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Pathological Implications of the Interaction Between Neurexins and Alpha-synuclein in Synucleinopathies

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Résumé

La maladie de Parkinson (PD) et la démence à corps de Lewy (DLB) sont les deuxième et troisième maladies neurodégénératives les plus communes et font partie d'une classe de maladies appelées synucléinopathies. Les synucléinopathies sont associées à une pathologie liée à l'α-synucléine (α-syn) laquelle se caractérise par une accumulation de cette protéine dans les neurones, formant ainsi les corps de Lewy. L'α-syn pathologique se retrouve aussi sous forme d'oligomères et de fibrilles, qui sont toxiques pour les neurones et leurs synapses. L'une des premières anomalies observables chez les patients atteints de synucléinopathies est la dysfonction synaptique, souvent combinée à une perte de synapses. Il a été rapporté que les oligomères d'α-syn retrouvés au niveau des synapses précèdent la formation de corps de Lewy dans les neurones et leur transmission semble être associée à la progression des symptômes. Pourtant, les mécanismes moléculaires sous-jacents la dysfonction synaptique causée par l'α-syn restent inconnus. D'autre part, le fonctionnement normal des synapses est fortement régulé par une famille de protéines appelées organisateurs synaptiques. Les organisateurs synaptiques, incluant la protéine neurexine, sont des molécules d'adhésion cellulaire qui régulent la synaptogenèse, la plasticité, la libération des neurotransmetteurs et les fonctions cognitives. De plus, nous avons préliminairement montré que l'α-syn interagit avec l'isoforme β des neurexines (NRXs) (β-NRXs). Mon projet avait donc pour but de caractériser l'interaction α-syn/β-NRX et d'évaluer comment celle-ci contribue à la pathologie liée à l'α-syn. **Nous avons émis l'hypothèse que cette interaction affecte la fonction synaptogénique liée aux NRXs et son trafic.** Dans un premier temps, pour tester notre hypothèse, l'interaction α-syn/β-NRX a été évaluée grâce à des analyses de liaison à la surface cellulaire. Il a été constaté que les oligomères d'α-syn se lient fortement à NRX1,2β de manière dépendante du domaine riche en histidine (HRD), caractéristique de l'isoforme β, et cela sans perturber sa liaison à ses ligands endogènes postsynaptiques, neuroligine 1 (NLG1) et « leucine rich repeat transmembrane neuronal 2 » (LRRTM2). De plus, à travers des essais d'internalisation, nous avons observé que les oligomères d'α-syn altèrent le trafic de NRX1β en augmentant son internalisation de façon dépendante au HRD et altèrent également la différenciation NRX-dépendante de la synapse en synapse inhibitrice. Par

conséquent, nous suggérons que cette internalisation accrue pourrait affecter la fonction synaptogénique associée aux NRXs. Ce travail contribue à une meilleure compréhension sur la façon dont l'α-syn provoque un dysfonctionnement synaptique, fournissant de nouvelles perspectives moléculaires et pharmacologiques sur les synucléinopathies.

Mots-clés : Alpha-synucléine, neurexines, synucléinopathies, maladie de Parkinson, démence à corps de Lewy, organisateurs synaptiques, dysfonction synaptique, toxicité synaptique.

Abstract

Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are the second and the third most common neurodegenerative disorders and are part of a class of diseases called synucleinopathies. Synucleinopathies are associated with an α-synuclein (α-syn) pathology which shows an accumulation of α-syn in neurons, forming Lewy bodies. This pathological α-syn can form oligomers and fibrils, which are toxic for neurons and their synapses. One of the first changes to occur in patients' brain with synucleinopathies is synaptic dysfunction often combined with synapse loss. Synaptic α-syn oligomers were revealed to precede the formation of Lewy bodies, and their transmission to other neurons to correlate with the progression of the symptoms. Yet, the molecular mechanisms underlying how α-syn leads to synaptic dysfunction are unknown. Synaptic function is highly regulated by a protein family called synaptic organizers. Synaptic organizers are cell adhesion molecules that regulate synaptogenesis, plasticity, neurotransmitter release, synaptic plasticity and cognitive functions. Of this family, we have found that α-syn interacts with the β-isoforms of the neurexins (NRXs) family members (β-NRXs). My project aimed to characterize α-syn/β-NRX interaction and to evaluate how this interaction contributes to α-syn pathology. **We hypothesized that this interaction affects NRX trafficking and its synaptic function.** Firstly, to test our hypothesis, the α-syn/β-NRX interaction was characterized by performing cell surface binding assays. I found that α-syn oligomers strongly bind to NRX1,2β in a histidine rich domain (HRD)-dependent manner, without disrupting NRX binding to its postsynaptic binding partners, neuroligin 1 (NLG1) and leucine rich repeat transmembrane neuronal 2 (LRRTM2). Moreover, using internalization assays, we discovered that α-syn oligomers impair NRX trafficking by increasing NRX1β internalization in an HRD-dependent manner and impair NRX-dependent inhibitory presynaptic differentiation. Thereby, we suggest that this increased internalization affects the inhibitory synaptogenic function of NRX-based synaptic organizing complexes. This work contributes to a better understanding of how α-syn causes synaptic dysfunction, providing promising new molecular mechanisms and pharmacological insights into synucleinopathies.

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Abbreviations

α-NRX: α-isoform of neurexin

α-syn: Alpha-synuclein

β-NRX: β-isoform of neurexin

γ-NRX: γ-isoform of neurexin

6-OHDA: 6-hydroxydopamine

AChE: Acetylcholinesterase

AD: Alzheimer's disease

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

ANOVA: Analysis of variance

APOE: Apolipoprotein E

APP: Amyloid precursor protein

ASO: Antisense oligonucleotide

ATP: Adenosine triphosphate

Aβ: Amyloid-beta

BSA: Bovine serum albumin

CAG: CMV early enhancer/chicken β actin

CFP: Cyan fluorescent protein

CMV: Human cytomegalovirus

CNV: Copy number variant

DIV: Days *in vitro*

DJ-1: Protein deglycase

DLB: Dementia with Lewy bodies

DLS: Dynamic light scattering

ECS: Extracellular solution

EGF: Epidermal growth factor

EM: Electron microscopy

EPSC: Excitatory postsynaptic current

ER: Endoplasmic reticulum

GABA: Gamma-aminobutyric acid

GBA: β-Glucocerebrosidase

GCI: Glial cytoplasmic inclusions

GFP: Green fluorescent protein

GST-tag: Glutathione S-transferase

GWAS: Genome-wide association studies

HRD: Histidine-rich domain

HRP: Horseradish peroxidase

Ig: Immunoglobulin-like

IgSF21: Immunoglobulin superfamily member 21

IL1RaP: Interleukin-1 receptor accessory protein

IL1RAPL1: Interleukin 1 receptor accessory protein like 1

IPSC: Inhibitory postsynaptic current

KO: Knockout

LAG3: Lymphocyte-activation gene 3

LAR-RPTP: Leukocyte common antigen-related receptor protein tyrosine phosphatase

LB: Lewy bodies

LBV/AD: Alzheimer's disease with Lewy bodies

LN: Lewy neurites

LNS: Laminin/neurexin/sex-hormone-binding globulin

LRRK2: Leucine-rich repeat kinase 2

LRRTM: Leucine rich repeat transmembrane neuronal protein

LTD: Long-term depression

LTP: Long-term potentiation

MAP2: Microtubule-associated protein 2

MAPT: Microtubule-associated protein tau

mEPSC: Miniature excitatory postsynaptic current

mGluR: Metabotropic glutamate receptor

MSA: Multiple system atrophy

MSN: Medium spiny neurons

NAC: Non-amyloid component

NBIA: Neurodegeneration with brain iron accumulation

NGL-3: netrin-G ligand 3

NLG: Neuroligin

NMDA: N-Methyl-D-aspartate receptors

NRX: Neurexin

PAF: Pure autonomic failure

PBM : PDZ-binding motif

PCR: Polymerase chain reaction

PD: Parkinson's disease

PDD: Parkinson's disease dementia

PFF: Preformed fibrils

PINK1: PTEN-induced kinase 1

PPR: Paired-pulse ratio

PrPc: Cellular prion protein

PSD-95: Postsynaptic density protein 95

REM: Rapid eye movement

RT: Room temperature

S4: Splicing site 4

SAP: Synapse associated protein

SDS-PAGE: Sodium dodecyl sulfate – polyacrylamide gel electrophoresis

SEM: Standard error of mean

SEP: Super-ecliptic pHluorin

Slitrk: SLIT and NTRK-like family member

SNAP-25: Synaptosomal-associated protein, 25 kDa

SNARE: Soluble NSF attachment receptor protein

SNc: Substantia nigra pars compacta

SNc DA: Dopaminergic neurons from the SNc

SNCA: Synuclein alpha

Sp: Signal peptide

TDP-43: TAR DNA-binding protein 43

TrkB: Tropomyosin receptor kinase B

TrkC: Tropomyosin receptor kinase C

TTX: Tetrodoxin

VGAT: Vesicular GABA transporter

VGlut1: Vesicular glutamate transporter

VPS35: Vacuolar protein sorting-associated protein 35

WT: Wild-type

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Chapter 1. Introduction

Neurodegenerative diseases are incurable diseases associated with the progressive loss of neurons in different brain regions. Many neurodegenerative disorders are characterized by the misfolding and accumulation of certain proteins which lead to the formation of insoluble aggregates in brain cells (neurons). Examples of such aggregates are amyloid-β (Aβ) and phosphorylated tau that accumulate in Alzheimer's disease (AD), α-synuclein in Parkinson's disease (PD) and Lewy body dementias, Huntingtin intranuclear inclusions in Huntington's disease and ubiquitin and TDP-43 inclusions in amyotrophic lateral sclerosis and frontotemporal dementia (1). Because of their association with these diseases, many studies have been carried out to understand the underlying mechanisms of these aggregates and how they result in cell toxicity. Strikingly, the pathogenesis in these diseases is thought to arise mostly from the smaller soluble aggregates of these misfolded proteins, called oligomers (2, 3). One of the most well studied proteins that accumulate in neurodegenerative diseases is α-synuclein (α-syn) which is present in structures called Lewy bodies (LB), Lewy neurites (LN) and glial cytoplasmic inclusions (GCI). First described by Fritz Heinrich Lewy in 1912 (4), LB and LN are the pathological hallmarks of Parkinson's disease and Lewy body dementias. α-syn accumulation-associated diseases are referred to synucleinopathies. These disorders are characterized by synaptic dysfunction in different brain regions, which can be linked to the observed clinical features, and likely to be due to α-syn oligomers and protofibrils (5, 6).

1.1 Synucleinopathies

Synucleinopathies are a diverse class of neurodegenerative disorders that share a common pathological inclusion of α-syn (7). This class of disorders includes PD, Parkinson's disease dementia (PDD), dementia with Lewy bodies (DLB), Alzheimer's disease with Lewy bodies (LBV/AD), neurodegeneration with brain iron accumulation (NBIA) type I, multiple system atrophy (MSA) and pure autonomic failure (PAF) (7). PD and DLB are the two most common synucleinopathies and are characterized by the accumulation of α-syn into insoluble aggregates which can be found in the soma (LB) and the processes of the neurons (neurites) (LN) (7). These

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accumulations are hallmarks in what is referred as Lewy pathology and are composed mainly of α-syn and ubiquitin, as well as other constituents such as neurofilaments observed by Shults C.W. (8). Surprisingly, the inclusions found in PD and DLB differ from those of α-syn found in MSA and NBIA type I which are composed of tubulofilamentous inclusions of α-syn found in oligodendrocytes called glial cytoplasmic inclusions (GCIs) (9).

Because synucleinopathies differ in term of symptoms, growing evidence has suggested that the localization and nature of the pathological inclusions of α-syn are linked to the clinical symptoms and therefore the diagnosis (9). In 2003, Braak et al. described a correlation between the topographic localization of the LBs and neuropathological stages in PD (10). In addition, LB can be present in different brain cells, such as neurons and glial cells, as well as in different brain regions which correlate with different diagnostics (7). This suggests that α-syn plays an essential role in the pathogenesis of these diseases. Furthermore, by using genome-wide association studies (GWAS) and candidate gene-based approaches, point mutations, duplications and triplications in *SNCA* gene, which codes for α-syn, have been identified in PD and DLB cases (11-17). Polymorphisms in this gene were also identified as high-risk factors for sporadic PD (18-26), DLB (27, 28), MSA (29, 30) and LBV/AD (28, 31).

1.1.1 Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disease and is characterized by four clinical features: bradykinesia, muscle rigidity, resting tremor and postural instability (9, 32), with the latter not always being present at the early stages of the disease (32). In addition to these symptoms, other clinical features are present in some cases, such as dysarthria, dysphagia, autonomic dysfunction, cognitive and/or neurobehavioral abnormalities, sleep disorders and sensory abnormalities (33). Prodromal stages of PD can include olfaction loss, sleep disorders (rapid eye movement (REM) sleep behavior disorder), constipation and depression (34). Other evidence suggests that visual changes, autonomic disorders and small cognitive changes can also occur in the prodromal stages (34).

A) Neuropathological Features

The neuropathological features of PD commonly include α-syn inclusions throughout the brain and the degeneration of neurons in different brain regions, particularly, the loss of dopaminergic neurons (DA) in the substantia nigra pars compacta (SNc) result in the characteristic motor dysfunctions in PD. Interestingly, it has been suggested that the distribution of LBs and LNs are correlated to PD's symptoms (10, 35). The disease progression has been divided into six stages (10). In stages 1 and 2 of the disease, LBs and LNs are found in the olfactory bulb, the dorsal motor nucleus of the vagal nerve, the intermediate reticular zone, the raphe nuclei, the locus coeruleus, the enteric nervous system and peripheral parasympathetic and sympathetic nerves (10, 35). These stages are associated with prodromal symptoms such as hyposmia, autonomic dysfunction and sleep disorders (10, 35). In stages 3 and 4, LBs and LNs are found in the tegmental pedunculopontine nucleus, the SNc, the magnocellular nuclei of the basal forebrain, the hypothalamic tuberomammillary nucleus, the amygdala, the thalamus, the hippocampus, the transentorhinal region and entorhinal region (10, 35). Early motor dysfunction can be observed at these stages: asymmetric tremor, rigidity, hypokinesia (10, 35). Stages 5 and 6 are characterized by LBs and LNs in superordinate cortical areas, motor areas, premotor areas, sensory areas and prefrontal cortex, and by late phase motor symptoms such as cognitive impairments and dementia (10, 35). Interestingly, it has been shown that exogenous α-syn promotes the aggregation of endogenous α-syn and the formation of LN-like pathology in axons, leading to synaptic dysfunction by decreasing synaptic protein levels, impairing neuronal excitability and connectivity, and neuron death (36). Furthermore, evidence has shown that the initial phase of PD could be associated with synaptic dysfunctions which precede neuronal death induced by α-syn (37, 38). Taken together, these studies suggest that α-syn can contribute to the progression of the symptoms through its distribution and its effects on synapses and neurons.

The underlying mechanisms behind the selective cell loss of dopaminergic neurons in the SNc are not yet well understood. However, it has been suggested that these cells are more vulnerable to misfolded α-syn toxicity, mitochondrial dysfunction, ubiquitin-proteasome pathway dysfunction and oxidative stress, which are thought to be the mechanisms behind PD pathogenesis (39).

Furthermore, the loss of function of mitochondrial genes (DJ-1, PINK1, parkin) is observed in familial forms of the disease, leading to an increase in oxidative stress (40).

B) Vulnerability

To explain this vulnerability of SNc DA neurons, there are three hypotheses. Firstly, that SNc DA neurons have a high energetic demand due to an important axonal arborization (41) associated with unmyelinated axons (42, 43) and a high number of neurotransmitter release sites (44). This high energetic demand increases mitochondrial oxidation stress, and interestingly, reducing the arborization size decreases the energetic burden (41). In addition, such high axonal arborization would exhibit a higher expression of α-syn, which could accentuate the α-syn pathology (40).

A second hypothesis arises from the unique electrical activity of the SNc DA neurons, as they have a slow pacemaker (2–10 Hz) activity combined with broad spikes. This electrical activity maximizes the entrance of calcium (45-47) through a high number of plasma membrane Cav1 calcium channels (48-51). In addition, SNc DA neurons have low levels of calcium buffering proteins, which give rise to high levels of intracellular calcium. By consequence, this high calcium level can alter the activity of other proteins (52) and can promote the entrance of calcium into the mitochondria, which stimulates oxidative stress and the production of ATP (49, 53).

The last hypothesis for this vulnerability is the use of dopamine as a neurotransmitter. It has been shown that dopamine oxidation by mitochondrial oxidative stress promotes the generation of dopamine quinones in humans, which alter the function of the mitochondria, the lysosome, the lysosomal enzyme and glucocerebrosidase (54). This lysosomal defect could then lead to the accumulation of cell debris and misfolded proteins such as α-syn.

C) Causes

The causes that lead to sporadic PD are unknown. However, studies on familial cases have identified genes implicated in this disease. Some point mutations or structural chromosome abnormalities on causative genes were identified in 5–10% of patients: *SNCA* (11, 13, 14, 17, 55-58), *LRRK2* (59-62), *VPS35* (63, 64), *PINK-1*(65, 66), *PARK7* (65, 67) and *PRKN* (68-70). The latter three may harbor autosomal recessive mutations, whereas the others may harbor autosomal dominant mutations. However, the vast majority of PD cases (71) are believed to be caused by a

combination of genetic, epigenetic and environmental factors. Genome-wide association studies identified 26 risk loci, including a high susceptibility loci in *SNCA*, *LRRK2*, *MAPT* (microtubule-associated protein tau) and *GBA* (glucosylceramidase beta) (25, 26). Besides genetic factors, meta-analysis studies have suggested a protective implication of cigarette smoking (71, 72) and caffeine consumption (73) in PD, while a higher risk when exposed to pesticides (71, 74, 75), organic solvents (75) or after a head injury (76).

1.1.2 Lewy body dementias

Lewy body dementias, including DLB and PDD, are the third most common neurodegenerative disorders and the second most common dementias following Alzheimer's disease. DLB and PDD differ in terms of dementia onset relative to parkinsonism onset. DLB is diagnosed when the dementia starts before or within a year of the parkinsonism's onset, but not all patients develop motor symptoms (77). In contrast, PDD is characterized by the onset of dementia one or more years after being diagnosed with PD (77). In both cases, they are characterized by four clinical features: progressive dementia with cognitive fluctuations, extrapyramidal signs (usually parkinsonism features like bradykinesia, rigidity and rarely tremor), visual hallucinations and increased sensitivity to neuroleptic drugs (9). Other clinical features include impairment in attention, memory, executive and visuospatial functions, changes in personality and mood, REM sleep behavior disorder, postural hypotension, daytime somnolence and urinary disturbance (78, 79).

A) Neuropathology Features

The neuropathological features in PDD and DLB are similar, both have LB and LN spread throughout the limbic and cerebral cortex (80), loss of dopaminergic neurons in the SNc (81) and loss of cholinergic activity in the midfrontal lobe (82, 83), likely due to neuronal loss (84). Most patients also have Aβ plaques and tau-containing neurofibrillary tangles (85-87), the two hallmarks of AD, and vascular pathology (85, 86). Although Aβ depositions are more prominent in DLB (88), in both cases, tau and Aβ pathologies are linked to more advanced dementia (89). The opposite can also exist, LBs can be found in AD patients' brain, known as Alzheimer's disease with Lewy bodies (LBV/AD) (90, 91). Cortical LBs also appear in late-stage patients of AD-like

dementia, which has been associated with extrapyramidal and neuropsychiatric symptoms (92, 93). This relation between Lewy body dementias and AD may suggest a common molecular pathway among the proteins implicated, including α-syn, Aβ and tau.

B) Causes

Similar to the other synucleinopathies, Lewy body dementias' causes remain unknown, but the genetic factors among DLB, PDD, PD and AD overlap (94). Rare autosomal dominant mutations were identified in *SNCA* and *LRRK2* genes (95). This genetic overlap suggests that PD, PDD and DLB are on a spectrum of diseases. A large multicenter study has identified mutations in *GBA* as a risk factor for DLB (96, 97). Moreover, the *APOE4* allele is another identified risk factor in sporadic Lewy body dementias and other synucleinopathies, which is also a risk factor in AD (98).

