The p75 neurotrophin receptor augments survival signaling in the striatum of pre-symptomatic Q175$^{WT/HD}$ mice, an animal model of Huntington's disease

by

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“Science can only ascertain what is, but not what should be, and outside of its domain value judgments of all kinds remain necessary.” Albert Einstein

“Truth in science can best be defined as the working hypothesis best suited to open the way to the next better one.” Konrad Lorenz

Dedication:

I would like to dedicate this dissertation to the people who have always believed in me and supported me no matter what, my family: my parents, Paul and Karen, my sister, Jennifer, my new brother-in-law Craig, and my aunts, uncles, and cousins. Also, to my niece and nephew, Morgan and Matthew, for reminding me how amazing and beautiful the world can be, for reminding me to appreciate every moment of life, and for giving me such love and joy. While my family has always supported my dreams, they have also always reminded me that I am more than my job. They have been the bedrock that has kept me grounded throughout my entire life, and also the support and faith to allow me to fly.

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Abstract

Huntington's disease (HD) is a dominantly-inherited neurodegenerative disorder characterized by a constellation of motor, cognitive, and psychiatric features. Striatal medium spiny neurons, one of the most affected populations, are dependent on brain-derived neurotrophic factor (BDNF) anterogradely transported from the cortex for proper function and survival. Recent studies suggest both receptors for BDNF, TrkB and p75, are improperly regulated in striata of HD patients and mouse models of HD. While BDNF-TrkB signaling almost exclusively promotes survival and metabolic function, p75 signaling is able to induce survival or apoptosis depending on the available ligand and associated co-receptor. We investigated the role of p75 in the Q175 knock-in mouse model of HD by examining the levels and activation of downstream signaling molecules, and subsequently examining $Q175^{WT/HD};p75^{-/-}$ mice to determine if p75 represents a promising therapeutic target. In $Q175^{WT/HD};p75^{+/+}$ mice, we observed enhanced survival signaling as evidenced by an increase in phosphorylation and activation of Akt and the p65 subunit of NFkB in the striatum at 5 months of age and an increase in XIAP expression compared to $Q175^{WT/WT};p75^{+/+}$ mice; this increase was lost in $Q175^{WT/HD};p75^{-/-}$ mice. $Q175^{WT/HD};p75^{-/-}$ mice also showed a decrease in Bcl-XL expression by immunoblotting compared to $Q175^{WT/HD};p75^{+/+}$ and $Q175^{WT/WT};p75^{+/+}$ littermates. Consistent with diminished survival signaling, DARPP-32 expression decreased both by immunoblotting and by immunohistochemistry in $Q175^{WT/HD};p75^{-/-}$

mice compared to *Q175$^{WT/WT}$;p75$^{+/+}$*, *Q175$^{WT/HD}$;p75$^{+/+}$*, and *Q175$^{WT/WT}$;p75$^{-/-}$* littermates. Additionally, striatal volume declined to a greater extent in *Q175$^{WT/HD}$;p75$^{-/-}$* when compared to *Q175$^{WT/HD}$;p75$^{+/+}$* littermates at 12 months, indicating a more aggressive onset of degeneration. These data suggest that p75 signaling plays an early role in augmenting pro-survival signaling in the striatum and that disruption of p75 signaling at a pre-symptomatic age may exacerbate pathologic changes in *Q175$^{WT/HD}$* mice.

Chapter 1: Introduction

Huntington's disease: Description, Pathology, and Genetics

Huntington's disease (HD) was described graphically, lucidly, and succinctly by George Huntington in 1872. Despite almost 150 years of research since his incisive description of its major clinical features, it remains, in his words, "one of the uncurables" (Huntington, 1872; reprinted in Huntington, 2003). HD is a devastating, progressive neurodegenerative disease characterized by a constellation of motor, psychiatric, and cognitive dysfunctions. HD is an autosomal dominant inherited disorder with a typical onset in midlife. Median age of onset is 40 years, and onset before 20 or after 65 is rare. There are approximately 30,000 cases of HD in the US and Canada, with 150,000 more at-risk individuals. Death usually occurs within 15-20 years post-diagnosis (Roos et al., 1993). While psychiatric and cognitive dysfunctions often appear earlier than the onset of motor symptoms, diagnosis is not made until the appearance of motor disturbances. The typical midlife onset results in a significant loss of productivity and, when combined with the slow progressive nature of the disease, generally means a high cost of care which affects the patient and the entire family. Additionally, the dominant inheritance of the disease affects multiple generations and tends to pull the family down the social scale. These factors combined cause HD to have an impact disproportionate to its prevalence.

HD classically consists of three categories of clinical symptoms: motor disturbance, most commonly chorea; progressive cognitive disturbance, often ending in dementia; and various behavioral disturbances. The classic motor sign of HD is chorea, derived from the Greek word for “dance”, and usually begins with fleeting, suppressible movements, eventually advancing to involve larger muscle groups. While chorea is the most classic motor sign, HD involves a wide range of movement disorders such as ataxia, dystonia, and bradykinesia. Many patients will experience several of these motor impairments during the course of their disease. Cognitive decline was classically thought to begin concurrently or shortly before manifestation of motor symptoms. More recent studies, however, indicate that cognitive impairment is among the earliest manifestations of the disease (Alyward, 2007; Alyward et al., 2000). Cognitive disturbance typically presents as a difficulty with multi-tasking, focus, short-term memory, and learning new skills. As opposed to Alzheimer’s disease, the cognitive decline of HD is largely subcortical as characterized by slow thought-processes, executive dysfunction, and problems with attention or sequencing. Many patients and families find the psychiatric disturbances the most troublesome. Behavioral disturbances range in features and vary greatly in severity. The most common are depression, obsessive-compulsive behavior, irritability, and outbursts.

Since Huntington’s description in 1872, the pathology of HD has been studied and described by many physicians and researchers (Alzheimer, 1911; Dunlap, 1927; McCaughey, 1961; Vonsattel and DiFiglia, 1998). HD is classically a striatal atrophy disorder and is characterized by significant dysfunction and degeneration of both the caudate and putamen. While striatal atrophy is a hallmark, careful analysis has revealed

a much more widespread atrophy. Post-mortem analysis of HD patients show 21-29% loss of cerebral cortex, 29-34% loss of telencephalic white matter, 64% loss of putamen, and 57% loss of caudate as compared to age-matched individuals (de la Monte, et al., 1988). The pattern of degeneration, most highly involving the striatum with a diffuse loss of cerebral white matter, is consistent with symptom development. Degeneration within the striatum typically progresses in caudal to rostral and dorsal to ventral gradients (Vonsattel et al., 1985). Of the cellular populations present in the striatum, the medium spiny neurons (MSNs) are the earliest and most severely affected. Selective degeneration among the MSNs also correlates well with typical motor symptom onset. Subpopulations of MSNs have been identified and categorized based on their projections, the neuropeptides they express, and their neurotransmitter receptors. MSNs which project to the external segment of the globus pallidus (GPe) and express enkephalins, dopamine D2 receptors, and adenosine A2a receptors, are the earliest to be lost in the disease course of HD. In contrast, MSNs that project to the internal segment of the globus pallidus (GPi) and express substance P and dopamine D1 receptors tend to be spared until later in the disease course (Reiner et al, 1988; Albin et al., 1992; Deng et al., 2004). In correlation with the presentation of HD symptoms, degeneration of striato-GPe MSNs leads to inhibition of the substantia nigra, which is associated with chorea. For many patients, as the disease progresses, symptoms transition to dystonia and bradykinesia which is characteristic of the loss of striato-GPi MSNs (Dayalu and Albin, 2015). Because of the highly specific pattern of degeneration observed in HD patients, it was believed that the discovery of the genetic cause would explain this pattern of neurodegeneration.

While the hereditary nature of HD was described by Huntington in 1872, it took over 100 years, vast improvements in positional cloning techniques, many dedicated research teams, and countless research hours to discover the genetic cause (Huntington's Disease Collaborative Research Group, 1993). HD is caused by a CAG repeat expansion in exon 1 of the *huntingtin (HTT)* gene on chromosome 4. *HTT* codes for huntingtin (htt), a large 350 kDa protein found in all metazoans, with the highest conservation among vertebrates. Structural studies suggest that htt forms an elongated superhelical solenoid structure that has several diverse cellular functions (Li et al., 2006; Schulte and Littleton, 2011). The CAG repeat codes for a poly-Q tract expressed near the N-terminus of the protein. A repeat length of greater than 40 glutamines has a 100% penetrance, whereas 6-26 repeats are considered normal, and 36-39 cause incomplete penetrance. While repeat lengths of 27-35 usually do not cause HD, it is important to note that alleles with 27 repeats or higher are unstable and prone to expansion in subsequent generations, an inheritance pattern known as genetic anticipation (Dayalu and Albin, 2015). The htt protein is ubiquitously expressed throughout the body, with the highest levels in brain and testes, and throughout the central nervous system without significant regional differences in virtually all neurons and glial cells (Li et al., 1993; Strong et al., 1993; Lawhrmeyer et al., 1995; DiFiglia et al., 1995; Sharp et al., 1996; Bhide et al., 1996; and Fusco et al., 1999). While the discovery of the gene which causes HD in 1993 has led to an explosion of important findings and potential therapeutic targets, the expression pattern of the protein did not explain the regional and cell type specific dysfunction and degeneration observed in the disease, as many had hoped it would.

Despite the ubiquitous expression of htt throughout the central nervous system, many potential causes for the selective vulnerability observed in HD have been proposed and explored using *in vitro* models and animal models of HD. Many functions of htt itself, as well as the mutated version observed in HD (mhtt), have been proposed since the discovery of the gene, and begin to suggest some possible causes for selective vulnerability. Most evidence supports a toxic gain-of-function role for mhtt, but there is evidence that loss-of-function of htt, which would be haploinsufficiency for most HD patients, also contributes to pathogenesis. Homozygous Htt knockout mice (*Hdh*$^{-/-}$) are embryonically lethal (Nasir et al., 1995; Duyao et al., 1995). Heterozygous animals for the *Hdh* allele also display physical defects and behavioral changes (White et al., 1997). Htt has a large number of protein-interacting domains, and has been shown to potentially interact with over 200 other proteins (Li & Li, 2004;Borrell-Pages et al., 2006). Many htt protein interactors are involved in microtubule-based transport such as Huntingtin-associated protein 1 (HAP1) which promotes the interaction between htt and kinesin, dynactin, and dynein (Engelender et al., 1997; Li et al., 1998; Rong et al., 2006; Caviston et al., 2007). These interactions suggest that htt is important for both retrograde and anterograde axonal transport, a finding which has been supported in several animal models and has implications for the experiments described here. Htt has also been reported to function in the movement of mitochondria (Trushina et al., 2004).

In addition to axonal transport, Htt may play a role in gene transcription. Wild-type htt binds transcriptional regulators, most notably and most relevant to this study, the Repressor Element -1 Transcription Factor/Neuron Restrictive Factor (REST/NRSF) (Zuccato et al., 2003). Htt interaction with REST/NRSF sequesters REST/NRSF in the

cytoplasm, preventing it from binding to neuron-restrictive silencer elements (NRSE) found within many transcriptional regulatory elements in the human genome, including the *brain-derived neurotrophic factor (BDNF)* gene and several other genes required for neuronal growth and electrophysiological function (Zuccato et al., 2003; Bruce et al., 2004). The potential role for BDNF transcription in HD is discussed further in subsequent sections.

While evidence suggests a loss-of-function role contributing to HD pathogenesis, the most prominent support points to a toxic gain-of-function role of mhtt. Recent evidence suggests that abnormal transcription of *HTT* and aberrant splicing of htt mRNA may produce increased levels of the polyQ-expanded allele and a truncated mRNA coding only for exon 1 (Sathasivam et al., 2013). Shorter N-terminal fragments, containing the poly-Q domain, can also be formed by protease activity post-translationally (Landles et al., 2010). These shorter fragments are thought to be highly toxic as individual misfolded molecules or as oligomers. Using animal models of HD as well as post-mortem human tissue, several methods by which these fragments may be toxic to cells have been identified (Ross and Tabrizi, 2011; Shoulson and Young, 2011). Axonal transport and transcriptional regulation, which are both affected by haploinsufficiency of htt as well, are also thought to be directly impaired by mhtt. The combination of both toxic gain-of-function effects as well as a loss of normal htt regulation of transcription and axonal transport may explain how this mutation can be more highly toxic to neuronal cells. Neurons typically have a higher level of transcription than other cell types in the body and also have to transport proteins tremendous distances from the nucleus to axons and dendrites. In addition, mhtt full length proteins

as well as shorter mhtt fragments lead to protein mishandling in which the ubiquitin proteasome system and autophagy-lysosome system are overwhelmed and cannot adequately clear misfolded proteins (Martinez-Vicente et al., 2010; Wong and Holzbaur 2014). Expression of mhtt also leads to altered mitochondrial dynamics, and excitotoxicity (Zeron et al., 2002; Weydt et al., 2006; Shirendeb et al., 2011). It has been argued that all of these toxic gain-of-function effects of mhtt contribute in a combinatorial fashion to HD pathogenesis.

In addition to understanding the pathological functions of mhtt and haploinsufficiency of normal htt in the pathogenesis of HD in a cell autonomous manner, it is equally important to consider whether non-cell autonomous mechanisms also play a role. Important insights from animal models have shown that cell types other than dying neurons themselves are critically involved in the degenerative process of HD, and these non-cell autonomous mechanisms may shed light on the selective vulnerability seen in HD (Lobsiger and Cleveland, 2007). Studies of conditional transgene expression of mhtt, utilizing the Cre-lox system, have demonstrated that expression of mhtt throughout the brain, far beyond the most affected cortico-striatal neurons, is necessary to produce the motor dysfunction and brain pathology seen in HD mouse models (Lobsiger and Cleveland, 2007). While an obvious non-cell autonomous mechanism involves production and transport of BDNF from the cortex to the striatum which will be discussed in more detail later, there is also evidence for the involvement of glial cells in HD pathogenesis (Myers et al., 1991; Zeron et al., 2002; Powers et al., 2007).

Rodent Models of HD

Since the discovery of the genetic cause of HD, a variety of rodent genetic models have been constructed utilizing various aspects of the known mutation. These rodent models vary significantly in their construction, their onset and progression of behavioral phenotypes, and their onset and severity of pathological changes. Studies using these rodent models have contributed significantly to the understanding of the potential pathogenesis of this disease. Despite this progress, however, a significant amount of study is still required in order to fully understand the mechanisms of degeneration and to discover, test, and validate therapeutic targets for treatment and prevention of disease. Rodent models of HD fall into two general categories: transgenic and knock-in models. Within these two broad categories, models vary as to level of expression, length of the mutant allele, and repeat length. I will briefly discuss several of the most widely used mouse models of HD below, indicating their similarities and differences, their face and construct validity as it relates to HD, and a brief description of their time course of major pathological and behavioral phenotypes. I will conclude this section with a description of the mouse model chosen for this project and the rationale for utilizing this particular model.

Transgenic models involve the ectopic, or non-endogenous, expression of the mutation that causes HD. There are two types of transgenic models, truncation and full-length. Several truncation models have been generated, all of which express truncated N-terminal fragments of mHtt. Of the truncation models, R6/2 is the most widely used. R6/2 mice express exon 1 of human *HTT*, originally with 144 CAG repeats (Mangiarini et al., 1996). The R6/2 mice, and indeed all of the truncation models, display an

aggressive phenotype. R6/2 mice develop behavioral and motor deficits by 5 weeks of age, striatal atrophy by 13 weeks, nuclear htt inclusions as early as post-natal day 1, and death within 12-15 weeks (Hickey et al., 2005; Morton et al., 2005; Stack et al., 2005). This rapidly progressing phenotype of R6/2 and the other truncation models makes them attractive to many studies testing preclinical interventions, due to the short time required to reach measurable outcomes. Because they express only an N-terminal fragment of htt, however, their construct validity with HD is not as high as other models, both because of the possibility of additional gain-of-function effects not relevant to human HD and the lack of any loss-of-function effects relevant to human HD. In addition, their face validity with human HD is also low as evidenced by the appearance of several phenotypes such as: epilepsy, diabetes, cardiac dysfunction, and neuro-muscular junction abnormalities, which are not characteristic of classic adult-onset HD (Hulbert et al., 1999; Ribchester et al., 2004; Mihm et al., 2007).

The second classification of transgenic models is full-length transgenic mice. These models were created using yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) technology (Hodgson et al., 1999; Gray et al., 2008). These models express human genomic mutant *HTT* transgenes, including all introns and exons as well as the regulatory sequences up to 23 kb upstream and 117 kb downstream of the gene. BACHD mice express 97 CAG repeats (Gray et al., 2008). A useful feature of the BACHD model is the presence of LoxP sites on either side of the expanded exon 1, allowing for the removal of mhtt expression in specific cell types expressing Cre recombinase (Branda and Dymecki, 2004). The BACHD mice have five copies of the transgene and express mhtt at about 1.5-2 fold the level of endogenous

Hdh (Gray et al., 2008). Motor symptoms appear around 2 months of age and become pronounced by 6-12 months (Brooks et al., 2011). These mice also exhibit some cognitive and behavioral abnormalities, such as enhanced anxiety in the elevated plus maze and enhanced depression in the forced swim test (Holmes et al., 2002; Menalled et al., 2009; Farrar et al., 2014). BACHD mice develop striatal atrophy by 56 weeks of age and nuclear inclusions (mostly cortical) also by 56 weeks (Brooks et al., 2011). Because the BACHD model expresses the full length human *HTT* under the control of the endogenous promoter and repressor sequences, it has higher construct validity with HD than the truncation models; the motor, behavioral, and cognitive symptoms observed in this model demonstrates high face validity as well. The gene is not, however, expressed in the endogenous locus, meaning there will be no loss-of-function effects, thereby these mice have lower construct validity than the knock-in mouse models.

Unlike the transgenic models, knock-in models express mhtt in an appropriate genomic context while also expressing endogenous levels of htt. Several knock-in models have been produced which differ in repeat length as well as the origin of the knocked-in exon 1 and poly-Q expansion. They were generated either by replacing exon I with a human *HTT* exon 1 along with an expanded CAG tract into the endogenous *Hdh* gene locus, or by knocking in only a poly-Q expansion into an otherwise endogenous mouse *Hdh* gene. While there are several knock-in models of varying repeat lengths, I will focus on two models. The $Hdh^{(CAG)150}$ mouse is completely murine and has approximately 150 CAG repeats inserted into exon 1 of the murine *huntingtin* homologue (*hdh*) (Lin et al., 2001; Tallaksen-Greene et al., 2005; Heng et al.,

2007). Heterozygous *Hdh*$^{(CAG)150}$ mice exhibit motor deficits by 100 weeks of age, as well as neuronal loss by 100 weeks of age. They develop neuronal inclusions in the striatum by 40 weeks of age, which are expressed in most striatal cells by 70 weeks of age. The repeat length in these mice has since been expanded to produce a *Hdh*$^{(CAG)200}$ line. An additional knock-in line, the *Q140* mouse was constructed by replacing the endogenous murine *Hdh* exon 1 with a chimeric mouse and human exon 1 with 140 glutamine repeats (Menalled et al., 2003). *Q140* mice develop motor abnormalities by 1 month of age as demonstrated by increased rearing. By 4 months of age, they become hypoactive in open field, and they develop gait abnormality by 12 months. Additionally, they develop selective striatal and cortical nuclear inclusions by 4 months in the striatum and 6 months in the cortex (Menalled et al., 2003; Brooks et al., 2011). The *Q140* model has been expanded to produce the *Q175* model used in our studies. Due to their endogenous expression, the knock-in models have the highest construct validity with HD of the available animal models, and the higher repeat length models display high face validity as well, developing progressive behavioral, motor, and neuropathological changes at midlife.

Heterozygous *Q175* mice develop some mild motor phenotypes in rotorod and grooming time by 6 months which become more severe at 12 and 16 months and expand to include deficits in coordination, nesting, and Y maze performance (Smith et al., 2014). They also develop age-specific progressive nuclear htt aggregates beginning in the striatum and motor cortex by 6 months of age and progressing into the prefrontal cortex and hippocampus at later ages. These mice display a significant loss of striatal volume and motor cortical thickness by 12 months and also have a significant decrease

in DARPP-32 immunoreactivity in the striatum by 12 months of age (Smith et al., 2014). Q175 mice also have significant weight loss by 31-37 weeks of age compared to WT littermates (Menalled et al., 2012) which is also observed in HD patients. The neuropathological changes in Q175 mice have been validated across multiple laboratories (Menalled et al., 2012; Heikkinen et al., 2012; Smith et al., 2014). We chose the *Q175* mouse model for this study due to its high construct and face validity to HD. In particular, we believe that the development of behavioral and neuropathological symptoms in heterozygous mice is essential, given the majority of human HD patients have one normal and one mutated allele of *HTT*. Furthermore, the compelling evidence for both gain- and loss-of-function effects in HD pathogenesis made the Q175 heterozygous mouse model attractive. As mentioned previously, neurotrophic factor dysregulation has been implicated in both the gain- and loss-of-function effects of mhtt, which prompted us to investigate their potential role in HD.

Introduction to neurotrophic factors

Neurotrophins and their receptors are essential to the proper development, physiologic function, and maintenance of the nervous system. They may also have therapeutic potential in the treatment of neurodegenerative disease. Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Huntington's disease (HD) have all had neurotrophins and/or their receptors implicated in their pathogenesis (Kraemer et al., 2014; Mariga et al., 2016). Some of these studies have advanced into clinical trials with varying, but generally limited levels of success

(Mariga et al., 2016). Most of this research, as well as the clinical trials, have focused on late symptomatic stages of disease in terms of palliative measures and/or slowing of disease progression. We believe that pre-symptomatic stages of neurodegenerative diseases represent a promising and under-studied direction for neurotrophin-based intervention.

There are four members of the neurotrophin family: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Each neurotrophin signals through a preferred high-affinity receptor from the specific family of tyrosine kinase receptors known as tropomyosin receptor kinases (Trk): NGF signals through TrkA; BDNF and NT-4 signal through TrkB; and NT-3 signals through TrkC (Barbacid 1995; Huang and Reichardt, 2003). Signaling of neurotrophins through their corresponding Trk receptor is essential for the development, growth, survival, and maintenance of many neuronal populations (Mariga et al., 2016). In addition to their corresponding Trk receptor, each neurotrophin can also bind to the p75 neurotrophin receptor (p75), which was the first high-affinity neurotrophin receptor to be identified. While signaling through Trk receptors almost exclusively promotes growth, survival, and maintenance, signaling through p75 is much more complex.

p75 signaling depends on the availability of ligands, co-receptors, and adaptor proteins (Kraemer et al., 2014). p75 often acts as part of a complex with TrkA which forms a high affinity binding site for NGF and enhances NGF's neurite growth-promoting effects. It was subsequently discovered that p75 also interacts with BDNF, NT-3, and NT-4 (Rodriguez-Tebar et al., 1990; Rodriguez-Teber et al., 1992; Ryden et al., 1995). Interestingly, p75 expression is not limited to cell types that express Trk receptors, and

Trk receptors exhibit a much more restricted pattern of expression than p75 (Cragnolini and Friedman, 2008). p75 has been intensively studied for over 30 years, and its functions are remarkably diverse, ranging from survival/growth promotion, to promoting myelination, and also to regulating and enhancing apoptosis (Kraemer et al., 2014). Due to our focus on the striatum and HD pathogenesis, I will focus subsequently on the neurotrophin signaling pathways associated with striatal maintenance and implicated in HD: BDNF, TrkB, and p75 signaling.

BDNF, TrkB and p75 signaling

BDNF was purified and cloned from pig brain in 1982 (Barde et al., 1982). BDNF, like the other neurotrophins, is biologically active as a homodimeric protein complex. It is initially synthesized as a larger precursor protein, proBDNF, and is cleaved by secretases to form mature BDNF. Both pro-BDNF and mature BDNF are biologically active (Park and Poo , 2013). BDNF is expressed widely throughout the central nervous system (Maisonpierre et al., 1990; Maisonpierre et al., 1991; Conner et al., 1997; Katoh-Semba et al., 1997), and has varied functions in different brain regions. Striatal neurons are highly vulnerable to BDNF deprivation, both during development and postnataly (Rauskolb et al., 2010; Baydyuk et al., 2013). BDNF signals primarily through its cognate survival and plasticity-promoting Trk receptor, TrkB, along with the shared neurotrophin receptor, p75.

TrkB is a type II transmembrane protein with an extracellular domain that is highly glycosylated and contains three leucine-rich repeats flanked by two cysteine

repeats and two immunoglobulin-C2 domains proximal to the transmembrane domain (Deinhardt and Chao, 2014). TrkB also contains a tyrosine kinase domain in its intracellular C-terminus, and BDNF binding to the extracellular domain leads to receptor homo-dimerization and autophosphorylation of the TrkB intracellular kinase domain (Huang and Reichardt, 2003). This phosphorylation leads to the recruitment of adaptor proteins and enzymes that trigger activation of downstream signaling cascades. These phosphorylated tyrosine residues serve as docking sites for intracellular adaptor proteins. One such docking site is Y515 which creates a Shc binding site. This binding can initiate downstream activation of extracellular signal-regulated kinase (ERK), which leads to axonal growth and CREB-mediated transcriptional events (Deinhardt and Chao 2014). This same Shc binding site can also lead to downstream activation of phosphatidylinositol 3-kinase (PI3K) and ultimately Akt activation (Deinhardt and Chao 2014). Akt activation, in turn, leads to enhanced protein translation, axonal growth, and neuronal survival. Phosphorylation of TrkB on the C-terminal tyrosine leads to recruitment and activation of PLC-γ. This pathway can also promote Erk signaling and CREB-related transcriptional changes. All of these pathways activated by TrkB predominantly promote cell survival, growth, and differentiation (Huang and Richardt, 2003; Reichardt, 2006). In addition to these three classical pathways common to all Trk receptors, activation of TrkB can lead to phosphorylation of Src on Tyr 416, inducing its catalytic activity and promoting cell survival (Huang and McNamara, 2010). TrkB signaling relevant to this study is summarized in Figure 1.1.

In addition to serving as a ligand for TrkB, BDNF can also bind to p75. Signaling through p75 can lead to more varied outcomes than signaling through TrkB and also

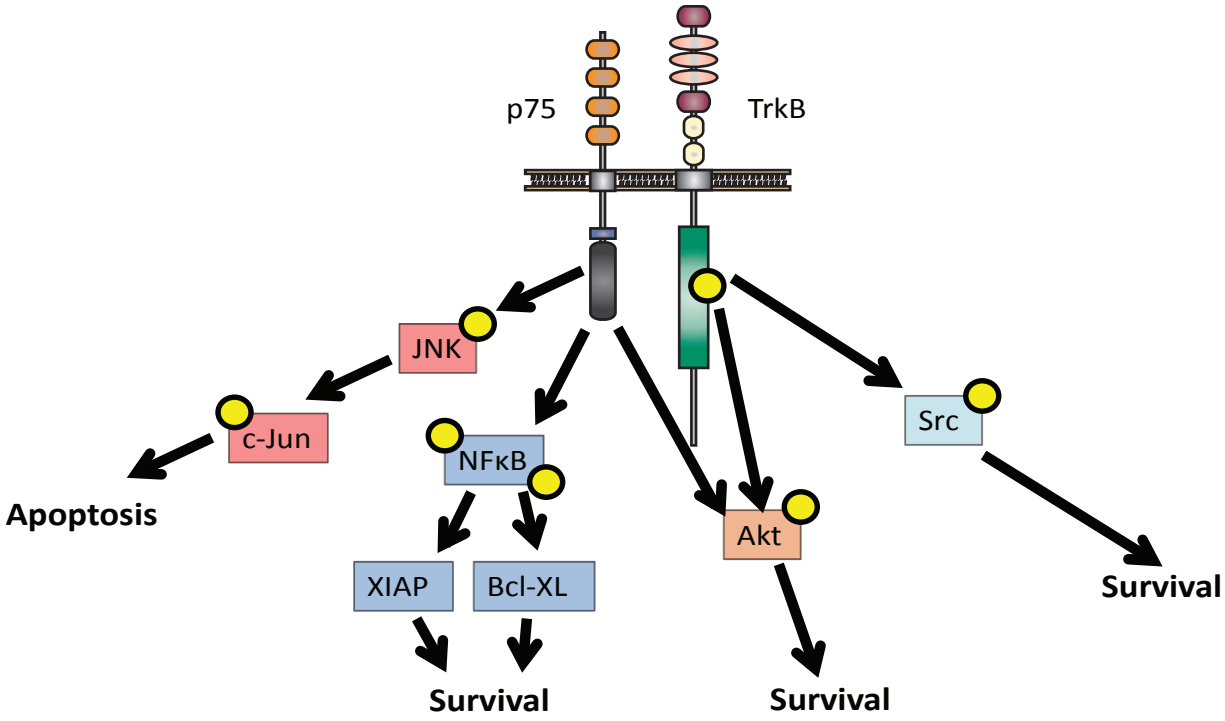

Figure 1.1. p75 and TrkB signaling schematic.
Schematic of the pro-survival and pro-apoptotic signaling downstream of TrkB and p75 measured in this study.

can lead to a wide range of downstream signaling events, depending on available co-receptors and intra-cellular adaptors (Kraemer et al., 2014). p75 was cloned in 1986 and became the founding member of a superfamily known as the tumor necrosis factor receptors (TNFR) (Chao et al., 1986; Radeke et al., 1987). The TNFR superfamily is classified by the presence of cysteine-rich domains (CRDs) in the extracellular domain. These CRD domains are required in p75 for neurotrophin binding (Barker, 2004). Similar to the Trk receptors, p75 binds soluble dimeric neurotrophin ligands (Grob et al., 1985; Gong et al., 2008).

Despite the discovery that p75 is a part of the high affinity binding site for the survival-promoting effects of NGF, perhaps the best studied role of p75 involves apoptotic cell death. Apoptosis mediated by p75 has been demonstrated in a variety of neuronal and non-neuronal cells (Cassacia-Bonnefil et al., 1996; Sedel et al., 1999; Syroid et al., 2000; Shrinivasan et al., 2004; Teng et al., 2005; Volosin et al., 2006; Volosin et al., 2008). The intracellular domain of p75 (ICD) contains a death domain, which can interact with adaptor proteins that lead to apoptosis (Kraemer et al., 2014). The ICD interacts with TNF receptor associated factor 6 (TRAF6), neurotrophin receptor interacting factor (NRIF), neurotrophin receptor-interacting MAGE homolog (NRAGE), and possibly other adaptor proteins (Khursigara et al., 1999; Salehi et al., 2002; Gentry et al., 2004; Linggi et al., 2005). These adaptors lead to activation of the stress kinase cJun N-terminal kinase (JNK) (Khursigara et al., 1999; Dempsey et al., 2003; Gentry et al., 2004; Bertrand et al., 2008). JNK activation phosphorylates pro-apoptotic proteins such as Bim (Lei and Davis, 2003) and Bad (Donovan et al., 2002), and JNK activation also leads to phosphorylation of cJun (Dhanasekaran and Reddy, 2008) resulting in

transcriptional upregulation of a variety of apoptotic proteins including Bax (Miyashita and Reed, 1995), PUMA (Nakano and Vousden, 2001), and Bak (Bogoyevitch and Kobe, 2006). This signaling cascade leads to cytochrome c release from the mitochondria, activation of Caspases 3 and 9, and ultimately apoptotic cell death (Barker, 2004; Boygoyevitch and Kobe, 2006). Interestingly, pro-neurotrophins induce p75-mediated apoptosis at very low concentrations (Lee et al., 2001). Apoptosis mediated by pro-neurotrophins is accomplished by binding to a complex consisting of p75 and Sortilin (Nykjaer et al., 2004). Importantly, in the absence of the relevant Trk receptor, mature neurotrophins have been shown to initiate p75-mediated apoptosis in peripheral and central nervous system neurons (Casaccia-Bonnefil, et al., 1996; Friedman, 2000).

While apoptotic signaling initiated by p75 has been most highly studied, p75 also plays a role in survival signaling through several mechanisms. The pro-survival role of p75 has been most clearly established in peripheral neurons, but has been reported in a wide range of cell types (Kraemer et al., 2014). The majority of the pro-survival function of p75 is thought to be through a high affinity receptor complex formed with members of the Trk family. This high-affinity binding complex was first demonstrated shortly after the identification of p75 (Hempstead et al., 1991). The presence of p75 in the neurotrophin-Trk complex was shown to increase the affinity of TrkA for NGF. The transmembrane and ICD of p75 are essential for formation of this high-affinity binding complex (Esposito et al., 2001). Co-expression of p75 with TrkB also increases the affinity and selectivity of TrkB for BDNF over NT-3 (Bibel et al., 1999). Importantly, not only does p75 increase the affinity and selectivity of Trk receptors for their neurotrophins, but p75 also

potentiates Trk survival signaling (Barker and Shooter, 1994; Verdi et al., 1994; Lachance et al., 1997; Ryden et al., 1997). While the exact mechanism of this potentiation is still being investigated, it may involve stabilizing the Trk receptor on the cell membrane and, therefore, prolonging its signaling, as has been shown for TrkA (Makkerh et al., 2005).

In addition to forming a high affinity binding complex with Trk receptors and augmenting their survival signaling directly, p75 is also capable of inducing survival signaling independently, which may serve to promote survival in the absence of Trk activation. One such survival signaling pathway is Akt. As described above, Akt can be phosphorylated and promote survival downstream of TrkB activation (Huang and Reichardt, 2003). Interestingly, several studies suggest that blocking p75 inhibits the activation of Akt following treatment with neurotrophins *in vitro* (Bui et al., 2002; Ceni et al., 2010), and that p75 activates Akt through the PI3K pathway (Roux et al., 2001). The ability of p75 to promote survival independently of Trk signaling has also been demonstrated in several neuronal populations (Longo et al., 1997; DeFrietas et al., 2001; Bui et al., 2002; Verbeke et al., 2010).

In addition to potentiating Akt activation either in combination with TrkB, or alone, p75 can also promote survival by activating the transcription factor nuclear factor kappa B (NFκB), which has been demonstrated to promote survival in a variety of cells types, both in the peripheral and central nervous systems (Carter et al., 1996; Mattson et al., 1997; Hamanoue et al., 1999; Gentry et al., 2000; Khursigara et al., 2001; Culmsee et al., 2002; Vicario et al., 2015). NFκB is activated by phosphorylation of its inhibitor IκB, after which it releases NFκB, allowing it to translocate into the nucleus (Baldwin, 2012).

Interestingly, the interaction of p75 with TRAF6 has been shown to mediate this activation of NFκB in some cell types (Yeiser et al., 2004), in addition to its role in p75-mediated activation of JNK. While the mechanisms by which TRAF6 mediates selectively survival or apoptosis remains an area of active research, some studies indicate that receptor-interacting protein 2 (RIP2) may be important in this process of choosing survival or apoptosis (Khursigara et al., 2001).

NFκB is a transcription factor composed of five possible subunits: p50, RelA/p65, c-Rel, RelB, and p52, which dimerize in several conformations, of which only the p65, RelB, and c-Rel subunits are able to directly activate transcription of target genes (Sarnico et al., 2009). The p65 subunit of NFκB can be phosphorylated on several different serine residues, which all lead to slightly different transcriptional outcomes. Importantly for our studies, phosphorylation at Ser276 and Ser 536 lead to potent and long-lasting NFκB transcriptional activity (Neumann and Neumann, 2007). Activation of NFκB leads to the upregulation of anti-apoptotic proteins, including B-cell lymphoma-extra large (Bcl-XL) and X-linked inhibitor of apoptosis (XIAP) (Stehlik et al., 1998; Chao et al., 2011). Signaling through p75 relevant to this study is summarized in Figure 1.1. The widely diverse functions of p75 indicate that it is a key signaling component in a wide assortment of biological responses. This diversity necessitates a careful examination of the function of p75 in neurodegenerative disease because the role p75 signaling may play depends on the specific circumstances and cellular context. Given the ability of p75 to promote both survival and apoptosis, it may be a novel target for disease treatment if its survival signal can be preferentially activated.

Role of BDNF, TrkB, and p75 in Neurodegenerative Diseases

Neurotrophins and their receptors have been implicated in a wide variety of nervous system disorders including developmental neuropathies, mood disorders, addiction, and neurodegenerative disease (Zuccato and Cattaneo, 2009; Ibanez and Simi, 2012; He et al., 2013; Cai et al., 2014; Meeker and Williams, 2014; Mariga et al., 2016). For the relevance to this study, this section will focus on the proposed roles of BDNF, TrkB, and p75 in neurodegenerative disease.

