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Protein misfolding and aggregation in Neurodegenerative disorders

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica sob orientação científica do Doutor Pedro Domingos e coorientação do Doutor Henrique Girão apresentada à Faculdade de Medicina da Universidade de Coimbra.

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Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica. Este trabalho foi realizado no laboratório de Cell Signaling in *Drosophila* no Instituto de Tecnologia Química e Biológica (ITQB) da Faculdade Nova da Universidade de Lisboa, sob orientação científica do Doutor Pedro Domingos e supervisão do Doutor Henrique Girão.

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ABBREVIATIONS

Ala, A Alanine

α-syn alpha-synuclein

BSA bovine serum albumin

CAG Cytosine-adenine-guanine trinucleotide

ER endoplasmic reticulum

HD Huntington's disease

Htt Huntingtin protein

Httex1 Huntingtin exon 1

NT17 N-Terminal sequence of 17 amino acids

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate buffer saline

PBS-T Phosphate buffer saline

PD Parkinson Disease

PolyQ Polyglutamine

Rpm rotations per minute

S13 Serine 13

S16 Serine 16

SNpc *substancia nigra pars compacta*

TBS-T Tris-HCl buffer saline-tween

T3 Threonine 3

UPR unfolded protein response

WT wild-type

ABSTRACT

Protein misfolding and aggregation is a common hallmark in neurodegenerative disorders, including Parkinson's disease (PD) and Huntington Disease (HD). The misfolding and aggregation of specific proteins contributes to neuronal degeneration in somewhat specific areas of the brain. However, the mechanisms by which these proteins lead to degeneration remains poorly understood. The main goals of this study was to elucidate and identify the molecular determinants of the subcellular localization of alpha-synuclein (α-syn) in PD and also to study the effect of N-terminal phosphorylation in the aggregation and toxicity of mutant huntingtin (Htt) in HD using *Drosophila melanogaster* as model.

Previous results from our lab have shown that the mutant form of α-syn protein (α-syn-EGFP A30P) fails to localize the photoreceptor's synapses. In this study we used RNAi knockdown screen to identify gene modulators of α-syn-EGFP A30P localization by performing head cryossections of adult flies using GMR-Gal4 driver in order to express the protein α-syn (WT and mutant form) in the *Drosophila* eye. We validated that α-syn-EGFP A30P has a mislocalized distribution throughout the photoreceptors cytoplasm. Using specific genetic knockdown for spaghetti squash, tomosyn isoform C and synaptotagmin 4, we saw an increase of α-syn-EGFP A30P localization at the synapse and these specific interactors may be possible modulators of this localization.

Phosphorylation pathways have been shown to modulate the toxicity of mutant Htt *in vitro* by affecting its oligomerization and aggregation dynamics. We used a *Drosophila* model where the amino acid residues T3, S13 and S16 of Httex1 97Q were mutated to aspartate (T3D, S13D and S16D - phosphomimics) or alanine (T3A, S13A and S16A - phosphoresistant). We did functional assays to evaluate the effect of N-terminal phosphorylation on motor abilities and survival rate and we also performed dissections of larval eye-imaginal discs and adult brains in order to characterize the pattern of aggregation of these mutants. Moreover, we studied the effect on Httex1 protein aggregation of particular phosphatases that may dephosphorylate NT17 using RNAi gene knockdown experiments in dopaminergic neurons, using TH-Gal4 driver.

From our results it was possible to confirm that single phosphorylation on NT17 domain modulates Htt aggregation and neurotoxicity in *Drosophila* since phosphomimic mutants showed an improvement in motor function with the exception of the T3D mutant.

Phosphomimic mutants also exhibited increased life span compared to the phosphoresistant mutants. Interestingly, phosphomimic mutants showed bigger aggregates than phosphoresistant mutants in two different cell types analyzed, the dopaminergic neurons of the adult brain and the photoreceptor cells of the larval eye-imaginal discs. We also test the effect of genetic knockdown for specific protein phosphatases on dopaminergic neurons and a significant reduction of mutant Htt aggregation was observed when PP1α-96A and PP1-87B protein phosphatases where downregulated.

These findings suggest the mislocalization of α-syn-EGFP A30P may due to its interaction with other proteins in the cell and impairs its traffic to the synapse. In HD, phosphorylation has an important role in modulating aggregation and toxicity of Httex1 and specific phosphatases may improve the outcomes.

Keywords: Neurodegenerative disorders, protein misfolding, protein aggregation, alpha-synuclein and huntingtin.

1. INTRODUCTION

1.1. Protein homeostasis

Maintenance of protein homeostasis, or proteostasis, requires a dynamic coordination to ensure the proper folding, transport and clearance of proteins that are critical for cell function and organismal health (1,2). The endoplasmic reticulum (ER) is a cellular organelle that is involved in numerous important mechanisms such as intracellular calcium homeostasis, correct protein folding and secretion, lipid biosynthesis, regulation of cellular redox state and post-translation modifications (3,4).

The ER can sense and transmit signals due its complex membranous network that extends throughout the cytoplasm and is contiguous with the nuclear envelope (5). Under physiological conditions, properly folded secretory proteins exit the ER and traffic to the Golgi apparatus for further maturation and distribution to their final destination (2). ER imbalance triggered by rising levels of protein synthesis and accumulation of misfolded proteins, nutrient deprivation or alterations in the calcium or redox balance can lead to a condition called ER stress (6). Under ER stress conditions, cells activate an adaptive response known as the unfolded protein response (UPR) (figure 1) that acts to restore ER homeostasis (7). UPR activation is observed in many human diseases such as type 1 and type 2 diabetes, myocardial infarction, stroke and neurodegeneration (4).

In response to the accumulation of unfolded/misfolded proteins in the ER, the UPR activates three signaling pathways: PERK (Protein Kinase RNA-like ER Kinase), IRE1 (Inositol requiring enzyme 1) and ATF6 (Activating transcription factor 6). Activation of these 3 signaling pathways leads to the genera shutdown in mRNA translation in combination with the up-regulation of genes encoding protein chaperones and ER-associated degradation (ERAD) machinery (8,9). The UPR is a complex stress response, which can lead to various outcomes on the cell depending of the intensity of the stress. Moderate ER stress may induce a pro-survival response (10) . However, in the presence of sustained and strong ER stress, activation of the UPR can lead to apoptosis (1).

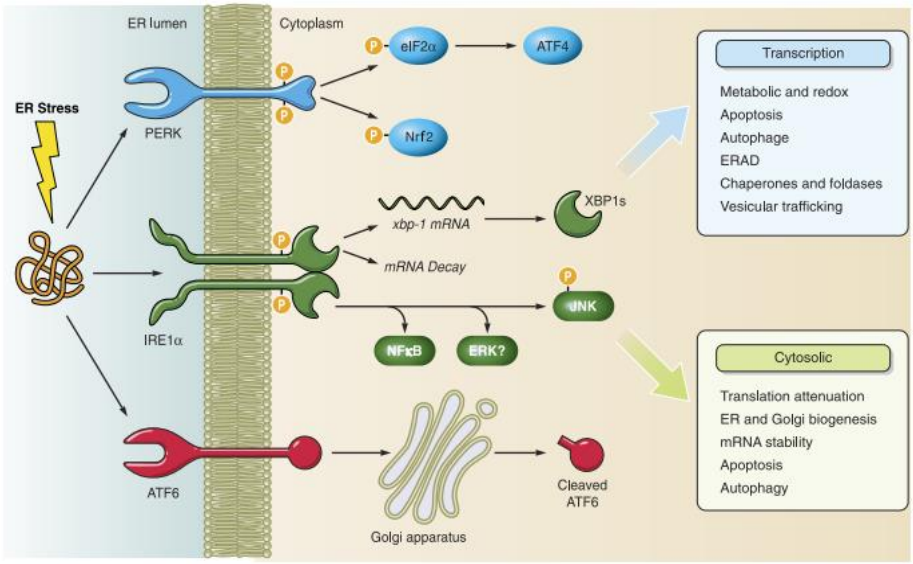

Figure 1 - The unfolded protein response. The UPR activates three major effectors signals: PERK, IRE1α and ATF6 (adapted from (8)).

Although the role of the UPR remains poorly understood in the nervous system, activation of the UPR may be involved in the modulation of neurodegenerative diseases and it is observed in several neurodegenerative disorders including amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, prion-related disorders and Alzheimer's disease (9). Since neurons are post-mitotic cells and due to absence of cell division, injured neurons cannot be replaced after apoptosis and deregulation neuronal cell death is implicated in most neurodegenerative disorders.

1.2. ER stress in Neurodegenerative disorders

Disruption of ER homeostasis impairs the protein folding process at the ER which leads to the accumulation of misfolded protein in its lumen causing ER stress, a common feature of several neurodegenerative disorders (8). The accumulation and deposition of misfolded proteins contribute to decreased neuronal function and viability, however the mechanism by which these proteins cause degeneration remains unknown.

Many efforts are being done to elucidate the causes underlying these disorders and although neurodegenerative disorders have different clinical manifestation, all share the phenomenon of misfolded pathological proteins accumulation in the form of small oligomers, aggregates or large-protein inclusions (2,11).

