NOVEL, PEPTIDE-MEDIATED NEUROPROTECTIVE STRATEGIES FOR HUNTINGTON'S DISEASE

by

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Abstract

Huntington's disease (HD) is an inherited neurodegenerative disease with progressive striatal loss. No treatments exist, though availability of predictive testing offers possibility for early intervention. Peptides represent an exciting way to interact with molecular signaling. My thesis investigates three potential early targets for preventative HD therapies: NMDAR-mediated PTEN nuclear translocation, caspase-6 activation, and peptide-mediated mutant huntingtin (mHTT) knockdown.

Excitotoxicity via N-methyl-D-aspartate receptor (NMDAR) over-activation has a role in HD pathogenesis. We recently demonstrated that nuclear translocation of phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a critical step in NMDAR-mediated excitotoxicity, and blocking PTEN nuclear translocation with peptide Tat-K13 prevents excitotoxic neuronal death. Given the role of NMDAR-mediated excitoxicity in HD, PTEN nuclear translocation may have a role in excitotoxic neuron death in HD. Here, PTEN nuclear translocation was associated with NMDAR-mediated death in cultured HD neurons. I also detected small increases in nuclear PTEN in HD transgenic mouse brains vs control. Interestingly, Tat-K13 effectively blocked PTEN translocation and prevented excitotoxicity in cortex/hippocampus, but not striatum, *in vitro* and *in vivo,* suggesting differential mechanisms of PTEN nuclear translocation.

Caspase activation downstream of NMDARs may be critically involved in excitotoxicity. Caspase-6 (casp6) particularly, has roles in pathogenesis of HD and other conditions. Using NMDA-induced excitotoxicity in cultured neurons, I demonstrated early increase in caspase profiles via mRNA, protein and activity. Casp6 is elevated and activated first, followed by caspase-8 and caspase-3. Casp6 substrate huntingtin, and novel casp6 substrates STK3 and DAXX, are cleaved in similar temporal patterns post-

NMDA, pointing to casp6 is an initiator caspase in NMDA-mediated apoptotic cascades and a potential therapeutic target.

Many studies suggest that reducing mHTT protein may be an effective HD therapy. Our lab recently developed a technique for targeted protein degradation using peptides that signal proteins for lysosome- or proteasome-mediated degradation. Utilizing mHTT-binding domains paired with degradation sequences, I designed and tested mHTT-degradation peptides. In cultured neurons from HD transgenic mice, mHTT-degradation peptides led to robust mHTT knockdown, at least in part due to degradation. mHTT degradation peptides were ineffective *in vivo*, but pointed to potential for development of peptide-mediated mHTT-lowering therapies.

Preface

All work in this thesis was jointly conceptualized by myself and Dr. Yu Tian Wang at the Centre for Brain Health University of British Columbia. Studies on Caspase activation after NMDAR excitotoxicity in chapter 3 were conceptualized by Dr. Rona Graham, from Sherbrooke University, along with Dr. Yu Tian Wang and me.

Part of Chapter 1 is adapted from an invited literature review. Girling, K., Wang, YT. (2016). Neuroprotective strategies for NMDAR-mediated excitotoxicity in Huntington's Disease, *Frontiers in Biology (In Press)*. All review and writing was done by Kimberly Girling, with edits from Dr. Yu Tian Wang.

A version of Chapter 2 has been submitted for publication and is in review (Girling KD, Demers MJ, Laine J, Zhang S, Wang YT & Graham RK. (2016). Activation of caspase-6 and cleavage of caspase-6 substrates is an early event in NMDA receptor-mediated excitotoxicity). This work was a collaboration with Dr. R. K. Graham at Sherbrooke University in Montreal, and research was co-collected with J. Laine, M. J. Demers, S. Zhang and R. Graham. In this work, I wrote and revised the manuscript, prepared, treated and collected cultured neurons and edited figures. MJD performed the RNA, protein and activity assays. JL provided support for quantification of data. SZ prepared, treated and collected first set of cell cultures. YTW advised conceptualization of study, supervised students. RKG conceptualized the study, performed data analysis, made figures and edited manuscript.

All other work in the thesis is unpublished. Student volunteer Scott Shyu (Dr. Yu Tian Wang Lab) conducted part of the nuclear fractionations and western blots in in Chapter 2. Lily Zhang (Dr. Lynn Raymond lab) Rujun Kang (Dr. Lynn Raymond lab) and Yuping Li (Dr. Yu Tian Wang lab) assisted with cell culture preparation. Lixia Wang, Lidong Liu and Loren Oshipok synthesized peptides (Dr. Yu Tian Wang lab). Peter Axerio-Ciles (Dr. Yu Tian Wang lab) helped design peptides. Ge Lu (Dr. Blair Leavitt lab) conducted Quinolinic Acid experiments, including injection, FluoroJade staining and analysis. All other experiments were conducted, analyzed and written up by me.

All experiments were approved by the UBC Animal Care Committee and the Canadian Council on Animal Care. The protocol numbers are as follows:

- Mouse primary culture: A13-0330 (Role of NMDA receptors, synaptic dysfunction in HD)
- Experiments for adult mice: A15-0069 (HD Physiology and Biochemistry)
- Rat primary culture: A13-0139 (Slice and Primary Culture Protocol)
- Rat kainic Acid injections: A16-0014 (Excitotoxicity Protocol 2016)

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List of Abbreviations

ASO – Antisense Oligonucleotide

ASK1 – Apoptosis-signal regulating kinase 1

BAC – Bacterial Artificial Chromosome

BDNF – Brain Derived Neurotrophin Factor

CMA – Chaperone Mediated Autophagy

DAPI - 4′,6-Diamidino-2-phenylindole dihydrochloride

DARPP-32 - Dopamine- and cAMP-regulated neuronal phosphoprotein 32

DAXX - Death domain associated protein

FAK – Focal Adhesion Kinase

FOXO – Forkhead Box O

HAP1 – Huntingtin Associated Protein 1

HIP14 – Huntingtin Interacting Protein 14

HTT - Huntingtin

HD - Huntington's Disease

KA – Kainic Acid

MSN - Medium Spiny Neuron (see also striatum projection neuron)

mHTT - Mutant Huntingtin

MMAC1 – Mutated in Multiple Advanced Cancers 1 (see also PTEN)

mTOR – Mammalian target of rapamycin

NEDD4-1 - Neural precursor cell expressed, developmentally downregulated-4-1

NMDA - N-methyl D-Aspartate

NMDAR - N-methyl D-Aspartate Receptor

NRSF - Neuronal restrictive silencing factor (Also see REST)

PDK1 – Pyruvade dehydrogenase kinase 1

PFA - Paraformaldehyde

PI(4,5)P_2 - Phosphatidylinositol-4,5-biphosphate

PI(3,4,5)P_3 – Phosphatidylinositol-4,5-triphosphate

PolyQ - Polyglutamine

PSD-95 – Post Synaptic Density 95

PTEN – Phosphatase and Tensin homolog deleted on chromosome ten

PI3K - Phosphoinositide-3-kinase

QA – Quinolinic Acid

RISC – RNA induced silencing complex

REST - RE1-silencing transcription factor (also see NRSF)

RNAi – RNA interference

siRNA – Small interfering RNA

SPN - Striatum Projection Neuron (see also medium spiny neuron)

STK3 - Serine/threonine kinase 3

UHDRS - Unified Huntington's Disease Rating Scale

UPS – Ubiquitin Proteasome System

WT - Wild Type

YAC - Yeast Artificial Chromosome (18, 72, 128 corresponds to number of CAG repeats)

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I wish to acknowledge the amazing support of my incredible lab mates and supervisors in Dr. Yu Tian Wang's lab. Throughout the years of my PhD thesis work, Yu Tian and his team provided an exceptional, stimulating and supportive space to learn, grow and expand as a scientist. By fostering creativity, collaboration, communication, independence and good scientific techniques, I was able to build myself into an effective scientist, and expand on the tools I will need for my future career. Thank you to Yu Tian, and my colleagues, for all your guidance, and for the honor of working with you all. Thank you especially to Shelly Fan, Wu Yang Jin, Jie Lu, Lidong Liu, Peter Axerio-Ciles, Taesup Cho, Yuping Lu, Agnes Wang and Rebecca Wiens for bouncing off ideas, helping me learn techniques, sharing experiments, and helping keep me on track. I could not have asked for a better lab.

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Dedication

This work is dedicated to my family. Your love and support over all these years has made my dreams possible. Thank you and I love you.

Chapter 1: Introduction

1.1 Huntington's disease

1.1.1 History and prevalence of Huntington's disease

Huntington's disease (HD) is an inherited, late onset neurodegenerative disease of cognitive, motor and behavioural dysfunction of which there are currently no effective treatments (Albin & Tagle 1995; Tan et al. 1976; Wilson et al. 1987). HD is relatively rare, and the incidence varies across the world, with a higher prevalence in the Western Hemisphere (Ghosh & Tabrizi 2015). For a long time it was believed that HD affected approximately 5-10 in 100,000 people in Western populations(Harper & Bates 2002) however, more updated data suggests the Western prevalence is closer to 12.3 in 100,000(Evans et al. 2013; Rawlins 2010; Fisher & Hayden 2014). HD affects approximately 0.5 in 100,000 in those of Asian descent (Walker 2007; Pringsheim et al. 2012). HD is believed to have spread globally during East-West migration into North America (Ghosh & Tabrizi 2015) with small pockets of the world demonstrating unusually high prevalence. In one small community in Venezuela, HD prevalence is as high as 700 in 100,000 (Gusella et al. 1983; HD collaborative & research group 1993). It was in this community where the causative gene mutation for HD was initially discovered.

1.1.2 Etiology and genetics of HD

HD is an inherited disease with a purely genetic causation. HD is caused by an expansion in a CAG triplet repeat at the 5' end of the huntingtin gene, located on the short arm of chromosome 4 (HD collaborative & research group 1993). This gene encodes the protein huntingtin (HTT), and the HD mutation leads to production of a mutated huntingtin protein (mHTT) with a subsequently expanded polyglutamine region near the N-terminus (HD collaborative & research group 1993).

In the normal huntingtin gene, the trinucleotide repeat region contains less than 36 CAG repeats (Snell et al. 1993). Age of onset of HD symptoms is linked with the size of the CAG expansion, with longer CAG repeats leading to earlier symptomatic onset (Langbehn et al. 2010; Duyao et al. 1993; Stine et al. 1993). Patients with ≥40CAG repeats demonstrate 'penetrant' HD mutations and are likely to show HD symptoms within a normal life span(Snell et al. 1993). Patients with repeats from 36-39 may not show symptoms, or may present very late in life(Rubinsztein et al. 1997). The average age of onset for HD is approximately 40, however, onset can be as early as age 4 or as late as age 80, with no apparent gender difference in disease prevalence (Reiner et al. 2011).

HD is inherited in an autosomal dominant fashion, thus, parents with a HD mutation have a 50% chance of passing the mutation on to their children(HD collaborative & research group 1993). The HD mutation is unstable during meiosis, which can lead to increases in the number of CAG repeats in successive generations, a phenomenon called "anticipation", shown to be more common in paternal inheritance of HD (Duyao et al. 1993; Kremer et al. 1995; Zühlke et al. 1993). Though relatively rare, this instability of the HD mutation can sometimes lead to sporadic HD cases where parents were non-HD mutation carriers (Siesling et al. 2000). Because of its purely genetic causation, HD can be genetically tested for, with aid from genetic counselors, along with some prediction of age of symptomatic onset (Lee et al. 2012; Craufurd & Harris 1989).

1.1.3 Clinical aspects of HD

Clinically, HD encompasses significant impairments in cognition, motor control and behavior. Generally, the earliest symptoms of HD are cognitive, sometimes presenting years before any other symptomatic onset (Paulsen et al. 2008). These symptoms are often very subtle, and may even be unrecognizable to patients. Common, early HD cognitive impairments include deficiencies in

higher executive function (organizational skills, planning, multitasking) (Novak & Tabrizi 2010) as well as impaired thought processes or memory formation (Craufurd & Snowden 2002). Psychiatric symptoms may often be present, pre-symptomatically. The most common of these is depression, and then anxiety, and some HD patients exhibit suicidal thoughts (Craufurd & Snowden 2002). Apathy is also common, as well as aggression and irritability (Craufurd & Snowden 2002; Ghosh & Tabrizi 2015). As HD progresses, characteristic motor impairments also occur. The earliest motor disturbances in HD are typically involuntary "dance-like" motor movements called "chorea", which often start subtle, but become more dramatic as the disease progresses (Ghosh & Tabrizi 2015). In later disease states, however, dystonia can be observed, along with bradykinesia (slowness of movement), akinesia (trouble initiating movement) and rigidity (Novak & Tabrizi 2010). Severe motor dysfunction in HD may impair gait and can lead to falls (Ghosh & Tabrizi 2015). Later in disease states, many HD patients exhibit very severe dementia (Craufurd & Snowden 2002) though this can sometimes be misdiagnosed when severe motor dysfunction renders patients unable to speak and move normally (Ghosh & Tabrizi 2015).

For most HD patients, the progression of HD occurs over the course of 15-20 years, from the appearance of earliest symptoms (Ghosh & Tabrizi 2015). The Unified Huntington's Disease Rating Scale (UHDRS) (Group 1996) has been developed to help assess and diagnose symptomatic HD patients, and encompasses cognitive, behavioral and motor assessments. Most HD patients die due to pneumonia or cardiovascular failure (Sørensen & Fenger 1992; Lanska et al. 1988)

1.1.4 Neurodegeneration in HD

The brains of HD patients are, on average 20-30% lighter than non-HD patients(Vonsattel & DiFiglia 1998). The most significant losses to brain tissue occur in the neostriatum, including the

caudate nucleus, putamen and other parts of the basal ganglia (Vonsattel et al. 1985; Graveland et al. 1985) These areas are primarily involved in integration and modulation of motor input from cortical and thalamic afferents, regulating motor behavior output (Albin et al. 1989). In particular, GABA-ergic medium spiny neurons(MSNs), making up >90% of cells in the striatum, are particularly affected in HD, leading to large losses of striatal tissue in HD, sometimes up to 95%(Vonsattel et al. 1985). The remaining 5-10% of non-MSNs in the striatum, primarily interneurons, are relatively spared in HD (Ferrante et al. 1985; Ferrante et al. 1987). In later stages of the disease, more widespread neuronal losses occur in HD brains, including reductions in the projection areas of the striatum such as the globus pallidus and substantia nigra pars reticulata(Waldvogel et al. 2015) as well as significant losses in the cerebral cortex(Nana et al. 2014), white matter (de la Monte et al. 1988) hippocampus (Spargo et al. 1993; de la Monte et al. 1988) thalamus (Kassubek et al. 2005), hypothalamus (Rosas et al. 2003) and cerebellum (Rüb et al. 2013).

1.2 The huntingtin protein

1.2.1 Structure and modifications of HTT

When the HD mutation on the huntingtin gene was discovered in 1993, it opened a field of research into the role of endogenous huntingtin. However, even after 23 years of research, there are still many unanswered questions about the role of wild type (WT) HTT in normal cell and body processes. WT HTT is a 348kDa protein, originating from the huntingtin gene on chromosome 4. Despite HD affecting primarily the striatum and brain, HTT (and mHTT, in HD) is actually found ubiquitously throughout the body(Van Raamsdonk et al. 2007). The sequence of HTT shares similar homology with other vertebrates (Baxendale et al. 1995; Tartari et al. 2008), however, there

are few similarities between HTT and other known proteins, making it difficult to draw inferences about the normal function of WT HTT (De Souza & Leavitt 2015). The HTT protein contains several putative HEAT domains, involved in protein-protein interactions (Takano & Gusella 2002) and HTT has shown to have a wide range of interactors and binding partners (Ratovitski et al. 2012; Shirasaki et al. 2012; Riechers et al. 2016). HTT is post-translationally modified in several ways, including palmitoylation by huntingtin interacting protein 14 (HIP14)(Young et al. 2012), shown to be involved in vesicle formation(Huang et al. 2004) as well as ubiquitination and sumoylation at N terminal lysine residues K6, K9, and K14(Kalchman et al. 1996; Steffan 2004). Ubiquitination and sumoylation of HTT inversely regulate degradation of HTT via the ubiquitin-ligase proteosomal degradation pathway and stabilization of HTT via sumoylation (Ehrnhoefer et al. 2011). HTT is also phosphorylated at several sites, shown to affect HTT cleavage, localization and function, as well as toxicity of mHTT(Wang et al. 2010; Ehrnhoefer et al. 2011; Maiuri et al. 2013; Greiner & Yang 2011). HTT contains a polyproline region, upstream of the polyglutamine stretch, involved in HTT folding and solubility (Steffan 2004; De Souza & Leavitt 2015) as well as a functional nuclear export and localization signal (Xia et al. 2003).

1.2.2 Function of HTT

Despite years of research, the underlying function of WT HTT is still relatively unknown, however many studies have pointed to insights into its functional role. Early research using complete HTT knockout mice (*Hdh nullizygous mice)* suggested that HTT may have a critical role in development, as this mutation is embryonic lethal as early as embryonic day 8.5 (Nasir et al. 1995; Duyao et al. 1995; Zeitlin et al. 1995). As this death occurs prior to gastrulation, this suggests an important role of HTT in both CNS and normal tissue development, and similar studies have

suggested that HTT may be involved in proper organization of tissues during development (Leavitt et al. 2001; Van Raamsdonk et al. 2005). Similarly, genetic lowering of HTT levels to ≤50% in mice results in significant developmental issues, primarily related to epiblast formation, resulting in reduced neurogenesis, and abnormal cortex and striatal development (White et al. 1997). Alongside its clear role in the developing brain, HTT has a role in vesicle transport, particularly in transport of Brain-Derived Neurotrophin Factor (BDNF), especially from cortical projection neurons to the striatum. In cultured cortical neurons, HTT associates directly with BDNF, and using RNAi to knockdown WT HTT, BDNF trafficking along microtubules is impaired(Gauthier et al. 2004). HTT facilitates BDNF transport on microtubules by interacting directly with a subunit of the dynein complex (Glued), via a complex of Huntingtin-Associated Protein 1 (HAP1), Glued and BDNF(Imarisio et al. 2008). HTT silencing has also shown to reduce transport of BDNF receptor TrkB, in striatum (Liot et al. 2013). This role of HTT in vesicle transport has also shown to play an important role in cellular protein degradation, with reductions in HTT leading to impaired autophagosome transport and impaired protein degradation via lysosomes (Wong & Holzbaur 2014). In addition to vesicle transport, HTT also appears to have a role in synaptic transmission. At the synapse, HTT interacts with a wide range of proteins involved in neurotransmission (De Souza & Leavitt 2015; Shirasaki et al. 2012). Similarly, HTT binds directly to the important synaptic protein PSD-95, involved in stabilization of glutamate receptors, such as NMDARs (discussed more in section 1.3.8.5) (Sheng & Kim 2002)

1.2.3 HTT and cell survival

Alongside the demonstrated functional roles of HTT in development and synaptic trafficking/transmission, several studies have shown that WT HTT may be particularly important

in promoting cellular survival. This was first demonstrated using stable striatum cell lines in which HTT was overexpressed(Rigamonti et al. 2000). In these cells, overexpressed HTT was neuroprotective against a wide range of toxic and apoptotic stimuli. Similar results have been shown *in vivo*. Mice overexpressing HTT show reduced brain insults when exposed to excitotoxic and ischemic stimuli (Zhang et al. 2003; Leavitt et al. 2006). Several mechanisms for the neuroprotective role of HTT have been hypothesized. Early studies suggest that HTT inhibits apoptotic caspase signaling pathways and prevents pro-apoptotic protein complex formation (Rigamonti et al. 2000; Rigamonti et al. 2001). The protective effects of HTT have also been strongly linked to HTT's role in BDNF trafficking, as discussed in section 1.2.2**.** BDNF, important for growth and cell survival, is not produced in the striatum. Rather, BDNF is produced in cortical neurons, then transported and released onto the striatal neurons (Ventimiglia et al. 1995; Mizuno et al. 1994). BDNF in the striatum has shown to be neuroprotective against excitotoxic quinolinic acid lesions in mice (Bemelmans et al. 1999) and reductions in striatal BDNF worsen HD symptoms in HD transgenic mice (Pineda et al. 2005). Given the important role of HTT in BDNF transport, HTT acts as an important player in ensuring effective BDNF delivery to the striatum. Similarly, HTT itself has shown to increase BDNF levels in the brain (Zuccato et al. 2001) by directly increasing BDNF transcription through interaction and sequestration of RE1-silencing transcription factor (REST or neuronal restrictive silencing factor -- NRSF) (Zuccato et al. 2003) and it has been suggested that HTT may similarly increase transcription of other pro-survival genes, (Zuccato et al. 2003; De Souza & Leavitt 2015).

1.3 Mechanisms of HD neuropathology

1.3.1 Gain-of-function vs loss-of-function

HD is caused by a genetic mutation that leads to a glutamine expansion in the huntingtin protein. This altered polyglutamine tract has significant impact on both the structure and the function of HTT. It is primarily believed that HD is a largely "gain-of-function" disease, in that the presence of mHTT leads to the majority of the cellular dysfunction and death characteristic to the disease, however, there is also evidence to support the fact that HD is also a "loss-of-function" disease, and the reduction in WT HTT caused by expression of mHTT also contributes to the neuropathological changes of HD. Despite significant impacts on normal function, mHTT still retains some of its function. For example, in Hdh knockout mice, embryonic lethality of the mutation can be rescued or reversed by supplementing HTT expression with mHTT, with normal development occurring, and HD symptoms only appearing later (Van Raamsdonk et al. 2005). Similarly HD patients with two mutated huntingtin alleles are viable, and in fact develop normally until HD symptoms develop later in life (Wexler et al. 1987; Myers et al. 1989), suggesting that mHTT can retain normal function during development, with deficits associated with the CAG expansion developing later in life. mHTT is considered a toxic protein species, and there are many neuropathological changes that occur as a result of its presence. In section 1.3 I will review some of the major known cellular changes that occur as a result of mHTT expression.

1.3.2 mHTT aggregation

As with many mutated proteins, one outcome of the presence of mHTT is the tendency of the protein to form aggregates. mHTT aggregates can be found in the brains of both transgenic HD animals and in the brains of human patients with HD (Davies et al. 1997; DiFiglia 1997). These

aggregates are primarily nuclear**,** are made up of insoluble mHTT and other proteins, and are often referred to as neuronal intranuclear inclusions (NIIs)(Lehrach et al. 2001; DiFiglia 1997). It has been shown that the rate and frequency of aggregate formation is dependent on polyglutamine length(Lakhani et al. 2010) and the size of mHTT aggregates increases with the onset of symptoms(Jones & Hughes 2011). Similarly, early studies showed that aggregates are common in the cortex and striatum, the areas of the brain primarily affected in HD (DiFiglia 1997). Based on other diseases where protein misfolding and aggregation is the primary cause of pathogenesis, such as Alzheimer's Disease, Parkinson's Disease and Amyotrophic Lateral Sclerosis (ALS) it is reasonable to presume that mHTT misfolding and potentially its aggregation, have a toxic, pathogenic role in HD and in fact, many studies have supported this (Lunkes & Mandel 1998; Yamamoto et al. 2000; Ehrnhoefer et al. 2006; Morton et al. 2001). However, more recent studies suggest that mHTT inclusion formation may actually be a neuroprotective mechanism, in an attempt to sequester mHTT and prevent its harmful effects (Gauthier et al. 2004; Ravikumar et al. 2004; Saudou et al. 1998; Van Raamsdonk et al. 2005; Arrasate et al. 2004; Arrasate & Finkbeiner 2012). Similarly, more recent experiments demonstrate lower levels of aggregates in striatal MSNs compared to less affected areas like the cerebellum (Gutekunst et al. 1999)further suggesting disconnect between aggregates and pathology and it is primarily believed that aggregates are not the primary mode of toxicity of mHTT.

1.3.3 mHTT cleavage

The proteolysis of mHTT has been demonstrated to have an important role in the pathogenesis of HD. HTT is a substrate for cleavage by several molecules, including caspases, calpains, cathepsins and matrix metalloproteinases (MMPs), all of which have been demonstrated to increase in

expression and activity in HD and be involved in HD pathogenesis (Gafni & Ellerby 2002; Gafni et al. 2004; Hermel et al. 2004; Graham et al. 2011; Miller et al. 2010; Qin et al. 2003). As a result, mHTT fragments can be detected in the brains of both animal models of HD and the HD patients (Ratovitski et al. 2009; Lunkes et al. 2002). In particular, N-terminal fragments of mHTT are believed to be the toxic species in HD, leading to many of the negative downstream effects (Goffredo et al. 2002). N-terminal mHTT fragments form nuclear inclusions and aggregate more readily than non-cleaved mHTT (DiFiglia 1997; Martindale et al. 1998) and have shown to be toxic to the cell(Warby et al. 2008; Cooper et al. 1998; Schilling et al. 1999; Martindale et al. 1998). This is demonstrated in mouse models of HD. Mice expressing short, N-terminal truncated mHTT demonstrate rapid onset of symptoms, increased neuropathology, worsened behavioural symptom severity and earlier death than mice with full-length mHTT (Ferrante 2009). Though the symptoms get worse more quickly in fragment models, the molecular and behavioral changes observed in these mice are similar to full-length mHTT HD models, and human patients, suggesting that the N-terminal fragment of mHTT is the toxic species (Jones & Hughes 2011). Caspases have been shown to play a particularly important role in the formation of toxic N-terminal mHTT fragment (Wellington & Hayden 2000; Graham et al. 2010; Graham et al. 2011). To show this, transgenic mice that express mHTT resistant to cleavage by caspase-3 or caspase-6 were created (Graham, Deng, et al. 2006). Caspase-3 mutations had no significant effect, however, caspase-6-resisitant mHTT mice exhibit dramatic resistance to behavioral and neuropathological HD phenotype (Graham, Deng, et al. 2006). HD transgenic mice with mutations preventing mHTT caspase-6 cleavage show protection against striatal degeneration and behavioral impairment, as well as rescue from HD-mediated NMDA receptor dysfunction (Wellington & Hayden 2000; Milnerwood et al. 2010; Graham et al. 2011; Warby et al. 2008; Mahmoud A. Pouladi et al. 2009).

Other studies have supported this, with genetic silencing (Wong et al. 2015; Uribe et al. 2012), chemical inhibition (Graham et al. 2010) and dominant-negative inhibition (Hermel et al. 2004) of caspase-6 showing neuroprotective effects against HD degeneration and symptoms. Caspases (in particular Caspase-6) and their role in HD pathogenesis will be discussed in more detail in chapter 3. Calpains have also been shown to play a role in cleaving mHTT, and inhibition of calpains has shown neuroprotective benefit in models of HD (Gafni & Ellerby 2002; Gafni et al. 2004). Similarly, mHTT is alternatively spliced in a way that increases translation of N-terminal mHTT in the striatum (Mende-Mueller et al. 2001). Cleaved mHTT N-terminal fragments have shown to be especially problematic in the nucleus, where it demonstrates enhanced toxicity(Saudou et al. 1998).

1.3.4 Impaired BDNF signaling

WT HTT has been shown to have an important role in regulating BDNF transcription, regulation and transport (see 1.2.3). In line with this, many studies have demonstrated that mHTT impairs HTT's ability to effectively interact with BDNF. mHTT's CAG expansion impairs the ability of huntingtin to interact with the REST/NRSF complex, preventing it from being sequestered in the cytoplasm, and allowing it to translocate to the nucleus, where it inhibits transcription of BDNF (Zuccato et al. 2001; Zuccato et al. 2003). Similarly, mHTT impairs the important role of HTT in BDNF transport. WT HTT normally interacts directly with many proteins involved in cellular transport, including HAP1(Li et al. 1995), HIP14 (Singaraja et al. 2002), PACSIN 1(Modregger et al. 2002) and synaptic fusion proteins of the SNARE complex (Kaltenbach et al. 2007). These interactions are central in facilitating transport of BDNF from cortical neurons to striatum, where BDNF is not synthesized (Imarisio et al. 2008; Gauthier et al. 2004). mHTT interaction with all of

these proteins is impaired, resulting in impaired BDNF transport from cortical afferents onto striatum (Sipione et al. 2002; Zuccato, Valenza & Cattaneo 2010). It is hypothesized that the reduction in BDNF reaching the striatum in HD plays an important role in HD pathogenesis. This is supported by experiments using transgenic mice engineered to produce reduced cortical BDNF (Baquet et al. 2004). These mice express behavioral and neuropathological phenotypes that recapitulate those seen in HD. Similarly, reducing BDNF in transgenic HD mice worsens the HD phenotype (Canals et al. 2004)

1.3.5 Transcriptional dysregulation

Via its disruption of REST/NRSF signaling, mHTT indirectly disrupts not only BDNF transcription but also the transcription of several other important genes controlled by REST/NRSF, which may contribute to neuronal dysfunction and death in HD(Zuccato, Valenza & Cattaneo 2010). The CAG expansion in mHTT also disrupts transcription in other ways by interacting inappropriately with other transcription factors, including TATA-binding protein, SP1 and p53 and CREB binding protein among others (Steffan et al. 2000; De Souza & Leavitt 2015; Zuccato, Valenza & Cattaneo 2010). This suppresses the transcription of several important genes, including many in the important CREB-dependent cell survival pathway (Steffan et al. 2000; De Souza & Leavitt 2015; Zuccato, Valenza & Cattaneo 2010). Importantly, more than 80% of striatally enriched genes are downregulated in brains expressing mHTT, pointing to a potential mechanism for striatum-specific degeneration in HD (Desplats et al. 2006)

1.3.6 Mitochondrial dysfunction

The presence of mHTT in HD has also been associated with mitrochondrial dysfunction. mHTT has been demonstrated to increase fragmentation of mitochondria, decrease mitochondrial membrane potential and disrupt mitochondria trafficking (Zuccato, Valenza, Cattaneo, et al. 2010). Enhanced oxidative stress as a result of mitochondria damage in HD has shown to play a role in disease pathogenesis(Butterfield et al. 2001). Mitochondria in the brains of HD transgenic mice have also shown impaired calcium handling, seen by reduced calcium uptake by HD mitochondria (Panov et al. 2002), an effect that is apparent early in disease progression, prior to symptom onset, and that is similar to effects seen in human HD patients (Zuccato, Valenza & Cattaneo 2010). This can enhance neurotoxicity in HD, by increasing cytplasmic Ca^{2+} and activating a wide range of cell death pathways(Panov et al. 2003; Zuccato, Valenza & Cattaneo 2010; Orrenius et al. 2003) and can exacerbate increased intracellular Ca^{2+} from enhanced glutamate excitotoxicity (as discussed in 1.3.8). It has been suggested that mitochondrial deficit in HD is a direct result of mHTT interaction with the mitochondrial membrane, as introduction of mHTT to normal mitochondria recapitulates this effect (Panov et al. 2003; Choo et al. 2004). The calcium handling deficits of mHTT on mitochondria are exacerbated in the striatum (Brustovetsky et al. 2005; Oliveira et al. 2006) further pointing to mechanisms for selective striatal effects in HD.

1.3.7 Impairments in cellular degradation

There are two major protein degradation pathways in the mammalian cell. The ubiquitin-proteasome system (UPS) is used to degrade ubiquitin-flagged, short lived, mislocalized, misfolded, or degraded proteins(Schwartz & Ciechanover 2009). In contrast, the autophagy pathway degrades large proteins, as well as protein complexes that would be too large to be

degraded by the UPS. Both the UPS and Autophagy pathways have shown to contribute to mHTT clearance in different ways. Similarly, both the UPS and the autophagy pathways are impaired in HD and may contribute to HD pathology.

1.3.7.1 Proteasome impairments in HD

The UPS utilizes a three step, ATP-dependent process in which the target protein is flagged with chains of ubiquitin (Ub), a small 76 amino acid protein(Hochstrasser 1996; Hegde 2013). First, Ub is activated by an E1 ubiquitination enzyme, then bound to an E2 conjugating enzyme, and finally attached by covalent linkage to a lysine residue on the target protein by an E3 ligase enzyme (Schipper-Krom et al. 2012a). This process repeats to create Ub chains, and the targeted protein is recognized by the 26S proteasome (Schwartz & Ciechanover 2009; Glickman & Ciechanover 2002). The 26S proteasome is comprised of two components, the 20S proteasome core and the 19S regulatory region. The 19S recognizes the ubiquitinated target protein, unfolds it, and feeds it into the 20S core (Voges et al. 1999). The 20S core is a cylindrical complex, whose inner components have catalytic activity, similar to caspases, trypsin and chemotrypsin, that degrade and break down protein chains (Arendt & Hochstrasser 1997). Targeted proteins that are fed into the 20S core are broken down and released as amino acids, or small fragments, where they can be recycled (Schipper-Krom et al. 2012a). In normal function, the UPS is utilized to maintain homeostasis; degrading short-lived proteins, like regulatory proteins or transcription factors, however it is also employed to degrade misfolded or erroneous proteins.

Much evidence exists to demonstrate that the UPS is used in the clearance of mHTT in HD (Schipper-Krom et al. 2012a). The ubiquitination ligase hE2-25K has shown to interact with mHTT, initially leading researchers to believe that mHTT may be targeted for degradation via the

proteasome (Kalchman et al. 1996). mHTT also contains a proteasome targeting motif (FQKLL), again suggesting that it may be a substrate for degradation via UPS(Chandra et al. 2008). Using an inducible model of HD (Yamamoto et al. 2000), activation of the HD transgene leads to formation of mHTT inclusions that colocalized with ubiquitin (Martín-Aparicio et al. 2001). When the HD transgene was shut off, the inclusions disappeared; however, mHTT inclusions were not cleared if the proteasome was inhibited. In human HD brains, transgenic models of HD, and when mHTT is transfected into cells, mHTT aggregates co-localize with ubiquitin, and many components of the UPS have also been found in mHTT nuclear inclusion complexes in HD (DiFiglia 1997; Davies et al. 1997; Jana et al. 2001; Kalchman et al. 1996), further supporting that this system is used to degrade mHTT.

Despite the suggested role of the UPS in degrading mHTT, the UPS is also impaired by the HD mutation (Jana et al. 2001; Lehrach et al. 2001; Waelter et al. 2001). For one, the 26S proteasome may not efficiently degrade expanded polyQ (Venkatraman et al. 2004) resulting in expanded polyQ peptides exiting the proteasome after mHTT UPS degradation, which can lead to further cellular dysfunction, or continual proteasome engagement (Schipper-Krom et al. 2012a). Proteasome impairment has been suggested to be a result of expanded polyQ clogging the proteasome, supported by FRET experiments showing mHTT bound to the proteasome core (Holmberg et al. 2004). Using HD knock-in mice, the activity of the 20S proteasome was shown to be impaired in HD, with its chemotrypsin-like and caspase-like activites downregulated as well as an increase in the half-life of Ub-linked reporter proteins (Hunter et al. 2007). Interestingly, in these experiments, the trypsin-like activity of the proteasome was increased in HD mice, however this effect is also seen in cellular stress and ATP depletion, suggesting it is a stress response. Similar proteasome activity impairments are detected in the tissues of HD patients (Seo et al.

2004). Another HD related UPS impairment is related to depletion of UPS machinery resources. In cells overexpressing mHTT, UPS components associate with mHTT aggregates, however this association is largely irreversible, which may cause depletion of Ub or proteasomes in HD, as well as proteasome blockage (Holmberg et al. 2004). Sequestration of the proteasome into mHTT aggregates has shown to impair the function of the proteasome, as well as enhance neurotoxicity (Jana et al. 2001).

Despite these studies, there is still controversy on the role of the UPS in degrading mHTT as well as the impairments in HD. In a recent study, in an *in vitro* system, mHTT inclusions and N-terminal mHTT were not extensively ubiquitated naturally (Hipp et al. 2012), suggesting that other clearance mechanisms are also employed. Also, some studies suggest that the proteasome is able to degrade polyQ. For one, inhibition of the proteasome increases levels of mHTT in cells overexpressing mHTT (Li et al. 2010). In the same experiment, using HD CAG-repeat knock-in mice, proteasome impairment increased accumulation of N-terminal mHTT, but not full-length mHTT(Li et al. 2010). Similarly, experiments using targeted degradation signals on polyQ proteins (Hoyt et al. 2005; Rousseau et al. 2009), forced nuclear localization of mHTT to exclude autophagy degradation (Iwata et al. 2009), and employment of the N-end rule test, which tests the ability of the proteasome to unfold Ub-tagged GFP (Michalik & Van Broeckhoven 2004), all demonstrated that the proteasome was able to degrade polyQ proteins. Similarly, proteasome impairment varies in HD models. For example, R6/2 mice carrying short GFP-tagged reporters to detect proteasome function demonstrate no significant proteasome impairments (Bett 2006), and in conditional HD94 mice, no impairments in proteasome activity are detected (Díaz-Hernández et al. 2003).

1.3.7.2 Autophagy impairments in HD

Another means by which cells degrade proteins is autophagy. Autophagy is a process in which cytoplasmic proteins and molecules are engulfed by double membrane structures called autophagic vacuoles/autophagosomes, originating from the endoplasmic reticulum, mitochondria or plasma membrane (Hayashi-Nishino et al. 2010; Hailey et al. 2010; Ravikumar et al. 2010). Autophagosomes are transported via microtubules to lysosomes, where they fuse with the lysosome membrane, and vacuole contents are subsequently degraded, via acidification, proteinase B and lipase Cvt17 (Klionsky & Emr 2000). The autophagy pathway is regulated by several important molecules, including positive regulation by PI3K and negative regulation by mTOR (Sarkar & Rubinsztein 2008). Autophagy has shown to be involved in the clearance of misfolded and aggregated proteins in several diseases, including Parkinson's Disease, Alzheimer's Disease, ALS, prion diseases and spinocerebellar ataxia. (Ravikumar et al. 2008; Ventruti & Cuervo 2007). Thus, it is not surprising that autophagy also plays a role in mHTT clearance in HD (Sarkar & Rubinsztein 2008; Ravikumar et al. 2004; Sarkar & Rubinsztein 2008). Induction of autophagy in cellular, fly, and mouse models of HD decreases levels of the toxic, monomeric mHTT and improves neurotoxicity (Rubinsztein 2006), whereas inhibition of autophagy in HD models results in increased mHTT aggregation and increased neurotoxicity (Ravikumar et al. 2002). Inhibition of mTOR, similarly, stimulating enhanced autophagy, leads to improved HD phenotype (Ravikumar et al. 2004)

Impairments in autophagy may be involved in HD neuropathology. Increased autophagy has been detected in HD. Transfected striatal cells expressing mHTT show that both HTT and mHTT localize to autophagic vacuoles (M Kim et al. 1999), and huntingtin-enriched autophagosomes are more numerous in cells expressing mHTT (Kegel et al. 2000). In HD patient

tissue, as well as in the brains of HD transgenic mice, increased autophagosomes, vesicular bodies and lysosomes can be detected (Sapp et al. 1997; Petersén A et al. 2001; Davies et al. 1997). However, it has been shown that these increased autophagosomes in HD are, in fact, largely devoid of content, which has shown to be attributed to an impairment in autophagosome cargo recognition as a result of mHTT (Martinez-Vicente et al. 2010). The result is that, despite enhanced autophagic response in HD, toxic mHTT does not get degraded, and instead remains in the cell, where it accumulates and contributes to neuropathology. This may also in turn initiate a feedback loop in which the cell responds by attempting to further upregulate autophagy, leading to worsen mHTT accumulation(Martin et al. 2015). mHTT's impairments on vesicle transport further exacerbate impairments in autophagy by impairing autophagosome motility(Wong & Holzbaur 2014). Fusion and machinery in the autophagosome pathway are also shown to be impaired in HD(Ge et al. 2014). The presence of mHTT is shown to interfere with expression of several genes relating to autophagy (Martin et al. 2015), subsequently influencing the level of autophagy-related proteins, particularly in the striatum (Hodges et al. 2006). The timing of autophagy impairments in HD is still relatively unknown and studies to determine the exact sequence of events will be necessary to better understand the potential for enhancing autophagy as a potential HD treatment (Martin et al. 2015).

1.3.8 NMDAR-mediated excitotoxicity

1.3.8.1 The excitotoxicity hypothesis of HD

The underlying cause of HD is the genetic mutation in the huntingtin gene, leading to expression of the glutamine-expanded mHTT. However, the presence of mHTT leads to many changes in neuron structure and function, hypothesized to underlie the neuropathological changes in HD. One

of the primary hypotheses is that mHTT induces enhanced glutamate excitotoxicity in HD. Striatal neurons receive glutamatergic input from several sources, importantly from the cortex and thalamus which stimulate glutamate receptors on striatal MSNs. Several decades of research have demonstrated that excessive glutamatergic stimulation of these receptors via impaired uptake, enhanced glutamate release, enhanced sensitivity of the receptors or impaired downstream signaling of glutamate receptors may contribute in important ways to striatal vulnerability in HD. Of note, "excitotoxic" changes seen in the synapse of HD neurons is, by definition, different than large-scale excitotoxic events seen in other neurological dysfunction, like stroke. As our knowledge of chronic NMDAR-mediated changes at the synapse of HD neurons develops, many researchers believe that the term "excitotoxicity" may not accurately describe the changes seen in HD and other chronic diseases. However, for the purpose of my thesis work, I will refer to these changes, and the subsequent vulnerabilities in HD as NMDAR-mediated excitotoxicity.

1.3.8.2 N-methyl-D-Aspartate receptor physiology

Glutamate is the primary excitatory neurotransmitter in the central nervous system(Kandel et al. 1995), exerting its actions either by activating metabotropic glutamate receptors (mGluRs) which couple to G proteins, or via ionotropic glutamate receptors (iGluRs), which, upon ligand binding, allow passage of cations through a receptor pore (Watkins & Evans 1981; Dingledine et al. 1999). N-methyl D-aspartate (NMDA) receptors are the most highly studied of the iGluRs due to their importance in both normal neuron physiology and in disease pathology. To open, NMDARs require dual ligand binding of glutamate and the co-agonist glycine (Johnson & Ascher, 1987) as well as removal of a Mg^{2+} block by membrane depolarization (Mayer et al. 1984). Activation of NMDARs causes influx of Ca^{2+} (MacDermott et al. 1986) which activates signal transduction

cascades. The slow activation and deactivation kinetics of NMDARs govern the duration of the excitatory post-synaptic potential(Lester et al. 1990), giving NMDARs an important role in synaptic strength modulation. Native NMDARs are heterotetrameric complexes of two GluN1 (NR1) with two GluN2 (NR2) and/or GluN3 (NR3) subunits (Benveniste & Mayer 1991; Clements & Westbrook 1991). Different NMDAR subunit combinations change the ion properties and pharmacology of the receptor allowing for wide functional diversity of NMDARs (Flint et al. 1997; Monyer et al. 1992; Ishii et al. 1993). GluN2 subunits in particular are encoded by four genes (GluN2A-D) which determine differences in NMDAR channel properties, pharmacology and distribution(Dingledine et al. 1999; Cull-Candy & Leszkiewicz 2004). GluN2 subunits are both spatially and developmentally regulated(Monyer et al. 1992; Akazawa et al. 1994). In the forebrain of adults, the majority of NMDARs contain GluN2A and GluN2B subunits, with most NMDARs being diheteromeric GluN1-GluN2A or GluN1-GluN2B or triheteromeric GluN1-GluN2A-GluN2B receptors (Sheng & Kim 2002; Li et al. 1998; Chapman et al. 2003). NMDA receptors are largely located at synaptic sites, but can also found extra- or perisynaptically. During development, up to 1/3 of NMDARs are extrasynaptically located, while synaptic NMDARs slowly increase in proportion as the brain matures. However a significant proportion of NMDARs remain extrasynaptic during adulthood (Tovar et al. 2013; Rosenmund et al. 1993; Cottrell et al. 2000; Petralia et al. 2010).

1.3.8.3 NMDAR-mediated excitotoxicity

NMDARs have an important role in many neurological functions, including critical roles in synaptic plasticity, brain development and normal synaptic transmission (Bliss & Collingridge 1993; Aamodt & Constantine-Paton 1999). However, in many pathological conditions,

overstimulation of NMDARs can trigger multiple neuronal death cascades, leading to apoptosis and necrosis(Berliocchi et al. 2005). This process is called **excitotoxicity** and has been implicated in a wide range of neuropathies and neurodegenerative diseases including stroke (Lai et al. 2014) Alzheimer's Disease (Hynd et al. 2004; Koutsilieri & Riederer 2007; Ong et al. 2013) Parkinson's Disease (Koutsilieri & Riederer 2007; Beal 1998) and neurotrauma (Marklund et al. 2004; Obrenovitch & Urenjak 1997; Johnston 2005). Initial research on excitotoxicity arose from studies demonstrating that monosodium glutamate (MSG), an additive commonly found in Chinese food, was neurotoxic in the retina of the mouse (Lucas et al. 1957). Subsequent studies demonstrated that this effect was not limited to the mouse retina, and similar effects were detected in both central and peripheral nervous system neurons in several other species (Burde et al. 1971; Freedman & Potts 1963; Olney & Sharpe 1969). Over several decades of research, it was discovered that the primary cause of neuronal death as a result of glutamate overstimulation was a result of excessive Ca^{2+} influx (Choi 1995) primarily through NMDARs (Choi et al. 1988). Subsequently, a large number of studies have investigated the potential for NMDAR antagonists to protect against excitotoxic insults in various nervous system disorders, however the majority of NMDAR-antagonist studies fail to show efficacy in human clinical trials, due to multiple factors, including side effects (Lipton 2004; Minnerup et al. 2012; Stroke Clinical Trials Registry 2016). The primary hypothesis for why NMDAR antagonists fail as therapeutics may be at least in part due to a paradoxical role of NMDARs, playing a pivotal role in both normal cellular function including survival and neuronal death (Hardingham & Bading 2003). Blockade of all neuronal NMDARs using antagonists, though effective at preventing cell death pathways associated with NMDAR-mediated excitotoxicity also block the necessary synaptic plasticity and cell survival pathways activated by NMDAR stimulation. These studies, and other led to research into the underlying

causes of the contradictory roles of NMDARs in hopes of developing better, more specific preventative therapies for nervous system disorders where NMDAR-mediated excitoxicity has a role.

1.3.8.4 Dichotomous roles of NMDARs in cell survival and cell death

NMDARs are critical players in numerous functions related to cell survival and maintenance of neuronal homeostasis, however NMDARs are also strongly involved in excitotoxic neuronal death. Although the underlying mechanisms of this dichotomous role for NMDARs is debated, there are many hypotheses as to the paradox of NMDAR function. For one, the role of the NMDAR can vary depending on activity level. For example, when stimulated with low doses of NMDA, cultured granule cells show enhanced cell survival, however, with high dose stimulation with NMDA, the same neurons undergo cell death (Balázs et al. 1988; Balázs et al. 1990; Balázs et al. 1989; Didier et al. 1989). Similarly, cultured spinal cord neurons treated with low doses of NMDAR antagonist demonstrate enhanced cell survival, where the same neurons undergo cell death with high doses of the same antagonist (Brenneman, Chang, et al. 1990; Brenneman, Forsythe, et al. 1990). This, and other similar studies prompted an early hypothesis that there may be an optimal amount of intracellular calcium, and over or under stimulation of highly calcium permeable NMDARs leads to cellular death (Choi 1995; Koike et al. 1989; Franklin & Johnson 1992). In a similar vein, subsequent and emerging studies have demonstrated that both NMDAR location and receptor subunit composition may contribute to the differential role of the NMDAR in cell death and cell survival.

1.3.8.4.1 The subcellular location hypothesis of NMDAR function

One hypothesis for the dichotomous role of NMDARs in cell survival and cell death is that different populations of NMDARs may trigger different downstream signaling pathways upon activation. Many proteins with significant roles in molecular signaling and scaffolding are located exclusively at the synapse, such as PSD-95. Thus, it is likely that synaptically-located NMDARs, interacting with synapse-specific proteins, may behave differently than NMDARs located peri- or extrasynaptically. Many studies have demonstrated differences in downstream signaling pathways of synaptically located NMDARs compared to extrasynaptic NMDARs, prompting a theory of excitotoxicity called the "subcellular location" model. These studies suggest that stimulation of synaptically-located NMDARs activates signaling pathways associated with cell survival and plasticity, whereas extrasynaptic NMDARs, trigger pathways associated with neuronal death(Lu et al. 2001; Hardingham & Bading 2003). This hypothesis was initially tested by selective enhancement of either synaptic or extrasynaptic NMDAR activity (Hardingham et al. 2002; Hardingham & Bading 2010; Choi et al. 1988; Lu et al. 2001) Synaptic NMDAR activity was enhanced pharmacologically, by blocking K^+ channels using 4-aminopyridine, applying NMDAR co-agonist glycine which only enhances synaptic NMDARs that are activated by presynaptically released glutamate (Lu et al. 2001) or blocking GABAergic inhibition using bicuculline(Hardingham & Bading 2010), or by electrical stimulation. Extrasynaptic NMDARs were selectively stimulated using synaptic NMDAR blockade with MK-801, then bath application of NMDA (Hardingham & Bading 2010); alternatively, their contribution was attenuated by using the extrasynaptic-preferential NMDAR antagonist memantine (Xia et al. 2010; Parsons & Raymond 2014). Synaptic NMDAR stimulation causes a calcium-dependent upregulation of several pro-survival genes, including several anti-apoptotic factors, and suppression of several

genes involved in cell death, leading to enhanced neuroprotection, reduced apoptotic ability and stimulating innate antioxidative properties of the cell (Hardingham et al. 2002; Hardingham & Bading 2010; Parsons & Raymond 2014). This synapse-specific NMDAR activity subsequently drives many cell-survival pathways, including extracellular signal-related kinase 1/2 activation, cAMP response elevated-binding protein (CREB) phosphorylation and enhanced expression of brain-derived neurotrophic factor (BDNF) (Hardingham et al. 2002; Xu et al. 2009; Hardingham & Bading 2010). On the contrary, specific stimulation of extrasynaptic NMDARs activates several molecular pathways that drive cell death, including CREB shut off, ERK1/2 inactivation and enhanced gene expression and activation of pro-apoptotic proteins such as Forkhead box protein O (Hardingham et al. 2002; Hardingham & Bading 2010; Xu et al. 2009). Similarly, calpains are differentially regulated by synaptic and extrasynaptic NMDARs. Synaptic NMDARs lead to activation of μ-calpain, whereas extrasynaptic NMDARs specifically activate m-calpain(Wang et al. 2013). m-calpain is preferentially involved in the cleavage of striatal-enriched tyrosine phosphatase (STEP), which subsequently activates p38 mitogen-activated protein kinase (p38MAPK) resulting in increased cell death (Hardingham et al. 2002; Hardingham & Bading 2010; Xu et al. 2009). In line with this data, stimulation of synaptic NMDA receptors is neuroprotective against neuronal insult caused by starvation or staurosporine (Hardingham et al. 2002; Papadia et al. 2005) whereas global NMDAR stimulation leads to neuronal death (Hardingham et al. 2002; Gouix et al. 2009; S. J. Zhang et al. 2007)

Synaptic and extrasynaptic NMDARs have also been shown to have opposite roles in regulation of synaptic plasticity (Lu et al, 2001). It is well known that NMDARs have an essential role in both long term potentiation (LTP) and long term depression (LTD), however recent data has demonstrated that increasing extracellular glutamate leads to impairments in LTP (Izumi et al.

2008; Katagiri et al. 2001; Li et al. 2011), an effect that is reversible using NMDAR antagonists. Similarly pre-blockade of synaptic NMDARs before bath NMDA application leads to LTD formation (Liu et al. 2013). These, and other data, suggest that synaptic NMDARs may be preferentially involved in LTP formation, whereas extrasynaptic NMDARs are necessary to facilitate LTD. Taken together, a wide range of data suggests that subcellular location of NMDARs may have large impact on the differential effects of these receptors with synaptic NMDARs preferentially involved in neuron survival machinery, and extrasynaptic NMDARs facilitating neuronal death.

1.3.8.4.2 The subunit hypothesis of NMDAR function

Another hypothesis for the paradoxical effects of NMDARs suggests that the physiological makeup of NMDARs contributes to the differential effects of the receptor. Most NMDARs are composed of two essential GluN1 subunit (Moriyoshi et al. 1991; Yamazaki et al. 1992) and two GluN2 subunits(Kutsuwada et al. 1992; Yamazaki et al. 1992; Mori & Mishina 1996). Variations in the GluN2 subunit leads to variations in receptor kinetics, properties and downstream signaling pathways due to differences in the carboxy- terminus of the receptor (Groc et al. 2006; Martel et al. 2009; Sanz-Clemente et al. 2013). Similar to the location hypothesis of NMDAR-mediated excitotoxicity, it has been proposed that subunit composition of NMDARs may underlie differential effects of NMDARs on cell survival and cell death. Using recently developed agonists and antagonists for GluN2A- and GluN2B-containing NMDARs, as well as genetic deletion of receptor subtypes, researchers have been able to investigate the particular role of each receptor subtype on neuron function, as well as the role in neuron survival vs death, and as a result, there has been support for a hypothesis that GluN2A-containing NMDARs have a primary role in cell

survival signaling, whereas GluN2B-containing NMDARs are largely involved in cell death signaling (Liu et al. 2007; Zhou & Baudry 2006; DeRidder et al. 2006). Using antagonists for GluN2A or GluN2B- containing NMDARs, it has been shown that specific stimulation of GluN2B-containing NMDARs triggers excitotoxic neuronal death and apoptotic cascades, while stimulation of GluN2A-containing NMDARs is neuroprotective both in vitro against NMDA and non NMDAR-mediated neuronal death, as well as *in vivo* (Liu et al. 2007a; Terasaki et al. 2010; Lai & Wang 2010). Similarly, specific stimulation of GluN2A-NMDARs is associated with activation of several downstream signaling pathways associated with cell survival such as CREB(Liu et al. 2007a), P13K and (kinase-D-interacting substrate of 220 kDa kinase-D-interacting substrate of 220 kDa (Kidins220) (López-Menéndez et al. 2009) whereas GluN2B-NMDAR stimulation activates numerous cell death specific pathways(Lai et al. 2014; Lai & Wang 2010; Martin & Wang 2010). Further, it has been shown that the subtype-specific differences in function of NMDARs are conferred by differences in the C-terminus of the NMDAR(Foster et al. 2010; Sprengel et al. 1998). To further test the differential role of NMDAR subtype on cell death and survival, Martel et al performed an experiment in which the C terminus of GluN2A was swapped with the C-terminus of GluN2B. In this experiment, the C-terminus of GluN2B enhanced neurotoxicity of the NMDAR when it replaced the GluN2A C-terminus(Martel et al. 2012), providing further evidence that GluN2B NMDARs are specifically involved in cell death, and GluN2A NMDARs enhance cell survival.

A controversial hypothesis of subunit-specific localization of NMDARs exists that suggests GluN2A- containing NMDARs are primarily located at synaptic sites in the forebrain, whereas GluN2B-containing NMDARs are mostly localized extra- and peri-synaptically (extrasynaptic sites)(Groc et al. 2006; Martel et al. 2009). This theory works in concert with

location-based theories of NMDAR-mediated excitotoxicity, suggesting that synaptic/GluN2A NMDARs lead to cell survival while extrasynaptic/GluN2B-NMDARs enhance cell death. Much evidence has supported this theory over the years (Groc et al. 2006; Sanz-Clemente et al. 2013; Martel et al. 2009; Tovar & Westbrook 1999) however, there are several studies that have shown contradictory findings, and it is known that GluN2B can be found at synaptic sites and GluN2A can also be found outside of the synapse(Groc et al. 2006; Sanz-Clemente et al. 2013; Martel et al. 2009; Tovar & Westbrook 1999; Petralia et al. 2010; Wang et al. 2004; Harris & Pettit 2007; Thomas et al. 2006). Similarly, recent studies have shown some support for scenarios in which GluN2A, as well as synaptically located NMDARs can be implicated in cell death signaling under specific conditions (Papouin et al. 2012; Wroge et al. 2012; Zhou et al. 2013). These findings and others make the generation of a unified hypothesis for location and subcellular hypotheses of NMDAR-mediated excitotoxicity difficult to definitively confirm. Similarly, variables such as the lack of very precise inhibitors for GluN2A and GluN2B, developmental changes in NMDAR subtype expression, growing knowledge of the importance heterotetrameric NMDARs containing both GluN2A and GluN2B (Tovar et al. 2013) and the fact that a disproportionate percentage of NMDARS in cultured neurons are extrasynaptically located (Xia et al. 2010; Gladding & Raymond 2011) lead to challenges in determining the true underlying cause for differences in NMDARs in controlling cell survival and cell death.

Based on a wide breadth of research on potential causes for dichotomy in NMDARs, it is likely that a combination of subtype, subcellular location, developmental stage and type of activation lead to the varieties in NMDAR role in cell survival and cell death (**See Figure 1-1**).

1.3.8.5 NMDAR-mediated excitotoxicity in HD

It has been widely demonstrated that NMDAR-mediated excitotoxicity has a role in the pathogenesis of Huntington's disease (HD). Evidence in human HD patients helped drive this theory, with the discovery that postmortem HD brains demonstrate reductions in NMDAR binding sites in the striatum (Albin et al., 1990; Young et al., 1988). Subsequently, brains of presymptomatic HD patients also showed increased expression in NMDARs in striatal MSNs, and it was found that these neurons were most vulnerable to death early on in disease progression (Graveland et al., 1985). Early studies using glutamate agonists quinolinic acid (QA) or kainic acid (KA) injected into the striatum of rats and later, to primates, led to neuropathological and behavioral changes similar to those seen in HD (Coyle & Schwarcz 1976; Beal et al. 1991; Sanberg et al. 1989; Hantraye et al. 1990; Burns et al. 1995; Ferrante et al. 1993; McGeer & McGeer 1976; Sanberg et al. 1978). Striatal neurons from pre-symptomatic transgenic HD mice show enhanced sensitivity to NMDA (Levine et al. 1999; Zeron et al. 2002) larger NMDAR currents(Li et al. 2004; Carlos Cepeda et al. 2001; Shehadeh et al. 2006; Zeron et al. 2002), enhanced apoptotic death after NMDA stimulation (Shehadeh et al. 2006; Fan et al. 2007) increased surface expression of NMDARs (Shehadeh et al. 2006; Fan et al. 2007; Milnerwood et al. 2010) increased calcium responses during NMDA stimulation (Tang et al. 2005) as well as larger striatal lesions after intrastriatal QA (Graham et al. 2009; Zeron et al. 2002). This enhanced NMDAR excitotoxicity in HD appears to be mediated by extrasynaptic, GuN2B-containing NMDARs. Enhanced expression of extrasynaptic GluN2B-containing NMDARs are seen in HD transgenic mice, and a low dose memantine, blocking extrasynaptic NMDARs is neuroprotective against neurodegeneration, synaptic dysfunction and behavioral dysfunction in mouse models of HD both *in vitro* and *in vivo* (Milnerwood et al. 2010; Lipton 2004). Similarly, elevated striatal

NMDAR current and excitotoxicity in primary striatal neurons and brain slices can be reversed with the GluN2B-selective antagonist ifenprodil (Zeron et al. 2002; Milnerwood et al. 2010; Fan et al. 2007; Tang et al. 2005)

Enhanced GluN2B expression and extrasynaptic localization in HD are, at least in part, regulated by enhanced forward trafficking and stabilization of GluN2B-NMDARs(Fan et al. 2007). The HTT protein has a wide variety of binding partners (Kaltenbach et al. 2007) many of which are disrupted by the presence of mHTT in HD(Zuccato, Valenza & Cattaneo 2010). mHTT disrupts normal binding of HTT to PSD-95 (Sun et al. 2001) and subsequently enhances PSD-95 binding to GluN2B, believed to lead to enhanced stabilization of GluN2B NMDARs in HD(Fan et al. 2009; Milnerwood et al. 2010). mHTT-mediated impariments in clathrin-mediated endocytosis (Harjes & Wanker 2003) and endosomal receptor recycling(Li et al. 2009) as well as impaired phosphorylation of both mHTT and NMDARs (Jarabek et al. 2004; Lan et al. 2001; Gladding & Raymond 2011) may also facilitate enhanced forward trafficking of NMDARs in HD (Gladding & Raymond 2011).

