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Alpha-Synuclein as a Regulator of Synaptic Signalling

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ANSSI PELKONEN

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ABSTRACT

Alpha-synuclein (α-syn) is a protein that becomes aggregated into Lewy bodies and other insoluble inclusions in Parkinson's disease and some other neurodegenerative diseases. Its expression is increased also in drug addiction. The principal physiological function of the protein appears to be regulation of synaptic dopamine (DA) signaling in the brain, but the protein also seems to regulate other neurotransmitter systems and is expressed throughout the body. The aim of the current work was to examine the neuromodulatory effects by which α-syn influences synaptic signaling in different scenarios.

The role of α-syn in development of addiction was studied by comparing α-syn protein expression between two rat lines bred for differential ethanol preference (study I). The ethanol preferring Alko, alcohol rats displayed significantly higher α-syn expression in nucleus accumbens core in comparison to their non-ethanol preferring Alko, non-alcohol counterparts. Constant potential amperometry (CPA) revealed that the increased α-syn expression in the ethanol preferring rats was associated with a decrease in stimulated DA overflow and facilitation of re-uptake.

Possible role of α-syn in peripheral cholinergic signaling and motor control was examined with electromyography in wild-type and α-syn knockout mice (study II). Lack of α-syn caused dysregulation in the neuromuscular junction, and the α-syn deficient mice showed signs of deteriorated motor learning, but displayed no significant defects in muscle strength or ambulation.

The effect of α-syn on general neuronal excitability and the resulting depolarization was determined by measuring the initiation and propagation of cortical spreading depression in wild-type and α-syn knockout mice using direct current potential recording (study III). However, no differences were revealed between the two lines.

α-Syn can be transferred from neuron to neuron via the extracellular space, but it is not known whether exogenous α-syn is able to influence synaptic DA signalling. Therefore α-syn protein was microinjected into the brain of wild-type and α-syn knockout mice, and the effects on DA signaling were measured with CPA (study IV). This revealed that the exogenous protein can affect stimulated DA overflow and that the effects depend on the presence of endogenous α-syn.

It can be concluded that α-syn is involved in the fine adjustment of synaptic signalling and its main function under normal conditions is to reduce DA release and facilitate its re-uptake, even though α-syn can have also paradoxical effect on these functions under certain conditions. At the behavioral level, this synaptic modulation can affect functions such as preference for substances of abuse and motor learning.

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TIIVISTELMÄ

Alfa-synukleiini (α-syn) on hermopäätteiden valkuaisaine joka kertyy Lewyn kappaleiksi ja muiksi liukenemattomiksi kertymiksi Parkinsonin taudissa ja erinäisissä muissa hermorappeumataudeissa. α-Syn:n ilmentyminen lisääntyy myös riippuvuudessa. α-Syn:n fysiologinen tehtävä vaikuttaisi olevan dopamiini (DA) – välitteisen synaptisen signaalinvälityksen säätely aivoissa, mutta se voi vaikuttaa myös muiden välittäjäainejärjestelmien toimintaan ja sitä ilmennetään läpi koko kehon. Tämän työn tavoitteena oli tutkia alfa-synukleiinin vaikutuksia synaptiseen signaalinvälitykseen erilaisissa yhteyksissä.

α-Syn:n roolin tutkimiseksi riippuvuuden kehityksessä sen ilmentämistä verrattiin kahdessa rottalinjassa jotka on jalostettu vapaaehtoisen etanolin kulutuksen suhteen (tutkimus I). Etanolista pitävät Alko, alcohol – rotat ilmensivät enemmän α-syn:a nucleus accumbensin ytimessä verrattuna etanolia välttäviin Alko, non-alcohol – rottiin. Vakiopotentiaaliamperometria (VPA) -tulosten mukaan lisääntynyt α-syn:n ilmentäminen etanolista pitävissä rotissa liittyi ärsytetyn DA:n ulosvirtauksen vähenemiseen ja DA:n takaisinoton nopeutumiseen.

α-Syn:n suhteen poistogeenisissä ja villityypin hiirissä tehtiin elektromyografisia mittauksia α-syn:n mahdollisen roolin tutkimiseksi ääreisosien kolinergisen signaalinvälityksen säätelyssä (tutkimus II). α-Syn:n puuttuminen aiheutti säätelyn puutoksia hermo-lihasliitoksen signaalinvälityksessä ja vaikeutti kehon hallinnan oppimista, mutta sillä ei ollut merkitystä lihasvoiman tai askelluksen kannalta.

α-Syn:n vaikutusta yleiseen hermosolujen ärtyvyyteen ja siitä johtuvaan depolarisaatioon tutkittiin mittaamalla aivokuoren laajenevan vaimentuman kynnystä ja etenemistä α-syn:n suhteen poistogeenisissä ja villityypin hiirissä jännitemittauksella käyttäen tasavirtakytkettyä vahvistinta (tutkimus III). Minkäänlaisia eroja ei kuitenkaan havaittu hiirilinjojen välillä.

α-Syn voi siirtyä hermosolusta toiseen soluvälitilan kautta, mutta muissa soluissa tuotetun α-syn:n vaikutuksia vastaanottajasolun synaptiseen signaalinvälitykseen ei tunneta. Niinpä α-syn:a mikroinjektoitiin villityypin ja α-syn:n suhteen poistogeenisten hiirten aivoihin ja vaikutukset DA-välitteiseen signaalinvälitykseen mitattiin VPA:lla. (tutkimus IV). Ulkosyntyisen α-syn:n havaittiin aiheuttavan muutoksia ärsytettyyn DA:n ulosvirtaukseen, ja vaikutukset riippuivat sisäsyntyisen α-syn:n ilmentämisestä.

Yhteenvetona voidaan todeta, että α-syn toimii synaptisen signaalinvälityksen hienosäädössä. Sen pääasiallinen tehtävä on vähentää DA:n vapautusta ja nopeuttaa sen takaisinottoa, vaikka sillä voi olla vastakkaisia vaikutuksia kyseisiin prosesseihin tietyissä yhteyksissä. Käyttäytymisen tasolla α-syn:n toiminta voi vaikuttaa esimerkiksi mieltymykseen huumaaviin aineisiin tai kehon hallinnan oppimiseen.

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Anssi Pelkonen

List of original publications

This dissertation is based on the following original publications:

I Pelkonen A, Hiltunen M, Kiianmaa K and Yavich L. Stimulated dopamine overflow and alpha-synuclein expression in the nucleus accumbens core distinguish rats bred for differential ethanol preference. *Journal of neurochemistry 114*: *1168-1176, 2010.*

II Pelkonen A and Yavich L. Neuromuscular pathology in mice lacking alpha-synuclein. *Neuroscience letters 487: 350-353, 2011.*

III Pelkonen A and Yavich L. Cortical spreading depression in alpha-synuclein knockout mice. *Synapse (New York, N.Y.) 66: 81-84, 2012.*

IV Pelkonen A, Kallunki P and Yavich L. Effects of exogenous alpha-synuclein on stimulated dopamine overflow in dorsal striatum. *Neuroscience letters 554: 141-145, 2013.*

Contents

Abbreviations

AcbC	Nucleus accumbens core
AcbSh	Nucleus accumbens shell
bFGF	Basis fibroblast growth factor
CMAP	Compound muscle action potential
CPu	Caudate putamen
CSD	Cortical spreading depression
CSPα	cysteine-string protein-α
DA	Dopamine
DAT	Dopamine transporter
DC	Direct current
DLB	Dementia with Lewy bodies
DS	Dorsal striatum
GABA	Gamma-aminobutyric acid
GATA-1	GATA binding factor 1
GATA-2	GATA binding factor 2
LTP	Long-term potentiation
LXRα	Liver X receptor alpha
LXRβ	Liver X receptor beta
MSN	Medium spiny neuron
NBIA1	Neurodegeneration with brain iron accumulation 1
NMDA	N-methyl-D-aspartic acid
NURR1	Nuclear receptor related 1 protein
PD	Parkinson's disease
PLD2	Phospholipase D2
R-CMAP	Repetitive compound muscle action potential
SNARE	Soluble N-ethylmaleimide–sensitive factor attachment protein receptor
SN	Substantia nigra
TH	Tyrosine hydroxylase
VMAT2	Vesicular monoamine transporter 2

VTA	Ventral tegmental area
ZNF219	Zinc finger protein 219
ZSCAN21	Zinc finger and SCAN domain containing 21
α-syn	Alpha-synuclein
β-syn	Beta-synuclein
γ-syn	Gamma-synuclein

1 Introduction

Alpha-synuclein (α-syn) is a small protein, best known for its aggregation into Lewy bodies and other insoluble inclusions found in Parkinson's disease (PD) which is the second most common neurodegenerative disease after Alzheimer's disease (de Lau and Breteler, 2006). In addition, α-syn inclusions are detected in dementia with Lewy bodies (DLB), multiple system atrophy (MSA) and neurodegeneration with brain iron accumulation 1 (NBIA1; Bennett, 2005). These neurodegenerative diseases are commonly known as synucleopathies (or synucleinopathies in some publications). The symptoms of synucleopathies range from severe loss of motor control to dementia and several other cognitive symptoms. The physiological role of α-syn is to regulate the chemical signal transduction between neurons, i.e. synaptic signaling, and it is disruption of these processes which is believed to be the foundation of the pathology of synucleopathies (Sidhu *et al*., 2004). α-Syn appears to modulate synaptic signaling particularly in those neuronal cells releasing the neurotransmitter, dopamine (DA), which is responsible for motivation of our actions (Salamone and Correa, 2012). The goal of the current work was to elaborate some of the basic principles by which α-syn could affect synaptic signaling in different scenarios.

DA release is affected by all drugs of abuse, either directly or indirectly (Hyman *et al*., 2006) and there is accumulating evidence suggesting that chronic substance abuse is associated with differential α-syn expression. Elevated levels of the protein have been found in the brains of cocaine users (Mash *et al.,* 2003) and serum of alcoholics (Bönsch *et al*., 2005a**)**. There are also findings implying that the level of α-syn expression may contribute to predisposition in an individual towards addictive behavior. For example, rats bred for differential ethanol preference were found to have significantly increased expression of α-syn specifically in the hippocampus, the area of the brain responsible for formation of memories (Liang *et al*., 2003). It is crucial to understand the neurobiology of substance abuse and addiction because of their massive socioeconomic impacts: in Finland alcohol related incidents are the most frequent cause of death in the age group 15-64 years (14.6 % in 2006; http://www.tilastokeskus.fi) and 9 out of 10 prisoners are addicted to some substance of abuse (http://www.porttivapauteen.fi). Addiction has been defined as a primary, chronic disease of brain reward, motivation, memory and related circuitry (The American Society of Addiction Medicine, http://www.asam.org). It is against this backround that we must learn to understand the association between α-syn and addiction.

It has been claimed that α-syn may be the initiating factor of disease in the synucleopathies (Luk *et al*., 2012). Furthermore, it is known that the protein can be exocytosed from neurons and taken up into neighbouring cells (Lee *et al*., 2005; 2008). In the case of pathological forms of the protein, this translates into a spread of pathology from neuron to neuron (Volpicelli-Daley *et al*., 2011) and from one brain region to its neighbours (Braak *et al*., 2003). This has made α-syn an interesting target for new therapies (Maguire-Zeiss, 2008). However, if pathological forms of α-syn are considered to be the enemy in the war against synucleopathies, the non-pathological role of the protein should first be understood.

What is already known about α-syn in synaptic signaling? α-Syn has been postulated to regulate the synthesis, storage, release and uptake of DA (Sidhu *et al*., 2004) but the published results on the mechanisms of function and effects of the protein are rather diverse and often contradictory (Burré *et al*., 2010, Nemani *et al*., 2010). Therefore, the exact function of α-syn in dopaminergic neurotransmission still needs further study. DA and α-syn are involved in addiction (Mash *et al*., 2003; Hyman *et al*., 2006) but the exact mechanisms and brain structures through which they affect the development of the condition still need to be elaborated. There are some histological findings pointing to

localization of the protein in peripheral acetylcholine-releasing neurons (Wakabayashi and Takahashi, 1997; Sharrad *et al*., 2013) but it is not clear whether α-syn has a functional role in peripheral cholinergic neurotransmission. α-Syn has also been shown to regulate glutamate (Liu *et al*., 2004) and noradrenaline release (Yavich *et al*., 2006) but it is not certain if this is because of α-syn's involvement in pre-synaptic modulation of these particular neurotransmitter systems, or because α-syn is able to modulate the general properties of neuronal excitability that are common to all neurons (Adamczyk and Strosznajder, 2006; Hettiarachchi *et al*., 2009). The protein's transfer from neuron to neuron has been shown to play a major part in the pathological degeneration of the dopaminergic system in synucleopathies (Volpicelli-Daley *et al*., 2011; Luk *et al*., 2012), but there is no evidence on whether the transferred, exogenous protein has any effect on pre-synaptic DA signaling.

The current research attempted to answer these questions by applying different electrochemisty and electrophysiology methods in rodent models. Constant potential amperometry was used to measure parameters of stimulated DA overflow in rats bred for differential ethanol preference, and western blot was used to compare the α-syn expression in different parts of the striatum (study I). Comparing the phenotype of knockout mice to wild-type is a useful and widely used tool for elucidating the functions of individual proteins and thus a mouse line that has lost α-syn as a result of a spontaneous mutation (Specht and Schoepfer, 2001) was investigated and a gene-targeted knockout line was used to verify the putative findings (Abeliovich *et al*., 2000). The peripheral cholinergic signalling in the neuromuscular junction of α-syn knockout and wild-type mice was measured using electromyography, and the motor control of these animals was compared in different behavioral tests (study II). The effect of α-syn on neuronal excitability was evaluated by initiating and measuring cortical spreading depression (CSD) in α-syn knockout and wild-type mice by direct current (DC) potential recording (study III). Exogenous α-syn protein was delivered by microinjection to the striatum of α-syn deficient and wild-type mice and constant potential amperometry was used to measure the effects on stimulated DA overflow (study IV). The aim was to evaluate the effects of new emerging treatments for synucleopathies which could directly decrease the levels of α-syn in the body or inactivate it. In particular, it was intended to increase the fundamental knowledge of the regulation of synaptic signaling through α-syn.

2 Review of the Literature

2.1 THE SYNUCLEIN PROTEIN FAMILY, AND STRUCTURE AND EXPRESSION OF α-SYN

The first section of the literature review will examine the factors that determine the biological activity of α-syn. First the main functions of all synucleins and possible redundancy between them will be reviewed. Then the structure of the protein will be investigated to determine which components are crucial in its synaptic localization and function. Finally the factors which regulate the expression of the protein will be reviewed as well as how their function translates into high α-syn expression in certain parts of the nervous system.

2.1.1 Synucleins

α-Syn belongs to a three-member protein family consisting of alpha-, beta- and gamma-synuclein (reviewed by George, 2003). If one wishes to understand the function of an individual protein, one needs to investigate which functions and features are so central that they are conserved throughout the whole protein family. More importantly, knowledge of the overlapping expression and properties of synucleins is crucial when interpreting results from knockout models lacking only one or two members of the family (Chandra *et al*., 2004; Senior *et al*., 2008; Burre *et al*., 2010). It should also be remembered that some changes seen especially in single knockouts may be compensatory in response to the lack of the protein.

At present only vertebrate orthologs of synucleins have been identified (George, 2003). The first member of the family to be found was gamma-synuclein (γ-syn), isolated from the cholinergic nerve terminals of of the pacific electric ray (*Torpedo californica)* electric organ (Maroteaux *et al*., 1988). Human (*Homo sapiens*) α-syn was originally identified when studying amyloid plaques in Alzheimer's disease, and it was named as the non-Aβ component of Alzheimer's disease amyloid precursor (NACP; Uéda *et al*., 1993). Later on, a second synuclein was extracted from human brain and named as beta-synuclein (β-syn; Jakes *et al*., 1994), even though the bovine β-syn ortholog had already been discovered earlier and named phosphoneuroprotein 14 kDa (PNP14; Nakajo *et al*., 1993). The human γ-syn gene was found when studying risk factors for breast cancer and originally named as *BCSG1* (breast cancer specific gene 1; Ji *et al*., 1997). The genes for human α-syn (*SNCA*), β-syn (*SNCB*) and γ-syn (*SNCG*) are located in chromosomes 4q21.3-q22 (Chen *et al*., 1995), 5q35 (Spillantini *et al*., 1995) and 10q23.2-q23.3 (Ninkina *et al*., 1998), respectively. The proteins encoded by these are evolutionarily conserved, and the amino-acid sequences of mouse (*Mus musculus*) and rat (*Rattus norwegicus*) α-syn are 95.7 % identical to human α-syn (Gerorge, 2003; Fig. 1). Therefore one can consider that mice and rats are suitable models for studying the functions of synucleins.

All synucleins are highly, but not exclusively, expressed in the brain, with β-syn being the most and γ-syn the least brain specific of the family (Lavedan, 1998). Within the brain, synucleins are expressed in the cerebral cortex, amygdala, substantia nigra (SN), striatum, hippocampus and thalamus (Lavedan, 1998; Ninkina *et al*., 1998; Ahmad *et al*., 2007). Synucleins are expressed also in other brain areas but their expression patterns are not identical (Li *et al*., 2002); the subthalamic nucleus (STN) expresses only α-syn and corpus callosum has α- and γ- but no β-syn (Lavedan, 1998).

α- and β-syn tend to be localized in the pre-synaptic terminals while γ-syn appears to be more widely dispersed throughout the cytosol (Jakes *et al*., 1994; Surguchov *et al*., 2001). α-Syn is present also in the nucleus, and the nuclear expression is elevated during prenatal

development (Zhong *et al*., 2010). At least human γ-syn does not appear to share the nuclear localization of α-syn, even though there were reports that this occurred in the electric ray (Maroteux *et al*., 1988; Specht *et al*., 2005).

In the peripheral nervous system, γ-syn is the most higly expressed member of the family and α-syn second most higly expressed while β-syn lies below the detection limit (Buchman *et al*., 1998; Papachroni *et al*., 2005). All synucleins are expressed in skeletal muscle, and α- and γ-syn are expressed in the heart, liver, pancreas and kidney (reviewed by Lavedan *et al*., 1998). α-Syn also has also been detected in the skin, lung, kidney, spleen and placenta (Beyer *et al*., 2008) and it also has a very strong expression in erythrocytes (Barbour *et al*., 2008). The systemic expression patterns of synucleins change during development and aging (Buchman *et al*., 1998; Ninkina *et al*., 1998; Baltic *et al*., 2004) and especially α-syn protein levels in the brain are significantly increased in aged individuals (Chu and Kordower, 2007).

The homology and similar expression patterns between α- and β-syn in the brain indicate that they share many functions in synaptic signaling (Chandra *et al*., 2004), e.g. both have both been shown to inhibit phospholipase D2 (PLD2) activity which is required for vesicle recycling in presynaptic terminals (Jenco *et al*., 1998; Lotharius and Brundin, 2002). On the other hand, α- and γ-syn have been reported to bind tubulins and promote their polymerization (Alim *et al*., 2004; Zhang *et al*., 2011). Alpha- and beta-tubulins are polymerized into microtubules, the dynamic network that forms the cytoskeleton and along which intracellular and pre-synaptic neurotransmitter vesicles are transported.

α- and γ-syn regulate cell cycle and survival, e.g. α-syn can inhibit apoptosis through regulation of p53 expression (Alves da Costa *et al*., 2002), and it may also exert a protective effect against oxidative stress (Quilty *et al*., 2006) and excitotoxicity (Leng and Chuang, 2006). The main function of γ-syn appears to be promotion of proliferation through interactions with the poles of the mitotic spindle (Surguchov *et al*., 2001) and the mitotic checkpoint protein kinase BUB1B (Gupta *et al*., 2003). γ-Syn is very highly expressed in many types of cancer, especially in its advanced stages (Ji *et al*., 1997; Ahmad *et al*., 2007). α- and β-syn appear to be involved also in axonal regeneration (Quilty *et al*., 2002). The functions in cell cycle and regeneration may be another way for the synucleins to modulate neuronal plasticity.

α-Syn has a tendency to aggregate into intracellular inclusions (Spillantini *et al*., 1997). β-Syn has been found in the same inclusions (Galvin *et al*., 1999; 2000) but it may also be protective in synucleopathies because of its ability to prevent the aggregation and inhibit the spread of harmful α-syn oligomers (Hashimoto *et al*., 2001; Israeli and Sharon, 2009). γ-Syn has been found in the same pathological inclusions as α- and β-syn (Galvin *et al*., 1999; 2000).

It can be concluded that redundancy between α- and β-syn is a potential problem when studying the pre-synaptic functions of α-syn in the brains of knockout models. γ-Syn is co-expressed with α-syn in the peripheral nervous system, but its cellular localization and functions in proliferation make redundancy in pre-synaptic functions less likely.

