Mitochondrial defects and neurodegeneration in mice overexpressing wild-type or G399S mutant HtrA2

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Abstract

The protease HtrA2 has a protective role inside mitochondria, but promotes apoptosis under stress. We previously identified the G399S HtrA2 mutation in Parkinson’s disease (PD) patients and reported mitochondrial dysfunction in vitro. Mitochondrial dysfunction is a common feature of PD and related to neurodegeneration. Complete loss of HtrA2 has been shown to cause neurodegeneration in mice. However, the full impact of HtrA2 overexpression or the G399S mutation is still to be determined in vivo. Here, we report the first HtrA2 G399S transgenic mouse model. Our data suggest that the mutation has a dominant-negative effect. We also describe a toxic effect of wild-type (WT) HtrA2 overexpression. Only low overexpression of the G399S mutation allowed viable animals and we suggest that the mutant protein is likely unstable. This is accompanied by reduced mitochondrial respiratory capacity and sensitivity to apoptotic cell death. Mice overexpressing WT HtrA2 were viable, yet these animals have inhibited mitochondrial respiration and significant induction of apoptosis in the brain leading to motor dysfunction, highlighting the opposing roles of HtrA2. Our data further underscore the importance of HtrA2 as a key mediator of mitochondrial function and its fine regulatory role in cell fate. The location and abundance of HtrA2 is tightly controlled and, therefore, human mutations leading to gain- or loss-of-function could provide significant risk for PD-related neurodegeneration.
Introduction

Mitochondrial dysfunctions are pathological features of Parkinson’s disease (PD), atypical PD and several other neurodegenerative diseases. The mitochondrial serine protease HtrA2 (also known as Omi) is crucial for mitochondrial and cellular quality control.

The neuroprotective role of HtrA2 was identified by the demonstration of a neurodegenerative phenotype with Parkinsonism in mice lacking HtrA2 (1). Furthermore, two mutations (S141A and G399S) adjacent to two putative phosphorylation sites were found in German PD patients (2). Genetic variability in the HtrA2 gene was later reported to contribute to the risk of PD in different populations worldwide (3,4), and recently the G399S mutation was found to cause essential tremor and Parkinsonism in a kindred with multiple affected individuals of Turkish descent (5). HtrA2 has also been implicated in the selective vulnerability of striatal neurons in Huntington’s disease (6).

HtrA2 is an essential protease responsible for maintaining proteostasis within mitochondria (7). The protein has low serine protease activity in its basal state (8) and employs a chaperone-like function (9). Like other members of the HtrA protein family, the protease activity of HtrA2 is increased in response to cellular stress (10). During initiation of mitochondrially mediated apoptosis, HtrA2 and other proteins such as cytochrome c can be released into the cytosol (11). In the cytosol, HtrA2 can interact with IAPs such as X-linked inhibitor of apoptosis (XIAP) via its exposed N-terminal tetrapeptide motif (AVPS) (12,13), releasing the IAP brake which is preventing the apoptotic cascade. HtrA2 activity enhances DEVD cleavage promoting the activation of effector caspases independent of IAP binding (8,14). HtrA2 activity is also required for caspase-independent apoptosis (15) and has been suggested to play a positive role in autophagy (16).

Mutations causing protein coding changes in HtrA2 at A141 and G399 cause mitochondrial dysfunction in vitro (2). The adjacent serine residues at S142 and S400, in the protease and PDZ (also known as Discs-large homologous regions (DHR) or GLGF) domains of HtrA2, respectively, were later found to be crucial for HtrA2 protease activity (2,17). The activity of HtrA2 is sensitively regulated by its requirement to form a pyramid-shaped homotrimer and by its C-terminal PDZ domain, which controls substrate binding and activity. The active site of HtrA2 is blocked by the PDZ domain, which forms a lid-like structure in its low-activity state, then binding by target proteins causes a large conformation change, opening up the trimeric assembly (8,18).

Phosphorylation of HtrA2 at S142 is required for its regulation by PD-associated protein PSEN-induced putative kinase 1 (17) and phosphorylation of HtrA2 at S400 by CDK5 regulates HtrA2 and mitochondrial function (19). Disruption of HtrA2 phosphorylation at S400 (19) greatly reduces its serine protease activity (17) probably because phosphorylation of this evolutionary conserved site within the PD2 domain is involved in the regulation of HtrA2 protease activity. The G399S mutation results in altered phosphorylation of HtrA2 at serine 400 (19). Overexpression studies in vitro concluded that the G399S mutation affects only the protease activity of HtrA2, but not its steady-state levels or formation of trimers (2). However, little is known about the functional relevance of the G399S mutation in vivo other than the finding of reduced HtrA2 phosphorylation at serine 400 in the brain tissue from transgenic (Tg) G399S mice compared with non-transgenic (non-Tg) animals (19).

Considering the importance of the PDZ domain for HtrA2 activity and function, it was important to ask whether the presence of the G399S mutation is relevant to HtrA2 function in vivo. Addressing this issue may give insight into the mechanism by which mitochondria are compromised and the pathways that lead to neuronal cell death in neurodegeneration. Although neuronal-specific overexpression of wild-type (WT) HtrA2 in mice has previously been reported to have no effect on development (20), no in depth functional or behavioral assessments were made, leaving us to question also whether gain of function of such a balancing act protein may also be detrimental in an in vivo situation.

