CHARACTERIZATION OF CONSTITUTIVE CASPASE-6 DEFICIENT MICE: INSIGHTS INTO AXONAL DEGENERATION, EXCITOTOXICITY AND AGE DEPENDENT BEHAVIORAL AND NEUROANATOMICAL CHANGES

by

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B.A., Cum Laude with distinction, Boston University 2005

A THESIS SUBMITTED IN PARTIAL FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Neuroscience)

THE UNIVERISTY OF BRITISH COLUMBIA

(Vancouver)

April 2011

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 cysteine-aspartic proteases that play a key role in programmed cell death. Aside from their roles during development, aberrant activation of caspases has been implicated in several human diseases. In particular, numerous findings implicate Caspase-6 (Casp6) in neurodegenerative diseases 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 neuropathological and behavioral characterization of constitutive Casp6-deficient (*Casp6* -/-) mice in order to understand the physiological function of Casp6 in brain development, structure and function and to establish if any biological effects are caused by ablation of Casp6.

We demonstrate that *Casp6* -/- neurons are protected against NMDA-mediated excitotoxicity and NGF-deprivation 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. These results provide further insights into the role of Casp6 in neurodegenerative diseases.

Preface

I designed and performed the experiments included in this thesis with guidance from my supervisor, Michael Hayden. A significant part of these experiments was done in collaboration with members of the Hayden lab and other academic laboratories. Dr. Rona Graham performed the excitotoxicity assays, Dr. Dagmar Ehrnhoefer assisted with the MEFs experiments and Niels Skotte with the mRNA analysis. The NGF-induced axonal degeneration studies were conducted in collaboration with Corey Cusack and Dr. Mohanish Deshmuckh, at the University of North Carolina, Chapel Hill (Department of Cell and Developmental Biology and Neuroscience). Technical support was provided by Dr. Nagat Bissada to perform the perfusions. Dr. Sonia Franciosi and Amanda Spreeuw assisted with stereological methods and Dr. Wei-ning Zhang with conducting some of the behavioral testing. All the experiments were performed according to the protocols approved by the University of British Columbia Committee on Animal Care (protocols A07-0106 and A07-0262).

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List of Abbreviations

aa	Amino acid
AD	Alzheimer Disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP-2	Activating protein 2
Apaf1	Apoptotic protease activating factor
APP	Amyloid precursor protein
AR	Androgen receptor
ATP	Adenosine triphosphate
Aβ	Amyloid beta
β-APP	Beta amyloid precursor protein
B6	C57Bl/6
BDNF	Brain-derived neurotrophic factor
C6R	Caspase-6 resistant
Ca2+	Calcium
CAG	Cytosine, Adenine, and Guanine
CARD	Caspase recruitment domain
Casp	Caspase
Casp6 -/-	Caspase-6 deficient mice
CBP	CREB-binding protein
CIF	Caspase inhibirory factor
Cys	Cysteine
Cyt c	Cytochrome C
DISC	Death inducing signaling complex
DNA	Deoxynucleic acid
DR6	Death receptror 6
DRPLA	Dentatorubropallidoluysian atrophy
ER	Endoplasmic rticulum
FasL	Fas ligand
Fig	Figure
FVB	FVB/NJ
HD	Huntington disease
HEK 293	Human Embryonic Kidney 293 cells
Hsp	Heat shock protein

htt	Huntingtin
IAP	Inhibitor of apoptosis protein
ITI	Inter-trial interval
KO	Knockout
LDH	Lactate Dehydrogenase
LTD	Long term depression
MEF	Mouse embryonic fibroblast
mhtt	Mutant huntingtin
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSN	Medium spiny neurons
NF-kB	Nuclear factor kB
NFTs	Neurofibrillary tangles
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NS5A	Nonstructural protein 5A
PARP	Poly(ADP-ribosyl) transferase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
QRTPCR	Quantitative real time polymerase chain reaction
Rho-GDI	Rho guanine nucleotide dissociation inhibitor
RT	Reverse transcription
SARS	Severe acute respiratory syndrome
SATB1	Special AT-rich sequence binding 1
SBMA	Spinal and bulbar muscle atrophy
SCA	Spinocerebellar ataxia
SCG	Superior cervical ganglion
SD	Standard deviation
SEM	Standard error of the mean
TGEV	Transmissible gastroenteritis virus
TNF	Tumor necrosis factor
TUNEL	Tdt-mediated dUTP-biotin nick and labeling
VCP	Valosin-containing protein
WT	Wild type
YAC	Yeast artificial chromosome

Acknowledgments

I would like to thank my supervisor, Dr. Michael Hayden, for his support and guidance and for all the challenges and experiences that made me grow not only professionally, but also personally while working on this project. I want to specially thank Dr. Rona Graham for her mentorship, unconditional encouragement and support, Dr. Mahmoud Pouladi, Dr. Jeffrey Carroll and Niels Skotte for their friendship and advice and Dr. Bibiana Wong for her enormous support during my Master's. I also want to acknowledge the insight and guidance that I received from the members of my supervisory committee, Dr. Blair Leavitt, Dr. Lara Boyd and Dr. Weihong Song. This work would not have been possible without the technical support provided by Nagat Bissada, Wei-ning Zhang, Sonia Franciosi, Amanda Spreeuw and I want to specially thank Robert Xie, for always being willing to help and doing it with a big smile. I also want to thank Dagmar Ehrnhoefer, Deborah Deng and Kuljeet Vaid. It was a pleasure to work with Corey Cusack and Dr. Mohanish Deshmuckh from the University of North Carolina. They are the best and most responsive and productive collaborators.

My family means everything to me and I could have never accomplished this without their love and inspiration. I want to thank my mom for her unconditional love, support and encouragement and for being not only a mom, but also a great friend. I also am so grateful to my dad, my hero and role model, for his enthusiasm, unconditional belief in me and for always encouraging my aspirations, and to my sister and best friend, Ana, for always being there and sharing every moment with me. Last and definitively not least I want to thank Ryan for his love patience and unconditional support.

Dedication

To my mom, dad and Ana and in loving memory of my grandparents.

1 Introduction

1.1 Caspases

1.1.1 Caspases and apoptosis

Apoptosis or programmed cell death is a ubiquitous and conserved cellular pathway involved in normal cell turnover, developmental tissue remodeling, embryonic development, cellular homeostasis maintenance and chemical-induced cell death among others (Arends and Wyllie, 1991) (Ellis et al., 1991). Diverse signaling events, that involve a broad array of protein networks and macromolecular complexes, converge upon the activation of caspases and are responsible for triggering apoptosis (Fuentes-Prior and Salvesen, 2004)

Caspases are a family of intracellular cysteine-aspartic proteases that play a key role in programmed cell death mainly during development (Troy and Salvesen, 2002). Following development, they are down regulated in the nervous system, but they remain involved in neurogenesis and synaptic plasticity. Different apoptotic signals converge into a common pathway that has been conserved throughout evolution; this is reflected by the presence of positive and negative regulators of caspase activation between caenorhabditis elegans and mammals (Ellis et al., 1991). The presence of multiple mammalian homologues of ced-3 and ced-9, the c. elegans proteins that mediate programmed cell death, indicates that the cell machinery has become more complex in higher organisms (Thompson, 1995) (Adams and Cory, 1998) (Zheng et al., 1999). While 14 mammalian caspase family members have been identified, only Ced-3 is required for all the programmed cell death events in C. elegans (Adams and Cory, 1998).

1.1.2 Structure and activation

Caspases have been classified as long or short prodomain caspases according to their structure, or as initiator and effector caspases depending on how and when they are activated and their role in apoptosis. Short prodomain

caspases -3, -6, -7 and -14 are typically considered the effectors of apoptosis and play an important role in the nervous system, with the exception of caspase-14 that is involved in keratinocyte differentiation. Long prodomain caspases -2, -8, -9 and -10 are in general responsible for initiating apoptosis and caspases -1 -4 -5 and -11 process cytokines and contribute to inflammation (Figure 1.1) (Nicholson, 1999) (Troy et al., 2011).

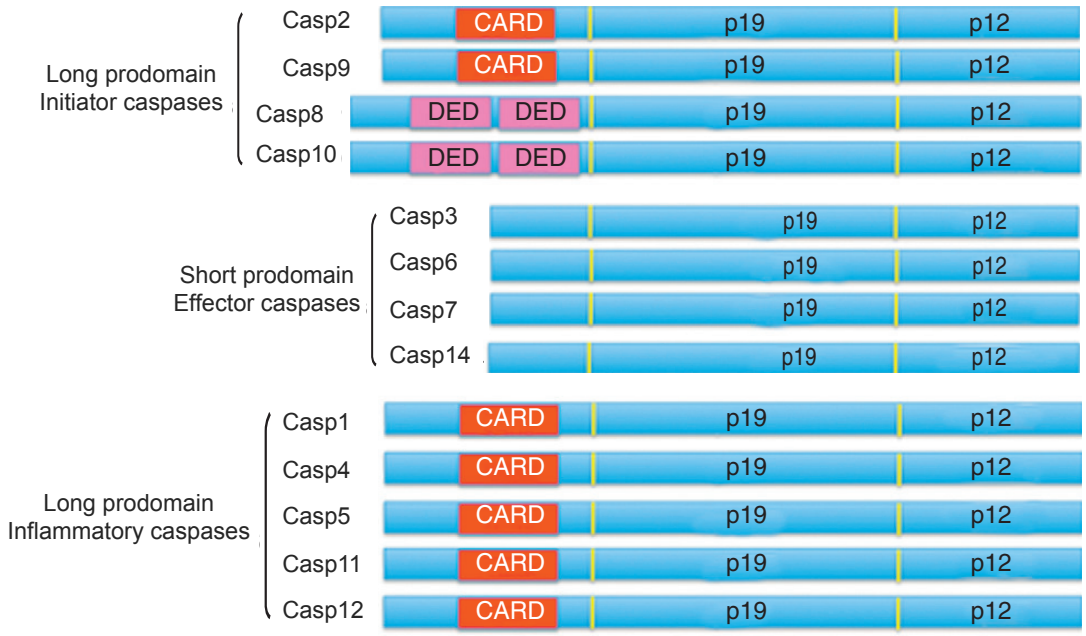

Figure 1.1 - Mammalian caspases. Mammalian caspases are schematically represented and grouped by structure and activity. Yellow lines indicate cleavage sites (adapted from Progress in Molecular Biology and Translational Science, Vol.99, Carol M. Troy, Nsikan Akpan and Ying Y. Jean. Regulation of Caspases in the Nervous System: Implications for Functions in Health and Disease. Pages 265-305, Copyright 2011, with permission from Elsevier).

Caspases are expressed as latent zymogens and they consist of a large (p20) and small (p10) domain, a N-terminal prodomain and a small linker region. Most long prodomain initiator caspases exist as monomers in solution and achieve catalytic maturity upon dimerization (Figure 1.2a) (Boatright et al., 2003). They can cleave and activate short prodomain effector caspases (Salvesen and Duckett, 2002), which are inactive dimers in solution (Boatright et al., 2003) (Figure 1.2b).

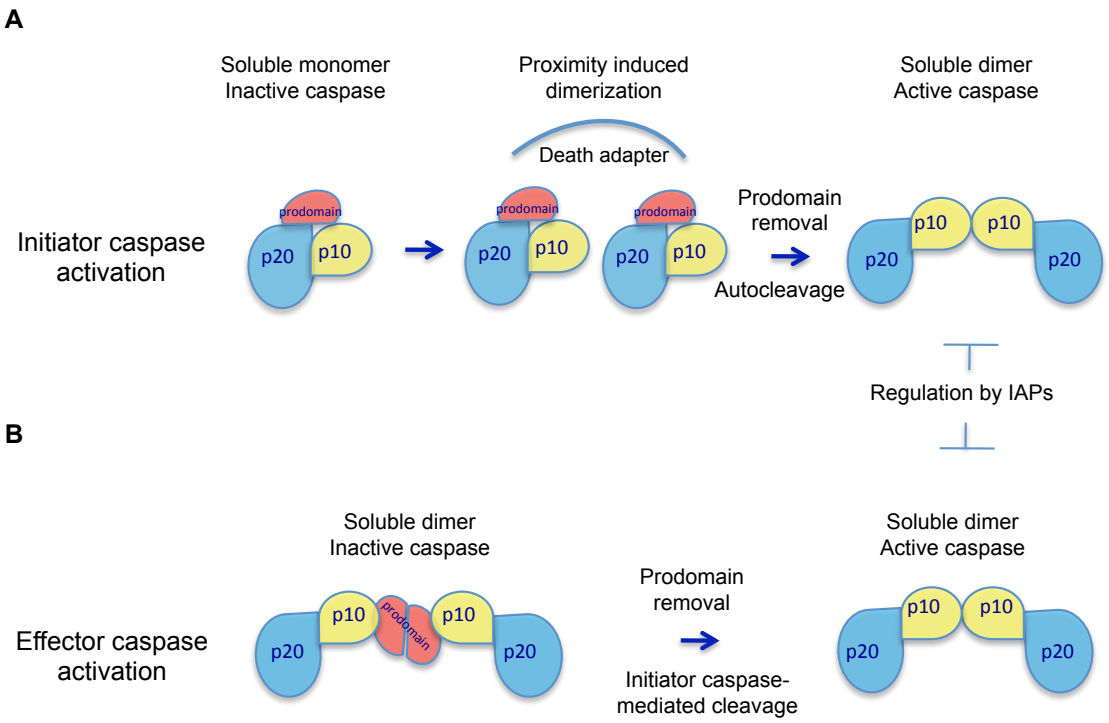

Figure 1.2 - Initiator and effector caspase activation mechanisms. A) Initiator caspases exist as monomers in solution and achieve catalytic maturity upon dimerization. B) Effector caspases are inactive dimers in solution. Their activation requires cleavage of the different subunits by initiator caspases, removal of the prdomain and structural changes.

The pathways involved in caspase activation depend on the initial cytotoxic stimulus and include excitotoxic stress, DNA damage and Ca^{2+} overload (Nicholson, 1999). After receiving an intracellular or extracellular apoptotic signal, caspases are activated by means of proteolytic cleavage and dimerization of their subunits at specific aspartic acid residues (Nicholson, 1999). There are two pathways involved in apoptosis that induce the activation of initiator caspases. The extrinsic, death receptor-mediated pathway gets activated when a ligand outside the cell binds to death receptors, this leads to recruitment of adaptor proteins, which in turn recruit caspase-8 and form the death inducing signaling complex (DISC). Once the DISC if formed caspase-8 is activated and cleaves effector caspases (Figure 1.3A) (Troy et al., 2011). The second pathway, the intrinsic, mitochondrial pathway is triggered by stress signals that originate within the cell (Riedl and Salvesen, 2007). It gets activated by mitochondrial permeabilization and leads to cytochrome c release, which causes an ATP

dependent recruitment of caspase-9 by the apoptotic protease activating factor (Apaf-1). The formation of the apoptosome, the caspase-9 activation platform formed by Apaf-1, cytochrome-C and casp9, leads to activation of effector caspases which cleave cellular proteins (Lassus et al., 2002) (Xanthoudakis et al., 1999) (Zheng et al., 1999) (Figure 1.3B). Cleavage of these proteins is the mechanism responsible for morphological and biochemical alterations, such as membrane blebbing, nuclear condensation, DNA fragmentation, pyknosis, and phagocytosis by immune cells (Arends and Wyllie, 1991).