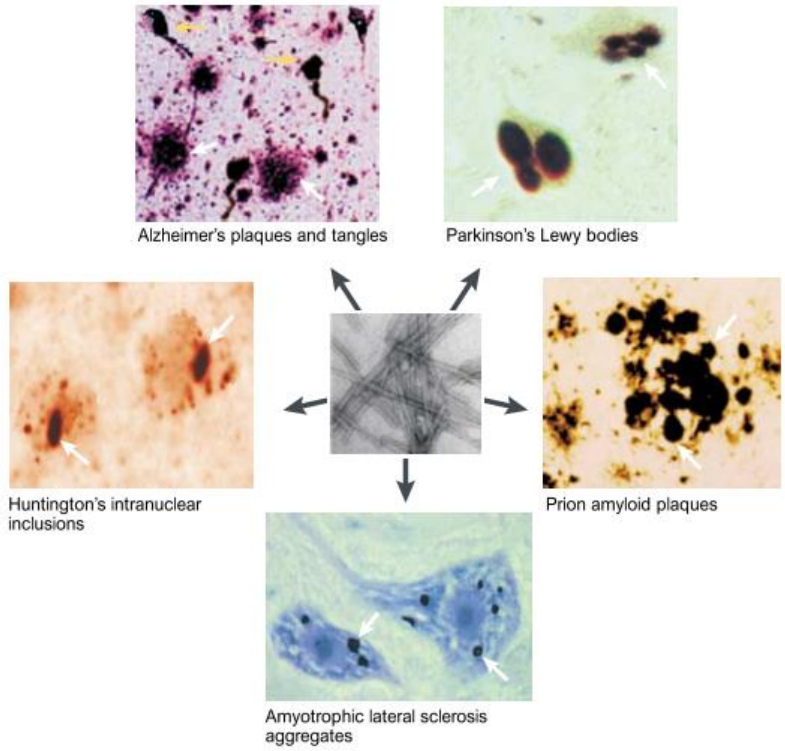

Figure 2- Protein aggregation in neurodegenerative disorders. The common pathological hallmark of the neurodegenerative disorders is the accumulation of intra- or extracellular misfolded protein that leads to cellular dysfunction and death in the long term (adapted from(11)).

Protein aggregates are dynamic structures and mutant proteins undergo covalent modifications such as proteolytic cleavage or post-translational modifications, leading to changes in protein conformation. The role of protein aggregates in neurodegenerative disorders is poorly understood and there is no consensus on whether protein aggregation constitutes a process which is protective or detrimental to cells. Increasing evidence considers that bigger insoluble protein inclusions are neuroprotective, while smaller soluble oligomers have neurotoxic effects (12,13).

1.2.1. Parkinson disease

Parkinson disease (PD) is the second most common neurodegenerative disease in the world and the major symptoms of PD are bradykinesia, rigidity, resting motor and postural instability (14,15). These symptoms are largely attributed to the selective loss of dopaminergic neurons in the *substancia nigra pars compacta* (SNpc) region of the brain and also the presence of intraneuronal proteinaceous inclusions (Lewy bodies) on surviving neurons (16). Alpha-synuclein (α-syn) is the major component of the amyloid

fibril form of Lewy bodies (13) and it is found in the brains of patients with Parkinson Disease and those with dementia with Lewy bodies (17).

Point mutations in the gene encoding α-syn (SNCA) are linked to an autosomal-dominant form of PD (15) and duplication/triplication of the α-syn gene cause a severe form of PD (18). Additionally, disease causing missense mutations (A30P, E46K and A53T) have been identified (19) in the N-terminal domain of α-syn, that forms an amphipathic helix important for membrane binding and for α-syn presynaptic localization (18). These mutations induce an increase in the propensity of α-syn to misfold and to form fibrillary aggregates enriched in β-sheet structure (19). These genetic and neuropathological findings suggest that α-syn plays an important role in PD pathogenesis.

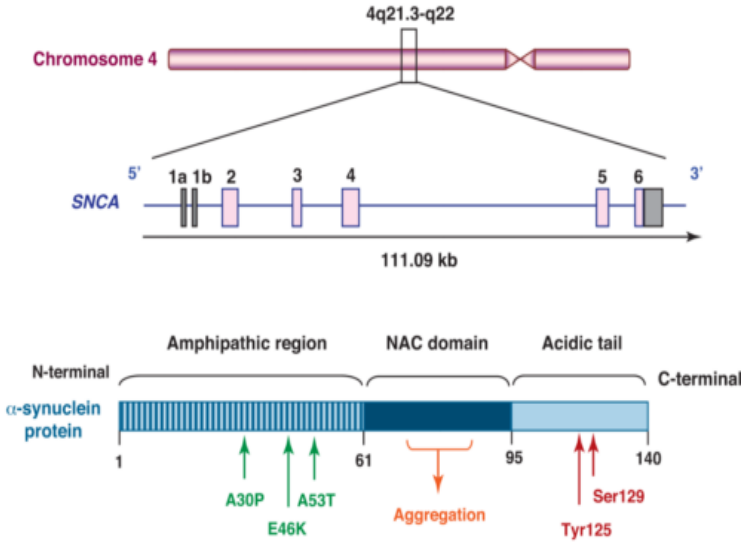

Figure 3 – Representation of human α-synuclein (α-syn). α-syn is encoded by the SNCA gene located on chromosome 4. α-syn consists of an amphipathic N-terminal region where are located the missense mutations, a hydrophobic core (also named as NAC domain) and an acidic C-terminal region. (adapted from (15)).

Loss of dopaminergic neurons leads to a depletion of dopamine within the striatum which causes dysregulation of the motor circuits, resulting in the clinical manifestations of PD. Some studies highlighted the role of α-syn accumulation as the early step of the disease and overexpression of α-syn in transgenic mice and *Drosophila melanogaster* has demonstrated the capacity of α-syn to aggregate and cause toxicity in the dopaminergic system (20,21).

1.2.1.1. Alpha-synuclein function and involvement in Parkinson Disease

α-syn is a 140 amino acid protein that localizes in the axon terminals in neurons and is involved in vesicular trafficking by interacting with the SNARE complex proteins at the presynaptic terminals (13,18,22), suggesting a role of α-syn in neurotransmitter release and maintenance of synapses (18,23). However, the physiological function of α-syn is still unknown and some studies have demonstrated that the increase of intracellular amount of α-syn is a risk factor for neurodegeneration, since it elicited motor and memory deficits and cognitive dysfunction in a transgenic mouse model with the formation of inclusion bodies and neuronal loss (24).

The α-syn protein is natively unfolded and adopts an alpha-helical structure upon binding to lipid membranes that mediates its association with membranes and its SNARE-complex chaperoning activity. Under pathological conditions, soluble α-syn protein can undergo conformational changes that start with the formation β-sheet oligomers which convert into amyloid fibrils, leading to its aggregation and deposit in Lewy bodies (figure 4) (25).

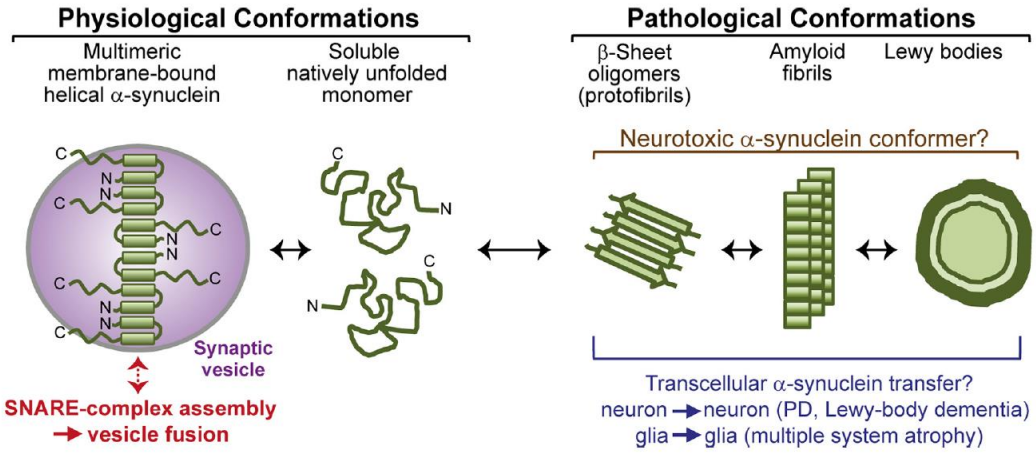

Figure 4- Schematic representation of α-syn self-assembly mechanism under normal and pathological conditions. Natively unfolded, monomeric α-syn undergoes self-assembly to form β-sheet-rich oligomers with different aggregations states. The oligomers become mature and convert to amyloid-like fibrils. Adapted from (25).

Moreover, it was demonstrated that synaptic dysfunction induced by a deficiency in CSPα in mouse model was ameliorated upon overexpression of α-syn and reversed the SNARE-complex assembly deficit (22,26). This result demonstrates that α-syn may have a

protective effect and it is important in the regulation of the synaptic pool size. Sudhof and co-workers have demonstrated that α-syn directly binds to synaptobrevin-2/vesicle-associated membrane protein 2 and promotes SNARE complex formation without changing the neurotransmitter release (22).

However, increasing evidence suggest that synucleins may modify neurotransmitter release (18,27) and, taking in account that α-syn has a tendency to form soluble oligomers and protein aggregates, it is highly possible that these species might interact with SNARE proteins and hamper normal synaptic transmission in a mechanism poorly understood. Furthermore, point mutations occurring in the N-terminal portion of α-syn have been linked to the impairment of α-syn binding to biological membranes and specifically regarding the A30P mutation, it was showed that this mutation modifies the subcellular localization of α-syn (28). However, it is also possible that some of the toxicity of a-syn A30P is due to spurious binding to biomolecules, namely protein partners, which may sequester a-syn A30P and hinder its normal axonal transport and subcellular localization at the synapses. We are interested in identifying such α-synA30P protein partners and investigate the molecular mechanisms which sequester α-synA30P, impairing its normal axonal transport and subcellular localization at the synapses.

1.2.2. Huntington disease

Huntington´s disease (HD) is an inherited autosomal dominant disorder that is characterized by motor dysfunction, psychiatric disturbances and intellectual decline, for which there is no treatment (29). HD belongs to the group of polyglutamine (polyQ) pathologies and is caused by an abnormal expansion of a triplet cytosine-adenine-guanine (CAG) repeats within the first exon of the IT15 gene that encodes the mutant huntingtin protein (Htt) (6). In normal conditions the Htt exon1 (Httex1) is constituted by 6-35 glutamine residues and an expansion of polyQ tract over 35 glutamines induces the misfolding and aggregation of mutant Htt, causing HD (30).