To shed light on these open questions, we overexpressed in mice WT human HtrA2 or human HtrA2 with the G399S mutation. We report that 10-fold overexpression of G399S mutant HtrA2 in mice affects fertility and therefore only low-expressing lines were able to be maintained. We found that G399S HtrA2 cannot form active trimers and its expression causes increased basal and state III respiration with reduced spare respiratory capacity. Several subunits of complexes I and III were less abundant in these mice compared with non-Tg littermates. The mitochondrial dysfunction observed leads to an increase in apoptotic cell death in the cerebellum, but motor function and behavior were not affected. Ten-fold overexpression of WT HtrA2 reduced the overall oxygen consumption rate (OCR) and significantly reduces maximal respiration and spare respiratory capacity. Complex I activity was reduced and at least four subunits of Complex I were less abundant in these mice compared with non-Tg animals. Although no morphological changes could be observed in the mitochondria across several brain regions, there was a significant increase in apoptotic cell death in the cerebellum, which may cause the motor dysfunction, observed in these animals over a 14-month period.

Our data indicate that the G399S mutation has a dominant-negative effect in vivo and that increased levels of WT HtrA2 exert a toxic gain of function, supporting the notion of HtrA2 as a rare variant with a substantial effect.

Results

The WT and G399S HtrA2 transgenes are integrated in the mouse genome and overexpressed

Human HtrA2 cDNA with a FLAG tag at the C-terminal was cloned in front of a mouse prion promotor (PRP), upstream of a poly-A signal in order to express the transgene primarily in neuronal cells (Fig. 1A). Transgenic animals overexpressing HtrA2 wild-type (Tg-HtrA2 WT) were generated and the line with the highest expression was chosen. Later the same cDNA PRP-construct containing HtrA2 cDNA, but containing the G399S mutation, was used to generate Tg animals. While establishing independent Tg-HtrA2 G399S mouse lines from Tg founders, only one progeny could be kept fertile for the Tg-HtrA2 G399S line. Primers that specifically detect human Tg-HtrA2 DNA in our animals revealed a relative copy number of ~1 for Tg-HtrA2 WT and ~4 for Tg-HtrA2 G399S. No signal was detected in non-Tg control animals (Fig. 1B and C). Although Tg-HtrA2 G399S animals had a high copy number of Tg DNA integrated in the genome, they presented a lower level of mRNA overexpression compared with the Tg-HtrA2 WT animals (Fig. 1D and E). There is substantially more Tg-HtrA2 WT mRNA than Tg-HtrA2 G399S mRNA in the respective Tg mice (Fig. 1D). This difference between Tg-HtrA2 WT overexpression and that of the G399S mutant was confirmed using primers detecting HtrA2-FLAG, where we detected a similar difference (Fig. 1E). Total HtrA2 RNA levels detected using a primer pair targeting a conserved region of HtrA2 common to humans and mice reveal that Tg WT HtrA2 animals express around five times the amount of endogenous HtrA2 in non-Tg animals. We were only able to detect ~15% overexpression of G399S RNA when we compared total levels of murine and Tg-HtrA2 RNA to
the non-Tg mice (Fig. 1F), despite the levels of murine HtrA2 were unchanged in both Tg lines (Fig. 1G). All RNA extractions were made from whole brain tissue and DNA was purified from ear biopsy. Expression of human WT or G399S HtrA2 in human embryonic kidney cells results in equal overexpression of both WT and G399S HtrA2 at the RNA and protein level (in the presence and absence of transcription and protein synthesis inhibitors, Supplementary Material, Fig. S1B). Since we detected high overexpression of G399S HtrA2 (∼10-fold at the protein level) in frozen brain tissue from a founder Tg-HtrA2 G399S line that was unable to breed (Supplementary Material, Fig. S1A), we concluded that in the remaining viable animals used for this study, the G399S mutant transgene was integrated in a low-expressing region of the genome or the stability of G399S HtrA2 mRNA is altered in vivo.

Tg-HtrA2 proteins are targeted to the mitochondria but the G399S HtrA2 protein levels are low

We assessed the levels of total and Tg-HtrA2 protein in several organs and brain regions of the founder mouse colonies and found overexpression of both Tg WT HtrA2 and Tg G399S HtrA2 in all major organs except spleen (Supplementary Material, Fig. S1C). Tg-HtrA2 protein is highly expressed in the brain, with the highest
expression in the cerebellum and olfactory bulb as expected from the PrP promoter (Fig. 2A) (21). In brain lysates, Tg WT HtrA2 protein expression in the cerebellum is ∼10-fold higher than that of the G399S HtrA2 mutant protein, detected using antibodies against either the FLAG tag or total HtrA2 (Fig. 2A). The HtrA2 antibody detects both endogenous and Tg-HtrA2, and the Tg-HtrA2 resolves slightly higher because of the FLAG peptide (Fig. 2A). The vast majority of Tg-HtrA2 remains in the mitochondria (Fig. 2B). Longer exposure of the western blots reveals that Tg-HtrA2 WT and to a lesser extent Tg-HtrA2 G399S is detectable in the cytosolic fraction (Fig. 2B).

As predicted from the expression pattern of the prion promoter used to drive the expression of recombinant HtrA2, Tg HtrA2 was detected in mouse brain tissue with the strongest staining within the granular layer of the cerebellum (Fig. 2C). Murine HtrA2 staining was weak, but detectable throughout all brain regions. We found low levels of total HtrA2 protein in brain tissue from TG G399S animals. Using immunohistochemistry, we found weak staining for total HtrA2 in Tg G399S animals in the cerebellum, basal ganglia, cortex, brain stem and olfactory bulb (Fig. 2C). It is possible that the HtrA2 epitope in tissue could be masked in Tg-HtrA2 G399S animals.