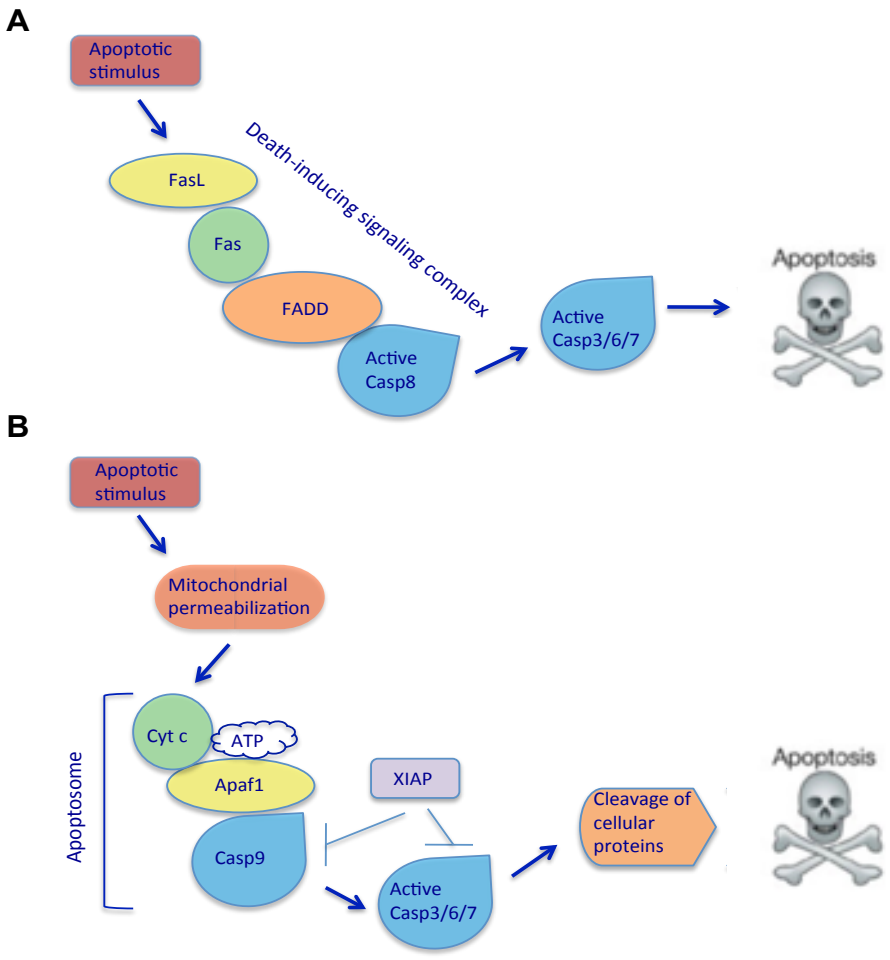

Figure 1.3 - Caspase activation pathways. A) The extrinsic, death receptor - mediated pathway is set off by ligands outside the cell (FasL) that bind to death receptors (Fas), leading to the formation of the death inducing signaling complex upon recruitment of adaptor proteins (FADD) and Casp8, leading to dimerization and activation of Casp8, which then cleaves effector caspases. B) The intrinsic pathway gets activated by mitochondrial permeabilization, which leads to cytochrome c release. The formation of the apoptosome activates effector caspases (adapted from Progress in Molecular Biology and Translational Science, Vol.99, Carol M. Troy, Nsikan Akpan and Ying Y. Jean. Regulation of Caspases in the Nervous System: Implications for Functions in Health and Disease. Pages 265-305, Copyright 2011, with permission from Elsevier).

Caspases may be activated by intracellular or extracellular signals; however, endogenous caspase inhibitors play an important role in controlling and localizing caspase activity in order to prevent aberrant cell death, and to adequately control the remodeling of synapses. Controlled caspase activation allows temporal, spatial and substrate specificity to the proteolytic pathways (Bingol and Sheng, 2011). The IAP (inhibitor of apoptosis protein) family is classified by the presence of the baculovirus IAP repeat domains. Of the eight IAPs that have been identified in mammals, three bind to caspases, CIAP1, CIAP2 and XIAP. However, XIAP is the only one capable of inhibiting caspase activity (Eckelman et al., 2006); however, it cannot inhibit Casp6.

1.1.3 Caspase deficient mice

Caspase deficient mice have been generated for more than half of the mammalian caspase genes (Table 1.1). The use of targeted caspase knockout mice has been instrumental for studying the involvement of caspases in apoptosis and to confirm the presence of the two apoptotic pathways. Death receptors can directly activate caspase-8; however, caspase-9 activation in response to apoptotic stimuli requires release of cytochrome c into the cytosol from the mitochondria (Zheng et al., 1999). *Casp8 -/-* mouse embryonic fibroblasts (MEFs) are resistant to extrinsic death signaling induced by death receptors including Fas, TNF receptor p55 and DR3, but are sensitive to intrinsic stimuli like dexamethasone and UV radiation (Adams and Cory, 1998). The *Casp9 -/-* mouse has validated the role of caspase-9 in intrinsic apoptotic pathways. Caspase-9 deficiency delays thymocyte apoptosis triggered by noxious stimuli such as dexamethasone, etoposide and staurosporine, but does not provide protection against apoptosis induced by death receptors, UV and heat shock (Kuida et al., 1998) (Hakem et al., 1998). Furthermore, caspase-8 and -9 knockout mice have confirmed that these enzymes are upstream initiator caspases.

Targeted knockout of caspase genes in mice has also revealed that different caspases play specific roles during mammalian development. Deletions

of the upstream initiator caspase-8, involved in receptor mediated apoptosis, result in embryonic lethality. Caspase-8 deficient mice show impaired formation of cardiac muscles and prominent abdominal hemorrhage due to hyperemia (Varfolomeev et al., 1998). Mice lacking caspase-9, the upstream initiator caspase of the mitochondrial pathway, die perinatally with robust brain malformations associated with supernumerary cells, multiple cerebral indentations and ectopic cell masses in the cortex (Kuida et al., 1996) (Kuida et al., 1998) (Hakem et al., 1998).

Caspase-2 deficient mice are born in Mendelian ratios and develop normally without an overt phenotype. However, they show dichotomous apoptotic defects in a tissue-specific fashion (Bergeron et al., 1998) and have a reduced life span, which is caused by age-dependent decreases in bone density, increased bone remodeling, reduced hair growth and increased levels of irreversibly oxidized protein (Zhang et al., 2007). In addition, these mice fail to gain fat mass due to altered basal metabolism and feeding behavior, and have pathological alterations in the liver and hypothalamus that may underlie these changes (Carroll et al., manuscript in preparation). Furthermore, the caspase-2 deficient mice are protected from beta-amyloid toxicity (Troy et al., 2000) and display reduced sensitivity to MPTP-induced toxicity, suggesting that caspase-2 may modulate MPTP-induced nigrostriatal dopaminergic system degeneration (Tiwari et al., 2011). A *Casp2 -/- ; Casp9-/-* mice was created to establish if the mild phenotype of the *Casp2 -/-* mice was caused by caspase-9 compensation. These mice died perinatally, like the *Casp9 -/-* mice, but displayed normal developmental phenotypes, suggesting that *Casp9 -/-* does not compensate for the lack of caspase-2 (Marsden et al., 2004).

Casp6 deficient mice have also been reported to be viable (Flavell unpublished data); however they have not been assessed in detail and have only been used for studying the role of Casp6 in immune responses. B cells from these mice display dysregulated entry into G1 phase of the cell cycle and accelerated differentiation into plasma cells, indicating that Casp6 may be

involved in balancing cell proliferation and differentiation through cleavage of substrates that are implicated in maintaining B cells latent (Watanabe et al., 2008).

Other caspase-deficient mice further demonstrate that not every caspase is crucial for normal mammalian development. Caspase-1 and -11 mediate inflammatory responses; however deleting them out does not have an effect on mouse development (Li et al., 1995) (Kuida et al., 1995) (Wang et al., 1998). Caspase-12 deficient mice not only develop normally, but they are resistant to endoplasmic reticulum (ER) stress induced apoptosis and amyloid-beta neurotoxicity, indicating that caspase-12 may mediate ER apoptosis and be involved in the mechanism behind amyloid beta neurotoxicity (Nakagawa et al., 2000).

Additionally, the phenotype of certain caspase deficient mice has been shown to be strain dependent. Mice of the 129S1/SvImJ (129) strain lacking caspase-3 are perinatally lethal; the few mice that survive are born at lower than expected Mendelian ratios, exhibit severe brain developmental defects resulting in brain overgrowth and die before 3 weeks (Kuida et al., 1996). However, on the C57Bl/6J (B6) background *Casp3-/-* mice develop normally (Houde et al., 2004).

Caspase-7 and -3 share nearly identical substrate preference (Thornberry et al., 1997), leading to the hypothesis that they were redundant. A study performed in order to understand the molecular mechanism responsible for the resistance of the *Casp3 -/-* mice on the B6 strain revealed that caspase-7 is expressed in brain development; however its expression is lower in the 129 strain compared to B6 mice. Murine caspase-7 cleaves ICAD (inhibitor of caspase-activation DNase) with the same efficacy that caspase-3, and it has been shown to be activated in proliferating precursor neurons from diverse strains after camptothecin-induced apoptosis. In addition, B6 *Casp3 -/-* cells with higher levels of caspase-7 are able to fragment their DNA *in vitro* (Houde et al., 2004) after camptothecin stress, suggesting that caspase-7 can compensate for the loss of caspase-3 and allows for healthy development of the brain. On the other hand,

lower levels of caspase-7 expression and activation in neurons from caspase-3 deficient mice on the 129 strain correlate with lack of DNA fragmentation (Houde et al., 2004).

These phenotypic differences observed in the array of caspase deficient mice serve to demonstrate that individual caspases play specific functional roles, and that their temporal and spatial expression changes during development. Even though caspase deficient mice have been generated for more than half of the mammalian caspases, caspase-4, -5, -10 and -14 null mice have not been reported. Generating and characterizing these mice would allow further understanding the role of these caspases in apoptotic and non-apoptotic pathways, as well as their specific role during development and how they interact with other caspases.

Table 1.1 - Caspase deficient mice viability and phenotypes

Caspase	Phenotype	References
Caspase-1	Viable	Kuida et al., 1995
	Resistant to lipopolysaccharide endotoxic shock	Li et al., 1995
	Impaired production of mature IL-1β and IL-1α	
Caspase-2	Viable	Zhang et al., 2007
	Tissue-specific dichotomous apoptotic defects	Bergeron et al., 1998
	Age-related reduced bone density and hair growth → reduced life span	Caroll et al., unpublished
	Increased levels or irreversibly oxidized protein	Troy et al., 2000
	Liver and hypothalamus pathology → altered basal metabolism and feeding behavior	Tiwari et al., 2011
	Resistant to Beta-amyloid toxicity and reduced sensitivity to MPTP-induced toxicity	Troy et al., 2000
Caspase-3	Perinatal lethality in 129 background	Kuida et al., 1998
	Supernumerary cells, cerebral indentations and ectopic cell masses in the cortex	Woo et al., 1996
	Viable in B6 background	Houde et al., 2004
Caspase-6	Viable	Uribe et al., unpublished
	Protection against NMDA-mediated excitotoxicity and NGF-induced axonal degeneration	Watanabe et al., 2008
	Age-dependent and region-specific behavioral and neuropathological alterations	
	Dysregulation of B cell entry into G1 phase of the cell cycle	
	Accelerated B cell differentiation into plasma cells	
Caspase-7	Embryonic lethality in 129 background	Kuida and Flavell, unpublished
	Viable in B6 background	Lamkanfi et al., 2009
	Resistant to lipopolysaccharide-induced apoptosis	
Caspase-8	Embryonic lethality	Varfolomeev et al., 1998
	Impaired cardiac muscle formation	
	Hyperemia → abdominal hemorrhage	
Caspase-9	Perinatal lethality	Kuida et al., 1998
	Supernumerary cells, cerebral indentations and ectopic cell masses in the cortex	Hakem et al., 1998
Caspase-11	Viable	Wang et al., 1998
	Resistant to lipopolysaccharide endotoxic shock and ICE overexpression- induced apoptosis	
	No production of IL-1β and IL-1α	
Caspase-12	Viable	Nakagawa et al., 2000
	Resistant to ER stress induced apoptosis and amyloid-beta protein neurotoxicity	

1.1.4 Non-apoptotic function of caspases

The pathways required for apoptosis have also been linked to non-apoptotic phenotypes. Caspase activity plays critical roles in the differentiation and proliferation of diverse cell types, axon guidance and synaptic activity (De Maria et al., 1999) (Kennedy et al., 1999). Inhibition of caspase activity blocks neurite extension and prevents differentiation of mouse neural stem cells (Fernando et al., 2005) (Aranha et al., 2009). In addition, the use of DEVDfmk, a caspase-3 inhibitor, has served to implicate caspase activity in long-term potentiation and in active avoidance learning (Gulyaeva et al., 2003).

It has been recently established that the molecular pathways involved in apoptosis are also used by neurons for synaptic plasticity (Li et al., 2010). Caspase-3 -7 and -9 are required by hippocampal neurons for long term depression (LTD) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor internalization. Furthermore, caspase-3 can be transiently activated via the mitochondrial pathway by stimulating NMDA receptors without causing cell death, and *casp3 -/-* hippocampal neurons fail to undergo NMDA receptor-dependent long-term depression. In addition, overexpression of the anti-apoptotic proteins XIAP or BCl-xL, and mutant Akt1 (a protein resistant to caspase-3 proteolysis) also prevented long-term depression, implicating the caspase mitochondrial pathway in synaptic depression (Li et al., 2010).

1.1.5 Caspases and neurodegeneration

Caspase activation is required for tissue sculpting during development and maintenance of homeostasis during adulthood (Raff et al., 1993); however, aberrant activation of apoptotic pathways leading to excessive or insufficient cell death has been implicated in many neurodegenerative diseases. Caspases have been implicated in the cleavage of disease-associated proteins. Two of the most prominent neurodegenerative diseases where caspases have been invoked are Huntington disease (HD) and Alzheimer disease (AD) (Hermel et al., 2004) (Wellington et al., 1998). (Graham et al., 2006a) (Zhang et al., 2010) (Gervais et al., 1999) (Albrecht et al., 2009). However, the role of caspases has been

implicated in other neurodegenerative disease including spinocerebellar ataxias and amyotrophic lateral sclerosis (ALS) (Kubodera et al., 2003) (Haacke et al., 2006) (Williams and Paulson, 2008).

1.1.5.1 Caspases and Huntington Disease

Huntington disease is an autosomal dominant neurodegenerative disorder characterized by onset of chorea and psychiatric disturbances, accompanied by a decline in cognition and eventual death within 15 to 20 years after initial symptoms (Harper, 1999). The underlying cause of HD is an expansion in the CAG trinucleotide repeat, resulting in an expanded polyglutamine tract in the N-terminus of the protein huntingtin (htt) (Borrell-Pagès et al., 2006) (Truant et al., 2006) (Brinkman et al., 1997).

Proteolytic cleavage of mutant huntingtin (mhtt) has been hypothesized to be a critical event in the pathogenesis of HD. TUNEL positive cells, which serve as markers of caspase activity, have been detected in HD human brains (Portera-Cailliau et al., 1995). In addition, the presence of htt fragments prior to clinical onset of the disease suggests that htt cleavage may be an early and crucial event (Wellington et al., 2002). Htt is proteolytically cleaved by caspases, releasing an amino terminal fragment containing the polyglutamine tract (Wellington et al., 2000) (Wellington et al., 2002) and it has been shown that caspases -2, -3, -6, -7, and -8 are capable of cleaving htt *in vitro* (Hermel et al., 2004) (Wellington et al., 1998). Caspase cleavage sites have been well defined for caspase-3 at amino acids 513 and 552, for caspase-2 at amino acid 552 and for Casp6 at amino acid 586. Casp6 specifically cleaves htt at amino acid 586 (Wellington et al., 2000); however, caspases -3 and -7 have similar substrate preferences and therefore cleave htt at amino acid 513 and 552 (Hermel et al., 2004).

Using caspase inhibitors and caspase resistant htt constructs has shown abrogation of htt cleavage and reduced htt toxicity in cells transfected with full-length htt (Wellington et al., 2000). In addition, novel antibodies developed to

specifically detect N-terminal htt fragments generated by caspase cleavage at aminoacids 513 or 552 have shown that caspase-cleaved htt at amino acid 552 is seen in the brain of HD patients and in control human brain. Cleaved htt is also found in WT and HD transgenic mouse brains before neurodegeneration is observed; suggesting that cleavage of htt may be a normal physiological event (Wellington et al., 2002).

1.1.5.2 Caspases and Alzheimer Disease

Alzheimer disease is a neurodegenerative disorder characterized by progressive impairment of cognitive function resulting in severe dementia and eventual death within 5 to 20 years after initial symptoms (Alzheimer et al., 1995). Senile plaques formed by extracellular deposits of Aβ peptide and neurofibrillary tangles (NFTs) consisting of intra-neuronal filamentous aggregates are the two histopathological hallmarks of AD.