1.1.3 Other synucleinopathies

In addition to the previously described synucleinopathies, α-syn also accumulates in rare synuclein-related disorders. These rare disorders are characterized by a variety of symptoms that include parkinsonism, autonomic failure, cerebellar and bulbar impairments. The patient's brain of these synucleinopathies shows α-syn accumulation throughout the central nervous system, especially at the level of the striatonigral and olivopontocerebellar systems, as well as in the peripheral nervous system (99).

A) Multiple System Atrophy

Multiple system atrophy (MSA) was first described by Graham and Oppenheimer as a spectrum of three disorders that have a variable combination of different neuronal atrophies (100). These three different clinicopathological disorders were called olivopontocerebellar atrophy (101), striatonigral degeneration (102) and Shy-Drager Syndrome (103). Shy-Drager syndrome is characterized by the atrophy of olivopontocerebellar and striatonigral system, in addition to a progressive autonomic failure (103, 104). It was later observed that all of these three sporadic syndromes within the appellation MSA had the same common glial cytoplasmic inclusions (GCIs) (104). GCIs are tubulofilamentous inclusions of α-syn present in oligodendrocytes (104). Therefore, the appellations were changed to MSA type parkinsonian (MSA-P) and type cerebellar (MSA-C) (105). The major clinical features of MSAs are a variable combination of parkinsonism,

cerebellar ataxia, autonomic and gait impairments as well as cognitive and corticospinal tract dysfunctions (9, 105). The two neuropathological hallmarks of MSA are olivopontocerebellar and striatonigral dysfunctions, due to neuronal loss, gliosis, myelin pallor or loss, and axonal degeneration, which result in atrophy of these systems (106). GCIs are found in the neocortex, the hippocampus, the brainstem, the spinal cord and the dorsal root ganglia (9). Point mutations, duplications and triplications in *SNCA* gene were identified in families that have manifestations of both MSA and PD (57, 107, 108). In addition, mutations in *SNCA* (24, 29), *COQ2* (109, 110) and *MAPT* (24, 111, 112) were identified to increase the risk for PD and MSA.

B) Neurodegeneration with Brain Iron Accumulation Type I

Another synucleinopathy is a rare early-onset neurodegenerative disorder named NBIA type I, also known as Hallervoden-Spatz syndrome or adult neuroaxonal dystrophy (113-115). It is characterized by parkinsonism, cognitive decline and cerebellar and bulbar abnormalities (115, 116). In this disorder, neuronal loss, gliosis and iron deposition are observed in patients' brains, resulting in cerebral atrophy and lesions to the regions affected by iron deposition (115). The iron deposits are found in the globus pallidus, the red nucleus, the SNc and the dentate nucleus (cerebellum) (115-117). In addition to this iron deposition, GCIs can be observed together with LB-like inclusions, axonal swelling called spheroids composed of α-syn and rare tau neurofibrillary tangles (113, 115, 118). Both familial and sporadic cases of NBIA type I exist (115). Autosomal recessive missense and nonsense mutations in the gene encoding for PANK2 (119) were identified as a cause of the disease.

C) Pure Autonomic Failure

Pure autonomic failure (PAF) is a rare sporadic neurodegenerative disease characterized by a progressive autonomic failure without neurological symptoms. The key features are neurogenic orthostatic hypotension and urinary and gastrointestinal dysfunctions (120). The neuropathology of this disease shows degeneration in the thoracic spinal cord (121) and the paravertebral ganglia (122) of the pre- and postganglionic sympathetic and parasympathetic neurons, which also show α-syn pathology (123, 124). In contrast to MSA, which presents both peripheric and central

neurological symptoms, PAF presents specifically peripheral autonomic nervous system abnormalities (125). No risk genes or environmental factors have been yet identified.

1.2 Alpha-synuclein

α-syn is a protein of 140 amino acids, encoded by the *SNCA* gene, and it is present in all subcellular compartments of neurons, particularly enriched at presynaptic terminals (126, 127). This protein, in its monomeric form, is associated with distal reserve pools of synaptic vesicles (126). Given its localization, several studies have suggested that its principal physiological role could be the trafficking of synaptic vesicles (128-130). α-syn was first identified in the presynaptic compartment and on the nuclear envelope of neurons in the electric organ of *Torpedo californica* (131). It was later identified within amyloid plaques as the "non-amyloid-β-component (NAC)" (132). This protein is composed of three regions: an N-terminal amphipathic region, a central hydrophobic NAC region and a C-terminal acidic region (133).

1.2.1 Physiological functions

The physiological functions of endogenous monomeric α-syn are not yet well understood. Some studies have suggested that it plays multiple roles including the suppression of apoptosis by decreasing the activity of protein kinase C (134), regulation of glucose levels (135-137), modulation of the activity of calmodulin (138) and/or the prevention of oxidation of unsaturated lipids from the vesicles (139, 140). In synapses, its roles include the trafficking and recycling of synaptic vesicles (130), the regulation of dopamine synthesis (141) and the maintenance and assembly of the SNARE complex acting as a chaperone protein (128, 129). The SNARE complex (soluble NSF attachment protein receptor) is critical for the release of synaptic vesicles (142). Due to its important role in synaptic vesicle regulation and its location at the presynaptic site, it is suggested that α-syn plays a role in neurotransmitter release, plasticity and synaptic function (143).

1.2.2 Pathological functions

The precise roles of pathological α-syn in synucleinopathies are still unknown. Several mechanisms have been suggested, but it is not well understood whether α-syn is a cause or

consequence of the disease, and how α-syn contributes to these diseases. However, it has been proposed that the oligomers and protofibrils of α-syn are the toxic forms (2, 3). Recent studies detected α-syn oligomer accumulation in PD (144) and DLB (145) patient's brains with undetected Lewy pathology, suggesting that α-syn oligomers are sufficient to cause toxicity. The terms oligomers and protofibrils are used to describe a wide spectrum of soluble aggregates of α-syn which differ in molecular weight and conformation with a large variation of beta-sheet content and hydrophobicity (146). α-syn form a helix-rich intermediate oligomeric structure before forming fibrils (147), and there are subgroups of oligomers that have a cylindrical morphology (148). According to the literature, distinct soluble oligomers could have different biological properties and toxicity (149). Small annular oligomers provoke calcium influx and caspase activation, leading to cell death, whereas large oligomers do not provoke these phenotypes and instead have high seeding capacity (149). Moreover, a form of α-syn that has a large concentration of serine-129 phosphorylation (α-syn-S129) is found in LB (150, 151) and is thought to be involved in the pathology and toxicity (151, 152).

Previous evidence suggests that an imbalance in α-syn synthesis and clearance, results in an increase of abnormal α-syn levels and thus increased accumulation of α-syn (153). Such imbalance is thought to be due to a mutation, a copy number variant (CNV) or dysfunction in the clearance mechanisms such as the autophagy-lysosome and ubiquitin-proteasome pathways. The increase in α-syn levels results in a higher accumulation and toxicity to neurons (153). Subsequently, α-syn oligomers have been shown to decrease the transmembrane potential of mitochondria, thus impairing their function and cellular respiration (154). Moreover, α-syn accumulation in the mitochondria would compromise the function of complex I of the respiratory chain (155). Moreover, oligomers and protofibrils of α-syn accumulate in the endoplasmic reticulum (ER) and cause ER stress (156). It has also been observed that a variant of α-syn induces neuronal death by a "pore-forming" mechanism (157, 158). An additional work has found that α-syn oligomers inhibit neuronal SNARE-mediated vesicle docking and thereby, they alter neurotransmitters release (159). By disrupting neurotransmitter release, α-syn oligomers would diminish dopamine release and negatively affect the nigrostriatal system (160-165). Finally, α-syn

can be transmitted from neuron to neuron although the pathways involved in this transmission are still unknown (166-168).

Several studies have suggested that oligomers and protofibrils of α-syn cause toxicity in cells of the nervous system, particularly in neurons (143, 153, 154, 156, 157, 169-173). Interestingly, α-syn oligomers accumulate in synaptic terminals and reduce GABAergic inhibitory transmission in a mouse model of MSA (174). Moreover, presynaptic accumulation of α-syn was detected in tissue samples from PD and DLB patient brains (145, 175) and in A53T α-syn transgenic mice (176). The majority of aggregates (50%-92%) occur at the presynaptic terminals in the cortex of DLB's brain (145). On the other hand, monomeric α-syn was also detected at presynaptic terminals (126, 127). This localization suggests that α-syn may play a role in normal synaptic function and its accumulation as oligomers would cause synaptic dysfunction.

1.2.3 Alpha-synuclein spreading

The hypothesis of α-syn self-propagation by cell-to-cell transmission has recently been suggested as a key mechanism behind the progression of symptoms in PD. It first arose from the observation of LBs in different brain regions as the disease progress (10). The first regions touched by α-syn aggregation would be in the peripheral nervous system, the medulla oblongata and the pons (10, 35). As the disease progresses, α-syn inclusions would be found in the midbrain and thalamic regions and later in the neocortical regions (10, 35). According to Braak et al. (2003), Parkinson's disease can be divided into six stages link to both symptoms' progression and LB spreading in a caudo-rostral pattern (10). Furthermore, it has been demonstrated that grafted wild-type neuronal cells in PD model mice develop LB-like inclusions, confirming that α-syn can be transmitted from neuron to neuron (177, 178). Additional *in vivo* studies have revealed that the injection of synthetic recombinant α-syn preformed fibrils (PFFs) induces the aggregation of soluble α-syn to insoluble aggregates in neurons (36, 178, 179). This aggregation results in impairment of neuronal excitability and connectivity, and in neuronal death (36). The finding of extracellular α-syn species in human plasma and cerebrospinal fluid (180, 181) has further supported this hypothesis since it suggests that α-syn can be secreted and therefore potentially uptaken. It has been suggested that α-syn is taken up by classical endocytic mechanisms (36, 178,

182, 183) such as dynamic-dependent receptor-mediated endocytosis (177, 178) or by passive diffusion through the cell membrane (184-186). Interestingly, two promising binding partners that could mediate the uptake have been identified: the lymphocyte-activation gene 3 (LAG3) and the neurexins (NRXs) (187, 188). LAG3 has been shown to be involved in α-syn endocytosis, transmission, and toxicity (187). However, it has been suggested that α-syn would retrogradely spread and would be transmitted from postsynaptic sites to presynaptic sites (35, 189, 190), and it remains unclear if LAG3 is expressed in synaptic sites (187). Therefore, the identification of a presynaptic binding partner, such as NRXs, and its characterization would be essential to better understand α-syn spreading and a possible trans-synaptic transmission.

1.2.4 Synapse and alpha-synuclein

Given that α-syn is located at presynaptic terminals under physiological conditions (126, 127), monomeric α-syn seems to play an essential role in presynaptic function. Knockdown of α-syn in primary hippocampal neurons using antisense oligonucleotide (ASO), resulted in a reduction in the number of vesicles at the presynaptic terminals in the distal pool, but the number of vesicles docked to the membrane was unchanged (161). This result was replicated in α-syn knockout (KO) mice that presented a significant impairment in the hippocampal response to prolonged repetitive low-frequency stimulation and a slower renewal of docked vesicles from the pool (162). Response to brief high-frequency stimulation that depletes docked vesicles, basal synaptic transmission and paired-pulse facilitation were unchanged, suggesting that α-syn is not implicated in the regulation of docked vesicles (162). Mice lacking α-syn also show an increase in evoked dopamine (DA) release, suggesting that α-syn may act as a negative regulator on DA release (160).

Furthermore, synaptic dysfunction is thought to be a pathophysiological mechanism behind the symptoms of synucleinopathies (190). Indeed, α-syn oligomers impact synaptic plasticity (133) through their presynaptic accumulation resulting in synapse degeneration (191). PDD patients present synaptic loss in the hippocampus (192), and synucleinopathy model mice present changes in presynaptic and axonal transport as well as synapse loss, which precedes neuronal death (193). In contrast to α-syn monomers that are not involved in the regulation of vesicles docking (162),

α-syn oligomers bind to synaptobrevin, which is part of the SNARE complex, preventing the formation of the complex and resulting in impairment of vesicle docking (159). This could result in presynaptic dysfunction and impairment in neurotransmitter release. Furthermore, overexpression of α-syn inhibits neurotransmitter release by reducing vesicle recycling pool (194).

Moreover, α-syn accumulation also causes electrophysiological changes. The corticostriatal neurons of mice overexpressing α-syn show a presynaptic form of long-term depression (LTD) after high-frequency stimulation (195). In addition, some studies have shown that transgenic mice expressing human α-syn or α-syn with the PD-linked A30P mutation exhibit alterations in short-term plasticity such as paired-pulse depression and reduced paired-pulse facilitation (196, 197). In the dentate gyrus perforant pathway, aged mice overexpressing α-syn A30P present a decrease in basal synaptic transmission and paired-pulse facilitation. They also present abnormal LTD in a protocol that usually causes long-term potentiation (LTP) (196). α-syn oligomers have been shown to impair hippocampal LTP, contrarily to α-syn monomers and fibrils (198, 199). This dysfunction is mediated by the activation of NMDA receptors, which alters calcium homeostasis (198, 200). Extracellular α-syn oligomers bind to cellular prion proteins (PrPc), which results in Fyn phosphorylation and consequently NMDAR subunit 2B activation via mGluR5 and alters calcium homeostasis by activation of NMDAR (200). Blocking mGluR5 phosphorylation rescues synaptic and cognitive deficits, and mice with PrPc KO rescues the impaired LTP (200). In addition, mutated α-syn with PD-related mutations enhances the firing rates in SNc DA neurons (201). However, the role of extracellular α-syn oligomers on presynaptic vesicle release impairment remains unknown.

1.2.5 Amyloid-beta and alpha-synuclein

Most individuals with DLB have an overlap between the pathologies of AD and DLB (202), and 50% of AD patients present α-syn pathology (203). Several studies have shown that Aβ and α-syn show binding to each other *in vitro* and *in situ* and that they can aggregate together (204-206). On the other hand, although α-syn is normally present intracellularly, α-syn can be present extracellularly in some conditions (180, 181, 186), suggesting a possible interaction with extracellular Aβ. Previous evidence supports that extracellular α-syn oligomers could play a role in neurodegeneration (207, 208). Strikingly, α-syn regulates the fibrillation of Aβ and tau. In fact,

α-syn has been shown to inhibit the formation of Aβ *in vivo* (209) but enhance tau fibrillization (210-212). However, another study has shown that α-syn stimulates Aβ aggregation *in vitro* (213). Meanwhile, Aβ was shown to enhance α-syn accumulation (214). Finally, tau was also identified as a risk factor in PD (24), suggesting an interplay between AD and PD. Further studies would be essential to investigate molecular and functional associations among α-syn and Aβ or tau.

1.3 Synapses

In 1888, Ramón y Cajal provided for the first time strong evidence for the neuron doctrine. Specifically, that the processes of neurons (dendrites and axons) establish connections between each other (215). He was intrigued by the mechanisms behind the growth of these processes in terms of appearance, migration and connections.

> What mysterious forces precede the appearance of the processes, promoting their growth and ramification, provoking the coherent migration of the cells and fibres in predetermined directions, as if obeying a wise architectonic plan, and finally establishing those protoplasmic kisses, the intercellular articulations that appear to constitute the final ecstasy of an epic love story? *Ramón y Cajal, 1917* (216)

Later in 1897, Sir Charles Sherrington named those protoplasmic kisses "synapses" (217). Until the discovery of acetylcholine transmission from the vagus nerve to the heart muscle, it had been thought that synapses were only electrical (218). Now it is known that while electrical synapses exist, the majority of synapses are chemical. However, while the synapse is chemical, the release of neurotransmitters is caused by an electric current, called an action potential, resulting in a change in the postsynaptic neuron's potential. To have a chemical synapse, there must be both neurotransmitter releasing and receiving sites, respectively named the presynaptic terminal and the postsynaptic site. The alignment and maintenance of presynaptic and postsynaptic sites are essential for brain functions and occurs through cell adhesion molecules called synaptic organizers.

1.3.1 Synaptic organizers and synaptogenesis

Synaptic organizers have essential roles in forming synapses, referred as synaptogenesis, and in stabilizing and modulating them. Besides their synaptogenic roles, these molecules play a role in synaptic plasticity, neurotransmitter release and cognitive functions (219-223). Together with

their postsynaptic binding partners, presynaptic organizers form a trans-synaptic complex that induces both retrograde and anterograde synaptogenic signals (223, 224). The retrograde signal results in the formation of the presynaptic active zone composed of synaptic vesicles and proteins implicated in the neurotransmitter release, while the anterograde signal recruits the postsynaptic assembly such as the accumulation of neurotransmitter receptors and their scaffold proteins (223, 224).

At the presynaptic site, synaptic vesicles are filled with either excitatory, inhibitory or modulatory neurotransmitters. In the brain, the main excitatory neurotransmitter is glutamate, whereas the main inhibitory neurotransmitter is gamma-aminobutyric acid (GABA). Synaptic vesicles can be found in a distal reserve pool and proximal to the presynaptic terminal membrane, where they will concentrate to form the active zone (225). In this active zone, a high number of voltage-dependent calcium channels are found (226-228). Once these channels are activated by an action potential, calcium enters the presynaptic terminals promoting vesicle fusion (226, 227). This fusion occurs through the SNARE complex. Vesicular SNARE proteins, synaptobrevins/VAMPs (229-232), interact with membrane SNARE proteins, Syntaxin-1 (233) and SNAP-25 (234), to dock the vesicles to the membrane. In the presence of high calcium concentrations, synaptotagmin undergoes conformational changes and interacts with the SNARE complex, resulting in vesicle fusion (235). Other proteins such as the presynaptic scaffolding proteins, bassoon (236) and piccolo (237), are also recruited to the presynaptic site.

At the postsynaptic site, the anterograde signal from a synaptic organizing complex results in the recruitment of postsynaptic proteins. For glutamate excitatory synapses, the postsynaptic density includes the glutamate receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and N-Methyl-D-aspartate receptors (NMDARs) (238) and the scaffolding proteins including PSD-93/95, SAP, Shank and Homer (239). These scaffolding proteins are essential for the clustering, trafficking and anchoring of the synaptic cell adhesion proteins and neurotransmitter receptors (240). For GABA inhibitory synapses, the anterograde signal recruits the GABA receptors and the scaffolding protein gephyrin (241). Few GABAergic postsynaptic specific proteins have been identified. In contrast to excitatory synapses, inhibitory synapses do not present a postsynaptic density structure of multiple proteins (241).

There are two major families of pre-synaptic organizers identified so far: Neurexin (NRX) and Leukocyte common antigen-related receptor protein tyrosine phosphatase (LAR-RPTP). NRXs bind to diverse postsynaptic organizers including neuroligins (NLGs) (242, 243), LRRTMs (244-247), GluD1/2-Cblns complex (248, 249), Igsf21 (250) and calsystenin (243), whereas LAR-RPTPs bind to Slitrks (251, 252), TrkC (253), NGL-3 (254, 255) and IL1RAPs (256-258).

1.3.2 Neurexins

Given their high expression level in the brain, NRXs are considered as essential proteins for normal brain functions. NRXs were first identified as receptors for the protein α-latrotoxin, which is present in the venom of widow spiders and stimulates massive neurotransmitter release (259). There are three *NRXN* genes in mammals (*NRXN 1, 2* and *3*), and each gene contains two promoters expressing two isoforms: the long form α-NRX and the short form β-NRX (259-261). In addition to these α/β-NRX isoforms, *NRXN1* also presents a third isoform called γ-NRX, which is transcribed from an internal promoter (262). α-NRXs have six laminin/neurexin/sex-hormone-binding globulin (LNS) domains separated by three epidermal growth factor (EGF)-like repeats, an O-glycosylation region, a short cysteine-loop domain, a transmembrane region and a cytoplasmic tail that contains a PDZ-binding motif (PBM) (259, 260, 263, 264) (Fig. 1). β-NRXs have only one LNS domain, which is identical to the sixth LNS (LNS6) of α-NRXs and importantly contain a unique N-terminal domain called histidine-rich domain (HRD) (261, 263) (Fig. 1). γ-NRX1 was recently described and only has a cysteine loop, a transmembrane domain and a C-terminal tail, lacking all LNS domains (262). Furthermore, NRX contains five canonical alternative splicing sites, five in α-NRX and two in β-NRX, and can be present in different combinations resulting in more than 1000 isoforms of NRXs (263). This high variability is suggested to contribute to synapse specification (265-267). Most postsynaptic NRX-binding partners interact with LNS6 domains, and multiple studies have shown the importance of splicing site 4 found in LNS6 for these interactions (242, 244-248, 268-271).