Therefore, we performed semi-native and native gels to detect all forms of HtrA2 using isolated mitochondria from the brains of non-Tg and Tg animals. Under semi-native conditions, we were able to detect FLAG-HtrA2 trimers in Tg-HtrA2 WT mice but not in Tg-HtrA2 G399S mice, whereas the FLAG-Tg monomers are present in both Tg animals (Fig. 2D, upper panel). In semi-native conditions, an antibody detecting endogenous and Tg-HtrA2 detected only endogenous HtrA2 trimers in Tg-HtrA2 G399S mice but endogenous, Tg and mixed endogenous/Tg-HtrA2 trimers in Tg-HtrA2 WT mice (Fig. 2D). We also prepared mitochondria from non-Tg and Tg animals and subjected them to blue-native PAGE following two different detergent treatments. Digitonin treatment allowed good detection of HtrA2 trimers and monomers in the TG WT HtrA2 animals, whereas HtrA2 monomers and trimers were hardly detectable in both non-Tg and Tg-HtrA2 G399S animals. Similar results were obtained using Triton X-100 as the detergent (Fig. 2E). Furthermore, we investigated homomeric HtrA2 interaction by immunoprecipitating FLAG-tagged Tg-HtrA2 and detecting co-immunoprecipitated proteins. FLAG pulldowns resulted in abundant pools of co-immunoprecipitated HtrA2 in the whole brain lysate of Tg-HtrA2 WT mice, but was just detectable in Tg-HtrA2 G399S animals (Supplementary Material, Fig. S2D). Because of the low level of overexpression in Tg-HtrA2 G399S mice, low sensitivity of detection with immunoblotting and immunoprecipitation cannot be ruled out. Taken together, the data support the hypothesis that the human Tg-HtrA2 in its WT form can form homomers. However, without sensitive antibodies specific for human or mouse HtrA2, it is difficult to specify that the G399S mutation categorically causes an absence or reduces the stability of the homomer formation.

**Overexpression of WT HtrA2 but not G399S HtrA2 causes a motor behavioral phenotype in mice**

The rotarod performance test can indicate both learning and motor deficits in rodents. Tg-HtrA2 WT mice consistently and significantly performed worse on the rotarod test than non-Tg animals and Tg-HtrA2 G399S mice from 6 months of age. We did not find an influence of ageing on the rotarod performance between animal groups using two-way analysis of variance (ANOVA), suggesting that the motor phenotype was already present at the beginning of the behavioral test. The Tg-HtrA2 WT mice performed worse than non-Tg and Tg G399S mice up to 14 months of age, indicating that Tg WT HtrA2 animals have a motor deficit (Fig. 3A). The impaired motor deficit observed in Tg-HtrA2 WT mice was not a result of changes in weight (Supplementary Material, Fig. S2A) and was accompanied by decreased motor skill learning (Supplementary Material, Fig. S2B).

Motor performance was also investigated using challenging beam walk, a locomotor test in which animals were videotaped while traversing a mesh beam. The time taken to traverse the beam and the number of times an animal limb slipped through the mesh was recorded. We found a significant increase in the number of slips made by Tg-HtrA2 WT animals compared with non-Tg animals (Fig. 3B). This implies that the Tg-WT HtrA2 mice have impaired motor balance and coordination.

We found no significant differences between the animal groups over a 14-month period using several other tests for locomotor activity or anxiety such as openfield or the automated home cage system (data not shown).

