Proteolytic Cleavage of Ataxin-7 Promotes SCA7 Retinal Degeneration and Neurological Dysfunction

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ABSTRACT

The neurodegenerative disorder spinocerebellar ataxia type 7 (SCA7) is caused by a polyglutamine (polyQ) expansion in the ataxin-7 protein, categorizing SCA7 as one member of a large class of heritable neurodegenerative proteinopathies. Cleavage of ataxin-7 by the protease caspase-7 has been demonstrated in vitro, and the accumulation of proteolytic cleavage products in SCA7 patients and mouse models has been identified as an early pathological change. However, it remains unknown if a causal relationship exists between ataxin-7 proteolysis and in vivo SCA7 disease progression. To determine if caspase cleavage is a critical event in SCA7 disease pathogenesis, we generated transgenic mice expressing polyQ-expanded ataxin-7 with a second site mutation (D266N) to prevent caspase-7 proteolysis. When we compared SCA7-D266N mice with SCA7 mice lacking the D266N mutation, we found that SCA7-D266N mice exhibited improved motor performance, reduced neurodegeneration, and substantial lifespan extension. Our findings indicate that proteolysis at the D266 caspase-7 cleavage site is an important mediator of ataxin-7 neurotoxicity, suggesting that inhibition of caspase-7 cleavage of polyQ-ataxin-7 may be a promising therapeutic strategy for this untreatable disorder.
INTRODUCTION

Spinocerebellar ataxia type 7 (SCA7) is an autosomal dominant, progressive neurodegenerative disorder. Affected individuals exhibit a marked loss of coordination due to cerebellar degeneration, and suffer vision loss due to retinal photoreceptor degeneration. SCA7 is one of nine CAG - polyglutamine (polyQ) repeat disorders, including Huntington’s disease (HD), spinobulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), and five other forms of cerebellar ataxia (SCA1, 2, 3, 6 & 17). These diseases are characterized by the inter-generational expansion of glutamine-coding CAG triplet repeats in diverse CNS-expressed proteins. In the case of SCA7, the glutamine tract is located in the N-terminal region of the ataxin-7 protein (1). Ataxin-7 is a highly conserved member of the Spt3-Taf9-Gcn5 acetyltransferase (STAGA) complex, a transcriptional co-activator complex that is fundamental to normal cellular function and may play a role in stress response (2, 3).

Expanded polyQ tracts are inherently toxic. When expressed at a sufficient level, they cause cellular dysfunction and death, even when removed from their normal protein context (4, 5). Despite this observation, the protein context in which the glutamine tract resides is a key modifier of polyQ-disease protein toxicity. This is underscored by an apparent lack of correlation between the expression patterns of polyQ disease proteins and the neuroanatomical areas of pathology observed in the different polyQ diseases (6-8). One well-recognized contextual factor is the site-specific cleavage of polyQ disease proteins by proteases (9). A role for proteolytic cleavage in polyQ disease pathogenesis was first suggested by the discovery that
huntingtin (Htt) protein can be cleaved *in vitro* by extracts taken from apoptotic cells (10). It was subsequently shown that only N-terminal epitopes of Htt are detectable in protein aggregates (11-14), and neo-epitopes resulting from cleavage events can be detected in presymptomatic and mildly affected HD patients (15). Fragments in HD can also form by non-proteolytic processes, such as aberrant splicing (16). The role of protein context in mediating the toxicity of polyQ disease proteins is particularly well established in HD. YAC128 mice that express full-length human Htt with 128 glutamine residues show HD-like signs, as do HD transgenic “fragment” models that express only Htt exon 1 (17). Furthermore, YAC128 mice that express a caspase-6 resistant version of human Htt do not develop neurodegeneration and motor deficits, while equivalent mice lacking this point mutation do (18).

