Manipulations of Protective Post-Translational Modifications of Ataxin-3 as a Possible Treatment of SCA3

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Abstract

Expansions of amino acids on ubiquitous proteins lead to many neurodegenerative diseases. Spinocerebellar ataxia 3 is a polyglutamine disorder in which an expansion of glutamine residues causes cerebellar neurodegeneration. SCA-3 is the most common autosomal dominant ataxia. It is associated with coordination and speech problems, as well as muscular atrophy. This progressive illness stems from dysfunction of ataxin-3, due to an abnormally long glutamine tract. Ataxin-3 is heavily modified after its translation. The literature suggests that ubiquitination and phosphorylation are protective, while proteolysis leads to harmful fibril accumulation. Previous studies have linked the individual effects of increasing ubiquitination, increasing phosphorylation, and blocking proteolysis to improvements in SCA3-like pathology. These post-translational modifications occur simultaneously in humans, but no research has investigated them in combination in model organisms. Here, we hypothesize that combining protective post-translational modifications (ubiquitination and phosphorylation) and inhibiting harmful ones (proteolysis) would lead to a decrease in SCA-3 pathology. Our goal is to study through genetic manipulation ubiquitination together with phosphorylation, phosphorylation with blocked truncation, ubiquitination with blocked truncation, and all three modifications together in three model systems: mice, flies, and cell lines. Data from this study could be used in the search for new treatments.

Background

A common characteristic of neurodegeneration is the accumulation of proteins that come out of solution, misfold, and form aggregates (1). This process could result from mutations in the protein that misfolds, breakdowns in autophagic pathways, or environmental factors (1). The spinocerebellar taxias are examples of neurodegenerative diseases (2). This family of disorders encompasses over 35 diagnoses, all of which are autosomal dominant and are related to coordination and balance problems (2). Spinocerebellar ataxia 3 (SCA-3, also called Machado-Joseph Disease) is the most common dominantly inherited ataxia (3). Symptoms begin in middle adulthood, but the appearance of initial signs does not correlate with age of diagnosis (4). Characteristics of this neurodegenerative disorder include poor coordination and balance, a rigidity of muscles, muscular atrophy, and problems with swallowing and speech (5). In the brain, the cerebellum, pons, and medulla oblongata decrease in size (6). The fourth ventricle often increases as the aforementioned regions atrophy (6). Neuronal nuclear inclusions and cytoplasmic inclusions characterize the pathology of SCA-3 (7). These aggregates are found throughout the brain, but they are most concentrated in the cerebellar dentate nucleus (8). Both types of inclusions stain for the protein ataxin-3 and for ubiquitin (9, 6).

SCA-3, like many other spinocerebellar ataxias and Huntington's disease, is a polyglutamine disorder (10). A genetic defect on chromosome 14q32.12 leads to a series of abnormally-expanded CAG repeats in the DNA (11). As a result, ataxin-3 contains abnormally long tracts of glutamine residues (10). While the healthy version contains 12-44 repeats, diseased ataxin-3 often exhibits 60-87 (12), causing the protein to misfold as antiparallel strands of beta sheets (13). Ataxin-3 generally functions as a deubiquitinase. The Josephin domain cuts ubiquitin-ubiquitin bonds on substrates, while the ubiquitin interacting motifs position the chain to be cleaved (6). Normal-length ataxin-3 restores eye degeneration in flies with knock-ins of polyQ ataxin-3 (14). Thus, the normal form of ataxin-3 may have a protective role, curbing the toxicity of itsberrant versions.

SCA-3 research has diverged in many directions. Promising studies have shown that in a mouse model, caloric restriction could prevent motor deficits through upregulation of sirtuins involved in the autophagic breakdown of ataxin-3 (15). Other researchers have focused on the interaction between parkin and ataxin-3, showing that aberrant ataxin-3 leads to a downregulation of parkin and contributes to the Parkinsonian symptoms often associated with SCA-3 (16). Another area of interest is the use of RNA interference to knock down aberrant ataxin-3 and reverse motor problems in mice (17). Although this research has yielded many insights, it has been conducted at the expense of more basic biological questions, such as the role that post-translational modifications play in the structure and function of ataxin-3. Ataxin-3 is heavily modified, with ubiquitination, phosphorylation, and proteolysis serving as the main examples (18).

