N-terminal ataxin-3 causes neurological symptoms with inclusions, endoplasmic reticulum stress and ribosomal dislocation

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Mutant ataxin-3 is aberrantly folded and proteolytically cleaved in spinocerebellar ataxia type 3. The C-terminal region of the protein includes a polyglutamine stretch that is expanded in spinocerebellar ataxia type 3. Here, we report on the analysis of an ataxin-3 mutant mouse that has been obtained by gene trap integration. The ataxin-3 fusion protein encompasses 259 N-terminal amino acids including the Josephin domain and an ubiquitin-interacting motif but lacks the C-terminus with the polyglutamine stretch, the valosin-containing protein binding region and part of the ubiquitin-interacting motif 2. Homozygous ataxin-3 mutant mice were viable and showed no apparent anatomical defects at birth. However, at the age of 9 months, homozygous and heterozygous mutant mice revealed significantly altered behaviour and progressing deficits of motor coordination followed by premature death at ∼12 months. At this time, prominent extranuclear protein aggregates and neuronal cell death was found in mutant mice. This was associated with disturbances of the endoplasmic reticulum-mediated unfolded protein response, consistent with the normal role of ataxin-3 in endoplasmic reticulum homeostasis. Thus, the ataxin-3 gene trap model provides evidence for a contribution of the non-polyglutamine containing ataxin-3 N-terminus, which mimics a calpain fragment that has been observed in spinocerebellar ataxia type 3. Consistent with the disease in humans, gene trap mice develop cytoplasmic inclusion bodies and implicate impaired unfolded protein response in the pathogenesis of spinocerebellar ataxia type 3.

Keywords: ataxin-3; calpain cleavage; endoplasmic reticulum stress; gene trap model; Josephin domain

Abbreviations: ERAD = endoplasmic reticulum-associated protein degradation; IBMPFT = frontotemporal dementia associated with inclusion body myopathy and Paget’s disease; SCA3 = spinocerebellar ataxia type 3; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
Introduction

Ataxin-3 protein consists of a highly conserved N-terminal Josephin domain followed by two ubiquitin-interacting motifs; the C-terminal region includes a valosin-containing protein-binding site, a polyglutamine stretch and a third ubiquitin-interacting motif depending on the isoform (Goto et al., 1997; Schmidt et al., 2002). Expansion of the polyglutamine stretch beyond 55 residues causes autosomal dominant spinocerebellar ataxia type 3 (SCA3), also known as Machado–Joseph disease (reviewed in Riess et al., 2008). The neurological disorder is associated with selective loss of neurons in distinct regions of the brain, although ataxin-3 protein is expressed ubiquitously (Ross, 1995; Nishiyama et al., 1996; Paulson et al., 1997a; Wang et al., 1997; Schmidt et al., 1998; Trottier et al., 1998).

A histological hallmark of the disease is intranuclear inclusion bodies in a subset of neurons, but mechanisms that lead to the formation of these aggregates are largely unknown. Intranuclear inclusion bodies contain the C-terminal part of ataxin-3 including the expanded polyglutamine stretch, ubiquitin, transcription factors, molecular chaperons and components of the 26S proteasome (Paulson et al., 1997b; Schmidt et al., 2002). In cellular and animal models, it has been shown that the C-terminal fragment of expanded ataxin-3 alone is more toxic than the full-length protein with the polyglutamine stretch, suggesting that ataxin-3 cleavage might be a contributing factor to SCA3 pathology (Ikeda et al., 1996; Paulson et al., 1997b; Warrick et al., 1998; Yoshizawa et al., 2000; Goti et al., 2004). Indeed, putative cleavage fragments of expanded ataxin-3 were identified in cell culture (Yamamoto et al., 2001; Berke et al., 2004) and in post-mortem brains of a SCA3 mouse model and patients (Goti et al., 2004; Colomer Gaud et al., 2006). Polyglutamine stretch-expanded ataxin-3 exhibits several potential cleavage sites for caspases (Wellington et al., 1998; Yoshizawa et al., 2000; Berke et al., 2004; Haacke et al., 2006; Mauri et al., 2006; Colomer Gaud et al., 2007) and calcium-dependent calpain proteases including those that have recently been identified at amino acid positions 60, 200 and 260 (Haacke et al., 2007). Particularly, the putative calpain cleavage site at amino acid position 260 seems to be important for high-level accumulation of ataxin-3 aggregates in vitro (Haacke et al., 2007).

Whereas in SCA3 the crucial pathogenic role of the polyglutamine stretch containing C-terminal ataxin-3 fragment is beyond question, the contribution of the N-terminal ataxin-3 fragment is ambiguous. However, the recent observation of widespread neuropil protein inclusions in patients with SCA3 (Seidel et al., 2004; Wellington et al., 1998) suggests a possible involvement of the N-terminal ataxin-3 fragment in the pathogenesis of SCA3. Here, we report on the identification and characterization of a novel ataxin-3 mutant allele in mouse that has been generated as part of a large-scale gene trap approach (Wiles et al., 2000; Hansen et al., 2003). Ataxin-3 mutant mice express a fusion protein between 259 N-terminal amino acids of ataxin-3 including the Josephin and one of the ubiquitin-interacting motif domains and β-galactosidase. Homozygous (ataxin-3<sup>350/350</sup>) and heterozygous (ataxin-3<sup>350/350</sup>) mutants die prematurely and display progressively abnormal behaviour and neurological symptoms that are reminiscent of transgenic SCA3 mouse models (expanded polyglutamine stretch) and patients with SCA3. The dominant mutant phenotype includes cytoplasmic inclusion bodies, significantly altered endoplasmic reticulum-mediated unfolded protein response and death of neurons. Thus, our SCA3 mouse model provides evidence that the N-terminal portion of ataxin-3 protein without the polyglutamine stretch can lead to toxic effects in cells that are similar to those observed with the expanded protein in the classical SCA3 model.

