UNIVERSITY OF CALIFORNIA SAN DIEGO

Accumulation of α-Synuclein Impairs Trafficking and Processing of Amyloid Precursor Protein in a Mouse Model of Parkinson's Disease

A Thesis submitted in partial satisfaction of the requirements for the degree

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in

Biology

by

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2020

The Thesis of Savannah YT Ding Fang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2020

DEDICATION

In recognition of her unending support throughout my academic career, this thesis is dedicated to my mom.

EPIGRAPH

"Be present in all things and thankful for all things."

Maya Angelou.

TABLE OF CONTENTS

Signature Page iii

Dedication iv

Epigraph v

Table of Contents vi

List of Figures vii

Acknowledgments viii

Abstract of the Thesis xi

Introduction 1

Methods 7

Results 11

Discussion 18

Figures 22

References 32

LIST OF FIGURES

Figure 1. Trafficking of amyloid precursor protein (APP) is disrupted in overexpressed α-synuclein (ASYN) neurons, but not in ASYN-knockout neurons 22

Figure 2. The presence of GFP-hASYN induced retrograde axonal transport deficits of β-secretase enzyme (BACE1) in E18 cortical neurons 23

Figure 3. Decreased levels of BACE1 were detected in E18 GFP-hASYN PD brain samples 24

Figure 4: Expression of GFP-ASYN impaired retrograde axonal transport for early endosomes (Rab5) in E18 cortical neurons 25

Figure 5: Expression of GFP-ASYN impaired retrograde axonal transport for late endosomes (Rab7) in E18 cortical neurons 26

Figure 6. GFP-hASYN colocalizes with Rab5 in E18 GFP-hASYN PD cortical neurons 27

Figure 7. Retrograde axonal trafficking of lysosomes is disrupted in GFP-hASYN PD E18 cortical neurons 28

Figure 8. Effect of GFP-hASYN on axonal transport of mitochondria. 29

Figure 9. Expression of GFP-hASYN disrupts synaptic formation 30

Figure 10. Expression of GFP-WT-ASYN or GFP-A53T-ASYN induced neuronal atrophy 31

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ABSTRACT OF THE THESIS

Accumulation of α-Synuclein Impairs Trafficking and Processing of Amyloid Precursor Protein in a Mouse Model of Parkinson's Disease

by

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Parkinson's Disease (PD) is the second most common neurodegenerative disease, which is characterized by the loss of dopaminergic neurons in the substantia nigra of the brain possibly due to the accumulation of α-synuclein (ASYN). Mutations or triplication of the ASYN gene (SNCA) contribute to synucleinopathies including PD, but studies have also noted that a significant portion of PD patients develop Alzheimer's Disease (AD) like dementia. Therefore, we hypothesized that excessive accumulation of ASYN may also impact the trafficking and processing of the amyloid precursor protein (APP), initiating the pathogenesis of AD. To test our hypothesis, we used a transgenic mouse model of PD that

over-expresses a green fluorescent protein fused human ASYN (GFP-hASYN) transgene. Our findings suggest that the over-expression of GFP-hASYN in mouse neurons i.e. PD neurons impaired axonal trafficking and processing of APP. Furthermore, APP carboxyl terminal fragment (APP CTF) levels were significantly higher in GFP-hASYN^{+} positive PD neurons than in GFP-hASYN^{-} neurons, suggesting impairment in downstream processes. Interestingly, in ASYN knockout (SynKO) neurons, APP trafficking and processing was largely unaffected as compared to wildtype (WT) neurons'. Based on these observations, we speculate that selective impairment of trafficking and processing of APP by ASYN may give rise to toxic CTFs, which may contribute to blockages, leading to endosomal enlargement, axonal transport impairment and eventually neuronal atrophy. This study provides an important molecular mechanism by which excessive accumulation of ASYN could potentially be linked to cellular events that lead to AD pathogenesis in addition to its role in inducing neurotoxicity in PD pathology.

INTRODUCTION

Parkinson's Disease (PD) General Overview

Parkinson's disease (PD) is one of the most common neurodegenerative disease in the world that affects 1% of the population above 60 years of age [1]. The main clinical symptoms of PD include rigidity in the limbs, delayed movement, and tremors [2-5]. These symptoms develop because of cell death in the deep parts of the brain, the substantia nigra and basal ganglia, which are located in the forebrain [6-8]. Specific neurons located in the basal forebrain produce neurotransmitters called dopamine. Dopamine send signals that are necessary for body movement, but are lost when the neurons degenerate, leading to the movement disorder in PD, but the mechanism underlying the neuronal degeneration is unclear. Therefore, research has been aimed to find ways to stop or slow down the death of these cells.

Treatments and Therapies for PD

Although there is currently no cure for PD, research has provided avenues for treatments and therapies to alleviate the progression and some symptoms of the disease [9, 10]. Common treatments for the motor symptoms include the use of pharmacological drugs that target the neurotransmitter, dopamine [9, 10]. Another alternative treatment is deep brain stimulation (DBS), where electrodes are implanted into the brain to mediate body movement [11]. Although DBS is an effective and FDA approved approach for treating motor symptoms, the invasive procedure of DBS only applies to a minority of PD patients, and there are also several adverse risks like psychiatric disturbance [11]. In addition, many of these treatments only help to alleviate the symptoms short term, but bring additional side effects; therefore, they are not ideal treatments for long-term usage. Taken together, many current treatments aim to address the motor symptoms, but no successful therapies target and correct the underlying molecular causes

of PD. Thus, we aim to investigate the underlying causes of these symptoms before they progress.

The main cause of dopaminergic neuronal death is still unknown in PD patients, but scientists have hypothesized that it may be related to α-synuclein (ASYN), a protein involved in the pathogenesis of PD. Therefore, ASYN would be a promising target to investigate because it may be a related to the loss of dopaminergic neurons. Studies have also shown that reducing ASYN could potentially prevent or slowdown the disease progression before it becomes irreversible [12-15].

Aggregates of alpha-synuclein (ASYN) are found in PD patients

Therefore, emerging studies using immunotherapy and gene therapy have been targeting ASYN. At the cellular level, neurons contain ASYN, a protein normally enriched in nerve terminals and involved in synaptic function, but ASYN tends to aggregate in PD patients [16, 17]. These aggregates occur either due to mutations or excessive accumulation of ASYN. Then, ASYN aggregates lead to the formation of fibrils called Lewy bodies, a major neuropathological hallmark in PD [16, 17].

Neurons exchange information through their axonal projections. Within the axonal projections, signaling molecules help the cell for differentiation or survival. However, studies have shown that axonal transport can be blocked by ASYN aggregates, leading to shrinkages of the axonal projections in pathological PD brains [18]. Subsequently, molecular motors that are involved with axonal transport are also decreased in levels of PD brains [19, 20].

Additionally, mutations or increased copies of the ASYN gene (SNCA) contribute to synucleinopathies including PD [21-23]. A particular type of familial PD is caused by pathogenic aggregates of ASYN [14, 24, 25]. ASYN aggregates occur due to missense mutations like A53T and A30P, but also from extra copies of the ASYN gene (SNCA). However, the exact pathogenic

mechanism of PD induced by ASYN is still unclear. Here, we see how the effect of ASYN may play a role in neurodegeneration when ASYN levels are abnormal [21-23].

Parkinson's Disease and Alzheimer's Disease may have overlapping mechanisms

Similarly to Parkinson's disease (PD), studies have shown that patients with Alzheimer's disease (AD) also accumulate Lewy bodies [26-28]. Alzheimer's disease (AD), which is one of the most common causes of age-related dementia, is manifested by β-amyloid plaques and neurofibrillary lesions that contain tau [26-28]. β-amyloid appeared to interact with ASYN, increasing neurotoxicity through oxidative stress [29]. There are many potential mechanisms by which excessive accumulation of ASYN contributes to AD pathogenesis; one of which is that aggregates of ASYN specifically alters endocytic trafficking and signaling leading to buildup of toxic aggregates of other proteins such as β-amyloid in the axons to compound the toxic effects, ultimately resulting in the demise of neurons [30, 31]. Studies have also suggested that ASYN-induced neuronal toxicity impairs cellular trafficking [30-35].

Intracellular Trafficking

The endocytic pathway sorts molecules from the plasma membrane for recycling or degradation using early endosomes (Rab5), late endosomes (Rab7), and lysosomes. In a recent study, expression of genes encoding endosomal trafficking was dysregulated in a panel of isogenic stem cell-derived neurons carrying familial AD mutations (APP and PS1) [36]. The data showing Rab5 and Rab7 in ASYN overexpressing cells were abnormally enlarged provide a possible hypothesis that ASYN aggregates may disrupt endocytic signaling. Interestingly, the endosomal malfunction is likely a result of β-C-terminal fragments (β-CTFs) of APP, not β-amyloid [36]. These new findings are consistent with previous studies, demonstrating that β-CTFs of APP affect endosomal trafficking in early phases of cellular pathogenesis in AD [37-

41]. Therefore, one of the potential mechanisms by which ASYN impacts the trafficking and processing of APP to promote the pathogenesis of AD in PD patients.

Increasing evidence has demonstrated impaired intracellular trafficking in early phases of PD [30, 31, 34]. In addition, an unbiased shRNA screening has uncovered many Rab proteins involved in intracellular transport and sorting of neurotrophic factors. These proteins serve as genetic modifiers for PD by mediating ASYN aggregation, secretion and toxicity [35]. Of particular interest, our lab has previously demonstrated that expression of ASYN induced hyper-activation of Rab5 (early endosome) [32, 33]. Moreover, ASYN also impaired retrograde axonal trafficking and trophic signaling mediated by brain-derived neurotrophic factor (BDNF) and its tyrosine receptor kinase B (TrkB), leading to neuronal atrophy in a PD mouse model [32, 33]. Neurotrophic factors, including BDNF, play an important role in retrograde transport of axons from the periphery to the survival of neurons and the growth of synapses [42, 43]. It has been shown that knocking down endogenous ASYN helped recover levels of Rab3a and Rab5 and prevented degeneration of cholinergic neurons in an APP transgenic mouse model of AD [44]. Therefore, we also implemented an ASYN knockout (SynKO) model in our study to investigate the function of ASYN and its role in axonal transport.

