Universidade do Algarve

Parkinson pathology propagation and long-distance transport in neurons

Joana Manuel Portela Domingues

Dissertação:

Mestrado em Ciências Biomédicas

Trabalho efetuado sob a orientação de:

Jia-Yi Li, MD., PhD.

Inês Araújo, PhD.

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Cover description: BiFC signal of rat brain section AAV-injected with human α-sinuclein linked to Venus fluorescence protein.

Parkinson pathology propagation and long-distance transport in neurons

Declaration of authorship of the work:

I hereby declare to be the Author of this work, which is original and unpublished. Authors and works consulted are properly cited in the text and listed in the list of references included.

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RESUMO

A doença de Parkinson é a segunda doença neurodegenerativa mais comum, afectando 1% da população acima dos 55 anos de idade [5]. A agregação da proteína α-sinucleína tem sido associada à sua etiologia. Em termos patológicos a doença de Parkinson é caracterizada pela perda progressiva de neurónios dopaminérgicos nigrostriatais e a presença de inclusões de proteína predominantemente compostas por α-sinucleína fibrilar, denominados corpos de Lewy, em neurónios dopaminérgicos. Apesar de os tratamentos atuais poderem melhorar os sintomas da doença de Parkinson, não há terapias disponíveis que retardem ou parem a progressão da doença. Desde 1997, quando foi descoberto que uma mutação *missense* no gene da α-sinucleína causa a forma familiar desta doença, o papel da α-sinucleína na etiologia e patologia tem sido extensivamente estudado. O desenvolvimento e caracterização de modelos animais baseados na α-sinucleína são passos cruciais para compreender a patogénese da doença, bem como para fornecer uma estrutura para testar estratégias terapêuticas. Neste estudo, e com o objectivo de estudar a propagação da patologia da doença de Parkinson, co-injectamos vírus adeno-associados que codificam α-sinucleína combinada com o N- ou C-terminal da proteína fluorescente Vénus em regiões do cérebro de ratos afectados na doença de Parkinson, tais como o córtex motor (Grupo CX), corpo estriado (Grupo ST) e na *substantia nigra* (Grupo SN) e descrevemos um novo modelo roedor basedo num vector viral com a capacidade de detetar e rastrear a agregação de α-sinucleína *in vivo*. Para este efeito, foi utilizada a técnica de complementação de fluorescência bimolecular. A co-injecção viral resultou em sinal Venus difundido na via corticoespinhal (Grupo CX) e na via nigroestriatal (Grupo ST e SN), incluindo nos corpos celulares nos locais de injecção e acumulação sináptica, sugestiva da formação de oligómeros de α-sinucleína. Os animais injectados com α-sinucleína apresentaram inchaços axonais. No entanto, não houve perda significativa de neurónios na *substantia nigra* do Grupo SN e nem perda significativa de terminais nervosos no corpo estriado do Grupo SN e ST. De acordo com estes resultados, ambos os grupos também não apresentaram nenhum comprometimento comportamental ou sinais de neuroinflamação. Desenvolvemos um modelo animal que fornece uma ferramenta para a pesquisa da doença de Parkinson, permitindo a deteção direta de oligómeros de α-sinucleína *in vivo* e consequentemente, o conhecimento que advirá, será essencial para o estudo desta patologia assim como para o desenvolvimento de novas estratégias terapêuticas para a intervenção na doença de Parkinson.

ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting 1% of the population over 55 years of age [5]. Alpha synuclein (α-Syn) aggregation has been closely linked to its aetiology. Pathologically PD is characterized by the progressive loss of nigrostriatal dopaminergic neurons and the presence of protein inclusions predominantly composed of fibrillar α-Syn termed Lewy bodies within dopaminergic neurons. Although current treatments can ameliorate the symptoms of PD, there are no available therapies that would slow-down or stop the progression of the disease. Since 1997, when it was discovered that a missense mutation in the gene for α-Syn caused familial PD, the role of α-Syn in the aetiology and pathology has been extensively studied. Development and characterization of α-Syn-based animal models are crucial steps to understand pathogenesis of the disease, as well as to provide a framework to test therapeutic strategies. Here, and with the main goal of study the Parkinson pathology propagation, we coinjected adeno-associated virus (AAV) vectors encoding α-Syn fused to either the N- or C-terminal of Venus in rat brain areas affected in PD such as motor cortex (CX group), striatum (ST group) or *substantia nigra* (SN group) and describe a novel viral vector rodent model with the ability to directly detect and track α-Syn aggregation in vivo. For this purpose we used the bimolecular fluorescence complementation (BiFC) assay. Viral coinjection resulted in widespread Venus signal within the corticospinal tract (CX group) and within the nigrostriatal pathway (ST and SN groups) including cell bodies in the injection sites and synaptic accumulation, suggestive of α-Syn oligomers formation. Transduced rats showed axonal swellings. However, there was no significant neuron loss in the *substantia nigra* of SN group and no significant loss of nerve terminals in the stiatum of SN and ST group. Concordant with this, both groups also did not exhibit any behavioural impairment or signs of neuroinflammation. We have developed an animal model that provides a tool for Parkinson's disease research allowing the direct detection of α-Syn oligomers in vivo and ultimately, the knowledge coming from it will be essential for the study of this pathology as well as for the development of novel therapeutic strategies for intervention in PD.

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ABBREVIATIONS

α-Syn – alpha synuclein

AAV – adeno associated virus

BiFC – bimolecular fluorescence complementation assay

BRET – bioluminescence resonance energy transfer

BSA – bovine serum albumin

Bzip – basic leucine zipper

CNS – central nervous system

CX – cortex

DA - dopamine

DAB - diaminobenzidine

DLB – dementia with Lewy bodies

DNA - deoxyribonucleic acid

DPX – distyrene, plasticizer, xylene

EEC – European Ethical Committee

FRET – fluorescence ressonance energy transfer

GFAP – glial fibrillary acidic protei

GFP – green fluorescent protein

GPint – globus pallidus internal segment

IBA1 – ionized calcium-binding adapter

LB - Lewy Bodies

LN – Lewy Neurites

LRRK2 – leucine rich repeat kinase 2

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

PBS – phosphate-buffered saline

PD- Parkinson's Disease

PDD – Parkinson's Disease with dementia

PDGFβ – platelet derived growth factor

PINK1 – phosphatise-induced novel kinase 1

PPIs – protein-protein interactions

RNA - ribonucleic acid

S129 – serine 129

SD – standard deviation

SNpc – *substatntia nigra pars compacta*

SNpr – *substantia nigra pars reticulata*

SNARE – soluble NSF(N-ethylmaleimide-sensitive factor) attachment protein receptor

SNCA - synuclein, alpha (non A4 component of amyloid precursor)

ST – striatum

SV2 - pAAV2-hSyn-Venus2CPart-WPRE (SV2)

TH – tyrosine hydroxilase

TX – Triton X

V1S - pAAV2-Venus1NPart-linker-hSyn-WPRE

WPRE – woodchuck hepatitis virus post-transcriptional regulatory element

WT – wild type

Chapter 1. INTRODUCTION

Parkinson`s disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder that was originally described by James Parkinson in 1817 [6]. It is the second most common neurodegenerative disease, affecting 1% of the population over 55 years of age[5]. PD belongs to a family of diseases called synucleinopathies, which also includes dementia with Lewy bodies (DLB) and PD with dementia (PDD) [7]. Synucleinopathies are neurological disorders, which although differing significantly in their symptomatic presentation, have in common α-synuclein (α-Syn) aggregation and neuronal degeneration [8, 9]. Bradykinesia, resting tremor, rigidity, and postural instability are the major characteristic motor symptoms of PD. The nigrostriatal pathway is the one mainly affected in PD giving rise to the motor symptoms, since it is the main dopaminergic pathway in the brain which accounts for ~75% of the dopamine in the brain [39] being involved in the coordination of movement. There have been reports of an early impairment in olfactory function, before occurrence of the motor symptoms in PD [10, 11]. In addition, many patients with PD have a loss of sympathetic innervation to the heart giving rise to cardiac dysfunction [12]; gastrointestinal disturbance, such as constipation, are a common early symptom [13]. As the disease progresses, other non-motor symptoms such as cognitive deficits and depression often occur [14, 15]. Other non-motor symptoms include sleep disorders, fatigue, psychosis and sexual dysfunction [16, 17]. (Fig.:1.1)

Parkinson's Disease: A sporadic and familial disease

There are two major forms of PD: idiopathic (sporadic) and hereditary (familial). Most PD cases are idiopathic, with unknown genetic component. Only about 5–10% of PD patients have familial patterns of inheritance [18]. A direct genetic connection between α-Syn and PD has been established by evidence that three missense point mutations (A30P [19], E46K [20], and A53T [21]) and multiplications of the gene locus (non A4 component of amyloid precursor) (SNCA), which encodes α-Syn, underlie autosomal dominant forms of PD [22]. Importantly, genome-wide association studies have shown clearly that SNCA is also linked to sporadic PD [23]. The aggregation of α-Syn into fibrillar assemblies in nerve cells is a molecular hallmark of the disease [24]. Unfolded monomers interact to form unstable dimmers, which grow slowly to generate oligomers of different morphologies that eventually convert to fibrils [2]. Moreover α-

Syn is found aggregated in Lewy bodies (LB) and Lewy neurites (LN) in both familial and idiopathic cases of PD. LB appear as cytoplasmic inclusions while LN correspond to abnormal neurites that contain filaments similar to those found in LB [8].

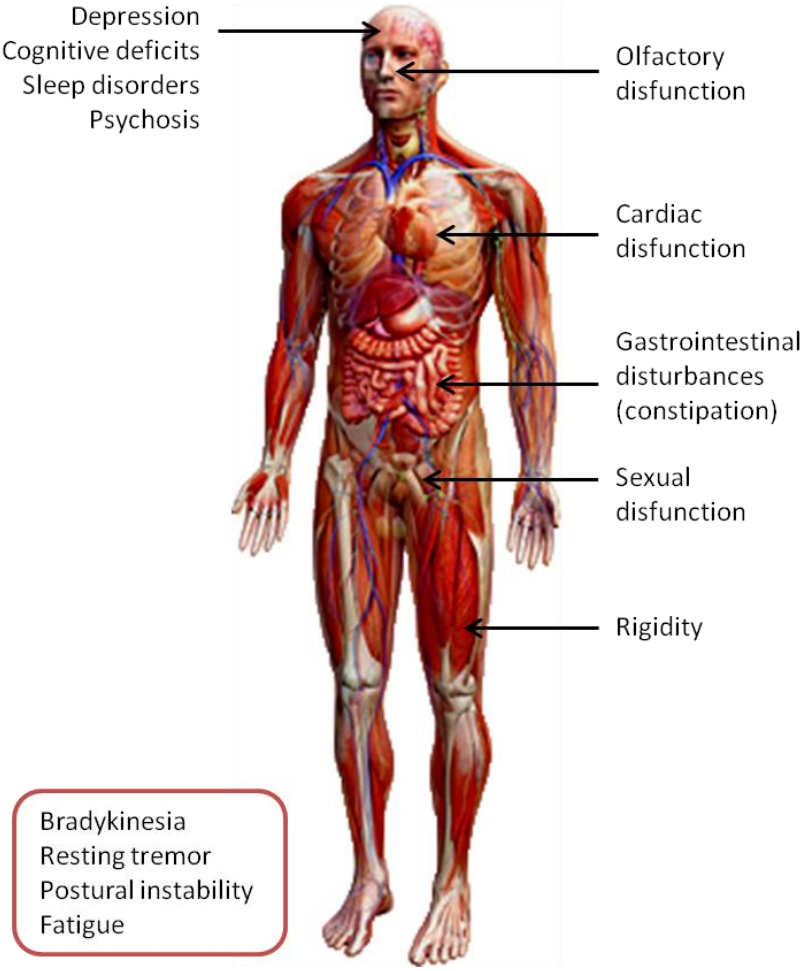


Fig.: 1.1 - **Representative schematization of the Symptomatology of Parkinson's Disease**. (Adapted from "3DScience")

The etiology of the idiopathic PD remains unknown so far. Aging is the major risk factor linked to the development of the disease but a combination of lifestyle, environmental and genetic factors have also been implicated. Epidemiological studies had also revealed several risk factors for developing idiopathic PD, including exposure to pesticides, herbicides, and some industrial chemicals [25].

In sporadic cases of PD, increased levels of α-Syn might be a consequence of impaired clearance of the protein. α-Syn is normally cleaned by the autophagy/lysosomal and ubiquitin proteasome systems [26-30], and defects in these systems have been detected in patients with sporadic PD [31, 32]. Elevated oxidative

and metabolic stresses are thought to contribute to the pathogenesis [33]. Impaired mitochondrial complex I can cause an increase on the concentration of reactive oxygen species, which can accelerate α-Syn aggregation [34, 35]. An impaired ubiquitin-proteasome degradation system may also play a role in initiating PD pathogenesis [33]. Increased misfolding of proteins due to oxidative damage could overwhelm and inhibit the proteasome degradation machinery. At the end, inefficient degradation may cause the accumulation of α-Syn and, by elevating α-Syn concentration, there is an increase in the rate of α-Syn aggregation [29].

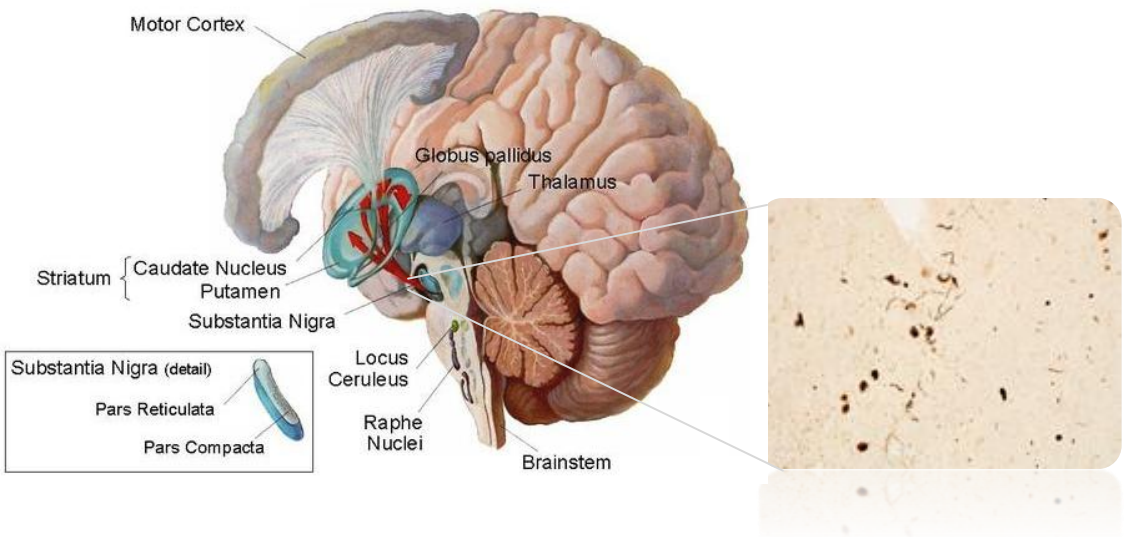


Fig.: 1.2 - **Brain regions affected in Parkinson's disease and Lewy Bodies in PD human brain tissue. Immunohistochemistry for α-Syn (right) shows the presence of LBs in the SN.** (Adapted from "Diseasespictures")

The major neuropathological hallmarks of PD are degeneration and cell death of dopaminergic neurons [9]. On a cellular level, PD is characterized by the formation and accumulation of proteinaceous intraneuronal inclusions, known as LB, in the perikarya, dendrites, and axons of dopaminergic nerve cells in the *substantia nigra pars compacta* (SNpc) as well as other regions of the central and peripheral autonomic nervous systems [36, 37]. (Fig.:1.2) LBs consist of a granular core that includes a wide variety of nitrated, phosphorylated, and ubiquitinated proteins surrounded by a filamentous halo primarily comprised of neurofilaments and α-Syn [36]. While initially thought to be toxic and contribute to neurodegeneration, LB might in fact represent a form of aggregosome that develops in response to increased levels of misfolded proteins to segregate and facilitate the clearance of these potentially toxic proteins [38].

However, identification of the toxic α-Syn species is still a matter of intense investigation in the scientific community.

α-Synuclein structure, function and localization

α-Syn is an abundant and highly conserved neuronal protein of 140 aminoacids that, under physiological conditions, is found mainly in neuronal presynaptic terminals in proximity to synaptic vesicles. It is a member of a conserved family of proteins that also includes β-synuclein and γ-synuclein, and was originally described as the precursor protein for the non-amyloid component of Alzheimer's disease senile plaques [39].

α-Syn is constituted by a positively charged N-terminal region (residues 1-60), a hydrophobic NAC region (residues 61-95) and a negatively charged C-terminal region (residues 96-140). (Fig.:1.3) The N-terminal half of α-Syn contains seven 11-amino acid repeats with a highly conserved motif KTKEGV. This domain is responsible for protein-protein interactions (PPIs) and for forming structural apolipoprotein-like class A2 amphipathic α-helical by binding of α-Syn to phospholipid vesicles [40]. The central hydrophobic NAC region of α-Syn is highly amyloidogenic and this confers the capacity to undergo a conformational change from random coil to β-sheet structure, protofibrils and fibrils. The acidic C-terminal region remains unfolded and does not associate with vesicles. This region contains serine 129 (S129) and tyrosine 125 (Y125) residues that can play a role in α-Syn fibrillization [41].

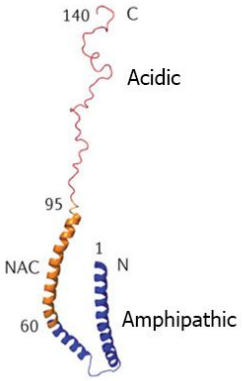


Fig.: 1.3 – **α-Syn structure and domains.** (Adapted from Lashuel et al. 2013 {Lashuel, 2013 [2]})

The protein is intrinsically unfolded, which means that in the purified form at neutral pH it lacks an ordered secondary or tertiary structure. Upon binding to membranes or synthetic vesicles containing acidic phospholipids, however, it assumes an α-helical structure [42]. α-Syn oligomers are known to bind and permeabilize vesicles composed of negatively charged lipids [41].