Huntingtin (Htt) is expressed throughout the body and Htt is essential for embryonic development in mice (31) but not in *Drosophila* (32). Htt knockout mice also showed neural degeneration in adults (31).

The huntingtin protein (3142 amino acids) contains a polyglutamine tract in its N-terminus, flanked N terminally by 17-amino acids (NT17 domain) and C-terminally by a

proline-rich region (33). Several studies have demonstrated that the N-terminal portion of mutant Httex1 is sufficient to cause severe HD-like phenotypes (34,35,30) and for this reason some *in vivo* HD models are based on the expression of mutant versions of Httex1.

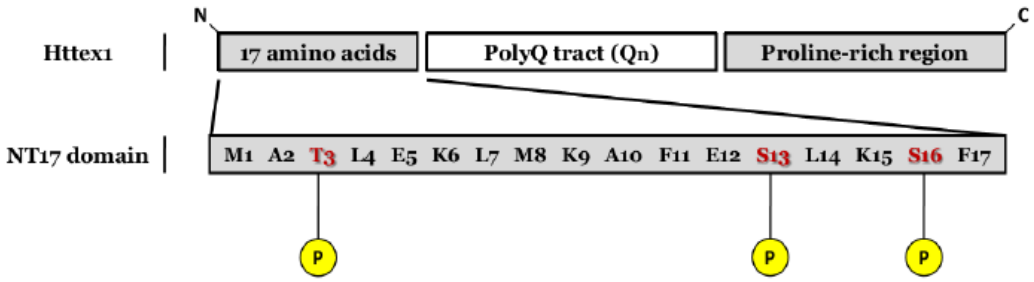

Figure 5 - Huntingtin exon 1 and its phosphorylation sites. The PolyQ tract is preceded by NT17 domain, which is highly conserved through mammalian evolution, suggesting an important function for this domain (adapted from (36)).

Within the NT17 domain, threonine 3 (T3), serine 13 (S13) and serine 16 (S16) are the three amino acid residues that can be modified by phosphorylation (figure 5) and it was demonstrated the phosphorylation of T3 occurs *in vivo* and alters Htt aggregation enhancing the formation of aggregates and the levels of insoluble Htt species in *Drosophila* HD model (30). Moreover, it was also observed a decrease in the amount of Htt aggregates on T3 phosphoresistant mutant (a substitution for alanine, T3A). Both phosphoresistant (T3A) and phosphomimic (a substitution to aspartic acid, T3D) mutants were able to reduce the neurodegeneration (30). A similar study demonstrated that the single phosphorylation of Ser13 and Ser16 reduced the formation of Htt fibrils and reduced the oligomerization in vitro. Moreover, studies in mice expressing phosphomimic Ser13/Ser16 mutations did not produced aggregates in the striatum and cortical layers of the brain (37). Phosphorylation of mutant Htt has a protective role in HD however, how phosphorylation regulates Htt aggregation and toxicity remains to be elucidated.

The activity state, intracellular localization and protein-protein interactions may be modulated by post-translational modifications (PTMs) and several modifications have been implicated in HD (38,39) however there is no evidence of these PTMs occurring within Httex1 (30). Since the first 17 amino acids are highly conserved throughout mammalian evolution and it is known that Httex1 is sufficient to cause HD-like phenotypes, modifications that occur in NT17 domain may affects the propensity of Httex1 to form protein aggregates or modify the biophysical interactions leading to the

pathology. Moreover, NT17 phosphorylation can be modified by protein phosphatase and so far it is unknown which phosphatases could be responsible for the dephosphorylation of NT17. In this work we aim to identify the phosphatases that could be involved on the dephosphorylation of these amino acid residues and understanding if and how the phosphorylation/dephosphorylation pathways acting on the NT17 domain could provide new insights for HD treatment.

*1.3.*Modelling Parkinson's and Huntington's diseases in *Drosophila*

In the last decade the fruit fly, *Drosophila melanogaster*, has contributed significantly to the understanding of the neurodegenerative disorders and homologs of human disease genes can be identified in the *Drosophila* genome (40). Many genes responsible for the human diseases have orthologs in *Drosophila* and the many experimental advantages of *Drosophila* can be used to study the function of these genes. Moreover, the *Drosophila* brain is capable of learning and memory and the CNS of invertebrates and vertebrates share a common evolutionary origin. The short life-span of flies makes *Drosophila* ideal for studying human neurological disorders (41).

To study human neurodegenerative disorders in flies, the target gene can be expressed using the binary GAL4-dependent upstream activating sequence (UAS/GAL4) (figure 6) (42).

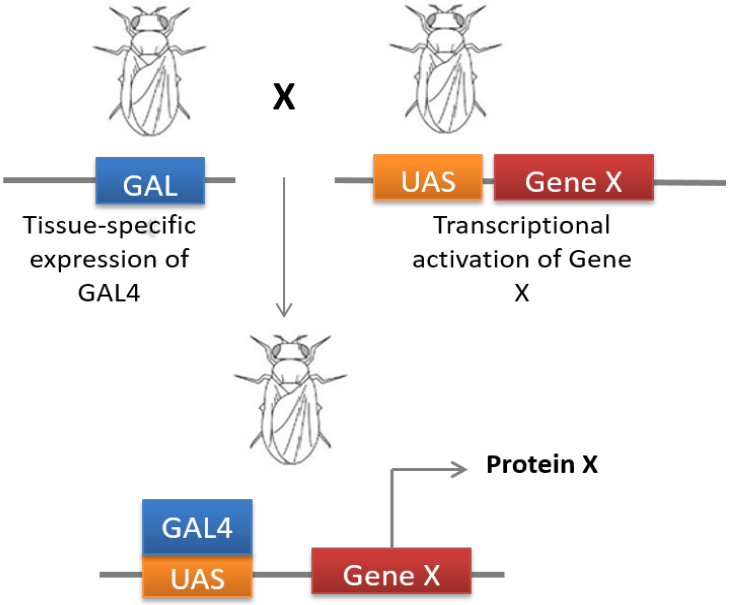

Figure 6 – Directed gene expression in Drosophila. To activate the target gene in a cell-or tissue-specific pattern, the flies carrying the target gene (UAS-Gene X) are crossed with flies expressing the GAL4 promoter. In the progeny will be possible activate UAS-Gene X in cells where the GAL4 is expressed and observe the effect of directed expression of the protein X.

The gene of interest is subcloned into an UAS expression construct, which is microinjected into fly embryos to establish the transgenic lines. When flies that carry the human transgene are crossed to flies that express the yeast transcriptional co-activator GAL4 in a specific tissue or cell type, the protein of interest will be expressed only in the tissues that have GAL4. Many GAL4 lines are available at Bloomington Drosophila Stock Center at Indiana University and other *Drosophila* stock centers. This system provides an advantage for studying neurodegenerative diseases, because it allows for the expression of genes in a cell-type specific manner and also for the expression of human genes which are absent in the *Drosophila* genome, as is the case of α-synuclein.

In the present work I used transgenic models for PD and HD which were previously established in the lab (figure 7). Concerning the PD model, we used transgenic flies expressing the human α-syn wild-type form (α-synWT) and the α-syn mutant form (α-synA30P) and both proteins were fused to EGFP tag (figure 7A). Related to HD model, it was used transgenic flies expressing the wild-type form (19QHttex1) and the mutant form (97QHttex1) in the polyQ tract fused both with mCherry tag (figure 7B).

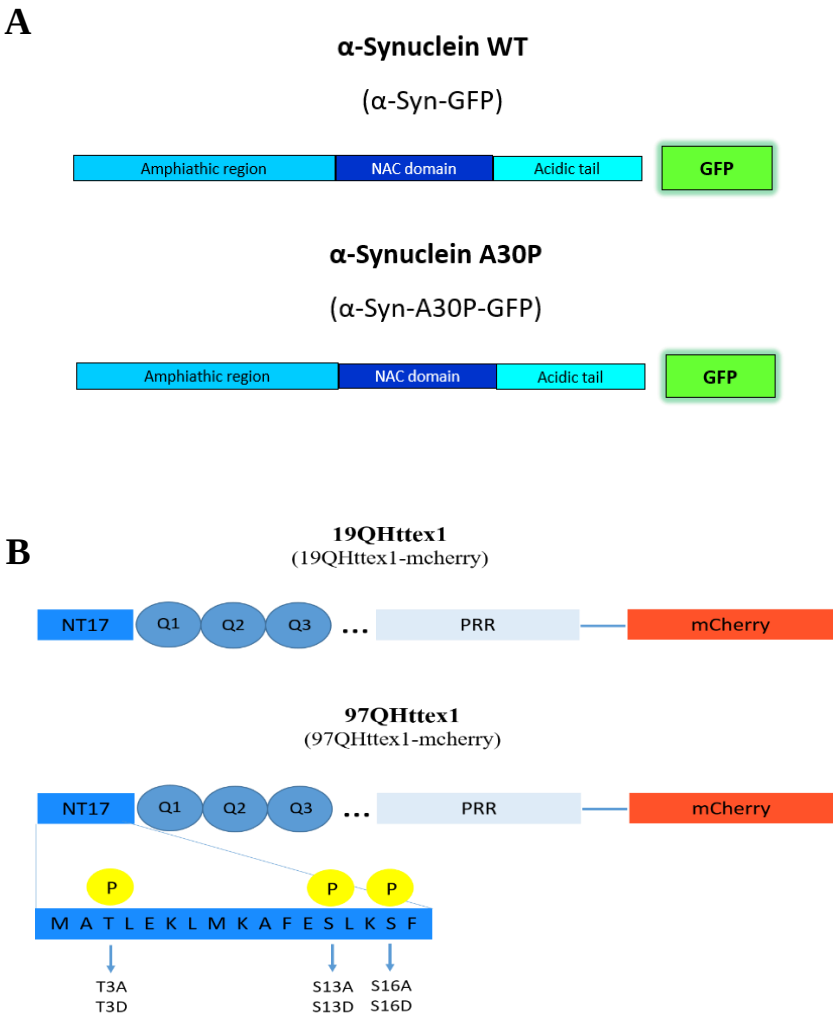

Figure 7- Schematic representation of the PD and HD models used. For PD were generated two constructs based on the expression of human α-syn and both constructs were fused with a EGFP tag for the wild-type form (α-syn-WT) and for the mutant form (α-synA30P). For HD were generated eight constructs based on the expression of Htt and all were fused with mcherry tag.