Intriguingly, studies have also pointed that ASYN may be associated with or contribute to the pathogenesis of AD because many PD patients develop AD-like dementia later on in their disease progression, suggesting overlapping mechanisms [45]. Previous studies have also suggested that the overlapping mechanisms involved with PD and AD are due to their connection with ASYN [26, 27, 46-54]. Increased levels of ASYN in the cerebrospinal fluid were found to be correlated with disease progression from mild cognitive impairment to AD [47, 54]. Additionally, the cerebrospinal fluid ASYN level exhibited an inverse relation to cognitive function as measured by Mini-Mental State Exam scores [50, 51, 54]. Moreover, clinical

examinations of autopsied brains from AD patients have revealed significant Lewy-related pathology (LRP) in various regions of the brain such as their neocortex, limbic system, and in the substantia nigra [26, 27, 46, 48, 49, 52, 53]. In addition, many patients with PD also develop β-amyloid plaques that underlie the potential cause for increased incidence of dementia in PD patients [55]. The observation of LRP in these different brain regions is correlated with degeneration of selected neuronal networks in AD and dementia with Lewy bodies patients, which may account for the behavioral deficits in these patients.

At the molecular level, ASYN may serve as a mediator of β-amyloid toxicity to selective neuronal populations in the neocortex and limbic system [12, 56]. ASYN and β-amyloid may also directly interact to form toxic hetero-oligomers that affect specific networks in APP/ASYN transgenic mice models as well as in the brains of patients with AD and PD [57]. On the other hand, β-amyloid interactions may trigger the misfolding and toxic conversion of ASYN [58]. Previous studies have shown that overexpression of ASYN in amyloid precursor protein (APP) transgenic mice resulted in greater cholinergic cell degeneration [59]. Furthermore, Overk and colleagues suggested that knocking down ASYN in APP/ASYN transgenic AD mouse model ameliorated the degeneration of hippocampal neurons, which provides some implication that ASYN-induced neurotoxicity can potentially be reversed [60]. On the other hand, in some studies, knocking down ASYN in rats and nonhuman primates increased neurotoxicity, which led to neurodegeneration suggesting that there is a level of ASYN to be maintained [13, 61-63]. These studies have provided strong evidence that ASYN is an important mediator of APP and β-amyloid toxicity in inducing neuronal vulnerability in selective neuronal populations, providing a possible linkage that ASYN may contribute to the pathogenesis of AD [12, 56-60]. Although the implications of these observations at present are unclear, these studies suggest a potential role for ASYN in AD because of its connection with PD [12, 56-60, 64].

Taken together, these studies have provided strong support of ASYN-induced neuronal toxicity that impairs cellular trafficking [30-35]. To further investigate and define the effect of ASYN in PD, we cultured primary neurons from a transgenic PD mouse model that overexpresses human GFP-tagged ASYN (hASYN). As an important control, we also used an ASYN knockout (SynKO) mouse model to generate insight to the necessity and function of ASYN. We performed biochemistry and live cell imaging studies. We then compared WT, hASYN and SynKO neurons for data obtained in parallel experiments. So far, our studies have demonstrated that excessive ASYN alters trafficking and processing of APP, giving rise to toxic β-CTFs, which may contribute to endosomal enlargement leading to axonal transport impairment and neuronal atrophy. Interestingly, we have also found differences when comparing the SynKO mouse model to the WT mice, suggesting that ASYN plays a critical role in these fundamental cellular functions.

METHODS

Ethical Statement: All experimental studies involving animals were approved by the Institutional Animal Care and Use Committee of University of California San Diego and performed in accordance with relevant guidelines and regulations established by NIH Guide for the Care and Use of Laboratory Animals.

Chemicals, reagents, media, antibodies and plasmids.

Hanks Balanced Salt Solution (HBSS), neurobasal media, trypsin, B27, GlutaMax, penicillin-streptomycin (PS), were purchased from Invitrogen. Fetal Bovine Serum (FBS) was purchased from Phoenix Research Products. HEPES, poly-L-lysine (PLL), DNase I, and GTP agarose beads were purchased from Sigma-Aldrich. Rabbit anti-Akt, rabbit anti-pAkt and rabbit anti-ERK1/2 were purchased from Cell Signaling Technologies. Rabbit anti-pTrkB (pY490) was kindly provided by Dr. Moses Chao (NYU). Mouse anti-TrkB and mouse anti-α-synuclein were purchased from BD. Mouse anti-pErk1/2, mouse anti-GFP, mouse anti-dynein (DIC-74) and rabbit anti-Rab5B were purchased from Santa Cruz. Mouse anti-Rab7 was purchased from Abmart (Shanghai, China). Mouse anti-GAPDH and mouse anti-β-actin were from GeneTex. Rabbit anti-Tau(pT181), rabbit anti-Tau(pS199), anti-Tau(pT205), anti-Tau(pT231), anti-Tau(pS396/404) and rabbit anti-Tau were all purchased from Genscript (Piscataway, NJ). Goat anti-rabbit or goat anti-mouse IgG–HRP conjugates were purchased from Jackson ImmunoResearch Laboratories Inc.

Animals.

The PD mouse model, Line 78 expresses a GFP-human ASYN (GFP-hASYN) transgene driven by the promoter of β-platelet-derived growth factor (βPDGF) [65]. The Line 78 pregnant mice carried a mixture of wild type and transgenic embryos. Transgenic GFP+ E18 embryos fluoresced “green” when screened with the “GFP flashlight” (Nightsea) and the remaining

littermates were categorized as non-transgenic to be used as controls. The synuclein knockout (SynKO) mouse model was obtained from Jackson laboratories. The SynKO pregnant mice only produced embryos with the SynKO genotype. All animals were maintained and bred according to standard procedures.

Preparation of Primary Neuronal Cultures.

Cortical neurons were collected from non-transgenic and transgenic PD mice embryos and from SynKO mice E18 embryos. Cortical neurons were isolated and plated with plating media (Neurobasal with 10% FBS, B27, GlutaMAX) on glass coverslips coated with poly-L-lysine (Invitrogen) in 12-well plates (VWR). Plating medium was replaced with maintenance medium (Neurobasal, B27, GlutaMAX) the following day. Only 2/3 of the media was replaced every other day until conclusion of the experiments.

Live Imaging of APP, BACE1, Rab5, Rab7, mitochondria and lysosomes.

For transient expression studies, we used LipoFectamine 2000 (ThermoFisher, Cat# 11668027) to transfect mouse E18 cortical neurons at DIV3 with APP-mCherry, Rab5-mCherry, or Rab7-mCherry vectors [32, 41, 66] and BACE1-mCherry (a generous gift from Dr. Utpal Das of UCSD). Images of cells were captured with a 100X objective lens using a Leica DMi8 Live Imaging Microscope, 24 hours post transfection. Time lapse series were collected and analyzed as published previously [32, 41, 66].

For imaging of axonal transport of mitochondria or lysosomes, cell culture medium was removed and washed with Gibco Minimum Essential Media (MEM) then incubated for 45 minutes at 37°C with working solution of either 50nM of MitoTracker Red (ThermoFisher, cat# M22425) or 50 nM of LysoTracker Red (ThermoFisher, cat# L7528) diluted in MEM. Working solution was then removed and replaced with CO2 independent media before live imaging microscopy.

Synaptic Double Staining.

DIV14 mouse E18 neurons cultured on coverglasses were washed three times with phosphate-buffered saline (PBS) (Invitrogen, Cat. No: 20012-027) and fixed with 4% paraformaldehyde (PFA). Following fixation, cells were rinsed three times with PBS and blocked in 5% goat serum (with 0.2% TritonX-100). Each well was incubated with rabbit monoclonal anti-synapsin (1:200) (Cell Signaling, D12G5 XP® Rabbit mAb #5297) and mouse anti-PSD95 (BioLegend, Clone K28/43, previously Covance cat# MMS-5182) (1:200) for 3-4 hours and washed with PBS after. Secondary antibodies were incubated overnight, washed and followed by DAPI (1:10,000) staining. Coverslips were mounted onto glass slides (VWR) using mounting media (1% n-propyl gallate in 90% glycerol) and prepared for imaging.

SDS-PAGE Immunoblotting.

The brain samples were homogenized in 3 times the volume of the brain weight (mg) in RIPA (50mM Tris pH 7.5, 150mM NaCl, 0.1%SDS, 1% sodium deoxycholate, 1%TritonX100, 1% NP40, 2mM phenylmethylsulfonyl fluoride). Samples were then centrifuged and measured for protein concentration using BCA Protein Assay kit (Lambda Biotech). Equal amounts of 40ug of protein from brain lysates were loaded and separated with 12.5% SDS-PAGE and electrotransferred to PVDF membranes. Membranes were blocked with 5% nonfat milk and probed with: 1) rabbit anti-C15 (1:1,000) primary antibody from Eddie Koo at UCSD; 2) mouse anti-APP (1:1,000) primary antibody from BioLegend; 3) rabbit anti-BACE (1:1,000) primary antibody from ProTeck; 4) rabbit anti-LAMP1 (1:1,000) primary antibody from ProSci; 5) rabbit anti-Rab5b (1:500) primary antibody from UC Santa Cruz. All blots were quantified against rabbit anti-Actin (1:1000) from Sigma as a loading control. This was followed by incubation with secondary antibodies conjugated to HRP. Blots were then washed and imaged using ChemiDoc XRS+ (Bio-Rad) and quantitated using ImageLab 6.0.1 software (BioRad).

Statistics.

All experiments were repeated at least 3 times independently. Data represent mean ± SEM. Statistical analyses and calculation of P values were performed using Prism5 (GraphPad Software, La Jolla, CA); One-Way ANOVA with Bonferroni's post-test was used for frequency distribution analysis. Student's t test was used for pairwise comparisons. P values less than 0.05 were considered statistically significant and P values less than 0.01 were considered highly statistically significant.

