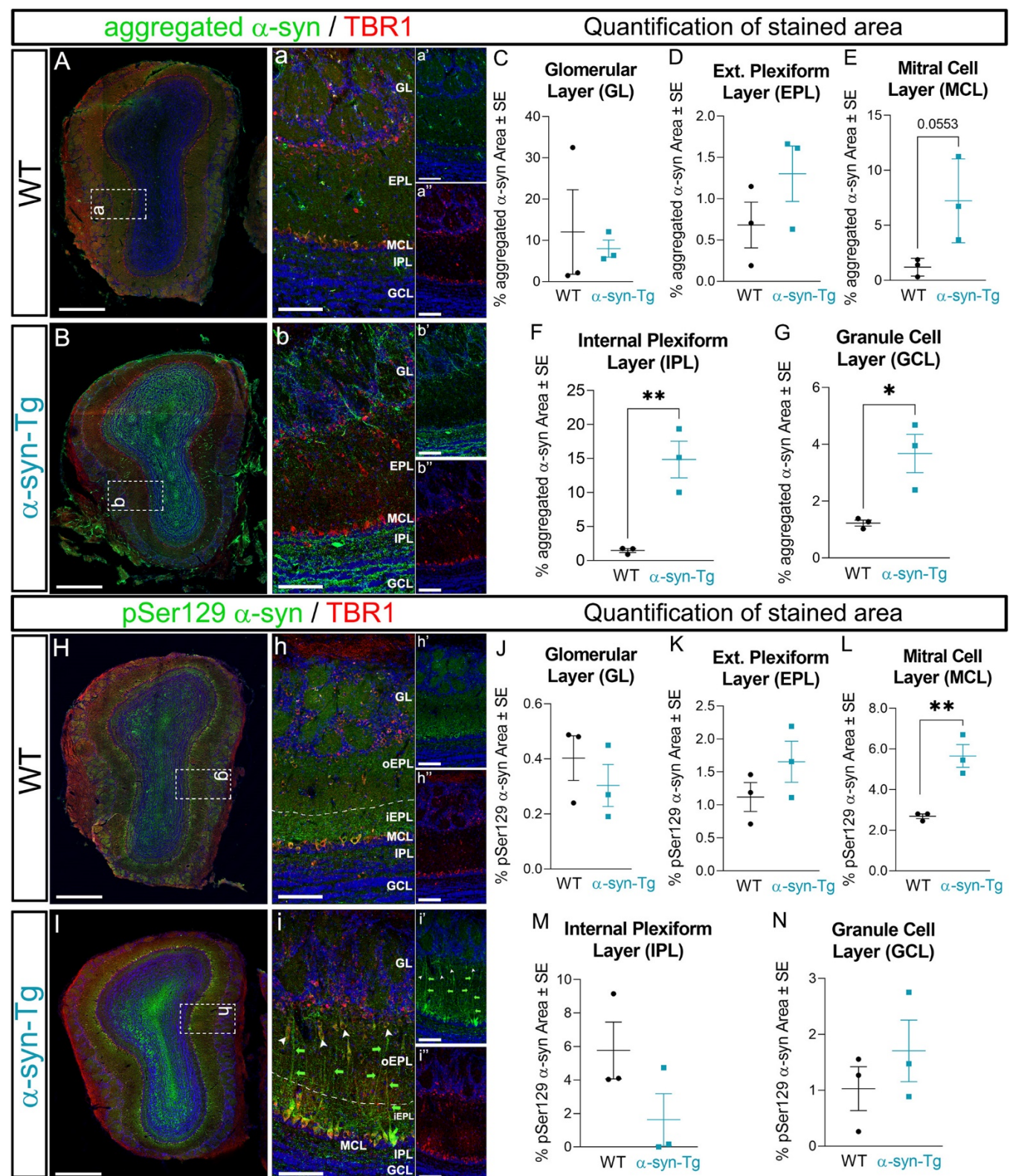


Figure 6. α -Synuclein pathology in the olfactory bulb (OB) of mice at 12-14 months. (A-a'', B-b'') Representative images of OB coronal sections of mice stained against aggregated α -syn (green, Mouse BioLegend, Table 1) and TBR1 (red) as a marker for projection neurons in WT (A-a'') and α -syn-Tg (B-b'') mice. Staining seems to be restricted to cell processes. Quantification of stained area shows a significantly higher accumulation of aggregated α -syn in α -syn-Tg mice in the IPL (F) and GCL (G), with a strong trend in MCL (E). No differences are detected between the groups in GL (C) and EPL (D). (H-

h'', I-i'') Representative images of OB coronal sections of mice stained against pSer129- α -syn (green, Mouse BioLegend, Table 1) and TBR1 (red) in WT (H-h'') and α -syn-Tg (I-i'') mice. Quantifications show significantly increased pSer129- α -syn in the MCL of α -syn-Tg mice compared to controls (L) with no statistical differences in the other layers (J, K, M, N). Remarkably, a strong accumulation of pSer129- α -syn is seen in tufted cells (i, i', white arrowheads) and in apical and lateral dendrites of mitral cells (i, i', green arrowheads) of α -syn-Tg. There is a characteristic staining of pSer129- α -syn in the GCL forming a pattern intermingled with granule cell islets that likely belong to M/Tc axons (I). Nuclei counterstained with Dapi (blue). 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, H, I; 100 μ m in a-a'', b-b'', h-h'', i-i''. * = $p < 0.05$; ** = $p < 0.01$. Statistics: Unpaired t-test.

