Calpain-mediated ataxin-3 cleavage in the molecular pathogenesis of spinocerebellar ataxia type 3 (SCA3)

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Spinocerebellar ataxia type 3 (SCA3) is pathologically characterized by the formation of intranuclear aggregates which contain ataxin-3, the mutated protein in SCA3, in a specific subtype of neurons. It has been proposed that ataxin-3 is cleaved by proteolytic enzymes, in particular by calpains and caspases, eventually leading to the formation of aggregates. In our study, we examined the ability of calpains to cleave ataxin-3 in vitro and in vivo. We demonstrated in cell culture and mouse brain homogenates that cleavage of overexpressed ataxin-3 by calpains and in particular by calpain-2 occur and that polyglutamine expanded ataxin-3 is more sensitive to calpain degradation. Based on these results, we investigated the influence of calpains on the pathogenesis of SCA3 in vivo. For this purpose, we enhanced calpain activity in a SCA3 transgenic mouse model by knocking out the endogenous calpain inhibitor calpastatin. Double-mutant mice demonstrated an aggravated neurological phenotype with an increased number of nuclear aggregates and accelerated neurodegeneration in the cerebellum. This study confirms the critical importance of calcium-dependent calpain-type proteases in the pathogenesis of SCA3 and suggests that the manipulation of the ataxin-3 cleavage pathway and the regulation of intracellular calcium homeostasis may represent novel targets for therapeutic intervention in SCA3.

INTRODUCTION

Neurodegenerative diseases such as Parkinson disease, Huntington disease or the spinocerebellar ataxia group (SCA) are proposed to share common pathogenic mechanisms, including generation of toxic fragments by cytoplasmic cleavage, aggregate formation and neurodegeneration in disease specific neurons (1,2). For polyglutamine diseases such as HD and SCA, pathogenesis models suggest cytoplasmic cleavage of the mutant protein leading to nuclear translocation of fragments containing the polyQ tract (3,4). Further studies revealed a much higher toxicity of fragments or truncated polyQ proteins with a shifted propensity to aggregate compared with expanded full-length polyglutamine proteins (reviewed in 5,6). Therefore, cleavage of disease proteins by proteases (e.g. caspases or calpains) is proposed to represent an important step in the pathogenesis of neurodegenerative diseases. For spinocerebellar ataxia type 3 (SCA3), caused by an expanded polyglutamine tract in the encoded ataxin-3 protein, cleavage by caspases (7–11) or calpains (12–14) is controversially discussed. The latter studies did, however, show that the calpain-dependent formation of polyglutamine containing fragments is triggered by calcium in neuroblastoma cells (12) and in patient-specific induced pluripotent stem cell (iPSC)-derived...
neurons (13). In iPSC cells, generation of fragments and aggregate formation were not suppressed after treatment with caspase-1, caspase-3, or pan-caspase inhibitors. In contrast, treatment with ALLN or calpeptin (both calpain inhibitors) resulted in complete disappearance of aggregates (13). Additionally, overexpression of calpastatin, the only known endogenous calpain inhibitor, inhibited cleavage and prevented aggregation of ataxin-3 in neuroblastoma cells (12) and mouse brain (14). Both studies indicated that the occurrence of specific cleavage products was closely correlated with the aggregation process and demonstrated that calpain proteolysis may initiate a harmful aggregation cascade.

The calpain system originally comprised three different important proteins in neurons: the two best characterized members of the calpain family, calpain-1 and calpain-2 and calpastatin, whose only known function is to inhibit these calpains (15). Calpain-1 is activated by calcium concentrations in the micromolar range, whereas calpain-2 is activated by millimolar levels of calcium in vitro. As the activation of calpains is an irreversible process, calpain activation must be strictly controlled. Calpastatin binds to the inhibitory domain on both sides of the active site cleft in a reversible manner to inhibit calpains in vivo (16). The calpain system is also critical for development, as mice lacking the common regulatory 30 kDa calpain subunit (17) or calpain-2 (18) die prenatally, whereas mice lacking calpain-1 (19) or calpastatin (20) are phenotypically normal.