Inappropriate apoptosis has been implicated in AD through cleavage of β-amyloid precursor protein (β-APP) (Yuan and Yankner, 2000) (Gervais et al., 1999) and numerous results implicate caspases in this process. Increased expression of caspases -1, -2, -3, -5, -6, -7, -8 and -9 and caspase substrates have been detected in the brains of AD patients compared to controls (Albrecht et al., 2007) (Gervais et al., 1999) (Guo et al., 2004) (Pompl et al., 2003). In particular, pyramidal neurons in AD brains show extensive caspase-3 and -6 activation (LeBlanc et al., 1999) (Stadelmann et al., 1999) and postmortem AD cortical and hippocampal tissues show increased cleaved caspase-8 expression (Rohn et al., 2001). In mouse models of AD, baseline caspase-3 activity has been shown to be elevated in hippocampal dendritic spines (D'Amelio et al., 2011). Furthermore, caspase-9 is enriched in synaptosomes prepared from AD frontal cortices, compared to healthy controls (Lu et al., 2000). It has also been shown that caspase activation precedes and leads to tangle formation (de Calignon et al., 2010), suggesting that caspase activation is an early event in the progressive neuronal cell dysfunction and death that occurs in AD.

In addition to the implication of caspases in HD and AD, caspase sites have been mapped in 5 of 9 poly-glutamine expansion disease proteins and mutation of the caspase cleavage sites has been shown to reduce cellular toxicity in different neurodegenerative disease models (Table 1.2) (Wellington et al., 2000) (Graham et al., 2006a) (Ellerby et al., 1999b) (Ellerby et al., 1999a) (Zhang et al., 2010) (Galvan et al., 2008) (Nguyen et al., 2008) (Saganich et al., 2006) (Banwait et al., 2008) (Mookerjee et al., 2009) (Jung et al., 2009) . Furthermore, inappropriate regulation of apoptosis, leading to excessive or insufficient cell death, has not only been implicated in neurodegenerative diseases, but has also been involved in autoimmune disorders and several forms of cancer (Thompson, 1995) (Nicholson, 1996).

Table 1.2 - Neurodegenerative disorders where prevention of caspase cleavage improves disease phenotypes

Disease	Protein	Protein function	Caspase	Experimental model	Rescue	Reference
HD	HTT	Possible scaffolding protein Linked to cellular pathways	Casp6	YAC128 HD mouse model HEK 293 T cells HN33 hippocampal cells	Preserved striatal volume Normal cognitive and motor function Resistant to excitotoxic stress Normal extrasynaptic NMDA receptors Reduced caspase activation, toxicity and aggregate formation	Graham et al., 2006 Pouladi et al., 2009 Milnerwood et al., 2010 Wellington et al., 2002
AD	APP	Membrane protein Involved in synapse formation, neural plasticity and iron export	Casp6	APP transgenic mouse	No synaptic loss, astrogliosis, dentate gyral atrophy or increased neuronal precursor proliferation Absence of behavioral abnormalities	Galvan et al., 2006 Saganichi et al., 2006 Galvan et al., 2008 Banwait et al., 2008 Nguyen et al., 2008 Zhang et al., 2008
SCA3	Ataxin-3	Deubiquitinating enzyme Involved in protein quality control	Casp1?	SCA3 dorsophila model	Decreased photoreceptor degeneration	Jung et al., 2009
SCA7	Ataxin-7	Component of histone acetyltransferase complex and transcriptional regulation	Casp7	SCA7 mouse model HEK 293 T cells	Absence of N-terminal truncation fragments Improvement of disease symptoms Reduced fragment accumulation	Guyenet et al., unpublished Mookerjee et al., 2009
DRPLA	Atrophin-1	Possible transcriptional corepressor	Casp3	HEK 293 T cells	Decreased cytotoxicity	Ellerby et al., 1999
SBMA	AR	Testosterone-activated steroid receptor	Casp3	HEK 293 T cells	Protection from cell death Absence of perinuclear aggregates	Ellerby et al., 1999

Huntington disease (HD), Alzheimer disease (AD), Spinocerebellar ataxia (SCA), Dentatorubropallidoluysian atrophy (DRPLA), Spinal and bulbar muscle atrophy (SBMA)
Huntingtin (HTT), Amyloid precursor protein (APP), Androgen receptor (AR)

1.2 Caspase-6

1.2.1 Caspase-6 expression, structure and function

Casp6 is expressed in the brain (Graham et al., 2010) (Hermel et al., 2004) and peripheral tissues and it localizes to the cytosol and nerve terminals (Singh et al., 2002). Limited microarray data also shows widespread expression of Casp6 (Rampon et al., 2000) (Jiang et al., 2001); however, a careful analysis of Casp6 mRNA and protein expression has never been done.

The alpha isoform of casp6 is synthesized as an inactive proenzyme of 34 kDA (Baumgartner et al., 2009). It is a dimeric zymogen with a short pro-domain, a large p20 subunit, which contains the Cys 163 catalytic cysteine, an inter-subunit linker and a small p10 subunit (Figure 1.4a) (Wang et al., 2010). There is also a beta isoform of Casp6, which lacks half of the p20 subunit and does not induce apoptosis (Figure 1.4b).

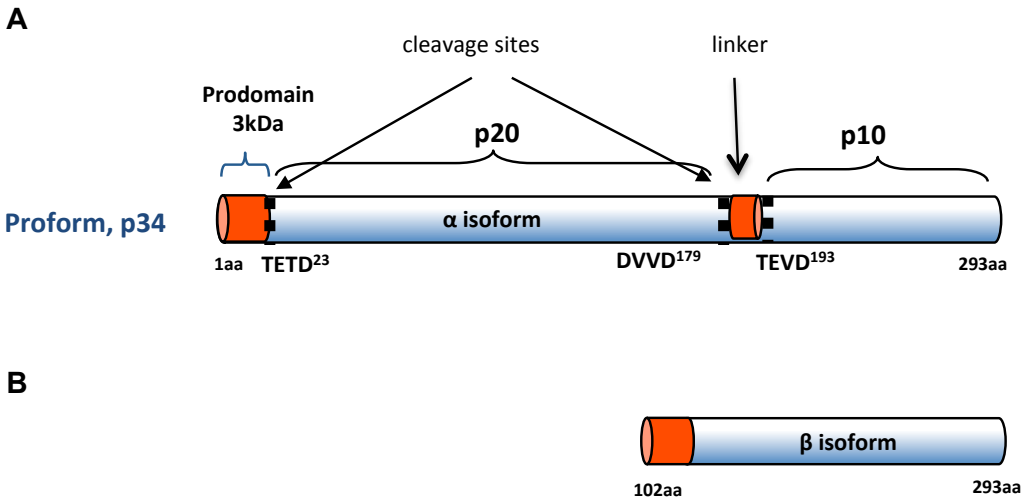

Figure 1.4 – Caspase-6 structure. A) The alpha isoform of Casp6 is synthesized as an inactive proenzyme of 34 kDA. It is a dimeric zymogen with a short pro-domain, a large p20 subunit, which contains the Cys 163 catalytic cysteine, an inter-subunit linker and a small p10 subunit. B) The Beta isoform of Casp6 lacks half of the p20 subunit and does not induce apoptosis. It acts as a dominant negative inhibitor of Casp6 by preventing activation of the proform.

Casp6 shares 35% sequence homology with initiator caspases-8 and -10 and 33% homology with executioner caspase-3 and -7 (Table 1.3) (Cohen, 1997) (Nicholson, 1999). Casp6 was originally considered an executioner caspase; however, it has been shown that it can activate effector caspases such as caspase-8 and -2 as well as executioner caspases (Xanthoudakis et al., 1999) (Allsopp et al., 2000) (Lassus et al., 2002). Capsase-3 has also been shown to be activated by Casp6 both *in vitro* and *in vivo* (Allsopp et al., 2000). Additionally, p53 and active Casp6 have been detected in the brains of Huntington (Graham et al., 2006a) (Hermel et al., 2004) (Bae et al., 2005) and Alzheimer disease (Gervais et al., 1999) (Zhang et al., 2010) patients before the onset of apoptosis and any signs of clinical symptoms, pointing towards a physiological role more upstream in the apoptotic cascade.

Table 1.3 - Sequence identity of the caspases. "This research was originally published in the Biochemical Journal. Gerald M. Cohen, Caspases: the executioners of apoptosis. Biochemical Journal. 1997; Volume 326: 1-16 © Biochemical Journal."

	Identity (%)										
Caspase	1	2	3	4	5	6	7	8	9	10	CED-3
1	100	22	30	55	50	22	26	22	25	22	29
2		100	22	27	22	28	22	26	33	28	28
3			100	33	30	33	52	33	37	33	34
4				100	77	28	22	20	22	21	26
5					100	22	25	22	24	22	25
6						100	33	35	33	35	35
7							100	33	32	33	33
8								100	22	41	26
9									100	33	29
10										100	25

1.2.2 Caspase-6 activation and regulation

Casp6 is activated by proteolytic cleavage at Asp 23, Asp 179 and Asp 193 (Srinivasula et al., 1996). In order to get activated, the N-terminal prodomain is removed by cleavage at the TETD site along with double cleavage in the linker region at the DVVD and TEVD sites. This results in a large 20 kDa and a small 10 kDA subunit which then dimerize to form the active Casp6 complex (Figure 1.5). Casp6 can also undergo self-processing and activation in vivo and in vitro (Klaiman et al., 2009) (Wang et al., 2010), and can be activated by caspase-1 and -3 (Srinivasula et al., 1996) (LeBlanc et al., 1999) (Allsopp et al., 2000) (Guo et al., 2006) (Singh et al., 2002). However, it has been recently demonstrated that Casp6 can also cleave and activate caspase-3 (Liu et al., 1996). Activation of caspase-3 and -6 during trophic factor deprivation-induced apoptosis shows that active Casp6 is capable of activating caspase-3 in rat cerebellar granule cells; however Casp6 was not activated by caspase-3. Consistent with these findings, the use of a Casp6 inhibitor (zVADfmk) prevented caspase-3 activation after trophic factor withdrawal, but adding a caspase-3 (CP-DEVD-cho) inhibitor failed to prevent Casp6 activation (Allsopp et al., 2000). These results indicate that aside from its role as an effector caspases Casp6 can act as an initiator caspase, interestingly activation of Casp6 can result in activation of caspase-3.

Casp6 is transcriptionally regulated by p53 through DNA binding of p53 to the third intron of Casp6 and transactivation and the threshold for Casp6 mediated apoptosis is decreased with increasing levels of p53 (MacLachlan and El-Deiry, 2002). Consistently, over-expression of p53 in cell culture increases the activity of both procaspase-6 and Casp6 and cleavage of its substrates (MacLachlan and El-Deiry, 2002). Furthermore, the death effector domain containing DNA binding protein (DEDD) co-localizes with Casp6 in nucleoli (Schickling et al., 2001) and activates Casp6 at the onset of apoptosis. Active Casp6 has been shown to co-localize to the nucleus (Warby et al., 2008) and many of its substrates are nuclear proteins. However, Casp6 also gets activated

in the cytosol and has many cytosolic substrates that include cytoskeletal proteins.

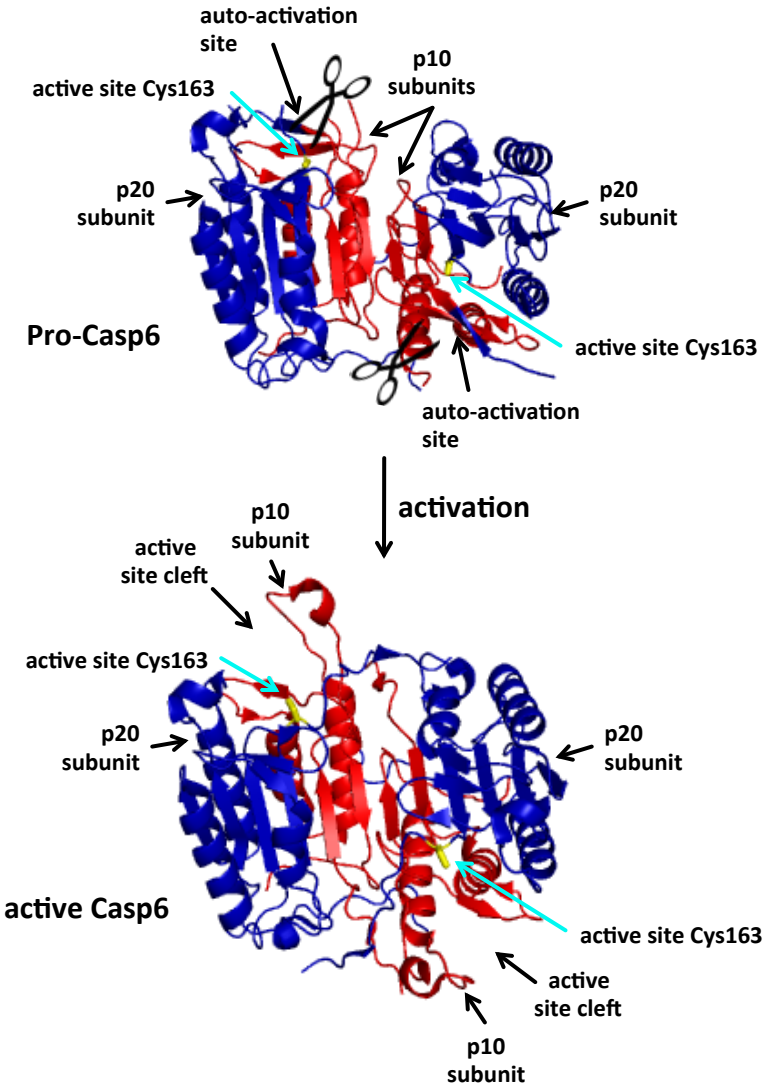

Figure 1.5 - Caspase-6 activation Large subunits (p20): blue and green, Small subunits (p10): yellow and pink. Pro-casp6 exists as a dimer with the linker region between the small and large subunits blocking the active site. Upon cleavage of the intersubunit linker and removal of the pro-domain, Casp6 shifts into its active conformation as a dimer with two opposite active site clefts (reprinted from Rona K. Graham et al. Caspase-6: Initiator and Executioner, a split personality of the protease type. Manuscript in preparation).

The beta isoform of Casp6 is capable of preventing activation of the Casp6 proform; however, it cannot inhibit it once it is already activated (Lee et al., 2010), suggesting that it acts as a dominant-negative regulator of Casp6 activation and could potentially serve as a therapeutic target for diseases where aberrant activation of Casp6 has been implicated. Additionally, the caspase inhibitory factor (CIF) degrades the active subunits of Casp6. CIF can be induced by estrogen treatment, making this an alternative strategy for inhibiting Casp6. In addition, procaspase-6 can also be inactivated through phosphorylation at Ser 257 by ARK5, a member of the AMP-kinase family.

1.2.3 Caspase-6 substrates

Casp6 has more than 60 substrates (Table 1.4) and/or interacting proteins (Table 1.5) (Graham et al., manuscript in preparation). It cleaves cytoskeletal and nuclear structural proteins, such as NuMA (Hirata et al., 1998), lamin proteins (lamin A is the best known caspase-6 specific substrate) (Chinnaiyan and Dixit, 1996) and transcription factors such as NF-kβ (Levkau et al., 1999), SATB1 (Galande et al., 2001), AP-2α (Nyormoi et al., 2001) and CBP (Rouaux et al., 2004), supporting the role of Casp6 in nuclear apoptosis.

Htt and amyloid precursor protein (APP) have been shown to be Casp6 substrates and they are implicated in HD and AD respectively (Wellington et al., 2002) (Saganich et al., 2006). Other substrates such as CBP and NF-kβ are also implicated in neurodegenerative diseases (Levkau et al., 1999) (Rouaux et al., 2004), and ischemia, brain trauma and HD lesion models show activation of NF-kβ and p53 (a Casp6 regulator) expression (Nakai et al., 2000). Cleavage of NF-kβ by Casp6 generates an inactive p65 molecule, which acts as an inhibitor of NF-kβ and leads to apoptosis. However, when p65 is resistant to Casp6 cleavage there is no apoptosis (Levkau et al., 1999), implicating cleavage of Casp6 substrates in mechanisms of disease.

Table 1.4 - Caspase6 substrates (reprinted from Rona K. Graham, et al. Caspase-6: Initiator and Executioner, a split personality of the protease type. Manuscript in preparation).

