

Sara Raquel Almeida Ferreira

Studying Alpha-Synuclein pathology in mouse striatal synaptosomes and primary neuronal cultures

Monografia realizada no âmbito da unidade de Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientada pela Professora Doutora Armanda Emanuela Castro Santos e pela Doutora Anne Panhelainen e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2016



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Coimbra, 7 de setembro de 2016.

(Sara Raquel Almeida Ferreira)

The Thesis Tutor:

(Professora Doutora Armanda Emanuela Castro Santos)

The Internship supervisor:

(Anne Panhelainen)

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I. Abbreviations

PD - Parkinson's disease

LBs - Lewy bodies

LNs - Lewy neurites

DA - Dopamine

SNpc - Substantia nigra pars compacta

DBS - Deep Brain Stimulation

α -Syn - Alpha-Synuclein

UPR - Unfolded Protein Response

ER - Endoplasmic Reticulum

DAT - Dopamine Transporter

DLB - Dementia with Lewy bodies

MSA - Multiple System Atrophy

PFFs - Pre-formed fibrils

CNS - Central Nervous System

SNARE - Soluble NSF (*N*-ethylmaleimide sensitive fusion) Attachment Protein Receptor

GRP78 - Glucose Regulated Protein 78

BiP - Binding immunoglobulin protein

HSP70 - Heat shock protein 70

ACSF - Artificial cerebrospinal fluid

PERK - Protein Kinase RNA-like endoplasmic reticulum kinase

P-PERK - Phosphorylated PERK

IRE1 - Inositol requiring enzyme I

ATF6 - Activating transcription factor 6

pelF2 α - phosphorylated eukaryotic translation initiator factor 2 α

GADD153 - DNA damage-inducible gene 153

CDNF - Cerebral dopamine neurotrophic factor

MANF - Mesencephalic astrocyte-derived neurotrophic factor

2. Resumo

Alfa-sinucleína (α -Syn) é uma proteína constituída por 140 aminoácidos que adota uma estrutura α -helicoidal quando ligada a membranas, no entanto, esta proteína pode facilmente dobrar-se numa estrutura β -pragueada que polimeriza em fibrilhas e agregados. A acumulação de corpos de Lewy e neurites de Lewy intra-neuronais contendo fibrilhas agregadas de α -Syn é um dos processos patogénicos que caracteriza a doença de Parkinson, uma perturbação neurodegenerativa motora relacionada com a idade.

A *glucose-regulated protein* (GRP78) de 78 kDa, um membro da família das *heat shock proteins* (HSP), é uma das principais *chaperones* que sob condições de *stress* do Retículo Endoplasmático (RE) é sobre-expressada e previne a agregação de proteínas, bem como facilita a degradação de proteínas deformadas. Recentemente, tem sido sugerido que a proteína GRP78 pode interagir com a α -Syn, e, eventualmente, transmitir algumas das suas ações patológicas nas células. Assim GRP78 pode ter um importante papel na acumulação de proteínas *misfolded* ou mutantes tais como α -Syn em relação à doença de Parkinson.

Nesta Monografia, foram criados vários métodos que permitam estudar a patologia da α -Syn e a sua localização sináptica, tal como da GRP78 que com ela interage. Foram utilizadas culturas neuronais primárias do hipocampo que foram, posteriormente, incubadas com fibrilhas pré-formadas de α -Syn, foram recolhidos sinaptossomas do estriado e posteriormente divididas as proteínas sinápticas em fracções citosólicas e membranares e foi também feito o estudo das proteínas extracelulares por um processo de biotinylação dos sinaptossomas. Em relação às culturas neuronais do hipocampo tratadas com fibrilhas de α -Syn, foram estudadas as formas patológicas, agregadas e fosforiladas de α -Syn, bem como feita a quantificação de vários marcadores de *stress* do retículo endoplasmático.

O estudo dos sinaptossomas durante o envelhecimento ou em modelos de doença permitirá detetar possíveis alterações nas quantidades, formas e localização sináptica de várias proteínas-chave patológicas relacionadas com a doença de Parkinson.

Palavras-chave: Doença de Parkinson; alfa-sinucleína; GRP78; sinaptossomas; culturas neuronais primárias do hipocampo; *stress* do RE.

3. Abstract

Alpha-synuclein (α -Syn) is a 140-amino acid protein that adopts an α -helical structure when bound to membranes; however, it can easily fold into a β -sheet-rich structure that polymerizes into fibrils and aggregates. The accumulation of intraneuronal Lewy-bodies/Lewy-neurites (LBs/LNs) containing misfolded fibrillar α -Syn is one of the disease processes that characterizes Parkinson's disease, a common age-related neurodegenerative motor disorder.

The 78 kDa glucose-regulated protein (GRP78), a member of the heat shock protein (HSP) family, is a major chaperone that under Endoplasmic Reticulum (ER) stress conditions is up-regulated and prevents protein aggregation as well as facilitates degradation of misfolded proteins. Recently, GRP78 has been suggested to interact with α -Syn, and possibly convey some of its pathological actions into the cells. Thus GRP78 might have an important role in accumulation of misfolded or mutant proteins such as α -synuclein also in relation to Parkinson's disease.

In this thesis work, several methods were set up to enable studying the α -Syn pathology and the synaptic localization of α -Syn and GRP78 that interacts with it. We used primary hippocampal neuronal cultures seeded with α -Syn preformed fibrils, collected striatal synaptosomes and further divided the synaptic proteins into cytosolic and membrane fractions and finally studied the extracellular proteins by biotinylating the synaptosomes. In α -Syn fibril-treated hippocampal neuronal cultures we studied the pathological aggregated and phosphorylated forms of α -Syn as well as levels of several ER stress markers. Studying the synaptosomes during aging or in disease models will allow to detect possible changes in the amounts, forms and synaptic localizations of key pathological proteins related to Parkinson's disease.

Key-words: Parkinson's disease; alpha-synuclein; GRP78; synaptosomes; hippocampal primary neuronal cultures; ER stress.

4. Introduction

Parkinson's disease (PD) is the most common age-related motor disorder and second most common neurodegenerative disorder after Alzheimer's disease (de Lau & Breteler, 2006; Van Den Eeden, 2003), affecting about 1% of the elderly population (de Rijk et al., 1995). There are 7-10 million patients worldwide with PD. As age is a significant risk factor, the prevalence of PD is predicted to rise sharply, doubling its numbers in 25 years (Reeve, Simcox, & Turnbull, 2014). Though the genetic background of the disease is well established, the etiology of PD remains unknown. Approximately 5-10% of patients have monogenic forms of the disease, exhibiting a classical Mendelian type of inheritance, but the majority PD cases are sporadic which is possibly caused by a combination of genetic and environmental risk factors (Lesage & Brice, 2009). PD is characterized by two major disease processes: the accumulation of intraneuronal Lewy-bodies/Lewy-neurites (LBs/LNs) containing misfolded fibrillar α -Synuclein (α -Syn) and the selective degeneration of midbrain dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fearnley & Lees, 1991) and striatum (nigrostriatal pathway), disrupting the neural circuitry that controls movements. This causes the major symptoms of PD: bradykinesia, slowness of movement, resting tremor, rigidity, gait dysfunctions and postural instability. Motor symptoms appear when there's approximately 60 % loss of dopaminergic neurons in SNpc and approximately 80 % reduction in striatal dopaminergic neurons (Lang & Lozano, 1998aa; 1998bb). Patients also suffer several non-motor symptoms such as impairment of smell, autonomic dysfunctions, depression, lack of motivation, sleep disorders and later on exhibit reduced cognitive abilities and dementia (Lindholm et al., 2015). PD is mainly described as a motor disease, but as the disease progresses, regions associated to autonomic, limbic, and somatomotor functions become affected (**Figure 1**) (Braak, Ghebremedhin, Rub, Bratzke, & Del Tredici, 2004).

Pathogenic mechanisms in PD include mitochondrial dysfunctions, calcium dysregulation, altered cell signalling, increased oxidative stress, as well as a disturbed protein handling (disturbed proteostasis) and ER stress with the accumulation and aggregation of unfolded or mutant proteins accompanied by defects in autophagy or in the ubiquitin–proteasome system (UPS) (Gupta, Dawson, & Dawson, 2008; Henchcliffe & Beal, 2008; Perier & Vila, 2012). In turn, the UPS can activate the unfolded protein response (UPR) on an attempt to restore ER homeostasis and correct protein folding.

Current therapeutic interventions for neurodegenerative diseases alleviate only the disease symptoms, while treatments that could stop or reverse actual degenerative processes are not available. Levodopa remains the most potent drug for controlling PD symptoms by restoring levels of dopamine in the brain, yet it is associated with significant complications such as the “wearing off” effect, levodopa-induced dyskinesias (Cotzias, Papavasiliou, & Gellene, 1969) and other motor complications. Catechol-o-methyl-transferase inhibitors, dopamine receptor agonists and non-dopaminergic therapies are alternative modalities in the management of PD and may be used concomitantly with levodopa or one another (Jankovic & Aguilar, 2008). Another promising therapy to treat the motor symptoms in PD is called Deep Brain Stimulation (DBS) that is delivered to areas in the brain controlling movement, through a surgically implanted, battery-operated neurostimulator. It’s considered as an option when optimal pharmacotherapy fails to control the motor symptoms and when those symptoms cause problems to the patient (National Parkinson’s Disease Foundation). Future therapies should focus on: i) slowing down or reverse the progression of neuronal degeneration by preventing the degeneration of dopaminergic neurons; ii) regenerating axons and stimulate branching and synaptogenesis; and iii) increasing the functional activity of the remaining dopaminergic neurons.

