Rescue from excitotoxicity and axonal degeneration accompanied by age-dependent behavioral and neuroanatomical alterations in caspase-6 deficient mice

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Abstract

Apoptosis, or programmed cell death, is a cellular pathway involved in normal cell turnover, developmental tissue remodeling, embryonic development, cellular homeostasis maintenance and chemical-induced cell death. Caspases are a family of intracellular proteases that play a key role in apoptosis. Aberrant activation of caspases has been implicated in human diseases. In particular, numerous findings implicate Caspase-6 (Casp6) in neurodegenerative diseases including Alzheimer’s and Huntington disease, highlighting the need for a deeper understanding of Casp6 biology and its role in brain development.

The use of targeted caspase deficient mice has been instrumental for studying the involvement of caspases in apoptosis. The goal of this study was to perform an in depth neuroanatomical and behavioral characterization of constitutive Casp6-deficient (Casp6 -/-) mice in order to understand the physiological function of Casp6 in brain development, structure and function.

We demonstrate that Casp6 -/- neurons are protected against excitotoxicity, nerve growth factor deprivation and myelin induced axonal degeneration. Furthermore, Casp6 deficient mice show an age-dependent increase in cortical and striatal volume. In addition, these mice show a hypoactive phenotype and display learning deficits. The age-dependent behavioral and region-specific neuroanatomical changes observed in the Casp6 -/- mice suggest that Casp6 deficiency has a more pronounced effect in brain regions that are involved in neurodegenerative diseases, such as the striatum in Huntington disease and the cortex in Alzheimer Disease.
Introduction

Apoptosis, or programmed cell death, is a highly regulated process involved in embryonic development, developmental tissue remodeling and normal cell turnover (1, 2). However, when dysregulation occurs in apoptotic pathways, excessive or insufficient cell death can lead to diseases such as cancers, autoimmune syndromes and/or neurodegenerative diseases (3, 4). Caspases are a family of intracellular cysteine-aspartic proteases that are not only essential for triggering programmed cell death (5, 6), but have also been shown to play key roles in non-apoptotic pathways, such as differentiation and proliferation of diverse cell types, axon guidance and synaptic activity and plasticity (7-11).

Caspases are divided into long prodomain caspases (caspases -2, -8, -9 and -10) that are initiators of apoptosis, and short prodomain caspases (caspases -3, -6, -7 and -14) that are generally termed the effectors of apoptosis (12). However, some caspases, including caspase-3 (Casp3) and caspase-6 (Casp6), appear to function as both initiators and effectors. For example, active Casp6 is observed in preclinical Alzheimer disease (AD) and Huntington disease (HD) brain years prior to overt cell death and can activate caspases-2, -3 and -8 (13-16). Similarly, activation of Casp3 has been detected concomitantly with memory loss in a murine model of AD and before obvious detection of cell death (17). Casp3 has also been shown to activate Casp6 in certain experimental paradigms (16).

Aberrant activation of caspases has been implicated in several neurodegenerative diseases, such as Alzheimer Disease (AD), Huntington Disease (HD), various ataxias and amyotrophic lateral sclerosis (ALS) (13, 18-22). Recently, Casp6 has also been implicated in ischemic brain injury (23). In mouse models of AD, baseline Casp3 activity has been shown to be elevated in hippocampal dendritic spines (17). Interestingly, inhibiting caspase cleavage of β-amyloid precursor protein (APP) at aa664, a described Casp6 cleavage site, results in the rescue of AD-like behavioral and neuropathological phenotypes (18, 24-30). Furthermore, cleaved APP binds to the death receptor 6 (DR6), which activates Casp6, resulting in axonal degeneration in an AD mouse model (31).
Casp6 cleavage of mutant huntingtin (mhtt) at aa586 has also been shown to be a rate-limiting step in the pathogenesis of HD (32, 33). Mice carrying mhtt resistant to cleavage by Casp6 maintain normal neuronal function, do not develop striatal neurodegeneration and are protected against neurotoxicity (14, 32, 34-36). Recently, Casp6 activity levels in the brain were also shown to be positively correlated with CAG size and to be increased in persons carrying the mutation but being asymptomatic (14).

Given the various findings implicating Casp6 in the progression of neurodegenerative diseases and the efforts underway to identify Casp6 inhibitors as a therapeutic strategy for neurological diseases (37, 38), understanding the normal role of Casp6 in brain development and structure is crucial. Therefore, the goal of this study was to perform an in depth neuropathological and behavioral characterization of mice lacking in Casp6 (Casp6 -/-).

We demonstrate that Casp6 -/- neurons are protected against both nerve growth factor (NGF) deprivation induced axonal degeneration and neurotoxic stimulus N-Methyl-D-aspartic acid (NMDA)-mediated excitotoxicity, thereby strengthening the evidence for a crucial role for Casp6 in this process. Similar to axons lacking p75 neurotrophin receptor (p75NTR) (39), Casp6 -/- axons were also observed in the white matter tract of the corpus callosum, which is uncommon in normal axons. These findings validate the role of Casp6 in myelin-dependent axon degeneration in the brain.

We show that Casp6-deficient mice show an age-dependent increase in cortical and striatal volume. In addition, they show a hypoactive phenotype and display a learning deficit. The age-dependent behavioral and neuroanatomical changes observed in the Casp6 -/- mice suggest that Casp6 deficiency has a more pronounced effect in brain regions including the striatum and cortex that are initially affected in HD and AD, respectively.
Results

Casp6 -/- brain and peripheral tissues show no Casp6 expression

Exons 2 to 5 of the Casp6 gene encode the catalytic domain of the Casp6 protein (40). These exons were removed to generate Casp6 knockout (Casp6 -/-) mice that express a truncated and inactive form of Casp6 (Supplementary Material, Fig. S1A and B). Two PCR assays were designed for genotyping: the wild type (WT) assay amplifies the WT allele from Casp6 WT and heterozygous (Casp6 +/-) mice; and the neo assay amplifies the knocked out allele from Casp6 +/- and Casp6 -/- mice. DNA extracted from Casp6 -/- mice shows PCR products generated with the neo primers. In contrast, no PCR products are detected in Casp6 -/- DNA using the WT primers (Supplementary Material, Fig. S1C).