To gain more insights into the impact of proteolytic cleavage of ataxin-3 in vitro, we investigated whether enhancing calpain cleavage modifies polyQ-expanded ataxin-3 in terms of cellular toxicity and formation of aggregates. Therefore, SCA3 transgenic mice overexpressing human ataxin-3 with 140Q (21) were crossed with mice lacking the intracellular calpain inhibitor calpastatin (Cast KO; 20). We focused on the question whether ataxin-3 is cleaved by calpain-1 or calpain-2 in mouse brain in vivo and how this process would influence aggregate formation and neuropathology. In summary, we demonstrate for the first time that ataxin-3 is a substrate of calpain-1 and calpain-2 in vitro and in vivo and that these proteases present promising targets for therapeutic intervention.

RESULTS

Ataxin-3 is a sensitive calpain substrate in vitro

To determine whether ataxin-3 is a calpain-sensitive substrate in vitro, we investigated the cleavage of overexpressed ataxin-3 with different polyglutamine lengths (15Q, 77Q, 148Q) in HEK293T cells. After incubation with 2 mM CaCl₂ and either recombinant calpain-1 or calpain-2 for up to 45 min, ataxin-3 was cleaved more efficiently with recombinant calpain-2. Whereas most of full-length ataxin-3 was still detectable after 45 min of calpain-1 incubation (Fig. 1A–C), the length of the polyglutamine tract modulates calpain-2 cleavage of ataxin-3 (Fig. 1D–F). Full-length ataxin-3 with 15Q is nearly fully cleaved by calpain-2 after 45 min of incubation (Fig. 1D). On the other hand, cell lysates with an expanded polyglutamine tract revealed a complete cleavage of full-length ataxin-3 after 30 min (77Q) or 15 min (148Q) of incubation, respectively (Fig. 1E and F). A clear N-terminal-derived cleavage fragment of around 30 kDa was detected after calpain-1 and -2 cleavage independently of the polyQ lengths (Fig. 1A–F). Inhibition of calpain cleavage by incubation with ALLN resulted in no fragmentation of full-length ataxin-3 regardless of the polyglutamine length, indicating that the observed cleaved ataxin-3 fragments were generated by calpain cleavage.

Generation of double-mutant Cast KO (−/−)/SCA3 mice

To further investigate the role of calpain cleavage in the pathogenesis of SCA3 in vivo, we crossbred calpastatin knock-out mice (20) with our SCA3 disease model (21). This SCA3 mouse model exhibits striking intergenerational instability of the expanded CAG stretch which modifies age at onset of symptoms and progression of the disease as it has been described for other polyQ models as well (22–26). SCA3 mice with 140Q demonstrated an ataxic and neurological phenotype as well as premature death, starting at the age of 6–9 months. In subsequent experiments, we therefore only used mice with 140Q, which are referred to as SCA3 in the following data. We confirmed that mutant ataxin-3 was expressed and calpastatin was knocked out in brains of double-mutant mice (dm-mice) by western blot and immunohistochemical analyses, whereas both mutant ataxin-3 and endogenous calpastatin were expressed in single-transgenic SCA3 mice (st-mice) (data not shown). We also confirmed for each mouse the polyQ size of 140.

