ABSTRACT OF THE DISSERTATION

Regulation of proNGF Processing and its Effects on p75NTR-Mediated Cell Death Following Seizure

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Nerve growth factor (NGF) has been known to play critical roles in neuronal survival and differentiation during development. Recent studies have discovered that its immature form, proNGF, is a ligand for the p75 neurotrophin receptor (p75NTR). proNGF binding to p75NTR activates apoptotic signaling, and this binding occurs with a five-fold higher affinity than that of mature NGF (Lee et al., 2001). This binding preference, along with the increased prevalence of proNGF after injury (Harrington et al., 2004), creates a cellular environment susceptible to cell death; thus, the balance between levels of pro- and mature NGF may be a key factor in determining whether a neuron lives or dies (Lee et al., 2001; Volosin et al., 2006). Using both in vitro and in vivo methods, this thesis examined the mechanisms that regulate the extracellular processing of proNGF and the consequences of that processing on p75NTR-mediated cell death following injury. The results discussed here demonstrate that 1) proNGF binding leads to cell death via the p75NTR signaling pathway; 2) after injury, proNGF is upregulated and preferentially secreted in a functional manner capable of activating the p75NTR-mediated apoptotic pathway; 3) the enzymes plasmin and
MMP7 extracellularly cleave proNGF, 4) after injury, plasmin activation and MMP7 activity are reduced, leading to increased proNGF-induced apoptosis, and 5) restoring plasmin or MMP7 activity following brain injury reduces proNGF levels and consequently, p75\textsuperscript{NTR}-mediated apoptosis. Overall, these data suggest that increased cell death following injury may be mediated in part by a change in the balance between extracellular proNGF and the activity of its processing enzymes, leading to increased cell death via p75\textsuperscript{NTR}.
Table of Contents

I. Abstract .........................................................................................................................ii

II. Table of Contents ........................................................................................................iv

III. List of Illustrations .....................................................................................................vi

IV. List of abbreviations .....................................................................................................ix

V. Introduction ..................................................................................................................1

1. The neurotrophins and their receptors ......................................................................2
   1.1 Nerve growth factor .................................................................................................2
   1.2 Neurotrophin receptors: trks and p75NTR .................................................................5

2. Enzymatic regulation of proneurotrophin cleavage .....................................................10
   2.1 Matrix metalloproteases and tissue inhibitors of metalloproteases .........................11
   2.2 The tissue plasminogen activator (tPA)-plasmin cascade .........................................15

3. CNS injury and kainic acid-induced seizures ...............................................................18

4. Significance ..................................................................................................................21

VI. Research Aims ............................................................................................................23

1. Determine the mechanisms of cell death following kainic acid treatment ..................23

2. Examine the expression and activity of MMP7 and TIMP1, and their effects on proNGF processing, in the hippocampus following kainic acid-induced injury .........................................................23

3. Determine the expression and activity of the tPA-plasmin cascade in the hippocampus following kainic acid-induced injury ........................................................................................................24

VII. Materials and Methods ............................................................................................25

1. Animals and kainic acid-induced seizures .................................................................25

2. Animal surgery and microinjection ..............................................................................25

3. Analysis of CSF ............................................................................................................26

4. Brain preparation for immunohistochemistry .............................................................26

5. In situ zymography .....................................................................................................28

6. Fluoro-Jade B labeling .................................................................................................29

7. Organotypic hippocampal slice cultures .....................................................................29

8. Propidium iodide assay ...............................................................................................30

9. Co-immunoprecipitation .............................................................................................31
10. Western blot analysis

11. Statistical analysis

VIII. Results

1. Chapter 1. Mechanisms of p75NTR mediated cell death in the hippocampus following kainic acid-induced injury
   1.1 Hippocampal cell death following kainic acid treatment is p75NTR-dependent
   1.2 proNGF release increases after kainic acid treatment and causes cell death via p75NTR
   1.3 Kainic acid induces proNGF and p75-mediated apoptosis in the hippocampus in vivo

2. Chapter 2. Expression and activity of MMP7 and TIMP1 from hippocampal cells after kainic acid-induced injury
   2.1 MMP7 and TIMP1 expression and activity following kainic acid treatment in vitro
   2.2 MMP7 and TIMP1 expression and activity following kainic acid treatment in vitro
   2.3 MMP7 protects hippocampal neurons from death following seizure in vivo

3. Chapter 3. Effects of the tPA-plasmin cascade on proNGF processing following kainic acid-induced injury
   3.1 tPA treatment reduces proNGF production following kainic acid treatment in vitro
   3.2 tPA activity decreases and proNGF and cell death increase following seizure in vivo

IX. Discussion

X. Conclusions and Future Directions

XI. Figures and Legends

XII. References

XIII. Curriculum Vitae
IV. List of Illustrations

Figure 1. Schematic of cleavage sites on proneurotrophins..........................60
Figure 2. Model of complex-receptor formation for pro- and mature NGF..................................................................................62
Figure 3. Cell death and $p75^{NTR}$ expression following KA treatment in organotypic hippocampal slice cultures.................................64
Figure 4. Cell death following kainic acid treatment is $p75^{NTR}$ dependent.............................................................................66
Figure 5. ProNGF is upregulated and released after kainic acid treatment in slice culture.................................................................68
Figure 6. Blocking proNGF activity results in reduced cell death and caspase 3 activation.................................................................................70
Figure 7. Secretion of proBDNF and proNT-3 from hippocampal cells following KA treatment in vitro.......................................................72
Figure 8. In vivo expression of $p75^{NTR}$ and cell death following KA-induced seizures...........................................................................74
Figure 9. Expression of MMP7 and TIMP1 in the CA1 following KA treatment in vivo.............................................................................76
Figure 10. MMP7 and TIMP1 secretion from the hippocampus following KA treatment......................................................................................78
Figure 11. Differential expression of cell death and extracellular proNGF in KA-treated slice culture.............................................................80
Figure 12. Expression of MMP7, TIMP1 and proNGF following KA-induced seizure in vivo………………………………………………………………………………..82
Figure 13. MMP7 activity in the hippocampus…………………………………………………………………………………………………………………………..84
Figure 14. KA-induced seizure cell death in the hippocampus in vivo…………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………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Figure 23. PAI-1 expression in the hippocampus following KA-induced seizures \textit{in vivo}…………………………………………………………………………104

Figure 24. Effect of exogenous tPA infusion on cell death in the CA1 following KA-induced seizure…………………………………………………106

Figure 25. Levels of proNGF in the CSF following KA-induced seizures following tPA infusion…………………………………………………..109
## V. List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BDNF</td>
<td>bran-derived neurotrophic factor</td>
</tr>
<tr>
<td>cc3</td>
<td>cleaved caspase 3</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine teraacetic acid</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’Diaminobenzidine</td>
</tr>
<tr>
<td>Erk</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FJ</td>
<td>fluoro-jade</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IC</td>
<td>intracerebral</td>
</tr>
<tr>
<td>HBSS</td>
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<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>KA</td>
<td>kainic acid</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-d-aspartate</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>NT-3</td>
<td>neurotrophin 3</td>
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<tr>
<td>NT4/5</td>
<td>neurotrophin 4/5</td>
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<tr>
<td>NTR</td>
<td>neurotrophin receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NgR</td>
<td>Nogo receptor</td>
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<tr>
<td>NADE</td>
<td>p75\textsuperscript{NTR}-associated death executor</td>
</tr>
<tr>
<td>NRAGE</td>
<td>neurotrophin receptor-interacting MAGE homolog</td>
</tr>
<tr>
<td>NRIF</td>
<td>neurotrophin receptor interacting factor</td>
</tr>
<tr>
<td>p75\textsuperscript{NTR}</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PAI</td>
<td>plamsinogen activator inhibitor</td>
</tr>
<tr>
<td>Pcsk6</td>
<td>proprotein convertase subtilisin/kexin type 6</td>
</tr>
<tr>
<td>PI-3k</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PLC-\gamma</td>
<td>phospholipase c gamma</td>
</tr>
<tr>
<td>SE</td>
<td>status epilepticus</td>
</tr>
<tr>
<td>serpin</td>
<td>serine protease inhibitor</td>
</tr>
<tr>
<td>SFM</td>
<td>serum free media</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
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<tr>
<td>Trk</td>
<td>tropomyosin receptor kinase</td>
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VI. Introduction

Neurotrophins are a family of growth factors that is abundantly expressed in a variety of cell populations in the nervous system. During development, they promote neuronal survival, proper synaptic formation, differentiation, and cell maintenance. Members of this family include nerve growth factor (NGF) (Levi-Montalcini, 1964a), brain-derived neurotrophic factor (BDNF) (Leibrock et al., 1989), neurotrophin-3 (NT3) (Hohn et al., 1990; Maisonpierre et al., 1990b) and neurotrophin 4/5 (NT4/5) (Hallbook et al., 1991; Ip et al., 1992; Wong et al., 1993). Each neurotrophin specifically binds with a particular member of the tropomyosin receptor kinase (trk) family of receptors to activate well-defined signaling pathways involved in cell differentiation and survival (Chao, 1992). Additionally, all neurotrophins may interact with the p75 neurotrophin receptor (p75\(_{\text{NTR}}\)), particularly when in their pro-form (Lee et al., 2001b; Roux and Barker, 2002), to induce apoptosis (Chao, 1992; Friedman, 2000). Neurotrophins can be released from cells in their mature or pro-form, suggesting that the extracellular balance between mature and proneurotrophins plays a critical role in determining whether they preferentially interact with trk or p75\(_{\text{NTR}}\) to promote survival or death, respectively. Thus, elucidating the mechanisms of neurotrophin processing is an important step towards understanding the signaling pathway that takes a cell towards survival or death. The work in this thesis examined changes in the extracellular environment following injury that affect the processing of proNGF, as well as the consequences of those changes on p75\(_{\text{NTR}}\)-mediated apoptotic signaling.
1. The Neurotrophins and their Receptors

1.1 Nerve Growth Factor

Neurotrophins are a family of growth factors that regulate a panoply of cellular activities, including development, migration, neurite outgrowth, synapse formation and proliferation. Mammals use four members of the neurotrophin family: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) (Levi-Montalcini, 1964b; Ernfors et al., 1990; Maisonpierre et al., 1990a; Hallbook et al., 1991). Two additional neurotrophins, neurotrophin 6 (NT-6) and neurotrophin 7 (NT-7) have been identified in fish (Gotz et al., 1994; Lai et al., 1998). All neurotrophins share approximately a 52% sequence identity at the amino acid level and function in several, often overlapping, cell populations throughout the nervous system (McDonald et al., 1991; Robinson et al., 1995; Heinrich and Lum, 2000).

NGF was the first identified neurotrophin and continues to be one of the more extensively characterized factors. It plays crucial roles in maturation, differentiation and survival throughout the nervous system, particularly during development (Levi-Montalcini, 1987). Early withdrawal of NGF in the developing embryo results in diminished limb growth and improper sympathetic ganglia innervation (Hamburger and Levi-Montalcini, 1949). In the adult brain, NGF retains essential functionality in various processes including learning (Koh et al., 1989; Henriksson et al., 1992), memory (Fischer et al., 1991; Fischer et al., 1994), and pain perception (Adler et al., 1984; Safieh-Garabedian et al., 1995;
Larsson et al., 2009). In addition, altered levels of NGF have been detected in various disease conditions such as schizophrenia, glaucoma, epilepsy and Alzheimer’s (Gall and Isackson, 1989; Mufson et al., 1991; Woolf et al., 1994; Chen et al., 1997; Fiore et al., 1999; Zeng et al., 2004; Sposato et al., 2008).

Mature neurotrophins are synthesized as 30-35 kDa pre-pro-proteins (Suter et al., 1991; Heymach and Shooter, 1995). The pre-domain contains a signaling peptide at its N-terminal that targets the pre-pro-neurotrophin to the endoplasmic reticulum where the pre-domain is removed, producing 26-28 kDa proneurotrophins. These pro-forms may then be sorted to the Golgi where the prodomain is glycosylated, and then undergoes further cleavage to produce 14 kDa mature neurotrophins (Bresnahan et al., 1990; Seidah et al., 1996; Marcinkiewicz et al., 1999) (figure 1). Earlier studies examining the sorting and release of neurotrophins found NGF, BDNF and NT-3 to be similarly sorted to a regulated secretory pathway (Heymach et al., 1996). However, others have found that NGF is generally sorted to the constitutive pathway, where it undergoes intracellular cleavage by convertases such as furin or proprotein convertase subtilisin/kexin type 6 (Pcsk6) before release (Seidah et al., 1996). BDNF was later confirmed to be released via the secretory pathway (Mowla et al., 1999). Because all of these studies used different overexpression systems to examine the localization and release of neurotrophins, and because the intracellular efficiency of neurotrophin processing, particularly BDNF, remains controversial (Matsumoto et al., 2008; Yang et al., 2009b), further investigation is required.
For many years, the pro-form of neurotrophins was thought to be merely a transient stage in the maturation process, and the fully processed neurotrophin believed to be the only biologically active and secreted form of the protein (Rattenholl et al., 2001). However, investigation into neurotrophin expression found that the pro-forms can also be released from cells (Heymach and Shooter, 1995; Lee et al., 2001b) and account for over 50% of secreted neurotrophins, particularly from neurons (Heymach et al., 1996; Farhadi et al., 2000; Mowla et al., 2001). Furthermore, proNGF was found to be extremely abundant in the CNS tissues of mouse, rat and human, often where mature NGF was undetectable (Fahnestock et al., 2001). This led to the discovery that proNGF can also be secreted from cells, functioning as a high-affinity ligand for the p75 neurotrophin receptor (p75\textsuperscript{NTR}) to induce apoptosis (Lee et al., 2001b; Beattie et al., 2002; Harrington et al., 2004).