NRXs are expressed from early stage in development, presumably for their synaptogenic role (272), and are constantly expressed through life for their importance in synapse maintenance

(267, 273, 274). In addition, NRXs are highly expressed through all the nervous system, especially in the hippocampus, and α-NRXs are more abundant than β-NRXs (273, 274).
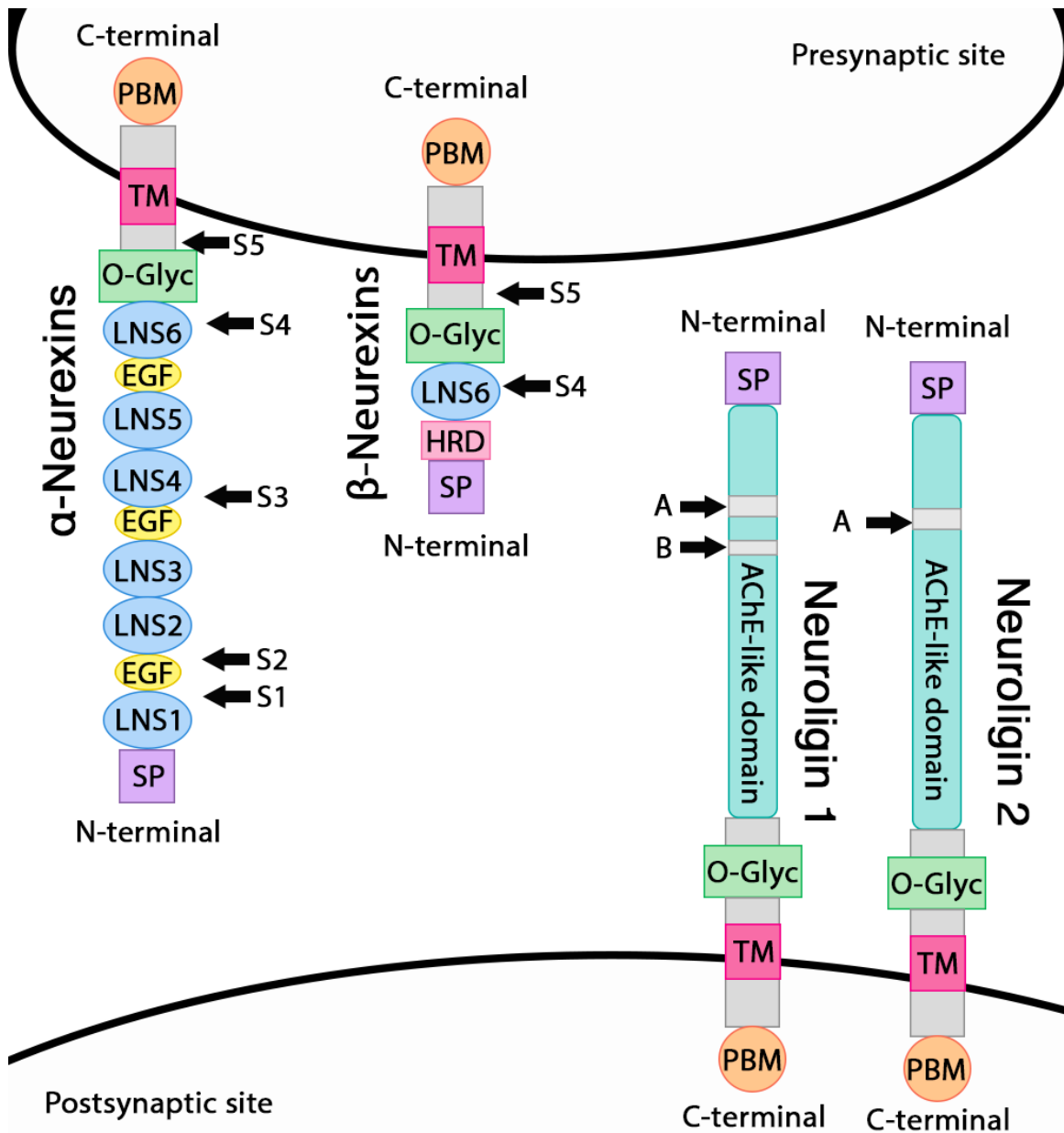
Figure 1. Neurexins and neuroligins structures show complex extracellular domains with several splicing sites

α-NRXs have five splicing sites (S1-5) and six LNS domains (LNS1-6) (275). β-NRXs have only two splicing sites (S4,5) and one LNS domain (LNS6) but contain a unique domain called a histidine-rich domain (HRD) (275). NLG1 contains two splicing sites (A and B), and NLG2 contains only one splicing site (A) (275). This figure was adapted from Li Q. et al. (2007) (275). SP, signal peptide; N, amino terminus; C, carboxyl terminus LNS,

laminin/neurexin/sex-hormone-binding globulin; EGF, epidermal growth factor-like repeats; O-Glyc, O-glycosylation region; TM, transmembrane domain; PBM, PDZ-binding motif.

A) Functions

NRX1β-overexpressing COS7 cells can induce excitatory and inhibitory postsynaptic differentiation when co-cultured with hippocampal neurons (276). Specifically, NRX1β with an insert at the splicing site 4 (NRX1β S4+) preferentially induces inhibitory synapses (277, 278). Furthermore, α-NRXs can recruit only inhibitory, but not excitatory, postsynaptic components (277). When all α-NRXs are deleted, mice die before birth due to respiratory impairment, and synapses show a decrease in inhibitory synapse density, leaving excitatory synapses number intact (279). However, both synapses show reduced neurotransmitter release (279, 280). On the other hand, KO of β-NRXs is not lethal but shows an important synaptic dysfunction (273). *In vivo* KO of β-NRXs reduces the release probability in the hippocampal neurons (273). When only α/β-NRX3 S4+ are constitutively expressed, the internalization of AMPAR is increased, which affects NMDAR-mediated LTP (274). Cell-specific deletion of all NRXs shows a decrease in synapse number and synaptic strength in parvalbumin-positive interneurons as well as a decrease in action potential-triggered Ca^{2+} influx and synaptic strength in somatostatin-positive interneurons (264). These studies indicate that NRXs regulate distinct cell-specific synapse functions.

1.3.3 Neurexin-binding partners

NRXs have multiple binding partners, including transmembrane proteins with either cis- or trans-interaction with NRXs and secreted adaptor proteins (222, 243, 272-274, 281-284). Major NRX partners are the postsynaptic organizers, which are transmembrane proteins essential for the normal function of NRXs in synaptogenesis, synaptic transmission, plasticity and synapse maintenance. Two well-described postsynaptic organizers interacting with NRXs are NLGs and LRRTMs.

A) Neuroligins

NLGs are encoded by five genes: *NLGN1,2,3,4X* and *4Y* (242, 284). NLG1 was first discovered in 1995 when identifying binding partners of β-NRXs in a rat brain lysate (242). NLGs are type I

transmembrane proteins, and they have an extracellular domain homologous to acetylcholinesterase (AChE) but lacking cholinesterase activity as well as a carbohydrate linker domain. They also have an intracellular C-terminal PDZ-binding motif tail (284) (Fig. 1). There are two alternative splicing sites in NLGs: splicing site A can be found in NLG1,2,3, and the splicing site B is only present in NLG1. Due to these splicing sites in *NLG* genes, there are multiple isoforms of NLGs, and they can be localized to distinct synapses even within the same neuron (284). The binding of NLGs to the LNS6 domain of NRXs is mediated by their AChE-like domain (243). The splicing sites in NLGs affect their interaction with α-NRXs and β-NRXs (285). NLG1 B+ binds only to NRX1β S4- but not to NRX1α. However, NLG1 B- binds to both α-NRXs and β-NRXs independently of the S4 site (243, 285). NLG1 is predominantly found at excitatory synapses (286) whereas NLG2 is predominantly found in inhibitory, cholinergic and dopaminergic synapses (276, 287-289). NLG3 is found in both excitatory and inhibitory synapses (290). NLG4 is localized to glycinergic synapses and a subclass of GABAergic synapses (291). Therefore, NLGs are considered essential for synapse specification.

Functions

NLGs, like NRXs, have many functions such as synaptogenesis, synapse maintenance and maturation (292). Overexpression of any NLG isoforms in neurons can induce the differentiation of both excitatory and inhibitory presynaptic terminals, although NLG2 preferentially induces presynaptic inhibitory terminals compared to NLG1 and 3 (293). Evidence suggests that NLG1 can drive both excitatory and inhibitory synapse formation (293, 294). It has been shown that the association of PSD-95 and NLG1 is involved in excitatory synapse differentiation (295). Because of their preferential synaptic localization, it is suggested that NLGs and their different binding partners play a critical role in excitatory and inhibitory synapse formation. NLG splicing also modulates synapse specificity, where the splicing site A of NLG1 decreases the excitatory synaptogenic activity of NLG1, whereas the splicing site B of NLG1 diminishes its inhibitory synaptogenic activity (278). In this artificial synapse formation assay, fibroblasts overexpressing NLG isoforms are co-cultured with neurons and NLGs in turn can bind to endogenous receptors such as NRXs, inducing presynaptic differentiation. It is still not well understood how these selective interactions and preferential synaptogenic activity play a role *in vivo*.

Constitutive NLG1,2,3 triple KO mice die after birth with severe synaptic transmission impairments but show no alteration in synapse density (292). Conditional KO of NLG1,2,3 in cerebellar Purkinje cells reduces excitatory and inhibitory synaptic transmission without any alteration in synapse number (296). Furthermore, NLG1 deletion blocks both NMDA-dependent LTP and NMDAR-independent LTP (297). NLG2 deletion reduces GABAergic and glycinergic inhibitory transmission (296, 298-300) and impairs selectively perisomatic inhibitory synapses number on CA1 hippocampal neurons (301). NLG3 deletion specifically reduces inhibitory transmission onto nucleus accumbens medium spiny neurons (302). NLG4 deletion leads to impaired GABAergic and glycinergic transmissions in the hippocampus and the brainstem, respectively (303, 304). These results suggest that NLGs are essential for synaptic transmission as well as synapse maintenance and specificity, but not in synapse development and establishment.

B) LRRTMs

LRRTM proteins are encoded by four genes (*LRRTM1*,2,3,4). LRRTM1 is the first identified LRRTM isoform as a synaptogenic molecule through an unbiased cDNA screen based on an artificial synapse formation assay (305, 306). All LRRTM isoforms share the same overall structure composed of 10 extracellular N-terminal leucine-rich repeats and a cytoplasmic tail (307). LRRTMs have been shown to be key organizers for excitatory synapses (305).

Functions

All LRRTMs induce excitatory presynaptic differentiation in artificial synapse formation assays (244, 246, 306, 308, 309). LRRTM2 exhibits the strongest synaptogenic activity and is localized to excitatory, but not inhibitory synapses (244, 245, 306). LRRTM1,2,3 bind to all NRX S4- isoforms, but not NRX S4+ isoforms (245, 246, 308). Interestingly, LRRTM4 binds to glypicans (308-310). Strikingly, LRRTMs compete with NLGs since they both interact with the LNS6 of NRXs (245, 247). *In vivo*, deletion of LRRTM1, LRRTM3, or LRRTM4 shows a small reduction of excitatory synapse density and evoked synaptic transmission (246, 308, 311). Furthermore, double KO of LRRTM1/2 reduces basal AMPAR-mediated synaptic transmission and LTP in the hippocampal CA1 pyramidal neurons (312).

1.3.4 Neurexins and diseases

Mutations in *NRXN* genes were identified as risk factors in autism spectrum disorder, Tourette's syndrome, learning disabilities, neurodevelopmental disorders and schizophrenia (313-323). In schizophrenia, both copy number variants in the promoter and in the first exon and micro-deletions within NRX1α have been identified (320, 324-328). Common genetic variation in NRX1 has been related to impact responsiveness to antipsychotics and antidepressant treatments as well as nicotine dependence (329-334). Deletions of NRX1 in humans were also found to predispose to a spectrum of neurodevelopmental disorders (335), and NRX1α deletion in mice correlates with behavioral changes and cognitive impairments (336).

NRXs have been also shown to be implicated in neurodegenerative diseases. Studies have previously described an interaction between NRXs and amyloid-β (Aβ) (337, 338). Aβ oligomers bind to the HRD of β-NRX1,2,3 and the S4 of α/β-NRX1,2. Importantly, Aβ binding to NRX1β reduces NRX1β surface level in its HRD-dependent manner (338). According to artificial synapse formation assays, Aβ oligomers also diminish synaptogenic activity of NLG1/2 and LRRTM2 to induce excitatory, but not inhibitory, presynaptic differentiation, which is mediated by presynaptic NRXs. Transgenic mice expressing the human form of mutated amyloid precursor protein (APP), have shown decreased synaptic expression of β-NRXs compared to wild-type mice (338). Another study has shown that Aβ binds to NLG1 and NRX2α and that these interactions mediate synapse damage and memory loss in mice (337). A recent study also found that NLG1 level in the hippocampus is decreased in AD patients and in AD model mice and that NLG1 KO mice display higher neuronal death in the dentate gyrus and greater spatial learning impairment after Aβ oligomers injections (339). Additionally, chromosomal alteration, splicing haplotypes and polymorphisms in NRXs and NLGs were identified in AD patients (340-342). Furthermore, the analysis of cerebrospinal fluid in patients with mild cognitive impairment, which is associate with AD prodromal stage, has revealed NRX1,2,3 as promising biomarkers, with NRX3 showing an especially high significance (343). An implication in PD was also described. Indeed, changes in NRX1 expression in the striatum of 6-OHDA-induced PD rat model were observed (344, 345). CNV polymorphisms in *CASPR2* (or *CNTNAP2*) gene, which is part of neurexin superfamily, show a positive association to AD and PD (346). However, no polymorphisms in *NRXNs* have been

identified in PD patients. On the other hand, NRXs were identified as a binding partner to α-syn in two distinct studies (187, 188). The first study showed a binding between α-syn preformed fibrils (PFF) and β-NRX1,2,3 or α-NRX1 with a nanomolar-range affinity (187). α-syn PFFs are obtained after five to seven incubation at 37 °C of α-syn monomers, and they are suggested to contain oligomers and protofibrils with variable size (347). The second study isolated α-NRX1,2 from a pull-down of whole neuron lysates using fibrillar α-syn as bait (188). However, the interaction between α-syn and NRXs is not fully understood, and its implication and effects on synapses remain unknown.

Chapter 2. Rationale and objectives

As mentioned above, α-syn oligomers cause synaptic dysfunction and synapse loss in synucleinopathy model mice (133, 191-193). Many previous studies have reported presynaptic impairment such as defects in presynaptic and axonal transport (193), in vesicle docking (159) and in vesicle recycling pool, resulting in impaired neurotransmitter release (194). Mouse models of synucleinopathies display a presynaptic form of LTD after high-frequency stimulation (195, 196) and short-term plasticity impairment such as paired-pulse depression or reduced paired-pulse facilitation (196, 197). Yet, little is known of the mechanisms behind presynaptic toxicity induced by α-syn oligomers. Extracellular oligomeric α-syn has been identified at the presynaptic terminal (180, 181, 186) and its accumulation is thought to result in synapse degeneration (191) and impaired synaptic plasticity (133). This suggests that presynaptic accumulation of extracellular α-syn is a key mechanism behind the pathogenesis(190). The identification of a pathway by which α-syn oligomers would cause presynaptic toxicity is essential to better understand synucleinopathies.

Interestingly, two independent studies have shown that NRXs bind to pathological α-syn (187, 188). NRXs are essential proteins for normal synaptic and cognitive functions; they are implicated in neurotransmitter release, synaptogenesis, plasticity and synapse maintenance. Thereby, they could be a key pathway for α-syn oligomers' toxicity. Furthermore, a previous study has uncovered a role for NRXs in the synaptic toxicity of pathological oligomeric proteins. Naito et al. have shown that Aβ oligomers bind to NRXs and diminish excitatory synaptogenic activity of NRX binding partners (338), further highlighting that NRXs are promising candidates for synaptic dysfunction in neurodegenerative diseases.

Although the binding between α-syn and NRXs has been identified, the implication of this interaction on α-syn-mediated synaptic impairment remains unknown. The overall objective of my master's project is to elucidate how the α-syn/β-NRX interaction contributes to α-syn pathology. We hypothesize that this interaction alters the function and trafficking of NRXs. To test our hypothesis, we first aimed to characterize α-syn/NRX interaction. To do so, we firstly

performed cell surface binding assays to identify the responsible domain for α-syn/β-NRX interaction and to determine its affinity and its competition with other binding partners. Secondly, we aimed to elucidate the effects of α-syn on NRX trafficking by internalization assays. Lastly, we aimed to reveal the effects of α-syn oligomers on NRX-mediated synaptogenic activity by performing artificial synapse formation assays. This study provides a promising new mechanism behind α-syn pathology and how α-syn can lead to synaptic toxicity.

In this project, I was able to start a completely new project on NRXs and synucleinopathies in our laboratory. The project and its aims were designed by Dr. Hideto Takahashi and were later modified together with me. I designed each experiment presented in this thesis in collaboration with Dr. Hideto Takahashi and my colleague Alfred Lee. In addition to the presented experiments, I designed new protocols for α-syn preparation and new experiments for the next steps of this project. Quantification and imaging were done by me, except for figure 3B that was done by an intern, Chloé Villedey.

Chapter 3. Methodology

3.1 Plasmids

As described previously (338), all isoforms of extracellular HA-tagged NRXs were generated from cDNA encoding NRX isoforms, then subcloned in the vector named spNRX1β-HA-C1, containing a human cytomegalovirus (CMV) promoter followed by the N-terminal signal peptide sequence, the HA sequence and multiple cloning sites. The NRX isoforms used for subcloning were intracellular CFP-tagged mouse NRX1α S4+, 1α S4-, 1β S4+, 1β S4-, 2α S4+, 2α S4-, 3α S4+, 3α S4- (kindly provided by Dr. Ann Marie Craig from University of British Columbia) and intracellular V5-tagged mouse NRX2β S4+, 2β S4-, 3β S4+ and 3β S4- (kindly provided by Dr. Takeshi Uemura from Shinshu University). For the HA-β-NRXs lacking the histidine-rich domain (ΔHRD), the mature coding sequences of NRX1β ΔHRD (aa 50–83), NRX2β ΔHRD (aa 54–87) and NRX3β ΔHRD (aa 48–81) were subcloned into spNRX1 β-HA-C1. The mutated NRX1β S4- D137A, which disrupts the interaction between NRX1β and NLG1 and LRRTM2 (247, 348), was kindly provided by Dr. Ann-Marie Craig.

The mature form of LRRTM2 (aa 34–515) was amplified by PCR from rat LRRTM2-CFP (kindly provided by Dr. Ann Marie Craig) and subcloned into spTrkC-HA-C1, as previously described (338). HA-NLG1 A+ B+, HA-NLG1 A+ B-, HA-NLG1 A- B+, HA-NLG1 A- B- were kindly provided by Dr. Peter Scheiffele from the University of Basel via Addgene. HA-NLG2 was kindly provided by Dr. Ann Marie Craig, LAR-CFP and NGL3-YFP by Dr. Eunjoon Kim from Korea Advanced Institute of Science and Technology (255), HA-GluRδ1 and HA-GluRδ2 by Dr. Michisuke Yuzaki from Keio University, and IL1RAPL1-pFLAG and IL1RAP-pFLAG by Dr. Tomoyuki Yoshida from Toyama University. HA-tagged glypicans were kindly provided by Dr. Ann-Marie Craig (308) and YFP-Caspr2 was kindly provided by Dr. Takashi Momoi (349). Mouse IgSF21 (aa 1-468, NM_198610.2) was cloned from mouse brain RNA extracts, subcloned in the vector pBluescript II SK and the HA tag was inserted between its second Ig domain and C-terminal signal sequence for GPI attachment (250). LAG3 plasmid (C-HA tag) was purchased from Sino Biological (RG80367-CY). All tags were inserted

within the coding sequence of the constructs. The other constructs used in the α-syn binding screening were described previously (251, 253). All constructs were verified by DNA sequencing.

