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STUDY OF PATHOLOGY PROPAGATION MECHANISMS IN NEURODEGENERATIVE DISEASES

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ΕΥΧΑΡΙΣΤΙΕΣ

Η παρούσα μεταπτυχιακή εργασία εκπονήθηκε στο Κέντρο Βασικής Έρευνας του Ιδρύματος Ιατροβιολογικών Ερευνών της Ακαδημίας Αθηνών την περίοδο “Μάρτης 2018-Φλεβάρης 2019”, ύπο την επίβλεψη του Δρ Βεκρέλλη Κώστα και στα πλαίσια του Προγράμματος Μεταπτυχιακών Σπουδών στις Νευροεπιστήμες της Ιατρικής Σχολής του Πανεπιστιμίου Κρήτης.

Θα ήθελα να ευχαριστήσω θερμά τον Δρ Βεκρέλλη για την επιστημονική του υποστήριξη και καθοδήγηση, καθώς και για την φιλική του διάθεση και το ενδιαφέρον του κατά τη διάρκεια της εργασίας μου. Στα ίδια πλαίσια, ευχαριστώ και την Δρ Καραμπέτσου Αδαμαντία για τη βοήθειά της και συνεργασία της.

Ευχαριστώ θερμά όλα τα μέλη του εργαστηρίου για την άριστη συνεργασία τους και τις όμορφες συνθήκες εργασίας που δημιουργούσαν και ειδικά τον υποψήφιο διδάκτορα Αναγνώστου Δημήτρη, του οποίου η προσφορά ήταν ιδιαιτέρως σημαντική.

Όλα τα μέλη της 3μελούς Επιτροπής, τον Δρ Στεφανή Λεωνίδα και την Δρ Σπανάκη Κλειώ για τον χρόνο τους και την καλή τους διάθεση και συνεργασία κατά την ολοκλήρωση της εργασίας αυτής.

Τέλος, ευχαριστώ θερμά όλα τα αγαπημένα μου πρόσωπα που το κάθε ένα με τον τρόπο του προσέφερε απλόχερα την υποστήριξή και ενθάρυνσή του, όχι μόνο στην εκπόνηση της παρούσας εργασίας, αλλά και καθ’ όλη τη διάρκεια φοίτησής μου. Ιδιαιτέρως ευχαριστώ πολύ τον Δρ Κακλαμάνο Αιμίλιο, του οποίου η ενεργή παρουσία ήταν καθοριστικής σημασίας και του οποίου η βοήθεια και η στήριξη μου δόθηκαν γενναιόδωρα, με ενθουσιασμό και αδειαλείπτως.

Contents

1. Introduction

1.1. A-synuclein

1.1.1. General

A-synuclein is a small soluble protein of 140 amino acids, member of the synuclein family, primarily found in the presynaptic nerve terminals in the central nervous system (HARTIKAINEN, 2017). It has been identified since 1988, when it was isolated from cholinergic vesicles of the *Torpedo californica* ray (Maroteaux et al., 1988). Human α-synuclein is largely sited in the neocortex, hippocampus, substantia nigra, thalamus and cerebellum (Emamzadeh, 2016) and it was originally discovered in the amyloid-plaques of Alzheimer's Disease (AD) patients' brains (Ueda et al., 1993). α-synuclein is intrinsically unstructured, a feature that attributes to the protein the ability to adopt various conformations linked to the given changes in the surrounding microenvironment and so interacting easily with different ligands, such as lipids (Uversky, 2003). It is encoded by the *SNCA* gene and its C-terminal tail regulates its nuclear localisation and its interactive behaviour with metals, small molecules and proteins (Eliezer et al., 2001, Ulmer et al., 2005). Truncation of the protein at the C-terminal induces the formation of aggregates, hinting that such changes might be involved in pathology (Venda et al., 2010). A-synuclein is considered a highly conserved protein, characterized by the presence of the incomplete repetition of the KTKEGV sequence along its N-terminus and by a C-terminus. Structurally, three distinct regions of the protein are identified (**Image1.1**):

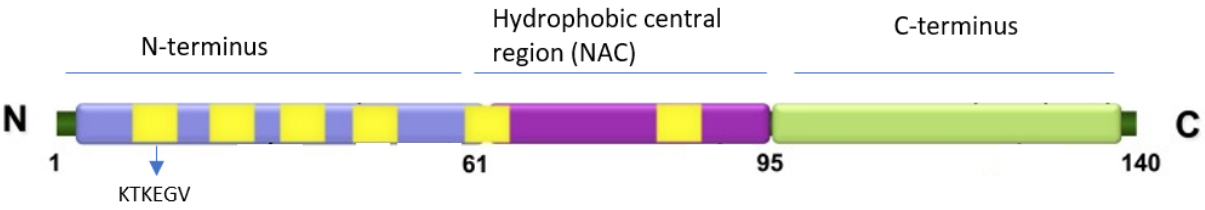


Image 1.1: A schematic representation of α-synuclein structure where its three main regions are depicted. The amphipathic N-terminus (light blue) including the incomplete repeats of the KTKEGV sequence (yellow), the hydrophobic central region (purple) and the C-terminus (green) [adapted from (Tenreiro et al., 2014)].

- Its N-terminus (1-61αα) which consists of 11 residue repeats of KTKEGV thought to be forming an amphipathic alpha-helix and playing a key role on protein binding to membranes and lipids (Perrin et al., 2000). These repeats are conserved not only across species, but also in the different isoforms of the protein and are thought to play a key role in disease.
- Its central region (62-95αα) which comprises the protein's hydrophobic body. The recombinant α-synuclein which is deprived of this region, demonstrates a decreased ability of oligomerisation (Giasson et al., 2001). Furthermore, it contains a domain called 'amyloid binding central domain' (NAC) which is involved in the formation of fibrils, as

well as in the protein aggregating ability (Rajagopalan and Andersen, 2001).

- Its C-terminus (96-140αα) which is the most variable part of the protein (Cookson, 2005). At this region lies the majority of the post-translational modifications, including the phosphorylation, the ubiquitination and the metal binding sites (Brown, 2007, Li et al., 2005, Oueslati et al., 2013).

1.1.2. Localisation

A-synuclein is mainly located in the hippocampus, the olfactory bulb, the striatum and the cerebellum, as well as in some internal organs, such as the heart and the kidneys (Barbour et al., 2008, Mori et al., 2002). Subcellularly, the protein is sited in higher quantities at presynaptic vesicles in the presynaptic nerve terminals without being engulfed in them (Clayton and George, 1999, Iwai et al., 1995). When the protein binds to the vesicles or membranes, its N-terminus is the first to bind to the lipid biolayer, taking the form of an alpha-helix, while the C-terminus remains on a free conformation (Fusco et al., 2014) **(Image 1.2)**. In humans, α-synuclein expression is being regulated during development. During adulthood, its expression is limited to the nerve terminals, whereas during normal ageing it is restricted to the cellular bodies (Raghavan et al., 2004).

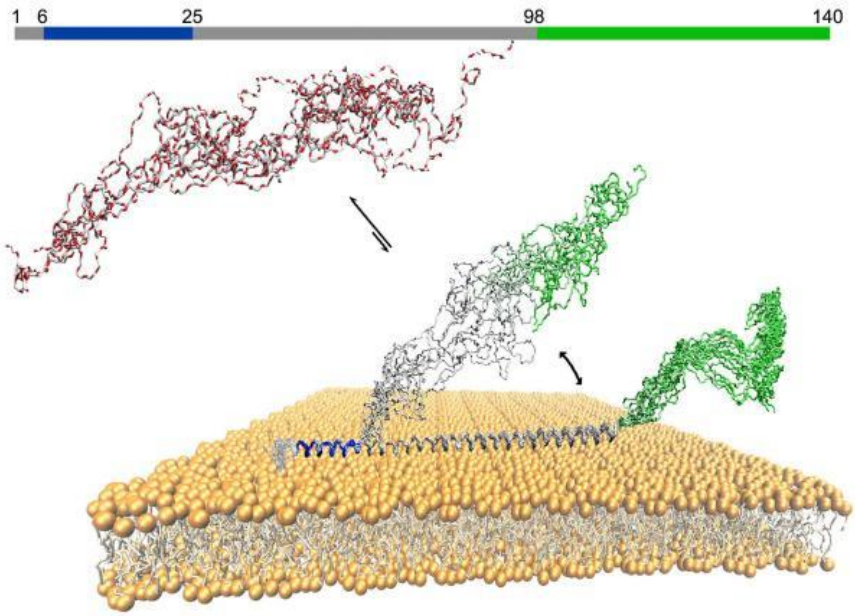


Image 1.2: Schematic representation of the three main domains of α-synuclein before and during its binding to lipid bilayers. The N-terminus (blue) gains an a-helix structure, while binding strongly to the membrane. The central domain (grey), has been suggested to play a key role in the protein's interaction with membranes. The C-terminus (green) retains a free conformation as it does not bind to the membrane [modified by (Fusco et al., 2014)].

1.1.3. Function

The function of α-synuclein is not yet well understood nor entirely unravelled. Nonetheless, research has suggested some attributes to the protein such as an indirect neuroprotection of dopaminergic cells by deactivating nuclear factor-κB which leads to reduction of the proapoptotic kinase PKCδ transcription (Jin et al., 2011). PKCδ has been identified as a key proapoptotic effector in different cell types and mediates dopaminergic neurodegeneration in cellular models of PD (Anantharam et al., 2002, Kanthasamy et al., 2003, Kaul et al., 2003). Inhibition of α-synuclein expression in primary hippocampal glutamatergic neurons, resulted in the reduction of the reserves as well as the numbers, of the presynaptic vesicles (Murphy et al., 2000). However, α-synuclein KO mice, do not exhibit notably altered neurological phenotypes, suggesting that α-synuclein plays no key role in synaptogenesis. This type of KO mice, interestingly show an elevation of dopamine secretion upon electrical stimulation (Abeliovich et al., 2000, Cabin et al., 2002). Additionally, it has been shown that under normal conditions α-synuclein interacts through its N-terminus with dopamine transporter (DAT) leading to decreased cellular dopamine intake (Wersinger et al., 2003, Wersinger and Sidhu, 2003). However, KO mice that lack α-synuclein, exhibit normal DAT activity (Dauer et al., 2002). Moreover, studies have shown that both hyperexpression and loss of α-synuclein in transgenic mice affect the mitochondrial function via various mechanisms (Ellis et al., 2005, Martin et al., 2006).

1.1.4. Mutations

A series of α-synuclein mutations, as well as, the duplications and triplications of the α-synuclein gene *SNCA*, have been shown to promote the formation of pathological α-synuclein formations that lead to familial forms of disease, called synucleinopathies **(Table 1.1).** The most common of these point-mutations are A53T, A30P, E46K, H50Q, G51D and A53E (Appel-Cresswell et al., 2013, Ibanez et al., 2004, Kruger et al., 1998, Lesage et al., 2013, Pasanen et al., 2014, Polymeropoulos et al., 1997, Singleton et al., 2003, Zarranz et al., 2004). Interestingly, most of these mutations have been observed in the 11 residue repeats of KTKEGV in the N-terminus of α-synuclein and are linked mainly to familial Parkinson's Disease (PD) (Appel-Cresswell et al., 2013, Kruger et al., 1998, Polymeropoulos et al., 1997, Zarranz et al., 2004). Additionally, genome-wide association studies have revealed that variants of *SNCA* are associated with sporadic PD (Satake et al., 2009, Simon-Sanchez et al., 2009).

Mutation	Disease		
	Parkinson's Disease	Lewy body dementia	Multiple system atrophy
A53T	X		
A30P	X		
E46K	X	X	
H50Q	X		
G51D	X		
A53E	X		X
Multiple copies of the *SNCA*	X		

Table 1.1: The most common α-synuclein mutations and their linked synucleinopathies.

Interestingly, all the above mentioned mutations have been linked to giving rise to different symptoms and shape the severity of the respective disease phenotype (Nussbaum, 2018) (**Table 1.2**). Moreover, they have been associated mainly with autosomal-dominant forms of the disease (Mullin and Schapira, 2015).

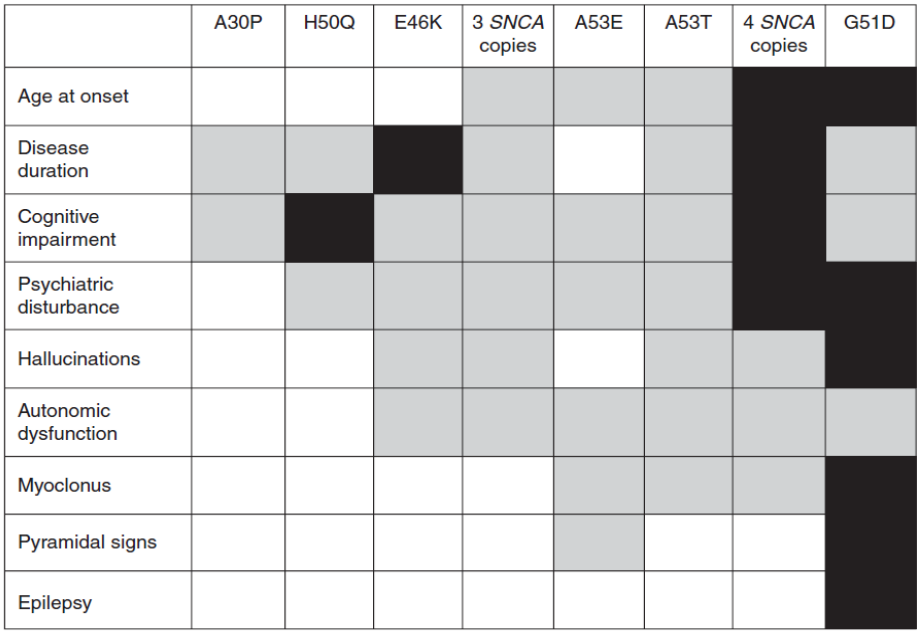


Table 1.2: Outline of clinical features associated with pathogenic SNCA mutations. Color scale from white to black is intended to denote increasing severity. For age at onset: (white) .60 yr, (gray) between 40 and 60 yr, and (black) ,40 yr; for disease duration: (white) .15 yr, (gray) between 10 and 15 yr, and (black) ,10 yr; for all other features: (white) absent, (gray) occasionally present, and (black) constantly present (Nussbaum, 2018).

1.2. Synucleinopathies – Parkinson's Disease

1.2.1. General

Synucleinopathies consist a group of neurodegenerative disorders in which the abnormal accumulation of α-synuclein aggregates is shared as a basic trait and observed in the neurons and/or glial cells (McCann et al., 2014). These aggregates have fibrillar formation and are insoluble. Since α-synuclein is the main protein component of Lewy bodies (LBs) **(Image 1.3)**, disorders that share the feature of LBs, such as Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), fall under the category of synucleinopathies.

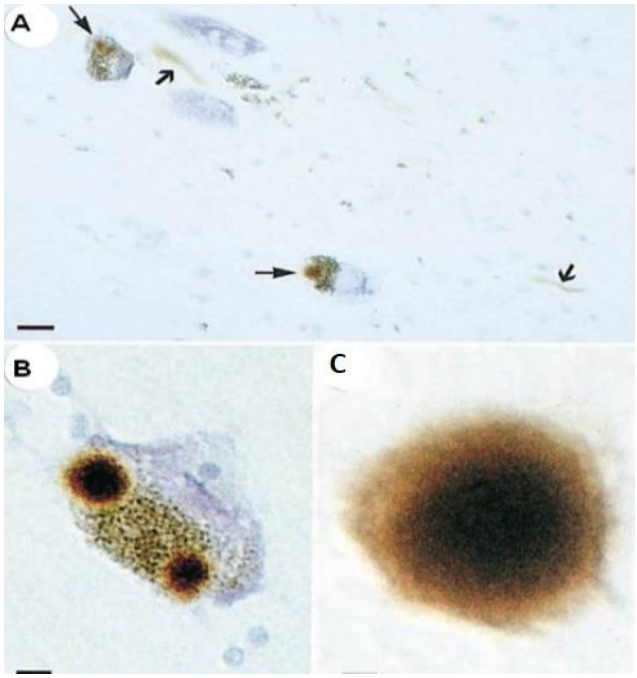


Image 1.3: A-synuclein aggregates in the substantia nigra of PD patients. **A)** Nerve cells positively stained for α-synuclein. Thin arrows pointing at Lewy bodies, thick arrows pointing at Lewy neurites (scale 20μm). **B)** Magnified neural cell with two α-synuclein positively stained Lewy bodies (scale 8μm). **C)** Extracellular Lewy body stained positive for α-synuclein (scale 4 μm) (Spillantini et al., 1997).

Parkinson's disease (PD) is an irreversible progressive neurodegenerative disorder and the most common synucleinopathy. It has been estimated that by 2030 there will be more than 8 million patients suffering from the disease (Dorsey et al., 2007). The disease affects mainly the motility of the patient, manifesting most commonly through resting tremor, bradykinesia and muscular stiffness. There is a plethora of symptoms associated with PD that have a non-motor nature including cognitive decline, hallucinations, depression, sleep disorders, olfactory decline, pain, as well as, dysfunction of the autonomic nervous system, such as constipation, hypotension, increased urinating frequency and perspiration (Dauer and Przedborski, 2003, Simuni and Sethi, 2008) **(Image 1.4)**. PD correlates with aging and among new PD patients, only 4% are adults younger than 40 years, when the disease is characterised as early-onset PD. At the ages of 65-69 years the percentage of PD patients accounts for 0.6% of the population, a percentage which rises up to 2,6% at 85-89 years (de Rijk et al., 2000).