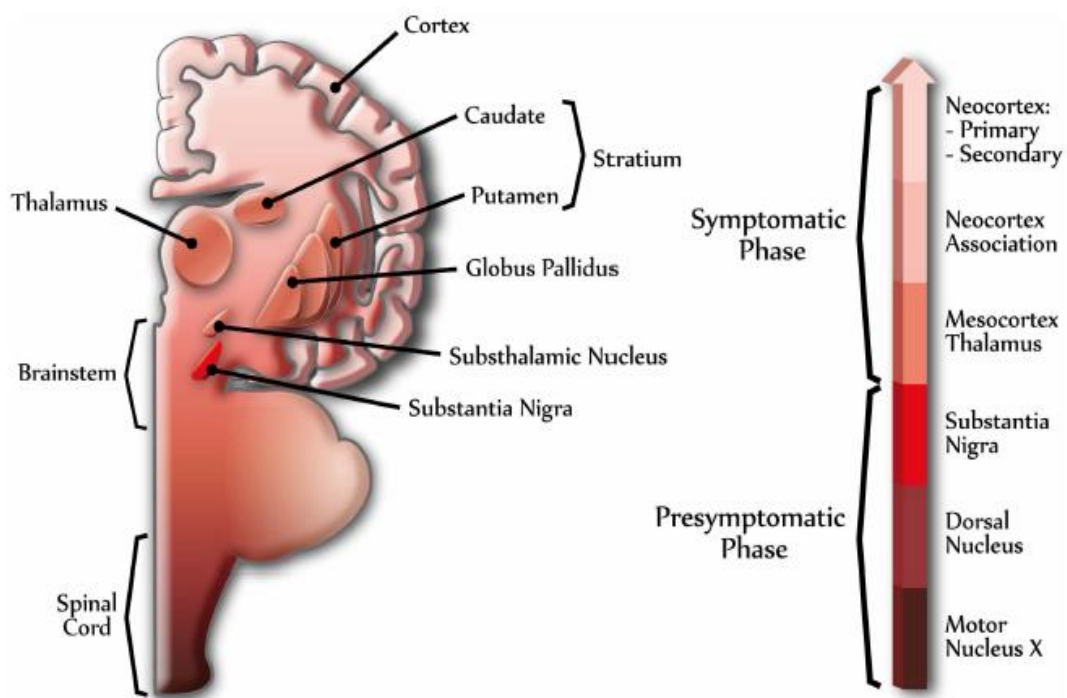


Fig.1 Different stages of Parkinson’s disease. The scheme shows the progression of PD in the human central nervous system. The right arrow shows the progression of PD, indicating some of the brain regions related to presymptomatic and symptomatic phases. Dark colours indicate early stages of PD and light colours indicate late stages of PD. Reprinted from: (Pacheco, Aguayo, & Opazo, 2012).

5. Alpha-Synuclein misfolding and aggregation on PD

One of the two major disease processes is the formation of eosinophilic inclusions in the soma of neurons named Lewy bodies (or Lewy neurites when present in the neurites), which are composed primarily of misfolded, fibrillary α -synuclein (Goedert, 2001). Post-mortem studies of PD-brains show that the extent of Lewy pathology it's directly correlated with the nature and severity of clinical symptoms (Kelvin C. Luk & Lee, 2014).

Abnormal deposition of α -Syn occurs early in the disease process and seems to follow a sequence of ascension from lower brainstem centres to limbic and wide cortical association areas (Braak et al., 2003). Accordingly to Braak's theory, in early disease stages 1 and 2, pathology occurs primarily in lower brainstem nuclei, olfactory nuclei, and peripheral neurons. Pathology in the midbrain, including substantia nigra, does not occur until stages 3 and 4 when classical PD motor symptoms become apparent. On stages 5 and 6 there's an involvement of neocortical regions. Interestingly, the neocortical LBs robustly correlate with dementia in PD, whereas LBs in the midtemporal cortex are associated to hallucinations (Braak et al., 2003; Harding, Broe, & Halliday, 2002; Irwin et al., 2012).

5.1 Structure and properties of α -Syn

Alpha-synuclein is a 140-amino acid protein that adopts an α -helical structure when bound to membranes; however, it can easily fold into a β -sheet-rich structure that polymerizes into fibrils and aggregates (Stefanis, 2012). This protein belongs to a family of proteins that also includes β -synuclein and γ -synuclein and it was the first gene linked to familial type of PD (Polymeropoulos et al., 1996). It was also originally described as the precursor protein for the non-amyloid component of Alzheimer's disease senile plaques (Ueda et al., 1993). It is abundantly expressed mainly within neurons, existing in a number of different forms - monomers, tetramers, oligomers and fibrils (**Figure 2**) (Lansbury & Brice, 2002; Moore et al., 2005), making up about 1% of the total brain protein.

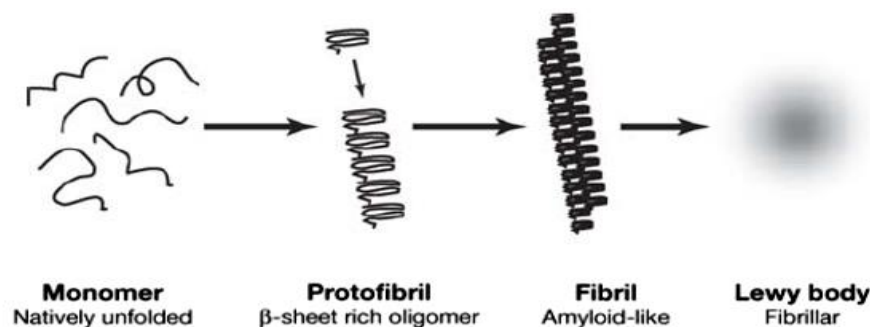


Fig.2 Representation of α -synuclein fibrillogenesis. Figure adapted from: (Lansbury & Brice, 2002; Moore, West, Dawson, & Dawson, 2005)

During disease, α -Syn suffers conformational changes to form oligomers and high molecular weight aggregates (pathological forms of phosphorylated α -Syn) which leads to an insoluble form of the protein (Bandopadhyay, 2016). The N-terminal domain of α -synuclein is almost wholly composed of eleven complete amino acid repeats that contain the consensus sequence, **XXKTKEGVXXXX**, spanning the first 89 residues of the protein, where three point mutations (A30P, E46K, and A53T) associated with early development of PD are localized. The central domain of α -synuclein is highly amyloidogenic, rich in hydrophobic aminoacids (60–95) and responsible for the aggregation and β -sheet formation (Bisaglia, Mammi, & Bubacco, 2009). Mainly acidic residues exist in the C-terminal region and they can modulate the α -synuclein aggregation depending on the pH of the environment (**Figure 3**) (Hoyer et al., 2002).

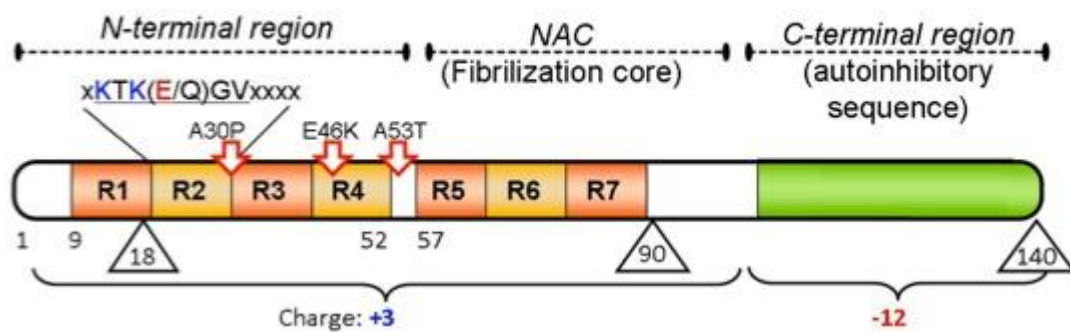


Fig.3 α -Syn aminoacid sequence. Red arrows indicate aminoacid mutations in familial PD patients, black triangles denote some alanine residues mutated to cysteine used for covalent attachment of dyes or ESR probes for biophysical experiments. The C-terminal region contains a autoinhibitory sequence that binds to the active site of α -Syn which leads to its autoinhibition. Reprinted from: Laboratory of Cellular Dynamics, Max Planck Institute for Biophysical Chemistry. (<http://www3.mpibpc.mpg.de/groups/jovin/index.php/ResearchSubjects/Alpha-synuclein>)

5.2 Physiologic role of α -Syn

α -Syn is a natively unfolded protein preferentially located in presynaptic terminals, where it dynamically associates with synaptic vesicle membrane and lipid rafts (Auluck, Caraveo, & Lindquist, 2010; Fortin et al., 2004). This suggests a role in synaptic maintenance and neurotransmitter release (Clayton & George, 1999; Maroteaux, Campanelli, & Scheller, 1988), but the precise physiological functions of α -Syn still remain undefined. Interestingly, α -Syn has been shown to depolymerize actin cytoskeleton, an important regulator of synaptic function (Cingolani & Goda, 2008), which raises the possibility that α -Syn affects vesicle dynamics through its interaction with actin (Bellani et al., 2014). In addition, α -Syn is capable of detecting

membrane curvature and lipid composition, having a preference for synaptic vesicle's small shape, high curvature, and charged lipid head groups (Bendor, Logan, & Edwards, 2013; Pranke et al., 2011).

One emerging hypothesis is that α -syn acts as a SNARE chaperone (Soluble NSF (N-ethylmaleimide sensitive fusion) Attachment Protein Receptor) to potentiate SNARE assembly for fusion of the synaptic vesicles with the axon terminal membrane (Burre et al., 2010). α -Syn can promote SNARE-mediated vesicle fusion in a manner that requires both lipid and SNARE interactions. The precise step modulated by α -Syn seems to be related to the final SNARE assembly during clustering, docking, and/or SNARE priming for assembly on the exocytosis process (Burre, Sharma, & Sudhof, 2014; Diao et al., 2013).

On the other hand, although localized in the cytosolic compartment, α -Syn is released from cultured neurons and *in vivo* studies showed it is present in biological fluids (Marques & Outeiro, 2012), which indicates that α -Syn may also act extracellularly, participating in cell-to-cell transmission of the pathology.