Since then, increased proNGF levels have been detected in human cortex during the prodromal stage of Alzheimer’s Disease (Pang et al., 2004), after spinal cord injury in vitro (Beattie et al., 2002), in the basal forebrain after kainic acid-induced seizure (Volosin et al., 2006) and in the hippocampus after pilocarpine-induced seizure (Volosin et al., 2008). Furthermore, disrupting the interaction between proNGF and p75\textsuperscript{NTR} after corticospinal axotomy (Harrington et al., 2004) or seizure (Volosin et al., 2008) results in significantly reduced cell death, confirming that endogenous, secreted proNGF is a death-inducing ligand for p75\textsuperscript{NTR}-mediated apoptotic signaling after injury.
1.2 Neurotrophin Receptors: Trks and p75\textsuperscript{NTR}

Neurotrophins can bind to two types of receptors: tropomyosin receptor kinases, or trks, and p75\textsuperscript{NTR}, a member of the tumor necrosis factor receptor (TNFR) superfamily (Friedman and Greene, 1999). In their processed, mature form, each neurotrophin preferentially binds particular trk receptors with high affinity. NGF binds trkA (Kaplan et al., 1991b), and cleavage of proNGF to NGF is required for trkA activation (Boutilier et al., 2008). BDNF and NT-4/5 bind trkB (Klein et al., 1991), and NT-3 preferentially activates trkC (Lamballe et al., 1991), but may also bind trkB (Glass et al., 1991; Squinto et al., 1991). NT-3 and NT-4/5 can also bind to trkA, but with a much lower affinity than NGF (Kaplan et al., 1991a). Binding to trk receptors results in receptor dimerization and the phosphorylation of tyrosine residues in the cytoplasmic domain (Jing et al., 1992). Following phosphorylation, trk signaling may activate PI-3 kinase, MAPK/Erk or PLC-γ, signaling pathways well known for their roles in mediating neuronal differentiation and survival (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001).

Neurotrophin signaling through trk receptors is a hallmark of proper development, while trophic withdrawal functions in parallel as a necessary cell death mechanism (Ernfors et al., 1992; Ringstedt et al., 1993; Deppmann et al., 2008). However, p75\textsuperscript{NTR}–induced signaling has also been shown to initiate programmed cell death during development, serving as an alternate method of neuronal pruning that is activated upon a lack of proper trk expression and
cognate neurotrophic support (Frade and Barde, 1999; Frade et al., 1999; Majdan and Miller, 1999; Singh et al., 2008).

Mature neurotrophins can bind p75NTR, but with a much lower affinity than to their respective trk receptor, a characteristic that led to p75NTR being initially named “the low-affinity neurotrophin receptor” (Chao et al., 1986). However, neurotrophin binding affinity can be modulated by trk interaction with p75NTR. For example, NGF binds with increased specificity to a trkA-p75NTR complex than to trkA alone (Hempstead et al., 1991; Benedetti et al., 1993; Esposito et al., 2001). Furthermore, following the discovery that the pro-forms of neurotrophins carry biologically active functions, it was revealed that proneurotrophins bind to p75NTR with a five-fold higher affinity compared to their respective trk receptors (Lee et al., 2001a; Chao and Bothwell, 2002) due to the formation of a complex with the co-receptor sortilin (Nykjaer et al., 2004; Tauris et al., 2011). Sortilin binds the pro-region of neurotrophins while p75NTR interacts with the mature domain, creating a ternary death receptor complex and greatly increasing the affinity between neurotrophins and p75NTR (Nykjaer et al., 2004; Teng et al., 2005). Sortilin knockout mice display reduced neuronal apoptosis, similar to levels observed in p75NTR knockout mice (Jansen et al., 2007), demonstrating that this receptor complex formation is critical for proper regulation of p75NTR-mediated programmed cell death during development (Koshimizu et al., 2009; Yang et al., 2009a). Moreover, sortilin signaling has also been shown to be essential for cell death following corticospinal lesion and age-dependent neurodegeneration
(Jansen et al., 2007), suggesting sortilin is a general p75\textsuperscript{NTR} signaling partner in proneurotrophin-induced apoptosis (figure 2).

Like the other members of the TNFR superfamily, p75\textsuperscript{NTR} contains an intracellular "death domain" near the C-terminus, and cleavage of this domain has been shown to be necessary for p75\textsuperscript{NTR} -mediated apoptotic signaling (Majdan et al., 1997; Kenchappa et al., 2006). However, p75\textsuperscript{NTR} itself lacks enzymatic activity and signals by recruiting various intracellular adapter proteins, many of which have been linked to apoptosis. For example, the zinc finger protein, neurotrophin receptor interacting factor (NRIF), has been shown to interact with p75\textsuperscript{NTR} to signal cell death (Casademunt et al., 1999). In vitro, p75\textsuperscript{NTR}-mediated cell death was not detected in neurons from NRIF/-/- mice following trophic withdrawal (Linggi et al., 2005) or treatment with proNGF (Volosin et al., 2008). Furthermore, following pilocarpine-induced seizures in vivo, NRIF/-/- mice demonstrated decreased cell death compared to wild-type mice, at a level comparable to those of p75\textsuperscript{NTR-/-} mice (Volosin et al., 2008). Other adapter proteins that are recruited to p75\textsuperscript{NTR} to initiate apoptotic signaling include the p75\textsuperscript{NTR}-associated death executor (NADE) (Yi et al., 2003) and the neurotrophin receptor-interacting MAGE homolog (NRAGE) (Salehi et al., 2000).

Neurotrophin-stimulated p75\textsuperscript{NTR} apoptotic signaling leads to the phosphorylation of c-Jun-N-terminal kinase (JNK) as first demonstrated in oligodendrocytes, which lack trkA (Casaccia-Bonnefil et al., 1996). However, investigation into the downstream events following neurotrophin signaling in cells containing both trks and p75\textsuperscript{NTR} have revealed a crosstalk between the survival
and apoptotic pathways elicited by each receptor. For example, in oligodendrocytes with virally expressed trkA, NGF treatment halted JNK phosphorylation, increased the extracellular signal-regulated kinase (ERK1) activity, and abolished p75\textsuperscript{NTR}-mediated apoptosis (Yoon et al., 1998). Similarly, in sympathetic neurons, which express trkA and p75\textsuperscript{NTR}, but not trkB, NGF treatment also prevented JNK phosphorylation and p75\textsuperscript{NTR}-mediated cell death (Bamji et al., 1998). Conversely, BDNF treatment did lead to JNK activation and p75\textsuperscript{NTR}-mediated apoptosis in the same cells, likely due to the lack of trkB and the presence of p75\textsuperscript{NTR} activation in the absence of competitive trophic signaling (Bamji et al., 1998; Kohn et al., 1999). Thus, the outcome of neurotrophin signaling is selectively influenced by the presence of particular trk receptors and their ability to inhibit JNK activation.

The convergence of trk and p75\textsuperscript{NTR} signaling pathways has also been examined in basal forebrain neurons, which, unlike sympathetic neurons, express all three trk receptors and p75\textsuperscript{NTR} throughout life. Also unlike sympathetic neurons, the activation of trk signaling alone in basal forebrain neurons was not enough to inhibit neurotrophin-induced cell death; activation of the PI3 kinase-Akt pathway by mature BDNF was also required to overcome apoptotic signaling (Volosin et al., 2006). Moreover, in the presence of both proNGF and BDNF, proNGF signaling through p75\textsuperscript{NTR} suppressed BDNF-trk activation of Akt, favoring cell death in basal forebrain neurons (Song et al., 2010). Taken together, these results indicate that the final outcome of neurotrophin signaling is highly dependent upon cellular context.
The importance of p75<sup>NTR</sup> signaling during development has also been illustrated using knockout mice. Two groups have generated p75<sup>NTR</sup> mutant mice that lack either exon III (Lee et al., 1992) or exon IV (von Schack et al., 2001) of the gene, resulting in a missing NGF-binding domain. Both knockout forms manifest posterior limb ataxia and a severe loss of peripheral sensory neurons and nerve volume. Mice carrying the exon III deletion have also been observed to have more cholinergic neurons in the basal forebrain (Van der Zee et al., 1996), as well as increased basal forebrain volume (Yeo et al., 1997) compared to wild-type mice. Furthermore, transgenic mice over-expressing p75<sup>NTR</sup> demonstrate extensive cell loss throughout the CNS (Majdan et al., 1997). Taken together, these studies illustrate the importance of p75<sup>NTR</sup> signaling in proper development.

In addition to trks and sortilin, p75<sup>NTR</sup> can form signaling complexes with other receptors to mediate a wide range of biological functions, such as Nogo receptor (NgR) to modulate neurite outgrowth or Ephrin-A to signal axon repulsion (Wang et al., 2002; Lim et al., 2008). In the hippocampus, p75<sup>NTR</sup> binding to proBDNF plays an important role in facilitating LTD (Woo et al., 2005). In the PNS, p75<sup>NTR</sup> signaling has also been shown to recruit Par-3 and to form a complex necessary for the establishment of polarity and proper Schwann cell myelination (Chan et al., 2006).

Nonetheless, the most extensively characterized role of p75<sup>NTR</sup> is in mediating apoptosis (Chao, 1992; Barrett and Bartlett, 1994). In addition to its function in developmental programmed cell death, p75<sup>NTR</sup>–mediated apoptosis
has also been described in other systems, including oligodendrocyte (Casaccia-Bonnefil et al., 1996) and photoreceptor cultures (Srinivasan et al., 2004), two different seizure models (Roux et al., 1999; Troy et al., 2002), ischemia (Park et al., 2000), multiple sclerosis (Nataf et al., 1998; Chang et al., 2000), Alzheimer's disease (Edwards et al., 1988; Kerwin et al., 1992), and corticospinal axotomy (Giehl et al., 2001; Harrington et al., 2004). Not coincidentally, these conditions correspond with those in which increased proneurotrophin levels are also detected. These studies and others have created a large body of research suggesting that the apoptotic consequence of \( p75^{\text{NTR}} \) signaling in pathological conditions is dependent upon the availability of proneurotrophins. If changes in the proportion of pro- and mature NGF, with consequent differences in \( p75^{\text{NTR}} \) binding affinity, create an environment more susceptible to cell death, particularly in disease states or after injury, it will be important to understand the mechanisms of pro- and mature NGF production under such conditions. We suggest that the regulation of that balance may be key in determining the difference between cell death or survival after injury.

2. Enzymatic regulation of proneurotrophin cleavage

The pro-forms of neurotrophins are potent, high affinity ligands for the \( p75^{\text{NTR}} \)-mediated apoptotic pathway. In pathological conditions, proneurotrophins are induced and released from cells, both \textit{in vitro} and \textit{in vivo}, suggesting that the extracellular proteolysis of proneurotrophins to their mature forms may be a
critical checkpoint in determining the extent of cell death after injury. To date, two enzymes have been identified that can extracellularly cleave proNGF to mature NGF: matrix metalloproteinase 7 (MMP7) and plasmin (Smith et al., 1995; Lee et al., 2001b; Watanabe et al., 2008). The existence of other enzymes capable of cleaving proneurotrophins to their mature forms remains a possibility. This section discusses the potential contributions of MMP7 and plasmin to extracellular proneurotrophin processing and cell death.

2.1 Matrix metalloproteinases and tissue inhibitors of metalloproteinases

The matrix metalloproteinases (MMPs) are a quickly expanding family of over twenty secreted Zn$^{2+}$ endopeptidases, classically described as the enzymes responsible for degrading all of the components of the extracellular matrix (Birkedal-Hansen, 1993; Wojtowicz-Praga et al., 1997; Malemud, 2006). They are found in all organisms and contain conserved pro- and catalytic domains. Each MMP is categorized into a sub-class defined by its substrate specificity (Ra and Parks, 2007).

Most MMPs are secreted as inactive pro-proteins. Classical activation of MMPs is referred to as the “cysteine switch,” in which the bond between a cysteine residue on the pro-region and a zinc ion on the catalytic site is disrupted (Springman et al., 1990). In vitro, full cleavage of the pro-domain is not always required to activate the enzyme, only disruption of the cysteine-zinc interaction.
However, it is believed that in vivo, MMPs must undergo additional cleavages, including proteolysis of the pro-domain, to generate a fully processed enzyme (Nagase et al., 1990; Suzuki et al., 1990). These extra processing steps may be autolytic (Suzuki et al., 1990) or performed by other MMPs or serine proteases (Nagase, 1997).

Once active, MMPs act upon a wide variety of substrates beyond extracellular matrix components. They can process cytokines, receptors and other peptides to modulate cell signaling in normal physiological processes such as reproduction, embryonic development and tissue remodeling (So et al., 1992; Harvey et al., 1995; Parks et al., 2004; Kessenbrock et al., 2010). In addition, MMP action has also been closely associated with cell migration (Ray and Stetler-Stevenson, 1995), tumor growth and metastatic activity (Liotta et al., 1991; Wojtowicz-Praga et al., 1997), and neurotoxic related death (Jourquin et al., 2003).

MMPs can also act upon secreted proneurotrophins. Specifically, proNGF and proBDNF both contain consensus sites for cleavage by MMP7, which effectively processes both proneurotrophins to their mature forms (Smith et al., 1995; Lee et al., 2001b). MMP7 (also known as matrilysin) is the smallest of the MMPs at 19 kDa due to the lack of a COOH-terminal domain. Also unlike most other MMP family members, MMP7 is constitutively expressed in most adult tissues (Wielockx et al., 2004). It can break down a wide range of substrates including casein, proteoglycans, fibronectin, and elastin (Woessner and Taplin, 1988; Miyazaki et al., 1990; Fosang et al., 1992; Hardingham and Fosang, 1992;
Imai et al., 1995), as well as activate the pro-forms of gelatinases MMP2 and MMP9 (von Bredow et al., 1998). It is secreted from cells in its pro-form, and although proMMP7 can be activated by enzymes such as trypsin and MMP3 \textit{in vitro}, its mechanism of activation \textit{in vivo} remains unclear (Imai et al., 1995; Ra and Parks, 2007).

MMP activity is endogenously inhibited by tissue inhibitors of metalloproteinases, or TIMPs. To date, four TIMPs have been identified and are named TIMP1-TIMP4 in order of discovery. To inhibit MMP activity, TIMPs bind MMPs in a 1:1 or 2:2 stoichiometric fashion (Nagase and Brew, 2003) forming a complex that covers the active site cleft (Gomis-Ruth et al., 1997). TIMPs may bind to either the mature or the pro-form of MMPs, and each TIMP has the capability to inhibit several different MMPs. In particular, TIMP1, which is the largest of the TIMPs at 28 kDa (Carmichael et al., 1986), can inhibit MMP7, as well as other MMPs, including MMP1, -2, -3, -9, -13, and -14. To inhibit MMP7 activity, TIMP1 binds to its pro-form, both blocking possible substrate interaction and disallowing the final cleavage steps required for its activation (Gogly et al., 2009).