2.1.2 Structure of α-syn

If one wishes to understand the role of α-syn in synaptic signaling and in related pathology, it is essential to elucidate the structural properties which dictate the cellular distribution and function of the protein. The 14 kDa α-syn protein appears to have no stable secondary structure in solution, but the N-terminal domain of α-syn (residues 1-92) is folded into two separate alpha-helixes after binding to phospholipid membranes (Davidson *et al*., 1998; Research Collaboratory for Structural Bioinformatics Protein Data Bank, http://www.rcsb.org; Fig. 1). β- and γ-syn have highly similar structures in their N-terminal regions (George, 2003). The helical region of α-syn consists mainly of six eleven-residue repeats that correspond with minor exceptions to the consensus sequence pKTKEGVaxaA,

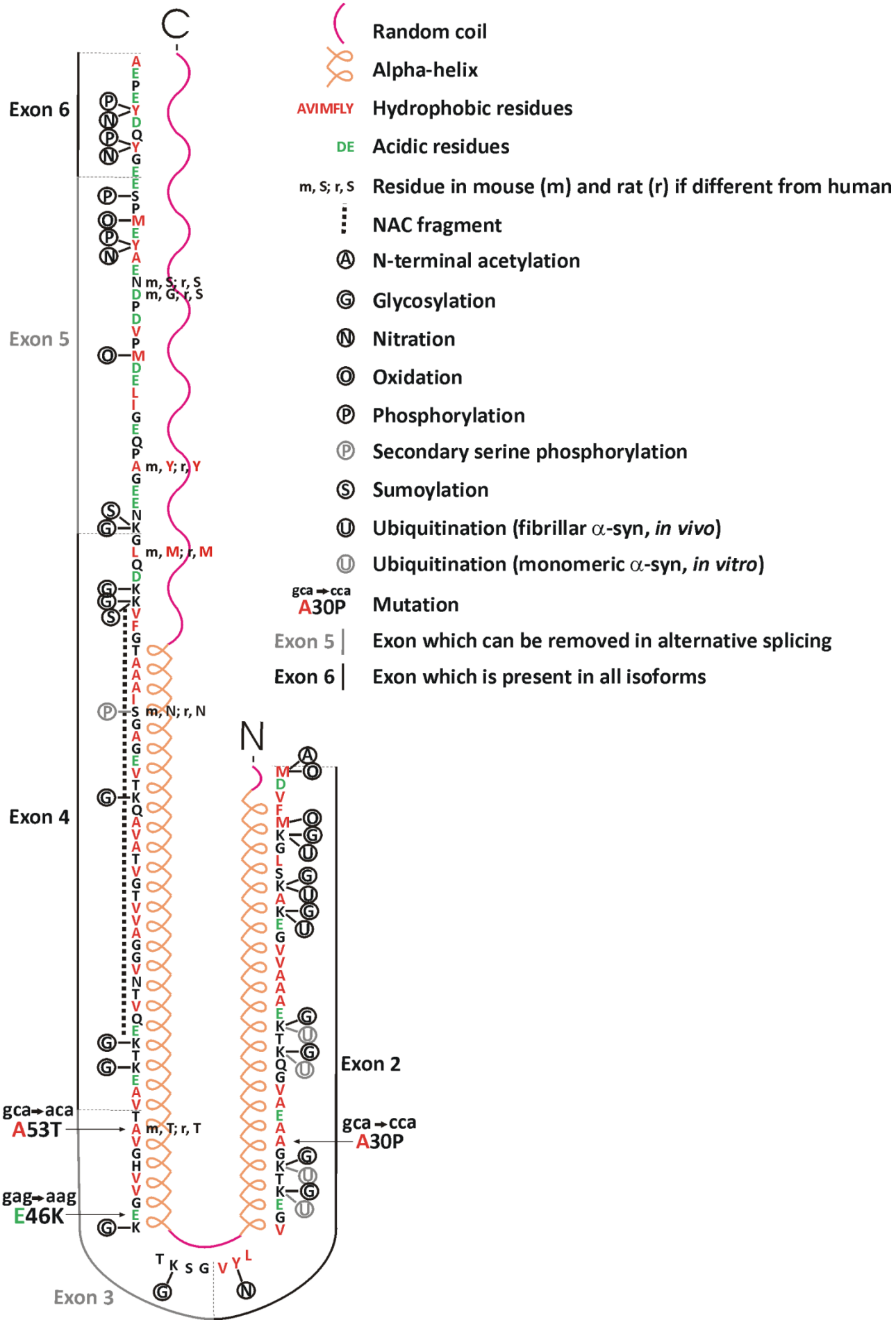

Figure 1. Simplified presentation of human alpha-synuclein with known missense mutations and post-translational modifications. See text for exact references

where p represents a polar residue, a refers to an apolar residue and x is any residue (Davidson *et al*. 1998; Perrin *et al*., 2000). Between repeats 5 and 6, there is also a hydrophobic sequence of 11 residues (68-78) which does not correspond to the consensus sequence but still contributes to the folding of the alpha-helix. The highly hydrophobic NAC fragment found from amyloid plaques consists of residues 61-95 (Ueda *et al*., 1993). The C-terminal domain of synucleins is unstructured and less conserved, comprising mostly of acidic residues (George, 2003) but it may contribute to folding of other proteins, i.e. chaperone function (Ostrerova *et al*., 1999; Souza *et al*., 2000).

Post-translational modifications can increase the size of α-syn from 14 to 19 kDa (Jakes *et al*., 1994) and they also affect the secondary structure and function of the protein. Human α-syn is acetylated at the N-terminal methionine (M) which may be crucial to the formation of the alpha-helix, lipid binding properties and oligomerization of the protein (Bartels *et al*., 2011; Maltsev *et al*., 2012; Trexler and Rhoades, 2012). Lysine (K) glycosylation, tyrosine (Y) nitration and serine (S) phosphorylation are clearly associated with α-syn aggregation and neurodegeneration (reviewed by Beyer, 2006; see also Munch *et al*., 2000 and Chen *et al*., 2010). S129 is the primary phosphorylation site, but there is also a secondary, non-conserved phosphorylation site at S87, which disrupts the alpha-helical structure and reduces the membrane binding of the protein (Beyer, 2006; Paleologou *et al*., 2010). Ubiquitination, which usually targets proteins for degradation, is a hallmark of Lewy bodies and other filamentous α-syn inclusions that resist degradation (Hasegawa *et al*., 2002). The ubiquitination in aggregated α-syn is concentrated around K residues 6, 10 and 12 (Nonaka *et al*., 2005). There are also some modifications which are potentially neuroprotective. Ubiquitination of K residues 21, 24, 32 and 34 has been detected in monomeric α-syn in *vitro* and may represent a way for targeting the protein for degradation *in vivo* (Nonaka *et al*., 2005). Addition of small ubiquitin-like modifier 1 (SUMO 1) to K96 and K102 (Krumova *et al*., 2011) and oxidation of M residues (Hokenson *et al*., 2004) appear to prevent α-syn aggregation and toxicity. Phosphorylation of the C-terminal Y residues is potentially neuroprotective and the ratio between S and Y phosphorylation may be significant to the development of synucleopathies (Negro *et al*., 2001; Chen *et al*., 2009).

The quaternary structure of α-syn has lately been the subject for a lively debate. Traditionally the unstructured monomer has been considered as the main physiological form of the protein while oligomeric species have been considered toxic and prone to fibrillization (Bennett, 2005; Uversky, 2007). This view was challenged by Bartels *et al.* (2011) who stated that α-syn could exist mainly as a helically folded tetramer that resisted further aggregation. Species of α-syn corresponding to the molecular weight of the tetramer have also been reported earlier (Baltic *et al*., 2004). The proposal that the tetramer was the main physiological form of α-syn was soon disputed (Fauvet *et al*., 2012) but the existence and properties of the tetrameric form cannot be completely excluded (Maltsev *et al*., 2012; Trexler and Rhoades, 2012).

It can be concluded that despite its relatively small size, α-syn has a complex, dynamic structure that allows it to perform different functions in relation to lipid membranes and as a soluble protein. The functionality of the protein can be affected by various post-translational modifications.

2.1.3 Cellular distribution of α-syn

α-Syn is mainly expressed in the brain and particularly in the presynaptic nerve terminals (Iwai *et al*., 1995; Baltic *et al*., 2004). It is associated with lipid rafts on membranes (Fortin *et al*., 2004) and preferentially with small pre-synaptic vesicles (Davidson *et al*., 1998; Jensen *et al*., 1998). However, the association with lipid membranes is reversible (Kahle *et al*., 2000) and there appears to be an equilibrium between the membrane associated and soluble fractions; in the rat brain, the membrane associated portion accounts for only 15 % of the total amount of the protein (Lee *et al*., 2002) and according to an amino-acid sequence based

prediction, 35.4 % of human α-syn is associated with secretory vesicles while 32.2 % is cytosolic (Devi *et al*., 2008).

In the fetus, α-syn expression is more evenly distributed also in other tissues and organs (Baltic *et al*., 2004) and data from neural progenitor cells and mice indicate that this expression is mostly nuclear (Schneider *et al*., 2007; Zhong *et al*., 2010). An amino-acid sequence based analysis has predicted that 16.2 % of human α-syn is located in the nucleus (Devi *et al*., 2008). It has been postulated that the pre-synaptic compartment is the primary site of α-syn expression and that increased expression drives the protein to the cell soma and also to the nucleus (Kahle *et al*., 2000; Masliah *et al*., 2000). In nigrostriatal neurons of adult mice, a toxic insult by the herbicide paraquat could increase α-syn expression, nuclear localization and association with histones (Goers *et al*., 2003). In addition, oxidative stress causes the protein to move to the nucleus where it binds to regulatory regions of several genes related to a variety of functions, including transcriptional regulation, differentiation of central nervous system neurons, and learning and memory (Siddiqui *et al*., 2012). The synaptic localization of the protein depends on the N-terminal region of the protein (residues 1-102) and the nuclear localization on the C-terminal region (residues 103-140; Specht *et al*., 2005; Burre *et al*., 2012). S129 phosphorylation appears to be associated with nuclear localization of the protein (Rieker, 2011; Amschl *et al*., 2013).

The N-terminal residues 1-32 also carry a signal sequence for mitochondrial localization and α-syn has been found to be located in the inner membrane of mitochondria in the dopaminergic neurons of SN and striatum (Li *et al*., 2007; Devi *et al*., 2008). At the inner membrane, α-syn interacts with mitochondrial complex I of the electron transport chain. It has been estimated that 16.2 % of human α-syn is associated with the mitochondria (Devi *et al*., 2008).

2.1.4 Regulation of α-syn expression

Differential expression of α-syn is believed to occur in addiction, PD and depression (Mash *et al*., 2003; Singleton *et al*., 2003; Chartier-Harlin *et al*., 2004; Martins-de-Souza *et al*., 2012). Therefore it is important to understand the mechanisms which regulate the level of α-syn expression and these are relevant also for understanding the systemic distribution of the protein. However, the subject has not been explicitly reviewed in the published literature.

Neural and other growth factors up-regulate α-syn expression, which explains the high prenatal expression (Stefanis et al., 2001; Wang *et al*., 2008; Clough *et al*., 2011). Basic fibroblast growth factor (bFGF) is able to upregulate α-syn expression in the midbrain dopaminergic neurons but not in gamma-aminobutyric acid (GABA) releasing or cortical neurons (Rideout *et al*., 2003). This is explained by the particularly high expression of the specific receptor of bFGF in dopaminergic neurons (Gonzalez *et al*., 1995). The extracellular signal-regulated kinase (ERK) pathway mediates the growth factor signal from the growth factor receptors to transcription factors like zinc finger and SCAN domain containing 21 (ZSCAN21) and zinc finger protein 219 (ZNF219; Clough *et al*., 2009). Leucine rich-repeat kinase 2 (LRRK2) upregulates α-syn expression through the same pathway and mutations in this protein are a frequent cause of autosomal-dominant PD (Carballo-Carbajal *et al*., 2010). The high expression of α-syn in erythrocytes (Barbour *et al*., 2008) is explained by direct transcriptional up-regulation by GATA binding factors 1 and 2 (GATA-1 and GATA-2), as both are essential transcription factors for blood cell development (Scherzer *et al*., 2008). GATA-2 is also expressed in the frontal cortex and SN which are vulnerable to degeneration in synucleopathies. Nuclear receptor related 1 (NURR1) guides the specialization and survival of the dopaminergic system (Saucedo-Garcenas *et al*., 1998; Sakurada *et al*., 1999), but surprisingly, it down-regulates *SNCA* transcription (Yang and Lachtman, 2008). Mutations in the gene coding NURR1 increase the risk of PD (Le *et al*., 2002).

Some important regulatory sites in and around the *SNCA* sequence have been recognized. Poly-(ADP-ribose) polymerase-1 (PARP-1) has been shown to down-regulate

SNCA transcription by binding to a regulatory region known as NACP-Rep1, located approximately 10 kb upstream of the *SNCA* gene (Chiba-Falek *et al*., 2005). The repeat length in this microsatellite is known to increase α-syn expression (Fuchs *et al*., 2008) and the risk of PD and alcoholism in certain populations (Farrer *et al*., 2001; Mizuta *et al*., 2002; Tan *et al*., 2004; Bönsch *et al*., 2005b). Other known regulatory regions are the NURR1 and ZNF219 binding elements in the 5′-promoter (Cheng *et al*., 2008; Clough *et al*., 2009) and the GATA-1, GATA-2 and ZSCAN21 binding elements in intron 1 (Scherzer *et al*., 2008; Clough *et al*., 2009). Epigenetic regulation of intron 1 may have pathological significance as the level of methylation of its cytosine-phosphate-guanine sites (i.e. epigenetic silencing) is reduced in the SN, putamen and cortex of patients with sporadic PD (Jowaed *et al*., 2010).

The expression of α-syn can also be post-transcriptionally regulated. *SNCA*'s translation into protein begins from exon 2 (Beyer *et al*., 2008). The full-length 140 residue α-syn is encoded by all protein coding exons (2-6), but the human protein has also three less expressed isoforms due to alternative splicing. The 126 residue α-syn lacks the residues coded by exon 3, the 112 residue α-syn exon 5 and the 98 residue α-syn exons 3 and 5 (Beyer *et al*., 2004; 2006; 2008; Fig 1). The 98 and 112 residue α-syn mRNAs are upregulated in the brain in DLB and also by oxidative stress (Beyer *et al*., 2004; 2008; Kalivendi *et al*., 2010) while the expression of the 126 residue α-syn appears to be reduced in DLB and Alzheimer's disease (Beyer *et al*., 2006). Two microRNAs, mir-7 and mir-153, have been found to inhibit the translation into protein by binding to the 3′-untranslated region of *SNCA* mRNA (Doxakis *et al*., 2010). The presence of iron appears to increase the stability of *SNCA* mRNA by binding to a specific responsive element at the 5′-untranslated region (Friedlich *et al*., 2007; Febbraro *et al*., 2012), and iron accumulation in the brain is a typical feature in neurodegenerative diseases and migraine (Welch *et al*., 2001; Kruit *et al*., 2009; Dusek and Schneider, 2012; Mochizuki and Yasuda, 2012).

There are environmental and life style factors which can affect α-syn expression. The post-transcriptional regulation of *SNCA* mRNA may explain why occupational exposure to iron increases the risk of PD (Lai *et al*., 2002). Exposure to pesticides and herbicides is also a PD risk factor (de Lau and Breteler, 2006) and the herbicide paraquat has been demonstrated to increase the expression of α-syn in nigrostratal neurons of mice (Manning-Bog *et al*., 2002; Goers *et al*., 2003). Valproic acid is used for treatment of epilepsy, migraine and mania and this drug up-regulates α-syn expression (Leng and Chuang, 2006). Liver X receptor α and β (LXRα and β) are transcription factors that use cholesterol metabolites as activating ligands and up-regulate *SNCA* transcription (Cheng *et al*., 2008). This suggests that cholesterol intake and/or its metabolism affect α-syn expression. Elevated levels of 27-hydroxycholesterol have been found in the blood and brains of PD patients (Lee *et al*., 2009; Seet *et al*., 2010; Cheng *et al*., 2011) and a high intake of animal fat and cholesterol is known to increase the risk of PD (Lai *et al*., 2002). LXRα expression is concentrated in the internal organs, whereas LXRβ is ubiqituous (Kidani and Bensinger, 2012).

2.1.5 Systemic distribution of α-syn

Between 0.5-1 % of the total protein in the brain is α-syn (Iwai *et al*., 1995). The expression is limited to neurons and α-syn is not endogenously expressed in astrocytes or astroglia, the cells that produce myelin, maintain brain homeostasis and provide support and protection to neurons (Ahn *et al*., 2012; Amschl *et al*., 2013). However, glial α-syn inclusions have been identified in synucleopathies (Tu *et al*., 1998; reviews by Jellinger, 2003, and Bennett, 2005). α-Syn expression is highest in dopaminergic and noradrenergic regions (Li *et al*., 2002) but it is also expressed in cholinergic (Dugger and Dickson, 2010) and glutamatergic neurons (Totterdell *et al*., 2010). Iwai *et al*. (1995) performed a comparative western blot analysis, according to which α-syn displayed highest protein expression in the hippocampus, striatum, frontal cortex and the olfactory bulb. In the same study, there was meagre expression at the thalamus and hypothalamus, and lowest expression in the midbrain, cerebellum, pontine tegmentum and medulla oblongata. Unfortunately it is not possible to

compare protein data from other brain areas reliably because of the methodological differences between studies, but the protein expression in the rat amygdala appears to be equal to or even more to that found in hippocampus (Jeannotte *et al*., 2009) whereas the expression in murine corpus callosum is low (Xu *et al*., 2011). The data on protein expression can be contradictory with mRNA expression because of several factors such as mRNA stability or the rate of protein degradation. In the case of α-syn, also the presynaptic localization can cause a mismatch between mRNA and protein expression in specific structures. This manifests itself as high protein expression in the striatum despite a low mRNA expression, as the protein is present mainly at the presynaptic terminals of dopaminergic neurons that project their axons to the area from SN and the ventral tegmental area (VTA) where *SNCA* mRNA expression is high but protein expression low (Mash *et al*., 2003; Allen Brain Atlas, human.brain-map.org).

As mentioned, α-syn, and especially its full length version, is known to be expressed also in various internal organs, heart and skeletal muscle (Lavedan, 1998; Beyer *et al*., 2008). α-Syn inclusions have been found within chromaffin cells of the adrenal medulla, which secrete adrenaline and noradrenaline into the systemic circulation (Wakabayashi and Takahashi, 1997). Data from mice suggest that α-syn expression in the adrenal medulla is concentrated in adrenaline producing cells (Khan *et al*., 2012). Askanas *et al*., (2000) reported that α-syn expression was concentrated to the postsynaptic side of the neuromuscular junction in skeletal muscle fibers and that the expression was high and diffuse across the cytoplasm in both regenerating and necrotic fibers. α-Syn inclusions have also been found in gastrointestinal mucosa of PD patients but in healthy controls α-syn in the gut is minimal (Forsyth *et al*., 2011). Even though α-syn is expressed in tyrosine hydroxylase (TH) expressing sympathetic nerve fibers of the heart (Marrachelli *et al*., 2010) the α-syn expressing neurons in the gastrointestinal tract are cholinergic (Sharrad *et al*., 2013). α-Syn inclusions have also been found in the cholinergic lower motor neurons (Mu *et al*., 2013).

Blood and cerebrospinal fluid contain α-syn and there have been several attempts to establish the protein level in these sources so they could act as a biomarker for different synucleopathies (reviewed by Kasuga *et al*., 2012). Neurons of the central nervous system have been verified as the source of the cerebrospinal fluid's α-syn (Mollenhauer *et al*., 2012); its level generally appears to be reduced in synucleopathies (Kasuga *et al*., 2012) and increased after traumatic brain injury (Su *et al*., 2010). It is easier to obtain blood samples than cerebrospinal fluid samples, but the results on serum and plasma α-syn as indicators of synucleopathies have been rather unconvincing (Kasuga *et al*., 2012). Since erythrocytes contain 99 % of the α-syn in blood (Barbour *et al*., 2008), whole blood samples might provide a better biomarker (Miller *et al*., 2004).

2.2 MECHANISMS OF α-SYN FUNCTION

The presynaptic localization and the high expression in the brain indicate that α-syn is important in regulating neurotransmitter release. This section of the literature review will summarize α-syn's molecular mechanisms of action, and how α-syn regulates the neural circuitries for motor control, reward, motivation, learning and memory. It does seem that the effects of a-syn on synaptic signaling are a very complex and fine-tuned process, and are very difficult to measure and characterize.

2.2.1. α-Syn in the control of neurotransmitter synthesis and storage

Before a neurotransmitter can be released in a controlled manner, it must be synthesized and packaged into small presynaptic vesicles (Fig. 3). α-Syn may reduce the total amount of presynaptic vesicles by decreasing their recycling rate due to the inhibition of PLD2, as PLD2 synthesizes phosphatic acid which is required for recruiting the necessary proteins for vesicle recycling (Jenco *et al*., 1998; Lotharius and Brundin, 2002; Nemani *et al*., 2010; Gaugler *et al*., 2012).

PLD2 mediates also choline synthesis, and therefore inhibition of PLD2 leads to reduced acetylcholine levels (Zhao *et al*., 2001). α-Syn may also antagonize the final step in acetylcholine synthesis, as pathological α-syn inclusions have been found to stain positive for choline acetyltransferse (Dugger and Dickson, 2010) and soluble α-syn has been shown to co-localize with choline acetyltransferse in transgenic mice overexpressing human α-syn (Magen *et al*., 2012). The α-syn overexpressing mice also displayed a ~30 % decrease in total acetylcholine concentration in the cortex. Acetylcholine is an important neurotransmitter in the periphery, but it is unknown whether α-syn has any functional significance in peripheral cholinergic signaling.

α-Syn has a more widely studied role in the control of DA synthesis. α-Syn physically interacts with TH which is the rate-limiting enzyme in catecholamine synthesis (Perez *et al*., 2002; Fig. 2 and 3). It was also found that TH phosphorylation, which determines the enzyme's activity, along with DA synthesis was reduced in response to α-syn overexpression (Peng *et al*., 2005). Serine 40 was identified as the main phosphorylation site determining the activation of TH and the modification was mediated by protein phosphatase 2A. Subsequently it was shown that silencing *SNCA* with siRNA could result in increased TH activity (Liu *et al*., 2008). Perez *et al*. (2002) did not find any changes in TH expression, but later studies have shown that increased expression of α-syn reduces TH mRNA and protein expression (Baptista *et al*., 2003; Yu *et al*., 2004; Chu and Kordower, 2007). In addition, transgenic mice expressing a C-terminally truncated human α-syn (1-120) have a lower striatal DA content (Tofaris *et al*., 2006). However, striatal DA levels in α-syn knockout mice are unchanged (Schlüter *et al*., 2003; Robertson *et al*., 2004) or decreased (Abeliovich *et al*., 2000; Al-Wandi *et al*., 2010) suggesting that there are compensatory mechanisms occurring in the complete absence of the protein. Al-Wandi *et al*. (2010) reported that the striatal DA content was reduced in aged α-syn knockout mice when no compensatory mechanisms were detected, but they were not able to confirm whether the observed changes were because of effects on TH activity or because of α-syn's protective effect on synaptic integrity.