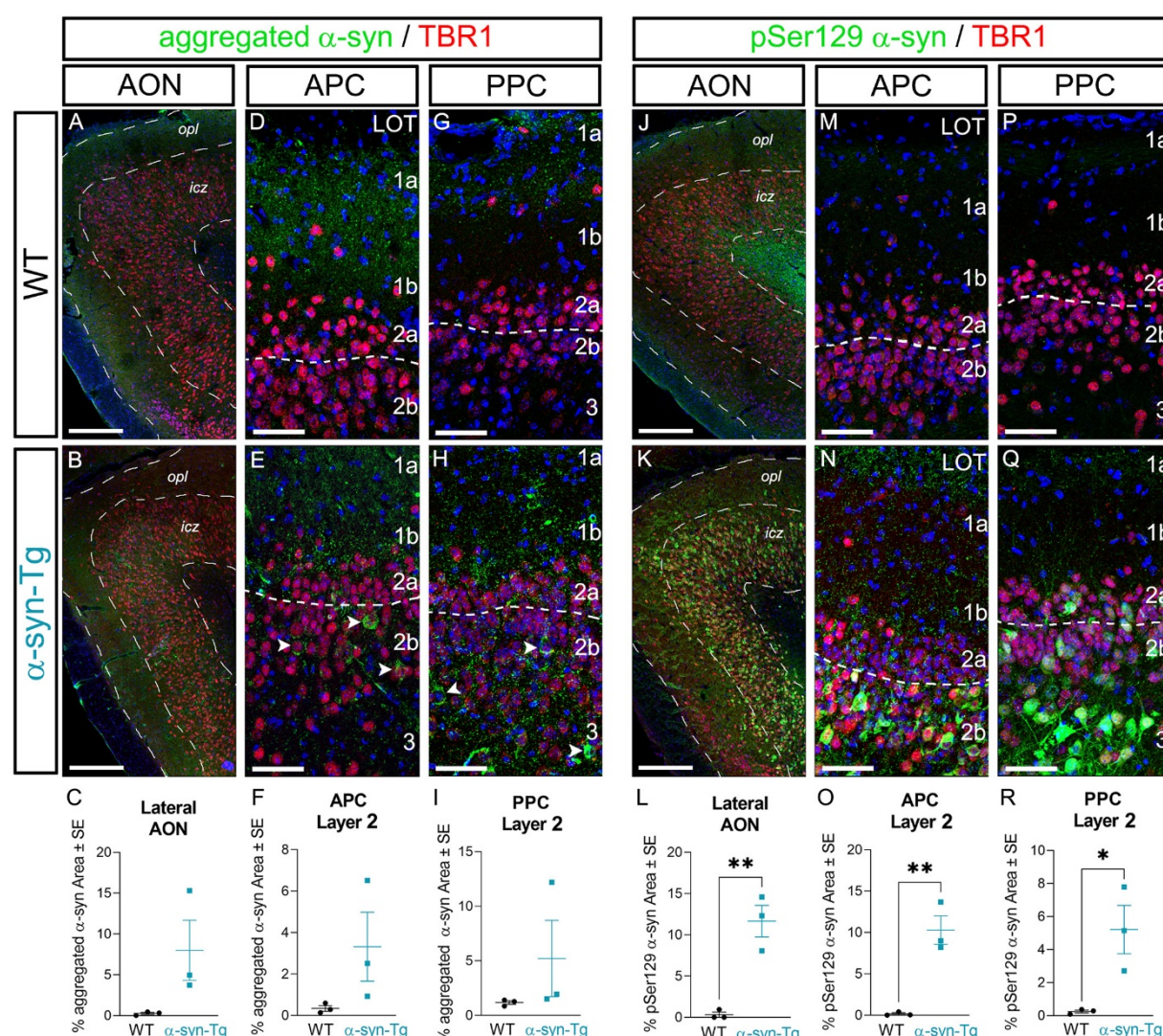


Figure 7. α -synuclein pathology in anterior olfactory nucleus and piriform cortex of mice at 12-14 months. (A, B) Representative images of AON coronal sections from WT and α -syn-Tg mice stained against aggregated α -syn (green, Mouse, BioLegend, Table 1) and TBR1 (red) as a marker for projection neurons. (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 2 (a + b) 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, Mouse BioLegend, Table 1) 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 layer 2 (a + b) 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 with Dapi (blue). Abbreviations: AON: anterior olfactory nucleus; APC: anterior piriform cortex; *icz*: inner cell zone; *opl*: outer plexiform layer; PPC: posterior piriform cortex; 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.