Calpastatin activity determines calpain cleavage rate of ataxin-3 ex vivo

To determine the cleavage rates of endogenous and overexpressed ataxin-3 ex vivo when calpastatin is normally expressed or fully abolished, we used brain homogenates from three independent animals of wild-type mice, homozygous calpastatin knock-out mice [Cast KO (−/−)] as well as SCA3 st- and dm-mice [Cast KO (−/−)/SCA3] in a cleavage assay. Here, we found that endogenous ataxin-3 in brain homogenates from wild-type mice was sensitively cleaved by calpain-1 and calpain-2 in the presence of 2 mM calcium, respectively. However, cleavage by calpain-2 occurs more rapidly and more efficiently. For both enzymes, calpain-1 and calpain-2, the same N-terminal-derived ataxin-3-specific cleavage fragments at around 30 kDa (Fig. 2A and E) were detected as seen before in the cell culture analyses (Fig. 1). Specificity of calpain cleavage was confirmed by incubation with the calpain inhibitor ALLN. Knocking out calpastatin completely, we observed a faster degradation of endogenous mouse ataxin-3 after incubation with calpain-1 (Fig. 2B) as well as an increased amount of the 30 kDa cleavage fragment (Fig. 2I). However, cleavage by calpain-2 occurred immediately and more efficiently and revealed a very strong 30 kDa cleavage fragment after 5 min of incubation (Fig. 2F and J). Analyzing mice with overexpressed human ataxin-3 demonstrated that after 5 min of incubation with calpain-1 and calpain-2, almost no overexpressed protein is detectable (Fig. 2C and G). Similar results were found after knocking out calpastatin (Fig. 2D and H). The 30 kDa N-terminal-derived cleavage fragment occurred independently from overexpressed
Quantification of the 30 kDa cleavage fragment demonstrated a more rapidly produced fragment if calpastatin is knocked out completely independently of the expression of a polyglutamine expanded human ataxin-3. The results were similar using either calpain-1 or calpain-2 (Fig. 2I and J).

Calpastatin knock-out increases mutant ataxin-3 fragmentation upon calcium stimulation

To quantify soluble mutant ataxin-3, we applied a highly sensitive time-resolved Förster resonance energy transfer (TR-FRET) assay, using the ataxin-3 antibody 1H9 (terbium cryptate-labeled) and the expanded polyQ-specific MW1 (D2-labeled; Fig. 3A). Both antibodies are flanking a previously described potential cleavage site of ataxin-3 at amino acid position 260 (12) and exhibit fluorescence energy transfer in close proximity. Using the TR-FRET assay, we assessed the amount of soluble-mutant ataxin-3 in the various genotypes in native cerebellar brain lysates, and additionally upon increased calpain-1 or calpain-2 activation via calcium stimulation over time (TR-FRET).

As expected, since the MW1 antibody is specific against an expanded polyQ stretch (≥6 CAGs), almost no ataxin-3 signal was observed in Cast KO (−/−) and wild-type mice. In contrast, in st-line SCA3 and in dm-mice a TR-FRET signal was detected. Notably, the signal intensity in the dm-mice was only half of that of the st-line SCA3. After calcium stimulation, the FRET signal decreases in cerebellar brain lysates of st-line and dm-mice to half of the normal level (Fig. 3B). This indicates that decreased detection of soluble-mutant ataxin-3 after calcium induction was due to increased calpain cleavage at amino acid position 260 (Fig. 3A).

Knock-out of calpastatin leads to an increased number of aggregates and neurodegeneration in SCA3 mice

Aggregate formation was analyzed in brain regions with high expression of calpain-1 and calpain-2 (confirmed by the ALLEN Brain Atlas; www.brain-map.org) as well as SCA3 transgene expression, including cerebellum and pons. Using ataxin-3 immunostaining of brain slices, we found the
highest number of ataxin-3-positive nuclear inclusions (NII) in dm-mice at the age of 12 months. Significantly fewer aggregates were detected in mice of the st-line SCA3 confirmed by quantitative analysis of aggregates per cell in the cerebellum and pons (Fig. 4A and B). Furthermore, even in SCA3 mice, in which calpastatin is knocked out heterozygously [Cast KO (+/−)/SCA3], the number of neurons with NII is dramatically increased compared with st-line SCA3 (Fig. 4), suggesting that loss of one calpastatin allele was sufficient to enhance the formation of aggregates. Immunofluorescence staining with calpain-1 or calpain-2 antibodies revealed no co-localization of calpains with aggregates (data not shown). To investigate whether the aggravated phenotype in dm-mice was due to alterations in neuronal pathology and neurodegeneration, we performed toluidine blue staining and immunostaining with an antibody against neurofilament in the cerebellum of all genotypes at the age of 12 months. Immunostaining with a neurofilament antibody and toluidine blue revealed a strong atrophy of Purkinje cells in the st-line SCA3 and the dm-mice, but nearly no aberrant cells in Cast KO (−/−) mice and wild-type controls, respectively (Fig. 5A). Quantitative analysis of the percentage of degenerated, atrophic cells of toluidine blue staining revealed significantly more neurodegeneration in dm-mice compared with st-line SCA3 (P ≤ 0.001), Cast KO (−/−) or wild-type mice (P ≤ 0.001). However, the neurofilament staining revealed only significant differences of both disease lines compared with the controls but not between st-line SCA3 and dm-mice (Fig. 5B).

