Cellular and Genetic Studies of α-Synuclein Propagation in the *C. elegans* Neuronal Circuit.

July 2021

XUAN WANG

Cellular and Genetic Studies of α-Synuclein Propagation in the *C. elegans* Neuronal Circuit.

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
(Doctoral Program in Life Sciences and Bioengineering)

XUAN WANG

Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative disorder in aged people. The disease is characterized by the loss of dopaminergic neuron in substantia nigra pars compacta and the formation of Lewy bodies and Lewy neuritis in the brain, which are composed of aggregated α-synuclein. Previous studies have shown that α-synuclein has high potential for aggregation via its hydrophobic domain, and its oligomers seem to be much more toxic than the monomer. To explore the pathogenesis and therapeutic development of PD, many animal models have been generated, using mice, fishes, flies and worms. In several model systems, the injection of fibrillar α-synuclein or overexpression of wild-type or PD-linked mutant forms of α-synuclein causes neurodegeneration and motor defects, which are the most common clinical symptoms observed in patients with PD. Furthermore, several studies reported that over-expression of α-synuclein inhibits both synaptic vesicle exocytosis by reducing the SNARE complex assembly and synaptic vesicle endocytosis. However, the current understanding of the native functions of α-synuclein and molecular mechanisms of its *in vivo* assembly is still uncertain.

In addition to cellular toxicity, previous studies have revealed that α-synuclein can be propagated from cell-to-cell in a prion-like manner. In several models, aggregated α-synuclein has been shown to travel along connected neuronal pathways, through endo/exocytosis, lysosomal and

autophagic mechanisms. However, the exact mechanism of α-synuclein propagation among cells still remains unclear.

In this study, genetic and cellular analyses were performed to explore the physiological roles of α-synuclein and the mechanism of α-synuclein trans-synaptic propagation in the *Caenorhabditis elegans* neuronal circuit. Compared to other animal models, *C. elegans* has several distinct advantages including a simple nervous system, a well-defined neuronal circuit, and the encoded proteins in its genome are highly conserved with human. Moreover, it is more practical to perform genetic manipulation and live-cell imaging in *C. elegans*, which are crucial to understand α-synuclein functions in both cellular and individual levels as well as the mechanism of prion-like propagation.

To understand the physiological roles of α-synuclein in the nervous system, human α-synuclein tagged with GFP was expressed specially in the ASER sensory neuron, which has a restricted number of synaptic connections and a controllable neuronal activity. By examining the effects of α-synuclein on the morphology of ASER, I found that both wild-type and A53T form of α-synuclein strongly accumulated at presynaptic regions on the axon and accelerated the age-associated changes in cellular morphology. Because α-synuclein accumulated as dots on the axon, I further examined whether α-synuclein affects synapse formation or synaptic vesicle trafficking. No gross difference was observed in synapse formation between control and α-synuclein expressing transgenic worms. However, larger synaptic vesicle clusters occurred on the axon in α-synuclein worms compared to that in the

control worms. Considering the possibility that larger synaptic vesicle clusters may also be caused by enlarged presynaptic sites due to the expression of α-synuclein, the effect of α-synuclein on the active zone was also investigated. However, no significant difference was found in the size or quantity of active zone between the control and α-synuclein worms, indicating that α-synuclein induced abnormal synaptic vesicle accumulation without changing the architecture of active zones. Then, to understand why synaptic vesicles accumulate at presynaptic sites, calcium imaging was carried out to examine the influence of α-synuclein on neuronal activity. Results showed that both wild-type and A53T forms of α-synuclein induced a significant lower Ca^{2+} response to the sensory stimulus for the ASER excitation. To understand which molecules are affected by α-synuclein in neuronal Ca^{2+} response, I examined calcium responses in two mutant animals of voltage-gated calcium channels, *cca-1* and *unc-2*. Compared to reduced Ca^{2+} response in the GFP control worms, such reduced Ca^{2+} response caused by α-synuclein was not observed in either *cca-1* and *unc-2* mutants. These results suggested that the reduction of Ca^{2+} response in α-synuclein-expressing wild-type worms is probably caused by the suppression of CCA-1 and UNC-2 calcium channel function. Therefore, the accumulation of synaptic vesicles in the presynaptic sites of transgenic animals is likely caused by decreased neuronal activity due to α-synuclein expression.

On the other hand, in order to understand the propagation mechanism of α-synuclein, I generated transgenic *C. elegans* in which α-synuclein propagation via connected neurons can be visualized. A split GFP system was

adopted in which each GFP fragment was fused with α-synuclein, and then expressed fluorescence was examined by the interaction of two α-synuclein when they were in the same neuron. The sensory neuron ASER and its prominent presynaptic neurons ADL as well as the prominent postsynaptic neurons AIY were chosen to express α-synuclein, and two pairs of BiFC transgenic lines, the ADL-ASER pair and ASER-AIY pair, were generated based on the synaptic direction and the number of synaptic connections. I found that Venus BiFC fluorescence was observed in presynaptic neurons of both two pairs (ADLs or ASER), and a weak fluorescence was observed in the postsynaptic neuron (ASER) only in the ADL-ASER pair. These finding suggested that α-synuclein can be transferred into synaptically connected neurons in both anterograde and retrograde way, while the retrograde transmission seemed to be dominant. Further analyses were performed in the endocytic mutants *unc-57* (endophilin) and *dyn-1* (dynamin) to explore the molecular mechanism of cellular uptake for the retrograde propagation of α-synuclein. In *dyn-1(ky51)* mutant worms, the Venus fluorescence in presynaptic neuron was greatly reduced compared to that in the wild-type worms. However, the Venus fluorescence in *unc-57(ok310)* mutant animals was not affected, suggesting that dynamin rather than endophilin was required to α-synuclein uptake in presynaptic neurons. The results of endocytic mutants suggested that the classical clathrin-mediated endocytosis may not be involved in the α-synuclein propagation into presynaptic neurons. In summary, based on the established *C. elegans* model, this study has provided new evidences of physiological role and propagation mechanism of

the α-synuclein. α-Synuclein expression may decrease the neuronal activity, resulting in the accumulation of synaptic vesicles in the presynaptic sites of transgenic animals. Findings in this study suggest a possible mechanism of α-synuclein propagation via synapses with uncharacterized, novel release and uptake pathways.

Contents

Figure List

Chapter 1

General Introduction

1.1 Neurodegenerative Disorders

With extending human lifespan by better health care and living conditions, the prevalence of neurodegenerative disorders is dramatically increasing in recent years. Neurodegenerative disorders are characterized by the progressive degeneration or death of neuronal cells, and common neurodegenerative disorders are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and prion diseases. These major neurodegenerative disorders are strongly linked with age [1], and often cause serious problems with cognitive functioning (dementias) and movement (ataxias) in different degrees.

As the recent progression of brain researches, many similarities are found in the pathology among these neurodegenerative disorders. First, almost all common neurodegenerative disorders occur in both sporadic and familial forms; the former contains multiple genetic and environmental factors, and the latter is caused by gene mutations that is likely to give rise to the risk of disease onset [2]. As for sporadic neurodegenerative disorders, although it is known that pathological protein aggregates are present in the patients' brain, knowledges on the molecular etiology is quite limited. Second, neurodegenerative disorders have a common pathological mechanism:

disease-specific proteins undergo misfolding from a native state to aggregates and these aggregates exhibit cellular toxicity, leading to cellular dysfunctions and brain damages [3]. Because the pathological proteins are variable in each disorder, affected neuronal populations and impacted regions in brain vary (Figure 1), causing disease-specific pathological symptoms. Third, in most neurodegenerative disorders, the regions affected by pathological protein aggregates gradually expand in the brain with time (Figure 2) [4]. Several studies indicate that pathological proteins misfold and aggregate into seeds, causing pathogenic assemblies ranging from small oligomers to large masses of aggregates [5], and travels between neurons to induce disease progression [6]. Currently, as there are no proven treatments to prevent or cure these neurodegenerative disorders, deep understandings of the causes and mechanisms underlying disease onset seem to be extremely important. Research progress in any neurodegenerative disorders may provide insight into the possible mechanisms and methods of treatment for many of these ailments.

1.2 α-Synuclein and Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorders next to Alzheimer's disease. PD usually begins around age 60 and affects 0.3% in the general population. The main motor symptoms of PD are rest tremor, bradykinesia, rigidity, and postural instability (Figure 3). In the brain, progressive loss of dopaminergic neurons and the formation of Lewy bodies and Lewy neuritis (Figure 4) are recognized as the pathological

hallmarks of PD [7, 8]. The selective loss of dopaminergic neurons in the substantia nigra (Figure 5), which are functionally involved in body movement and reward behaviors as a part in the basal ganglia, leads to the reduction in dopamine secretion that contributes to the motor symptoms of PD. Lewy bodies are also seen in neurons in the cerebral cortex of the late stage of PD patients, suggesting the possibility that a prion-like propagation in the brain is associated with PD. Most cases of PD are sporadic while approximately 5% of PD are familial. In familial PD, mutations in the genes *SNCA, parkin, LRRK2, PINK1, GBA* have been identified to be causes for the disease onset [9-11]. At present, the exact etiologies of sporadic PD have not been understood yet. However, numerous studies have reported that environmental risk factors such as pesticide exposure, head injury [12] and genetic risk factors [11] are probably associated with PD.

In these situations, α-synuclein, which is a major component of Lewy bodies (LBs) and Lewy neurites (LNs), has been focused on the PD study [8]. α-Synuclein is closely linked to PD because duplications and triplications of the gene dosage can cause familial Parkinson's disease [13-16]. In addition, several substitutional mutations in α-synuclein are associated with parkinsonism, including A30P, E46K, H50Q, G51D, A53T and A53E (Figure 6), in which E46K, H50Q, and A53T α-synuclein have a higher aggregation propensity than the wild-type protein [17-24].

α-Synuclein is a 140-amino acid protein primarily localized in presynaptic terminals and is constituted of three domains (Figure 6). The N terminus (1–60 residues) contains the KTKEGV motif repeats allowing for the formation

of an alpha-helical structure [25]. The central hydrophobic domain known as the non-amyloid component (NAC) region (61–95 residues) is crucial for its aggregation [26, 27]. The C terminus (96–140 residues) is highly negative charged and unstructured. Based on the structure, α-synuclein has been reported to associate with membranes and result in pore formation in the membranes by its oligomerization. Although α-synuclein is thought to be natively unfolded and soluble, it has a propensity to form toxic soluble oligomers and ultimately aggregates into insoluble fibrils [28]. Both oligomers and fibrils have been shown to display a toxicity to the cell (Figure 7) [29, 30].

1.3 *C. elegans* is a valuable animal model for PD study

Caenorhabditis elegans is one of the useful animal models that can be used for studying the mechanisms involved in human diseases. This animal has some advantages compared to other models, including its transparency, fast life cycle, easy to create transgenic animals, and low cost of maintenance.

C. elegans is a species of nematode worm and grows to be 1-2mm in length as adults. Because *C. elegans* body is transparent, proteins of interest can be visualized in a live worm by fusing with fluorescent proteins, which is of benefit to confirm their localization and measure expression levels. *C. elegans* displays two sexes, male and hermaphrodite, and the latter is able to self-fertilize. The adult hermaphrodite has 959 cells in total and contains 302 neurons in which all connections have been mapped by electron micrograph analyses [31, 32]. Additionally, the genome of *C. elegans* has been fully sequenced and about 80% of genes are homologues to human genes [33].

Deletion and point mutants can be obtained from the *Caenorhabditis Genetics Center*, which is a central repository for *C. elegans* strains. Also, it is easy to generate transgenic animals by a microinjection within one month. Furthermore, it is possible to combine double mutants or transgenic animals with the mutant background to examine interaction of each other. It can be achieved by only one cross using male animals and then self-fertilizing to generate homozygous worms with both phenotypes. The combination of these properties makes *C. elegans* as a valuable model organism to study human diseases.

As for the PD research, *C. elegans* has been successfully used as a valuable animal model. *C. elegans* has several genetic components related to the Parkinson's disease. Since the orthologue of α-synuclein is not found in *C. elegans* genome, it is favorable to use *C. elegans* as a model organism to study the effects of α-synuclein by expressing it in the animals without having to consider the effects of background endogenous α-synuclein expression.

1.4 Goals

The goals of this study are to:

1. Examine the influence of α-synuclein on the neurons in *C. elegans*.
2. Identify the molecules altered by α-synuclein expression for the activity in *C. elegans* neuronal cells.
3. Examine the relationship with synaptic direction and strength on α-synuclein propagation.
4. Identify the molecules involved in the α-synuclein propagation process in *C. elegans*.

In Chapter 2, I explored the events occurred in the *C. elegans* nervous system due to the expression of α-synuclein, to reveal how α-synuclein does influence on the neuronal functions at the synapse (referred to the goals 1 and 2). In Chapter 3, I generated a *C. elegans* model to explore the mechanism of α-synuclein propagation in the *C. elegans* neuronal circuits, and examined the association with the synaptic connectivity (referred to the goals 3 to 4).

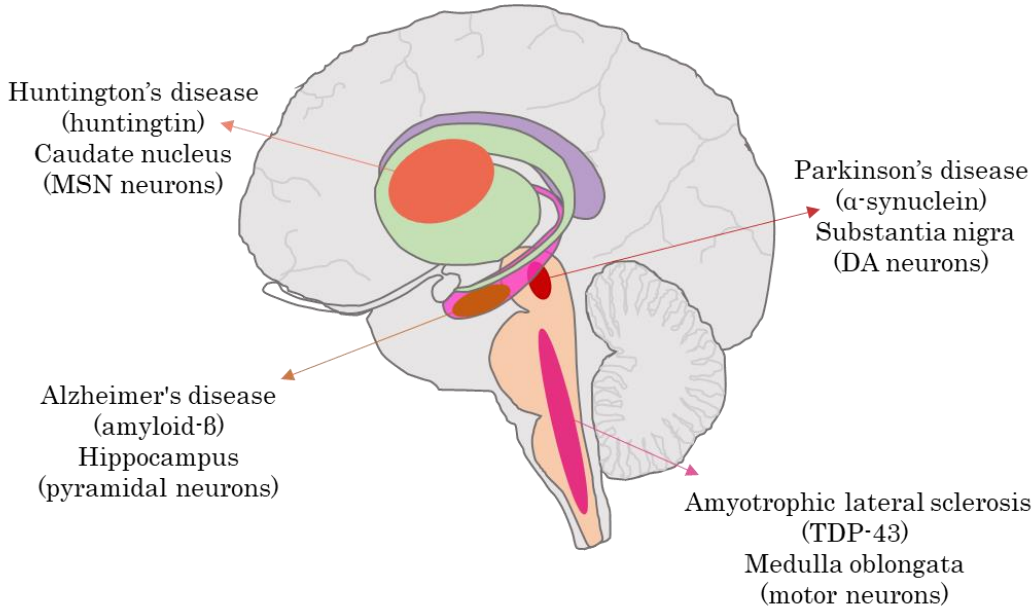


Figure 1. Schematic representation of vulnerable regions in neurodegenerative diseases.

Huntington's disease is caused by the mutation in the huntingtin gene and the caudate nucleus is the primary affected region. In Parkinson's disease, pathological aggregation of α-synuclein is commonly observed in the brain and the dopaminergic neurons of substantia nigra are particularly vulnerable. Alzheimer's disease is caused by the deposition of amyloid-β peptide as plaques in the brain and hippocampus is especially vulnerable to the damage. Amyotrophic lateral sclerosis (ALS) is characterized by motor neuron damage in the bulbar region and TDP-43 has been identified as the major pathological protein in ALS. Adapted from Fu et al. (2018).

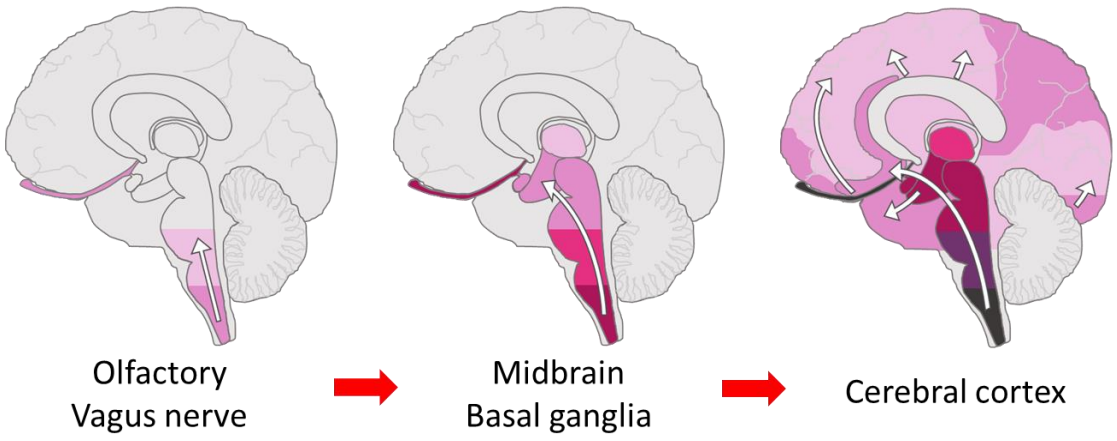


Figure 2. Spreading of pathology in neurodegenerative diseases.
The progression of α-synuclein inclusion (Lewy body) in the brain of patients with Parkinson's disease over time. Adapted from Jucker and Walker (2013),

Figure 3. Motor symptoms of Parkinson's disease.

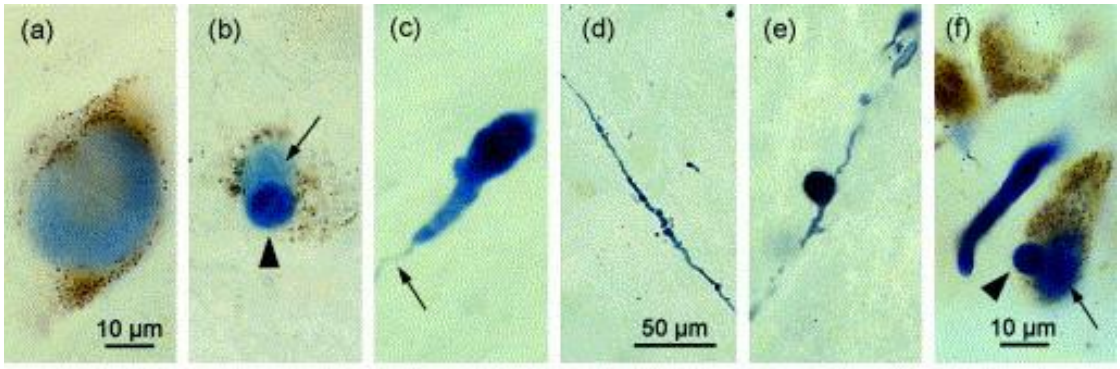


Figure 4. PD-related brain lesions.

The figure is cited from Braak et al. (2003).

Intraneuronal lesions in the form of (a) a large pale body, (b and f) a combination of a pale body (arrows) and a small LB (arrowheads) in melanized projection cells of the substantia nigra. (c) A thread-like LN (arrow) terminating in a club-shaped enlargement, (d) a filiform LN decorated with small spine-like appendages with or (e) a large globular swelling, as well as (f) coarse plump LNs. Bar in (a) is valid for (b and c), bar in (d) is also valid for (e). Cited from Braak et al. (2003) with the permission.

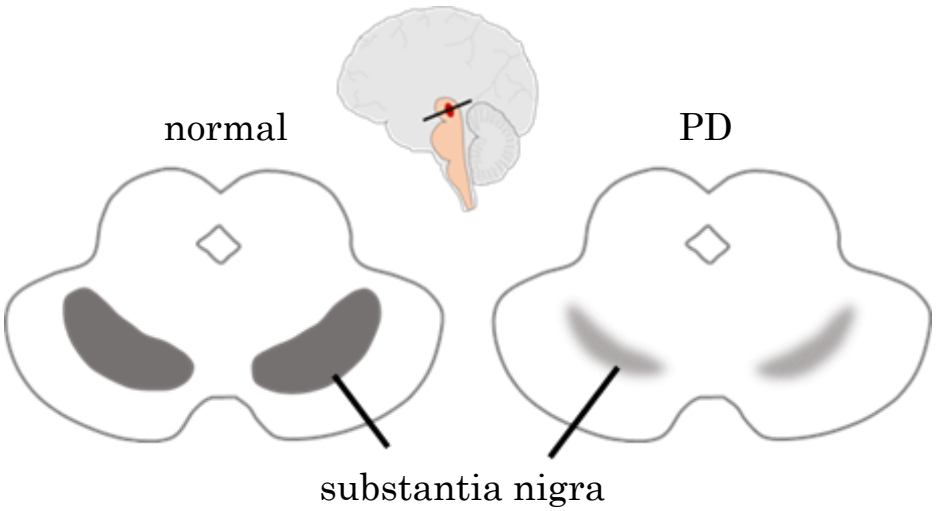


Figure 5. The loss of dopaminergic neurons in the substantia nigra of patients with Parkinson's disease.

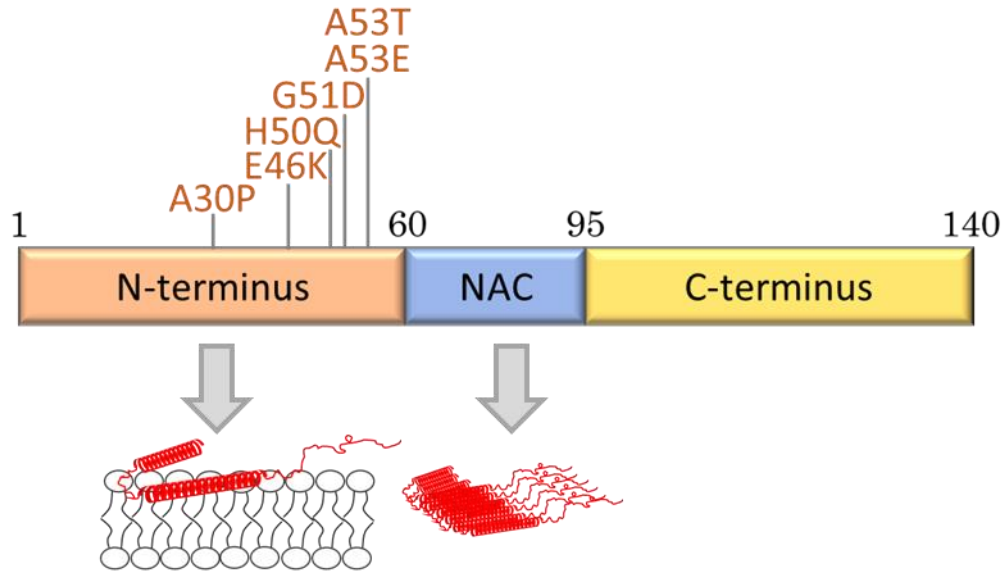


Figure 6. The protein structure of α-synuclein.

The N-terminal region and the non-amyloid-component (NAC) domain contribute to membrane binding and aggregation, respectively. The mutations linked to the familial Parkinson's disease (A30P, E46K, H50Q, G51D, A53E, and A53T) are located in the N-terminal region.