These mice were then examined by quantitative reverse transcriptase PCR (qRT-PCR) and western analysis to confirm the absence of Casp6 expression and Casp6 protein in brain and peripheral tissues of Casp6 -/- mice. Quantitative RT-PCR shows no Casp6 mRNA expression in Casp6 -/- and reduced Casp6 mRNA expression in Casp6 +/- brain tissue (Fig. 1A; ANOVA p=0.0001). Western blotting using a Casp6 antibody demonstrates absence of the Casp6 protein in Casp6 -/- cerebellum, hippocampus, striatum, cortex, kidney, liver, spleen and post-natal day 0 (P0) peripheral tissue (Fig.1B). These findings confirm that the Casp6 -/- mice used in this study constitutively lack Casp6.

Casp6 -/- sympathetic neurons show protection against axonal degeneration

Axonal degeneration is a crucial process for normal neurodevelopment (41, 42) and is disturbed in neurological disorders (31, 43, 44). It has been recently established that axonal degeneration in both an AD mouse model and after growth factor deprivation at axon terminals is mediated through activation of Casp6 (31, 39).
Enhanced axonal degeneration may also explain the early white matter loss that is seen in individuals with HD and AD (45-47).

We investigated if Casp6 -/- sympathetic neurons are protected from axonal degeneration after nerve growth factor (NGF) withdrawal. Sympathetic neurons were cultured in microfluidic chambers to allow deprivation of NGF from the axon compartment. While axons of WT sympathetic neurons degenerate after NGF deprivation (Fig. 2A and C; t-test p < 0.0001), NGF withdrawal did not induce axonal degeneration in Casp6 -/- sympathetic neurons (Fig. 2B and C; t-test p=0.24) (n=3 chambers/condition/genotype).

Medium spiny neurons from Casp6 +/- and Casp6 -/- mice show protection against NMDA-mediated excitotoxicity in a Casp6 dose dependent manner

Casp6 inhibitors and/or dominant-negative inhibition of Casp6 provide protection against excitotoxic stress (14, 15). Therefore, we hypothesized that medium spiny neurons (MSNs) derived from Casp6-deficient mice might be protected from NMDAR-mediated excitotoxicity. Casp6 -/- MSNs demonstrate a significant decrease in LDH levels (Fig. 3A; one-way ANOVA p=0.01, post-hoc Tukey WT vs. Casp6 -/- p<0.05, n=10 cultures), a significant increase in levels of ATP (Fig. 3B; one-way ANOVA p=0.03, post-hoc Tukey WT vs. Casp6 -/- p<0.05, n=10 cultures) and reduced TUNEL-positive cells (Fig. 3C and SI2; one-way ANOVA p=0.02, post-hoc Tukey WT vs. Casp6 -/- and WT vs. Casp6 +/- p<0.05, n=9-12 cultures) compared to WT MSNs post-NMDA treatment, indicative of protection against NMDAR-mediated excitotoxicity in Casp6 -/- MSNs. Furthermore, post-hoc linear trend test reveals a dose-dependent effect; where the Casp6 +/- mice also demonstrate partial rescue from NMDAR-mediated excitotoxicity (post-hoc test for linear trend, LDH: p=0.005; ATP: p=0.01; TUNEL: p=0.02).

Casp6 is necessary for myelin-induced degeneration of septal cholinergic axons in vivo

A recent study demonstrated that axon degeneration was an ongoing process in the mature adult brain and implicated a p75NTR-mediated pathway in this process.
Immunostaining of choline acetyltransferase (ChAT) allowed visualization of cholinergic axons that projected from the forebrain to the hippocampus by way of the supracallosal pathway, which is immediately adjacent to the corpus callosum.

In wild type mice any axons of septal cholinergic neurons that sprouted on to the myelinated corpus callosum degenerated, while axons in p75NTR-null mice grew and survived on this myelin tract (39). Since p75NTR-mediated axon degeneration requires Casp6 in vitro (39), we determined if genetic ablation of Casp6 inhibited axonal degeneration in vivo.

Axon number and length were measured in 3- and 8-month old mice. In Casp6 +/- mice, only a few short ChAT-positive axons were observed on the white matter of the corpus callosum (Fig. 4A), which is consistent with WT littermate controls and previous WT data from the previous study (39) (data not shown). Therefore, WT and Casp6 +/- data were combined as one control group. In contrast, in brains of Casp6 -/- mice, more ChAT-positive axons were evident in the corpus callosum and many of these were very long (Fig. 4A).

Quantification of these results verified that at 3 and 8 month of age more ChAT-positive axons grew on the corpus callosum in Casp6 -/- versus control mice (Fig. 4B; two-way ANOVA genotype: p=0.037, age: p<0.0001, interaction p=0.76, n= 3-4 control, 3-4 Casp6 -/-), and that the total length of these axons was significantly increased (Fig. 4C; two-way ANOVA genotype: p=0.0015, age: p<0.0001, interaction p=0.88, n= 3-4 control, 3-4 Casp6 -/-). Even after normalizing for the increased number of axons growing on the corpus callosum, axons in Casp6 -/- mice were significantly longer than those in control mice at both ages (Fig. 4D; two-way ANOVA genotype: p=0.037, age: p=0.86, interaction p=0.96, n=3-4 control, 3-4 Casp6 -/-). Moreover, while many ChAT-positive axons grew continuously over the white matter in Casp6 -/- brains, axons growing in the control brains appeared to be degenerating (Fig. 4A).