Recombinant α-Syn incubated under certain conditions in vitro assumes an oligomeric conformation and is gradually converted to β-sheet-rich, fibrillar structures that resemble the LB and neurites found in human neuropathological samples. This process is termed aggregation and is thought to underlie the toxic potential of α-Syn [42].

Although the precise functions of α-Syn remain uncertain, the preferential localization to presynaptic nerve terminals and its interaction with phospholipids and proteins suggests regulatory functions associated with synaptic activity, dopamine metabolism and lipid vesicle trafficking [40, 43-48], having a stabilising effect on complexes of soluble NSF(N-ethylmaleimide-sensitive factor) attachment protein receptor (SNARE) family proteins [49-52]. (Fig.:1.4) The stabilising effect of α-Syn on the SNARE-complex assembly is made by a non-enzymatic mechanism that involves simultaneous binding of α-Syn to phospholipids via its N-terminus, and to synaptobrevin-2 via its C-terminus [46].

Relative loss of α-Syn function, for example by sequestration of α-Syn in LB or by increased truncation of α-Syn during aging, may thus contribute to neurodegenerative diseases such as PD and LBD.

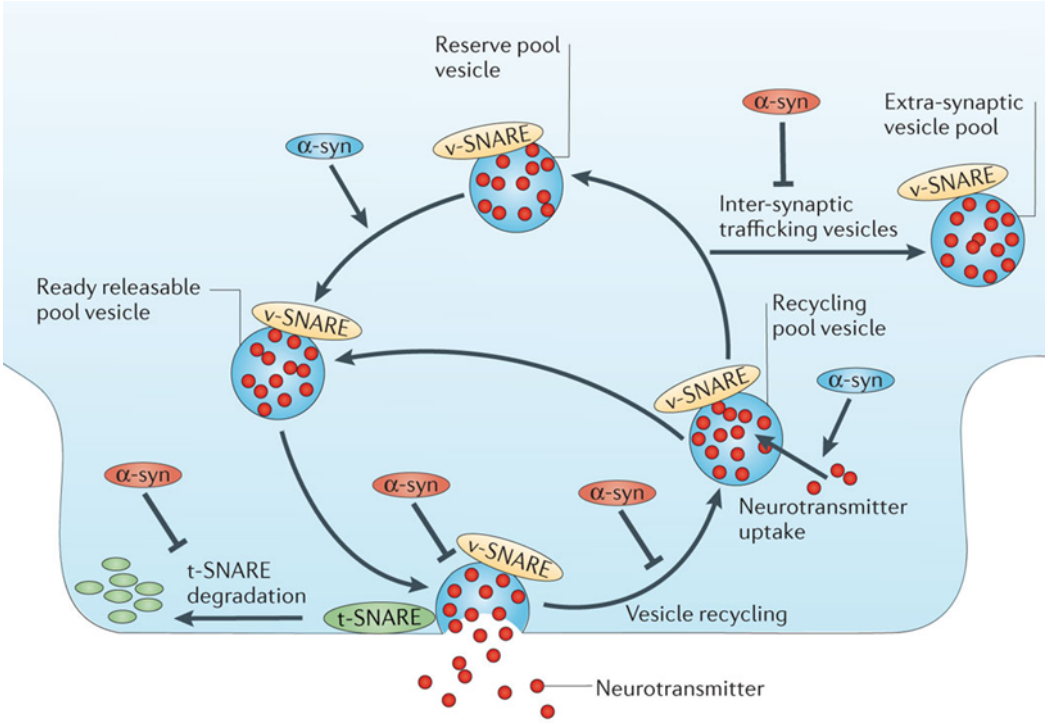


Fig.: 1.4 - **Function of α-Synuclein**. α-Syn has key role in the regulation of neurotransmitter release, synaptic function and plasticity. At the pre-synaptic terminal it exerts its function in the regulation of veside trafficking and veside refilling, besides interactions between target-associated SNARE and veside-associated SNARE proteins and neurotransmitter release. In case of accumulation of α-Syn, occurs an impairment of these mechanisms. (Adapted from Irwin.DJ,.et al., 2013 [2])

α-Synuclein in PD (and other synucleinopathies)

The molecular mechanisms by which α-Syn aggregation contributes to neurodegeneration, the nature of the toxic forms of α-Syn and the cellular pathways that are affected by α-Syn remain unknown. An increasing body of evidence from animal models as well as data from genetic, biochemical and biophysical studies sustain the hypothesis that the processes of α-Syn oligomerization [53], and fibril growth [54, 55] have central roles in the pathogenesis of PD and other synucleinopathies [56]. In addition, it is possible that the α-Syn monomers might also have a role in synucleinopathies by their displacement from their physiological location, resulting in a loss of cellular function, or by disrupting the activity of other molecular or signalling pathways [57].

There are a number of different α-Syn conformers, including oligomers, protofibrils and fibrils, which have been associated with the pathogenesis [58, 59]. The fibrillar forms of α-Syn are detected mostly in LB [53, 60, 61] and are thought to reflect an attempt by the neurons to isolate and/or convert toxic α-Syn oligomers to fibrils, which are stable, less dynamic structures that exhibit reduced toxicity. In contrast to fibrillar α-Syn, oligomeric aggregates are most likely to be located in axons and presynaptic terminals, where they might damage synapses and dendrites [58, 62-66], and destabilize cytoskeletal units which in turn might accelerate the formation of α-Syn oligomers and further cytoskeletal disruption resulting in neuritic degeneration [39]. Spherical oligomers 2–6 nm in diameter may be the toxic forms of α-Syn, as they promote abnormal calcium currents and neuronal degeneration in cultured primary neurons [67]. There is still the possibility that α-Syn oligomerization may alter the distribution of functional forms of monomeric α-Syn or result in sequestration into non-functional oligomeric forms, thus resulting in partial loss of α-Syn function [68]. Or even, the native or misfolded forms of the monomeric protein may contribute to α-Syn toxicity and PD pathogenesis via aggregation-independent mechanisms, including aberrant interactions with membranes, proteins and small molecules, retention in specific cellular compartments, and disruption of specific cellular processes. (Fig.:1.5)

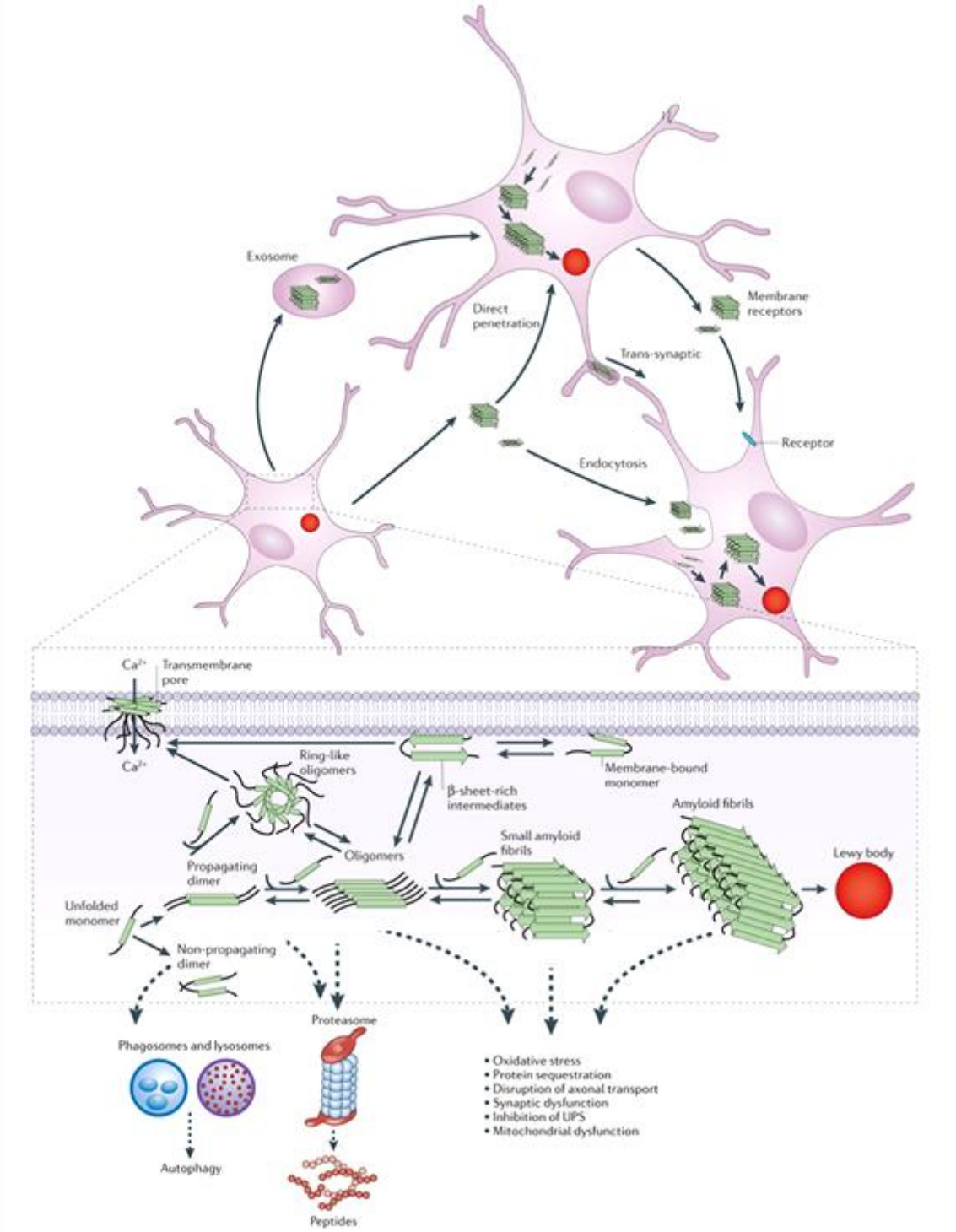


Fig.: 1.5 - **Mechanisms of α-Synuclein aggregation, propagation and clearance**. α-Syn aggregation can occur in the cytoplasm or with the cellular membrane. In the cytosol, unfolded monomers form dimers, which grow to generate oligomers — including transient spherical and ring-like oligomers — that convert to fibrils. The accumulation of amyloid fibrils leads to the formation of Lewy bodies. Membrane-bound monomeric α-Syn adopts a mostly α-helical conformation, but at high concentrations it undergoes a conformational change to form membrane-bound β-sheet-rich structures that form oligomers. During α-Syn fibrillogenesis and aggregation, the intermediate species (oligomers and amyloid fibrils) are highly toxic, affecting mitochondrial function, endoplasmic reticulum-Golgi trafficking, protein degradation and/or synaptic transmission, inducing neurodegeneration. Quality-control systems (chaperones, ubiquitin proteosomes and phagosome-lysosome systems) that prevent or reverse protein misfolding or eliminate misfolded proteins are overwhelmed by oligomeric species of α-Syn. α-Syn oligomers and fibrils, as well as the monomers, can be transferred between cells and induce disease dissemination to other brain regions. Spreading mechanisms can occur via endocytosis, direct penetration, trans-synaptic transmission or membrane receptors. Once inside the host cells, α-Syn aggregates can nucleate aggregation and propagate. (Adapted from Irwin, D.J., et al., 2013 and Lashuel, HA., et al., 2013 [2, 3])

Toxicity of particular α-Syn species has been debated but not yet resolved in a satisfactory manner. In any case the levels of α-Syn in the central nervous system (CNS) depend on the balance between the rates of α-Syn synthesis, aggregation and clearance. The ubiquitin proteasome system and the autophagy-lysosome pathway, (which involves microautophagy, macroautophagy, and chaperone-mediated autophagy) are the two major quality-control systems postmitotic neurons use to maintain intracellular proteostasis [69]. An imbalance between these mechanisms, caused by dysfunction of one or more of these pathways, can result in abnormal levels of α-Syn that might favour the formation and/or accumulation of oligomeric and fibrillar species. (Fig.:1.5) Whether accumulation of α-Syn precedes the impairment of autophagic pathways or vice versa remains as well unclear [39]. Damage to proteasomal and lysosomal systems could cause α-Syn to accumulate, as well as the increased levels of α-Syn could inhibit the proteasome and lysosomal systems, ultimately leading to the formation of oligomers and aggregates with resulted neurodegeneration and clinical dysfunction [70]. Additionally α-Syn might suffer a conformational transition from an α-helix–rich structure to one high in β-sheet structure. If such a stochastic process were to escape physiological control by the protein clearance systems, this could result in increased levels of misfolded protein, which could then act as a template to promote misfolding of native wild type α-Syn and a prion-like chain reaction leading to neurodegeneration [36].

Protein post-translational modifications are fairly significant in the regulation of the protein structure and function. These modifications result in alterations in protein size, structure, charge, and folding, which may interfere with their activity, binding affinity and hydrophobicity of proteins [71]. Protein phosphorylation is the most studied and considered the most important post-translational modification. Data from immunohistochemical and biochemical studies demonstrated that most of α-Syn within LB, isolated from PD patients and others synucleinopathies, is phosphorylated at Serine 129 (S129). Protein phosphorilation of α-Syn at S129 is believed to be intrinsically linked to PD pathogenesis [72-74]. In addition, the detection of phosphorylated S129 has been one of the key criteria to identify LB in human brains as well as in animal models [72, 74]. Phosphorylation play a role in modulation of α-Syn aggregation, LB formation and toxicity in vivo [73].

Prion-like hypothesis of spread of pathology in PD

Recent studies highlighted possible similarities in mechanisms underlying the propagation of α-Syn with that of prions [75, 76]. The fundamental event in the biology of a prion is a conformational transition that converts the normal cellular protein to a misfolded isoform. These, in turn, polymerize into oligomers and amyloid fibrils that coalesce into plaques and cause neurodegeneration [36, 77].

Under physiological conditions, α-Syn has been traditionally considered to be an exclusively intracellular synaptic protein. However, evidence suggests that under pathological circumstances, toxic α-Syn oligomers could be eliminated from neurons via unconventional secretory mechanisms [78-80].

Abnormal deposition of α-Syn occurs early in the disease process, and seems to follow a sequence of ascension from lower brainstem centres to limbic and wide cortical association areas [81]. LB pathology propagates in a sequential and predictable fashion, beginning in the olfactory system, peripheral autonomic nervous system, and dorsal motor nucleus of the vagus; extending to involve dopamine neurons of the *SNpc* in the mid stage of the disease; and affecting the cerebral hemispheres in the later stages of the illness [82]. (Fig.:1.6) Lewy pathology affects norepinephrine neurons of the locus coeruleus, cholinergic neurons of the nucleus basalis of Meynert, serotonin neurons of the median raphe, and specific sets of nerve cells in the olfactory system, neocortex of the cerebral hemispheres, upper and lower brainstem, spinal cord, and peripheral autonomic nervous system [83]. It has been hypothesised that this pathology can be initiated and propagated by an unknown pathogen, aggregated form of α-Syn being the major candidate [4, 36].

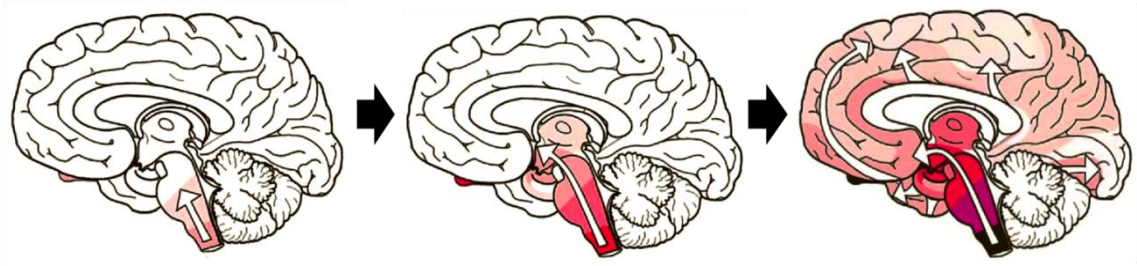


Fig.:1. 6 – **Lewy Body pathology propagation.** The first lesions appear in the olfactory bulb, anterior olfactory nucleus, and dorsal motor nucleus of the vagus nerve. From there, the pathology follows a predominantly ascending path affecting the locus coeruleus, gigantocellular reticular nucleus, and caudal raphe nuclei. The pathology continues its ascent and reaches the central subnucleus of the amgygdala, the cholinergic nuclei of the basal forebrain, and the pars compacta of the substantia nigra. The cerebral cortex becomes involved in a later stage, commencing with the anteromedial temporal mesocortex. The higher order association areas of the neocortex become involved later, followed by the first-order association areas and primary fields. Growing severity of the lesions is shown by increasing degrees of shading (red, violet, black (Adapted from Braak, H., et al., 2003) [4]

There is also an increasing body of evidence showing the transfer of α-Syn between cells and propagation of the disease both *in vitro* and *in vivo*. *In vitro* studies demonstrate that α-Syn monomers and aggregates can be secreted from affected neurons via exocytosis and taken up by unaffected neurons via endocytosis [84, 85]. Brains from PD subjects transplanted with fetal mesacenphalic dopaminergic neurons exhibit that the disease can propagate from host brain to grafted cells [86].

Pathways leading to the release of toxic α-Syn oligomers include exocytosis in clear vesicles [85], exosomal release [79, 87], and penetration [88, 89] from the donor cell membrane. Interestingly, these extracellular α-Syn aggregates can then transfer from neuron to neuron or from neuron to glial cell [85], where they can nucleate further intracellular aggregation and/or trigger neuroinflammation to exacerbate the neurodegenerative process [90, 91].