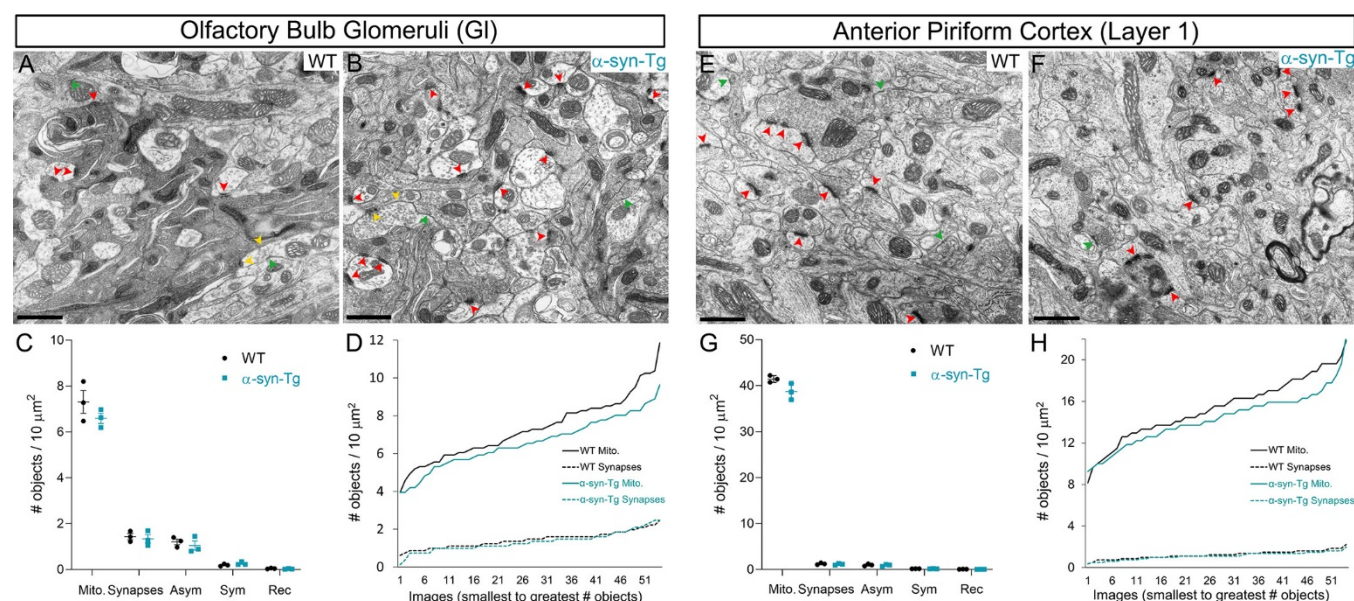


Figure 8. Ultrastructure of synapses and mitochondria in the OB glomeruli and APC layer I. (A, B) Representative images of electron microscopy in the glomeruli of OBs from WT and α -syn-Tg mice. Number of mitochondria (Mito) and different types of synapses are quantified and highlighted as: asymmetric (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 1 parenchyma at EM showing asymmetric (red arrowheads) and symmetric (green arrowheads) synapses. (G) Quantification of mitochondria and synapses in APC showing no statistical differences between WT and α -syn-Tg mice. (H) Representation of the distribution of mitochondria 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.