RESULTS

Trafficking of APP is disrupted in hASYN-PD neurons, but not in SynKO neurons

Recent studies have demonstrated that disruption of axonal trafficking and processing of amyloid precursor protein (APP) contributes to the early phase of Alzheimer's Disease (AD) pathogenesis [67-70]. Our previous study demonstrated that Rab proteins were dysregulated [71, 72] and axonal transport of brain-derived neurotrophic factor (BDNF) was impaired in a transgenic mouse model of Parkinson's Disease (PD) [71]. Therefore, we speculated that α-synuclein (ASYN) may also alter axonal trafficking and processing of APP in this mouse model. To test whether or not ASYN interacts and affects APP, we cultured neurons from mice that express the GFP-human ASYN (GFP-hASYN) driven by the β-platelet-derived growth factor (βPDGF) promoter. The presence of GFP signal helped distinguish between neurons that expressed no or very little hASYN [71]. To define a role of endogenous mouse ASYN, we also prepared neuronal cultures from an ASYN knockout (SynKO) mouse model. To visualize APP, we transfected neurons with an mCherry-APP construct [71]. 24 hrs following transfection, live imaging experiments were performed to capture time-lapse series of axonal transport of mCherry-APP in WT, GFP-hASYN and SynKO neurons as described in materials and methods [71, 72]. Kymographs were generated from the time lapse series and transport parameters were quantitated [71, 72]. Kymographs provide a visual representation of the acquired images over time, where the y-axis displays the total time recorded while the x-axis displays the distance traveled. The slope, distance over time, represents the velocity. Generally, a straight vertical line denotes no movement while an angled line shows movement within the axon that can be measured and recorded as retrograde moving velocity. Calculations from these kymographs showed that in WT (Figure 1D), the APP retrograde moving velocity (0.72±0.07μm/sec) was

significantly higher compared to the APP retrograde moving velocity of the GFP-hASYN PD neurons (0.30±0.03μm/sec).

There were also significant differences in retrograde moving velocity between SynKO neurons (0.71±0.07μm/sec) and GFP-hASYN PD neurons. Interestingly, there were no observed significant differences in retrograde moving velocity between WT and SynKO neurons, suggesting that APP trafficking is selectively impaired in GFP-hASYN PD neurons with overexpressed ASYN.

Average velocity encompasses the total time of movement including the short intervals of rest/pause times. We speculated that pause times may have an effect on average velocity; therefore, average and retrograde velocity were measured separately to gain a more holistic approach. Trends for the average velocity were inconsistent with the retrograde moving velocity trends, suggesting slight mechanistic differences between average and retrograde moving velocities. The average APP moving velocities (Figure 1E) were significantly different across all neurons. WT neurons had an average APP retrograde moving velocity (0.31±0.054μm/sec), which was faster, compared to GFP-hASYN PD neurons (0.05±0.0079μm/sec) and SynKO neurons (0.167±0.024μm/sec).

Taken together, our results showed that the absence of endogenous ASYN did not show adverse effects on retrograde moving axonal transport of APP since no significant differences were observed between the SynKO and WT neurons (Figure 1). However, increasing expression of hASYN significantly affected axonal transport of APP (Figure 1). Based on these results, we conclude that excessive accumulation of ASYN impairs axonal transport of APP.

Trafficking of BACE1 is disrupted in hASYN-PD neurons, but not in SynKO neurons

Given that axonal trafficking of APP is impaired by excessive ASYN in GFP-hASYN PD neurons, we asked if ASYN also affected β-secretase enzyme (BACE1) trafficking. The

proteolytic processing of APP is first mediated by BACE1 to generate the β-carboxyl terminal fragment (β-CTF) of APP [73]. Accumulation of β-CTF negatively impacts axonal trafficking and function [69, 74-76]. We transfected DIV5 cortical neurons with mCherry-BACE1 into WT, SynKO and GFP-hASYN PD neurons and performed live imaging experiments to quantitate the transport of BACE1 as described in APP transport (Figure 1). Consistent with the observations for APP, our results demonstrated that axonal trafficking velocity of BACE1 is also reduced in GFP-hASYN PD neurons (Figure 2). Interestingly, the change in axonal transport of BACE1 is also observed in SynKO neurons, suggesting that ASYN may play a role in regulating axonal transport of BACE1.

ASYN enhances APP processing by BACE to produce toxic APP β-CTF

Given that expression of the transgene hASYN impaired axonal trafficking of both APP and BACE1 and that APP is cleaved by BACE1 intracellularly to yield the β-carboxyl terminal fragments (β-CTF) of APP [73], we hypothesized that the expression of hASYN increases the level of β-CTF. To this end, we harvested brain lysates from WT, GFP-hASYN PD and SynKO E18 embryos. We used immunobloting to assay for the full-length APP, APP cleavage products and BACE1. Our results show higher expression levels for the full-length APP while BACE1 decreased in expression levels (Figure 3). ASYN aggregation is often accompanied by β-amyloid deposition, and it has been shown that ASYN promotes β-amyloid production from APP [29]. There seems to be more expression of full length APP when BACE1 has lower expression levels (Figure 3). There were no significant differences in the expression levels of APP, APP-CTF, and Rab5 between KO, WT, PD neurons (Figure 3). Both TrkB and LAMP1 levels were significantly lower in KO neurons, suggesting that expression of these proteins may be down-regulated either transcriptionally or translationally or post-translationally when ASYN is missing. Our results suggest that overexpression of ASYN not only impairs retrograde axonal transport of lysosomes,

but also suppresses LAMP1 in both the GFP-hASYN PD and SynKO neurons. Of note, there were trends, but the differences were not significant, which were inconsistent with another study possibly due to the fact that these diseases are age related and we harvested the whole brains of E18 mice, which could have led to excess compensatory signaling/processing.

Effect of ASYN on endosomal trafficking.

Members of the Rab GTPase family such as Rab5 and Rab7 are involved in regulating axonal trafficking. Recent evidence has suggested that the levels and/or activity of Rab proteins are dysregulated in GFP-hASYN PD [71, 72, 77, 78]. Furthermore, we have previously demonstrated that expression of ASYN altered both the level and activities of Rab proteins that regulate endocytic trafficking [71, 72]. We then examined transport of Rab5-early endosomes and Rab7-late endosomes. Using the same approach, we transfected an expression vector of mCherry-Rab5 into E18 cortical neurons of WT, SynKO, hASYN PD. Axonal transport of mCherry-Rab5 was quantitated as described.

Our results showed that the retrograde moving velocity of Rab5-early endosomes (Figure 4) in WT neurons (0.7800±0.06682 μm/sec) was significantly faster than the GFP-hASYN PD neurons (0.3251±0.05469 μm/sec). There was also a significant difference in retrograde moving velocity (Figure 4D) between SynKO neurons (0.6924±0.05956 μm/sec) and GFP-hASYN PD neurons, but no statistical significant differences were seen between WT and SynKO. Overall for retrograde moving velocity, WT and SynKO moved at comparable levels while GFP-hASYN PD neurons were significantly slower. The average velocity (Figure 4E) showed significant differences between WT and GFP-hASYN PD neurons. WT neurons had an average velocity (0.6457±0.1225 μm/sec) that was the fastest overall. SynKO neurons (0.3615±0.04197 μm/sec) showed some significant differences for average velocity but not as significantly as GFP-hASYN

PD neurons (0.07548±0.02234μm/sec) compared to WT neurons. Unlike in retrograde moving velocity, there were significant differences between WT and SynKO neurons for the average retrograde velocity, but not between SynKO and GFP-hASYN PD neurons (Figure 4).

We used mCherry-Rab7 to quantitate axonal transport of late endosomes. Rab7 retrograde moving velocity (0.6949±0.04984 μm/sec) in WT neurons and was significantly faster in GFP-hASYN PD neurons (0.4042±0.03930 μm/sec) (Figure 5D). In SynKO neurons, the retrograde moving velocity (0.2646±0.03955μm/sec) was the slowest overall (Figure 5). In the WT neurons, the average velocity (0.5743±0.06354 μm/sec) was comparatively faster in the GFP-hASYN PD neurons (0.01592±0.03269 μm/sec) (Figure 5). SynKO neurons had an average velocity (0.08579±0.01718 μm/sec) that was also faster than GFP-hASYN PD neurons (Figure 5E). Surprisingly, overall Rab7 axonal transport was significantly reduced in both SynKO and GFP-hASYN PD neurons as compared to WT neurons (Figure 5). However, there were no observed significant differences between SynKO and GFP-hASYN PD neurons (Figure 5). These results suggest that ASYN homeostasis is critical to maintaining axonal transport of late endosomes.

Rab5 colocalized with ASYN

Since we found endocytic pathway axonal transport to be compromised, specifically for early endosomes (Rab5), late endosomes (Rab7), and lysosomes (Lamp1), we wanted to see if there is specificity between ASYN. Small GTPases such as Rab5 and Rab7 play a crucial role in regulating axonal trafficking. Since APP has been shown to colocalize with Rab5 in previous studies, the subcellular distribution of ASYN thus overlaps likely with that of APP [72]. To identify if there is specificity of ASYN, immunofluorescence staining was performed and visualized on a confocal laser scanning microscope. The GFP-ASYN labels colocalized with the

early endosomal marker (Rab5), but not with late endosomes (Rab7) or lysosomes (Lamp1) (Figure 6).

Effect of ASYN on axonal trafficking of lysosomes

We ask if ASYN also affected axonal trafficking of lysosomes, so we used LysoTracker to label, track and quantitate axonal movement of lysosomes in WT, SynKO, and GFP-hASYN PD neurons [79]. The retrograde moving velocity of lysosomes (Figure 7) in WT neurons (1.183±0.101μm/sec) was significantly faster than in GFP-hASYN PD neurons (0.4806±0.05738μm/sec). The data for SynKO neurons (0.8765±0.1117μm/sec) was significantly higher than in GFP-hASYN PD neurons, but no statistical significance was observed from WT neurons (Figure 7).

Effect of ASYN on axonal trafficking of mitochondria

We also used MitoTracker to label, track and quantitate axonal movement of mitochondria in WT, SynKO, and GFP-hASYN PD neurons [79]. The average retrograde moving velocity for mitochondria: 0.9713±0.1466μm/sec for WT, 0.3408±0.1047μm/sec for SynKO and 0.2326±0.03477μm/sec for GFP-hASYN PD neurons (Figure 7). While no significant differences between WT and SynKO neurons for the average retrograde velocity of mitochondria, the data for GFP-hASYN PD neurons is lower compared to both WT and SynKO neurons (Figure 7).