5.3 Pathologic role/Toxicity of α -Syn

There is an extensive amount of evidence indicating that mutated or misfolded (i.e., oligomers) α -Syn species are responsible for the neurotoxicity, and the final spread of this neurodegenerative disease throughout the brain (Winner et al., 2011). The neuronal toxicity of α -Syn over-expression or mutation seems to involve a wide range of pathways and cellular functions such as ER-to-Golgi transport, Golgi homeostasis, mitochondrial function, presynaptic trafficking, endocytosis, autophagy, the ubiquitin-proteasome system, and the ER function on proteostasis (Snead & Eliezer, 2014). Usually, α -Syn is degraded by the autophagy-lysosome system including chaperon-mediated autophagy (Cuervo, Stefanis, Fredenburg, Lansbury, & Sulzer, 2004) and disturbances in this system may lead to aggregation and accumulation of this protein in the cell, triggering ER stress (Chu, Dodiya, Aebischer, Olanow, & Kordower, 2009). An over-expression of α -Syn causes ER stress and activation of the unfolded protein response (UPR) in cellular models which can cause apoptosis if unresolved. (Smith et al., 2005). The dopamine transporter (DAT) is one secretory cargo of particular importance to dopaminergic neurons. Interestingly, α -Syn attenuates the reuptake of dopamine by interaction with DAT, in a manner dependent on expression levels of α -Syn, showing that under normal expression conditions, α -Syn negatively modulates dopamine uptake by DAT (Wersinger & Sidhu, 2003).

It is believed that dopaminergic neurons have a high-energy request that is reflected by an enhanced rate of oxidative phosphorylation and with increased production of reactive oxidative species (ROS), which causes oxidative stress (Pacelli et al., 2015). Curiously, cytosolic DA can interact with α -Syn and lead to the formation of adducts that stabilize α -Syn protofibrils (Conway, Rochet, Bieganski, & Lansbury, 2001), a process that might explain the susceptibility of dopaminergic neurons to degenerate in PD.

Cytoplasmic α -Syn inclusions are also characteristic of several other neurodegenerative conditions, most remarkably Dementia with Lewy bodies (DLB) and Multiple System Atrophy (MSA). Furthermore, α -Syn was also identified as a component of amyloid from brain tissues of Alzheimer's disease (AD) patients. These disorders share the accumulation of α -Syn aggregates as a pathological feature and are together known as synucleinopathies (Marques & Outeiro, 2012).

5.4 Seeding α -Syn pathology with pre-formed fibrils (PFFs)

Studies have demonstrated that the injection of exogenously accumulated α -Syn fibrils into the cytoplasm of cultured cells leads to intracellular α -Syn aggregation in various cells engineered to overexpress α -Syn. Although monomeric and oligomeric α -Syn showed a very slight effect, α -Syn fibrils rapidly recruited endogenous soluble α -Syn protein, altering into detergent-insoluble inclusions (K. C. Luk et al., 2009). Later on, experiments in cell culture and animals models have given direct indication that α -Syn pathology is transmissible to recipient cells. Particularly, it is now clear that brain homogenates enriched in α -Syn pathology can induce Lewy pathology in the CNS of recipient animals (Mougenot et al., 2011; Recasens et al., 2014; Watts et al., 2013). An equivalent process occurs also in non-transgenic mouse primary neurons when delivering α -Syn fibrils extracellularly (Kelvin C. Luk & Lee, 2014), which suggests that α -Syn fibrils can be uptaken by neurons and endogenous α -Syn is sufficient to support LB/LN formation (**Figure 4**).

It was demonstrated that a single intracerebral inoculation of pathological α -Syn, including α -Syn PFFs assembled from recombinant protein, is enough to induce widespread CNS α -Syn pathology and accelerate disease *in vivo*. It was also observed that inoculation into the cortex and striatum also resulted in strong α -Syn inclusions in brainstem and cerebellar nuclei, regions that do not share direct innervation with the injection sites, and that the path of transmission of pathological α -Syn does not appear to be restricted by either the presence or number of intermediary connections, which suggests that trans-synaptic spreading is a possible mode of propagation for pathological α -Syn species (K. C. Luk et al., 2012).

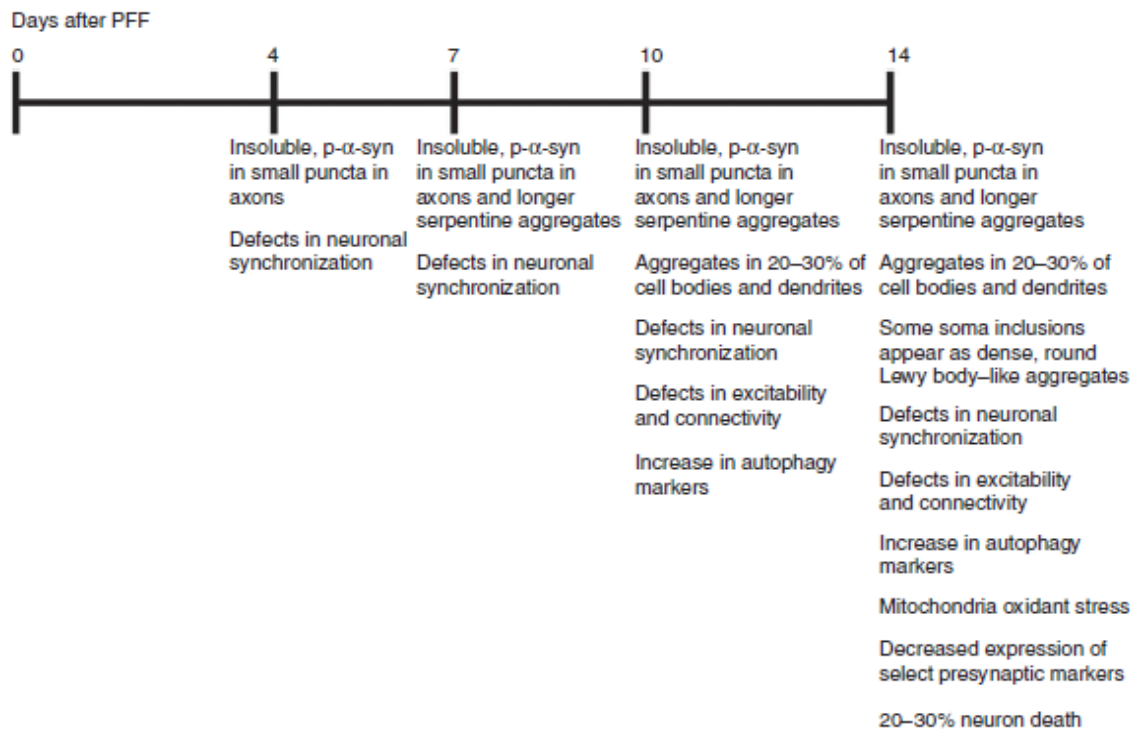


Fig.4 Timeline of known events that occur in the PFF model of α -Syn aggregation and accumulation. In this model, small seeds of PFFs generated from recombinant α -Syn were added directly to primary neurons derived from wild-type nontransgenic mice and endocytosed by them, inducing the recruitment of endogenously expressed α -Syn into abnormal phosphorylated aggregates of the protein. Reprinted from: (Volpicelli-Daley, Luk, & Lee, 2014)

6. Glucose Regulated Protein 78 and the relation with α -Synuclein

The glucose-regulated protein, GRP78 (also referred to as BiP or HSPA5), is a member of the heat shock protein 70 (HSP70) superfamily and is evolutionarily conserved from yeast to human (Ni & Lee, 2007). The ER is an essential organelle for the synthesis and processing of plasma membrane and secretory proteins. As a major ER chaperone protein with ATPase activity, GRP78 complexes with nascent polypeptides and is critical for their folding and maturation in the ER compartment. Under ER stress conditions, when misfolded proteins accumulate in the ER, GRP78 is up-regulated and prevents protein aggregation as well as facilitates degradation of misfolded proteins. GRP78 is a key regulator of the UPR as it binds and maintains the transmembrane ER stress sensors (Protein Kinase RNA-like endoplasmic reticulum kinase (PERK), inositol requiring protein I (IRE1) and activating transcription factor 6 (ATF6)) in their inactive forms. Upon ER stress, GRP78 is released resulting in the activation of these signalling pathways, impacting both cell survival and apoptosis (Bertolotti, Zhang, Hendershot, Harding, & Ron, 2000; Luo & Lee, 2013; Salganik et al., 2015).

Defects in vesicle transport are important mechanisms in PD, and mutant α -Syn was shown to reduce ER to Golgi trafficking as well as to aggravate ER stress (Colla et al., 2012; Thayanidhi et al., 2010). A possible mechanism for α -Syn induced ER stress in dopaminergic neurons was related to the accumulation of toxic α -Syn oligomers within the ER lumen and binding to chaperones such as GRP78 (Lindholm et al., 2015).

It was shown that the binding *in vitro* of extracellular Syns to GRP78 occurs at the cell surface of hippocampal neurons and leads to its clustering in discrete membrane domains, possibly as a consequence of inhibition of its lateral movement along the plasma membrane, and of its decreased recycling (Bellani et al., 2014). Alternatively, α -Syn might induce clustering of GRP78 at the cell surface through changes in membrane composition, curvature and integrity (Westphal & Chandra, 2013).

7. Possible treatments to prevent α -Syn pathology

Neuroprotective strategies should be directed to the pre-symptomatic phase of the disease so as to restrain the continuous loss of viable neurons in PD. For this, the development of novel biomarkers and early diagnostic procedures are urgent and pre-requisites for a better treatment of PD in the future (Lindholm et al., 2015).