Cortical and striatal volume is increased in older Casp6 -/- mice

Mendelian ratios and necropsy were examined prior to neuroanatomical studies to assess viability and gross peripheral abnormalities in Casp6-deficient mice. Casp6 -/-
mice are viable, breed normally and are born in appropriate Mendelian ratios (Supplemental Table 1). Additionally, necropsy performed on Casp6 -/- mice reveal that testis, kidney, liver, heart, spleen, stomach, intestine, cecum and colon of the Casp6 -/- mice are normal compared to WT mice (Data not shown; n=1).

Deletions of specific caspases can result in robust brain malformations associated with supernumerary cells, multiple cerebral indentations and ectopic cell masses in the cortex (48-51). To determine if Casp6 -/- mice have any brain malformations, brain and cerebellum weights were measured and more detailed structural and volumetric analyses were examined through stereology in Casp6 -/- mice at 3 and 8 months of age. Casp6 -/- mice display normal brain architecture at 3 months of age (Supplementary Material, Fig. S2; one-way ANOVA cortical volume p=0.49, striatal volume p=0.66, striatal neuronal counts p= 0.23, brain weight p=0.10, cerebellum weight p=0.50, n=20). However, neuropathological analysis at 8 months reveals a significant increase in cortical (Fig. 5A; one-way ANOVA p=0.004, post-hoc Tukey WT vs. Casp6 -/- and Casp6 +/- vs. Casp6 -/- p<0.05, n=6) and striatal volume (Fig. 5A; one-way ANOVA p=0.02, post-hoc Tukey WT vs. Casp6 -/- p<0.05, n=6) and in striatal neuronal counts (Fig. 5A; one-way ANOVA p=0.03, post-hoc Tukey WT vs. Casp6 -/- p<0.05, n=6). Furthermore, post-hoc test for linear trend reveals a dose-dependent effect; the Casp6 +/- mice demonstrate an increase in cortical and striatal volume and in striatal neuronal counts compared to WT, but to a lesser extent than the Casp6 -/- mice (post- hoc linear trend, cortex volume: p=0.001; striatum volume: p=0.006; striatal neuronal counts: p=0.01). However, no differences were observed in brain (Fig. 5B; one-way ANOVA p=0.36, n=20) and cerebellum weight (Fig. 5B; one-way ANOVA p=0.38, n=20) in Casp6 -/- mice compared to WT at 8 months of age.

**Casp6 -/- mice demonstrate a hypokinetic phenotype and decreased novel object preference**

Before conducting behavioral tests we investigated body weight in Casp6 -/- mice. Longitudinal recording of body weight shows no differences in body weight between Casp6 -/- and WT mice from 2 to 8 months of age (Females: RM ANOVA genotype:
p=0.40, age: p<0.0001, interaction p=0.95, n=7 WT, 9 Casp6 -/-; Males: RM ANOVA genotype: p=0.20, age: p<0.0001, interaction p=0.42, n=4 WT, 10 Casp6 -/-).

In light of the neuroanatomical data, rotarod, total activity and object recognition tasks were conducted to elucidate whether Casp6 -/- mice show behavioral deficits. Two independent cohorts were tested for each behavioral task and since the results from the cohorts were not significantly different for each test (ANOVA p>0.05), the cohorts were pool, analyzed and presented below.

Casp6-deficient mice display motor coordination indistinguishable from WT mice during the accelerating rotarod testing at all time-points assessed (Fig. 6A; factorial ANOVA genotype: p=0.27, age: p<0.0001, interaction p=0.86, n=19 WT, 21 Casp6 -/-). However, Casp6 -/- mice are hypoactive compared to WT mice during the 30-minute open field trial that measures locomotor activity (Fig. 6B; factorial ANOVA genotype: p=0.0017, age: p=0.053, interaction p=0.75, n=17 WT, 20 Casp6 -/-).

Furthermore, Casp6 -/- mice display a deficit in the novel object preference task when tested at 12 months (Fig. 6C; factorial ANOVA genotype: p=0.37, trial: p<0.0001, interaction: p=0.0014, n= 13 WT, n= 19 Casp6 -/-). In trial 2, WT animals spend more time exploring the novel object that was not present during trial 1 (Fig. 6C; post-hoc Tukey p=0.00016), indicating that they are able to distinguish the known object from the novel one. However, Casp6-deficient mice did not spend significantly more time exploring the novel object during trial 2 (Fig. 6C; post-hoc Tukey p=0.58), indicative of a learning deficit at this time-point.
Discussion

In this study we provide evidence that Casp6 activity in vivo is essential for axonal degeneration, as Casp6 -/- neurons are completely protected from NGF-deprivation induced axonal loss in vitro and do not undergo axonal degeneration on the myelin in vivo. We further establish a role of Casp6 in NMDAR-mediated excitotoxic cell death by demonstrating that Casp6 -/- and Casp6 +/- neurons are protected against NMDA stimulation in a dose dependent manner. In addition, this study is the first to examine the neuroanatomical and behavioral effects of ablating Casp6 in mice, which has identified age-dependent and region-specific changes in Casp6 -/- mice.

Previous studies have demonstrated increased activity of Casp6 in neurological diseases, including HD, AD, and ischemia (13-15, 23, 31, 32). Casp6 inhibitors and/or dominant-negative inhibition of Casp6 have also suggested a role for Casp6 in axonal degeneration (31, 39).

Axonal degeneration is a key mechanism involved in developmental axonal pruning (41, 42). However, it also occurs in the mature nervous system as a consequence of neuronal damage and contributes to neuronal loss in neurodegenerative disorders (31, 43, 44). Patients with early HD display markedly altered white matter in the corpus callosum, thalamus, sensorimotor and prefrontal regions (45, 46, 52). Interestingly, white matter pathways of the sensorimotor cortex and corpus callosum are already reduced in pre-manifest HD subjects compared to control subjects (46), indicating that axonal degeneration is an early pathogenic event.