Effect of ASYN on synaptic formation

Given that expression of the hASYN transgene disrupted axonal trafficking and synaptic formation is critically dependent on axonal transport, we cultured E18 WT or GFP-hASYN PD cortical neurons on coverglasses to measure the number of synapses. Neurons were fixed at DIV14, at which point mature synapses are expected to be formed in WT neurons. We used anti-Synapsin I to stain pre-synapses and anti-PSD95 to stain for post-synapses. The samples were

examined using confocal microscopy. Colocalization between Synapsin I and PSD95 were quantitated using ImageJ and Pearson's colocalization coefficients were computed using the MOSAIC SUITE Plugin. As shown in Figure 8, the Pearson's colocalization coefficient for GFP-hASYN PD neurons is significantly less than that of WT neurons at DIV14. The deficits persisted even at DIV21 (Figure 8B, D), suggesting that ASYN affects synaptic formation in GFP-hASYN PD neurons. These results have demonstrated that ASYN causes disorders in retrograde axonal transport and affects synapse formation in endosomes and lysosomes of GFP-hASYN PD neurons. Therefore, demonstrating that ASYN causes endosomal/lysosomal retrograde axonal transport disorders and in turn affects synaptic formation of GFP-hASYN PD neurons.

Expression of ASYN induced neuronal atrophy

To investigate if accumulation of ASYN induced cellular phenotypes, we transfected WT E18 cortical neurons at DIV3 with GFP-ASYN^{WT} or GFP-ASYN^{A53T} using GFP as a control. After 72hrs, the WT neuron soma sizes shrunk (894.0±49.41 μm^2) and with addition of ASYN the soma size was 564.9±37.37 μm^2 (Figure 9). In the A53T cells at 72hrs, the soma size also shrunk (758.5±28.23 μm^2) and with ASYN it was 438.2±46.93 μm^2 (Figure 9). Consistent with our previous findings [71], the results confirm that over-expression of either GFP-ASYN^{WT} or GFP-ASYN^{A53T} induces neuronal atrophy.

DISCUSSION

Differences in overexpression of ASYN and ASYN knockout neurons

Given that axonal transport of brain-derived neurotrophic factor (BDNF) was impaired in a transgenic mouse model of Parkinson's Disease (PD) in a previous study [71], we asked if α-synuclein (ASYN) may also alter axonal trafficking and processing of APP. To test whether or not ASYN interacts and affects APP, we examined APP trafficking in mass cultures of E18 cortical neurons derived from Line 78 transgenic mice and their littermate non-transgenic WT controls. Increasing evidence suggests defective axonal transport is associated with the early pathogenesis of PD, but the underlying mechanism remains to be determined.

Here, we show that overexpression of ASYN impairs retrograde axonal transport in cortical neurons and also demonstrated that overexpression of ASYN induces synaptic deficits, eventually leading to neuronal death. Interestingly, we have also found differences when comparing the SynKO mouse model to the WT mice, suggesting ASYN plays a critical role in these fundamental cellular functions. Activated Rab proteins (Rab 5 and Rab7) showed levels of transport impairment in both SynKO and GFP-hASYN PD neurons, which may suggest endosomal dysfunction. Consistent with our previous study [32], this study reveals a mechanism by which excessive accumulation of ASYN induces axonal transport deficits and causes neuronal atrophy in the very early stages of PD.

Increased levels of ASYN inhibits APP trafficking

Previous studies have suggested that disruption of axonal trafficking and processing of amyloid precursor protein (APP) contributes importantly to the early phase of Alzheimer's Disease (AD) pathogenesis [67, 69, 70, 80]. Taken together, our results showed that increased expression of hASYN significantly impaired axonal transport of APP, which further proves the interactions that ASYN has with APP. Alterations and changes in axonal transport behaviors of

APP indicate axonal dysfunctions. Interestingly, the absence of endogenous ASYN had some impact on axonal transport retrograde moving velocity of APP, since there was some difference observed between the SynKO and WT neurons. This led us to hypothesize that pause times may play a significant role in average velocities because certain vesicles may take longer pauses, which may affect the overall average velocity, but may have no phenotypic difference when looking at retrograde moving velocity. Therefore, we looked at different behaviors of movement, retrograde moving velocity and average velocity. Retrograde moving velocity focused on just the moving speed within the axons while average velocity encompassed the movement duration across all frames. Average velocity includes the times when the movement paused, and this difference in behavior could lead to a hypothesis that behavior movement within the axon is potentially affected when ASYN levels are abnormal, as observed in GFP-hASYN and SynKO models.

When ASYN levels are altered, axonal trafficking may be impaired, which may lead to a decrease in synaptic formation and eventually neuronal death. Further studies to explore the mechanisms involved with ASYN and aim to address the homeostatic level of ASYN could prove promising for translational medicine to target before onset of synucleinopathies. There were limitations to our study it being that the mouse model used expressed human ASYN (A53T mutation), so it cannot directly provide insight and be translated into human models because the A53T mutation is actually a wild type in mice. Therefore, we do cannot conclude with certainty that similar mechanisms are happening in the patients with the A53T mutation.

In our study, we cultured cortical neurons because they are easier to maintain and visualize transport, but the disease progression mainly happens in the midbrain. This could also prove promising to investigate since we already see disease pathology in other regions of the brain. Since age is a risk factor, using older models and/or models that are more closely related

to human neurons such as neurons derived from human patients with the neurological diseases and mutations that alter ASYN expression levels could provide further validations of the role of ASYN in axonal trafficking and function. These studies could provide more insight on ASYN-induced neurotoxicity with regards to the pathology of PD.

Deficits in synapses likely result from deficits in transport

Consistent with our previous findings that expression of the hASYN transgene impaired retrograde axonal transport of BDNF [32]. Here, we have demonstrated that disruption of axonal trafficking by the expression of the hASYN transgene is more wide-spread, a result that likely accounts for the deficits in synaptic formation in PD neurons, since synaptic formation and maintenance is critically dependent on axonal delivery. Currently, it is unclear how increased ASYN accumulation impairs axonal trafficking. Among many different possible causes, we speculate there are several possibilities: 1) ASYN interferes with motor protein functions; 2) ASYN alters mitochondrial functions; and 3) ASYN increases the size of cargo vesicles. Future studies will be needed to investigate the mechanism.

Implications of findings

Since axonal trafficking of APP is impaired by excessive ASYN in GFP-hASYN PD neurons, we asked if ASYN also affected β-secretase enzyme (BACE1) trafficking. Given that expression of the transgene hASYN impaired axonal trafficking of both APP and BACE1 (Figure 1 and 2), we hypothesized that the expression of hASYN increases the level of β-CTF because APP is cleaved by BACE1 intracellularly to yield the β-carboxyl terminal fragments (β-CTF) of APP [81].

Consistent with previous findings, our data strongly suggest that axonal dysfunction is an early manifestation of ASYN toxicity in PD. Our findings suggest that APP, BACE1, Rab5

likely localize in the same compartments (Figure 6). To further our investigation, we propose to do another staining with hASYN neurons along with APP and BACE1.

In summary, my study has provided evidence that excessive accumulation of ASYN disrupts axonal trafficking and processing of APP mediated by endosomes, and impairment of axonal trafficking in conjunction with production of toxic APP CTFs compromises critical neuronal functions such as synaptic function and plasticity, ultimately, resulting in neuronal degeneration.

FIGURES

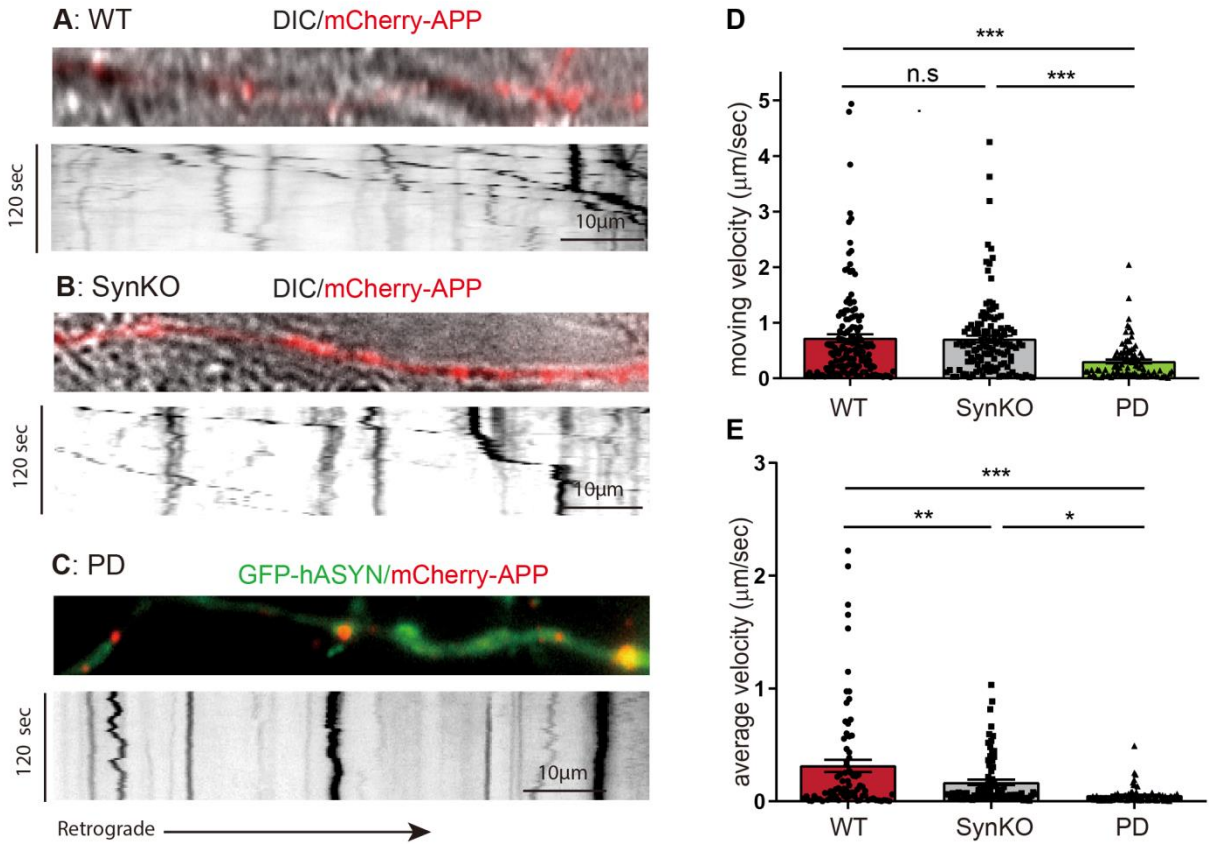

Figure 1. Trafficking of amyloid precursor protein (APP) is disrupted in overexpressed α-synuclein (ASYN) neurons, but not in ASYN-knockout neurons.