Given the role of neurotrophins, especially BDNF, in the function and maintenance of the central nervous system, it is not surprising that reduced expression of BDNF has been observed in several neurodegenerative diseases. Alzheimer's disease (AD) has been investigated in terms of the role of neurotrophins both in the pathogenesis and as a potential therapeutic approach. AD is characterized neuropathologically by degeneration in the cholinergic neurons of the basal forebrain (Teipel et al., 2005), the limbic system (Arnold et al., 1991; Klucken et al., 2003), and the neocortex (Terry et al., 1981), as well as the deposition of plaques containing amyloid-β (Aβ) and tau tangles. Analysis of post-mortem AD brains and animal models of AD display reduced levels of BDNF mRNA and protein (Phillips et al., 1991; Narisawa-Saito et al., 1996; Durany et al., 2000; Hock et al., 2000; Lee et al., 2005). This decrease is particularly apparent in the hippocampus, an area which degenerates early in AD (Phillips et al., 1991; Ginsberg et al., 2010).

It is important to note, however, that there are some conflicting studies that demonstrated increased levels of BDNF in the hippocampus of post-mortem AD brains

and in the APP model of AD (Murer et al., 2001; Burbach et al., 2004; Tapia-Arancibia et al., 2008). Despite the conflicting results regarding BDNF levels in AD, some studies indicate a positive effect of BDNF on learning and memory; deficits in both are hallmarks of AD (Ando et al., 2002). In addition to its effects on learning and memory, administration of BDNF in animal models of AD has led to neuroprotective effects against amyloid-β (Aβ) induced toxicity, which is also a hallmark of AD neuropathology (Arancibia et al., 2008). BDNF gene delivery has also been demonstrated to have protective effects on cortical and hippocampal degeneration in AD-like animal models, leading to improvement of cognitive and behavioral phenotypes (Nagahara et al., 2009). These conflicting results on the expression of BDNF during the progression of AD argue that further study is needed to aid in the evaluation of the potential therapeutic benefit of BDNF treatment.

A link has also been proposed between p75 and AD. p75 is highly expressed in the cholinergic cells of the basal forebrain which degenerate early in AD. There have also been studies indicating Aβ can serve as a ligand for p75, thereby inducing apoptosis via p75 (Yaar et al., 1997; Hashimoto et al., 2004; Costnatini et al., 2005). The studies examining the proposed pro-apoptotic role of p75 in AD have, however, also reported conflicting results. Some studies, for example, have shown protective effects of p75 deletion in animal models of AD (Sotthibundu et al., 2008), while others indicate p75 expression is protective (Zhang et al., 2003; Benhoechea et al., 2009) against Aβ-induced toxicity. While these studies represent only a small portion of the literature relating to the potential role of p75 in AD pathogenesis, they argue that future research is necessary to determine the exact functions of p75 in AD.

While AD has received significantly more attention, there have been some indications of neurotrophin involvement in Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS). PD is characterized by progressive loss of dopaminergic neurons in the substantia nigra important for control of movement. While the evidence for involvement of neurotrophins in PD is limited, reduced BDNF mRNA has been observed in the substantia nigra of post-mortem PD patients (Parain et al., 1999; Murer et al., 2001). There is also evidence linking mutations in α-synuclein, which are associated with familial forms of PD, to BDNF production (Kohno et al., 2004; Zuccato and Cattaneo, 2009). Several studies also report some success with BDNF administration into animal models of PD (Levivier et al., 1995; Isacson et al., 1995; Tsukahara et al., 1995; Murer et al., 2001). While these studies demonstrate that future research on the role of BDNF in PD may be beneficial, glial cell line-derived neurotrophic factor (GDNF) remains the most studied trophic factor in PD pathogenesis to date.

BDNF and p75 have both been investigated in ALS research as well. ALS is characterized by progressive degeneration of both upper and lower motor neurons. Many of the motor neurons that degenerate in ALS express both BDNF and TrkB (Nishio et al., 1998). Additionally, BDNF treatment has been shown to prevent lesion-induced degeneration of spinal motor neurons and motor neuron degeneration in animal models of ALS (Mitsumoto et al., 1994; Tuszynski et al., 1996). Clinical trials using BDNF treatment for ALS have had mixed success (Ochs et al., 2000). Researchers have also reported an increased level of p75 expression in spinal motor neurons of a mouse model of ALS, as well as in spinal cord samples from ALS patients (Seeburger

et al., 1993; Lowry et al., 2001; Copray et al., 2003). Manipulation of p75 signaling has also shown to ameliorate some disease-related phenotypes in animal models of ALS (Turner et al., 2003). Collectively, these studies warrant additional investigation of BDNF as well as its receptors, p75 and TrkB, as potentially promising therapeutic targets for the treatment of ALS.

Perhaps the best studied role of BDNF in neurodegenerative disease is in HD. Reduced levels of BDNF protein have been reported in the putamen and caudate of human post-mortem brains and in several animal models of HD (Ferrer et al., 2000; Zuccato et al., 2001; Zuccato and Cattaneo, 2007; Zuccato et al., 2008). BDNF has been reported by multiple laboratories to be produced in the cortex, anterogradely transported to the striatum, and released to promote growth and survival of the MSNs which are selectively vulnerable in HD (Altar et al., 1997; Baquet et al., 2004; Baydyuk et al., 2011; Baydyuk et al., 2013). Loss of this anterograde transport of BDNF has been shown to lead to deficits in dendritic maturation and branching, as well as subsequent neuronal loss in the striatum (Baquet et al., 2004). A mechanistic link between BDNF transcription and htt was first proposed in 2001 (Zuccato et al., 2001). Wild-type htt was shown to increase cortical production of BDNF, and mutated htt caused a reduction of BDNF in the striatum (Zuccato et al., 2001). Wild-type htt has since been shown to sequester RE1-silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) in the cytoplasm, preventing its translocation to the nucleus (Zuccato et al., 2003). This interaction, however, appears to be indirect through an interaction between htt and REST/NRSF-interacting LIM domain protein (RILP) (Shimojo, 2008). Mutated htt is unable to sequester REST/NRSF in the cytoplasm, allowing it to

translocate to the nucleus where it binds to repressor element 1/neuron-restrictive silencer elements (RE1/NRSE) throughout the genome. This binding silences transcription (Timmusk et al., 1999). There is an RE1/NRSE site in promoter II of the *BDNF* gene (Palm et al., 1998; Timmusk et al., 1999), indicating that release of REST/NRSF from its sequestration in the cytoplasm inhibits the production of BDNF transcripts from promoter II.

In addition to affecting transcription of BDNF, mhtt has also been shown to impair anterograde transport of BDNF. As mentioned above, wild-type htt interacts with kinesin motor complexes and is thought to play a role in vesiclular transport along axons. Gauthier et al. used BDNF tagged with green fluorescent protein (GFP-BDNF) to demonstrate that ectopic expression of htt led to increased transport of BDNF vesicles. The same effect was not seen following ectopic expression of mhtt (Gauthier et al., 2004). These studies provide support for a mechanistic link between mhtt and the observed decrease in BDNF protein in the striatum of post-mortem HD patients and in animal models of HD. BDNF levels have also been demonstrated to the reduced in the striatum of the *Q175* mouse line by 6 months of age and in the cortex by 12 months of age (Ma et al., 2015).

Both receptors for BDNF, TrkB and p75, have also been implicated in the pathogenesis of HD. The vesicle transport role of htt has been proposed to also function in the retrograde transport of TrkB-containing vesicles from the dendrites to the soma of MSNs. Upon activation by BDNF stimulation, TrkB-positive endosomes have been shown to be internalized and transported along microtubules to the cell body to induce survival signals (Watson et al., 1999; Heerssen et al., 2004; Ha et al., 2008). A study

from the Saudou laboratory used microfluidic devices to demonstrate that BDNF stimulation leads to TrkB association with htt and dynein, resulting in transport in cultured striatal neurons. This transport was inhibited by silencing htt and the expression of mhtt in wild type striatal neurons, and was also inhibited in striatal neurons from a mouse model of HD (Liot et al., 2013). This study suggests that mhtt may decrease striatal BDNF/TrkB survival signaling through not only decreased BDNF production and transport from the cortex, but also through reduced transport of activated TrkB to the cell body of MSNs. Additional studies in animal models of HD have also suggested altered levels of TrkB expression the striatum. These studies, however, have often been contradictory with some finding increased levels of TrkB at various ages while others report decreased levels of TrkB, and yet others report no change (Brito et al., 2013; Simmons et al., 2013; Smith et al., 2014; Ma et al., 2015). There has, however, been consistent evidence of a decrease in TrkB activation in the striatum of HD mouse models (Brito et al., 2013; Simmons et al., 2013; Plotkin et al., 2014; Ma et al., 2015). Importantly, both the Hallett and Hempstead labs found no change in levels of TrkB at 6 and 12 months, respectively, in the striatum of the *Q175* mouse model of HD (Smith et al., 2014; Ma et al., 2015).

While the studies exploring the involvement of BDNF and TrkB in HD pathogenesis began over fifteen years ago, p75 is only beginning to be appreciated for its potential role. The involvement of p75 in HD was first suggested in 2008 when Zuccato et al. described increased levels of p75 mRNA in the caudate, but not cortex, of post-mortem brains of HD patients (Zuccato et al., 2008). Since publication of this finding, p75 protein levels have since been further examined in post-mortem brains of

HD patients and in several animal models of HD. The Gines laboratory reported an increased expression of p75 protein in the striatum of homozygous *Q111* mice beginning at 2 months as well as in heterozygous *Q111* mice by 6 months (Brito et al., 2013; Brito et al., 2014). Additionally, this same laboratory reported an increase in p75 expression in the striatum of R6/1 mice beginning at 12 weeks of age, as well as in the putamen of post-mortem HD patients (Brito et al., 2013). Based on these results, in combination with the decrease they reported in TrkB levels in the striatum, the Gines laboratory proposed an imbalance in p75/TrkB expression as a potential pathogenic mechanism in HD.

They also observed a similar increase in p75 expression in the hippocampus of the *Q111* and R6/1 mice at 2 months of age, but not in the cortex (Brito et al., 2014). As a test of their hypothesis, the Gines laboratory made *Q111* mice heterozygous for p75, reporting decreased p75 levels in these mice. Using this mouse line, they reported a rescue in learning and memory deficits (Brito et al., 2014). In addition to these studies, the Surmeier laboratory has also explored the role of p75 in the BACHD animal model of HD. This study examined signaling dysfunction in iSPNs in the striatum, and they reported that plasticity was rescued in these neurons by knocking down or inhibiting p75 (Plotkin et al., 2014). While these studies have focused on the potentially apoptotic/pathogenic role of p75, our knowledge of the function of p75 in HD is limited. As discussed previously, p75 signaling is also capable of promoting neuronal survival, both in the presence and independently of Trk receptors. This potential survival or compensatory role for p75 in HD has not been investigated. The role p75 may play in the pathogenesis of HD is likely to be more complex than has been initially appreciated,

and our experiments aimed to address the potential contributions of p75 in both apoptotic and survival signaling in the striatum of *Q175*$^{WT/HD}$ mice.

Overview of Study

As just described in the previous section, p75 has been implicated in the pathogenesis of HD. Because p75 can function in many diverse signaling pathways (Fig 1.1), we sought to explore which of these functions p75 is performing in the *Q175* mouse model of HD at various time points during disease progression. The three time points examined were chosen to represent an early time point (Chapter 3), a time point right before the onset of behavioral phenotypes (Chapter 2), and a post-symptomatic time point (Chapter 4). First, p75 and TrkB levels were measured in the striatum, and the protein-protein association between p75 and TrkB was also investigated. Then, to test the potential survival signaling effects of p75 in the striatum Akt activation and phosphorylation of the p65 subunit of NFκB were measured, as well as expression levels of XIAP and Bcl-XL. To investigate potential apoptotic signaling downstream of p75, JNK and cJun activation were measured. Finally, function and degeneration of the striatum were measured by DARPP-32 expression and striatal volume calculations. To determine the involvement of p75 in the changes observed, these same tests were performed in *Q175* mice bred into a germline p75 knockout mouse line and compared.

Collectively, our results indicate that p75 plays an important role in augmenting survival signaling in the striatum of pre-symptomatic *Q175*$^{WT/HD}$ mice and that disruption of this signaling leads to earlier onset of dysfunction and degeneration. We did not

observe changes in expression of p75 or TrkB in the striatum at any age examined. We did, however, observe an association between p75 and TrkB in the striatum at all three ages examined, and this association was significantly increased in *Q175*$^{WT/HD}$ mice at 2 months of age compared to WT littermates. We also observed increases in Akt activation at both 2 and 5 months in the *Q175*$^{WT/HD}$ mice, increases in phosphorylation of NFκB at 5 months, and increases in XIAP expression at 5 months of age. Importantly, these increases were abolished in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice. We also observed a decrease in Bcl-XL and DARPP-32 expression in the striatum of *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice at 5 months. This decrease in DARPP-32 expression continued in 10/12 month old mice. We did not observe any changes in survival signaling at 10/12 months, nor any changes in JNK activation at any age examined. Finally, we saw a decrease in striatal volume at 12 months in both *Q175*$^{WT/HD}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice. Importantly, the striatal volume of *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice was also significantly lower than the striatal volume of *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice. These results, taken together, indicate that p75 plays a role in augmenting survival signaling in the striatum of *Q175*$^{WT/HD}$ mice at pre-symptomatic ages, which wanes as the animals become increasingly symptomatic. If this increase in survival signaling is abolished by deletion of p75, the mice develop earlier striatal dysfunction and more severe striatal degeneration, suggesting an important role for p75 in inhibiting striatal degeneration during the progression of HD. The results from Chapter 2 and Chapter 4 (Fig. 4.9) have been published in *Neuroscience* and the results from the Appendix have been published in *Development*.

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Chapter 2: Examining the role of p75 signaling in the striatum of *Q175*$^{WT/HD}$ mice at 5 months of age

Introduction

Huntington's disease (HD) is an adult-onset, progressive, and invariably fatal neurodegenerative disease characterized by a constellation of motor, cognitive, and psychiatric features. HD is dominantly-inherited and caused by a CAG repeat expansion in the *huntingtin* gene (Group, 1993). There are only limited symptomatic treatment options and no disease-modifying therapies. Striatal medium spiny neurons (MSNs) are one of the earliest and most affected populations of neurons (Walker, 2007). MSNs are dependent on cortically-derived neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), anterogradely transported to the striatum for normal development, function, and maintenance (Baydyuk, et al., 2013; Baquet et al., 2004; Mizuno et al., 1994).

Huntingtin protein (HTT) regulates cortical BDNF transcription, with mutated HTT (mHTT) being impaired in its ability to regulate BDNF transcription (Zuccato et al., 2001). The *BDNF* gene contains as many as eight promoters, with promoters I, II, and IV being most highly transcriptionally active and well-studied (West et al., 2014). BDNF transcription from promoter II depends largely on the activity of the repressor element 1/neuron-restrictive silencer element (RE1/NRSE). This sequence recruits RE1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF), which actively silences transcription (Timmusk et al., 1999). HTT sequesters REST in the

cytoplasm, preventing it from binding to the RE1/NRSE, and subsequently leading to higher levels of BDNF transcription from promoter II. mHTT is less able to sequester REST in the cytoplasm, leading to reduced transcription of BDNF from promoter II (Zuccato et al., 2003). Consistent with these studies, in several mouse models of HD and in post-mortem HD brains, lower levels of cortical BDNF expression were reported (Zuccato et al., 2008). In addition, HTT may be involved in the anterograde trafficking of BDNF from the cortex to the striatum (Gauthier et al., 2004), which is also consistent with the lower levels of BDNF observed in the striatum of several animal models of HD and in post-mortem HD brains (Her & Goldstein, 2008; Ferrer et al., 2002). BDNF protein levels can be difficult to measure due to the lack of antibodies of sufficient specificity and sensitivity. To overcome this difficulty and investigate the level of BNDF protein in the striatum, Ma et al. crossed the Q175 mouse line to a line expressing BDNF with an HA epitope tag. Using highly sensitive and specific HA antibodies, a decrease in BDNF protein levels in the striatum of Q175 mice at 6 months of age was observed (Ma et al., 2015). A decrease in BDNF levels and signaling in the striatum is proposed to contribute to the pathogenesis of HD (Zuccato & Cattaneo, 2007).

There are two known BDNF receptors, Tropomyosin receptor kinase B (TrkB) and p75 neurotrophin receptor (p75). Both TrkB and p75 are expressed in the striatum. Signaling downstream of TrkB activation almost exclusively promotes survival and metabolic function, and disruption of TrkB signaling is likely detrimental to MSN functional integrity. While TrkB levels and trafficking were studied in multiple animal models of HD and in post-mortem HD brains, p75 has been examined only recently. p75 mRNA levels were reported to be elevated in post-mortem HD striata (Zuccato et

al., 2008), and striatal p75 protein levels were reported as elevated in two mouse models of HD (Brito et al., 2013). Unlike TrkB, signaling downstream of p75 is highly context-dependent and may support neuronal survival or contribute to neuronal dysfunction and death. p75 can be activated by all four neurotrophins, pro-forms of neurotrophins, and other ligands (Hempstead, 2002; Nykjaer et al., 2005).

A member of the TNF family of death receptors, p75 is capable of inducing apoptosis when complexed with pro-apoptotic co-receptors or adaptor proteins, such as sortilin and NRIF (Nykjaer et al., 2004). p75 can also form receptor complexes with Trk receptors, however, and these interactions are crucial for maximal survival signaling in several neuronal populations (Ceni et al., 2010; Kommaddi et al., 2011; Matusica et al., 2013; Negrini et al., 2013). p75 was shown to stabilize activated Trk receptors on the membrane, extending survival signaling (Makkerh et al., 2005). p75 is also capable of promoting survival signaling independently of Trk receptors through activation of nuclear factor kappa B (NFκB) (Carter et al., 1996). Based on the ability of p75 to complement Trk signaling and to promote survival signaling independently of Trk activation, we hypothesized that p75 signaling augments survival signaling in the striata of HD mouse models.

To explore the role of p75 in HD, we crossed a germline p75 knock-out mouse into the Q175 mouse model of HD. The Q175 mouse is a knock-in model containing approximately 200 glutamine repeats in the closely related murine homologue of *htt* (*hdh*). These mice develop behavioral phenotypes and pathological changes beginning at 6-12 months of age (Smith et al., 2014). In order to investigate signaling changes and the function of p75 prior to the onset of visible pathology, we examined mice at 5

months of age to determine whether p75 had a compensatory protective function for MSNs. We examined the effect of p75 deletion on both pro-survival and pro-apoptotic signaling in the following genotypes: *Q175*$^{WT/WT}$*;p75*$^{+/+}$, *Q175*$^{WT/HD}$*;p75*$^{+/+}$, *Q175*$^{WT/HD}$*;p75*$^{-/-}$, and *Q175*$^{WT/WT}$*;p75*$^{-/-}$. Our data suggested that p75 plays an early role in augmenting survival signaling in the striatum and that disruption of p75 signaling at a pre-symptomatic age may exacerbate pathologic changes in *Q175*$^{WT/HD}$ mice.

Methods

Mice

Heterozygous Q175 (*Q175*$^{WT/HD}$) knock-in mice (C57Bl/6 background) and wild type (*Q175*$^{WT/WT}$) littermate controls were obtained from Jackson Laboratories (Stock #027410). CAG repeat numbers were in the range of 176-201 (reported by Jackson Laboratories). Mice arrived in one cohort at 5 months of age, with an equal number of males and females used for biochemical analyses. A separate cohort of *Q175*$^{WT/HD}$ mice were used for breeding. p75 deletion was achieved by crossing *Q175*$^{WT/HD}$ mice with *p75*$^{-/-}$ mice in a mixed background (C57Bl/6 and 129/Sv) (Bogenmann et al., 2011) for two successive generations to produce the necessary genotypes. All mice examined were littermates. All mice were housed with access to food and water ad libitum under a 12:12 hr light/dark cycle. All animal procedures were performed in accordance with National Institutes of Heath guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

Striata dissections

Mice were anesthetized by isoflurane, euthanized by decapitation, and striata were rapidly isolated using a Zivic instruments mouse brain slicer which produces 1 mm thick slices. The striata were isolated as individual hemispheres from two successive slices. Each hemisphere of the striata was immersed in 300 µL of immunoprecipitation buffer (10% glycerol, protease inhibitors, sodium vanadate in TBS, pH 6.8) with each hemisphere treated individually. Tissues were homogenized rapidly (Tissuelyser II, Qiagen) and detergent-extracted (Addition of nonidet P-40 to 1%) by incubation for 30 minutes, with rotation, at 4°C. To produce whole cell lysates (WCLs), one hemisphere from each animal was mixed with 300 µL 2X SDS sample buffer (20% glycerol, 4% SDS, 1% β-ME, and bromophenol blue in TBS, pH 6.8), boiled for 6 min, resolved by 4-20% SDS-PAGE and transferred to PVDF membranes. Following detergent extraction, the second hemisphere was treated as follows: insoluble debris was removed by centrifugation and the supernatants subjected to immunoprecipitation using 2 µg of anti-p75 (rabbit, Millipore, catalog 07-476). The immune complexes were washed and denatured in 25 µL 2x SDS sample buffer, and processed as mentioned above.

Immunoblotting and antibodies

Membranes were incubated with blocking solution (3% BSA or 4% milk) in TBS-T (Tris buffered Saline, pH 7.4, with 0.1% Tween-20) and incubated overnight with the appropriate primary antibodies in blocking solution. The blots were washed, incubated with appropriate HRP-linked secondary antibodies (Jackson ImmunoResearch), washed again, and visualized using an enhanced chemiluminescent substrate (Advansta and

ThermoScientific). The following primary antibodies were used: anti-p75 (Promega, catalog G323A), anti-p75 (Millipore, catalog 07-476), anti-p75 (Advanced Targeting Systems, AB-N01AP), anti-TrkB (BD biosciences, catalog 610101), anti-actin (Iowa Hybridoma Bank, catalog JLA 20-9), anti-Bcl-XL (Abcam, catalog ab 178844), anti-XIAP (Abcam, catalog ab28151), anti-DARPP-32 (Abcam, catalog ab40801), anti-phospho-c-Jun (catalog 9261), anti-phospho-JNK (catalog 9251), anti-phospho-Akt (Y243/241, catalog 4058), anti-phospho-Src (Y416, catalog 2101), and all non-phosphorylated versions of these proteins (Cell Signaling Technologies). All images were quantified using ImageJ software (NIH) and graphed as means +/- SEM.

Perfusion and immunohistochemistry

7-8 month old mice were terminally anesthetized with isofluorane and perfused transcardially with heparinized saline (0.1%) followed by 4% paraformaldehyde (PFA) in phosphate buffer. Brains were removed and post-fixed in 4% PFA for 1 hr before cryoprotection in 30% sucrose for 48 hours. Brains were frozen following cryoprotection in isopentane chilled for 2 hours over dry ice and stored at -80°C until use. Coronal sections were cut at 60 µm on a cryostat (CM1950, Leica), and stored in cryoprotection solution at -20°C until use. For immunohistochemistry, sections were rinsed in PBS and endogenous peroxidases were quenched in 1% hydrogen peroxide for 10 min. After rinses, sections were incubated in 2.5% normal donkey serum (Jackson labs) and MOM (Vector, for NeuN-staining) in 0.3% Triton X-100 in PBS. Sections were then incubated for 15-18 hours at RT with either anti-NeuN (1:10,000; Millipore) or anti-DARPP-32 (1:300,000; Abcam). After washing, sections were incubated with a biotinylated secondary antibody (anti-mouse or rabbit, 1:500; Jackson Immunoresearch). Staining of

tissue-bound antibodies was visualized using a standard peroxidase-based method (Vectastain Elite, Vector Labs) with the 3, 3-diaminobenzadine chromogen (DAB, Sigma).

Image analysis and cell counting

Immunohistochemical images were collected using a Zeiss Axiovert 200M inverted microscope at 10X and 64X. All conditions were imaged using identical microscope settings and entire striata were tiled together for analysis. From a randomized starting point, every sixth slice (60µm thickness) containing the striata was imaged (an average of 6 slices per animal). For volume measurements, contours were drawn around the striatum on every sixth slice, and volume was estimated using the Cavalieri volume estimation method. For neuronal density measurements, every NeuN+ cell was counted from randomly chosen regions on every sixth slice containing the striata. Sampling was evenly distributed from rostral to caudal and medial to lateral. Every NeuN+ cell in an unbiased selection of 8 boxes of defined volume were counted per slice and divided by the total area. All cells were counted by an experimenter blinded to genotype.

Statistics

All results are expressed as mean ± SEM. Experimental data from *Q175;p75* mice were analyzed by one-way ANOVAs followed by post hoc Tukey-Kramer minimum significant differences comparison. Experimental data from *Q175* mice were analyzed by Student t-tests. A value of $p<0.05$ was accepted as denoting statistically significant differences. To justify parametric testing, all data sets were tested for normality using

the Shapiro-Wilk test for normality. Sampling was random, and all samples are independent. Homogeneity of variance was determined using the Brown-Forsyth test.

Results

p75 associates with TrkB in the striatum of Q175 mice

Although the levels of p75 were reported to be increased in several animal models of HD, levels of p75 have not been examined in *Q175*$^{WT/HD}$ mice. We examined p75 protein levels in striata of *Q175*$^{WT/WT}$ and *Q175*$^{WT/HD}$ mice both following immunoprecipitation (IP) and from whole cell lysates (WCLs) at 5 months of age (Fig. 2.1). This age was chosen because it is a pre-symptomatic time point and may provide insight into pathophysiological changes occurring before onset of overt pathology. p75 levels ascertained from IPs were not significantly decreased in *Q175*$^{WT/HD}$ mice, although a trend appeared to be emerging ($p=0.06$; Fig. 2.1 A, B). p75 levels from WCLs were not altered (Fig. 2.1 A, D). We also examined potential association between TrkB and p75 to determine whether p75 forms a complex with TrkB. TrkB co-immunoprecipitated with p75 to a similar extent in all animals examined (Fig. 2.1 A, C). TrkB levels were also measured independently of their p75 association in WCLs, and no significant difference in TrkB protein levels was observed in striata of *Q175*$^{WT/WT}$ and *Q175*$^{WT/HD}$ mice (Fig. 2.1 A, E). Taken together, in striata of 5 month-old *Q175*$^{WT/HD}$ mice, p75 and TrkB were associated, and there was no significant change in striatal levels of either p75 or TrkB when compared to *Q175*$^{WT/WT}$ mice.

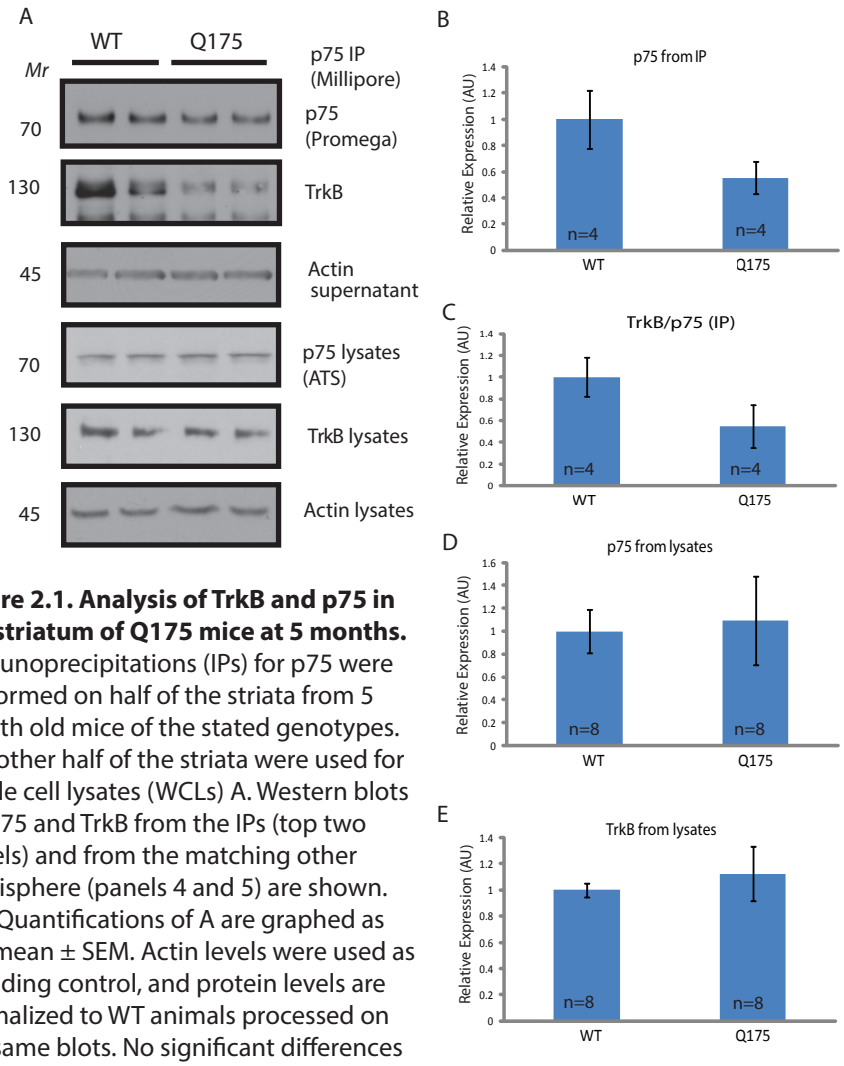

Figure 2.1. Analysis of TrkB and p75 in the striatum of Q175 mice at 5 months. Immunoprecipitations (IPs) for p75 were performed on half of the striata from 5 month old mice of the stated genotypes. The other half of the striata were used for whole cell lysates (WCLs) A. Western blots for p75 and TrkB from the IPs (top two panels) and from the matching other hemisphere (panels 4 and 5) are shown. B-E. Quantifications of A are graphed as the mean ± SEM. Actin levels were used as a loading control, and protein levels are normalized to WT animals processed on the same blots. No significant differences in p75 or TrkB levels between WT and Q175 mice were observed (t-test; $p>0.05$).

Apoptotic signaling pathways were not altered in the striatum of *Q175$^{WT/HD}$* mice at 5 months

In some cellular contexts, signaling through p75 leads to activation of apoptotic signaling pathways, caspase activation, and programmed cell death (Kraemer, 2014). To determine if apoptotic signaling was enhanced at 5 months in *Q175$^{WT/HD}$* mice as compared to *Q175$^{WT/WT}$* mice, we examined the level of activation of JNK and c-Jun, as well as expression levels of PTEN. Activation of JNK by phosphorylation leads to phosphorylation and activation of c-Jun, which then leads to transcription of pro-apoptotic genes (Freeman et al., 2004; Putcha et al., 2001). Activation of JNK also leads to activation of pro-death members of the Bcl-2 family, such as Bim and Bax (Bogoyevitch & Kobe, 2006). PTEN inhibits the PI3K/Akt signaling pathway (Datta et al., 1999). PTEN levels and JNK and c-Jun phosphorylation are increased by pro-apoptotic p75 activation (Kraemer et al., 2014). WCLs of *Q175$^{WT/WT}$* and *Q175$^{WT/HD}$* mice were examined by p-JNK, JNK, and p-c-Jun immunoblotting. Phosphorylation of c-Jun leads rapidly to its own transcriptional upregulation and therefore, normalizing to total c-Jun levels can lead to an underrepresentation of the extent of c-Jun activation (Eilers et al., 1998). The 46 KD isoform of JNK was analyzed as it was significantly more abundant in our samples than the 54 KD isoform. When comparing *Q175$^{WT/HD}$* mice to *Q175$^{WT/WT}$* littermates, there were no differences in striatal JNK or c-Jun phosphorylation (Fig. 2.2 A, B, C). Likewise, PTEN immunoblotting did not reveal any significant differences between *Q175$^{WT/HD}$* mice and *Q175$^{WT/WT}$* mice (Fig. 2.2 A, D). Given that the activation of JNK and c-Jun and the levels of PTEN were unchanged, there does not appear to be

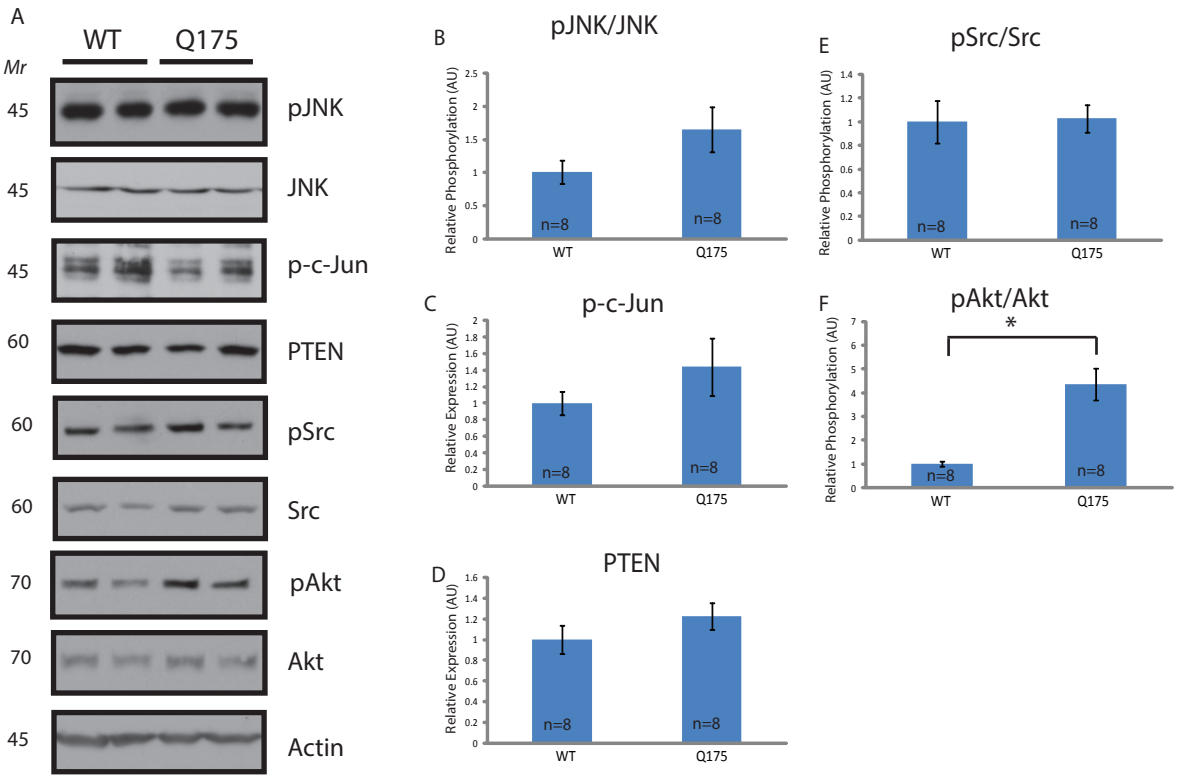

Figure 2.2. Biochemical analysis of apoptotic and survival signaling in the striatum of Q175 mice at 5 months. WCLs of the striata from 5 month old mice of the stated genotypes were analyzed by immunoblotting. A. Western blots were run on WCLs using the stated antibodies. B-F. Quantifications of A. were performed as in Figure 1. No significant differences were seen between WT and Q175 mice in JNK, cJun, or Src activation, or in PTEN levels. A significant increase in Akt activation was observed. (t-test $p<0.05$).

an increase in p75-related apoptotic signaling in the striatum of *Q175*$^{WT/HD}$ mice at 5 months of age.