Magnetic resonance imaging (MRI) analysis of the YAC128 full length mouse model of HD shows progressive white matter loss that corresponds to alterations observed in human HD (53). Axonal white matter is also reduced in AD patients at the earliest stages of disease (47). Similarly, AD mouse models show age-dependent axonal degeneration in the cortex, hippocampus, midbrain and hindbrain that likely contributes to the motor and cognitive behavioral deficits observed in these mice (54,
In an AD mouse model, axonal degeneration occurs through activation of Casp6 (31, 39, 56), where the N-terminal fragment of cleaved APP binds to DR6, activating Casp6 and resulting in axonal degeneration (31). The use of Casp6 inhibitors rescued the APP-induced axonal degeneration thereby implicating a critical role for Casp6 in this process. Casp6 activation also leads to neurite degeneration in a process independent of amyloid β-peptide (Aβ) production from APP cleavage. APP mutants that cannot generate Aβ still induced neurite beading and cell death, which are inhibited by Casp6 inhibitors (56).

It has also been established that Casp6 is involved in focal non-pathogenic axonal pruning along myelin tracks, such as in the corpus callosum, in mice (39). Our findings show that Casp6-/- sympathetic neurons are protected against NGF deprivation–mediated axonal degeneration in vitro and Casp6 -/- cholinergic neurons are protected from pruning of their axons on the myelin-rich corpus callosum in vivo, These data are consistent with Casp6 being necessary for myelin-dependent axon degeneration in the intact adult brain.

Casp6 activity has been shown to be increased in the brains of both early stage HD patients and in an HD mouse model (14). In addition to axonal degeneration, we examined whether Casp6 activity may play an important role in NMDAR-induced excitotoxicity. Over-activation of glutamate receptors is involved in the early stages of HD (57, 58) and several HD mouse models demonstrate enhanced susceptibility to glutamate and/or NMDAR-mediated excitotoxic stress (14, 57, 59, 60). The direct link between Casp6 activity and enhanced NMDAR-mediated excitotoxic stress in HD mouse models was demonstrated when MSNs expressing mHtt showed Casp6 activation post-NMDA stimulation (14) and protection against excitotoxic stress was observed with use of Casp6 inhibitors and/or dominant negative inhibition of Casp6 (14, 15). Furthermore, mice expressing mHtt resistant to cleavage by Casp6 do not show enhanced Casp6 activation and demonstrate protection from excitotoxic stress (14, 32) and alterations in extrasynaptic NMDA receptors in vivo (35).
To further demonstrate that Casp6 has a crucial role in this process, here we show that MSNs derived from Casp6 +/- and Casp6 -/- mice show protection against NMDAR-mediated excitotoxicity in a Casp6 dose dependent manner. The Casp6 dosage effect suggests that even partial inhibition of Casp6 activity can be beneficial in reducing cell death under excitotoxic conditions. This has important implications for the potential therapeutic efficacy of Casp6 inhibitors in neurological diseases where enhanced susceptibility to NMDAR-mediated excitotoxicity has been shown. Our findings provide further support that inhibition of Casp6 may represent a possible therapeutic target for neurodegenerative diseases (37).

Given the importance of Casp6 in axonal degeneration and excitotoxicity, both processes involved in HD and AD, Casp6 -/- mice were examined for neuroanatomical alterations in regions affected in these neurodegenerative diseases. Interestingly, we identified age-dependent and region-specific neuroanatomical changes in the Casp6 -/- mice. Neuroanatomical analysis at 8 months of age reveals a significant increase in cortical and striatal volume and striatal neuronal counts in Casp6-deficient mice compared to WT mice, which were not observed at 3 months of age. Since increased Casp6 activity is implicated in the pathogenesis of HD and AD, where selective degeneration of neuroanatomical structures is progressive and becomes more severe and widespread with advancing age (61-63), the age-dependent increase in cortical and striatal volume observed in Casp6 -/- mice is congruent with evidence showing that Casp6 is required in processes leading to neurodegeneration. The neuroanatomical alterations observed in Casp6-deficient mice also provide evidence that specific brain regions, such as the striatum and cortex, are particularly sensitive to Casp6 activity in an age-dependent manner.

Casp6 does not act alone and plays a crucial role in the regulation of cell death pathway members. Although details of apoptosis pathways are currently being elucidated, Casp9, 3 and 8 are shown to be key regulators and effectors of Casp6 activity (23, 64). Casp6 has recently been identified as a downstream target of active Casp9, where inhibition of Casp9 activity results in a lack of Casp6 activation and protects against ischemic insult (23). Active Casp6 can then initiate cell death pathways
by activating Casp3 and 8 (64, 65). Casp3 has been implicated in cell death; however during axonal pruning Casp6 can activate Casp3 without inducing cell death (31). Since Casp6 signals through other caspases in the apoptosis and axonal pruning pathways, the expression profiles of Casp6 and other caspases in the brain may contribute to the region-specific neuroanatomical changes observed in Casp6 -/- mice. Casp6 expression is observed predominantly in neurons, with higher expression in MSNs in the striatum, compared to neurons in the cortex, hippocampus and cerebellum (13-15, 20, 66, 67) (Allen brain atlas). Striatal MSNs also show high Casp7 expression compared to other brain regions, such as the cortex. Interestingly Casp7, in the absence of Casp3, can also functionally compensate and ameliorate brain phenotypes seen in Casp3-null mice (51). Unlike Casp6, Casp3 expression is mostly expressed in glia (15), while Casp9 is detected in cholinergic neurons and in pyramidal cells located in cortical layer V and Casp8 is ubiquitously expressed in different brain regions. Therefore, a potential mechanism leading to initial region-specific neurodegeneration in HD and AD could potentially be caused by the regional and selective expression of Casp6 and its interaction with other caspases involved in regulating cell death.