Materials and methods

Generation of the ataxin-3 gene trap mouse

In a large-scale gene trap approach, PT<i>1</i>|geo plasmid DNA was transferred by electroporation into mouse embryonic stem cells. Individual clones were then selected and expanded to identify trapped genes by rapid amplification of complementary DNA ends-polymerase chain reaction and nucleotide sequence determination of fusion transcripts, as described previously (Wiles et al., 2000; Hansen et al., 2003). The embryonic stem cell clone A041D06, which was found to contain the gene trap vector integration in the ataxin-3 gene, was used to generate chimeric mice by morula aggregation with wild-type embryos (E2.5) obtained from superovulated CD1-females (Charles River). Following overnight culture of aggregates, blastocysts were transferred to foster mothers and chimeric offspring were mated to albino CD1 mice to generate heterozygous ataxin-3<sup>350/350</sup> progeny. Mutant mice were analysed after a minimum of seven backcrosses to C57BL/6 mice.

Genotyping by Southern blot analysis and polymerase chain reaction

For genotyping, DNA was isolated from tail biopsies by overnight incubation in lysis buffer (100 mM Tris–HCl, pH 8.5, 200 mM NaCl, 0.2% sodium dodecyl sulphate, 5 mM EDTA, 0.5 mg/ml proteinase K) at 55°C followed by extraction with phenol/chloroform/isooamyl alcohol (25:24:1), precipitation with ethanol and dissolving the DNA pellets in Tris–EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Southern blot analysis was performed using DNA digested with PstI restriction endonuclease and a <sup>32</sup>P-labelled hybridization probe (957 bp) that was generated by polymerase chain reaction amplification of exon8/intron8 sequence (forward primer: 5’-CAT AAG TCG CCA GGA ATT CG-3’; reverse primer: 5’-CAA GGC ATT CCT AAC AAC CT-3’) of the ataxin-3 gene. This probe detects fragments of 7.2 kb resembling wild-type alleles and of 6.7 kb detecting mutant gene trap fragments, respectively. Genotyping by polymerase chain reaction was performed using Qiagen Taq polymerase (Qiagen). The forward primer 5’-GTG CTG AGA CAC TCA AAG C-3’ localized at the beginning of intron 8 was combined with two reverse primers: R-WT 5’-CAT GTG CAC TAT GTG TCT GTG-3’ localized in exon 9, which amplifies the wild-type allele, and R-GT 5’-CTT CGG TAC TAT CAC GAC AGC TG-3’ localized in the PT1|geo vector, which specifically amplifies the mutant allele. Polymerase chain reaction conditions were as follows: 5 min at 95°C, 35 cycles of 45 s at 94°C, 60 s at 55°C and 90 s at 72°C, and a final extension for 5 min at 72°C.
Generation of deletion constructs

Plasmid constructs were made according to the standard cloning procedures (Sambrook and Russell, 2001). To generate the deletion construct of ataxin-3, which includes the first eight exons of the ataxin-3 gene, we used the ataxin-3 complementary DNA Machado–Joseph disease 1-1 as template (Goto et al., 1997; Ichikawa et al., 2001). To generate an exact copy of the gene trap model, which lacks the lacZ and neomycin-resistant cassette of the PTBgene vector, we added new restriction sites for XhoI and HindIII by using primers ataxin-3_F 5’-CCG TTC GAG ATG GAG TAC TTC CA-3’ and ataxin-3_R 5’-CCC AAG ATT CCT ACC TTG CAT ACT TAG CGT-3’. The resulting N-terminal fragment of the ataxin-3 gene was cloned into the pEGFP-N1 vector via XhoI/HindIII restriction sites.

Histochemical β-galactosidase staining

The brain and part of the thoracic vertebral column were dissected, rinsed in phosphate-buffered saline and fixed in 2% paraformaldehyde and 0.25% glutaraldehyde overnight. The brain was then cut sagittally and the spinal cord in transverse direction. Tissues were washed in 0.01% sodium deoxycholate, 0.02% Nonidet P40 and 2 mM magnesium chloride in phosphate-buffered saline. Staining for β-galactosidase activity of the fusion protein was performed at 30°C overnight in X-gal staining solution (2 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.02% NP40, 0.01% sodium deoxycholate, 1 mg/ml X-gal in dimethylformamide and 20 mM Tris, pH 8.0, in 1× phosphate-buffered saline).

Western blot analysis

Western blot analyses were performed as described previously (Bichelmieier et al., 2007). Antibodies were used at the following dilutions: mouse anti-ATXN3 (1:4000; MAB 5360, 1H9), mouse anti-β-galactosidase (1:2000, MAB 3468) (both from Chemicon); rabbit anti-ATXN3 (1:500, Schmidt et al., 1998); mouse anti-p97 ATPase (alternative name valosin-containing protein; 1:200, Cat No. 65278, Progen); mouse anti-β-actin (1:50.000, A5441) and goat anti-rabbit (1:10.000, A9169) (both from Sigma Aldrich, Inc.), as well as goat anti-mouse (1:2000, 115-035-003, Jackson ImmunoResearch) and rabbit anti-elF2α phospho-Ser52 (1:500, KAP-CP131, Stressgen). The antibodies against VIMP and Derlin-1 are described previously (Ye et al., 2004; Wang et al., 2006).

Immunohistochemistry

Immunohistochemistry was performed as described previously (Bichelmieier et al., 2007). The following antibodies were used: mouse anti-β-galactosidase (1:400, #2372 from Cell Signalling Technology, Inc.), rabbit anti-β-galactosidase (1:250; #200-4136, Rockland Immunochemicals, Inc.), mouse anti-β-galactosidase (1:1000, MAB 3468), mouse anti-ATXN3 (1:2000; MAB 5360) (both from Chemicon), phosphorylated mouse anti-neurofilament (1:250, M0762, DakoCytomation), rabbit anti-ubiquitin (1:500, Z-0458, from DakoCytomation), mouse anti-ubiquitin (1:1000, sc-8017, Santa Cruz Inc.).

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

Quantitative real-time polymerase chain reaction

For the detection of differentially regulated genes, we isolated RNA from the whole mouse brain using RNeasy® Midi Kit (Qiagen). RNA quality was validated using a RNA 6000 NanoChip (Agilent Technologies) and subjected to complementary DNA synthesis using the Transcriptor First Strand complementary DNA Synthesis Kit (Roche Applied Science). Complementary DNAs amplified using a LightCycler® TaqMan® Master Kit (Roche Applied Science) were analysed in triplicates using a LightCycler® 480 system (Roche Applied Science) and Universal Probe Library probes (Roche Applied Science) for detection. Primer pairs used for polymerase chain reaction amplification are listed in Supplementary Table 1.