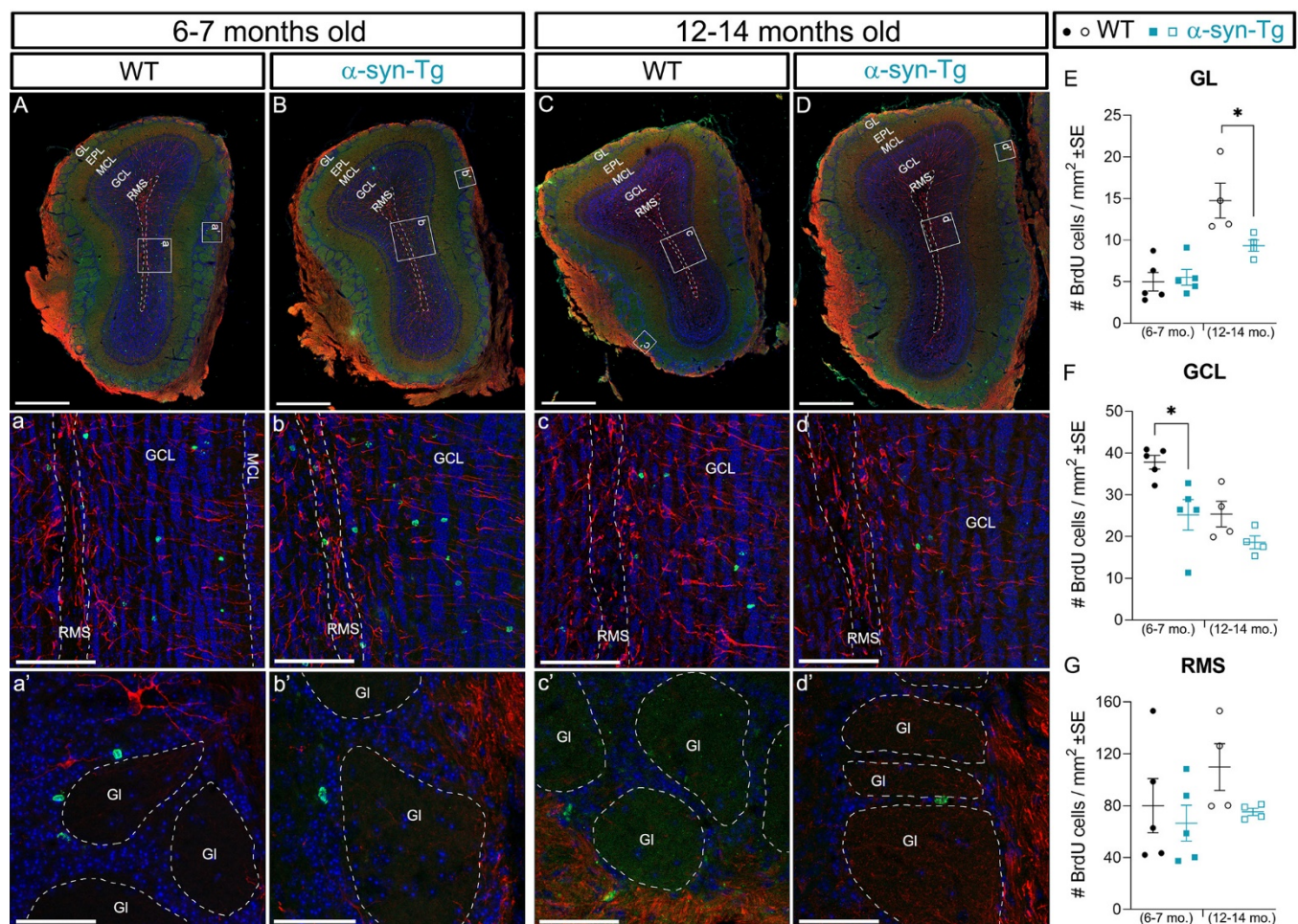


Figure 9. Effect in adult neurogenesis in the olfactory bulb glomerular and granule cell layers of α -syn-Tg mice. Neurogenesis is studied by counting newly generated cells 25 days after they incorporated the thymidine analog BrdU during cell division at the moment of injection. BrdU staining is shown in green, while immature neurons (neuroblasts) are identified with doublecortin (DCX) marker (red). (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. Nuclei counterstained with Dapi (blue). 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 μ m in A-D; 100 μ m in a-d; 50 μ m in a'-d'. * = p < 0.05. Statistics: Unpaired t-test.