Neurodegenerative disease proteins	Proposed cleavage site(s)	Reference
Huntingtin	IVLD	Graham et al, 2006
Amyloid Precursor Protein	VEVD, DNLD	Pellegrini et al, 1999 Galvan et al, 2006
Presenilin 1, 2	AQRD, ENDD, DSYD	LeBlanc et al 1999 Van de Craen, 1999,
DJ-1	VEKD	Giaime et al, 2009
Cytoskeletal proteins	**Proposed cleavage site(s)**	**Reference**
Tau	VSED, VMED	Guo et al, 2004
Keratin -14, -15, -17, -18	VEMD, VEVD	Horowitz et al, 2004, Badock et al, 2001 Caulin et al, 1997
Desmoplakin		Aho et al, 2004,
Plectin		Aho et al, 2004,
Vimentin	IDVD	Byun et al, 2001
Periplakin	TVAD	Kalinin et al, 2005, Aho et al, 2004,
Desmin	VEMD	Chen et al, 2003,
hDlg	YEVD	Inesta-Vaquera et al, 2009
Drebrin	EDID, IETD	Klaiman et al, 2008
Beta actin		Klaiman et al, 2008
Spinophilin	LDAD, EEVD, LEED, EEDD, DDED, DEDD, DEED, EEED, EDYD, EDVD, LEKD, VEVD, TDED, EEMD	Klaiman et al, 2008
Alpha actinin -1, -4	EDWD, IEED, DQWD, LEGD	Klaiman et al, 2008
Capping protein alpha		Klaiman et al, 2008
ezrin	LEAD	Klaiman et al, 2008
cofilin		Klaiman et al, 2008
Glial fibrillary acidic protein	VERD, VELD	Klaiman et al, 2008
Alpha tubulin	IQPD, LEKD	Klaiman et al, 2008
Signalling proteins	**Proposed cleavage site(s)**	**Reference**
14-3-3 zeta, epsilon	TQGD	Klaiman et al, 2008
cFlip	LEVD	Srinivasula et al, 1997
Inhibitor-2 of PP2A	EEDD, EDDD, DDDD,EDID, EEGD, EDED, DEDD	Klaiman et al, 2008
eNos		Tesauro et al, 2006
Guanylate cyclase		Payne et al, 2003
Notch I	EEED, ANRD, DITD, HMD, CLLD	Cohen et al, 2005
FAK	VSWD	Gervais et al, 1998
NEDD 4	DQPD	Harvey et al, 1998
PKCzeta	EETD	Smith et al, 2000
TRAF1	LEVD	Leo et al, 2001

Cell cycle proteins	Proposed cleavage site(s)	Reference
Cyclin B1	ILVD	Chan et al, 2009,
Retinoblastoma protein	DSID	Lemaire et al, 2005,
Chaperones	**Proposed cleavage site(s)**	**Reference**
Hsp90 alpha	IDED, DEDD, LEGD, EEVD	Klaiman et al, 2008
Hsp gp96 precursor	DEVD, VDVD, VEED, LELD, EESD, VEED, VDSD, EDED, DEDD, IDPD, TEQD, EEMD	Klaiman et al, 2008
VCP	VAPD	Klaiman et al, 2008 Halawani et al., 2010
Autophagy related proteins	**Proposed cleavage site(s)**	**Reference**
Atg3	LETD	Norman et al., 2010,
Beclin 1/Atg6	TDVD	Norman et al., 2010,
p62	KEVD, GDDD, IEVD	Norman et al., 2010,
Transcription factors/coactivators	**Proposed cleavage site(s)**	**Reference**
NFκB	VFTD	Levkau et al, 1999
SATB1	VEMD	Galande et al, 2001
AP-2α	DRHD	Nyormoi et al, 2001
CBP		Rouaux et al, 2003
PPARγ	TTVD	Guilherme et al, 2009
Serum response factor	EATD, SASD	Drewett et al, 2001
Nuclear matrix proteins	**Proposed cleavage site(s)**	**Reference**
NuMa		Hirata et al, 1998
Lamin A	VEID	Srinivasula et al, 1996 Takahashi et al, 1996 Orth et al, 1996
DNA repair/binding	**Proposed cleavage site(s)**	**Reference**
PARP		Miyashita et al, 1998 Orth et al, 1996 Orth and O'Rourke, 1996
Topoisomerase I	PEED, EEED	Samejima et al, 1999
Protein synthesis and conjugation	**Proposed cleavage site(s)**	**Reference**
Elongation factor 1γ	VDSD, EEMD	Klaiman et al, 2008
UFD2p	VDVD	Mahoney et al, 2002
Metabolism	**Proposed cleavage site(s)**	**Reference**
5' lipoxygenase	IQFD	Werz et al, 2005
Inorganic pyrophosphatase	DDPD, TDVD	Klaiman et al, 2008
Glyceraldehyde-3-phosphate dehydrogenase		Klaiman et al, 2008
Phosphate cytidylyltransferase 1 choline alpha isoform	TEED	Lagace et al, 2001

Proteases and cofactors	Proposed cleavage site(s)	Reference
Neurolysin	TEAD, TDDD, VETD	Klaiman et al, 2008
Prolyl endopeptidase	EDPD	Klaiman et al, 2008 Srinivasula et al, 1996 Xanthoudakis et al, 1999
Caspases -3, -6, -8	IETD, TETD, TEVD, VETD, VEVD, DVVD	Van de Craen et al, 1999 Thornberry et al, 1997, Allsopp et al, 2000, Talanian et al, 1997 Cowling et al, 2002 Slee et al, 2001
Proteasome activator 28 subunit PA28γ	DGLD	Araya et al, 2002
Membrane and lipid binding	**Proposed cleavage site(s)**	**Reference**
Rab GDP dissociation factor inhibitor α	EEYD, TEND	Klaiman et al, 2008
Brain fatty acid binding protein	EEFD, LDGD	Klaiman et al, 2008
Annexin V	LEDD, VEQD	Klaiman et al, 2008
Viral proteins	**Proposed cleavage site(s)**	**Reference**
TGEV nucleocapsid protein	VVPD	Eleouet et al, 2000
SARS nucleocapsid protein	PAAD, DMDD	Diemer et al, 2008
NS5A protein (hepatitis C virus)		Kalamvoki et al, 2006

Table 1.4 - Casp6 Interacting Proteins (reprinted from Rona K. Graham, et al. Caspase-6: Initiator and Executioner, a split personality of the protease type. Manuscript in preparation).

Interacting protein	identified by	Reference
Chromodomain helicase DNA binding protein 3	Yeast 2 Hybrid	Stelzl et al, 2005
Hsp 60	co-IP	Xanthoudakis, 1999
αA-Crystallin	Pull-down	Morozov et al, 2005
Ark5	phosphorylation	Suzuki et al, 2004

1.2.4 Caspase-6 and neurodegeneration

1.2.4.1 Caspase-6 and neurodegenerative disorders

Casp6 has been implicated in HD, AD and other human disorders, such as Ischemia, Parkinson disease and spinocerebellar ataxia, where proteolytic cleavage of disease associated proteins has been implicated (Zhang et al., 2010) (Gervais et al., 1999) (Albrecht et al., 2007) (Albrecht et al., 2009) (Kubodera et al., 2003) (Haacke et al., 2006) (Nakai et al., 2000).

1.2.4.1.1 Caspase-6 and Huntington Disease

It has been shown that caspase inhibitors are capable of abrogating htt cleavage and reducing its toxicity (Wellington et al., 2002) (Leyva et al., 2010). Since the reduced toxicity could be attributed to either inhibition of pro-apoptotic caspase activity or to caspase cleavage prevention, Wellington et al. generated a site-directed htt mutant that is resistant to caspase-3 cleavage at amino acids 513 and 552 and to Casp6 cleavage at amino acid 586. Neuronal and non-neuronal cells expressing caspase resistant htt showed reduced caspase activation, less toxicity and turned out to be less susceptible to aggregate formation compared with caspase cleavable huntingtin (Wellington et al., 2000).

The YAC128 transgenic mouse model of HD displays an age and CAG-dependent phenotype that accurately recapitulates many features of the human disease including protein cleavage and nuclear localization of htt, prior to detection of cognitive, motor deficits and selective striatal degeneration (Slow et al., 2003) (Graham et al., 2006a) (Van Raamsdonk et al., 2005). In order to validate that caspase cleavage was responsible for cellular toxicity of mutant htt, YAC128 mice expressing caspase-3- and/or caspase-6-resistant mutant htt (C6R) were created and served to demonstrate that eliminating cleavage at the 586aa Casp6 site, but not the 513 and 552aa caspase-3 sites of mhtt was sufficient to preserve striatal volume and cognitive and motor function in the C6R HD mouse model. Furthermore, the C6R were resistant to excitotoxic stress (Graham et al., 2006a).

In addition to the findings in the C6R mice, a number of results support Casp6 as the protease responsible for cleavage of htt at aa586 and as an important mediator of neuronal dysfunction and neuro-degeneration. These include increased Casp6 activation in early grade human HD brain and in the YAC128 HD model (Graham et al., 2010) in addition to enhanced immunoreactivity for active Casp6 in the brain of end stage human HD patients Hermel, 2004, p00109}. Furthermore Casp6 physically interacts with htt (Hermel et al., 2004) and dominant-negative and/or peptide inhibition of Casp6 activity in

primary striatal neurons protects neurons from degeneration (Hermel et al., 2004) (Graham et al., 2010).

1.2.4.1.2 Caspase-6 and Alzheimer Disease

Several findings implicate Casp6 in the neurotoxicity observed in AD. Increased caspase-6 mRNA expression is observed in AD brain tissues compared to controls (de Calignon et al., 2010) and active Casp6, Casp6 cleaved-tau and Casp6-cleaved-αtubulin are abundant in neuropil threads, neurofibrillary tangles and neuritic plaques of AD brains (Klaiman et al., 2008) (Guo et al., 2004). In addition, amyloid plaques display high caspase-cleaved APP expression (Koffie et al., 2009). Interestingly, serum deprivation in neuronal cell cultures causes Casp6 activation, and Casp6 mediated processing of APP generates an Aβ containing fragment (LeBlanc et al., 1999).

Caspase cleavage sites can be found in β-APP and it has been shown that Casp6 can cleave APP (Gervais et al., 1999) (LeBlanc et al., 1999). Cleavage of β-APP at aa664, a described Casp6 cleavage site, is detected early in human AD brain tissue, suggesting that this protease could be an early instigator of neuronal dysfunction (Albrecht et al., 2007) (Banwait et al., 2008). Most importantly, in a transgenic mouse model of AD that features senile plaques and synapse and memory loss, mutation of the caspase cleavage site at aa664 in the amyloid precursor protein completely suppressed synapse loss, dentate gyral atrophy, astrogliosis and memory loss (Saganich et al., 2006) (Galvan et al., 2008).

1.2.4.2 Caspase-6 and axonal degeneration: implications for Huntington and Alzheimer Disease

Axonal degeneration is a key mechanism involved in developmental axonal pruning (Raff et al., 2002) (Buss et al., 2006). However, it also occurs in the mature nervous system as a consequence of neuronal damage and in neurodegenerative disorders (Nikolaev et al., 2009) (Singh et al., 2008) (Luo and O'Leary, 2005).

Axonal degeneration has been shown to be involved in the early stages of HD. Selective loss of striatal projection neurons is the neuropathological hallmark of HD (Ferrante et al., 1985) (Graveland et al., 1985) and neuronal degeneration is most prominent in the lateral globus pallidus (LGP) and substantia nigra (SN), where the projections of axons from neurons in the striatum terminate (Graybiel, 1990). Mouse models of HD display neuropil aggregates and axonal degeneration in the LGP and SN followed by intranuclear accumulation of mutant huntingtin. In addition, cultured striatal neurons that express mutant huntingtin also contain neuritic aggregates that block protein transportation in neurites and cause their degeneration (Li et al., 2001). Interestingly, pre-symptomatic HD patients also display neuropil degeneration (Albin et al., 1990), indicating that axonal degeneration is implicated in the early neuropathological changes observed in HD.

The corpus callosum is the largest white matter brain structure, consisting of densely packed myelinated axonal fibers that arise from large pyramidal neurons in layers III and V that give rise to long-reaching intra-cortical projections and whose main purpose is to connect homologous areas of the cerebral cortex (Conti and Manzoni, 1994) (Innocenti, 1994) (Innocenti et al., 1995). The structure of the corpus callosum was analyzed through a study spanning over 20 years of HD progression and reveal that the corpus callosum displays locally selective anatomical alterations a decade before the onset of behavioral symptoms (Rosas et al., 2010). These findings further validate the involvement of axonal degeneration early in HD and have significant implications for the cognitive deficits observed in the disease since the degeneration of pyramidal neurons projecting from the cortex as well as by loss of cortico-cortical connectivity lead to deficits in associative cortical processing (Rosas et al., 2010).

Different lines of research have provided insights into the mechanisms involved in the axonal degeneration observed in HD and other neurodegenerative diseases such as AD. Caspase-3 is known as the main

effector caspase during development (Ranger et al., 2001), and more specifically as responsible for cell body degeneration (Nikolaev et al., 2009). The death receptor 6 (DR6), a member of the TNF receptor family, that has a cytoplasmic death domain and is highly expressed in neurons during the pro-apoptotic state, signals via BAX and caspase-3 to trigger cell body degeneration (Nikolaev et al., 2009) (Finn et al., 2000) (White et al., 1998). Interestingly, caspase-3 inhibitors are capable of preventing cell body degeneration; however, axonal degeneration is not prevented by caspase-3 inhibition (Finn et al., 2000).

It has been recently established that BAX is also required for axonal degeneration, but in conjunction with Casp6 instead of caspase-3. APP and DR6 are responsible for triggering axonal degeneration through Casp6 after trophic deprivation. When Casp6 activation is induced by trophic factor deprivation it occurs in a punctate pattern and the sites of activation are the same sites where axonal microtubule fragmentation occurs. These data suggest that an extracellular fragment of APP, acting via DR6 and Casp6, contributes to axonal degeneration in AD (Nikolaev et al., 2009).

Axonal degeneration can also be triggered by extrinsic signals through pro-apoptotic receptors such as the tumor necrosis factor (TNF) receptor super-family, including the p75 neurotrophin receptor (p75NTR), Fas and TNFRSF1A (Raff et al., 2002) (Buss et al., 2006). p75NTR has been implicated in developmental sympathetic axon pruning (Singh et al., 2008) (Nikolaev et al., 2009) (Singh and Miller, 2005) (Luo and O'Leary, 2005) and in aberrant axonal degeneration (Plachta et al., 2007).

Furthermore, Casp6 has been implicated in degeneration of cholinergic axons that occurs through p75NTR and a myelin dependent mechanism. Neurotrophins bind to p5NTR, which interacts with Rho guanine nucleotide dissociation inhibitor (Rho-GDI), which in turn activates Rho and Casp6 and results in degeneration of axons that aberrantly grow into the corpus callosum (Park et al., 2010). Altogether, these findings highlight the importance of Casp6 in axonal degeneration and neurodegenerative diseases.

1.3 Thesis objectives

Caspases are involved in programmed cell death (Raff et al., 1993) (Troy and Salvesen, 2002) and non-apoptotic pathways (De Maria et al., 1999) (Kennedy et al., 1999) (Li et al., 2010). Importantly, aberrant activation of caspases has been implicated in several human diseases. As caspases are substrate-recognizing enzymes with constrained structural features that can be targeted by small molecule approaches, this makes them potential drug targets.

Given the various findings implicating Casp6 in the progression of neurodegenerative diseases (Nikolaev et al., 2009) (Graham et al., 2006a) (Saganich et al., 2006) (Zhang et al., 2010) (Gervais et al., 1999) (Albrecht et al., 2007) (Albrecht et al., 2009) (Kubodera et al., 2003) (Haacke et al., 2006) (Nakai et al., 2000) and the efforts underway to identify Casp6 inhibitors as a therapeutic strategy for neurological diseases (Leyva et al., 2010), gaining a deeper understanding of the role of Casp6 in brain development is crucial.