Akt activation was increased in the striatum of *Q175*$^{WT/HD}$ mice at 5 months

While p75 signaling can lead to apoptosis, it can also lead to survival when activated independently or when paired with the correct co-receptors and downstream effectors. To examine whether survival pathways were affected in *Q175*$^{WT/HD}$ striata at 5 months of age, we examined phosphorylation of Src on Tyr416 and Akt on Ser473. Src can be phosphorylated on Tyr416 following TrkB activation and this phosphorylation event induces Src catalytic activity, enhancing cell survival (Huang & McNamara, 2010). By immunoblotting, there was no change in Src activation in the striata of *Q175*$^{WT/HD}$ mice as compared to *Q175*$^{WT/WT}$ mice (Fig. 2.2 A, E). Akt activation, which can be monitored via Akt phosphorylation at Ser473, promotes survival by inactivating Bad and Caspase 9, among other pro-apoptotic proteins (Datta et al., 1999). We examined Akt phosphorylation because it can be activated by TrkB and p75 independently (Datta et al., 1999; Kraemer et al., 2014). Phosphorylation of Akt at Ser473 was significantly increased in the striata of *Q175*$^{WT/HD}$ mice when compared to *Q175*$^{WT/WT}$ littermates (Fig. 2.2 A, F). This increase in activation of Akt in the striata of *Q175*$^{WT/HD}$ mice at 5 months of age suggests augmented survival signaling.

Analysis of p75 and TrkB in *Q175;p75* mice

After surveying survival and apoptotic signaling pathways in *Q175*$^{WT/HD}$ mice and *Q175*$^{WT/WT}$ littermates, we evaluated the functional role of p75 in pro-survival and pro-apoptotic signaling. To address this question, *Q175*$^{WT/HD}$ mice were crossed into a germ-line knock-out of p75 (Bogenmann et al., 2011), and the following genotypes were examined at 5 months of age: *Q175*$^{WT/WT}$*;p75*$^{+/+}$, *Q175*$^{WT/HD}$*;p75*$^{+/+}$, *Q175*$^{WT/HD}$*;p75*$^{-/-}$, and *Q175*$^{WT/WT}$*;p75*$^{-/-}$. In this mixed background, we again examined p75 levels by immunoprecipitation (IP) and by direct immunoblotting (WCL). In attempting to probe the WCLs for p75 using a commonly reported p75 antibody (Promega), our results yielded a non-specific band at the same size as p75 which was present equally in *p75*$^{+/+}$ and *p75*$^{-/-}$ extracts (Fig. 2.3 A panel 4). To combat this issue, we used an alternative antibody (Advanced Targeting Systems, ATS) to measure levels of p75 in WCLs that detected this non-specific protein at a significantly lower level than p75 itself (Fig. 2.3 A panel 5). At 5 months, *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice had decreased p75 levels with IP, but not in WCLs, when compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ littermates (Fig. 2.3 A, B, D). This decrease in p75 with IP, but not in WCLs, may indicate that p75 is forming a novel complex in the striata of *Q175*$^{WT/HD}$ mice and that this complex prevented the IP antibody from recognizing and pulling down p75. Additionally, there was an association between p75 and TrkB in both genotypes to a similar extent (Fig 2.3 A, C). There was no p75 protein detected either by IP or by direct immunoblotting of WCLs from *Q175*$^{WT/WT}$*;p75*$^{-/-}$ or *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice as expected (Fig. 2.3 A panels 1, 5). The levels of TrkB were also examined in these four genotypes by immmunoblotting WCLs

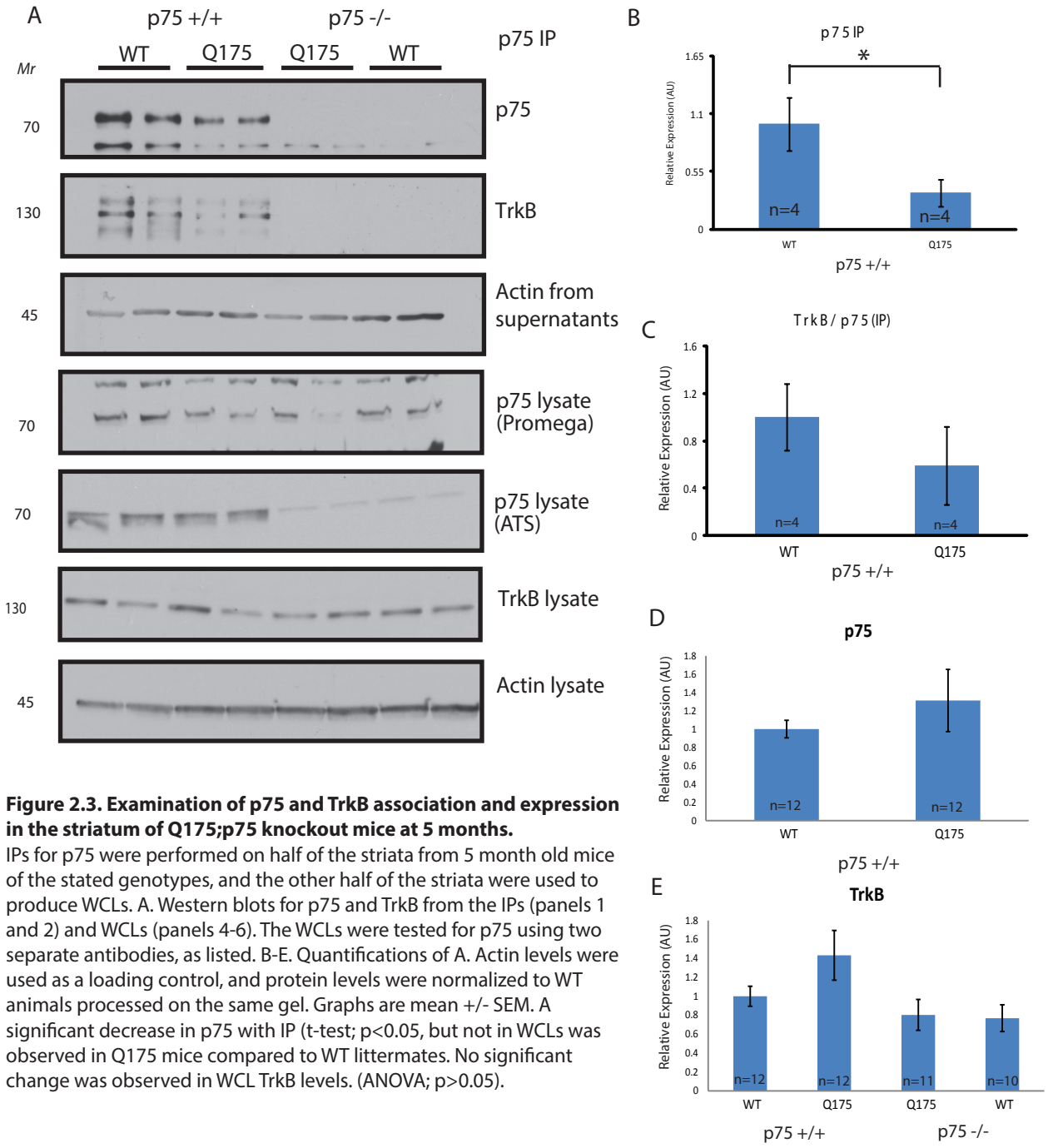

Figure 2.3. Examination of p75 and TrkB association and expression in the striatum of Q175;p75 knockout mice at 5 months.
IPs for p75 were performed on half of the striata from 5 month old mice of the stated genotypes, and the other half of the striata were used to produce WCLs. A. Western blots for p75 and TrkB from the IPs (panels 1 and 2) and WCLs (panels 4-6). The WCLs were tested for p75 using two separate antibodies, as listed. B-E. Quantifications of A. Actin levels were used as a loading control, and protein levels were normalized to WT animals processed on the same gel. Graphs are mean +/- SEM. A significant decrease in p75 with IP (t-test; $p<0.05$, but not in WCLs was observed in Q175 mice compared to WT littermates. No significant change was observed in WCL TrkB levels. (ANOVA; $p>0.05$).

for TrkB. As seen in Figure 1, there was no change in the levels of TrkB in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ mice.

p75 does not regulate apoptotic signaling in the striatum of Q175 mice at 5 months of age

To determine whether p75 signaling altered striatal pro-apoptotic signaling pathways, JNK and c-Jun activation were examined. Similar to what was observed in *Q175*$^{WT/HD}$ mice (Fig. 2.2), there was no change in JNK or c-Jun activation in the striata of *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice when compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ mice (Fig. 2.4). Additionally, there was no significant difference in JNK or c-Jun activation in the striata of *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice when compared to *Q175*$^{WT/WT}$*;p75*$^{-/-}$ mice (Fig. 2.4).

Increased survival signaling in Q175 mice is abolished by p75 deletion

To determine whether p75 was important for the increase in survival signaling observed in *Q175*$^{WT/HD}$ mice at 5 months of age, Akt activation was examined as in Figure 2.2. Similar to the increase seen in *Q175*$^{WT/HD}$ mice, Akt activation was also increased in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ control littermates (Fig. 2.5 B). This increase was completely abolished in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice (Fig. 2.5 A, B). Because Akt activation was increased in *Q175*$^{WT/HD}$ mice (Fig. 2.2 A, F), we decided to also examine activation of NFκB. Due to the time-elapsed and amount of freeze-thaw cycles performed, we were unable to reliably investigate NFκB

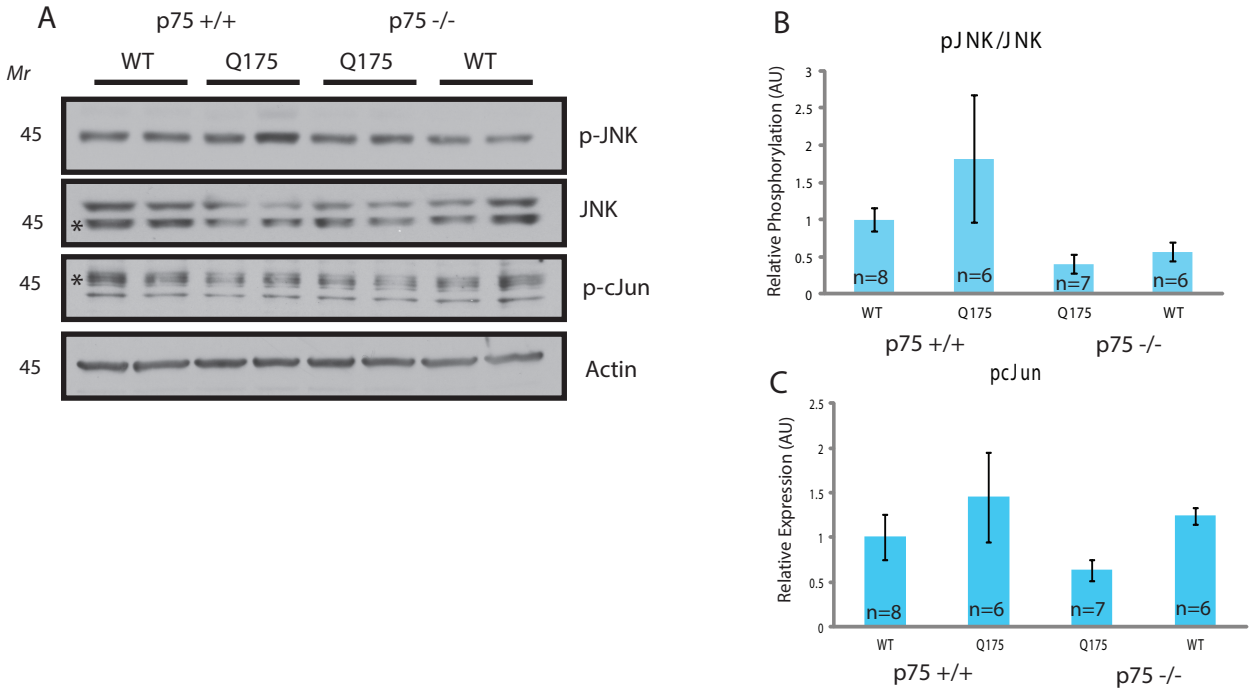

Figure 2.4. Apoptotic signaling markers are unchanged in the striatum at 5 months.
WCLs were examined on half of the striata from 5 month old mice of the stated genotypes. A. Western blots were run on WCLs using the stated antibodies. B, C. Quantifications of A. Normalization was performed as in Figure 1. No significant difference in JNK or cJun phosphorylation was observed in any genotype (ANOVA; $p>0.05$).

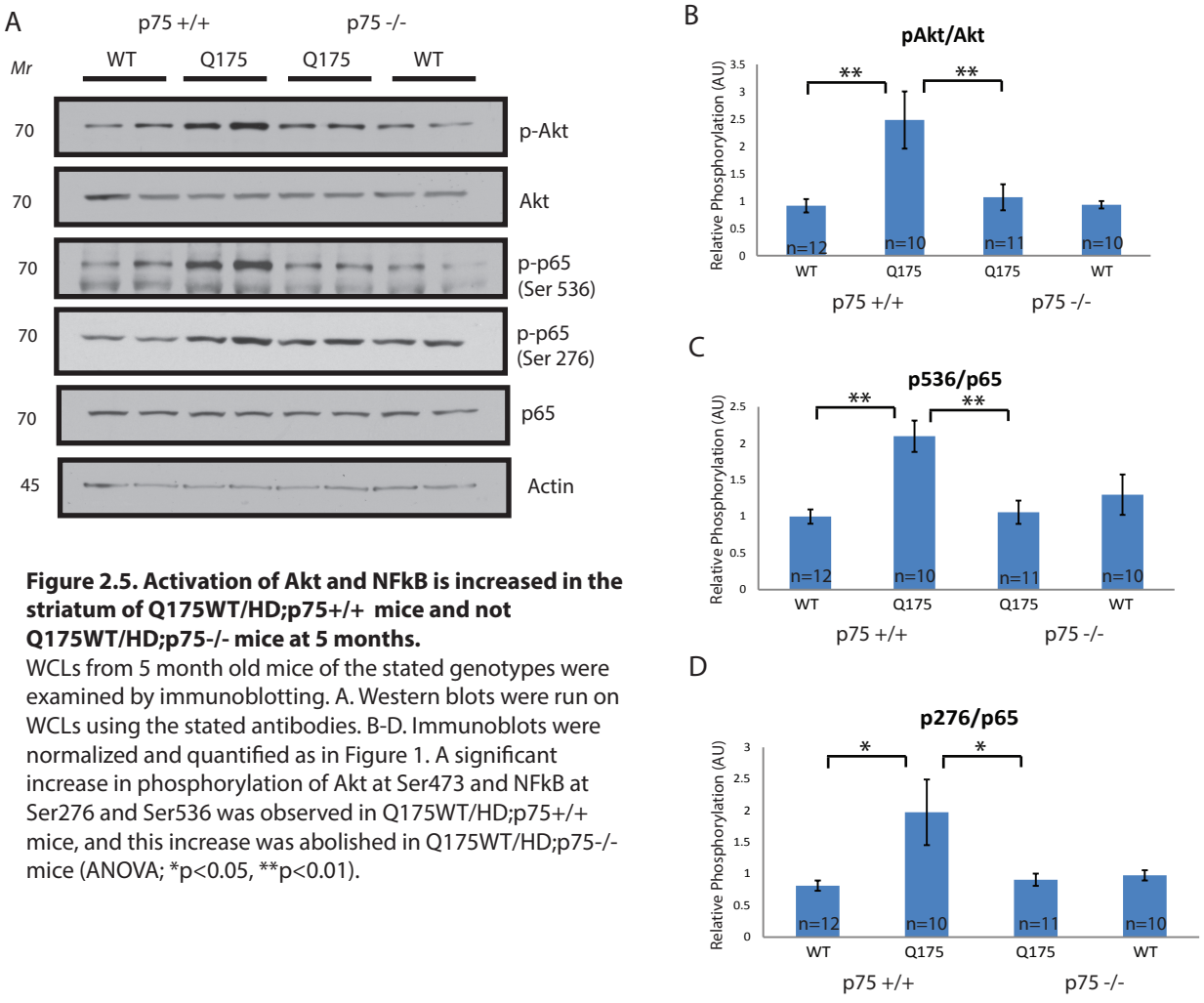

Figure 2.5. Activation of Akt and NFkB is increased in the striatum of Q175WT/HD;p75+/+ mice and not Q175WT/HD;p75-/- mice at 5 months.

WCLs from 5 month old mice of the stated genotypes were examined by immunoblotting. A. Western blots were run on WCLs using the stated antibodies. B-D. Immunoblots were normalized and quantified as in Figure 1. A significant increase in phosphorylation of Akt at Ser473 and NFkB at Ser276 and Ser536 was observed in Q175WT/HD;p75+/+ mice, and this increase was abolished in Q175WT/HD;p75-/- mice (ANOVA; *p<0.05, **p<0.01).

phosphorylation in the *Q175*$^{WT/HD}$ and *Q175*$^{WT/WT}$ samples. NFκB activation is capable of promoting survival downstream of p75 activation in multiple cell types (Mattson et al., 1997; Middleton et al., 2000; Vicario et al., 2015), but does so independently of TrkB activity (Carter et al., 1996). The p65 subunit of NFκB can be phosphorylated on several different serine residues, all leading to slightly different transcriptional outcomes. We examined phosphorylation of p65 at Ser276 and at Ser536 because these two serines are important for more potent and longer NFκB-dependent transcriptional activation (Neumann & Naumann, 2007). p65 subunit phosphorylation at both residues was increased in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ mice. This increase was completely abolished in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice (Fig. 2.5 A, C, D). B-cell lymphoma-extra large (Bcl-XL) and X-linked inhibitor of apoptosis (XIAP) are inhibitors of apoptosis known to be transcriptionally up-regulated by activation of NFκB (Chao et al., 2011; Stehlik et al., 1998). Interestingly, levels of XIAP were also increased in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice, and this increase was abolished in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice (Fig 2.6). Additionally, expression of Bcl-XL was significantly lower in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice when compared to *Q175*$^{WT/HD}$*;p75*$^{+/+}$ and *Q175*$^{WT/WT}$*;p75*$^{+/+}$ mice (Fig. 2.6). Taken together, these data suggest that at pre-symptomatic ages in *Q175*$^{WT/HD}$ mice p75 upregulates pro-survival signaling pathways, and that loss of this p75-mediated augmentation leads to decreased survival signaling.

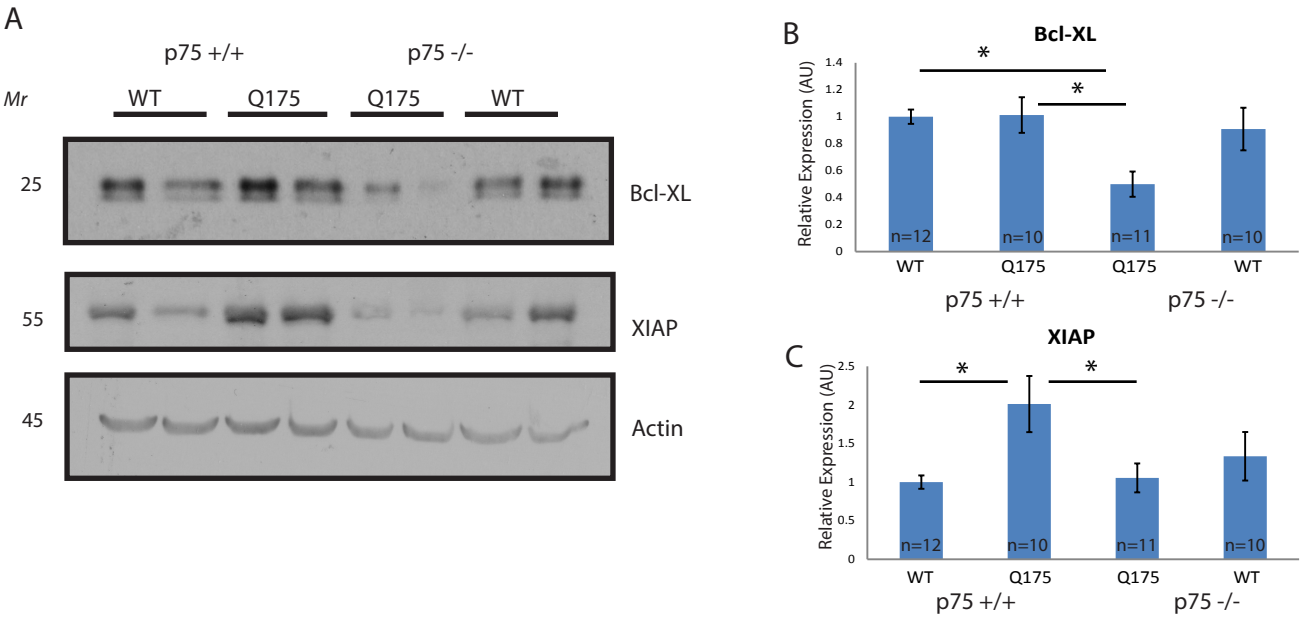

Figure 2.6. XIAP and Bcl-XL exression decreased in the striatum of Q175WT/HD;p75-/- mice compared to Q175WT/HD;p75+/+ mice at 5 months.

A. WCLs from half of the striata of the stated genotypes at 5 months of age were tested using the listed antibodies. B, C. Quantifications of A. A significant decrease in XIAP and Bcl-XL expression was observed in Q175WT/HD;p75-/- mice compared to Q175WT/HD;p75+/+ littermates (ANOVA; $p<0.05$).

Striatal dysfunction is accelerated in *Q175$^{WT/HD}$;p75$^{-/-}$* mice

Striatal dysfunction and eventual degeneration is a hallmark in patients with HD and in some animal models of HD. Healthy MSNs express high levels of DARPP-32, which plays a critical role in the processing of dopamine signaling in the striatum. Decreased levels of DARPP-32 are a pathological marker of several diseases affecting striatal function, including HD (Bibb et al., 2000; Dellen et al., 2000). Previous examination of DARPP-32 levels in *Q175$^{WT/HD}$* mice showed DARPP-32 to be significantly reduced in *Q175$^{WT/HD}$* mice compared to *Q175$^{WT/WT}$* littermates at 12 months of age, but not at earlier time points (Smith et al., 2014). In agreement with prior work, we did not observe a change in DARPP-32 expression in *Q175$^{WT/HD}$;p75$^{+/+}$* mice at 5 months by western blot nor at 7 months by immunohistochemistry (IHC), when compared to *Q175$^{WT/WT}$;p75$^{+/+}$* littermates (Fig. 2.7). Interestingly, DARPP-32 expression decreased significantly in *Q175$^{WT/HD}$;p75$^{-/-}$* mice both by western blot at 5 months and by IHC at 7 months compared to littermates of all three other genotypes examined, suggesting an earlier onset of striatal dysfunction (Fig. 2.7).

Neuronal density unchanged in striatum at 7 months of age

We evaluated striatal volume and neuronal density using NeuN IHC at 7 months of age. Differences in striatal volume have been reported at 12 months of age in this mouse line, but not earlier (Smith et al., 2014). No significant difference was observed in striatal volume or in neuronal density in any genotype examined (Fig. 2.8). As the

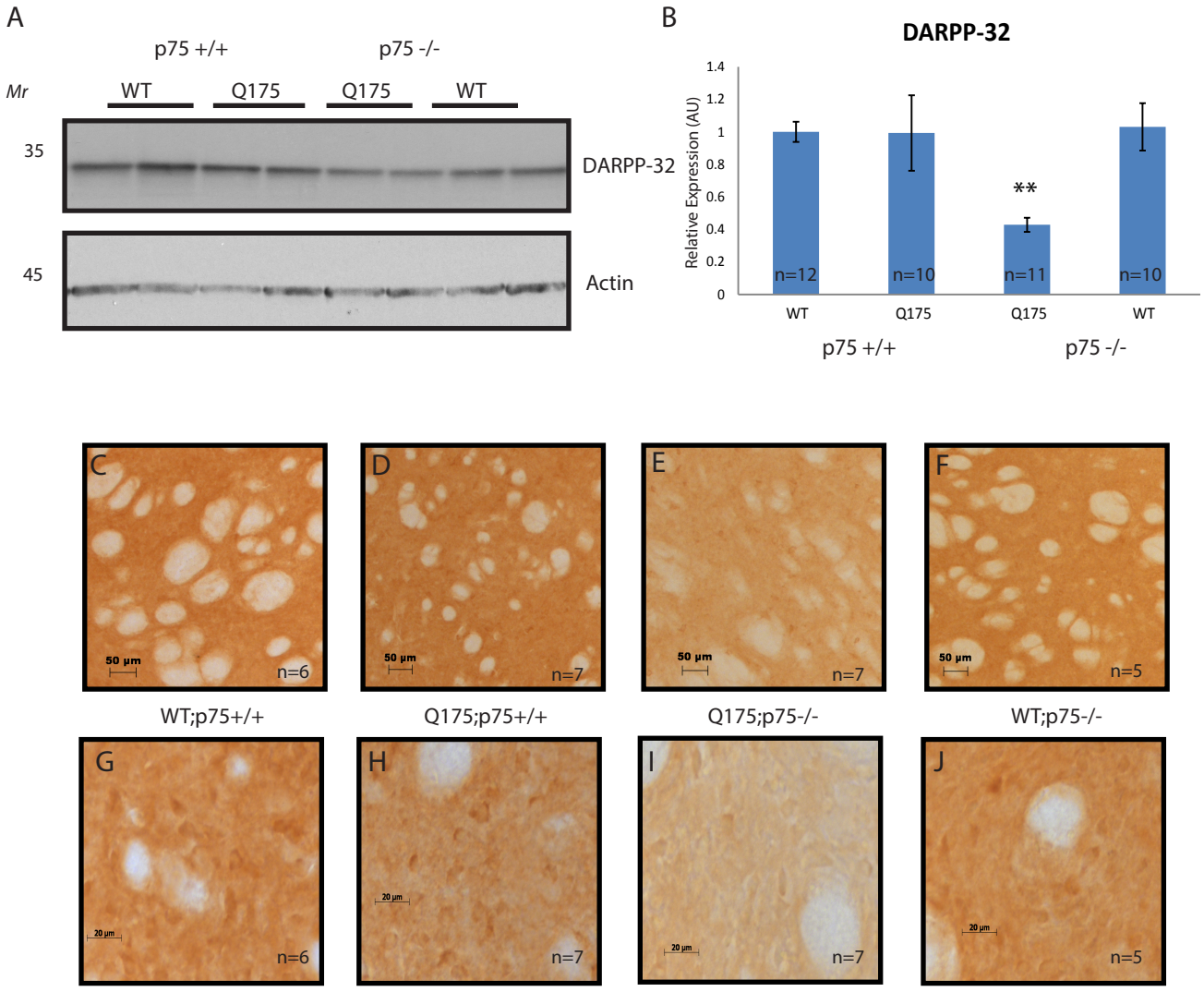

Figure 2.7. DARPP-32 expression decreased in the striatum of Q175WT/HD;p75-/- mice compared to Q175WT/HD;p75+/+ mice.

A. WCLs from half of the striata of the stated genotypes at 5 months of age were probed for the listed antibodies. B. Quantification of A. C-J. Immunohistochemistry was performed on striata of 7 month old mice of the stated genotype for DARPP-32 at 10X (C-F) and 64X (G-J) as described in the methods. Decreased levels of DARPP-32 were observed with WB and IHC in the striatum of Q175WT/HD;p75-/- mice (ANOVA; $p<0.01$).

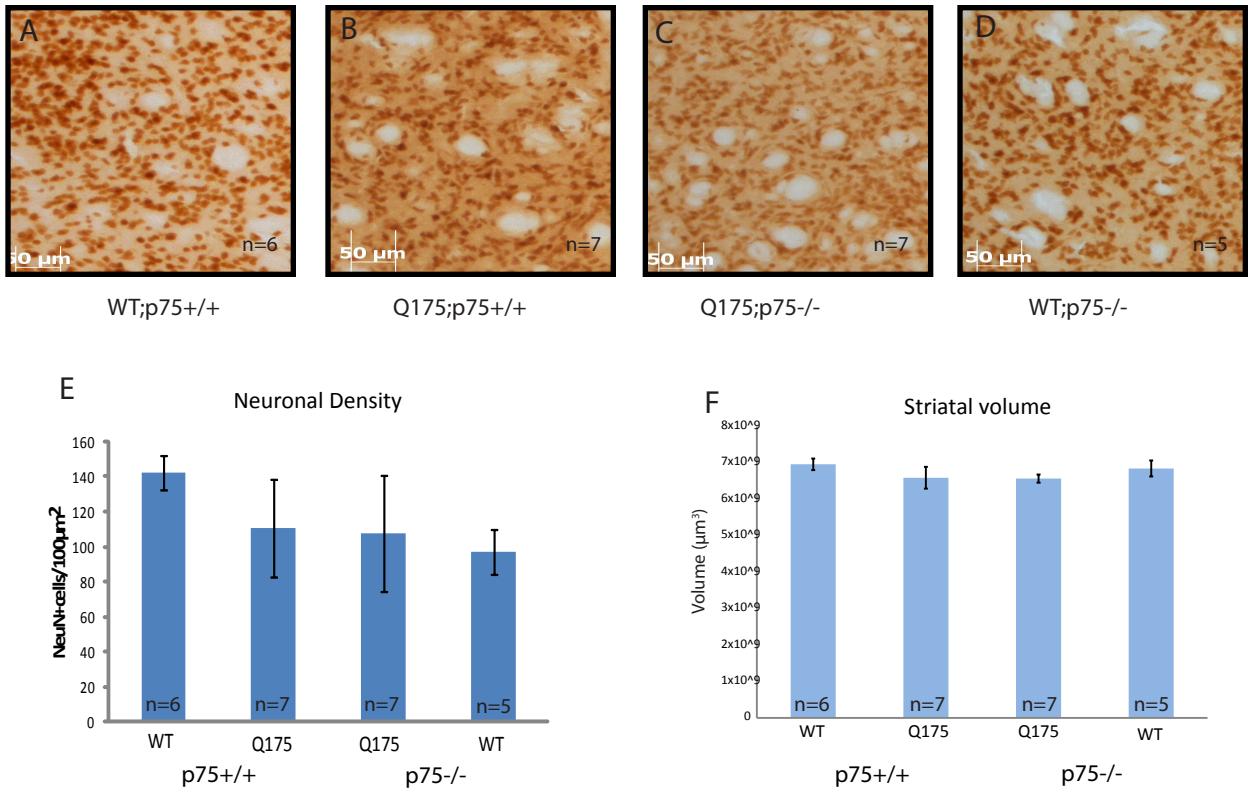

Figure 2.8. Neuronal density and volume are unchanged in the striatum at 7 months.
A-D. NeuN immunohistochemistry was performed on striata of 7 month old mice of the stated genotypes as described in the methods. E. Cell density quantifications of A-D. F. Striatal volume quantifications of A-D. No significant differences were observed in either neuronal density or striatal volume in any genotype. (ANOVA; $p>0.05$).

density of NeuN+ cells does not change, overt neuronal degeneration does not seem to have begun in these mice at this time point.

Discussion

In this study, we examined the extent to which p75 is involved in the early striatal response to mutant *Hdh*. *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice showed increased activation of Akt, increased phosphorylation of the p65 subunit of NFκB at two sites, and increased expression of XIAP at 5 months of age when compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ littermates. These increases were abolished in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice, suggesting that p75 is essential for this enhanced survival signaling. Additionally, *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice showed a decrease in Bcl-XL expression compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{+/+}$ littermates, as well as a decrease in DARPP-32 expression, consistent with a loss of survival signaling and an earlier onset of dysfunction in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice. These data suggest a role for p75 in augmenting survival signaling in the striata of *Q175*$^{WT/HD}$ mice, and that the loss of this signaling leads to an earlier onset of striatal dysfunction.

While these data support a role of p75 in augmenting survival signaling prior to onset of dysfunction in the striata of *Q175*$^{WT/HD}$ mice, it is possible that the role of p75 changes with disease progression and/or acts in a region-specific manner. Plotkin et al. demonstrated that inhibition of p75 rescued the loss of synaptic plasticity seen in the striatum of symptomatic BACHD mice (2014). In addition, Brito et al. reported rescued learning and memory deficits in Q111 mice by decreasing the level of p75 expression in

the hippocampus (2014). These two studies suggest a potentially pathogenic role for p75 in HD at later disease stages and/or possibly in a regionally-specific fashion. To address whether p75 signaling can switch from survival-promoting to dysfunctional later in disease progression, or whether p75 functions differently in different populations of cells, conditional p75 mice should be utilized in future experiments. Crossing conditional p75 mice into varied Cre recombinase-expressing strains will allow for cell-type-specific and temporal-specific deletion. It is important to acknowledge that, unlike our results, other studies have reported an increase in p75 protein and mRNA levels in the striatum of mouse models and HD post-mortem brains, respectively (Brito et al., 2013; Zuccato et al., 2008). The mouse models used in these prior studies were a truncation model (R6/1) and a homozygous knock-in model (Q111), which differ considerably from the heterozygous *Q175*$^{WT/HD}$ mice. R6/1 is more rapidly progressive, and homozygous Q111 mice lack any normally-functional Hdh protein (Brooks et al., 2012; Pouladi et al., 2013). Interestingly, in the Q175 model of HD used here, p75 levels were decreased at 12 months of age in both the heterozygous and homozygous mutants when compared to WT animals (Ma et al., 2015). p75 levels and its effects may therefore be affected by how the animal model was constructed.

Although surprising that we observed a decrease in p75 levels in *Q175*$^{WT/HD}$ mice by IP, but not in WCLs, one possible explanation is that p75 is forming a different signaling complex in *Q175*$^{WT/HD}$ mice compared to *Q175*$^{WT/WT}$ mice, and that this complex is blocking the relevant epitope(s), preventing the antibody from immunoprecipitating p75 to the same extent in these mice. Determining what the composition of this complex is will be important for gaining insight into the p75-specific

signaling events leading to increased survival signaling in *Q175*$^{WT/HD}$ mice. It is possible that interaction with TrkB makes it more difficult for the antibody to bind with p75. The antibody we use for IP recognizes the entire intracellular domain (ICD) of p75 which is thought to be essential for interaction with Trk receptors (Ceni et al., 2010; Matusica et al., 2013). Futhermore, it is possible that p75 also forms a complex with TrkC, which has recently been reported to be expressed in Drd1-expressing MSNs of the striatum (Baydyuk et al., 2013). If this complex is increased in *Q175*$^{WT/HD}$ mice, this finding would be particularly interesting because Drd1-expressing MSNs tend to become dysfunctional later than Drd2-expressing MSNs in HD (Albin et al., 1992; Richfield et al., 1995).

Considering these data along with the growing literature involving p75 and HD, it is clear that p75 signaling in HD mouse models is more complicated than initially appreciated. There is interest in developing p75 antagonists as a disease-modifying treatment for HD. Antagonizing p75 prior to dysfunction may be counter-productive, however, and developing p75-specific antagonists may only be helpful later in the course of the disease. An effective early intervention could take advantage of augmenting and/or prolonging this pro-survival signaling via p75. Additionally, it will be important to determine which cell type(s) express p75 in the striatum of *Q175*$^{WT/HD}$ mice. The simplest model would be cell-autonomous signaling in MSNs leading to increased activation of Akt and NFκB and followed by an increase/normalization in survival targets of Akt and NFκB, such as XIAP and Bcl-XL. This process, however, may not necessarily be cell-autonomous. Other non-neuronal cell types present in the striatum, such as astrocytes, microglia, and oligodendrocytes could express p75 and provide some type

of extrinsic support for the MSNs. In fact recent studies have reported expression of p75 in oligodendrocytes and neurons in the striatum (Brito et al., 2013; Ma et al., 2015). Determining which cell type expresses p75 will be essential for discovering if this pathway can be utilized to prolong normal striatal function in these mice. If this potential compensatory mechanism can be better understood, it may be possible to find novel targets to augment the brain's natural defenses, prolonging normal function, and delaying, or even preventing, the onset of dysfunction.

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Chapter 3: Examining the role of p75 signaling in the striatum of *Q175$^{WT/HD}$* mice at 2 months of age

Introduction

The experimental results suggesting a pro-survival function of p75 in the striatum of *Q175$^{WT/HD}$* mice observed at 5 months, especially the increase in NFkB and Akt activation and the changes in XIAP/Bcl-XL levels, prompted the question of when these signaling changes begin. We sought to determine whether this increase in survival signaling was present throughout the lifespan or developed over time. To address this question, we chose to examine mice at 2 months of age. We examined the same signaling markers of both apoptotic and survival signaling as we had investigated in 5/7 month animals. Interestingly, based on the experiments reported here, at least some of the increase in p75-related survival signaling observed in the striatum of *Q175$^{WT/HD}$* mice at 5 months of age appears to develop as the animal ages. At 2 months of age, we observed an increase in association between p75 and TrkB not observed at 5 months, and no change in XIAP or Bcl-XL expression. We did see an increase in Akt phosphorylation, similar to what we observed at 5 months, which may be downstream of the observed p75-TrkB complex. There is no decrease in DARPP-32 expression in the striatum of *Q175$^{WT/HD}$;p75$^{-/-}$* mice at 2 months of age, which argues that the decrease observed at 5/7 months is an earlier onset of dysfunction and not a developmental deficit. If there had been a defect in striatal development, leading to an innate DARPP-

32 deficit in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice, we would have expected to see a DARPP-32 decrease at 2 months of age as well.