Evidence for ubiquitination could be found in intranuclear inclusions, which stain positive for ubiquitin (9). In Drosophila, blocking ubiquitination prevents ataxin-3 from restoring eye degeneration caused by the polyQ versions (19). Thus, this posttranslational modification has a protective role. Decreasing ubiquitination through a knockout of the U box of E4 increases the aggregation of ataxin-3, providing further evidence that ubiquitination is protective (20). Mutating the catalytic cysteine on the Josephin domain both inactivates ataxin-3 and increases its ubiquitination (21). Dysfunction likely recruits ubiquitination as a defense.

Phosphorylation also appears to be protective. In rats, mutating the twelfth residue (serine) of ataxin-3 to aspartic acid mimics constitutive phosphorylation and reduces aggregation, neuronal loss, and synaptic loss (22). In vitro, CK2-dependent phosphorylation controls the nuclear localization, aggregation, and stability of ataxin-3 (23, 24). This modification acts to stabilize the protein, and helps decrease the formation of inclusions.

Finally, the cleavage of ataxin-3 may lead to the accumulation of harmful fragments (25). When ataxin-3 is truncated at the 221st amino acid, a stable 37kDa fragment is formed (26). Ataxin-3 cleavage is mediated by calcium dependent enzymes such as calpains and caspases (27 & 28). The fragment that accumulates includes the nuclear localization signal, resulting in neuronal nuclear inclusions. The polyQ expansion increases the toxicity of the truncated fragments in mice (27). In fruit flies, the caspase inhibitor zVAD-fmk reduces neurotoxicity and the number of 37kDa fragments (28). In addition, the orally administered calpain inhibitor BDA-410 prevents ataxin-3 cleavage and correlates with a reduction in the size of intranuclear inclusions (29). Thus, truncation of ataxin-3 could lead to pathology, while blocking it is correlated with protective effects.

In vivo, post-translational modifications rarely occur one at a time, but the combined effects of ubiquitination, phosphorylation, and truncation on ataxin-3 toxicity have not been investigated. In this proposal, we delineate the creation of double and triple mutants that combine post-translational modifications in their protective forms in mice, flies, and cell cultures. We hypothesize that phosphorylation together with ubiquitination will be a protective combination. Similarly, we expect to observe protective effects when ubiquitination is combined with blocked truncation, phosphorylation is combined with blocked truncation, and with all three simultaneous modifications (upregulation of ubiquitination and phosphorylation with blocked proteolysis). This study may spur future research focusing on therapeutic targets for SCA-3.

Relevance

Broader Relevance: SCA-3 is the most common form of dominant spinocerebellar ataxia, but it is a rare disease. It affects fewer than 3 out of 100,000 people worldwide, but in the Azores Islands, 39 out of 100,000 are at risk (30). SCA-3 and related ataxias are extremely debilitating, with symptoms ranging from nystagmus to nearly complete loss of the ability to coordinate motor movements. The behavioral tests outlined for the fly and mouse models in this proposal could help determine whether targeting simultaneous post-translational modifications of ataxin-3 could alleviate symptoms and reduce disease-associated mortality.

Intellectual Relevance: Post-translational modifications are common ways to manipulate the structure and function of protein. By observing the effects of ubiquitination, phosphorylation, and truncation ataxin-3 in model systems, we hope conclude the interplay between them. This research will allow us to pinpoint the mechanisms by which the toxicity of aberrant ataxin-3 could be alleviated. The results obtained by combining the three manipulations would be more realistic than the findings of previous studies, as ataxin-3 is modified simultaneously. We hope that by identifying the combinations of post-translational modifications that yield the best reversal of the disease phenotype, we would be

able to identify targets for non-invasive therapy. Additionally, as SCA-3 is a poly-Q disease, our new understanding of protective mechanisms could generalize to other poly-Q disorders, such as HD, contributing to the development of treatments.

Specific Aims

The goal of this study is to investigate the effects of simultaneously facilitating protective post-translational modifications of ataxin-3 and blocking those that are harmful. We predict that combining post-translational modifications in a way that reinforces their beneficial effects would decrease the pathology induced by the polyQ expansions in our model organisms. If realized, these findings would set the groundwork for potential therapies of SCA-3.