In our first transgenic mouse model of SCA7, we detected an N-terminal proteolytic truncation fragment of ataxin-7 that migrated at a molecular mass of ~ 55 kDa (19). To fully characterize ataxin-7 proteolysis, we tested the ability of various caspases to cleave ataxin-7, and we found that recombinant caspase-7 cleaves ataxin-7 *in vitro* (20). Site-directed mutagenesis of two putative caspase-7 cleavage sites (D266N and D344N) eliminated caspase-7 proteolysis, diminished polyQ-ataxin-7 toxicity and aggregation, and ameliorated polyQ-ataxin-7 transcriptional interference in cell culture. These studies suggested that cleavage at the aspartic acid residue at amino acid position 266 is primarily responsible for the reduction of toxicity. In this study, we examined the role of ataxin-7 proteolytic cleavage in SCA7 disease pathogenesis in transgenic mice, and found that an intact proteolytic cleavage site at amino acid position 266 is required for polyQ-ataxin-7 neurotoxicity.
RESULTS

Production and Characterization of Transgenic Mouse Lines

In our earlier work, we created a SCA7 transgenic mouse model by placing an ataxin-7-92Q cDNA under the control of the murine prion protein (MoPrP) promoter, as this transgenic vector typically drives neuronal and retinal expression at levels similar to endogenous murine SCA7 (21, 22). To determine if caspase cleavage of ataxin-7 contributes to SCA7 neurotoxicity in vivo, we began with a similar MoPrP-ataxin-7 construct and mutated the aspartic acid residue at amino acid position 266 to an asparagine (D266N), as prior work demonstrated that caspase-7 can cleave ataxin-7 protein at this amino acid residue, and expression of ataxin-7-92Q-D266N yields reduced toxicity in a SCA7 neuron cell culture model (20). Prior to microinjection, we confirmed that both PrP-ataxin-7 cDNA transgenic constructs produce full-length ataxin-7 protein by Western blot analysis of transiently transfected mouse neuroblastoma (Neuro2A) cells (Fig. 1B). After we identified transgene-positive PrP-ataxin-7-92Q and PrP-ataxin-7-92Q-D266N founders, we crossed the founders onto a C57BL/6J strain background and measured the expression of human transgenic ataxin-7 relative to murine endogenous ataxin-7 by competitive RT-PCR (22). We chose this RNA-based quantification method because ataxin-7 protein is known to form insoluble aggregates that are difficult to quantify by Western blot analysis. In this way, we obtained five lines of transgenic mice, and established four lines of transgenic mice (i.e. two lines from each genotype) with similar ataxin-7-92Q expression levels (Fig. 2A, Table 1). We confirmed full-length protein expression of ataxin-7 by Western blot analysis of cerebellum and retina (Fig. 2B). In light of the nearly identical transgene expression levels in PrP-ataxin-7-92Q line 1963 (hereafter referred to as the “SCA7” line) and in PrP-ataxin-7-92Q-
D266N line 1239 (hereafter referred to as the “D266N” line), we focused primarily upon these two lines during the course of this study (Table 1).

The D266N Mutation Prevents Cleavage of Ataxin-7 and Attenuates Retinal Dysfunction

To determine if the D266N mutation affects caspase-7 cleavage of ataxin-7 ex vivo, we incubated cerebellar lysates from SCA7 and D266N lines with recombinant caspase-7. SCA7 lysate without the addition of caspase-7 exhibited very little fragment, while the addition of caspase-7 caused the appearance of an ~55 kDa fragment that was immunoreactive with an antibody raised against the N-terminus of ataxin-7 (Fig. 2C). This cleavage fragment is readily apparent in line 283 (Fig. 2C), a high-expressing SCA7 transgenic line that we could not maintain due to the severity of the phenotype (Table 1). Lysate from D266N mice lacked this cleavage fragment after recombinant caspase-7 treatment (Fig. 2C), indicating that the D266N mutation prevents caspase-7 cleavage of ataxin-7. As reported previously (20), we also observed a minor 65 kDa cleavage product in the presence of the D266N mutation (Fig. 2C, noted by *).

To assess the effect of the D266N mutation on retinal function, we evaluated retinal histopathology and performed electroretinogram (ERG) analysis. Retinal tissue from 24 week-old SCA7 mice exhibited a loss of cone photoreceptor cells and outer nuclear layer thinning, while D266N and WT retinas appeared normal (Fig. 3A-C). Quantification of retinal photoreceptor number revealed a significant reduction in SCA7 mice relative to the D266N line and WT controls (Fig. 3D). Upon ERG analysis, SCA7 mice at 22 weeks of age exhibited a significantly impaired rod photoreceptor depolarization response, while the response of D266N rod photoreceptors was not statistically different from non-transgenic littermates (Fig. 4). This
indicates that the D266N mutation substantially attenuates retinal dysfunction, indicating that cleavage of ataxin-7 is a required step for manifestation of the full SCA7 retinal phenotype.

