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Authors

Lin, Suzhen
Leitão, André
Fang, Savannah
et al.

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Overexpression of alpha synuclein disrupts APP and Endolysosomal axonal trafficking in a mouse model of synucleinopathy

Suzhen Lin[a,b,1], **André D.G. Leitão**[b,1], **Savannah Fang**[b], **Yingli Gu**[b], **Sophia Barber**[b], **Rhiannon Gilliard-Telefoni**[b], **Alfredo Castro**[b], **Kijung Sung**[b], **Ruinan Shen**[a,b], **Jazmin B. Florio**[b], **Michael L. Mante**[b], **Jianqing Ding**[a], **Brian Spencer**[b], **Eliezer Masliah**[b], **Robert A. Rissman**[b,c,*], **Chengbiao Wu**[b,*]

[a]Institute of Neurology, Ruijing Hospital, Shanghai JiaoTong University School of Medicine, Shanghai, China

[b]Department of Neurosciences, University of California San Diego, La Jolla, CA, USA

[c]VA San Diego Health System, La Jolla, CA, USA

Abstract

Mutations or triplication of the alpha synuclein (ASYN) gene contribute to synucleinopathies including Parkinson's disease (PD), Dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). Recent evidence suggests that ASYN also plays an important role in amyloid-induced neurotoxicity, although the mechanism(s) remains unknown. One hypothesis is that accumulation of ASYN alters endolysosomal pathways to impact axonal trafficking and processing of the amyloid precursor protein (APP). To define an axonal function for ASYN, we used a transgenic mouse model of synucleinopathy that expresses a GFP-human ASYN (GFP-hASYN) transgene and an ASYN knockout ($ASYN^{-/-}$) mouse model. Our results demonstrate that expression of GFP-hASYN in primary neurons derived from a transgenic mouse impaired axonal trafficking and processing of APP. In addition, axonal transport of BACE1, Rab5, Rab7, lysosomes and mitochondria were also reduced in these neurons. Interestingly, axonal transport of these organelles was also affected in $ASYN^{-/-}$ neurons, suggesting that ASYN plays an important role in maintaining normal axonal transport function. Therefore, selective impairment

*Corresponding authors at: Department of Neurosciences, University of California San Diego, Medical Teaching Facility, 9500 Gilman Drive, La Jolla, CA 92093-0624, USA. rrissman@health.ucsd.edu (R.A. Rissman), chw049@ucsd.edu (C. Wu).
[1]Suzhen Lin and André D.G. Leitão contributed equally to this work and share first authorship.

CRediT authorship contribution statement

Suzhen Lin: Conceptualization, Methodology, Investigation, Formal analysis. **André D.G. Leitão:** Formal analysis, Data curation, Software, Writing – original draft, Writing – review & editing, Visualization. **Savannah Fang:** Investigation. **Yingli Gu:** Investigation. **Sophia Barber:** Investigation. **Rhiannon Gilliard-Telefoni:** Investigation. **Alfredo Castro:** Investigation. **Kijung Sung:** Investigation. **Ruinan Shen:** Investigation. **Jazmin B. Florio:** Investigation. **Michael L. Mante:** Investigation. **Jianqing Ding:** Investigation. **Brian Spencer:** Writing – review & editing. **Eliezer Masliah:** Supervision, Writing – review & editing, Project administration, Funding acquisition. **Robert A. Rissman:** Supervision, Writing – review & editing, Project administration, Funding acquisition. **Chengbiao Wu:** Supervision, Writing – review & editing, Project administration, Funding acquisition.

Appendix A. Supplementary data

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of trafficking and processing of APP by ASYN may act as a potential mechanism to induce pathological features of Alzheimer's disease (AD) in PD patients.

Keywords

Alzheimer's disease; Parkinson's disease; Alpha synuclein; APP; Rab5; Rab7; Mitochondria; Lysosomes; Axonal trafficking; beta amyloid

1. Introduction

Alpha-synuclein (ASYN), is a small 14 kDa protein that is encoded by the *SNCA* locus (4q23) in humans (Chen et al., 1995) and is highly abundant in presynaptic terminals (Iwai et al., 1996; Maroteaux et al., 1988; Twohig and Nielsen, 2019a). Although the precise physiological function of ASYN is still unknown, studies have made considerable progress over the last decade. ASYN is now thought to play an important role in SNARE-complex formation (Burré et al., 2010) by binding to and assembling synaptic vesicles at the synaptic cleft, thus enabling a coordinated neurotransmitter release (Carnazza et al., 2022). The tendency for ASYN to multimerize at the lipid interface has been shown to regulate the clustering of synaptic vesicles, thus early multimerization of native ASYN could be essential for its physiological function (Burŕe et al., 2014; Diao et al., 2013).

However, multimerization at the lipid interface can also initiate a cascade of pathological aggregation that is associated with disease. Mutation(s), triplication and polymorphism(s) in the *SNCA* gene that accelerate aggregation of ASYN also lead to synucleinopathies such as Parkinson's disease (PD), Dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) (Houlden and Singleton, 2012; Mullin and Schapira, 2015).

Intriguingly, studies have also indicated that ASYN may be associated with or contribute to the pathogenesis of AD (Crews et al., 2009; Masliah et al., 2001; Roberts et al., 2017; Twohig and Nielsen, 2019b). Increased cerebrospinal fluid ASYN levels is correlated with disease progression from mild cognitive impairment (MCI) to AD (Berge et al., 2016; Twohig et al., 2018) and inversely correlated with cognitive function as measured by Mini-Mental State Exam (MMSE) scores (Korff et al., 2013; Majbour et al., 2017; Twohig et al., 2018). AD neuropathology is characterized by accumulation of Aβ and tau but both these proteins have been shown to interact with ASYN and promote its aggregation and toxicity in vitro and in vivo (Ferman et al., 2018; Masliah et al., 2001; Tsigelny et al., 2008). Although the implications of these observations are presently unclear, these studies suggest a potential role for ASYN in aggregation or accumulation of Aβ.

Clinical neuropathology studies demonstrate that <50% of autopsied AD brains have revealed lewy-related pathology (LRP) in their neocortex, limbic system, and substantia nigra (Hamilton, 2000; Hansen et al., 1990; Hansen et al., 1993; Iwai et al., 1996; Marui et al., 2000; Mikolaenko et al., 2005). The observation of LRP is correlated with degeneration of selected neuronal networks in the neocortex, limbic system, and the cholinergic nucleus basalis of Meynert (NbM) in AD and DLB, that may account for the behavioral deficits reported in these patients (Sahin et al., 2006). At the molecular level, ASYN may serve

as a mediator of Aβ toxicity to selected neuronal populations (Crews et al., 2009; Larson et al., 2012). ASYN and Aβ may directly interact to form toxic hetero-oligomers that affect specific networks (Tsigelny et al., 2008). In line with this, Aβ has been shown to trigger the misfolding and toxic conversion of ASYN (Bachhuber et al., 2015). We have previously shown that overexpression of ASYN in APP transgenic mice results in greater cholinergic cell degeneration (Masliah et al., 2001) and knocking-down ASYN in the same APP transgenic mice prevents cholinergic loss (Spencer et al., 2016). Likewise, knocking down ASYN in APP/ASYN double transgenic mice ameliorates the degeneration of pyramidal neurons in the CA3 region (Overk et al., 2014). These studies provide strong evidence that ASYN is an important mediator of APP/Aβ toxicity in inducing neuronal vulnerability in selective neuronal populations.