Previously Casp6 deficient mice were reported to be viable, (Zheng et al., 1999) but have not been assessed in detail and have only been used for studying the role of Casp6 in immune responses (Watanabe et al., 2008) (Kobayashi et al., 2011). Targeted caspase knockout mice have been instrumental for studying the involvement of caspases in apoptotic and non-apoptotic pathways, and provide an ideal pre-clinical tool for studying the role of caspases in neurodegenerative diseases.

The overall objective of this study is therefore to examine the physiological function of Casp6 in brain development, structure and function by performing an in depth neuropathological and behavioral characterization of mice deficient in Casp6.

The specific objectives are:

Objective 1: To determine if complete ablation of Casp6 causes phenotypic alterations in mice

Deletions of specific caspases can result in robust brain malformations associated with supernumerary cells, multiple cerebral indentations and ectopic cell masses in the cortex (Varfolomeev et al., 1998) (Kuida et al., 1998) (Hakem et al., 1998) (Houde et al., 2004), highlighting the importance of establishing if *Casp6* -/- mice display any neuropathological or behavioral changes compared to wild type (WT) mice.

Questions that will be addressed are:

Does Casp6 ablation cause neuropathological phenotypes in mice?

Do Casp6 deficient mice display any behavioral changes compared to WT mice?

Objective 2: To understand whether Casp6 plays a role in excitotoxic cell death and axonal degeneration pathways

N-methyl-D-aspartate (NMDA)-mediated excitotoxicity has been repeatedly linked to HD. Mice expressing mhtt resistant to cleavage by Casp6 are protected from NMDA-induced excitotoxicity *in vitro* (Graham et al., 2006a) (Graham et al., 2010) and from alterations in extrasynaptic NMDA receptors *in vivo* (Milnerwood et al., 2010). Furthermore, it has also been shown that Casp6 inhibitors and/or dominant-negative inhibition of Casp6 provide protection against excitotoxicity (Hermel et al., 2004) (Graham et al., 2010). Axonal degeneration has also been implicated in HD and AD and it has been demonstrated that it occurs through activation of Casp6 (Nikolaev et al., 2009) (Park et al., 2010) (Sivananthan et al., 2010). Interestingly, Casp6 inhibitors rescue APP-induced axonal degeneration. In addition, axonal white matter is reduced in HD and AD patients (Paulsen et al., 2010) (Salat et al., 2009). These findings implicate Casp6 in neurodegenerative disease, and indicate that Casp6 deficient mice may

be protected against NMDA-mediated excitotoxic stress and NGF-induced axonal degeneration.

Questions that will be addressed are:

Does Casp6 deficiency cause improved neuronal health and protection against NMDA-induced cell death?

Are Casp6 deficient mice protected against NGF-induced axonal degeneration?

2 Experimental procedures

2.1 Generation of mutant Casp6 -/- mice

The Casp6 -/- mouse was created by Xenogen using retroviral gene trap methods**.** BAC clones lacking exons 2 to 5 of the *Casp6* gene, which encode the catalytic domain of the Casp6 protein, were introduced to embryonic stem cells by homologous recombination to generate *Casp6* knockout (*Casp6* -/-) mice. The homologous recombination strategy was validated by Southern analysis. Two PCR assays were designed for genotyping: the 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. The following primers were used:

WT assay forward: 5' - AGGGTGGGTTACACCAGGTT - 3'
WT assay reverse: 5'- TCCAGCTTGTCTGTCTGGTG - 3'
neo assay forward: 5'- CCTGTGGGGTCAAAAGACTTTCACAG - 3'
neo assay reverse: 5'- GCAAGCTGCTAACAGCCAACACAAC - 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 DH_2O. 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.

2.2 Breeding and housing

The *Casp6 -/-* 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 and A07-0262). The mice were housed in groups of maximum five mice per cage as previously described (Slow et al., 2003).

2.3 Caspase-6 expression levels

2.3.1 mRNA analysis and quantitative real-time 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'

2.3.2 Protein analysis and western blotting

Protein was extracted as previously described (Wellington et al., 2002) from whole brains, peripheral tissues and/or MEFs from casp6-/- and WT mice and/or MEF cultures and its concentration measured by Bio Rad DC Protein Assay. 4-12% Bis-Tris polyacrylamide gels from Invitrogen were used to load 70μg of protein for brain and peripheral tissues and 50ug for MEFs 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 Casp6 antibody (Cell Signaling 9762, 1:500) or a lamin antibody (Cell Signaling 2032, 1:1000).

2.3.3 Mouse embryonic fibroblasts

Embryos were dissected from E12.5 pregnant *Casp6 +/-* females who had been bred with *Casp6 +/-* males. DNA was isolated from bodies post-dissection

for genotyping. The spleen, liver, lung, heart, head and limbs were removed from each embryo and the remaining tissues were minced into ~2mm pieces in 5mL of trypsin to make the mouse embryonic fibroblasts (MEFs). After 15 minutes of digestion at 37° C, cell suspensions were dissociated by pipetting and media was added (DME + 10% FBS) and then collected by centrifugation (1000 RPM/5'). Cell pellets were re-suspended in 10mL DME + 10% FBS and plated in 10cm dishes ("passage 0"). Experiments were done with genotype pairs of cells of the same passage number (always P0-P7). Staurosporine stress was induced by adding stauropsorine to the MEF media to a 50nM final concentration. Treatment was done for 24 hours.

2.4 Necropsy

Kidney, liver, heart, spleen, stomach, intestine, cecum, colon and testis tissues were collected 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.

2.5 Body weight

Mice were weighed at 2, 4, 6, 8, 10 and 12 months of age using a digital scale and weight recorded. Male: *Casp6 -/-* (n=10), WT (n=4) Female: *Casp6 -/-* (n=9) and WT (n=8).

2.6 NMDAR excitotoxicity

Cultures (9 -12 individual cultures) of primary MSNs were prepared from newborn pups of *Casp6 -/-* and WT mice in a procedure described previously (Graham et al., 2006a) (Zeron et al., 2002)**.** 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 (n=3), 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, 75ul media was removed for LDH quantification, according to the manufacturers instructions (Roche, cytotoxicity detection kit). The media was then mixed 150ul 1:1 with lysis reagent (Cell Titre Glo, Promega). For ATP assessment, cells were lysed on an orbital rotator at room temperature for 10 minutes. 100ul of reagent: media was removed to a black walled 96-well plate and luminescence detected with an OMEGAstar plate reader. In order to obtain the LDH and ATP data, the raw value of each well was normalized to mock-treated cells/neurons on the same plate, and expressed as a fraction of this value.

2.7 NGF induced axonal degeneration

2.7.1 Cell culture

Dissociated superior cervical ganglion (SCG) neurons of P0-1 WT and *Casp6 -/-* mice were NGF-maintained (50 ng/ml NGF) for 5 days before treatment. For NGF deprivation, the medium was exchanged to medium lacking NGF that contained anti-NGF antibody for 24 hours. The Casp9 inhibition condition required the addition of 40 uM Casp9 inhibitor (Z-LEHD-FMK).

2.7.2 Microfluidic chambers

Sympathetic neurons were cultured in microfluidic chambers as previously described (Potts et al., 2003) (Taylor et al., 2005). Briefly, PDMS replica-molded microfluidic chambers were placed onto glass coverslips coated with poly-D-lysine (50 ug/ml) and lamin (1 ug/ml). Dissociated SCG neurons of P0-1 WT and *Casp6 -/-* mice (~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 3x 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. The Casp9 inhibition condition required the addition of 40 uM Casp9 inhibitor (Z-LEHD-FMK) to both compartments during local deprivation.

2.7.3 Immunofluorescence

Neurons were 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.

2.7.4 Quantification

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 6^{th} 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 (n=3).

2.8 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 (Mullen et al., 1992), as described previously (Slow et al., 2003).

The area of the striatum was traced with Stereoinvestigator 10.0 software (Microbrightfield). 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 probed was used for neuronal size. A minimum of 200-300 cells per animal were counted or 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 (Mayhew and Olsen, 1991) (Sonmez et al., 2010) (n=20 at 3 months, n=6 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 (Mayhew and Olsen, 1991) (Sonmez et al., 2010) (n=20 at 3 months, n=6 at 8 months).

2.9 Behavior

Behavioral analysis was done blind to genotype. Both male and female *Casp6 -/-* and WT mice were tested.

2.9.1 Novel object recognition

Mice (n= 11 WT (8 female, 3 male); n= 18 C6-/- (9 female, 9 male)) 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.

2.9.2 Open-field

Mice (n= 8-12 WT female, 4-7 WT male, 9-10 C6-/- female, 10-12 C6-/- male) were assessed using an open-field activity monitor (Med Associates Inc., St Albans, VT, USA) during the dark cycle every two months from 2 to 12 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.

2.9.3 Accelerating rotarod

Motor coordination and learning were examined using an accelerating rotarod (UGO Basile, Comerio, Italy). For training, naïve 2-month-old mice (n= 20 WT (13 female, 7 male); n= 21 C6-/- (10 female, 11 male)) 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 longitudinal accelerating rotarod assessment, mice (n= 8-13 WT female, 2-7 WT male, 9-10 C6-/- female, 9-11 C6-/- male) were tested every two months from 2 to 12 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= 8-13 WT female, 2-7 WT male, 9-10 C6-/- female, 9-11 C6-/- male).

2.10 Statistics

Statistical analysis of the LDH, ATP and TUNEL data, as well as of the stereology data was performed using one-way ANOVA followed by an unpaired t-test to determine if there were any significant differences between WT and Casp6 deficient mice. In case of significant genotype effects, post hoc comparisons between genotypes were performed using linear trend post hoc test. p values, means, and SDs were calculated using Prism version 4.0 (GraphPad Software). Differences between means were considered statistically significant if $p<0.05$.

In order to establish if there was a significant difference in axon distance before and after NGF deprivation, fold change was calculated by dividing the mean post-treatment axon distance 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 (n=3). An unpaired t-test was used to determine if there was a statistically significant difference between treatment conditions.

Statistical analysis of the body weight and behavioral testing data, which had two or more independent variables, was performed using a two-way ANOVA model. In the case of the Novel object recognition task, an unpaired t-test was used to determine if WT mice displayed a significant difference in percentage

investigation to target between the first and second trial. p values, SEM (behavior), means, and SDs (body weight) were calculated using Prism version 4.0 (GraphPad Software). Differences between means were considered statistically significant if $p<0.05$.

3 Characterization of constitutive caspase-6 deficient mice

Aside from the role of caspases during development, aberrant activation of caspases has been implicated in several human diseases, such as Alzheimer Disease (AD), Huntington Disease (HD), cerebellar ataxias, amyotrophic lateral sclerosis (ALS) and ischemic brain injury (Zhang et al., 2010) (Gervais et al., 1999) (Albrecht et al., 2007) (Albrecht et al., 2009) (Kubodera et al., 2003) (Haacke et al., 2006). In particular, numerous findings implicate Casp6 mediated apoptosis in neurodegenerative diseases (Nikolaev et al., 2009) (Graham et al., 2006a) (Saganich et al., 2006) (Zhang et al., 2010) (Banwait et al., 2008) (Galvan et al., 2008) (Nguyen et al., 2008) highlighting the need for a deeper understanding of Casp6 biology.

Previously Casp6 deficient mice were reported to be viable, (Zheng et al., 1999), but have not been assessed in detail (Watanabe et al., 2008) (Kobayashi et al., 2011) and they exhibit significant levels of Casp6 protein expression in the brain (Graham and Hayden, unpublished data), rendering them unsuitable for studying the physiological function of Casp6 in the central nervous system. This study is the first to examine neuropathological and behavioral effects of deleting Casp6 in mice. The complete ablation of proteins implicated in neurodegenerative diseases provides a unique *in vivo* system for understanding their involvement in these disorders. We generated *Casp6* -/- mice and verified the absence of Casp6 transcript and protein in brain and peripheral tissues. Our results further demonstrated that *Casp6*-/- mice do not suffer from gross abnormalities or peripheral phenotypes that would interfere with neuroanatomical and behavioral analyses.

Interestingly, we show that *Casp6* -/- neurons are protected against both excitotoxicity, a process that has repeatedly been linked to HD, and axonal degeneration, which has previously been implicated in HD and AD. In addition, we detect region-specific and age-dependent neuroanatomical and behavioral changes in brain areas that are most affected in neurodegenerative diseases, such as the striatum in HD and the cortex in AD.

3.1 Generation of *Casp6 -/-* mice

The *Casp6 -/-* mouse was created by Xenogen using retroviral gene trap methods**.** BAC clones lacking exons 2 to 5 of the *Casp6* gene, which encode the catalytic domain of the Casp6 protein were introduced to embryonic stem cells by homologous recombination (Figure 3.1A and B). The homologous recombination strategy was validated by Southern analysis. Two PCR assays were designed for genotyping: the wild type (WT) assay that amplifies the WT allele from *Casp6* WT and heterozygous (*Casp6* +/-) mice; and the neo assay that amplifies the knocked out allele from *Casp6* +/- and *Casp6* -/- mice (Figure 3.1C).

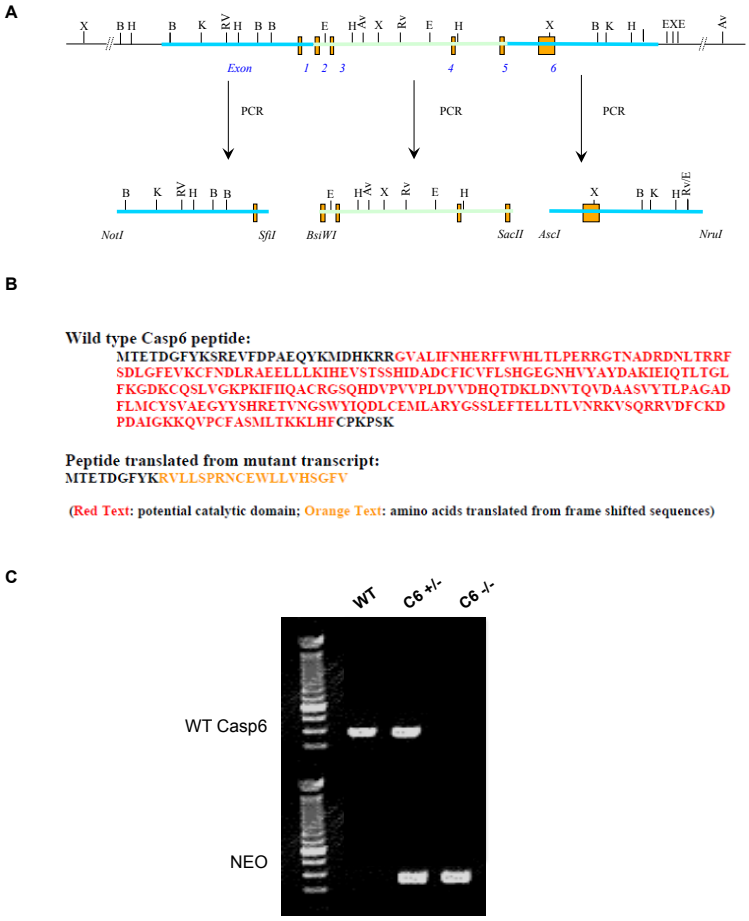

Figure 3.1 - *Casp6 -/-* construction. A) The catalytic domain of Casp6, coded by exon 2 to 5, was deleted from the wild type Casp6 peptide sequence to create the *Casp6* -/- mouse. B) The potential catalytic domain of Casp6 is shown in red and the amino acids translated from the mutant transcript in orange. C) WT Casp6 PCR assay shows presence of Casp6 in WT and *Casp6* +/- mice. In contrast, neo cassette primers show absence of Casp6 in *Casp6* +/- and *Casp6* -/- mice.

3.2 Verification of caspase-6 deficiency

3.2.1 Casp6 mRNA and protein expression

The *Casp6* -/- mice were examined by reverse transcriptase PCR (RT-PCR) and western analysis to confirm the absence of Casp6 expression in brain and peripheral tissues. Quantitative RT-PCR shows no Casp6 mRNA expression in *Casp6* -/- and reduced *Casp6* mRNA expression in *Casp6* +/- brain tissue (Figure 3.2A; ANOVA p=0.0001). Western blotting using a Casp6 antibody demonstrates absence of the Casp6 protein in *Casp6* -/- whole brain, cerebellum, hippocampus, striatum, cortex kidney, liver spleen and fetal tissue (Figure 3.2B). These findings confirm that the *Casp6* -/- mice constitutively lack Casp6.

