



Characterization of the neuroprotective effect of anti-aggregation molecules in a *Drosophila* model of Machado-Joseph Disease

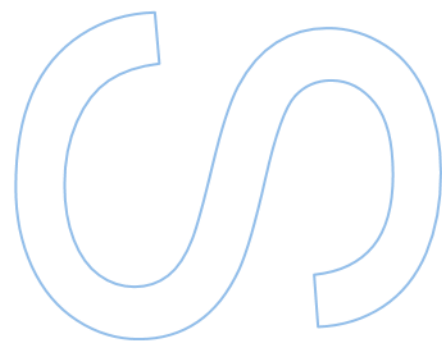
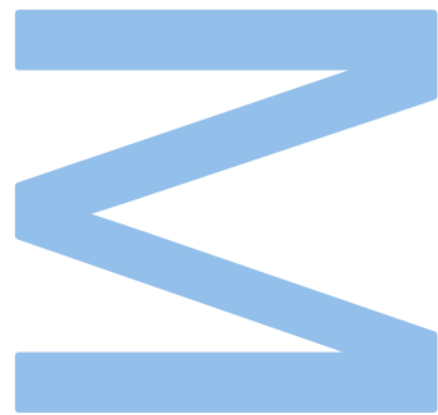
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2022-2023

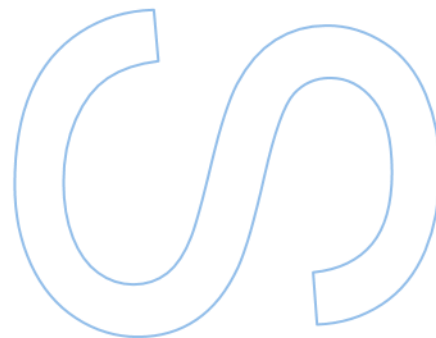
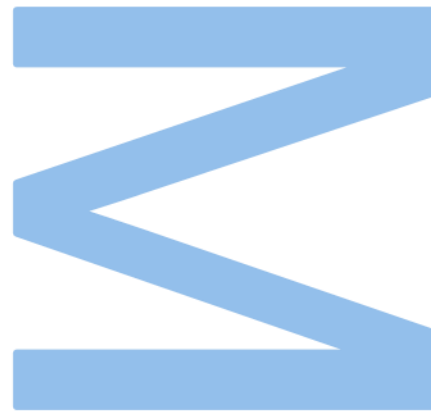
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Sworn Statement

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Acknowledgements

Thanks.

To Dr. Sandra Ribeiro for welcoming me into her group, her availability, and her support.

To my supervisor Dr. Rita Vilaça, who enabled me to overcome difficulties that have arisen, and for all the patience shown.

To my co-supervisor Dr. Carla, for all the contributions made to the development of the project.

To the other members of the group, especially Sára and Martyna for all the help and Dr. Joana Santos for all availability.

To all my family, friends and colleagues who have shared this journey with me.

To my parents, a special thank you, for supporting all my decisions and for always encouraging me.

To Isa, for being an amazing company and the best confidant.

Finally, the biggest thanks to Renato, for being by my side all the time and for being my biggest support.

This study is part of the project “Development of brain-targeted nanobodies for application in spinocerebellar ataxia type 3 therapy - NanoSCA3” funded by Ataxia UK, Associazione Italiana per la lotta alle Sindromi Atassiche, Catalan Association of Hereditary Ataxias and Swedish SCA-network. This study is also part of the PhasAGE project that has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No 952334.

Resumo

A ataxia espinocerebelosa tipo III (SCA3), também conhecida por doença de Machado-Joseph (DMJ), é uma doença neurodegenerativa rara e incapacitante com elevada incidência em Portugal, para a qual apenas está disponível terapia sintomática. A SCA3 resulta de uma expansão anormal da sequência de poliglutaminas (poliQ) na proteína ataxina-3 (Atx3), resultando na sua acumulação em depósitos amiloides em regiões específicas do cérebro, que contribuem para a neurodegeneração. Com o objetivo de neutralizar a agregação patogénica da Atx3, avaliamos o potencial neuroprotetor de quatro *nanobodies* (NBs, anticorpos com um único domínio) previamente selecionados pelo grupo de trabalho, num modelo de SCA3 em *Drosophila*. Foram realizadas técnicas moleculares com o objetivo de caracterizar o efeito dos NBs nos níveis totais de proteína e de fibras de Atx3 resistentes a SDS, em extratos proteicos obtidos a partir de cabeças de *Drosophila* que co-expressam as variantes patogénicas de Atx3 com NBs. Adicionalmente, foram realizados ensaios funcionais para avaliar a capacidade neuroprotetora dos NBs na sobrevivência e no comportamento motor de moscas SCA3. Os nossos resultados demonstram que os nanobodies NB20, NB02 e NB01 têm um efeito neuroprotetor em moscas que expressam Atx3 patogénica, prolongando o tempo de vida e melhorando a capacidade motora. Estudos futuros vão permitir avaliar se os NBs selecionados atravessam a barreira hematoencefálica (BHE) por transcitose e caracterizar as propriedades de anti-agregação da Atx3 utilizando um modelo celular neuronal SCA3. Estes avanços permitirão selecionar qual o NB com mais potencial terapêutico aplicado à SCA3.

Palavras-chave: Doenças neurodegenerativas; Doenças de poliglutaminas; Ataxia espinocerebelosa tipo III; Ataxina-3; *Nanobodies*.

Abstract

Spinocerebellar ataxia type III (SCA3), also known as Machado-Joseph disease (MJD) is a rare and incapacitating neurodegenerative disorder with high incidence in Portugal, for which only symptomatic therapy is available. SCA3 is triggered by the expansion of a polyglutamine (polyQ) segment in the protein ataxin-3 (Atx3), resulting in its accumulation as amyloid deposits at specific brain regions, leading to neurodegeneration. Aiming to counteract Atx3 pathogenic aggregation, we evaluated the neuroprotective potential of four nanobodies (antibodies with a single domain) previously selected by the i3S research working group, in a well-established SCA3 *Drosophila* model. Molecular techniques were performed to understand the effect of NBs on Atx3 total protein levels and Atx3 SDS-resistant fibrils in head extracts collected from *Drosophila* co-expressing Atx3 pathogenic variants and NBs. Additionally, functional assays were performed to evaluate the neuroprotective effect of NBs in the survival and climbing behavior of SCA3 flies. Our results demonstrate that NB20, NB02 and NB01 have a neuroprotective effect on flies that express pathogenic Atx3, prolonging their lifespan and improving their climbing ability. Future work will focus on the evaluation of their capacity to cross blood–brain barrier (BBB) by transcytosis across epithelia and characterize their anti-aggregation effect using a SCA3 neuronal cell model. These studies will contribute to a deepen characterization of NBs as potential novel therapies for SCA3.

Keywords: Neurodegenerative diseases; Polyglutamine diseases; Spinocerebellar ataxia type III; Ataxin-3; Nanobodies.

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List of Abbreviations

Atx3	Ataxin-3
BSA	Bovine serum albumin
CAG	Cytosine-Adenine-Guanine
CDR	Complementarity-determining region
Cf	Partition coefficient
Cy	Curly mutation
CyO	Curly of Oster
DB	Dot-blot
DRPLA	Dentatorubral-pallidoluysian atrophy
DTT	Dithiothreitol
eGFR	Estimated glomerular filtration rate
ELAV	Embryonic lethal abnormal visual system
FT	Filter trap
GFP	Green Fluorescent Protein
GMR	Glass multiple reporter
HCAb	Heavy-Chain Antibody
HD	Huntington's disease
HMW	High molecular weight
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IPTG	Isopropyl β -D-1-thiogalactopyranoside
JD	Josephin domain
LB	Lysogeny broth
LMW	Low molecular weight
MJD	Machado Joseph Disease
mRNA	Messenger Ribonucleic Acid
myrRFP	Myristoylated red fluorescent protein
NB	Nanobody
OD	Optical density
OrR	Oregon-R
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PI	Protease Inhibitor

PolyQ	Polyglutamine
PVDF	Polyvinylidene difluoride
REP	Rough eye phenotype
RT	Room temperature
SB	Sample buffer
SBMA	Spino and bulbar muscular atrophy
SCA	Spinocerebellar ataxias
SDS	Sodium dodecyl sulphate
SEC	Size-exclusion chromatography
Ser	Serrate
SM	Second Multiple
TB	Terrific Broth
Thio-T	Thioflavin-T
TTBS	Tween Tris buffered saline
UAS	Upstream activation sequence
UIM	Ubiquitin interacting motif
VHH	Single variable domain on a heavy chain
WB	Western blot

Introduction

1. Protein Misfolding Disorders

The linear sequence of amino acids encoded in mRNA is translated by ribosomes to produce proteins, which must then fold into their native three-dimensional structure, a requirement for their biological function. The majority of the time, the cell synthesizes proteins in their native structure, but occasionally, during translation, the newly translated proteins are exposed to environmental stresses that can lead to misfolding and aggregation. Genetic mutations may facilitate the misfolding process (1).

Protein misfolding and aggregation can be prevented by the protein quality control system. This system encompasses an extensive proteostasis network. One of the specialized groups of proteins that are part of this network is called chaperones. These molecules can help newly generated proteins to fold, as well as help partially misfolded proteins to refold into their proper three-dimensional shape and regain their functional activity. However, the protein quality control system can be insufficient resulting in the accumulation of misfolded proteins in intracellular or extracellular compartments (1,2).

Misfolded proteins that escape degradation can accumulate and self-assemble into aggregates with an amyloid conformation. Structurally, amyloid fibrils have β -strands organized perpendicularly to the fiber's axis and present highly organized, elongated aggregates. The amyloid fibrils kinetic formation is based on three separate stages at the macroscopic level: Lag phase, growth phase (elongation), and the final phase (steady state). In the first phase, misfolding causes the protein to adopt a local or more global conformational change leading to the initial self-assembly and the formation of oligomers. During the growth phase, the oligomers further develop to protofibrils which ultimately will lead to the assembly of irreversible amyloid fibers in the last phase. Characterizing the molecular events that take place in each aggregation phase, how to avoid the elongation of protofibrils or how to prolong the lag phase has been the focus of intensive research (1). The production of amyloid fibrils is associated with several biological processes such as hormone storage and templating for small chemical molecules (2) although they are more studied in the context of human illnesses (3,4).

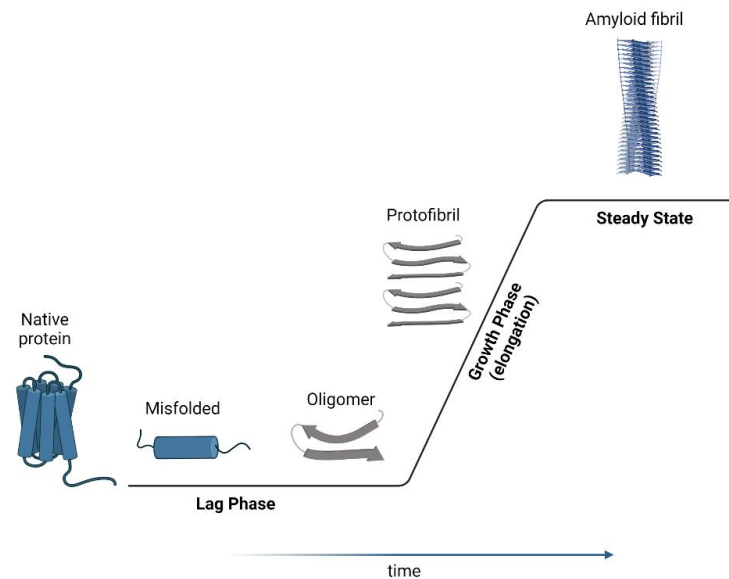


Figure 1. Amyloid Protein Fibrillation Pathway. Image created with BioRender.com

Some of the most prevalent neurodegenerative diseases are characterized by the accumulation of amyloid fibrils in organs and tissues. Parkinson's and Alzheimer's diseases are some of the most well-known amyloid diseases. They can be inherited or manifest sporadically. However, the likelihood of developing any of these rises sharply with age. The delicate balance of protein synthesis, folding, and degradation is disturbed throughout time, resulting in the generation and accumulation of misfolded proteins, leading to aggregation (5).

2. Polyglutamine diseases

Polyglutamine (PolyQ) diseases belong to a group of ten hereditary neurodegenerative disorders. The list includes Spino and Bulbar muscular atrophy (SBMA) (also known as Kennedy disease), Huntington's disease (HD), Dentatrubal-pallidoluysian atrophy (DRPLA), Spinocerebellar ataxia type 1 (SCA1), Spinocerebellar ataxia type 2 (SCA2), Spinocerebellar ataxia type 3 (SCA3), Spinocerebellar ataxia type 6 (SCA6), Spinocerebellar ataxia type 7 (SCA7), Spinocerebellar ataxia type 17 (SCA17) (6) and a novel Spinocerebellar ataxia disease caused by CAG repeat expansion in *THAP11* gene (7). All these diseases have an autosomal dominant inheritance with amplification of the CAG trinucleotide repeat in the coding region of each gene (8). This results in the expansion of the glutamine tail originating misfolding and aggregation of

the encoded protein into large insoluble protein aggregates (9). The clarification about the potential neuroprotective or neurotoxic function of those aggregates in their various assembly states needs deeper knowledge about the molecular features of each disease-causing protein.

Table 1. The Polyglutamine Diseases with their causative genes. Also, the CAG number range in the normal gene and CAG number that leads to full penetrance disease (6).

Disease	Causative gene	Normal number of CAGs	Number of CAGs leading to clinical presentation
SBMA	Androgen receptor	9-36	≥ 38
HD	Huntingtin	17-20	≥ 40
DRPLA	Atrophin1	6-35	≥ 48
SCA1	Ataxin1	6-39	≥ 39
SCA2	Ataxin2	13-31	≥ 37
SCA3	Ataxin3	12-44	≥ 55
SCA6	α _{1A} -voltage-dependent calcium channel subunit	7-17	≥ 21
SCA7	Ataxin7	7-17	≥ 47
SCA17	TATA-binding protein	25-40	≥ 50
Novel SCA disease	THAP11	20-38	≥ 45

3. Spinocerebellar Ataxia type 3 (SCA3)

3.1. General overview

Spinocerebellar Ataxia type 3 (SCA3), commonly known as Machado-Joseph Disease (MJD) is a rare neurodegenerative disorder for which there is no cure. Currently, only treatments to help manage the symptoms are available (10). This disorder results from the expansion of the trinucleotide repeat CAG at exon 10 of the *ATX3* gene located on chromosome 14q32. This expansion consequently leads to the production of a pathogenic version of the ataxin-3 protein with an expanded glutamine stretch. The abnormal protein accumulates in the form of toxic aggregates in the brain and spinal cord cells, leading to the appearance of disease (11). The larger the sequence of glutamines becomes, the more the protein aggregation propensity and disease severity upsurge.

According to neuropathologic and brain imaging research, atrophy has been reported in many parts of the nervous system especially in the spinal cord, brain stem and cerebellum, which is the region that aids in controlling muscle movement and balance (12,13). The clinical symptoms include progressive ataxia, muscle weakness, progressive death of neurons, and many cognitive dysfunctions (11).

3.2. Epidemiology

In general, polyQ diseases have a relatively low prevalence (1 per 100.000 people) worldwide. Nonetheless, the distribution and prevalence of these disorders vary between ethnicities and geographic regions (14).

According to the first reports, SCA3 was first described in 1972, in members of the families of Machado, Thomas and Joseph from Azores, who were residing in North America (15). Thus, it has always been considered a disease originating in the Azores, probably spread by Portuguese navigations in the late 15th and 16th centuries. Currently, there are new theories, that SCA3 was probably originated in Asia before spreading to Europe, and the high prevalence of the disease in Portugal can be explained by the founder effect (16–18).

Regardless of its origin, SCA3 has been recognized as the most common type of autosomal dominant ataxia in the world (12,19). Currently, this disease has intermediate prevalence rates in Portugal, Brazil, Netherlands, Germany, Japan, China, North America, and India. However, it remains with the highest prevalence in Azores, more precisely at Flores Island (1 per 239 people) (10).

3.3. Clinical presentation

Overall, this disorder has a late onset and starts to manifest around 40 years old leading to the premature death of the patients. But there are cases reported in patients between 4 and 70 years old. In the majority of cases, gait ataxia is the first symptom reported (10). Over the years, many other motor symptoms appear, such as muscle atrophy, loss of coordination and balance, dysarthria, oculomotor disturbance, and paralysis due to the progressive death of neurons in the central nervous system (11). Other non-motor symptoms have been related to SCA3, including sleep disorders, and cognitive, olfactory, psychiatric and nutritional dysfunctions (12).

Different repeat sizes result in different illness onset rates, normally larger repeats lead to earlier disease and interfere with the phenotype of SCA3. Based on this diversity, this disorder was divided into three clinical categories. The "Type I" condition has an earlier onset (age of onset is between 10-30 years) and is distinguished by bradykinesia (slowness of movement) (20), bradyphasia (slow speech), and a bit of ataxia. The most prevalent form of the illness is the "Type II", it starts between the age of 20-50 years and is marked by upper motor neuron symptoms and progressive ataxia. "Type III" is the latest-onset version of the illness (between 40-70 years), and it is noticeable by the ataxia, substantial peripheral nerve involvement, amyotrophy (irregular motor neuropathy of the lower limbs), and general areflexia. These classifications emphasize the tremendous heterogeneity of SCA3 (19).

4. Ataxin-3

4.1. Molecular features

Ataxin-3 (Atx3) is a protein ubiquitously expressed in peripheral and brain tissue cells. This protein has a molecular mass of ~45 kDa, and is evolutionarily conserved (21). It is known that Atx3 participates in diverse cellular processes and appears to be crucial for the ubiquitin-proteasome system. This protein has been described as a deubiquitinating enzyme, but it also controls the expression of several genes (22).

The full-length structure of the Atx3 protein is still unsolved. However, using methods like magnetic resonance and X-ray crystallography, it was possible to structurally characterize the Josephin Domain (JD) (23). JD is a flexible helical hairpin formed by a 32-residue insertion that is divided in two subdomains: one forms a hairpin containing four α -helices and the other is a β -sheet catalytic subdomain that flank and stabilizes the conformation of the loop (24). Additionally, this domain has an intrinsic tendency to form fibrils and to aggregate (25). The Atx3 C-terminal region can be subdivided into four regions, the polyQ tract and the three ubiquitin interacting motifs (UIMs), with a high propensity to fold into helices and traits of a natively unfolded or partially structured system (24). The three UIMs are short sequences with a α -helical structure that have the function of binding ubiquitin. Summarizing, Atx3 presents a globular JD with an aggregation-prone region (73' GFFSIQVISNALKVWGLELILFNS '96) (24),(25) and a flexible C-terminal tail (26) which contains two or three ubiquitin interaction motifs (UIMs) and a polyQ tract (27) (**Figure 2**).

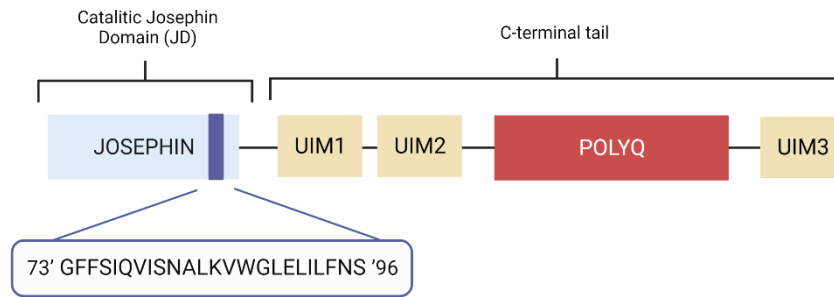


Figure 2. Schematic representation of the domain architecture of ataxin-3; the globular Josephin domain (JD) with the aggregation-prone region (73' GFFSIQVISNALKVWGLELILFNS '96) and the C-terminal tail with variable polyQ region and ubiquitin-interacting motifs (UIMs).

4.2. Aggregation pathway

Normal Atx3 has a sequence between 12 and 44 repeats of glutamines. When the polyQ tract of Atx3 reaches a value above a threshold of 55 glutamines, protein aggregation propensity upsurges resulting in SCA3 pathophysiology. In cases in which Atx3 has between 43 and 54 continuous glutamines, the disease has incomplete penetrance (9).

Atx3 follows a two-step-aggregation pathway *in vitro* (**Figure 3**). The first step is common for both expanded and non-expanded Atx3 which is mediated by the aggregation-prone region of the globular JD that shows an intrinsic amyloidogenic tendency to form aggregates (25). The aggregation through the JD slowly forms soluble oligomers and small protofibrils, which are sensitive to denaturing sodium dodecyl sulfate (SDS). The second step is exclusive for the polyQ expansion, it involves side-chain hydrogen bonds that make aggregation irreversible and leads to the generation of mature, insoluble and SDS-resistant fibrils (9,28).

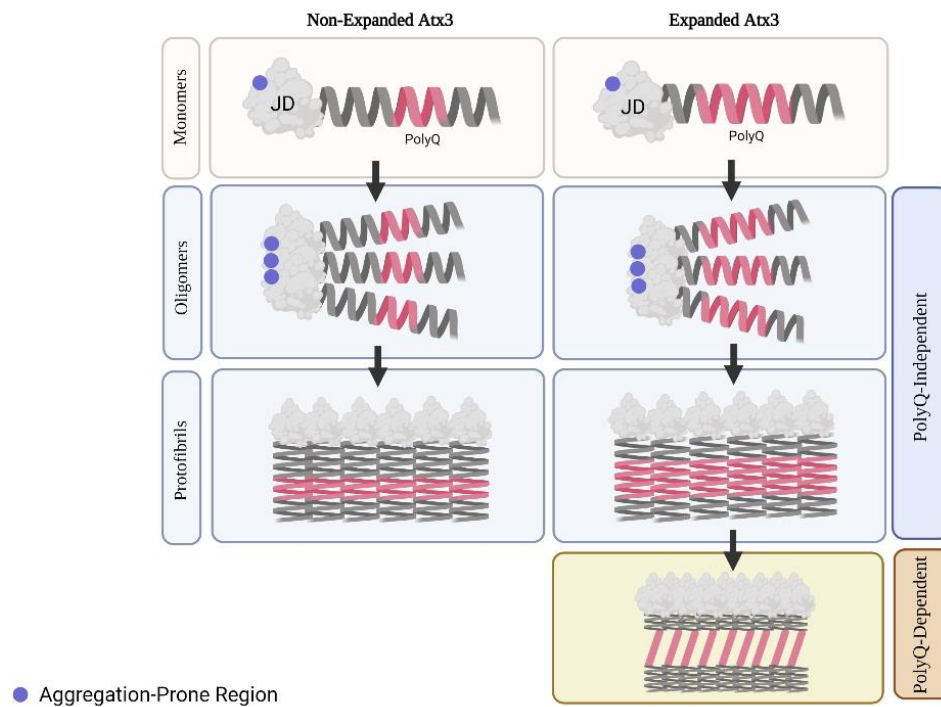


Figure 3. Schematic representation of the two-step-aggregation pathway of ataxin-3. Image created with BioRender.com

The characterization of self-assembly mechanisms and their modification by point mutations, interaction partners, or inhibitors of aggregation is particularly difficult due to the complexity of the Atx3 aggregation pathway. Even slight changes in the buffer conditions can drastically impact the kinetics of ataxin-3 aggregation *in vitro*. These changes affect both the oligomerization processes and the ratio of aggregation to ordered fibrillar structures (27).

Thioflavin-T (Thio-T, an amyloid sensitive dye) fluorescence assay (29), transmission electron microscopy (TEM) (30), and filter trap assay (FT) (31) are often used to measure the formation of amyloid aggregates and SDS-resistant fibers. In this regard, examining the *in vitro* mechanisms of Atx3 aggregation under well-controlled experimental conditions can provide useful information to assess how different compounds and molecules influence the creation of ordered fibrillar structures. These studies serve as the basis for further research using SCA3 cellular and animal models (27), to evaluate the correlation between structural features of Atx3 aggregates and toxicity. Altogether, these studies set the framework for the development of different strategies to inhibit the build-up of neurotoxic Atx3 aggregates.

5. Nanobodies

5.1. Description

In the decade of 90s, we witnessed the revolutionary development of recombinant bispecific prototype immunoglobulin (IgG) molecules with two identical variable heavy chains and two variable light chains, as antibodies, with the aim of creating new treatments (32). For individuals who experience recurrent infections, as a result of inadequate antibody production in their bodies, immunoglobulin replacement therapy can be lifesaving. This treatment has been used in disorders such as: Kawasaki Disease (related to an inflammation of the small and medium-sized blood vessels) (33), Guillain-Barre syndrome (form of polyneuropathy that causes muscle weakness) (34), Chronic inflammatory demyelinating polyneuropathy (also related with progressive weakness and to the limited senses in upper and lower members) (35) and other neurologic disorders (36). Additionally, despite some initial controversy due to their safety, a few months ago antibody was approved as a therapy for Alzheimer's disease (37). On the other hand, another type of antibodies (monoclonal) have been used on cancer treatment (38). However, the use of these innovative solutions in treatment of rare diseases was limited by some unfavorable characteristics of antibodies associated with their production and application, such as high costs, big size, and instability (39).

In 1989, Professor Raymond Hamers-Casterman found that the blood of Camelidae contains specific, useful, homodimeric heavy chain-only antibodies (HCAbs). These antibodies, as in IgG, have a small single domain known as VHH (variable domain of heavy chain antibody) (40) (**Figure 4**). Over the years, the discovery of highly specific VHH, also known as Nanobodies (NBs), led to the development of new cellular research tools for therapeutic applications (41). They have the smallest form of an antibody but still retain the useful feature of full antigen-binding. In addition, they have characteristics that make them more promising than conventional IgG antibodies for clinical purposes. After injection, nanobodies can bind faster to their binding sites on the target tissues, while renal clearance quickly removes the unbound fraction from the bloodstream (42). Also, when compared to conventional antibodies, NBs have a lot more tolerance to extreme temperatures (even 90 °C) and pH (between 3.0–9.0) conditions (43).