An important consequence of enhanced NMDAR-mediated excitotoxicity in HD is the activation of calcium-dependent calpain, shown to cleave NMDARs and affect receptor trafficking and diffusion between the synapse and extrasynaptic sites (Guttmann et al. 2002; Guttmann et al. 2001). In HD, calpain activity is increased, leading to enhanced GluN2B cleavage (Cowan et al. 2008; Gafni & Ellerby 2002). Similarly, Striatal-Enriched protein tyrosine Phosphatase (STEP) has been shown to reduce synaptic NMDAR localization by dephosphorylation of GluN2B, and STEP activation is increased in early stage HD due to early increases in calcineurin activity (Paul et al. 2002). It has been recently suggested in a study using transgenic HD mice that these changes work together to enhanced NMDA excitotoxic vulnerability in HD, with increased STEP61

activity leading to reductions in synaptic NMDARs while calpain cleavage of GluN2B enhances expression of extrasynaptic NMDARs(Gladding et al. 2012).

Although the striatum is the most widely studied brain area in HD, NMDAR dysfunction may also have a role in cognitive deficits in HD. Cognitive and mood disturbances are present in early stages of HD in humans and animal models, often preceding motor symptoms (Murphy et al. 2000; Van Raamsdonk 2005; Klapstein et al. 2001). Hippocampal loss is also observed in HD (Spargo et al. 1993b; Usdin et al. 1999) and NMDAR-dependent hippocampal synaptic plasticity and transmission in CA1 pyramidal neurons is altered in early and presymptomatic stages in HD mice suggesting a potential role for NMDARs in altered hippocampal function in HD (Murphy et al. 1999; Cummings et al. 2006; Milnerwood & Raymond 2007; Klapstein et al. 2001). Hippocampal neurons from YAC HD mice show hyper-excitability, reversed with NMDAR antagonists as well as increased resting cytosolic Ca^{2+} (Hodgson et al. 1999). Similarly, late stages of HD are accompanied by dementia and memory loss (MacDonald et al. 1993; Hansson, Guatteo, Mercuri, Bernardi, X. J. Li, et al. 2001) These data suggest that NMDAR excitotoxicity may also have a role in neuronal death and dysfunction in the hippocampus in HD, contributing to cognitive decline and memory deficit in HD.

Enhanced sensitivity to NMDA in HD models is present from birth and likely is an early underlying mechanism in HD pathogenesis (Zeron et al. 2002; Cepeda et al. 2001; Shehadeh et al. 2006). Conversely, in later, symptompatic disease stages, HD transgenic mice of several strains develop resistance to NMDA both *in vitro* (Hansson et al. 2001) and *in viv o*(Graham et al. 2009) which may represent compensatory mechanisms in response to elevated calcium (Ca^{2+}), reduced spine density(Hansson et al. 2001; Sun et al. 2002) or other neurological changes. An overview of NMDAR-mediated changes in HD synapses can be seen in **Fig 2-2)**

1.4 Animal models of HD

In order to effectively study any disease, having good non-human models that mimic molecular and behavioural changes are imperative for both investigating and testing potential therapeutic targets. Historically, the first animal models of HD were not genetic. As discussed in 1.3.8, early studies of HD demonstrated that injections of glutamate receptor agonists kainic acid or quinolinic acid led to changes that recapitulated symptoms of HD, molecularly, neuropathologically and behaviorally, experiments important to pointing to excitotoxicity as a key player in HD pathogenesis (Coyle & Schwarcz 1976; Beal et al. 1991; Sanberg et al. 1989; Hantraye et al. 1990; Burns et al. 1995; Ferrante et al. 1993; McGeer & McGeer 1976; Sanberg et al. 1978). Similarly, the toxin 3-nitro-proprionic acid has been used to model HD non-genetically. 3-NP, irreversibly inhibiting the mitochondrial enzyme succinate dehydrogenase, also mimics cell death and neuropathology in HD, demonstrating the important role of mitochondrial dysfunction in HD (Browne et al. 1997; Gu et al. 1996; Tabrizi et al. 1999) However, a unique advantage of HD, being a disease with a known genetic cause, is the potential to create good animal models whose genetic makeup mimics the CAG repeat of HD patients, and where disease progression mimics the slow onset observed in humans, rather than acute toxin models. Simple genetic models, using *drosophila melanogaster* fruit flies or *C. elegans* have been used to model HD, desirable due to their short lifespan and high throughput testing ability. Small, non-rodent models with HTT gene CAG expansions similar to HD patients lead to polyglutamine aggregates, motor impairments and neurodegeneration (Jackson et al. 1998; Brignull et al. 2006). By far the most common animal models for HD are rodents. One of the most common mouse models of HD is the R6/2 model (Mangiarini et al. 1996). These mice express only exon1 of the human huntingtin gene with 144 CAG repeats, leading to expression of mHTT N-terminal fragments. Due to the known toxicitiy

of mHTT fragments, these mice have very aggressive neuropathology and behavioral symptoms, with onset even at 4 weeks, and most of these mice don't live past 14 weeks. Though R6/2 provides a valuable asset, being a genetic model with rapid disease development, the actual progression of HD in human patients is much longer and milder, thus mouse models that better recapitulate the true progression of HD may be preferable in some cases. The N171-82Q mouse is another, similar HD model, also expressing N-terminal mHTT (82 repeats). N171-82Q mice demonstrate cognitive and motor impairments and significant loss of striatal neurons, as well as a shortened lifespan (McBride et al. 2006), however onset is milder and later than R6/2. Following N-terminal mouse models, full-length mHTT models were developed. The first way full-length mHTT was integrated into mice was by knocking in human exon1 HTT into the mouse huntingtin gene locus. A wide variety of knock-in mouse models have been generated, including HdhQ111, CAG140 and HdhQ144 (White et al. 1997; Lin et al. 2001; Menalled et al. 2002; Guidetti et al. 2006). Knock-in mice have also been shown to replicate HD symptoms, developing neurological and neurodegenerative phenotypes. Full-length mHTT was then incorporated into transgenic mice using either a bacterial artificial chromosome (BAC) or a yeast artificial chromosome (YAC) (Hodgson et al. 1999; Slow et al. 2005; Slow et al. 2003; Gray et al. 2008; Yu-Taeger et al. 2012). YACHD and BACHD mice show loss of striatal neurons, motor, cognitive and behavioral phenotypes similar to HD patients, as well as many molecular and neuropathological defects similar to HD patients, including sensitivity to NMDAR-mediated excitotoxicity(Graham et al. 2009; Gladding et al. 2012; Milnerwood et al. 2012; Fernandes et al. 2007; Van Raamsdonket al. 2005; Gray et al. 2008). YACHD and BACHD mice live significantly longer than N terminal fragment mice and their lifespan and symptomatic patterns are more characteristic of HD patients, making them a good model for long-term therapeutic testing experiments. More recently, a fully

humanized mouse model of HD has been developed, crossing YAC and BAC HD mice to create the first mouse model that recapitulated having two human huntingtin genes, without mouse huntingtin alleles and heterozygocity of the mHTT gene (Southwell et al. 2013). Lastly, large animal models are often the last stop before clinical testing of potential treatments for neurodegenerative diseases. At present there exist a few transgenic animal models such as a transgenic monkey (Chan et al. 2010) and a transgenic pig (Yang et al. 2010).

In all, a broad range of HD model systems exist in which to investigate the molecular underpinnings of HD, as well as test potential therapeutic treatments. The choice of model will depend on the particular goal of the experiment.

1.5 Therapeutic strategies for HD

Despite a knowledge of the underlying genetic causes of HD and good insight into the pathological changes associated with the disease, there still remains a distinct lack of effective HD therapeutics, and those that do exist are primarily focused on symptom management, rather than prevention of cell death and dysfunction. Given the potentially large window between diagnosis and onset of symptoms, many groups are currently investigating potential therapeutic targets for HD. Section 1.5 will overview some of the current research and strategies for HD therapeutics.

1.5.1 Huntingtin and mutant huntingtin lowering

As discussed, the underlying cause of HD is a CAG expansion in the huntingtin gene leading to expression of a mutated huntingtin protein (mHTT). The presence of mHTT leads to a wide range of cellular dysfunctions and many attempts to develop therapies for HD have focused on targeting events downstream of mHTT, or focus on relieving HD symptoms; however, an obvious

therapeutic strategy would be to try and remove mHTT by reducing its expression. There have been two major avenues for reducing huntingtin expression, which I will discuss in section 1.5.1: Preventing transcription and translation of HTT/mHTT, or enhancing degradation of HTT/mHTT.

1.5.1.1 Targeting huntingtin transcripts

HD is a unique neurodegenerative disease in that its underpinnings are purely genetic, caused by an autosomal dominant, CAG expansion mutation in the huntingtin gene. Given this, and our increasing understanding of transcriptional regulation and manipulation, reducing mHTT expression at the transcriptional level is an appealing strategy. Early studies using conditional mice expressing an HTT transgene with expanded CAG under control of a doxycycline-responsive promoter showed that an HD-like phenotype resulted with expression of the expanded gene that could be reversed following conditional mHTT transgene suppression (Yamamoto et al. 2000). This was the first evidence that removal of mHTT could be beneficial in improving the HD phenotype. Subsequently, there have been two major strategies for therapeutic reduction of mHTT transcription – RNA interference (RNAi) and antisense oligonucleotides (ASO). In RNAi, a small interfering RNA (siRNA) brings target mRNA into a complex of proteins called the RNA induced silencing complex (RISC). One strand of the siRNA duplex (the guide strand), loads the siRNA onto the RISC complex, then, through complementary binding of the siRNA, the RISC complex is guided to the target mRNA. Proteins in the RISC complex then cleave the mRNA target, preventing it from being translated into protein (Aronin & Difiglia 2014). siRNA targeting works via inherent cellular machinery expressed in mammalian cells, using similar proteins as endogenous microRNA gene silencing (Aronin & Difiglia 2014). Short hairpin RNA (shRNA) have also been tested to silence huntingtin transcripts in HD. Similar to siRNA, shRNA works to

degrade target mRNA via the RISC complex, however, unlike siRNA, which must be introduced to the cytosol via cell transfection, shRNA is transcribed in the nucleus via polymerase promoters (Moore et al. 2010). The resultant pre-shRNA is exported from the nucleus, processed by the Dicer protein, then loaded onto the RISC complex. The passenger (sense) strand is degraded, and the antisense strand guides the target mRNA for degradation

RNAi has shown therapeutic benefit in HD models both *in vitro* and *in vivo*. Huntingtin lowering with RNAi to reduce both HTT and mHTT expression has, numerous times, been shown to significantly improve neuropathology and behavioral deficits in HD mice (DiFiglia et al. 2007; Wang et al. 2005; Rodriguez-Lebron et al. 2005; Boudreau et al. 2009; Machida et al. 2006; Drouet et al. 2009; S. Harper et al. 2005) as well as in primate models of HD (Stiles et al. 2012). Interestingly, despite the embryonic lethality of HTT lowering (Zeitlin et al. 1995; Nasir et al. 1995) and importance of HTT in cell survival (discussed in 1.2) targeting both mHTT and HTT post-development appears to be relatively well tolerated. However, given the known neuroprotective qualities of HTT through adulthood, allele-specific silencing of mHTT may be preferable. Several mechanisms have been developed to attempt allele-specific silencing in HD. For example RNAi targeting the CAG repeat domain has been attempted (Hu, et al. 2009; Hu et al. 2010) The CAG expansion in mHTT vs HTT is not a unique sequence, as CAG repeats are a common motif across many genes, including WT HTT(Caplen et al. 2002) and specificity for expanded CAG decreases in repeat lengths common in HD population (Kay et al. 2014) . Instead, targeting single nucleotide polymorphisms (SNPs) found specifically in the mHTT allele have allowed for allele-specific transcript targeting using RNAi. mHTT-specific siRNA and shRNA against mHTT have subsequently proven beneficial, leading to significant improvements in neuronal dysfunction, cell loss and behavioral impairments in HD models (Zhang et al. 2009;

Lombardi et al. 2009; Pfister et al. 2009) as well as in cells from human Juvenile HD patients(Hu et al. 2010). An iteration of siRNA that has also been useful for HD drug design is single stranded RNA(ssRNA)(DiFiglia et al. 2007; Yu et al. 2012). ssRNA is more potent than siRNA, allow for mHTT-specific silencing and may be another way to specifically silence mHTT. Experiments using both allele-specific and non-specific RNAi for huntingtin show long lasting effects, with lowering of HTT/mHTT protein lasting weeks after single treatment, and, interestingly, behavioral and neuropathological improvements extending beyond knockdown, up to 14 weeks post treatment (Wang et al. 2005; Machida et al. 2006).

Unfortunately, RNAi has limitations. The efficacy of RNAi silencing depends on specificity, however many show off-target effects, resulting from binding of RNAi to similar sequences in non-targeted mRNA, immunostimulation, or inhibitory RNA overexpression which leads to disruptions in normal miRNA production(Harper 2009; McBride et al. 2008; Denovan-Wright et al. 2008). Secondly, though long-term lowering of mHTT/HTT may be required for effective treatment, and can be achieved relatively well with RNAi, the treatment is irreversible. Considering the potential for off-target effects, and the lack of good understanding about long-term use of RNAi vectors, better control would be ideal for mHTT silencing therapies. Also, administration of RNAi requires direct delivery into the affected area, primarily the striatum. Improved technology using pumps have allowed more steady delivery into HD primate brain(Stiles et al. 2012). However, the delivery system is invasive, and often requires viral vectors for deliver, the long term use of which is still largely unknown(Harper 2009; Aronin & DiFiglia 2014). Also, recent evidence suggests there are non-striatal and peripheral effects of mHTT such as skeletal muscle wasting and oxidative stress in blood(Zielonka et al. 2014; Koroshetz et al.

1997; Chen et al. 2007). The brain-specific lowering of huntingtin transcripts would do little to rescue mHTT-effects on peripheral issues.

ASOs have been another major strategy for targeted mHTT silencing in HD. ASOs are single stranded DNA molecules designed to interact with target RNA sequences, leading to steric hindrance and prevention of translocation, as well as induction of RNaseA, which cleaves the target mRNA (Bennett & Swayze 2010). Like RNAi, ASOs are blood brain barrier impenetrable, however, they are able to cross cellular membranes, allowing for more flexible delivery, for example by intraparenchymal bolus injection or via intrathecal delivery (Aronin & DiFiglia 2014). ASOs have shown exciting promise as potential therapeutics for HD. Similar to RNAi, in both HD mice and primates, broad HTT ASOs led to significant lowering of HTT and mHTT in brain and spinal cord, and improved neuropathological and behavioral effects of HD (Kordasiewicz et al. 2012). More recently, allele-specific ASOs have been developed using CAG expansion (Gagnon et al. 2010) or, more effectively, SNPs (Carroll et al. 2011; Bečanović et al. 2015; Southwell et al. 2014; Skotte et al. 2014; Ostergaard et al. 2013). In pre-clinical testing, candidate mHTT-specific ASOs lead to specific lowering of mHTT in the brains of HD mice that lasts for 18 weeks after a single ICV injection, without significant toxicity, and resulting in significant improvements in neuron dysfunction, degeneration, morphology and behavioral performance (Southwell et al. 2014; Skotte et al. 2014). Non-allele-specific silencing of mHTT using ASOs has recently moved into clinical testing for human HD patients, and may provide therapeutic benefit.

Again, despite powerful evidence that allele-specific ASOs may be an effective, long-term therapy for HD, there still exist limitations. Similar to RNAi, there exists possibilities for off-target effects, and delivery, though less invasive than RNAi, still requires direct delivery to the brain/cerebrospinal fluid and doesn't distribute outside of the CNS. Also, long-lasting effects,

desirable if effective, may lead to issues with slow-reversibility if toxicity occurs, difficult to predict from patient to patient.

Similar to RNAi and ASO lowering of mHTT, similar strategies to molecularly lower levels of mHTT at the transcript level have included using microRNA (miRNA), endogenously produced, small, non-coding RNA sequences that silence RNA transcripts via the RISC complex (Grondin et al. 2012; Kozlowska et al. 2013; Sinha et al. 2011) and newly developed CAG-directed zinc finger proteins, restriction enzymes which target mHTT DNA via fusion of a DNA binding domain to a DNA cleaving domain (Garriga-Canut et al. 2012). These strategies have also showed therapeutic benefits in late pre-clinical experiments in HD models (Mrzljak & Munoz-Sanjuan 2013), further supporting the idea that lowering levels of mHTT may be an effective therapeutic route for HD.

1.5.1.2 Enhancing clearance of huntingtin and mutant huntingtin

Another way that researchers have attempted to therapeutically lower levels of mHTT in HD is by enhancing endogenous protein degradation. As discussed in section 1.3.7.1 and 1.3.7.2, both the ubiquitin proteasome system (UPS) and autophagy are involved in the clearance of mHTT, and both have been shown to be impaired in HD. Thus, it has been hypothesized that enhancing UPS or autophagy activity may help increase mHTT clearance, helping to relieve the negative downstream effects in HD. Early experiments demonstrated that mTOR is a negative regulator of autophagy, and used rapamycin to inhibit mTOR, and subsequently increase autophagy (Ravikumar et al. 2004). Rapamycin, and a similar analog, effectively reduced huntingtin accumulation and mHTT aggregate formation and led to improvements in behavior in both fly and mouse HD models. Unfortunately, given the important role of mTOR in normal cell

function, and immunosuppressive side effects, rapamycin and mTOR inhibitors are not ideal therapeutics (Zuccato, Valenza & Cattaneo 2010). Subsequently, the same group demonstrated that lithium was also able to induce mTOR-independent autophagy, by inhibition of inositol monophosphatase, reducing inositol and IP3 levels (Sarkar et al. 2005; Sarkar & Rubinsztein 2006). Unfortunately, lithium can also inhibit glycogen synthase kinase-3beta (GSK-3beta), which normally stimulates mTOR and attenuates autophagy (Sarkar et al. 2008). To counter any potential anti-autophagy side effects via GSK-3beta after lithium, this group used combination lithium and rapamycin treatment, enhancing autophagy via both mTOR-dependent and mTOR-independent pathways(Sarkar et al. 2008), leading to significantly improved mHTT clearance and phenotypic improvements in HD drosophila. Using screening techniques, researchers are continually discovering new autophagy enhancers as potential therapies for HD (Sarkar 2013) and a wide range of compounds have been discovered that are able to enhance autophagy such as mTOR-independent drugs like trehalose (Sarkar et al. 2007) and rilmenadine (Rose et al. 2010) and many more. Many of these show therapeutic benefit in HD models.

Along the same lines, several groups have investigated increasing activity of the UPS as a means of enhancing mHTT clearance. Unfortunately, though many pharmacological inhibitors of the proteasome exist, some of which have reached the clinic for treatments in cancer (Richardson et al. 2006) there are no effective proteasome activators (Ortega & Lucas 2014). Current experiments are underway to develop proteasome activators, or enhance UPS protein clearance in other ways, such as pharmacological chaperones that stabilize properly folded proteins,or agents to help direct misfolded proteins to the proteasome(Balch et al. 2008).

It has been suggested that therapeutic strategies to enhance degradation of mHTT via autophagy or UPS degradation should be initiated early in disease progression, ideally pre-

symptomatically, to maximize turnover and prevent accumulation and aggregation of mHTT (Martin et al. 2015).

1.5.2 Targeting NMDAR-mediated excitotoxicity

NMDAR-mediated excitotoxicity, primarily via GluN2B-containing, extrasynaptic NMDARs has a key role in the pathogenesis of HD. Yet, broad NMDAR antagonists are generally not ideal therapeutics for due to the important role of NMDARs in cell survival and function (Ikonomidou & Turski 2002; Lai et al. 2011; Lai et al. 2014). Thus, many studies have investigated the potential of developing better HD therapeutics that target NMDAR-mediated excitotoxicity by inhibiting GluN2B-containing, extrasynaptic NMDARs and the associated downstream signaling, while preserving GluN2A, synaptic NMDAR activity and signaling pathways. The following section will outline some of the major efforts.

1.5.2.1 Targeting NMDARs directly

1.5.2.1.1 Blocking GluN2B, extrasynaptic NMDAR activity

Given the broad evidence supporting the role of GluN2B-containing, extrasynaptically located NMDARs in HD, NMDAR antagonism seems a natural goal in the development of therapeutics. Early studies investigating excitotoxic insults in stroke and neurotrauma attempted to block NMDARs using broad NMDAR antagonists. Unfortunately, these drugs led to undesirable side effects in clinical application, largely because they also block the essential, neuroprotective role of NMDARs in normal function including synaptic plasticity and cell survival (Ikonomidou & Turski 2002; Lipton 2004; Lai et al. 2014). Similarly, in HD, drugs that block the NMDAR, such as Lamotrigine, a non-competitive glutamate receptor antagonist, and Remacemide, a non-specific

NMDAR antagonist, though both showing benefit in HD animal models (Ferrante et al. 2002; Mary et al. 1995; Schilling et al. 2001) were ineffective in human trials and led to undesirable side effects. (Kieburtz et al. 1996; Mary et al. 1995, Kremer et al 1999). Thus, developing potent and specific antagonist drugs for GluN2B-containing, extrasynaptic NMDARs is an area of intense interest. Ifenprodil and other similar drugs are a class of NMDAR antagonists showing selective, non-competitive binding for GluN2B NMDARs(Williams 1993; Fischer et al. 1997; Gallagher et al. 1996; Kew et al. 1996). Initial experiments demonstrated effectiveness of these GluN2B antagonists in NMDAR-mediated excitotoxic models of stroke both *in vivo* and *in vitro* (Wang & Shuaib 2005; Liu et al. 2007a; DeRidder et al. 2006; O'Donnell et al. 2006; Chen et al. 2008; Gotti et al. 1988) suggesting that they may be more therapeutically relevant drugs for excitotoxic conditions. Recent studies have demonstrated the potential therapeutic benefit of GluN2B antagonism in HD, using a co-cultured system of MSNs and cortical neurons, a more physiologically relevant culture system for studying glutamatergic synapses in HD (Kaufman et al. 2012; Milnerwood et al. 2012). Enhanced whole cell and extrasynaptic NMDAR-currents, increased sensitivity to NMDA and increased cell-death specific signaling can be detected in YAC128 HD MSNs compared to wild type in this system (Milnerwood et al. 2012). However, blocking GluN2B activity in these cultures using ifenprodil protects against NMDAR insults and mHTT-mediated CREB shutoff, whereas blocking GluN2A NMDARs does not (Milnerwood et al. 2012; Zeron et al. 2002). Similarly, enhanced NMDAR currents seen in MSNs from YAC HD mice can be attenuated using ifenprodil (Zeron et al. 2002, Milnerwood et al. 2010), as well as toxic mHTT nuclear inclusions (Okamoto et al. 2009) suggesting that GluN2B NMDAR antagonism may help alleviated neuropathological changes in HD. Despite promising effects of GluN2B antagonists in HD and other NMDA excitotoxic disease models, there are still limitations,

namely 1) potential inefficacy of GluN2B antagonism without additional potentiation of GluN2A and subsequent cell survival signaling. 2) lack of strong subunit specificity of current GluN2B antagonists, 3) restrictive therapeutic window for GluN2B antagonists alone after an excitotoxic event(Yuan et al. 2015); these may explain the limiting results. In addition, the dose-limiting side effects of GluN2B antagonism in clinical applications of excitotoxicity(Yuan et al. 2015) and potential for negative outcomes by blocking GluN2B, which also has a role in synaptic plasticity, could contribute to lack of efficacy in improving neurological outcomes. For example, despite promising effects of ifenprodil in the YAC128 HD model, GluN2B-specific antagonists have shown varied effects. Subcutaneous injections of three different GluN2B antagonists, ifenprodil, RO25,6981 and CP101,606, failed to show benefits in an R6/2 HD model *in vivo* (Tallaksen-Greene et al. 2010) suggesting that effective therapeutics for HD may need to expand upon GluN2B antagonism. Subsequent studies have attempted to improve efficacy of GluN2B antagonists in *in vivo* models of NMDAR-mediated excitotoxic neuronal death. A recent study attempted to maximize GluNB antagonist effectiveness in ischemia based on the idea that ischemia is associated with acidification of tissues (Katsura et al. 1992; Yuan et al. 2015; Matsumoto et al. 1990). Subsequently this study developed pH sensitive GluN2B antagonist compounds using medicinal chemistry to help limit NMDAR antagonism to ischemic tissue, while reducing effect in healthy brain (Yuan et al. 2015). These compounds provided significant neuropathological and behavioral improvements with minimal side effectssuggesting that limiting GluN2B NMDAR antagonism to areas of the brain undergoing excitotoxicity may help mitigate some of the negative consequences of NMDAR antagonism, and pave the way to effective therapies.

Concurrent with GluN2B antagonism, another potential antagonism-based therapy for excitotoxicity in HD is memantine, the only current clinically-approved NMDAR antagonist.

Memantine is a non-competitive NMDAR antagonist with fast on-off kinetics that, at low doses, has shown to preferentially block tonically-activated, extrasynaptically located NMDARs but not phasically-activated synaptic NMDARs (Xia et al. 2010). Memantine is currently approved as a prescription medication for Alzheimer's Diseases patients, and has shown to delay onset of behavioral and cognitive symptoms in human patients (McShane et al. 2006; Howard et al. 2012). Given the critical role of extrasynaptic NMDARs in HD pathogenesis (Parsons & Raymond 2014; Milnerwood et al. 2012; Kaufman et al. 2012) researchers have investigated the potential of memantine to act as a therapeutic strategy against excitotoxicity in HD. Given at a low dose, shown to preferentially block extrasynaptic NMDARs, memantine was able to abolish the early sensitivity to NMDA seen in YAC128 HD mouse striatum *in vivo* (Okamoto et al. 2009). Memantine treatment also lead to motor improvement and reduced striatal loss (Okamoto et al. 2009; Milnerwood et al. 2010). More recently, memantine treatment in YAC128 HD mice was shown to also rescue synaptic dysfunction and downstream cell death signaling in HD, normalizing enhanced extrasynaptic NMDAR expression, reducing calpain activtion, reducing p38 MAPK activation and rescuing CREB shutoff (Dau et al. 2014). Synaptic NMDAR activity and signaling were unaffected by low-dose memantine (Dau et al. 2014).

While broad NMDAR antagonism isn't ideal for HD therapeutic development, these studies demonstrate that disease- or area-specific GluN2B- or extrasynaptic-specific NMDAR antagonism may provide neuroprotective benefit in HD.

1.5.2.1.2 Enhancing GluN2A, synaptic NMDAR activity

Based on evidence demonstrating that synaptically-located, GluN2A-containing NMDARs are preferentially tied to several fundamental cell-survival pathways (Giles E. Hardingham & Bading

2003; Hardingham et al. 2002; Hardingham & Bading 2010; Martel, Tomás J. Ryan, et al. 2012; Lai et al. 2014; Liu et al. 2007b) and that blockade of GluN2A-containing NMDARs using the antagonist NVP-AAV077 significantly worsens apoptosis, cell death and behavioral outcomes *in vitro* or *in vivo* models of stroke (Liu et al. 2007) a better option may be to enhance the function of GluN2A-containing, synaptic NMDARs to improve cell survival and function after excitotoxic insults. It has been shown that brief bath application of suprasaturating doses of glycine leads to selective stimulation of synaptically located NMDARs (Man et al. 2003; Lu et al. 2001). As glycine is a co-agonist of NMDARs, bath glycine application enhances activation of NMDARs selectively located in the synapse that are stimulated by spontaneous presynaptic glutamate release (Man et al. 2003; Lu et al. 2001) and not extrasynaptic NMDARs, which are not active in an unstimulated state. Glycine enhancement of synaptic GluN2A-containing NMDARs leads to enhancement of cell survival signaling and reduces apoptotic cell death in *in vitro* NMDAR-mediated excitotoxicity (Liu et al, 2007). Similarly, in an MCAO stroke model in rats, glycine given post-stroke significantly reduced infarct size, an effect that was enhanced when animals were co-treated with GluN2B antagonist Ro-25–698(Liu et al, 2007) .One limitation of GluN2A agonism as a potential target for excitotoxic death is that there is a lack of specific, effective GluN2A agonists available. Of note, recent studies suggest that synaptic and extrasynaptic NMDARs may have different co-agonists, with glycine being more specific for extrasynaptic NMDARs and d-serine co-gating synaptic NMDARs (Papoudin et al. 2012). Using receptor crystal structure and modeling, several studies are now attempting to develop more potent GluN2A-containing NMDAR positive modulators. Similarly, despite many studies supporing a survival role of synaptic NMDARs, some studies refute this and suggest synaptic NMDARs can also mediate cell death (Wroge et al 2012). However, based on data in excitotoxic stroke models, it is possible

that these compounds to enhance synaptic, GluN2A NMDARs may show neuroprotective benefit against NMDAR excitotoxicity and may be a promising next step in the search for better HD therapeutics, especially if used in conjunction with extrasynaptic GluN2B antagonism.

1.5.2.2 Targeting glutamate release and uptake

Another way in which striatal neurons may be particularly prone to NMDAR mediated excitotoxicity in HD is through impaired glutamate release and uptake. Though not observed in all mouse models (Li et al. 2004), some transgenic mouse models, such as the R6/2 HD model, show increased spontaneous EPSCs in striatum MSNs in acute slices in pre-symptomatic animals (Cepeda et al. 2003) indicative of enhanced glutamate release. Similarly, in HD patients and transgenic HD mice, metabolites 3-hydroxykyneurine, enhancing oxidative stress or quinolinic acid, stimulating NMDARs, are both augmented (Guidetti et al. 2004; Guidetti et al. 2006) further suggesting upstream changes that could enhance excitotoxic vulnerability in MSN NMDARs. Along these lines, impaired glutamate transport may also play a key role in HD excitotoxicity. Glutamate transporters play an important role in preventing glutamate buildup at the synapse, re-uptaking excess to prevent excitotoxicity. However, in HD, some studies have suggested that glutamate re-uptake is impaired. In R6/2 HD mice, very early deficits in mRNA expression of the important glial transporter GLT-1 can be detected in both cortex and striatum tissues(Liévens et al. 2001) as well as decreased protein expression (Faideau et al. 2010; Liévens et al. 2001). Impaired basal glutamate uptake by GLT-1 in HD mouse models has also been reported by using microdialysis (Miller et al. 2008), measurement of impaired glutamate uptake in synaptosomes (Huang et al. 2010; Liévens et al. 2001) and D-aspartate binding (Liévens et al. 2001). Human HD brains similarly show impaired glutamate uptake , using a [^{3}H]-glutamate uptake assay or

measuring [^{3}H]-aspartate binding(Cross et al. 1986; Hassel et al. 2008). Subsequent studies have investigated the potential of reversing impairments in GLT-1 in HD models. The antibiotic ceftriaxone, shown to elevate expression of GLT-1, was recently used in R6/2 HD mice, given for 5 days (Miller et al. 2008). The drug effectively enhanced GLT-1 levels, reversed impaired glutamate uptake in HD animals vs wild type and led to improvements in HD phenotype (Miller et al. 2008). However, recent data using a more physiological *in situ* model of glutamate uptake demonstrated no impairments in glutamate clearance following synaptic release in YAC128 or R6/2 mice (Parsons et al. 2016).

Along the same lines, suppressing enhanced glutamate release in HD has been investigated as a potential therapeutic strategy for HD. In early studies of HD, the drug Riluzole, shown to inhibit glutamateric transmission in the CNS, was one of the first compounds to show therapeutic benefit in HD models(Seppi et al. 2001; Schiefer et al. 2002; Guyot et al. 1997; Mary et al. 1995). These findings led to clinical tests of Riluzole in human HD patients; however, results were disappointing, neither slowing onset or reducing symptoms(Seppi et al. 2001; Landwehrmeyer et al. 2007)

1.5.2.3 Targeting cell-death specific signaling of NMDARs

Given the ineffectiveness of using broad NMDAR antagonism as a therapeutic target, a potential druggable strategy for targeting NMDAR-mediated excitotoxic death focuses on downstream pathways activated by NMDAR stimulation. As discussed, synaptic, GluN2A-NMDAR stimulation is specifically associated with many cell-survival signaling pathways. These pathways are antagonized during NMDAR-excitotoxicity by extrasynaptic GluN2B-NMDAR stimulation, which trigger downstream signaling associated with cell death. By developing therapeutic targets

that specifically shut down cell death signaling associated with GluN2B, extrasynaptic NMDARs, the cell-survival signaling machinery of synaptic, GluN2A NMDARs may remain intact, preventing the negative side effects of NMDAR antagonism.

1.5.2.3.1 PSD-95

One way in which NMDARs confer differential outcomes on cell death and cell survival is by coupling directly with different interacting proteins. By interacting with proteins located specifically at synaptic or extrasynaptic locations, NMDARs may be functionally linked to different downstream signaling pathways for cell survival or cell death. Similarly, GluN2A and GluN2B NMDARs have shown to interact preferentially with cell survival and cell death-specific signaling molecules, respectively, by differential direct coupling via the NMDAR C-terminus (Foster et al. 2010; Sprengel et al. 1998; Martel et al. 2009) These important differences have allowed scientists opportunity to develop therapeutic potential drugs for NMDAR excitotoxic conditions that aim to disrupt direct or indirect NMDAR interaction with cell-death specific molecules, such as neuronal nitric oxide synthase(nNOS) (Aarts et al. 2002; Zhou et al. 2010; Sattler & Tymianski 2000), death-associated protein kinase 1 (DAPK1) (Tu et al. 2010; Fan et al. 2014) and PTEN (Zhang et al. 2013a). A particularly important target of NMDAR-mediated interaction protein studies is PSD-95, a membrane-associated guanylate cyclase (MAGUK) that is found concentrated at the postsynaptic density of glutamatergic synapses, with essential roles in synapse stabilization and plasticity (El-Husseini et al. 2000). PSD-95 binds directly to GluN2B via PDZ domains(Kornau et al. 1995; Brenman et al. 1996), which has shown to facilitate excitotoxic death signaling, by linking GluN2B-NMDARs with nNOS (Aarts et al. 2002; Sattler & Tymianski 2000). mHTT has shown to exacerbate GluN2B-NMDAR interaction, with enhanced

GluN2B-PSD-95 binding detected in striatal tissue from YAC transgenic HD mice at times when enhanced NMDA sensitivity is present(Fan et al. 2009) whereas this effect is gone later, when NMDA resistance occurs (Jarabek et al. 2004). Uncoupling of PSD-95 with GluN2B using a small interfering peptide NR2B-9c (Aarts et al. 2002) reduced striatal sensitivity to NMDA to levels observed in WT MSNs (Fan et al. 2009). However, this mHTT-PSD-95-NMDAR mechanism in HD is thought to be independent of nNOS, and rather dependent on enhanced activation of p38 MAPK cell death signaling (Fan et al. 2012) as enhanced p38 MAPK can also be rescued with GluN2B-9c in HD transgenic models (Fan et al. 2012).

Another important role of PSD-95-GluN2B interaction in HD is through regulation of NMDAR trafficking and localization. PSD-95 interaction with NMDARs via the C-terminal tail has previously been shown to regulate NMDAR stabilization at synapses (Roche et al. 2001; Lin et al. 2004; Prybylowski et al. 2005). In the striatum of YAC transgenic models of HD, altered NMDAR trafficking includes enhanced surface expression of NMDARs (Fan et al. 2009) as well as increased expression of PSD-95 at extrasynaptic sites (Milnerwood et al. 2010), shown to sensitize HD mice to NMDA excitotoxic cell death. Wild type huntingtin itself has also been shown to interact strongly with PSD-95 directly, linking it to NMDARs (Shirasaki et al. 2012), however, in HD, expanded polyglutamine weakens the HTT-PSD-95 interaction (Sun et al. 2001), leading to enhanced sensitivity of existing NMDARs to NMDA. Similarly, impaired mHTT-PSD-95 interaction increases extrasynaptic expression of PSD-95, a normally synapse-specific protein (Fan et al. 2012). Taken together, PSD-95's strong interaction with GluN2B, strengthened by the presence of mHTT is thought to facilitate extrasynaptic expression of GluN2B-containing NMDARs by stabilizing them via PSD-95 (Fan et al. 2012; Parsons & Raymond 2014; Parsons et al. 2014). The NR2B-9c peptide further shows benefit to HD models by partially correcting

mistaken trafficking of NMDARs, mildly reducing the enhanced surface expression of NMDARs in HD transgenic MSNs (Fan et al. 2009).

Several studies have investigated other potential ways to correct impaired PSD-95 and NMDAR localization in HD. Mislocalization of PSD-95 and extrasynaptic NMDARs in HD has been shown to be linked to post-translational modifications of NMDARs such as caspases, calpains, phosphorylation and palmitoylation (Gladding & Raymond 2011; Parsons & Raymond 2014, Gladding et al. 2012). HD is linked with impairments in palmitoylation (Young et al. 2012; Sanders et al. 2015). Wild type HTT, along with its strong interaction with PSD, also interacts with the palmitoylacytransferase DHHC17, otherwise known as Huntingtin Interacting Protein 14 (HIP14) (Huang et al. 2011; Singaraja et al. 2011; Sutton et al. 2013). In HD, mHTT-HIP14 interaction is impaired(Huang et al. 2011; Singaraja et al. 2011), and genetic knockout mice for HIP14 or the HIP14-like protein (HIP14L) share similar phenotypical features with HD(Singaraja et al. 2002). Palmitoylation of NMDARs can impact their surface expression and trafficking(Hayashi et al. 2009; Mattison et al. 2012), and similarly, palmitoylation of PSD-95 is important for synaptic targeting of the GluN2B-PSD-95 complex (Craven & Bredt 2000; Parsons & Raymond 2014). Along with HD-like phenotype, Hip14-/- mice also show reduced palmitoylation of PSD-95 (Singaraja et al. 2002). Current studies are investigating the role of impaired palmitoylation in HD on PSD-95 and NMDAR expression and localization.

1.5.2.3.2 Calpains and STEP

Calcium influx as a result of NMDAR stimulation activates a wide variety of downstream molecular mechanisms. Calpains, a class of calcium-dependent cysteine proteases have been shown to have a role in NMDAR-mediated HD pathogenesis. Excitotoxic NMDAR activation of

calpains(Siman & Carl Noszek 1988) has been shown to have a key role in proteolysis, impaired synaptic function and neuronal damage after an NMDAR-mediated excitotoxic event(Arai et al. 1991; Lee et al. 1991; Arlinghaus et al. 1991; Lai et al. 2014; Rami & Krieglstein 1993). NMDAR-mediated calpain activity can also cause further calcium overload after NMDAR calcium entry, due to calpain-mediated cleavage of the sodium-calcium exchanger (NCX) (Bano et al. 2005), creating enhanced cell vulnerability to calcium-mediated cell death pathways (Lai et al. 2014). Calpain activation after NMDAR excitotoxicity appears to be GluN2B-specific, triggering cell death signaling such as the 35-kDa regulatory activator (p35) of cdk5(Patrick et al. 1999; Lee et al. 2000), cleaving it into the toxic p25 fragment, and can be blocked using antagonists for GluN2B but not GluN2A. Calpains are also differentially regulated by synaptic and extrasynaptic NMDARs, with synaptic NMDARs activating μ-calpain while extrasynaptic NMDARs activate m-calpain (Wang et al. 2013). m-calpain in particular cleaves striatal enriched tyrosine phosphatase (STEP), which subsequently activates p38-activated mitogen activated kinase (p38MAPK) (Wang et al. 2013; Xu et al. 2009), further contributing to differential NMDAR effects(Parsons & Raymond 2014)

In mouse models of HD, early, pre-symptomatic enhancement of calpain activation can be detected, along with a subsequent increase in the synaptic-specific STEP61(Gascón et al. 2008; Cowan et al. 2008; Gafni & Ellerby 2002; Gladding et al. 2012; Dau et al. 2014). Calpain and STEP61 activation significantly contributes to enhanced NMDA sensitivity in HD by dephosphorylating the GluN2B NMDAR subunit, leading to enhanced expression of extrasynaptic and not synaptic NMDARs (Gladding et al. 2012). STEP61 also contributes to neuronal death in HD by dephosphorylation and deactivation of the survival-specific proteins ERK1/2 (Gladding et al. 2014) as well as STEP33 (a cleavage product of STEP61)-mediated activation of p38 MAPK,

leading to p38 activation and subsequent cell death (Gladding et al. 2014). Activated p38MAPK can be detected in the striatum of transgenic HD mice both pre-symptomatically, during enhanced sensitivity to NMDA, and in later, NMDA resistant stages (Fan et al. 2012; Saavedra et al. 2011). Inhibition of calpain has shown to reverse the enhanced extrasynaptic NMDAR expression seen in the YAC128 HD model(Gladding et al. 2012; Gladding & Raymond 2011; Parsons & Raymond 2014). Calpain effects appear to be particularly important at early stages of the disease, as enhanced calpain is only detected at early, pre-symptomatic stages(Dau et al. 2014). These data point to calpain inhibition as potential early target to prevent enhanced sensitivity to NMDA as well as prevent p38 activation in later disease stages (Gladding et al. 2012). However, calpains are ubiquitously expressed and necessary for many aspects of cell survival (Lai et al. 2014) thus they should be viewed with caution when developing therapeutics.

1.5.2.3.3 Caspases

A major downstream pathway associated with NMDAR-mediated excitotoxic and subsequent apoptotic death is the activation of caspases. Caspases are cysteine-aspartic proteases, activated as a part of the apoptotic cascade that act to post-translationally modify proteins by cleaving at specific sites, which may act as a gain- or loss- of function modification(Pop & Salvesen 2009). Caspases play an important role in apoptosis, and initiator caspases, activated both stress- and cell-type dependently, can initiate proteolytic caspase cascades, leading to recruitment and activation of executioner caspases and apoptotic cell death (Pop & Salvesen 2009; Graham et al. 2011). Caspase activation and apoptosis have shown to play an important role in HD pathogenesis. TUNEL staining in the affected areas of HD brains (Dragunow et al. 1995) indicates that apoptotic cell death contributes to HD neuropathy (Fan et al. 2007). Similarly,

activated caspase-8 and caspase-6 can be detected in the tissues of transgenic HD animal models, as well as in the brains of both early and late stage HD patients (Sánchez et al. 1999; Graham, Slow, et al. 2006; Hodges, Andrew D. Strand, et al. 2006; Graham et al. 2010; Hermel et al. 2004) and increased proform casp3 can be detected in later stage HD human brain tissue (Graham et al. 2010) The huntingtin protein contains several consensus sites for caspase cleavage(Wellington et al. 1998; Wellington et al. 2000) and is proteolytically cleaved by caspases in both HD and normal tissues. In HD, caspase cleavage of the mutated mHTT creates toxic fragments, shown to have a key role in HD pathogenesis both *in vitro* and *in vivo* (Graham et al. 2006; Graham et al. 2010; Wellington et al. 2002; Mangiarini et al. 1996). Caspase-mediated apoptosis in HD appears to be NMDA excitotoxicity-dependent. Cultured MSNs from transgenic YAC128 HD mice brains have enhanced casp6 mRNA (Graham et al. 2006) and cultured YAC46 and YAC72 HD striatal neurons have increased levels of active casp3 after NMDA stimulation (Zeron et al. 2002). Similar increases in casp3 are detected in human HD lymphoblasts when stimulated with mitochondrial stressors (Sawa et al. 1999). Caspase activation effects in HD appear to be mediated by the intrinsic apoptotic cascade, rather than the extrinsic death-receptor mediated pathway(Zeron et al. 2004).

Several studies have investigated the potential for caspase inhibition as a means of protecting against HD-induced neuropathologies. In cultured HD neurons, inhibition of casp3, casp6 or casp9 protects cells against NMDAR-mediated apoptotic death (Tang et al. 2005; Graham, Deng, et al. 2006). Similarly, minocycline treatment, reducing casp1 and casp3 mRNA, slows HD progression in mouse HD mode ls *in vivo* (Chen et al. 2000). However, casp3 inhibition alone doesn't work in all HD models (M. Kim et al. 1999). Casp6, in particular, has been proposed as an initiator caspase in apoptotic HD cell death cascades (Graham et al. 2011). Levels of activated casp6 correlate positively with the size of the CAG repeat and inversely with age of HD onset.

Transgenic HD mice engineered with a casp6 resistent mHTT (C6R mice) show neuroprotection against HD striatal atrophy and neurodegeneration (Graham, Deng, et al. 2006). Similarly, C6R striatal neurons show neuroprotection against NMDA and staurosporine *in vitro* and C6R mice have reduced quinolinic acid-induced striatal lesions, improved behavioral outcomes and improvements in neurological HD changes (Graham, Deng, et al. 2006; Mahmoud A Pouladi et al. 2009; Warby et al. 2008; Graham et al. 2011; Graham et al. 2010; Milnerwood et al. 2010). Similarly, chemical casp6 inhibition (Graham et al. 2010), dominant-negative caspase inhibition (Hermel et al. 2004) or genetic silencing of casp6 (Uribe et al. 2012; Wong et al. 2015) are neuroprotective and behaviorally beneficial in several models of HD. These data, as well as data demonstrating early activation of casp6 in pre-symptomatic and early stage HD brains, while casp3 isn't active until later in disease progression (Graham et al. 2010) suggest that casp6 may be activated early, followed by casp3 activation, and may be a possible therapeutic target for HD (Graham et al. 2011).

1.5.3 Targeting dopaminergic signaling

The striatum is an important area in the brain for dopaminergic neurotransmission, with dopamine released on nigrostriatal terminals. However, injection of dopamine directly into the striatum is neurotoxic (Jakel & Maragos 2000; Hastings et al. 1996; Ben-Shachar et al. 1995) and it has been suggested that over-active dompaminergic striatal signaling may be an underlying cause in HD, relating to chorea and neurotoxicity (Jakel & Maragos 2000; André et al. 2011; Pineda et al. 2005; Cyr et al. 2006; Charvin et al. 2005; Benchoua et al. 2008). Subsequently, inhibitors of dopaminergic signaling, such as tetrabenazine, have been shown to be beneficial in HD, reducing the motor deficits and improving striatal loss in HD mice (Tang et al. 2007). Tetrabenazine has

been tested in controlled clinical HD trials in humans (Frank et al. 2008; Huntington Study Group 2006), and has been approved as a treatment for HD, to improve chorea symptoms. However, tetrabenazine has been shown to cause side effects, such as depression, sedation and Parkinson's Disease-like symptoms (Frank et al. 2008; Huntington Study Group 2006). Another drug, ACR16, or pridopidine, has also been tested to target dopaminergic signaling in HD. This D2-targeting compound acts as a dopamine stabilizer *in vivo* and has been suggested to improve cortico-striatal synaptic function that is impaired in HD (Ponten et al. 2010). ACR16 is currently in clinical trials in human patients (Lundin et al. 2010)

1.5.4 Strategies to increase BDNF

As outlined in section 1.3.4, reduced BDNF in HD contributes to impairments and neuropathology seen in the disease. As a result, increasing BDNF has been explored as a means to treat HD. Many studies have investigated ways to enhance BDNF clinically in patients, including recombinant BDNF (Kasarskis et al. 1999), viral vector delivery of gene targets (Zuccato & Cattaneo 2007), intrastriatal cell grafts that release BDNF (Pérez-Navarro et al. 2000; Ryu et al. 2004; Zuccato & Cattaneo 2007) drugs that mimic BDNF action on Trk receptors (Massa et al. 2010), diet and environmental enrichment (Sullivan et al. 2001) or small molecules that enhance BDNF through other pathways (Conforti et al. 2013). Though some of these therapies have been met with disappointment in clinical trials, many are still underway and the potential to enhance BDNF in HD remains of interest.

1.5.5 Other small molecule therapeutics

The loss of MSNs in HD appears to be a major neuropathological mechanism of the diseas. Significant changes in MSN structure and function, as well as striatal dysfunction has been shown to play an important role in HD pathology (Mrzljak & Munoz-Sanjuan 2013). Impairments in cortico-striatal synaptic signaling is an early impairment in both HD animal models as well as in patients, and significant impairments in cannabinoid, dopamine and cholinergic signaling pathways have also been implicated in HD (Heikkinen et al. 2012; Switonski et al. 2012; André et al. 2011; Cepeda et al. 2003; Eidelberg & Surmeier 2011; Horne et al. 2013; Pisani et al. 2007; Vlamings et al. 2012). Thus, an ongoing strategy of HD therapeutic development is the better understanding of the precise impairments to both cortico-striatal as well as thalamo-striatal synapses to develop drugs to interact with and restore the synaptic deficits of HD (Mrzljak & Munoz-Sanjuan 2013). A wide array of small molecule therapeutics are currently under investigation for HD. Loss of CB1 cannabinoid receptors in HD has been one of these targets (Blazquez et al. 2011) For example, a recent study investigated several CB1 cannabinoid receptor activating drugs in a mouse model of HD (Dowie et al. 2010). Though therapeutic results were limited, researchers are expanding on this work to determine potential effects. Similarly, THC delivery, to activate CB1 receptors has shown to attenuate neuropathology and behavioral effects in HD mice (Blazquez et al. 2011). Cannabinoid receptor targeting in HD has also been shown to potentially benefit several other synaptic impairments in HD, such as adenosine receptor signaling and cortico-striatal receptor signaling, as cannabinoids have an important modulatory role(Mrzljak & Munoz-Sanjuan 2013). Along with CB1 targets, many research groups are investigating a wide range of potential small molecule therapeutics that act on impaired signaling pathways and synaptic dysfunction in HD, such as mitochondrial impairment and cellular energy dysfunction

(Hickey et al. 2012; Van Raamsdonk et al. 2005), phosphodiesterases (Rutten et al. 2006), and HDACs (Benn et al. 2009; Mrzljak & Munoz-Sanjuan 2013) Initiatives such as the Cure Huntington's Disease Initiative, in partnership with several pharmaceutical partners are currently working together with researchers to better understand the diverse network of synaptic impairment in HD and develop novel molecules or repurpose current drugs to help develop new therapeutic approaches (Mrzljak & Munoz-Sanjuan 2013).

1.6 Cell-penetrating peptide therapies for neurological disease

In recent years, peptide drugs have been surfacing as potential therapeutic tools for treating neurological disease. Peptide drugs are small, single chains of amino acids that can be designed and synthesized to act as signaling molecules to specifically and efficaciously bind to molecular targets and influence their function (Fosgerau & Hoffmann 2015). Peptides represent a particularly interesting therapeutic tool for many reasons that may give them some advantage over small molecule and other drug mechanisms. For one, because peptides drugs are similar to small, naturally-occurring peptides in the body, they have been shown to often translate with good safety and tolerability in humans (Fosgerau & Hoffmann 2015). Similarly, the predictable metabolic breakdown of peptide drugs in the body, with high turnover and short half-life, make them particularly desirable, especially for novel therapeutic targets in which non-reversible effects may not be ideal, and allowing the potential for better clinical control(Fosgerau & Hoffmann 2015; McGregor 2008). Peptides naturally have a very short half-life, and studies using peptide drugs have shown that peptides can be cleared from the body by natural degradation within several hours to a day(Zhang et al. 2013b; Fan et al. 2014; Taghibiglou et al. 2009). Similarly, peptides are relatively simple to synthesize in a lab environment and can be designed to interact specifically

with molecular targets, giving broad therapeutic flexibility (Fosgerau & Hoffmann 2015; McGregor 2008). Though, in their natural state, peptides cannot cross plasma membranes, using cell-penetrating peptide sequences such as TAT, isolated from the HIV virus (Schwarze 1999), peptide-based drugs can be made to cross both the blood brain barrier and the plasma membrane of cells. These attributes make them relatively easy to deliver, and have been shown to distribute widely through the body through intramuscular, intraperitoneal or intranasal delivery (Zhang et al. 2013b; Taghibiglou et al. 2009; Fan et al. 2014) Many studies have demonstrated the therapeutic benefits of peptide-based therapies in animal models for neurological disorders such as stroke (Zhang et al. 2013; Taghibiglou et al. 2009; Aarts et al. 2002; Fan et al. 2014), some of which have moved into clinical trials with positive outcome (Hill et al. 2012). Similarly, several peptide based drugs have been approved in clinical trials(Kaspar & Reichert 2013), including glucagon-like peptide 1 receptor (GLPR1) agonists which have shown benefit in treating diabetes, as well as some positive benefits in HD mouse models (Martin et al. 2009) as well as several other peptides to treat conditions such as anemia, Cushing's Disease and constipation (Kaspar & Reichert 2013)

Along with the positive benefits of peptide therapeutics, there do exist potential risks and downfalls as well. By the same token, the short half-life and chemical instability of peptide drugs may be detrimental for long-term or chronic treatments where long lasting effects are necessary. Similarly, most peptide drugs are not orally bioavailable (Fosgerau & Hoffmann 2015) which means regular injections may be necessary. Similarly, introducing novel peptides into the body has the potential to induce immunogenic response(Kaspar & Reichert 2013; Fosgerau & Hoffmann 2015; McGregor 2008). However, current research is underway to help mediate many of these potential downfalls. For example, extension of peptide half-life can be achieved by structural analysis, identifying enzymatic cleavage sites, and substituting amino acids(Fosgerau & Hoffmann

2015). Similarly, attaching linker proteins or inserting protective sequences or molecules to the peptide can also help extend the half-life, as well as modifying structure by cyclization(Timmerman et al. 2009; Houston et al. 1996; Werle et al. 2006). High cost of peptide synthesis may also be a potential downfall of this therapy, however, as technology improves, it is like that a combination of increased stability and technology advancement will help lower synthesis cost.

In general, peptide therapies have provided an exciting new way to think about treatment of many diseases, and recent advancements in peptide-based therapeutics for stroke suggest that peptide therapies may be a new way to approach treatment for neurological disease.

1.7 Hypothesis and Rationale

Despite a wide range of studies investigating molecular and neurological changes in HD and many studies working on developing therapeutics, there is still a distinct lack of effective treatments for HD, and currently, the only approved treatments target symptom management, rather than delaying or preventing onset. Since HD is a genetic disorder, for which there exists predictive testing, there is opportunity to detect the disease early, leaving a potentially large therapeutic time window for preventative medicine. At present, there is a clear need for effective early, preventative drugs for Huntington's Disease. Luckily, scientists have discovered many early molecular changes in HD brains that are implicated in HD pathology, many of which occur far before symptom onset, leaving opportunity for the development of therapeutics acting on these early molecular targets. In particular, peptides, with their capacity to interact specifically and effectively with molecular targets, quick reversability and their non-invasive delivery route, may be a novel way to think about early preventative medicine for HD. In the current study, I investigate three potential early

preventative therapies for Huntington's disease, based on early molecular changes associated with neuronal death and dysfunction, in the hopes of creating novel, primarily peptide-based therapeutics for HD. My thesis work is separated into three sections, based on three distinct, early preventative therapeutic targets.

Target 1: The role of PTEN nuclear translocation in NMDAR-mediated neuronal death in HD

The first target is based on strong evidence supporting early sensitivity of HD neurons to NMDAR-mediated excitotoxicity (discussed in detail in section 1.3.8). These experiments will be discussed in Chapter 2. Excitotoxicity due to NMDAR over-activation has a key role in HD pathogenesis, with enhanced NMDAR currents (Zeron et al. 2002; Cepeda et al. 2003), increased sensitivity to NMDAR-mediated excitotoxicity(Zeron et al. 2002; Shehadeh et al. 2006; Graham et al. 2009), enhanced forward trafficking of NMDARs (Fan et al. 2007) and increased NMDAR surface expression observed in HD models, even pre-symptomatically, indicating a role of NMDARs in disease progression. NMDAR-mediated excitotoxicity in HD appears to be primarily mediated by GluN2B-containing, extrasynaptically located NMDARs (Zeron et al. 2002; Tang et al. 2005; Fan et al. 2007; Cowan et al. 2008; Milnerwood et al. 2012; Milnerwood et al. 2010). Unfortunately, broad NMDAR antagonists may pose difficulties as therapeutics (Ikonomidou & Turski 2002; Lai et al. 2014; Tymianksi 2014), due to the important role of NMDAR signaling in normal cell function. An alternative to NMDAR antagonists is to target cell death-specific signaling pathways downstream of GluN2B, extrasynaptic NMDARs, while preserving the positive, cell-survival signaling of GluN2A-containing, synaptic NMDARs. Determination of downstream molecular targets associated with NMDAR-mediated cell death signaling may allow

for the possibility to develop peptide-based therapeutics to interact specifically with excitotoxic signaling to silence them. This approach has been successful in other models of NMDAR-mediated excitotoxicity, such as ischemic stroke, with peptides being designed to act on cell-death specific targets such as cleavage of sterol-regulated element binding protein 1 (SREBP1), activated Death Associated Protein Kinase 1 (DAPK1) and disruption of GluN2B interaction with PSD-95(Aarts et al. 2002). Given the important role of excitotoxicity in HD, it is possible that utilizing a similar approach may be a viable way to specifically target the detrimental GluN2B, extrasynaptic NMDAR signaling that is enhanced in the disease.

We have recently demonstrated that nuclear translocation of phosphatase and tensin homolog deleted on chromosome ten (PTEN), a tumor suppressor protein, is a critical step in NMDAR mediated excitotoxic neuron death in ischemic stroke models (Zhang et al. 2013). In our studies, blocking PTEN nuclear translocation with our lab-designed interference peptide, Tat-K13, prevents NMDA-induced neuronal death *in vitro* and *in vivo*. (To be discussed in more detail in Chapter 2, introduction). Given the role of NMDAR-mediated excitoxicity in HD, we hypothesize that **PTEN nuclear translocation may have a role in NMDAR-mediated excitotoxic neuron death in HD**. Project 1 will investigate the mechanisms of NMDAR-mediated excitotoxicity in a mouse model of HD, and investigate the possibility of targeting PTEN-nuclear translocation as a potential therapeutic.

Target 2: Activation of caspase-6 and cleavage of caspase-6 substrates is an early event in NMDA receptor-mediated excitotoxicity.

Chapter 3 represents a set of experiments that investigate caspase activation after an NMDAR-mediated excitotoxic event. Significant research suggests that activation of caspases downstream

of NMDAR-mediated excitotoxic events in HD play a critical role in disease pathogenesis (see section 1.5.2.3.3). Activated forms of caspase 8, -3 and -6 can be found in the brains of both animals models of HD and human HD patients(Sánchez et al. 1999; Graham, Slow, et al. 2006; Hodges, Andrew D. Strand, et al. 2006; Graham et al. 2010; Hermel et al. 2004) (Graham et al. 2010) and caspase cleavage of mHTT contributes to the formation of toxic N-terminal mHTT fragments(Graham, Deng, et al. 2006; Graham et al. 2010; Wellington et al. 2002; Mangiarini et al. 1996). Caspase-6 is particularly important in HD pathogenesis, and is related to NMDAR-mediated excitotoxcity in HD (Graham et al. 2011). Several studies have investigated casp6 as a potential therapeutic target for HD with exciting promise, suggesting that silencing casp6, genetically or chemically, may provide therapeutic benefit in HD (Graham, Deng, et al. 2006; Pouladi et al. 2009; Warby et al. 2008; Graham et al. 2011; Graham et al. 2010; Milnerwood et al. 2010; Uribe et al. 2012; Wong et al. 2015; Hermel et al. 2004). Caspase activation has also shown to be an important event in neuronal death after NMDAR-mediated excitotoxicity in other neurological disorders, such as ischemic stroke (Akpan et al. 2011; Akpan & Troy 2013; Harrison et al. 2001; Krupinski et al. 2000), Alzheimer's Disease (Galvan et al. 2006; Graham et al. 2011; Blandini et al. 2006; Su et al. 2001; Cotman et al. 2005; Avila 2010), Parkinson's Disease(Hartmann et al. 2000; Blandini et al. 2006; Hartmann et al. 2001; Viswanath et al. 2001) and traumatic brain injury(Chen et al. 2003; Sullivan et al. 2002; Sanchez Mejia et al. 2001). Similarly, in these other conditions where NMDAR-mediated exciotoxicity plays a role, casp6 has shown promise as a therapeutic target (Akpan et al. 2011; Graham et al. 2011; Blandini et al. 2006), suggesting that it may be a critical player in neuronal death after NMDAR-mediated excitotoxicity, not only in HD, but in many conditions, and may be acting as an initiator caspase.