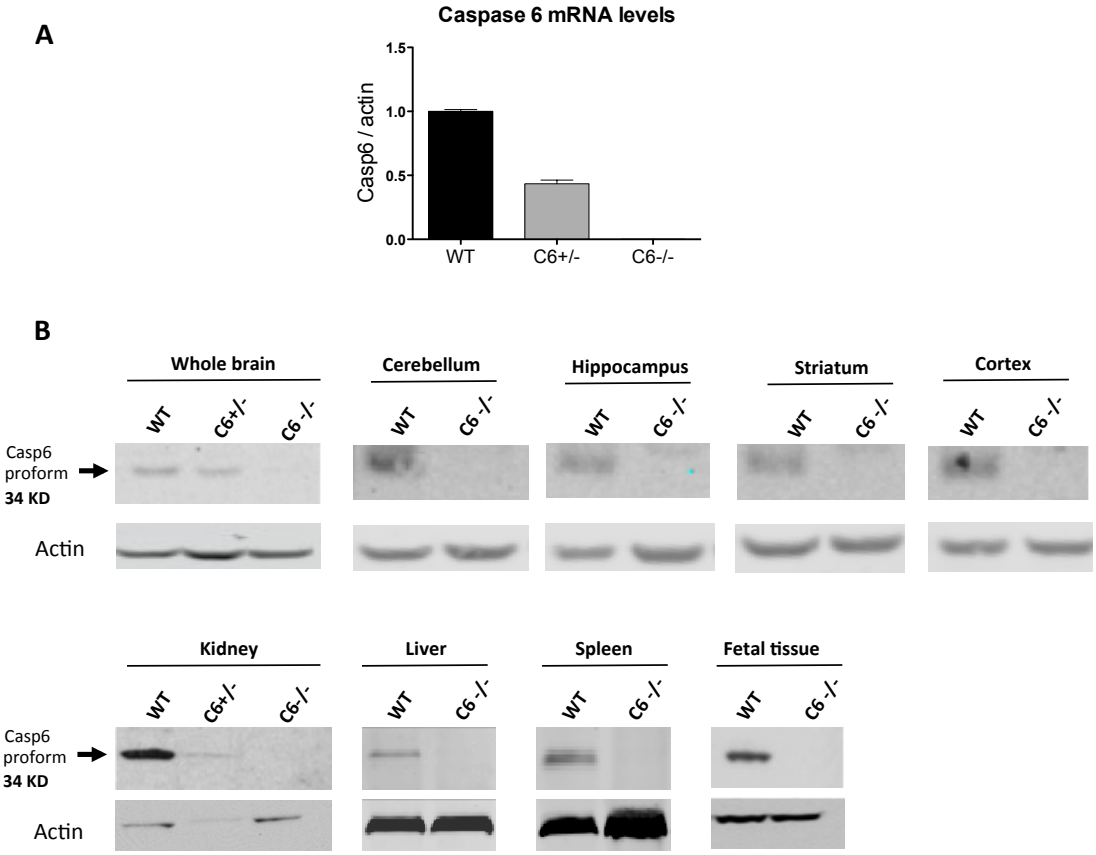

Figure 3.2 - No Casp6 expression is observed in *Casp6* -/- brain and peripheral tissues. A) Quantitative RT-PCR shows absence of Casp6 mRNA expression in *Casp6* -/- brain tissue and reduced expression in *Casp6* +/- brain tissue when compared to WT. B) Western blots using Casp6 antibody show absence of Casp6 protein in *Casp6* -/- whole brain, cerebellum, hippocampus, striatum, cortex, kidney, liver, spleen and fetal tissue.

3.2.2 Lamin cleavage

Lamin A has been shown to be a Casp6 specific substrate (Chinnaiyan and Dixit, 1996) and Casp6 cleavage is observed rapidly after treatment with staurosporine, a broad-spectrum kinase inhibitor (Warby et al., 2008). We used staurosporine induction of apoptosis to assess Casp6-mediated cleavage of lamin A in MEFs from *Casp6 -/-* and WT mice. *Casp6 -/-* fibroblasts show absence of Casp6-specific lamin cleavage 24 hours after staurosporine treatment. In contrast, the Casp6 cleaved-lamin A+C fragment is detected in WT MEFs 24 hours post-stress. The antibody that recognizes full length lamin A at 70 kDa can also detect the 60kDA lamin C protein, which shares sequence homology with lamin A (Fisher et al., 1986); the N-terminal fragments of both cleaved lamin A and C can be observed at 28kDA (Ehrnhoefer and Hayden, manuscript in preparation) (Figure 3.3).

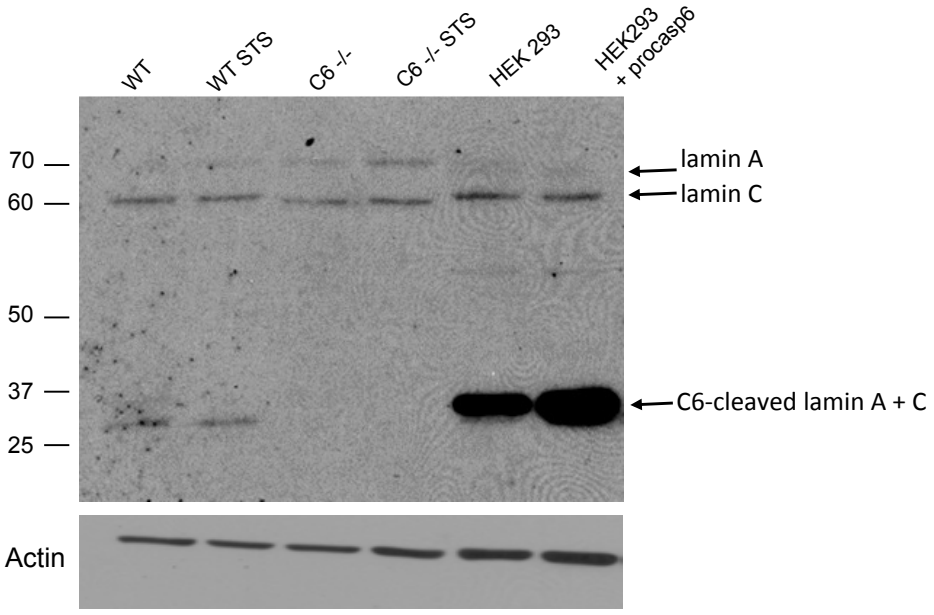

Figure 3.3 - Absence of lamin cleavage in Casp6 -/- fibroblasts. Casp6 -/- mouse embryonic fibroblasts show absence of Casp6-specific lamin cleavage after 24hr staurosporine treatment. In contrast, the casp6 cleaved-lamin A fragment is detected in WT MEFs 24hr post stress.

3.3 Peripheral phenotypes

3.3.1 Mendelian ratios and necropsy

The majority of the *Casp6* -/- mice characterization was performed on the FVB background. However, since the role of certain caspases has been shown to be strain dependent (Houde et al., 2004), we performed necropsy and examined Mendelian ratios on both B6 and FVB backgrounds to assess viability in both strains. Both B6 and FVB *Casp6* -/- mice are viable, breed normally and are born in Mendelian ratios (Table 3.1). Additionally, necropsy performed on *Casp6* -/- mice from both B6 and FVB backgrounds 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).

Table 3.1 - Casp6 -/- mice Mendelian rations on FVB and BL6 background

***Casp6 -/-* on FVB background**				
	WT	HET	KO	Total
Actual	164	348	146	658
Expected	164.5	329	164.5	658
P value = 0.45				

***Casp6 -/-* on B6 background**				
	WT	HET	KO	Total
Actual	55	101	37	193
Expected	48.25	96.5	48.25	193
P value = 0.38				

3.3.2 Body weight

After establishing that *Casp6* -/- mice are viable and do not show severe abnormalities we investigated body weight alterations that have been previously observed in *Casp2* -/- mice, who fail to gain fat mass (Carroll et al., manuscript in preparation). Longitudinal recording of body weight shows no differences in body weight between female *Casp6* -/- and WT mice from 2 to 12 months of age (two-way ANOVA genotype: p = 0.31, age: p=0.0001, interaction p=0.99 male, n= 8-12 WT, 9-10 C6-/-) (Figure 3.4a). In addition, male *Casp6* -/- mice show normal body weight from 4 to 12 months of age (two-way ANOVA genotype: p = 0.08, age: p=0.0001, interaction p=0.95 n= 4-7 WT, 10-13 C6-/-) (Figure 3.4b).

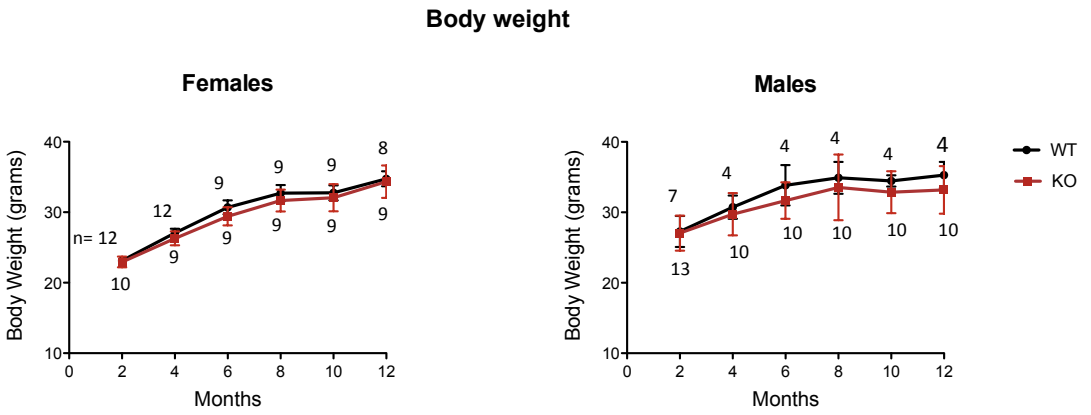

Figure 3.4 - No alterations in body weight in female and male *Casp6* -/- mice A) Female *Casp6* -/- mice show normal body weight compared to WT mice from 2 to 12 months of age (two-way ANOVA genotype: p = 0.31, age: p=0.0001, interaction p=0.99 male, n= 8-12 WT, 9-10 C6-/-). B) Male *Casp6* -/- mice show normal body weight from 4 to 12 months of age (two-way ANOVA genotype: p = 0.08, age: p=0.0001, interaction p=0.95 n= 4-7 WT, 10-13 C6-/-).

3.4 Central phenotypes

3.4.1 Susceptibility to excitotoxic stress

It has been previously demonstrated that Casp6 inhibitors and/or dominant-negative inhibition of Casp6 provides protection against excitotoxic stress (Graham et al., 2010) (Hermel et al., 2004). Therefore, we hypothesized that medium spiny neurons (MSNs) derived from *Casp6*-deficient mice would be protected against NMDA-mediated excitotoxicity. *Casp6* -/- MSNs demonstrate a significant decrease in LDH levels (one-way ANOVA p=0.01, t-test WT vs. C6-/- p=0.003 n=10 cultures) (Fig 3.5A), a significant increase in levels of ATP (one-way ANOVA p=0.03, t-test WT vs C6-/- p=0.01 n=10 cultures) (Fig 3.5B) and reduced TUNEL-positive cells (one-way ANOVA p=0.02, t-test WT vs. C6-/- p=0.03 n=9-12 cultures) (Fig 3.5C) compared to WT MSNs post-NMDA treatment, indicative of protection against NMDA-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 NMDA-mediated excitotoxicity (post- hoc linear trend, LDH: p=0.005; ATP: p=0.01; TUNEL: p=0.02).

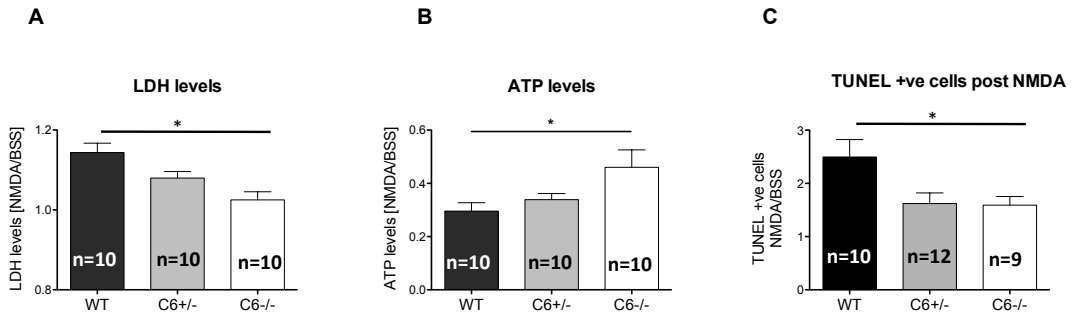

Figure 3.5 - **Medium spiny neurons from *Casp6* +/- and *Casp6* -/- mice show protection against NMDA-mediated excitotoxicity in a Casp6 dose dependent manner**. Assessment of susceptibility to excitotoxic stress demonstrates A) a significant decrease in LDH levels (one-way ANOVA p=0.01, t-test WT vs. C6-/- p=0.003 n=10 cultures), B) a significant increase in levels of ATP (one-way ANOVA p=0.03, t-test WT vs. C6-/- p=0.01 n=10 cultures) and C) a significant decrease in number of TUNEL positive cells (one-way ANOVA p=0.02, t-test WT vs C6-/- p=0.03 n=9-12 cultures) 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).

3.4.2 Axonal degeneration

Axonal degeneration is a key mechanism involved in developmental axonal pruning (Raff et al., 2002) (Buss et al., 2006). However, it also occurs in the mature nervous system as a consequence of neuronal damage and is observed in brains of individuals with neurodegenerative disorders (Nikolaev et al., 2009) (Singh et al., 2008) (Luo and O'Leary, 2005). It has been recently established that axonal degeneration occurs through activation of Casp6 (Nikolaev et al., 2009) (Park et al., 2010).

In order to further validate the role of Casp6 in axonal degeneration we investigated if *Casp6* -/- sympathetic neurons are protected from axonal degeneration after nerve growth factor (NGF) withdrawal. Dissociated cervical ganglion neurons of P0-P1 WT and *Casp6* -/- mice were plated in the somatic compartment of microfluidic chambers and the axonal compartment was NGF deprived. Neurons were probed by immunofluorescence with an alpha-tubulin antibody. Our results show that axons of WT sympathetic neurons degenerate after NGF deprivation (t-test $p < 0.0001$) (Fig 3.6A and C), but NGF withdrawal did not induce axonal degeneration in *Casp6* -/- sympathetic neurons (t-test p=0. 24) (n=3 chambers/condition/genotype) (Fig 3.6B and C).

Furthermore, caspase-9 (Casp9) has recently been shown to act as an upstream mediator of Casp6 (Akapan et al., manuscript in preparation). Therefore we used a Casp9-specific inhibitor, Z-LEHD-FMK, to determine if inhibiting Casp9 activity would prevent Casp6 activation and protect WT sympathetic neurons from axonal degeneration. Our data show that Casp9 inhibition prevents axonal degeneration in WT mice post-NGF removal (Appendix A. Figure A.1). These findings suggest that Casp6-mediated axonal degeneration is dependent on Casp9 activation.