Figure 7. Representative intracellular targets of α-synuclein toxicity.
Pore formation: α-synuclein oligomers are proposed to form pore-like structures that could act as non-selective channels in the plasma membrane, resulting in dramatic perturbations of membrane permeability. ER stress: cellular accumulation of misfolded proteins can lead to chronic endoplasmic reticulum stress. α-synuclein can associate with the ER membrane and cause ER stress by altering intracellular protein traffic, synaptic vesicles transport, and Ca^{2+} homeostasis. Mitochondrial dysfunction: α-synuclein can interact with mitochondria and induce mitochondrial fragmentation.

Chapter 2

The physiological functions of α-synuclein in *C. elegans* neuronal system

2.1 Introduction

α-Synuclein is a major component of Lewy bodies, and mutations in the α-synuclein gene have been suggested to be associated with Parkinson's disease. The presynaptic location of synuclein suggests a role in synapse such as regulator of neurotransmitter release. There have been many attempts to understand the role of α-synuclein in vivo by using knockout mice and transgenic animals over-expressing human α-synuclein.

Abeliovich and colleagues reported that α-synuclein-knockout mice are viable, exhibit normal brain architecture and the dopamine neurons appear morphologically indistinguishable from wildtype mice [34]. However, the knockout animals exhibit altered paired stimulus depression when neurons are stimulated with two paired electrical pulses (Figure 8). In wildtype mice, the amount of DA released is less after the second stimulus than after the first one. However, in α-synuclein-knockout mice DA neurons released more DA after a second electrical pulse and accelerated the recovery of DA release following the initial stimulus, indicating that α-synuclein might be a negative regulator of activity-dependent DA release. Additionally, DA content was significantly reduced in the striatum of α-synuclein-knockout mice, and this

reduction may result from decreased DA synthesis or storage [34]. These findings indicate that α-synuclein might be a regulator of a form of DA plasticity, PSD, as well as a regulator of DA release, synthesis or storage.

Transgenic mice overexpressing human α-synuclein exhibit age-dependent loss of dopamine neurons and motor defects similar to the common clinical symptoms observed in patients with PD (Figure 9) [35-37]. One of the studies showed that neuronal loss was observed independently of inclusions, suggesting neurodegeneration is not driven by aggregate formation [36]. Furthermore, it has been reported that modest over-expressing α-synuclein in cultured neurons and the neurons of transgenic mice inhibits synaptic vesicle exocytosis which may result from the specific reduction in size of the synaptic vesicle recycling pool and reducing synaptic vesicle density at the active zone (Figure 9) [38]. In addition, mice over-expressing synuclein show reductions in synapsin [38]. Recent work also suggests that α-synuclein facilitates synaptic vesicle clustering and attenuates recycling (Figure 9), which require synapsin [39], suggesting the inhibition of synaptic vesicle exocytosis by α-synuclein. Moreover, an new evidence shows that α-synuclein-VAMP2 interactions are necessary for α-synuclein-induced synaptic attenuation [40], supporting an effect of α-synuclein on the inhibition of synaptic vesicle release. However, another seemingly divergent view is also important to note. Burre and colleagues found that α-synuclein directly binds to VAMP2 and promotes SNARE-complex assembly (Figure 9) [41]. The exact mechanism by which α-synuclein affects neurotransmitters release remains unclear and controversial.

Since *C. elegans* does not have a homolog to α-synuclein, human α-synuclein have been expressed in several tissues (Figure 10). Because the loss of dopamine neurons and the aggregation of α-synuclein are the hallmarks of PD, many studies focus on measuring the survival of dopamine neurons and α-synuclein aggregation in *C. elegans*. By taking advantage of a transparent body and cell-type specific promoter of *C. elegans*, neuron survival can be assessed in live animals by expressing a fluorescent protein in specific neurons. Expression of human α-synuclein in the dopaminergic neurons and pan-neuron induces dopaminergic neuronal and dendritic loss. Motor deficits were also observed when α-synuclein was overexpressed in pan-neuron and motor neuron [42]. To visualize aggregation, α-synuclein has been fused to fluorescent protein and expressed in body wall muscle cells which are large and better for detecting the subcellular localization of aggregation. The progressive, age-dependent accumulation of aggregations were observed in α-synuclein expressing body wall muscles, which is consistent with the pathological inclusions in PD patients [43]. *C. elegans* also has been used to study other genetic and environmental factors and has been utilized to the incorporation of genetics and environment or chemical compound to understand the molecular basis and search for therapeutic agents of PD.

Considering the subcellular localization of α-synuclein, it can be supposed that α-synuclein may be involved in synaptic function, suggesting the importance of detailed analysis at synaptic sites. However, due to the complex neural networks of vertebrata brain, the analysis could be disturbed by many factors and be difficult to look into the events in synaptic sites. Therefore, *C.*

elegans model provides a good opportunity for studying the function of α-synuclein in synaptic sites, such as the relatively simple and well-defined structure of nervous system and physiological methods for monitoring neural activity in vivo. In this study, a new transgenic *C. elegans* model was developed, which is available to express and monitor α-synuclein in a sensory neuron and analysis its influence on synaptic and neuronal activity (Figure 11).

2.2 Materials and methods

2.2.1 *C. elegans* strains

All *C. elegans* strains were grown on standard Nematode Growth Medium agar plates seeded with *Escherichia coli* (OP50) at 20°C. The N2 Bristol strain was used as the wild-type strain. The mutant lines *cca-1(ad1650)* and *unc-2(e55)* were acquired from the Caenorhabditis Genetics Center.

2.2.2 Plasmid construction

For the expression of α-synuclein or other synaptic marker proteins in the ASER neuron, a 3 kb upstream region from the ATG start site of the *gcy-5* gene was inserted between the SphI and SmaI sites of pPD95.77 plasmid DNA. To express α-synuclein in ASER neuron and visualize its localization, the human α-synuclein sequence (wild type or A53T) and GFP sequences were inserted in the frame downstream of the *gcy-5* promoter.

To visualize presynaptic sites of the ASER neuron, the mCherry sequence and the full-length r*ab-3* genomic DNA sequence were inserted in the frame downstream of the *gcy-5* promoter (P*gcy-5*::mCherry::*rab-3*).

To visualize the active zone, the P*gcy-5*::mCherry::*syd-2* plasmid was generated by amplifying the *syd-2* genomic DNA sequence from the pJH23 plasmid (kindly provided by Prof. Mei Zhen) and then replacing the *syd-2* sequence with *rab-3* sequence in the above P*gcy-5*::mCherry::*rab-3* DNA by an infusion cloning reaction (Takara).

For generation of transgenic *C. elegans* to visualize the localization of α-synuclein and neuronal morphology, plasmid DNA encoding P*gcy-5*::α-synuclein (wild-type)::GFP or P*gcy-5*::α-synuclein (A53T)::GFP or P*gcy-5*::GFP (as control) (each 25 ng/ul), P*gcy-5*::mCherry (25 ng/ul), and the marker P*lin44*::mCherry (40 ng/ul) were co-injected into the gonads of young adult worms.

For calcium imaging, a red-fluorescent calcium sensor R-GECO was used [44]. The R-GECO sequence was amplified and inserted between the KpnI and EcoRI sites of the pPD95.77 vector, and the *gcy-5* promoter sequence was inserted upstream of R-GECO.

Each of the three plasmid DNA (P*gcy-5*::mCherry::*rab-3*, P*gcy-5*::mCherry::*syd-2* and P*gcy-5*::R-GECO are 30 ng/ul, 30 ng/ul and 50 ng/ul, respectively) was co-injected with P*gcy-5*::α-synuclein (wild-type)::GFP or Pgcy-5::α-synuclein (A53T)::GFP or Pgcy-5::GFP (each 25 ng/ul) into the gonads of young adult worms with a selection marker P*lin44*::tdTomato (20 ng/ul), to generate transgenic line expressing the RAB-3 or SYD-2 or R-GECO with α-synuclein.

2.2.3 Transgenic animals

DK5522 *taEx [Pgcy-5:: GFP; Pgcy-5:: mCherry; Plin44:: mCherry]*

DK5525 *taEx [Pgcy-5:: α-syn (wild-type):: GFP; Pgcy-5:: mCherry; Plin44:: mCherry]*

DK5526 *taEx [Pgcy-5:: α-syn (A53T):: GFP; Pgcy-5:: mCherry; Plin44:: mCherry]*

DK5527 *taEx [Pgcy-5:: GFP; Pgcy-5:: mCherry:: rab-3; Plin44:: tdTomato]*

DK5602 *taEx [Pgcy-5:: α-syn (wild-type):: GFP; Pgcy-5:: mCherry:: rab-3; Plin44:: tdTomato]*

DK5528 *taEx [Pgcy-5:: α-syn (A53T):: GFP; Pgcy-5:: mCherry:: rab-3; Plin44:: tdTomato]*

DK5633 *taEx [Pgcy-5:: syb-2; Pgcy-5:: mCherry:: syd-2; Plin44:: tdTomato]*

DK5634 *taEx [Pgcy-5:: α-syn (wild-type):: GFP; Pgcy-5:: mCherry:: syd-2; Plin44:: tdTomato]*

DK5635 *taEx [Pgcy-5:: α-syn (A53T):: GFP; Pgcy-5:: mCherry:: syd-2; Plin44:: tdTomato]*

DK5529 *taEx [Pgcy-5:: GFP; Pgcy-5:: R-GECO; Plin44:: tdTomato]*

DK5530 *taEx [Pgcy-5:: α-syn (wild-type):: GFP; Pgcy-5:: R-GECO; Plin44:: tdTomato]*

DK5531 *taEx [Pgcy-5:: α-syn (A53T):: GFP; Pgcy-5:: R-GECO; Plin44:: tdTomato]*

DK5617 *cca-1(ad1650); taEx [Pgcy-5:: GFP; Pgcy-5:: R-GECO; Plin44:: tdTomato]*

DK5616 *cca-1(ad1650); taEx [Pgcy-5:: α-syn (wild-type):: GFP; Pgcy-5:: R-GECO; Plin44:: tdTomato]*

DK5615 *cca-1(ad1650); taEx [Pgcy-5:: α-syn (A53T):: GFP; Pgcy-5:: R-GECO; Plin44:: tdTomato]*

DK5612 *unc-2(e55); taEx [Pgcy-5:: GFP; Pgcy-5:: R-GECO; Plin44:: tdTomato]*

DK5613 *unc-2(e55); taEx [Pgcy-5:: α-syn (wild-type):: GFP; Pgcy-5:: R-GECO; Plin44:: tdTomato]*

DK5614 *unc-2(e55); taEx [Pgcy-5:: α-syn (A53T):: GFP; Pgcy-5:: R-GECO; Plin44:: tdTomato]*

2.2.4 Microscopy

Worms in M9 solution containing NaN3 (100 mM) were mounted onto slides with 2% agarose pads and covered with a coverslip. All images were obtained using an Olympus FV1000 confocal laser scanning microscope with a 60×x objective lens. RAB-3 puncta were scored as abnormal when any large dots were observed somewhere on the axon.

2.2.5 Calcium imaging

For the imaging of calcium responses, 5-day-old adult transgenic worms were used. The worms were immobilized on a microfluidic device fabricated from polydimethylsiloxane. An inverted fluorescent microscope (Olympus IX71) with ORCA-Flash 4.0 CCD camera (Hamamatsu Photonics) and HCImage software installed were used to capture time-lapse images. Images were captured at a rate of 10 fps within 3 min after the removal of food. The stimulation process and data analysis were according to a previous study (Kuramochi and Doi, 2017). Furthermore, $\Delta F/F_0 = (F-F_0) / F_0$ was calculated to represent the fluorescence intensity change, where F_0 is the average fluorescence in a 10 sec window before stimulation. Fmax represents the

maximum fluorescence intensity. The total fluorescence intensity was measured from cell body of the ASER neuron after background subtraction.

2.2.6 Statistical analyses

The statistical analyses of data from morphological change were performed using Chi-square test and data from calcium imaging were carried out using the ANOVA test followed by a Tukey's multiple comparisons test using GraphPad Prism 7. The data were regarded as statistically significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.3 Results

2.3.1 α-Synuclein expression causes age-related morphological changes in neuronal cells

To understand the effect of α-synuclein expression on the neuronal functions, I expressed α-synuclein in ASER neuron (Figure 12A) and analyzed its influence related to presynaptic functions. To investigate the influence of α-synuclein on ASER, I generated transgenic *C. elegans* by expressing wild-type or mutant form (A53T) of α-synuclein fused with GFP, or GFP only as a control (Figure 12B and 13). Previous studies have shown that the degeneration of dopaminergic neurons was accelerated by the expression of α-synuclein in *C. elegans* [45, 46]. I compared morphological features of GFP control worms to α-synuclein expressing worms in ASER, including the integrity and the guidance of dendrite, cell body and axon. I found that while α-synuclein accumulated as dots on the axon, especially in aged animals (Figure 13), α-synuclein worms did not show significant neurodegeneration such as loss of structure, guidance defects or cell death compared to GFP control worms. Although α-synuclein worms exhibited the neuronal integrity, several age-related changes in neuronal morphology were accelerated. In the nervous system of *C. elegans*, aged worms exhibit physiological changes including branching from the cell body and axon, as well as blebbing and beading along the dendrite and axon (Figure 14) [47-49]. Representative images of the changes in α-synuclein worms are shown in Figure 14A. Morphological changes such as cell-body branching, axon branching and

dendrite blebbing, increased with age, along with expression both wild-type and A53T mutant α-synuclein compared to control worms at the same stage (Figure 14B).

2.3.2 α-Synuclein expression induces age-dependent accumulation of synaptic vesicles

Because α-synuclein is known to localize at the presynaptic region of *C. elegans* neurons and α-synuclein puncta were observed along the axon of the ASER neuron, I further assessed the effect of α-synuclein on the presynapse. To visualize the ASER presynaptic sites, a presynaptic protein RAB-3 was fused with mCherry and co-expressed with α-synuclein (WT)::GFP or α-synuclein (A53T):: GFP or control GFP in ASER neuron. RAB-3 is a Ras-related protein localized to presynaptic vesicles (Figure 15A) [50], which adopts a punctate pattern of localization along the axon. L4 and 5-day-old adult animals were visualized (Figure 15B and 16) and assessed for defects in RAB-3 localization and distribution on axons, including in the number of RAB-3 puncta and their sizes. Both wild-type and A53T α-synuclein showed a high degree of colocalization with RAB-3 puncta (Figure 15B and 16), suggesting that α-synuclein in the ASER neuron also localizes to the presynaptic region. I found no changes in number of RAB-3 puncta in wild-type and A53T α-synuclein worms, suggesting that α-synuclein did not affect synapse formation. At the L4 stage, although the number of both wild-type and A53T mutant α-synuclein-expressing animals with larger RAB-3 punctate was slightly increased, no significant difference was observed

compared to GFP-expressing control worms (Figure 15B and 17). However, on day-5, RAB-3 puncta were significantly larger in wild-type and A53Tα-synuclein animals (Figure 16). Therefore, I scored the animals with the larger size of RAB-3 puncta in the axon and statistic results showed that the quantity of animals with larger size RAB-3 puncta was significantly increased in α-synuclein worms (Figure 17). It is considered that synaptic vesicles were abnormally accumulated at synapses due to the expression of α-synuclein. However, considering that the larger RAB-3 puncta may also be caused by enlarged presynapse due to the expression of α-synuclein, I also investigated the active zone to determine whether the size of the presynapse is altered in the α-synuclein-expressing animals. SYD-2 is required for synaptic active zone assembly in *C. elegans* [51], which localizes strictly in active zones and can be used as an active zone marker (Figure 18A) [52]. To visualize the active zones in ASER, I expressed the mCherry::SYD-2 fusion protein under the control of the *gcy-5* promoter. No significant difference was observed in the size and quantity of SYD-2 fluorescence between the control and α-synuclein animals on both L4 and day 5 (Figure 18B). Altogether, these data suggest that α-synuclein induces abnormal synaptic vesicle accumulation at presynaptic sites in an age-dependent manner without changing the presynaptic architecture.

2.3.3 Age-dependent calcium channel dysfunction by α-synuclein expression

To explore the reason why synaptic vesicles accumulate in presynaptic sites, I focused on neuronal activity in the ASER neuron. Calcium imaging is a suitable technique to monitor activity of living cells in *C. elegans* and has been used as an indicator of neuronal activity in several neurons [53-55]. Since the ASER neuron is excited by the decrease in NaCl concentration [56], I applied the stimulation of NaCl concentration from 50mM to 0mM (Figure 19B) to transgenic animals expressing R-GECO protein (Figure 19A) [44] and α-synuclein specifically in the ASER neuron. When worms are exposed to 0mM NaCl, ASER excitement causes the Ca^{2+} concentration to increase in the cytoplasm, which leads to an increase in RFP fluorescence. The RFP fluorescence intensity of cell body was measured to estimate the changes in cytoplasmic Ca^{2+} concentration. The traces of mean fluorescent intensity increase rate and statistical analysis are shown in Figure 20. At L4 stage, no significant difference weas observed in α-synuclein worms compared to the control animals expressing only GFP (Figure 21A). However, at day 5 both wild-type and A53T α-synuclein-expressing worms showed significantly reduced fluorescence intensity with a decrease in NaCl concentration (Figure 21B). Approximately 20–50% reduction in Ca^{2+} response was observed in day-5 animals, indicating that reduced Ca^{2+} influx was induced by α-synuclein expression. To identify molecules responsible for this reduced Ca^{2+} signaling by α-synuclein, I examined the calcium response of voltage-gated calcium channels (VGCC) mutants. *C. elegans* has three genes encoding VGCC α1

subunits, *egl-19*, *cca-1*, and *unc-2*, expression of which contributes towards configuration of the L-, T-, and N, P/Q, R-type of VGCC (Figure 22A), respectively [57-59]. Since *egl-19* is expressed and functions in muscle cells [57], I selected two VGCC mutant *cca-1(ad1650)* [60] and *unc-2(e55)* [61], and crossed them into the transgenic lines expressing R-GECO protein and α-synuclein. The recordings were made using the same protocol as for the original transgenic lines. As expected, both mutant animals showed reduced responses to the decrease in NaCl concentration in control GFP animals (Figure 22B to 24). Compared to the significant reduction in Ca^{2+} response in the α-synuclein-expressing wild-type animals, both *cca-1(ad1650)* and *unc-2(e55)* mutant animals expressing wild-type or A53T mutant α-synuclein did not show a significant reduction in Ca^{2+} response (Figure 24). These data suggest that both channels are involved in the reduction of the Ca^{2+} response caused by α-synuclein molecules. Therefore, lower Ca^{2+} responses in α-synuclein-expressing animals may cause an accumulation of synaptic vesicles in the presynapse.

2.4 Discussion

To understand the role of α-synuclein in vivo, multiple transgenic models over-expressing or directly injecting human α-synuclein were produced. Recent studies showed that overexpression of α-synuclein resulted in degeneration of dopaminergic neurons in several mouse models [36, 62]. As a *C. elegans* model, Lasko et. al. reported that overexpression of α-synuclein in dopaminergic neurons induces the neuron loss [45]. However, in this study, the ASER neuron overexpressing α-synuclein did not exhibit overt degeneration such as neuronal loss in any ages but showed only age-dependent mild morphological changes. As shown in several studies, this study also supported that α-synuclein probably has a strong toxicity specifically in dopaminergic neurons. Although the native function of α-synuclein in the nervous system has not been elucidated yet, its presynaptic localization suggests a role in neurotransmitter release. In several models, overexpression of synuclein showed an inhibitory effect on neurotransmitter release [63, 64]. Furthermore, a recent study showed that α-synuclein directly interacts with the vesicle-associated membrane protein VAMP2 and facilitates SV clustering and α-synuclein–induced synaptic attenuation [40]. In this study, I also observed lager clustering of RAB-3 that is indicative of excess synaptic vesicle accumulation or clustering at synapse by the α-synuclein expression. Because the ASER neuron seems to be a glutaminergic neuron, this kind of α-synuclein's role in synaptic vesicle release may not be specific in dopaminergic neurons. In addition, I found that α-synuclein decreased Ca^{2+} response by blocking the activity of voltage-activated Ca^{2+}

channel, resulting in decreased neuronal activity. Therefore, I propose that α-synuclein may decrease the frequency of synaptic vesicle fusion by inhibiting the neuronal activity via voltage-gated Ca^{2+} channels. On the other hand, α-synuclein is known to trigger the increase of cytosolic Ca^{2+} by inducing Ca^{2+} release from ER [65, 66]. Since Ca^{2+} from the ER may be involved in synaptic neuropeptide release [67], there is a possibility that α-synuclein may play a role in regulating the release of synaptic vesicle and dense core vesicle ,both of which are triggered by activity-induced Ca^{2+} entry, inhibiting synaptic vesicle release and enhancing dense core vesicle release. Although this study has not shown whether the inhibition of Ca^{2+} channel is a direct or indirect effect of α-synuclein, further analyses will identify the exact roles of α-synuclein in cellular Ca^{2+} signaling. Nevertheless, by using advantages of *C. elegans* genetics, this model system also helps to identify the molecular machinery for synaptic-connection-independent retrograde propagation of pre-fibril α-synuclein.

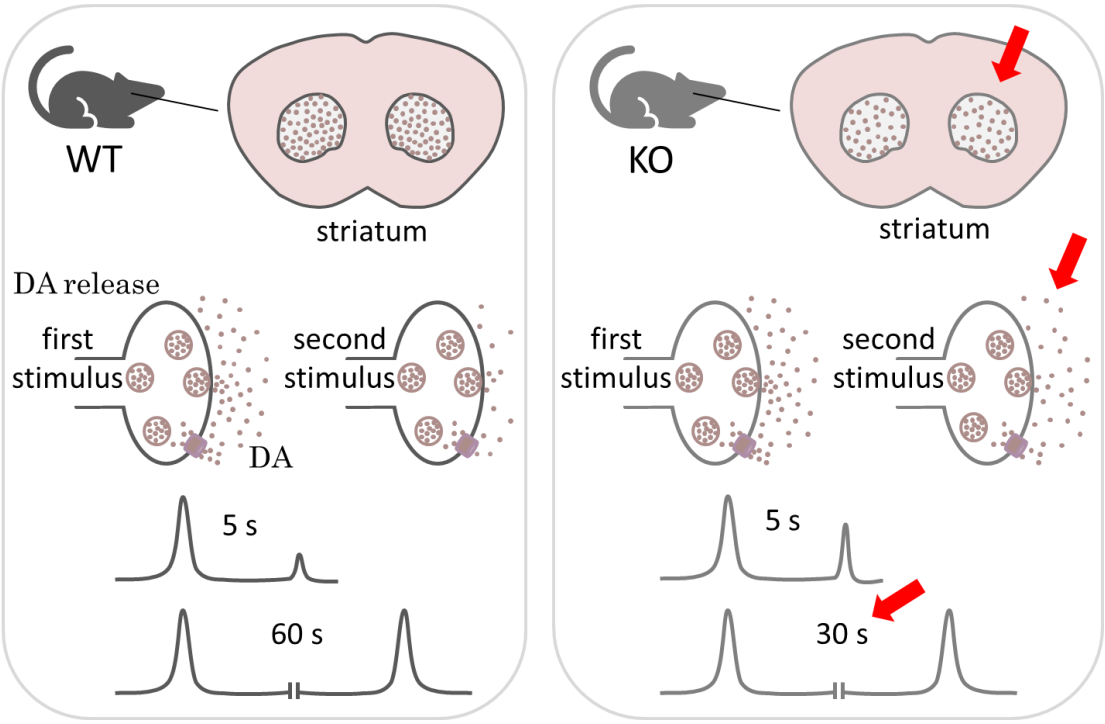


Figure 8. Comparison of DA content and synaptic plasticity between wild-type and α-synuclein-knockout mice.