The mechanisms through which extracellular α-Syn oligomers transfer to other cells include endocytosis [92], direct penetration [93], trans-synaptic dissemination [79] and membrane-receptor-mediated access [85]. (Fig.: 1.6) Once inside the recipient cells, α-Syn oligomers could act as a seed for intracellular aggregation or the protein could be targeted for degradation. Although the exact process of intracellular oligomer and fibril propagation remains unknown, evidence from *in vitro* biophysical studies has consistently shown that fibrillization of α-Syn follows a nucleated polymerization mechanism [94]. Moreover an *in vivo* study has shown that inoculation of α-Syn transgenic mice with homogenates containing α-Syn protofibrils and fibrils results in enhancement of the α-Syn pathology and propagation [95]. These observations led once more to the hypothesis that extracellular α-Syn seeds might behave in a prion-like fashion.

Basal ganglia

The basal ganglia consist of a set of brain structures in the telencephalon, diencephalon, and mesencephalon. The forebrain structures comprise the caudate nucleus, the putamen, the nucleus accumbens (or ventral striatum) and the globus pallidus. Collectively, these structures are named the corpus striatum. The subthalamic nucleus is part of the diencephalon and it is placed under the thalamus. The *substantia nigra* is a midbrain structure, composed of two parts: the *pars compacta* (SNpc) and the *pars reticulata* (SNpr). The *substantia nigra* is sited between the red nucleus and

the cerebral peduncle on the ventral part of the midbrain. The *pars compacta* is the source of a dopaminergic pathway to the striatum. [96, 97]

The striatum is the major receiver of afferents to the basal ganglia [98]. These excitatory afferents arise from all cerebral cortex and from the intralaminar nuclei of the thalamus. The main output structures of the basal ganglia are the globus pallidus internal segment (GPint) and the SNpr. Both of these structures make GABAergic, inhibitory connections on their targets.

An essential pathway in the modulation of the direct and indirect pathways is the dopaminergic, nigrostriatal projection from the SNpc to the striatum. Direct pathway striatal neurons have D1 dopamine (DA) receptors, which depolarize the cell in response to DA. On the contrary, indirect pathway striatal neurons have D2 DA receptors, which hyperpolarize the cell in response to DA. The nigrostriatal pathway has the dual effect of exciting the direct pathway while inhibiting the indirect pathway. Because of this dual effect, excitation of the nigrostriatal pathway leads to the excitation of cortex by two routes, by exciting the direct pathway and inhibiting the indirect pathway.

The neurodegeneration in the nigrostriatal pathway results in the motor symptoms in PD [99]. Since the nigrostriatal pathway is both excitatory and inhibitory, the loss of this input tips the balance in benefit of activity in the indirect pathway. Therefore, the GPint neurons are abnormally active, maintaining the thalamic neurons inhibited. Without the thalamic input, the motor cortex neurons are not as much excited, and consequently the motor system lags behind to execute the motor plans in response to the patient's volition.

Animal models of PD

Development of relevant animal models of PD is crucial for elucidating the etiopathology of the disease as well as for evaluation of potential therapeutic targets. Several animal models are currently used; however, there are certain drawbacks in each particular model. A model representing all the PD hallmarks together with consistent symptomatology is still lacking.

The development and characterization of α-Syn-based animal models might bring important insights into PD-linked pathology [100]. There are several models, being the genetic and toxin-induced ones the most used. Numerous α-Syn-based

models including toxin-induced animal models, being in use in primates and rodents have been developed and characterized. Although these models can bring valuable insights into the disease pathology processes, they present several drawbacks. More recently, adeno associated virus (AAV)-based models were developed and, compared to the previous models they bring some advantages.

Neurotoxin Models

6-hydroxydopamine (6-OHDA) induced model of PD was first used in 1968 [101]. Injection of this toxin in the SNpc results in about 60% tyrosine hydroxilase (TH)-neurons cell loss in the midbrain dopaminergic system, with subsequent loss of TH-positive terminals in the striatum [102], however 6-OHDA does not produce or induce proteinaceous aggregates or Lewy-like inclusions like those seen in PD.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is used in primates [69] and mice [103], however cannot be used in rats since they are found to be resistant to this toxin [104]. It replicates almost all of the hallmarks of PD in monkeys, however it does not induce formation of LB [105].

Pesticide/herbicide Models

Paraquat (N,N-dimethyl- 4–4–4-bipiridinium) is a herbicide. It induces its effects throughout oxidative stress mediated by redox cycling, which in turn generates reactive oxygen species that result in the damage of lipids, proteins, DNA and RNA. It leads to an increase in α-Syn and presence of LB in DA neurons of the SNpc [105, 106].

Rotenone is both an herbicide and an insecticide. It seems to replicate almost all of the hallmarks of PD including complex I blockade, behavioural alterations, inflammation, α-Syn aggregation, Lewy-like body formation, oxidative stress and gastrointestinal problems [107]. However, it does not cause major depletion of DA in the nigrostriatal system [108].

Genetic Models

Although genetic mutations in PD are rare and represent only about 10% of all PD cases [1], animal models of these mutations [α-Syn and LRRK2 (leucine rich repeat kinase 2), autosomal dominant PD, and PINK1 (phosphatase- induced novel kinase

1)/Parkin and DJ-1, autosomal recessive PD] are important as they represent potential therapeutic targets. Moreover, in the case of α-Syn there is evidence from genome-wide association study linking variations in SNCA gene with higher risk of PD [109, 110] and it is also known that aggregation of α-Syn is present in sporadic PD [61].

Nowadays, there are several transgenic mouse models, for the wild type or the mutated α-Syn, under promoters strictly neuronal or not strictly neuronal. In 2000 the first α-Syn knockout mice was created [111]. In 2002 it was shown for the first time that α-Syn knockout mice exhibits DA neuron loss in the presence of MPTP [112]. In 2000 Masliah and co-workers developed the first α-Syn transgenic mice, using a platelet derived growth factor (PDGFβ) promoter [113]. One year later the [A53T] α-Syn [114] and [A30P] α-Syn [115] transgenic mouse models were created. Up to date [wt] α-Syn or a double-transgenic [A53T, A30P] α-Syn mouse models are also available for the study of PD [116].

Most of the rat models of PD have been developed based on the connection between α-Syn and PD. However the first PD rat models employed neurotoxins such as 6-OHDA or MPTP, that acutely degenerate DA neurons in *substantia nigra* but don not outcome a significant α-Syn pathology [101]. Models using viral vectors to target α-Syn expression in the *substantia nigra* were recently developed. Those α-Syn-overexpressing rat models show neuronal loss and LB inclusion formation [100].

Overexpression of α-Syn in transgenic rodents can lead to loss of nigral dopaminergic neurons and the accumulation of α-Syn aggregates [113]. Finally, overexpression of α-Syn via gene delivery to the region of the SNpc leads to the formation of inclusion bodies immuno-positive for α-Syn, degeneration of dopamine neurons, and parkinsonian motor disturbances in both rats and monkeys [117]. Those results, with accompanied dopaminergic cell loss, resemble more closely the chronic pathology of PD [118-121].

Despite all the advantages of these models, the detection of α-Syn oligomers is based on indirect approaches, without the possibility of direct *in vivo* detection. Recently a new rat PD model, which enabled the direct detection and visualization of α-Syn oligomers along the nigrostriatal pathway by using bimolecular fluorescence complementation assay (BiFC), was presented [122]. BiFC assay is based on the chemiluminescence signal from fluorescent protein conjugates which enable the detection and imaging of α-Syn oligomers *in vivo*.

Bimolecular fluorescence complementation (BiFC)

Protein-protein interactions (PPIs) play significant roles in various biological processes. To study PPIs, several methods, such as canonical yeast two-hybrid assay, in vitro pull-down assay, in vivo immunoprecipitation assay, fluorescence resonance energy transfer (FRET) assay, bioluminescence resonance energy transfer (BRET) assay, and BiFC assay, have been used [123].

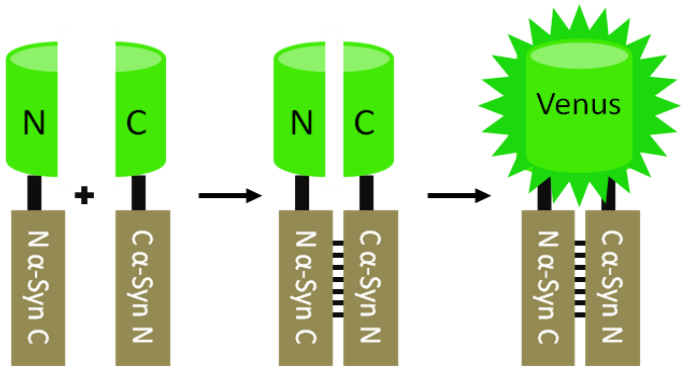


Fig.: 1.7 - Schematic representation of the BiFC assay. A part of Venus protein is fused with N-terminal of α-Syn and the other part is fused with C-terminal of α-syn. When two complementary non-fluorescent fragments are brought together due to α-Syn dimerization, the fluorophore is reconstituted.

Over the past decade, BiFC has emerged as a key technique to visualize PPIs in a variety of model organisms. The BiFC assay is based on structural complementation of an intact fluorescent protein when two complementary non-fluorescent fragments are brought together by a pair of interacting proteins [123]. (Fig.:1.7) When the proteins interact in a suitable orientation and conformation, the fluorescent protein is non-covalently reconstituted and fluorescence is generated. If the proteins do not interact, or do not do so in the appropriate orientation, no fluorescence is emitted [124]. BiFC was introduced in 2002 to study interactions between basic leucine zipper (Bzip) and Rel family transcription factors in cellular environment using the COS-1 cell line [125]. Since then, BiFC has been used successfully in different model organisms, such as mammalian cell lines, plants, nematodes, yeast, bacteria and even rodents [122, 126, 127]. One of the limitations of BiFC is its inability for visualizing dynamic interaction changes [128-130]. However, the fact that reconstitution of the fluorophore lead to stabilization of the protein complex turns out to be useful because it allows the selective enrichment of dimeric/oligomeric species. More precisely, for the study of neurodegenerative diseases, the stabilization of certain proteins could be useful because it enables the study of species that might be transient, such as those generated in the protein aggregation process. In the field of

PD, BiFC has been used to visualize α-Syn oligomers and to observe their modulation by HSP70 [128], to investigate the effect of CHIP on α-Syn oligomerization [128, 130]. Multicolor BiFC has been used to investigate dopamine D_2 and adenosine A_{2A} receptor oligomerization [131]. Very recently a new animal AAV-based model using the BiFC assay allowing the direct detection of α-Syn oligomers *in vivo* was developed [122].

In this project, taking into account the potential of this technique we used the BiFC assay in order to study α-Syn oligomers long-distance transport in neurons in vivo.

Objective

As outlined in the Introduction chapter, development and characterization of α-Syn-based animal models are crucial steps to understand pathogenesis of the Parkinson's disease. In recent years, studies have pointed to α-Syn oligomers as the toxic species of α-Syn [94, 132], playing a key role in PD pathogenesis. Previous studies have also shown the applicability of protein complementation assay to detect α-Syn oligomers in vitro [128-130, 133] and in vivo [122], providing a tool to dissect the role of α-Syn oligomers in PD. Thus, the major goal of this study was to develop and characterise a new animal model for detection of α-Syn oligomers *in vivo* as well as investigate the Parkinson pathology propagation. More specifically our aim was to study whether and how different forms of a-syn (especially oligomeric and fibril) are transported in neurons, whether transporting a-syn oligomers and fibrils can contribute to aggregation. To pursue this goal we overexpressed the human α-Syn in different brain regions affected in the PD, such as *substantia nigra*, striatum or motor cortex, using an AAV-mediated gene delivery of two constructs containing complementary halves of a fluorescent protein linked to human α-Syn. This approach enables the study of α-Syn oligomers formation and transport in neurons and neuronal pathways.

Chapter 2. METHODS

Virus preparation

The viral vectors pAAV2-Venus1NPart-linker-hSyn-WPRE (V1S) and pAAV2-hSyn-Venus2CPart-WPRE (SV2) were constructed by inserting the human *SNCA* gene, fused to either the N-terminus half of venusYFP (V1S) or the C-terminus half of venusYFP (SV2) (Fig.:2.1) into the EcoRV and NheI sites of the pAAV2-WPRE vector [122]. pAAV2-Venus-W PRE was constructed by inserting the venusYFP gene into the XhoI and NheI sites of pAA V2-WPRE vector. The expression of the transgene is driven by the synapsin-1 promoter and enhanced using a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). Briefly, transfer plasmids carrying AAV2 ITRs coding for a human wt α-Syn, downstream to a synapsin-1 promoter, were generated as described in [134]. The transfection into HEK 293 cells was carried out using the calcium-phosphate method, and included the packaging plasmids encoding AAV6 capsid proteins.

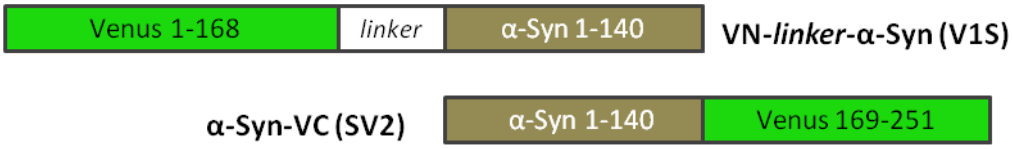


Fig.: 2.1 - **Schematic representation of the BiFC constructs**

Animals

Adult female Sprague Dawley rats, weighing 225-250 g at the time of surgery, were housed two to three per cage with *ad libitum* access to food and water through a 12h light/dark cycle. All procedures were approved and conducted in accordance with guidelines set by the Ethical Committee for the use of laboratory animals in the Lund-Malmö region and the European Ethical Committee (EEC). In order to assess whether and how oligomerized α-Syn transports throughout the CNS *in vivo* we set 3 experimental groups of animals:

- ✓ CX Group: AAV V1S+SV2 or Venus injection into the **motor cortex**
- ✓ SN Group: AAV V1S+SV2 or Venus injection into the ***substantia nigra***
- ✓ ST Group: AAV V1S+SV2 or Venus injections into the **striatum**

Surgical Procedure

All surgical procedures were performed under general anaesthesia using a 20:1 mixture of fentanylcitrate (Fentanyl) and medetomidin hypochloride (Dormitor) (Apoteksbolaget, Sweden) injected intra peritoneal (IP). Rats were placed in a stereotaxic frame (Stoelting), the surgical site was shaved and sterilized with ethanol and a 2 cm incision was made along the midline. Solution containing the AAV was injected using a 5 µl Hamilton syringe fitted with a glass capillary (outer diameter of 250 µm). The groups of animals injected in motor cortex, *substantia nigra* or striatum comprised 8, 17 and 23 rats, respectively.

AAV injection: 2µl of the pAAV2-Venus1NPart-linker-hSyn-WPRE (V1S) plus pAAV2-hSyn-Venus2CPart-WPRE (SV2) (mixture 1:1), or pAAV2-Venus-WPRE solution were infused at a rate of 0.2 µl/min and the needle was left in place for an additional 3 minute period before it was slowly retracted at a rate of 1mm per minute. Injection was carried out unilaterally on the right side, targeting the *substantia nigra* at coordinates: antero-posterior: - 5.3 mm, medio-lateral: -1.7 mm , dorso-ventral: -7.2 mm, the striatum: antero-posterior: +0.5 mm, medio-lateral: -3.5 mm , dorso-ventral: -4.8 mm, (Fig.:2.2) or the motor cortex: antero-posterior: +2.0 mm, medio-lateral: -1.8 mm , dorso-ventral: -1.5 mm below dural surface, with bregma as a point of reference. The viral concentration used was of $1,25*10^{13}$ gc/ml. The total viral load per injection (2µl)/animal was $2,5*10^{10}$ gc. Equal number of gc/ml were used for α-Syn (SV1+SV2) and control (Venus) AAV injection.

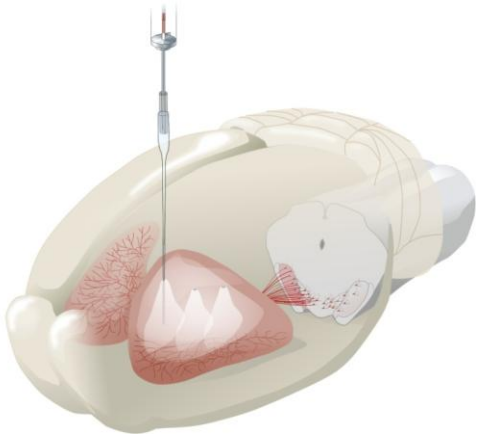


Fig.: 2.2- **Schematic representation of the AAV stereotaxic injection.** Stereotaxic injection of the AAV was performed targeting the striatum. (Courtesy of Itzia Ferrer)

Sciatic Nerve crush was performed bilaterally in the group of animals injected in the motor Cortex as described previously [135]. Briefly, animals were placed in prone position. After asepsis a 2 cm incision was made over the lateral aspect of the hind limb, muscles were separated in order to expose the sciatic nerve and then, the crush was done with a glass rod and silk suture (10 seconds each). The crush procedure included double crushes, with a distance of 1-2 mm between them. (Fig.: 2.3) Lastly the skin was sutured with (4-0) stitches.