**Overexpression of WT and G399S mutant HtrA2 disrupt mitochondrial respiration**

Subcellular analyses of neurons throughout all brain regions using transmission electron microscopy revealed no organellar abnormalities in either Tg-HtrA2 WT or Tg-HtrA2 G399S mutant mice compared with non-Tg animals (Fig. 4A). However, mitochondrial membrane potential in cells isolated from the brains of Tg-HtrA2 G399S HtrA2 but not Tg-HtrA2 WT mice was ∼35% lower than in cells from non-Tg animals (Fig. 4B), but the difference was not statistically significant. Mitochondria from brains of both HtrA2 Tg animals revealed different patterns of respiration compared with non-Tg animals (Fig. 4C, upper panel). The mitochondria from animals overexpressing WT HtrA2 consumed less oxygen in response to adenosine diphosphate (ADP) (lower state III respiration) and in response to the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (maximal respiration) compared with non-Tg animals. This results in a significantly lower spare respiratory capacity in Tg-HtrA2 WT animals. In contrast, mitochondria from animals expressing HtrA2 G399S had a higher basal respiration rate and state III respiration rate compared with non-Tg controls, which, in combination with a slightly higher state IV respiration rate, resulted in a mild respiratory phenotype where spare respiratory capacity is significantly reduced compared with non-Tg controls. In line with these data, we consistently found a reduction of Complex 1 activity in Tg-HtrA2 WT mice (Fig. 4E). The overall level of oxidized proteins in brain cell lysates and isolated mitochondria are unchanged in our Tg animals compared with non-Tg controls (Fig. 4D), suggesting no significant alterations in the oxidative modification of proteins that could indicate increased reactive oxygen species. In parallel we analyzed by mass spectrometry the abundance of proteins in brain samples from our Tg-HtrA2 mice at 3 and 6 months of age, using a global approach. We found changes in the abundance of mitochondrial proteins at both 3 and 6 months of age. In Tg-HtrA2 WT animals, we observed a general reduction of subunits and core subunits abundance of electron transport chain complexes I and III coupled with an increase in an assembly factor for Complex V and regulatory enzymes of the glutathione-antioxidant system at 6 months of age (Fig. 4F).
Figure 2. Tg-HtrA2 proteins are targeted to the mitochondria, but the G399S HtrA2 protein levels are low. (A) Western blot showing levels of endogenous (En) and Tg-HtrA2 in brain lysates prepared from the cerebellum (CBM), cortex (CTX), basal ganglia (BG), olfactory bulb (OB) and brain stem (BS) of non-TG and Tg animals. The FLAG antibody detects only Tg HtrA2, whereas the HtrA2 antibody detects both En-HtrA2 and Tg-HtrA2, which resolves slightly higher. (B) Western blot showing brain lysates from non-Tg and Tg animals subfractionated to obtain total soluble extract, cytosol, mitochondria (Mito), matrix and mitochondrial inner membrane (Mat/IMM) and mitochondrial intermembrane space and outer membrane (IMS/OMM). The membrane stained with copper phthalocyanine shows the total protein input for each fraction. En- and Tg HtrA2 is visible at short (upper panel) and long (lower panel) exposures to film. (C) Immunohistochemistry of 7 µm thick paraflin brain sections, with heat-induced epitope retrieval in an EDTA buffer, using an antibody, which detects both human and murine HtrA2 and counterstained with hematoxylin. The specific signal was observed in multiple brain regions, but was prominent in the BS, CBM and OB. (D) Semi-native gel loaded with mitochondrial extracts from mouse brain made under native conditions and partially solubilized in a sample buffer containing 1% SDS and electrophoresed on an acrylamide gel containing no SDS. The FLAG antibody only detects transgenic trimers (Tg-trimer) and transgenic monomers (Tg-mono). The HtrA2 antibody detects endogenous HtrA2 trimers (En-tri), endogenous and transgenic trimers (Tg/En-tri), Tg-trimer, endogenous (En-mono) and transgenic monomers (Tg-mono). TOM20 was used as a loading control. (E) The blue-native gel loaded with mitochondrial extracts from mouse brain made under native conditions and subjected to solubilization with either 1% triton or 1% digitonin. The sample buffer contains no detergent and samples electrophoresed on a blue-native gradient gel containing no SDS. The HtrA2 antibody detects HtrA2 trimers and monomers. All western blots shown are representative and a minimum of three age- and gender-matched animals were used for each group in each experiment. Male and female animals aged 6 months were used, where n = 3.
significant increase in the amount of cell death in the cerebellum of Tg-HtrA2 WT animals (Fig. 5A). Overexpression of WT HtrA2 resulted in a significant (~50%) increase in cell death compared with baseline levels in non-Tg animals (Fig. 5A). In Tg-HtrA2 G399S animals, there was also an increase in cell death in the cerebellum by ~35%, but this was not statistically significant to P = 0.05 (Fig. 5A). A similar trend toward increased apoptosis in the cortex was observed (Fig. 5A). No changes in apoptotic cell death were detected between Tg and non-Tg animals in the basal ganglia region. The apoptotic cell death observed in Tg-HtrA2 WT animals is accompanied by a significant increase in cleaved caspase-9 detected by western blot (Fig. 5B), an apoptotic cleavage event that is mediated by the release of cytochrome c from mitochondria. A reduction of tyrosine hydroxylase (TH, a marker of dopaminergic neurons) in Tg-HtrA2 WT, but not in Tg-HtrA2 G399S, mice (Fig. 5B) might point to the relevance of dopaminergic cell death specifically leading to motor deficit in Tg-HtrA2 WT mice. We also observed upregulation in steady-state levels of heat shock protein 60 (Hsp60), total ubiquitin and XIAP in both Tg mice compared with non-Tg controls (Fig. 5B), indicating a cellular stress response.

To investigate the increased steady-state levels of the cytosolic HtrA2 substrate XIAP, we performed co-immunoprecipitation to test whether the XIAP-HtrA2 interaction is increased in HtrA2 Tg mice. XIAP co-immunoprecipitates with HtA2 significantly more in Tg-HtrA2 WT animals compared with the endogenous interaction in non-Tg or Tg-HtrA2 G399S animals (Supplementary Material, Fig. S2G).

Discussion

There is support for the relevance of HtrA2 in the pathways that are known to cause PD both in vivo (1,22,23) and in vitro models (2,17,24). To date, much of the in vivo work has been carried out in mice with HtrA2 deletion (1,25) and in Drosophila (26). Here, we provide evidence for a pivotal role of HtrA2 in cellular quality control and describe a mechanism and functional relevance of the G399S HtrA2 mutation in mice in vivo.

In our study, we expressed either human WT HtrA2 or human G399S mutant HtrA2 in mice based on the concept of a dominant-negative effect of mutant HtrA2 from our in vitro studies (2). Both transgenes integrated in the mouse genome but RNA expression of the transgene was ~10-fold less for fertile G399S HtrA2 mice. Tg-HtrA2 G399S mutant RNA is expressed but steady-state levels are much less than Tg-HtrA2 WT RNA. Our in silico analysis of the G399S mutation predicts that the RNA secondary structures for the native sequence are only slightly more energetically favorable, both for the predicted structures with minimum free energy and for the thermodynamic ensemble average across alternative structure predictions. No formation of new hairpin or loop structures that could interfere with translation was predicted by any of the in silico methods. To investigate whether the G399S mutation may instead affect the accessibility of the adjacent phosphorylation site (S400) as part of a conserved binding site on the protein surface, a visual analysis of the protein structure using colored conservation scores derived from a multiple sequence alignment (MSA) was conducted, showing that the mutated glycin residue is exposed on the protein surface and is highly conserved. The visual analysis also reveals that the replacement of G99 by a bulkier serine residue creates a bulge in the protein surface, which may sterically hinder access to the neighboring S400 phosphorylation site (see Supplementary Material, Fig. S2B). Overall, these observations suggest that the mutation is more likely to result in a modification of a binding site on the HtrA2 protein surface, rather than affecting mRNA degradation or translation. HtrA2 turnover has been reported in vitro (2) and in Drosophila (26), where the G399S mutation apparently had no effect on protein steady-state levels. Therefore, species differences, specific regulatory loops effective in mammals or effects related to site of integration may account for the observed differences.