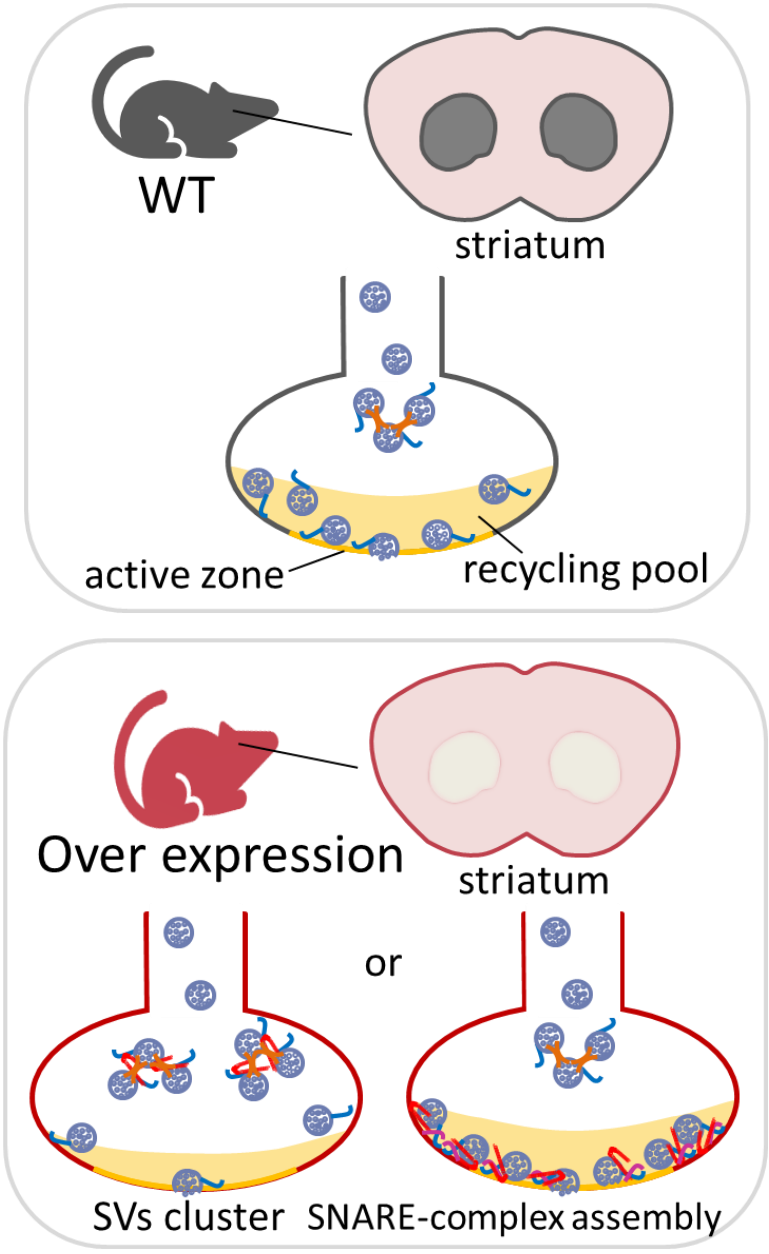


Figure 9. Comparison of DA neurons and synaptic vesicle features between wild-type and human α-synuclein transgenic mice.

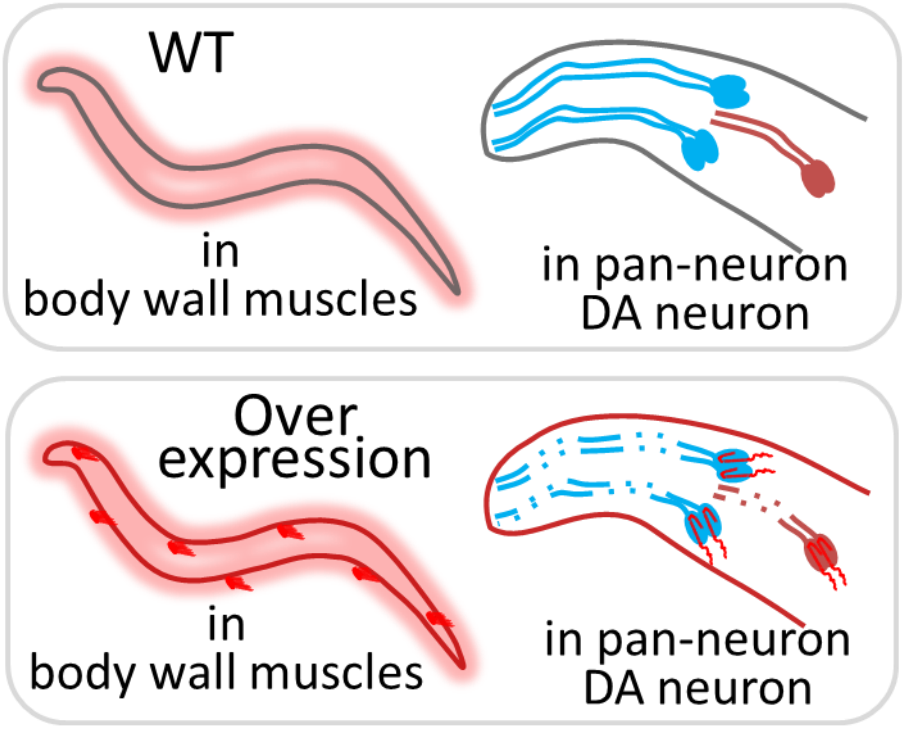


Figure 10. Comparison of body-wall muscles and DA neuron morphology between the wild-type and human α-synuclein transgenic worms.

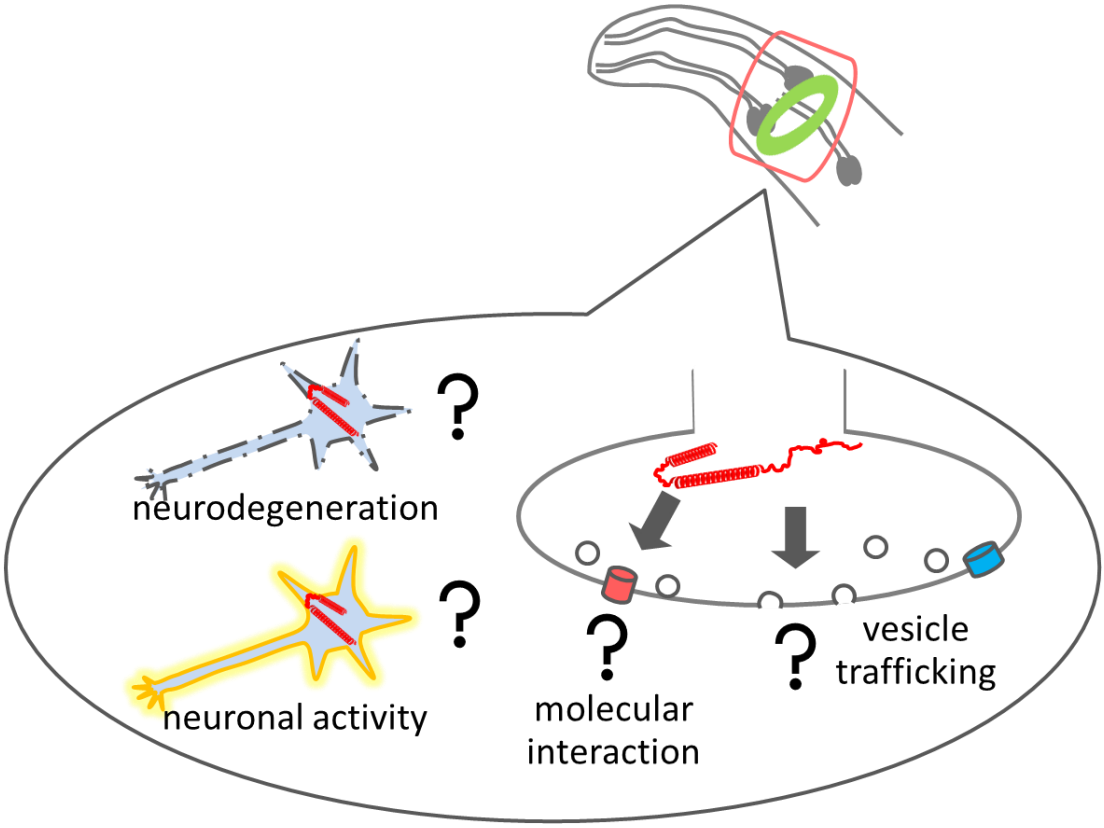
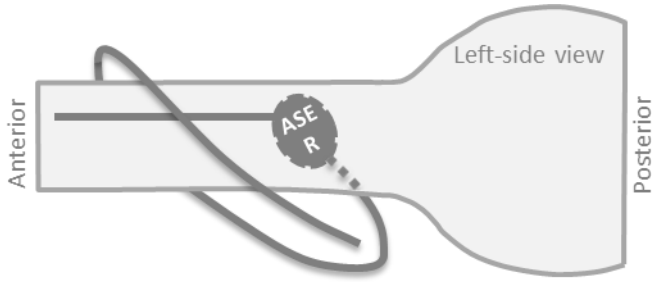


Figure 11. The possible functions of α-synuclein.

A



B

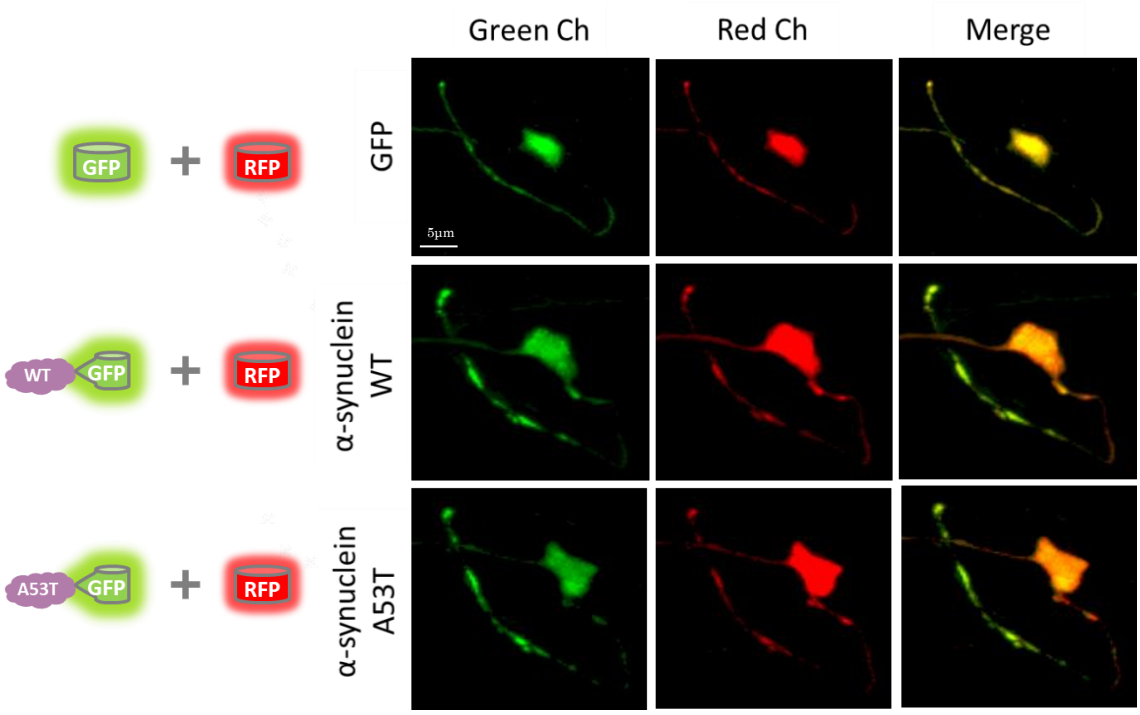


Figure 12. Expression patterns of α-synuclein in the ASER neuron of young animals.

(A) Schematic representation of the position and morphology of ASER. (B) Expression and localization patterns of the GFP, wild-type α-synuclein, and A53T mutant α-synuclein in the ASER neuron. All images are from L4 stage worms co-expressing mCherry. Anterior is left and dorsal is top. Arrows indicate the dotted accumulations of α-synuclein on the axon.

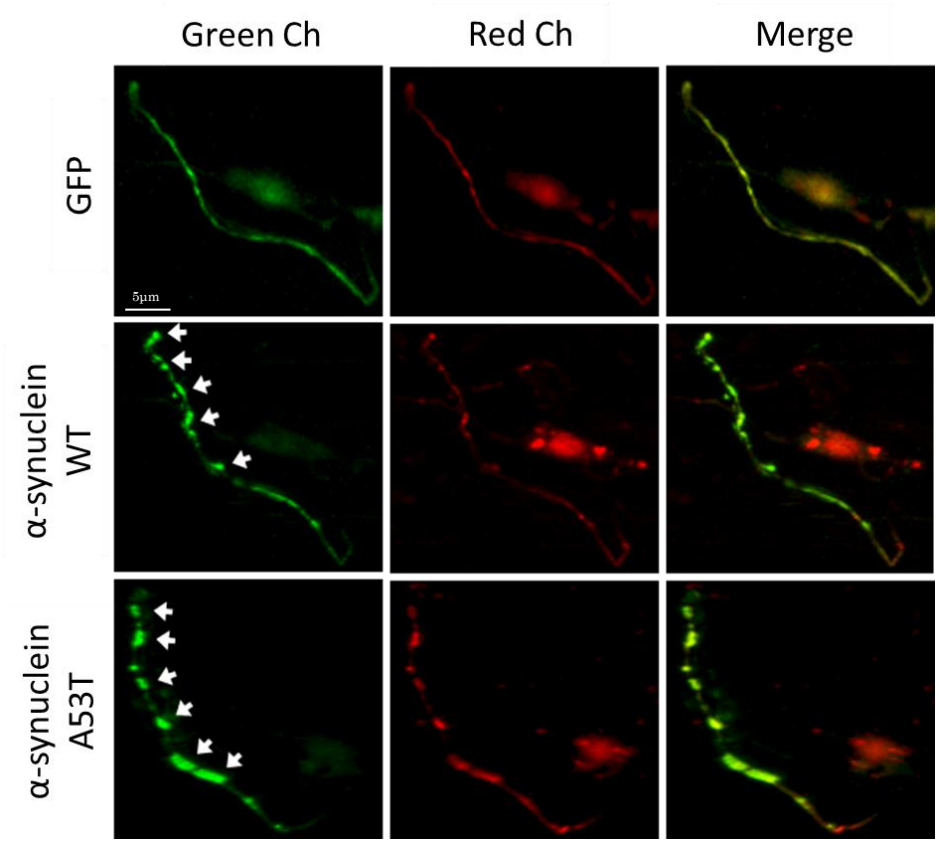
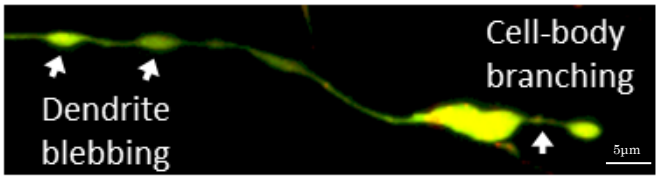


Figure 13. Expression patterns of α-synuclein in the ASER neuron of aged animals.

Expression and localization patterns of the wild-type or A53T mutant α-synuclein in the ASER neuron. All images are from 5-day-old worms co-expressing mCherry. Anterior is left and dorsal is top. Arrows indicate the dotted accumulations of α-synuclein on the axon.

A



B

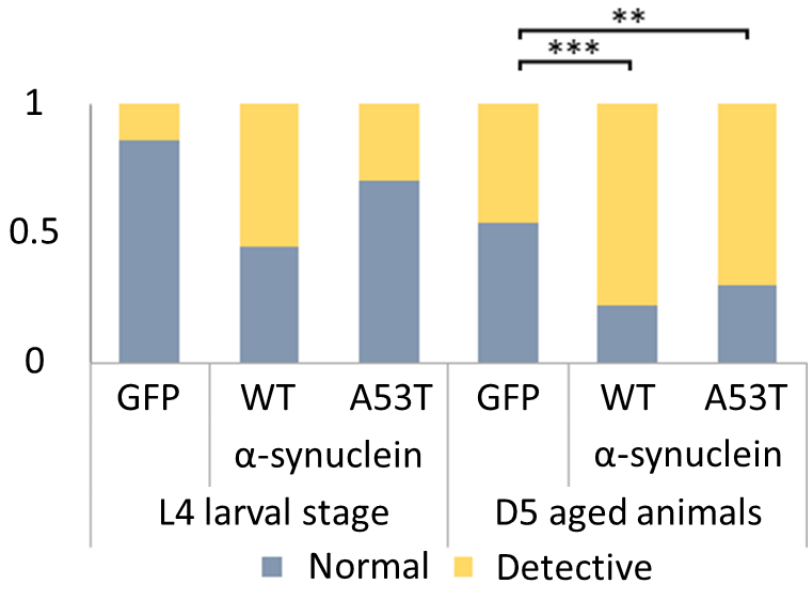
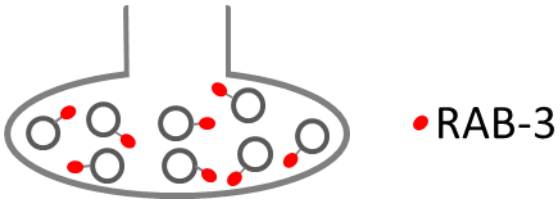


Figure 14. Effect of α-synuclein expression on neuronal morphology.
(A) Examples of morphological change in the ASER neuron of a 5-day-old animal by the expression of α-synuclein. Arrows point out the blebbed dendrite and additional cell-body branching, respectively. (B) Quantification of the neuronal morphological changes in worms expressing α-synuclein. Animals expressing GFP only was used as control. 'Defective' animals were counted if either dendrite blebbing or cell-body branching was observed. Statistical significance was determined by Chi-square test, ** $P < 0.01$ and *** $P < 0.001$. n = 22 - 51 animals for L4 stage, and n = 62 - 79 animals for Day5 stage.

A



B

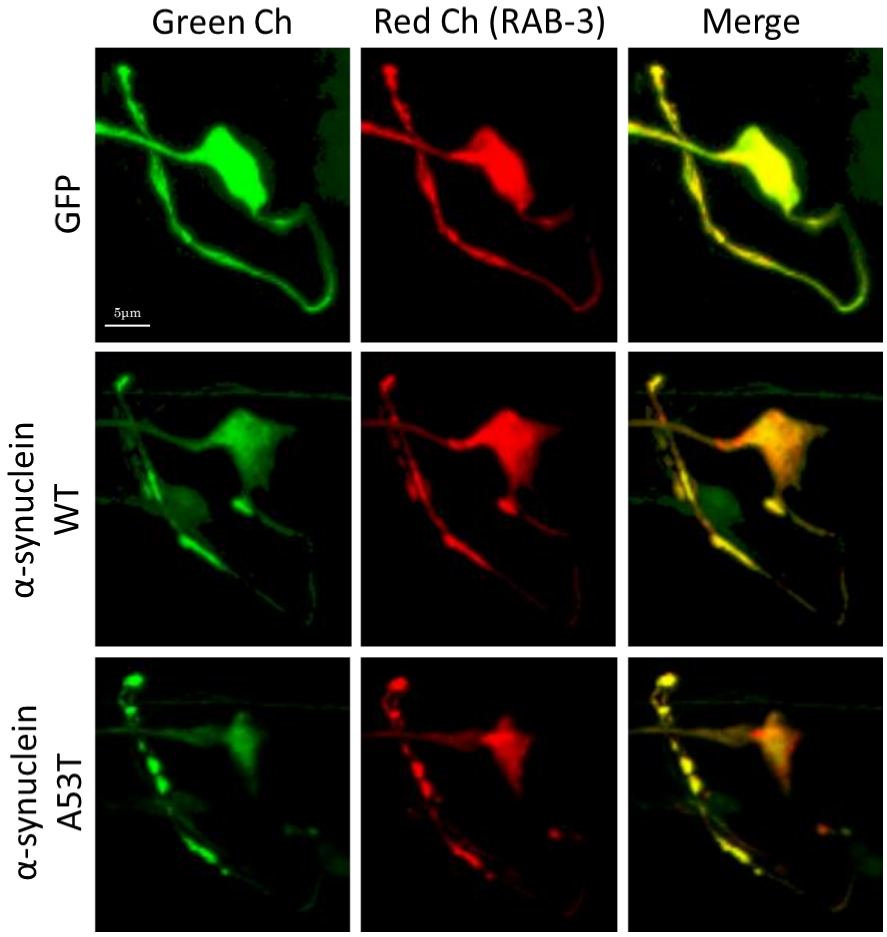


Figure 15. Effect of α-synuclein expression on synaptic vesicle accumulation in young animals.

(A) Schematic representation of RAB-3 protein in presynaptic sites. (B) Confocal micrograph images of ASER presynaptic sites labeled with the synaptic vesicle marker mCherry::RAB-3 in GFP and α-synuclein worms at L4 stage.

