Elucidating the Neuroprotective Role of Teneurin C-terminal Associated Peptide (TCAP)-1

By

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ABSTRACT

The physiology of neuroprotection includes the mechanisms and strategies utilized to guard against neuronal injury or degeneration in the central nervous system (CNS). Degenerative disorders may be generally classified as either acute, such as stroke or nervous system injury/trauma or chronic such as Parkinson's (PD) or Alzheimer's (AD) The goal of neuroprotection is to limit neuronal dysfunction and death after such injury and attempt to maintain the highest possible integrity of cellular interactions in the brain resulting in undisturbed neural function. There is a wide range of agents currently under investigation and some can potentially be used in more than one disorder, as many of the underlying mechanisms of damage to neural tissues, in both acute disorders and in chronic neurodegenerative diseases, are similar. These agents include: free radical trappers/scavengers, anti-excitotoxic agents, apoptosis/ programmed cell death (PCD) inhibitors, anti-inflammatory agents, and neurotrophic factors. The objective of this research is to characterize a novel family of neuroprotective peptides, teneurin C-terminal associated peptide (TCAP), and to elucidate their protective mechanism .

I have investigated the possibility of TCAP-1 as having the ability to protect neurons during times of pH induced cellular stress due to a decreased incidence of

necrosis. I show that TCAP may be upregulating reactive oxygen species (ROS) scavengers to confer neuroprotection during necrotic cell death. I have also shown that TCAP plays a neuroprotective role during apoptotic and glutamate induced stress by decreasing cleavage of death executers and increasing ERK activation and superoxide dismutase. Together, the data indicate that TCAP-1 may play a significant role in preventing neuronal loss in times of neurotoxin stress. Moreover, I have shown that TCAP increases brain derived neurotrophic factor (BDNF) protein and gene expression in cell culture and consequently may confer survivability to cells under stress, thus demonstrating that TCAP may play a pivotal role in maintaining cell viability via modulating growth factor transcription and translation. Thus TCAP-1 may be implicated in activating essential survival pathways leading to the stabilization of the cell. This may prevent neuronal damage caused by neurodegenerative disorders and brain trauma.

Acknowledgements

Carpe Diem is a notable term that has been the quintessential keystone for my journey as a PhD student and an indicative reflection of my lifestyle for the past four years. I now believe that to forge ahead and to grasp every opportunity with conviction, despite risk and potential failure were fundamental tenets that have allowed me to succeed. Dotted with memories of persistence, stubbornness, anarchy and triumph my time in graduate school has been rewarding on various levels.

I would first like pay homage to my "famiglia" and thank them for their endless patience. My wonderful parents, Cellito and Angel have provided unconditional encouragement and humor and have never failed to lift my spirits even in the darkest of times. I will forever be grateful and humbled by your love and support.

I would like to give thanks to my supervisor, Dr. David A. Lovejoy, whom I have always viewed as a superb scientist with modern flair and infinite vision. My time as your graduate student will always be remembered as a period of integral personal growth and I will always be grateful for your guidance, insight and understanding. I would also like to thank my committee member, Dr. Susan Rotzinger who has offered valuable contributions to my research and mental well-being.

To my fellow lab mates who have each contributed to my liberation and salvation throughout this journey: Claudio, a beacon of strength and meticulousness; Laura, artistic and "digitally" gifted; Andrew, a complete riot; Tanya, a stealth interpretive dancer; Dhan, on Caribbean time and "lovin' it"; Arij, as the other half of Arigina, my travel buddy, partner in crime, hallway chair racing team and chocolate fondue accomplice, I would like to thank you for endless amusing rants and most importantly for your friendship.

I would like to attribute my skills of fortitude and controlled demeanor in times crisis to Rose and the Graduate Students' Union (GSU). My experience as a student activist at the GSU has forged everlasting bonds with incredible characters who have taught me to "productively focus my political fervor" and to moderately control "the beast within".

Great thanks are owed to my external support network of friends. The journey towards the completion of a doctorate is fraught with tribulations and sometimes an outside viewpoint provides much needed clarity and perspective. Finally, an ode to my dearest "Consigliera" and fellow cheese connoisseur, what words can describe our bond of rage and mirth? I believe these pearls of wisdom will suffice:

"Be who you are and say what you feel because those who mind don't matter and those who matter don't mind."
"I have heard there are troubles of more than one kind. Some come from ahead and some come from behind. But I've bought a big bat. I'm all ready you see. Now my troubles are going to have troubles with me!"
"I like nonsense; it wakes up the brain cells." **-Dr. Seuss**

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Abbreviations

3-MA 3-methyladenine
5-FU 5-fluorouracil
AD Alzheimer's disease
ADNF activity-dependent neurotrophic factor
AIF apoptosis-inducing factor
APAF-1 apoptotic protease activating factor 1
ATP adenosine triphosphate
BAD Bcl-2-associated death promoter
BAX Bcl-2–associated X protein
BBB blood–brain barrier
BCL2 B cell leukemia 2
BDNF brain derived neurotrophic factor
BH3 Bcl-2 homology (BH) domain 3
cAMP cyclic adenosine monophosphate
c. elegans Caenorhabditis elegans
CNS central nervous system
CREB cAMP responsive element binding protein
CRF corticotrophin releasing factor
CYT C cytochrome c
DISC death-inducing signaling complex
DR death domain receptors
EGF epidermal growth factor
ER endoplasmic reticulum
ERK extracellular signal regulated protein kinase
FADD Fas-associated death domain
FAS Apoptosis Stimulating Fragment
FAS L Fas-ligand (FasL)
FGFs fibroblast growth factors
FKHRL1 forkhead transcription factor
GGF2 neuregulin glial growth factor-2
HI hypoxia-ischemia
HD Huntington's disease
ICV intracerebroventricular
IGF insulin-like growth factors
IKK IkappaB kinase
ION isthmo-optic nucleus
JAK janus kinase
JNK1 c-Jun N-terminal kinase
MAPK mitogen-activated protein kinase
MCAO middle cerebral artery occlusion
MPT mitochondrial permeability transition
MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MR magnetic resonance
mRNA messenger RNA

MS multiple sclerosis
mTOR mammalian target of rapamycin
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NE neonatal encephalopathy
NEC-1 necrostatin-1
NF-κB nuclear factor-kappa B
NGF nerve growth factor
NMDA N-methyl-D-aspartic acid
NPY neuropeptide Y
NRIF neurotrophin-receptor-interacting factor
NT3 neurotrophin 3
PD Parkinson's disease
PCD programmed cell death
pHe extracellular pH
pHi intracellular pH
PI propidium iodide
PI-3K phosphatidylinositol-3 kinase
PKC protein kinase C
PTK protein tyrosine kinase
p75NTR p75 neurotrophin receptor
RIP receptor interacting protein
ROS reactive oxygen species
RTKs receptor tyrosine kinases
SET serial endosymbiosis theory
SIMPs soluble mitochondrial intramembrane proteins
siRNA short interfering ribonucleic acid
SNc sustantia nigra pars compacta
SOD 1 superoxide dismutase 1
tBID truncated BH3 interacting domain death agonist
TBP tatabox binding protein
TCAP teneurin C-terminal associated peptide
TGF transforming growth factors
TNF tumor necrosis factor
TNFR tumor necrosis factor receptor
TRAD TNF-receptor associated death domain
TRAIL tumor necrosis factor (TNF)-related apoptosis-inducing ligand
TRK receptor tyrosine kinase
VIP vasoactive intestinal peptide

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Chapter 1: Introduction: Investigating the potential role of Teneurin C-terminal associated peptide as a novel neuroprotective agent

Neuroprotection is defined as protecting neurons from trauma and is undoubtedly a vital and relevant field of research. The central nervous system (CNS) is complex and essential yet still remains a mystery in so many regards. Our brain controls vital functions including voluntary movements and perception but also mediates learning, creativity, abstract thinking and emotions. These complex networks are orchestrated via multiple pathways of highly specialized neurons. The process of evolution has provided a skull and backbone to protect the CNS from outside mechanical injuries, and a molecular obstacle, the blood-brain barrier (BBB), to protect it from endogenous chemical agents and yet the brain remains vulnerable to neurodegenerative diseases associated with dramatic increases in human live expectancy. Diseases of the brain may be characterized by specific patterns of neuronal damage and degeneration but the molecular bases of these specific neuronal losses are not yet fully understood. The objective of contemporary research focuses on the necessity to find therapeutic solutions to avert the deleterious outcome of these diseases. The objective of this chapter is to examine the current understanding of cell death and the necessity to explore new methods of combating neuronal loss in brain disease. The data presented in this thesis indicates that TCAP peptides may represent a highly conserved system to protect and modulate neural circuitry of key regions during times of stress. The application of TCAP as a therapeutic agent may provide a novel means by which to protect the brain from otherwise irreversible damage.

1.1 Neurodegeneration

The history of cell death

Cell death is a phenomenon that was first observed and understood in the 19th century. Further experimental evidence was not attained until the mid-20th century. Most notable was in the 1950s the lab of Sydney Brenner at Cambridge, UK succeeded in tracing the entire embryonic cell lineage of *Caenorhabditis elegans (Kerr et al., 1972).* Brenner's original interests were in genetics and development of the nervous system, but cell lineage and differentiation inevitably led him to the study of cell fate and one member of his laboratory, H. Robert Horvitz, commented on the unusual absence of certain cells in the adult:

"One aspect of the cell lineage particularly caught my attention: in addition to the 959 cells generated during worm development and found in the adult, another 131 cells are generated but are not present in the adult. These cells are absent because they undergo programmed cell death" – Horvitz (Hodgkin et al., 1979)

Beginning in the 1960s, several laboratories demonstrated that cell death was biologically controlled, now known as programmed cell death, today characterized as apoptosis (Kerr et al., 1972). By 1990, the genetic basis of programmed cell death had been established, and the first components of the cell death machinery, such as caspase 3, bcl-2, and Fas, had been identified, sequenced, and recognized as highly conserved in evolution (Wyllie et al., 1980). The rapidly growing field of neurodegeneration has provided ample understanding of the intricate process of cell termination and has broadened our appreciation of the multiple pathways to cell death. Moreover, the complex and convoluted manner through which a cell may experience death and how the

execution of such processes are controlled remain the focus of research aimed at preventing death and the correlative injurious effects on brain function. Although our comprehension of cell death has increased exponentially, we have yet to deduce how a cell commits to death and in what way it chooses a path to die. This mystery adds a complex dimension to neuroprotective research attempting to control neuronal death thus conferring survivability to compromised neurons, such as in debilitating neurodegenerative diseases.

Understanding the ambiguity of cell death

Mammalian neurons are amongst the most robust cell types, however, they are not invincible (Finklestein et al., 1987, David, 1988). Neuronal cell death occurs during the development of the CNS as well as in situations of physiological stress and trauma, such as acute injury and progressive degenerative diseases such as brain ischemia (Mattson et al., 2001, Friedlander, 2003). There is still much debate concerning the mechanisms of neuronal cell death during brain trauma due to the complexity of the cellular pathways. Moreover, neurons can undergo various forms of death such as apoptosis, autophagy, necrosis, necroptosis (Dunn, 1990, Yuan and Yankner, 2000). Apoptosis can be described as programmed cell suicide whereas necrosis is considered to be reactive cell homicide. Autophagy, on the other hand acts as a means of cell degradation, whereas necroptosis exhibits characteristics of all three types of cell death. However, these overlap greatly, for instance in the late stages of apoptosis cells will exhibit the same characteristics of necrosis, necroptosis and autophagy. For example, the cell death seen in the core of an

ischemic infarct is necrotic, whereas in the surrounding penumbra region the death is probably apoptotic (Friedlander, 2003) (Figure 1.1.). Regardless of the degenerative pathway, damaged or dead neurons are a hallmark of many neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's disease as well as brain ischemia and multiple sclerosis (Siao and Tsirka, 2002).

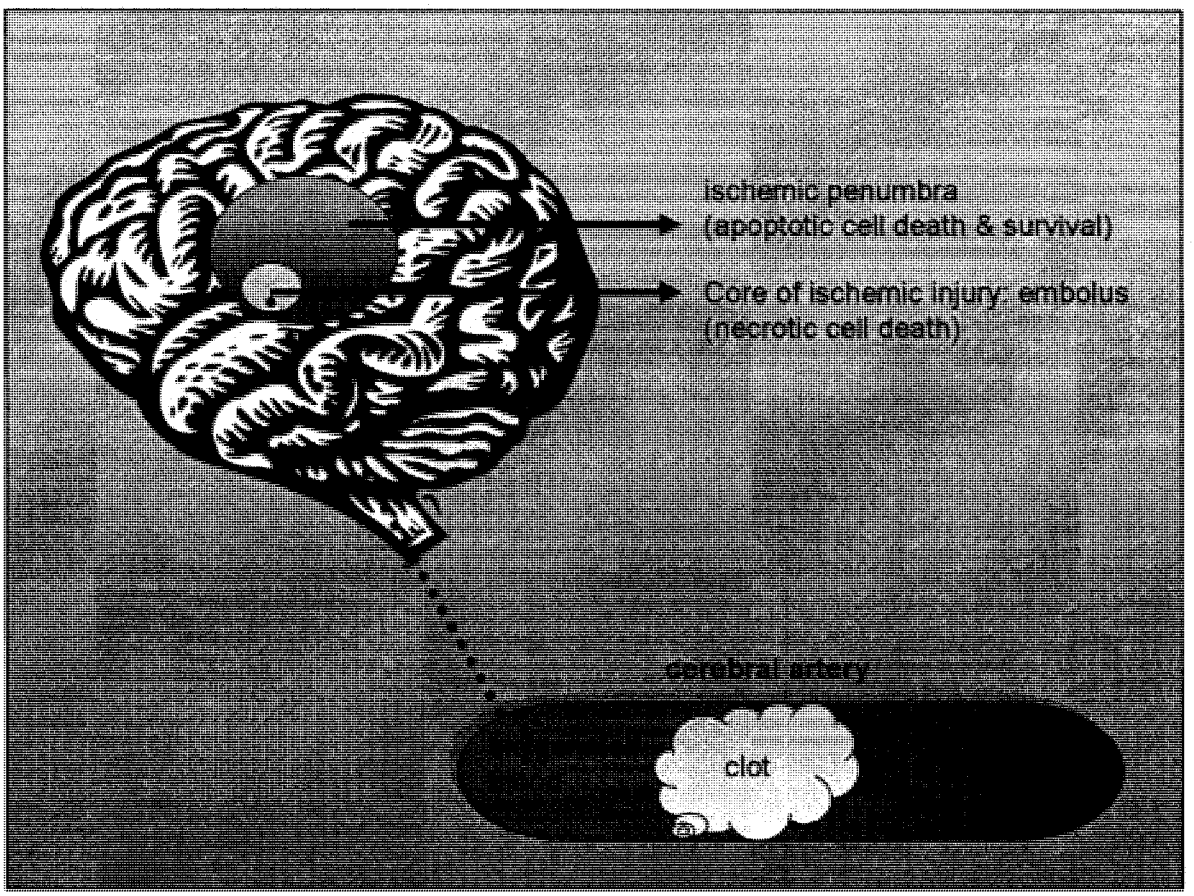

Figure .1.1. An embolus with both apoptotic and necrotic cell death features. An embolus in the brain may be caused by a thrombus or other form of blockage in the blood vessels, resulting in little or no collateral flow. This eventually leads to hypoxia and necrotic cell death characterized by inflammation. In the penumbra, where there is some degree of collateral blood flow, a gradient of tissue perfusion establishes a threshold among necrosis, apoptosis and tissue survival.

These neurodegenerative conditions are characterized by their deleterious effects on brain function resulting from deterioration of neurons. The destruction of neurons in

these conditions may be regulated by various forms of cell death and can be caused by damaged mitochondria, increased levels of excitotoxins such as glutamate, which increases calcium influx into the neurons and activates calcium dependent variables such as calpain, caspases radical oxygen species (ROS) and pH (Brorson et al., 1995a, Brorson et al., 1995b, Brorson et al., 1995c, Rathore et al., 2006, Wang et al., 2007). Fluctuations in brain homeostasis during times of neurodegenerative stress remains ambiguous. There are fluxes of calcium, pH and radical oxygen species that may occur and the interplay between variables is complex and overlapping. For instance, during both respiratory and metabolic alkalosis, increases in calcium occur in rat neurons due to intracellular calcium accumulating structures such as the mitochondria which in turn impinge on ROS production (Potapenko et al., 2004, Wang et al., 2007). Also, variations of intracellular and extracellular pH can influence diverse cellular functions such as glycolysis, protein synthesis, cell volume, receptor affinities, and membrane channel activity in turn affecting growth factor regulation and cell synthesis (Siao and Tsirka, 2002). Thus, several factors play a concomitant role in the brain throughout the cell death process.

1.2 Necrosis

Necrosis: cell death

Necrosis has been traditionally seen as the "unregulated" pathological cell death (Nicotera et al., 1999). The stimulus of death, such as ischemia, is itself often the direct cause of the demise of the cell. Recent studies have also suggested that in certain conditions, necrosis may also be a regulated cellular mechanism. Given that necrosis is a

rather ambiguous process, it will be defined here as the caspase-independent cell death that occurs under pathological conditions (Columbano, 1995, Nicotera and Lipton, 1999).

Some studies have revealed that when caspases are inhibited, apoptosis stimulating fragment (Fas) and tumor necrosis factor (TNF) can induce cell death with necrotic features including blebbing of the nuclear envelope, the formation of intracellular vacuoles and inflammation of the mitochondria and endoplasmic reticulum (ER) (Vercammen et al., 1997, Vercammen et al., 1998a, Vercammen et al., 1998b, Matsumura et al., 2000). What is intriguing is that this process entails the loss of mitochondrial membrane potential but no release of cytochrome c, indicating that the damage inflicted on the mitochondria by necrosis is different from that caused by apoptosis. However, the mechanism of receptor-mediated necrosis requires both adaptor protein Fas-associated death domain (FADD), which is also necessary for apoptosis and Ser/Thr receptor interacting protein (RIP), which is not required in apoptosis (Stanger et al., 1995, Holler et al., 2000). RIP has been shown to mediate TNFα-induced nuclear factor-kappa B (NF-κB) activation by recruiting the IkappaB kinase (IKK) complex to TNF receptor(TNFR) during apoptosis, however, during necrosis it must recruit IKK in a kinase-dependent fashion (Ting et al., 1996, Holler et al., 2000). Thus, it seems that FAS induced apoptosis/necrosis is mediated in a similar manner and subsequently bifurcate into two distinct signaling pathways (Figure 1.2.).

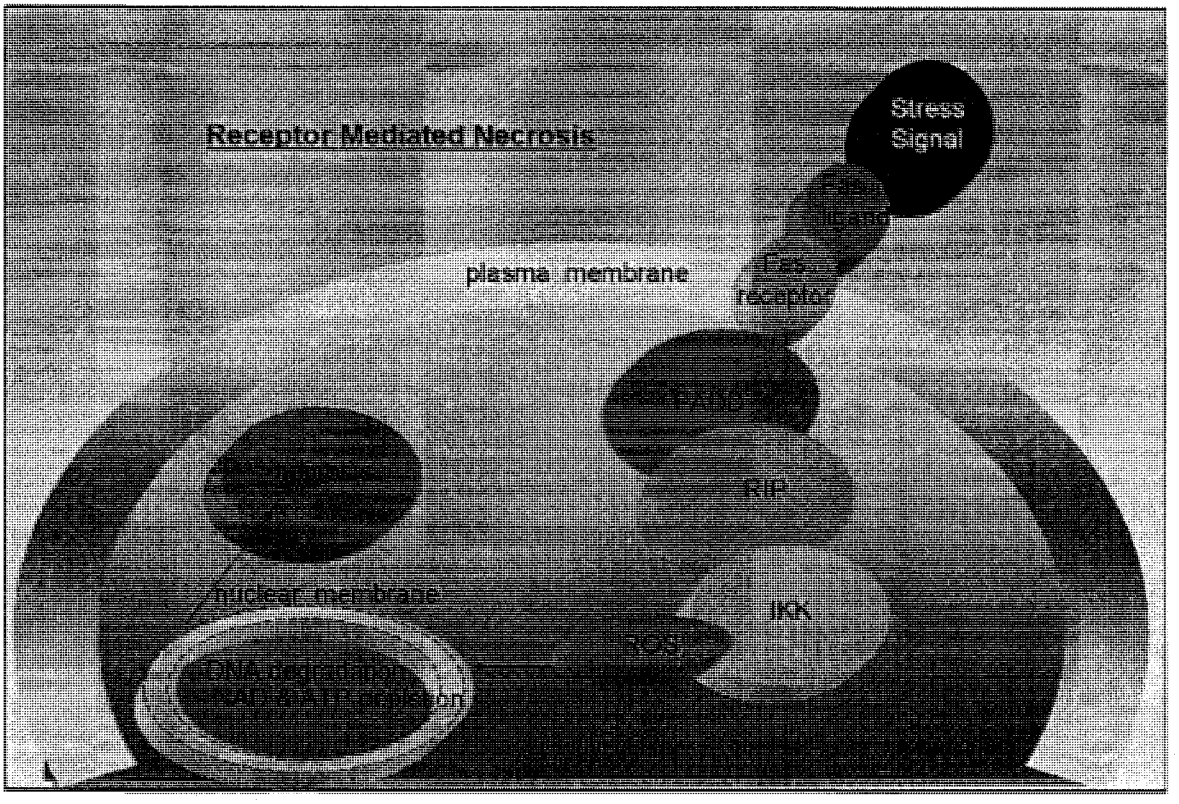

Figure 1.2. receptor mediated necrosis. The mechanism of receptor mediated necrosis requires FADD and RIP. RIP in turn, must recruit IKK in a kinase-dependent manner activating ROS generation eventually leading to NAD and ATP depletion causing necrosis to occur.

Necrosis in neuronal cell death

Blocking of Ca^{2+} channels in the ER can suppress necrotic degeneration, thus an increase in the concentration of cytoplasmic Ca^{2+} excreted by the ER stores may play a major role in necrosis in neurons (Bonfoco et al., 1995). Elevated cytoplasmic Ca^{2+} has been previously implicated in neuronal cell death, which is not surprising as Ca^{2+} has multiple roles in the regulation of various cell processes (Xue et al., 1999). However, it is clear that when intracellular Ca^{2+} concentrations exceed normal physiological levels, cell death does occur (Bonfoco et al., 1995).

Rises in intracellular Ca^{2+} may be induced by excitotoxicity caused by the accumulation of glutamate as a result of ischemic brain injury and subsequent over stimulation of post-synaptic glutamate receptors (Bonfoco et al., 1995). Calpains and cathepsins have been implicated in neuronal cell death under various neurotoxic conditions, including brain ischemia (Yamashima, 2000). According to this hypothesis, ischemic brain injury-induced N-methyl-D-aspartic acid (NMDA) activation leads to intracellular Ca^{2+} overload, which activates calpain, which in turn activates lysosomal cathepsin and induces necrosis; however, lack of specific calpain and cathepsins inhibitors and the multiple roles of both these proteins in cellular function place this hypothesis in question.

Intracellular Ca^{2+} also regulates necrosis and apoptosis through Bcl-2–associated X protein (Bax/Bak), two pro-apoptotic members of the B cell leukemia 2 (Bcl-2) family that control mitochondria integrity and resting concentrations of Ca^{2+} in the ER (Scorrano et al., 2003). In Bax/Bak double knockout cells, low ER Ca^{2+} levels cause resistance to proapoptotic factors that release Ca^{2+} stores, such as oxidative stress. Both Bax/Bak act as receptors for Bcl-2 homology domain 3 (BH3)-only proteins such as Bcl-2-associated death promoter (BAD) and truncated BH3 interacting domain death agonist (tBid), in the mitochondria, so the possibility that an unknown BH3-only factor could be activating Bax/Bak, thus releasing ER Ca^{2+} stores is a possibility. Thus, it seems that Bax/Bak could be regulating apoptosis and necrosis as both require the release of intracellular Ca^{2+} stores (Figure 1.3).

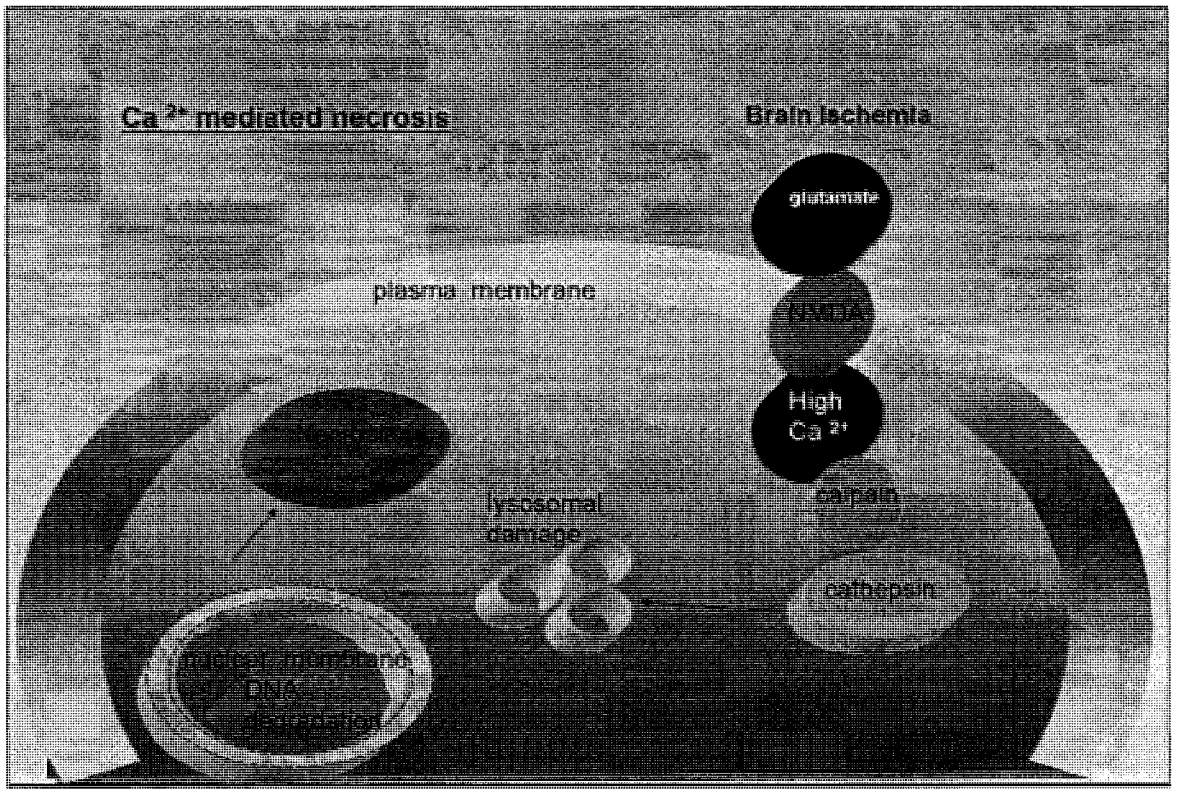

Figure 1.3. Ca $^{2+}$ mediated necrosis in mammalian neurons. Brain injury such as ischemia induces NMDA activation leading to intracellular increase in Ca $^{2+}$ causing calpain dependent activation of cathepsin. This leads to lysosomal damage, nuclear membrane disintegration and eventually necrosis.

Necrosis in neurodegenerative diseases

Necrotic cell death in the CNS follows acute ischemia or traumatic injury to the brain or spinal cord (Linnik et al., 1993, Martin, 2001). It occurs in areas that are most severely affected by abrupt biochemical collapse, which leads to the generation of free radicals and excitotoxins (e.g., glutamate, cytotoxic cytokines, and Ca $^{2+}$). The histologic features of necrotic cell death are mitochondrial and nuclear swelling, dissolution of organelles, and condensation of chromatin around the nucleus (Martin, 1999). These events are followed by the rupture of nuclear and cytoplasmic membranes and the degradation of DNA by random enzymatic cuts in the molecule (Martin-Villalba et al.,

2001). Given these mechanisms and the rapidity with which the process occurs, necrotic cell death is extremely difficult to treat or prevent.

The role of pH in necrosis

Studies on pH have shown that brain alkalinization induces an increase of Ca^{2+} in neurons due to Ca^{2+} sequestering structures, such as the mitochondria and endoplasmic reticulum (Potapenko et al., 2004). Elevated cytoplasmic Ca^{2+} is implicated in neuronal cell death, more specifically, necrosis during brain ischemia (Deitmer and Rose, 1996, Kostyuk et al., 2003). Such excessive rises in Ca^{2+} may be induced by excitoxicity caused by brain ischemia, subsequently overstimulating postsynaptic glutamate receptors. Of these glutamate-gated channels, NMDA receptor channels play a key role in excitotoxicity as they conduct both Na^{+} and Ca^{2+} (Shishkin et al., 2002, Voitenko et al., 2004).

pH and cell death during brain injury

Various neurodegenerative diseases exist contingent upon various forms of cell death that in turn are mediated by their environments' surrounding pH. One of the logistical problems in understanding cell death and its corroborating factors is the ambiguity surrounding cell death. The current research indicates that many characteristics that were once thought to pertain only to apoptosis, now apply to necrosis as well.

Furthermore, novel functions have been identified within the cell death pathway, such as autophagy and necroptosis (Lemasters et al., 1998). The role of pH during trauma is essential as the body is continuously attempting to maintain physiological homeostasis, especially during times of stress. The current consensus is that following the initial insult, brain cells die by necrosis, apoptosis or a combination of the two and pH plays a pivotal role during these times (Traynelis and Cull-Candy, 1990).

Although the literature on brain acidosis is extensive, brain alkalosis, is not well understood. In the case of brain acidosis, this is a result of either an increase in tissue PCO_2 or an accumulation of acids produced by metabolism (Gennari et al., 1972). Severe hypercapnia, such as arterial PCO_2 around 300 mm Hg, may cause a fall in tissue pH to around 6.6 (Zauner et al., 2002). In severe ischemia and tissue hypoxia, anaerobic glycolysis leads to lactic acid accumulation (Xu et al., 1998). The deleterious effect of excessive lactic acidosis may be related to an influence on the following: synthesis and degradation of cellular constituents; mitochondrial function; cell volume control; post-ischemic blood flow; and stimulation of pathologic free radicals (Mabe et al., 1983, Levine et al., 1992, Lascola and Kraig, 1997).

In the case of high pH, intracellular alkalinization has been observed in cells undergoing cytokine deprivation (Khaled et al., 1999), gamma radiation (Dai et al., 1998), neuronal apoptosis (Ostermann et al., 2003) as well as hypoxia-ischemia (HI) (Robertson et al., 2002).

Studies on brain energy metabolism using phosphorous and proton magnetic resonance (MR) spectroscopy have allowed an understanding of energy changes within the brain following HI (Moon and Richards, 1973, Thornton et al., 1998). A phenomenon

named the "secondary energy failure" has been investigated. This energy failure is seen as the decline in high-energy phosphates occurring 6 to 8 hours after birth in infants with neonatal encephalopathy (NE) and the magnitude of this disruption can be compared with the eventual neurodevelopmental outcome (Thornton et al., 1998, Robertson et al., 2002). A similar relationship between intracellular alkalosis and the severity of brain injury in term infants has also been found. Infants with the most alkaline brain cells had more severe changes on MR imaging within the first 2 weeks of life and the worst neurodevelopmental outcome at one year. This brain alkalosis was seen to persist for some months in those with the worst outcome (Robertson et al., 2002).

The return of pH_i to normal values may be deleterious to cells that have undergone HI due to the pH paradox (LaManna, 1996, LaManna et al., 1996a, LaManna et al., 1996b). Possible mechanisms leading to the pH-dependent injury include the onset of the mitochondrial permeability transition, leading to uncoupling of oxidative phosphorylation and aggravation of adenosine triphosphate (ATP) depletion, (Lemasters et al., 1998) and an exacerbation of excitotoxic neuronal injury due to increased NMDA activation at alkaline pH_i (Traynelis and Cull-Candy, 1990). An understanding of transmembrane transporters leading to brain alkalosis after HI, therefore, may be important in defining possible neuroprotective strategies.

Intracellular pH, volume and cell proliferation are influenced by the electroneutral Na^+/H^+ transporter, which is thought to normalize pH_i on reperfusion (Grinstein et al., 1989, Wakabayashi et al., 1992). At low pH_i, binding of H^+ to the modifier site activates the exchanger (Jakubovicz et al., 1987). The Na^+/H^+ transporter has been implicated in post-ischemic alkalinization after experimental HI. The time course of pH_i recovery after

cardiac arrest was altered by amiloride, a Na^+/H^+ exchange blocker (Ferimer et al., 1995). In vitro experiments suggest that Na^+/H^+ exchanger blockade may be beneficial to cell survival after transient HI. A slower rate of pH_i recovery with amiloride was associated with neuronal protection (Vornov et al., 1996).

One of the other major regulators of pH_i, the Na^+/HCO_3 cotransporter, is unique to glial cells and in times of acute experimental stroke in penumbral and peri-infarct areas, intracellular brain alkalinization occurs, characterized by depolarization in astrocytes due to delayed expression of glial fibrillary acidic protein mRNA (a marker of reactive astroglia) (Lascola and Kraig, 1997). Suppression of peri-infarct depolarizations with dizocilpine decreased the frequency of alkaline pH_i shifts (Back et al., 2000).

Experimental studies have demonstrated elevated brain tissue lactate despite restoration of ATP after transient HI; in fact, high lactate levels persist while brain pH_i has become alkalotic (Kogure et al., 1978, Kogure et al., 1980). There is increasing evidence that the high levels of lactate during this postischemic phase aid neuronal survival; however, lactate clearance and pH_i increase occur at different time courses (Schurr and Rigor, 1998). For example, clearance of lactate occurs over approximately 30 minutes of reperfusion (Lei and Peeling, 1998). After this, there is a secondary increase in brain lactate occurring after 12 to 24 hours (Penrice et al., 1996, Penrice et al., 1997), thought to be caused by renewed production of lactate in brain tissue (Rothman et al., 1991). Exaggerated astrocyte glycolysis may be a possible mechanism leading to this increase in brain lactate. Brain pH_i neutralizes within 5 to 15 minutes of reperfusion and subsequently may become alkaline (Mabe et al., 1983, Allen et al., 1988). This can be explained by the observation that the hyperemic washout of CO_2 is complete by 5

minutes of perfusion and that ionic gradients are reestablished within 2 to 5 minutes. Exchangers may then be upregulated leading to a rapid pH_i recovery (Robertson et al., 2002).

In vitro studies have indicated that intervention to prevent rebound alkalosis immediately after HI may be neuroprotective (Vornov et al., 1996). As brain alkalosis may persist for weeks after an insult, intervention might be possible in both the subacute and chronic phase. To further substantiate this finding the role of pH in early human focal ischemia using phosphorus-31 nuclear magnetic resonance spectroscopy was investigated. Serial ischemic brain pH levels indicated a progression from early acidosis to subacute alkalosis (Levine et al., 1992). The data suggest a link between high- energy phosphate metabolism and brain pH, especially during the period of ischemic brain acidosis.

The relationship between ROS, pH and brain stress

It is apparent that pH plays a fundamental role in brain homeostasis; in particular brain alkalosis has been shown to be detrimental to neurons. Previous studies have supported a link between alkalosis and radical oxygen species (ROS) (Majima et al., 1998, Susa and Wakabayashi, 2003). ROS have been shown to accumulate in the brain especially under neurodegenerative conditions. In one study, cells cultured under alkaline conditions can increase ROS levels, mitochondrial damage and cell death. Over-expression of SOD2 (Mn SOD) was shown to prevent cell death (Majima et al., 1998). There is little that is known about pH and ROS concentrations in the microenvironments

surrounding cells and in tissue interstices, although pH concentrations appear to vary widely in local microenvironments and ROS may follow suite. A clearer understanding of ROS and pH in neuronal populations afflicted with brain damage may provide a fundamental link between the need for oxidative stress relief which may in turn attenuate alkalotic induced cell death.

1.3 Necroptosis

Necroptosis in neurodegenerative disease

While the long standing dogma has been that caspase activation is essential for apoptosis to occur, there have been recent studies suggesting that this is not the case. In fact, in some situations, caspase inhibition does not prevent death domain receptor (DR) cell death and the morphology is characteristic of necrosis as opposed to apoptosis (Schulze-Osthoff et al., 1994, Vercammen et al., 1998b, Holler et al., 2000, Matsumura et al., 2000). The distinct necrotic morphological characteristics: irregular disintegration of the cell, small surface evaginations caused by the loss of control of the water influx through the plasma membrane and inflammation, is exhibited by multiple cell types when undergoing Fas/TNFR family of death domain receptors (DR) cell death without caspase activation (Rello et al., 2005). This would indicate the existence of an alternative non-caspase dependent apoptotic cell-death-pathway. The difficulty in such an assumption is that the mechanisms of the DR-induced non apoptotic pathway are unclear. It has been demonstrated that there is the existence of a common necrotic cell death pathway activated by a DR signal in the absence of cellular damage, suggesting that necrosis *in*

vivo might be regulated at the cellular level (Degterev et al., 2005). By identifying necrostatin-1 (Nec-1), a small molecule inhibitor of cell death caused by DR stimulation in the presence of caspase inhibition, detection of DR signaling that triggers an alternative apoptotic pathway was elucidated and termed necroptosis. Nec-1 inhibited all published examples of necrotic cell death induced by DR activation in the presence of caspase inhibitors. Nec-1 had no effect on apoptotic cells, meaning it did not affect the number of Annexin V-positive and propidium iodide (PI)-negative cells, an indicator of apoptosis. Nec-1 did not induce any change in apoptotic morphology, cytoplasm condensation, chromatin marginalization and nuclear fragmentation. It completely inhibited the appearance of necrotic morphology, nuclear condensation and loss of membrane integrity. Also, autophagy, the process of protein degradation via phagocytosis, has been implicated in caspase independent cell death (Klionsky and Emr, 2000, Gozuacik and Kimchi, 2004). The characteristic double membrane enclosed vesicles were observed in necroptotic cells; however, necroptosis proceeded normally in the presence of 3-methyladenine (3MA), an inhibitor of autophagy. This suggests that autophagy is a downstream result of necroptosis as opposed to a contributing factor (Gozuacik and Kimchi, 2007).

Necroptosis in neuronal cell death

Necroptosis has been shown to be a delayed component of ischemic neuronal injury. Neuronal cell death caused by ischemic brain injury has been documented as involving DR (Martin-Villalba et al., 1999, Rosenbaum et al., 2000, Martin-Villalba et

al., 2001) and having a non apoptotic component (Gwag et al., 1995, Lo et al., 2003), thus making necroptosis a likely component of cell death during brain ischemia. By using a variant of Nec-1 it was demonstrated that necroptosis was blocked, while caspase-dependent apoptosis was not. This could signify that necroptosis may function as a mechanism to expel damaged cells under stress conditions when apoptosis is inhibited.

1.4 Autophagy

Autophagy: cell death

Autophagy is a subset of physiological cell death in higher organisms associated with extensive formation of intracellular vacuoles (Schweichel and Merker, 1973). Thus, proteins participating in autophagy undergo a process of intracellular bulk degradation in which cytoplasmic components including organelles are sequestered within double-membrane vesicles that deliver the contents to the lysosome/vacuole for degradation (Figure 1.4). Autophagy is tightly regulated by the serine/threonine protein kinase mammalian target of rapamycin (mTor) (Ravikumar et al., 2003, Ravikumar et al., 2004, Jaboin et al., 2007, Sarkar et al., 2007, Yokoyama et al., 2007, Zeng and Zhou, 2008). There are three primary forms of autophagy: chaperone-mediated autophagy, microautophagy and macroautophagy (Klionsky and Emr, 2000).

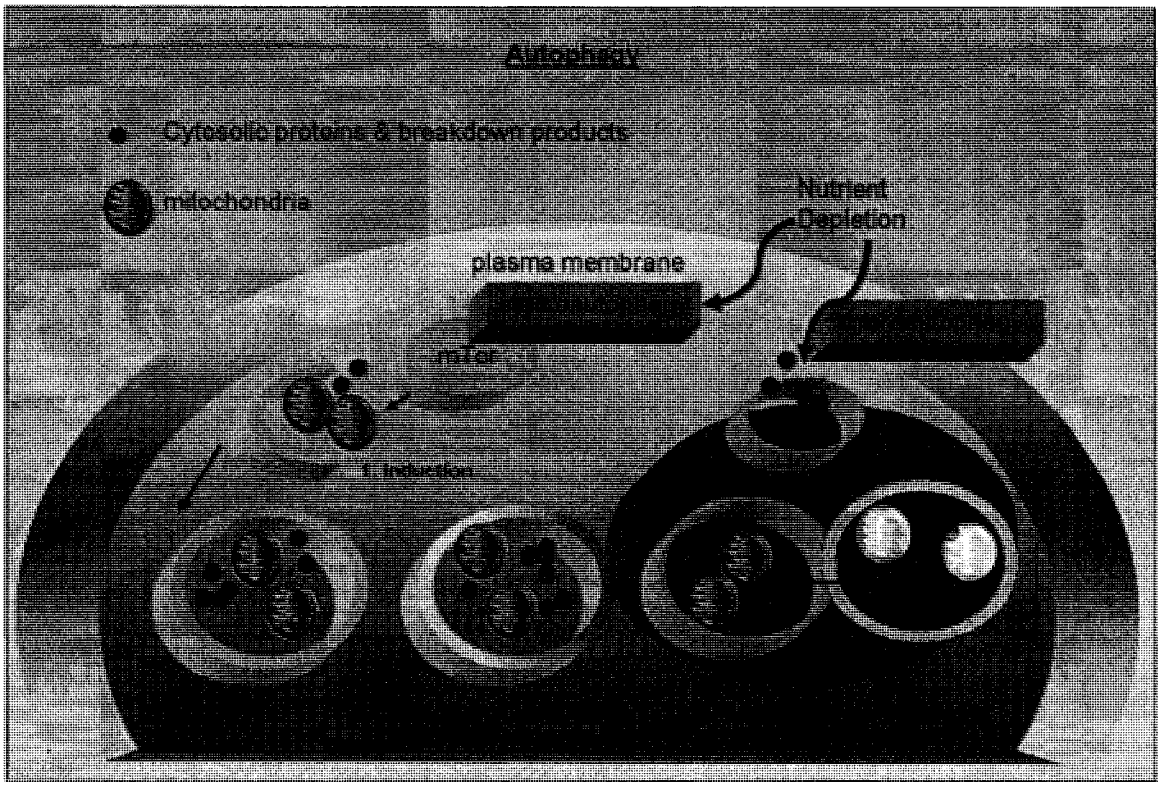

Figure 1.4. Autophagy. A generalized schematic depicting the catabolic process of autophagy, involving the degradation of cell components through lysosomes.

Chaperone-mediated autophagy results from the delivery of the proteins with the signature sequence of KFERQ to lysosomes through designated lysosomal transporters and, therefore, may be primarily involved in the degradation of specific proteins (Dice, 1990). Microautophagy occurs when a protruding or invaginating portion of pre-existing vacuolar membrane occurs in order to engulf cytosol or organelles (Dice and Terlecky, 1990). During macroautophagy, the sequestering vesicles, termed autophagosomes, fuse with the lysosome or vacuole resulting in the delivery of an inner vesicle (autophagic body) into the lumen of the degradative compartment and is the main mechanism involved in the degradation and recycling constituent of intracellular organelles (Dice et al., 1990).

Autophagy and neuronal cell death

Neuronal cell death with features of autophagy has been described in vertebrate development, such as the naturally occurring developmental death of neurons in chick isthmo-optic nucleus (ION) (Clarke, 1990). Both anterograde signals from the tectum and retrograde signals from the retina is required for the survival of developing chick (ION). The neurons of ION that make harmful projections are eliminated through formation of autophagic vacuoles. Nerve growth factor (NGF) deprivation-induced sympathetic neuronal cell death and serum deprivation-induced death of PC12 cells also seem to exhibit autophagic features (Xue et al., 1999).

A conclusive demonstration of involvement of autophagy in neuronal cell death is based on the premise that a loss of function mutation is one of the genes required for autophagy, which can also prevent neuronal cell death. Unfortunately, due to the lack of understanding of autophagy in mammalian cells such an assumption is difficult to prove. However, a functional role of autophagy in neuronal cell death has been suggested through the inhibition of autophagy and delay of neuronal cell death by 3-MA, which has also been shown to inhibit *in vitro* class III phosphatidylinositol-kinase activity, found to be required for the sequestration step of autophagy in yeast (Klionsky, 2005). Evidence supporting the role of autophagy in neuronal cell death has also implicated 3-MA, in that it has the ability to delay apoptosis in various systems, such as NGF deprivation induced death of sympathetic neurons (Xue et al., 1999); however, given the poor specificity of 3-MA this assumption must not be considered conclusive.

Autophagy in neurodegenerative diseases

Autophagy is responsible for the degradation of normal proteins and is involved in cellular remodeling found during metamorphosis, aging and differentiation, as well as for the digestion and removal of abnormal proteins that would otherwise accumulate following toxin exposure, cancer or degenerative disease (Xue et al., 1999). Autophagy is correlated with the formation of autophagosomes, autolysosomes, electron-dense membranous autophagic vacuoles (with double membranes), membranous whorls and multivesicular bodies, as well as the engulfment of entire organelles (i.e. mitochondria), which can be seen at the ultrastructural level (Klionsky and Kumar, 2006). A number of studies have demonstrated the presence of autophagy in the brain in Huntington's disease (HD). Similarly, autophagic degeneration was observed in AD patients. In 1997, several PD patients' ultrastructural examinations revealed characteristics of apoptosis and autophagic degeneration in melanized neurons of the sympathetic neurons (Stefanis et al., 2001). The results suggest that even at the final stage of the disease, the dopaminergic neurons are undergoing an active process of cell death.