Despite much research supporting casp6 as a key player in HD pathogenesis, as well as many other excitotoxic conditions, many caspases are activated downstream of NMDAR-mediated apoptotic cascades, and there is still a lack of understanding in the distinct topographical caspase cascade following an excitotoxic event. In order to design effective therapeutics targeting caspase activation following NMDAR-mediated excitotoxicity, a better understanding of these caspase cascades are needed. In Chapter 3 I conduct some exploratory, mechanistic experiments using an *in vitro* model of NMDAR-mediated excitotoxicty to examine capase activation and expression patterns at very early time points following an excitotoxic event. Based on previous studies implicating casp6 in a wide number of excitotoxic conditions I hypothesize that **caspase 6 may be an initiator caspase in NMDAR-mediated excitotoxic cascades**. These experiments aim to pave the way to development of more effective therapeutics for HD, including peptide-mediated therapies targeting caspase-6, and could be extended to other neurological diseases where NMDAR-mediated excitotoxicity plays a role in pathogenesis.

Target 3: Peptide-mediated mutant HTT knockdown

Though there are many molecular changes associated with neuropathology of HD, at its root, HD is caused by expression of a CAG repeat in the huntingtin gene, leading to expression of mutated huntingtin protein. As a result, a primary therapeutic target in the current literature for HD has been aiming to lower mHTT levels in the brains of HD patients(See section 1.5.1). However, normal HTT has shown to be neuroprotective (section 1.2.3), thus total HTT knockdown is not ideal. A better therapy for HD may be to remove mHTT, while allowing WT HTT to function normally. Enhancing degradation of mHTT by enhancing autophagy and proteasome function using chemical compounds in HD has been shown to improve HD phenotypes in animal models,

suggesting that mHTT lowering may be an effective therapeutic target for HD. Also, RNAi and ASO silencing of mHTT and HTT transcripts have shown great promise as therapeutics for HD, with the potential for allele-specific silencing, showing significant improvements in rodent and non-human primate HD models, and currently in clinical trials.

One major downside to current mHTT lowering strategies is the lack of therapeutic control in transcript targeting strategies. Most mHTT-reducing studies use intrastriatal or intraventricular injection. These techniques are challenging in therapeutic application. First, though several studies using ASOs or RNAi demonstrate long-lasting knockdown effects, sometimes up to several months, it is likely that repeat treatments will be necessary in patients. Injections into the brain are invasive and may not be a preferable route of administration in humans. Also, long lasting treatments with RNAi virus or ASO may be dangerous if off-target or side effects occur. Conversely, peptides can be delivered IV or even IP, and can be cleared within several hours, allowing for control over treatment. Similarly, HD is likely non cell-autonomous as cell-cell interactions are critical to the neuronal dysfunction and neurodegeneration in HD(Gu et al. 2007), suggesting that mHTT knockdown in striatum alone may not be sufficient to completely protect against mHTT toxicity.

Our lab has recently developed a novel technique for targeted protein degradation for using peptides that signal proteins for lysosome- or proteasome-mediated degradation(Fan et al. 2014). Peptides contain 3 functional domains: 1) *Tat-sequence* for blood-brain-barrier permeability(Schwarze 1999); 2) *protein binding domain*, containing a binding sequence specific to the targeted protein; and 3) *degradation sequence*, either a KFERQ-like motif, recognized by chaperone proteins that signal for chaperone mediated autophagy (CMA) lysosomal degradation (Kaushik & Cuervo 2012) or an RRRG Degron tag, for degradation via the proteasome(Bonger et

al. 2011). Both degradation pathways have important roles in mHTT clearance. CMA is upregulated in HD in response to deficits in macroautophagy to clear aggregated mHTT (Koga et al. 2011). Similarly, the UPS has an important role in clearing N-terminal, cleaved mHTT (Li et al. 2010; Wu et al. 2003; Schipper-Krom et al. 2012b). Thus, they may provide efficient clearance pathways for mHTT. Our peptide-mediated knockdown technique has been successful in our lab, using degron- or CMA-fused peptides designed to degrade alpha-synuclein, DAPK1 and PSD-95 in cell culture and *in vivo* (Fan et al. 2014) with peptides reducing targeted protein levels by >50% in mouse tissues (Fan et al 2014, Jin et al, 2016). In Chapter 4, I use this technique to design and test mHTT-targeting peptides, that use mHTT-preferential binding sequences to target mHTT for degradation via CMA or UPS degradation. My hypothesis is that **short synthetic peptides containing an mHTT binding domain and a degradation sequence will enhance mHTT degradation via CMA or UPS degradation systems without significant HTT degradation.** I hypothesize that peptide-mediated knockdown of mHTT may provide a novel avenue for clinically applicable neuroprotection in HD.

By testing these three putative targets, NMDAR-mediated PTEN nuclear translocation, NMDAR-mediated caspase activation and mHTT knockdown, I open the door to potential new, preventative therapies for HD, where peptides may be useful as early strategies to slow neuronal cell death in HD.

Figure 1-1 Dichotomous role of NMDARs in cell survival and cell death

Activation of synaptically located, primarily GluN2A-containing NMDARs is associated with a wide range of cell survival and plasticity-promoting signaling pathways. Activation of these receptors leads to increased CREB phosphorylation in the nucleus, driving transcription of a range of cell survival-promoting genes. GluN2A, synaptic NMDAR activation also activates calcium-dependent calcineurin, shown to be involved in LTP and plasticity. Similarly, synaptic, GluN2A-NMDARs promote signaling of several cell survival molecular pathways including ERK1/2, PI3K and BDNF. Conversely, extrasynaptic, primarily GluN2B-NMDARs are related to cell death signaling. These receptors inhibit many of the synaptic, GluN2A pathways, including CREB shutoff, inhibition of ERK1/2, BDNF and P13K, and signals many molecular pathways involved in cell death, including JNK, and p38 MAPK. Extrasynaptic GluN2B NMDARs also activate caspases and m-calpain, involved in protein cleavage, apoptosis and cell death.

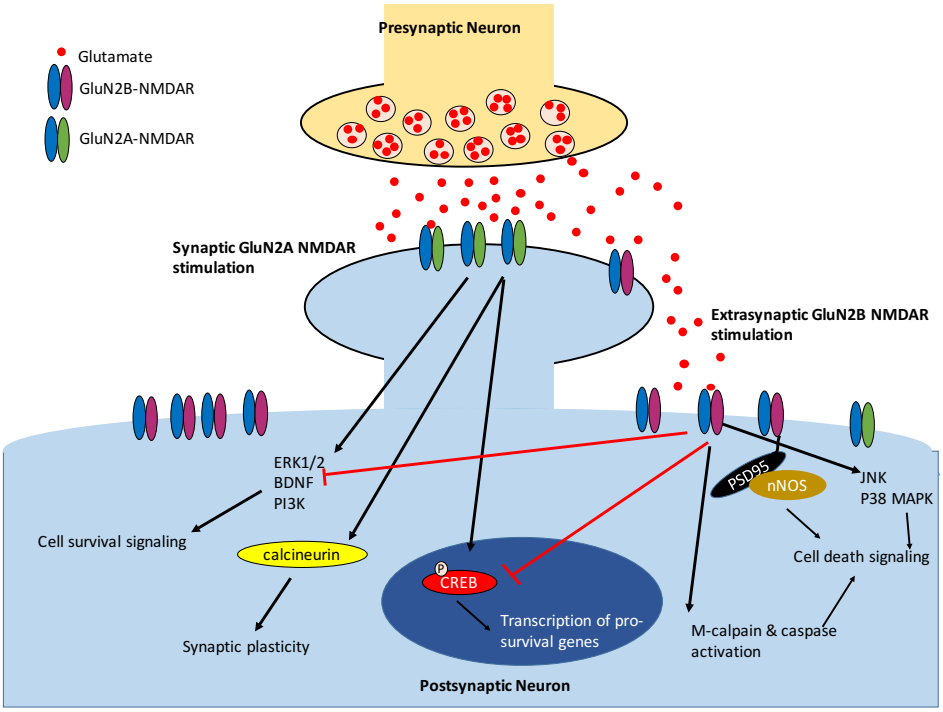
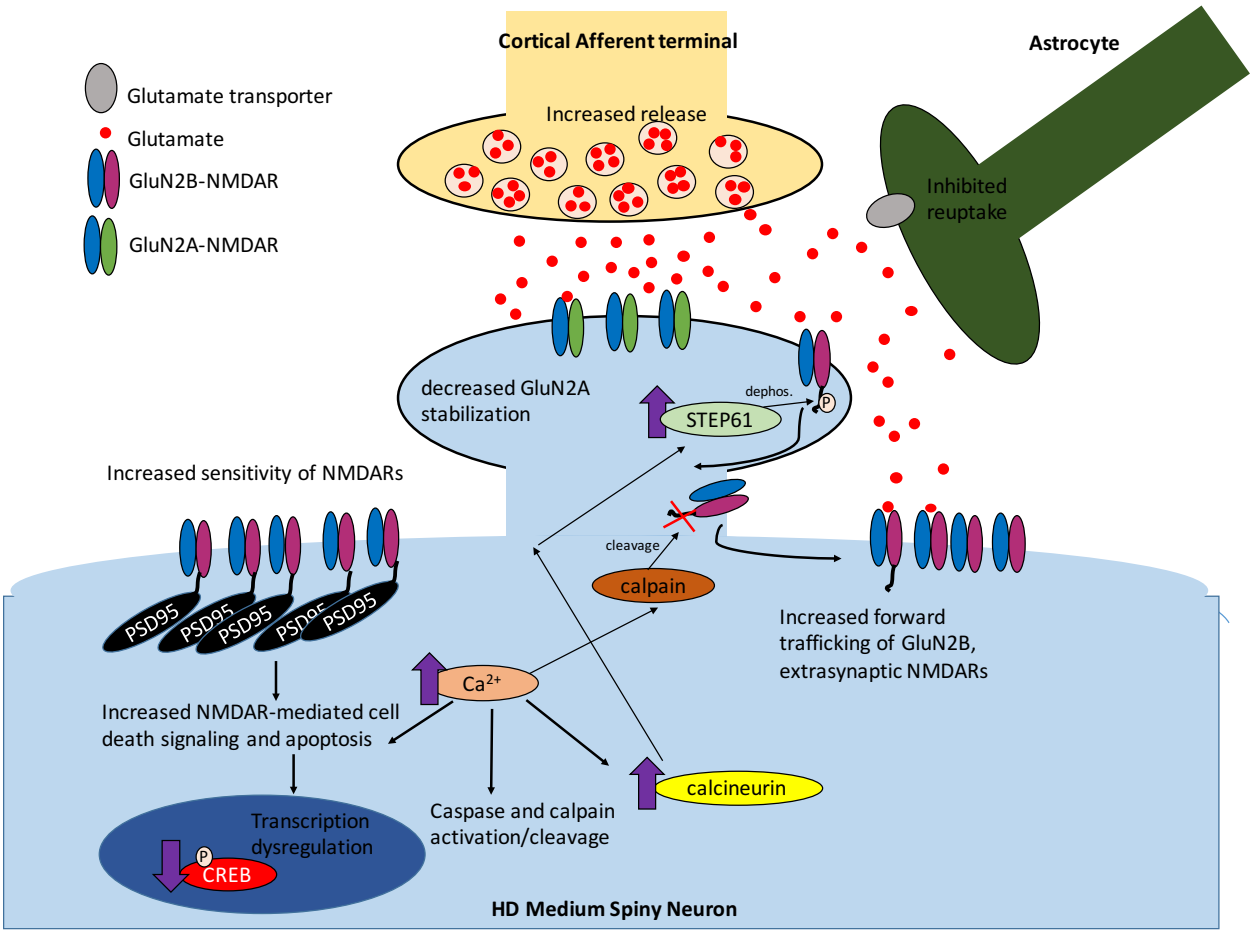


Figure 1-2 NMDAR-mediated synaptic dysfunction in HD

Schematic diagram of mHTT-dependent dysregulation in NMDAR expression and signaling in HD. Increased release from cortical (or thalamic) afferents stimulates NMDARs on medium spiny neurons in the striatum. Increased release and reduced reuptake from astrocytes leads to increased glutamate at the synapse, causing spillover and stimulating extrasynaptic, GluN2B-containing NMDARs, shown to be associated with cell death signaling. Increased NMDAR stimulation leads to increases in intracellular calcium, which stimulates increase in apoptotic signaling pathways, as well as activation of calcium-dependent molecules, such as caspases and calpains, which cleave mHTT into toxic fragments and stimulate cell death signaling. Calpain activation also leads to increased cleavage of GluN2B NMDARs, facilitating their movement from synaptic to extrasynaptic sites. Ca^{2+} also activates the calcium dependent molecule calcineurin, which subsequently activates STEP, which dephosphorylates and destabilizes synaptic NMDARs, reducing their expression. STEP also leads to the clathrin vesicle endocytosis of NMDARs, and their recycling to extasynaptic sites (not shown). mHTT influences PSD-95 interaction with NMDARs, interacting less strongly with GluN2A-NMDARs in the synapse, while stabilizing GluN2B-NMDARs extrasynaptically. Increased expression and stabilization of extrasynaptic NMDARs leads to increased sensitivity to NMDA, increased cell death and apoptotic signaling and dephosphorylation of CREB in the nucleus, leading to transcriptional dysregulation.


Glutamate transporter
Glutamate
GluN2B-NMDAR
GluN2A-NMDAR
Cortical Afferent terminal
Increased release
Astrocyte
Inhibited reuptake
decreased GluN2A stabilization
dephos.
STEP61
P
Increased sensitivity of NMDARs
cleavage
calpain
PSD95
PSD95
PSD95
PSD95
PSD95
Increased forward trafficking of GluN2B, extrasynaptic NMDARs
Ca2+
Increased NMDAR-mediated cell death signaling and apoptosis
calcineurin
Caspase and calpain activation/cleavage
Transcription dysregulation
P
CREB
HD Medium Spiny Neuron

Chapter 2: The Role of PTEN Nuclear Translocation in NMDAR-mediated Neuronal Death in Huntington's Disease

2.1 Introduction

As discussed in detail in chapter 1 (In particular, sections 1.3.8 and 1.5.2), there exists ample evidence that excitotoxicity, primarily via GluN2B-containing, extrasynaptic NMDARs, plays a critical role in neuronal death and dysfunction in HD. NMDAR-mediated excitotoxicity in HD appears to be an early event, happening pre-symptomatically(Levine et al. 1999; Cowan et al. 2008; Graham et al. 2009) making it a tempting target for early, preventative therapies for HD. Broad NMDAR antagonists have proven imperfect therapeutics for NMDAR-mediated excitotoxicity(Ikonomidou & Turski 2002 Lai et al, 2014, Tymianski 2013), which is due to the dual role of NMDARs in cell survival and cell death. Synaptic, GluN2A-containing NMDARs have been implicated in cell survival, whereas extrasynaptic, GluN2B-contianing NMDARs have been demonstrated to primarily activate cell death signaling. As a result, potential therapeutic targets for NMDAR-mediated excitotoxicity in HD have focused on either blocking GluN2B-containing NMDARs (Milnerwood et al. 2012; Dau et al. 2014; Okamoto et al. 2009), enhancing glutamate uptake (Miller et al. 2008) or targeting downstream cell-death signaling of NMDARs (Gafni et al. 2004; Wong et al. 2015; Graham et al. 2010; Fan et al. 2009)(See section 1.5.2).

Recently, in our lab, we demonstrated that nuclear translocation of the tumor suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome ten) is a critical step in NMDAR-mediated excitotoxic neuronal death in ischemic stroke (Zhang et al, 2013). PTEN, a dual lipid/protein phosphatase, normally antagonizing the phosphatidylinositol-3-kinase (PI3K)-

Akt pathway, was shown in our work to be mono-ubiquitinated at lysine r esidues K13 and K289, playing a role in subcellular localization(Trotman et al. 2007; Baker 2007). In cancer studies, mutations at either K13 or K289 retained the phosphatase ability of PTEN, but prevented nuclear translocation(Trotman et al. 2007; Baker 2007). Reducing PTEN's nuclear translocation impaired PTEN's tumor suppressive ability, leading to reduced apoptosis during tumorigenesis, suggesting that PTEN nuclear translocation may be linked to PTEN's cell death promoting activity. Following on these studies, previously in our lab, we measured rapid nuclear translocation of PTEN after an NMDAR-mediated excitotoxic event (Zhang et al, 2013). This PTEN mechanism appears to be specifically linked to GluN2B-mediated NMDAR signaling and can be blocked by NR2B-NMDAR antagonists. Using site-directed mutagenesis, we found that PTEN nuclear translocation in neurons after NMDA stimulation is mediated by mono-ubiquitination of K13 and not K289(Zhang et al, 2013). We subsequently designed 2 interference peptides to flank the K13 and K289 residues on PTEN (Tat-K13 and Tat-K289 respectively) and act as competitive substrates for PTEN mono-ubiquitination(**Fig 2-1)**. These peptides are fused to a TAT sequence to render them membrane permeable(Schwarze 1999). In these studies, Tat-K13, but not Tat-K289 prevents NMDA-induced PTEN nuclear translocation and effectively prevents NMDA-induced neuronal death in cultured neurons *in vitro* as well as in a rat stroke model suggesting that PTEN nuclear translocation may be a critical step in NMDAR-mediated excitotoxicity (Zhang et al, 2013).

Based on these findings, the Tat-K13 peptide may be useful to extend into other forms of neurotoxicity and cell death. In our previous studies, Tat-K13 provided neuroprotection against ischemic injuries (Zhang et al, 2013) and PTEN nuclear translocation occurs after stimulation of neurons with hydrogen peroxide (Howitt et al. 2012) PTEN nuclear translocation is associated with neuron death in cancer cells (Trotman et al. 2007; Baker 2007) suggesting that this PTEN

nuclear translocation may be associated with a wide range of death pathways. These studies constitute a new mechanism of protection against NMDAR-mediated neuronal death, by targeting downstream cell death related to GluN2B-mediated excitotoxicity. We now want to determine if Tat-K13 may be an effective therapy for other neurological disorders that implement NMDAR excitotoxicity as a cause, namely HD.

2.1.1 PTEN

A recently discovered downstream target of NMDAR-mediated neuronal death is the mono-ubiquitination and nuclear translocation of PTEN (Zhang et al. 2013a). Chapter 2.1.11 will discuss the function and regulation of PTEN and the potential of inhibiting nuclear translocation of PTEN as a potential neuroprotective strategy in Huntington's disease.

2.1.1.1 The PTEN protein

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor-suppressing protein located on chromosome 10q23.31 (Govender & Chetty 2012) that was discovered in 1997 (Steck et al. 1997; Li 1997). The PTEN protein is a dual lipid protein phosphatase(Di Cristofano & Pandolfi 2000). Also known as Mutated in Multiple Advanced Cancers 1 (MMAC1) mutations or deletions of PTEN have been implicated in a wide range of cancers including glioblastoma, melanoma, and prostate, breast and ovarian cancer (Sansal & Sellers 2004). Despite its wide distribution around the body, PTEN is expressed highly in brain tissue, and along with its well-known role in cancer, recent studies have also linked PTEN to NMDAR-mediated excitotoxic neuron death(Zhang et al. 2013; Ning 2004). The PTEN protein has three major regulatory domains. The first is the phosphatase domain (aa352-403), central to PTEN's function as a

phosphatase protein. Mutations to cysteine 124 inhibit PTEN's phosphatase abilities(Ali et al. 1999). The second functional domain of PTEN is the C2 domain (aa6-15) at the N terminus, which is important for PTEN membrane interaction. Lastly, the C terminal tail domain of PTEN contains several common regulatory domains such as PDZ and PEST(Wu et al. 2000; Tolkacheva et al. 2001). PTEN has a highly conserved sequence, especially in vertebrates (Makałowski et al. 1996). Though PTEN is primarily cytoplasmic, it can also be found in the nucleus (Leslie et al. 2016).

2.1.1.2 PTEN function

The primary function of PTEN is its role as a dual lipid and protein phosphatase(Di Cristofano & Pandolfi 2000). PTEN is most commonly known for its cytoplasmic tumor-suppressing phosphatase activity, acting to dephosphorylate PI(3,4,5)P3 into PI(4,5)P2, antagonizing the PI3K/Akt cell survival pathway(Vivanco & Sawyers 2002; Déléris et al. 2006); however PTEN can also move between the nucleus and the cytoplasm and is involved in many cellular activities beyond the Akt pathway, including roles in NMDAR-mediated excitotoxicity(Zhang et al. 2013a; Ning 2004) arresting cell cycle(Chung et al. 2005; Chung & Eng 2005) relationships with p53 (Stambolic et al. 2001; Li et al. 2006) and several other important functions.

2.1.1.2.1 Cytoplasmic role of PTEN

PTEN's major functional role is its antagonism of class I phosphoinositide-3-kinases (PI3Ks), acting to inhibit the Akt cell survival signaling pathway. Upon activation, primarily by the binding of growth factors to receptor tyrosine kinases (Vivanco & Sawyers 2002), PI3K is recruited to the plasma membrane where it acts to phosphorylate the 3-position of phosphatidylinositol-4,5-biphosphate (PI(4,5)P_2) to produce second messenger phosphatidylinositol-3,4,5-triphosphate

($PI(3,4,5)P_3$) (Vanhaesebroeck et al. 2001) and subsequently activates pyruvate dehydrogenase kinase 1 (PDK1) and Akt, leading to increases in cell growth, survival and proliferation(Alessi et al. 1997). PTEN, utilizing its abilities as a lipid phosphatase, antagonizes this pathway by dephosphorylating the 3- position of the inositol ring of PIP_3, catalyzing its conversion into PIP_2 (Maehama & Dixon 1998) and blocking the survival signaling of PI3K/Akt as well as Akt/ mammalian target of rapamycin (mTOR). PIP_3 signaling has an important role in many cell survival, proliferation and growth activities. Upon its conversion from $PI(4,5)P_2$ to $PI(3,4,5)P_3$ by P13K at the plasma membrane, PIP_3 activates Akt signaling by binding to the PH (plectsrin homology) domain of Akt and recruiting it to the plasma membrane. This results in a conformational change and phosphorylation of Akt, leading to its activation (Vivanco & Sawyers 2002; Papadia et al. 2005). Once phosphorylated, Akt acts to further phosphorylate several important targets, simultaneously promoting cell survival signaling and inhibiting cell death signaling pathways. These Akt targets include cell survival molecules eNOS (Bell & Yellon 2003) MDM2 (Mocanu & Yellon 2007) and c-AMP element response binding protein (CREB) (Papadia et al. 2005), which, upon phosphorylation by Akt become activated and trigger cell survival, proliferation, transcription-promition and growth signaling pathways (Mocanu & Yellon 2007). Activated Akt also acts to phosphorylate and subsequently deactivate several pro-death molecules, including GSK3β(Cross et al. 1995), Bcl-2(Raftopoulou et al. 2004)BAD (Jonassen et al. 2001), Forkhead Box O (FOXO) (Brunet et al. 1999) as well as proteins in the c-Jun N-terminal kinase (JNK) cell death pathway (Kim et al. 2001). Akt also activates the mTORC1 complex, leading to enhanced protein translation (Guertin & Sabatini 2007; Ma & Blenis 2009) Together, Akt activation by PIP_3 signaling plays an important role in driving cell survival, proliferation angiogenesis and growth, while simultaneously inhibiting death. PTEN, antagonizing formation

of PI(4,5)P_2 to PI(3,4,5)P_3 therefore, plays a critical role in tumor suppression by prevention of anomalous cell growth, proliferation and survival.

Recent evidence has further linked the cytoplasmic activity of PTEN to the regulation of several other important cellular processes via inhibition of PI(3,4,5)P_3-Akt-mTORC1 signaling such as glucose metabolism(Horie et al. 2004; Song et al. 2012) and regulation of the tumor micro-environment (Trimboli et al. 2009). PTEN has also been shown to inhibit cellular motility, both through its lipid phosphatase activity (Liliental et al. 2000) and through its protein phosphatase activity, by dephosphorylation and inactivation of the focal adhesion kinase (FAK) (Tamura et al. 1981). Recently, PTEN has also shown to enhance surface expression of NMDARs through its protein phosphatase activity(Ning 2004). PTEN interacts directly with GluN2B-containing NMDARs, and downregulation of PTEN inhibits extrasynaptic NMDAR function, as well as reduces surface expression of NMDARs, suggesting it has an important role in NMDAR function and excitotoxicity(Ning 2004).

2.1.1.2.2 Nuclear role of PTEN

Although the primary function of PTEN has been demonstrated to be the tumor suppressive properties vis its lipid phosphatase activity through the PI3K pathway, much data demonstrates that PTEN can move from the cytoplasm to the nucleus and that PTEN has several important nuclear functions (Baker 2007; Planchon et al. 2008; Song et al. 2012). PTEN maintains a regular nuclear expression both in normal cells, as well as in early stage cancer, however, advanced-stage tumor development has shown to be associated with a complete lack of nuclear PTEN, and is actually a useful indicator for prognosis (Lindsay et al. 2006; Lian & Di Cristofano 2005; Trotman

et al. 2007; Leslie et al. 2016; Perren et al. 1999). This suggests that nuclear PTEN must serve a role in tumor suppression, as well as other cell functions.

Much recent evidence supports this claim. The PI3K/Akt signaling cascade has been shown to similarly exist in the in the nucleus as it does in the cytoplasm(Déléris et al. 2006) and nuclear PTEN has similarly dephosphorylates nuclear PI(3,4,5)P_3 to PI(4,5,)P_2 (Déléris et al. 2003) suggesting that part of nuclear PTEN's protective role may come from antagonizing nuclear P13K/Akt signaling. This was further supported by experiments showing enhanced DNA fragmentation and apoptotic signaling with PTEN exposure to PC12 nuclei, which could be reversed by exposing nuclei to PIP(3,4,5)P_2 (Ahn et al. 2004; Ahn et al. 2005). Similarly, reductions of nuclear PTEN in advanced stage cancer is accompanied by similar increases in nuclear p-Akt expression and resultant increases in tumor invasion (Vasko et al. 2004). Conversely, increasing PTEN expression lowers levels of activated nuclear Akt (Trotman et al. 2007). These experiments, and others demonstrate that nuclear PTEN's tumor suppressive role is also important in the nucleus, by similarly antagonizing P13K/Akt cell survival signaling.

Nuclear PTEN has also been shown to have an important role in cell cycle regulation and genomic stability. By up-regulating the protein RAD51, an important molecule for repairing double-stranded DNA breaks, nuclear PTEN has shown to positively regulate chromosomal integrity(Shen et al. 2007). Conversely, a lack of PTEN leads to Akt phosphorylation and subsequent cytoplasmic sequestration of checkpoint kinase 1 (CHK1), an important cell cycle regulator protein(Puc et al. 2005). This leads to disruptions in the checkpoint of cell cycle G2-M and results in chromosome instability and double stranded DNA breaks (Puc et al. 2005). PTEN loss has also been linked to complete cell senescence (Leslie et al. 2016). Nuclear PTEN, utilizing its protein phosphatase ability, downregulates phosphorylated MAPK and cyclin D, leading to cell

cycle arrest(Chung & Eng 2005; Chung et al. 2006; Radu et al. 2003). This could be blocked by preventing PTEN movement to the nucleus(Radu et al. 2003; Chung & Eng 2005; Chung et al. 2006) further demonstrating that nuclear PTEN has a critical role in the balance of cell survival and cell death, and prevention of cancer formation.

The role of PTEN in the nucleus has also been linked to its interaction with another tumor suppressor, p53. These two proteins reciprocally influence one another, with PTEN increasing p53 stability, and p53 leading to enhanced transcription of PTEN (Stambolic et al. 2001; Li et al. 2006) and the two proteins are similarly regulated (M. Li et al. 2003). At baseline, non-pathologic conditions, PTEN is highly expressed, and it has been suggested that it serves to function as a "housekeeping" cancer suppressive protein (Trotman et al. 2007; Wang & Jiang 2008). Conversely, p53 becomes active and upregulated in response to cellular stress (Brooks & Gu 2006) suggesting that its role as a tumor suppressor is more important in pathological conditions. PTEN has shown to influence p53 expression in a phosphatase dependent way, by antagonizing Akt/Mdm2 signaling (Zhou et al. 2001; Ogawara et al. 2002; Mayo & Donner 2001) and as well as independently of its phosphatase ability through direct PTEN p53 protein-protein interaction (Freeman et al. 2003; Tang & Eng 2006) and by increasing p53 acetylation (Li et al. 2006). PTEN and p53 form a complex in the nucleus which prevents sequestration of p53 by Akt-Mdm2, and increases the transcriptional activity and stability of p53(Freeman et al. 2003; Tang & Eng 2006). In addition, p53 has shown to bind upstream of the PTEN gene, leading to enhanced transcription of PTEN (Stambolic et al. 2001). This reciprocal PTEN and p53 relationship has been suggested to contribute to cellular senescence seen after loss of nuclear PTEN (Wang & Jiang 2008).

2.1.1.3 Regulation of PTEN

PTEN, located on chromosome ten is constitutively active in most healthy tissues. However, PTEN is both positively and negatively transcriptionally regulated under various conditions. Several positive regulators of PTEN, including early growth response protein 1 (EGFR-1), peroxisome proliferator-activated receptor γ (PPAR-γ), and tumor protein 53 (Tp53) have shown to bind directly to the PTEN promoter, enhancing PTEN transcription (Bermúdez Brito et al. 2015). Similarly, negative regulators of PTEN include members of the Ras/Raf/MEK/ERK pathway, nuclear factor kappa B (NF-κB) and several transcription factors such as mitogen-activated protein kinase kinase-4 (MKK4), transforming growth factor beta (TGF-β), and the polycomb group (PcG) protein BMI1 can decrease PTEN transcription(Bermúdez Brito et al. 2015). PTEN can also be transcriptionally regulated by epigenetic silencing through DNA methylation or histone modification (J. M. García et al. 2004; Kang et al. 2002) as well as both positive and negative re gulation by NOTCH signaling (Palomero et al. 2007; Leissring et al. 2002). A wide range of miRNAs have also been identified that can alter PTEN expression, many of which have shown links to cancer progression (Bermúdez Brito et al. 2015).

PTEN is also post-translationally regulated, which can alter PTEN function and cellular localization. PTEN has two important clusters of phosphorylation sites on the C terminus (Al-Khouri et al. 2005; Vazquez et al. 2000; Vazquez et al. 2001) which are phosphorylated primarily by casein kinase 2 (CK2) and S6K (Vazquez et al. 2001; Bermúdez Brito et al. 2015). PTEN phosphorylation can influence many factors, including stability, localization and activity. Phosphorylated PTEN is unable to interact with the phospholipid membrane (Rahdar et al. 2009; Vazquez et al. 2006), as well as membrane proteins (Wu et al. 2000) and this prevention of PTEN anchoring by phosphorylation has shown to prevent PTEN from antagonizing the PI3K/Akt

pathway(Bermúdez Brito et al. 2015). Under normal conditions, PTEN exists cytoplasmically, in a phosphorylated, inactive state (Vazquez et al. 2000), and when it is needed, becomes dephosphorylated, allowing it to move to the plasma membrane where it can act via the P13K/Akt pathway. Phosphorylation of the PTEN c-tail has also shown to influence PTEN cellular localization, with phosphorylation leading to enhanced nuclear export (Gil et al. 2006). Interestingly, though functionally, PTEN is negatively impacted by C-tail phosphorylation, PTEN stability may be increased by this modification, rendering PTEN less prone to degradation(Wu et al. 2014). A second set of kinases, RAK and Rho-Associated Kinase (ROCK) phosphorylation further influence PTEN, by preventing interaction of PTEN with its ubiquitination E3 ligase (discussed in 2.1.11.5)(Yim et al. 2009) which can similarly influence PTEN stability, localization and function. Along with phosphorylation, PTEN is both oxidized(Cho et al. 2004) and acetylated(Okumura et al. 2006), both of which largely lead to PTEN loss-of-function.

Another important post-translational modification of PTEN is ubiquitination. Ubiquitination is an process in which small 34kDa ubiquitin proteins are covalently attached to lysine (K) residues on a target protein(Komander & Rape 2012). Ubiquitination, primarily polyubiquitination, is commonly used to trigger an enzymatic cascade that flags proteins for degradation via the proteasome. Ubiquitination, especially by mono-ubiquitination, can a lso be used to change protein localization, function, trafficking and many other factors (Komander & Rape 2012). PTEN has shown to be both mono- and polyubiquitinated. Early ubiquitination studies of PTEN demonstrated that it is classically polyubiquitinated and degraded by the proteasome complex (Tolkacheva et al. 2001; Wu et al. 2003). PTEN contains two C-terminal PEST motifs, common in short-lived, quickly protasomally-degraded proteins, which may be surprising, given PTEN's long-lasting, constitutive expression (Wang et al. 2008), however, this has been shown to

be an adaptive mechanism of PTEN to help finely regulate PTEN expression levels(Wang & Jiang 2008). Alongside classical degradation-based ubiquitination, PTEN is also mono-ubiquitinated, which importantly regulates PTEN shuttling from cytoplasm to nucleus (Trotman et al. 2007). PTEN has been found to have two major mono-ubiquitination sites at K13 and K289 (Trotman et al. 2007; Baker 2007; Fouladkou et al. 2008; Song et al. 2008; Leslie et al. 2016). Though several enzymes have been found to catalyze PTEN ubiquitination, the primary ubiquitin ligase for PTEN is neural precursor cell expressed, developmentally downregulated-4-1 (NEDD4-1)(Wang et al. 2007). NEDD4-1 mediates both mono- and polyubiquitination of PTEN. Other ubiquitin ligase candidates for PTEN have been proposed, such as XIAP(Van Themsche et al. 2009) WWP2 (Maddika et al. 2011) and RFP (Lee et al. 2013). Although it is likely that PTEN is differentially ubiquitinated by various ligases in different conditions, the general consensus is that NEDD4-1 represents PTEN's primary E3 ligase.

2.1.1.4 PTEN nuclear translocation

As discussed, many modifications of PTEN can influence its subcellular localization. PTEN can be shuttled in and out of the nucleus by several factors, including Ran-GTPase-dependent nuclear import by a nuclear localization signal on PTEN's N-terminus(Gil et al. 2006; Gil et al. 2007), active, calcium-dependent transport, with help of molecular vault protein MVP(Chung & Eng 2005) as well as some passive diffusion of PTEN through the nuclear membrane(Liu et al. 2005). Nuclear localization of PTEN has been shown to be largely regulated by mono-ubiquitination of PTEN by NEDD4-1 at its two ubiquitination sites, K13 and K289(Trotman et al. 2007; Plafker et al. 2004; M. Li et al. 2003; Wang et al. 2007). In a study by Trotman et al in 2007, it was demonstrated that preventing mono-ubiquitination of PTEN by NEDD4-1 knockdown retains

PTEN cytoplasmically, whereas overexpression of NEDD4-1increases PTEN's nuclear localization (Trotman et al. 2007). Similarly, overexpression of GFP-linked PTEN plasmids with mutations at K13 or K289 (GFP-PTEN$_{K13R}$ and GFP-PTEN$_{K289R}$), retain lipid phosphatase ability of PTEN but prevent mono-ubiquitination, and lead to cytoplasmic PTEN retention in PC3 cancer cells(Trotman et al. 2007), whereas non-transfected cells, or cells transfected with WT PTEN showed both cytoplasmic and nuclear expression of PTEN. This suggests that nuclear import of PTEN is in large part regulated by mono-ubiquitination. This study also demonstrated that this nuclear shuttling of PTEN can influence its stability. Nuclear PTEN can be de-ubiquitinated in the nucleus, preventing its nuclear export, and can also protect PTEN from degradation by cytoplasmic poly-ubiquitination and proteosomal degradation (Trotman et al. 2007).

Several events may cause PTEN to shuttle from the cytoplasm into the nucleus. In developing cells, PTEN nuclear translocation has been linked with cellular differentiation, with treatments of BDNF and Nerve growth factor (NGF) leading to increased PTEN nuclear expression and increased neuronal differentiation in CNS stem cells (Lachyankar et al. 2000). Nuclear PTEN may also be regulated by cell cycle stage, with increased nuclear PTEN during G0-G1 and decreased nuclear PTEN in S phase (Ginn-Pease & Eng 2003). Interestingly, many cellular stress or apoptotic stimulations trigger PTEN nuclear import, including TNF-α and sorbitol(Gil et al. 2006), suggesting that PTEN nuclear translocation may be involved in cellular death signaling. PTEN contains a GTPase dependent nuclear localization signal which can also lead PTEN to move into the nucleus, as well as nuclear exclusion signals which force PTEN out(Gil et al. 2006; Gil et al. 2007). Mutations in these signals lead to increases in PTEN nuclear localization and subsequent increases in apoptosis (Gil et al. 2006). ATP deficiency also has been shown to drive PTEN nuclear translocation, accompanied by cell death (Lobo et al. 2008).

2.1.1.5 PTEN nuclear translocation in NMDAR-mediated excitotoxicity

Along with its important role in cancer cells, PTEN is highly expressed in neurons, and several recent studies have implicated that PTEN may play a role in NMDAR-mediated excitotoxic neuronal death (Gary & Mattson 2002; Ning 2004; Wu et al. 2006; Zhang et al. 2013; Q. G. Zhang et al. 2007). Initial experiments linking PTEN to excitotoxicity came from studies in ischemia. Immediately after ischemia, the phosphorylated, inactive form of PTEN decreases, thought to lead to increased inhibition of P13K/Akt signaling, preceding activation of apoptotic signaling in a stroke (Omori et al. 2002; Zhao et al. 2006; Choi et al. 2005). P-Akt levels also change following stroke, with immediately early decreases immediately following stroke and subsequent increases in the hours following, thought to be a neuroprotective mechanism (Zhao et al. 2006). In this vein, siRNA knockdown or pharmacological lowering of PTEN has shown to provide some neuroprotective benefit in animal models of ischemia (Ning 2004; Wu et al. 2000; Q. G. Zhang et al. 2007). Similarly, several other putative stroke treatments have subsequently shown to work via PTEN inhibition or enhancement of p-Akt signaling (Yoshimoto et al. 2001; Jin et al. 2003; L. García et al. 2004; Lee et al. 2009) suggesting that PTEN not only has a role in ischemic cell death, but also blocking PTEN signaling may be neuroprotective against ischemic cell death signaling.

Much evidence has supported the hypothesis that NMDAR-mediated excitotoxicity has a critical role in the cell death associated with ischemia(Lai et al. 2014), thus several groups have also investigated a potential link between NMDA receptors and PTEN. Importantly, PTEN has been shown to bind directly to NMDARs, and is recruited particularly to GluN2B- but not GluN2A-containing NMDARs where they form a novel neuronal death complex (NDC) with NMDARS via PSD-95 (Ning 2004; Jurado et al. 2010; Q. G. Zhang et al. 2007). This cell-death specific activity is dependent upon Ca^{2+} flow through NMDARs, linking it directly with NMDAR-

mediated excitotoxicity. As discussed in Chapter 1, GluN2B and PSD-95, by binding together, link GluN2B-NMDAR activation with several important cell death signaling pathways (Aarts et al. 2002; Hardingham et al. 2002; Liu et al. 2007b). Given this, and the neuroprotective benefits of PTEN silencing both *in vitro* and *in vivo* in animal models of stroke and NMDAR-mediated excitotoxicity (Gary & Mattson 2002; Ning 2004; Wu et al. 2006; Q. G. Zhang et al. 2007) thus it has been hypothesized that PTEN may have a critical role in NMDAR-mediated excitotoxic signaling pathways downstream of GluN2B activation.

Unfortunately, given the critical role of PTEN in tumor suppression and normal cell function, PTEN inhibition may not by the most viable neuroprotective strategy for NMDAR-mediated excitotoxic injury, especially for chronic diseases such as Huntington's Disease which would require long-term treatment. This has potential to lead to cell dysfunction, cell death, or tumor formation. Thus, in the past several years, researchers in our lab have investigated other ways in which to antagonize NMDAR-mediated PTEN cell death signaling without complete knockdown.

Nuclear PTEN, in particular, has been suggested to have a role in apoptotic cell death, PTEN mutants that get retained in the nucleus enhance apoptotic signaling, and apoptotic-inducing stimuli leads to increased nuclear accumulation of PTEN (Gil et al. 2006). Similarly, nuclei isolated with PTEN demonstrate DNA fragmentation (Ahn et al. 2004; Ahn et al. 2005). In recent years, nuclear translocation of PTEN has also shown to be linked to NMDAR-mediated excitotoxic death. In an exciting study from Zhang et al, in our lab in 2013, we measured rapid nuclear translocation of PTEN, 6-9 hours after an NMDAR-mediated excitotoxic event (Zhang et al. 2013) that is specifically linked to GluN2B-mediated NMDAR signaling. Although nuclear translocation of PTEN in cancer cells depends on mono-ubiquitination at K13 and K289, using site-directed

mutagenesis, Zhang et al found that PTEN nuclear translocation in cortical or hippocampal neurons after NMDA stimulation is mediated by mono-ubiquitination of K13 but not K289(Zhang et al. 2013a). We subsequently designed 2 interference peptide to flank the K13 and K289 residues on PTEN, (Tat-K13 and Tat-K289, respectively) and act as competitive substrates for PTEN mono-ubiquitination (**Fig 2-1)**. These peptides are fused to a cell-membrane transduction domain of the HIV-type1 Tat protein to render then membrane permeable(Schwarze 1999). In this study, Tat-K13, but not Tat-K289 prevented NMDA-induced PTEN nuclear translocation. Tat-K13 was effective at preventing NMDA-induced neuronal death in cultured neurons *in vitro* as well as in a medial carotid artery occlusion stroke model suggesting that PTEN nuclear translocation may be a critical step in NMDAR-mediated excitotoxicity. These studies constituted a completely new mechanism of protection against NMDAR-mediated neuronal death, by targeting downstream cell death mechanisms specifically related to GluN2B-mediated excitotoxicity (Zhang et al. 2013).

2.1.2 Rationale and specific aims

As discussed in detail in Chapter 1.3.8 and 1.5.2, much evidence supports the hypothesis that NMDAR-mediated excitotoxicity has a pivotal role in the pathogenesis of Huntington's disease, primarily via Glu2B, extrasynaptic NMDARs. Based on this, and the known critical role of PTEN nuclear translocation in NMDAR-mediated neuron death in ischemic stroke, I hypothesized that **nuclear translocation of PTEN may have a critical role in NMDAR-mediated excitotoxicity in HD.** HD neurons, in particular in the striatum as a part of cortico-striatal or thalamo-cortical synapses, are particularly vulnerable to NMDAR-mediated excitotoxicity, thus, if PTEN nuclear translocation plays the same critical role in NMDAR-mediated excitotoxicity in HD as it does in stroke, Tat-K13 may be a potential, novel early

preventative therapeutic target for HD. Although the striatum is the most commonly discussed affected area in HD, and has been shown to be sensitive to NMDAR-mediated excitotoxicity in HD, I also examined PTEN nuclear translocation and Tat-K13 neuroprotection in other HD neuron types, namely the cortex and hippocampus. Later in HD disease progression, loss of cortical and hippocampal tissue is detected(Nana et al. 2014) (Spargo et al. 1993a; de la Monte et al. 1988) and NMDAR dysfunction has been implicated in impairments both in cortico-striatal synaptic interaction (Ponten et al. 2010; Carlos Cepeda et al. 2001; Cepeda et al. 2003; Milnerwood et al. 2010; Milnerwood & Raymond 2007) as well as hippocampal plasticity and function in CA1 neurons (Murphy et al. 1999; Cummings et al. 2006; Klapstein et al. 2001). Similarly, PTEN nuclear translocation has been implicated in other types of neuron death (Howitt et al. 2012; Baker 2007; Trotman et al. 2007) suggesting that blocking PTEN nuclear translocation using Tat-K13 may be a useful therapeutic for many types of neuron loss in HD.

To test my hypothesis, I utilized the following specific aims:

1) *Determine the effects of Tat-K13 on PTEN nuclear translocation and NMDA excitotoxicity in cultured neurons from HD transgenic mice:*
2) *Investigate baseline changes in PTEN nuclear translocation in tissue and brain slices from WT and HD transgenic mice at various timepoints during disease progression*
3) *Determine neuroprotective ability of Tat-K13 in vivo*
4) *Investigate PTEN nuclear translocation and Tat-K13 effects in different cell types*

Overall, the studies in this chapter aimed to provide new insight into the role of PTEN nuclear translocation in NMDAR-mediated excitoxicity in HD, not only in the striatum, but also

in other brain areas, as well as investigate the potential of Tat-K13 as a potential early preventative HD therapeutic.

2.2 Materials and methods

2.2.1 Antibodies and reagents

Mouse anti-PTEN antibody (#sc-7974) was ordered from Santa Cruz (Santa Cruz, CA). HSP90 (#610418) was obtained from BD Transduction labs (San Jose, CA). LaminB1 (#16048), GFP (#13970) and DARPP-32 (#EP720Y) antibodies were purchased from Abcam (Cambridge, UK). DAPI nucleic acid stain (#D9542) was ordered from Sigma Aldrich (St. Louis, Missouri). Secondary antibodies for immunostaining were the AlexaFluor series and were ordered from Thermo Fischer Molecular Probes (Portland, OR). Mounting media Fluoromount-G (0100-01) was bought from Southern Biotech (Birmingham, AL). NMDA (Acs-052) and Kainic Acid (Asc-100) were purchased from Ascent Scientific (Victoria, UK). Protein assays were conducted using the D-C protein assay kit (#5000111, Bio-Rad, Hercules, CA). Cell Toxicity Colormetric Assay Kit (TOX#1) was purchased from Sigma-Aldrich (St. Louis, Missouri). Complete Protease Inhibitor Cocktail Tablets (#04693116001) and Phosphatase Inhibitor Cocktail Tablets (04906845001) were obtained from Roche Applied Science (LSC). Cell Transfection kits (ProFection Mammalian Transfection System #E1200) were ordered from Promega (Madison, Wisconsin). Fluoro-Jade staining kits (#AG310) came from Millipore (Billerica, MA). Tat-fused peptides were synthesized in the Yu Tian Wang lab at the Brain Research Centre, UBC, Peptide synthesis facility.

2.2.2 Buffers

All buffers were made in the laboratory and were sterile filtered before use using vacuum or syringe filtration systems (Corning, Polyethersulfone with 0.22 µm pore size). Nuclear Translocation Buffer A (Cytoplasmic Buffer) contained 10mM HEPES-KOH, 10mM KCl, 10mM EDTA, 0.4% NP40, 1.5mM MgCl2, 1mM DTT (added immediately when using) protease inhibitor cocktail (added immediately when using), pH 7.9. and Buffer B (Nuclear Buffer) contained 20mM HEPES-KOH, 400mM NaCl, 1mM EDTA, 10% Glycerol, 1mM DTT (added right when using), protease inhibitor cocktail (added right when using), pH 7.9. Phosphate Buffered Saline (PBS) contains 137mM NaCl, 2.7mM KCl, 8.1mM Na2HPO4, 1.76mM KH2PO4, pH 7.4.. Buffer B (for nuclei fractionation) contains 20mM HEPES-KOH, 400mM NaCl, 1mM EDTA, 10% Glycerol, 1mM DTT (add right before using), protease inhibitor cocktail (add right before using), pH to 7.9.. 4x Sample Buffer for western blots was made up of 4 X Sample Buffer (SB) contains 50% Glycerol, 125mM Tris- HCl (pH6.8), 4% SDS, 0.08% Bromophenol Blue, and 5% β-mercaptoethanol, added right before use. Stripping buffer for westerns was made up of 2% SDS, 62.5mM Tris-HCl (pH 6.7) and 100mM β-mercaptoethanol. Poly-D-Lysine Coating Solution for cell culture contains 10µg/ml poly-D-lysine. HBSS Dissection Buffer contained 5.0g/L glucose, 1.25g/L Sucrose, 0.89g/L Hepes, pH to 7.4, osmolarity to 310-320 mOsm, and is aliquoted and stored at - 20°C. Neurobasal Media for most cell culture contains 487.75mL Neurobasal Media (Gibco Thermo Fisher), 0.5mM GlutaMAXTM-I Supplement, 2% B27 supplement, 25µM of glutamic acid. For postnatal cultures, Neurobasal Media contained 200ml Neurobasal Media-A (Gibco ThermoFisher) 4.5ml B27 supplement, 2ml of 200nM stock glutamine and 2ml of penicillin/streptomycin. Poly-D-Lysine coating buffer for postnatal cultures

contained 5mg poly-D-lysine and 5ml sterile H_20, stored at -20C. Before use, aliquots were diluted 50 times and kept at 4°C.

2.2.3 Equipment

Microscopes: For DAPI staining and transfection studies, images were taken on a Zeiss Inverted Fluorescent microscope. For all other immunocytochemistry or immunohistochemistry experiments, confocal microscopes were used, either an Olympus FV1000, or a Leica SP8 WLL.

Nucleofector: Transfection experiments were done using an Amaxa nucleofector, from Lonza.

Peptide facility: All peptides were synthesized in Dr. Yu Tian Wang's lab UBC Peptide Synthesis Facility

Cryostat: Brain slicing was done on a Leica CM 3050 cryostat.

2.2.4 Plasmids

GFP-fused plasmids (GFP-$PTEN_{WT}$ and GFP-$PTEN_{K13R}$) were initially a kind gift from Dr. Alonzo H. Ross (University of Massachusetts Medical School, USA). The coding sequence of human PTEN was inititally cloned into pcDNA3 plasmid, with an enhanced Green Fluorescent Protein (GFP) attached into the 5' end of PTEN. The GFP-$PTEN_{K289R}$ plasmid was constructed in Dr. Yu Tian Wang's lab by Shu Zhang using site-directed mutagenesis with Pfu 68 DNA polymerase. YFP plasmids were a gift from Dr. Lynn Raymond's lab

2.2.5 Postnatal primary mouse neuronal culture

Prior to culture, plates were coated with PDL and put in incubator for at least 2 hours. Prior to cell plating, plates were washed 3 times with sterile water, and dried in the culture hood. Newborn

mouse pups were removed from their mother at postnatal day P0-P1. Mouse pups were anesthetized by freezing at -20 for 10minutes. Brains were removed from pups and placed into a 10mm dish of ice-cold HBSS (kept on ice). Using a dissecting scope in the culture hood, cortex, striatum and/or hippocampal tissue was dissected out and cut into small pieces, then transferred, with HBSS into a 15ml tube and spun down at 1600g for 2min. Supernatant was removed and tissue was incubated at 37°C in papain solution (10ml HBSS (ThermoFisher), 10µl 1M $CaCl_2$, 10µl 500mM EDTA, 475ul Papain (Sigma-Aldrich), 2mg L(+)-cysteine HCl (Sigma) and 20µl 1M NaOH, made fresh and warmed to 37°C) for 15minutes, making sure all tissue is adequately covered. Tubes were then spun down for 2min at 1600g. Supernatant was removed and trypsin inhibitor solution was added to cells (25mg Bovine serum albumin, 25mg trypsin inhibitor (Sigma), 10ml Neurobasal Media (ThermoFisher), 100ul DNAse (Sigma), warmed to 37°C). Cells were then triturated, first 4-5 times with a 10ml pipette, then 10 times with a fire-polished Pasteur pipette, until solution is smooth. Cells were then spun at 1600g for 2 min. Supernatant was removed and cells were re-suspended in 10ml plating media. Cells were plated at $2.5x10^5$ cells/well in 24 well plates, or $5x10^6$ per plate for 10cm dishes. Media was changed every 3-4 days by removing half of conditioned media and replacing with half volume fresh NBM plating media. Cortical and hippocampal cells were used at 12-14DIV, while striatal neurons were used 10-12DIV.

2.2.6 Embryonic primary mouse neuronal culture

Prior to starting cultures, an aliquot of dissection buffer (DB) was removed from the -20°C and thawed on ice. Time-pregnant mice were approximately E16.5 on the day of culture. Mothers were

sacrificed with a dose of 25% urethane (approx. 1ml). Lower abdomen of the mouse was sprayed with 70% ethanol and the uterus was removed and placed in a chilled 10cm dish on ice, filled with sterile DB. Using sterile tools, embryos were transferred to another pre-cooled dish of DB on ice. Brains were removed and transferred to another pre-cooled 35mm dish of ice cold DB (on ice). Using a dissecting scope, cortex, striatum or hippocampal tissue was carefully dissected out, and cut into small pieces. Carefully with a pipette, DB was removed and and replaced with 2-5ml pre-warmed, sterile 0.25% Trypsin-EDTA (Invitrogen) then incubated at 37°C for 30minutes. Cells were then removed from the incubator and trypsin was inactivated by adding 8-10ml of DMEM (Thermo-Fisher) containing 10% Fetal Bovine Serum (FBS) (Invitrogen). Cells were gently mixed and transferred to a 15ml Falcon tube. The cells were left to settle for a few minutes, then the DMEM was removed, save for ~1ml, and fresh DMEM/FBS was replaced. This was repeated with 3 washes. Cells were then triturated gently in NBM 3-4 times with a 10ml pipette, then again several more times with a glass-polished Pasteur pipette until the mixture is smooth. Cells were spun down for 45-50s at 2500RPM then re-suspended in NBM Plating (NP) media. Cells were plated at 2-2.5x10^5 cells/well in a 24 well plate, 1.5x10^5 cells/well for 6 well plates, or 4-6x10^6 wells/plate for 10cm dishes.

2.2.7 Nucleofection of plasmids and MSN-cortical co-culture preparation

For co-cultured MSN-cortical cultures, cells were first prepared for primary embryonic neuron culture, as above. For a 24 well plate, 2x10^6 cells of each cell type were counted and separated, prior to plating. Either cell type was then prepared to be nucleofected with YFP plasmid to distinguish between cell types. YFP plasmid was prepared by was adding 2ug of plasmid to electroporation buffer. Cells to be transfected were spun down gently for ~30s and supernatant

was aspirated. Using a fire-polished Pasteur pipette, electroporation buffer containing plasmid was added to cell pellet and gently pipetted up and down to mix until mixture is homogenous with no bubbles. Buffer and cells were then transferred with the pipette into a nucleofection cuvette (Lonza) and placed into an Amaxa Nucleofector (Lonza). For transfection of MSNs, channel 5 was used, and for cortical neurons, channel 3 was used. The machine was turned on, and the cuvette was removed from the machine. Using the Pasteur pipette, buffer and cells were returned to a 15ml tube and DMEM containing 10% FBS was added to cells as a recovery buffer. Untransfected cells (in DMEM+serum) were then added to transfected cells. 2 million cells each MSN and cortex were used per 24 well plate. Cells were gently pipetted up and down to mix. Cells were then plated into 24 well plates (500ul/well) and recovered in the 37C incubator for 3hours. After 3 hours, DMEM was removed, and NBM plating media (NP) was added to cells. Culture media was changed every 3-4 days. Co-cultured cells were used between 18-21 DIV. The same nucleofection protocol was utilized for transfection of GFP-PTEN plasmids into cultured striatum or cortical neurons, except cells were plated as a mono-culture, instead of co-culture.

2.2.8 NMDAR-mediated *in vitro* excitotoxicity

Primary mono-cultured neurons (cortex/hippocampus at 13-15DIV and striatum at 11-12 DIV) as well as co-cultured MSN-cortical cultures (18-21DIV) were used for excitotoxicity experiments. Prior to NMDA treatments, ½ of the conditioned media was removed and saved in a 50ml Falcon and kept at 37°C. Any peptides, specified by experiment, were delivered to culture media 30 minutes before NMDA treatment. NMDA was then delivered by bath application to cells, at doses dependent on cell type (cortex/hippocampal monoculture 25μM, striatum monoculture 100μM, co-culture 50μM). Monocultured neurons were treated for 1hour at 37°C, while co-cultures were

treated for 10 minutes. After NMDA treatment, media was aspirated, and pre-saved, warm conditioned media was returned to cells, along with any additional peptides. Cells were allowed to recover for various times between 0-24 hours after treatment, depending on experiment, before collection or additional assays.

2.2.9 DAPI nuclear cell death staining

Apoptotic cell death was measured by using nucleic acid stain to identify and count condensing or fragmenting nuclei, a common method for classifying cells undergoing apoptotic cell death ((Hardingham et al. 2002; Wang et al. 2004; Zhang et al. 2013b). PFA-fixed neurons were washed 3x with PBS then stained briefly with DAPI nuclear dye (1:1000 in PBS) for 5minutes at room temperature. Cells were then rinsed gently 3 times with PBS and mounted on glass slides. 5 visual fields were randomly selected on each coverslip, and imaged on a Zeiss Inverted fluorescence microscope at 20x magnification. Images were analyzed using ImageJ software. Condensed/fragmented nuclei were counted as “apoptotic” whereas healthy, non-condensed nuclei were considered “healthy”. Condensing/fragmented nuclei were determined by both size and brightness in comparison to diffusely stained, large nuclei to differentiate. Cell death was calculated as a percentage of “apoptotic” to “healthy” cells to determine the displayed “% Dead Cells”. All experiments were conducted as a double blind.

2.2.10 LDH assays

Lactate dehydrogenase (LDH) is a cytoplasmic-located enzyme that converts NAD into NADH. During cell death, the plasma membrane becomes compromised and LDH is released from the cell. Calculating the amount of LDH in the media of cultured cells can thus be used a means of

measuring cell death. Colormetric LDH assay kits (Sigma) were used to determine cell death, by measuring extracellular LDH in conditioned cell culture media. Kits work to determine extracellular LDH concentration in the following way: LDH in culture medium converts NAD to NADH, which stoichiometrically converts a tetrazolium dye. The resulting color change can be measured using a spectrophotometer plate reader at 490nm, subtracting out a 690nm background. In my study, sample media was collected from cultured neurons and 50ul of media was loaded, in triplicate on 96 well plates. 100ul of the kit mixture of LDH substrate, enzyme and dye (1:1:1) was then added to each well, and plates were shaken at 300rpm, covered with foil, for 30-40 minutes. Plates were then read on the spectrophotometer. Cell death was recorded as a (%) difference between treatment groups and control groups.

2.2.11 Nuclear fractionation

Cell culture nuclear fractionation: Cultured neurons in 10cm dishes, plated densely at $6x10^6$ cells/well. Prior to experiments, Buffer A and Buffer B were prepared and chilled to 4°C on ice (see **Buffers** section) Cells were washed 2x with PBS, then 900ul of Buffer A was added to plates. Plates were rocked, on ice, on a belly-dancer shaker for 30minutes, until, under a microscope, all cells were detached from the plate. Neuron lysates were then gently pipetted up and down to further release cells, then collected into autoclaved 1.5ml Eppendorf tubes, on ice. Tubes were spun down at 14,000g for 3 minutes at 4°C. The supernatant of this spin is the cytosolic fragment. Cytosolic fragments were spun down again at top speed at 4°C for 10minutes to remove any extra debris, keeping the supernatant. Remaining pellets from the first spin are intact nuclei. Pellets were washed 2x with Buffer A, spinning down at 14000g for 3 minutes each time, removing as much supernatant as possible. Pellets were then re-suspended with 150ul Buffer B, pipetting up and

down and vortexing to break up the pellet. Tubes were buried in ice and placed on a rocker for 2 hours, vortexing every 30minutes for 10 seconds. Nuclear lysates were then spun down at 14000g for 5minutes at 4°C. Supernatant is the nuclear fraction, and was transferred to a fresh 1.5ml Eppendorf tube. Protein concentrations were measured using a DC protein assay, and samples were measured using western blot. To assure purity of cytoplasmic and nuclear fractions, corresponding subcellular marker proteins were probed for in western blot. (Song et al. 2008; Zhang et al. 2013b). In my studies, the cytoplasmic protein Heat Shock Protein 90 (HSP90) was used as a cytoplasmic maker, and the nuclear envelope protein LaminB1was used as a nuclear marker.