1. Investigate the interaction of modifications in Mus musculus: The following combinations are to be investigated in mice expressing ataxin-3: increasing levels of phosphorylation and ubiquitination, increasing ubiquitination while decreasing proteolysis, increasing phosphorylation while decreasing proteolysis, and increasing phosphorylation and ubiquitination while decreasing proteolysis. These combinations were chosen as all three individual manipulations have protective effects. Transgenic mice for upregulated ubiquitination and phosphorylation will be crossed to create double mutants. Reduced proteolysis will be achieved by administration of calpain and caspase inhibitors.

2. Investigate the interaction of modifications in Drosophila melanogaster: We will replicate the same combinations in a fly model. We will cross flies transgenic to create double or triple mutants.

3. Investigate the interaction of modifications in a cell-based model: The same combinations are to be investigated in cell lines of iPSC neurons. We will convert fibroblasts from SCA-3 patients and healthy controls into cerebellar dentate nuclear neurons.

Research Methods and Design

1. Investigate the interaction of modifications in Mus musculus

Rationale: Mouse models have been used previously to investigate the effects of post-translational modifications of ataxin-3. Multiple studies have shown that mice accurately display the behavioral and neuropathological deficits of SCA-3. Furthermore, the effects of decreased ataxin-3 cleavage in transgenic SCA-3 mice were shown to improve the disease phenotype (26, 27, 29). The protective effects of artificial phosphorylation of S12 were demonstrated in a rat model of SCA-3 (22). Ubiquitination of expanded ataxin-3 also appears to be protective against SCA-3 progression. However, combinations of multiple protective post-translational modifications of ataxin-3 have yet to be investigated in mouse models. We expect that by mimicking combinations of these post-translational modifications in transgenic SCA-3, mice would exhibit a recovery from disease.

1. Description of Experiments:

We will use a transgenic knock-in mouse model adapted from Ramani et al., 2014 (31), which utilized homologous recombination to generate homozygous expanded ataxin-3 mice. These mice present SCA-3 like motor deficits and increased levels of the C-terminal ataxin-3 fragment found in human intranuclear inclusions (31). We intend to mimic disease by knocking in human ataxin-3 with a polyglutamine expansion of 82 repeats (Atx3Q82) to make mutants following the procedure of Ramani et al., 2014 (31). Knock-in of human ataxin-3 with 27 repeats (Atx3Q27), within the non-disease range, will serve as a control. To mimic increased phosphorylation within Ramani et al.'s targeting vector for diseased mice, the serine at position 12 will be changed to aspartic acid (Atx3Q82/S12E), as demonstrated by Matos et al. 2016 (22). To mimic increased ubiquitination of expanded ataxin-3, E3 Gp78 will be upregulated via incorporation of Ying et al.'s (2009) Gp78 upregulation vector (32) which will be subcloned into the Ramani ataxin-3 vector (Atx3Q82/Gp78). To mimic decreased proteolytic cleavage of expanded ataxin-3, calpain inhibitor BDA-410 and caspase inhibitor zVAD-fmk will be administered following the protocol of Simoes et al., 2014 (29) and Jung et al., 2009 (26). All single mutant modifications will be replicated in the healthy non-expanded ataxin-3 mice. PCR will be used to confirm that the target mutations were created successfully.

2. Generating Multiple Mutants:

To demonstrate the effects of manipulated post-translational modifications double mutant groups will be generated for both diseased mice and non-expanded control mice. Combining increased phosphorylation and ubiquitination will yield an Atx3Q82/S12E/Gp78 mutant. To combine increased phosphorylation and reduced proteolytic ataxin-3 cleavage, we will use an Atx3Q82/S12E single mutant that will be administered BDA-410 and zVAD-fmk. To combine increased ubiquitination and reduced proteolytic cleavage, a single Atx3Q82/Gp78 mutant will be administered BDA-410 and zVAD-fmk. Finally, the Atx3Q82/S12E/Gp78 double mutant will be administered BDA-410 and zVAD-fmk to combine all three modifications.