The D266N Mutation Ameliorates the Neurobehavioral Phenotype in SCA7 Mice

We used multiple behavioral measures to compare the phenotypes of SCA7, D266N and littermate WT mice. In humans, SCA7 causes a progressive loss of coordination due to cerebellar degeneration. We used an accelerating rotarod apparatus to assess the coordination function of these three cohorts of mice. At 8 weeks of age, there was no difference between SCA7, D266N, and WT mice in latency to fall off the rotarod (Fig. 5A). At 14 weeks, however, the SCA7 mice had a shorter latency to fall than either WT or D266N mice (Fig. 5A). To further explore the behavioral phenotype, we used multiple measures of physical coordination and neurological function. We determined the degree of clasping and kyphosis in each line. D266N mice were not different from WT mice in either clasping or kyphosis, while SCA7 mice were significantly impaired in both measures (Fig. 5B). We then assessed the physical coordination of each line using the ledge test. By this measure, WT and D266N mice were not statistically different at 8 or 14 weeks, while SCA7 mice exhibited a robust and statistically significant impairment (Fig. 5B). Finally, as an additional measure, we evaluated the extent of kyphosis as an indication of neuromuscular dysfunction and found that SCA7 transgenic mice scored significantly higher compared to D266N and WT mice (Fig. 5B). Together, this indicates that the D266N mutation confers a substantial degree of protection from SCA7 neurological dysfunction in transgenic mice.
To determine the extent of cerebellar neuropathology in the SCA7 transgenic mice, we performed immunohistochemistry on SCA7, D266N, and WT mice at 24 weeks of age. We observed a modest decrease in Purkinje cell neuron dendritic arborization in the cerebellar molecular layer in SCA7 mice, while Purkinje cell dendritic arborization appeared normal in the D266N mice (Fig. 6A-C). We also measured glutamate uptake in cerebellar synaptosome preparations from SCA7, D266N, and WT mice, and observed a significant decrease in GLAST-mediated glutamate uptake in SCA7 transgenic mice relative to WT and D266N mice (Fig. 6D). D266N transgenic mice displayed glutamate uptake that was not significantly different from WT controls (Fig. 6D).

The D266N Mutation Significantly Prolongs Survival of SCA7 Transgenic Mice

To determine the mean lifespan of SCA7 and D266N mice, we aged mice from matched generations and compared their age of euthanasia due to severe morbidity. SCA7 mice lived an average of 17.1 weeks, while D266N mice survived 57.3 weeks on average (Fig. 7, Table 1). This greater than 3-fold extension of survival time in D266N mice indicates a substantial protection from SCA7 pathology. To statistically analyze the life span extension observed in the D266N mice, we compared the life spans of the SCA7, D266N, and WT cohorts using a Kaplan-Meyer plot (Fig. 7). Statistical analysis of the Kaplan-Meyer plot revealed a highly significant increase in lifespan for the D266N line (p < 0.0001), as >90% of the D266N mice outlived all of their SCA7 counterparts. SCA7 transgenic mice with a second site D344N mutation blocking this proteolytic site were also created and characterized as part of this study, but were not protected from SCA7 behavioral abnormalities, neuropathology, or decreased survival, despite comparable transgene-expression levels (data not shown).
DISCUSSION