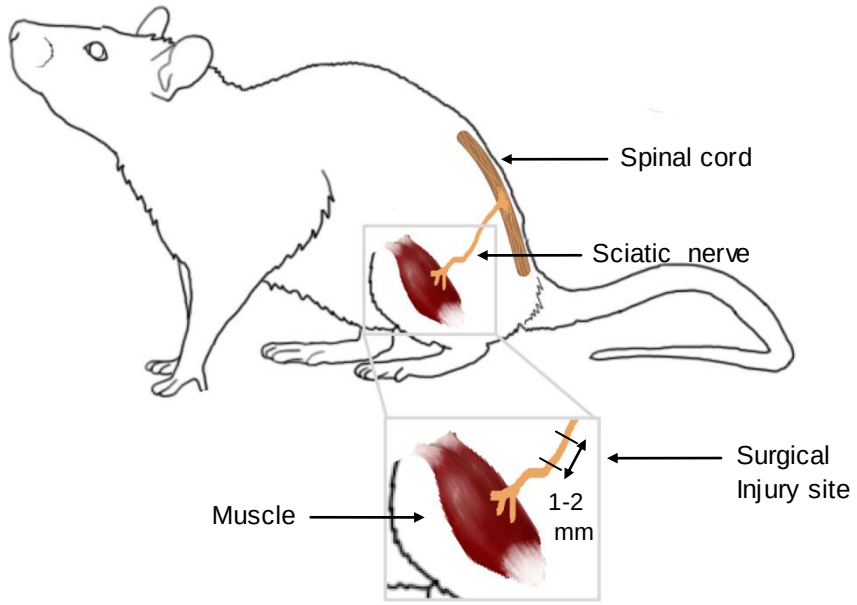


Fig.: 2.3 - **Shematic representation of the Sciatic nerve crush.**

Behavioural Testing

Assessment of behavioural function was performed in the groups of animals that were injected in *substantia nigra* and striatum 4, 8 and 12 weeks after injection of the viral vector using three different tests. Rats were food restricted and maintained at 85% free-feeding bodyweight throughout habituation and testing.

Corridor Task: Lateralised sensorimotor integration was measured using a task that was first established in 2005 [136]. At the first time point, rats were habituated to the corridor by scattering sugar pellets (40 mg; TestDiet) along the floor and allowing them to freely explore for 10 min on 5 consecutive days prior to testing. When testing began, fasted rats were first placed in an identical, but empty, corridor for habituation for 5 min,

before being transferred to one end of the testing corridor (Fig.: 2.4). The number of ipsilateral and contralateral retrievals made by each rat was counted and trials were terminated when the rat made a total of 20 retrievals or a maximum time of 5 min had elapsed. A 'retrieval' is defined as the animal poking its nose a pot, whether or not a pellet is eaten, and a new retrieval can only be made by investigating a new pot. Data are expressed as percentage of contralateral retrievals of the total retrievals made from both sides of the body axis.

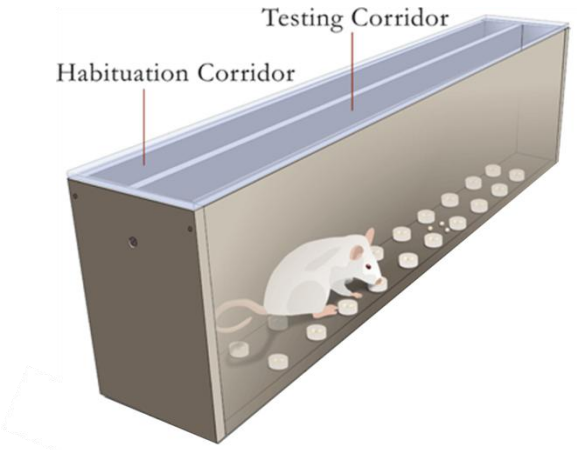


Fig.:2.4 - **Corridor Task.** A mouse is first placed into an identical but empty habituation corridor for 5 min prior to being placed at the start of the testing corridor. The testing corridor has adjacent pairs of pots that contain sugar. (Adapted from Grealish, S., et al., 2010) [1]

Cylinder Task: Forelimb use asymmetry during exploratory activity was analysed by videotaping rats. They were put in a transparent glass cylinder, a mirror was placed to the side of the cylinder at an angle to enable the recording of forelimb movements even when the animal was turned away from the camera (Fig.: 2.5A). Trials were terminated when the rat made a total of 20 forepaw touches or a maximum time of 5 min had elapsed. The number of ipsilateral and contralateral touches made by each rat was counted. Data are presented as a percentage of contralateral paw touches.

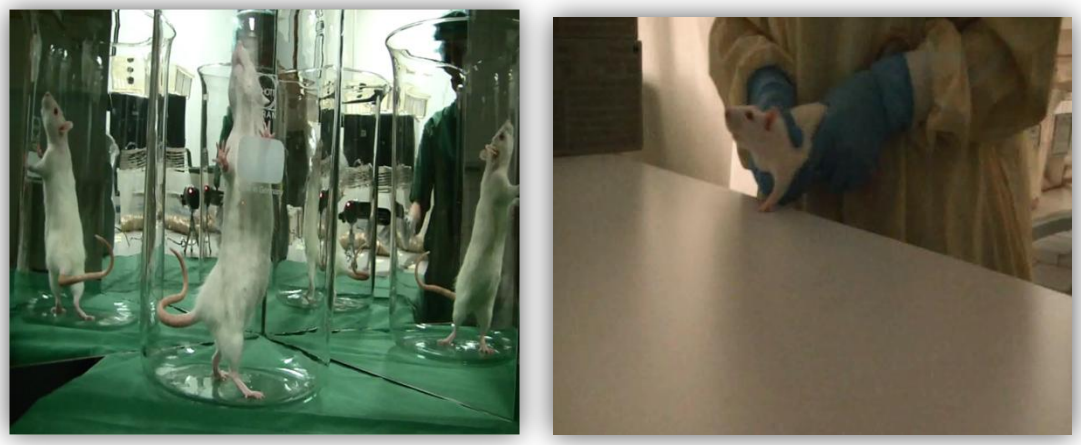


Fig.:2.5 – A) **Cylinder Task; B) Stepping Task.** A) A rat's forelimb use during exploratory activity. B) A rat being held by the experimenter fixing both hind limbs with one hand and the forelimb not to be monitored with the other hand.

Stepping Task: Forelimb akinesia was analysed videotaping rats performing the stepping task. On 3 days preceding the test, animals were handled by the experimenter to familiarise them with the test procedure. The test was performed on three consecutive days. Briefly, the rat was held by the experimenter fixing both hind limbs with one hand and the forelimb not to be monitored with the other [137], while the unrestrained forepaw was touching the table (Fig.: 2.5B). The rat was moved sideways along the table surface (90 cm in 5 s). This procedure was repeated three times for each forelimb. The number of adjusting steps in the forehand direction was counted. The mean of data obtained on three testing days constituted the final dependent variable.

Tissue processing and immunohistochemistry

All animals from the groups injected in the *substantia nigra* and striatum were sacrificed 5 days after the last behavioural test, and the ones from the group injected in the motor cortex were sacrificed 7 hours after the sciatic nerve crush. The rats were deeply anaesthetised with 0.6 ml sodium pentobarbital IP. (Apoteksbolaget, Sweden) and then perfused through the ascending aorta with 50 ml saline (0.9% w/v), followed by 250 ml ice-cold paraformaldehyde (4% w/v in 0.1 M phosphate buffered saline). The brains of the SN, ST and CX group and, spinal cord and sciatic nerve from the CX group were removed, post-fixed for 12 h in 4% paraformaldehyde and cryoprotected in sucrose (25% w/v in 1% phosphate buffered saline) before being sectioned on a

microtome (Microtome Leica SM 2010R) in the case of brain and spinal cord, or in a cryostat (Cryostat Leica CM 3050 S) in the case of the sciatic nerve. Regarding the brain, coronal sections were collected in 8 series at a thickness of 40 µm. In the case of the spinal cord coronal and sagittal sections were collected from the cervical, thoracic and lumbar regions at a thickness of 40 µm. The sciatic nerve samples were sectioned at 16 µm in the cryostat and collected to the glass slide. All the collected samples were kept at 4°C.

Immunohistochemical staining was performed on free floating sections (brain and spinal cord) or on mounted slides (sciatic nerve) using antibodies raised against tyrosine hydroxilase (TH) (mouse, 1:10000; Immunostar), tyrosine hydroxilase (TH) (rabbit, 1:500; Pelfreeze), Green Fluorescent Protein (GFP) (Chicken, 1:20000; Abcam), ionized calcium-binding adapter (IBA1) (rabbit, 1:500; Wako Chemicals), glial fibrillary acidic protein (GFAP) (rabbit, 1:500 ; Dako) for brain staining , Synuclein-1(mouse, 1:400; BD Biosciences), for sciatic nerve staining, α-synuclein (211) (mouse, 1:1000; Santa Cruz Biotechnology), Phospo-Ser129 α-synuclein (rabbit, 1:1000; Abcam) for brain, sciatic nerve and spinal cord staining, and Synaptophysin (G96) (rabbit, 1:2000; R.Jahn), Rabphilin-3A antisera full length (rabbit, 1:5000; Jia-Yi Li) , Rab-3A (mouse, 1:2000; Synaptic Systems) for sciatic nerve staining. Sections were rinsed three times in PBS between each incubation period and all steps, except the incubation with the primary antibody, were performed at room temperature with three 10 min washes in PBS-TX (0,1 – 0,5% triton X-100) between each step. Primary antibody was incubated at 4ºC overnight. Sections were mounted and coverslipped using 50% glycerol (PBS) and kept at 4°C.

For diaminobenzidine (DAB) staining sections were treated with 100% methanol and 3%H_2O_2 for 30 min to inhibit endogenous peroxidases, permeabilized in PBS-TX for 30 min and blocked in 5% BSA in PBS-TX for 1 h. Sections were incubated with primary antibody in 2% BSA over night at 4°C followed by incubation with biotinilated secondary antibody for 2 h at room temperature and avidin-biotin (Vectastain kit; Vector) for 1 h at room temperature. Sections were incubated with 3,3-diaminobenzidine (DAB; Vector), rinsed in PBS, mounted, dehydrated and coverslipped using DPX mounting medium.

Microscopy and stereology

Fluorescence images were obtained using a confocal laser-scanning microscope (Zeiss LSM 510, Jena, Germany) using ZEN software or a fluorescence microscope (Olympus BX53) using Cell Sens Dimension software. All images were analysed using ImageJ. For an overview of the distribution of total α-Syn and pSer129 α-Syn in the brain of AAV-injected animals, sections from different brain regions were scanned using EPSON perfection V750 PRO with Silver Fast software.

Assessment of the total number of TH+ neurons in the *substantia nigra* was made according to the optical fractionator principle, using a Leica Leitz DM RBE and the Stereo Investigator software. All the sections covering the *substantia nigra* from two series were included in the counting procedure. A coefficient of error <0.10 was accepted. Striatal TH+ fibre density was measured by densitometry using the ImageJ software (Version 1.47v, National Institute of Health, USA). The striatum, ipsilateral and contralateral, was outlined and the mean intensity in the region of interest was determined. The measured values were corrected for non-specific background staining by subtracting values obtained from the *corpus calosum*. The data are expressed as a percentage of the corresponding area from the contralateral side.

Statistical Analysis

Data is expressed as a group mean ± SD (standard deviation) unless stated otherwise. Statistical analysis was performed using GraphPad Prism software, except for the behaviour assessment where statistical analysis was performed using SPSS 21 software. Datasets from Behaviour assessment were tested for statistical significance using t-test to compare between timepoints, Friedman test to compare the differences between the timepoints within one group of animals, and Man-Whitney test as non-parametric test. A paired Student's t-test was used to compare the number of midbrain TH+ neurons on the contra- and ipsilateral-side of the injection. Linear regression was performed on the densitometric values.

Chapter 3. RESULTS

In this study we developed and characterized a novel animal model of PD that enables us to monitor α-Syn oligomers *in vivo*. We used a mix of two AAV constructs expressing human wt α-Syn fused with either the N- or C-terminus half of Venus protein (AAV V1S+SV2). An AAV construct expressing Venus alone was used as a control. In order to assess whether and how oligomerized α-Syn transports throughout the CNS *in vivo* we set 3 experimental groups of animals:

- ✓ CX Group: AAV V1S+SV2 or Venus injection into the **motor cortex**
- ✓ SN Group: AAV V1S+SV2 or Venus injection into the ***substantia nigra***
- ✓ ST Group: AAV V1S+SV2 or Venus injections into the **striatum**

AAVs were stereotactically injected into the respective brain target area of Sprague Dawley rats.

CX Group (motor cortex-injected)

Transport of oligomerized α-synuclein via the corticospinal tract

The CX group constituted of 8 animals (4 V1S+SV2 + 4 Venus). All animals were subjected to stereotactic AAV injection weighting 225-250 g targeting the motor cortex. Sciatic nerve crush was performed 9 weeks after the AAV injection with the objective of investigating the accumulation of the human α-Syn near the site of the crush. Animals were sacrificed 9 weeks post AAV injection and brain, spinal cord and sciatic nerve samples were prepared for analysis.

9 weeks post viral injection we were able to observe the BiFC signal at the place of injection (motor cortex) (Fig.:3.1 A). Moreover, we have detected the BiFC signal in the entire cortico-spinal tract, (Fig.:3.1) being evident in brain regions including the striatum (Fig.:3.1 B), thalamus (Fig.:3.1 C), midbrain (Fig.:3.1 D), pons (Fig.:3.1 E) and even in the spinal cord(Fig.:3.1 H).

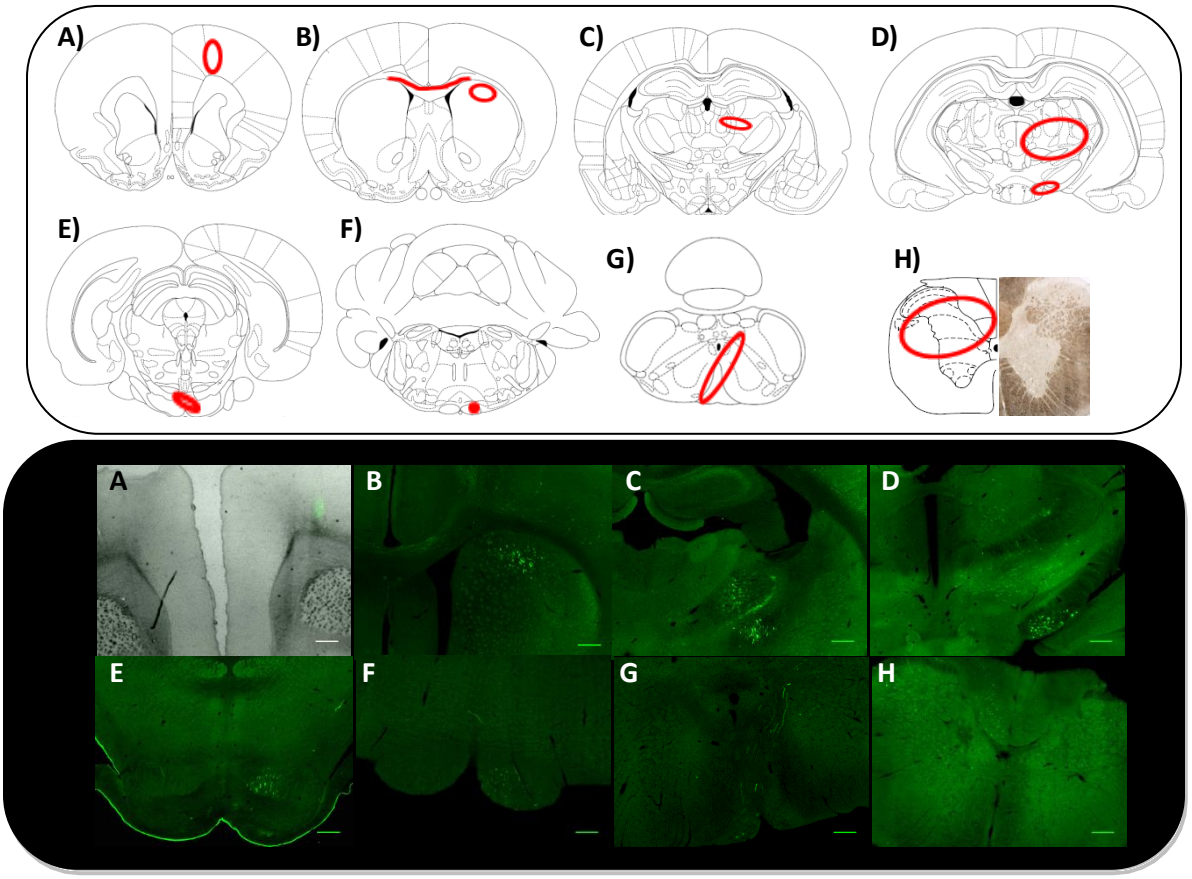


Fig.:3.1 - **Detection of α-Syn oligomerization in the Corticospinal tract using protein complementation assay.** Schematic representation of the brain areas where the BiFC signal was detected (top). Coronal sections from rats injected with AAV V1S+SV2 were mounted onto glass slides and directly imaged under a fluorescence microscope.(Bottom) Reconstituted Venus fluorescence is visible in the ipsilateral side of the brain in the injection site (A), Striatum (B), Thalamus (C), Midbrain (D), Pons (E), Medulla oblongata (F, G) and in the contralateral side in the spinal cord (H). Scale bar: 500 µm in the images A-E and 250µm in the images F-H.

More specifically, we were able to detect oligomerized α-Syn in the cell bodies and dendrites at the site of injection and (Fig.:3.2 A) in the neuronal projections in the subsequent brain regions (Fig.:3.2 B and C). Interestingly, we have observed axonal accumulation of α-Syn oligomers in the midbrain (Fig.:3.2 D), pons (Fig.:3.2 E) and spinal Cord (Fig.:3.2 F).