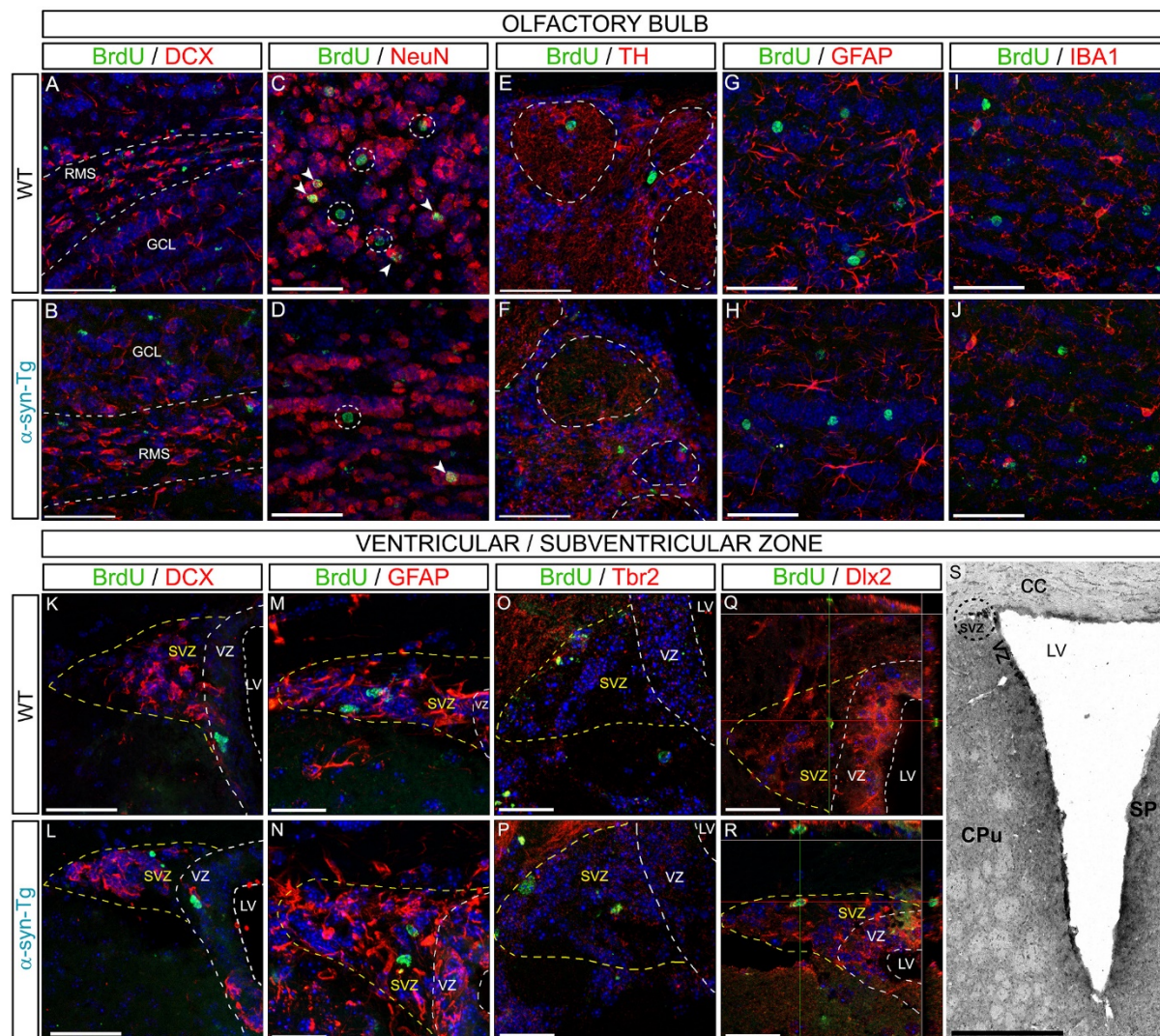


Figure 10. Characterization of cellular phenotypes of BrdU-expressing cells in the olfactory bulb and subventricular zone after 25 days of injection. BrdU staining (green) is shown with different markers typical of neuronal and glial cells (red). (A-J) Phenotypic characterization in the OB. (A, B) no BrdU⁺ cell expresses the neuroblast marker DCX in either the GCL or RMS. (C, D) Identification of mature neurons with NeuN in the GCL, which accounts for roughly 43% and 30% of the BrdU⁺ cells in WT controls and α -syn-Tg mice, respectively. (E, F) Detection of dopaminergic neurons in the GL using the TH markers. No colocalization is seen with BrdU⁺ cells. (G, H) GFAP marker is used to identify astrocytes shows no colocalization with BrdU⁺ cells. (I, J) Microglial cells expressing IBA1 do not colocalize with BrdU⁺ cells either. (K-S) Phenotypic characterization in the SVZ of WT control and α -syn-Tg mice. (K, L) DCX marker is used to label neuroblast or “A” cells which do not colocalizes with BrdU⁺ cells. (M, N) GFAP is used to label radial glia neural stem cells or “B” cells do not colocalizes with BrdU⁺ cells. (O, P) TBR2 used to identify SVZ-dorsal TACs do not colocalizes with BrdU⁺ cells. (Q, R) Dlx2 is used to label TACs or “C” cells which colocalizes with adult resident BrdU⁺ cells at the time of study (25 days post-BrdU injection). (S) Greyscale image of the analyzed region showing the anatomical location of the SVZ next to the ventricular zone (VZ) and the lateral ventricle (LV). Nuclei counterstained with Dapi (blue). Abbreviations: DCX: doublecortin; Dlx2: Distal-Less Homeobox 2; GCL: granule cell layer; GFAP: glial fibrillary acidic protein; IBA1: ionized calcium-binding adapter molecule-1; NeuN: neuronal nuclei marker; OB: olfactory bulb; RMS: rostral migratory stream; SVZ: subventricular zone; TACs: transient amplifying cells; TBR2: T-Box brain transcription factor-2; TH: tyrosine hydroxylase. Scale bars of 400 μ m in S; 50 μ m in A-J; 25 μ m in K-R.