Caspase activation can also be regulated by neurotrophic factors (68). For example, deprivation of brain derived neurotrophic factor (BDNF) has been shown to trigger cell death pathways by activating caspases through increased p75NTR signaling (69). The presence of BDNF provides protection against staurosporine-induced excitotoxic cell death in vitro by activating the PI3K/Akt pathway through Trk receptor signaling (70). BDNF has also been implicated in neuronal maturation, axonal and dendritic branching and regeneration, and in synaptic transmission and plasticity (71-73). Although the pathways by which BDNF affects these processes are still being elucidated, BDNF is known to regulate transcription of Sprouty proteins, which modulate neuronal differentiation and survival and axonal branching by inhibiting BDNF signaling in a negative feedback loop (74, 75). BDNF has also been shown to play a key role in cognition and behavior by modulating learning, anxiety and depression-like behaviors (76-78). Interestingly, similar to Casp6 -/- mice, Bdnf -/- mice also show structure-
specific neuroanatomical alterations and behavioral abnormalities. They display decreased dendritic complexity and spine density in the cortex and hippocampus and to an even greater extent in the striatum, where 90% of the affected cells are GABAergic MSNs (79, 80). Behavioral changes associated with BDNF are also region dependent, infusion of BDNF in the hippocampal dentate gyrus reduces depression-like behaviors (81); however, infusion into the nucleus accumbens increases depression (82) and social aversion (83). These findings suggest that the diverse responses to BDNF and/or Casp6 by different brain structures may be mediated by region-intrinsic programs (79).

We further examined Casp6 -/- mice using behavioral tests to elucidate whether the neuroanatomical alterations observed could result in cognitive changes. Casp6 -/- mice are hypoactive compared to WT mice during a 30-minute open field test that measures locomotor activity. Striatal damage by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to impair performance in an open field test, causing severe behavioral inactivity in distance and speed of locomotion, peripheral activity, and frequency and duration of rearing (84). Interestingly, hypoactivity is also observed in the full length YAC HD mouse model, where degeneration of the striatum is a key pathogenic feature (61, 62). These findings suggest that the striatum may be involved in planning and executing pathways of movement. We also show that by 12 months Casp6 -/- mice demonstrate a deficit in the novel object recognition task. Excitotoxic lesions of the perirhinal cortex have been shown to cause deficits in object recognition tasks (85, 86). Furthermore, studies of neuronal activation in rats and monkeys suggest that cortical neurons are involved in object recognition tasks (87, 88). Interestingly, AD mouse models also show age-dependent cortical neuron pathology and deficits in object recognition tasks (89-91), while intrastriatal quinolinic acid (QA) injection in mice, which is an acute, in vivo HD model, also causes object recognition impairment (92). Altogether, these data suggest that the altered cortical and striatal volume observed in the Casp6-deficient mice could compromise the normal function of these brain regions, and that these changes could possibly translate into the learning impairment and hypoactivity phenotype observed in the Casp6 -/- mice.
Our data show that the behavioral and neuroanatomical alterations observed in the Casp6 -/- mice are age dependent. This is consistent with data showing active Casp6 in striatal and cortical neurons of WT mice starting at 9 and 12 months respectively, with increased activation at 18 months of age. Microarray studies on the cortex and hypothalamus of BALB/c mice also show that Casp6 is significantly up-regulated with age (93) and age-related activation of Casp6 is also observed in human brain tissue (14). The normal age-dependent activation of Casp6 may explain the neuropathological phenotype observed in the Casp6 -/- mice, which only manifests with advanced age. Ablating Casp6 while it is predominantly inactive may not cause any brain alterations. However, its absence during a time when Casp6 normally becomes activated could cause a decrease in apoptosis and enlarged brain structures.

Our data highlight the importance of assessing whether Casp6 inhibitors may offer protection against excitotoxic stress and axonal degeneration in neurodegenerative diseases, such as HD and AD. Although detrimental neuropathology is only observed later in life, we have not yet ruled out whether Casp6 deficiency during embryogenesis or development may contribute to the behavioral abnormalities observed in Casp6-deficient mice. Future studies with a conditional Casp6-deficient mouse would allow examination of the morphological and behavioral impact of Casp6 deletion not only in specific brain structures, but also at specific time-points.
Materials and Methods

Generation of mutant Casp6 -/- mice

The Casp6 -/- mouse was created by Taconic Biosciences by targeted gene disruption of Casp6 gene in embryonic stem cells. Exons 2 to 5 of the Casp6 gene, which encode the catalytic domain of the Casp6 protein, were disrupted in embryonic stem cells by homologous recombination to generate Casp6 -/- mice (Supplementary Material, Fig. S1A & B). The homologous recombination strategy was validated by Southern analysis. Two PCR assays were designed for genotyping: the wild type (WT) assay amplifies the WT allele from Casp6 WT and heterozygous (Casp6 +/-) mice; and the mt allele assay amplifies the knocked out allele from Casp6 +/- and Casp6 -/- mice. The following primers were used:

WT assay forward: 5′- AGGGTGAGGTAGCCTTGTCTTGTCTTGTTG -3′;
WT assay reverse: 5′- TCCAGCTTGTCTTGTTCTTGTTG -3′;
mt assay forward: 5′- CCTGTGGGGTCAAAAAGACTTTACACAG -3′;
mt assay reverse: 5′- GCAAGCTGCTAACACAGCCAACAAC -3′.

The PCR was performed in a 20 /μL volume using 1.5 μL of genomic DNA in 10X PCR buffer, 50mM MgCl2, 10mM dNTP, 2% formamide, 50% glycerol, Taq polymerase and DH2O. A complex temperature profile was adopted to ensure maximum specificity in the early rounds of amplification. An initial DNA denaturation for 3 min at 94°C was followed by 35 cycles at 94°C for 30 sec, 60°C for 1 min, 72°C for 1 min and finally a 7 min extension at 72°C.

Breeding and housing

The Casp6 knockout mouse, originally generated on the C57Bl/6 (B6) strain, was backcrossed for 5 generations to the FVB/NJ (FVB) strain. All the experiments, with the exception of the necropsy and the Mendelian ratios, which were performed and the data recorded on both the B6 and FVB strains, were conducted on the incipient congenic mice on the FVB strain and according to the protocols approved by the University of British Columbia Committee on Animal Care (protocol # A07-0106). The mice were
housed in groups of maximum five mice per cage as previously described (61).