Proteolytic cleavage is a common theme in neurodegenerative disease, ranging from inherited polyQ disorders to idiopathic disorders such as Alzheimer’s disease (23). Here we found that mutating the caspase-7 cleavage site of ataxin-7 at an aspartic acid residue at amino acid position 266 significantly attenuates the SCA7 disease phenotype in transgenic mice. D266N mice displayed an extended life span, a mild behavioral phenotype, and only modest abnormalities of retinal histopathology and visual function when compared to SCA7 mice expressing ataxin-7-92Q protein without the D266N mutation. Indeed, even when the ataxin-7-92Q D266N transgene was expressed in a second independent line of mice (Line 2357) at levels that were ~70% greater than ataxin-7-92Q, these D266N transgenic mice still lived twice as long as their SCA7 counterparts and exhibited reduced retinal disease and neurological dysfunction (Table 1). This suggests that proteolytic cleavage of ataxin-7 is a crucial event in the SCA7 disease process, and provides further support for the “toxic fragment hypothesis” which posits that proteolytic processing of polyQ disease proteins is required for full toxicity (24, 25).

In the case of SCA7, caspase-7 has been implicated as the likely protease responsible for cleaving the ataxin-7 protein (20). Caspase-7 is an executioner caspase whose activation typically occurs downstream of so-called initiator caspases (26); however, non-apoptotic activation of executioner caspases has been observed under conditions of CNS stress (27). While mutation of the aspartic acid residue at position 266 of ataxin-7-92Q protein substantially ameliorated the SCA7 disease phenotype, the D266N mice did ultimately develop progressive SCA7 disease that led to their demise. The slow progression of SCA7 polyQ neurotoxicity upon mutation of the putative caspase-7 cleavage site suggests that full-length polyQ-expanded ataxin-
protein is capable of eliciting some degree of cellular toxicity, but that proteolytic cleavage is required for more dramatic neurotoxicity – at least in mice. Our findings thus support a “positive feedback loop” model in which polyQ-expanded ataxin-7 produces an initial cytotoxic insult that elicits a cellular stress response that culminates in caspase-7 activation (9). Consistent with this model, polyQ-expanded proteins have been shown to produce endoplasmic reticulum stress, activate JNK, and thereby de-repress Bax (28, 29). In this way, apoptotic pathway activation could yield activated caspase-7 that cleaves ataxin-7 at the aspartic acid residue at position 266, generating a truncated ataxin-7 fragment that is more toxic than the full-length protein. Cell culture and in vivo models of polyQ diseases suggest that certain truncated polyQ-containing fragments are highly potent in eliciting cell stress responses and apoptotic activation, and this would increase caspase-7 activation in a feed-forward fashion, accelerating the progression to severe neuronal dysfunction and cell death.

Although this new generation of SCA7 transgenic mice recapitulated the cone-rod dystrophy phenotype, neurological disease, and reduced life span of our previous PrP-SCA7 transgenic mice (22), we did not observe abundant polyQ-ataxin-7 aggregates or nuclear inclusions in these PrP-ataxin-7-92Q transgenic mice upon immunohistochemical analysis. Lack of visible ataxin-7 protein aggregates might be attributed to an effect of the FLAG tag upon the N-terminal conformation. This suggests that polyQ-ataxin-7-mediated SCA7 retinal degeneration, neurological dysfunction, and shortened lifespan do not require the production of visible protein aggregates, and is consistent with work done on HD, where soluble levels of diffuse mutant huntingtin protein in a primary neuron model was found to be the best predictor of likelihood of cell death (30). Our results thus further reinforce the view that microscopically visible
aggregates are not the cause of polyQ disease; rather, insoluble conformers that form oligomers are likely the primary pathogenic agent.