Terminal dUTP nick-end labelling

To demonstrate neurodegeneration in the cerebellum of 12-month old SCA3-mutated mice, we used a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay kit conjugated with TMR red (Roche Applied Science) as directed in the manufacturer’s instructions. The brains of three male mice per genotype were prepared after sacrificing the mice with CO2 and directly frozen to −20°C. The unfixed brains were embedded in tissue freezing medium (Leica Microsystems GmbH) and sectioned sagittally (14 μm) in a cryotome (JUNG CM3000, Leica). For the microscopic analyses, a Zeiss Imager Z1 Apotome Microscope with a Zeiss Axiocam digital camera was used, and data analysis was performed with the Zeiss Axiosvision 4.7 software.

Conventional electron microscopy

Electron microscopy of mouse embryonic fibroblast cells and brains of SCA3 mutated and wild-type mice were performed as previously described (Lundkvist et al., 2004). One mouse per genotype was analysed at the ages of 3 and 12 months. The procedure followed the description in Bichelmieier et al. (2007).

Electron microscopy with immunogold labelling

Post-embedding immunogold labelling was performed on ultrathin sections of Lowicryl®-embedded specimens. The tissues were fixed with 3% paraformaldehyde plus 0.1% glutaraldehyde in phosphate buffer, pH 7.2, for 4 h, then dehydrated and embedded in Lowicryl® K4M at low temperature in a Balzers low-temperature embedding apparatus (LTE 020; Balzers), followed by ultraviolet polymerization at low temperature in a Balzers freezer (FTP 010; Balzers). The sections were cut on a Leica Ultracut R at 50–70 nm and mounted on Pioloform-coated nickel grids (SCI). For on-section labelling, grids were floated on drops of the following solutions and transferred using a nickel-coated copper loop. Initially, the grids were floated for 5 min on Tris-buffered saline followed by blocking buffer (5% (w/v) bovine serum albumin, 0.1% (v/v) cold water fish skin gelatin (Biotrend), 5% (v/v) normal goat serum (Biotrend), and 0.04% NaN₃ in Tris-buffered saline) for 30 min to minimize non-specific binding. The primary monoclonal antibody (mouse anti-ATXN3; Chemicon; MAB 5360) was diluted (1:50) in incubation buffer (0.8% (w/v) bovine serum albumin, 0.1% (v/v) cold water fish skin gelatin, and 0.04% NaN₃ in Tris-buffered saline), and the sections were incubated overnight at 4°C. The sections were washed several times in incubation buffer. In order to ease the observation of gold grains, grids were incubated for 1 h with two secondary antibodies (diluted 1:50 in
ataxin-3gt/gt mutant genotypes were generated from Day 12.5 to Day 14.5 mouse embryos (Joyner, 1993). Uterine horns were removed and portions of the embryos were minced with a razor blade into small fragments. These fragments were seeded into 25 cm² cell culture dishes (Greiner bio-one).

All cells were cultured in modified Eagle medium (Gibco) in the presence of 10% foetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). To analyse endoplasmic reticulum stress, cells were treated with tunicamycin (Sigma-Aldrich). All concentrations and times of treatment are listed for each experiment in the figure legends.

For the transfection with the pDsRed2-endoplasmic reticulum vector (Clontech) and/or pEGFP-N1 (with and without deletion construct), lipofectamine (Invitrogen) was used under standard procedure.

Motor analysis was performed with 12 animals per group using different behavioural tests, including footprint analysis, rotarod (TSE), beam walking and the Labmaster behavioural system (TSE).

A standard group of 97 wild-type, 105 ataxin-3wt/wt and 91 ataxin-3gt/ gt mice was measured every second week.

To detect abnormalities in gait, front and hind paws were dipped in red and blue water-based dye, respectively. The mice were then placed on a sheet of white paper and allowed to walk through a tunnel, leaving a footprint pattern on the white paper. For each time point, three complete runs per mouse were used for analysis.

Mice were placed on an accelerating rotating rod (rotarod) (TSE) at the same time of day for five consecutive days. Three training sessions (each with acceleration from 4 to 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.

To examine the co-ordination and balance capabilities of the mice, we performed beam walking tests. Briefly, mice were trained to traverse 80 cm long beams. After a training session, two test runs on beams of different shapes and sizes (square: 28, 12 and 5 mm width; round: 28, 17 and 11 mm diameter) were performed. Beam transversal time was recorded.

To examine the movement, drinking and feeding activity of mice for 22 h. One mouse was placed in each cage with a defined amount of water, food and litter. A standard dark-light cycle (12 h each) and defined housing conditions were maintained at all times. Over 22 h, drinking and feeding activity was recorded every minute and the amount of drinking and feeding was detected by a computer supporting system.

Mouse embryonic fibroblasts with wild-type, ataxin-3wt/wt or ataxin-3gt/gt mutant genotypes were generated from Day 12.5 to Day 14.5 mouse embryos (Joyner, 1993). Uterine horns were removed and rinsed with 1 × phosphate-buffered saline, pH 7.4. Each embryo was separated from its placenta and surrounding membranes. Head and internal organs were removed from the embryos. The remaining
parts of ubiquitin-interacting motif-2 and the valosin-containing protein-binding domain as well as the polyglutamine stretch tract (Fig. 1A). Real-time polymerase chain reaction with messenger RNA from mutant mice confirmed the expression of ataxin-3 exons 1 through 8, while exons 9–11 were not expressed (data not shown).