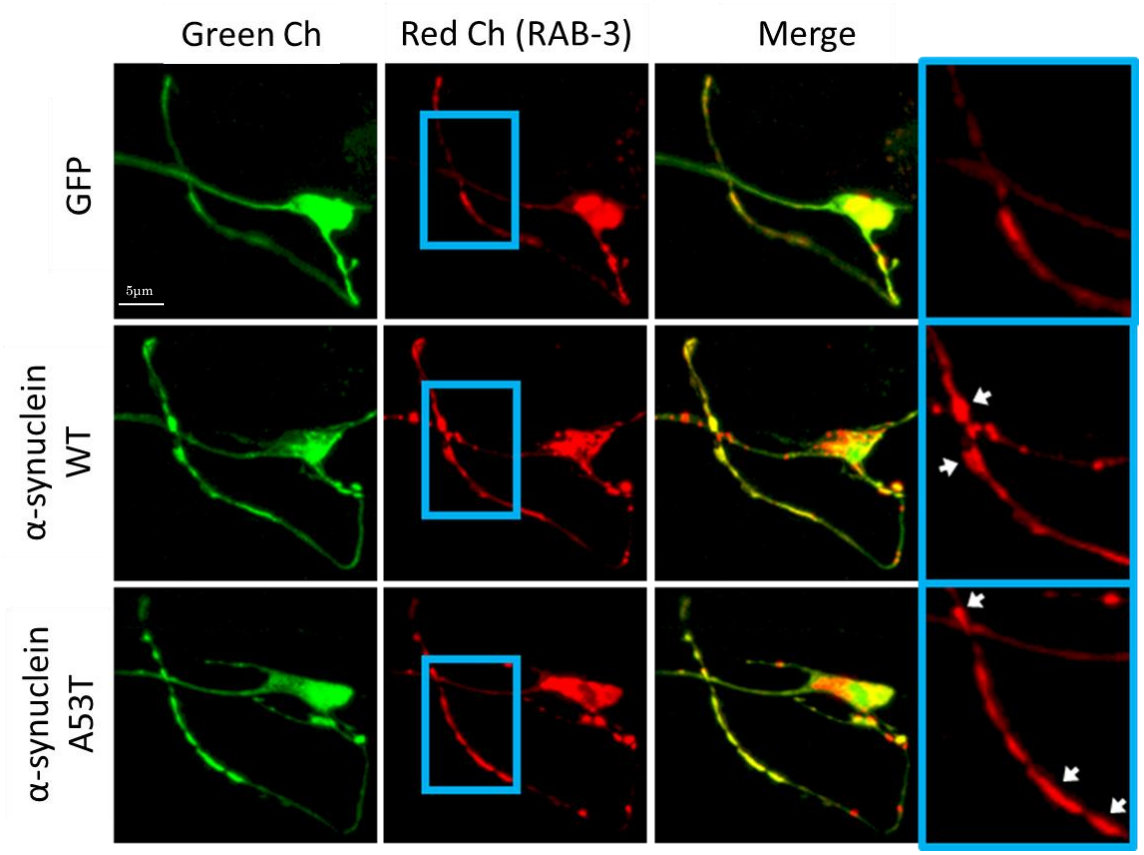


Figure 16. Effect of α-synuclein expression on synaptic vesicle accumulation in aged animals.

Confocal micrograph images of ASER presynaptic sites labeled with the synaptic vesicle marker mCherry::RAB-3 in GFP and α-synuclein worms at day 5. Blue squires Arrows show enlarged mCherry::RAB-3 puncta observed on the axon in α-synuclein-expressing worms.

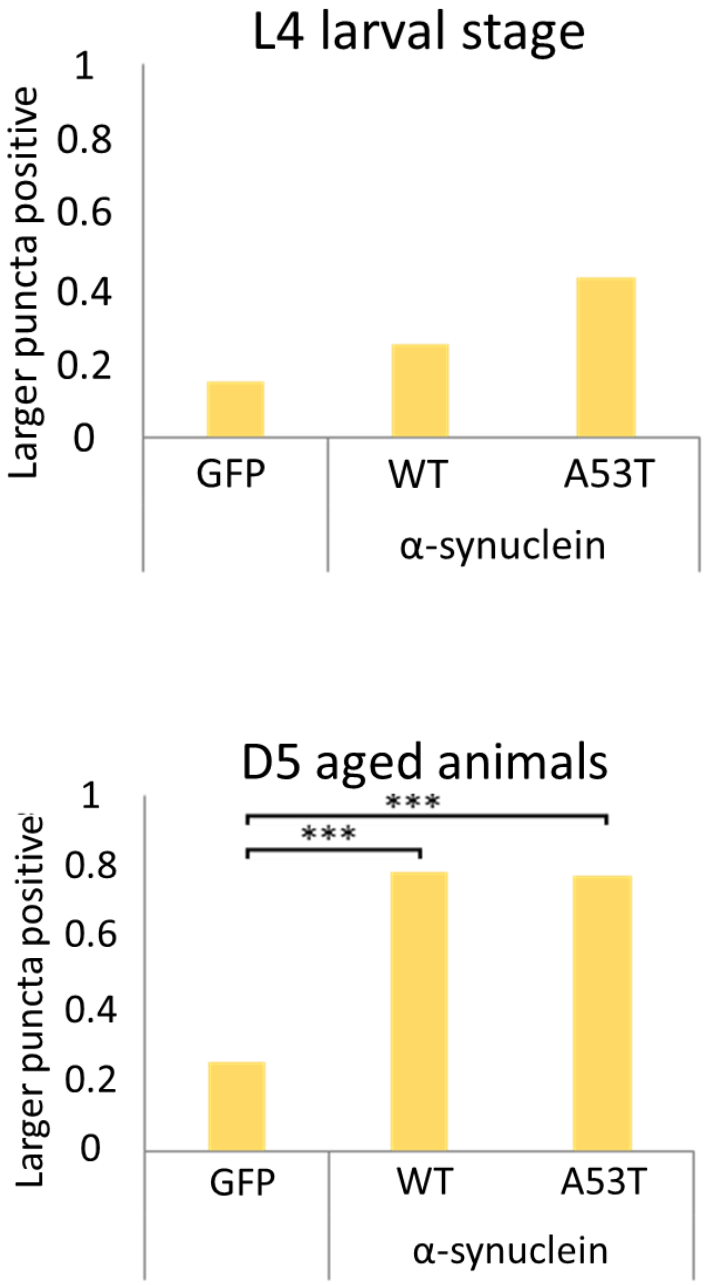


Figure 17. Quantification of large mCherry::RAB-3 puncta in α-synuclein transgenic worms.

Frequency of the larger mCherry::RAB-3 puncta in L4 (upper) and 5-day-old (below) worms. Statistical significance was determined by Chi-square test, *** $P < 0.001$. L4: GFP, n = 14- 31.

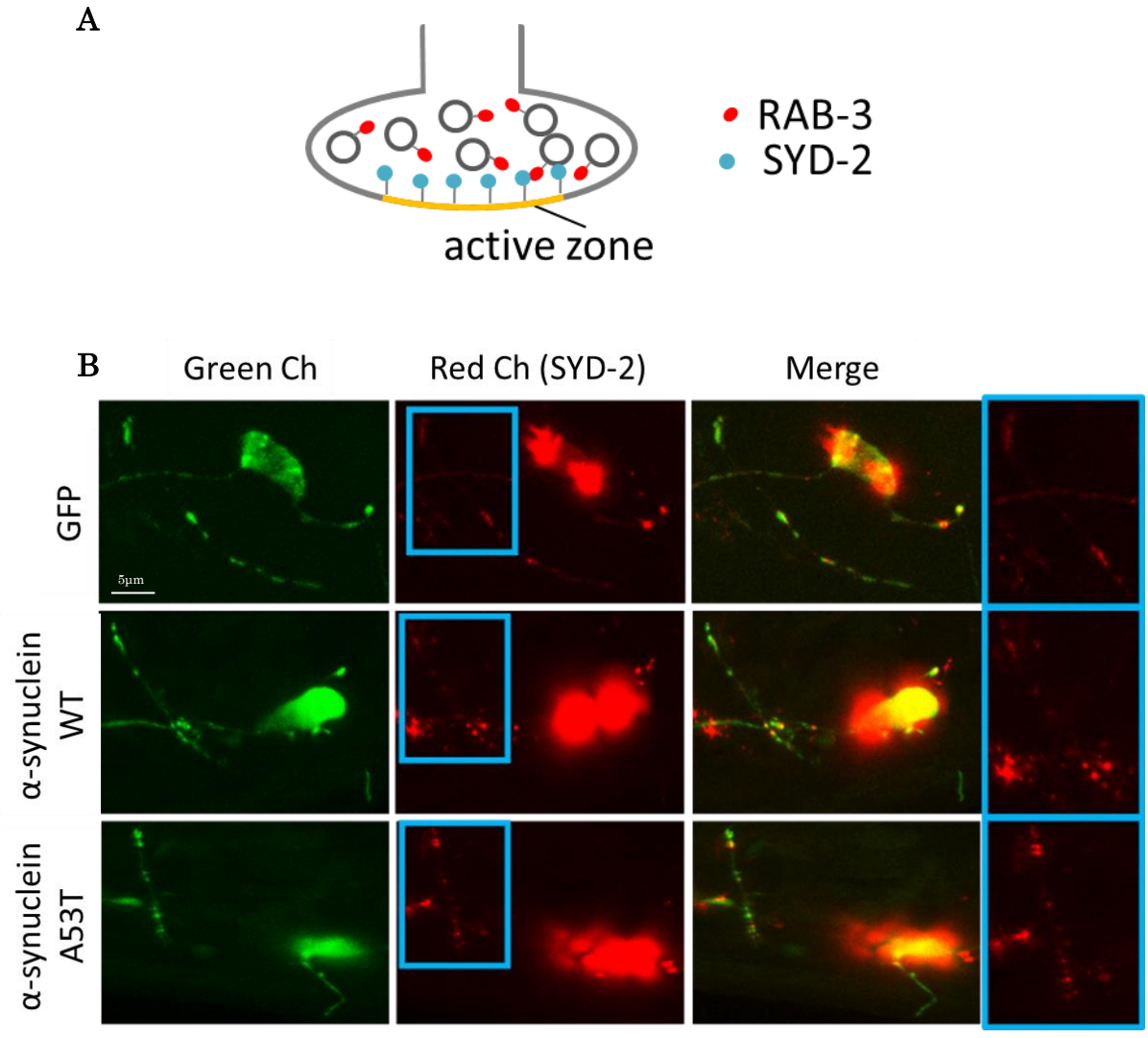
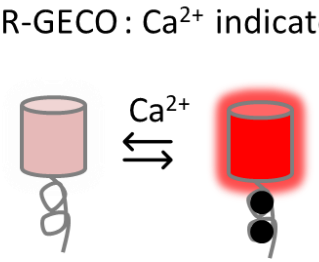


Figure 18. Effect of α-synuclein expression on the activity zone formation.
(A) Schematic representation of SYD-2 and active zone in presynaptic sites. (B) Confocal micrograph images of ASER active zone labeled with active zone protein mCherry::SYD-2 in GFP and α-synuclein worms at day 5.

A



B

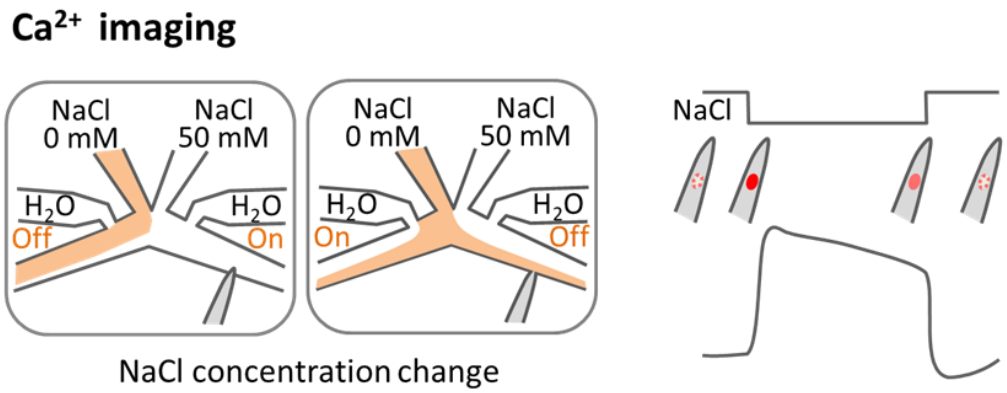


Figure 19. Schematic representation of R-GECO and calcium imaging in the ASER neuron.

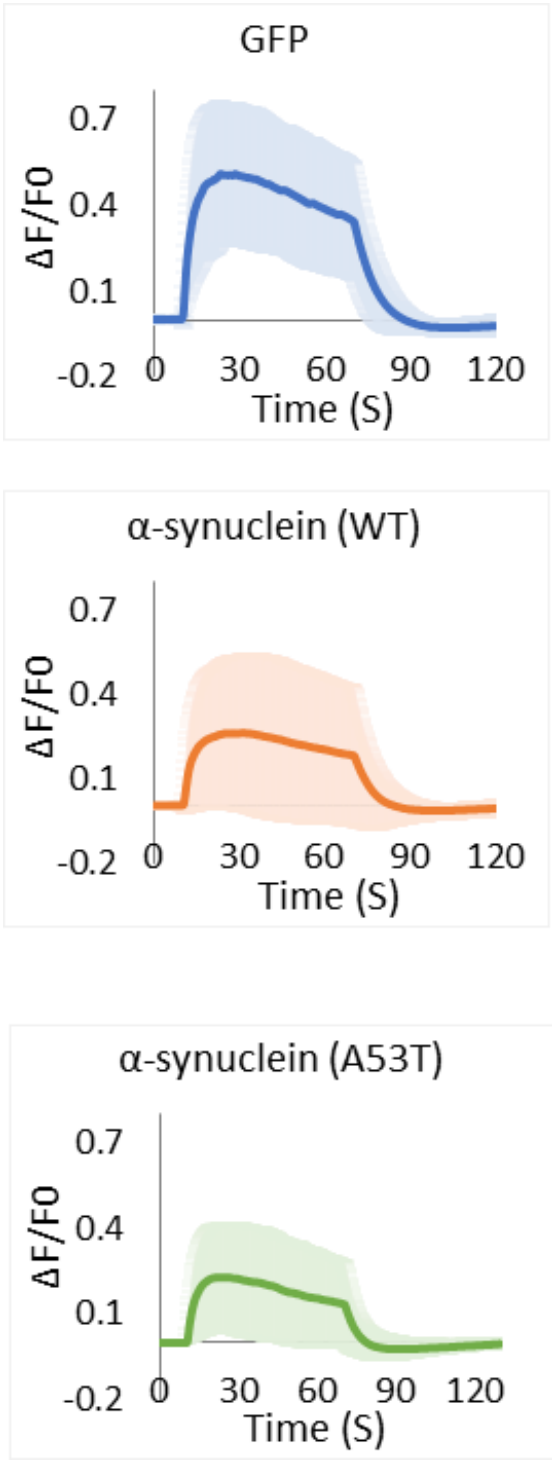
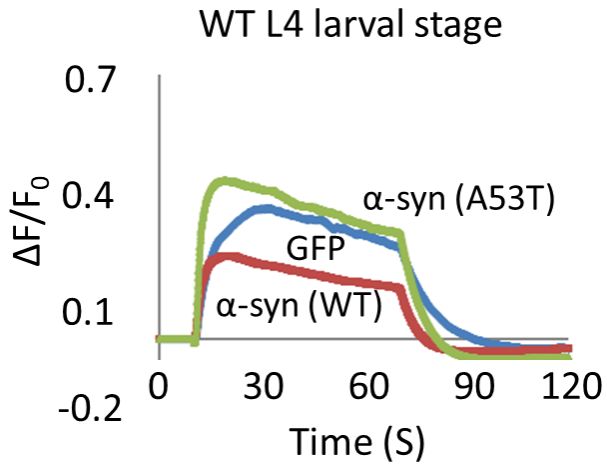


Figure 20. Effect of α-synuclein expression on calcium response in the ASER neuron.

Normalized calcium dynamics in the cell body of ASER neuron for GFP and α-synuclein worms at day 5. 10 − 70s time period denotes the stimulation of reducing NaCl concentration from 50 mM to 0 mM, and 70 − 130s time period denotes the NaCl concentration recovers back to 50 mM. Solid lines represent average data, and the lightly colored region around each line shows the SEM.

A



B

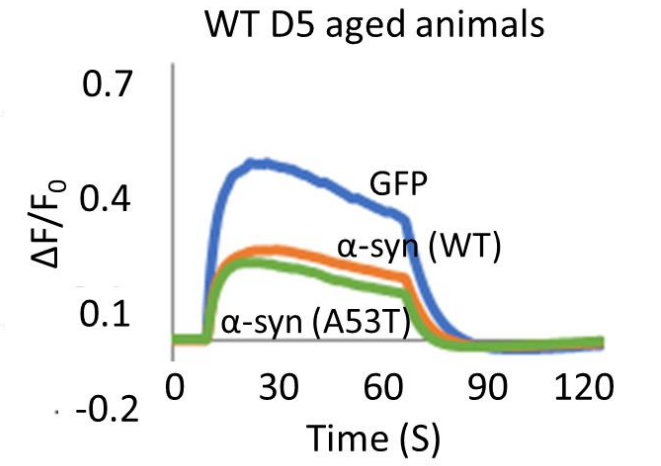
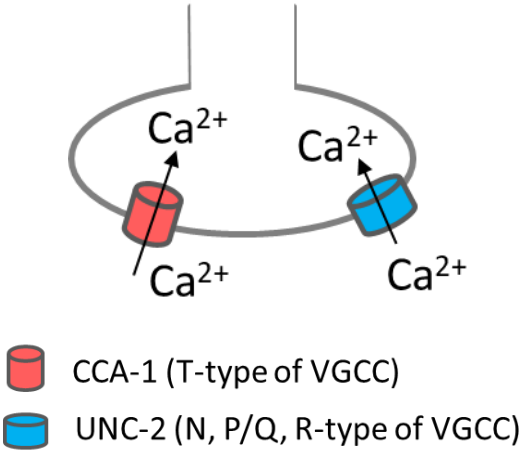


Figure 21.α-synuclein expression alters calcium response in the ASER neuron with age.

Normalized calcium dynamics in the cell body of ASER neuron at L4 stage (A) and day 5 (B) worms expressing α-synuclein at day 5. Blue, red and green lines indicate the dynamics in GFP (control), wild-type and A53T α-synuclein, respectively.

A



B

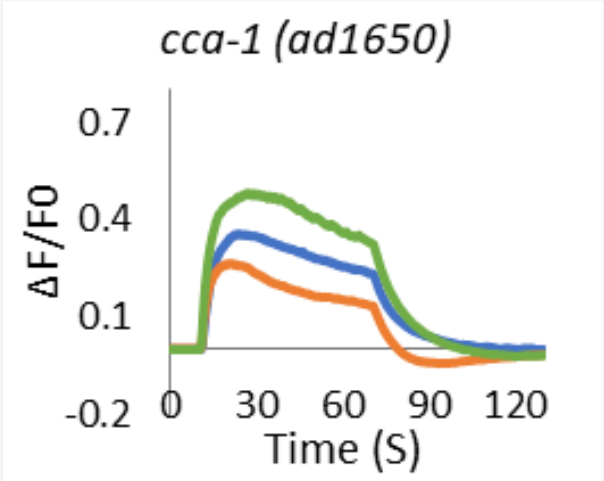


Figure 22. Effect of α-synuclein expression on the calcium response in the *cca-1* mutant animals.

(A) Schematic representation of CCA-1 and UNC-2 calcium channels in presynaptic sites. (B) Normalized calcium dynamics in the cell body of ASER neuron for *cca-1* mutant worms expressing α-synuclein at day 5. Blue, red and green lines indicate the dynamics in GFP (control), wild-type and A53T α-synuclein, respectively.

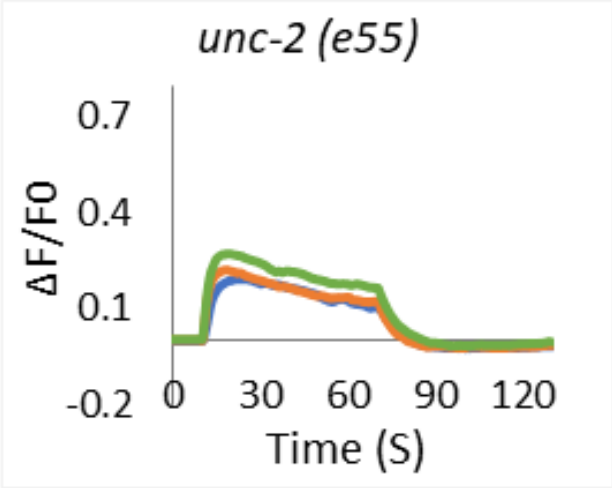


Figure 23. Effect of α-synuclein expression on the calcium response in the *unc-2* mutant animals.

Normalized calcium dynamics in the cell body of ASER neuron for *unc-2* mutant worms expressing α-synuclein at day 5. Blue, red and green lines indicate the dynamics in GFP (control), wild-type and A53T α-synuclein, respectively.

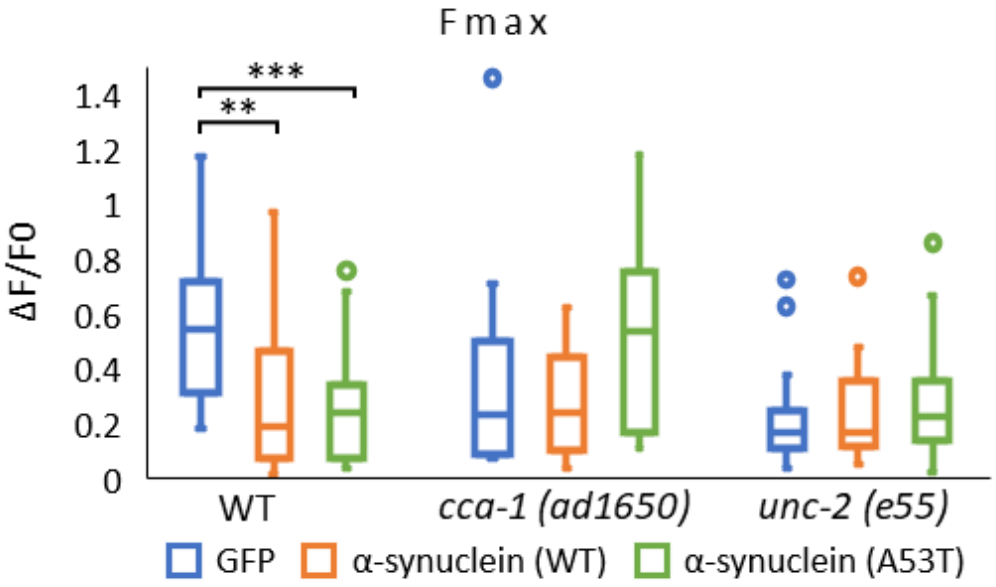


Figure 24. The box and whiskers plot of the peak values of wild-type and mutant worms at day 5.

Statistical significance was determined by the ANOVA test followed by the Tukey's multiple comparisons test, ** $P < 0.01$ and *** $P < 0.001$. Wild-type: GFP, n = 10 - 28.

Chapter 3

The propagation of α-synuclein in *C. elegans* neuronal circuits

3.1 Introduction

Several experiments performed in vitro (exposing cells to α-synuclein oligomers or fibrils) and in vivo (injection of recombinant α-synuclein fibrils or aggregates derived from diseased mice or PD patients) suggest that α-synuclein aggregates can be transmitted between cells (Figure 25) and facilitate the formation of the seed that eventually turns into toxic aggregates [68-76]. These findings support the "prion-like" propagation hypothesis by Braak et al. 2003, that the pathology of α-synuclein inclusion bodies in PD occurs in a gradual progression, starting in the olfactory system and the dorsal motor nucleus of the vagus nerve and then propagating through neuroanatomically connected pathways to the neocortex (Figure 2) [77]. Therefore, studies of α-synuclein propagation are important to unravel insights into the molecular basis of pathology of PD, and blocking α-synuclein propagation represents one of the effective strategies to prevent α-synuclein pathology formation and treat PD.