Depletion of calpastatin induces an advanced and more severe behavior phenotype in SCA3 mice

To assess the ataxic and neurological phenotype of the mouse models, we performed rotarod tests and monitored the survival rate as well as the body weight. Measuring the body weight every second week, a significant decrease in body weight in the st-line SCA3 and both dm-lines [Cast KO (+/−)/SCA3 and Cast KO (−/−)/SCA3] compared with controls was found starting at the age of 24 weeks. Whereas wild-type mice and mice of Cast KO (−/−) line continuously gained weight up to the age of 60 weeks, the st-line SCA3 and both dm-lines progressively lost weight, in the end more than one-third of the body weight of control mice. On the other hand, we did not find any significant differences between

Figure 2. Calpastatin deficiency increases endogenous ataxin-3 cleavage. Whole-brain homogenates of the indicated animals were incubated up to 15 min with 2 mM calcium and recombinant calpain-1 (A–D) or 2 (E–H), respectively. Stars highlight endogenous mouse ataxin-3, arrows indicate human overexpressed full-length ataxin-3 with expanded polyQ and arrowheads point to an N-terminal-derived cleavage fragment. (A–D) Cleavage of ataxin-3 by recombinant calpain-1 occurs slowly; after 15 min of incubation only little endogenous or overexpressed ataxin-3 is cleaved. (E–H) Ataxin-3 cleavage by recombinant calpain-2 revealed an increased cleavage rate, which is enhanced when calpastatin (Cast) is knocked out. Both calpain-1 and calpain-2 cleavage result in a 30 kDa N-terminal-derived fragment, respectively. Densitometric quantification of the 30 kDa fragment relative to time point zero and loading control GAPDH revealed more accumulation of the cleavage fragment if calpastatin is knocked out completely over time (I, J). SEM = standard error of mean.
both control groups [wild-type and Cast KO (\(2/2\))] and between the disease lines (SCA3 and both dm-lines, Fig. 6A), respectively. The progressive loss of body weight resulted in a premature death of all disease lines, shown in the Kaplan–Meier curve (Fig. 6B). Although, there was a clear tendency that heterozygous or homozygous knock-out of calpastatin in SCA3 mice leads to a decreased life span compared with st-line SCA3, this difference did not reach significance. In confirmation to the published data of Takano et al. (20), the Cast KO (\(2/2\)) showed no premature death (Fig. 6B). Furthermore, the disease lines (SCA3, both dm-lines) showed neurological symptoms with tremor, clasp ing and gait abnormalities from the age of 40 weeks onwards. To detect motor coordination abnormalities in the different lines, we performed rotarod tests. Dm-mice showed impaired motor capabilities on the rotarod with a significant difference starting at the age of 32 weeks for Cast KO (\(+/-\))/SCA3 mice and at the age of 44 weeks for Cast KO (\(-/-\))/SCA3 mice, whereas no significant coordination deficits were detectable in the st-line SCA3 or Cast KO (\(-/-\)) mice at this age (Fig. 6C). While it is surprising that Cast KO (\(+/-\))/SCA3 mice seemingly show an earlier onset of motor deficit in the rotarod test at the age of 32 weeks, it should be noted that there was no significant difference between Cast KO (\(+/-\))/SCA3 and Cast KO (\(-/-\))/SCA3 mice. Also, it has to be kept in mind that Cast KO (\(-/-\))/SCA3 mice started to die earlier (Fig. 6B). This has reduced the number of available mice for the rotarod test in the Cast KO (\(-/-\))/SCA3 mice group to a larger extent and therefore, has reduced the statistical power in this group. Moreover, the