One possible intervention is associated with the emerging role of extracellular α -Syn in disease progression. Interfering with either the process of α -Syn secretion or with the neuronal uptake of extracellular α -Syn might be beneficial for stopping or slowing down the propagation of α -Syn pathology in PD and other synucleinopathies. Interestingly, removal of α -Syn from the extracellular space might decrease detrimental inflammatory processes (Marques & Outeiro, 2012). One promising approach is immunotherapy, as immunization with human α -Syn reduces the accumulation of aggregated α -Syn and reduces neurodegeneration in transgenic mice overexpressing the protein (Masliah et al., 2005). Immunotherapy with monoclonal anti- α -Syn antibodies promoting the removal of extracellular α -Syn species might delay the progression of the disease (Marques & Outeiro, 2012).

Another promising approach to prevent this pathology is the use of neurotrophic factors such as CDNF (Cerebral dopamine neurotrophic factor), since recent studies have shown that this neurotrophic factor can protect DA neurons from the damage caused by α -Syn oligomers which induces ER stress, as is evident in PD (Latge et al., 2015). In addition, another neurotrophic factor called MANF (Mesencephalic astrocyte-derived neurotrophic factor) that was characterized and demonstrated to promote the survival of embryonic dopamine neurons

in culture (Petrova et al., 2003), together with CDNF, can regulate ER stress, modulate UPR, restore homeostasis and detain cell death by their interaction with GRP78, contributing to the maintenance and functioning of dopamine neurons (Lindholm et al., 2015).

8. Materials and Methods

8.1 Studying alpha-synuclein pathology in primary neuronal cultures

8.1.1 Preparation of Hippocampal primary neuron cultures from mice embryos brains

One six-well plate was coated with 1,5ml of 0,5mg/ml poly-DL-ornithine (in 0,15M boric acid, pH 8,7) and left overnight. Culture medium was prepared using Neurobasal medium, B27, L-Glutamine and Primocin. Embryonic E16 brains were dissected under microscopy into Dulbecco + 0,2% BSA solution. Subsequently, hippocampus was dissected out and cut in small pieces into culture medium, which was after removed and added Hank's balanced salt solution (HBSS) solution. Hippocampus was transferred into 15ml falcon tube and incubated 10 minutes on ice. Then, 100µl of 2,5% Trypsin were added and the hippocampus explants were incubated at 37°C in a water bath for 15 minutes. Afterwards, prewarmed HBSS + 10% FBS was added to the tube, as well as 5µl of Mg^{2+} and 25µl of Dnase (10mg/ml). After inverting the tube 3 times for dissociation of the hippocampus pieces, a 30 seconds centrifugation at 500 rpm was made. The supernatant was taken away and HBSS+10% FBS was added again, following another centrifugation, repeating the wash in a total of 3 times. After the last wash and the removal of the supernatant, culture medium was added and pipetted gently up and down for about 20 times for neurons dissociation. The amount of neurons were diluted (1:10) and counted using a Neubauer-Chamber. Afterwards, the neurons were plated with 2ml per well (approximately 1 million cells per well) and the medium changed after 3 days (**Figure 5**).

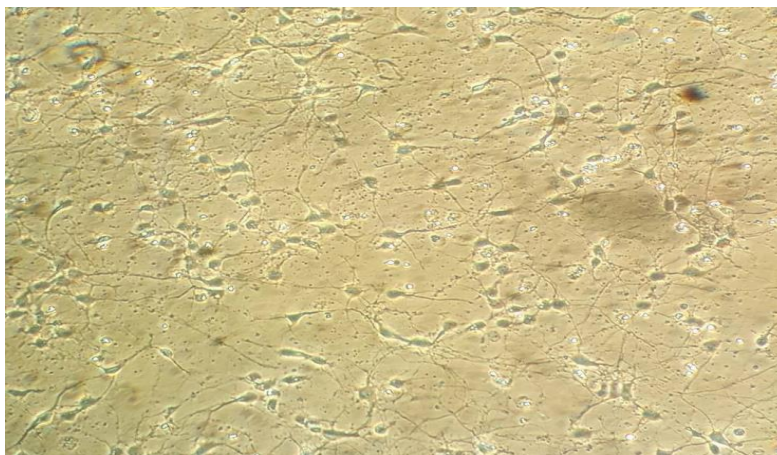


Fig.5 Neurons after 3 days of plating.

8.1.2 Addition of α -Syn PFF

After one week of growth, PFFs (5mg/ml) were added to sterile PBS to make a final concentration of 0,1mg/ml (50x dilution). The critical step follows with sonication with 60 pulses at 10% power (total 30s, 0,5s on, 0,5s off) using a microprobe tip sonicator. For a six-well plate, 80 μ l of PFFs were added into 2ml of neuronal medium (per well) for a 1:25 dilution. As vehicle, an equivalent volume of PBS was diluted in neuronal medium. 80% of the medium in each well was aspirated and the medium containing PFFs was added to three wells, as well as vehicle to the remaining wells (Volpicelli-Daley et al., 2014). The neurons were incubated with PFFs for one week.

8.1.3 Sequential extraction of proteins

SDS is an anionic strong detergent and solubilises α -Syn oligomers that can include membrane bound forms of α -Syn, whereas TX-100 is a non-ionic and thus a milder detergent. By using these detergents sequentially for protein extraction, it is possible to first harvest the more easily soluble forms of α -Syn and next the more insoluble forms of it.

While placing the six-well plate on ice, the neurons were rinsed twice with PBS, which was after aspirated and 250 μ l of ice-cold 1,1% (vol/vol) of TX-100/TBS with protease and phosphatase inhibitors were added to each well. Subsequently, all neurons were carefully scraped from each well using a cell scraper. The neurons were placed in two polycarbonate tubes (for a tabletop ultracentrifuge), one for the neurons that contain the vehicle and other for the neurons with the PFFs. The tubes were kept on ice. Afterwards, the tubes were sonicated ten times at a 0.5-s pulse and at 10% power and then incubated on ice for 30 min. After that time, the neurons were centrifuged at 100,000g at 4 °C for 30 min. The TX-100 extract of each tube was collected into two Eppendorf tubes respectively and 50 μ l 5x Laemmli buffer was added to 200 μ l of the supernatant in each tube. Twenty μ l of the TX-100 extracts were saved for protein assay and the remaining extracts were kept in a -20°C freezer. To the pellet was added 250 μ l of the ice-cold 1,1% (vol/vol) TX-100/TBS solution with protease and phosphatase inhibitors. The same sonication was made, keeping the tip of the probe toward the bottom of the tubes to prevent frothing and making sure that the pellet was dispersed completely. Afterwards and in the same conditions, a centrifugation was made for 30min. The supernatants were discarded and 125 μ l of 2,2% (wt/vol) SDS/TBS with protease and phosphatase inhibitors were added to the pellet in each tube. Later on, the tubes were sonicated 15 times, at a 0.5-s pulse and at 10% power always making sure that the pellet was

completely dispersed and keeping the tip of the probe toward the bottom of the tubes. Thereafter, the SDS extract was placed into a new Eppendorf tube and 5x Laemmli buffer was added. Twenty μ l of the SDS extract were also saved for protein assay. (Volpicelli-Daley et al., 2014).

8.1.4 Western Blotting

The TX-100 Vehicle, TX-100 PFFs, SDS Vehicle and SDS PFFs samples were boiled at 95°C for 5 min and then loaded on a 8-16% (wt/vol) polyacrylamide gel (depending on the expected size of the protein of interest) where they were subjected to SDS-PAGE. After electrophoresis, the proteins from the gel were transferred to a nitrocellulose membrane using Power Pac Basic (Bio-Rad) at 100 V constant for 1-1,5h. During this step, the apparatus was placed on ice. According to Sasaki et al (2015), when doing α -Syn antibody labelling, if the membrane is fixed with 4% Paraformaldehyde and 0,01-0,1% Glutaraldehyde for 30 min, the bands will be more clear for visualization (Sasaki, Arawaka, Sato, & Kato, 2015) and so, this procedure was followed after blotting. Then, the membrane was washed in TBS containing 0,01% (v/v) Tween 20 (TBS-T 0,1%) 3x10 min. The membrane was blocked in TBS containing 3% skim milk for 60 minutes before antibody incubations. Afterwards, the membrane was incubated in TBS-T containing 3% skim milk and the correspondent primary antibody at a proper dilution overnight in the cold room (4°C) and then washed 3x15min with TBS-T. The membrane was incubated in the same buffer containing the corresponding secondary antibody at 1:10000 dilution for 1h at room temperature and then washed 3x15min with TBS-T. The secondary antibodies used were IRDye[®] from LI-COR (Lincoln, Nebraska, USA). This fluorescent antibody has absorption and emission wavelengths in the near-infrared spectrum. The membrane was revealed at Odyssey[®] CLx imaging system from LI-COR that measures infrared fluorescence signal which would be directly proportional to the amount of target protein. The signal intensities of the revealed bands were quantified using the Odyssey[®] CLx software. One of the advantages of using near-infrared antibodies is the ability to label different proteins on the same membrane without having to strip it out of the previously used antibody due to the use of a secondary antibody with a different absorption and emission wavelength enabling two-color imaging. The same samples were used to detect (the antibody used is given in parentheses) the non-phosphorylated (Abcam, ab6162) and phosphorylated (Abcam, ab51253) forms of α -synuclein and different ER stress markers such as GRP78 (Santa Cruz Biotechnology Inc, sc-1052), p α F-2 α (Cell Signalling, 9721), P-PERK (Cell Signalling, 3179), GADD 153 (F-168) (Santa Cruz Biotechnology Inc, sc-575) and IRE1 alpha (p Ser 724) (Novus Biologicals, NBI00-2323), using the same methodology for each.