How does the truncated ataxin-7-92Q protein fragment produce neurotoxicity? Akin to a number of other polyQ disease proteins with normal functions in transcription, ataxin-7 is known to shuttle into and out of the nucleus, as it possesses functional nuclear localizations signals and a functional nuclear export signal (NES) at amino acid position 341 – 352 (31). When we tested the effect of mutation of the NES domain upon ataxin-7 toxicity in primary cerebellar granule neurons, we noted increased toxicity of NES mutant, full-length polyQ-expanded ataxin-7 that was accompanied by markedly increased nuclear aggregation (31), suggesting that impaired nuclear export of polyQ-expanded ataxin-7 promoted nuclear accumulation of ataxin-7 and cellular toxicity. As activated caspase-7 interacts with ataxin-7 in the nucleus (20), our findings are consistent with a model in which proteolytic cleavage of full-length polyQ-expanded ataxin-7 in the nucleus prevents its egress from the nucleus, promoting its retention and accumulation, thereby driving neurotoxicity. Of particular relevance to this model of nuclear toxicity, ataxin-7 is a component of the STAGA transcription co-activator complex, and studies have shown that polyQ-expanded ataxin-7 can impair the GCN5-dependent histone acetyltransferase activity of the STAGA complex (32, 33). Furthermore, ataxin-7 also serves as an adapter protein between the STAGA transcriptional co-activator complex and the USP22 histone deubiquitinase module, and may serve as a scaffold linking these two chromatin-remodeling activities (34, 35). Both STAGA acetyltransferase activity and USP22 deubiquitination activity are important for the regulation of specific genes, including retinal genes (32, 34). In yeast, loss-of-function of Sgf73, the ataxin-7 orthologue, disrupts the interaction between the yeast STAGA complex (SAGA) and
the yeast USP22 module (Ubp8). This yields a marked reduction in Ubp8-mediated histone H2B deubiquitination, increasing the level of ubiquitinated H2B (36, 37). As H2B ubiquitination regulates gene silencing at specific loci in the yeast genome (37), this likely is a functionally deleterious alteration. As the aspartic acid residue at position 266 in human (and mouse) ataxin-7 is located between two highly conserved regions known respectively as “conserved block I” and “conserved block II”, cleavage at this position may sever the interaction and cross-talk between histone deubiquination and acetylation and thus exert a profound toxic effect upon STAGA complex function. Thus, accumulation of truncated polyQ-ataxin-7 could alter regulation of the USP22 deubiquitination activity or Gcn5 acetyltransferase activity within neurons and glia.

Our results indicate that inhibition of polyQ-ataxin-7 proteolytic cleavage could be a highly effective therapeutic intervention. The D266N transgenic line survived approximately 3 times longer than the SCA7 line, exhibited a substantially attenuated behavioral phenotype, and displayed minimal retinal histopathology and visual dysfunction. If caspase-7-mediated proteolytic cleavage is a critical element of SCA7 disease pathogenesis, then inhibition of caspase-7 cleavage of ataxin-7 may hold promise as a treatment for human SCA7 patients. Therapeutic strategies would include pharmacological options (38), if a specific caspase-7 inhibitor capable of crossing the blood-brain barrier could be identified, or genetic interventions, as targeted delivery of viral vectors expressing a caspase-7 specific RNA interference sequence or of caspase-7 antisense oligonucleotides might be plausible. Since the proteolytic cleavage event occurs early in the pathogenic cascade, our findings in SCA7, together with studies of HD
and AD (23), suggest that therapeutic protease inhibition is worthy of consideration for a broad range of neurodegenerative disorders.

MATERIAL AND METHODS

Transgenic Constructs

Constructs were created from human SCA7 cDNA vectors using site-directed PCR mutagenesis as described previously (20). In contrast to the previous generation of SCA7 transgenic mice, the current constructs carry N-terminal FLAG and C-terminal c-myc epitope tags.

Production of Transgenic Mice

All animal work was approved by the IACUC at the University of Washington, Buck Institute, and UCSD. Constructs were verified for correct electrophoretic migration and expression level by transient transfection into the murine neuroblastoma N2a cell line using Lipofectamine 2000 (Invitrogen). NotI digestion followed by agarose gel separation was used to isolate the expression cassette from the vector backbone. The SCA7 cassette was then microinjected into the pronuclei of oocytes from C57BL/6J – C3H/HeJ F1 hybrid mice. Founders were identified using the following primers designed to selectively amplify the SCA7 cDNA: SCA7-440 F, CCGAGA ATGGATGGCACACT; SCA7-440 R, TGCGGTGGTTGCTGAGAGT. The following primers were designed to amplify a fragment of mouse genomic DNA, and were included in genotyping reactions as an internal control: mPrp-957 F, AAAGGGCTGTTCTCAGAGGCAC; mPrp-957 R, CTGCATGACCTGAAGCAAAATCA. Transgenic mice were backcrossed with C57BL/6J mice for at least ten generations.
Expression Analysis of Transgenic Mice