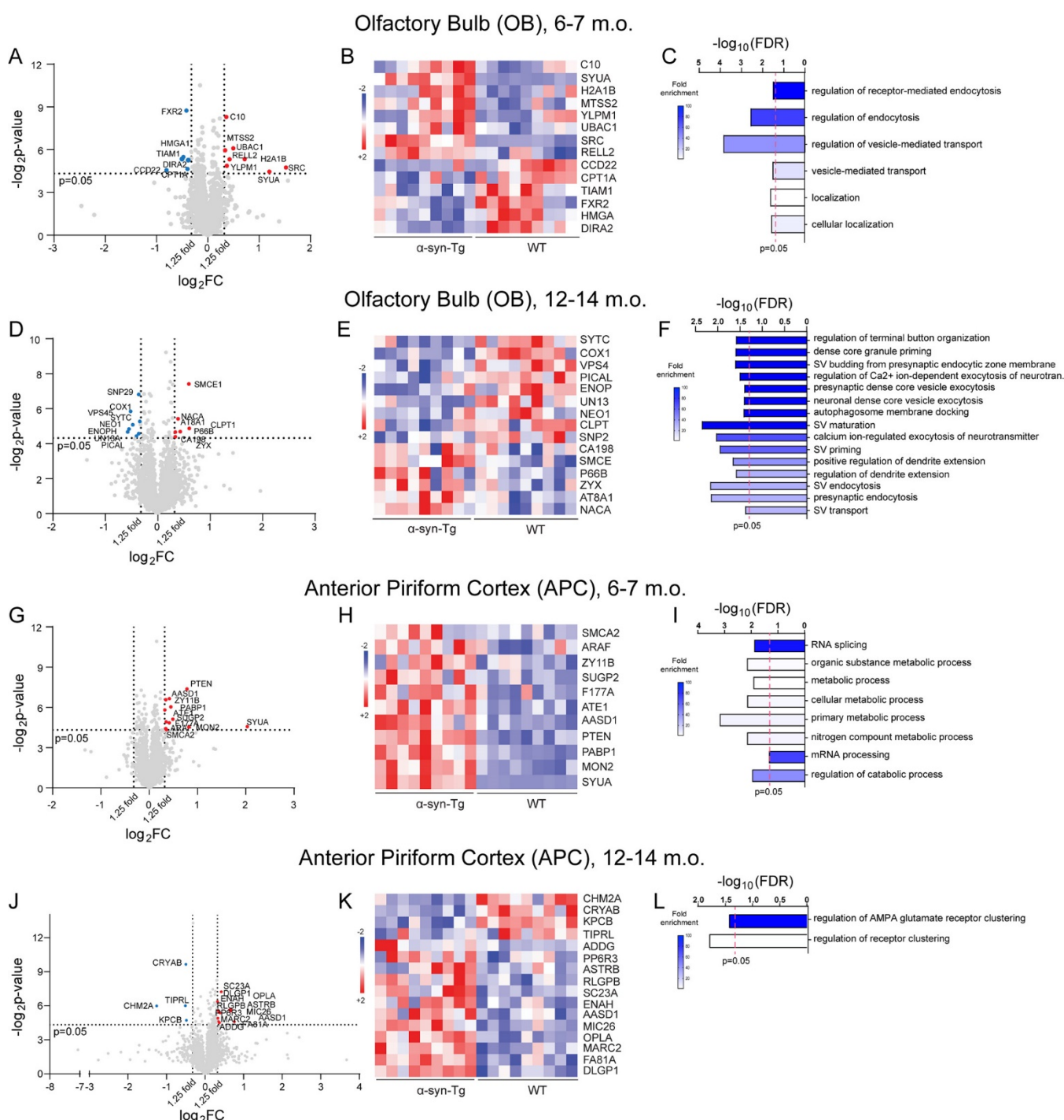


Figure 11. Proteomics of OB and APC revealed defects in synaptic endo- and exocytosis pathways in the OB, but minimum alterations in APC of α -syn-Tg mice. (A) Volcano plot of fold change in the OB proteome of 6-7 months α -syn-Tg compared to control wild type (WT) mice (n=3 mice/genotype). Proteins 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. Abbreviations: APC: anterior piriform cortex; GO: Gene Ontology analysis; OB: olfactory bulb.

Tables

Table 1. Primary and secondary antibodies

Antigen	Primary Ab	Source (Cat. #)	RRID	Dilution	Secondary Ab	Source	Dilution
AC3	Rabbit Poly.	ThermoFisher (PA5-35382)	AB_2552692	1:300	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
α -synuclein endo (M, H)	Mouse IgG1	BD Bioscience (610786)	AB_398107	1:500 (IHC)	Goat anti-Ms IgG1 Alexa 555	Invitrogen	1:1000
				1:1000 (WB)	Goat anti-Ms IgG1 IRDye 800	LICOR	1:10000
α -synuclein (H)	Mouse IgG1	Millipore Sigma (MABN824)	AB_2921328	1:1000	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
α -synuclein, aggregated	Mouse IgG1	BioLegend (847801)	AB_2632811	1:500	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
α -synuclein, aggregated	Mouse IgG1k	EMD Millipore (MABN389)	AB_2716647	1:300	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
α -synuclein, aggregated	Rat IgG2a, k	BioLegend (864901)	AB_2810763	1:300	Goat anti-Rat IgG Alexa 546 Donkey anti-Rat IgG Alexa 647	Invitrogen	1:1000
α -synuclein pSer129	Mouse IgG2a	BioLegend (825702)	AB_2734593	1:1000	Goat anti-Ms IgG2a Alexa 488	Invitrogen	1:1000
α -synuclein pSer129	Rabbit IgG	Abcam (ab51253)	AB_86997	1:500	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000
β -actin	Mouse IgG1	GeneTex (GTX629630)	AB_2728646	1:1000	Goat anti-Ms IgG1 IRDye 800	LICOR	1:10000
BrdU	Rat IgG	Abcam (ab6326)	AB_305426	1:300	Goat anti-Rat IgG Alexa 488	Invitrogen	1:1000
Caspase 3 cleavage	Rabbit Poly.	Cell Signaling (9661)	AB_2341188	1:500	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
c-Src/Src	Mouse IgG2a	Santa Cruz (sc-5266)	AB_627308	1:250	Goat anti-Ms IgG2a Alexa 555	Invitrogen	1:1000
Dlx2	Rabbit Poly.	EMD Millipore (AB5726)	AB_2093141	1:200	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
Doublecortin (DCX)	Guinea Pig	EMD Millipore (AB2253)	AB_1586992	1:500	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
GAP43	Mouse IgG2a	ThermoFisher (33-5000)	AB_2533123	1:100	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000
GFAP	Rabbit Poly.	BioLegend (840001)	AB_2565444	1:1000	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
Iba1	Rabbit Poly.	Wako (016-20001)	AB_839506	1:200	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
NQO1	Goat IgG	Abcam (ab2346)	AB_302995	1:100	Donkey anti-Goat Alexa 555	Invitrogen	1:1000
OMP	Goat Poly.	Wako (019-22291)	AB_664696	1:500	Donkey anti-Goat Alexa 546	Invitrogen	1:1000
Phospho-S6 (pS6)	Rabbit IgG	Cell Signaling (4858)	AB_916156	1:400	Goat anti-Rb IgG Alexa 555	Invitrogen	1:1000
TBR1	Rabbit Poly.	Abcam (ab31940)	AB_2200219	1:500	Goat anti-Rb IgG Alexa 546/647	Invitrogen	1:1000
TBR2	Rabbit Poly.	Abcam (ab23345)	AB_778267	1:250	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
TH (clone LNC1)	Mouse IgG1	EMD Millipore (MAB318)	AB_2201528	1:500	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
VGLUT2	Rabbit Poly.	SySy (135-403)	AB_887883	1:200	Donkey anti-Rb IgG Alexa 488	Invitrogen	1:1000