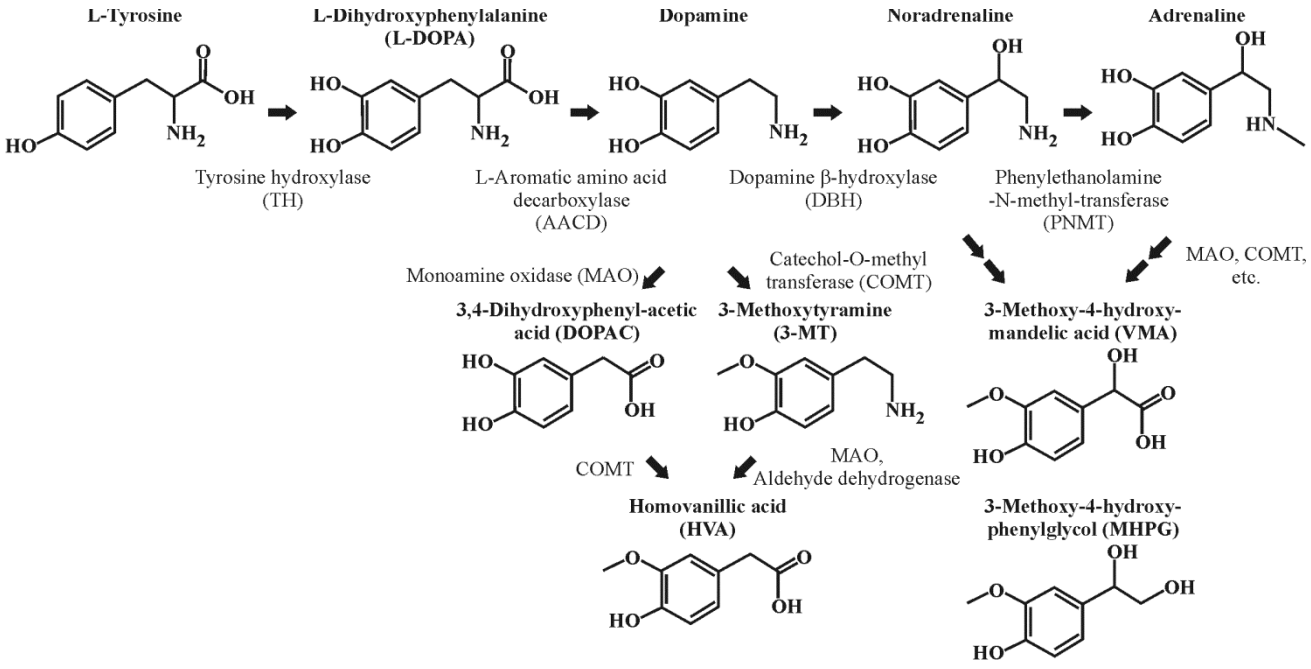

Figure 2. Biosynthesis of catecholamines and the main end-products of their degradation. Activity of TH determines not only the level of DA, but also of the other catecholamines. Adapted from Nestler *et al*., 2009

The synthesized neurotransmitters are packaged into the presynaptic vesicles by specific transporter proteins, e.g. in the central nervous system catecholamines are packaged by

vesicular monoamine transporter 2 (VMAT2; Parsons, 2000; Fig. 3). It was reported by Guo *et al*., (2008) that VMAT2 activity was decreased in SH-SY5Y cells, in which there was stable overexpression of α-syn. The expressions of VMAT2 mRNA and protein were decreased in the α-syn transfected cells, but it remained unclear whether the decrease in activity was simply because of changes in expression since α-syn and VMAT2 are known to form a complex. The regulation of expression was verified later by siRNA silencing of *SNCA* which increased the VMAT2 expression per vesicle by 2.8-fold and total expression by two-fold (Fountaine *et al*., 2008).

2.2.2 α-Syn in the organization of synaptic vesicle pools

Determining the exact function of α-syn in neurotransmitter release (Fig. 3) does not appear to be an easy task, as the literature is full of more or less contradictory reports on the subject. The first proof of a functional role of α-syn in neurotransmitter release came from Abeliovich *et al*. (2000). They discovered that in striatal slices of α-syn knockout mice, paired-pulse depression of stimulated DA release was decreased, i.e. α-syn would negatively regulate neurotransmitter release. DA overflow in response to repeated bursts of electrical stimulation suggested that α-syn could reduce the refilling rate of the readily releasable pool (Yavich *et al*., 2004). The readily releasable pool consists of vesicles that are located at the active zone of the terminal, in other words of vesicles that are docked to the pre-synaptic membrane. The results were similar with noradrenaline when its release was measured in knockout mice (Yavich *et al*., 2006). In chromaffin cells, overexpression of α-syn reduced the number of catecholamine vesicles that were released per stimulus, suggesting that α-syn reduces the size of the readily releasable pool (Larsen *et al*., 2006).

The regulatory effect of α-syn in neurotransmitter release is not restricted to catecholamines (Cabin *et al*., 2002; Liu *et al*., 2004; Gureviciene *et al.* 2007). Murphy *et al*., (2000) proposed that α-syn positively regulated the size of the storage pool of synaptic vesicles in a hippocampal cell culture; a 47 % decrease in α-syn expression by antisense oligonucleotide treatment reduced the amount of vesicles that were distally located from the pre-synaptic membrane. Nemani *et al*. (2010) came to an opposite conclusion; they showed that α-syn overexpression evoked a reduction in the size of the readily releasable pool which they attributed to reduced size of the recycling pool. The recycling pool consists of vesicles that have been reformed from the plasma membrane and are located near the active zone. In contrast to the earlier results (Murphy *et al*., 2000; Larsen *et al*., 2006) they also showed that the overexpression of α-syn in mouse neurons lowered the amount of vesicles at the active zone (Nemani *et al*., 2010). There was also a reduction in the general amount of vesicles at the pre-synaptic terminals. Similar changes after α-syn overexpression were also seen by Gaugler *et al*. (2012) and Yavich *et al*., (2005). Electronmicroscope images revealed that in α-syn overexpressing neurons vesicles appeared diffused across the axonal space instead of clustering near the presynaptic membrane (Nemani *et al*., 2010). It is possible that α-syn achieves this effect by binding the vesicles to microtubules (Alim *et al*., 2004; Wersinger and Sidhu, 2005). The general conclusion from all these results was that α-syn could inhibit the reclustering of vesicles near the active zone after exocytosis (Nemani *et al*., 2010).

2.2.3 α-Syn and the SNARE-complex

α-Syn might affect not only the vesicle pool organization, but also the actual release event (Fig. 3). Electronmicroscope images indicated that the amount of vesicles in the active zone was increased in response to α-syn overexpression in PC12 cells, a cell line originating from a tumor of the rat adrenal medulla (Larsen *et al*., 2006). Since overexpression decreased neurotransmitter release in the same study, it was postulated that α-syn could facilitate docking of vesicles but inhibit a later stage in the exocytosis process called priming. Priming consists of conformational changes in proteins of the soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)-complex that are required for fusion

of the vesicle and the presynaptic terminal membrane (reviewed by Südhof, 2012). There are three proteins, or sub-sets of proteins in the core of the SNARE-complex: soluble N-ethylmaleimide–sensitive factor attachment protein 25 (SNAP-25) and syntaxin 1 at the terminal membrane (t-SNAREs), and synaptobrevins (1 and 2) at the vesicles (v-SNAREs). There are also several other proteins that guide the transient, extremely rapid folding and refolding of these proteins. Lundblad *et al*. (2012) overexpressed human α-syn in rat striatum, and reported that α-syn was able to inhibit a late stage of stimulated DA release when measured with chronoamperometry. Darios *et al*. (2010) performed assays in chromaffin and PC12 cells and determined that arachidonic acid, which is essential for the SNARE assembly process, was bound and isolated from the SNARE-complex by α-syn, resulting in a reduced amount of SNARE-complexes. They also revealed that synapses isolated from the brain tissue (synaptosomes) of α-syn single knockout mice displayed increased glutamate release. Anwar *et al*. (2011) did not detect any changes in the amount of SNARE-complexes in the striatum of α, β- and γ-syn triple knockout mice.

There are some results suggesting that α-syn could also facilitate SNARE-complex assembly. Mice with a deletion in a known SNARE-folding protein, cysteine-string protein-α (CSPα), exhibited a decreased amount of SNARE-complexes, severe neuronal degeneration and reduced survival (Chandra *et al*., 2005). Overexpression of α-syn in CSPα knockout mice was able to counteract these phenotypes. Furthermore, deletion of α- and β-syn exacerbated the effects of CSPα deletion. Later on it was also shown that overexpression of α-syn was able to increase the amount of assembled SNARE-complexes in cell models (Burre *et al*., 2010; 2012). Mice lacking all three synucleins have a reduced amount of SNARE-complexes in the brain, shortened lifespan, age-dependent reduction in the expression of synaptobrevin 2 and a dramatic increase in the expression of CSPα (Burre *et al*., 2010). It was also shown that α-syn underwent a direct interaction with the SNARE-proteins and it was hypothesized that α-syn had a chaperone function in folding them so that they were ready for priming. The functional and structural similarity between α-syn and the 14-3-3 proteins suggests that all synucleins might have a chaperone-like activity (Ostrerova *et al*., 1999), which has been confirmed (Souza *et al*., 2000). It appears that the N and C-terminal regions of α-syn are required for the chaperone function in SNARE-complex assembly, while only the NAC fragment is necessary for the neurodegenerative effects of the protein (Burre *et al*., 2012). The results of Cabin *et al*., (2002), Liu *et al*. (2004) and Gureviciene *et al.* (2007) indicated that the suspected facilitation of SNARE-complex assembly could translate into increased hippocampal glutamate release. However, the evidence is indirect and it remains unclear if α-syn is able to facilitate neurotransmitter release and if so, under which circumstances.

2.2.4 α-Syn in the control of neurotransmitter re-uptake

α-Syn has a role in the control of neurotransmitter uptake, or re-uptake as the process is also referred to (Fig. 3, reviewed by Oaks and Sidhu, 2011). Uptake by the DA transporter (DAT) appears to be the main mechanism responsible for clearing the released DA from the extracellular space (Wightman and Zimmerman, 1990; Benoit-Marand *et al*., 2000; Budygin *et al*., 2002). Lee *et al*. (2001) were the first investigators to show a physical interaction with α-syn and DAT. They also showed that α-syn overexpression accelerated the maximal velocity of DA uptake (V_{max}) in a fibroblast model. It was hypothesized that α-syn could bind DAT and anchor it to the extracellular membrane, as the main mechanism for controlling the activity of DAT is to sequester it to the cytosol, away from the cell surface (Melikian and Buckley, 1999). Both proteins have later been shown to be associated with membrane rafts (Fortin *et al*., 2004; Foster *et al*., 2008) and siRNA mediated silencing of *SNCA* in a dopaminergic neuroblastoma cell line (SH-SY5Y) resulted in a ~50 % decrease in DAT surface expression and a corresponding reduction in DA uptake (Fountaine *et al*., 2008). The increased uptake discovered by Lee *et al*. (2001) evoked an increase in DA

induced cell death, as DA and its metabolites (Fig. 2) are capable of causing oxidative stress and apoptosis (reviewed by Miyazaki and Asanuma, 2008).

The direct interaction between DAT and α-syn has not been questioned since, but it was shown that the facilitation of uptake may be due to the loss of cell adhesion in the experiment setup (Wersinger *et al.*, 2003a). Cells that were attached to the surface of the container showed a significant reduction in V_{max} after α-syn overexpression, but even a

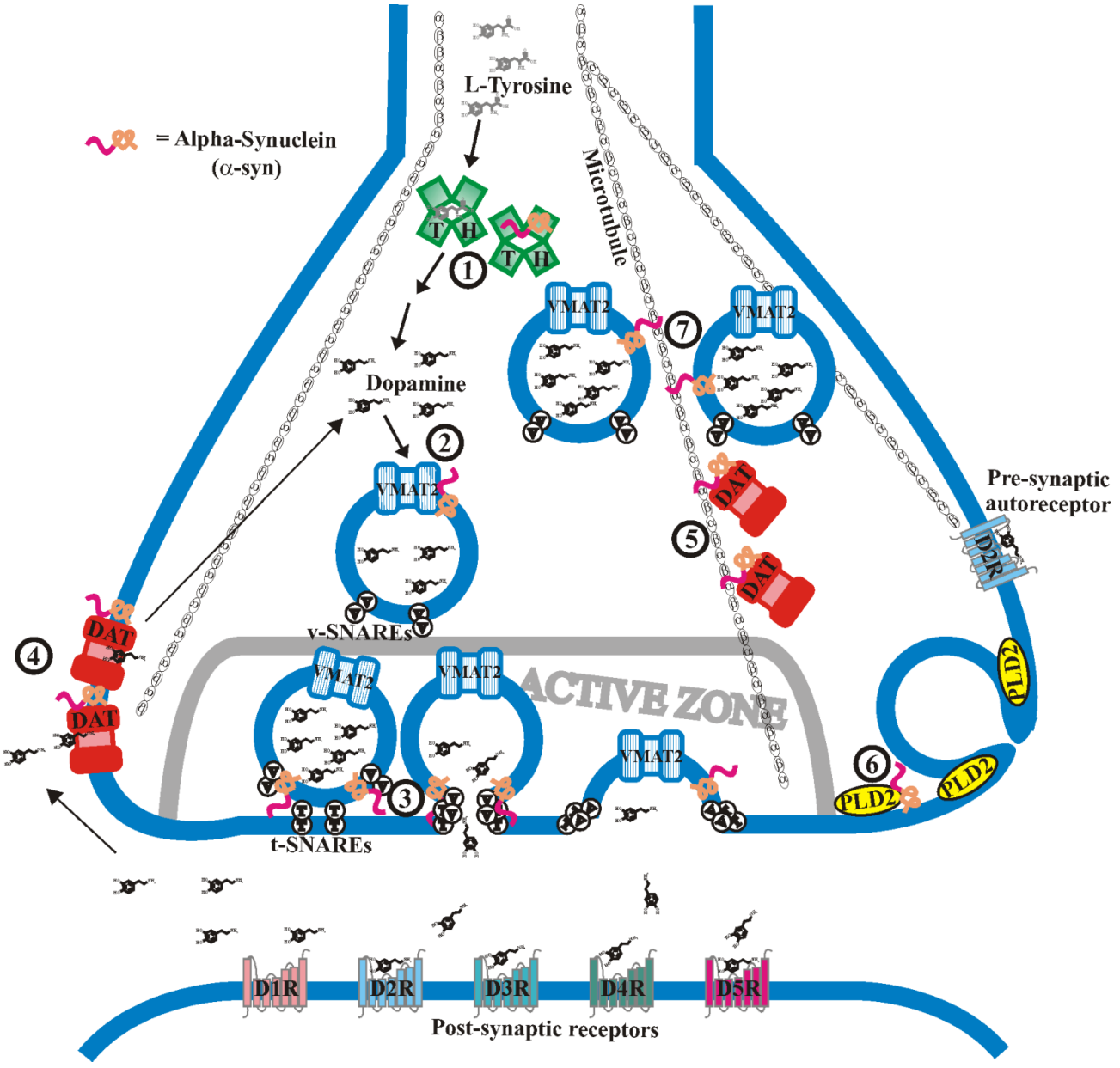

Figure 3. Proposed pre-synaptic functions of α-syn in a dopaminergic synapse. 1) α-Syn binds to TH and inhibits DA synthesis. 2) α-Syn inhibits the activity of VMAT2, thus attenuating the packaging of DA into pre-synaptic vesicles. 3) α-Syn inhibits a late stage in DA release, even though it has also been suggested to promote the assembly of the SNARE-complex which is necessary for neurotransmitter release. Release may happen in a kiss-and-run –fashion or by full merger of the vesicle into the extracellular membrane. 4) α-Syn has been suggested to promote DA re-uptake by clustering DAT to the extracellular membrane. 5) α-Syn appears to bind DAT also to the microtubular network, which inhibits membrane trafficking and activity of the transporter. 6) α-Syn inhibits PLD2 activity, which has several functions including an involvement in vesicle recycling. 7) α-Syn inhibits the clustering of vesicles near the active zone of DA release, possibly by binding them to microtubules. See text for detailed references

slight disruption of the adhesion by trypsination could reverse the effect of the protein and V_{max} became increased. DA induced cell death was also reduced in non-trypsinized cells overexpressing α-syn. Subsequently it was shown that α-syn could prevent the membrane trafficking of DAT by binding it to microtubules (Wersinger and Sidhu, 2005). α-Syn overexpression slowed down DA uptake as earlier (Wersinger *et al*., 2003a), but when the microtubular network was disrupted pharmacologically, α-syn was again able to increase V_{max}, and in fact it could form an even stronger complex with DAT (Wersinger and Sidhu, 2005). It was shown that the interaction between α-syn and DAT occurred through the NAC fragment of α-syn, but also other parts of the α-syn transmembrane region were able to affect DA uptake (Wersinger *et al*., 2003b).

Despite the clear changes in V_{max}, which depend mainly on the surface expression of DAT, no statistically significant changes in the affinity of DAT towards DA (depicted by the K_m value) have been seen in cell models in response to α-syn overexpression (Lee *et al*., 2001; Wersinger *et al*., 2003a; b; Wersinger and Sidhu, 2005). However, α-syn overexpression has caused a slight increase in the K_m value consistently in all studies, suggesting that α-syn is able to reduce the affinity of DAT.

The three monoamine transporters, DAT, noradrenaline transporter and serotonin transporter are highly homologous and show considerable redundancy (Oaks and Sidhu, 2011). For example, in the frontal cortex of mice where DAT expression is low, DA is taken up through the noradrenaline transporter, even though the affinity of the other transporters towards DA is considerably less than that of DAT itself (Morón *et al*., 2002). It has been shown that both serotonin (Wersinger *et al*., 2006a) and noradrenaline transporters (Wersinger et al., 2006b) have a direct interaction with α-syn and that α-syn regulates their surface expression and thus their activity by binding them to the microtubular network. β-Syn has not been shown to undergo a physical interaction with the monoamine transporters (Oaks and Sidhu, 2011), but γ-syn can have a direct interaction with the serotonin transporter and a negative effect on its activity, even though the effect is not as robust as α-syn's (Wersinger and Sidhu, 2009).

Even though there is clear evidence from cell models that α-syn can modulate monoamine uptake, functional studies performed either in brain slices (Abeliovich *et al*., 2000; Senior *et al*., 2008; Anwar *et al*., 2011) or whole brains (Yavich *et al*., 2004; 2006) of single, double or triple knockout animals have not been able to confirm this proposal. It was also reported that DAT expression is unchanged in α-syn knockout mice (Schlüter *et al*., 2003). However, it was later shown by Bellucci *et al.* (2011) that mice with a spontaneous deletion of α-syn have a 34-44 % reduction of total DAT expression in the striatum. Furthermore, the transgenic mice expressing truncated human α-syn displayed a 59-95 % increase in DAT expression in comparison to wild-type mice. Lundblad *et al*. (2012) showed that overexpression α-syn exerted a functional effect on DA re-uptake in a physiological environment, but the results were contradictory to the reported effects on DAT expression by Bellucci *et al,* (2011), as rats with a virally induced overexpression of human α-syn in their nigrostriatal neurons showed significant reduction of the DA re-uptake rate in the striatum after K^+-induced release (Lundblad *et al*., 2012). The reduction of the rate was ~50 % ten days after vector injection and ~80-90 % at the subsequent time points (3-16 weeks). It was shown that the dramatic decrease in re-uptake was not only because of changes in DAT activity but also because of axonal degeneration. The evidence described above shows that α-syn is able to affect DA re-uptake, but it remains unclear which of the two proposed mechanisms of function (Lee *et al*., 2001; Wersinger and Sidhu, 2005; Fig. 3) is the primary one, and if the control of re-uptake is part of the pathological properties of α-syn or the normal regulation of synaptic signaling.

2.2.5 Control of striatal dopamine release by other neurotransmitter systems

There is a complex circuitry within the basal ganglia which controls the activity of the dopaminergic neurons. The connectivity of the striatum is important for understanding its function, and for clarifying the role of α-syn in broader concepts.

Almost all, 90-95 %, of striatal post-synaptic neurons are GABA releasing medium spiny neurons (MSNs; Kawaguchi, 1993; Bolam *et al*., 2000; Nestler *et al*., 2009; Wichmann and DeLong, 2010). Type 1 MSNs express the D_1 DA receptor and release substance P and dynorphin as co-transmitters. They have direct projections to the SN, and because activation of D_1 receptors is stimulatory (G_s-coupled), their activation leads to release of GABA in the SN, which in turn is inhibitory. Thus, type 1 MSNs form a negative feedback loop known as the direct pathway. Type 2 MSNs express the D_2 DA receptor and release enkephalin as a co-transmitter. Type 2 MSNs have projections to globus pallidus externa which has inhibitory projections to the SN and globus pallidus interna. Since the activation of D_2 receptors is inhibitory ($G_{i/o}$ coupled), binding of DA to D_2 receptors of type 2 MSNs leads to disinhibition of inhibitory input to the SN, i.e. inhibition of DA release in the striatum. This is known as the indirect pathway. Globus pallidus externa has a regulatory loop to nigral DA neurons also through the STN which provides an excitatory (glutamatergic) input to the SN.

Dopaminergic projections in the striatum possess pre-synaptic nicotinic receptors (Champtiaux *et al*., 2003) but only 1-2% of striatal neurons are tonically active non-spiny cholinergic interneurons (Kawaguchi, 1993) and dopaminergic neurons synapse with some of them (Chang, 1988). Despite their low number, cholinergic interneurons exert important modulatory actions on DA release (reviewed by Threlfell and Cragg, 2011). It appears that cholinergic activation is required for the dopaminergic neurons to modify their release according to the inputs which they are receiving (Rice and Cragg, 2004). The cholinergic modulation of striatal DA release through the nicotinic receptors may be linked to the postulated protective role of smoking against PD (Lai *et al*., 2002; de Lau and Breteler, 2006).

2.2.6 Significance of α-syn to motor functions

Motor symptoms are frequent in synucleopathies and PD is often defined as a motor syndrome (McKeith *et al*., 2004; Nestler *et al*., 2009; Ubhi *et al*., 2011; Fritsch *et al*., 2012; Dusek and Schneider, 2012). The motor symptoms in PD begin asymmetrically but start to affect both sides of the body as the disease progresses, and include resting tremor, bradykinesia, Parkinsonian gate, postural instability and micrographia. Motor symptoms in the face and neck include facial impassivity, dysphagia and difficulties in producing speech (Logemann *et al*., 1978; Hartelius and Svensson, 1994; Johnston *et al*., 1995).

Human motor functions are controlled by two main systems, the pyramidal and extrapyramidal systems (reviews by Bolam *et al*., 2000 and Obeso *et al*., 2008). The pyramidal system, or corticospinal tract as it is also called, consists of upper and lower motor neurons. The glutamatergic upper motor neurons project from the motor cortex to lower motor neuron somas in the anterior horn of the spinal cord. The cholinergic lower motor neurons innervate the striated skeletal muscles. The striated muscles of the face and neck are controlled by the corticobulbar system that has its lower motor neuron somas in the brain stem. Degeneration and α-syn inclusions can be detected in the lower bulbar motor neurons in PD (Mu *et al*., 2013). The cholinergic lower motor neurons activate skeletal muscles through a specialized synaptic structure known as the neuromuscular junction. The functionality of the neuromuscular junction can be evaluated using repetitive stimulation of motor axons and measuring the amplitudes of the resulting compound muscle action potentials (CMAPs) with electromyography (AAEM Quality Assurance Committee, 2001; Boërio *et al*., 2009). This methodology is widely used for diagnosing diseases of the neuromuscular junction, such as myasthenia gravis and Lambert-Eaton myasthenic syndrome.