Overexpression of Tg-HtrA2 RNA is reflected at the protein level in our mouse models, where WT HtrA2 is overexpressed ~10 times more than the G399S mutant in the cerebellum (the highest expressing brain region in line with the PrP promoter used to drive Tg-HtrA2). Still both WT and G399S mutant HtrA2 transgenes are highly expressed in the cerebellum compared with non-Tg animals.

Since we were only able to detect weak staining of HtrA2 in brain tissue (where the protein is not fully denatured), in Tg G399S mice by immunohistochemistry, we hypothesized that the G399S mutant protein might have a dominant-negative effect since the presence of mutant HtrA2 may affect trimeric assembly. Our in silico analysis of the G399S HtrA2 mutation predicts decreased stability of the RNA secondary structure with high confidence using Sfold (27). The glycine residue at position 399 has a high solvent accessibility, is part of a coil secondary structure...
Figure 4. Overexpression of WT HtrA2 and the presence of G399S mutant HtrA2 disrupt mitochondrial respiration. (A) Representative electron micrographs from the cerebellum of non-Tg and Tg mice where n = 3. No ultrastructural differences were observed in any of the animals in any of the brain regions prepared (cerebellum, cortex, brain stem, basal ganglia and olfactory bulb). (B) Mitochondrial membrane potential (Tetramethylrhodamine, Ethyl Ester, Perchlorate fluorescence) was measured by FACS using freshly isolated cells from the whole brains of non-Tg and Tg animals, where n = 6. (C) The top panel shows the respiratory analysis of non-Tg and Tg animals by measuring the OCR in freshly isolated mitochondria from the whole brain of mice, where n = 3. In each experiment, mitochondria were isolated, normalized and titrated in replicates of six for use in a Seahorse XF96 Analyzer™. In the basal state, Complex I is inhibited with rotenone and succinate is given as a substrate. Sequential injection of mitochondrial toxins allows measurement of OCR in state III respiration (stimulated by the addition of ADP), state IV respiration (addition of oligomycin), maximal respiration (addition of the uncoupler FCCP) and inhibition of the respiratory chain (antimycin A). The six lower panels illustrate the data with statistics for each group of animals in each state and the calculation of spare respiratory capacity and ATP output (n = 3, *P = 0.05, **P = 0.01, ***P = 0.001, n/s = not significant, one-way ANOVA, post hoc Dunnett). (D) Western blots of whole brain samples (left panel) and isolated mitochondria from the whole brain (right panel) labeled for the modification of proteins as a result of oxidation. A representative blot is shown from three replications using two animals from each group. (E) Complex I activity normalized to citrate synthase activity measured from the whole brain mitochondria isolated from three animals from each group (n = 3, ***P = 0.001, one-way ANOVA). (F) Relative spectral abundance of mitochondrial proteins, where differences were found between non-Tg and Tg groups (n = 3, *P = 0.05, **P = 0.001, one-way ANOVA).
and is evolutionary conserved (Supplementary Material, Fig. S2B), so it may not only be important for interaction with other proteins and post-translational modification, but also structural stability. We speculate that the instability of the HtrA2 protein trimer caused by the presence of the G399S mutation even at low levels is able to alter HtrA2 function, and may also affect the RNA pool through feedback mechanisms in mice in vivo. To our knowledge, the effect of the G399S mutation at the protein structural level has been investigated to date in vitro in human embryonic kidney and human neuroblastoma cells overexpressing FLAG-tagged HtrA2 and via FLAG immunoprecipitation (2). Transfected FLAG-tagged G399S mutant HtrA2 was shown to physically interact with transfected FLAG-tagged WT HtrA2 (2). It is possible that in acute overexpression models in vitro, complexes can form but in the chronic physiological situation in vivo, trimers might be disrupted and cause impaired function. Co-immunoprecipitation data support this hypothesis since very high amounts of total HtrA2 was pulled down with FLAG-tagged HtrA2 WT, but it was barely detectable in FLAG-tagged HtrA2 G399S pulldowns. However, we cannot conclude that G399S monomers cannot physically interact with WT HtrA2 monomers since the detection limits using FLAG antibodies on low-expressing Tg-HtrA2 G399S animals are minimal.

Figure 5. Overexpression of WT and to a lesser extent, G399S mutant HtrA2 causes cell death in the brains of Tg mice. (A) Apoptosis detected by TUNEL staining in cryosections from the mouse cerebellum, basal ganglia and cortex (n = 3, *P = 0.05, one-way ANOVA). (B) Representative western blots (left panels) and band quantification normalized to loading control in each case (right panels) of TH, cleaved caspase-9 (Casp 9), TOM20, heat shock protein 60 (Hsp60), XIAP and total ubiquitin protein levels shown in each case above β-actin or tubulin loading controls (n = 3–6, *P = 0.05, **P = 0.01, one-way ANOVA, post hoc Dunnett).
However, since we were unable to detect trimeric HtrA2 containing Tg G399S monomers on semi-native and native gels, we suggest that the presence of G399S mutant HtrA2 protein even at low levels could be enough to destabilize the trimeric structure of HtrA2, which is crucial for activity and function. These data may give further insight into the disease situation in humans, since heterozygous mutations in HtrA2 statistically could result in an ~75% loss of trimeric and therefore functional HtrA2.