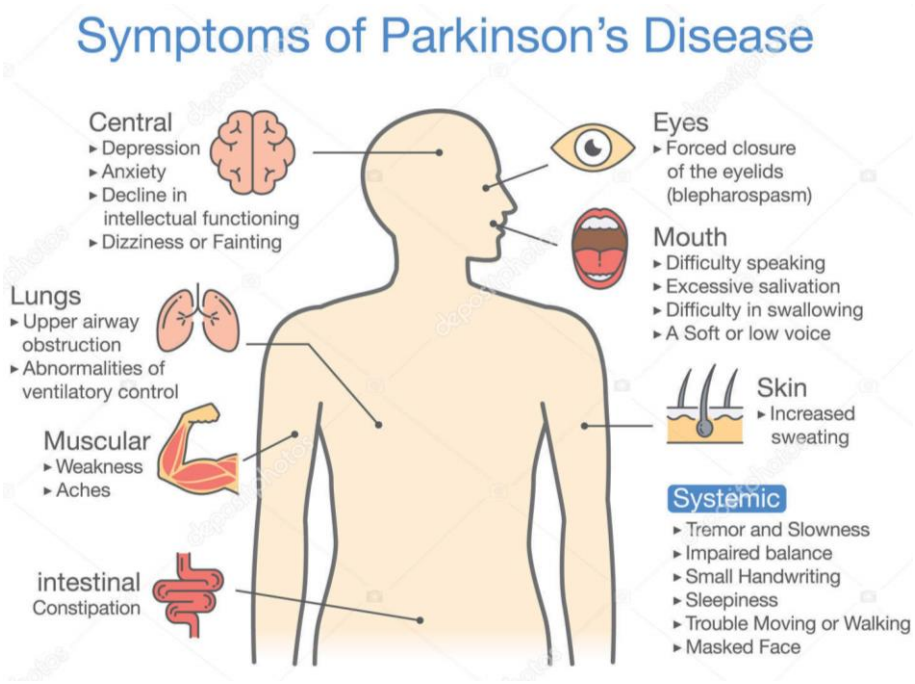


Image 1.4: Main PD symptoms linked to different organs-systems (adapted from AmericanHomeCare.org)

The key pathophysiological trait of the disease is the extensive dopaminergic neuronal death in the substantia nigra pars compacta (SNpc). These neurons project to the striatum, where dopamine is released. As the neuronal death proceeds, the circuitry is interrupted leading to deficits in motor synchronisation (Jankovic, 2008). At the regions of the brain that are affected by the disease, cytoplasmic protein inclusions called Lewy bodies (LB) are found **(Image 1.5)**. These inclusions are rich in aggregates of α-synuclein and in other proteins, parts of the ubiquitin-proteasome degradation system, molecular protein chaperones and lipids (Forno, 1996, Halliday et al., 2005, Spillantini et al., 1998, Uryu et al., 2006, Uversky, 2007). The majority of PD cases are characterised as sporadic, however about 10% of the diagnoses are found to have a genetic basis. Aging is the greatest risk factor for developing PD. Epidemiology, as well as life style, contribute to the expression of the disease. A series of different genes, like the *SNCA,* have been associated with familial forms of PD (autosomal-dominant or recessive) or with an increased susceptibility towards PD (Houlden and Singleton, 2012, Mullin and Schapira, 2015, Wirdefeldt et al., 2011).

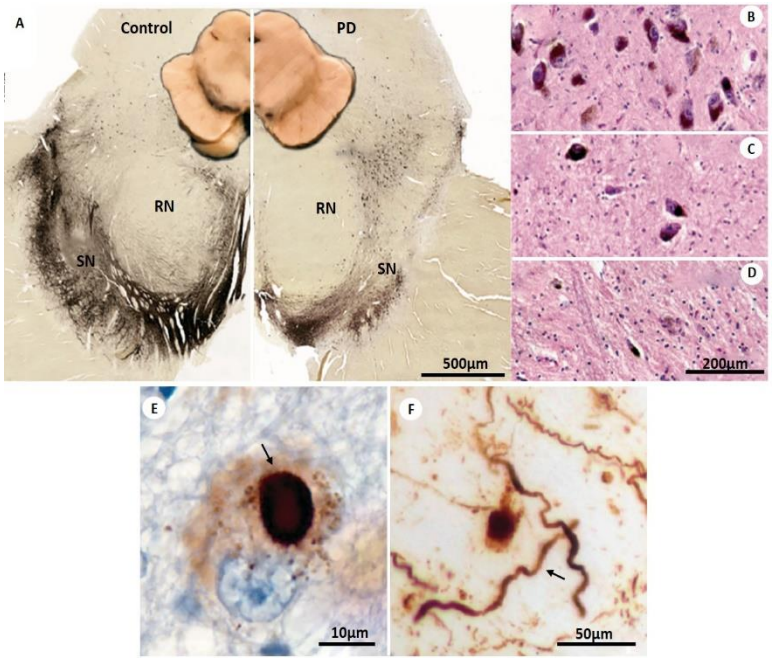


Image 1.5: Pathological changes in SN in PD. A) PD brain section depigmented compared to control due to dopaminergic neuronal loss, stained against tyrosine hydroxylase. **B-D)** Progressive moderate (C) and severe (D) loss of pigmented neurons in the SN of PD brain section in comparison to the healthy control (B). **E-F)** Localisation of α-synuclein in the cytoplasmic inclusions (E-arrow) and neuronal axes (F-arrow) in Lewy neurites [modified by (Poewe et al., 2017)].

1.2.2. Pathophysiology

Under specific circumstances, α-synuclein can be toxic for the cells by affecting different pathways. The ability of α-synuclein to inhibit the main pathways responsible for protein degradation plays an important role in the induction of toxicity, since it drives accumulation and further oligomerisation of α-synuclein (Wong and Krainc, 2017). It has been shown that α-synuclein oligomers can inhibit proteasomal activity and drive pathogenesis due to protein accumulation both of α-synuclein and other proteins (Emmanouilidou et al., 2010b, McNaught et al., 2002). Additionally, the mutated α-synuclein forms A53T and A30P, as well as, the modified-after its interaction with dopamine molecules- α-synuclein , bind strongly to lysosomal receptor LAMP2a inhibiting the lysosomal pathway and leading to further accumulation of α-synuclein and of other substrates (Cuervo et al., 2004, Martinez et al., 2003, Xilouri et al., 2016). Hyperexpression of α-synuclein can contribute to the macroautophagy activation, leading to neuronal death (Xilouri et al., 2009). Moreover, hyperexpression of mutated A53T α-synuclein induces oxidative stress and increases ROS levels, effects which lead in turn to mitochondrial damages and cell death (Smith et al., 2005).

Under normal conditions, α-synuclein monomers tend to fold and form intermediate unstable conformations, which in turn can lead to the formation of high molecular weight toxic aggregates. This procedure is controlled by homeostatic mechanisms at a cellular level in order to prevent toxicity (Peelaerts and Baekelandt, 2016). Such mechanisms include the proteasome and lysosome. At a cellular level, the aggregates are colocalised with components such as the proteasomal subunit 20S, the ubiquitin and p62 protein, the LC3, a protein indicator of autophagy (Luk et al., 2009). However, in pathology it has been observed that after α-synuclein levels reach a critical threshold, the formation of stable oligomers and amyloid fibrils is favoured. These abnormal formations demonstrate the ability to attract and attach extra monomers of the protein, thus promoting the formation of larger and more stable aggregates (Lorenzen et al., 2014, Uversky et al., 2001) **(Image 1.6)**. There is strong evidence supporting that these aggregates play a key role in the pathogenesis of degenerative diseases with LB (Lashuel et al., 2013). Additionally, biochemical and imaging studies of LB, isolated from human patients' brains suffering from synucleinopathies, also show different forms of α-synuclein aggregates (Tsigelny et al., 2008). Furthermore, it has been suggested that α-synuclein oligomers, interact with the plasma membrane creating pores through which Ca^{2+} ions enter from the extracellular space. Increased Ca^{2+} concentration damages the neurons (Danzer et al., 2007, Furukawa et al., 2006, Tsigelny et al., 2012). The A30P and A53T mutated forms of α-synuclein tend to form protofibrils increasing the membrane permeability, reinforcing the wild-type protein toxicity (Lashuel et al., 2002).

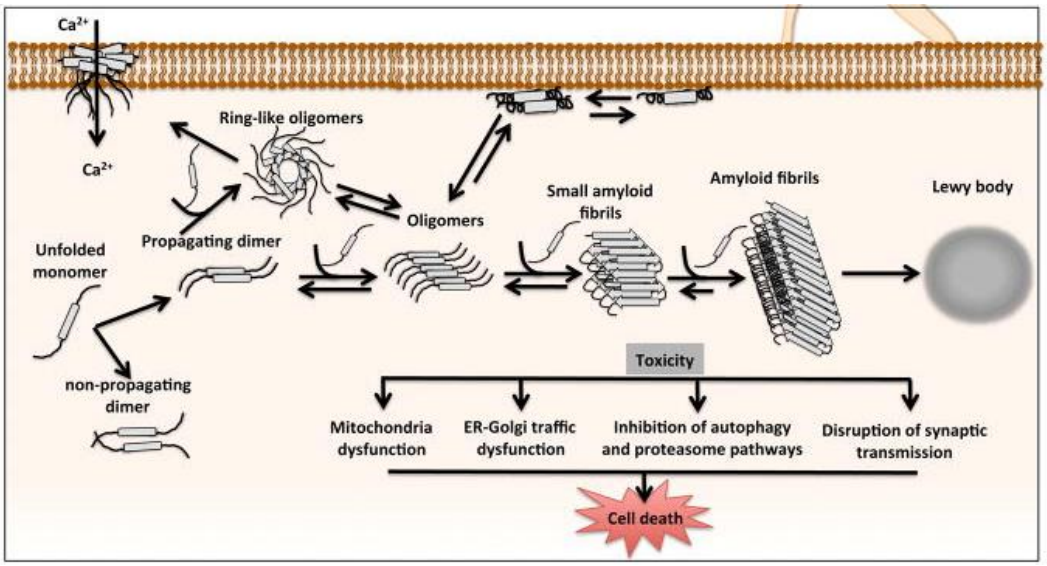


Image 1.6: Suggested model of α-synuclein aggregation. Interaction of unfolded α-synuclein monomers leads to the formation of two different dimers: parallel dimers that have the ability to propagate and anti-parallel dimers that lack this ability. This process, which can take place either on the membrane or in the cytoplasm of the neuron, is controlled under a dynamic equilibrium. The addition of unfolded monomers on propagating dimers of α-synuclein leads to the growth of these dimers and the generation of ring-like oligomers as well as plain oligomers. On the one hand, the interaction of ring-like oligomers with the cellular membrane result in the formation of trans-membrane pores that in turn result in abnormal intracellular calcium influx. On the other hand, soluble monomers α-synuclein are added to cytoplasmic oligomers, forming first small and then longer amyloid fibrils. Further accumulation of such fibrils results in the formation of intracellular inclusions called Lewy Bodies (LB). During this procedure, the intermediate forms of α-synuclein aggregates (fibrils and oligomers) are highly toxic via different mechanisms (mitochondrial dysfunction, endoplasmic reticulum-Golgi trafficking deregulation, protein degradation and/or synaptic transmission). The result of these effects is regarded to be neurodegeneration and neuronal loss. [modified by (Lashuel et al., 2013)]

Respectively, studies in mouse models of PD have detected the development of pathological α-synuclein high molecular weight aggregates (Luk et al., 2012b). Inside the cell these α-synuclein toxic aggregates are usually found either in insoluble cytoplasmic forms (Lashuel et al., 2013) or in oligomeric and ring-like forms which can interact with and cross the cellular membrane (Lashuel et al., 2002).

1.2.3. Propagation mechanisms

The intricate network of intracellular propagation of the pathological fibrillar forms of α-synuclein includes a) uptake, b) copying of the pathological form acting as a mold, c) transportation among neurons and glia, d) secretion (**Image 1.7**). The mechanisms involved are:

Transmembrane receptors and extracellular proteins

Fibrilar forms of α-synuclein bind on the surface of various cell types. Their uptake is probably mediated from transmembrane receptors and proteins of the membrane. Recently, it has been shown that α-synuclein fibrils specifically bind to the LAG-3 receptor (lymphocyte activating gene-3), which is expressed on the surface of neurons. Endocytosis via LAG-3 is mediated from clathrins, whereas different substrates, such as tau protein, exhibit weak non-specific binding (Mao et al., 2016). Moreover, it has been shown that α-synuclein fibrils can bind directly to the membrane, causing a rapture and thus allowing for more fibrillar forms to be uptaken (Jiang et al., 2017).

Tunneling nanotubes, (TNTs)

The TNTs are a suggested way of intracellular transport. Actin fibrils form a tube which connects the cytoplasm of two different cells, allowing the exchange of cellular content. The interaction includes the connection of either the same or different types of cell types, eg neurons and astrocytes (Abounit et al., 2016, Sun et al., 2012). It has been shown that lysosomes with fibrillar forms of α-synuclein are transported via TNTs and induce aggregation of the endogenous protein to the receiving cell. Furthermore, the accumulation of α-synuclein at the astrocytes inhibits the lysosome and induces damage on the mitochondria. The astrocytes send TNTs to other healthy astrocytes in order to be released from the α-synuclein (Dieriks et al., 2017, Rostami et al., 2017)

Endocytosis

In cells, the uptake of recombinant fibrils is inhibited at low temperature (4^0C). The same effect produces the simultaneous expression of the mutated form of dynamin-1, a small GTPase, which is necessary for endosomal formation (Lee et al., 2008). Moreover, in primary hippocampal neuronal cultures the simultaneous incubation with recombinant fibrils and wheat germ agglutinin (WGA), which accelerates endocytosis, lead to increased pathology of phosphorylated α-synuclein inclusions. WGA-specific inhibitor decreases drastically the pathological accumulation of α-synuclein (Volpicelli-Daley et al., 2011).

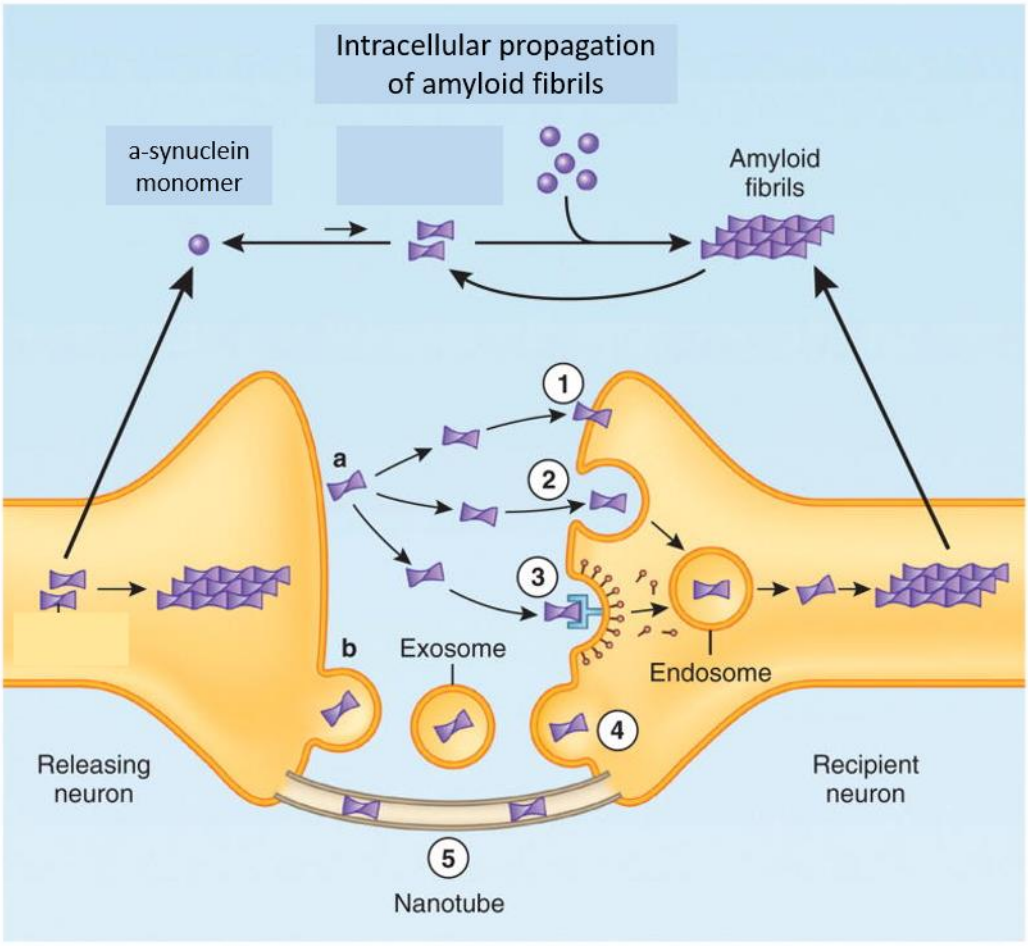


Image 1.7: Proposed mechanisms of intracellular α-synuclein fibrils propagation. Pathological form of α-synuclein (oligomers, fibrils) which are formed from the aggregation of the endogenous protein (primary aggregation), or from the shedding of amyloid aggregates and the subsequent nucleation of the monomer to a new cell (secondary aggregation), can be transferred between cells: 1) Through direct binding on the membrane, 2) Through endocytosis, 3) Through a receptor, 4) Through exosomes (either as an inclusion or bound to the membrane), 5) Through tunnelling nanotubes (TNTs) which create a bridge for the cytoplasmic interchange between cells [adapted from (Guo and Lee, 2014)].

Glia

Astrocytes uptake recombinant α-synuclein fibrils and lead them towards lysosomal degradation. Contrary to neurons, astrocytes degrade effectively the α-synuclein fibrils. However, when their degradation ability is saturated, due to incomplete digestion, there is mitochondrial dysfunction and cytotoxicity (Lindstrom et al., 2017). Pathological forms of α-synuclein bind to microglia and oligodendrocytes and become phagocytosed subsequently (Fellner et al., 2013, Ihse et al., 2017). Additionally, pathological forms of α-synuclein activate glia cells resulting in the secretion of inflammatory mediators (pro-inflammatory cytokines) which contribute to a further toxic environment in the brain (Buck et al., 2015).