TIMP1 function has been linked to myriad biological processes throughout the body, including tissue remodeling and growth factor induction, as well as pathological conditions, including inflammation, angiogenesis and tumor cell metastasis (Gomez et al., 1997; Crocker et al., 2006). In the CNS, TIMP1 is associated with neuroprotection, such as maintaining the blood-brain barrier and mediating excitotoxic stress (Tan et al., 2003). TIMP1 also manifests inhibitory
effects on tumor growth and angiogenesis (London et al., 2003), and transfection of TIMP1 into tumor cells has been shown to inhibit invasive metastatic activity in cell lines (Kawamata et al., 1995; Jee et al., 2006). Due to their modulating effects on malignant cell progression, the use of MMP inhibitors has been widely hypothesized as a possible treatment for cancer (Yoon et al., 2003).

However, TIMP1 expression is not always associated with beneficial consequences. TIMP1 mRNA and protein levels are strongly upregulated in the forebrain and hippocampus following kainic acid-induced seizure, and this expression coincides with maximal levels of cell death (Rivera et al., 1997). Likewise, TIMP1 knockout mice demonstrate resistance to cell death (Jourquin et al., 2005). TIMP1 expression is also strongly correlated with malignancy in particular forms of glioma (Groft et al., 2001), and is also elevated in the brains and CSF of patients with Parkinson’s disease (Lorenzl et al., 2003) and multiple sclerosis (Kouwenhoven et al., 2001).

The upregulation of TIMP1 found in these and other disease states suggests that an imbalance between MMP activity and associated TIMP inhibition may be a direct cause of decreased cell viability in pathological conditions. In the case of MMP7 and TIMP1, increased MMP7 levels have been shown to be necessary for pro- to mature NGF conversion in vivo, and these levels inversely corresponded to TIMP1 expression (Kendall et al., 2009). Furthermore, MMP7 production and activity was found to be significantly decreased in both rat and clinical patient models of diabetic retinopathy, leading to proNGF accumulation and enhanced retinal neurodegeneration via p75NTR (Ali
et al., 2011). Together, these data suggest that the activity of MMP7 and its inhibitor, TIMP1, is a system that directly influences the proapoptotic or prosurvival effect of p75NTR signaling.

2.2 The tissue plasminogen activator (tPA)-plasmin cascade

Another enzyme system linked to the extracellular proteolysis of proneurotrophins is the tPA-plasmin cascade (Lee et al., 2001a; Bruno and Cuello, 2006). tPA, or tissue plasminogen activator, is a serine protease found in various mammalian tissues, including the brain (Tsirka et al., 1995; Bruno and Cuello, 2006). Once secreted from a cell, tPA can catalyze the conversion of the single-chain protein plasminogen to the double-chain active protease plasmin. TPA and plasmin are most commonly associated with the pharmacological lysis of blood clots, particularly following embolic or thrombotic stroke (Papadopoulos et al., 1987). However, tPA therapy has also demonstrated neuroprotective effects in a mouse model of multiple sclerosis (East et al., 2005), on β-amyloid neurotoxicity (Ledesma et al., 2000; Jacobsen et al., 2008), on remyelination and axonal regeneration after sciatic nerve crush (Zou et al., 2006), and following hypoxic injury (Echeverry et al., 2010).

tPA-plasmin cascade activity has been shown to be important in motor learning in adults (Seeds et al., 1995), tissue remodeling (Liu et al., 1995), responding to stress (Skrzypiec et al., 2008), and proper development of binocular vision during the critical period (Mataga et al., 2004). These studies
and others emphasize the role of tPA in neuronal plasticity (Basham and Seeds, 2001; Nagappan et al., 2009) and in fact, plasmin cleavage of proBDNF to BDNF is essential for hippocampal long-term potentiation (LTP) (Pang et al., 2004). Conversely, proBDNF was found to produce a physiologically opposite effect, as perfusion of uncleavable proBDNF induced p75\textsuperscript{NTR}-dependent long-term depression (LTD) in hippocampal slices (Woo et al., 2005). These physiologically opposing effects between the pro- and mature forms of the protein demonstrate that elucidating the mechanisms of plasmin activity on neurotrophin processing will be crucial for understanding synaptic modulation.

The tPA-plasmin cascade is inhibited at the level of tPA by the serine protease inhibitor (serpin) neuroserpin (Hastings et al., 1997; Osterwalder et al., 1998). In the adult, neuroserpin has been detected in neurons throughout the brain and spinal cord, particularly where tPA is also located (Hastings et al., 1997), and neuronal depolarization results in the concomitant release of tPA and neuroserpin (Gualandris et al., 1996; Berger et al., 1999). Furthermore, tPA and neuroserpin also colocalize with plasminogen and proNGF (Bruno and Cuello, 2006), all of which are secreted in conjunction upon stimulation, indicating a tightly regulated system between plasmin cleavage of proNGF, its activation by tPA, and a closely corresponding inhibitory loop regulated by neuroserpin (Bruno and Cuello, 2006). Thus, proNGF processing may also depend upon the balance between tPA and neuroserpin activity.

Two other serpin family members are also capable of inhibiting tPA activity: plasminogen activator inhibitor (PAI) -1 and -2. PAI-2 is produced by the
placenta and thus only detectable in the blood during pregnancy (Kruithof et al., 1987). PAI-1 is expressed throughout the body and binds directly to tPA in a 1:1 stoichiometric complex to suppress activity (Lindahl et al., 1990). PAI-1 mRNA and protein levels have been found to be elevated twenty-four hours following ischemic injury and kainic acid-induced seizures, corresponding with decreased tPA activity (Salles and Strickland, 2002; Kim et al., 2011). Furthermore, tPA:PAI-1 complexes are found throughout multiple sclerosis lesions, suggesting a role for PAI-1 in exacerbating axonal injury (East et al., 2005). However, the functional roles of PAI-1 extend beyond that of inhibiting plasminogen activation. Independent of tPA expression, PAI-1 is rapidly increased in response to injury and inflammation (Kawasaki et al., 2000; Del Signore et al., 2006). Also in response to inflammation, PAI-1 can act as a chemoattractant, mediating cell migration by inducing changes in cell morphology, receptor expression, and cytoskeletal organization (Degryse et al., 2001). In opposition to the thrombolytic effects of tPA, PAI-1 activates the coagulation pathway (Chambers, 2003), but may also function in conjunction with tPA to coordinate macrophage migration (Cao et al., 2006), an effect that is also seen in the invasive nature of certain types of cancer (Caccamo et al., 1994; Chazaud et al., 2002). In a mouse model of Alzheimer’s disease, elevated proNGF and p75NTR levels (Cuello et al., 2007; Capsoni et al., 2010), as well as decreased tPA axis activity (Melchor et al., 2003), suggest that PAI-1 upregulation contributes to cell death in this disease. Although heightened levels of PAI-1 have been found in the CSF of Alzheimer’s patients (Sutton et al., 1994), conflicting studies suggest that PAI-1 is not
consistently associated with (Hino et al., 2001), or involved at all (Fabbro and Seeds, 2009), in the pathological phenotype. Considering that all of the components of the tPA-plasmin cascade, including its inhibitors, PAI-1 and neuroserpin, display both protective and detrimental effects in the brain, it will be relevant to examine the relationship between the tPA axis and neurotrophic activity following injury, clarifying the mechanisms of plasmin-dependent neuronal survival.

Unlike with proBDNF, the relationship between plasmin and proNGF processing has remained relatively unexplored. However, this common processing pathway suggests that improper proNGF cleavage by the tPA-plasmin cascade may also significantly affect several aspects of cellular function. We suggest that decreases in tPA activity following injury may prompt a reduction in proNGF processing and thus an increase in the pro- to mature NGF ratio, negatively impacting cell viability via increased p75\textsuperscript{NTR} signaling.

3. CNS Injury and Kainic Acid-Induced Seizures: The Role of Neurotrophins and p75\textsuperscript{NTR}

NGF protein expression is increased after various forms of neuronal injury, including corticospinal axotomy (Harrington et al., 2004), seizure (Ballarin et al., 1991; Katoh-Semba et al., 1999) and Alzheimer's Disease (Fahnestock et al., 2001; Cuello and Bruno, 2007). Although NGF is known to promote cell survival in neurons expressing trkA, pro- and mature NGF release is still increased after
injury in areas where this receptor is lacking (Ernfors et al., 1989; Roux et al., 1999; Fahnestock et al., 2001). Additionally, $p75^{NTR}$ expression is also increased after injury in these same areas that lack trk expression (Roux et al., 1999; Oh et al., 2000), implicating a role for $p75^{NTR}$-mediated signaling in the injury response.

Seizure models are commonly used to study injury-induced damage in the brain (Turski et al., 1985; Vincent and Mulle, 2009), and cell death following seizures has been strongly tied to $p75^{NTR}$ signaling. For example, pilocarpine-induced seizures have been shown to increase $p75^{NTR}$ expression in apoptotic hippocampal neurons (Roux et al., 1999), and mice lacking $p75^{NTR}$ demonstrate significantly reduced neuronal death compared to wild-type mice (Troy et al., 2002).

Kainic acid (KA), a glutamatergic agonist, is another widely used neurotoxin for studying seizure-induced cell death. To induce seizures, kainic acid can be injected intraperitoneally (IP) or intracerebrally (IC), including into the ventricles (ICV) or amygdala (ICA). IC injection results in the most rapid and severe seizure effects, including full body tremors, foaming at the mouth, bulging eyes, necrosis at the injection site and in the pyriform cortex, and extensive degeneration, gliosis and atrophy throughout the hippocampal region (Schwarz et al., 1978). IP injection of kainic acid produces similar motor and EEG symptoms, but culminates in more mild sequelae, particularly reduced neuronal damage that is also more symmetrical compared to IC injections (Ben-Ari et al., 1980). The *in vivo* experiments in this thesis utilized IP injections to examine the
mechanisms that regulate apoptosis following seizure, as IC injections obscure the difference between seizure-induced cell death and direct KA toxicity.

Kainic acid acts through AMPA/kainate receptors, and continued stimulation of these receptors can lead to extended kindling and epileptic behavior (Ernfors et al., 1991). KA-induced seizures evoke upregulation of neurotrophin mRNA and protein, as well as cell death, in the dentate and CA1 areas of the hippocampus (Gall and Isackson, 1989; Frade et al., 1996; Rao et al., 2006). Moreover, studies from our lab have confirmed that kainic acid-induced seizures elicit the production of proNGF, leading to p75\textsuperscript{NTR}-mediated signaling and, consequently, increased cell death (Volosin et al., 2006).

Although increased mRNA and protein levels in tissue are indicative of neurotrophin production, they do not necessarily evince what form of the neurotrophin is released from cells following seizure. Examination of the CSF of rats that had undergone seizure treatment revealed increased levels of proNGF compared to rats that received vehicle treatment, indicating that proNGF is produced and secreted under pathological conditions \textit{in vivo} (Volosin et al., 2008). More interesting, infusion of a blocking antibody to proNGF into the hippocampus for three days following seizure induction dramatically reduced cell death, further illustrating that endogenously secreted proNGF promotes apoptotic signaling after seizures (Volosin et al., 2008).

Once released from a cell, proneurotrophins can be further processed to their mature forms. What remains unclear, however, is what ultimately
determines the form of the neurotrophin available for signaling. Because the production and secretion of proneurotrophins and their cleaving enzymes are regulated after injury, the extracellular proteolysis of proneurotrophins to their mature forms represents a critical checkpoint in determining whether a cell lives or dies.

4. Significance

Since their discovery over sixty years ago, the subject of neurotrophins has been recognized as a matter of great importance, particularly for their roles in growth, differentiation and survival in the developing nervous system. Their wide functionality throughout the nervous system has made neurotrophins targets of particular interest for their potential therapeutic functions in numerous disorders including ischemia (Han and Holtzman, 2000), spinal cord trauma (Ye and Houle, 1997), Alzheimer’s Disease (Lad et al., 2003), and depression (Shirayama et al., 2002). Recent findings indicating that NGF induces cell death via the p75NTR, especially in its pro- form, tell us that the consequences of neurotrophin signaling also depend on a number of other factors, including the ratio of pro- and mature neurotrophins and the enzymes that regulate that balance.

Although enhancing proneurotrophin cleavage in injury or disease conditions appears to be a potential avenue for the restoration of cell viability, the pleiotropic properties of proneurotrophin processing enzymes complicate this
idea. For example, MMP7 is a biomarker for the invasive phenotype of several types of cancer, such as ovarian (Tanimoto et al., 1999), breast (Barrett et al., 2002), prostate (Pajouh et al., 1991), pancreatic (Ohnami et al., 1999), colorectal (Brabletz et al., 1999), hepatocellular (Ozaki et al., 2000), astro- and oligodendroglialomas (Thorns et al., 2003; Rome et al., 2007), and others (Amalinei et al., 2010). However, cancers are different from brain injury, and the role of MMP7 in neuropathological conditions remains to be determined. tPA contributes to blood-brain barrier damage following ischemic stroke (Yepes et al., 2003) and cell death following spinal cord injury (Abe et al., 2003). More interesting, tPA has been shown to be both neuroprotective (Kim et al., 1999) and necessary for cell death following kainic acid-induced seizures (Tsirka et al., 1995). This range of effects purports that if altered enzyme activity contributes to improper trophic support, and if these enzyme systems are to be targeted in clinical treatments, it will be crucial to understand the influence of enzymatic regulation and the potential outcomes of shifting enzyme levels on neurotrophin processing in the central and peripheral nervous systems.
VI. Research Aims

1. Specific Aim 1: Determine the mechanisms of cell death following kainic acid treatment.

Previous results from our lab and others have demonstrated increased p75<sub>NTR</sub> expression and cell death after seizure <i>in vivo</i> (Roux et al., 1999; Troy et al., 2002; Yi et al., 2003; Volosin et al., 2008). To examine more thoroughly the mechanisms of injury-induced cell death, a reduced hippocampal slice culture system was utilized. This method allows for the manipulation of cellular conditions and provides a simple way to measure protein secretion into the media, all while maintaining cellular network structure. Furthermore, kainic acid was used to induce cell death as it can be applied both <i>in vitro</i> and <i>in vivo</i> to simulate epileptiform activity and injury (Routbort et al., 1999; Jourquin et al., 2003). The experiments in this aim defined the mechanisms of p75<sub>NTR</sub> activation and their effects on cell death after kainic acid treatment.