Cortical neurons from wildtype (WT), PD mouse model Line 78 (PDGF-β-GFP-hASYN) and ASYN-knockout (SynKO) were dissected and cultured from E18 mice embryos. The PD mouse model, Line 78 expresses a GFP-human ASYN (GFP-hASYN) transgene driven by the promoter of β-platelet-derived growth factor (βPDGF). After 4 days in vitro (DIV4) neurons were transfected with mCherry-APP. After 24hrs, axonal transport of mCherry-APP was captured using live imaging microscopy. Representative images of mCherry-APP within axons of WT (A), SynKO (B) and GFP-hASYN PD neurons (C) are shown. Kymographs directly shown below axons were generated from time-lapsed image series using ImageJ. Vertical axes depict total time took to capture axonal transport of a single axon and horizontal axes depict distance. Scale bar of 10µm is the same throughout images (A-C). Retrograde moving velocity (D) and average velocity (E) within the axon were calculated from generated kymographs. Data were obtained with 20 WT neurons, 20 SynKO neurons and 15 GFP-positive transgenic PD neurons. The p-values were obtained using student t-test. $p < 0.05$ (**); $p < 0.01$ (***); n.s. = non-significant.

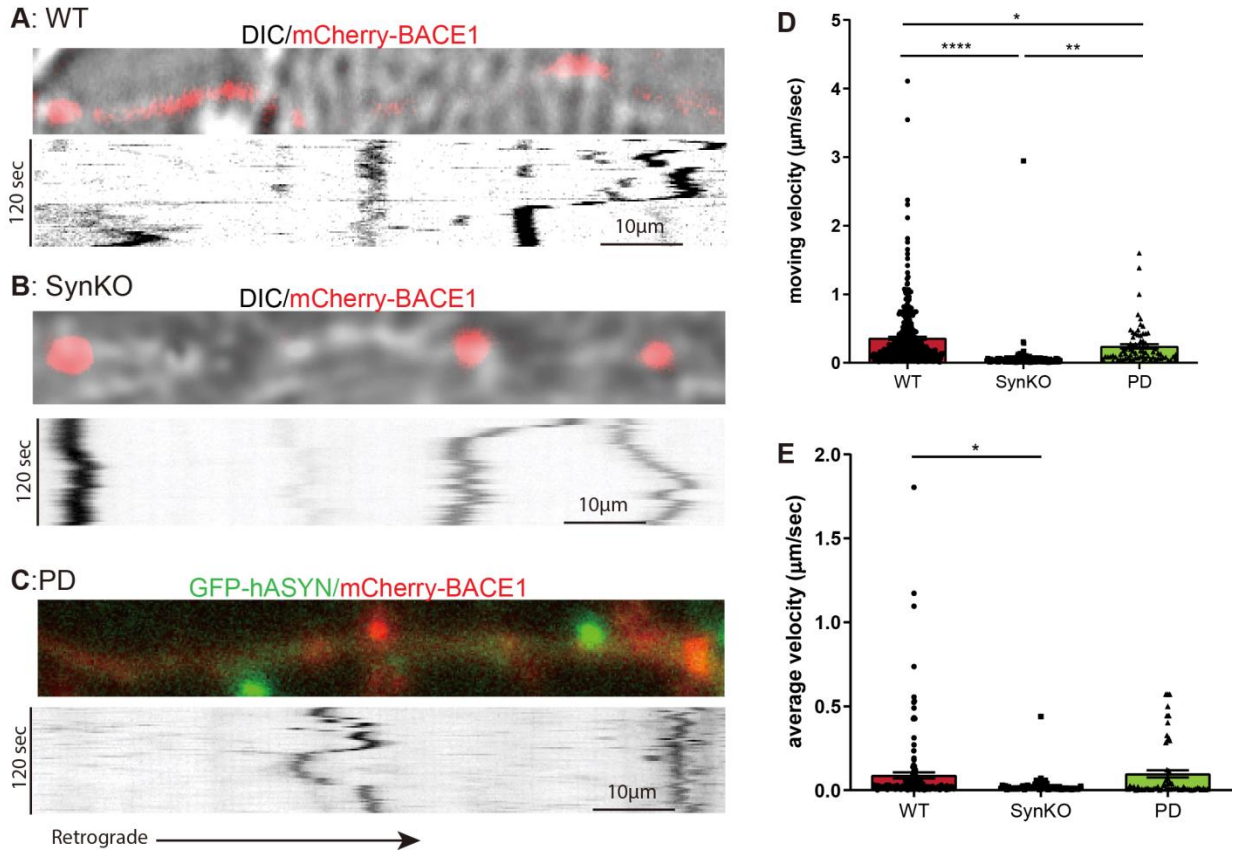

Figure 2. The presence of GFP-hASYN induced retrograde axonal transport deficits of β-secretase enzyme (BACE1) in E18 cortical neurons.

Cortical neurons from wildtype (WT), PD mouse model Line 78 (PDGF-β-GFP-hASYN) and ASYN-knockout (SynKO) were dissected and cultured from E18 mice embryos. The PD mouse model, Line 78 expresses a GFP-human ASYN (GFP-hASYN) transgene driven by the promoter of β-platelet-derived growth factor (βPDGF). After 4 days in vitro (DIV4) neurons were transfected with mCherry-BACE1. After 24hrs, axonal transport of mCherry-BACE1 was captured using live imaging microscopy. Representative images of mCherry-BACE1 within axons of WT (A), SynKO (B) and GFP-hASYN PD neurons (C) are shown. Kymographs directly shown below axons were generated from time-lapsed image series using ImageJ. Vertical axes depict total time took to capture axonal transport of a single axon and horizontal axes depict distance. Scale bar of 10µm is the same throughout images (A-C). Retrograde moving velocity (D) and average velocity (E) within the axon were calculated from generated kymographs. Data were obtained with 20 WT neurons, 20 SynKO neurons and 15 GFP-positive transgenic PD neurons. The p-values were obtained using student t-test. $p < 0.05$ (**); $p < 0.01$ (***); n.s. = non-significant.

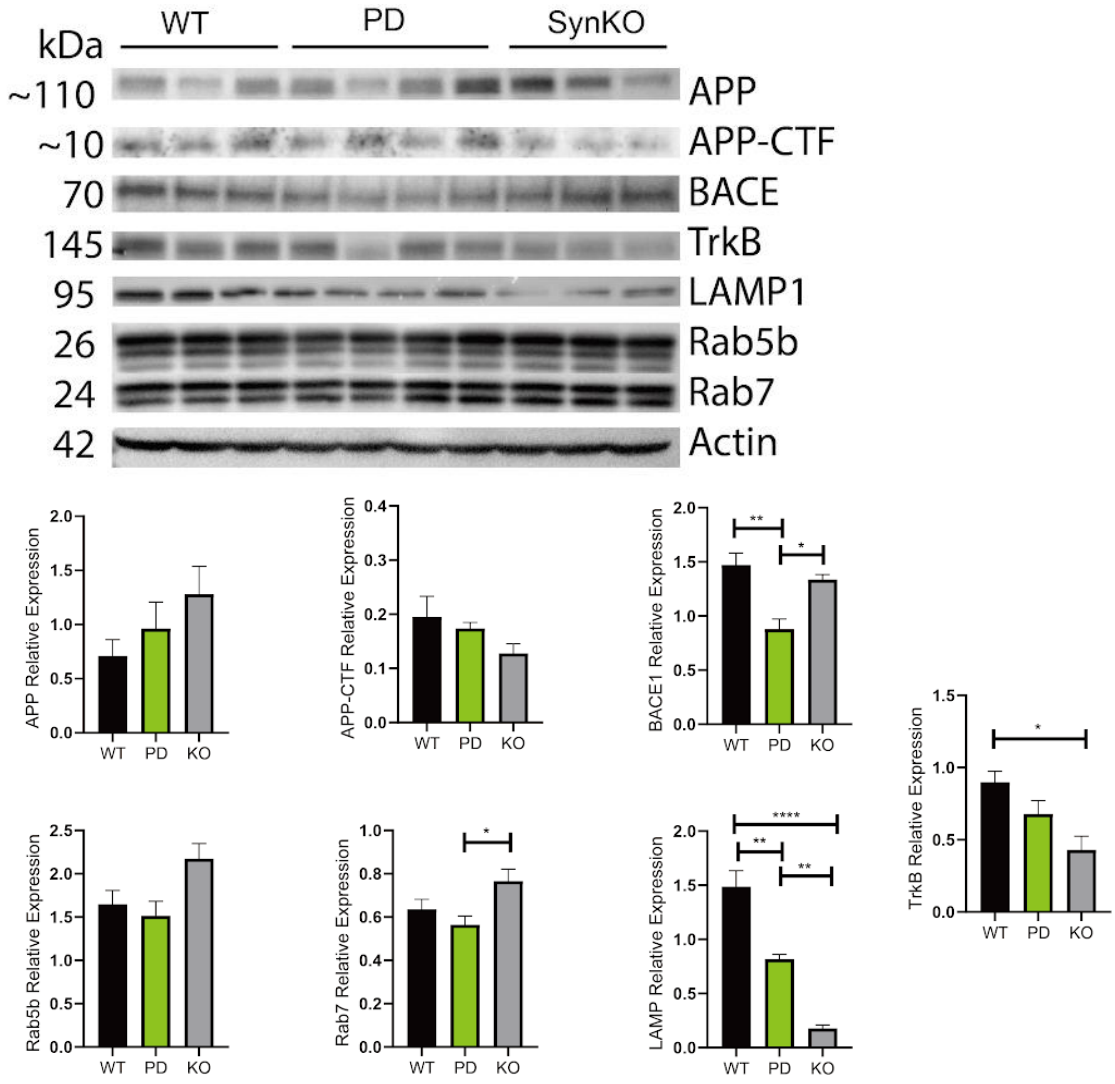

Figure 3. Decreased levels of BACE1 were detected in E18 hASYN PD brain samples.