8.2 Studying synaptic proteins from striatal synaptosomes

Synaptosomes are isolated synaptic terminals from neurons that are created during nerve tissue homogenization under isotonic conditions and subsequent fractionation using differential and density gradient centrifugation, giving origin to an enriched fraction of synaptic proteins (**Figure 6**). Liquid shear detaches the nerve terminals from the axon and the plasma membrane surrounding the nerve terminal particle reseals. Synaptosomes contain the complete presynaptic terminal, including mitochondria, dense core vesicles and synaptic vesicles mainly in the cytosol-vesicular fraction, with the postsynaptic membrane attached to the presynaptic active core and the postsynaptic density mostly in the membrane-endosomal fraction. The soluble fraction of lysed synaptosomes contains primarily material corresponding in size to actin and tubulin. These proteins may originate from the synaptoplasm or they may be removed from the presynaptic membrane during lysis (Blitz & Fine, 1974). Synaptosomes are commonly used to study synaptic function because they have functional ion channels, receptors, enzymes and proteins, as well as the intact molecular machinery for the release, uptake and storage of neurotransmitters.

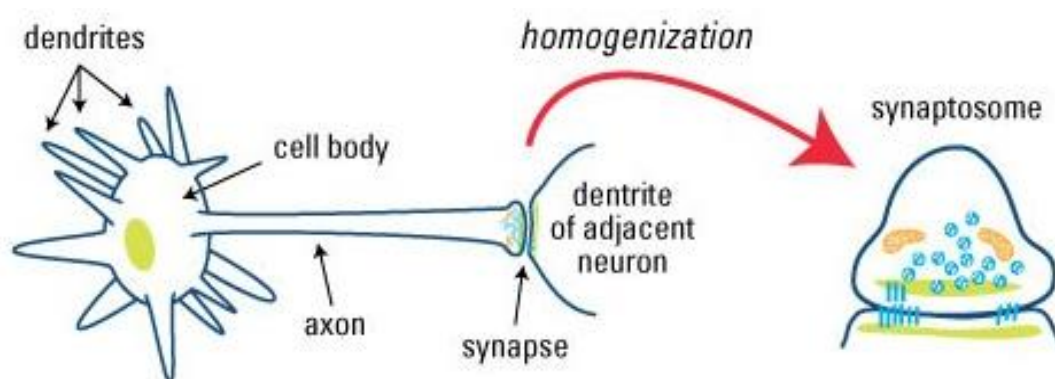


Fig.6 Representation of a synaptosome formed from the detached nerve terminal and part of the postsynaptic membrane during mechanical homogenization. Reprinted from: ThermoFisher Scientific, Method to isolate functional Synaptosomes.

8.2.1 Dissection of a mouse striatum

The brains of mice were dissected using a brain matrix (**Figure 7**) to insure reproducible sections of 2.00mm. The brains were removed quickly and kept on ice-cold plate immediately. Then, brains were washed in ice-cold cutting artificial cerebrospinal fluid (ACSF) (124 mM NaCl; 2.5 mM KCl; 2.0 mM MgSO₄; 1.25 mM KH₂PO₄; 26 mM NaHCO₃; 10 mM glucose; 4

mM sucrose; 2.5 mM CaCl₂) pH7.4 and the medial and dorsal striatum of each brain was isolated from those sections and collected into 1,5ml Eppendorf tubes (two brains per tube) with 500µl of 0.32M sucrose solution.

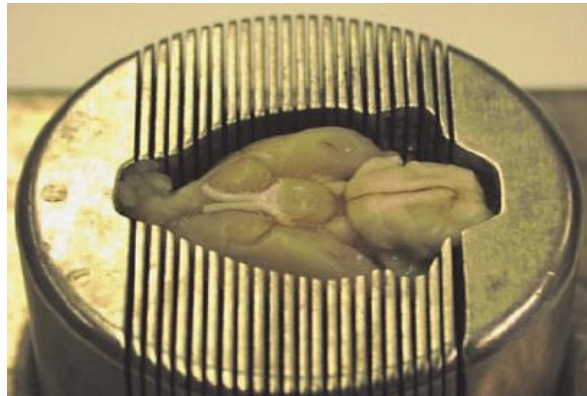


Fig.7 Image of a brain matrix for dissection of mouse brain. Scientific Figure reprinted from ResearchGate. Available from: https://www.researchgate.net/8331229_fig10_Fig-81c-Coronal-Brain-Matrix-System-supplier-A-Sem

8.2.2 Sample Homogenization and Differential Centrifugation

To obtain an enriched synaptosomal fraction, suspended tissue in 0.32M sucrose solution was homogenized using a hand-helmed homogenizer with approximately 15 up-and-down even strokes. Further, the homogenates were centrifuged at 4°C for 15 min at 3200rpm to remove cell debris. The supernatants containing the synaptosomes were then collected, the pellets were discarded and another centrifugation at 4°C for 20 min at 13300rpm was made. The supernatant was discarded and the pellets containing the synaptosomes were gently resuspended in 1ml of ice-cold ACSF pH7.4 solution. The suspension was further centrifuged at 13000rpm for 20min at 4°C and the supernatant discarded. The pellet containing the synaptosomes was then resuspended in 100 µl distilled deionized water, and centrifuged at 13300 rpm 20 min. The supernatant containing the cytosolic-vesicular proteins was aliquoted and stored in -20 °C, and the pellet containing the synaptosomal membranes, was resuspended to homogenization buffer (Tris–hydrogen chloride 50 mM, pH 7.4, Igepal 1%, sodium-deoxycolate 0.25%, sodium chloride 150 mM, ethylenediaminetetraacetic acid 1 mM, with protease and phosphatase inhibitors) to get the membrane protein sample, which was aliquoted and stored to -20 °C (Chauhan, Killinger, Miller, & Moszczynska, 2014). 10µl of each sample were taken for measurement of protein concentration.

8.2.3 Measurement of Protein Concentration with DC Protein assay (Biorad)

One crucial step for the Biotinylation of synaptosomes and to identify the correct amount of protein to load for Western Blotting is the protein concentration determination using the Bio-Rad DC Protein Assay Kit that consists in a colorimetric assay for protein concentration following detergent solubilization. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent leading to the development of colour primarily due to the presence of tyrosine and tryptophan amino acids (Bio-Rad, CA, USA). The protein standards were prepared from 1.4mg/ml standard protein stock solution and the samples were diluted 2x in ACSF pH7.4 solution. 5µl of protein standards and samples were pipetted in duplicate on a regular 96-well plate. Afterwards, 25µl of A' (1ml of reagent A (alkaline copper tartrate solution) +20µl of reagent S) were added on wells, following the addition of 200µl of reagent B (dilute Folin Reagent) in each well. The plate was then placed on a shaker and after 15min the absorbance at 750nm was measured with Victor equipment. Later, a calibration curve was made on Excel for protein concentration calculation. For all the measurements, a new calibration curve was made using protein standards and the protein concentration was determined accordingly to its calibration curve.

8.2.4 Biotinylation of cell surface proteins

Cell surface proteins play a very significant role in physiology and pathology and are receiving an increased attention by the pharmaceutical industry as important targets for development of new therapeutic strategies. Biotinylation is the process of covalently attaching biotin to a protein, and it can be used to enrich and purify proteins in different conditions due to biotin's extremely high binding affinity to streptavidin, avidin or Neutravidin. The Pierce[®] Cell Surface Protein Isolation Kit (Thermo fisher Scientific) labels proteins that have accessible lysine residues and sufficient extracellular exposure. The isolation procedure uses a cell-impermeable, cleavable biotinylation reagent (Sulfo-NHS-SS-Biotin) to label exposed primary amines of proteins on the surface of intact adherent or suspension cells. Treated cells are then harvested, lysed and the labeled surface proteins are affinity-purified using Thermo Fisher Scientific NeutrAvidin Agarose Resin. Elution of the proteins bound to the resin is done using the kit's elution buffer. The isolated cell surface proteins contain a small, nonreactive tag of the originally labeled primary amines but are no longer biotinylated (biotin remains bound to the resin).and the protein concentration was determined on the DC protein assay. The

biotinylation solution was added to the pellet from the last centrifugation on step 8.2.2. and left for 30 minutes at 4°C. Thereafter the suspension was centrifuged at 13000 rpm for 20 min at 4°C and the supernatant was further discarded. Afterwards, 500 µl of the biotin quenching solution (0,1M glycine in ACSF pH7.4) was added to the pellet, mixing gently. The suspension was centrifuged at 13000rpm for 20 min at 4°C and the resultant pellets resuspended into 500µl of Lysis buffer with protease and phosphatase inhibitors. Therefore, a 5x one second sonication was made and the cell lysate was further incubated on ice for 10 minutes, following a centrifugation at 10200rpm for 2 minutes at 4°C. The remaining steps of the procedure, i.e. the affinity purification of biotinylated proteins and their elution from the column were performed following the Kit's protocol and the resulting samples were further analysed by Western Blot, following the same methodology described at 8.1.4. step.

9. Results and Discussion

9.1 Measuring α -Syn, phosphorylated α -Syn and ER stress proteins on TX-100 and SDS extracts from primary neuronal cultures

By adding PFFs to cultured hippocampal neurons we wanted to study its effects on endogenous α -Syn and ER stress markers expression. It was also of our interest to test the protein extraction from the neurons with two different detergents: SDS and TX-100. In order to do this, a Western blot was performed on the extracted samples and membranes were incubated with antibodies for α -Syn, p- α -Syn and ER stress markers such as GRP78, GADD and p-IRE1-alpha.

9.1.1 Alpha-Synuclein

In order to study if PFFs would induce expression of α -Syn and phosphorylated α -Syn and which extraction method is the most reproducible, the proteins of the neurons incubated with the PFFs were extracted using two different detergents: TX-100 and SDS.

Regarding α -Syn, (Fig.8 – 1) after hippocampal neurons treatment with PFFs, there is an appearance of a substantial amount of α -Syn in the SDS-soluble fraction (SDS PFFs) that typically appear as a series of bands predominately within the SDS fraction (slight bands on the TX-100 fraction) and probably correspond to α -Syn oligomers. On the PBS-treated control neurons (VEHicle) there isn't that much α -Syn expression.