There are many potential mechanisms by which excessive ASYN accumulation contributes to PD and AD pathogenesis; one of which is that toxic ASYN species alter endocytic trafficking and signaling (Fang et al., 2017; Gonçalves and Outeiro, 2017; Hunn et al., 2015). Recent work demonstrates that expression of genes encoding endosomal trafficking is dysregulated in a panel of isogenic stem cell-derived neurons carrying familial AD mutations (i.e. APP and PS1) (Kwart et al., 2019). As a result, early endosomes in these cells were abnormally enlarged. Interestingly, this endosomal malfunction is likely resulted from accumulation of the carboxyl-terminal fragments cleaved by BACE1 (β-CTFs) of APP, not Aβ (Kwart et al., 2019). This finding is consistent with previous studies demonstrating that β-CTFs of APP impair endosomal trafficking in an early phase of cellular pathogenesis in AD (Cataldo et al., 2003; Israel et al., 2012; Jiang et al., 2019; Kim et al., 2016; Xu et al., 2016). Various other studies have demonstrated that impaired intracellular trafficking also occurs in early stages of Parkinson's Disease (Gonçalves and Outeiro, 2017; Hunn et al., 2015) and in primary embryonic neurons cultured from a transgenic mouse model expressing human ASYN (Fang et al., 2017). Evidence abounds to support the fact that endocytic dysfunctions play a decisive role in mediating synuclein pathology. First, an unbiased shRNA screening has uncovered that many Rab proteins are genetic modifiers of PD by mediating ASYN's aggregation, secretion and toxicity (Gonçalves et al., 2016). Second, we and other investigators have demonstrated using a mouse model of PD that over-expression of ASYN induces hyper-activation of Rab5, impairing retrograde axonal trafficking mediated by brain-derived neurotrophic factor (BDNF) and its TrkB receptor, leading to neuronal atrophy (Fang et al., 2017; Kang et al., 2017). Third, knocking down endogenous ASYN in an APP transgenic mouse model of AD prevented the degeneration of cholinergic neurons and recovered the levels of Rab3a and Rab5 proteins involved in intracellular transport and sorting of neurotrophic factors (Spencer et al., 2016). Finally, the seeding of ASYN aggregates within primary neurons by administration of ASYN pre-formed fibrils was shown to accurately reproduce synucleinopathies in cell models (Volpicelli-Daley et al., 2014b) and lead to defects in endolysosomal trafficking by effectively blocking endosomes (Mazzulli et al., 2016; Volpicelli-Daley et al., 2014a). Taken together, these studies have provided strong support that axonal function, and indeed the endolysosomal system are prime targets for ASYN toxicity.

To further investigate the possible link between ASYN and AD pathogenesis, we examined the role of excessive accumulation of ASYN in axonal transport and function using primary

neurons from a ASYN transgenic mouse (SYN-Tg) model of synucleinopathy and compared these results with primary neurons from ASYN-knockout ($SYN^{-/-}$) and WT mice. Our study demonstrates that excessive ASYN dramatically alters trafficking and processing of APP and BACE1, which may contribute to defects in APP processing and accumulation of intermediate APP and/or synuclein species.

2. Materials and methods

2.1. 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 under protocol #S02221 to RAR.

2.2. 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 were purchased from Sigma-Aldrich.

2.3. Animals

The SYN-Tg mouse model used in this work- Line 78 (also known as PDNG78) – expresses a GFP-human ASYN fusion (GFP-hASYN) transgene driven by the β-platelet-derived growth factor (βPDGF) promoter (Shults et al., 2005). 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 ($ASYN^{-/-}$) mouse model was obtained from Jackson laboratories. Pregnant mice from this line only produced embryos with the $ASYN^{-/-}$ genotype. All animals were maintained and bred according to standard procedures.

2.4. Preparation of primary neuronal cultures

Cortical neurons were harvested from non-transgenic and transgenic PDNG78 mouse embryos and from SYN−/− mouse E18 embryos as previously described (Fang et al., 2017). Briefly, cortical neurons were isolated and plated with plating media (Neurobasal with 10% FBS, B27, GlutaMAX) on (size) glass coverslips coated with poly-L-lysine (company) in 12-well plates (company). 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.

2.5. Live imaging of APP, BACE1, Rab5, 7, mitochondria and lysosomes

Transient expression studies were performed as previously described (Fang et al., 2017). Briefly, we used Lipofectamine 2000 (ThermoFisher, Cat# 11668027) to transfect mouse E18 cortical neurons at DIV3 with APP-mCherry, Rab5-mCherry, or Rab7-mCherry

vectors(Fang et al., 2017; Xu et al., 2016; Zhang et al., 2013) and BACE1-mCherry (a generous gift from Dr. Utpal Das). Images of cells were captured with a 100× objective lens using a Leica DMi8 Live Imaging Microscope, 24 h post transfection. Time lapse series were collected and analyzed as published previously (Fang et al., 2017; Xu et al., 2016; Zhang et al., 2013).

For imaging of axonal transport of mitochondria or lysosomes, cell culture medium was removed and washed with Gibco Minimum Essential Media (MEM) then incubated in working solution with either 50 nM of MitoTracker Red (ThermoFisher, cat# M22425) or 50 nM of Lysotracker Red (ThermoFisher, cat# L7528) diluted in MEM for 45 min in 37 °C. Live cell imaging was carried out using a 100× objective lens using a Leica DMi8 Live Imaging Microscope with a capturing rate of 1 frame every two seconds. We quantitated retrograde axonal movement in two different ways: first we calculated the instantaneous moving speeds that exclude pauses between two moving segments and defined it as 'retrograde moving velocity'. Then we measured average speed, including the pausing times for the 'retrograde average velocity'. All measurements were performed during 120 s timescales. Videos were acquired from axonal segments which were located at least one field of view away from the soma.

2.6. Synaptic double staining

Mouse E18 neurons cultured in vitro for 14 days (DIV14) on cover glasses 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 h and washed with PBS after. Secondary antibodies conjugated to Alexa 488, 568 (1/800) were for 1 h at room temperature, washed, and followed by DAPI (1:10,000) staining (5 min at room temperature). Coverslips were mounted onto glass slides using mounting media and prepared for imaging by confocal microscopy (a Leica SP confocal microscope under a 63× oil objective lens with a 1.6× zoom factor).

2.7. Immunofluorescence

For immunofluorescence experiments, DIV14 primary neurons from lines 78 (PDNG78 overexpressing hSYN-GFP) and line 61 (over-expressing hSYN) were fixed following the following protocol. We treated the cells with 4% paraformaldehyde for 20 min to fix them, rinsed with DPBS and permeabilized with 0.3% Triton-X (diluted in DPBS) for 5 min at RT. We coated the cells with 5% goat serum and incubated for 60 min at RT. We performed primary staining with selected antibodies (see below) and incubated overnight. After rinsing with DPBS we treated the cells with the secondary antibody (Alexa Fluor 488 goat-anti mouse (H + L), 10 μg/mL, and/or Alexa Fluor 594 goat-anti rabbit (H + L), 10 μg/mL) diluted in 5% goat serum solution. After 1 h incubation and rinsing we stained the cells with a DAPI solution (3 ng/mL) for 10 min, mounted the cells with ProLong Gold Anti-fade Reagent and observe them under the microscope. For primary and secondary staining steps the following antibodies were used. Rab5 and Rab7 were stained

with anti-mouse Rab5 rabbit primary antibody (abcam EPR21801 and EPR7589), followed by anti rabbit AF-594-tagged secondary antibody (Invitrogen). ASYN was stained with Syn211 mouse antibody (ThermoFisher Scientific) followed by anti-mouse AF-488-tagged secondary antibody (Invitrogen). ASYN was stained as above and LAMP1 was stained with anti-mouse LAMP1 rabbit primary antibody (abcam), followed by anti-rabbit AF-594-tagged secondary antibody.