In the past, cell-to-cell propagation of α-synuclein was evaluated by direct injection of preformed exogenous α-synuclein fibrils. These studies, so far,

failed to resolve the following issues: α-synuclein species variability in their propagation, whether the propagation is trans-synaptic or synaptic-independent, whether there is a directionality for propagation, how the neutral activity is affected, and the regulators of α-synuclein propagation. (Figure 26). To address those knowledge gaps, I applied a well-established bimolecular fluorescence complementation (BiFC) (Figure 27A) [78] system to the *C. elegans nervous* system to assess the relationship between α-synuclein transmission and synaptic properties. I generate a new transgenic *C. elegans* model which expresses human α-synuclein in synaptically connected neurons specifically to study the cell-to-cell transmission of α-synuclein (Figure 27B). Compared to complex human or mouse neural system, *C. elegans* model exhibits several advantages in studying the propagation of α-synuclein, including well-defined synaptic connection direction and strength and controllable neuronal activity. By using this *C. elegans* model, I aim to evaluate the effects of both synaptic direction and strength on α-synuclein transmission, as well as define the molecules involved in propagation process.

3.2 Materials and methods

3.2.1 *C. elegans* strains

All *C. elegans* strains were grown on standard Nematode Growth Medium agar plates seeded with *Escherichia coli* (OP50), at 20°C, except for *dyn-1(ky51)* mutant animals, which were maintained at 15°C. The N2 Bristol strain was used as wild-type strain. The mutant lines *unc-57(ok310)* and *dyn-1(ky51)* were acquired from the Caenorhabditis Genetics Center.

3.2.2 Plasmid construction

To apply the BiFC system, plasmids containing either V1S or SV2 were generated by amplifying first the V1S and SV2 sequences from their corresponding vectors (kindly provided by Prof. Pamela J. McLean) and inserting either V1S or SV2 between the XmaI and MfeI sites of the pPD95.77 vector (kindly provided by Prof. Andy Fire) to generate pPD95.77/V1S or pPD95.77/SV2. Then, each cell-specific promoter sequence was inserted between the SphI and XmaI sites of the pPD95.77/V1S or pPD95.77/SV2 plasmid. The following DNA sequences were used as promoters for cell-specific expression: the *gcy-5* promoter (3 kb) for ASER, the *ttx-3* 2nd intron (0.4 kb) for AIY, and the *sre-1* promoter (1 kb) for ADL. Resulting V1S and SV2 plasmids (each 30 ng/ul) were co-injected into the gonads of young adult hermaphrodite N2 worms with a selection marker *rol-6* (50 ng/ul), to generate transgenic line expressing the BiFC pair with α-synuclein.

3.2.3 Transgenic animals

DK5608 *taEx [Psre-1:: V1S; Pgcy-5::SV2; rol-6]*

DK5609 *taEx [Pgcy-5:: V1S; Psre-1::SV2; rol-6]*

DK5605 *taEx [Pgcy-5:: V1S; Pttx-3::SV2; rol-6]*

DK5606 *taEx [Pttx-3:: V1S; Pgcy-5::SV2; rol-6]*

DK5611 *taEx [Psre-1:: mCherry; Plin44:: tdTomato]*

DK5610 *taEx [Pgcy-5:: mCherry; Plin44:: tdTomato]*

DK5620 *taEx [Psre-1:: V1S; Pgcy-5::SV2; rol-6]; taEx [Psre-1:: mCherry; Plin44:: tdTomato]*

DK5619 *taEx [Pgcy-5:: V1S; Psre-1::SV2; rol-6]; taEx [Pgcy-5:: mCherry; Plin44:: tdTomato]*

DK5618 *taEx [Pgcy-5:: V1S; Pttx-3::SV2; rol-6] ; taEx [Pgcy-5:: mCherry; Plin44:: tdTomato]*

DK5625 *unc-57(ok310); taEx [Pgcy-5:: V1S; Pttx-3::SV2; rol-6] ; taEx [Pgcy-5:: mCherry; Plin44:: tdTomato]*

DK5626 *dyn-1(ky51); taEx [Pgcy-5:: V1S; Pttx-3::SV2; rol-6] ; taEx [Pgcy-5:: mCherry; Plin44:: tdTomato]*

3.2.4 Microscopy

Worms in M9 solution containing NaN3 (100 mM) were mounted onto slides with a 2% agarose pads and covered with a coverslip. All images were obtained using Olympus FV1000 confocal laser scanning microscope with a 60× objective lens.

3.2.5 Statistical analyses

The statistical analyses of data from α-synuclein propagation were performed using Chi-square test. The data were regarded as statistically significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3 Results

3.3.1 Generation of *C. elegans* model for trans-synaptic propagation of α-synuclein

In order to monitor α-synuclein propagation via synaptically connected neurons in the *C. elegans* neuronal circuit, I used a bimolecular fluorescence complementation (BiFC) assay. To generate *C. elegans* transgenic lines visualizing α-synuclein propagation, the N-terminal fragment of Venus (named V1) was fused to the N terminus of α-synuclein (V1S), and the C-terminal fragment of Venus (V2) was fused to the α-synuclein C terminus (SV2), and each fusion protein was expressed cell-specifically using corresponding promoters. Theoretically, when α-synuclein in each fusion protein forms oligomers, the fused split Venus fragments combine and reconstitute a full, fluorescent complex (Figure 27). I here focused on the sensory neuron ASER and its prominent presynaptic neurons ADL as well as the prominent postsynaptic neurons AIY (Figure 28A). I generated two pairs of BiFC transgenic lines: one expressed fusion protein in the ADL-ASER pair and the other in the ASER-AIY pair. For the ADL-ASER pair, V1S was expressed in the presynaptic ADL sensory neurons under the control of an ADL specific promoter P*sre-1* while SV2 was expressed in the postsynaptic ASER sensory neuron using the *gcy-5* promoter (P*gcy-5*) (P*sre-1*::V1S; P*gcy-5*::SV2 or ADL::V1S; ASER::SV2), as well as its reverse combination (P*sre-1*::SV2;P*gcy-5*::V1S or ADL::SV2;ASER::V1S). For the ASER-AIY pair, V1S was expressed in the ASER as a presynaptic neuron while SV2 was expressed

in AIY inter neurons using the *ttx-3* 2nd intron (P*ttx-3*) as a postsynaptic neurons to ASER (P*gcy-5*::V1S; P*ttx-3*::SV2 or ASER::V1S; AIY::SV2), and its verse vasa (P*gcy-5*::SV2;P*ttx-3*::V1S or ASER::SV2;AIY::V1S). Because the strength of synaptic connections in these two pairs is largely distinct (Figure 28B), I can analyze both the direction and strength of synaptic connections in α-synuclein propagation.

Expressing either V1S or SV2 alone did not cause any BiFC fluorescence in any of the three types of neurons. However, a clear BiFC signal was observed when both V1S and SV2 were co-expressed in the same neurons (Figure 29).

3.3.2 α-Synuclein propagation occurs in both anterograde and retrograde directions, but retrograde propagation is the predominant mode

In ADL-ASER pair, Venus fluorescence was observed in both ADL::V1S; ASER::SV2 and ADL::SV2; ASER::V1S lines. In ADL::V1S; ASER::SV2 line, strong Venus fluorescence was observed in both ADLR and ADLL, but not in ASER, via the propagation of SV2 from postsynaptic ASER neuron (Figure 30). In order to confirm that Venus fluorescence was really observed in ADLs, I crossed worms ADL::V1S; ASER::SV2 worms with those expressing mCherry specifically in the ADL neurons (P*sre-1*::mCherry). As expected, I found that the Venus fluorescence colocalized with mCherry, indicating that BiFC signal was expressed in the ADLs (Figure 30). It may be possible that the smaller molecule SV2 can be released easily from ASER, but the larger V1S is not sufficiently released from ADLs to emit clear Venus signal in the

ASER neuron. Thus, I examined fluorescent signal in the ADL::SV2; ASER::V1S animals and found that weak fluorescence was observed only in ASER, which was caused by the propagation of SV2 from presynaptic neuron ADLs (Figure 31). However, this propagation from presynaptic to postsynaptic neurons was significantly weak than that from postsynaptic neurons to presynaptic neurons (Figure 32).

In the ASER-AIY pair, Venus fluorescence was observed only in ASER of the ASER::V1S; AIY::SV2 animals (Figure 33). No signal was observed in either ASER or AIY of ASER::SV2; AIY::V1S animals (Figure 34). These data suggested that α-synuclein spread in both anterograde and retrograde direction among synapse. In both pairs, presynaptic neurons showed significantly brighter fluorescent signals than postsynaptic neurons, suggesting that the retrograde propagation of α-synuclein is dominant. I also found that the strength of neuronal connectivity did not seem to affect the efficacy of α-synuclein propagation. Although the number of synaptic connections between the ASER-AIY pair is much larger than that between the ADL-ASER pair, the BiFC signal in ASER (from AIY propagation) is weaker than that in ADLs (from ASER propagation) (Figure 35).

3.3.3 DYN-1 Dynamin but not UNC-57 Endophilin is involved in α-synuclein propagation

Previous evidence has suggested that several cellular mechanisms, such as vesicle exocytosis/endocytosis or exosomal pathways, are involved in the cell-to-cell transmission of α-synuclein such. Because exosome signaling is not a

major releasing machinery in *C. elegans*, I focused on identifying potential signaling molecules involved in vesicle endocytosis. To validate the role of endocytosis in the predominant retrograde propagation of α-synuclein in *C. elegans*, I examined the propagation efficacy of AIY on ASER neurons in *unc-57* and *dyn-1* endocytic mutants. The *unc-57* and *dyn-1* genes encode endophilin and dynamin (Figure 36A) homologue in *C. elegans*, respectively. Since the null *dyn-1* mutation is lethal at the embryonic stage of the animal, we used a temperature-sensitive *dyn-1(ky51)* mutant allele, which displays defects in the recycling of synaptic vesicles at the restrictive temperature [79, 80]. Additionally, the Venus fluorescence in ASER was greatly reduced in *dyn-1(ky51)* mutant animals compared to that in the wild-type worms (Figure 36B), suggesting that DYN-1 is required for the uptake of α-synuclein in presynaptic neurons. The *unc-57(ok310)* mutant animals also exhibited defects in synaptic vesicle endocytosis; however, the Venus fluorescence in *unc-57* mutant animals was not altered compared to that in the wild-type worms (Figure 37). Since endophilin has been proposed to play a role in clathrin-mediated endocytosis [81, 82], these results suggest that classical clathrin-mediated endocytosis may not be involved in α-synuclein propagation into presynaptic neurons.

3.4 Discussion

Retrograde propagation of α-synuclein via synaptic connections

Recently, much attention has been paid to the direction of α-synuclein propagation in the pathology of PD. Evidences from autopsies of human patients suggested afferent propagation of pathogenic forms in the brain-gut nervous system [77, 83-86]. Several studies using preformed fibril (PFF) - injected mouse models of PD have argued the direction and synaptic strength in spreading of PFF. In fact, both the anterograde and retrograde directions of spreading have been shown, but recent findings support that retrograde spreading is probably dominant and that synaptic connectivity is not a primary determinant for spreading of PFFs at initial stages of neuronal vulnerability [74, 84, 85, 87]. However, in both human autopsy analyses and mouse PFF-injected model, clear connectivity analysis of pathogenic PFF propagation in specific cells is quite difficult for several reasons. First, the large number of innervating neurites makes it difficult to promptly evaluate the strength of connectivity. Thus, most studies have hypothesized that the number of synapses per neurite is the same in all the neurons. Second, evaluating the region of spreading from the injected site requires a long period, causing uncertain results because some cells may have been affected by α-synuclein and may have been lost during spreading. To the best of my knowledge, there is no direct evidence that α-synuclein is propagated in the retrograde direction through confirmed synaptic connections. Importantly, the BiFC *C. elegans* model in this study allows us to perform a quantitative, real-time analyses in certain neurons during propagation, and we were able

to explore the impact of synaptic strength based on the complete map of synaptic connectivity. Since this strategy is advantageous over earlier analyses, my data strongly indicated that the retrograde propagation from postsynaptic to presynaptic neurons is dominant for natively expressed α-synuclein, and the strength of connections is not the determinant of propagation. Quantitative analyses of α-synuclein propagation using a well-characterized synaptic connectivity in *C. elegans* will represent an important framework for understanding and treating progressive neurodegenerative diseases.

Different conclusions have been drawn regarding the contribution of synaptic connections in α-synuclein propagation. For example, Henrich et al. suggested that the strength of connections does not consistently correlate with the magnitude of the α-synuclein pathology [88], while the other two studies concluded that the strength is the primary determinant of spreading [85, 89]. However, in this study, the neuronal pair with fewer synaptic connections (i.e., ADL-ASER) showed more effective propagation than the pair with more synaptic connections (i.e., ASER-AIY), suggesting that the strength of connections does not positively correlate with the propagation. However, it may be possible that the different expression levels in transgenic lines may affect the BiFC signal intensity. So I also compared the signal intensity in ADLL and ADLR from the ASER neuron in a single transgenic line. Although ADLL has more synaptic connections with ASER than ADLR (6 and 1, respectively), no difference in the BiFC signals was observed (Figure 30). These findings indicate that the efficacy of α-synuclein propagation is

probably not dependent on the strength of synaptic connection, but might be influenced by other factors. Few studies have showed that the BiFC system works in the *C. elegans*, nervous system and that α-synuclein is transmitted from cells to cells. However, these analyses do not focus on the connectivity of neuronal cells in which α-synuclein is propagated, and it is not clear how and where synuclein is spread in the nervous system. In this study, I first indicated the strong propagation of α-synuclein into the synaptically connected presynaptic neurons, but there is no correlation between the amount of synuclein propagation and strength of synaptic connectivity.

In this model, only the SV2 propagation was observed while no V1S propagation was seen, which might depend on the distinct characteristics of V1S and SV2. A recent study showed that Vn-α-syn (corresponding to V1S) exhibited a high propensity to form oligomers and higher-order aggregates and only a small fraction of Vn-α-syn was involved in the formation of the fluorescent BiFC complex [90]. It might be possible that oligomers and higher-order aggregates are not the predominate state to be transmitted from cells to cells. Alternatively, transmitted V1S may be difficult to exhibit BiFC signal by forming complex with SV2 when V1S oligomers or aggregates are present during transmission. Thus, the choice of fusion protein in target cells may alter the conclusion on the direction and propensity of transmission of α-synuclein. To avoid this misleading, in this study, I generated both transgenic animals expressing V1S in pre- and SV2 in postsynaptic neurons, and its vice versa. Both analyses showed that retrograde transmission is dominant in two pairs of connected neurons. I strongly believe that the direction of

transmission did not result from differed expression level nor transmission efficacy of these two proteins.

Mechanisms of α-synuclein secretion and uptake

Another important aspect is the cellular mechanisms that might contribute to α-synuclein propagation. Although α-synuclein does not contain a signal for extracellular release, several studies on trans-synaptic α-synuclein propagation suggest that its release by exocytosis and exosomes [91-93], uptake by endocytosis [94-97], and spread from cell death could be involved in the α-synuclein propagation. Because the exosomes uptake is through clathrin-mediated endocytosis [98] and cell death was not observed in α-synuclein expressing neurons, exo/endocytosis is the most reasonable interpretation for the propagation observed in this study. As α-synuclein propagation was observed in a retrogradely manner, from postsynaptic to presynaptic neurons, I presume that α-synuclein is released from synaptic neuron via exocytosis of dense core vesicle. In fact, a previous study also confirmed that α-synuclein is released from cells by exocytosis and is enriched in large dense core vesicles [99]. Thus, I carefully observed that GFP signals did not co-localize with presynaptic RAB-3 puncta on the neurites of ASER, but failed to observe such non-presynaptic localization of α-synuclein::GFP protein. Since α-synuclein is known as a presynaptic protein, the density of vesicles containing α-synuclein should be quite low at postsynaptic sites. Using another approach to visualize postsynaptic α-synuclein may help us understand the mechanism of retrograde secretion through synapses.

Moreover, the results suggest a novel endocytic mechanism for α-synuclein uptake in presynaptic neurons. I found that *dyn-1* (dynamin), and not *unc-57* (endophilin), is involved in retrograde propagation of α-synuclein. Endophilin is an essential protein involved in clathrin-mediated endocytosis. This suggests that α-synuclein may be taken up to presynaptic sites via nonclassical clathrin-independent endocytosis. I believe that the *C. elegans* model in this study will contribute to the identification of other genetic factors involved in postsynaptic release and presynaptic uptake of α-synuclein using cell-specific gene knockdown, in addition to genome-wide mutant screening.

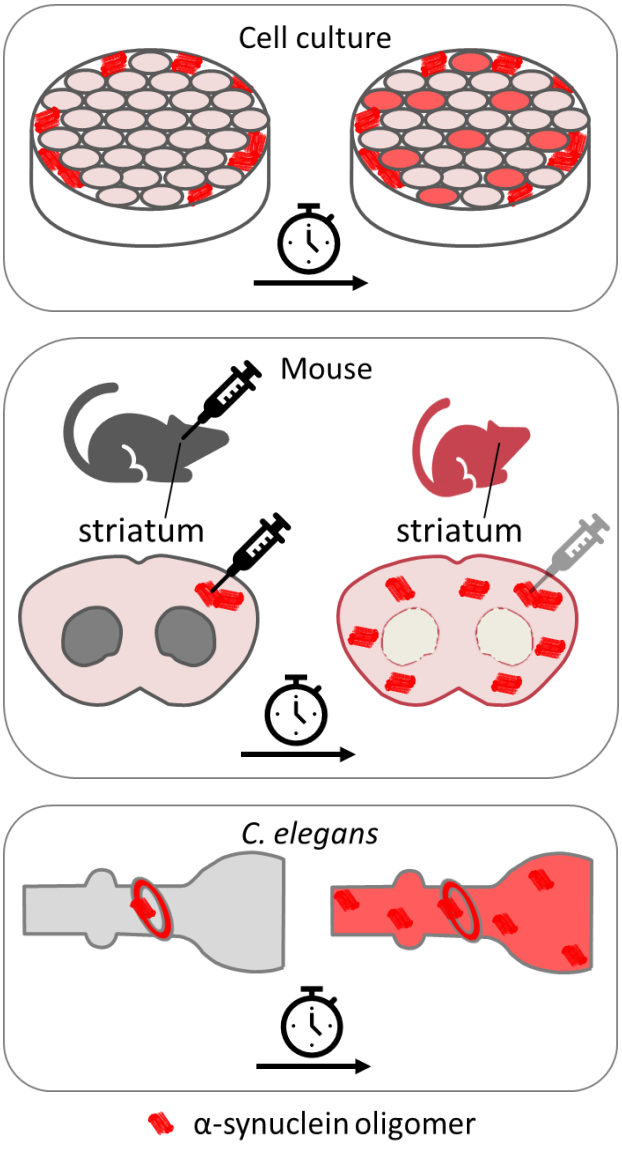


Figure 25. Examples of α-synuclein propagation in model studies.

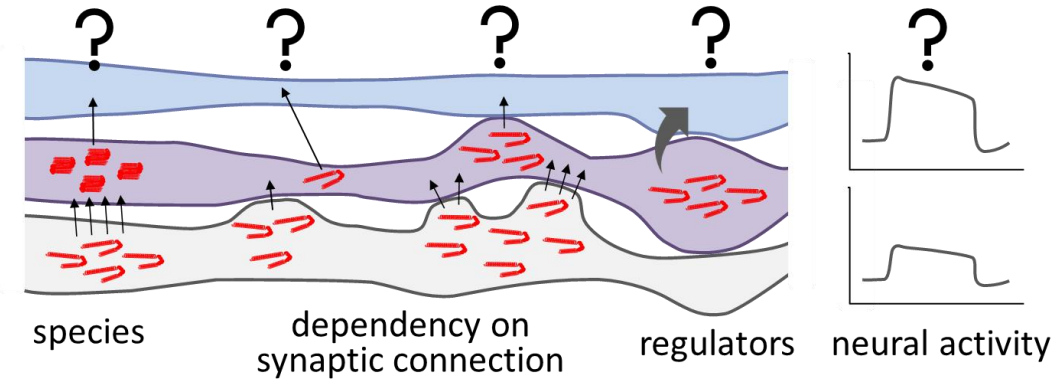


Figure 26. The possible mechanism of α-synuclein propagation in the synaptically-connected neurons.

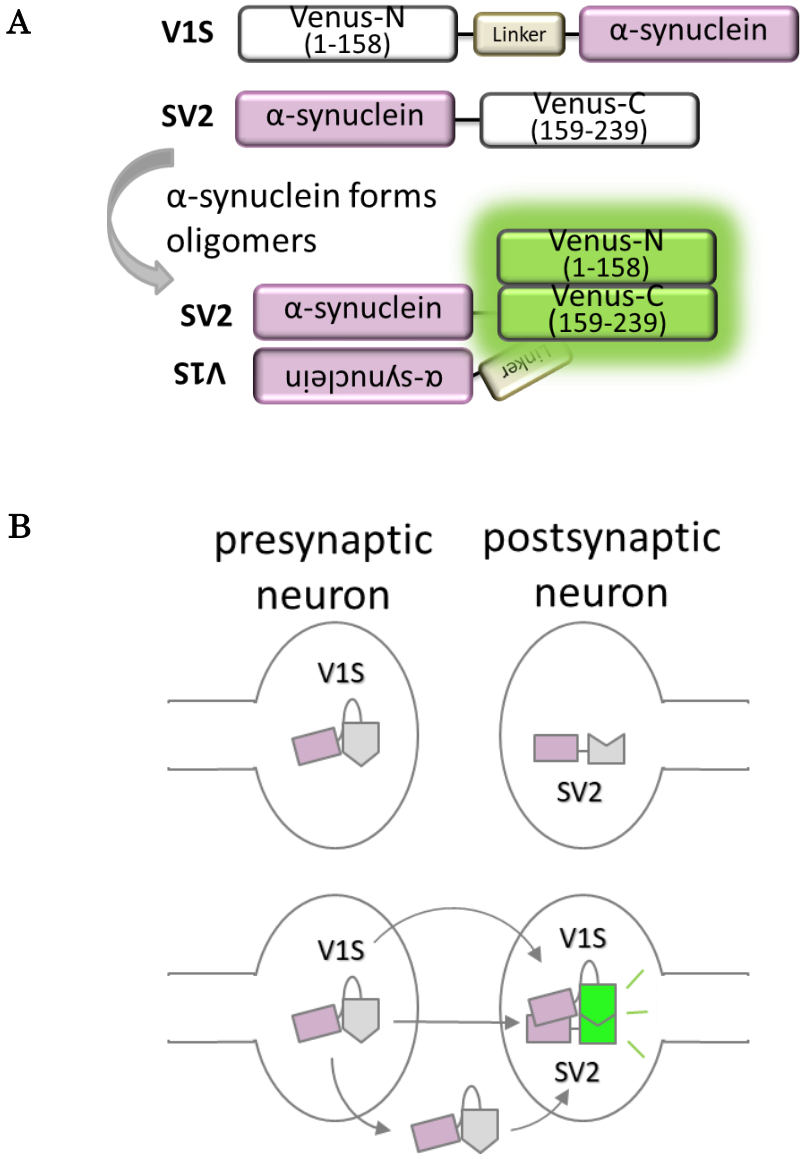
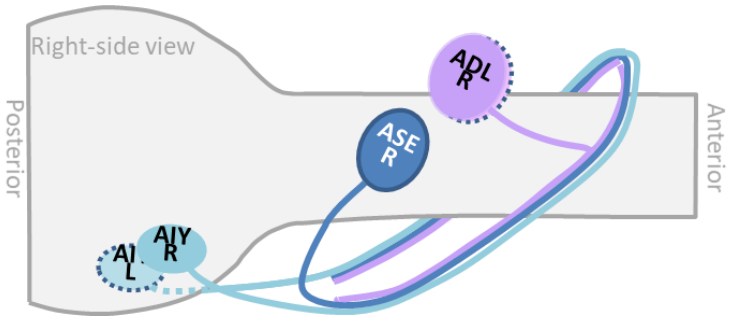


Figure 27. Scheme of Bimolecular Fluorescence Complementation assay (BiFC).