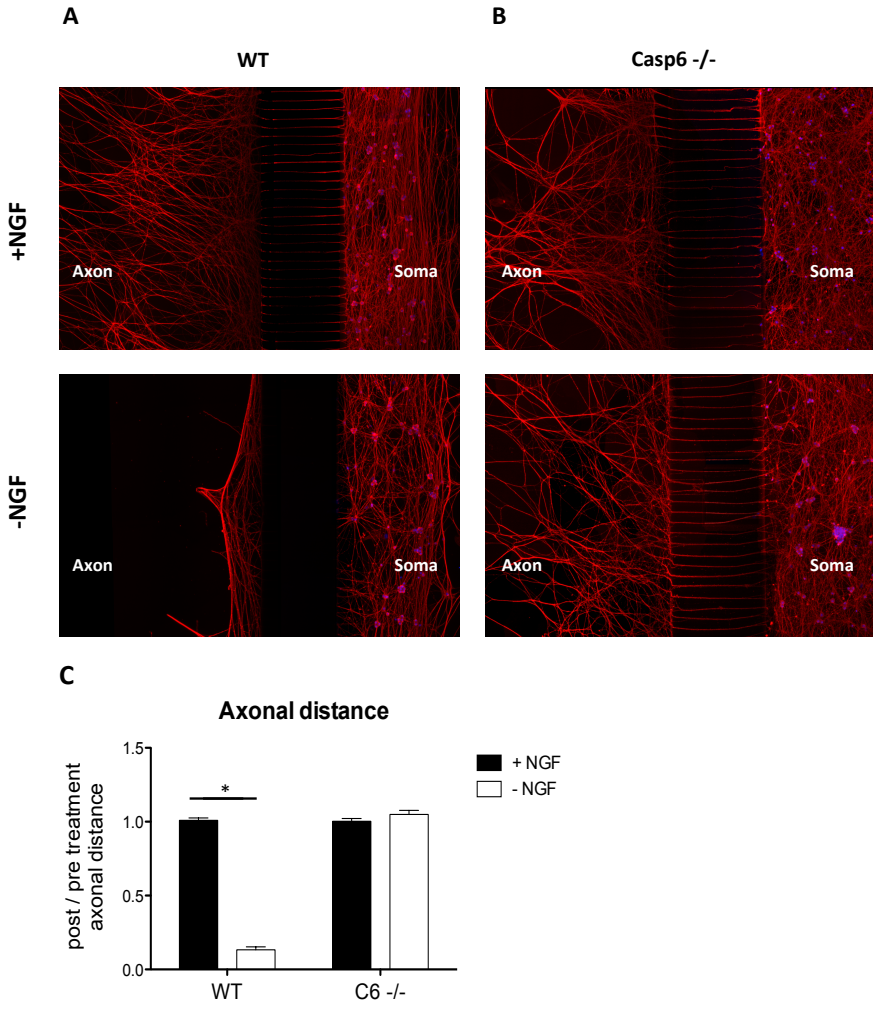

Figure 3.6 – *Casp6* -/- sympathetic neurons show protection against axonal degeneration. A) WT axons from sympathetic cervical ganglion neurons degenerate after NGF deprivation; in contrast, B) NGF deprivation does not induce degeneration in axons from *Casp6* -/- mice. C) Quantification of axonal distance reveals a significant difference between control and NGF deprived axons from WT mice (t-test $p < 0.0001$) and no difference in the axons from *Casp6* -/- mice regardless of treatment conditions (t- test $p=0.24$) (n= 3 chambers/condition/genotype).

3.4.3 Neuropathological phenotypes

Deletions of specific caspases can result in robust brain malformations associated with supernumerary cells, multiple cerebral indentations and ectopic cell masses in the cortex (Kuida et al., 1996) (Varfolomeev et al., 1998) (Kuida et al., 1998) (Hakem et al., 1998) (Houde et al., 2004).

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 male and female *Casp6* -/- mice at 3 and 8 months of age. *Casp6* -/- mice display normal brain architecture at 3 months of age (one-way ANOVA brain weight p=0.10, cerebellum weight p=0.50, cortical volume p=0.49, striatal volume p=0.66, striatal neuronal counts p= 0.23 n=20) (Appendix A, Fig. A.2). However, neuropathological analysis at 8 months reveals a significant increase in cortical (one-way ANOVA p=0.004; t-test WT vs. C6-/- p=0.009 n=6) and striatal (one-way ANOVA p=0.02; t-test WT vs. C6-/- p=0.02 n=6) volume and in striatal neuronal counts (one-way ANOVA p=0.03; t-test WT vs. C6-/- p=0.02 n=6) (Figure 3.7A). Furthermore, 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 were observed in brain (one-way ANOVA p=0.36; t-test WT vs. C6-/- p=0.10 n=20) and cerebellum (one-way ANOVA p=0.38; t-test WT vs. C6-/- p=0.50 n=20) weight in *Casp6* -/- mice compared to WT (Fig 3.7B).

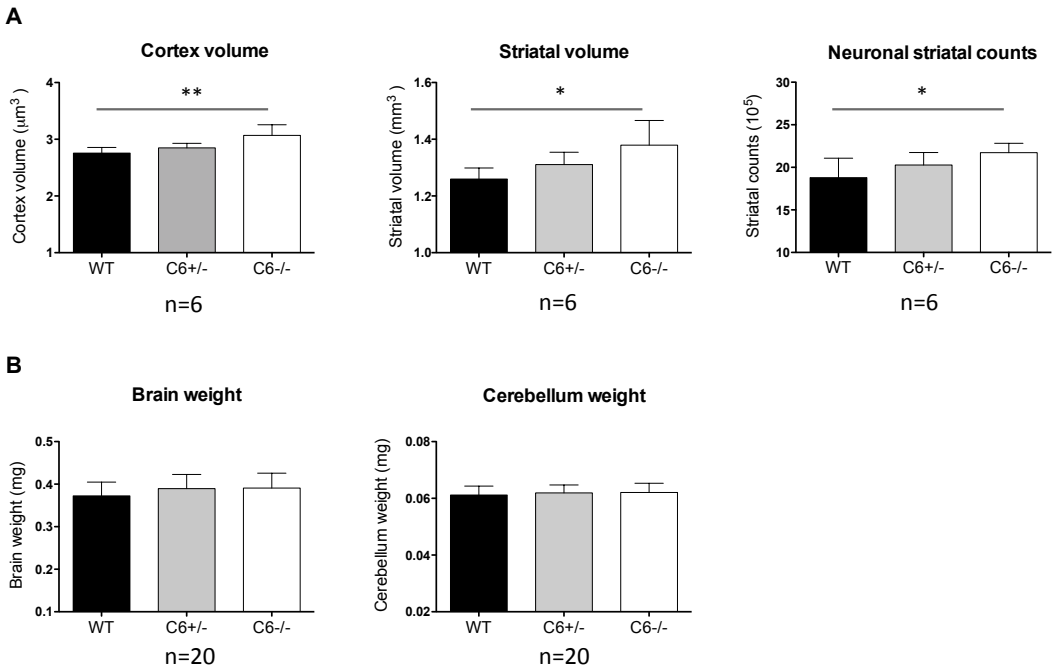

Figure 3.7 - Cortical and striatal volume is increased in *Casp6* -/- mice. Neuropathological analysis at 8 months reveals A) a significant increase in cortical (one-way ANOVA p=0.004; t-test WT vs. C6-/- p=0.009 n=6) and striatal (one-way ANOVA p=0.02; t-test WT vs. C6-/- p=0.02 n=6) volume and in striatal neuronal counts (one-way ANOVA p=0.03; t-test WT vs. C6-/- p=0.02 n=6). 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 (one-way ANOVA p=0.36; t-test WT vs. C6-/- p=0.10 n=20) and cerebellum (one-way ANOVA p=0.38; t-test WT vs. C6-/- p=0.50 n=20) weight in *Casp6* -/- mice compared to WT.

3.4.4 Behavioral phenotypes

3.4.4.1 Novel object recognition

In order to assess novel object preference the mice were place in an open box with two different novel objects in the upper corners and frequency and duration of investigations were scored. The experiment was repeated 5 minutes later; however, the top right corner object was replaced with a new unfamiliar object and preference for the new object was measured by calculating the percentage of frequency and duration of investigations to the new object.

No gender difference were observed during the novel object recognition task, both male and female *Casp6 -/-* mice display a deficit in the novel object preference task when tested at 12 months (two-way ANOVA genotype: p = 0.94, trial: p=0.02, interaction: p=0.14; t-test WT trial 1 vs. trial 2 p = 0.03 n= 11 WT (8 female, 3 male), n= 18 C6-/- (9 female, 9 male)) (Figure 3.8). WT animals spend more time exploring the object with which they have no prior experience, indicating that they are able to distinguish the known object from the novel one. However, the Casp6 deficient mice spend significantly less time exploring the novel object, indicative of a learning deficit at this time-point.

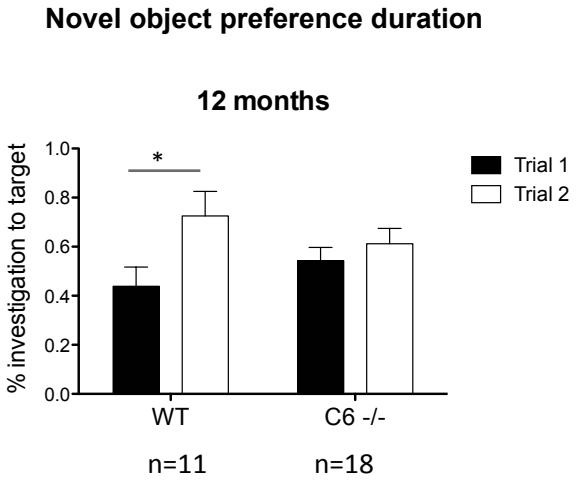

Figure 3.8 - *Casp6* -/- mice display decreased novel object preference. *Casp6* -/- mice demonstrate a deficit in a novel object preference task at 12 months of age (two-way ANOVA genotype: p = 0.94, trial: p=0.02, interaction: p=0.14; t-test WT trial 1 vs. trial 2 p = 0.03 n= 11 WT (8 female, 3 male), n= 18 C6-/- (9 female, 9 male)).

3.4.4.2 Total activity

Mice were placed in an open box and total activity was assessed using an activity monitor. No gender differences were observed during the total activity test, from 4 to 10 months of age both male and female *Casp6* -/- mice are hypoactive compared to WT mice during the 30-minute open field trial that measures locomotor activity (two-way ANOVA genotype: p = 0.0001, age: p=0.90, interaction p=0.68 n= 8-12 WT female, 4-7 WT male, 9-10 C6-/- female, 10-12 C6-/- male) (Fig 3.9).

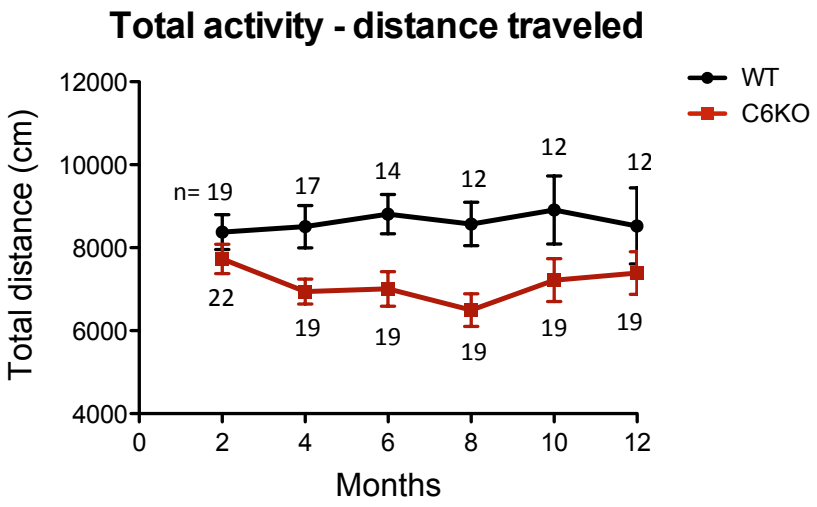

Figure 3.9 - *Casp6* -/- mice demonstrate a hypokinetic phenotype. Male and female *Casp6* -/- mice are hypoactive commencing at 4 months of age compared to WT during a 30-minute open field trial (two-way ANOVA genotype: p = 0.0001, age: p=0.90, interaction p=0.68 n= 8-12 WT female, 4-7 WT male, 9-10 C6-/- female, 10-12 C6-/- male).

3.4.4.3 Accelerating rotarod

In contrast to the decreased novel object preference and the hypokinetic phenotype, male and female Casp6 deficient mice display motor coordination indistinguishable from WT mice during the accelerating rotarod testing at all time-points assessed and no gender differences were observed (two-way ANOVA genotype: p = 0.25, age: p=0.001, interaction p=0.96 n= 8-13 WT female, 2-7 WT male, 9-10 C6-/- female, 9-11 C6-/- male) (Fig 3.10).

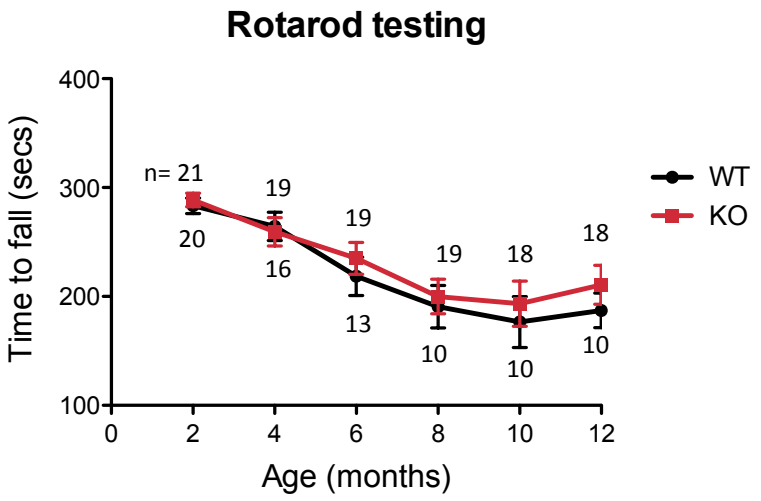

Figure 3.10 - *Casp6* -/- mice have normal motor coordination. Male and female *Casp6* -/- mice display motor coordination indistinguishable from WT mice during rotarod testing at all time-points assessed (two-way ANOVA genotype: p = 0.25, age: p=0.001, interaction p=0.96 n= 8-13 WT female, 2-7 WT male, 9-10 C6-/- female, 9-11 C6-/- male).

4 Discussion and conclusions

This study is the first to examine the neuropathological and behavioral effects of removing Casp6 in mice. The use of targeted caspase knockout mice has been instrumental for studying the involvement of caspases in apoptotic and non-apoptotic pathways, and provides an ideal tool in the context of human diseases. The ablation of proteins implicated in neurodegenerative diseases provides a unique *in vivo* system for understanding their involvement in these disorders. We generated and characterized a *Casp6* -/- mouse and verified the absence of both Casp6 transcript and protein in brain and peripheral tissues. Our results further demonstrated that *Casp6*-/- mice do not suffer from gross abnormalities that would interfere with neuropathological and behavioral analyses.

We began by examining the role of Casp6 during NMDA-mediated excitotoxicity. Glutamate excitotoxicity is driven by Ca^{2+} influx through NMDA receptors. This influx of Ca^{2+} leads to mitochondrial permeabilization, resulting in caspase activation and apoptosis (Fernandes et al., 2007) (Tang et al., 2005). Over-activation of glutamate receptors is also involved in the early stages of HD (Levine et al., 1999) (Zeron et al., 2002) and several HD mouse models demonstrate enhanced susceptibility to glutamate and/or NMDAR mediated excitotoxicity (Graham et al., 2006b) (Graham et al., 2010) (Zeron et al., 2004). Since Casp6 activity has been shown to be increased in the brains of both early stage HD patients and in an HD mouse model, Casp6 activation may play an important role in NMDA-induced excitotoxicity. The direct link between Casp6 activity and enhanced NMDA-induced excitotoxicity in HD mouse models was further validated when MSNs expressing mutant Htt (mHtt) showed Casp6 activation post-NMDA stimulation (Graham et al., 2010). In contrast, mice expressing mhtt resistant to cleavage by Casp6 do not show enhanced Casp6 activation and demonstrate protection from excitotoxic stress (Graham et al., 2006a) (Graham et al., 2010) and alterations in extrasynaptic NMDA receptors *in*

vivo (Milnerwood et al., 2010). Furthermore, it has also been shown that Casp6 inhibitors and/or dominant-negative inhibition of Casp6 provide protection against excitotoxicity (Hermel et al., 2004) (Graham et al., 2010). Our findings show that MSNs from *Casp6 +/-* and *Casp6 -/-* mice show protection against NMDA-mediated excitotoxicity in a Casp6 dose dependent manner. These findings provide further validation for a critical role for Casp6 in NMDA-mediated excitotoxic stress.