The extrapyramidal system consists mainly of the basal ganglia, cerebellum and several brain stem nuclei (Bolam *et al*., 2000 and Obeso *et al*., 2008). The basal ganglia and thalamus are in a feedback loop with the motor and other parts of the cerebral cortex, which enables generation and initiation of voluntary movement. Within the basal ganglia, the DS and globus pallidus have connections with each other and the STN, SN and mediodorsal nucleus of the thalamus. The death of dopaminergic cells in the SN pars compacta leads to the cardinal motor symptoms in PD (Fritsch *et al*., 2012).

Since α-syn can be found throughout the pyramidal and extrapyramidal systems (Iwai *et al*., 1995; Beyer *et al*., 2008; Mu *et al*., 2013) and motor symptoms are prevalent in synucleopathies, it would seem plausible that α-syn serves some function in the control of movement. This kind of function might be revealed by performing basic behavioral tests for motor control in α-syn knockout models. However, the first published results investigating this topic detected no differences between wild-type and α-syn knockout mice in motor performance (Cabin *et al*., 2002). Clear motor impairments were not detected in knockout mice in later studies (Yavich *et al*., 2005; Senior *et al*., 2008), even though reduced movement velocity was recorded in α-syn knockout mice freely exploring in an open field test (Kokhan *et al*., 2012). When the topic was studied in transgenic mice expressing mutated, truncated and wild-type human α-syn, it was found that they developed age-dependent motor impairments (van der Putten *et al*., 2000; Yavich *et al*., 2005; Tofaris *et al*., 2006; Amschl *et al*., 2013). Interestingly, these impairments may be related to an age-dependent increase of human α-syn protein levels alone, while the total α-syn expression is unchanged (Amschl *et al*., 2013). Mice overexpressing their own version of the protein did develop motor impairments after 7 months of age, as measured by Rotarod test, but the α-syn protein level in these mice was as much as six fold higher in comparison to wild-type (Rieker *et al*., 2011). Their initial motor performance at two months of age was also worse than their controls, but after a few training sessions they were able to improve it to the level of their wild-type counterparts, suggesting that they might have problems in learning motor skills. Nonetheless, it is not clear if these changes are attributable to the neurodegenerative effect of α-syn, or if α-syn has a functional role in the control of movement. The observation that α-syn knockout mice have no phenotype related to motor function may be due to redundancy and the mildness of the phenotype.

2.2.7 Dopamine and motivation

α-Syn modifies the function of dopaminergic synapses in the striatum (Abeliovich *et al*., 2000; Yavich *et al*., 2004), and thus if one wishes to understand the putative impact of α-syn on behavior and its pathology, it is essential to examine the behavioral significance of striatal DA signaling. Striatal DA is known to motivate actions and activities by reinforcing positive action outcomes (revieved by Salamone and Correa, 2012) and also α-syn has been shown to modulate reward processes (Oksman *et al*., 2006). However, experiments with crab-eating macaques (*Macaca Fascicularis*) indicated that DA does more than simply encode reward (Ljungberg *et al*., 1992; Schultz *et al*., 1993; Hollerman and Schultz, 1998). Most of the DA neurons of the SN pars compacta and VTA were activated by reward primarily during the early stages of the learning process when it was not yet known which action would yield a reward. The number of activated neurons lessened gradually as the action-reward relationship became clear to the macaques, and only ~1/3 of the DA neurons were activated consistently with each presentation of the reward. Furthermore, the activity of the DA neurons was reduced when the animals knew to expect reward but it was subsequently denied. The conclusion was that DA codes for reward prediction error (Hollerman and Schultz, 1998).

When the matter was studied in the ventral striatum of rodents (Fig. 4), it was noted that the extracellular DA level increased in response to unexpected reward in nucleus accumbens shell (AcbSh), suggesting that the neurons activated primarily by novel reward projected to this region (Corbit *et al*., 2001; Bassareo *et al*., 2002). In addition, an aversive

stimulus (taste of quinine) and stimulus signifying potential danger (smell of red fox urine) caused DA level to be elevated in AcbSh when presented to the animal for the first time, suggesting that the DA release in this region codes novelty. DA level in nucleus accumbens core (AcbC) increases also in response to a previously known reward. When the known reward is associated with a preceeding cue, the cue alone is sufficient to trigger DA release in the neurons projecting to AcbC (Schultz, 1986; Ljungberg *et al*., 1992; Cacciapaglia *et al*., 2011). DA release in AcbC appears to be necessary for initiation (motivation) of actions which require relatively high effort (Cousins and Salamone, 1994; Salamone *et al*., 2007; Yin *et al*., 2008; reviewed by Salamone and Correa, 2012).

The dorsal striatum (DS) also controls motivation of different activities. In rodents, DS has been traditionally referred to as the caudate putamen (CPu; Fig. 4) and it corresponds to two clearly separate structures in primates: caudate nucleus and putamen (Joel and Weiner, 2000; Grahn *et al*., 2008). The functions and connectivity of the rodent medial DS correlate roughly with those of the putamen while lateral DS corresponds to the caudate nucleus. Lateral DS (or caudate nucleus) appears to be important to initial acquisition of motor functions and habit formation (Pisa and Cyr, 1990; Yin *et al*., 2004), while medial DS (or putamen) is essential for reversal learning and adapting to new situations by switching strategies (Pisa and Cyr, 1990; Ragozzino *et al*., 2002). Medial DS may have some significance also in spatial learning (Devan and White, 1999).

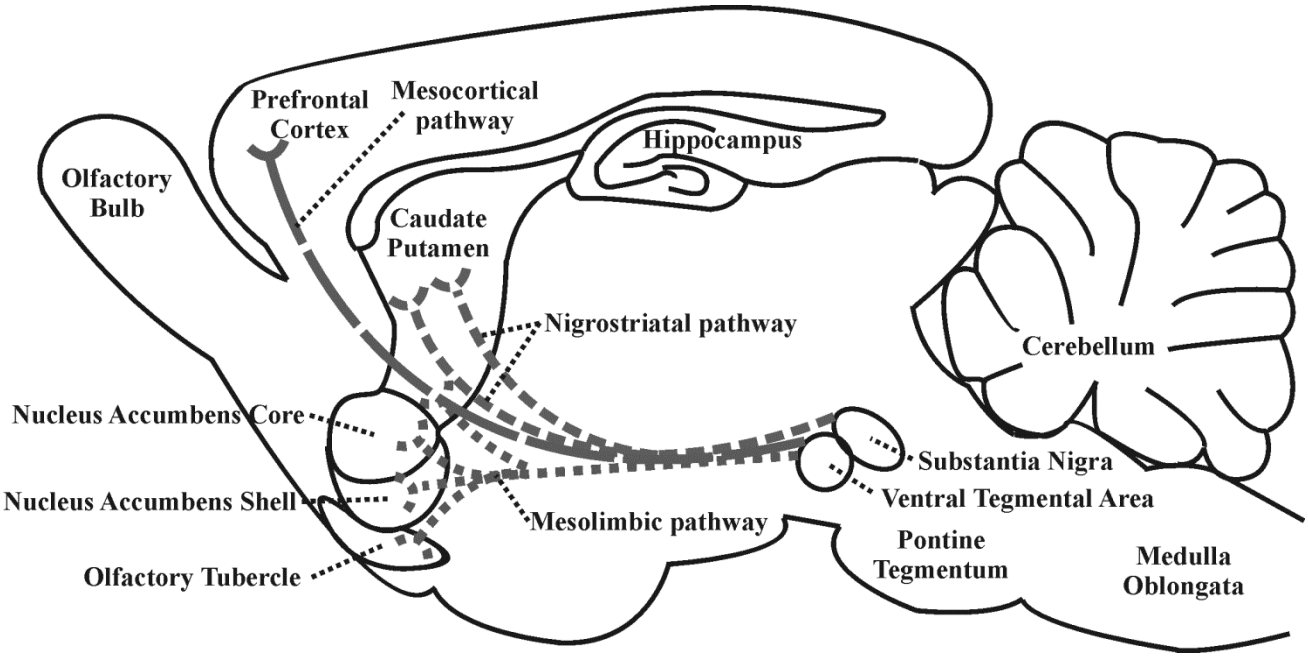

Figure 4. Schematic presentation of dopaminergic pathways ascending from the sunstantia nigra pars compacta and ventral tegmental area and their main projection areas in rodent brain. Adapted from Honkanen (1999) and Hyman *et al*. (2006)

2.2.8 α-Syn, learning and long-term memory

Learning means acquiring new or modifying old memories, behavioral patterns, skills, values or preferences. It requires neurons to modify the activity of their synapses, i.e. synaptic plasticity (reviewed by Cooke and Bliss, 2006). It is believed that α-syn modifies the signaling of dopaminergic synapses in the striatum (Abeliovich *et al*., 2000; Yavich *et al*., 2004) and the experiments on reward prediction error have suggested that striatal DA release has important functions in learning processes (see section 2.2.7). PD is a synucleopathy defined by the loss of dopaminergic connections to the striatum, and in addition to dementia its cognitive symptoms include difficulties in some functions essential to learning, such as set-switching, problem solving, concept formation, decision making,

working memory and attention (Nestler *et al*., 2009; Fritsch *et al*., 2012; Svenningsson *et al*., 2012). Attentional deficits have been described also in another synucleopathy, NBIA1, which is associated with attention deficit hyperactivity disorder (ADHD; Assami *et al*., 2011). If one takes all these conciderations into account, then it seems likely that α-syn function and related pathologies are connected to learning and memory.

The effects of α-syn on learning have been studied in humans. The duplication of the *SNCA* locus can be a great tragedy to its carriers (Chartier-Harlin *et al*., 2004) but it does provide a unique opportunity to study the significance of α-syn in cognitive functions (Keri *et al*., 2010). The duplication inhibited reward-oriented learning in a simple two-choice task. This concurs with the results from mice which indicated that in the absence of α-syn reward-motivated behavior was increased (Oksman *et al*., 2006). However, it was also shown that in α-syn knockout mice punishment-oriented learning was impaired in passive and active avoidance tests (Kokhan *et al*., 2012).

The first evidence that α-syn would be able to affect learning and long-term memory in a non-pathological manner came from the zebra finch (*Taeniopygia guttata*), as mRNA of the α-syn zebra finch homolog synelfin was differentially expressed in brain areas crucial to song learning depending on the phase of the learning process (George *et al*., 1995). The formation of long-term memory depends on a type of synaptic plasticity known as long-term potentiation (LTP) and of its inverted equivalent, long-term depression (LTD; Bliss and Collingridge, 1993; Cooke and Bliss 2006). In LTP, the synaptic signaling between two neurons is enhanced when both neurons are stimulated synchronously (Bliss and Lomo, 1973; Gustafsson *et al*., 1987). LTP in the hippocampus is essential for the formation of long-term spatial memory (Morris *et al*., 1986). Because α-syn has high expression (Iwai *et al*., 1995) and functional significance (Abeliovich *et al*., 2000; Cabin *et al*., 2002; Nemani *et al*., 2010) in the hippocampus and medial DS, it would seem plausible that the protein has some effect on spatial learning and memory. α-Syn is also able to facilitate hippocampal LTP (Liu *et al*., 2004; Gureviciene *et al*., 2007).

Initial results from Morris water maze tests revealed no difference in spatial learning between α-syn null and wild-type mice when they were 6 months old (Chen *et al*., 2002). When spatial learning was measured in a similar test in 10 month old mice of different lineage, α-syn knockouts displayed impaired performance (Kokhan *et al*., 2012). The impairment of spatial learning was very mild, and the same was detected in double knockout mice lacking α- and γ-syn (Senior *et al*., 2008). Subsequently, it was recently reported that improved spatial memory in rats, measured by t-maze test, was associated with a ~1.5-fold increase in hippocampal α-syn expression after perilla (*Perilla frutescens*) diet (Lee *et al*., 2012). Perilla seeds and oil are very high in α-linoleic and omega-3 fatty acids which are essential for neural development (reviewed by Karr *et al*., 2011). However, dementia and learning deficits are prevalent in synucleopathies (McKeith *et al*., 2004; Svenningsson *et al*., 2012) and several mouse models that overexpress α-syn develop pathological α-syn inclusions, and display impaired LTP and memory function (Lim *et al*., 2011; Costa *et al*., 2012; Magen *et al*., 2012). Overall, it appears that α-syn has a function in promoting learning and memory, but the effect is mild, difficult to detect in knockout models and easily masked by the neurodegenerative effects of α-syn overexpression.

2.3 α-SYN IN DISEASE

This section of the literature review will examine the evidence that links altered α-syn expression to substance abuse and addiction but it will also review α-syn's aggregation and how α-syn and its inclusions spread across the brain in PD. The mechanisms by which α-syn triggers neurodegeneration in synucleopathies will be reviewed as well as how these mechanisms relate to the non-pathological function of α-syn in synaptic signaling.

2.3.1 α-Syn in substance abuse and addiction

Drugs of abuse are both rewarding and reinforcing, and they target the ascending dopaminergic pathways from the VTA, either directly or indirectly (for review, see Koob and Moal, 2001 and Hyman *et al*., 2006). The reward system of the brain has evolved to motivate individuals to seek natural reinforcers, such as food, water and sex, but drugs of abuse can easily take over the system. It can be said that substance abuse harnesses and overdrives the neural circuits meant for reward-oriented learning, which ultimately leads to addiction.

The first evidence that α-syn might be involved in the neurobiology of substance abuse and addiction came from *post mortem* samples of cocaine abusers, which displayed elevated α-syn mRNA and protein expression in the SN and VTA (Mash *et al*., 2003). Qin *et al*. (2005) found that the expressions of α-syn and DAT were elevated in the DS of cocaine abusers. Brenz Verca *et al*. (2005) reported that in rats repeated doses of cocaine over a period of six hours (4 × 30 mg/kg i.p.) were sufficient to elevate *Snca* mRNA in the hippocampus, DS and tegmentum when measured 24 h later. Viral overexpression of α-syn in the nucleus accumbens of rats induced an increase in cocaine self-administration and cocaine induced locomotor activity (Boyer and Dreyer, 2007). Accumbal α-syn protein expression might be linked to drug craving, since it was found to be increased in rats which did not stop their drug seeking behaviour after conditioned cocaine treatment, in comparison to rats that did stop (del Castillo *et al*., 2009). Drug craving also correlated with serum levels of α-syn in recently abstinent cocaine users, and the serum levels were significantly higher in comparison to control subjects (Mash *et al*., 2008). Kobayashi *et al*., (2004) reported that polymorphisms in intron 1 of *SNCA* were associated with metamphetamine dependence in Japanese female population. These results suggest that differential expression of α-syn in the reward system of the brain may be both a cause and a consequence of drug abuse.

Cocaine and metamphetamine act mainly by increasing synaptic DA levels, cocaine by blocking the function of DAT, and metamphetamine by reversing the function of VMAT2, which leads to an increase in intracellular DA and reversal of DAT function (Budygin *et al*., 2002; Hyman *et al*., 2006). However, even the abuse of substances which do not affect the dopaminergic system directly, such as opiates, appears to have an association with α-syn. Opiate use (or abstinence from opiates) was reported to have long-term affects on α-syn expression in rat brain (Ziolkowska *et al*., 2005). Ethanol is known to affect the dopaminergic system indirectly by facilitating $GABA_A$ receptor function and inhibiting N-methyl-D-aspartic acid (NMDA) glutamate receptor function, even though ethanol's exact mechanisms of function are not fully understood (Nestler *et al*., 2009). α-Syn protein levels have been found to be higher in the serum of alcoholics in comparison to controls, and the protein level was positively correlated with alcohol craving among the addicts (Bönsch *et al*., 2005a). Foroud *et al*. (2007) reported that 8 single nucleotide polymorphisms in the *SNCA* locus were associated with alcohol craving, but not with addiction. Addiction was instead found to be associated with repeat length in the *SNCA* regulatory region NACP-Rep1, suggesting that differential expression of the protein might be a confounding factor also in alcohol dependence (Bönsch *et al*., 2005b; Fuchs *et al*., 2008). Changes of α-syn expression linked with alcohol abuse do not appear to contribute to α-syn aggregation, as the amount of α-syn inclusions was not found to be different between heavy consumers and control subjects in a *post mortem* histopathological study (Aho *et al*., 2009).

Genetic vulnerability factors to substance abuse have been studied using various animal models, including pairs of rodent lines that have been bred based on their preference for ethanol (reviewed by Green and Grahame, 2007). In one such pair (P/nP rats), high *Snca* mRNA expression was detected in the alcohol preferring (P) line (Liang *et al*., 2003). The increased expression was linked to a mutation in the 3'-untranslated region of *Snca* mRNA which is believed to increase the stability of the mRNA. The high mRNA expression translated into significantly elevated (1.7-fold) α-syn protein expression selectively in the hippocampus. There was a trend towards increased protein expression (1.6-fold) also in the CPu of the alcohol preferring rats. However, it was recently reported that mice with a spontaneous deletion of α-syn show dramatically increased preference for 10 % ethanol when compared to α-syn expressing controls (López-Jiménez *et al*., 2013). The authors attributed this to compensatory mechanisms or downstream effects of the deletion, such as an increase in cannabinoid receptor 1 (CB1) expression in the hippocampus (~3-fold) and amygdala (~8-fold). The expression levels of these receptors remained unchanged in the striatum and cortex.

Another pair of rat lines bred for differential ethanol preference is readily available in Finland; the Alko, non-alcohol (ANA) and Alko, alcohol (AA) rats. Their breeding program was started in the Alko Research Laboratories (Helsinki, Finland) by Dr. Kalervo Eriksson in the 1960's (Eriksson, 1968; reviewed by Sommer *et al*., 2006). Nowadays the lines are maintained by the National Institute for Health and Welfare (Helsinki, Finland). Their neurochemistry has been widely studied, and the striatal DA content has been postulated to be higher in the ethanol preferring AA rats in comparison to non-preferring ANA rats (Ahtee and Eriksson, 1975; Kiianmaa *et al*., 1991). However, *in vivo* microdialysis in the nucleus accumbens of the two lines showed identical extracellular DA levels and elevations in DA levels after an ethanol challenge (Kiianmaa *et al*., 1995). The maximal amount of D_2 DA receptors is lower in the brain of AA rats (Kiianmaa *et al*., 1991) but there are no significant differences between the rat lines in the affinity of D_1 and D_2 DA receptors (Syvälahti *et al*., 1994). The rapid dynamics of DA release and re-uptake or α-syn expression have not been studied in this particular model in the past.

It is clear that changes in α-syn expression are associated with the development of addiction. However, the association of α-syn with genetic background of addiction is uncertain, and data on the exact neurotransmitter systems and brain structures linking α-syn to addiction are inconsistent.

2.3.2 α-Syn in other psychiatric disorders

There is some evidence that depression is linked with α-syn associated disorders. Alcoholism doubles the risk of depression and *vice versa* (Boden and Fergusson, 2011), and 56 % of PD patients can be diagnosed with depression (Yamanishi *et al*., 2013). Depression is frequent also in other synucleopathies (reviews by McKeith *et al*., 2004; Ubhi *et al*., 2011 and Dusek and Schneider, 2012). In some PD cases, depression preceeds the onset of other symptoms (Fukunishi *et al*., 1991; Merschdorf *et al*., 2003) and a previous history of depression more than doubles the risk of PD (Leentjens *et al*., 2003). Furthermore, non-synucleopathy related Lewy body pathology may increase the risk of depression (Lopez *et al*., 2006; Iritani *et al*., 2008). Beck depression inventory scores correlate with serum α-syn mRNA levels in females with eating disorders (Frieling *et al*., 2008) and with NACP-Rep1 repeat length in reportedly healthy individuals (Lenz *et al*., 2011). Martins-de-Souza *et al*., (2012) reported that α-syn protein expression was 1.5 fold in dorsolateral prefrontal cortex of patients with major depression in comparison to healthy controls. The monoamine theory states that depression is caused by disrupted DA, noradrenaline and especially serotonin signaling, and most of the current pharmacological treatments are designed to increase the tone of these neurotransmitters within the synapse (reviewed by Nutt, 2008). However, more modern theories suggest that loss of synaptic plasticity lies at the core of the disease and that it can be fought by promoting growth factor function (for review, see

Thakker-Varia and Alder, 2009). Considering the role of α-syn in synaptic signaling and plasticity, its implication in depression is not surprising.

α-Syn appears to have little involvement with other psychiatric disorders. Lewy body pathology has been suggested to be less frequent in schizophrenia patients (Jellinger, 2009) but there do not seem to be significant changes occurring in total α-syn expression during the course of the disease (Scarr *et al*., 2006; Pennington *et al*., 2008; Gray *et al*., 2010; Noori-Daloii *et al*., 2010).

2.3.3 α-Syn aggregation in synucleopathies

Synucleopathies are a group of neurodegenerative disorders which share similar intracellular proteinaceous lesions with aggregated, insoluble α-syn (Spillantini *et al*., 1998; Jellinger, 2003). The inclusions contain also other proteins and they can be found in perikarya and axonal processes of practically all neuron types of the brain, and they are also present in glia. α-Syn inclusions have also been found in peripheral neurons (Wakabayashi and Takahashi, 1997; Orimo *et al*., 2007; Mu *et al*., 2013). At least five different α-syn inclusions defined by morphology and localization have been identified: Lewy bodies, Lewy neurites (also known as dystrophic neurites), glial cytoplasmic inclusions, neuronal cytoplasmic inclusions and axonal spheroids (Spillantini *et al*., 1997; Arima *et al*., 1998; Tu *et al*., 1998; Braak *et al*., 1999; Galvin *et al*., 2000). Lewy bodies can be further divided into classical (or brain stem) and cortical Lewy bodies (Jellinger, 2003).

Monomeric α-syn oligomerizes when it folds into a beta-sheet like structure (El-Agnaf *et al*., 1998). Small oligomers then aggregate into larger protofibrils and later into fibrils which form the large inclusions detected in synucleopathies. Iron, calcium and some other metals are able to promote α-syn aggregation (Uversky *et al*., 2001; Lowe *et al*., 2004; Kostka *et al*., 2008). Three missense mutations of *SNCA* causing rare forms of familial PD also facilitate the oligomerization of the protein (Fig. 1). The first mutation leads to a substitution of alanine with threonine at residue 53 (A53T; Polymeropoupolos *et al*., 1997), the second in an alanine to proline change at residue 30 (A30P; Kruger *et al*., 1998) and the third to a glutamate being replaced lysine at residue 46 (E46K; Zarranz *et al*., 2004). All three mutations promote α-syn oligomerization, but only the A53T and E46K mutations appear to increase its fibrillization (Conway *et al*., 1998; 2000; Greenbaum *et al*., 2005). The mutations lead to disruption of the secondary structure, revealing the hydrophobic residues within the alpha-helixes (Bussell and Eliezer, 2001; Eliezer *et al*., 2001; Bodner *et al*., 2010). This conformational change promotes the assembly of α-syn into intermolecular beta-sheets and thus facilitates the protein's aggregation.