Brain tissue nuclear fractionation: FVB control, YAC transgenic mice, or Sprague Dawley rats were used to generate tissue for nuclear fractionation experiments. Prior to experiments, Buffer A and Buffer B were prepared and chilled to 4°C on ice (see **Buffers** section). Also prior to experiment, cortical, striatal and hippocampal brain tissues were isolated, and snap frozen in dry ice and kept at -80°C until experiment date. Before experiment, tissues were placed, in sterile Eppendorf tubes on ice. Tissues were finely chopped, keeping them cold on ice, using small, sterile scissors. Once chopped, 50ul of chilled buffer A was added and tissues were homogenized 15-20 strokes with a plastic homogenizer, on ice. Another 950ul of chilled Buffer A was added to the tissue lysates and they were homogenized again for 15-20 strokes, on ice. Homogenates were then incubated on ice for 15minutes, then spun down at 850g for 10 minutes at 4°C. Supernatant was kept as a full cell lysate control. At this point, most of the cells have not been lysed. Cell pellets were re-suspended in another 1ml of Buffer A, and homogenized for 15-20 strokes, and pipetted up and down to create a homogenous mixture. Homogenates were incubated on ice for another

15minutes, then spun down at 14,000g for 3 minutes at 4°C. The supernatant of this spin is the cytosolic fragment. Cytosolic fragments were spun down again at top speed at 4°C for 10minutes to remove any extra debris, keeping the supernatant. Remaining pellets from the first spin are intact nuclei. Pellets were washed 2x with 1ml Buffer A, spinning down at 14000g for 3 minutes each time, removing as much supernatant as possible. Pellets were then re-suspended with 300ul Buffer B, pipetting up and down and vortexing to break up the pellet. Tubes were buried in ice and placed on a rocker for 2 hours, vortexing every 30minutes for 10 seconds. Nuclear lysates were then spun down at 14000g for 5minutes at 4°C. Supernatant is the nuclear fraction, and was transferred to a fresh 1.5ml Eppendorf tube. Protein concentrations were measured using a DC protein assay, and samples were measured using western blot, as described above. For Brain Tissue fractionations, an additional layer of analysis was added. First, bands were normalized using respective nuclear and cytoplasmic control bands. Then, nuclear band density was compared to cytoplasmic band density to determine the fold difference of nuclear PTEN to cytoplasmic PTEN for each condition. FVB was normalized to 1 for all experiments.

2.2.12 Western blotting

Samples for western blot were prepared by measuring equal amounts of protein, then making all samples equal volume using PBS and adding 4x Sample buffer. Samples were then boiled at 95°C for 5 minutes and spun down briefly. Proteins were separated by electrophoresis using 10% acrylamide SDS-PAGE gels, then transferred for 1.5hours onto polyvinylidene diflouride (PVDF) membranes. After transfer, membranes were incubated for 1hour in 5% fat free milk to block non-specific protein binding. Membranes were incubated overnight at 4°C in antibody to the target

protein. In the morning, membranes were washed 3 times with Tris-Buffered Saline, containing 0.5% Tween (TBS-T) then incubated for 1hour at room temperature with corresponding horseradish peroxidase (HRP) –tagged secondary antibody in 3-5% skim milk-TBS-T. Membranes were then washed 3 times with TBS-T and developed using enhanced chemiluminescence system (Pierce ECL). If other target proteins were to be probed on the same membrane, blots were stripped at 30°C for 20minutes, rinsed 5x times under running water, then washed 3 times for 10 minutes with TBS-T before adding the next primary antibody. Blots were imaged on a Bio-Rad ChemiDoc™ and analyzed using Bio-Rad QuantityOne® or ImageLab® imaging software by measuring raw intensity of the target bands. Levels of target protein were determined by comparing the intensity of the target bands to those of loading control proteins. HSP90 (1:1000 concentration) was used for cytosolic proteins, and LaminB1 (1:1000 concentration) was used for nuclear proteins(Zhang et al. 2013b).

2.2.13 Immunocytochemistry and image analysis

Cultured neurons on coverslips were washed two times with PBS, then fixed with 4% Paraformaldehyde in PBS for ten minutes at room temperature, then washed three times for 5 minutes each wash on a slow shaker with PBS. Cells were then permeabilized for 3 minutes with 0.2% Triton-X in PBS then rinsed 3 times with PBS. Cells were blocked to prevent non-specific antibody binding with 10% BSA, standing, at room temperature, then incubated overnight to a few days at 4°C with primary antibody to the target protein. In the morning, cells were washed 3 times with PBS containing 0.5% BSA, for 5 minutes each wash, on a slow shaker. AlexaFluor conjugated secondary antibodies corresponding to the target protein (1:1000 in 1% BSA) were then applied to cells and incubated overnight at 4°C, or room temperature for 2 hours. Cells were then washed

three times with 0.5% BSA for 5 minutes on a slow shaker. If DAPI staining was required, it was then done, as outlined in Chapter 2.9. Cells were then mounted on glass slides using Fluormount-G, and left to dry overnight, in the dark. Cells were imaged on a Zeiss Confocal Microscope at 64x magnification, taking 5 images per coverslip, separated into quadrants to randomize. Images were analyzed using ImageJ software after being converted to greyscale. Nuclear translocation of PTEN was determining by creating Regions of Interest (ROIs) of the nucleus and the cytoplasm and comparing the PTEN fluorescence intensity of the nucleus vs cytoplasm. Nuclear ROI was determined using DAPI nuclear stain while cytoplasmic ROI was determined using GFP or PTEN images. Fluorescence intensity of PTEN was measured as mean grey value (MGV). For each cell, MGV of the nuclear ROI was divided by the MGV of the cytoplasmic ROI, then divided by 100 to give an estimation of the fold difference of PTEN in the nucleus to PTEN in the cytoplasm.

2.2.14 Kainic acid CA1 hippocampal lesions

Sprague Dawley rats (age ~3months) were used for kainic acid experiments. 1 hour prior to kainic acid treatment, animals were treated with 10mg/kg K13 peptide or saline vehicle (300ul) through IV tail vein delivery. This is a dose previously shown effective *in vivo* against a model of stroke(Zhang et al. 2013b). Prior to all IV injections, animals were briefly subdued using isoflurane anesthesia, to ensure effective IV injections and reduce stress. This was especially important as animals receiving kainic acid treatment were very stressed in the subsequent treatment days. Kainic acid was then delivered IP (10mg/kg, 300ul) or saline vehicle. Kainic acid treatments induced seizure behavior in many animals. Seizures were monitored after KA. Tat-K13 peptide or saline vehicle was subsequently delivered at 24hours and 28hours (10mg/kg, 300ul, IV tail vein)

after kainic acid treatment. 7 days after kainic acid, animals were sacrificed by urethane and brains were harvested for immunostaining or western blot.

2.2.15 Quinolinic acid striatal lesions

FVB-N or YAC128 mice (aged 3 months) were used for quinolinic acid (QA) experiments. 1 hour prior to QA, animals were injected IV with Tat-K13 (10mg/kg, dissolved in saline) or saline vehicle. QA Sigma, Sweden) was dissolved in 0.1M Phosphate Buffered Saline. Under isoflurane anesthesia, mice were given intrastriatal injections of 8nmol QA (0.8 µl). A 2µl Hamiton microsyringe fitted with a glass micropipette (60-80µm diameter) was used to deliver QA into the right striatum at stereotaxic coordinates of 0.9mm rostral to bregma, 2.0mm lateral to midline, 3.2mm ventral from bone surface, and tooth-bar set to zero. QA injections were delivered for 5minutes, then the cannula was left untouched for 5min to reduce leakage. Mouse body temperature was maintained at 37°C with a heating pad. Following QA, peptide or saline vehicle was delivered at 1hour post-surgery by IV injection (10mg/kg) and also delivered at 24hr and 48hr after surgery. 7 days after surgery, mice were sacrificed for lesion volume analysis by FluoroJade.

2.2.16 Tissue perfusion and slicing

Prior to rodent perfusions, saline was run through perfusion tubing to adequately flush the system. Animals (rats or mice) were anesthetized by IP urethane (10% in phosphate buffered saline). Once animals were in a surgical plane of anesthesia, confirmed by toe pinch, animals were moved to a fume hood and their limbs were taped down to secure. An incision was made through the skin, the length of the diaphragm. Sharp scissors were used to cut through the connective tissue at the bottom of the diaphragm to allow access to the ribcage. The thoracic cavity was opened by using

scissors to cut through the ribcage and clamps were used to keep the cavity open. Using forceps to steady the heart, still beating, a small, blunted needle, attached to the perfusion rig, was inserted gently into the protrusion of the left ventricle, about 5mm. Position of the needle was secured using a small clamp. Once needle was secured, the right atrium was cut using small scissors. Perfusion valve was then slowly released to allow flow of 0.9% saline solution. Animals were perfused with saline until blood was cleared from the body, at which point, the perfusion lines were switched to 4% paraformaldehyde (PFA) in PBS. Approximately 200-300ml of PFA were used for rats, and about 30-40ml was used for mice. After animals were adequately perfused (confirmed by firm, white tissues), brains were removed and kept overnight at 4°C in 4%PFA. The next day, brains were moved to a dehydration solution of 30% sucrose in PBS at 4°C until brains no longer floated. After dehydration, brains were snap frozen in dry ice, then kept for at least 1hour at -80°C. Brains were sliced at 30µm on a cryostat kept at -20°C, and slices were kept in PBS containing azide at 4°C until ready for staining.

2.2.17 Immunohistochemistry

Rat or mouse brain slices were prepared as in 2.2.17. Slices were transferred between washes using small, mesh-bottomed cups (Netwell, VWR) to avoid damaging tissue. Slices were washed 3x for 10minutes in PBS in 12well plates, with slow shaking. Slices were then blocked and permeabilized for 30min in 0.5% Triton-X in 5% BSA- containing PBS. Slices were then washed 3 times with PBS for 10minutes. Primary antibody was added to slices for 3-5 days at 4°C to ensure tissue penetration. Following primary antibody incubation, slices were washed 3x with 0.5% BSA-containing PBS, then incubated overnight with secondary antibody (1:1000, fluorescent Alexa

series in 1% BSA) at 4°C. Any other primary antibodies were done subsequently in the same way. Prior to mounting, slices were washed 3x with 0.5% BSA PBS, and using a paintbrush, slices were gently laid on glass slides, then dried. Fluoromount-G, containing DAPI stain for nuclear identification was dropped onto dried slices, and then covered with a rectangular glass coverslip. Mounted slices were dried overnight before imaging.

2.2.18 Fluoro-Jade B staining

Tissues to be stained were prepared as in 2.2.17. Prior to staining, slices were washed 3x with PBS, then gently mounted on gelatinized glass slides using a paintbrush and air dried. Slides were then placed in a staining rack and immersed in 100% EtOH for 3min, then 70% EtOH for 1min, then ddH_20 for 1min. Slices were then immersed in 0.06% Potassium Permanganate ($KMn0_4$) for 15min, shaking gently, then immersed in ddH_20 for 1min. From this step on, the protocol was performed in the dark. Slices were immersed in 0.001% Fluoro-Jade B (Millipore) prepared from a 0.01% stock solution (prepared ahead of time but stored no more than 2 months) in ddH_20. Working solution of Fluoro-Jade B also contained 0.1% acetic acid. Slices were stained in Fluoro-Jade B for 30min, then washed 3x for 1min with ddH_20, again using immersion. Slides were then removed from the rack, and dried at room temperature, in the dark. Slides were placed back in the rack when dried and immersed in 3 separate baths of xylene for 2min each, then coverslipped with DPX (Electron Microscopy Sciences)

2.2.19 Animal models

Animals used in the current studies were HD YAC128 transgenic mice, originally designed by Michael Hayden's lab at UBC(Slow et al. 2003). These mice express a full-length, human

huntingtin gene with 128 CAG repeats, as discussed in section 1.4. Mouse background is FVB-N, line 55. Control animals were non-transgenic FVB-N. In some experiments control animals were YAC18 mice, which also express a YAC transgene with human huntingtin, carrying a non-HD CAG expansion of 18 repeats. Also, in a few experiments, a mid-range HD model was used, which expresses 72 CAG repeats instead of 128. This model is a less extreme model of HD than the YAC128 and was used to test CAG-dependency on PTEN nuclear translocation over time. All animals were bred, raised and maintained by Dr. Lynn Raymond's lab (University of British Columbia).

2.2.20 Statistical analysis

All measurements were performed blindly where possible. Results are expressed as a mean and error bars represent standard error of the mean. One way ANOVA was used for multiple group comparison, followed by post-hoc (Tukey) test where appropriate. Students *t*-tests were used for comparison between two groups. Data lacking normal distribution were analyzed by a nonparametric Rank Sum Test (Mann-Whitney). Statistical significance was defined as $*P < 0.05$, $** P < 0.01$ ***P<0.001 ****P<0.0001.

2.3 Results

In our previous studies, we demonstrated that blocking PTEN nuclear translocation using the Tat-K13 peptide provided significant neuroprotection against NMDAR-mediated excitotoxicity both *in vitro* and *in vivo* using cortical and/or rat hippocampal neurons. Based on the strong evidence for NMDAR-mediated excitotoxicity as a key player in neuron death in HD, particularly in the striatum, my first experiments aimed to test, first, if PTEN nuclear is also associated with

NMDAR-mediated excitotoxicity in HD neurons, and next, if the Tat-K13 peptide could provide therapeutic benefit against NMDAR-mediated cell death in cultured neurons from HD mice.

2.3.1 NMDA stimulation causes excitotoxic neuronal death in cultured HD neurons

For my initial experiments, I used cultured striatal neurons from YAC128 HD mice and FVB-N WT mice. Though excitotoxicity is most prevalent in HD striatum, I also tested cultured neurons from cortex and hippocampus in my studies, as the cortex plays an important role in excitotoxicity in HD(Cepeda et al. 2007; Cepeda et al. 2003; Milnerwood et al. 2012) and NMDAR dysfunction in the hippocampus is apparent in HD (Hodgson et al. 1999) Similarly, both areas show cell loss in late stages of HD. It has been shown in previous experiments that this culture protocol for striatal neurons yields ~95% inhibitory GABA-ergic medium spiny neurons (MSNs), the primary cell type affected by HD(Zeron et al. 2002; Fan et al. 2012; Fan & Raymond 2007) whereas cortical and hippocampal cultures contain primarily excitatory, glutamatergic neurons(Zhang et al, 2013, Taghibiglou et al, 2011). In these experiments, I used an *in vitro* model of NMDAR-mediated excitotoxicity previously used in our lab(Zhang et al. 2013b; Taghibiglou et al. 2009; Fan et al. 2014), and in YAC128 primary cultured neurons(Zeron et al. 2002; Fan et al. 2012; Fan et al. 2007). In this model, cultured neurons are treated with NMDA in culture medium to induce excitotoxicity. As I was using a variety of cell types, I first tested the NMDA treatment protocol in a dose response to determine optimum treatment conditions. Cultured cells were treated with various doses of NMDA (between 1-1000uM) in cultured media for 1hour, then allowed to recover for 24hours to allow excitotoxicity to develop. LDH cell death assays were used to determine neuron death. The broad NMDAR antagonist APV was used (50uM) to test NMDA-dependency of cell death. NMDA provided dose-dependent neuronal death in all neurons

tested that was reversible using NMDAR-antagonist APV (Data not shown). NMDA was more toxic at lower doses in cortex and hippocampal neurons, whereas higher doses of NMDA were required to reach similar excitotoxicity levels in striatal neurons. This is similar to previous studies, which demonstrate that cultured MSNs are more resistant to NMDA than excitatory cortical/hippocampal neurons (Fan et al. 2010). Based on these experiments, I decided to use 25uM NMDA for 1 hour when treating cortex or hippocampal neurons, whereas striatal neurons were all treated with 100uM NMDA for 1 hour.

2.3.2 NMDAR-mediated excitotoxicity causes rapid PTEN nuclear translocation in cultured HD neurons

Once the *in vitro* NMDA excitotoxicity protocol was established, I next aimed to test if NMDA excitotoxicity is associated with subsequent PTEN nuclear translocation in HD neurons. Also, I wanted to text whether Tat-K13 could prevent PTEN nuclear translocation, as we previously demonstrated. All experiments also used Tat-K289 as a control peptide, which we expected to not block PTEN nuclear translocation and thus act as a control peptide. Similar to our previous Tat-K13 studies (Zhang et al, 2013), I pretreated neurons in culture medium for 1 hour with 10uM Tat-K13 or Tat-K289 (dissolved in sterile water), before stimulating neurons for an hour with NMDA. After NMDA treatment, culture media was aspirated and medium containing Tat-K13 (10uM) was added to cells. In our previous studies, PTEN nuclear translocation peaks 6-9hrs after an excitotoxic event, thus, neurons were removed from the incubator at 7 hours post NMDA to assess PTEN translocation. Nuclear fractionations were conducted to separate nuclear material from cytoplasmic cell material, and western blot was used to test nuclear translocation of PTEN. As seen in our previous studies, NMDAR-mediated excitotoxicity was associated with a

robust increase in nuclear translocation of PTEN that was associated with a decrease in cytoplasmic PTEN in both WT and HD neurons (**Fig 2-2).** As hypothesized, Tat-K13 peptide, but not Tat-K289 prevented PTEN nuclear translocation in cortical and hippocampal neurons (**Fig 2-2 A-D).** However, unexpectedly, PTEN nuclear translocation was not rescued by the Tat-K13 peptide in both WT and HD striatal neurons (**Fig 2-2 E-F).** The Tat-K289 peptide had no effect on PTEN nuclear translocation. To further study PTEN nuclear translocation after NMDA excitotoxicity in these neuron types, the same peptide/NMDA treatment paradigm was used and neurons were fixed using 4% PFA and immmunostained for PTEN and nuclear marker DAPI. In support of my western blots, in nuclear fractionation experiments, NMDA stimulation led to a clear nuclear translocation of PTEN in all neurons studied (**Fig 2-3).** Using immunostaining, Tat-K13 peptide successfully blocked PTEN nuclear translocation in hippocampal neurons (**Fig 2-3 A)**, whereas Tat-K13 did not significantly prevent NMDAR-mediated PTEN nuclear translocation in the striatum in either WT or YAC128 cells **(Fig 2-3 B, C).** Interestingly, NMDA lead to more dramatic PTEN nuclear translocation in WT neurons compared to YAC128 neurons. To test any dose dependent effects of Tat-K13, dose response experiments were conducted in WT striatum. Although Tat-K13 slightly lowers PTEN nuclear translocation in both WT and YAC128 neurons, blockade never reached control levels even by increasing dose (**Fig 2-3 D)**. Although my hypothesis was confirmed, that PTEN nuclear translocation appears to be a downstream effect in HD neurons associated with an NMDAR-mediated excitotoxic event, I found, unexpectedly, that the Tat-K13 peptide only blocked this translocation in cortex and hippocampal neurons, but not the striatum. This suggests there may be differential factors involved in PTEN nuclear translocation in the striatum.

2.3.3 Tat-K13 peptide is neuroprotective against NMDAR-mediated excitotoxic neuronal death in HD cortical/hippocampal neurons *in vitro* but not in striatal neurons

In my first experiments, NMDA stimulation was associated with rapid PTEN nuclear translocation following NMDAR-mediated excitotoxicity in all cells tested, suggesting it may be involved in neuron death following an excitotoxic event. Interestingly, the Tat-K13 provided differential roles in preventing PTEN nuclear translocation in cortex/hippocampal neurons vs striatal neurons. My next experiments aimed to determine if blocking PTEN nuclear translocation in HD neurons could provide neuroprotective benefit against NMDAR-mediated excitotoxic death. Similar to my initial experiments, cells were pretreated with Tat-K13 for an hour, then stimulated with NMDA, and treated with Tat-K13 during recovery. 24 hours after NMDA treatment, cells were removed from the incubator. A sample of culture media was collected to conduct LDH cell-death assays, and cells were then fixed using 4% PFA. Apoptotic cell death was assessed using either DAPI nuclear stain to assess nuclear condensation and fragmentation, commonly used to identify apoptotic nuclei. Based on my initial experiments demonstrating blockade of PTEN nuclear translocation in cortex and hippocampal HD neurons, I expected that Tat-K13 would protect against NMDA excitotoxicity in the same cell types. Indeed, Tat-K13 provided complete neuroprotection against neuronal death in both cortex and hippocampal neurons from WT and YAC128 mice, while Tat-K289 had no effect, demonstrated using LDH assays (**Fig 2-4 A, B**) and DAPI staining (**Fig 2-4 E, F)**. However, Tat-K13 did not completely protect against NMDAR-mediated excitotoxicity in both HD and WT striatal neurons (**Fig 2-4 C, G-H).** As some neuroprotection was seen with Tat-K13 in LDH assays, I tested whether different doses of Tat-K13 could improve neuroprotection in striatal neurons. Dose response experiments showed a slightly increasing neuroprotection of Tat-K13 between 1-30uM in striatal neurons in LDH assays,

however, neuroprotection hits a plateau, never completely rescuing striatal neurons, as demonstrated using LDH assay (**Fig 2-4 D).** Tat-K13 was not protective at any dose using DAPI nuclear staining to measure apoptotic condensing nuclei in either WT or YAC128 (**Fig 2-4 G, H).** Tat-K289 had no neuroprotective effect in any cells. Neither peptide caused any significant toxicity at low doses, however as dose of Tat-K13 increased, neuronal death increased, starting at 20-30uM of peptide. This is likely due to toxicity of the TAT sequence, shown previously in other studies. These data demonstrate that Tat-K13, blocking PTEN nuclear translocation effectively, can provide significant neuroprotection against NMDAR-mediated excitotoxicity in HD cortex/hippocampus, however, Tat-K13 is be ineffective at blocking this NMDAR-mediated PTEN nuclear translocation in the striatum, and is not efficiently neuroprotective. This could provide insight into some of the differential mechanisms of excitotoxicity in striatum vs other cell types. This data also suggests that PTEN nuclear translocation may be associated with neuronal death after NMDAR-mediated excitotoxicity, including in HD neurons.

Following these experiments, I speculated on reasons why the striatum may be only partially protected by Tat-K13. My initial experiments were performed in mono-cultured striatal neurons. Although these cultures have been useful for learning much about NMDAR-mediated excitotoxicity in HD in many studies (Zeron et al. 2001; Zeron et al. 2002; Fan & Raymond 2007), in their natural setting, medium spiny neurons (MSNs), receive glutamatergic input from cortical and thalamic afferents, and when cultured without these supportive input neurons, mono-cultured MSNs do not develop normal, full spiny morphology (Segal et al. 2003). Similarly, striatal mono-cultures do not live as long as long as cortical/hippocampal mono-cultured neurons, meaning they need to be used at an earlier time point in order to obtain healthy neurons (~9-12 DIV vs 14-16DIV) (Zeron et al. 2001; Zeron et al. 2002; Fan & Raymond 2007). Thus, it is possible that

monocultured striatal neurons may not provide the ideal model for testing NMDAR-mediated excitotoxicity, Tat-K13 neuroprotection and PTEN nuclear translocation. To address this, Dr. Lynn Raymond's lab at UBC developed a co-culture system to study glutamatergic signaling in cortico-stiratal circuits (Kaufman et al. 2012; Milnerwood et al. 2012). In these cultures, cortical and striatal neurons from embryonic mice are harvested and plated at a 1:1 ratio. One cell type is nucleofected prior to plating with YFP plasmid for identification. Alternatively, MSNs can be identified by staining with antibodies against the MSN-specific protein DARPP-32. These cultures have been useful for improving the health and morphology of MSNs *in vitro*, as they are more representative of MSN physiology in the brain (Kaufman et al. 2012; Milnerwood et al. 2012). MSNs in these cultures demonstrate excellent, representative MSN spiny morphology and allow all neurons to live much longer, with healthy neurons between 18-21DIV. Using cortex/MSN co-cultures, I next investigated PTEN nuclear translocation and Tat-K13 neuroprotection following NMDAR-mediated excitotoxicity.

My first experiments in co-cultures looked at PTEN nuclear translocation over a range of doses of NMDA in cultured WT neurons. Co-cultures were treated with doses of NMDA between 1-1000uM NMDA for only 15minutes in culture media, as experiments using these cells in the past have demonstrated that co-cultured cortex/MSNs are more sensitive to NMDA than their monocultured counterparts(Kaufman et al. 2012; Milnerwood et al. 2012). 7 hours after NMDA treatment, cells were fixed with 4% PFA. Immunostaining revealed trending nuclear translocation in both cortical (**Fig 2-5 A).** and striatal (**Fig 2-5 B)** neurons after NMDA treatment that was reversed by treatment with ifenprodil, an NMDAR antagonist that has specificity for GluN2B-containing NMDARs (**Fig 2-5 A, B).** This data supports my previous findings, demonstrating that NMDAR-mediated excitotoxicity is associated with rapid PTEN nuclear translocation in both WT

and HD neurons. This data also supports the idea that this translocation is GluN2B-dependent, supporting previous studies demonstrating that GluN2B-containing, extrasynaptic NMDAR activity is associated with neuronal death in HD. Based on these experiments, I selected a dose of NMDA of 60uM for 15minutes, as this dose led to clear PTEN nuclear translocation without compromising cell integrity (**Fig 2-5 C**). Next, I repeated my experiments using Tat-K13 peptide in the co-culture system for both WT and YAC128 neurons. Various doses of Tat-K13 (1uM-30uM) were used to determine any dose-dependencies of Tat-K13 in the co-culture. Similar to my monoculture experiments, neurons were pretreated for 1hour with Tat-K13 or Tat-K289 peptide, then treated with NMDA, before being recovered with peptide/vehicle. Based on dose response experiments, 60uM NMDA was used for 15 minutes. Media was then aspirated and cells were treated again with peptide in the culture media. Cells were collected at 7 hours for immunostaining. Despite the more physiologically relevant co-culuture system, and improved baseline morphology of MSNs in co-culture, striatum MSNs from both WT (**Fig 2-5 D)** and YAC128 HD mice (**Fig 2-5 E)**, PTEN nuclear translocation following NMDA was not reversed with Tat-K13 peptide, at any dose. Tat-K289 had no effects on PTEN nuclear translocation following NMDA. Similar to mono-culture, TAT peptides demonstrate toxicity beginning around 20-30uM, at slightly lower doses in YAC128, (confirmed using LDH assays, not shown) accompanied by increased nuclear PTEN, preventing any further dose increase of Tat-K13. Interestingly, similar to my monoculture experiments, PTEN nuclear translocation after NMDA was less dramatic in YAC128 neurons, potentially demonstrating differential signaling in HD vs non-HD striatal neurons. In contrast, similar to my experiments in mono-culture, cortical neurons in co-culture (**Fig 2-5 E)** demonstrated complete rescue of PTEN nuclear translocation following Tat-K13, but not Tat-K289 peptide treatment. This was associated with improvements in cellular

morphology, suggesting that this reversal of PTEN nuclear translocation was associated with neuroprotection in cortical neurons.

These experiments demonstrate that PTEN nuclear translocation appears to be associated with NMDAR-mediated excitotoxicity and cell death in both WT and HD neurons, and show some exciting prospect in the potential of Tat-K13 peptide to block PTEN nuclear translocation and protect against NMDAR-mediated excitotoxicity in cortical and hippocampal HD cells. However, Tat-K13 was ineffective at significantly blocking PTEN nuclear translocation in the striatum in both WT and YAC128 neurons. This is an interesting finding that might demonstrate some differences between striatum and cortex in response to NMDAR-mediated excitotoxicity in terms of PTEN nuclear translocation.

2.3.4 Nuclear PTEN is elevated in the brains of HD transgenic mice

My next set of experiments investigated baseline changes in PTEN nuclear translocation in the brains of HD mice. There is much evidence that demonstrates that NMDAR-mediated excitotioxicity, primarily through GluN2B-containing, extrasynaptically-located NMDARs, plays a major role in cell death and dysfunction in HD brains, especially in cortico-striatal circuits (see section 1.3.8). This is an early event in HD pathogenesis, with neurons in the striatum demonstrating enhanced sensitivity to NMDA excitotoxicity via enhanced NMDA current, increased Ca^{2+} influx in response to NMDA, enhanced forward trafficking and expression of GluN2B, extrasynaptic NMDARs and greater NMDA-induced neuronal death vs WT control, prior to symptom onset(Milnerwood et al. 2010; Benn et al. 2007; Fan et al. 2012; Chen et al. 1999; Carlos Cepeda et al. 2001; Fan & Raymond 2007; Graham et al. 2009; Gladding et al. 2014; Milnerwood et al. 2012; Zeron et al. 2004; Okamoto et al. 2009; Gu et al. 2007). Based on our

studies showing that PTEN nuclear translocation appears to be a critical downstream step associated with cell death following GluN2B, extrasynaptic NMDAR activation(Zhang et al, 2013), and the fact that acute NMDA excitotoxicity is associated with PTEN nuclear translocation in HD neurons *in vitro*, I hypothesized that PTEN nuclear translocation may be elevated in the brains of HD mice in areas where NMDAR-mediated excitotoxicity may be occurring, primarily in the striatum and the cortex. I would expect these changes to occur early in disease pathogenesis, at times when sensitivity to NMDAR-mediated excitotoxicity is present(Graham et al. 2009; Zeron et al. 2002). On the contrary, later in disease pathogenesis, striatal neurons become resistant to NMDA in HD(Graham et al. 2009). Based on experiments demonstrating that PTEN nuclear translocation may be associated with cell death in other conditions, outside of NMDAR-mediated excitotoxicity, it is also possible that PTEN in the nucleus may be elevated in the areas of the brain experiencing cell loss in later stages of the disease, such as the cortex and hippocampus. If NMDAR-mediated excitotoxicity and neuronal death is associated with increased nuclear PTEN in HD brains, it may be possible to use Tat-K13 as a means to prevent nuclear translocation of PTEN and potentially rescue neuronal loss in HD tissues.

To test my hypothesis, I raised mice to various ages. Mice tested were of four strains. Wild type mice were either FVB-N (non-transgenic) or YAC18, expressing a full YAC transgene, but with only 18 CAG repeats (non-HD length). I also tested two strains of HD transgenic mice, the milder YAC72, with 72 CAG repeats and YAC128 with 128 CAG repeats. This was to determine any CAG repeat length dependencies of nuclear PTEN. Mice were raised to 2 months (pre-symptomatic, NMDA sensitive), 6 months (early symptomatic, still NMDA sensitive) and 12 months (late symptomatic, NMDA resistant) (Graham et al. 2009). My first experiments used tissue collected from the brains of these animals from the striatum, cortex and hippocampus.

Nuclear fractionations were performed on tissues to separate the nuclear material from the cytoplasmic material and western blots were performed to detect PTEN expression in various cell compartments. At 2 months of age, although most data did not reach significance, there was a trend towards increased PTEN nuclear expression in all tissues tested in YAC128 mice, with a slight increase in YAC72. This increase was significant, interestingly, in hippocampus, and in the cortex compared to YAC18 control, with trends in cortex and striatum compared to FVB-N (**Fig 2-6 A)**. At 6 months, no significant increase in nuclear PTEN is observed in striatum or hippocampus, but YAC128 mice continued to show elevated nuclear PTEN in the cortex, reaching significance (**Fig 2-6 B)**. These findings suggest that HD mice, at time-points correlating with increased sensitivity to NMDAR-mediated excitotoxicity, may also experience increased cellular nuclear PTEN, an effect, surprisingly, detected primarily in the cortex. At 6 months, nuclear PTEN in the hipppocampus trends towards decrease in YAC mice vs control. At 12 months there were no clear differences between mice in nuclear PTEN expression (**Fig 2-6 C**). 12 months is a time when YAC HD mice have shown to be resistant to NMDA, thus, this may be a reflection of these changes. This may also represent compensatory mechanisms in late disease stage. In all, though changes were very subtle, these data suggest that increases in PTEN nuclear translocation may be an early effect in the brains of HD mice, that could be associated with enhanced NMDAR-mediated excitotoxicity in HD brains, particularly with longer CAG repeats. This effect, interestingly, appears to be most prevalent in the cortex and the hippocampus at early stages, suggesting that, surprisingly, perhaps PTEN nuclear translocation is associated more with excitotoxicity and/or cellular dysfunction in these brain areas in HD, than the striatum.

2.3.5 Tat-K13 is neuroprotective against NMDAR-mediated excitotoxicity in CA1 Hippocampus *in vivo*

My experiments in non-stimulated HD tissues demonstrated that there are small, but significant increases in nuclear PTEN in HD brains. Based on the role of NMDAR-mediated excitotoxicitiy in HD pathogenesis, and my observations *in vitro* that PTEN nuclear translocation is associated with cell death in both WT and HD neurons, I next wanted to test if Tat-K13 could provide any therapeutic benefit against NMDAR mediated excitotoxicitiy *in vivo*. Previously, our lab demonstrated that Tat-K13 was effective at protecting against NMDAR-mediated excitotoxic neuron death in an MCAO model of stroke, in cortical neurons(Zhang et al, 2013), which supports my findings demonstrating significant neuroprotection in WT and HD cortex *in vitro*. However, cell death in HD occurs in the striatum, as well as later loss in hippocampus and cortex, thus, I wanted to also test if Tat-K13 could block NMDAR-mediated PTEN nuclear translocation in hippocampus and striatum, and protect against neuron death in live animals. If successful, these studies could point the way to novel mechanisms for delaying or preventing neuron loss in HD. As Tat-K13 was significantly effective at reversing PTEN nuclear translocation and protecting against neuron death in WT and HD hippocampus, and since there was a significant increase in nuclear PTEN in YAC128 hippocampus at 2 months, I first tested an acute model of NMDAR-mediated excitotoxicitiy in the hippocampus, to determine the neuroprotective ability of Tat-K13 in CA1 neurons. Although the striatum is most vulnerable in striatum in HD, several studies have demonstrated neuronal dysfunction and cell loss in other brain areas, including the hippocampus (Spargo et al, 1993; Kassubek et al, 2004;Usdin et al, 1999; Murphy et al, 2000; Milnerwood et al, 2006). In the hippocampus, activity-dependent plasticity and changes in synaptic efficacy, many of which are NMDAR-dependent, have been widely investigated as a cellular substrate of

learning and memory(Bliss & Collingridge 1993; Morris 1989). In HD, degeneration is seen in HD hippocampus and impaired NMDAR-dependent synaptic plasticity at CA3-CA1 synapses is seen in several mouse model of HD (Usdin et al. 1999; Murphy et al. 2000; Cummings et al. 2006) a mechanism hypothesized to contribute to abnormalities in cognition and memory in HD(Rosas et al, 2008; Usdin et al., 1999). CA1 hippocampal loss is also apparent in late HD, at times when cognitive deficit and dementia are apparent(Spargo et al. 1993b; Usdin et al. 1999). Thus NMDAR-dependent dysfunction and degeneration in the hippocampus may have an underlying role in HD pathogenesis.

In my study, an acute model of hippocampal excitotoxicity was used to test the ability of Tat-K13 to protect hippocampal neurons against NMDAR-mediated damage. Kainic Acid (KA) is an agonist for ionotrophic glutamate receptors that mimics glutamate excitotoxicity in neurodegenerative diseases (Sperk et al. 1983). Previously, direct injections of KA were used as early animal models of HD, and helped pave the way for our current understanding of the role of excitotoxicity in HD(Coyle & Schwartz 1976; McGeer & McGeer 1976; Sanberg et al. 1978). Intraperitoneal (IP) injection of KA causes neuronal death and aberrant signaling in several brain areas including prominent damage in CA1 hippocampal pyramidal neurons (Sperk et al. 1983; Brandt et al. 2003; Vincent & Mulle 2009). KA is used as an epilepsy model, as treatment is accompanied by catatonia, “wet dog shakes”, and tonic-clonic convulsions (Sperk et al. 1983). IP KA-induced CA1 neuronal death can be blocked with NMDAR antagonist MK-801, suggesting that neuronal death in the hippocampus is mediated at least in part by NMDAR-mediated excitotoxic mechanisms (Brandt et al. 2003). Based on evidence of hippocampal death and dysfunction in HD and its role in cognitive decline, I hypothesized that Tat-K13 peptide would protect against KA-incluced hippocampal neuronal death.

My experiments used 3 month old Sprague-Dawley(SD) rats to test the KA-induced excitotoxic model, as this animal model has been widely used, historically, for KA injections (Sperk et al. 1983). First, I tested the ability of Tat-K13 to protect against CA1 hippocampal death after KA. Rats were treated with 10mg/kg Tat-K13 or saline, by IV tail vein injection one hour prior to 10mg/kg intraperitoneal KA or saline(Sperk et al. 1983; Vincent & Mulle 2009). Subsequent IV injections of peptide/saline were given 24 and 48 hours after KA. 7 days after KA treatment, a time-point previously associated with CA1 hippocampal death following KA (Sperk et al. 1983), rats were sacrificed, perfused with 4% PFA, and hippocampal slices were stained with Fluoro-Jade to assess neuronal death. Similar to previous studies, KA injection led to significant neuron loss in CA1 hippocampal neurons (**Fig 2-7 B)** As hypothesized, animals who had been injected with Tat-K13 pre- and post-KA demonstrated significant neuroprotection against KA-induced CA1 lesions (**Fig 2-7 C, D).** Saline injected animals showed no significant change in cell death following KA (**FIG 2-7 A)**, suggesting that Tat-K13 was able to effectively prevent NMDAR-mediated neuronal death in CA1 hippocampus. As seizure activity is common with KA treatment(Sperk et al. 1983; Brandt et al. 2003; Vincent & Mulle 2009), seizure activity was also monitored and recorded for 5 hours after KA, and for a half hour each subsequent day, according to the Racine seizure grading scale (Racine 1972). (data not shown) Seizure behavior was compared to biochemical results to control for changes in cell death resulting from seizures. Interestingly, all mice injected with KA demonstrated seizure activity, and Tat-K13 injection did not impact seizure activity, despite neuroprotection in the CA1 hippocampus. These exciting results demonstrate that Tat-K13 can effectively protect the CA1 hippocampus against NMDAR-mediated neuronal death

2.3.6 Tat-K13 blocks KA-induced PTEN nuclear translocation *in vivo*

My experiments using KA demonstrated that Tat-K13 could significantly reduce KA-induced neuronal death in the CA1 hippocampus. However, though this cell death protocol has shown to be NMDAR dependent, I wanted to identify the role of PTEN nuclear translocation in KA-induced CA1 neuronal death, to determine if Tat-K13 is working through PTEN-based mechanism. To do this, 3 month old rats were injected IP with 10mg/kg KA or saline vehicle. Following injection, hippocampal brain slices were used to detect PTEN nuclear translocation immunohistochemically using PTEN antibody and DAPI nuclear marker in hippocampal pyramidal neurons from CA1, to determine specific loci of PTEN nuclear translocation and effect of Tat-K13 (**Fig 2-8)**. As systemic KA has shown to cause NMDAR-dependent CA1 neuronal death (Brandt et al., 2003), and PTEN nuclear translocation is a common event after neurotoxicity(Zhang et al. 2013b; Trotman et al. 2007; Baker 2007) I hypothesized that CA1 neuron death following KA is accompanied with increases in nuclear PTEN. This was supported by immunohistochemical staining, demonstrating increased nuclear PTEN in CA1 9 hours after KA injection (**Fig 2-8).** Thus, it is likely that Tat-K13 protects CA1 hippocampus following KA by blocking PTEN nuclear translocation following an excitotoxic event.

These experiments demonstrate the potential of Tat-K13 to block PTEN nuclear translocation and protect CA1 hippocampus against NMDAR-mediated excitotoxicity. In line with my earlier experiments showing increases in PTEN nuclear translocation in HD tissue in the cortex and hippocampus, this data suggests that Tat-K13, and blockade of PTEN nuclear translocation, could potentially have therapeutic potential for excitotoxic neuronal loss in HD hippocampus or cortex.

2.3.7 Tat-K13 is not neuroprotective against NMDAR-mediated excitotoxicity in WT or YAC128 striatum *in vivo*

Given the important role of NMDAR-mediated excitotoxicity in neuronal dysfunction and cell loss in the striatum in HD pathogenesis, I next wanted to assess the ability of Tat-K13 to protect against NMDAR-mediated excitotoxicity in the HD striatum. My *in vitro* experiments demonstrated that Tat-K13 could not block NMDAR-mediated PTEN nuclear translocation and did not rescue cell death in striatal neurons, thus, I speculated that it Tat-K13 may similarly be ineffective in striatum *in vivo*. However, these experiments were important to explore, both to examine potential neuroprotective effects in live cells *vs* cultured striatal neurons, and also to explore differential effects of NMDAR-mediated PTEN nuclear translocation in cortical/hippocampal neurons vs striatal neurons. Acute intrastriatal quinolinic acid (QA) was used to examine NMDAR-excitotoxicity in the striatum. Intrastriatal QA has shown to cause neuronal death in MSNs, producing neuron damage and behavioral deficits similar to HD(Beal et al. 1991). YAC128 mice show enhanced sensitivity to QA in early, pre-symptomatic phases(Graham et al. 2009) thus it is a good model to use for our purpose. In my experiments, YAC128 HD or FVB WT mice (3months old) were treated via intravenous tail vein injection with Tat-K13 or Tat-K13R (a control peptide containing the PTEN sequence with a K13R mutation that does not block PTEN nuclear translocation(Zhang et al, 2013) one hour prior to unilateral intrastriatal injection of QA. Uninjected hemispheres were used as a control. In previous studies, IV Tat-K13 effectively penetrates the brain within 30min, and remains at functional levels for several hours(Zhang et al, 2013). QA injections (6nM) were done stereotaxically under 1.5% isofluorane. After peptide pretreatment and QA, mice were treated again with TAT-13/Tat-K13R 24 and 48 hours after QA. 7 days after QA, mice were sacrificed and neuronal death in the striatum was assessed by Fluro-

Jade. Initial dose response experiments using QA demonstrate dose-dependent lesion size increase, showing the method is reliable and replicable **(Fig 2-9 A)** However, in line with my experiments demonstrating only partial or even no neuroprotection or block of PTEN nuclear translocation in WT and YAC128 MSNs *in vitro*, neither Tat-K13 or Tat-K13R protected against QA-induced striatum lesions in any mice tested (**Fig 2-9 B)** In FVB WT mice, TAT peptides had no effect on lesion size following QA (**Fig 2-9 B)** However, in YAC128 HD mice, Tat-K13 actually led to increases in lesion size. These data further support my findings demonstrating differential roles of Tat-K13 in striatum and hippocampus/cortical neurons.

2.3.8 Other mechanisms may underlie PTEN nuclear translocation and retention in striatal vs cortical neurons

My last experiments in this study attempted to tease out potential underlying reasons why Tat-K13 is more effective in hippocampus and cortex compared to striatal neurons. Although I have demonstrated that PTEN nuclear translocation occurs 6-9 hours following GluN2B-containing, extrasynaptic NMDAR stimulation in all cells tested, and that this appears to be associated with excitotoxic neuron death in both WT and YAC128 mice, Tat-K13 was much more effective at reversing this translocation protecting against excitotoxicity in excitatory cortical or hippocampal neurons than in striatal neurons. As discussed previously in section 2.1.1.4 and 2.1.1.5, nuclear PTEN has an important role in regulating neuronal death not only in NMDAR-mediated excitotoxicity, but also in cancer, tumor suppression and following stimulation with other toxins(Gil et al. 2007; Gil et al. 2006). Alongside mono-ubiquitination of PTEN, other factors may regulate nuclear import and retention in various cell types, including Ran-GTP-ase dependent nuclear import (Gil et al. 2006; Gil et al. 2007), calcium dependent transport via MVP(Chung &

Eng 2005) or passive diffusion (Liu et al. 2005). Although our previous studies characterizing PTEN nuclear translocation in stroke demonstrate that mono-ubiquitination of K13, but not K289, regulate nuclear translocation following NMDAR-mediated excitotoxicity (Zhang et al, 2013), it is possible that PTEN nuclear translocation is regulated via different mechanisms in striatal neurons. To test this, I utilized GFP-fused plasmids for PTEN, used previously in our lab (Zhang et al., 2013). In these previous studies, transfection of cortical or hippocampal neurons with plasmids for normal PTEN (GFP-$PTEN_{WT}$) or PTEN with a mutation at K289 (GFP-$PTEN_{K289R}$) demonstrated GFP expression throughout the nucleus and cytoplasm, indicating that PTEN translocation had occurred. However, transfection with GFP-PTEN with a mutation at K13, preventing mono-ubiquitination (GFP-$PTEN_{K13R}$) led to cytoplasmic retention of the GFP signal, indicating that PTEN nuclear translocation had been blocked. This led to the knowledge that mono-ubiquitination of PTEN at K13, but not K289 regulated nuclear translocation in these cells. In my experiments, I repeated these studies using mono-cultured cortical or striatal neurons from FVB-N mice to compare nuclear translocation of PTEN with these mutations. Neurons were nucleofected with DNA plasmids at the time of plating using a nucleofector, then grown for 18 DIV. At 18 days, neurons were fixed and stained for GFP, DARPP-32 as a striatum marker, and DAPI nuclear stain. As seen in **Fig 2-10 A** I replicated the previous findings in our lab in cortical neurons, with both WT PTEN and PTEN-K289R showing expression throughout both the nuclear and cytoplasmic compartments, and PTEN-K13R being cytoplasmically retained. However, in the striatum (**Fig 2-10 B)**,all GFP-fused PTEN plasmids were expressed in both cytoplasm and nucleus, and neither mutation of mono-ubiquitination sites K13 or K289 had any impact. This suggests that, although PTEN can and does move into the nucleus following NMDAR-mediated stimulation in the striatum, other, non-ubiquitination based mechanisms may mediate this

molecular change aside from K13 mono-ubiqutination. These studies help elucidate the underlying reason into the difference in Tat-K13 neuroprotection in these different neurons types and may suggest a differential role of PTEN nuclear translocation in mediating neuron death in cortical and hippocampal neurons compared to striatal neurons.

2.4 Discussion

In my experiments in project 1, I aimed to examine PTEN nuclear translocation in NMDAR-mediated neuronal death in HD. Based on strong evidence that GluN2B, extrasynaptic NMDAR-mediated excitotoxicity plays a pivotal role in early-stage HD pathogenesis, and recent evidence suggesting that PTEN nuclear translocation is a key player in cell death following GluN2B-containing, extrasynaptic NMDAR excitotoxicity in stroke, I hypothesized that blocking PTEN nuclear translocation using our Tat-K13 peptide may be a potential early preventative target to protect against cell death in HD. My first experiments tested this theory using cultured neurons from WT or YAC128 HD mice. Using an *in vitro* model of NMDAR-mediated excitotoxicity, shown previously to stimulate GluN2B-containing, extrasynaptic NMDARs (Zhang et al. 2013b; Taghibiglou et al. 2009; Liu et al. 2007b) (Fan et al., 2007), I demonstrated, using nuclear fractionation and immunostaining, that NMDAR-mediated excitotoxicity and neuron death is accompanied by a clear increase in nuclear translocation of PTEN in cortical, hippocampal and striatal neurons from both WT and YAC128 mice, indicating that this appears to be a universal change associated with GluN2B, extrasynaptic NMDAR-mediated neuronal death following excitotoxicity. As predicted by previous studies in stroke, Tat-K13, preventing mono-ubiquitination of PTEN at K13, was able to effectively prevent PTEN nuclear translocation in cortical and hippocampal neurons from both WT and HD mice, accompanied by nearly complete

neuroprotection against NMDAR-mediated cell death. The control peptide, Tat-K289, blocking mono-ubiquitination of K289, had no effect. Conversely, and surprisingly, in striatal MSNs from both WT and YAC128 mice, the neurons primarily affected in HD, although clear PTEN nuclear translocation was associated with NMDAR stimulation, Tat-K13 did not effectively block nuclear translocation of PTEN, or provide neuroprotective benefit. Although Tat-K13 provided some neuroprotection against NMDAR-mediated excitotoxic neuronal death using LDH assay, it never reached complete neuroprotection, even over increasing doses of Tat-K13. Similarly, nuclear fractionation and immunostaining experiments in WT and YAC128 striatal neurons following NMDA stimulation demonstrate that Tat-K13-treated neurons retain PTEN in the nucleus, suggesting that the peptide does not effectively prevent or reverse nuclear translocation of PTEN. The Tat-K289 peptide also had no significant effect. These results were replicated *in vivo*, with TAT-13 showing significant block of PTEN nuclear translocation and neuroprotective effects against KA-induced CA1 hippocampal lesions, a mechanism that has shown previously to be NMDAR-mediated. However, Tat-K13, nor a control peptide had any significant neuroprotective effects against QA-induced striatum lesions in either WT or YAC128 mice, with Tat-K13 showing a trend towards increased lesion size in YAC128 HD mice. These data provide three important and novel findings; 1) that PTEN nuclear translocation appears to be associated with NMDAR-mediated excitotoxic neuron death in both WT and HD neurons, pointing to PTEN nuclear translocation as a potential candidate for early HD therapies for NMDA excitotoxicity 2) that the Tat-K13 peptide may be an effective way to significantly reduce nuclear levels of PTEN and potentially improve neuroprotection in both WT and HD cortical/hippocampal neurons and 3) that differential mechanisms underlie NMDAR-mediated PTEN nuclear translocation in striatum vs cortical and hippocampal neurons.

Given that these findings were assessed using acute NMDAR-mediated excitotoxicity, I also assessed baseline changes in nuclear PTEN in HD mice compared to WT animals. HD is a disease of chronic NMDAR-mediated excitotoxicity, which may be different than acute NMDAR-mediated excitotoxic events, thus I wanted to know if HD animals, undergoing early sensitivity and exposure to NMDA excitotoxicity, demonstrate any changes in nuclear levels of PTEN. This information could help reveal both potential time point for targeting PTEN nuclear translocation as a neuroprotective strategy against excitotoxicity in HD, as well as help elucidate the role of nuclear PTEN in cell death and dysfunction in various brain areas in HD. Using nuclear fractionation techniques I demonstrated that there are small but significant changes in nuclear levels of PTEN in YAC128 HD mice compared to WT mice. My western blot studies demonstrate that, at 2 months of age, YAC128 mice have increased nuclear PTEN expression compared to WT mice. This increase reached significance in hippocampus and cortex. In HD models, including YAC128, the most dramatic NMDAR-mediated excitotoxic changes occur at early time points, including increased striatal sensitivity to NMDA and expression of GluN2B-containing, extrasynaptic NMDARs(Zeron et al. 2002; Milnerwood et al. 2010; Graham et al. 2009; Dau et al. 2014). My studies suggest that at this same sensitive time point, YAC128 mice may also have increases in nuclear PTEN compared to control, though, surprisingly, this effect was not striatal, but rather cortical and hippocampal. Though cell loss in more widespread brain areas, including the cortex and striatum, do not occur until later in disease pathology(Nana et al. 2014; Spargo et al. 1993b; de la Monte et al. 1988) my studies show significant increases in nuclear PTEN in these areas in YAC128 mice as early as 2 months. Given a known role of PTEN nuclear translocation in cell death, this suggests that the presence of mHTT may lead to increased vulnerability or cell dysfunction in a non-cell autonomous manner even at early time points. At 6 months of age, a time

when HD neurons are still NMDA-sensitive but symptom onset has begun, significantly increased nuclear PTEN in YAC128 cortex persisted. Interestingly, though the striatum is the primary affected region in HD, the cortex showed most consistent baseline increases in nuclear PTEN in YAC128 at pre- and early symptomatic time points in western blot. This is in line with previous and emerging evidence suggesting that the cortex may actually play a larger role than previously thought in HD pathogenesis (Estrada-Sánchez & Rebec 2013) via cortico-striatal circuitry impairments including impaired glutamate release and subsequent striatal NMDA excitotoxicity, impaired plasticity and altered BDNF signaling (Cepeda et al. 2003; Cepeda et al. 2007; Parsons et al. 2014; Joshi et al. 2009; Zuccato & Cattaneo 2007). Increased early nuclear PTEN could be a sign of altered excitotoxicity in HD cortex and/or hippocampus, something that will need future study. Given the demonstrated ability of Tat-K13 to block PTEN nuclear translocation and rescue cell death, Tat-K13 could be a useful tool for this. By 12 months of age, all detectable changes in nuclear PTEN were gone in all tissues. This lines up nicely with previous studies demonstrating resistance to NMDA at these later time points (Zeron et al. 2002; Milnerwood et al. 2010; Graham et al. 2009; Dau et al. 2014) and may be associated with impairments in cell integrity or function at late, symptomatic HD stages, or may represent a neuroprotective attempt of the cell to protect against neuron death at these stages. No significant changes in YAC72 mouse tissue were detected at any of these time points, suggesting that these PTEN-mediated changes may be mHTT CAG-length dependent, with longer repeats, associated with worsened phenotype, showing more dramatic effects.

My studies investigating changes in PTEN in HD begin a novel discussion surrounding the role of nuclear PTEN in a chronic excitotoxic disease model. Based on our knowledge of the role of nuclear PTEN in cell death not only in excitotoxicity but also in cancer, tumor progression and

other cell stresses, in HD, PTEN nuclear translocation may be a chronic change associated with sensitivity to excitotoxicity, or other mHTT-dependent neuronal stressors. All baseline changes in nuclear PTEN were subtle, unlike dramatic changes following acute NMDA stimulation. This could indicate that small increases in nuclear PTEN, chronically, are associated with cellular dysfunction, rather than death in HD. Conversely, given the 6-9 hour window of PTEN nuclear translocation in acute excitotoxicity studies (Zhang et al., 2013) it is possible that cells where PTEN nuclear translocation occurs in HD are targeted for cell death, making them difficult to detect. Further studies looking at more detailed time points or more sensitive nuclear expression techniques, as well as studies testing long term Tat-K13 treatment in YAC128 *vs* WT mice may help elucidate the role of PTEN in the nucleus at a baseline in HD. In all, these studies suggest that nuclear PTEN may have some role in HD, primarily in the cortex and hippocampus, rather than striatum. It is also worth noting that as the knowledge of NMDAR-mediated synaptic changes in HD becomes more known, the semantic definition of these NMDAR-mediated changes is changing. Although the phrase "excitotoxicity" has been used to describe the enhanced susceptibility to neuronal death in HD, these changes are not the same as the acute excitotoxicity experienced in a large glutamatergic release, such as in a stroke. Excitotoxicity, by definition, generally refers to the large and sudden over-activation of NMDA receptors and the subsequent cell death that occurs, such as excitotoxicity in a stroke, or brain injury. However, in HD, though many similar signaling pathways are activated, the cell is succeptible to NMDAR-mediated cell death via many synaptic changes (outlined in **Fig 1-2)** and researchers are now reframing the way we discuss excitotoxicity in chronic disease vs acute excitotoxicity. These differences in acute excitotoxicity vs NMDAR-mediated changes at the synapse over time in HD may also outline some of the differences we see here.

Slight differences in PTEN nuclear translocation following acute NMDA stimulation in YAC128 vs WT cultured neurons may also suggest differential roles of nuclear PTEN in HD vs non HD neurons. It is worth noting that Tat-K13 had slightly different effects in WT vs YAC128 brains. In both cultured YAC128 neurons and in the striatum of YAC128 mice following treatment with Tat-K13 sometimes led to increases in cell death without NMDA stimulation (in culture), or exacerbated NMDAR-mediated lesions (in QA brains), an effect that I did not detect in WT brains. Though I did not examine this effect in detail, it is something that might be worth investigating in future studies and may point to differences in PTEN signaling following NMDAR-mediated excitotoxicity in HD *vs* WT neurons. Similarly, though I expected YAC128 striatum neurons to show enhanced PTEN nuclear translocation following NMDA treatment, given early sensitivity to NMDA in YAC128 striatum, NMDAR-mediated PTEN nuclear translocation was actually lower in YAC128 than in WT striatum in cultured cells. This demonstrates the need for better understanding of excitotoxic PTEN nuclear translocation in WT vs HD striatal neurons, and could also suggest that PTEN has differential roles in NMDA excitotoxicity in striatum compared to cortex and hippocampus.

An unexpected aspect of my studies was the finding that Tat-K13 was ineffective at blocking PTEN nuclear translocation and protecting against NMDAR-mediated excitotoxicity in the striatum. This was, initially, a disappointing finding, as the striatum is the primary affected area in HD, and is most vulnerable to NMDAR-mediated excitotoxicity. However, this points to a new and exciting finding that PTEN nuclear translocation may be mediated differentially in different neuron types. Using GFP-fused PTEN plasmids, I identified that, while mutation of the K13 mono-ubiquitination site on PTEN prevented nuclear translocation of PTEN in cortical neurons, the same mutation does not prevent PTEN nuclear translocation in striatum, similar to

my findings with the Tat-K13 peptide. This indicates that, though PTEN nuclear translocation appears to be a universal response of all neurons following NMDAR-mediated excitotoxicity, preventing mono-ubiquitination of K13 (or K289) is not sufficient to prevent this translocation. Based on this finding, several factors may be implicated. First, it appears that another mechanism may be driving PTEN into the nucleus in striatal neurons following excitotoxicity, independent of mono-ubiquitination at K13 or K289. PTEN has shown to translocate into the nucleus via several mechanisms, so it is possible that another of these mechanisms is activated by NMDAR-mediated excitotoxicity, such as activation of a nuclear localization signal, nuclear import, or passive diffusion, perhaps due to weakening of the nuclear envelope. Alternatively, PTEN may be retained in the nucleus following translocation. This has been shown in other cells, where PTEN in the nucleus is de-ubiquitinated and blocked from nuclear export, as well as from polyubiquitination and degradation (Trotman et al., 2007). Also, it is possible that PTEN in striatal neurons is not ubiquitinated at K13 or K289, and other post-translational modification of PTEN may be involved in cellular compartmentalization of PTEN in these neurons. Subsequent studies should investigate PTEN ubiquitination following NMDA in striatum vs cortex//hippocampus. Similarly, a combination of several of these factors may be at play. PTEN, both nuclear and cytoplasmic, may also play differential roles in striatum comparted to cortical and hippocampal neurons. Using immunostaining and western blot, I noted that PTEN expression in striatal neurons is much lower than cortical neurons. Also, at a baseline level, there is very little PTEN in the nucleus in all cortical or hippocampal neurons tested, with most of the PTEN expressed cytoplasmically, however, as seen in **Fig 2-5 D-E,** baseline PTEN in the nucleus of striatal neurons is always much higher (at levels of ~80-90% comparing nuclear PTEN to cytoplasmic PTEN) suggesting that nuclear PTEN may be more prevalent in striatal neurons normally than in cortex/hippocampus. Many cells

express high levels of nuclear PTEN(Trotman et al. 2007; Baker 2007) and nuclear PTEN has been linked to cell cycle regulation, nuclear P13K-Akt signaling, balance with p53 nuclear signaling, chromosomal integrity and tumor suppression (discussed in detail in 2.1.1.2.2) . It is possible that the nuclear role of PTEN varies in striatum vs cortex and hippocampus. Also, interestingly, I noticed that in striatal neurons, particularly in YAC128 neurons, Tat-K13 treatment actually appeared to exacerbate NMDA-mediated neuronal death. This can be seen in immunostained co-culture, as well as *in vivo* after QA treatment. This further supports my data suggesting that Tat-K13 is not effectively blocking PTEN nuclear translocation in the striatum, and also suggests that blocking PTEN ubiquitination at K13 may cause neuronal stress in other ways in HD striatum. Future studies will need to examine PTEN function and nuclear translocation both in striatum vs other neuron types, as well as in the context of HD. The translocation of PTEN following an excitotoxic event and differences in the ability to prevent this with Tat-K13 (or even worsen the neuronal damage) may also open up novel discussion surrounding potential reasons why the striatum is more vulnerable in HD compared to other cells, despite ubiquitous mHTT expression. Perhaps the nuclear expression and/or retention of PTEN following excitotoxicity is an underling mechanism of cell death in the striatum specifically in HD. Similarly, it would be worth investigating potential differential roles of PTEN in general in striatum vs cortex and in WT vs HD cells. siRNA experiments looking at PTEN knockdown in each cell type may help elucidate these findings.