While looking at the membrane targeted for the phosphorylated α -Syn (Fig.8 – 2), PBS-treated control neurons do not show phosphorylated α -Syn immunoreactivity in the TX-100

or the SDS fraction. PFF-treated neurons show pathologic phosphorylated α -Syn immunoreactivity only in the SDS-soluble fraction, which again appears as a series of bands.

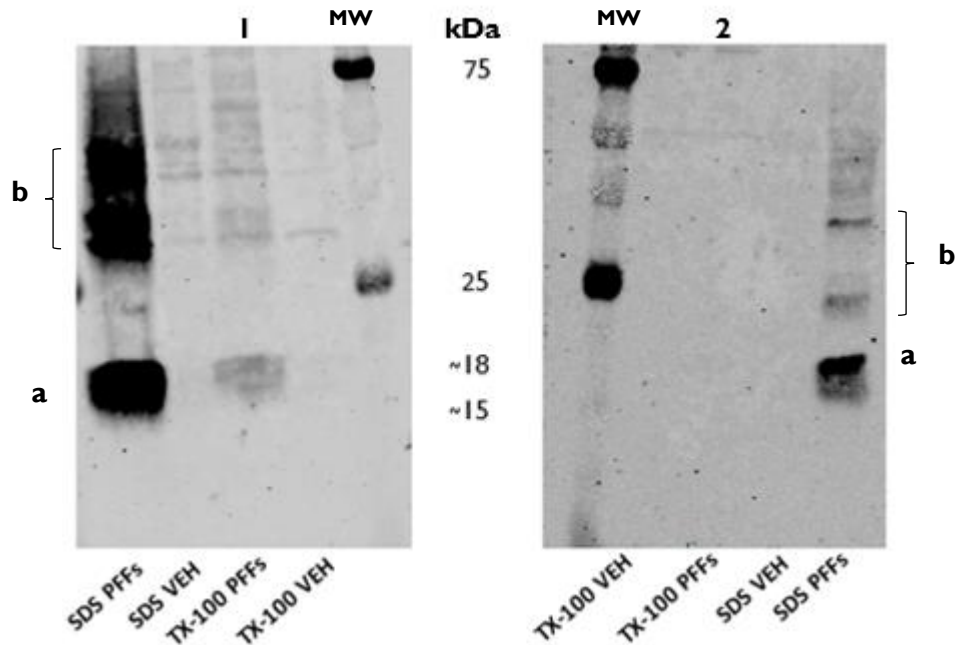


Fig.8 α -Syn (1) and Phosphorylated α -Syn (2) labelling. MW (molecular weight). Incubation with PFFs induces overexpression of both monomeric (a) and oligomeric (b) forms of phosphorylated and non-phosphorylated protein.

In conclusion and according to Nature's Protocol by (Volpicelli-Daley et al., 2014), we can clearly see that incubation with PFFs induces the expression of different forms of α -Syn and that the extraction using SDS is a better method than extraction with TX-100.

9.1.2 ER stress markers

9.1.2.1 GRP78

As GRP78 is up-regulated under ER stress conditions to prevent protein aggregation as well as facilitate degradation of misfolded proteins, we wanted to study the expression of this ER stress marker after exposing neurons to PFFs.

Ratio (in %) between PFF-treated neurons and PBS-treated neurons (band intensities normalized to actin) :

- TX-100: 121%
- SDS: 98%

After normalization of the values to actin (protein loading controls), 21% more GRP78 is present on the fibril treated sample extracted with TX-100 than on the PBS treated sample extracted with the same detergent. Regarding SDS fraction, approximately the same amount of GRP78 is present on the PBS treated sample and in the fibril treated sample. However, this experiment should be repeated to clarify whether the GRP78 is expressed significantly more in response to PFF-treatment in neurons.

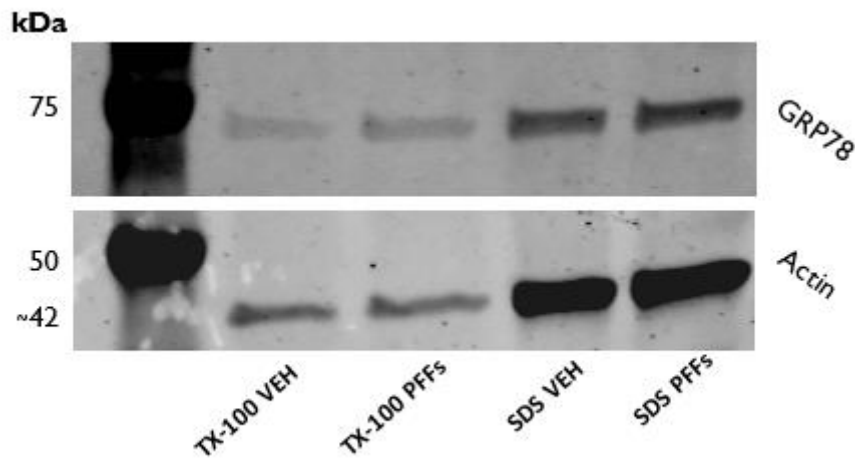


Fig.9 GRP78 labelling.

9.1.2.2 GADD153

Ratio (in %) between PFF-treated neurons and PBS-treated neurons on SDS fraction: 129 %

There aren't clear bands with TX-100 detergent, so it was not possible to quantify on these samples. Also the bands in the SDS fractions are weak, but their signal could however be detected. After normalizing the band intensities of the SDS fraction to actin, it is shown that there's 29% more GADD on the PFF-treated sample than on the PBS-treated sample.

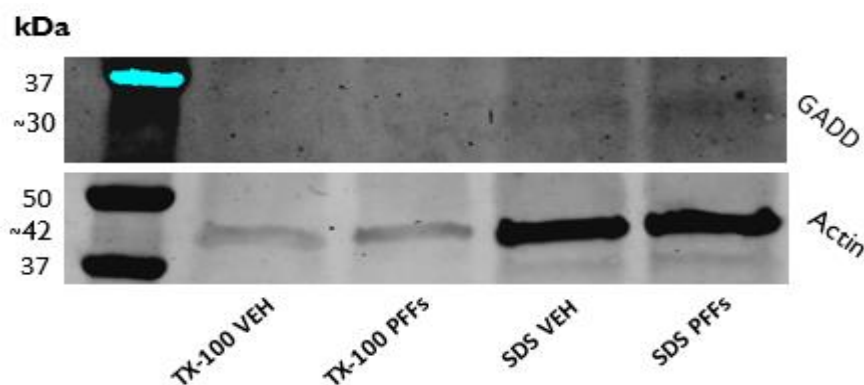


Fig.10 GADD labelling.

Even though there is a trend to increase the ER-stress marker expression after neurons treatment with PFFs, the GADD153 marker is the least expressed and this might be because the other ER stress markers promote cell survival and GADD153 is responsible for signaling apoptosis. In this way, the one week incubation of the neurons with the PFFs might not be enough to cause apoptosis (Wang, Wey, Zhang, Ye, & Lee, 2009) and that is why the expression of GADD153 is still very low. Also here, this experiment should be repeated to confirm the increase in GADD153 expression in response to PFF-treatment.

9.1.2.3 P-IREI-alpha

IREI is activated in response to accumulation of misfolded proteins within the ER as part of the UPR. It is a unique enzyme, possessing both kinase and RNase activity that is required for specific splicing of Xbp1 mRNA leading to UPR activation and promotion of cell survival (Prischi, Nowak, Carrara, & Ali, 2014). The same methodology was used to quantify the amount of this ER stress marker on its phosphorylation form expressed after PFFs addition to neurons.

Ratio (in %) between PFF-treated neurons and PBS-treated neurons on SDS fraction: 144%

There aren't clear bands with TX-100 detergent, so it was not possible to quantify on these samples. After normalizing the band intensities of the SDS fraction to actin, it is shown that there's 44% more P-IREI α on the PFF-treated sample than on the PBS-treated sample.

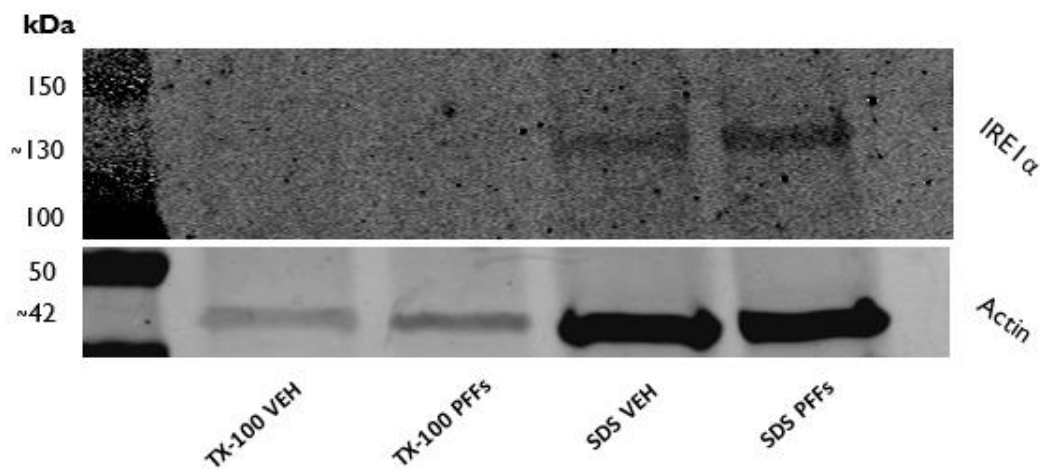


Fig.11 P-IREI α labelling.

When ER stress increases, this phosphorylation increases the activity of this protein, upon its dimerization. Again, to confirm the suggested increase, it is required to repeat the experiment.

9.1.2.4 Other ER stress markers

Some other ER stress markers were also studied on these samples such as P-PERK and pelf2 α . It was not possible to see any signal for P-PERK, but a lot of unspecific bands. The antibodies used might not be specific enough for detection of these markers and also the gels used started giving some problems in visualization of the bands.