2.8. Colocalization analysis

For colocalization analysis, ImageJ software was used with Coloc-2 plugin (https://imagej.net/plugins/coloc-2). Background was subtracted and regions of interest were selected to isolate the cell bodies around the nuclei. Pearson's correlation coefficients were acquired based on correlation plots and output images obtained, as described in the protocol above available online for Coloc-2 plugin.

2.9. Statistics

All experiments were repeated for at least 3 times independently. Data represent mean ± SEM. Statistical analyses and calculation of *p* values were performed using Prism 6.0 (GraphPad Software, La Jolla, CA); One-Way ANOVA with Bonferroni's pos*t*-test was used for frequency distribution analysis. Student's t-test was used for pairwise comparisons. p values <0.05 were considered statistical significance, and p values <0.01 were considered highly statistical significance.

3. Results

3.1. Trafficking of APP is disrupted in SYN-Tg (PDNG78) neurons, but not in ASYN$^{-/-}$ neurons

Recent studies have demonstrated that disruption of axonal trafficking and processing of APP contribute to the early phase of AD pathogenesis (Brunholz et al., 2012; Rodrigues et al., 2012; Stokin et al., 2008; Stokin et al., 2005). Since our previous study has demonstrated that Rab proteins were dysregulated (Fang et al., 2017; Spencer et al., 2016) and axonal transport of brain-derived neurotrophic factor (BDNF) was impaired in a transgenic mouse model of PD (Fang et al., 2017), we speculated that ASYN may also alter axonal trafficking and processing of APP in this model.

To test whether or not this is the case, we dissected and cultured E18 cortical neurons from a mouse model of PD following established protocols (Fang et al., 2017). These mice expressed GFP-human ASYN (GFP-hASYN) driven by the promoter of β-platelet-derived growth factor (βPDGF) – mouse line henceforth mentioned as PDNG78 or SYN-Tg. Various studies have shown that the PDNG78 transgenic mouse line develops biochemical and neuropathological features consistent with DLB/PD and has been used previously for imaging ASYN in the CNS and retina (Osterberg et al., 2015; Price et al., 2018; Price et al., 2016; Rockenstein et al., 2005; Shults et al., 2005). PDNG78 ice showed accumulation of ASYN in the inner layers of the neocortex and limbic system and to a lesser extent in the striato-nigral system (Rockenstein et al., 2005). In addition, these mice display hippocampal synaptic dysfunction, as well as behavioral impairments (Scott et al., 2010). Together these

findings suggest that the α-ASYN-GFP tg model might mimic some of the non-motor aspects of LBD.

ASYN expression in cortical neurons in cell models of overexpression (e.g. line 78 or line 61) is variable so the presence of GFP signal helped in distinguishing from neurons that expressed high levels of hASYN from those that expressed no or very low hASYN (Fang et al., 2017). To define a role for endogenous mouse ASYN, we also prepared neuronal cultures from a ASYN knockout mouse model ($SYN^{-/-}$) and compared our results with neurons from wild-type mice (C57BL6) – Fig. S1 shows examples of cortical neurons used throughout this work to acquire timelapse data of axonal transport.

To visualize APP, we used LipoFectamine2000 to transfect an APP-mCherry construct into neurons at DIV14 (Xu et al., 2016). 24 h following transfection, live imaging experiments were performed to capture time-lapse series of axonal transport of APP-mCherry in WT, GFP-hASYN (PDNG78) and ASYN knockout neurons ($SYN^{-/-}$) as described previously (Fang et al., 2017; Xu et al., 2016). Kymographs were generated from the time-lapse series (Fig. 1A–C) and transport parameters were quantitated as described in previous work (Fig. 1D–E) (Fang et al., 2017; Xu et al., 2016). Fig. S2 represents ten examples of axons imaged for each mouse line (WT, Syn−/− and PDNG78), with respective kymographs for quantification of retrograde transport speeds.

Our results showed that in WT, the retrograde moving velocity (not counting the pausing times) of APP-mCherry was 0.72 ± 0.07 μm/s and was significantly higher compared to the PDNG78 neurons with 0.30 ± 0.03 μm/s retrograde moving velocity (Fig. 1D). The retrograde-instantaneous moving velocity of APP-mCherry in $SYN^{-/-}$ neurons was 0.71 ± 0.07 μm/s, and non-significantly different from WT. The average retrograde velocity (including pausing times) showed significant differences across all neurons. WT neurons had an average retrograde moving velocity of 0.31 ± 0.054 μm/s. These values for PDNG78 neurons and $ASYN^{-/-}$ neurons were 0.05 ± 0.0079 μm/s and 0.167 ± 0.024 μm/s, respectively.

Taken together, our results indicate that the absence of endogenous ASYN had no impact on axonal transport of APP since no difference was observed between the $ASYN^{-/-}$ and WT neurons (Fig. 1D). However, increased expression of hASYN significantly affected axonal transport of APP (Fig. 1D). Based on these results, we conclude that excessive accumulation of human ASYN impaired axonal transport of APP in primary mouse neurons.

3.2. Trafficking of BACE1 is disrupted in ASYN-PDNG78 neurons, but not in $ASYN^{-/-}$ neurons

The proteolytic processing of APP is first mediated by β-secretase (BACE) to generate the β-carboxyl terminal fragment (β-CTF) of APP (Das et al., 2013). Accumulation of β-CTF negatively impacts axonal trafficking and function (Kwart et al., 2019; Rodrigues et al., 2012; Xu et al., 2018; Xu et al., 2016). Given that axonal trafficking of APP is impaired by excessive ASYN in PDNG78 neurons, we asked if ASYN also affected BACE1 trafficking. We transfected E18 cortical neurons at DIV14 with BACE1-mCherry into WT, $SYN^{-/-}$ and PDNG78 (SYN-Tg) neurons and carried out live imaging experiments to quantitate

the transport of BACE1 as described for APP. Consistent with the observations for APP, kymographs of axonal transport (Fig. 1F–H) demonstrated that axonal trafficking velocity of BACE1 is also reduced in PDNG78 neurons overexpressing ASYN (Fig. 2I–J). Interestingly, the change in axonal transport of BACE1 is also observed in $SYN^{-/-}$ neurons, suggesting that ASYN may play a role in regulating axonal transport of BACE1. Double imaging for the GFP protein of the GFP-hASYN and the mCherry of BACE1-mCherry suggested no co-localization of synuclein and BACE in the axons of these neurons in contrast to that observed for APP and hASYN (Fig. S3).

3.3. 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 PD (Fang et al., 2017; Gao et al., 2018; Shi et al., 2017; Spencer et al., 2016). Further, we have previously demonstrated that expression of ASYN altered both the level and activities of Rab proteins that regulate endocytic trafficking (Fang et al., 2017; Spencer et al., 2019). We then examined the axonal transport of Rab5-early endosomes and Rab7-late endosomes. Using the same approach described above, we transfected an expression vector of mCherry-Rab5 into E18 cortical neurons of WT, $SYN^{-/-}$ and PDNG78 mouse line (SYN-Tg) at DIV14. Axonal transport of mCherry-Rab5 was quantitated as described below.