Expression was determined by competitive PCR. RNA was extracted from cerebellar tissue and reverse transcribed using random hexamer primers. A portion of the cDNA was amplified in the presence of $^{33}$P-labeled dCTP (1.5 $\mu$Ci per 20 $\mu$L reaction) using primers that anneal equally to human and murine ataxin-7 to produce an amplicon of 1322 bp from both the murine gene and human transgene. The primer sequences are 5’-GGAAAAACCGCGAAGTCATGGGGCTCTG-3’ and 5’-CCATAGCTGTGCAT-TCAGATGCTTCTC-3’. The resulting amplicons were digested using Xmn I, which cleaves the murine and human amplicons differently, and then resolved by polyacrylamide gel electrophoresis. The gel was dehydrated and analyzed by phosphorimager (Molecular Dynamics).

Behavioral Analysis of Transgenic Mice

All behavioral experiments were performed in a blinded manner. To measure coordination, mice were challenged on an Economex Accelerating Rotarod apparatus and the latency to fall was recorded. Speed was set to 4 rpm and increased at a rate of 0.1 rpm per second, to a maximum time of 5 minutes. Latency to fall was recorded. This procedure was repeated five times per day for four consecutive days. Statistical significance was determined for each day by one-way ANOVA. Three additional measures were used to assess SCA7-like neurodegenerative signs: the degree of clasping, the ledge test, and the degree of kyphosis. The ledge test has been described previously (19). Clasping, kyphosis, and the ledge test were recorded on a scale of 0-3, with larger numbers indicating a more pronounced phenotype (39). These measures were performed once per day for five consecutive days, and results from each week were averaged.
Statistical differences between groups were examined using one-way ANOVA and Turkey’s multiple comparison test.

**Western Blot Analysis**

Mouse cerebella or retinas were homogenized in sample buffer (62.5 mM Tris-HCl pH 6.8, 4% SDS, 200 mM dithiothreitol, 10% glycerol, 0.001% Bromophenol blue) and heated for 10 min at 95°C. Samples were run on 8% SDS-PAGE gels, transferred to a nitrocellulose membrane and probed using antibody K (22). Samples for *in vitro* digestion were prepared in TPER with complete protease inhibitors and cleavage was performed with recombinant caspase-7 at 37°C for 1 h as previously described (40, 41). For these experiments, we used two lines SCA7 mice-1963 and B283 (higher ataxin-7 expressor).

**Immunohistochemistry**

Mice were deeply anesthetized by intraperitoneal injection of Avertin (240 mg/kg) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brain and eyes were removed and placed in 4% paraformaldehyde for 4 hrs. The brain was cryoprotected in 10% sucrose in PB, then 30% sucrose in PB. 30 μm parasagittal sections were cut from cryoprotected brain tissue using a sliding microtome. Free-floating sections were incubated in blocking solution (10% goat serum, 1% BSA, 0.3% Triton X-100 in PBS, pH 7.4). For retinal IHC, fixed eyes were cut into 12 μm sections after mounting in OCT compound and freezing. Retinal sections were blocked in 2% horse serum, 1% BSA, and 0.1% Triton X-100 in PBS. Cerebellar and retinal sections were probed with primary antibodies in 0.3% Triton X-100 in PBS. Primary antibodies used for IHC in this study were rabbit α-red cone opsins 1:200.
(Chemicon, #AB5405); rabbit α-ataxin-7 1:300 (Affinity BioReagents, #PA1-749); mouse α-calbindin D-28K 1:500 (Swant, #300); and rabbit α-GFAP 1:400 (Dako Cytomation, #Z0334). Primary antibody staining was visualized with fluorophore-conjugated secondary antibodies. For cerebellar IHC, we generated coronal sections from the midpoint of the cerebellum at a thickness of 40 μm for all mice, and compared sets of sections from 3 mice / genotype.

Electroretinograms

Mice were anesthetized using isoflurane gas. A gold wire contact electrode was placed on the corneas, and a reference electrode was inserted subdermally on the dorsal side of the neck. Rod responses were recorded in response to white light flashes from -20 to +20 dB at 10 dB intervals.

Glutamate uptake

Cerebellar synaptosome preparations and glutamate uptake assays were performed as previously described (42).