(A) Scheme of the generation of BiFC fluorescence by α-synuclein oligomerization. (B) The application of BiFC in this study.

A



B

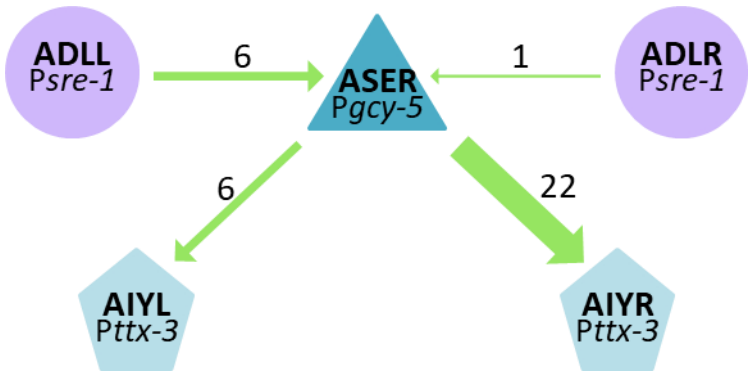


Figure 28. Schematic representation of the neuronal circuit examined in this study.

(A) Schematic representation of the position and morphology of studied neurons. (B) Strength of synaptic connectivity among the studied neurons in the cell-to-cell propagation of α-synuclein. Arrows represent the synaptic directions, and the numbers above arrows represent the amount of synaptic connections between neurons.

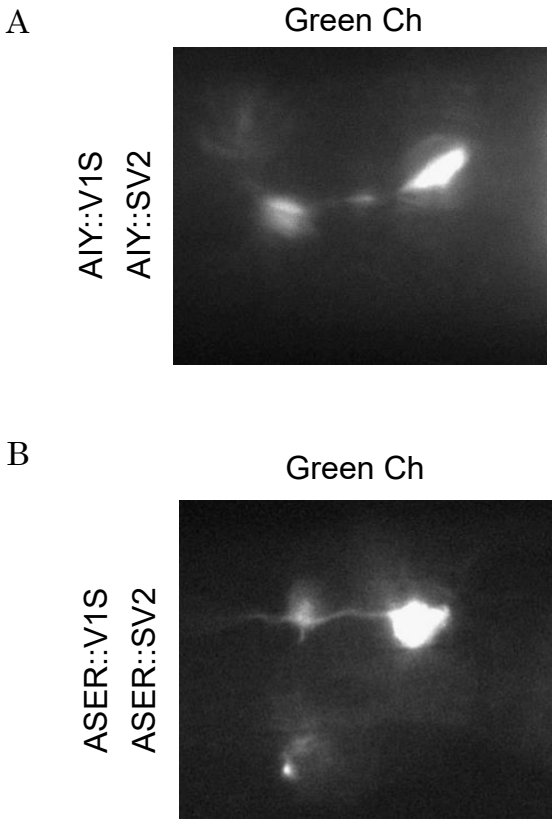


Figure 29. Representative images of BiFC signal in *C. elegans* neurons.
(A) Strong Venus BiFC fluorescence in AIY when both V1S and SV2 were expressed in AIY neurons. Left side view. Anterior is left dorsal is top. (B) Strong Venus BiFC fluorescence in the ASER when both V1S and SV2 were expressed in AIY neurons. Top view. Anterior is left.

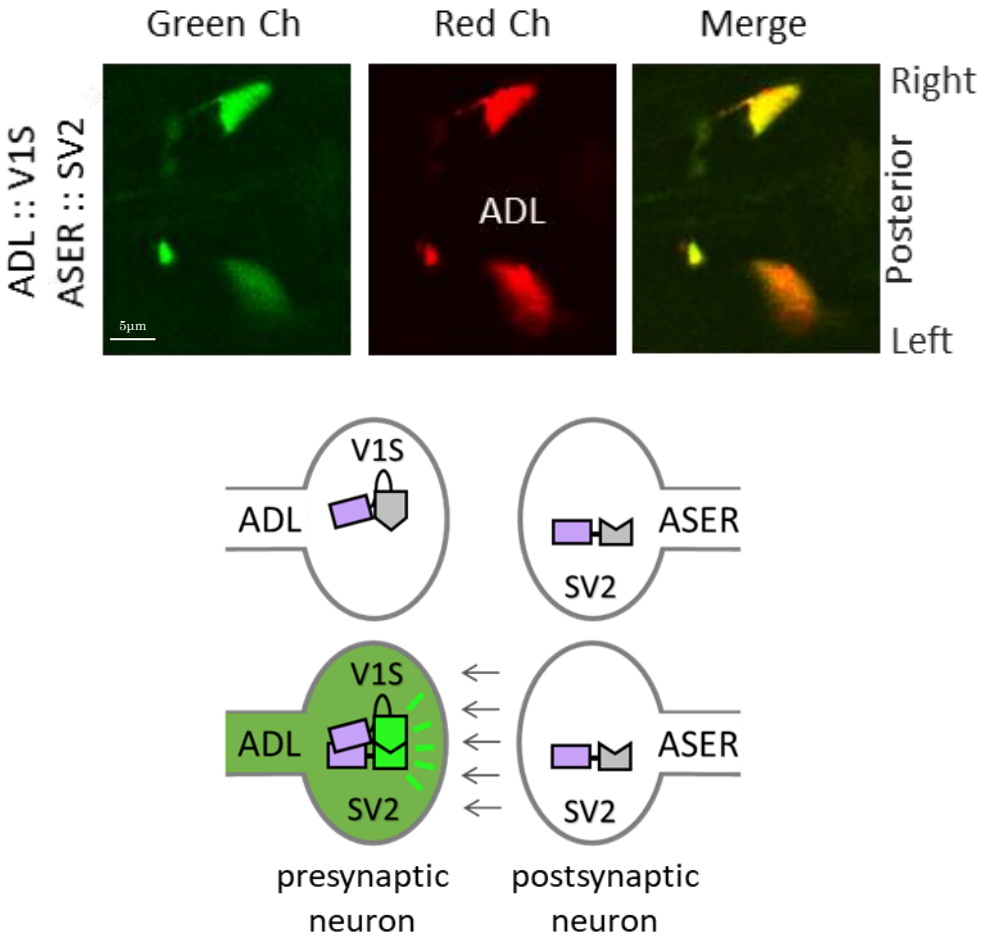


Figure 30. The retrograde propagation of α-synuclein in the ADL-ASER pair. Strong Venus BiFC fluorescence in the ADL neurons of the ADL::V1S; ASER::SV2 animals. Top view. Anterior is left. The lower figure is the scheme of the α-synuclein propagation in ADL::V1S; ASER::SV2 animal.

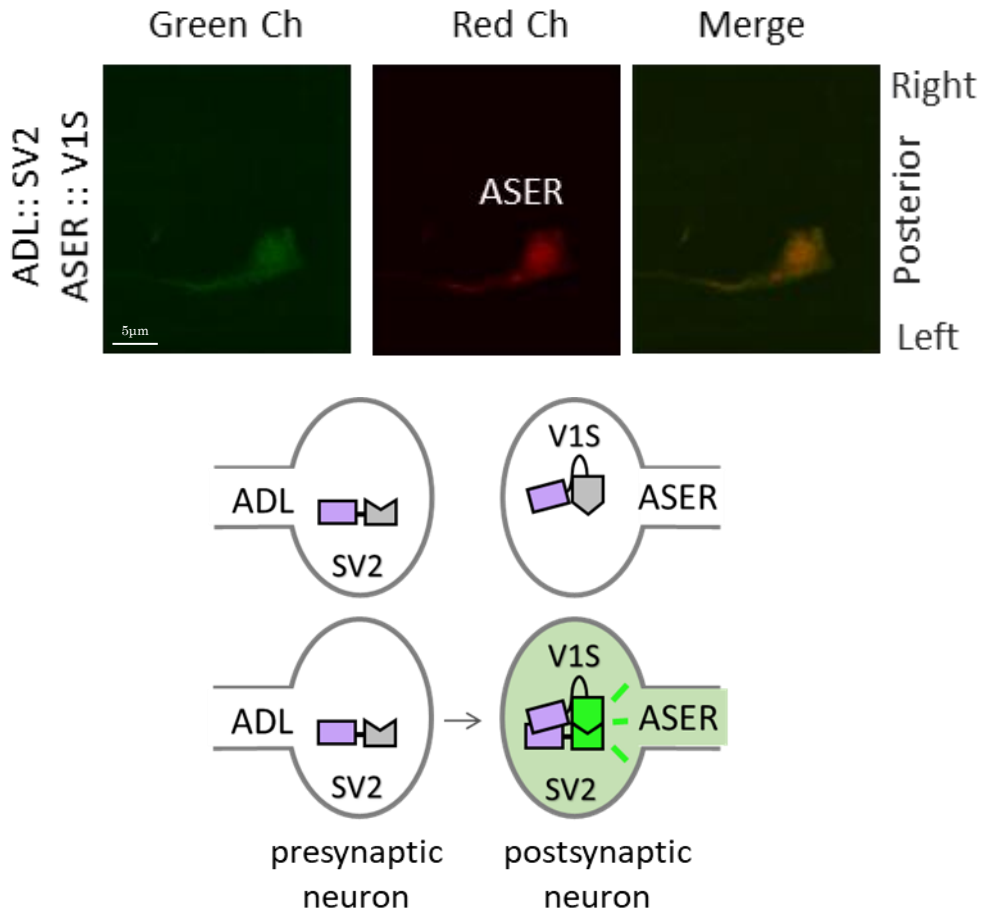


Figure 31. The anterograde propagation of α-synuclein in the ADL-ASER pair. Weak Venus BiFC fluorescence in the ASER neuron of the ADL::SV2; ASER:: V1S animals. Side view. Anterior is left and dorsal is top. The lower figure is the scheme of the α-synuclein propagation in ADL::SV2; ASER:: V1S animal.

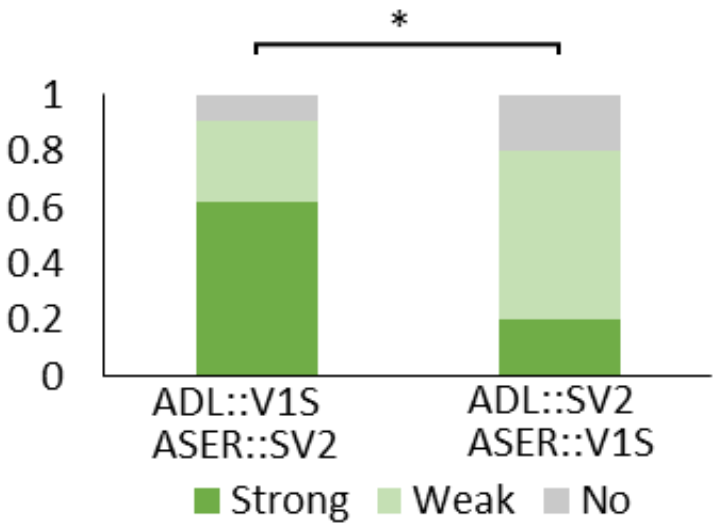


Figure 32. Quantification of Venus BiFC fluorescence intensity in the ADLs or ASER neuron in the ADL-ASER pair.

The color bars represent the ratio of animals showing strong, weak or no Venus BiFC intensity in each BiFC transgenic line. Twenty young adult worms were examined in each line, and the statistical significance was determined by Chi-square test, * $P < 0.05$.

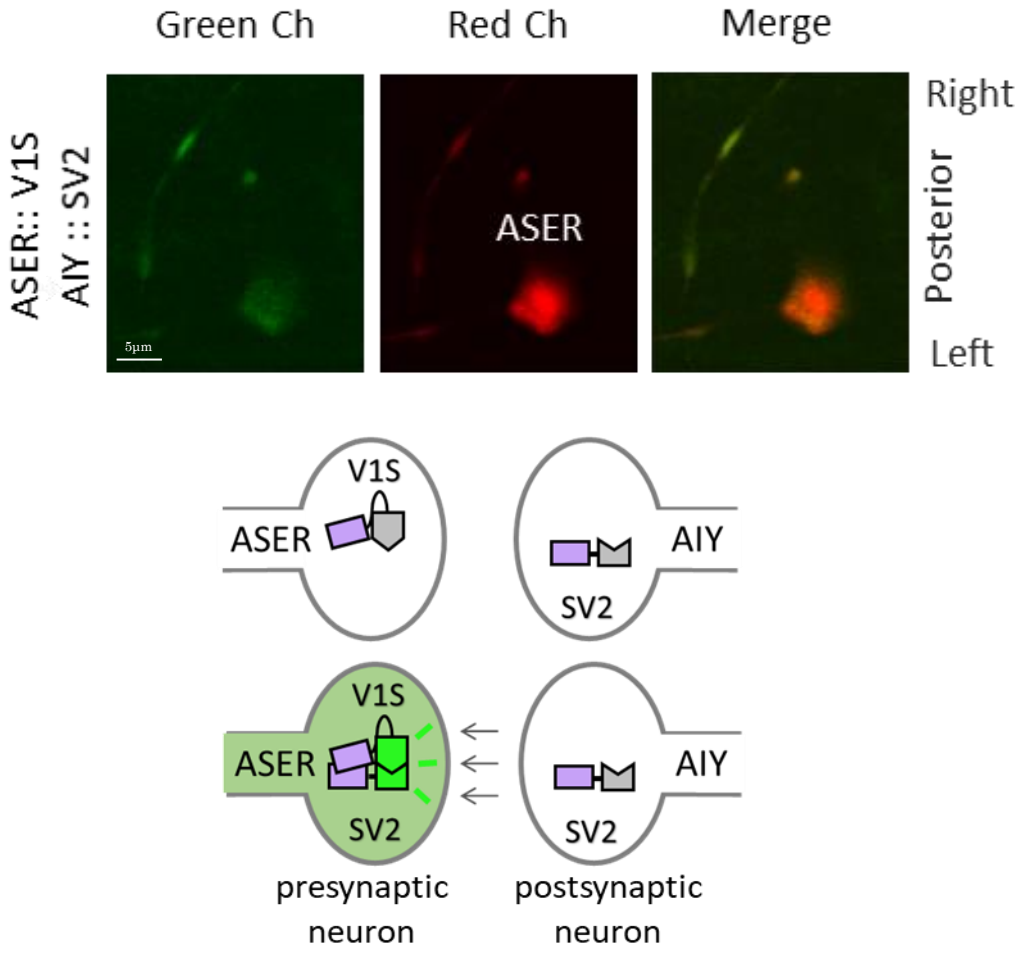


Figure 33. The retrograde propagation of α-synuclein in the ASER-AIY pair. Venus BiFC fluorescence was only observed in the ASER neuron of the ASER::V1S; AIY::SV2 animals. The lower figure is the scheme of the α-synuclein propagation in ASER::V1S; AIY::SV2 animal.

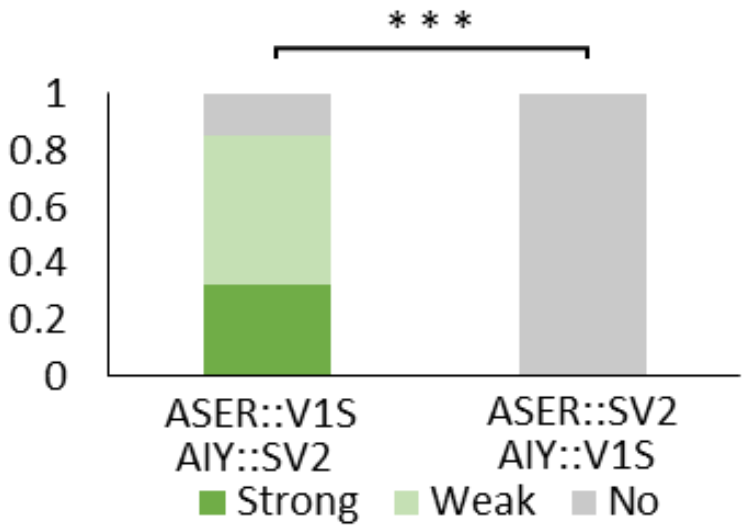


Figure 34. Quantification of Venus BiFC fluorescence intensity in the ASER or AIY neurons in ASER-AIY pair.

Eighty worms were examined in each line. *** $P < 0.001$.

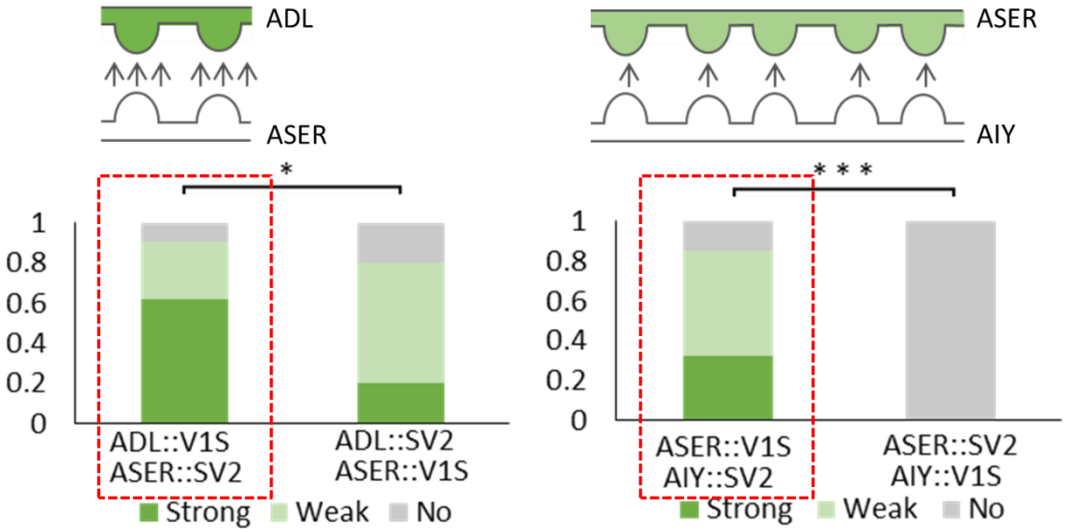
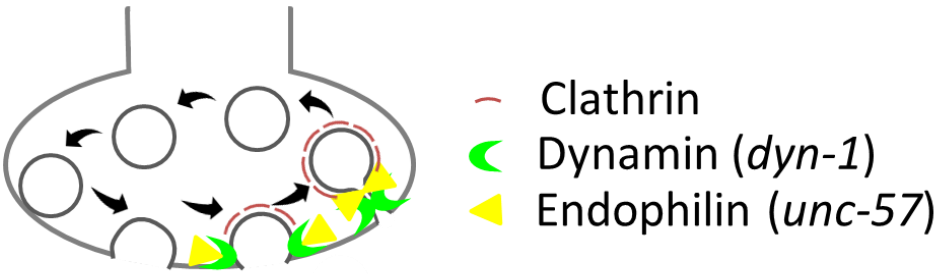


Figure 35. Comparison of Venus BiFC fluorescence intensity in the ADLs neuron in ADL-ASER pair and the ASER neurons in ASER-AIY pair.

A



B

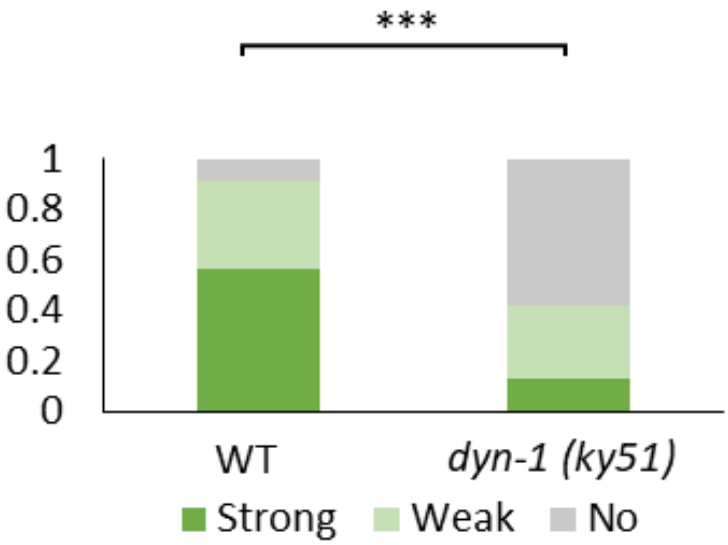


Figure 36. The weakened propagation of α-synuclein in the *dyn-1* mutant animals.

(A) Schematic representation of endocytosis. (B) Quantification of Venus BiFC fluorescence intensity in the wild-type or *dyn-1* mutant worms containing the ASER::V1S; AIY::SV2 transgene. The color bars represent the ratio of animals showing strong, weak or no Venus BiFC intensity in the ASER neuron. N = 23 or 96 for the wild-type and *dyn-1* mutant animals, respectively. The statistical significance was determined by Chi-square test, *** $P < 0.001$.

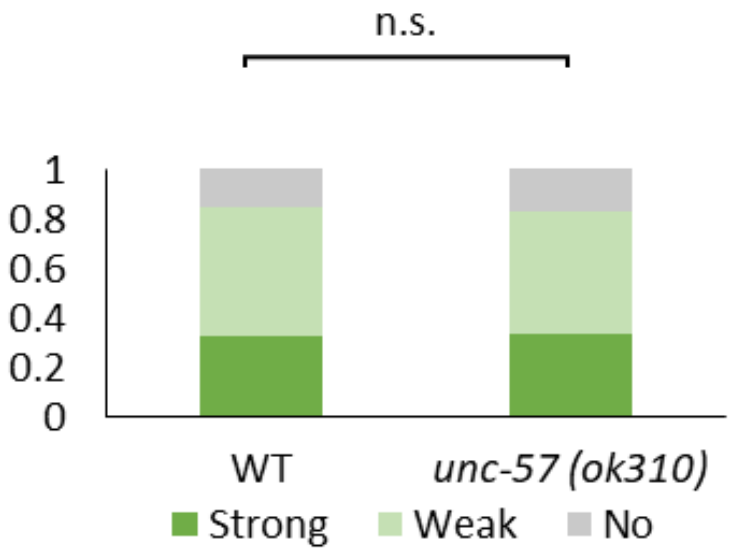


Figure 37. The normal propagation of α-synuclein in the *unc-57* mutant animals.