Our results showed that the retrograde moving velocity of Rab5 (early endosomes) in WT neurons was 0.78 ± 0.06682 μm/s, which was significantly faster than the PDNG78 neurons at 0.3251 ± 0.05469 μm/s (Fig. 2D). There was also a significant difference in retrograde moving velocity between $SYN^{-/-}$ neurons with 0.0.3251 ± 0.05469 μm/s and PDNG78 neurons, but no statistically significant differences were seen between WT and $SYN^{-/-}$ (Fig. 2D). The average retrograde velocity showed significant differences between WT and PDNG78 neurons. WT neurons had an average retrograde velocity of 0.6457 ± 0.1225 μm/s, $SYN^{-/-}$ neurons at 0.3615 ± 0.04197 μm/s, and PDNG78 neurons at 0.07548 ± 0.02234 μm/se (Fig. 2E). There were significant differences between WT and $SYN^{-/-}$ neurons for the average retrograde velocity, but not between $SYN^{-/-}$ and PDNG78 neurons (Fig. 2E).

We used mCherry-Rab7 to investigate the axonal transport of late endosomes. The retrograde moving velocity of Rab7-decorated vesicles was 0.6949 ± 0.04984 μm/s in WT neurons, significantly faster than PDNG78 neurons (0.4042 ± 0.03930 μm/s) and $SYN^{-/-}$ neurons (0.2646 ± 0.03955 μm/s) (Fig. 2I). In WT neurons, the average retrograde velocity was 0.5743 ± 0.06354 μm/s, significantly faster than PDNG78 neurons (0.08579 ± 0.01718 μm/s) and $SYN^{-/-}$ neurons (0.01592 ± 0.03269 μm/s) (Fig. 2J). In sum, Rab7 axonal transport was significantly reduced in both $SYN^{-/-}$, and PDNG78 neurons as compared to WT neurons. However, there were no observed significant differences between $SYN^{-/-}$ and PDNG78 neurons (Fig. 2I–J). These results suggest that ASYN homeostasis is critical to maintaining axonal transport of late endosomes.

3.4. Rab5 colocalized with ASYN

Since our results suggest that the axonal transport of *endo*-lysosomal vesicles is compromised when hASYN is overexpressed, we set out to investigate whether ASYN colocalizes preferentially with Rab5-, Rab7- or LAMP-1-decorated vesicles (early-endosomes, late-endosomes or lysosomes, respectively). 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 disease (Kim et al., 2016), the subcellular distribution of ASYN may also overlap with specific endocytic vesicles. To identify if this is the case, immunofluorescence was performed on DIV14 PDNGN78 mouse cortical neurons, and visualized on a confocal laser scanning microscope. The GFP-ASYN labels overlapped with the early endosomal marker Rab5, less so with Rab7 and no colocalization with Lamp1, a marker for lysosomes (Fig. 3). We quantified colocalization using image analysis software ImageJ and the Coloc-2 plugin, obtaining a Pearson's coefficient from pixel correlation analysis in the two channels, shown in Fig. S4.

To check whether the overexpression of α-synuclein leads to changes in the size of endolysosomal vesicles, BV2 cells were co-transfected with the following vectors (GFP, GFP-WT-Syn, GFP-A53T-Syn) for 24 h. Cells were fixed and imaged by confocal microscopy and histograms of sizes were obtained for co-expressions with Rab5-mCherry (Fig. S5). Results show a significant increase in the population of larger early endosomes (Rab5-mCherry) when ASYN is overexpressed both in its WT and A53T (aggregation-prone) forms. This suggests that ASYN, like other aggregation-prone proteins, colocalizes and builds up within early endosomes leading to enlargement of these vesicles, corroborating findings by other authors (Chassefeyre et al., 2021; Teixeira et al., 2021).

3.5. Effect of ASYN on axonal trafficking of lysosomes and mitochondria

Since α-synuclein degradation is mostly lysosomal-dependent, lysosomal impairment can affect α-synuclein turnover, contributing to its accumulation and aggregation(Navarro-Romero et al., 2020). Lysosomes exist mostly in the soma, where cellular degradative function is exerted. Lysosomes can also be transported throughout the cell and this phenomenon correlates with efficiency in degradation of cargo derived from the endocytic and autophagosomal pathways (Hunn et al., 2015). For this reason, we decided to investigate whether the intracellular trafficking of lysosomes to the soma is impaired in our neuronal model of synucleinopathy (PDNG78 neurons) – Fig. 4. At DIV14, neurons were treated with Lysotracker for 30mins, and axonal transport of lysosome was captured by live imaging. Our results reveal that lysosomes from PDNG78 have significantly lower retrograde moving velocities (counted only when moving) as well as retrograde average velocities (pausing times included), as compared to WT neurons – Fig. 4D,E. Interestingly, neurons from $SYN^{-/-}$ line also show slower retrograde moving velocities of lysosomes than WT, suggesting that α-synuclein might play a role in the lysosome turnover and degradative process altogether.

Mitochondria are dynamic organelles, and essential for energy conversion and neuron survival. Several mitochondrial imbalances have been found to play a role in neurodegeneration, e.g. mitochondrial fragmentation, impaired mitophagy and blocked

transport of mitochondria along axons (Sheng and Cai, 2012). In Parkinson's disease, dysregulation of contacts between mitochondria and the endolysosomal system resulted in disrupted organelle distribution within axons (Kim et al., 2021). Therefore, we set out to investigate whether mitochondria transport defects could explain the axonopathies occurring in our model of PD. We used MitoTracker to label, track and quantitate axonal movement of mitochondria in WT, $SYN^{-/-}$, and PDNG78 neurons, as described in previous work (Zhao et al., 2016). Our results showed the following average retrograde moving velocities for mitochondria: 0.9713 ± 0.1466 μm/s for WT, 0.3408 ± 0.1047 μm/s for $SYN^{-/-}$ and 0.2326 ± 0.03477 μm/s for PDNG78 neurons (Fig. S7). While mitochondria transport in PDNG78 showed a tendency to be decreased, this difference was not statistically significant against WT neurons. Interestingly, mitochondria were transported significantly faster in neurons that were deprived of SYN ($SYN^{-/-}$), as compared to both WT and SYN-Tg neurons. We did not observe statistically significant differences in the average retrograde velocity values between the three groups.

3.6. Effect of ASYN on synaptic formation

Given that expression of the hASYN transgene delayed axonal trafficking, and synaptic formation is critically dependent on axonal delivery of presynaptic components (Guedes-Dias and Holzbaur, 2019), we set out to investigate whether the hASYN transgene would affect synapse formation. To that end, we cultured E18 WT or PDNG78 cortical neurons on coverslips and 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 for post-synapses. Colocalizations between Synapsin I and PSD95 were quantitated using ImageJ and Pearson's colocalization coefficients were computed using the MOSAIC SUITE Plugin. Co-localization of Synapsin I and PSD 95 was significantly reduced in primary neurons derived from the PDNG78 mice compared to WT littermates (Fig. 5). The Pearson's colocalization coefficient between Synapsin and PSD95 in PDNG78 neurons was significantly lower than in WT DIV14 neurons (Fig. 5A, C). The deficits persisted at DIV21 (Fig. 5B, D). These data suggest that overexpression of hASYN significantly disrupts synapse formation in PDNG78 neurons.

4. Discussion

In the present study, we investigated how expression levels of ASYN affected axonal trafficking of APP and endolysosomal vesicles in a mouse model of synucleinopathy. Consistent with published results (Araki et al., 2019; Roberts et al., 2017), we demonstrate that expression of GFP-hASYN in the PDNG78 neurons (a mouse model that has been shown to accurately replicate pathological features of DLB/PD) impaired axonal trafficking of APP. In addition, axonal transport of BACE1, Rab5, Rab7, lysosomes and mitochondria were also reduced in these neurons. ASYN knockout neurons also showed a deficit in axonal movement of these organelles. Thus, our data suggest that ASYN plays an important role in maintaining homeostatic endolysosomal function, i. e. either increased or reduced expression of ASYN protein can disrupt axonal trafficking.