**mRNA analysis and Quantitative reverse transcriptase PCR**

RNA was extracted from Casp6 -/- and WT mice whole brain using the RNeasy mini kit (Qiagen, 74104). cDNA was prepared using 250ng total RNA and the superscript-III first-strand synthesis kit with oligo-dT priming (Invitrogen, 11752- 050). SYBR Green PCR master mix (Applied Biosystems, 4309155) in the ABI7500 instrument was used to perform the quantitative real-time PCR with the absolute quantification standard curve method. The following primers were used:

Mouse Casp6 forward: 5′- CAACGCAGACAGAGACAACCT -3′;
Mouse Casp6 reverse: 5′- TCGACACCTCGTGAATTTTGAG -3′;
Mouse actin forward: 5′- ACGGCCAGGTCATCACTATTG -3′;
Mouse actin reverse: 5′- CAAGAAGGAAGGCTGGAAAAGA -3′

**Protein analysis and Western blotting**

Protein was extracted as previously described (94) from brain and peripheral tissues from Casp6 -/- and WT mice and its concentration measured by Bio Rad DC Protein Assay. 4-12% Bis-Tris poyacrylamide gels from invitrogen were used to load 70g of protein for brain and peripheral tissues in LDS sample buffer (Invitrogen, NP0008) after it was denatured by heating it to 70°C. Proteins were transferred to an Immobilon-PVDF-FL membrane and probed with a caspase-6 antibody (Cell Signaling 9762, 1:500) and calnexin antibody (Sigma C4731, 1:5000).

**Necropsy**

Kidney, liver, heart, spleen, stomach, intestine, cecum, colon and testis tissues were collected from 1 B6 and 1 FVB Casp6 -/- mice and stored in 10% formalin. A #15 scalpel blade was used to cut the tissues into 3-4mm sections; they were placed into cassettes and then set in a paraffin block and cut into 1um slices using a microtome. They were mounted into slides and a hematoxylin and eosin stain was applied in order to perform a macroscopic examination of the different tissues cell structure.
NMDAR-mediated excitotoxicity

Cultures (9-12 individual cultures) of primary MSNs were prepared from P0-P1 Casp6 -/- and WT pups in a procedure described previously (32, 58). Cultures were maintained in vitro for 9-10 days, after which they were exposed to balanced salt solution (BSS) or 500μm NMDA (Sigma) in BSS for 10 minutes. Twenty-four hr post NMDA, cultures were fixed and assessed for apoptotic cell death using TUNEL staining (Roche) and morphological criteria (small, condensed nuclei) by propidium iodide (Sigma) counterstaining. For each experiment, all treatments were done blind, in triplicate, and a minimum of 1,000 cells counted.

LDH and ATP were assessed in separate cultures. 24-hours post stress, 50ul of culture media was used for LDH quantification, according to the manufacturer’s instructions (Roche, cytotoxicity detection kit). For ATP assessment, CellTiter-Glo Luminescent Cell Viability Assay from Promega was used. Cells were lysed on an orbital rotator at room temperature for 10 minutes. 100ul of cell lysis was removed to a black 96-well plate and luminescence recorded with PolarStar Omega plate reader. In order to obtain the LDH and ATP data, the raw value of each well was normalized to BSS-treated cells/neurons on the same plate.

NGF induced axonal degeneration in microfluidic chambers

Sympathetic neurons were isolated as previously described (95) from P0-1 WT and Casp-6 -/- mice. Briefly, PDMS (Sylgard 183, Dow Corning) replica-molded microfluidic chambers (96) were placed onto glass coverslips coated with poly-D-lysine (50 ug/ml) and laminin (1 ug/ml). Dissociated sympathetic cervical ganglion (SCG) neurons (~20,000 cells) were plated into the somatic compartment and maintained in NGF-containing (50 ng/mL) media for 5-6 days. For localized NGF deprivation, the axon compartment was rinsed 3 times with medium lacking NGF and then maintained in 70 uL of NGF-free media containing an anti-NGF neutralizing antibody. 100 uL of NGF-containing media remained in the somatic compartment to create a 30 uL volume differential between the two compartments. The volume differential was carefully
maintained during local deprivation to prevent any medium exchange between soma and axon compartments.

Fixed neurons were then probed with tubulin (Sigma T9026, 1:400) using standard immunofluorescence techniques. Nuclei were stained with Hoechst 33258 (Molecular Probes). Images were acquired by an ORCA-ER digital B/W CCD camera (Hamamatsu) mounted on a DMIRE2 inverted fluorescence microscope (Leica) using Metamorph version 7.6 software (Molecular Devices). Adobe Photoshop was used to scale down and crop images to prepare the final figures.

Metamorph version 7.6 software was used to measure horizontal axon distance (µm) from the left outer edge of the central channels (where the axons exit the channels and enter the axon compartment) to the farthest point of axon growth inside the axon compartment. Unbiased measurements were obtained by measuring axon distance at every 6th channel within the same chamber both before and after treatment. To calculate fold change, the mean post-treatment axon distance was divided by the mean pre-treatment axon distance. Fold change in axon distance was calculated for three chambers per condition. Graph values represent the average fold change in axon distance ± standard error of the mean (SEM) (n=3).

Stereology

Quantitative analysis was done blind to genotype. Mice were terminally anesthetized by intra-peritoneal injection of 2.5% avertin and perfused with 3% paraformaldehyde/0.15% glutaraldehyde in phosphate buffered saline (PBS). Mouse brains were post-fixed in the same solution for 24 hr at 4°C and then cryoprotected in 30% sucrose prior to coronal sectioning on a cryostat (MICROM HM 500 M, MICROM, Heiderberg, Germany) at 25 µm. Every eighth section throughout the striatum from Bregma 1.34 mm to -0.94 mm was collected and stained with an antibody reactive to NeuN (Chemicon), a marker of neuronal nuclei (93), as described previously (61).