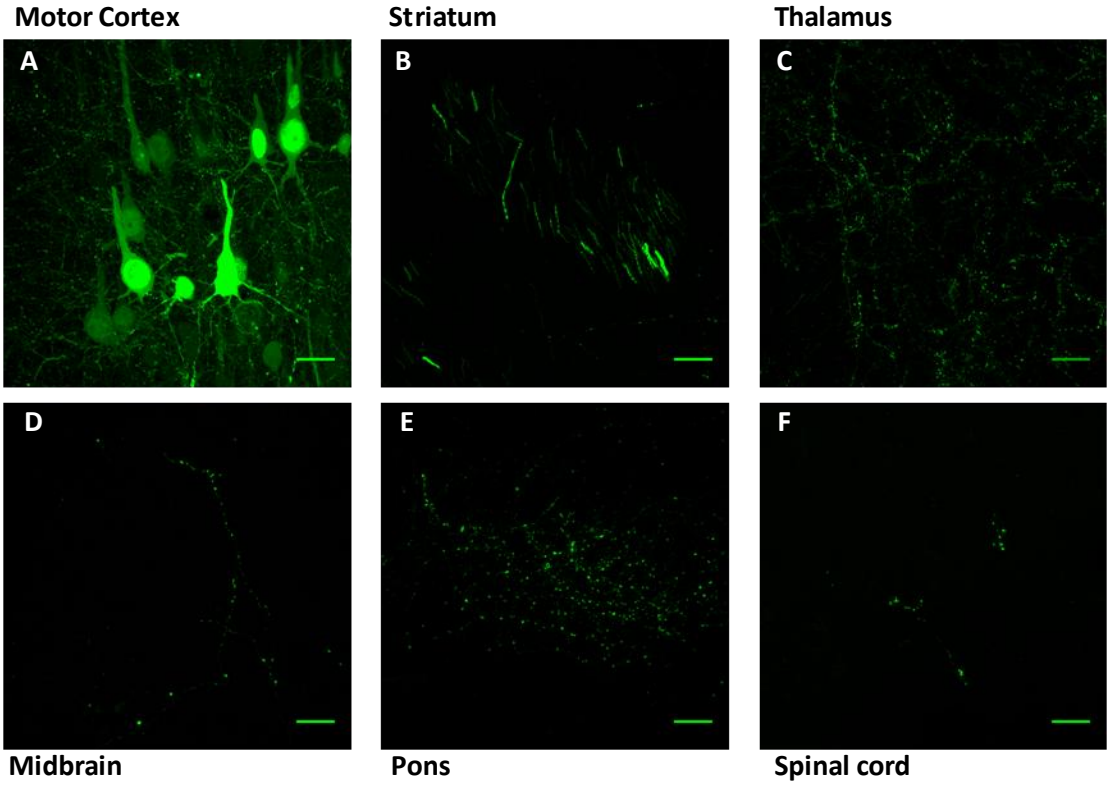


Fig.: 3.2 – **Detection of α-Syn oligomerization in different brain areas using protein complementation assay.** Coronal sections from rats injected with AAV V1S+SV2 were mounted onto slides and directly imaged under a fluorescence microscope. Reconstituted Venus is visible in cell bodies at the injection site as well as in the neuronal projections through the corticospinal tract. Axonal accumulation of α-Syn oligomers is seen in regions such as midbrain (D), pons (E) and spinal cord (F). Scale bar: 25µm

Microscopic analysis of the brain samples enabled us to conclude that the AAV injection was well targeted and that there was formation of α-Syn oligomers readily observed by reconstitution of Venus fluorescence (BiFC signal). Moreover, oligomeric a-Syn forms were transported from the motor cortex via the corticospinal tract into the spinal cord.

Through the BiFC signal we were able to detect the fraction of human α-Syn that was dimerized or oligomerized. In order to detect the total human α-Syn protein load in the brain, we performed immunohistochemistry (IHC) analysis using an antibody against human α-Syn (Fig.:3.3). Human α-Syn was found throughout the corticospinal tract. As expected, we found more α-Syn positive neurons/neuronal projections in the specific brain areas when compared to BiFC, however, the overall pattern of distribution in the corticospinal tract was similar to BiFC (Fig.:3.3). No α-Syn-positive structures were observed in the Venus-injected animals, showing the specificity of the α-Syn antibody to human α-Syn. Additionally, we performed an IHC analysis using an anti-GFP antibody to visualize the distribution of the Venus protein in the control group (Venus-injected animals). The overall distribution profile of the Venus was similar to the human α-Syn.

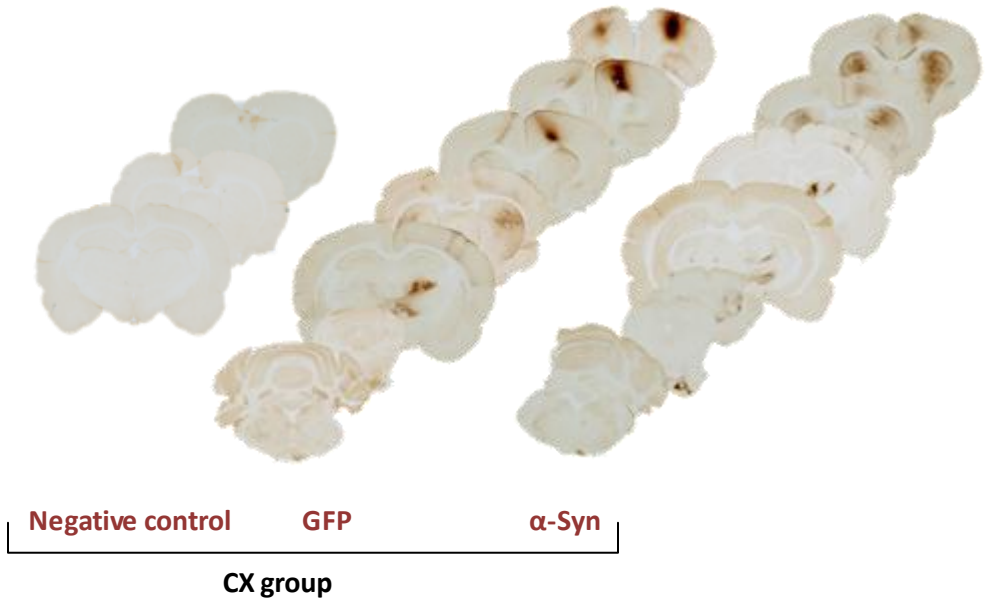


Fig.: 3.3 – **Histological overview of transduced brain regions.** Coronal brain sections were stained with antibodies recognizing the syn211 against human α-Syn and an antibody against GFP. Negative control animals were stained with human α-Syn (211) antibody to confirm specificity. Transduction patter was very similar in the AAV V1S+SV2 (stained for human α-Syn) and the AAV Venus group (stained for GFP) with a very intense immunoreactivity in the cortex. No staining was observed in the Negative control animals (AAV Venus stained for human α-Syn), confirming the specificity of this antibody towards human α-Syn.

Several studies suggest that phosphorylation at serine 129 (pS129) may play an important role in regulating α-Syn fibrillization, aggregation, LB formation and toxicity in different model systems [54, 72, 73, 138, 139]. In order to evaluate the presence of phosphorylated α-Syn in our model, we performed an IHC assay using an antibody against human α-Syn pSer129. As shown in the (Fig.:3.4), we were able to detect pSer129 α-Syn in the cell bodies and neurites in the cortex (Fig.:3.4 A), as well as in the neuronal projections of other zones of the corticospinal tract. We were also able observe accumulated α-Syn pSer129 in some of the axons as well (Fig.:3.4 B, E and F)

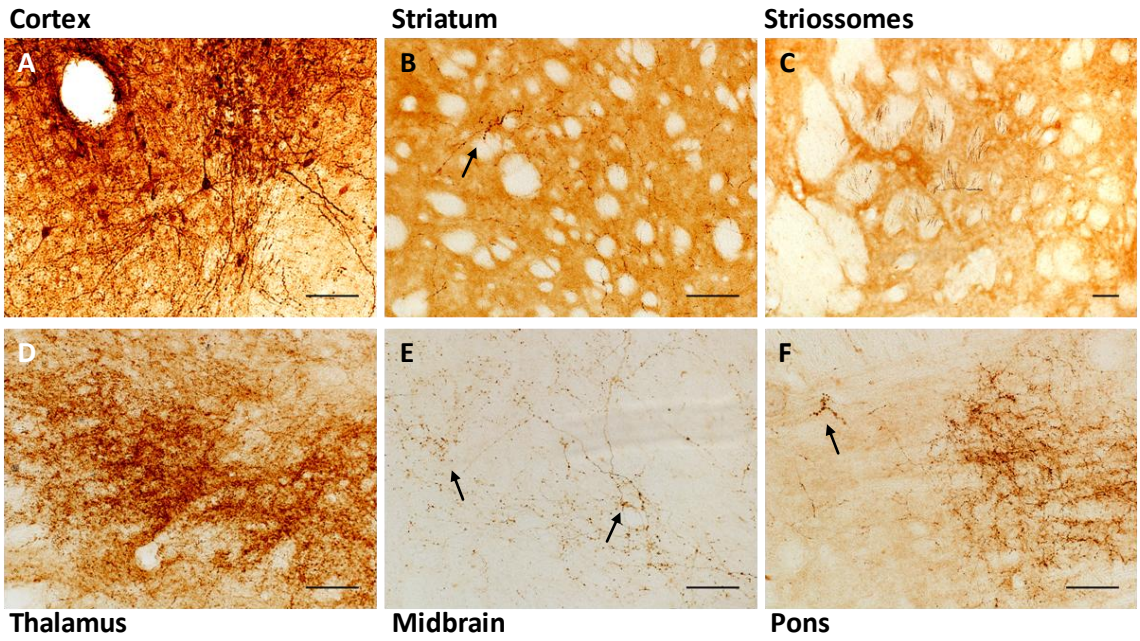


Fig.: 3.4 –**Histological analysis of phosphor Serine 129 α-Syn expression.** Histological analysis of the brain sections revealed high expression of phosphor Serine 129 α-Syn (pS129 α-Syn) in different regions of the corticospinal tract. Neurons of the site of injection (A) displayed a high incidence of pSer129 within the cell body and axons while the other regions presented high incidence of pS129 within the axonal projections. Large pS129 positive structures, believed to be axonal swellings, were present throughout the striatum (B and C), thalamus (D), midbrain (E) and pons (F). Scale bar: 50µm

Because we have seen presence of total human α-Syn and even pSer129 α-Syn in the thoracic part of the spinal cord, reflecting its transport/spreading from the cortex, it prompted us to check for the accumulation of proteins near the sciatic nerve crush site. In case we would be able to see accumulation of human α-Syn, it will suggest that there had been trans-synaptic transfer of these oligomeric species from the upper motor neuron to the lower motor neuron. Initially, and to define the proximal and distal side of the crushes we stained for Rabphilin and Rabphilin-3A antibodies, the proteins involved in the regulation of synaptic vesicle fusion and that are known to be

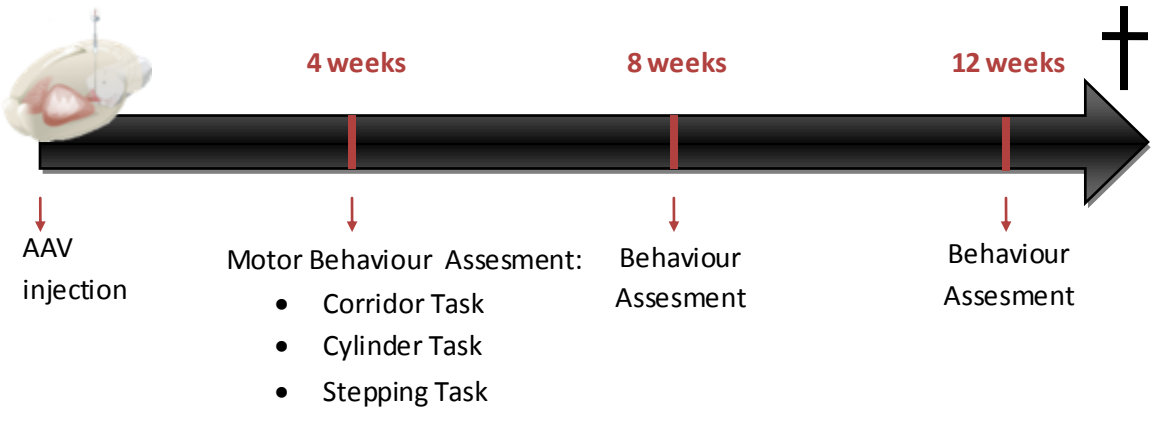
primarily transported in an anterograde manner, which means that we can use them as markers of the proximal site. Than we tried to stain by immunofluorescence the human α-Syn, but the result was not conclusive. (Data not shown)

In conclusion, we were able to observe transport of oligomerized α-Syn via the corticospinal tract after the AAV injection into the motor cortex. Additionally, the phosphorylated form of α-Syn on serine 129 was detected in the axonal profiles where the oligomerized α-Syn was transported as well as axonal accumulation, reflecting phenomena of neurodegeneration.

SN and ST Groups (s*ubstantia nigra*- and striatum- injected)

Transport of oligomerized α-Synuclein via the nigrostriatal pathway with signs of axonal degeneration

The nigrastriatal dopaminergic system is mainly affected in PD. The nigrostriatal pathway projects from the *substantia nigra pars compacta* to the striatum. Taking this into consideration, in the next step we performed AAV injection targeting the nigrostriatal pathway, more precisely *substantia nigra* and striatum. We had 2 groups of animals according to the area targeted with the AAV injection. The SN group was constituted of 23 animals (12 V1S+SV2 and 11 Venus) and the ST group was constituted of 17 animals (9 V1S+SV2 and 8 Venus). According to the following schematic representation, all animals were subjected to AAV injection weighting 225-250 g targeting the *substantia nigra* or striatum, respectively. Motor function was assessed in both groups 4, 8 and 12 weeks after viral injection using three different tests, the Cylinder, Corridor and Stepping test (for detailed description see Chapter2. Methods, section Behavioural Testing). Animals were sacrificed 12 weeks post-AAV injection and brain samples were collected for analysis.



SN group presented strong BiFC signal in cell bodies and dendrites of the SNpc (injection site) (Fig.:3.5 A) and also in the axonal projections in the striatum, (Fig.:3.5 B) which strongly suggests the spreading of the α-Syn oligomeric forms through the nigrostriatal pathway, the main dopaminergic pathway. We also observed BiFC signal in other brain regions such as thalamus and midbrain, showing that apart from our main target – the nigrostriatal dopaminergic pathway, we targeted other systems to a certain extent.

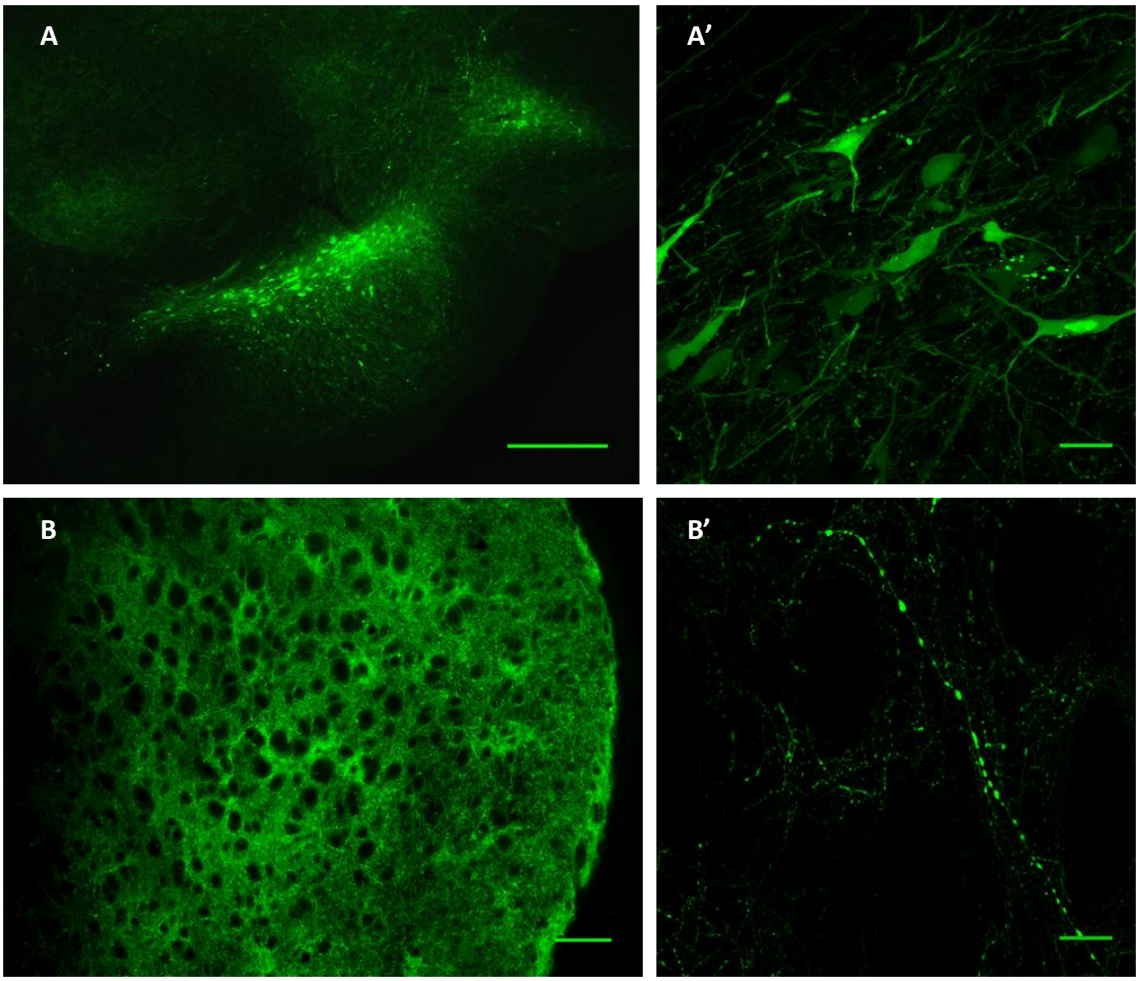


Fig.: 3.5 – **Detection of α-Syn oligomerization in the nigrostriatal pathway using protein complementation assay.** Coronal sections from rats injected in *substantia nigra* with AAV V1S+SV2 were mounted onto slides and directly imaged under a fluorescence microscope. Reconstituted Venus is visible in cell bodies in *substantia nigra pars compacta* (injection site) (A) and in the neuronal projections in Striatum (B). Scale bar: 500µm (A), 250µm (B), 25µm (A' and B')

The ST group showed strong BiFC signal in the cell bodies of the striatum (injection site) (Fig.:3.6 B) and in the SNpr (Fig.:3.6 A). This group also showed some BiFC signal in the thalamus too, but not as wide spread in the midbrain as the SN group.