Axonal degeneration is a key mechanism involved in developmental axonal pruning (Raff et al., 2002) (Buss et al., 2006). However, it also occurs in the mature nervous system as a consequence of neuronal damage and during neurodegenerative disorders (Nikolaev et al., 2009) (Singh et al., 2008) (Luo and O'Leary, 2005). In AD mouse models, age-dependent axonal degeneration is observed in the cortex, hippocampus, midbrain and hindbrain and likely contributes to the motor and cognitive behavioral deficits observed in these mice (Dawson et al., 2010) (Jawhar et al., 2010). It has been recently demonstrated that axonal degeneration occurs through activation of Casp6 (Nikolaev et al., 2009) (Park et al., 2010) (Sivananthan et al., 2010). The N-terminal fragment of cleaved APP binds to DR6, activating Casp6 and resulting in axonal degeneration (Nikolaev et al., 2009). APP-induced axonal degeneration was rescued by Casp6 inhibitors, demonstrating the role of Casp6 in this process. Casp6 activation leading to neurite degeneration can also occur in a process independent of amyloid ß-peptide (Aß) production from APP cleavage. APP mutants identified in familial AD patients that cannot generate Aß still activate Casp6 and induced neurite beading and Casp6-dependent cell death (Sivananthan et al., 2010). Furthermore, it has been established that Casp6 is involved in focal non-pathogenic axonal pruning along myelin tracks, such as the corpus callosum, in mice (Park et al., 2010). Interestingly, pre-manifest HD subjects and early symptomatic HD patients display altered white matter microstructure in the corpus callosum (Rosas et al., 2010) and axonal white matter is reduced in AD patients at the earliest stages of disease (Salat et al., 2009). Our findings show that *Casp6*-/- sympathetic neurons are protected

against NGF deprivation–mediated axonal degeneration, further confirming the crucial role of Casp6 in this process.

Deletions of specific caspases can result in robust brain malformations associated with supernumerary cells, multiple cerebral indentations and ectopic cell masses in the cortex (Varfolomeev et al., 1998) (Kuida et al., 1998) (Hakem et al., 1998) (Houde et al., 2004). Interestingly, we identified age-dependent and region-specific neuroanatomical and behavioral changes in the *Casp6*-/- mice. Neuroanatomical analysis at 8, but not at 3 months of age reveals a significant increase in cortical and striatal volume and striatal neuronal counts in Casp6-deficient mice compared to WT mice. These findings suggest that Casp6 deficiency has a more pronounced effect in brain regions that are involved in neurodegenerative diseases, such as the striatum in HD, where cleavage and nuclear localization of mutant huntingtin precedes striatal degeneration and cognitive and motor deficits, and the cortex in AD, where accumulation of amyloid plaques and neurofibrillary tangles are observed congruently with progressive loss of synapses and neuronal atrophy. In HD mouse models, the expression of mutant full-length human huntingtin results in a slowly progressive phenotype (Slow et al., 2003) (Van Raamsdonk et al., 2005) and similarly, in a mouse model of AD with APP over-expression functional deficits become predominant with advancing age (Hsia et al., 1999). Therefore, the age-dependent and region-specific neuroanatomical effects observed in *Casp6*-deficient mice may help to understand why the striatum and cortex are more affected in neurodegenerative diseases such as HD and AD. Collectively, these findings have significant implications for the role of Casp6 in these neurodegenerative diseases.

Region-specific alterations have been previously observed in other caspase deficient mice. The Casp3 and Casp9 null mice showed brain malformations caused by ectopic cell masses that were predominantly present in the cerebral cortex and to a lesser extent in the cerebellum and retinal neuroepithelium (Kuida et al., 1996). In addition, Casp2 deficient mice displayed

accelerated cell death of facial motor neurons during development, but no overt phenotypes in other regions such as the vestibular, geniculate, nodose and superior cervical ganglia (Bergeron et al., 1998). These findings indicate that different caspases act in a tissue specific fashion.

It has been shown that each individual caspase has a unique pattern of expression in different brain areas. Casp6 expression has been demonstrated in the mouse striatum, cortex, hippocampus and cerebellum (Hermel et al., 2004) (Graham et al., 2010) (Henshall et al., 2002) (Narkilahti and Pitkänen, 2005) (Albrecht et al., 2007) (Albrecht et al., 2009) (Allen brain atlas). Significantly increased levels of Casp6 protein expression have been identified in the mouse cerebellum compared to other brain regions such as the cortex, striatum and hippocampus (Uribe and Hayden, unpublished data). In addition, region-specific expression of different caspases has been observed within the striatum and cortex of mice (Hermel et al., 2004) (Graham et al., 2010). Immunostaining in the mouse striatum revealed expression of caspases-6, -7 and -9 with higher Casp6 and Casp7 expression in MSNs, and higher Casp9 expression in cholinergic neurons. Furthermore, labeling with an active Casp6 antibody revealed that most cells immunoreactive to Casp6 were neurons, in contrast to Casp3 that is mostly expressed in glia (Hermel et al., 2004). The expression of Casp7 is also higher in the striatum compared to other brain regions, such as the cortex. Aside from expression in cholinergic neurons, Casp9 is also expressed in pyramidal cells located in cortical layer V, whereas Casp8 was ubiquitously expressed in different brain regions. Considering that Casp6 interacts with these different caspases, their differential patterns of expression could in part explain the increased cortical and striatal volume, as well as the presence of additional neurons in the striatum of the *Casp6*-/- mice.

Caspase activation can also be regulated by neurotrophic factors (Nguyen et al., 2009). For example, deprivation of brain derived neurotrophic factor (BDNF) has been shown to trigger cell death pathways by activating caspases (Yu et al., 2008). BDNF has been implicated in neuronal maturation, axonal and

dendritic branching and regeneration, and in synaptic transmission and plasticity (Abidin et al., 2008) (Tanaka et al., 2008) (Poo, 2001). It has also been shown to play a key role in cognition and behavior by modulating learning, anxiety and depression-like behaviors (Gorski et al., 2003) (Bekinschtein et al., 2008a) (Bekinschtein et al., 2008b). Interestingly, similar to *Casp6*-/- mice, BDNF-/- mice also show structure-specific behavioral and neuropathological alterations. They display decreased dendritic complexity and spine density in the cortex and to an even greater extent in the striatum, where 90% of the affected cells are GABAergic MSNs. In contrast, minimal changes are observed in the dendrites of CA1 pyramidal neurons from these mice, indicating that BDNF is necessary for the postnatal growth of striatal neurons, but is not as essential for the development of the hippocampal neurons (Rauskolb et al., 2010). Behavioral changes associated with BDNF are also region dependent. Infusion of BDNF in the hippocampal dentate gyrus reduces depression-like behaviors (Shirayama et al., 2002), while infusion into the nucleus accumbens increases depression (Eisch et al., 2003) and social aversion (Berton et al., 2006). These findings may suggest that the diverse responses to BDNF and/or Casp6 by different brain structures maybe mediated by region-intrinsic programs (Rauskolb et al., 2010). Therefore, a potential mechanism leading to region-specific neurodegeneration in HD and AD could be caused by the structures' specific pattern of caspase expression and/or activation by trophic factors.

We further examined *Casp6*-/- mice using behavioral tests to elucidate whether the neuroanatomical alterations observed could result in cognitive changes. We show that 12 month *Casp6*-/- mice demonstrate a deficit in the novel object recognition task.

It has been shown that the hippocampus is implicated in object recognition memory (Eichenbaum, 1999); therefore, several studies have attempted to understand the role of the hippocampus in object learning tasks and have demonstrated that lesions to the hippocampus impair object recognition performance in rats when long retention intervals are involved (Clark et al., 2000)

(Gaskin et al., 2003) (Vnek and Rothblat, 1996), suggesting that the hippocampus is implicated in object recognition memory in a delay-dependent manner. A study where acute lidocaine administration was used to temporarily inactivate the hippocampus prior to training in the spontaneous object recognition task revealed that lidocaine-treated mice displayed impaired object recognition memory after 24 h, but not after a 5 min retention interval (Hammond et al., 2004). Contrary to these data, the Casp6 -/- deficient mice display impaired object learning during a novel object recognition task that took place after a 5 min retention interval, suggesting that other brain structures besides the hippocampus might be involved in the learning deficit observed in these mice.

The medial temporal lobe has also been implicated in object recognition memory in primates and humans (Squire and Zola, 1996) and studies with primates and rodents have demonstrated that the parahippocampal regions of the temporal lobe are involved in visual object recognition memory (Gilbert and Kesner, 2003) (Murray et al., 2000). Excitotoxic lesions of the perirhinal cortex have been shown to cause deficits in object recognition tasks (Aggleton et al., 1997) (Liu and Bilkey, 2001). Furthermore, studies of neuronal activation in rats and monkeys suggest that cortical neurons, but not hippocampal neurons are involved in object recognition tasks (Brown and Aggleton, 2001) (Xiang and Brown, 1999). Altogether, these data suggest that the increase in cortical volume observed in the Casp6 deficient mice could compromise the normal function of the cortex, and that these alterations could possibly translate into the learning impairment observed in the *Casp6 -/-* mice during the novel object recognition task.

Additionally, *Casp6*-/- mice are also hypoactive from 4-10 months during a 30-minute open field test that measures locomotor activity. The striatum is known to be involved in planning and executing pathways of movement. 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 (Bazzu et al., 2010). Given that the striatum has been

shown to be implicated in open field performance, the increased striatal volume observed at 8 months of age could alter normal striatal function and could possibly account for the hypoactive phenotype observed in these mice at later ages.

Our data show that the behavioral and neuroanatomical alterations observed in the *Casp6*-/- mice are age dependent. We previously performed an experiment to examine the timeline of Casp6 activation in WT murine brains and detected active Casp6 in MSNs of the striatum starting at 9 months, with increased activation at 18 months of age. In addition, active Casp6 was not observed in WT murine cortex at 3 months of age (Graham et al., 2010). Microarray studies on the cortex and hypothalamus of BALB/c mice have shown that Casp6 is significantly up regulated in both brain structures at 22, but not at 2 months of age (Jiang et al., 2001). This is consistent with the age-related effects observed in humans, where only the inactive p34 proform of Casp6 is observed in the striatum and cortex of human controls under 50 years of age. However, after 50 years, a decrease in the inactive proform is accompanied by an increase in the active p20 fragment of Casp6 (Graham et al., 2010). 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.

Casp6 is known as an effector caspase. However, it has been shown that it can also activate effector caspase-3 (Liu et al., 1996) (Allsopp et al., 2000). Future studies to determine if there is altered expression of different caspases in the Casp6 deficient mice would serve to gain a better understanding of the caspase activation pathways and the role of Casp6 in the apoptotic cascade and neurodegenerative diseases.

Overall our results have implications for the development of Casp6 inhibitors for neurodegenerative diseases. We show that *Casp6*-/- neurons are

protected against both NMDA-mediated excitotoxicity, a process that has consistently been linked to neurodegenerative diseases, and axonal degeneration, which is also implicated in the pathogenesis of HD and AD. Our findings provide further support that inhibition of Casp6 could be a possible therapeutic target for neurodegenerative diseases, and efforts are already underway to identify Casp6 inhibitors (Leyva et al., 2010). Interestingly, our results show that MSNs from *Casp6 +/-* and *Casp6 -/-* mice show protection against NMDA-mediated excitotoxicity in a Casp6 dose dependent manner. Even though the present study did not analyze the effects of trophic factor deprivation in *Casp6 +/-* mice, future studies analyzing the effects of NGF deprivation in these mice would allow assessment as to whether partial ablation of Casp6 would be protective against NGF-induced axonal degeneration in a dose dependent manner.

Interestingly, we detect region-specific and age-dependent neuropathological and behavioral changes in brain areas that are most affected in neurodegenerative diseases, such as the striatum in HD and the cortex in AD. Results from this study provide further insights into how Casp6 may contribute to the region- and age-specificity of various neurodegenerative diseases. The *Casp6*-/- mice can serve as a pre-clinical tool for further validation of the role of Casp6 in these diseases. Future studies could involve crossing the Casp6 deficient mice to well established mouse models of neurodegenerative diseases, such as the YAC128 mouse model of HD and the PDAPP mouse model of AD to determine if there is rescue of disease phenotypes.

Our data suggest that the use of Casp6 inhibitors during embryogenesis or development may result in detrimental neuropathology and abnormal behaviors observed later in life. However, as HD and AD are late-onset disorders and treatment would occur in adult life, Casp6 inhibitors may offer protection against excitotoxic stress and axonal degeneration in these neurological diseases without the abnormalities resulting from Casp6 deficiency during development. Future studies with a conditional Casp6-deficient mouse would

allow examination of morphological and behavioral impacts of Casp6 deletion not only in specific brain structures, but also at specific time-points.

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Appendix A – Supplemental figures

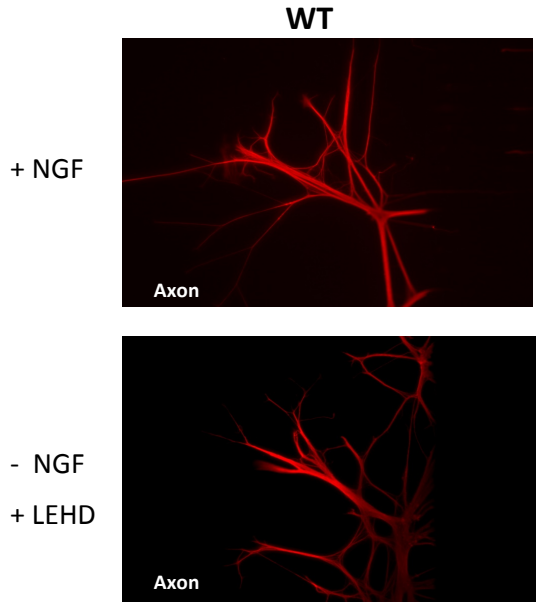

Figure A.1 - Casp9 inhibition prevents axonal degeneration. WT axons from sympathetic cervical ganglion neurons treated with Z-LEHD-FMK, a Casp9 inhibitor, are protected from axonal degeneration post NGF removal.

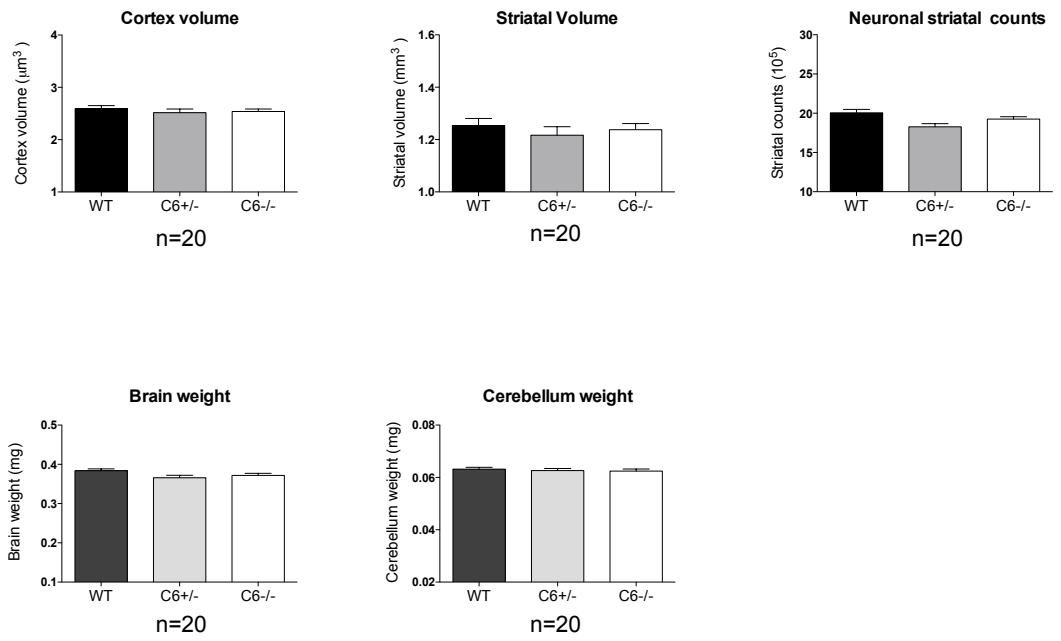

Figure A.2 – Male and female *Casp6* -/- mice display normal brain architecture at 3 months of age. Neuropathological analysis at 3 months reveals A) no significant differences in cortical and striatal volume and in striatal neuronal counts (one-way ANOVA cortical volume p=0.49, striatal volume p=0.66, striatal neuronal counts p= 0.23 n=20) and B) normal brain and cerebellum weight in the *Casp6* -/- mice compared to WT at 3 months of age (one-way ANOVA brain weight p=0.10, cerebellum weight p=0.50 n=20).