Quantification of Venus BiFC fluorescence intensity in ASER of wild-type and *unc-57* mutant worms. N = 80 worms.

Chapter 4

General discussion and conclusion

α-Synuclein aggregates are the neuropathological feature of PD, which are suggested to exhibit neurotoxicity and propagate in neuronal networks. Besides the neurotoxic, it could also be considered that α-synuclein aggregates are merely a consequence of the disease. Thus, understanding the relationship between α-synuclein aggregation and PD progression is essential to develop therapeutic strategies for this disease. In this thesis, I have developed new transgenic *C. elegans* models to study the physiological function and cell-to-cell propagation of α-synuclein.

In chapter 2, I found that the expression of α-synuclein causes the abnormal accumulation of synaptic vesicles at presynaptic sites and the decreased extracellular Ca^{2+} influx by inhibiting the function of voltage-dependent calcium channel *cca-1* and *unc-2* (Figure 38). These findings may reveal some of the physiological functions of α-synuclein in neuronal cells. In chapter 3, to evaluate the propagation mechanism of α-synuclein in synaptically connected neurons, I focused on well-established synaptic neuronal pairs in the *C. elegans* neuronal circuit and showed evidences on the bidirectional propagation of α-synuclein through synaptic connections. However, I also found that the retrograde propagation from postsynaptic to presynaptic neurons is dominant. This finding helps to explain why α-synuclein pathology is commonly found in the peripheral autonomic nervous system and

progressively spread to the central nervous system in the disease course. Furthermore, endophilin was not involved in α-synuclein propagation, suggesting that a nonclassical exo/endocytotic mechanism may regulate the propagation process (Figure 39). The quantitative analyses of α-synuclein transmission using a well-characterized synaptic connectivity in *C. elegans* established an important framework for understanding and treating progressive neurodegenerative diseases.

Requirement a trigger to cause α-synuclein aggregation and neurodegeneration

In chapter 2, I attempted to evaluate native physiological roles of α-synuclein by expressing in a single neuron ASER of *C. elegans*. Although other studies using *C. elegans* DA neurons showed that overexpression of α-synuclein often caused dopaminergic neurodegeneration, no significant degeneration such as loss of the dendrite was observed in ASER neuron. Since previous studies have suggested that dopamine facilitates α-synuclein oligomeric conformation [100], it is conceivable that a trigger may be required for α-synuclein oligomerization, which results in the transfer of pathogenic α-synuclein strains. Due to a lack of such trigger in my *C. elegans* model, overexpression of α-synuclein in ASER just accelerated the aging process, without changing normal α-synuclein into pathological structure.

Such trigger seems to play an important role in defining the mechanism underlying PD progression. To date, many studies have been carried out to identify factors that potentially associate with the onset and progression of

PD, including aging, genetics and environmental factors. PD usually begins over the age of 60 and shows a rising prevalence with age. Although it is only a few cases, several genes have been identified to associated with early-onset and familial PD, including α-synuclein (SNCA), Parkin (PRKN), and LRRK2. Since most patients are sporadic, environmental factors have been suggested to increase the risk of PD, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which can induce dopaminergic neuron death, air pollutants and pesticides. Under normal conditions, although α-synuclein have a potential to aggregate, the aggregates can be cleared by proteasome mechanism without amyloid deposition. However, under the influence of aging, genetic and environmental factors, α-synuclein becomes easier to accumulate or hard to be cleared, leading to the accumulation and spread of pathological strains of α-synuclein and eventually resulting in the development of PD. On the other hand, although age-associated morphological changes were accelerated in α-synuclein expressing worm compared to control animals in this study, no significant neurodegeneration was observed in ASER, even for aged animals. This result may suggest a possibility that those risk factors are not enough in the ASER neuron of *C. elegans*.

A hypothesis of physiological accumulation of α-synuclein triggered by infection

An interesting observation that needs to be paid attention is that LBs and LNs are initially found in the dorsal motor nucleus of the glossopharyngeal

(interfaces with tongue and pharynx), vagal (interfaces with the parasympathetic control of the heart, lungs, and digestive tract) nerves and anterior olfactory nucleus (involved in odor) before neurologic symptoms appear [101]. This anatomical distribution of Lewy pathology can provide a new insight into the possible triggers of PD pathology. Gastrointestinal tract and nasal cavity are organs which are faced with the outside and have high possibility of being infected by foreign substances such as bacteria and viruses. In human patients with intestinal inflammation, accumulation of α-synuclein in enteric neurites was found [102]. Furthermore, recent study indicates that patients who had undergone truncal vagotomy seem to have lower risk of PD [103]. Therefore, the infection-triggered PD hypothesis can be proposed, in which the expression of α-synuclein can be increase after pathogen infection and inflammation, leading to pathological accumulation of α-synuclein. Similar views have been also proposed for Alzheimer's disease [104]. Aß has been shown to disrupt membranes in vitro studies [105] and may play a role similar to antimicrobial peptides (AMPs) which function in the innate immune system, exhibiting antimicrobial activity against several microorganisms [106]. Recent studies also reported that Aß functions as an innate immune protein that protects against bacterial and viral infections in mice model [107, 108]. Beyond Aß, other neurodegeneration-associated proteins including tau and prion have been shown to have antimicrobial activities [109, 110]. These findings imply the physiological functions of neurodegeneration-associated proteins to play a protective role in host

defense, although under certain conditions, they could be dysregulated and form toxic amyloid oligomers leading to pathological processes.

Based on the infection-triggered PD hypothesis, it can be further interpreted that membrane binding and channel formation of α-synuclein may contribute to increase the bacterial membrane permeability. Neuro-invasive viral infections that invade into the nervous system is known, and these transmit progeny virions or virion components within axons and spread to post-synaptic cells [111]. The function of α-synuclein in inhibiting neuronal activity, synaptic vesicle release and propagation between synaptically connected neurons, found in this thesis, could be important to block the infection route to prevent the spread of infection. To test this presumption, it is necessary to perform a large number of experiment and to provide a large amount of corroborative evidence as future works. I believe that a deeper understanding of the role of α-synuclein in the immune system could further create new treatments for Parkinson's disease and other neurodegenerative disorders.

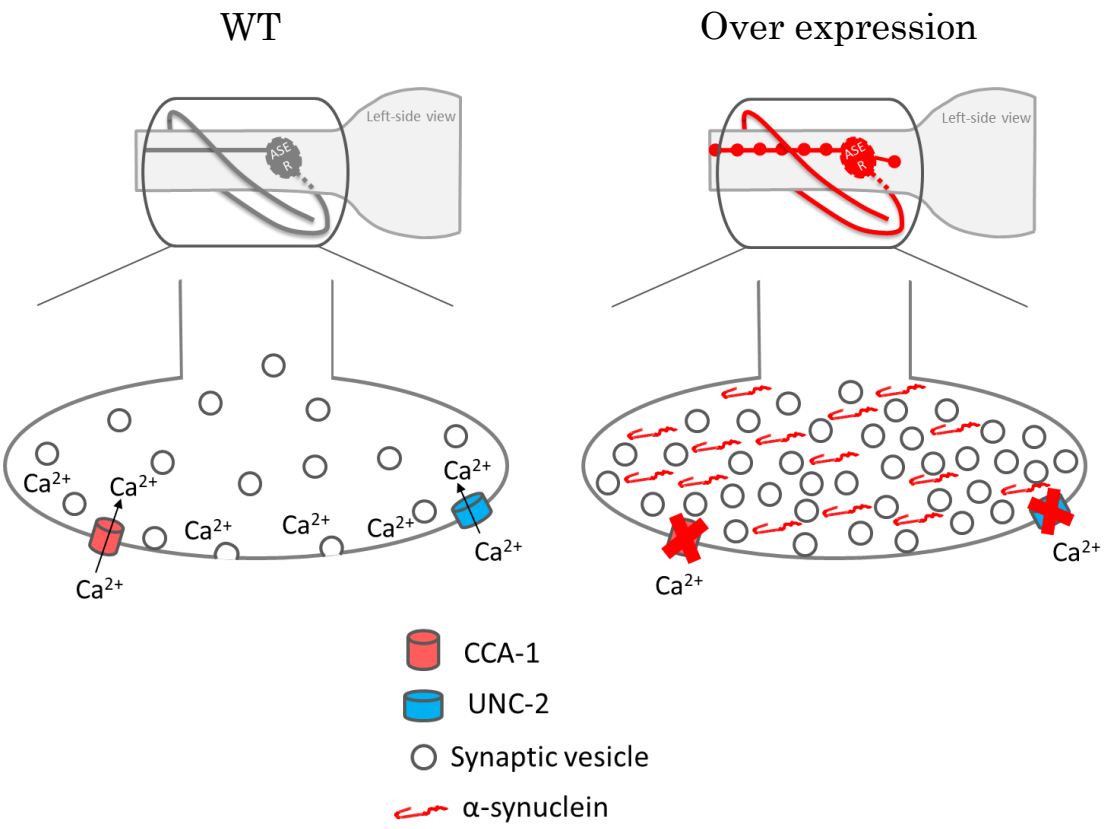


Figure 38. Model of the physiological function of α-synuclein in the *C. elegans* neuronal system.

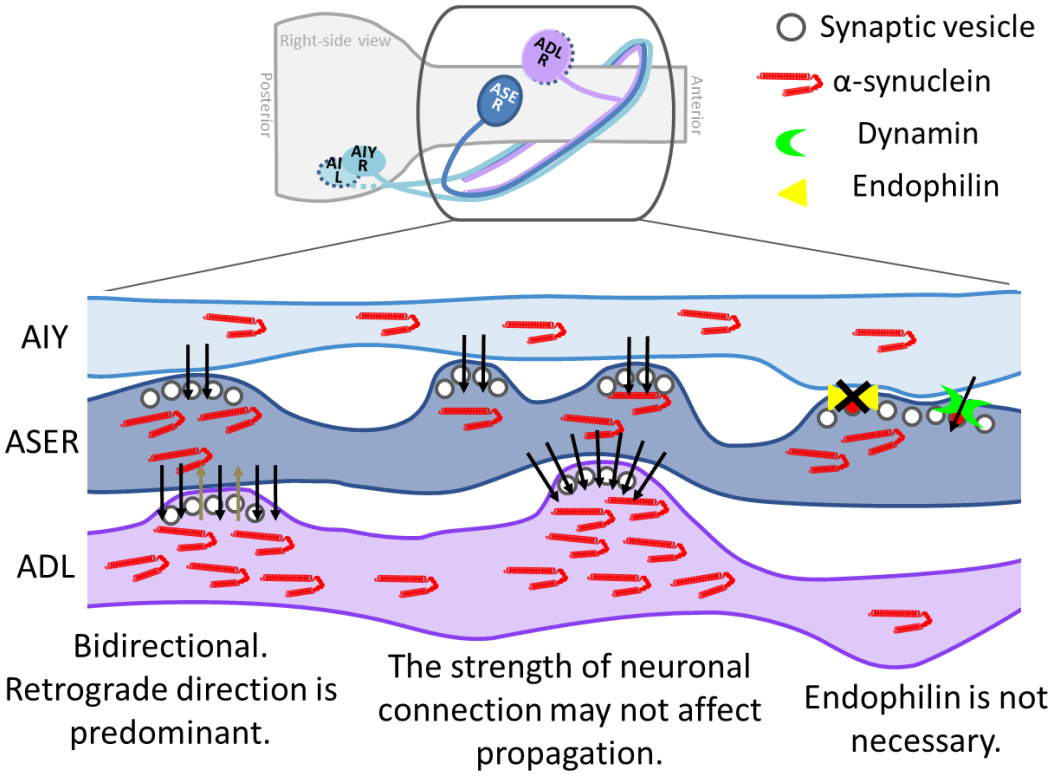


Figure 39. Model of the propagation of α-synuclein in *C. elegans* neuronal circuits.

Acknowledgements

I would like to express my deep gratitude to my advisor Dr. Motomichi Doi for his thoughtful guidance in research planning and for countless of times and patience for mentoring me. He cheers me up throughout the program. This thesis would never be accomplished without his precious help. I would like to give special thanks to my dissertation committee Dr. Akiyoshi Fukamizu, Dr. Keiji Tanimoto and Dr. Hiroaki Daitoku for being so accommodating and for providing valuable reach advice and critical thinking for this study.

I would like to thank Megumi Sato, Masako Otobe and Emiri Abe for technical assistance and Dr. Yoichi Shinkai and other members in Molecular Neurobiology RG in AIST for critical discussion for this study. I would like to thank Dr. Pamela J. McLean and Dr. Mei Zhen for providing plasmids. Worm strains were provided by the CGC which is funded by NIH Office of Research Infrastructure Programs (P40OD010440).

I would like to thank my family members Dr. Pengxiang Hu and May chan for always supporting and accompanying me. Finally, I would like to thank my mother Suwei Wang for her understanding, support and encouragement.

References

[1] T. Wyss-Coray, Ageing, neurodegeneration and brain rejuvenation, Nature, 539 (2016) 180-186.

[2] S. Tsuji, Genetics of neurodegenerative diseases: insights from high-throughput resequencing, Human Molecular Genetics, 19 (2010) R65-R70.

[3] C. Soto, S. Pritzkow, Protein misfolding, aggregation, and conformational strains in neurodegenerative diseases, Nature Neuroscience, 21 (2018) 1332-1340.

[4] H. Fu, J. Hardy, K.E. Duff, Selective vulnerability in neurodegenerative diseases, Nature Neuroscience, 21 (2018) 1350-1358.

[5] M. Jucker, L.C. Walker, Self-propagation of pathogenic protein aggregates in neurodegenerative diseases, Nature, 501 (2013) 45-51.

[6] D.M. Walsh, D.J. Selkoe, A critical appraisal of the pathogenic protein spread hypothesis of neurodegeneration, Nature Reviews Neuroscience, 17 (2016) 251-260.

[7] P. Damier, E.C. Hirsch, Y. Agid, A.M. Graybiel, The substantia nigra of the human brain: II. Patterns of loss of dopamine-containing neurons in Parkinson's disease, Brain, 122 (1999) 1437-1448.

[8] M.G. Spillantini, M.L. Schmidt, V.M. Lee, J.Q. Trojanowski, R. Jakes, M. Goedert, Alpha-synuclein in Lewy bodies, Nature, 388 (1997) 839-840.

[9] H. Deng, H. Liang, J. Jankovic, F-Box Only Protein 7 Gene in Parkinsonian-Pyramidal Disease, JAMA Neurology, 70 (2013) 20-24.

[10] J. Hardy, Genetic analysis of pathways to Parkinson disease, Neuron, 68 (2010) 201-206.

[11] D. Chang, M.A. Nalls, I.B. Hallgrímsdóttir, J. Hunkapiller, M. van der Brug, F. Cai, G.A. Kerchner, G. Ayalon, B. Bingol, M. Sheng, D. Hinds, T.W. Behrens, A.B. Singleton, T.R. Bhangale, R.R. Graham, C. International Parkinson's Disease Genomics, T. andMe Research, A meta-analysis of genome-wide association studies identifies 17 new Parkinson's disease risk loci, Nature genetics, 49 (2017) 1511-1516.

[12] R. Martino, H. Candundo, P.v. Lieshout, S. Shin, J.A.G. Crispo, C. Barakat-Haddad, Onset and progression factors in Parkinson's disease: A systematic review, NeuroToxicology, 61 (2017) 132-141.

[13] M.C. Chartier-Harlin, J. Kachergus, C. Roumier, V. Mouroux, X. Douay, S. Lincoln, C. Levecque, L. Larvor, J. Andrieux, M. Hulihan, N. Waucquier, L. Defebvre, P. Amouyel, M. Farrer, A. Destée, Alpha-synuclein locus duplication as a cause of familial Parkinson's disease, Lancet (London, England), 364 (2004) 1167-1169.

[14] M. Farrer, J. Kachergus, L. Forno, S. Lincoln, D.S. Wang, M. Hulihan, D. Maraganore, K. Gwinn-Hardy, Z. Wszolek, D. Dickson, J.W. Langston, Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications, Annals of neurology, 55 (2004) 174-179.

[15] A.B. Singleton, M. Farrer, J. Johnson, A. Singleton, S. Hague, J. Kachergus, M. Hulihan, T. Peuralinna, A. Dutra, R. Nussbaum, S. Lincoln, A. Crawley, M. Hanson, D. Maraganore, C. Adler, M.R. Cookson, M. Muenter, M.

Baptista, D. Miller, J. Blancato, J. Hardy, K. Gwinn-Hardy, alpha-Synuclein locus triplication causes Parkinson's disease, Science, 302 (2003) 841.

[16] J. Fuchs, A. Tichopad, Y. Golub, M. Munz, K.J. Schweitzer, B. Wolf, D. Berg, J.C. Mueller, T. Gasser, Genetic variability in the SNCA gene influences alpha-synuclein levels in the blood and brain, FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 22 (2008) 1327-1334.

[17] S. Appel-Cresswell, C. Vilarino-Guell, M. Encarnacion, H. Sherman, I. Yu, B. Shah, D. Weir, C. Thompson, C. Szu-Tu, J. Trinh, J.O. Aasly, A. Rajput, A.H. Rajput, A. Jon Stoessl, M.J. Farrer, Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease, Movement disorders : official journal of the Movement Disorder Society, 28 (2013) 811-813.

[18] A.P. Kiely, Y.T. Asi, E. Kara, P. Limousin, H. Ling, P. Lewis, C. Proukakis, N. Quinn, A.J. Lees, J. Hardy, T. Revesz, H. Houlden, J.L. Holton, α-Synucleinopathy associated with G51D SNCA mutation: a link between Parkinson's disease and multiple system atrophy?, Acta neuropathologica, 125 (2013) 753-769.

[19] R. Krüger, W. Kuhn, T. Müller, D. Woitalla, M. Graeber, S. Kösel, H. Przuntek, J.T. Epplen, L. Schöls, O. Riess, Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease, Nature genetics, 18 (1998) 106-108.

[20] S. Lesage, M. Anheim, F. Letournel, L. Bousset, A. Honoré, N. Rozas, L. Pieri, K. Madiona, A. Dürr, R. Melki, C. Verny, A. Brice, G51D α-synuclein

mutation causes a novel parkinsonian-pyramidal syndrome, Annals of neurology, 73 (2013) 459-471.

[21] P. Pasanen, L. Myllykangas, M. Siitonen, A. Raunio, S. Kaakkola, J. Lyytinen, P.J. Tienari, M. Pöyhönen, A. Paetau, A novel α-synuclein mutation A53E associated with atypical multiple system atrophy and Parkinson's disease-type pathology, Neurobiology of Aging, 35 (2014) 2180.e2181-2180.e2185.

[22] M.H. Polymeropoulos, C. Lavedan, E. Leroy, S.E. Ide, A. Dehejia, A. Dutra, B. Pike, H. Root, J. Rubenstein, R. Boyer, E.S. Stenroos, S. Chandrasekharappa, A. Athanassiadou, T. Papapetropoulos, W.G. Johnson, A.M. Lazzarini, R.C. Duvoisin, G. Di Iorio, L.I. Golbe, R.L. Nussbaum, Mutation in the alpha-synuclein gene identified in families with Parkinson's disease, Science, 276 (1997) 2045-2047.

[23] R. Sakai, M. Suzuki, M. Ueyama, T. Takeuchi, E.N. Minakawa, H. Hayakawa, K. Baba, H. Mochizuki, Y. Nagai, E46K mutant α-synuclein is more degradation resistant and exhibits greater toxic effects than wild-type α-synuclein in Drosophila models of Parkinson's disease, PloS one, 14 (2019) e0218261.

[24] J.J. Zarranz, J. Alegre, J.C. Gómez-Esteban, E. Lezcano, R. Ros, I. Ampuero, L. Vidal, J. Hoenicka, O. Rodriguez, B. Atarés, V. Llorens, E. Gomez Tortosa, T. del Ser, D.G. Muñoz, J.G. de Yebenes, The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia, Annals of neurology, 55 (2004) 164-173.

[25] W.S. Davidson, A. Jonas, D.F. Clayton, J.M. George, Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes, The Journal of biological chemistry, 273 (1998) 9443-9449.

[26] H.-N. Du, L. Tang, X.-Y. Luo, H.-T. Li, J. Hu, J.-W. Zhou, H.-Y. Hu, A Peptide Motif Consisting of Glycine, Alanine, and Valine Is Required for the Fibrillization and Cytotoxicity of Human α-Synuclein, Biochemistry, 42 (2003) 8870-8878.

[27] B.I. Giasson, I.V. Murray, J.Q. Trojanowski, V.M. Lee, A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly, The Journal of biological chemistry, 276 (2001) 2380-2386.

[28] H.A. Lashuel, C.R. Overk, A. Oueslati, E. Masliah, The many faces of α-synuclein: from structure and toxicity to therapeutic target, Nat Rev Neurosci, 14 (2012) 38-48.

[29] G. Fusco, S.W. Chen, P.T.F. Williamson, R. Cascella, M. Perni, J.A. Jarvis, C. Cecchi, M. Vendruscolo, F. Chiti, N. Cremades, L. Ying, C.M. Dobson, A. De Simone, Structural basis of membrane disruption and cellular toxicity by α-synuclein oligomers, Science, 358 (2017) 1440-1443.

[30] W. Peelaerts, L. Bousset, A. Van der Perren, A. Moskalyuk, R. Pulizzi, M. Giugliano, C. Van den Haute, R. Melki, V. Baekelandt, α-Synuclein strains cause distinct synucleinopathies after local and systemic administration, Nature, 522 (2015) 340-344.

[31] C.I. Bargmann, Neurobiology of the Caenorhabditis elegans Genome, Science, 282 (1998) 2028-2033.

[32] J.G. White, E. Southgate, J.N. Thomson, S. Brenner, The structure of the nervous system of the nematode Caenorhabditis elegans, Philosophical Transactions of the Royal Society of London. B, Biological Sciences, 314 (1986) 1-340.

[33] C.H. Lai, C.Y. Chou, L.Y. Ch'ang, C.S. Liu, W. Lin, Identification of novel human genes evolutionarily conserved in Caenorhabditis elegans by comparative proteomics, Genome Res, 10 (2000) 703-713.

[34] A. Abeliovich, Y. Schmitz, I. Fariñas, D. Choi-Lundberg, W.-H. Ho, P.E. Castillo, N. Shinsky, J.M.G. Verdugo, M. Armanini, A. Ryan, M. Hynes, H. Phillips, D. Sulzer, A. Rosenthal, Mice Lacking α-Synuclein Display Functional Deficits in the Nigrostriatal Dopamine System, Neuron, 25 (2000) 239-252.

[35] E. Masliah, E. Rockenstein, I. Veinbergs, M. Mallory, M. Hashimoto, A. Takeda, Y. Sagara, A. Sisk, L. Mucke, Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders, Science, 287 (2000) 1265-1269.