Methods

All methods were performed identically as described in Chapter 2, with the exception that all experiments were performed on mice of 8 weeks of age.

Results

Levels of p75 and TrkB are unchanged in the striatum of Q175 mice at 2 months of age

As described in the methods section of chapter 2, striata were isolated from *Q175*$^{WT/WT}$ and *Q175*$^{WT/HD}$ 2 month old mice. Half of each striatum was used to make whole cell lysates (WCLs). These lysates were then probed for p75 and TrkB levels. No differences were observed in either p75 or TrkB at 2 months of age between *Q175*$^{WT/WT}$ and *Q175*$^{WT/HD}$ mice (Fig. 3.1).

Phosphorylation of c-Jun is decreased at 2 months of age in Q175 mice

As discussed in chapter 2, signaling through p75 can lead to activation of apoptotic signaling pathways, caspase activation, and programmed cell death (Kraemer, 2014). To determine if apoptotic signaling was enhanced at 2 months of age

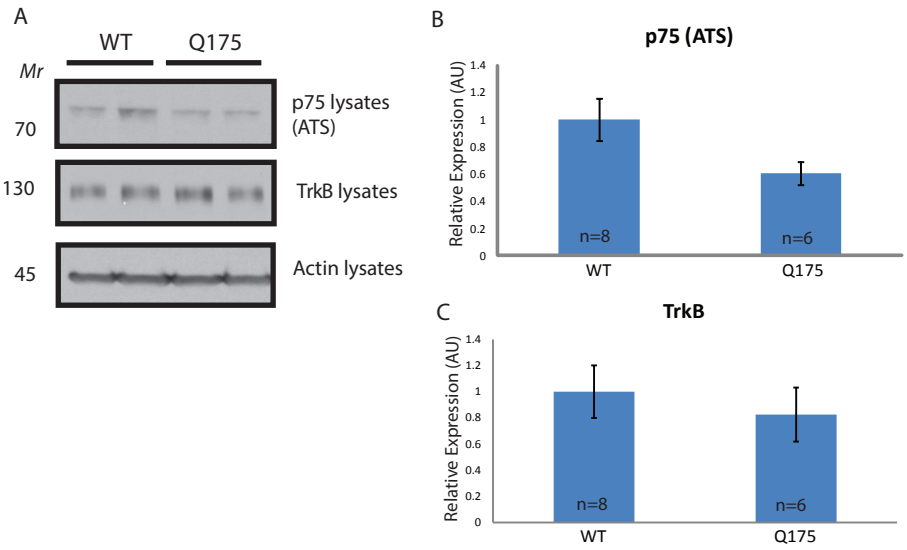

Figure 3.1. Analysis of TrkB and p75 in the striatum of Q175 mice at 2 months. The striata of 2 month old mice of the stated genotype were used to produce whole cell lysates (WCLs) A. Western blots for p75 and TrkB are shown. B-C. Quantifications of A are graphed as the mean ± SEM. Actin levels were used as a loading control, and protein levels were normalized to WT animals processed on the same blots. No significant differences in p75 or TrkB levels between WT and Q175 mice were observed (t-test; $p>0.05$).

in *Q175*$^{WT/HD}$ mice as compared to *Q175*$^{WT/WT}$ mice, we examined the level of activation of JNK and c-Jun, as well as expression levels of PTEN. WCLs of *Q175*$^{WT/WT}$ and *Q175*$^{WT/HD}$ mice were examined by p-JNK, JNK, p-c-Jun, and PTEN immunoblotting. Phosphorylation of c-Jun leads rapidly to its own transcriptional upregulation and therefore, normalizing to total c-Jun levels can lead to an underrepresentation of the extent of c-Jun activation (Eilers et al., 1998). The 46 KD isoform of JNK was analyzed because it was significantly more abundant in the striatum than the 54 KD isoform. Unlike at 5 months of age, there was a significant decrease in p-c-Jun levels in the striatum of *Q175*$^{WT/HD}$ at 2 months of age (Fig. 3.2 A and C). Similar to what was observed at 5 months of age, JNK and PTEN immunoblotting did not reveal any significant differences between *Q175*$^{WT/HD}$ mice and *Q175*$^{WT/WT}$ mice (Fig. 3.2 A, B, D). Taken together, unlike at 5 months, there appears to be a decrease in apoptotic signaling via c-Jun in the striatum of *Q175*$^{WT/HD}$ mice at 2 months of age.

Akt activation is increased in the striatum of *Q175*$^{WT/HD}$ mice at 2 months

While p75 signaling can lead to apoptosis, it can also lead to survival independently or when paired with certain co-receptors such as Trk receptors. At 5 months of age, an increase in Akt phosphorylation was observed in the striatum of *Q175*$^{WT/HD}$ mice, indicating increased survival signaling. To examine whether this increased signaling was present from development into adulthood or developed over time, we examined the phosphorylation of Akt at 2 months of age. Similar to 5 months of age, there was an increase in the levels of Akt phosphorylation in the striatum of

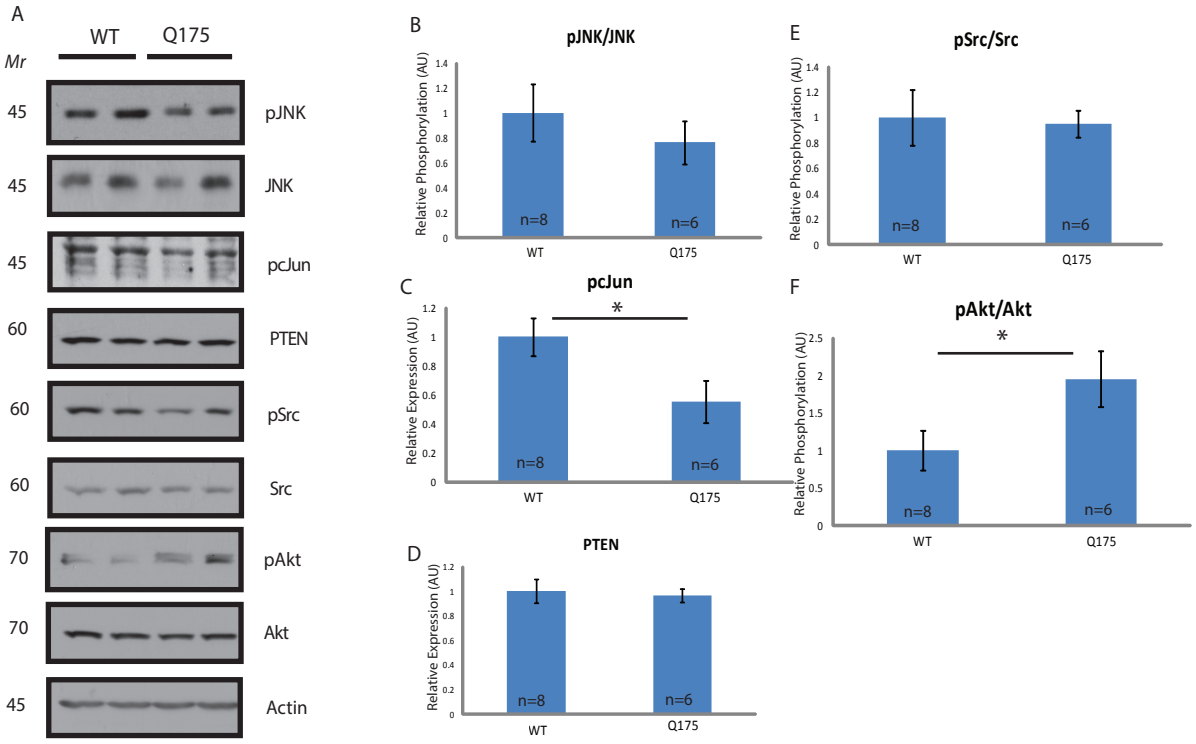

Figure 3.2. Biochemical analysis of pro-apoptotic and survival signaling in the striatum of Q175 mice at 2 months. WCLs of the striata from 2 month old mice of the stated genotypes were analyzed by immunoblotting. A. Western blots were run on WCLs using the stated antibodies. B-F. Quantifications of A were performed as in Figure 1. No significant differences were seen between WT and Q175 mice in JNK or Src activation, or in PTEN levels. A significant increase in Akt activation, and a significant decrease in p-cJun were observed (* t-test $p<0.05$, ** $p<0.01$).

Q175$^{WT/HD}$ mice at 2 months of age, indicating increased survival signaling (Fig 3.2 A, F). Src can be phosphorylated on Tyr416 following TrkB activation and this phosphorylation event induces Src catalytic activity, enhancing cell survival (Huang & McNamara, 2010). Similar to 5 months of age, there was no change in Src activation in the striata of *Q175*$^{WT/HD}$ mice as compared to *Q175*$^{WT/WT}$ mice at 2 months (Fig. 3.2 A, E). This increase in activation of Akt in the striata of *Q175*$^{WT/HD}$ mice at 2 months of age suggests there is augmented survival signaling even at early adulthood.

XIAP and Bcl-XL are both inhibitors of apoptosis known to be transcriptionally upregulated by activation of NFkB. The levels of Bcl-XL were found to be decreased in the striatum of *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice at 5 months of age compared to *Q175*$^{WT/HD}$*;p75*$^{+/+}$, *Q175*$^{WT/WT}$*;p75*$^{-/-}$, and *Q175*$^{WT/WT}$*;p75*$^{+/+}$ mice, however they were not significantly affected in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice when compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ littermates. Additionally, the levels of XIAP were found to be increased in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice compared to both *Q175*$^{WT/WT}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice. To determine whether this altered expression was present at 2 months of age, their expression levels were examined by immunoblotting striata from 2 month old mice, and no significant differences in BCL-XL and XIAP expression were observed between *Q175*$^{WT/WT}$ and *Q175*$^{WT/HD}$ mice (Fig 3.3 A-C). DARPP-32 expression, which is commonly used as a general measure of the health of medium spiny neurons of the striatum, was also decreased in the striatum of *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice at 5 months but not in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice. Our analysis indicated that there was no significant difference in DARPP-32 expression at 2 months of age between *Q175*$^{WT/WT}$ and *Q175*$^{WT/HD}$ mice (Fig

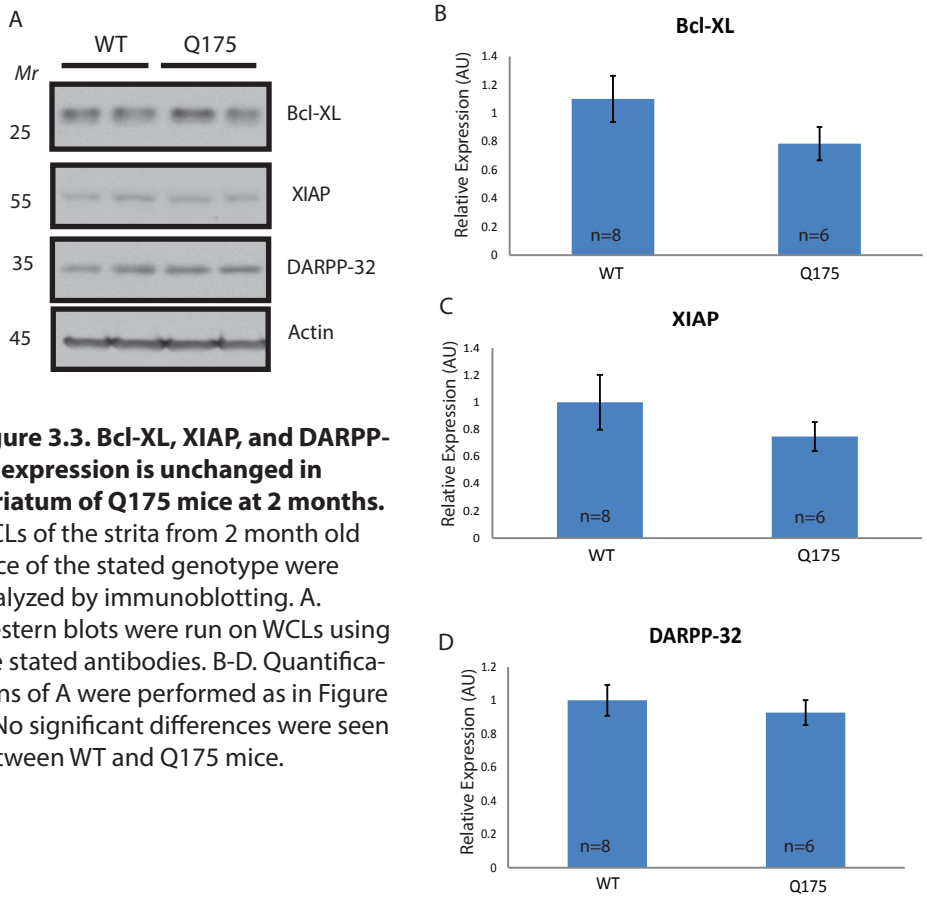

Figure 3.3. Bcl-XL, XIAP, and DARPP-32 expression is unchanged in striatum of Q175 mice at 2 months. WCLs of the strita from 2 month old mice of the stated genotype were analyzed by immunoblotting. A. Western blots were run on WCLs using the stated antibodies. B-D. Quantifications of A were performed as in Figure 1. No significant differences were seen between WT and Q175 mice.

3.3 A, D). These observations are not surprising, given there is no difference in DARPP-32 expression between these two genotypes at 5 months of age.

Examination of p75/TrkB association and levels in the striatum at 2 months

After surveying survival and apoptotic signaling pathways in *Q175*$^{WT/HD}$ mice and *Q175*$^{WT/WT}$ littermates, we evaluated the functional role of p75 in pro-survival and pro-apoptotic signaling at 2 months to compare to the results from 5 months. In this mixed background at 2 months of age, we again examined p75 levels by immunoprecipitation (IP) and by direct immunoblotting (WCL). At 5 months, *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice had decreased p75 levels with IP, but not in WCLs, when compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ littermates. In comparison, at 2 months of age, there was no significant difference in p75 levels either by IP or in WCLs (Fig 3.4 A, B, D), although we observed a trend via p75 IP ($p=0.06$). As at 5 months of age, there was an association between p75 and TrkB observed in the striatum at 2 months (Fig 3.4 A). In contrast to 5 months, however, there was an increase in the amount of TrkB associated with p75 at 2 months in the striatum of *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ littermates (Fig 3.4 A, C). Importantly, even though there was an increase in TrkB associated with p75, there was no change in overall TrkB levels, as measured by TrkB immunoblotting of WCLs (Fig 3.4 A, E). This increased association between p75 and TrkB supports the hypothesis that p75 may support survival signaling in *Q175*$^{WT/HD}$ mice by augmenting TrkB signaling, and possibly by stabilizing TrkB on the membrane to enhance signaling, as shown previously (Makkerh et al., 2005).

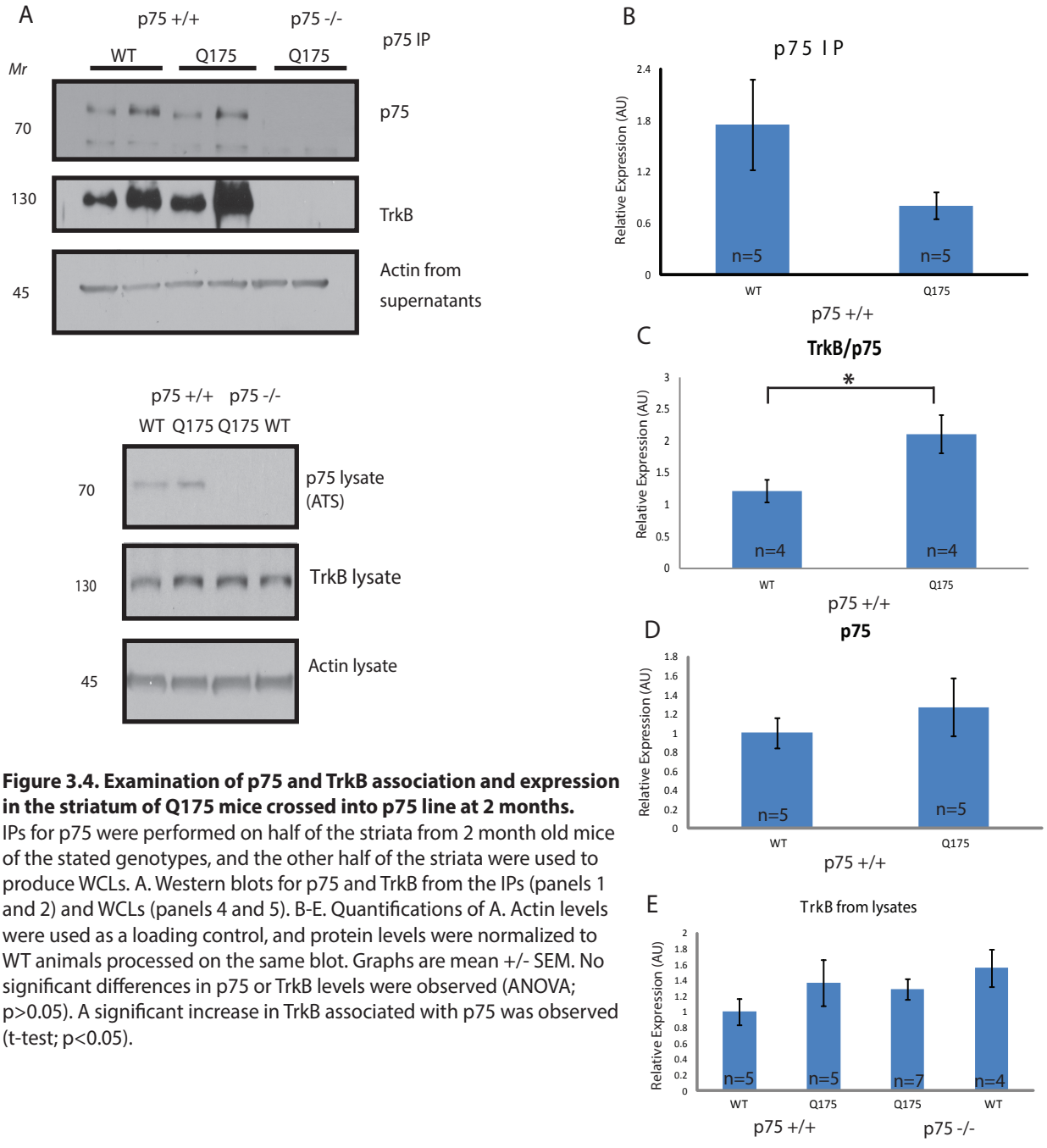

Figure 3.4. Examination of p75 and TrkB association and expression in the striatum of Q175 mice crossed into p75 line at 2 months. IPs for p75 were performed on half of the striata from 2 month old mice of the stated genotypes, and the other half of the striata were used to produce WCLs. A. Western blots for p75 and TrkB from the IPs (panels 1 and 2) and WCLs (panels 4 and 5). B-E. Quantifications of A. Actin levels were used as a loading control, and protein levels were normalized to WT animals processed on the same blot. Graphs are mean +/- SEM. No significant differences in p75 or TrkB levels were observed (ANOVA; $p>0.05$). A significant increase in TrkB associated with p75 was observed (t-test; $p<0.05$).

Striatal volume and apoptosis regulators are not affected by p75 deletion at 2 months of age

In order to examine survival signaling pathways at 2 months of age, the expression levels of XIAP, Bcl-XL, and DARPP-32 were measured, as described previously in chapter 2. At 5 months, there was a significant increase in expression of XIAP in the striatum of *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice when compared to all other examined genotypes, and a significant decrease in Bcl-XL expression in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice when compared to all other genotypes examined. In contrast to those findings, we did not observe any significant difference in expression of either Bcl-XL or XIAP in the striatum at 2 months of age (Fig 3.5). Striatal dysfunction and eventual degeneration is a hallmark in patients with HD and in some animal models of HD. Healthy MSNs express high levels of DARPP-32, which plays a critical role in the processing of dopamine signaling in the striatum. Decreased expression of DARPP-32 is a pathological marker of several diseases affecting striatal function, including HD (Bibb et al., 2000; Dellen, 2000). At 5 months of age, there was a significant decrease in DARPP-32 expression in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice when compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ littermates, both by WB and IHC. To examine whether these changes in DARPP-32 occurred earlier than 5 months of age, we examined DARPP-32 in mice at 2 months of age. At 2 months, there was no significant difference in DARPP-32 expression, either by WB or IHC (Fig 3.6). We also examined striatal volume, as described in chapter 2, and we did not detect a difference in striatal volume at 2 months (Fig 3.7). Taken together, as there is no difference in XIAP, Bcl-XL, or DARPP-32 expression in the striatum at 2

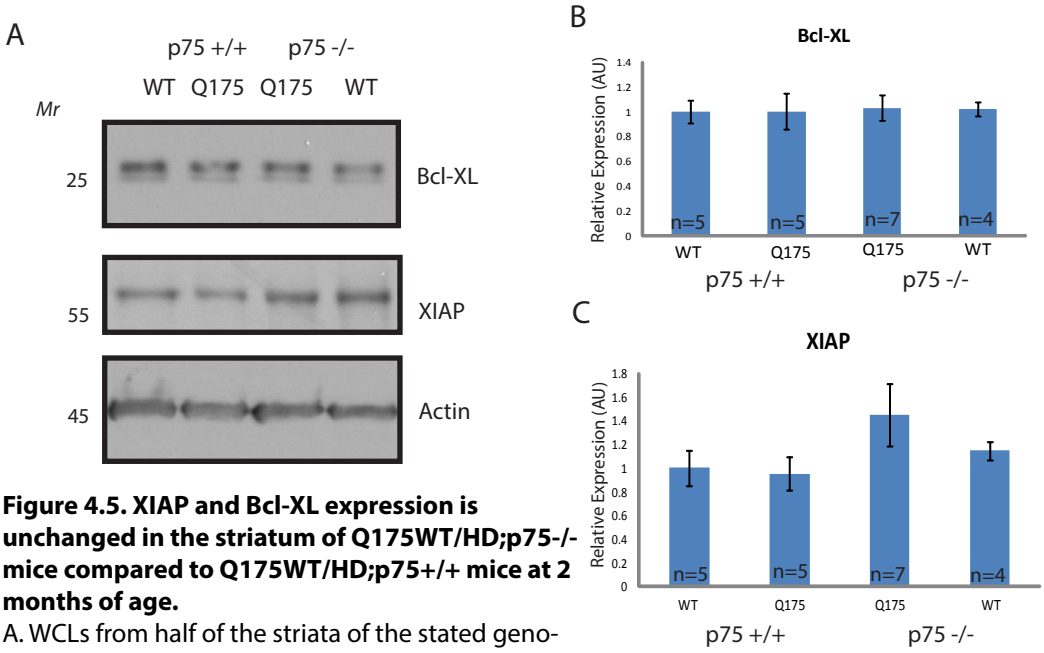

Figure 4.5. XIAP and Bcl-XL expression is unchanged in the striatum of Q175WT/HD;p75-/- mice compared to Q175WT/HD;p75+/+ mice at 2 months of age.

A. WCLs from half of the striata of the stated genotypes at 2 months of age were examined using antibodies to Bcl-XL and XIAP. Actin served as a loading control. B, C. Quantifications of A. No significant change in XIAP and Bcl-XL expression was observed (ANOVA; $p>0.05$).

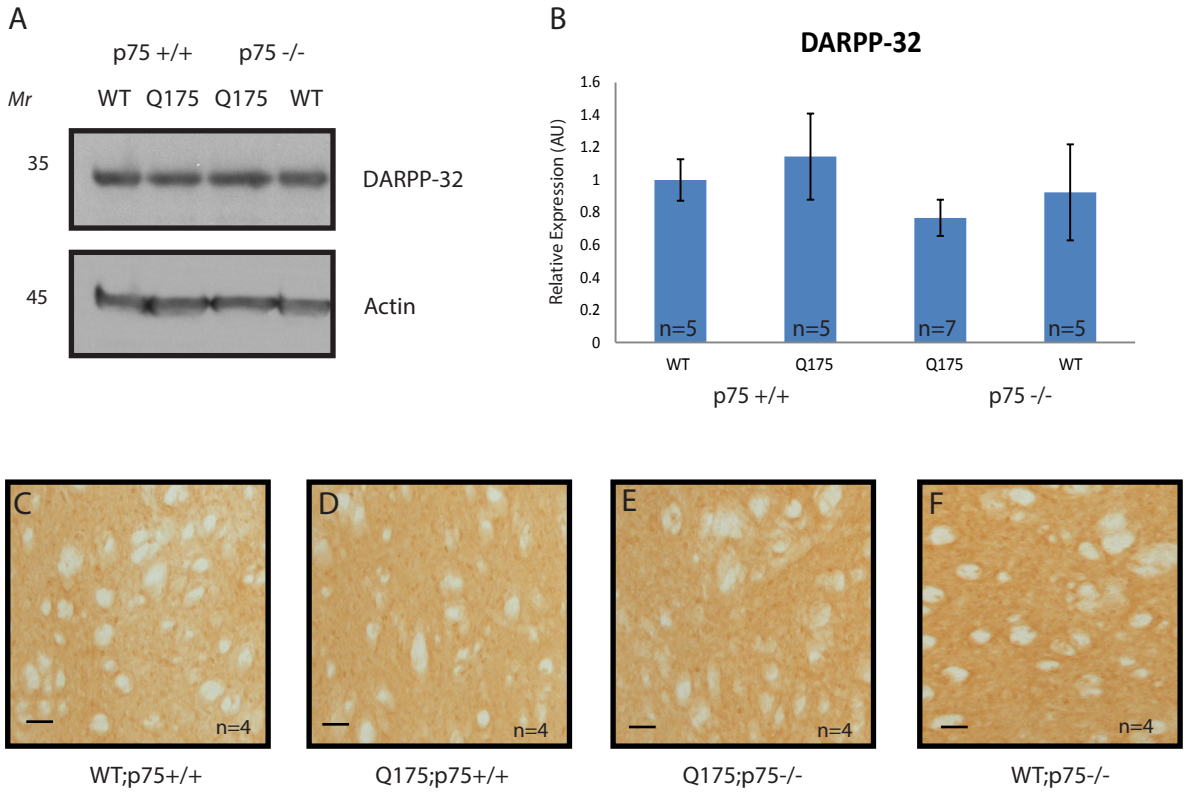

Figure 3.6. DARPP-32 expression is unchanged in the striatum of Q175WT/HD;p75-/- mice compared to Q175WT/HD;p75+/+ mice.

A. WCLs from half of the striata of the stated genotypes at 2 months of age were probed with DARPP-32 and actin antibodies. B. Quantification of A. C-F. Immunohistochemistry was performed on striata of 2 month old mice of the stated genotype for DARPP-32 as described in the methods. Scale bar: 50 μm

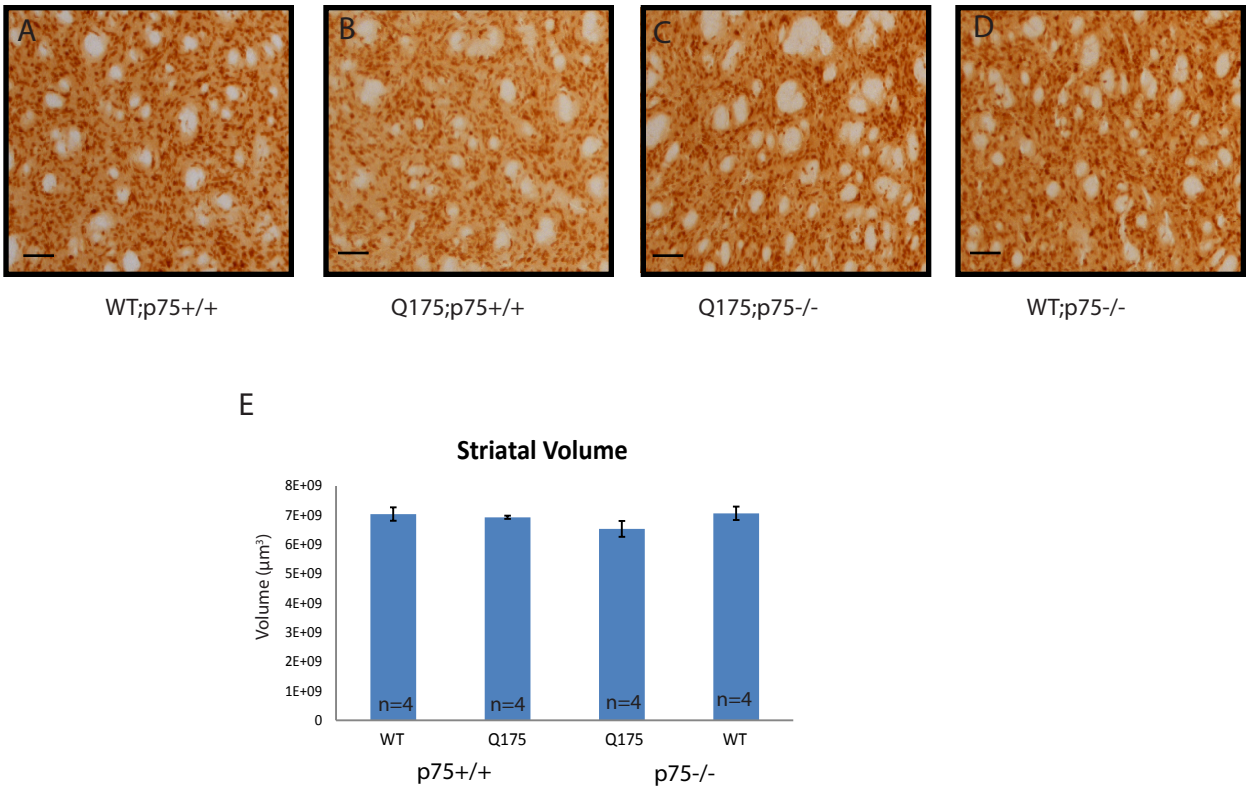

Figure 3.7. Neuronal volume is unchanged in the striatum at 2 months of age.
A-D. NeuN immunohistochemistry was performed on striata of 2 month old mice of the stated genotypes as described in the methods. E. Striatal volume quantifications of A-D. No significant differences were observed in striatal volume in any genotype. (ANOVA; $p>0.05$). Scale bar: 50µm

months, nor in striatal volume, dysfunction does not appear to have begun. These observations support the hypothesis that the decrease in DARPP-32 expression observed in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice at 5 months of age is a marker of an earlier onset of dysfunction and not a developmental deficit due to p75 deletion.

Discussion

In this part of the study, we examined the extent to which p75 is involved in the very early striatal response to mutant *Hdh*. As at 5 months of age, *Q175*$^{WT/HD}$ mice showed increased activation of Akt, when compared to *Q175*$^{WT/WT}$ littermates. Unlike 5 month old mice, however, 2 month *Q175*$^{WT/HD}$ mice also showed decreased phosphorylation of c-Jun. Both of these markers are indications of increased survival signaling and decreased apoptotic signaling. During the course of these studies, it became clear that the WCLs from the mice crossed in the p75 knockout background had not been stored in a manner that would preserve the phosphorylation status of the proteins. Therefore, these phosphorylation changes in Akt and cJun were unable to be studied in *Q175;p75* mice, unfortunately removing the possibility of asking whether p75 is essential for this enhanced survival signaling. Interestingly, in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice, there was an increased association of p75 with TrkB in the striatum. This increase in association is interesting as p75/Trk complexes have been shown to enhance survival signaling in many neuronal populations (Ceni et al., 2010; Kommaddi et al., 2011; Matusica et al., 2013; Negrini et al., 2013), and this increased association in Q175 mice may underlie the increase in Akt phosphorylation observed. Interestingly, also in

contrast to 5 months of age, *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice showed no change in XIAP, Bcl-XL, or DARPP-32 expression compared to *Q175*$^{WT/HD}$*;p75*$^{+/+}$ littermates. Due to the storage problems with the 2 month samples described above, NFkB activation was unable to be accurately measured. Nevertheless, this lack of change in XIAP and Bcl-XL expression supports the idea that NFkB signaling may not be as significant at 2 months of age as at 5 months given the direct involvement of NFkB signaling in regulating XIAP and Bcl-XL levels. This potential lack of Bcl-XL/XIAP involvement supports the hypothesis that the increase in Akt activation observed at 2 months of age may be caused by the increased association between p75 and TrkB in *Q175*$^{WT/HD}$ mice compared to *Q175*$^{WT/WT}$ mice, which is not observed at 5 months. Futhermore, one could speculate that this enhanced survival signaling shifts to a more p75-supported survival signaling through NFkB activation at older ages. Gaining a fuller picture of the role of p75 in the striatum of Q175 mice over time is essential to properly addressing the value of p75 has as a therapeutic target, and for identifying proper times during the disease course in which targeting p75 may be effective. It is possible that early in the disease, p75 and/or TrkB could be targeted with agonists to enhance survival signaling, and at later time points this therapy may be less effective.

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Chapter 4: Examining the role of p75 signaling in the striatum of *Q175$^{WT/HD}$* mice at 10/12 months of age

Introduction

Based on the increase in survival signaling observed in *Q175$^{WT/HD}$;p75$^{+/+}$* mice at 5 months, coupled with the decrease in DARPP-32 expression in *Q175$^{WT/HD}$;p75$^{-/-}$* mice at 5/7 months, we wanted to examine a later, fully symptomatic time point to investigate how these differences change with disease progression. We chose 12 month old mice to address these questions because behavioral and pathological changes have been reported in *Q175$^{WT/HD}$* mice at this age (Smith et al., 2014). We examined the same apoptotic markers, survival signaling markers, and pathological markers in these mice to ascertain whether the increased survival signaling observed at 5 months persisted after symptom onset. We also wanted to investigate whether there was more severe striatal degeneration in *Q175$^{WT/HD}$;p75$^{-/-}$* mice, and whether the decrease in DARPP-32 observed in these mice at 5 months was indicative of a more severe time course of dysfunction in the striatum of these mice compared to *Q175$^{WT/HD}$;p75$^{+/+}$* mice, or whether the dysfunction would stabilize at a later time point and not progress further. Finally, studying older symptomatic mice allowed us to examine whether apoptotic signaling dependent on p75 expression becomes more apparent at later stages in *Q175$^{WT/HD}$;p75$^{+/+}$* mice, since no changes were observed in the apoptotic pathways we examined at 5 months.

From our examination of mice at 10/12 months of age, surprisingly, we saw no changes in survival or apoptotic signaling in any genotype examined. We did observe, however, a decrease in DARPP-32 expression in both *Q175*$^{WT/HD}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice compared to WT littermates. This decrease in DARPP-32 expression was not different between *Q175*$^{WT/HD}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice. We also observed a decrease in striatal volume in both *Q175*$^{WT/HD}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice compared to WT littermates. Interestingly, the striatal volume of *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice was also significantly smaller than both the *Q175*$^{WT/HD}$*;p75*$^{+/+}$ littermates at 12 months of age and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice at 2 and 5 months of age. These data suggest that onset of striatal degeneration is earlier and/or more severe in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice compared to *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice, prompting the hypothesis that the increased survival signaling observed in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice at earlier, pre-symptomatic time points may be important for delaying the onset of dysfunction/degeneration in these mice.

Methods

Methods were performed as discussed in Chapter 2, except that all experiments were performed on mice at 10-13 months of age.