**Figure 3.** Calpastatin knock-out increases mutant ataxin-3 fragmentation upon calcium stimulation. (A) Schematic representations of antibody-binding sites in the context of the ataxin-3 protein and the principles of the TR-FRET assay. (B) TR-FRET analysis of cerebellar protein lysates of mice with indicated genotypes at the age of 12 months, without calcium stimulation or with appropriate calcium levels for calpain-1 or calpain-2 activation. In wild-type and Cast KO (\(-/-\)) mice only a background signal is detectable. Specific signals were found in st-line SCA3 and dm-mice. Importantly, the signal intensity in the dm-mice was only half of that of SCA3 single transgenic mice. Upon calcium stimulation, the ataxin-3-specific signal decreases significantly in dm-mice. Bars represent means of three different mice per genotype. **P \(\leq 0.01.\)
surviving mice in the Cast KO (−/−)/SCA3 group might have a milder phenotype leading to this slightly better performance on the rotarod.

DISCUSSION
Caspases (7–11) and calpains (12–14) have been demonstrated to be involved in the proteolysis of ataxin-3. To gain more insights into the role of proteolytic cleavage in the pathogenesis of SCA3, we focused on calpain cleavage, specifically if ataxin-3 is a calpain-1 or calpain-2-sensitive substrate, as it was previously shown for huntingtin (27). Identification of the key protease that cleaves ataxin-3 is of particular importance as this will pave the way for developing a targeted therapy. We demonstrated that overexpressed ataxin-3 is cleaved more efficiently by calpain-2 than by calpain-1 and that expanded ataxin-3 seems to be more sensitive to calpain degradation. That calpain-2 can cleave ataxin-3 more efficiently can be further supported by the expression pattern of calpain-1 and calpain-2 in wild-type C57Bl/6 mice as shown in the database of the ALLEN Brain Atlas (www.brain-map.org). Calpain-2 is highly expressed in the most affected brain areas by SCA3 including cerebellum, pons and medulla oblongata (expression value ≥2.5). In contrast, calpain-1 shows only a very low expression in the cerebellum (expression value ≤0.5) but normal expression values in the pons and medulla (≤2.0), respectively.

Our in vitro results were confirmed in vivo as we showed that endogenous wild-type murine and overexpressed human-expanded ataxin-3 are calpain-2-sensitive substrates independently of the polyQ length. Furthermore, by knocking out calpastatin, endogenous and polyQ-expanded ataxin-3 is further destabilized. These results suggest that modifying the level of calpains (in particular, calpain-2) or of calpastatin will influence the rate of proteolysis of ataxin-3 and subsequently the generation of potentially toxic fragments ultimately leading to neurodegeneration. Indeed, knocking out calpastatin in our SCA3 transgenic mouse model (21) leads to a worsening of behavioral and neuropathological abnormalities. This is in agreement with reports that in the brain of patients, transgenic mice and cell models of Alzheimer disease, as well as in the animal models for retinal neurodegeneration, calpastatin depletion can act upstream of calpains to activate a calcium-

Figure 4. Increased number of nuclear inclusions in the dm-lines. (A) Sections stained with an ataxin-3-specific antibody (1H9) revealed ataxin-3-positive nuclear inclusions in the cerebellum and pons (indicated by an arrow). Notably, in the dm-lines with heterozygous or homozygous loss of calpastatin function, an increased number of aggregates were detected. No nuclear inclusions were found in wild-type and Cast KO (−/−) mice. Scale bar = 20 μm. (B) Quantitative analysis of aggregates per cells in the cerebellum and pons revealed significantly less aggregates in the st-line SCA3 compared with both dm-lines (**P ≤ 0.001). Results are expressed as means ± SEM.
dependent cascade of protein kinase activation, hyperphosphorylation and proteolysis resulting in neurodegeneration (28,29). Therefore, it has been proposed that calpastatin acts as a positive regulator against unwanted protein cleavage during calpain activation (15) and under pathological conditions as a negative regulator of calpains (20,30). Calpastatin, therefore, is an attractive target for developing therapeutic approaches in SCA3, in particular as neither overexpression (28) nor ablation of calpastatin (20) has any effect on normal nervous system’s function in mice under physiological conditions.