9.2 Measuring α -Syn and GRP78 on synaptosomal membrane and cytosol fractions

Synaptosomal fractions were collected two independent times, measuring on each the described proteins. The localization of proteins of interest were compared between the fractions, to evaluate whether in synaptosomes they are located more on the cytosol or more bound to synaptic membranes.

9.2.1 Alpha-Synuclein

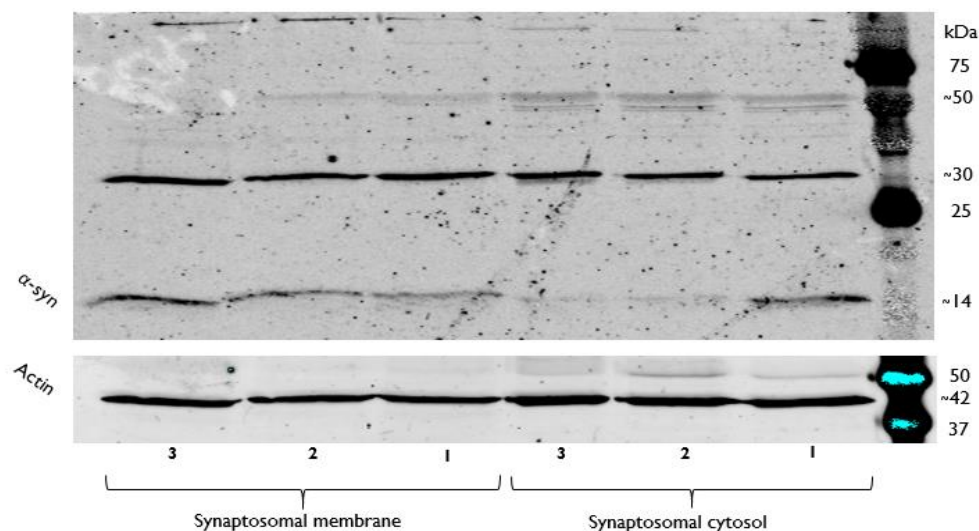


Fig.12 α -Syn expression on synaptosomal fractions extracted from mouse striatum.

Samples 1, 2 and 3 are from three 1-year-old female c57bl/6j mice.

Ratio between fractions (membrane / cytosol, given in %) (band intensities normalized to actin):

- α -Syn monomers (appr. 14 kDa):
 1. 100 % - Same quantity of α -Syn monomers on synaptosomal cytosol-vesicular fraction 1 and synaptosomal membrane-endosomal fraction 1
 2. 1077 % - approximately 10x more on synaptosomal membrane-endosomal fraction 2 than on synaptosomal cytosol-vesicular fraction 2
 3. 1320 % approximately 13x more on synaptosomal membrane-endosomal fraction 3 than on synaptosomal cytosol-vesicular fraction 3

This suggests, that monomeric α -Syn can be found in both the cytosol and bound to synaptic membranes. Since its division between the fractions varies between the samples from similar mice, this method might not be very consistent to study the α -Syn localization at synaptosomes, or it can be that in sample number 1 the synaptosomes were not so successfully separated into fractions.

- α -syn dimers (appr. 30 kDa):
 1. 51,5 %
 2. 237,5 %
 3. 244,4 %

Again, the division of α -Syn dimers between the fractions varies between the samples, as in sample 1 the localization is more on the cytosol but in samples 2 and 3 in the membrane fraction. Interestingly, there is clearly more α -Syn dimers in all samples in the synaptosomes in both the cytosol-vesicular fraction and the membrane-endosomal fraction. Even though our results suggest otherwise, α -Syn is found mostly in the nerve terminals in its monomeric form (Betzer et al., 2015). The reason why this could be so, may have something to do with the mice advanced age which can be leading to α -Syn aggregation.

9.2.2 GRP78

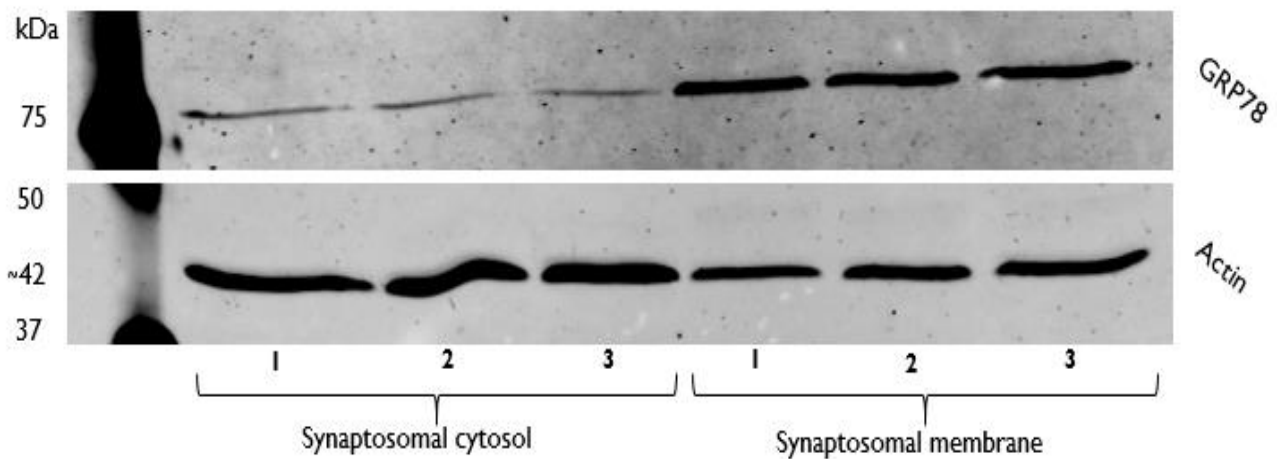


Fig.13 GRP78 expression on the synaptosomal fractions.

The blot shows, that although most of the actin is in the synaptosomal cytosol fraction, we have clearly more GRP78 on the synaptosomal membrane fraction than on the cytosol. This suggests that interestingly most of the GRP78 protein in synapses is located in the membranes.

9.3 Measuring α -Syn, GRP78 and Dopamine Transporters on biotinylated synaptosomes

The fractionation of synaptosomes into membrane and cytosolic fractions doesn't yet clarify whether the protein of interest is localized to the cell surface. To study this in more detail, we used cell-surface biotinylation to label the extracellular proteins with biotin in the synaptosomes.

9.3.1 First biotinylation experiment

- **Alpha-Synuclein**

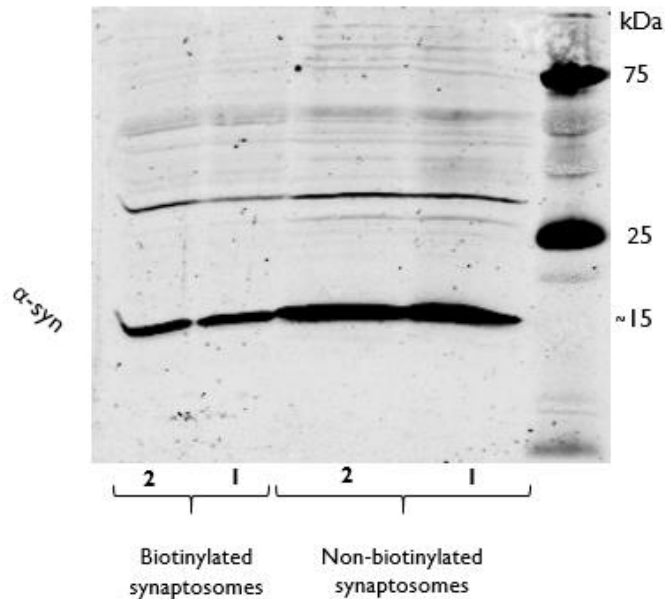


Fig.14 α -Syn expression in the biotinylated synaptosomes.

Part of α -Syn got biotinylated to the surface:

It is clear from the blot that most of the α -Syn monomers did not get biotinylated (only 10% of the monomer got biotinylated according to the bands intensities), meaning that most of it is located inside the synapses. Also a clear α -Syn dimer (30 kDa) band was seen. Band intensities quantification revealed that at least 28% of the dimer got biotinylated, a bigger ratio than the

monomeric form. This suggests that dimer form of α -Syn could be more probable to be exposed at the cell surface.

- **GRP78**

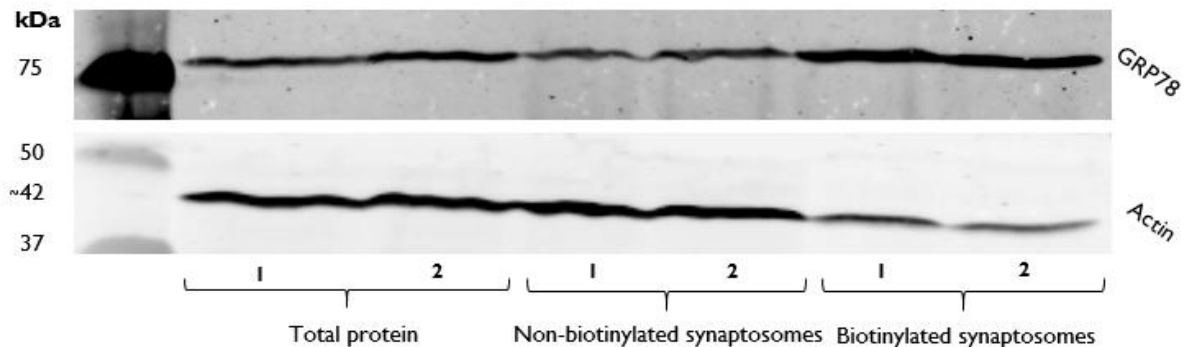


Fig.15 GRP78 expression in the biotinylation synaptosomes.