Increased levels of autophagy were also found in neuronal cell lines expressing mutant proteins associated with diseases such as PD and HD. Expression of PD-associated A53T, but not wild type, α-synuclein in PC12 cells caused extensive autophagic vacuole formation (Stefanis et al., 2001). Excess amounts of vesicles were also observed in biopsy tissue from HD patients (Bahr and Bendiske, 2002). Over expression of the protein mutant, huntington, which causes the disease, was found to result in the formation of cathepsin D-positive autophagic vacuoles, which subsequently

accumulated huntington (Kegel et al., 2000). The mechanism of such formations remains unclear.

The role of pH in autophagy

Autophagy is a phenomenon that cannot be compartmentalized in context of one kind of cell death and given its ambiguous nature the role of pH remains allusive as well. However, there have been documented cases of pH playing a role in autophagic activity. During tissue ischemia, acidosis develops followed by mitochondrial permeability transition (MPT) leading to downstream pathway activation of cell death. The MPT occurs in several forms of necrotic cell death, including oxidative stress, pH-dependent ischemia/reperfusion injury and Ca^{2+} ionophore toxicity (Hunter et al., 1976, Bernardi, 1996). After the initial insult, a small fraction of mitochondria spontaneously depolarize and enter an acidic lysosomal compartment, suggesting that the MPT precedes the normal process of mitochondrial autophagy. A model is proposed in which onset of the MPT to increasing numbers of mitochondria within a cell leads progressively to autophagy, apoptosis and necrotic cell death (Dunn, 1990). Because of acidosis, anoxic cell death is substantially delayed (Lemasters et al., 1998). However, when pH is restored to normal after reperfusion (reoxygenation at pH 7.4), cell death occurs rapidly, termed the "pH paradox". This death is caused by pH-dependent onset of the MPT, which is blocked by reperfusion at acidotic pH, cell death then takes the form of autophagy (Lemasters et al., 1998).

1.5 Apoptosis

Apoptosis: programmed cell death

The term apoptosis was coined in the early 1970s, although the phenomenon of apoptosis was described already in the late 1700s (Buja et al., 1993). The word, apoptosis, is derived from Greek roots meaning, "dropping off" such as in the case of falling of leaves. Since its original morphological characterization as a distinct form of cell death, apoptosis has been widely studied for its incidence, biochemistry and genetics (Nagata, 1997). In order to characterize apoptosis, there first has to be some kind of causative factor, which activates the apoptotic pathway (Nagata, 1994). This activation is followed by induction of the apoptotic signaling, and an initiator phase, including changes in mitochondrial permeability, and activation of upstream caspases (Arends and Wyllie, 1991). The apoptotic process is then further spread to an effector phase, which ultimately leads to the actual degradation of the cell, including cytoplasmic blebbing, endonuclease activity and nuclear fragmentation and cell death (Honig and Rosenberg, 2000).

The diversity of stimuli that can activate and regulate apoptosis is extensive, and so will be the diversity of subsequent signaling pathways. A trigger for apoptosis can be a complex external signal resulting from depletion of growth factors or nutrients, accumulation of toxic metabolites, pH shifts, oxygen deprivation or excessive formation of reactive oxygen species (Arends and Wyllie, 1991, Buja et al., 1993). It can also be a more specific signal mediated through signaling, such as Fas and its corresponding receptor Fas/TNFR1. The process leading to apoptosis is regulated through various

kinases and second messengers, such as protein tyrosine kinases (PTKs), mitogen-activated protein kinase (MAPK) and Ras signaling pathways, protein kinase C (PKC), and cyclic AMP (cAMP) and Ca^{2+} related pathways (Arends and Wyllie, 1991, Hale et al., 1996). Also, loss of cell attachment in a normal healthy cell can initiate apoptotic death (Anglade et al., 1997a, Anglade et al., 1997b). The cell surface death receptor pathway and the mitochondria-initiated pathway are the major systems known to activate the executioners of caspase-dependent apoptosis.

The Fas-ligand (FasL) activated pathway is one of the best-understood initiators of apoptosis, and the corresponding receptors are widely distributed throughout the body. It is often active in cells from the immune system, and it is important in the downregulation of the immune response and elimination of self-reactive lymphocytes (Arnoult et al., 2003). The first stage of apoptosis signaling principally occurs through the interaction between extracellular ligands, FasL, TNF-α, or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and their cognate receptors such as members of the TNF receptor family, Fas, TNFR1, and TRAIL receptors, respectively (Arends and Wyllie, 1991). This is followed by the receptor trimerization and activation of the death-domain and recruitment of TNF-receptor associated death domain (TRADD) and/or FADD. These components form the death-inducing signaling complex (DISC) that recruits and activates procaspase -8, which subsequently activates downstream caspases, such as caspase-3, and finally results in apoptotic cell death (Cecconi et al., 1998). However, even if the death signal is propagated from the death receptor, it can be suppressed at several points along the pathway. There are proteins that directly inhibit the procaspase recruitment and /or activation at the level of DISC. These include death

effector domain containing proteins, FADD-like ICE inhibitory proteins (FLIP) that compete with procaspases of FADD binding (Cryns and Yuan, 1998) (Figure 1.5).

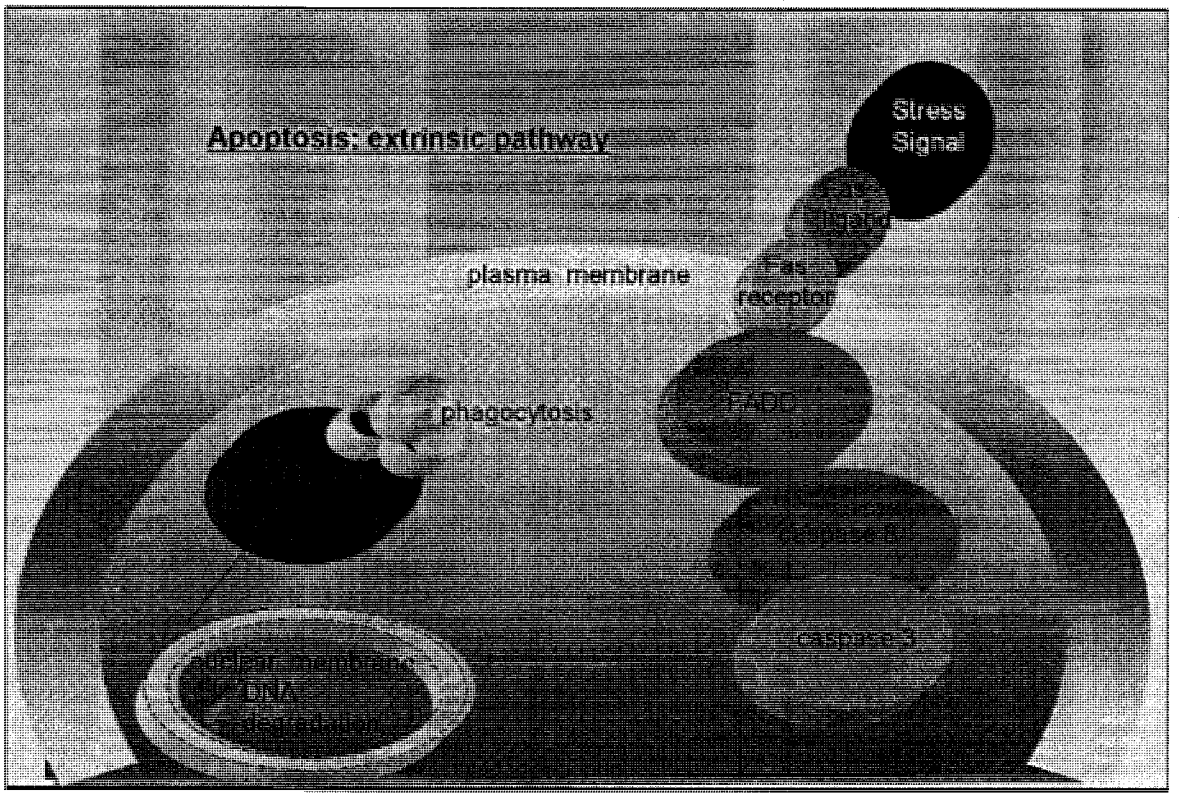

Figure 1.5. Apoptosis: the extrinsic pathway. The extrinsic pathway begins outside a cell, when conditions in the extracellular environment determine that a cell must die. Binding of Fas ligand (FasL or CD95L) to the Fas receptor (CD95) results in clustering of receptors and initiates the extrinsic pathway. Fas is a member of the so-called "death receptor" family, which includes the TNF receptor. Fas clustering recruits FADD and pro-caspase 8 to the complex. Concentration of pro-caspase 8 results in its autocatalysis and activation; activated caspase 8 cleaves pro-caspase 3, which then undergoes autocatalysis to form active caspase 3, a principle effector caspase of apoptosis.

Apoptosis is an energy-based process and the mitochondria plays a pivotal role in apoptosis. The functions of the mitochondria include the MPT and disruption of essential mitochondrial functions and release of soluble mitochondrial intramembrane proteins (SIMPs), such as cytochrome c (cyt c), procaspase-3 and apoptosis-inducing factor (AIF) (Clarke, 1990, Deshmukh et al., 2002). The suggested role of the mitochondria as the "sensor of cellular stress" might derive from their ancient endosymbiotic bacterial origin

(Petit et al., 1995, Margulis, 1996). Margulis was a supporter of the `serial endosymbiosis theory' (SET) whereby endosymbiosis could explain the origin of both microtubules and mitochondria. Margulis argued that eukaryotes originated first from a symbiotic relationship between a host archaeon (with a nucleoid membrane) and an endosymbiotic spirochete, with the spirochete providing mobility to the archaeon. The chimeric organisms generated by this symbiosis only later acquired a respiring bacterial symbiont that eventually evolved into extant mitochondria. This second endosymbiosis is well supported by the structure of mitochondria, the presence of a circular mitochondrial genome (in many eukaryotes) and the sequence of mitochondrial DNA.

The mitochondria can trigger apoptotic cell death by at least three general mechanisms: 1) disruption of electron transport and energy metabolism, 2) release of pro-apoptotic caspase activators, or 3) alteration of cellular redox potential (Shimizu et al., 1998). When cyt c is released into cytoplasm, it can activate caspases and induce apoptosis (Liu et al., 2004). Another consequence of the release may also be disruption of the electron transport chain and energy production of mitochondria (Yang et al., 1997). This, in turn, depends on the cell type and the amount of cyt c in the mitochondria; if there is excess of cyt c, the mitochondria may tolerate this release, and the electron transport chain will remain functional. Also caspase activation by cyt c depends on the amount of endogenous caspase inhibitors, which can prevent consequences of cyt c release, even when it is released in large quantities (Desjardins et al., 1985, Yang et al., 1997). The mitochondria is implicated as an important player in several neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, in

which the mitochondrial function is often disturbed (Green and Kroemer, 1998, Green and Reed, 1998) (Figure 1.6.).

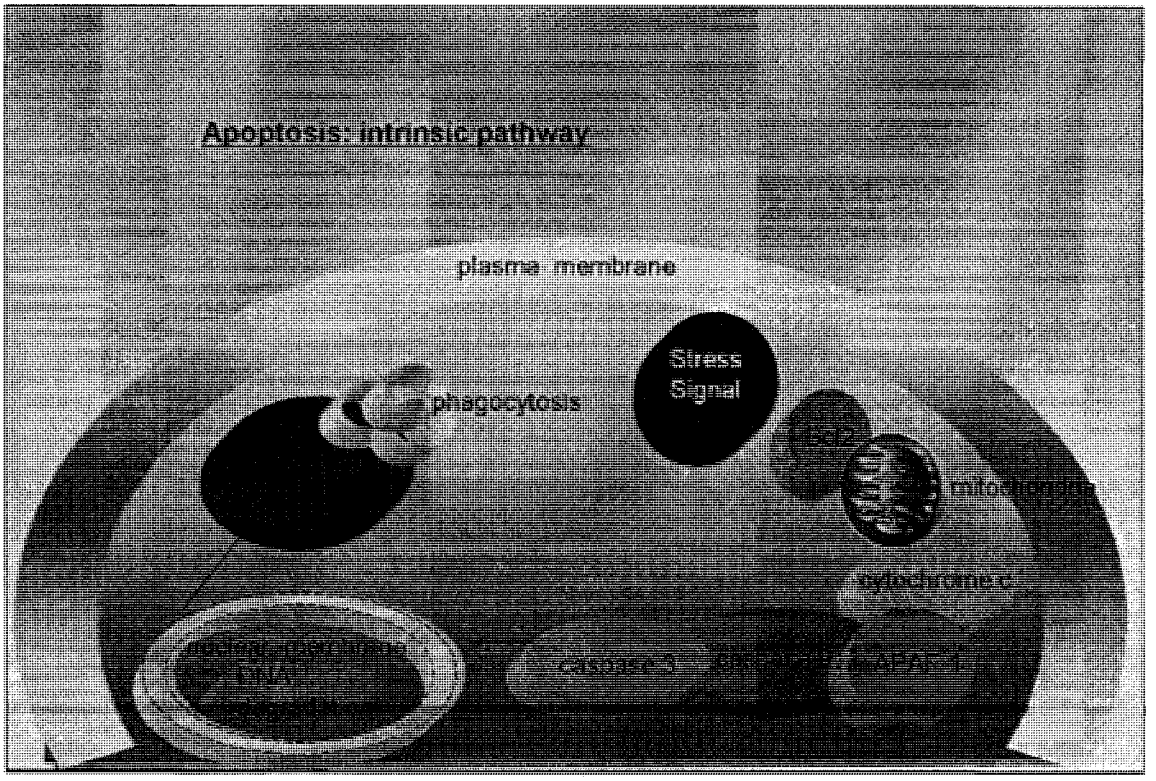

Figure 1.6. Apoptosis: the intrinsic pathways. The intrinsic apoptosis pathway begins when an injury occurs within the cell. The injury could result in necrosis and produce an inflammatory response, but the apoptotic machinery is in place to ensure that the damaged cell is packaged and removed cleanly, in order to prevent inflammation. Mitochondrial damage, for example, can initiate the intrinsic pathway, overcoming the effect of pro-survival protein Bcl2. Cytochrome c, released from damaged mitochondria, binds Apaf1, which then activates an initiator caspase, in this case caspase 9, which then activates the effector caspase, caspase 3. Other proteins released from damaged mitochondria, Smac/DIABLO and Omi/HtrA2, counteract the effect of IAPs (inhibitor of apoptosis proteins), which normally bind and prevent activation of caspase 3

One family of proteins that is critical to regulating both extracellular and mitochondrial induction of apoptosis is the Bcl-2 family (Du et al., 2000, Pawlowski and Kraft, 2000). Bcl-2 was shown to be the mammalian homolog of CED-9, recognized for its anti-apoptotic function in *Caenorhabditis elegans (c. elegans)* (Yuan et al., 1993, Jabbour et al., 2004). All members of the Bcl-2 family contain at least one of the four Bcl-2 homology domains (BH1-BH4) that are important to the homo- and

heterodimerization and thus to their function on cell survival (Leung and Wang, 1999). The anti-apoptotic proteins of the family include: Bcl-x_L, Bcl-w and Mcl-1, in addition to Bcl-2. Pro-apoptotic members of the family include the more closely related proteins that contain more than one BH-domain such as Bax and Bak, and also more distantly related proteins that contain only the BH3 domain, such as Bik, Blk, Bad and Bid (Brambilla et al., 1996). Heterodimerization is not required for anti-apoptotic function but it is essential for the pro-apoptotic function in the group of proteins that contain only the BH3 domain (Ghanem et al., 2001).

The pro-survival Bcl-2 is a 26-kDa protein that is localized in the mitochondrial, endoplasmic reticulum and perinuclear membranes (Antonsson et al., 1997). Bcl-2 belongs to the family of proto-oncogenes, but the ability of Bcl-2 to block apoptosis is different from other oncogenes; it can support cells that are destined to die without affecting the proliferation rate of the cell (Zhang et al., 2000). It is possible that the location of Bcl-2 might be a key feature of its function. Because Bcl-2 is involved in maintaining the homeostatic status, including the mitochondrial membrane status and balance of the interactions between the members of the Bcl-2 protein family (e.g. Bax) and interactions between Bcl-2 and other proteins, such as direct interaction with cyt c or apoptotic protease activating factor 1 (apaf-1), it may be one of the crucial players in the mitochondria-based apoptosis sensing system (Ghanem et al., 2001).

Caspase-dependent neuronal apoptosis in neurodegenerative diseases

Apoptotic cell death can be a feature of both acute and chronic neurologic diseases (Martin, 1999, Yuan and Yankner, 2000, Martin, 2001). After acute insults, apoptosis occurs in areas that are not severely affected by the injury. For example, after ischemia, there is necrotic cell death in the core of the lesion, where hypoxia is most severe, and apoptosis occurs in the penumbra, where collateral blood flow reduces the degree of hypoxia (Allen et al., 1988). Apoptotic death is also a component of the lesion that appears after brain or spinal cord injury (Rink et al., 1995, Crowe et al., 1997, Lei and Peeling, 1998). In chronic neurodegenerative diseases, such as Huntington's disease and Alzheimer's disease, it is the predominant form of cell death (Smale et al., 1995, Thomas et al., 1995, Stadelmann et al., 1999).

Apoptosis exhibits specific morphological characteristics including nuclear condensation and fragmentation, cytoplasmic shrinkage, plasma membrane blebbing, and exposure of phosphatidylserine (Cohen, 1997). The fragmented portions of the cells are then transported out of the cell via phagocytosis by macrophages, without inducing an inflammatory response. These same characteristics are also observed in neurons, caused by acute and chronic injury (Fadeel and Orrenius, 2005).

In the vertebrate nervous system, up to 50-80% or more of different types of neurons die before embryonic development is complete (Cecconi et al., 1998). Despite massive death during development, mature neurons are among the most long-lived cell types in mammals. Therefore, because neurons are post-mitotic cells, meaning they are not able to divide, a constant survival support is considered to be necessary for the

maintenance of neuronal cells and the nervous system (Yuan and Yankner, 2000). Apoptosis is regulated by survival and death signals such as the NGF-regulated neuronal survival pathways. NGF binding to its receptor tyrosine kinase (TrkA), activates prosurvival proteins such as phosphatidylinositol-3 kinase (PI-3K), Akt, and MAPK, which regulate apoptosis by inhibiting pro-apoptotic proteins such as Bax and Forkhead and by activating pro-survival proteins such as cAMP responsive element binding protein (CREB) and NF-κB (Yuan and Yankner, 2000). The balance is maintained between the "survival factors" that constantly reduce and inactivate the amount or activity of "death factors" cell death effector proteins to harmless levels (Conradt and Horvitz, 1998). Eventually, at a so-called cell death and survival checkpoint, the ratio between anti-apoptotic Bcl-2 and pro-apoptotic Bax determines whether neurons will survive or die by apoptosis (Datta et al., 1999). If the survival factors, such as growth factors, are inadequate, the amount of anti-apoptotic restraining force might be insufficient, and consequently the cell may proceed into apoptosis following cytochrome c release and caspase activation.

The role of growth factors in apoptosis

Important neurotrophic factors include NGFs, neurotrophins -3 and -4, neuropoietins, insulin-like growth factors (IGFs), transforming growth factors (TGFα), BDNF and fibroblast growth factors (FGFs). These factors, as well as others, regulate the growth and survival of neurons, both during development and adult life, by signaling through specific cell surface receptors and several downstream messengers and pathways

(Motoyama et al., 1995). The major survival pathways that are activated by these factors, and that have been repeatedly implicated in neuronal survival are the PI-3K and MAPK, or extracellular signal regulated protein kinase (ERK) pathways (Putcha et al., 2001) (Figure 1.7).

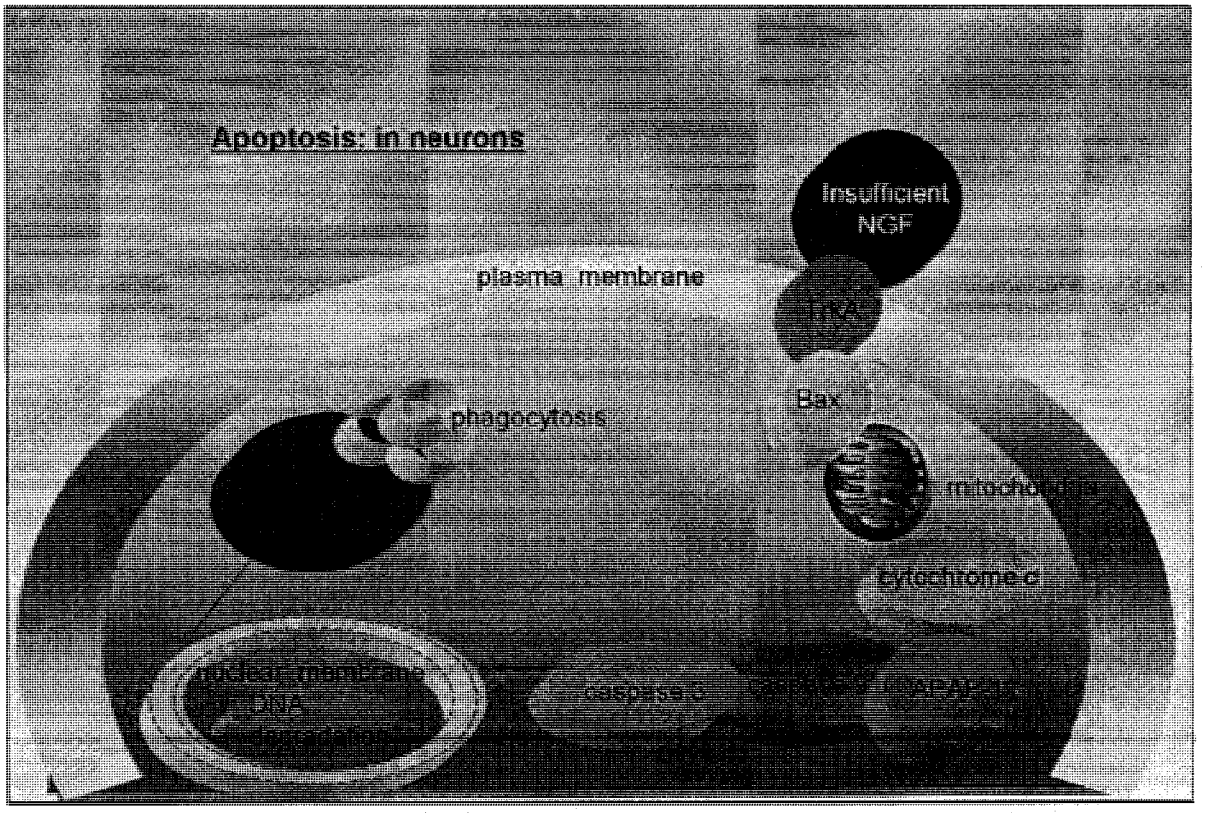

Figure 1.7. Apoptosis in neurons. Apoptosis in neurons is regulated by survival and death signals such as the nerve growth factor (NGF)-regulated neuronal survival pathways. If the survival factors (e.g. growth factors) are inadequate, the amount of anti-apoptotic restraining force might be insufficient, and consequently the cell may proceed into apoptosis following cytochrome c release and caspase activation.

1.6 Growth factors

Implications for growth factors in neuroprotection

The neurotrophin family of proteins plays an important role in determining the survivability of neurons during embryonic development as well as in the adult (Castren,

2004). Neurotrophins such as BDNF can mediate neuronal survival and activity-dependent plasticity by binding to two receptors, either the Trk family of RTKs (receptor tyrosine kinases) and the p75NTR (p75 neurotrophin receptor)(Beck et al., 1994). The receptors may be activated by the mature or precursor neurotrophin and, therefore, elucidating the cellular mechanisms associated with neurotrophins is multifarious and remains ambiguous (Tabakman et al., 2004).

Neurotrophins: Trk and p75 receptors

Embryologists in the early 1950s discovered and characterized a family of proteins expressed in the mammalian brain, they coined the neurotrophins, and consisted of NGF, BDNF, NT3 (neurotrophin 3) and NT4 (Andrews, 2003). They postulated that these neurotrophins mediated processes in the development of the nervous system as well as controlled functions in the adult brain (Ebadi et al., 1998). These impacts on the brain were classified into two categories: the first being that they act as growth factor support for adult neurons as well as activity-dependent plasticity (Pardridge, 2002b).

Two types of receptors are implicated in mediating neurotrophic function, the Trk family of RTKs and p75NTR (Samii et al., 1994). The process is as follows: the Trk receptor dimerizes and becomes catalytically active and undergoes autophosphorylation once the ligand has been bound. This results in activation of the MAPK, PI3K and phospholipase C-γ1 pathways(Bibel and Barde, 2000), which act to intercept nuclear and mitochondrial cell-death programs(Chao, 2003).

The activation of the MAPK pathway is essential for the neurotrophin-induced differentiation of neuronal and neuroblastoma cells. Several pathways can lead from Trk receptors to MAPK activation, most involving the phosphorylation of tyrosine residues in the Trk receptors. The various MAPKs activated through Ras and Rap1 have different downstream targets that mediate gene transcription via activation of several different transcription factors resulting in diverse biological responses (Alberch et al., 2002, Chao, 2003, Castren, 2004).

The recruitment of PI3K to activated Trk receptors is critical for receptor-mediated survival signalling via second messengers that activate the serine threonine kinase Akt (also known as protein kinase B). Substrates of Akt include proteins involved in several steps of the cell death pathway including: forkhead transcription factor (FKHRL1), Bad, Caspase-9, GSK3β, and NFκB), all of which can be intercepted by Akt (Ibanez, 1995, Kaplan and Miller, 2000).

In contrast, activation of the p75NTR receptor has been implicated in neuronal cell death through its activation of the JNK1 (c-Jun N-terminal kinase) pathway and the p53 pathway, as well as through the neurotrophin-receptor-interacting factor (NRIF) (Haapasalo et al., 1999). However, the opposite has also been found, where the p75NTR receptor has been shown to promote neuronal cell survival through its activation of the NF-κB transcription factor, following its interaction with NGF. p75NTR can also promote neurite outgrowth and cell migration (Ebadi et al., 1998). Trk receptors can play a part in neuronal cell death. Therefore, neuronal cell fate seems to be a balancing act that is in part determined by the associated signalling molecules elicited upon activation of the receptor.

Neurotrophins and neuronal fate

Neurodegeneration has been at the forefront of research for decades, however the mechanism through which a neuron survives under stress remains uncertain. Neurotrophins have been implicated in mediating death and survival in both the developing and adult brain. In particular, a decrease in Trk receptor expression and subsequent decrease in trophic support has been shown to increase cell loss (Wu and Pardridge, 1999). Also, the p75NTR has been shown to have increased expression during brain injury causing degenerative effects following the trauma (Huang and Reichardt, 2003). Expression of p75NTR is increased in rat motor neurons following sciatic nerve lesion (Huang and Reichardt, 2003), in the hippocampus following seizure and is also associated with neuronal degeneration following experimentally induced ischemia and AD associated with apoptotic cell death due to binding of b-amyloid (Hennigan et al., 2007).

Neurotrophins and neuroprotection

The neurotrophins have been shown to be crucial mediators of neuroprotection against several deleterious diseases leading to neuronal cell loss (Pardridge, 2002a). For instance, Huntington's disease is a neurodegenerative disorder characterized by a selective degeneration of striatal projection neurons (Gratacos et al., 2001). Neuroprotective therapy could be achieved with the knowledge of the specific trophic requirements of these neuronal populations (Alberch et al., 2002). Thus, the induction of

endogenous trophic response or the exogenous administration of neurotrophic factors may help to prevent or stop the progression of the illness (Hennigan et al., 2007).

Excitotoxicity has been implicated in the etiology of HD, because intrastriatal injection of glutamate receptor agonists reproduces some of the neuropathological features of this disorder (Castren, 2004). Neurotrophins, BDNF, NGF, NT-3 and NT-4 have been investigated as potential candidates to provide selective protection for basal ganglia circuits, which are affected in striatonigral degenerative disorders, such as HD or multisystem atrophy (Huang and Reichardt, 2003).

NGF act as an endogenous neurotrophic factor for central cholinergic neurons, which are depleted in AD (Kazanis et al., 2004). In animal studies mainly involving rodents, intracerebroventricular (ICV) administration of NGF can rescue the cholinergic neurons, stimulate axonal growth, and improve cholinergic function (Skaper et al., 1998). Similarly, intracerebroventricular injection of NGF attenuated neuronal death in the hippocampi of gerbils subjected to brain ischemia (Wu, 2005).

Although the underlying mechanisms of PD remain unknown, one promising approach involves the potential therapeutic use of neuregulin glial growth factor-2 (GGF2), a neural growth/differentiation factor as a neuroprotective agent for the damaged dopaminergic nigrostriatal system (Wu, 2005). Evidence, *in vitro*, shows that GGF2 can function as a neurotrophic factor for nigrostriatal dopaminergic neurons by enhancing striatal dopamine release (Zhang et al., 2004).

1.7 Stress and cell death

The need for therapeutic cures

As more people become afflicted with debilitating neurodegenerative diseases the need to counteract neuronal stress becomes a pivotal step in the research realm. In particular, the literature indicates that there is a direct link between alkalosis the generation of ROS and cell death (Loeper et al., 1991, Klein and Ackerman, 2003). Results have shown that that the loss of antioxidant genes, such as SOD, can lead to neuron loss highlighting the need to further explicate agents with antioxidant capacities. Moreover, increases in ROS have been directly linked to cell cycle arrest and neurodegenerative diseases as ROS may directly cause the upregulation of anti-survival genes (Feddersen et al., 1992). The brain and nervous system are prone to oxidative stress, due to high energy demands and O_2 consumption thus, therapeutic applications which may bolster the antioxidant defense systems to prevent irreversible oxidative damage may attenuate the effects of neurodegenerative diseases. Oxidative stress may lead to mitochondrial dysfunction, accumulation of oxidized aggregated proteins, inflammation, and defects in protein clearance due to deleterious gene transcription. This complicated interplay displays the dire need to investigate therapeutic agents which may be able to attenuate cell degeneration.

The potential neuroprotective role of peptides during times of stress

The potential for neuropeptides to regulate brain processes during times of stress is an important facet in the search for novel ways of coping with neurodegenerative diseases and physiological stress and examples of neuropeptides being connected with therapeutic uses are plentiful.

For instance, studies have shown that the neuropeptide vasoactive intestinal peptide (VIP) is implicated as a potent dilator of blood vessels, increases blood flow and improvement of bodily functions. Its expression is reduced in the aging brain and its inhibition in gene activity resulted in loss of memory, decreased learning abilities and inhibition of sexual function (Glazer and Gozes, 1994, Gozes et al., 1994). Based on these findings it became apparent that studies concerning VIP gene functions could lead to the synthesis of novel neuropeptide-based drugs. These specific peptides are designed to penetrate barriers to the brain and skin and VIP has been shown to be essential for embryonic brain development, with its inhibition resulting in severe microcephaly (Gozes et al., 1994). Furthermore, VIP inhibiting molecules may play important roles in breast, lung, neuroblastoma and colon cancer therapy (Gozes et al., 1994).

A key invention in those studies is the use of hybrid molecules, such as hybrids between proteins and fatty substances allowing better biological availability. For instance, activity-dependent neurotrophic factor (ADNF) acts in extremely low concentrations (10-100 molecules/cell) making it an attractive candidate for drug design (Gozes and Brenneman, 1996). A short fragment derived from ADNF, a peptide ADNF-14, prevented neuronal cell death from the beta amyloid peptide, a substance implicated

in the death of nerve cells in Alzheimer's disease. Another promising study indicated that nerve cells grown in culture were protected by ADNF-14 from a toxic protein (gp120) shed by the human immunodeficiency virus, the causative agent in AIDS (Gozes et al., 1999).

Neuropeptide Y (NPY), a 36-amino-acid peptide widely expressed in the brain (de Quidt and Emson, 1986) is involved in many physiological responses, including hypothalamic control of food intake and cardiovascular homeostasis. NPY was shown to reduce blood pressure and catecholamine release and provided novel evidence for an important physiological role of this endogenous peptide in blood pressure regulation and protection against sympathetic hyperexcitation (Michalkiewicz et al., 2001, Michalkiewicz et al., 2003). NPY mediates its effects through binding to the Y1, Y2 and Y5 G-protein-coupled receptors (Rose et al., 1995). Little is known of the role of the Y2 receptor in mediating the different NPY effects. However, upon inactivating the Y2 receptor subtype in mice it was observed that these mice developed increased body weight, food intake and fat deposition. The null mutant mice showed an attenuated response to leptin administration but a normal response to NPY-induced food intake and intact regulation of re-feeding and body weight after starvation. An absence of the Y2 receptor subtype also affected the basal control of heart rate, but did not influence blood pressure (Naveilhan et al., 1999). These findings indicate an inhibitory role for the Y2 receptor subtype in the central regulation of body weight and control of food intake. NPY is also expressed in the neurons that control blood pressure and upon administration, NPY reduced blood pressure and anxiety, together with lowering sympathetic outflow. The generation of neuropeptide Y transgenic rats overexpressing this peptide, under its

natural promoter, has allowed for the examination of endogenous neuropeptide Y in the long-term control of blood pressure by the sympathetic nervous system (Michalkiewicz et al., 2003).

In context of brain ischemia, neurotrophins such as BDNF are potential neuroprotective agents that could be used in the treatment of acute stroke, should these proteins be made transportable through the blood–brain barrier (BBB) *in vivo* (Zhang and Pardridge, 2001). One approach to the BBB problem is to attach the nontransportable peptide to a brain targeting vector, which is a peptide or peptidomimetic monoclonal antibody (MAb), which is transported into brain from blood via an endogenous BBB transport system, which has shown to significantly reduce stroke volume. Thus, brain ischemia can be treated by neuropeptides with non-invasive intravenous administration of neurotrophins such as BDNF, providing the peptide is conjugated to a BBB drug targeting system.

The literature has revealed that certain peptides may act in a neuroprotective capacity; therefore the objective of this research has been to investigate TCAP as a potential candidate. This peptide is located on the extracellular face of a type II transmembrane protein, teneurin, and exhibits bioactive characteristics which implicate it as a potential endogenous agent which may procure survivability to compromised neurons.

1.8 Teneurin

Introduction to teneurins

The function of the invertebrate homologues of the glycoprotein tenascin-C was investigated in the early 1990s (Tucker et al., 2006). A low stringency screen of a *Drosophila* library using a tenascin-C probe revealed two novel molecules: Ten-a (tenascin-like molecule accessory) and Ten-m (tenascin-like molecule major), however, beyond a series of tenascin-type epidermal growth factor (EGF)-like repeats in both Ten-a, Ten-b there is no similarity with vertebrate tenascins (Tucker et al., 2007). The name, teneurin, came from a blend of Ten-a and the fact that there is common neuronal expression during development (Minet et al., 1999a). Ten-a and Ten-b were then subsequently characterized in embryonic mouse brain via in situ hybridization followed by homologues in zebrafish, chicken, rat and *C. elegans* (Mieda et al., 1999, Minet et al., 1999a, Oohashi et al., 1999, Otaki and Firestein, 1999, Minet and Chiquet-Ehrismann, 2000, Drabikowski et al., 2005). Altogether, 4 teneurins have been found, teneurin 1-4 (Minet and Chiquet-Ehrismann, 2000).

Characterization of the teneurin protein

All four teneurins share certain characteristics including a molecular mass of ~300kDa composed of 2500-2800 amino acids with a single membrane-spanning helical domain and a predicted Type II transmembrane protein structure (Tucker et al., 2006). The intracellular domain contains two EF-hand-like putative Ca^{2+} binding sites and a

proline rich stretch characteristic of SH3- binding sites for CAP/ponsin and potentially links the intracellular domain to the actin-based cytoskeleton, which may be mediated by vinculin (Nunes et al., 2005).

On the extracellular domain there are eight conserved tenascins-type EGF-like repeats characterized by replacement of cysteines at repeats 2 and 5 with tyrosines leading to dimerization (Rubin et al., 1999a). This stretch is followed by a region of 700-800 amino acids (aa) common to only teneurins containing 17 cysteines. This is subsequently followed by 26 YD repeats described elsewhere only in some prokaryotic proteins such as the *rhs* elements of *Escheria coli* (Feulner et al., 1990). In the most distal region of the C-terminus there is a consensus sequence RXRR, which leads to the possibility that this region is a phylogenetically conserved proteolytic processing site, presumably for furin. (Tucker et al., 2001) (Figure 1.8).

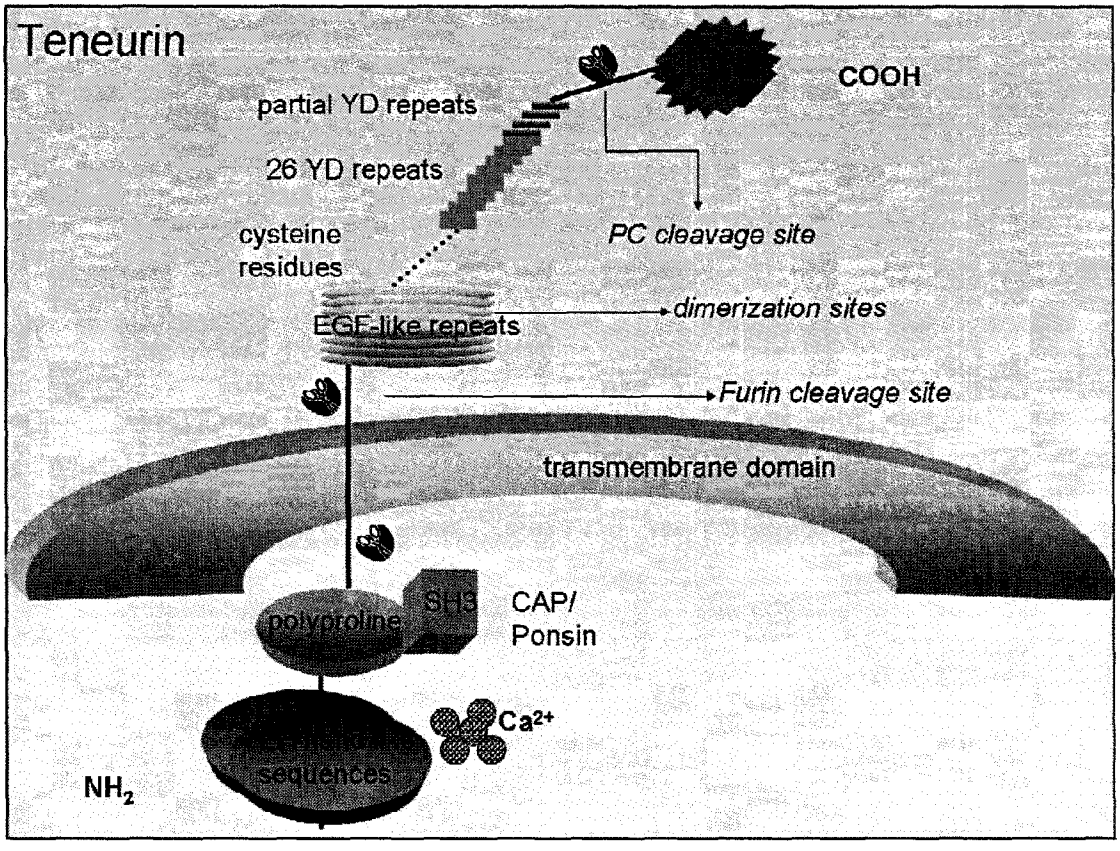

Figure 1.8. Teneurin. Schematic representation of teneurin domains and potential cleavage sites.

As the name suggests the expression patterns of the teneurins have been localized to subpopulations of neurons predominantly throughout development, in particular neurons of the developing visual system (Minet et al., 1999a, Rubin et al., 1999a). To a lesser extent, expression has been reported in other tissues relating to pattern formation, cell migration and muscle attachment points (Baumgartner et al., 1994, Levine et al., 1994, Drabikowski et al., 2005). Interestingly, certain X-linked mental retardation syndromes have been mapped to chromosome Xq25 of the teneurin-1 gene, this may caused by a teneurin-1 mutation (Minet et al., 1999a). Also, ten-1 expression knocked down by RNAi in *Caenorhabditis elegans* led to deficits in neuronal migration and cell growth (Drabikowski et al., 2005).

The teneurin system consist of 4 conserved genes that are expressed in all bilateral metazoans examined to date however, little is known about the roles of these genes during ontological development ((Rubin et al., 1999b, Lovejoy et al., 2006, Tucker and Chiquet-Ehrismann, 2006, Kenzelmann et al., 2007, Tucker et al., 2007). The teneurins have been best studied in embryonic tissue. All four teneurins are expressed in the developing brain. For instance teneurin-1 mRNA in the murine cerebellum is primarily expressed in the granular layer, whereas teneurin-2 is also expressed in the Purkinje cells along with Teneurin-3, which shows predominant expression in the same region, however; Teneurin-4 is expressed in both Purkinje cells as well as cerebellar white matter (Zhou et al., 2003). Both teneurins 3 and 4 are expressed earlier in development, between E7.5-E12.5, whereas teneurin 1 is expressed initially at E15.5 in both mice and chicken (Minet et al., 1999b, Zhou et al., 2003). At E15.5 the pituitary is undergoing formation, in particular Rathke's pouch (Scully and Rosenfeld, 2002).

1.9 The teneurin C-terminus associated peptides (TCAP)

Introduction to the TCAP family

The teneurin C-terminus associated peptides (TCAP) are a recently discovered class of corticotrophin releasing factor (CRF)- related neuropeptides with a conserved amidation signal sequence GKR located at the C-terminus of all 4 teneurins. TCAP was originally discovered using a low-stringency screening of rainbow trout (*Onchorynchus mykiss*) hypothalamic cDNA library with a mammalian stress hormone probe, urocortin, characterized by having affinity to CRF receptors (Qian et al., 2004).

The neuromodulatory effects of TCAP

TCAP has been implicated in the regulation of stress and anxiety in adult rats (Qian et al., 2004, Wang et al., 2005, Lovejoy et al., 2006). For instance, repeated intracerebralventricular (ICV)TCAP-1 injections produce enduring changes in behavioural responses to CRF in rat models of anxiety. TCAP-1 was administered into the cerebral ventricles once per day for 5 days to rats. At 1-3 weeks after the last TCAP-1 treatment, the rats were tested in the elevated plus maze, open field test, or the acoustic startle test, with or without an acute CRF injection 30 min prior to the test. The results showed a difference in behavioral response between TCAP-treated and saline-treated rats, but only when an acute CRF challenge is delivered prior to testing. In the plus maze and open field tests, acute CRF effects were enhanced by prior TCAP-1 treatment, whereas in the acoustic startle test, the acute CRF effects were diminished by prior TCAP-1 administration (Tan et al., 2008).

Furthermore, TCAP-mediating anxiety-related behaviour in male rats was investigated following repeated intravenous (IV) TCAP-1 administration with either an acute ICV or IV CRF challenge. TCAP-1 significantly affected CRF-regulated behaviours depending on CRF's mode of injection. In both the elevated plus-maze and the open field tests, TCAP-1 had an anxiolytic effect on ICV CRF responses as indicated by decreased stretched-attend postures in the elevated plus maze and increased center time and center entries in the open field. However, prior TCAP-1 treatment has an anxiogenic effect on the IV CRF-induced behaviours (decreased center entries and total distance in the open field). TCAP-1's actions are not mediated through acute changes in

glucocorticoid levels and may occur via a central action in the brain. A fluorescently (FITC)-labeled TCAP-1 analog was IV-administered to investigate whether IV TCAP-1 has the potential to regulate central mechanisms by crossing the blood-brain barrier. FITC-TCAP-1 was detected in blood vessels and fibers in the brain indicating that uptake into the brain is a possible route for its interaction with CRF and its receptors. Thus, TCAP may modulate CRF-associated behaviours by a direct action in the CNS (Al Chawaf et al., 2007b).

TCAP-1 has also been shown to play a role in the regulation of neurite growth in immortalized mouse hypothalamic neurons and rat hippocampal primary cultures. Synthetic mouse/rat TCAP-1 was used to treat cultured immortalized mouse hypothalamic cells, to determine if TCAP-1 could directly regulate neurite and axon growth. TCAP-1-treated cells showed a significant increase in the length of neurites accompanied by a marked increase in beta-tubulin transcription and translation. Primary cultures of E18 rat hippocampal cells were also treated with 100 nM TCAP-1. The TCAP-1-treated hippocampal cultures showed a significant increase in both the number of cells, dendritic branching and the presence of large and fasciculated beta-tubulin immunoreactive axons (Al Chawaf et al., 2007a). These data suggest that TCAP acts, in part, as a functional region of the teneurins to regulate neurite and axonal growth of neurons.

Further neuromodulatory effects of TCAP-1 were also observed in *in situ* hybridization studies which showed that the teneurin-1 mRNA containing the TCAP-1 sequence is expressed in regions of the forebrain and limbic system regulating stress and anxiety. TCAP-1 induces a dose-dependent change in cAMP accumulation and MTT

activity in immortalized mouse neurons (Wang et al., 2005). Administration of synthetic TCAP-1 into the basolateral amygdala significantly increases the acoustic startle response in low-anxiety rats and decreases the response in high-anxiety animals in a dose-dependent manner. When 30 pmol TCAP-1 is administered into the lateral ventricles each day for 5 days, the sensitization of the rats to the acoustic startle response is abolished (Wang et al., 2005).