Even though Lewy bodies and other large α-syn inclusions are the defining characteristics of synucleopathies, they do not appear to be the toxic species and may be even neuroprotective (Tompkins and Hill, 1997; Rideout *et al*., 2001; Tanaka *et al*., 2004). It is believed that the smaller oligomeric and protofibrillar species are the actively harmful form of the protein, and aggregation into larger bodies might be one way to neutralize these species (Bennett, 2005; Uversky, 2007). The α-syn protofibrils have been shown to form pore-complexes which affect membrane permeability (Volles *et al*., 2001; Lashuel *et al*., 2002; Lowe *et al*., 2004; Kostka *et al*., 2008). In conjunction with the other obvious defects in cellular integrity, this may cause leakage of vesicular DA into the cytosol where it can cause oxidative stress and cell death (Miyazaki and Asanuma, 2008).

Disruption of the native pre-synaptic functions of the protein by misfolding and oligomerization may well contribute to the pathology, since it was discovered that in DLB, 90 % of α-syn is concentrated in small aggregates outside Lewy bodies in the pre-synaptic terminals (Kramer and Schulz-Schaeffer, 2007). Recently it was reported that large α-syn oligomers inhibit neurotransmitter exocytosis in PC12 cells by binding to synaptobrevin 2 and thus prevent the formation of the SNARE-complex (Choi *et al*., 2013). It remains unclear how much of the neurodegeneration in synucleopathies is due to loss of native α-syn function, and to what extent there is gain of toxic function through oligomerization.

2.3.4 α-syn and neuronal excitability

An interesting aspect in the pathology related to oligomeric forms of α-syn is the potential effect on postsynaptic excitability. Diogenes *et al*. (2012) and Martin *et al*., (2012) reported that α-syn oligomers (but not monomers or fibrils) were able to facilitate calcium influx in hippocampal slices and cell cultures. This was postulated to happen through activation of glutamate activated calcium channels, but it remains unclear if the pore-structures comprised of oligomeric α-syn are able to contribute to this effect (Volles *et al*., 2001; Lashuel *et al*., 2002; Kostka *et al*., 2008). Since calcium is able to facilitate the formation of α-syn oligomers (Lowe *et al*., 2004) abrupt strong increases in intracellular calcium levels may initiate a vicious cycle of α-syn aggregation and neurodegeneration. A constantly activated calcium influx was shown to disrupt normal LTP, which might explain the deterioration of learning and memory in synucleopathies.

There are also results suggesting that facilitation of calcium influx is a part of the normal, non-pathological function of α-syn. In experiments reported by Adamczyk and Strosznajder (2006), α-syn and especially its NAC-fragment were able to facilitate calcium influx, whereas β-syn had no significant effect. Pharmacological evidence and the rapid mechanism of action suggested that α-syn could potentiate the function of N-type voltage-gated calcium channels. The results of Hettiarachi *et al*. (2009) suggested that the facilitation by α-syn occurred primarily through up-regulation of L-type voltage-gated calcium channels. The effect on calcium influx was reduced by pathological mutations of α-syn. However, it remains unclear whether the postulated facilitation of calcium influx can translate into modulation of neuronal excitability *in vivo*. As mentioned earlier (section 2.2.3), there is indirect evidence suggesting that α-syn could facilitate neurotransmitter release. This evidence is largely based on the measurement of post-synaptic potentials, and therefore it is essential to determine whether the measured effects depend on pre-synaptic mechanisms of neurotransmitter release or on changes in neuronal excitability.

Neuronal excitability can be evaluated *in vivo* by measuring the properties of an electrophysiological phenomenom known as cortical spreading depression (CSD) with DC potential recording (reviewed by Charles and Brennan, 2009; Dreier, 2011 and Eikermann-Haerter *et al*., 2012). CSD is a slowly propagating wave of hyperexcitability followed by total neuronal depolarization. It can be inhibited by glutamate antagonists and calcium channel blockers. It is considered as the electrophysiological manifestation of migraine aura, and it can also be triggered in traumatic brain injury, hypoxic conditions and epilepsy. The primary parameters of excitability in stimulus evoked CSD are threshold of initiation and speed of progression, but also other properties of the depolarization wave can be measured.

2.3.5 α-Syn degradation

Disruption of α-syn elimination is a potential cause of synucleopathies, and differences in degradation processes may explain differences in α-syn protein expression levels. Misfolded, otherwise damaged or excess proteins are typically targeted to proteasomal degradation by ubiquitination, but in synucleopathies, the activity of the proteasome protein complex might be inhibited (McNaught and Jenner, 2001; Rideout *et al*., 2001). Mutations in two genes coding proteins of the ubiquitine-proteasome system (*UCHL1*, *PARK2*) have been found to be causes of familial PD (Nestler *et al*., 2009). α-Syn can be degraded also through autophagocytosis, a process where damaged proteins or organelles are packaged within lipid vesicles, and merged with lysomomes which contain acidic hydrolases that break down the content of the vesicle (Webb *et al*., 2003). A loss of function –mutation in a gene coding the lysosomal protein probable cation-transporting ATPase 13A2 has been identified as a cause of familial PD (Ramirez *et al*., 2006). Cytosolic α-syn can be taken up directly into lysosomes, but the A30P and A53T mutations appear to inhibit this process (Cuervo *et al*., 2004). If ubiquitination is inhibited, α-syn starts to be eliminated mainly by autophagocytosis (Rott *et al*., 2012). However, autophagosomes may not be as

efficient in α-syn degradation as proteasomes, and having to resort to autophagocytosis can lead to accumulation of α-syn.

2.3.6 Spread of α-syn inclusions in synucleopathies

Lewy bodies and neurites spread across the brain over the course of PD and this phenomenom has been described by a scale known as Braak-staging (Braak *et al*., 2003). In the non-symptomatic stage, the first inclusions appear to the medulla oblongata, pontine tegmentum and the olfactory bulb. The symptoms begin to appear when inclusions spread to SN, areas of the midbrain and basal forebrain and the cortical regions are those last to be affected. There is little variation in the order of the spread between individuals with sporadic PD. A similar orderly spread is seen with amyloid-beta pathology in AD, even though the order of affected regions is different (Braak and Braak, 1991).

In 2005 it was shown by Lee *et al*. that monomeric and oligomeric α-syn could be exocytosed from SH-SY5Y cells. It was also claimed that the α-syn in secretory vesicles was more prone to aggregate than the cytosolic protein. Subsequently, these workers reported that the exocytosed protein could be taken up by other cells (Lee *et al*., 2008). This fueled the idea that the reason for Braak-staging was seeding of α-syn from one cell to the next, although a theory of seeding in neurodegenerative diseases had been proposed even earlier (Lansbury, 1997). According to the pathological protein transfer theory, misfolded, aggregation prone α-syn is secreted and taken up into a recipient cell, where it is able to propagate the misfolding and aggregation of the recipient cells own α-syn, much like the prion protein in the transmissible spongiform encephalopathies (Luk *et al*., 2012; Angot *et al*., 2012). Convincing proof for the theory was provided by the observation that α-syn inclusions spread also to neurons which had been grafted to SN of PD patients (Kordower *et al*., 2008; Li *et al*., 2008). Volpicelli-Daley *et al*. (2011) stated that exposure of axonal terminals to pre-formed α-syn fibrils caused the terminals to develop α-syn inclusions, and that the inclusions later spread also to perikarya of the cells. The theory provides a reasonable explanation to why α-syn inclusions end up in glial cells which do not express the protein endogenously (Arima *et al*., 1998; Tu *et al*., 1998; Ahn *et al*., 2012; Amschl *et al*., 2013).

The oligomeric and fibrillar forms of the protein appear to be taken up through endocytosis and subjected to lysosomal degradation (Lee *et al*., 2008; Hansen *et al*., 2011; Steiner *et al*., 2011; Angot *et al*., 2012). However, lysosomes are not necessarily very effective in destroying aggregated or mutated α-syn (Cuervo *et al*., 2004; Rott *et al*., 2012). The monomeric form is suspected to incorporate itself into the cell membrane and pass through it, spread across the cytosol and escape the degradation by autophagocytosis (Lee *et al*., 2008; Hansen *et al*., 2011, Steiner *et al*., 2011). It was recently reported that a single injection of pre-formed α-syn fibrils to dorsal striatum of wild-type mice evoked the spread of Lewy pathology, death of nigrostriatal DA neurons and motor deficits over a time course of 3 to 6 months (Luk *et al*., 2012). Injection of the monomeric form of the protein was not reported to have neurodegenerative effects during the same period and expression of endogenous α-syn was needed for the development of pathology. However, it is not known if the exocytosed and taken up protein is able to have the same effects on synaptic signaling as the recipient cell's own endogenous α-syn.

2.3.7 Putative treatment strategies targeting α-syn

α-Syn has been considered as drug targets in synucleopathies and especially in PD (reviewed by Maguire-Zeiss, 2008). Prevention of α-syn misfolding and aggregation has been demonstrated to be protective in cell models (Outeiro *et al*., 2008; Sultana *et al*., 2011). siRNA mediated silencing of α-syn expression has also been postulated as a potential treatment (Lewis *et al*., 2008). Passive immunization is one interesting treatment strategy (Masliah *et al*., 2011), and antibodies specific to α-syn can prevent the intercellular transmission of α-syn aggregates (Bae *et al*., 2012). Antibodies specific for oligomeric and

protofibrillar α-syn are being developed (Fagerqvist *et al*. 2013). Furthermore, a clinical trial of active immunization with short α-syn resembling peptides is underway (Schneeberger *et al*., 2012).

Naturally the α-syn targeting treatments, as any new therapies, are not without major concerns. The potential problem with passive immunization is that if it is used after the onset of symptoms, it may already be too late as a crucial amount of neurons will have been already lost, and the cascade of neurodegeneration involving also many other factors may still continue. It is not enough simply to create antibodies against α-syn, but the truly harmful forms of the protein should first be identified (Beyer *et al*., 2008; Bartels *et al*., 2011; Fagerqvist *et al*. 2013). Preventing α-syn misfolding and aggregation could be a valid strategy (Outeiro *et al*., 2008; Sultana *et al*., 2011), but achieving this with traditional small-molecule compounds might prove difficult. Reducing the systemic amount of the protein with siRNA or immunization may have detrimental effects on the dopaminergic neurons (Gorbatyuk *et al*., 2010; Khodr *et al*., 2011) since native α-syn exerts its own neuroprotective role (Alves da Costa *et al*., 2002; Leng and Chuang, 2006; Quilty *et al*., 2006). Active immunization against the body's own proteins also carries a threat of triggering autoimmune disease (Schneeberger *et al*., 2012). The protein may have important functions in synaptic plasticity, learning, motivation and control of movement. These functions can be compensated in knockout models where the protein has never existed in the first place, but it is not known what happens to these functions if the protein levels are suddenly dramatically reduced in adult individuals. Before reducing the systemic amount of the protein, its normal physiological functions need to be fully elucidated.

3 Aims

The overall aim of this thesis work was to study the neuromodulatory effects by which α-syn affects synaptic signaling in different scenarios. The more specific aims were as follows:

1) To find possible link between differential α-syn expression, dopaminergic neurotransmission and genetic predisposition to drug abuse and alcoholism (Study I). One particular interest was α-syn and dopaminergic signaling in striatum of alcohol-preferring rats. Striatum appears to be an important region for experiences of reward, as well as learning new preferences and behavioral patterns. Another focus was to examine if systemic ethanol would be able to affect the same pre-synaptic mechanisms of DA signaling as α-syn.

2) To determine whether lack of α-syn in knockout mice would affect the cholinergic signaling in the neuromuscular junction by using electrophysiology and behavioral tests (Study II).

3) To investigate if the lack of α-syn in knockout mice was able to affect neuronal excitability (Study III).

4) To clarify whether locally applied, exogenous α-syn affected DA release and re-uptake *in vivo* (Study IV).

4 Materials and Methods

4.1 ANIMALS

4.1.1 ANA and AA rats (Study I)

The ANA and AA rats used for study I were ethanol naïve, male and 3–5 months old. They were maintained at the National Laboratory Animal Centre (Kuopio, Finland; nowadays known as Lab Animal Centre) in an ambient temperature of 22–23°C and on 12/12 h dark/light cycle (lights on at 7 AM). Food and water were available *ad libitum*. The experiments on the rats were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland.

4.1.2 Mice (Studies II-IV)

The substrain of C57Bl/6J mice from Harlan Olac (Bicester, UK) has been found to carry a spontaneous deletion of the *SNCA* locus, in other words they are "natural" α-syn knockouts (Specht and Schoepfer, 2001). Here they are referred to as b6-. No compensatory up-regulation of β- or γ-syn has been detected in the strain. The genomic deletion spans also to a neighbouring gene which codes multimerin 1, a protein expressed in platelets and endothelium that is involved in hemostasis (Specht and Schoepfer, 2004; Hayward, 1997). The most commonly used control for the b6- mice is the substrain of C57Bl/6J mice from Charles River Wiga (Sulzfeld, Germany) that express α-syn normally (Yavich *et al*., 2004; 2005; 2006; Gureviciene *et al*., 2007; Chadchankar and Yavich, 2011; Chadchankar *et al*., 2011; 2012). Here they are referred to as b6+. The b6- and b6+ mice have been bred separately for generations, and therefore any difference in their phenotype that is suspected to result from α-syn deletion needs to be verified using other models. The B6;129X1-Snca^{tm1Rosl}/J mice are a gene-targeted α-syn knockout line (Abeliovich *et al*., 2000). They were used in study II and they are referred to as b6-Ros. They originate from knockouts in F2 of 129SV/j × C57/Bl6 hybrid mice that were originally created by Dr. Arnon Rosenthal. A breeding pair of these mice was obtained from the Jackson laboratory, cleaned by embryo transfer and bred in the Lab Animal Centre similarly as the b6+ and b6- mice. All mice used in these studies were males in the age range 8 to 25 weeks. Their general housing conditions were similar to those of the rats and their use had been sanctioned similarly as the rats (section 4.1.1).

4.2 CONSTANT POTENTIAL AMPEROMETRY (STUDIES I AND IV)

Stimulated DA release and re-uptake in the striatum were evaluated *in vivo* with constant potential amperometry (Fig. 5; Benoit-Marand *et al*., 2007). The animals were anesthesized and fixed in a stereotaxic frame (David Kopf, Tujunga, CA, USA) for the duration of the experiments. ANA and AA rats (study I) were anesthesized with urethane (1.4 g/kg i.p., 10 ml/kg). b6+ and b6- mice (study IV) were anesthetized with chloral hydrate (420 mg/kg i.p., 10 ml/kg) and the anesthesia was maintained with one quarter of the original dose every 60-90 minutes. These anesthetics were chosen based on their minimal effects on dopaminergic synapses (Maggi and Meli, 1986; Lu and Greco, 2006). Local anesthesia was performed with s.c. injections of lidocaine (10 mg/ml), after which the skin and muscles were retracted, the skull was exposed and drilled to allow the insertions of working, stimulating and auxiliary electrodes. The auxiliary electrode (stainless steel screw) was

placed in the skull behind lambda, and the reference electrode (silver/silver chloride) was placed against the exposed skull which was always covered by a layer of saline. The working electrode was positioned in the striatum and the stimulating electrode in the medial forebrain bundle at the level of lateral hypothalamus. The coordinates for the electrode postitions in relation to bregma and the skull surface were obtained from rat (Paxinos and Watson, 1986) and mouse (Franklin and Paxinos, 2007) brain atlases. The exact position of the stimulating electrode was determined from the maximal DA overflow in response to stimulation.

The working electrode was a single carbon fiber with a diameter of 30 μm (World Precision Instruments, Sarasota, FL, USA) fixed with epoxy glue inside a glass capillary. Then, 350 ± 50 μm of the tip of the fiber was exposed from the capillary. A custom-built three-electrode potentiostat maintained the working electrode at 0.4 V versus a silver/silver chloride reference electrode, which is sufficient to cause the near complete oxidation of DA (Dugast *et al*., 1994). Data were digitized at 1 kHz and stored in a computer using Invilog Voltammetry System (Invilog Research, Kuopio, Finland). The exact sensitivity of individual working electrodes had been measured beforehand in an unstirred solution with 1, 2 and 3 μM of DA in the presence of 200 μM of ascorbic acid. Ascorbic acid was included in the calibration solution since it is electrochemically active and present in the brain at concentrations of 200-300 μM (Basse-Tomusk and Rebec, 1991). No post-calibrations of the working electrodes were performed because in some animals the electrodes were used for electrolytic marking of their positions within striatum. The actual measurements were always started 1.5 h after lowering the working electrode into the brain, since carbon fiber electrodes lose sensitivity exponentially during this time (Michael *et al*., 1987). If the working electrode was moved within striatum, it was allowed to remain for 10-12 min in its new position before performing measurements in order to allow the baseline signal to stabilize.

The stimulating electrode was a bipolar tungsten wire with diameter of 350 μm. Biphasic constant current pulses (1 ms each polarity) were applied on the tungsten electrode from a battery-operated constant current unit (A365; World Precision Instruments) controlled by the Invilog Voltammery System through a personal computer. In ANA and AA rats (study I) DA overflow was evoked with 400 μA pulses delivered in 20 (5 s) and 50 Hz (2 s) frequencies. The interval between the 20 and 50 Hz stimulations was 3 min. In b6+ and b6- mice (study IV), 10 (5s), 20 (2 s), 30 (2s) and 50 Hz (2s) stimulations were used and the current of the pulses was 180 μA. The interval between stimulations of different frequency increased from 1-2 min to 3-4 min as the frequency of the stimulations increased in order to prevent exhaustion of the DA stores.

In AA and ANA rats (study I), the responses to the stimulations were measured from three different structures in the striatum; CPu, AcbC and AcbSh. The working electrode was lowered from 1.8 mm lateral and 1.6 mm anterior to bregma to depths of 5.5 mm (CPu), 7.2 mm (AcbC) and 8.3 mm (AcbSh) from the skull surface. An additional set of stimulations (20 and 50 Hz) was applied 12 min after the first stimulation to ensure a stable DA overflow when the working electrode was in its most ventral position in the AcbSh. Immediately after this second measurement, the rats were injected i.p. with a 0.1 g/kg dose of 10 % (v/v) ethanol. Evoked DA overflow was measured 15, 30, 45 and 60 min after the injection. After the last measurement, a 3g/kg dose of 50 % (v/v) ethanol was injected and measurements of evoked DA overflow were conducted again with 15 min intervals for 1 h after the treatment. It should be recalled that 0.1 g/kg of ethanol is a very small dose that is eliminated rapidly, whereas 3 g/kg is a very large, hypnotic-sedative dose (Lopez-Jimenez *et al*., 2013).

The exact stimulation and measurement protocols in b6+ and b6- mice (study IV) are described in section 4.5. Electrolytic lesions were made via the working electrodes (6 V, 15 s for rats and 5 s for mice) when the lesions did not interfere with following tissue analysis.

Brains were removed, frozen and cut with a microtome for histological analysis. In some rats, striatal structures were dissected, frozen and used for western blot analysis.

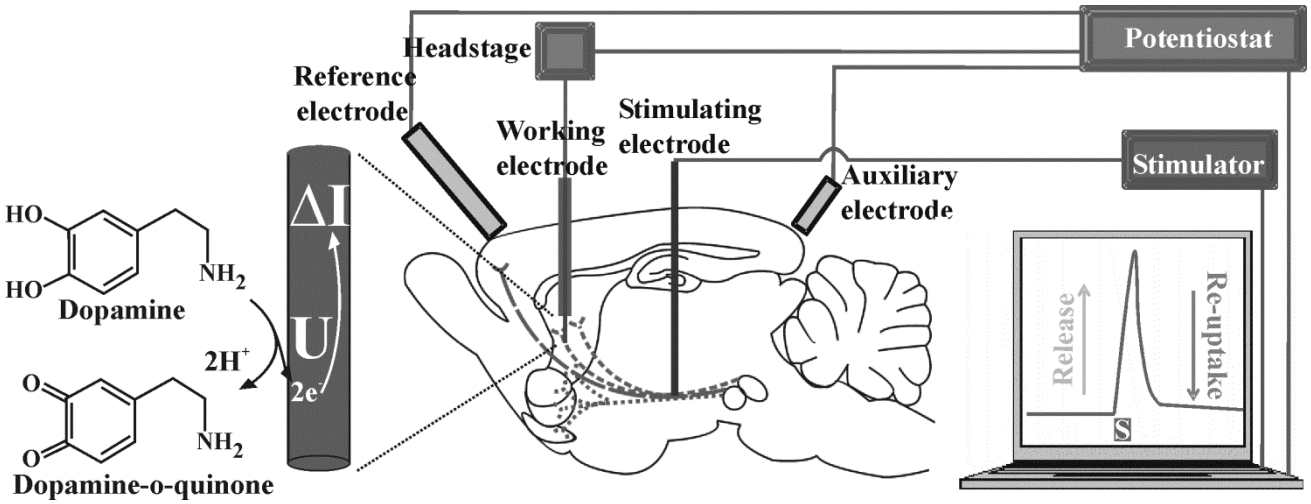

Figure 5. Schematic presentation of the setup for constant potential amperometry. Constant potential (U) which is sufficient to oxidise dopamine is applied to the working electrode. The oxidation reaction causes a change of the Faradaic current of the working electrode (ΔI) which is directly proportional to the concentration of dopamine. The current is transformed to voltage by the head stage, digitized by digital-to-analog converter and stored on a hard drive for further analysis. The potential of the working electrode is maintained by means of potentiostat with its reference and auxiliary electrodes. Stimulation (S) is applied through the stimulating electode to the dopaminergic axons, which results in measurable changes in DA concentration near the dopaminergic terminals. The changes in concentration are detected practically in real time (Benoit-Marand *et al*., 2007).

4.3 ELECTROMYOGRAPHY (STUDY II)

Repetitive electrical stimulation and electromyography were used to measure the functionality of the neuromuscular junction in b6+, b6- and b6-Ros mice (Fig. 6). The animals were anesthetized with chloral hydrate (450 mg/kg i.p., 10 ml/kg) and the anesthesia was sustained by injecting one quarter of the initial dose after every 45-60 min. The animals were fixed to a heated stand by their limbs and tail using tape. The electrodes were 26-gauge injection needles made from stainless steel. Their exact positions in relation to each other are indicated in fig. 6. The two stimulating electrodes were placed against the caudal motor axons at the base of the tail. The recording and reference electrodes were inserted into the caudal muscles on the lateral side of the tail. The ground electrode was implanted under the skin at the back of the mouse.