Future studies using the Tat-K13 peptide should investigate long term treatment of YAC128 mice and WT controls with the Tat-K13 peptide to determine if Tat-K13 can reverse baseline changes in nuclear PTEN and protect against early NMDAR-mediated excitotoxicity and behavioral deficit in HD. Also, no one has investigated the long term effects of limiting nuclear

PTEN in the neuron. It would be imperative to also determine any potential negative effects, as nuclear PTEN has a key role in some neuron function.

2.5 Conclusion

In all, my studies using HD mice demonstrate an interesting finding, that PTEN nuclear translocation may be associated with early HD, not in the excitotoxic-sensitive striatum, but in more widespread affected brain areas, such as the cortex and hippocampus. Also, though PTEN nuclear translocation was associated with NMDAR-mediated neuronal death in all neurons, blocking PTEN mono-ubiquitination using Tat-K13 peptide was only effective at preventing NMDA-induced PTEN nuclear translocation and protecting HD cortex and in the hippocampus, and not striatum, both *in vitro* and *in vivo*. This suggests that the Tat-K13 peptide may be useful to target cortical/hippocampal death and dysfunction in HD. Also, I discovered differential roles of NMDAR-mediated PTEN nuclear translocation in striatum compared to cortex and hippocampus. PTEN nuclear translocation following an excitotoxic event appears to be mediated by different mechanisms other than K13 mono-ubiquitination, a novel finding.

Given the link between cortical and hippocampal impairments in HD and cognitive impairments in HD, as well as the importance of the cortico-striatal circuit impairments in HD, Tat-K13 may provide a novel therapeutic target where nuclear PTEN is a goal. Also, having shown that PTEN nuclear translocation is associated with NMDAR-mediated excitotoxicity in the striatum, these studies pave the way for future studies looking at ways to lower nuclear PTEN in the striatum in HD, as this may be a viable strategy for neuroprotection, as well as better understand the role of nuclear PTEN in the striatum in general.

Figure 2-1: Design and sequence of Tat-K13 and Tat-K289 peptides

Tat-K13 peptides were designed by Dr. Shu Zhang and colleagues in Dr. Yu Tian Wang's lab, UBC and synthesized by our technicians in the UBC Peptide synthesis facility. TAT-linked peptides contained a TAT cell penetrating sequence (YGRKKRRQRRR) to allow plasma membrane penetration, and an amino acid sequence borrowed from WT PTEN that shares homology across many species. The Tat-K13 peptide sequence flanks the K13 region of PTEN, and by competitive binding, prevents mono-ubiquitination of PTEN at K13. Similarly, the Tat-K289 peptide flanks the K289 mono-ubiquitination site of PTEN and prevents K289 mono-ubiquitination. In previous studies in rat cortex, Tat-K13 prevents nuclear translocation of PTEN while Tat-K289 does not, and is thus used as a control peptide.

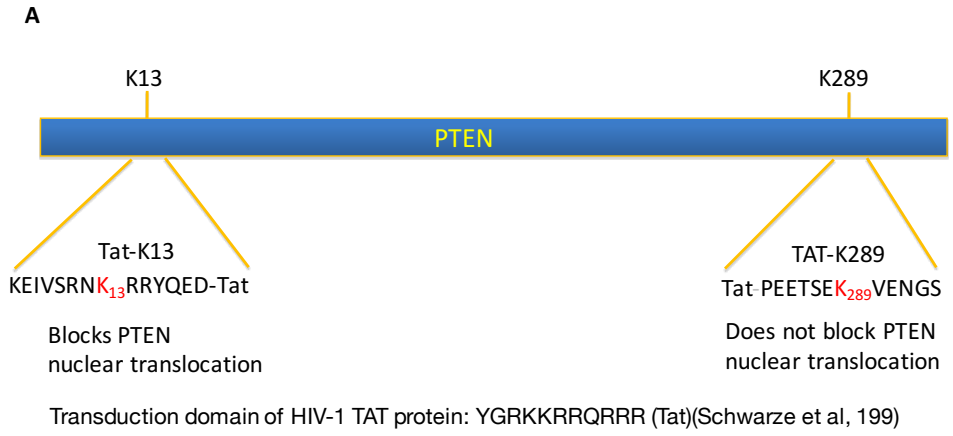
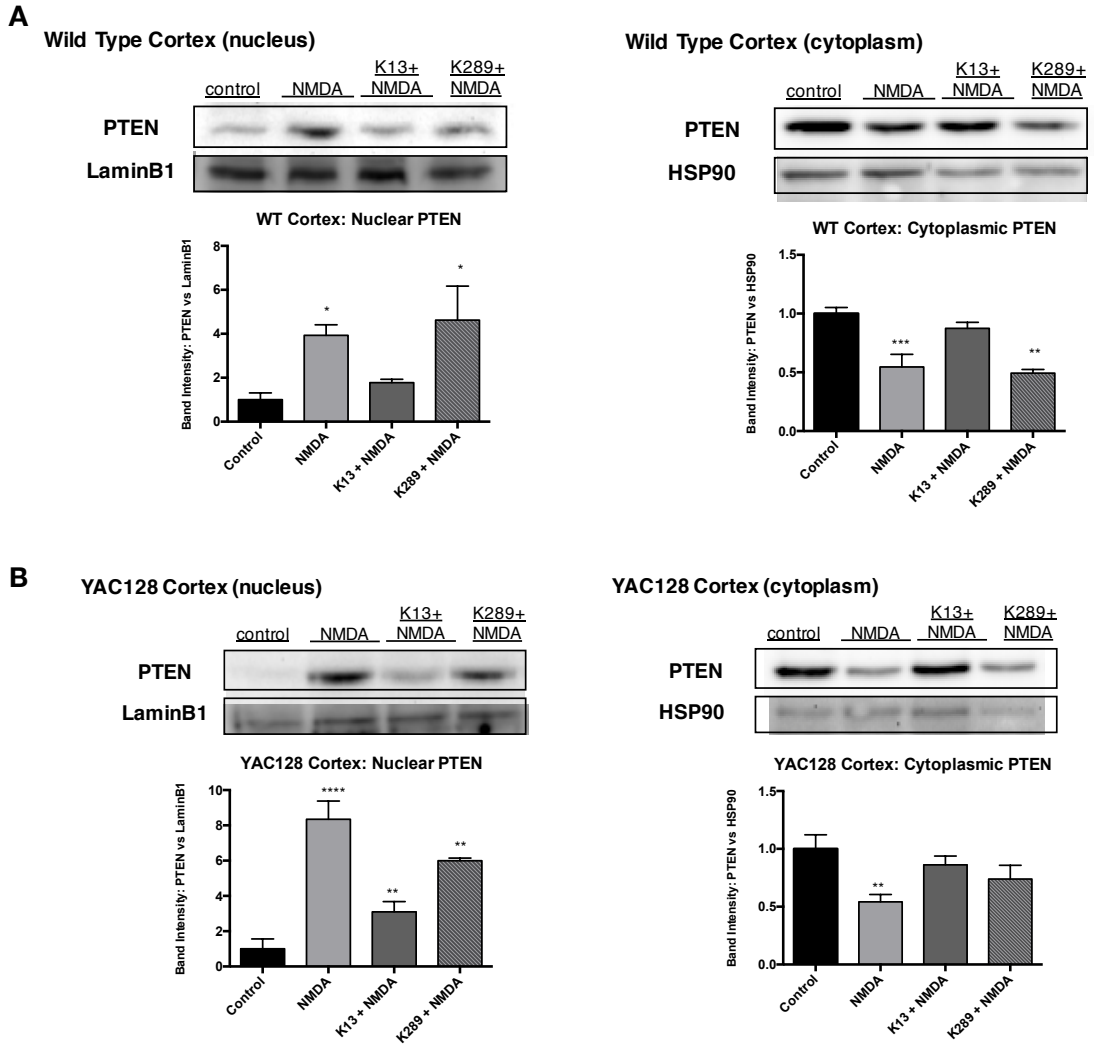


Figure 2-2 Tat-K13 blocks NMDAR-mediated nuclear translocation of PTEN in WT and HD cortex and hippocampus but not striatum: nuclear fractionation

Cultured FVB-N WT or YAC128 neurons were pretreated with Tat-K13, Tat-K289 or vehicle control then stimulated with excitotoxic NMDA. 6-9 hours following NMDA, nuclear fractionations were conducted and western blots investigated nuclear translocation of PTEN. There was clear increase in nuclear PTEN expression in all neurons tested, including WT cortex (**A**) (n=4 cultures, One Way ANOVA, $F(3,8)=8.104$ $p<0.001$), YAC128 cortex (**B)** (n=4 cultures, One-Way ANOVA $F(3,11)=22.6$ $p<0.0001$) WT hippocampus (**C)** (n=2-3, One-way-ANOVA $F(5,9)=1.574$, $p=.2609$ but clear trend) YAC128 Hippocampus (**D)**(n=2-4 Student's t-test control vs NMDA, $p<0.0001$) WT striatum MSNs (**E**) (n=2-6, One-Way ANOVA $F(4,61)=9.961$, $p<0.005$) and YAC128 striatum MSNs (**F**) (n=4, One-Way ANOVA $F(2,9)=2.372$, $p=.1488$ with a clear trend). As hypothesized, Tat-K13 effectively prevented NMDAR-mediated PTEN nuclear translocation in both WT and YAC128 neurons from cortex and hippocampus (**A-D)** while control peptide Tat-K289 peptide had no effect. However, in WT and YAC128 striatal MSNs, Tat-K13 did not significantly block PTEN nuclear translocation. Similar results were seen in cytoplasmic samples, with NMDA treatment leading to reductions in cytoplasmic PTEN expression in all neurons tested (WT cortex, One-Way ANOVA $F(3,8)=8.104$, $p=.1426$); YAC128 Cortex n=3-4, $F(3,11)=6.616$ $p<0.0081$; WT Hippocampus n=2-3 cultures, One-way ANOVA $F(5,9)=.5833$, $p=.4728$; YAC128 Hippocampus n=2-4, Student's t-test vs control p=0..963; WT striatum n=3-9, One-way ANOVA $F(5,27)=6.126$, $p<0.001$; YAC128 striatum n=4 cultures, One-way ANOVA(2,9)=2.372). Tat-K13 reversed these cytoplasmic changes to control levels in Cortex and Hippocampus (**A-D**) however Tat-K13

did not rescue this cytoplasmic reduction in PTEN in WT or YAC128 striatum (**E-F**). Tat-K289 had no effect. (Tukey's Post hoc vs control * = p<0.05, ** = p<0.01, *** = p<0.001, ****p<0.0001)



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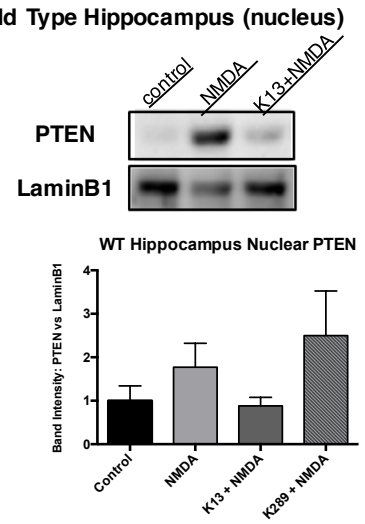
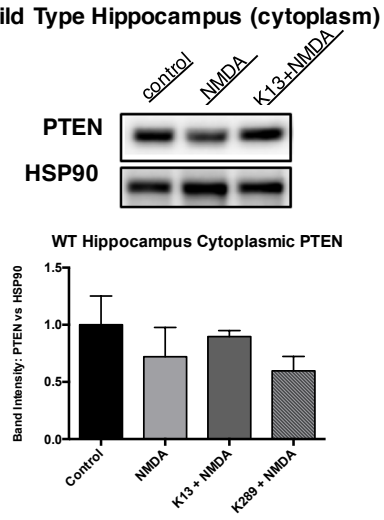

Wild Type Hippocampus (nucleus)
control
NMDA
K13+NMDA
PTEN
LaminB1
WT Hippocampus Nuclear PTEN
Band Intensity: PTEN vs LaminB1
0
1
2
3
4
Control
NMDA
K13 + NMDA
K289 + NMDA


Wild Type Hippocampus (cytoplasm)
control
NMDA
K13+NMDA
PTEN
HSP90
WT Hippocampus Cytoplasmic PTEN
Band Intensity: PTEN vs HSP90
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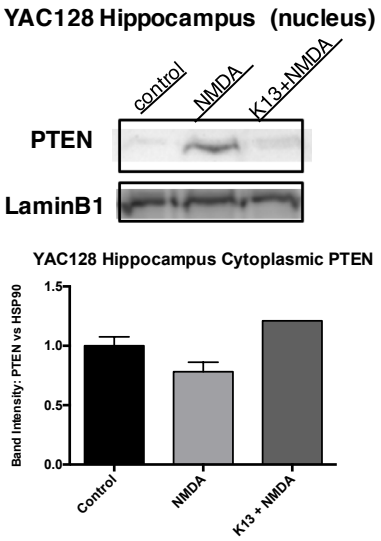
0.5
1.0
1.5
Control
NMDA
K13 + NMDA
K289 + NMDA

D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control
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K13+NMDA
PTEN
LaminB1
YAC128 Hippocampus Cytoplasmic PTEN
Band Intensity: PTEN vs HSP90
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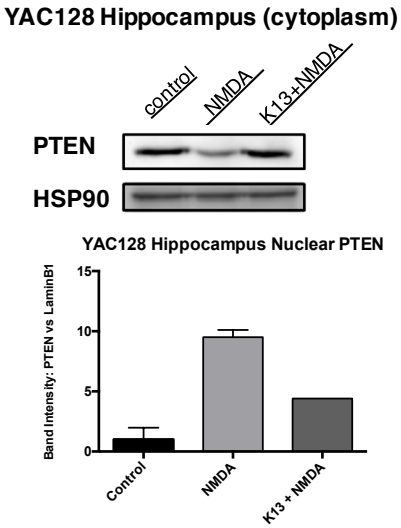
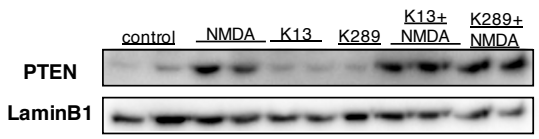
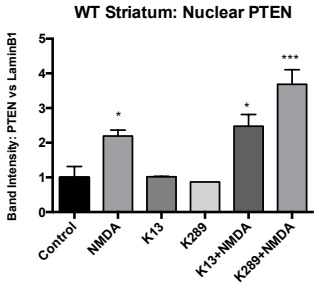
0.5
1.0
1.5
Control
NMDA
K13 + NMDA

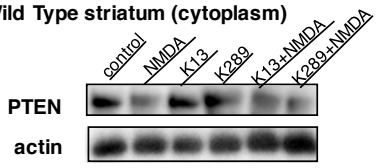

YAC128 Hippocampus (cytoplasm)
control
NMDA
K13+NMDA
PTEN
HSP90
YAC128 Hippocampus Nuclear PTEN
Band Intensity: PTEN vs LaminB1
0
5
10
15
Control
NMDA
K13 + NMDA

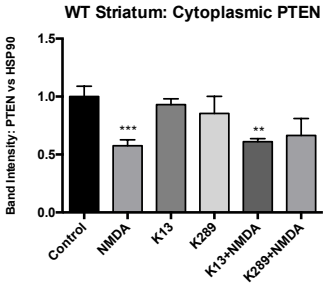
E Wild Type striatum (nucleus)



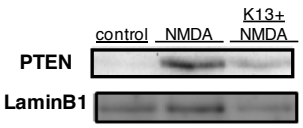


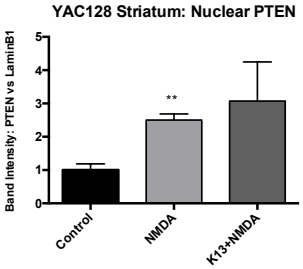
Wild Type striatum (cytoplasm)



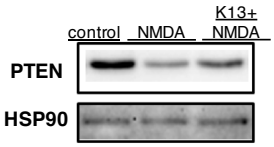


F YAC128 striatum (nucleus)





YAC128 striatum (cytoplasm)



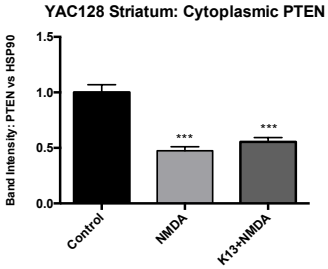
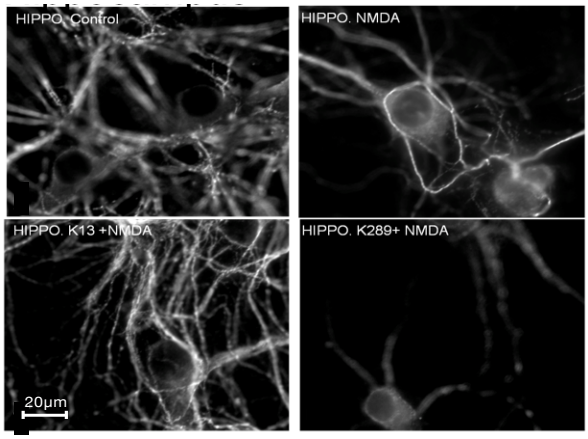


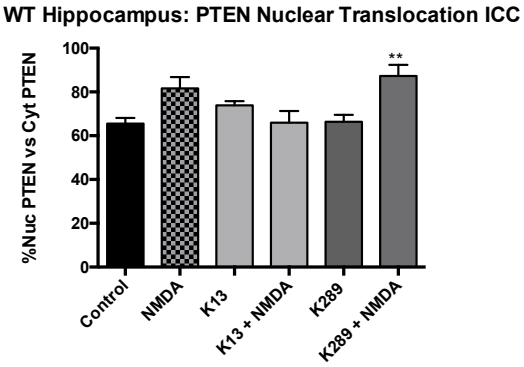
Figure 2-3 Tat-K13 blocks nuclear translocation of PTEN in WT and HD hippocampus but not striatum: Immunocytochemistry

Cultured hippocampal neurons or striatal MSNs were pre-treated with Tat-K13, Tat-K289, or vehicle, then stimulated with NMDA and recovered with peptide/vehicle for 6-9 hours. Immunocytochemistry was used to assess subsequent PTEN nuclear translocation and blockade by Tat-K13. Similar to nuclear fractionations, excitotoxic NMDA stimulation led to significant increases in PTEN nuclear translocation in all neurons tested (**A-C**) (WT hippocampus, n=1 culture, 4-10 replicates, one-way ANOVA $F(5,41)=4.876$, $p<0.01$; WT striatum, n=3 cultures, ~40 cells, one-way ANOVA $F(2,976)=10.30$, $p<0.0001$; YAC128 striatum, n=2 cultures, ~50 cells, one-way ANOVA $F(5,298)=11.63$, $p<0.0001$). Tat-K13, but not Tat-K289 significantly blocked PTEN nuclear translocation in hippocampal neurons (**A**). However, Tat-K13 only partially lowered nuclear PTEN translocation following NMDA in both WT striatum (**B)** and YAC128 striatum (**C**). Tat-K289 did not reverse PTEN nuclear translocation in any condition. To determine if increasing the dose of Tat-K13 could increase neuroprotection, dose response experiments were done in WT striatum (**D**). Even over increasing doses, Tat-K13 only provided moderate blockade of PTEN nuclear translocation in striatum (n=3 cultures, ~40 cells, one-way ANOVA $F(2,976)=10.30$, $p<0.0001$). (Tukey's Post hoc vs control * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$)

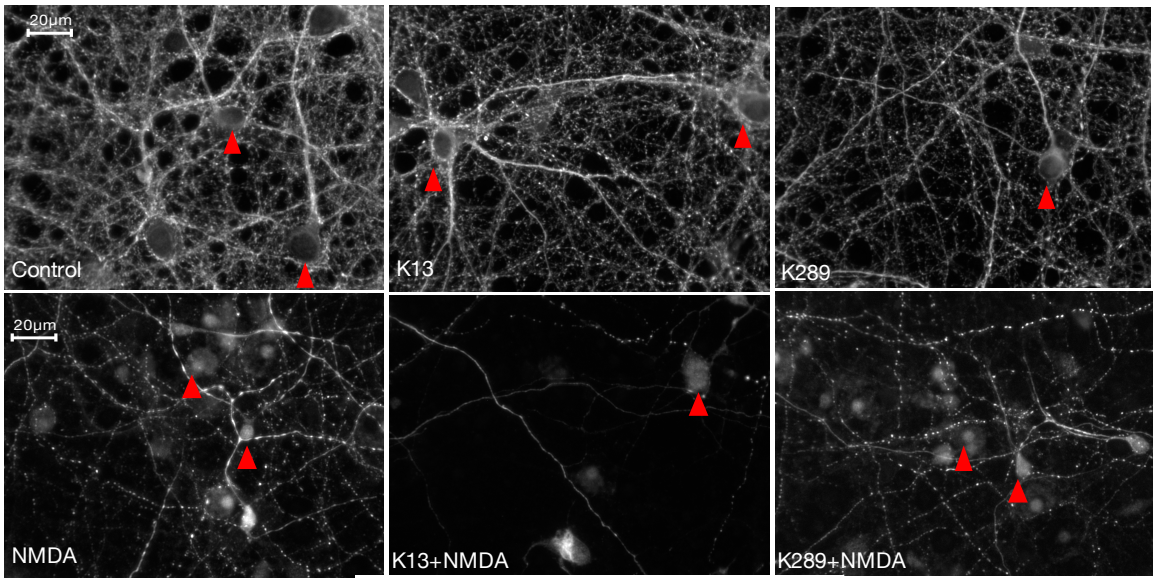
Wild Type hippocampus

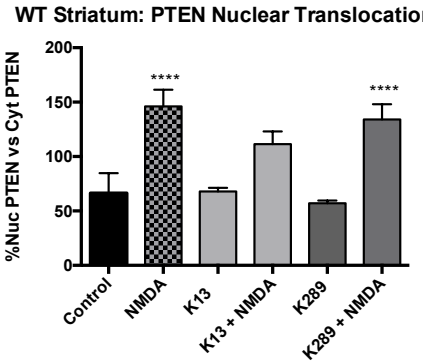
A



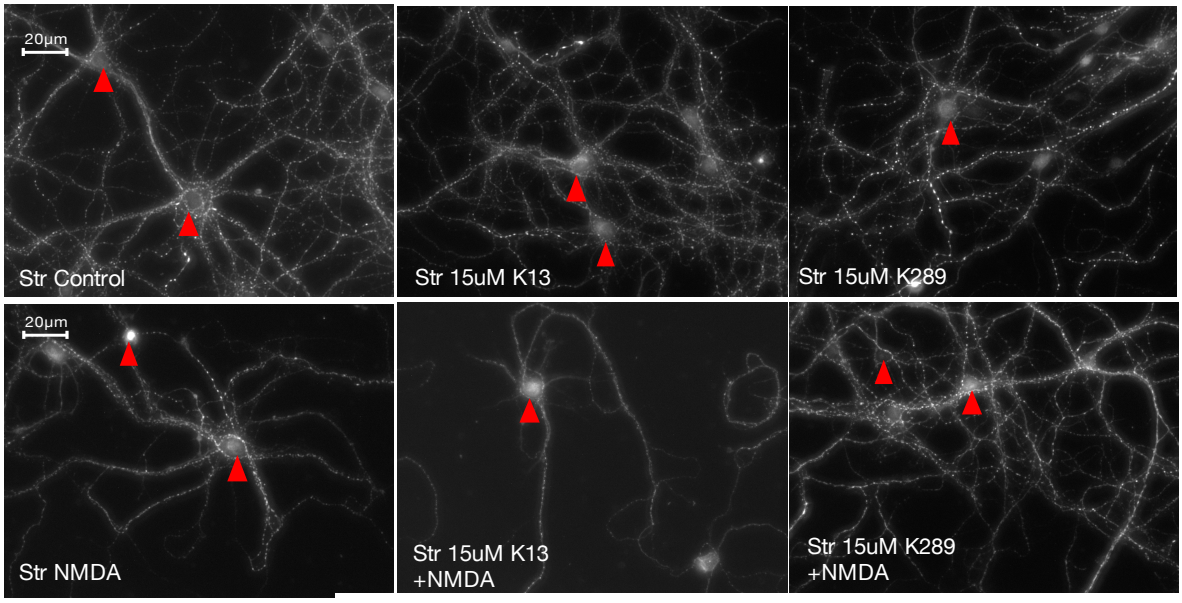


B **Wild Type striatum**

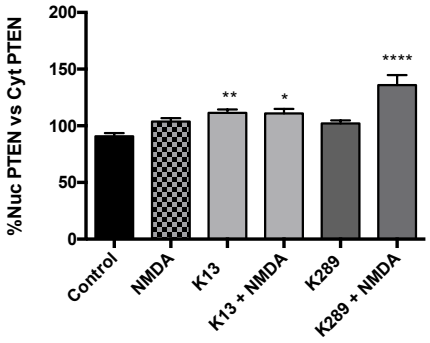




C **YAC128 striatum**



YAC128 Striatum: PTEN Nuclear Translocation ICC



D **WT Striatum: PTEN Nuclear Translocation ICC**
K13 Dose Response

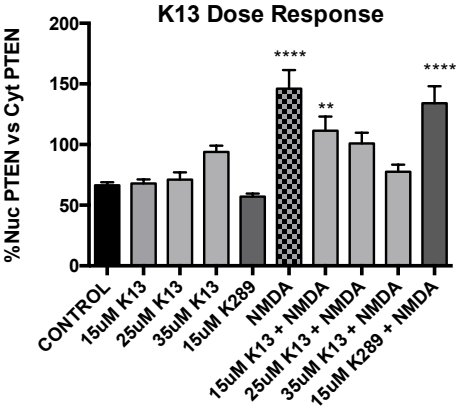
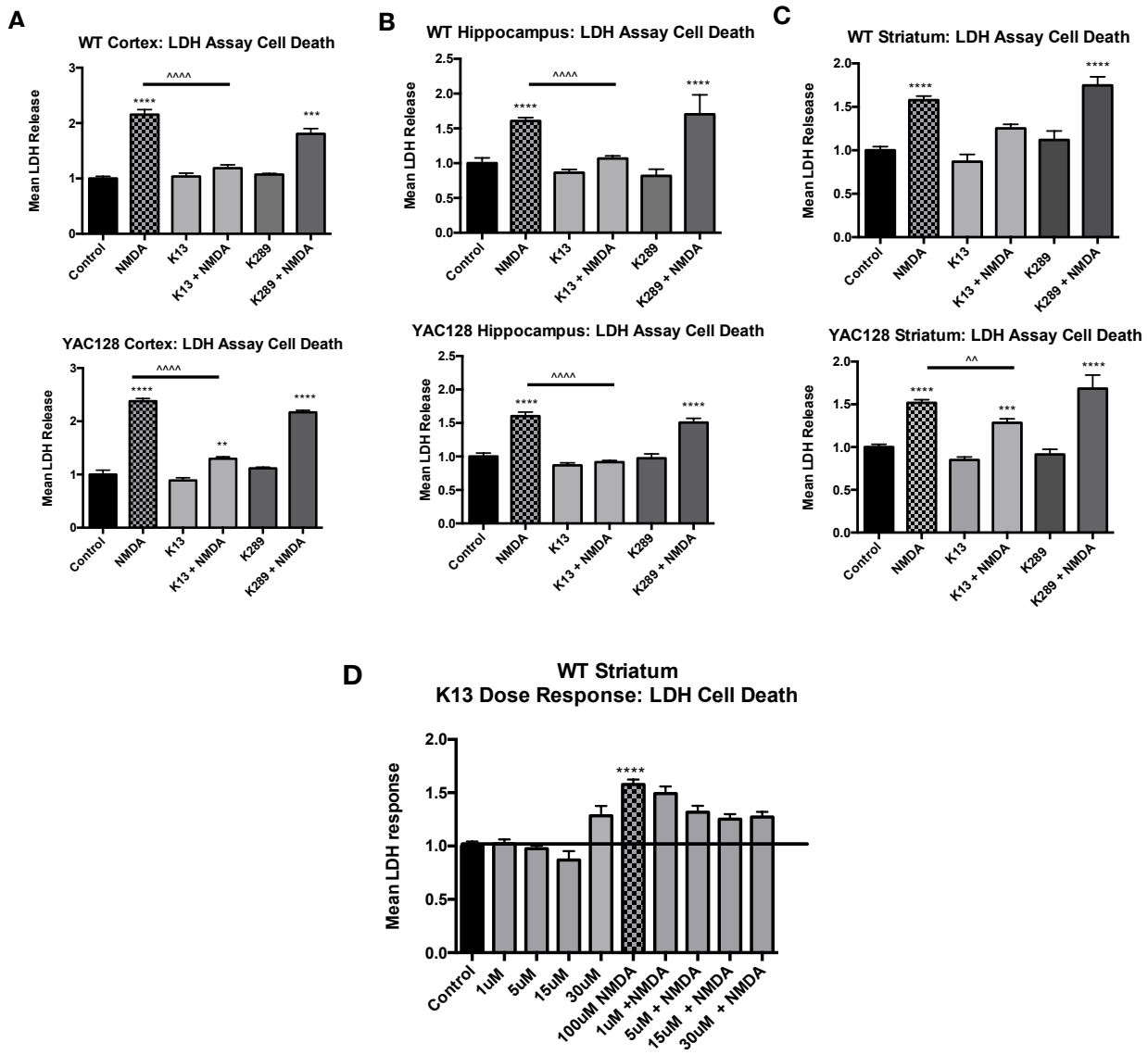
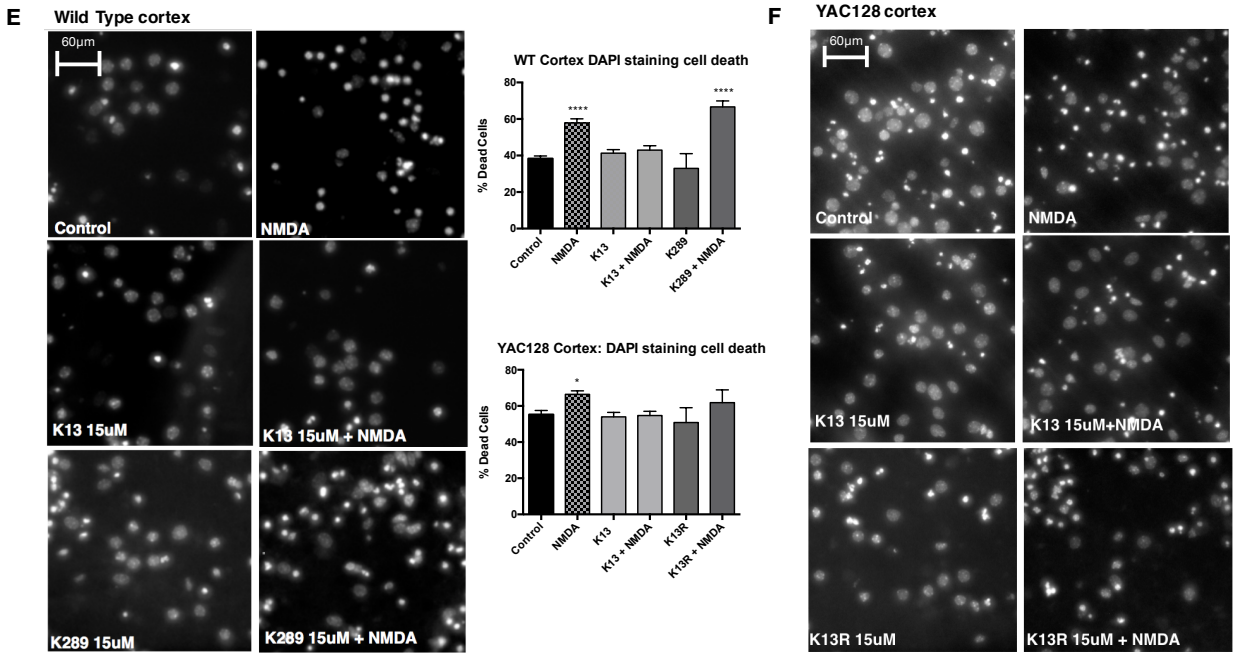


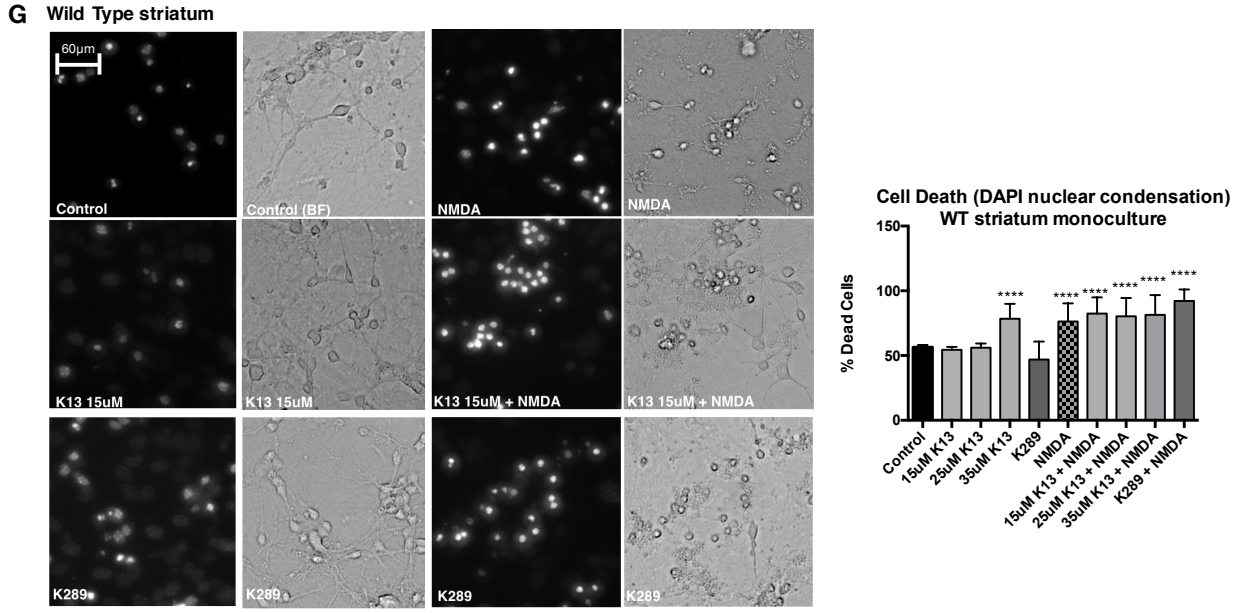
Figure 2-4 Tat-K13 is completely neuroprotective against NMDAR-mediated neuron death in HD cortex and hippocampus, but incompletely in striatum

Cultured neurons from FVB-N WT or YAC128 cortex, hippocampus or striatum were pre-treated with Tat-K13, Tat-K289 or vehicle, stimulated with excitotoxic NMDA, then recovered for 24 hours with peptide/vehicle. First using LDH assays to assess neuron death, it was shown that Tat-K13, but not Tat-K289 peptide could effectively lower cell death levels in both WT and HD cortex (**A**) (WT cortex n=6 cultures, one-way ANOVA $F(5,76)=46.67$, $p<0.0001$; YAC128 cortex n=6 cultures, one-way ANOVA $F(5,79)=96.55$, $p<0.0001$) and in both WT and HD hippocampus (**B**) (WT hippocampus n=7 cultures, one way ANOVA $F(5,105)=25.25$, $p<0.0001$; YAC128 hippocampus, n=6 cultures, one-way ANOVA $F(5,86)=46.02$, $p<0.0001$). However, Tat-K13 only partially lowers NMDAR-mediated neuron death in both WT and HD striatum (**C**) (WT striatum, n=14 cultures, one-way ANOVA $F(14,253)=6.199$, $p<0.0001$; YAC128 striatum n=8 cultures, one-way ANOVA $F(9,137)=35.98$) whereas Tat-K289 did not protect striatal neurons at all. Dose dependency of Tat-K13 was tested using WT striatal neurons. Even with increased doses of Tat-K13, neuroprotection in striatal neurons was only partial (**D**). Similar results were seen using DAPI staining to assess cell death, at the same time point following NMDA. In WT (**E**) and YAC128 (**F**) cortical neurons, Tat-K13, but not Tat-K289 significantly reduced nuclear condensation/fragmentation to control levels (WT cortex n=2 cultures, ~25 replicates, one-way ANOVA $F(7,185) = 17.52$, $p<0.0001$; YAC128 cortex n=1 culture, ~25 replicates, one-way ANOVA $F(5,91)=2.929$, $p<0.01$). However, in both WT (**G**) and YAC128 (**H**) striatum Tat-K13 does not protect against cell death, measured by nuclear condensation, even with increasing dose. Tat-K289 did not have any protective effects in any cells tested.

(Tukey's Post hoc vs control * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, and vs NMDA ^ = p<0.05, ^^ = p<0.01, ^^^ = p<0.001, ^^^^ = p<0.0001).




E
Wild Type cortex
60µm
Control
NMDA
K13 15uM
K13 15uM + NMDA
K289 15uM
K289 15uM + NMDA
WT Cortex DAPI staining cell death
% Dead Cells
Control
NMDA
K13
K13 + NMDA
K289
K289 + NMDA
YAC128 Cortex: DAPI staining cell death
K13R
K13R + NMDA
F
YAC128 cortex
K13 15uM+NMDA
K13R 15uM
K13R 15uM + NMDA


G
Wild Type striatum
60µm
Control
Control (BF)
NMDA
K13 15uM
K13 15uM + NMDA
K289
Cell Death (DAPI nuclear condensation)
WT striatum monoculture
% Dead Cells
Control
15uM K13
25uM K13
35uM K13
K289
NMDA
15uM K13 + NMDA
25uM K13 + NMDA
35uM K13 + NMDA
K289 + NMDA

H

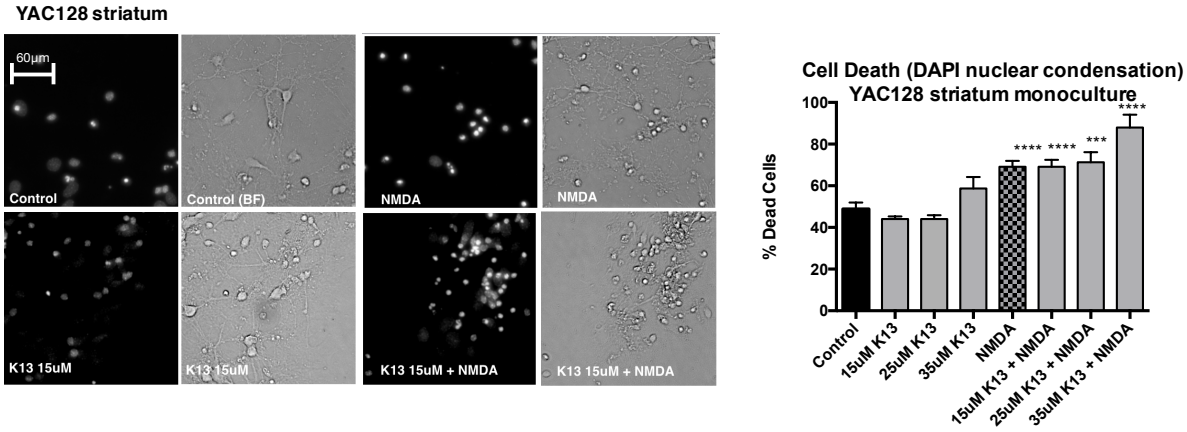
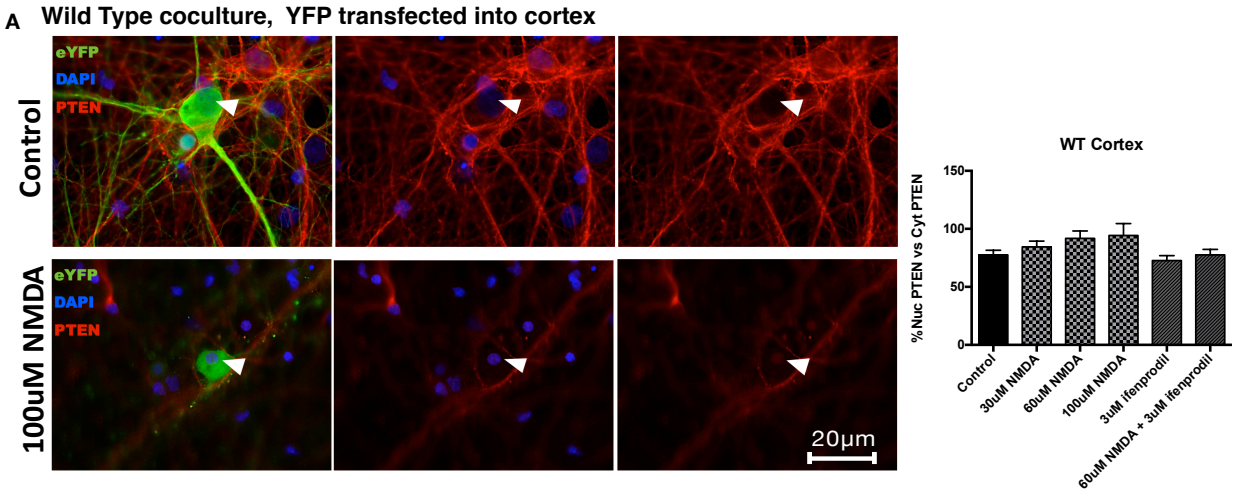
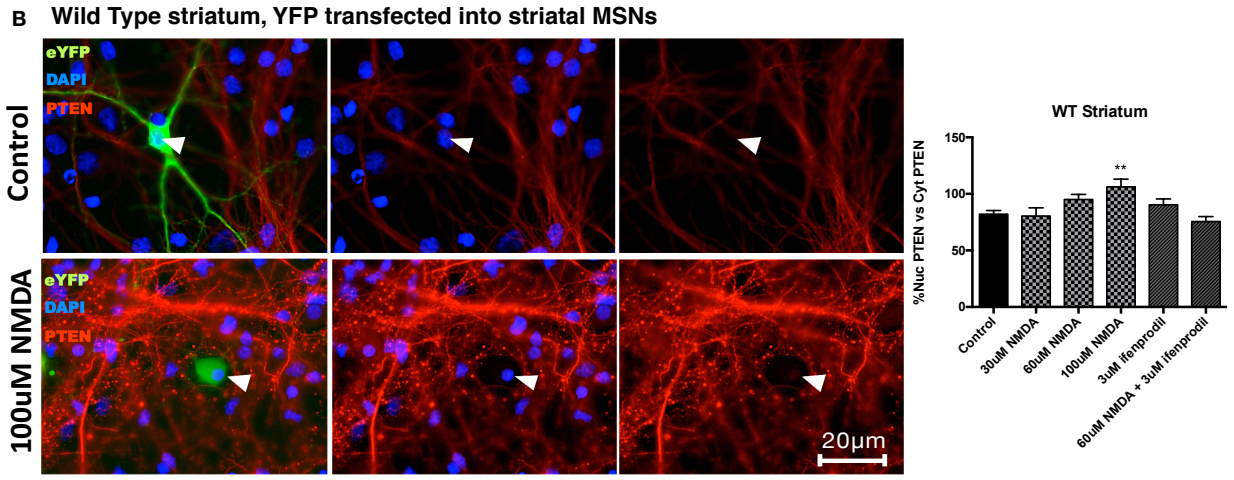

YAC128 striatum
60µm
Control
Control (BF)
NMDA
NMDA
K13 15uM
K13 15uM
K13 15uM + NMDA
K13 15uM + NMDA
Cell Death (DAPI nuclear condensation)
YAC128 striatum monoculture
% Dead Cells
100
80
60
40
20
0
****
****
****
***
Control
15uM K13
25uM K13
35uM K13
NMDA
15uM K13 + NMDA
25uM K13 + NMDA
35uM K13 + NMDA

Figure 2-5 Tat-K13 blockade of PTEN nuclear translocation in cortico-striatal co-culture

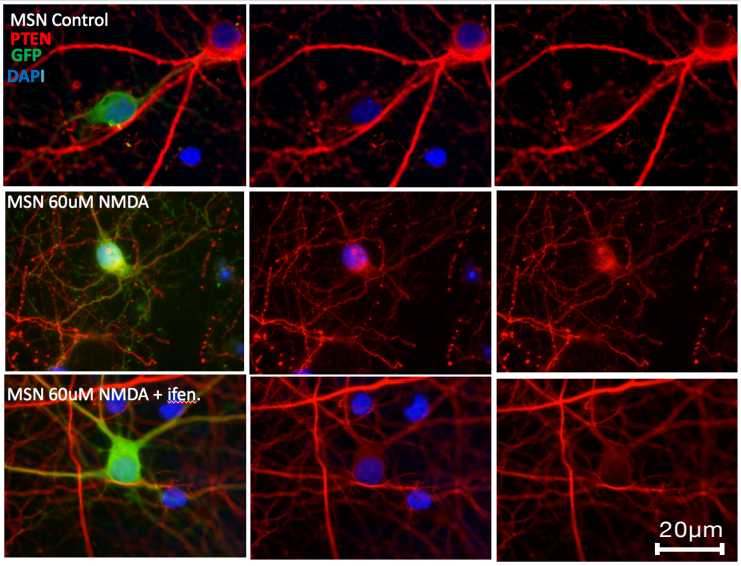
Striatal MSNs and cortical neuron co-cultures were generated from FVB-N or YAC128 neurons and cultured in a 1:1 ratio. Cell types were identified either by nucleofection with YFP DNA plasmids into one cell type at time of plating, or, when working with MSNs, using DARPP-32 staining as a marker of striatal neurons. Initial experiments characterized PTEN nuclear translocation following increasing doses of NMDA at 18-21DIV using immunostaining for PTEN and a DAPI nuclear marker and showed that in both WT cortex (**A**) and striatum (**B**) PTEN nuclear translocation increased in an NMDA dose-dependent way (Cortex n=2 cultures, ~25 cells, one-way ANOVA $F(5,103) = 1.80$, not significant but trending, $p=.1193$; Striatum n=3 cultures, one-way ANOVA $F(5,138) = 4.725$, $p<0.005$). This was reversible in both cell types using GluN2B NMDAR antagonist ifenprodil (**A, B**). Based on these experiments, a 60uM dose of NMDA was selected, as there was clear nuclear translocation, without loss of cell integrity in MSNs (**C**). Next, I tested the ability of Tat-K13 to block PTEN nuclear translocation in cortex and striatal neurons in co-culture in both WT and YAC128 cells (18-21DIV). Cultures were pre-treated with peptide/vehicle, stimulated for 15min with NMDA, then recovered with peptide/vehicle for 7 hours. Using DARPP-32 staining to identify MSNs, alongside PTEN and DAPI staining, I demonstrated that, even in more physiologically relevant co-cultures, NMDAR-mediated PTEN nuclear translocation in PTEN could not be rescued in either WT MSNs (**D**) or YAC128 MSNs (**E**) even over increasing doses of Tat-K13 (**D, E).** (WT striatum n=3 cultures, ~40 cells, one-way ANOVA $F(7,141) =4.806$, $p<0.0001$; YAC128 striatum n=4 cultures, ~30 cells, one-way ANOVA $F(9,423)= 4.407$, $p<0.0001$). On the contrary, in cortical neurons in co-culture (WT, 18-21 DIV, nucleofected with GFP), Tat-K13 completely blocked PTEN nuclear translocation, whereas Tat-K289 had no effect **(F)** (WT cortex, n=3 cultures, ~25cells, one-way

ANOVA F(7,279) = 6.954, p<0.0001). (Tukey's Post hoc vs control * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001)

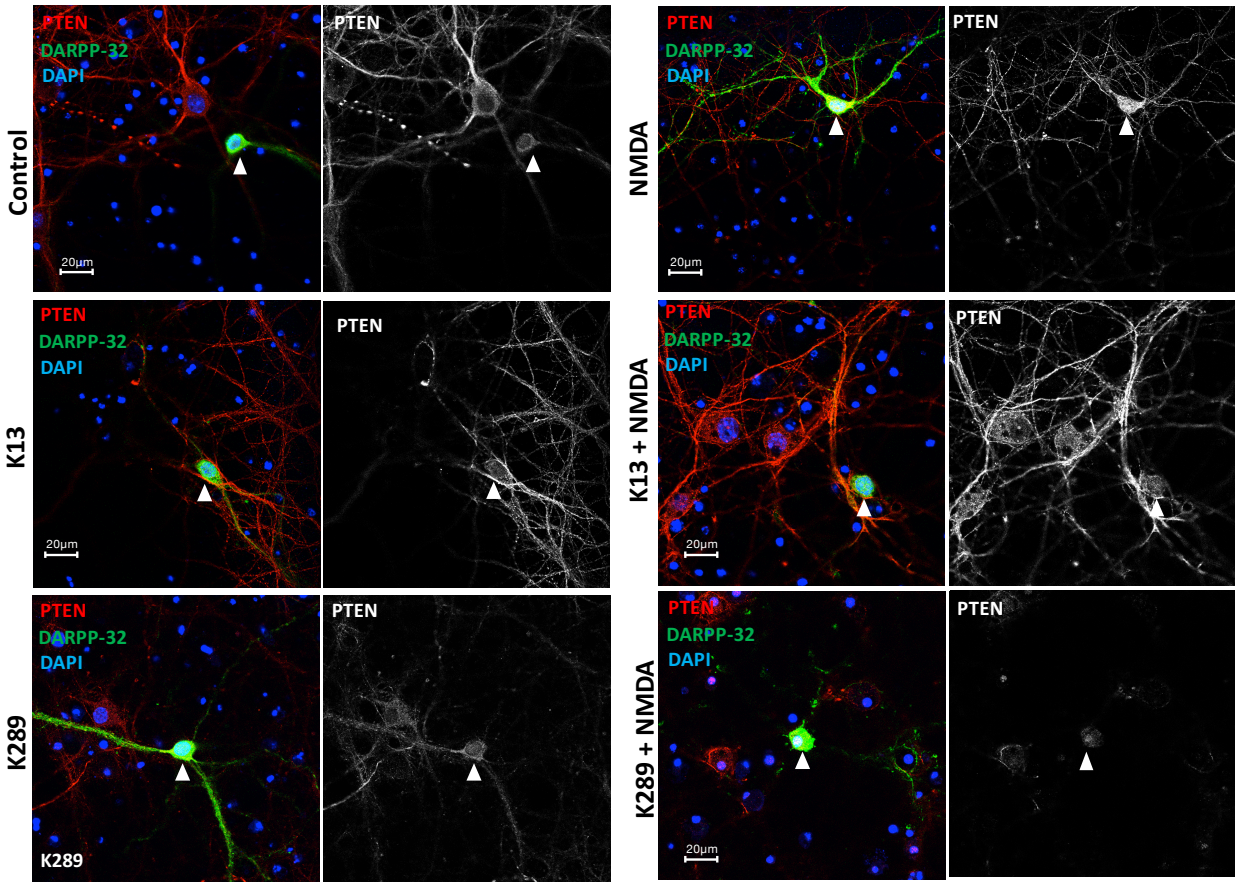


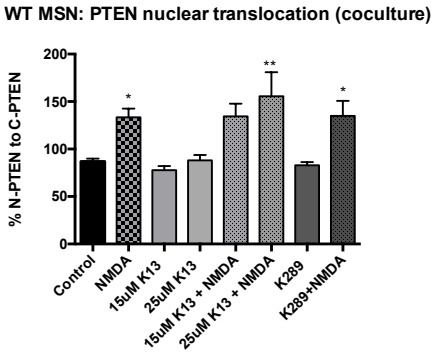


C **Wild Type coculture, YFP transfected into striatal MSNs**

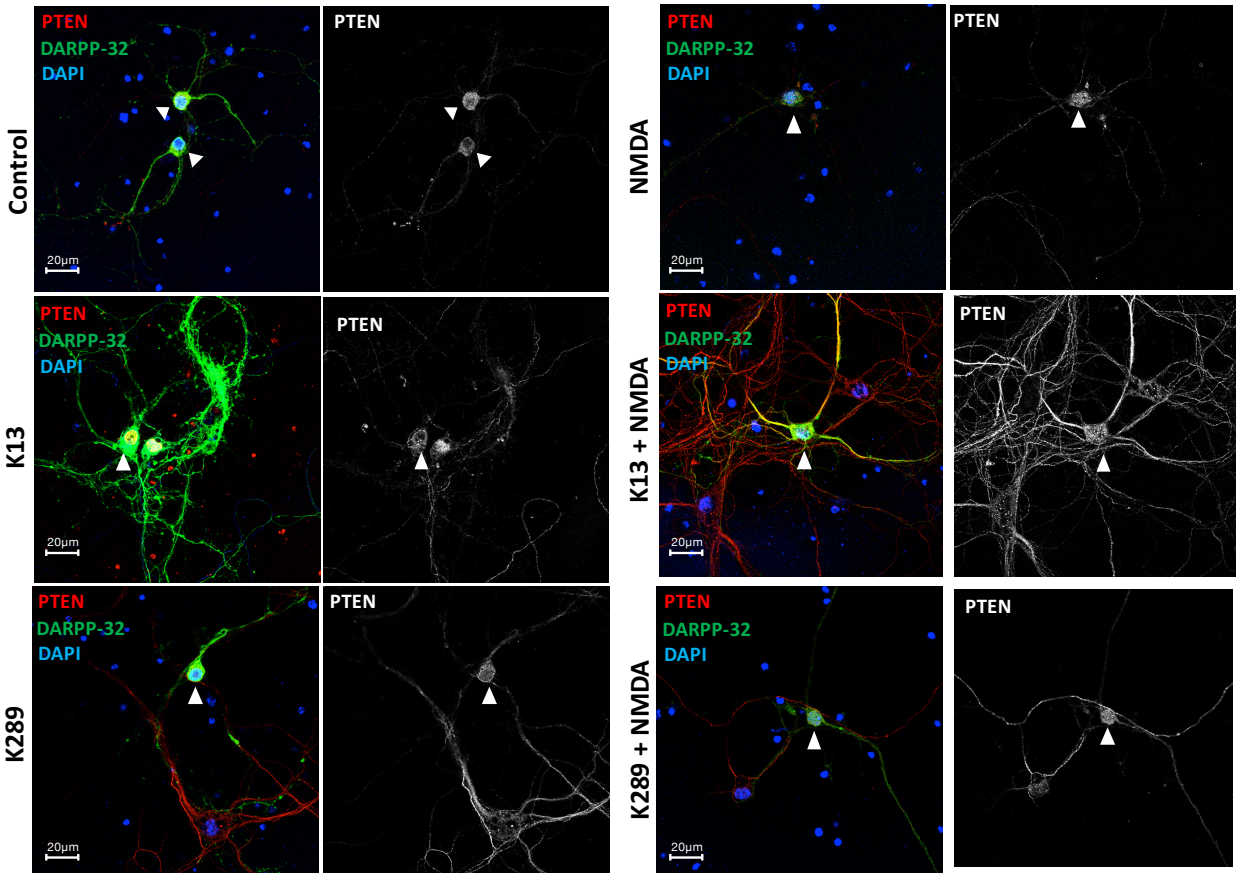


D Wild Type striatum

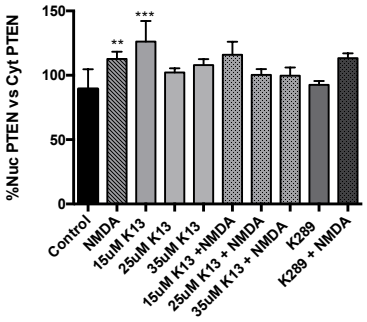




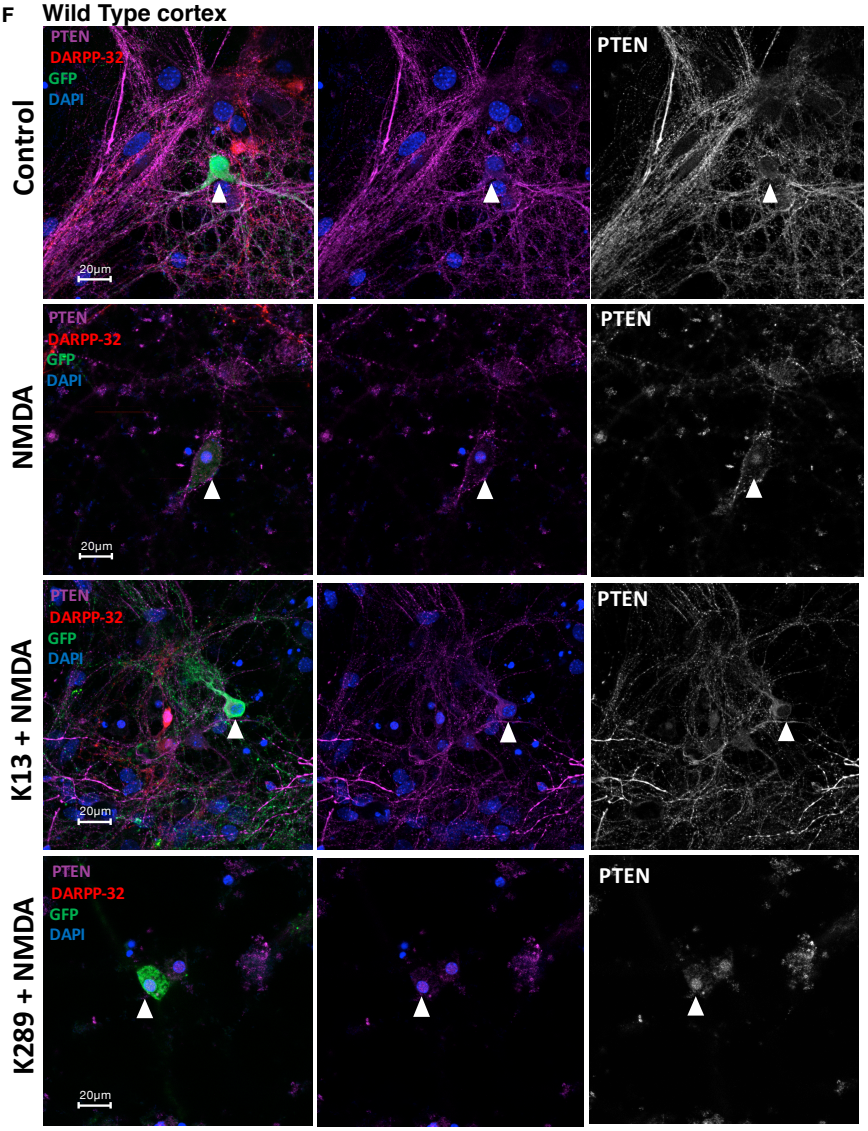
E YAC128 striatum



YAC128 MSN: PTEN nuclear translocation (coculture)