3. Immunohistochemical and Behavioral analyses:

To examine the effects of these post-translational modifications of ataxin-3 on disease symptoms, tissue analyses, and behavioral tests will be performed. Neurodegeneration will be quantified through measurements and comparison of volume and mass of brain regions. Tissue sections from the cerebellum, striatum, and cortex will be stained with the ataxin-3 specific antibody 1H9 (33). Intranuclear neuronal inclusions and cytoplasmic inclusions will be quantified and compared to controls. Balance beam, rotarod, and open field assessments will be used to compare conditions and quantify the degree of motor deficits or improvements. Results will be compared across conditions using the appropriate ANOVAs in SPSS.

Prediction: Previous research investigated the consequences of manipulating post-translational modifications of ataxin-3 individually and found alleviation of behavioral and neuropathological symptoms. We predict that by combining the beneficial post-translational modifications in a mouse SCA-3 model, the disease phenotype will be normalized. By increasing phosphorylation and ubiquitination and by decreasing ataxin-3 cleavage, we expect to see improved health.

2. Investigate the interaction of modifications in Drosophila melanogaster

Rationale: Fly models of SCA-3 have been used to define the abnormalities induced by poly-Q expansions (34). We hope to build on Warrick et al.'s and Tsou et al.'s work (14, 19). These studies suggest that ubiquitination of ataxin-3 is protective, but they do not observe this modification in conjunction with other post-translational alterations. We believe that by combining ubiquitination with either a phosphorylation mimic, a proteolysis blocker, or both, we would be able to reinforce the protective effects of ubiquitination alone. We propose using more two separate manipulations for each modification, as the fly model allows for a large sample size, and the possibility that our data is due to a confounding effect would be reduced.

1. Manipulations of Post-Translational Modifications of Ataxin-3 in Drosophila:

We will use a fly model similar to the one in Warrick et al. 1998, where flies express ataxin-3 with 78 glutamine repeats in their eyes under a Gal-4 UAS system for all genetic manipulations (14). We will repeat all experiments in control flies expressing ataxin-3 with 27 polyQ repeats (35). To model constitutive ubiquitination, we will breed transgenic flies that express ubiquitin-COOH-terminal aldehyde (Ub-Al), a nonhydrolyzable ubiquitin analog (36). Flies in which CHIP (the E3-ubiquitin ligase of ataxin-3) is upregulated in the eyes could serve as a second model of enhanced ubiquitination (19). To constitutively mimic phosphorylation, we will create a Drosophila strain in which serine 12 and serine 256, the two main phosphorylation sites of ataxin-3 (22) are mutated to glutamic acid, as suggested in Shiba-Fukushima et al., 2014 (37). An alternative constitutive phosphorylation mimic would be a transgenic fly expressing upregulated casein kinase 2, which phosphorylates ataxin-3 (38). To block the proteolysis of ataxin-3, we will use calpain A RNA interference (CalpA knockdown) directed selectively to the eyes via a Gal-4 UAS system (39). Alternatively, calpain inhibition could be achieved by treatment of the flies with BDA-410 (29). We will evaluate the success of each manipulation in all models with the same methods. Constitutive ubiquitination could be detected through the assay described in Choo et al., 2009 (40). The S12E/S256E mutations could be visualized through gel electrophoresis after the mutagenesis experiments, and the success of calpain knockdown and inhibition could be determined by a Western blot. As an additional control, we will observe flies expressing unmodified wild-type ataxin-3 with 78 glutamine expansions.

2. Creation of Multiple Mutants:

Once these single mutants are established, we will cross flies to create multiple mutants. In organisms expressing ataxin-3 with 78 repeats (disease condition) and 27 repeats (control condition), we will create a Ub-AI/S12E/S256E mutant in which constitutive ubiquitination and phosphorylation are combined (flies in which CHIP and CK2 are upregulated will be bred as a second source of this manipulation). We will also create a Ub-AI/CalpA KD mutant (and a corresponding CHIP mutant treated with BDA-410, a calpain inhibitor), investigating the effects of constitutive ubiquitination and blocked proteolysis. We will use a S12E/S256E/CalpA KD mutant (and a CK2 mutant treated with BDA-410) to model constitutive phosphorylation together with blocked truncation. Finally, we will create a triple Ub-AI/S12E/S256E/CalpA KD mutant (and a corresponding CHIP/CK2 mutant treated with BDA-410), in which all three manipulations are combined.