The expression of these two proteins in different neuronal tissues was done using the Gal4/UAS system. We induced the expression of the target protein in the whole nervous system using the pan-neuronal driver nsyb-GAL4, in the eye retina using the sGMR-GAL4 driver and in the dopaminergic neurons using the TH-GAL4 driver.

2. OBJECTIVES

The main goals of this study using the new established *Drosophila* models of neurodegenerative disorders in our lab are:

- Elucidate and identify the molecular determinants of the subcellular localization of α-syn in Parkinson Disease;
- Highlight the effect of N-terminal phosphorylation in the aggregation and toxicity of mutant Htt in Huntington Disease

3. MATERIAL AND METHODS

3.1. *Drosophila melanogaster* constructs and stocks

Huntingtin transgene flies' lines were generating after cloning of Htt into pWalium-10-roe, using the Gateway cloning technology (Thermo Fisher Scientific, USA) and using phiC31 integrase-mediated DNA integration. The DNAs were injected into embryos of the BestGene Strain #9723, with the attP acceptor site in 28E7. For HD work eight constructs were generated, each encoding different versions of UAS-Htt-mCherry lines. One encoding a wild-type version of Htt with a polyQ tail containing 19 glutamines, another encoding a mutant version of Htt with a 97 glutamines in the polyQ tail. Other six lines encoding phosphomutant versions (T3A/D, S13A/D and S16A/D) in a background of Htt containing a polyQ tract with 97 glutamines.

PD transgene flies' lines were cloned into pUAST using BglII and Acc65I restriction sites and the flies were generated by BestGene, USA. Two different lines were generated, one encoding the wild type α-syn version, UAS α-synWT-EGFP, and another encoding the familial mutation of α-synA30P, UAS α-synA30P-EGFP.

Drosophila stocks were maintained at 25°C on standard cornmeal media in an incubator with a 12 h light/dark cycle.

3.1.1. Directed gene expression in *Drosophila melanogaster*

The Gal4/UAS system was used for ectopic gene expression in *Drosophila*, and the following Gal4 lines were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN, USA): GMR-GAL4 (for eye imaginal disc expression, under the control of glass multiple reporter); TH-GAL4 (active in DA neurons, under the control of the tyrosine hydroxylase promoter) and nSyb-GAL4 (active in the entire nervous system, under the control of the Synaptobrevin promoter).

3.2. Locomotion and Survival assays

We determined the locomotor ability with a negative geotaxis assay as described previously (43). Groups of ten flies from different genotypes were placed in a vertical plastic vial (18-cm-long) at 25ºC and after acclimatization they were gently tapped down to the bottom of the vial (a minimum of 50 flies per genotype were assayed). We measured time that took for five flies to climbing across the 15-cm mark in the vial, after the tap.

For each genotype we tested three independent groups of males and performed five trials for each time point and at least 50 males' flies were used for each genotype tested. Results are the average climbing time ± SEM of three independent experiments.

Female flies were used for survival assay and they were placed in groups of ten per vials at 25ºC for each genotype and transferred to a fresh vial every 3 days (a minimum of 30 flies per genotype). The lifespan for each genotype was measured during three months' maximum.

3.3. Microscopy

3.3.1. Whole-mount dissection and immunofluorescence of adult fly brains

The heads of 8 days old flies were separated from the body and a cut was made between the proboscis and the eye to maximize the entry of the paraformaldehyde (PFA) 4% into each head during 40 minutes on ice. After this period, the flies' heads were removed from the fixed solution and transferred to phosphate buffered saline (PBS) 1x. The brains were isolated from the head and fixed in PFA 4% and after 15 minutes of incubation at room temperature with agitation, the brains were washed three times in phosphate buffered saline Tween-20 (PBST). Dopaminergic neurons were stained with mouse anti-TH antibody (Immunostar, Hudson, WI, USA) diluted 1:100 in PBST during 48h at 4ºC. After this period, more three washes with PBST were done and the brains were incubated with a secondary anti-mouse Cy5 antibody (1:200) also diluted in PBST overnight at 4ºC. Brain samples were mounted between two glasses cover-slips using glycerol as mounting agent. The images were acquired using a LSM10 Meta Zeiss confocal microscope and were taken with a resolution of 1024x1024, with a slice thickness of 1µm. The z-projections were generated using LSM Image Browser and the images were processed using Adobe Photoshop.

3.3.2. Immunofluorescence of Eye Imaginal Discs

Eye imaginal disc were dissected from 3rd instar larvae and fixed with 4% paraformaldehyde during 1h at room temperature. Three 15-min washes with PBST were done and eye imaginal discs were stained with rat anti-Elav antibody (7E8A10, Developmental Studies Hybridoma Bank, University of Iowa, USA) diluted in 1:100 in PBST overnight at 4ºC. The eye imaginal discs were washed three more times in PBST

and incubated with a secondary antibody anti-rat Cy5 (1:200 in PBST) at room temperature during 2h. The images were acquired using a LSM10 Meta Zeiss confocal microscope with a resolution of 1024x1024. The z-projections were generated using LSM Image Browser and the images were processed using Adobe Photoshop.

3.4.Statistical analysis

Results are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 6.0 for Windows (GraphPad Prism Software, USA). For climbing assays, we performed a two-way ANOVA followed by a Bonferroni post-test. For survival assays, we performed a Log-rank (Mantel-Cox) test.

4. RESULTS

4.1. Mutant α-synA30P has a mislocalized localization in the synaptic terminals of *Drosophila* photoreceptors

The protein α-syn is involved in the neurotransmitter release and it is mainly localized to the axon terminal in neurons (18). In order to investigate the subcellular localization of α-syn in the *Drosophila* eye, we induced the expression of α-syn-EGFP WT and α-syn-EGFP A30P mutant using the GMR-Gal4 driver. Cryossections of adult fly heads with 5 days old were made to observe the subcellular localization of α-syn-EGFP WT- and α-syn-EGFP A30P (figure 8).

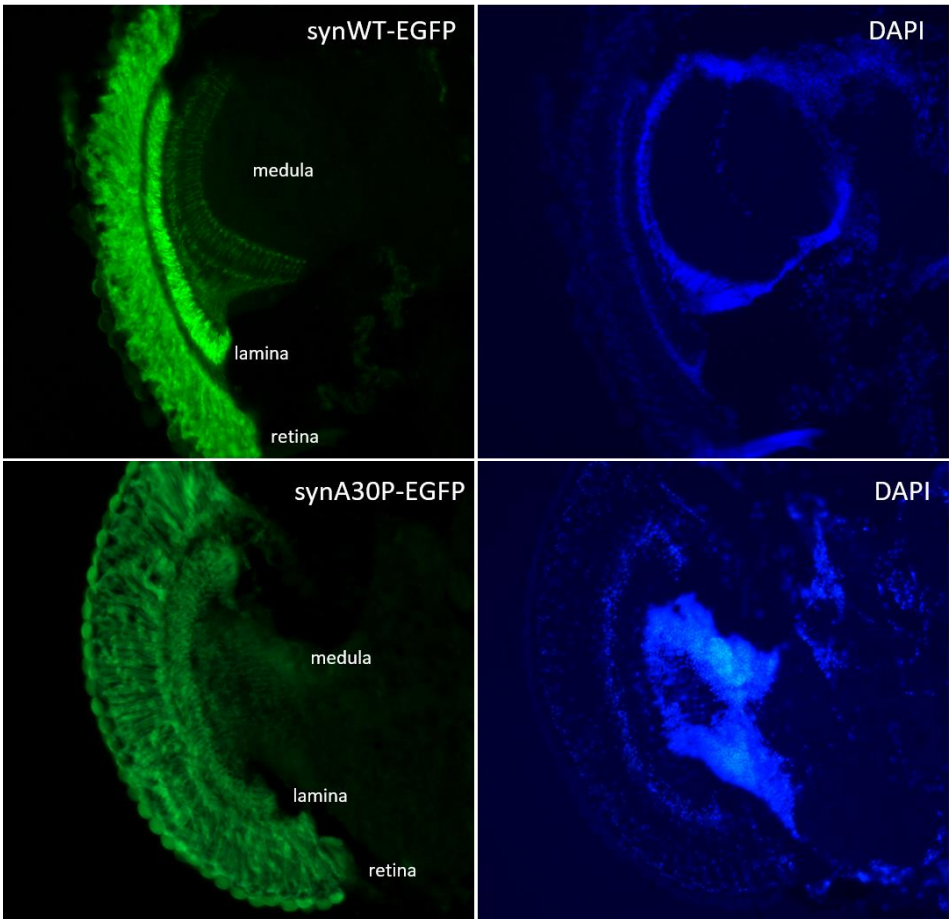

Figure 8 – Subcellular localization of α-synWT and α-synA30P in *Drosophila* photoreceptors. Horizontal retinal cryossections showing the localization of the α-synWT and α-synA30P protein in the photoreceptors.

As α-syn has an important role in neurotransmitter release it is expected a mainly synaptic localization of this protein in the terminal of photoreceptors 'axons. However, the α-syn-EGFP A30P protein has a different pattern of distribution comparing to the α-syn-EGFP

WT. α-syn-EGFP A30P protein was observed throughout the cell bodies and axons of the photoreceptors with a diminished localization in the synapse.

To determine the levels of protein expression in the two independent UAS lines (syn-EGFP WT and α-syn-EGFP A30P), we performed an immunoblot assay using fly head extracts from these two different lines. We found similar levels of protein expression in both UAS lines (figure 9).