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CONFLICT OF INTEREST

None declared.
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FIGURE LEGENDS

Fig. 1. Generation and validation of transgenic constructs. (A) Schematic of construct utilized in these studies. MoPrP, blue; FLAG tag, yellow; CAG tract, red; c-myc tag, green. (B) Western blot analysis of Neuro2A cells, transfected with the indicated constructs, confirms expression of full-length ataxin-7 protein (arrows).

Fig. 2. Characterization of SCA7 transgenic mice expressing a proteolytic cleavage site mutation. (A) mRNA expression analysis by competitive RT-PCR. Bands marked with an arrow represent the full-length, murine ataxin-7 amplification product at 1322 bp. Bands marked with an asterisk (414, 420 and 488 bp) represent Xmn I restriction fragments of the human ataxin-7 cDNA. For results of band intensity quantification, see Table 1. (B) Western blot analysis of retinas at 6 months of age. Retinal lysates from non-transgenic (NT), ataxin-7-92Q transgenic (SCA7), and ataxin-7-92Q-D266N transgenic (D266N) mice were immunoblotted with anti-ataxin-7 antibody K to confirm comparable expression of full-length ataxin-7 protein. Loading control = β-actin. (C) Caspase-7 cleavage assay. Mouse cerebellar brain lysates from three different SCA7 lines (SCA7-1963, SCA7-B283 [high-expressor], and SCA7 D266N; see Table 1) were incubated with or without recombinant caspase-7 (indicated with – or + sign), separated by SDS PAGE, and immunoblotted with anti-ataxin-7 antibody K. Loading control = tubulin. Bands marked with an arrowhead correspond to full-length ataxin-7, while bands marked with an arrow represent N-terminal cleavage fragments of ataxin-7. The minor cleavage product generated by cleavage of ataxin-7 at D344 is indicated by an asterisk. Loading control = β-actin. “SCA7” refers to line 1963, and “D266N” refers to line 1239 throughout the text.
Fig. 3. D266N proteolytic cleavage site mutation prevents retinal degeneration in SCA7 transgenic mice.

(A-D) Retinal sections from 28 week-old mice were analyzed by confocal microscopy after propidium iodide staining (cyan). Non-transgenic controls (A) exhibit normal retinal photoreceptor number, while SCA7 transgenic mice (B) display marked loss of retinal photoreceptors, as demonstrated by a thinning of the outer nuclear layer. SCA7 transgenic mice expressing ataxin-7-92Q with the D266N alteration (C) retain a normal complement of retinal photoreceptors. Quantification (D) confirms that outer nuclear layer thickness is diminished in SCA7 mice and normal in D266N mice (n = 4 per group; p < 0.01; one-way ANOVA with Tukey post-test). “SCA7” refers to line 1963, and “D266N” refers to line 1239.

Fig. 4. Electroretinogram analysis indicates that cone photoreceptor function is preserved in D266N transgenic mice.

(A-C) We recorded the retinal response to 10 dB flashes of white light after dark adaptation, and noted reduced amplitude responses in ataxin-7-92Q transgenic mice (B) in comparison to non-transgenic (A) and ataxin-7-92Q-D266N transgenic mice (C). Arrowheads indicate the timing of the light flash (n=3-4 mice per group; 22 weeks of age). “SCA7” refers to line 1963, and “D266N” refers to line 1239.
Fig. 5. Behavioral analysis indicates rescue of neurological disease in D266N transgenic mice.

(A) Rotarod analysis. Mice were tested for coordination deficits using an accelerating rotarod apparatus. Latency to fall was recorded for SCA7 mice (line 1963), D266N mice (line 1239) and non-transgenic controls at 8 weeks and 14 weeks of age. SCA7 transgenic mice exhibit rotarod impairment at 14 weeks of age, however this is not observed in D266N mice (p = 0.006; one-way ANOVA with Tukey post-test). Error bars = SEM. (B) Composite phenotype score. Mice were examined in a blinded fashion for signs of SCA7 neurodegeneration. Clasping, ledge test, and kyphosis were measured on a scale of 0 – 3, with 0 being unaffected and 3 being severely affected. A highly significant difference in performance was noted for SCA7 transgenic mice compared to NT and D266N transgenic mice (p < 0.0001; one-way ANOVA with post-hoc analysis). Using the Kruskal-Wallis non-parametric test, all differences were statistically significant. NT and D266N groups, however, performed comparably, underscoring the protective effect of the proteolytic cleavage mutation upon polyglutamine-expanded ataxin-7 neurotoxicity (n = 7-10 mice / group). Error bars = SEM. “SCA7” refers to line 1963, and “D266N” refers to line 1239.