The mechanism through which TCAP confers these cellular modulations remains illusive. Although TCAP and teneurin do have overlapping expression in the hippocampus, olfactory bulb and the cerebellum there are tissues which express only TCAP; for example, the cortex and thalamus. Therefore, TCAP may act independently from the teneurins, possibly via cleavage from the teneurin pro-protein at either the teneurin cleavage site or at dibasic cleavage sites in the last exon of teneurin. Also, studies have shown that TCAP does not mediate signal transduction shifts via the CRF receptor system. It may be the case that TCAP may potentially act via a separate receptor or as a tethered portion of the teneurin molecule, it may also act on another teneurin molecule and/or mediate signal transduction via regulated intramembrane proteolysis.

The literature has revealed that the teneurin proteins act in several capacities to modulate neuronal development act as well as affect certain transcription factors. These large proteins may therefore act as a means by which the neurons maintain growth and stability. Furthermore, our laboratory has discovered a bioactive segment of the teneurins, TCAP, which has been shown to mediate neuronal viability, regulation and morphology. These neuropeptides could therefore play an integral protective role during times of physiological stress. By analyzing TCAP's various roles in the prevention of cell death, a

better understanding of neuronal processes and novel therapies for treating neurodegenerative diseases may be achieved.

1.10 Thesis Rationale

Deducing the potential neuroprotective effects of TCAP

The current literature on TCAP reveals it to be an efficacious neuromodulatory agent. TCAP has been shown to be expressed in regions associated with the limbic system and, therefore, may act in a homeostatic fashion to regulate vital functions such as stress and how the body copes with it. Furthermore, TCAP has been shown to affect the physiology of cells. In particular, the proliferative effects of this peptide *in vitro* reveal that TCAP may be an effective neuroprotective agent against certain types of neuronal cell stress, which would otherwise detrimentally decrease neuronal populations.

Working hypothesis & Objectives

It is the objective of this thesis work to investigate the potential neuroprotective capacities of TCAP-1 under neuronal stress. By duplicating several form of stress and cell death in neuronal cultures and subsequently treating these cells with TCAP, a better understanding of whether it may act in a neuroprotective fashion may be deduced and an understanding of how it confers survivability may be attained. The elucidation of the neuroprotective capacities of TCAP may potentially reveal a novel therapeutic agent which may be used to attenuate neuronal death. I have utilized a series of immortalized

and hippocampal cells derived from primary cells to achieve 4 objectives outlined below characterizing the protective capacities of TCAP-1 against several forms of neuronal cell stress as well as investigates the potential mechanism by which TCAP may confer cell survival.

Objective 1 – Characterizing the role of TCAP-1 in preventing alkalotic-induced necrotic cell death by stimulating superoxide dismutase and catalase activity in immortalized mouse hypothalamic cells

The first objective was to identify and characterize the neuroprotective effects of TCAP-1 in pH induced-necrotic death. Under necrotic cell death, fluxes in pH mediate a series of events that eventually lead to compromised cell integrity; thus it was my aim to deduce the mechanism through which TCAP-1 confers neuroprotection under this oxidative stress paradigm. I report that TCAP-1 upregulates radical oxygen species (ROS) scavenging systems, namely SOD1 and catalase, in order to confer neuroprotection.

Objective 2 – Investigating the effects of TCAP-1 in attenuating etoposide and 5-FU induced apoptosis in immortalized mouse hypothalamic and hippocampal cells

The second objective was centered on deducing TCAP's potential neuroprotective effects against an apoptotic-induced cell death and in particular whether TCAP-1 mediated survival via preventing the caspase cascade as well as maintaining cell

synthesis. I have shown that the TCAP-1 induces cell survival during periods of etoposide and 5-FU induced cellular death in immortalized mouse hypothalamic and hippocampal cells in a dose response manner by inhibiting caspase 3 cleavage and upregulating cell synthesis. Furthermore, TCAP's effects on neurogensis may be accountable for the production of pro survival factors such as Bcl-2 or may upregulate growth factors which in turn activate radical oxygen scavenging systems to prevent cell degradation during apoptotic stress.

Objective 3 – Utilizing a TCAP siRNA to deduce whether TCAP-1 may confer neuroprotection against induced programmed cell death by increasing BDNF and decreasing caspase 3 expression *in vitro*: implications for survivability and morphology during neurodegenerative stress

The third objective was to investigate the possibility of TCAP-1 acting on neurotrophic factors to confer a neuroprotective effect. I have shown that the chronic TCAP-1 increases BDNF expression in vitro. Furthermore I utilized a TCAP siRNA in order to determine the effects of TCAP on cell development and morphology and to explore whether knocking down TCAP would augment neurodegeneration by 5-fluorouracil induced apoptosis by degrading neurite growth and proliferation.

I have shown that two TCAP siRNA designs effectively degenerate cell morphology. In addition, I have shown that TCAP-1 significantly increasing the survivability of cells under PCD via increasing BDNF and decreasing caspase 3 protein and mRNA

expression, thus conferring survivability to neurons against cell proliferation inhibition and neurodegeneration.

Objective 4- Deducing whether TCAP-1 may confer neuroprotection against glutamate excitotoxicity *in vitro*

Glutamate-induced neuronal damage contributes to several neurological diseases including ischemia and several studies have focused on characterizing methods of antagonizing glutamate neurotoxicity (Choi, 1990). I wanted to determine whether chronic pre incubation with TCAP-1 would attenuate glutamate excitotoxicity which has been implicated in a series of deleterious diseases. I have shown that several neuronal cell lines that have been pre incubated with TCAP-1 show attenuated cell degeneration during glutamate excitotoxicity via SOD upregulation.

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Chapter 2: Characterizing the role of TCAP-1 in preventing alkalotic induced necrotic cell death by stimulating superoxide dismutase and catalase activity in immortalized mouse hypothalamic cells

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TCAP is implicated in the regulation of neuron growth and differentiation. However, evidence suggest that TCAP-1 may also have a neuroprotective action during times of pH-induced cellular stress in the brain such as during hypoxia-ischemia and brain alkalosis. To test this hypothesis, a TCAP-1-responsive mouse hypothalamic cell line was cultured (N38), using media buffered at pHs 6.8, 7.4, 8.0 and 8.4 subsequently treated with 100nM TCAP-1. TCAP-1 significantly inhibited the decline in cell proliferation at pHs 8.0 and 8.4 as determined by direct cell viability assays and decreased the incidence of cells showing necrotic morphology. In addition, TCAP-1 decreased the number of cells undergoing necrosis by 4- to 5-fold as measured by uptake of ethidium homodimer III. Moreover, TCAP-1 significantly decreased the incidence of superoxide radicals and increased SOD1 expression. These results were accompanied by an increase in SOD copper chaperone expression and increased catalase activity and expression. The results indicate that TCAP may play a neuroprotective role during periods of pH stress by up-regulating oxygen radical scavenging systems. Thus, the TCAP/teneurin system may be part of a mechanism to protect neurons during trauma, such as hypoxia and ischemia.

2.1. Introduction

We have reported that TCAP, is active *in vivo* and *in vitro*. Furthermore, we have observed that TCAP-1-treated cells exhibit higher survivability under stressful conditions such as high confluency compared to vehicle-treated cells (Wang and Lovejoy, unpublished observations). This observation is consistent with the findings of Wang and associates (1998) who reported that a number of stress-inducing situations such as alkylating agents and UV light can trigger the activation of mouse teneurin-4. Taken together, these studies suggest that TCAP may induce long-term changes in neuron function and may confer enhanced survivability during stressful conditions.

Although the molecular and cellular mechanisms associated with this loss of neural function includes a number of apoptotic, necrotic and excitotoxic processes, metabolic perturbation of the CNS will necessarily involve disruptions in pH. For example, ischemia or myocardial infarct can result in neuronal acidosis (pH<7.4) due to the low pO_2 relative to the high metabolic activity of tissues (high pCO_2). On the other hand, hypoglycemia (low lactate concentrations), hyperventilation (high pO_2) or renal alkalosis (high plasma HCO_3^-) can all induce neural alkalosis (pH>7.4) (Mabe et al., 1983, Giffard et al., 1992, Ostermann et al., 2003, Auer, 2004, Emerit et al., 2004). External pH (pH_e) will eventually affect intracellular pH (pH_i) concentrations 20 to 70% of pH_o depending on the sensitivity of the neuron (Bouyer et al., 2004). Neural excitability in most regions of the brain is suppressed by acidosis and enhanced by alkalosis (Vornov et al., 1996, Xu et al., 1998, Huang and Dillon, 1999, Emerit et al., 2004). Alkalosis generally evokes increased activity leading to aberrant afferent and

efferent firing. Previous studies have shown a relationship of changes in reactive oxygen species metabolism and signal transduction pathways using pH ranges of 6.8 to 8.8 (Giffard et al., 1992, Majima et al., 1998, Susa and Wakabayashi, 2003, Potapenko et al., 2004).

The aim of the current study is to determine the pathway through which TCAP-1 induces cell survival in the brain during periods of pH-induced cellular stress. These data indicate that mouse TCAP-1 enhances the survival of immortalized mouse hypothalamic cells at pH of 8.0 and 8.4, in part, by inhibiting oxidative necrosis. TCAP-1-treated cells show a decrease in the intracellular superoxide radical concentrations along with a concomitant increase in superoxide dismutase 1 protein expression. Moreover, the superoxide dismutase copper chaperone mRNA and catalase mRNA and activity is enhanced with TCAP-1 treatments.

2.2 Materials & Methods

Peptide synthesis

Mouse TCAP-1 was prepared by solid-phase synthesis as previously described (Qian et al., 2004). The peptide was solubilized in phosphate buffered saline (PBS) at a stock concentration of $2x10^{-5}$ M before being diluted in the appropriate medium. Non-functional scrambled TCAP with the same amino acid composition as TCAP-1 (pEESQRMFHINNVSLVFYGDYGQVRGTGLLQASDSLELYQEI-NH_2) was

synthesized as described previously (Qian et al., 2004, Al Chawaf et al., 2007) and used as a negative control in the vehicle.

Cell morphology analysis

The effect of TCAP-1 on cell morphology was conducted in quadruplicate using the N38 immortalized mouse hypothalamic cell line (Belsham et al., 2004). The cells were grown in culture plates with 2 mL of Dulbecco's Modified Eagle Medium (DMEM) containing high glucose, L-glutamate, 25mM HEPES buffer, pyridoxine hydrochloride in the absence of sodium pyruvate and 5ml penicillin with 10% fetal bovine serum (FBS) at pH 7.4 (Gibco-Invitrogen, Burlington, Canada). At 24 and 48 hrs, the medium was replaced with medium buffered at pH 6.8, 7.4, 8.0 or 8.4. Half of the cell groups received 10^{-7}M TCAP-1, whereas the other half received 0.01 M PBS (pH 7.4) containing 8g NaCl, 0.2gKCl, 1.4g Na_2HPO_4 and 0.2g KH_2PO_4 in 800mL ddH_2O. Digital images were recorded at 24, 48 and 72 hrs using an Olympus IX&1 inverted microscope at 100x magnification using ImageMaster Ver 5.0 software.

Cell proliferation and viability analysis

The effect of TCAP-1 on cell proliferation at each pH was examined at 24 and 48 hr directly by cell counts using a hemocytometer, and indirectly using a colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. For hemocytometer counts, the cells were suspended using 1mL of 0.25% trypsin with EDTA

(Gibco-Invitrogen, Burlington, Canada), centrifuged at 1600 RPM for 4 min, and resuspended with PBS and counted as 50μL aliquots. The MTT assay was conducted using the In Vitro Toxicology Assay Kit: MTT based (Sigma, St. Louis). The background absorbance of the samples determined at 690nm and subtracted from the 570nm measurement.

Colorimetric Caspase Assays

Caspase 8 and 3 colorimetric assays were performed on the N38 cells at all pH conditions. The assay was based on the detection of the chromophore pNA after cleavage from the labeled substrates, IETD-pNA, and DEVD-pNA, for caspase 8 and 3, respectively. Comparison of the pNA absorbance from the experimental sample was compared to the un-induced neutral pH sample. Caspases 8 and 3 were analysed using the Caspase- 3 Colorimetric Activity Assay (Chemicon, Temecula USA) and Caspase- 8 Colorimetric Activity Assay (Chemicon, Temecula USA). The cells were removed using a cell scraper and centrifuged at 1500 rpm for 10 min. The cells were resuspended in 350μL of chilled cell lysis buffer containing 500μL PBS, 5μL 1% Triton X-100 (Sigma, St. Louis), 25μL proteinase inhibitor cocktail set III (VWR, Mississauga), 0.5μL 1M dithiothreitol (DTT) (Sigma, St. Louis) and 2.5μl phenylmethylsulphonylfluoride (PMSF), diluted in 1mL of methanol (EM Science, Gibbstown), incubated on ice for 10 min and centrifuged for 5 min at 10,000 rpm. A bicinchoninic acid (BCA) protein assay (Pierce, Rockford) was conducted to determine total protein concentration in the supernatants. The absorbance of each sample was measured on a SPECTRAmax

microplate spectrophotometer at 405nm after an incubation period of 2 hr at 37° C. Changes in caspase 3 activity were determined by comparing the absorbance reading from the induced sample with the level of the un-induced control. Background absorbance from the buffer was subtracted from the absorbance of both the induced (pH 6.8, 8.0, 8.4) and un-induced (pH 7.4) samples before calculating changes in caspase 3 activity. The same was done for the detection of caspase 8. As a control, apoptosis was induced using 10μM/mL etoposide and lysed according to the above protocol. All assays were performed with 4 replications

Caspase 3 and poly (ADP-ribose) polymerase (PARP) cleavage by immunoblot

The detection of caspase cleavage was determined at 48 hrs. The samples at each pH and control (see above) were lysed using the total protein isolation lysis buffer (described above). An aliquot of 25μL of each sample was combined with 25μL of 2x 20% sodium dodecyl sulphate (SDS) sample buffer and loaded onto a 4-10% HCL-Tris pre-cast polyacrylamide gel (BioRad, Mississauga). The gel ran at 200 v for 35 min and proteins were electro-transferred to a Hybond-C nitrocellulose membrane (Amersham, Baie d'Urfé) for 75min at 100 v. After transfer, the membrane was washed with 10 mL of PBS with 0.05% Tween 20 (PBST) for 5 min at room temperature (RT) and the membrane was incubated in 10 mL of PBST-milk for one hour at RT followed by three 5-min washes with 10 mL of PBST. The membrane was then incubated with the cleaved caspase 3 primary antibody (Cell Signaling Technology, Beverly CA) at a titre of 1:500 in 6mL of PBST-milk, with gentle agitation overnight at 4°C. Using the same protocol,

changes in PARP expression were determined at 48 hrs. The membrane was incubated with PARP primary antibody (Cell Signaling Technology, Beverly, CA) at a titre of 1:100. The membranes were washed 3 times for 5 min with 10mL of PBST followed by incubation with the anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham, Baie d'Urfé) at 1:3000 in 6mL of PBST-milk with gentle agitation for 1 hr at RT. The membranes were then washed 3 times for 5 min with 10mL of PBST then exposed to Kodak X-OMAT Blue scientific imaging film (Perkin Elmer Canada Inc, Vaudreuil-Dorion) for 30 min. The total optical density of the blots was quantified using LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.).

Protein kinase B/Akt cell survival pathway

The western blots, using Akt and phosphorylated Akt (P-Akt) primary antisera, were conducted on all pH conditions of the cultured N38 cells to determine whether TCAP prevented cell death by activating the survival pathway by phosphorylation of Akt. The same western blot procedure outlined above was repeated with an Akt primary antiserum (Cell Signaling Technology, Beverly, CA) at a titer of 1:500. P-Akt expression at 48hrs was determined using the method described above with a P-Akt primary antibody (Cell Signaling, Beverly,CA) at 1:1000 followed by membrane incubation with anti-rabbit HRP-conjugated secondary antibody (Amersham, Baie d'Urfé) at 1:2000 followed by exposure on film overnight. The cultured N38 cells were serum-starved for 48 hours in order to induce phosphorylation, and following the same protocol above,

were loaded as a control. The total optical density of the blots, were quantified as described previously.

Bromodeoxyuridine (BrdU) Incorporation Assay

Bromodeoxyuridine (BrdU) (Calbiochem, Canada) incorporation into newly synthesized DNA of actively proliferating cells was conducted to determine whether TCAP affected the DNA synthesis (S) phase of the cell cycle. The N38 cells were grown in a 96-well culture plate using 100-μL aliquots at an initial density of $2x10^5$cells/ml. The controls consisted of a blank; DMEM with no cells and background; and DMEM with cells but with no BrdU label added. A working stock of BrdU was prepared by diluting the BrdU label 1:2000 into fresh DMEM and subsequently, 20μL of the working stock solution was added to each well to be labelled. The BrdU was allowed to incubate with the cells for 2 hrs at 37°C. The media was removed and 200μL of the enclosed fixative/denaturing solution was added to each well and incubated for 30 min at RT. This solution was removed, the BrdU antiserum (1:100) was added to each well and the contents incubated for 1hr at RT. Then, the wells were washed 3 times with buffer. The conjugate was prepared by diluting the reconstituted peroxidase goat anti-mouse IgG horseradish (HRP) conjugate in 1xPBS and filtering through a 0.2 μm filter unit. An aliquot of 100μL of this solution was transferred to each well and incubated for 30 min at RT. The wells were washed with the buffer, and the entire plate flooded with double deionized water and the excess solution removed. An aliquot of 100μl of BrdU substrate solution was added to each well and the plate was incubated in the dark at RT for 15 min.

An aliquot 100µl of stop solution containing 2.5N sulphuric acid was added to each well in the same order as the previously added substrate solution. Absorbance was measured at at 450 and 540nm.

Determination of necrosis and apoptosis activity

The presence of normal, apoptotic and necrotic cells were investigated using together using Hoechst 3342 (4',6-diamidino-2-phenylindole, DAPI), An annexin V antibody labelled with fluorescein (FITC) and ethidium homodimer III (EtD-III), respectively. The N38 cells were cultured on poly-D-lysine-treated cover-slips (VWR, Mississauga) in each of the four pH conditions. The cells were washed twice with PBS before 5 µL of each fluorochrome were added. The samples were then incubated in the dark for 15min, washed, and mounted. The cells were viewed and recorded using a Leica DM4500 inverted fluorescent microscope using OpenLab software.

The proportion of viable cells in the samples was also determined by measuring Trypan Blue uptake. At 48 hrs, the cells from the four pH treatments were suspended using 1mL of trypsin-EDTA, centrifuged and resuspended in 1mL of Hank's Balanced Salt Solution (HBSS) (Sigma, St. Louis). An aliquot of 0.5mL of 0.04% Trypan Blue was combined with 0.2mL of the cell suspension, mixed, allowed to stand for 10 min, then counted on a hemocytometer.

Superoxide dismutase detection and measurement

A Cu-Zn superoxide dismutase 1 assay (SOD1) assay (Cell Technology, Mountain View, CA) was performed in quadruplicate on the N38 cells at 48hrs. The cells were cultured in a 75cm^3 culture flask, treated with trypsin, centrifuged at 1600rpm and. resuspended in chilled cell lysis buffer containing 500µL PBS, 5µL 1% Triton X-100 (Sigma, St. Louis), 25µL proteinase inhibitor cocktail set III (VWR, Mississauga), 0.5µL 1M dithiothreitol (DTT) (Sigma, St. Louis) and 2.5µl phenylmethylsulphonylfluoride (PMSF). Mn-Fe superoxide dismutase (SOD2) activity was inactivated using ice-cold chloroform/ethanol (37.5/62.5 (v/v)). The experimental and SOD1 standard curve samples were incubated at 37°C for 20min and the absorbance measured at 450nm.

A direct detection of SOD1 in cell samples was determined at 48 hrs. The cells were removed, centrifuged and resuspended in chilled cell lysis buffer (see above) diluted in 1mL of methanol (EM Science, Gibbstown). The mixture was then incubated on ice, and recentrifuged. Total protein concentration in the supernatants was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford). Samples (20µl) were loaded onto 4-10% HCl-Tris pre-cast polyacrylamide gels (BioRad, Mississauga). In addition, 20ng of a control sample, SOD1 (1-154), expressed as a 44kDa tagged fusion protein, corresponding to amino acids 1-154 of the full length SOD1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was loaded onto the gel with a 1:1 ratio of SDS buffer. After running for 40 min, the proteins were transferred to a Hybond-C nitrocellulose membrane (Amersham, Baie d'Urfé). The membrane was then incubated with SOD1 (FL-154) rabbit polyclonal antibody raised against the full-length SOD1 (Santa Cruz Biotechnology Inc,

Santa Cruz, CA) at a titre of 1:500 followed by incubation with the anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham, Baie d'Urfé) at 1:3000. The optical density of the blots was measured using LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.).

H_2O_2 toxicity and catalase activity

The cells were incubated with 50μM of H_2O_2 containing medium for 0, 6, 12, 24 and 48 hours at pH 7.4. Cell activity was assessed using the MTT assay described previously. A catalase colorimetric assay (Cayman Chemical, Ann Arbor, MI) was performed on the N38 cells at all pH conditions. The cells were harvested and collected by centrifugation. The pellet was homogenized on ice in 1ml of cold 50mM potassium phosphate, pH 7.0, containing 1mM EDTA. The absorbance of the supernatant determined at 540nm .

Real-time PCR gene expression analysis

Total RNA was isolated with Trizol (Invitrogen) and 5 μg of total cellular RNA reverse-transcribed using Superscript II Reverse Transcriptase and OligoDTs (Invitrogen). Quantitative real-time PCR was performed using SYBR Green (Sigma) on the ABI PRISM 7900-HT (Applied Biosystems). A total of $1/1000^{th}$ of the cDNA reaction was used for each PCR triplicate. The reactions were activated at 95°C for 3 minutes followed by 40 cycles of 95°C (15 s) and 60°C (60 s). The transcript number/ng

cDNA was obtained using standard curves generated with mouse genomic DNA. The data was normalized to control TBP transcript levels. Two to 4 independent treatment sets were used and data presented as means and SEMs. The SOD1 primers consisted of: forward, 5'-AGCGGTGAACCAGTTGTGTTG-3' and reverse, 5'-TACTGATGGACGTGGAACCCA-3'; the catalase primers consisted of forward, 5'-GCGGGAACCCAATAGGAGATA-3' and reverse, 5'-CAGGAATCCGCTCTCTGTCAA-3'; and the copper chaperone (CCSD) primers consisted of forward, 5'-AGCTGAAGGTGTGGGATGTGA-3' and reverse 5'-GGCCAACCTCTTCCCAGAAT-3'. The TBP primers consisted of forward, 5'-CAGACCCCACAACTCTTCCATT-3' and reverse 5'-TCTCAGAAGCTGGTGTGGCA-3'.

2.3 Results

Cell morphology and proliferation

TCAP-1 did not induce any observable morphological changes in the cells cultured at pH 7.4 up to 72 hours (Figure 2.1A,B,C). However, there was a significant increase in the number of small, round cell types in the vehicle-treated cultures at pH 6.8 ($p<0.05$), 8.0 ($p<0.001$) and 8.4 ($p<0.001$) as compared to the TCAP-treated samples at 48 hrs. At 72hrs, TCAP significantly decreased the number of rounded cells in pH 8.0 ($p<0.001$) and pH 8.4 ($p<0.001$) relative to the vehicle-treated cells as determined by a two-way analysis of variance (ANOVA) with a Bonferroni's post hoc test.

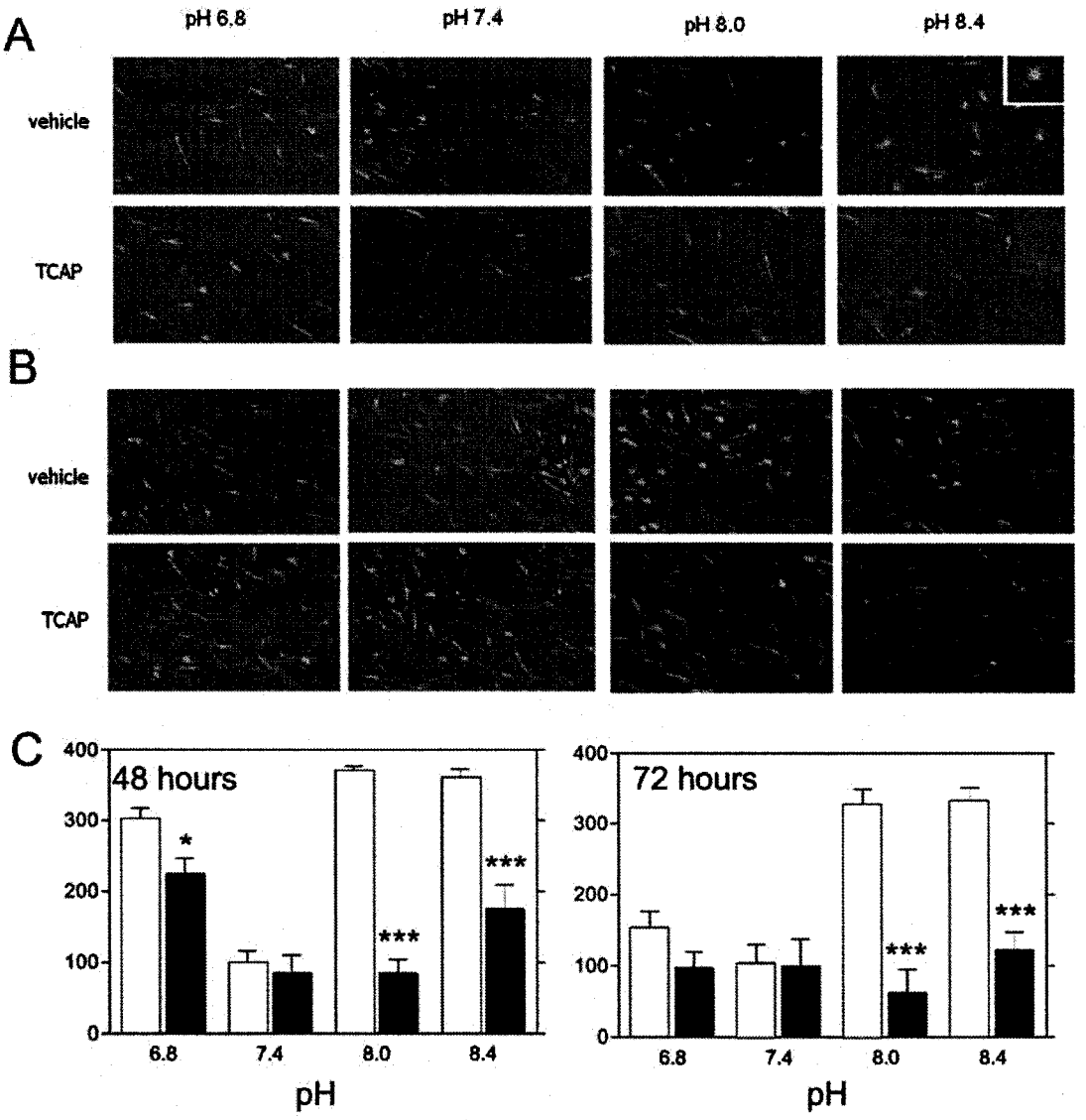

Figure 2.1. Changes in cell morphology as a function of pH treatment. A. photomicrographs of N38 cells at 48 hours. B. photomicrographs of N38 cells at 72 hours. C. Quantification of small, crenated cells (see inset in 'A'). The levels of significance were determined by two-way ANOVA using Bonferroni's post hoc test. n=4 for all analyses. The white and black bars indicate vehicle-treated and TCAP-1-treated cells, respectively.(* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

There were no significant differences in the total number of cells, as determined by direct hemocytometer counts, between the vehicle- and TCAP-1-treated cells at 24 hrs

under 6.8, 7.4 and 8.4 pH conditions, although a significant ($p<0.05$) increase in TCAP-1 treated cells was noted at pH 8.0 (Figure. 2.2). There was a marked reduction in the number of total cells at pH 8.0 and 8.4 in the vehicle-treated cells relative to the TCAP-1-treated cells at 48 hrs. TCAP inhibited the decrease in total cell numbers relative to the vehicle-treated cells at 8.0 ($p<0.05$) and 8.4 ($p<0.01$) after 48 hrs of incubation as determined by a two-way ANOVA. In addition, the TCAP-1-treated cells showed an overall increase in the number of cells at pH 6.8 ($p<0.01$), relative to the vehicle-treated cells at this pH.

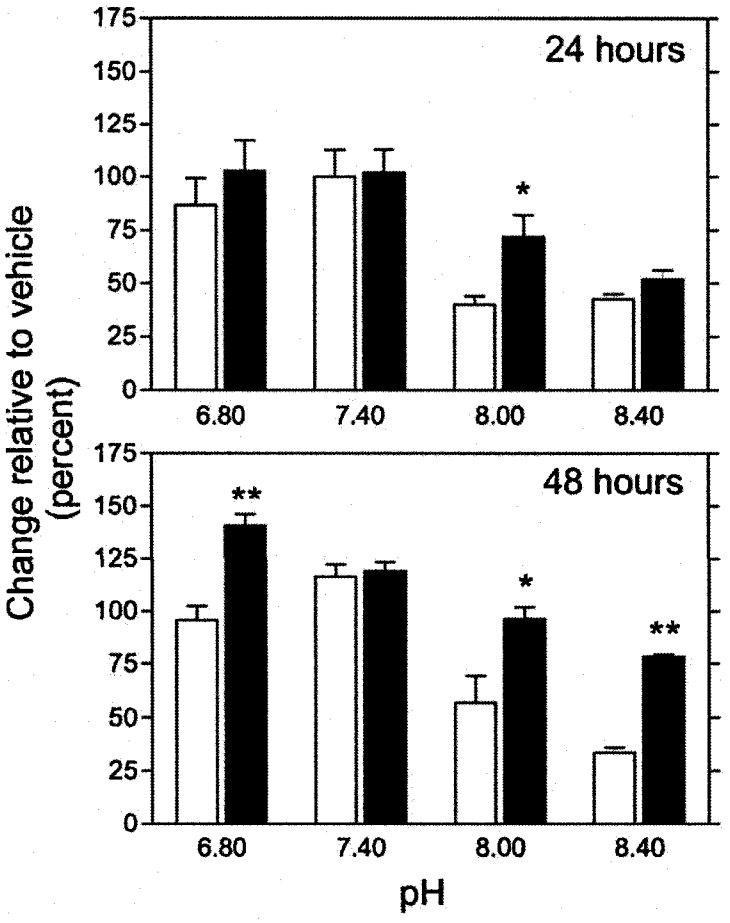

Figure 2.2. Changes in cell number as a function of pH at 24 and 48 hrs. TCAP-1(10^{-7} M) increased the number of cells at 48hrs after treatment at pHs 6.8, 8.0, 8.4. The level of significance was determined using a two-way analysis of variance (ANOVA) using Bonferonni's post hoc test. n=4 for all analyses. The white and black bars indicate vehicle-treated and TCAP-1-treated cells, respectively. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

TCAP-1 did not induce a significant effect on MTT activity at any of the pH conditions at 24 hours. There was, however, a significant increase in optical density at 48hrs in TCAP-1-treated samples cultured in pH 8.0 ($p<0.01$) and pH 8.4 ($p<0.001$) (Figure 2.3). The MTT values were elevated in both the vehicle and experimental groups at the higher pHs due to interference with the assay although the relationship between the vehicle and experimental groups remained consistent.

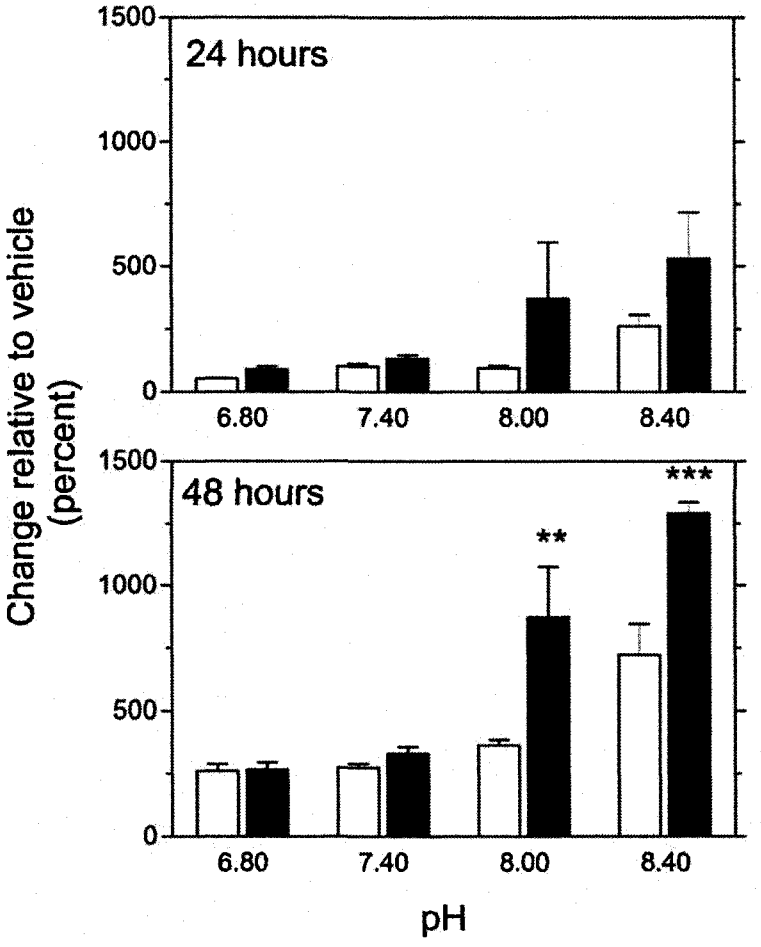

Figure 2.3. Proliferation of N38 cells over the pH range at 24 and 48 hrs as assessed using an MTT assay. TCAP-1 (10^{-7} M) increased the number of viable cells at 48hrs after treatment at pHs 8.0 and 8.4, however there were no significant differences at 24 hrs. The level of significance was determined using a two-way analysis of variance (ANOVA) using Bonferonni's post hoc test. n=4 for all analyses. The white and black bars indicate vehicle-treated and TCAP-1-treated cells, respectively. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Apoptosis (caspase and PARP) markers

Etoposide was used to determine the amount of caspase 8 (Fig 2.4A) and caspase 3 (Fig 2.4B) activation under apoptotic conditions. Etoposide induced a greater than 3-fold increase in caspase 8 and 3.5-fold increase in caspase-3 relative to the vehicle-treated cells at pH 7.4. Although TCAP-1 increased caspase 8 activity in pH 7.4 samples ($P<0.001$) and increased caspase 3 activity in pH 6.8 samples ($P<0.05$), the relative level of caspase activity was about 70% and 40% of the etoposide-induced increase for caspase 8 and 3, respectively as determined by the two-way ANOVA described previously. There were no significant differences in caspase 8 and 3 activity between the TCAP-1- and vehicle-treated cells at pH 8.0 and 8.4. As a further determination of caspase 3 activity, western blots were conducted on pH-treated N38 cells at 48hrs in order to detect the cleaved and activated caspase 3 (17/19 kDa) (Fig 4C). The 19 kDa caspase 3 cleavage product was visible in the protein extracts of the etoposide-treated cells but could not be observed in any of the TCAP-1 or vehicle-treated cells at any of the pH conditions.

Protein levels, as assessed by western blots, were also conducted on pH-treated N38 cells at 48 hrs in order to detect endogenous levels of full-length PARP (115 kDa), as well as the smaller fragment (89 kDa) of PARP resulting from caspase cleavage (Fig 4 D,E). The western blot revealed the presence of endogenous PARP in cell extracts from all pH treatments as well as vehicle-treated samples. Based on a two-way ANOVA, using Bonferroni's post hoc test, there were no significant differences between vehicle and TCAP-1 treated samples (Fig 4 D,F). The cleaved 89 kDa fragment was only consistently, and strongly, detected in the cells cultured at pH 8.4, however there were no

significant differences between the expression of this band in the vehicle or TCAP-1-treated cells (Figure 2.4 E,F).

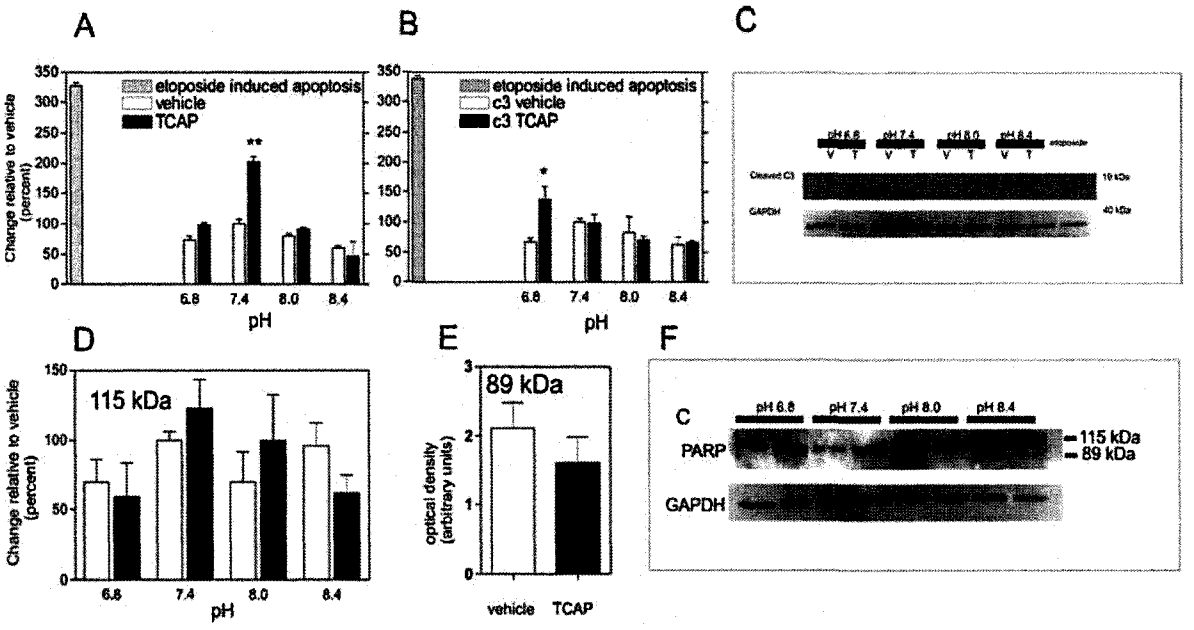

Figure 2.4. Apoptosis biomarker activity. A. Caspase 8 activity as determined by a colorimetric assay. B. Caspase 3 activity as determined by a colorimetric assay. C. Detection of caspase 3 cleavage by western blot. D. Changes in 115 kDa PARP protein expression quantification. E. Changes in 89 kDa PARP fragment at pH 8.4. F. A representative of one of the western blots showing both immunoreactive PARP forms. The level of significance was determined using a two-way analysis of variance (ANOVA) using Bonferonni's post hoc test. The level of significance for the 89 kDa fragment was determined using Student's t-test. n=4 for all analyses. The white and black bars indicate vehicle-treated and TCAP-1-treated cells, respectively.

Survival pathway and Protein Kinase B/Akt

There were no significant differences between the levels of total Akt in the vehicle and the TCAP-treated samples as established by a two-way ANOVA (Fig. 2.5) although there was a non-significant tendency for TCAP-1 to induce an attenuation of the Akt response at pHs 6.8 and 7.4 (Fig 2.5A). There was a significant decrease ($p<0.05$; $p<0.01$) at pHs 8.0 and 8.4) in Akt protein expression in the vehicle-treated group (Figure 2.5A,B). The data, describing the same effect was not significant in the TCAP-1-treated

samples due to the higher variance of the means although the same trend was indicated. Western blots were also conducted using a phospho-Akt antiserum, which detects Akt only when phosphorylated at serine 473. The blot revealed no bands in any samples, suggesting that that phospho-Akt was undetectable under the conditions established by the cultures (Fig 5C). Phospho-Akt was significantly increased ($p<0.05$) in positive control cells where serum was added, as determined by a Student's t-test.

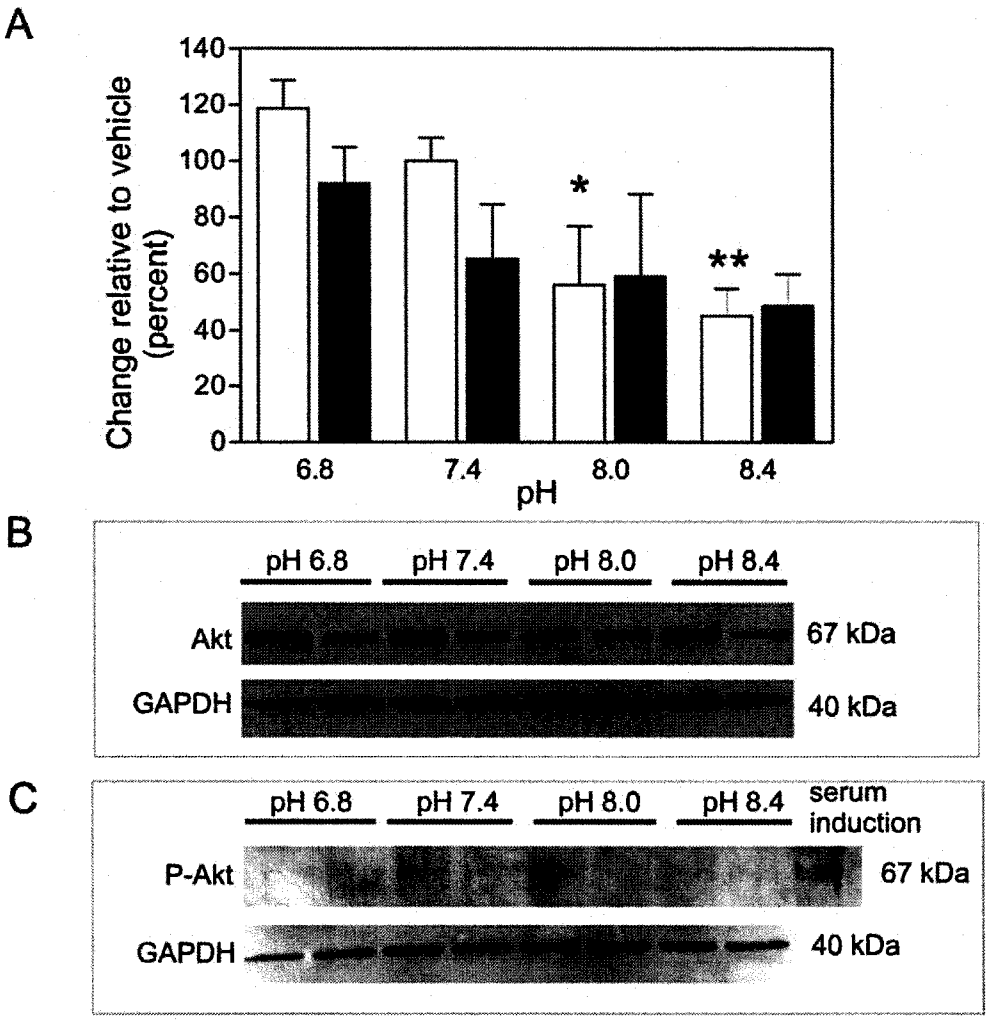

Figure 2.5. Detection of Akt and phospho-Akt. A. Changes in total (phosphorylated and non phosphorylated Akt over the pH range. B. Representative western blot with antiserum recognizing both Akt forms. C. Representative western blot with antiserum that recognizes only the phosphorylated form of Akt. There was no indication of Akt phosphorylation in any sample except for the control. The level of significance was determined using a two-way analysis of variance (ANOVA) using Bonferonni's post hoc test. n=4 for all analyses. The white and black bars indicate vehicle-treated and TCAP-1-treated cells, respectively.

Cell cycle and BrdU Uptake

After 24 hrs, BrdU incorporation did not differ between the vehicle and TCAP-1 treated cells at pH 6.8, 7.4, 8.0 or 8.4, as determined by a two-way ANOVA (Figure 2.6). At 48 hrs, there was a significant ($p<0.05$) reduction in BrdU incorporation in both vehicle and TCAP-1-treated groups relative to the analog uptake after 24 hrs in the pH 8.4 group. The minor increase observed at 48 hrs in the pH 6.8 vehicle group was not significant. There were no significant differences between the vehicle or TCAP-1 treated cells at any time or pH condition.

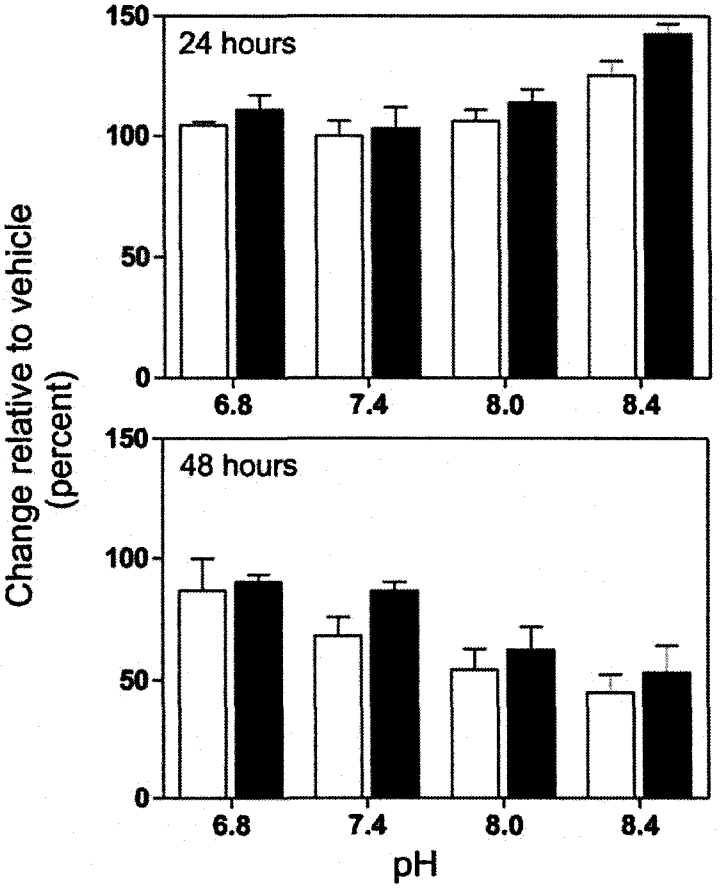

Figure 2.6. BrdU uptake by cells at all pHs at 24 and 48 hours. There were no significant changes between vehicle and TCAP-1-treated cells. n=4 for all analyses. n=4 for all analyses. The white and black bars indicate vehicle-treated and TCAP-1-treated cells, respectively

Fluorescent microscopy of necrosis and apoptosis markers

TCAP-1 decreased the number of necrotic rhodamine-fluorescing cells at pH 6.8 ($p<0.01$), 8.0 ($p<0.001$) and 8.4 ($p<0.001$) but not in the pH 7.4 samples (Figure 2.7A,B). There few FITC-labelled apoptotic cells located intermittently throughout samples, where only 3 green cells were counted (see inset, Figure 2.7).