3.2 Preparation of alpha-synuclein oligomers

EndoClear recombinant human untagged α-syn (AS-55555-1000, 1 mg) and recombinant human biotin-labelled α-syn (biotin-α-syn) (1–140) (AS-55581, 200 μg) were used to generate α-syn PFFs. Adapted from Michael J. Fox Foundation protocol (350), the recombinant α-syn diluted in sterile 1X PBS was centrifuged at 15,000 rpm for 10 minutes and incubated for one day at 37 °C, 1000 rpm in an Eppendorf Thermomixer C (Eppendorf, model 5382). After incubation, the α-syn preparation was sonicated for 10 cycles of 30 seconds ON/30 seconds OFF, at high power, in a 10 °C water using a sonication bath (Bioruptor®Plus sonication device with soundproof metallic box (B01200001) and water cooler (B02010003), Diagenode). The characterization of the size was done by SDS-PAGE using gradient gel (4-20% Mini-Protean TGX Precast Protein Gels, 4561096), by Dynamic light scattering (DLS) (ZetasizerNano S (Malvern Panalytical) with Malvern Zetasizer software for Zetasizer Nano S, 7.13 (Malvern Panalytical)) and by electron microscopy (Tecnai 12 BioTwin 120 kV transmission electron microscope). The incubated recombinant α-syn was diluted to 50μM for easier calculation for future experiments and store at -80 °C.

Our α-syn preparation showed oligomers/protofibrils-like lengths (347) and molecular weights using electron microscopy and gel gradient SDS-PAGE (Fig. 2 A-B, E-F). The protein separation by SDS-PAGE was performed using gradient gels on different preparations starting from zero-days incubation to seven-days incubation at 37 °C, 1000 rpm (Fig. 2A). To avoid disrupting α-syn oligomeric structure, no boiling was performed and no 2-Mercaptoethanol was applied. Given that the majority of molecular weights observed correspond to monomers (14.9 kDa) and oligomers of biotin-α-syn (between 50 kDa and 150 kDa) (Fig. 2A), we surmised that this one-day preparation contained mostly oligomers. Due to variation in fibril size, molecular weight, shape and heterogeneity, it is suggested that the sonication of five- or seven-days preparation of α-syn PFF samples forms protofibrils and/or oligomers (347). Monomers were observed for each preparation (14.9 kDa) likely due to the use of detergent (SDS) in the running buffer. Surprisingly, higher molecular weights are also detected in the zero-days preparation, likely due to the

instability of α-syn which may easily form oligomers at room temperature. The molecular weights of α-syn observed in longer incubation samples were in majority higher than 250 kDa while one-day preparation shows in majority strains between 50 kDa to 150 kDa (Fig. 2A) which could correspond to the appearance of protofibrils with longer incubation. This result supports that our preparation of one-day incubation contains mostly oligomers of α-syn. The one-day preparation for untagged α-syn also showed higher molecular weights compared to zero-day preparation (Fig. 2B) similar to the case of biotin-α-syn preparation. Native gel was used for untagged α-syn, because normal SDS-PAGE did not allow to observe any α-syn bands, and thereby it is not possible to determine the molecular weight of these strains due to the use of Native gel (Fig. 2B). A cell surface binding assay with NRX1β S4- was done to identify the best preparation condition that gives us the highest binding of biotin-α-syn (Fig. 2C, D). The one-day preparation was selected for further experiments as it shows the highest binding (Fig. 2C, D). Electron microscopy on the one-day preparation shows strains of 40 nm to 100 nm in majority (Fig. 2E, F). Evidence shows that majority of α-syn strains should be around 50 nm to have a good seeding and that common PFFs preparation displays a similar distribution (347).
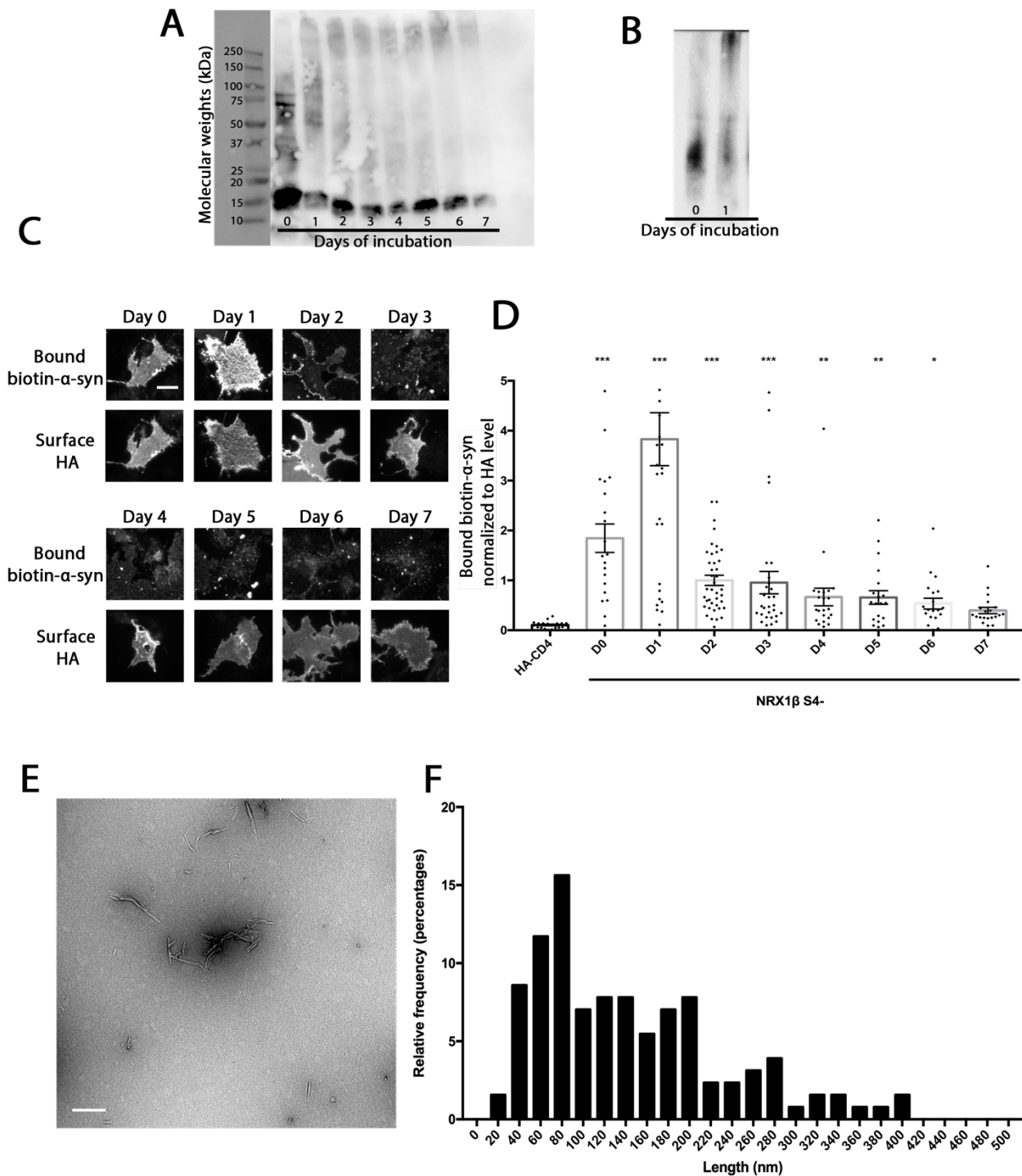
Figure 2. One-day preparation of biotin-α-syn has a high affinity with HA-NRX1β S4- and shows α-syn oligomer-like lengths and molecular weights

(**A**) Immunoblotting of biotin-conjugated α-synuclein (biotin-α-syn) preparations incubated for indicated days (zero days to seven days) and stained by HRP-conjugated streptavidin. Incubation was performed at 37 °C with shaking at 1000 rpm, and the same quantity (10 μl of 25 μM, monomer equivalent) was added

to each well. The immunoblot image shows the presence of biotin-α-syn oligomers with variable molecular weights (approximately between 37 kDa and 250 kDa). From the second day, the observed molecular weights of biotin-α-syn were mostly higher than 250 kDa. Monomers (14.9 kDa) can be identified in all samples. (**B**) Immunoblotting using native-PAGE of untagged α-syn preparations incubated for zero days and one day and stained by anti-α-syn antibodies confirmed the presence of larger strains in one-day preparation compared to zero-day preparation as they show slower migration. (**C**) Representative images of COS7 cells expressing extracellular HA-tagged NRX1β isoform lacking its S4 splicing site (HA-NRX1β S4-) incubated for 1h at 4°C with zero-days to seven-days preparations of biotin-α-syn. Each preparation was done at 37°C with shaking at 1000 rpm for the indicated number of days. Scale bar represents 30 µm. (**D**) Quantification of the cell surface binding of biotin-α-syn preparations to COS7 cells expressing HA-NRX1β S4-. The highest binding was observed at one-day incubation condition. n = 15-20 cells for each construct from 2 independent experiments using a Kruskal-Wallis test, N.S.: not significant, *P ≤0.05, **P ≤0.01 and ***P ≤0.001 compared with a negative control HA-CD4 by Bonferroni multiple comparisons tests. Data are presented as mean ± SEM. (**E**) Representative image of biotin-α-syn preparation using transmission electron microscopy showing the formation of α-syn aggregates, some of which look like oligomers and others of which exhibit a fibril-like structure. Scale bar represents 200 nm (**F**) Quantification of the length of all segments in electron microscopy images. Short segments (between 50 nm and 200 nm) are found in majority which corresponds to normal α-syn PFFs size distribution. Quantification was done on the total segments of two independent experiments.

3.3 Neuron and cell cultures and transfection

Cultures of COS7 cells and HEK293T cells and embryonic (E18) rat primary hippocampal neuron cultures were performed as described previously (251, 253). COS7 (CV-1 in Origin, and carrying the SV40 genetic material) cells are well suited for cell surface staining because of their tolerance of exogenous gene expression, their flat morphology and their resemblance to human fibroblasts (351). HEK293T (human embryonic kidney) cells are often used for artificial synapse formation assays (243, 245, 248, 254, 255) as they are human fibroblasts and have high protein production, they are accessible for transfection, they do not self-aggregate and they have a small size which optimizes the accumulation of synaptic proteins (352). However, both of these cell types lack neuronal phenotypes. Primary hippocampal neuron cultures were used for their high expression

level of NRX1β (267, 353) and because LB/LN are also found in the hippocampus (10, 35, 354). COS7 and HEK293T cells were transfected with lipofection using TransIT-LT1 (Mirus Bio. LLC), while calcium-phosphate transfection was performed on rat hippocampal neurons using the phosphate-mediated ProFection Mammalian Transfection System (Promega). For artificial synapse formation assays, HEK293T cells were first transfected with indicated plasmids by Mirus transfection for one day and then co-cultured with high-density hippocampal neuron cultures (300K cells/well of 12-wells culture dish).

3.4 Immunocytochemistry and Fluorescent Imaging

For all experiments, all conditions for each repetition were stained simultaneously. Cultures were fixed with parafix solution (4% paraformaldehyde and 4% sucrose in PBS 1X, pH 7.4) for 12 minutes at room temperature (RT) and then permeabilized with PBST (PBS 1X and 0.2% Triton X-100) for five minutes at RT. No permeabilization was performed for cell surface staining. Cultures were incubated for one hour at RT with a blocking solution (PBS1X, 3% bovine serum albumin (BSA) and 5% normal donkey serum). Primary antibodies were diluted in blocking solution and incubated overnight at 4° C, while secondary antibodies in blocking solution were applied for one hour at RT. Primary antibodies used were: anti-HA (1:2000, rabbit IgG, ab9110, Abcam), anti-HA (1:1000, mouse IgG2b, 12CA5, Roche), anti-VGlut1 (1:2000, guinea pig, AB5905, Millipore), anti-VGAT (1:1000, rabbit, 131,003, Synaptic Systems), anti-flag (1:2000, mouse IgG1, F1804, Sigma), anti-Synapsin I (1:2000, rabbit, AB1543P, Cedarlane), anti-alpha-synuclein (1:1000, mouse IgG1, 328100, Thermo Fisher Scientific) and anti-MAP2 (1:2000, chicken, AB5392, Abcam). Secondary antibodies used were: AMCA and Alexa-dye conjugated secondary antibodies generated in donkey against IgG of the same species as primary antibodies and having minimal cross-reactivity with the serum protein of many other species were used (Alexa-488, Alexa-594 and Alexa-647) (1:500; Jackson ImmunoResearch). AMCA (1:500; Jackson ImmunoResearch) and Alexa-594 (1:2000; Jackson ImmunoResearch) conjugated anti-streptavidin were used to stain biotin-α-syn and Alexa594-conjugated donkey anti-human IgG(H+L) (1:500; Jackson ImmunoResearch) was used to stain Fc-tagged proteins. After the application of secondary antibodies, coverslips were washed and mounted using Elvanol. Fluorescent images were captured by Volocity software (Perkin Elmer) using a Leica DM6000 fluorescent microscope with a 40X 0.75 NA air objective (for

cell line-based experiments) or a 63X 1.4 NA oil objective (for neuron-based experiments) and a Hamamatsu cooled CCD camera. All sets of cells were imaged on the same day using identical settings. Images were exported as 12-bit grayscale and figures were created with Adobe Photoshop CC 2019.

3.5 Cell surface binding assay

The binding capacity of biotin-α-syn to the indicated constructs expressed on COS7 cells was measured by cell surface binding assay. HA-CD4 was used as a negative control as it lacks the binding ability of biotin-α-syn. Plasmid constructs were transfected by lipofection in COS7 cells and maintained for 24 hours at 37 °C. Next, the cells were washed with extracellular solution (ECS) (168 mM NaCl, 2.4 mM KCL, 20 mM HEPES (pH 7.4), 10 mM D-glucose, 2 mM $CaCl_2$ and 1.3 mM $MgCl_2$) with 100 µg/ml BSA (ECS-BSA) and biotin-α-syn oligomers in ECS-BSA were applied to the transfected cells at the indicated concentration (monomer equivalent) for one hour at 4°C to allow the binding of biotin-α-syn on cell surface and prevent its endocytosis. After washing with ECS, the cells were fixed with parafix solution for 12 minutes at RT. For cell surface binding assay permeabilization was skipped, therefore allowing only surface binding. The blocking solution was applied for 1h at RT and the primary antibodies were then applied overnight at 4°C. Alexa594-conjugated streptavidin (1:2000; Jackson ImmunoResearch) and Alexa488-conjugated anti-rabbit IgG (H+L) (1:500) against rabbit anti-HA were used for most cell surface binding assay to detect bound biotin-α-syn and HA-tagged constructs, respectively. For our screening, in addition to the previously described antibodies for HA staining, anti-flag (1:2000) was used together with Alexa488 anti-mouse IgG (H+L) (1:500). Secondary antibodies were applied for one hour at RT.

A competitive NRX binding assay between biotin-α-syn and NLG1-Fc or LRRTM2-Fc was also done by cell surface staining. COS7 cells were transfected with indicated HA-NRX isoforms and incubated for 24 hours at 37 °C. Both biotin-α-syn (500nM) and NLG1-Fc (50nM) or LRRTM2-Fc (50nM) were applied together to the cells for one hour at 4° C. The recombinant proteins of NLG1-Fc and LRRTM2-Fc were obtained from HEK293T cells transfected with pc4-NLG1 A-B+ -Fc and pc4-LRRTM2-Fc vectors, as essentially described before (251, 253). Alexa594-conjugated donkey

anti-human IgG (H+L) (1:500; Jackson ImmunoResearch) was used to label bound Fc proteins and AMCA-conjugated streptavidin (1:500; Jackson ImmunoResearch) was used to label bound biotin-α-syn.

For all cell culture-based experiments, at least 10 cells with similar surface HA level expression were selected for imaging (Supplementary data, Fig. S1), and the identical microscope settings were applied for each experiment repetitions. All experiments were performed three times for a total of at least 30 cells.

3.6 Internalization assay

Hippocampal rat neuron cultures were transfected at one week *in vitro* with calcium phosphate transfection method to overexpress HA-CD4, HA-NRX1β S4- or HA-NRX1β S4- ΔHRD. Two weeks after transfection, neurons at 21–24 days *in vitro* (DIV) were washed with cold neuron media and incubated with anti-HA (rabbit, 1:500) in cold media for 1h at 4° C under live condition to allow the binding of anti-HA antibodies to HA-tagged constructs expressed on cell surface and prevent endocytosis. The cultures were washed and incubated in media with biotin-α-syn (500nM) diluted in 1X PBS or with 1X PBS for 1h at 37 °C to allow internalization of HA-tagged proteins bound with anti-HA antibodies. An acid wash (MilliQ, 10% 5M NaCl, 1.2% acetic acid) was carried out to remove the remaining anti-HA antibodies on cell surface and keep only internalized HA-tagged proteins bound with anti-HA antibodies. The cultures were then fixed by parafix solution (12 min, RT), permeabilized (5 min, RT) and blocked (1h, RT) with the blocking solution. Anti-MAP2 (1:2000, Abcam) was used to identify axons (MAP2-negative neurite segments) and assure the quality of the neuron culture. Alexa488-conjugated anti-rabbit (1:500, Jackson ImmunoResearch) was used to label the HA tag, AMCA-conjugated anti-chicken (1:500, Jackson ImmunoResearch) labelled MAP2 and Alexa594-conjugated streptavidin (1:2000, Jackson ImmunoResearch) labelled biotin-α-syn. Biotin-α-syn staining allowed to select the α-syn positive segments. The axons were identified as MAP2-negative neurites and were selected for the quality of the HA staining and the biotin-α-syn staining on axons. At least 10 neuronal segments with similar conditions were selected for the imaging. Identical microscope settings were applied for each experiment

repetition. All experiments were independently done three times for a total of at least 30 segments.

3.7 Artificial synapse formation assay

HEK293T cells were transfected by Mirus transfection with NRXs binding partners (HA-NLG1 A-B- and HA-NLG2) and a negative control (HA-CD4) and maintained for 24 hours. Afterwards, the cells were trypsinized, harvested and applied onto high-density rat hippocampal neurons in a 12-well dish. Sterile PBS, the negative control, or untagged α-syn (500nM) were applied soon after applying the HEK293T cells to the neurons. After 24 hours, the co-cultures were fixed with parafix solution (12 min, RT) and blocked (1h, RT). Only anti-HA mouse IgG2b (1:2000, Roche) was first applied at 4° C overnight to stain the surface HA of the HEK293T cells. The cultures were then permeabilized (5 min, RT) and blocked again (1h, RT). Chicken anti-MAP2 (1:2000, Abcam), guinea pig anti-VGlut1 (1:2000, Millipore) and rabbit anti-VGAT (1:1000, Synaptic Systems) were applied overnight at 4° C. The secondary antibodies used were: AMCA-conjugated anti-chicken (1:500, Jackson ImmunResearch), Alexa488-conjugated anti-mouse (1:500, Jackson ImmunResearch), Alexa594-conjugated anti-guinea pig (1:500, Jackson ImmunResearch) and Alexa647-conjugated anti-rabbit (1:500, Jackson ImmunResearch). At least ten HEK293T cells with similar HA level were selected for the imaging and the same microscope settings were applied for each repetition. All experiments were independently done three times for a total of at least 30 cells.

3.8 Fluorescence quantification

Imaging and image analysis were carried out under blinded conditions. For cell line-based experiments, the analysis was done with Volocity software (Perkin Elmer). For neuronal experiments, Metamorph 7.8 software (Molecular Devices) was used. For all experiments, Microsoft Excel and GraphPad Prism 7 were used for collecting data, calculations and statistical tests. For binding quantification, after subtracting background intensity from each channel, the average intensity of bound proteins in a selected area was normalized to the average intensity of the surface HA in this area. For internalization assay, after subtraction of the background and threshold application, the total HA puncta intensity in a selected segment was normalized to the distance of the axon segment. For artificial synapse formation assays, the HA-positive HEK293T

cell area for each repetition was selected and transferred on VGlut1 and VGAT channels. The total VGlut1 and VGAT puncta intensities were measured and normalized by the region area previously transferred. All raw data from Volocity and MetaMorph were conserved on Excel sheets.

3.9 Statistical analysis

Values on Excel sheets were transferred on GraphPad Prism 7 to prepare the graphs and perform statistical analysis. As the majority of the obtained data did not show normal distribution, non-parametric tests were used. Specifically, statistical tests were carried out using Kruskal-Wallis test with post hoc Dunn's multiple comparisons test for multiple groups comparisons or unpaired Mann-Whitney tests for two-groups comparison. All data are represented as the mean ± standard error of the mean (SEM) and statistical significance was defined as P ≤0.05 (N.S.: not significant (P> 0.05), *P ≤0.05, ** P ≤0.01 and ***P ≤0.001).