Brain lysates from E18 mice were denatured and prepared for immunoblotting as described in Materials and Methods. The expression levels in these samples were quantitated and normalized against actin, the internal control. A significant decrease of BACE1 is seen in GFP-hASYN PD mice as compared to WT controls and SynKO mice. At least three independent experiments were performed for all the studies presented.

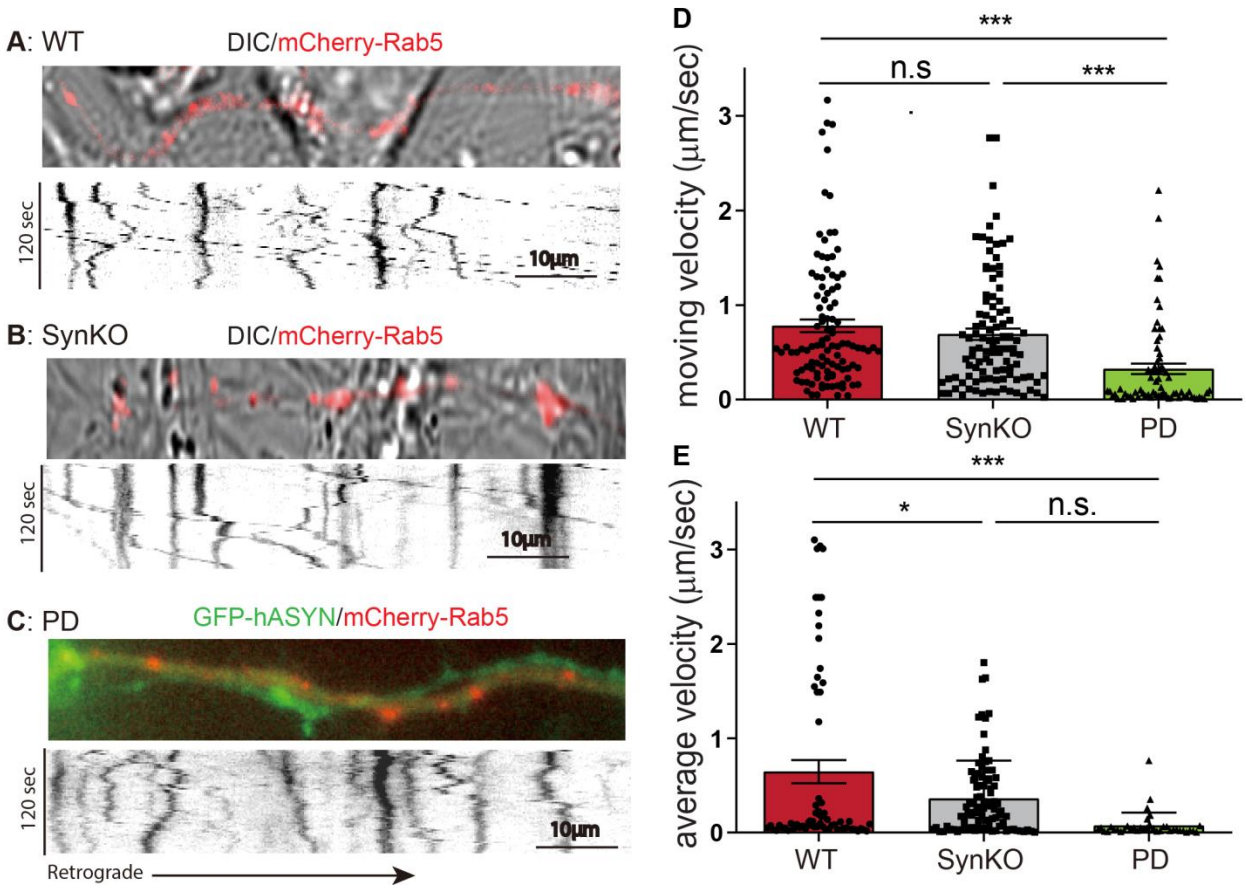

Figure 4. Expression of GFP-ASYN impaired retrograde axonal transport for early endosomes (Rab5) in E18 cortical neurons.

Cortical neurons from wildtype (WT), PD mouse model Line 78 (PDGF-β-GFP-hASYN) and ASYN-knockout (SynKO) were dissected and cultured from E18 mice embryos. The PD mouse model, Line 78 expresses a GFP-human ASYN (GFP-hASYN) transgene driven by the promoter of β-platelet-derived growth factor (βPDGF). After 4 days in vitro (DIV4) neurons were transfected with mcherry-Rab5 (early endosome). After 24hrs, axonal transport of mcherry-Rab5 was captured using live imaging microscopy. Representative images of mcherry-Rab5 within axons of WT (A), SynKO (B) and GFP-hASYN PD neurons (C) are shown. Kymographs directly shown below axons were generated from time-lapsed image series using ImageJ. Vertical axes depict total time took to capture axonal transport of a single axon and horizontal axes depict distance. Scale bar of 10µm is the same throughout images (A-C). Retrograde moving velocity (D) and average velocity (E) within the axon were calculated from generated kymographs. Data were obtained with 20 WT neurons, 20 SynKO neurons and 15 GFP-positive transgenic PD neurons. The p-values were obtained using student t-test. $p < 0.05$ (**); $p < 0.01$ (***); n.s. = non-significant.

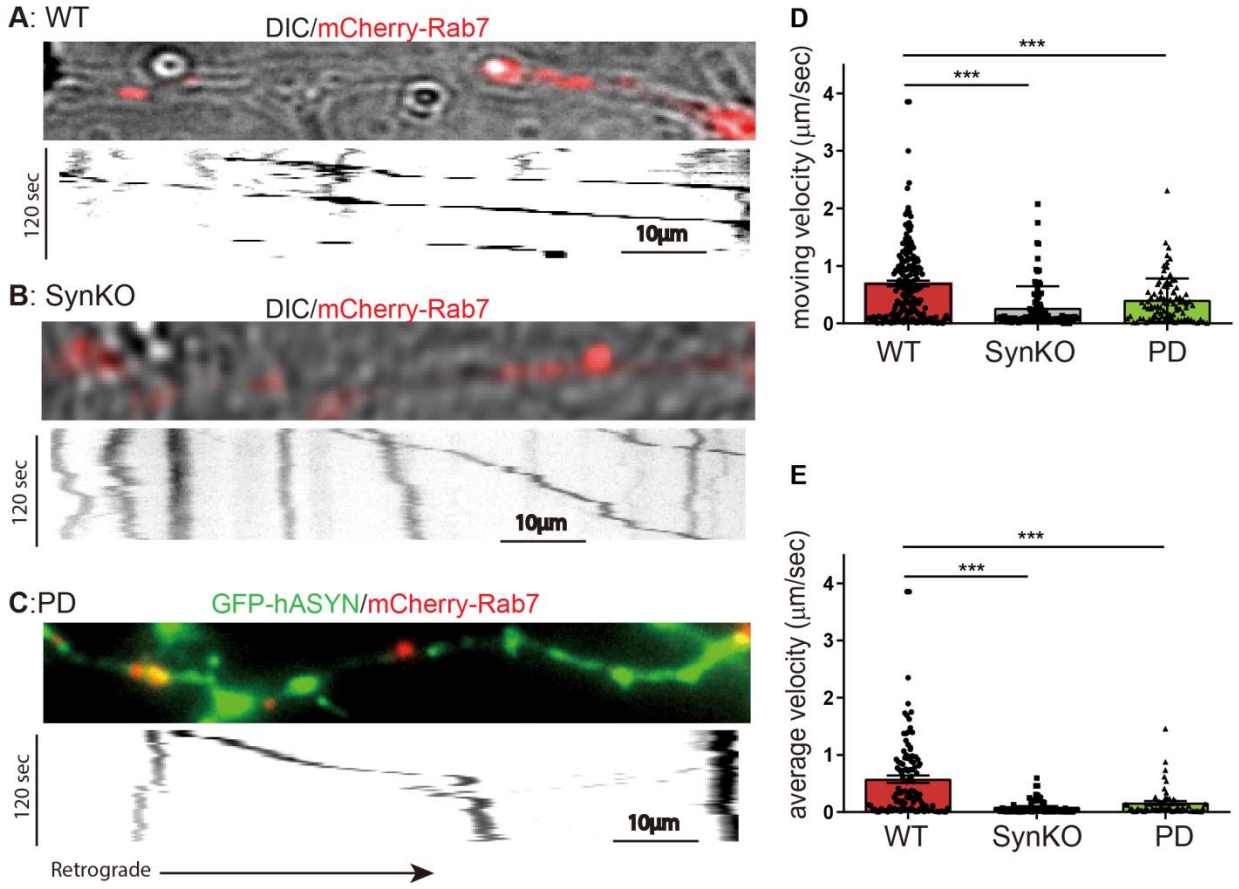

Figure 5. Expression of GFP-ASYN impaired retrograde axonal transport for late endosomes (Rab7) in E18 cortical neurons.

Cortical neurons from wildtype (WT), PD mouse model Line 78 (PDGF-β-GFP-hASYN) and ASYN-knockout (SynKO) were dissected and cultured from E18 mice embryos. The PD mouse model, Line 78 expresses a GFP-human ASYN (GFP-hASYN) transgene driven by the promoter of β-platelet-derived growth factor (βPDGF). After 4 days in vitro (DIV4) neurons were transfected with mcherry-Rab7 (late endosome). After 24hrs, axonal transport of mcherry-Rab7 was captured using live imaging microscopy. Representative images of mcherry-Rab7 within axons of WT (A), SynKO (B) and GFP-hASYN PD neurons (C) are shown. Kymographs directly shown below axons were generated from time-lapsed image series using ImageJ. Vertical axes depict total time took to capture axonal transport of a single axon and horizontal axes depict distance. Scale bar of 10µm is the same throughout images (A-C). Retrograde moving velocity (D) and average velocity (E) within the axon were calculated from generated kymographs. Data were obtained with 20 WT neurons, 20 SynKO neurons and 15 GFP-positive transgenic PD neurons. The p-values were obtained using student t-test. $p < 0.05$ (**); $p < 0.01$ (***); n.s. = non-significant.