ASYN is an abundant presynaptic protein and increased expression of ASYN leads to excessive accumulation of ASYN aggregates in axons. Therefore, it is possible that these ASYN aggregates could disrupt axonal transport of endosomes and other organelles. Recent studies using a similar mouse model of PD have found that ASYN overexpressing mice show decreased spine density and abnormalities in spine dynamics in an age-dependent manner, which corroborates our results on the loss of synaptic integrity (Blumenstock et al., 2017). Furthermore, seeded ASYN aggregates have a catastrophic effect on dendritic architecture as well as dystrophic deformation of dendritic shafts in pyramidal neurons (Blumenstock et al., 2017). Reduced axonal trafficking likely impacts neuronal functions in many different aspects and could partially arise from the disruption of normal cell architecture imparted by synuclein aggregates (Froula et al., 2018).

Axonal transport impairments in synucleinopathies have been observed before by previous authors. One of the first studies to reveal pathologic α-synuclein aggregates in PD and DLB axon terminals showed they are associated with and block synaptic vesicle proteins (Galvin et al., 1999). Later, studies using A30P and A53T ASYN mutations revealed that levels of the molecular motors associated with axonal transport were dramatically reduced in PD brains, well before neuronal death (Chu et al., 2012). More recently, breakthrough studies were able to replicate and follow the formation of Lewy Bodies in primary neurons after exposure to fibrils made from recombinant α-synuclein (Volpicelli-Daley et al., 2014b; Volpicelli-Daley et al., 2011). It was found that the presence of axonal inclusions of ASYN in axons reduces velocities and mobilities of Rab7-positive late endosomes, TrkB neurotrophin receptors, and LC3-positive autophagosomes. This agrees with the results we present in this study, as well as previous results from our lab on the axonal transport of BDNF (Fang et al., 2017). As to the more mechanistic question of *'How do ASYN aggregates cause axonal transport impairments?'*, we and other authors have raised several possibilities. It is possible that ASYN aggregates may sequester or impair the function of motor proteins or associated adaptors. Indeed, proteomics of purified Lewy bodies showed enrichment of dynein and dynactin (Shen et al., 2018). In Huntington's disease, for example, huntingtin forms a complex that regulates the motor activity of kinesin and dynein (Caviston and Juliane, 2007). Other studies revealed that ASYN aggregates could be detrimental for the endolysosomal and macroautophay pathways, which are involved in their clearance (Tanik et al., 2013; Winslow et al., 2010; Yan et al., 2014). This could occur by direct binding of toxic ASYN aggregates or by sequestration of proteins and vesicles by insoluble aggregates. Indeed, oligomers and fibrils of ASYN have been shown to bind directly to LC3 (Leitão et al., 2021). Our work as well as previous authors suggested that ASYN colocalizes with endosomes within the axon – inclusions could then grow from these membranes (Galvagnion et al., 2015) and selectively trap more vesicles and organelles, explaining the heterogeneity of proteins found in inclusions.

Although it is unclear how APP and ASYN interact and how their interaction affect each other's biological functions (Jellinger, 2011), recent evidence has shown that both accumulate on early endosomes (Spencer et al., 2016) and on mitochondria (Devi and Anandatheerthavarada, 2010). Since BACE1 is also trafficked in endosomes, it is possible that reduced axonal trafficking of APP will increase the likelihood that APP is cleaved by BACE1 to yield toxic APP C-terminal fragments as well as Aβ (Das et al., 2013; Fang et

al., 2017; Shen et al., 2020; Xu et al., 2018; Xu et al., 2016). In sum, exactly how ASYN triggers Aβ pathology (or vice-versa) is still unknown, but we propose in this work that the disruption of normal somatic and synaptic architecture of neurons (by either ASYN aggregates or toxic aggregates of APP itself) could offer an explanation into how APP (as well as BACE1, Rab5, Rab7 and lysosomes) are defectively transported along axons. Defective transport has been shown by several studies to lead to accumulation of neuronal proteins into pathological lesions, and that too has been associated with the *endo*-lysosomal system as a hotspot for aggregate formation, leading to so called 'enlarged endosomes' (Chassefeyre et al., 2021; Hu et al., 2015; Soper et al., 2011; Volpicelli-Daley et al., 2014a). In support of these ideas, both Aβ and tau have been shown to have higher aggregation propensities under the acidic environment of endosomes and lysosomes (Schützmann et al., 2021; Vingtdeux et al., 2007) and enlarged endosomes have been consistently found in both cellular and in vivo models of neurodegenerative diseases (Chassefeyre et al., 2021; Pensalfini et al., 2020; Podinovskaia et al., 2021; Xu et al., 2016; Yim et al., 2015). Whether aggregates precede or succeed transport deficits is still a topic of debate, yet our work offers more evidence that both are inter-related and that transport deficits could ultimately build a positive feedback loop into the formation of pathological inclusions.

In addition to APP and BACE1, the axonal transport of Rab5, Rab7, lysosomes and mitochondria was also delayed under conditions whereby an ASYN human transgene is overexpressed, mimicking the pathology of PD in neurons (Fig. 6A,B). Since lysosomes are mostly located in the soma, our data raise the hypothesis that slower retrograde axonal transport could theoretically lead to a positive feedback loop for an accumulation of ASYN, since its clearance is slower in the first place. Furthermore, this accumulation could be enhanced within endosomes (indeed we see colocalization of ASYN-GFP and Rab5 but less so with LAMP1) (Fig. 6B). Over time, the overexpressed hASYN is not cleared effectively and could accumulate within early-endosomes, where it colocalizes (and could interact) with APP. ASYN and Aβ have been shown before to cross-seed each other's amyloidogenic properties (Ono et al., 2012) and thus further aggregation could ensue, potentially leading to further disruptions of cellular architecture and/or endocytic fusion machinery. For example, some studies have shown that pathological ASYN aggregates can destabilize microtubules both by binding directly to its subunits and by promoting hyperphosphorylation of its associated protein, tau, leading to its irreversible aggregation (Carnwath et al., 2018). These aggregation phenomena could therefore lead to transport deficits of organelles such as mitochondria and lysosomes, which are critical for cellular homeostasis. We indeed see less synaptic formation (as shown by lower colocalization between pre- and post-synapse markers) and more cellular atrophy (smaller average soma sizes) when ASYN is overexpressed – Fig. 6B and (Fang et al., 2017). Though this hypothetical model needs further elucidation, it corroborates several of our findings (see Fig. 6) and could partially explain the fact that concurrent ASYN and Aβ lesions are found in patients with AD and PD, within the endolysosomal system.

Our study shows colocalization between ASYN and Rab5, yet these results were obtained in primary neurons overexpressing GFP-tagged hASYN. The question should be asked as to whether endogenous untagged ASYN would also colocalize with endosomes. In fact, previous work has successfully characterized the association of endogenous untagged ASYN

with various sorts of endomembranes, from multivesicular bodies to endosomes, lysosomes or autophagosomes (Boassa et al., 2013; Fakhree et al., 2021; Masaracchia et al., 2018). Boassa et al. (2013) used the Line 61 untagged hASYN overexpression mouse model and reported that enlarged presynaptic terminals in these mice show extensive membranous structures and tubulovesicular architecture. The authors established that ASYN associates with membranes in early stages of the sorting process and argue that it could therefore play an important role in membrane structure modification and/or collection of cargos for degradation, ultimately becoming entrapped in membranous aggregates that arise due to degradation dysfunction or overburdening over time.