Table 2: 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-palmitoyl transferase 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

Table 3: 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	Neol	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

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 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

Table 5: 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

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Abstract: Parkinson's Disease (PD), considered a movement disorder, is being increasingly studied as a cognitive disorder. Cognitive dysfunctions are predominant in PD patients with GBA mutations, a gene that encodes Gcase1 and regulates glycosphingolipid metabolism. Mutations in the gene SNCA that encodes α -synuclein is well studied in PD. Aggregation of α -synuclein is the neuropathological hallmark of PD. We developed a mice model that carries a GBA mutation, and crossed it with a mice model of PD with a SNCA mutation to evaluate how GBA mutations might exacerbate cognitive dysfunctions of PD. Mice that carry both GBA and SNCA mutations revealed exacerbated age-related cognitive behavior deficits compared to single mutants. Interestingly, the GBA mutant mice alone had cognitive deficits compared to wild-type in novel object recognition, suggesting the GBA mutation alone may exacerbate cognitive phenotypes. Western blots revealed that mice carrying both mutations possess higher pathological α -synuclein in their brains compared to mice that carry only the SNCA mutation. Immunohistochemistry revealed an increase in pathological α -synuclein in double mutants, especially in cognitive brain areas, including frontal cortex and hippocampus. Thus, it appears that dysfunctional glycosphingolipid metabolism in GBA mutations synergistically acts with dysfunctional α -synuclein to exacerbate PD pathology in cognitive brain areas.

Conference: Annual Biomedical Research Conference for Minoritized Scientists (ABRCMS), virtual, November 9-12, 2022

Title: Pathogenic mechanisms for cognitive dysfunctions of Parkinson's disease: insights from studies on GBA mutations

Authors: Risha Chakraborty; Vidyadhara DJ, Ph.D.; David Backstrom, Ph.D.; Jamie Park; Jeff Jiapeng Ruan, Ph.D.; Pramod Mistry, M.D., Ph.D.; and Sreeganga Chandra, Ph.D.

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Abstract: Parkinson's Disease (PD), considered a movement disorder, is being increasingly studied as a cognitive disorder. Cognitive dysfunctions are predominant in PD patients with GBA mutations, a gene that encodes Gcase1 and regulates glycosphingolipid metabolism. Mutations in the gene SNCA that encodes a-synuclein is well studied in PD. Aggregation of a-synuclein is the neuropathological hallmark of PD. We developed a mice model that carries a GBA mutation, and crossed it with a mice model of PD with a SNCA mutation to evaluate how GBA mutations might exacerbate cognitive dysfunctions of PD. Mice that carry both GBA and SNCA mutations revealed exacerbated age-related cognitive behavior deficits compared to single mutants. Interestingly, the GBA mutant mice alone had cognitive deficits compared to wild-type in novel object recognition, suggesting the GBA mutation alone may exacerbate cognitive phenotypes. Western blots revealed that mice carrying both mutations possess higher pathological alpha-synuclein in their brains compared to mice that carry only the SNCA mutation. Immunohistochemistry revealed an increase in pathological alpha-synuclein in double mutants, especially in cognitive brain areas, including frontal cortex and hippocampus. Thus, it appears that dysfunctional glycosphingolipid metabolism in GBA mutations synergistically acts with dysfunctional alpha-synuclein to exacerbate PD pathology in cognitive brain areas. Preliminary investigations of synaptic defects as possible mechanisms of cognitive decline suggest that both presynaptic and postsynaptic markers are significantly upregulated in the double mutant cortices compared to cortices of mice with just the SNCA mutation, suggesting that GSL accumulation exacerbates synaptic defects resulting from synuclein accumulation, specifically in synaptic vesicles.

Conference: International Congress of Parkinson's Disease and Movement Disorders, September 15-18, 2022

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Abstract:

Title: Presynaptic dopamine compartmentalization defects initiate auxilin-mediated parkinsonism

Objective: To evaluate the pathogenic mechanisms for auxilin-mediated Parkinson's disease (PD).

