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Quantification of Ataxin-3 and Ataxin-7 aggregates formed *in vivo* in *Drosophila* reveals a threshold of aggregated polyglutamine proteins associated with cellular toxicity

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ABSTRACT

Polyglutamine diseases are nine dominantly inherited neurodegenerative pathologies caused by the expansion of a polyglutamine domain in a protein responsible for the disease. This expansion leads to protein aggregation, inclusion formation and toxicity. Despite numerous studies focusing on the subject, whether soluble polyglutamine proteins are responsible for toxicity or not remains debated. To focus on this matter, we evaluated the level of soluble and insoluble truncated pathological Ataxin-3 *in vivo* in *Drosophila*, in presence or absence of two suppressors (*i.e.* Hsp70 and non-pathological Ataxin-3) and along aging. Suppressing truncated Ataxin-3-induced toxicity resulted in a lowered level of aggregated polyglutamine protein. Interestingly, aggregates accumulated as flies aged and reached a maximum level when cell death was detected. Our results were similar with two other pathological polyglutamine proteins, namely truncated Ataxin-7 and full-length Ataxin-3. Our data suggest that accumulation of insoluble aggregates beyond a critical threshold could be responsible for toxicity.

1. Introduction

Polyglutamine (PolyQ) diseases are fatal dominantly inherited neurodegenerative disorders provoked by the expansion of a polyQ domain in a disease-specific protein prone to aggregate. With Huntington's disease, Spinobulbar Muscular Atrophy, Dentatorubral Pallidoluysian Atrophy, Spinocerebellar Ataxia (SCA) type 1, 2, 6 and 17, SCA3 and SCA7 are two of the nine polyQ diseases identified to this date. When the size of their polyQ domain exceeds a threshold of 54 glutamines for the Atx3 protein or 36 for the Atx7 protein, patients respectively develop the SCA3 or SCA7 disease [1].

A common feature to polyQ diseases is the presence of inclusions detectable by microscopy on histological brain slices from patients or in cellular and animal models. Inclusions are mainly nuclear [2,3] and contain numerous proteins, including the polyQ protein responsible for the pathology and chaperone proteins such as Hsp70 [4]. Neither the conformation nor the oligomerization states of the pathological polyQ proteins can be fully characterized *in vivo* whether inside or outside inclusions. Therefore the link between aggregates and inclusions remains unclear.

Recent studies have focused on oligomers that can be formed by elongated polyQ proteins. Oligomers have been detected both *in vitro* and *in vivo* as multiple different oligomeric conformations [5–8]. They can be considered as soluble [6,9] or insoluble [8] but the notion of solubility is not defined on common grounds. Depending on the study, soluble oligomers are either toxic [10,11] or not [12], and reciprocally, insoluble oligomers can be either toxic [13] or not [8].

In the present work we sought to investigate the toxicity of the SDS-soluble or -insoluble species of Atx3 and Atx7 polyQ proteins formed *in vivo*.

2. Materials and methods

2.1. Fly genetics and phenotype observation

All crosses were grown at 25 °C or 19 °C on standard medium, which was changed every other day. Glass-Mediated Response-GAL4 (GMR-gal4) was used as a GAL4 driver. *Drosophila* eyes were photographed with a Leica MZFL III microscope.

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2.2. Western and dot blotting

Twenty male and twenty female flies were sacrificed in liquid nitrogen. Their heads were crushed in denaturing buffer (TE pH 8.5; 2% SDS, 0.05M DTT; 10 μM Protease Inhibitor Cocktail AEBSF, Roche) and incubated at 96 °C for 12 min. Part of the protein extracts was dot blotted on a nitrocellulose membrane (Schleicher and Schuell, Whatman, OPTITRAN BA-S85, 0.45 μm) without aspiration to quantify the SDS-insoluble fraction named hereafter aggregates. Another part of the same extracts was separated by Western Blot to quantify the SDS-soluble fraction on NuPAGE 4–12% Bis-Tris polyacrylamide gels following standard protocols [14]. Immunoreactive bands were detected by ImmobilonTM western Chemoluminescent HRP Substrate (Millipore) and chemoluminescent signals were quantified with a BioRad Chemidoc™ XRS+ or Molecular Imager® System GS505, allowing saturation-free measures. The glass protein was used as a loading control, since it is specifically expressed in the cells of interest.

Western blot quantifications are often considered as non-linear. To make sure that our western blotting conditions allow linearity of protein quantifications, we compared the signals observed for Glass immunodetection with two-fold dilutions of 54 independent samples. The average ratio between dilution quantifications was exactly equal to 2 with a standard error of 0.12. Therefore, our protocol allows linear quantification of SDS-soluble proteins.

2.3. Antibodies and reagents

Primary antibodies used were mouse anti-Myc (9E10, 1/1000, Developmental Studies Hybridoma Bank), rabbit anti-Myc (1/100, #2272, Cell Signaling), mouse anti-Ha (Ha.11 16B12, 1/5000, Eurogentec), rabbit anti-Atx7 (PA1-749, 1/400, OZYME), mouse anti-Glass (9B2.1, 1/250, Developmental Studies Hybridoma Bank), Horseradish peroxidase conjugated secondary antibodies (1/10000, Jackson ImmunoResearch).