2. Specific Aim 2: Examine the expression and activity of MMP7 and TIMP1, and their effects on proNGF processing, in the hippocampus following kainic acid-induced injury.

proNGF has been shown in various systems to stimulate apoptotic signaling via p75<sub>NTR</sub> (Beattie et al., 2002; Harrington et al., 2004; Nykjaer et al., 2004; Domeniconi et al., 2007; Volosin et al., 2008; Rogers et al., 2010; Song et al., 2010). The experiments in this aim investigated the expression and activity of
the proNGF extracellular processing enzyme, MMP7, in the hippocampus following kainic acid treatment to determine if an association existed between the levels of enzyme available for proNGF processing and proNGF-induced cell death. The expression of the inhibitor to MMP7, TIMP1, was also examined to determine whether inhibitor levels affected changes in MMP7 activity. Levels of proNGF, p75^{NTR} and cell death were measured to determine whether enhancing enzyme activity would lead to augmented proNGF processing and reduced cell death following kainic acid treatment.

3. **Specific Aim 3: Determine the expression and activity of the tPA-plasmin cascade in the hippocampus following kainic acid-induced injury.**

The third aim evaluated the connection between proNGF/p75^{NTR}-mediated cell death and another proNGF processing enzyme, plasmin, following kainic acid-induced injury. Both *in vitro* and *in vivo*, we examined whether levels of tPA/plasmin were altered following kainic acid treatment, and the relationship between enzyme and inhibitor expression. Similar to Aim 2, we examined the consequences of artificially increasing proNGF processing using exogenous tPA treatment. Finally, we examined whether altering the levels of proNGF processing by increasing tPA after kainic acid treatment would reduce p75^{NTR}-mediated cell death.
VII. Materials and Methods

1. Animals and Kainic Acid-Induced Seizures.

Male Sprague Dawley rats (250–275 g) were injected with kainic acid (KA) (10 mg/kg, i.p.) for induction of status epilepticus (SE). After 1 hour of demonstrating SE behavior, rats were treated with diazepam (10 mg/kg, i.p.; Henry Schein, Melville, NY) and phenytoin (50 mg/kg, i.p.; Sigma) to stop seizure activity. Control rats received all the same treatments except they were injected with saline instead of KA. Animals not displaying this seizure behavior were not included in this study. During recovery, the animals were injected subcutaneously with Hartmann’s solution (130 mM NaCl, 4 mM KCl, 3 mM CaCl, 28 mM lactate; 1 ml/100 g) twice daily until they were capable of eating and drinking freely.

2. Animal Surgery and Microinjection

In some cases, rats were cannulated 1 week before the induction of seizures. Rats were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic apparatus for cannula implantation into the dorsal hippocampus with the following coordinates: bregma, −3.1 mm; lateral, 2.0 mm; and depth, 3.1 mm (Paxinos and Watson, 1994). Cannulas were fixed to the skull with a screw and dental cement. MMP7 (1 µg) or tPA (1 µg) was infused on one side of the brain and saline on the other side at a rate of 0.5 µl/minute immediately following the seizure, and twice daily thereafter until the rats were sacrificed, for a total of 3 infusions over the course of 24h. Animals found to have an incorrectly placed cannula were excluded.
All animal studies were conducted using the National Institutes of Health guidelines for the ethical treatment of animals with approval of the Rutgers Animal Care and Facilities Committee.

3. **Analysis of CSF.**

Twenty-four hours after KA-induced seizure, animals were anesthetized with ketamine/xylazine. CSF (40–100 µl per animal) was collected from the cerebello-medullarcisterna using a 25 gauge needle into tubes containing protease inhibitors, snap frozen, and stored at −80°C until analysis. Only CSF samples that did not contain blood contamination were used for Western blot analysis.

4. **Brain Preparation for Immunohistochemistry.**

Animals were anesthetized with ketamine/xylazine and perfused transcardially with saline followed by 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde for 2 h, and cryoprotected in 30% sucrose overnight. Sections (12 µm) were cut on a cryostat (Leica) and mounted onto charged slides. Sections were blocked in PBS/10% goat serum and permeabilized with PBS/0.3% Triton X-100, and then exposed to primary antibodies overnight at 4°C in PBS/0.3% Triton X-100. Slides were then washed three times in PBS. Sections for fluorescent microscopy were exposed for 1h at room temperature to secondary antibodies coupled to appropriate fluorophores (Alexa Fluor, Invitrogen; 1:1000 dilution), and washed again in PBS in the presence of 4',6'-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich; 1:1000 dilution).
dilution) to identify apoptotic neurons. Sections were coverslipped with anti-fading medium (ProLong Gold; Invitrogen) and analyzed by fluorescence microscopy (Nikon). Sections were alternatively prepared for immunohistochemical staining using the Vectastain ABC kit (Vector Labs, Burlingame, CA) followed by incubation in Sigmafast 3,3’Diaminobenzidine (DAB) substrate (Sigma Aldrich, St. Louis, MO) according to the manufacturers’ instructions. Endogenous peroxidase was quenched by incubating tissue in 0.3% H$_2$O$_2$ and MeOH for 30 minutes prior to staining. Sections were cleared in xylene and coverslipped with Permount mounting media (Daigger, Vernon Hills, IL). Images were captured digitally (ORCA-R2, Hamamatsu Photonics, Japan) and assembled in Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA).

Primary antisera were as follows: anti-cleaved caspase-3 (pAb; 1:500; Cell Signaling Technology), anti-NGF (pAb; Sigma-Aldrich; 1:1000 dilution), anti-MMP7 (pAb; GeneTex; 1:1000 dilution), anti-TIMP1 (mAb; Abcam; 1:500 dilution), anti-p75 (pAb; 9651; 1:500 dilution) (Huber and Chao, 1995) or 192 IgG (mAb or pAb; Millipore Bioscience Research Reagents; 1:1000 dilution), anti-proNGF (kindly provided by Dr. Barbara Hempstead; 1:1000 dilution). Specificity of each antibody was determined by immunoblot analysis of whole tissue lysates; antibodies demonstrating non-specific binding were not used for histochemistry. No immunostaining was seen in controls with omission of the primary antibodies or secondary antibodies.
5. In Situ Zymography.

24h following KA treatment, rats were anesthetized with ketamine/zylazine, decapitated and their brains removed, flash frozen, and cryosectioned (12µm). To assess MMP7 activity, sections were coated with an in situ zymogram assay buffer (50mM Hepes, 200 mM NaCl, 1mM CaCl$_2$, 0.01% Brij-35, pH 7.5) containing 1% low-melting point agarose and 10µg/ml MMP7 fluorogenic substrate (Calbiochem, San Diego, CA) specific to MMP7 activity, then incubated overnight in a humidified chamber at 37°C. MMP7 activity was observed using fluorescence microscopy (Nikon) as fluorescent holes on a black background and calculated as a percentage of the entire hippocampal surface area using Adobe Photoshop CS4 Adobe Systems Inc., San Jose, CA). Ethylene diamine tetraacetic acid (EDTA), a chelating agent that inhibits MMP activity, was added to the assay solution as a negative control and abolished enzymatic activity. To determine substrate specificity, 10µg/ml MMP7 fluorogenic substrate was incubated with varying concentrations (1-100ng/µl) of active MMP2, MMP3 and MMP7 in in situ zymogram assay buffer at 37°C overnight, placed on a coverslip, and imaged using fluorescence microscopy. Activity was only observed in MMP7-incubated samples.

tPA activity was measured by overlaying fresh-frozen sections with an assay solution containing 1% low-melting point agarose (BioRad, Hercules, CA), 0.1 M Tris, pH 7.5, 2.5% milk and 25 µg/mL plasminogen. Activity was observed as dark areas on a light background using Bright field microscopy. Aprotinin was added to the assay solution as a negative control.
6. Fluoro-Jade B Labeling

The number of dying neurons in rats after KA-induced seizures was assessed by labeling with Fluoro-Jade B according to published protocol (Schmued and Hopkins, 2000). Briefly, sections were air-dried on a slide warmer at 50°C for 30 minutes following histochemical labeling. Sections were then rehydrated for 2 minutes in distilled water and transferred to 0.06% potassium permanganate for 10 minutes. The sections were then rinsed in distilled water for 2 minutes and transferred to Fluoro-Jade B working solution (0.0001% Fluoro-Jade B in 0.1% acetic acid) for 10 minutes, followed by three rinses of two minutes each in distilled water. The sections were air-dried on a slide warmer at 50°C for 5 minutes, then cleared in xylene and coverslipped with Permount mounting media (Daigger, Vernon Hills, IL). Images were captured digitally (ORCA-R2, Hamamatsu Photonics, Japan) and assembled in Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA).

7. Organotypic hippocampal slice cultures

Organotypic hippocampal cultures were prepared as previously described (Stoppini et al., 1991). Postnatal day 7 rat pups were sacrificed by exposure to CO₂ and soaked in 80% ethanol for ten minutes. Their brains were removed under sterile conditions and placed into Hanks Balanced Salt Solution plus glucose (100ml HBSS with 0.6g glucose). Hippocampi were removed in HBSS, set on sterile Whatman chromatography paper and sectioned to 300 µm using a McIlwain tissue chopper. Slices that retained the cytoarchitecture of the hippocampus were placed onto Millipore filters suspended in 1.5 ml of serum-
containing media (25% horse serum, 75% MEM, 0.6g glucose, 1 uM penicillin-streptomycin). Cultures were incubated at 37°C in 95% O₂ and 5% CO₂ for 7 days to eliminate the initial inflammatory response. Afterwards, the media was changed to Serum Free Media (SFM) containing 1:1 Minimum Essential Medium (MEM):F12+ (6 mg/ml d-glucose, 100 µg/ml transferrin, 25 µg/ml insulin, 20 nM progesterone, 60 µM putrescine, 30 nM sodium selenide) and incubated for 24 hours. Slices were treated with MK-801 (10 µM; Tocris Bioscience) for 30 min to block NMDA receptor activity, then 5 µM KA. After 1h, KA was washed out and slices returned to fresh SFM. Slices were lysed for Western analysis 2, 8 and 24h following their return to SFM. Some slices were fixed in 4% PFA at room temperature, followed by preparation for immunofluorescent staining as described for brain sections. Control slices were prepared similarly, but treated with vehicle instead of kainic acid. Additional controls included treating slices with CNQX (10 µM; Tocris Bioscience) to block AMPA/kainite receptor activity.


Propidium iodide (PI) nucleic acid stain (2.5 µl/ml) was added to SFM to label cells with compromised membrane structure, an indication of cell death. The amount of cell death was measured as the difference between PI fluorescence intensity at each timepoint, subtracted from the intensity of the slice after saturation with KA for 24h (max intensity). Fluorescence intensity was measured using Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA). For all samples at all timepoints, images were taken of the whole
hippocampus at equal magnification, exposure times and camera parameters (Hammamatsu Orca).

9. Co-immunoprecipitation

Protease inhibitor tablets (Roche Diagnostics, Indianapolis, IN) were added to slice culture media. Media was pre-cleared for 1h using Protein A/G magnetic beads (New England Biolabs), then immunoprecipitated for 2h with anti-BDNF (N-20) (Santa Cruz Biotechnology), anti-NT-3 (gift from David Kaplan), or anti-TIMP1 (Abcam) at 4°C. 25µl of magnetic beads were added to media and incubated 1h at 4°C. The supernatant was removed, and the beads were rinsed three times with TNE buffer (10 mM Tris; 0.2 M NaCl; 1 mM EDTA; pH 7.4). The beads were incubated for 5 min at 70°C in 3x SDS sample loading buffer (187.5 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 150 mM DTT, 0.03% (w/v) bromophenol blue, 2% β-mercaptoethanol), then the proteins were subjected to electrophoresis using SDS polyacrylamide gels, followed by immunoblot analysis.

10. Western Blot Analysis

Hippocampal tissue or slice tissue was lysed in RIPA buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P40, 0.5% deoxycholic acid and 0.5% SDS) supplemented with a protease inhibitor mixture (Roche). Protease inhibitors were also added to slice culture medium and CSF. Medium was concentrated in Pierce Protein Concentrators, 9K MWCO (Thermo Scientific) to 30% of its original volume. Total protein was quantified by the
Bradford assay (Bio-Rad, Hercules, CA). Twenty µg of protein from lysates and culture medium were subjected to Western blot analysis and probed with antibodies to MMP7 (Abcam; 1:500), TIMP1 (Abcam; 1:500), p75NTR (Upstate; 1:1000), cleaved caspase 3 (Cell Signaling; 1:1000), NGF (Sigma; 1:1000), anti-proBDNF (gift from Dr. Carlos Ibanez) and anti-NT-3 (gifts from Dr. David Kaplan; 1:1000). Positive controls utilized were NGF (gift from Genentech), BDNF (gift from Dr. Carlos Ibanez) and NT-3 (Genentech). To ensure equal protein loading, blots were stained with Ponceau and stripped/reprobed for actin. All Western blot analyses were performed at least three times with samples from independent experiments.