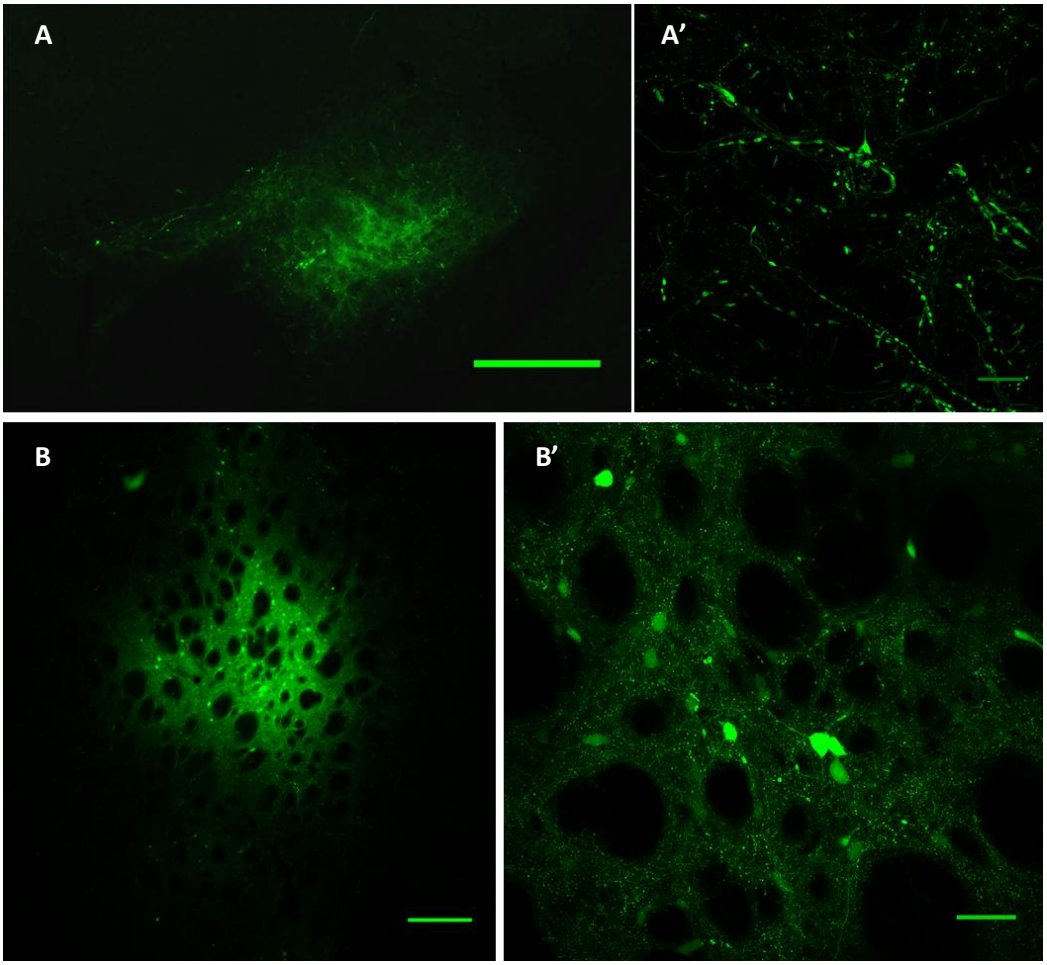


Fig.: 3.6 – **Detection of α-Syn oligomerization in the nigrostriatal pathway using protein complementation assay.** Coronal sections from rats injected in striatum with AAV V1S+SV2 were mounted onto slides and directly imaged under a fluorescence microscope. Reconstituted Venus is visible in cell bodies of striatum (injection site) (B) and in *substantia nigra pars reticulata* (B). Scale bar: 500µm (A), 250µm (B), 25µm (A'), 50µm (B')

As in the case of the CX group, through the BiFC signal we were only able to detect the fraction of human α-Syn that was dimerized or oligomerized. In order to detect the total load of human α-Syn protein, brain sections were subjected to IHC using an antibody against human α-Syn. Control animals were additionally subjected to IHC using an antibody against GFP (recognizes Venus protein). In the ST group,

human α-Syn was found in the striatum (cell bodies and neuronal projections) and in SNpr.

As expected, the extent of total α-Syn expression was higher than what was observed through the BiFC, however the overall distribution was similar to the BiFC. Regarding the SN group, the spread of the α-Syn protein is much wider than in the ST group. (Fig.:3.7) Human α-Syn was found throughout the nigrostriatal pathway, but moreover was found in other brain regions such as thalamus and midbrain, being detected as caudal as the cervical part of the spinal cord. This confirms once again that apart from our main target – the nigrostriatal dopaminergic pathway, we targeted other systems to a certain extent as well. For both groups and as expected the overall distribution profile of the Venus was similar to the human α-Syn.

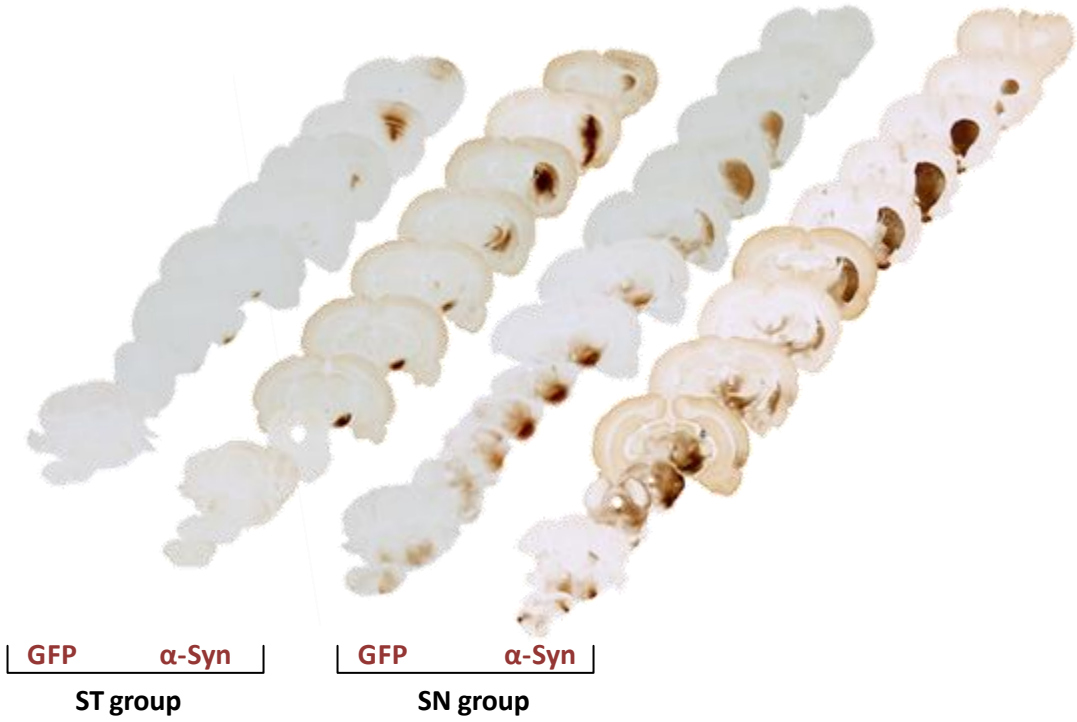


Fig.: 3.7 – **Histological overview of transduced brain regions.** For both ST and SN goups coronal brain sections were stained with antibodies recognizing the syn211 against human α-Syn and an antibody against GFP. Negative control animals were stained with syn211 antibody to confirm specificity. (not shown) Transduction pattern was very similar in the AAV V1S+SV2 (stained for human α-Syn) and the AAV Venus group (stained for GFP) with a very intense immunoreactivity in the cortex. No staining was observed in the Negative control animals (AAV Venus stained for human α-Syn), confirming the specificity of this antibody towards human α-Syn.

In SN injected animals, closer examination revealed the presence of human α-Syn in cell bodies and neuronal fibers in the ipsilateral *SNpc* (Fig.:3.8 A) contrasting with the lack of α-Syn in the contralateral s*ubstantia nigra* (Fig.:3.8 C) . Similarly to what we observed in the CX group we were also able to detect pSer129 α-Syn, the pathology-linked form of α-Syn, in the ipsilateral *substantia nigra* cell bodies and neurites (Fig.:3.8 B).

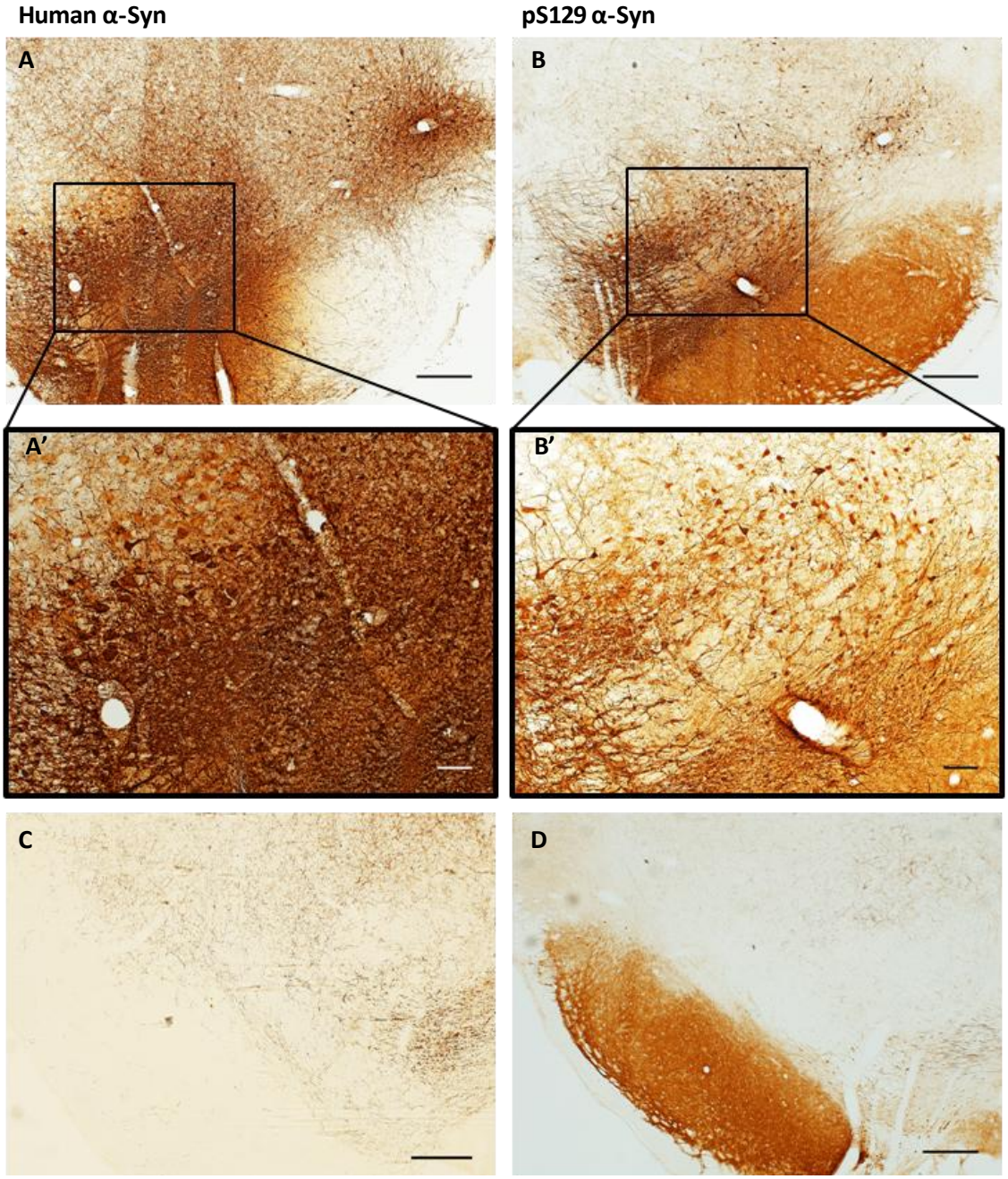


Fig.: 3.8 - **Histological analysis of α-Syn expression in *substantia nigra*.** Histological analysis of the brain sections of AAV injected animals of SN group revealed high expression of human α-Syn (211)(left) and phosphor Serine 129 α-Syn (pS129 α-Syn)(right) in the cell bodies and fibers of the ipsilateral *substantia nigra*. (A, B, A' and B') The contralateral side presented no expression of α-Syn. (C and D) Scale bar: 200µm (A ,B, C and D), 50µm (A' and B')

Furthermore, total human α-Syn (Fig.:3.9 A) as well as pSer129 α-Syn (Fig.:3.9 B) was detected in axonal projections in the striatum fibers. And accumulation of the phosphorylated form of α-Syn in the cell bodies and axons in striatum was detected (Fig.:3.9 B, arrow).

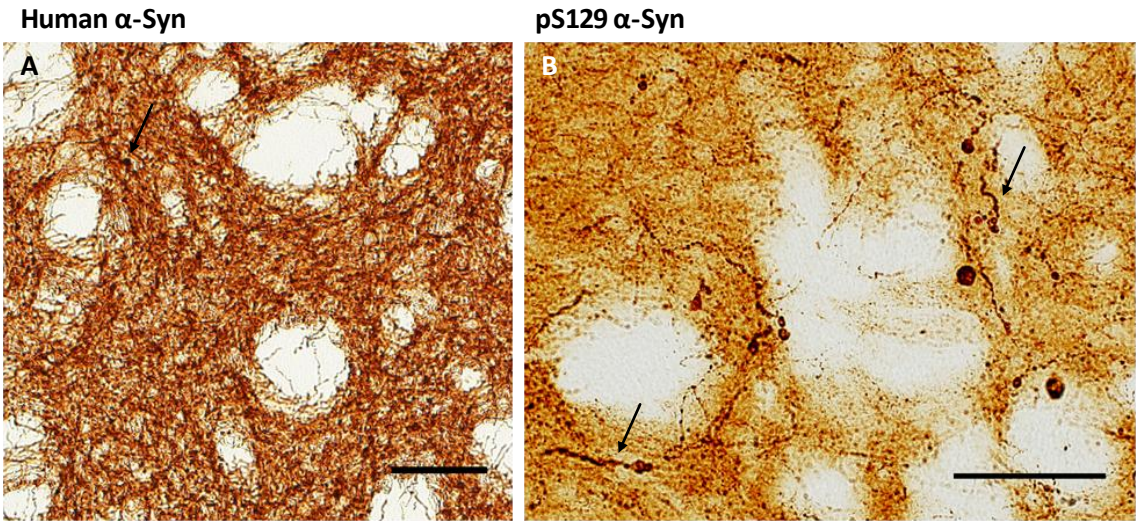


Fig.: 3.9 - **Histological analysis of α-synuclein expression in the striatum.** Histological analysis of the brain sections of rats AAV injected in *substantia nigra* revealed high expression of human α-Syn (211)(A) and phosphor Serine 129 α-Syn (pS129 α-Syn)(B) in striatum fibers with some visible signs of axonal accumulation. (arrow) Scale bar: 50µm

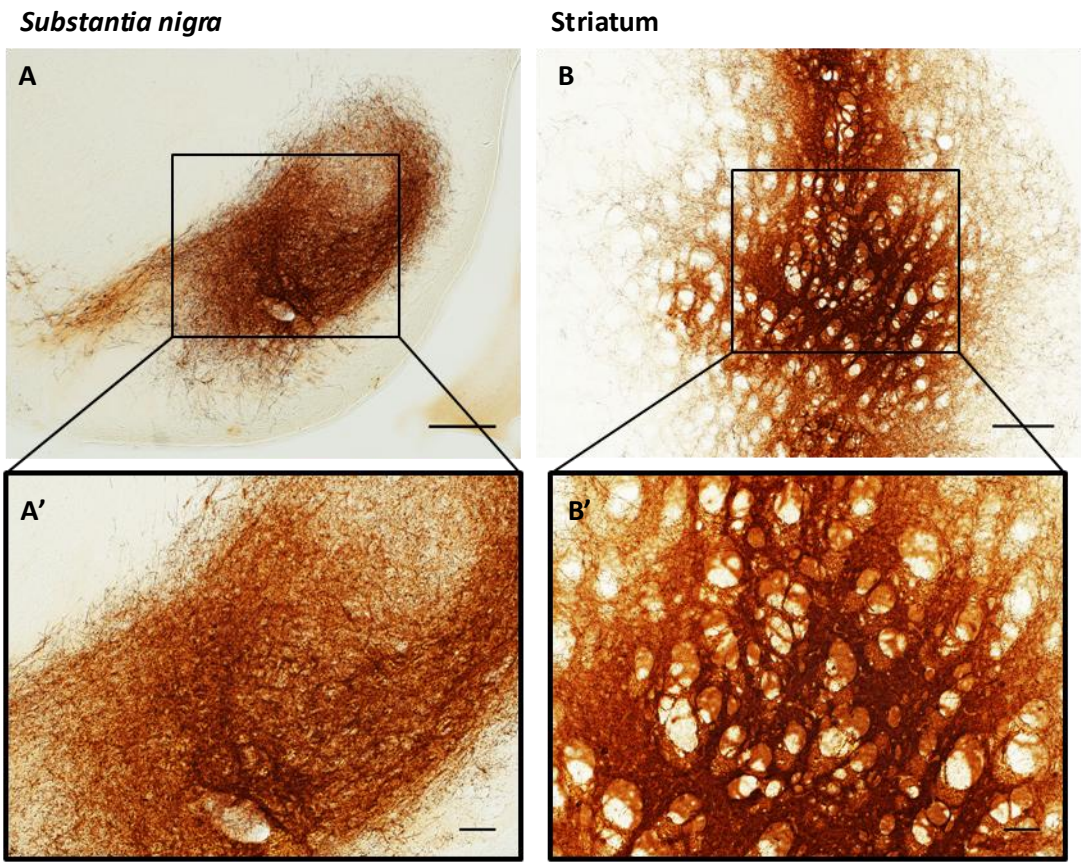


Fig.: 3.10 - **Histological analysis of α-Synuclein expression in *substantia nigra* and striatum.** Histological analysis of the brain sections of AAV injected animals of ST group revealed high expression of human α-Syn (211) in the striatum (injection site) (B) and in *substantia nigra pars reticulata* (A) Scale bar: 200µm (A and B), 50µm (A' and B')

In sections from animals of ST group, closer examination revealed the presence of human α-Syn in the striatum (injection site) (Fig.:3.10 B) and *SNpr* (Fig.:3.10 A). Similarly to what we observed in the CX and SN group we were also able to detect pSer129 α-Syn, the pathology-linked form of α-Syn, in the ipsilateral *substantia nigra* (Fig.:3.11 A) and striatum (Fig.:3.11 B), contrasting with the lack of pSer129 α-Syn in the contralateral side (Fig.:3.11 C and D).