Results

Levels of p75 and TrkB are unchanged in the striatum of Q175 mice at 10 months of age

As described in the methods section of chapter 2, striata were isolated from 10 month old *Q175*$^{WT/WT}$ and *Q175*$^{WT/HD}$ mice. Half of each striatum was used to perform p75 immunoprecipitations (IP) and half was used to produce whole cell lysates (WCLs). These samples were resolved using SDS-PAGE and transferring to PVDF membranes. The IPs were tested for p75 levels (using the ATS antibody) and TrkB levels. The WCLs were also probed for p75 (also using the ATS antibody) and TrkB levels. Probing both the IPs and WCLs for p75 levels enabled us to test whether the decrease seen at 5 months between levels of p75 able to be immunoprecipitated from *Q175*$^{WT/HD}$ mice compared to *Q175*$^{WT/WT}$ mice, that was not observed in WCL p75 levels, was still present at 10 months. In these 10 month samples, no difference between the levels of p75 present in the IP sample was detected in either genotype (Fig. 4.1 A, B). Additionally, no difference in total p75 levels in WCLs was detected in either genotype (Fig. 4.1 A, D). As at 5 months, TrkB was able to be detected in p75 immunoprecipitations, indicating an association between p75 and TrkB in the striatum at 10 months, irrespective of Q175 genotype (Fig. 4.1 A, C). There was no difference in TrkB levels either associated with p75 or in the WCLs (Fig. 4.1 A, C, E). Taken together, at 10 months of age, *Q175*$^{WT/HD}$ mice did not display any alterations in p75 or TrkB levels, or their association, as compared to *Q175*$^{WT/WT}$ mice.

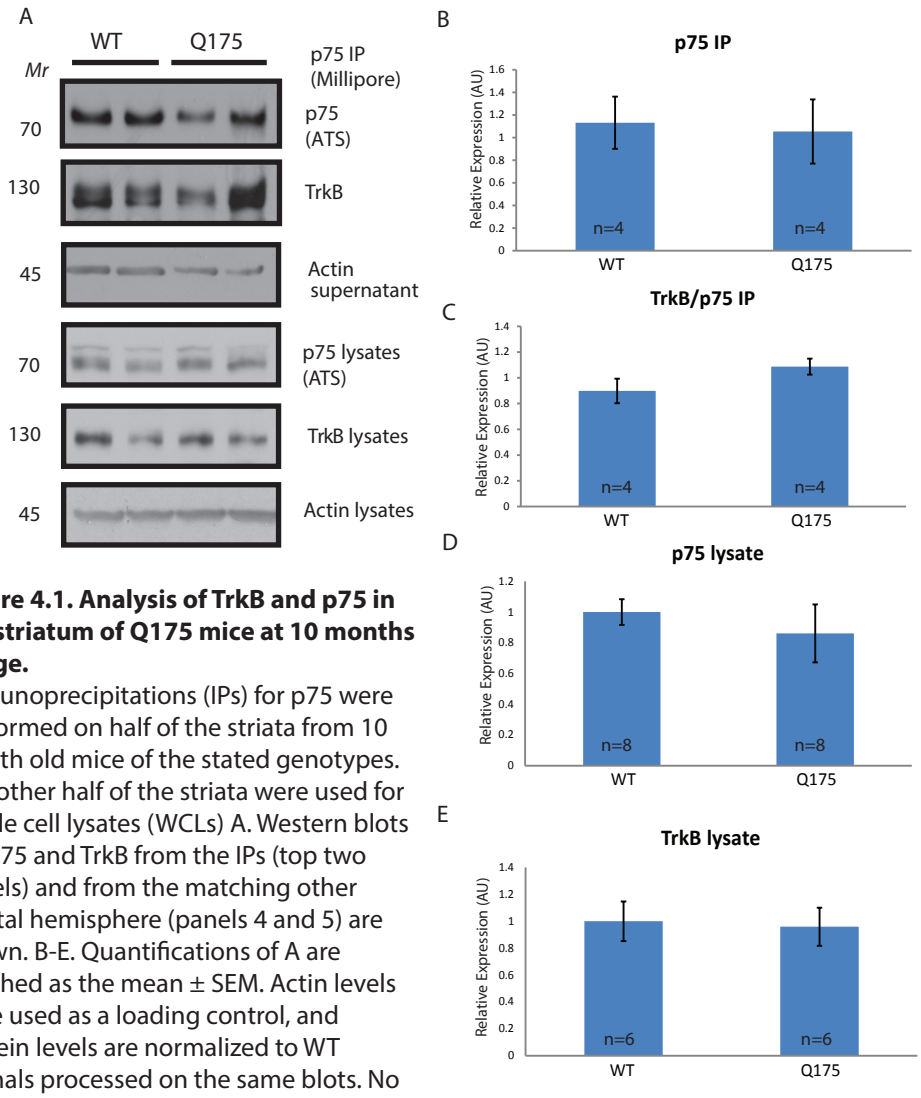

Figure 4.1. Analysis of TrkB and p75 in the striatum of Q175 mice at 10 months of age.

Immunoprecipitations (IPs) for p75 were performed on half of the striata from 10 month old mice of the stated genotypes. The other half of the striata were used for whole cell lysates (WCLs) A. Western blots for p75 and TrkB from the IPs (top two panels) and from the matching other striatal hemisphere (panels 4 and 5) are shown. B-E. Quantifications of A are graphed as the mean ± SEM. Actin levels were used as a loading control, and protein levels are normalized to WT animals processed on the same blots. No significant differences in p75 or TrkB levels between WT and Q175 mice were observed (t-test; $p>0.05$).

There are no apparent differences in apoptotic or survival signaling in the striatum at 10 months of age in Q175 mice

Activation of JNK and c-Jun, as well as the levels of PTEN, were examined in WCLs as a general measure of pro-apoptotic signaling. Quantitative immunoblotting indicated that there was no difference by any of these measures between genotypes at 10 months (Fig. 4.2 A-D). Additionally, Src and Akt activation were examined as was done at 2 and 5 months, and no significant differences were detected between genotypes in the proportion of either Src or Akt activation (Fig. 4.2 A, E, F). This lack of difference in the levels of Akt activation differs from both the 2 month and 5 month samples, where a significant increase in Akt activation was observed in the striatum *Q175*$^{WT/HD}$ mice when compared with *Q175*$^{WT/WT}$ mice. There is a trend towards this same increase, but it is no longer significant (p=0.07), suggesting that Akt activation is waning at later ages in the striatum. The levels of Bcl-XL and XIAP, both anti-apoptotic proteins regulated by NFκB, were also examined in these mice, and no significant differences were observed between genotypes (Fig. 4.3 A-C). There was, however, a significant decrease in DARPP-32 expression in the striatum of *Q175*$^{WT/HD}$ mice compared to *Q175*$^{WT/WT}$ mice (Fig. 4.3 A, D). This decrease was not observed at either 2 months or 5 months in these mice, but has been reported at 12 months before. This decrease in DARPP-32 levels indicates that dysfunction has begun in the striatum at 10 months of age.

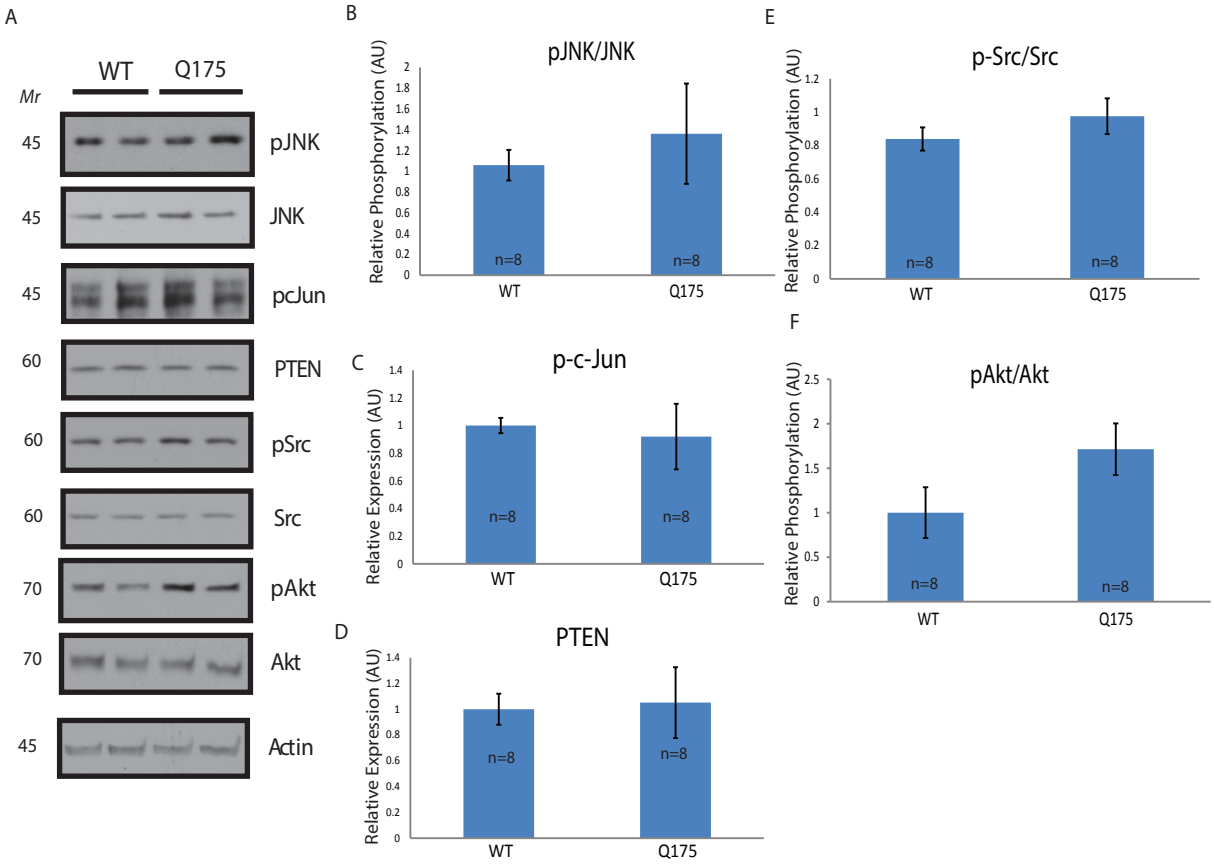

Figure 4.2. Biochemical analysis of pro-apoptotic and survival signaling in the striatum of Q175 mice at 12 months.

WCLs of the striata from 12 month old mice of the stated genotypes were analyzed by immunoblotting. A. Western blots were run on WCLs using the stated antibodies. B-F. Quantifications of A. were performed as in Figure 1. No significant differences were seen between WT and Q175 mice.

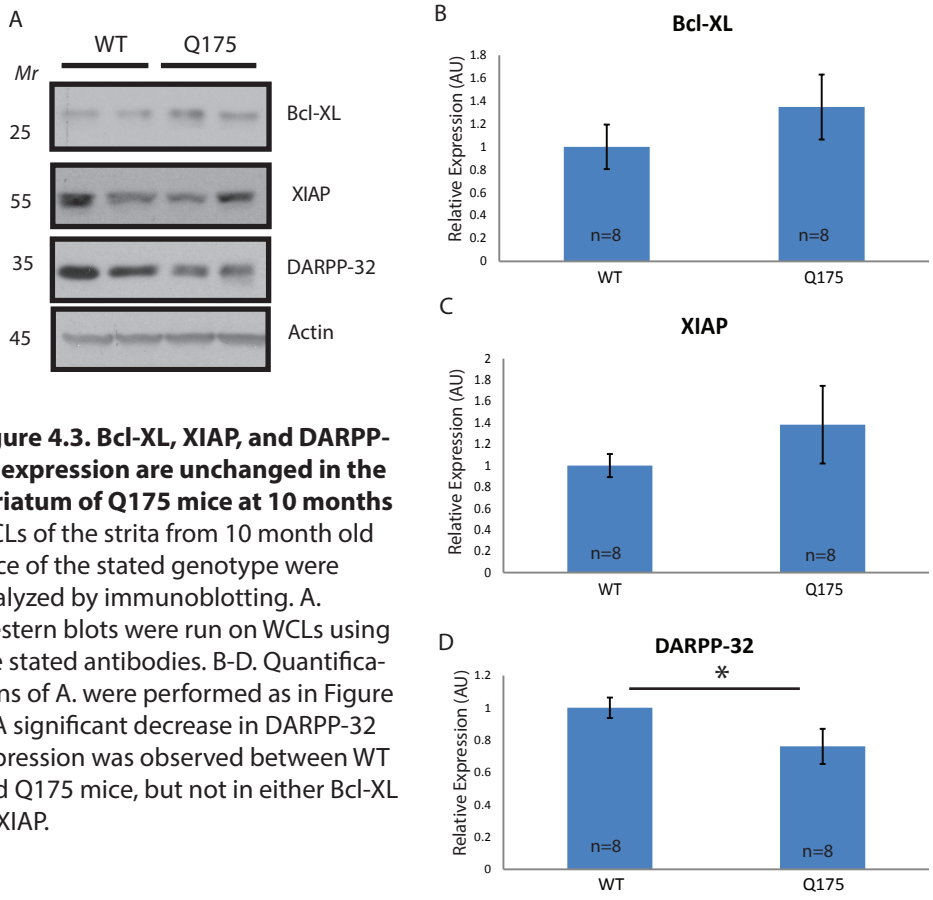

Figure 4.3. Bcl-XL, XIAP, and DARPP-32 expression are unchanged in the striatum of Q175 mice at 10 months
WCLs of the strita from 10 month old mice of the stated genotype were analyzed by immunoblotting. A. Western blots were run on WCLs using the stated antibodies. B-D. Quantifications of A. were performed as in Figure 1. A significant decrease in DARPP-32 expression was observed between WT and Q175 mice, but not in either Bcl-XL or XIAP.

Examination of p75 and TrkB levels in the striatum at 12 months

The same questions investigated above were also probed in *Q175;p75* mice at 12 months of age, similar to our analyses of 5 months and 2 months old mice. Conclusions regarding all of the signaling questions for the 12 month time point, however, are hampered by the small experimental sample sizes. In fact, no conclusions can be drawn about $Q175^{WT/HD};p75^{+/+}$ mice at this time point, because only 2 animals were harvested from this genotype. Despite this limitation, however, we have included the available 12 month data here for the sake of completeness. This smaller data set will direct plans for future studies and may reveal potentially interesting signaling changes at this age. As with our analysis of 5 months and 2 months of age, striata were used to prepare WCLs, and the levels of p75 and TrkB were examined in these WCLs. Because of the small sample size of $Q175^{WT/HD};p75^{+/+}$ mice, a statistical comparison between the $Q175^{WT/WT};p75^{+/+}$ and $Q175^{WT/HD};p75^{+/+}$ mice was unable to be made, although there appeared to potentially be a decrease in p75 expression in the WCLs of the $Q175^{WT/HD};p75^{+/+}$ mice (Fig. 4.4 D). There was no p75 detected in the $Q175^{WT/HD};p75^{-/-}$ and $Q175^{WT/WT};p75^{-/-}$ mice, again confirming the specificity of our p75 antibody (Fig. 4.4 A). There was no significant difference observed in TrkB levels in the WCL of any genotype examined (Fig. 4.4 A, E).

Pro-apoptotic and survival signaling at 12 months of age

To examine whether apoptotic signaling via the JNK pathway was altered, activation of JNK and c-Jun were examined in the WCLs of the 12 month mice. No

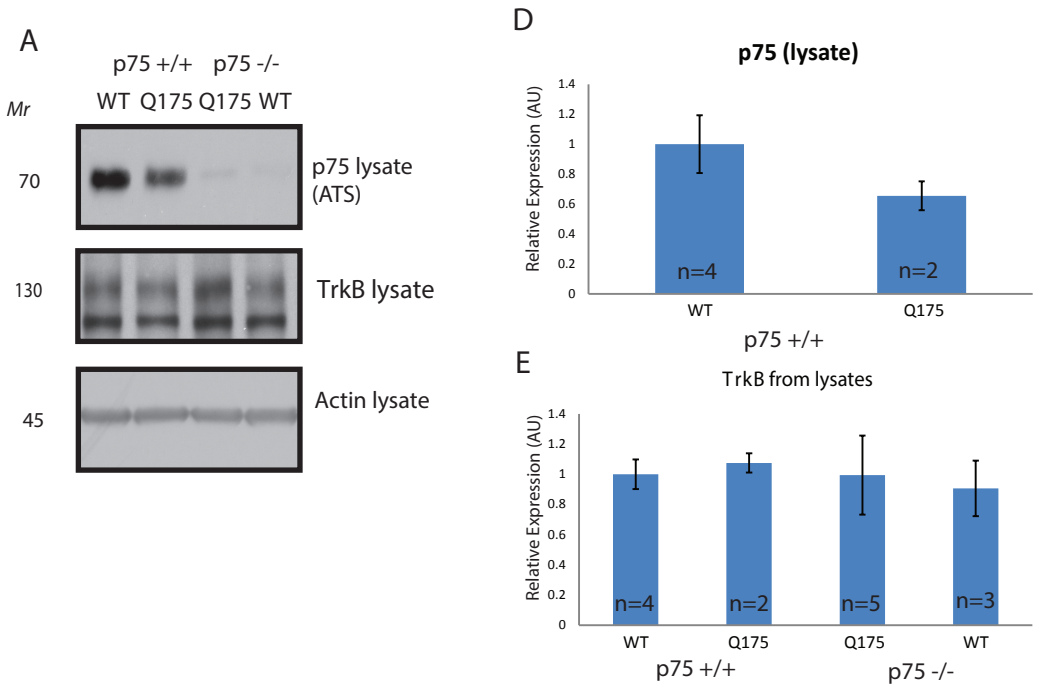

Figure 4.4. Examination of p75 and TrkB expression in the striatum of Q175 mice crossed into the germline p75 knockout at 12 months.

WCLs were made from half of the striata from 12 month old mice of the stated genotypes. A. Western blots for p75, TrkB, and actin were performed. B, C. Quantifications of A. Actin levels were used as a loading control, and protein levels were normalized to WT animals processed on the same blot. Graphs are mean +/- SEM. No significant differences in TrkB levels were observed (ANOVA; $p>0.05$).

significant differences were observed between any genotypes examined (Fig. 4.5). This lack of apparent change in JNK and c-Jun activation, combined with a similar lack of difference at 10 months, suggests that apoptotic signaling, by this pathway, is not playing an active role in the degeneration of the striatum of *Q175*$^{WT/HD}$ mice, at least at this age. One caveat to this argument, however, is that we may not be able to detect an increase in p-c-Jun and p-JNK if it is occurring only in a subset of cells present. To examine survival signaling, the activation of Akt and NFkB were examined, as in chapter 2. In contrast to 5 months, in which significant increases in activation of Akt and both sites measured on NFκB were observed in the striatum of *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice compared to all three other genotypes, no apparent differences in NFkB activation are observed in the striatum at 12 months (Fig. 4.6 A, C, D). There does appear to be a qualitative increase in Akt activation in the *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice (Fig. 4.6 A, B); however, the small sample size and the lack of a similar increase in p-Akt at 10 months (Fig. 4.2) argues that this potential increase should be contemplated with skepticism. Consistent with the lack of differences in NFkB activation, there was no observed difference in levels of Bcl-XL or XIAP expression in the striatum of any genotype examined (Fig. 4.7). Taken together, these preliminary observations argue that p75-related NFkB activity evident at 5 months of age may no longer be playing a role at 12 months of age.

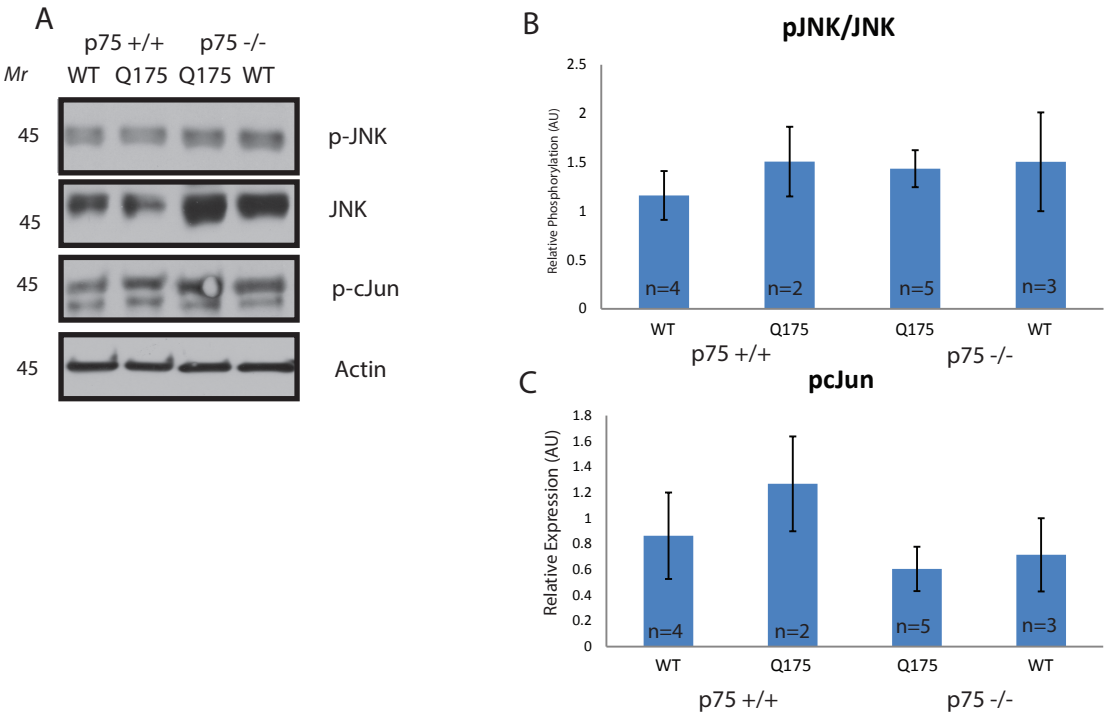

Figure 4.5. Pro-apoptotic signaling markers are unchanged in the striatum at 12 months. WCLs were examined on half of the striata from 12 month old mice of the stated genotypes. A. Western blots were run on WCLs using the stated antibodies. B, C. Quantifications of A. Normalization was performed as in Figure 1. No significant difference in JNK or c-Jun phosphorylation was observed in any genotype for statistically testable genotypes (n=3 or above) (ANOVA; $p>0.05$).

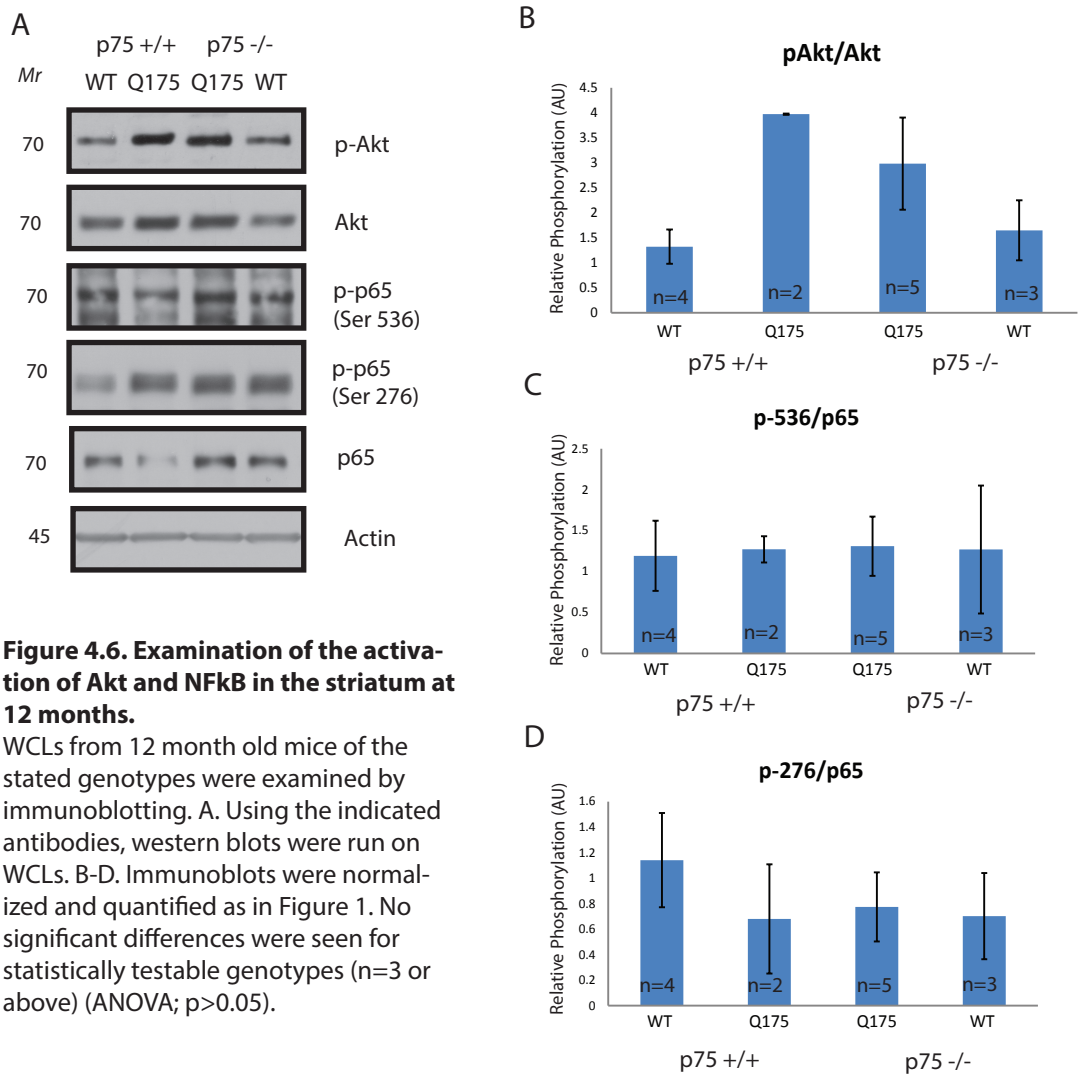

Figure 4.6. Examination of the activation of Akt and NFkB in the striatum at 12 months.
WCLs from 12 month old mice of the stated genotypes were examined by immunoblotting. A. Using the indicated antibodies, western blots were run on WCLs. B-D. Immunoblots were normalized and quantified as in Figure 1. No significant differences were seen for statistically testable genotypes (n=3 or above) (ANOVA; p>0.05).

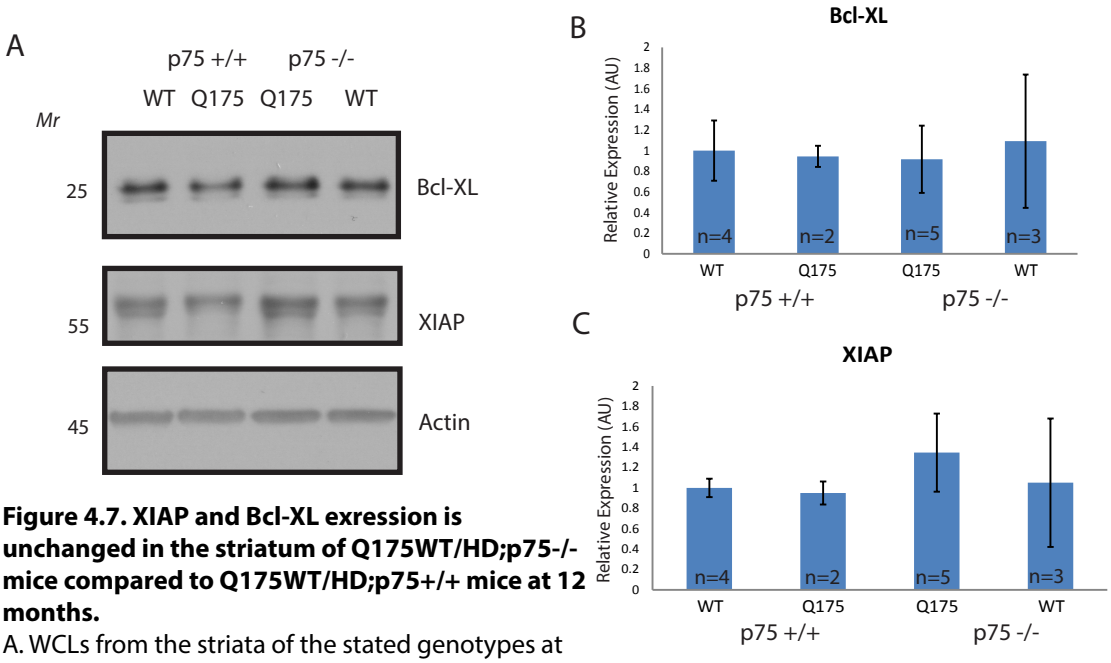

Figure 4.7. XIAP and Bcl-XL exression is unchanged in the striatum of Q175WT/HD;p75-/- mice compared to Q175WT/HD;p75+/+ mice at 12 months.

A. WCLs from the striata of the stated genotypes at 12 months of age were tested using Bcl-XL, XIAP, and Actin antibodies. B, C. Quantifications of A. No significant change in XIAP and Bcl-XL expression was observed (ANOVA; $p>0.05$).

Striatal DARPP-32 levels and volume are decreased at 12 months of age

As a general measure of striatal function, the expression of DARPP-32 was examined by two methods: immunoblotting of WCLs and IHC on striatal slices from perfused brains. Interestingly, a significant decrease in DARPP-32 expression by immunoblotting was again observed between *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice and *Q175*$^{WT/WT}$*;p75*$^{+/+}$ littermates, as we observed at 5 months (Fig. 4.8 A, B). Similarly, while significance is unable to be determined due to the small sample size, there appears to be a similar decrease in DARPP-32 expression in the striatum of *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice (Fig. 4.8 A, B). While this potential decrease compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ mice is not surprising, and has been observed before, the observation that the level of decrease appears the same between *Q175*$^{WT/HD}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice, as compared to the data on 5 month old mice (Fig. 2.7), is interesting because it suggests that the dysfunction observed at 5 months in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice stabalizes to *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice instead of continuing to progress. There was also a qualitative decrease in DARPP-32 expression by IHC in both *Q175*$^{WT/HD}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ and *Q175*$^{WT/WT}$*;p75*$^{-/-}$ mice, although *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice seem to qualitatively have less DARPP-32 expression than their *Q175*$^{WT/HD}$*;p75*$^{+/+}$ littermates (Fig. 4.8 C-F). Finally, striatal volume was measured using the Cavilieri method on brain slices stained for NeuN, as described in chapter 2. While there was no apparent difference in neuronal density, there was a decrease in striatal volume in both *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice (6.35×10^9 +/- 0.067 μm^3) and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice (6.00×10^9 +/- 0.13 μm^3) when compared to either *Q175*$^{WT/WT}$*;p75*$^{+/+}$ mice (6.70×10^9 +/- 0.13 μm^3) or *Q175*$^{WT/WT}$*;p75*$^{-/-}$

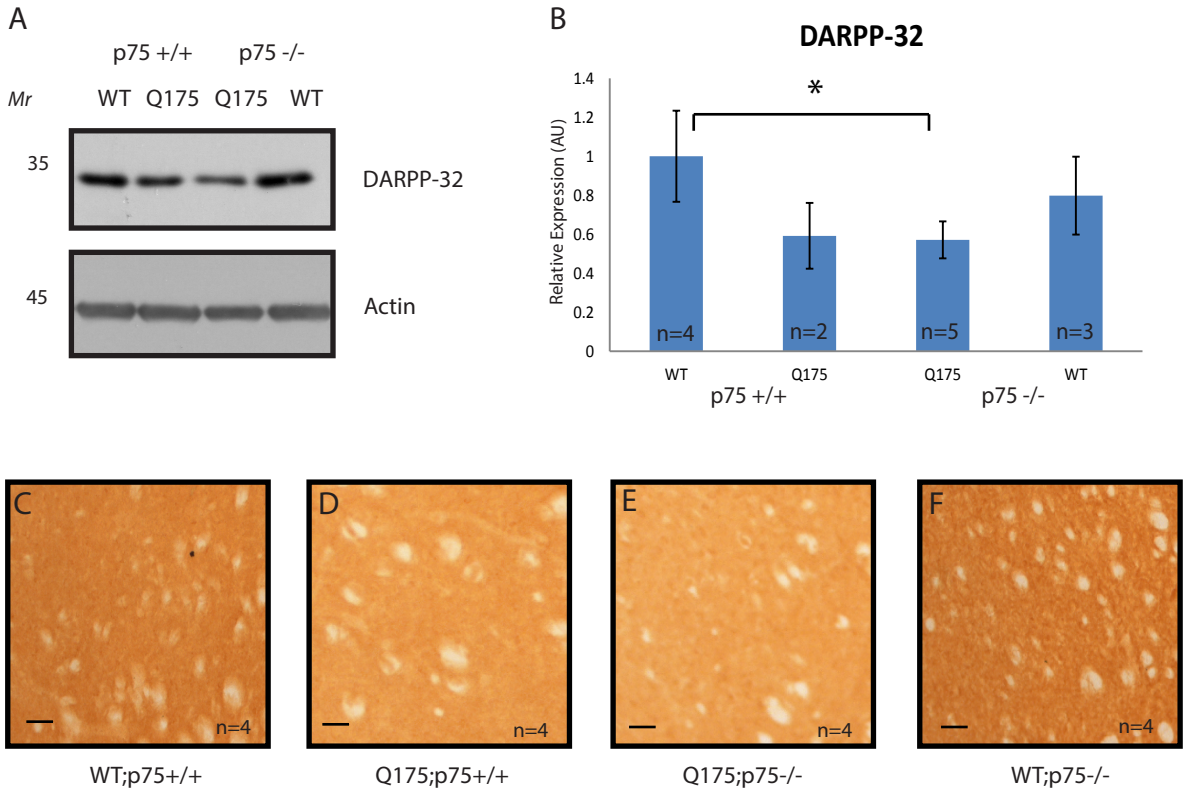

Figure 4.8. DARPP-32 expression decreased in the striatum of Q175WT/HD;p75-/- mice compared to WT;p75+/+ mice. A. WCLs from half of the striata of the stated genotypes at 12 months of age were probed using the listed antibodies. B. Quantification of A. C-F. Immunohistochemistry was performed on striata of 12 month old mice of the stated genotype for DARPP-32 as described in the methods. Statistics were unable to be run on Q175;p75+/+ mice for western, due to sample size. * $p<0.05$; t-test. Scale bar: 50 μm

mice ($6.71x10^9$ +/- 0.27 μm^3) (Fig. 4.9). A decrease in striatal volume has been reported in *Q175*$^{WT/HD}$ mice at 12 months of age previously which agrees with what we observe (Smith et al., 2014). Interestingly, there is also a significant decrease in striatal volume of *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice compared to *Q175*$^{WT/HD}$*;p75*$^{+/+}$ littermates, indicating an earlier and/or more severe onset of degeneration in the striatum of *Q175*$^{WT/HD}$ mice in the absence of p75.