A Trypan Blue uptake assay was conducted in order to estimate the proportion of viable cells in a population (Figure 2.7C). TCAP-1 treatment caused a significant increase in the number of cells that took up the Trypan Blue stain at 48hrs in cells cultured at pH 6.8 ($p<0.05$), pH 8.0 ($p<0.001$) and at pH 8.4 ($p< 0.01$) but not pH 7.4. relative to the vehicle-treated cells. The statistical analysis was performed using a two-way ANOVA with a Bonferroni's post hoc test.

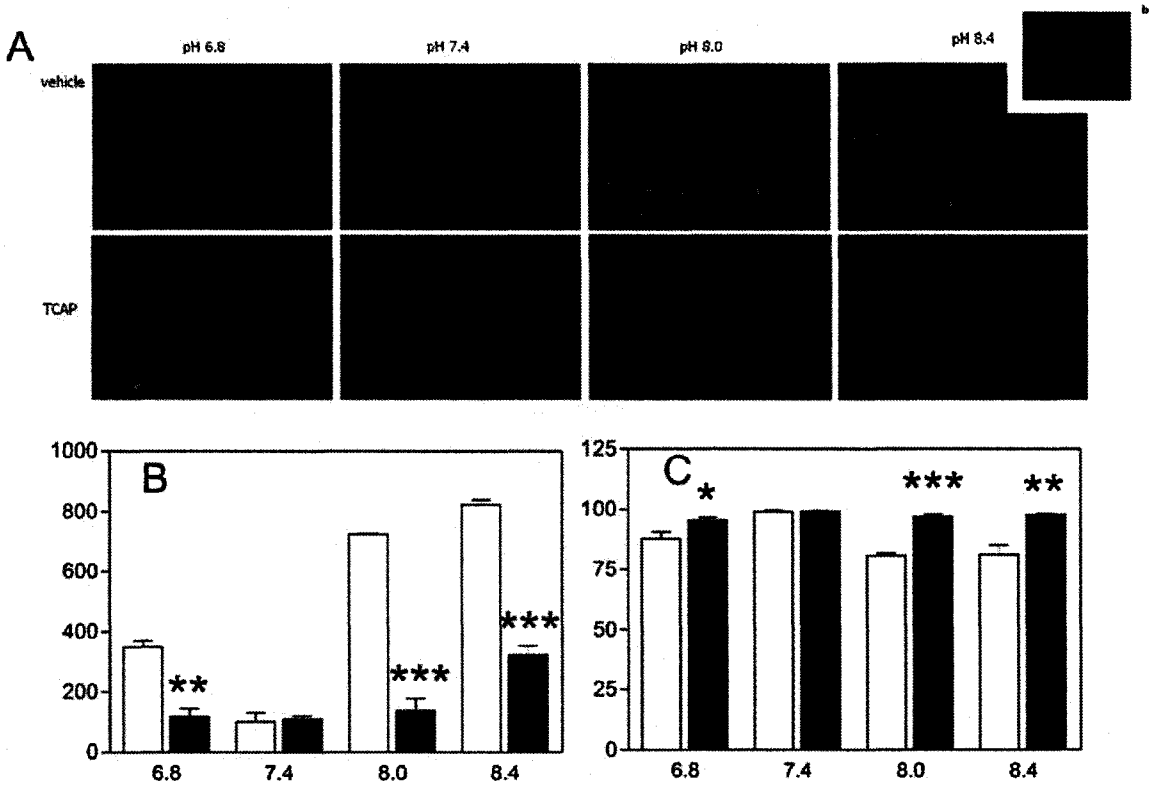

Figure 2.7. Determination of apoptotic, necrotic and healthy cells. A. Cell types are characterized by color: apoptosis (green) necrosis (red/pink) healthy (blue). The inset shows an example of apoptotic cell. Only 3 apoptotic cells were observed in all sets of replicates. B. Incidence of necrotic and healthy cells. TCAP-1 significantly decreased the amount of necrotic cells at 48hrs at pHs 6.8, 8.0 and 8.4. C. Trypan Blue uptake in cells cultured in the various pHs at 48 hrs. TCAP-1 increased the number of viable cells at pH 6.8, 8.0 and 8.4. The level of significance was determined using a two-way analysis of variance (ANOVA) using Bonferonni's post hoc test. n=4 for all analyses. The white and black bars indicate vehicle-treated and TCAP-1-treated cells, respectively. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Superoxide dismutase detection and measurement

Elements of the Cu-Zn superoxide dismutase (SOD1)-associated system were investigated as a possible mechanism via which TCAP-1 confers neuroprotection against pH induced necrotic cell death. The presence of the superoxide radical was measured indirectly by the conversion of a soluble tetrazolium salt in cells after 48 hours (Figure 2.8A). The TCAP-1 treated cells showed a 40% ($p<0.05$) and 60% ($p<0.01$) decrease in the absorbance of the substrate, which is proportional to superoxide radical activity, at

pHs 8.0 and 8.4, respectively. However, because this method shows only the indirect presence of the superoxide radical, we wished to corroborate that SOD1 is inversely related to superoxide radical. Therefore, we also examined the presence of SOD1 enzyme protein directly by western blot (Figure 2.8B,C). Relative to the vehicle-treated cells at pH 7.4, SOD1 levels in the vehicle-treated cells showed a significant ($p<0.05$) decrease as a function of pH, as determined by a one-way ANOVA. In contrast, at pH 8.0 and 8.4, TCAP-1 significantly ($p<0.05$ and $p<0.01$, respectively) reduced the pH-induced decline in SOD1 levels. The SOD1 protein expression levels at pH 8.0 and 8.4 were not significantly different than that of the vehicle-treated cells at pH 7.4.

SOD1 gene expression, as measured by real-time PCR, indicated a significant ($p<0.01$) increase over the vehicle-treated cells at pH 7.4 and 8.4 (Figure 2.8D). A greater effect on gene expression was noted in the copper chaperone for SOD1 (CCSD) expression where CCSD expression levels in the TCAP-1 treated cells at pH 8.4 was increased almost 4.5 fold ($p<0.001$) over the vehicle-treated cells (Figure 2.8E).

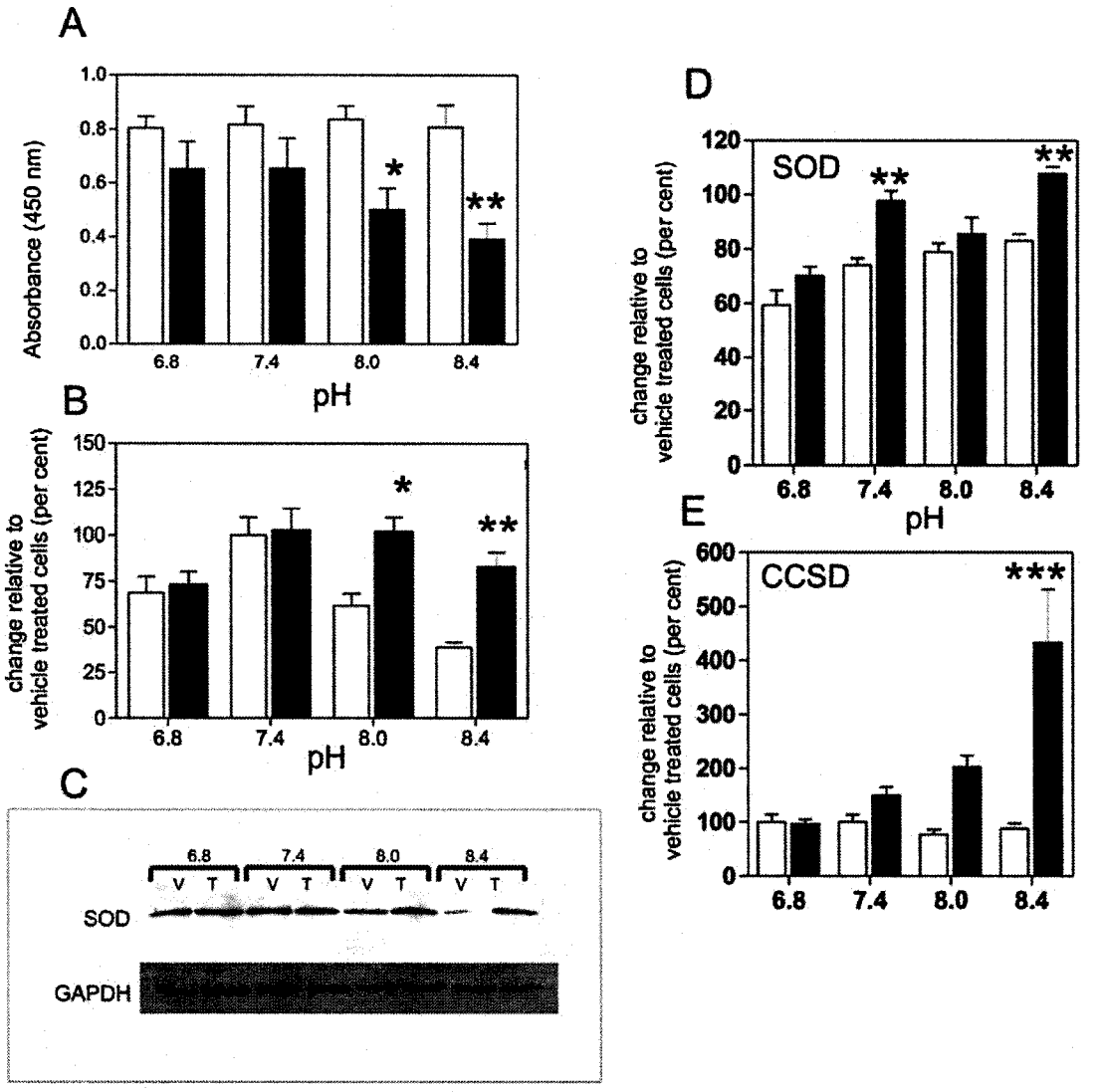

Figure 2.8. Changes in superoxide, superoxide dismutase and copper chaperone activity as a function of TCAP treatment at 48 hrs. A. Superoxide production. The presence of the superoxide radical is directly proportional to absorbance. B Expression of superoxide dismutase protein as assessed by western blot. C. Representative western blot. D. Superoxide dismutase mRNA expression as determined by real-time PCR. E Copper chaperone (CCSD) activity as determined by real-time PCR. The level of significance was determined using a two-way analysis of variance (ANOVA) using Bonferonni's post hoc test. n=4 for all analyses. The white and black bars indicate vehicle-treated and TCAP-1-treated cells, respectively.(* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

H_2O_2 toxicity and catalase activity

In cells treated with 50 μM H_2O_2, TCAP-1 significantly increased mitochondrial activity at 6, 12 and 48 hours ($p<0.001$) as compared with the vehicle-treated cells

(Figure 2.9A). There was also a less significant effect at 24 hours ($p<0.05$) and no effect at 0 hours.

A catalase assay was performed on the pH-treated cells in order to determine whether TCAP-1 was conferring survivability to the cells via upregulation of catalase and thus increasing H_2O_2 breakdown into H_2O and O_2 (Figure 2.9B). The results indicate that TCAP-1 significantly increased catalase levels at pHs 8.0 ($p<0.01$) and 8.4 ($p<0.001$) as compared to the vehicle-treated cells according to a two-way ANOVA with a Bonferroni's post hoc test. Bovine liver was assayed as a positive control. Catalase gene expression, as determined by real-time PCR, indicated that TCAP induced mRNA levels by 3 fold ($p<0.001$) and 5 fold ($p<0.001$) at pHs 8.0 and 8.4, respectively (Figure 2.9C). A small increase in mRNA expression in the TCAP-1-treated group observed at pH 7.4 was not significant.

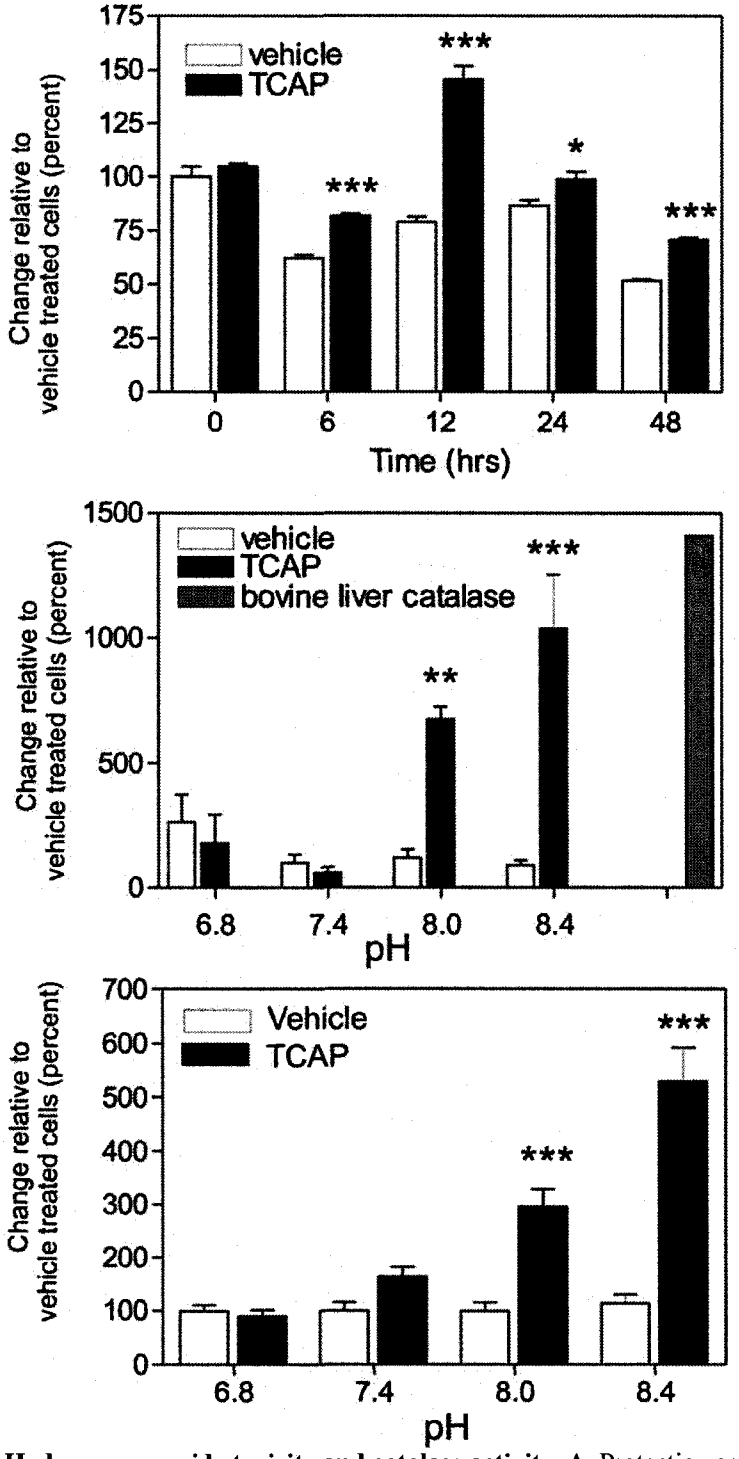

Figure 2.9. Hydrogen peroxide toxicity and catalase activity. A. Protection against hydrogen peroxide induced cell death by TCAP as measured by MTT assay. B. Changes in catalase activity as a function of pH in vehicle and TCAP-treated groups. Bovine liver catalase was used as a positive control to determine the full range of the assay. C. Catalase mRNA expression as determined by real-time PCR. The level of significance was determined using a two-way analysis of variance (ANOVA) using Bonferonni's post hoc test. n=4 for all analyses. The white and black bars indicate vehicle-treated and TCAP-1-treated cells, respectively. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

2.4 Discussion

The data described in this study suggest a significant neuroprotective role for TCAP during times of pH-induced cellular stress. Several lines of evidence point to this. Based on hemocytometer cell counts, Trypan Blue uptake and MTT assays, TCAP-1 reduces the decrease in viable cells and mitochondrial activity induced by high and low pH extremes. Morphologically, TCAP-1-treated cells displayed a significant decrease in the number of small, crenated cells characteristic of some types of cell death. Moreover, TCAP-1 reduced the membrane permeability of the nucleic acid probe, ethidium homodimer III (EtD III) suggesting a decrease in the number of necrotic cells. TCAP-1 increases cytosolic Cu-Zn superoxide dismutase 1 (SOD1) protein and mRNA expression, SOD copper chaperone mRNA expression and reduces the incidence of superoxide activity. In addition, TCAP-1 protects cells from H_2O_2 activity and increases catalase activity. Thus, TCAP and the teneurins may represent a novel molecular mechanism to protect cells from necrosis by upregulation of the SOD1-catalase pathway.

Relevance of Brain injuries related to alkalosis

I show that TCAP-1 is most efficacious at high pH stress. Insults to the brain can quite often lead to shifts in pH and based on the data presented it appears that TCAP is rescuing neurons from necrosis consistently at high pH extremes, specifically pH 8.0 and 8.4. Alkalosis can take on more than one form including: respiratory alkalosis (RA) and metabolic alkalosis (MA) (Gennari et al., 1972, Ostermann et al., 2003). However, in this

chapter we will focus on the occurrence of brain alkalosis and the implications of the trauma associated with it specifically the death of neurons. Dying neurons are a clear indication of many neurodegenerative diseases including Alzheimer's, Parkinson's, Huntington's, brain ischemia and multiple sclerosis (Siao and Tsirka, 2002). These neurodegenerative conditions are characterized by their deleterious effects on brain function resulting from deterioration of neurons. The destruction of neurons in these conditions may be regulated by various forms of cell death and can be caused by damaged mitochondrion, increased levels of excitotoxins such as glutamate, which increases calcium influx into the neurons and activates calcium-dependent enzymes such as calpain and caspases (Brorson et al., 1995a, Brorson et al., 1995b, Brorson et al., 1995c) and pH. Brain pH during times of neurodegenerative stress is not well understood, however, calcium and pH are not mutually exclusive, during both respiratory and metabolic alkalosis, increases in calcium occur in rat neurons due to intracellular calcium accumulating structures such as the mitochondrion (Shishkin et al., 2002, Kostyuk et al., 2003, Potapenko et al., 2004, Voitenko et al., 2004), this is also substantiated by the fact that glutamate induced neuron death requires mitochondrial calcium uptake (Stout et al., 1998).

TCAP is not preventing alkalosis induced cell death via the apoptotic death pathway

I have previously discussed the apoptotic pathway and that it is mediated by a family of death proteins, caspases, these signaling proteins are proteolytic enzymes that when inactive, lay dormant as zymogens, procaspase molecules which must be activated

by various triggers (Hengartner, 2000). Briefly, upon activation of caspase 3 certain nuclear proteins are cleaved such as PARP. PARP, a 116kDa nuclear polymerase, is involved in DNA repair usually in response to environmental stress (Kerr et al., 1972, Wyllie et al., 1980).

Based on the fluorescent and caspase colorimetric data it is clear that TCAP is not rescuing through the apoptotic pathway. Further immunoblot studies utilizing caspase 3 and PARP antibodies corroborate that TCAP is mediating protease cleavage in order to prevent cell death.

Cell Survival: AKT pathway

AKT, also referred to as PKB or Rac, plays a critical role in controlling the balance between cell survival and cell death in neurons (Dudek et al., 1997). This protein kinase is activated by insulin and various growth and survival factors (Akbar et al., 2005). AKT is activated by phospholipids binding and activation loop phosphorylation at Thr308 by PDK1 and also by phosphorylation within the C-terminus at Ser473(Downward, 2004). AKT functions to promote cell survival by inhibiting cell death by means of its ability to phosphorylate and inactivate several targets, including Bad, Forkhead transcription factors and caspase-9 (Crowder and Freeman, 1998).

Akt functions to promote cell survival through two distinct pathways. Akt inhibits apoptosis by phosphorylating, the Bcl-2 family member Bad, which then interacts with 14-3-3 and dissociates from Bcl-xL allowing for cell survival(Endo et al., 2006). Alternatively, Akt activates IKKα that ultimately leads to NFκB activation and

cell survival. Interestingly, AKT has also been shown to mediate neuronal survival against several brain injuries via SOD1(Endo et al., 2006, 2007). Proapoptotic Bcl-2 family members, such as Bax and Bak can promote mitochondrial permeability, while Bcl-2 can inhibit their effects (Datta et al., 1999). Based on results from the western blots conducted, it is clear that TCAP is not acting through this particular survival pathway as AKT is not being phosphorylated. However, TCAP does seem to be affecting endogenous levels of AKT at pH extremes, this could signify that TCAP plays a minor role in cell survival acting through another AKT pathway, which does not require phosphoylation.

Cell Cycling

The evaluation of cell cycle progression is important when assessing the viability of a cell population. The cell cycle is a sequence of stages that a cell passes through between one division and the next. The cell cycle oscillates between mitosis and the interphase, which is divided into G, S, and G 2 (Cameron and McKay, 2001). In the G phase there is a high rate of biosynthesis and growth; in the S phase there is the doubling of the DNA content as a consequence of chromosome replication; in the G 2 phase the final preparations for cell division (cytokinesis) are made (Raza and Preisler, 1985). In order to determine whether TCAP was increasing cell cycle efficiency, a BrdU non-isotopic enzyme immunoassay was conducted to quantify cell cycle progression. Previously, measurements of [^{3}H] thymidine incorporation as cells enter the synthesis (S) phase were used as a means of detecting cell proliferation. In this method

bromodeoxyuridine (BrdU), a thymidine analog replaces [^{3}H] thymidine (Raza et al., 1985a). BrdU is incorporated, into newly synthesized strands of DNA and is detected immunocytochemically and the population of cells of cells entering the S phase is quantified (Gratzner, 1982, Raza et al., 1985b). Based on the results TCAP has no statistically significant effect.

TCAP is mediating cell survival in alkalosis conditions by preventing necrosis

As discussed prior, necrosis occurs when cells are exposed to a number of injurious extracellular events such as hypothermia, hypoxia or inflammation that damages the plasma membrane (Majno and Joris, 1995). Briefly, necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis) (Linnik et al., 1993). Due to the ultimate degeneration of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (Emery et al., 1998). Necrosis can include a number of morphological alterations affecting the plasma membrane, such as including massive production of small surface evaginations caused by the cells inability to control water influx through the plasma membrane (Rello et al., 2005). At pHs 6.8, 8.0 and 8.4, there was a 3- to 8-fold increase in the number of rhodamine-fluorescing cells relative to the vehicle-treated cells at pH 7.4. At pHs 6.8 and 8.0, the number of of rhodamine-fluorescing cells in the TCAP-1-

treated groups were statistically indistinguishable from the vehicle-treated cells at pH 7.4. In addition, at pH 8.4, TCAP-1 reduced the incidence of rhodamine-fluorescing cells by about 60%. Thus, taken together, TCAP-1 may act to maintain cell populations subjected to a pH-induced stress, in part, by inhibiting the incidence of necrosis.

Superoxide dismutase-1, catalase and alkalkosis

Cytosolic Cu-Zn superoxide dismutase (SOD1) is responsible for catalyzing the highly reactive oxygen radical, superoxide (O_2^-), into hydrogen peroxide (H_2O_2). The superoxide radical is generated from a variety of sources, however the majority of it is produced as a function of the respiratory activity of mitochondria (Cadenas and Davies, 2000). SOD1 is bound to copper atoms for full activity. The protein superoxide dismutase copper chaperone (CCSD) is the primary route by which copper ions are transferred to SOD1 (Furukawa et al., 2004). CCSD also possesses additional sulfhydryl oxidase and protein disulfide isomerase activity that has been associated with increased physiological adaption to oxidative stress (Culotta et al., 2006). Hydrogen peroxide is, in turn, catalyzed to water by the enzyme, catalase. Although SOD is a significant source of H_2O_2, the enzymatic breakdown of dopamine by monoamine oxidase B, for example, also constitutes a major source of this metabolite in dopaminergic systems and may play a role in the neurodegenerative effects associated with Parkinson's Disease (Jenner and Olanow, 1996). Together, SOD1, CCSD and catalase act to protect the cells from the toxic effects of ROS. High concentrations of ROS are implicated in the destruction of cellular membranes and proteins and play a significant role in the onset of

neurodegenerative disorders, cancers, diabetes and schizophrenia (Jenner and Olanow, 1996, Mahadik and Mukherjee, 1996, McCord and Edeas, 2005). The brain is particularly susceptible to ROS due to the sensitivity of polysaturated fatty acids in the plasma membrane. ROS and other molecular radicals can disrupt the structure of these fatty acids leading to a destruction of the plasma membrane and subsequent cell death.

Alkalosis and ROS

Previous studies have supported a link between alkalosis and radical oxygen species (ROS) (Majima et al., 1998, Susa and Wakabayashi, 2003). In one study, cells cultured under alkaline conditions can increase ROS levels, mitochondrial damage and cell death. Over-expression of SOD2 (Mn SOD) could prevent cell death (Majima et al., 1998). Little is know about pH concentrations in the microenvironments surrounding cells and in tissue interstices, although pH concentrations appear to vary widely in local microenvironments. These changes become exaggerated under pathological conditions such as spreading depression or ischemia. The magnitude of the pH shifts ultimately depends on the buffering properties of the extracellular compartment. We have developed the present model to address some of these concerns. We have utilized the N38 mouse immortalized hypothalamic cell line (Belsham et al., 2004) as a model system to understand the basic molecular mechanisms of TCAP-1. In previous studies, we have utilized these cells to investigate changes in cAMP-levels mediated by TCAP-1 (Wang et al., 2005).

Our data indicate that under physiological pH conditions (7.4,) TCAP-1 significantly upregulates the SOD gene but not protein levels. At high pH, TCAP-1 up-regulates expression of the SOD1 and CCSD gene and this is associated with a significant drop in the presence of superoxide radicals. One interpretation of this observation may be that TCAP primes the cell for the anticipated pH or metabolic threat in response to the TCAP signal. However, only when the metabolic perturbation occurs, is the SOD mRNA translated into the protein levels that we have observed at pH 8.0 and 8.4 where both high TCAP and a metabolic challenge occurs. This effect is reflected by the increase in the copper chaperone that also occurs at pH 8.0 and 8.4. Although this pathway remains a viable physiological mechanism to reduce the incidence of necrosis additional mechanisms may be involved. For example, TCAP-1 may increase the efficiency of pH regulation by inducing changes in Na^+/H^+ exchange, Na^+ independent Cl^-/HCO_3^- exchange or Na^+/HCO_3^- co transport mechanism. On the other hand, it may act to regulate carbonic anhydrase transcription or incorporation of Zn^{2+} required to activate carbonic anhydrase. Alternatively, the TCAP-1 molecular mechanism may be associated with a general decrease in metabolic activity, and a concomitant reduction of the conductance of a number of these transporter and channel systems thereby rendering the cells more refractile to the actions of extracellular pH changes.

Necrotic cell death in the CNS follows acute ischemia or traumatic injury to the brain or spinal cord (Linnik et al., 1993, Emery et al., 1998). It occurs in areas severely affected by abrupt biochemical collapse, which leads to the generation of free radicals and excitotoxins (e.g., glutamate, cytotoxic cytokines, and calcium). The histologic features of necrotic cell death are mitochondrial and nuclear swelling, dissolution of organelles,

and condensation of chromatin around the nucleus. These events are followed by the rupture of nuclear and cytoplasmic membranes and the degradation of DNA by random enzymatic cuts in the molecule (Basu et al., 2000). Given these mechanisms and the rapidity with which the process occurs, necrotic cell death is extremely difficult to treat or prevent.

ROS, hypoxia and brain injury

The relationship among ROS, hypoxia and brain injury are not well understood, particularly with respect to alkalosis. A number of studies have reported that intracellular alkalosis occurs after ischemia (Chopp et al., 1990, Chen et al., 1992, Mitsufuji et al., 1995, Back et al., 2000). In addition, more recent evidence implicates a relationship between ROS, hypoxia and ischemia (Stvolinsky et al., 1999, Zauner et al., 2002, Honda et al., 2005). As TCAP-1 confers a neuroprotective effect during alkalotic stress via up-regulating oxygen radical scavenging systems, TCAP-1 may potentially play a role during ischemic stress as this is associated with alkalosis and an increased generation of ROS. Calcium also plays an important role during pH fluxes in the brain. During both respiratory and metabolic alkalosis, increases in calcium occur in rat neurons due to intracellular calcium accumulating structures such as the mitochondrion (Potapenko et al., 2004). This is also substantiated by the fact that glutamate-induced neuron death requires mitochondrial calcium uptake (Stout et al., 1998). Thus, a means of identifying neuropeptides with pH protective properties would be a pivotal finding as it would provide novel therapeutic treatments.

This research shows that TCAP-1 may prevent cellular damage via upregulating ROS scavenging systems such as SOD and catalase. This is significant as TCAP may play a role in preventing the detrimental effects of aging. Oxidants play a pivotal role in physiological regulation; however it is imperative that a balance is maintained. Any shift may cause a detrimental increase in oxidant generation causing an accumulation of oxidative damage in the mitochondria and eventual irreparable effects in the brain (Liu et al., 2003). Oxidative stress has been associated with aging effects, in particular, deterioration of cognitive performance in learning and memory processes (Gallagher and Nicolle, 1993, Ingram et al., 1994, Grady and Craik, 2000, Liu et al., 2003). Studies show that ROS production in the mitochondria is directly related to long-lived species and in particular it has been shown that antioxidants may reverse the effects of aging in the brain (Barja, 2004).

The data described in this study suggest a significant neuroprotective role for TCAP during times of pH-induced cellular stress. Several lines of evidence point to this. Based on hemocytometer cell counts, Trypan Blue uptake and MTT assays, TCAP-1 reduces the decrease in viable cells and mitochondrial activity induced by high and low pH extremes. Morphologically, TCAP-1-treated cells displayed a significant decrease in the number of small, crenated cells characteristic of some types of cell death. Moreover, TCAP-1 reduced the membrane permeability of the nucleic acid probe, ethidium homodimer III (EtD III) suggesting a decrease in the number of necrotic cells. TCAP, however, did not affect the apoptotic pathway as indicated by the minor effect on caspase 8 and 3 activity, PARP cleavage or membrane phosphotidylserine turnover. Further, the protective effect of TCAP-1 does not corroborate with Akt-mediated survival pathway

activation. In contrast, TCAP-1 increases cytosolic Cu-Zn superoxide dismutase 1 (SOD1) protein and mRNA expression, SOD copper chaperone mRNA expression and reduces the incidence of superoxide activity. In addition, TCAP-1 protects cells from H_2O_2 activity and increases catalase activity. TCAP and the teneurins may represent a novel molecular mechanism to protect cells from necrosis by upregulation of the SOD1-catalase pathway.

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Chapter 3: Characterizing the neuroprotective role of TCAP-1 in apoptosis by preventing Etoposide and 5-FU induced inhibition of neurogenesis and decreasing caspase 3 cleavage

This chapter is in preparation for submission to *Molecular Pharmacology*

Gina **Trubiani,** Denise D. Belsham, and David A. Lovejoy. Etoposide and 5-FU induced apoptosis in immortalized mouse hypothalamic and hippocampal cells is attenuated by Teneurin carboxy (C)-terminal associated peptide-1

The investigation of neuronal cell death associated with neurodegenerative diseases and in particular the role of apoptotic cell death and its associated biochemical pathways has become increasingly important as our population ages. The teneurin C-terminal associated peptides (TCAP) have been implicated in the regulation, as well as the growth and differentiation of neurons and current observations suggest that TCAP-1 may also have neuroprotective actions during times of apoptosis induced cellular stress in the brain. To test this hypothesis, we cultured a TCAP-1-responsive mouse hypothalamic, N38, and E14 mouse hippocampal cell lines using media buffered with either 10μM etoposide or 50μg/ml 5-fluorouracil (5-FU) and subsequently treated with $10^{(-7)}$ - $^{(-15)}$M TCAP-1. TCAP-1 significantly inhibited the decline in cell proliferation as determined by direct cell viability MTT assay in a dose response manner. In addition, TCAP-1 significantly decreased the number of cells undergoing apoptosis as measured by uptake of annexin-V. Moreover, TCAP-1 significantly decreased the incidence of caspase-3 cleavage and increased BrdU uptake. The results indicate that TCAP may play a neuroprotective role during periods of apoptosis induced stress by up-regulating cell synthesis, inhibiting caspase 3 cleavage and subsequent cell death. Thus, TCAP may be a valid candidate for therapeutic applications in the protection of neurons against neurodegenerative trauma by inhibiting the apoptotic death pathway.

3.1 Introduction

As previously discussed, the term apoptosis was coined in the early 1970s, although the phenomenon of apoptosis was described already in the late 1700s and originally derived from Greek roots meaning, "dropping off" e.g. falling of leaves (Kerr et al., 1972). The apoptotic pathway was originally identified in *C. elegans* and is evolutionarily conserved throughout the metazoans and represents a default mechanism in mammalian development (Danial and Korsmeyer, 2004). Apoptosis is a normal component of the development and health of multicellular organisms where cells die in response to a variety of stimuli in a controlled, regulated fashion. This differentiates apoptosis from necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and potentially irreversible damage to neurons (Wyllie et al., 1980).

In chapter two TCAP-1 was shown to confer neuroprotection to necrotic induced cells via scavenging radical oxygen species (ROS) (Trubiani et al., 2007). Unlike necrosis however, apoptosis may occur in localized regions without damaging adjacent cells and has been implicated during the course of various neurodegenerative disorders, such as Alzheimer's, Huntington's, Parkinson's and Lou-Gehrig's disease (Portera-Cailliau et al., 1995, Troost et al., 1995, Mattson, 2000, Sathasivam and Shaw, 2005) . ROS are also implicated in apoptotic cell death as the activation of caspases may be triggered by ROS formation (Machiavelli et al., 2007). In particular, radical scavengers can attenuate apoptosis and caspase activation during periods of hypoxic stress, ischemia and stroke (Mattson, 2000, Mattson et al., 2001, Ho et al., 2006).

Current *in vitro* experiments performed on immortalized hypothalamic and hippocampal cells indicate that etoposide and (5-fluorouracil) are useful models to investigate aspects of apoptosis. Etoposide binds to and inhibits topoisomerase II and functions in ligating cleaved DNA molecules, resulting in the accumulation of single- or double-strand DNA breaks thereby inhibiting of DNA replication and transcription leading to apoptotic cell death. Etoposide acts primarily in the G2 and S phases of the cell cycle (Mizumoto et al., 1994, Nakajima et al., 1994, Pritchard et al., 1998, Takeda et al., 1999). Application of 5-FU leads to an accumulation of cells in the S-Phase and is also implicated in induction of p53-dependent apoptosis in both mouse and humans (Inada et al., 1997, Takeda et al., 1999).

The aim of this chapter is to determine the pathway through which TCAP-1 induces cell survival in the brain during periods of apoptotic induced death. We show that mouse TCAP-1 enhances the survival of immortalized mouse hypothalamic and hippocampal cells at physiological concentrations of TCAP $10^{(-7)}$ - $^{(-15)}$ M. TCAP-1 may be preventing apoptosis by inhibiting caspase 3 cleavage and cell synthesis arrest.

3.2 Materials & Methods

Peptide Synthesis

Full length mouse TCAP-1 and a non-functional truncated TCAP variant (TCAP-$1_{(9\text{-}41)}$), denoted as $TCAP_i$, was prepared by solid-phase synthesis as previously described (Qian et al., 2004). The peptide and fragment were solubilized in phosphate buffered saline (PBS) at a stock concentration of $2x10^{-5}$ M before being diluted in the appropriate medium. Non-functional scrambled TCAP (pEESQRMFHINNVSLVFYGDYGQVRGTGLLQASDSLELYQEI-NH_2) (Qian et al., 2004, Al Chawaf et al., 2007) was used as a negative control in the vehicle.

Cell Culture

The effect of TCAP-1 on cell viability during etoposide induced stress was conducted using the N38 immortalized mouse hypothalamic cell line and E14 immortalized mouse hippocampal cell line. The cells were grown in six-well culture plates with 2 mL of Dulbecco's Modified Eagle Medium (DMEM) containing high glucose, L-glutamate, 25mM HEPES buffer, pyridoxine hydrochloride in the absence of sodium pyruvate and 5ml penicillin with 10% fetal bovine serum (FBS) at pH 7.4 (Gibco-Invitrogen, Burlington, Canada).

At 24 hrs, the medium was replaced with medium buffered at 10μM etoposide (Sigma, St. Louis) or 50μg/ml 5-fluorouracil (Sigma, St. Louis). Replicates of four wells

received either TCAP-1 10^{-7},10^{-9},10^{-11},10^{-13}M, 10^{-7}M TCAP-1 9-41 fragment, whereas a separate group received no treatment.

Viability Analysis: MTT assay

The effect of TCAP-1 on cell viability was examined at 24 hrs post-apoptosis induction indirectly by assessing mitochondrial activity using a colourimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. The MTT assay was conducted using the In Vitro Toxicology Assay Kit: MTT based (Sigma, St. Louis). The cells were incubated at 37° C in 5% CO_2 for 3hrs in the presence of MTT 200μL/2mL medium. The samples were mixed by shaking the plate horizontally for 30 min. The background absorbance of the samples was determined at 690nm and subtracted from the 570nm measurement. A one-way ANOVA with Tukey post test was used to determine significance.

Determination of apoptosis activity: fluorescence study

The presence of normal, apoptotic and necrotic cells were initially investigated together using a mixture of specific reagents. An annexin V antibody labelled with fluorescein (FITC) was used to identify apoptotic cells in green. Ethidium homodimer III (EtD-III) is a positively charged nucleic acid probe, which is impermeable to live or apoptotic cells but stains necrotic cells with red fluorescence when tagged with rhodamine. Cells undergoing partial stages of apoptosis show as a mixture of blue and

green. Hoechst 3342 (4',6-diamidino-2-phenylindole, DAPI) emits blue fluorescence upon binding to DNA in living cells.

The cells were cultured on poly-D-lysine-treated cover-slips (VWR, Mississauga) in each of the conditions. The cells were washed twice with PBS before 5 μL of each fluorochrome were added: fluorescein (FITC)-Annexin V in Tris EDTA buffer containing 0.1% bovine serum albumin (BSA) and 0.1% NaN_3, pH 7.5, rhodamine EtD-III 200μM in PBS; and 5μL 4',6-diamidino-2-phenylindole (DAPI) Hoechst 33342 5μg/mL in PBS (Biotium, Inc. Hayward). The samples were incubated in the dark for 15min, washed, and then mounted on slides. The cells were viewed under a Carl Zeiss Axiovert 200M inverted fluorescent microscope using AxioVision Image Viewer software version 4.2. The images were recorded using each filter, for FITC, DAPI and rhodamine and then superimposed.

The data was analyzed by observers blind to treatments to quantify FITC, DAPI and rhodamine cells. A one way ANOVA with Tukey's multiple comparison post hoc analysis was used to determine significance. Statistical analysis of all data was carried out with GraphPad Prism® version 4.03.

Detection of cleaved caspase 3 by immunoblot

The detection of caspase cleavage was determined at 24 hrs. The samples at each condition and control were removed using a cell scraper and centrifuged at 1500 rpm for 10 min. The cells were resuspended in 350μL of chilled cell lysis buffer containing 500μL PBS, 5μL 1% Triton X-100 (Sigma, St. Louis), 25μL proteinase inhibitor cocktail

set III (VWR, Mississauga), 0.5µL 1M dithiothreitol (DTT) (Sigma, St. Louis) and 2.5µl phenylmethylsulphonylfluoride (PMSF), diluted in 1mL of methanol (EM Science, Gibbstown), incubated on ice for 10 min and centrifuged for 5 min at 10,000 rpm. An aliquot of 25µL of each sample was combined with 25µL of 2x 20% sodium dodecyl sulphate (SDS) sample buffer and loaded onto a 4-10% HCL-Tris pre-cast polyacrylamide gel (BioRad, Mississauga). The gel ran at 200 v for 35 min and proteins were electro-transferred to a Hybond-C nitrocellulose membrane (Amersham, Baie d'Urfé) for 75min at 100 v. After transfer, the membrane was washed with 10 mL of PBS with 0.05% Tween 20 (PBST) for 5 min at room temperature (RT) and the membrane was incubated in 10 mL of PBST-milk for one hour at RT followed by three 5-min washes with 10 mL of PBST. The membrane was then incubated with the cleaved caspase 3 primary antibody (Cell Signaling Technology, Beverly CA) at a titre of 1:500 in 6mL of PBST-milk, with gentle agitation overnight at 4°C. The membrane was washed 3 times for 5 min with 10mL of PBST followed by incubation with the anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham, Baie d'Urfé) at 1:3000 in 6mL of PBST-milk with gentle agitation for 1 hr at RT. The membrane was then washed 3 times for 5 min with 10mL of PBST then exposed to Kodak X-OMAT Blue scientific imaging film (Perkin Elmer Canada Inc, Vaudreuil-Dorion) for 30 min. The total optical density of the blots was quantified using LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.). TIFF files of blots were imported into CorelDRAW 12.0® and then arranged in sequence accordingly, please see Appendix 1A for a generalized representation of a sample blot, all subsequent western blots were treated in the same manner.

Bromodeoxyuridine (BrdU) Incorporation Assay

Bromodeoxyuridine (BrdU) (Calbiochem, Canada) incorporation into newly synthesized DNA of actively proliferating cells was conducted to determine whether TCAP was affecting the DNA synthesis (S) phase of the cell cycle. N38 and E14 cells were grown in a 96-well culture plate using 100-μL aliquots at an initial density of $2x10^5$cells/ml. The controls consisted of a blank; DMEM with no cells and background; and DMEM with cells but with no BrdU label added. A working stock of BrdU was prepared by diluting the BrdU label 1:2000 into fresh DMEM and subsequently, 20μL of the working stock solution was added to each well to be labelled. The BrdU was allowed to incubate with the cells for 24 hrs at 37°C. The contents of the wells were then removed and 200μL of the enclosed fixative/denaturing solution was added to each well and incubated for 30 min at RT. The contents of the wells were removed and the BrdU antiserum (1:100) was added to each well and incubated for 1hr at RT. Wells were washed 3 times with wash buffer and the plate blotted on paper towel. The conjugate was prepared by diluting the reconstituted peroxidase goat anti-mouse IgG horseradish (HRP) conjugate in 1xPBS and filtering through a 0.2 μm filter unit. An aliquot of 100μL of this solution was transferred to each well and incubated for 30 min at RT. The wells were washed with the buffer, and the entire plate flooded with double deionized water and the excess solution removed. An aliquot of 100μl of BrdU substrate solution was added to each well and the plate was incubated in the dark at RT for 15 min. An aliquot 100μl of stop solution containing 2.5N sulphuric acid was added to each well in the same order as

the previously added substrate solution. Absorbance was measured on a SPECTRAmax Microplate spectrophotometer at dual wavelengths of 450 and 540nm.

Real-time PCR gene expression analysis: caspase 3

Total RNA was isolated with Trizol (Invitrogen) and reverse-transcribed using OneStep RT-PCR (Qiagen). Quantitative real-time PCR was performed using SYBR Green (Sigma) on Rotor-Gene 6 software (Corbett Research). In order to activate the reverse transcriptase the samples were treated at 50°C for 30 minutes and the initial PCR activation step consisted of 15 minutes at 95°C followed by 40 cycles at 94°C for 1 minute, 50° for 1 minute and 72° for 1 minute. The transcript number/ng cDNA was obtained using standard curves generated with mouse brain RNA. The data was normalized to control TBP transcript levels. All reactions were run in triplicate and the data is presented as means and SEMs. The caspase 3 primers consisted of: forward, 5'-TGGAGAAATTCAAAGGACGGGTCG-3' and reverse, 5'-GTACAGTTCTTTCGTGAGCATGGA-3'; The TBP primers consisted of forward, 5'-CAGACCCCACAACTCTTCCATT-3'and reverse 5'-TCTCAGAAGCTGGTGTGGCA-3'.