Offspring of heterozygous parents was genotyped by polymerase chain reaction or Southern blot analysis (Fig. 1B) and yielded the expected Mendelian distribution of genotypes (23% wild-type, 49% ataxin-3wt/gt and 28% ataxin-3 gt/gt) with no apparent phenotypic differences among littermates. The ataxin-3-specific antibody 1H9, which recognizes an epitope in the N-terminal half of the protein on western blots, identified the 45 kDa ataxin-3 protein in brain extracts of wild-type and heterozygous ataxin-3wt/gt mice but not in homozygous ataxin-3 gt/gt mutants (Fig. 1C). The same antibody also recognized the ~200 kDa fusion protein (Δataxin-3/β-galactosidase/neomycinphosphotransferase) in brains of ataxin-3wt/rt and ataxin-3gt/rt mutant but not in wild-type mice (Fig. 1C). The presence of the large fusion protein was also detected with the anti-β-galactosidase antibody. Conversely, the anti-ataxin-3 antibody that recognizes a C-terminal epitope in ataxin-3 protein (Schmidt et al., 1998), identified ataxin-3 protein in wild-type and heterozygous mutant mice confirming the loss of the ataxin-3 C-terminus in the homozygous gene trap mutant (Fig. 1C).

**Ataxin-3 mutant protein is highly expressed in the central nervous system**

To investigate the neuronal expression pattern of the ataxin-3 mutant allele during mouse development, LacZ staining was
performed on sections of brain and spinal cord from ataxin-3\(^\text{wt/gt}\) and ataxin-3\(^\text{gt/gt}\) mutant mice. Particularly, strong expression was observed in the bulbus olfactorius, cortex, striatum, hippocampus (Fig. 2A and C), and in the grey matter of spinal cord (Fig. 2B). Expression in the cerebellum was generally weaker and limited to deep cerebellar nuclei and Purkinje cells (Fig. 2A and D). Except for more intense staining in homozygous mutants, the expression patterns in ataxin-3\(^\text{wt/gt}\) and ataxin-3\(^\text{gt/gt}\) mutant mice were essentially indistinguishable from each other (data not shown) and very similar to the published pattern of the wild-type \(\text{atnx3}\) gene in mouse (compare to Allen brain atlas database: http://mouse.brain-map.org). These observations confirm the preferential expression of ataxin-3 in certain neuronal cells and demonstrate that the neuronal expression pattern of ataxin-3 has not been affected by the gene trap insertion.

**Age-dependent symptoms in ataxin-3 mutant mice**

Careful observation of ataxin-3 mutant mice did not reveal any overt phenotype for several months after birth. However, at the age of \(\sim 9\) months, most homozygous and heterozygous mutant mice displayed a scruffy hair coat and wet sticky eyes (Fig. 3A and B), an unusual clasping reaction when suspended by the tail (Fig. 3C), abnormal gait (Fig. 3D), impaired beam walking ability (Fig. 3E) and an abrupt loss of body weight at \(\sim 12\) months (Fig. 4B). The neurological symptoms appeared to be progressive and similar in ataxin-3\(^\text{wt/gt}\) and ataxin-3\(^\text{gt/gt}\) mutant mice, as demonstrated by significant defects in motor coordination and locomotion on the rotarod (Fig. 3F and Supplementary video 1) and in balanced beam tests (Fig. 3E). Following the dramatic reduction of \(20-30\) g of body weight within 2 weeks (Fig. 4B), both ataxin-3\(^\text{wt/gt}\) and ataxin-3\(^\text{gt/gt}\) mutant mice died prematurely at the age of \(\sim 12\) months with no significant difference \((P = 0.6)\) between heterozygous and homozygous mutant genotypes (Fig. 4A). To analyse the cause of the reduction of the body weight, we performed home cage behaviour studies. This study revealed no differences in the drinking and feeding activity between yet non-phenotypical mutant mice and wild-type controls at the age of 12 months (just before an overt phenotype was observed). However, with manifestation of severe neurological symptoms a few weeks later, both phenotypical ataxin-3\(^\text{wt/gt}\) and ataxin-3\(^\text{gt/gt}\) mutant mice showed significantly reduced drinking and feeding activity (Fig. 4C).

**Neuronal cytoplasmic inclusion bodies and neuron degeneration in ataxin-3\(^\text{wt/gt}\) and ataxin-3\(^\text{gt/gt}\) mutant mice**

Immunohistochemistry on cerebellar sections using the ataxin-3-specific antibody 1H9 led to the detection of cytoplasmic inclusion bodies (Fig. 5A and B) that stained strongly positive in a halo-like pattern (Fig. 5B inset) in ataxin-3\(^\text{wt/gt}\) and ataxin-3\(^\text{gt/gt}\) mutant mice. Similar aggregates were not found in wild-type animals. Besides cerebellum, cytoplasmic inclusions were present in...
multiple other brain regions of ataxin-3 mutants, such as dentate nucleus, hippocampus, cortex, pons and brainstem (data not shown). Notably, we failed to detect ubiquitin and β-galactosidase in cytoplasmic inclusions using several different antibodies (data not shown). Staining of the cerebellum with neurofilament antibody (Fig. 5C and D) and toluidine blue (Fig. 5E–H) indicated significant degradation of Purkinje cells in ataxin-3wt/gt and ataxin-3gt/gt mutants at 3 and 12 months of age in comparison to wild-type littermates (*P < 0.05; **P ≤ 0.001). (F) The rotarod test reveals significant age-dependent deficits in motor coordination in mutant mice at the age of 9 (*P = 0.02) and 12 months (**P = 0.001). No differences were detectable between ataxin-3wt/gt and ataxin-3gt/gt mice (P = 0.094).

Electron microscopic images exhibited a lamellar ultrastructure of the inclusion bodies, which were found in Purkinje cells of 3 month, and even more frequently in 12-month-old ataxin-3gt/gt mutant mice (Fig. 6C–F). This lamellar structure was not present in the few lipofuscin granules of wild-type controls (Fig. 6A and B). Lamellar inclusion bodies were also observed in heterozygous ataxin-3 mutants (Supplementary Fig. 1A–D). Staining with Nile blue sulphate (Supplementary Fig. 1E and F) and the morphology (Fig. 6D and F) suggested that the inclusions in mutant cells arose
from lipofuscin granules but differed from the classical amorphous pigment and lipid components containing structures seen in wild-type cells (Fig. 6A and B). In addition, there were also round cytoplasmic inclusion bodies in mutant mice that had no lamellar but a granular appearance of their interior and lacked any surrounding membrane (Fig. 6G and H). These characteristic inclusion bodies were, by immunogold staining with 1H9 antibody, identified as consisting of the mutant ataxin-3 protein (Fig. 6I and J).