Discussion

In this chapter, we examined the role of p75 at later, fully symptomatic ages in the Q175 mouse model of HD. In contrast to our analyses of mice at both 2 months and 5 months, there was no significant increase in Akt activation in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice. Additionally, unlike at 5 months, there is also no observed increase in NFkB activation or XIAP expression in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice and no decrease in Bcl-XL expression in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice at this age. There was an interaction observed between p75 and TrkB in the striatum of both *Q175*$^{WT/WT}$ and *Q175*$^{WT/HD}$ mice at 10 months, as at both 2 and 5 months. Similar to our data from both 2 and 5 month old mice, there was no observed change in the expression levels of p75 or TrkB and no difference in the activation of Src, c-Jun, or JNK. Interestingly, there was a decrease in DARPP-32 expression observed in the striatum of *Q175*$^{WT/HD}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice compared to WT littermates at this age. This decrease in DARPP-32 expression was also observed in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice but not *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice at 5 months of age. Intriguingly, the decrease in DARPP-32 expression does not appear to be different

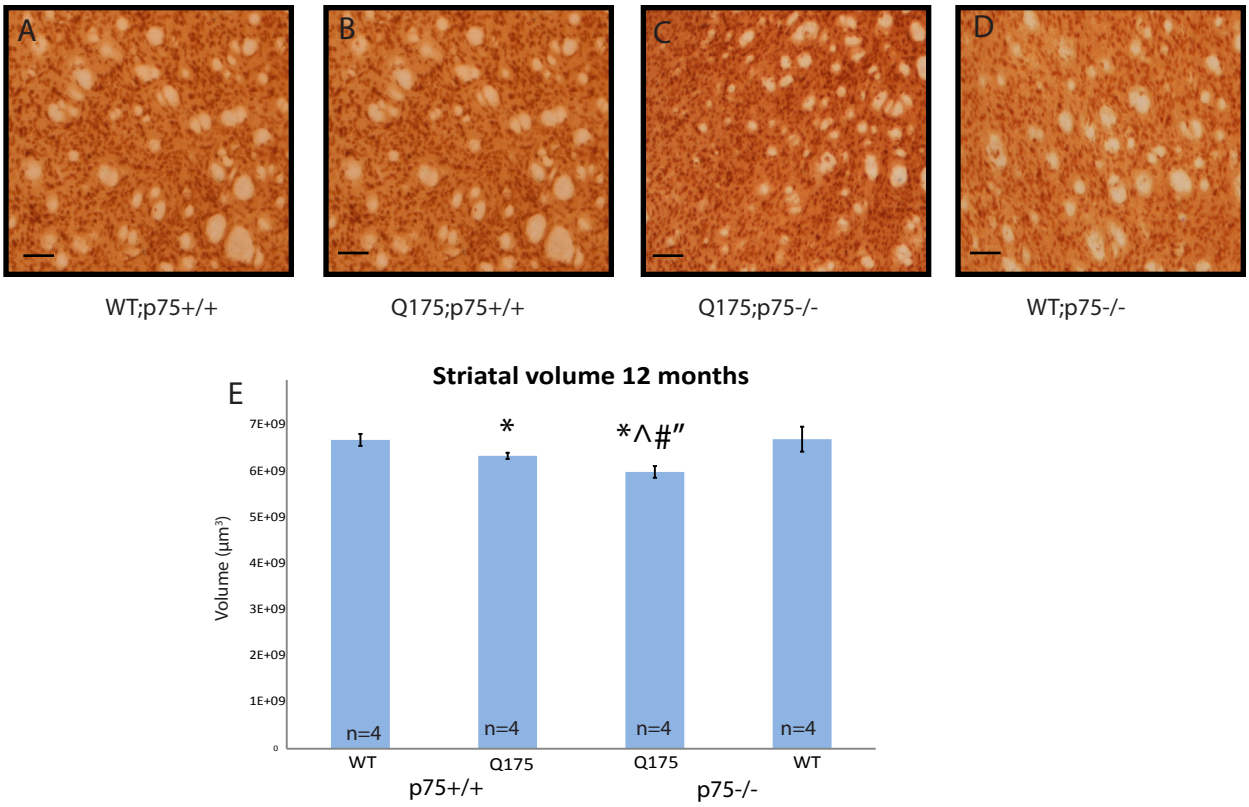

Figure 4.9. Neuronal volume is decreased in the striatum of Q175;p75-/- at 12 months of age. A-D. NeuN immunohistochemistry was performed on striata of 12 month old mice of the stated genotypes as described in the methods. E. Striatal volume quantifications of A-D. Striatal volume is decreased in both Q175;p75+/+ and Q175;p75-/- mice compared to WT;p75+/+ mice. Striatal volume is also decreased in Q175;p75-/- mice when compared to Q175;p75+/+ littermates. * indicates significantly decreased compared to WT;p75+/+, ^ indicates significantly decreased compared to Q175;p75+/+, # indicates significantly decreased compared to WT;p75-/-," indicates significantly decreased compared to Q175;p75-/- at 2 and 7 months (ANOVA; $p<0.05$). Scale bar: 50µm

between these two genotypes at 12 months of age, suggesting that dysfunction may now be equivalent between the two genotypes. All of the observed protein expression levels at 12 months, however, are hampered by the small sample size available for the *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice. Due to some difficulties with breeding and survival of these mice, we were unable to obtain a larger sample size of 12 month animals. Therefore, the results should be validated using a larger sample size in future studies. Finally, and perhaps most interestingly, there is a decrease in striatal volume in both the *Q175*$^{WT/HD}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice, and the decrease observed in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice is significantly greater than the decrease observed in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice, indicating an earlier and/or more severe onset of degeneration in *Q175*$^{WT/HD}$ mice in the absence of p75. These results suggest the hypothesis that the increased survival signaling observed in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice at 5 months of age is important for delaying onset of dysfunction/degeneration of the striatum, and that disruption of p75 signaling, by germline deletion in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice, leads to earlier and/or more severe pathological changes.

References

Smith, G. A., Rocha, E. M., McLean, J. R., Hayes, M. A., Izen, S. C., Isacson, O., & Hallett, P. J. (2014). Progressive axonal transport and synaptic protein changes correlate with behavioral and neuropathological abnormalities in the heterozygous Q175 KI mouse model of Huntington's disease. *Human Molecular Genetics, 23*(17), 4510-4527.

Chapter 5: Conclusions and Discussion

In summation, we examined the role of p75 signaling across various ages in the Q175 mouse model of Huntington's disease (HD). By comparing the data collected from 2 months, 5/7 months, and 10/12 months, we can begin to assess how p75 regulates pro-apoptotic and pro-survival signaling pathways, and thereby affects pathological changes in the striatum over time (Fig 5.1). These three ages represent a very early time point, a later but still pre-symptomatic time point, and a time point past symptom onset. Akt activation is increased in $Q175^{WT/HD};p75^{+/+}$ mice relative to $Q175^{WT/WT};p75^{+/+}$ and $Q175^{WT/HD};p75^{-/-}$ mice as early as 2 months of age and remains increased at 5 months, but is no longer significantly increased at 10/12 months. While we were not able to examine NFκB activation at the 2 month time point, phosphorylation is increased at 5 months of age on two separate serine residues, and this increase appears to be lost by 12 months of age. In further support of the important role of p75 in survival signaling through Akt and NFκB at early ages in $Q175^{WT/HD};p75^{+/+}$ mice, there is an increase in expression of XIAP, an anti-apoptotic protein known to be regulated by NFκB, which correlates with the increased activation of NFκB. This increase in XIAP expression is absent in $Q175^{WT/HD};p75^{-/-}$ mice. Additionally, expression of Bcl-XL, an anti-apoptotic member of the Bcl-2 family, is decreased at 5 months of age specifically in the $Q175^{WT/HD};p75^{-/-}$ mice. These changes suggest that the pro-survival effects of p75 in the striatum of $Q175^{WT/HD};p75^{+/+}$ mice begin early and taper off later in life, around

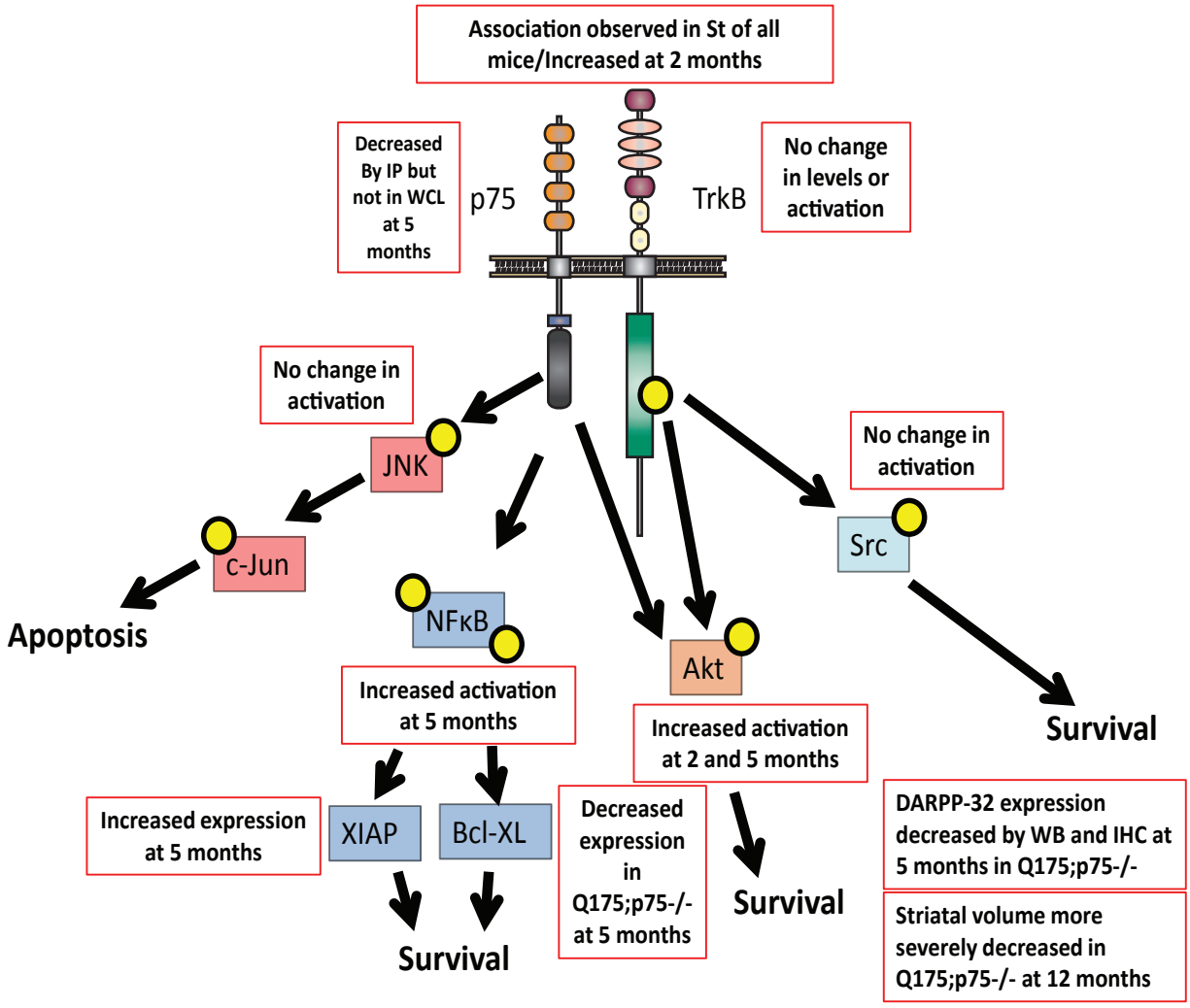

Figure 5.1. p75 and TrkB signaling changes schematic.
Schematic of the results pro-survival and pro-apoptotic signaling downstream of TrkB and p75 measured in this study.

the time of symptom onset. The significance of this increased survival signaling is supported by the earlier onset of striatal dysfunction and degeneration in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice compared to their *Q175*$^{WT/HD}$*;p75*$^{+/+}$ littermates. This earlier onset of striatal dysfunction and degeneration is evidenced by an earlier decrease in DARPP-32 expression and a more severe striatal volume decrease in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice.

At 2 months of age, in addition to the increased activation of Akt, there was also a significant decrease in p-c-Jun observed in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ littermates. There was, however, no change in p-c-Jun expression at either 5 or 12 months of age. 2 month *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice also displayed a significant increase in the amount of TrkB associated with p75 in the striatum compared to their *Q175*$^{WT/WT}$*;p75*$^{+/+}$ littermates. While this association between p75 and TrkB was also observed at 5 and 10 months, the levels of association were not significantly different between genotypes at these later ages. This increased p75/TrkB association may account for some of the increased survival signaling observed at 2 months, and may also explain the decreased p-c-Jun observed at this age.

Another important observation which differed with age was the comparison of p75 levels by WCL and by IP. At 2 and 10 months, there was no change in p75 levels between *Q175*$^{WT/HD}$*;p75*$^{+/+}$ and *Q175*$^{WT/WT}$*;p75*$^{+/+}$ mice by analyzing either IPs or whole cell lysates (WCLs). In the 5 month mice, however, there was a significant decrease in the amount of p75 observed in the IP sample of the *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice compared to their *Q175*$^{WT/WT}$*;p75*$^{+/+}$ littermates, while in the WCLs there was no difference in p75 levels between genotypes. This decrease in IPs and not in WCLs may indicate formation of a p75 signaling complex unique to this age. This idea will be further

discussed later in this chapter. Finally, there was no change in Src activation or TrkB total levels in any genotype examined at any age, arguing for the selective augmentation of some survival pathways but not others. Our proposed model for pro-survival and pro-apoptotic signaling changes and onset of dusfunction/degeneration is summarized in Figure 5.2.

Possible mechanism of survival signaling

While these experiments support a role for p75 in enhanced survival signaling in the striatum of *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice at pre-symptomatic ages, it does not address how this enhanced survival signaling occurs. Furthermore, it is possible that the mechanism by which p75 promotes survival signaling changes over time. The increased association between p75 and TrkB observed in the striatum of *Q175*$^{WT/HD}$*;p75*$^{+/+}$ as compared to *Q175*$^{WT/WT}$*;p75*$^{+/+}$ mice at 2 months of age supports the idea that a p75/TrkB complex may be formed to compensate for the lower levels of BDNF transported to the striatum from the cortex in *Q175*$^{WT/HD}$ mice (Ma et al., 2015). The increased Akt activation observed at 2 months could be downstream of this complex (Bui et al., 2002; Ceni et al., 2010; Verbeke et al., 2010; Deinhardt and Chao, 2014). By 5 months of age, however, this increased p75/TrkB association is no longer observed, but there is still an increase in Akt activation. This increase in Akt activation, along with the observed increase in NFκB activation, and the changes in XIAP and BCL-XL expression also observed at 5 months of age, raise the possibility that the increased survival signaling in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice at this age is not downstream of TrkB activation. It is important to

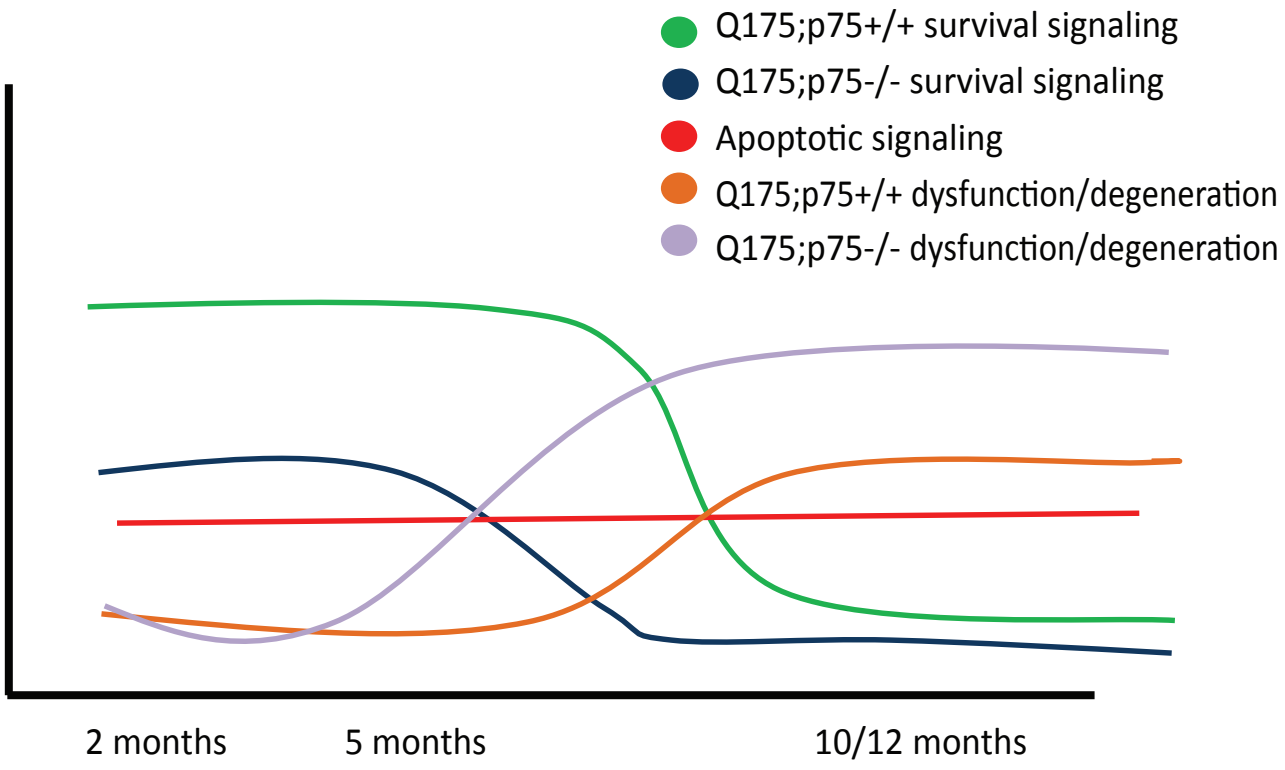

Figure 5.2. Proposed model of pro-survival and pro-apoptotic signaling changes and dysfunction/degeneration. In Q175;p75+/+ mice survival signaling is enhanced pre-symptomatically and declines at later ages, leading to onset of dysfunction and degeneration. In Q175;p75-/- mice, this enhanced survival signaling is not as high pre-symptomatically, leading to an earlier onset of dysfunction and degeneration.

note, however, that we still observe an association between p75 and TrkB at 5 months of age, and it is likely that some of this survival signaling is due to TrkB activity.

Increased activation of both NFκB and Akt has been shown to be downstream of p75 activation alone, independently of TrkB (Carter et al., 1996; Longo et al., 1997; Mattson et al., 1997; DeFrietas et al., 2001; Khursigara et al., 2001; Roux et al., 2001; Verbeke et al., 2010; Vicario et al., 2015). On the other hand, it is also possible that p75 is forming a complex with another receptor, and the formation of this complex may explain our conflicting IP/WCL results. Recent studies have reported the presence of TrkC as well as TrkB in the striatum of both young and adult mice (Baydyuk et al., 2011; Baydyuk et al., 2013; Baydyuk et al., 2014). Interestingly, these two receptors seem to be mostly segregated according to MSN cell type. The D1-expressing MSNs appear to express predominantly TrkC while the D2-expressing MSNs appear to express mostly TrkB (Baydyuk et al., 2013). These potential associations between p75-TrkB and p75-TrkC, and their potential involvement in p75-dependent survival signaling, have interesting implications for the pathology observed in HD. In HD, the D2-expressing MSNs become dysfunctional earlier than the D1-expressing MSNs (Albin et al., 1992). If p75 is able to form complexes with both TrkB and TrkC to enhance survival signaling in MSNs, the p75/TrkC complex may be more successful at prolonging normal functioning of the neurons due to the presence of neurotrophin-3 in the striatum (Baydyuk et al., 2013). The p75/TrkB complexes, meanwhile, would be less successful due to the decrease in striatal BDNF, causing the D2-expressing neurons to degenerate more rapidly. To address this issue, D1 and D2-specific Cre lines could be utilized. These lines express Cre recombinase under the *Drd1* and *Drd2* promoters, respectively

(Jackson Labs). If they were crossed into a mouse model of HD, along with conditional p75 mice in which p75 is flanked by *loxp* sites (Bogenmann et al., 2011), they could be used to selectively delete p75 in the D1 or D2-expressing neurons and examine whether p75 deletion has a larger effect on one population or opposing effects on the two populations. If this p75/TrkC complex is being formed and promotes longer survival of D1-expressing neurons, they would be expected to degenerate more quickly when p75 is specifically deleted in these neurons. This examination of pathologic changes, in order to make valid conclusions, would need to accompany biochemical measurements of TrkB and TrkC levels, phosphorylation, and interaction with p75.

Possible role of p75 in apoptotic signaling

The nature of the $p75^{-/-}$ mouse line used in our studies makes conclusions about the potential role of p75 in apoptotic signaling pathways at later, symptomatic ages problematic. There were no changes in JNK activation in the striatum of $Q175^{WT/HD};p75^{+/+}$ or $Q175^{WT/HD};p75^{-/-}$ mice at any ages examined, and c-Jun activation was only affected at 2 months of age. Combined with the increased survival signaling observed in the striatum of $Q175^{WT/HD};p75^{+/+}$ mice at 2 and 5 months, these observations suggest little role for JNK and c-Jun-mediated apoptosis at early, pre-symptomatic time points. One caveat to this immunoblotting method, however, is that differences in individual neurons could be difficult to detect if there is an overall basal level of JNK and c-Jun phosphorylation, which we have observed (Fig 2.2, 2.4, 3.2, 4.2, 4.6). The survival signaling activity of p75, however, appears to have tapered off by the

10/12 month time point. Despite the lack of observed JNK and c-Jun activation changes at this time point, it is possible that p75 is playing a role in apoptotic signaling later in the disease course, and we are unable to detect this effect due to the nature of the germline deletion of p75. Because the *Q175$^{WT/HD}$;p75$^{-/-}$* mice begin dysfunction and degeneration in the striatum at an earlier time point than *Q175$^{WT/HD}$;p75$^{+/+}$* mice, a potential protective role of p75 deletion at later ages may be missed. In order to address this problem, time-specific deletion of p75 will be required. We attempted to address this issue using a p75 conditional line in which exons 4, 5, and 6 of p75 are flanked by *loxp* sites. We crossed this line into a knock-in mouse model of HD similar to the Q175 line used in this study which has approximately 200 glutamine repeats (Q200). We then crossed these mice into a line which expresses Cre under a ubiquitin promoter, which is induced by treating the mice with tamoxifen (*UBC-Cre^{ERT2}, Jackson labs)*. The goal of this experiment was to allow the mice to develop and age with normal amounts of p75 and then delete p75 at 7-8 months of age. Unfortunately, while the p75 deletion was successful in peripheral neuronal populations, we were not able to achieve consistent deletion of p75 in the striatum, and therefore, we were not able to address the issue of whether p75 plays a protective or pro-apoptotic role during disease onset in HD. This question, however, remains critical to understanding the role of p75 signaling in the striatum at later ages. To combat this problem, more powerful Cre lines should be examined to determine if other promoters (Actin, CMV), or other induction systems (Tet-on, for example), can achieve a higher level of striatal deletion. Additional methods of Cre-induced deletion could also be explored, such as the use of a Cre line under an endogenous promoter which does not require tamoxifen activation, although finding a gene which “turns on” at

relevant times and in the relevant neurons has been difficult. We are currently not aware of any striatum-enriched Cre lines that turn on after development.

Direct injection of an AAV-Cre viruses into the striatum of animals at the relevant ages is also a possible alternative, although there are challenges with this approach as well. The striatum is a deep structure, and reaching it in order to perform injections would disrupt other brain areas and connections, possibly causing complications that would impair interpretation of the study results. Additionally, the deletion would need to be spread throughout the entire striatum, and would likely require several injections. Another interesting way to tackle the possible temporal control of p75 signaling in the striatum of Q175 mice would be to allow the *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice to age until they have reached 5-7 months (after we observed dysfunction but prior to degeneration onset), and then re-introduce p75 potentially using viral expression. If p75 plays an apoptotic role once the striatum begins to become dysfunctional, then we would anticipate that these mice will degenerate even faster than *Q175*$^{WT/HD}$*;p75*$^{+/+}$ and *Q175*$^{WT/HD}$*;p75*$^{-/-}$ littermates.

Cell types expressing p75 in the striatum

While our experiments clearly support a role for p75 in survival signaling at pre-symptomatic ages, a limitation of the study design is the inability to determine which cell type(s) are essential for this protective role. Whole striatal extracts were used to produce the WCLs and IPs which were used in these experiments, and many cell types were present in these extracts: neurons and multiple types of glia. The MSNs of the

striatum (especially the D2-expressing MSNs) are the predominant and earliest affected population in the striatum of HD patients (Reiner et al., 1988; Albin et al., 1992; Deng et al., 2004). The observed DARPP-32 reduction by western and IHC at 5/7 and 10/12 months is expressed in these MSNs, but the increased survival signaling through Akt and NFκB need not necessarily come from the MSNs themselves. While there may be a cell-autonomous function of p75 in survival signaling, it is possible that there are also non-cell-autonomous functions of p75 in promoting longer survival of MSNs in *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice. The cell types which express p75 in the striatum are unclear. Studies have suggested p75 is expressed by MSNs and also by oligodendrocytes (Brito et al., 2013; Ma et al., 2015). We attempted to determine which cell types express p75 in the striatum using both IHC and immunofluorescence (IF). The autofluorescence of the white matter tracks in the striatum themselves, however, made double-label IF difficult for us to achieve the level of specificity necessary to have confidence in the validity of the staining. To combat this issue, we attempted to use enzymatic IHC. While we were able to achieve specific p75-staining using a DAB substrate in the striatum, we were unable to achieve specific co-labeling for cell types with another enzymatic marker, making any conclusions about the cell type expressing p75 speculative.

Based on the staining we achieved for p75 (Fig 5.3), the majority of the p75 we observed appears to be expressed either in astrocytes or in white matter tracks. For all of our p75 IHC experiments, we included not only a secondary antibody alone control, but also a *p75*$^{-/-}$ negative control to confirm the specificity of the labeling we observed (Fig 5.3). We have also performed DAB-IHC for myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule 1 (Iba1), and

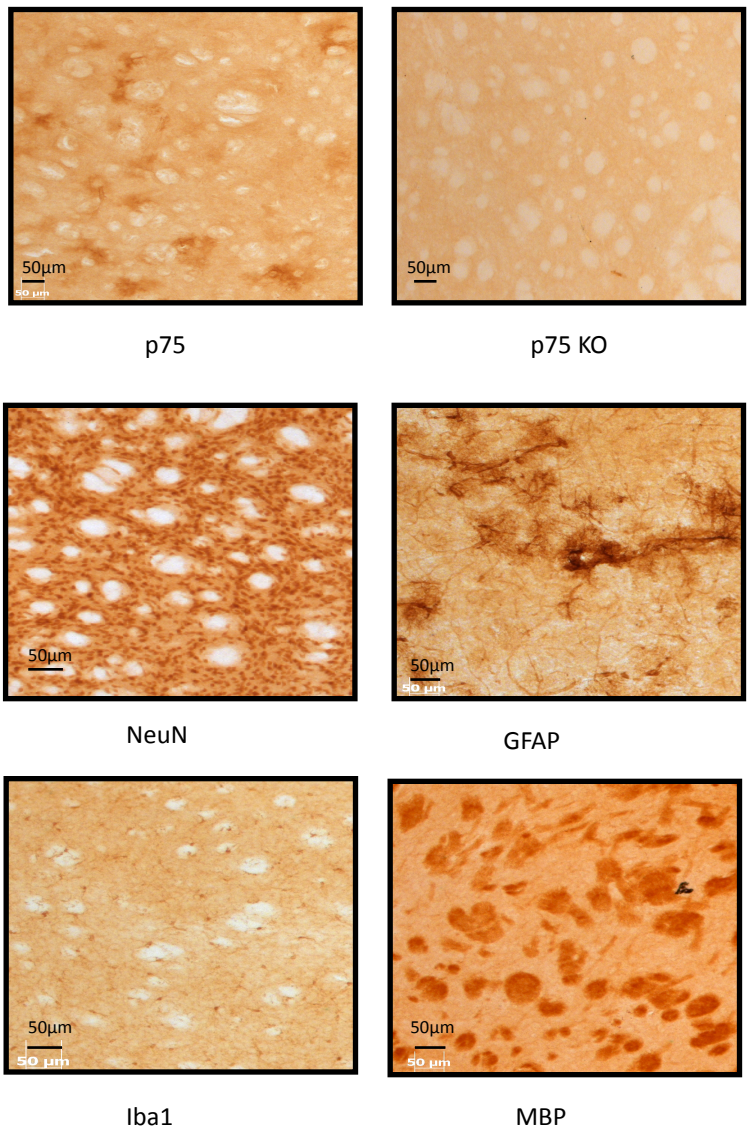

Figure 5.3 Expression of p75 and various cell types in the striatum of Q175 mice. Immunohistochemistry was performed on brain slices from 7 month old Q175WT/HD mice using the listed antibodies.

NeuN (Fig 5.3). These four proteins label oligodendrocytes (white matter tracks), astrocytes, microglia, and neurons, respectively (Mullen et al., 1992; Benjamins and Morell, 1978; Eng and Ghrinikar, 1994; Imai et al., 1996). Based on visual comparisons of the staining pattern in the striatum of these four cell-type markers with the expression pattern we observe for p75, we hypothesize that p75 is expressed either in astrocytes or white matter tracks. Without being able to perform co-labeling, however, this conclusion is difficult to state with confidence. This difficulty can be addressed in several ways. A mouse line expressing Cre under the p75 locus would allow us to create a p75 reporter line that would allow for visualizing cell types expressing p75, although the auto-fluorescence of the striatum would still be a challenge. Additional methods of co-labeling using different enzymatic assays could also be performed. There are many other enzymatic assays available for IHC (such as alkaline phosphatase and BCIP, Vector labs), and it is likely that two of these could be combined to yield reliable staining for both p75 and specific cell types in order to test co-expression.

One additional way to examine the importance of p75 signaling within specific striatal populations is to utilize the Cre recombinase system and p75 conditional mice, as just discussed. By using the p75 conditional line crossed into the Q200 mouse model of HD, cell-type specific Cre recombinase lines could be utitlized to investigate the importance of p75 expression in various cell types. A Cre line specific for dopaminergic neurons (DAT-Cre, Drd1-Cre, Drd2-Cre) could be used to test the role of cell-autonomous p75 signaling from the MSNs themselves in regards to increased survival signaling. GFAP-Cre and Olig2-Cre systems could be used to test the non-cell-autonomous functions that p75 signaling in astrocytes and oligodendrocytes,

respectively; these cell types may play a role in the increased survival signaling and in the development of dysfunction and degeneration of MSNs. As these experiments are designed to only remove p75 from certain cell types, a WCL would likely not show substantial differences in p75 expression and could therefore not be used to validate the effectiveness of the Cre-mediated recombination. A separate experiment would need to be performed using a Rosa-26 reporter line, or a similar approach, to measure how much recombination is being achieved using different promoters in order to accurately assess the results observed. The timing of Cre-mediated recombination and p75 deletion would also be important to control. Initial studies could use a system in which Cre is expressed in the target cell type as soon as the marker is expressed without experimenter induction being necessary, unlike tamoxifen-mediated excision. In order to address the relevance of p75 signaling at specific times in specific cell types, a system would need to be utilized in which p75 deletion can be achieved in that cell type at various time points of the experimenter's choosing using tamoxifen-mediated excision or the tet-on system. Quite surprisingly, there are very few dopaminergic neuron-specific Cre^{ERT2} mouse lines available that have been well-characterized, making this more of a long-term goal.

AAV-Cre injections into the area of interest to delete p75 is also a possibility to achieve region-specific p75 deletion, and similar experiments have been used in HD mouse models using an AAV-shRNA to p75 (Brito et al., 2014; Plotkin et al., 2014). One of the major limitations of AAV-shRNA experiments is that one does not typically achieve complete deletion of the protein of interest, and usually varying levels of deletion across experiments are observed. In addition, there is the potential

complication of either not fully penetrating your tissue of interest or that the AAV diffuses into areas outside the region of interest. For AAV-shRNA experiments, a rigorous verification of knock down on the individual cell level needs to be built into the project design. This type of experiment, though, if performed carefully could be used to address many interesting questions.

Our results raise the question of whether p75 signaling in other areas of the brain (such as the cortex) can affect the health and signaling of distant areas of the brain (such as the striatum). In the experiments reported here, p75 was deleted from all cells in the brain. Therefore, not only is our ability to make conclusions about which cell type(s) are essential for p75's survival promoting effects limited, but we also cannot be certain that p75 signaling is necessary in the striatum itself for this increased survival signaling. The p75-dependent increased survival signaling we observe in the striatum may actually originate in the cortex and have an indirect effect on striatal MSNs through the anterograde transport of BDNF, or some other factor, from the cortex to the striatum. Using the powerful tissue-specific p75 deletion methods described here, this question could be addressed.

Role of p75 in behavioral phenotypes

This study focused on biochemical and pathological changes in the striatum of pre- and post-symptomatic *Q175*$^{WT/HD}$ mice and how the expression of p75 affected these changes. We chose not to measure behavioral phenotypes or the effect of p75 on motor behavior. While behavioral phenotypes are widely used in the field as a measure

of the effectiveness of drugs or other interventions (Brooks et al., 2011), there are many conflicting opinions on the ability of behavioral tests in rodents to faithfully predict successful treatment in humans. Indeed, many treatments which have shown promising behavioral results in rodents have had very little or no clinical effect when used for clinical trials (Ochs et al., 2000; Mariga et al., 2016). Even after considering these limitations, however, behavioral phenotypes are still regarded by many in the field as an essential measure of the effectiveness of a drug or the validity of a new animal model. Given the earlier onset of dysfunction and degeneration in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice when compared to their *Q175*$^{WT/HD}$*;p75*$^{+/+}$ littermates, I would predict an earlier onset of behavioral symptoms. One limitation with our use of germline *p75*$^{-/-}$ mice is that they develop an age-dependent peripheral neuropathy (Bogenmann et al., 2011; Lee 1992). Indeed, these mice will be impaired in the rotorod and balance bean tests at older ages, potentially making it difficult to observe changes in *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice. Nevertheless, at younger ages, it would be interesting to generate a cohort large enough to run behavioral tests and measure rotorod performance as well as gait analysis. After the behavioral tests, the brains could then be examined for biochemical and pathological changes. For these experiments, we would choose at least three time points: one early age (no later than 3 months) to see if the animals begin at a similar baseline of motor and cognitive behavior, one age before the normal onset of symptoms in *Q175*$^{WT/HD}$ mice (at 5 months) to determine whether *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice develop symptoms earlier, and one at 18 months once *Q175*$^{WT/HD}$*;p75*$^{+/+}$ mice become fully symptomatic to see if *Q175*$^{WT/HD}$*;p75*$^{-/-}$ mice continue to perform more poorly, or if the two genotypes equalize.

Validation of these findings in other animal models of HD and human HD tissues

As mentioned in chapter 2, it is important to note that some of our results conflict with previously published results. For example, we see no change in p75 or TrkB protein levels in the striatum at any of the ages we examined. A 2013 study from the Gines lab reported a significant increase in p75 and a decrease in TrkB in the striatum of R6/1 mice and homozygous Q111 mice (Brito et al., 2013). The R6/1 model is significantly different than the heterozygous Q175 mice used in this study in construct design, phenotype onset, and pathological progression (Brooks et al., 2011). As mentioned in the introduction, R6/1 mice are a truncation mouse model in which exon 1 and the expanded polyQ region of the human *htt* were transgenically expressed in the mouse genome (Brooks et al., 2011). This model develops rapid behavioral phenotypes and has a much shorter life span than the Q175 mouse model. Due to the nature of the transgenic overexpression of the expanded exon 1 of poly-Q region of the protein, physiological and biochemical changes that are observed in this model only represent changes caused by the presence of the mutated section of htt, and the loss of function alterations associated with mutant Htt seen in humans are not present.

While these changes in p75 and in TrkB expression were also reported in a homozygous knock-in mouse (Q111), the lack of functional “wild type” hdh in this model may explain the decreased TrkB levels. The same lab also reported an increase in p75 levels in the striatum of heterozygous Q111 mice (Brito et al., 2014). While the Q111 and Q175 mouse models differ in the number of glutamines, they are fairly similar in construct design, phenotype, and development of pathologic changes (Brooks et al., 2011; Smith et al., 2014). Both of these studies, however, used a single p75 antibody

from Promega. We also attempted to use this same p75 antibody, but we were unable to obtain specific labeling, and always detected bands of equal intensity in our *p75*$^{+/+}$ and *p75*$^{-/-}$ mice (Fig 2.3). Given these limitations, we ultimately used a different p75 antibody obtained from Advanced Targeting Systems (ATS) to determine total p75 levels, which did not detect a 75 kD band in *p75*$^{-/-}$ samples. Therefore, it is difficult to directly compare our study to these previous studies. To address these differences, this p75 antibody from ATS should be used to measure p75 levels in the striatum of heterozygous Q111 mice. It is also important to note that our results do agree with the Hempstead laboratory that reported a decrease in p75 expression in the striatum of Q175 mice at 12 months of age (Ma et al., 2015). The Hempstead study also did not use the p75 antibody from Promega to measure their p75 protein levels, and this study was performed in the Q175 mouse line (Ma et al., 2015). While our 12 month results are inconclusive due to small sample size, we see a slight decrease in p75 expression in the mice we did analyze, as reported in this previous study (Ma et al., 2015).