3.3 Results

MTT Assay: TCAP viability analyses

The MTT assay was used to examine cell viability in response to etoposide, 5-FU or TCAP in both N38 hypthalamic cells or the E14 mixed hippocampal cutures (Figure 3.1). Overall, TCAP-1 co-treatment with the cell toxins significantly reduced the loss in cell viability compared to the toxin treatment alone. Treatment of the E14 cells with 5-FU resulted in almost a complete loss in cell viability after 24 hours (Figure 3.1A). Co-incubation of 5-FU with TCAP9-41 (TCAPi) did not induce any significant improvement in viability. However, when 5-FU was co incubated with full-length TCAP-1, there was a dramatic improvement in cell viability. At the lowest concentration of 10^{-13} M, MTT hydrolysis activity was about 60% relative to the negative control ($p<0.001$) and the 10^{-13} M TCAP-1-treated cell group. At 10^{-11} M TCAP and 5-FU combined resulted in a MTT hydrolysis activity of 50% greater than the negative control ($p<0.001$) and about 40% greater that the 10^{-11} M TCAP-treated cells. The 10^{-9} M and 10^{-7} M TCAP-1 concentrations showed an MTT activity of 80% ($p<0.001$) and 50% ($p<0.001$) compared to the negative control. Treatment of the cells with TCAP-1 but without 5-FU did not show a major increase in MTT hydrolysis activity although the 10^{-11} M and 10^{-9} M TCAP-1 concentration showed non-significant MTT activity that was 15% and 10% greater than the control cells containing vehicle and scrambled peptides. A similar pattern emerged with the cells treated with etoposide (Figure 3.1B). The toxin induced a complete loss of MTT activity which was not significantly reversed by the co-treating the

cultures with TCAPi. Co-treatment of the cells with both etoposide and TCAP-1 yielded a similar pattern as seen for 5-FU and TCAP-1 (Figure 3.1A) co-treated cultures. Concentrations of 10^{-13} M, 10^{-9} M and 10^{-7} M TCAP showed MTT hydrolysis activity of about 50% ($p<0.001$), 75% ($p<0.001$) and 35% ($p<0.001$) relative to the vehicle-treated cells respectively. There was no significant difference of 10^{-11} TCAP-1-treated cells with that of the vehicle-treated cells.

Similar MTT activity profiles were observed when N38 cells were treated with both apoptotic agents and TCAP-1. The co-treatment of TCAP 1 with either 5-FU (Figure 3.1C) or etoposide (Figure 3.1D) did not significantly affect MTT activity relative to cells treated with either toxin. Co-treatment of TCAP-1 with 5-FU induced a significant increase over the 5-FU treated cells at all concentrations (Figure 3.1C). Concentrations of 10^{-13}, 10^{-9} and 10^{-7} induced increased MTT activity that were less than the vehicle-treated controls by 50% ($p<0.001$) 15% ($p<0.01$) and 55% ($p<0.001$) respectively. Co-incubation of 10^{-11} M TCAP-1 with 5-FU resulted in a 60% increase in MTT over the vehicle-treated controls. Treatment of the cultures with any of the four concentrations of TCAP-1 did not affect MTT activity significantly different from the vehicle-treated cells. For etoposide treated cells, MTT activity was reduced about 95%, similar to previous experiments. The TCAP-1 fragment co treated with etoposide did not cause a significant difference in MTT activity. Co-treatment of TCAP-1 of 10^{-13}, 10^{-9} and 10^{-7} M induced MTT assay activity of 50% 25% and 60% less than the vehicle-treated controls but all significantly greater than the etoposide-treated cells. The 10^{-11} M TCAP co-treatment concentration was not significantly different from the vehicle-treated controls. MTT assay activity in cells treated only with TCAP was similar to the vehicle-treated cells.

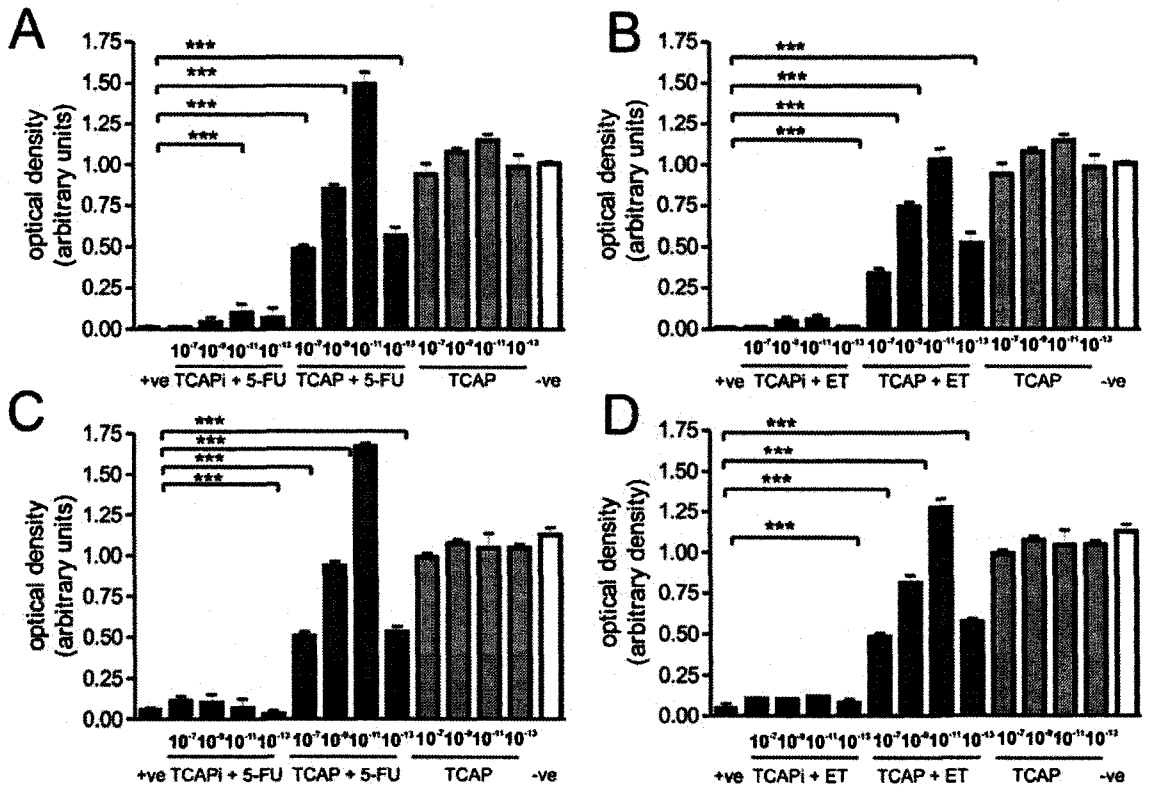

Figure 3.1. Viability of E14 and N38 cells treated with 5-FU, etoposide and TCAP-1 as determined by MTT activity. A. 5-FU-treated E14 cells; B. Etoposide-treated E14 cells; C. 5-FU-treated N38 cells; D. Etoposide-treated N38 cells. All TCAP-1 treated cells increased the number of viable cells at 24hrs after treatment, however there were no significant differences between the positive and TCAPi fragment control. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=4 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Determination of programmed cell death induction: fluorescence study

The incidence of apoptosis was examined by the presence of annexin V to determine if the TCAP-1 increase in cell viability was associated with a decrease in the number of apoptotic cells (Figure 3.2). A concentration of 10^{-7} M TCAP was chosen in order to produce a target of about 50% apoptotic cells with the co-incubation of TCAP-1 and the toxin based on the experiments described in Fig 1. TCAP-1, at 10^{-7} M, significantly decreased the number of apoptotic annexin V cells. Treatment of E14 cells

with etoposide induced about 85% cells showing signs of fluorescence. In contrast, vehicle-treated or TCAP-1- treated cells showed about 5-10% cells with evidence of aopotosis ($p<0.001$ relative to the 5-FU treated cells). The TCAPi fragment, when co-treated with 5-FU showed no significant difference from the cells treated with 5-FU alone. However, when the cells were treated with both TCAP-1 and 5-FU, only about 45% of the cells could be classified as aopototic indicated by a significant ($p<0.001$) decrease in the number of apoptotic cells relative to the cells treated with 5-FU only. A similar pattern was evident when TCAP-1 and etoposide were used to treat the E14 cells (Figure 3.2B). The combination of both components produced an incidence of about 48% apoptotic cells ($p<0.001$) whereas etoposide alone induced 92% apoptotic cells. The vehicle or TCAP-1 treated cells showed an apoptotic population of about 4 and 6 % respectively. The TCAP-1 fragment, when co-treated with etoposide did not show a response that was significantly different from cells treated with etoposide only. The same pattern of response held for the interaction of TCAP-1 with either 5-FU or etoposide, although the efficacy of TCAP-1 to inhibit apoptosis was somewhat stronger (Figure 3.2 CD). For both 5-FU and etoposide the apoptotic populations were 85% to 90% where a similar value was obtained for the co-incubation of the toxin and the TCAP-1 fragment. Cells co-treated with TCAP-1 and either 5-FU or etoposide showed that the number of fluorescing cells were 34 and 22 %, respectively.

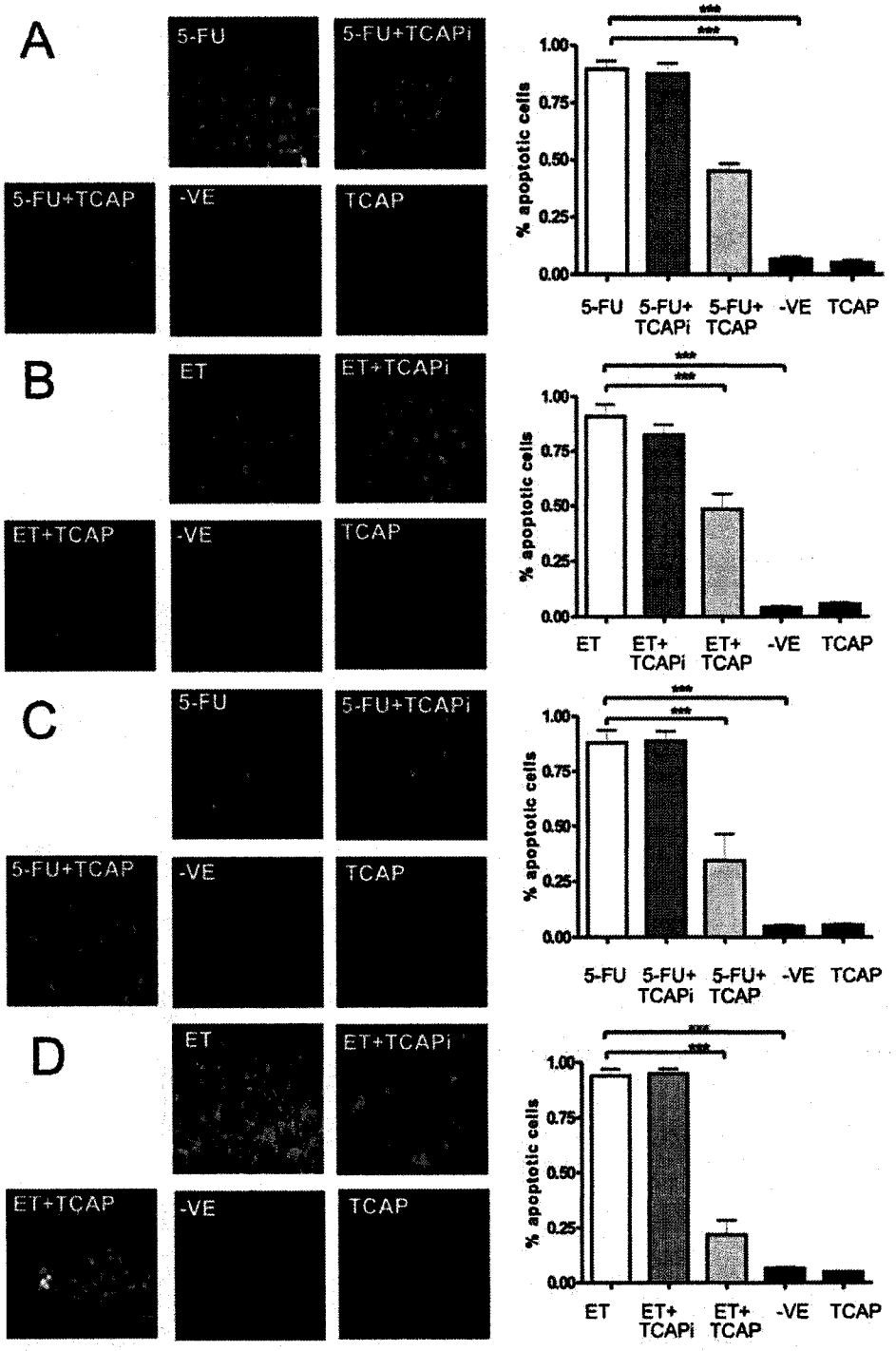

Figure 3.2. Determination of apoptotic and healthy cells in E14 and N38 cultured cells with 5-FU, etoposide and TCAP-1 treatments for 24 hrs. A. 5-FU-treated E14 cells; B. Etoposide-treated E14 cells; C. 5-FU-treated N38 cells; D. Etoposide-treated N38 cells. Cell types are characterized by color: apoptosis (green) necrosis (red) healthy (blue). TCAP-1 significantly decreased the amount of apoptotic cells at 24hrs at all TCAP dosages in a dose-response manner. There were no significant differences between the positive and TCAPi fragment control. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=4 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Bromodeoxyuridine (BrdU) Incorporation Assay: TCAP effects on cell synthesis

TCAP-1 induced a significant increase in BrdU incorporation for both 5-FU- and etoposide- treated E14 hippocampal and N38 hypothlamic cell lines for all concentrations of TCAP used (Figure 3.3). The effect of TCAP-1 was similar in both cases with all four concentrations of the peptide inducing a significant increase ($p<0.001$; Tukey's post hoc test) in BrdU uptake relative to the 5-FU treated cells. A similar efficacy of TCAP-1 occurred with the treatment of the toxins on E14 hippocampal cells where all concentrations showed a significant ($p<0.001$) increase in BrdU uptake with the exception of 10^{-7}M TCAP-1 which was less effective ($p<0.05$) in etoposide treated cells. The TCAPi analog was generally not effective at inducing BrdU uptake in any of the toxin treated cells, however there was some stimulatory action of the peptide at 10^{-7} M in 5-FU treated N38 and E14 cells, and for etoposide-treated N38 cells.

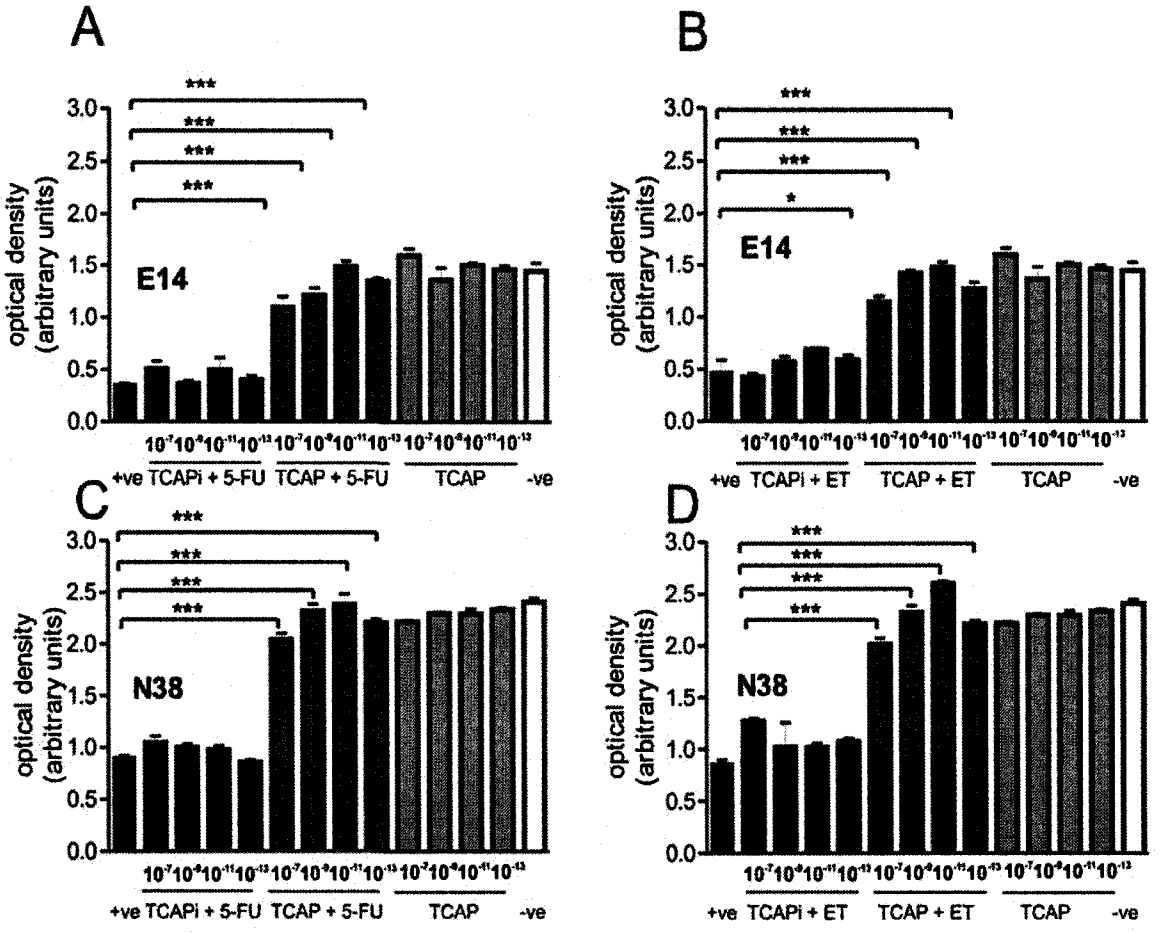

Figure 3.3. BrdU uptake by E14 and N38 cultured cells with 5-FU, etoposide and TCAP-1 treatments after 24 hrs. A. 5-FU-treated E14 cells; B. Etoposide-treated E14 cells; C. 5-FU-treated N38 cells; D. Etoposide-treated N38 cells. There were no significant differences between the positive and the TCAPi fragment control. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=4 for all analyses. (* $p<0.05$; **$p<0.01$; ***$p<0.001$)

Regulation of Caspase protein and mRNA expression by TCAP-1

TCAP-1 was effective at inhibiting the expression of cleaved caspase 3 (Figure 3. 4). Significance was determined by one-way ANOVA using Tukey's multiple comparison post hoc test. A concentration of 10^{-11} M was the most effective ($p<0.001$) at reducing the caspase 3 product for both cell lines treated with 5-FU and etoposide. All other concentrations significantly reduced the expression of cleaved caspase 3. The effect

of TCAP-1 was generally greater for the E14 cells where all concentrations induced a highly significant ($p<0.001$) decrease. There was no significant effect of TCAPi on the reduction of cleaved caspase 3 expression. In cells treated with TCAP-1 but without either apoptotic agent, there was no significant change relative to the vehicle-treated cells.

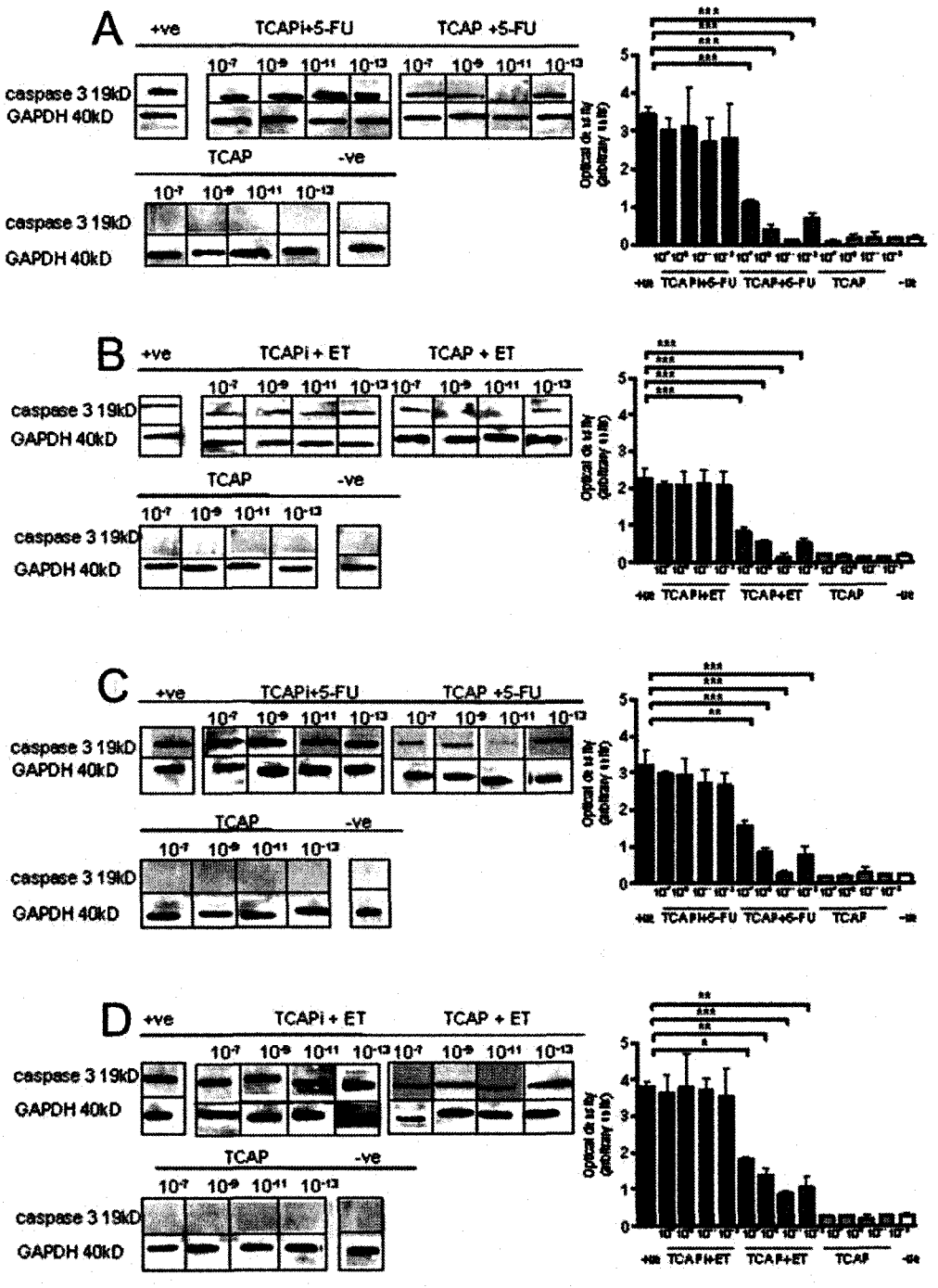

Figure 3.4. Caspase 3 cleavage in E14 and N38 cultured cells with 5-FU, etoposide and TCAP-1 treatments after 24 hrs. Detection of the cleaved caspase protein was detected by the formation of the 19 kDa caspase product on western blot and quantified relative to the detection of the 40 kDa GAPDH protein product. A. 5-FU-treated E14 cells; B. Etoposide-treated E14 cells; C. 5-FU-treated N38 cells; D. Etoposide-treated N38 cells. Detection of caspase 3 cleavage by western blot. B. A representative of one of the western blots showing immunoreactive caspase 3 cleavage forms. There were no significant differences between the positive and the TCAPi fragment control. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=3 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

TCAP-1 at concentrations of 10^{-13}, 10^{-11} and 10^{-9} M was also effective at reducing the expression of caspase mRNA in the E14 and 5-FU-treated cells (Figure 3.5). The highest concentration of TCAP-1 used, 10^{-7}M, significantly reduced caspase 3 mRNA expression in etoposide treated N38 cells but was subsignificant for the other cells and treatments. TCAP-1 had no effect on caspase mRNA expression on cells that were not treated with the toxins. TCAPi did not have a significant effect on the reduction of caspase 3 mRNA in cells treated with either of the apoptotic agents.

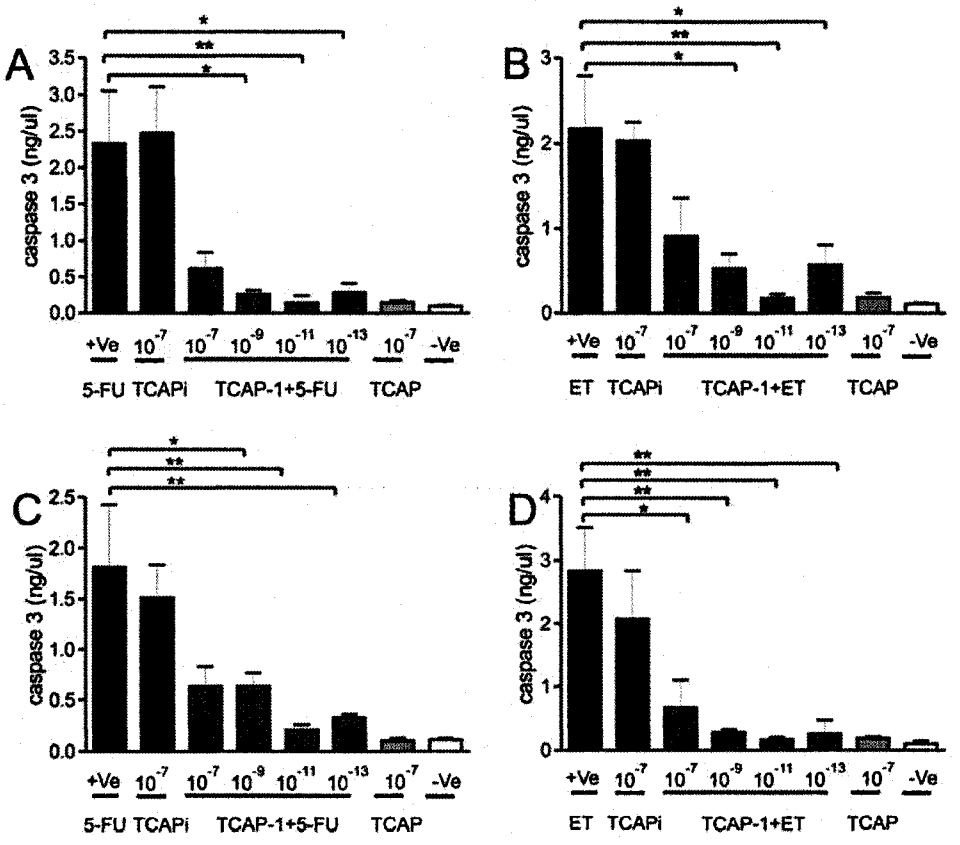

Figure 3.5. Caspase 3 mRNA expression in E14 and N38 cultured cells with 5-FU, etoposide and TCAP-1 after 24 hrs. Caspase 3 mRNA expression was determined by real-time PCR. A. 5-FU-treated E14 cells; B. Etoposide-treated E14 cells; C. 5-FU-treated N38 cells; D. Etoposide-treated N38 cells. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=4 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

3.4 Discussion

The data presented in this study indicate that teneurin C-terminal associated peptide (TCAP)-1 has an ability to interfere with the apoptotic agents, 5-FU and etoposide to reduce the incidence of apoptosis. This study builds on previous investigations that show TCAP-1 can reduce the incidence of cell death and increase the viability of neurons (Al Chawaf et al., 2007; Trubiani et al., 2007). We have used two different apoptotic agents to determine if TCAP-1 acts trhough a common caspase-3 mediated pathway to inhibit apoptotic cell death.

Due to the fact that the teneurins are type II transmembrane proteins, the carboxy terminus is directed extracellularly. The TCAP region is flanked by an amidation motif on its carboxy terminus and a prohormone convertase like cleavage motif at its amino terminus (Lovejoy et al., 2006). Thus, TCAP is structurally poised to be cleaved into the extracellular space or act as a tethered ligand to a receptor in the extracellular matrix or on the plasma membrane on a juxtaposed cell. Specific antisera raised to the TCAP sequence can detect immunoreactivity in brain, immortalized neuron extracts and blood serum (Al Chawaf et al., 2008a). Moreover, fluorescence resulting from FITC-labelled TCAP-1 can be detected in capillaries and fibers in the rat brain after intravenous injection (Al Chawaf et al., 2007). Taken together, these studies support the hypothesis that TCAP-1 can be released from the cell in a soluble form. However, the processing mechanism of the TCAPs may differ among the four paralogs. Recent northern blot studies indicate that antisense probes that hybridize to the TCAP mRNA sequence can detect short mRNAs corresponding to the size of the exon encoding TCAPs 1 and 3 but

not 2 and 4 (Al Chawaf et al., 2008). These studies indicate that TCAPs -1 and -3 may be transcribed independently from their teneurin proproteins.

Synthetic TCAP-1 confers a variety of neuroprotective effects *in vitro*. In primary cultures of embryonic rat hippocampal neurons TCAP-1 increases neurite outgrowth and axon fasciculation without changing the rate of proliferation (Al Chawaf et al. 2007). In immortalized mouse hypothalamic neurons (Belsham et al., 2004) TCAP-1 inhibits high pH induced cell death in part by upregulation of superoxide dismutase (SOD), SOD copper chaperone, and catalase while significantly reducing the incidence of superoxide radicals (Trubiani et al., 2007). *In vivo*, synthetic TCAP can modulate dendritic outgrowth and branching in the rat hippocampus (Al Chawaf et al., 2008b) and modulate stress-induced behavior (Wang et al., 2005; Al Chawaf et al 2007; Tan et al., 2008). The current chapter sought to determine if the neuroprotective effects of TCAP-1 extend to the mechanisms associated with apoptosis-inducing agents.

Etoposide, an epipodophyllotoxin, has been a clinically useful anti-neoplastic agent that inhibits the nuclear enzyme topoisomerase II of replicating cells, which binds to DNA forming a cleavable complex of the enzyme and the 5' cleaved ends of the DNA molecule (Drlica and Franco, 1988). This enzyme subsequently modifies DNA by breaking both strands and passing double-helical DNA though this break prior to rejoining. Thus, topoisomerase II prevents "knots" from forming in DNA by allowing the passage of an intact segment of the helical DNA through a transient double stranded break. Topoisomerase II inhibitors, such as etoposide, interfere with DNA religation by stabilizing the covalently bound complexes formed between the enzyme and the DNA, thus forming nonrepairable protein-linked DNA double strand breaks (Roy et al., 1992)

leading to inhibition of cell proliferation followed by DNA fragmentation and apoptosis(Drlica and Franco, 1988). This has been reported in mouse fibroblast and various types of hematopoietic cells such as lymphocytes and leukemic cells (Lennon et al., 1990). Etoposide-induced apoptosis has been associated with changes in the levels of c-Fos and c-Jun transcription factors, both of which have been shown to mediate neuronal apoptosis (Estus et al., 1994, Saura et al., 1997, Solovyan et al., 1998). Increased expression of these genes has been observed after withdrawal of growth factors as well as etoposide treatment (Hollander and Fornace, 1989, Colotta et al., 1992). Other studies indicate that etoposide toxicity is achieved via an AP-1 independent process where changes in c-fos and c-jun and subsequent AP-1 activity is a consequence of the cell death process rather than the cause of it (Jarvis et al. 1999). However, depending on the cell type, condition and type of apoptosis induced, both an increase and reduction in AP-1 activity has been linked with increased and decreased apoptosis (Herdegen and Waetzig, 2001). There are some indications that TCAP-1 treatment may affect AP-1 activity. Corticotropin-releasing factor (CRF) induction of c-fos in the raphe nucleus and hippocampus is reduced to basal levels with concomitant treatment administration with synthetic TCAP-1 (Tan et al., 2008b). Moreover, AP-1 reporter activity is reduced in immortalized hypothalamic cells (Nock and Barsyte-Lovejoy, unpublished observations). Repeated low concentrations of lithium has been shown to reduce AP-1 activity while conferring neuroprotective actions on cells, although the mechanism by which this occurs is not known (Xu et al., 2002)

5-fluorouracil (5-FU) is a fluorine-substituted analog of pyrimidine uracil and its main function is to block DNA synthesis by reducing thymidine monophospate formation

via inhibition of the enzyme thymidylate synthetase (Cho et al., 2004). 5-FU has been shown to trigger apoptosis is several types of cells by mediating the caspase cascade, induction of Bax or suppression of NF-κB (Aota et al., 2000, Mirjolet et al., 2000, Ohtani et al., 2000, Wu et al., 2001). Oxidative toxicity has also been linked by 5-FU induced apoptosis which is mediated by the decrease in superoxide dismutase (SOD) and glutathione peroxidase activity and thereby rendered the system incapable of coping with free radical attack (Szczepanska et al., 1988, Hara et al., 1990). Further studies have suggested that apoptosis and free radical injury may be involved in 5-FU induced brain trauma including cerebral white matter injury (Cho et al., 2004). In cases of p53 induced apoptosis, mitochondria derived reactive oxygen species (ROS) have been implicated as mediators of 5-FU toxicity. Thus, antioxidant therapy may prove advantageous under 5-FU neuronal stress. We have shown that TCAP may be preventing etoposide induced cell death via S-phase mediation. The data show that TCAP significantly increases cell proliferation during times of both etoposide and 5-FU induced cell stress. TCAP may be acting via an AP-1 dependent signaling cascade in order to confer neuroprotection to neurons. We have shown that TCAP-1 is successful in upregulating radical oxygen scavenging systems, such as SOD(Trubiani et al., 2007). It may be the case that TCAP-1 is acting in an antioxidant enhancing capacity to prevent 5-FU apoptotic induced death as well.

Apoptosis may be divided into two stages. The first stage is characterized by the activation of biochemical mediators in order to attempt to repair cell damage. Should this attempt not be successful, the cell then enters the second or "execution phase" in which structural damage leads to eventual cell death including, caspase 3 cleavage (Cohen,

1997). There is extensive evidence to show that inhibition of the caspases reduces cellular apoptosis initiated by a number of stimuli (Cohen, 1997, Denis et al., 1998).

There is ample evidence which suggests that apoptosis contributes to cell death in several neurodegenerative diseases and that activation of the cysteines protease caspase 3 appears to be a key mediator in the execution of apoptosis in the CNS (Arends and Wyllie, 1991, Robertson et al., 2000). Caspase 3 activation has been observed in stroke, spinal cord trauma, Alzheimer's, Parkinson's disease and several other forms of head trauma (Cohen, 1997, Crowe et al., 1997, Cryns and Yuan, 1998, Carracedo et al., 2002a, Carracedo et al., 2002b). The current literature reveals that this neuronal cell death may be linked to oxidative stress, mitochondrial defects and neurotoxic agents that increase the cells susceptibility to pro apoptotic factors (Mattson, 2000, Arnoult et al., 2003, Ho et al., 2006, Machiavelli et al., 2007). Current pharmacological approaches aim at reducing apoptosis associated with neurodegeneration via glutamate or calcium channel blockers; however caspase inhibitors have also begun to be assessed for potential therapeutic applications (Thompson, 1995). Consequently, interest has emerged in employing various therapeutic approaches to specifically target apoptosis signaling pathways.

The present chapter indicates that synthetic mouse TCAP-1 can protect mouse immortalized neurons from apoptotic induced death. This study supports previous observations that the teneurins have a neuroprotective effect against necrotic cell death and may do so, via upregulation of radical oxygen scavenging systems. Our study suggests that this neuroprotective effect occurs by the inhibition of mechanisms associated with caspase-3 cleavage and cell synthesis. Western blot analyses on active

caspase 3 reveal that TCAP is inhibiting cleavage and preventing subsequent apoptotic induced death.

Initial cell proliferation MTT studies indicated that TCAP is likely acting to inhibit cell death, these observations were further substantiated by increase in cell proliferation between the vehicle and TCAP treated samples suggesting that cell cycle may be a major factor in how TCAP confers a neuroprotective effect.

A bromodeoxyuridine (BrdU) uptake assay was performed to determine if TCAP-1 was having a direct effect on the DNA synthesis (S) phase of the cell cycle. The evaluation of cell cycle progression is important when assessing the viability of a cell population. The cell cycle is a sequence of stages that a cell passes through between one division and the next. The cell cycle oscillates between mitosis and the interphase, which is divided into G, S, and G2 (Gratzner, 1982). In the G phase there is a high rate of biosynthesis and growth; in the S phase there is the doubling of the DNA content as a consequence of chromosome replication; in the G2 phase the final preparations for cell division (cytokinesis) are made (Raza and Preisler, 1985). In the BrdU assay, BrdU is a thymidine analog that is incorporated into newly synthesized strands of DNA. BrdU is detected immunocytochemically and the population of cells of cells entering the S phase is quantified (Raza et al., 1985a, Raza et al., 1985b). In the present study, there was a reduction in BrdU as a function of increased cell stress resulting from apoptotic-induced cell death. There were significant differences between BrdU uptake in 5-FU/etoposide and TCAP-1 treated cells post 24hrs of treatment. Thus, TCAP appeared to be inhibiting the attenuation of proliferation via increased efficiency of cell cycle progression at the same time conferring a neuroprotective effect.

There is extensive evidence that points to a link between apoptosis and cell proliferation suggesting that there may be a sizeable advantage for cells undergoing the death cascade during neurodegenerative trauma to have high proliferative tendencies to either increase or decrease specific cell types which modulate death or survival. In particular, caspase-3 cleaves p21, p27 and PARP via induction of cell-cycle arrest in cells activated for 48 hours by tumour growth factor (TGF)-[beta]1 treatment (Kim et al., 2001). It is also suggested that caspase-3 activation by TGF-[beta]1 may initiate the conversion of cell-cycle arrest to apoptosis (Kim et al., 2001, Katsuda et al., 2002). Several studies have also shown that that anti-Fas may also cause increased cell growth by causing death of certain cell types which then allows for the proliferation of remaining cells (Stoffers et al., 2000, Ling et al., 2001, Zulewski et al., 2001, Rolin et al., 2002). In other studies it has been proposed that caspase activity is required for cancer cell proliferation, although in some instances caspase blockers have been shown to partially inhibit T cell proliferation while potentially increasing proliferation of other cell types. (Susini et al., 1998, Buteau et al., 1999).

Current clinical findings have recently shown that hippocampal volume in patients with depression is significantly reduced when compared to healthy persons (Morishita and Watanabe, 1997). Also that increased levels of neurogenesis are observed in the hippocampus with administration of antidepressant drugs such as desipramine (Huang et al., 2007). In particular, certain studies have shown that anti depressant agents increase viability of cells as well and proliferation in stressed conditions (Santarelli et al., 2003). Specifically, it has recently been shown that desipramine treated neural stem cells can significantly prevent apoptotic events such as caspase 8 and 3 cleavage via

upregulation of the anti apoptotic protein, Bcl-2. Bcl-2 has been identified as both a neuronal cell death repressor and modulator of cell death caused by mitochondrial damage and hypoxic stress (Mizuguchi et al., 1994, Myers et al., 1995). Major depression has also been linked with the activation of the inflammatory response system, thus the diminished production of proinflammatory cytokines due to Bcl-2 activation highlights the importance of identifying effective anti apoptotic agents, which may be essential not only for neuroprotection but for the prevention of cell cycle arrest during periods of neurodegenerative stress and mood disorders (Kubera et al., 2001a, Kubera et al., 2001b).

Corroborations between cell death and proliferation have been made in hypoxic damage as well and in these instances certain growth factors have been implicated in mediating cell death through proliferation. Vascular endothelial growth factor (VEGF) has been shown to have therapeutic capacities in ischemia, particularly strokes (Hayashi et al., 1997, Plate et al., 1999). During cerebral ischemia, this angiogenic protein has been shown to inhibit death in cultured hippocampal neurons from glutamate and N-methyl-D-aspartate (NMDA) toxicity. VEGF has also been implicated in increasing neurogenesis in the adult brain, possibly via the release of brain derived neurotrophic factor from endothelial cells, which may subsequently upregulate antioxidant factors (Svensson et al., 2002). Therefore, the expression of this protein in the ischemic brain may exert a neuroprotective role stroke stress.

TCAP has been implicated in the regulation of stress and anxiety. However, this chapter seeks to establish that TCAP may also have a neuroprotective effect from programmed cell death such as during neurodegenerative trauma. The evidence presented here indicates that TCAP may play an important role in preventing apoptotic

induced cell death via mediating cell synthesis. In addition TCAP's effects on neurogenesis may be accountable for the production of pro- survival factors such as Bcl-2 or may upregulate growth factors which in turn activate radical oxygen scavenging systems to prevent cell degradation during apoptotic stress.

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Chapter 4: TCAP-1 protects neurons from glutamate excitotoxicity via mediation of ERK activation and SOD1 regulation

This chapter is in preparation for submission to *Regulatory Peptides*

Gina **Trubiani,** Denise D. Belsham, and David A. Lovejoy. Chronic treatment of teneurin carboxy (C)-terminal associated peptide-1 (TCAP-1) protects neurons from glutamate excitotoxicity through ERK 1 activation and SOD1 upregulation and gene expression

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and has also been associated as being excitotoxic in neurons during certain types of brain trauma. In the present study I examined the effects of glutamate on hippocampal and hypothalamic cell lines in order to deduce the potential neuroprotective effects of chronic TCAP-1 treatment. Neuronal cell cultures were exposed to TCAP-1 treatment paradigm of 72hrs and excitotoxicity was then induced by glutamate treatment at 10μM for 12hrs. TCAP-1 significantly increased viability of cells under toxic glutamate treatment compared to the control group. Furthermore, investigation of endogenous and active forms of ERK revealed that TCAP-1 may be conferring neuroprotection via the MAPK pathway. TCAP-1 treatment did not change endogenous levels of ERK however, it did cause a significant increase in the levels of phosphorylated ERK (PERK). Recent studies have also revealed that ROS may impinge on the MAPK pathway and effect survival. Chronic TCAP-1 treatment significantly increased SOD1 protein expression as compared to the control groups. Taken together, these data reveal that TCAP-1 may act in a neuroprotective capacity via mediating the activation of ERK and attenuating the deleterious effects of ROS, which play a concomitant role in glutamate excitotoxicity.

4.1 Introduction

In general, excitotoxicity refers to the ability of excitatory amino acids, such as glutamate, to induce death of neurons (Choi, 1990). The implications of toxic levels of glutamate in the extracellular space are severe including: pathological changes in cell morphology, degradation of intracellular organelles eventually leading to either necrotic or apoptotic cells death (Choi, 1992). At basal levels, glutamate is present in mammalian central grey matter and exhibits a ubiquitously excitatory effect on neurons of the CNS, thus acting in a neurotransmitter capacity (Choi, 1987b, Choi et al., 1987). However, at higher levels glutamate becomes a neurotoxin as in the case of several neurodegenerative disorders including ischemia, Alzheimer's, Huntington's and Parkinson's disease (Choi and Rothman, 1990, Casper et al., 2000).

Glutamate acts through two types of receptors, ionotropic and metabotropic. The ionotropic glutamate receptor subtypes are NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) (Brorson et al., 1995). Both receptors have been observed to play a role in trauma associated with glutamate excitotoxicity, such as hypoxia-ischemia (Bazan-Peregrino et al., 2007). Excessive stimulation of these glutamate receptors causes an increase in intracellular calcium concentrations, if sustained for a prolonged period of time this calcium deregulation leads to mitochondrial overload and subsequent production of ROS eventually leading to the demise of the cell (Dice and Terlecky, 1990). Mitochondrial stress plays a vital role in glutamate excitotoxicity and in order to prevent deleterious cell death a means of attenuating ROS production would be a key component to any therapeutic strategy.

Protecting neurons from glutamate toxicity has been an important scientific directive as decreasing the mortality of neurons in the CNS during ischemic and other insults may lead to preventing irreversible and permanent damage. Several studies have indicated that certain agents may be applicable therapeutic agents in preventing damage due to glutamate excitotoxicity (Choi, 1987a). Specific growth factors have also been implicated in preventing glutamate induced death such as vascular endothelial growth factor (VEGF) as well as BDNF (Jin et al., 2000, Jiang et al., 2005). In addition, radical oxide scavenging systems, such as CuZn-superoxide dismutase have also been shown to attenuate glutamate induced neuronal swelling and toxicity (Chan et al., 1990).

The mammalian family of MAPKs contains three distinctive members: ERK, stress activated protein kinase (SAPK) and p38 (Park et al., 2004b). ERK has been reported to affect mitogenic responses and SAPK and p38 have been shown to influence the stress response via downstream players: JNK, and mitogen- and stress-activated kinase (MSK) 1 respectively. The ERK pathway has been specifically observed to influence cell survival via regulation by neurotrophic factors as well as confer neuroprotection in response to oxidative stress. Studies have also shown that pre-incubation with BDNF attenuates glutamate excitotoxicity induced-apoptotic cell death via ERK and PI3-kinase pathways (Almeida et al., 2005). Also, inhibition of ERK activation has been shown to significantly decrease SOD1 expression (Scorziello et al., 2007). Thus, a neuroprotective pathway consisting of BDNF, SOD1 and PERK may be an effective mechanism through which TCAP mediates neuroprotection.

The work in this thesis has reported that TCAP-1 may impart survival to compromised cells potentially via radical oxygen scavenging and growth factor

regulation. This study seeks to deduce through which pathway chronic TCAP application may confer neuroprotection and whether ROS scavenging regulation may act as a general mechanism of neuroprotection for the novel peptide.

4.2 Materials and Methods

Viability Analysis (MTT assay): glutamate excitotoxicity induction

N38, N3, N46 hypothalamic and E14 immortalized hippocampal cells were cultured for 3 days prior glutamate treatment with either TCAP-1 10^{-7}, 10^{-9}M, TCAP-1 10^{-7}M 9-41 fragment or 10^{-7}M urocortin, there was also a no treatment group. The medium was then replaced with medium buffered at 10μM glutamate in each of the four replicate wells (Sigma, St. Louis).

The effect of TCAP-1 on cell viability was examined at 12 post glutamate induction indirectly by assessing mitochondrial activity using a colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. The MTT assay was conducted using the In Vitro Toxicology Assay Kit: MTT based (Sigma, St. Louis). The cells were incubated at 37° C in 5% CO_2 for 3hrs in the presence of MTT 200μL/2mL medium. The samples were mixed by shaking the plate horizontally for 30 min. The background absorbance of the samples was determined at 690nm and subtracted from the 570nm measurement. A one-way ANOVA with Tukey post test was used to determine significance.