Based on our findings, we propose a role of ataxin-3 cleavage by calpains in the pathogenesis of SCA3. By applying a new assay (TR-FRET) to detect the level of soluble-mutant ataxin-3, we found less soluble-mutant ataxin-3 in dm-mice compared with st-line SCA3, suggesting cleavage of ataxin-3 within the 63 amino acids separating the binding sites of the antibodies 1H9 and MW1. Interestingly, Haacke et al. (12) proposed a cleavage site for calpain-2 in ataxin-3 at amino acid position 260, which is positioned between the two antibodies we used. The fragments derived from cleaving ataxin-3 around this position have been shown to be highly susceptible to aggregation (12) and are associated with toxicity (31). As well, we have previously described a genetrap mouse model, which expresses 259 amino acids N-terminal of ataxin-3 and which partially mimics a possible cleavage at position 260. In these mice, we observed SCA3-like symptoms and a strong cytoplasmic accumulation of the N-terminal part of the protein in the brain (32). Additionally, a calculation of the molecular weight of the first 259 amino acids of the mouse and human ataxin-3 protein using a Protein Molecular Weight calculator (Science gateway) revealed a fragment size of 29.7 kDa for mouse and 29.93 kDa for human N-terminal ataxin-3 (first 259 amino acids). The calculated size is consistent with the detected 30 kDa N-terminal fragment in the presented cleavage assays of overexpressed ataxin-3 in cell culture and mouse brain homogenates. In addition, the C-terminal fragments including the polyQ stretch have a much higher toxicity and propensity to aggregate than expanded full-length polyglutamine proteins (31). In support of this hypothesis, we found a significantly increased number of aggregates in the granular layer of the cerebellum in dm-mice.

In summary, our results indicate that endogenous murine ataxin-3 and human-expanded ataxin-3 are cleaved by calpains, especially by calpain-2, and that increased proteolytic cleavage of ataxin-3 results in a more severe and faster progressing neurological phenotype. Therefore, aberrant activation of calpains and consequently enhanced proteolytic

Figure 5. Calpastatin knock-out leads to enhanced neurodegeneration in the cerebellum of SCA3 mice. (A) In 12 months old mice striking atrophy of Purkinje cells was detected in the st-line SCA3 and the dm-line indicated by empty baskets in the neurofilament staining and by dark blue staining of Purkinje cells by toluidine blue. Scale bar = 20 μm. (B) Quantitative analysis revealed more severe neurodegeneration in the dm-mice and st-line SCA3 compared with controls. Furthermore, toluidine blue staining demonstrated significantly more neurodegeneration of Purkinje cells in dm-mice compared with the st-line SCA3 (**P ≤ 0.01, ***P ≤ 0.001). Results are expressed as means ± SEM.
cleavage of ataxin-3 may play a pivotal role in SCA3 pathogenesis. Thus, the ataxin-3 cleavage pathway and the molecules involved in it, in particular calpastatin, may represent potential targets for therapeutic intervention in SCA3. This perspective is particularly exciting since calpain inhibitors entering the brain are already in clinical trials (33).

Figure 6. Calpastatin knock-out aggravates behavioral deficits in SCA3 mice. (A) Body weight measurements revealed a significant reduction of body weight starting from the age of 24 weeks in the st-line SCA3 and dm-lines compared with controls. No significant differences were found between the disease lines. (B) Cumulative survival using Kaplan–Meier estimator revealed premature death in the disease lines (st-line SCA3 and the dm-lines) compared with controls. Between the disease lines, no significant differences in the survival rate were detected ($P = 0.226$). (C) Rotarod analysis demonstrated abnormalities in balance and motor coordination in animals of dm-lines. No differences were found between wild-type and the st-line for SCA3 ($P = 0.899$). Results are expressed as means ± SEM. ($^*P \leq 0.05$, $^{**}P \leq 0.01$).
MATERIALS AND METHODS

Expression constructs

In order to generate ataxin-3 constructs with the C-terminal V5 tag, the stop codon of ataxin-3 (ataxin-3c isoform containing a third ubiquitin interacting motif (UIM), NM_004993.5) was replaced by an XbaI restriction site via PCR using the reverse primer MJDnoStopXbaRev (TAAAGGctgtaTTTTTTTCTCTGTGTTT). Full-length ataxin-3 was then cloned into the vector pcDNA 3.1/V5-His (Life Technologies) using BamHI and XbaI. Expanded CAG repeats (77 and 148 CAG, respectively) were inserted into the constructs by employing Esp3I and PpuMI.