F Wild Type cortex



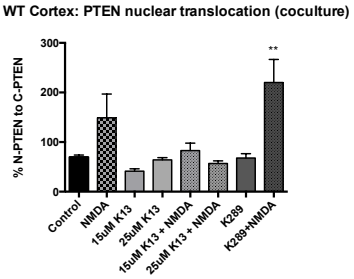
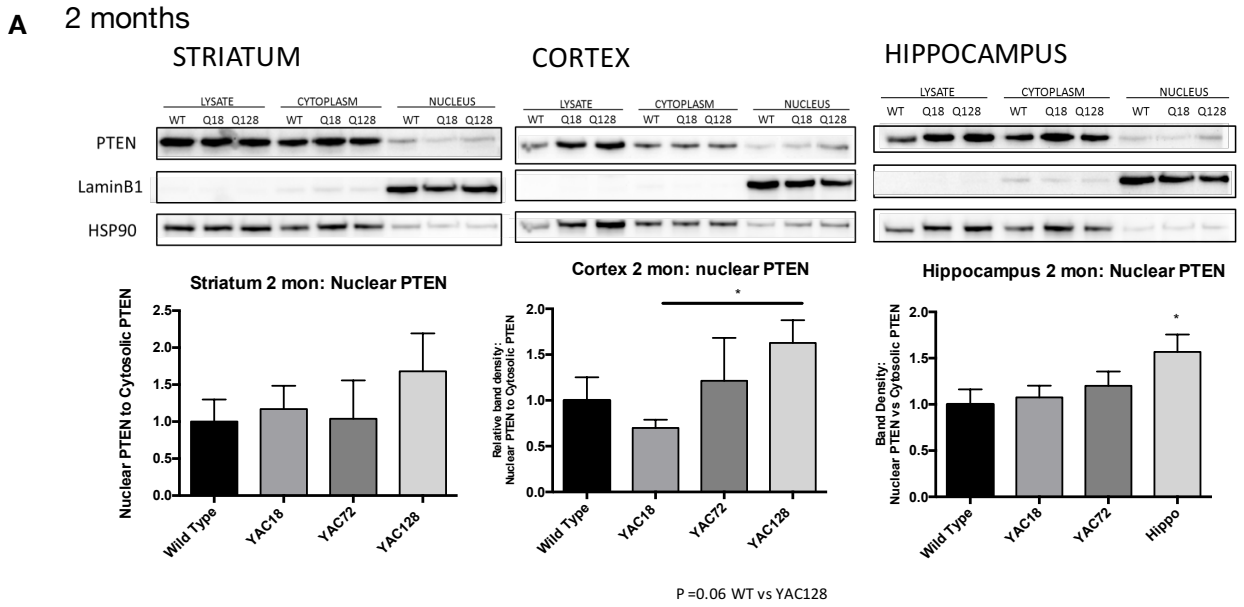
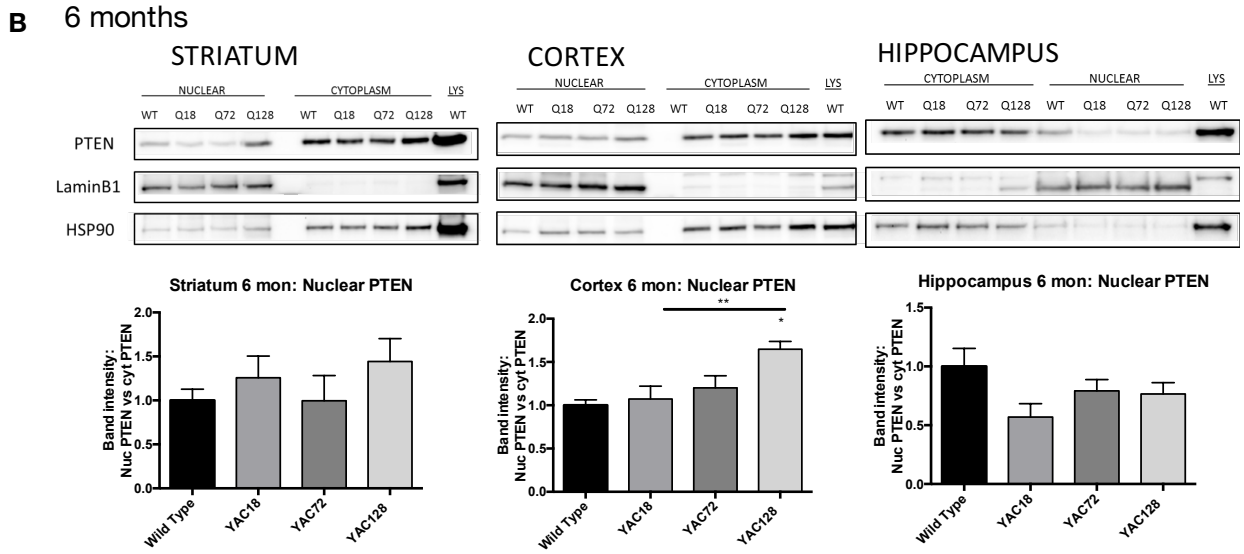


Figure 2-6 Baseline increases in nuclear PTEN in HD brain tissue compared to WT animals FVB-N or YAC18 WT mice and YAC72 and YAC128 HD mice were raised to 3 age time points; 2 months (pre-symptomatic, NMDA sensitive), 6 months (early symptomiatic, NMDA sensitive) or 12 months (late symptomatic, NMDA resistant). At these time points, animals were sacrificed to assess baseline levels of nuclear and cytoplasmic PTEN in cortex, striatum and hippocampus to determine if nuclear translocation of PTEN is associated with HD at various time points. Initial experiments used nuclear fractionation and western blot to look at protein expression. Nuclear PTEN was assessed by comparing nuclear to cytoplasmic expression of PTEN then normalizing control to 1. At 2 months (**A**) there was a trend towards increased nuclear PTEN in all YAC128 tissues, reaching significance in YAC128 hippocampus, and in YAC128 cortex vs YAC18 control (Striatum n=4-7, one-way ANOVA $F(3,20) = .92$, $p=.4491$; cortex n=4-7, one way ANOVA $F(3,20) = 3.8$, $p<0.05$; hippocampus n=4-7, one-way ANOVA (3,2), 2.598, $p<0.05$). At 6 months (**B**), similar effects were seen in YAC128 cortex, with significant increases in nuclear PTEN vs control, and a trend towards increased nuclear PTEN in YAC128 striatum. No effects at 6 months were seen in hippocampus (striatum n=5-9, one-way ANOVA $F(3,25) = .9766$, $p=.4915$; cortex n=5-9, one-way ANOVA $F(3,22)= 11.21$, $p<0.0001$; hippocampus n=5-9, one-way ANOVA $F(3,2) = 1.907$, $p=.1579$) at 12 months (**C**) there were no significant changes in nuclear PTEN in HD compared to WT tissues (striatum n=3-6, one-way ANOVA $F(3,15) = .7687$, $p=.5292$; cortex n=2-5, one-way ANOVA $F(3,11)= 10.92$; hippocampus n=3-5, one-way ANOVA $F(3,12) = 2.167$, $p=.1450$). (Tukey's Post hoc * =

p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001)





C 12 months

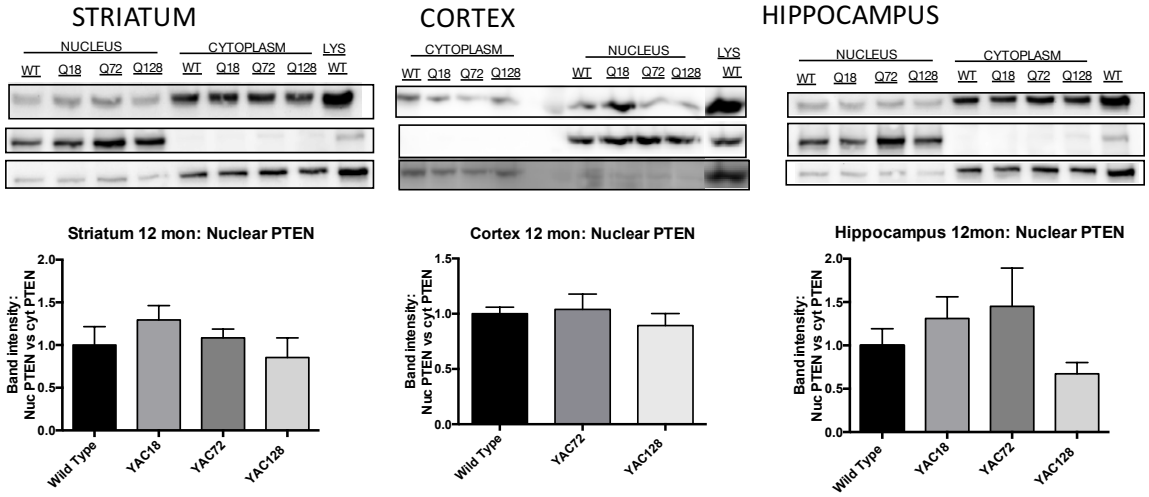

STRIATUM
CORTEX
HIPPOCAMPUS
NUCLEUS
CYTOPLASM
LYS
WT Q18 Q72 Q128 WT Q18 Q72 Q128 WT
Striatum 12 mon: Nuclear PTEN
Cortex 12 mon: Nuclear PTEN
Hippocampus 12mon: Nuclear PTEN
Band intensity: Nuc PTEN vs cyt PTEN
Wild Type
YAC18
YAC72
YAC128

Figure 2-7 Tat-K13 effectively protects CA1 hippocampal neurons against kainic acid-induced lesions

Effects of K13 peptide on kainic acid (KA)-induced CA1 hippocampal lesions in rat. IP kainic acid (10mg/kg) produces CA1 specific lesion in rat brain (**B**) compared to saline injections IP (**A**). IV injection of K13 1 hour prior, as well as 24 and 48 hours post IP injection of KA significantly reduces CA1 lesion (**C, D)**. The majority of animals tested showed complete lesion protection (**C**) Quantification (**D**). Animals were treated with IV Tat-K13 or IV saline 1 hour prior to 10mg/kg IP KA, then subsequently injected with IV Tat-K13 or IV saline at 24hours and 48hours after KA. 7 days after KA, animals were sacrificed, brains were fixed and sliced and stained with Fluoro-Jade to assess cell death. Analysis was done using ImageJ, by creating a threshold, and analyzing relative intensity of fluorescent stain. * = comparison to control group (Students T-Test, $p<0.05$) # = comparison to KA treatment group (Students T-Test, $p<0.05$).

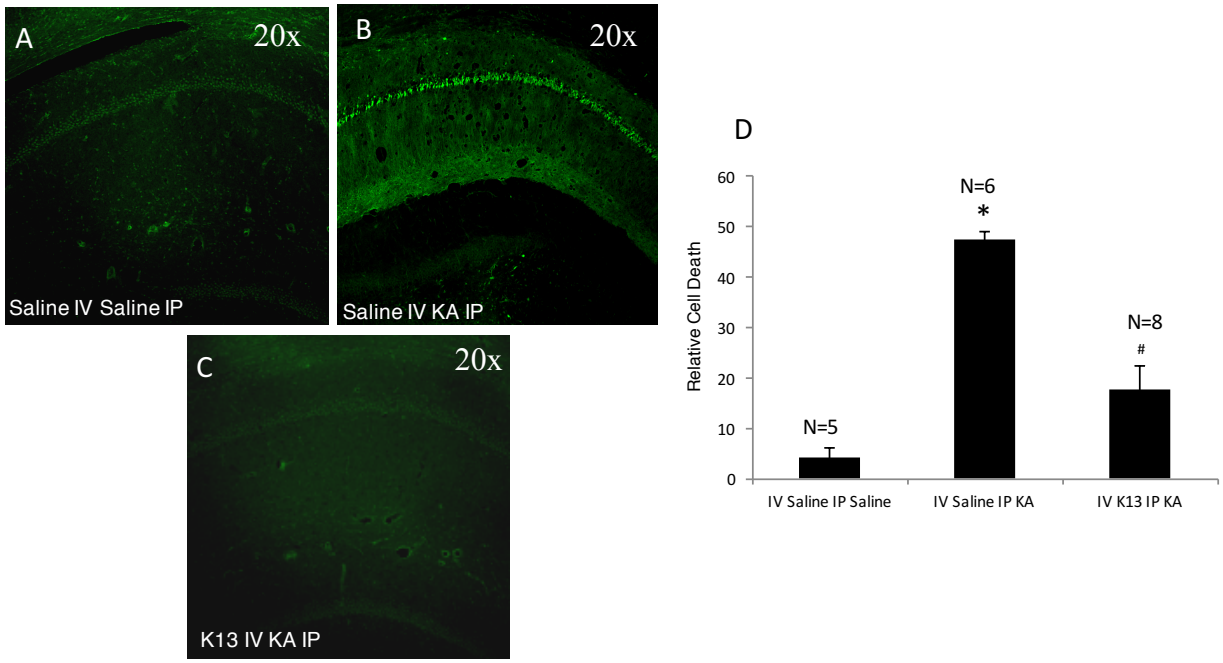


Figure 2-8 IP Kainic Acid causes PTEN nuclear translocation in CA1 hippocampus 9 hours after injection

Immunohistochemistry on fixed brain slices from rats injected with saline vehicle or 10mg/kg KA was used to investigate PTEN nuclear translocation following KA-induced excitotoxicity. Following KA injection, animals were sacrificed at 6-9 hours post KA. Significant increase in PTEN nuclear translocation was detected at 9 hours following KA (Preliminary data, n=1-2, 60 cells, Student's T-test $p<0.05$).

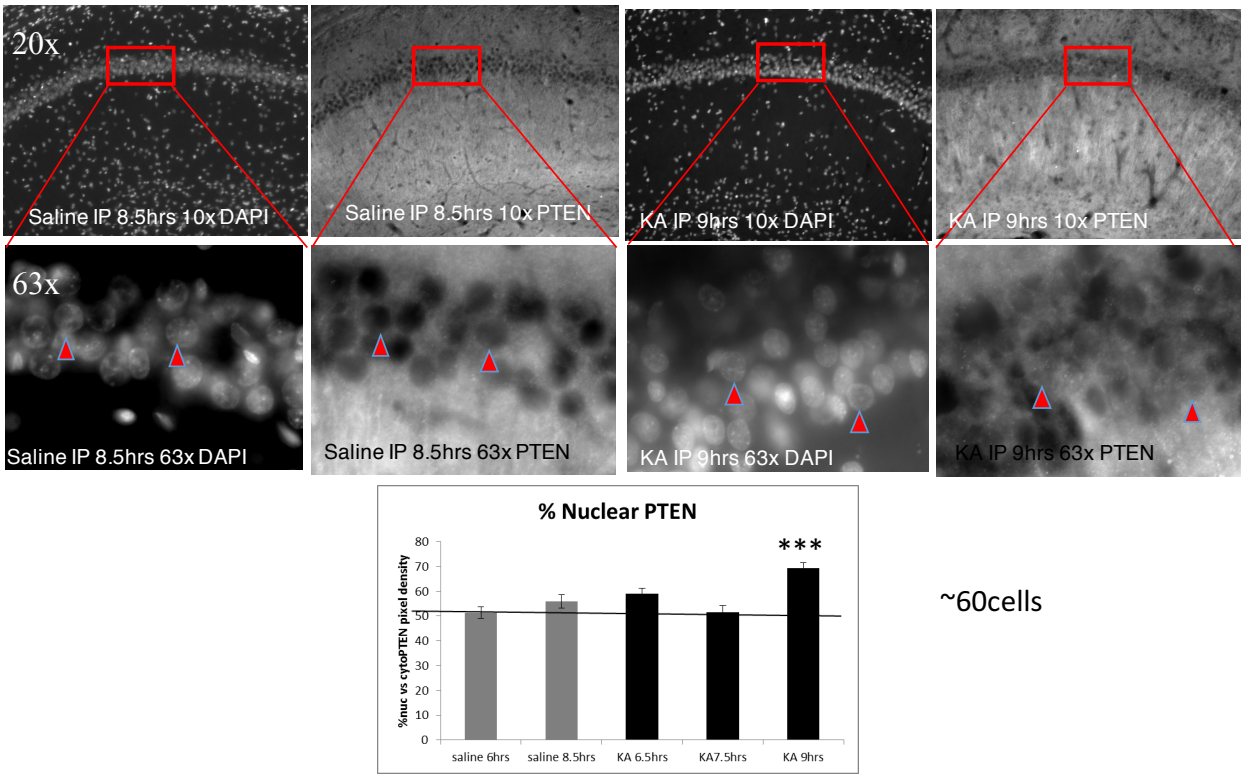


Figure 2-9 Tat-K13 is not neuroprotective against Quinolinic Acid-induced striatum lesions in WT or YAC128 mice

FVB-N WT or YAC128 mice were used to test the ability of Tat-K13 to protect against NMDAR-mediated excitotoxicity in the striatum *in vivo*. Mice were pre- injected with (10mg/kg) Tat-K13, Tat-K13R, a control peptide with mutated K13, or saline vehicle. 1 hour later, QA was sterotaxically injected into the striatum to induce NMDAR-mediated neuron death. Un-injected hemispheres were used as a control and lesion volume was calculated by comparing FluorJade positive neurons in QA hemisphere vs non-QA hemisphere. QA injections lead to dose-dependent increases in striatal QA lesion volume(**A**). Interestingly, Tat-K13, nor Tat-K13R had any effect on lesion volume in any mice tested, with lesion volumes the same as mice injected with IV saline, suggesting that Tat-K13 is not neuroprotective against NMDAR mediated neuronal death in the striatum *in vivo* (**B**). In fact, an increase in lesion volume appears to occur in YAC128 mice compared to WT mice injected with Tat-K13 or Tat-K13R vs saline vehicle, suggesting that peptide may actually worsen the lesion.

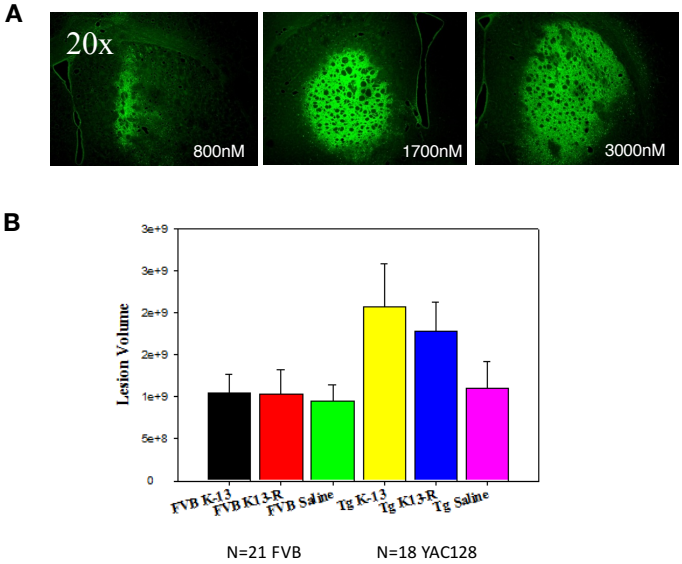
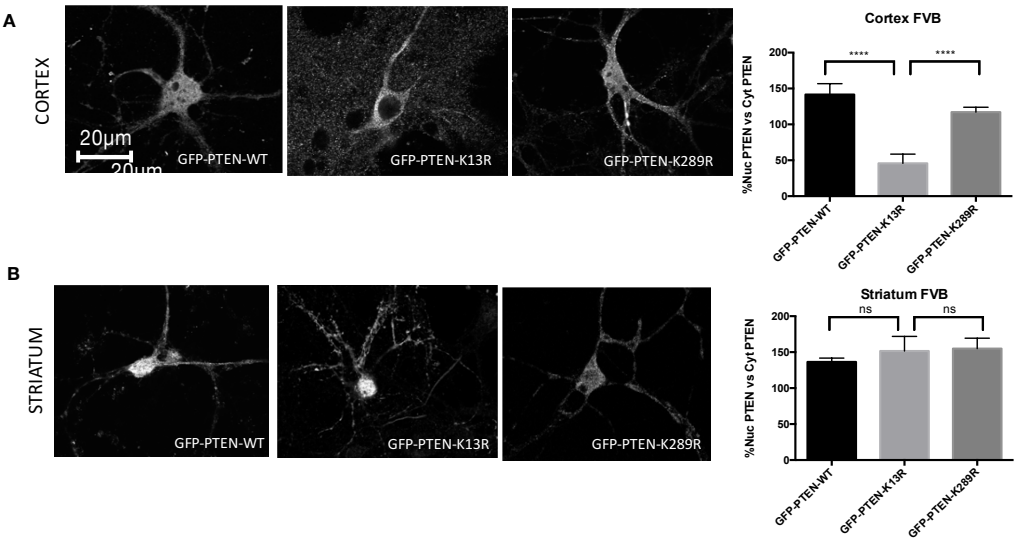


Figure 2-10 PTEN nuclear translocation is blocked by PTEN K13R mutation in cortex but not striatum

Mono-cultured cortical or striatal neurons were nucleofected with GFP-fused PTEN plasmids at time of plating (2ug/well in 12 well plates, or 1ug/well in 24 well plates). Plasmids were either WT PTEN or contained mutations at K13 or K289 (GFP-PTEN-K13R and GFP-PTEN-K289R) to block mono-ubiquitination. At 18DIV, neurons were fixed and stained for GFP and DAPI to investigate nuclear localization of PTEN. Comparison of nuclear to cytoplasmic PTEN was used to investigate PTEN nuclear translocation. As shown previously (Zhang et al, 2013), GFP-PTEN-WT and GFP-PTEN-K289R are expressed diffusely throughout the cytoplasm and nucleus in WT cortex, indicating that PTEN nuclear translocation can occur freely (**A).** However, GFP-PTEN-K13R is retained in the cytoplasm, indicating that, in the cortex, K13 monubiquitination is required for PTEN nuclear translocation (n~ 25cells, one-way ANOVA $F(2,76) = 18.47$, $p<0.0001$). However, in the striatum, all three PTEN plasmids could be detected in the nucleus and cytoplasm, with no significant differences between them (**B**) (n~25 cells, one-way ANOVA, $F(2,48) = 1.002$, $p=.3747$) suggesting that other mechanisms may regulate PTEN nuclear translocation in striatum.


A
CORTEX
20µm
20µm
GFP-PTEN-WT
GFP-PTEN-K13R
GFP-PTEN-K289R
Cortex FVB
%Nuc PTEN vs Cyt PTEN
200
150
100
50
0
****
****
GFP-PTEN-WT
GFP-PTEN-K13R
GFP-PTEN-K289R
B
STRIATUM
GFP-PTEN-WT
GFP-PTEN-K13R
GFP-PTEN-K289R
Striatum FVB
%Nuc PTEN vs Cyt PTEN
200
150
100
50
0
ns
ns
GFP-PTEN-WT
GFP-PTEN-K13R
GFP-PTEN-K289R

Chapter 3: Activation of Caspase-6 and Cleavage of Caspase-6 Substrates is an Early Event in NMDA Receptor-Mediated Excitotoxicity

3.1 Introduction

As people live longer, the prevalence of age-related neurodegenerative disorders represents a primary cause of public health concern, currently affecting millions of people worldwide. Excitotoxicity, caused by over-activity at glutamate receptors, plays a primary role in neuronal death in the brain in a large number of neurological disorders. N-methyl-D-Aspartate receptors (NMDARs) are particularly important in mediating glutamatergic excitotoxic neuronal death event, in part due to its high level of calcium permeability; a fact that has been widely demonstrated in many scientific studies(Lai et al. 2014; Germano et al. 1987; Goldberg et al. 1987; Ikonomidou & Turski 2002; Steinberg et al. 1988; Steinberg et al. 1989; Weiss et al. 1986). NMDA excitotoxicity has a major role in neuronal death after ischemic stroke (Lai et al. 2014b; Martin & Wang 2010; Lai et al. 2011) After an ischemic event, NMDARs are excessively activated by uncontrolled increases in extracellular glutamate, leading to enhanced calcium influx and activation of cell death signaling pathways. (Choi, 1998; Lai et al., 2014; Lipton & Rosenberg, 1994; Martin & Wang, 2010) NMDAR-mediated excitotoxicity plays an important role in several neurodegenerative conditions. In Huntington's disease the mutant huntingtin protein causes enhanced NMDA sensitivity, NMDAR expression and impaired glutamate transport(Dau et al. 2014; Fan et al. 2012; Fan & Raymond 2007; Milnerwood et al. 2010; Shehadeh et al. 2006; Graham et al. 2009) (discussed in 1.3.8) In Alzheimer's Disease(Hynd et al. 2004; Olney et al. 1999; Danysz & Parsons 2012) toxic Aβ enhances excitotoxic potential by enhancing presynaptic

glutamate release, inhibiting glutamate reuptake and increasing extracellular glutamate concentration in hippocampal neurons (Abramov et al. 2009; Danysz & Parsons 2012) (Abramov et al., 2009; Danysz et al., 2012), as well as triggering glutamate release from glial cells (Danysz et al., 2012; Noda et al., 1999). NMDAR-mediated excitotoxicity has been implemented in Parkinson's Disease (Ambrosi et al. 2014; Beal 1998) and neurotrauma (Arundine et al. 2003; Kuroda et al. 1992; Johnston 2005; Yu et al. 2015). Based on the literature, it is clear that NMDAR-mediated excitotoxic signaling represents an important common mechanism of neuronal death and disorder in many diseases of aging.

NMDAR-mediated excitotoxic events have been shown to initiate neuronal death in numerous ways, including activation of apoptotic cascades (Lai et al., 2014) and thus it is not surprising that strong evidence supports a role of apoptosis in pathogenesis disorders where NMDAR-mediated excitotoxicity plays a role (Lai et al. 2014; Akpan et al. 2011; Gafni et al. 2012; Krajewska et al. 2011; Aarts et al. 2002; Peng et al. 2002; Cheng et al. 2003). Despite this knowledge of underlying causes of neuronal death after excitotoxic events, there remains a lack of effective treatments for neurodegenerative excitotoxic diseases. Caspases, activated as part of the apoptotic cascade, may represent a potential target for reducing neuronal damage after NMDAR excitotoxic stress(Aarts et al. 2002; Akpan et al. 2011; Cheng et al. 2003; Gafni et al. 2012; Krajewska et al. 2011; Lai et al. 2014; Peng et al. 2002; Wong et al. 2015). Initiator caspase(s) are activated upon induction of the programmed cell death pathway (Pop & Salvesen 2009; Graham et al. 2011). Initiator caspases are stress- and cell-type dependent and initiate proteolytic cascades, with the inevitable activation of executioner caspase activation, death and cell removal. Caspases act by post-translationally modifying their substrates through cleavage at specific

recognition sites, causing either protein inactivation or gain of function through generation of active proteolytic fragments.

Many studies have demonstrated a role of caspases in NMDAR-mediated neuronal death in neurodegenerative conditions. Genetic deletion of caspases 1, 3, 6, 8 and/or 9 in *in vivo* mouse models of stroke (Akpan et al. 2011; Graham et al. 2011; Krajewska et al. 2011; Le et al. 2002; Schielke et al. 1998; Shabanzadeh et al. 2015) or caspase inhibition using chemical inhibitors in *in vitro* stroke models(Krupinski et al. 2000; Peng et al. 2002; Uribe et al. 2012)have both shown neuroprotective effects. Similarly, caspase deletion or inhibition of cleavage of specific caspase substrates has also been linked neuropathological and behavioral improvements in HD(Albrecht et al. 2007; Gafni et al. 2012; Graham et al. 2010; Milnerwood et al. 2010; Wellington et al. 2002; Wong et al. 2015; Mahmoud A Pouladi et al. 2009; Graham, Slow, et al. 2006)(see section 1.5.2.3.3 and 1.3.3) and AD(Galvan et al. 2008; Galvan et al. 2006; Nguyen et al. 2008; Saganich et al. 2006). Much evidence suggests that caspase activation and substrate cleavage may be associated with both neuronal damage and behavioural decline after an excitotoxic event.

In particular, casp6 may be an important component of neurodegeneration and cognitive decline after NMDAR-mediated excitotoxicity(Akpan et al. 2011; Krupinski et al. 2000; Ambrosi et al. 2014; Beal et al. 1986; Leavitt et al. 2006; Graham et al. 2010; Milnerwood et al. 2010; Harrison et al. 2001; LeBlanc et al. 2014; Wong et al. 2015) . Casp6 has been described as both an initiator and executioner caspase during apoptosis and cleaves other initiator caspases including caspases -2 and -8(Cowling & Downward, 2002; Slee, 1999; Van de Craen, Declercq, Van den brande, Fiers, & Vandenabeele, 1999). This initiates an apoptosis cascade, causing permeabilization of the mitochondrial membrane, subsequent release of cytochrome c and activation of executioner caspases(Cowling & Downward 2002; Slee et al. 1999; Van de Craen et

al. 1999). Casp6 has also been known to cleave caspase-3 (Allsopp et al. 2000; Liu et al. 1996; Xanthoudakis et al. 1999), and cleavage of several casp6 substrates, such as NF-κß, have been shown to generate toxic fragments involved in apoptosis(Levkau et al. 1999). Activation of casp6 has been a common finding in many animal and *in vitro* models of ischemic stroke (Akpan et al. 2011; Harrison et al. 2001; Krajewska et al. 2011; Krupinski et al. 2000). Similarly, in human brain tissues of post-mortem patients who died of ischemic stroke, elevated levels of activated casp6 are observed(Friedlander & Yuan 1998; Harrison et al. 2001) Activation of caspase-9 (Akpan et al. 2011; Krajewska et al. 2011) as well as caspase-3 (Le et al. 2002; M. Kim et al. 1999) are also observed both *in vitro* and *in vivo* in animal stroke models, and animals with targeted genetic deletion of casp6, 8 or 9 show improved behavioural and neuropathological outcomes after stroke (Akpan et al. 2011; Krajewska et al. 2011). These data suggest that caspases have important roles in ischemic stroke, and that casp6 may be acting as an important initiator in apoptotic cascades in ischemia, upstream of other caspase-mediated apoptotic events.

Casp6 has similarly important implications in other neurodegenerative diseases where excitotoxicity and apoptosis are involved such as HD (Graham et al. 2010; Graham et al. 2011). In HD, the mutant huntingtin (mHTT) protein is proteolytically cleaved by caspases, leading to release of toxic fragments critical in HD pathogenesis (Wellington et al. 2002; Graham et al. 2010; Graham, Slow, et al. 2006). HD transgenic mice with mutations preventing casp6 cleavage of mHTT show protection from striatal loss and behavioral deficits as well as rescue of HD-mediated NMDAR dysfunction (Milnerwood et al. 2010; Wellington et al. 2002; Graham et al. 2010; Graham et al. 2011; Graham, Slow, et al. 2006; Warby et al. 2008; Mahmoud A Pouladi et al. 2009). Similarly, genetic silencing (Wong et al. 2015; Uribe et al. 2012), chemical inhibition (Graham et al. 2010) or dominant-negative inhibition(Hermel et al. 2004) of casp6 shows

significant neuroprotective effects in HD models. NMDAR-mediated excitotoxicity also has a key role in AD. Casp6 is enhanced and activated in the brains of both familial and sporadic AD patients (Albrecht et al. 2007; Albrecht et al. 2009; Pompl et al. 2003) Active casp6 as well as casp6 cleavage products are found in human AD plaques, tangles and neuropil (Albrecht et al. 2007; Guo et al. 2004). Casp6 activity correlates with decreases in cognitive function and neuropathology, and both precedes and leads to formation of tau neurofibrillary tangles (LeBlanc et al. 2014; LeBlanc 2013; de Calignon et al. 2010; Fisher 2015; Gamblin et al. 2003; Rissman et al. 2004; Zhao et al. 2003). In AD, APP is cleaved by caspases, a process thought to lead to toxic Aβ(Tesco et al. 2007). Casp6 cleavage of APP at asp664 is an early event in human AD brains (Zhao et al. 2003) and prevention of APP cleavage at asp664 by casp6 improves behavior and neuropathology in mouse AD models (Galvan et al. 2006; Saganich et al. 2006; Nguyen et al. 2008) suggesting that casp6 may be a critical target for therapeutic development in AD.

In order to better understand the role of caspases in excitotoxic neuron death, it is also critical to understand the substrates of caspase and the potential role of the proteolytic fragments produced. Stress-induced generation of caspase-generated proteolytic fragments have been shown to trigger toxicity and amplify the cell death response in numerous experimental paradigms (Breckenridge et al. 2003; de Calignon et al. 2010; Halawani et al. 2010; Levkau et al. 1999; Mazars et al. 2009; Tang et al. 2001; Warby et al. 2008). Furthermore, proteolytic cleavage of specific caspase substrates is an important cellular event in the pathogenesis of several neurodegenerative diseases (Galvan et al. 2008; Galvan et al. 2006; Banwait et al. 2008; Graham, Slow, et al. 2006; Graham et al. 2010; Milnerwood et al. 2010; Mookerjee et al. 2009; Nguyen et al. 2008; Mahmoud A Pouladi et al. 2009). Serine/threonine kinase 3 (STK3) and death domain associated protein (DAXX) are recently described casp6 substrates with defined roles in

apoptosis(Riechers et al. 2016). DAXX induces Fas-mediated cell death and is implicated in the pathogenesis of AD (Bojunga et al. 2004; Y.-L. Niu et al. 2011; Song & Lee 2004). Furthermore, DAXX destabilizes ASK1 causing release of GRX1 and TRX1, redox proteins that are involved in stabilizing ASK1 and inhibiting the DAXX-ASK1-JNK toxic cell death pathway (Bojunga et al., 2004; Lee et al., 1998; Niu et al., 2011; Song & Lee, 2004). Proof of principle studies reveal a marked reduction in infarct size and apoptosis in ischemia-induced cell death is observed with knockdown of DAXX (Bojunga et al., 2004; Lee et al., 1998; Niu et al., 2011; Song & Lee, 2004). STK3 is a stress-activated pro-apoptotic kinase that, upon activation and caspase cleavage, enters the nucleus and induces cell death pathways. Under normal conditions the neuroprotective protein, AKT (a potential casp6 substrate)(Medina et al. 2005) phosphorylates STK3 leading to inhibition of its kinase activity and pro-apoptotic functions (Graves et al. 1998; Bojunga et al. 2004; Lee et al. 1998; Y.-L. Niu et al. 2011; Song & Lee 2004). Interaction of these two molecules with casp6 may similarly link them to apoptotic pathways in NMDAR-mediated excitotoxicity a mechanism that has not been well studied.

3.2 Hypothesis and specific aims

Despite extensive evidence that caspases have a role in excitotoxic neuronal death, there is a strong need for better elucidation of the mechanisms underlying activation of these apoptotic pathways and of the specific substrates cleaved by caspase cascades as well as the function of the proteolytic fragments produced by caspase cleavage. In the current study I aimed to establish the role of caspases and casp6 substrate proteolysis in NMDAR-mediated excitotoxicity. I used a common *in vitro* model of excitotoxicity known to activate apoptotic cascades(Zhang et al. 2013b; Fan et al. 2014; Lai et al. 2014; Taghibiglou et al. 2009). Using this model, we aimed to determine

a time-course of caspase activation after an excitotoxic event in order to better understand apoptotic pathways that may lead to neuronal death in stroke, and other excitotoxic ailments. Similarly, I aimed to study caspase expression after excitotoxicity at the level of mRNA, as transcriptional regulation of caspases in ischemic stroke and excitotoxicity is still poorly understood. We also investigated expression and cleavage of the casp6 substrates huntingtin, STK3 and DAXX post NMDA treatment in NMDAR-mediated excitotoxic pathways to determine their cleavage profiles to further elucidate their potential roles in excitotoxic apoptotic cascades. A better understanding of the mechanisms and timeframe of caspase activation in an NMDAR-mediated excitotoxic event provides potential targets for novel neuroprotective strategies for neurodegenerative diseases.

3.3 Methods

3.3.1 Antibodies and reagents

NMDA was purchased from Ascent Scientific (Cambridge, MA, USA, Asc-052). Reagents for protein concentration assay (reagent A no. 500-0113, reagent B no. 500-0114) were obtained from Bio-Rad Laboratories (Redmond, WA, USA). Antibodies used were the following: caspase-8 (Santa Cruz, sc-7890, Dallas, TX, USA, 1:100), caspase-6 (Cell Signaling, Danvers, MA, USA #9762, 1:500), caspase-3 (Cell Signaling, Danvers, MA, USA #9662, 1:1000), caspase-9 (Cell Signaling, Danvers, MA, USA #9508, 1:1000), HTT (Millipore, Etobicoke, ON, Canada, MAB2166, 1:500), STK3 (Abcam, Cambridge, UK, ab52641, 1:1000) and/or DAXX (Abcam, Cambridge, UK, ab105173, 1:1000). All reagents were recently authenticated and tested.

3.3.2 Animal models

Animals used to generate cultures were pregnant female Sprague Dawley rats (*Rattus norveticus)*. Rats were purchased from Charles River Laboratories (Montreal, QC, Canada). All animal protocols were approved by UBC's Animal Care Committee and research was overseen by the Canadian Council for Animal Care (A13-0139).

3.3.3 Buffers

All buffers were sterilized by autoclave or filtration (Corning, polyethersulfone with 0.22µm pore size, Corning, NY, USA). PBS contains 137 mM NaCl(Sigma, St Louis, Missouri, USA), 2.7 mM KCl(Sigma), 8.1 mM Na2HPO4(Sigma), 1.76 mM KH2PO4(Sigma), pH 7.4. poly-D-Lysine coating solution contains 10g/ml poly-D-lysine(Sigma). HBSS dissection buffer (DB, Thermo-Fisher) contains 5.0 g/L glucose (Sigma), 1.25 g/L sucrose (Sigma), 0.89 g/L HEPES, pH 7.4, osmolality to 310 –320 mOsm, stored at 20°C. Neurobasal plating (NP) media) contains 487.75 ml neurobasal media (Thermo-Fisher, Carlsbad, CA, USA), 0.5 mM GlutaMAXTM-I supplement (Thermo-Fisher), 2% B27 supplement,(Thermo-Fisher) 25µm of glutamic acid(Sigma).

3.3.4 Primary cortical neuron culture

Time-pregnant rats (E18) were euthanized by overdose using 3.5ml of 25% urethane solution. Using a dissection microscope, cortex tissue was isolated from rat pups in ice-cold HBSS dissection buffer then digested using 2-4ml of pre-warmed 0.25% trypsin-EDTA (37°C for 30min) then the dissociated cells were washed with warm DMEM(Gibco, Carlsbad, CA, USA) (containing 10% FBS) three times, followed by gentle pipetting with gradually smaller tips, to ensure a

homogenous cell suspension. Cells were then resuspended in NP and counted using a hemocytometer. Cells were plated on poly-D-lysine coated culture dishes (coated overnight at room temperature) at a density of 6.0 x 10^6 per 100ml dish or 2 x 10^5 in 24 well plates. Cells were then cultured in a 37°C incubator with 95% O_2 and 5% CO_2. 2 days after plating, 1/3 of plating media was replaced by equal volume of fresh plating media. Media culture was repeated every four days until cells were used for experiments. All experiments were done at 13-15DIV.

3.3.5 *Ex vivo* NMDAR-mediated excitotoxicity

Primary cultured mature cortical neurons were used in this study (13-15DIV). Prior to NMDA stimulation, half of the conditioned media in the culture dishes was collected and saved at 37°C. Neurons were stimulated with bath application of 25uM NMDA into conditioned media for 1hour at 37°C. After stimulation, media containing NMDA was aspirated and replaced with saved conditioned media. Control cell culture media was changed similar to NMDA treated plates. Neurons were collected at various time-points after NMDA stimulation. Control, unstimulated neurons were collected at 0hr. NMDA stimulated neurons were collected either 1, 4 or 24hours after NMDA, or 30minutes into the 1hour NMDA treatment.

3.3.6 Neuron collection protocol

At specified time points, cells were removed from 37°C incubator and placed on ice. 500ul of conditioned media was collected for LDH assay and frozen at -80°C. Media was then aspirated and cells were washed 1x with ice cold PBS, then scraped down gently using a cell scraper in 100ul PBS, containing no inhibitors. Cells were then spun down at 140,000rpm for 5min. Cell pellets were kept for protein and mRNA analysis. Supernatant was also collected in the case of

any cell damage and component release into supernatant. All samples were immediately frozen at -80°C to store for further analysis.

3.3.7 Lactate dehydrogenase assay

To assess cell death and confirm excitotoxicity we used a lactate dehydrogenase (LDH) assay. In this study, an *in vitro* colormetric LDH kit was used (Sigma-Aldrich, St Louis, Missouri, USA, TOX-7). In this kit, LDH reduces NAD into NADH, which is used in stoichiometric conversion of a tetrazolium dye. The resulting color change is measured using a spectrophotometer at 490nm (subtracting out a control reading at 690nm).

3.3.8 Real-Time quantitative RT-PCR

Total RNA was extracted from primary rat cortical neurons post NMDA with RNeasy mini kit (QIAGEN, Hilden, Germany) and cDNA was prepared using ProtoScript Reverse Transcriptase II (#M0368X, New England BioLabs, Ipswitch, MA, USA). Quantification was done using Mx3005P QPCR Systems (Stratagene, La Jolla, CA, USA) with rat-specific β-actin primers (Forward 5' GCTACAGCTTCACCACCACA and Reverse 5' GCCATCTCTTGCTCGAAGTC), , casp3 (Forward 5'-GGACCTGTGGACCTGAAAAA-3' and Reverse 5'-GCATGCCATATCATCGTCAG-3'), casp6 (Forward 5'-ACGTGGTGGATCATCAGACA-3' and Reverse 5'-GGAGCCGTTCACAGTCTCTC-3'), casp8 (Forward 5'-GGGGATGGCTACTGTGAAAA-3' and Reverse 5'-CATGTTCCTCGGGTTGTCTT-3') and casp9 (Foward 5'-AAGACCATGGCTTTGAGGTG-3' and Reverse 5'-CAGGAACCGCTCTTCTTGTC-3') Amplification of β-actin was used to standardize the amount

of sample RNA in the reaction. Gene-expression levels were measured using MxPro QPCR Software (Stratagene, La Jolla, CA, USA).

3.3.9 Western blot analysis

Primary rat cortical neurons ± NMDA treatment were homogenized in lysis buffer (0.32 mM Sucrose, 20 mM Tris pH 7.2, 1 mM $MgCl_2$, 0.5 mM EDTA pH 7.2) with a protease inhibitor cocktail (Roche, Basal, Switzerland), PefaBloc SC (Roche, Basal) and Z-Vad-FMK (Enzo Lifes Sciences, Brockville, ON, Canada) then cleared by centrifuged at 13,000. The protein concentration was determined using BCA (bicinchoninic acid) protein assay kit (Pierce). Protein lysates (50µg) were separated on SDS-PAGE gel and transferred to PVDF membrane (PerkinElmer). The membranes were probed with caspase-8 (Santa Cruz, sc-7890), caspase-6 (Cell Signaling, #9762), caspase-3 (Cell Signaling, #9662), caspase-9 (Cell Signalling #9508), HTT (Millipore, MAB2166,), STK3 (Abcam, ab52641) and/or DAXX (Abcam, ab105173) antibodies. The membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (#7074, Cell Signaling) or anti-mouse IgG (#7076, Cell Signaling). Peroxidase activity was detected and densitometric values obtained with the Odyssey Fc imaging system (LiCor) using Luminata Crescendo Western HRP substrate (Millipore). Quantification of, calnexin (Abcam, ab75801) was used to standardize loading and densitometric values obtained with the Odyssey Fc imaging system (Li-Cor, Lincoln, Nebraska, USA).

3.3.10 Fluorogenic caspase activity assay

The protein concentration of neuronal lysates (prepared as described above without protease inhibitors) was measured using the Bradford assay. The lysate was incubated with the reaction

mix (as per manufactures instructions) containing fluorogenic casp6 substrate (Ac-VEID-AFC, from BIOMOL, Brockville, ON, Canada) at 50µM in 100µl reaction volume at 37°C for 1 hour. The fluorescent units were read at excitation 405nm and emission 535nm. The µM AFC was calculated against the AFC standard curve and then divided by the protein concentration. The relative casp6 activity is expressed as µM AFC/mg protein.

3.3.11 Statistical analysis

Statistical analysis was done using Student's *t*-test, one-way ANOVA (in cases of significant effect of genotype, post-hoc comparisons between genotypes were performed using Turkey or linear trend *post hoc* test). P values, SEM, means and standard deviations were calculated using Graphpad Prism version 6.0. Linear regression analysis for r^2 and p values was calculated by Pearson correlation coefficient. Differences between means were considered statistically significant if $p<0.05$.

3.4 Results

3.4.1 Early increase in caspase-6 expression and activity post NMDA-induced excitotoxicity in primary cultured rat cortical neurons

Initial experiments investigated activity and expression levels of several caspases in a well-characterized *in vitro* model of NMDAR-mediated excitotoxicity. Primary cultured rat cortical neurons were stimulated for 1 hour with 25uM NMDA in culture media, then allowed to recover and cells collected at several time points post NMDA treatment. This model of NMDAR-mediated excitotoxicity has been previously used in our lab to investigate downstream pathways associated with excitotoxicity(Zhang et al. 2013b; Fan et al. 2014; Taghibiglou et al. 2009; Lai et al. 2014).

We validated that this model effectively created excitotoxic conditions using a lactate dehydrogenase (LDH) cell death assay. Samples of culture media collected 0, 1 and 24 hours after NMDA stimulation confirmed a time dependent increase in LDH release, signifying increased cell death over time (ANOVA $p<0.001$), (**Fig 3-1 A**). Once excitotoxic cell death was confirmed, this *in vitro* model was used to investigate caspase activation and expression after NMDAR-mediated excitotoxicity. Control cells, treated with vehicle, were collected at 0hr. Cell pellets were then probed for various caspases previously shown to be involved in excitotoxic events using RT-PCR. As expected, upregulation of caspases appears to be an early event after excitotoxic stress. Casp3 mRNA is significantly elevated 24 hours after NMDA, in line with observed cell death profiles, though no changes were seen at earlier time-points (ANOVA $p=0.03$ post hoc 1 vs 24hr $p<0.05$) (**Fig 3-1 B**). Of note, a significant increase in casp6 mRNA expression was seen at 1 and 24 hours post NMDA treatment (ANOVA $p=0.016$, post hoc 0hr vs 24hr $p<0.05$, t-test: 0hr vs 1hr $p=0.009$) suggesting that casp6 upregulation is an early event of NMDAR-mediated excitotoxicity (**Fig 3-1 C**). No changes in casp8 (ANOVA $p=0.109$) or casp9 (ANOVA $p=0.558$) mRNA levels were observed at any of the time-points assessed (**Fig 3-1 D, E)**. Supporting early increases in casp6 mRNA, casp6 activity assays post-NMDA also demonstrate increased casp6 activity 1hour post NMDA (ANOVA $p=0.011$ post hoc 0hr vs 1hr $p<0.05$,) These data demonstrate that caspases are indeed upregulated after NMDAR-mediated excitotoxic events, both at the mRNA level and in activity with earliest changes seen in casp6 and subsequent increase in casp3 and a trend increase in casp8. These data point to casp6 as a potential initiator caspase in the apoptotic cascade after an NMDAR-mediated exctotoxic event , with changes in mRNA expression occurring even one hour after NMDA and, increased expression of casp3 occurring further along in the cascade.

As increased casp6 mRNA was detected even one hour after NMDA excitotoxicity I speculated that changes in caspase expression and activity might be very early events in NMDAR-mediated apoptotic cascades. Thus a more detailed time course study was done to investigate early changes in caspases known to be involved in neurodegeneration. Similar to our previous experiment, primary rat cortical neurons (13-15DIV) were stimulated with 25uM NMDA or vehicle control and cells were collected at 30min or 1 hour into the NMDA treatment. After NMDA treatment, media was aspirated and replaced with conditioned media. A second excitotoxicity time point was collected 4hours after NMDA. All control cells were untreated and collected at 0hr. LDH assays confirmed a subtle time-dependent increase in cell death post NMDA (ANOVA $p<0.0001$), confirming excitotoxicity (**Fig 3-2 A**). Assessment of caspase mRNA expression at these early time-points demonstrate early changes in caspases after excitotoxicity. In support of our initial experiments, casp6 mRNA was significantly elevated post-NMDA. In line with my initial time-course experiment, no early changes were seen in mRNA expression of casp3 (**Fig 3-2 B**). In contrast, a linear trend in casp6 mRNA increases post-NMDA ($R^2=0.6604$, $p=0.0018$) was observed, beginning at 30min and becoming very significant at 4hours, supporting the idea that increased casp6 expression may be an early event after NMDAR-mediated excitotoxicity (**Fig 3-2 C**). A trend towards increased casp8 mRNA increase was also detected at 4 hours after NMDA, suggesting that casp8 may be subsequently upregulated after casp6 (ANOVA $p=0.08$), (**Fig 3-2 D**). There was no change in casp9 mRNA (Fig 2 E) at early time-points, suggesting it may be recruited later in the apoptotic cascade**.** These results are supported by casp6 activity assays, which demonstrate a linear trend towards increase in casp6 activity even at these early time-points (linear trend $R^2=0.1825$, $p=0.04$, t-test Ctrl vs. 4h $p=0.06$). (**Fig 3-2 F).**

These data further support the hypothesis that casp6 may be an initiator caspase in NMDAR-mediated apoptosis after an excitotoxic event.

NMDA influences proform caspase expression levels in primary rat cortical neurons at early time points.

Results of the mRNA expression analysis demonstrate early increases in caspase mRNA expression and activity after an NMDAR-mediated excitotoxic event *in vitro*, with very early changes seen for casp6, and accompanying increases in casp6 activity**.** Though mRNA increase suggests an upregulation of caspases, I next wanted to investigate whether NMDAR-mediated excitotoxicity leads to changes in the protein expression of caspases. Increased caspase protein expression may influence caspase activation, through increased availability and autoactivation(Klaiman et al, 2009) which would support increases in casp6 activity observed. Using western blotting, expression of the pro- and active form of caspases was analyzed using the same NMDA excitotoxicity time course study. In particular interest was cleavage of caspases known to be substrates of casp6, such as casp3 and casp8. Western blotting of casp3 demonstrates a significant decrease in expression of the casp3 proform 4 hours post NMDA, suggesting early activation and cleavage of casp3 after an excitotoxic event (ANOVA p=0.009,) (**Fig 3-3 A,B**). Casp6, which showed early changes in mRNA expression and is known to autoactivate with sufficient expression(Klaiman et al. 2009) also showed significant cleavage 4 hours post NMDA, supporting the previous data that it is activated early in ischemia (ANOVA p=0.008, linear trend R^2=0.1694, p=0.03) (**Fig 3-3 C, D**). Similar changes were seen for casp8, with significant reductions in proform expression by 4 hours after NMDA (ANOVA p=0.02, linear trend R^2=0.3881, p=0.007) (**Fig 3-3 E, F**) and for casp9 with a very significant reduction in its full-

length form as early as 30min after NMDA (ANOVA $p<0.0001$, linear trend $R^2=0.8369$, $p<0.0001$) (**Fig 3-3 G, H**). These data demonstrate that, even at early time points, increases in caspase mRNA induction and activity correspond with expected cleavage products, pointing to very early activation after NMDA excitotoxicity, especially casp6.

Decreased full-length expression levels of caspase-6 substrates following NMDAR-mediated excitotoxic stress in rat cortical neurons

Experiments using *in vitro* excitotoxic time course studies demonstrate very early changes in casp6 profiles after an NMDAR-mediated excitotoxic event at the level of mRNA, protein expression, activity and expected caspase cleavage. Based on this, and previous studies suggesting that casp6 may have a role as an initiator caspase in neurodegenerative apoptotic pathways, I investigated whether these changes in casp6 profiles are associated with alterations of known casp6 substrates.

Casp6 is known to have an important role in the generation of a toxic fragment of the huntingtin protein in HD(Graham, Deng, et al. 2006; Graham et al. 2010; Graham et al. 2011; Milnerwood et al. 2010; Mahmoud A Pouladi et al. 2009; Wellington et al. 2002) thus changes in huntingtin mRNA and protein expression were investigated using the *in vitro* excitotoxic model. Interestingly, after NMDA stimulation, a significant increase in huntingtin mRNA levels was detected in both the initial time point experiment (0h, 1h and 24h, ANOVA, $p=0.03$, linear trend $R^2=0.4129$, $p=0.02$, **Fig 3-4 A**) and in the subsequent early time points (0, 0.5h, 4h ANOVA $p=0.008$, linear trend $R^2=0.3713$, $p=0.016$, **Fig. 3-4 B**). Of interest, a significant linear trend is observed between casp6 and huntingtin mRNA expression levels post-NMDA ($R^2=0.8657$, $p=0.007$, Fig.4C). In contrast to the mRNA results, a significant decrease in the full-length huntingtin protein at both 30min and 4 hours after NMDA stimulation was observed (ANOVA

p=0.008, linear trend R^2=0.5845, p=0.0.003), suggesting that, despite increases in huntingtin transcription after NMDA, cleavage is still occurring on the same timeframe as casp6 activation (**Fig 3-4 E**).

Next, two newly discovered casp6 targets, STK3 and DAXX, were examined, known to be involved in neurodegenerative diseases and apoptosis(Riechers et al. 2016). Using a similar NMDAR-mediated time course study, we investigated cleavage of these casp6 substrates at various time points after NMDA stimulation of cortical neurons.

STK3 is only the third known kinase to be a substrate of casp6(Riechers et al. 2016; van Raam et al. 2013; Medina et al. 2005) however, caspases have been shown to target kinases and phosphatases, which can lead to fine regulation of subsequent caspase activation pathways(Graves et al. 1998; Lee et al. 1998; Tang et al. 2001). In the current study, a significant decrease of full-length STK3 protein levels was observed by 30min that continued out to 4 hours (ANOVA p<0.001 linear trend R^2=0.6728, p<0.001, post hoc **Fig 3-5 A, B**). DAXX, previously implicated in stroke-mediated cell death(Y. L. Niu et al. 2011; Roubille et al. 2007) was originally identified by interaction with the death receptor Fas. Cleavage fragments of DAXX are pro-apoptotic and activate the JNK pathway in an *in vitro* model(Song & Lee 2004). After NMDA stimulation, a time dependent reduction in levels of full-length phospho-DAXX was seen, becoming significant by 4 hours, suggesting that cleavage of phospho-DAXX occurs post- excitotoxic stress (**Fig 3-5 C, D**). Similarly, an accompanying increase in toxic DAXX cleavage products was detected that correspond to previously shown casp6 cleavage by DAXX (**Fig 3-5 C, D**). To confirm that the detected changes in DAXX and STK3 expression were not due to changes in transcription levels of these proteins, mRNA expression of these targets was assessed at the time points of interest after NMDA. Neither DAXX nor STK3 showed any significant changes in mRNA expression after

NMDA stimulation, suggesting that the changes are a result of caspase cleavage (data not shown). This is supported by studies in our lab directly demonstrating similar STK3 and DAXX cleavage by casp6(Riechers et al., 2016).

The profiles of the casp6 substrates assessed all showed significant decreases in the full-length form of the protein and a trend increase in fragments generated. These data fit well with the increase in casp6 mRNA and activity and suggest that casp6 may be an early, initiator caspase in apoptotic cascades after an NMDAR-mediated excitotoxic event. Data investigating substrates of casp6 further implicate an early role for casp6 in NMDAR-mediated excitotoxicity and further point to casp6 as an initiator caspase in apoptotic cascades after ischemia. Similarly, these results point to HTT, DAXX and STK3 as potential substrates of casp6 that may have a role in apoptotic cascades in NMDAR-mediated excitotoxicity.

3.5 Discussion

Using an *in vitro* model of NMDAR-mediated excitotoxicity(Zhang et al., 2013;Fan et al., 2014; Lai et al., 2014; Taghibiglou et al., 2009;) time-course studies, I investigated the activity and expression patterns of several caspases previously shown to have a role in ischemia and several other neurological conditions. We aimed to develop a timeline for caspase expression after an NMDAR-mediated excitotoxic event in order to determine if caspase activation is an early event, and in particular which caspase may be a potential therapeutic target for stroke and other conditions where NMDAR-mediated excitotoxicity contributes to pathology. Using an *in vitro* model, signs of early activation of caspases was found, even 30 minutes into NMDAR-mediated excitotoxic stimulation at the level of mRNA, caspase activity and cleavage of caspase substrates. At the mRNA level, early and constant transcriptional elevation of casp6 was observed post-NMDA

treatment, suggesting early upregulation. mRNA elevations at later time points were observed for casp3 and casp8 post-excitotoxic stress, suggesting their upregulation later in the cascade. At the protein level, western blots demonstrate reduced expression of the proforms of all caspases assessed post NMDA suggesting their cleavage and potential activation at very early time points after NMDA. This is complimented by data demonstrating increases in casp6 activity early post NMDA, and previous data demonstrating casp6 autoactivation(Klaiman et al. 2009) Based on this pattern of early upregulation and activity of casp6 in an *in vitro* model of excitotoxicity, and based on previous studies implicating casp6 in pathogenesis of neurodegenerative diseases, I hypothesize that casp6 may be acting as an initiator caspase in the apoptotic cascades associated with NMDAR-mediated excitotoxicity. Investigation of known casp6 substrates support this hypothesis. Novel casp6 interactors STK3 and DAXX(Reichers et al., 2016) both show significant decreases in the full-length protein expression over time post NMDA suggesting their cleavage by active casp6 at very early time points post -NMDA. To further explore this, expression of pro-apoptotic DAXX cleavage products was investigated using western blot. There was a trend towards a time dependent increase in the 40kDa cleavage product of DAXX, a known, toxic casp6 cleavage product of DAXX(Reichers et al, 2016) 30min post NMDA treatment, suggesting that casp6 may be acting to increase expression of pro-apoptotic cleavage fragments of DAXX, very early after an excitotoxic event. Similarly, the known casp6 substrate huntingtin also demonstrated decreased full-length levels despite increased mRNA expression as early as 30min into NMDAR-mediated excitotoxicity, further supporting our hypothesis that casp6 may be an initiator caspase in excitotoxic neuron death.

Based on the results of this study, myself and colleagues suggest a proposed signaling pathway for caspase activation post NMDAR-mediated excitotoxicity to expand on known

apoptotic signaling events after excitotoxic events (**Fig 3-6**). After excitotoxic NMDAR stimulation, calcium influx has previously shown to induce activation of p53(Cheng et al. 2003) as well as activate casp6 (Akpan et al., 2011; Harrison et al., 2001; Krajewska et al., 2004; Krupinski et al., 2000, Graham et al, 2010) which would be expected to lead to cleavage of casp6 substrates. The current data demonstrates that reduced full-length levels of casp6 substrates are observed post NMDA supporting that cleavage of casp6 substrates may be occurring post an ischemic event, via NMDAR-mediated excitotoxicity. It was shown that the cleavage of huntingtin and novel casp6 interacting proteins STK3 and DAXX, both previously known to have roles in apoptosis, occurs early during excitotoxicity. Similarly, the data showing reduced full-length protein levels of the casp6 substrate, casp8, on the same timeframe of increasing casp6 activity suggests that casp8 may also be a potential substrate of casp6 in ischemia. Casp8 has previously been shown to cleave casp9(McDonnell, Wang et al. 2003), which may explain the decrease in the proform of casp9 post NMDA. We also propose that, after casp6 upregulation and activation immediately after NMDA stimulation, casp3 may be subsequently recruited and activated, leading to executioner caspase activation and cell death(Ashkenazi, Avi, & Guy, 2014; Graves et al., 1998; Le et al., 2002; Slee, 1999). Based on this data, casp6 may be an early initiator of the apoptotic cascade after NMDAR-mediated excitotoxicity. This suggests that targeting caspases, primarily casp6, may be a potential target for novel therapeutics as it may be a fundamental, early process in the apoptotic cascades after excitotoxic stress(Akpan et al. 2011).

The findings of this study provide important insight into apoptotic cascades after an excitotoxic event. Importantly, it suggests casp6 as the possible initiator caspase in a series of events involving caspase cleavage and activation ultimately leading to neuron death. Casp6 is upregulated and activated very soon post NMDA treatment. Similarly, this study demonstrates

important time course data at the level of mRNA for various caspases after NMDAR-mediated excitotoxicity, showing very early increases in mRNA expression for casp6, followed by subsequent increase in casp8, then casp3. This provides novel temporal data for transcriptional caspase regulation in excitotoxicity. Elevations in casp6 mRNA have been observed prior to up-regulation of other caspases in a rat model of focal cerebral ischemia (permanent middle cerebral artery occlusion)(Harrison et al. 2001) Strong upregulation of casp6 mRNA at earlier time points than caspase 1, 3 and 7 post excitotoxic injury points to its involvement as an upstream initiator, as well as having a clear role in the execution phase, of ischemic induced apoptotic cell death. Additionally, our data helps link activation of caspases in NMDAR-mediated excitotoxicity with two newly discovered substrates of casp6, namely STK3 and DAXX. Despite previous knowledge of their role in apoptosis, they are now confirmed casp6 substrates(Riechers et al., 2016) and the current protein expression studies demonstrate reduced levels of the full-length protein expression on a timescale similar to casp6 activation and upregulation. These data suggest that they are cleaved by casp6 after NMDAR-mediated excitotoxicity, strengthening data indicating their role in apoptosis and helping to further elucidate apoptotic signaling cascades in ischemia and other disorders of excitotoxicity. Importantly, as NMDAR-mediated excitotoxicity has a role in neuronal death in a wide range of neurodegenerative conditions such as AD, HD, Parkinson's Disease and brain injury(Ambrosi et al. 2014; Arundine & Tymianski 2003; Beal 1998; Carlos Cepeda et al. 2001; Dau et al. 2014; Fan et al. 2012; Galvan et al. 2008; Galvan et al. 2006; Graham, Slow, et al. 2006; Milnerwood et al. 2010; Graham et al. 2011; Hermel et al. 2004; Inglis et al. 1992; Koutsilieri & Riederer 2007; Olney et al. 1999; Saganich et al. 2006; Sepers & Raymond 2014; Shehadeh et al. 2006; Wong et al. 2015; Zhao et al. 2003), this data may help to further understand apoptotic signaling cascades in many diseases and neurological conditions. This

could aid in the search for better therapeutic targets for various neurological conditions where NMDAR-mediated excitotoxicity has a role.