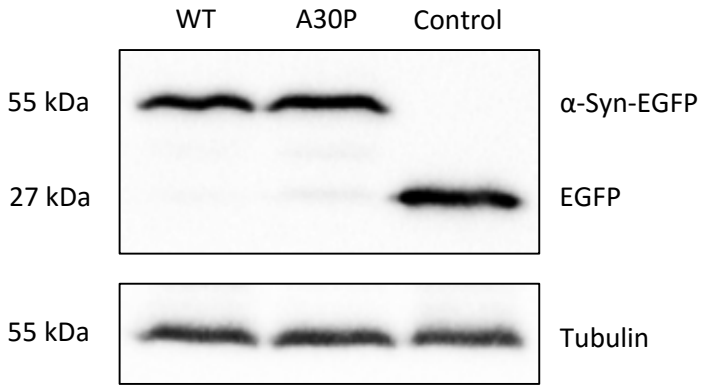

Figure 9 – Similar protein levels are expressed in the two transgenic UAS lines encoding for the wild-type and mutant form of α-syn. Performed an immunoblotting (anti-EGFP) of the total protein extracts from head flies expressing the transgenes (α-synWT-EGFP, α-synA30P-EGFP or EGFP), under the control of sGMR-Gal4 driver.

To better understand the differences observed in terms of α-syn subcellular localization in the photoreceptors, several experiments were performed previously in the lab, to evaluate the state of α-syn-EGFP WT and α-syn-EGFP A30P aggregation in our PD model. However, it was not possible to observe differences in terms of protein aggregation between the α-synWT and α-synA30P lines (44). Given these results, it was decided to identify specific protein interactors for the α-synWT and α-synA30P in our PD model using co-immunoprecipitation and mass spectrometry analysis. This approach led to the identification of 304 protein partners specific for α-syn-EGFP WT and 303 that were specific for α-syn-EGFP A30P.

In order to validate the results obtained by immunoprecipitation/mass spectrometry, we decided to perform a reverse RNAi knock down for the genes encoding specific protein interactors for the α-synA30P form and possibly identify modulator genes that could promote the synaptic localization of the α-syn -EGFP A30P upon gene knock down. For that purpose, we induced the expression of the α-syn-EGFP A30P in the *Drosophila* eye

using the GMR-GAL4 driver and then crossed with specific RNAi lines from TRiP (Harvard Medical School) (figure 10).

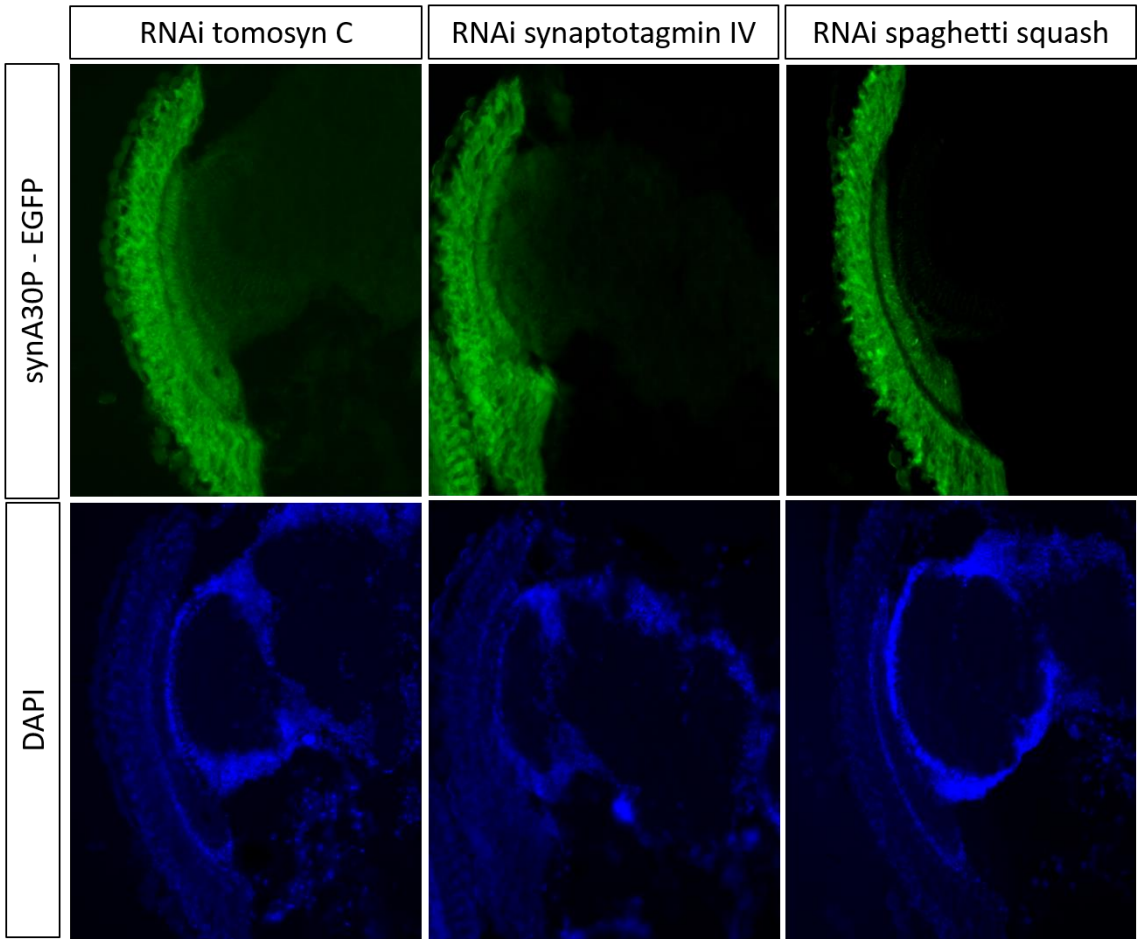

Figure 10 – Tomosyn C, Synaptotagmin 4 and Spaghetti squash are modulators of α-synA30P axonal localization in Drosophila photoreceptors. Performed RNAi knockdown of specific proteins identified by Co-IP and mass spectrometry on flies expressing the mutant form (α-synA30P).

The mislocalization of α-synA30P was ameliorating after knocking down of *spaghetti squash* because there was an increase of α-synA30P localization at the synapse, although α-synA30P is still observed at the cell bodies of the photoreceptors. This result was obtained by RNAi knock down for only one interactor. This experimental setup may be insufficient to have a significant rescue of α-synA30P localization, mainly because α-synA30P could still be interacting with other proteins in the cell bodies of the photoreceptors.

4.2. Motor dysfunction and decreased life span induced by α-synWT expression on PAM cluster

One of the mostly used assay in Drosophila to monitor aging-related behaviour is to study the loss of the climbing response. Normal *Drosophila* flies display a strong geotactic response (21), and Feany and Bender have shown a strong locomotor impairment in a *Drosophila* model of PD, upon expression of the mutant form α-synA30P in all *Drosophila* neurons (using the elav GAL4 driver) (21). In agreement with Feany and Bender results, Riemensperger and collaborators showed an accelerated age-dependent decline on the motor abilities when α-synA30P is expressed in all neurons. Moreover, these authors identified a group of 15 dopaminergic neurons (mentioned as the PAM cluster) whose disruption correlates with climbing impairments and surprisingly they did not observe a significant motor deficit when expressed α-synA30P using the dopaminergic driver TH-GAL4 (45).

Taking in account this results, we decided to use our PD model in order to study the behavioural effects of expressing α-syn-EGFP A30P in the PAM cluster using the NP6510-GAL4 driver (figure 11).

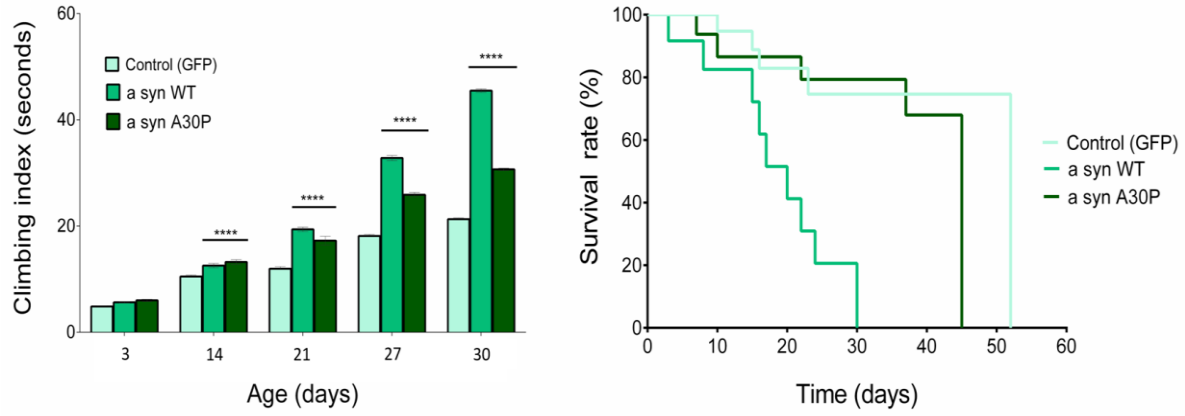

Figure 11 - Flies expressing the wild-type form of α-syn under the control of NP6510-Gal4 showed a strong impairment of the motor abilities and a strong decrease on life span.
The y-axis represents the time (in seconds) it took for male flies to climb 15 cm (mean±SEM).
Statistical analysis was performed using two-way ANOVA with Bonferroni post-test for the climbing assay and used log-rank, Mantel-Cox test for the survival assay of two independent experiments, $p < 0{,}0001$.

Surprisingly, when we induce the expression of the α-syn-EGFP A30P in NP6510-positive neurons, it caused an accelerated decline with age on geotactic response, but this effect was stronger in flies expressing the wild type form of α-syn-EGFP. Moreover, the

flies expressing α-syn EGFP A30P demonstrated a longer life span when compared to the wild type form of α-syn EGFP.