We observe ~10-fold overexpression of WT human HtrA2 in our mouse model, with the highest expression in the cerebellum. Overexpression of HtrA2 caused significant motor dysfunction in our mice. We applied a classical approach to monitor several behavioral aspects of both mice expressing WT or G399S HtrA2 over a 14-month period. Two motor function tests revealed significant deficits in mice with overexpressed HtrA2, but not G399S mutant HtrA2. We cannot rule out that the differences in overexpression levels of the transgenes might account for the lack of motor dysfunction and milder phenotype in the Tg-HtrA2 G399S mice. It would be, therefore, extremely useful to investigate the G399S function and milder phenotype in the Tg-HtrA2 G399S mice. It would be, therefore, extremely useful to investigate the G399S function and milder phenotype in the Tg-HtrA2 G399S mice.

To our knowledge, only one WT HtrA2 overexpressing mouse model has been described earlier, where human WT HtrA2 was overexpressed in neurons using a rat neuron-specific enolase promoter (20). As our mice, these animals grew with normal weight gain and reproductive capacity, however, in contrast to our observations; no motor phenotype was reported in these lines. We speculate that the level of expression of the WT HtrA2 transgene may be responsible for the lack of motor phenotype, as the level of expression with ~2.8-fold compared with non-Tg animals was lower compared with the our model with ~10-fold overexpression of human HtrA2. Unfortunately, the authors did not specify, how ‘normal behavior’ was formally assessed and no data on motor tests were shown, so that motor phenotypes observed on rotarod or challenged beam walk as performed in our study cannot be excluded.

The overexpressed WT HtrA2 is mostly contained inside mitochondria with a small proportion detected in the cytosol. The levels of HtrA2 have to be kept in check for mitochondrial and cellular quality control (28). Processed functional HtrA2 in the cytosol is pro-apoptotic and loss of neurons could in part explain the observed motor phenotype. Indeed increased levels of apoptotic cell death were observed in mice overexpressing WT HtrA2 (Fig. 5A and B). Therefore, not only loss of HtrA2 function (1,22,28), but also overexpression of HtrA2 may cause neurodegeneration in mice via increased release from mitochondria or toxic gain of function. This indicates a critical regulation of levels of HtrA2 within neurons in order to maintain cellular homeostasis.

Increased HtrA2 levels inside mitochondria might also be damaging since HtrA2 is one of few regulatory proteases of mitochondrial as well as a chaperone. Loss-of-function studies have shown that mitochondrial substrates of HtrA2 are likely to be critical components of oxidative phosphorylation affecting respiration (24). Therefore, we assessed mitochondria in brain tissue of our mice overexpressing HtrA2 and G399S mutant HtrA2. We found no structural or morphological changes of mitochondria from both mice by transmission electron microscopy. Fitting with the different concepts of mechanism for each Tg line (gain of function for WT HtrA2 overexpression and reduces function for G399S overexpression), we found distinct respiration patterns in WT and G399S Tg mice compared with their non-Tg littermates. Mice overexpressing HtrA2 have a low-state III (ADP-stimulated) and maximal respiration; whereas G399S mutant mitochondria appear to be consuming more oxygen on a basal level, have higher state III respiration. However, both alterations in respiration come at a cost since their spare respiratory capacity is compromised. The data from Tg-HtrA2 G399S mice are in line with the previous respiratory report from the primary neurons of HtrA2 knockout mice (24), except that here we do not observe change in respiratory control ratio, rather spare respiratory capacity, which may be related to the differences in measuring oxygen consumption in whole cells compared with isolated mitochondria. In isolated mitochondria measurements, Complex I of the respiratory chain is inhibited and succinate given as substrate.

The enzyme activity of Complex I was already significantly reduced in Tg-HtrA2 WT mice and in this case may mask to some extent a phenotype whereby reduced substrates are provided by Complex I. Therefore, more work is needed (ideally in primary cell cultures or using the iPSC technology) that might reveal further the complex mechanism of the metabolic defects and confirmation of theoretical adenosine triphosphate (ATP) output with a luciferase-based assay. We hypothesized that levels of ROS in Tg animals might be elevated due to an altered mitochondrial redox state, but we could not observe any changes in the state of oxidative protein modification in mitochondrial or whole cell fractions. Further work is required in cell models, where live microscopy can be employed to determine the redox status and quantification of mitochondrial and cytosolic ROS.

In order to find potential correlates between function and abundance of components of the respiratory chain, we used a mass spectrometry approach to determine the abundance of all proteins in our Tg models. We looked for changes in the abundance of mitochondrial proteins. In Tg-HtrA2 WT animals, we observed a general reduction of subunits and core subunits abundance of electron transport chain complexes I and III coupled with an increase in an assembly factor for Complex V. One could speculate that a significant increase in functional HtrA2 inside mitochondria might be detrimental since the finely balanced assembly, folding and degradation of respiratory chain complexes could be disrupted by ‘over surveillance’ by HtrA2. Furthermore, oxidative phosphorylation is required to continually respond to cellular needs and this may put mitochondria under stress if HtrA2 is itself involved in modulating such metabolic responses. Compensatory mechanisms might explain increases in assembly factors and enzymes involved in maintaining mitochondrial redox.