Background: Auxilin participates in uncoating of clathrin-coated vesicles (CCVs), thereby facilitating synaptic vesicle (SV) re-generation in presynaptic termini. Loss-of-function mutations of auxilin (PARK19) cause early-onset parkinsonism. Here, we utilized auxilin-knockout (KO) mice to elucidate the mechanisms through which auxilin deficiency, and more broadly clathrin-uncoating deficits lead to parkinsonism.

Methods: We performed behavioral and histochemical evaluations to understand if auxilin KO mice develop PD-like abnormalities. To get an unbiased insight into the early mechanisms, we performed proteomic analysis of the whole-brain, synaptosomes, and CCVs in auxilin-KO and WT mice. Taking clues from proteomics, we evaluated dopamine release and reuptake kinetics *in vivo* in the dorsal striatum using fast-scan cyclic voltammetry. Dopamine transporters (DAT) were evaluated by immunostaining and *ex-vivo* imaging with a dichloropane probe. The SVs, CCVs, autophagosomes and axonal membrane in the dorsal striatum were evaluated using electron microscopy.

Results: We demonstrate that auxilin KO mice display the cardinal features of PD, including progressive motor deficits, synucleinopathy, nigral dopaminergic loss, and neuroinflammation. Through unbiased proteomic and neurochemical analysis, we determined disrupted dopamine homeostasis in auxilin KO mice. We measured dopamine reuptake kinetics *in vivo* and confirmed that they were decreased, potentially due to dopamine transporter (DAT) misrouting into axonal membrane deformities of the dorsal striatum. We also showed that elevated synaptic autophagy and defective SV protein sorting contribute to ineffective dopamine sequestration and homeostasis.

Conclusions: Parkinsonian pathology in auxilin deficiency begins with a disruption of clathrin uncoating leading to fewer functional SVs for neurotransmitter filling. While these deficits occur at all synapses, it is particularly detrimental to nigrostriatal dopaminergic synapses due to the toxicity of cytosolic dopamine, dopaminergic axonal deformity, and aberrant DAT trafficking mediated dysfunction in dopamine reuptake. Thus, investigating auxilin loss-of-function mutations have also helped decipher the mechanisms for dopaminergic specific vulnerability of PD. This study also enhances our understanding of how SV endocytosis deficits lead to PD.

Conference: Society for Neuroscience (SfN) meeting, November 12-16, 2022

Authors: Vidyadhara D J¹, Mahalakshmi Somayaji², Nigel Wade¹, Betül Yücel¹, Helen Zhao¹, Shashaank N², Joseph Ribaud¹, Jyoti Gupta¹, TuKiet T. Lam¹, Dalibor Sames², Lois E. Greene³, David L. Sulzer², and Sreeganga S. Chandra^{1*}

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Abstract:

Background: Auxilin, a co-chaperone, participates in the uncoating of clathrin-coated vesicles (CCVs) and facilitates synaptic vesicle (SV) regeneration at presynapses. Auxilin (DNAJC6/PARK19) loss-of-function mutations cause early-onset Parkinson's disease (PD). Here, we utilized auxilin knockout (KO) mice to elucidate the mechanisms through which auxilin deficiency and clathrin-uncoating deficits lead to PD.

Methods: We performed longitudinal behavioral and histopathological analysis of wildtype (WT) and auxilin KO mice and evaluated development of PD characteristics. To obtain unbiased insights into mechanisms, we performed proteomic analysis of the whole brain, synaptosomes, and CCVs derived from 3-month-old auxilin KO and WT mice. Taking cues from the proteomic results, we performed neurochemical analyses to measure levels of dopamine and its metabolites, and in vivo fast-scan cyclic voltammetry along with a new computational analysis to quantitate striatal dopamine release and reuptake kinetics. Dopamine transporters (DAT) membrane localization was evaluated by immunostaining, novel dichloropane-based ex-vivo imaging, and conventional and immuno-electron microscopy (EM). Monoaminergic neurotransmitter transporters and endocytic proteins were also evaluated by immunostaining. The SVs, CCVs and autophagic vacuoles in the dorsal striatum were evaluated using EM.

Results: We show that auxilin KO mice display the cardinal features of PD, including progressive motor deficits, α -synuclein pathology, nigral dopaminergic neuron loss, decreased striatal dopamine levels, and gliosis. Through proteomic and neurochemical analyses, we demonstrate that dopamine homeostasis is disrupted in auxilin KO brains, including slower dopamine reuptake kinetics in vivo, an effect associated with DAT misrouting into axonal membrane deformities in the dorsal striatum. We also show that elevated macroautophagy and defective SV protein sorting contribute to ineffective dopamine sequestration and homeostasis, ultimately leading to neurodegeneration.

Conclusions: Our findings indicate that auxilin mutations and clathrin uncoating deficits lead to PD through four distinct mechanisms: cytoplasmic dopamine accumulation, DAT mis-trafficking, SV sorting deficits and autophagic overload, which collectively lead to dopamine compartmentalization defects. This study advances our knowledge of how presynaptic endocytosis deficits lead to dopaminergic vulnerability and pathogenesis of PD.