Sub-maximal electrical stimuli, 2–3 mA in amplitudes, were produced using an A320 current isolation unit (World Precision Instruments). Master-8 pulse stimulator (A.M.P.I., Jerusalem, Israel) was used to set the pulse length (0.1 ms) and intervals (10–300 ms). DAM 70 Differential Amplifier (World Precision Instruments) amplified and filtered (300 Hz to 3 kHz) the signals, which were transferred to a personal computer using Digidata 1321A analog-digital converter and Clampex 8.2 software (Axon Instruments, Sunnyvale, CA, USA). The sampling rate was 10 kHz. Five trains of six pulses, each at different between-pulses intervals, were used to stimulate the caudal motor axons. The intervals between pulses were 300, 100, 50, 20 and 10 ms, and data were collected over a period of 2 s. Each stimulation train was repeated three times per mouse with a 1 min pause after each train. The anesthesized animals were sacrificed after the measurements.

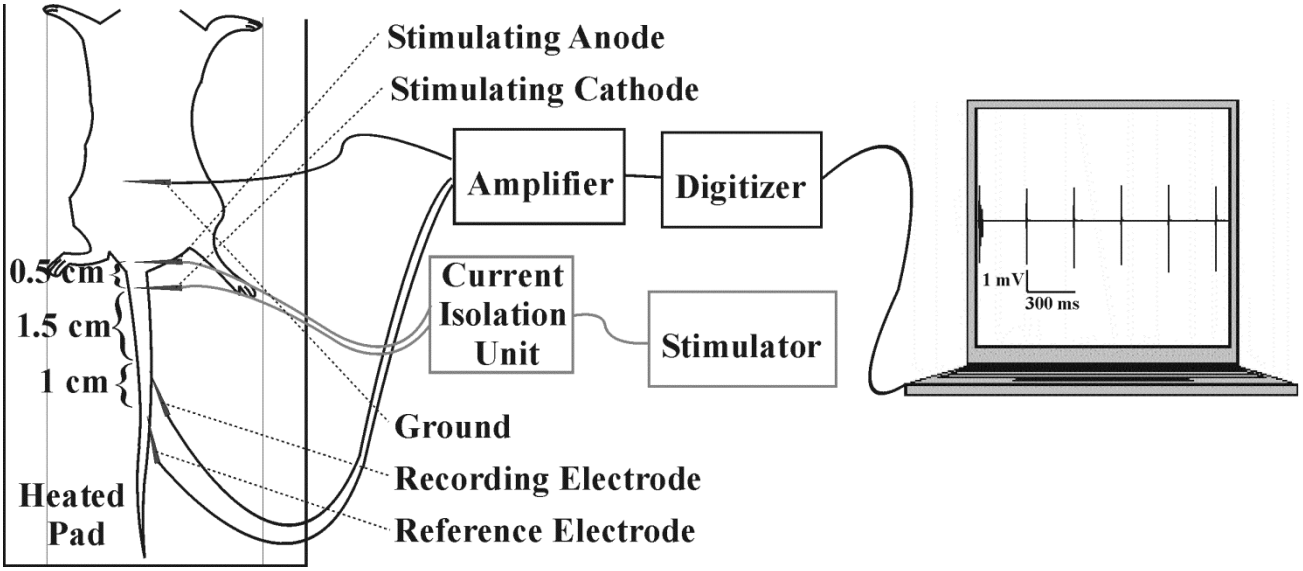

Figure 6. Schematic presentation of the electromyography setup. Electrical stimulation of the tail's motor axons causes the lower motor neurons to release acetylcholine at the neuromuscular junction. This produces compound muscle action potentials (CMAPs) at the muscles of the tail which can be measured using electromyography. The example shows six CMAPs acquired from a b6-Ros mouse using a stimulation train with 300ms intervals between stimulation pulses (for more detailed visualization of the CMAPs, see figure 10 A). Method was adapted from Boërio *et al*., 2009.

4.4 DIRECT CURRENT POTENTIAL RECORDING (STUDY III)

CSD was initiated with single pulse electrical stimulation and measured with DC potential recording in b6+ and b6- mice (Fig. 7). The mice were anaesthetized with chloral hydrate (420 mg/kg i.p., 10 ml/kg) and fixed in a stereotaxic frame (David Kopf). Anaesthesia was maintained with one sixth of the original dose administered every 45–60 min. The skin and muscles were retracted, the skull was exposed and trepanations for the working and stimulating electrodes were made to the positions indicated in Fig. 7 without puncturing the dura mater. The recordings were made with two miniature silver/silver chloride electrodes (Invilog Research, Kuopio, Finland) in plastic capillaries (0.5 mm in diameter) filled with 2% (w/v) gelatin in saline. The two electrodes are referred to as channels 1 and 2. The tips of the capillaries were lowered 0.3 mm down from the skull surface against the dura. The current was recorded against a silver/silver chloride reference electrode. A 26 G stainless steel needle functioned as the ground electrode and it was punctured through the skin at the back of the mouse. The stimulating electrode was a tungsten bi-wire with two ball-shaped tips in a distance of 1.5 mm from each other (Invilog Research), and it was lowered on the dura 0.4 mm from the skull surface.

The DC potential recording was performed using a Model 440 Instrumentation Amplifier (Brownle Precision Co., Palo Alto, CA, USA). The method measured changes in the potential in the DC mode, meaning that there was no highpass filter in use (Peeters *et al*., 2007; for review, see Eikermann-Haerter *et al*., 2012). The low-pass filter was set to 100 Hz. Data were digitized at 1000 Hz with a Digidata 1321A analog-digital converter and the program Clampex 8.2 (Axon Instruments). The stimulation pulses were produced with a type 215/I stimulator (Hugo Sachs Elektronik - Harvard Apparatus GmbH, March-Hugstetten, Germany) and transmitted to the electrodes through a SIU5 Stimulus Isolation Unit (Grass Technologies, West Warwick, RI). The pulse width was set at 0.1 s.

The stimulating electrode was placed on the dura and was always covered by a layer of saline on the skull, and thus a field stimulation approach was used, and data on threshold is expressed as voltage. The CSD threshold was determined by applying electrical pulses with intervals of 100 s and at increasing voltage. The voltage of the first pulse was 10 V, the

increment from pulse to pulse 10 V and the maximal stimulation intensity 100 V. The stimulations for evoking CSD were done twice. In order to make sure that the surgical procedures did not cause CSD, baseline signal was recorded for 30 min before and between stimulations and 15 min after. The anesthesized animals were sacrificed after the experiments.

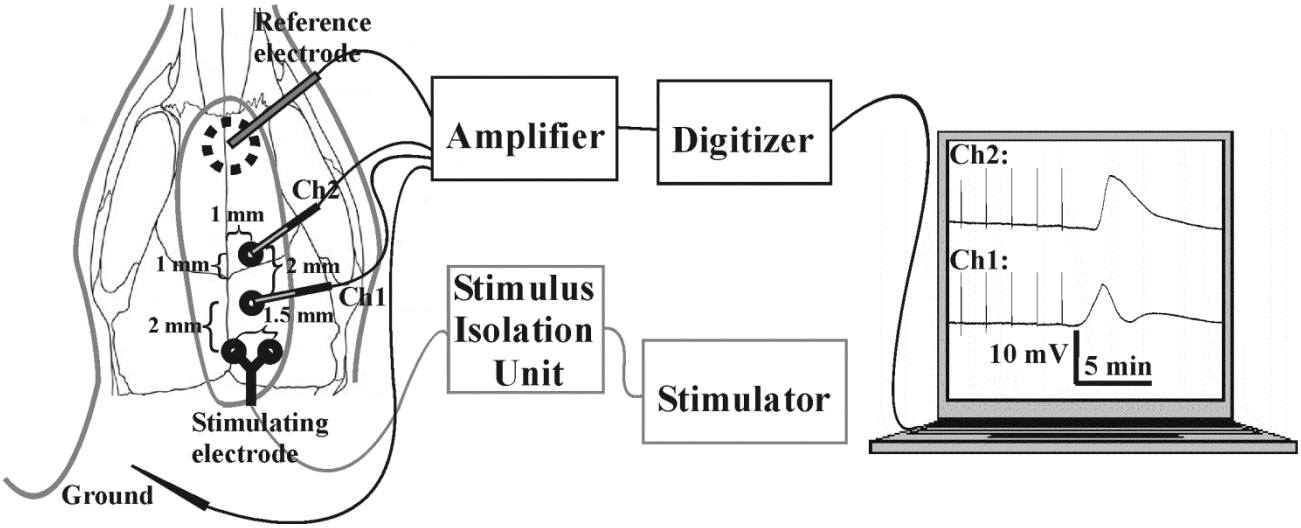

Figure 7. Schematic presentation of the direct current potential recording setup. The positions of trepanations are indicated with black circles and the dashed circle indicates the position of the reference electrode. Ch1 (channel 1) and Ch2 (channel 2) refer to the working electrodes. The ground electrode was punctured through the skin in the back of the mouse. The example data shows a typical recording with artefacts of stimulation and the cortical spreading depression (CSD) waves.

4.5 MICROINJECTION (STUDY IV)

For studying the delayed effects of exogenous α-syn on DA overflow, the protein was injected into the DS of b6+ and b6- mice and constant potential amperometry was performed six days later (Fig. 8 A). Stock solution of monomeric Alexa Fluor 488–labeled α-syn protein (1 μg/μl, 30 mM Tris-HCl and 325 mM NaCl, pH 7.5; provided by H. Lundbeck A/S, Valby, Denmark) was diluted to a concentration of 0.1 μg/μl with phosphate buffered saline (0.1 M, pH 7.4). Mice were anesthetized with a medetomidine-ketamine mixture (0.6 mg/kg and 45 mg/kg i.p. respectively, 5 ml/kg) and fixed into a stereotaxic frame (David Kopf). Local anaesthesia was performed with s.c. injections of lidocaine (10 mg/ml). Microinjections were performed using glass capillaries with a tip diameter of 40 μm. Animals were injected into the dorsal striatum with 0.1 μg of α-syn protein over a period of 17 min. The needle was kept in place for an additional 15 min after the flow of liquid was stopped. A similar injection with diluent only was performed to the contralateral hemisphere. The injections were performed at the following coordinates respective to bregma: anterior +1.4 mm; lateral ± 1.3 mm; ventral -3.3 mm (Franklin and Paxinos, 2007). Post-operative care was sought to according to the general instructions of the lab animal centre and the mice were single housed until the measurements of stimulated DA overflow were performed six days later. For these measurements, the mice were anesthesized and fixed to the frame as described earlier (section 4.2). The working electrode was lowered into the dorsal striatum at an angle of 16 ° from vertical at coordinates 1.4 anterior and ± 2.1 lateral to bregma. The ventral positions of the electrode are described in Fig. 8 A. DA overflow was evoked at each position of the working electrode with 50 Hz stimulation, except at the depth of -3.3 mm where also 10, 20 and 30 Hz stimulations were applied. After

the measurements, the anesthesized mice were sacrificed, their brains removed, immersion-fixed in 4 % (w/v) paraformaldehyde in phosphate buffered saline for 36 h (+4 °C) and stored in phosphate buffered saline containing 0.02 % (w/v) Na-azide (+4 °C).

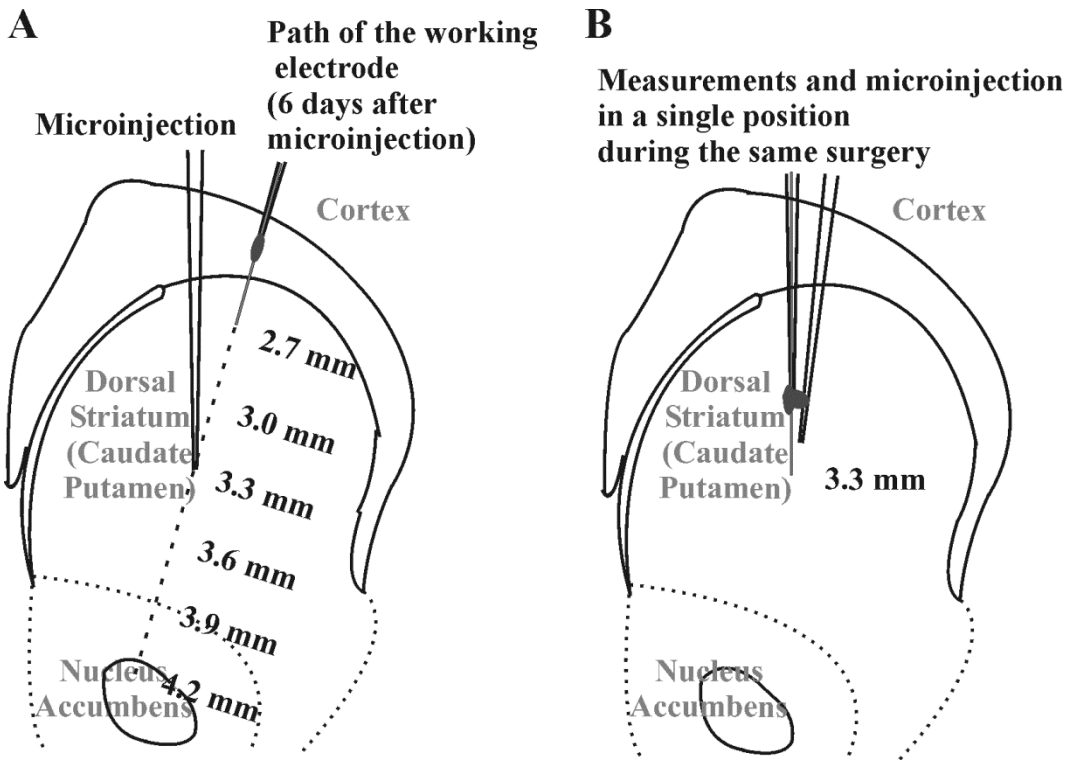

Figure 8. Schematic presentation of the positions of the microinjection capillaries and the working electrodes in the measurement of delayed (A) and immediate (B) effects of α-syn microinjection.

Only b6- mice were used for studying the immediate effects of the protein, and the microinjections and constant potential amperomery were performed during the same surgical procedure (Fig. 8 B). The tip of the microinjection capillary was fixed to a distance of 46 ± 3 μm from the center of the working electrode using epoxy glue. This assembly was lowered into the brain (1.4 mm anterior, 1.3 mm lateral and 3.3 ventral from bregma) where it was kept for the entire duration of the experiment. The injection of protein (monomeric human recombinant α-syn, 1 μg/μl, Nordic Biosite, Täby, Sweden) or diluent (20 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 1mM $MgCl_2$) was made immediately after the pre-injection stimulations. The amount of injected protein was 2 μg and the injection time was 20 min. Responses to 10, 20, 30 and 50 Hz stimulations were measured before and after the microinjection. The post-injection stimulations were repeated every 30 min for 3 h. The animals were sacrificed after the experiment and their brains stored as described above.

4.6 BEHAVIORAL TESTING (STUDY II)

4.6.1 Rotarod

The motor coordination and balance in b6+, b6- and b6-Ros mice were analyzed using an accelerating Rotarod (Ugo Basile, Comerio, Italy). The animals were allowed accustom themselves with the device for 1 min before testing, 30 s on a motionless bar and 30 s on a bar that was rotating at minimal speed (4 rpm). After this adaptation period, the apparatus was set to accelerate (from 4 to 40 rpm) and timing was started. The time that the animals were able to run on the rotating bar without falling down from the rod was recorded. The mouse could grab hold on the bar for one or two consecutive turns, but after the third consecutive turn the animal was removed from the rod. The maximum time that the

animals were allowed to remain on the rod was 480 s (8 min). The test was repeated three times at two day intervals.

4.6.2 Grip strength

Neuromuscular function in b6+, b6- and b6-Ros mice was assessed by measuring the front paw grip strength. The animals were positioned on a normal home cage roof with parallel round metal bars (2 mm in diameter and 7 mm from each other). The animals were confined to a space of 15 x 18 cm with 10 cm high plastic walls. The animal's tail was taped tightly to a calibrated spring which could be raised upwards at a steady speed. Before the actual testing, the animals were allowed to familiarize themselves with the surroundings for 30 s and one or two rehearsal lifts were performed to make sure that the animals grabbed the bars. During the actual measuring the animals were lifted so that they were able to grab the bars only with their front paws. When lifting, the apparatus added 0.95 Newtons of force per second. The maximal force the animals were able to grab against the bars against was recorded. Three measurements were conducted per animal from which an average was calculated. The apparatus was cleaned in the same way as the Rotarod between animals. The results were calculated as force per unit of body mass (N/kg).

4.6.3 Footprint pattern

The gait of b6+, b6- and b6-Ros mice was assessed with a footprint pattern test. The front paws of the animals were dipped in red nontoxic ink and the hind paws in blue nontoxic ink. The animals were placed at the brightly lit end of a transparent tunnel that had its bottom lined with paper. At the far end of the tunnel there was a dark cylinder with some home cage bedding. The animals walked through the tunnel so that consecutive steps could be recorded without the animal turning back or stopping too often. A consecutive step pair was chosen from each animal for analysis, excluding the first and last recorded steps. Fore- and hindpaw stride length, hind- and front base width between left and right paws and forepaw/hindpaw overlap were measured. The tunnel was cleaned after each individual animal.

4.7 WESTERN BLOT (STUDY I)

The tissue samples from the striatum of ANA and AA rats were mechanically homogenized in 150 µl of Dulbecco's phosphate-buffered saline (Invitrogen, Carlsbad, CA, USA). Protein degradation was prevented with proteinase (100× cocktail; Thermo Scientific, Waltham, MA, USA) and phosphatase inhibitors (Halt phosphatase inhibitor; Thermo Scientific). Fifty µl of the tissue homogenate was mixed with an equal volume of protein extraction detergent (T-PER, Pierce, Rockford, IL, USA) in order to extract also the membrane bound proteins. The remaining membrane particles were separated from the protein fraction by incubating the samples on ice for 10 min and by centrifugating with 15700 g for 10 min at +4 °C. Total protein concentration was measured with BCA Protein Assay Kit (Thermo Scientific). The separation of the proteins in the sample was achieved by loading 30 µg of total protein which was run on 4–12 % sodium dodecyl sulphate–page gels (NuPAGE 4–12% BT 1.5; Invitrogen). The proteins were then transferred from the gels to polyvinylidene difluoride-membranes (Hybond-P; Amersham Bio-sciences, Amersham Place, UK) using a semi-dry electro-transfer apparatus (TE77 ECL-semidry Transfer unit, Amersham Biosciences). The membranes were blocked with 5% (v/v) milk powder in Tris-buffered saline with 0.05% Tween-20 (TBST) and then incubated in primary α-syn antibody (ab6162, 1:1000 dilution; Abcam, Cambridge, UK) overnight with rotation at +4°C. The membrane was washed for 4 × 10 min with TBST, incubated in horseradish peroxidase-labelled secondary antibody (ab6900, 1:15000 dilution; Abcam) for 1 h with rotation at +4°C and washed as before. Depending on the properties of the individual membrane, either ECL+ or ECL Advance

reagent (Amersham Biosciences) were used to visualize the horseradish peroxidase-label in the secondary antibody. The membranes were photographed with ImageQuant RT ECL-camera (GE healthcare, Buckinghamshire, UK). The α-syn antibody recognized a specific band at ~13 kDa. The membranes were stripped from the antibodies using Restore Western Blot Stripping Buffer (Thermo Scientific) after imaging and milk blocked as before. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as the control protein and it was detected using specific primary (ab8245, 1:15000 dilution; Abcam) and secondary (NA931V, 1:12000 dilution; Amersham Biosciences) antibodies.

4.8 IMMUNOHISTOCHEMISTRY (STUDY IV)

The brains of α-syn and/or diluent injected b6+ and b6- were cut into 35 μm coronal sections with a freezing-sliding microtome. The sections were immunostained by free floating technique. They were first heat treated at +80 °C in 0.05 M Na-citrate for 30 min in order to reveal the antigen, and blocked in 1% bovine serum albumin (BSA) for 1 h. The solution used for diluting the BSA and antibodies, and for rinsing the sections between treatments (3 × 5 min) was 0.5 M Tris buffered saline (pH 7.6) with 0.5% of Triton X-100. The sections were transferred to a solution containing the primary rabbit antibody against human α-syn (SA 3400, Enzo Life Sciences, Farmingdale, NY, USA, 1:1000 dilution) for overnight incubation on a shaker table. After this, the sections were transferred for 2 h to a solution containing a biotinylated secondary antibody (BA-1000 anti-rabbit IgG, Vector Labs, Burlingame, CA, USA, 1:500 dilution), and for 2 h to a solution containing streptavidin-horseradish peroxidase (RPN1231V; GE Healthcare, Little Chalfront Buckinghamshire, UK, 1:1000 dilution). Following rinsing, the sections were incubated for 3 min in 3,3-diaminobenzine tetrahydrochloride for making the horseradish peroxidase label visible. The stained sections were mounted on slides and coverslipped.

4.9 DATA ANALYSIS

When studying DA overflow data, it should be remembered that peak concentration of DA after stimulation does not reflect only DA release, but also the effectiveness of re-uptake can affect the maximal concentration, and that maximal velocity of DA re-uptake can be reliably assessed only after release which is sufficient to saturate the DAT (Wightman and Zimmerman, 1990; Benoit-Marand *et al*., 2007). In study I, the peak levels of evoked DA overflow were calculated in molar concentrations (μM) or as a percentage of peak overflow before ethanol administration. Constant potential amperometry can follow the changes in extracellular DA concentration in real time and makes it possible to evaluate DA re-uptake from the downward slope of the DA overflow curve. The slope was approximated by non-linear regression following the equation $y(x)= y0 \cdot e^{kx}$. The fit was performed using the software package StimFit 0.8.15 (University of Fribourg, Fribourg, Switzerland). In the equation, a high value of k represents a faster disappearance of DA from the extracellular space.

In the western blot analysis in study I, the intensities of the α-syn and control protein bands were measured using Quantity One software (Bio-Rad, Hercules, CA, USA). Samples were divided into random ANA–AA pairs so that the comparable samples were placed on the same membrane. The percentage of α-syn band intensity from that of the control protein was calculated for each sample. ANA values were chosen as the baseline.

The electromyography data in study II and DC potential recording data in study III were analyzed off-line with Clampfit, version 8.2 (Axon Instruments).