Chapter 4. Results

4.1 Oligomers of alpha-synuclein bind to β-neurexins

Oligomers of α-syn have been shown to impair synaptic function and enhance synapse loss (133, 191-193). Furthermore, as extracellular α-syn oligomers can be found in the brain of patients with synucleinopathies especially PD (180, 181, 186, 207, 208), we wanted to identify a cell-surface binding partner of α-syn that acts as its receptor to cause α-syn synaptic impairments. Since synaptic organizers are membrane proteins that play an essential role in normal synaptic function (267, 272-274, 279, 280), we hypothesized that some of them would be implicated in α-syn pathology as α-syn receptors. The identification of synaptic organizers that work as α-syn receptors will help to better understand molecular mechanisms behind α-syn-induced synaptic dysfunction and behind the spreading of α-syn pathology in synucleinopathies.

4.1.1 Biotinylated alpha-synuclein oligomers bind to the synaptic neuronal surface, likely through an interaction with neurexins.

We first investigated whether extracellularly applied α-syn oligomers bind to the cell surface of neurons, particularly synaptic membrane regions. To answer this question, we applied biotin-α-syn oligomers to neurons and co-stained for a synaptic marker. The oligomeric biotin-α-syn used for all experiments was incubated for one day at 37 °C, 1000 rpm as previously described (Method, Fig. 2). Previous studies have shown that majority of α-syn strains should be around 50nM to have a good seeding (347). As Figure 2 illustrates, our preparations of one-day incubation exhibit a wide range of sizes which corresponds to previously described size distribution of α-syn PFFs (Fig. 2E, F) (347) and oligomer-like molecular weights (50 kDa to 150 kDa) (Fig. 2A). The difference in sizes of oligomers and protofibrils has not been determined, so typically it is assumed that an α-syn PFF preparation contains both (347). Then we applied biotin-α-syn oligomers to cultured hippocampal neurons at 21 days *in vitro* (DIV) for 1 hour at 4°C to prevent internalization processes and labelled the neurons for both bound biotin-α-syn oligomers and Synapsin I, a presynaptic marker. We observed a co-localization between biotin-α-syn and

Synapsin I signals (Fig. 3A, right panel). In addition, when the neurons were not permeabilized, the signal for Synapsin I was absent, whereas the signal for biotin-α-syn was still detected (Fig. 3A, middle panel), suggesting that biotin-α-syn binds to the neuronal surface. Lastly, when biotin-α-syn was not applied, no staining signal for biotin was detected, indicating that the Alexa-conjugated streptavidin signal for biotin detection is specific to detect biotin-α-syn without any significant non-specific signals (Fig. 3A, left panel). These results suggest that α-syn oligomers can bind synaptic neuron surface.

Since synaptic organizers are membrane proteins essential for normal synaptic function (222, 223), they are good candidates for α-syn binding partners that could mediate this synaptic surface binding. Therefore, we next performed a protein interaction screening for a total of 29 synaptic organizers as well as a negative control CD4 and a positive control LAG3 (187) using a cell surface binding assay to test whether and which pre- or postsynaptic organizers bind to α-syn oligomers (Fig. 3). This assay allows us to identify cell-surface binding partners because biotin-α-syn is extracellularly applied to COS7 cells at 4 C˚, which blocks internalization events, and because no permeabilization of cell membranes is performed at staining steps. A concentration of 250 nM (monomer equivalent) was used for our screening as Mao et al. have measured a Kd of 260 ± 44 nM for NRX1β in their previous study (187). At a concentration of 250 nM (monomer equivalent), COS7 cells expressing HA-NRX1β S4-, but not those expressing the other tested synaptic organizers, showed significant binding to biotin-α-syn (Fig. 3C). A negative control, HA-CD4 showed no binding, whereas a known binding partner, LAG3-HA showed significant binding, supporting the binding specificity of biotin-α-syn (Fig 3B). The binding of biotin-α-syn to HA-NRX1β S4- is the strongest when biotin-α-syn is incubated for one day (Fig. 2C, D), likely forming oligomers-like-molecular weight in our preparation protocol. All experiments using cell lines were done using cells with similar HA level (Supplementary data, Fig. S1), suggesting that when we observed a higher biotin-α-syn signal that was normalized on HA surface level, it was coming from a higher affinity and not from a higher or lower HA signal.
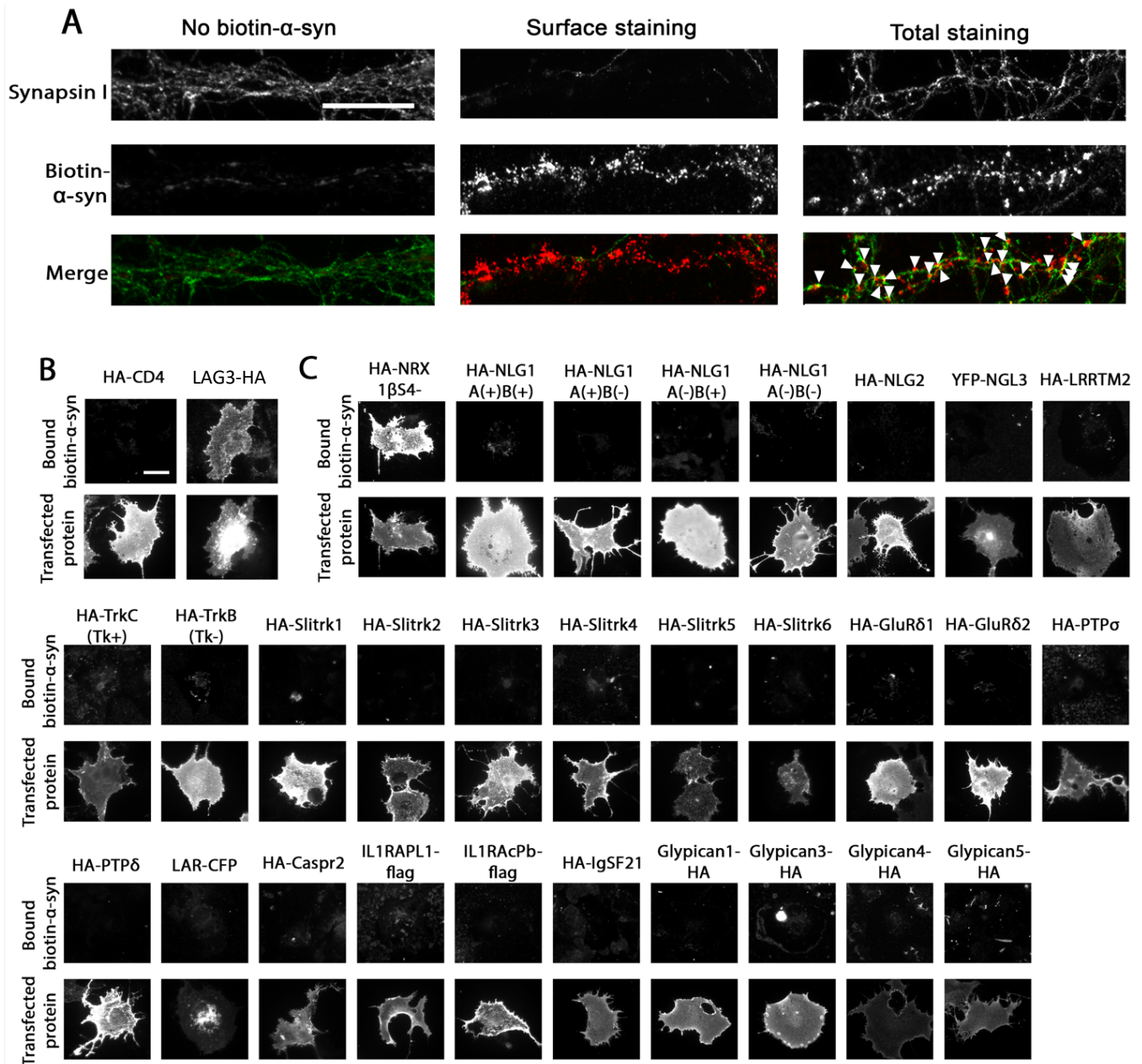
Figure 3. Oligomeric biotinylated α-synuclein binds to the synaptic neuronal surface, and a candidate screening identified NRX1β S4- as a major binding partner of α-syn

(A) Representative images of hippocampal neurons at three weeks in culture treated with biotin-α-syn oligomers (250nM, monomer equivalent) for 1h at 4°C and then stained for Synapsin I and biotin-α-syn. Neurons untreated by biotin-α-syn (left panel) show no Alexa594-conjugated streptavidin signal. Neurons treated by biotin-α-syn without permeabilization in staining (middle panel) show Alexa594-conjugated streptavidin signals, but not Synapsin I immunoreactivity. Neurons treated with biotin-α-syn with permeabilization in staining showed significant signals for both synapsin I and biotin-α-syn, which

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colorized with each other (right panel). Scale bar represents 20 µm. **(B, C)** Screening of synaptic organizers to identify a candidate binding partner for biotin-α-syn (250 nM, monomer equivalent). Biotin-α-syn was extracellularly added on COS7 cells transfected with each synaptic organizer plasmid. Bound biotin-α-syn was detected on HA-NRX1β S4- transfected cells **(C)** HA-CD4 was used as a negative control and HA-LAG3 as a positive control. Scale bar represents 30 µm**.**

4.1.2 Alpha-synuclein oligomers bind to β-neurexins in a histidine-rich domain (HRD)-dependent manner

Next, we investigated whether other NRX isoforms bind to α-syn oligomers. Therefore, cell surface binding assays were performed using COS7 cells expressing extracellular HA-tagged NRX1α, 2α 3α, 1β, 2β or 3β isoform with the application of biotin-α-syn oligomers (250nM monomer equivalent). Given that the splicing site 4 (S4) is important for the binding of NRXs with several NRX-binding proteins such as NLGs (242, 247), LRRTMs (244, 245, 247, 269) and amyloid-β (338), we decided to test the binding of biotin-α-syn to both S4-positive NRXs (S4+, Fig. 4A, top panels) as well as S4-negative ones (S4-, Fig. 4A, lower panels). We found that COS7 cells expressing HA-NRX1,2β, but not HA-NRX1α/2α/3α, showed a significant binding of biotin-α-syn oligomers regardless of the presence or absence of S4 site (Fig. 4). Interestingly, HA-NRX3β binding to biotin-α-syn was dependent on the S4 site insert as HA-NRX3β S4+ showed low binding to biotin-α-syn.

We further determined the NRX domain responsible for α-syn interaction. Since our previous study has shown that Aβ oligomers bind to β-NRXs in their histidine-rich domain (HRD)-dependent manner (338), we focused on testing the involvement of HRD domain by deleting it in β-NRX1,2,3 (ΔHRD). HRD is a unique domain of β-NRXs and does not exist in α-NRXs (261, 263, 267). When the HRD was deleted from HA-NRX1,2β, the signals of bound biotin-α-syn were fully abolished regardless of S4 insertion (Fig. 4A, B). Although HA-NRX1,2β S4+ seems to show a higher binding signal of biotin-α-syn than HA-NRX1,2β S4-, there was no difference between them (Fig. 4B). These data suggest that the HRD of NRX1,2β is primarily responsible for α-syn binding. Notably, the binding signal of biotin-α-syn to HA-NRX3β S4+ was higher than that to a negative control HA-

CD4 although this signal was generally very weak and significantly lower than with HA-NRX1,2β, and HA-NRX3β S4- did not show significant binding (Fig. 4B). These results further suggest that the insertion S4 may positively modulate the binding of α-syn either due to conformation changes and/or adding low-affinity binding site through the S4. The concentration of 250 nM, which we used for the domain analysis, might be too low to detect a binding to S4. This is supported by previous papers that have identified interaction with α-NRX1,2 by a pull-down assay using 4.8 μM of α-syn (188) and identified a Kd of 933nM ± 657 nM for NRX1α and 340nM ± 45 nM for NRX3β (187). Further experiments, applying higher concentrations of biotin-α-syn, are required to address this question. Given that α-syn binds to NRX1,2β in an HRD-dependent manner (Fig. 4A, B), we decided to focus on this domain for further experiments by using HA-NRX1β S4-.

4.1.3 Alpha-synuclein oligomers bind to HA-neurexin1β S4- with high affinity

To determine the binding affinity of α-syn with NRX1β S4-, we applied serial dilutions of biotin-α-syn oligomers (0 nM to 3000nM monomer equivalent) to COS7 cells expressing HA-NRX1β S4- and performed binding curve analysis and Scatchard plot analysis. The binding curve reached a plateau level from 1000 nM (Fig. 5A). According to a nonlinear regression (Binding curve – Saturation, Specific binding with Hill Slope) on GraphPad Prism 7, the dissociation constant (Kd) was in nanomolar range (Kd = 523 ± 34.4 nM, Fig. 5), indicating a nanomolar range interaction affinity between α-syn oligomers and NRX1β S4-. The binding curve shows a sigmoidal shape, and the Scatchard plot shows a concave-down distribution. Given that in the Scatchard plot analysis, a linear distribution implicates simple one-site binding mode whereas a concave-down distribution generally implicates a positive cooperative binding mode, in which binding of a ligand facilitates binding of more ligands (355, 356), our results suggest that an interaction between α-syn oligomers and NRX1β S4- is not based on a simple one-site binding mode, instead may be based on a positive cooperative binding mode.
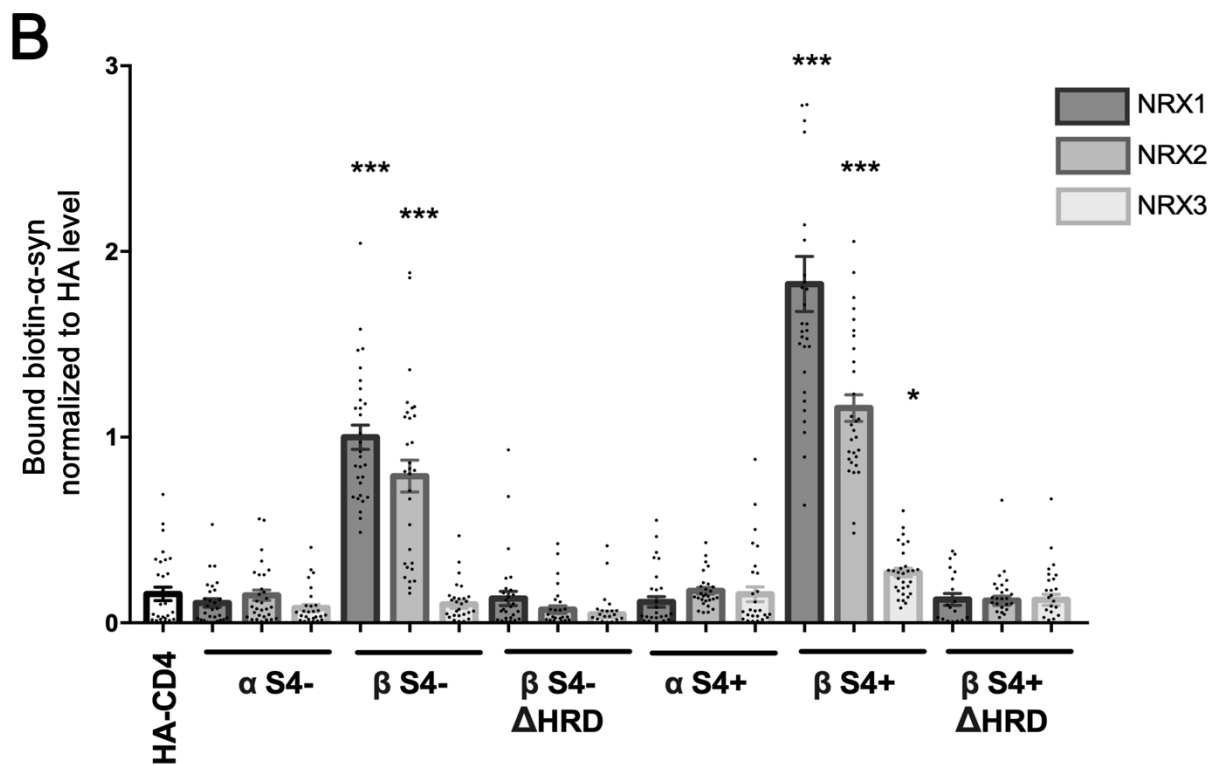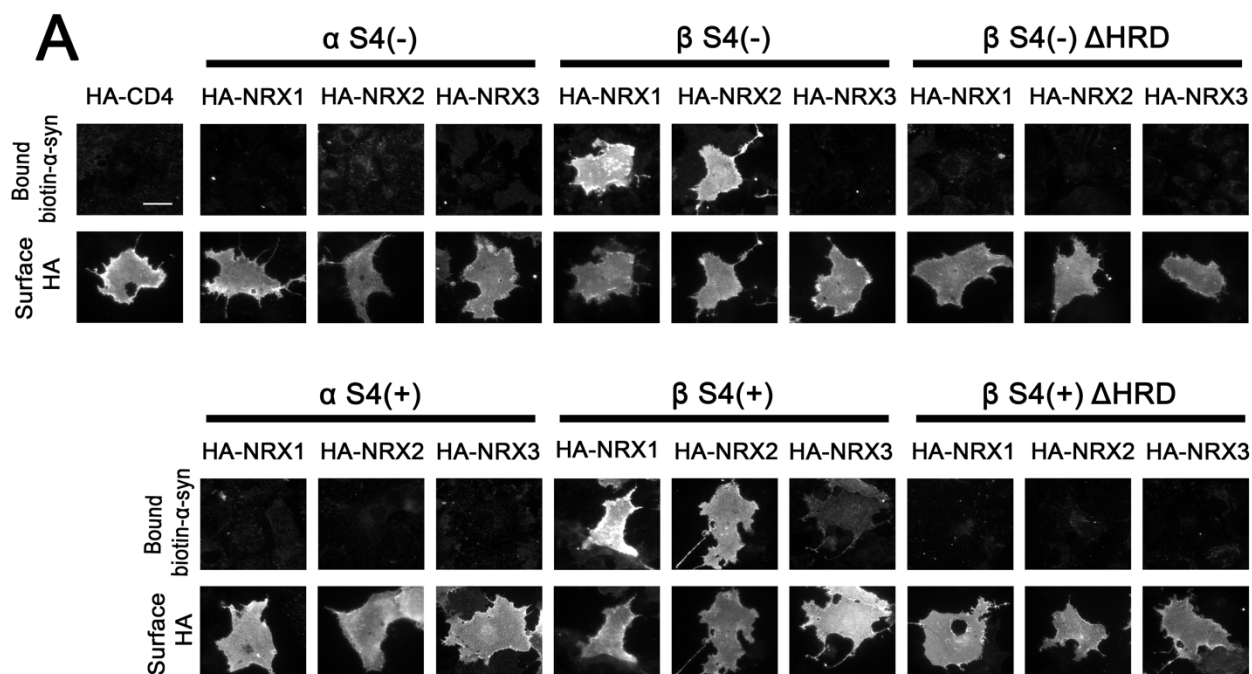
Figure 4. Oligomeric biotinylated α-synuclein binds to NRX1,2β in a histidine-rich domain (HRD)-dependent manner