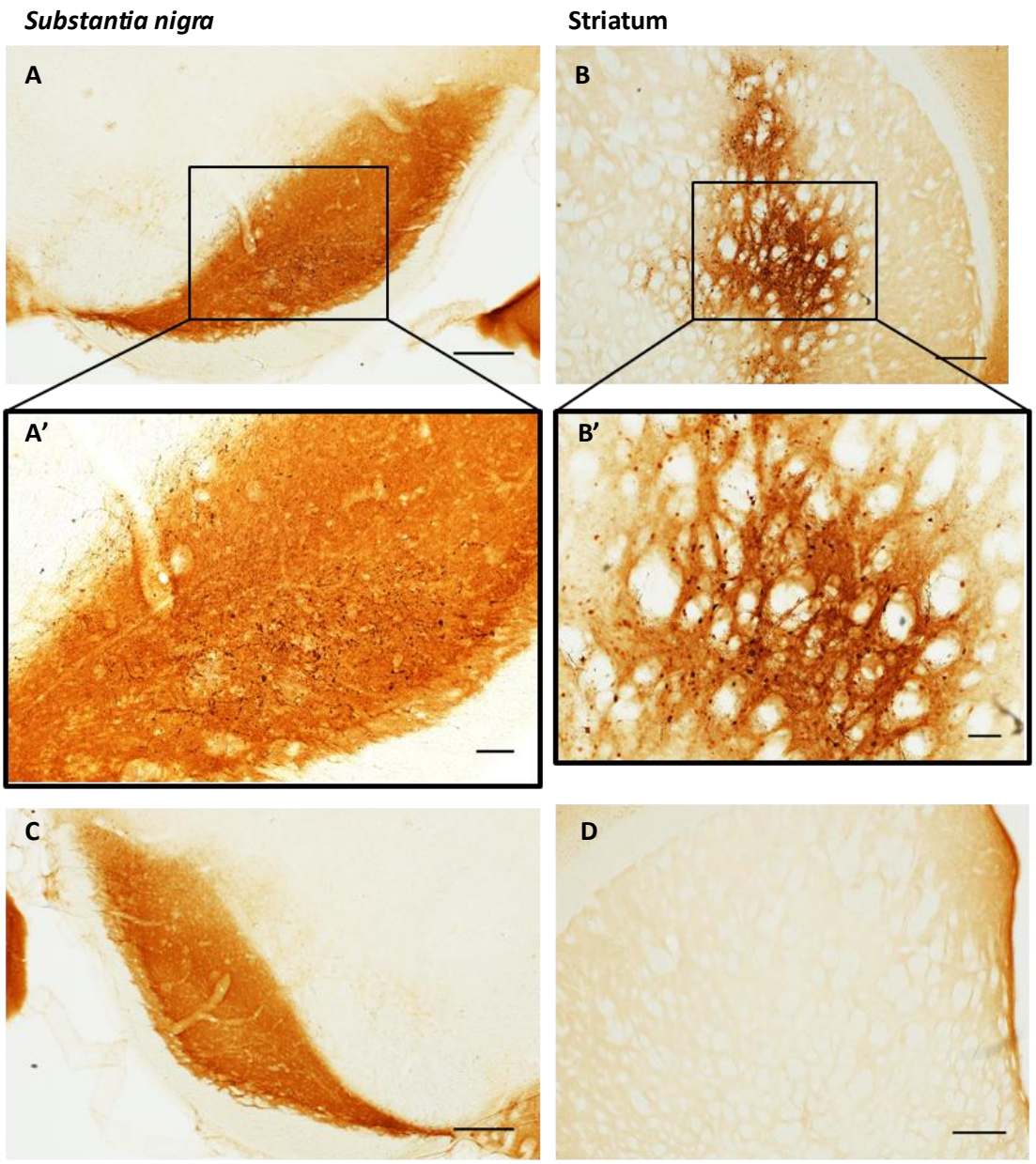


Fig.: 3.11 - **Histological analysis of pathology-linked form of α-Synuclein expression in *substantia nigra* and striatum.** Histological analysis of the brain sections revealed high expression of phosphor Serine 129 α-Syn (pS129 α-Syn) in the cell bodies of striatum (injection site) (B) and fibers of the ipsilateral *substantia nigra pars reticulate* (A). The contralateral side presented no expression of pS129 α-Syn. (C and D) Scale bar: 200µm (A, B, C and D), 50µm (A' and B')

Interestingly, we have observed altered axonal morphology – axonal swellings- in the striatum of the animals injected with AAV V1S+SV2 (Fig.:3.12 A) when compared to the control Venus group (Fig.:3.12 B). The axonal swellings present in the animals injected with AAV V1S+SV2 might be due to block of axonal transport and axonal

degeneration. Altogether these results show that α-Syn oligomers were formed and transported antero-gradely through the nigrostriatal pathway.

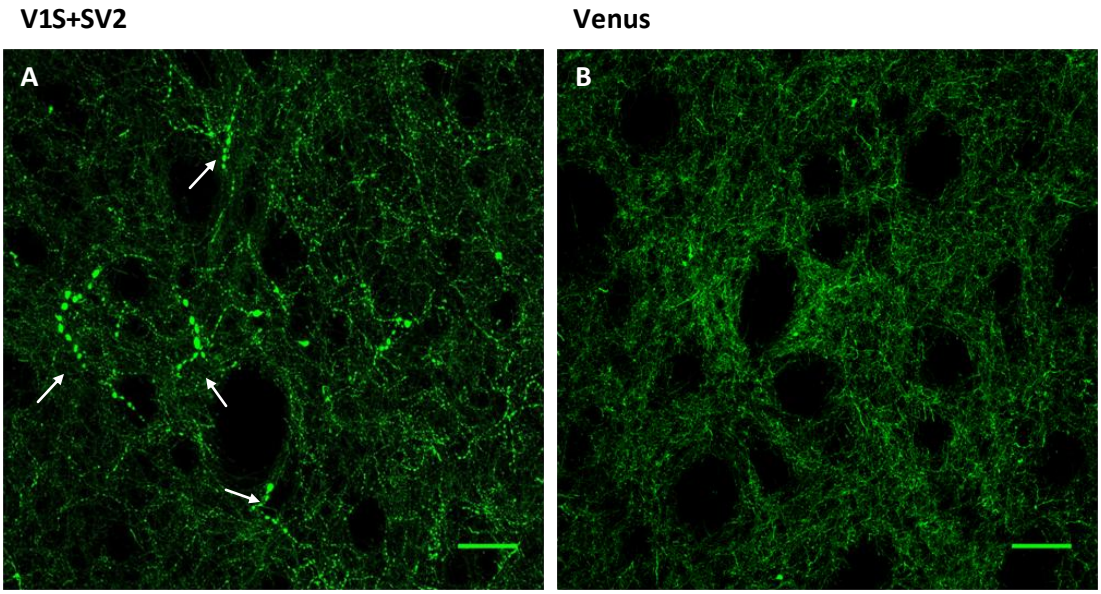


Fig.: 3.12 – **Detection of α-Synuclein oligomers in the striatum.** Coronal sections from the striatum of rats injected with AAV V1S+SV2 and AAV Venus in the *substantia nigra* were imaged for the presence of Venus fluorescence. Venus fluorescence is detected in both AAV V1S+SV2 (A) and AAV Venus (B) injected animals. However, only the animals injected with the AAV V1S+VS2 presented punctuate pattern resembling axonal accumulation (A) (arrow). Scale bar: 50µm

The nigrostriatal pathway is the main dopaminergic pathway in the brain and is the one mainly affected in PD giving rise to the motor symptoms [39]. To investigate whether there were any deficits in motor function in our model, we performed three distinct tests of spontaneous motor performance at 4, 8 and 12 weeks post AAV injection. (for detailed description see Chapter2.Methods, section Behavioural Testing) There was no significant difference between the Venus and α-Syn groups in any of the timepoints in corridor and cylinder task. However, in the stepping task at 4 weeks the α-Syn group showed a significantly reduced number of adjusting steps by the left paw (Fig.:3.13), suggesting impairment in the motor function of the limb controlled by the side of the brain where the injection was made. However, this impairment was not observed at later time-points, suggesting a compensatory mechanism.

Dopamine has been described as an important neurotransmitter involved in movement control due to the association between the depletion of striatal DA and motor deficits observed in PD. Synthesis of DA is limited by the activity of enzyme tyrosine hydroxylase.

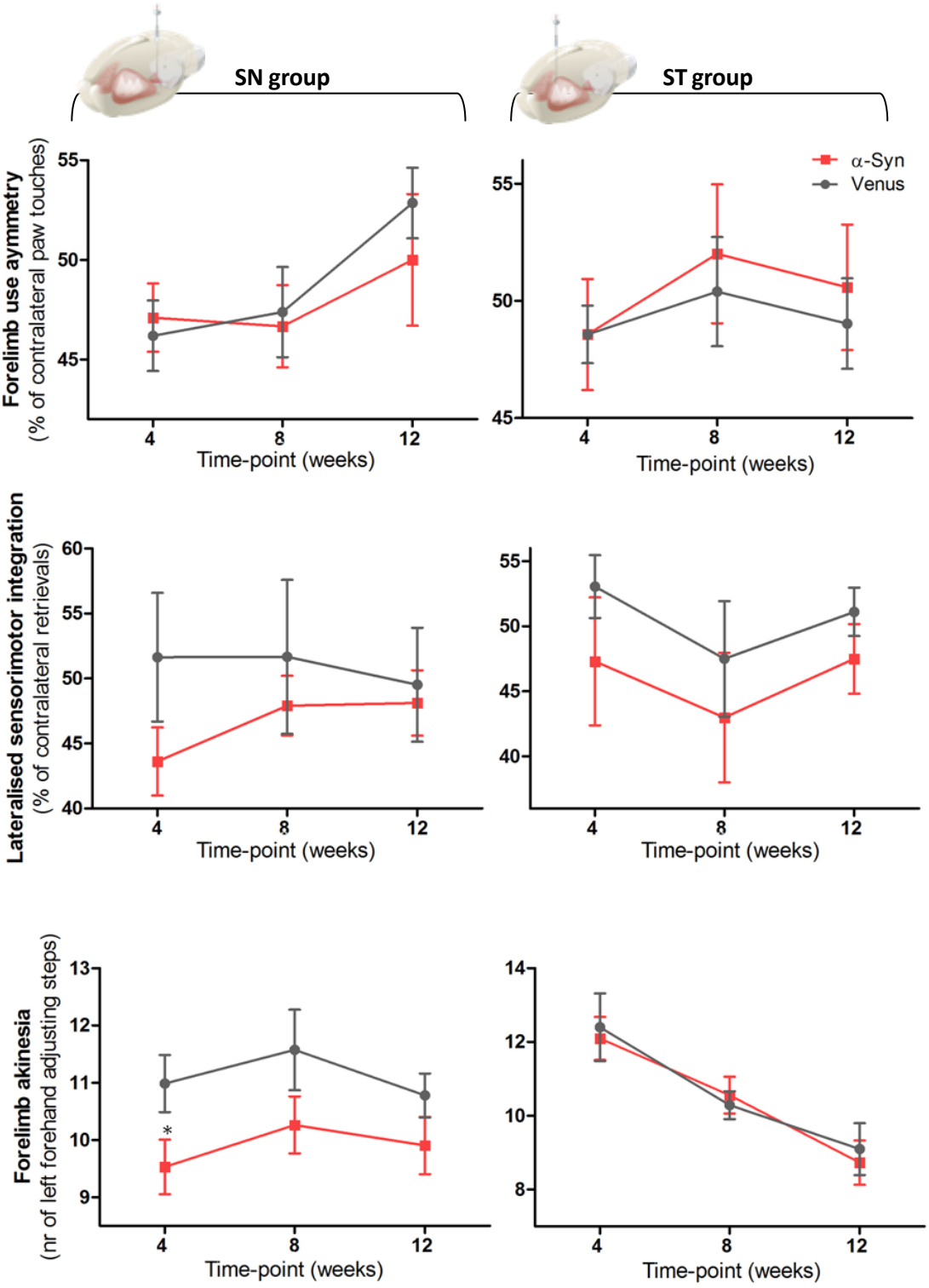


Fig.: 3.13 – **Behavioural assessment of motor functions.** At 4, 8 and 12 weeks post viral injection, animals were subjected to 3 tests of motor performance. Error bars +/- 1 SE

Overexpression of α-Syn in cells has been shown to lead to a significantly reduced TH activity and DA synthesis [140, 141]. To determine if the presence of α-Syn oligomers in the *substantia nigra* resulted in dopaminergic cell death we examined neuronal cell death 12 weeks after viral injection. Stereological analyses revealed no significant death of the TH^+ cells in the animals injected with α-Syn when compared to the control (Fig.:3.14). Furthermore, we analysed the TH levels in the dorsolateral striatum to examine if there was a degeneration of the axons of the dopaminergic cells from SNpc projecting to striatum. Although there was a trend of decrease in the TH-positive fibers of the dorsolateral striatum of the ipsilateral side in the α-Syn injected animals, it did not reach statistical significancy (Fig.:3.14).

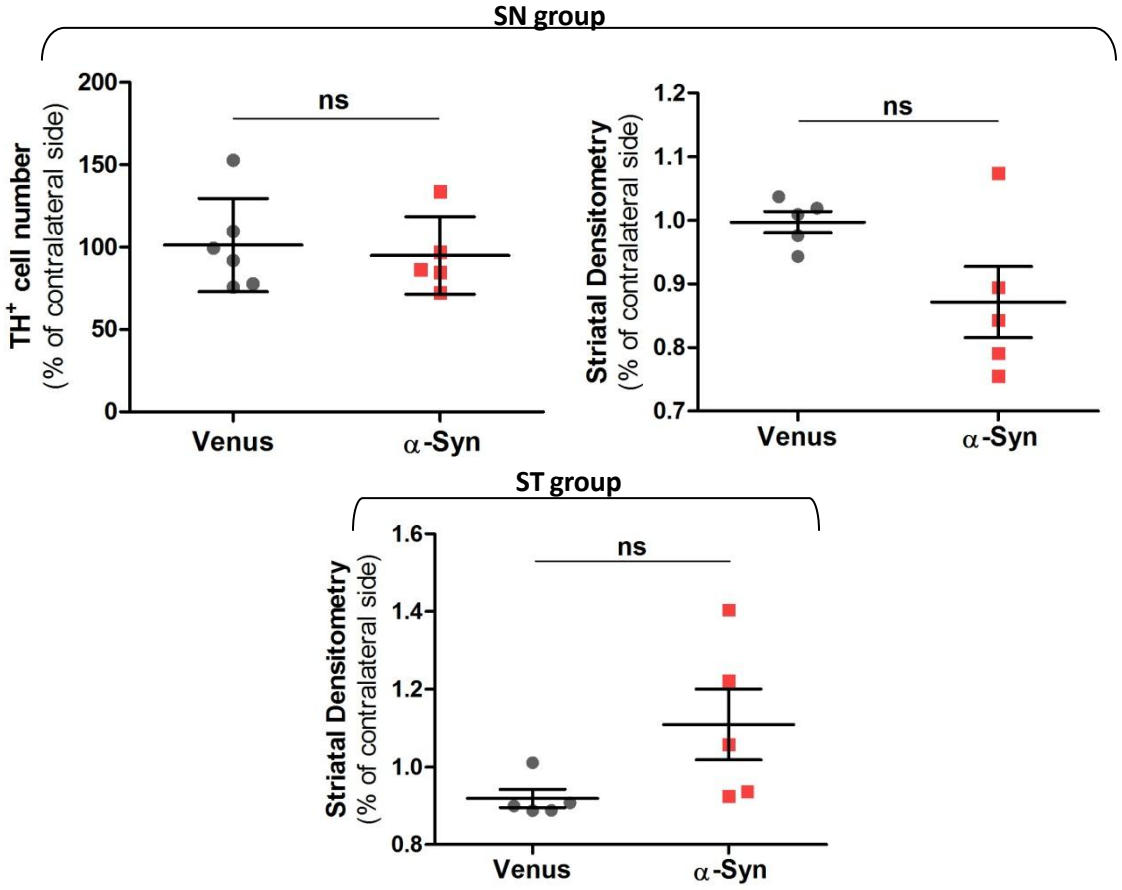


Fig.: 3.14 – **Assessment of midbrain dopaminergic TH+ cells and TH+ innervation in striatum.** Unbiased stereological analysis of TH immunopositive cells in coronal sections across the s*ubstantia nigra* of SN group animals was performed using DAB. TH+ innervation in striatum of SN and ST groups animals was measured using semiquantitative densitometry. Regarding SN group analyses revealed no significant death of the TH^+ cells in the AAV V1S+SV2 injected animals when compared to AAV Venus injected ones; striatum densitometry revealed no significant differences but a slight tendency of decrease in the AAV V1S+VS2 injected animals compared with the AAV Venus ones. ST group revealed no significant differences in the TH+ fibers of striatum, showing an interesting decrease in the AAV venus injected animals. ns : non significant

There is evidence suggesting that inflammation contributes to the pathophysiology and aetiology of neurodegenerative disorders [142-144]. Moreover, inflammatory reactions involving microglia, astrocytes, and lymphocytes have been described in several animal models of PD [145-147] and in PD patients [148, 149]. Several studies link striatal neuroinflammation to neurotoxicity and disease progression as a result of α-Syn aggregation [147, 150]. To assess striatal inflammation and/or gliosis in our model striatal sections were subjected to IHC analysis for GFAP, an astrocytic marker and Iba1, a microglial marker, to determine whether the presence of α-Syn oligomers in our model resulted in an inflammatory response and gliosis.