Endogenous ERK, PERK and SOD1 detection by immunoblot

The detection of endogenous and active ERK and SOD1 was determined at 12 hrs post glutamate treatment as described above. The samples at each treatment were lysed using total protein isolation lysis buffer (described above). An aliquot of 20μL of each sample was combined with 25μL of 2x 20% sodium dodecyl sulphate (SDS) sample buffer and loaded onto a 4-10% HCL-Tris pre-cast polyacrylamide gel (BioRad, Mississauga). The gel ran at 200 v for 35 min and proteins were electro-transferred to a Hybond-C nitrocellulose membrane (Amersham, Baie d'Urfé) for 75min at 100 v. After transfer, the membrane was washed with 10 mL of PBS with 0.05% Tween 20 (PBST) for 5 min at room temperature (RT) and the membrane was incubated in 10 mL of PBST-milk for one hour at RT followed by three 5-min washes with 10 mL of PBST. The membrane was then incubated with the ERK, PERK and SOD1 primary antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at a titre of 1:1000 in 6mL of PBST-milk, with gentle agitation overnight at 4°C. GAPDH was used as a control antibody at a titer of 1:1000 and treated in the same manner as mentioned above. The membranes were washed 3 times for 5 min with 10mL of PBST followed by incubation with the anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham, Baie d'Urfé) at 1:3000 in 6mL of PBST-milk with gentle agitation for 1 hr at RT. The membranes were then washed 3 times for 5 min with 10mL of PBST then exposed to Kodak X-OMAT Blue scientific imaging film (Perkin Elmer Canada Inc, Vaudreuil-Dorion) for 5 min. The total optical density of the blots was quantified using LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.). TIFF files of blots were

imported into CorelDRAW 12.0® and then arranged in sequence accordingly, please see Appendix 1B for a generalized representation of a sample blot using the ERK primary antibody, all subsequent western blots were treated in the same manner.

Real-time PCR gene expression analysis: SOD1

Total RNA was isolated with Trizol (Invitrogen) and reverse-transcribed using OneStep RT-PCR (Qiagen). Quantitative real-time PCR was performed using SYBR Green (Sigma) on Rotor-Gene 6 software (Corbett Research). In order to activate the reverse transcriptase the samples were treated at 50°C for 30 minutes and the initial PCR activation step consisted of 15 minutes at 95°C followed by 40 cycles at 94°C for 1 minute, 50° for 1 minute and 72° for 1 minute. The transcript number/ng cDNA was obtained using standard curves generated with mouse brain RNA. The data was normalized to control TBP transcript levels. All reactions were run in triplicate and the data is presented as means and SEMs. The SOD1 primers consisted of: forward, The SOD1 primers consisted of: forward, 5'-AGCGGTGAACCAGTTGTGTTG-3' and reverse, 5'-TACTGATGGACGTGGAACCCA-3'; The TBP primers consisted of forward, 5'-CAGACCCCACAACTCTTCCATT-3'and reverse 5'-TCTCAGAAGCTGGTGTGGCA-3'.

4.3 Results

Viability Analysis (MTT assay): TCAP and glutamate toxicity

TCAP-1 induced a significant effect on MTT activity for glutamate treated E14 hippocampal and N38 hypothalamic cell lines (Figure 4.1). Concentrations of TCAP 10^{-7}, 10^{-9}M were significant when compared to the control sample using a one way ANOVA with Tukey's multiple comparison post hoc test. Replicates were completed at an n=4 for each sample.

Significance for the glutamate treated E14 hippocampal cells is as follows: there was overall significance of (F =116.0) according to the one way ANOVA. Tukey's post test reveals that positive control samples including those samples treated with the TCAP 9-41 fragment and urocortin 10^{-7}M were not significant with relation to each other. There was a significant increase in TCAP 10^{-7}, 10^{-9}M +glutamate treated samples in relation to glutamate positive controls ($p<0.01$) ($p<0.001$) respectively.

For glutamate treated N38 hypothalamic cells there was overall significance of (F=193.5) according to the one way ANOVA. Tukey's post test reveals that positive control samples including those samples treated with the TCAP 9-41 fragment and urocortin 10^{-7}M were not significant with relation to each other. There was a significant increase in TCAP 10^{-7}, 10^{-9}M +glutamate treated samples in relation to glutamate positive controls ($p<0.001$) ($p<0.001$) respectively.

For glutamate treated N3 hypothalamic cells there was overall significance of (F=140.6) according to the one way ANOVA. Tukey's post test reveals that positive control samples including those samples treated with the TCAP 9-41 fragment and urocortin 10^{-7}M were not significant with relation to each other. There was a significant increase in TCAP 10^{-7}, 10^{-9}M +glutamate treated samples in relation to glutamate

positive controls ($p<0.001$) ($p<0.001$) respectively.

For glutamate treated N46 hypothalamic cells there was overall significance of ($F=80.85$) according to the one way ANOVA. Tukey's post test reveals that positive control samples including those samples treated with the TCAP 9-41 fragment and urocortin 10^{-7}M were not significant with relation to each other. There was a significant increase in TCAP 10^{-7}, 10^{-9}M +glutamate treated samples in relation to glutamate positive controls ($p<0.01$) ($p<0.001$) respectively.

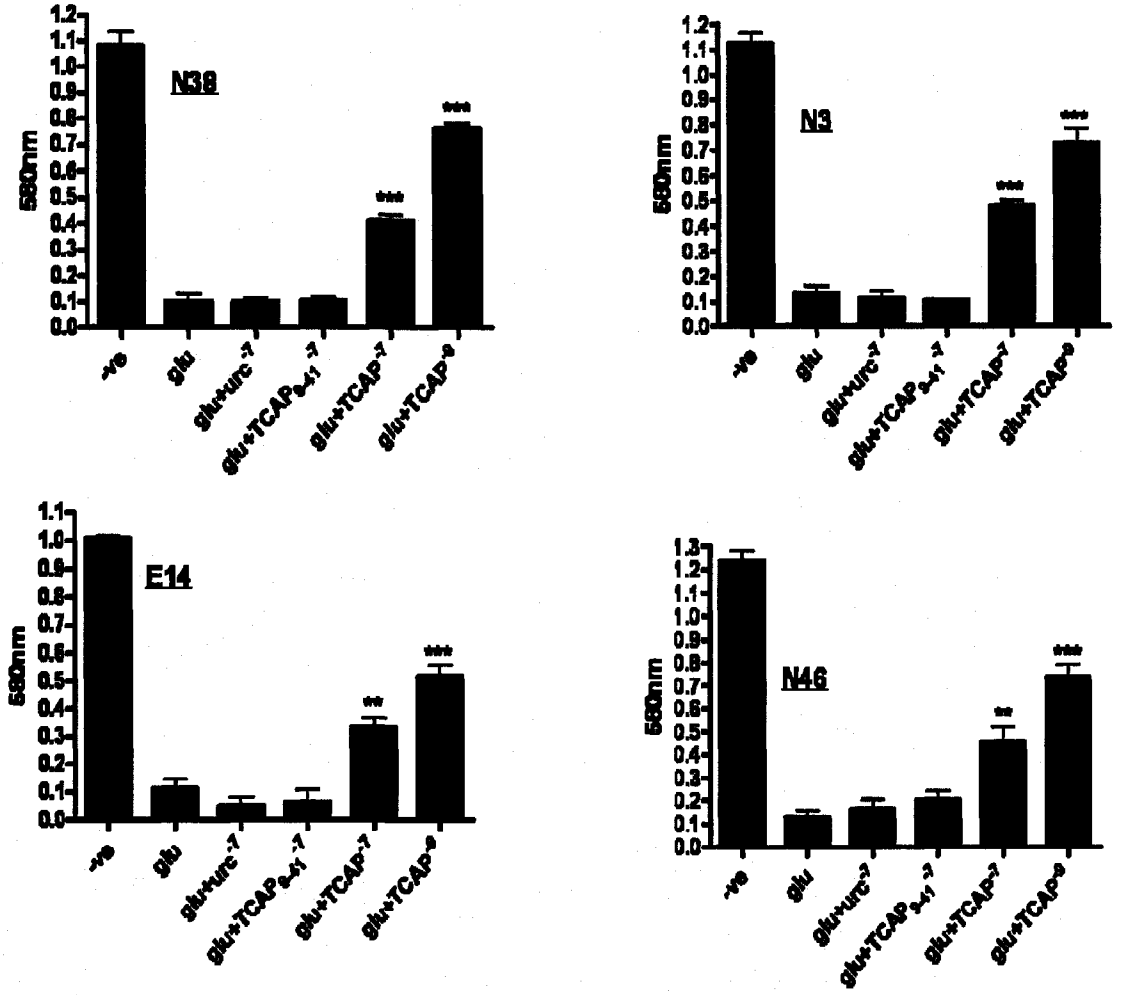

Figure 4.1. Viability of E14 hippocampal, N38, N3 and N46 hypothalamic cells with chronic TCAP-1 treatments of TCAP-1 10^{-7}, 10^{-9}M and glutamate as determined by MTT assay. All TCAP-1 treated cells increased the number of viable cells, however there were no significant differences between the negative controls. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=4 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

ERK and PERK protein expression

The effects of glutamate were used a means to determine whether chronic TCAP-1 treatment could differentially affect levels of endogenous ERK and PERK. There was no significant change in the endogenous levels of ERK. In all 4 cell lines there was no differential effect of chronic TCAP-1 treatment (Figure 4.2). One way ANOVA using Tukey's post test showed F values as follows: N38 F=1.066, N3 F=0.7982, N46 F=2.870, E14 F=0.7809.

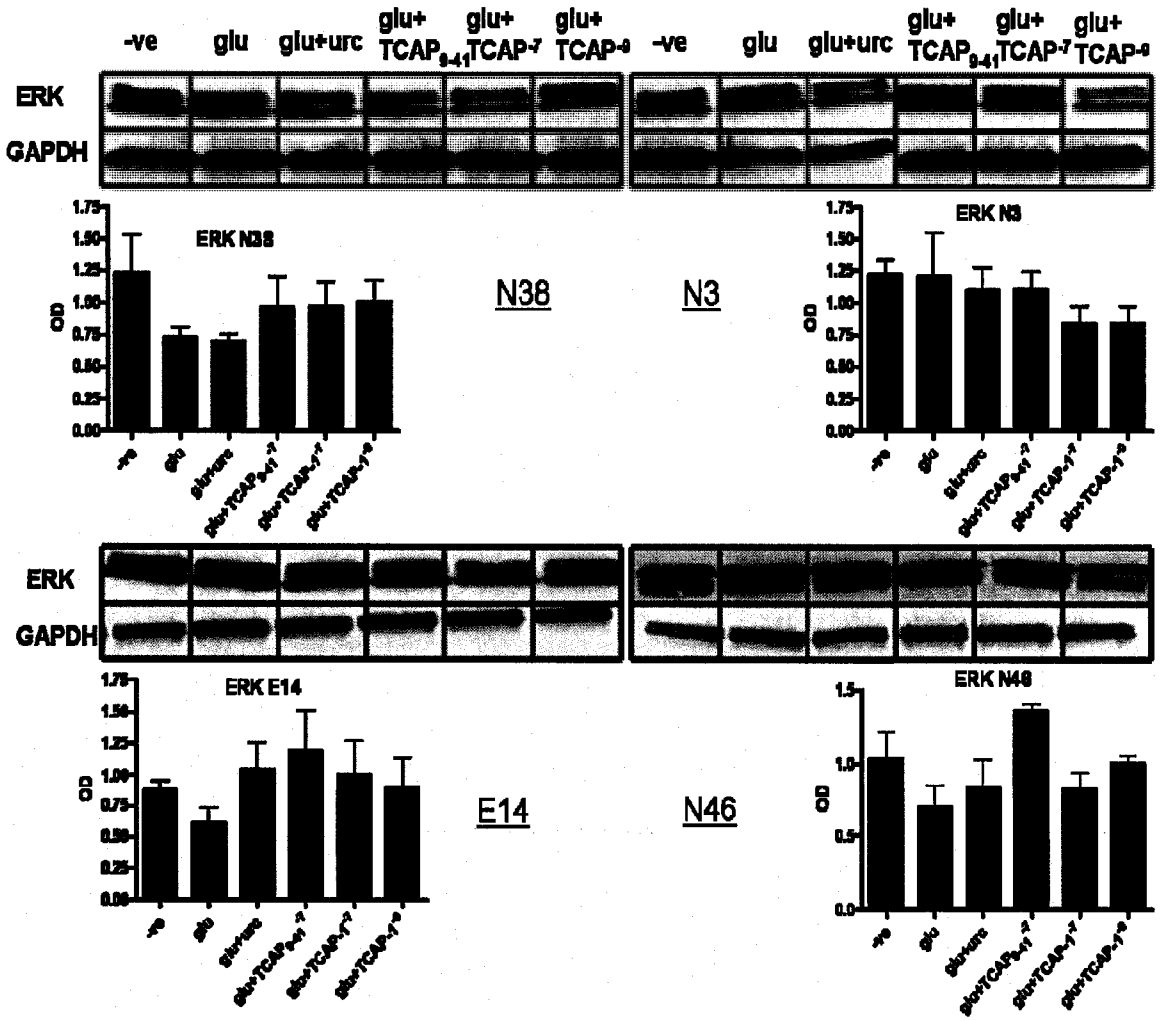

Figure 4.2. ERK biomarker activity. Detection of endogenous ERK (40kDa) by western blot in hippocampal, N38, N3, N46 and E14 hypothalamic cells with chronic TCAP-1 treatments of TCAP-1 10^{-7}, 10^{-9}M and glutamate as determined by western blot. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. (* $p<0.05$; **$p<0.01$; ***$p<0.001$)

Further analysis revealed that there was a significant change in the active form of ERK (Figure 4.3). TCAP-1 significantly increased PERK expression. In N38 cells chronic TCAP-1 treatment significantly increased PERK in a dose response fashion. Although TCAP-1 10^{-7} did not significantly increase PERK, there was overall significance ($p<0.001$) across treatments and TCAP-1 10^{-9} increased PERK expression by 70% ($p<0.001$) and a 30% increase over negative control samples. There was no significance between glutamate cells treated with urocortin or the truncated version of TCAP-1. Analyses of N3 cells reveals that both TCAP-1 10^{-7} and 10^{-9} significantly increased PERK expression, ($p<0.05$) for both, as compared to glutamate and glutamate + urocortin and TCAP-1 $_{9\text{-}41}$ treated cells. TCAP treated cells did not significantly increase PERK expression as compared to the negative control nor was there any significant change amongst the glutamate and glutamate + urocortin and TCAP-1 $_{9\text{-}41}$ treated cells. The N46 and E14 cells show that TCAP-1 10^{-9} only was significant in increasing PERK expression as compared to the glutamate positive control. Again, there was no significance amongst the other glutamate-treated cultures.

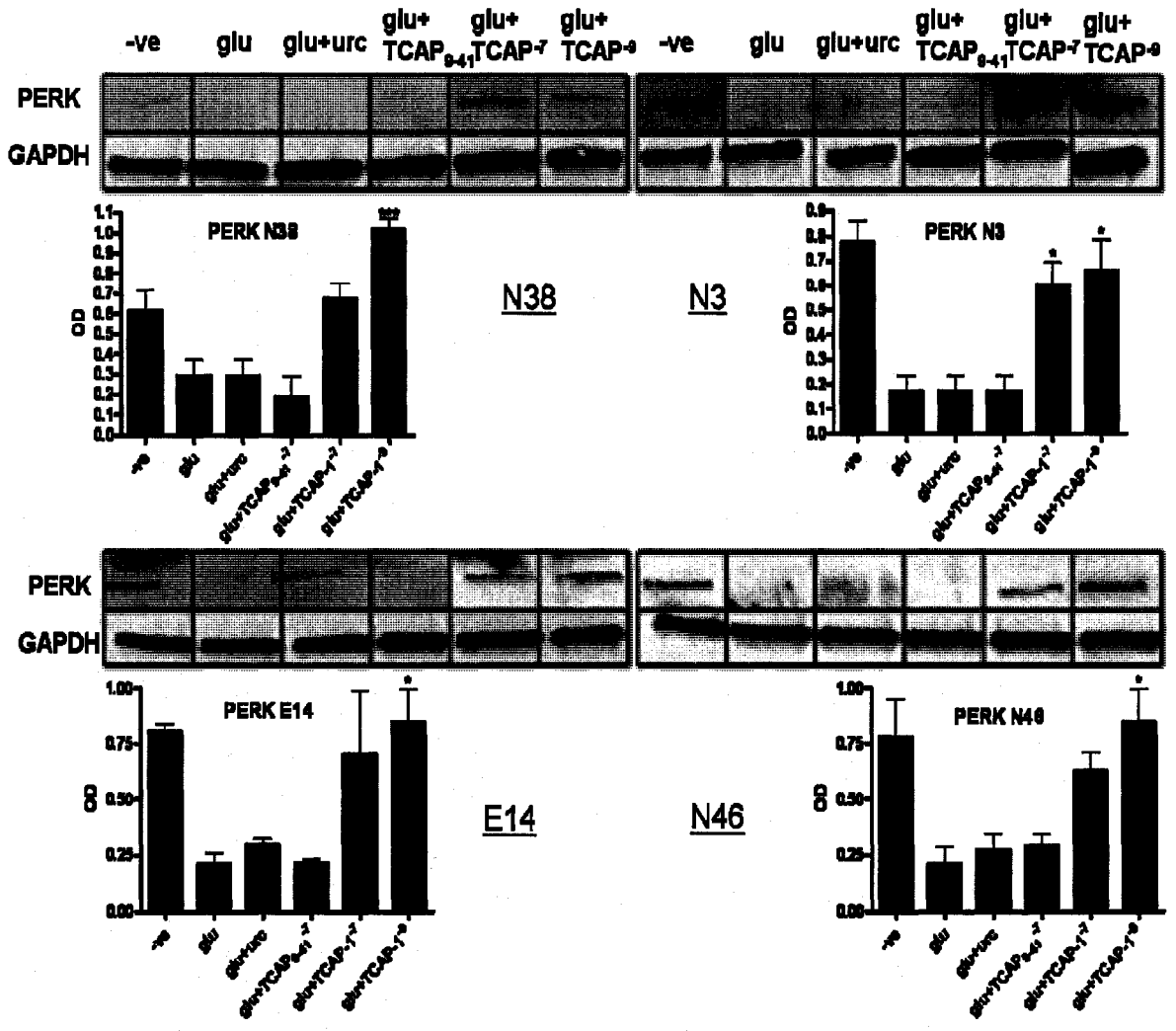

Figure 4.3. PERK biomarker activity. Detection of active PERK (125kDa) by western blot in hippocampal, N38, N3, N46 and E14 hypothalamic cells with chronic TCAP-1 treatments of TCAP-1 10^{-7}, 10^{-9}M and glutamate as determined by western blot. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Superoxide dismutase detection and measurement

Protein levels, as assessed by western blots, were also conducted on glutamate treated cells in order to detect whether chronic TCAP-1 treatment differentially affected endogenous levels of SOD1. The western blot revealed that TCAP-1 significantly increased the presence of SOD 1 in cell extracts (Figure 4.4). N38 cells reveal that

TCAP-1 10^{-9} only was significant in increasing SOD1 expression as compared to the glutamate positive control ($p<0.05$) whereas in N3 cells both . TCAP-1 10^{-7} and 10^{-9} significantly increased SOD1 expression, ($p<0.05$) for both, as compared to glutamate and glutamate + urocortin and TCAP-1 $_{9-41}$ treated cells. TCAP-1 10^{-7} and 10^{-9} also significantly increased SOD1 expression in N46 cells, ($p<0.05$) and ($p<0.01$) respectively as well as in E14 cells ($p<0.01$) and ($P<0.001$). In all cell lines, there was neither significant difference in TCAP-1 treated cells and the negative control nor any significant difference amongst the positive glutamate control samples.

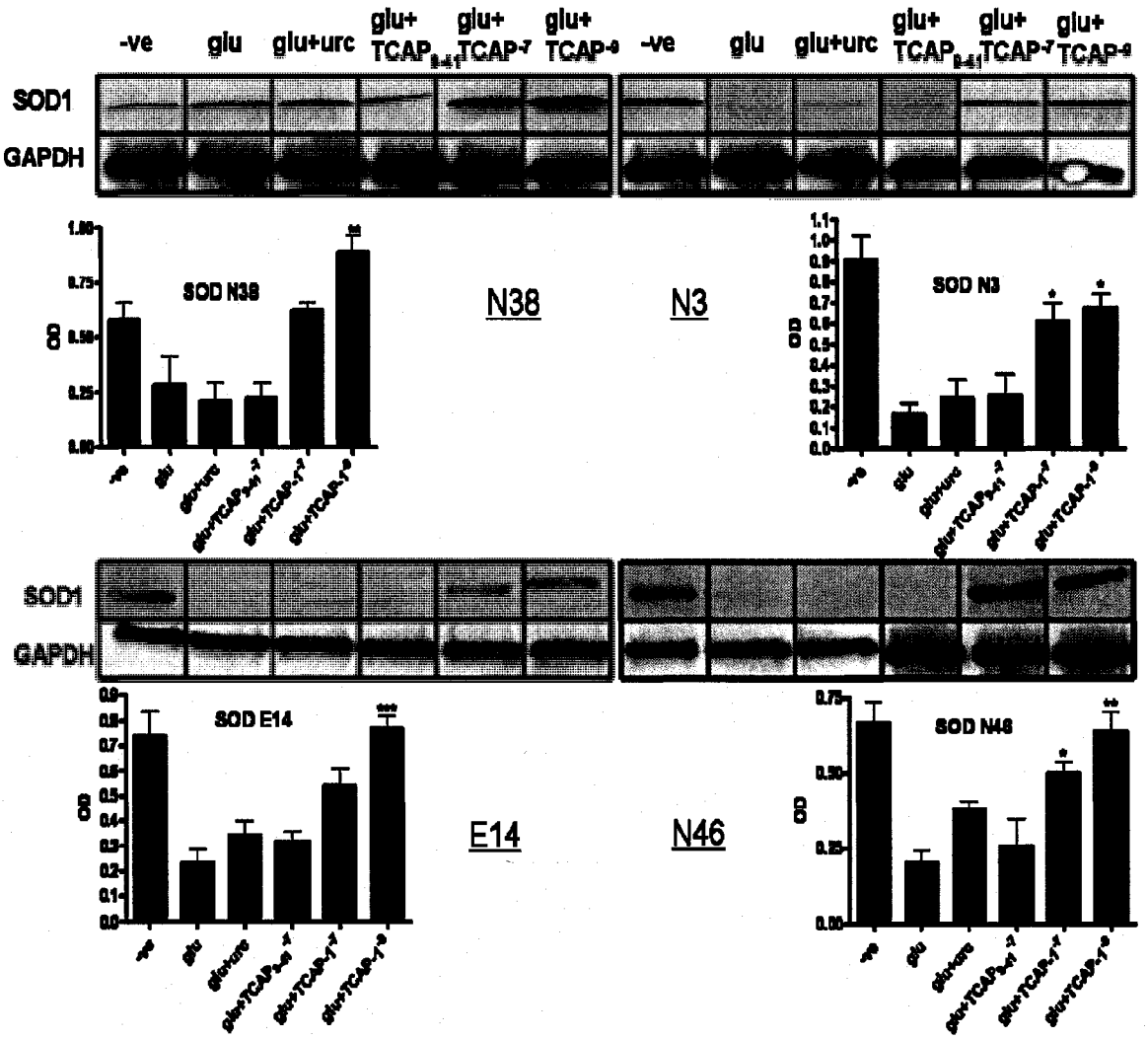

Figure 4.4. SOD1biomarker activity. Detection of SOD1 (23kDa) by western blot in hippocampal, N38, N3, N46 and E14 hypothalamic cells with chronic TCAP-1 treatments of TCAP-1 10^{-7}, 10^{-9}M and glutamate as determined by western blot. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

SOD1 gene expression, as measured by real-time PCR, indicated a significant TCAP-1 effect at both 10^{-7} and 10^{-9} in N3 ($p<0.05$ for both) and N46 cell lines ($p<0.05$) and ($p<0.01$) respectively. TCAP-1 10^{-9} chronic treatment in N38 and E14 cell lines showed significant increases in SOD 1 expression ($p<0.01$) and ($p<0.001$) respectively. In all cell lines there was no significance amongst the glutamate and glutamate + urocortin or TCAP-1 $_{9-41}$ treated cells (Figure 4.5).

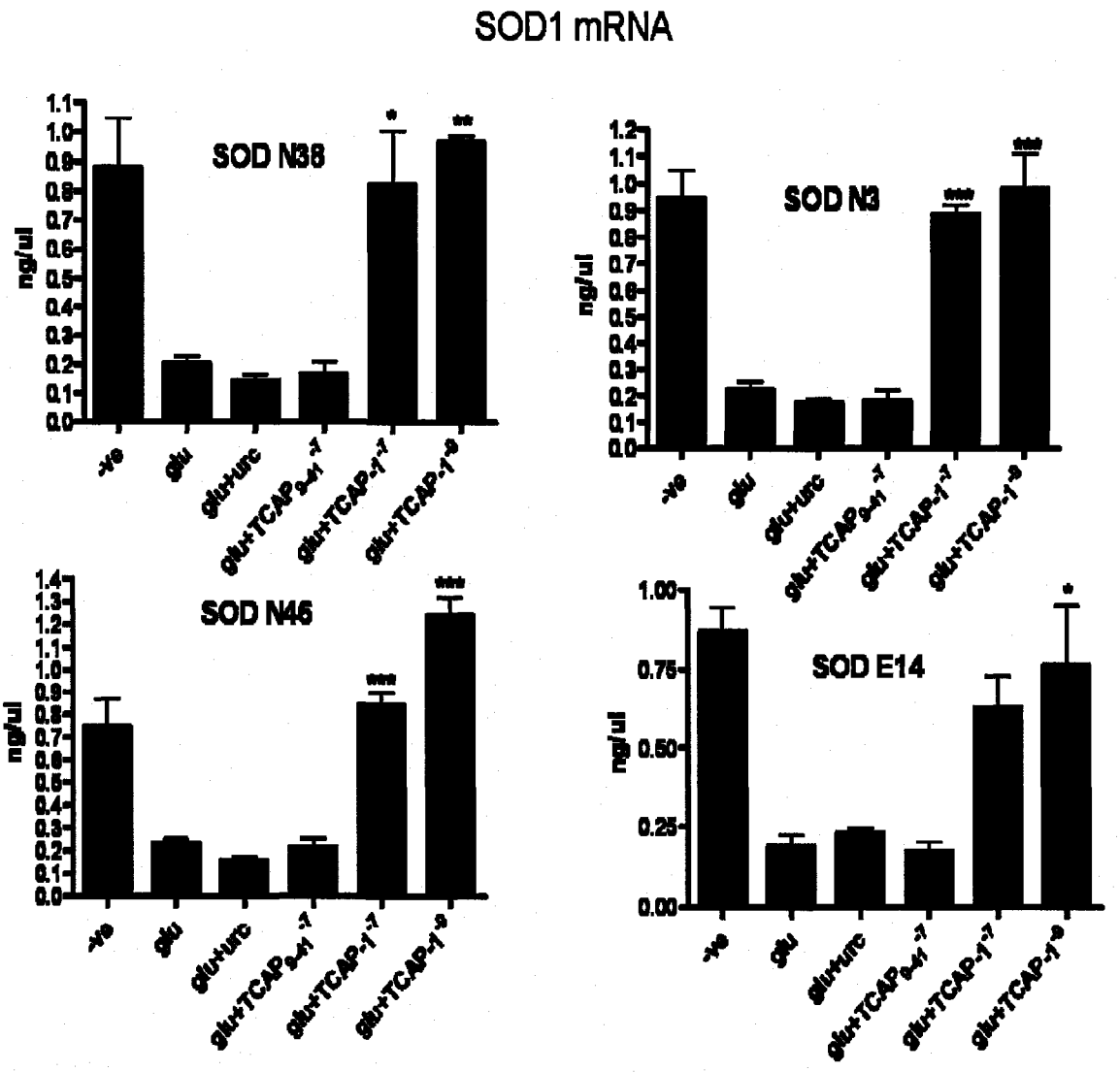

Figure 4.5. SOD1mRNA. Detection of SOD1 by real-time PCR in hippocampal, N38, N3, N46 and E14 hypothalamic cells with chronic TCAP-1 treatments of TCAP-1 10^{-7}, 10^{-9}M and glutamate. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

4.4 Discussion

The data in this chapter reveals that TCAP-1 treatment given prior to glutamate insult confers neuroprotection to neurons. TCAP-1 treated cells exhibit higher levels of

PERK than control treated cells indicating that TCAP-1 may be mediating the Ras-Raf-ERK signal transduction pathway in order to procure survivability. Furthermore, ROS generation has been shown to increase under glutamate stress and TCAP-1 treated cells were shown to upregulate SOD1 protein and gene transcription. Urocortin was also co-treated with glutamate. Urocortin shares sequence similarity with CRF and has also been shown to confer neuroprotection to compromised neurons via CRF receptor 1 (Pedersen et al., 2002). Thus, urocortin was utilized as an additional peptide control. Although the data reveal that urocortin did not confer protection this may be because the immortalized cell lines used do not exhibit either CRF receptor. Thus, TCAP-1 may be acting as a radical oxygen scavenger as a means of mediating survival through ERK phosphorylation.

Growth factors, through receptor tyrosine kinases, recruit a large network of signaling proteins to execute their cellular programs. The first of these networks to be discovered was the Ras-Raf-ERK signal transduction cascade, defined by extracellular signal-regulated kinase-1 (ERK1) and ERK2 (Seger and Krebs, 1995). One of four mitogen-activated protein kinase (MAPK) signaling pathways, the ERK phosphorylation cascade's importance in intracellular signaling has been compared to the role of the Krebs cycle in energy metabolism (Reszka et al., 1995). The ERK cascade functions in cellular proliferation, differentiation, and survival, and its inappropriate activation is a common occurrence in human cancers (Alberola-Ila et al., 1995, Eldar-Finkelman et al., 1995). During growth factor stimulation, the ERK phosphorylation cascade is linked to cell surface RTKs and other upstream signaling proteins with known oncogenic potential (Campbell et al., 1995). Activation of all MAPKs is regulated by a central three-tiered

core signaling module, comprised of an apical MAPK kinase kinase (MAP3K), a MAPK kinase (MEK or MKK), and a downstream MAPK (Schlessinger, 2000). Like Ras, the MAP3K Raf was first discovered in the form of a mutant retroviral transforming agent, v-raf. Raf is a Ser/Thr protein kinase, catalyzing the phosphorylation of hydroxyl groups on specific Ser and Thr residues (Rapp et al., 1983).

Phosphorylated Raf activates MAPK/ERK kinase 1 and 2. Disruption of mouse *mek1* is lethal *in utero*, with mutant embryos dying from defective placental vascularization, suggesting a role for MEK in angiogenesis (Giroux et al., 1999). MEK1 and MEK2, about 45 kDa each, share 80% sequence identity. It is unclear why two MEKs exist, although conservation of both forms throughout eukaryotic species suggests non-redundant functions, as does MEK1 gene disruption. Both MEKs are expressed ubiquitously in mammalian cells at micromolar levels, although some tissue-specific variation has been noted (Brott et al., 1993).

Raf family activation of MEK1 and MEK2 occurs through phosphorylation of two Ser residues at positions 217 and 221 found in the activation loop (Alessi et al., 1994). MEKs can be partially activated by phosphorylation at either site, and substitution of these sites with acidic amino acids enhances basal activity (Rosario et al., 1999). While Raf isoforms are enzymes of relatively low abundance, the high concentration of MEKs allows for amplification of signaling (Alessi et al., 1994). Different Raf isoforms activate MEK1 and MEK2 differentially: A-Raf is a weak activator; B-Raf activates MEK1 preferentially; and Raf-1 efficiently activates both MEKs (Seternes et al., 1998). Raf-1 contains two separate MEK binding sites, with substrate interaction greatly enhanced following phosphorylation of Raf-1 at Ser338. Two regulatory phosphorylation

sites on MEK outside the activation loop either positively or negatively regulate the MAPKK. The first, at Ser298 and phosphorylated by PAK1, may help prime MEK1 for activation by Raf-1. Conversely, *in vivo* phosphorylation by an unknown kinase at Ser212, a site conserved in all MAPKKs, sharply decreases MEK1 activity (Paasinen-Sohns and Holtta, 1997).

Also known as MAPK3 and MAPK1, the MAP kinases ERK1 and ERK2 are 44- and 42-kDa Ser/Thr kinases, respectively, with 90% sequence identity in mammals (Jaiswal et al., 1996, Hughes et al., 1997). Initially isolated and cloned as kinases activated in response to insulin and NGF, ERK1 and ERK2 are both expressed in most, if not all, mammalian tissues, with ERK2 levels generally higher than ERK1 (Jaiswal et al., 1996, Weiglein et al., 1997). Knock-out studies in mice demonstrate that either ERK may at least partially compensate for the other's loss, although ERK1 has been found to specifically regulate thymocyte maturation (Pages et al., 1999).

Several studies have identified ERK isoforms have having neuroprotective capacities. The best studied of these isoforms is ERK 1/2 , which have been shown to protect cells from DNA damage, trophic factor deprivation and oxidative stress (Hetman et al., 1999). Studies have identified a role for MAPK/ERK or MEK, an upstream kinase for CREB, as effective in protecting neurons during cerebral ischemia. In particular, N-acetyl-O-methyldopamine (NAMDA), induced neuroprotection occurs via the activation of ERK and downstream CREB phosphorylation in hippocampal neurons exposed to oxygen-glucose deprivation (Park et al., 2004a) as well as upregulation of SOD1 transcription (Scorziello et al., 2007). The neuroprotective effects were reversed with a MEK kinase inhibitor and accordingly NAMDA treated cells following ischemia showed

a significant increase in ERK expression. Thus, the activation of this pathway would be a beneficial means by which to counter ischemic insult. This study reveals that TCAP-1 effectively increases activation of ERK in order to confer survivability to neurons compromised by glutamate excitotoxicity. This identifies TCAP-1 as a potential mediator of the MEK pathway and subsequent neuroprotective agent against potential brain damage during ischemia.

Due to the brain being highly aerobic, its oxygen dependency is significantly more pronounced than any other vital organ. One of the pitfalls of this dependency is the production of O_2^-, which are very detrimental to neurons. The literature reveals that protection against such O_2^- stress is imperative for neuronal survival (Perez-Asensio et al., 2005). In chapter 2, I elucidated the importance SOD1, which is predominantly associated with O_2^- scavenging. Several *in vitro* studies have shown that high oxygen stress induced significantly higher levels of neuronal death in embryonic as opposed to post-natal cell cultures. Future studies may lead to a better understanding of TCAP's role in the embryo under oxidative stress conditions.

Interestingly, data show that antioxidants are neuroprotective in experimental stroke by delaying ischemia induced decreases in and subsequent glutamate release (Cardenas et al., 2000). Therefore, antioxidants may be neuroprotection agents by increasing ATP levels and preventing decreases in ATP in ischemia. Administration of a SOD analogue was significantly effective in improving stroke outcome as demonstrated by reduction in infarct volume and general improvement of neurological scores (Hurtado et al., 2003).

This thesis has identified TCAP-1 as being able to protect cells from both apoptotic and necrotic cell death and that increasing ROS scavenging systems, such as SOD, may act as the main mechanism. The generation of ROS has been implicated on several occasions to play a role in glutamate excitotoxicity as well as mediate both apoptotic and necrotic cell death pathways. This study reveals that TCAP effectively inhibits cell death in glutamate treated cells via regulation of SOD1 and we have previously shown that TCAP-1 has successfully prevented both apoptotic and necrotic cell death in stressed neurons. Both forms of cell death have been implicated in stroke disease and in ischemic stress there is a large increase in glutamate release causing neuronal injury, however changes in development and injury determines whether apoptotic or necrotic stress are the main players in cell death (Nicotera et al., 1999, Nicotera and Lipton, 1999, Liu et al., 2004). As ROS is a common player in several forms of cells death the role of TCAP-1 may be to act in an anti oxidant capacity to procure survivability to compromised neurons during periods of brain trauma in particular when glutamate levels become neurotoxic.

4.5 References

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Chapter 5: TCAP-1 confers neuroprotection by mediating growth factor regulation

This chapter is in preparation for submission to *Journal of Neuroscience*

Gina **Trubiani,** Dalia Barsyte, Claudio Casatti, Denise D. Belsham, and David A. Lovejoy. Teneurin carboxy (C)-terminal associated peptide-1 regulates BDNF expression and neuronal morphology *in vitro*

Growth factors have been implicated in protecting neurons from various forms of cell injury including several neurodegenerative disorders in the brain. In particular, BDNF has been reported to influence neural growth during episodes of cell stress, effect cell proliferation and play an important role in the formation and maintenance of vertebrate neurons. Previous data has shown that TCAP-1 mediates several cellular signaling processes, which in turn confers a neuroprotective effect in vitro. However, the physiological importance of TCAP remains ambiguous. We explored the effects of reduced TCAP expression in a siRNA transfected immortalized hypothalamic cell line under normal and 5-fluorouracil (5-FU) stressed conditions. 5-FU induced apoptosis has been characterized by a detrimental attenuation in cell proliferation by inhibiting the cell cycle in s-phase eventually leading to apoptotic cell death in several cell lines. TCAP knockdown cells show a decrease in cell proliferation, number of connecting cells, number of neurites, neurite length, cell size and cell length; furthermore, TCAP knockdown also revealed an exacerbation of 5-FU toxicity and addition of TCAP rescues a neuroprotective effect although the knockdown reveals an attenuated neuroprotective effect in comparison to the control.

Chronic treatment of TCAP-1 at concentrations 10^{-7} and 10^{-9} caused a significant increase in BDNF expression. The treated cells were then scored based on different

morphological characteristics. TCAP-1 treated cells exhibited significant increases in neurite length, number of neuritis, cell size, cell length, number of cells and number of connecting cells compared to the control treatment. TCAP-1 treatment also attenuated 5-FU (5-fluorouracil) induced death as compared to the control treatments in TCAP siRNA cells. The ability of TCAP-1 to increase BDNF expression, decrease caspase 3 cleavage, mediate cell morphology and confer neuroprotection under induced cell injury indicates a potential therapeutic application against traumatic stress such as stroke and ischemia as well as chronic neurodegenerative diseases such as Alzheimer's and Parkinson's associated with uncontrolled and deleterious cell death by regulating growth factors such as BDNF.

5.1 Introduction

Growth factors have been implicated in mediating cell survival in neurons and several have been known to confer neuroprotection in a myriad of toxic environments. BDNF is predominantly, though not exclusively, localized in the CNS, is highly expressed in the hippocampus and exhibits prevalent protein expression in the granule cells of the dentate gyrus (Poo, 2001). It supports the survival of neuronal populations that are all located either in the central nervous system or directly connected to it; for instance, cerebellar and hippocampal regions (Wu and Pardridge, 1999). It is expressed within mature peripheral ganglia and depending on the injury may act in either an autocrine or paracrine manner immediately following neuronal insult sometimes characterized by a rapid influx of growth factors near the site of trauma (Bella et al., 2006). It also regulates synaptic

transmission and plasticity at adult synapses in many regions of the CNS (Haapasalo et al., 1999). The versatility of BDNF is characterized by its ability to modulate a range of adaptive neuronal responses including long-term potentiation, long-term depression, certain forms of short-term synaptic plasticity, as well as homeostatic regulation of intrinsic neuronal excitability (Poo, 2001, Hanada et al., 2004).

Neurotrophins act on signaling pathways via two cell surface receptors: low affinity p75 receptor, high affinity tyrosine kinase receptor, TrkB, the most abundant Trk receptor in the brain (Bibel and Barde, 2000, Kaplan and Miller, 2000, Huang and Reichardt, 2003). Unlike the p75 receptor, which is coupled to the janus kinase (JAK) pathway and is associated with the death cascade, the TrkB receptor is linked to the MAPK/ERK pathways associated with cell survival (Han and Holtzman, 2000). Once activated the TrkB receptors autophosphorylate tyrosine residues, which act as interactions sites for PTB/SH2 protein domains (Bibel and Barde, 2000). Consequently, Ras is also activated along with TrkB which is affected by Shc/Grb2/SOS subsequently activating ERK, MAPK and P13-K which binds to 1,4,5-trisphosphate forming 3'-phosphorylated phosphoinositides then activating Akt/PKB causing cell survival pathways to be active (Kaplan and Miller, 2000). Akt phosphorylates Bad causing it to be sequestered by 14-3-3 in the cytosol and prevents cytochrome c dispersion from the mitochondria as well as translocates to the nucleus where it acts with ERK to phosphorylate transcription factors thus regulating cell survival gene expression (Rabizadeh et al., 1993, Han and Holtzman, 2000). This is particularly pertinent in the protection of cultured hippocampal neurons from glutamate induced apoptotic cell death, specifically by reducing caspase 3 cleavage (Almeida et al., 2005).

BDNF exhibits neuroprotective effects on cell morphology including promoting neurite length, cell size, number of neuritis, axon sprouting and axon branching (Kerrison et al., 2005). In particular, BDNF has been shown to increase neurite growth in cultured hippocampal cells under prenatal hypoxia conditions, which have been identified as adversely affecting neural growth (Briscoe et al., 2006). Further studies have shown that antibody sequestration of BDNF attenuated neurite growth and increased Trk receptor expression following AMPA receptor potentiation administration indicating that AMPA induced increases in neurite length *in vitro* are mediated by a BDNF mechanisms (Voss et al., 2007).

In chronic neurodegeneration, such as Alzheimer's, decreased levels of BDNF have been reported in specific regions of the brain (Kahle and Haass, 2001, Lee et al., 2005). In Parkinson's disease, BDNF mRNA is significantly reduced in part due to loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) which express BDNF (Howells et al., 2000). Taken together, these studies suggest that neurons expressing particularly low levels of BDNF may result in degeneration by increasing the risk and effects of injury. Behavioral and neurochemical data have shown that BDNF may act on dopamine neurons *in vivo* highlighting a therapeutic function for BDNF in the treatment of Parkinson disease (Altar et al., 1992, Howells et al., 2000). Thus, agents that may upregulate BDNF expression, especially during times of brain injury, may play pivotal roles in preventing neuronal damage.

In vivo BDNF has been shown to rescue different types of neurons from ischemic, traumatic and toxic brain injury (Schabitz et al., 1997, Yamashita et al., 1997, Wu and Pardridge, 1999, Schabitz et al., 2000, Zhang and Pardridge, 2001, Kazanis et al., 2004).

Recent data show that BDNF is an effective agent upon activation of glutamate receptors (Almeida et al., 2005) as there is an increase in the presence of radical oxygen scavengers due to abundant ROS products produced in the mitochondria from the high intracellular calcium levels.

5-FU is part of a group of drugs known as the anti-metabolites and is currently being used as an oral prodrug to achieve optimal response and postoperative survival in oral adjuvant chemotherapy of cervical cancer patients (Inada et al., 1997). Conversely, it is also used a means to induce uncontrolled cell death (Pritchard et al., 1998, Borralho et al., 2007). 5-FU must be enzymatically activated to its cytotoxic form by the targeted tumor cells and is an analogue of pyrimidine nucleosides that blocks the synthesis of deoxythymidylic acid by thymidylate synthetase and disrupts normal RNA function (Werner et al., 2007, Wu et al., 2007). Dihydropyrimidine dehydrogenase is the enzyme for the rate-limiting step for 5-FU catabolism that accounts for more than 80% of its elimination. The effective cytotoxic level of available 5-FU is thus principally determined by its catabolism by dihydropyrimidine dehydrogenase and experimental data suggests the involvement of p53, the Bcl-2 family and BAG-1 in chemotherapy-induced apoptosis (Jaattela, 1999b, a, Mathiasen et al., 1999, Hengstermann et al., 2001).

This chapter seeks to show that chronic treatment of TCAP-1 up-regulates BDNF expression in hypothalamic and hippocampal immortalized cultured cells. Furthermore, we observed that TCAP-1 has significant effects on morphology in cultured cells. In addition we show that TCAP siRNA has detrimental effects on cell morphology and neuronal survivability under 5-FU apoptotic conditions and subsequent treatment with TCAP-1 attenuates cell degeneration thus, indicating that TCAP-1 may be modulating

cell activity in order to confer a neuroprotective effect during times of brain trauma via mediating BDNF. These findings suggest that TCAP-1 may act endogenously to activate cell signaling in times of brain insult. Taken together, the current study uncovers the essential function of TCAP expression as well as establishes it as an important physiological neuroprotective agent against induced cell death.

5.1 Materials & Methods

Peptide Synthesis

Mouse TCAP-1 was prepared by solid-phase synthesis as previously described (Qian et al., 2004). The peptide was solubilized in phosphate buffered saline (PBS) at a stock concentration of $2x10^{-5}$ M before being diluted in the appropriate medium. Non-functional TCAP fragment was synthesized as described previously (pEESQRMFHINNVSLVFYGDYGQVRGTGLLQASDSLELYQEI-NH_2) (TCAP 9-41) (Qian et al., 2004, Al Chawaf et al., 2007) and used in conjunction with 10^{-7}M urocortin as negative controls in the vehicle.

Cell Culture: pre incubation treatment

The effect of TCAP-1 on cell morphology was investigated using E14 immortalized mouse hippocampal cell line. The cells were grown in 12-well culture plates with 1 mL of Dulbecco's Modified Eagle Medium (DMEM) containing high glucose, L-glutamate, 25mM HEPES buffer, pyridoxine hydrochloride in the absence of sodium pyruvate and 5ml penicillin with 10% fetal bovine serum (FBS) at pH 7.4 (Gibco-Invitrogen, Burlington, Canada). At 24 hrs replicates of four wells received either TCAP-1 10^{-7}, 10^{-9}M, TCAP-1 9-41 fragment, 10^{-7}M urocortin, whereas a separate group received no treatment. The cells were incubated until they reached 80% confluency, approximately 4 days from their initial culture. The cells were viewed under a Carl Zeiss Axiovert 200M inverted microscope using AxioVision Image Viewer software version 4.2.