These data underscore the role of HtrA2 in mitochondrial function via fine tuning of mitochondrial proteostasis. Altered proteostasis of respiratory substrates compromises mitochondrial function impacting on ageing (28), mitochondrial dynamics (25), PINK-1 dependent quality control (17) autophagy (16) and of course regulation of cell death. Indeed, we observed reduced mitochondrial membrane potential in mixed cells from the cerebellum of mice with overexpressed mutant G399S HtrA2. These data point toward a pivotal role for HtrA2 where the G399S mutation leads to reduced function with negative effect. In contrast the overexpression of HtrA2 leads to a toxic gain of function in vivo. HtrA2 serine protease activity is instrumental under normal conditions and crucial under stress conditions for control of proper cellular responses, but first and foremost as a regulator of mitochondrial quality control. Since mitochondria are central to the initiation of apoptosis, HtrA2 plays a central role at several different levels of quality control. In our models, increased apoptosis was coupled with a general reduction in TH levels and increased XIAP levels highlighting the vulnerability of the mitochondria of dopaminergic neurons and the requirement for cells to protect overall integrity by using different levels of
cellular quality control including modulation of apoptosis. The increased steady-state levels of XIAP in Tg animal lysates is not directly related to HtrA2 binding since XIAP levels are significantly increased in the lysates of Tg-HtrA2 G399S mice, but the physical interaction between HtrA2 and XIAP is not. It is possible that increased XIAP levels are a result of the stress response or apoptotic feedback in the Tg animals.

This mutation in a critical position in the PDZ domain of HtrA2 should be considered relevant if we consider the extremely complex regulation of HtrA2 through its allosteric interactions (18,29) as well as post-translational modification (17,19) and conformational changes leading to its activity (8,18) both inside and out of the mitochondria.

Our data provide new insights into the fine-tuned regulation of HtrA2 function in vivo as they add the G399S Tg phenotype in mice to previous loss-of-function models and provide first evidence for a neurodegenerative motor phenotype related to over-expression of WT HtrA2. Our data support a role of HtrA2 in mitochondrial and cellular quality control related to neurodegeneration in vivo and, underscore the relevance of molecular and cellular quality control mechanisms during ageing.

Material and Methods

Animals

Mice were housed in a group of maximum five animals per cage on a 12:12 h light-dark cycle. All the procedures used were followed according to international ethical guidelines for the use of laboratory animals and all procedures, and husbandry was approved by the local Animal Welfare and Ethics committee of the County Commission, Tübingen, Germany.

Generation of HtrA2 Tg mice

FLAG-tagged human WT and G399S HtrA2 cDNA clones were generated and described previously (2). Both constructs were cloned into pBluescript II KS (+) vector (Agilent Technologies, 212208) downstream of a murine Prp promoter [a gift from Thorsten Schmidt (21)]. Poly-adenylated residues were added after the HtrA2 DNA sequence. DNA fragments were injected into fertilized as described previously (32) by evaluating the progression and the tests took place over 4 days. Motor skill learning was analyzed by the average of all trials per day and using two-way ANOVA. Motor coordination and fine movement alterations were tested in challenging beam walk during dark phase as described previously (33).

Conventional electron microscopy

For conventional electron microscopy, tissues were fixed with 2.5% glutaraldehyde (Paesel-Lorei, Frankfurt, Germany) buffered in 0.1 M cacodylate buffer (pH 7.4). Thereafter, the tissues were post-fixed in the identical fixative for 4 h, and then stored in cacodylate until further processed as previously described in detail (34). Ultra-thin sections were analyzed with a Zeiss EM-10 electron microscope (Zeiss, Oberkochen, Germany).

Immunohistochemical studies

Immunohistological analysis was performed as reported previously (31) and adapted for HtrA2 staining [Af1458, R&D Systems; diluted 1:500 in phosphate-buffered saline (PBS), 1% normal serum]. The TUNEL method was applied on tissue sections to visualize DNA fragmentation (in situ Cell Death Detection Kit, TMR red, #12 156 792 910, Roche Diagnostics). According to the manufacturer’s instructions, 6 µm sections of 12-month-old mice were incubated 4% paraformaldehyde in PBS for 20 min and washed 30 min in PBS. Sections were then incubated in permeabilization buffer (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice and washed in PBS. Enzyme solution mix was then applied on sections for 1 h at 37°C, washed in PBS and then mounted in Mowiol (475904, Calbiochem) supplemented with 25 mg/ml of antifade (DABCO, D27802, Sigma-Aldrich). Staining was visualized using an AxioCam MR camera (Zeiss). Cell counting was performed by a blinded unbiased method, using three mice per group, four slices per mice and eight non-overlapping counting frames per slices. Counting frames were taken at ×20 magnification (270 × 200 mm). Values obtained were normalized by the total number of nuclei per field.

RNA isolation and cDNA preparation

The total RNA from 3- to 6-month-old whole mouse brain was isolated using the GeneJet RNA purification kit (#K073, Thermoscientific). RNA quality and concentration was determined using a spectrophotometer (NanoDrop 2000, ThermoScientific). Synthesis of cDNA was performed with 200 ng of total RNA using the SuperScript III Reverse Transcriptase Kit (Life Technologies) and Oligo(dt) primer (Life Technologies).