By the time that study IV was conducted a more sophisticated custom-made software for analyzing DA overflow data was available in our lab. The results given by the program are based on the principles described below. The extracellular DA levels in the terminal fields after brief electrical stimulation are a result of the balance between release and re-uptake and can be described by the following Michaelis-Menten equation (Wightman and Zimmerman, 1990): $d[DA]/dt = [DA]_p * f - V_{max}/ (K_m/[DA] + 1)$. In the equation, [DA] represents instantaneous DA concentration, $d[DA]/dt$ the rate of re-uptake, $[DA]_p$ the DA release per stimulus pulse, f is the frequency of stimulation, and V_{max} and K_m refer to the maximal rate of re-uptake and apparent affinity, respectively. After the end of stimulation, the release component is absent and the change in DA concentration can be described by another equation: $d[DA]/dt = -V_{max}/(K_m/[DA] +1)$. The custom made software solves both these equations, and determines $[DA]_p$, V_{max} and K_m providing the best fit between experimental data and the data simulated by the model.

4.10 STATISTICAL ANALYSIS

Statistical analysis was performed using the statistical tools of Prism 5 for Windows (GraphPad Software Inc., San Diego, CA, USA), and IBM SPSS Statistics 19 and its earlier versions (IBM Corporation, Armonk, NY, USA). In study I, differences between ANA and AA rats in absolute levels of peak DA overflow, α-syn expression and the value of k (representing rate of re-uptake) were evaluated with unpaired, two-tailed Student's t-test. Peak DA overflow and k were evaluated also with ANOVA for repeated measures, as were also the effects of ethanol on evoked DA overflow. In study II, the Rotarod results were evaluated with ANOVA for repeated measures while other results were evaluated with one-way ANOVA. Bonferoni's test was used in the *post hoc* analysis. In study III, the results on CSD initiation and propagation were evaluated with ANOVA for repeated measures. In study IV, ANOVA for repeated measures was used for evaluating data from measurements which were performed repeatedly in a single position in the striatum. Otherwise two-way ANOVA was used. *Post hoc* analysis was performed with unpaired, two-tailed Student's t-test since it is capable of comparing only two groups. The error bars in all results signify standard error of mean.

5 Results

5.1 STIMULATED DOPAMINE OVERFLOW AND α-SYN EXPRESSION IN THE STRIATUM OF ANA AND AA RATS (STUDY I)

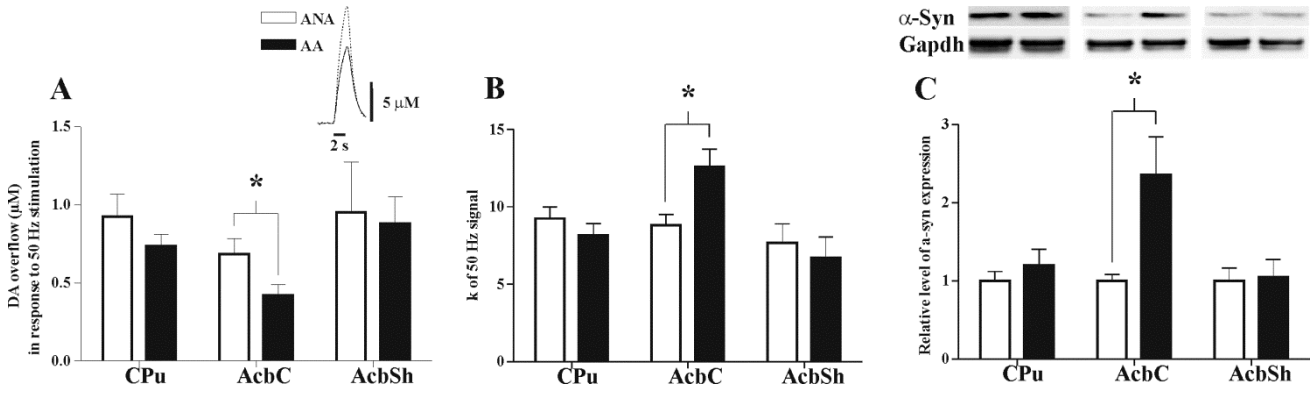

Figure 9. Stimulated dopamine (DA) overflow, re-uptake and α-syn expression in striatum of ANA and AA rats (Study I). A: DA overflow in the caudate putamen (CPu), nucleus accumbens core (AcbC) and nucleus accumbens shell (AcbSh) in ANA and AA rats in response to 50 Hz stimulations of the medial forebrain bundle. n_{ANA} = 6–8 and n_{AA} = 7–8. B: Re-uptake of DA (represented by the value of k) was determined from the right-sided slope of the DA overflow curve following stimulation at 50 Hz in the CPu, AcbC and AcbSh. C: Comparison of α-syn expression between AA and ANA rats in the CPu, AcbC and AcbSh (n = 4). * designates $p < 0.05$ (t-test)

Constant potential amperometry revealed no differences in the parameters of stimulated DA overflow between ANA and AA rats when examining the striatum as a whole. However, peak DA overflow after 50 Hz stimulation was lower and re-uptake faster in AA rats in comparison to ANA rats when concentrating AcbC alone (Fig. 9 A and B,). Data from 20 Hz stimulations confirmed the result on peak DA overflow but not on re-uptake (Study I, table 1) suggesting that the reduction in peak DA overflow was not entirely due to more efficient re-uptake. When comparing α-syn expression in the same structures with western blot, no differences were seen in the CPu or AcbSh, but it was found that in the AcbC AA rats exhibited higher expression than the ANA rats (Fig. 9 C). No differences were seen between the rat lines in stimulated DA overflow after the ethanol injections (study I, Fig. 3). The results suggest that increased α-syn expression in specific regions is associated with decreased DA release and more efficient re-uptake, and that these factors in the AcbC may predispose an individual to substance abuse.

5.2 EXCITABILTY OF SKELETAL MUSCLES AND MOTOR PERFORMANCE IN WILD-TYPE AND α-SYN DEFICIENT MICE (STUDY II)

No α-syn dependent differences were seen in the amplitudes of compound muscle action potentials (CMAPs) in response to repetitive stimulation, but instead, repetitive CMAPs (R-

CMAPs, Fig. 10 A) were detected in 4 out of 5 b6- mice and 3 out of 5 b6-Ros mice, while only 1 out of 5 b6+ mice displayed a single R-CMAP (Fig. 10 B). The R-CMAPs were typically detected in α-syn deficient mice after the first pulse (39.5 % of all R-CMAPs). Next it was tested whether this abnormality would reflect a defect in motor control. The α-syn deficient mice performed initially worse in the Rotarod test, but they were able to improve their performance as the test was repeated so that all three lines were indistinguishable by the third day of testing (Fig. 10 C; training × genotype interaction $F(4, 30) = 3.2$, $p = 0.026$). The time spent running on the rod was significantly different between the three lines ($F(2, 15) = 10.5$, $p = 0.020$) but *post hoc* analysis revealed a significant difference only between b6+ and b6-Ros mice ($p = 0.003$) and between b6- and b6-Ros mice ($p = 0.004$). b6+ and b6- mice were able to stay on the non-slippery rod of the Rotarod apparatus during a full turn but in contrast to this behavior, b6-Ros mice could not cling on to the rod and dropped off. Clinging on to the bar was seen in 83% of all trials with b6+ mice, 89% with b6- mice but only in 6% with b6-Ros mice. This was not due to any deficiency in muscle strength or control of ambulation, since the results of the grip strength and footprint pattern tests were the same in all three lines (study II, Fig. 2 B and table 1). The occurrence of R-CMAPs in b6- and b6-Ros mice suggests that the lack of α-syn influences the excitability of muscles. However, the effect is not strong enough to cause any major motor dysfunction. The slow improvement of b6- and b6-Ros mice to the level of b6+ mice in the Rotarod test suggests that lack of α-syn causes a disability in motor learning.

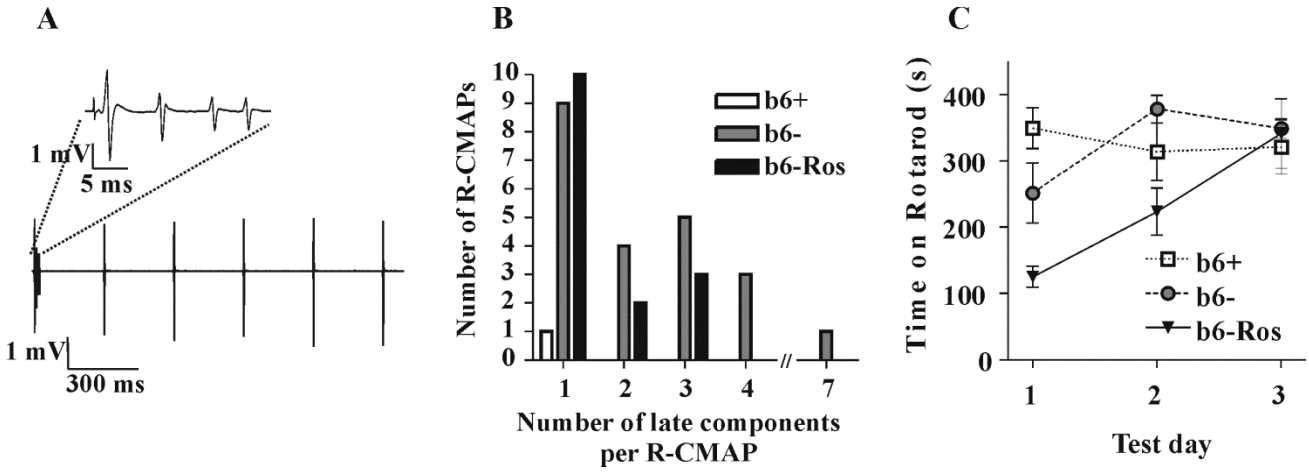

Figure 10. Occurrence of repetitive compound muscle action potentials (R-CMAPs) and performance of the α-syn deficient (b6- and b6-Ros) and wild-type (b6+) mice in the Rotarod test (Study II). A: Example of a train with 300 ms intervals between pulses from a b6-Ros mouse showing a R-CMAP after the first pulse. The inset shows the artifact of stimulation (the first small peak), the primary compound musle action potential (CMAP) and three late components. B: Number of detected R-CMAPs with different amounts of late components (n = 5). C: The Rotarod test was repeated three times every third day (n = 6).

5.3 INITIATION AND PROPAGATION OF CORTICAL SPREADING DEPRESSION IN WILDTYPE AND α-SYN DEFICIENT MICE (STUDY III)

CSD did not differ between b6+ and b6- mice in any of the analyzed parameters (Fig. 11), nor was there any interaction between genotype and stimulation or channel. No spontaneously occurring CSD waves were detected in the recordings. The average speed of the CSD wave was 2.1 mm/min. The results suggest that the lack of α-syn does not effect the initiation or propagation of CSD.

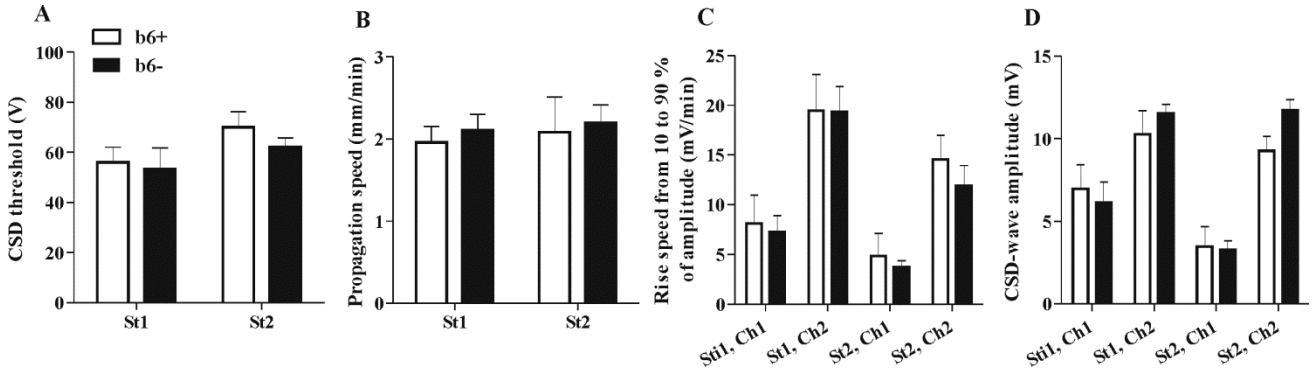

Figure 11. Properties of cortical spreading depression (CSD) waves in wild-type (b6+, n = 5) and α-syn deficient (b6-, n = 6) mice (Study III). A: Threshold of CSD in the two mouse lines. St1 refers to stimulation 1 and St2 to stimulation 2. B: Propagation speed of the CSD wave. C: Rising speed of the CSD wave measured from the left slope. Ch1 stands for channel 1 and Ch2 for channel 2. D: Peak amplitudes of the waves.

5.4 DELAYED EFFECTS OF α-SYN MICROINJECTION ON STIMULATED DOPAMINE OVERFLOW IN WILD-TYPE AND α-SYN DEFICIENT MICE (STUDY IV)

Human α-syn could not be detected from the striata of mice six days after microinjection (Fig. 12 A-D), even though some difference in staining could be seen between control and α-syn injected hemispheres in b6+ mice (Fig. 12 C). Nonetheless, there was a general trend for reduced peak DA overflow and maximal velocity of DA re-uptake in the α-syn injected mice (Fig. 12 E-J). The reduction in stimulated DA overflow in response to 50 Hz stimulations was significant in b6- mice [Fig. 12 H, $F(1,57) = 9.0$, $p = 0.004$]. The *post hoc* analysis revealed a significant difference only at a depth of -3.9 mm. No significant changes were seen in b6+ mice (Fig. 12 G). The peak amplitudes of DA overflow after 50 Hz stimulations were different between b6+ and b6- mice after control treatment ($F[1,57] = 4.7$, $p = 0.034$) but because the damage from the control treatment alone may affect the dopaminergic tone in the region, these results should not be used to draw conclusions on the intrinsic differences between the mouse lines. The effect of α-syn microinjection on DA overflow after 10, 20, 30 and 50 Hz stimulations at depth -3.3 mm was not statistically significant (Fig. 12 E-H, $F[1,20] = 2.1$, $p = 0.165$).

The injection of α-syn decreased V_{max} when examining all data from b6+ and b6- mice (Fig. 12 I and J, $F[1,129] = 14.26$, $p < 0.001$). The effect remained significant when looking at b6+ (Fig. 12 I, $F[1,60] = 4.4$, $p = 0.040$) or b6- (Fig. 12 J, $F[1,57] = 10.3$, $p = 0.002$) mice alone. The *post hoc* analysis revealed a significant difference only at a depth of -3.0 mm in the b6- mice. V_{max} was significantly different between b6+ and b6- mice after control treatment ($F[1,57] = 4.8$, $p = 0.033$). There were no significant differences in K_m (Fig. 12 I and J, insets) resulting from genotype, treatment or their interaction. The results indicate that exogenous α-syn is capable of causing changes in pre-synaptic DA release and re-uptake six days after microinjection even though α-syn itself is no longer present at the pre-synaptic terminals in any detectable amounts.

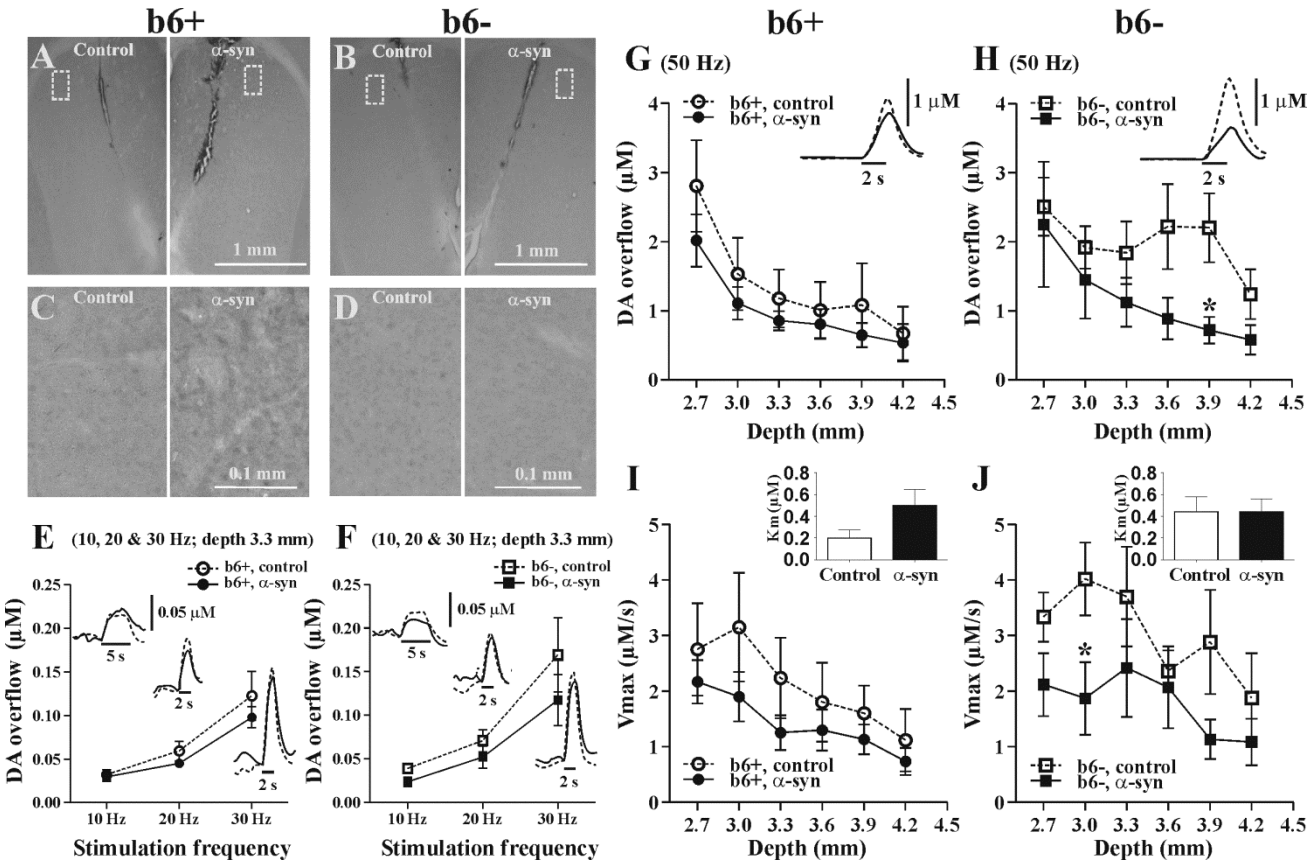

Figure 12. Delayed effects of α-syn microinjection in b6+ and b6- mice (Study IV). A, B: Sections showing the control-injected (left) and α-syn-injected (right) hemispheres of b6+ (A) and b6- (B) after staining with an antibody specific for human α-syn. The tracks of the voltammetry electrode are visible in the hemispheres. The magnified areas shown in C and D are indicated with the white, dashed rectangles in A and B, respectively. E, F: Dopamine (DA) release in response to 10, 20 and 30 Hz stimulations was measured at a depth of -3.3 mm in the ventral striatum of b6+ (E) and b6- (F) mice. n = 6 per group. G, H: DA overflow in response to 2 s, 50 Hz stimulation was measured in six different positions in the striatum of b6+ (G) and b6- (H) mice. Maximal velocities of DA re-uptake (V_{max}) in b6+ (I) and b6- (J) mice were calculated from the DA peaks after 50 Hz stimulations. The K_m values, (insets in I and J) describing the affinity of DAT, were calculated from the DA peaks after 30 Hz stimulations. * designates $p < 0.05$ in the *post hoc* analysis (t-test)

5.5 IMMEDIATE EFFECTS OF α-SYN MICROINJECTION ON STIMULATED DOPAMINE OVERFLOW IN α-SYN DEFICIENT MICE (STUDY IV)

Since it was not possible to detect the injected α-syn protein from the striatum six days after microinjection, (Fig. 12 A-C), it was decided to inject a larger dose of the protein into b6- mice and to measure the effects on stimulated DA overflow immediately (Fig. 13). As a result, the injected α-syn was seen to have diffused around the injection site (Fig. 13 E), and α-syn accumulating cell bodies were also detected (Fig. 13 F). In contrast to the delayed effects of α-syn microinjection (Fig. 12), the immediate effects included a trend for increased peak DA overflow, especially after low frequency stimulations (Fig. 13 A-D), and an increase in maximal velocity of DA re-uptake (Fig. 13 H). There was also a trend for increase in the value of K_m (Fig. 13 G). However, the effects did not reach statistical significance, not even the overall interaction between stimulation frequency, time and treatment in peak DA overflow [$F(1.8,10.7) = 2.8$, $p = 0.112$]. Some of the effects could be

seen even before the actual injection (Fig. 13 A, B, G, H) indicating that the protein had leaked from the tip of the electrode-capillary assembly during the 1.5 h period that the electrode had to stay in the brain before measurements (see section 4.3).