4.3. NT17 residues phosphorylation modulates the aggregation of mutant Htt in *Drosophila* eye imaginal discs and dopaminergic neurons

Previous results from our collaborators have shown that the phosphorylation of NT17 residues affects Htt oligomerization and formation of large aggregates in H4 cells (data not shown).

Taking these results from human cell studies, we decided to study the effect of single-residue phosphorylation of Httex1 in the context of Htt aggregation and neurotoxicity in *Drosophila*. In order to study the effects of phosphorylation / dephosphorylation of different mutant's forms of Httex1, we generated phosphomimic and phosphoresistant mutants in which T3, S13 and S16 were mutated to aspartate (T3D, S13D and S16D) or alanine (T3A, S13A and S16A), respectively. These mutants were generated in the expanded Httex1 97Q background.

We induced the expression of all different versions of Htt fused to mcherry tag, under the control of GMR-GAL4 and TH-GAl4 driver, to evaluate the Htt aggregation in eye imaginal discs and dopaminergic neurons, respectively.

In eye imaginal disc expressing Htt19Q-mCherry did not shown inclusion formation, while Htt97Q-mCherry showed high number of protein aggregates. The phosphomimic and phosphoresistant mutants showed a decrease in the number of protein aggregates compared to Htt97Q-mCherry, with the exception of the S13D phosphomimic which has a pattern of aggregation identical to Htt97Q-mCherry (figure 12).

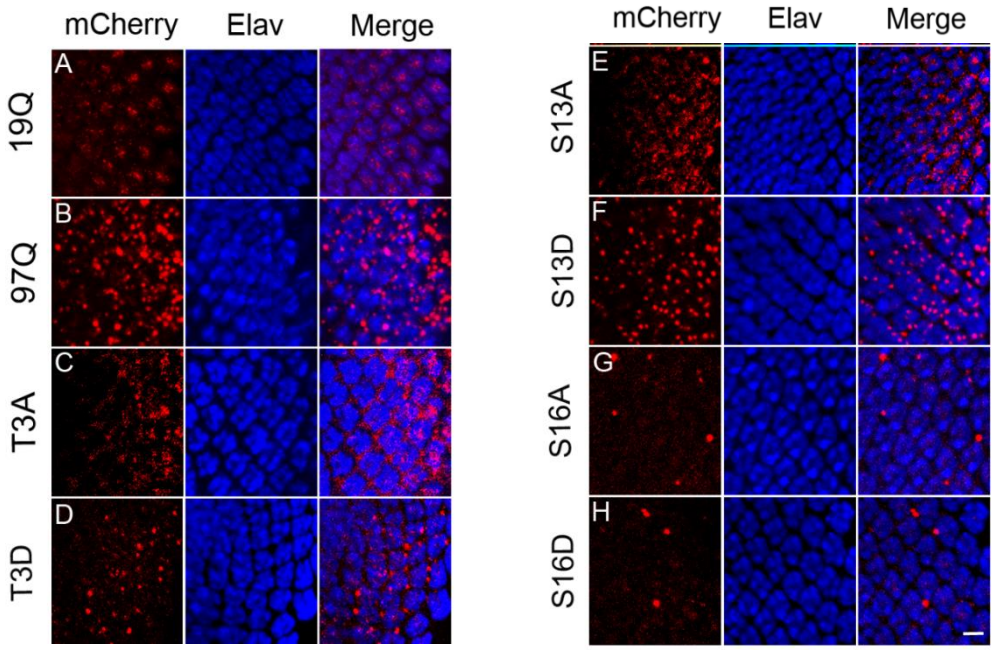

Figure 12- NT17 phosphorylation modulates Drosophila Htt aggregation in eye imaginal disc.
Flies expressing the phosphoresistant mutations T3A, S13A and S16A (left panel) and the phosphomimic mutations T3D and S16A (right panel) under the control of GMR-Gal4 showed a decrease in the number of protein aggregates compared non-mutated Htt97Q-mCherry.

In dopaminergic neurons, Htt19Q-mCherry also did not produced protein aggregates and Htt97Q-mCherry showed the formation of protein aggregates mainly localized in the cytoplasm. The phosphoresistant mutants have patterns of aggregation very similar to Htt97Q-mCherry with a tendency to generate higher number of aggregates with smaller sizes (figure 12 C, E, G). The phosphomimic mutants showed the formation of bigger aggregates (figure 12 D, F, H) compared to Htt97Q-mCherry and the S13D phosphomimic exhibited substantially more protein aggregates than Htt97Q-mCherry (figure 13).

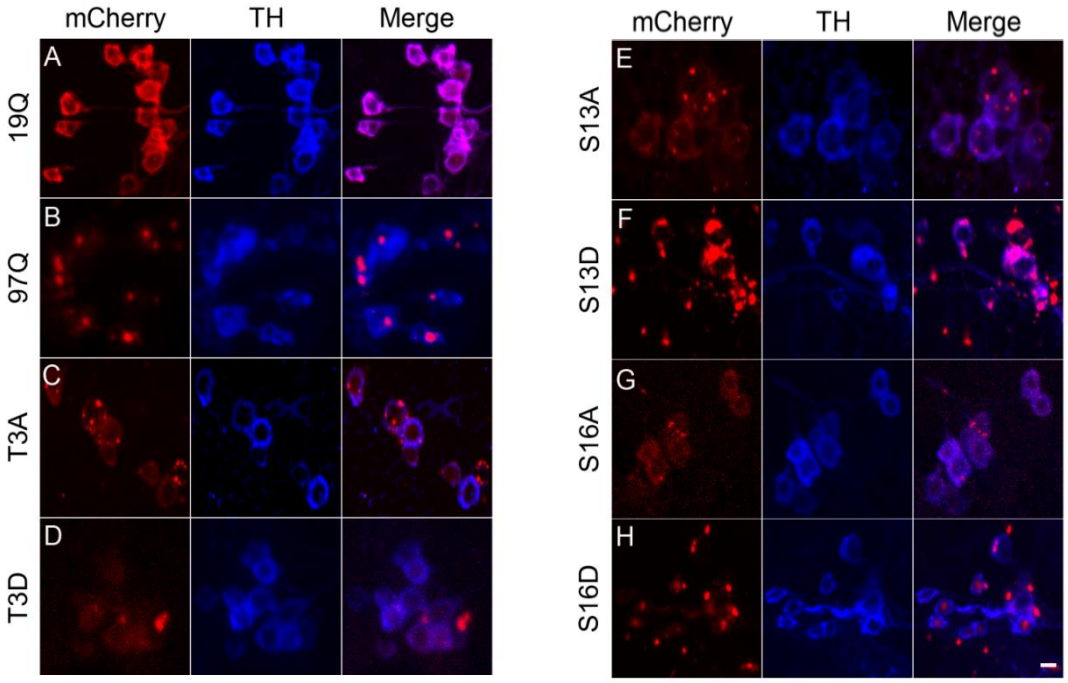

Figure 13 - NT17 phosphorylation modulates Drosophila Htt aggregation in dopaminergic neurons. Flies expressing the phosphoresistant mutations T3A, S13A and S16A exhibited a higher number of aggregates of smaller size and the phosphomimic mutations T3D, S13D and S16A showed bigger aggregates compared to the non-mutated Htt97Q-mcherry. The expression of the different versions of Htt were expressed in the DA neurons using the TH-GAL4 driver.

Regarding the results obtained, we demonstrated that single NT17 residue phosphorylation modulates the pattern of Htt aggregation in a Drosophila model. These results are consistent with a previous study where the phosphomimic T3D mutant exhibited an increase in aggregation and SDS-insoluble protein compared to the phosphoresistant T3A and to the non-mutated 97QHtt (30). From our results, it is difficult to define whether the aggregates from phosphoresistant and phosphomimic mutants are located in the nucleus or cytoplasm, since several aggregates are located outside the cells. This could be due to the fact that Htt species are toxic to the cells and in some cases may cause cells to entry in apoptosis. How protein aggregation can induce neurotoxicity is still unknown and there is no scientific consensus regarding which species are the most toxic for the cells, but growing consensus believes that intermediate oligomeric structures may be the toxic form and that the larger inclusions are the protective form (46).

4.4. Motor dysfunction and decreased life span induced by dephosphorylation of NT17 residues

Drosophila models of Huntingtin disease have demonstrated several phenotypes when the expanded form of Httex1 is expressed, including reduced viability and lifespan, neurodegeneration and protein aggregation (47) (48).

Since NT17 phosphorylation can modulate Htt aggregation in two different cell types, we decided to study the behavioural effects of single NT17 phosphorylation residue in *Drosophila*. To assess the role NT17 phosphorylation behaviour, we expressed the different versions of Htt fused to mCherry tag, under the control of nSyb-GAL4, a driver that targets all neurons in the central nervous system of the adult organisms.

The climbing assays were performed to evaluate the motor abilities of flies expressing the different Htt constructs during one month. The results obtained demonstrated an age-dependent impairment in motor function in general, with the phosphoresistant mutants S13A and S16A exhibiting a significant decrease in motor function compared to the phosphomimic mutants T3D, S13D and S16D and also to the non-mutated 97QHtt-mcherry (figure 14 A). The phosphomimic mutants S13D and S16D showed an improvement in motor function compared to the non-mutated 97QHtt-mcherry (figure 14 B). However, the flies expressing the T3D phosphomimic mutant were unable to climb from the third week of age (approximately 23-days old) showing a total loss of geotactic response that was registered as the maximum score (60 minutes).