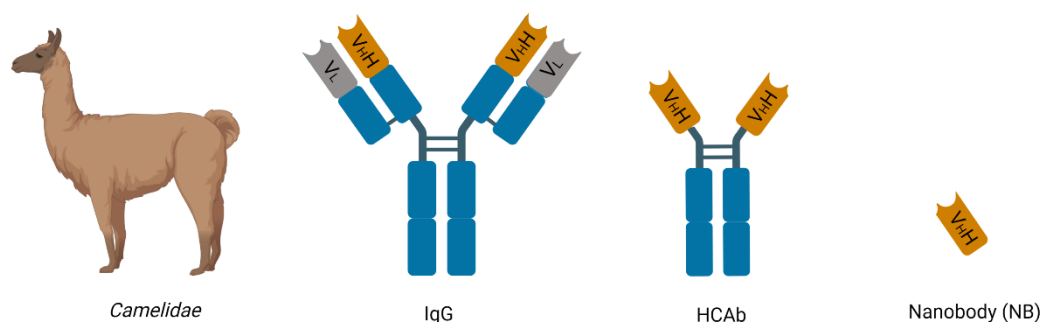


Figure 4. Schematic representation of conventional immunoglobulin (IgGs), heavy chain-only antibodies (HCAbs) and nanobodies. VH, variable heavy; VL, variable light; VHH, VH of HCAbs. Image created with BioRender.com

5.2. Biochemical, biophysical properties and applications

Nanobody structures can be explored for novel applications because of their small size (12 to 15 kDa (42)), strict monomeric state, robustness, easy customization (43), and the fact of being easily produced through cloning and recombinant expression in *E.coli* (44). NBs single-domain facilitates the genetic manipulation to yield fused molecules with high solubility and low aggregation potential. Their increased hydrophilicity and high sequence stability allow for large expression yields, high solubility, proteolytic stability and easiness to be tailored for biotechnological applications (45). Nanobodies are composed of complementarity determining regions – CDR loops - that are involved in binding and recognition, while efficiently refold after chemical or thermal denaturation, which gives them a high stability (45).

The use of NBs has been reported in several applications in different areas. Due to their great water solubility, thermostability and specificity affinity they are suitable probes for molecular imaging applications (42), and are used as biomarkers for some diseases, like cancer (46). They are used as antitumor agents, due to their specificity and capacity of inhibit epidermal growth factor receptor (EGFR) which has a significant role in the progression of tumors (47). Another potential application is their use as supporting materials to protein crystallization. They can be utilized as crystallization chaperones for the structural analysis of various protein conformational states (48). Additionally, some reports showed that nanobodies are able to effectively prevent plaque development, dissolve oligomers or pre-fibrillar aggregates, and interfere with protein pathogenic amyloid fibrillation (49), (50). These findings support their use as potential

modulators of Atx3 aggregation. Recently nanobodies have been studied to be used as diagnosis and treatment of neurodegenerative disorders, such as Alzheimer's disease. With nanobodies, it has been possible to delay the apoptosis of nerve cells, reduce inflammation, and enhance drug delivery to the brain, compared to current neurodegenerative disease treatment protocols, which only address symptoms (51).

Nanobodies have been widely studied, with numerous articles referring to them as possible cures for neurodegenerative disorders (51,52), autoimmune and inflammatory diseases (53) and cancer (54). However, it was with Thrombotic Thrombocytopenic Purpura that, for the first time, there was a clinical trial to assess the efficacy and safety of a Nanobody to treat the disease. This is a deadly, uncommon blood condition, where blood clots develop in the patient body's small blood vessels. The clots may restrict blood flow to the heart, kidneys, brain, and other vital organs. Patients received either placebo or anti-vWF Nanobody, via intravenous injection, as adjunctive therapy to plasma exchange. The study has already results and is finalized (55), coming to demonstrate the promising potential of NBs in the area of disease treatment.

However, despite the valuable potential of nanobodies for therapeutic applications, there are some limitations that need to be further addressed. For instance, their inability to directly induce effector functions, the fact that their binding to some peptides requires multiple steps or the activation of a possible inflammatory process might require long retention times (45). That is why the study of these emerging molecular therapies has been a challenge for researchers.

6. *Drosophila melanogaster*: a model organism for SCA3

6.1. *Drosophila* genetics and biological model

Drosophila melanogaster belongs to the *Drosophilidae* family, and it is commonly known as the fruit fly. Their life cycle is divided in four stages: embryo, larva (with 3 instars), pupa, and adult (**Figure 5**). *Drosophila*'s maximum life span varies from 60 to 80 days, and depends on the growth conditions (56). More specifically, it takes between 9 and 12 days from fertilized eggs to reach adult state when maintained at 25°C (57), but when growing at 18°C this time interval can increase. Their fast generation time, low cost, small size (2 mm), and the fact of being an exceptional genetic tool, have made it a crucial model for fundamental basic research on biochemical pathways (58). Moreover, *Drosophila* has been used to test and enhance development of new therapeutic drugs as they are suitable for high-throughput screening assays (57). Mainly, it has a simple

nervous system which makes it an ideal model for studying behavior mechanisms of brain-related diseases (59,60).

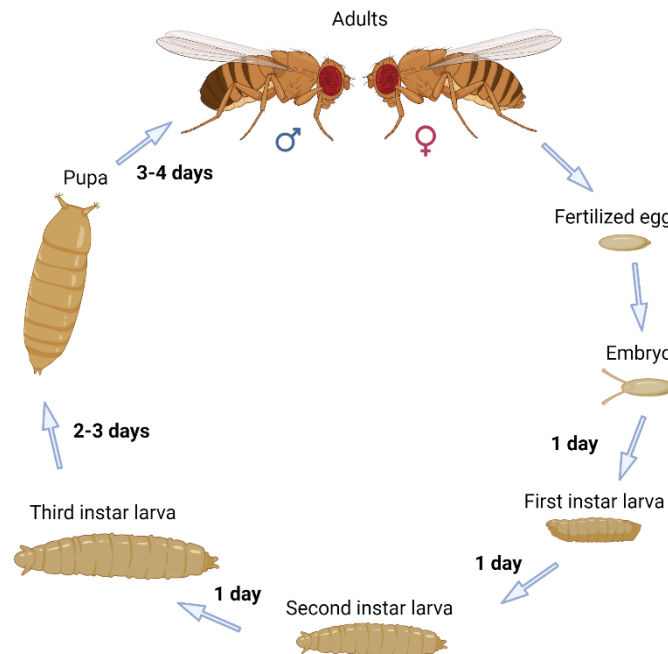


Figure 5. *Drosophila melanogaster* life span. Four stages: embryo, larva (with 3 instars), pupa, and adult. Image created with BioRender.com

6.2. *Drosophila* and the er chromosomes

Throughout the past century, *Drosophila* genetic tools and methods have undergone a significant evolution. However, the balancer chromosome is a particular tool that remains until our days and it is crucial in genetic research, for maintaining and studying desired mutations in a population of flies (61). The artificially created chromosomes contain multiple inversions, which prevent recombination with the normal chromosome during meiosis and ensure that the desired mutation is always present in the population. Also, this tool is particularly useful for maintaining recessive mutations, in heterozygous state and which otherwise would be lost. By ensuring that the mutation is always present in a heterozygous state, balancer chromosomes allow researchers to study the effects of the mutation more easily (62).

Firstly, it was demonstrated that a crossover suppressor on chromosome 3 called CIII was actually an inverted segment and was eventually given the new name In(3R)C.

The majority of third chromosomal balancers in use today have this inversion, which affects the distal portion of chromosome arm 3R. In(3R)C, which carries a recessive fatal allele, was in a stock with a homolog bearing a recessive lethal allele of Serrate (Ser), which had a dominant visible wing phenotype known as Beaded (Ser^{Bd-1}).

By exposing the third chromosome, that carries two inversions (In(3LR)sep and In(3R)C), to X-rays, three additional inversions were produced, resulting in the creation of Third Multiple 3 (TM3). In another instance, a different chromosome that already had three inversions (In(3L)P, In(3LR)P88 and In(3R)C), when exposed to radiation, led to the formation of TM6. Later on, the left arm of TM6 was replaced with another existing inversion, which led to the creation of TM6B. TM6B is represented with the phenotype of extra humeral bristles in flies.

Almost all of the second chromosome balancers currently in use have descended from two paracentric inversions that occurred naturally. Within this chromosome, the curly mutation (Cy) was discovered, and it underwent X-irradiation to create new inversions. A whole-chromosome inversion occurred, resulting in Second Multiple 1 (SM1), which was further irradiated to produce SM5. SM5 carried an additional duplication and inversions. Furthermore, a new balancer known as Curly of Oster (CyO) was created by separately irradiating [In(2L)Cy and In(2R)Cy]. While SM6 was later produced by single exchange events between SM1 and CyO (61).

As these alleles present in balancer chromosomes are easily observed with a magnifying glass (**Figure 6**), they became markers to distinguish flies that carry the balancer from those that do not and allow researchers to track the inheritance of the desired trait through breeding experiments. They have been used extensively in the study of developmental biology, behavior, and evolution, and continue to be an important tool for genetic research (62).

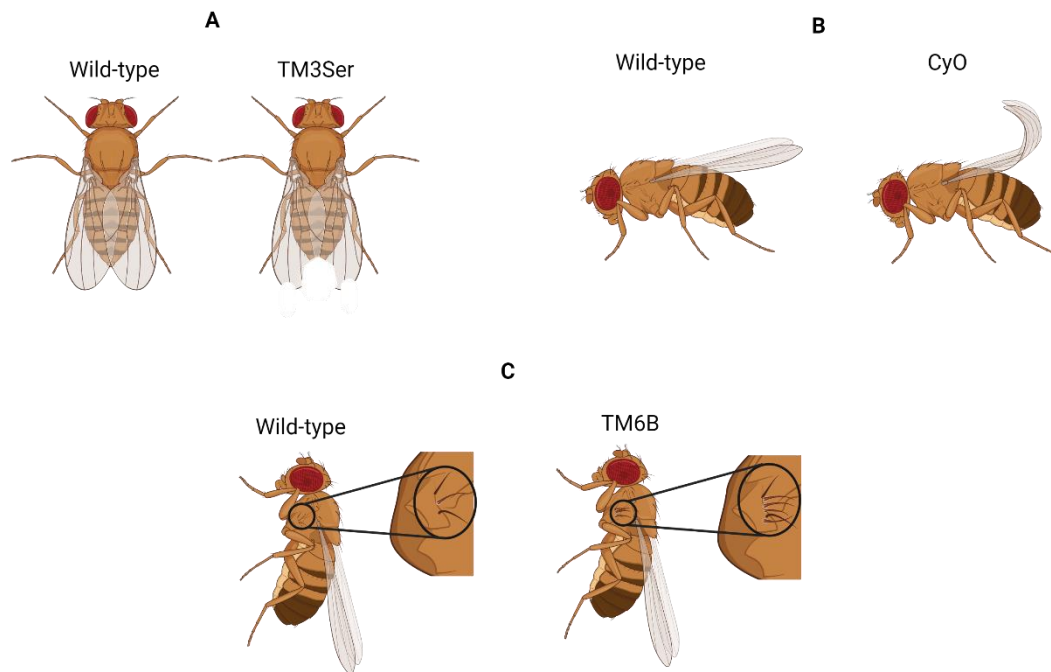


Figure 6. *Drosophila* balancer chromosomes phenotype. (A) TM3Ser corresponds to serrate wings; (B) CyO leads to curly wings; (C) TM6B corresponds to extra humeral bristles. Image created with BioRender.com

6.3. GAL4-UAS transcription activation system

GAL4 was initially described in *Saccharomyces cerevisiae* model as a transcriptional activation protein regulator (63). GAL4 binds to UAS enhancer sequences found in DNA to activate the transcription of Galactose metabolism genes (64). In the 80s, it was discovered that GAL4 system could be used, as well, in *Drosophila* to drive the expression of genes (65). Although *Drosophila* does not display UAS-linked genes, they can be genetically engineered to express genes under UAS enhancer sequences. (63). The combination of stably integrated UAS-based responder designs and tissue-specific GAL4 drivers, designated as GAL4-UAS system (66) (**Figure 7**), has been widely used in many research fields, especially for functional analysis. This gene expression system enables the temporal and spatial expression of any sequence in a controlled manner, and at any time of the development of the fly.

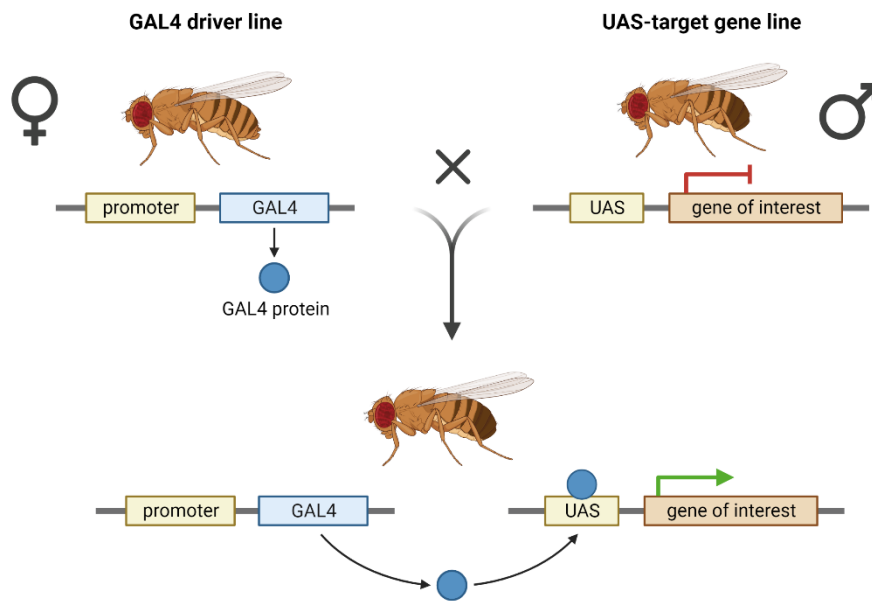


Figure 7. *Drosophila* exogenous GAL4-UAS gene expression system. Image created with BioRender.com

6.3.1. GMR-GAL4 and ELAV-GAL4 drivers

The retina of *Drosophila melanogaster* displays a highly organized lattice structure. Some mutations will translate into alterations in this organized structure giving rise to a phenotype, that is easily observed under a stereoscope and scanning electron microscopy. Apoptosis, programmed cell death, neural connection, tissue patterning, and cell cycle control are among the processes that can be studied using the anatomical alterations of the fly eye (67). One of the most common tools used by geneticists for this type of study is the GMR-GAL4 driver, which is used to express target transgenes in the developing eye (68). It expresses the transcription factor GAL4 under the control of the glass multiple reporter (GMR).

Another widely used tool is ELAV-GAL4. The *elav* gene codes for the RNA-binding protein, ELAV (Embryonic Lethal, Abnormal Vision), which is expressed in all neuronal cells and is widely used as a pan-neuronal cell-specific driver (69). Since ELAV protein was discovered, it was known as a neuronal and postmitotic marker. Later, it was found that it is also transiently expressed in embryonic glial cells. Furthermore, it is transiently expressed in embryonic neuroblasts (70). In summary, ELAV-GAL4 driver begins to be expressed earlier in the fly and has broader expression at the neuronal level when compared to GMR-GAL4.

6.4. *Drosophila* SCA3 models

Knowledge about the pathophysiological basis of neurodegeneration can be provided by researching neurodegenerative disorders resorting to transgenic animal models. Mouse is typically used in those studies, but creating and analyzing such models can be expensive and time-consuming (67).

Despite flies and humans have divergent evolutionary histories, around 70 % of human genes involved in disease have a fly counterpart (71). Furthermore, both human and fly central nervous systems are composed of neurons and glial cells (72), use a similar circuit and organization including synaptic plasticity, neurotransmission, and neurogenesis. So, there is a reasonable similarity between them. Due to their compact brain and the low prevalence of duplicate genes, straightforward genetic investigation is possible without major functional redundancy (73). *Drosophila* recapitulates the same age-related deterioration in brain performance in terms of motor function and cognitive decrease as in humans. Therefore, the fly and human brains can be compared in terms of anatomy, function, and aging (71). Because of those features, *Drosophila* has emerged as a valuable genetic tool to study the pathophysiological underpinnings of neurodegenerative diseases.

The study of genes involved in human neurodegenerative diseases using *Drosophila* as a model system has been made possible by the analyses of their phenotype and by behavioral assays. Most of these disorders are characterized by a decline in learning score, memory, and motor coordination. Behavioral assays on flies provide insight into how genetic and environmental variables may contribute to the development of neurodegeneration (74).

Initially, J. M. Warrick *et al.* used truncated forms of Atx3, lacking the N-terminal part of the protein, in an attempt to describe how the protein's function might be related to toxicity. For this, they expressed non-pathogenic (Atx3-27Q) and pathogenic (Atx3-78Q and 84Q) truncated forms of the protein in *Drosophila*. Expression of Atx-27Q showed no change in phenotypic behaviors, while both pathogenic forms led to accelerated and progressive neuronal degeneration in adult flies (75). Later, work from S. L. Johnson *et al.* showed similar results after expression of full-length variants of pathogenic Atx3, with repeats range of 77Q–80Q in *Drosophila*. Both studies showed that, expression of Atx3 with more than 77Q is lethal for the fly (76).

6.4.1. Phenotypic traits of SCA3 fly models.

Previous reports showed that expression of polyQ-expanded human Atx3 in *Drosophila* recapitulates molecular features of SCA3, including the development of nuclear inclusions and neurodegeneration in the fly retina (77). The expression of pathogenic Atx3 in fly eyes with the GMR-GAL4 system leads to alterations of the retina morphology, characterized by de-pigmentation and roughness (71), known as rough eye phenotype (REP). Since it is simple to identify under a regular microscope, this feature has been utilized as a readout of SCA3 (73). Previous studies from other research groups demonstrated that it is possible to evaluate how the expression of expanded Atx3 affect through the use of photos of the external eye morphology of adult flies, under the control of GMR-GAL4 (78,79). Flies expressing Atx3 with ~20Q exhibited normal retina, which means normally diffuse, well organized without any degeneration. On the other hand, flies expressing Atx3 with 78Q and 84Q repeats displayed severe retinal degeneration, associated with collapsed and depigmented eye (**Figure 8**) (75).

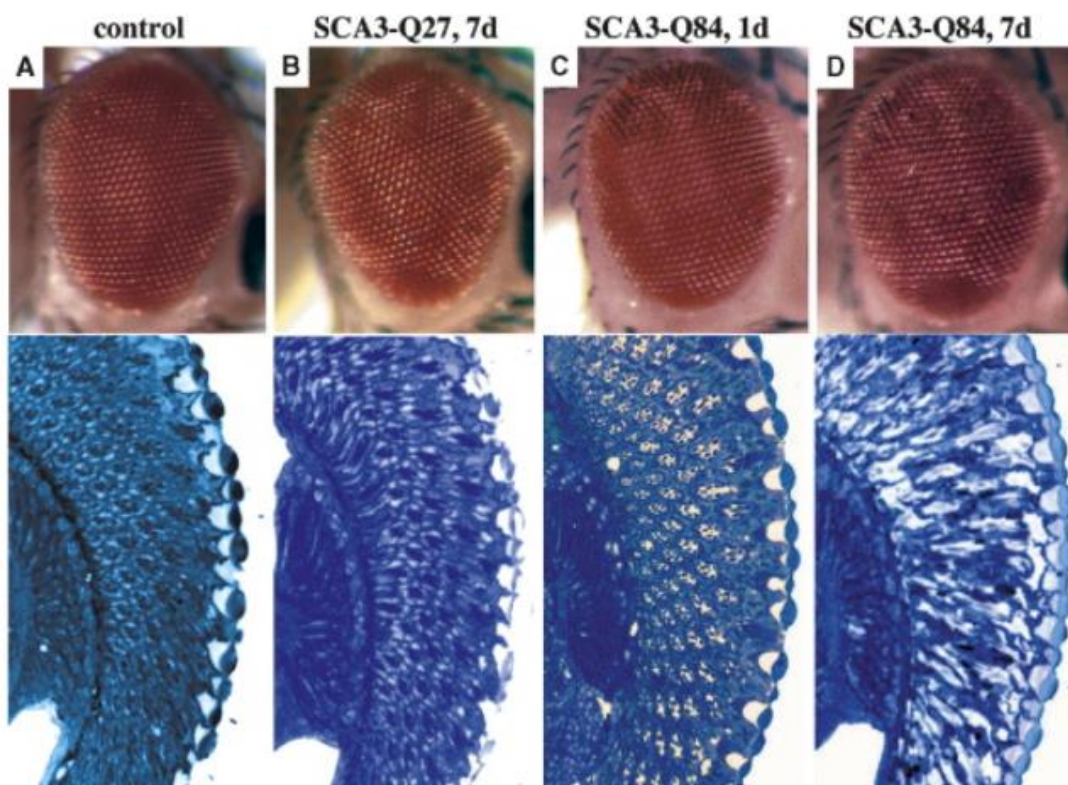


Figure 8. Expression of the pathogenic Atx3 leads to retinal neurodegeneration; Eye from control flies without Atx3 expression (A) and with non-expanded Atx3 expression (B); (C) Atx3-84Q induces eye degeneration, condition that is dramatically progressive over time (D). Image adapted from (75).

It is possible to obtain protein extracts from heads of the flies with the genotype of interest to understand whether there is a reduction in the levels of the pathogenic protein or its aggregates *in vivo* (79,80). The head protein extracts can be analyzed through blotting techniques (western blot and filter trap) to specifically evaluate the presence of Atx3 SDS-resistant or soluble species (31).

Previous studies demonstrated that SCA3 *Drosophila* models expressing Atx3 in the same chromosomal region driven by the same ubiquitous driver (*sqh*-GAL4), present significant differences between 77Q and 80Q pathogenic lines (76). The expression of Atx3-80Q is more toxic than expression of Atx3-77Q. The authors proposed that these differences could be related with the use of an optimized Kozak sequence in Atx3-80Q, which consequentially leads to higher Atx3 protein levels, leading to more toxicity.

In ataxias, such as SCA3, motor decline appears more quickly than in a healthy person, leading to poor quality of life for patients. To understand how molecular aspects of aging impact disease progression and to provide new ideas for strategies to promote a healthier lifespan for patients, simple model organisms have been used to study longevity (81). Previous report demonstrated that lifespan of transgenic flies expressing expanded human Atx3 driven by ELAV-GAL4 were significantly shortened (3 weeks after eclosion the females' mortality rate was above 50 %) when compared with the control flies (expressing non-expanded Atx3), that lived more than 50 days. The frequency of premature death of adults often coincided with the onset of locomotor impairment, with pathogenic Atx3 flies dying substantially earlier than wild-type flies. Also, the rates of survival were gender specific. In the same conditions, males had a shorter lifespan when compared to females (82). ELAV-GAL4 is positioned on the X chromosome, so this discrepancy is likely the result of a dosage effect. The mammal's dosage compensation is performed by inactivating one of the two X females' chromosomes. However, in *Drosophila* it is achieved by overexpressing the X male' chromosome, leading to a more pronounced toxic effect in males than in females (82,83).

Climbing assay is a physiological test used to characterize the fly's motor capacity. The climbing ability of *Drosophila* is known to decline with age; old flies tend to make short abortive climbs and then fall back to the bottom of the tube. This loss of climbing response is commonly used to monitor age-related changes in *Drosophila* (84). Previous report (82) showed that both Atx3 transgenic and control flies climbed normally but after one day of eclosion the climbing response of the flies expressing Atx3-78Q was declined. In conclusion, this accelerated, gradual loss in climbing response

demonstrated that expression of pathogenic Atx3 in the nervous system of *Drosophila* model resulted in a functional motor impairment.

7. Previous work

New strategies to predict the distribution of fibrillar and oligomeric Atx3 species during aggregation have been developed in our lab over the past few years as a result of the intensive time-resolved analysis of Atx3 structure and aggregation routes. From previous work, our group also aims to develop therapeutic strategies to inhibit Atx3 oligomerization and prevent SCA3 (77,85).

From a library of 63 Atx3-targeting NBs (48), our lab has already identified a small set of promising NBs with Atx3 anti-aggregation properties that were further characterized *in vitro*. The NBs that delayed pathogenic Atx3 self-assembly without compromising Atx3 function were further characterized by standard biophysical methods. This list was narrowed to four NBs (NB01, NB02, NB05 and NB20) as candidates for further evaluation. *In vitro* analyses showed that selected NBs bind to the pathogenic Atx3 and inhibit its self-assembly (**Figure 9**; A. Silva *et al.*, manuscript in preparation). Also, through analysis of the Atx3 aggregation samples by Size Exclusion Chromatography (SEC) it was possible to determine that some NBs had a significant impact on the stabilization of pathogenic and monomeric Atx3. The selected NBs demonstrated to be able to alter fibril morphology and obstruct the maturation of the fibrils created by the expanded Atx3. After validating that they are promising as inhibiting molecules to prevent amyloid fiber formation and protein aggregation, future work aims to characterize their neuroprotective potential *in vivo* using *Drosophila* and cellular models of SCA3.