2.4. Statistical analyses

Each experiment was realized at least thrice independently and two dilutions of the samples were used to verify the linearity of the quantification process. The quantitative results were analyzed by ANOVA. To work on more normal data, we applied a log transformation to the measures or a square root transformation when focusing on the threshold of protein levels involved in triggering cell death.

3. Results

3.1. Quantification of SDS-soluble and -insoluble Atx proteins by western and dot blotting

The definition of protein aggregates often varies. They are defined in our study as oligomers that resist boiling in a 2% SDS reducing solution. Aggregate quantities are often evaluated from the amount of proteins trapped at the top of western blots. However, only small aggregates can enter stacking gels, while large aggregates cannot. An alternative method to quantify aggregates is dot blotting. To compare the accuracy of western and dot blotting quantifications of protein aggregates, we first used a SCA3 model based on the expression of truncated human Atx3 (Atx3tr, Fig. S1). Driven by the UAS-gal4 expression system and using the GMR-gal4 driver, the transgene was expressed in all cells posterior to the morphogenetic furrow in the eye tissue, including photoreceptor neurons. We compared the ratio of signals detected for two dilutions of forty independent samples with both techniques. The average ratio was similar for western and dot blots. Interestingly, the variance of the ratio was 2.75 times higher with western blots than with dot blots (Fig. S2, F test: $p < 2 \cdot 10^{-3}$). Therefore, dot blotting is more reproducible for aggregate quantification than western blotting.

Relative soluble and aggregated Atx protein levels per cell were determined in the following experiments by normalizing Atx levels with the Glass protein level. *Glass* gene expression is restricted to few cells of the central nervous system and to the differentiated cells of the eye tissue [15]. As Glass directly controls the expression of *GAL4* with the GMR-gal4 construct used to drive the expression of the different transgenes in this study, Glass levels roughly characterize the number of Atx-expressing cells that remain alive. Hence, we used Glass levels to normalize the quantity of Atx proteins per Atx-expressing cell, rather than usual loading controls (such as tubulin), which would have evaluated the amount of Atx proteins per cell, whether cells expressed Atx or not.

3.2. Aggregated truncated Atx3 protein level is correlated with toxicity

As described by the group of Nancy Bonini [16,17], while the non-pathological Atx3Q27tr that carries a repeat of 27 glutamines does not induce toxicity, pathological Atx3Q78tr induces cell death (Fig. S3A–B). The Atx3Q78tr model is of particular interest since truncated forms of Atx3 can be observed *in vivo* [18,19] and could be more relevant for the study of polyQ protein-induced toxicity since they reveal more toxic than the corresponding full-length protein [20–24].

Atx3Q78tr-induced apoptosis is opposed by human Hsp70 and non-pathological full-length Atx3Q27 as observed by cross-sectioning [16,17] and through a loss of pigmentation of the eye (Fig. S3B) that has been shown to be due to the death of pigment cells [25]. Interestingly, cell death is not fully suppressed by Hsp70 and Atx3Q27, but is only slowed down since loss of pigmentation appeared in 45-day-old flies (Fig. S3B). As shown in Fig. S3E, the effect of suppressors is not due to GAL4 titration.

To better understand the relationship between aggregation and toxicity, we compared the amount of aggregated and SDS-soluble Atx3Q78tr in different genetic backgrounds that modulate toxicity. Suppressing Atx3Q78tr-induced toxicity decreased the quantity of aggregated Atx3Q78tr (Fig. 1A, left panel) without affecting its soluble level (Fig. 1A, right panel) (Representative western and dot blots are shown in Fig. S4A). The UAS-GAL4 system is temperature-sensitive and decreasing the temperature lowers the expression of transgenes. To reduce the levels of Atx3Q78tr and thus toxicity that was extreme and only left few cells to study, we lowered the temperature at which flies were raised from 25 °C to 19 °C. The quantity of aggregated Atx3Q78tr remained reduced by both Atx3Q27 and Hsp70 but these suppressors also significantly reduced the soluble Atx3Q78tr level (Fig. 1B and Fig. S4B). Globally, the data obtained at 25 °C and 19 °C suggest that Atx3Q78tr soluble levels are not linked to the toxicity whereas its aggregated level could be.

3.3. A threshold of aggregated polyQ protein characterizes cell death onset

As previously indicated, phenotypes aggravate while flies producing Atx3Q78tr age. We thus wondered whether the level of aggregates of pathological polyQ proteins increased with aging. Interestingly, neither aggregated nor soluble levels of Atx3Q78tr significantly changed between 2- and 30-day-old flies (Fig. 1C, black bars and Fig. S5A). As assessed by eye degeneration, toxicity was already detectable in two-day-old Atx3Q78tr-expressing flies