The effects of p75 signaling on certain HD-like phenotypes were also examined by the Surmeier laboratory using predominantly the BACHD mouse model of HD. While this research group did not directly report p75 expression levels, they did not see a change in p75 mRNA levels between WT and BACHD mice. This study used an shRNA to knock down p75 protein levels in the striatum and also inhibitors of ROCK, PTEN, and the interaction of RhoA with p75 (Plotkin et al., 2014). These interventions rescued the percentage of potentiating spines and EPSPs in the striatum in BACHD mice (Plotkin et al., 2014). While this study supported a role for p75 in electrophysiological dysfunction of iSPNs in the BACHD mouse model of HD, it does not conflict with our

results. The Plotkin study examined electrophysiological properties of 6 month old BACHD mice only. The BACHD model differs considerably from the Q175 model in both phenotype onset and progression (Brooks et al., 2011), and the 6 month time point examined in BACHD mice corresponds with a considerably later time point in Q175 mice. As mentioned before, the design of our study unfortunately does not allow us to comment on the potential apoptotic/dysfunctional role of p75 at later time points. As reported in these previous studies, it is possible p75 contributes to the dysfunction of the striatum at later ages, and this does not negate the importance of the pro-survival role of p75 at earlier ages. In addition, the Plotkin study only measured electrical dysfunction and not biochemical dysfunction or degeneration (Plotkin et al., 2014). It is possible that the mechanisms that underlie these different phenotypes may be different. To address these possibilities, survival and apoptotic signaling should be examined in BACHD mice at 2-3 months of age, a time point more comparable to 5 months in Q175 mice, and the effect of p75 deletion on these signaling pathways should also be examined. Additionally, the effect of p75 deletion on development of pathology in these mice should be investigated. Finally, it would be interesting to do a thorough and well-controlled study of electrical properties in Q175 mice and the effect of p75 deletion on these electrical properties.

In addition to validating our results supporting a role of p75 in augmenting survival signaling in pre-symptomatic mice, it will also be an important future direction to validate these results in human post-mortem brains as much as possible. While animal studies are an important step in identifying possible therapeutic targets, the goal of this research always ultimately is to improve patient treatments. Therefore, after identifying

a potential target in animal models, this target should be examined in human tissues from HD patients when possible. Human tissue can be obtained from certain brain banks to perform tests in post-mortem human HD tissue (such as: the Michigan Brain Bank, the Center for Neurodegenerative Disease Research, the Neurodegenerative Disease Brain Bank, and the New York Brain Bank). Because our results represent a pre-manifest age, however, it will be difficult to correlate any results from human post-mortem HD tissue with our observed results. Due to this complication, the availability of human tissue from pre-manifest HD patients is likely to be rare. Following additional validation in animal models of these biochemical changes, however, potential therapeutic interventions can be tested and validated in animals, and if they achieve positive results should be considered for clinical trials in HD patients.

Testing modulators of p75

The laboratory of Frank Longo at Stanford has been pioneering the use of Trk receptor and p75 receptor small molecule “modulators” over the last twenty years (Longo et al., 1997; Longo and Massa, 2013). These modulators are designed to mimic certain structural loops of the ligands for each receptor (Longo et al., 1990; Xie and Longo, 2000; Longo and Massa. 2013). Dr. Longo is careful not to classify these modulators as “agonists” or “antagonists” because they are intended to modify the activity of the receptor and not simply mimic the known ligands (Longo and Massa, 2013). This laboratory is currently in the process of testing the use of several of these p75 modulators in animal models of neurodegenerative diseases, including Alzheimer’s

and HD (Longo and Massa, 2013; Nguyen et al., 2014; Simmons et al., 2014). Two such small molecule p75 modulators (LM11A-31 and LM11A-24) have shown some therapeutic benefit in *in vitro* and mouse models of ALS and AD (Massa et al., 2006; Pehar et al., 2006; Yang et al., 2008; Knowles et al., 2013; Longo and Massa, 2013). The Longo laboratory has also shown improvement in several behavioral measures, including the rotorod, balance beam, and gait analysis following treatment with a small molecule TrkB modulator, and this modulator also decreases several pathological measures in mouse models of HD (Simmons et al., 2013). While LM22A-4, a TrkB small molecule modulator, has been tested in BACHD and R6/1 mice, the modulator has not been tested in a knock-in model of HD (Simmons et al., 2013), and the p75 modulators have not been tested in HD mouse models. This testing represents an important next step, as the knock-in models of HD share more construct validity with the human disease. Using the Q175 animal model, the effect of these modulators should be tested at various time points, including both pre- and post-symptomatic time points. In addition to the behavioral and pathological tests performed previously, the effects of these modulators on survival and apoptotic signaling pathways known to be affected by p75 should also be examined. It is possible that these modulators may be able to specifically trigger the pro-survival downstream signaling of p75 at the expense of apoptotic activation of JNK and c-Jun. If these modulators can be used in this capacity, perhaps the early pro-survival function of p75 described in this study could be further augmented or could be prolonged to lead to longer striatal function and increased lifespan.

If the use of these modulators is successful in slowing or preventing disease onset in multiple animal models of HD, possibly through the survival signaling functions

of p75 examined here, they would be potential candidates for future clinical trials in HD patients. LM11A-31 is able to be administered peripherally and crosses the blood-brain barrier, and it is well-tolerated by the mice for several months (Knowles et al., 2013). Both of these qualities are important for drugs administered to humans. If this treatment is going to be maximally effective in patients, however, it would likely need to be administered earlier in the course of disease than is typical for early stage clinical trials. As with most therapies based on the use of neurotrophic factor pathways, this strategy would serve to maintain neuronal integrity and synaptic connections. By the late disease stages, which is more common for Phase 2 Clinical trials, most HD patients have already lost the majority of the MSNs in their striatum, and those MSNs that remain have lost many of their dendrites and axons connecting them to other neuronal areas (Vonsattel and DiFiglia, 1998). For a p75-modulator-based therapy to be maximally successful, pre-manifest patients would most likely need to receive treatment in order to have the best hope of delaying the onset of disease. HD itself is an ideal disease in which to employ this type of clinical testing because the genetic cause of the disease is unambiguous and genetic testing can be used to identify patients who will eventually develop the disease. Genetic testing can also predict age of onset using the repeat length (Andrew et al., 1993; Stine et al., 1993; Ashizawa et al., 1994; Kieburtz et al., 1994).

An important cautionary note about the use of disease modifying treatments, such as p75 modulators, was described in a recent article (Albin and Burke, 2015). For a chronic disease, such as HD, if the therapy both delays the onset of disease but also prolongs disease course, the net outcome may be that more patients will be living for a

longer period with manifest HD (Albin and Burke, 2015). The most effective therapy would be one that delays onset of disease while having no impact on the progression of the disease following the onset of symptoms. Whether p75-modulation therapy could be effective in this manner is unclear.

Final Conclusions

One conclusion from this study which seems relatively unambiguous is that the role p75 is playing in mouse models of HD is more complicated than originally thought. Several studies have proposed using p75 antagonists as potential treatments for HD (Brito et al., 2013; Brito et al., 2014; Plotkin et al., 2014). Indeed, research groups are performing early stage experiments testing this possibility. The experiments reported here indicate that, at least at pre-symptomatic ages, this type of treatment may actually be detrimental. If p75 is predominantly functioning in a survival promoting capacity prior to symptom onset, antagonizing p75 too early may actually cause an earlier onset of dysfunction. The complex function of p75 in the striatum needs to be studied more thoroughly before such therapies should be considered.

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Appendix: Semaphorin 3A is a retrograde cell death signal in developing sympathetic neurons

Abstract

During the development of the peripheral nervous system excess neurons are generated, most of which will be lost by programmed cell death due to a limited supply of neurotrophic factors produced by their targets. Other environmental factors, such as “competition factors” produced by neurons themselves, and axon guidance molecules, have also been implicated in developmental cell death. Semaphorin 3A (Sema3A), in addition to its function as a chemorepulsive guidance cue for motor, sensory and autonomic axons, can also induce death of sensory neurons *in vitro*. Whether Sema3A acts locally on the cell bodies to induce cell death or acts on growing axons to retrogradely induce apoptosis, is unclear. Moreover, the extent to which Sema3A regulates developmental cell death *in vivo* is debated. We show that in compartmentalized cultures of sympathetic neurons, a Sema3A-initiated apoptosis signal was retrogradely transported from axon terminals to cell bodies to induce cell death. Sema3A-mediated apoptosis utilizes the extrinsic pathway and requires both neuropilin-1 and plexinA3. Importantly, deletion of either *Neuropilin-1* or *PlexinA3* significantly reduces developmental cell death in the superior cervical ganglia. Taken together, a Sema3A-initiated apoptotic signaling complex likely regulates

the apoptosis of sympathetic neurons during the period of naturally occurring cell death.

Introduction

In the developing vertebrate nervous system neurons are generated in excess. Many of these neurons are nonessential, or inappropriately connected, and are eliminated by programmed cell death (Oppenheim, 1991). In peripheral neurons, apoptosis is in large part due to the limited supply of neurotrophic factors produced by their targets of innervation. Neurons that make appropriate connections and successfully compete for neurotrophic factors survive, while unsuccessful neurons are eliminated by developmental apoptosis (Levi-Montalcini, 1987; Oppenheim, 1991). Some neurons undergo apoptosis at early developmental stages, while extending their axons, before reaching their final targets (Coggeshall et al., 1994; White and Behar, 2000). It is therefore conceivable that the environment contains cues that not only guide developing axons, but also actively induce neuronal apoptosis. Moreover, it has been speculated that apoptosis at early stages of development may be related to axon pathfinding errors, possibly as a mechanism to eliminate neurons whose axons have wandered astray and innervate non-target tissues (Oppenheim, 1991).

It has recently become apparent that neuronal apoptosis can be induced in response to some axon guidance molecules, including ephrin-A5, Semaphorin 4D and Semaphorin 3A (Sema3A) (Gagliardini and Fankhauser, 1999; Yue et al.,

1999; Giraudon et al., 2004). Sema3A, a secreted glycoprotein that belongs to the semaphorin family of axon guidance molecules, plays a crucial role in axonal pathfinding and neuronal patterning of numerous populations of peripheral nervous system (PNS) and central nervous system (CNS) neurons (Luo et al., 1993; Messersmith et al., 1995; Behar et al., 1996; Varela-Echavarria et al., 1997). *In vitro* Sema3A induces apoptosis of CNS neurons such as cerebellar granule neurons (CGNs) (Shirvan et al., 1999) and retinal ganglion cells (RGCs) (Shirvan et al., 2002a), as well as PNS neurons such as paravertebral sympathetic ganglion neurons (Shirvan et al., 1999) and sensory DRG neurons (Gagliardini and Fankhauser, 1999; Ben-Zvi et al., 2006). Neuropilin-1 (Npn-1) supports high-affinity binding of Sema3A (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997) and functions as a co-receptor in a complex with PlexinA family members (Takahashi et al., 1999; Tamagnone et al., 1999; Yaron et al., 2005). The PlexinA cytoplasmic domain is essential for Sema3A signaling (Takahashi et al., 1999; Tamagnone et al., 1999). Although Sema3A can induce apoptosis when applied to cultured neurons *in vitro*, to what extent Sema3A directly induces apoptosis *in vivo* via Npn-1 and PlexinAs is debated (Ben-Zvi et al., 2008; Haupt et al., 2010).

In the context of target-derived survival mediated by neurotrophic factors, nerve growth factor (NGF), together with its receptor tyrosine kinase TrkA, is internalized and retrogradely transported as a complex from the axon terminal to the cell body to support survival of sympathetic and sensory neurons (Cosker et al., 2008). Using compartmentalized cultures of superior cervical ganglia (SCG)

sympathetic neurons, we report here that Sema3A is similarly retrogradely transported from distal axons to cell bodies to induce apoptosis. Importantly, this retrograde Sema3A death signal triggers the extrinsic apoptosis pathway, requires both Npn-1 and PlexinA3 and contributes significantly to programmed cell death of SCG neurons *in vitro* and *in vivo*.

Materials and Methods

Cultures of superior cervical ganglia (SCG) neurons. The production of primary sympathetic neurons from rat Sprague-Dawley embryos has been described previously (Tsui-Pierchala and Ginty, 1999). Glial cells were eliminated from the cultures by including aphidicolin (5µg/ml, Sigma) to the culture medium. Compartmentalized cultures of sympathetic neurons were produced as described previously (Tsui-Pierchala and Ginty, 1999). In both mass and compartmentalized cultures, neurons were maintained for 5–21 days *in vitro* (DIV) before treatment.

Sympathetic neuron treatments. Stimulation of SCG neurons with Sema3A was performed using tdT-Sema3A or AP-Sema3A (5nM) along with NGF (25ng/ml) for 6 hrs or overnight (37°C, 8% CO_2).To inhibit caspases, the pan-caspase inhibitor Boc-Asp(OMe)-fluoromethyl ketone (BAF, 50 µM, Sigma) or the caspase-8 specific inhibitor (Caspase-8 Inhibitor II, 5 µM, Calbiochem) were applied concomitant with Sema3A. In compartmentalized cultures, only the distal axons were stimulated, and controls consisted of either tdT alone or AP-Fc

(5nM). For retrograde labeling, FluoSphere carboxylate beads (Yellow-Green, Invitrogen) were applied to the distal axons at a 1:2000 dilution of the stock, and cell bodies imaged 24 hrs later.

Immunofluorescence labeling. Some of the *PlexinA3*$^{-/-}$ mice used in this study were generously provided by Alex Kolodkin (Cheng et al., 2001). For conditional Npn-1 knockout mice, neuron-specific *Npn-1*$^{f/f}$;Syn-CreP1 mice were generated from heterozygous matings (Gu et al., 2003). For the analysis of whole SCGs, P1 mice were collected and the neck regions were fixed with 4% PFA in PBS overnight at 4°C, washed in PBS and cryoprotected. Tissue cryosections (10µm) were used for immunofluorescence labeling of activated caspase-3. SCG sections were incubated in PBS containing 1% BSA (Sigma), 0.1% Triton X-100 (Sigma), MOM (Vector Labs) and 5% goat serum for 1 hr at room temperature (RT), followed by incubation with the primary antibody against activated caspase-3 (1:250 dilution, Cell Signaling Technology) or TH (1:250 dilution, Millipore). For immunofluorescence labeling of primary SCG neurons, the cells were washed in cold PBS and fixed in 4% PFA for 20 min at RT. Neurons were permeablized in PBS containing 0.3% Triton X-100 (5 min, RT), next incubated in PBS/1% BSA/5% NGS (30 min, RT) and then incubated with activated caspase-3 antibodies (1:500 dilution) or RFP antibodies (for tdT-Sema3A; 1:250 dilution, Rockland Inc.) in the blocking solution overnight. The labeling was detected by using Alexa-488-conjugated donkey anti-rabbit and Alexa-633-conjugated donkey anti-sheep antibodies (Biotium, 2 hrs, RT). The terminal

deoxynucleotidyltransferase-mediated dUTP nicked end labeling assay (TUNEL) was performed on 10 µm sections of SCGs using the ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Millipore) following the manufacturer's protocol.

Immunoblotting. Western blotting was performed as described previously (Tsui-Pierchala and Ginty, 1999). Antibodies and dilutions used for immunoblotting were as follows: anti-Npn-1 (Cell Signaling, 1:1000), anti-actin (Santa Cruz, 1:1000), anti-DsRed antibody (Clonetech, 1:1000 dilution) and anti-β-actin (Sigma, 1:2000). Immunoblots were visualized using enhanced chemiluminescence substrates (Supersignal, ThermoFisher).

Microscopy and quantification of apoptosis. Immunofluorescence was performed using a fluorescence inverted microscope (Carl Zeiss, AxioVert 200M and Olympus, IX71, Germany). The tdT-Sema3A and FluoSphere retrograde transport in SCG compartmentalized cultures were visualized using a confocal microscope (Leica microsystems, TCS SP5II, Germany). Images were analyzed by using the image acquisition Zeiss Axiovision or Leica LAS AF software (Carl Zeiss and Leica microsystems, respectively). For activated caspase-3 immunolabeling, every third 10 µm section throughout the entire ganglion was analyzed for activated caspase-3-positive cells (predicted soma size of an SCG neuron at this age is roughly 30 µm) to avoid double counting of cells. Total neuron counts in the SCG were obtained by counting every TH+ cell in every third 10 µm section throughout the entire ganglion as well. Apoptosis was

quantified for activated caspase-3 immunofluorescence in cultured SCG neurons as a percentage of activated caspase-3-positive cells divided by the total number of DAPI-positive SCG neurons. Quantifications were typically performed by an observer that was naïve to the conditions and data were graphed as the mean ± standard error (SEM).

Construction and analysis of Sema3A fusion proteins. The tandem dimerized tomato-Semaphorin3A fusion protein (tdT-Sema3A) was constructed by standard PCR cloning using the *Tth* DNA polymerase (Applied Biosystems) and sequences from tdT (a generous gift from Samuel L. Pfaff) and rat Sema3A. As a negative control, the tdT-Sema3A expression plasmid was modified such that the tdT stop codon was included. Thus, this protein (referred to as tdT) consists of an expressed tdT and non-expressed Sema3A. Conditioned cell culture supernatants from transfected HEK293 cells were collected and concentrated using centrifugal filter units (MWCO 30kDa, Millipore).

*Bioactivity of Sema3A fusion proteins.*To confirm the functionality of the tdT-Sema3A fusion protein, HEK293T cells were transiently transfected with either pIRES-EGFP (GFP) or full-length rat Npn-1 in pIRES-EGFP (GFP-Npn-1). After 24 hours, the cells were rinsed with PBS and incubated with 5 nM of either tdT or tdT-Sema3A for 90 minutes at RT. Following three additional washes with PBS, the cells were fixed with 4% paraformaldehyde (PFA) for 40 min, rinsed 3 times with PBS, and analyzed by fluorescence microscopy. To assess the bioactivity of

tdT-Sema3A, a neurite repulsion assay on E18 dorsal root ganglion (DRG) explants was performed as described previously (Kolodkin et al., 1997). Briefly, HEK293T cells were transiently transfected with either tdT-Sema3A or tdT. After 24 hours, the cells were trypsinized and collected in “hanging droplets” and the droplets were incubated for 5 hrs at 37°C to form cell aggregates. In a collagen matrix, a DRG explant was placed in close proximity to aggregated HEK293T cells. After 30 min at RT, neuronal growth medium (Neurobasal medium with B-27 supplement, glutamine, penicillin/streptomycin, and 15ng/ml NGF) was added and the co-cultures were incubated at 37°C for 48 hrs. The co-cultures were then fixed with 4% PFA for 1 hour, washed 3 times with PBS, and immunolabeled with the 2H3 anti-neurofilament antibody (1:500 dilution, Developmental Studies HybridomaBank, University of Iowa). For quantification, the length of axonal projections on the proximal (P) and distal (D) sides of the DRG explant was measured and used to calculate the P/D ratio. 16 explants were analyzed for each condition (from 3 independent experiments). Results are presented as mean ± SEM ($P < 0.001$, unpaired t-test).

Immunoprecipitations. After stimulation of compartmentalized sympathetic neurons, the dishes were placed on ice, gently washed twice with PBS, and the CB and DA were lysed separately with immunoprecipitation buffer (IP) as done previously (Tsui-Pierchala and Ginty, 1999). Npn-1 was immunoprecipitated using Npn-1 antibodies (Cell Signaling) along with protein A and protein G (Roche). After 24 hrs of gentle agitation at 4°C, the immunoprecipitates were

then washed three times with IP buffer and the complexes were prepared for sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) by adding 2X sample buffer (125mM Tris pH 6.8, 20% Glycerol, 4% SDS, 0.016% bromophenol blue) and heating this mixture for 6 min at 100°C.These extracts were subjected to SDS-PAGE followed by electroblotting onto PVDF membranes (Millipore) and Westernblot analysis.

Results

Sema3A is retrogradely transported from the distal axons to the cell bodies of sympathetic neurons

In the developing PNS, Sema3A plays a critical role as a chemorepulsive axon guidance molecule for postganglionic sympathetic neurons and repels developing axons from non-target tissues (Nakamura et al., 2000; Kolodkin and Tessier-Lavigne, 2011). Upon receptor binding on the neuronal growth cone, the Sema3A ligand-receptor complex undergoes rapid endocytosis (Fournier et al., 2000). The fate of the internalized complex, however, has not yet been investigated. To determine whether Sema3A internalized at the axon terminal is retrogradely transported to the cell body, compartmentalized cultures of sympathetic neurons were used (Fig. A.2A). Campenot chambers were prepared and dissociated SCG neurons were plated in the central compartment and left to grow their axons into fluidically-isolated, side compartments. To visualize Sema3A, a fluorescent tandem dimerized tomato (tdT)-Sema3A fusion protein, as well as tdT alone, were generated (Fig. A.1A,B). The bioactivity of tdT-

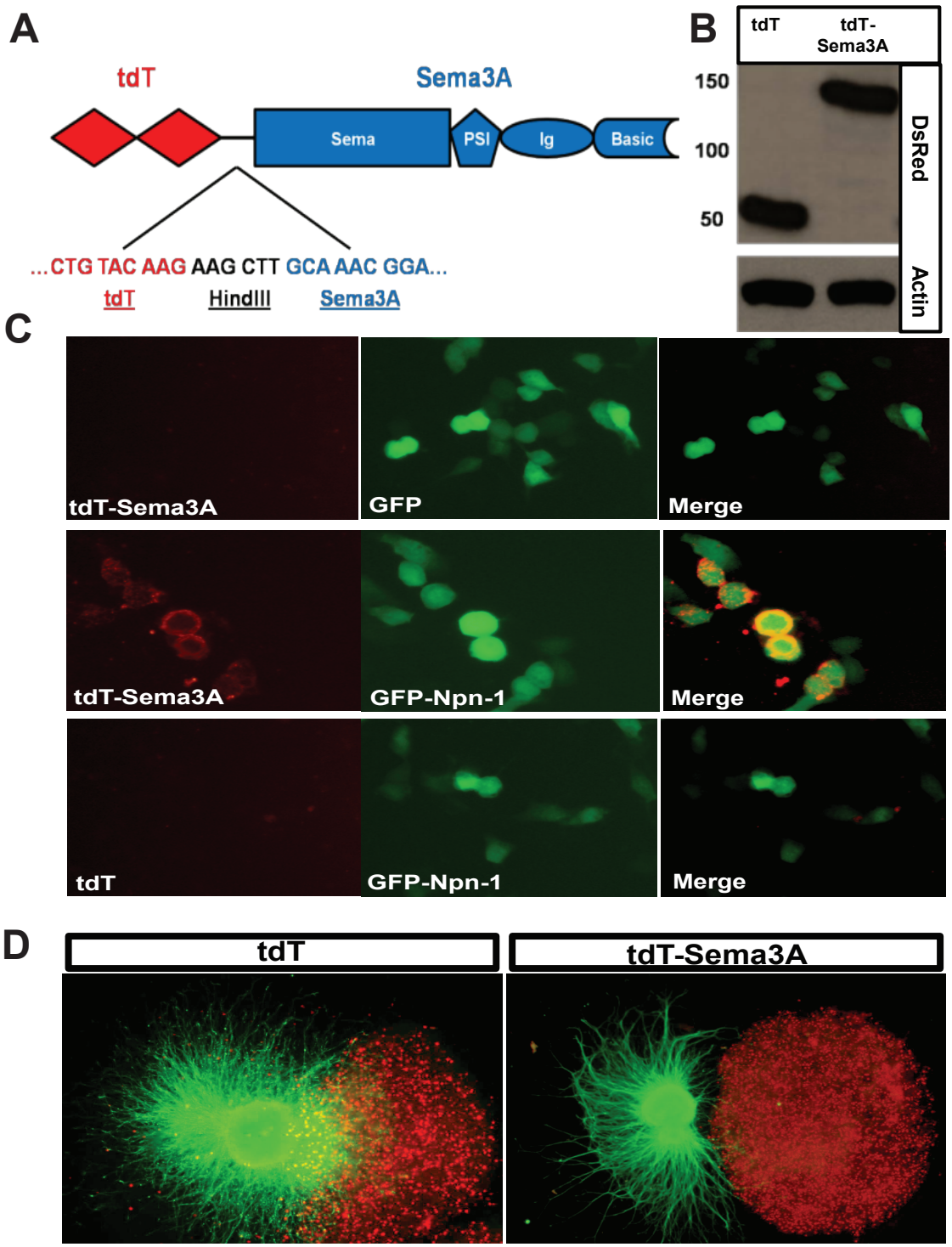

Fig. A.1. Confirmation of tdT-Sema3A functionality. (A) Schematic diagram of the tdTomato-Semaphorin3A fusion protein (tdT-Sema3A). Tandem dimerized tomato was fused in frame to the amino terminus rat Sema3A (23 amino acids after the start codon - blue). Sema is the amino-terminal semaphorin domain; PSI is the plexin-semaphorin-integrin domain; Ig is the immunoglobulin-like domain; Basic is the basic C-terminal domain. (B) Immunoblotting using conditioned media from HEK293T cells transfected with either tdT or tdT-Sema3A revealed the expected protein sizes when probed with an anti-DsRed antibody. Actin immunoblotting confirmed analysis of equal amounts of protein. (C) HEK293T cells were transfected with either GFP or GFP-Npn-1, and binding of tdT or tdT-Sema3A was assessed 24 hours later. Binding is only observed between the tdT-Sema3A fusion protein and GFP-Npn1-transfected cells. (D) E18 rat dorsal root ganglion (DRG) explants were co-cultured in a collagen matrix with HEK293T cells expressing either tdT or tdT-Sema3A and grown for 48 hours. DRG axonal projections were labeled with 2H3 anti-neurofilament antibody. DRG axons are repelled by the tdT-Sema3A-expressing HEK293Ts, but not by the tdT-expressing cells. All experiments for this figure were performed by Travis Dickendesher.

Sema3A was tested by examining its binding to Npn-1 (Fig. A.1C) and axon repulsion of NGF responsive DRG neurons (Fig.A.1D). Unlike tdT alone, tdT-Sema3A was able to bind to Npn-1 (Fig. A.1C) and induced axon repulsion of DRG neurons (Fig. A.1D).

To determine whether Sema3A can be retrogradely transported, the distal axons of SCG neurons grown in compartmentalized cultures for 7 days, a time at which there is robust axon growth into the side compartments, were exposed to tdT-Sema3A, or tdT alone, for 24 hours. Interestingly, some of the cell bodies in the central compartment displayed tdT fluorescence, but only in cultures where tdT-Sema3A, and not tdT, was added to the axon compartments. The neurons that displayed the accumulation of tdT-Sema3A appeared to be atrophic and were possibly undergoing apoptosis (data not shown). Because the induction of apoptosis could potentially underestimate the number of neurons that were retrogradely transporting tdT-Sema3A, these experiments were repeated in neurons maintained in a pan-caspase inhibitor (BAF) to eliminate cell death. Immunofluorescence labeling of tdT with tomato antibodies was also performed on these cultures to better detect the retrogradely transported tdT-Sema3A. Interestingly, the majority of NGF-maintained SCG neurons appeared to retrogradely transport tdT-Sema3A under these conditions after 7 DIV (Fig. A.2 B-D). To quantify the percentage of SCG neurons capable of retrogradely transporting tdT-Sema3A from distal axons to the cell body, we determined the percentage of neurons that projected into the side compartments where the tdT-Sema3A was applied. This was accomplished by applying fluorescently-labeled

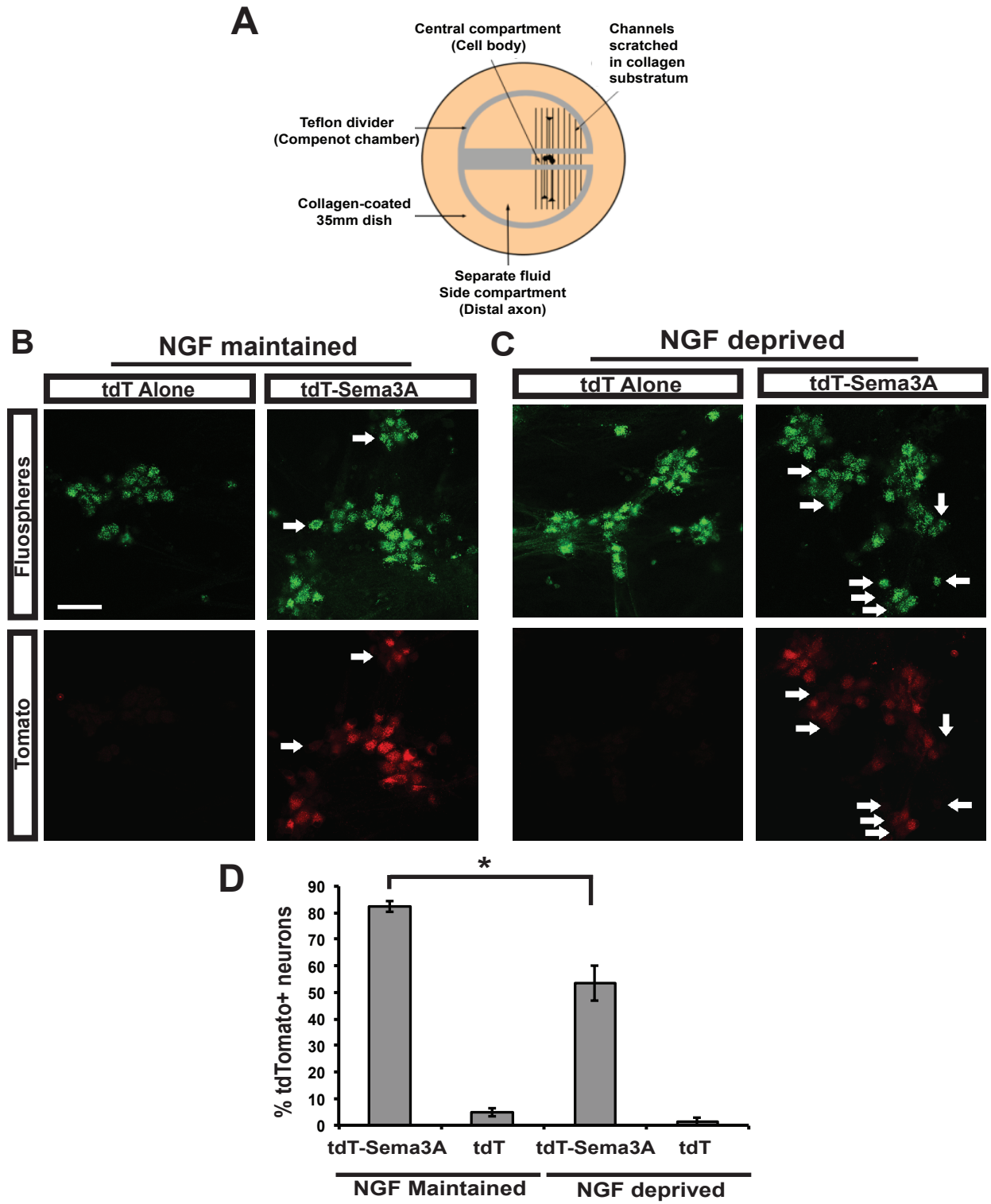

Fig. A.2. Semaphorin3A is retrogradely transported from the distal axons to the cell bodies of sympathetic neurons. (A) Schematic diagram of a compartmentalized culture containing neuronal cell bodies and growing distal axons in separate fluid compartments. (B) Compartmentalized cultures of P5 SCG neurons provided with tdT-Sema3A, or tdT alone in the presence of BAF, along with FluoSpheres to indicate cell bodies from neurons projecting axons into the side compartment (green fluorescence, top panels) and retrogradely transporting tdT-Sema3A (Red fluorescence, bottom panels). These transport studies were performed on neurons that were either maintained or deprived of NGF (indicated on top of images). (D) Quantification of the data in B and C, with the bars representing tdT-Sema3A+ neurons/FluoSphere+ neurons. Results are presented as mean ± SEM from three independent experiments. (*) $P < 0.05$, using student's t-test.

carboxylate beads that are internalized and retrogradely transported (FluoSpheres, 515 nm emission) to the distal axons along with tdT-Sema3A, or tdT alone. When compared to the total number of neurons that were FluoSphere labeled, and thus projecting into the side compartments (Fig. A.2B, C), the cell bodies of 82.2% ±2.0% of these NGF-maintained neurons were tdT-Sema3A positive. This was a significant increase compared to neurons treated with tdT alone ($P< 0.01$), in which only 4.7% ±1.5% of cell bodies were tdT positive (Fig. A.2D). Thus, most SCG neurons (82.2%) were able to internalize and retrogradely transport Sema3A from distal axons to the cell body in compartmentalized cultures after 7 DIV.

To determine whether the retrograde transport of Sema3A was affected by the trophic status of the neurons, Sema3A retrograde transport studies were performed on neurons that were deprived of NGF 24 hours prior to the application of tdT-Sema3 to the distal axons. tdT-Sema3A was also retrogradely transported in NGF-deprived sympathetic neurons, but to a lesser extent (Fig. A.2C, D). Quantification of the number of NGF-deprived neurons that retrogradely transported tdT-Sema3A revealed that 53.4% ± 6.7% of these neurons were tdT-Sema3A+, as compared to only 1.3% ± 1.4% of NGF-deprived neurons that transported tdT alone (significantly different, $p<0.01$). When tdT-Sema3A transport was compared between NGF-maintained neurons (82.2%) and NGF-deprived neurons (53.4%), this difference was statistically significant ($p<0.05$), indicating that neurons that were atrophic and beginning to undergo apoptosis transported less Sema3A. This is likely due to a generalized reduction

in axonal integrity and, therefore, retrograde transport in the NGF-deprived neurons.