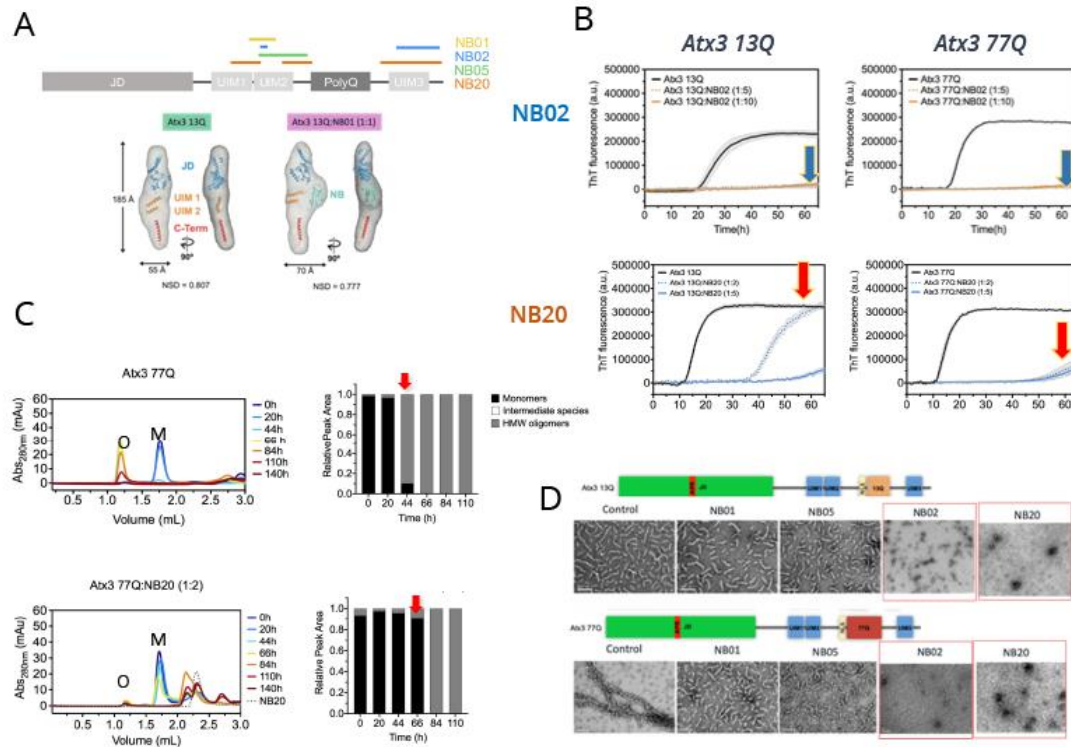


Figure 9. Impact of NBs on Atx3 structure and amyloid assembly in vitro. A) Schematic representation of Atx3 domain architecture. Colored lines highlight the binding sites of NB01, NB02, NB05 and NB20 identified by NMR. Bottom image: SAXS-derived ab initio envelope and structural model of Atx3-13Q>NB01 complex. B) Analysis of Atx3-13Q and Atx3-77Q aggregation kinetics in the absence and presence of NB02 and NB20 (ThT assay) at different molar ratios. Blue arrows denote the full inhibition of Atx3 aggregation by NB02; red arrows highlight the different inhibitory activity of NB20 towards Atx3-13Q and Atx3-77Q aggregation. C) Time-course of Atx3-77Q aggregation monitored by SEC; integration of the monomer (M) and oligomer (O) peaks shows stabilization of the Atx3-77Q monomers in the presence of NB20. D) The morphology of the protofibrils/fibrils was observed by transmission electron microscopy (TEM). Results show that NBs prevent/delay the formation of Atx3-77Q mature fibrils and affect the morphology and size of the protofibrils, with NB02 and NB20 favoring the appearance of more spherical aggregates. This is in striking contrast to NB01 and NB05, which do not abolish the assembly of Atx3 protofibrils.

8. Objectives

This work aims to characterize the neuroprotective effect of four previously selected Nanobodies with Atx3 anti-aggregation properties using *Drosophila* and mammalian cell-based models of Machado-Joseph disease. To achieve this, we define two main objectives:

- 1) Evaluate how NBs impact the occurrence of Atx3 SDS-sensitive and resistant aggregates and fibrils in flies expressing pathogenic forms of human Atx3;
- 2) Evaluate the neuroprotective effect of selected NBs through functional assays using SCA 3 *Drosophila* models.

For future work, we aim to:

- 1) Evaluate if selected NBs can cross the blood-brain barrier;
- 2) Evaluate the anti-aggregation properties of selected NBs and their ability to modulate Atx3 aggregates using neuronal cell models expressing normal and expanded Atx3.

Material and Methods

1. Tables:

Table 2. Description of the *Drosophila* genotypes used in this work.

NAME	GENOTYPE	SOURCE
GMR-GAL4		(86)
ELAV-GAL4	P{w[+mW.hs]=GawB}ELAV[C155]	Bloomington Drosophila Stock Center #458
UAS-Atx3- myrRFP	w[1118]; P{w[+mC]=UAS-myrRFP-mRFP}1	Bloomington Drosophila Stock Center #7118
UAS-Atx3-22Q	w-; UAS-Atx3-22Q-his6; +	(76)
UAS-Atx3-77Q	w-; Sp/CyO; UAS-Atx377Q(CAG)	(76)
UAS-Atx3-80Q	w-; UAS-Atx380Q(CAGCAA)-HA	(76)
UAS-NB20; UAS-Atx3-77Q	w-; UAS-NB20/CyO; UAS-Atx3-77Q(CAG)/TM6B	In-house
UAS-NB02; UAS-Atx3-77Q	w-; UAS-NB20/CyO; UAS-Atx3-77Q(CAG)/TM6B	In-house
UAS-NB01; UAS-Atx3-77Q	w-; UAS-NB01/CyO; UAS-Atx3-77Q(CAG)/TM6B	In-house
UAS-NB05; UAS-Atx3-77Q	w-; UAS-NB05/CyO; UAS-Atx3-77Q(CAG)/TM6B	In-house
UAS-NB20; UAS-Atx3-80Q	w-; UAS-NB20/CyO; UAS- Atx3-80Q(CAGCAA)-HA /TM6B	In-house
UAS-NB02; UAS-Atx3-80Q	w-; UAS-NB02/CyO; UAS- Atx3-80Q(CAGCAA)-HA /TM6B	In-house
UAS-NB01; UAS-Atx3-80Q	w-; UAS-NB01/CyO; UAS- Atx3-80Q(CAGCAA)-HA /TM3Ser	In-house
UAS-NB05; UAS-Atx3-80Q	w-; UAS-NB05/CyO; UAS- Atx3-80Q(CAGCAA)-HA /TM3Ser	In-house

Table 3. Complete and abbreviated genotypes used in this work.

Genotype Group	Complete Genotype	Abbreviated Genotype
GMR-GAL4; UAS-Atx3-variants	GMR-GAL4; OrR	GMR-GAL4; OrR
	GMR-GAL4; UAS-Atx3-myrRFP	GMR-GAL4 > Atx3-myrRFP
	GMR-GAL4; UAS-Atx3-22Q	GMR-GAL4 > Atx3-22Q
	GMR-GAL4; UAS-Atx3-77Q	GMR-GAL4 > Atx3-77Q
	GMR-GAL4; UAS-Atx3-80Q	GMR-GAL4 > Atx3-80Q
GMR-GAL4; UAS-NBs; UAS-Atx3-80Q	GMR-GAL4; UAS-NB20; UAS-Atx3-80Q	GMR-GAL4 > NB20; Atx3-80Q
	GMR-GAL4; UAS-NB02; UAS-Atx3-80Q	GMR-GAL4 > NB02; Atx3-80Q
	GMR-GAL4; UAS-myrRFP; UAS-Atx3-80Q	GMR-GAL4 > myrRFP; Atx3-80Q
	GMR-GAL4; UAS-NB01; UAS-Atx3-80Q	GMR-GAL4 > NB01; Atx3-80Q
	GMR-GAL4; UAS-NB05; UAS-Atx3-80Q	GMR-GAL4 > NB05; Atx3-80Q
GMR-GAL4; UAS-NBs (no Atx3)	GMR-GAL4; UAS-NB20	GMR-GAL4 > NB20
	GMR-GAL4; UAS-NB02	GMR-GAL4 > NB02
	GMR-GAL4; UAS-myrRFP	GMR-GAL4 > myrRFP
	GMR-GAL4; UAS-NB01	GMR-GAL4 > NB01
	GMR-GAL4; UAS-NB05	GMR-GAL4 > NB05
ELAV-GAL4; UAS-Atx3-variants	ELAV-GAL4; UAS-Atx3-myrRFP	ELAV-GAL4 > Atx3-myrRFP
	ELAV-GAL4; UAS-Atx3-22Q	ELAV-GAL4 > Atx3-22Q
	ELAV-GAL4; UAS-Atx3-77Q	ELAV-GAL4 > Atx3-77Q
	ELAV-GAL4; UAS-Atx3-80Q	ELAV-GAL4 > Atx3-80Q
ELAV-GAL4; UAS-NBs; UAS-Atx3-77Q	ELAV-GAL4; UAS-NB20; UAS-Atx3-77Q	ELAV-GAL4 > NB20; Atx3-77Q
	ELAV-GAL4; UAS-NB02; UAS-Atx3-77Q	ELAV-GAL4 > NB02; Atx3-77Q
	ELAV-GAL4; UAS-myrRFP; UAS-Atx3-77Q	ELAV-GAL4 > myrRFP; Atx3-77Q
	ELAV-GAL4; UAS-NB01; UAS-Atx3-77Q	ELAV-GAL4 > NB01; Atx3-77Q
	ELAV-GAL4; UAS-NB05; UAS-Atx3-77Q	ELAV-GAL4 > NB05; Atx3-77Q
ELAV-GAL4; UAS-NBs; UAS-Atx3-80Q	ELAV-GAL4; UAS-NB20; UAS-Atx3-80Q	ELAV-GAL4 > NB20; Atx3-80Q
	ELAV-GAL4; UAS-NB02; UAS-Atx3-80Q	ELAV-GAL4 > NB02; Atx3-80Q
	ELAV-GAL4; UAS-myrRFP; UAS-Atx3-80Q	ELAV-GAL4 > myrRFP; Atx3-80Q
	ELAV-GAL4; UAS-NB01; UAS-Atx3-80Q	ELAV-GAL4 > NB01; Atx3-80Q
	ELAV-GAL4; UAS-NB05; UAS-Atx3-80Q	ELAV-GAL4 > NB05; Atx3-80Q

Table 4. List of antibodies used in this work.

Primary Antibodies	Dilution	Host
Anti-Atx3 1H9 (MAB5360, Millipore)	1:5000 in TTBS with 3 % BSA	Mouse
HA-Tag (F-7) (sc-7392, Santa Cruz Biotech)	1:1000 in TTBS with 3 % BSA	Mouse

Anti-Amyloid -Oligomer A11 (AB9234, Millipore)	1:1000 in TTBS with 3 % BSA	Rabbit
Anti-Amyloid Fibrils OC (AB2286, Millipore)	1:10000 in TTBS with 3 % BSA	Rabbit
Anti-Camelid VHH Antibody [HRP] (A01861, GenScript)	1:2000 in TTBS with 3 % powdered milk	Rabbit

Secondary Antibodies	Dilution
Anti-Mouse-HRP (A4416, Sigma-Aldrich) 1:5000	1:5000 in TTBS with 3 % BSA
Anti-Rabbit-HRP (A6154, Sigma-Aldrich) 1:10000	1:10000 in TTBS with 3 % BSA

2. Solutions and buffers

- **1x Tris-Buffered Saline TBS**

150 mM NaCl;
25 mM Tris pH 7.5;

- **1x TTBS**

150 mM NaCl;
25 mM Tris pH 7.5;
0,1 % (w/v) Tween 20

2.1. Filter trap and dot-blot buffers

- **Blocking solution**

10 % (w/v) Bovine Serum Albumin in TTBS

- **1x Phosphate-buffered saline (PBS)**

10 mM Sodium Phosphate;
150 mM NaCl, pH 7.4

2.2. Western-blotting buffers

- **Blocking solution**

5 % (w/v) Bovine Serum Albumin in TTBS

- **Antibodies solution**

3 % (w/v) Bovine Serum Albumin in TTBS

- **Running buffer**

25 mM Tris Base;
192 mM Glycine;
0.1 % (w/v) SDS

- **Wet transfer buffer**

25 mM Tris,
192 mM Glycine, pH 8.3
20 % (w/v) Methanol

- **5x Sample loading buffer (SB)**

0.625 M Tris-HCl, pH 6.8
100 % Glycerol
20 % SDS
0.01 % Bromophenol Blue

- **Urea buffer**

50 mM TRIS pH 7.5;
100 mM NaCl;
6M Urea

2.3. *Drosophila* protein extracts

- **NETN buffer**

50 mM Tris, pH 7.5;
150 mM NaCl;
0.5 % Nonidet P-40

2.4. Nanobody purification buffers

- **Cell lysis buffer (TES buffer)**

30 mM Tris-HCl,
0.1 mM EDTA
20 % (w/v) Sucrose

- **HisTrap buffer A**

50 mM Sodium Phosphate pH 7.5;
500 mM NaCl;
10 mM Imidazole

- **HisTrap buffer B**

50 mM Sodium Phosphate pH 7.5;
 500 mM NaCl;
 300 mM Imidazole

- **Gel filtration buffer**

50 mM Sodium Phosphate pH 7.5
 150 mM NaCl

2.5. Cell culture media for *E. coli* (Expression of Nanobodies):

- **Terrific Broth (TB)**

1.2 % (w/v) Tryptone;
 2.4 % (w/v) Yeast Extract;
 0.4 % (w/v) Glycerol;
 0.23 % (w/v) Potassium Phosphate Monobasic (KH₂PO₄);
 1.64 % (w/v) Potassium Phosphate Dibasic (K₂HPO₄)

- **Luria broth (LB plates)**

1mM MgCl₂
 0.1 % Glucose
 100 µg/mL Ampicillin

3. *E. coli* Strains (Expression of Nanobodies)

Table 5. *E. coli* strains used in this work.

Strain	Application
<i>E. coli</i> DH5α [F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 Φ80dlacZΔM15 deoR Δ(lacZYA-argF) U169 nupG, hsdR17(rK-mK+), λ-]	Plasmid Replication
<i>E. coli</i> WK6(su-) [Δ lac-proAB), GalE, strA/F' lacIq ,lacZΔM15, proA+B+]	Expression of Nanobodies

4. Plasmids

Table 6. Information about mammalian Atx3-13Q expression plasmid.

Vector ID	VB220810-1264nzc
Vector Name	pRP[TetOn]-TRE>{EGFP-Atx3-13Q-XhoI/SalI}-rev(mPGK>tTS:T2A:rtTA)
Vector Size	7062 bp
Vector Type	Mammalian Tet-On Inducible Gene Expression Vector (All-In-One)
Inserted Promoter	mPGK
Inserted ORF	{EGFP-Atx3-13Q- XhoI/SalI}
Plasmid Copy Number	High
Antibiotic Resistance	Ampicillin
Cloning Host	VB UltraStable (or alternative strain)

Table 7. Information about mammalian Atx3-77Q expression plasmid.

Vector ID	VB220808-1463cyn
Vector Name	pRP[TetOn]-TRE >{Atx3-77Q-optimized}-rev(mPGK>tTS:T2A:rtTA)
Vector Size	7254 bp
Vector Type	Mammalian Tet-On Inducible Gene Expression Vector (All-In-One)
Plasmid Copy Number	High
Antibiotic Resistance	Ampicillin
Cloning Host	VB UltraStable (or alternative strain)

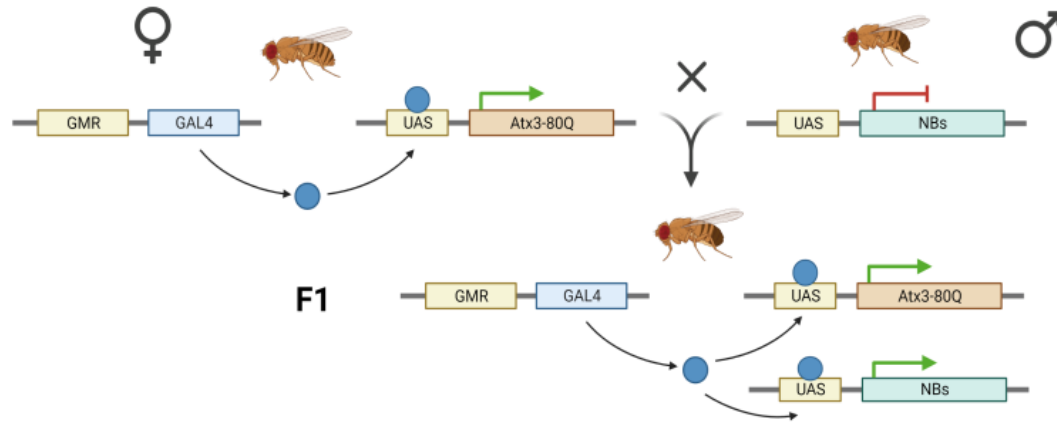
5. Fly maintenance and genetics

Fly stocks and crosses were usually kept at 25 °C. For fly crosses it is necessary that the females are virgins because they naturally have the ability to store the sperm after mating, so if they have already bred before and we selected them for the cross, the new descendants may not have the genotype of interest.

The virgin collection was done about 8-10 hours after hatching. However, in some cases, the culture was also kept at 18 °C as it slows down development and virgins could be collected up to 16 hours after hatching. The identification of these flies was done visually, as the virgin females have an inflated abdomen and with a whiter coloration than older females. Another selective indicator is the presence of meconium, a dark spot on the abdomen of virgin females as a result of the flies' last meal before pupating.

Flies expressing UAS-Atx3-80Q and UAS-Atx3-77Q in the retina or pan-neurally, were obtained after crossing UAS-Atx3-80Q and UAS-Atx3-77Q male flies with females from the GMR-GAL4 or ELAV-GAL4 lines. Control flies (UAS-myrRFP or UAS-lacZ – expressing of a neutral UAS) and flies expressing Atx3 with different polyQ length (22Q, 77Q and 80Q), were also used in this study. For co-expression of pathogenic Atx3 variants with nanobodies, females from GMR-GAL4 or ELAV-GAL4 were crossed to males from the different lines carrying UAS-NBs; UAS-Atx3-77Q or UAS-NBs; UAS-Atx3-80Q (Table 2). Virgin females expressing the desired driver, were crossed to males with the genotype of interest. After about 12 days the first adults (F1) were selected (**Figure 10**).

A



B

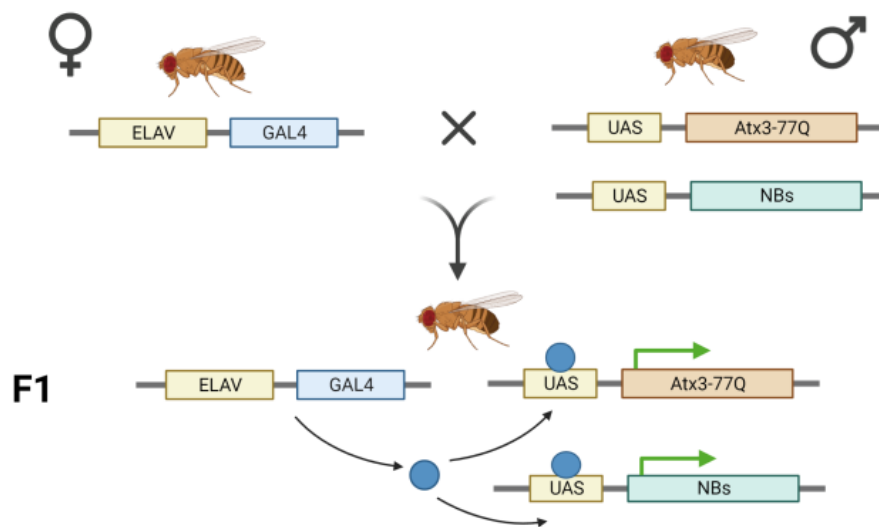


Figure 10. Representative image of the genetic crosses used. A) Schematic example of genetic cross between GMR-GAL4>Atx3-80Q female flies with males with the NB of interest. Originating the F1 adults: GMR-GAL4>NB; Atx3-80Q. B) Schematic example of a genetic cross between ELAV-GAL4 female flies with males with the Atx3-77Q and the NB of interest, originating the F1 adults: ELAV-GAL4>NB; Atx3-77Q. Image created with Biorender.com.

However, when the F1 adults start to appear, it was necessary to identify flies with the correct genotype for subsequent selection. For that, we made the identification through the use of chromosome balancers (**Figure 11**). Balancer chromosomes translate themselves into visible markers, making this task easier. UAS-Atx3 transgenes are inserted on the third chromosome. Therefore, the markers TM3Ser (serrate wings) or TM6B (extra humeral bristles) which are in the third chromosome, are used for selection. On the other hand, UAS-NBs are inserted on chromosome 2. In this case, we used the marker CyO (curly wings) present in the second chromosome. For the correct selection of flies, we had to discard the flies that presented either serrate wings, or extra humeral bristles and curly wings, in this way we were sure that the selected flies had the genotype of interest (e.g., ELAV-GAL4>NB05; Atx3-80Q). Briefly, our objective was to collect female flies that phenotypically do not present any markers. After selection, the female flies were snap-frozen in liquid nitrogen and stored at -80°C for later analysis.

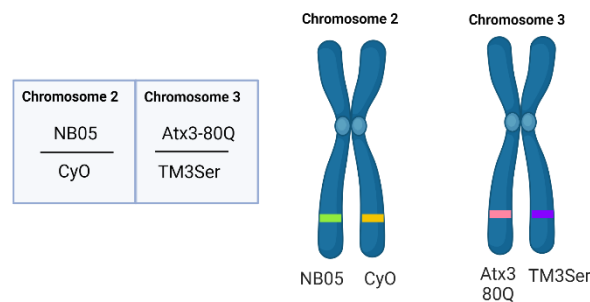


Figure 11. Balancer chromosomes. Chromosome 2 with NB05 in one arm and CyO in the other. Chromosome 3 with Atx3-80Q in one arm and TM3Ser in the other. Image created with Biorender.com.

6. Analysis of protein extracts by western blot

After snap-frozen, the adult flies' heads were separated from the body by vortexing. Ten fly heads were resuspended in 60 µL SB 1x (diluted in PBS) supplemented with 10 mM DTT and bath sonicated for 4 minutes for cell lysis. The head lysates were boiled in a two-step gradient of temperature (10 min at 65 °C followed by 10 min at 95 °C) for sample denaturation and centrifuged at 13000 rpm for 12 minutes. The supernatant was collected to a new tube (soluble fraction) and the pellet was resuspended in the same volume of SB 1x supplemented with 10 mM DTT. Both soluble and pellet fractions were snap-frozen and stored at -20 °C.

6.1. Western blotting

For analysis of protein expression levels, protein extracts from fly heads were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were prepared according to the molecular weight of the protein of interest. To analyze Atx3 protein levels, 20 µL of soluble protein and 20 µL of pellet samples were loaded and resolved in a 10 % acrylamide SDS-PAGE gel at 30 mV per gel. The gels were transferred to a nitrocellulose membrane (87) in a semi-dry system (TransBlot® Turbo™ Transfer System) at 72 mA for 2 h. After transfer, the membranes were stained with Ponceau S to visualize protein bands and the gels with Blue Coomassie (to evaluate the transfer efficiency). The membranes were washed with TTBS and placed in a blocking solution for 1 h (5 % milk supplemented with TTBS). For detection of Atx3, membranes were incubated with anti-Atx3 1H9 (1:5000, overnight at 4 °C) or with anti-HA (1:1000, overnight at 4 °C). Membranes were washed twice with TTBS and incubated with the secondary mouse antibody conjugated with HRP (1:5000, 1 h at room temperature). Bands were visualized by chemiluminescence with ECL™ Prime Western Blotting System (Cytiva RPN2232) using ChemiDoc™ MP System with Image Lab™ Software

For detection of Atx3 species (soluble and SDS-resistant high-molecular weight species (HMW)) samples were resolved in a 4-20 % Mini-PROTEAN precast protein gels (BioRad) and transferred overnight to a nitrocellulose membrane in a wet transfer system at 4 °C. After the transfer, the membrane was stained with Ponceau S for analysis of Atx3 total protein. The membrane was blocked with 5 % BSA in TTBS for 1 h, incubated with primary 1H9 antibody (1:5000, overnight at 4 °C), and finally with secondary antibody mouse HRP (1:5000, 1 h at RT). Bands were visualized by chemiluminescence with ECL™ Prime Western Blotting System (Cytiva RPN2232) using ChemiDoc™ MP System with Image Lab™ Software

7. Analysis of protein extracts by dot-blot and filter trap

To evaluate the Atx3 total protein level and the presence of Atx3 SDS-resistant fibrils in fly head extracts we performed dot-blot and filter trap (FT) assays, respectively. Extracts for dot-blot and filter retardation assays were prepared as follows: after snap-frozen the flies, ten fly heads were separated from the body by vortexing and resuspended in 200 µL of NETN [150 mM NaCl, 50 mM Tris-HCl pH 7.5 and 0.5 % Nonidet-40] lysis buffer supplemented with protease inhibitor cocktail (PI). The tubes

were placed in liquid nitrogen and then, bath sonicated for 4 minutes. The sample was centrifuged at 9000 rpm for 2 minutes to remove cell debris. Half of the supernatant was collected to a new tube (100µL for DB) while the other half of the supernatant (100 µL) was collected to another tube and an equal volume of PBS with 0.5 % SDS was added. The protein extracts were processed immediately after, or snap-frozen in liquid nitrogen and stored at -20 °C.

7.1. Dot-blot

For dot-blot, 3 µl of serial dilutions of protein samples (prepared in PBS supplemented with 1 % glycerol) were spotted on a nitrocellulose membrane and let dry for at least 24 h. Membranes were blocked with 10 % BSA in TTBS for 1 h at RT and further incubated with one of the primary antibodies: anti-Atx3 monoclonal antibody 1H9 (1:5000), anti-HA (1:2000), anti-amyloid oligomers A11 (1:1000) or anti-amyloid fibrils OC (1:10000) overnight at 4 °C. After double washing with TTBS, membranes were incubated with the corresponding secondary antibody mouse (1:5000) for 1 h at RT. Bands were visualized by chemiluminescence with ECL™ Prime Western Blotting System RPN2232 (Cytiva) using ChemiDoc™ MP System with Image Lab™ Software.

7.2. Filter trap

The filter trap assay was performed in the presence of SDS to evaluate the presence of SDS-resistant Atx3 fibrils. Protein samples were thawed and diluted 5 times with PBS (80 µL of sample plus 320 µL of PBS). Samples were loaded into a Bio-dot slot format microfiltration system (BioRad) previously assembled. Firstly, two filter papers and the nitrocellulose membrane were soaked in PBS with 0.1 % SDS and the system was assembled as represented in **Figure 12**. After system assembly, the membrane was pre-washed three times by placing 100 µL PBS 0.1 % SDS on each of the wells. After, 70 µL of each sample were loaded into each well (in replicates) and filtered through the membrane by applying vacuum to the system. The membrane was re-washed three times with PBS 0.1 % SDS. Then, the membrane was blocked with 10 % BSA in TTBS for 1 h at RT and incubated with the primary antibody anti-Atx3 1H9 (1:5000) for 1 h at RT. After double washing with TTBS, the membrane was incubated with the secondary antibody anti-mouse (1:5000) for 1 h at RT. The membrane was developed, and bands were visualized by chemiluminescence with ECL™ Prime Western Blotting System RPN2232 (Cytiva) ChemiDoc™ MP System with Image Lab™ Software

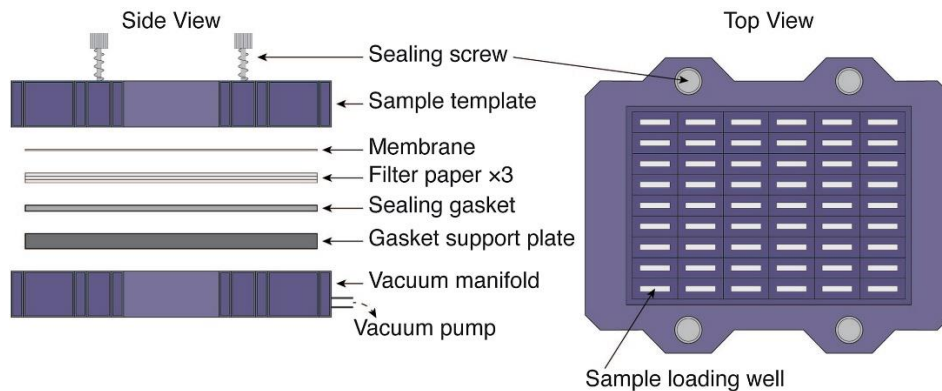


Figure 12. Schematic representation of filter trap apparatus assembly. The apparatus is composed by sealing screws, forty-eight sample loading wells, sealing gasket, gasket support plate and a vacuum manifold for connection to a pump vacuum system. Both the membrane and filter papers were immersed in PBS 0.1 % SDS before the assembly of the system and sample loading (88).