Fig. 6. Cerebellar histopathology and glutamate uptake defects are rescued in D266N transgenic mice.

(A-C) Cerebellar sections from 28 week-old NT, SCA7, and D266N transgenic mice were immunostained with an anti-calbindin antibody (green) and counterstained with DAPI (blue). As shown here in representative sections, NT mice exhibit a normal cerebellar cytoarchitecture with Purkinje cell dendritic arbors extending to the distal surface of the molecular layer (A). Purkinje
cell dendritic arborization in D266N transgenic mice (C) is comparable to the appearance of the molecular layer in control mice, but Purkinje cell dendritic calbindin immunoreactivity appears reduced in SCA7 transgenic mice (B). (D) We prepared cerebellar synaptosomes from 28 week-old NT, SCA7, and D266N mice, and measured glutamate uptake in the presence of the GLT-1 inhibitor dihydrokainate (DHK) in order to isolate the activity of GLAST. While SCA7 transgenic mice displayed a significant reduction in glutamate uptake compared to either NT or D266N transgenic mice (p < 0.05; one-way ANOVA with Tukey post-hoc analysis), D266N transgenic mice exhibited glutamate uptake that was comparable to NT mice. “SCA7” refers to line 1963, and “D266N” refers to line 1239.

**Fig. 7. The ataxin-7 proteolytic cleavage mutation markedly extends life span in D266N transgenic mice.** Kaplan-Meier survival analysis. Survival data from NT, SCA7, and D266N transgenic mice are shown here for cohorts of mice (n = 12 / group), matched for generation number. A dramatic increase in life span was afforded by the presence of the D266N proteolytic cleavage mutation in cis to the polyglutamine expansion tract in the D266N lineage (p < 0.0001 for SCA7 vs. D266N; Logrank test). “SCA7” refers to line 1963, and “D266N” refers to line 1239.
Table 1. Expression Levels and Average Life Span of SCA7 Transgenic Lines

<table>
<thead>
<tr>
<th>Line #</th>
<th>Transgene</th>
<th>Expression level *</th>
<th>SD</th>
<th>Average Lifespan (wks)</th>
<th>SEM</th>
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<tbody>
<tr>
<td>1963</td>
<td>Ataxin-7-92Q</td>
<td>2.13</td>
<td>0.28</td>
<td>17.1</td>
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<tr>
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<td>Ataxin-7-92Q</td>
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<td>0.21</td>
<td>38.3</td>
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<td>B283</td>
<td>Ataxin-7-92Q</td>
<td>4.19</td>
<td>0.60</td>
<td>7.5</td>
<td>ND</td>
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<tr>
<td>1239</td>
<td>Ataxin-7-92Q-D266N</td>
<td>2.15</td>
<td>0.12</td>
<td>57.3</td>
<td>1.8</td>
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<td>Ataxin-7-92Q-D266N</td>
<td>3.55</td>
<td>0.42</td>
<td>37.2</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*RT-PCR analysis comparing the human ataxin-7 transgene level to endogenous murine ataxin-7 was performed, as previously described (La Spada et al., 2001). All samples were run in triplicate and the average of the three runs was rounded to the nearest 0.01.

We were unable to maintain the high-expressing B283 line beyond the N2 generation.

ND = not determined

ABBREVIATIONS

Spinocerebellar ataxia type 7 (SCA7)

polyglutamine (polyQ)

Huntington’s Disease (HD)

spinobulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA),

cerebellar ataxia (SCA1, 2, 3, 6 & 17).

CNS central nervous system

Spt3-Taf9-Gcn5 acetyltransferase (STAGA)