11. Statistical Analysis

All data were expressed as mean ±SEM from three or more independent experiments. Student’s t-test was used to analyze two comparison groups. One-way analysis of variance (ANOVA) followed by Tukey post-hoc test were used to analysis of three or more groups. For all statistical analyses, a value of p<0.05 was considered significant.
VIII. Results

Overview

Previous work has identified increased $p75^{NTR}$-mediated cell death following various forms of seizure in vivo (Roux et al., 1999; Troy et al., 2002; Yi et al., 2003; Volosin et al., 2008). However, the mechanisms of $p75^{NTR}$ activation following this type of injury-induced cell death remain unclear. Using hippocampal slice cultures to assess the role of $p75^{NTR}$ in kainic acid-induced cell death, activation of $p75^{NTR}$ by proNGF was found to be necessary for kainic acid-induced cell death, independent of NMDA-mediated glutamate toxicity (Chapter 1). As the upregulation of extracellular proNGF was observed to be a part of the injury response, the processing of proNGF to NGF represented a critical step in determining the extent of cell death after injury. The expression and activity of the proNGF processing enzyme MMP7 was found to inversely correspond with the expression levels of proNGF, indicating that the reduction of proNGF processing by MMP7 is associated with increased proNGF levels after injury. Furthermore, restoring MMP7 activity rescued hippocampal cells from kainic acid-induced death, both in vitro and in vivo, demonstrating a crucial connection between the processing of proNGF by MMP7 and cell survival (Chapter 2). The effects of the tPA-plasmin cascade were also examined with regard to their effects on proNGF processing after injury. Like MMP7, tPA activity decreased following kainic acid treatment, and expression of its inhibitors, PAI-1 and neuroserpin, increased. In vitro, exogenous tPA completely prevented kainic acid-induced cell death in hippocampal slices. However, exogenous application of tPA following seizure in
vivo did not result in as dramatic a rescue from cell death. tPA infusion into the hippocampus following kainic acid treatment resulted in reduced cell death in the CA1, but not the dentate. Thus, tPA-induced proNGF processing is region-specific and ultimately influenced by the prevalence of its inhibitor (Chapter 3). These findings are consistent with the idea that enhanced proNGF levels are due in part to a reduction in the availability of the enzymes that cleave it. Together, the two proNGF processing enzymes examined here demonstrated similar, but not identical effects on cell viability after seizure.

Chapter 1. Mechanisms of p75<sup>NTR</sup>-mediated cell death in the hippocampus following kainic acid-induced injury

1.1 Kainic acid treatment induces p75<sup>NTR</sup> mediated cell death in organotypic hippocampal slice cultures

To examine the mechanisms by which hippocampal neurons undergo cell death following kainic acid-induced injury, an organotypic slice culture preparation was employed. Cultured hippocampal slices were pretreated with MK-801 to block NMDA-mediated toxicity, then treated with vehicle or 5 µM kainic acid, a glutamate agonist that can be used both <i>in vivo</i> and <i>in vitro</i> to stimulate injury, for one hour and monitored 24 hours later for cell death by measuring propidium iodide (PI) uptake. KA treatment induced cell death, especially throughout the CA1, dentate gyrus and hilus, as demonstrated by PI uptake that was absent in vehicle-treated slices (figure 3a). Kainic acid treatment
also induced \( p75^{\text{NTR}} \) expression in the same areas, particularly in the CA1 (figure 3b).

To further confirm whether kainic acid treatment caused \( p75^{\text{NTR}} \) induced cell death in hippocampal slices, cultured tissue was lysed and analyzed by Western blot for \( p75^{\text{NTR}} \) and cleaved caspase-3, a downstream effector of \( p75^{\text{NTR}} \)-mediated apoptosis (Troy et al., 2002). \( p75^{\text{NTR}} \) expression levels as well as caspase-3 activation were increased two-fold by 8h following KA treatment, and this level was sustained through 24h (figure 4a). To ascertain whether the increased cell death was due to \( p75^{\text{NTR}} \)-mediated signaling, a blocking antibody to \( p75^{\text{NTR}} \) was applied to hippocampal slices prior to KA treatment. Cleaved caspase-3 (cc3) was not significantly different from control in anti-\( p75 \)-treated cultures (figure 4b), suggesting that kainic acid-induced death in hippocampal slice cultures is \( p75^{\text{NTR}} \)-dependent.

1.2 proNGF increases after kainic acid treatment and causes cell death via \( p75^{\text{NTR}} \)

Numerous reports have observed seizure-induced upregulation of NGF mRNA in the hippocampus (Gall and Isackson, 1989; Rocamora et al., 1992; Morimoto et al., 1998; Katoh-Semba et al., 1999). However, these expression studies did not indicate which form of the protein was secreted, or whether proNGF directly functioned as a death-inducing ligand for \( p75^{\text{NTR}} \) after seizure. To assess whether proNGF might induce cell death following KA treatment, hippocampal slices from KA-treated cultures were fixed and immunostained with
an antibody to the pro-region of proNGF. ProNGF immunodetection was upregulated after kainic acid treatment, particularly in the CA1 and dentate, areas that also demonstrated high levels of PI-positive cells (figure 5a-b). Previous studies have demonstrated that proNGF is induced and secreted following corticospinal axotomy (Harrington et al., 2004) and pilocarpine-induced seizure (Volosin et al., 2008). To determine if proNGF is also released from cells in hippocampal slices, tissue lysates and medium from kainic acid-treated slice cultures were subjected to Western analysis and probed with an antibody to NGF in order to detect both the mature and pro-forms. ProNGF was detected in both the tissue and culture medium, reaching a 1.5-fold increase by 8h that was maintained through 24h, indicating that proNGF is produced and secreted from hippocampal cells following kainic acid treatment (figure 5c-d). Mature NGF was detected only in the tissue, but not the media, indicating that following KA-treatment, proNGF is the preferentially secreted form of the protein.

In other injury systems, proBDNF and proNT-3 have also been shown to act as death-inducing ligands via p75NTR (Teng et al., 2005; Koshimizu et al., 2009; Tauris et al., 2011). To ascertain that the increased cell death observed in KA-treated slice cultures was due to proNGF activity, a proNGF blocking antibody was applied to culture media prior to kainic acid treatment. Slices treated with anti-proNGF together with KA demonstrated a 60% decrease in PI uptake, compared to cultures treated with KA alone (figure 6a-b). Western analysis also demonstrated that anti-proNGF treatment lead a reduction of caspase-3 activation to levels comparable to control (figure 6c-d). To determine
whether proBDNF or proNT-3 is secreted from the hippocampus following KA treatment, slice culture media was analyzed for the presence of either proneurotrophin. A conventional Western analysis was unable to detect BDNF or NT-3 in culture media (data not shown). To enhance neurotrophin detection, media was immunoprecipitated with antibodies to BDNF and NT-3, followed by Western blot. Bands were detected at ~30 kDa, which correlate to proBDNF and proNT-3 (Lee et al., 2001b; Matsumoto et al., 2008), but demonstrated no regulated changes following KA treatment (figure 7). Interestingly, the mature form of either neurotrophin was not detected, possibly due to the limitations of detection. Taken together, these results suggest that, in response to KA treatment, proNGF is the primary proneurotrophin to be secreted and act as a death-inducing ligand for p75NTR in hippocampal slice culture.

1.3 Kainic acid induces proNGF and p75-mediated apoptosis in the hippocampus in vivo

We previously demonstrated that pilocarpine-induced seizures increased proNGF expression and p75NTR-mediated apoptosis in the hippocampus (Volosin et al., 2008). To determine if seizures induced by KA also elicited these effects, adult rats were injected IP with KA (10 mg/kg) to evoke status epilepticus. Twenty-four hours following treatment, KA-treated rats showed increased p75NTR staining that colocalized with cleaved caspase 3, suggesting that KA-induced seizures stimulate p75-mediated apoptosis, similar to pilocarpine-induced seizures (figure 8a). In accordance with previous findings (Volosin et al., 2008),
proNGF levels also increased (figure 8b). Taken together, these data suggest that, following seizure, proNGF is upregulated and stimulates apoptotic signaling via p75NTR.

Chapter 2. Expression and activity of MMP7 and TIMP1 in the hippocampus after kainic acid-induced injury

2.1 MMP7 and TIMP1 expression and activity following kainic acid treatment *in vitro*

Increased levels of proNGF have been detected in the CSF after injury (Harrington et al., 2004; Volosin et al., 2006). To determine the mechanisms that stabilize proNGF and prevent its cleavage to NGF, we examined the expression and release of MMP7, an enzyme known to cleave proNGF, and its inhibitor TIMP1, in hippocampal slice cultures.

To assess whether MMP7 and TIMP1 expression patterns were affected in a manner that could facilitate increased proNGF and cell death following KA treatment in slice culture, PI-labeled, KA-treated slices were fixed and immunostained with antibodies to MMP7 or TIMP1. MMP7 expression was observed throughout the hippocampus in normal conditions, but demonstrated a global decrease 24h following kainic acid treatment (figure 9a). Additionally, TIMP1 levels increased following kainic acid treatment, and this expression colocalized with PI-positive cells (figure 9b).

Western analysis of culture media was also performed to measure the amount of protein released from cells following KA treatment. Twenty-four hours
after KA treatment, extracellular TIMP1 and proMMP7 levels were both detected, and mature MMP7 was undetected (figure 10a). To determine whether secreted TIMP1 interacted with pro- or mature MMP7, co-immunoprecipitation of culture media was performed. A pull-down for TIMP1 and western analysis for MMP7 revealed increased proMMP7 bound to TIMP1 through 24h. Western analysis of the supernatant was unable to detect either form of MMP7, suggesting that although proMMP7 was released, it was inhibited by TIMP1 binding (figure 10b). Mature MMP7 was not detected following TIMP1 immunoprecipitation, indicating that TIMP1 bound only to the pro-form and inhibited MMP7 activation. These data suggest that following kainic acid-induced injury, proMMP7 is released from cells, but is bound to TIMP1, reducing the enzyme activity available for neurotrophin processing.

If the endogenous inhibition of MMP7 contributes to increased proNGF and cell death, then enhancing MMP7 activity should manifest opposite effects. To determine if exogenously supplied MMP7 could prevent neuronal loss, hippocampal culture medium was supplemented with 2.5µg MMP7 or the equivalent volume of saline (5µl), 30 minutes prior to kainic acid treatment. After 24h, KA-treated slices in MMP7-supplemented medium demonstrated comparable PI uptake to non-treated controls, indicating that exogenous MMP7 can protect cells from KA-induced death in slice culture (figure 11a). Furthermore, Western analysis of culture medium from MMP7-supplemented slices demonstrated proNGF levels similar to control (figure 11b), suggesting that
exogenously supplied MMP7 increases proNGF cleavage and simultaneously decreases cell death.

2.2 MMP7 and TIMP1 expression and activity following kainic acid treatment in vivo

Post-seizure rats were examined for changes in TIMP1 and MMP7 expression to determine whether these proteins' levels were affected in a manner that could lead to increased proNGF in vivo. TIMP1 was found to be upregulated while MMP7 was downregulated in the hippocampal tissue 24h after seizure (figure 12a), suggesting that inhibition of MMP7 may lead to increased proNGF. Interestingly, TIMP1 and proNGF were found to be tightly colocalized throughout the CA1 following seizure (figure 12b), indicating a relationship that may correspond with cell death after injury (arrows).

To assess whether MMP7 activity, as well as its expression, decreased following seizure, in situ zymography was performed on fresh frozen hippocampal sections taken from adult rats with and without kainic acid treatment. 24h following seizure, MMP7 activity was reduced throughout the hippocampus, particularly in the CA1 and dentate, areas that coincide with seizure-induced cell death (figure 13a). As a negative control, zymograms from each animal were incubated in the presence of EDTA, a metal chelator that inhibits MMP catalytic activity. No activity was detected in the presence of EDTA (figure 13b).
2.3 MMP7 protects hippocampal neurons from death following seizure *in vivo*

To determine whether the seizure-induced reduction of MMP7 activity contributes to neuronal death after seizures, rats were given a direct infusion of MMP7 into one hippocampal hemisphere immediately following seizure treatment. The opposite hemisphere received an equal volume of saline. *In situ* zymography confirmed MMP7 activity on the infused side, which showed almost complete rescue from cell death compared with the vehicle-infused hemisphere, as demonstrated by FluoroJade-B (FJ) labeling (figure 14a-b). Consistent with increased cell death, both p75\textsuperscript{NTR} staining and caspase 3 activation were found throughout the saline-treated hemisphere of the hippocampus. Interestingly, detection of p75\textsuperscript{NTR} and cleaved caspase 3 was nearly abolished in the MMP7-injected hemisphere (figure 15).

To ascertain whether the neuroprotective effect of MMP7 was due to reducing proNGF, hippocampal sections from post-seizure rats were also analyzed for proNGF expression. As with p75\textsuperscript{NTR}, proNGF was only detected in the saline-treated hemisphere of KA-treated rats, and was almost completely eliminated on the MMP7-injected side (figure 16a). To determine whether extracellular proNGF levels were affected, proNGF secretion was measured by Western analysis of the CSF from cannulated, KA-treated rats, with and without MMP7 infusions. ProNGF was not detected in rats that were not subjected to seizures. ProNGF was strongly detected in the CSF of rats that underwent KA-induced seizures, but levels were reduced by 54% when KA-treated rats also
received MMP7 infusions following seizure treatment (figure 16b). Mature NGF was undetected in MMP7-injected rats, compared to both seizure and non-seizure treated rats.

Because MMP7 can also cleave proBDNF to its mature form, CSF from vehicle and KA-treated rats was also analyzed for BDNF expression. Interestingly, mature BDNF, but not proBDNF, was detected in the CSF (figure 17a). However, there was no detectable difference between the level of BDNF in KA or vehicle-treated rats.

proNT-3 also acts as an apoptosis-inducing ligand through p75NTR activation and has recently been shown to be released from cerebellar granular neurons following membrane depolarization (Yano et al., 2009). To determine whether proNT-3 was released following seizure treatment, CSF from KA-treated rats was immunoblotted for pro- and mature NT-3 expression. As with BDNF, only the mature form of NT-3 was detected in CSF samples, and the level of expression between KA and vehicle-treated rats was not significantly different (figure 17b). The size of mature neurotrophins detected was lower than that of the purified positive controls, possibly due to processing at alternate cleavage sites.