8. Functional assays

For functional assays, ELAV-GAL4 was used to drive the co-expression of Atx3-80Q, or Atx3-77Q with NBs in the fly nervous system. Important control models (UAS-myrRFP and UAS-lacZ) and Atx3 variants (22Q, 77Q and 80Q), were included. The expression of Atx3 in the central nervous system is useful to characterize the effect that NBs have on the protein and how this translates into physiological changes, specifically in fly's lifespan and motor ability. We accessed the lifespan through survival assay and the locomotor performance through climbing assay, which is a powerful *in vivo* indicator for neuromuscular integrity.

8.1. Survival assay

The lifespan assay was based on the article written by Linford, N. J *et al.* (81) (**Figure 13**). After flies reached the adult stage and the genotype of interest selected (as described previously, in point 5), we let them mate for 2 days in the bottle. These 2 days will prevent unintentional selection of early emergent and enable the collection of the most synchronized flies possible. Then, we anesthetized a group of flies on the

anesthetic pad. With the help of a paintbrush, we collected about 8 to 15 females with the right genotype (with no balancer chromosomes) per tube, for a total of 100 flies per genotype. When using CO₂, it's important to limit exposure to avoid any long-term health problems in the fly that can compromise the survival and validity of the experiment. To eliminate bias related to the vial location in the incubator and to hide the identity of the vial from the researcher, it is advised to randomly assign each tube position rather than grouping tube vials by experimental condition. To do this, first, we gave each vial a randomly generated numerical ID, and then arrange the vials on trays according to the ID number.

Flies were transferred onto new vials containing fresh food every two days during the experimental period, and every three days for females older than three weeks. By taking this step, we ensured that larvae did not interfere with the females' lifespan. For each transfer, the vials carrying new food were maintained at room temperature. Anesthesia should not be used for this transfer because it can cause abrupt mortality, especially in older flies. The dead, the carried, and the censored flies were noted during each vial transfer. A fly is censored if it left the experiment before dying naturally, by escaping, by accidental death, or in the case flies remain stuck to the food in the old vial but alive.

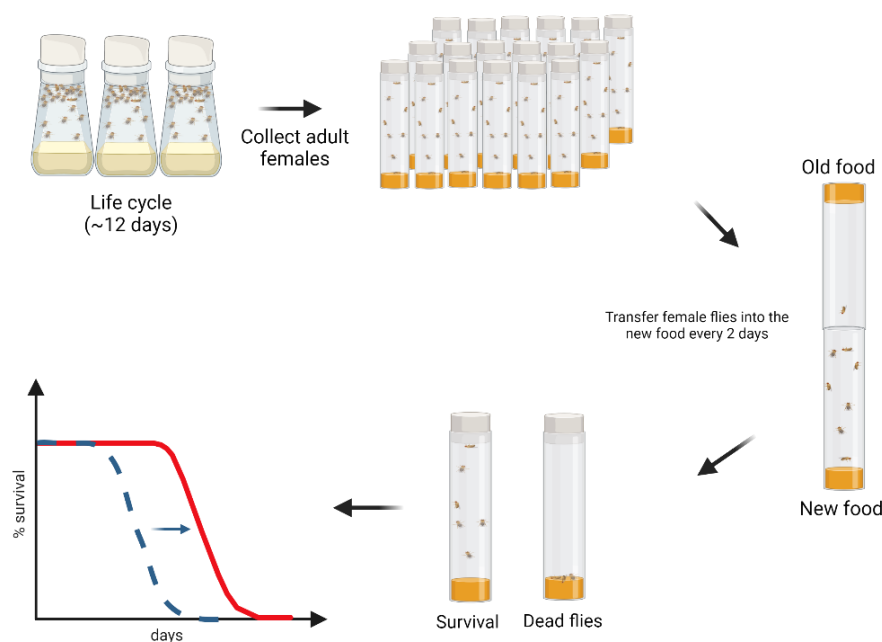


Figure 13. Schematic example of the lifespan assay with the protocol steps. Image created with Biorender.

8.1.1. Survival assays statistical analysis

The lifespan experience was analyzed using the Kaplan-Meier survival analysis method with GraphPad Prism (89). The days of each analysis were placed in the first column of the table, while the different genotypes were placed in the first row. The system will automatically calculate the difference between the entered dates and provide the elapsed days. For dead flies, the number “1” was placed in their respective genotype and experimental day, while for flies that were censored, it was entered the value “0”. On the day of the analysis, there may not have been any dead or censored flies, so it is not mandatory that all spaces in the table are filled. For the analysis, these values were discarded. The comparison of survival curves was done with Log-rank (Mantel-Cox) test and with Gehan-Breslow-Wilcoxon test in GraphPad Prism.

8.2. Climbing assay

For the climbing assay, the genotype of interest was collected by using the method described previously, in point 5. But, in this case, the females were initially selected and placed in new tubes. After that, flies were aged for 5 or 10 days prior to the experimental assay. In any case, during this period, it was also necessary to change the tubes so that the larvae did not interfere with the female’s viability. After those 5 or 10 days, the flies were anesthetized and those that appear to be in good condition for the experiment were collected. We waited at least 30 minutes for them to fully recover from the anesthesia and then followed on to the procedure. The tube with the flies was placed at the bottom left of the countercurrent apparatus which has 5 tubes in the top and 6 tubes in the bottom (**Figure 14**). The apparatus was tapped 3 times and after 15 seconds, the tubes were shifted, and the flies tapped again and allowed to climb-up into the second tube. This process was repeated 5 times. In the end, the number of flies in each of the bottom tubes was counted.



Figure 14. Climbing assay apparatus. (Source: https://www.youtube.com/watch?v=vmR6s_WAXgc)

8.2.1. Climbing assay statistical analysis

The six bottom tubes were grouped into three fitness groups, two tubes per fitness group. Flies that remained in the tube 1 and 2 belong to the fitness group I, flies in the tube 3 and 4 belong to the fitness group II, and the flies which reach the last 2 tubes belong to the fitness group III (**Figure 15**). Flies that managed to reach the last group are in a better fitness condition. The ratio of each fitness group was calculated with this formula: $\frac{N_{tube(i)} + N_{next(i+1)}}{N_{total}} \times 100$, where N is the number of flies in the tube, i is the number of the tube and N_{total} is the total number of analyzed flies of that genotype (≥ 100). This formula was applied for all fitness groups.

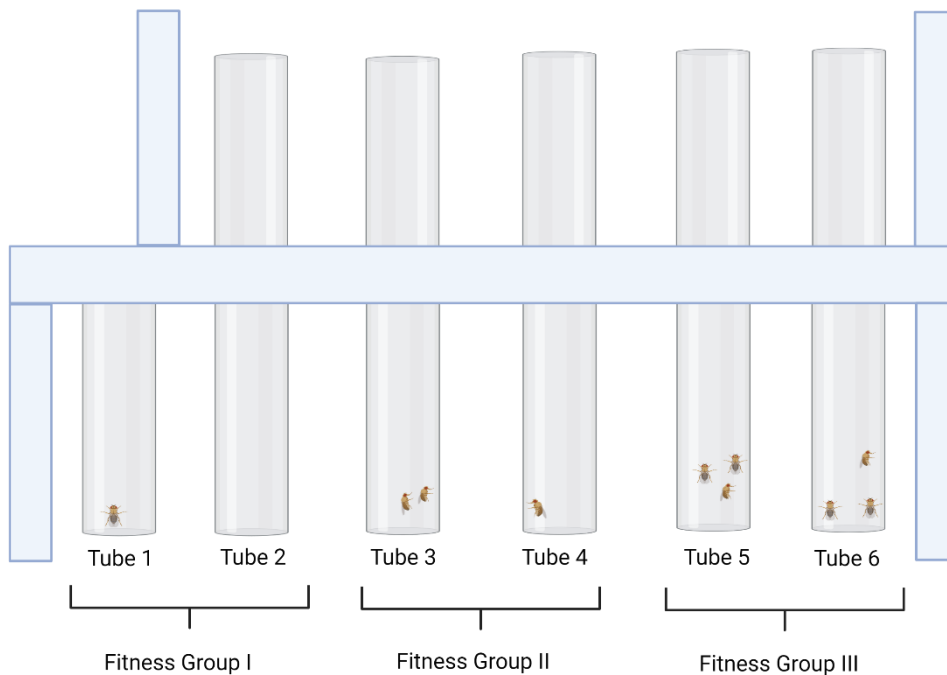


Figure 15. Representative image of a climbing assay apparatus and the division into fitness groups. Flies remaining on tube 1 and tube 2 belong to the fitness group I; flies on tubes 3 and 4 belong to fitness group II; and flies that reach tubes 5 and 6 belong to the fitness group III. Example of final counting: 1 fly in the fitness group I, 3 flies in the fitness group II and 6 flies in the fitness group III.

What is expected from a population of flies that is genetically homogeneous and that always has the same probability of going up the tube in all 5 attempts, is that its data distribution is binomial. Therefore, it is possible to also analyze this data with the use of the partition coefficient (Cf) to quantify the negative gravitation of flies. Cf is defined as the real probability that the flies climb the tubes and is given by the formula $Cf = \frac{(N_2 + 2N_3 + 3N_4 + 4N_5 + 5N_6)}{5(N_1 + N_2 + N_3 + N_4 + N_5 + N_6)}$ where N is the number of flies in the tube. The final values were analyzed using GraphPad Prism. Cf data was analyzed with Ordinary one-way ANOVA and with Mixed-effects analysis. Fitness groups data was analyzed with 2 way ANOVA (59,90).

9. Expression of NBs

The expression of four of the most promising nanobodies with Atx3 anti-aggregation properties (NB01, NB02, NB05, NB20) with a minor modification - addition of a cysteine on the C-terminal (NBcys) - was performed according to previously described protocol (48). First, *E. coli* WK6(su-) cells were transformed with the NBcys expressing plasmids (**Table 3**). Cells were premixed with 10 ng of each plasmid and incubated on ice for 30 min. After 45 sec of heat shock at 42 °C cells were incubated 2 min on ice and then recovered in TB medium for 1 h at 37 °C. Cells were plated on LB plates supplemented with 1 mM MgCl₂, 0.1 % glucose and 100 µg/mL ampicillin and incubated overnight at 37 °C.

For small-scale tests, one isolated colony was pre-inoculated on 50 mL of TB supplemented with 1 mM MgCl₂, 0.1 % glucose and 100 µg/mL ampicillin and grown overnight at 37 °C at 200 rpm in baffled flasks.

In the following day, the optical density at 600 nm (OD_{600nm}) of the cell culture was measured in a spectrophotometer (Shimadzu). Cell culture was diluted to an OD_{600nm} of 0.01 in 500 mL of TB medium supplemented with 1 mM MgCl₂, 0.1 % glucose and 100 µg/mL ampicillin. Cells were grown at 37 °C at 200 rpm until an OD_{600nm} of 0.8. Protein expression was induced by adding 1 mM IPTG at different temperatures (25 °C and 28 °C) for 16 h. After this time, the OD_{600nm} of cell culture was measured by spectrophotometry.

The protein expression was confirmed by SDS-PAGE analysis with lysates from equal number of cells collected before and after induction. For that, 1 mL of cell culture was collected by centrifugation before the induction and corresponding number of cells was collected after induction (normalized with OD_{600nm}). Cell pellets were resuspended in Urea Buffer for 5 min at RT and samples were boiled for 5 min with SB 5x. Lysates were loaded into 15 % SDS-PAGE gels and resolved by running at 25-30 mA/gel. The gel was stained with Safe Blue Coomassie (Bio-Rad) for visualization of the protein bands.

Alternatively, proteins were transferred to a nitrocellulose membrane presoaked in Trans-Blot Turbo Transfer Buffer supplemented with 20 % of Ethanol using Trans-blot turbo transfer system (Bio-Rad) at 1.3 A, 25 V for 7 min. The membrane was washed with TTBS, and then blocked with non-fat powdered milk (5 % in TTBS) for 1 h at RT. The membrane was incubated with antibody anti-VHH (1:2000) for 1 h and after three

washes with TTBS, the NBs corresponding bands were visualized by chemiluminescence with ECL™ Prime Western Blotting System RPN2232 (Cytiva) using ChemiDoc™ MP System with Image Lab™ Software.

10. Expression and purification of NB20cys in larger scale

Glycerol stocks of *E. coli* WK6(su-) cells transformed with each of the NBcys expressing plasmids were made and stored at -80 °C. For large scale purification of NB20cys, a few uL of each glycerol stock were inoculated in 50 mL TB medium supplemented with 1 mM MgCl₂, 0.1 % glucose and 100 µg/mL ampicillin at left shaking overnight at 200 rpm at 37 °C. The next day the pre-inoculum was diluted to an OD_{600nm} of 0.01 into twelve baffled flasks containing 500 mL of TB medium supplemented with 1 mM MgCl₂, 0.1 % glucose and 100 µg/mL ampicillin (total of 6 L cell culture). The cell culture was grown at 37 °C until an OD_{600nm} of 0.8 and induction of NB20cys expression was performed by adding 1 mM IPTG to cell culture. After 16 h at 28 °C, cells were pelletized by centrifugation at 4000 rpm at 4 °C for 25 min. The supernatant was discarded, and the pellet was resuspended in 70 mL of filtered lysis buffer (ice-cold TES buffer). Cell suspension was split into two tubes and left shaking gently for 1 h at 4 °C. The volume of each tube was split into half for two new tubes and equal volume of 4x diluted TES buffer was added to each tube and left shaking gently for 1 h at 4 °C. After this, supernatant was collected by centrifugation at 17000 rpm for 45 minutes at 4 °C and stored at -20 °C until required.

The supernatant was thawed under tap water, filtered through a PVDF 0,45 µm low binding filter and loaded into a pre-equilibrated Ni²⁺-charged HiTrap™ IMAC HP 5 mL Column (GE Healthcare). The column was washed with ice-cold HisTrap Buffer A and connected to the AKTA Prime Plus (GE Healthcare) chromatography system. Elution of NB20cys was induced with 50 % of elution buffer B corresponding to 150 mM imidazole. The fractions containing the NB20cys were pooled together and filtered with a 0.22 µm low protein binding PVDF filter (Millipore Corporation). Next, the protein sample was loaded to a size exclusion chromatography (SEC) column (HiPrep 26/60 Sephacryl S-100 High Resolution), eluted in gel filtration buffer and purified fractions were selected by following Abs_{280 nm}. The fractions containing purified NB20cys were pooled together and concentrated using an Amicon Ultra-15 concentrator with 10 kDa

cut-off. Concentrated protein was centrifuged for 10 min at 13000 rpm at 4 °C to remove any aggregates. Final concentration of purified NB20cys was determined by measuring the absorbance at 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and small aliquots were flash-frozen with liquid nitrogen and stored at -80 °C.

Cell pellets collected during expression were resuspended in Urea Buffer. Cell lysates and samples collected throughout the purification procedure were denaturated at 95 °C for 5 min with Sample loading buffer (SB 1X; 10mM DTT). To detect the successful expression and purification, samples were resolved in a 15 % acrylamide SDS-PAGE gel at 30 mV, and then stained with Blue Coomassie to detect the bands corresponding to NBs.

11. Mammalian Atx3 expression plasmids preparation

Two plasmids for expression of Atx3 variants in mammalian cells were designed and synthesized using Vector Builder (<https://en.vectorbuilder.com>):

- pRP-[TetOn]-TRE-EGFP-Atx3-13Q for expression of normal Ataxin-3 with 13Q (**Figure 16A**).
- pRP-[TetOn]-TRE-EGFP-Atx3-77Q for expression of Ataxin-3 with 77Q (**Figure 16B**).

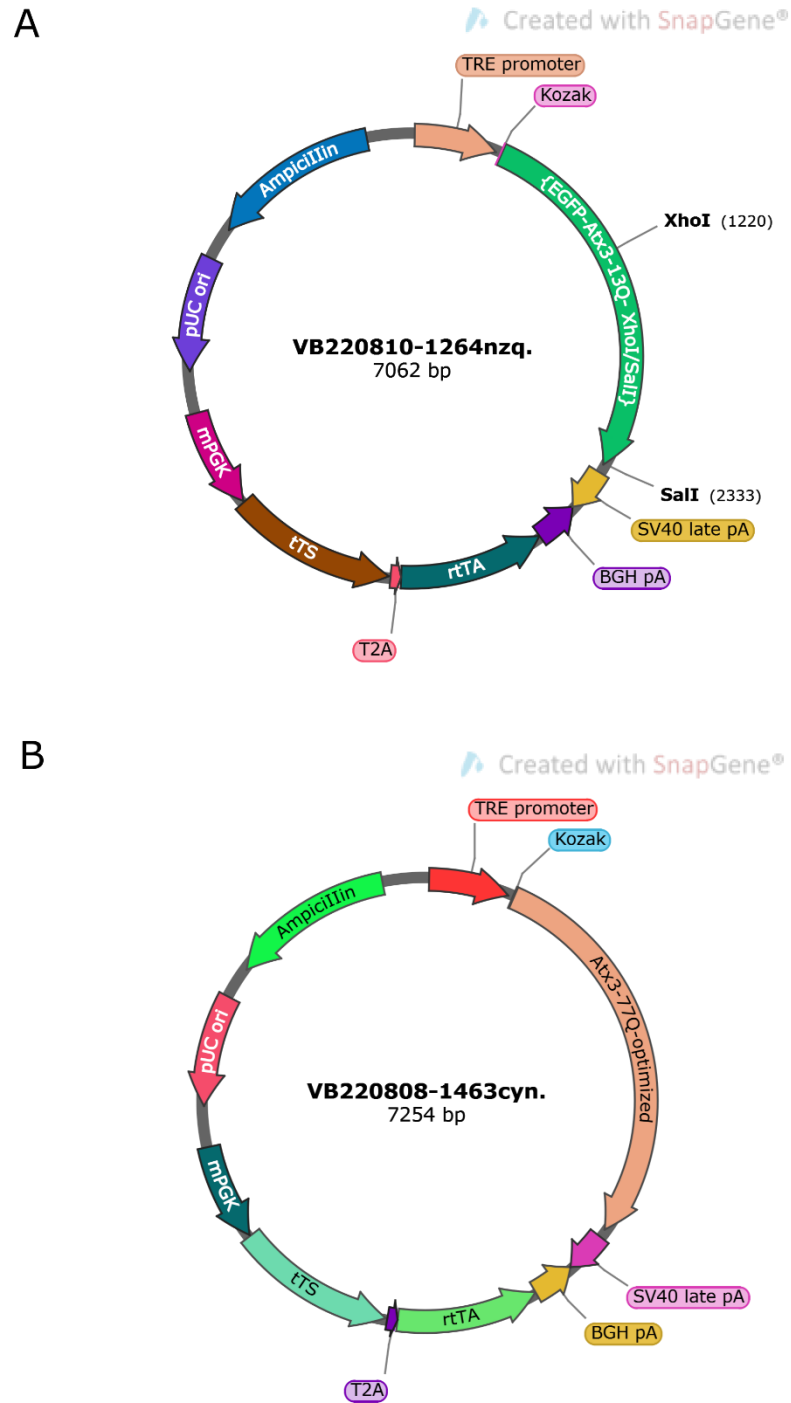


Figure 16. Schematic representation of plasmids designed for expression of Atx3 variants in mammalian cells, designed and synthesized using Vector Builder. A) Plasmid for Atx3-13Q expression. B) Plasmid for Atx3-77Q expression.

To obtain the control plasmid expressing only EGFP, 500 ng of the plasmid pRP-[TetOn]-TRE-EGFP-Atx3-13Q was double digested with two compatible sticky ends restriction enzymes XhoI and SalI at 37 °C for 2 h. The enzymes were inhibited at 65 °C for 20 min. After digestion, the plasmid was separated from the insert in 1% agarose gel and the band corresponding to the plasmid was purified with NZYtech GelPure following commercial protocol. Self-circularization of linear plasmid was performed with T4 DNA ligase (ThermoScientific) according to commercial standard protocol. *E. coli* DH5 α competent cells were transformed with 5 μ L of ligation reaction by heat shock. Briefly, cells and plasmid were incubated on ice for 30 min. After 45 sec of heat shock at 42 °C, cells were incubated 2 min on ice and then recovered in LB medium for 1 h at 37 °C. Cells were plated on LB plates containing 100 μ g/mL ampicillin and incubated overnight at 37 °C. Finally, plasmids were extracted from two positive colonies with NZYtech plasmid miniprep kit. Plasmids were confirmed by sequencing to ensure that sequence of pRP-[TetOn]-TRE-EGFP empty vector is correct.

Results and Discussion

Previous studies carried out by our research group led to the identification and selection of four NBs among sixty-three candidate NBs with Atx-3 anti-aggregation properties. The selection of these four NBs was based on previous results showing a high binding affinity of each NB to pathogenic full length Atx3 and a clear impact on Atx3 self-assembly *in vitro*. The work herein described aims to complement these studies and focuses on evaluating the potential neuroprotective effect of the four selected NBs in well-characterized *Drosophila* models of SCA3. Two SCA3 fly models were used based on expression of pathogenic human Atx3 variants (Atx3-80Q; Atx3-77Q) in the retina (using GMR-GAL4) or throughout the nervous system, using the pan-neural driver ELAV-GAL4.

1. Analysis of Atx3 expression in *Drosophila*

We started by characterizing the expression levels of the Atx3 variants under study to confirm the reproducibility of results previously described for these SCA3 models (75,76). For that, we obtained protein extracts from flies expressing UAS-Atx3 full-length variants (22Q, 77Q, and 80Q) under the control of GMR-GAL4 (**Figure 17**) or ELAV-GAL4 (**Figure 19**). Atx3-22Q corresponds to the non-pathogenic version, while Atx3-77Q and Atx3-80Q are associated with SCA3 phenotypes. Additionally, flies expressing an unrelated protein, myrRFP, were used as control. The protein extracts prepared from heads of flies of each genotype were analyzed through dot-blot, and western-blot. The presence of Atx3 SDS-resistant fibrils was analyzed through filter trap. This molecular characterization was performed to identify the presence of high molecular and insoluble aggregates of pathogenic Atx3 in fly models.

First, we analyzed the extracts from flies that express Atx3 variants (22Q, 77Q and 80Q) in the eye (under the control of GMR-GAL4) (**Figure 17A**) by dot-blot (**Figure 17B**). The total Atx3 protein levels were detected with Atx3 specific antibody 1H9. As expected, we do not detect antibody reactivity in the control (GMR>myrRFP). A comparison of signal intensity between the Atx3-22Q, Atx3-77Q and Atx3-80Q extracts revealed that Atx3 protein levels were reduced in Atx3-77Q expressing flies when compared to Atx3-22Q and Atx3-80Q. These analyses revealed that GMR>Atx3-Q80

flies have the highest levels of total Atx3. Because protein extracts are spotted on a membrane in native conditions, performing dot-blot with anti-Atx3 1H9 does not allow us to differentiate the type of HMW aggregates – oligomers or fibrils – neither to evaluate the levels of soluble and insoluble Atx3 species, we can only detect total protein levels.

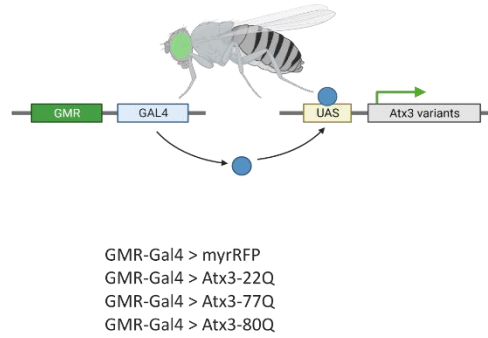
The protein extracts were also analyzed by western blotting. Western blot under denaturing conditions allows us to distinguish the presence of high-molecular-weight (HMW) and low-molecular-weight (LMW) Atx3 species with 1H9 antibody. As expected, in the control line (GMR>myrRFP) we do not detect Atx3. However, in all replicates, we were able to observe the presence of HMW species in extracts from flies expressing Atx3-80Q. Remarkably, we observed lower expression/levels of HMW species in Atx3-77Q expressing flies, and only one very well-marked band close to the 50kDa (LMW) corresponding to soluble Atx3 in Atx3-22Q expressing flies (**Figure 17C** and supplementary **Figure 30C**). This data is in accordance with results described previously, showing that expression of Atx3-77Q or Atx3-80Q results in different protein levels in flies (76); the expression of Atx3-80Q is “stronger” while the expression of Atx3-77Q is “weaker”. According to the authors this difference is due to the inclusion of an improved Kozak sequence in Atx3-Q80 sequence that leads to expression of higher ataxin-3 protein levels and consequently to the presence of higher levels of HMW species. We hypothesized that Atx3-77Q might not be fully translated due to the intracellular depletion of glutaminyI-tRNA^{GUC} in *Drosophila* cells which is supported by the absence of a visible band corresponding to the soluble form of Atx3 (**Figure 17C**). The codon optimization of Atx3-80Q sequence in the genotype used in this study, by interspersing the codons CAG and CAA, could explain the noticeable presence of higher levels of soluble Atx3-80Q and also the presence of HMW aggregates when compared to Atx3-77Q.

Moreover, the strong band corresponding to soluble Atx3 in GMR>Atx3-22Q samples (**Figure 17C**) might justify the presence of the strong bands on the dot-blot (**Figure 17B**). We also analyzed the pellet of these samples, and we did not detect significant levels of Atx3 insoluble species, confirming that the protein was mostly in the supernatant soluble fraction. With this result, we decided to only use the supernatant fraction of the samples in the following experiments.