To investigate whether enhanced cell death of neurons ensues in mutants, TUNEL assays on the cerebellum of 12-month-old ataxin-3\textsuperscript{wt/gt} and ataxin-3\textsuperscript{gt/gt} mutant mice were performed. Numerous apoptotic neurons were detected in the molecular layer of the cerebellum in ataxin-3\textsuperscript{gt/gt} mutant mice (Fig. 7G–I). Compared with age- and sex-matched wild-type littermates (Fig. 7A–C), a small but statistically significant increase of apoptotic neurons in the cerebellum of ataxin-3\textsuperscript{gt/gt} mutants ($P = 0.022$) was found, suggesting that programmed neuronal cell death is part of the ataxin-3 mutant phenotype (Fig. 7J). Heterozygous mutant mice also displayed more apoptotic neurons compared with wild-type, but this did not reach significance (Fig. 7D–F).

**Ataxin-3 mutant mice display inducible endoplasmic reticulum stress and attenuated response to unfolded proteins**

Ataxin-3 is involved in the degradation of misfolded proteins by the endoplasmic reticulum-associated protein degradation (ERAD) system (Wang et al., 2006; Zhong and Pittman, 2006). It interacts with components of ERAD, such as p97 (valosin-containing protein) and hHR23 for transport of misfolded and polyubiquitinated proteins from the endoplasmic reticulum to the...
Figure 5  Immunohistochemistry revealed cytoplasmatic inclusions and neurodegeneration. Cerebellar pathology in ataxin-3^{wt/gt} and ataxin-3^{gt/gt} mutant mice at the age of 12 months. (A) Staining with the 1H9 antibody (counterstained with toluidine) showed diffuse staining of the cell body in slices of wild-type mice. In contrast, cytoplasmatic inclusion bodies (arrows in B), which were not β-galactosidase- and ubiquitin-positive, were detected in ataxin-3^{wt/gt} and ataxin-3^{gt/gt} mutant mice. (C) Neurofilament staining revealed
ubiquitin-proteasome. Since the valosin-containing protein-binding site is deleted in the ataxin-3<sup>Gr/Gr</sup> allele, the mutant protein may not efficiently clear misfolded endoplasmic reticulum proteins. We investigated the accumulation of critical ERAD components in heterozygous and homozygous ataxin-3 mutants on western blots and found no significant differences in protein levels of valosin-containing protein, VIMP and Derlin-1 compared with wild-type animals (Fig. 8A). These results suggest that acute endoplasmic reticulum stress response was not elicited in ataxin-3<sup>Gr/Gr</sup> mutant mice.

The unfolded protein response constitutes a second mechanism to reduce misfolded proteins from the endoplasmic reticulum. We analysed messenger RNA levels for the endoplasmic reticulum stress-responsive genes namely BiP (Hspa5, heat shock 70 kDa protein 5) and CHOP (DDIT3, DNA-damage inducible transcript 3), two important components of the unfolded protein response. Interestingly, expression of both transcripts was significantly upregulated in ataxin-3<sup>Gr/Gr</sup> mutant mice at the age of 12 months and to a lesser extent at 3 months (Fig. 8B and C). Moderate increase of BiP and CHOP expression was also observed in heterozygous mutant mice. These results provide evidence that ataxin-3 mutant mice may suffer from chronic endoplasmic reticulum stress.

To explore endoplasmic reticulum dysfunction in mutant cells in more detail, mouse embryonic fibroblasts were treated with the endoplasmic reticulum stress inducer tunicamycin. Ataxin-3<sup>WT/WT</sup> and ataxin-3<sup>Gr/Gr</sup> mutant cells showed considerable swelling of the endoplasmic reticulum lumen 24 and 48 h after treatment (Fig. 9C–F), while tunicamycin did not elicit any major effect on the endoplasmic reticulum lumen and was not seen in wild-type mouse embryonic fibroblasts. Detachment of ribosomes from the endoplasmic reticulum membrane of ataxin-3<sup>Gr/Gr</sup> mouse embryonic fibroblasts was demonstrated additionally by centrifugation of microsomes on sucrose gradients. As shown in Fig. 11F, endoplasmic reticulum membranes isolated from ataxin-3<sup>Gr/Gr</sup> cells were significantly less dense than those from wild-type based on the increased fraction of Sec61α containing membranes in lower concentrated sucrose (∼57% in ataxin-3<sup>Gr/Gr</sup> cells versus 28% in the wild-type cells). A similar density shift was seen in a control experiment with microsomes isolated from 293T cells upon treatment with EDTA that chelates Mg<sup>2+</sup>, thereby leading to dissociation of ribosomes from the endoplasmic reticulum membrane (Fig. 11E). Loss of membrane-bound ribosomes was not stimulated further by the addition of tunicamycin in this experiment. Taken together, these results suggest that endoplasmic reticulum membranes from ataxin-3<sup>Gr/Gr</sup> mouse embryonic fibroblasts are occupied by fewer ribosomes compared with those from wild-type cells. To exclude that membranes of ataxin-3<sup>Gr/Gr</sup> mouse embryonic fibroblasts were generally less dense, the Golgi membrane was analysed in a similar centrifugation experiment using β-COP, a subunit of the coat protein complex-I coatamer as marker (reviewed in Nickel and Wieland, 1997). We found that the density of Golgi membranes was not affected by the ataxin-3 mutation (Supplementary Fig. 2).