Secretion

Interestingly, while it was thought that the different forms of α-synuclein remain in the cytoplasm, α-synuclein has been detected in the cerebrospinal fluid and the serum of both healthy human subjects and PD patients (El-Agnaf et al., 2003, Miller et al., 2004). Additionally, human neuroblastoma cell lines, as well as, primary cortical neuron cultures that overexpress human α-synuclein have been shown to secrete monomeric and oligomeric forms of α-synuclein (Emmanouilidou et al., 2010a, Lee et al., 2005). Although, the exact mechanism of α-synuclein secretion has not been elucidated yet, it has been suggested that part of the cytoplasmic protein is engulfed in vesicles and subsequently excreted via exocytosis (Lee et al., 2005). Furthermore, monomeric and oligomeric forms of α-synuclein have also been shown to enter in or bound on the membranes of exosomes so to be transported to the extracellular space, affecting the viability of healthy neighbouring neurons (Danzer et al., 2012, Emmanouilidou et al., 2010a, Vekrellis et al., 2011).

Dysfunction of the autophagy mechanism leads to increased exosomal secretion (Alvarez-Erviti et al., 2011). In a recent study, exosomes from PD patients and Lewy body dementia patients were isolated. In their carriage, pathological forms of α-synuclein were detected and could act as molds for the accumulation of α-synuclein monomers in the cell targets (Stuendl et al., 2016).

This exosomal secretion of α-synuclein aggregates agrees with the prion-like propagation model of α-synuclein. This model posits that once α-synuclein aggregates have formed in a neuron, they can be transported intra-axonally to other brain regions, be released into extracellular space, be taken by neighbouring neurons and seed aggregation of endogenous α-synuclein once inside their new cellular host (Angot et al., 2010, Brundin et al., 2010). The model suggested by Braak *et al*. states that α-synuclein pathology gradually engages more brain regions as the disease progresses (Braak et al., 2003) (**Image 1.8**)

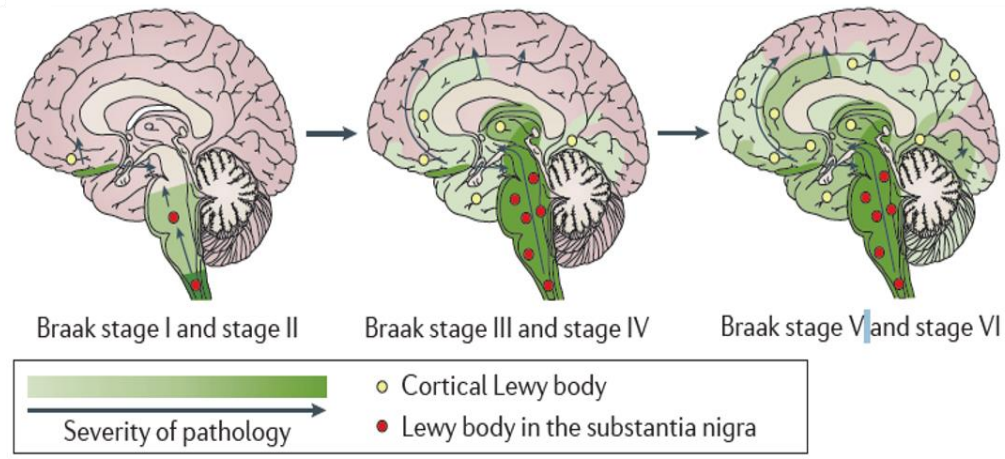


Image 1.8: The theorized progression of α-synuclein aggregation in PD. α-synuclein inclusions occur in cholinergic and monoaminergic lower brainstem neurons in asymptomatic cases (Braak stage I and stage II), infiltrate similar neurons in the midbrain and basal forebrain in those with the motor symptoms of PD (Braak stage III and stage IV), and then are found later in limbic and neocortical brain regions with disease progression (Braak stage V and stage VI) (Braak et al., 2003, Poewe et al., 2017)

As mentioned earlier, α-synuclein is the main component of LB which have been shown to spread between cells and cause disease in other brain regions in a prion-like manner. In two studies, PD patients were treated with grafts of embryonic dopaminergic neurons in their striatum aiming to the relief of their symptoms. Post mortem analysis (11-16 years later) showed the presence of α-synuclein aggregates resembling LB. The conversion of the healthy grafts to pathological neurons as the disease progressed, was attributed to the transference of the pathological form of the protein from the original patient tissue to the grafts and the extended gliosis observed (Kordower et al., 2008, Li et al., 2008). The same cell-to-cell transmission was also observed for different forms of α-synuclein (independently of LBs) via multiple mechanisms such as endocytosis, direct penetration, transynaptic transmission and via membrane receptors (Lashuel et al., 2013). This cell-to-cell spread of pathologic α-synuclein further supports the similarities between α-synuclein pathology and prion pathology (Desplats et al., 2009). Interestingly, it has very recently been shown in vitro, that astrocytes with impaired chaperon mediated autophagy due to mutations in LRRK2 accumulate α-synuclein aggregates which can then be transferred to nearby neurons and lead to neurodegeneration (diDomenico et al., 2019).

1.2.4. Serine S129 phosphorylation

There has been observed a variety of post-translational alterations of α-synuclein. The most common one, is that of the phosphorylation of the protein which levels are linked directly to the progress of PD (Fujiwara et al., 2002, Zhou et al., 2011). Studies have shown that almost 90% of the insoluble α-synuclein accumulated in DLB patient brains is the phosphorylated protein at the serine S129. The same phosphorylated protein has been observed also in PD and MSA patients' brains (Anderson et al., 2006, Nishie et al., 2004). Although this type of phosphorylation seems to affect α-synuclein oligomerisation, hence the toxicity levels, in PD models, it is still unclear whether the phosphorylation has an enhancing or inhibiting effect on the protein aggregation, since the studies undertaken exhibit contradicting results. In healthy human brains, the percentage of the phosphorylated protein drops to only 4% and it is found primarily in monomers. This percentage can rise with age (McCormack et al., 2012). The low percentage of the phosphorylated α-synuclein in healthy states could be related to its high pasted degradation due to its phosphorylated state. Indeed, when synthesis of the protein was inhibited in SHSY-5Y cells, the phosphorylated α-synuclein depicted a half-life of approximately 60 minutes, compared to the 240 minutes of the total protein, showing that the degradation mechanisms are targeted selectively to the phosphorylated state. Both proteasomal and lysosomal inhibition resulted in the accumulation of the phosphorylated α-synuclein in SHSY-5Y cells in primary cultures of cortical rat neurons (Machiya et al., 2010).

There is contradicting evidence about the role of the S129 phosphorylation of α-synuclein on the induction of toxicity. The evidence comes from animal models of PD. Viral hyperexpression of the mutated S129A α-synuclein in the SN of rats, resulted in the formation of pathological inclusions, thus in increased toxicity of the dopaminergic neurons. Whereas, the toxicity induced from the wild type sequence is smaller. Hyperexpression of the mutation S129D, which mimics phosphorylation, results in fewer, but larger aggregates and it does not induce toxicity (Azeredo da Silveira et al., 2009). Respective viral hyperexpression of the S129A α-synuclein, led to early loss of dopaminergic neurons and decreased dopamine in the striatum in comparison to the wild type protein. Agreeing with the previous mentioned study, this one also showed that hyperexpression of S129D α-synuclein does not lead to toxicity (Gorbatyuk et al., 2008). Contradicting these results, another study performed viral hyperexpression of both wild type α-synuclein and the mutated S129D and S129A, exhibited no difference in the inclusions formation and the induction of toxicity (Kuwahara et al., 2012).

Hyperexpression of both mutated S129D α-synuclein and the wild type in *D.melanogaster* led to dopaminergic neuronal death. Contrary to mice, hyperexpression of mutated S129A showed no toxicity. It was concluded that phosphorylation is necessary for toxicity induction. Further confirmation came from the simultaneous hyperexpression of the homologous to the α-synuclein, kinase GRK2, in drosophila, where the normally phosphorylated α-synuclein led to the same increased toxicity, as the hyperexpression of S129D α-synuclein (Chen and Feany, 2005).

On a different approach, A53T α-synuclein was virally hyperexpressed alongside the kinase GRK6 in the SN of rats. The phosphorylation of A53T α-synuclein on the residue S129 led to early increased toxicity of the dopaminergic neurons, compared to the hyperexpression of A53T α-synuclein. Moreover, hyperexpression of the double mutated sequence A53T/S129A did not affect toxicity compared to the A53T (Sato et al., 2011). Simultaneous phosphorylation of the wild type α-synuclein alongside the kinase PLK2, led to the reduction of α-synuclein aggregates in the dopaminergic neurons of rats. Furthermore, hyperexpression of PLK2 restricted the cell death, as well as, the motility deficits that the rats exhibited due to the hyperexpression of the wild type α-synuclein. This observation was related to the selective lysosomal degradation of α-synuclein, which was lead from its natural contact with the PLK2, thus reducing its levels (Oueslati et al., 2013).

On an alternative approach for the study of the role of S129 phosphorylation, the activation of the phosphatase PP2A, in transgenic mice hyperexpressing the wild type α-synuclein, led to significant reduction of both the α-synuclein aggregates and the phosphorylated protein. The reduction of the levels of the phosphorylated α-synuclein, was accompanied by improved neuronal function, reduced astroglioses and improved motility in mice. This study is of critical significance regarding the pathogenicity of the S129 phosphorylation on α-synuclein, due to the therapeutic

impact of its reduction (Lee et al., 2011). Another very important study confirming the important role of S129 phosphorylation in the pathogenicity of α-synuclein showed that phosphorylated PFF (P-PFF) injection in mouse brains promoted pathological α-synuclein accumulation in the SN of the mice and impaired their motor coordination. This effect was much stronger in comparison to the one observed after injection with non-phosphorylated PFF (wt-PFF). Moreover, P-PFF injections induced a more robust seeding of α-synuclein pathology in healthy cortical regions in comparison to wt-PFF injection. Finally, primary neurons showed significantly increased uptake of P-PFF in comparison to wt-PFF (Karampetsou et al., 2017). All these observations strongly support the critical role of S129 phosphorylation of α-synuclein in enhancing its pathogenicity.

1.2.5. Degradation of α-synuclein

Regarding its degradation, it has been proposed that both the proteasome and the lysosome play a key role for α-synuclein (Cuervo et al., 2004, Webb et al., 2003). When proteasomal activity of the SN in rats was inhibited, α-synuclein formed aggregates and dopaminergic neurons showed degradation (McNaught et al., 2002). A-synuclein oligomers are directed to proteasome for degradation and result in proteasomal inhibition (Emmanouilidou et al., 2010b), whereas lysosomal degradation via autophagy by the lysosome is seen in wild type α-synuclein monomers (Vogiatzi et al., 2008). Mutations in the gene encoding for leucine-rich repeat kinase 2 (LRRK2) are responsible for defective autophagy, by binding to LAMP2, the receptor responsible for chaperone-mediated autophagy, resulting in defective α-synuclein degradation and consequent accumulation, leading to cell death and eventual PD pathogenesis (Cookson, 2017, Orenstein et al., 2013). Notably, such mutations in LRRK2, are responsible for an autosomal-dominant form of PD, accounting for 5% of the familial cases and 2% of the sporadic ones (Gilks et al., 2005, Paisan-Ruiz et al., 2006).

1.2.6. Animal models for studying the propagation of α-synuclein pathology

Injection of synthetic pre-formed fibrils (PFF) of α-synuclein in the brain and the induction and propagation of pathology, constitutes the most recent model on intracellular transport. Injection of PFF, induces the formation of pathological inclusions and the progressive aggregation of the endogenous protein and its gradual propagation in the brain (Abdelmotilib et al., 2017, Bernis et al., 2015, Luk et al., 2012a, Luk et al., 2012b, Paumier et al., 2015, Peelaerts et al., 2015).

Accumulation of the pathological inclusions, having as a main trait the positive staining against the antibody of the phosphorylated α-synuclein, it is often accompanied by the death of dopaminergic neurons, reduced dopamine levels in the striatum and motor deficits, that are directly related to the timepoint of the animals'

analysis, since the progression of pathology unfolds in a time-dependent manner (Luk et al., 2012a, Sacino et al., 2013) **(Image 1.9)**. The expression of endogenous α-synuclein is a prerequisite for the formation of pathological inclusions, given the absolute absence of pathology in mice that are deprived of the endogenous protein expression (Osterberg et al., 2015).

Moreover, injection of PFF of α-synuclein, both in the musculature and intravenously, penetrating the blood-brain barrier, induces pathological inclusions which are identified in the brain (Ayers et al., 2017, Sacino et al., 2014). According to Braak's model, which places the commencement of pathology in PD at the intestine and the olfactory bulb, injection of PFF of α-synuclein in the intestinal wall and the olfactory bulbs, leads to the pathology of the phosphorylated α-synuclein inclusions in the brain (Breid et al., 2016, Rey et al., 2018, Uemura et al., 2018). Observation of the mice after the injection in the olfactory bulbs, shows that the aggregates are transported slowly, in a time point of 1 year, to anatomically connected regions, as well as, to distal ones (more than two synapses farther from the point of injection). At the same time, the animals exhibit olfactory deficits. Gradually, the pathology appears in the dopaminergic neurons of the SN. This model is perceived that mimics better the clinical image of PD in its precursor form, since hyposmia in patients occurs some years before the onset of the motor deficits (Rey et al., 2016).

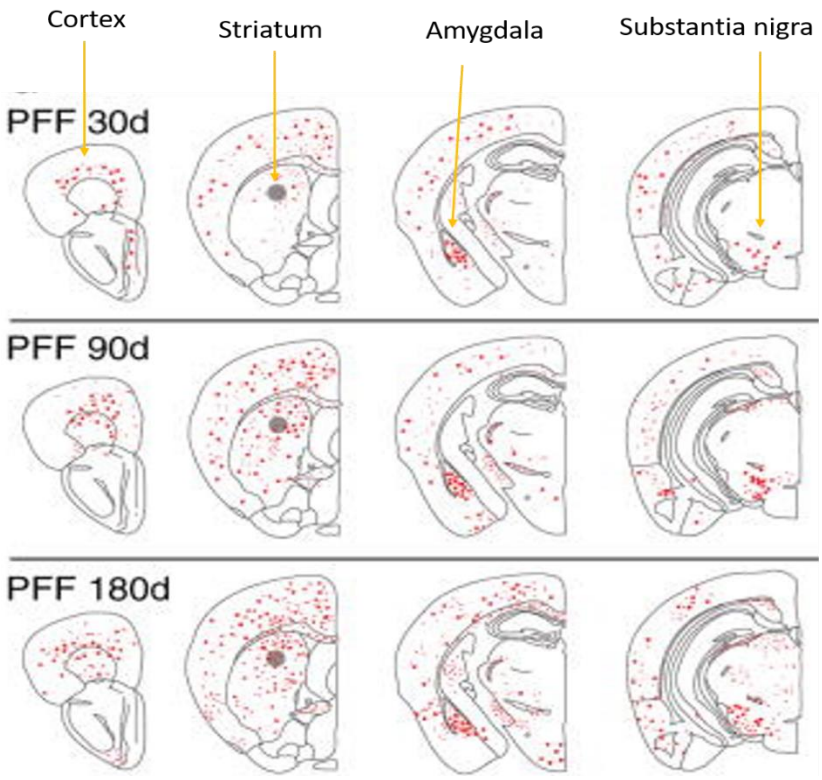


Image 1.9: Mapping of the development of pathological inclusions of α-synuclein in mouse brain. Distribution of the phosphorylated α-synuclein inclusions (red), in mouse brain, following injection of pre-formed fibrils of α-synuclein (PFF) in the striatum (grey dot). Animals were analysed after 30/90/180 days of injection. Pathology can bre traced in the cortex, striatum, amygdala and substantia nigra, while rising overtime (180days). Arrows indicate respective brain region [Adapted from (Luk et al., 2012a)]

1.2.7. Immune system and Parkinson's Disease

1.2.7.1. Innate immunity

Extensive research has proposed again and again the key role that immunity plays in synucleinopathies, hence in PD. As early as in 1988, activated microglia was detected closely to degenerated dopaminergic neurons in post mortem tissue of PD patients, giving the first evidence of the participation of the immune system in PD (McGeer et al., 1988). Additionally, the MHC-II activation marker was linked to the α-synuclein aggregates (Croisier et al., 2005). Later on, there were more studies confirming the presence of glia, as well as, the presence of activated astrocytes in PD patients' SN (Braak et al., 2007, Cabezas et al., 2014). Additionally, neuromelanin release from the degenerated dopaminergic neurons can be characterised as an extra factor inducing the accumulation of microglia in PD patients' SN (Halliday et al., 2005, Zhang et al., 2011).

The secreted α-synuclein is phagocytosed by microglia. The aggregated forms of the protein that are released from the degenerated neurons, act as an activation signal for the microglia, with simultaneous secretion of pre-inflammatory factors (Zhang et al., 2005). Despite the still not completely identified role of astrocytes, it has been proposed that the aggregation of α-synuclein at then, initiates the assembly of activated glial cells (Halliday and Stevens, 2011). Moreover, it has been shown that microglia have the ability to take in fibrillar forms of α-synuclein (Barcia et al., 2011). The presence of α-synuclein, sensitises the glia against further toxic inputs, both from the brain and from the periphery.