Finally, the levels of different APP processing by-products has also been correlated with the large levels of iron that are seen in older Parkinson's and Alzheimer's patients (Kenkhuis et al., 2021). APP plays a role in the facilitation of iron export from the brain by interacting with ferroprotein, a transmembrane protein that is known to transport iron (Duce et al., 2010). Thus, if this function is impaired, brain iron accumulation could exacerbate cellular homeostasis and neurodegeneration, however, further studies are necessary to understand if the high levels of ASYN do indeed affect APP processing and, if so, whether or not this can be conducive to the accumulation of iron in the brain.

Interestingly, axonal trafficking was also impaired in SYN−/− neurons, albeit to a lesser degree than PDNG78 neurons. Although the exact mechanism(s) is presently unclear, the data suggest a protective role of ASYN at normal expression levels. Future studies are needed to further define the exact role of ASYN but we argue that ASYN is critical for axonal homeostasis as a total absence of ASYN seems to expose axons vulnerable to insults.

To our knowledge this is the first study to suggest that over-expression of ASYN could affect APP transport, a link that was previously unreported. Axonal transport deficits are also observed for markers of early- and late-endosomes as well as lysosomes. We raise the discussion point that defective APP transport could impact its function and processing into Aβ species, and that defective transport of endolysosomal vesicles could represent a positive feedback loop into the formation of inclusions. These two mechanistic questions remain, at this stage, hypotheses that warrant extensive investigation in the future.

5. Limitations of the study

In this study we discovered widespread axonal transport impairments in neurons from a GFP-hASYN overexpression model. Considering that transport deficits were common to many proteins and vesicles, the question could be asked as to whether this is indeed what happens in disease or a direct consequence of this PDNG78 mouse model, i.e. the overexpression of hASYN itself or the presence of a GFP-tag. We have previously shown that, in PDNG78 mice, expression of ASYN-GFP under the control of the PDGFβ promoter results in the accumulation of ASYN in the inner layers of the neocortex and limbic system and to a lesser extent in the striato-nigral system (Rockenstein et al., 2005). In addition, these mice display hippocampal synaptic dysfunction, as well as behavioral impairments (Scott et al., 2010). Together these findings suggest that the α-ASYN-GFP tg model might mimic some of the non-motor aspects of LBD. However, neurons from different mouse lines

could have physiological alterations that are unaccounted for in this work. Whether some of the alterations such as the increased aggregation of ASYN-GFP in PDNG78 neurons or the lack of ASYN in Syn−/− could trigger widespread alterations in neuronal function beyond the ones found in this work is a strong possibility. Further, despite some clues that the PDNG78 overexpression model could be useful in understanding the cellular mechanism of disease in PD, it is possible that the accelerated state of ASYN aggregation does not accurately reflect the detailed mechanistic aspects of disease in non-familial Parkinson's Disease, where ASYN gene copies are not mutated or duplicated/triplicated.

A question could also be asked as to whether the overexpression of GFP-fused ASYN could affect normal folding, physiological function and aggregation of ASYN. The latter is of particular interest since accumulation and aggregation of ASYN are a pathological hallmark in PD. In order to be sure that the PDNG78 model accurately reproduces the pathology seen in PD, it should also replicate the aggregation pattern of ASYN. Despite some studies characterizing hASYN-GFP mouse lines, it is still largely unknown whether GFP could affect protein-protein interactions in such a heterogeneous environment as are Lewy Body inclusions and the overall cellular environment in primary neurons. Hansen et al. (2013) observed ASYN-GFP accumulation and aggregation in this mouse line that correlated with the progressive behavioral deficits. Other authors have measured ASYN-GFP aggregation and characterized it over time: evidence showed the formation of early microaggregates exclusively at the pre-synapse (Spinelli et al., 2014) and centripetal formation of aggregates at longer timescales, i.e. a pattern of aggregate formation that is similar to other human ASYN mouse overexpression lines (Line 61, untagged hASYN) with accumulation of phosphorylated and pK resistant ASYN in somatic inclusions over time (Osterberg et al., 2015).

Another important aspect to mention is that our study was exclusively done using mouse cortical neurons. However, recent studies have shown that neurons within different regions show very different vulnerability to insults by toxic ASYN aggregates and that inclusions themselves form preferential and sequentially in specific brain regions (Kaul et al., 2011; Taguchi et al., 2019). In mice and in humans both the hippocampus and the cortex are the main areas where ASYN is highly expressed. Although ASYN expression is higher in the hippocampus, we decided to use cortical neuron cultures throughout this work because levels of ASYN in this region are still high relative to other regions and due to the technical aspects, to isolate more efficiently the frontal cortex from other regions in brains of E18 mouse embryos. However, we expect that neurons from other brain regions could have different results in the axonal transport of APP and endolysosomal vesicles. Testing this hypothesis could shed light on how and why synuclein aggregates spread throughout the brain.

Mechanistically, our work was not designed to discover a model that explains the axonal transport impairments observed. The complexity of the pathways involved led us to limit the scope of our work to investigating axonal transport speeds under three different conditions of ASYN expression (ASYN overexpression, ASYN knock-out and wild-type). Our work suggests that ASYN overexpression and axonal function are interrelated, however, more detailed studies are required in order to understand what this means for: a) the

relation between ASYN over-expression and changes in processing of APP; b) the rate of endolysosomal clearance and its relation with neuronal dysfunction and c) the relation between the increase in endosomal size and disease. As part of the discussion of this work, we included a hypothetical model in the GFP-hASYN overexpression line, however investigating the three questions above would be necessary in the future in order to elucidate the mechanism for axonal impairments in synucleinopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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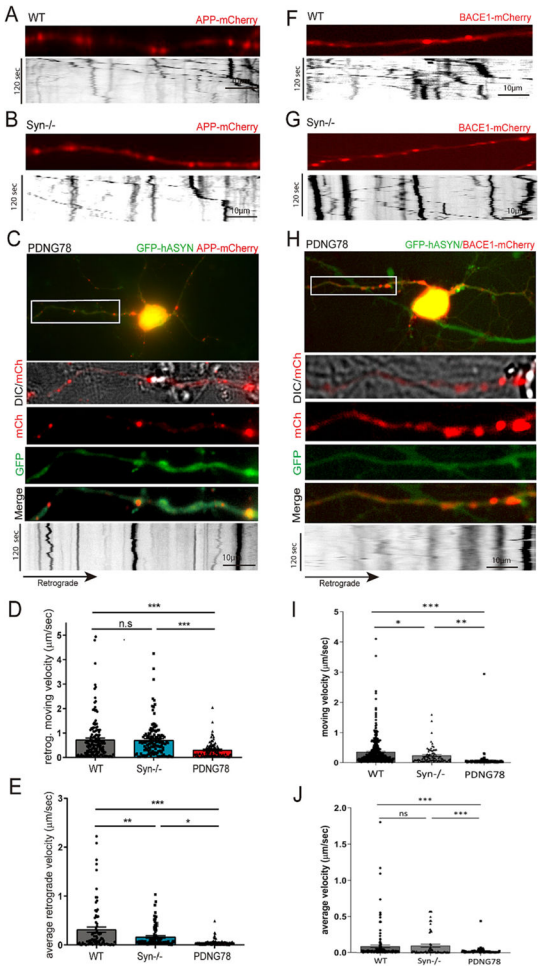

Fig. 1.