Translational regulation by misfolded endoplasmic reticulum proteins involves the activation of the endoplasmic reticulum membrane-bound kinase Perk [PKR (double-stranded RNA-dependent protein kinase)-like endoplasmic reticulum kinase] and phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) at serine 51 (reviewed in Harding et al., 2002). eIF2α controls binding of initiator transfer RNA to the 40S ribosomal subunit and phosphorylation reduces the rate of translation initiation. Analysis of phosphorylated eIF2α on western blots

**Figure 5 Continued**

normal morphology of Purkinje cells in wild-type controls, but empty baskets and/or atrophy of Purkinje cells in the ataxin-3<sup>WT/WT</sup> and ataxin-3<sup>Gr/Gr</sup> mice (D). Furthermore, slices stained with toluidine detected striking differences between mutant ataxin-3 mice and controls (E–H). In ataxin-3<sup>WT/WT</sup> and ataxin-3<sup>Gr/Gr</sup> mice, an increased number of toluidine-positive Purkinje cells was found indicating neurodegenerative processes (F and in a higher magnification H). Purkinje cells were not stained by toluidine in control animals (E and G). Scale bar = 50 μm. (I) Quantification of toluidine-positive cells at the age of 3 and 12 months. Cells were assessed as ‘normal’ when the cells were not stained, ‘intermediate’ when a light blue staining was visible or ‘atrophic’ when a dark blue toluidine staining was evident. At both ages, a significantly increased number of ‘atrophic’ and ‘intermediate’ cells were detected in ataxin-3<sup>WT/WT</sup> and ataxin-3<sup>Gr/Gr</sup> mice (*P < 0.001). Results are means ± SEM.
indicated increased levels of phospho-eIF2α in 3- and 12-month-old heterozygous and homozygous ataxin-3 mutant mice in comparison to wild-type (Fig. 12). These observations further support the hypothesis that the ataxin-3 wt mutant allele causes endoplasmic reticulum stress possibly by unfolded proteins and unfolded protein response associated with inhibition of protein synthesis.

**Discussion**

In this study, we report on a gene trap mutation in the mouse ataxin-3 gene that leads to the expression of a ~200 kDa fusion protein between the C-terminally truncated ataxin-3 protein and the β-geo (β-galactosidase and neomycin-phosphotransferase) protein. Notably, this ataxin-3 fusion protein retains the highly conserved Josephin and the ubiquitin-interacting motif-1 domains but lacks the valosin-containing protein-binding site and the polyglutamine stretch. The mutant mouse represents a model in which loss of the ataxin-3 C-terminus and potential roles of the ataxin-3 N-terminal fragment in the pathogenesis of SCA3 can be investigated. Both ataxin-3 wt/gt and ataxin-3 gt/gt mutant mice are viable with no apparent phenotype in early postnatal life, suggesting that the polyglutamine stretch and the valosin-containing protein-binding site of ataxin-3 are not absolutely required for functions during embryonic and early postnatal development. This is in good agreement with observations in the ataxin-3-knockout mouse that also has no overt embryonic phenotype and develops no signs of SCA3 (Schmitt et al., 2007). In contrast to the recessive ataxin-3 loss-of-function mutation (KO), the ataxin-3 mutant allele described here is dominant and causes typical neurological symptoms and premature death at the age of 12 months. The pathology of this mutant mouse possibly reflects characteristics of two different neurodegenerative diseases, e.g. frontotemporal dementia associated with inclusion body myopathy and Paget’s disease (IBMPFT) (Watts et al., 2004) and SCA3. IBMPFT is caused by mutations in the valosin-containing protein gene encoding a binding partner of ataxin-3 (Doss-Pepe et al., 2003; Zhong and Pittman, 2006). Valosin-containing protein is

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**Figure 6** Ultrastructural changes. Electron microscopy images of Purkinje cells of the cerebellum from (A) a wild-type mouse at the age of 3 months. The cytoplasm is filled with rough endoplasmic cisterns, Golgi apparatus and mitochondria. The arrow points to a single lipofuscin granule. (B) Wild-type mouse at the age of 12 months. The number of lipofuscin granules has increased (arrows). Atxn3gt/gt mouse at the age of 3 months (C and D) or 12 months (E and F). (D) Higher magnification image of C. The arrow points to the identical inclusion. All inclusions exhibit a typical lamellar structure, which could be identified as lipofuscin granules. Mitochondria (asterisk in E) appear to be normal. No classical lipofuscin inclusions, as detected in wild-type animals, were found in ataxin-3 wt/gt mice indicating that the lamellar inclusions are indeed lipofuscin granules. (G–J) Representative images from ataxin-3 wt/gt mutant mice at the age of 12 months. The arrow in G points to an inclusion that is different from the lamellar inclusions as shown

**Figure 6 Continued**
in A–F and characterized by the absence of any membrane around or within the inclusion body and which has a granular appearance of its interior. In (H) showing a higher magnification of (G), asterisks point to lamellar inclusions that are identical to the other inclusions in (C–F). (I and J) Immunogold staining with gold particle labelled ataxin-3. In order to ease the observation of gold grains, grids were incubated with two secondary antibodies of the same species but conjugated to different gold sizes (10 and 20 nm), thus facilitating the observation of the sites of immunoreactivity by large grains and allowing a better antigenicity of small gold grains. (J) is a higher magnification of (I), the arrow indicates the ataxin-3-positive cytoplasmatic inclusion, which could also be found in (G) and is shown in a higher magnification in (H) and J. Scale bars in A, C, E, G, I = 2 μm, B, D, F, H, J = 0.5 μm. N = nucleus.
also called p97 and plays a role in the ERAD of misfolded proteins. Valosin-containing protein mutations that cause IBMPFT fail to support the release of misfolded proteins and lead to the formation of inclusion bodies (Ju et al., 2008). Notably, patients with IBMPFT develop both intranuclear inclusion bodies and cytoplasmic aggregates with characteristics of lipofuscin particles (Schröder et al., 2005), similar to what we observed in the ataxin-3 gt/gt mutant. Although lipofuscin pigment increases in the cytoplasm of ageing neurons (Goyal, 1982a, b), its accumulation is accelerated in neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease (reviewed in Jung et al., 2007; Eichhoff et al., 2008). Since the formation of cytoplasmic inclusions in the ataxin-3 gene trap mouse displays a dominant phenotype, it is unlikely that the aggregates solely result from loss of the valosin-containing protein-binding site in the ataxin-3 mutant allele but they might rather reflect a gain-of-function by the ataxin-3 fusion protein.