Cell culture

HEK293T cells were maintained in Dulbecco’s modified eagle medium supplemented with 10% fetal calf serum, 1% non-essential amino acids and 1% penicillin/streptomycin at 37°C in 5% CO2. Transient transfections with V5-tagged ataxin-3 constructs with different polyglutamine lengths (15Q, 77Q, 148Q) were performed using the Attractene transfection reagent (QIAGEN) following the manufacturer’s instructions followed by 72 h incubation. To obtain protein lysates, HEK293T cells were mechanically homogenized in calpain reaction buffer (2 mM HEPES/KOH pH 7.6, 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol) using a Qiashredder homogenizer (QIAGEN) according to manufacturer’s instructions.

Preparation of brain homogenates

Dissected mouse brains were homogenized in 1× PBS buffer with 1% Triton-X 100 and complete protease inhibitor without EDTA (Roche Applied Science) using a Dounce homogenizer (Fisher Scientific).

Calpain activation assay

For in vitro calpain activation assays of cell culture lysates or brain homogenates, 200 ng of total protein were incubated with calpain-1 or calpain-2 in calpain reaction buffer containing 2 mM CaCl2 at room temperature for indicated time points. To inhibit calpain activity, 100 μM N-acetylleucylleucylnorleucinal (ALLN; Sigma Aldrich, Inc.) were added to the respective reactions. All reactions were quenched by adding 5× Laemmli buffer and denatured for 5 min at 95°C prior to western blot analysis. For quantification of the 30 kDa fragment, three brain samples were inserted into the constructs by employing Esp3I and PpuMI.

Generation and genotyping of the double-mutant mouse model

Hemizygous SCA3 mice overexpressing human ataxin-3 containing 140 CAGs under the control of the Prp promoter were crossbred with homozygous calpastatin knock-out mice (Cast KO (+/−), st-SCA3, double-mutant Cast KO (+/−)/SCA3 and double-mutant Cast KO (+/−)/SCA3 mice. Offspring with the expected Mendelian distribution of genotypes for both breeding steps were achieved (data not shown). For genotyping, we used the primers and conditions described in the respective publications. The number of CAG repeats for all animals was analyzed using the primers pre-CAG for (5′-GCTAAGTGATGC AAGGTAGTTCC-3′) and post-CAG-rev (5′-CAAGTGCTC TGAACCTGGT-3′). The forward primer is labeled at the 5′ end with the fluorescent dye Cy5. The PCR amplicon length was then analyzed on the Beckman coulter sequencer (CEQ 8000 Cycle Sequencer, Krefeld, Germany).

Western blot analysis

Western blot analyses were performed as previously described (21). Antibodies were used at the following dilutions: mouse anti-ATXN3 (1:4000; MAB 5360; clone 1H9; Millipore), rabbit anti-calpain-1 (1:2500 in 3% BSA; ab39170), rabbit anti-calpain-2 (1:2500; ab39168) (both from Abcam), rabbit anti-murine calpastatin (1:2000; Ref. 20), mouse anti-GAPDH (1:5000; sc-47724; Santa Cruz Biotechnology, Inc.), as well as peroxidase-conjugated secondary antibodies goat anti-mouse (1:2000; 115-035-003; Jackson Immuno-Research) and donkey anti-rabbit (1:3333; NA934; GE Healthcare Biosciences).

Immunohistochemistry

Immunohistochemistry was performed as previously described (21). The following antibodies were used: rabbit anti-ATXN3 (1:1000; Ref. 34), rabbit anti-calpain-1 (1:100; ab39170), rabbit anti-calpain-2 (1:100; ab39168) (both from Abcam), rabbit anti-murine calpastatin (1:2000; Ref. 20), mouse anti-GAPDH (1:5000; sc-47724; Santa Cruz Biotechnology, Inc.), as well as fluorescence-coupled antibodies rabbit anti-Cy2 (1:50) and mouse anti-Cy3 (1:100) (both from Dianova).