Morphology Analysis: pre incubation treatment

Using LabWorks 4: Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.) the neurons were scored based on a series of morphological characteristics: number of soma, number of connecting cells, number of neurites, length of neurites, cell size, cell length. The data was analyzed by observers blind to treatments to quantify each morphological characteristic. One cell located in the upper right hand quadrant of the image from each replicate was quantified. A one-way ANOVA with

Tukey's multiple comparison post hoc analysis was used to determine significance. Statistical analysis of all data was carried out with GraphPad Prism® version 4.03.

BDNF immunoblot detection in hypothalamic & hippocampal cells

The detection of BDNF was determined at 24 hrs in N38, N3, N46 hypothalamic and E14 immortalized hippocampal cells were cultured as described earlier. The samples at each condition and control were removed using a cell scraper and centrifuged at 1500 rpm for 10 min. The cells were resuspended in 350μL of chilled cell lysis buffer containing 500μL PBS, 5μL 1% Triton X-100 (Sigma, St. Louis), 25μL proteinase inhibitor cocktail set III (VWR, Mississauga), 0.5μL 1M dithiothreitol (DTT) (Sigma, St. Louis) and 2.5μl phenylmethylsulphonylfluoride (PMSF), diluted in 1mL of methanol (EM Science, Gibbstown), incubated on ice for 10 min and centrifuged for 5 min at 10,000 rpm. An aliquot of 25μL of each sample was combined with 25μL of 2x 20% sodium dodecyl sulphate (SDS) sample buffer and loaded onto a 4-10% HCL-Tris pre-cast polyacrylamide gel (BioRad, Mississauga). The gel ran at 200 v for 35 min and proteins were electro-transferred to a Hybond-C nitrocellulose membrane (Amersham, Baie d'Urfé) for 75min at 100 v. After transfer, the membrane was washed with 10 mL of PBS with 0.05% Tween 20 (PBST) for 5 min at room temperature (RT) and the membrane was incubated in 10 mL of PBST-milk for one hour at RT followed by three 5-min washes with 10 mL of PBST. The membrane was then incubated with the mature BDNF primary antibody (Santa Cruz) at a titre of 1:1000 in 6mL of PBST-milk, with gentle agitation overnight at 4°C. The membrane was washed 3 times for 5 min with

10mL of PBST followed by incubation with the anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham, Baie d'Urfé) at 1:3000 in 6mL of PBST-milk with gentle agitation for 1 hr at RT. The membrane was then washed 3 times for 5 min with 10mL of PBST then exposed to Kodak X-OMAT Blue scientific imaging film (Perkin Elmer Canada Inc, Vaudreuil-Dorion) for 30 min. The total optical density of the blots was quantified using LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.) and a ratio of the BDNF versus GAPDH OD was computed. A one way ANOVA with Tukey's multiple comparison post hoc analysis was used to determine significance. Statistical analysis of all data was carried out with GraphPad Prism® version 4.03.

Real-time PCR gene expression analysis: BDNF

Total RNA was isolated with Trizol (Invitrogen) and reverse-transcribed using OneStep RT-PCR (Qiagen). Quantitative real-time PCR was performed using SYBR Green (Sigma) on Rotor-Gene 6 software (Corbett Research). In order to activate the reverse transcriptase the samples were treated at 50°C for 30 minutes and the initial PCR activation step consisted of 15 minutes at 95°C followed by 40 cycles at 94°C for 1 minute, 50° for 1 minute and 72° for 1 minute. The transcript number/ng was obtained using standard curves generated with mouse brain RNA. The data was normalized to control TBP transcript levels. All reactions were run in triplicate and the data is presented as means and SEMs. The BDNF primers consisted of: forward, 5'-GGTATCCAAAGGCCAACTG-3' and reverse, 5'-CTTATGAATCGCCAGCCAA-3';

The TBP primers consisted of forward, 5'-CAGACCCCACAACTCTTCCATT-3'and reverse 5'-TCTCAGAAGCTGGTGTGGCA-3'. A ratio of the BDNF versus GAPDH OD was computed. A one way ANOVA with Tukey's multiple comparison post hoc analysis was used to determine significance. Statistical analysis of all data was carried out with GraphPad Prism® version 4.03.

siRNA mediated TCAP knockdown

siRNA sequences for TCAP and the control were synthesized by Genosys, annealed, phosphorylated and cloned into pLKO.1puro (Sigma). SiRNA 270 was designed to recognize TCAP 2,3,4 and siRNA 721 to TCAP 3. Both siRNAs were co-transfected with a puromycin resistant gene. The sequences used were:

270 sense: CCGGGGACACACACTACTTCATCAATTCAAGAGATTGATGAAGTAGTGTGTGTCCTTTTTG

270 antisense: AATTCAAAAAGGACACACACTACTTCATCAATCTCTTGAATTGATGAAGTAGTGTGTGTCC

721 sense: CCGGCCAGTTCTTGCGACAAAGTTTCAAGAGAACTTTGTCGCAAGAACTGGTTTTTG

721 antisense AATTCAAAAACCAGTTCTTGCGACAAAGTTCTCTTGAAACTTTGTCGCAAGAACTGG

Negative control sense

CCGGGGATTCCAATTCAGCGGGAGCCACCTGATGAAGCTTGATCGGGTGGCTCTCGCTGAGTTGGAATCCTTTTTG

Negative control antisense

AATTCAAAAAGGATTCCAACTCAGCGAGAGCCACCCGATCAAGCTTCATCAGGTGGCTCCCGCTGAATTGGAATCC

The lentiviruses were packaged in the 293 cells using Mission Lentiviral packaging mix plasmids (Sigma). 48h later, filtered supernatants with viral particles were

transferred to N38 cells and incubated with 8μg/ml hexadimethrine bromide (Sigma) for 16h.

SiRNA mediated knockdown of TCAP and teneurin

The effect of TCAP-1 siRNA 270 and 721 on cell morphology was investigated compared to a non transfected N38 immortalized mouse hypothalamic cell line. All 3 cell lines were grown in 6-well culture plates with 2 mL of Dulbecco's Modified Eagle Medium (DMEM) containing high glucose, L-glutamate, 25mM HEPES buffer, pyridoxine hydrochloride in the absence of sodium pyruvate and 5ml penicillin with 10% fetal bovine serum (FBS) at pH 7.4 (Gibco-Invitrogen, Burlington, Canada).

The cells were washed and incubated in the media for 24h before puromycin selection at 2μg/ml (Sigma). The selection was monitored using the control uninfected cells. For higher stringency another subset of the cells were subsequently treated with puromyocin (4mg/ml) 28hrs post incubation at 80% confluence. In passage 3 the antibiotic concentration was increased to 10 mg/ml and after 72 hours the cells were harvested at 80-90% confluency. The cells remained in culture, from P0 to harvest in P3 was 13 days for control cells luciferase, 17 days for 721 and 28 days for 270.

The cells were harvested and total RNA was isolated using the RNeasy Mini Kit (74124, Qiagen) followed by mRNA purification with Oligotex kit (70022, Qiagen). The quality and quantity of samples were evaluated by spectrophotometer at 260 and 280 nm. The mRNA was submitted to DNase treatment (1907, Turbo DNA-free Kit, Ambion) in order to remove any persistent DNA contamination.

Sets of degenerate primers were designed for TCAPs, teneurins and histone H3 (Table 1). About 60-80 ng of mRNA from cell cultures were submitted to one-step RT-PCR (OneStep RT-PCR kit, Qiagen) 50 µl final volume reaction, containing 400 µM of each dNTP, 10 µl of 5× QIAGEN OneStep RT-PCR buffer, 10 µl of 5x Q-solution, 2 µl of QIAGEN OneStep RT-PCR enzyme mix and 0.2 µM each primer to TCAPs and teneurins or 0.1 µM to histone. The thermal cycler conditions were 30 min at 94°C to reverse transcription, 15 min at 95 C to initial PCR activation, 20 to 33 cycles to denaturation (1 min at 94°C), annealing (1 min at 59°C) and extension (1 min at 72°C) following with final extension of 10 min at 72°C. The RT-PCR products were examined by 1.5% agarose gel electrophoresis using a UVP System 8000 digital imaging system with the Laboratory Imaging and Analysis System software (Ver. 4) (UVP, Upland, CA, USA).

The cells were cultured as described above and grown in 6 well plates. Their morphology was analyzed at the 24hr and 48hr mark. Using LabWorks 4: Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.) the neurons were scored based on a series of morphological characteristics: number of soma, number of connecting cells, number of neurites and length of neurites. The data was analyzed by observers blind to treatments to quantify each morphological characteristic. One cell located in the upper right hand quadrant of the image from each replicate was quantified. A one-way ANOVA with Tukey's multiple comparison post hoc analysis was used to determine significance. Statistical analysis of all data was carried out with GraphPad Prism® version 4.03.

Rescue experiment: 270 TCAP siRNA

The cells were cultured as described above for 48hrs at which point replicates of four wells received 50μg/ml 5-fluorouracil (Sigma, St. Louis) and either TCAP-1 10^{-11}M, or $TCAP_{9-41}10^{-11}$M whereas a separate group received no treatment. The cells were incubated for 24hrs post-treatment.

The cells morphology was subsequently analyzed as described above. A one-way ANOVA with Tukey's multiple comparison post hoc analysis was used to determine significance. Statistical analysis of all data was carried out with GraphPad Prism® version 4.03.

Viability Analysis (MTT assay5-FU cell death induction): 270 TCAP siRNA

The effect of TCAP-1 10^{-11}M on non-transfected, Luc transfected and 270 siRNA transfected cell viability was examined at 24 post 5-FU induced stress indirectly by assessing mitochondrial activity using a colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. The MTT assay was conducted using the In Vitro Toxicology Assay Kit: MTT based (Sigma, St. Louis). The cells were incubated at 37° C in 5% CO_2 for 3hrs in the presence of MTT 200μL/2mL medium. The samples were mixed by shaking the plate horizontally for 30 min. The background absorbance of

the samples was determined at 690nm and subtracted from the 570nm measurement. A one-way ANOVA with Tukey post test was used to determine significance.

Table 1. primers used for RT-PCR to detect TCAPs and Teneurins.

Gene product	forward	reverse
TCAP-1	ttcatttccttggatcagcttcctatg	aagctgctgcttttctccctctgtcca
Teneurin 1	gtgtcacctgatggcaccctctat	tcctgggtatgtcatcaaggccaa
TCAP-2	gacaagatgcactacagcatcgag	ccatctcattctgtcttaagaactgg
Teneurin 2	atcctgaactcgccgtcctcctta	ctccaggttctgagtggacacggc
TCAP-3	caacaacgccttctacctggagaac	cgatctcactttgtcgcaagaact
Teneurin 3	agtggaatacccggtggggaagcac	gtgagtaccgttgatgtcaaagatg
TCAP-4	tttgcctccagtggttccatctt	tggatattgttggcgctgtctgac
Teneurin 4	atcgaccaattcctgctgagcaag	catgttctgagtgttcaggaaagg
Histone H3	gcaagagtgcgccctctactg	ggcctcacttgcctcgtgcaa

BDNF and caspase 3 protein expression in 270 TCAP siRNA

The detection of BDNF and caspase 3 protein expression was determined at 24 hrs post treatment in 270 TCAP siRNA, luciferase and N38 cells as described earlier. Briefly, the samples at each condition and control were removed, centrifuged resuspended in 350μL of chilled cell lysis buffer An aliquot of 25μL of each sample was loaded onto a 4-10% HCL-Tris pre-cast polyacrylamide gel (BioRad, Mississauga), transferred, washed, blocked then incubated with the mature BDNF or caspase 3 (Santa Cruz) or GAPDH (Abcam) primary antibody at a titre of 1:1000 overnight at 4°C followed by a wash and incubation with the anti-goat horseradish peroxidase (HRP)-conjugated secondary

antibody (Amersham, Baie d'Urfé) at 1:3000 for 1 hr at RT. The membrane was then washed then exposed for 30 min. The total optical density of the blots was quantified using LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.) and a ratio of the BDNF/caspase 3 versus GAPDH OD was computed. A one-way ANOVA with Tukey's multiple comparison post hoc analysis was used to determine significance. Statistical analysis of all data was carried out with GraphPad Prism® version 4.03. TIFF files of blots were imported into CorelDRAW 12.0® and then arranged in sequence accordingly, please see Appendix 1C for a generalized representation of a sample blot using the caspase primary antibody, all western blots were treated in the same manner.

Real-time PCR gene expression analysis: BDNF and caspase 3 in 270 TCAP siRNA

The detection of BDNF and caspase 3 mRNA expression was determined at 24 hrs post treatment in 270 TCAP siRNA, luciferase and N38 cells as described earlier. Briefly, total RNA was isolated with Trizol (Invitrogen) and reverse-transcribed using OneStep RT-PCR (Qiagen) followed by quantitative real-time PCR The data was normalized to control TBP transcript levels. All reactions were run in triplicate and the data is presented as means and SEMs. The BDNF primers consisted of: forward, 5'-GGTATCCAAAGGCCAACTG-3' and reverse, 5'-CTTATGAATCGCCAGCCAA-3'. The caspase 3 primers consisted of: forward, 5'-TGGAGAAATTCAAAGGACGGGTCG-3' and reverse, 5'-GTACAGTTCTTTCGTGAGCATGGA-3'. The TBP primers consisted of forward, 5'-

CAGACCCCACAACTCTTCCATT-3'and reverse 5'-TCTCAGAAGCTGGTGTGGCA-3'. A ratio of the BDNF or caspase 3 versus GAPDH OD was computed. A one way ANOVA with Tukey's multiple comparison post hoc analysis was used to determine significance. Statistical analysis of all data was carried out with GraphPad Prism® version 4.03.

5.3 Results

Morphology Analysis: E14 hippocampal cells with chronic TCAP-1 treatment

Four replicates of each sample were analyzed (Tukey one-way ANOVA) for the following morphological characteristics in order to determine chronic effects on BDNF expression of TCAP10^{-7}M and TCAP 10^{-9}M treated E14 hippocampal cells: number of soma, number of connecting cells, number of neurites, length of neurites, cell size, cell length. (Figure 5.1). TCAP 10^{-7}M ($p<0.05$) and TCAP 10^{-9}M ($p<0.001$) showed overall significance in both cell number and connecting cells as compared to vehicle-treated cultures at 24, 48 and 72 hrs. There was a marginally significant decrease in the number of neurites as compared to vehicle-treated cultures for both concentrations of TCAP 10^{-7}M ($p<0.05$) and TCAP 10^{-9}M ($p<0.05$) at 24 and 72hrs. There was overall significant increase in the neurite length as compared to vehicle-treated cultures for both concentrations of TCAP 10^{-7}M ($p<0.001$) and TCAP 10^{-9}M ($p<0.001$) at 24, 48 and 72 hrs. There was overall significant increase in cell size and cell length as compared to vehicle-treated cultures for TCAP 10^{-9}M ($p<0.05$) at 48 and 72hrs for both.

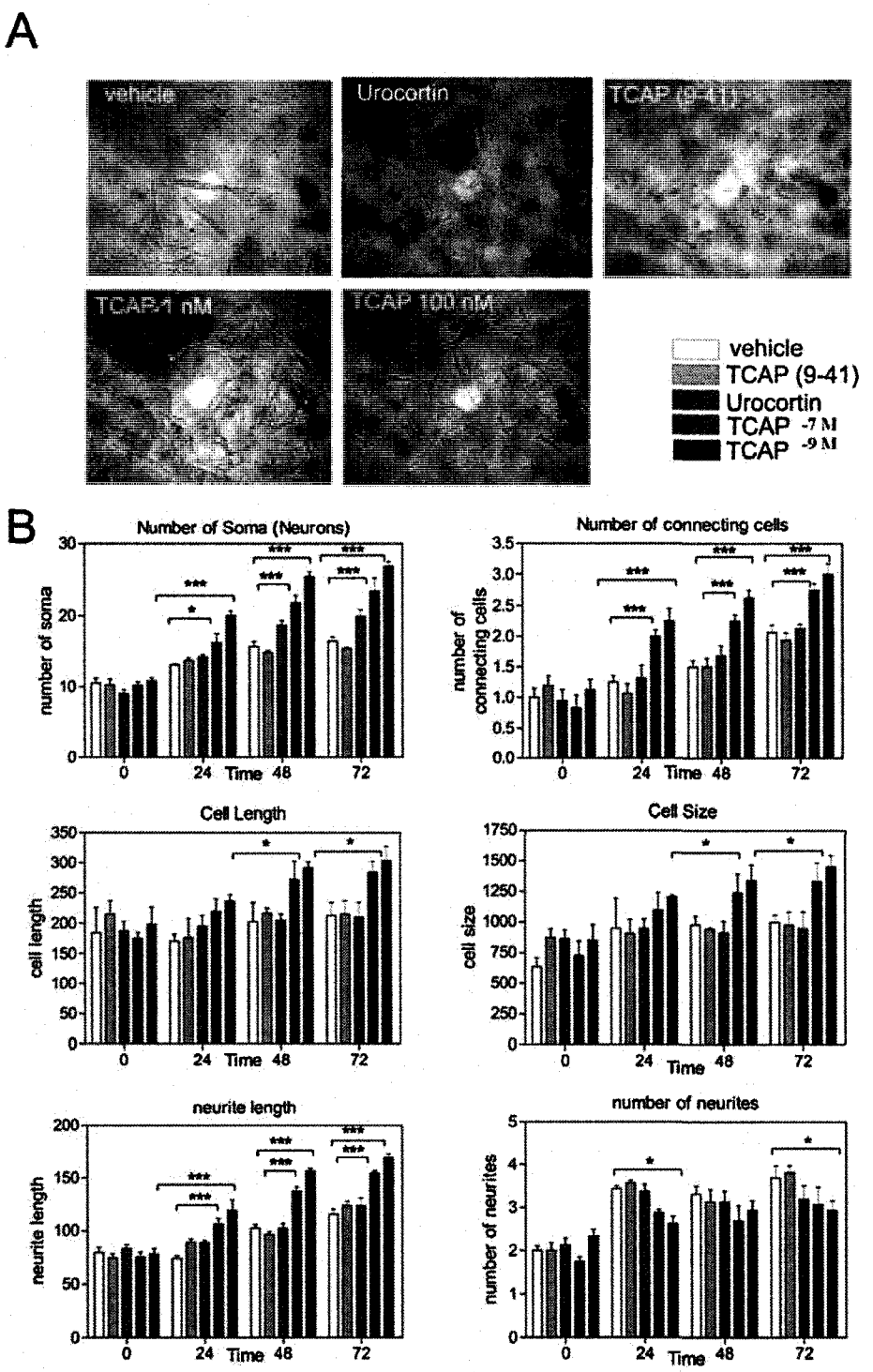

Figure 5.1. A.Neuroprotective morphology of immortalized E14 hippocampal, N38, N3 and N46 hypothalamic cells with chronic TCAP-1 treatments of TCAP-1 10^{-7}, 10^{-9}M was assessed by scoring cells for neurite length, number of neurites, cell size, cell length, number of cells and number of connecting cells. TCAP-1 treated cells significantly increased all 6 morphological characteristic post 4 days of treatment, however there were no significant differences between the negative controls. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=4 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) B) Panel depicting representative morphological effects for each treatment.

Detection of BDNF by immunoblot in E14 cells post chronic TCAP treatment

Three replicates of western blots were conducted on TCAP-1 treated E14 hippocampal and N38, N3 and N46 hypothalamic cell lines at the 4 day mark in order to detect BDNF expression. Concentrations of TCAP10^{-7}M and TCAP 10^{-9}M were significant as determined by one way ANOVA using Tukey's multiple comparison post hoc test (Figure 5.2A). There was overall significance of ($p<0.001$) according to the one-way ANOVA. Tukey's post test reveals that TCAP 9-41 fragment and urocortin 10^{-7}M, and no treatment control were not significant with relation to each other in E14 hippocampal cells. There was a significant increase in TCAP10^{-7}M and TCAP 10^{-9}M treated samples compared with the controls scores ($p<0.05$) ($p<0.01$) respectively. Analyses on N38 hypothalamic cells reveal overall significance of ($p<0.001$) according to the one-way ANOVA. Tukey's post test reveals that TCAP 9-41 fragment and urocortin 10^{-7}M, and no treatment control were not significant with relation to each other. There was a significant increase in TCAP10^{-7}M and TCAP 10^{-9}M treated samples compared with the controls scores ($p<0.01$) ($p<0.001$) respectively. In N3 and N46 hypothalamic cells overall significance was attained when comparing TCAP10^{-7}M and TCAP 10^{-9}M treated samples with control treated groups ($p<0.01$) ($p<0.001$). However, TCAP 9-41 fragment and urocortin 10^{-7}M, and no treatment control were not significant with relation to each other.

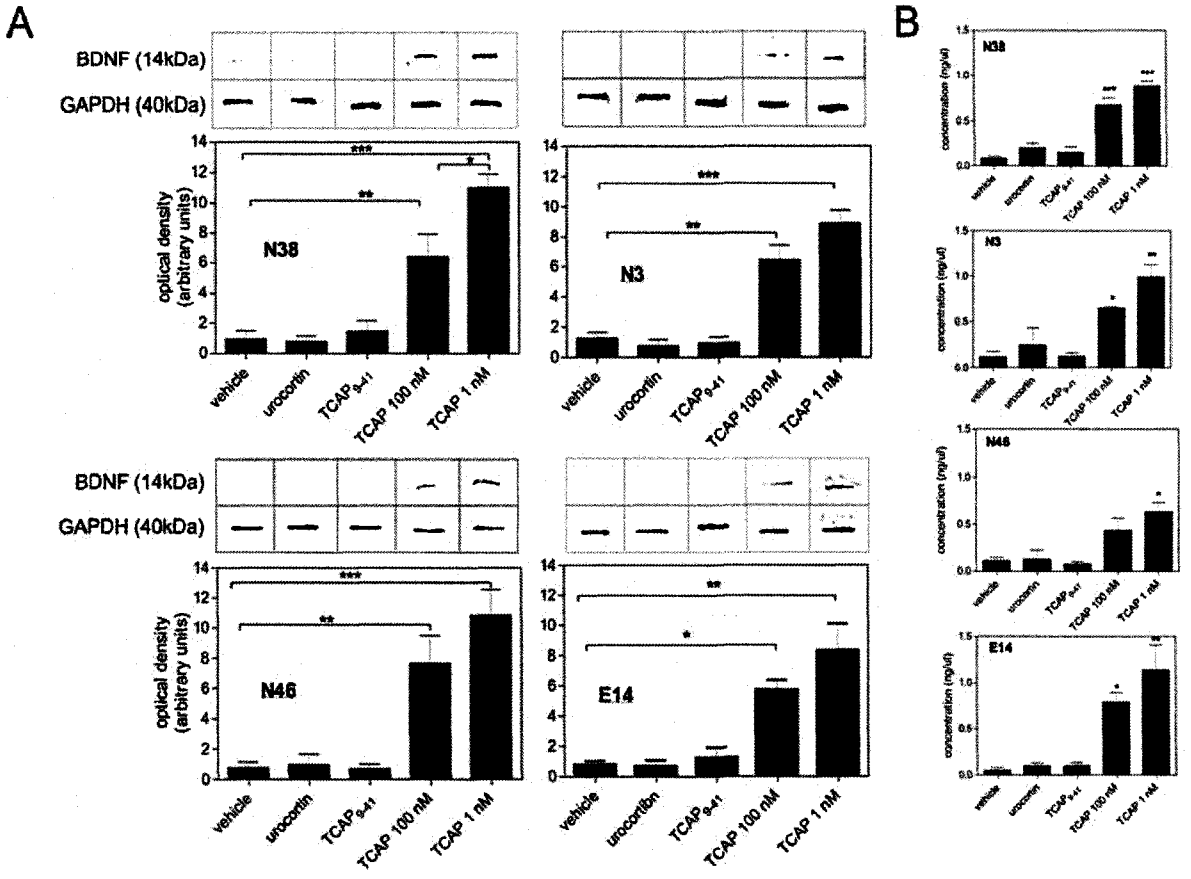

Figure 5.2. BDNF biomarker activity and mRNA expression in E14 hippocampal, N38, N3 and N46 hypothalamic cells with chronic TCAP-1 treatments of TCAP-1 10^{-7}, 10^{-9}M. A. Detection of mature BDNF by western blot. TCAP-1 treatments significantly increased BDNF expression post 4 days of treatment, however there were no significant differences between the negative controls. B. BDNF mRNA expression in E14 hippocampal, N38, N3 and N46 hypothalamic cells with chronic TCAP-1 treatments of TCAP-1 10^{-7}, 10^{-9}M as determined by real-time PCR. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=3 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Real-time PCR: BDNF in E14, N3, N38 & N46 cells post chronic TCAP treatment

TCAP-1 induced a significant effect on BDNF mRNA expression on E14 hippocampal and N38 hypothlamic cell lines. Concentrations of TCAP-1 10^{-7}, 10^{-9}M were significant when compared to the control samples using a one way ANOVA with Tukey's multiple comparison post hoc test. Replicates were completed at an n=3 for each sample (Figure 5.2B). For E14 and N3 cells there was overall significance of ($p<0.001$)

according to a one-way ANOVA. Tukey's post test reveals that TCAP 9-41 fragment and urocortin 10^{-7}M, and no treatment control were not significant with relation to each other but there was a significant increase in TCAP 10^{-7}, 10^{-9}M treated samples compared with the controls scores ($p<0.05$) ($p<0.01$) respectively. N38 cell analyses reveal a significant increase in TCAP 10^{-7}, 10^{-9}M treated samples compared with the controls scores ($p<0.001$) ($p<0.001$) respectively. However, N46 cells show an overall significance of ($p<0.001$) with only a significant increase in, 10^{-9}M treated samples compared with the controls scores ($p<0.05$).

SiRNA mediated knockdown of TCAP and teneurin

Both siRNA vectors (p270 and p721) showed similar profiles at down-regulating TCAP and teneurin expression in N38 cells (Figure 5.3). The TCAP-3 and teneurin-3 message was reduced to about 20% of the control values. The p270 vector was effective at reducing TCAP-4/teneurin-4 mRNA levels to about 50% of the control values. The p721 vector showed a similar effect at reducing TCAP-4 levels but only reduced teneurin-4 levels to 70% of the control value. TCAP-2 and teneurin-2 mRNA was the least effective with p270 and reducing TCAP-2 and teneurin-2 message to about 75 and 90% of the control values, respectively. The p721 vector had no significant effect on the teneurin-2 mRNA but reduced the TCAP-2 level to about 80% of the control value. N38 cells do not express either TCAP or teneurin 1 in significant amounts.

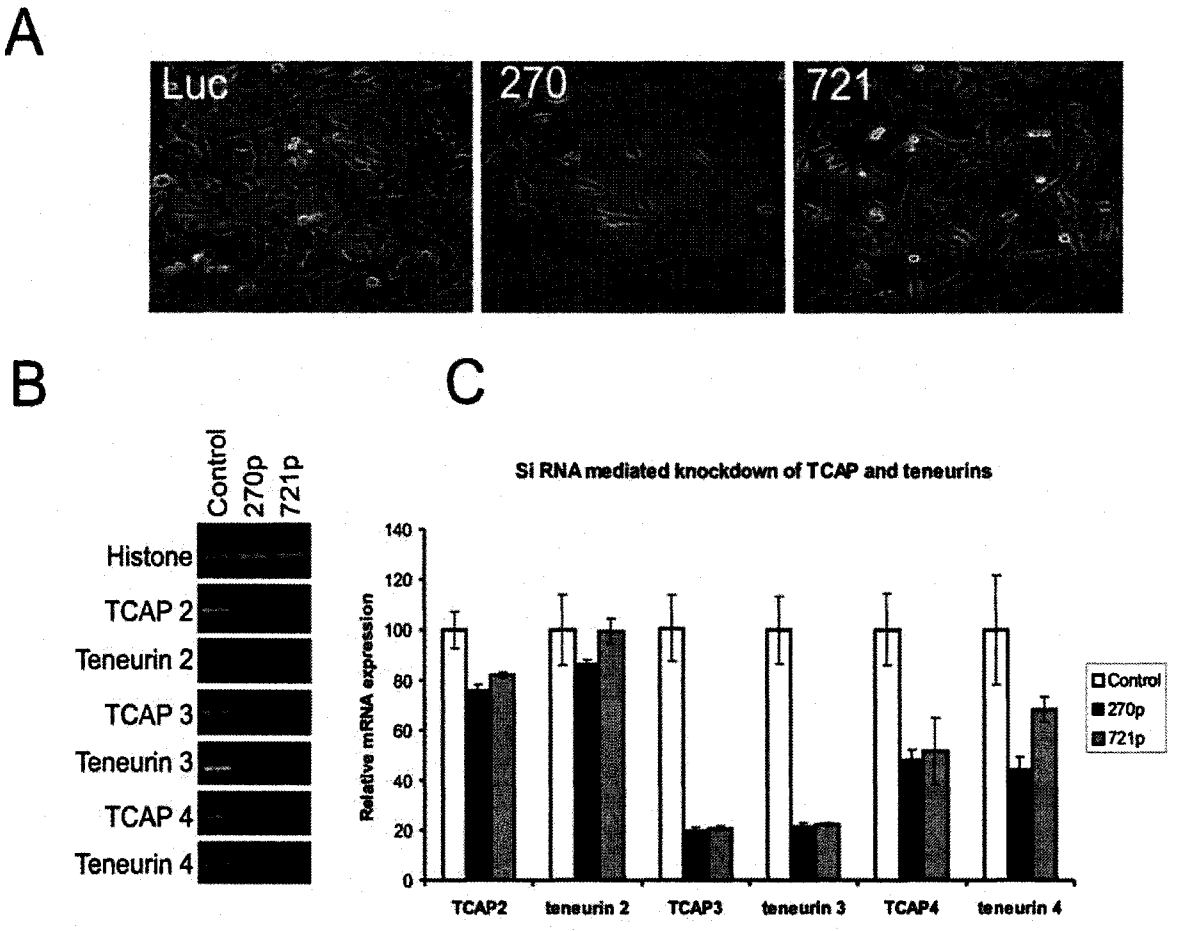

Figure 5.3. Effect of TCAP and Teneurin expression on N38 cells transfected with TCAP-based siRNAs. A. N38 cells after transfection with either the pLuc negative control, or p270 and p721 siRNA containing plasmids. B. Expression product of histone H3, TCAP and teneurin after transfection. C. Quantification of the data provided in 'B'. TCAP-1 and teneurin-1 is not expressed by N38 cells.

Morphology Analysis: 270 and 721 TCAP siRNA

Four replicates of each sample were analyzed for the following morphological characteristics in order to determine effects on luciferase transfected negative control (Luc) and 270 and 721 TCAP siRNA: number of soma, number of connecting cells, number of neurites and length of neurites. Significance was determined by one way ANOVA using Tukey's multiple comparison post hoc test. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Puromyocin selected cells

Significance for the puromyocin selected cells showed that siRNA 270 and 721 effectively decreased number of soma, number of connecting cells, number of neurites and length of neurites at both the 24 and 48hr time points (Figure 5.4).

There was an overall significant change in the number of soma $p<0.0001$ ($F = 43.72$), number of connecting cells $p<0.0001$ ($F = 45.25$), number of neurites $P<0.0001$ ($F = 13.57$) and length of neurites $P<0.0001$ ($F = 32.06$) between 721, 270 and Luc transfected cells. At 24hrs and 48hrs 721 showed a significant decrease in number of soma ($p<0.01$), number of connecting cells ($p<0.01$), number of neurites and length of neurites ($p<0.01$) as compared with Luc transected negative control. At 24hrs and 48hrs 270 showed a significant decrease in number of soma ($p<0.001$), number of connecting cells ($p<0.001$), number of neurites and length of neurites ($p<0.001$) as compared with Luc transected negative control.

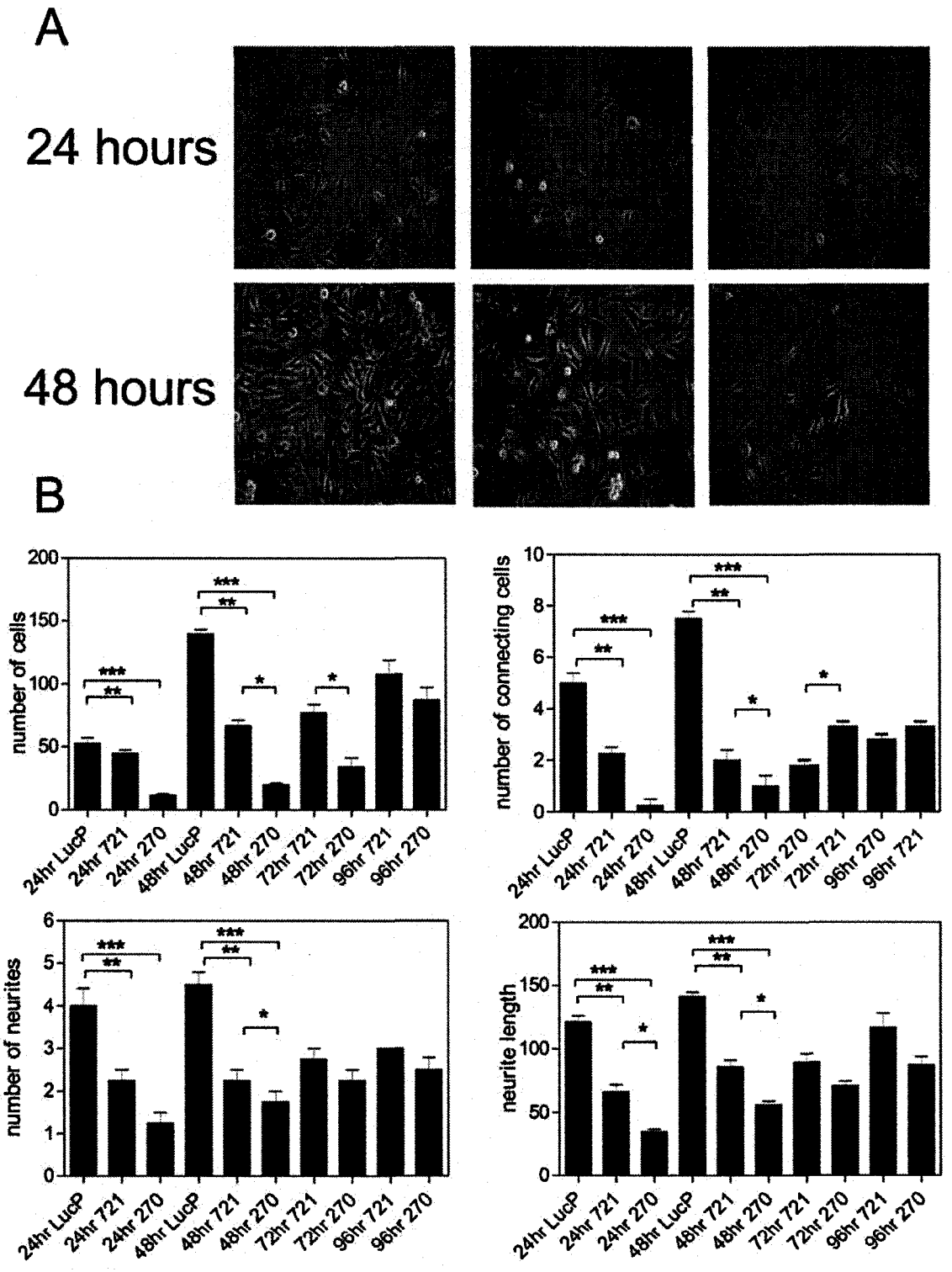

Figure 5.4. Puromyocin selected cells. Morphology effects of TCAP 270, 721 siRNA and Luc transfected cells at 24 and 48hrs. A. Representative pictures of siRNA transfected neurons. B. Quantification of number of cells, connecting cells, neurites and neurite length at 24, 48 and 96hrs. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=3 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Stringent puromyocin selected cells

Significance for the stringent puromyocin selected cells showed that siRNA 270 and 721 effectively decreased number of soma, number of connecting cells, number of neurites and length of neurites at both the 48hr time points. An exception is that 270 showed significantly shorter neurite length at the 24hr mark ($p<0.01$) (Figure 5.5).

There was an overall significant change in the number of soma $p<0.0001$ ($F = 29.60$), number of connecting cells $p<0.0001$ ($F = 11.71$), number of neurites $P<0.0001$ ($F = 7.374$) and length of neurites $P<0.0001$ ($F = 11.95$) between 721, 270 and Luc transfected cells at 48hrs. However, at 48hrs 721 showed a significant decrease in number of soma ($p<0.01$), and length of neurites ($p<0.01$) as compared with Luc transected negative control as compared with the 270 cells, which showed a significant decrease in number of soma ($p<0.001$), number of neurites ($p<0.05$) and length of neurites ($p<0.01$) as compared with Luc transected negative control at 48hrs.

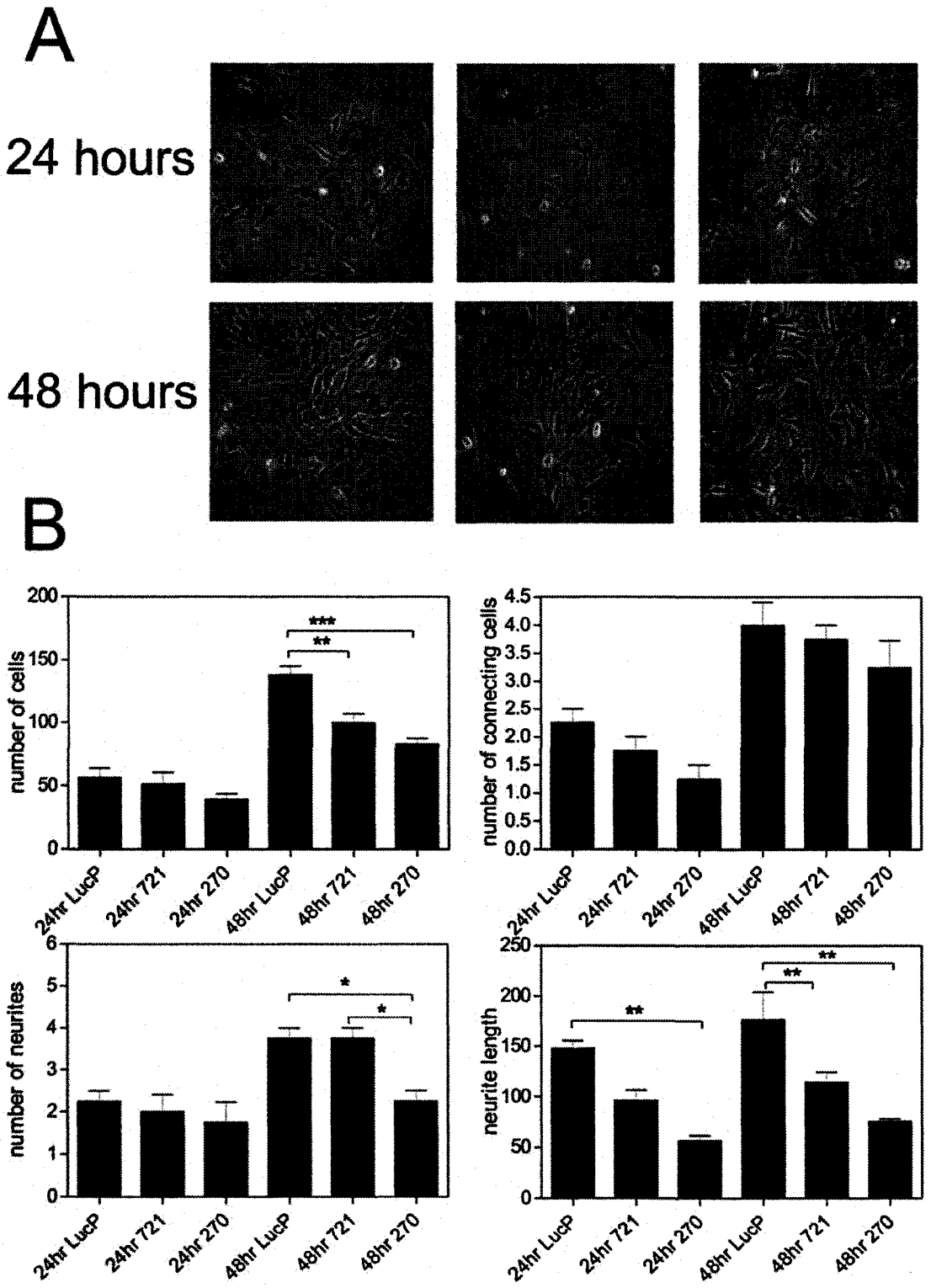

Figure 5.5. Stringent puromyocin selected cells. Morphology effects of TCAP 270, 721 siRNA and Luc transfected cells at 24 and 48hrs. A. Representative pictures of siRNA transfected neurons. B. Quantification of number of cells, connecting cells, neurites and neurite length at 24 and 48hrs. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=3 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Rescue Experiment: 270 TCAP siRNA

Viability Analysis: MTT assay

TCAP-1 10^{-11}M induced a significant effect on MTT activity for 5-FU and or $TCAP_{9-41}10^{-11}$M treated non-transfected N38, Luc transfected and 270 siRNA transfected cells using a one way ANOVA with Tukey's multiple comparison post hoc test. Replicates were completed at an n=4 for each sample (Figure 5.6). There was an overall significant change in viability when comparing sample treatments ($P<0.0001$) ($F= 106.8$). There was no significant difference in negative, $TCAP_{9-41}10^{-11}$M or positive 5-FU treated samples; however, there was significance between the N38 non transfected 5-FU positive control and 5-FU + TCAP-1 (10^{-11}M) samples ($p<0.001$), Luc transfected 5-FU positive control and 5-FU + TCAP-1 (10^{-11}M) samples ($p<0.001$), and 270 5-FU positive control and 5-FU + TCAP-1 (10^{-11}M) samples ($p<0.001$).

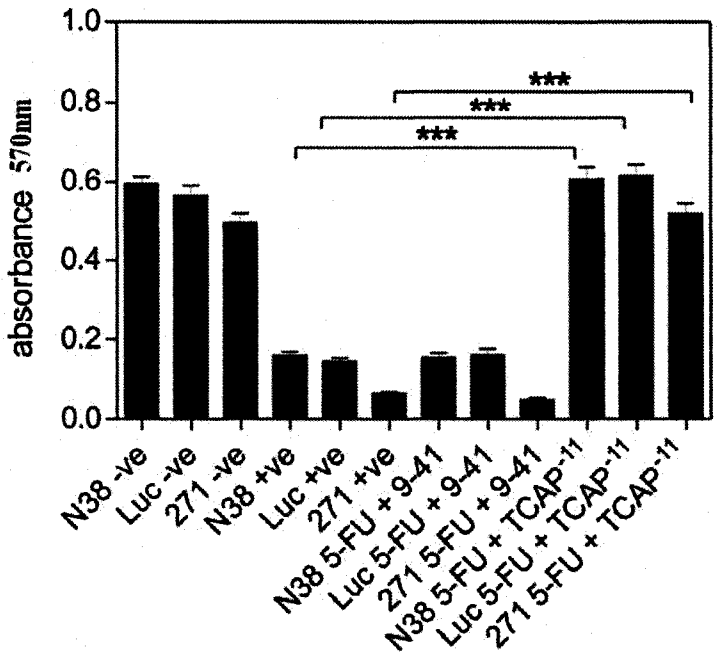

Figure 5.6. Cell viability as assessed by an MTT assay in siRNA transfected cells after treatment with 5-FU and/ or TCAP-1. The MTT assay was conducted 24 hours after treatment. Both 5-FU and the siRNAs significantly reduced MTT activity. Treatment of 10^{-11} M TCAP-1 restored MTT activity within normal levels. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=4 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Morphology Analysis: 5-FU rescue

TCAP-1 10^{-11}M effectively attenuated 5-FU induced cell degradation in non-transfected N38, Luc transfected and 270 cells. In particular, TCAP increased the number of soma, number of connecting cells, number of neurites and length of neurites post 24hrs of 5-FU treatment (Figure 5.7). There was an overall significant change in the number of soma $p<0.0001$, number of connecting cells $p<0.0001$, number of neurites $P<0.0001$and length of neurites $P<0.0001$ between N38, 270 and Luc transfected cells.

At 24hrs post 5-FU treatment TCAP-1 10^{-11}M treated cells showed a significant increase in number of soma ($p<0.001$), number of connecting cells ($p<0.001$), number of neurites ($p<0.001$) and length of neurites ($p<0.001$) in non-transfected N38, Luc

transfected and 270 cells as compared with the positive controls. There was no significant difference in $TCAP_{9\text{-}41}10^{-11}M$ or positive 5-FU treated samples, nor amongst any of the non-treated cells.