An interesting finding in the current study was a surprising increase in huntingtin mRNA despite decreased protein expression levels after NMDAR stimulation that correlated with casp6 mRNA expression levels. As huntingtin is a known substrate of casp6, changes in the full-length form of the huntingtin protein was anticipated due to cleavage as casp6 is activated. This increase in mRNA expression of huntingtin could be an indication of compensatory neuroprotective strategies of the cell. Previous studies have suggested that wild type huntingtin protein is neuroprotective in HD(Feng et al. 2006; Rigamonti et al. 2000; Leavitt et al. 2006) so transcriptional upregulation of huntingtin may be an attempt of the cell to activate survival pathways after an excitotoxic event. This activity may be mediated by the transcription factor p53 as p53 regulates the expression of huntingtin gene expression(Feng et al. 2006). Activated casp6 is a well-known molecular phenotype of HD both in animal models and in human patient striatum(Graham et al., 2010). It was recently discovered that p53, a transcriptional activator of casp6(MacLachlan & El-Deiry 2002), is upregulated in HD post mortem tissue and transgenic mice, which may lead to the large increases in expression and activity of casp6 observed in HD(Ehrnhoefer et al. 2014) This increase is exacerbated when mutant huntingtin protein is present, suggesting that mutant huntingtin protein might enhance p53 and lower the threshold for apoptosis, demonstrating p53's role in regulating both HTT and casp6 activity(Ehrnhoefer et al. 2014).

3.6 Conclusion

The current study helps to elucidate the pattern of caspase expression after an NMDAR-mediated excitotoxic event and opens the doors to several potential studies to help further

understand this pathway. This work highlights therapeutic approaches for not only ischemic stroke but also HD, AD, neuronal injury and other disorders of NMDAR-mediated excitotoxicity, with casp6 as a potential therapeutic target.

Figure 3-1 Increase in caspase-6 expression and activity in acute excitotoxicity model

Primary cortical neurons were treated with 25uM NMDA for 1 hour (h). Neurons were allowed to recover for 0, 1, 24 hrs, respectively. (n=4 cultures) **A)** Media were collected at the end of treatment and subjected to LDH assay. A significant increase in LDH activity is observed at 24 hrs post NMDA (ANOVA p<0.0001, post hoc control, 0h or 1h vs. 24hrs, p<0.0001). **B)** Assessment of caspase mRNA levels demonstrates casp3 mRNA is significantly increased at 24 hrs post NMDA (ANOVA p=0.03, post hoc 1h vs. 24h, p<0.05). **C)** Increased casp6 mRNA is also observed post NMDA (ANOVA p=0.016, post hoc 0h vs. 24h p<0.05 [t-test 0h vs. 1h p=0.009]) whereas no differences in **D)** casp8 or **E)** casp9 mRNA levels were observed. **F)** Correlating with the increased casp6 mRNA levels, at 1hr post NMDA a significant increase in casp6 activity is observed (ANOVA p=0.055, post hoc 0h vs. 1h p<0.05).

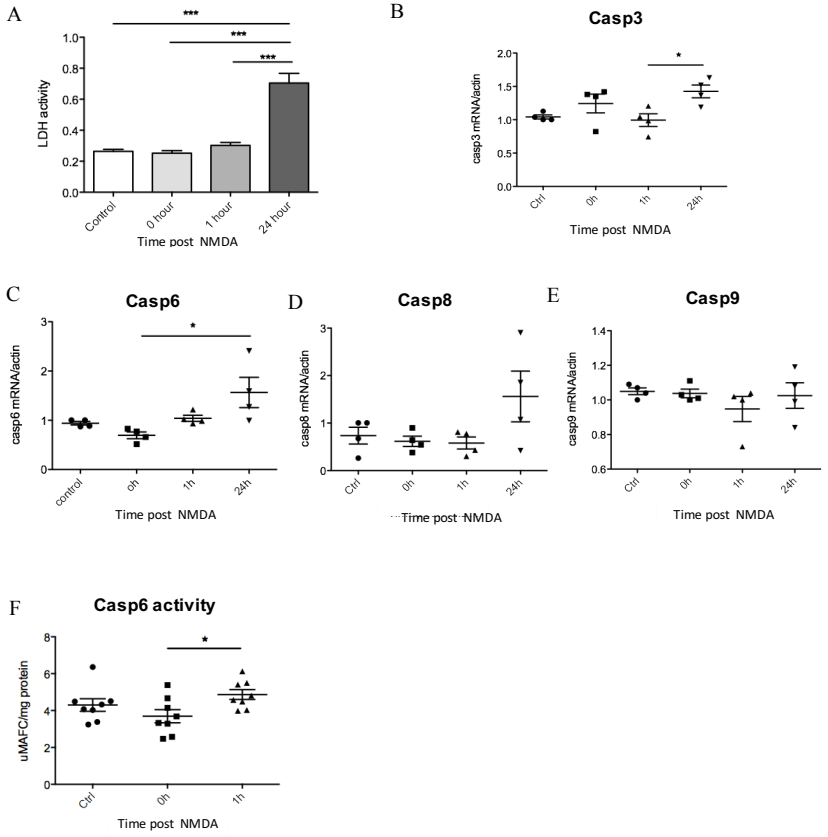


Figure 3-2 NMDA influences casp6 mRNA expression levels and cell death in primary rat cortical neurons

A) In order to confirm our original findings and assess additional time points we assessed caspase profiles at 0.5 and 4hrs post NMDA. Using a well-characterized *in vitro* model of stroke (rat primary cortical neurons ± NMDA) we demonstrate a subtle but significant increase in cell death at 0.5 hr ($p<0.01$) and 4 hr ($p<0.0001$) time point post NMDA (ANOVA $p<0.0001$). **B)** Assessment of caspase mRNA expression levels demonstrates no change in casp3 mRNA levels post NMDA at these early time points. However, **C)** an increase in casp6 mRNA levels is observed post NMDA (ANOVA $p=0.005$, post hoc 0.5hr vs. 4hr $p<0.01$, t-test Ctrl vs. 30mins $p=0.0002$; linear trend $R^2=0.6602$, $p=0.0018$). **D)** A trend increase is observed for casp8 mRNA expression levels (ANOVA $p=0.088$). **E)** There is no change in casp9 mRNA levels. **F)** Correlating with the increase in casp6 mRNA levels, an increase in casp6 activity is observed post NMDA (linear trend $R^2=0.1825$, $p=0.04$; t-test Ctrl vs. 4h $p=0.06$).

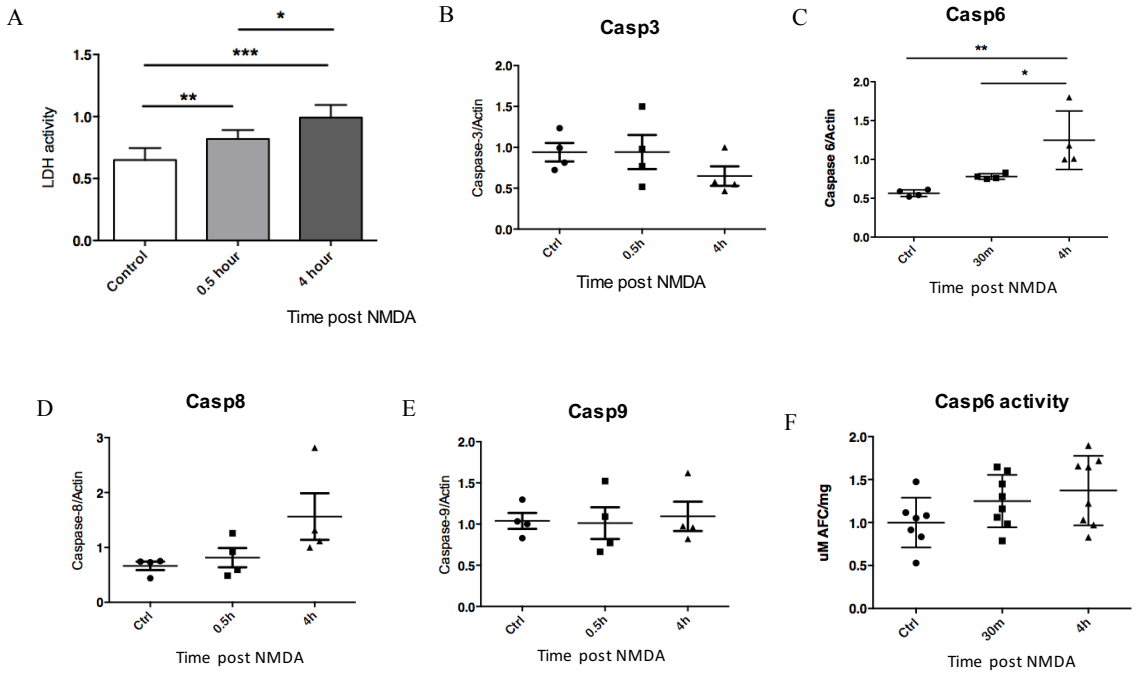


Figure 3-3 Alterations in caspase protein expression levels post NMDA in rat cortical neurons.

Western blots were performed on cortical cell lysates 0.5 and 4hrs post NMDA to assess cleavage of various caspases. Significant decrease in casp3 proform (n=4-8 cultures) **(A B)** is seen 4hours after NMDA (ANOVA p=0.009). A similar decrease is seen in casp6 proform **(C, D)** (ANOVA p=0.008, linear trend R^2=0.1694, p=0.03) and casp8 proform **(E, F)** (ANOVA p=0.02, Linear Trend R^2=0.3881, p=0.007) at 4hours post NMDA. A decrease in casp9 proform expression **(G, H)** was seen 30min post NMDA that persisted to the 4hr time point. (ANOVA P<0.0001, Linear Trend R^2=0.8369, p<0.0001).

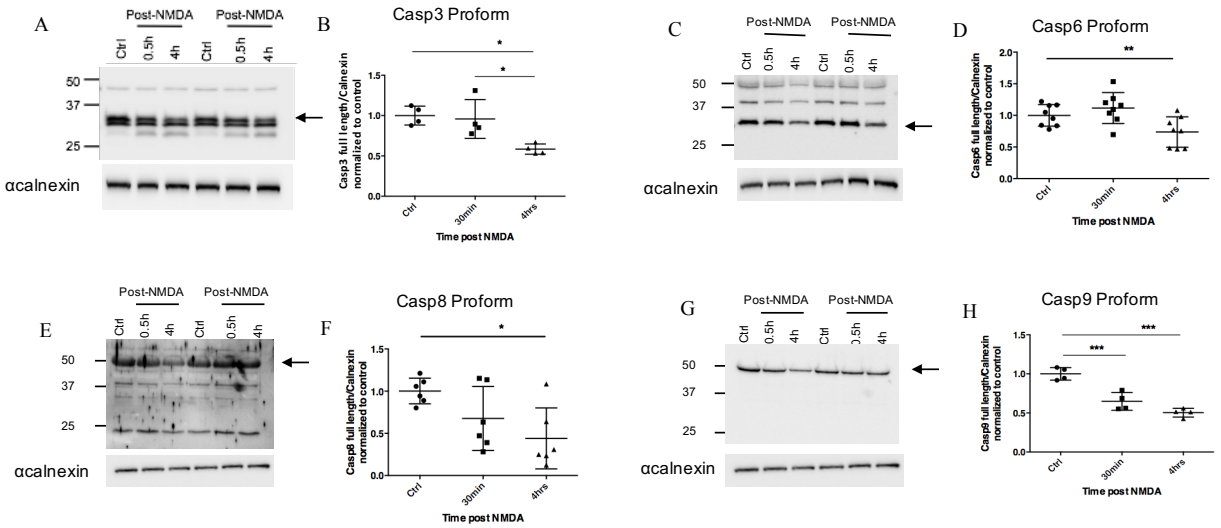


Figure 3-4 Excitotoxic stress influences casp6 substrate expression levels.

A) A significant increase in HTT mRNA expression levels is observed post NMDA in rat cortical neurons both at earlier 30min and 4hour post NMDA (ANOVA p=0.03, linear trend R^2=.4129 p=0.02) and **B)** 24hr post NMDA time point (ANOVA p=0.008, linear trend R^2=0.3713, p=0.016). **C)** A significant linear trend was detected between casp6 and HTT mRNA levels after NMDA (linear trend R^2=0.8657, p=0.007). **D,E)** Despite overall increases in mRNA expression of HTT, decreases in full-length HTT was detected 0min and 4hrs post NMDA (ANOVA p=0.008 linear trend R^2=0.5845, p=0.003) suggesting cleavage by caspases. HTT=Huntingtin, C6=casp6, Ctrl=control, h=hour. (n=4 cultures)

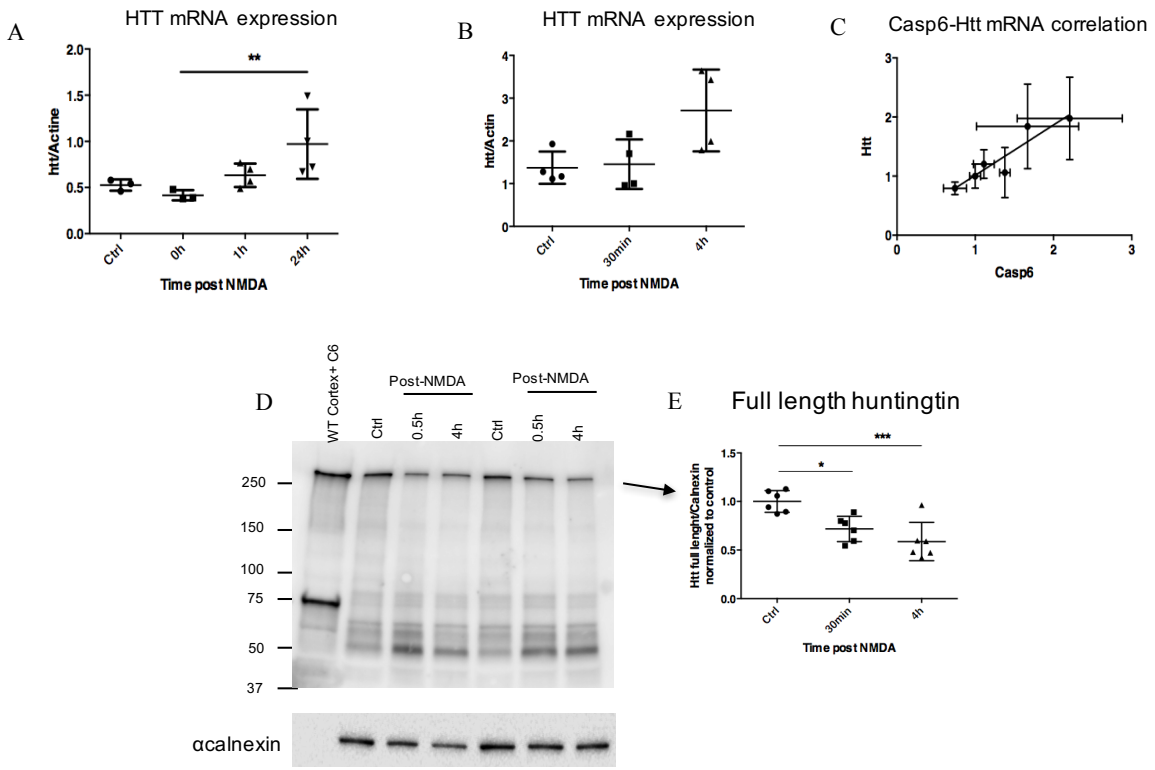


Figure 3-5 Alterations in casp6 substrate expression levels post-NMDA in rat cortical neurons

(n=6 cultures) **A,B)** Using western blot on cell lysates collected post NMDA, very significant decreases in expression of full-length STK3, a newly discovered casp6 substrate, were observed 30min and 4hours post NMDA (ANOVA $p<0.001$ linear trend $R^2=0.6728$, $p<0.001$). **C, D)** Similarly, significant decreases in protein levels of the casp6 substrate phospho-DAXX were detected, becoming significant by 4hrs post NMDA (ANOVA $p=0.0053$, linear trend $R^2=0.5029$, $p<0.01$).

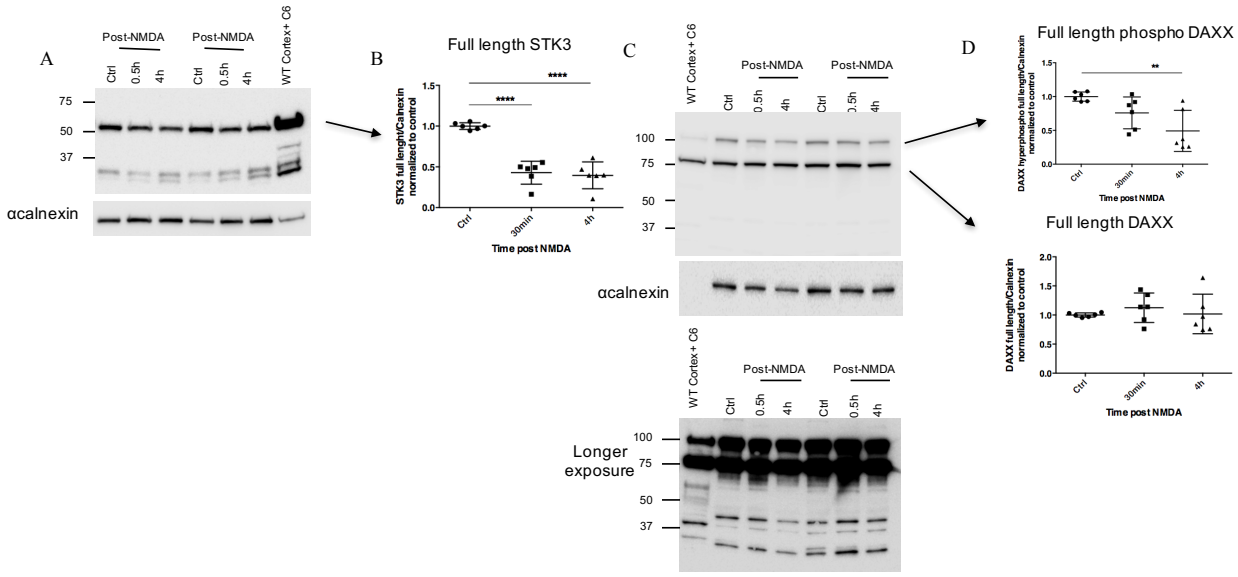
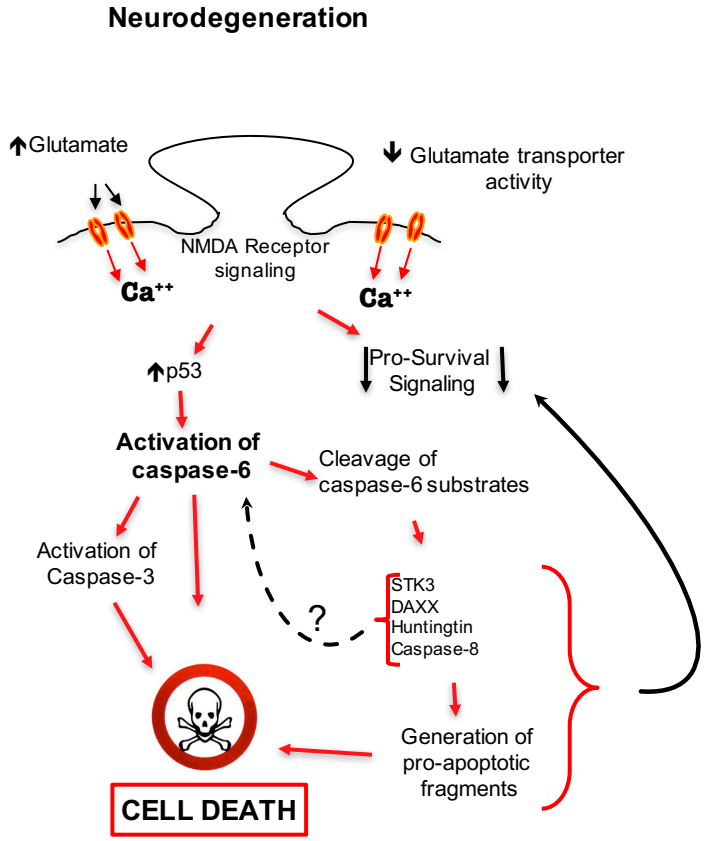


Figure 3-6 Proposed signaling pathway leading to activation of caspase-6 and substrate cleavage in the pathogenesis of neurodegenerative diseases.

NMDA receptor signaling leads to increased intracellular calcium levels, induction of p53, casp6 activation and cleavage of casp6 substrates. Cleavage of NF-κß is observed in stroke, generating a transcriptionally inactive p65 molecule that acts as a dominant-negative inhibitor of NF-κß and promotes apoptosis. These studies suggest cleavage of other casp6 substrates may contribute to the pathogenic process in stroke and other neurodegenerative disorders. Using an *in vitro* model of stroke we show that decreases in the full-length form of the casp6 substrates DAXX, STK3 and huntingtin occurs early during excitotoxicity. The cleavage fragments generated may contribute to the toxic cascade and lower the threshold for apoptosis to occur.



Chapter 4: Enhancing Degradation of Mutant Huntingtin Protein Using Small TAT-linked Peptides as a Potential Therapeutic Strategy for Huntington's Disease

4.1 Introduction

Huntington's disease (HD) is a late-onset hereditary neurodegenerative disease that affects ~5-10 per 10,000 people in the Western Hemisphere (Albin & Tagle 1995; Tan et al. 1976; Wilson et al. 1987; Harper & Bates 2002). HD patients experience cognitive and behavioral symptoms, significant motor impairments and suffer significant brain cell and tissue loss, primarily in the striatum, later spreading to broader brain areas such as the cortex, white matter, substantia nigra, thalamus, hippocampus and cerebellum (Nana et al. 2014; de la Monte et al. 1988; Spargo et al. 1993a; Kassubek et al. 2005; Rosas et al. 2003; Rüb et al. 2013). HD is caused by a genetic, autosomal-dominant CAG expansion mutation in the huntingtin gene, leading to expression of a mutated huntingtin protein mHTT (HD collaborative & research group 1993). Despite the ability to test for HD, there is, at present, a lack of effective early, preventative therapies for HD. The presence of mHTT has shown to cause a wide range of downstream cellular dysfunctions, discussed in detail in section 1.3. Based on this, many attempts to develop therapeutics for HD have focused on mediating these downstream changes, some with promising effects (see section 1.5). However, at the core of HD pathology is the mHTT mutation, and thus, a better therapeutic strategy for HD may, be to reduce or prevent the presence of mHTT.

Several research groups have worked actively on lowering mHTT in HD (see section 1.5.1), with various strategies, including targeting the huntingtin transcript using vector-based

RNA interference (RNAi) molecules (Franich et al. 2008; Boudreau et al. 2009; DiFiglia et al. 2007; S. Q. Harper et al. 2005) or non-vector dependent antisense oligonucleotides (ASOs)(Boado et al. 2000; Nellemann et al. 2000; Kordasiewicz et al. 2012), or by increasing mHTT degradation by enhancing cellular degradation of mHTT (Ravikumar et al. 2004; Sarkar & Rubinsztein 2008; Sarkar et al. 2007; Sarkar 2013; Bauer et al. 2010; Wong et al. 2008). These studies have proven exciting potential for therapeutic lowering of mHTT, showing improvements in neuropathological and behavioral changes in animal models of HD. Interestingly, in many of these studies, levels of mHTT and HTT were both lowered, and despite an embryonic lethality of HTT/mHTT knockout during development (Duyao et al. 1995; Nasir et al. 1995; Zeitlin et al. 1995) and important roles in cell survival, later in life, lowering levels of both proteins appears to be tolerated (Boudreau et al. 2009; Kordasiewicz et al. 2012) However, much evidence demonstrates that the wild type huntingtin protein (HTT) may play a role in normal cell function, with important roles in brain development (Leavitt et al. 2001; Van Raamsdonk, et al. 2005; Nasir et al. 1995; Zeitlin et al. 1995) vesicular transport (Gauthier et al. 2004; Imarisio et al. 2008) and cell survival (Zuccato et al. 2001; Leavitt et al. 2006; Van Raamsdonk et al. 2006; Leavitt et al. 2001; Van Raamsdonk et al. 2005; Rigamonti et al. 2000). Thus, strategies to lower mHTT should, ideally, be allele-specific, reducing mHTT without impacting levels of WT HTT. Several studies have achieved this using RNAi or ASO treatments that target single nucleotide polymorphisms specific to mHTT vs HTT, or by targeting the CAG expansion (Yu et al. 2012; Skotte et al. 2014; Ostergaard et al. 2013; Southwell et al. 2014; Hu, Matsui & Corey 2009; Lombardi et al. 2009; Carroll et al. 2011b). These strategies have also shown great therapeutic potential, with HD ASOs now moving into clinical testing.

mHTT lowering strategies have great clinical potential and could change the way we treat HD dramatically. However, they still have their challenges. For one, mHTT lowering strategies, particularly transcript targeting, have long-lasting, non-reversible effects that extend sometimes beyond a few months. If the strategy works, this is a great benefit, however with many RNAis showing off-target effects (Denovan-Wright et al. 2008), and lots of variation from patient to patient, this could be problematic if unexpected side effects occur. Similarly, many of these therapies disseminate poorly through the body, meaning they require invasive delivery directly to spinal cord or brain, likely multiple times for long term treatment, and also don't address the potential non cell-autonomous effects of HD (Zielonka et al. 2014; Koroshetz et al. 1997; Chen et al. 2007). Thus, a better therapy targeting allele-specific silencing of mHTT would also be reversible, be more easily clinically deliverable, and would disseminate widely through the body.

4.1.1 Peptide-mediated protein knockdown

The need for better, more clinically applicable protein knockdown strategies is not limited to HD. Many proteins are caused by mutated proteins, and developing ways to clear them represents a major challenge in neurodegenerative disease research. To address this, our lab recently developed a novel technique for rapid and reversible, peptide-mediated knockdown of proteins by making use of specific binding sites on target proteins and hijacking endogenous cellular degradation machinery. Our small peptides are made up of three distinct regions. The first region is a cell-penetrating sequence (specifically TAT, in my experiments), allowing the peptide to cross the plasma membrane easily (Schwarze 1999). The second sequence is a binding sequence, designed, based on analysis of protein binding partners, to bind specifically to the protein targeted for degradation. The third sequence is a degradation sequence, that flags the bound protein and peptide

and targets it for degradation, either through recognition by chaperone proteins and subsequent degradation via the lysosome, or via a degron sequence that targets the flagged protein/peptide complex for degradation via the ubiquitin-proteasome system (UPS). This technique has recently been used in our lab to degrade several small proteins, including PSD-95, DAPK1 and alpha-synuclein(Fan et al. 2014), both *in vitro* using cultured cells, and *in vivo*. In these exciting studies, our peptides provide substantial knockdown of the target proteins within hours of delivery. Results were sustained for several hours, and reversed within 24 hours. The peptides did not lead to off-target knockdown of other proteins, including binding partners of the target protein, or proteins with similar sequences. Excitingly, the peptides, when delivered systemically, provided dramatic protein knockdown with therapeutic benefits in a mouse model of stroke(Fan et al. 2014), as well as in a mouse model of Parkinson's Disease (Jin et al. 2016).

Based on these exciting findings, my studies in chapter 4 aim to extend upon this technique and design small peptides to target mHTT, but not HTT for degradation in a rapid and reversible way.

4.1.2 Degradation pathways activated by peptides

As discussed in detail in section 1.3.7, mHTT (and HTT) are both degraded naturally by the UPS as well as via lysosomal degradation. The UPS, generally targeting small proteins for degradation, works via attachment of poly-ubiquitin chains to a target protein, that get recognized by the proteasome. The target protein is fed into the proteasome where it is unfolded and broken down by enzymes, then released as small fragments and amino acids to be recycled by the cell(Schipper-Krom et al. 2012b). The UPS is actively utilized in mHTT clearance, and in particular has been shown to be an important player in clearance of toxic mHTT fragments in HD (Schipper-Krom et

al. 2012b) (DiFiglia 1997; Davies et al. 1997; Jana et al. 2001; Kalchman et al. 1996). However, the UPS has shown in several studies to be impaired in HD(Jana et al. 2001; Lehrach et al. 2001; Waelter et al. 2001) and thus, enhancing mHTT degradation via the UPS with peptides may be able to provide some therapeutic benefit. In the current study, we use a –RRRG degron sequence as our UPS-targeting degradation sequence for mHTT-degron peptides (Bonger et al. 2011), shown to target proteins for proteasomal degradation, and previously used in our lab to target alpha-synuclein for degradation both *in vitro* and *in vivo* (Jin et al. 2016).

The second degradation system we aim to harness to degrade mHTT using peptides in our lab is the lysosomal autophagy system, and in particular, chaperone mediate autophagy. mHTT (and HTT) have both been shown previously to be degraded via the lysosome (reviewed in(Martin et al. 2015)). In autophagy, targeted proteins are engulfed by autophagosomes and transported to the lysosome, where the autophagosome fuses and proteins are degraded inside the lysosome(Hayashi-Nishino et al. 2010; Hailey et al. 2010; Ravikumar et al. 2010. Like the UPS, autophagy is impaired in HD, and previous studies have demonstrated that enhancing autophagy in models of HD increased mHTT clearance and provides therapeutic benefit, both at the cellular and behavioral level(Sarkar & Rubinsztein 2008; Ravikumar & Rubinsztein 2006). Another similar form of protein degradation is chaperone mediated autophagy (CMA), a more specific and selective form of autophagy (Kaushik & Cuervo 2012). In CMA, proteins targeted for degradation are flagged with a KFERQ or KFERQ-like motif that is recognized by chaperone proteins HSC70, along with other chaperones/co-chaperones, then transported to the lysosome, where they bind with receptor Lamp2A (Cuervo & Dice 1996). At the lysosome, the targeted proteins are transported across the lysosome membrane and then degraded (Cuervo & Dice 1996; Kaushik & Cuervo 2012). CMA has been suggested to play a role in the degradation of mHTT and mHTT

fragments(Qi & Zhang 2014; Bauer et al. 2010; Cuervo & Wong 2014) as well as mHTT-interacting proteins(Qi & Zhang 2014) and in fact, it has been suggested that CMA upregulation may be a compensatory mechanism in HD to make up for losses in cellular degradation machinery and help aid in mHTT clearance(Koga et al. 2011), suggesting that CMA may be a viable therapeutic target for mHTT lowering. Also, a recent study used viral vector-mediated small fusion molecules to bind to mHTT and demonstrated that flagging mHTT with KFERQ-like motifs could enhance CMA-dependent degradation of mHTT (Bauer et al. 2010). Intrastriatal delivery of these vectors improved the HD phenotype in R6/2 mice. In my experiments, I designed mHTT-CMA peptides with a similar KFERQ-like motif as our degradation sequence to target mHTT CMA-mediated lysosomal degradation, in a rapid, reversible, peptide mediated way. A schematic overview of the design for peptide-mediated knockdown using mHTT-degradation peptides can be see in **Fig 4-1.** Specifics of peptide design are discussed in results section 4.3.1.

4.1.3 Rationale and Hypothesis

A potentially viable therapeutic strategy for treating HD is the systemic lowering of the mHTT protein, however, current approaches have clinical limitations. Previously in our lab, we successfully lowered the levels of several proteins both rapidly and reversibly in cell culture, as well as in animal models using small peptides targeting proteasomal or CMA-mediated lysosomal degradation machinery. Both the UPS and the CMA lysosomal degradation systems have been shown to play a role in mHTT degradation, and enhancing both of these pathways has been suggested to lower mHTT levels and lead to improvements in HD phenotype in animal models. Based on these data, I hypothesize that **small peptides designed to bind specifically to mHTT over HTT can successfully lower mHTT protein levels in a mouse model of HD via**

proteasomal/lysosomal degradation. If successful, these peptides could provide a novel avenue for mHTT lowering that is rapid, reversible and clinically applicable, that also distributes widely thorough the body.

4.2 Materials and methods

4.2.1 Antibodies and reagents

Mouse anti-huntingtin (#MAB 2166) was ordered from Millipore (Billerica, MA, USA). (1:1000 in western blot, 1:100 in immunostaining). Mouse monoclonal anti-Vinculin (V9131) was from Sigma (St Louis, Missouri, USA) (1:2000 western blot). HSP90 (#610418) was obtained from BD Transduction labs (San Jose, CA) (1:1000 western blot). DAPI nucleic acid stain (#D9542) was ordered from Sigma Aldrich (St. Louis, Missouri). Secondary antibodies for immunostaining were the AlexaFluor series and were ordered from Thermo Fischer Molecular Probes (Portland, OR). Secondary antibodies for western blot were HRP-tagged secondary series from Perkin-Elmer. Mounting media Fluoromount-G (0100-01) was bought from Southern Biotech (Birmingham, AL). Acrylamide and Bis reagents for making gels were purchased from Bio-Rad Complete Protease Inhibitor Cocktail Tablets (#04693116001) and Phosphatase Inhibitor Cocktail Tablets (04906845001) were obtained from Roche Applied Science (LSC).Cell Toxicity Colormetric Assay Kit (TOX#1) was purchased from Sigma-Aldrich (St. Louis, Missouri). Ammonium Chloride was purchased from sigma Aldrich. Tat-fused peptides were synthesized in the Yu Tian Wang lab at the Brain Research Centre, UBC, Peptide synthesis facility. Sample buffer for low-bis westerns was from BioRad (4x Sample buffer).

4.2.2 Buffers

All buffers were made in the laboratory and were sterile filtered before use using vacuum or syringe filtration systems (Corning, Polyethersulfone with 0.22 µm pore size). Running buffer (5x) for Low Bis Western blots contained 250mM Tris, 1.9M Glycine and 1% SDS. 20x Transfer buffer contained 25mM BICINE, 25mM BIS TRIS and 1mM EDTA. Low Bis gels for allelic separation of mHTT and HTT contained 10% Acrylamide, .05% Bis, .375M 1.5M Tris (pH 8.8), .075% TEMED and 0.75% APS for resolving gels. Stacking gels contained 30% acrylamide, 0.5M Tris (pH 6.8), 0.075% TEMED and 0.075% APS. Lysis buffer for both cultured neurons and mouse brain tissue was SDP lysis buffer, optimized for allelic separation (recipe from Dr. Michael Hayden's lab) (50mM Tris pH 8.0, 150mM NaCl, 1% Igepal, 40mM B-glycerophosphate, 10mM NaF, 1x protease inhibitor cocktail (Roche), 1mM sodium orthovanadate and 800mM PMSF).

4.2.3 Primary embryonic mouse neuron culture

See section 2.2.6

4.2.4 Peptide-mediated knockdown of mHTT *in vitro*

Cultured neurons were treated with various doses of mHTT-CMA or mHTT-degron peptides in the same volume of culture media, then left to recover in the incubator for various time-points to allow for peptide-mediated knockdown. At determined time points, neurons were removed from the incubator and either lysed with SDP buffer (including rocking on ice for a few minutes, then scraping down) or fixed with 4% PFA for immunostaining. In NH_4Cl lysosome inhibitor experiments, neurons were co-treated at the same time as peptide application with NH_4Cl, dissolved in water (20mM).

4.2.5 Western blots

4.2.5.1 Non-low bis

Early experiments in this study utilized our lab's standard western blot protocol, outlined in 2.2.12.

4.2.5.2 Low bis for improved allelic separation of mHTT/HTT

Subsequent experiments utilized Dr. Michael Hayden's lab protocol for better allelic separation of mHTT and HTT using western, which requires careful gel preparation. In this protocol, all gel reagents (excluding TEMED and APS) were combined and brought to room temperature for 15 minutes. Then, resolving gel solution was degassed for 15 minutes by vacuum suction before quickly adding APS and TEMED and pouring. Gels were layered with ethanol and set for at least 30-40 minutes. After this, ethanol was washed off and APS & TEMED were added to stacking gel solution and poured onto the resolving gel, with clean, ethanol-washed combs inserted. Stacking gel was set for at least 2 hours. When running gels, samples were loaded quickly to avoid diffusion, and gels were run 20-25min at 110V, then at 190V for 2-2.5hours. Running buffer in the centre compartment of buffer tanks included 750ul of B-mercaptoethanol per litre of running buffer. Following gel running, gels were washed gently 3 times for 5 min with transfer buffer (rocking) then transferred for 2 hours at 11V onto PVDF membrane. Membranes were blocked for 1hour with 5% milk before antibody application.

4.2.6 Peptide-mediated knockdown of mHTT *in vivo*

3 month old mice (FVB-N or YAC128) were treated with 18mg/kg mHTT-degron or mHTT-CMA peptide (based on Fan et al, 2014). Controls were either control scrambled peptide, or saline vehicle. Peptides/vehicle were delivered either IP (300ul) or intranasally via small drops on the nostril (5ul/nostril). Following peptide delivery, mice were recovered for various times, before sacrifice by urethane overdose IP. Brains were removed as quickly as possible, and cortical and striatal tissue was removed and snap frozen on ice. When running western blots, tissues were lysed using SDP buffer + 0.5% SDS, then lysed using a sonicator for multiple short pulses. Full lysates were run in gels to assess mHTT and HTT protein expression compared to loading control.

4.2.7 LDH assays

See 2.2.10

4.2.8 Immunocytochemistry

See 2.2.14

4.2.9 Tissue perfusion, fixation and immunohistochemistry

See 2.2.17 and 2.2.18

4.2.10 Fluro-Jade B staining

See 2.2.19

4.2.11 Animal models

See 2.2.21

4.2.12 Statistical analyses

All measurements were performed blindly where possible. Results are expressed as a mean and error bars represent standard error of the mean. One-way ANOVA was used for multiple group comparison, followed by post-hoc (Tukey) test where appropriate. Students *t*-tests were used for comparison between two groups. Data lacking normal distribution were analyzed by a nonparametric Rank Sum Test (Mann-Whitney). Statistical significance was defined as $*P < 0.05$, $** P < 0.01$ ***P<0.001 ****P<0.0001.

4.3 Results

4.3.1 Design of TAT-mHTT degradation peptides

In order to design peptides for mHTT degradation, I first needed to find a binding sequence that might show preferential binding to mHTT over HTT, to design peptides that lead to degradation of mHTT but not HTT. To do this, I searched the literature for sequences that preferentially bind mHTT more strongly than WT HTT. Preferential binding was focused on expanded polyQ on mHTT vs HTT. Three sequences were chosen (**Table 1**). The first comes from MW1, a polyQ-binding antibody that binds pathogenic polyQ(Li et al. 2007). The second sequence comes from mHTT binding peptoid, HQP09, originally pulled from a non-biased screen using N-terminal mHTT as bait(Chen et al. 2011) The third sequence is derived from polyglutamine binding protein 1 (QBP1), which was pulled from a combinatorial peptide library screen to find peptides that bind pathologic length polyQ(Nagai et al. 2000) Though the original binding regions sometimes

contained multiple repeats of the binding domain for increased specificity, I started with a single repeat of the binding sequence, for ease of synthesis and general screening. All mHTT peptide sequences are fused to a TAT sequence(Schwarze 1999) and a degradation targeting sequence. Two sets of peptide were synthesized to target mHTT for degradation via the proteasome or the lysosome. Initial peptides to screen binding sequences were UPS-targeting peptides (mHTT-degron peptides). All degron peptides contain a -RRRG degron as the degradation sequence(Bonger et al. 2011), which target its fused protein for degradation via the proteasome. All three putative binding sequences were synthesized and tested using the degron tail, to help determine the best for lowering levels of mHTT. Conversely, mHTT-CMA peptides contained a KFERQ-like motif. This tag signal is a commonly used motif in CMA for recognition by chaperone HSC70, and has been used in our lab using peptides to target the flagged protein complex for degradation via CMA (Fan et al, 2014). Peptides were synthesized in house and purity was examined with MS-HPLC. Control peptides were also made containing the TAT sequence, a scrambled binding sequence and corresponding degradation signal. As initial CMA peptides resulted in some toxicity *in vitro*, a second set of CMA peptide/scrambled control were synthesized with a shortened KFERQ motif. A breakdown of all peptides their amino acid sequences can be found in **Table 2**.

4.3.2 mHTT-degron peptides significantly lower levels of mHTT and HTT in cultured neurons

Initial experiments using mHTT degradation peptides aimed to determine if treatment of cultured cortical and striatal neurons from HD mice would result in significant lowering of mHTT *in vitro*. In our lab previously, treating cultured cells using small protein-targeting peptides for DAPK1,

alpha synuclein, resulted in significant knockdown of target protein within only a few hours(Fan et al. 2014; Jin et al. 2016). Thus, I hypothesized that my small mHTT-targeting peptides could significantly lower levels of mHTT, rapidly following treatment. My initial experiments utilized degron peptides, signaling for mHTT degradation via the ubiquitin proteasome system (UPS). All three putative mHTT-binding sequences (mHTT-DYY-degron and mHTT-WFP-degron) were tested in time-course studies to determine if mHTT degradation peptides resulted in dose-dependent mHTT knockdown (**Fig 4-2).** In these experiments, cultured cortical or striatal neurons from YAC128 HD mice were treated with degron peptide at doses between 1-30μM. Control peptides were a degron peptide containing a scrambled binding sequence, taken from alpha-synuclein(Jin et al. 2016). Cells were treated with peptide into the culture medium, then left to recover for 6 hours, a time point where robust knockdown of other target proteins have been seen in our previous experiments(Fan et al. 2014; Jin et al. 2016). As seen in **Fig 4-2 A and B,** all three mHTT-degron peptides demonstrated dose-dependent knockdown of huntingtin protein, becoming signficant at 10μM. Degron control peptide, though also lowering HTT at high doses, did not impact huntingtin levels at low doses where mHTT/HTT knockdown was apparent, suggesting that the lowering effect is due to mHTT-degron, rather than broad peptide-mediated effects. Although mHTT-degron peptides were designed to bind specifically to mHTT rather than HTT, all three peptides led to similar reductions in HTT in both WT and YAC128 cells, suggesting their mHTT-specificity is low. However, many experiments in mHTT lowering technologies have demonstrated that, despite the negative implications of removing HTT in development, the lowering of both mHTT and HTT in adulthood provides significant therapeutic benefit in HD models, without significant side effects (see section 1.5.1). Thus, I decided to proceed with experiments, despite apparent lack of mHTT specificity. Next, I determined if mHTT/HTT

lowering was specific to cell type. Treating cultured YAC128 striatal neurons with mHTT-degron or controls also demonstrated clear lowering of mHTT/HTT on a timeframe similar to cortical neurons (**Fig 4-2 C)** suggesting that mHTT-degron peptides can effectively penetrate the cell membrane to rapidly decrease levels of HTT/mHTT. It is worthy to note that the SNW-degron peptide, in both cortex and striatal neurons, while lowering huntingtin levels, also led to significant reduction in actin, suggesting a loss of cell integrity or other cell materials. Thus, the SNW-degron peptide was removed from further experiments. Next, I demonstrated time-dependent knockdown of mHTT/HTT. Using cultured cortical or striatal neurons, I used a 25µM dose of mHTT-degron, applied to the culture medium of cells, and lysed cells for western blot at 0, 4, 8 and 24 hours following peptide application. As seen in **Fig 4-2 D-E,** mHTT-degron peptides DYY-degron and WFP-degron led to a clear lowering of mHTT compared to a vehicle control at each time point, increasing over time. Again, no specificity for mHTT over HTT was shown. LDH assays were used to determine cell death from peptide treatment over increasing doses (**Fig 4-2 F**) and over time (**Fig 4-2 G)**. Unfortunately, mHTT-degron peptides caused significant neuron death (shown here in YAC128 cortical neurons), becoming significant at 10uM and at 8hours post treatment. (DYY-degron is shown as a representative figure in Figure 4-2 F, as all peptides showed similar cell death patterns over increasing dose). Cell death with mHTT-degron peptides was significantly higher than a corresponding control at each time point, with a scrambled degron peptide not causing any significant neuron death. This could suggest several things. For one, lowering of both mHTT and HTT using degron peptides could be causing cell dysfunction, given the neuroprotective role of HTT (see section 1.2.3). Also, the proteasome does not normally degrade large proteins like HTT (Schipper-Krom et al. 2012a), and there is evidence of mHTT-dependent proteasome impairment in HD (as discussed in section 1.3.7.1) so it is possible that enhancing

mHTT-degradation via the proteasome may cause proteasome impairment and cell dysfunction. To attempt to combat the increased cell death with mHTT-degron peptides, the next experiments tested whether using a KFERQ-like tag and signaling mHTT for degradation via the CMA lysosomal degradation system could lead to mHTT degradation with less dramatic cell death.

4.3.3 mHTT-CMA peptides significantly lower levels of mHTT and HTT in cultured neurons

The next set of experiments set out to determine whether mHTT targeting peptides utilizing an mHTT degradation sequence and a CMA degradation signal could enhance mHTT degradation *in vitro*. As discussed, the CMA pathway has shown to be upregulated in HD and play a role in the degradation of mHTT and HTT (Qi & Zhang 2014; Bauer et al. 2010; Cuervo & Wong 2014). As all thre putative degradation sequences using degron peptides demonstrated similar knockdown patterns, I chose only one of the mHTT binding sequences to test using CMA peptides. The sequence I chose was the WFP- sequence, isolated from HQP09, as WFP-degron experiments demonstrated slightly better mHTT knockdown and slightly less toxicity in cultured neuron experiments than the DYY-degron peptide. (CMA peptide sequences can be seen in **Table 2**). Experiments to test WFP-CMA *in vitro* were simlar to those used to test mHTT-degron peptides. Cultured cortical or striatal neurons from WT or YAC128 mice were treated with WFP-CMA or a scrambled CMA control (containing the mHTT binding sequence, scrambled). First, dose response experiments were done, collecting cells again at 6 hours following treatment. Samples were run in western blot. To increase allelic separation of mHTT and HTT on the gels, a low bis protocol was used, previously shown to improve resolution between thes proteins of similar weight. Using doses between 1-30μM, I demonstrated that WFP-CMA significantly lowered levels of mHTT in

YAC128 mice (**Fig 4-3 A)**. However, there was also significant lowering of HTT, similar to what was shown with the degron peptides. Scrambled control was not as dramatic, suggesting that knockdown may be specific for mHTT and not a non-specific effect of CMA peptides. Interestingly, in WT neurons, which do not contain mHTT, though there was a clear trend towards lowered levels of HTT, knockdown of HTT was not as dramatic with WFP-CMA treatment over increasing doses, suggesting there may be some specificitiy for mHTT over WT HTT with my CMA targeting peptide.

4.3.4 mHTT-CMA degradation occurs, at least in part, via the lysosome

Next, I wanted to determine if WFP-CMA mHTT targeting peptide's effects on mHTT and HTT expression *in* vitro were, in fact, due to lysosomal degradation. To test this, at the time of peptide simulation, I treated cultured cortical neurons from WT or YAC128 mice with 20mM NH_4Cl, shown to inhibit lysosomal degradation (Fan et al, 2014.). Results can be seen in **Fig 4-3 C, D.** As shown previously, in YAC128 cells, WFP-CMA led to significant reduction in both mHTT and WT HTT over increasing dose of WFP-CMA. Although there was an apparent decrease in baseline mHTT/HTT expression with NH_4Cl treatment, the lysosome inhibitor partially reversed mHTT and HTT knockdown. This was especially apparent when accounting for NH_4Cl-mediated lowering of baseline protein. Comparing WFP-CMA + NH_4Cl treatments over increasing doses to control cells treated with NH_4Cl shows no significant knockdown with WFP-CMA in YAC128, suggesting that mHTT/HTT lowering is, at least in part, due to lysosomal degradation. Similar effects were seen in WT neurons. Although HTT is lowered slightly by WFP-CMA in non-NH_4Cl conditions, this effect is totally reversed with treating cells with lysosome inhibitor (**Fig 4-3 C**). To further these experiments, LDH assays were conducted to assess cell death following peptide

treatments, with and without controls (**Fig 4-3 D)**. As seen in mHTT-degron experiments, WFP-CMA led to significant increase in cell death with increasing dose, becoming significant at 10μM This effect was not as dramatic with scrambled CMA controls. Interestingly, treatment of cells with NH_4Cl reversed cell death following WFP-CMA treatment. Similar to experiments using mHTT degron, this may suggest that the cell death observed using mHTT degradation peptides occurs due to the negative effects of both mHTT and HTT loss, or could also result as a consequence of overloading the endogenous degradation machinery.

4.3.5 mHTT-degradation peptides provide only small knockdown *in vivo*

Although both my mHTT-degron and mHTT-CMA peptides caused significant cell death *in vitro*, I did demonstrate significant lowering of mHTT (and HTT) that appears to, at least in part, occur due to activation of cellular degradation machinery and clearance of huntingtin proteins. Thus, I decided to test the peptides in their ability to lower mHTT (or HTT) in the brains of intact HD mice. I wondered if, perhaps, the cell death impairments I observed *in vitro* may be a result of fragile cellular machinery in cell culture, which don't always perfect represent how drugs work in living animals. Initial experiments *in vivo* tested the peptides using 20mg/kg IP injection into 3-month-old YAC128 mice, a dose similar to previous peptide-mediated knockdown studies in our lab(Fan et al, 2014; Jin et al, 2016). Animals were injected once with 300ul peptide (mHTT-degron or WFP-CMA or a degron scrambled control) or saline control and recovered for 6-8hours, a time point based on previous studies in our lab. IP injections demonstrated that there was no significant reduction in either mHTT or HTT in either cortex or striatum in YAC128 mice (**Fig 4-4 A, B).** Though there was a trend to increased mHTT with IP WFP-CMA, knockdown was not significant. To try and improve this outcome, I repeated these experiments using WFP-CMA and WFP-degron

in YAC128 mice, but used intranasal peptide delivery of 20mg/kg peptide or saline vehicle. Intranasal peptide delivery of knockdown peptide in our lab has shown to increase target protein knockdown in the brains of mice (Jin et al, 2016). However, in my experiments, intranasal delivery of WFP-degron or WFP-CMA did not lead to any significant knockdown of WT HTT or mHTT in YAC128 cortex or striatum (**Fig 4-4 C, D)**. These experiments were further confirmed in FVB WT mice. Intranasal delivery of WFP-CMA, compared to saline control, showed that 6-8 hours following peptide delivery, no knockdown of HTT could be detected, with even a trend towards increased HTT expression with peptide treatment.

Lastly, although my time point for mHTT degradation peptide delivery was determined based on past experiments, it is possible that the 6-8 hour time point of peptide delivery missed the critical time point for mHTT knockdown. To test this, I conducted time course studies in YAC128 mice to determine if there is a time point following mHTT degradation peptide treatment where mHTT is lowered *in vivo*. 3month old YAC128 mice were given 20mg/kg mHTT-CMA or a scrambled control (or saline vehicle) and brains were collected for protein analysis at various time points following peptide delivery, between 3-24 hours (**Fig 4-4 G, H)**. However, timecourse experiments showed that, even over a wide range of time points, no significant knockdown of mHTT could be detected at any time point following peptide treatment with WFP-CMA or WFP-CMA-scr suggesting that the peptides do not effectively lower mHTT or HTT *in vivo.*

4.4 Discussion

In Chapter 4, I attempted to expand on a new technology in our lab for rapid and reversible peptide-mediated protein knockdown, developed by Fan et al, in 2014. This technique utilizes a three-part, short synthetic peptide that can 1) cross the plasma membrane after systemic delivery, due to a

cell-penetrating TAT sequence, 2) bind to a specific target protein that we want to degrade, through specific binding sequences and 3) flag that target protein for degradation via proteasome of lysosome (CMA) degradation. Given the promising successes of novel peptides that target DAPK1, PSD-95 and α-synuclein (Fan et al., 2015, Jin et al., 2016) both rapidly, a few hours after application both *in vitro* and *in vivo*, specifically, and with benefit against neurodegenerative disease, I hypothesized that, by harnessing the same technique, and utilizing previously discovered mHTT-preferential binding sequences, I could design degradation peptides that flagged mHTT for degradation in a rapid and reversible way. Based on previous data indicating a therapeutic benefit of mHTT lowering in many animal models of HD, I hypothesized that, if these peptides successfully lowered mHTT in YAC128 HD cells, that they might pave the way for novel mHTT lowering therapeutics that are more clinically deliverable, easier to control, and more widely distributed through the body than current mHTT knockdown techniques.

My initial experiments tested both mHTT-degron and mHTT-CMA degradation peptides in cultured neurons from WT and YAC128 mice. As predicted, mHTT-degron and –CMA peptides specific for mHTT demonstrated dose- and time-dependent reduction in mHTT in HD neurons. However, despite designing peptides to be specific for mHTT over HTT, I was surprised to discover that all peptides led to significant reductions in WT HTT as well, in both YAC128 and WT neurons. Although this was disappointing, in terms of clinical relevance, this may not be completely detrimental. Although many studies have demonstrated a neuroprotective role of HTT in cell survival and normal cell function (see section 1.2.3) and the evidence that removal of WT HTT during embryonic development leads to serious disability or death(Zeitlin et al. 1995; Nasir et al. 1995; Duyao et al. 1995), many studies investigating huntingtin lowering using RNAi strategies or general enhancement of protein degradation machinery lower levels of both mHTT

and HTT, with neuropathological and behavioral improvement, without significant detectable negative impact (DiFiglia et al. 2007; Wang et al. 2005; Rodriguez-Lebron et al. 2005; Boudreau et al. 2009; Machida et al. 2006; Drouet et al. 2009; Harper et al. 2005) (Stiles et al. 2012). This suggests that, post-development, non-allele specific huntingitn silencing, though not an ideal therapy, is relatively well tolerated. Interestingly, though very dramatic lowering of WT HTT was seen for both mHTT-degron and mHTT-CMA peptides in YAC128 neurons, loss of WT HTT in non-HD FVB-N WT mice, particularly, in experiments using WFP-CMA peptide, was always slightly less dramatic than in YAC128. This was surprising, given that both YAC128 and FVB mice express similar levels of endogenous, mouse HTT protein. It is possible that the presence of mHTT in YAC128 mice makes them more vulnerable to protein degradation, given previous experiments demonstrating upregulation of protein degradation machinery, particularly CMA, in HD(Koga et al. 2011), as a potential compensatory mechanism for impaired protein degradation. If peptides are not especially selective for mHTT over HTT, this could explain more dramatic HTT lowering in HD vs non-HD neurons. Also, previous studies have demonstrated that cultured HD neurons are more sensitive to cell death compared to WT neurons(Zeron et al, 2009). Given some toxicity of mTTT degradation peptides *in vitro*, it is possible that the small difference in HTT protein expression in YAC128 vs WT cells results due to slight differences in sensitivity to cell death following peptide treatment. In subsequent studies, several modifications to mHTT-degradation peptides may help to increase specificity. For one, only a single repeat of the mHTT-binding domain was used in my studies, for ease of peptide synthesis. Repeating the binding sequence my help increase specificity for expanded polyQ mHTT over WT HTT, especially for larger sequences. Secondly, my peptides worked by targeting the expanded polyQ region. Because both HTT and mHTT contain a polyQ, it is possible that the specificity isn't maximized. By

redesigning the peptide to bind to different mHTT characteristics, such as targeting morphological changes of the mutated protein compared to the WT protein, due to misfolding or aggregation, or targeting SNPs, better mHTT specificity might be reached and might also help to target pathogenic misfolded mHTT.

In my *in vitro* characterization of degradation peptides for UPS and CMA degradation, I demonstrated that the lowering of mHTT appears to be specific to the degradation peptides and not due to non-specific effects. In mHTT-degron peptide expeirments, signaling for mHTT proteasomal degradation, a scrambled control peptide of similar weight and size, containing the same degron flag and TAT sequence, but designed not to bind to huntingtin, showed no significant impact on mHTT/HTT expression at early doses where mHTT/HTT was significantly lowered. Similarly, using lysosome inhibitors during WFP-CMA mHTT degradation peptide treatment, it was shown that, though NH_4Cl alone led to some loss of mHTT/HTT, comparing WFP-CMA + NH_4Cl treatment conditions to Control + NH_4Cl conditions indicated that lysosome inhibitor wipes out the dose-dependent lowering of mHTT. Similarly, treatment of cells with NH_4Cl reduces WFP-CMA-dependent cell death, suggesting, first, that enhancing degradation of mHTT/HTT may cause some cellular stress and that, NH_4Cl treatment may both inhibit lysosome activity and slow cell death.

Along with this, it is important to discuss the fact that all mHTT-degron and –CMA peptides targeting mHTT/HTT degradation were associated with significant dose- and time-dependent cell death, as measured using LDH assay. This cell death was on a similar time and dose frame as mHTT/HTT knockdown in all conditions. Despite the detected peptide-specific mHTT and HTT lowering in my initial experiments, that are, at least in part, occuring via their targeted degradation pathways, it is possible that some of the reduction in mHTT/HTT observed

in vitro are due to cell death. However, no significant loss in loading control protein was seen, suggesting that cell integrity is not completely lost. It is possible that increasing mHTT or HTT degradation using degron or CMA peptides is, in itself, conducive to increasing cell death in this system. For one, many previous studies have indicated impairments in both the UPS proteasomal degradation machinery and lysosomal degradation in HD (discussed in section 1.3.7 and 4.1). Both mHTT and HTT are very large proteins, and both highly expressed in the cell types studied. By increasing the degradation load for the proteasome and/or lysosome using our peptides, without also increasing the function of these pathways, it is possible that my peptides may overload the endogenous degradation pathways, clogging and slowing an already overloaded system in HD. This could, in principle, not only impair mHTT or HTT degradation, but also impair the normal degradation of other important proteins. This is supported by evidence that NH_4Cl treatment rescues some of the cell death observed. Also, since mHTT and HTT are so large, it is possible that proteasome or lysosome degradation of these massive targets cannot fully complete, leading to fragments of mHTT (or HTT) which may actually increase cellular toxicity, similar to mHTT N-terminal fragment. Similarly, although the majority of our protein-targeting peptides do not demonstrate significant off-target effects, it is possible that my peptides are binding to non-specific targets, which could cause aberrant protein degradation, or interfere with normal signaling pathways. Subsequent experiments using these pathways will have to investigate these possibilites if these peptides will be used therapeutically. Another future important experiment that would be essential to test these peptides is their ability to reduce neuropathological changes in HD neurons compared to WT neurons (including the several factors discussed in section 1.3, mechanisms of HD neuropathology). Initial experiments were attempted to determine if mHTT degradation peptides could rescue morphological cell arbour changes seen in HD MSNs compared to WT

MSNs, however, cell death caused by long term peptide treatment rendered these experiments difficult. Future studies with less toxic knockdown will be utilized to test the ability of mHTT lowering to rescue cellular deficit in HD.