Staining with toluidine blue (Sigma Aldrich, Inc.) was done for 10 min in 0.2% toluidine blue in sodium acetate buffer. Dehydration steps with 70, 96 and 100% ethanol were then performed for 5 min each.

Aggregates and neurodegenerative cells were manually counted from three independent individuals in the cerebellum and pons from three animals of each indicated genotype at the age of 12 months. For quantifying aggregates or neurodegenerative cells, four fields per brain region were counted on three different sections of each mouse.

Time-resolved FRET assay

Cerebellar samples from indicated genotypes were homogenized in homogenization buffer (10% PBS, 1% Triton X, complete without EDTA). After diluting lysates 1:1 in homogenization buffer, the reactions were carried out by adding 3 μM or 2 mM CaCl2 to activate calpain-1 or calpain-2,
respectively, and incubated for 5 min at 30°C. The reactions were terminated by adding 100 pmol ALLN and 2.5 µmol EDTA. As control, the samples were incubated under the same conditions with 3 µM or 2 mM CaCl2 and 100 µM ALLN. The reactions were stopped by addition of pure 2.5 µmol EDTA.

Polyglutamine-specific antibody MW1 was developed by Patterson et al. (35) and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute for Child Health and Human Development and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA, USA). Ataxin-3 antibody was obtained from Millipore (1H9; Mab5360). MW1 antibody was labeled with acceptor fluorophore D2. Ataxin-3 antibody was labeled with terbium-cryptate (Tb) as donor fluorophore. A TR-FRET detection protocol was performed as previously described (36). In short, 5 µL sample and 1 µL detection buffer (50 mM NaH2PO4, 400 mM NaF, 0.1% BSA and 0.05% Tween + antibodies) was pipetted into low-volume wells of an opaque 384-microtiter plate and incubated for 20 h at 4°C. The final amounts of detection antibody per well were 0.3 ng ataxin-3-Tb and 10 ng MW1-D2. TR-FRET quantification was performed with an Envision reader (PerkinElmer). The terbium donor fluorophore was excited at 320 nm. After a time delay of 100 µs, an emission signal of D2 was detected at 665 nm. All signals are reported as the percentage signal intensity over the lysis buffer background signal.

**Phenotype analysis**

Body weight was determined every second week for 8–10 animals per group starting at the age of 4 weeks. Behavioral experiments were performed in the dark cycle when mice were more active. Behavioral testing began at the age of 8 weeks and was repeated every 8 weeks until the age of 44 weeks. For rotarod tests, mice were placed on an accelerating rotating rod at the same time of the day for 5 consecutive days. Three training sessions (each with acceleration from 4–16 rpm in 2 min) were followed by two test sessions. Each test trial started with rotation at 4 rpm and accelerated to a maximum of 40 rpm within 5 min. The amount of time that elapsed before the mouse fell off the rod was recorded. The trials within the same day were performed ~1 h apart. Eight to ten age- and sex-matched animals were analyzed per group. All experiments were conducted in a blind-coded manner with respect to genotype until an overt phenotype was visible.

The survival rate was measured by recording the life span of mice that died naturally. As the license to carry out experiments on mice requires that mice are euthanized when disease exceeds defined, moderately severe, humane end points, only a relatively small number of animals were used to determine the survival rate: wild-type: n = 28; Cast KO (−/−): n = 8; SCA3: n = 5; Cast KO (+/−)/SCA3: n = 16; Cast KO (−/−)/SCA3: n = 7.

**Statistical analysis**

All data were analyzed by using JMP® version 8.0 (SAS Institute, Inc., Cary, NC, USA). Rotarod tests, survival rate, measurement of body weight, TR-FRET assay and immunohistochemistry were investigated using an unpaired Student’s t-test with a significance threshold of P < 0.05. All results are presented as means ± standard error mean.

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**Conflict of Interest statement.** None declared.

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