The overexpression of GFP-hASYN induced retrograde axonal transport deficits of APP and BACE1 in E18 cortical neurons. E18 cortical neurons from wildtype (WT), the PD mouse model Line 78 (PDGF-β-GFP-hASYN) and ASYN-knockout (SynKO) mouse embryos were dissected and cultured as described in Materials and Methods. At DIV14, neurons were transfected with APP-mCherry (A-E) or BACE1 (F-J). After 24 h, axonal transport of APP-mCherry and BACE1-mCherry was captured by live imaging. Kymographs were generated from time-lapsed image series. Representative images of APP-mCherry and BACE1-mCherry within axons of WT (A, F), Syn−/− (B, G) and PDNG78 neurons (C, H) are shown. Axonal transport parameters: the retrograde moving velocity (D, I) and average velocity (E, J) are quantitated and presented. Data were obtained from 20 WT neurons, 20 Syn−/− neurons and 15 GFP-positive transgenic PDNG78 neurons. Representative images of axons correspond to the first frame of the kymograph. All data are analyzed using Prism GraphPad 6.0. The *p* values were obtained using student *t*-test. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); n.s. = non-significant.

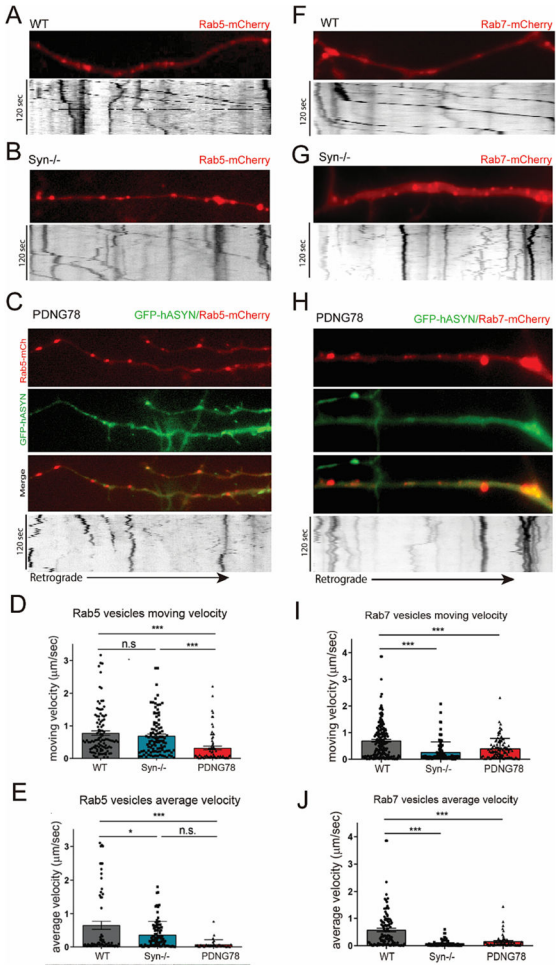

Fig. 2.

The overexpression of GFP-ASYN induced retrograde axonal transport deficits of Rab5 and Rab7 in E18 cortical neurons. E18 cortical neurons from Line 78 (PDGF-β-ASYN-GFP) and SynKO mouse embryos were dissected and cultured in microfluidic chambers as described in Materials and Methods. At DIV14, neurons were transfected with mCherry-WT-Rab5 (A-E) and mCherry-WT-Rab7 (F-J). After 24hs, axonal transport of Rab5 and Rab7 was captured by live imaging. Kymographs were generated from time-lapsed image series. Representative image of Rab5 and Rab7 within axons of non-transgenic (A, F), ASYN-knockout (B, G) and PDNG78 transgenic neurons (C, H) are shown. Axonal transport parameters: the retrograde moving velocity (D, I) and average velocity (E, J) are quantitated and presented. Data were obtained from 20 non-transgenic neurons, 20 ASYN-knockout neurons and 15 GFP-positive transgenic neurons. Representative images of axons correspond to the first frame of the kymograph. All data are analyzed using Prism GraphPad 6.0. The p values were obtained using student t-test. $p < 0.05$ (*); $p < 0.001$ (***); n.s. = non-significant.

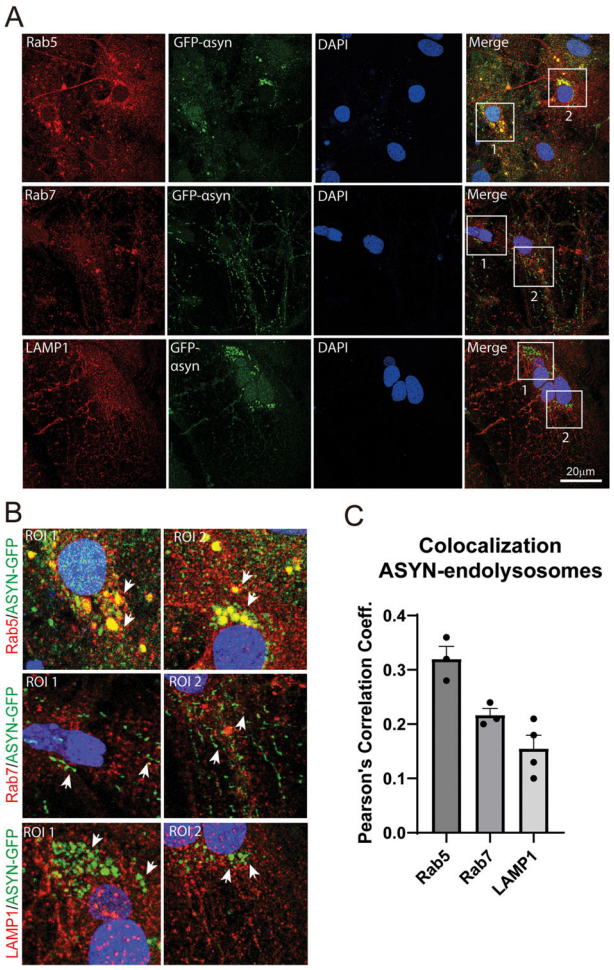

Fig. 3.

Colocalization of GFP-hASYN with Rab5 in E18 PDNG78 cortical neurons. E18 cortical neurons were cultured and fixed for immunostaining with specific Abs against Rab5 (red), Rab7, Lamp1. (A) Representative images for GFP-hASYN (green), Rab5 (red), Rab7 (red), and Lamp1 (red) are shown. All nuclear staining (DAPI) is shown in blue. (B) Insets were enlarged to better visualize the colocalization between hASYN and these three different markers. (C) Colocalization was quantified as Pearson's correlation coefficients for at least three cells for each experiment.