(A) Representative images of biotin-α-syn oligomers binding (250 nM, monomer equivalent) to the indicated extracellular HA-tagged NRXs expressed on COS7 cell surface. The binding through the splicing

site 4 (S4) and the histidine-rich domain (HRD) were evaluated. S4- indicates the lack of this site. No binding is observed in α-NRXs S4+, suggesting that unlike Aβ oligomers binding to NRXs (338), the S4 site may not have significant responsibility for α-syn binding. NRX1β- and NRX2β-expressing cells show a significant binding compared to the negative control HA-CD4, regardless of the S4 insert. No binding to biotin-α-syn was observed in cells expressing β-NRX1,2 lacking the HRD (ΔHRD), suggesting that the HRD is required for α-syn oligomers binding. Scale bar represents 30 µm. **(B)** Quantification of biotin-α-syn oligomer binding (250 nM monomer equivalent) to each indicated NRX construct. Quantification shows that biotin-α-syn binds to NRX1,2β S4+ and S4- in an HRD-dependent manner. The binding with HA-NRX3β S4+ is statistically significant when compared to the negative control HA-CD4, but significantly lower than with HA-NRX1,2β. Although a tendency of the binding enhancement by S4 insert is observed in comparison between NRX1β S4+ and S4- and between NRX2β S4+ or S4-, no statistically significant difference was measured. *n* = 30 cells for each construct from three independent experiments using a Kruskal-Wallis test, N.S.: not significant (P> 0.05), *P ≤0.05. and ***P ≤0.001 compared with HA-CD4 and comparing between NRX1,2,3β S4+ and S4- by post hoc Dunn's multiple comparison tests. Data are represented as mean ± SEM.
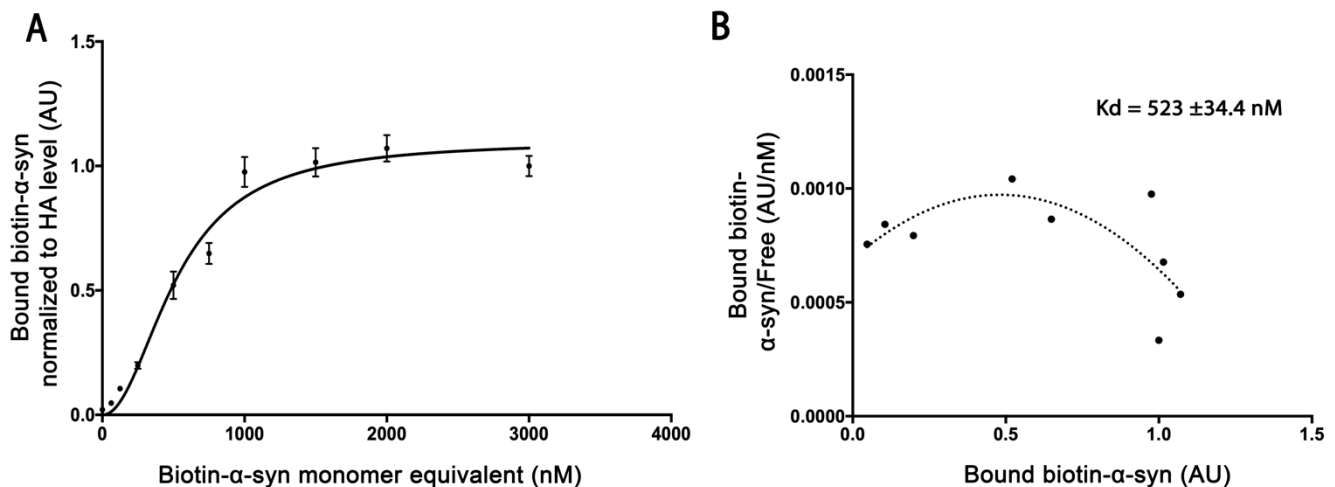
Figure 5. Biotinylated α-synuclein oligomers bind to HA-NRX1β S4- in a nanomolar range affinity

(A) Saturable binding of biotin-α-syn to COS7 cells expressing HA-NRX1β S4-. Concentrations of 0 nM to 3000nM of biotin-α-syn were applied. Using the nonlinear regression function of GraphPad Prism 7, a saturation binding curve (Equation: Specific binding with Hill Slope) was done to measure a dissociation constant (Kd) of 523 ± 34.4 nM. Data are represented as mean ± SEM. **(B)** Scatchard plot of binding data showing a concave downward curve. n = 30 cells for each plot, from three independent experiments.

4.1.4 Alpha-synuclein/neurexin1β interaction does not disrupt neurexin1β interaction to neuroligin1 or LRRTM2

Given that biotin-α-syn oligomers bind to NRX1β depending on its HRD (Fig. 4), a distinct binding domain from its postsynaptic binding partners such as NLG1 and LRRTM2 (LNS domain) (242, 244, 245, 247, 269), we determined if an interaction between α-syn oligomers and NRX1β would disrupt the interaction between NRX1β and its postsynaptic binding partners. To answer this question, we performed a competitive binding assay, in which recombinant proteins of NLG1-Fc (Fc-tagged NLG1 A-B+) or LRRTM2-Fc were applied with or without biotin-α-syn oligomers (500 nM, monomer equivalent) to COS7 cells expressing HA-NRX1β S4-, HA-NRX1β S4- D137A mutant (NLG1- and LTTRM2-binding dead mutant (247, 357)) or HA-CD4 (a negative control without any binding to α-syn) (Fig. 6A, B). We used NLG1 A- B+ isoform because NLG1 B+ strongly binds only to NRX1β S4-, but not NRX1β S4+, regardless of the insert A; therefore, it is helpful to better characterize the potential competition of α-syn and NLG1 A- B+ which has high affinity to NRX1β S4-. HA-NRX1β S4- showed significant binding to NLG1-Fc and LRRM2-Fc as expected, and its binding was not changed even when biotin-α-syn oligomers were added (Fig. 6C, D). As expected, HA-CD4 and HA-NRX1β S4- D137A show no binding of NLG1-Fc or LRRTM2-Fc (Fig. 6C, D). Furthermore, biotin-α-syn oligomers show their binding to HA-NRX1β S4- and HA-NRX1β S4- D137A, but not to HA-CD4, as expected (Fig. 6A, B, top panels). These results suggest that the NRX1β/α-syn interaction does not affect the binding of NRX1β S4- to trans-synaptic NRX binding partners such as NLG1 A-B+ or LRRTM2.

4.2 Oligomers of alpha-synuclein impair neurexin1β S4- trafficking on axons.

As mentioned previously, NRXs are presynaptic organizers that interact with postsynaptic organizers through their extracellular domain (272-274, 281-283). Their roles include synapse formation, synapse maintenance, synaptic plasticity, neurotransmitter release and cognitive function. In order to play these roles, NRXs are to be expressed on the presynaptic membrane surface of axons for a trans-interaction with postsynaptic organizers. Therefore, normal cell-surface composition of NRXs at presynaptic sites is essential for normal synaptic function.

4.2.1. Alpha-synuclein oligomers increase the internalization of neurexin1β S4- on axon surface

As α-syn oligomers do not impair the interaction between NRX1β S4- and its postsynaptic binding partners (Fig. 6), we next tested if α-syn oligomers could affect its trafficking and surface level. Higher internalization of NRXs than normal would result in a reduction of the surface expression level of NRXs and thereby dampen their synaptic roles. To assess this, we performed an internalization assay using cultured hippocampal neurons expressing extracellularly HA-tagged NRX1β. This allows us to stain only internalized antibody-tagged proteins. To do so, the neuron cultures are incubated at 4°C for 1h to stop the receptor internalization with anti-HA to immunolabel extracellularly HA-tagged plasmids, such as HA-CD4, HA-NRX1β S4- and HA-NRX1β S4- ΔHRD. The neuron cultures were next incubated at 37 °C for 1h with or without biotin-α-syn oligomers to allow internalization and then put on ice for an acid wash which removes all remaining surface HA antibodies that bind to the HA-tagged proteins still expressed on the cell surface. Using this method, we confirmed that after applying biotin-α-syn oligomers (500nM) for 60 minutes, HA-NRX1β S4- internalization increased around twofold. On the other hand, α-syn application did not affect the internalization of HA-NRX1β S4- ΔHRD or that of HA-CD4 (Fig. 7). These results suggest that the binding of α-syn oligomers to NRX1β S4- enhances its internalization. Furthermore, the deletion of the HRD was able to occlude α-syn-induced increase in NRX1β internalization, indicating that α-syn enhancement of HA-NRX1β S4- internalization is in an HRD-dependent manner.
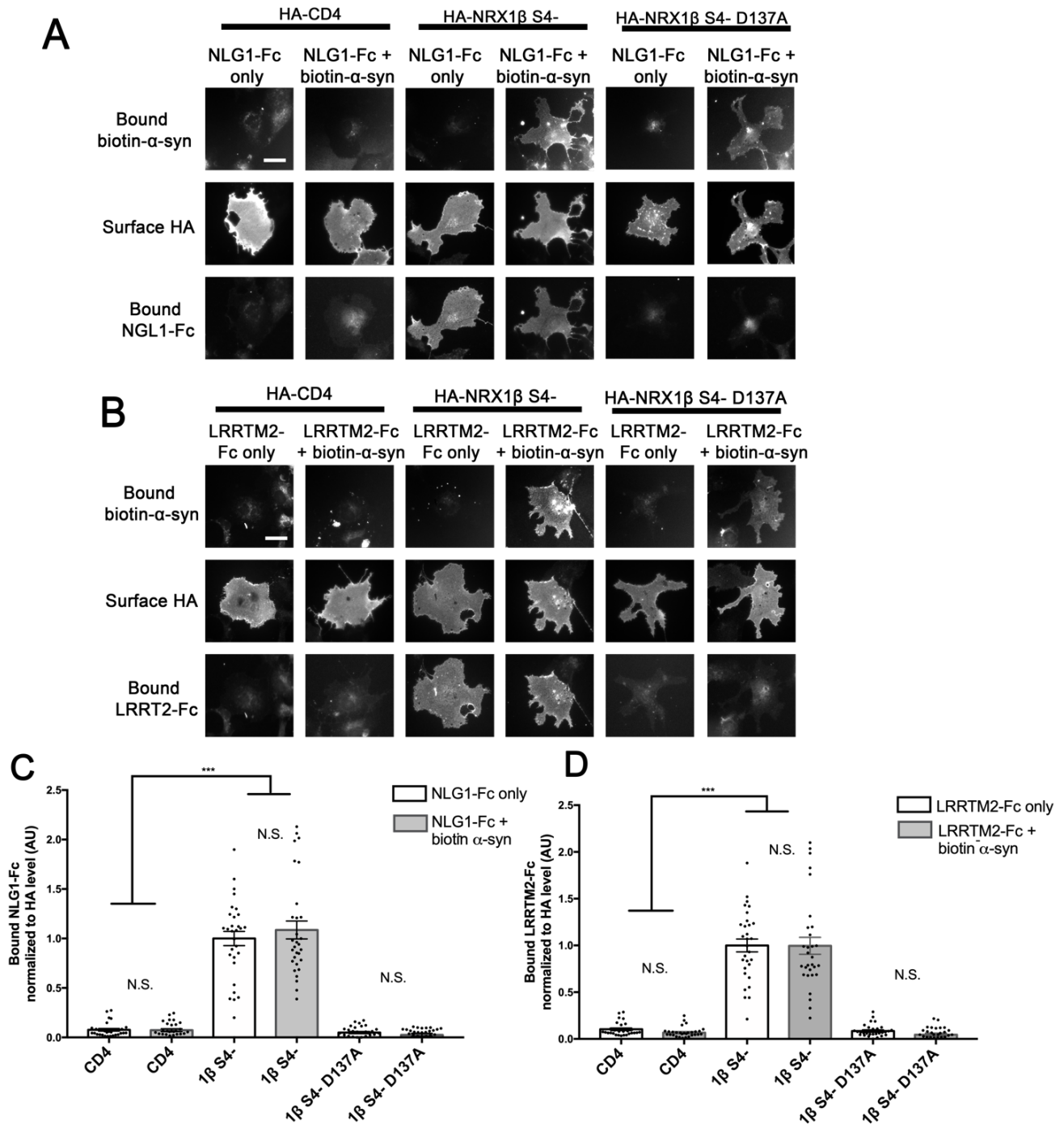
Figure 6. Oligomeric biotinylated α-synuclein does not affect the interaction between NRX1β S4- and its binding partners, NLG1 A-B+ and LRRTM2

(A) Representative images of COS7 cells transfected with HA-tagged NRXs or CD4 and stained for bound biotin-α-syn oligomers (upper panels), surface HA (middle) and bound NLG1-Fc (Fc-tagged NLG1 A-B+) (bottom). Biotin-α-syn oligomers at 500 nM monomer equivalent and NLG1-Fc at 50nM were applied to

the indicated transfected COS7 cells. Biotin-α-syn binds to HA-NRX1β S4- and HA-NRX1β S4- D137A, but not to HA-CD4 (upper panels). As previously reported, NLG1-Fc binds to HA-NRX1β S4-, but not HA-NRX1β S4- D137A or HA-CD4 (bottom panels). **(B)** Representative images of HA-tagged NRXs or CD4 transfected into COS7 cells binding to biotin-α-syn oligomers (500 nM) and LRRTM2-Fc (50nM). COS7 cells were stained for biotin-α-syn oligomers (upper panels), surface HA (middle) and bound NLG1-Fc (bottom). As expected, HA-NRX1β S4- and HA-NRX1β S4- D137A show a binding to biotin-α-syn oligomers, but not to HA-CD4 (upper panels). LRRTM2-Fc binds to HA-NRX1β S4-, but not HA-NRX1β S4- D137A and CD4 (bottom panels). (a,b) Scale bar represents 30 μm. **(C)** Quantification of NLG1-Fc (50 nM) binding to each construct shows that it binds to HA-NRX1β S4- regardless of the presence of biotin-α-syn. **(D)** Quantification of LRRTM2-Fc (50 nM) binding to each construct shows that it binds to HA-NRX1β S4- and that its binding does not change after the application of biotin-α-syn oligomers. (c,d) n = 30 cells for each construct from three independent experiments using a Kruskal-Wallis test, N.S.: not significant (P>0.05) and ***P ≤0.001 compared with HA-CD4 and comparing between "NLG1-Fc or LRRTM2-Fc only" and "NLG1-Fc or LRRTM2-Fc + biotin-α-syn" conditions by post hoc Dunn's multiple comparison tests. Data are presented as mean ± SEM.
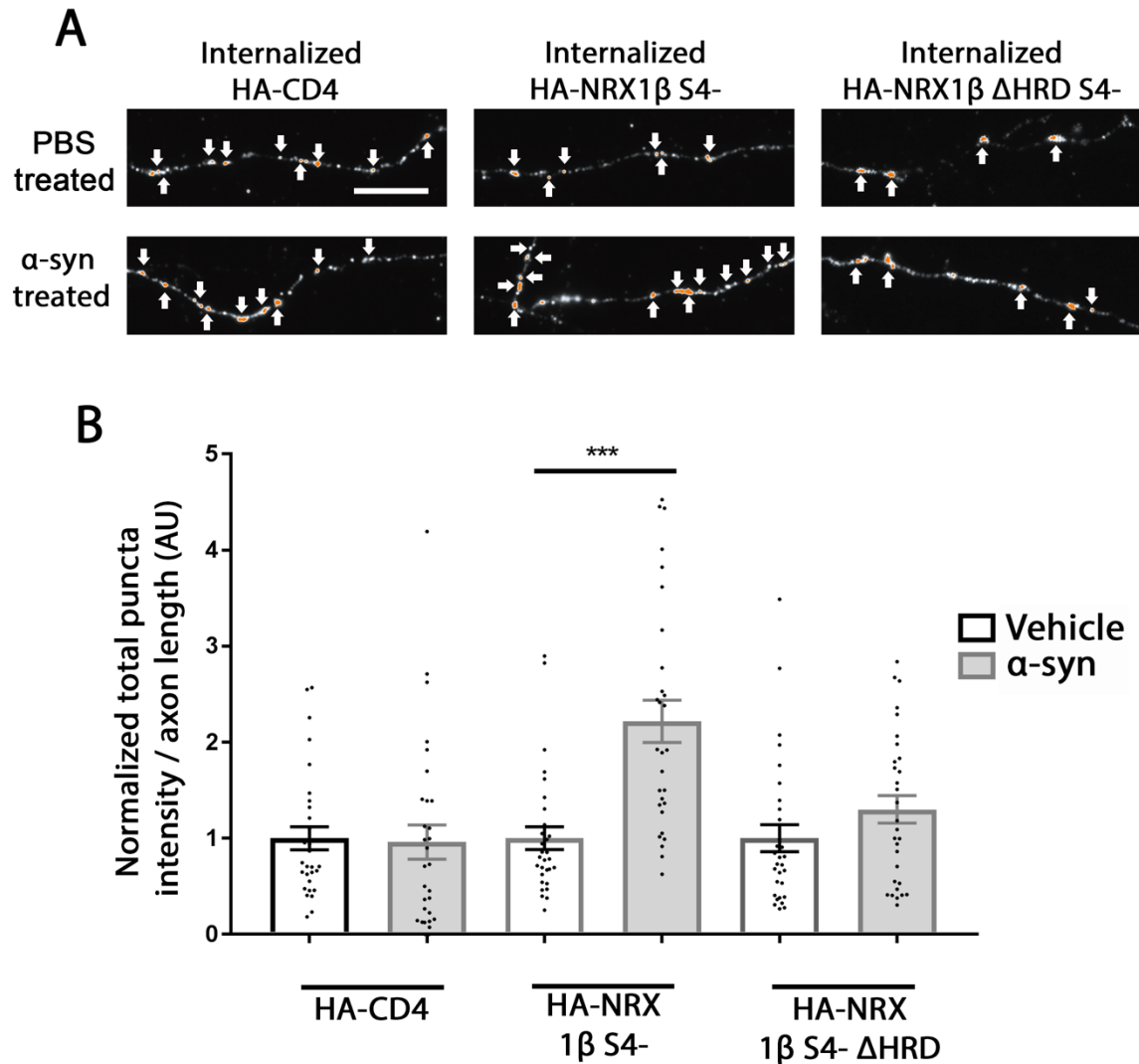
Figure 7. Biotinylated α-synuclein oligomers promote the internalization of NRX1β S4- in axons in a histidine-rich domain (HRD)-dependent manner

(**a**) Representative images of internalized HA-tagged constructs transfected on hippocampal neurons at two weeks *in vitro*. A threshold was used on Metamorph 7.8 software to identify puncta (arrow heads) of antibody-tagged proteins. Neurons were transfected with HA-CD4 (negative control), HA-NRX1β S4- and HA-NRX1β S4- lacking the histidine-rich domain (HA-NRX1β S4- ΔHRD) and then treated with biotin-α-syn (500nM) or with PBS. Scale bar represents 10 μm. **(b)** Quantification of the total intensity of internalized HA-tagged constructs per length of axons (μm). After biotin-α-syn treatment, only HA-NRX1β S4- shows a significant increase in total intensity per axon length. Both HA-NRX1β S4- ΔHRD and HA-CD4 show no significant increase. n = 30 axonal segments from three independent experiments using a Mann-Whitney

test for non-parametric, N.S.: not significant (P>0.05) and ***P ≤0.001 comparing between PBS-treated group and α-syn-treated group. Data are presented as mean ± SEM.

4.3 Alpha-synuclein affects NRX functions

Given that NRXs play their functions at the neuron surface through their trans-synaptic interaction with postsynaptic partners (272-274, 281-283), an enhancement of NRX internalization on axons by α-syn oligomers is likely to affect its synaptic functions. One important function of NRXs is to mediate synaptogenesis, especially presynaptic differentiation induced by NRX-binding partners such as NLGs and LRRTMs (272-274, 281-283).

4.3.1: Alpha-synuclein diminishes inhibitory presynaptic differentiation induced by neurexin interactors neuroligin1/2

Next, we tested if the interaction of α-syn with NRXs could affect NRX functions. As we found that α-syn increases the internalization of NRX1β S4- (Fig. 7), we hypothesized that enhanced internalization would be translated by an alteration in NRX-mediated synaptogenic activity. To investigate this, we decided to take advantage of an artificial synapse formation assay (183, 245, 250, 251, 253, 276, 277, 338, 348) for assessing the synaptogenic activity of NLGs to induce presynaptic differentiation through their trans-interaction with endogenous NRXs expressed on axons of neurons. To do so, NLG-expressing fibroblasts were co-cultured together with primary hippocampal neuron cultures.