Stereotactical injection of recombinant α-synuclein in the SN of mice, lead to increase of inflammatory markers and activation of microglia (Couch et al., 2011). Additionally, sterotactical injections with P-PFF in the SN of mice markedly decreased the percentage of $CD45^{high}CD11b^{+}$ macrophages that infiltrate the CNS. Macrophage infiltration in CNS is regarded as an inherent protective mechanism against neuroinflammation/ neurodegeneration, which therefore suggests that P-PFF might abolish this protective effect from the mouse brain at least partially (Bossu et al., 2015, Karampetsou et al., 2017). Finally, P-PFF injections also significantly decreased IL-10 production by both macrophages and microglia in mouse CNS, in comparison to mice injected with wt-PFF or untreated mice, suggesting a decreased capacity of the animal to activate protective immunosuppressive mechanisms (Karampetsou et al., 2017).

1.2.7.2. Adaptive immunity

It has been shown that the participation of cells from the peripheral lymphatic system, can contribute to the progression of PD. Under normal conditions, the presence of T lymphocytes in the brain is negligible. In PD patients' SN there are increased levels of these lymphocytes, specifically of CD4+ and CD8+ (Brochard et al., 2009). Specific peptides that are seated at the N-terminus and C-terminus of α-synuclein, can act as antigen epitopes, inducing a cytotoxic response to T lymphocytes, which were isolated from PD patients (Sulzer et al., 2017). Increased levels of T lymphocytes have been detected in PD models and their presence correlates to the neurodegeneration (Theodore et al., 2008). In an MPTP mouse model, deletion of $CD4^+$ cells, decreased significantly cellular death (Brochard et al., 2009). The same conclusion was supported by another study, showing that mice depleted from B and T lymphocytes, exhibited resistance against the MPTP induced toxicity (Benner et al., 2008).

1.2.7.3. Inflammation

Activation of the innate immunity leads to the production and release of pro-inflammatory mediators, such as cytokines and chemokines, which create a toxic environment for the neurons. Pro-inflammatory cytokines, such as TNF-α, interleukins IL-1β, IL-6, interferon IFN-γ and chemokines CCL3 and CCL5 are found in elevated concentrations in PD patients' cerebrospinal fluid and blood, as well as in intestinal tissue biopsies (Allen Reish and Standaert, 2015, Brodacki et al., 2008, Devos et al., 2013, Mogi et al., 1994). Respective increase of the cytokines has been identified in intestine biopsies of PD patients (Devos et al., 2013).Moreover, PD animal models also exhibit increased inflammation markers and secretion of pro-inflammatory agents, like TNF-α (Ling et al., 2006, Wu et al., 2002, Karampetsou et al., 2017).

All the above help confidently conclude that neuroinflammation plays a critical role to the development and progression of PD. In recent years, the promising pathway of administrating growth factors for supporting the survival of the remaining neurons, as well as enhancing the growth of immature neurons, has justifiably been gaining weight and capturing the interest of science (Stayte et al., 2015). Transforming growth factor β superfamily (TGF-β) is a pleiotropic cytokine with potent regulatory and inflammatory activity (Li and Flavell, 2008a, Li and Flavell, 2008b). One such very promising and still not well studied cytokine is ActivinA.

1.2.8. ActivinA

ActivinA is, as mentioned above, a member of the TGF-β superfamily essential in embryonic development, hematopoiesis and stem cell pluripotency (Kariyawasam et al., 2011). Like most members of the TGF-β superfamily, ActivinA mediates its biological effects through complex of transmembrane receptor serine/threonine kinases. ActivinA initially binds to ActivinA receptors type II (ActRIIA or ActRIIB) and then recruits ActivinA receptor, type IB (ALK-4) (Abe et al., 2004, Larsson and Karlsson, 2005). ALK-4 interacts with and phosphorylates adaptors of SMAD family member 2 and 3 (SMAD2 and SMAD3). Subsequently, SMAD family member 4

(SMAD4) bounds to phosphorylated SMAD2 and SMAD3 and this complex is translocated to the nucleus (Abe et al., 2004). After translocation into the nucleus, SMAD2 and SMAD3 may activate transcription of different genes (**Image 1.10**).

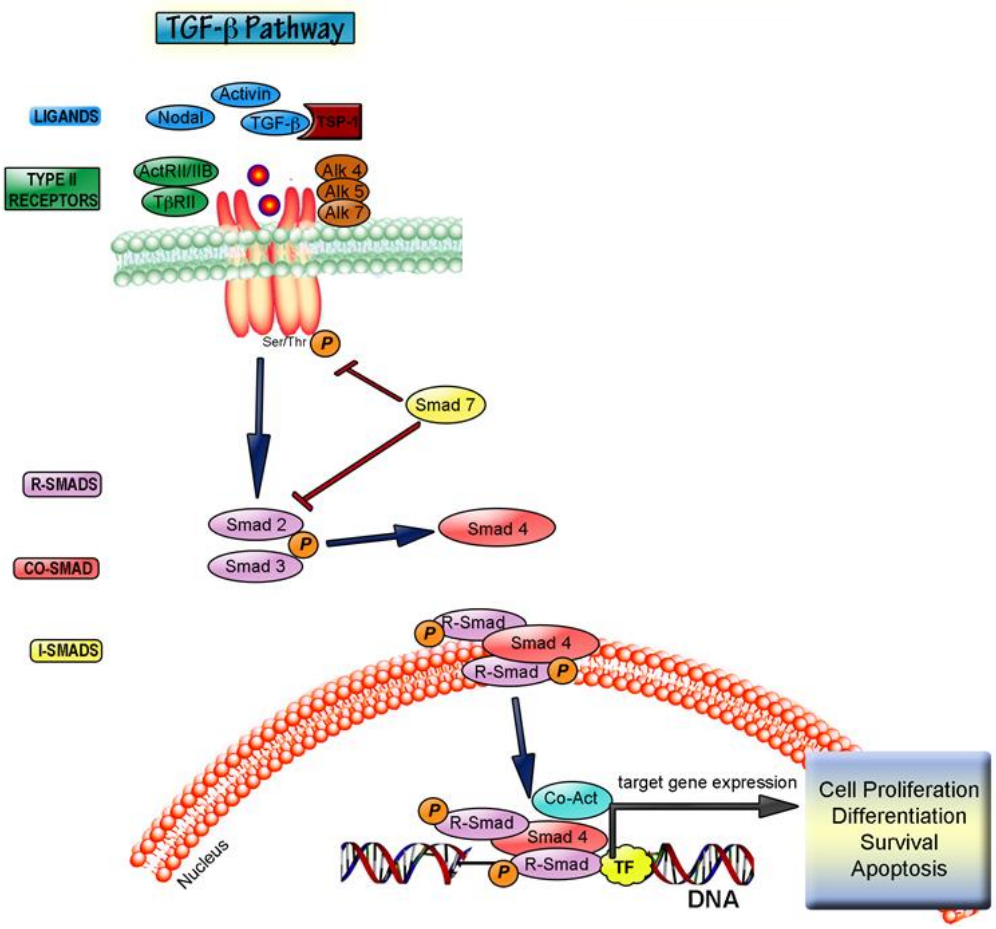


Image 1.10: Activin signalling pathways through SMAD. Activin ligand binds to receptors type II recruiting the Activin receptor type IB. Recruitment of type I receptors phosphorylate SMAD 2/3, complexing with SMAD 4, transclocating to the nucleus to bind at specific DNA motifs. SMAD proteins activate or repress transcription through Co-Act proteins. This pathway is inhibited by Smad7 [Adapted from (Sonia Villapol, 2013)]

ActivinA is rapidly upregulated in the brain upon high frequency neuronal activity and expressed by neurons following excitotoxicity and infection (Brackmann et al., 2013, Jones et al., 2007), and it exhibits some strong neuroprotective effects in animal models of neurodegenerative diseases. Even since 1999, ActivinA, after being administered in a Huntington's disease rat model, was suggested to have the ability to rescue both striatal interneurons and striatal projection neurons from excitotoxic lesioning (Hughes et al., 1999). When ActivinA was studied alongside the basic fibroblast growth factor (bFGF) – the most well-documented cytokine for its neuroprotective and neurotrophic effects that has also been tested in stroke patients- in an acute excitotoxic brain injury animal model, it was reported that its action depends on the induction of ActivinA, while the recombinant ActivinA also exhibited a neuroprotective effect of its own (Tretter et al., 2000). Recombinant ActivinA also exhibited neuroprotective effects against both serum deprivation and 6-OHDA -the parkinsonism-inducing neurotoxin- induced neuronal death in SH-SY5Y human

neuroblastoma cells. Furthermore, in the same study, when the SH-SY5Y cells were transfected with activin beta, they were protected against serum withdrawal-induced apoptosis (Kupershmidt et al., 2007). Moreover, ActivinA promoted tissue survival after focal cerebral ischemia/reperfusion injury in mice, and the treatment of adult mice with ActivinA reduced the activation of the stress responsive c-Jun N-terminal promoting apoptosis kinase after stroke (Mukerji et al., 2009). Intraventricular infusion of ActivinA increased the number of newborn neurons in the dentate gyrus, CA3 and CA1 layers of the normal adult hippocampus in mice, as well as inhibited glia and microglia proliferation in vivo and in vitro (Abdipranoto-Cowley et al., 2009). All these findings nominate ActivinA as a very promising candidate in the research of neurodegenerative diseases and synucleinopathies.

1.3. Aim

Preliminary experiments in our lab, showed that ActivinA was protective against the pathophysiology produced by pre-formed fibrils of α-synuclein in mice. To this end, we also wanted to investigate:

1) whether such protection had an effect on behaviour.

2) the possible mechanism of action of ActivinA in vitro using the SH-SY5Y human neuroblastoma cell line.

2. Methods and Materials

2.1. Animals

For the purposes of the present study, male and female wild-type C57B16/C3H (Jackxon Laboratory, Bar Harbor, Main) were used. A minimum of 4 animals were used per treatment. Animals were housed in the animal facility of the Biomedical Research Foundation of the Academy of Athens in a room with a controlled light-dark cycle (12hrs light – 12hrs dark) and free access to food and water. Unilateral injections had been previously performed in our lab, under general isoflurane anaesthesia by an apparatus adjusted to the stereotactic frame (Kopf Instrument, USA). Right dorsal striatum had been targeted using the following coordinates of bregma: anteroposterior +0.5mm, mediolateral -2mm and dorsolateral in two depths -3,4mm and -3,6mm, according to mouse brain atlas. A total of 4,25μg (4μl) of human pre-formed α-synuclein fibrils (PFF) at a constant flow rate of 0,27μl/min were injected. Some of them received 2μg/ml (4μl) of intraperitoneal ActivinA treatment twice a week for 2 months starting at 2 months post injection (PFF+ActivinA). Equal volume of PBS was used for control animals. An interval of 5 minutes was maintained between the two dorsoventral depths and the needle was slowly removed 5 minutes after the injection procedure was completed. Both the behavioural assays and the sacrifice took place 4 months post injection.

2.2. Immunofluorescence

Fluorescent immunohistochemistry was performed in pre-cut free-floating brain sections of 35mm from mice that had been stereotactically injected with pre-formed α-synuclein fibrils as described above. Brain sections were washed with PBS, then blocked with buffer (5% NGS, 0,1% Triton and 1xPBS) for 1hr in RT and incubated with primary antibodies against phosphorylated α-synuclein and Tuj-1 for 48hrs at 4^0C. Consequently, sections were washed with PBS and incubated with a mixture containing both secondary antibodies, 5% NGS, 0,1% Triton and 1:2000 DAPI for 2hrs, before being washed again with PBS and mounted. The sections were examined using the inverted confocal microscope.

2.3. Behavioural analysis

Mice were tested under behavioural assays, so the effects of pathology on their motor ability could be determined. The assays included the pole test, the beam test, the rotarod and the footprint assay. The animals, a week prior to commencing the assays, spent 2-3 minutes with the handler, so they could familiarise themselves with the handler's touch and odour. Prior to each test, the animals were placed in the assay

room for 30 minutes and the room's temperature was set at 21-22^{0}C. Both the beam and the pole assay were undertaken at the same day, whereas the footprint assay took place on a different one.

2.3.1. The pole assay

The pole descending assay is used in order to assess coordination and balancing in pathologies that are connected with the basic ganglia (Fleming et al., 2004, Ogawa et al., 1985). Each animal is placed facing upwards on the top of the wooden pole (50cm height, 1cm diameter), which is in the cage of the animal ((Fleming et al., 2004). The mice are trained 2 days prior to the testing. The time required for the mouse to turn on the top in order to start descending is measured (turn time). The average score of 5 trials is calculated.

2.3.2. The beam assay

The beam assay is a test designed to assess the fine motor skills and it requires fine tuning and balancing (Fleming et al., 2004). Each mouse gets trained for 2 days to cross a plexiglass beam, which consists of 4 separate sections of different width (3.5cm-2.5cm-1.5cm-0.5cm). The mouse's cage is being placed at the end of the beam with the bedding exposed. On the third day, a wire mesh gets placed 1cm on top of the beam and the animals crossing is being recorded on video. The test is repeated 5 times and the 4 best performances are analysed. One mistake is counted every time one of the animal's four paws is hanging in the air. One step is the forwarding successive movement of the front paws.

2.3.3. The rotarod assay

This assay tests the movement coordination and balance. The mice are placed on a rotarod of 7cm diameter. They are left for 1minute to get used to the apparatus and find their balance on a forward motion, on low speed and steady velocity of 4rpm. There are 3 trials of 5 minutes each, when under increasing velocity up to 40rpm, the time for which the mice can follow the movement of the rotarod is recorded. The test ceases, once the animals drop, lose their balance, or start turning in a passive manner. Between each trial, there is a resting time of 45minutes. The final score is the averaged time of all 3 trials.

2.3.4. The footprint assay

The footprint assay is often used to assess gait. The front and hind paws of the mouse are painted with non-toxic dyes of different colours. The mice are placed on a passageway, made on its bottom of paper and enclosed on the sides, and are allowed to walk on one direction leaving their footprints on the paper. Three parameters are measured 1) overlap between forepaw and hindpaw placement, 2) hind-base width and front-base width and 3) left and right stride length.

2.4. Cell culture

For the needs of the present study, a stable inducible Tet-off SH-SY5Y human neuroblastoma cell line overexpressing WT α-synuclein was used. Cell-stock was kept under doxycycline treatment of 1μg/ml [dox(+) cells], whereas the undertaken experiments were performed in cells from which doxycycline was withheld for 4 days [dox(-) cells]. Cells were grown in 1% pen/strep+10% Foetal Bovine Serum (FBS) RPMI medium. G418 (250μg/ml) and hygromycine B (50μg/ml) were added in the full medium once per two weeks to preserve the Tet-off system expression. Passaging of the cells followed the standard protocol of trypsin-EDTA:

- Pre-warming of the trypsin solution and growth medium to 37˚C
- Examination of the cells to ensure viability and absence of contamination
- Removal and discarding of the culture media from petri-dish
- Gentle rinsing of the cells with room-temperature PBS and removal of the solution.
- Addition of 1ml pre-warmed trypsin solution to the side wall of the petri-dish. Gentle swirling of the contents to cover the cell layer.
- Incubation of the dish at 37˚C for 2-3 minutes. The detached cells appeared rounded and refractile under microscope. If less than 90% of cells were detached incubation at 37˚C for another 2 minutes followed.
- Once cells appeared detached addition of 2 volumes of pre-warmed complete growth media inactivated trypsin.
- Transfer of the cell suspension to a 15ml falcon and gentle centrifugation at 15,000rpm for 5min. After removal of the supernatant, gentle resuspension of the cell pellet in pre-warmed complete growth medium was performed.
- 1/3 of the resuspended solution was pipetted to 3ml full growth medium in a 15ml falcon and then transferred to a new dish and placed back to the incubator.

2.4.1. Experimental conditions

For all experiments performed, cells were plated in 60mm plates in 3ml full growth medium. Five different experimental conditions were applied when 60% of confluency was reached. 1) Control cells were untreated and full growth medium was changed every other day. 2) 60 μl of Epoxomycin 1μM (20nM final concentration) were introduced per plate 24hrs before harvest. 3) Treatment with 3μl of 50μg/ml ActivinA overnight was followed by treatment with 60 μl of Epoxomycin 1μM per plate 24hrs before harvest. 4) 30 μl of Bafilomycin 5μM (50nM final concentration) were introduced per plate 24hrs before harvest. 5) Treatment with 3μl of 50μg/ml ActivinA overnight was followed by treatment with 30 μl of Bafilomycin 5μM per plate for 24hrs before harvest. For each condition 3 replicates were generated. (**Image 2.1**)

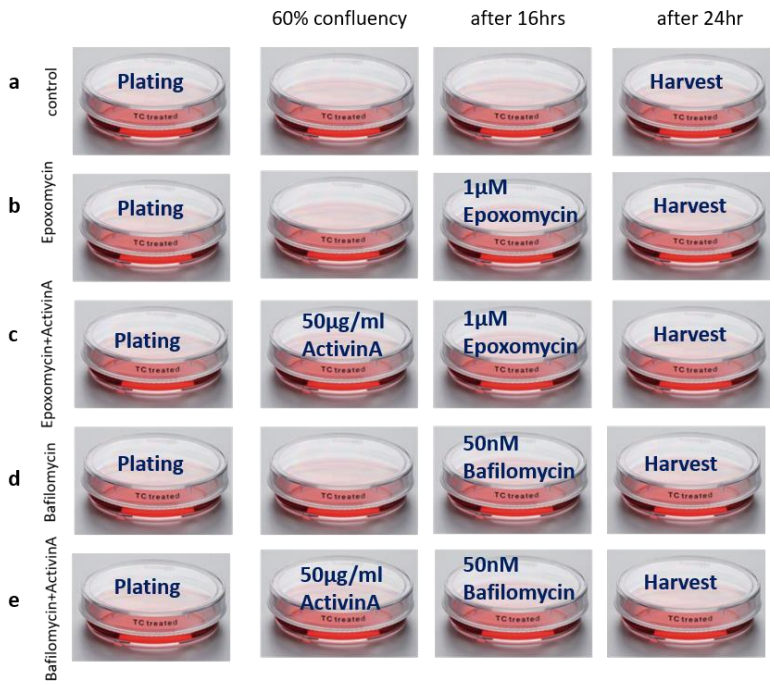


Image 2.1: Experimental conditions of the cell lines. a) untreated, **b)** 1μM Epoxomycin were added 24hrs before harvest, **c)** at 60% confluency 50μg/ml ActivinA were introduced in the medium for an overnight pre-treatment, after which 1 μM Epoxomycin were added 24hrs prior harvest, **d)** 5 μM Bafilomycin were added 24hrs before harvest, **e)** at 60% 50μg/ml confluency ActivinA were introduced in the medium for an overnight pre-treatment, after which 5 μM Bafilomycin were added 24hrs prior to harvest.