Phenotypic resemblance of the ataxin-3 gene trap mouse with SCA3 transgenic mouse models (Bichelmeier et al., 2007; Boy et al., 2009, 2010) is demonstrated by common neurological symptoms, such as tremor, clumping and gait ataxia and dramatic weight loss at the age of 12 months. However, whereas in brains from ataxin-3 wt/gt and ataxin-3 gt/gt mutant mice aggregates are located in the cytoplasm of neurons and contain the C-terminally truncated ataxin-3 fusion protein that lacks polyglutamine stretch, in SCA3 one finds polyglutamine stretch containing aggregates predominantly in the nucleus of distinct neurons (Schmidt et al., 2002; reviewed in Riess et al., 2008). Nevertheless, neuropil aggregates have also been detected in brains of patients with SCA3 (Seidel et al., 2010) and in several rodent models of other

**Figure 7** TUNEL staining revealed neurodegeneration and apoptosis in the cerebellum. As expected, TUNEL staining demonstrated degenerating cells in the molecular layer of the cerebellum of ataxin-3 wt/gt mice (D–F) and ataxin-3 gt/gt mutants (G–I) at the age of 12 months. Less TUNEL-positive cells were detected in age- and sex-matched wild-type littermates (A–C). (J) Quantification of TUNEL-positive cells showed significantly more neurodegenerative cells in ataxin-3 gt/gt mice as compared with controls (*P = 0.022). Scale bar = 20 μm.
Involvement of ERAD system and unfolded protein response. (A) Expression of the ERAD proteins valosin-containing protein (VCP), VIMP and Derlin-1 as analysed by western blots was not altered in mutant mice. Quantification of western blot analysis revealed <5% difference between protein levels in the different genotypes (data not shown). (B) Messenger RNA expression level of BiP, an important chaperone in the unfolded protein response, is upregulated in ataxin-3\textsuperscript{gt/gt} mice at 3 and 12 months. In the ataxin-3\textsuperscript{wt/gt} mice, however, BiP expression is upregulated at 12 months, although to a lesser extent than in ataxin-3\textsuperscript{gt/gt} mice. (C) The messenger RNA expression level of CHOP is also upregulated in ataxin-3\textsuperscript{wt/gt} brains at the age of 3 and 12 months and in ataxin-3\textsuperscript{gt/gt} mice at the age of 12 months. Results are presented as means ± SEM.

Increased susceptibility of ataxin-3\textsuperscript{wt/gt} and ataxin-3\textsuperscript{gt/gt} to endoplasmic reticulum stress. Electron microscopic analyses of tunicamycin (1 μg/ml) treated and untreated mouse embryonic fibroblast cells revealed differences in endoplasmic reticulum morphology between the different genotypes. In contrast to tunicamycin treated wild-type (wt) cells (A and B), mouse embryonic fibroblast cells derived from ataxin-3\textsuperscript{wt/gt} (C and D) and ataxin-3\textsuperscript{gt/gt} (E and F) mice reacted with swelling of the endoplasmic reticulum filled with an increased amount of proteins. This observation was confirmed by transfecting mouse embryonic fibroblast cells with a pDsRed2-endoplasmic reticulum vector and by subsequent analyses with immunofluorescent microscopy. Treated wild-type cells showed a homogenous distribution of the red fluorescent signal in the cytoplasm (G), whereas swelling of the endoplasmic reticulum membranes was detected in treated ataxin-3\textsuperscript{wt/gt} and ataxin-3\textsuperscript{gt/gt} cells, indicated by a ‘bubble-like’ structure and a strong red fluorescent signal in these areas (H and J). Untreated wild-type, ataxin-3\textsuperscript{wt/gt} and ataxin-3\textsuperscript{gt/gt} cells showed a homogenous distribution without swelling of endoplasmic reticulum membrane areas (data not shown). Bars in the electron micrographs represent 2 μm.
Figure 10 Immunofluorescence analysis of the endoplasmic reticulum. (A) The transfection with the pDsRed2-endoplasmic reticulum vector of SCA3 knockout, ataxin-3<sup>gt/gt</sup> and wild-type mouse embryonic fibroblast cells after 24 h tunicamycin treatment. In the ataxin-3<sup>gt/gt</sup> cells, the described ‘bubble-like’ structure of the endoplasmic reticulum is clearly visible, whereas mouse embryonic fibroblast cells of SCA3 knockout and wild-type mice showed a normal staining of the endoplasmic reticulum. (B) Analysis of the endoplasmic reticulum in wild-type mouse embryonic fibroblast cells transfected with a deletion construct, expressing the N-terminal part of ataxin-3 that resembles the aberrant protein of ataxin-3 mutant mice. To exclude transfection artefacts, wild-type and ataxin-3<sup>gt/gt</sup> cells were transfected with an empty pEGFP-N1 vector. Cells transfected with the deletion construct showed the same ‘bubble-like’ structure after 24 h tunicamycin treatment as we found in ataxin-3<sup>gt/gt</sup> mouse embryonic fibroblast cells. In contrast, untransfected wild-type cells performed normally. (C) The graph showed the number of counted cells with ‘bubble-like’ structure or normal distribution of the endoplasmic reticulum of all analysed cells from (A) and (B). In total, ~500 cells per genotype were analysed. In cells transfected with the deletion construct or in ataxin-3<sup>gt/gt</sup> mouse embryonic fibroblast cells, significantly fewer cells with normal endoplasmic reticulum (*P < 0.01) and more cells with ‘bubble-like’ structure were found (**P < 0.001). No differences were found between wild-type and SCA3 knockout mouse embryonic fibroblast cells, where we never observed any cells with ‘bubble-like’ structure after 24 h tunicamycin treatment (P = 0.74). Furthermore, no significant differences were found between untreated cells from ataxin-3<sup>gt/gt</sup> mice or wild-type cells transfected with the deletion construct. ER = endoplasmic reticulum.
polyglutamine stretch diseases such as Huntington’s disease, in which expanded huntingtin (htt) was part of cytoplasmic inclusions (Li et al., 1999; von Hörsten et al., 2003; Wang et al., 2008). Most interestingly, a putative calpain cleavage site at amino acid residue 260 of ataxin-3 is activated in SCA3 (Haacke et al., 2007; Jung et al., 2009) separating two active nuclear export signals at the N-terminus from one C-terminal nuclear import signal (Antony et al., 2009). As the Josephin domain alone is able to form insoluble amyloid fibrils in vitro (Masino et al., 2004), cytoplasmic aggregates containing N-terminal ataxin-3 fragments as well as intranuclear polyglutamine stretch containing inclusions can be explained in SCA3 (proposed pathogenesis model Fig. 13). Indeed, cleavage of ataxin-3 has already been shown in vitro (Graham et al., 2006) and in vivo in SCA3 mouse models (Goti et al., 2004) and human patients (Berke et al., 2004). Whereas the toxicity of the polyglutamine stretch containing fragments has been clearly demonstrated for a number of polyglutamine stretch diseases (reviewed in Ross et al., 1999; Ross and Poirier, 2004), the pathogenic significance of the resulting protein fragments, in particular of the non-polyglutamine stretch containing N-terminal fragment of ataxin-3, has not been elucidated. As the ataxin-3 gene trap mouse mutant expresses 259 N-terminal amino acids of ataxin-3 as part of a cytoplasmic fusion protein and is mimicking major effects of polyglutamine stretch expanded ataxin-3, it provides a model to study the potential contribution of the N-terminal ataxin-3 fragment to SCA3 symptoms. The pathogenic effects of the Josephin domain containing N-terminal ataxin-3 fragment include endoplasmic reticulum stress and cellular responses to unfolded or misfolded proteins, such as the ERAD system and unfolded protein response. In the ERAD system, valosin-containing protein binds to ataxin-3 (Doss-Pepe et al., 2003; Boeddrich et al., 2006) as part of a larger complex at the endoplasmic reticulum membrane that mediates transport of misfolded proteins to the proteasome for degradation.