To address the toxicity in mHTT-CMA peptides specifically, in my studies, I wondered if shortening the CMA degradation tail might help reduce cell death. The current KFERQ-like tail on my peptides, based on Fan et al, 2014, contains three KFERQ-like domains, and it is possible that one is sufficient, or that the long tail is causing toxiciy. To test this, I synthesized TAT-WFP-CMAT, which is the same peptide as WFP-CMA, but with a shortened KFERQ tail (see **Table 2)**. A simple experiment in cultured YAC128 cortical neurons demonstrated that, indeed, changing the tail of CMA peptides to a shorter sequence reduces cell death (**Fig 4-5 A)**, suggesting that the CMA tail is not, in itself, toxic, however, WFP-CMAT peptide, nor it's scrambled control could significantly lower levels of mHTT or HTT, compared to the full-length WFP-CMA (**Fig 4-5 B)**. These experiments demonstrate that further experiments using mHTT degradation peptides may need to rework the structure of the protein itself, manipulating the degradation sequence to reduce toxicity, while also allowing degradation to occur.

Lastly, I tested my mHTT degradation peptides *in vivo*, using both intraperitoneal and intranasal delivery methods. These methods have successfully delivered protein degradation targeting peptides to the brains of mice in previous studies in our lab(Fan et al, 2014; Jin et al, 2016), shown both through demonstrated therapeutic and *in vivo* knockdow effects of peptides in living animals, and also by mass spectrometer analysis of peptide sequences in the brains of rats (data not published). In my experiments, I used a dose of peptide that has been used in all of our previous *in vivo* peptide experiments (Fan et al, 2014; Jin et al, 2016) so I felt confident that the peptides could successfully reach the brain. However, despite signficant knockdown *in vitro,* none

of my mHTT degradation peptides demonstrated significant lowering of mHTT or HTT *in vivo,* even over time. This could be due to several reasons. First, compared to the small proteins targeting in our previous work, mHTT and HTT are large, highly expressed proteins. It is possible that, under current conditions, targeting for degradation of such large proteins via endogenous machinery that is already hard at work in the brain is not sufficent to break down these massive targets. Subsequent experiments could try supplementing peptide treatment with compounds that enhance degradation machinery to test if mHTT/HTT can be enhanced. Also, though our previous peptides seem to get into the brain effectively, it is possible that these peptides do not work as efficiently. Also, since all degradation peptides contain degradation tags, for UPS or CMA peptides, they are, by design, going to be degraded by the proteasome or the lysosome. It is possible that this may occur very quickly, with peptides being degraded before they can effectively bind to their huntingtin targets.

4.5 Conclusion

In all, my experiments demonstrate exciting potential of mHTT degradation peptides to rapidly lower levels of mHTT in HD neurons *in vitro* by activating endogenous degradation sequences. Subsequent experiments in future studies will aim to reduce toxicity of mHTT degradation peptides, test long-term and side effects of the peptides, as well as increase potency in live animals. If successsful, the potential of these peptides to protect against mHTT-dependent neuropathies and behavioral deficits in HD will be examined, both at a cellular level, as well as in live animals. This could point to a novel direction in mHTT lowering that is rapid, reversible, non-cell autonomous and perhaps more clinically applicable than current technologies.

Figure 4-1 Schematic for mHTT-degradation peptide design

Schematic diagram of mHTT degradation peptides (adapted from design by Fan et al, 2014). mHTT degradation peptides contain three functional domains. First, a cell penetration amino acid sequence (TAT, sequence YGRKKRRQRRR), then a domain to bind mHTT over HTT (see Table 1), and lastly a degradation sequence. Degradation sequences are either RRRG degron, signaling for proteasomal degradation, or a KFERQ-like motif, recognized by chaperone proteins to transport proteins to the lysosome via chaperone mediated autophagy. Peptides first cross the cell plasma membrane, via the cell penetration sequence (1). Then, peptides recognize mHTT via the mHTT binding domain (2). Peptides are designed to bind more strongly to mHTT via HTT. Bound peptide-mHTT complexes are then flagged for degradation via the proteasome (degron peptides), or recognized by chaperone proteins (CMA peptides). mHTT bound to degron peptides are then degraded by the proteasome, while mHTT bound to CMA peptides are taken to the lysosome and transported via Lamp2A, then degraded. As mHTT binding domains are designed for preferential binding of mHTT over HTT, HTT should remain relatively untouched.

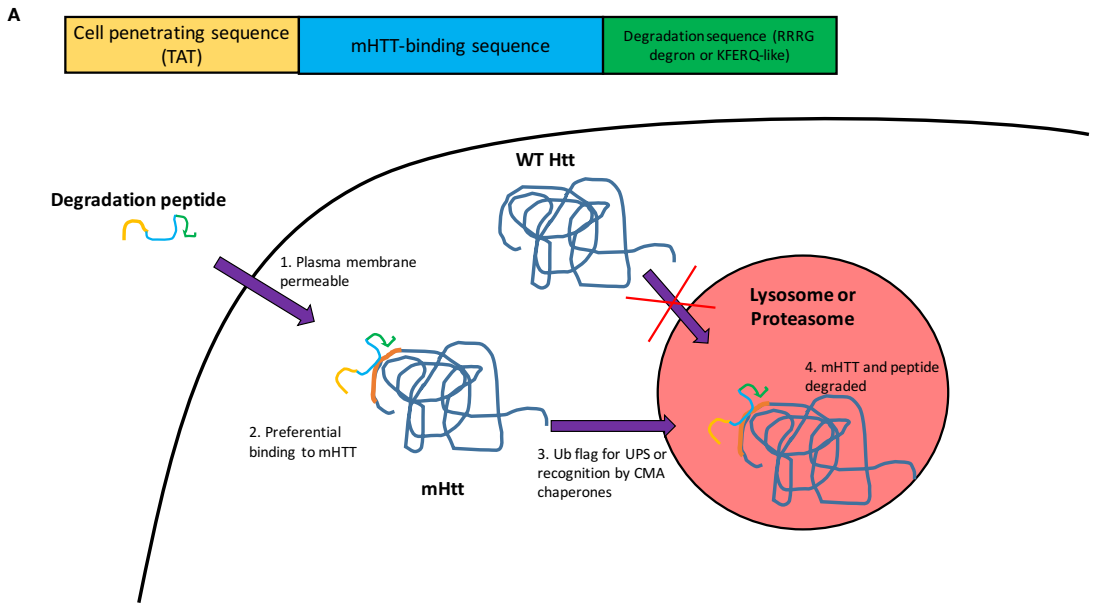


Table 4-1 mHTT binding sequences for mHTT degradation peptide design

mHTT binding sequences for mHTT degradation peptides were chosen based on previous studies showing preferential binding with polyQ expanded HTT. Sequences were designed to target the polyQ region of mHTT. Some sequences originally contained multiple repeats of the binding domain for optimum specificity for mHTT over HTT. For ease of synthesis, all peptides synthesized in our lab contained one repeat of the binding sequence.

mHtt binding sequence	# of repeats	Source	Reference
DYYYGRGYVW	3	MW-1 polyQ binding antibody	Li et al, Nat Struct Mol Biol, 2007)
WFPMLKS	1	mimics the mutant HTT-binding molecule HQP09	Chen et al, Chem Biol, 2011)
SNWKWWPGIFD	2	known mutant HTT-binding peptide QBP1	Nagai et al, Journal of Biological Chemistry, 2000)

Table 4-2 mHTT degradation peptides and controls

The following are the synthesized peptides for mHTT degradation, or peptide controls, for degradation via the UPS proteasome system, or via CMA degradation. All peptides contain a TAT cell penetrating domain, an mHTT binding domain, and a dedgradation sequence. Scrambled peptides contain a scrambled binding domain; to ensure that peptide effects are not due to TAT or the degradation signal. Degron sequences contain an RRRG degron sequence, signaling for UPS degradation, and CMA peptides contain a KFERQ-like motif, recognized by chaperones for CMA lysosomal degradation.

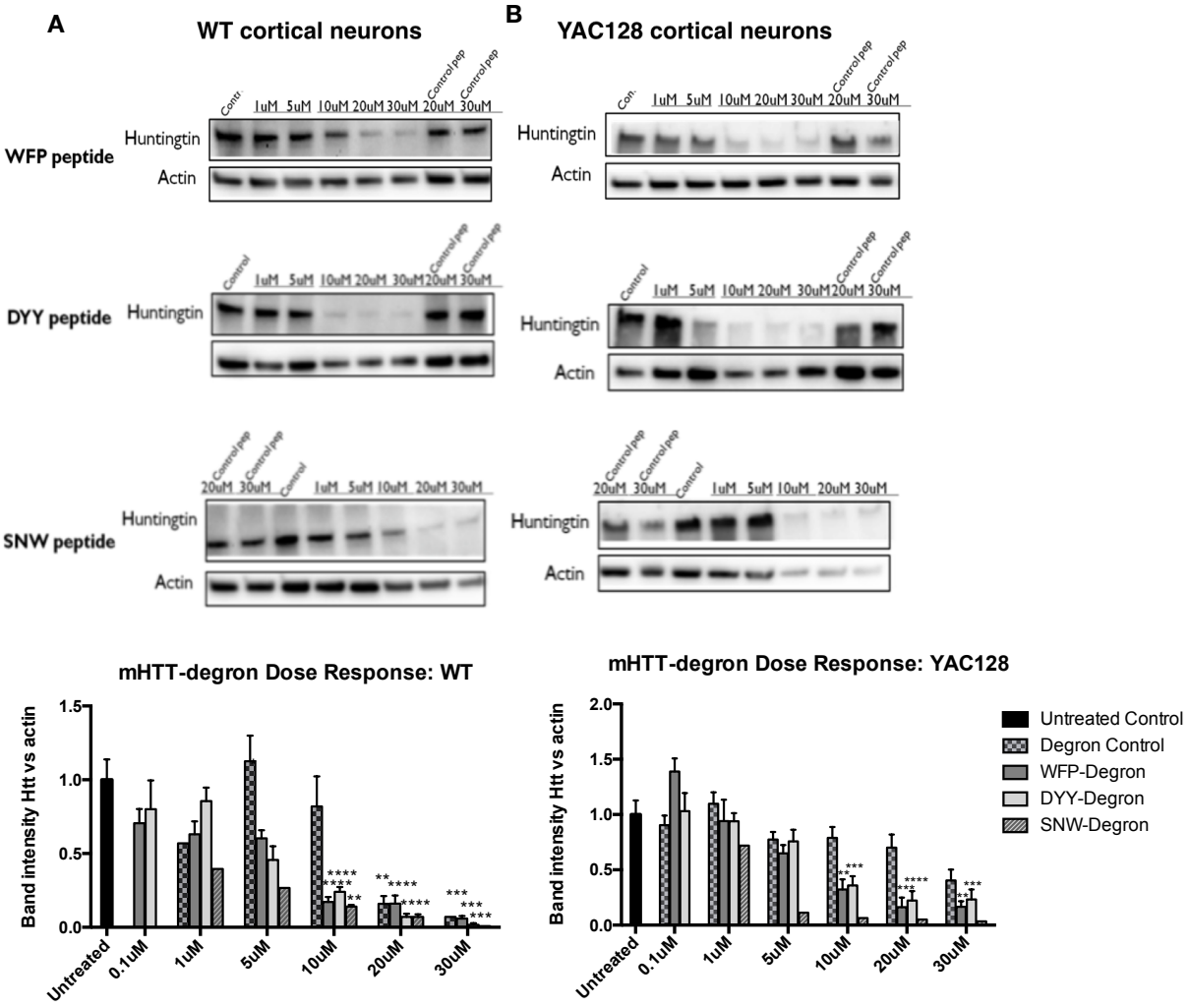
Degron Peptides (signal for degradation via the proteasome)		
Abbreviated name	**Use**	**Peptide sequence**
TAT-DYY-Degron	mHTT-targeting degron peptide from MW-1	YGRKKRRQRRR-DYYYGRGYVW-RRRG
TAT-WFP-Degron	mHTT-targeting degron peptide from HQP09	YGRKKRRQRRR-WFPMLKS-RRRG
TAT-SNW-Degron	mHTT-targeting degron peptide from QBP1	YGRKKRRQRRR- SNWKWWPGIFD-RRRG
TAT-Degron-scr	Scrambled control for degron peptides (using a scrambled binding sequence from α-synuclein and degron sequence)	YGRKKRRQRRR-KGYVRTSVLG-RRRG
CMA Peptides (signal for degradation via the proteasome)		
TAT-WFP-CMA	mHTT-targeting CMA peptide from HQP09	YGRKKRRQRRR-WFPMLKS- KFERQKILDQRFFE

CMA Peptides (signal for degradation via the proteasome) (cont.)		
Abbreviated name	**Use**	**Peptide sequence**
TAT-WFP-CMA-scr	Scrambled control for TAT-mHTT-CMA	YGRKKRRQRRR-SWKFLPM- KFERQKILDQRFFE
TAT-WFP-CMAT	mHTT-targeting CMA peptide with truncated CMA tail, aiming to reduce toxicty	YGRKKRRQRRR-WFPMLKS-QREFK
TAT-WFP-CMAT-scr	Scrambled control for TAT-mHTT-CMAT	YGRKKRRQRRR-SWKFLPM-QREFK

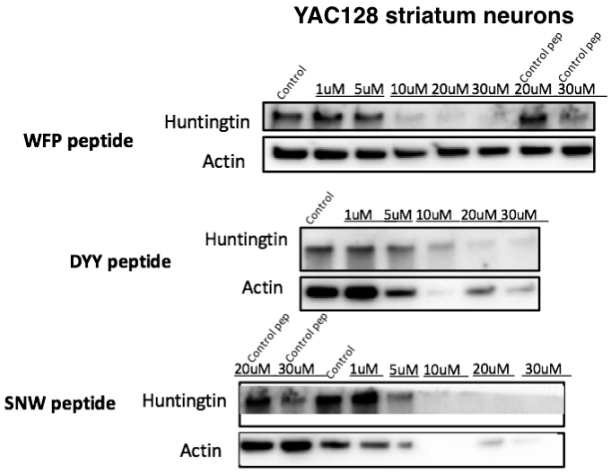
Figure 4-2 mHTT-degron peptides lower levels of both mHTT and HTT *in vitro*

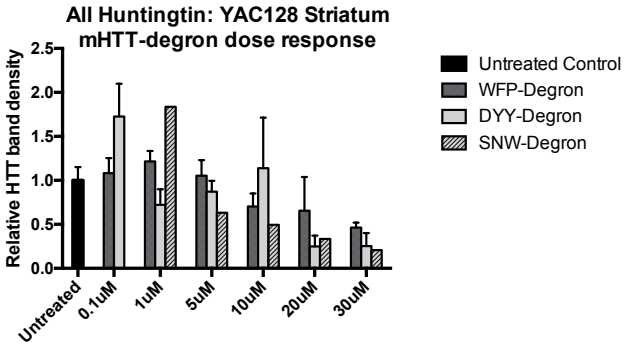
mHTT-degron peptides (3 putative sequences) were delivered to cultured cortical or striatal neurons from WT FVB-N or YAC128 HD mice. Dose response experiments tested all three peptides at doses between 1-30μM and examined HTT expression 6 hours later. Although peptides were designed to bind more strongly to mHTT than HTT, all three mHTT-degron peptides led to reductions in HTT in both WT (**A**) and YAC128 (**B**) cells. (WT n=3 cultures, 2-10 replicates, one-way ANOVA $F(16,50) = 8.778$, $p<.0001$; YAC128 n=6 cultures, 3-13 repliates $F(18, 106) = 9.389$, $p<0.0001$) Dose responsivity of all three peptides was similar, with significant knockdown appearing at 10μM. Control peptides showed less impact on mHTT, suggesting knockdown is mHTT-degron specific. mHTT-degron peptides showed similar knockdown profile in YAC128 striatum (**C**) (n=3 cultures, 1-7 replicates, one-way ANOVA $F(12,46) = .6543$, $p=.7845$, but clear trend). Time course experiments were also conducted, using 25μM of mHTT-degron peptides or a vehicle control. A clear time-dependent decrease in HTT and mHTT was seen in both YAC128 cortex (**D**) and striatum (**E**), but not vehicle. (Cortex, n=2 cultures, 3-4 replicates, one-way ANOVA $F(11,35)=24.82$, $p<.0001$; Striatum, n=2, 3-4 replicates, One-way ANOVA $F(11,36)=3.867$, $p<0.05$, for both, significance marked is Student's t-test for mHTT degron vs control at each time-point). LDH assays conducted in YAC128 neurons using mHTT-degron peptides demonstrate significant cell death in both a dose dependent (**F**) and time-dependent (**G**) way. Only DYY-degron is shown for dose-dependent cell death, as all peptides showed similar patterns (dose dependent, n=2 cultures, one way ANOVA $F(12,55) = 32.46$, $p<0.0001$; time dependent n=2 cultures, one way ANOVA F((11,12)=11.25,

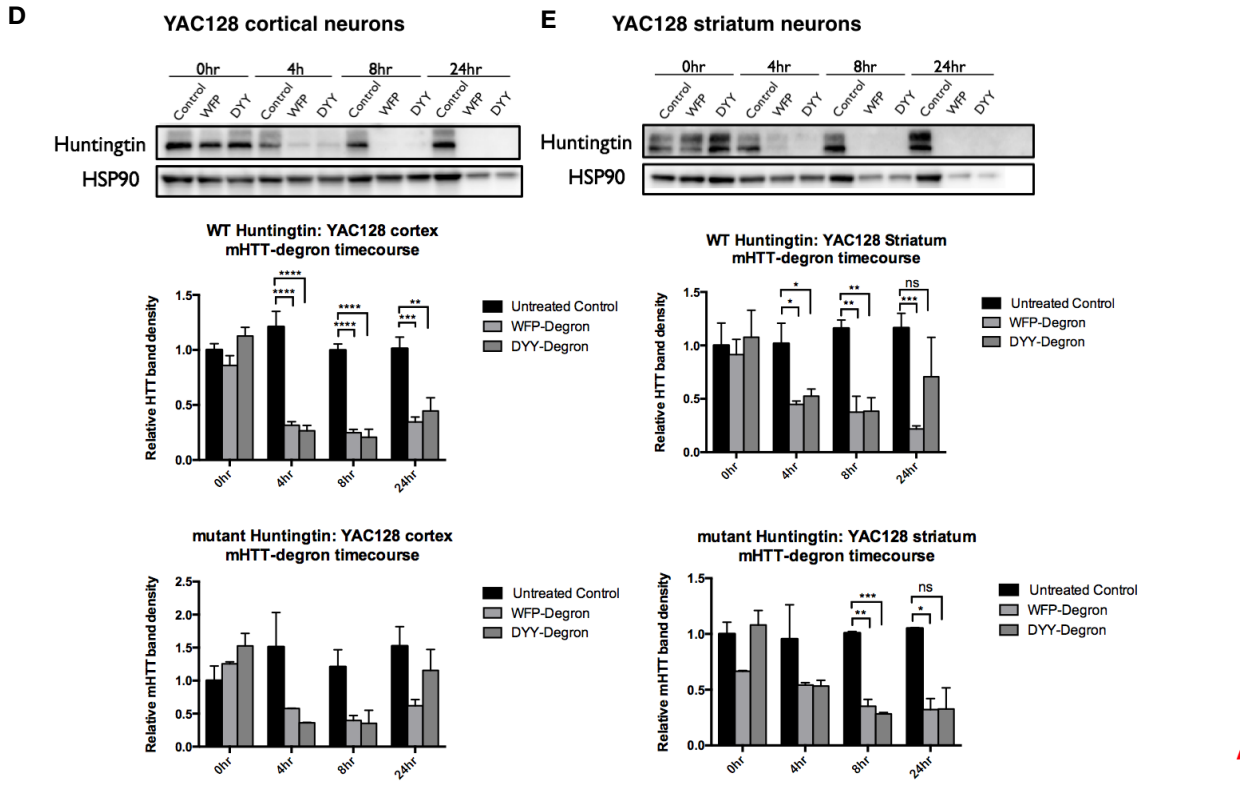
p<0.0001). (For all non-indicated, Tukey's Post hoc vs control * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001).



C


YAC128 striatum neurons
Control
1uM 5uM 10uM 20uM 30uM
Control pep
Control pep
20uM 30uM
WFP peptide
Huntingtin
Actin
Control
1uM 5uM 10uM 20uM 30uM
DYY peptide
Huntingtin
Actin
Control pep
Control pep
Control
20uM 30uM
1uM 5uM 10uM 20uM 30uM
SNW peptide
Huntingtin
Actin


All Huntingtin: YAC128 Striatum
mHTT-degron dose response
Relative HTT band density
2.5
2.0
1.5
1.0
0.5
0.0
Untreated
0.1uM
1uM
5uM
10uM
20uM
30uM
Untreated Control
WFP-Degron
DYY-Degron
SNW-Degron


D
YAC128 cortical neurons
0hr
4h
8hr
24hr
Control
WFP
DYY
Huntingtin
HSP90
E
YAC128 striatum neurons
WT Huntingtin: YAC128 cortex
mHTT-degron timecourse
WT Huntingtin: YAC128 Striatum
mHTT-degron timecourse
mutant Huntingtin: YAC128 cortex
mHTT-degron timecourse
mutant Huntingtin: YAC128 striatum
mHTT-degron timecourse
Relative HTT band density
Relative mHTT band density
Untreated Control
WFP-Degron
DYY-Degron
ns

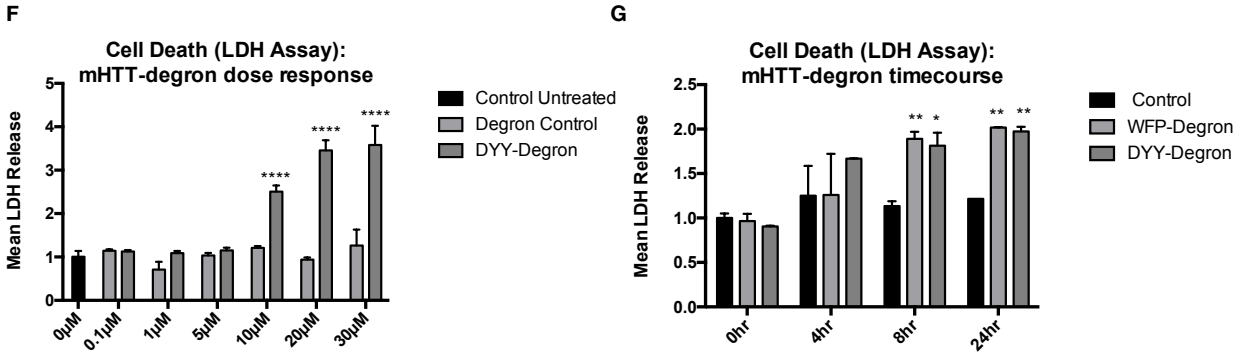
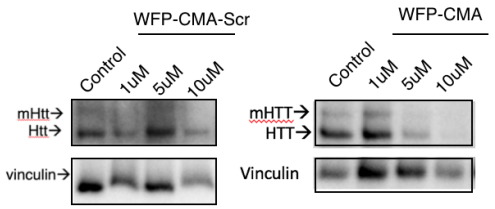
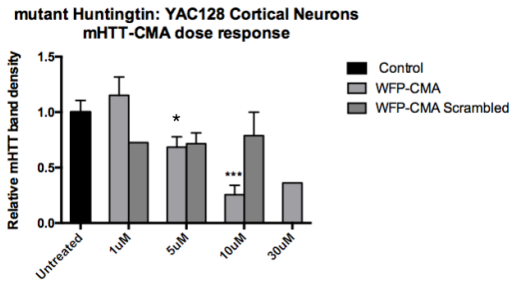

F
Cell Death (LDH Assay):
mHTT-degron dose response
Mean LDH Release
Control Untreated
Degron Control
DYY-Degron
0μM
0.1μM
1μM
5μM
10μM
20μM
30μM
G
Cell Death (LDH Assay):
mHTT-degron timecourse
Control
WFP-Degron
DYY-Degron
0hr
4hr
8hr
24hr

Figure 4-3 mHTT-CMA peptides lower levels of both mHTT and HTT *in vitro*

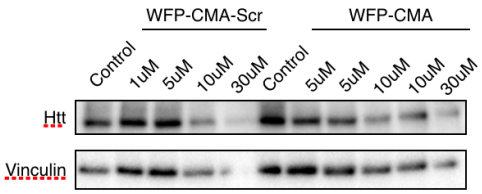
Cultured cortical neurons from YAC128 HD or control FVN-N mice were treated with WFP-CMA or a scrambled control. First, dose response experiments were conducted. WFP-CMA significantly lowered both mHTT and HTT in YAC128 neurons, more dramatically than scrambled control (**A**) (mHTT: n=7 cultures, 3-7 replicates, One-way ANOVA $F(12, 67)=4.366$, $p<0.05$; WT HTT n=7 cultures, 3-6 replicates, one-way ANOVA $F(12,66)=4.984$, $p<0.001$). In WT cortical neurons from FVB mice, although there was a clear decrease in HTT expression, it was not as dramatic as in YAC128 (**B**) (n=6 cultures, 5-17 replicates, one-way ANOVA $F(12,102)=1.788$, $p=0.0598$). Rescue experiments were then conducted using a lysosome inhibitor NH_4Cl, applied at the time of peptide treatment. In YAC128 neurons, while WFP-CMA leads to clear dose-dependent lowering of mHTT and HTT, treatment with NH_4Cl in part rescues this knockdown (**C**) (mHTT: n=7 cultures, 3-7 replicates, One-way ANOVA $F(12, 67)=4.366$, $p<0.05$; WT HTT n=7 cultures, 3-6 replicates, one-way ANOVA $F(12,66)=4.984$, $p<0.001$). In WT neurons (**D**), dose-dependent HTT lowering was also reversed by NH_4Cl, suggesting mHTT/HTT knockdown occurs, at least in part, via CMA lysosomal degradation (n=6 cultures, 5-17 replicates, one-way ANOVA $F(12,102)=1.788$, $p=0.0598$). LDH assays were again used to assess cell death following peptide treatment (**E**). WFP-CMA led to significant increase in neuron death in cultured YAC128 neurons, that was less dramatic with scrambled CMA peptide. Interestingly, NH_4Cl treatment significantly reversed cell death following WFP-CMA treatment (n=2, one way ANOVA $F(9,10)=4.887$, $p<0.05$)(For all non-indicated, Tukey's Post hoc vs control * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

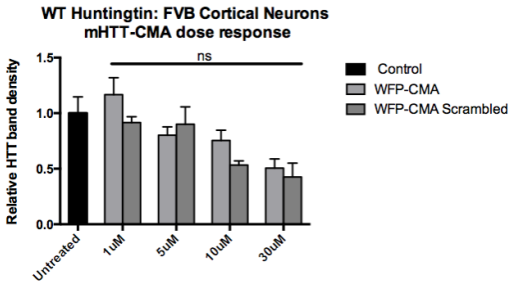
A **YAC128 cortical neurons**



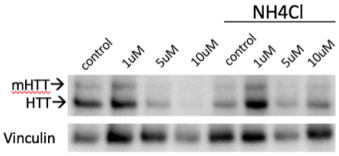


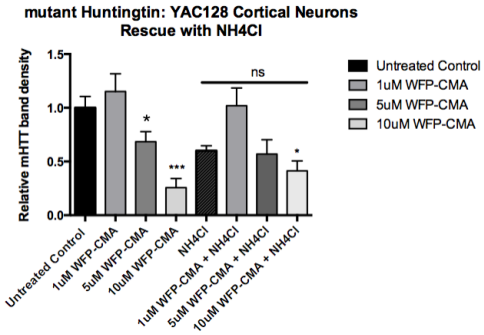
B **FVB cortical neurons**

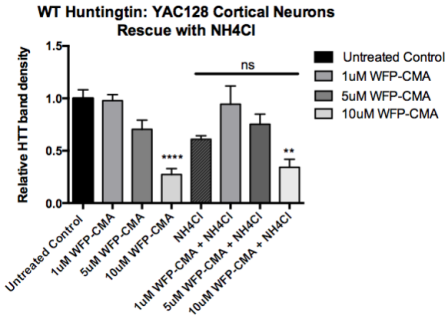




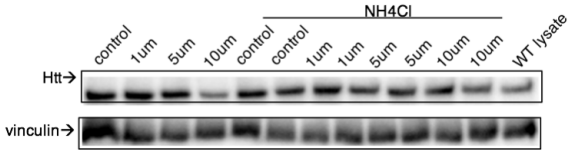
C **YAC128 cortical neurons**

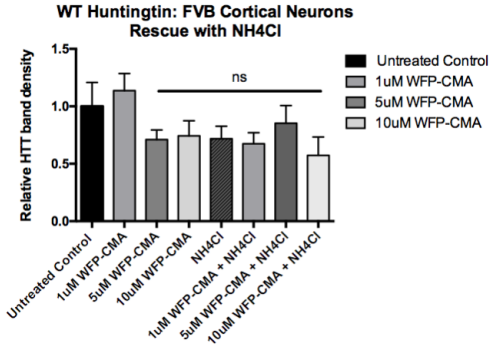






D **FVB cortical neurons**





E

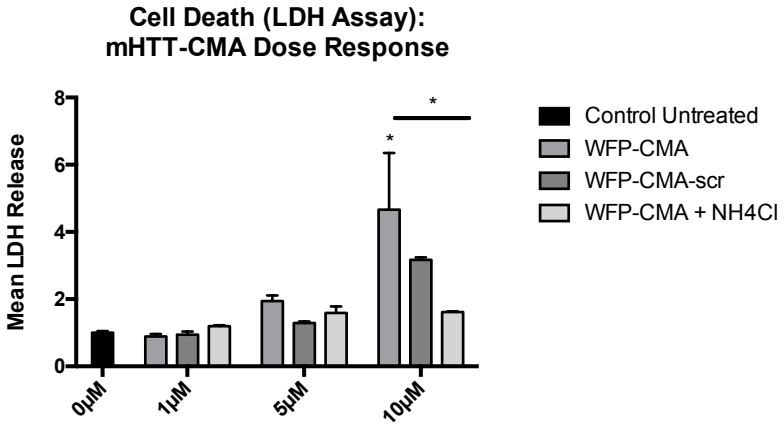
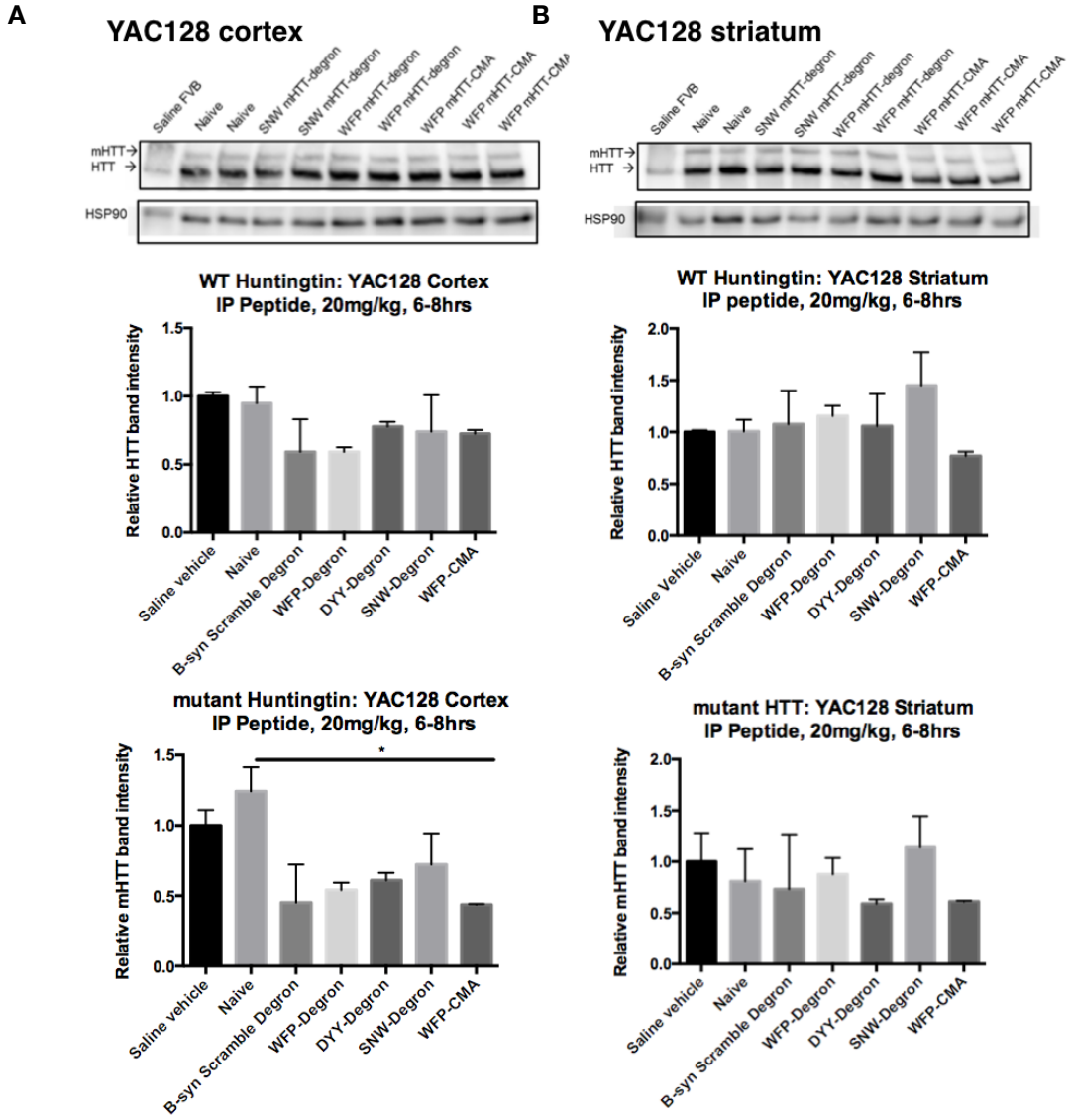
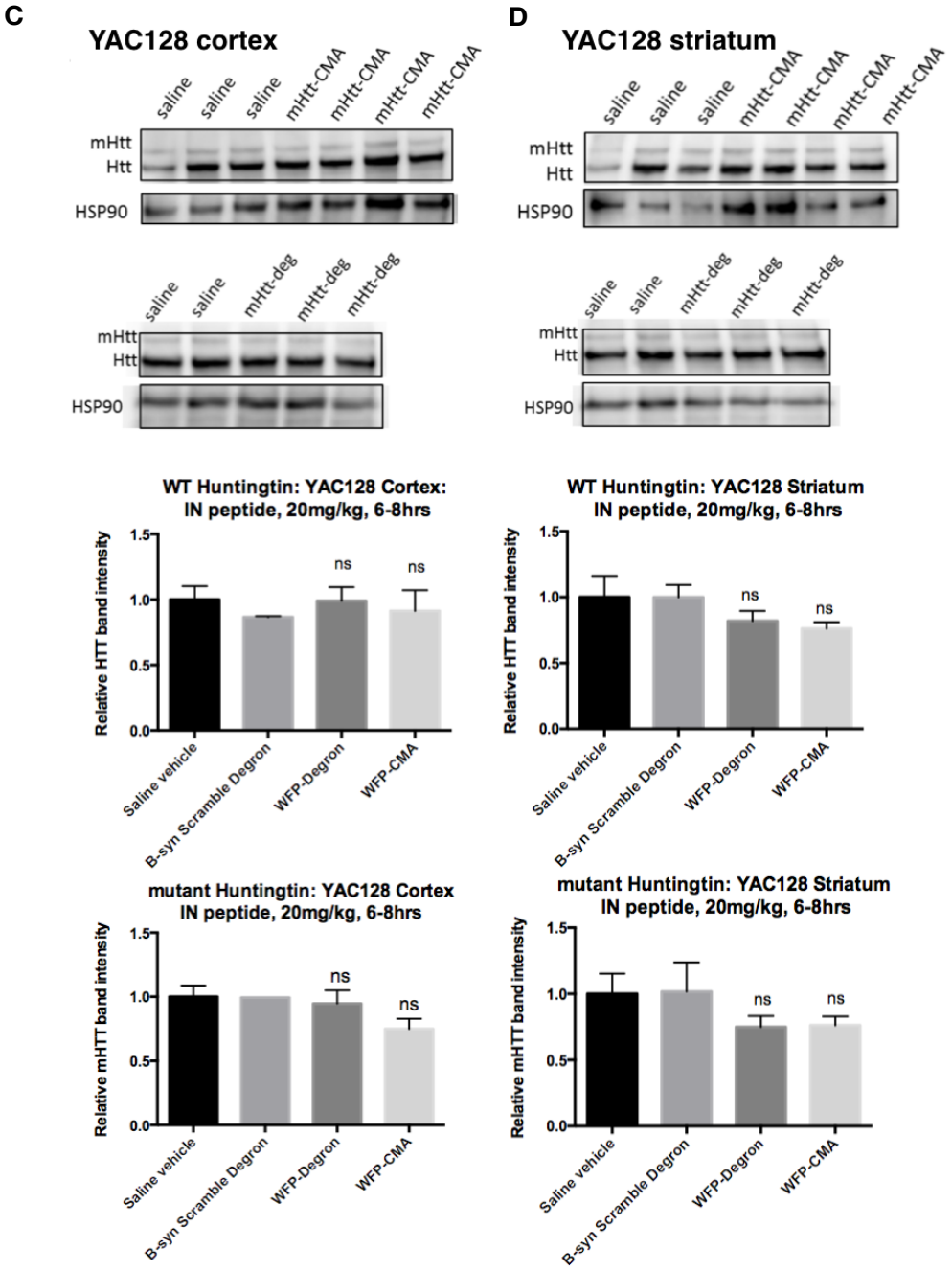

Cell Death (LDH Assay):
mHTT-CMA Dose Response
Mean LDH Release
8
6
4
2
0
0μM
1μM
5μM
10μM
*
*
Control Untreated
WFP-CMA
WFP-CMA-scr
WFP-CMA + NH4Cl

Figure 4-4 mHTT degradation peptides do not dramatically lower mHTT or HTT *in vivo*

YAC128 mice (aged 3mon) were injected IP with mHTT-degron peptide, WFP-CMA peptide, a scrambled control degron peptide (all peptides 20mg/kg), vehicle control, or uninjected, then recovered for 6-8hours. Brains were removed, and striatum and cortical tissues were dissected and flash frozen in dry ice. Frozen tissue was lysed and analyzed using western blot to determine concentration of mHTT and HTT. No significant difference between control peptide, saline vehicle and experimental peptides could be detected for mHTT or HTT in either cortex (**A**) or striatum (**B**) (Cortex n=2-4, one way ANOVA F(6,13)=.6464, p=.6927; Striatum n=2-4, one-way ANOVA F(6,13)=.5655, p=.7507). Using Student's T-Test, a small but significant decrease in mHTT vs WT HTT could be detected in YAC cortex compared to naïve animals, but overall there was no significant mHTT or HTT lowering with IP Peptide. Experiments were repeated with intranasal peptide delivery in an attempt to increase knockdown. Again, animals were treated wih 20mg/kg peptide or control intranasally via drops of 5-10µl on the nostril. Mice were recovered for 6-8hours, then brains were removed, and striatum and cortical tissues were used to assess mHT and HTT using western. No significant difference between control peptide, saline vehicle and experimental peptides could be detected for mHTT or HTT in either cortex (**C**) or striatum (**D**) (Cortex, n=2-10, one-way ANOVA F(3,20)=.1405, p=.9306; striatum n=2-10, one-way ANOVA F(3,20)=1.103, p=.3710) though a trend towards lowered mHTT was seen with both mHTT degron and CMA degradation peptides. To test HTT knockdown in control animals, WFP-CMA, CMA scrambled peptide or saline control was delivered (20mg/kg) to WT animals and assessed at 6-8 hours using western blot. There was no significant reduction in HTT expression in either cortex (**E**) or striatm (**F**) with a slight trend towards increased HTT expression with peptide treatment (Cortex n=2-6, one-way ANOVA, F(2,9)=.6114, p=0.5637;

Striatum n=2-6, one-way ANOVA F(2,9)=1.380, p=0.3001). Lastly, CMA peptides, scrambled control (20mg/kg each) or saline vehicle were tested in a time course to determine if there was any time dependent lowering of mHTT or HTT in brains *in vivo*. As seen in (**G**) and (**H**), there were no significant knockdowns of mHT or HTT in YAC128 Cortex or Striatum with WFP-CMA or scrambled control. (n=2-6, one-way ANOVA: mHTT cortex F(9,4)=1.302, p=.2866; WT cortex F(9,24)=1.678, p=0.1496; mHTT striatum F(9,24)=1.302, p=0.2866; WT HTT striatum F(9,24)=0.7848, p=0.6325).




C
YAC128 cortex
saline
saline
saline
mHtt-CMA
mHtt-CMA
mHtt-CMA
mHtt-CMA
mHtt
Htt
HSP90
saline
saline
mHtt-deg
mHtt-deg
mHtt-deg
mHtt
Htt
HSP90
D
YAC128 striatum
saline
saline
saline
mHtt-CMA
mHtt-CMA
mHtt-CMA
mHtt-CMA
mHtt
Htt
HSP90
saline
saline
mHtt-deg
mHtt-deg
mHtt-deg
mHtt
Htt
HSP90
WT Huntingtin: YAC128 Cortex:
IN peptide, 20mg/kg, 6-8hrs
Relative HTT band intensity
1.5
1.0
0.5
0.0
ns
ns
Saline vehicle
B-syn Scramble Degron
WFP-Degron
WFP-CMA
WT Huntingtin: YAC128 Striatum
IN peptide, 20mg/kg, 6-8hrs
Relative HTT band intensity
1.5
1.0
0.5
0.0
ns
ns
Saline vehicle
B-syn Scramble Degron
WFP-Degron
WFP-CMA
mutant Huntingtin: YAC128 Cortex
IN peptide, 20mg/kg, 6-8hrs
Relative mHTT band intensity
1.5
1.0
0.5
0.0
ns
ns
Saline vehicle
B-syn Scramble Degron
WFP-Degron
WFP-CMA
mutant Huntingtin: YAC128 Striatum
IN peptide, 20mg/kg, 6-8hrs
Relative mHTT band intensity
1.5
1.0
0.5
0.0
ns
ns
Saline vehicle
B-syn Scramble Degron
WFP-Degron
WFP-CMA

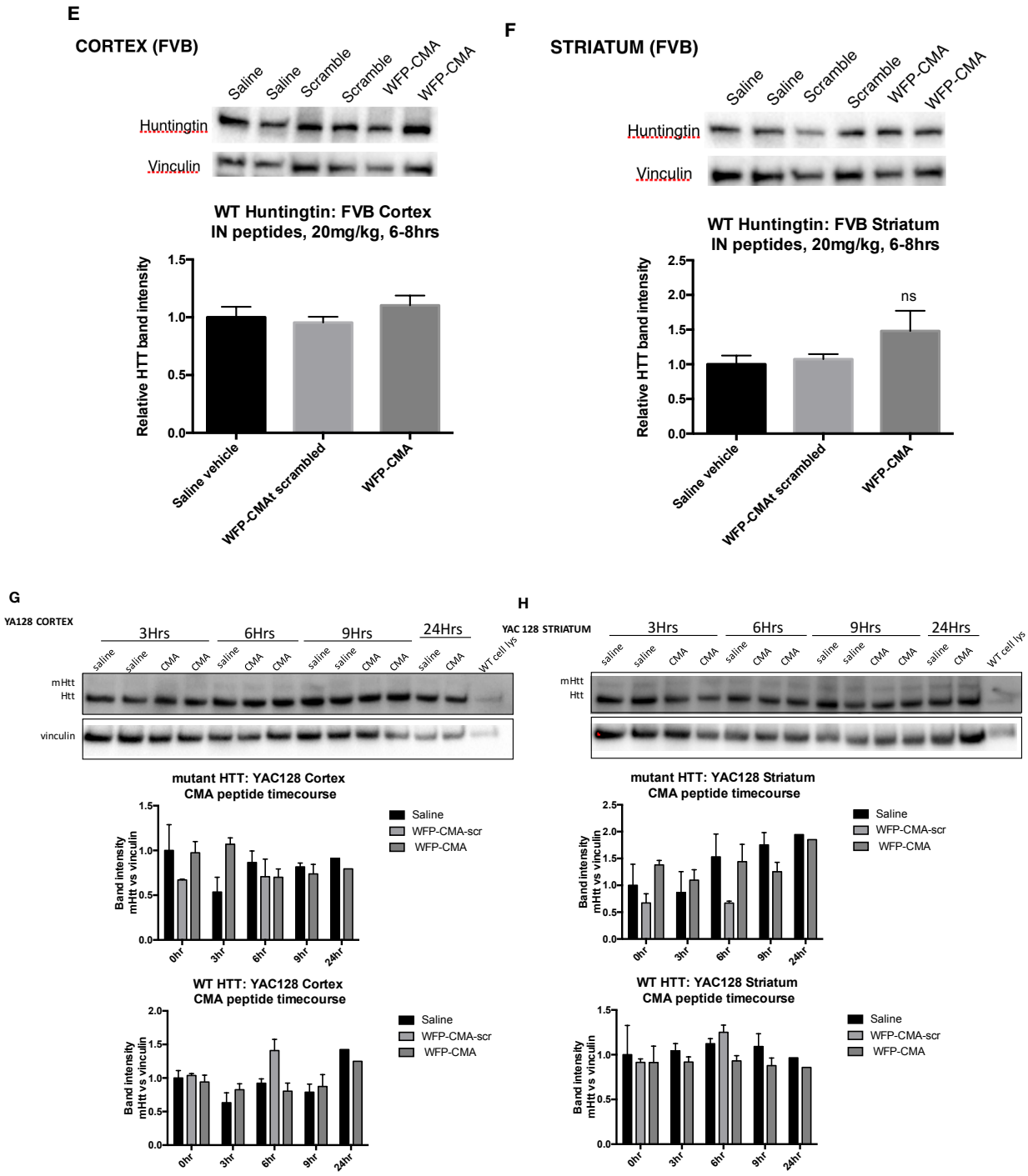
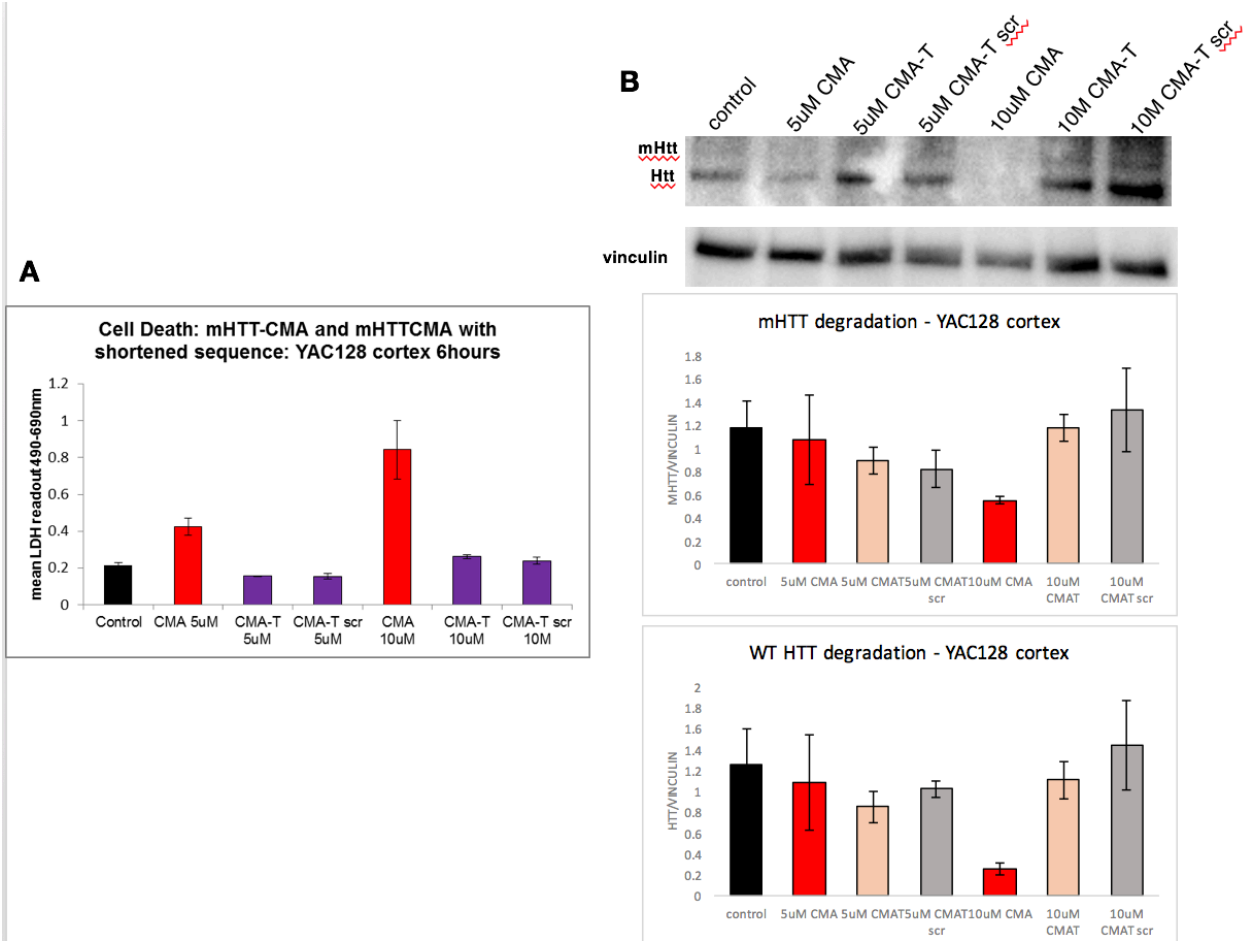

E
CORTEX (FVB)
Saline
Saline
Scramble
Scramble
WFP-CMA
WFP-CMA
Huntingtin
Vinculin
WT Huntingtin: FVB Cortex
IN peptides, 20mg/kg, 6-8hrs
Relative HTT band intensity
Saline vehicle
WFP-CMAt scrambled
WFP-CMA
F
STRIATUM (FVB)
WT Huntingtin: FVB Striatum
IN peptides, 20mg/kg, 6-8hrs
ns
G
YA128 CORTEX
3Hrs
6Hrs
9Hrs
24Hrs
WT cell lys
mHtt
Htt
vinculin
mutant HTT: YAC128 Cortex
CMA peptide timecourse
Band intensity mHtt vs vinculin
Saline
WFP-CMA-scr
WFP-CMA
WT HTT: YAC128 Cortex
CMA peptide timecourse
H
YAC 128 STRIATUM
mutant HTT: YAC128 Striatum
CMA peptide timecourse
WT HTT: YAC128 Striatum
CMA peptide timecourse

Figure 4-5 Test of truncated KFERQ tail on CMA peptides to reduce toxicity

A brief test experiment was done in cultured cortical neurons from YAC128 to test a putative new mHTT-CMA degradation peptide. WFP-CMA-T peptide contains a TAT sequence, the WFP binding sequence for mHTT and truncated KFERQ motif. A test experiment was done to determine if shortening the CMA tail could reduce toxicity of CMA mHTT targeting peptides *in vitro*. Neurons were treated with 5µM and 10µM peptide, either WFP-CMA, WFP-CMA-T or a control CMA-T peptide containing a scrambled binding domain. As seen in **(A)**, truncated CMA peptide did clearly reduce cell death that was evident in WFP-CMA down to control levels, however western blot **(B)** demonstrates that CMA-T peptides did not significant lower levels of mHTT or HTT in cultured neurons. (Only one experiment, n=3 replicates).



Chapter 5: Overall Discussion and Conclusions

5.1 Discussion

In my PhD thesis work, I investigated novel, early, preventative targets related to neuron death and dysfunction in HD, with the hopes of developing novel, peptide-mediated therapies for HD. This devastating disease, for which there is no effective treatment, has the unique feature of having a genetic cause, allowing for early testing and detection, as well as a late onset, meaning there is a potentially large therapeutic window for treatment with preventative medicine that could slow or prevent the onset of neuronal loss, cellular dysfunction and cognitive and behavioral deficits. However, although the genetic cause of HD is known, the resulting molecular changes and dysfunctions as a result of the HD mutation are numerous, and researchers are still discovering early potential druggable targets in HD. At present, there are no good early preventative medicines for HD.

In my study, I aimed to investigate some of the early molecular changes associated with HD pathology, with the hopes of designing early, mostly peptide-mediated treatment strategies. My first experiments looked at downstream signaling relating to NMDAR-mediated excitotoxicity. GluN2B, extrasynaptic NMDAR-mediated excitotoxicity has a key role in HD pathogenesis through several mechanisms, however, NMDAR antagonists are not the best therapies, given the critical role of NMDARS in basic cell function and cell survival. Instead targeting downstream signaling pathways associated with cell death in an excitotoxic event may instead be preferable. Basing my experiments on studies in our lab in stroke, I hypothesized that PTEN nuclear translocation may have a key role in NMDAR-mediated neuronal death in HD, and tested if blockade of PTEN nuclear translocation in HD neurons, using our lab-designed Tat-K13 peptide could provide neuroprotective and therapeutic benefit. As predicted, I detected PTEN

nuclear translocation following NMDAR-mediated excitotoxic events in all HD and WT neurons, that was reversible using inhibitors designed to block GluN2B-containing, extrasynaptic NMDARs, suggesting that PTEN nuclear translocation is associated with excitotoxicity in all neurons tested. As predicted, Tat-K13 effectively blocked PTEN nuclear translocation and protected against neuron death in cortical and hippocampal neurons, however, in both WT and HD striatum, Tat-K13 did not have any PTEN translocation blocking or neuroprotective effects. These events were demonstrated both in cultured neurons, as well as *in vivo*, with Tat-K13 effectively protecting against CA1 hippocampal NMDAR-mediated lesions following KA injection, but unable to protect against QA-induced striatum lesions. Using GFP-fused plasmids for PTEN I demonstrated that mutations to K13, but not K289 in cortical neurons lead to cytoplasmic retention of PTEN in cortical neurons, indicating that ubiquitination of K13 is necessary for PTEN nuclear translocation, however mutations to either K13 or K289 in striatal neurons did not block PTEN nuclear translocation. This demonstrates that ubiquitination of PTEN is not likely mediating its nuclear import and explains the lack of efficacy of Tat-K13. Lastly, in project 1, I demonstrated that in YAC128 brain tissue, there is a small, but significant increase in nuclear translocation of PTEN compared to WT tissue, primarily outside of the striatum. This may suggest that PTEN nuclear translocation is associated with increased vulnerability to cell death and dysfunction in HD neurons, and may point to Tat-K13 as a potential way to both study the role of PTEN nuclear translocation in non-striatal neurons, as well as protect against broad cellular death in HD.

These studies, though initially disappointing in their lack of support for my initial hypothesis, provide some thought provoking findings. For one, they point to PTEN nuclear translocation as a potential target for NMDAR-mediated neuronal death in all neurons, including striatal MSNs. Although blocking mono-ubiquitination of PTEN in MSNs did not protect them

against NMDA mediated cell death, it appears that PTEN nuclear translocation is linked to excitotoxic NMDAR stimulation. Given the knowledge that HD striatal neurons are susceptible to over-activity at GluN2B-NMDARs, PTEN nuclear translocation, or PTEN generally, may be a target worth pursuing in the future. Lastly, the finding that PTEN nuclear translocation is mediated differently in MSNs vs cortex/hippocampal neurons is a fascinating finding that may help us understand the differential role of PTEN in each neuron type, especially pertaining to cell death and nuclear expression. Further studies can expand on this work in many ways. Future studies should investigate the baseline function of nuclear PTEN in striatum vs cortical or hippocampal neurons, as well as how this might change in HD. The finding that PTEN nuclear translocation may be modulated by differential mechanisms in different neuron types may help explain different vulnerabilities too, not only NMDAR-mediated excitotoxic cell death, but also other cell death sensitivities. Also, given the fact that PTEN nuclear translocation appears to be linked to cell death in all cells studied, future experiments should investigate what mediates nuclear translocation of PTEN in the striatum, if not ubiquitination, then perhaps PTEN translocates via a nuclear localization signal, or transporters, or interaction with other nuclear proteins. Figuring out what leads to this nuclear change might help us to design therapeutics for disorders like HD where striatum is particularly vulnerable, and help us to better understand the role of nuclear PTEN in chronic diseases like HD. At present, future studies should investigate the role of PTEN nuclear translocation in cortex and hippocampus in HD, and investigate the ability of Tat-K13 to block translocation, and potentially rescue neuron loss or HD behavior.

In project 2, related to my initial experiments on NMDAR-mediated excitotoxicity in HD, I carried out experiments to study another downstream event related to NMDAR-mediated neuronal death: caspase activation. Caspase activation, especially via casp6 activation and

cleavage, has an important role in HD pathogenesis, as well as other acute and chronic neurodegenerative conditions. Following NMDAR-mediated excitotoxicity, caspases are activated, and initiate apoptotic cascades, as well as cleave substrates such as mHTT, which can lead to cellular dysfunction and death. Caspase 6 is a key player in apoptotic cascades following NMDAR-mediated excitotoxicity in in HD and other neurodegernative disesase, however, the precise activation pattern of caspase activation following an NMDAR-mediated excitotoxic event is still relatively unknown. In my study, I used NMDAR-mediated excitotoxicty in cultured neurons to study the patterns of caspase activation following an excitotoxic event. I demonstrated that casp6 is activated very early following NMDAR-mediated excitotoxicity, suggesting it is an initiator caspase in NMDAR-mediated excitotoxic cascades. This was supported with mRNA, protein and caspase activation assays, as well as examination of casp6 substrate huntingtin, as well as two new substrates DAXX and STK3. These studies support previous experiments and point to casp6 as a potential initator caspase, and a potential target for NMDAR-mediated excitotoxic conditions such as HD. Future experiments may aim to develop peptide-mediated interference with casp6 in HD to test the ability of interfering with NMDAR-mediated casp6 activation, expression and cleavage to protect against neuron death and dysfunction in HD. Peptide-based studies could also help to examine targeting casp6 in other models for diseases and conditions where NMDAR-mediated excitotoxicity has a role.

In Chapter 4, I investigated another early neuroprotective strategy in HD clinical development. Based on the idea that mHTT is, at its root, a disease caused by a CAG expansion mutation, and following on exciting research demonstrating the therapeutic benefits of mHTT lowering using RNAi and ASO therapies, I investigated the potential for mHTT lowering using targeted degradation peptides that flag mHTT for proteasomal or lysosomal degradation.

Excitingly, in these studies, I was able to rapidly and effectively lower levels of mHTT in cultured HD neurons, albeit non-specifically, with significant HTT reduction as well. This knockdown was, at least in part, due to endogenous degradation of mHTT/HTT, suggesting this strategy may be a viable way to lower mHTT levels in HD neurons. However, knockdown was associated with significant neuronal death, which could be due to overloading of degradation machinery, detrimental effects of loss of HTT, or off target effects. Further experiments with these peptides will be necessary to attempt to reduce toxicity *in vitro*. If successful toxicity is reduced, these studies will also be used to test the ability of mHTT knockdown peptides to reverse baseline neuropathological changes in HD neurons, such as sensitivity to NMDA, mitochondrial deficit, changes in BDNF expression and cellular morphological changes. In my work, I also conducted *in vivo* experiments to test the ability of my peptides to knock down mHTT in intact brains, however, knockdown in live animals was very mild. Future experiments will need to expand on these studies and investigate ways to increase peptide-mediated knockdown in intact brains. Future studies will investigate the therapeutic potential of mHTT degradation peptides to reverse cellular dysfunction in HD, as well as rescue neuron loss and behavioral phenotype in HD mice.

5.2 Conclusions

My results provided some novel insight into early preventive medicine for HD, utilizing peptide-mediated therapies, and examined three potential targets for the development of early therapies for HD that aim to prevent or delay onset of cellular dysfunction and neuronal death; PTEN nuclear translocation, NMDAR-mediated caspase 6 activation, and mHTT silencing. Peptides point to an exciting potential way of treating neurodegenerative disease, such as HD, where long term treatment is likely necessary, due to their ease of delivery, being relatively well-tolerated in

patients, reversible in approximately a day, and ease of synthesis. Though all three targets still require future work to move into clinical testing, my experiments provide important insight into molecular underpinnings of HD, and may help with future studies looking at early preventative therapies for HD, where good early medicines are still desperately needed.

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