2.4.2. SDS-PAGE and Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 5mM EDTA, 0.5% sodium deoxycholate), containing protease inhibitors and phosphatase inhibitors. Total protein content was determined using DC protein assay and samples were subjected to SDS-PAGE 12% acrylamide gel and subsequently transferred to a nitrocellulose membrane. After blocking with either 5% BSA in PBS for all phosphorylated proteins of interest, or 5% milk in TBS-T for the rest of the proteins, membranes were incubated with primary antibodies against GAPDH, c-Jun, p62, total α-synuclein/BD and phosphorylated α-synuclein. Secondary antibodies were conjugated with HRP. Quantification of the blots was achieved with the GelQuant.NET programme and the values were normalised according to the GAPDH of each blot.

2.4.3. Proteasomal Assay

For this assay 3 different cell batches under conditions 1,2 and 3 (3μl of 50μg/ml ActivinA) were used in duplicates, were scraped in PBS and lysed in resuspension buffer (25mM Tris pH 7.6, 1mM DTT, 2mM ATP and 2mM $MgCl_2$). They were then sonicated twice for 5sec, centrifuged at 13.000 rpm for 30min at 4^{o}C and the supernatant underwent Bradford Protein Assay. 3.5 μg of protein from each sample were mixed with reaction buffer (250mM Tris pH 7.6, 50mM DTT, 50mM $MgCl_2$ and 10mM ATP) and incubated at 37^{o}C for precisely 10min. Termination of the reaction was achieved at 10min by adding 500μl 5% SDS. Sample fluorescence was measured at 437 nm using a PerkinElmer LS-55 luminescence spectrophotometer.

2.5. Statistical analysis

For data analysis, GraphPad Prism5 software was used. Statistics were performed using the one-way ANOVA test followed by the Tuckey's Multiple Comparison test, and the Dunnett's Multiple Comparison test. Statistical significance is depicted on the graphs as *$*p<0.05$, **$p<0.001$, ***$p<0.001$*.

3. Results

3.1. ActivinA treatment leads to decreased phosphorylated α-synuclein accumulations in the cortex and striatum of mouse brains injected with PFF

Previous studies in our lab have shown that the stereotactical injection of (exogenous) pre-formed phosphorylated α-synuclein fibrils (P-PFF) in mouse brains induce neuronal dysfunction and exacerbated pathology (Karampetsou et al., 2017). In an effort to uncover the possible effect of ActivinA in this mouse model, mice injected with PFF (PFF mice) were intraperitoneally treated with 2μg of ActivinA twice/week for 2 months (PFF+ActivinA mice) and sacrificed at 4 months of age. After sacrifice, two 35mm brain sections from these mice underwent immunofluorescent staining against phosphorylated α-synuclein and Tuj-1. The comparison between P-PFF and PFF+ActivinA mice showed decreased phosphorylated α-synuclein accumulations in both the cortex (**Image 3.1**) and the striatum (**Image 3.2**) of these mice.

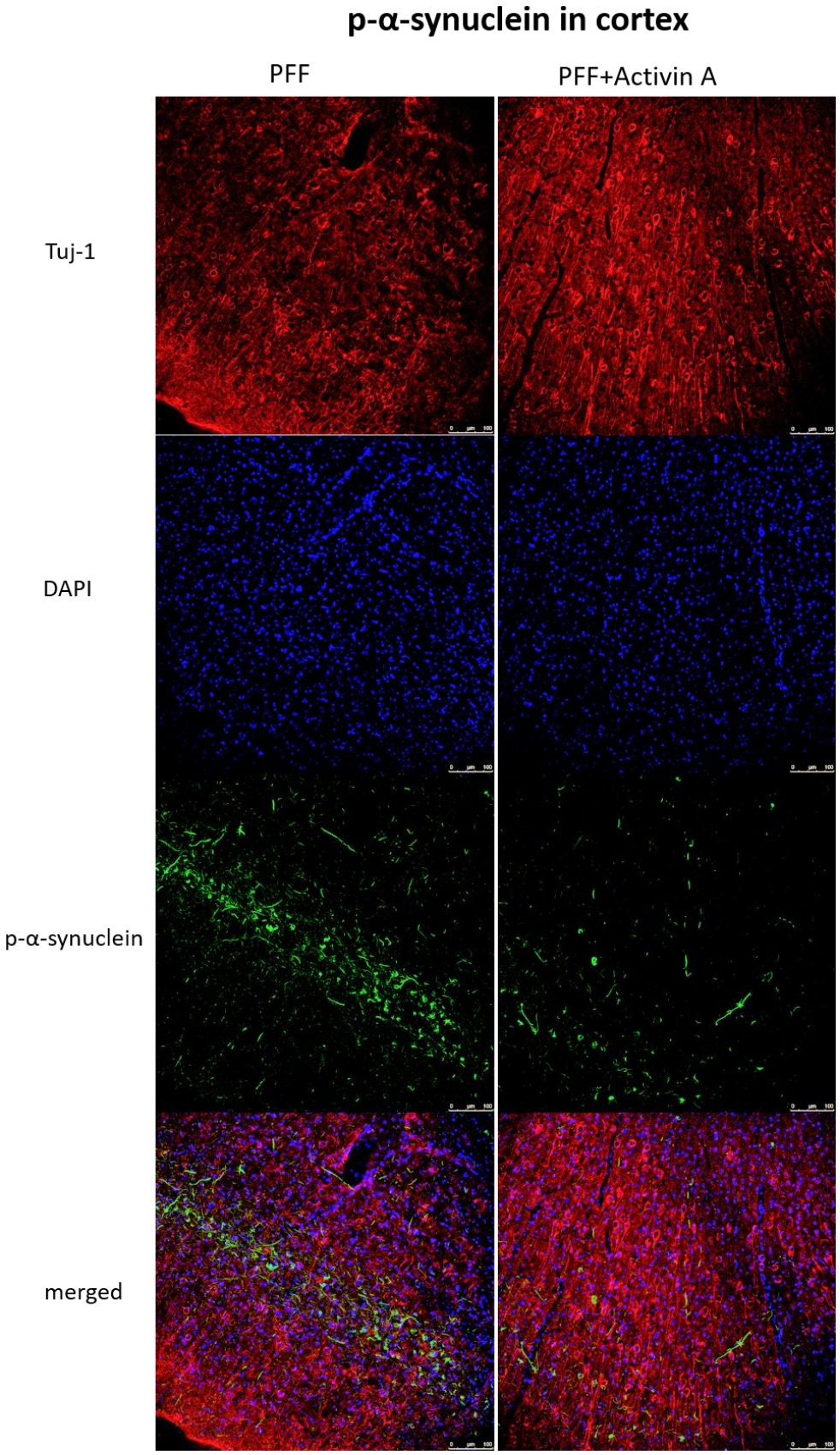


Image 3.1: Phosphorylated α-synuclein aggregates are decreased in the cortex of PFF injected mouse brains after ActivinA treatment. Immunofluorescent images were taken with confocal microscope. Phospho -α-synuclein stains with green (right panel versus left depict the decrease in accumulation). Tuj-1 (red) stains for neuronal cells and DAPI (blue) stains for nuclei. Magnification x 20.

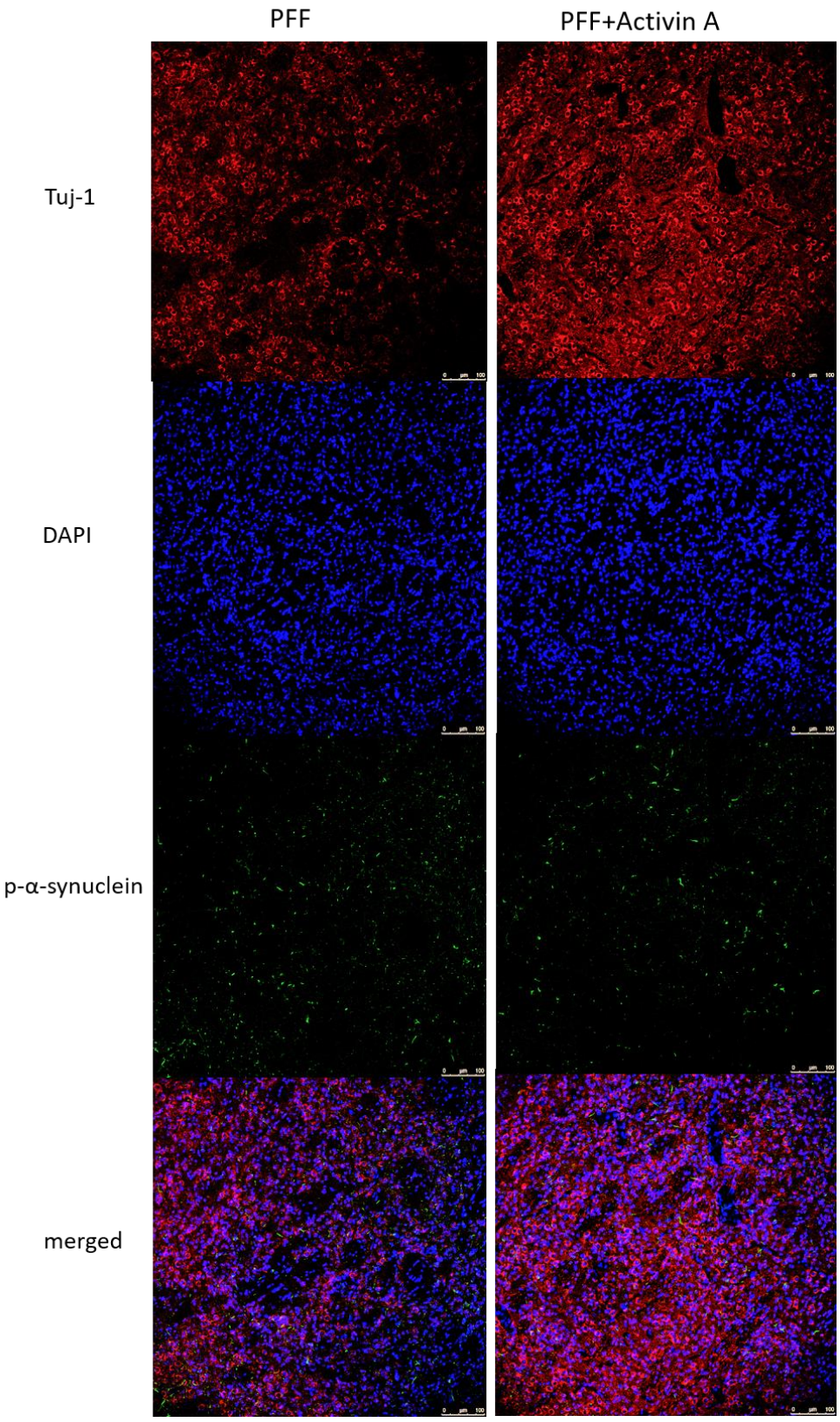


Image 3.2: Phosphorylated α-synuclein aggregates are decreased in the striatum of PFF injected mouse brains after ActivinA treatment. Immunofluorescent images taken with confocal microscope, reveal that phosphorylated α-synuclein (green) aggregates decrease after treatment with ActivinA (right panel versus left). Tuj-1 (red) stains for neuronal cells and DAPI (blue) stains for nuclei. Magnification x 20.

3.2. ActivinA treatment reverses the motor effects on the pole assay performance of PFF mice

After confirming the effect of ActivinA treatment in the histopathology of PFF injected mouse brains, a series of behavioural assays were conducted in order to examine whether this effect is also translated on the animals' behaviour.

Initially, the pole assay was performed which tests the coordination and the balance of the animal. The turn time that was recorded and analysed is indicative of both these abilities. Three groups of mice were examined: 1) PBS injected mice (control), 2) PFF mice and 3) PFF+ActivinA mice. There is a slight, yet significant increase of the time required for the mice to turn in the PFF group (1.920±0.080sec) compared to the controls (1.457±0.1702sec) indicating that the injection of the fibrils, not only induced pathology in the brain (**Image 3.1**), but also affected the behaviour of the animal. Moreover, the opposite significant effect (decreased turn time) was seen in the PFF+ActivinA group (1.439±0.1171sec) compared to the PFF group, indicating that the ActivinA treatment did improve the behaviour of the animals up to a level where no significant difference can be detected compared to the healthy control (**Figure 3.1**).

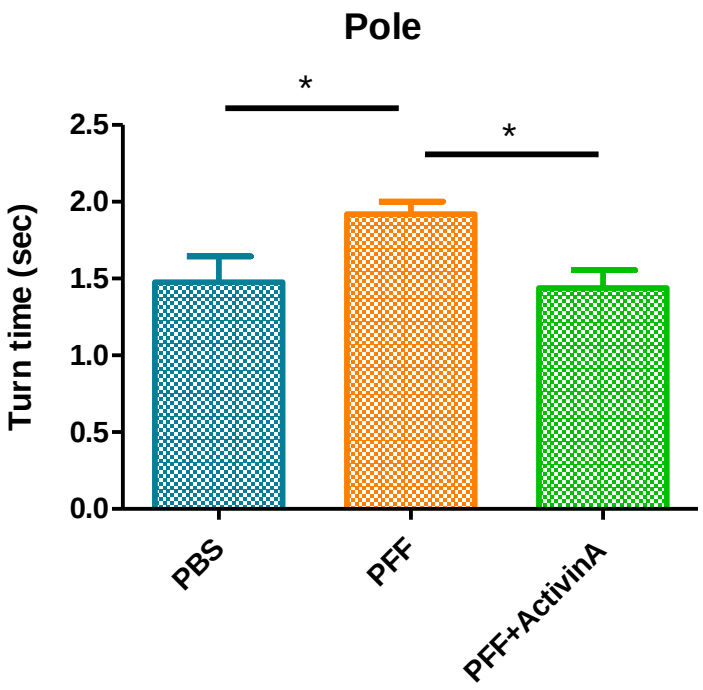


Figure 3.1: ActivinA treatment reverses the effects in the pole assay performance of PFF mice. PFF mice showed increased turn time, whereas ActivinA treatment decreased this time in levels similar to that of the control group (PBS). For the control group n=4, for the PFF group n=5 and for the PFF+ActivinA group n=5. Data represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

3.3. ActivinA treatment enhances performance in the beam assay in PFF mice

Next, the beam assay was performed, which tests motor fine tuning and balancing of mice. The same three groups (PBS, PFF, PFF+ActivinA) of animals were tested. This assay showed a very significant improvement in the performance of the animals that had been treated with ActivinA compared to the PFF injected group (PBS 0.3252±0.05460, PFF 0.3810±0.02155, PFF+ActivinA 0.26667±0.01182 errors/step) (**Figure 3.2**). Notably, probably due to the small number of animals employed, no significant difference was detected between the control and the PFF group.

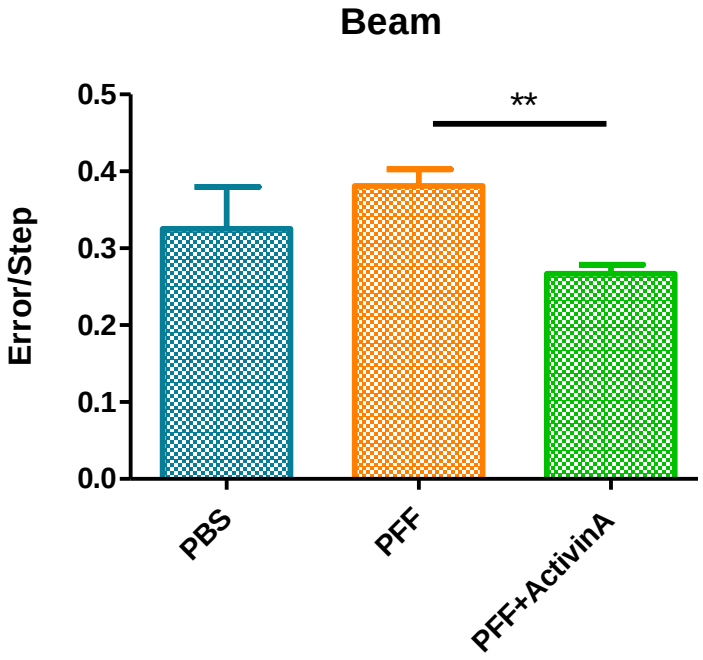


Figure 3.2: **ActivinA treatment enhanced the performance on the beam assay of PFF mice by lowering their Error/Step score.** PFF injected mice had a very significantly increased average score on this assay, compared to the ActivinA treated group. For the control group n=4, for the PFF group n=5 and for the PFF+ActivinA group n=5. Data represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

3.4. ActivinA did not significantly affect the impaired performance on the rotarod assay of the PFF mice

As a next step, the rotarod assay was undertaken, examining coordination and balance of the mice. The same three groups (PBS, PFF, PFF+ActivinA) of mice were used. Analysis of the time that the mice could keep following the rotating rod, showed a very significant impairment of the PFF group in compared to the control group (PBS). However, no difference was detected between the PFF and the PFF+ActivinA groups, as well as between the PBS and the PFF+ActivinA groups (PBS 180.6±10.69, PFF 117.1±10.42, PFF+ActivinA 147.6±17.41 sec) (**Figure 3.3**). This observation suggests that probably ActivinA treatment could not significantly alleviate the effects of PFF injections in the rotatrod assay performance of mice examined.