After GRP78 labelling, actin was labelled on the same membrane, for control of biotinylation efficacy, i.e. by comparing the amount of biotinylated intrasynaptic protein to the protein of interest. Regarding actin, minority of this intracellular protein got biotinylated, suggesting that biotinylation of extracellular/extrasynaptic proteins was quite effective, showing some promising results. It could be possible that small part of the biotin-linked complex got broken and few biotin was able to enter the synaptosomes or, as actin can be released from injured cells into the extracellular space, before biotinylation some of the synaptosomes might have

been ruptured releasing actin to the extrasynaptic space, which could explain the slight bands of actin expressed on this samples. Interestingly, the blot shows clearly that GRP78 became biotinylated to a large extend, suggesting that there was a lot of GRP78 exposed extracellularly. It is also clear, as expected, that there was similar amounts of total protein and biotinylated GRP78 between the samples, since the mice were in similar conditions.

- **Dopamine transporter (DAT)**

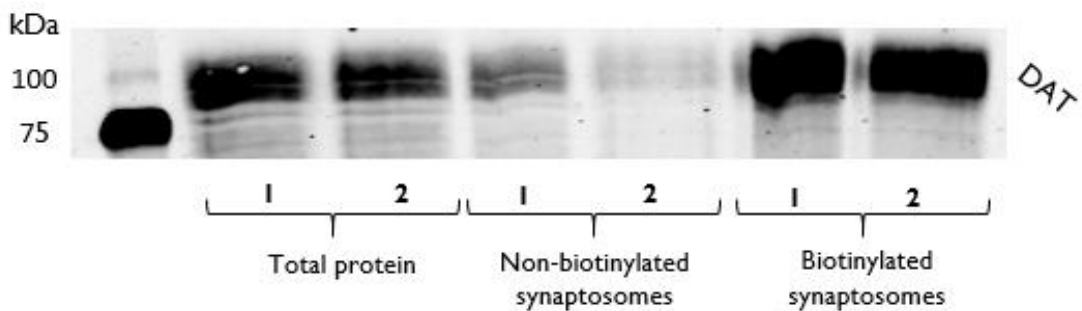


Fig.16 DAT expression in the biotinylated synaptosomes.

After biotinylation and as expected, it is clearly visible the superior amount of Dopamine Transporter levels on biotinylated proteins-fraction, as we can find them on the pre-synaptic membrane. Regarding the non-biotinylated proteins-fraction, we can see some signal from the intracellular dopamine transporter and part of it is due to biotinylation of actin from the samples, representing the non-specific biotinylation of the intracellular proteins.

9.3.2 Second biotinylation experiment:

- **α -Syn**

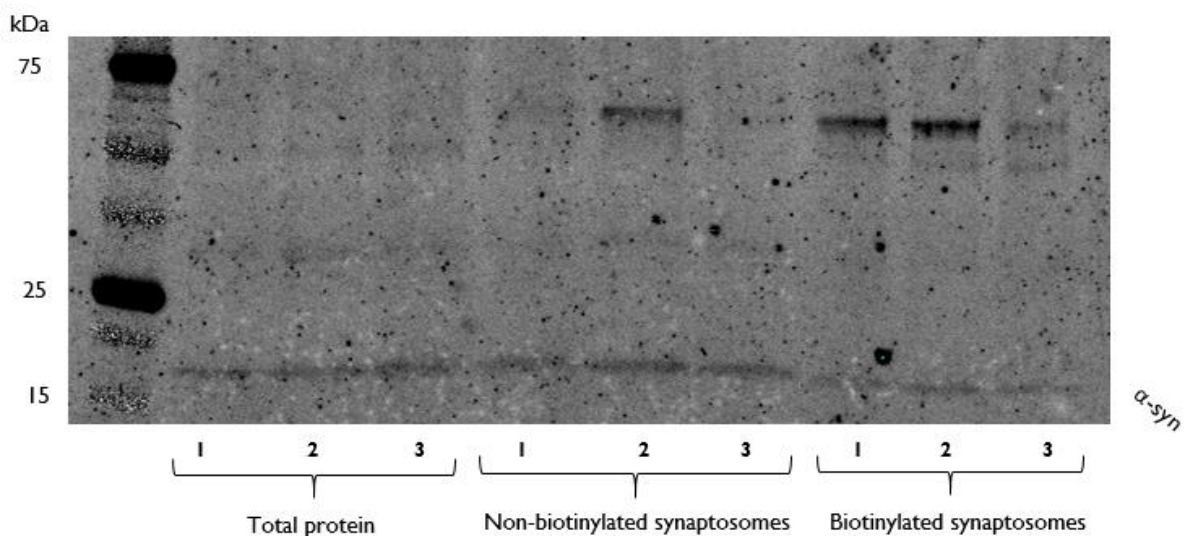


Fig.17 α -Syn expression in the biotinylated synaptosomes.

1. 2 young males (9 weeks)
2. 2 older males (8 months)
3. 2 older females (8 months)

The total protein sample was collected before biotinylation of the synaptosomes. Oligomers of large sizes were seen in both non-biotinylated and biotinylated protein-fractions of older males but mainly in the biotinylated proteins-fraction of young males and older males, which indicates the presence of the pathogenic form of α -Syn mainly on the synaptosomes surface (oligomers got biotinylated).

Contrary to the first synaptosomal samples biotinylated, there is no 30kDa oligomers presenting the dimeric size of α -Syn. This could be due to fact that the signals from these samples were very weak, suggesting that the collection of proteins did not succeed as well as previously, and thus it could also be that the dimer forms of α -Syn are simply not seen due to low signal.

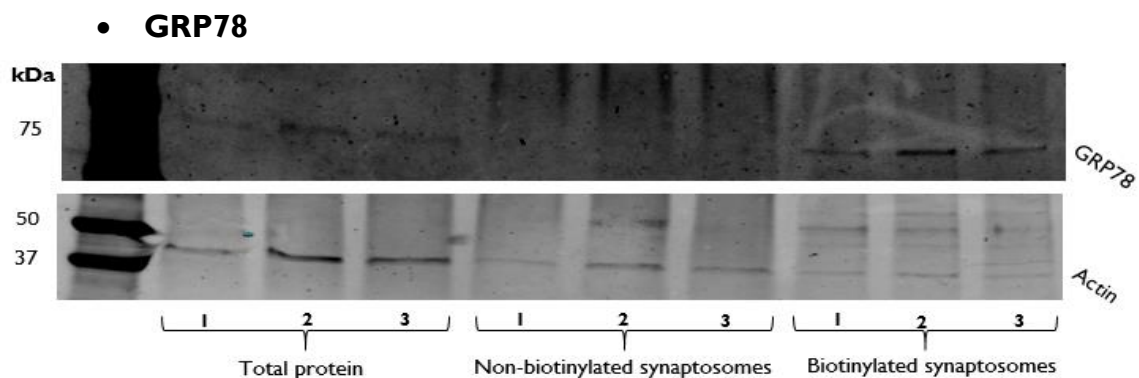


Fig.18 GRP78 expression in the biotinylated synaptosomes.

As mentioned earlier, these second biotinylation samples did not succeed so well, thus giving bad signals in western blots, making it difficult to analyse the results. However, it seemed that also in these samples only a minority of the intracellular protein actin got biotinylated suggesting that the biotinylation of only extrasynaptically exposed proteins mostly worked.

The actin bands on total protein samples reveal that the samples from young males (1) have less protein loaded on the gel. Taking this into account it seems that there are similar levels of GRP78 in all mouse groups. However, most of the GRP78 protein got biotinylated in all samples, suggesting that it was exposed extrasynaptically, and that way supporting the findings from the first biotinylation experiment.

10. Conclusion

The main aims for this study were to set up methods to study synaptic proteins in striatum, where nigral dopamine neurons project, and the more specific synaptic localizations of proteins of interest, such as α -Syn, p- α -Syn, GRP78 and other ER stress proteins. Consistent with the research developed, α -Syn is presented as a valuable target for the treatment of PD and possibly other related synucleinopathies. Here I have presented possible methods to study the α -Syn pathology in primary neuronal cultures seeded with α -Syn preformed fibrils and to study the synaptic localization of α -Syn and GRP78 that interacts with it, and possible changes in their amounts, forms and synaptic localization during aging or in disease models.

Regarding the expression of the proteins on hippocampal primary neuronal cultures, it was shown that the neurons incubated with PFFs of α -Syn and extracted with SDS revealed increased ER stress levels with the consequent increased expression of those ER stress proteins, which support the events that occur on the disease pathogenesis. However, the repetition of this experiment is required for more enlighten conclusions.

On synaptosomal fractions, the experiments were not conclusive since the expression of α -Syn varies between fractions showing that the first method to separate fractions was not suitable to study the localization of the protein. However, biotinylation of those samples proved that as described in the literature, α -Syn is mostly an intracellular protein. Interestingly, we found that it was more probable to be biotinylated i.e. exposed extracellularly when it is on its pathogenic aggregated form. After repetition of the experiment with different mice samples, it was shown an increased expression of α -Syn oligomers in older males samples which is also consistent with the prevalence of PD in elderly human males. However, this finding should be still repeated and confirmed. Regarding the synaptic localization of GRP78, it was mostly found on the synaptosomal membrane fraction and became biotinylated on a large extend in the synaptosomes. With the second biotinylation experiment, with different mice samples, despite the bad conditions of the gels that lead to bad signal images, it was also visible the bigger localization of the protein extracellularly, supporting the findings of the first biotinylation experiment. Biotinylation also confirmed the presence of DAT mostly on the membrane, exposed extracellularly.

Eventually in the future, an experiment that I would like to do would be to incubate the PFFs with primary DA neurons together with CDNF or MANF and study their effects on cell survival.

With this work I was able to show the various tasks that a Pharmacist can undertake in his/hers career, not only as a drug specialist but also as a research professional capable of

conducting experiments in different science fields, thus contributing to society with the constant effort to discover new therapeutic strategies.

11. References

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