[36] S. Janezic, S. Threlfell, P.D. Dodson, M.J. Dowie, T.N. Taylor, D. Potgieter, L. Parkkinen, S.L. Senior, S. Anwar, B. Ryan, T. Deltheil, P. Kosillo, M. Cioroch, K. Wagner, O. Ansorge, D.M. Bannerman, J.P. Bolam, P.J. Magill, S.J. Cragg, R. Wade-Martins, Deficits in dopaminergic transmission precede neuron loss and dysfunction in a new Parkinson model, Proceedings of the National Academy of Sciences of the United States of America, 110 (2013) E4016-4025.

[37] M. Oliveras-Salvá, A. Van der Perren, N. Casadei, S. Stroobants, S. Nuber, R. D'Hooge, C. Van den Haute, V. Baekelandt, rAAV2/7 vector-mediated overexpression of alpha-synuclein in mouse substantia nigra induces protein aggregation and progressive dose-dependent neurodegeneration, Molecular neurodegeneration, 8 (2013) 44.

[38] V.M. Nemani, W. Lu, V. Berge, K. Nakamura, B. Onoa, M.K. Lee, F.A. Chaudhry, R.A. Nicoll, R.H. Edwards, Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis, Neuron, 65 (2010) 66-79.

[39] M. Atias, Y. Tevet, J. Sun, A. Stavsky, S. Tal, J. Kahn, S. Roy, D. Gitler, Synapsins regulate α-synuclein functions, Proceedings of the National Academy of Sciences, 116 (2019) 11116-11118.

[40] J. Sun, L. Wang, H. Bao, S. Premi, U. Das, E.R. Chapman, S. Roy, Functional cooperation of α-synuclein and VAMP2 in synaptic vesicle recycling, Proceedings of the National Academy of Sciences, 116 (2019) 11113-11115.

[41] J. Burré, M. Sharma, T. Tsetsenis, V. Buchman, M.R. Etherton, T.C. Südhof, Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro, Science, 329 (2010) 1663-1667.

[42] M. Lakso, S. Vartiainen, A.-M. Moilanen, J. Sirviö, J.H. Thomas, R. Nass, R.D. Blakely, G. Wong, Dopaminergic neuronal loss and motor deficits in Caenorhabditis elegans overexpressing human α-synuclein, Journal of neurochemistry, 86 (2003) 165-172.

[43] T.J. van Ham, K.L. Thijssen, R. Breitling, R.M.W. Hofstra, R.H.A. Plasterk, E.A.A. Nollen, C. elegans model identifies genetic modifiers of alpha-synuclein inclusion formation during aging, PLoS Genet, 4 (2008) e1000027-e1000027.

[44] Y. Zhao, S. Araki, J. Wu, T. Teramoto, Y.F. Chang, M. Nakano, A.S. Abdelfattah, M. Fujiwara, T. Ishihara, T. Nagai, R.E. Campbell, An expanded palette of genetically encoded Ca^{2+} indicators, Science (New York, N.Y.), 333 (2011) 1888-1891.

[45] M. Lakso, S. Vartiainen, A.M. Moilanen, J. Sirviö, J.H. Thomas, R. Nass, R.D. Blakely, G. Wong, Dopaminergic neuronal loss and motor deficits in Caenorhabditis elegans overexpressing human alpha-synuclein, Journal of neurochemistry, 86 (2003) 165-172.

[46] A.J. Harrington, A.L. Knight, G.A. Caldwell, K.A. Caldwell, Caenorhabditis elegans as a model system for identifying effectors of α-synuclein misfolding and dopaminergic cell death associated with Parkinson's disease, Methods, 53 (2011) 220-225.

[47] E.M.H. Tank, K.E. Rodgers, C. Kenyon, Spontaneous Age-Related Neurite Branching in Caenorhabditis elegans, The Journal of Neuroscience, 31 (2011) 9279-9288.

[48] C.-L. Pan, C.-Y. Peng, C.-H. Chen, S. McIntire, Genetic analysis of age-dependent defects of the Caenorhabditis elegans touch receptor neurons, Proceedings of the National Academy of Sciences of the United States of America, 108 (2011) 9274-9279.

[49] M.L. Toth, I. Melentijevic, L. Shah, A. Bhatia, K. Lu, A. Talwar, H. Naji, C. Ibanez-Ventoso, P. Ghose, A. Jevince, J. Xue, L.A. Herndon, G. Bhanot, C. Rongo, D.H. Hall, M. Driscoll, Neurite Sprouting and Synapse Deterioration in the Aging Caenorhabditis elegans Nervous System, The Journal of Neuroscience, 32 (2012) 8778-8790.

[50] M.L. Nonet, J.E. Staunton, M.P. Kilgard, T. Fergestad, E. Hartwieg, H.R. Horvitz, E.M. Jorgensen, B.J. Meyer, Caenorhabditis elegans rab-3 Mutant Synapses Exhibit Impaired Function and Are Partially Depleted of Vesicles, The Journal of Neuroscience, 17 (1997) 8061-8073.

[51] M. Zhen, Y. Jin, The liprin protein SYD-2 regulates the differentiation of presynaptic termini in C. elegans, Nature, 401 (1999) 371-375.

[52] E. Yeh, T. Kawano, R.M. Weimer, J.-L. Bessereau, M. Zhen, Identification of Genes Involved in Synaptogenesis Using a Fluorescent Active Zone Marker in Caenorhabditis elegans, The Journal of Neuroscience, 25 (2005) 3833-3841.

[53] M.A. Hilliard, A.J. Apicella, R. Kerr, H. Suzuki, P. Bazzicalupo, W.R. Schafer, In vivo imaging of C. elegans ASH neurons: cellular response and adaptation to chemical repellents, EMBO J, 24 (2005) 63-72.

[54] K.D. Kimura, A. Miyawaki, K. Matsumoto, I. Mori, The C. elegans Thermosensory Neuron AFD Responds to Warming, Current Biology, 14 (2004) 1291-1295.

[55] H. Suzuki, R. Kerr, L. Bianchi, C. Frøkjær-Jensen, D. Slone, J. Xue, B. Gerstbrein, M. Driscoll, W.R. Schafer, In Vivo Imaging of C. elegans Mechanosensory Neurons Demonstrates a Specific Role for the MEC-4

Channel in the Process of Gentle Touch Sensation, Neuron, 39 (2003) 1005-1017.

[56] H. Suzuki, T.R. Thiele, S. Faumont, M. Ezcurra, S.R. Lockery, W.R. Schafer, Functional asymmetry in Caenorhabditis elegans taste neurons and its computational role in chemotaxis, Nature, 454 (2008) 114-117.

[57] R.Y.N. Lee, L. Lobel, M. Hengartner, H.R. Horvitz, L. Avery, Mutations in the α1 subunit of an L-type voltage-activated Ca2+ channel cause myotonia in Caenorhabditis elegans, EMBO J, 16 (1997) 6066-6076.

[58] W. Schafer, C. Kenyon, A calcium-channel homologue required for adaptation to dopamine and serotonin in Caenorhabditis elegans, Nature, 375 (1995) 73-78.

[59] B. Shtonda, L. Avery, CCA-1, EGL-19 and EXP-2 currents shape action potentials in the Caenorhabditis elegans pharynx, Journal of Experimental Biology, 208 (2005) 2177-2190.

[60] K.A. Steger, B.B. Shtonda, C. Thacker, T.P. Snutch, L. Avery, The C. elegans T-type calcium channel CCA-1 boosts neuromuscular transmission, The Journal of experimental biology, 208 (2005) 2191-2203.

[61] E.A. Mathews, E. García, C.M. Santi, G.P. Mullen, C. Thacker, D.G. Moerman, T.P. Snutch, Critical residues of the Caenorhabditis elegans unc-2 voltage-gated calcium channel that affect behavioral and physiological properties, The Journal of neuroscience : the official journal of the Society for Neuroscience, 23 (2003) 6537-6545.

[62] P. Thakur, L.S. Breger, M. Lundblad, O.W. Wan, B. Mattsson, K.C. Luk, V.M.Y. Lee, J.Q. Trojanowski, A. Björklund, Modeling Parkinson's disease

pathology by combination of fibril seeds and α-synuclein overexpression in the rat brain, Proceedings of the National Academy of Sciences, 114 (2017) E8284-E8293.

[63] V.M. Nemani, W. Lu, V. Berge, K. Nakamura, B. Onoa, M.K. Lee, F.A. Chaudhry, R.A. Nicoll, R.H. Edwards, Increased Expression of α-Synuclein Reduces Neurotransmitter Release by Inhibiting Synaptic Vesicle Reclustering after Endocytosis, Neuron, 65 (2010) 66-79.

[64] D.A. Scott, I. Tabarean, Y. Tang, A. Cartier, E. Masliah, S. Roy, A Pathologic Cascade Leading to Synaptic Dysfunction in α-Synuclein-Induced Neurodegeneration, The Journal of Neuroscience, 30 (2010) 8083-8095.

[65] C. Betzer, L.B. Lassen, A. Olsen, R.H. Kofoed, L. Reimer, E. Gregersen, J. Zheng, T. Calì, W.-P. Gai, T. Chen, A. Moeller, M. Brini, Y. Fu, G. Halliday, T. Brudek, S. Aznar, B. Pakkenberg, J.P. Andersen, P.H. Jensen, Alpha-synuclein aggregates activate calcium pump SERCA leading to calcium dysregulation, EMBO Rep, 19 (2018) e44617.

[66] T. Calì, D. Ottolini, A. Negro, M. Brini, α-Synuclein controls mitochondrial calcium homeostasis by enhancing endoplasmic reticulum-mitochondria interactions, The Journal of biological chemistry, 287 (2012) 17914-17929.

[67] D. Shakiryanova, G.M. Zettel, T. Gu, R.S. Hewes, E.S. Levitan, Synaptic neuropeptide release induced by octopamine without Ca²⁺ entry into the nerve terminal, Proceedings of the National Academy of Sciences, 108 (2011) 4477-4481.

[68] K.M. Danzer, W.P. Ruf, P. Putcha, D. Joyner, T. Hashimoto, C. Glabe, B.T. Hyman, P.J. McLean, Heat-shock protein 70 modulates toxic extracellular α-synuclein oligomers and rescues trans-synaptic toxicity, The FASEB Journal, 25 (2011) 326-336.

[69] B. Dehay, M. Vila, E. Bezard, P. Brundin, J.H. Kordower, Alpha-synuclein propagation: New insights from animal models, Movement disorders : official journal of the Movement Disorder Society, 31 (2016) 161-168.

[70] P. Desplats, H.-J. Lee, E.-J. Bae, C. Patrick, E. Rockenstein, L. Crews, B. Spencer, E. Masliah, S.-J. Lee, Inclusion formation and neuronal cell death through neuron-to-neuron transmission of α-synuclein, Proceedings of the National Academy of Sciences, 106 (2009) 13010-13015.

[71] C. Hansen, E. Angot, A.L. Bergström, J.A. Steiner, L. Pieri, G. Paul, T.F. Outeiro, R. Melki, P. Kallunki, K. Fog, J.Y. Li, P. Brundin, α-Synuclein propagates from mouse brain to grafted dopaminergic neurons and seeds aggregation in cultured human cells, The Journal of clinical investigation, 121 (2011) 715-725.

[72] S. Holmqvist, O. Chutna, L. Bousset, P. Aldrin-Kirk, W. Li, T. Björklund, Z.-Y. Wang, L. Roybon, R. Melki, J.-Y. Li, Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats, Acta Neuropathologica, 128 (2014) 805-820.

[73] K.C. Luk, V. Kehm, J. Carroll, B. Zhang, P. O'Brien, J.Q. Trojanowski, V.M.-Y. Lee, Pathological α-Synuclein Transmission Initiates Parkinson-like Neurodegeneration in Nontransgenic Mice, Science, 338 (2012) 949-953.

[74] N.L. Rey, S. George, J.A. Steiner, Z. Madaj, K.C. Luk, J.Q. Trojanowski, V.M. Lee, P. Brundin, Spread of aggregates after olfactory bulb injection of α-synuclein fibrils is associated with early neuronal loss and is reduced long term, Acta neuropathologica, 135 (2018) 65-83.

[75] N.L. Rey, G.H. Petit, L. Bousset, R. Melki, P. Brundin, Transfer of human α-synuclein from the olfactory bulb to interconnected brain regions in mice, Acta Neuropathologica, 126 (2013) 555-573.

[76] Laura A. Volpicelli-Daley, Kelvin C. Luk, Tapan P. Patel, Selcuk A. Tanik, Dawn M. Riddle, A. Stieber, David F. Meaney, John Q. Trojanowski, Virginia M.Y. Lee, Exogenous α-Synuclein Fibrils Induce Lewy Body Pathology Leading to Synaptic Dysfunction and Neuron Death, Neuron, 72 (2011) 57-71.

[77] H. Braak, U. Rüb, W.P. Gai, K. Del Tredici, Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen, Journal of Neural Transmission, 110 (2003) 517-536.

[78] T.F. Outeiro, P. Putcha, J.E. Tetzlaff, R. Spoelgen, M. Koker, F. Carvalho, B.T. Hyman, P.J. McLean, Formation of toxic oligomeric alpha-synuclein species in living cells, PloS one, 3 (2008) e1867.

[79] K.R. Schuske, J.E. Richmond, D.S. Matthies, W.S. Davis, S. Runz, D.A. Rube, A.M. van der Bliek, E.M. Jorgensen, Endophilin is required for synaptic vesicle endocytosis by localizing synaptojanin, Neuron, 40 (2003) 749-762.

[80] S.G. Clark, D.L. Shurland, E.M. Meyerowitz, C.I. Bargmann, A.M. van der Bliek, A dynamin GTPase mutation causes a rapid and reversible

temperature-inducible locomotion defect in C. elegans, Proceedings of the National Academy of Sciences of the United States of America, 94 (1997) 10438-10443.

[81] H. Gad, N. Ringstad, P. Löw, O. Kjaerulff, J. Gustafsson, M. Wenk, G. Di Paolo, Y. Nemoto, J. Crum, M.H. Ellisman, P. De Camilli, O. Shupliakov, L. Brodin, Fission and Uncoating of Synaptic Clathrin-Coated Vesicles Are Perturbed by Disruption of Interactions with the SH3 Domain of Endophilin, Neuron, 27 (2000) 301-312.

[82] N. Ringstad, H. Gad, P. Löw, G. Di Paolo, L. Brodin, O. Shupliakov, P. De Camilli, Endophilin/SH3p4 Is Required for the Transition from Early to Late Stages in Clathrin-Mediated Synaptic Vesicle Endocytosis, Neuron, 24 (1999) 143-154.

[83] A. Lionnet, L. Leclair-Visonneau, M. Neunlist, S. Murayama, M. Takao, C.H. Adler, P. Derkinderen, T.G. Beach, Does Parkinson's disease start in the gut?, Acta neuropathologica, 135 (2018) 1-12.

[84] A.J. Schaser, T.L. Stackhouse, L.J. Weston, P.C. Kerstein, V.R. Osterberg, C.S. López, D.W. Dickson, K.C. Luk, C.K. Meshul, R.L. Woltjer, V.K. Unni, Trans-synaptic and retrograde axonal spread of Lewy pathology following pre-formed fibril injection in an in vivo A53T alpha-synuclein mouse model of synucleinopathy, Acta neuropathologica communications, 8 (2020) 150.

[85] C. Mezias, N. Rey, P. Brundin, A. Raj, Neural connectivity predicts spreading of alpha-synuclein pathology in fibril-injected mouse models: Involvement of retrograde and anterograde axonal propagation, Neurobiology of disease, 134 (2020) 104623.

[86] T. Uchihara, An order in Lewy body disorders: Retrograde degeneration in hyperbranching axons as a fundamental structural template accounting for focal/multifocal Lewy body disease, Neuropathology : official journal of the Japanese Society of Neuropathology, 37 (2017) 129-149.

[87] D.M. Mason, N. Nouraei, D.B. Pant, K.M. Miner, D.F. Hutchison, K.C. Luk, J.F. Stolz, R.K. Leak, Transmission of α-synucleinopathy from olfactory structures deep into the temporal lobe, Molecular Neurodegeneration, 11 (2016) 49.

[88] M.T. Henrich, F.F. Geibl, H. Lakshminarasimhan, A. Stegmann, B.I. Giasson, X. Mao, V.L. Dawson, T.M. Dawson, W.H. Oertel, D.J. Surmeier, Determinants of seeding and spreading of α-synuclein pathology in the brain, Science advances, 6 (2020).

[89] M.X. Henderson, E.J. Cornblath, A. Darwich, B. Zhang, H. Brown, R.J. Gathagan, R.M. Sandler, D.S. Bassett, J.Q. Trojanowski, V.M.Y. Lee, Spread of α-synuclein pathology through the brain connectome is modulated by selective vulnerability and predicted by network analysis, Nature neuroscience, 22 (2019) 1248-1257.

[90] B. Frey, A. AlOkda, M.P. Jackson, N. Riguet, J.A. Duce, H.A. Lashuel, Monitoring alpha-synuclein oligomerization and aggregation using bimolecular fluorescence complementation assays: What you see is not always what you get, Journal of neurochemistry, (2020).

[91] E. Emmanouilidou, K. Melachroinou, T. Roumeliotis, S.D. Garbis, M. Ntzouni, L.H. Margaritis, L. Stefanis, K. Vekrellis, Cell-Produced α-Synuclein

Is Secreted in a Calcium-Dependent Manner by Exosomes and Impacts Neuronal Survival, The Journal of Neuroscience, 30 (2010) 6838-6851.

[92] K.M. Danzer, L.R. Kranich, W.P. Ruf, O. Cagsal-Getkin, A.R. Winslow, L. Zhu, C.R. Vanderburg, P.J. McLean, Exosomal cell-to-cell transmission of alpha synuclein oligomers, Molecular Neurodegeneration, 7 (2012) 42.

[93] J.A. Steiner, E. Angot, P. Brundin, A deadly spread: cellular mechanisms of α-synuclein transfer, Cell death and differentiation, 18 (2011) 1425-1433.

[94] A.T. Medeiros, L.G. Soll, I. Tessari, L. Bubacco, J.R. Morgan, α-Synuclein Dimers Impair Vesicle Fission during Clathrin-Mediated Synaptic Vesicle Recycling, Frontiers in cellular neuroscience, 11 (2017) 388.

[95] F. Longhena, G. Faustini, C. Missale, M. Pizzi, P. Spano, A. Bellucci, The Contribution of α-Synuclein Spreading to Parkinson's Disease Synaptopathy, Neural Plast, 2017 (2017) 5012129.

[96] J. Lautenschläger, C.F. Kaminski, G.S. Kaminski Schierle, α-Synuclein - Regulator of Exocytosis, Endocytosis, or Both?, Trends in Cell Biology, 27 (2017) 468-479.

[97] H.-J. Lee, E.-J. Bae, S.-J. Lee, Extracellular α-synuclein—a novel and crucial factor in Lewy body diseases, Nature Reviews Neurology, 10 (2014) 92-98.

[98] T. Tian, Y.L. Zhu, Y.Y. Zhou, G.F. Liang, Y.Y. Wang, F.H. Hu, Z.D. Xiao, Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery, The Journal of biological chemistry, 289 (2014) 22258-22267.

[99] H.-J. Lee, S. Patel, S.-J. Lee, Intravesicular localization and exocytosis of alpha-synuclein and its aggregates, The Journal of neuroscience : the official journal of the Society for Neuroscience, 25 (2005) 6016-6024.

[100] D.E. Mor, E. Tsika, J.R. Mazzulli, N.S. Gould, H. Kim, M.J. Daniels, S. Doshi, P. Gupta, J.L. Grossman, V.X. Tan, R.G. Kalb, K.A. Caldwell, G.A. Caldwell, J.H. Wolfe, H. Ischiropoulos, Dopamine induces soluble α-synuclein oligomers and nigrostriatal degeneration, Nature Neuroscience, 20 (2017) 1560-1568.

[101] H. Braak, K.D. Tredici, U. Rüb, R.A.I. de Vos, E.N.H. Jansen Steur, E. Braak, Staging of brain pathology related to sporadic Parkinson's disease, Neurobiology of Aging, 24 (2003) 197-211.

[102] E. Stolzenberg, D. Berry, D. Yang, E.Y. Lee, A. Kroemer, S. Kaufman, G.C.L. Wong, J.J. Oppenheim, S. Sen, T. Fishbein, A. Bax, B. Harris, D. Barbut, M.A. Zasloff, A Role for Neuronal Alpha-Synuclein in Gastrointestinal Immunity, Journal of innate immunity, 9 (2017) 456-463.

[103] E. Svensson, E. Horváth-Puhó, R.W. Thomsen, J.C. Djurhuus, L. Pedersen, P. Borghammer, H.T. Sørensen, Vagotomy and subsequent risk of Parkinson's disease, Annals of neurology, 78 (2015) 522-529.

[104] A. Abbott, Are infections seeding some cases of Alzheimer's disease?, Nature, 587 (2020) 22-25.

[105] E.E. Ambroggio, D.H. Kim, F. Separovic, C.J. Barrow, K.J. Barnham, L.A. Bagatolli, G.D. Fidelio, Surface Behavior and Lipid Interaction of Alzheimer ß-Amyloid Peptide 1–42: A Membrane-Disrupting Peptide, Biophysical Journal, 88 (2005) 2706-2713.

[106] S.J. Soscia, J.E. Kirby, K.J. Washicosky, S.M. Tucker, M. Ingelsson, B. Hyman, M.A. Burton, L.E. Goldstein, S. Duong, R.E. Tanzi, R.D. Moir, The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide, PloS one, 5 (2010) e9505.

[107] D.K.V. Kumar, S.H. Choi, K.J. Washicosky, W.A. Eimer, S. Tucker, J. Ghofrani, A. Lefkowitz, G. McColl, L.E. Goldstein, R.E. Tanzi, R.D. Moir, Amyloid-ß peptide protects against microbial infection in mouse and worm models of Alzheimer's disease, Science Translational Medicine, 8 (2016) 340ra372-340ra372.

[108] W.A. Eimer, D.K. Vijaya Kumar, N.K. Navalpur Shanmugam, A.S. Rodriguez, T. Mitchell, K.J. Washicosky, B. György, X.O. Breakefield, R.E. Tanzi, R.D. Moir, Alzheimer's Disease-Associated ß-Amyloid Is Rapidly Seeded by Herpesviridae to Protect against Brain Infection, Neuron, 100 (2018) 1527-1532.

[109] N. Kobayashi, J. Masuda, J.U.N. Kudoh, N. Shimizu, T. Yoshida, BindingSites on Tau Proteins as Components for Antimicrobial Peptides, Biocontrol Science, 13 (2008) 49-56.

[110] M. Pasupuleti, M. Roupe, V. Rydengård, K. Surewicz, W.K. Surewicz, A. Chalupka, M. Malmsten, O.E. Sörensen, A. Schmidtchen, Antimicrobial Activity of Human Prion Protein Is Mediated by Its N-Terminal Region, PloS one, 4 (2009) e7358.

[111] M.P. Taylor, L.W. Enquist, Axonal spread of neuroinvasive viral infections, Trends Microbiol, 23 (2015) 283-288.