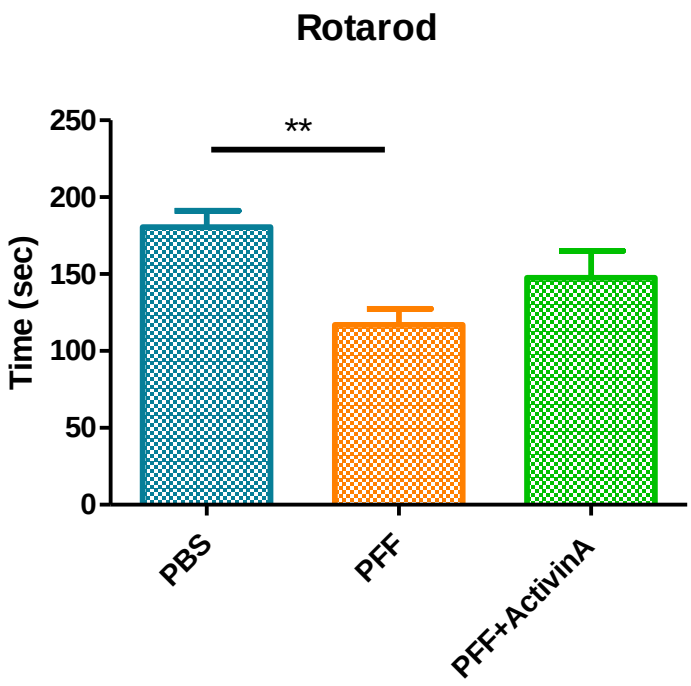


Figure 3.3: ActivinA treatment did not significantly affect the reduced performance on the rotarod assay of the PFF injected mice. Compared to the control group, PFF injected mice showed a very significant impairment on their score. This effect was not significantly altered with the ActivinA treatment. For the control group n=9, for the PFF group n=9 and for the PFF+ActivinA group n=8. Data represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

3.5. PFF injections, irrespective of subsequent treatment with ActivnA, did not affect the overall gait of the mouse subjects

Following the changes in motor performance that were observed in the PFF mice, we wanted to examine whether PFF injections could also affect normal gait of these mice and the subsequent effects that ActivinA treatment could exhibit. For this reason, the footprint assay was performed during which a series of measurements was recorded: 1) The **overlap index** which measures the distance between the front and hind paws of each side of the animal, 2) the **width index** which measures the distance between the left and right front/or hind paws, 3) the **left stride index** and 4) the **right stride index** which measure the distance covered by the front/or hind paw in subsequent steps of each side respectively (**Figure 3.4**).

No significant changes were observed among groups in the overlap index (left paws: PBS 0.62±0.05066, PFF 0.6225±0.03470, PFF+ActivinA 0.6505±0.04240cm – right paws: PBS 0.8484±0.07255, PFF 0.7585±0.07502, PFF+ActivinA 0.6902±0.1036cm) (**Figure 3.5**) or in the width index (front paws: PBS 1.524±0.02347, PFF 1.696±0.05686, PFF+ActivinA 1.565±0.06051cm – hind paws: PBS 2.735±0.1009, PFF 2.959±0.08571, PFF+ActivinA 2.839±0.09239cm) (**Figure 3.6**). Additionally, no difference was detected either in the left stride index (front paws: PBS 6.792±0.2839, PFF 6.982±0.3045, PFF+ActivinA 7.145±0.2244cm – hind paws: PBS 6.538±0.3456, PFF 6.827±0.3132, PFF+ActivinA 6.964±0.2620cm) (**Figure 3.7**) or the right stride index (front paws: PBS 6.909±0.3051, PFF 6.938±0.2810, PFF+ActivinA 7.170±0.2240cm – hind paws: PBS 7.121±0.1624, PFF 6.961±0.1922, PFF+ActivinA 7.204±0.1699cm) (**Figure 3.8**). This result suggests that both PFF injection as well as subsequent treatment of the mice with ActivinA, does not affect their overall gait.

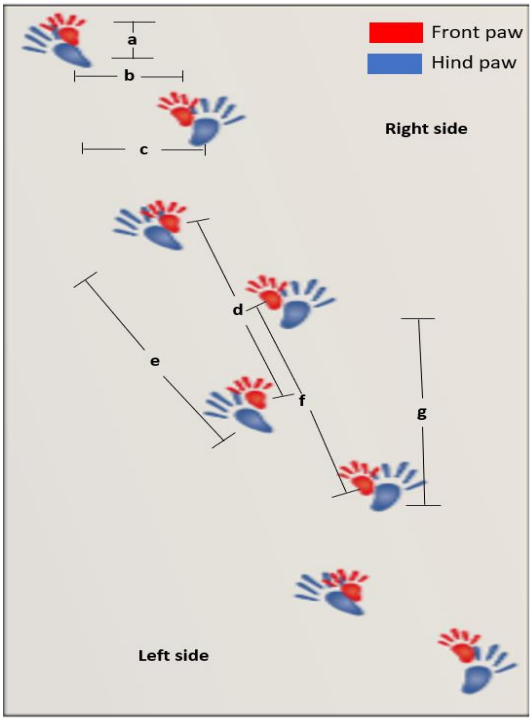


Figure 3.4: The different indexes measured in the footprint assay. (a) overlap, **(b)** front width, **(c)** hind width, **(d)** front left stride, **(e)** hind left stride, **(f)** front right stride, **(g)** hind right stride. [Adapted and modified by (Brooks and Dunnett, 2009)]

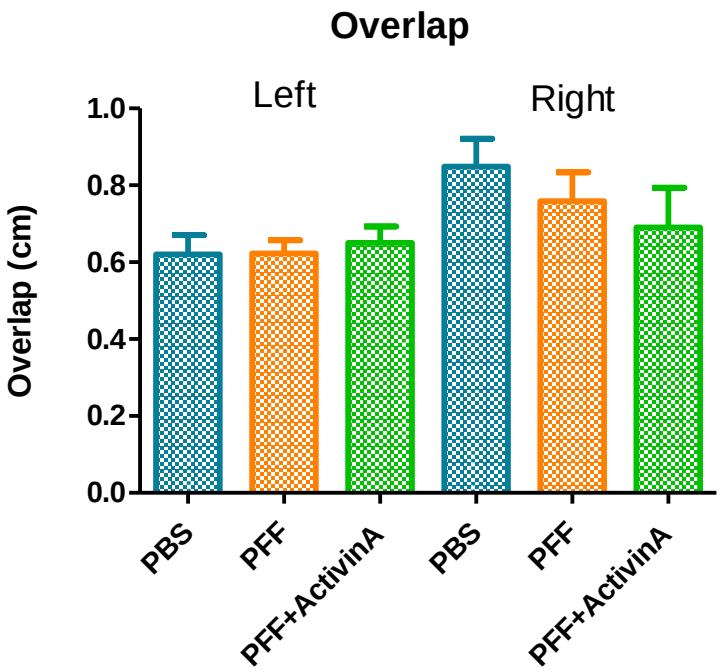


Figure 3.5:-PFF injection did not significantly affect the overlap between front and hind paws of mice tested, irrespective of their treatment with ActivinA. All comparisons made, even between the two sides, showed no significant difference. For the control group n=4, for the PFF group n=5 and for the PFF+ActivinA group n=5. Data represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

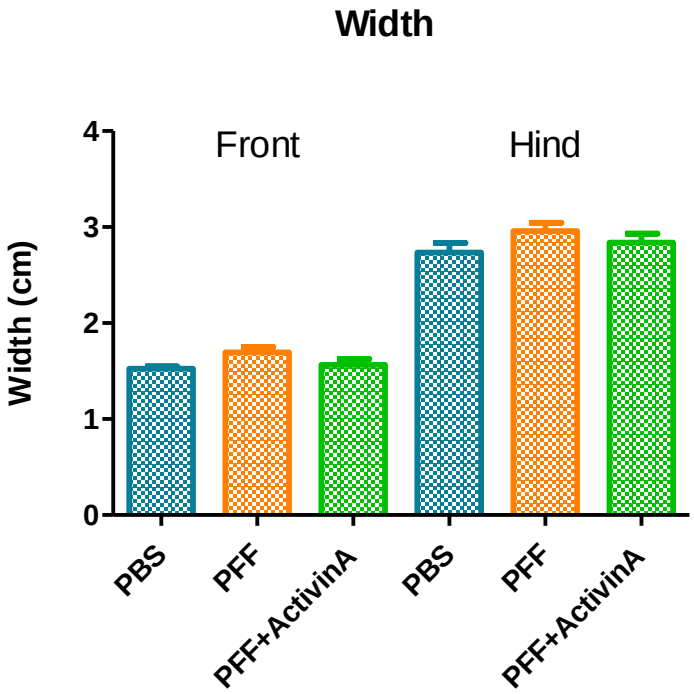


Figure 3.6: PFF injection irrespective of ActivinA treatment, did not significantly affect the width between left and right paws of mice tested. All comparisons made among front paws and among hind paws showed no significant difference. This measurement does not compare the distance between front to hind paws, rather the distance between front to front and hind to hind for each step. For the control group n=4, for the PFF group n=5 and for the PFF+ActivinA group n=5. Data represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

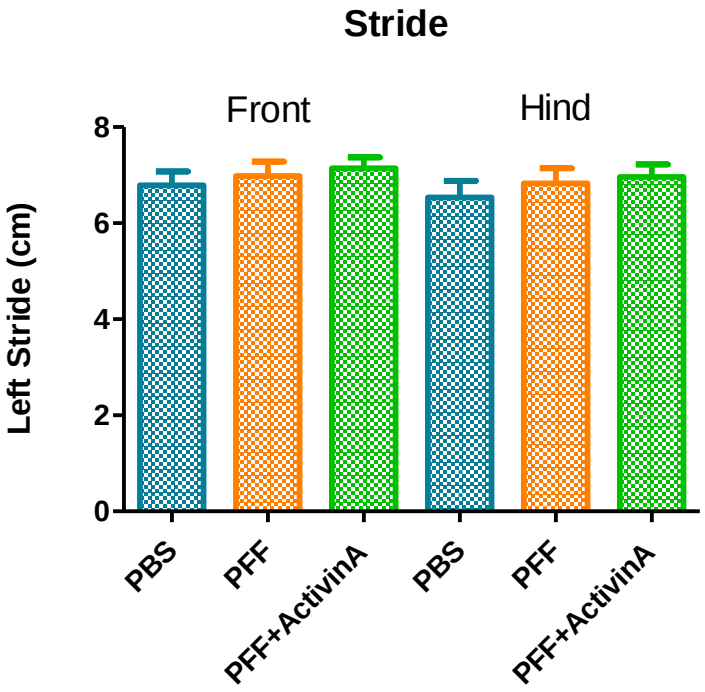


Figure 3.7: PFF injection, irrespective of subsequent ActivinA treatment, did not significantly affect the left stride between neither front, nor hind paws in subsequent steps. All comparisons showed no significant difference. For the control group n=4, for the PFF group n=5 and for the PFF+ActivinA group n=5. Data represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

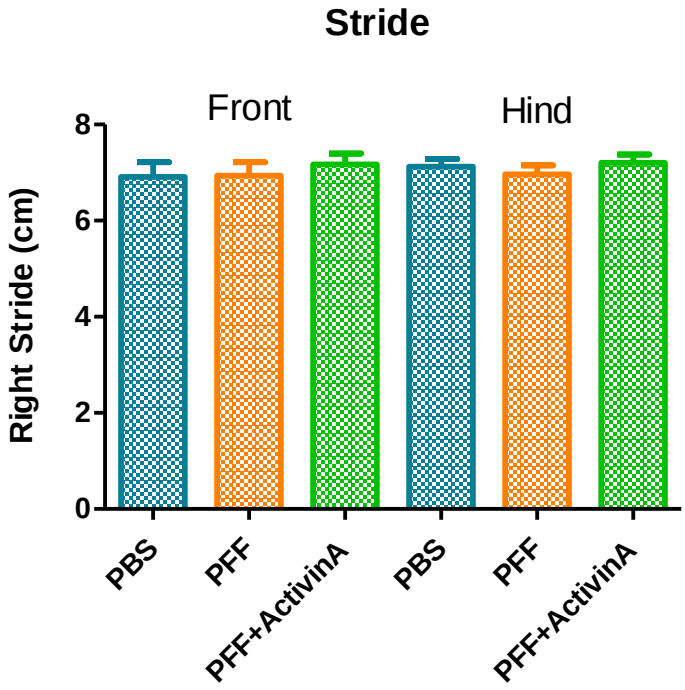


Figure 3.8: PFF injection, irrespective of subsequent ActivinA treatment, did not significantly affect the right stride between neither front, nor hind paws in subsequent steps. All comparisons showed no significant difference. For the control group n=4, for the PFF group n=5 and for the PFF+ActivinA group n=5. Data represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

3.6. Increased phosphorylation of α-synuclein due to proteasomal inhibition is not reduced by ActivinA pre-treatment in vitro

The next step was to attempt to elucidate the molecular mechanisms underlying these phenotypical changes observed by the behavioural assays. For this reason, the SH-SY5Y overexpressing wt-α-synuclein cell line was employed.

Initially, we wanted to examine which system/pathway was responsible for the degradation of phosphorylated α-synuclein. Therefore, both the ubiquitin-proteasomal system and the autophagy-lysosmal pathway were separately and independently inhibited in the SH-SY5Y cell line. Epoxomycin and Bafilomycin were used on separate experiments, in order to inhibit the proteasome and the lysosome respectively. At the same time, in order to uncover the molecular mechanisms that mediate the effects of ActivinA observed on histopathological and behavioural levels, cells were also treated overnight with 50μg/ml ActivinA, before either 1μM Epoxomycin or 5μM Bafilomycin treatment. Proteasomal inhibition due to Epoxomycin treatment significantly increased phosphorylation of wt-α-synuclein. Simultaneously, ActivinA pre-treatment did not significantly alter the increased levels of phosphorylated α-synuclein. On the other hand, neither Bafilomycin, nor Bafilomycin with ActivinA pre-treatment had any effect on the levels of the phosphorylated α-synuclein (control 1, Epoxomycin 2.670±0.7383, Epoxomycin+ActivinA 1.776±0.4122, Bafilomycin 0.9839±0.02990, Bafilomycin+ActivinA 0.9204±0.1699 arbitrary units after normalization to GAPDH and control samples) (**Figure 3.9**). These results suggest that phosphorylated α-synuclein is degraded at the proteasome in our model, while ActivinA does not seem to significantly affect this degradation.

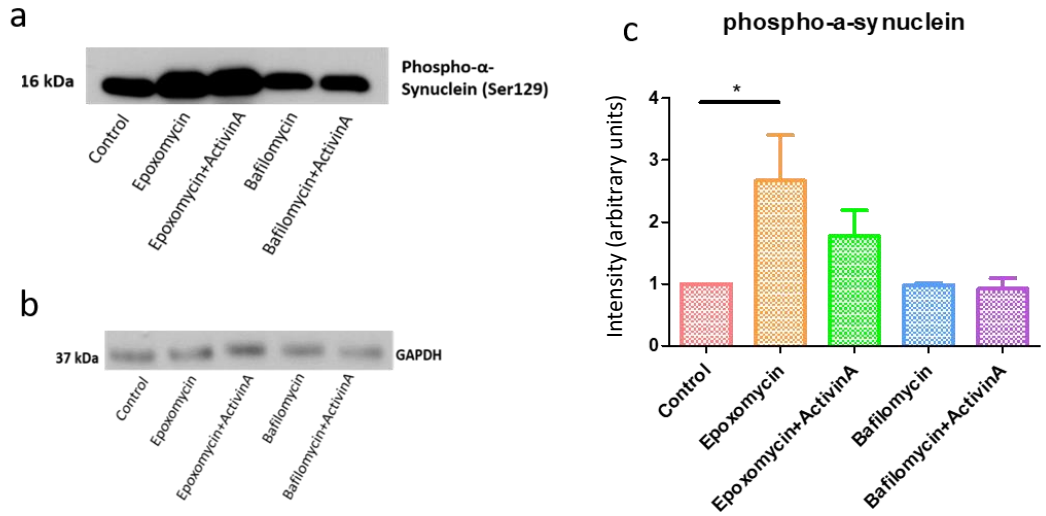


Figure 3.9: Proteasomal inhibition with Epoxomycin increased the levels of phosphorylation of α-synuclein in vitro. a) Western blot analysis of phosphorylated α-synuclein at Ser129 residue b) Western blot analysis of GAPDH from the same samples was used for the normalisation of phosphorylated α-synuclein c) Quantification of the phosphorylated α-synuclein levels from (a). Data in (c) represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

At the same time, total α-synuclein levels remained unchanged upon any treatment (Control 1, Epoxomycin 1.016±0.1667, Epoxomycin+ActivinA 0.9948±0.2536, Bafilomycin 0.7273±0.3249, Bafilomycin+ActivinA 0.9973±0.5858 arbitrary units after normalization according to GAPDH and control samples) (**Figure 3.10**)