Quantitative PCR

Quantitative PCR reactions were performed to assess the expression levels of endogenous and Tg-HtrA2 and the respective housekeeping genes using FastStart SYBR green Master mix (Roche) to amplify 2 ng of genomic DNA using 10 µM of each primer. β-actin was used as an internal control (Supplementary Material, Table S1). To calculate relative copy numbers, efficiency of the both HtrA2 and β-actin were investigated via serial dilution of genomic DNA and used to correct delta–delta Ct method (30).
For sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), mouse tissue was prepared for western blot according to Casadei et al. (31). All membranes were stained with 0.1% copper phthalocyanine in 12 mm HCl to stain for total protein loading prior to blocking. Proteins were detected by immunoblotting with antibodies against HtrA2 (AF1458, R&D Systems), FLAG® (F3165, Sigma-Aldrich), β-actin (A1978, Sigma-Aldrich), TH (AB5425, Chemicon), XIAP (120116377, Biocarta) and LC3-I/II (Q9GZQ8, Novus). For semi-native PAGE, mitochondrial pellets were resuspended in 30 µl sample buffer [125 mm Tris/HCl pH 6.8, 10% (v/v) glycerol, 1% (v/v) SDS (10% w/v), bromophenol blue]. Samples were resolved by electrophoresis in a 10% gel lacking SDS at 4°C and further analyzed by immunoblotting with HtrA2 (AF1458, R&D Systems), FLAG® (F3165, Sigma-Aldrich) and TOM20 as loading control (sc-17764, Santa Cruz). For blue-native PAGE, mitochondria were lysed in 50 µl solubilization buffer (20 mM Tris/HCl pH 7.2, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaCl, 10% (v/v) glycerol, 1 mM PMSP) containing 1% of either Triton X-100 or digitonin. Insolubilized material was spun down (30 000 g, 30 min, 2°C) and 5 µl sample buffer (5% (w/v) Coomassie blue G, 500 mM 6-aminono-7-acetic acid, 100 mM Bis–Tris pH 7.0) were added to the supernatant. Samples were analyzed on a 6–13% gradient blue-native gel and proteins were detected by immunoblotting with HtrA2 (AF1458, R&D Systems).

Mitochondrial membrane potential
Intact cells from mouse brain were isolated according to Derecki et al. (35) and mitochondrial membrane potential measured using a fluorescence-activated cell sorting (FACS) method previously reported (36).

Isolation of mitochondria and cellular subfractionation
Mitochondria were isolated by homogenization in a sucrose buffer followed by differential centrifugation, according to a protocol described by Burte et al. (37). Cellular subfractionation was carried out using differential centrifugation described previously (19).

Complex I activity
Following isolation of mitochondria from whole mouse brain (37), Complex I activity was measured according to Hargreaves et al. (38). The activity of Complex I was normalized to citrate synthase activity and, therefore, data are expressed as a ratio of Complex I/citrate synthase. Citrate synthase activity measurements were also based on the protocol by Hargreaves et al. (38). All substrates and chemicals required for Complex I and citrate synthase activity measurements were purchased from Sigma-Aldrich.

Respiratory analysis
OCR was measured in isolated mitochondria from whole animal brains using a Seahorse™ XF96 Extracellular Flux Analyzer according to Rogers et al. (39). The basal respiration state was measured in the presence of the Complex I inhibitor rotenone (Sigma-Aldrich) and succinate (Sigma-Aldrich) was given as a substrate. ADP (Sigma-Aldrich) was injected to stimulate state III respiration, oligomycin (Santa Cruz Biotechnology) (state IV), followed by the addition of FCCP (Santa Cruz Biotechnology) to maximize respiration and finally inhibition of Complex III by antimycin A (Santa Cruz Biotechnology). All substrates and mitochondrial toxins were titrated and used in a concentration range recommended by Rogers et al. (39). Mitochondria were titrated in each experiment at 4, 6, 8 and 10 µg per well. All substrates were used per line in total. The respiratory control ratio was calculated: state III/state IV respiration, ATP output: basal respiration-state IV respiration, proton leak: state IV respiration-Antimycin A inhibition and spare respiratory capacity: maximal respiration-state IV respiration.

Mass spectrometry
Briefly, mouse brains were disrupted in urea–solution and lysates cleaned-up by separation on a 12% polyacrylamide Bis–Tris gel. Coomassie stain gel pieces were excised, destained and subsequently used for an in-gel digest with 0.15 µg trypsin (Serva Electrophoresis GmbH, Heidelberg, Germany) in 50 mM ammonium bicarbonate at 37°C overnight. Quantitative label-free analyses was performed with an UltiMate 3000 RSLC nano Liquid Chromatography (LC) system ( Dionex, Idstein, Germany) coupled to a Q Exactive quadrupole-orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as described by Molina et al. (40). The spectral counting method was used to identify unique peptides. From the feature list in Progenesis LC MS, containing m/z values of all peptides, only those charged positively 2-, 3- or 4-fold were used for quantification. To correct experimental variation between the runs, the raw abundances of each feature were normalized as previously published (41). Proteins were identified by Mascot (v.2.3.0.2) (Matrix Sciences Ltd). Label-free quantification by spectral counting was completed according to (42). To detect differential-regulated proteins and for multiple testing an adjusted ANOVA was used (43) (for a detailed description of the mass spectrometry method and analysis, see Supplementary Material).

In silico analyses
Prediction of the RNA structure was obtained across three different bioinformatics approaches, RNA-fold (44), Mfold (45) and Consurf (46). The visualization and the MSA using homologues of the HtrA2 sequence from the UNIREF90 database were generated using the Consurf software (46).

Statistical analyses
All other statistical analyses were performed using ANOVA or Student’s t-test (stated in the figure legends), where independent experimental n was a minimum of 3 and *P < 0.05, **P < 0.005 and ***P < 0.001.

Supplementary Material
Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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