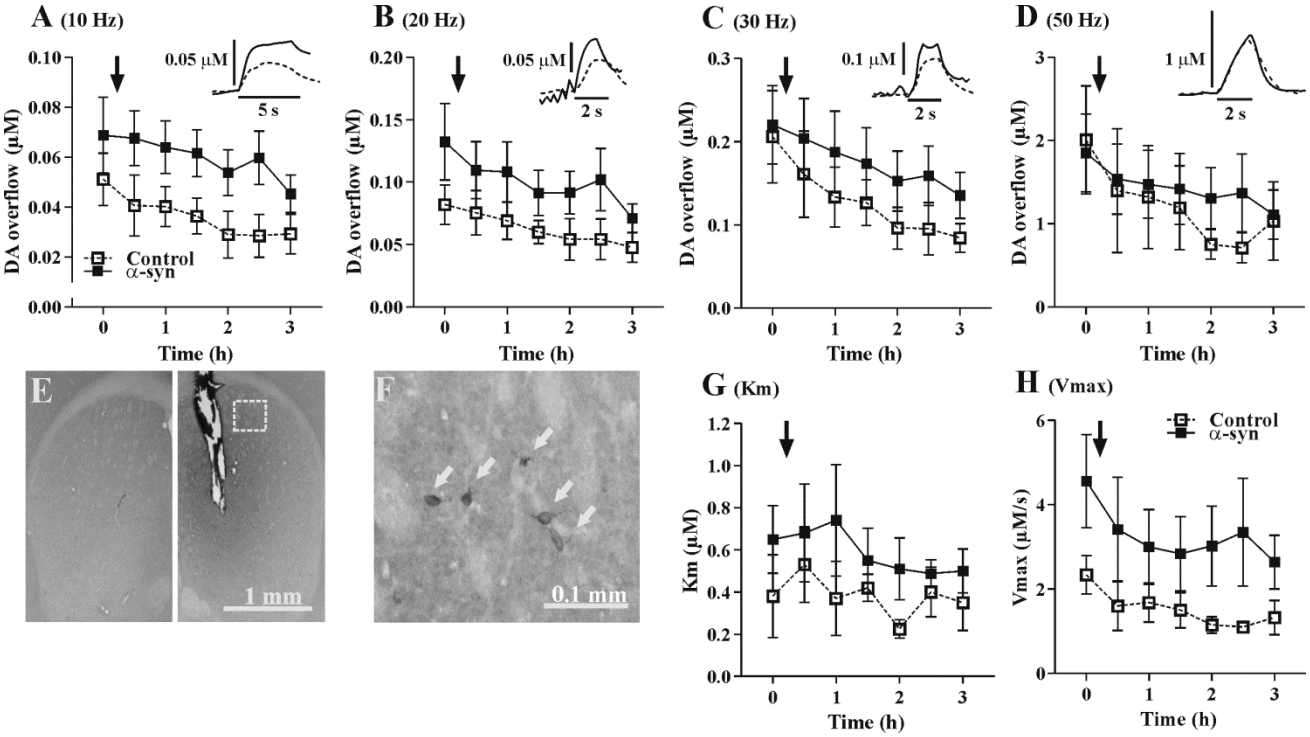

Figure 13. Immediate effects of α-syn microinjection in b6- mice (Study IV). A-D: Dopamine (DA) release was measured in response to 10 (A), 20 (B), 30 (C) and 50 Hz (D) stimulations of the medial forebrain bundle. n = 5 per group. The black arrow indicates when the microinjection pump was turned on. E: Section showing the non-injected (left) and α-syn-injected (right) hemispheres after staining with an antibody specific for human α-syn. The track of the microinjection capillary-voltammetry electrode -assembly is visible in the right hemisphere. F: The area shown in F is indicated with the white, dashed rectangle in E. Individual cells stained by the α-syn antibody are indicated with white arrows. G: The K_m values were calculated from the DA peaks after 30 Hz stimulations. H: Maximal velocities of DA re-uptake (V_{max}) were calculated from the DA peaks after 50 Hz stimulations

6 Discussion

6.1 STIMULATED DOPAMINE OVERFLOW AND α-SYN EXPRESSION IN THE NUCLEUS ACCUMBENS CORE DIFFERENTIATE ANA AND AA RATS (STUDY I)

In the first part of this work (study I) the presynaptic DA release and re-uptake along with α-syn expression were studied in the striatum of two rat lines that have been bred for differential ethanol consumption (Fig. 9). The result that higher α-syn expression was found in the AcbC of the ethanol preferring AA rats is in line with the results from human subjects and other animal models which show that high α-syn expression is associated with substance abuse (Mash *et al*., 2003; Bönsch *et al*., 2005a; b). Substance abuse can cause α-syn expression to increase (Brenz Verca *et al*. 2005); this might be due to disinhibition of transcription as cocaine abusers have a low expression of NURR1 (Bannon *et al*., 2002) which downregulates *SNCA* expression (Yang and Latchman, 2008). Cocaine abusers also display decreased expression of striatal VMAT2 (Little *et al*., 2003) which may be a downstream effect of the increased α-syn expression (Mash *et al*., 2003; Guo *et al*., 2008; Fountaine *et al*., 2008). The current results concur with other reports which suggest that increased α-syn expression can also contribute to vulnerability to substance abuse (Liang *et al*., 2003; Boyer and Dreyer, 2007). An increased α-syn level may be a part of a vicious cycle which leads to addiction by modulating the neurotransmitter tone in certain areas of the brain to a state where an individual experiences heightened drug craving (Bönsch *et al*., 2005a; Mash *et al*., 2008; del Castillo *et al*., 2009).

The localization of expressional and functional differences between ANA and AA rats to AcbC concurs with the reports which show that increased drug self-administration and drug-seeking is associated with increased α-syn expression in this area (Boyer and Dreyer, 2007; del Castillo *et al*., 2009). However, it was reported by Liang *et al*. (2003) that the differences in α-syn expression between the alcohol preferring (P) and non-preferring (nP) rats were localized to the hippocampus and CPu (DS), while the expression in the AcbC was identical. This could indicate that the differential ethanol consumption in these line pairs is driven by slightly different behavioral mechanisms, as DA release in the AcbC is essential for motivation of effort requiring actions and the CPu drives more automated, habitual behavior (Yin *et al*., 2004; Salamone and Correa, 2012).

The high α-syn expression in the AcbC of AA rats coincided with increased peak DA overflow and faster re-uptake after stimulated release in comparison to the situation in the ANA rats. Methodological restrictions do not allow to conduct any direct correlations between α-syn expression and parameters of DA overflow, but the current results suggest that in a physiological environment the moderately increased α-syn expression leads to inhibition of DA release and facilitation of re-uptake. However, the differences in DA overflow between ANA and AA rats can be seen as contradictory to earlier results showing identical resting DA levels in the AcbC of these rats (Kiianmaa *et al*., 1995). This may be explained by the different firing rates of dopaminergic neurons between ANA and AA rats, since it has been reported that in other rodent line pairs with differential ethanol preference, the intrinsic firing rates of dopaminergic neurons in the SN and VTA tend to be higher in ethanol preferring animals in comparison to their ethanol avoiding counterparts (Risinger *et al*., 1994; Morzorati and Marunde, 2006; Beckstead and Phillips, 2008). The higher firing rate might be able to compensate for the inhibition of release and facilitation of re-uptake in the AcbC of AA rats and make the extracellular DA level identical with that in ANA rats. At present, the intrinsic activity of dopaminergic neurons has not been studied in ANA and AA rats and it would make a very interesting and important topic for future work. The reduced DA overflow in AA rats is unlikely to result from autoregulation by pre-

synaptic D_2 receptors, since their levels are lower in AA rats in comparison to ANA rats (Kiianmaa *et al*., 1991).

Stimulated DA overflow in the AcbSh did not differ between ANA and AA rats after ethanol challenge (study I, Fig. 3) which concurs with previous data on extracellular DA levels after an ethanol challenge (Kiianmaa *et al*., 1995). In fact, the ethanol challenge evoked rather mild changes in DA overflow in comparison to the results reported by Budygin *et al*. (2001) which showed that ethanol causes a dramatic dose dependent reduction in stimulated DA release (but did not affect re-uptake) in the CPu of Sprague-Dawley rats. The discrepancy could be explained by anesthesia used in the experiment setup, as Budygin *et al*. measured stimulated DA overflow in non-anesthesized, freely-moving animals. Similar studies performed in mice have revealed that effects of ethanol on stimulated DA overflow were clearer in freely moving mice than in chloral hydrate anesthesized mice (Jones *et al*., 2006). In study I, the rats were anesthesized with urethane which induces a long-lasting surgical anesthesia, deeper than that achieved with chloral hydrate (Maggi and Meli, 1986; Lu and Greco, 2006). Thus, the results are in line with the general consensus that the effects of ethanol on the dopaminergic system are indirect and require fully functional connections to other neurotransmitter systems (see section 2.2.5) which can be severely affected by the applied anesthetics (Lu and Greco, 2006; Dalo and Hackman, 2013).

6.2 LACK OF α-SYN MODULATES FUNCTION OF THE NEUROMUSCULAR JUNCTION BUT DOES NOT AFFECT MOTOR CONTROL (STUDY II)

α-Syn is localized to peripheral and motor neurons in its native and pathological forms (Papachroni *et al*., 2005; Mu *et al*., 2013). The absence of α-syn does not affect the viability of peripheral neurons (Papachroni *et al*., 2005) but α-syn's role in their function is unknown. In order to study the role of α-syn in lower motor neurons, electromyography and behavioral testing were performed in α-syn deficient and wild-type mice in the second part of this work (study II). The applied electrical stimulation produced R-CMAPs almost exclusively in the α-syn deficient mice (Fig. 10 B). The R-CMAPs can be of synaptic or neural origin (van Dijk *et al*., 1996). Synaptic R-CMAPs originate from an excessive acetylcholine effect in the synaptic cleft, whereas neural R-CMAPs are attributable to abnormal motor axons that generate impulse trains in the wake of a single passing impulse. In humans, synaptic R-CMAPs have typically one or two late components and the shape of the components is very regular, which fits quite well to the current findings (Fig. 10 A and B). Neural R-CMAPs are more irregular in shape and have up to 25 components. This suggests that α-syn may have a regulatory effect on the cholinergic tone at the neuromuscular junction. The regulation may occur at the level of vesicle pool organization (Nemani *et al*., 2010) or acetylcholine synthesis (section 2.2.1). However, other explanations for the R-CMAPS cannot be excluded; acetylcholinesterase terminates the signaling at the neuromuscular junction by breaking down the neurotransmitter into acetyl and choline groups, and acetylcholinesterase inhibitors can cause synaptic R-CMAPs (van Dijk *et al*., 1996). Synaptic R-CMAPs have been detected also in a mouse-model of slow-channel syndrome where they are caused by slow closing of the Na+/K+ channels in the muscular acetylcholine receptors (Gomez *et al*., 1997). This is interesting because α-syn has been reported to be localized to the post-synaptic side of the neuromuscular junction in human skeletal muscle fibers (Askanas *et al*., 2000).

Despite the pathological electromyography finding in the α-syn deficient mice, no significant differences were seen between the mouse lines in grip strength or ambulation (study II, Fig. 2 B and table 1). Even the initial difference detected in the Rotarod test appeared to be more because of a defect in motor learning and not in motor function (Fig. 10 C). This is understandable since the effect of α-syn deficiency on the CMAPs did not

manifest itself consistently in all stimulation sets, indicating that the effect of α-syn in the neuromuscular junction is rather mild. However, other explanations for the differeces seen in the Rotarod test cannot be completely ruled out. It is possible that the hyperactivity detected in double (Senior *et al*., 2008) and triple knockout mice (Anwar *et al*., 2011) could contribute to the results. Deteriorated motor learning in α-syn deficient mice is still an interesting possibility, since overexpression of mouse α-syn has been reported to have a similar effect in young mice (Rieker *et al*., 2011). This suggests that a certain, controlled level of α-syn expression is required for optimal synaptic function and efficient motor learning (see section 2.2.8), and that these might be interfered by α-syn targeting therapies. The behavioral consequences of α-syn modulating therapies are difficult to predict since a complete lack of α-syn can have the same behavioral effect as a modest, local increase in the protein level, as shown by the increased ethanol preference in knockout mice (study I and Liang *et al*., 2003 vs. López-Jiménez *et al*., 2013).

6.3 LACK OF α-SYN HAS NO EFFECT ON CORTICAL SPREADING DEPRESSION (STUDY III)

Since α-syn modulates glutamatergic neurotransmission (Liu *et al*. 2004), facilitates calcium influx (Hettiarachchi *et al*., 2009; Martin *et al*., 2012) and is highly expressed in the cortex (Iwai *et al*., 1995) it was postulated that there might be differences in the initiation and/or propagation of CSD between wild-type and α-syn deficient mice (study III). However, no differences were seen between the mice (Fig. 11). Initiation and propagation of CSD reflect neuronal excitability (Eikermann-Haerter *et al*., 2012), and the current results suggest that facilitation of neuronal excitability is not part of the native physiological function of α-syn. Some functions of α-syn can be masked in knockout mice by the other synucleins, especially β-syn (see section 2.1.1), but according to Adamczyk and Strosznajder (2006) β-syn is not able to influence calcium influx significantly. However, it still remains possible that oligomeric forms of the protein can affect excitability in a pathological manner (Diogenes *et al*., 2012, Martin *et al*., 2012). CSD causes massive glutamate release, but the mechanisms of release in CSD appear to include also the reversal of glutamate transporter function (Basarsky *et al*., 1999; Charles and Brennan, 2009). The current results support the idea that α-syn modulates particularly pre-synaptic mechanisms of vesicular neurotransmitter release (Sidhu *et al*., 2004), but the modulation is so subtle that it is unable to affect a phenomenon as strong as CSD which includes complete neuronal depolarization and alternative mechanisms of neurotransmitter release.

CSD is a pathological phenomenon and considered as the electrophysiological manifestation of the migraine aura (Charles and Brennan, 2009; Dreier, 2011). It has been long debated whether CSD is a cause or a consequence of a migraine attack, but the current opinion is that it triggers the migraine headache (Eikermann-Haerter *et al*., 2012). One peculiar feature of migraine is the accumulation of iron into the basal ganglia (Welch *et al*., 2001; Kruit *et al*., 2009). Since iron is able to promote α-syn expression (Febbraro *et al*., 2012) and aggregation (Uversky *et al*., 2001), and iron accumulation is a feature of synucleopathies (Dusek and Schneider, 2012; Mochizuki and Yasuda, 2012) there might be some connection between migraine and synucleopathies. The first case-control study published on this topic indicated that male PD patients have an increased prevalence of moderate to severe migraine (Lorentz, 1989). In contrast to these results, van Hilten (1992) reported a preliminary finding from 5 PD patients suggesting that onset of PD seemed to alleviate migraine, especially in patients with no family history of migraine. Similar observations were made by Barbanti *et al*., (2000) from a larger set of patients, but their interpretation of data has been subjected to criticism (Serrano-Duenas, 2001). Since D_2 antagonists are capable of alleviating migraine (Snow *et al*., 2002) PD therapy may easily obscure the results on migraine prevalence. The current results suggest that if there is any

causality between migraine and PD, brain α-syn is not the connecting factor, and that α-syn targeting therapies will not necessarily increase migraine vulnerability. Changes in the peripheral sympathetic nervous system in migraine, such as reduced plasma noradrenaline and increased sensitivity of peripheral adrenergic receptors, indicate that common factors with PD are more likely to be found in the periphery (Peroutka, 2004; Orimo *et al*., 2007).

6.4 EXOGENOUS α-SYN AFFECTS STIMULATED DOPAMINE OVERFLOW (STUDY IV)

According to the pathological protein transfer theory different forms of α-syn are exocytosed and taken up by neighbouring cells (Luk *et al*., 2012). In an attempt to study the effects of exogenous monomeric α-syn on DA release and re-uptake processes, recombinant human α-syn was injected into the DS of wild-type and α-syn deficient mice, and stimulated DA overflow was measured six days later and immediately after the microinjection (study IV). The injected α-syn was not detected from the striatum six days after microinjection, which might be due to degradation (Webb *et al*., 2003) or slow axonal transport to the cell perikaryon (Utton *et al*., 2005; Volpicelli-Daley *et al*., 2011). The inability to determine the fate of the injected α-syn between the two possibilities is a limitation of this present study. Nevertheless, the injection of exogenous α-syn caused a reduction in peak DA overflow induced by 50 Hz stimulations, detectable selectively only in the α-syn deficient mice (Fig. 12 G and H). A reduction in the maximal velocity of DA re-uptake was detected also in wild-type mice, even though the effect was more robust in the α-syn deficient mice (Fig. 12 I and J).

The effects of the α-syn microinjection did not localize to the exact site of the microinjection, but as shown by the stained cells in Fig. 12 E and F, the injected protein can diffuse quite distant from the injection site. The delayed effects of human α-syn microinjection on stimulated DA overflow in mice were similar to those of human α-syn overexpression in rats induced by viral vectors (Gaugler *et al*., 2012, Lundblad *et al*., 2012). The effects of the overexpression were partly due to axonal degeneration and neuronal loss in the nigrostriatal dopaminergic system, but the reduction in TH activity, DA release and re-uptake manifested even before the neurodegeneration (10 days after vector injection; Lundblad *et al*., 2012) and the magnitude of these changes exceeded that of the neuronal loss (Gaugler *et al*., 2012). Therefore human α-syn has also functional effects on the dopaminergic neurons, and since the changes in stimulated DA overflow were similar in the current study, it appears that also exogenous, injected α-syn can have the same effects. Stimulated DA release can be affected by reduced activity of TH and VMAT2 (section 2.2.1, Fig. 3), and α-syn is known to inhibit not only the function but also the expression of both proteins (Yu *et al*., 2004; Guo *et al*., 2008). The changes in activity that arise from the expression of the proteins may linger long after α-syn itself has been removed from the system.

The immediate effects of α-syn did not include a reduction in peak DA overflow, but instead there was a trend towards increased DA overflow, manifesting especially with the low-frequency stimulations (Fig. 13 A-D). Considering the dynamic and modifiable structure of α-syn (section 2.1.2), multiple and even opposing mechanisms of action of the protein are feasible (Sidhu *et al*., 2004). Previously published results suggest that there are three explanations for the increase in DA release: 1) The slightly reduced affinity of the DAT (described by the K_m value; Fig. 13 G) causes the increase in peak DA overflow (Wightman and Zimmerman, 1990; see also section 2.2.4). 2) The striatal cells that take up α-syn (Fig. 13 F) are cholinergic interneurons, and α-syn affects their output which in turn affects the release of DA (Rice and Cragg, 2004; Section 2.2.5). 3) Exogenous α-syn facilitates the assembly of the SNARE-complex at the terminal membrane, which translates into increased DA release (Burre *et al*., 2010; Section 2.2.3).

The credibility of the first explanation is hampered by the increase in maximal velocity of DA re-uptake (Fig. 13 H), showing that even if affinity of DAT were to be reduced, it still could be compensated by increased membrane expression of the protein. The second explanation is plausible but uncertain since the exact nature of the stained cells remains unknown. Their characterization remains a necessary and interesting topic for future work. The problem with the third explanation is that all studies done in catecholaminergic models indicate that α-syn inhibits SNARE complex assembly or reduces DA release through other mechanisms (Larsen *et al*., 2006; Darios *et al*., 2010; Choi *et al*., 2013). There are some results from glutamatergic regions that suggest that α-syn could promote neurotransmitter release (Cabin *et al*., 2002, Liu *et al*. 2004; Gureviciene *et al.* 2007), and the primary neurons which were used by Burre *et al*. (2010; 2012) for studying the SNARE-complex assembly were obtained from mainly glutamatergic areas (cortex, hippocampus) of mouse and rat brain. Similar studies by Chandra *et al*. (2005) were performed using whole-brain samples, and glutamate is by far the most abundant neurotransmitter in the brain (Bear *et al*., 2007; Nestler *et al*., 2009). It has been shown that α-syn binds to intracellular, catecholaminergic markers such as TH (Perez *et al*., 2002) and VMAT2 (Guo *et al*., 2008) which are not present in glutamatergic neurons. The facilitation of release appeared to be mild and transient (immediate vs. delayed effects) and it might be that the exogenous protein slowly incorporates itself with the catecholaminergic markers and microtubules (Alim *et al*., 2004) at the striatal dopaminergic terminals, and loses the association with the SNARE-complexes. It might be that if catecholaminergic markers are absent from the pre-synaptic terminal, α-syn is available for facilitation of the SNARE-complex assembly.

The delayed (Fig. 12 I and J) and immediate (Fig. 13 H) effects of exogenous α-syn on maximal velocity of DA re-uptake were also quite opposite. The results on the immediate effects of the protein suggest that α-syn can speed up DA re-uptake also in a physiological environment (Lee *et al*., 2001; Fountaine *et al*., 2008). This mechanism of function is supported by the results of study I where an endogenous increase of α-syn expression was associated with more efficient re-uptake (Fig. 9 B and C). Considering the role of α-syn as a negative regulator of DA release (Abeliovich *et al*., 2000; Lundblad *et al*., 2012), facilitation of DA re-uptake is a very logical function for the protein, since both functions reduce the concentration of the neurotransmitter at the synaptic cleft.

The delayed effects of α-syn microinjection showed that exogenous α-syn is also able to reduce the maximal velocity of DA re-uptake (Fig. 12 I and J; Wersinger and Sidhu, 2005) but the results of Lundblad *et al*. (2012) and Bellucci *et al*. (2011) suggest that this function is associated with neurodegeneration and aggregation of α-syn. The exogenous α-syn may reduce the rate of re-uptake not only by binding DAT to the intracellular compartment (Wersinger *et al*., 2005; Bellucci *et al*. 2011), but it is also capable of causing inflammation (Lee *et al*., 2010) which may interfere with the re-uptake process (L'episcopo *et al*., 2010). It appears that human α-syn is particularly toxic to the rodent brain and causes severe degeneration within a few weeks (Lundblad *et al*., 2012), whereas significantly higher overexpression of mouse α-syn in mouse brain is tolerated for several months (Rieker *et al*., 2011). Luk *et al*. (2012) were able to initiate neurodegeneration with pre-formed mouse α-syn fibrils three months after exposure, but injection of monomeric mouse α-syn had no degenerative effects at any of the tested time points up to five months. Taken together, the results suggest that exocytosed α-syn can affect DA release and re-uptake, but it remains uncertain whether the effect is pathological or a part of normal control of synaptic signaling.

7 Conclusions and Summary

The general aim of this thesis work was to clarify the neuromodulatory effects by which α-syn could affect synaptic signaling under different scenarios. Based on the results, the following conclusions can be drawn:

1) The ethanol-preferring AA rats have a higher expression of α-syn in AcbC than their non-preferring ANA counterparts. They also display lower maximal DA overflow and faster re-uptake in response to stimulation of the dopaminergic axons in the same region of the striatum. This suggests that there is a link between differential α-syn expression, dopaminergic neurotransmission and genetic predisposition to drug abuse and alcoholism. It also appears that ethanol does not modulate pre-synaptic mechanisms of DA signaling directly.

2) The lack of α-syn in mice can result in dysregulation of cholinergic signaling at the neuromuscular junction, but there is no definite evidence that it could significantly affect the motor performance of these animals. However, lack of α-syn does appear to impair motor learning.

3) Lack of α-syn does not affect the initiation or propagation of CSD, suggesting that the native function of α-syn does not include facilitation of neuronal excitability.

4) Locally applied exogenous human a-syn affects DA overflow, and the effects depend on the presence of endogenous a-syn.

Taken together, it can be concluded that α-syn can affect dopamine release and re-uptake in striatum with various mechanisms that can oppose each other, but in the normal physiological environment and level of expression, the net outcome of the effects is reduced release and accelerated re-uptake. Elevated expression of the protein in specific areas of the reward circuitry of the brain may function as a predisposing factor to drug and ethanol abuse, as shown by the high expression of α-syn in the AcbC of AA rats associated with decreased peak DA overflow and facilitation of DA re-uptake. Even though α-syn can modulate such behaviors as ethanol preference or motor learning, the protein's role is only to fine-tune neurotransmitter signaling, as the slight modulation of the neuromuscular junction in skeletal muscles yields little effect on motor performance and α-syn is unable to influence a phenomenon as strong as CSD which produces massive depolarization in the cortex.

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Anssi Pelkonen

Alpha-Synuclein as a Regulator of Synaptic Signalling

Alpha-synuclein is a small pre-synaptic protein, best known for its aggregation into Lewy bodies in Parkinson's disease, but it is also up-regulated in addiction. This thesis examined how alpha-synuclein influences synaptic signalling. It appears that the main physiological function of alpha-synuclein is to regulate dopamine signalling in the brain. At the behavioral level, the synaptic modulation by alpha-synuclein can affect functions such as preference for substances of abuse and motor learning.

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