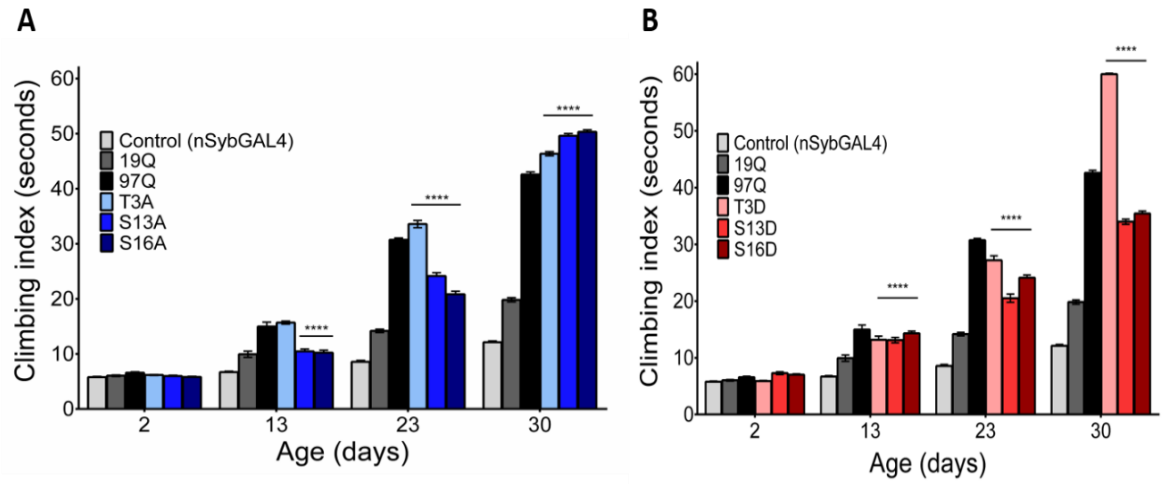

Figure 14- NT17 phosphorylation reduces flies' motor deficits.
Flies expressing the phosphoresistant mutations T3A, S13A and S16A (left panel) under the control of nsyb-Gal4 showed a strong impairment of the motor abilities compared to the phosphomimic mutations T3D, S13D and S16A (right panel) and also to the non-mutated Htt97Q-mcherry.
The y-axis represents the time (in seconds) it took for male flies to climb 15 cm (mean±SEM). Statistical analysis was performed using two-way ANOVA with Bonferroni post-test of three independent experiments, $p < 0,0001$.

Female flies were used to study the effects of NT17 phosphorylation on the survival rate and the phosphoresistant mutants T3A, S13A and S16A demonstrated a significant decrease in flies' life span when compared to the non-mutated 97QHtt-mcherry and also to the phosphomimic mutants T3D, S13D and S16D (figure 15).

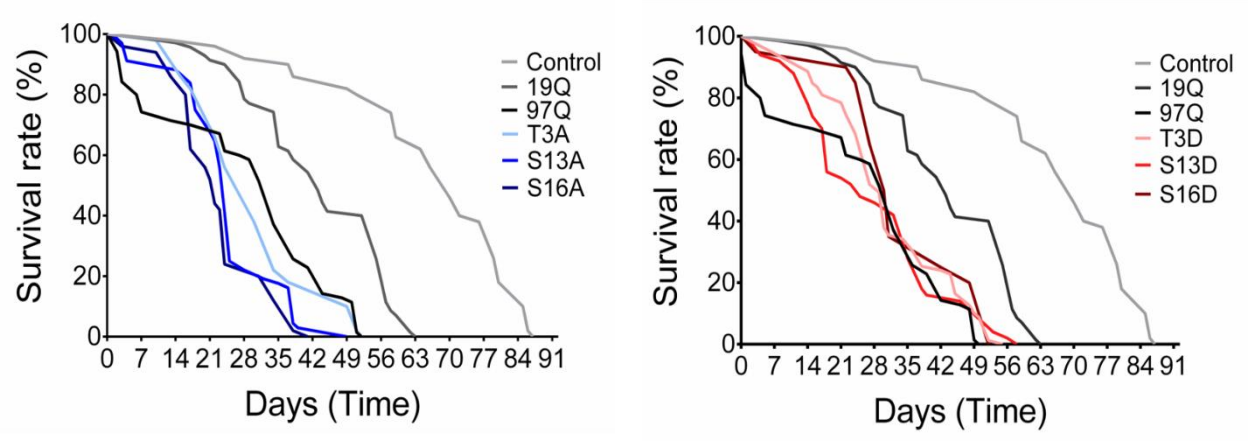

Figure 15- NT17 phosphorylation increases *Drosophila* life span.
Flies expressing the phosphoresistant mutations T3A, S13A and S16A (left panel) under the control of nsyb-Gal4 exhibited a significant decrease on flies' life span compared to the phosphomimic mutations T3D, S13D and S16A (right panel) and to the non-mutated Htt97Q-mcherry.
The y-axis represents the percentage of flies alive at each time point analysed. The mean maximum survival (in days) is indicated for each genotype: control=82; 19Q=60; 97Q=49; T3A=33; T3D=54; S13A=45; S13D=52; S16A=39 and S16D =47.
Statistical analysis was performed using log-rank, Mantel-Cox test of three independent experiments.

It is known that expression of N-terminal fragments containing Httex1 is sufficient to produce HD-like features due to its propensity to aggregate and modifications in these specific domain (NT17) has an important role on Htt aggregation and toxicity. Our results demonstrate that phosphorylation of huntingtin within NT17 domain can modulate aggregation and neurotoxicity, since flies expressing the phosphoresistant mutant versions of Htt generated a higher number of aggregates with smaller sizes, showed a strong impairment of the motor abilities and induced a decrease in the life span of flies, compared to the non-mutated 97QHtt-mcherry. Additionally, these results suggest that the phosphorylation state of Htt protein may contribute to the decrease of neurotoxicity

and aggregation. Therefore, the identification of specific phosphatases involved in the dephosphorylation of NT17 residues could constitute a promising approach to target HD pathology.

4.5. Protein Phosphatase 1 (PP1) reduced the aggregation of mutant Htt in *Drosophila* dopaminergic neurons

Phosphorylation pathways have been shown to modulate the toxicity of mutant Htt *in vitro* by affecting its aggregation dynamics and several kinases may be involved in NT17 phosphorylation (33) (37). Less is known, about the identity of the phosphatases that may dephosphorylate NT17. Previous results from our collaborators showed that chemical inhibitors of specific phosphatases, such as Cdc25 and PP1/PP2A, significantly decreased the percentage of cells with Htt inclusions, resulting in lower toxicity and aggregation (data not shown). Based on these results, we decided to investigate the inhibition of these phosphatases in *Drosophila*, by doing genetic knockdown experiments.

In these experiments we expressed the Htt97Q-mCherry in dopaminergic neurons using the TH-GAL4 driver and then crossed with individual RNAi lines for each phosphatase mentioned above (figure 16).

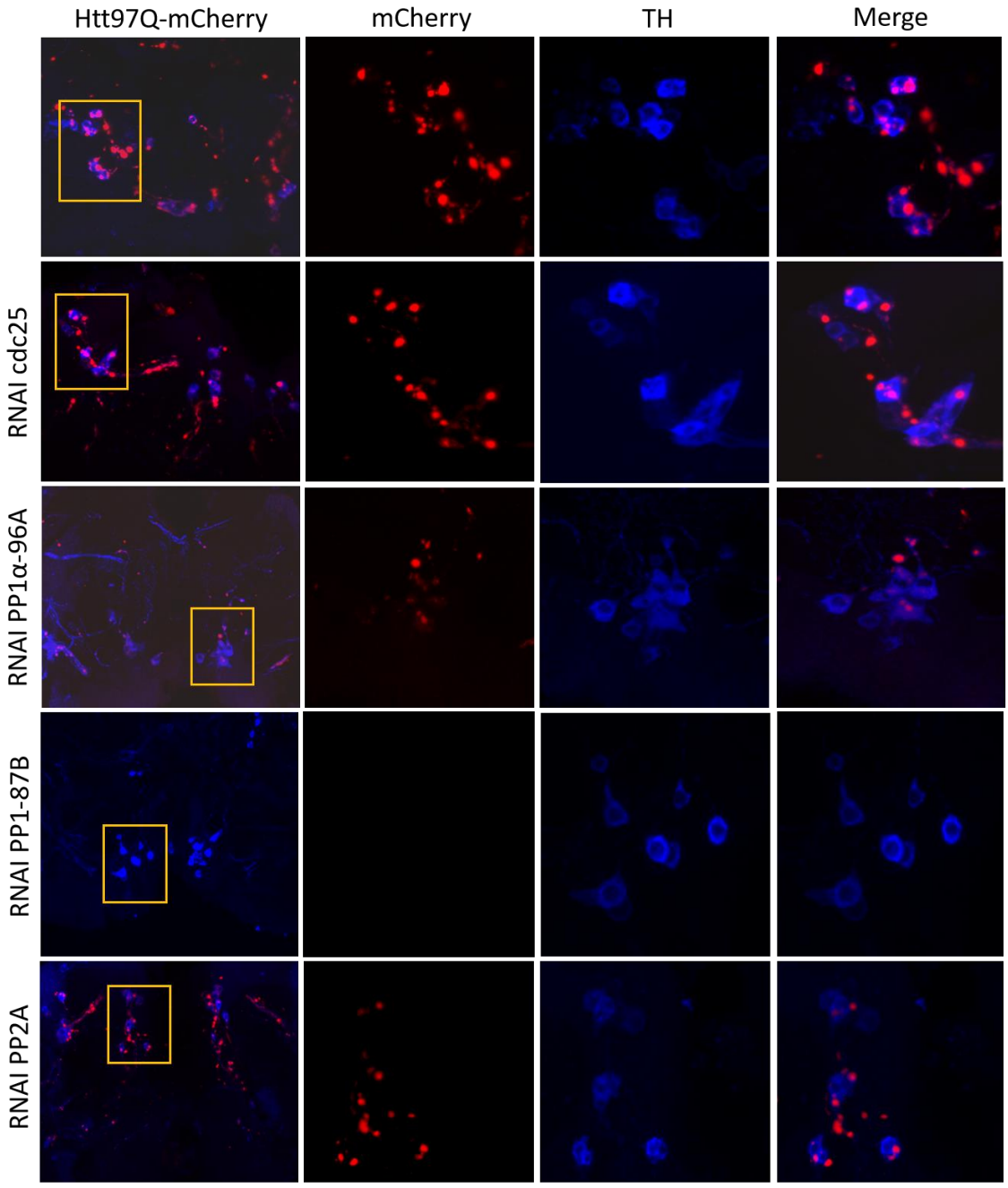

Figure 16- Genetic knockdown for PP1α-96A and PP1-87B reduced the aggregation of mutant Htt in dopaminergic neurons. Representative images of the expression of Htt97Q-mCherry in all brain (upper panel) and using genetic knockdown for Cdc25, PP1α-96A, PP1 87B and PP2A on Htt97Q-mCherry background. Boxes drawn in the whole-brain (left column) shows co-expression of Htt97Q-mcherry in dopaminergic neurons, except for PP1 87B which did not produced aggregates (mCherry signal).