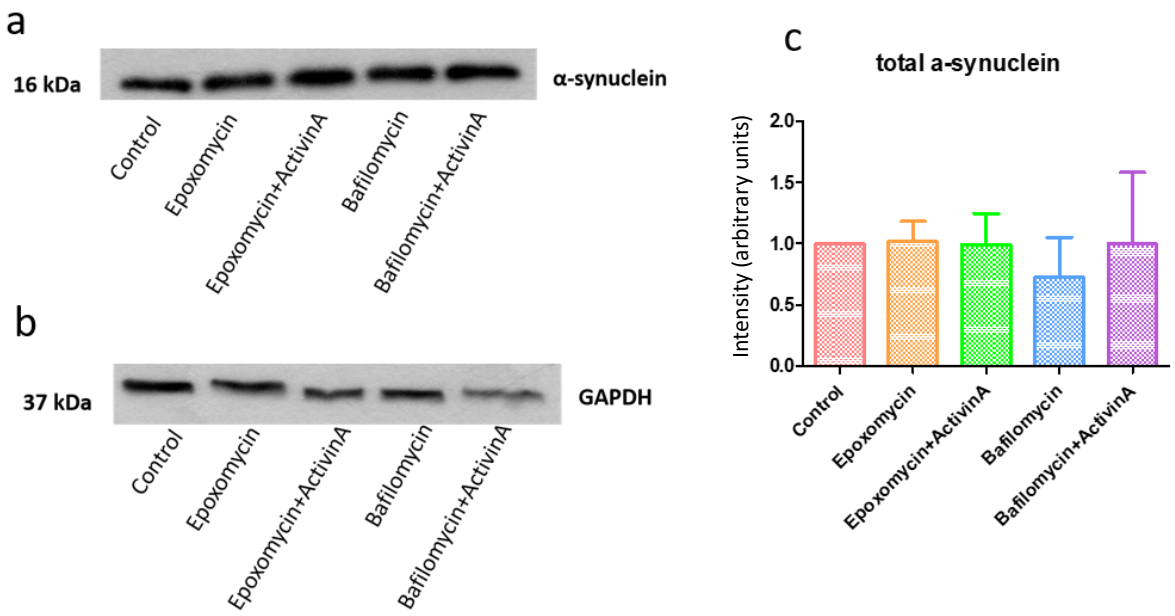


Figure 3.10: In vitro treatment of the SH-SY5Y cell line with Epoxomycin +/- ActivinA and Bafilomycin +/- ActivinA, does not alter levels of total α-synuclein. a) Western blot analysis of total α-synuclein b) Western blot analysis of GAPDH from the same samples was used for the normalisation of total α-synuclein c) Quantification of the total α-synuclein levels from (a). Data in (c) represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

3.7. ActivinA pre-treatment failed to significantly alleviate Epoxomycin-mediated proteasomal inhibition

In order to verify that Epoxomicin indeed inhibited the proteasome, and to further study the possible effects of ActivinA pre-treatment on that inhibition, two approaches were applied. Firstly, the levels of c-Jun, as a marker of proteasomal inhibition (Emmanouilidou et al., 2010b), were compared among SH-SY5Y cells treated with Epoxomycin +/- ActivinA pre-treatment and control cells. As expected, increased levels of c-Jun were detected in the cells treated with Epoxomycin, thus verifying proteasomal inhibition/dysfunction, while pre-treatment with ActivinA failed to alter this inhibition (control 1, Epoxomycin 28.79±0.5091, Epoxomycin+ActivinA 12.49±5.926 arbitrary units after normalization according to GAPDH and control samples) (**Figure 3.11 a-c**). Secondly, proteasomal activity was assessed using the Proteasomal Assay under the same conditions. Proteasomal activity was significantly decreased upon Epoxomycin treatment regardless of ActivinA pre-treatment (control

681.0±28.78, Epoxomycin 222.9±7.381, Epoxomycin+ActivinA 218.1±2.208nm) (**Figure 3.11 d**). Taken together, these results confirm that Epoxomycin treatment inhibits the proteasome, while ActivinA pre-treatment is not sufficient to overcome this effect.

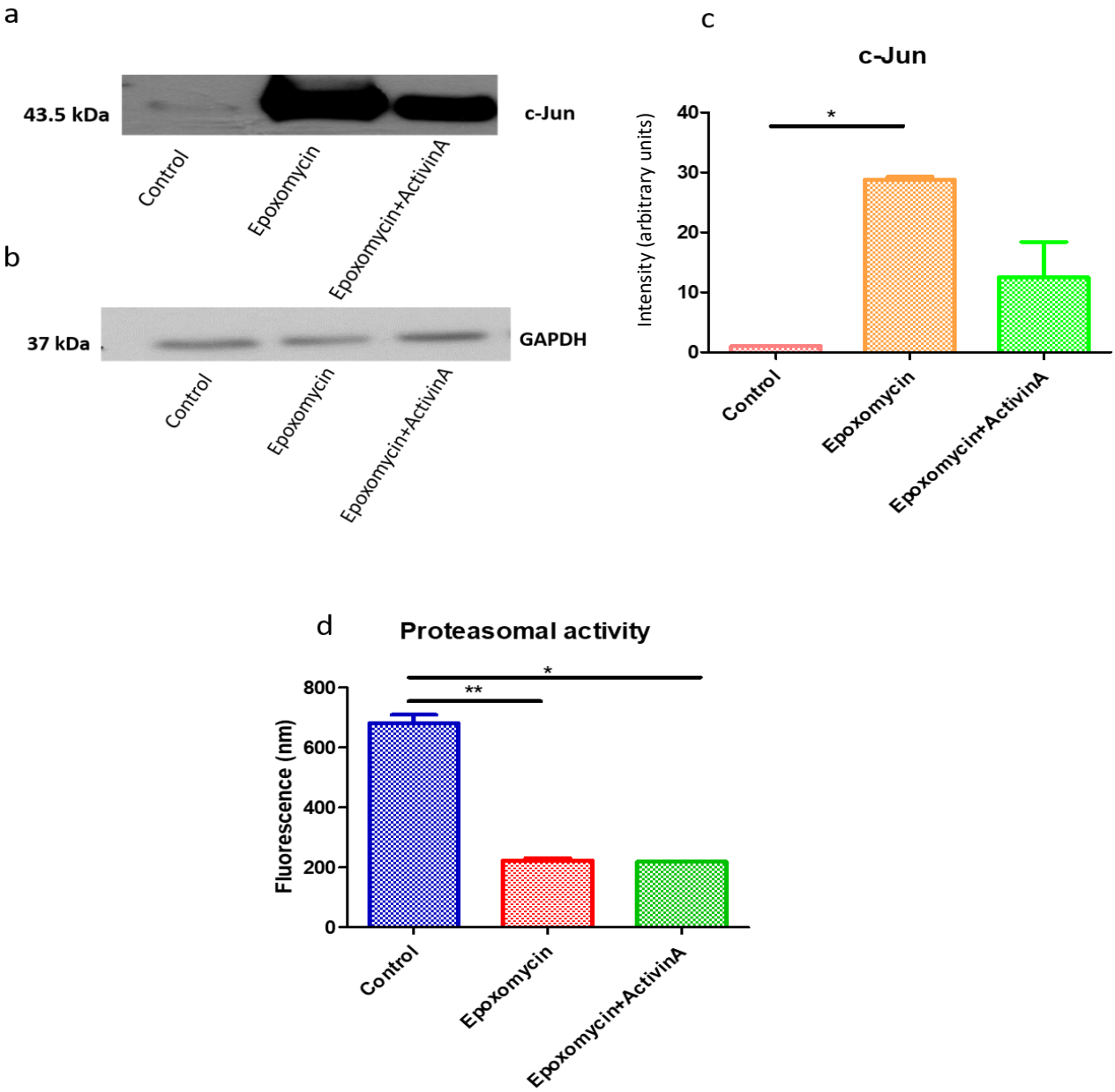


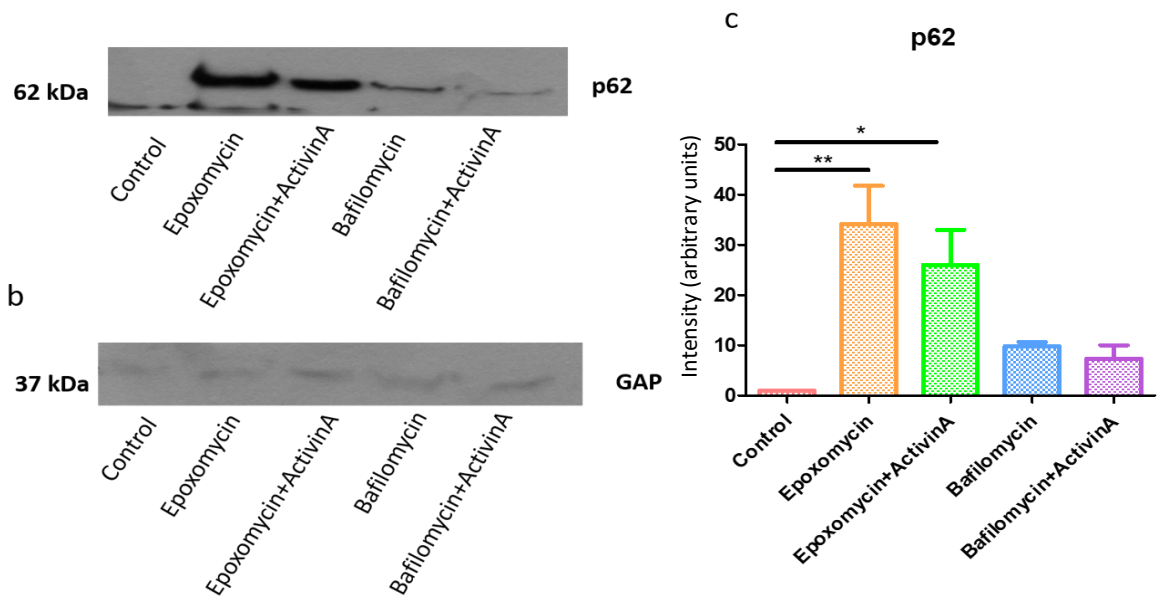
Figure 3.11: ActivinA pre-treatment is not sufficient to prevent the Epoxomycin-mediated proteasomal inhibition. a) Western blot analysis of c-Jun b) Western blot analysis of GAPDH from the same samples was used for the normalisation of c-Jun c) Quantification of c-Jun levels from (a). d) Proteasomal activity assay. Data in (c) and (d) represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.

3.8. Increased p62 levels due to proteasomal inhibition are not reduced by ActivinA pre-treatment in vitro

The ubiquitin-proteasome system, autophagy and p62 are the interdependent elements that act in a networked manner to maintain proteostasis. p62 has been identified as an autophagy substrate and has been used as a reporter of autophagy activity; however, recently p62 was also identified to deliver ubiquitinated proteins to the proteasome for degradation. It has been shown that proteasomal protein degradation, as well as autophagy, act synergistically in order to protect the cell, and dysfunction in one of them can be -at least partially- compensated by the other (Liu et al., 2016). Additionally, it has been proposed that in synucleinopathies, the accumulation of phosphorylated α-synuclein (probably as a result of defective degradation at the proteasome) increases autophagy and leads to pathology (Lilienbaum, 2013).

In order to further explore this hypothesis, in our case, p62 was measured in the above mentioned experimental conditions. Indeed, cells of which proteasome was inhibited by the use of Epoxomycin and thus the levels of phosphorylated α-synuclein were increased, also exhibited elevated levels of p62. Pre-treatment with ActivinA did not seem to significantly prevent this increase in p62 levels. On the contrary, inhibition of lysosome (using Bafilomycin +/- ActivinA pre-treatment), did not affect the levels of p62 (control 1, Epoxomycin 34.18±7.591, Epoxomycin+ActivinA 26.02±6.945, Bafilomycin 9.822±0.8216, Bafilomycin+ActivinA 7.368±2.632 arbitrary units after normalization according to GAPDH and control samples) (**Figure 3.12**).

Figure 3.12: Epoxomycin-mediated proteasomal inhibition results in increased p62 levels which are not decreased by ActivinA pre-treatment. a) Western blot analysis of p62 b) Western blot analysis of GAPDH from the same samples was used for the normalisation of p62 c) Quantification of the total p62 levels from (a). Data in (c) represent Mean±SEM. Differences were estimated using one-way ANOVA followed by Tuckey's post-hoc test.



4. Discussion

Synucleinpathies are a group of neurodegenerative disorders which share the common pathological feature of the abnormal accumulation of different forms of α-synuclein aggregates in the neurons (McCann et al., 2014). Many post-translational modifications have been identified at the molecule of α-synuclein, its phosphorylation being the most common one. The phosphorylated form of the protein is linked directly to the progress of PD, has been found in great percentages in DLB patients brains and in MSA patients brains and is the main components of Lewy bodies (Anderson et al., 2006, Nishie et al., 2004, Zhou et al., 2011). Moreover, 90% of the aggregated form of α-synuclein is found phosphorylated at the S129 residue (Anderson et al., 2006, Fujiwara et al., 2002, Kahle et al., 2002). Across the literature, there are contradicting results of in vitro and in vivo studies concerning its role on the properties of α-synuclein (Fiske et al., 2011, Paleologou et al., 2008). Nonetheless, the toxic species of the protein found across pathology are the phosphorylated forms. Injections of pre-formed α-synuclein fibrils in the brain of mice induced the formation of pathological inclusions and the progressive aggregation of the endogenous protein, as well as the gradual propagation of pathology (Karampetsou et al., 2017, Luk et al., 2012a, Peelaerts et al., 2015). Furthermore, the phosphorylated α-synuclein undergoes degradation by the proteasomal pathway (Machiya et al., 2010). This study showed that the inhibition of the proteasome by MG132 or lactacystin and the protein phosphatase 2A/1 by okadaic acid accumulated the Ser-129-phosphorylated α-synuclein in SH-SY5Y cells, without having an effect on total α-synuclein. Moreover, the short half-life of Ser-129-phosphorylated α-synuclein was blocked as well by MG132 to a greater extent compared to the okadaic acid. Furthermore, treatment with either MG132, lactacystin, or okadaic acid, also accumulated Ser-129-phosphorylated α-synuclein in rat primary cortical neurons. Thus, exhibiting the degradation role of the proteasome in phosphorylated α-synuclein.

Preliminary experiments in our laboratory revealed that ActivinA treatment was protective against the pathophysiology produced by injection of pre-formed fibrils of α-synuclein (PFF) in mice, decreasing the protein aggregation in the SNpc, the cortex and the striatum (Karampetsou et al., 2017). ActivinA is a pleiotropic cytokine that exerts neuroprotective and anti-inflammatory functions in animal models of neurodegenerative diseases (Abdipranoto-Cowley et al., 2009, Kupershmidt et al., 2007, Mukerji et al., 2009). The first study to demonstrate that the anti-inflammatory role of ActivinA may contribute to neuroprotective effects in vivo, the MPTP model as well as the instranigral administration of LPS in C57B16/6 mice were used (Stayte et al., 2017). The MPTP model of PD remains the most widely used model to study potential neuroprotective therapeutic targets as it replicates the selective death of dopaminergic neurons within the SN. ActivinA promoted survival of dopaminergic and total neuron populations in the SNps both 8 days and 8 weeks prior to MPTP induced degeneration. However, there was no corresponding protection of striatal dopamine

levels and Acrtivin A failed to protect against loss of striatal dopamine transporter expression in the striatum, suggesting the neuroprotective action of ActivinA may be localised to the SN. However important these results might be, the limitation of the MPTP being a pharmacological model still exists. Thus, it was important to study the possible therapeutic effects of ActivinA in our PFF model as well. Despite not having verified that these PFFs are indeed secreted in human brains, it definitely resembles the disease model better. Furthermore, our model has a more preventative therapeutic approach, since the administration of ActivinA proceeds PFF injection, following the real-time events of pathology development arising prior to treatment administration.

ActivinA is rapidly upregulated in the brain upon high frequency neuronal activity and expressed by neurons following excitotoxicity and infection (Brackmann et al., 2013, Jones et al., 2007). When ActivinA was studied alongside the basic fibroblast growth factor (bFGF) – the most well-documented cytokine for its neuroprotective and neurotrophic effects that has also been tested in stroke patients- in an acute excitotoxic brain injury animal model, it was reported that its action depends on the induction of ActivinA, while the recombinant ActivinA also exhibited a neuroprotective effect of its own (Tretter et al., 2000). ActivinA is produced in elevated levels during inflammation and following various neuronal insults (Foster et al., 2004, Mukerji et al., 2007, Sulyok et al., 2004) supporting its neuronal protective role. Our preliminary data also revealed that ActivinA is highly expressed by neuronal cells in the striatum, cortex and midbrain of the PFF injected mice and it increases the frequencies of $GFAP^{+}$ astrocytes and $CD45^{low}CD11b^{+}$ microglia cells in the cortex and striatum. The positive effects of ActivinA are associated with the suppression of inflammatory cell activation in the brain (Karampetsou et al., 2017).

The endogenous occurrence of ActivinA in the human brain, its neuroprotective and neurotrophic effects on a series of models, its interaction with the brain's inflammatory response, and most importantly the therapeutic effects that arose in the MPTP-PD model mentioned above, attribute to the molecule the candidacy of a possible PD drug-target. The MPTP's authors rationale, was to search for a factor that could protect the remaining neurons after the extended degeneration that occurs before PD symptomatology arises. Selecting the ActivinA -an inflammatory molecule- not only succeeded in producing a therapeutic effect protecting the residual cells from further degeneration, but also verified the great role that inflammation holds in PD pathogenesis.

To this end, we proceeded to investigate whether such protection exerted its effects to behaviour in vivo. Initially, we confirmed that PFF injections resulted in accumulation of phosphorylated α-synuclein in the cortex and striatum of the mice and that ActivinA treatment reduced this accumulation. This suggested that in a

histopathological level, ActivinA may offer a therapeutic effect against α-synuclein pathogenesis. Intensity analysis of the histochemistry should have been performed in order to further verify this observation, as well as phosphorylated-fibrillar quantification under both conditions.