Together, these data suggest that proNGF is the primary apoptotic proneurotrophin produced following seizure. Furthermore, providing MMP7 leads to the extracellular proteolysis of proNGF and reduction in cell death, indicating that following seizure, the absence of available MMP7 directly enhances proNGF, creating a cellular environment more vulnerable to apoptosis.
Chapter 3. Expression and activity of the tPA-plasmin cascade following kainic acid-induced injury

3.1 tPA treatment reduces proNGF production following kainic acid treatment \textit{in vitro}

The tPA-plasmin cascade was also analyzed with regard to its role in proNGF production and p75-mediated apoptosis following injury (Lee et al., 2001b; Bruno and Cuello, 2006). Cultured hippocampal slices were pretreated with MK-801 to block NMDA mediated toxicity, then treated with 5 µM kainic acid or vehicle for one hour and monitored for 24 hr and assessed for cell death by measuring propidium iodide (PI) uptake. Some slices received recombinant tPA in culture media 30 min prior to KA treatment. As shown previously, slices treated with kainic acid demonstrated increased cell death compared to vehicle-treated slices, especially throughout the CA1, dentate gyrus and hilus, whereas slices that received additional tPA treatment did not show a significant increase in cell death compared to control (figure 18a). Culture medium was also analyzed to determine whether proNGF release was affected by KA-induced injury and tPA activity. proNGF levels were found to be increased following KA treatment, but were not detected in samples collected from tPA + KA treated slices, indicating that increased tPA activity can reduce extracellular proNGF and protect cells from KA-induced death in slice culture (figure 17b).

tPA activity is endogenously regulated by the serine protease inhibitors neuroserpin and PAI-1. To examine whether neuroserpin or PAI-1 expression were affected in a manner that could influence proNGF levels following KA
treatment, tissue and medium from slice cultures was collected and examined for neuroserpin and PAI-1 expression. Cultured slices demonstrated increased neuroserpin immunodetection that colocalized with PI-positive cells following KA treatment, particularly in the dentate (figure 19a-b). Secreted neuroserpin was found to be increased in the media as early as 2h following KA treatment (figure 19c). Similarly, PAI-1 also increased in hippocampal tissue and culture media, but reached maximum levels by 2h that were sustained through 24h, suggesting different regulatory systems for each inhibitor (figure 20a-b).

Taken together, these data suggest that decreased tPA levels, as well as increased levels of their inhibitors, PAI-1 and neuroserpin, may contribute to decreased proNGF processing and thus, increased proNGF-induced cell death following kainic acid treatment.

3.2 tPA activity decreases and proNGF and cell death increase following seizure in vivo

tPA activity was measured using in situ zymography on fresh frozen hippocampal sections taken from adult rats with and without kainic acid treatment. 24h following seizure, tPA activity was reduced throughout the hippocampus, particularly in the CA1-3 and hilus, areas that coincide with increased proNGF and cell death after seizure (figure 21a). As a negative control, zymograms from each animal were incubated in the presence of aprotinin, a competitive serine protease inhibitor, to prevent tPA activity. No activity was detected in the presence of aprotinin (figure 21b). Sections taken
from the same brains were subjected to immunostaining to examine levels of neuroserpin and PAI-1, endogenous inhibitors of tPA activity. Neuroserpin staining was most strongly detected in the dentate, with very little immunostaining appearing in the CA1 (figure 22a-b). PAI-1 was similarly upregulated after seizure, with additional immunostaining detected in the CA3 (figure 23a-b).

To determine whether the seizure-induced reduction of tPA activity contributes to neuronal death after seizures, rats were given a direct infusion of tPA into one hippocampal hemisphere immediately following seizure treatment. The opposite hemisphere received an equal volume of saline. In situ zymography confirmed increased tPA activity only on the infused side (figure 24a). Additionally, the tPA-injected hemisphere showed an almost complete rescue from cell death in the CA1, but not the dentate, when compared to the saline-injected hemisphere (figure 24b-c), in accordance with increased neuroserpin and PAI-1. Increased p75\textsuperscript{NTR} was detected in both the CA1 and dentate of both hemispheres. The CA1 area of the tPA-injected hemisphere, however, showed an almost complete rescue from cell death compared to the vehicle-injected side, and despite increased p75\textsuperscript{NTR} staining in both hemispheres. Conversely, the hilus did not show a significant difference in cell death compared to control (figure 24c), a finding in accordance with the increase in neuroserpin and PAI-1 staining as shown in figures 22-23. These data suggest that endogenous tPA inhibitor expression is sufficient to inhibit tPA activity, but regional differences lead to differential vulnerability.
To verify whether the neuroprotective effect of tPA was through a reduction of extracellular proNGF, proNGF secretion was measured by Western blot analysis on CSF from cannulated, kainic acid-treated rats, with and without tPA infusions. Increased proNGF was found in the CSF of seizure-injured rats that had not received exogenous tPA, but was reduced in tPA-injected rats (figure 25). These data suggest that decreased tPA activity, along with increased neuroserpin following seizure lead to reduced proNGF processing and thus, more available proNGF, creating a more vulnerable environment susceptible to cell death.
IX. Discussion

Neurotrophins have long been recognized for their crucial roles in growth, differentiation and survival in the developing nervous system. Recent findings demonstrating that the pro-form of NGF induces cell death via the p75NTR suggest that the consequences of neurotrophin signaling depend on various factors, including the ratio of pro- and mature neurotrophins and the regulation of that balance. Thus, understanding neurotrophin processing is an important step towards discerning the pathway that a cell takes towards survival or death.

Proneurotrophin processing by MMP7 after seizure is neuroprotective

This study investigated components of the extracellular environment that can regulate whether the apoptosis-inducing ligand proNGF is processed to its mature form. Once released from a cell, proNGF can be cleaved to mature NGF by its processing enzyme MMP7 (Smith et al., 1995; Lee et al., 2001b). However, after injury, we found that proNGF was not only upregulated in the extracellular environment but MMP7 decreased in both expression and activity. Moreover, TIMP1, which inhibits MMP7, was increased in expression and colocalized with both proNGF and cell death following kainic acid treatment, indicative of a functional association between TIMP1, proNGF and cell death after injury. In slice cultures, release of TIMP1 and proNGF into the media were both dramatically increased following kainic acid treatment, suggesting that proNGF and TIMP1 are not only upregulated in cells, but also released from them as a response to injury. Furthermore, restoring MMP7 by supplementing culture
medium or by direct infusion into the hippocampus resulted in a loss of proNGF in the CSF and an almost complete rescue of hippocampal neurons from cell death, suggesting that changes to the enzyme-inhibitor ratio may directly affect the balance between pro- and mature NGF after injury, contributing to increased cell death. More specifically, an increase of TIMP1 after injury inhibits MMP7 activity, leading to reduced proteolytic processing of proNGF and thus more available ligand for the p75NTR-mediated apoptotic pathway.

It is worth noting that when post-seizure rats were treated with exogenous MMP7, not only did proNGF and dying cells decrease, but p75NTR expression decreased as well. These changes are in accordance with previous studies from our lab showing that blocking proNGF activity in the hippocampus after seizure reduced p75NTR expression (Volosin et al., 2008), suggesting that the presence of proNGF may be necessary for the upregulation of its receptor.

We also observed that when recombinant MMP7 was infused into the hippocampus following seizure, not only did the levels of proNGF decrease, but so did mature NGF. A possible explanation for this lies in the intertwined network of matrix metalloproteases. In inflammatory conditions such as seizure, a number of MMPs are released from cells, including MMP9 (Nico et al., 2009; Girgenti et al., 2010), an enzyme linked to the degradation of mature NGF (Bruno and Cuello, 2006; Cuello and Bruno, 2007). However, MMP9 is released as a pro-enzyme, requiring further processing for activation; MMP7 is one of the many enzymes capable of cleaving the prodomain from MMP9 (Imai et al., 1995). The influx of recombinant MMP7 may have also activated endogenous proMMP9,
increasing the degradation of mature NGF in the extracellular space, resulting in lower levels of detection.

Because proNGF and mature NGF have differential effects on neuronal survival, and because proNGF levels increase in parallel with neuronal apoptosis following various forms of injury or pathological conditions, the processing of pro- to mature NGF is critical in determining neuronal fate. Our findings on the MMP7-proNGF relationship following excitotoxic injury demonstrate that the enzymatic conversion of proNGF to NGF by MMP7 is important for cell survival and suggest a novel mechanism by which MMP7 may function in the injury process. Changes in MMP7 expression have been detected in an animal model of multiple sclerosis (Buhler et al., 2009) and in the brains of Alzheimer’s patients (Ethell et al., 2002), and the use of MMP inhibitors has been proposed as a treatment for diseases including Alzheimer’s (Backstrom et al., 1996) and ALS (Lorenzl et al., 2003). Elevated proneurotrophin levels have also been found during these conditions, suggesting the MMP7-proNGF relationship as a possible therapeutic target for disease or injury. But the precise roles of MMP7 and TIMP1 in these and other disorders have not been elucidated. If alterations to the MMP7/TIMP1 balance contribute to the increased proNGF and improper trophic support seen in Alzheimer’s and other neurodegenerative disorders, it will be crucial to continue examining the influence of enzymatic regulation and the potential outcomes of altering enzyme activity on neurotrophin cleavage and the consequences for neuronal survival or death.
Proneurotrophin processing by plasmin

MMP7 is not the only enzyme involved in the extracellular proteolysis of proNGF. This study also examined the role of the tPA-plasmin cascade in the regulation of proNGF processing after injury. tPA is a serine protease that catalyzes the conversion of the inactive zymogen plasminogen to plasmin. The active enzyme plasmin can then cleave proNGF to its mature form (Lee et al., 2001b; Bruno and Cuello, 2006; Larsson et al., 2009). Plasminogen, tPA, and its inhibitor, neuroserpin, are released from neurons in conjunction with proNGF upon stimulation, indicating a relationship between the activation of plasmin, the subsequent cleavage of proNGF, and a closely associated inhibitory loop regulated by neuroserpin (Bruno and Cuello, 2006). Additionally, an increase in p75\textsuperscript{NTR} expression following sciatic nerve injury has been shown to suppress tPA activity by upregulating another tPA inhibitor, PAI-1 (Sachs et al., 2007). Taken together, these studies suggest that a reduction in tPA-plasmin activity following injury leads to decreased proneurotrophin processing and subsequently, increased p75\textsuperscript{NTR} expression, creating an environment more susceptible to cell death.

The work in this thesis found that tPA activity was downregulated following seizures, particularly in areas that coincided with cell death. The expression of neuroserpin and PAI-1 following injury also corresponded with dying cells, both \textit{in vitro} and \textit{in vivo}. However, restoring tPA activity by direct infusion following seizure did not result in a complete rescue of cell death as seen with MMP7. Cells in the hilus of kainic acid-treated rats demonstrated no difference in the
amount of cell death between the hemisphere injected with tPA or vehicle. CA1 area cells, however, demonstrated a dramatic decrease in cell death compared with vehicle-injected hippocampi. Neuroserpin expression was found to be strongly increased throughout the hilus, but not the CA1, following seizure, supporting the finding that only CA1 cells demonstrated increased survival following tPA infusion. PAI-1 also increased following seizure in the dentate but not the CA1, suggesting neuroserpin and PAI-1 play similar roles in mediating plasminogen activation (Siconolfi and Seeds, 2001; Fabbro and Seeds, 2009). Furthermore, proNGF levels were also sustained in the hilus, but not detected in the CA1, after tPA treatment. Taken together, these results indicate that differential expression of the enzymes and their inhibitors lead to differential vulnerability of specific populations of neurons after injury, and that restored tPA/plasmin activity could rescue cells from apoptosis by decreasing proNGF. However, the protective effect of proNGF processing by the tPA-plasmin cascade is restricted to regions expressing lower levels of inhibitor.

Similar to our findings, pharmacological upregulation of tPA activity has also demonstrated beneficial effects following peripheral nerve injury (Zou et al., 2006), and endogenous tPA following ischemic insult protects hippocampal neurons from cell death (Echeverry et al., 2010). Furthermore, the progression of cell death in mild cognitive impairment and Alzheimer’s disease has been connected to downregulated proNGF processing by plasmin and subsequently increased p75_{NTR}-mediated cell death (Cuello and Bruno, 2007; Fabbro and Seeds, 2009).
Based on established time courses from our lab and others (Roux et al., 1999; Troy et al., 2002; Yi et al., 2003; Volosin et al., 2006), post-KA treated rats were examined 24 hours after seizure, corresponding with elevated levels of proNGF, p75NTR expression and cell death. Similar to other groups, we found that 24 hours after KA-induced seizure, tPA activity falls to nearly undetectable levels, an effect complemented by the simultaneous upregulation of endogenous PAI-1 and neuroserpin (Salles and Strickland, 2002; Yepes et al., 2002; Siao et al., 2003). More interesting, the cell death observed at this time point can be rescued by application of exogenous tPA, an effect also observed by others (Kim et al., 1999). Additionally, the activity timecourse of tPA is biphasic: tPA protein expression sharply rises one hour following seizure induction, but falls to basal levels within 8 hours (Salles and Strickland, 2002), in accordance with when we were first able to detect significantly increased extracellular proNGF (figure 3) and possibly accounting for lower levels of detected extracellular proNGF from 2-6 hours following seizure induction. Although exogenous tPA resulted in significantly reduced neuronal death, both in vitro and in vivo, we cannot eliminate the possibility that supplying tPA may have only delayed cell death. Examination of proNGF, p75NTR and cell death at later time points will be necessary to elucidate the role of tPA in neuroprotection after seizure. Taken together, this suggests a complex deregulation of the plasminogen system following pathological conditions that can result in, among other events, a reduction in proneurotrophin processing and subsequent cell death.
It is interesting to note that tPA injection also resulted in reduced mature NGF detected in the CSF. As mentioned previously, proMMP9 is known to be upregulated following various forms of stimulation and injury (Nico et al., 2009; Girgenti et al., 2010). In addition, plasmin is a known activator of MMP9 (Baramova et al., 1997). Thus, the influx of recombinant tPA may activate plasmin, which could in turn, activate MMP9 to facilitate the degradation of NGF. This system illustrates the intricacy of extracellular matrix enzyme systems and emphasizes the complexity in elucidating the consequences of altering any step of the enzyme process.

*The roles of proneurotrophin processing extend beyond regulating apoptosis*

The basic tenet of the neurotrophic hypothesis states that, during development, neurons that receive sufficient trophic support from target tissue will form functional synapses with those targets, and those that do not will eventually undergo programmed cell death. Thus, cells must receive a threshold level of trophic support to counteract their innate apoptotic signaling. With the discovery of proneurotrophin functionality, the neurotrophic hypothesis has gained another dimension, one in which proneurotrophins bind to p75NTR to initiate an active programmed cell death mechanism, both during development and in response to pathological conditions (Frade et al., 1996; Majdan et al., 1997; Bamji et al., 1998).
However, the functional significance of proneurotrophin signaling extends beyond a simple decision between life and death. BDNF and the tPA axis are known to play important roles in synaptic modulation, and TrkB stimulation by BDNF is essential for various forms of synaptic plasticity (Seeds et al., 1995; Xu et al., 2000). Moreover, because proBDNF is released from cells in a regulated manner, the proteolysis of proBDNF to mature BDNF represents an important step in proper synaptic transmission and in fact, extracellular proBDNF conversion by plasmin is required for LTP (Pang et al., 2004). These data suggest that the regulation of neurotrophin processing directly influences the synaptic efficacy of proneurotrophins. However, a role for proNGF in synaptic function remains to be determined.