In our protocol, HEK293T cells were transfected with extracellular HA-tagged NLG1 A-B- and HA-NLG2, or HA-CD4 as a negative control. The cells were then co-cultured with wild-type hippocampal rat neuron cultures, and the co-cultured samples were treated with PBS (a negative control) or untagged α-syn oligomers (500 nM, 24h). Like biotin-α-syn, the oligomeric form of untagged α-syn was prepared by an incubation for one day at 37 °C. We double-stained the treated cocultured samples for VGlut1 and VGAT to distinguish between excitatory presynaptic differentiation and inhibitory one. We found that the treatment of α-syn oligomers decreased VGAT accumulation induced by HA-NLG1 A-B- and HA-NLG2, suggesting a decrease in NRX-mediated inhibitory presynaptic differentiation (Fig. 8). Surprisingly, the treatment of α-syn

oligomers did not significantly suppress excitatory presynaptic differentiation induced by either NLG1 A-B- or NLG2 although there seems to be a trend towards a decrease (Fig 8b). These data suggest that the α-syn/β-NRX interaction impairs NRX-mediated synaptogenic activity of NLGs, especially in inhibitory synapses.
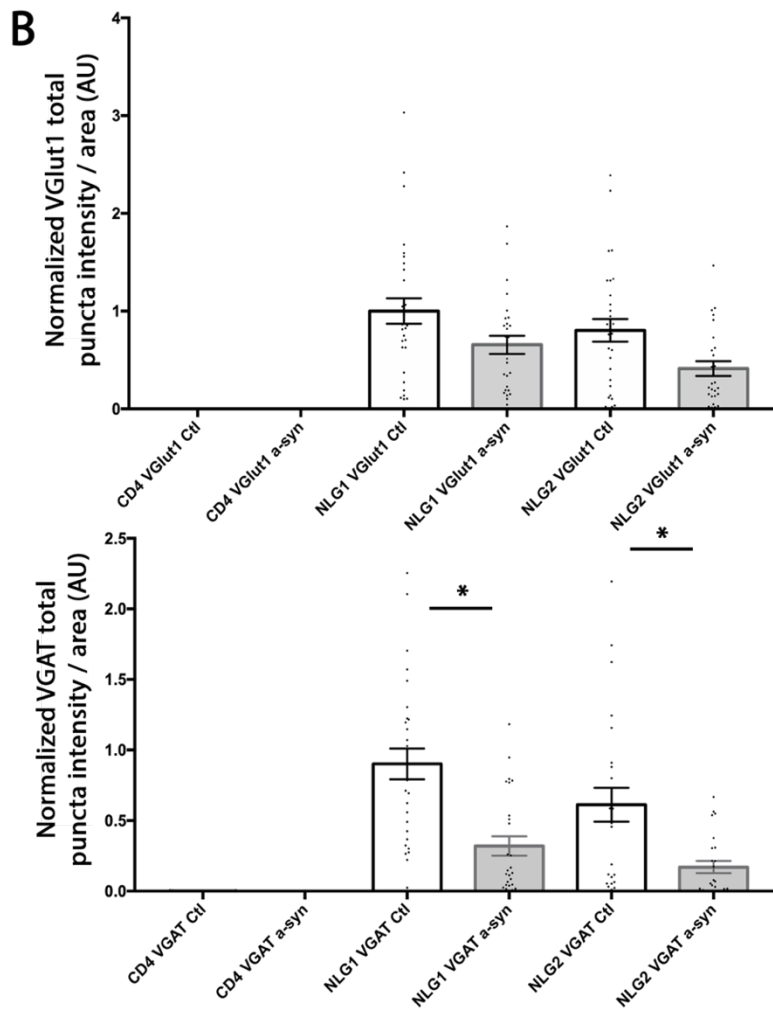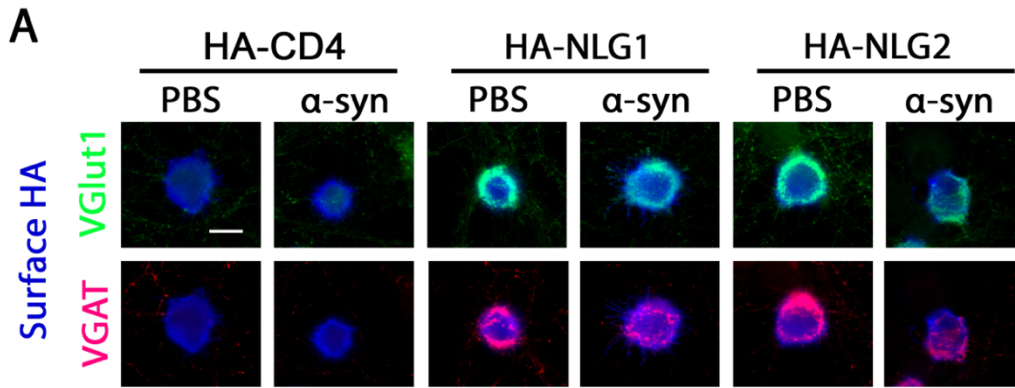
Figure 8. Oligomeric α-synuclein diminishes inhibitory presynaptic differentiation induced by NRX interactors neuroligin 1/2

(a) Representative images of HEK293T cells overexpressing the indicated HA-tagged constructs co-cultured with hippocampal neurons and treated with untagged α-syn oligomers (500 nM, monomer equivalent) or PBS (a

negative control) followed by triple immunolabelling for surface HA (blue), VGlut1 (green) and VGAT (red). α-syn treatment significantly decreases inhibitory presynaptic induction (observed as a VGAT accumulation) by NLG1 A-B- and NLG2. In contrast, α-syn treatment doesn't significantly affect excitatory presynaptic induction (observed as a VGlut1 accumulation) induced by NLG1 A-B- (P = 0.6755) and NLG2 (P = 0.2613) although a decreasing tendency can be observed. Scale bar represents 20 μm. **(b,c)** Quantification of the total intensity of VGlut1 **(b)** and VGAT **(c)** puncta on HEK293T cells expressing the indicated HA-tagged proteins divided by the cell area. n = 30 cells for each construct from three independent experiments using a Kruskal-Wallis test for non-parametric data, N.S.: not significant (P>0.05) and *P ≤0.05 in comparing between PBS- and α-syn-treated groups by post hoc Dunn's multiple comparison tests. Data are presented as mean ± SEM.

Chapter 5. Discussion

In this study, we screened a total of 29 synaptic organizers to isolate a potential receptor for α-syn oligomers and found that β-NRXs are the only synaptic organizers that bind to biotin-α-syn oligomers (250nM). Specifically, NRX1β S4- showed a nanomolar range affinity with a dissociation constant of 523 ± 34.4 nM. We further found that α-syn oligomers strongly bound to NRX1,2β S4± in an HRD-dependent manner and weakly bound to NRX3β S4+. Despite no significant effect on the binding of NRX1β S4- to NLG1 and LRRTM2, the α-syn interaction increased the internalization of NRX1β S4- on axon surface and therefore dampened NRX-mediated synaptogenic activity, especially the induction of inhibitory presynaptic differentiation induced by NLG1 and LRRTM2 (Fig. 9).

5.1 Our alpha-synuclein preparation differs from other protocols

Although most studies use α-syn PFFs prepared by five to seven-days incubation (12, 179, 347, 358-360), we used biotin-α-syn oligomers prepared by one-day preparation. This selection was determined by a cell surface binding assay using the preparation of biotin-α-syn oligomers with different incubation periods, which showed that one-day preparation has the highest binding of biotin-α-syn to NRX1β. To avoid any sample variability among the different indication period conditions, we first prepared eight aliquots by taking the supernatant at eight different time points from the identical source of biotin-α-syn that was incubated for seven days. However, we observed that incubation for more than two days increased the insoluble fraction (pellet), suggesting that for two- to seven-days preparations, the effective concentration of biotin-α-syn that works for NRX binding in a cell surface binding assay may be decreased as this assay allows us to measure only the binding of soluble ligands on cell surface. In general, the increase in the insoluble fraction by longer incubation would result in a decrease of soluble fraction in the supernatant. Therefore, when a part of the supernatant was aliquoted at the different time points, the actual concentration of soluble biotin-α-syn in the aliquots would be lower than the monomer equivalent. Instead of using the monomer equivalent, a standard curve of BSA protein would be more helpful to measure more precisely the concentration of soluble α-syn in each

aliquot. Another possibility to explain the observation of lower α-syn binding in case of two- to seven-days preparation than one-day preparation is that α-syn forms generated by longer incubation may be different from those generated by one-day incubation, and these forms may have a different binding affinity to NRX1β. Indeed, previous studies using a thioflavin T (ThT) fluorescence assay have shown that α-syn aggregates more and forms larger aggregates after longer incubation (361, 362). Generally, oligomers are thought to be an intermediate form between monomers and protofibrils (363) and distinct α-syn conformations result in different phenotypes *in vitro* and *in vivo* (212, 364). This suggests that NRXs may have a higher binding affinity to oligomeric forms of α-syn than its protofibril forms, this idea would be consistent with our observations of biotin-α-syn condition and also by several independent assays. Indeed, our immunoblotting confirmed that high molecular weights in oligomer range (50-150 kDa, corresponding to trimers to decamers as α-syn molecular weight is 14.9 kDa) was detected most in one-day incubation, and that longer incubation resulted in higher molecular weights (more than 250 kDa). Additionally, our assay using a dynamic light scattering (DLS) (collaboration with Dr. Thomas Durcan and his team), which allows measuring an approximate size distribution of small particles, confirmed that the size of biotin-α-syn was around 30 nm (Table. S1, Supplementary Data), which corresponds to the oligomer size (365, 366).

5.2 Our screening only identified LAG3, NRX1,2β S4 ± and NRX3β S4+ as alpha-synuclein oligomer binding partners

Our cell surface binding assay using biotin-α-syn oligomers at 250 nM detected their significant binding to NRX1,2β S4± and their weak binding to NRX3β S4+. Several previous studies have detected significant binding of α-syn PFFs to other NRX isoforms such as NRX1,2α (187, 188) and to other synaptic organizers such as TrkB (367) and glypican1/2/4 (188), while our cell surface binding assay could not detect them. Specifically, Shrisvastava et al., isolated NRX1α and NRX2α as α-syn interactors by pull-down of whole-cell lysate of neurons using S-tagged α-syn as bait (188). Another study has shown that NRX1α as well as NRX1,2,3β bind to biotinylated α-syn PFFs by a cell surface binding assay (187). Furthermore, a previous pull-down assay using GST-tagged α-syn as bait has found a binding between pathological α-syn strains and TrkB in a whole-cell

lysate (367). Glypican 1,2,4 were also isolated from a whole-cell lysate after the application of S-tagged α-syn oligomers followed by the detection with nanoliquid chromatography and mass spectrometry (188) although the binding of α-syn to glypicans could not be confirmed by our binding assay (Fig. 3C) or direct pull-down (188).

These results suggest several possibilities. First, given a previous study showing that NRX1β/α-syn PFF interaction (Kd: 260 ± 44 nM) exhibits higher affinity than NRX1α/α-syn PFF interaction (Kd: 933 ± 657 nM) (187), the binding affinity of α-syn to NRX1,2α, TrkB and glypican 1,2,4 might be lower than the one to NRX1β S4- or LAG3, therefore their binding signals might be below measurable levels by our cell surface binding assay. The concentration of 250 nM, which we used for our candidate screening, might not be enough to detect α-syn binding to NRX1,2α, TrkB or glypican 1,2,4. Thus, additional binding assays using a higher concentration of α-syn would be necessary. The second possibility is that specific forms of α-syn, which would be different from those in our α-syn preparation, may possess the binding ability to NRX1,2α, TrkB and/or glypicans. As described above, our preparation seems mainly to contain oligomeric form of α-syn, whereas other groups used α-syn PFFs obtained with longer incubation which could contain bigger oligomers and protofibrils. Lastly, to check α-syn binding, we used a cell surface binding assay, but other studies used a pull-down assay in whole brain or neuron lysates (188, 367). Thus, different α-syn preparation and/or different approaches to detect α-syn binding might affect the measurable limit of each study, leading to inconsistent results. Further studies based on several independent α-syn preparations and binding detection methods would be required to verify α-syn binding to such key synaptic proteins.

5.3 Our binding curve displays a positive cooperative binding mode

The binding curve that we obtained for α-syn/NRX1β S4- interaction displayed a sigmoidal shape with a plateau level, and the Scatchard plot showed a concave-downward distribution, suggesting that this interaction may be based on a positive cooperative mode (355, 356). Positive cooperativity results from an increasing affinity between a ligand to a receptor as binding sites become occupied (355, 356). Previous studies reported that positive cooperativity can be observed in an aggregating system (368-370). Furthermore, it has been described that α-syn

aggregates show a positive cooperative binding mode to molecules and proteins such as benzothiazole molecules, dopamine and prolyl oligopeptidase (371-373). Interestingly, NLG1 was shown to bind to Aβ and act as a nucleating factor during its aggregation (374). Nucleation is the first step by which proteins aggregate and initiate the polymerization into bigger structures (375), and it has been shown that α-syn aggregation is nucleation-dependent (376). Therefore, considering our positive cooperative mode observed in the Scatchard plot for the binding of α-syn and NRX1β S4-, NRX1β could act as a nucleating factor for α-syn oligomers, thereby binding to more oligomers as the concentration increases until it reaches a plateau. To determine whether NRX acts as a nucleating factor, it would be interesting to follow α-syn accumulation on NRX1β S4- by ThT fluorescence assay.

5.4 Alpha-synuclein does not disrupt neurexin1β binding to its partners, but rather increase its internalization.

As previously shown, the interaction of NLG1 and LRRTM2 to β-NRXs requires their LNS domain (242, 244, 245, 247, 269), while we found that the interaction of α-syn with NRX1,2β requires their HRD. Given distinct domains responsible for each protein binding, our results showing that the α-syn binding did not disrupt the interaction between NRX1β S4- and NLG1-Fc or LRRTM2-Fc conformed with what we expected. Our previous study also showed that Aβ oligomers do not compete with NLG1 and LRRTM2 (338), proposing that both pathological proteins do not affect NRX synaptic function by disrupting its interaction with its postsynaptic binding partners. Since both proteins bind to HRD, it would be interesting to see if they compete with each other for binding to HRD.

Because α-syn does not disrupt NRX interactions with NLG1 A-B+ and LRRTM2, we next evaluated if α-syn would affect NRX trafficking and surface expression levels as a molecular consequence of α-syn-NRX interaction. Our results show that α-syn treatment facilitates NRX1β S4-internalization, which would presumably result in a decrease in its surface expression level. Our previous study has demonstrated that Aβ oligomers interact with NRXs (like α-syn oligomers) and also decrease the surface expression level of NRX1β S4- on hippocampal axons in an HRD-dependent manner (338). As we showed the increased internalization, it would be essential to

confirm if α-syn treatment decreases the surface level of NRX1β S4- on axons by time-lapse imaging of neurons expressing NRX tagged with a pH-sensitive GFP named Superecliptic pHluorin (SEP). When a SEP-tagged protein is expressed at the neuronal surface, as it is under neutral conditions, SEP protein emits green fluorescent light when exposed to light in the blue range, but it is non-fluorescent when exposed to lower pH (pH <6) as it is internalized. Using time-lapse imaging of hippocampal neurons expressing SEP-tagged CD4, NRX1β S4- or NRX1β S4- ΔHRD, we could quantify GFP intensity before and after the bath application of α-syn oligomers, as we did previously for Aβ studies (338).

5.4.1 NRX internalization could play a key role in alpha-synuclein uptake

Given that α-syn facilitates NRX internalization, extracellular α-syn oligomers could be internalized together with NRXs by forming a complex with NRXs, this leads to the interesting possibility that NRX may act as an α-syn receptor to uptake α-syn oligomers into neurons. Furthermore, as Braak's model has indicated, α-syn is known to be transmitted from neurons to other neurons through neuronal internalization of α-syn (10). Therefore, it is also possible that NRXs mediate neuron-to-neuron spreading of α-syn as its functional receptor. To address the first possibility, the internalization assay using pH-sensitive dye (e.g., pHRodo)-tagged α-syn peptides applied to neurons overexpressing NRXs or those with NRX knockdown or knockout would be interesting for future studies. For the second possibility, it would be worthwhile to investigate neuronal spreading of α-syn using a microfluidic three chambers system with NRX-knockdown/knockout neuron cultures as an *in vitro* experiment and performing α-syn injection into the striatum of NRX mutant mice and wildtype littermates followed by assessing neuronal pathology in SNc as *in vivo* experiments.

Using these experimental approaches *in vitro* and *in vivo*, a previous study has nicely demonstrated that LAG3 acts as a receptor of α-syn PFFs to mediate their neuronal uptake and spreading (187). However, it remains unclear if LAG3 acts as an α-syn receptor at synaptic level because it has not been elucidated if LAG3 is expressed and/or localized at synapses. Importantly, it has been previously demonstrated that the accumulation of α-syn oligomers in synapses is observed in 50-92% of DLB patients (145) and that these aggregates are progressive in the disease

and precedes LB (176). Thereby, the identification of a synaptic α-syn receptor could be essential to better understand synaptic accumulation of α-syn and synaptic spreading of α-syn pathology. Given our findings, NRXs could be promising candidates as synaptic α-syn receptors. Future experiments would be crucial to elucidate if NRXs mediate α-syn uptake and spreading.

5.5 Alpha-synuclein and neurexin interaction is a promising pathway behind synaptic dysfunction in synucleinopathies

5.5.1 Alpha-synuclein affects NRX-mediated inhibitory presynaptic differentiation

Furthermore, since NRXs play their roles at synaptic surface (272-274, 281-283), NRX internalization facilitated by α-syn oligomers could largely affect its synaptic functions, consequently affecting normal cognitive functions. In this study, our artificial synapse formation assays demonstrate that α-syn oligomers significantly dampen NRX-dependent inhibitory presynaptic differentiation. In addition, we observed a decrease in VGAT level in hippocampal neuron cultures treated by α-syn oligomers for one DIV compared to a PBS negative control treatment (Supplementary data, Fig. S3).

Inhibitory control is indispensable in all networks throughout the central nervous system (377, 378). Inhibitory synaptic circuits are crucial to create a balance between motor activation and inhibition (379-381). As inhibitory circuits are present throughout the brain, the effect of α-syn oligomers on NRX-dependent functions should be tested in other regions such as the cortex, the striatum and the midbrain. It has been previously shown that NRXs are widely expressed in the cerebral cortex, the hippocampus, the striatum, the substantia nigra and the cerebellar nuclei (242), all of which are affected in synucleinopathies. One interesting region is the striatum, because motor symptoms in Parkinson's disease result from the impairment of the SNc/striatum circuit (382). Its major population is inhibitory neurons called medium spiny neurons (MSNs) (383, 384). It has been described that MSNs from the ventral striatum (also known as nucleus accumbens) express high levels of NRX1α,β and NRX3α,β (267, 353, 385) and low levels of NRX2α,β (267). Interestingly, NRX1β was revealed to be highly expressed in the first and the second layers of the neocortex, the thalamus, the hippocampus and the granular cells, but also

expressed in the striatum, the olfactive bulb and the dorsal motor nucleus of the vagal nerve which are regions affected in PD (10, 266, 353). Furthermore, previous studies have found a decrease of NRX1 levels in primary mesencephalic cultures treated by 6-OHDA (345) and in the striatum of PD model animals (6-OHDA treated)(344). Given that striatal neurons express high levels of NRX1β, which is a major NRX isoform for α-syn binding, it would be interesting to check whether and how α-syn/NRX binding affects the functions of striatal neurons.

5.5.2 Alpha-synuclein does not affect NRX-dependent excitatory presynaptic differentiation

An unexpected finding in our artificial synapse formation assay is that α-syn is not likely to significantly affect NRX-mediated excitatory presynaptic differentiation. It has been previously described that NRX1β S4+ preferentially induces inhibitory synapses (277, 278) even if NRX1β-expressing COS7 cells cocultured with neurons are able to induce both excitatory and inhibitory synapses (276). Given that we observed the strongest binding of biotin-α-syn oligomers to NRX1β S4+ and that the differential expression of NRXs in different neuron-type and region is likely to yield insight into synapse sensitivity (385, 386), it is possible that the neuron-type specific expression of NRX1β S4+ may be involved in the preferential effects of α-syn to GABAergic, rather than glutamatergic, axons. Indeed, a higher level of S4 insertion in NRX1 is detected in PV-positive interneurons compared to excitatory pyramidal neurons in the hippocampus although NRX1β expression in pyramidal neurons and in PV-positive interneurons is comparable (386). Therefore, NRX expression patterns in PV-positive interneurons and pyramidal neurons could be the key behind inhibitory synapse sensitivity to α-syn. Since we observed a small tendency towards decreasing VGlut1 puncta intensity in α-syn-treated neurons, it is possible that a longer treatment of α-syn may be necessary to detect a significant effect on NRX-mediated excitatory presynaptic differentiation.