The area of the striatum was traced with Stereoinvestigator 10.0 software (Microbrightfield, Williston, VT). For neuronal counts, the physical fractionator probe was used with a grid size of 500 x 500 and counting frame of 25 x 25 and the nucleator
probe was used for measurement of neuronal size. A minimum of 200-300 cells per animal were counted and analyzed. For striatal volume, the Cavalieri principle was employed where the total striatal area was multiplied by section thickness (25 µm) and sectional sampling interval (8) as previously described (97, 98) (n=20/genotype at 3 months, n=6/genotype at 8 months). The cortex was delineated using the corpus callosum as the ventral boundary in the same sections used for striatal analyses. Cortical volume was determined according to the Cavalieri principle as previously described (97, 98) (n=20/genotype at 3 months, n=6/genotype at 8 months).

For analysis of cholinergic axons in the CNS, adult mice were anesthetized, intracardially perfused with ice-cold 0.9% NaCl and fixed with 4% paraformaldehyde (Electron Microscopy Sciences). Brains were postfixed for 6 h in 4% paraformaldehyde at 4 °C and cryoprotected in 30% (wt/vol) sucrose in PBS, and sagittal serial 40-µm sections were collected in cryoprotectant. For diaminobenzidine staining, we analyzed every third section from 0–480 µm away from the midline using standard immunohistochemical procedures (39). Briefly, sections were rinsed three times for 10 min each in TBS, incubated in 0.6% H2O2 for 15 min, and blocked in Tris buffer containing 5% donkey serum and 0.25% Triton X-100. Sections were incubated overnight at 4 °C in primary antibody, anti-ChAT (1:200, Chemicon), diluted in blocking solution. Sections were rinsed three times for 10 min each in TBS and incubated with a biotinylated donkey-anti-goat secondary antibody for 2 h at room temperature. Sections were then rinsed and processed with avidin-biotin complex (Vectastain Elite, Vector Laboratories) and 3’,3’-diaminobenzidine (Vector). Sections were rinsed, mounted onto gelatin-coated glass slides, dried overnight, dehydrated in an alcohol gradient, washed in xylene and cover-slipped using Permount (Fisher). For immunofluorescence staining, random sections from 0–480 µm away from the midline were rinsed three times for 10 min each in PBS, blocked in PBS containing 10% donkey serum, 2% horse serum and 0.25% Triton X-100, incubated overnight at 4 °C in blocking solution plus primary antibody, rinsed three times for 10 min each in PBS, incubated with secondary antibody
for 2 h at room temperature, rinsed and mounted using Fluoromount (Sigma-Aldrich) (n=3-4/genotype at 3 and 8 months).

**Body weight**

Mice were weighed at 2, 4, 6 and 8 months of age using a digital scale and weight recorded. Female: WT (n=7) and Casp6 -/- (n=9). Male: WT (n=4) and Casp6 -/- (n=10).

**Behavior**

Behavioral analysis of Casp6 -/- and WT mice was done blind to genotype.

**Rotarod**

Motor coordination and learning were examined using an accelerating rotarod (UGO Basile, Comerio, Italy). For training, naïve 2-month-old mice were given three trials of 2 minutes on a fixed speed (18RPM) task per day for three days (9 trials total). The inter-trial interval was 2 hours. Mice falling from the rod were returned, to a maximum of 10 falls/trial. The time to first fall and total number of falls per trial were recorded. For accelerating rotarod assessment, mice were tested at 2, 4 and 8 months of age on a rod accelerating from 5 to 40 RPM over 300 seconds. Latency to fall from the rod was recorded. 3 trials in 1 day were averaged to give mean performance for each mouse at each age (n=19 WT, 21 Casp6 -/-).

**Open-field**

Mice (n=17 WT, 20 Casp6 -/-) were assessed using an open-field activity monitor (Med Associates Inc., St Albans, VT, USA) during the dark cycle at 2, 6 and 8 months of age. Mice were placed in the testing chamber for 30 minutes, total activity was recorded and measurements were calculated by accompanying software (Med Associates). The testing chambers were wiped clean with water between mice.
Novel-object recognition

Mice (n=13 WT, 19 Casp6 -/-) were placed in the lower left corner of a 50x50 cm open grey acrylic box with a 20X20 cm center in a room brightly lit by fluorescent ceiling lights. Open field activity was recorded for 10 min by a ceiling-mounted video camera. Distance traveled, mean velocity, entries into the 20x20 cm center and time spent in the center of the center point of the mouse were scored using Ethovision 7.0 XT software (Noldus).

After open field exploration, which allows acclimation to the testing arena, mice were returned to their home cage for a 5 min inter-trial interval (ITI). Two different novel objects of sufficient height and weight to prevent mice from moving or climbing on them were placed in the upper two corners of the box, far enough from the sides so as to not impede movement around the outer edge (~8 cm). Mice were reintroduced to the box in the lower left corner and recorded for 5 min, during which the number of investigations of the objects was scored as frequency and duration of the nose point of the mouse entering the zone immediately around the object by Ethovision XT software. Mice were then removed from the box for a 5 min interval, and the object at the top right corner of the box was replaced by a different unfamiliar object in the same location. Mice were reintroduced to the box and recorded for 5 min and the number and duration of investigations of the objects was scored. For novel object preference testing, the percentage of the investigations to the target object (the unfamiliar one) was computed. For novel object location, the experiment was repeated on the subsequent day, but rather than replacing the object with an unfamiliar one, the object at the top right corner of the box was moved to the lower right corner of the box. The percentage of the investigations to the target object (the one in the new location) was computed.

Statistics

Statistical analysis (i.e. p values, means, and standard error of the mean (SEMs)) were performed using Prism version 4.0 (GraphPad Software). Differences between means were considered statistically significant if p<0.05.
Axon distance before and after NGF deprivation were measured and fold change was calculated by dividing the mean post-treatment axon distance by the mean pretreatment axon distance. Fold change in axon distance was calculated for three chambers per condition. Graph values represent the average fold change in axon distance ± SEM (n=3). An unpaired Student’s t-test was used to determine if there was a statistically significant difference between treatment conditions. The t-test was also used to analyze the number and length of ChAT-positive axons in the corpus callosum.