Sema3A induces apoptosis of sympathetic neurons via the extrinsic pathway

NGF deprivation of sympathetic neurons triggers apoptosis via the intrinsic pathway, which requires gene expression that induces cytochrome-c (Cyt-c) release from mitochondria leading to activation of caspases (Chang et al., 2002; Kirkland and Franklin, 2003; Freeman et al., 2004; Jacobs et al., 2006). The extrinsic pathway, in contrast, is typically initiated by death receptors such as TNFRs that initiate caspase8 activation at the plasma membrane (Benn and Woolf, 2004; Kantari and Walczak, 2011). The intrinsic pathway requires gene expression of several proapoptotic mediators such as cyclin D1, c-Jun, BIM and SM-20 (Freeman et al., 2004), and NGF-deprivation induced apoptosis of sympathetic neurons is inhibited by cycloheximide (Deckwerth and Johnson, 1993; Estus et al., 1994; Freeman et al., 2004). To determine whether inhibition of protein synthesis also protected neurons from Sema3A-induced cell death, SCG neurons were treated with AP-Sema3A, or AP-Fc, in the presence or absence of cycloheximide. Nuclear condensation and apoptotic morphology (axon degeneration, loss of phase bright appearance of cell bodies) were measured from images of the same fields of neurons before and after the stimulations in order to determine the total percentage of neurons that die in the presence Sema3A (Fig. A.3A). There was not a significant difference in the

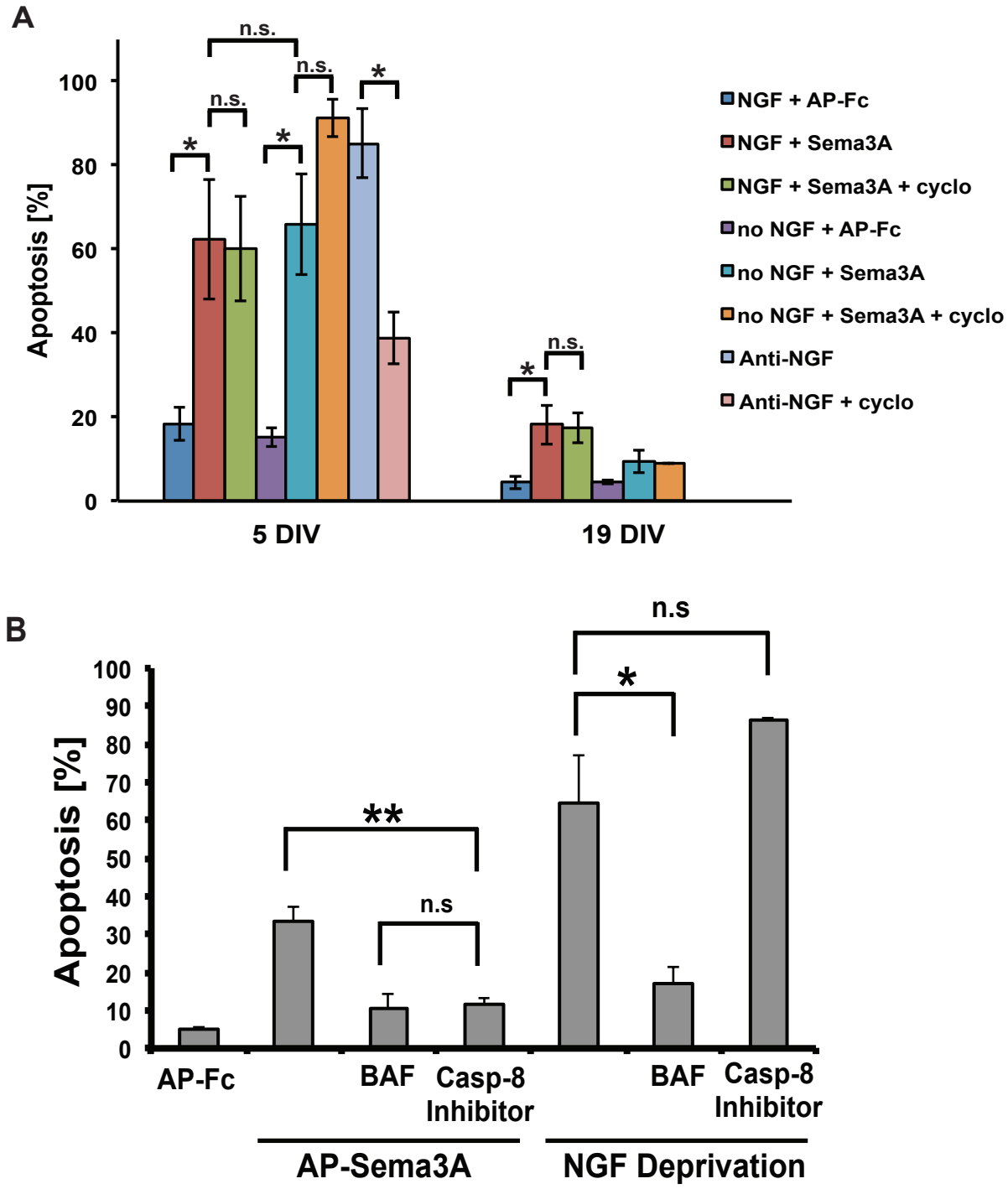

Fig. A.3. Sema3A induces apoptosis via the extrinsic pathway. (A) SCG neurons were maintained for 5 DIV or were maintained for 19 DIV, a time at which they are fully mature. The neurons were then deprived of NGF, deprived of NGF in the presence of cycloheximide, given Sema3A in the presence or absence of NGF, or given Sema3A in the presence of cycloheximide for 24 hours. The percentage of neurons that underwent apoptosis was quantified by measuring both cellular morphology and nuclear morphology. Results were graphed as the mean ± SEM from three independent experiments. (B) SCG neurons were maintained in vitro for 5 DIV and then exposed to AP-Fc, AP-Sema3A or deprived of NGF in the presence or absence of the pan-caspase inhibitor BAF (50 μM) or caspase-8 inhibitor II (5 μM). Apoptosis was ascertained 24 hours later as described for (A). Results were graphed as the mean ± SEM from three independent experiments, except for the AP-FC treatment (N=2). In both panels, n.s: not significant, (*) $P< 0.05$, (**) $P< 0.01$ using student's t-test.

number of neurons that underwent Sema3A-induced apoptosis in the presence or absence of cycloheximide, suggesting that protein synthesis is not required for Sema3A induced death (Fig. A.3A). Consistent with previous reports, cycloheximide did rescue a large percentage of neurons from undergoing apoptosis upon NGF withdrawal (Fig. A.3A)

In addition to examining the effect of protein synthesis inhibitors, we examined whether the age of the sympathetic neurons influenced their sensitivity to Sema3A-induced apoptosis. Sympathetic neurons undergo a transition from acutely requiring NGF for survival to becoming largely insensitive to the removal of NGF (Easton et al., 1997). This transition, known as maturation, occurs *in vivo* as well as in primary SCG neurons *in vitro* (Angeletti et al., 1971; Goedert et al., 1978; Easton et al., 1997). Sympathetic neurons at 5 DIV (immature), or 19 DIV (fully mature), were exposed to Sema3A and cell death was assessed. AP-Sema3A induced the death of a significantly higher percentage of immature neurons than mature neurons (Fig. A.3A). However, as much as 20% of mature SCG neurons remained Sema3A sensitive and still underwent apoptosis (Fig. A.3A). To determine whether the loss of sensitivity to Sema3A with age was due to a loss of expression of the receptor for Sema3A, Neuropilin-1 (Npn-1), mass cultures of sympathetic neurons were matured in culture to the equivalent of P0, P5 and P20. The neurons were then detergent extracted, Npn-1 was immunoprecipitated from these extracts, and these immunoprecipitates were subjected to Npn-1 immunoblotting to determine the protein expression levels of Npn-1. The level of Npn-1 did not decline with age and, in fact, the expression

increased by P20 (Fig. A.4). It is unlikely, therefore, that the loss of Sema3A sensitivity in mature neurons (Fig. A.3A) is due to the downregulation of Npn-1. Interestingly, the retrograde transport of tdT-Sema3A did decline in compartmentalized cultures of mature neurons (Fig. A.5). In fact, no mature neurons were observed to retrogradely transport tdT-Sema3A to the extent observed in young, NGF-dependent neurons (Fig. A.2B-D). Although there was some specific, but very faint, labeling in tdT-Sema3A treated neurons as compared to tdT treated neurons (Fig. A.5), this amount of labeling could not be accurately quantified. Taken together, mature sympathetic neurons are significantly less sensitive to Sema3A-induced cell death, which may be due to their inability to internalize and retrogradely transport Sema3A.

It is possible that Sema3A could function to enhance, or accelerate, apoptosis in sympathetic neurons that are not receiving sufficient NGF. To test this hypothesis, NGF was removed from sympathetic cultures, but we did not include an anti-NGF antibody in the medium during this deprivation (which is used to block NGF that remains on the collagen and cell surface). This NGF deprivation method allows the neurons to survive in this very low amount of NGF for 24-48 hours before apoptosis is initiated. AP-Sema3A, or AP alone, was added to these NGF-deprived neurons, and the extent of cell death measured after 24 hours. As before, Sema3A induced robust and significant apoptosis in NGF deprived sympathetic neurons, as compared to AP alone (Fig. A.3A, $p<0.05$). Interestingly, there was no difference in the level of apoptosis induced by Sema3A in NGF-maintained neurons versus NGF-deprived neurons (Fig.

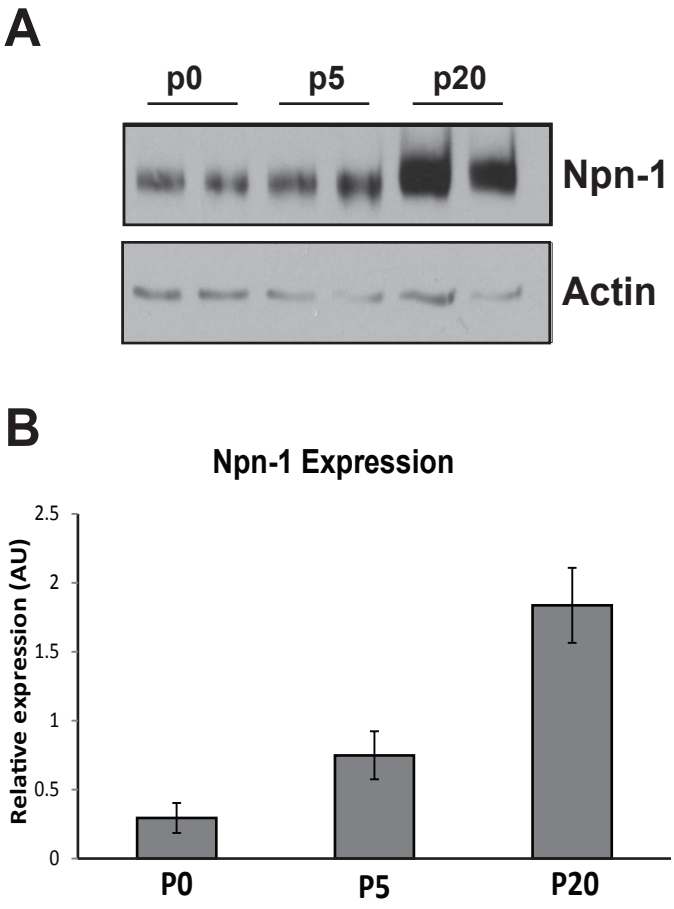

Figure A.4. Neuropilin expression is higher in mature neurons. (A) Sympathetic neurons maintained in culture to the equivalent of P0, P5 or P20 were detergent extracted and subjected to Npn-1 immunoblotting after the immunoprecipitation of Npn-1. Note that Npn-1 expression increased by P20 relative to actin, which was used as a loading control. (B) The experiments in (A) were quantified from three independent cultures and graphs as the mean ± SEM.

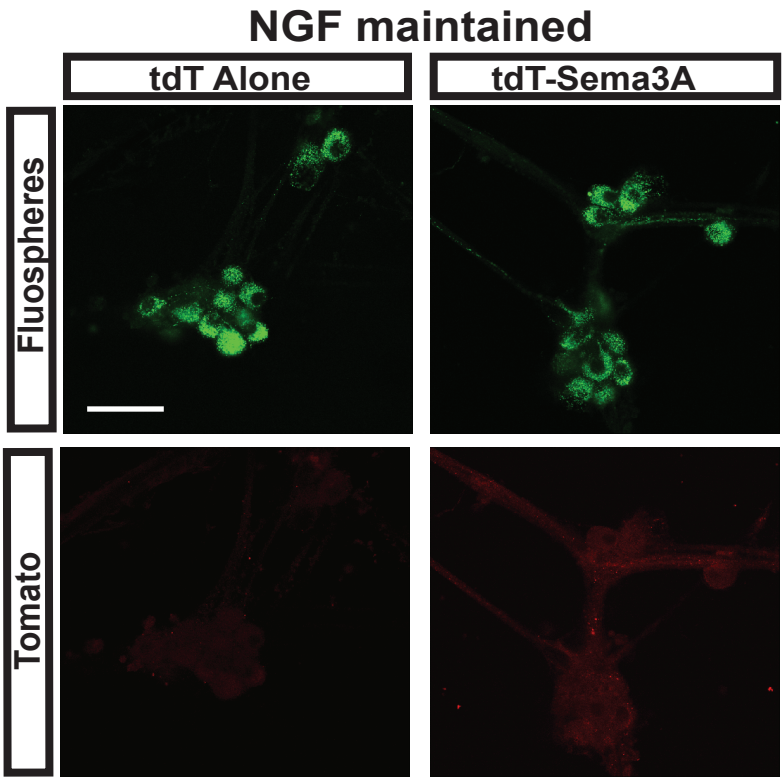

Figure A.5. Less tdT-Sema3A is retrogradely transported in mature neurons. Retrograde Sema3A transport studies of compartmentalized cultures of P18 SCG neurons. Note that at this age little tdT-Sema3A was retrogradely transported, as compared to the transport at P5 in Fig. A.2.

A.3A), indicating that Sema3A does not act to enhance or trigger apoptosis induced by NGF withdrawal.

As further support for Sema3A acting via the extrinsic pathway to induce apoptosis, we examined whether Sema3A-induced apoptosis could be blocked by a selective inhibitor of Caspase-8. As expected, a pan-caspase inhibitor blocked cell death of sympathetic neurons that were either deprived of NGF, or exposed to Sema3A (Fig. A.3B). Specific inhibition of caspase-8, however, selectively blocked Sema3A-mediated apoptosis, but did not inhibit NGF-deprivation induced apoptosis (Fig. A.3B), indicating that caspase-8 is required for Sema3A-induced, but not NGF deprivation-induced, cell death. Taken together, the data presented in Figure A.3 support the hypothesis that Sema3A-induced apoptosis occurs via the extrinsic pathway.

PlexinA3 and Npn-1 are required for developmental cell death of sympathetic neurons *in vivo*

Sema3A mediates axon growth collapse *in vitro* and axon guidance in sympathetic and sensory neurons *in vivo* via Npn-1 and Plexin-A4 (Yaron and Zheng, 2007; Pasterkamp and Giger, 2009).The observation that the Npn-2 ligand Sema3F does not induce apoptosis of SCG neurons suggested that binding of Sema3F to Npn-2 does not signal apoptosis (data not shown). Previous work from our lab as well as previously published papers have reported neuropilin1 (Npn-1) and Plexin-A3 as being important for Sema3A-induced apoptosis *in vitro* and in other cell types (H. Abdesselem, not shown; Ben-Zvi et

al., 2008). To determine whether Plexin-A3 and Npn-1 were involved in developmental cell death of sympathetic neurons *in vivo*, the extent of apoptosis in the SCG was examined in *PlexA3*$^{-/-}$ knockout mice and in *Npn-1*$^{f/f}$;synapsin-Cre (syn-Cre) mice. Apoptotic cells were detected in SCG sections of E18.5, P0 and P20 *PlexA3*$^{-/-}$ mice by immunofluorescence labeling for activated caspase-3 (Fig. A.6A,B). Interestingly, the number of activated caspase-3-positive SCG neurons per ganglion was reduced significantly in E18.5 *PlexA3*$^{-/-}$mice as compared to *PlexA3*$^{+/+}$mice (763 ± 76 versus 985 ± 55 activated caspase-3-positive cells/ganglion, respectively).There were also significantly fewer activated caspase-3 positive neurons in the SCGs of P0 *PlexA3*$^{-/-}$ mice compared to *PlexA3*$^{+/+}$ mice (571 ± 46 versus 804 ± 50 activated caspase-3-positive cells/ganglion, respectively). By P20, an age when programmed cell death is largely complete in the SCG, there was no difference in the amount of apoptosis between *PlexA3*$^{-/-}$mice and *PlexA3*$^{+/+}$ mice (184 ± 53 versus 195 ± 110, respectively). The reduction of activated caspase-3-positive (E18.5 and P0) neurons in *PlexA3*$^{-/-}$mice was significantly different than in *PlexA3*$^{+/+}$ mice ($p<0.05$). Npn-1 was deleted from sympathetic neurons by using *Npn-1*$^{f/f}$;syn-Cre in order to avoid the embryonic lethality of *Npn-1*$^{-/-}$ mice due to vascular defects, which would have precluded analysis of programmed cell death in the SCG (Kitsukawa et al., 1997). The loss of Npn-1 was confirmed in the SCG of *Npn-1*$^{f/f}$*;*syn-Cre mice by the absence of AP-Sema3A binding to sections of SCGs from *Npn-1*$^{f/f}$*;*syn-Cre mice as compared to *Npn-1*$^{f/f}$ mice (Fig. A.7A). Similar to *PlexA3*$^{-/-}$ mice, there were fewer apoptotic neurons in the SCGs of P1-

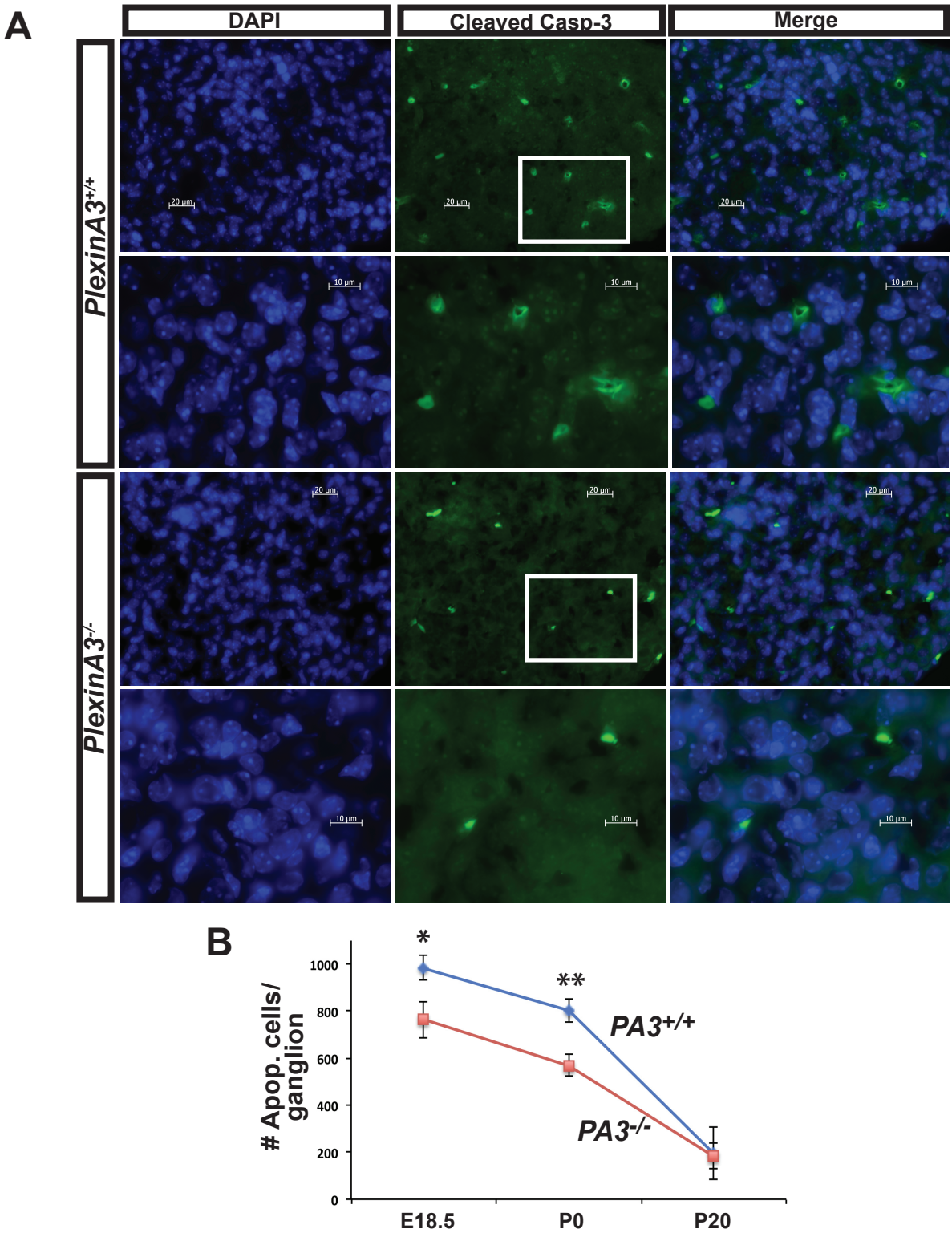

Fig. A.6. Programmed cell death is reduced in the SCG of PlexinA3 knockout mice. (A) Activated caspase-3 immunofluorescence labeling of P0 SCGs of PlexinA3+/+ (green, top 6 panels) and PlexinA3-/- mice (bottom six panes) revealed a decrease in the number of apoptotic neurons observed in plexinA3-/- mice. Nuclei are shown in blue with DAPI staining. White boxes indicate regions shown at higher magnification in the lower row of 3 images in each genotype. (B) The analysis in A was performed on E18.5 (n=5 for each genotype), P0 (n=4 for each genotype) and P20 (n=4 for PlexinA3+/+ and n=5 for PlexinA3-/-) SCGs and the quantifications of activated caspase-3-positive cells per ganglion are graphed as the mean ± SEM. (*) P< 0.05, (**) P< 0.01 using student's t-test.

P2 *Npn-1*$^{f/f}$;syn-Cre mice as compared to *Npn-1*$^{f/f}$ mice (3 ± 2.5 versus 51 ± 10.8 caspase-3-positive neurons/ganglion, P< 0.05; Fig. A.7B). Taken together, these data indicate that both PlexinA3 and Npn-1 are involved in developmental cell death of SCG neurons, most likely via their interaction with Sema3A.

Based on these observations, we sought to determine whether there was a sustained increase in the number of neurons in the SCG after the period of programmed cell death. In some knockout mice, the deletion of a molecule involved in apoptosis results in an initial protection from cell death, but the eventual elimination of these neurons, such as in *p75*$^{-/-}$ mice (Deppmann et al., 2008). In the BAX knockout mice, in contrast, all neurons are rescued from apoptosis permanently (Deckwerth et al., 1996), indicating that BAX is absolutely required for the apoptosis pathway in SCG neurons. To distinguish between these two possibilities, the number of neurons in the SCGs of P20 *PlexA3*$^{-/-}$ mice and *PlexA3*$^{+/+}$ mice were compared. Tyrosine hydroxylase (TH) immunolabeling, which labels greater than 95% of SCG neurons, was performed on serial sections of SCG ganglia, and the total number of neurons in each ganglia were counted (Fig. A.8A,B). There was no significant difference in the number of TH+ neurons between *PlexA3*$^{-/-}$SCGs and *PlexA3*$^{+/+}$ SCGs (6244 ± 924 neurons versus 7072 ± 97 neurons, respectively). Therefore, the neurons that are protected from apoptosis in *PlexA3*$^{-/-}$mice between E18.5-P2 eventually go on to die and do not persist beyond P20.

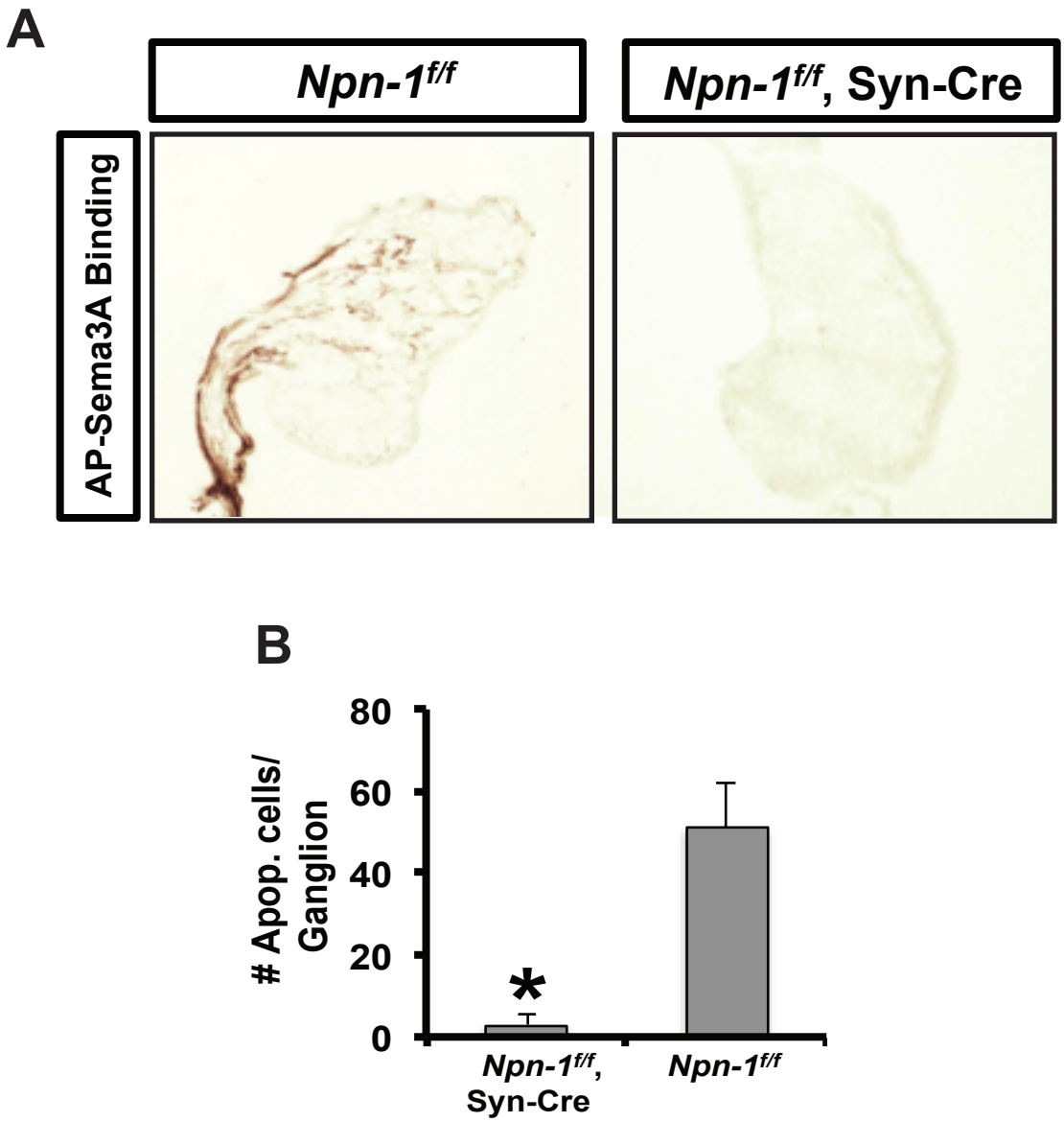

Fig. A.7. Neural deletion of Npn-1 rescues neurons from apoptosis. (A) Confirmation of Npn-1 deletion from sympathetic neurons was achieved using a Sema3A binding assay. Assays were performed on sections of SCGs isolated from either Npn-1f/f or Npn-1f/f;Syn-Cre mice at P1-P2. Npn-1f/f;Syn-Cre SCGs did not bind Sema3A (right panel), in contrast to Npn-1f/f SCGs (left panel), functionally confirming the deletion of Npn-1. (B) SCGs from either Npn-1f/f (n=3) or Npn-1f/f;Syn-Cre (n=3) mice were serially sectioned and the number of activated caspase-3-positive cells per ganglion were quantified as in Fig. 6. All quantifications are displayed as the mean ± SEM and the asterisk denotes $P < 0.05$. Mice were generated and processed for AP-Sema3A binding by Travis Dickendesher and apoptotic counts were performed by Houari Abdesselem.

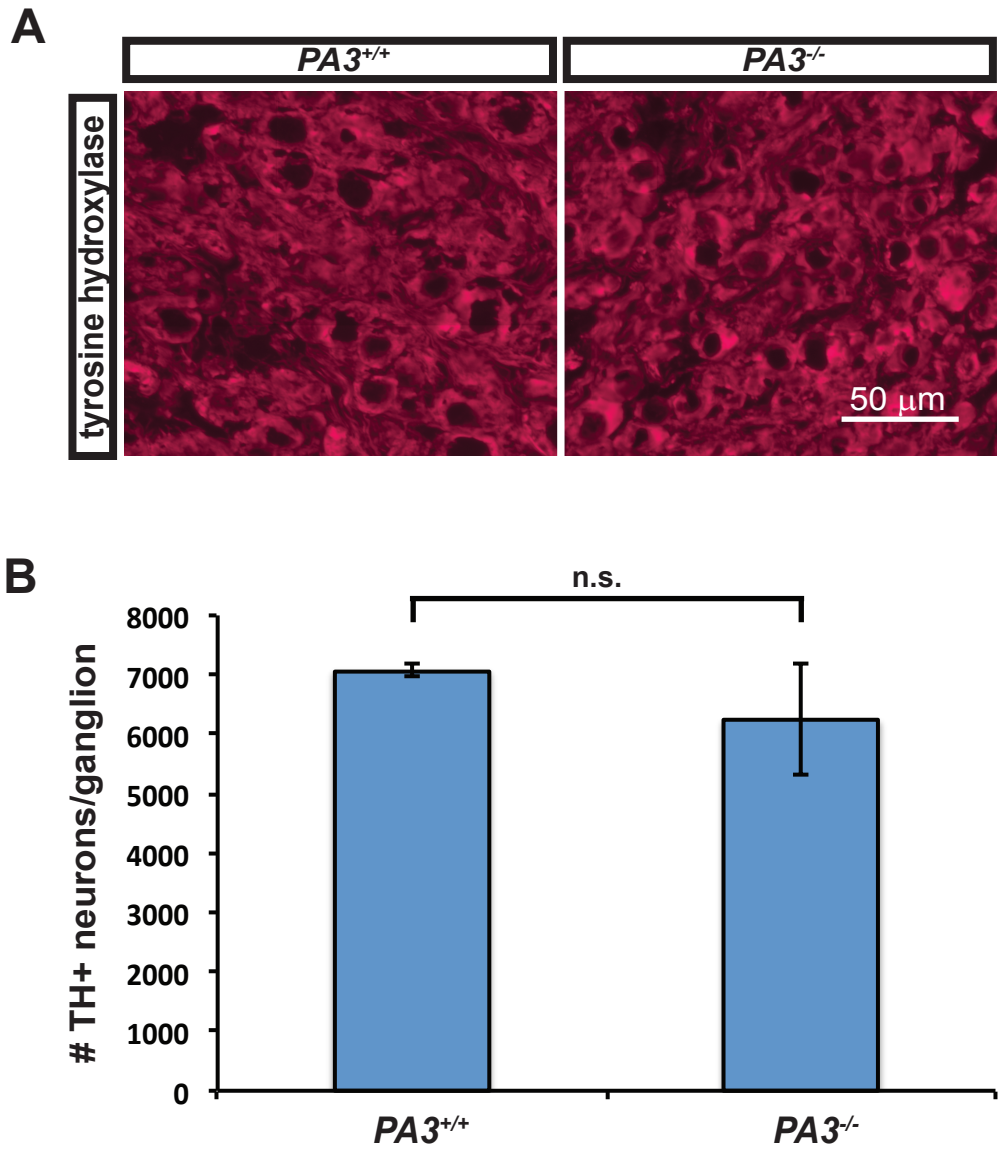

Fig. A.8. Programmed cell death in the SCG is eventually complete by P20 in PlexinA3-/- mice. (A) Tyrosine hydroxylase (TH) immunolabeling was performed on serial sections of SCGs from P20 PlexinA3+/+ mice (left panel) and PlexinA3-/- mice (right panel). (B) The total number of TH+ neurons in each SCG was ascertained by counting the number of TH+ cells in every third section throughout the entire ganglia (n=3 for both genotypes). These quantifications are displayed as the mean ±SEM, and there was no significant difference by t-test (n.s., not significant). TH counts were performed by Houari Abdesselem.

Discussion

Sema3A is best known for its role as a repulsive axon guidance cue for developing PNS and CNS neurons (Luo et al., 1993; Messersmith et al., 1995; Behar et al., 1996; Varela-Echavarria et al., 1997). Besides its role in regulating neuronal morphology, accumulating evidence suggests that Sema3A induces neuronal apoptosis (Gagliardini and Fankhauser, 1999; Shirvan et al., 1999; Shirvan et al., 2002a; Ben-Zvi et al., 2006). Despite recent progress, whether Sema3A, Npn-1 and plexins are physiologically relevant in developmental cell death *in vivo*, and the mechanisms underlying semaphorin-induced apoptosis, remain poorly understood (Ben-Zvi et al., 2006; Jiang et al., 2007; Ben-Zvi et al., 2008; Haupt et al., 2010; Jiang et al., 2010). Here we identify Sema3A as a retrograde death signal for developing sympathetic neurons. In compartmentalized cultures of SCG neurons, Sema3A applied to distal axons is retrogradely transported to the cell soma and, upon its arrival, triggers caspase-3 and caspase-8 dependent cell death. Sema3A-initiated apoptosis is not NGF sensitive and progresses via the extrinsic cell death pathway. Importantly, deletion of either *PlexA3* or *Npn-1* protects SCG neurons from naturally occurring cell death during early postnatal development, indicating that retrograde Sema3A signals the programmed cell death of sympathetic neurons by a PlexinA3/Npn-1 receptor complex.

Eliminating excessively produced neurons by programmed cell death is an important step during nervous system development and patterning. In the PNS, it is well established that remote signals at nerve terminals influence the final

number of neurons through regulation of cell death and survival (Levi-Montalcini, 1987; Oppenheim, 1991). Pro-survival neurotrophic factors are produced in limiting amounts by their peripheral targets and neurons compete for access to neurotrophic factors. Mounting evidence suggests the existence of pro-apoptotic signals, such as precursor forms of neurotrophins, that trigger cell death through activation of p75 (Lee et al., 2001; Teng et al., 2010). Evidence for Semaphorin induced cell death stems predominantly from experiments with neural tumor cells and cultured neurons (Gagliardini and Fankhauser, 1999; Shirvan et al., 1999; Bagnard et al., 2001; Ben-Zvi et al., 2006). The extent to which Sema3A is involved in developmental cell death of embryonic sensory and motor neurons is debated (Ben-Zvi et al., 2008; Haupt et al., 2010; Ben-Zvi et al., 2013). During embryonic development, Sema3A is broadly expressed in the PNS and is often found along the trajectory of sensory, motor and sympathetic fibers (Giger et al., 1996). Whether Sema3A primarily functions in axon guidance of sympathetic neurons or also contributes to their programmed cell death, however, has remained unknown. Here we show that Sema3A induced developmental-stage dependent cell death of sympathetic neurons. We propose two possible models of how Sema3A may regulate the developmental cell death of sympathetic neurons. First, Sema3A released from non-target areas is endocytosed at the growth cone and retrogradely transported to the cell body to trigger cell death. Prolonged exposure to Sema3A may trigger cell death and eliminate neurons whose axons have wandered astray and failed to reach their appropriate target. Consistent with this idea, the PNS of *Sema3A*$^{-/-}$ mice shows profound

defasciculation of sensory, motor and sympathetic projections. Second, sympathetic neurons themselves that have innervated their correct targets and receive sufficient NGF, i.e. “winning neurons,” express Sema3A as a competition factor and release it into target areas or the ganglion itself. This Sema3A then induces the apoptosis of neurons that are not obtaining sufficient amounts of NGF, i.e. “losing neurons.” The observations that NGF-deprived neurons are not more susceptible to Sema3A, and that Sema3A induces the apoptosis of neurons even in the presence of NGF, provide some evidence contrary to this competition model. An important future direction will be to determine what cell types express Sema3A, and when during development they do so, to aid in the prediction of whether Sema3A acts to eliminate off-target neurons, or acts as a competition factor.

The observation that tdT-Sema3A was visible in the cell body compartment of compartmentalized cultures after being applied only to axons indicates that the death-inducing signal is retrogradely transported from distal axons to cell bodies. When coupled with the previous observation that Sema3A binding to Npn-1 triggers rapid endocytosis at the neuronal growth cone (Fournier et al., 2000), the retrograde apoptosis signal may be comprised of a Sema3A-Npn-1-PlexinA3 ligand-receptor complex, rather than Sema3A alone. Similar to Sema3A, growth inhibitory molecules belonging to the ephrin family (Jurney et al., 2002; Zimmer et al., 2003),or the reticulon family member Nogo-A (Joset et al., 2010), have been shown to undergo rapid endocytosis, furthering the likelihood that axon guidance complexes may also affect cellular events

remotely from their initial location of receptor binding. Additional studies are needed to determine the molecular composition of the retrogradely transported death complex, as well as the mechanism by which it is internalized and trafficked to cell bodies. The signaling events subsequent to Sema3A/Npn-1/PlexinA3 receptor assembly that induce apoptosis are not well understood. Semaphorins have been reported to inhibit NGF-mediated Akt and Erk signaling (Atwal et al., 2003), suggesting that they could potentially antagonize NGF signaling and induce apoptosis via the intrinsic pathway. The observations, however, that Sema3A-mediated apoptosis did not require new protein synthesis and required caspase-8 activation, suggest that death occurred via an extrinsic pathway. There were similarities between NGF deprivation-induced death and Sema3A-induced death, such as the loss of sensitivity in mature neurons. The extrinsic pathway often induces JNK/c-Jun signaling (Benn and Woolf, 2004; Kantari and Walczak, 2011), but maturation suggests that there may be common components between these two death pathways, such as Apaf-1 (Wright et al., 2007). Sema3A receptor components or downstream components of the Sema3A-initiated apoptotic signaling cascade could be downregulated as sympathetic neurons mature. We did not observe a decrease in Npn-1 expression, but the retrograde transport of Sema3A was reduced dramatically in mature sympathetic neurons, suggesting that the loss of sensitivity to Sema3A may be at the level of receptor internalization and transport. Interestingly, the pro-apoptotic serine/threonine kinase NDR was recently identified as a signaling target of MICAL, which associates with Plexins and is required for Sema3A-

mediated axon repulsion (Terman et al., 2002; Zhou et al., 2011). It will be important to determine whether any of these signaling molecules are altered during maturation of sympathetic neurons. Lastly, it is well established that Sema3A and other members of the semaphorin family are upregulated following injury to the PNS and CNS (Pasterkamp and Giger, 2009). Axonal injury to the mammalian optic nerve, for example, triggers death of the majority of retinal ganglion cells (Dickendesher et al., 2012) and correlates with an upregulation of Sema3A. Remarkably, intra-ocular injection of blocking antibodies to Sema3A inhibits injury induced death of RGCs (Shirvan et al., 2002b), suggesting that a detailed understanding of the Sema3A cell death mechanisms may aid the development of therapeutic strategies that block neuronal cell death following nervous system injury or disease.

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