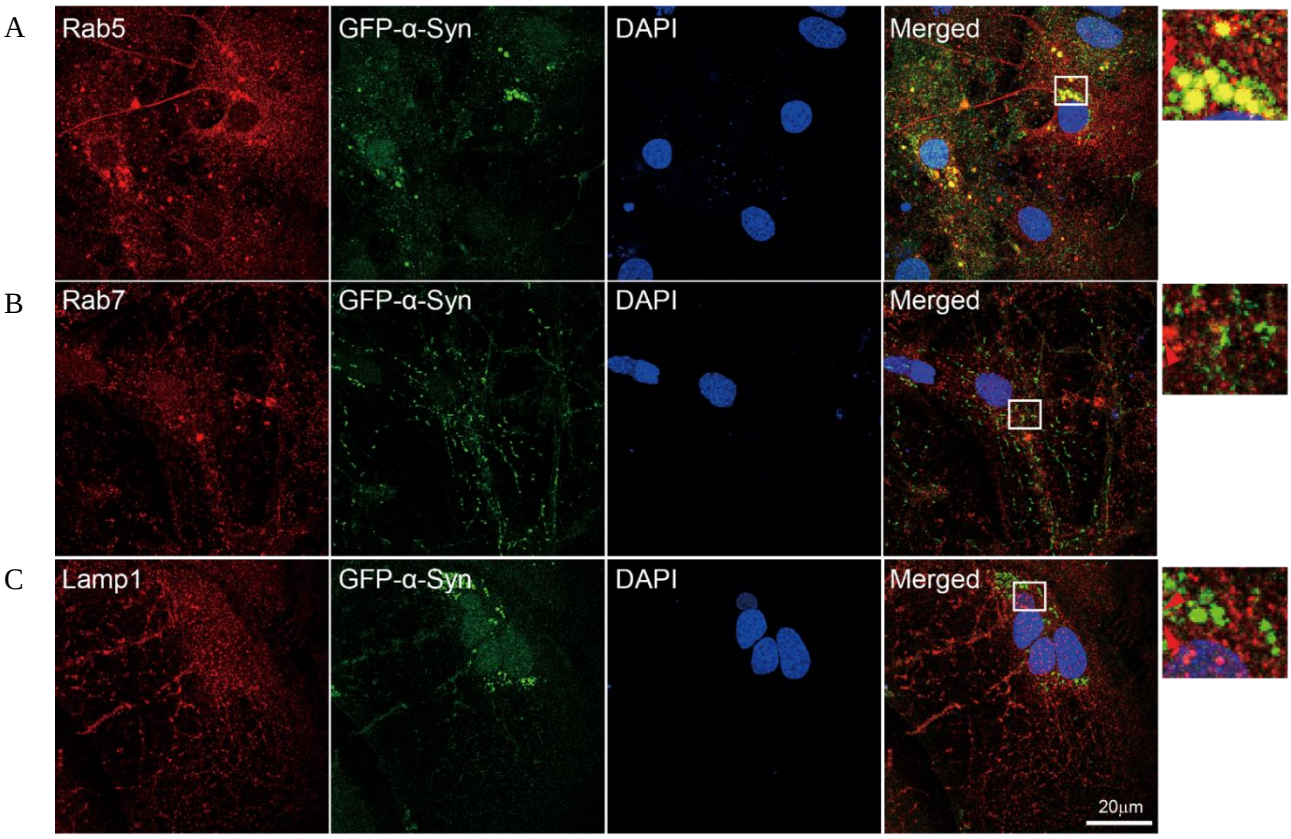

Figure 6. GFP-hASYN colocalizes with Rab5 in E18 GFP-hASYN PD cortical neurons.

E18 cortical neurons were cultured and fixed for immunostaining with specific Abs against Rab5 (red), Rab7, Lamp1. Representative images for GFP-hASYN (green), Rab5 (red, A), Rab7 (red, B), and Lamp1 (red, C) are shown. All nuclear staining are shown in blue using DAPI. Insets were enlarged to better visualize the colocalization between hASYN and these three different markers.

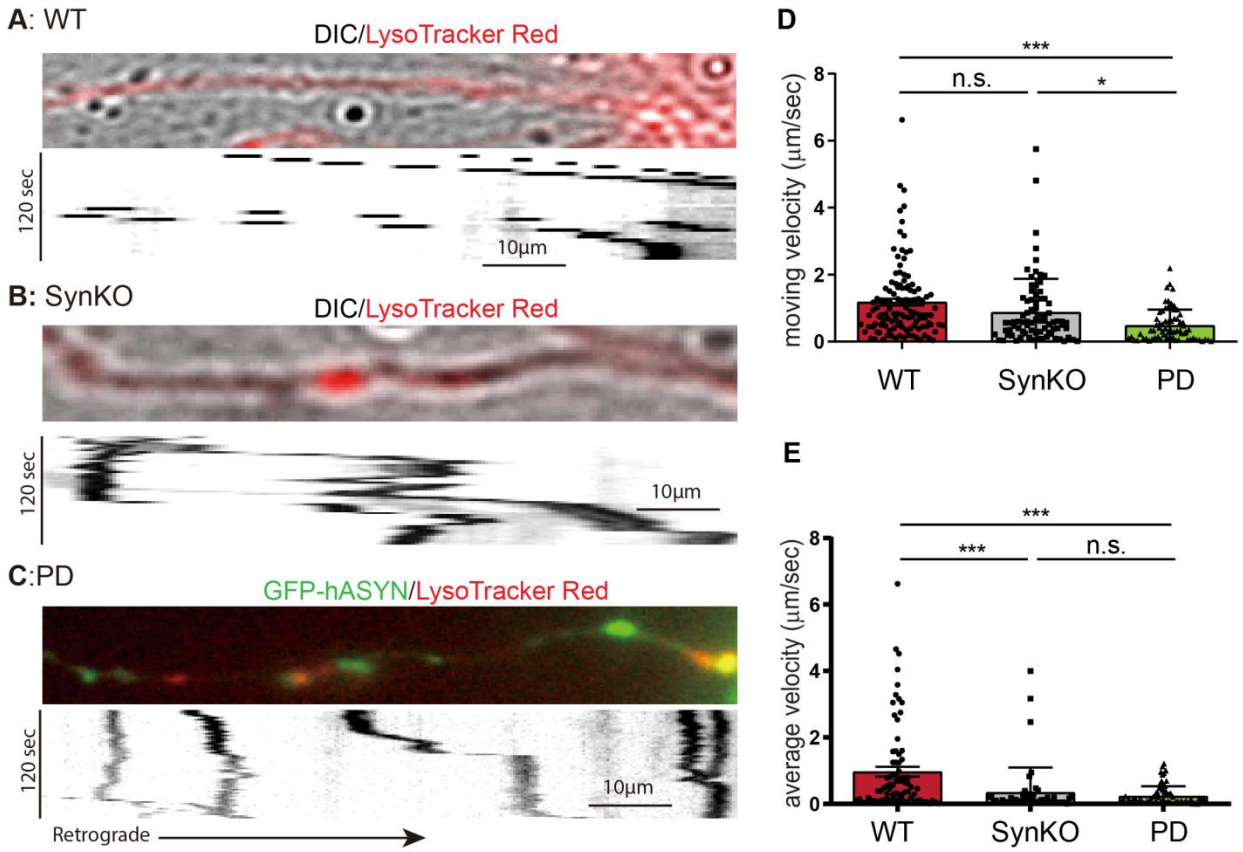

Figure 7. Retrograde axonal trafficking of lysosomes is disrupted in GFP-hASYN PD E18 cortical neurons.

E18 cortical neurons from Line 78 (PDGF-β-ASYN-GFP) and ASYN-knockout mouse embryos were dissected and cultured as described in Materials and Methods. At DIV4, neurons were treated with Lysotracker for 30mins and axonal transport of lysosomes were captured by live imaging. Representative images of lysosomes within axons of WT (A), SynKO (B) and GFP-hASYN PD neurons (C) are shown. Kymographs directly shown below axons were generated from time-lapsed image series using ImageJ. Vertical axes depict total time took to capture axonal transport of a single axon and horizontal axes depict distance. Scale bar of 10µm is the same throughout images (A-C). Retrograde moving velocity (D) and average velocity (E) within the axon were calculated from generated kymographs. Data were obtained with 20 WT neurons, 20 SynKO neurons and 15 GFP-positive transgenic PD neurons. The p-values were obtained using student t-test. $p < 0.05$ (**); $p < 0.01$ (***); n.s. = non-significant.