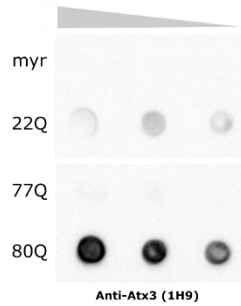
We also performed filter trap assay to quantify the presence of Atx3-SDS-resistant species. In the control (GMR>myrRFP), we did not identify the presence of SDS-resistant fibrils. We observed an increase in the intensity of the bands associated with an increase in the number of glutamines, from Atx3-22Q to Atx3-80Q samples

(**Figure 17D**). In agreement with what we observed previously by WB, the protein samples from flies expressing Atx3-80Q showed higher levels of SDS-resistant species. Although the differences between the control (GMR>myrRFP), Atx3-22Q and Atx3-77Q samples are not statistically significant, the Atx3-80Q sample had significantly higher levels of SDS-insoluble aggregates when compared to GMR>myrRFP control sample (**Figure 17E**).

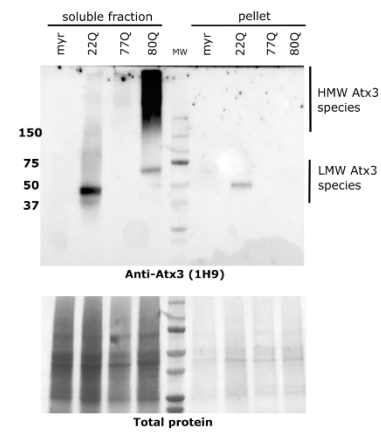
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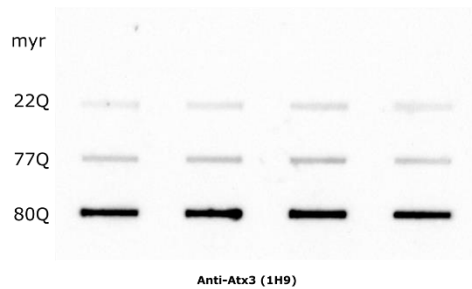
B



C



D



E

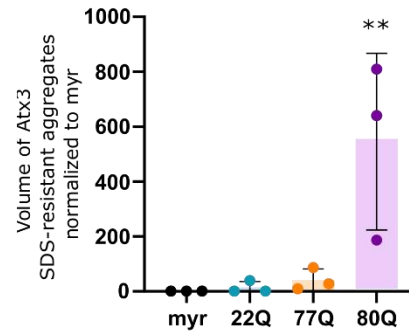


Figure 17. Analysis of the levels of Atx3 variants under the control of GMR-GAL4. A) Representative image of the GMR-GAL4 > UAS gene expression system, with the four genotypes used (GMR-GAL4 > myrRFP; GMR-GAL4 > Atx3-22Q; GMR-GAL4 > Atx3-77Q; GMR-GAL4 > Atx3-80Q). B) Analysis of Atx3 total levels in protein extracts (with decreasing dilutions) from adult head flies detected with anti-Atx3 (1H9) antibody by dot-blot. One representative blot is shown (n=2). C) Representative image of western blot analysis of Atx-3 species with anti-Atx3 (1H9). The loading control was evaluated with Ponceau S staining after transfer and before the blocking step (n=3). D) Analysis of Atx3 SDS-resistant aggregates in protein extracts from adult head flies detected with anti-Atx3 (1H9) by filter trap (the four bands correspond to four replicates of the same protein extract) (n=3). E) Quantification by densitometry of the filter trap bands. Mean values of the densitometry of the four bands of each genotype from three replicates were normalized to the line of control sample (GMR > myrRFP), **p-value < 0.01 (Ordinary one way ANOVA).

Aiming to specifically differentiate the presence of Atx3 amyloid fibrils and/or oligomers, we conducted dot-blots using specific antibodies for amyloid fibrils (anti-OC) and amyloid oligomers (anti-A11). However, we detected signal in extracts from control samples (GMR-GAL4>myrRFP) with both antibodies (**Figure 18**), which might indicate the presence of other protein aggregates in *Drosophila* head extracts. Therefore, we conclude that Anti-OC and Anti-A11 are not ideal for the specific detection of Atx3 amyloid fibrils and oligomers, respectively in SCA3 fly models. Consequently, we did not follow-up the analysis of Atx3 oligomers or fibrils in this study using this approach.

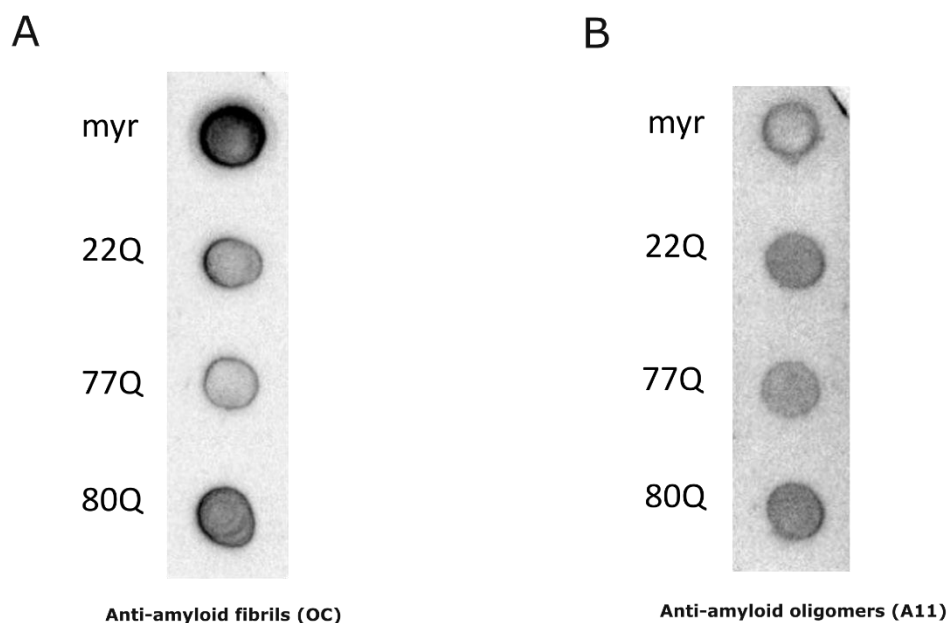


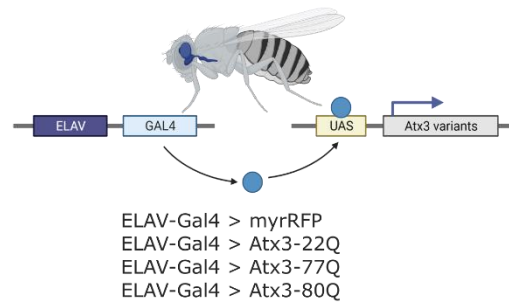
Figure 18. Detection of Atx3-amyloid fibrils (A) and oligomers (B) in *Drosophila* heads extracts of GMR-GAL4 Atx3-variants expressing flies using dot-blot. Membranes were incubated with Anti-OC and Anti-A11, respectively (n=1).

Atx3 is ubiquitously expressed in human cells and has a particularly important role on the nervous system. Accordingly, we expressed UAS-Atx3 variants (22Q, 77Q and 80Q) and UAS-myrRFP (control) in the nervous system, under the control of the pan-neuronal driver ELAV-GAL4 (**Figure 19A**). We started by performing dot-blot analysis of serial dilutions of protein extracts from adult fly heads and we observed higher levels of total Atx3 protein in ELAV>Atx3-80Q extracts when compared to ELAV>Atx3-77Q, and ELAV>Atx3-22Q (**Figure 19B**). As expected, Atx3 protein was not detected on control flies expressing UAS-myrRFP.

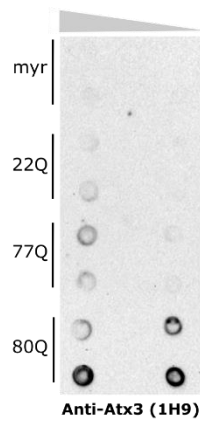
Using western blot, we observed the presence of HMW (SDS-resistant) species in the Atx3-80Q protein samples, lower levels of HMW Atx3-77Q species and the absence of HMW species in Atx3-22Q expressing flies. However, we observed again the presence of an intense band close to 50 kDa corresponding to soluble Atx3-22Q. In the control (ELAV>myrRFP) Atx3 expression was not detected, as expected (**Figure 19C** and supplementary **Figure 31C**). These results are in agreement with the previous results obtained with samples from Atx3 variants expressed in fly retina using GMR-GAL4 (**Figure 17C**).

We also performed filter trap assay to detect Atx3 SDS-resistant aggregates in pan-neuronal tissues and we observed a higher signal for SDS-insoluble aggregates in Atx3-80Q expressing flies when compared to control (**Figure 19D**) which is statistically significant (**Figure 19E**).

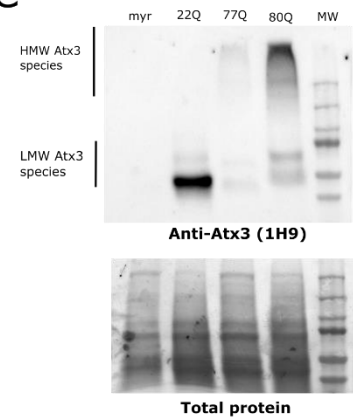
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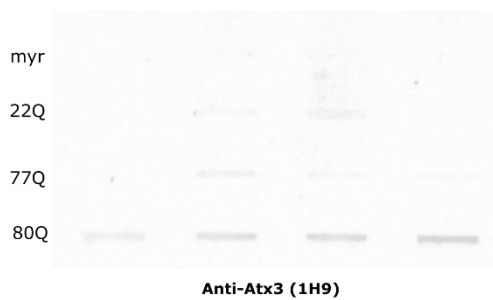
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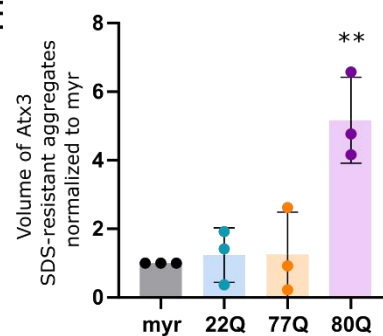


Figure 19. Analysis of the expression of Atx3 variants under the control of ELAV-GAL4. A) Representative image of the ELAV-GAL4>UAS gene expression system, with the four genotypes used (ELAV-GAL4>myrRFP; ELAV-GAL4>Atx3-22Q; ELAV-GAL4>Atx3-77Q; ELAV-GAL4>Atx3-80Q). B) Analysis of Atx3 total levels in protein extracts (with two replicates of each dilution) from adult head flies detected with primary antibody anti-1H9 by dot-blot. C) Western blot for detection of Atx-3 species with primary antibody anti-1H9. For loading control, membranes were reversibly stained with Ponceau S after transfer and before blocking step (n=3). D) Analysis of SDS-resistant Atx3 fibrils in protein extracts from adult head flies by filter trap (the four bands correspond to four replicates of the same protein extract) (n=3). E) Quantification by densitometry of the filter trap bands. Mean values of the densitometry of the four bands of each genotype from three replicates were normalized to the line of control sample (ELAV-GAL4>myrRFP), ***p*-value<0.001 (Ordinary one way ANOVA).

When comparing the levels of pathogenic and non-pathogenic variants of Atx3 in the fly eye (GMR expression driver) or the nervous system (ELAV expression driver) we observed higher levels of SDS-resistant Atx3 aggregates on the eye (e.g., GMR-GAL4>Atx3-80Q flies had ~600 arbitrary units detected in the filter trap assay – **Figure 17E** - while ELAV-GAL4>Atx3-80Q had ~6 arbitrary units in the same assay – **Figure 19E**). Moreover, we observed that Atx3-80 has higher levels of Atx3 HMW and soluble species when compared to Atx3-77Q, as described previously. This strongly suggests that uninterrupted CAG codon repetition in Atx3-77Q can result in lower protein expression. This problem might not occur in Atx3-80Q because of codon optimization and usage of alternate CAG and CAA codons, as already described previously in this section.

1.1. Impact of Atx3 expression on flies' lifespan and locomotor function

Next, we characterized how the expression of Atx3 with different polyQ lengths impact on motor coordination and flies' survival, by expressing Atx3 variants (22Q, 77Q and 80Q) under the control of the pan-neuronal driver ELAV-GAL4. This system allows expression of non-pathogenic and pathogenic variants of Atx3 throughout all nervous system, making it possible to assess the impact of polyQ expansion in flies at a functional level. With that end, survival (lifespan) and climbing assays were performed.

We analyzed the survival rate of flies with the different genotypes over time by transferring flies into new tubes containing fresh food every two days and later, every three days, during the experiment. The dead, the carried, and the censored flies were noted during each vial transfer, and then analyzed. The survival curves showed that flies expressing Atx3-22Q tend to die a few days later when compared to control ELAV>myrRFP expressing flies, although not statistically significant. The lack of statistical significance might be justified because this analysis gives more importance to deaths at early time points than deaths at later time points.

When comparing the mean survival of flies expressing ELAV>myrRFP (35 days) and flies expressing ELAV>Atx3-77Q (28 days) we observed a shorter lifespan for Atx3-77Q expressing flies; these die earlier than control flies and than flies expressing the non-

pathogenic form of Atx3. Finally, in flies expressing Atx3-80Q we detected a very sharp decrease in survival in the initial days of the experiment and a median survival of 13 days. These results indicate that expression of Atx3 with expanded polyQ tail results in shortened lifespan of *Drosophila* SCA3 models, especially above the threshold of 77 glutamines (**Figure 20A**).

Next, we aimed to evaluate if the lower survival rates of flies expressing pathogenic variants of Atx3 were related with the appearance of motor coordination impairment at earlier stages. We measured locomotor ability at days 5 and 10 in Atx3 expressing flies using the climbing assay. We used this assay to collect data about the partition coefficient (Cf) which is defined as the real probability that a fly has to climb the tubes (**Figure 20B**) and the fitness profile of each genotype according to the final distribution of the flies in the countercurrent apparatus.

When comparing the Cf data over time we observed that Atx3-22Q expressing flies showed a tendency for reduction of the motor capacity (~10%) between day 5 and day 10 (**Figure 20B**). The same reduction was observed for Atx3-80Q expressing flies when comparing day 5 and day 10, although not statistically significant. However, flies expressing Atx3-77Q showed a statistically significant increase in climbing rate from 5 to 10 days.

Then we analyzed the Cf within each time point (e.g., 5 days) and we compared each Atx3-variant with the control (ELAV-GAL4>myrRFP). The analysis of CF at day 5 reveals a significant difference between flies expressing the non-pathogenic Atx3 variant and the control ELAV>myrRFP flies. This was also observed for Atx3-80Q expressing flies when compared to the control. Both Atx3-22Q and Atx3-80Q expressing flies had significantly less probability of climbing the tube than control ELAV>myrRFP expressing flies. Moreover, the climbing ability of Atx3-80Q expressing flies was significantly reduced relative to ELAV>Atx3-22Q expressing flies. No significant differences were observed between Atx3-77Q expressing flies and the control (ELAV>myrRFP). We observed similar results when analyzing the Cf of 10-day-old flies, with a significant reduction of Cf for Atx3-22Q and Atx3-80Q expressing flies (**Figure 20B**). These results showed that the reduction of Cf for flies expressing Atx3-80Q in comparison with control flies is more accentuated over time.

We also evaluated the fitness profile of each genotype at day 5 and day 10. After the final counting of flies in each tube, the flies that remained in tubes 1 and 2 (the fitness group I) were considered to have the worst climbing ability. The flies included in the

fitness group II (tubes 3 and 4) had a relative better motor ability than those in the first tubes, and the flies included in tubes 5 and 6 - fitness group III - were those that had better performance in the climbing assay. When analyzing the fitness groups at day 5, we observed that control, ELAV>myrRFP expressing flies, are the ones that show less retention in fitness group I (tubes 1 and 2) and have higher percentage of flies reaching the last 2 tubes (5 and 6), fitness group III. As for Atx3 expressing flies, we observed a significant increase in the percentage of flies in fitness group I, for Atx3-22Q and Atx3-80Q expressing flies when compared to the control (**Figure 20C**).

The percentage of Atx3-77Q expressing flies retained in group I is also increased, relative to control (ELAV>myrRFP), however this difference is not statistically significant. We also did not detect significant differences when comparing the Atx3 expressing flies with the control (ELAV>myrRFP) regarding behavior in fitness groups II and III. However, from this type of analysis, we can observe that, on average, the percentage of ELAV>Atx3-80Q flies in fitness group I is higher when compared to all other genotypes. In agreement, the percentage of ELAV>Atx3-Q80 flies reaching the last tubes is the most reduced, when comparing all genotypes. These results suggest that expression of Atx3-80Q significantly reduces locomotor ability at early stages of adult life in *Drosophila* SCA3 model.

When analyzing fitness groups in 10-day old flies, we observed that the control flies had the best fitness, since most of the flies reached the last two tubes and were grouped in the fitness group III (**Figure 20D**). As expected from the 5-day analysis, flies expressing Atx3-80Q showed the worst fitness behavior, as more flies stayed in the first two tubes (fitness group I) resulting in less flies in the fitness group III. Similar to the 5-day analysis, we also observed a significant increase in the percentage of flies expressing Atx3-22Q retained in fitness group I when compared to control. The number of flies expressing Atx3-22Q and Atx3-80Q and retained in fitness group I was significantly higher compared to the 5-day analysis, revealing that there is a deterioration of locomotor function over time, as suggested by the CF analysis.

At day-10, we observed a significant reduction in the percentage of Atx3-80Q expressing flies in fitness group II, relative to control flies (ELAV>myrRFP). Importantly at this time point, all Atx3 expressing flies (Atx3-22Q, Atx3-77Q and Atx3-80Q) showed a significant reduction in locomotor function as indicated by the reduced number of flies reaching the last tubes.

Therefore, although Atx3-80Q has a higher impact on flies' locomotor fitness, expression of Atx3-77Q also induces a significant reduction in climbing ability by day-10. These results showed that expression of pathogenic and non-pathogenic Atx3 in the nervous system of flies' result in a locomotor deficit over the time (between days 5 and 10). Because we also observe a reduction of the fitness for flies expressing Atx3-22Q, we suggest that locomotor impairment is not only mediated by polyQ-dependent aggregation of Atx3.

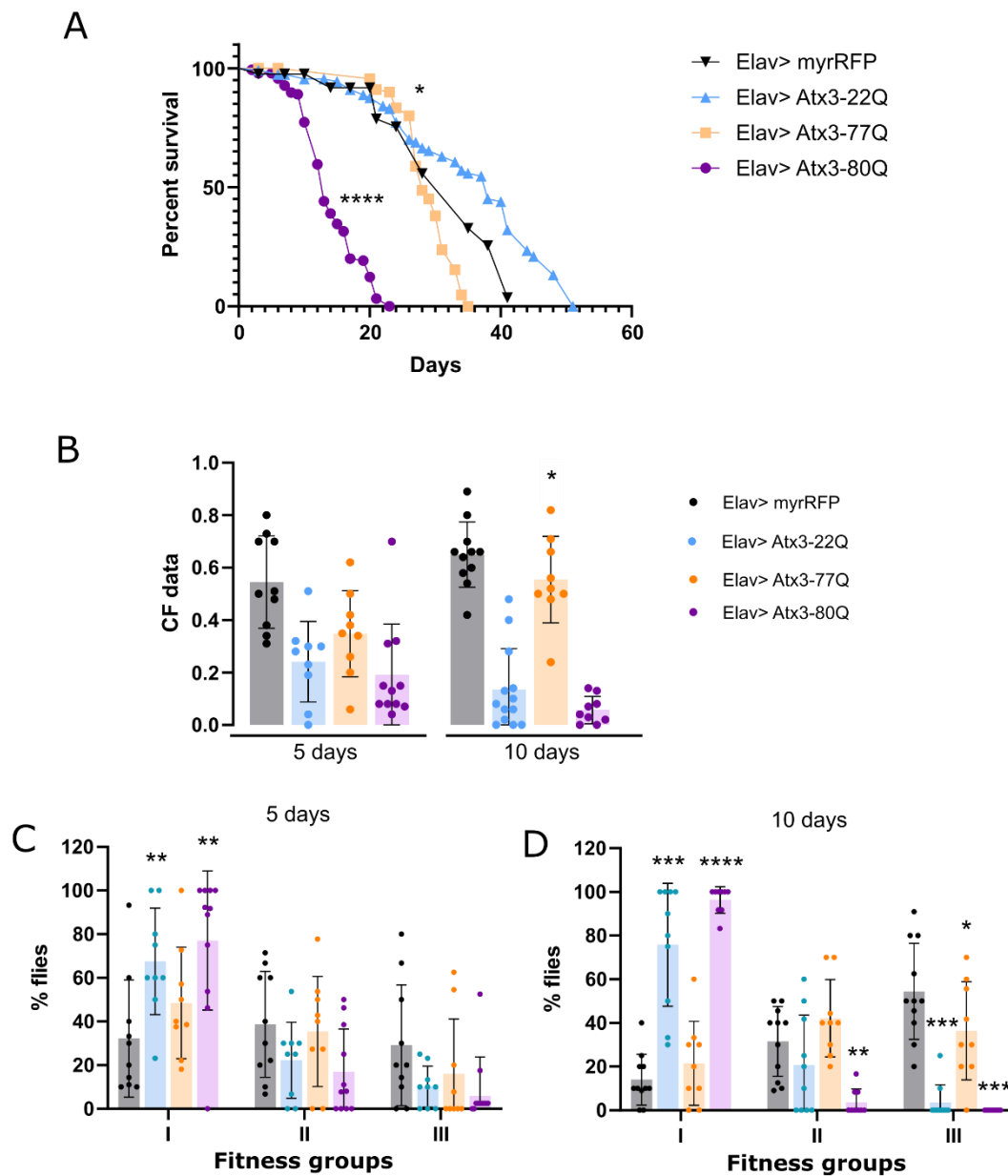


Figure 20. Functional analysis of flies expressing Atx3 variants under the control of ELAV-GAL4. A) Survival (lifespan) curve using the Kaplan-Meier survival method analyzed with Gehan-Breslow-Wilcoxon test comparing all variants with ELAV>myrRFP, *p-value<0.05 and ****p-value<0.0001 B) Climbing assay: Cf

data ($Cf = (N2 + 2N3 + 3N4 + 4N5 + 5N6)/5$ ($N1 + N2 + N3 + N4 + N5 + N6$), N is the number of flies in the tube) analysis of 5 and 10-days old flies. The comparison analysis was made for each genotype between 5 and 10 days and was evaluated with Mixed-effects analysis, *p-value<0.05. C) Climbing assay: Fitness groups data analysis of 5-days-old flies. Data were evaluated inside each fitness group and compared with the ELAV>myrRFP (control), *p-value<0.05 (Two-way ANOVA) D) Climbing assay: Fitness groups data analysis of 10-days-old flies. Data were evaluated inside each fitness group and compared with the ELAV>myrRFP (control), *p-value<0.05, **p-value<0.01, ***p-value<0.001 and **** p-value<0.0001 (Two-way ANOVA). Note: at least 100 flies of each genotype were analyzed for each of both functional assays (survival and climbing)

The functional analysis of flies expressing the different UAS-Atx3 full-length variants under ELAV-GAL4 led us to conclude that the Atx3-80Q pathogenic variant reduces the survival rate and the locomotor function in flies. These results can be associated with an increase of Atx3 HMW and SDS-resistant aggregates in the nervous system, as observed with previously described biomolecular analysis.

With these results, we decided to evaluate the impact of selected NBs on the functional behavior of Atx3-80Q expressing flies, as they present the worst survival and locomotor phenotype. Moreover, as the Atx3-77Q expressing flies also demonstrated reduced lifespan and climbing fitness at day 10, we also evaluate the neuroprotective effect of NBs in ELAV-GAL4>Atx3-77Q expressing flies.

2. Analysis of the impact of NBs expression on Atx3-80Q aggregation

As described in the previous section, we decided to proceed with the molecular analysis to evaluate the effect of NBs on the expression and aggregation of Atx3-80Q. We made this analysis with GMR-GAL4>Atx3-80Q flies because they had significantly more insoluble aggregates when compared to the other pathogenic variant (Atx3-77Q). Therefore, we analyzed Atx3 total protein levels in flies co-expressing UAS-Atx3-80Q and UAS-NBs (NB20, NB02, NB01 or NB05) under the control of GMR-GAL4 (**Figure 21A**).

Initially, we performed dot-blot analysis of native protein extracts from fly heads and Atx-3 total protein levels were detected with Atx3 specific antibody 1H9. As control we co-expressed Atx3-80Q with myrRFP under the control of GMR-GAL4 (GMR>myrRFP; Atx3-80Q). We also used extracts from GMR>OrR; Atx3-80Q control flies in this experiment, which only express one UAS-transgene (Atx3-80Q) under GMR-GAL4. After a critical analysis, we concluded that wild-type OrR is not the most suitable

control for analysis of molecular differences. The availability of the GAL4 protein at the UAS sequence can alter depending on the quantity of UAS transgenes present in the genetic background. All the flies that we were using in this study had two UAS, while the GMR>OrR; Atx3-80Q control only has one. The results from dot-blot showed differences in the total amount of Atx3-80Q species when co-expressed with different NBs (**Figure 21B**). However, the replicates showed high variability, making it difficult to conclude which of the NBs promotes reduction of Atx3 protein levels. Despite this high variability, we observed that expression of NB05 seems to increase Atx3 total protein levels.

The results obtained by dot-blot analysis were not informative, in addition to obtaining variable results between dot blot replicates, they do not allow a distinction between HMW and LMW Atx3 species. For this reason, we decided to evaluate the protein profile by WB, as we can detect the total protein levels and also the differences between HMW and LMW Atx3 species.

Protein extracts from flies co-expressing Atx3-80Q and each of the NBs were resolved by SDS-PAGE and Atx3 species was evaluated using Atx3-specific antibody 1H9 allowing the visualization of HMW and soluble Atx3 species within each genotype. The results showed a high variability of the protein profile among different replicates for the same genotype (**Figure 21C** and supplementary **Figure 32C**). While in some protein extracts replicates certain nanobodies (e.g., NB20 and NB01) seem to modulate the levels of Atx3 and reduce the amount of insoluble aggregates, in others the expression of the same nanobody does not seem to have the same effect. However, to normalize and quantify the data, despite being arbitrary values, a ratio was made between the volume of the band corresponding to soluble Atx3 (between 50 kDa - 75 kDa) over the total Atx3 arbitrary units (total volume of Atx3). The values from all replicates were averaged and normalized to control (GMR>myrRFP; Atx3-80Q). Ratios greater than 1 would indicate that there is a tendency for the nanobody to reduce the amount of Atx3 insoluble aggregates. We were able to identify ratios greater than 1 in the NB01 and NB05, however, without statistical significance (**Figure 21D**).