Figure 11 continued

gradient centrifugation revealed that ribosomal-bound endoplasmic reticulum membranes remained at the bottom of the tube due to their higher density (E, top). By incubation of microsomes with EDTA, a reduced association of ribosomes with the endoplasmic reticulum was observed. Consequently, ribosome peaks were shifted towards the bottom, while more endoplasmic reticulum membranes were observed in the lower density fractions (increased from ~10–40%) (E, bottom). Confirming the data of the electron microscopy studies, endoplasmic reticulum membranes ataxin-3gt/gt cells were significantly lighter than those of wild-type (WT) cells as more endoplasmic reticulum membranes were able to migrate into the low-density fractions (~57% in ataxin-3gt/gt cells versus 28% in the wild-type cells). (F) Similar results were obtained when these cells were treated with a low concentration of the endoplasmic reticulum stressor tunicamycin (1 μg/ml).
degradation (Wang et al., 2006; Zhong and Pittman, 2006). Since mutant ataxin-3 lacks the interaction with valosin-containing protein, it is reasonable to assume that the ERAD system might be affected in mutant mice, although the level of the components valosin-containing protein, Derlin-1 and VIMP appeared unchanged. Activation of the unfolded protein response leads initially to attenuation of protein synthesis and enhanced production of chaperones, like BiP, or ultimately to apoptosis (reviewed in Kaufman, 1999; Szegedi et al., 2006; Zhang and Kaufman, 2006). The elevated level of BiP messenger RNA and the upregulation of the pro-apoptotic marker CHOP in brains of ataxin-3<sup>gt/gt</sup> mutant mice suggested that the unfolded protein response was activated. This was further supported by the observation that the alpha subunit of eIF2 was highly phosphorylated in ataxin-3<sup>wt/gt</sup> and ataxin-3<sup>gt/gt</sup> mice resulting in the inhibition of translation initiation (Kouroku et al., 2007 and reviewed in de Haro et al., 1996; Kimball, 1999; Harding et al., 2002). The in vivo data were supported by observations in mouse embryonic fibroblasts that have been treated with the endoplasmic reticulum stress inducer tunicamycin, which blocks N-linked protein glycosylation and causes cell cycle arrest in the G1 phase (Hoyer-Hansen and Jäättelä, 2007). Electron microscopy and immunofluorescent analysis revealed that cells expressing mutant ataxin-3 in the presence of tunicamycin suffer from endoplasmic reticulum stress elicited by swelling of the endoplasmic reticulum lumen and reduced binding of ribosomes to the endoplasmic reticulum membrane.

In conclusion, the ataxin-3 mutant mouse investigated in this study provides, for the first time, in vivo evidence that the N-terminal region of ataxin-3 harbouring the Josephin domain can contribute to the symptoms of SCA3. A cellular hallmark in this mouse model is the age-dependent accumulation of cytoplasmic protein inclusion bodies containing the N-terminal ataxin-3 fragment. We propose that cytoplasmic protein aggregation and cellular dysfunction might be mediated by the Josephin domain when separated from the valosin-containing protein-binding domain. Whether or not this reflects the pathogenic activity of naturally occurring ataxin-3 fragments remains to be elucidated. We furthermore suggest that biochemical mechanism(s) underlying the pathogenicity in the ataxin-3 gene trap mutant include endoplasmic reticulum stress and inappropriate response to partially or totally denatured proteins.

**Figure 12** Differential expression of phosphorylated eIF2α <i>in vivo</i>. Western blot staining with an antibody against phosphorylated eIF2α revealed differences in staining intensity. In ataxin-3<sup>wt/gt</sup> and ataxin-3<sup>gt/gt</sup> brains, an increased level of phosphorylated eIF2α was detected at the age of 3 and 12 months in comparison to wild-type controls.

**Figure 13** Proposed pathogenesis model. Pathogenesis model including calpain cleavage site at amino acid residue 260 and localization signal of ataxin-3. Normally, the two nuclear export signals (NES) on the N-terminal part of the protein keep the full-length non-expanded ataxin-3 in the cytoplasm. A possible calpain II cleavage site at amino acid residue 260 separates the two nuclear export signals (N-terminus) from the nuclear localization signal (NLS) and the polyglutamine stretch tract (QQ) located on the C-terminus of the ataxin-3 protein. Former studies demonstrated that the expanded polyglutamine stretch translocates into the nucleus polyglutamine tract expanded C-terminus translocates into the nucleus via the nuclear localization signal to form intranuclear inclusion bodies. In the present study, we showed that the cleaved N-terminal part, especially the Josephin domain, of ataxin-3 (marked in grey) stays in the cytoplasm to form cytoplasmatic aggregates.

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Supplementary material

Supplementary material is available at Brain online.

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