Currently, it is unclear how $\text{p75}^{\text{NTR}}$ can distinguish between proNGF and proBDNF to execute distinct downstream events, but it appears as though proNGF binding favors apoptotic signaling and proBDNF stimulates processes related to synaptic transmission. Along with apoptosis and synaptic communication, $\text{p75}^{\text{NTR}}$ signaling can also function in mediating neuronal outgrowth. $\text{p75}^{\text{NTR}}$ has been shown to bind RhoA to negatively modulate neurite expansion, which is reversed by mature neurotrophin binding to $\text{p75}^{\text{NTR}}$, resulting in RhoA dissociation and subsequent neuronal growth (Yamashita et al., 1999). Overexpression of $\text{p75}^{\text{NTR}}$ leads to stunted dendritic arborization and reduced spine density in the adult mouse, and blocking $\text{p75}^{\text{NTR}}$ signaling manifests the opposite results (Zagrebelsky et al., 2005). Additionally, $\text{p75}^{\text{NTR}}$–mediated axonal retraction can be overcome with concurrent TrkA activation by NGF (Singh and
Miller, 2005). Although these studies did not address whether p75NTR requires proneurotrophin binding to execute these effects, proBDNF has been shown to be a critical ligand for inducing presynaptic terminal retraction (Yang et al., 2009a). Moreover, p75NTR is not limited to neurons, and its activation on other cell types facilitates a variety of functions that do not affect apoptosis, including enhancing Schwann cell myelination (Cosgaya et al., 2002) or regulating glial cell activation in response to injury (Gschwendtner et al., 2003). Indeed, NGF activation of p75NTR on astrocytes displayed no effect on cell death, but rather attenuated mitogen-induced proliferation (Cragnolini et al., 2009). Taken together, these data suggest that neurotrophin signaling through p75NTR functions in shaping several aspects of the cellular network, that these functions are highly dependent upon cellular context, and that death is not the only potential consequence of p75NTR signaling.
X. Conclusion and Future Directions

The work in this thesis was designed to clarify some of the circumstances that affect the stimulation of p75\textsuperscript{NTR}-mediated apoptosis under pathological conditions. Neurotrophins have long been targeted for their possible therapeutic properties in numerous disorders including multiple sclerosis (Caggiula et al., 2006), spinal cord trauma (Ye and Houle, 1997), and Alzheimer’s disease (Lad et al., 2003). ProNGF in particular has been found to accumulate in the brains of Alzheimer’s patients (Fahnestock et al., 2004; Peng et al., 2004; Pedraza et al., 2005), while levels of the mature NGF receptor, TrkA, diminish and p75\textsuperscript{NTR} levels remain (Ginsberg et al., 2006). The regulation of proNGF processing in the progression of this and other nervous system disorders awaits further investigation.

Although the role of proNGF in mediating cell death following injury has been well-characterized, we cannot rule out the involvement of other neurotrophins as well, and future studies would examine the regulation of proBDNF and proNT-3 processing, elucidating their roles in cellular pathology and the injury response. To date, the majority of proneurotrophin research has focused on the role of proNGF in pathology and proBDNF in physiology. Indeed, the experiments in this thesis were unable to detect regulated release of proBDNF \textit{in vitro} or \textit{in vivo} in response to injury. These results were surprising because BDNF mRNA has been shown to be dramatically upregulated following seizure activity (Gall and Isackson, 1989; Ernfors et al., 1991; Kokaia et al., 1996). Possible explanations for these discrepancies include the time points at
which protein levels were examined, increased breakdown of proBDNF and/or BDNF by various proteases, or perhaps that changes in BDNF regulation were too small to detect using Western analysis. Furthermore, although proBDNF has been shown to be an apoptotic ligand in an overexpression study (Teng et al., 2005), examination in physiologically relevant conditions revealed that proBDNF is released following low and high frequency stimulation to induce LTD and LTP, respectively (Nagappan et al., 2009). More specifically, low frequency stimulation resulted in proBDNF secretion and LTD, whereas high frequency stimulated release of proBDNF and the components of the tPA axis, resulting in plasmin-dependent pro- to mature BDNF conversion and LTP (Pang et al., 2004; Nagappan et al., 2009). These results, as well as our findings that proBDNF release was not detected in the CSF following seizure, suggest that it is unlikely that MMP7 acts upon proBDNF in vivo to mitigate apoptotic signaling. This hypothesis is supported by previous work that found BDNF and NGF to be differentially regulated following certain types of nerve injury (Matsuoka et al., 1991; Meyer et al., 1992; Harrington et al., 2004). Future experiments will examine whether endogenous proBDNF plays a role in post-injury and disease pathology.

ProNGF contains three possible sites of cleavage: a site recognized by plasmin and furin that releases a 13.5 kDa product, and a site on each side of that site, both recognized by MMP7, that releases a smaller or larger product, depending on the target (Lee et al., 2001b). Interestingly, Western analysis of CSF taken from sham-treated rats often detected smaller forms of NGF,
suggesting that the majority of secreted NGF in vivo is the pro-form (Reinshagen et al., 2000; Fahnestock et al., 2001; Hasan et al., 2003; Bierl et al., 2005), which is extracellularly processed by MMP7. The experiments in this thesis examined the extracellular processing of proNGF, as the pro-form is reported to be the predominant isoform in sympathetic neurons (Bierl et al., 2005), cortical cells (Fahnestock et al., 2001), dorsal root ganglion neurons (Reinshagen et al., 2000), and other neuronal and non-neuronal cell populations (Dicou et al., 1986; Yamamoto et al., 1992; Skaper et al., 2001) under basal conditions. However, it is important to note that proNGF may also be processed intracellularly by furin and other proconvertases (Seidah et al., 1996). Thus, the cleavage of proNGF by intracellular proconvertases also reflects a step in proNGF processing that may affect the final form of the protein available for signaling. Examination of any changes to the intracellular biosynthesis of NGF after injury awaits further investigation.

To date, the cells responsible for the synthesis and secretion of proneurotrophins have not been ascertained. Under different physiological and pathophysiological conditions, studies from our lab and others have observed an upregulation of higher neurotrophin isoforms in glia as well as neurons (Yune et al., 2007; Volosin et al., 2008; Yano et al., 2009; Marler et al., 2010). Moreover, the intracellular processing system in glia is not as active as in neurons (Mowla et al., 1999), suggesting that glial cells may be a rich source of secreted proneurotrophins. Further investigation would determine which form of the
protein is released under basal conditions, which cells make and secrete proNGF, and which enzymes are most actively involved in its processing.

The data presented in this thesis strongly support the idea that the regulation of proneurotrophin processing activity is an important means of mediating cellular responses to injury. Exogenous application of MMP7 and tPA were shown to alter the amount of proNGF processing, illustrating two separate but overlapping mechanisms involved in regulating NGF activation of p75NTR. The existence of redundant systems represents a complex, buffered network that functions to perpetuate efficient synaptic communication. Thus, a downregulation of proneurotrophin processing following injury may function as part of a complex system to stimulate the retraction and pruning of injured cells, as well as to rewire the parts of the cellular network that remain, via increased p75NTR signaling.
Figure 1

Lee et al., 2001
Figure 1. Schematic illustrating putative, highly conserved proteolytic cleavage sites in proneurotrophins for serine proteinases and MMPs. Note that although a processing site for MMP3 is indicated, only BDNF has been shown to cleaveable by this enzyme. “A” and “B” designate previously characterized cleavage sites by unknown proteases.

Adapted from Lee et al., 2001
Figure 2

Nykjaer et al., 2004
Figure 2. Schematic model of receptor-complex formation. Sortilin is a necessary co-receptor for p75$^{NTR}$ to create a high-affinity binding site for proNGF and to signal cell death. Regulated activity of extracellular proteases may cleave proNGF to mature NGF to instead promote trk-mediated survival signaling.

Adapted from Nykjaer et al., 2004
Figure 3

A

Pretreatment

Vehicle

24h KA

24h

B

Vehicle

24h KA

p75 PI merge
**Figure 3.** Organotypic hippocampal slice cultures show increased cell death in the presence of kainic acid (KA). **A)** Slices for p7 rat pups were cultured for 7 days in serum with 50% media changes every 3 days. On day 7, slices were placed into serum free media (SFM) containing 2.5 µl/ml propidium iodide (PI). 24h later, pretreatment photos of slices were taken (left column). Slices were treated 24h later with 10µl MK-801 and 1.5 µl vehicle or 5 µM KA. After 1h, slices were placed into fresh SFM. Photos were again taken 24h following return to SFM. Only slices that received KA treatment demonstrated propidium iodide (PI) uptake 24h after kainic acid treatment, particularly in the CA1. **B)** Magnification of CA1 showing p75NTR immunodetection and PI staining only after kainic acid treatment. Note the colocalization between p75NTR and PI positive staining, indicating dying cells are also positive for p75NTR. Photos are representative staining from 5 independent experiments. Scale bar = 25 µm.
Figure 4

A

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- 75 kDa
- 18 kDa

B

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- 18 kDa

Relative intensity

C  KA  ap75  ap75 + KA

* denotes significant difference.
**Figure 4.** Cell death following kainic acid treatment is p75<sup>NTR</sup> -dependent. (A) Following KA treatment, slice culture tissue was harvested at 2, 4, 6, 8 and 24h timepoints, lysed in buffer containing protease inhibitors and subjected to Western analysis to examine levels of p75<sup>NTR</sup> and cleaved caspase-3. By 8h post-KA treatment, levels of both p75<sup>NTR</sup> and activated caspase had doubled compared to basal levels. (B) Slice cultures treated with a blocking antibody to p75<sup>NTR</sup> in conjunction with KA treatment show levels of caspase-3 activation comparable to control, indicating p75<sup>NTR</sup> is involved in KA-induced caspase activation. Each graph represents the normalized densitometric index of immunoblots from 3 independent experiments. Error bars represent SEM. Data were analysed using ANOVA, followed by Tukey post-hoc test. *P<0.05 relative to control.
Figure 5

A

B

C

D

proNGF

PI

merge

Veh

KA

proNGF

NGF

Culture Tissue

Relative Densitometric Units

Culture Media

Relative Densitometric Units

0

0.5

1

1.5

2

2.5

3

3.5

0

0.5

1

1.5

2

2.5

3
Figure 5. proNGF is upregulated and released after kainic acid treatment in slice culture. (A) proNGF is undetected in CA1 area cells under control conditions (top row). In response to KA treatment, proNGF is highly upregulated throughout the CA1, near areas of PI-positive staining. Western analysis (B) and quantification (C) of tissue from slice cultures detects very low levels of proNGF in control conditions. However, KA induces a 1.5-fold increase in proNGF detection by 8h following treatment. Mature NGF is strongly detected in both control and treated conditions, but does not manifest significant differences in expression levels in response to KA. (D) In culture medium, proNGF is found in control conditions, but is nearly doubled by 8h following KA stimulation, reaching 2.5-fold increase by 24h. Extracellular mature NGF was not detected. Each graph represents the normalized densitometric index of immunoblots from 3 independent experiments. Error bars represent SEM. Data were analyzed using ANOVA, followed by Tukey post-hoc test. *P<0.05 relative to control.
Figure 6

A

Veh
KA
KA+aproNGF

B

Relative Fluorescence Intensity

Veh  KA  KA + anti-proNGF

C

cc3  actin

Veh  KA  KA + aproNGF  aproNGF

D

Relative Densitometric Units

veh  KA  KA + anti-proNGF  proNGF
Figure 6. Blocking proNGF activity results in reduced cell death and caspase 3 activation. (A) Slices treated with KA demonstrate a 68% increase in PI uptake as measured by fluorescence intensity of the whole slice. Slices treated with a blocking antibody to proNGF 30 min prior to KA demonstrated decreased cell death that was insignificant from control. Results are the normalized fluorescence index of slice images from 3 independent experiments. (B) Quantification of (A). Error bars represent SEM. Data were analyzed using one-way ANOVA, followed by Tukey post-hoc test. *$P<0.01$ relative to control. (C) Western analysis of slice culture tissue demonstrating that blocked proNGF activity results in decreased KA-induced caspase 3 activation, comparable to control. Activated caspase 3 was 67% higher than control levels. (D) Quantification of (C). Bars represent SEM. Data were analyzed using one-way ANOVA, followed by Tukey post-hoc test. *$P<0.01$ relative to control. Images and blots are each representative of 3 independent experiments.
Figure 7

A

C 2 8 24

- 25 -

kDa

- 15 -

IP: BDNF mAb
IB: BDNF pAb

C 2 8 24

IP: NT-3 pAb
IB: NT-3 pAb

B

C 2h 8h 24h

Relative Densitometric Units

0 0.2 0.4 0.6 0.8 1 1.2 1.4

c 2h 8h 24h

Relative Densitometric Units

C 2h 8h 24h

Relative Densitometric Units

0 0.2 0.4 0.6 0.8 1 1.2 1.4

c 2h 8h 24h
**Figure 7.** Secretion of proBDNF and proNT-3 is not regulated following kainic acid treatment *in vitro*. Western analysis was unable to detect BDNF or NT-3 from hippocampal slice culture media (data not shown). To enhance signal detection, media was immunoprecipitated and immunoblotted with antibodies to BDNF (left) and NT-3 (right) as indicated. The pro-forms of each protein was detected, but no significant difference was detected at any timepoint. Note the mature forms were not detected even following immunoprecipitation. N=3, p>0.05. Graphs represent the normalized densitometric index of blots from 3 independent experiments. Error bars represent SEM. Data were analyzed using one way ANOVA, followed by Tukey post-hoc test.
Figure 8. *In vivo* expression of p75NTR and cleaved caspase 3 increase following kainic acid-induced seizures. Male Sprague Dawley rats (250–275 g) were injected with kainic acid (KA) (10 mg/kg, i.p.) for induction of status epilepticus (SE). After 1 hour of SE behavior, rats were treated with diazepam and phenytoin to stop seizure activity. Control rats received the same treatments but were injected with saline instead of KA. Twenty-four hours after stopping seizure activity, the rats were anesthetized and brains prepared for histochemistry. (A) Hippocampal sections were co-immunolabeled with anti-p75NTR (green) and anti-cleaved caspase 3 (cc3, red). Arrows indicate colocalization of p75NTR and cleaved caspase-3 in response to KA-induced seizure. (B) Histochemical analysis for expression of proNGF shows proNGF induction 24h after KA-induced seizure. Arrows indicate colocalization of proNGF with p75NTR. All images are taken from the CA1 area of the hippocampus and are representative images from 5 independent experiments. Scale bars = 50um.
Figure 9