Additionally, we also performed filter trap assays as an attempt to evaluate the presence of SDS-resistant aggregates, but the same variability was observed between replicates (**Figure 21E** and supplementary **Figure 32D**) resulting in no significant differences between genotypes (**Figure 21F**).

Overall, using this analysis and although we observe changes in expression levels, we cannot conclude on the impact of the selected NBs on the levels of Atx3-80Q

by dot-blot, WB or FT. These might result from the fact that extracts used for each technique corresponded to approximately three heads per lane and dot. In future experiments, we will prepare the protein extracts from a higher number of fly head (to correspond, for example, to approximately ten heads per lane and dot).

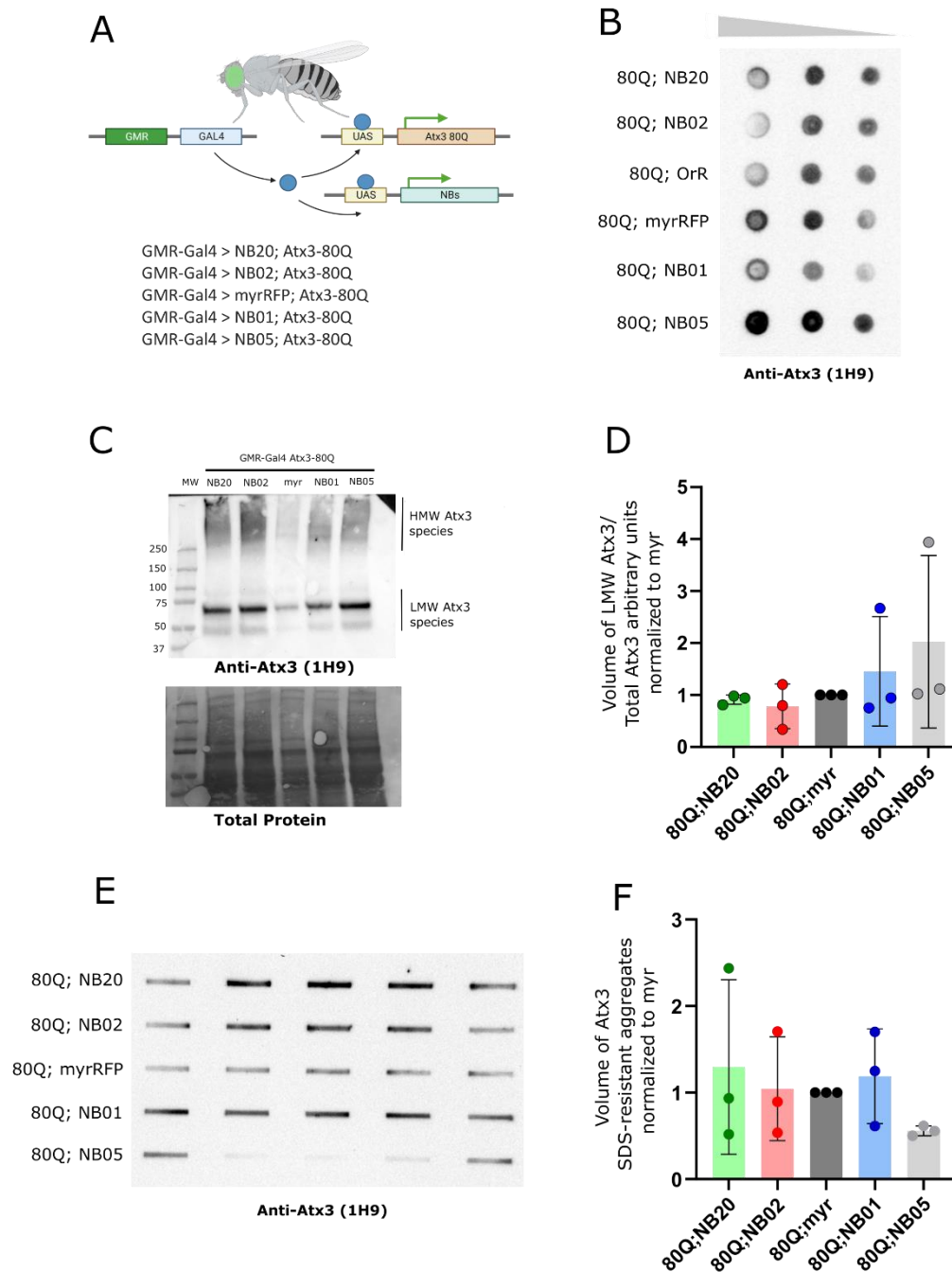


Figure 21. Molecular analysis of NBs impact in Atx3-80Q protein levels soluble and aggregates in *Drosophila* eye. A) Representative image of the GMR-GAL4>UAS gene expression system, with the five genotypes

used GMR-GAL4>NB20; GMR-GAL4>NB02; GMR-GAL4>myrRFP; GMR-GAL4>NB01; GMR-GAL4>NB05, all co-expressed with Atx3-80Q. B) Analysis of Atx3 total levels in protein extracts (with decreasing dilutions) from fly heads by dot-blot with primary antibody anti-1H9 (n=2). C) Western blot for detection of Atx3 species with primary antibody anti-1H9. The loading control was evaluated with Ponceau S staining after transfer and before blocking step (n=3). D) Quantification of the densitometry of WB lanes from three replicates (Volume of LMW Atx3/Total Atx3 arbitrary unites normalized to GMR>myrRFP), non-significant results (Ordinary one way ANOVA). E) Analysis of Atx3 SDS-resistant aggregates of protein extracts from adult head flies by filter trap (the five bands correspond to five replicates of the same protein extract) (n=3). F) Quantification by densitometry of the filter trap bands. Mean values of the densitometry of the five bands of each genotype from three replicates were normalized to the line of control sample (GMR>myrRFP; Atx3-80Q), non-significant results (Ordinary one way ANOVA).

3. Analysis of the impact of NBs in Atx3-80Q pan-neuronal expression and aggregation

Although we were not able to obtain data about the effect of NBs in the protein expression of GMR-GAL4>Atx3-80Q lines, we decided to further evaluate the effect of NBs on the levels of pathogenic Atx3 protein expression in the nervous system, using the driver ELAV-GAL4.

We started by evaluating the protein profile in flies co-expressing UAS-Atx3-80Q and UAS-NBs (NB20, NB02, NB01 or NB05) under the control of ELAV-GAL4. From our previous survival analysis (**Figure 20A**) we observed that ELAV>myrRFP control flies showed reduced survival. Accordingly, we decided to use UAS-lacZ as a new control, for both molecular and functional assays (**Figure 22A**).

Firstly, the results obtained with WB analysis showed that replicates were again very variable. In fact, it was not possible to identify Atx3-80Q insoluble and soluble species in all of the experimental replicates, so we did not performed the quantification using the ratio of the total volume of each lane over the volume of the band corresponding to soluble Atx3 (**Figure 22B** and supplemental **Figure 33B**). Still, from WB it is possible to identify a tendency for NB20 and NB01 to reduce the amount of SDS-soluble and HMW Atx3 species while co-expression of NB05 seems to increase them.

The FT analysis also revealed variability between replicates (**Figure 22C** and supplemental **Figure 33C**). Unexpectedly, the results showed a tendency for all NBs to increase the formation of Atx3-SDS-resistant aggregates when compared to the control (ELAV>lacZ; Atx3-80Q) (**Figure 22C** and **Figure 22D**).

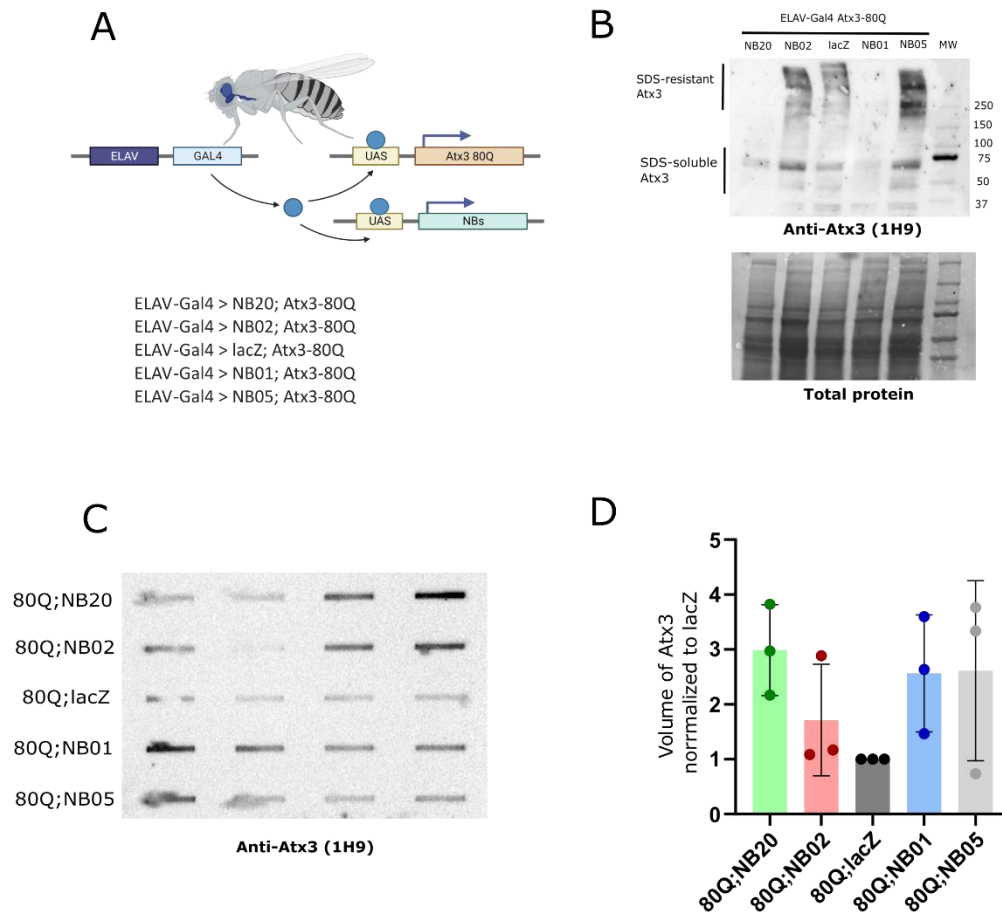


Figure 22. Molecular analysis of NBs impact in the protein levels of soluble and aggregated of Atx3-80Q species in *Drosophila* nervous system. A) Representative image of the ELAV-GAL4>UAS gene expression system, with the five genotypes used ELAV-GAL4>NB20; ELAV-GAL4>NB02; ELAV-GAL4>lacZ; ELAV-GAL4>NB01; ELAV-GAL4>NB05, all co-expressed with Atx3-80Q. B) Western blot for detection of Atx-3 species with primary antibody anti-1H9. The loading control was evaluated with Ponceau S staining after transfer and before blocking step (n=3). C) Analysis of SDS-resistant Atx3 aggregates of protein extracts from adult head flies by filter trap (the four bands correspond to four replicates of the same protein extract) (n=3). D) Quantification by densitometry of the filter trap bands. Mean values of the densitometry of the four bands of each genotype from three replicates were normalized to the line of control sample (ELAV>lacZ; Atx3-80Q), non-significant results (Ordinary one way ANOVA).

As we previously observed, the expression of Atx3-77Q in the *Drosophila* nervous system leads to a mild impairment of motor coordination and a slightly reduced lifespan (Figure 20A and Figure 20B). For this reason, we also evaluated the effect of NBs on Atx3 protein levels in crossing flies co-expressing ELAV-GAL4 with UAS-Atx3-77Q and with UAS-NBs (NB20, NB02, NB01 or NB05), UAS-lacZ was again used as control (Figure 23A).

Similar to what was observed upon the expression of NBs and Atx3-80Q when driven by GMR-GAL4 and ELAV-GAL4, here, we also observed high variability on the protein profile between WB replicates (Figure 23B and supplemental Figure 34B). In

this case, we were not able to identify the SDS-soluble bands corresponding to Atx3 in none of the replicates (**Figure 34B**). However, we can observe a tendency for NB05 co-expression to increase the levels of HMW species when compared to the other NBs.

The analysis of FT to evaluate the levels of SDS-resistant fibrils also revealed high variability between replicates (**Figure 23C** and supplemental **Figure 34C**). However, we also observed a tendency for each NB to increase the volume of Atx3-SDS-resistant species when compared to the control (ELAV>lacZ; Atx3-77Q). Again, the co-expression of NB05 showed the highest tendency to increase the levels of Atx3 insoluble species (**Figure 23C** and **Figure 23D**).

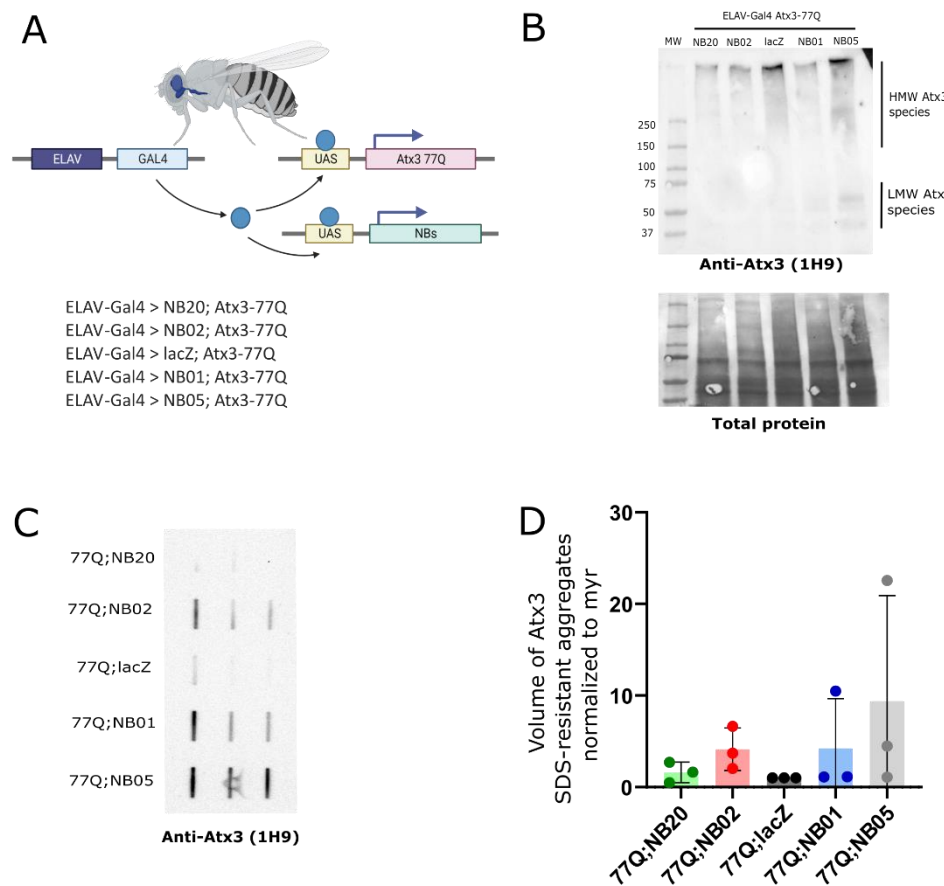


Figure 23. Molecular analysis of NBs impact in the protein levels of soluble and aggregates of Atx3-77Q in *Drosophila* nervous system. A) Representative image of the ELAV-GAL4>UAS gene expression system, with the five genotypes used ELAV-GAL4>NB20; ELAV-GAL4>NB02; ELAV-GAL4>lacZ; ELAV-GAL4>NB01; ELAV-GAL4>NB05, all co-expressed with Atx3-77Q. B) Western blot for detection of Atx3 species with primary antibody anti-1H9. The loading control was evaluated with Ponceau S staining after transfer and before blocking step (n=3). C) Analysis of SDS-resistant Atx3 aggregates of protein extracts from adult head flies by filter trap (the three bands correspond to three replicates of the same protein extract) (n=3). D) Quantification by densitometry of the filter trap bands. Mean values of the densitometry of the three bands of each genotype from three replicates were normalized to the line of control sample (ELAV>lacZ; Atx3-77Q), non-significant results (Ordinary one way ANOVA).

From the results collected, we were not able to conclude the impact of NBs co-expression in *Drosophila* SCA3 models. Several factors could explain our results. We hypothesized that the weaker expression of Atx3-77Q could contribute to the difficulty in detecting both soluble and HMW species by WB and FT analysis. Due to having such low expression it is difficult to observe differences between the effect of different NBs in Atx3-77Q. On the other hand, we detect a strong signal of HMW species of Atx3-80Q when expressed on the nervous system and more specifically on the eye. This suggest that Atx3-80Q might be overexpressed and with high propensity to aggregate. Because we are using a fly model with codon optimization in the polyQ encoding region integrated in the genome, this might lead to higher levels of protein with high aggregation propensity while the co-expressing levels of NB might be too low to have impact on the levels of Atx3-80Q species. Additionally, to analyze the effect of NBs on Atx3 pathogenic lines, flies with two UASs were used (one for NBs and another for Atx3), so, the activating capacity of GAL4 will have to be divided by these two UASs. This could mean a lower expression of both NBs and Atx3, thus making the evaluation more difficult.

To overcome these issues, in future work we will increase the number of replicates of each technique to obtain more robust data, and probably increase the number of heads used to prepare protein extracts for each technique, as previously discussed. We are currently using the corresponding amount of 3.3 heads per well for both WB and FT to avoid waste of living material while we were optimizing the protocol and the number of replicates. Also, *Drosophila* genetic crosses and maintenance is time consuming, especially for functional analysis of locomotion and survival which results in the death of a high number of flies. However, we are aware that further optimization of the WB protocol will involve an increase in the number of heads used for protein extract preparation. To counteract the lower expression of NBs, we could increase the number of UAS-NBs copies in the genetic background, or to combine the action of two NBs. In fact, this is supported by previous *in vitro* studies by our lab group showing that higher ratio of NB:Atx3 is relevant for Atx3 aggregation pathway modulation (**Figure 9**).

Despite this, it was possible through biochemical analysis to verify a tendency of NB05 to increase the amount of insoluble aggregates. That way, we will probably focus more on the other three NBs on future experiments.

3.1. Analysis of impact of NBs on SCA3 flies' survival and locomotor function

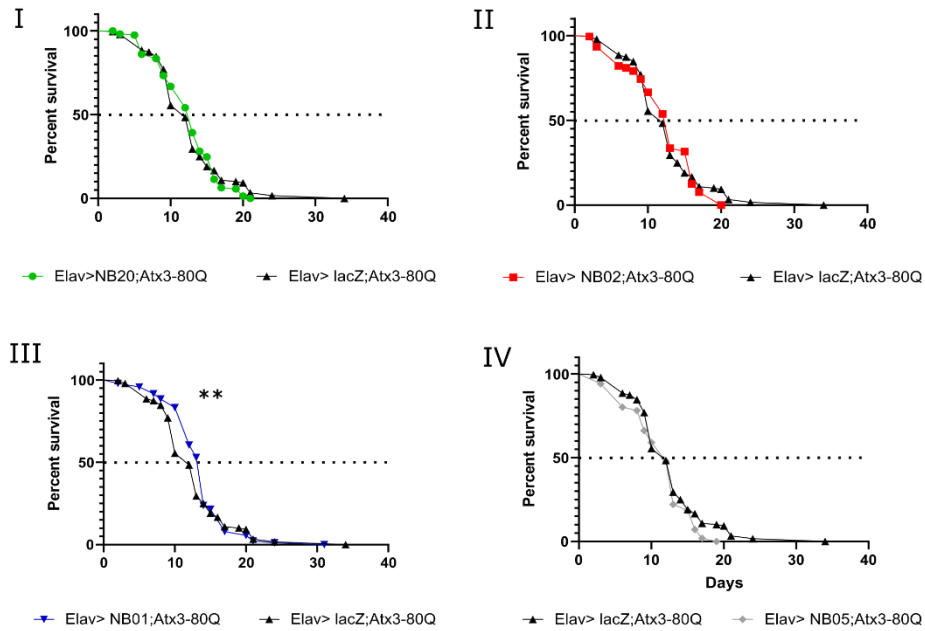
Even though we were not able to quantify biochemical effect of NBs on the levels of pathogenic species of Atx3 variants of *Drosophila*, we decided to evaluate how NBs impact the lifespan of flies expressing pathogenic Atx3 (**Figure 24**).

We started by evaluating the effect of NBs on the survival of Atx3-80Q expressing flies. For this, survival assays were performed in flies co-expressing UAS-Atx3-80Q and UAS-NBs (**Figure 24A**). Our results showed no significant differences between NB20, NB02 and NB05 when compared to the control (ELAV>lacZ; Atx3-80Q) survival curves. However, co-expression of NB01 seems to slightly improve the mean survival (14 days) of Atx3-80Q flies when compared to the control (12 days) (**Figure 24A.III**).

We also evaluated the effect of NBs on the lifespan of Atx3-77Q expressing flies (**Figure 24B**). We observed that all Atx3-77Q flies co-expressing NBs induced a significant increase in the average days of survival [ELAV>NB20; Atx3-77Q (31 days); ELAV>NB02; Atx3-77Q (28 days); ELAV>NB01; Atx3-77Q (31 days) and ELAV>NB05; Atx3-77Q (31 days)] when compared to the control [ELAV>lacZ; Atx3-77Q (26 days)]. All NBs extended the time point at which 50 % of the population is lost, however NB01 was the one that increased the most this period.

The overall results showed that NBs have a noticeable effect improving the lifespan of flies expressing Atx3-77Q when compared to flies expressing Atx3-80Q. This also supports the theory that NBs are not being sufficiently expressed to overcome the expression and aggregation of Atx3-80Q variant. Indeed, when we analyze the survival level of flies co-expressing Atx3-77Q and each NB we observe that all four NBs demonstrated a neuroprotective effect by improving motor coordination and extending lifespan of SCA3 fly model. These results endorse our hypothesis that NBs are promising neuroprotective strategies for SCA3, and we have to optimize the molecular techniques to evaluate how they act on Atx3 aggregation pathway.

A



B

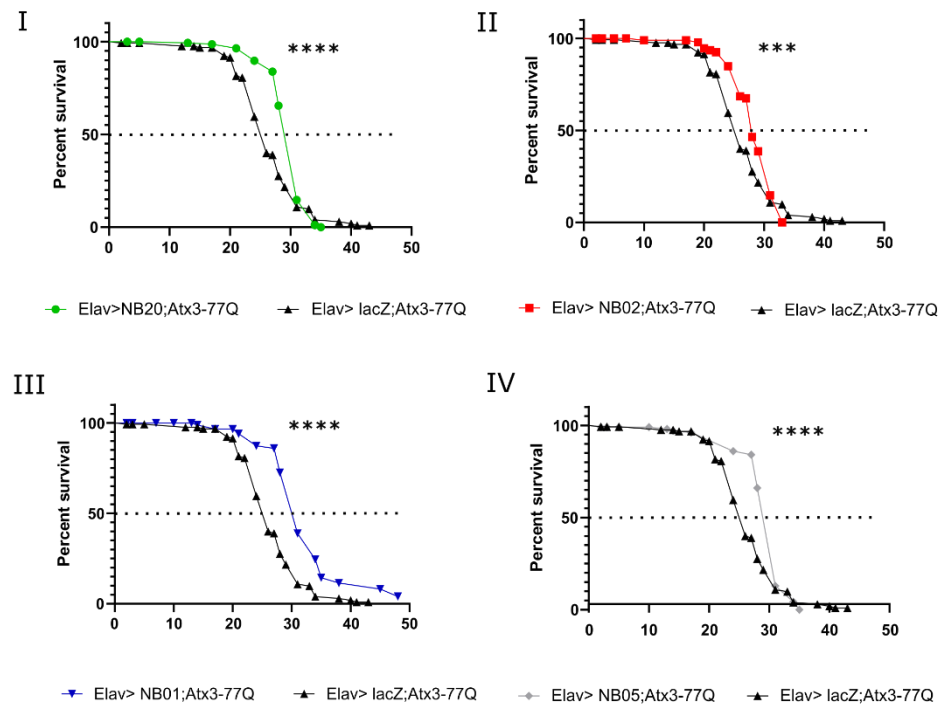


Figure 24. Survival (lifespan) curves using the Kaplan-Meier survival method analyzed with Gehan-Breslow-Wilcoxon test A) Comparing the effect of all ELAV>NBs; Atx3-80Q with ELAV>lacZ; Atx3-80Q, **p-value<0.01 and B) Comparing the effect of all NBs with ELAV>NBs; Atx3-77Q with ELAV>lacZ; Atx3-77Q, ***p-value<0.001 and ****p-value<0.0001.

Next, we wanted to understand the effect of NBs on the climbing behavior of flies that express the pathogenic Atx3.

We started by analyzing the effect on Cf after co-expressing NBs and Atx3-80Q over time (**Figure 25A**, **Figure 25B** and **Figure 25C**). When comparing the Cf between day 5 and day 10 of ELAV-GAL4>NBs; Atx3-80Q flies (**Figure 25A**), we observe a tendency for decreased motor capacity in all the genotypes. Moreover, there was a significant decrease in the Cf of flies expressing NB02 and NB01 at day 10 when comparing the same genotypes at day 5. Then we analyzed the Cf within each time point (e.g., 5 days) and we compared each genotype (ELAV-GAL4>NB; Atx3-variant) with the control (ELAV-GAL4>lacZ; Atx3-80Q). However, from the analysis within at day 5 or day 10 we did not identify significant differences between Atx3-80Q flies co-expressing NBs and the control. Nevertheless, there is a tendency of all NBs to improve the climbing probability of Atx-80Q expressing flies. This tendency was most marked in 5-day old flies, but it is also noticeable in the 10-day old flies.