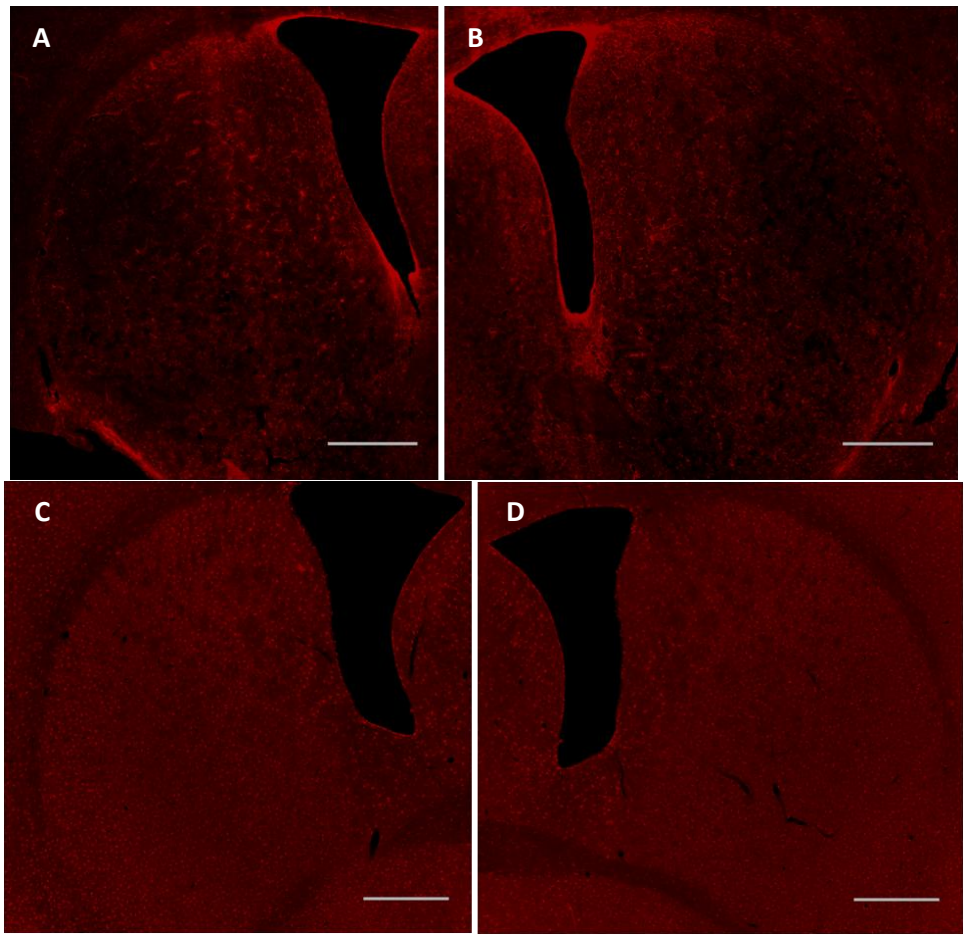


Fig.: 3.15 – **Absence of neuroinflammation in the striatum of SN group.** Striatal coronal sections were immunostained for Iba1 (C and D) or GFAP (A and B) expression. Comparing Ipsi- (B and D) and contralateral-side (A and C) of AAV V1S+SV2 injected animals there is no difference in the Iba1 or GFAP expression. Scale bar: 500µm

Immunofluorescence analysis revealed no significant difference in the expression of GFAP (Fig.:3.15/16 A and B) or Iba1 (Fig.:3.15/16 C and D) in AAV V1S+SV2 injected animals of SN (Fig.:3.15) and ST (Fig.:3.16) groups comparing ipsilateral to the contralateral side. Therefore, we did not observed any signs of gliosis or microglia-driven inflammation in our model 12-weeks post injection.

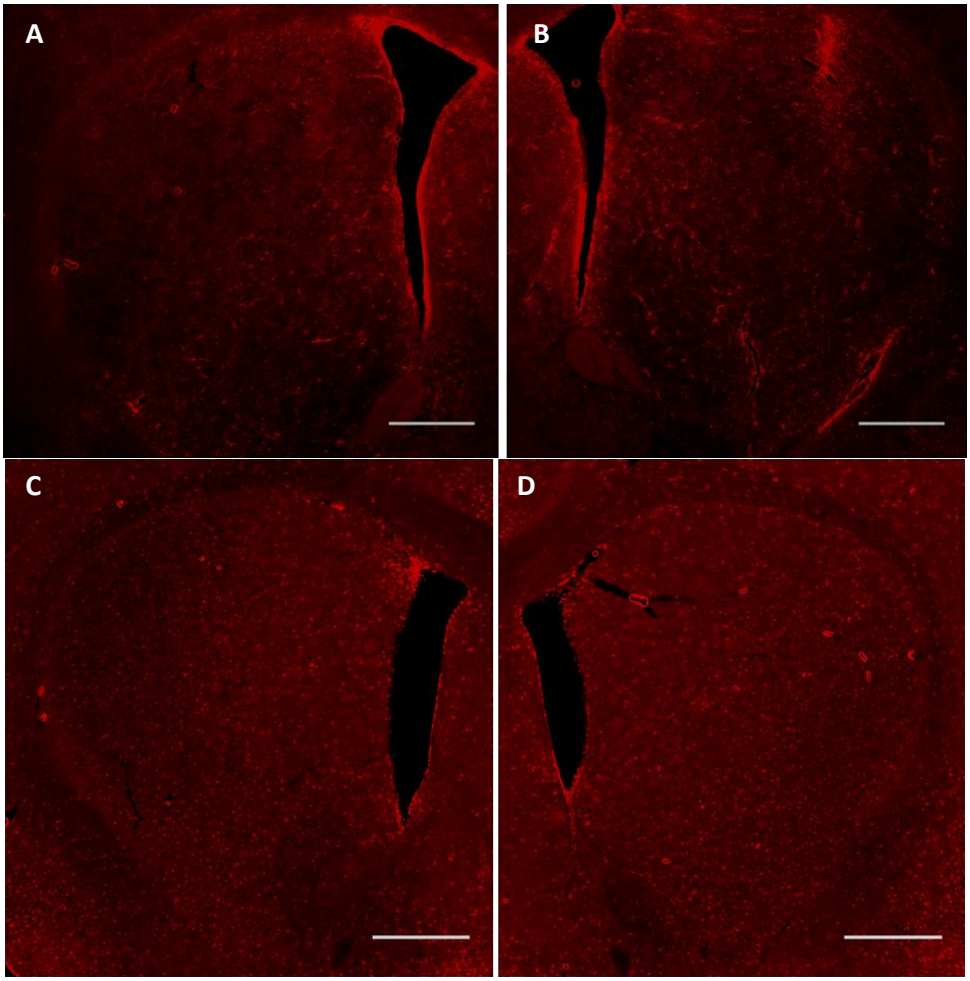


Fig.: 3.16 – **Absence of neuroinflammation in the striatum of ST group.** Striatal coronal sections were immunostained for Iba1 (C and D) or GFAP (A and B) expression. Comparing Ipsi- (B and D) and contralatera-l side (A and C) of AAV V1S+SV2 injected animals there is no difference in the Iba1 or GFAP expression. Scale bar: 500µm

Chapter 4. DISCUSSION

In recent years, studies have pointed to α-Syn oligomers as the toxic species of α-Syn [94, 132], playing a key role in PD pathogenesis. Previous studies have shown the applicability of protein complementation assay to detect α-Syn oligomers in vitro [128-130, 133] and in vivo [122], providing a tool to dissect the role of α-Syn oligomers in PD. Several animal models of synucleinopathies have been valuable for studying the etiopathology, including models overexpressing α-Syn [113, 117, 118, 121]. However, all the available models so far only allowed the detection of α-Syn oligomers based on indirect biochemical approaches. In this study we developed and characterised a new animal model for detection of α-Syn oligomers *in vivo.* We overexpressed the human α-Syn in different brain regions affected in the PD, such as *substantia nigra*, striatum or motor cortex, using an AAV-mediated gene delivery of two constructs containing complementary halves of a fluorescent protein linked to human α-Syn. This approach enables the study of α-Syn oligomers formation and transport in neurons and neuronal pathways. A similar approach was employed in a recent study [122].

AAV-mediated α-Syn delivery to the, motor cortex, *substantia nigra* or striatum of Sprague Dawley rats was performed and incubated for a period of 9 weeks (CX) or 12 weeks (SN and ST), respectively. In the CX group, we performed a sciatic nerve crush 9 weeks after viral injection to assess possible trans-synaptic transfer of α-Syn. In the SN and ST groups we performed behaviour tests at 4, 8 and 12 weeks after viral injection to examine the functional impacts of α-Syn expression in the brain. Then, histological analysis was conducted to validate the detection of α-Syn oligomers. Venus fluorescence was detected in the three injection areas, which demonstrate the direct delivery and expression of 2 viruses resulting from an *in vivo* α-Syn oligomer formation. Control animals injected with Venus alone exhibited stronger fluorescence than V1S+SV2 injected animals, as expected, given that in the V1S+SV2 injected animals not all the halves injected come together and reconstitute the fluorescence.

Regarding the CX group, injected in the motor cortex, α-Syn oligomers were found throughout the corticospinal tract, from the place of injection until the thoracic part of the spinal cord indicating the oligomers transport throughout this neuronal pathway. Interestingly, we were able to detect the pathological-linked form of α-Syn, pS129, which means our model presents signs of pathology. However, IHC analysis of sciatic nerves did not reveal presence of human α-Syn. The motor cortex area is divided in several areas, and each one of these sub-areas is responsible for the motor control of specific parts of the body. In order to be able to detect human α-Syn near the site of

the crush in the sciatic nerve, the injection of the AAV vector would have to target specifically the motor cortex area responsible for the control of the hind limbs. In our case this motor cortex area was most probably not targeted, and that could explain why we could not detect human α-Syn near the site of the crush in the sciatic nerve.

In all the three groups of the AAV-injected animals (CX, SN and ST), we detected presence of α-Syn oligomers in neurites and neuronal cell bodies. We were also able to detect great amounts of pSer129 in the cell bodies and neurites, linking this model to the pathology of PD, since pSer129 is known to have a role in α-Syn aggregation and to be present in LB of PD patients.

Overexpression of α-Syn in cells has been shown to lead to a significantly reduced TH activity and DA synthesis [140, 141]. Quantification of TH-immunopositive neurons in the *substantia nigra* of SN group revealed no statistical differences, in agreement with the negative results from the motor behaviour tasks but contrary to previous studies [118-122, 151]. This could be due to differences in the virus titer or even in the time-point. However, the V1S+SV2 group of SN group, at 4 weeks showed impairment in the limb controlled by the side of injection in the stepping task. In the animals injected with the V1S+SV2 constructs the TH levels in the striatum, presented a tendency of decrease, however this did not reach statistical significance. The ST group revealed no statistical differences in the behaviour tasks as well as in the striatal fiber density, revealing still interestingly a tendency of increase in the TH levels in the striatum in the V1S+SV2 injected animals compared to the Venus-injected animals. The SN and ST groups had respectively 23 and 17 animals, however during the microscopy analysis of the place of injection, only the animals that had a precise targeting and a good expression of Venus fluorescence were chosen for the behavioural analysis and for the TH analysis. This process decreased significantly the number of animals. In the future, and in order to have more reliable results it would be valuable to have a bigger set of animals.

Degeneration to axons and synapses in the striatum has been shown to precede the loss of DA neurons in the *substantia nigra* both in PD patients and in animal models of PD [152]. Evidences from rodent models of PD indicate that degeneration of the neurons starts in the axons, and not in the cell body. In a mouse model of PD dopaminergic presynaptic terminals of the striatum appeared reduced before cell body loss [153]. In our model we observed beaded axons in the striatum of rats of SN group injected with the V1S+SV2 construct, which is a sign of dystrophic axons consistent with reported in viral vector models [119, 122]. Moreover, animals

from the same group injected with the V1S+SV2 construct presented a trend of decrease in the TH levels of striatum, but no difference in the TH+ cells of *substantia nigra,* concordant with the hypothesis that neuronal degeneration starts in the axons. We might need a later time-point in order to see some cell loss or a higher virus titer in order to obtain a more severe phenotype; however our main goal was to visualize the pattern and transport of α-Syn oligomers, and the increase of virus titer is a risky approach since we do not want to overload the system with viral particles and cause severe degeneration.

In a recent study, where rats were injected with AAV carrying α-Syn targeting the *substantia nigra* [122], the authors report TH+ cell loss in the *substantia nigra* of animals injected with V1S+SV2 as well as a reduction in TH+ levels and presence of neuroinflammation in the striatum. Our results contrast with this study, but the apparent differences in the experimental design between the studies could account for the different results. Namely, the AAV serotype, depending on the main goal of the study, different AAV serotypes can be chosen according to their pattern of spread and effectivity. In our study AAV vectors pseudotyped with a serotype 6 capsid were chosen because of their high tropism for nigral dopaminergic neurons [154-156], while in the study by Dimant et al. the AAV used was the AAV serotype 8. The AAV8 is known to be more efficient for widespread gene deliver [157], but it is also neurotoxic to dopamine neurons in the *substantia nigra* at higher vector doses [158]. The difference in the serotype of the AAV used could then explain why using the same kind of approach, these reported results present neuronal cell loss while ours do not. Another factor that can contribute to a difference in the results is the titer of the virus/total viral load per animal; while in our model we used equal viral load for V1S+ SV2 and for Venus ($1{,}25*10^{13}$ gc/ml) control virus, the other group used a different titre for V1S+VS2 ($8{,}3*10^{12}$ gc/ml + $8{,}7*10^{12}$ gc/ml) and for Venus ($1*10^{12}$ gc/ml). In future studies, in order to obtain phenotype, it would be interesting to increase the titre of the virus. However, as said before, this approach needs to be pondered since an increase in the titre of the virus could lead to an overload of the system, and in that case it would be difficult to resolve if the phenotype was due to the α-Syn oligomerization or due to the excess of virus particles.

Since there is still an ongoing discussion about the fluorescence properties of the separate halves of the fluorescence protein [123, 124, 159] it would also be interesting and valuable in the future to have more controls. The two halves of the Venus alone could be separately injected in the same animal (to study if or how the two halves interact without being linked to another protein) or in different animals (in order

to understand better the fluorescent properties of the separate halves) and the two halves of the construct (V1S and SV2) could be separately injected in different animals (in roder to study he fluorescent properties of the constructs).

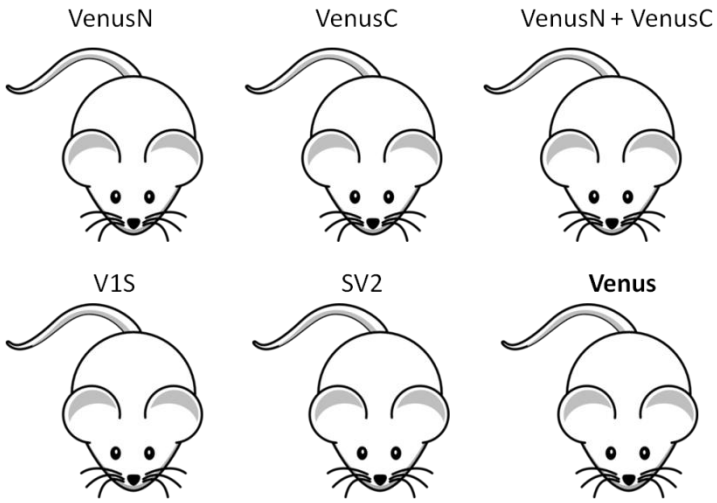


Fig.:4.1 - **Ideal controls**. In an optimal study there should exist 6 different controls. The two halves of the Venus alone could be separately injected in the same animal (VenusN + VenusC) or in different animals (VenusN or VenusC) and the two halves of the construct (V1S and SV2) could be separately injected in different animals. Besides those, it's important to inject Venus alone.

Chapter 5. Conclusion

Viral vector gene delivery has shown great promise in development of novel models replicating human disease conditions in animals. Using this technique we have created a novel animal model, based on over-expression of human α-Syn, which replicates the α-Syn aggregation and pathology seen in PD patients and furthermore it allows the detection of α-Syn oligomers *in vivo*. This study has for the first time shown that AAV6 delievery of α-Syn linked to a fluorescence protein in the motor cortex, striatum and *substantia nigra* is a good approach to study the pathology propagation. The pathology in this model was present as the phosphorylated form of α-Syn. Therefore, this rat model could become very useful for the study of the spreading of the disease and even for the study of therapies that could prevent this process. Although we used human wild-type α-Syn in our study, the gene delivery approach described here may be used to investigate other, mutated forms of α-Syn. Taken together, this model exhibiting α-Syn over-expression localized to motor cortex, striatum and *substantia nigra* provides significant advantages over current models for evaluating the oligomers formation and spreading as well as its effect on the pathology mechanisms observed in PD. Despite the results of the present work, it is still necessary to perform additional studies in order to optimize the viral injection and titre of the virus, and also have a bigger set of animals. It would be also interesting to perform a viral injection of the V1S and SV2 separately in distinct but connected regions of the brain (striatum and *substantia nigra*) in order to see if the two halves meet at some point, and if so, where.

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