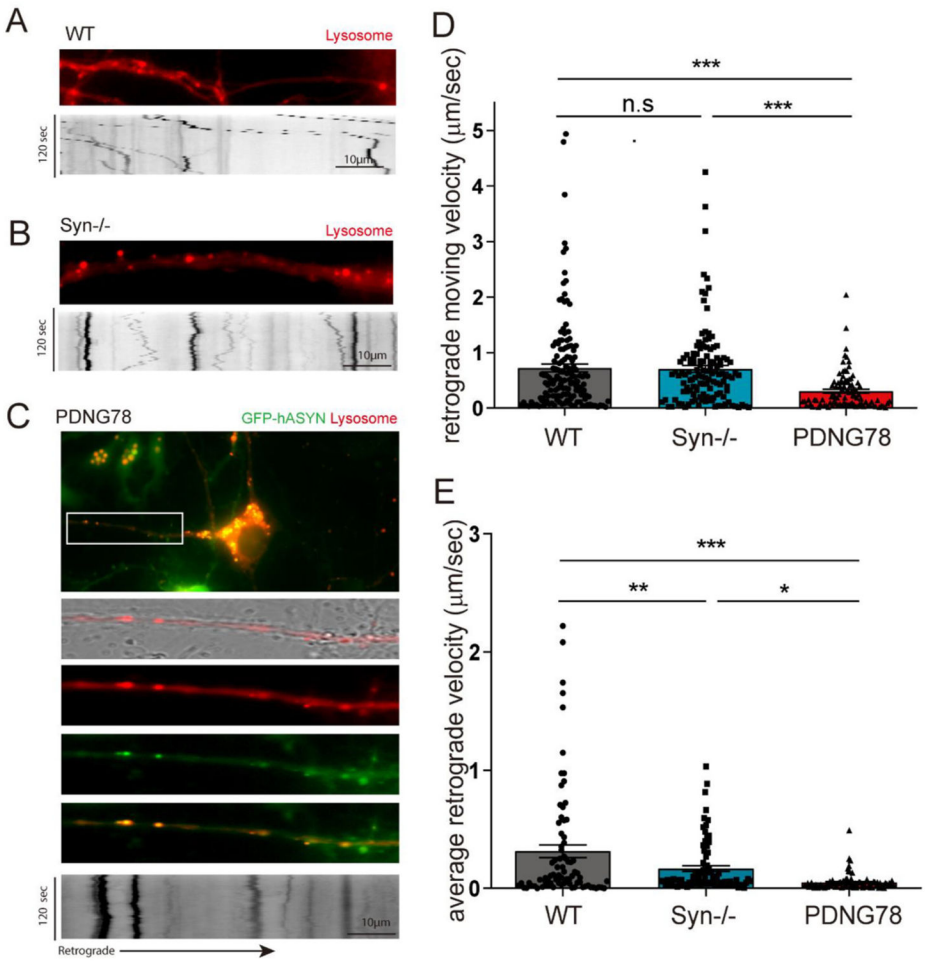

Fig. 4.

The overexpression of GFP-ASYN induced retrograde axonal transport deficits of lysosomes in E18 mouse cortical neurons. E18 cortical neurons from Line 78 (PDGF-β-ASYN-GFP) and ASYN-knockout mouse embryos were dissected and cultured in microfluidic chambers as described in Materials and Methods. At DIV14, neurons were treated with Lysotracker for 30 min, and axonal transport of lysosomes was captured by live imaging. Kymographs were generated from time-lapsed image series. Representative image of lysosome within axons of non-transgenic (A), ASYN-knockout (B) and PDNG78 (Syn-Tg) neurons (C) are shown. Axonal transport parameters: the retrograde moving velocity (D) and average velocity (E) are quantitated and presented. Data were obtained from 20 non-transgenic neurons, 20 ASYN-knockout neurons and 15 GFP-positive transgenic neurons. 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.

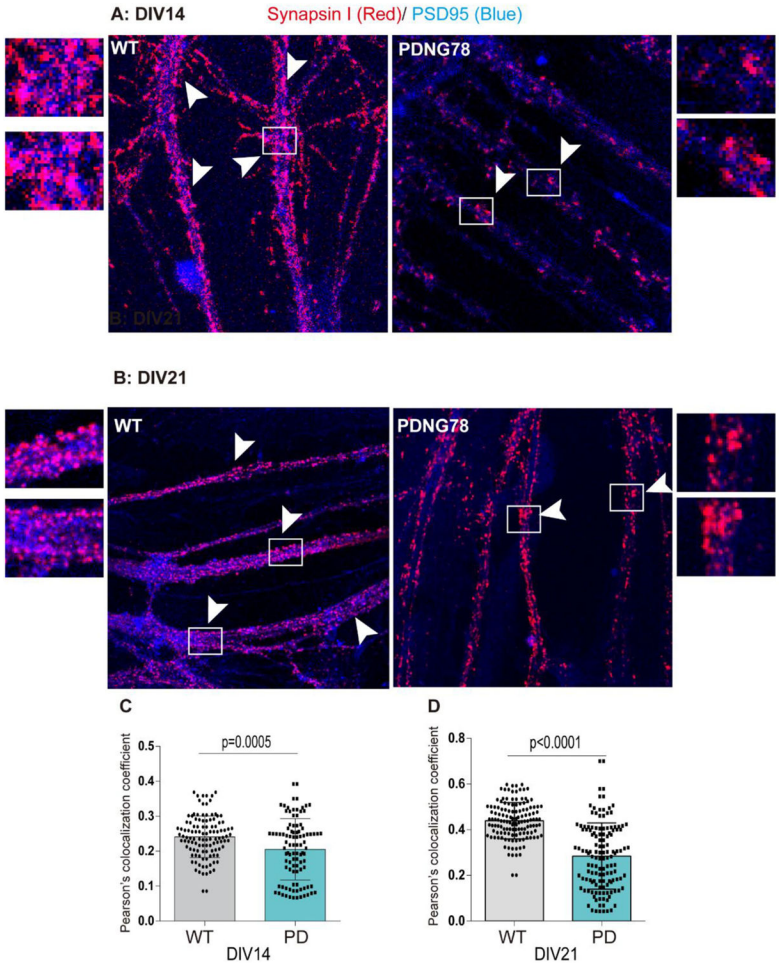

Fig. 5.

Effect of ASYN on 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 colocalization 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.

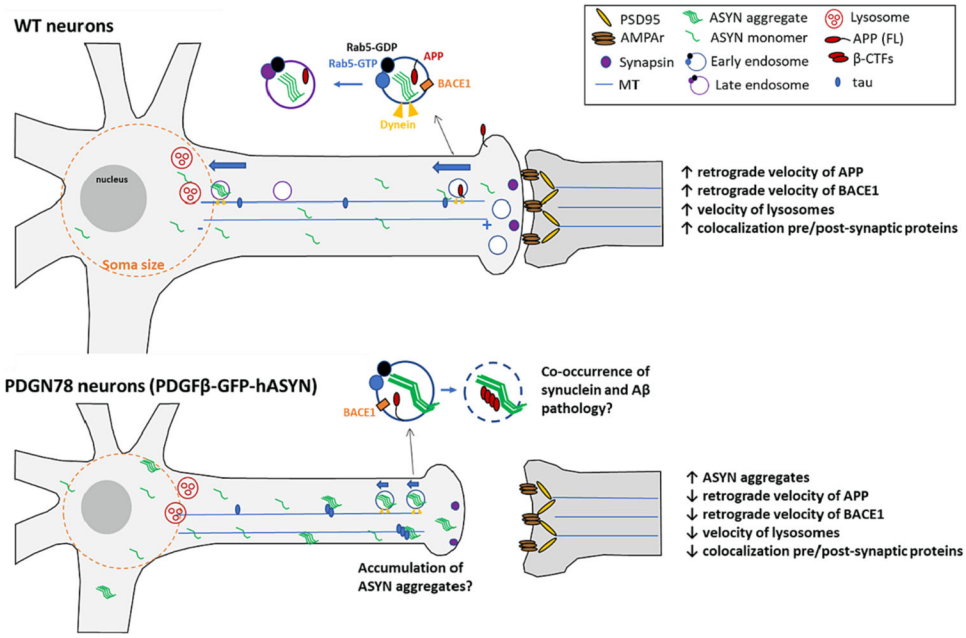

Fig. 6.

Proposed model of axonal transport impairment in PDNG78 synuclein-overexpressing neurons. (A) Schematical representation of retrograde axonal transport in WT neurons. In normal WT neurons, synapses are well established, with the co-localization of pre- and post-synaptic proteins (synapsin and PSD95 respectively). The endocytic pathway is involved in internalizing and recycling proteins from both the cell membrane (APP) and the presynapse, where ASYN is involved in neurotransmitter recycling. Eventual protein aggregates colocalize with early endosomes and transported retrogradely to the soma, where lysosomes are present. (B) In neurons overexpressing ASYN, retrograde transport of APP is slower. Retrograde transport and recycling of transport is slower, potentially accumulating within early endosomes, where APP resides and is processed. Slow transport also leads to endosomal enlargement and smaller soma sizes for PDNG78 neurons as shown in Fig. S5 and in previous publications (Fang et al., 2017).