When analyzing the fitness groups at day 5 in Atx3-80Q expressing flies (**Figure 25B**), some significant differences were observed. NB02 induced a significant reduction in the percentage of flies in fitness group I, when compared to the control (ELAV>LacZ; Atx3-80Q). Co-expression of all NBs significantly increased the number of flies in the fitness group III when compared to the control. With these data we were able to demonstrate that co-expression of the nanobodies appears to have a neuroprotective effect in 5-day-old flies, allowing them to reach the last tubes more efficiently than the ELAV>lacZ; Atx3-80Q expressing flies. When we analyzed the climbing behavior at day 10, we observed that the co-expression of NB05 significantly improved the climbing ability of Atx3-80Q expressing flies (**Figure 25C**); there was a significant increase in the number of flies reaching the last tubes (fitness group III). Although co-expression of the other NBs also led to an increase in the number of Atx3-80Q in fitness group III, this was not significant.

Then, we also decided to analyze the effect of NBs on the climbing behavior of flies that express Atx3-77Q (**Figure 25D**, **Figure 25E** and **Figure 25F**) to address if we could detect similar improvement in lifespan and climbing ability. When comparing the Cf between 5 and 10-days-old ELAV-GAL4>NBs; Atx3-77Q flies (**Figure 25D**), we observed that co-expression of NBs improves motor capacity in Atx3-77Q expressing flies. Actually, there was a significant increase by day 10 in flies expressing NB20 and

NB02 when compared to these same genotypes at day 5. However, we could not identify significant differences in the Cf between Atx3-77Q co-expressing NBs and the control (ELAV-GAL4>lacZ; Atx3-77Q flies) at either day 5 or day 10.

When analyzing the fitness groups of the 5-day-old Atx3-77Q flies (**Figure 25E**) we did not observe any significant difference between flies expressing NBs and the control in any of the three groups. Demonstrating that there is similar climbing behavior among all genotypes. Regarding the fitness groups at day 10 (**Figure 25F**), we detected a significant increase in the number of NB20; Atx3-77Q co-expressing flies reaching the last tubes when compared to control flies (ELAV-GAL4>lacZ; Atx3-77Q). Therefore, from this analysis we can conclude that NB20 may have a beneficial impact on 10-days old Atx3-77Q expressing flies, translating into a noticeable increase in their motor capacity.

What we can conclude from the climbing analysis is that all NBs tend to improve Cf in 5- and 10-days-old Atx3-80Q expressing flies when compared to the control (ELAV>lacZ; Atx3-80Q). On the other hand, only NB20 and NB02 significantly improve the Cf of Atx3-77Q expressing flies by day 10, comparing to the control.

In the fitness groups analysis, all NBs seem to tend to improve the climbing of Atx3-80Q expressing flies. However, all NB (especially NB02) seem to improve the fitness in a much more accentuated way in 5-days-old Atx3-80Q flies, when comparing to 10 days old flies. The effect of all NBs on the fitness of Atx3-77Q expressing flies, seems to be more pronounced by day 10 of the assay. Through this analysis we can see that NB20 is the one with the most pronounced beneficial effect on the fitness of Atx3-77Q expressing flies, allowing them to reach the last two tubes.

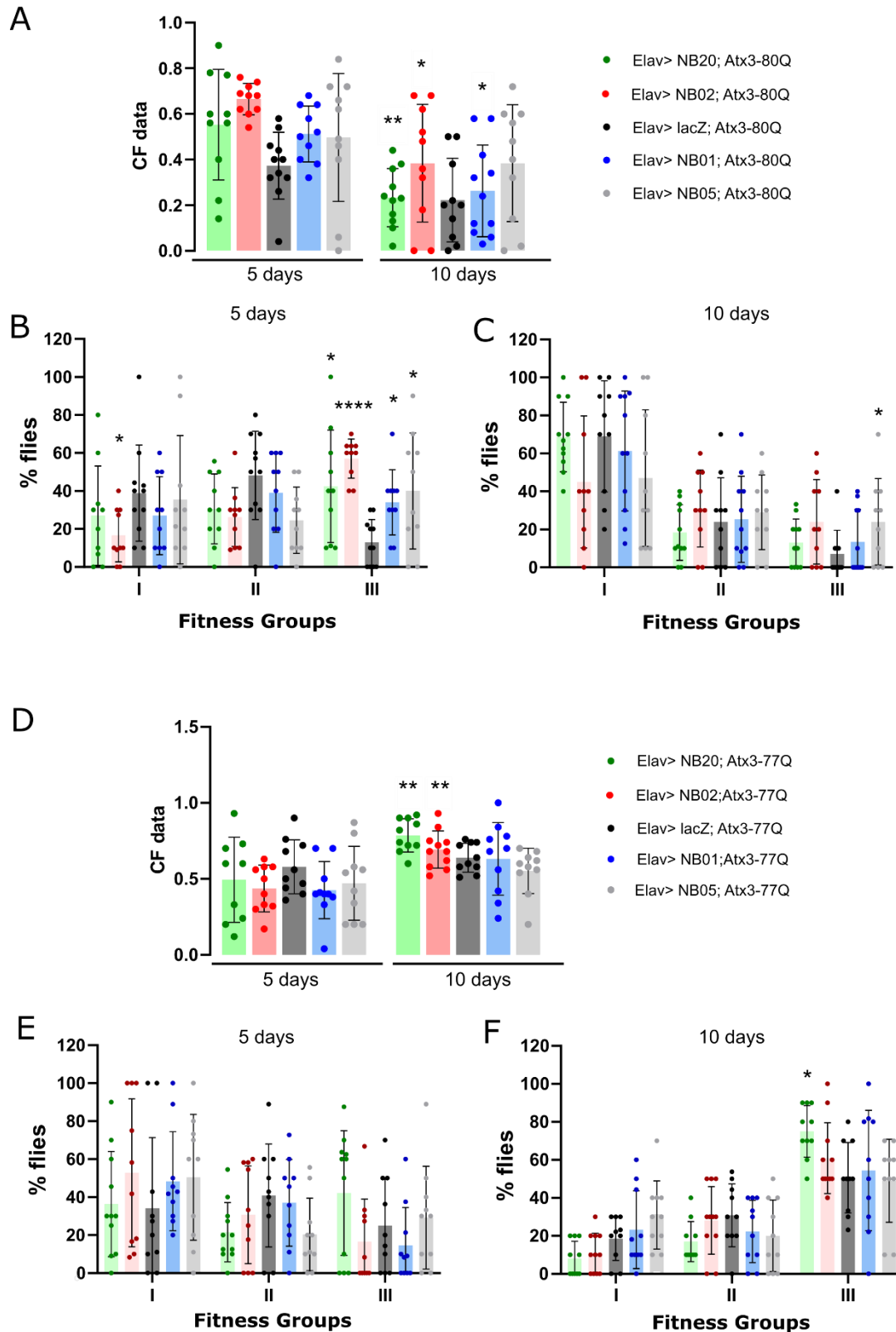


Figure 25. Climbing assay analysis of ELAV-GAL4>NBs; Atx3-80Q (A, B and C) and of ELAV-GAL4>NBs; Atx3-77Q (D, E and F). A) Cf data analysis ($Cf = (N2 + 2N3 + 3N4 + 4N5 + 5N6)/5$ (N1 + N2 + N3 + N4 + N5 + N6) where N is the number of ELAV-GAL4>NBs; Atx3-80Q flies in the tube) with 5 and 10-days old. The comparative analysis was made between 5 and 10 days and was evaluated with Mixed-effects analysis, *p-value<0.05 B) Fitness groups data analysis of 5-days-old ELAV-GAL4>NBs>Atx3-80Q flies. Data were evaluated within each fitness group and compared with the ELAV>lacZ; Atx3-80Q (control), *p-value<0.05

and ****p-value<0.0001 (Two-way ANOVA) C) Fitness groups data analysis of 10-days-old ELAV-GAL4>NBs; Atx3-80Q flies. Data were evaluated within each fitness group and compared with the ELAV>lacZ; Atx3-80Q (control), *p-value<0.05 (Two-way ANOVA). D) Cf data analysis of ELAV-GAL4>NBs; Atx3-77Q flies with 5 and 10-days old. The comparative analysis was made between 5 and 10 days and was evaluated with Mixed-effects analysis, **p-value<0.01 E) Fitness groups data analysis of 5-days-old ELAV-GAL4>NBs; Atx3-77Q flies. Data were evaluated within each fitness group and compared with the ELAV>lacZ; Atx3-77Q (control), no significant results (Two-way ANOVA) F) Fitness groups data analysis of 10-days-old ELAV-GAL4>NBs; Atx3-77Q flies. Data were evaluated within each fitness group and compared with the ELAV>lacZ; Atx3-77Q (control), *p-value<0.05 (Two-way ANOVA). Note: at least 100 flies of each genotype were analyzed for each of both functional assays (survival and climbing)

Through functional analysis and the biochemical results, we could observe that NB20 and NB01 are the ones who reduced the levels of HMW and SDS-soluble Atx3-species, especially in Atx3-80Q expressing flies. NB01 extended the lifespan, and NB20, NB02 and NB05 were the ones who contributed the most to improve flies climbing ability. Summarizing, although it is preliminary data, all NBs seem to increase the functional behavior of the SCA3 flies.

4. Implementation of cell-based methods to evaluate Atx3 anti-aggregation properties of NBs

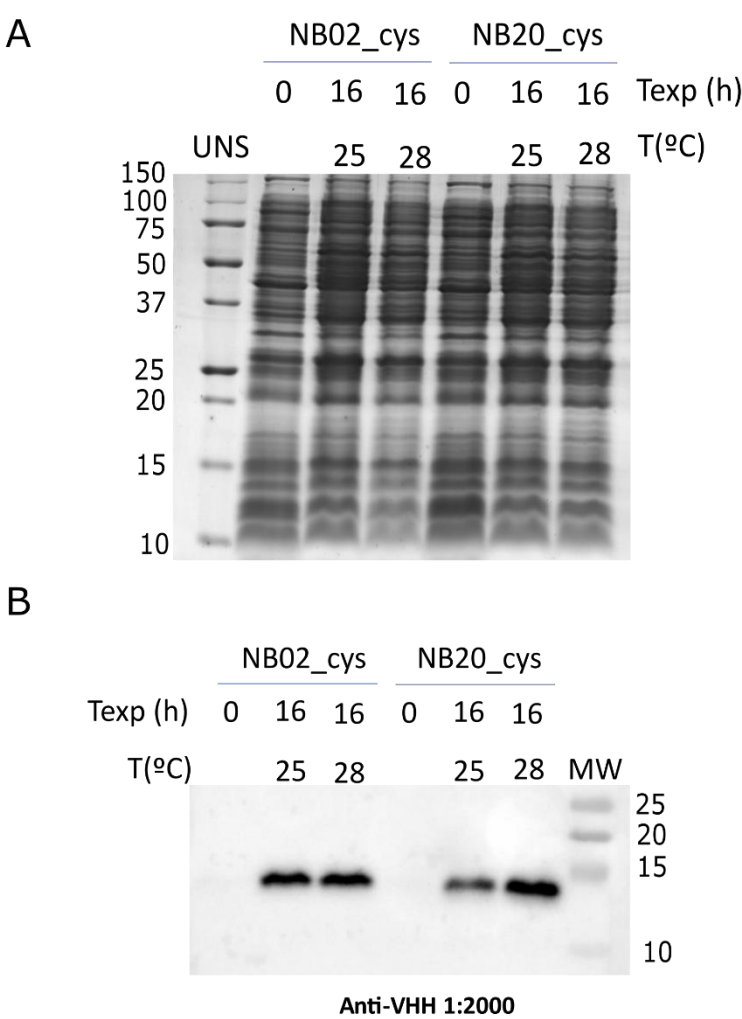
4.1. Purification of nanobodies

For the development of nanobody brain-targeting therapeutics, it is of most importance to address if the selected NBs used in this work can cross the blood–brain barrier (BBB) by transcytosis across epithelia.

We aim to advance this goal in the future, by analyzing the permeability of BBB to the selected NBs and the Atx3 anti-aggregation effect of each NB on neuronal cell models. If required, NBs can be engineered by introducing mutations to reduce immunogenicity and increase polycationic resurfacing to improve their intracellular penetration. As a first approach, we want to label each NB with small fluorescent maleimide probes such as Cy3 or Cy5 through binding to the C-terminal cysteine of a modified version of each NBs (91). After labeling, we can use commercial BBB Kit, which are normally composed of primary cultures of rat brain cells and a polyethylene terephthalate membrane allowing to evaluate the BBB permeability to small compounds.

To initiate this part of the future work, we performed smaller expression tests for NB20 and NB02 mutated with a cysteine in the C-terminal (NBcys) using WK6 (su-) *E. coli* cells. For that, expression of each NB was induced in cells transformed with plasmids expressing each NBcys with 1 mM IPTG for 16 hours at 25 °C and 28 °C, to evaluate which would be ideal for later use on a large-scale expression (**Figure 26**). We confirmed

the induction of each NB expression by SDS-PAGE, by running equal amounts of cell mass before and after induction time (**Figure 26A**). The visualization of NB corresponding bands with Coomassie Blue is challenging due to low yield of expression but also due to the small molecular weight of the NB (15 kDa) making it difficult to detect in the final part of the gel with lower resolution (**Figure 26A**). To better improve the detection of NBs, we also evaluate the expression levels by WB, using a specific anti-VHH antibody to detect NB. We detect similar levels of NB02cys expression at 25 °C and 28 °C. For NB20cys, we observe a more intense band after expression at 28 °C (**Figure 26B**).



Further, we also evaluate the expression of NB05cys and NB01cys after induction with 1 mM IPTG at 28 °C for 16 h (**Figure 27**). Once again, visualization of NB corresponding bands with Coomassie Blue was difficult (**Figure 27A**) but we were able to detect NB corresponding bands (15 kDa) by WB (**Figure 27B**). Despite the results demonstrated less signal in NB01cys and NB05cys when compared to NB20cys and NB02cys, this difference does not necessarily mean less expression. We know from previous studies in the lab that anti-VHH detection of these two NBs is suboptimal. Therefore, taking these results into account, we can conclude that induction of NB expression at 28 °C for 16 h is the best protocol to further proceed with large scale NB expression and purification.

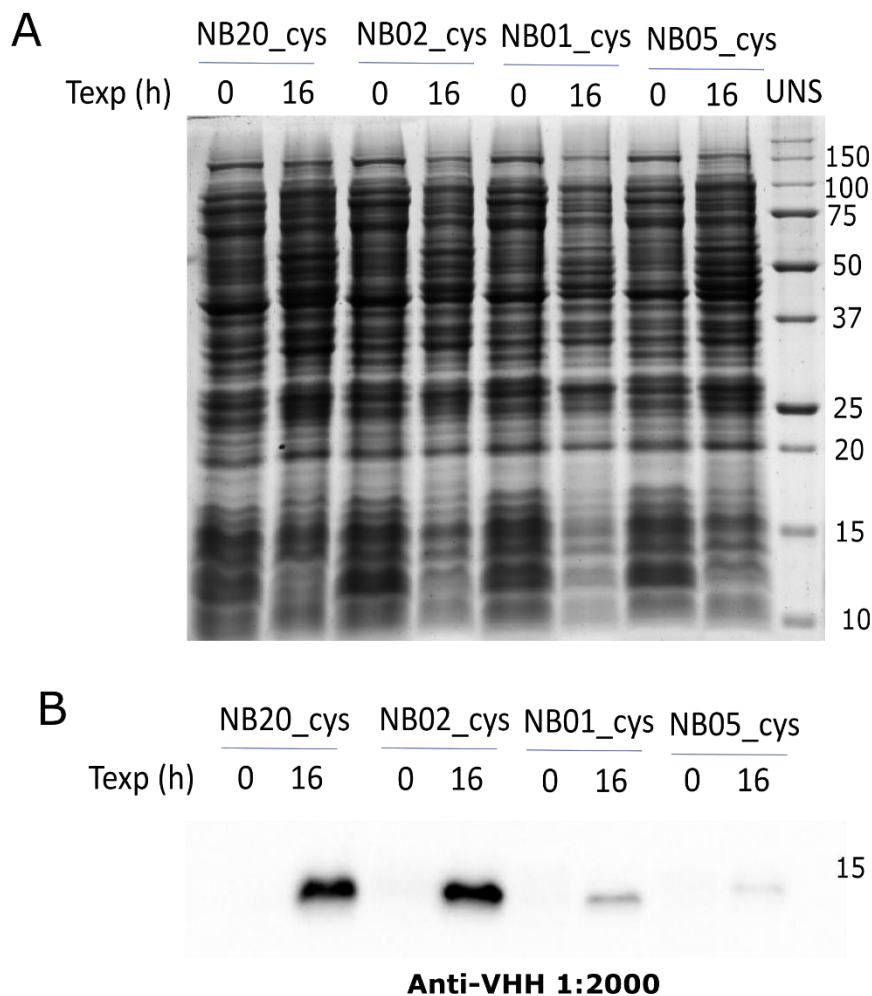


Figure 27. Small-scale expression test for NB20, NB02, NB01 and NB05 mutated with cysteine at the C-terminal. Expression was induced for 16h with 1mM IPTG at 28°C and confirmed by SDS-PAGE stained with Coomassie Blue (A) or detected by western-blotting with anti-VHH (B)).

For the lack of time and based on our previous results from molecular and functional analyses, we decided to only advance with the expression and purification of NB20cys at 28 °C for 16 h (**Figure 28A, B**). Expression of NB20cys was induced with 1mM IPTG in 3 L of WK6 (su-) *E. coli* cell culture previously transformed with NB20cys expressing plasmid for 16 h at 28 °C. After cell lysis (following the protocol detailed in material and methods), supernatant was snap-frozen and stored at -20 °C until required. To proceed with the two-step purification of the NB20cys, filtered supernatant was loaded into Ni²⁺-charged HiTrap™ IMAC HP column. The SDS-PAGE analysis of protein fractions collected during the ion immobilized affinity chromatography (IMAC) (**Figure 28C**) showed that NB20cys was loaded and almost fully retained in the column as we can only observe a residual band on the flow through sample (**Figure 28E**). After, NB20cys was eluted with 150 mM imidazole (Buffer HisTrap B) as confirmed by SDS-PAGE analysis (**Figure 28E**, strong band at fraction 10). The fraction corresponding to the NBcys20 eluted from the IMAC column was further purified by size exclusion chromatography (SEC) using a HiPrep 26/60 Sephacryl S-100 High Resolution. As observed in the SEC chromatogram (**Figure 28D**) the A_{280nm} peak corresponding to the monomeric form (fractions 23 to 27) of the NB was preceded by a smaller peak (fraction 6) but was not detectable as NB in SDS-PAGE analysis (**Figure 28E**). Fractions with the NB20cys (sec23-27) were pool together and concentrated, snap frozen and stored at -80 °C.

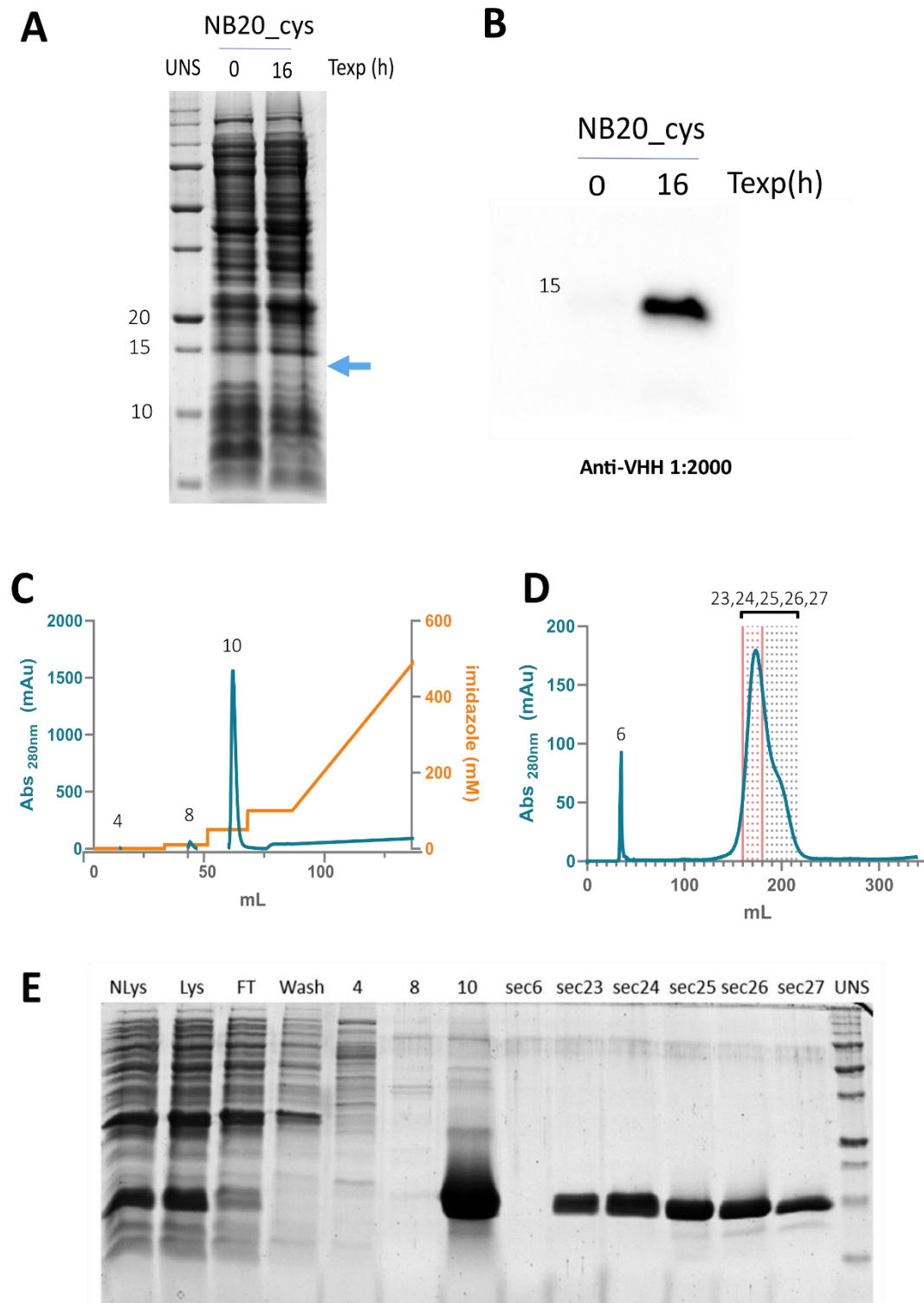


Figure 28. Expression and purification of NB20cys. Large-scale expression was performed at 28°C for 16h. Analysis of NB20cys expression by SDS-PAGE with Coomassie Blue (A) and western-blot. (B) Chromatograms obtained for IMAC (C) and SEC (D). SDS-PAGE analysis of NB20cys purification by IMAC and SEC (E).

4.2. Mammalian Atx3 plasmids

In the future work, we also aim to explore the Atx3 anti-aggregation properties of the selected NBs using neuronal cell models. For that, synthetic plasmids were produced for expression of mammalian Atx3 with 13Q or 77Q tract fused with GFP on the N-terminal and an empty vector for GFP expression as a control. These plasmids will be transfected on a human neuroblastoma cell line SHSY-5Y. This cell line is relatively easy to manipulate, it is low cost and it can be differentiated into a functionally mature neuronal-like cell under the catalysis of retinoic acid (92). These cellular models can be further used to evaluate how NBs impact the aggregation of normal and expanded Atx3 in a neuronal-like intracellular environment.

After receiving the synthetic plasmids, we transformed DH5α *E.coli* cells by heat shock protocol (described on methods section) and stored glycerol stocks for further use.

To obtain the control empty plasmid for EGFP expression, we use the original plasmid pRP-[TetOn]-TRE-EGFP-Atx3-13Q and double digested with two compatible sticky ends restriction enzymes XhoI and Sall to remove Atx3-13Q gene. After digestion, the digested plasmid was separated from the insert by agarose gel. There were two bands, one with 5949 bp corresponding to the plasmid (cut with XhoI and Sall) and another band with 1100 bp corresponding to Atx3-13Q insert (**Figure 29**). The band corresponding to the plasmid was purified being collected from the gel and using a commercial kit (NZYtech GelPure).

Self-circularization of linear plasmid was performed with T4 DNA ligase. The DH5α *E. coli* cells were transformed with ligation reaction. Finally, we isolated plasmids from two positive colonies that were further sequenced to confirm the full sequence of pRP-[TetOn]-TRE-EGFP empty vector.

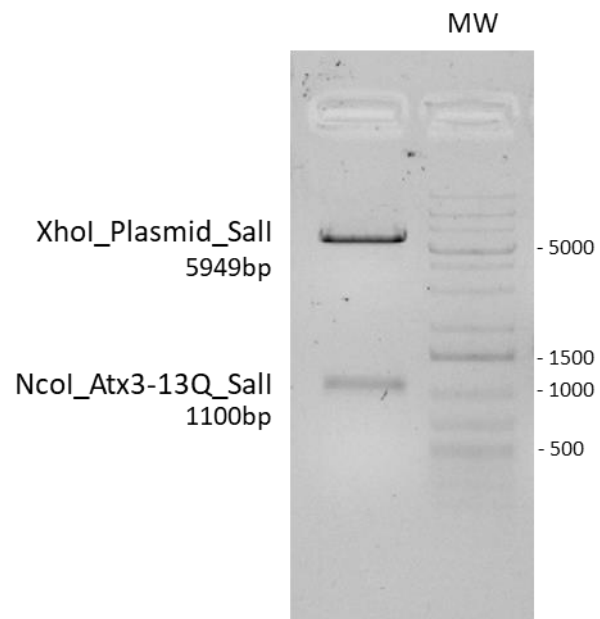


Figure 29. Confirmation of double digestion of the plasmid to remove Atx3-13Q gene in agarose gel. The band with 5949bp corresponds to the plasmid and band at 1100bp corresponds to Atx3-13Q insert.

Conclusion

With this work we intended to characterize 4 NBs previously described to have the potential to modulate pathogenic Atx3 self-assembly *in vitro*. For this, we first started to characterize the non-pathogenic (Atx3-22Q) and pathogenic Atx3 variants (Atx3-77Q and Atx3-80Q), to understand which would be the most suitable SCA3 model for co-expression with the NBs, to later evaluate the neuroprotective effect of the NBs.