Expression of the Htt97Q-mCherry in the dopaminergic neurons induced the formation and accumulation of big protein aggregates, as expected (figure 16). Co-expression of Htt97Q-mCherry with Cdc25 RNAi did not reduced Htt aggregation and the pattern of Htt expression was similar to the Htt97Q-mCherry brain. Hence, the knockdown of Cdc25 phosphatase in *Drosophila* had no specific effect in reducing Htt aggregates and

these results are different from those obtained in mammalian cells (data not shown). Co-expression of Htt97Q-mCherry with PP1α-96A and PP1-87B RNAi had a significant effect on reducing Htt aggregates when compared to the Htt97Q-mCherry. Surprisingly, in the case of protein phosphatase PP1 87B RNAi no Htt97Q-mCherry aggregates were observed in the brain. Co-expression of Htt97Q-mCherry with PP2A RNAi did not diminished Htt97Q-mCherry aggregates formation.

Considering that Httex1 does not have any other residue capable of being phosphorylated beyond the NT17 domain, the results obtained from PP1α-96A and PP1-87B RNAi phosphatases showed that Htt can be modified by protein phosphatases and possibly as a direct effect on these 3 residues present in NT17. Further experiments should be done in order to address which specific residue in NT17 domain is regulated by which specific protein phosphatases.

5. DISCUSSION

The accumulation of misfolded mutated proteins, a common sign of many neurodegenerative disorders, affects several cell signaling pathways, the neuronal connectivity and cell death (3), however the mechanism by which these proteins cause degeneration remains unknown.

In this work we show results from two different neurodegenerative diseases such as PD and HD and for that we used *Drosophila melanogaster* as our model study. Taking advantage of the GAL4/UAS system we induced the overexpression of EGFP-tagged versions of wild-type human α-synuclein (α-synWT) and mutant form (α-synA30P) that renders PD model. For the HD model, we induced the overexpression of mCherry-tagged versions of wild-type (Htt19Q) and mutant (Htt97Q) form of Htt.

The major player of PD is the α-syn protein which is a presynaptic neuronal protein. We observed a strong localization of the α-syn-EGFP wild-type version in the *Drosophila* photoreceptor's synapses, while α-syn-EGFP A30P mislocalized throughout the photoreceptors cytoplasm. Based on these results, our PD model based on the expression of α-syn in the photoreceptors constitute a useful tool to study the α-syn's biology, since is consistent with the normal localization of this protein in the vertebrate nervous system (49). The mechanism underlying the mislocalization of the A30P mutant is unknown, but could be explain due its promiscuous binding with other proteins which could may have an effect on its aggregation and accumulation, leading to a less efficient axonal transport of the α-synA30P protein and to an accumulation in the cytoplasm of the photoreceptors. The cytoplasmic localization of α-synA30P could be a consequence of the disruption of ER-Golgi traffic (50). The phenotypes observed were not due to different levels of protein expression (figure 9). The mislocalization of α-synA30P may cause synaptic dysfunction and may contribute to abnormal neurotransmission. Considering that α-syn-EGFP A30P may interact with other protein partners, previous results in our lab have shown the interaction of the α-syn-EGFP A30P mostly with proteins associated to mitochondria and/or ribosomes (44) and may explain the localization of this protein at the cytoplasm and not at the synapse. Taking in account the results from Co-IP and mass spectrometry, we decided validate these results using genetic RNAi knockdown for three specific protein interactors: tomosyn isoform C, spaghetti squash and synaptotagmin 4. The knockdown of these 3 proteins ameliorated the localization of α-syn-EGFP A30P at the synapse. The protein tomosyn, also known as a syntaxin-binding protein, decreases

synaptic transmission and inhibits the vesicle's fusion competence, modulating the synaptic transmission in neurons (51). Tomosyn is also involved in the SNARE complex assembly regulation and is a negative regulator of secretion since it competes directly with synaptobrevin, blocking the release of pools of synaptic vesicles (52). Spaghetti squash is required for cytokinesis in Drosophila and is critical to ensure efficient migration of cells (53). The synaptotagmin 4 protein is ubiquitously present at the synapse and is a vesicular CA^{2+} sensor that controls synaptic growth and presynaptic release properties (54) (55). The three modulators identified play an important role in the neurotransmission and a possible explanation for the α-synA30P toxicity may due its abnormal interaction with other neuronal proteins which are involved in this mechanism. Future work will address if there is a physical interaction between these proteins and α-syn-EGFP A30P, using IP (immunoprecipitation) and WB (Western Blott) assays. Functional assays, such as climbing and survival will be used in order to understand the effect of these modulators in terms of motor ability and survival rate on flies.

The most frequent motor disorder of PD's patients is due mainly associated with the loss of midbrain dopaminergic neurons in the substantia nigra pars compacta. In *Drosophila*, Riemensperger and colleagues identified a subset of 15 dopaminergic neurons in the protocerebral anterior medial (PAM) dopaminergic cluster whose disruption correlates with severe locomotor deficits. Taking in account these results, we decided to study the behavior effect on the expression of the α-syn-EGFP WT and α-syn-EGFP A30P under the control of NP6510-GAL4, a driver that targets the 15 dopaminergic neurons within the PAM cluster. Surprisingly, our results are not consistent with those described for Riemensperger and colleagues, since the expression of the wild-type version resulted in the stronger impairment of the motor abilities and also showed a decrease life span when compared to the flies expressing α-syn-EGFP A30P (figure 11). A possible explanation for these contradictory results could be the different lines used and an evaluation of the expression levels of these two lines will be necessary and may clarify these phenotypes.

Huntington disease is a polyQ disease and the disease occurs when the number of CAG repeats is over 35 glutamines inducing the misfolding and aggregation of mutant Htt (56). In this work we induced the expression of 19QHtt-mcherry and 97QHtt-mcherry (non-mutated) and the phosphomutants versions (phosphomimic and phosphoresistant) under the of GMR-GAL4 and TH-GAl4 driver, to evaluate the Htt aggregation in eye imaginal

discs and dopaminergic neurons, respectively. Our results have shown that single residue phosphorylation in the NT17 domain modulates Htt aggregation and neurotoxicity in *Drosophila*. More precisely, NT17 dephosphorylation events enhanced Htt toxicity since the phosphoresistant mutants showed a stronger deficit in motor abilities and shorter life span. These results are consistent with previous studies showing that phosphorylation of NT17 residues is associated with reduced toxicity (33) (37). Moreover, we showed that NT17 dephosphorylation reduced the Htt aggregation in eye imaginal discs and also in dopaminergic neurons, compared to the non-mutated 97QHtt-mcherry. In addition, the phosphoresistant mutants seem to produce smaller aggregates compared to the phosphomimic ones. There is a growing consensus that the intermediate soluble structures might be more toxic than the bigger insoluble aggregates (46). Further work should be done to clarify the aggregation state of Htt on phosphoresistant and phosphomimic mutants performing a Triton-X assay which will allow us to identify soluble and insoluble aggregates.

So far, there is no study where the effect of a single NT17 phosphorylation is analyzed on mutant Htt aggregation and toxicity and our results brings new insights into HD pathology, since most of the studies had demonstrated protective role on double phosphorylation at serine 13 and serine 16 residues (33) (37).

Taking in account that NT17 phosphorylation might be enhanced by activation of particular kinases or the inhibition of specific phosphatases, we evaluated the effect of RNAi genetic knockdown for specific protein phosphatases on dopaminergic neurons of *Drosophila*. These experiment suggest that the PP1α-96A and PP1-87B protein phosphatases may modulate Htt phosphorylation since PP1α-96A was able to reduce the Htt aggregates and PP1-87B did not produced aggregates. The same effect was not observed when we did cdc25 RNAi knockdown which had no difference in terms of Htt aggregate formation compared to the Htt97Q-mCherry. A slightly reduction on Htt aggregation was observed upon PP2A RNAi knockdown but there is no significant effect compared to the Htt97Q-mCherry. These results are quite similar with those from our collaborators, where they showed PP1/PP2A protein phosphatases inhibitors prevented Htt toxicity by quantifying the degeneration of the rhabdomeres (the specialized light-sensing organelles of the photoreceptors), while the cdc25 was toxic data not shown). Given our results on Drosophila the PP1 protein phosphatases has a major role on prevented Htt aggregation and further investigation will be necessary in order to clarify

which specific residues on the NT17 domain are substrates of these specific protein phosphatases. Additionally, functional assays should be performed to test the behavior and survival rate and also evaluate the neurotoxicity.

6. CONCLUSION

Regarding the results obtained from Parkinson Disease in the present study, we demonstrate that:

- α-synA30P mutant form has a mislocalized distribution throughout the photoreceptors cytoplasm while α-synWT localizes at the photoreceptor's synapses.
- Validated three candidate modulators of the subcellular localization of α-synA30P.

Regarding the results obtained from Huntington Disease, we demonstrate that:

- Single phosphorylation on NT17 domain modulates Htt aggregation and neurotoxicity in *Drosophila*.
- PP1α-96A and PP1-87B protein phosphatases inhibitors reduced the mutant Htt aggregation.

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