Statistical analysis of the LDH, ATP and TUNEL data, as well as of the stereology data was performed using one-way ANOVA. Significant genotype effects were followed up with post hoc Tukey comparisons and post hoc linear trend tests. Statistical analysis of the body weight was performed using a repeated measures ANOVA model. For rotarod data, a factorial ANOVA was used to determine genotype, age and genotype-age interaction effects. Similarly for the novel object recognition test, a factorial ANOVA was used to determine genotype, trial and genotype-trial interaction effects. Significant effects were followed by post hoc Tukey comparison test.
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References


Figure legends

Figure 1 – No Casp6 expression is observed in Casp6 -/- brain and peripheral tissues. A) Quantitative RT-PCR shows absence of Casp6 mRNA expression in Casp6 -/- brain tissue and reduced expression in Casp6 +/- brain tissue when compared to WT (n = 3-4/genotype). B) Western blots using Casp6 antibody show absence of Casp6 protein in Casp6 -/- cerebellum, hippocampus, striatum, cortex, kidney, liver, spleen and post-natal P0 peripheral tissue (n = 3/genotype).

Figure 2 – Casp6 -/- sympathetic neurons show protection against axonal degeneration. A) WT axons from sympathetic neurons grown in microfluidic chambers degenerate after NGF deprivation; in contrast, B) NGF deprivation does not induce degeneration in axons from Casp6 -/- mice. C) Quantification of axonal distance reveals a significant difference between control and NGF-deprived axons from WT mice (*p < 0.0001) and no difference in the axons from Casp6 -/- mice regardless of treatment conditions (n=3 chambers/condition/genotype).

Figure 3 – Medium spiny neurons from Casp6 +/- and Casp6 -/- mice show protection against NMDA-mediated excitotoxicity in a Casp6 dose dependent manner. Assessment of susceptibility to excitotoxic stress demonstrates A) a significant decrease in LDH levels (n=10 cultures/genotype), B) a significant increase in levels of ATP (n=10 cultures/genotype) and C) a significant decrease in number of TUNEL positive cells (n=9-12 cultures/genotype) in Casp6 +/- MSNs compared to WT post-NMDA stimulation. Post-hoc linear trend tests reveal a dose dependent effect; partial rescue from NMDA is observed in Casp6 +/- mice (LDH: p=0.005; ATP: p=0.01; TUNEL: p=0.02).

Figure 4 – Casp6 is necessary for myelin-dependent axon degeneration in vivo. A) Sagittal sections through the corpus callosum and the adjacent supracallosal pathway of adult control and Casp6 -/- mice immunostained for ChAT. Arrows denote ChAT-positive axons projecting into the corpus callosum and the boxed areas in the top panels denote regions of the corpus callosum that are shown at higher magnification in
the lower panels. Scale bar, 100 μm. Quantification of the number (B), length (C) and average length (D) of ChAT-positive axons projecting vertically into the corpus callosum anterior to the splenium in control versus Casp6 -/- mice. Error bars represent standard error of the mean. ANOVA: *Geno p<0.05, **Age p<0.001; Post-hoc Tukey: *P<0.05; n = 3-4 each.

Figure 5 – Cortical and striatal volume is increased in 8 month old Casp6 -/- mice. Neuropathological analysis at 8 months reveals A) a significant increase in cortical (n=6/genotype) and striatal (n=6/genotype) volume and in striatal neuronal counts (n=6/genotype). Post-hoc linear trend test reveals a dose-dependent effect; the Casp6 +/- mice demonstrate an increase in cortical and striatal volume and in striatal neuronal counts compared to WT, but to a lesser extent than the Casp6 -/- mice (post-hoc linear trend, cortex volume: p=0.001; striatum volume: p=0.006; striatal neuronal counts: p=0.01). However, no differences are observed in B) brain (n=20/genotype) and cerebellum (n=20/genotype) weight in Casp6 -/- mice compared to WT. **ANOVA p<0.01; *ANOVA p<0.05.

Figure 6 – Casp6 -/- mice demonstrate normal motor coordination, a hypokinetic phenotype and decreased novel object preference. A) Casp6 -/- mice display motor coordination indistinguishable from WT mice during rotarod testing at all time-points assessed (n=19 WT, 21 Casp6 -/-). B) Casp6 -/- mice are hypoactive compared to WT during a 30-minute open field trial (ANOVA **Geno p<0.005, *Age, n=17 WT, 20 Casp6 -/-). C) Casp6 -/- mice demonstrate a deficit in a novel object preference task at 12 months of age (*Post-hoc Tukey p<0.001, n=13 WT, 19 Casp6 -/-).
Figure 1

A  
Caspase 6 mRNA levels

B

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Figure 2

A

WT

+NGF

Axon

Soma

B

C6 -/-

+NGF

Axon

Soma

-NGF

Axon

Soma

C

Axonal distance

post / pre treatment

axonal distance

WT

C6 -/-

* p < 0.0001

+NGF

-NGF
Figure 4

A

Control  C6 -/-

B

Axon number

*Geno (p<0.05); **Age (p<0.001)

Number of ChAT+ axons

3 month  8 month

C

Axon length

*Geno (p<0.05); **Age (p<0.001)

Total length of ChAT+ axons (µm)

3 month  8 month

D

Average axon length

*Geno (p<0.05)

Average length of ChAT+ axons (µm)

3 month  8 month
Figure 5

A

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B

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Figure 6

A. Rotarod

- **Age (p<0.0001)**
- WT
- C6 -/-

B. Open field

- **Geno (p<0.005)**; **Age (p<0.05)**
- WT
- C6 -/-

C. Novel object preference - 12 month

- **p<0.001**
- WT
- C6 -/-