Following, the behavioural assays were conducted which revealed impairments in the coordination and balance of the PFF mice linking the phosphorylated α-synuclein aggregation to behavioural discrepancies. More specifically, the pole assay and the rotarod assay exhibit pathological behaviour by defective coordination and balance, whereas the beam assay testing the fine tuning of the mice did not reveal such impairment. Literature reveals that the deficits in fine tuning have been studied during later stages of pathology {Luk, et al., 2012a, Mao et al., 2016}. The fine tuning of motor function arises when dopaminergic loss exceeded the 35%, whereas the mice used for this study demonstrated pathology at 23% {Karampetsou et al., 2017). Additionally, the authors did not observe alterations in overall gait even at further extended pathology, an observation that agrees with our mice gait analysis. It could be suggested that in this model the gross motor behaviour remains unaffected. In our study, some of these impairments in fine motor skills were alleviated by ActivinA pre-treatment revealing that histological reduction of phosphorylated α-synuclein is linked to improved behaviour. Replication of these assays should be performed using a greater number of animals to further verify our results and unravel any possible trends in behaviour that could have been hidden due to insufficient number of animals. At a cellular level, the phosphorylated α-synuclein aggregates are colocalised with components such as the p62 protein which is a receptor of autophagy but is also involved in the proteasomal degradation and the LC3, a protein indicator of lysosomal-autophagy (Luk et al., 2009). Moreover, both proteins are colocalised with Lewy Bodies in pathology (Paumier et al., 2015). These two markers could be also investigated in our in vivo model in order to a) further assign LB-imitating pathology arising due to PFF injections, b) attempt to reveal whether ActivinA exerts its effects in association with the markers of the proteasomal and lysosomal pathways. Furthermore, stereology could have been performed as well in order to analyse the survival ratio of neurons. Another hypothesis to test could be whether instead of ActivinA aiding the fibrils degradation, it inhibits their further formation. This could be tested if we treated the PFF with ActivinA before phosphorylating them and investigated whether phosphorylation has succeeded.

In vitro studies have revealed that ActivinA can promote survival of neurogenic clonal cell lines and retinal neurons, midbrain dopaminergic mad hippocampal neurons (Iwahori et al., 1997, Schubert et al., 1990). It has also been observed that it protects neurons against toxicity, while also regulating the phenotype of sympathetic neurons (Fann and Patterson, 1994, Krieglstein et al., 1995).

Subsequently, we tried to unravel the mechanisms underlying these effects of ActivinA treatment in the SH-SY5Y human neuroblastoma cell line. Literature suggests that Ser126-phosphorylated α-synuclein is targeted to the proteasome pathways in a ubiquitin-independent manner (Machiya et al., 2010). This cell line does not express high levels of the aggregated α-synuclein. Therefore, the proteasomal inhibitor Epoxomycin was introduced in the cells in order to at least partially inhibit the proteins degradation, thus enhancing the phosphorylated α-synuclein levels and subsequently examine whether ActivinA pre-treatment could reverse this enhanced phosphorylation. For the same reason of small aggregation, it would have been invalid to create a condition of control cells pre-treated with ActivinA.

Our in vitro system confirmed that proteasomal inhibition with Epoxomycin lead to increased levels of phosphorylated α-synuclein indicating that phosphorylated α-synuclein could be at least partially degraded at the proteasome. ActivinA pre-treatment did not aid the proteins degradation when the proteasome was inhibited, failing to reverse phosphorylation. There could be a number of reasons for this lack of ActivinA neuroprotective effect. Firstly, the Epoxomycin concentration levels could have been too high, transcending the in vivo debility of the proteasomal degradation pathway thus, the concentration levels could be decreased. Alternatively, the time of Epoxomycin treatment could be moderated as well. Following the same syllogism, ActivinA concentration levels could have been too low in order to compete with the Epoxomycin proteasomal inhibition, thus either a greater concentration could be used and/or increase time of pre-treatment. Furthermore, ActivinA as an inflammatory molecule, could possibly require the presence of glia, the main player of the inflammatory response in the brain, in order to unfold its full neuroprotective and anti-inflammatory effect. Last but not least, the proteasomal inhibitor could be altogether replaced. A possible candidate could be the proteasome-specific inhibitor clasto-lactacystin-beta-lactone (cLbetaL) since its inhibitory effects are less harsh and have shown to effectively hinder proteasomal activity in the SH-SY5Y cell line (Dasgupta et al., 2014). Bafilomycin lysosomal inhibition did not alter the phosphorylated α-synuclein levels, suggesting that the lysosome is not significantly involved in the degradation of the protein. Pre-treatment with ActivinA showed no effect on phosphorylated α-synuclein levels, prior to Bafilomycin. This comes to no surprise, since Bafilomycin failed to create an effect in order for ActivinA to attempt to counteract it. Under the same conditions, levels of total α-synuclein remained unchanged.

Lastly, proteasomal inhibition with Epoxomycin lead to increased levels of p62 far beyond the levels induced by lysosomal inhibition. As mentioned earlier, p62 is a multifunctional protein located throughout the cell, used to be identified as a classic receptor of autophagy, but later discovered to be involved in the proteasomal degradation. Proteasome inhibition may further activate autophagy, in which interaction p62 acts as a bridge between the processes (Liu et al., 2016, Cortes and La

Spada, 2015) since p62 along with the proteasome can modulate activity of the HDAC6 deacetylase, thus influencing the autophagic degradation. p62 and the proteasome are colocalised under basal conditions in situ (Choe et al., 2014, Myeku and Figueiredo-Pereira, 2011). Moreover, p62 aggregates contain inactive proteasomal ubiqutinated-proteins and autophagosomes upon proteasomal degradation (Myeku and Figueiredo-Pereira, 2011). These findings suggest an intricate interaction between p62 and the proteasome, making it complicated to confidently name their separate effects. ActivinA yet again did not have a significant effect on levels of p62. Bafilomycin lysosomal inhibition either with or without ActivinA pre-treatment, failed to affect p62 levels, possibly due to small concentration used. In any case, levels of LC3 should be measured in order to verify lysosomal behaviour at the same system. The question of how ActivinA mediates its alleviating effects on pathology remains unanswered, since it seems not to interact with either the proteasome nor the lysosome. However, this hypothesis could be studied by using a tagged ActivinA in order to observe its possible localisation with the proteasome or the lysosome. Moreover, p62 is a key sensor of the mToR pathway. p62 knockdown likely activates autophagy through mTORC1 inhibition in response to starvation, since p62 is a positive regulator of mTORC1. This creates a feed-forward loop in which mTORC1 activation increases p62 levels, further promoting mTORC1 activity (Duran et al., 2011). Additionally, ActivinA has been shown to interact with mTOR in different models (Hino et al., 2017, Hino et al., 2018, Yu and Cui, 2016). A possible good suggestion would be to attempt to identify whether ActivinA mechanism involves the mTOR in our model and if possibly exerts its effect on p62 in such a way. Ideally, any suggestions on our in vitro model would be implemented on cortical neurons, which our laboratory has been trying for a year to extract them with no success so far.

Another study in vitro, showed that recombinant ActivinA is neuroprotective against the parkinsonism-inducing neurotoxin 6-OHDA in SH-SY5Y cell line. It was the first to show that transient transfection with ActivinA significantly protect the SH-SY5Y cells. Moreover, it was seen that the survival effect was mediated by the Bcl-2 family members involving inhibition of caspase-3 activation, reduction of cleaved poly-ADP ribose polymerase and phosphorylated H2A.X protein levels (Kupershmidt et al., 2007). This apoptotic pathway could be studied in our SH-SY5Y cell model to observe whether this apoptotic inhibition serves as a possible ActivinA mechanism. Such pathway could be also studied in the PFF mouse model.

Concluding, our in vivo model demonstrated that both the PFF induced histopathology and the AcrtivinA treatment post injection enhancing effect, translate to an impaired and improved behaviour respectively. Could this mean that ActivinA has a reversal effect on pathology, or due to its interaction with glia it simply delays the heavy formation of the phosphorylated α-synuclein fibrils? What effect would we observe if the conditions had more time to act in the mouse brain? Our in vitro model did work but failed to exhibit a reversal effect of ActivinA pre-treatment in the phosphorylation of α-synuclein.

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6. Supplementary Data

Dr Karampetsou performed analysis of immunofluorescence on brain sections of the same mice groups used in Section 3.1 in order to measure the intensity of the immunostaining so to obtain a more accurate result of the differences observed. Dr Karampetsou performed a double immunostaining for phosphorylated α-synuclein (Ser129) and TH (**Figure 6.1**).

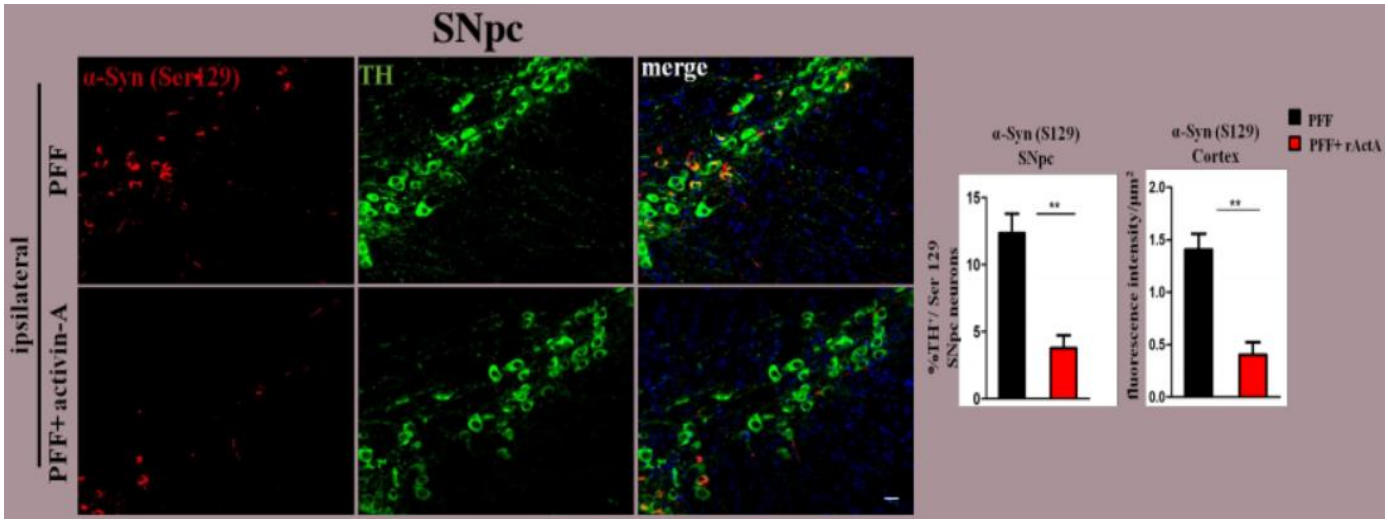

Figure 3.1: Confocal images showing double immunostaining for α-synuclein (Ser129) and TH in nigral sections of PFF injected animals. ActivinA administration resulted in reduced accumulation of phosphorylated α-synuclein. Graphs show the absolute numbers of α-synuclein (Ser129) accumulation that were formed within the TH positive neurons in the SNpars compacta and the mean fluorescence intensity of phosphor Ser129 in the ipsilateral cortex of injected animals normalised to the measured area (intensity/μm^2) (n=4 animals.group). TO-PRO-3 (blue) was used as a cell nuclear marker. Scale bar represent 25μm.

7. Abstract

Synucleinopathies are a group of neurodegenerative disorders in which the abnormal accumulation of insoluble α-synuclein fibrilar aggregates is shared as a basic trait and observed in the neurons and glial cells. Since α-synuclein is the main protein component of Lewy bodies, disorders that share the feature of LBs fall under the category of synucleinopathies. A-synuclein is a small soluble protein of 140 amino acids which is primarily found in the presynaptic nerve terminals in the central nervous system. Subcellularly, the protein is sited in higher quantities at the presynaptic vesicles in the presynaptic nerve terminals without being engulfed in them. The Serine 129 (S129) phosphorylation of the protein is linked directly to the progress of PD. Regarding its degradation, it has been proposed that both the proteasome and the lysosome play a key role for α-synuclein.

In general, the most accepted hypothesis of the pathological mode of action of α-synuclein is its ability to form oligomers and fibrils that can inhibit the proteasome and the lysosome**,** leading to further accumulation of the protein and induction of toxicity. It is widely accepted that a-synuclein is both physiologically and pathologically secreted and exerts its action on neighbouring cells, both neurons and glia following a pattern which agrees with the prion-like propagation model.

Preliminary experiments in our lab, showed that the cytokine ActivinA was protective against the pathophysiology produced by pre-formed fibrils of α-synuclein (PFFs) in mice. To this end, we also wanted to investigate, whether such protection had an effect on behaviour and so male and female wild-type C57B16/C3H mice were used, which had been stereotactically injected with PFFs. The possible mechanism of ActivinA action was investigated in vitro using the SH-SY5Y human neuroblastoma cell line.

Our results confirmed that PFF injections resulted in accumulation of phosphorylated α-synuclein in the cortex and striatum of the mice and that ActivinA treatment reduced this accumulation. The behavioural assays revealed impairments in the coordination and balance of the PFF mice linked to the phosphorylated α-synuclein aggregation. Some of these impairments were alleviated by ActivinA treatment revealing that histological reduction of phosphorylated α-synuclein is linked to improved behaviour. In vitro ActivinA failed to reduce the phosphorylated α-synuclein levels, or a reverse the proteasomal activity inhibition and had no effect on the lysosome.

8. Περίληψη

Οι συνουκλεϊνοπάθειες είναι μια ομάδα νευροεκφυλιστικών διαταραχών των οποίων βασικό γνώρισμα είναι η μη φυσιολογική συσσώρευση αδιάλυτων ινιδικών συσσωματωμάτων α-συνουκλεΐνης που παρατηρείται στους νευρώνες και τα γλοιακά κύτταρα. Δεδομένου ότι η α-συνουκλεϊνη είναι το κύριο πρωτεϊνικό συστατικό των σωματίων Lewy (LB), οι διαταραχές που μοιράζονται το χαρακτηριστικό των LBs εμπίπτουν στην κατηγορία των συνουκλεϊνοπαθειών. Η α-συνουκλεϊνη είναι μια μικρή διαλυτή πρωτεΐνη των 140 αμινοξέων, η οποία βρίσκεται κυρίως στα προσυναπτικά νευρικά τερματικά στο κεντρικό νευρικό σύστημα. Υποκυτταρικώς, η πρωτεΐνη απαντάται σε υψηλότερες ποσότητες πάνω στα προσυναπτικά κυστίδια στα προσυναπτικά νευρικά τερματικά. Η φωσφορυλίωση της πρωτεΐνης στη σερίνη 129 (S129) συνδέεται άμεσα με την εξέλιξη της νόσου του Πάρκινσον. Όσον αφορά την αποικοδόμησή της, έχει προταθεί ότι τόσο το πρωτεάσωμα όσο και το λυσοσώμα διαδραματίζουν βασικό ρόλο για την α-συνουκλεΐνη.

Γενικά, η πιο αποδεκτή υπόθεση του παθολογικού τρόπου δράσης της α-συνουκλεΐνης είναι η ικανότητά της να σχηματίζει ολιγομερή και ινίδια που μπορούν να αναστείλουν το πρωτεάσωμα και το λυσοσώμα, οδηγώντας σε περαιτέρω συσσώρευση της πρωτεΐνης και επαγωγή τοξικότητας. Είναι ευρέως αποδεκτό ότι η α-συνουκλεΐνη εκκρίνεται φυσιολογικά και παθολογικά και ασκεί τη δράση της σε γειτονικά κύτταρα, αμφότερα στους νευρώνες και τα γλοία, ακολουθώντας ένα πρότυπο το οποίο συμφωνεί με το μοντέλο διάδοσης τύπου πρίον.

Προκαταρκτικά πειράματα στο εργαστήριό μας έδειξαν ότι η κυτοκίνη Ακτιβίνη-Α ήταν προστατευτική έναντι της παθοφυσιολογίας που παράγεται από προ-σχηματισμένα ινίδια της α-συνουκλεΐνης (PFFs) σε μύες. Για το σκοπό αυτό, θέλαμε επίσης να διερευνήσουμε εάν αυτή η προστασία είχε επίδραση στη συμπεριφορά και έτσι χρησιμοποιήθηκαν αρσενικοί και θηλυκοί μύες C57B16 / C3H άγριου τύπου, οι οποίοι είχαν εγχυθεί στερεοτακτικά με PFFs. Ο πιθανός μηχανισμός της δράσεως της Ακτιβίνη-Α ανιχνεύθηκε in vitro με χρήση της SH-SY5Y κυτταρικής σειράς ανθρώπινου νευροβλαστώματος.

Τα αποτελέσματά μας επιβεβαίωσαν ότι οι εγχύσεις με PFF είχαν ως αποτέλεσμα τη συσσώρευση φωσφορυλιωμένης α-συνουκλεΐνης στον φλοιό και το ραβδωτό σώμα των μυών και ότι η θεραπεία με ActivinA μείωσε αυτή τη συσσώρευση. Οι δοκιμασίες συμπεριφοράς αποκάλυψαν αλλοιώσεις στον συντονισμό και την ισορροπία των PFF μυών, οι οποίες συνδέονται με τη φωσφορυλιωμένη συσσωμάτωση α-συνουκλεΐνης. Ορισμένες από αυτές τις αλλοιώσεις μετριάστηκαν με τη θεραπεία με Ακτιβίνη-Α αποκαλύπτοντας ότι η ιστολογική μείωση της φωσφορυλιωμένης α-συνουκλεΐνης συνδέεται με βελτιωμένη συμπεριφορά. Η in vitro Ακτιβίνη-Α απέτυχε να μειώσει τα επίπεδα φωσφορυλιωμένης α-συνουκλεΐνης ή να αναστρέψει την αναστολή της πρωτεασωματικής δραστηριότητας και δεν είχε καμία επίδραση στο λυσόσωμα.