A

MMP7

PI

merge

Ctrl

24h KA

B

TIMP1

PI

merge

Ctrl

24h KA
**Figure 9.** Expression of MMP7 and TIMP1 in the CA1 following KA treatment *in vitro*. Following 7 days in culture, slices were placed into fresh serum-free medium (SFM) containing 2.5 µl/ml propidium iodide (PI). The next day, slices were treated with 5µm KA for 1 hour, then placed back into SFM. Twenty-four hours following treatment, slices were fixed in 4% PFA, rinsed in PBS, and subjected to immunolabeling with anti-MMP7 or anti-TIMP1. *(A)* MMP7 expression is found throughout cell bodies and processes in the hippocampus under basal conditions, but is barely detectable in tissue 24h after KA treatment. *(B)* TIMP1 expression is detected at relatively low levels under basal conditions, but is strongly upregulated throughout hippocampal tissue in response to KA treatment. Photos are from CA1 and are representative of 3 independent experiments. Scale bar = 50µm.
Figure 10

A

B

IP: TIMP1
IB: MMP7, TIMP1
Figure 10. MMP7 and TIMP1 secretion following KA treatment. (A) Western analysis of culture medium shows increased secretion of TIMP1 and proMMP7 2-24h following KA treatment. Note mature MMP7 was undetected. (B) Medium from slice cultures was co-immunoprecipitated for TIMP1 and immunoblotted for MMP7 and TIMP1. Extracellular proMMP7 was detected in TIMP1 precipitate, but not in the supernatant, and mature MMP7 was not detected in either sample set, indicating that secreted proMMP7 is bound to TIMP1. All experiments were repeated three times. Images are representative from 4 independent experiments.
**Figure 11.** Exogenously supplied MMP7 reduces the presence of extracellular proNGF and cell death in slice culture. (A) After 7 days in culture, hippocampal slice cultures were treated with KA or with 1 µg/ml active MMP7 in conjunction with KA. Slices that received KA treatment alone demonstrated 70% increase in PI uptake as calculated by fluorescence intensity. Slices that received exogenous MMP7 demonstrated significant rescue from cell death, as depicted by diminished PI uptake, comparable to control levels. Graph represents are the normalized fluorescence index of slice images from 3 independent experiments. Error bars represent SEM. Data were analyzed using one-way ANOVA, followed by Tukey post-hoc test. *P<0.05 relative to control. (B) Western analysis of culture media from slices treated with vehicle, KA, or KA+MMP7 and probed with anti-proNGF to determine the effect of exogenous MMP7 on extracellular proNGF levels. ProNGF was nearly abolished in medium from slices that received 1 µg/ml active MMP7 in conjunction with KA compared with those that received KA alone. Images and blot are representative of three independent experiments.
Figure 12

A

Ctrl

24h KA

MMP7

B

Ctrl

24h KA

proNGF

TIMP1
**Figure 12.** Expression of MMP7, TIMP1 and proNGF 24h following KA-induced seizure *in vivo*. *(A)* Hippocampal tissue from post-seizure rats immunolabeled with anti-MMP7. Vehicle-treated rats demonstrated wide MMP7 expression throughout cell bodies and processes, but is greatly diminished throughout the hippocampus after seizure. *(B)* Expression of TIMP1 and proNGF are strongly induced and are found in the same cells throughout the CA1 following KA-induced seizure. Images are from the CA1 area of the hippocampus and are representative of 5 independent experiments. Scale bars = 50um.
Figure 13

A

Ctrl

KA

B

+EDTA

Relative Fluorescence Intensity
**Figure 13.** MMP7 activity is reduced in response to KA-induced seizure. Twenty-four hours following seizure, rats were anesthetized with, decapitated and their brains removed, flash frozen, and cryosectioned (12µm). To assess MMP7 activity, sections were coated with an *in situ* zymogram assay buffer containing 10µg/ml MMP7 fluorogenic substrate that is specific to MMP7 activity. Following overnight incubation in a humidified chamber at 37°C, MMP7 activity can be observed using fluorescence microscopy, appearing as fluorescent areas on a black background. *(A)* In situ zymogram demonstrating 60% reduction in MMP7 activity in the hippocampus, particularly in the CA1 and dentate, areas that coincide with seizure-induced cell death, and calculated as a percentage of the entire hippocampal surface area. Relative fluorescence intensity, normalized to background fluorescence, is quantified below. N=5. Error bar represents SEM. Data were analyzed using Student’s t-test. *P*<0.01. *(B)* In situ zymogram performed in the presence of EDTA, a metal chelator that inhibits MMPs, demonstrating abolished activity. Scale bar = 100µm.
Figure 14

A

Vehicle  MMP7

B

Vehicle  MMP7
Figure 14. Exogenous MMP7 protects hippocampal neurons from death following seizure in vivo. (A) Panoramic montage of photos illustrating both hippocampal hemispheres from a single rat following seizure and unilateral infusion of active MMP7 (1µg) at a rate of 0.5 µl/min. In situ zymography was performed on sections to confirm MMP7 activity in the infused hemisphere, as observed by bright, fluorescent areas on a dark background. (B) FluoroJade B staining showing decreased cell death in the hippocampal hemisphere ipsilateral to MMP7 injection (arrows).
Figure 15

Vehicle  MMP7

p75

cc3

merge
Figure 15. Sections from a single, bilaterally cannulated, seizure-induced rat that received infusions of active MMP7 (1µg) into one hippocampal hemisphere, as well as vehicle infusions (0.5µl) into the opposite hemisphere, immediately following seizure treatment and every 12 hours thereafter. The left column represents the vehicle-infused side, and the right column represents the MMP7-infused side of the same rat. Twenty-four hours following seizure, immunostaining for cleaved caspase 3 and p75NTR is diminished on the MMP7-infused side compared to the vehicle-infused hemisphere, indicating MMP7 protects against KA-induced, p75NTR–mediated cell death. Images are from the CA1 area of the hippocampus and are representative of 6 independent experiments. Scale bar = 50um.
Figure 16

A

Vehicle     MMP7

proNGF

FJ

Merge

B

|            | KA
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NGF

Relative Densitometric Units

proNGF

![Image of gel electrophoresis and densitometry analysis](image)
**Figure 16.** MMP7 infusion reduces proNGF protein levels after seizure. *(A)* Image from opposite hippocampal hemispheres of a single, bilaterally cannulated rat that had undergone seizure treatment, followed by unilateral infusions of MMP7 and vehicle. Sections were immunolabeled for proNGF detection as well as subjected to Fluoro-Jade B staining (FJ) to label dying cells. MMP7 infusions resulted in diminished proNGF as well as FJ-positive cells compared to the contralateral, vehicle infused hemisphere. Images are representative of 6 independent experiments. *(B)* Western analysis of CSF from cannulated, seizure-treated rats that received MMP7 or vehicle infusions. Rats that received seizure treatment and MMP7 infusions demonstrated a 54% reduction in extracellular proNGF compared with those that received vehicle infusions. Graph represent the normalized densitometric index of blots from 3 independent experiments. Error bars represent SEM. Data were analyzed using one way ANOVA, followed by Tukey post-hoc test. **p<0.05, ***p<0.001. Scale bar = 50µm.
Figure 17

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 Probe: BDNF

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 Probe: NT-3
**Figure 17.** Secreted proBDNF and proNT-3 are not detected after KA-induced seizures. Twenty-four hours following seizure, rats were anesthetized and their CSF collected for Western analysis. Each lane represents 15µg of CSF. Only rats from the same experimental group were used for comparisons. *(A)* Western analysis of CSF (15µg) from vehicle and KA-treated rats did not reveal the presence of proBDNF. *(B)* proNT-3 was also not detected following seizure. Purified BDNF and NT-3 were loaded as positive controls. Blots are representative of 3 independent experiments.
Figure 18

A

Pretreatment

24h KA

C

Ctrl

KA

tPA + KA

B

C

KA

KA+tPA

tPA

proNGF

-36kDa

ponceau

*p

Veh

KA

KA+tPA

tPA

0

0.5

1

1.5

2

0

0.5

1

1.5

2
**Figure 18.** tPA reduces PI uptake and cell death in slice culture. (A) Hippocampal slices were cultured for one week, then subjected to KA treatment, with and without a pretreatment of exogenous tPA. Only slices that received KA treatment alone demonstrated PI uptake. (B) Western analysis of culture medium from slices that received vehicle, KA, KA+tPA or tPA. Medium that was supplemented with recombinant tPA did not demonstrate a significant difference in proNGF levels compared to control, indicating exogenous tPA reduces extracellular proNGF. Graph represents the normalized densitometric index of blots from 3 independent experiments. Error bars represent SEM. *p<0.001 Data were analyzed using one way ANOVA, followed by Tukey post-hoc test.
Figure 19

A

Nsp

CA1

Ctrl

24h KA

merge

B

Dentate

Ctrl

24h KA

merge

C

Nsp

-55 kDa

ponceau

C 2 8 24h
Figure 19. Neuroserpin expression is induced in hippocampal cells in response to KA treatment *in vitro*. (A) Confocal image of CA1 cells displaying increased neuroserpin immunostaining and PI uptake 24h after KA treatment. (B) Neuroserpin increase is particularly strong throughout the hilus in response to KA. (C) Time course demonstrating increasing neuroserpin release into culture medium 2-24h following KA treatment. Scale bar = 50um.
Figure 20

A

PAI-1

PI

merge

Veh

24h KA

B

PAI-1

ponceau

C  2  8  24

-46kDa
Figure 20. Expression and release of PAI-1 in the dentate. (A) Twenty-four hours after KA treatment, slices demonstrated moderate induction of PAI-1. Images are representative of 3 independent experiments. Scale bar = 50um.

(B) Western analysis of culture medium was performed to examine secretion of PAI-1. PAI-1 was not detected in culture medium of vehicle-treated slices, but was induced by 2h following KA treatment and sustained through 24h.
Figure 21

A

Ctrl

24h

B

+aprotinin
Figure 21. tPA activity is downregulated following seizure. Twenty-four hours following seizure, rats were anesthetized with, decapitated and their brains removed, flash frozen, and cryosectioned (12µm). To assess endogenous tPA activity, sections were subjected to in situ zymography. Fresh-frozen sections were coated with an in situ zymogram assay buffer containing 1% casein and 10µg/ml plasminogen and incubated overnight in a humidified chamber at 37°C. Endogenous tPA activates the plasminogen, which then digests the casein, creating dark areas in the overlayed substrate buffer and visualized using dark field microscopy. (A) In situ zymogram demonstrating tPA activity in the hippocampus with and without seizure treatment. Note the decrease in areas of activity particularly in the CA1-CA3 and dentate. (B) In situ zymogram in the presence of aprontinin, a serine protease inhibitor, demonstrating abolished tPA activity.
Figure 22

A

Ctrl 24h post-seizure

B

CA1  Dentate

24h post-seizure

C

Ctrl  KA

nsp  actin
Figure 22. Neuroserpin expression is induced by seizures *in vivo*. *(A)* DAB staining for neuroserpin shows upregulated expression in the dentate and CA3 areas of the hippocampus 24h following KA-induced seizures. *(B)* High magnification of CA1 and dentate areas from *(A)*, showing differential neuroserpin expression following KA treatment. *(C)* Western blot of whole hippocampal lysates demonstrating upregulated neuroserpin in response to KA-induced seizures.
Figure 23

A

Ctrl  KA

B

<table>
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<tr>
<th></th>
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<tbody>
<tr>
<td>PAI-1</td>
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**Figure 23.** Neuroserpin expression is induced by seizures *in vivo*. *(A)* DAB staining demonstrating PAI-1 expression is upregulated 24h following KA treatment. Note that expression is particularly localized to the dentate and CA3, similar to the pattern of neuroserpin upregulation as seen in figure 22. *(B)* Western blot showing differential PAI-1 levels from whole hippocampal lysates 24h following KA-induced seizures.
Figure 24
Figure 24. Exogenous infusion of recombinant tPA (1µg every 12 hours) leads to decreased cell death in the CA1 24h following KA-induced seizure. 

(A) In situ zymogram demonstrating increased tPA activity in the injected hemisphere and confirming tPA infusion. (B) Co-staining for p75NTR and FJ demonstrates decreased p75NTR immunoreactivity and cell death in the CA1 ipsilateral to the tPA infusion. (C) FJ and p75NTR staining reveal no difference in the dentate gyrus compared between vehicle or tPA injections, suggesting regional differences in the rescue effect of tPA. All images are taken bilaterally from the same rat and representative of images from 5 independent experiments. Scale bar = 50um.
Figure 25

[Image: Western blot showing proNGF and NGF bands with molecular masses of 37 kDa and 15 kDa, respectively.]

Legend:
- KA: Kainate
- Veh: Vehicle
- tPA: Tissue Plasminogen Activator
- NGF: Neurotrophin-3
Figure 25. Exogenous tPA infusions (1µg every 12 hours) reduce extracellular proNGF 24h following KA-induced seizures. Western analysis of CSF from tPA-injected rats demonstrate decreased proNGF detection compared with non-seizured or vehicle-injected rats. Only rats from the same experimental group were used for comparisons. Blot is representative of 3 independent experiments.
XII. References


neurotrophic activity but is less active than mature nerve growth factor. J Neurochem 89:581-592.


Suter U, Heymach JV, Jr., Shooter EM (1991) Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. EMBO J 10:2395-2400.


Woo NH, Teng HK, Siao CJ, Chiaruttini C, Pang PT, Milner TA, Hempstead BL, Lu B (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat Neurosci 8:1069-1077.


XI. Vita

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1999 Graduated from Grossmont High School, La Mesa, California

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