Analysis of flies expressing the pathogenic and non-pathogenic Atx3 variants in the retina (GMR-GAL4) or pan-neurally (ELAV-GAL4), allowed us to conclude that Atx3-77Q is expressed at lower levels, irrespective of the driver, and presents reduced amounts of HMW Atx3 aggregates and SDS-insoluble aggregates/fibrils when compared to Atx3-80Q. This data is in accordance with previous results showing that Atx3-80Q is the "stronger" and the Atx3-77Q is the "weaker" line (76). This difference is due to the inclusion of an improved Kozak sequence in Atx3-Q80 construct that leads to higher expression levels of Atx3 protein. Additionally, we were able to conclude that lines driven by GMR-GAL4 always presented a much higher expression of SDS-resistant Atx3 aggregates in all Atx3 variants, when compared to ELAV. As GMR-GAL4>Atx3-80Q is the genotype with the highest levels of HMW Atx3 aggregates, we concluded this would be the most favorable genotype for observing the effect of NBs at a biochemical level.

Due to the high variability between replicates when expressing Atx3-80Q together with NBs in the retina, we cannot conclude which NBs lead to the reduction of Atx3 expression.

Therefore, we also decided to analyze the total protein levels in the flies that co-expressed pathogenic Atx3 and NBs under the transcriptional control of ELAV-GAL4. Despite the variability between replicates in Atx3-77Q and Atx3-80Q samples, we were able to observe an overall tendency towards a reduction in the HMW Atx3 species upon co-expression of NB20 and NB01 and an increase of these same species induced by NB05 in Atx3-80Q expressing flies. In agreement, filter trap assays showed an increase in the formation of SDS-resistant aggregates by co-expression of NB05.

The functional assays in flies expressing the pathogenic and non-pathogenic Atx3 variants showed that the Atx3-80Q variant had the strongest impact on flies' survival, and severely affected flies climbing ability. These results demonstrated that is important to test NBs impact in the functional behavior of ELAV-GAL4>Atx3-80Q

expressing flies. Furthermore, as ELAV-GAL4>Atx3-77Q expressing flies also showed reduced lifespan compared to the control and defective climbing behavior by day 10, we also considered interesting to evaluate the neuroprotective effect of NBs in ELAV-GAL4>Atx3-77Q expressing flies. All NBs improved the average lifespan of Atx3-77Q expressing flies when compared to the control. On the other hand, only NB01 extended the lifespan of Atx3-80Q expressing flies.

All NBs trend to improve the climbing ability of Atx3-80Q expressing flies at day 5 and day 10. However, this tendency is much more marked in ELAV>NB02; Atx3-80Q expressing flies at day 10. We can also conclude that NB20 significantly improves the climbing of Atx3-77Q expressing flies at day 10. Summarizing, NB02 and NB20 are the ones who most improve the climbing ability in both Atx3-77Q and Atx3-80Q expressing flies. However, their impact performance is more evident in Atx3-77Q flies by day 1 and in Atx3-80Q flies by day 5.

Despite the variable results regarding the protein levels of Atx3 when co-expressed with the NBs, we can conclude that all NBs extended the SCA3 flies' lifespan and improved their climbing ability. In addition, NB20 and NB02 reduced the levels of soluble and resistant Atx3-species. On the other hand, NB05 increased the Atx3 aggregates. However, it also extended the lifespan of the Atx3-77Q expressing flies and improved the climbing ability in both pathogenic Atx3 variants expressing flies. Thus, we proposed that the increase of insoluble Atx3 levels, that we observed in the biochemical results, may represent less toxic aggregates. Those may induce less harmful effect in the functional capacities of the SCA3 flies when compared to the highly damaging functional effects caused by the aggregates found in SCA3 flies without NBs action.

Future studies are required to analyze whether the NBs can cross the blood–brain barrier (BBB) by transcytosis across epithelia, and if necessary, genetically improve them. We also want to further characterize the effect of the NBs on the Atx3 aggregation using a SCA3 neuronal cell model, to understand which of them could be used in human SCA3 therapies. Overall, we can conclude that further research is needed, but NBs might be promising molecules to improve motor impairment and lifespan quality in SCA3 patients.

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Attachment 1

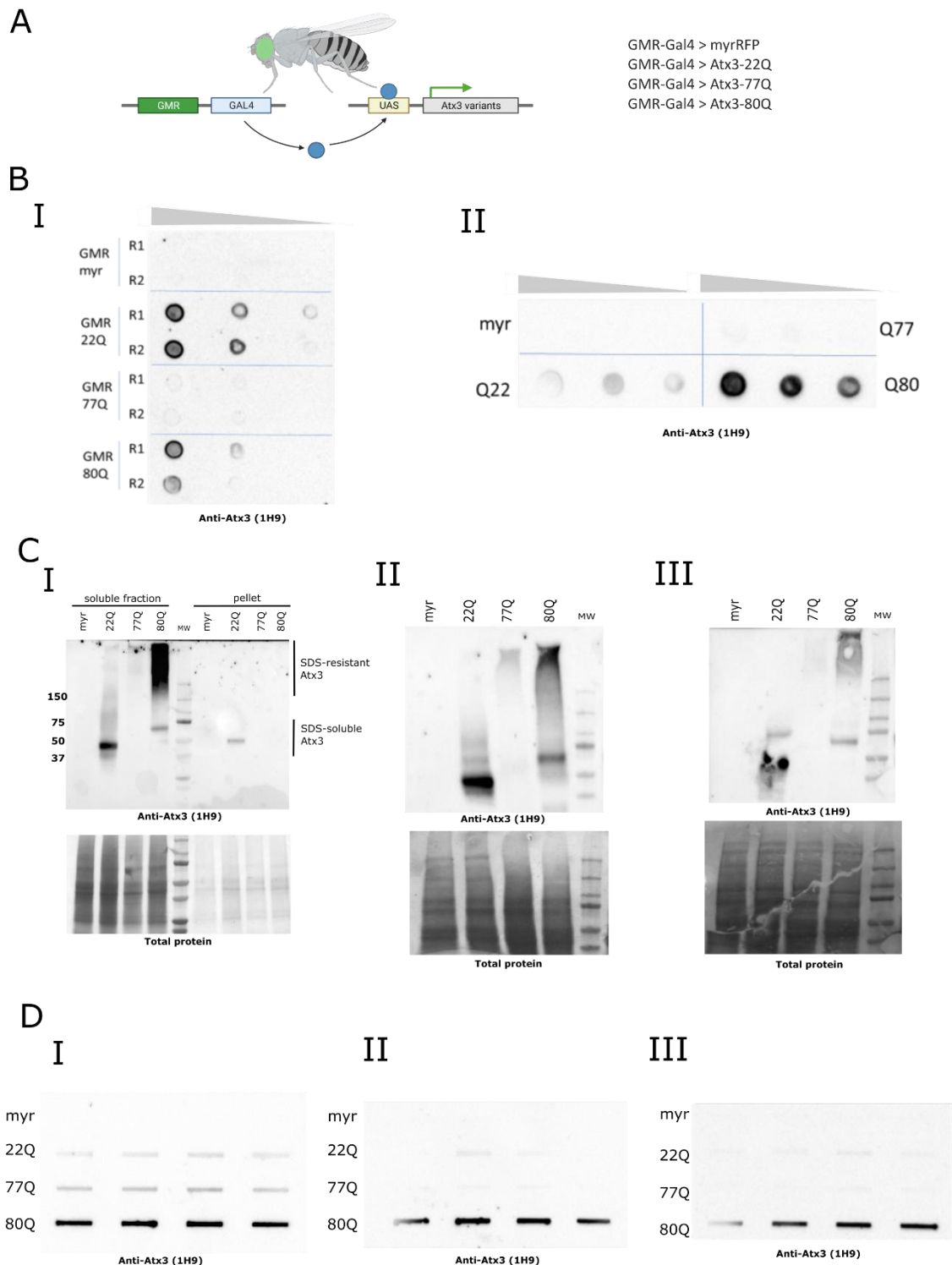


Figure 30. All replicates of the analysis of Atx3 variants expression under the control of GMR-GAL4. A) Representative image of the GMR-GAL4>UAS gene expression system, with the four genotypes used (GMR-GAL4> myrRFP; GMR-GAL4> Atx3-22Q; GMR-GAL4> Atx3-77Q; GMR-GAL4> Atx3-80Q). B) Analysis of Atx3 total levels in protein extracts from adult head flies by dot-blot with anti-Atx (1H9) antibody, two replicates represented. C) Western blot analysis of Atx-3 species with anti-1H9. The loading control was

evaluated with Ponceau S staining after transfer and before blocking step, three replicates represented. D) Analysis of SDS-resistant Atx3 aggregates of protein extracts from adult head flies by filter trap, three replicates represented.

Attachment 2

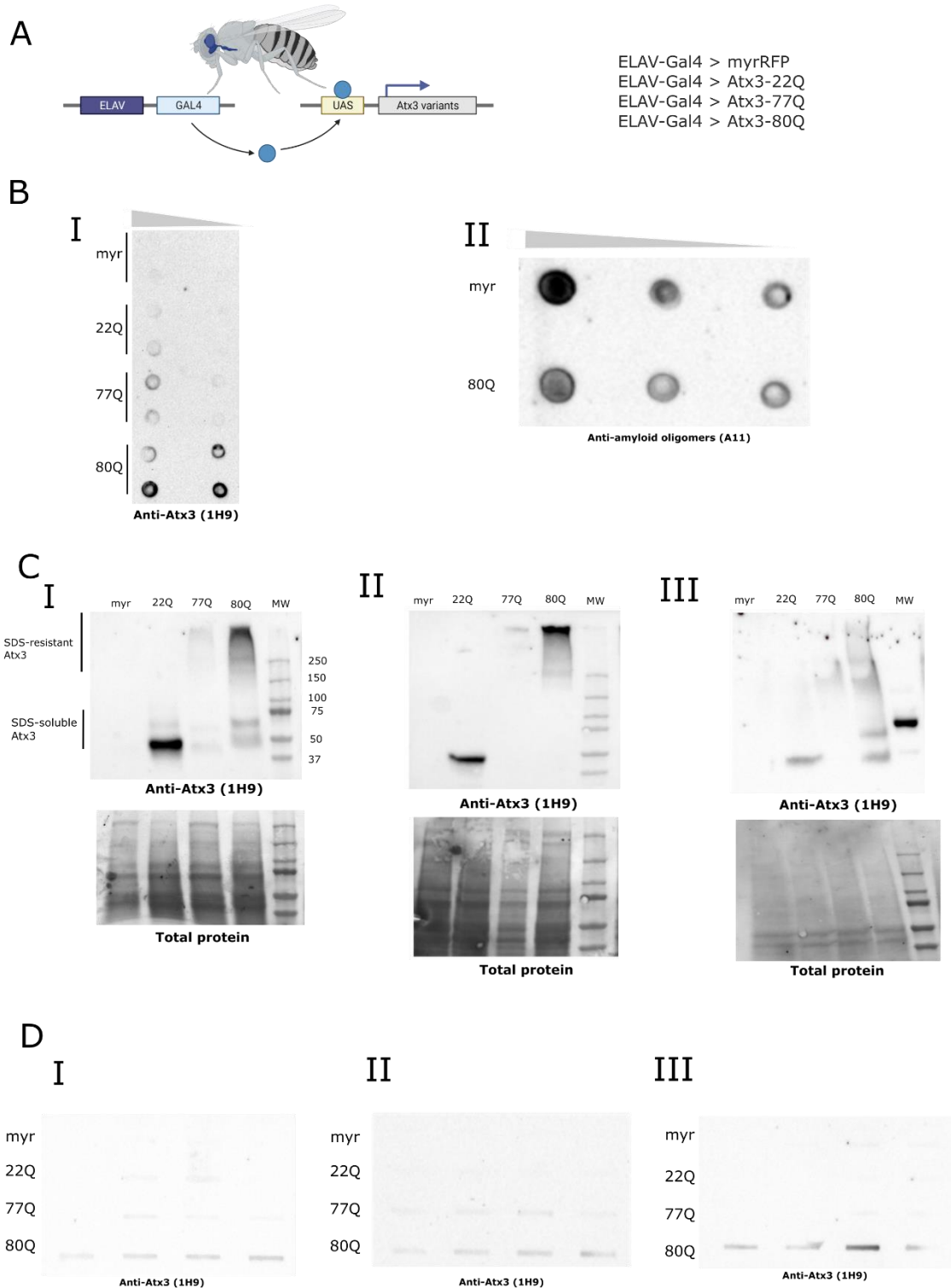


Figure 31. All replicates of the analysis of the expression of Atx3 variants under the control of ELAV-GAL4 A) Representative image of the ELAV-GAL4-UAS gene expression system, with the four genotypes used (ELAV-GAL4> myrRFP; ELAV-GAL4> Atx3-22Q; ELAV-GAL4> Atx3-77Q; ELAV-GAL4> Atx3-80Q). B) Analysis of Atx3 total levels in protein extracts from adult head flies by dot-blot with primary antibody anti-1H9 (I) and another with antibody anti-A11(II). C) Western blot for detection of Atx3 species with primary

antibody anti-1H9. The loading control was evaluated with Ponceau S staining after transfer and before blocking step, three replicates represented. D) Analysis of SDS-resistant Atx3 aggregates of protein extracts from adult head flies by filter trap, three replicates represented.

Attachment 3

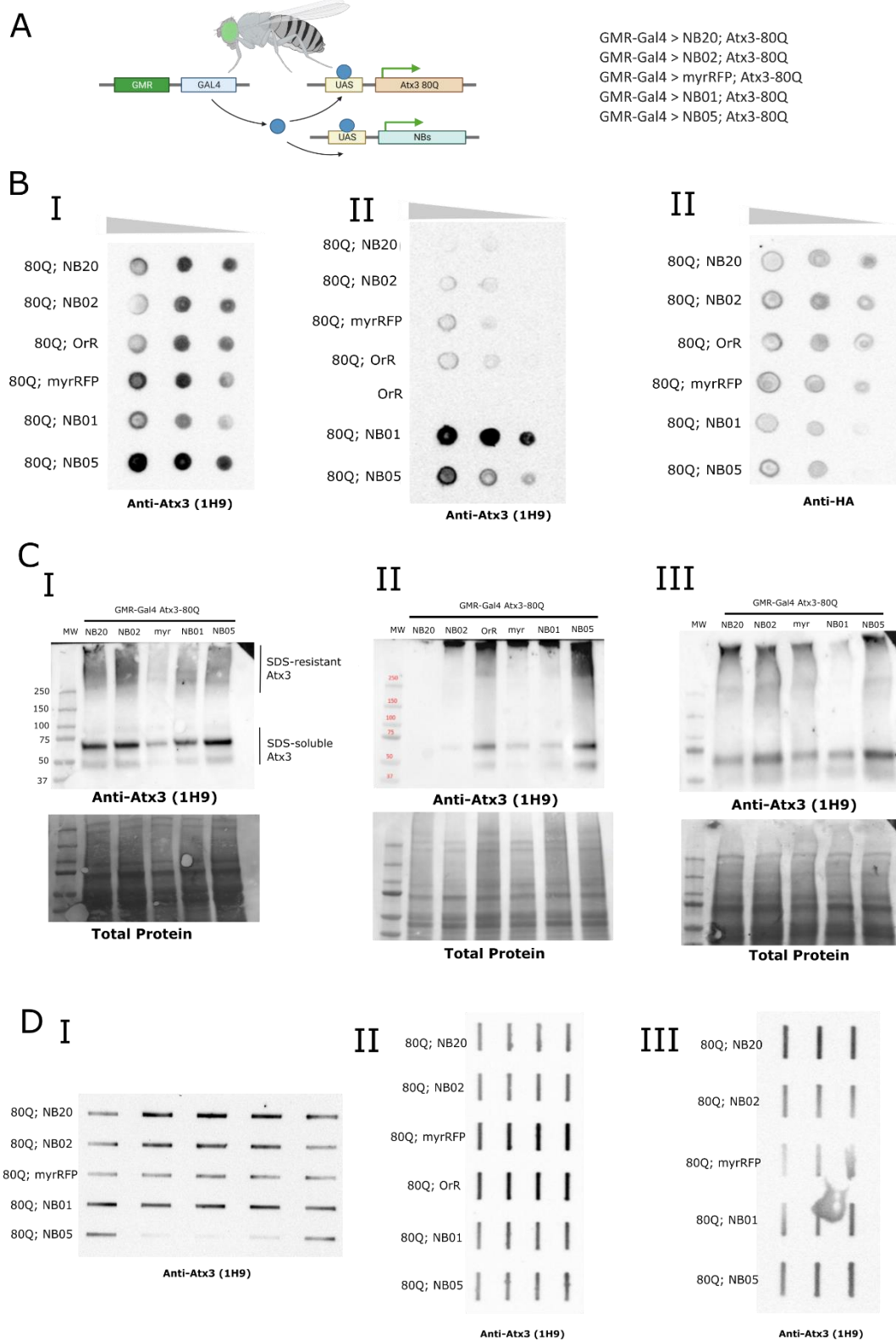


Figure 32. All replicates of the molecular analysis of NBs impact in the protein levels of soluble and aggregates of Atx3-80Q in *Drosophila* eye. A) Representative image of the GMR-GAL4>UAS gene

expression system, with the five genotypes used (GMR-GAL4> NB20; GMR-GAL4> NB02; GMR-GAL4> myrRFP; GMR-GAL4> NB01; GMR-GAL4> NB05 all co-expressed with Atx3-80Q). B) Analysis of Atx3 total levels in protein extracts from adult head flies by dot-blot with primary antibody anti-1H9, two replicates (I) and (II); another incubated with anti-HA (III). C) Western blot for detection of Atx3 species with primary antibody anti-1H9. The loading control was evaluated with Ponceau S staining after transfer and before blocking step, three replicates represented. D) Analysis of SDS-resistant Atx3 aggregates of protein extracts from adult head flies by filter trap, three replicates represented.

Attachment 4

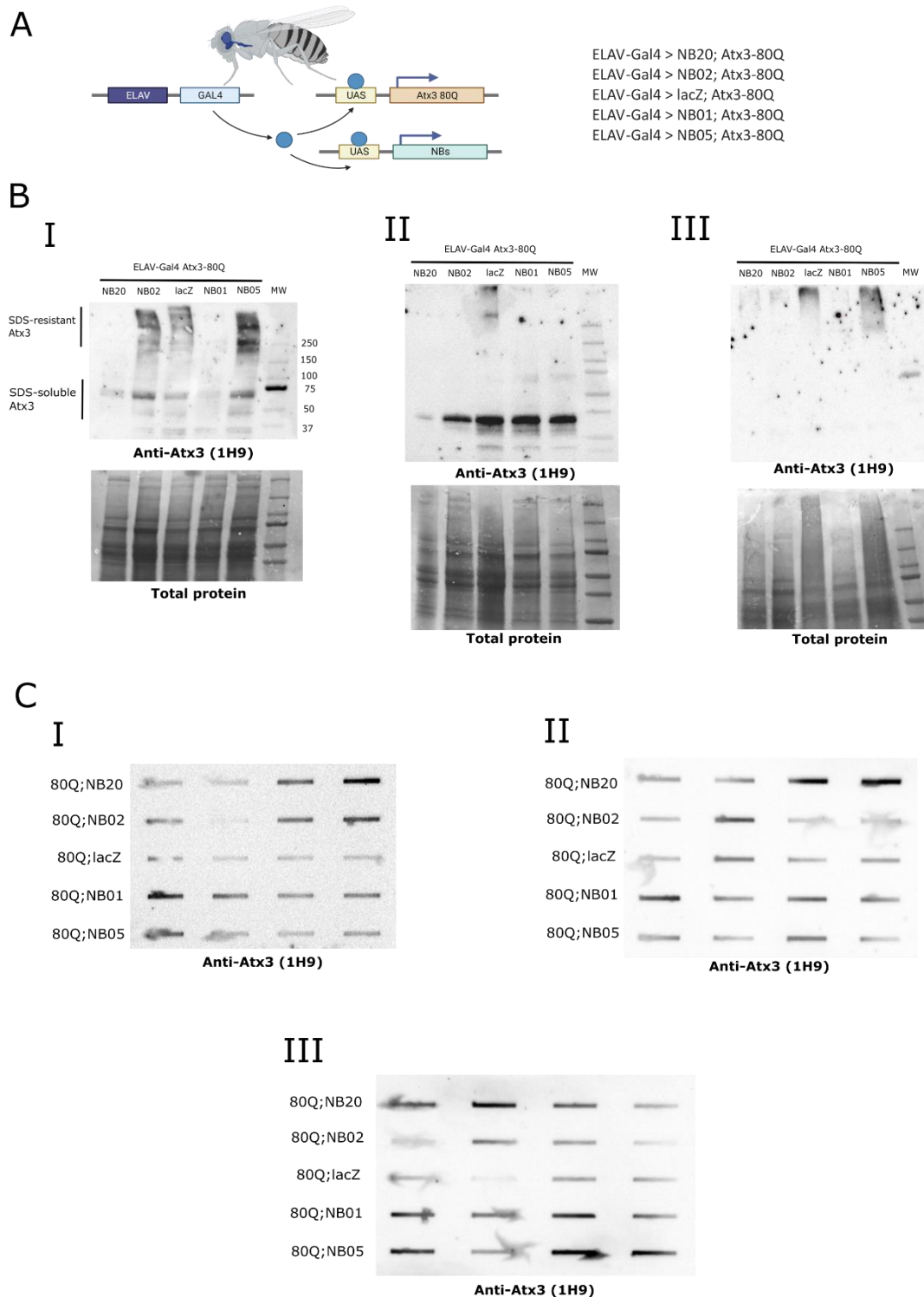


Figure 33. All replicates of the molecular analysis of NBs impact in the protein levels of soluble and aggregates of Atx3-80Q in *Drosophila* nervous system. A) Representative image of the ELAV-GAL4>UAS gene expression system, with the five genotypes used (ELAV-GAL4> NB20; ELAV-GAL4> NB02; ELAV-GAL4> lacZ; ELAV-GAL4> NB01; ELAV-GAL4> NB05, all co-expressed with Atx3-80Q). B) Western blot for detection of Atx-3 species with primary antibody anti-1H9. The loading control was evaluated with Ponceau

S staining after transfer and before blocking step, three replicates represented C) Analysis of SDS-resistant Atx-3 aggregates of protein extracts from adult head flies by filter trap, three replicates represented.

Attachment 5

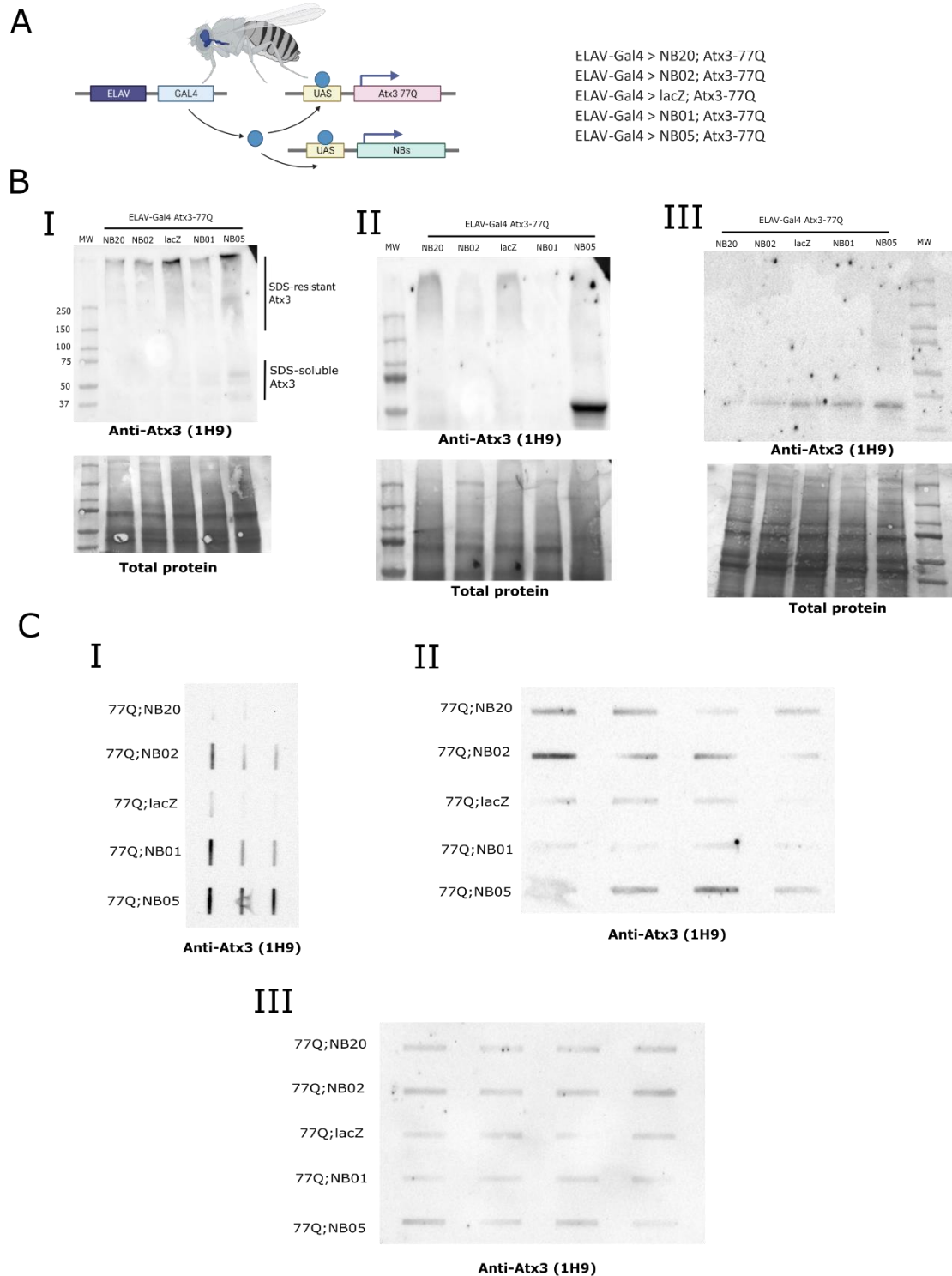


Figure 34. All replicates of the molecular analysis of NBs impact in the protein levels of soluble and aggregates of Atx3-77Q in *Drosophila* nervous system. A) Representative image of the ELAV-GAL4-UAS gene expression system, with the five genotypes used (ELAV-GAL4> NB20; ELAV-GAL4> NB02; ELAV-GAL4> lacZ; ELAV-GAL4> NB01; ELAV-GAL4> NB05, all co-expressed with Atx3-77Q). B) Western blot for detection of Atx-3 species with primary antibody anti-1H9. The loading control was evaluated with Ponceau

S staining after transfer and before blocking step, three replicates represented. C) Analysis of SDS-resistant Atx-3 aggregates of protein extracts from adult head flies by filter trap, three replicates represented.