3. Functional Assays:

In the 78 and 27 repeat conditions, the three single and four multiple mutants are to be subjected to the same functional assays. In Drosophila, Poly-Q expanded ataxin-3 leads to degeneration of the rhabdomere lattice in the pupil (34). We will use light and scanning electron microscopy to characterize the effects of our manipulations and to measure retinal depth. Immunostaining with the anti-HA antibody will be used to visualize the presence of polyQ repeat aggregates in inclusions (14). Although flies develop no brain pathology, we will quantify the number of individuals that develop blindness (by measuring action potentials through the optic nerve), tremors, and early death (14). The results of the assays are to be analyzed using SPSS. For each assay, we will compare the results for both experimental and control flies by running ANOVAs appropriate for each condition.

Prediction: Previous research suggests that the single mutants expressing constitutively ubiquitinated and phosphorylated ataxin-3 would show a rescue of eye structures and retinal depth, more cytoplasmically diffuse ataxin-3, and a lower rate of blindness, tremors, and death, compared to controls (19). The single mutant blocking truncation should display a similar phenotype. All double mutants should exhibit greater improvements than the single mutants.The triple mutant would yield even more protection against pathogenesis. One alternative explanation for data that follow this pattern is that flies endogenously express many cysteine-based deubiquitinases. These proteins may have a similar function to ataxin-3, making it difficult to determine if combining post-translational modificationsrescued the function of ataxin-3. It would not be possible to prevent this confound by knocking down these proteins, as flies would not survive without them.

3. Investigate the interaction of modifications in iPSC neurons

Rationale: Patient-derived neurons have been used to establish a model for researching the abnormalities associated with SCA-3 (41). Expanding upon the work that was done by Koch et al. in 2011, we will use patient-derived human neurons in the last stage of our research. Human neurons have been used to study the effects of proteolysis on ataxin-3, but these effects have not been studied in conjunction with other ataxin-3 alterations (42). We believe that combining a proteolysis blocker with either a phosphorylation mimic, ubiquitination, or both, will further reinforce the protective effects of just one protective modification taken individually.

Description of Experiments: To further expand upon current research we will use a human neuron model similar to the one proposed in Koch et al., 2011 (41). Diseased human fibroblasts will be harvested and converted into IPSCs which will be used to create excitatory human dentate neurons, based on the methods outlined in Zhang et al., 2013 (43). A control group will be created, in which neurons will be formed in the same way but from non-diseased patients. The same mutations will be repeated in both the control group and the experimental condition. Gel electrophoresis of extracted DNA will be used to confirm the successful genetic modification of the cell.

Creation of Multiple Mutants: The effects of increased phosphorylation, blocked proteolysis, and increased ubiquitination will be assessed in vitro. Constitutive phosphorylation will be modeled by creating a point mutation on the S12 residue of the ataxin gene by changing the serine to aspartic acid (22). Furthermore, we will use protein casein kinase 2 (CK2) to increase phosphorylation in serine 340 and serine 352 as a second source of increasing phosphorylation (43). These mutations are shown to mimic the net effects of phosphorylation. We will decrease proteolysis by administering calpastatin which has been shown to decrease proteolysis (44). Lastly, we will increase ubiquitination by overexpressing CHIP (the E-3 ubiquitin ligase of ataxin-3), resulting in net constitutive ubiquitination of ataxin-3. Non-diseased neurons will also receive the same genetic manipulations to serve as a control. Furthermore, a group of diseased neurons not receiving any treatment will be created to serve as an additional control.

We plan to create the following experimental conditions: mimicking ubiquitination together with phosphorylation, mimicking ubiquitination together with blocking proteolysis, mimicking phosphorylation together with blocking proteolysis, and mimicking ubiquitination and phosphorylation together with blocking proteolysis.

Functional Assays: Pathology in the model will be analyzed through TH staining to analyze neuronal function. In addition, the model will also be analyzed through Western blotting in order to analyze whether or not the protein is forming aggregates. The results of the assays are to be analyzed using SPSS software. We will run the appropriate ANOVAs for each assay, comparing the control and experimental conditions.

Prediction: Previous research leads us to believe that all single mutants that increase phosphorylation, increase ubiquitination, and block proteolysis will show increased neuronal function as well as less aggregation of Ataxin-3. Double mutants will likely show the same neuronal protection but to a higher degree. Furthermore, we believe that the triple mutant will show even more protection against the harmful effects of the poly-Q expansion in SCA3.

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