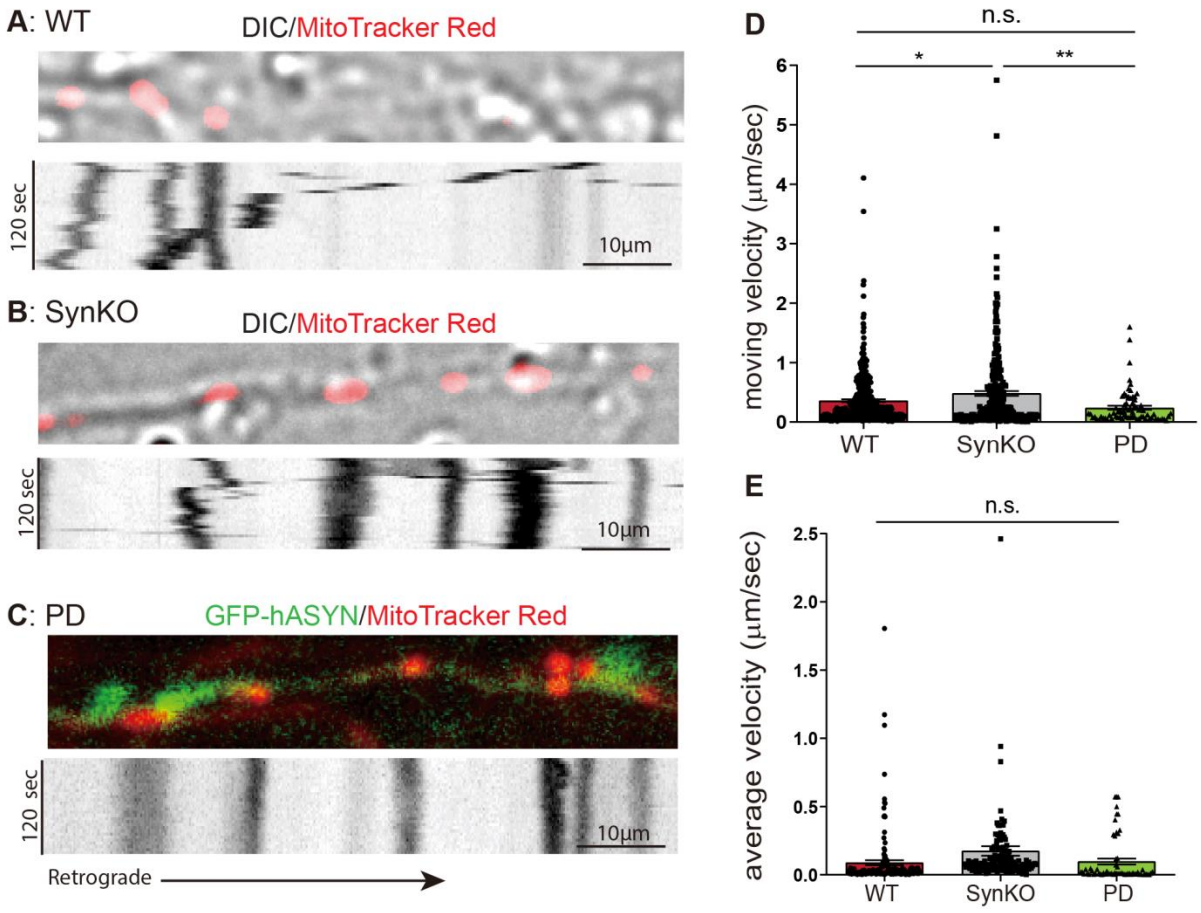

Figure 8. Effect of GFP-hASYN on axonal transport of mitochondria.

E18 cortical neurons from Line 78 (PDGF-β-ASYN-GFP) and ASYN-knockout mouse embryos were dissected and cultured as described in Materials and Methods. At DIV4, neurons were treated with Mitotracker for 30mins and axonal transport of mitochondria were captured by live imaging. Representative images of mitochondria within axons of WT (A), SynKO (B) and GFP-hASYN PD neurons (C) are shown. Kymographs directly shown below axons were generated from time-lapsed image series using ImageJ. Vertical axes depict total time took to capture axonal transport of a single axon and horizontal axes depict distance. Scale bar of 10μm is the same throughout images (A-C). Retrograde moving velocity (D) and average velocity (E) within the axon were calculated from generated kymographs. Data were obtained with 20 WT neurons, 20 SynKO neurons and 15 GFP-positive transgenic PD neurons. The p-values were obtained using student t-test. $p < 0.05$ (**); $p < 0.01$ (***); n.s. = non-significant.

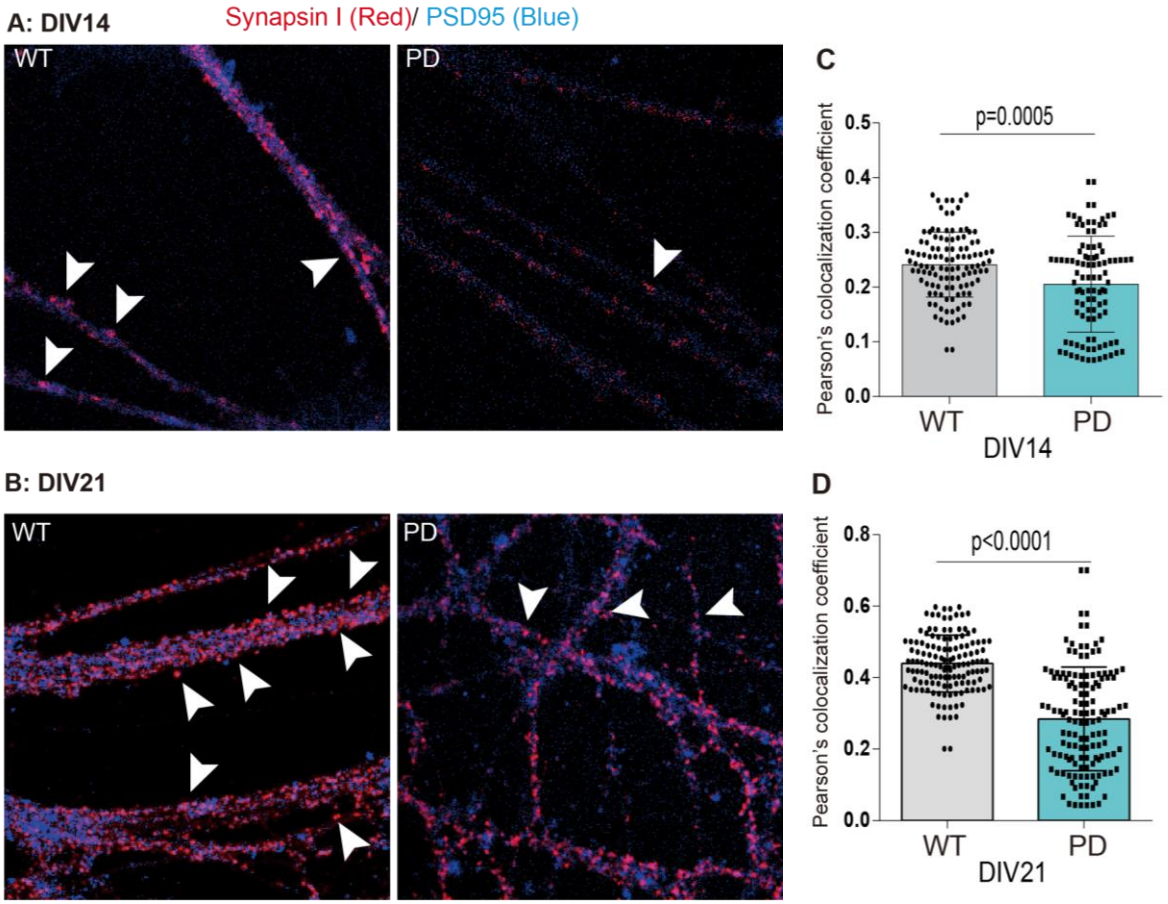

Figure 9. Expression of GFP-hASYN disrupts synaptic formation.

Co-localization of PSD95 and synapsin was decreased in GFP-ASYN transgenic mice. E18 cortical neurons from Line 78 (PDGF-β-ASYN-GFP) mouse embryos were dissected and cultured in 12-well plate as described in Materials and Methods. At DIV14 (A) and DIV21(B), immunostaining using PSD95 (Blue) and synapsin (Red) to visualize synapse. The count of colocolization is conducted by ImageJ (C-D). All data are analyzed using Prism Graphpad 6.0. The p values were obtained using student t-test. $p < 0.05$ (**); $p < 0.01$ (***); n.s. = non-significant

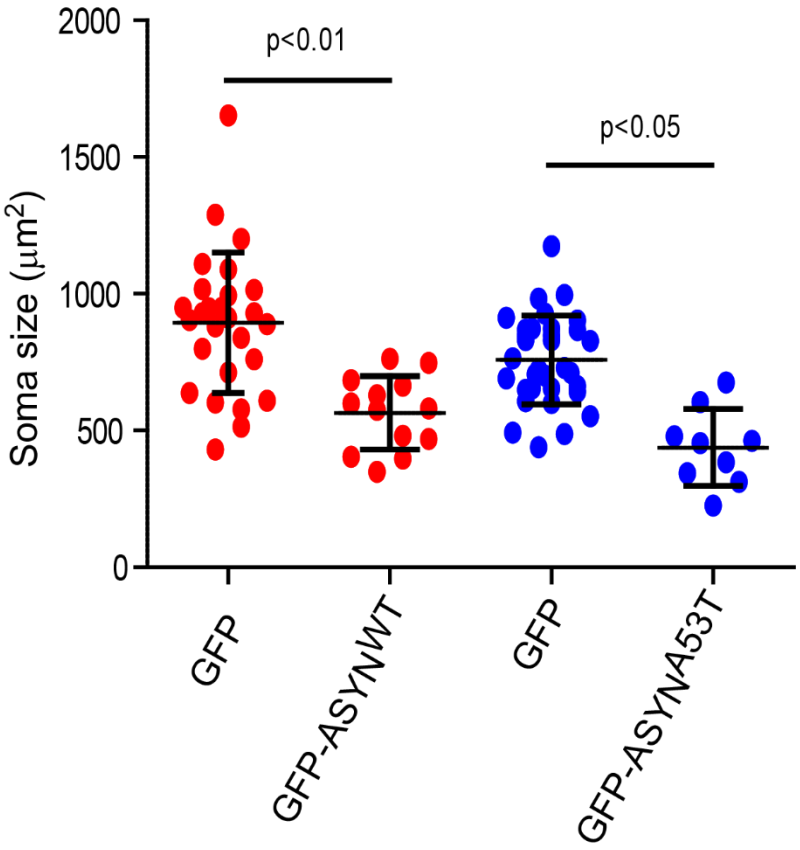

Figure 10. Expression of GFP-WT-ASYN or GFP-A53T-ASYN induced neuronal atrophy.

Primary cortical neurons were transfected at DIV3. Expression of GFP-WT-ASYN or GFP-A53T-ASYN induced cell shrinkage in E18 WT cortical neurons. At DIV3, E18 WT cortical neurons were transfected with GFP-WT-ASYN or GFP-A53T-ASYN. After 72hs, image of cells were captured by live imaging. Cells with GFP-green or not were counted respectively. All data was analyzed using Prism Graphpad 6.0. The p-values were obtained using student t-test.

This thesis, in part, is currently being prepared for submission for publication of the material. Alpha Synuclein Impairs Axonal Trafficking of Amyloid Precursor Protein in a Mouse Model of Parkinson's Disease. Suzhen Lin, Savannah Fang, Kijung Sung, Ruinan Shen, Alfredo Castro, Esther Na, Jazmin B. Florio, Michael L. Mante, Brian Spencer, Jianqing Ding, Robert A Rissman, Chengbiao Wu. The thesis author will be first co-author with Lin, Suzhen.

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