Surprisingly, previous experiments have shown that Aβ decreases specifically NRX-mediated excitatory presynaptic differentiation (338). Even if both aggregated proteins bind to HRD, they have two different effects on NRX function. In this experiment, we used NLG1 A-B- because it was shown that NLG1 A+ decreases excitatory synaptogenic activity and NLG1 B+ almost abolishes

inhibitory synaptogenic activity (278). To avoid a decrease in synaptogenic activity we decided to use NLG1 A-B-. However, since NLG1 B- binds to both α-NRXs and β-NRXs (243, 285), the decrease could be due to α-syn binding to other isoforms. The use of NLG A- B+ that only binds NRX1β S4- (243, 285) would have isolated the effect on our isoform of interest. Both NLG A-B- and NLG2 can bind to NRXs S4+ and S4-. One difference between Aβ and α-syn is that Aβ, but not α-syn, can bind to NRX3β S4± and NRX1,2α S4+. All these NRX isoforms are expressed in all hippocampal cells with distinct expression intensity and patterns (267). Particularly, NRX3β is highly expressed in pyramidal cells (267). Therefore, it is possible that Aβ could preferentially impair NRX-mediated excitatory presynaptic differentiation through interacting with NRX3β in pyramidal cells. Given that Alzheimer's disease and synucleinopathies are similar diseases in respect to deposits of protein aggregates and their association with synaptic dysfunction and synapse loss, it would be essential to understand how Aβ and α-syn differentially affect NRX functions to provide new insight on common and distinct molecular pathways in neurodegenerative diseases.

In this study and our previous study on the roles of NRXs in α-syn and Aβ pathologies, respectively, we have focused on the roles of NRXs in synaptic differentiation. However, NRXs play other important roles such as roles in synaptic transmission and synaptic plasticity (219, 273, 274, 283, 387, 388). Interestingly, like Aβ oligomers treatment and AD model mice, LTP is impaired by α-syn treatment and in PD model mice line overexpressing α-syn (198, 199). Besides, some PD model mice display cognitive deficits, which would be behavioral consequences of LTP impairment (389-393). Therefore, to promote translational research, it would be interesting to test if NRXs mediate the α-syn-induced impairments of synaptic plasticity by electrophysiology and/or cognitive dysfunctions by characterizing NRX mutant mice crossed with the PD model mice.

5.6 Limitations

In this study, the majority of the experiments were done on non-neuronal cells called COS7 cells that were overexpressing different plasmids. In consequence, all binding measures were indirect measures and could have been influenced by the cell type since α-syn and NRX are neuronal proteins. The overexpression does not represent the physiological levels of expression of these

proteins. The other cellular model used was primary hippocampal neuron culture. One type of neuron highly affected in synucleinopathies is the dopaminergic neurons from the SNc, thereby the use of nigral neuron cultures would have been preferable to better illustrate the disease and highlight the importance of NRX1β in these neurons. However, hippocampal neurons have high levels of NRX1β (267, 353) and also show α-syn inclusions (354), therefore are a great model for this study. Another limitation is the use of internalization assays which are indirect measures of the surface level of NRX1β S4-. Moreover, internalization of α-syn together with NRX1β S4- was not confirmed. Lastly, synucleinopathies are age-related diseases, thus artificial synapse formation assays are not ideal to portray the impact of α-syn/NRX interaction in neurodegenerative disorders, even if it nicely illustrates impairment in NRX functions. In addition, both artificial synapse formation assays and internalization assays, where HA-tagged plasmids were overexpressed, do not represent physiological conditions.
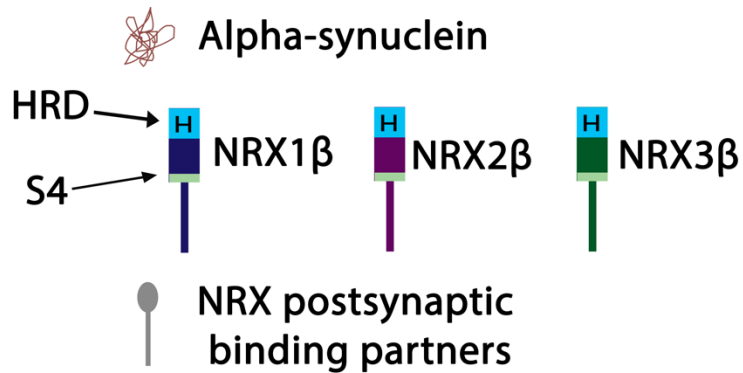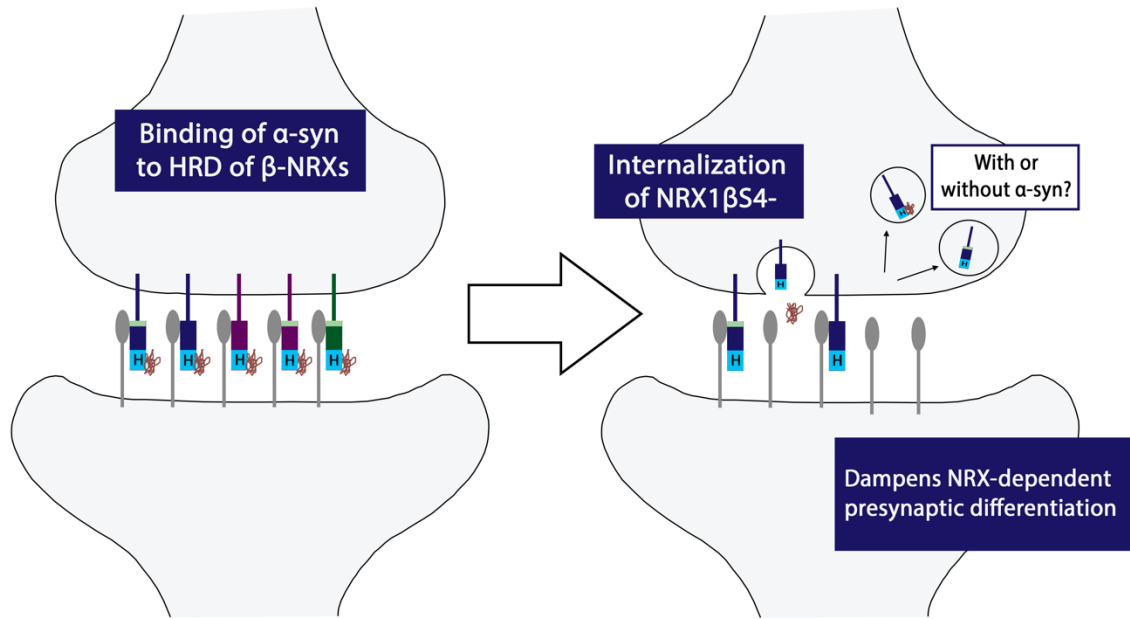
Figure 9. Scheme of potential mechanisms of neurexin implication in α-synuclein pathology

α-syn binds to the HRD of NRX1,2β S4± and NRX3β S4+. Although α-syn binding does not directly affect the binding of NRX1β to its postsynaptic partners NLG1 and LRRTM2, α-syn binding facilitates NRX1β S4-internalization in an HRD-dependent manner and consequently dampens NRX-dependent inhibitory presynaptic differentiation. The formation of the complex of α-syn oligomers and NRX1β S4- might result in the neuronal uptake of α-syn, which may trigger synaptic and neuronal toxicity and also be involved in neuron-to-neuron spreading.

Chapter 6. Conclusion and future perspectives

Throughout this study, we show that α-syn oligomers bind to NRX1,2β in an HRD-dependent manner and increase β-NRX internalization, which results in the impairment of NRX-mediated inhibitory presynaptic differentiation (Fig. 9). Our results present a key molecular mechanism underlying α-syn-induced synaptic dysfunction and perhaps neuronal spreading of α-syn pathology. Given that β-NRXs are expressed throughout the brain, β-NRXs are also good candidates that mediate α-syn pathology spreading (267, 353). Furthermore, β-NRXs could be key molecules in neurodegenerative disease as they are essential proteins for normal synaptic function, and as they are affected by both Aβ oligomers and α-syn oligomers, two pathological aggregates in Alzheimer's disease (AD) and synucleinopathies, respectively. Interestingly, both AD and synucleinopathies patients can present with dementia. Thus, the study of β-NRXs contributes to a better understanding of a common mechanism underlying synaptic dysfunctions, in two major neurodegenerative diseases, offering a promising pharmacological target.

The next important future perspective should be to characterize *in vivo* roles of NRXs in α-syn pathology. In particular, to elucidate the *in vivo* roles of NRX1,2β, we recently generated two new mouse lines expressing either the mutant NRX1β lacking its HRD (NRX1β ΔHRD) or the mutant NRX2β lacking its HRD (NRX2β ΔHRD). It would be interesting to cross these mice with synucleinopathy model mice such as transgenic mice overexpressing wild-type human α-syn under the Thy-1 promoter(394-398) to check if the NRX1,2β HRD deletion affects α-syn pathology *in vivo*. From around 4.5 months after birth (399), these mice show α-syn accumulation in synapses and neurons throughout the brain, including the substantia nigra, the hippocampus, the basal ganglia, the thalamus, the cortex and the brainstem (394). This line also exhibits synaptic and neuronal pathologies such as the alteration of synaptic activity in the striatum, the hippocampus and the cortex (195, 389, 400-402), LTP impairment in the hippocampus, striatum and SNc (200, 402) and neuronal loss in the SNc (389, 401). Therefore, this model would be ideal to assess the *in vivo* effects of HRD deletion in NRX1,2β in α-syn accumulation and α-syn-induced synaptic and neuronal toxicity.

Finally, since the Thy1-α-syn mice show motor and non-motor features (394-398), it would be ideal to assess if the deletion of HRD could rescue these behavioral abnormalities in this line. Firstly, motor symptoms would be evaluated using the pole test and the challenging beam test that are commonly used in PD *in vivo* studies, and these mice show decreased performance in both of these tests (389, 396, 397). In the pole test, the mice are installed at the top of a pole and the time they take to turn and descend the pole are measured separately (389, 396, 397). In the challenging beam test, the mice are put on one side of a beam and the number of slips, the number of steps and the time they take to cross the beam are measured (389, 396, 397). Cognitive deficits could be evaluated by one-trial object-place recognition test, Y-maze test and novel object recognition test which were shown to be altered in Thy1-α-syn mice (389). Performance in the Morris water maze test, which evaluates hippocampus-dependent memory and learning, is impaired in Thy1-α-syn mice. Yet, it could be argued that this performance is due to motor deficits but not cognitive deficits (390). However, given that NRXs are highly expressed in the hippocampus (253, 254), it would be essential to do this test by measuring the time passed in the right cadran on total swimming time. These mice also show PD-early disease symptoms such as olfactory and sleep alterations, which could also be evaluated by measuring the latency to find a buried and a surface pellet and by measuring the EEG (electroencephalogram) and EMG (electromyogram) activity as it was previously described (389, 395, 397, 403). The roles of NRX1,2β in these tests could be assessed by comparing wild-type mice to Thy1-α-syn mice, NRX1,2β ΔHRD and NRX1,2β ΔHRD crossed to Thy1-α-syn mice.

Although Thy1-α-syn mouse line is useful to study α-syn pathology (389, 394, 398), this line may have a disadvantage for us to investigate neuronal spreading of α-syn pathology because Thy-1 promoter drives α-syn overexpression very broadly throughout the brain (389, 394). To more preciously elucidate whether and how the NRX1,2β HDR deletion affects the α-syn spreading *in vivo*, it would be worthy to perform a stereotactic injection of α-syn PFFs in the striatum of the NRX1,2β ΔHRD mutant mice and then investigate α-syn pathology in the SNc. According to previous works (166, 179, 404, 405), after injection of α-syn PFFs in the dorsal striatum in wild-type mice, this line displays PD-like LB/LNs and cell-to-cell transmission to interconnected regions. The accumulation of α-syn was associated with progressive loss of dopaminergic neurons from

the SNc, reduced dopamine levels in the dorsal striatum and motor abnormalities. Therefore, this model is suitable to test the *in vivo* roles of NRX HRD in neuronal spreading of α-syn pathology (in this case, from the striatum to SNc) and α-syn-induced motor dysfunction.

Finally, I believe that the use of these mice will provide new insights into how α-syn and NRXs interaction can contribute to *in vivo* phenotypes. First, the crossing of Thy1-α-syn mice with NRX HRD-deleted model mice will allow us to distinguish the implication of α-syn/NRX interaction in the different phenotypes observed in synucleinopathies, such as motor, cognitive, behavioral and sleep abnormalities (5, 9, 33). With these *in vivo* studies, we will identify which symptoms in synucleinopathies, if any, could be mediated by NRX1,2β and their binding to α-syn. Furthermore, the use of the HRD-deleted model mice will allow a better understanding of the implications of the HRD in α-syn pathology and synaptic pathology in synucleinopathies and could yield a promising region of interest for pharmaceutical research. Finally, these studies would address the question whether NRX1,2β through their HRD play a key role in α-syn pathology spreading and would contribute to translational research and therapeutic strategies against the disease progression in synucleinopathies.

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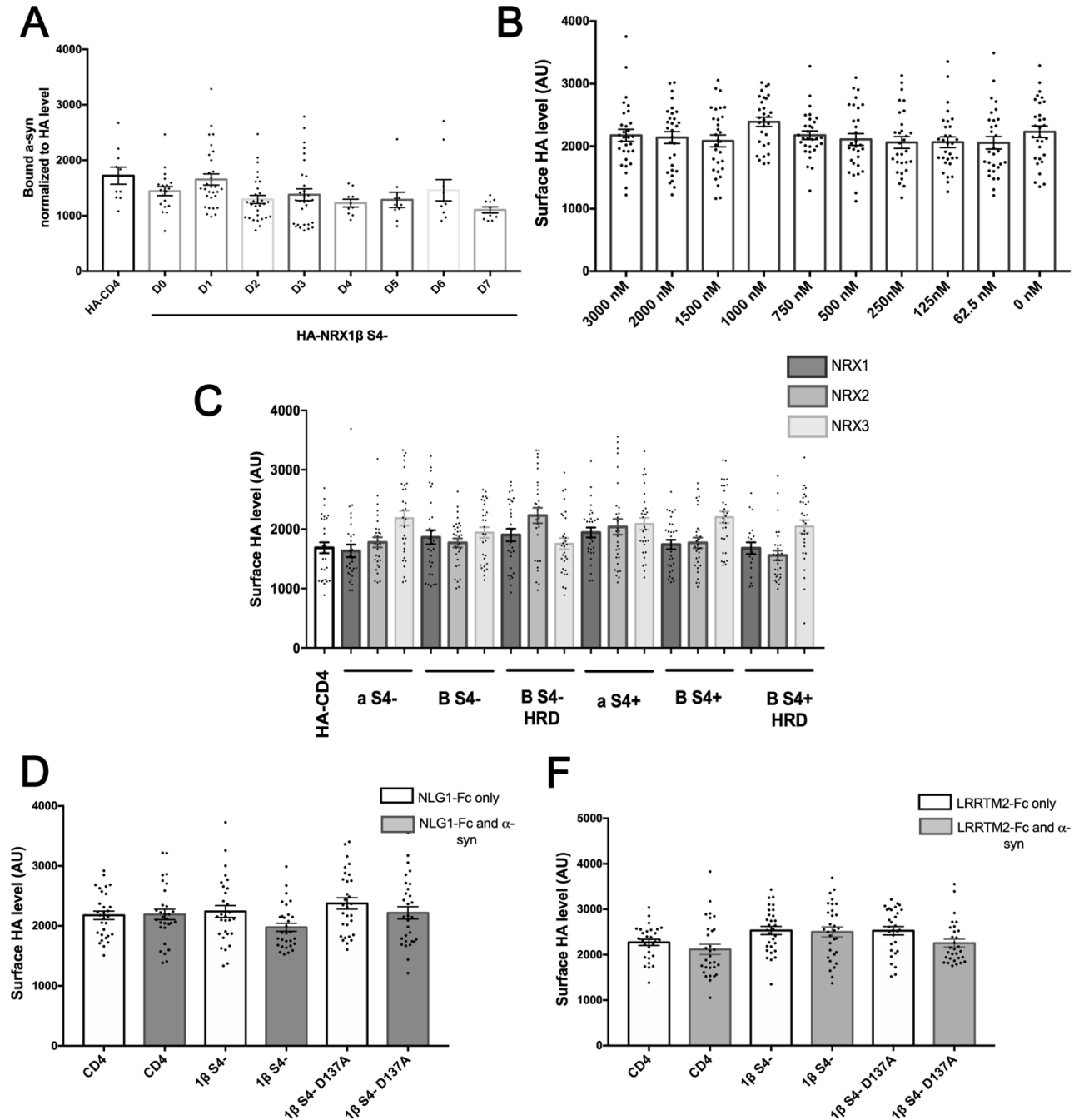
Annexes

Supplementary data

Figure S1. All cells selected for analysis for each experiment have similar levels of surface HA signal

(a) Cells selected for cell surface binding assays of zero- to seven-days preparations have similar levels of surface HA signals. **(b)** Cells selected for binding curve analysis show similar levels of surface HA signals. **(c)** Cells

expressing HA-tagged NRX isoforms used for the analysis have similar levels of surface HA signals. **(d)** The HA

levels of cells expressing NRX1β isoforms treated by NLG1-Fc only or NLG1-Fc + biotin-α-syn were comparable.

(e) The cells expressing NRX1β isoforms treated by LRRTM2-Fc only or LRRTM2-Fc + biotin-α-syn have

comparable levels of surface HA signal. **(a-e)** *n* = 30 cells for each construct from three independent experiments

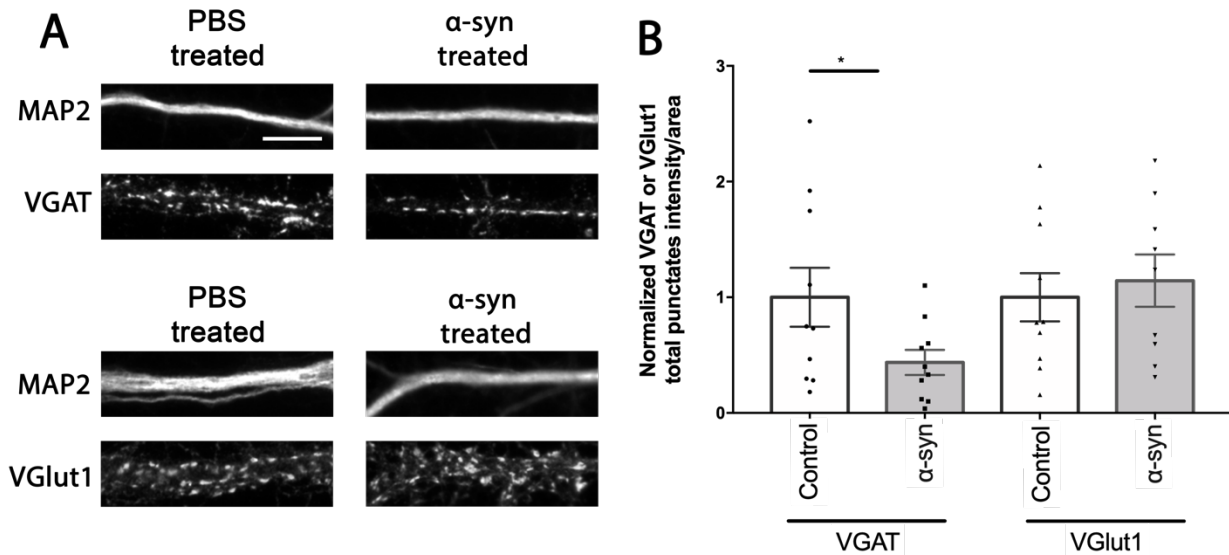using a Kruskal-Wallis test for non-parametric data, N.S.: not significant. Data are presented as mean ± SEM.

Figure S2. Application of untagged α-syn decreases VGAT level, but not VGlut1 level in cultured hippocampal neurons

 (a) Representative images of hippocampal neurons at three weeks *in vitro* treated with α-syn oligomers (500

nM, monomer equivalent) or PBS (a negative control) and labelled by anti-VGAT or anti-VGlut1. Scale bar

represents 10 μm. **(b)** Neurons treated with α-syn oligomers show a decrease in VGAT total puncta intensity,

but not in VGlut1 total puncta intensity. *n* = 10 cells for each construct from one independent experiment using

unpaired Mann-Whitney tests for two groups comparison, N.S.: not significant, *P ≤0.05, **P ≤0.01 and ***P

≤0.001 in comparing between PBS- and α-syn-treated groups. Data are presented as mean ± SEM.

Table S1. Examples of size measures in our oligomer preparations of biotin-α-syn and untagged α-syn by Dynamic Light Scattering.

The measured samples of the indicated α-syn were prepared for one-day incubation. These samples show smaller sizes as major species than what is reported with seven-days incubation (between 29 nm to 195 nm) (347).

	Size (nm)	% volume
Biotin-α-syn	24.34	99.0
	25.38	99.2
Untagged α-syn	33.55	94.8
	29.59	100.00