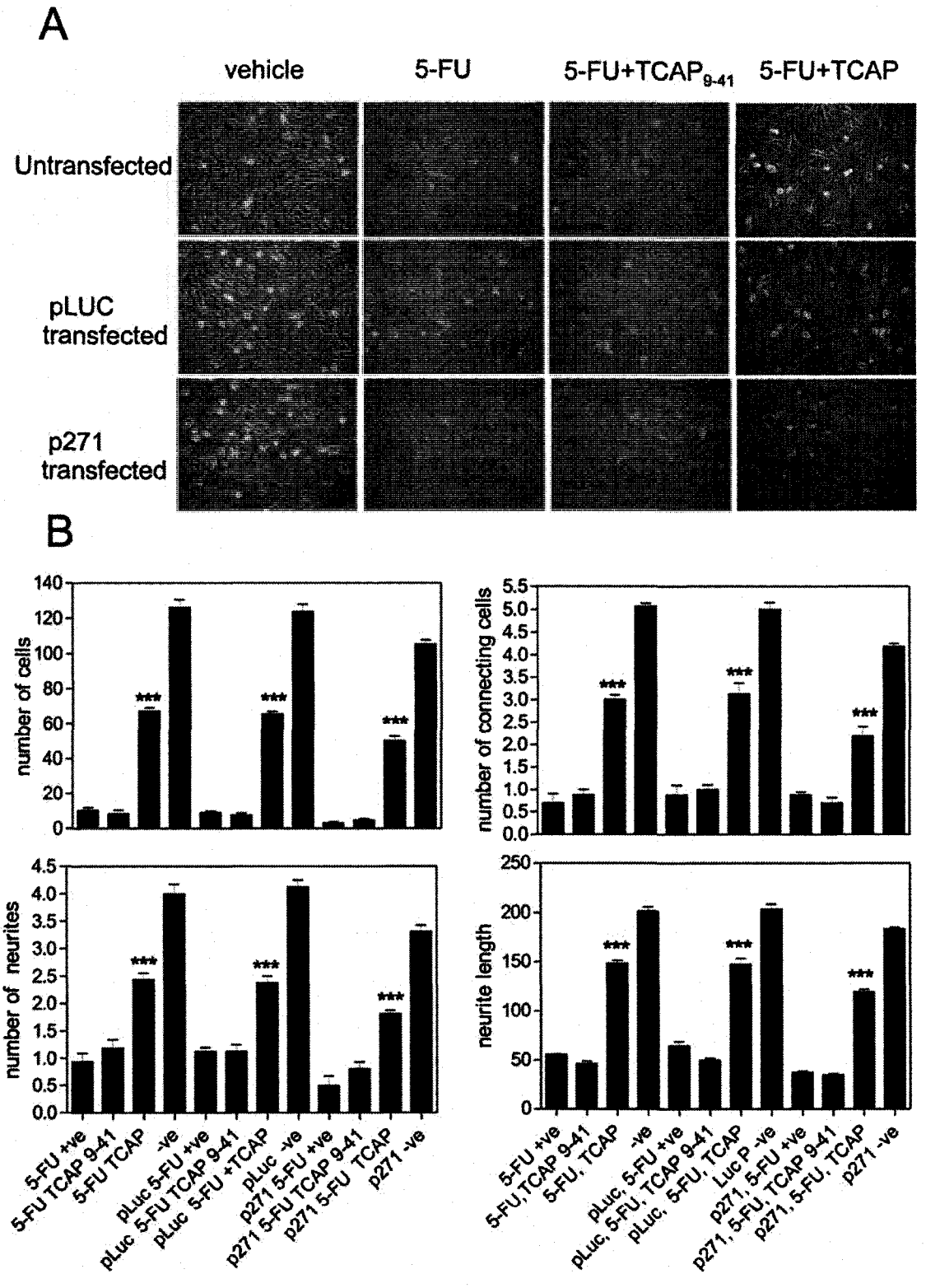

Figure 5.7. Effect of TCAP-1 treatment on siRNA-transfection and 5-FU-exposure on cell morphology. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=4 for all analyses. (* $p<0.05$; **$p<0.01$; ***$p<0.001$)

BDNF protein expression in 270 TCAP siRNA

Three replicates of western blots were conducted on TCAP-1 treated 270, luciferase and N38 cell lines post 24hrs of 5-FU &/or $TCAP^{-11}$ treatment in order to detect BDNF expression. TCAP treated cells showed a significant difference compared to control as determined by one way ANOVA using Tukey's multiple comparison post hoc test ($F = 28.68$) ($p<0.0001$) (Figure 5.8). In N38 cells TCAP 9-41 fragment and positive control were not significant with relation to each other. There was a significant decrease in positive and 9-41 treated cells compared to negative control treated cells ($p<0.001$) and a significant decrease in $TCAP^{-11}$ + 5-FU treated cells as compared to negative control treated cells ($p<0.01$). There was a significant increase in $TCAP^{-11}$ + 5-FU treated samples compared with the positive and 9-41 control with scores of ($p<0.01$) for both. Also, there was a significant decrease in BDNF protein expression when comparing the N38 and luciferase negative controls with the 270 negative control, ($p<0.05$) for both. For luciferase cells TCAP 9-41 fragment and positive control were not significant with relation to each other. There was a significant decrease in positive and 9-41 treated cells compared to negative control treated cells ($p<0.001$) and a significant decrease in $TCAP^{-11}$ + 5-FU treated cells as compared to negative control treated cells ($p<0.01$). There was a significant increase in $TCAP^{-11}$ + 5-FU treated samples compared with the positive and 9-41 control with scores of ($p<0.01$) for both. In 270 TCAP siRNA cells TCAP 9-41 fragment and positive control were not significant with relation to each other. There was a significant decrease in positive and 9-41 treated cells compared to negative control treated cells ($p<0.001$) and a significant decrease in $TCAP^{-11}$ + 5-FU

treated cells as compared to negative control treated cells ($p<0.01$). There was a significant increase in $TCAP^{-11}$ + 5-FU treated samples compared with the positive and 9-41 control with scores of ($p<0.01$) for both.

Caspase 3 protein expression in 270 TCAP siRNA

Three replicates of western blots were conducted on TCAP-1 treated 270, luciferase and N38 cell lines post 24hrs of 5-FU &/or $TCAP^{-11}$ treatment in order to detect caspase 3 expression. TCAP treated cells showed a significant difference compared to control as determined by one way ANOVA using Tukey's multiple comparison post hoc test ($p<0.0001$) (Figure 5.8). For N38, luciferase and 270 cells TCAP 9-41 fragment and positive control were not significant with relation to each other. There was a significant increase in positive and 9-41 treated cells compared to negative control treated cells ($p<0.01$) and no significant change in $TCAP^{-11}$ + 5-FU treated cells as compared to negative control treated cells. There was a significant decrease in $TCAP^{-11}$ + 5-FU treated samples compared with the positive and 9-41 control with scores of ($p<0.01$) for both.

BDNF mRNA expression in 270 TCAP siRNA

Three replicates of Real-Time PCR were conducted on TCAP-1 treated 270, luciferase and N38 cell lines post 24hrs of 5-FU &/or $TCAP^{-11}$ treatment in order to detect BDNF gene expression. TCAP treated cells showed a significant difference

compared to control as determined by one way ANOVA using Tukey's multiple comparison post hoc test ($p<0.0001$) (Figure 5.8). In N38 and luciferase cell lines TCAP 9-41 fragment and positive control were not significant with relation to each other. There was a significant decrease in positive and 9-41 treated cells compared to negative control treated cells ($p<0.001$) and no significant change in $TCAP^{-11}$ + 5-FU treated cells as compared to negative control treated cells. There was a significant increase in $TCAP^{-11}$ + 5-FU treated samples compared with the positive and 9-41 control with scores of ($p<0.01$) for both. However, for 270 TCAP siRNA cells There was a significant decrease in positive and 9-41 treated cells compared to negative control treated cells ($p<0.001$) and no significant decrease in $TCAP^{-11}$ + 5-FU treated cells as compared to negative control treated cells. There was a significant increase in $TCAP^{-11}$ + 5-FU treated samples compared with the positive and 9-41 control with scores of ($p<0.001$) for both.

Caspase 3 mRNA expression in 270 TCAP siRNA

Three replicates of western blots were conducted on TCAP-1 treated 270, luciferase and N38 cell lines post 24hrs of 5-FU &/or $TCAP^{-11}$ treatment in order to detect caspase 3 expression. TCAP treated cells showed a significant difference compared to control as determined by one way ANOVA using Tukey's multiple comparison post hoc test ($p<0.0001$) (Figure 5.8). In N38, luciferase and 270 cell lines TCAP 9-41 fragment and positive control were not significant with relation to each other. There was a significant increase in positive and 9-41 treated cells compared to negative control treated cells ($p<0.001$) and no significant change in $TCAP^{-11}$ + 5-FU treated cells

as compared to negative control treated cells. There was a significant decrease in TCAP^{-11} + 5-FU treated samples compared with the positive and 9-41 control with scores of ($p<0.05$) for both.

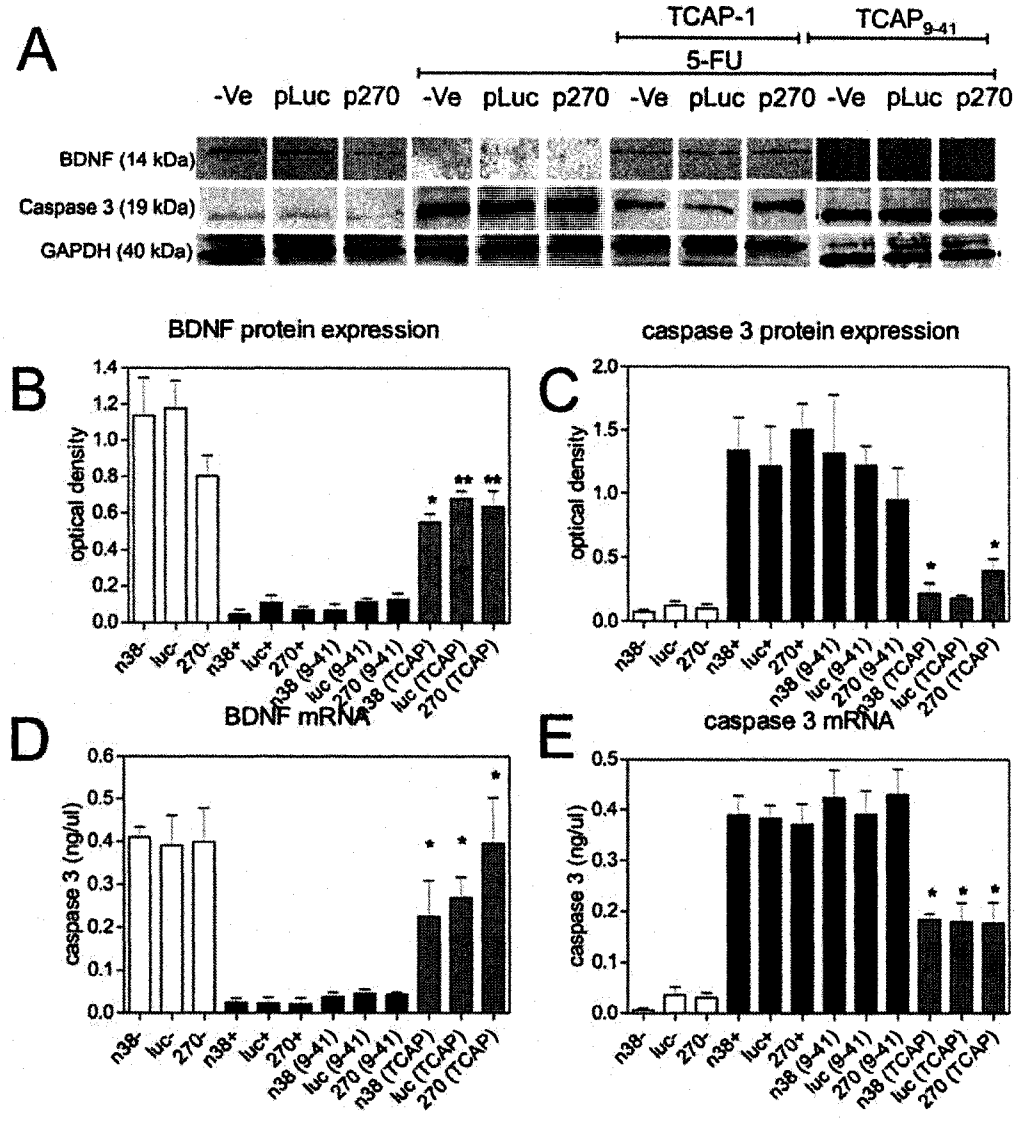

Figure 5.8. BDNF and cleaved caspase 3 biomarker activity and mRNA expression in N38, luciferase and 270 with treatments of TCAP-1 10^{-11} and/or 5-FU. A. Detection of mature BDNF and cleaved caspase 3 by western blot. B. BDNF protein expression quantification. TCAP-1 treatments significantly increased BDNF expression post 24hrs of treatment, however there were no significant differences between the negative controls nor amongst the positive control and 9-41 fragment treated cells. C. Caspase 3 protein expression quantification. TCAP-1 treatments significantly decreased caspase 3 expression post 24hrs of treatment, however there were no significant differences between the negative controls nor amongst the positive control and 9-41 fragment treated cells. D. BDNF mRNA quantification based on Real-Time PCR. E. Caspase 3 mRNA quantification based on Real-Time PCR. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. n=3 for all analyses. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

5.4 Discussion

This chapter seeks to investigate how TCAP confers neuroprotection to compromised cells. I have shown that TCAP-1 treatment increases BDNF expression as well as increases number of cells, neurites and neurite length. Furthermore, interference of the TCAP gene significantly diminishes cell number and neurites. However, subsequent TCAP-1 treatment rescues these cell morphological traits as well as increases viability under stress induced conditions. This may indicate that TCAP-1 may act to regulate BDNF and potentially other neurotrophins to confer survivability to compromised neurons.

The neurotrophins are a family of structurally related molecules that include: nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin -4/5 (NT-4/5) and neurotrophin-6 and BDNF (Ibanez, 1995). Collectively, the neurotrophins promote the differentiation and survival of various types of neuron populations in both the peripheral and central nervous system (Ibanez and Ryden, 1995). In particular, BDNF has been shown to protect neurons from spinal cord injury, focal brain ischemia and stress in adult rats (Snider, 1994, Husson et al., 2005). Traditionally research has centered on investigating the mechanisms of neuronal damage, while recently it has become clear that endogenous mechanisms of protection are equally important for therapeutic applications and neuronal survival. It would be beneficial to clarify endogenous agents, which may confer protection to neurons and death and disruptions to cellular function in specific disease states. The purpose of this study is to highlight the potential endogenous role of

TCAP as a neuroprotective agent in immortalized hippocampal and hypothalamic cells by mediating BDNF expression *in vitro* by using siRNA.

It has been shown that early increases in neuronal BDNF expression may modulate neuronal resistance and recovery during ischemic stress, in particular apoptotic induced death (Nicotera et al., 1999). In addition, an increase in BDNF associated neuronal survivability may be indicative of synaptic plasticity required to confer neuroprotective morphology such as extended neurite length, number of neurites and cell size in the hippocampus (Briscoe et al., 2006).

Related, is a study that shows BDNF as increasing soma size of GABAergic neurons in fetal rat hippocampal cultures substantiating data indicating that upregulation of BDNF causes increases in neuron size (Sato et al., 1994). Although, mechanisms through which this occurs remains ambiguous. Also, BDNF has been proven to increase total neurite length in placental insufficient growth compromised fetuses potentially by GTPases (Sato et al., 1994, Hayashi et al., 2002, Yamada et al., 2002).

In the present study we showed that TCAP-1 promotes survival of cultured immortalized hypothalamic and hippocampal cells defined by apoptotic injury. 5-FU induced stress was significantly reduced in cell populations that were incubated with TCAP-1 indicating agents that selectively target BDNF regulation via survival pathways may be clinically beneficial in the protection of neurons from brain toxicity.

Specific pathways that are activated in BDNF mediated neuroprotection remain ambiguous. Studies show that phosphorylation of ERK 1/2 and the PI3-kinase substrate AKT was induced by intracerebroventricular administration of BDNF to postnatal rats; however, subsequent inhibition of ERK as opposed to PI3-kinase inhibited BDNF from

neonatal hypoxia ischemia (H-I) suggesting that ERK activation is integral for protection against H-I brain injury (Cheng et al., 1998, Han and Holtzman, 2000). Others suggest that the ERK signaling pathway plays a pivotal role in BDNF protection against camptothecin, a commonly used as a model to study neuronal apoptosis induced by DNA damage eventually leading to neurodegenerative and aging related neuronal toxicity (Morris and Geller, 1996, Park et al., 1997, Park et al., 1998). Alternatively the PI-3 kinase pathway is activated for BDNF protection against serum deprivation (Hetman et al., 1999). Further studies have shown that NMDA attenuates glutamate toxicity via BDNF dependent mechanism whereby hippocampal cultures exposed to 50μM of NMDA caused a marked increase in BDNF secretion followed by TrkB receptor activation and subsequent increases in BDNF mRNA expression (Jiang et al., 2005). The studies suggest that NMDA receptor stimulation increases survival in cerebellar granule neurons through the influx of extracellular Ca2+, which may cause the release of BDNF and the activation of TrkB activating the subsequent anti apoptotic mechanism (Bazan-Peregrino et al., 2007).

Studies have shown that neuroprotective action in animal models of ischemia and other injuries may act by down-regulation of free radical production through the p38MAPK/ERK pathway during NMDA induced excitotoxicity (Park et al., 2004). We have previously shown that TCAP-1 mediates ROS production by upregulating superoxide dismutase-1 and catalase this may be correlated with an ERK/PI-3 kinase pathway to mediate BDNF activity as well.

These various modes of pathway activation in BDNF mediated protection of neurons depend on cell type, stress stimulus/injury and culture parameters. Future studies will

entail elucidating the particular pathway through which TCAP-1 confers a neuroprotective effect via BDNF upregulation.

Regardless of the diffuse nature of BDNF it confines its regulation to specifically targeted synapses without impeding on surrounding cell activity (Balkowiec and Katz, 2000). This is due to local and activity-dependent secretion. Various studies have shown that BDNF is sorted into the regulated pathway as opposed to the constitutive pathways as other neurotrophins. ProBDNF is mediated by the sorting signal motif pro-opiomelanocortin (POMC) which interacts with membrane carboxypeptidase E (CPE), which acts as either a sorting or retention receptor to target prohormones to the regulated pathways (Cool and Loh, 1994, Cool et al., 1995, Lou et al., 2005). This has important implications if BDNF is not sorted to the regulated pathway as it may cause irregular BDNF secretion, thus hippocampal function impairment and retardation of dendrite growth, which may be the case in brain injury (Balkowiec et al., 2000). Our data reveal that TCAP-1 confers significant effect to hypothalamic and hippocampal cells morphology and may act to ensure that BDNF is sorted to the regulative pathway.

Evidence suggests that specific neuropeptides may be used therapeutically to attenuate brain damage during periods of brain ischemia or other injuries. VIP was first utilized as a chimeric peptide that showed a neuroprotective effect in the CNS after noninvasive peripheral administration, which was effective in crossing the BBB (Said and Mutt, 1970, Wu, 2005). VIP has been shown to activate Akt and MAPK causing axonal sprouting in a neuroprotective manner and TCAP-1 may play a similar role in decreasing brain insult (Jefferies et al., 1984, Lee et al., 2000). The data reveals that TCAP increases BDNF expression, which in turn may evoke a neuroprotective effect

against induced cell death. These findings suggest that TCAP may represent a promising new approach to neuroprotection following injury.

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Chapter 6: Conclusion

The TCAP family of peptides is derived from the extracellular terminal of the teneurins, a large transmembrane protein, which may be processed in several ways in order to modulate cellular signaling. In vertebrates there are four paralogs, all of which are expressed prominently in the developing central nervous system (Baumgartner et al., 1994, Minet et al., 1999). The teneurins are essential for normal development and disruption of the teneurin gene is lethal in the embryo. Subsequently, the TCAP family was discovered to be located on the final exon of the teneurin genes and a synthetic version of the peptide was found to effect stress behaviour and cell proliferation (Qian et al., 2004, Wang et al., 2005). The objective of this research has been to investigate the role of TCAP-1 in times of cellular induced stress and to elucidate the mechanism through which it may confer neuroprotection. By investigating the potential function of TCAP in times of cell toxicity a better understanding of how to attenuate neuronal loss may be garnered. This chapter will introduce a potential TCAP model for neuroprotection. Furthermore, this chapter will also introduce future experiments relevant and complementary to the research presented in this thesis. Novel peptides and the unraveling of their mechanisms of action have and will continue to open new horizons in the study of the processes leading to nerve cell survival and death. These mechanisms are of importance to our understanding of brain diseases and the neuronal stress response and potential therapeutic roles for neuropeptides.

6.1 Summary

Establishing TCAP as an effective mediator of cell survival

In this thesis work I have established that TCAP-1 is an effective mediator of neuroprotection under a multitude of cellular stressors. I have attempted to investigate the functional significance of TCAP-1 *in vitro* as well as attempted to elucidate an understanding of its cellular mechanism. To summarize, TCAP-1 has been shown to increase survivability in neurons under several different types of induced cell death. In chapter 2, TCAP-1 was shown to reduce the effects of alkalosis induced necrosis by upregulating ROS scavenging systems such as SOD and catalase. ROS are also implicated in apoptotic cell death and in chapter 3 further studies conducted revealed that TCAP-1 is able to prevent apoptotic programmed cell death by preventing caspase 3 cleavage and cell proliferation inhibition in the s-phase of cell synthesis. In chapter 4, I discuss glutamate excitotoxicity, in particular as a defining feature of several forms of cell death, including apoptosis and necrosis and may lead to the generation of deleterious ROS and intracellular calcium levels. I have shown that TCAP-1 may be acting in a neuroprotective manner under glutamate toxic conditions via the ERK pathway possibly in tandem with mediating ROS scavengers, such as SOD1, which was also upregulated. In chapter 5 I attempt to investigate the pathway through which TCAP may confer survivability. Cell death, including apoptosis, necrosis and glutamate mediated death have been shown to be attenuated by the presence of growth factors such as BDNF. Therefore in chapter 5, I investigated the possibility that TCAP may be mediating

neurotrophins to prevent cell mortality. I show that the disruption of the TCAP gene detrimentally effects cell morphology and survival and that TCAP-1 treatment subsequently rescues the attenuated cell features, such as cell number, number of neurites and neurite length. I also show that TCAP-1 has the ability to upregulate growth factors, such as BDNF, under times of induced cell death as well as prevents the caspase cascade by preventing the cleavage of caspase 3. Apoptosis, necrosis, glutamate and growth factors are all relevant factors in cell survival. I have shown that TCAP-1 is effective in preventing death elicited by several types of deleterious exogenous factors (high pH, PCD inducers, glutamate) and have identified possible executers of survival (SOD, BDNF, neurogenesis, ERK) acting under the control of this small peptide. Future experiments will further explicate the intricate cellular signaling pathway through which this novel peptide acts leading to the possibility of applying this knowledge *in vivo* and potentially devising therapeutic application for humans.

Elucidating a neuroprotective pathway for TCAP-1

Sequence similarity between all 4 TCAP members reveal highly conserved cleavage motifs which indicate that they may be processed independently or as part of the teneurin protein. The manner through which TCAP becomes independently active remains elusive. However, it is possible that TCAP may be cleaved within vesicles prior to fusion with the membrane or at the membrane itself. Once released into the extracellular space TCAP may then bind a specific membrane bound receptor in order to illicit cellular signal transduction pathways in order to confer neuroprotection. Upon

binding to a specific receptor, TCAP may cause a signal cascade leading to the upregulation of BDNF transcription and translation. BDNF is then released extracellularly at which time it may act on its high affinity trkB receptor in order to activate an ERK dependent survival pathways eventually leading to CREB activation and pro-survival gene transcription. In times of neurodegenerative stress, TCAP mediated BDNF regulation may increase ERK activation and inhibiting ROS generation through SOD1/catalase scavenging. Studies have shown that SOD may confer neuroprotection via an ERK dependent pathway, therefore upon activation of ERK a significant increase in SOD2 was observed which was abolished when ERK1/2 was inhibited by its selective inhibitor PD98059 (Scorziello et al., 2007). Consequently this may also increase pro-survival factors, such as Bcl2, in order to inhibit pro-apoptosis executer Bax and the subsequent death cascade. Further studies have show that ROS directly impinge upon the death cascade by decreasing the phosphorylation of the Bcl-2 family proteins, resulting in increased pro-apoptotic protein levels and decreased anti-apoptotic protein expression (Li et al., 2004). Therefore, TCAP induced ERK phosphorylation may increase SOD1 transcription and inhibit ROS dependent attenuation of Bcl-2 phosphorylation. Together, these findings indicate that TCAP may play a valuable role in neurotrophin and anti-oxidant regulation in order to prevent cell death and eventual neuronal degeneration. Future experiments are required to deduce downstream targets of TCAP-1 mediated BDNF regulation, including Bcl-2 activation as well as intracellular Ca^{2+} levels. In particular, future experiments may focus on characterizing a TCAP specific receptor in order to better understand how it may induce intracellular signaling and neurotrophin regulation.

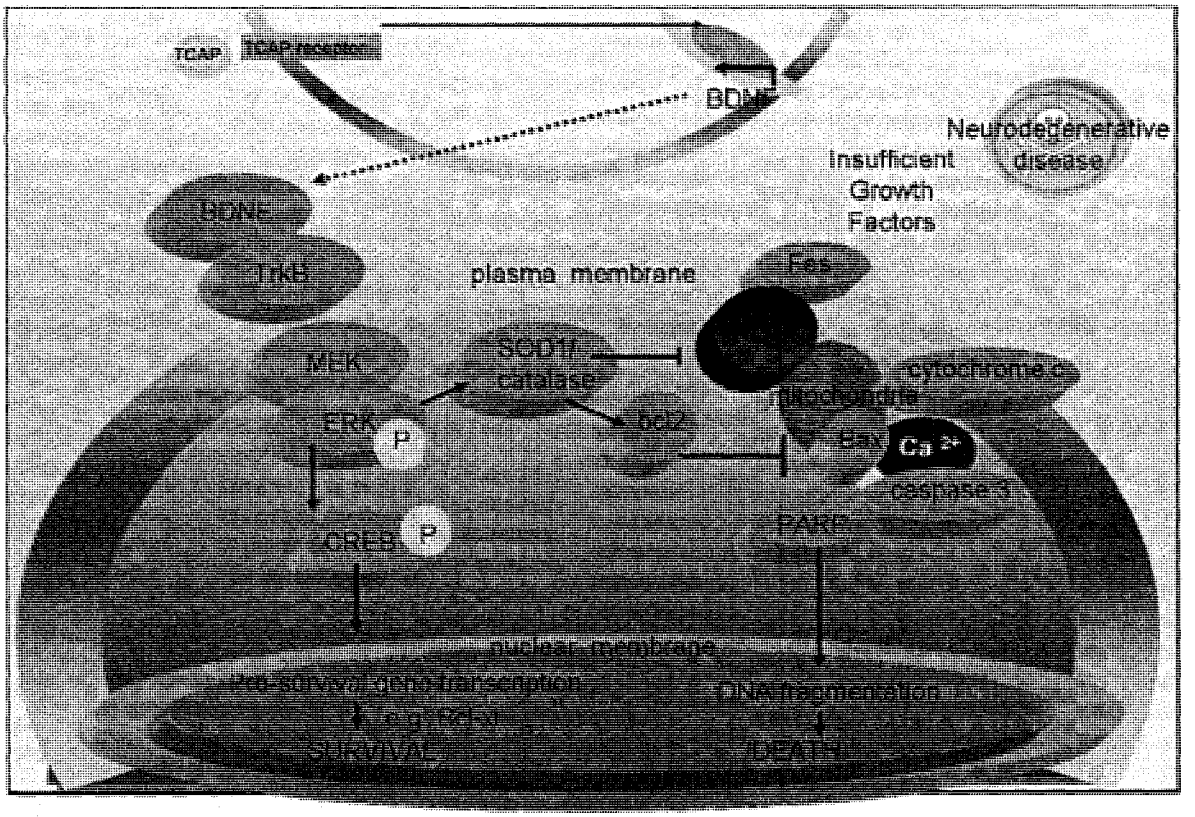

Figure 6. 1. Teneurin C-Terminal Associated Peptide model for neuroprotection. Upon being cleaved at the membrane surface or secreted from vesicles free TCAP may then interact with its specific receptor in order to induce intracellular signaling leading to BDNF transcription and translation. Secreted BDNF will bind to its high affinity receptor, TrkB, causing the activation of the MEK pathway leading to ERK activation and SOD upregulation. This subsequently leads to the suppression of ROS as well as to an increase in pro-survival factors such as Bcl2, inhibiting downstream death targets such as caspase 3 and PARP cleavage.

6.2 Future Directions

The neuroprotective ability of TCAP-1 to mediate cell proliferation *in vivo*

Thus far, I have shown that TCAP-1 is an effective moderator of neuroprotection in the face of various forms of cellular stress. I have also sought to determine the cellular signaling pathway in order to better understand how it achieves its cell survival potential. Future directives should be focused on investigating the neuroprotective capacities of TCAP *in vivo* in order to substantiate the *in vitro* data. Specifically, it would be useful to

investigate the role of TCAP-1 in the *in vivo* modulation of calcium as related to brain stress and trauma. Furthermore, a thorough understanding of how TCAP-1 may mediate cell proliferation and survival under certain neurodegenerative stressors would be beneficial in devising effective therapeutic treatments for humans and preventing neuronal degeneration, the hallmark of several debilitating diseases and trauma such as ischemia, Parkinson's disease and depression.

Future direction #1: Can TCAP-1 mediate calcium to prevent neuronal deficits in an *in vivo* model of Parkinson's disease?

One distinctive attribute of Parkinson's disease (PD) is the dysfunction of the mitochondria due to reduced complex 1 activity in the of the sustantia nigra pars compacta (SNc) and the subsequent sensitivity of dopamine neurons in this area to this reduces mitochondrial activity (Schapira et al., 1989). Both oxidative stress, glutamate and calcium toxicity have been shown to be affected by deficits in mitochondrial activity and are governed by a complex array of signaling pathways that impinge upon neuronal survival or death. The role of calcium in nigral dopamine neurons is complex however, calcium dependent proteases, such as calpains may be play a role in the pathogenesis of PD and other neurodegenerative disorders (Crocker et al., 2003). Calpain activation under pathological conditions is characterized as hyper-activation that involves sustained calcium overload and is generally associated with severe cellular challenge or damage (Liu et al., 2008). I have shown that TCAP-1 is successful in preventing the cleavage of caspase 3 and inhibiting ROS species as well as glutamate toxicity under cellular stressed

conditions. It has also been shown that calpains also mediate apoptotic cell death and are upregulated by increased ROS and Ca^{2+} under induced apoptosis in neurons (Persson and Karlsson, 1991, Ray et al., 1999a, Ray et al., 1999b, Ray et al., 2000, Varghese et al., 2001, Ray and Banik, 2003, Danial and Korsmeyer, 2004, Liu et al., 2008). Thus, determining whether TCAP-1 inhibits calpain cleavage products or even increases the calpain inhibitor, calpastatin, may provide useful information on TCAP's calcium cellular signaling activities in the presence of neurodegenerative processes or neuronal injury.

My proposed study would entail the use of Sprague Dawley rats co- injected with MPTP, 25 mg/kg, i.p and TCAP-1 for 5 consecutive days with a negative control group receiving an equivalent volume of saline (0.9%) once daily and a positive control group receiving MPTP only(Crocker et al., 2003). MPTP or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine has been consistently used as an inducer of PD and appears to be selectively toxic to the cells in the substantia nigra, and is capable of producing virtually all the signs and symptoms of idiopathic PD including sustained levels of cytoplasmic Ca^{2+} levels (Cassarino and Bennett, 1999, Cassarino et al., 1999). Brain tissues from rats would then be subjected to immunohistochemistry using antibodies for tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines (to determine loss of neurons in the SNc), as well as antibodies for calpain and calpastatin in order to determine whether any TCAP mediated dopaminergic neuronal survival is due to a decrease is calpain presence in those areas via calpastatin upregulation.

Future direction #2: Can TCAP-1 prevent the inhibition of cell proliferation *in vivo* to avert neuronal degeneration under restraint stress via BDNF regulation?

Several studies have revealed that the dysregulation of adult neurogenesis may contribute to the pathogenesis of neurodegenerative disorders including: Huntington's, Alzheimer's, Parkinson's as well as depression disorders. Furthermore, recent evidence suggests that promoting neurogenesis may modify the onset and progression of such disorders by upregulating neurotrophic factors such as BDNF, which has been shown to be essential in regulating basal levels of neurogenesis. Several studies have shown that repeated restraint stress suppresses neurogenesis and may even induce biphasic polysialylated neural cell adhesion molecule (PSA-NCAM) expression, a marker for neuronal plasticity, in the adult rat dentate gyrus (Pham et al., 2003). Also, further studies reveal that adult neurogenesis may be regulated by antidepressant treatment, which could block or reverse the effects of stress on hippocampal neurons via the cAMP signal transduction pathway (Malberg et al., 2000, Duman et al., 2001a, Duman et al., 2001b, Nakagawa et al., 2002, Malberg, 2004, Malberg and Schechter, 2005, Gur et al., 2007). These finding reveal that neurogenesis may be an important mediator in depressive mood disorders as well.

I would like to complete the current research work with an *in vivo* study which would determine whether TCAP attenuates the deleterious effects on neurogenesis by chronic restraint stress. Previous studies have shown that TCAP-1 mediates stress behaviour in rats via modulating the ASR, this future goal is to determine whether the mechanisms associated with TCAP neuroprotection that I have identified *in vitro* translate to an *in vivo* system which may treat degenerative and mood disorders.

The study would entail Sprague-Dawley rats undergoing chronic restraint stress (6hr/day for 14 days). The rats would receive a daily dose of TCAP injected subcutaneously and on the 14^{th} day would receive 50mg/kg of BrdU and would be sacrificed using chloral hydrate (500mg/kg) and perfused with 250ml of 0.1M phosphate buffered saline (PBS) followed by 300ml of 4% paraformaldehyde in PBS. The brains would be subsequently post fixed, cryoprotected in 20% sucrose for 2-3 days at 4°. Serial coronal sections through the lateral ventricles and hippocampus would be attained at 10μm and BrdU flurorescent conjugated antibody would be used to visualize the level of cell synthesis in conjunction with a DAPI counterstain. I would also be interested in determining whether TCAP may mediate neurogenesis via growth factors such as BDNF as I have shown in cultured cells. Thus, probing with a fluorophore conjugated secondary antibody against a BDNF primary antibody would be an effective means of elucidating whether this is the case.

Future direction #3: Can TCAP-1 intercede to induce cell survival in an *in vivo* model of ischemia?

Brain ischemia is multi-modal in nature. There are at least three distinct modes of cell death that characterize ischemic cell death including apoptosis, autophagy and necrosis (Allen et al., 1988). Ischemia is initiated by inhibition of oxidative phosphorylation, decreased intracellular pH, decreased ATP and cell proliferation, ROS production, increased cell Na^{+}, Ca^{2+} and subsequent membrane depolarization (Bonfoco et al., 1995, Honda et al., 2005). Calcium is an important mediator in cell damage sustained during ischemia as sustained levels of cytosolic Ca^{2+} generate ROS, damage the

mitochondria and activate cysteines proteases such as caspases and calpain (Cheng et al., 1998). Due to the important role of Ca^{2+}, the role of glutamate has also come under scrutiny. Several studies have shown that glutamate levels increase several fold during times of ischemic insult and cause deleterious lesions (Honda et al., 2005). I have previously shown that TCAP-1 is capable of preventing ROS under a necrotic, alkalosis cell death paradigm as well as inhibiting caspase 3 dependent apoptosis. I have also shown that chronic pre-incubation of TCAP-1 inhibits glutamate dependent cell death as well as a role for TCAP-1 in mediating cell survival through growth factors such as BDNF. It would be pertinent to investigate whether TCAP-1 is able to mediate these players within an *in vivo* model of ischemia as studies have shown that BDNF may help to protect neurons against ischemia and that protection against high pH stress reduces neuronal damage by decreasing cell proliferation inhibition (Choi and Rothman, 1990, Levine et al., 1992, Han and Holtzman, 2000, Robertson et al., 2002, Endo et al., 2007).

The classical approach to an *in vivo* ischemia model was put forth by Levine, in which hypoxia is combined with unilateral carotid occlusion and has since been established as the basis for the systemic study of brain cell damage (Levine, 1960, Levine et al., 1992, Lipton, 1999). I would propose a similar study which utilizes a rat model of hypoxia ischemia following chronic pre treatment with TCAP-1. Briefly, rats would be subjected to 2% isolurane anesthesia followed by permanent ligation of the left common carotid artery. After placement in their respective dams for several hours, rats would then be placed in an 8% oxygen chamber partially immersed in a water bath at 36 for ~75minutes (Smrcka et al., 2003, Bartley et al., 2005). 48 hrs post hypoxic-ischemic injury the rats would receive 50mg/kg of BrdU prior to sacrifice to allow for one cell

cycle of labeling (Cameron and McKay, 2001). The rats would be sacrificed using chloral hydrate (500mg/kg) and perfused with 250ml of 0.1M phosphate buffered saline (PBS) followed by 300ml of 4% paraformaldehyde in PBS. The brains would be subsequently post fixed, cryoprotected in 20% sucrose for 2-3 days at 4°. Serial coronal sections through the lateral ventricles and hippocampus would be attained at 10μm and BrdU, BDNF and ROS flurorescent conjugated antibody would be used to visualize the level of cell synthesis in conjunction with a DAPI counterstain.

Current preliminary data show that TCAP may be an effective therapeutic agent in preventing stroke damage in an induced focal iscaemic paradigm. Briefly, the rats were given a two week acclimatization period prior to surgery. Upon completion of middle cerebral artery occlusion (MCAO), rats were then given an additional two weeks of recovery. Subsequent TCAP-1 treatment at 500 and 1000pmol at two weeks post stroke induction reveal higher labeling of dividing and developing neurons, rats were sacrificed two weeks post TCAP treatment (Figure 6.2). This preliminary data reveal that TCAP-1 may be an efficacious therapeutic agent against stroke induced neuronal degeneration. Future experiments should corroborate these findings and potentially work towards deducing an *in vivo* mechanism through which TCAP-1 may be conferring survivability to compromised neurons.

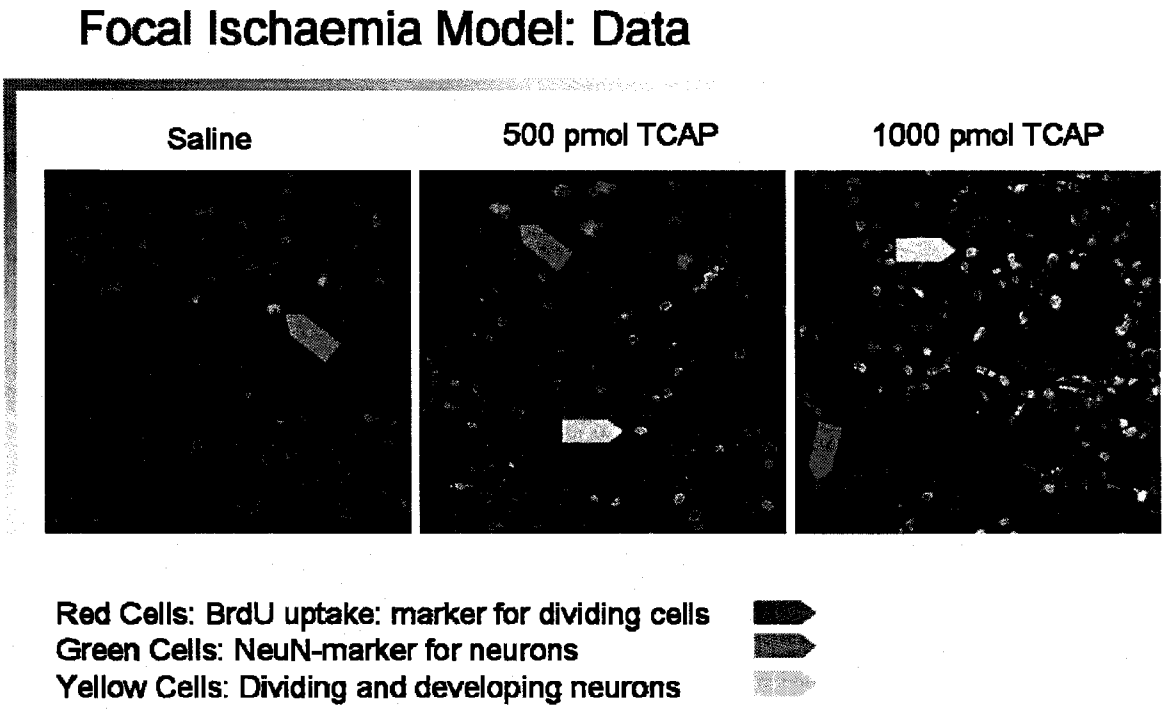

Figure 6.2. TCAP-1 prevents neuronal degradation in rat ischemic model. Preliminary data showing a TCAP-1 induced dose response effect at 500 and 1000pmol TCAP-1 treatment. Red cells correspond to dividing cells, Green cells correspond to cells destined to become neurons and yellow cells correspond to dividing and developing neurons. Courtesy of Dr. Xia Zhang, University of Ottawa. Institute of Mental Health Research.

6.3 Concluding remarks

The research work presented here seeks to deduce the mechanism of neuroprotection which may be conferred to compromised neurons by a novel family of highly conserved bioactive neuropeptides, TCAP. This integrative approach has entailed the investigation of various forms of neuronal cell death as well has identified overlapping characteristics that define the phenomenon in neurodegenerative diseases. This research has lead to a better understanding of the ambiguous nature of cell death and

in particular an appreciation for the complex multifaceted approach that is required to successfully and effectively circumvent deleterious brain trauma. Further advances in the field may lead to a broader understanding of neuronal death in context of the brains physiological environment; particularly in relation to oxidative stress, aging, death signaling, neurotrophins and neurogenesis. I have identified TCAP as a potential therapeutic agent in the rescuing of neurons during several forms of stress whereby a significant attenuation of brain damage may be achieved.

The objective of this thesis research has been to investigate the role of TCAP in preventing cell death. Particularly, with an attempt to understand the significance of deleterious brain fluxes in ROS, proliferation and growth factors relating to brain stress in order to elucidate the potential role of peptide neuroprotection during times of neural stress and brain injury.

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Appendix

western blot data

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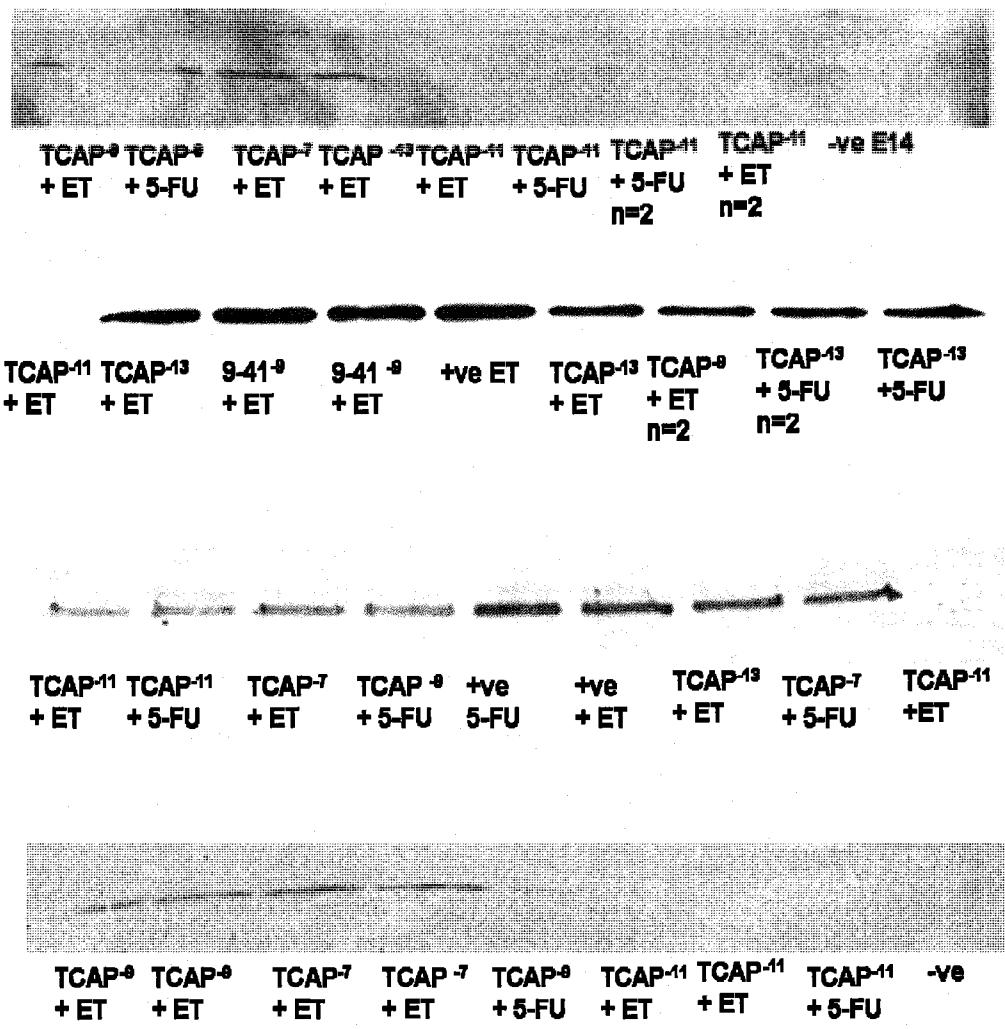
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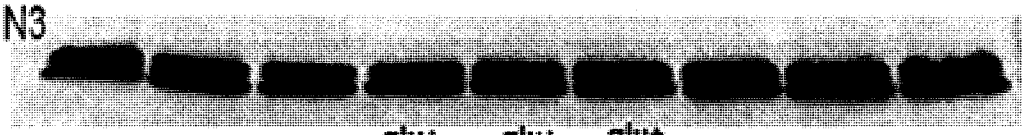

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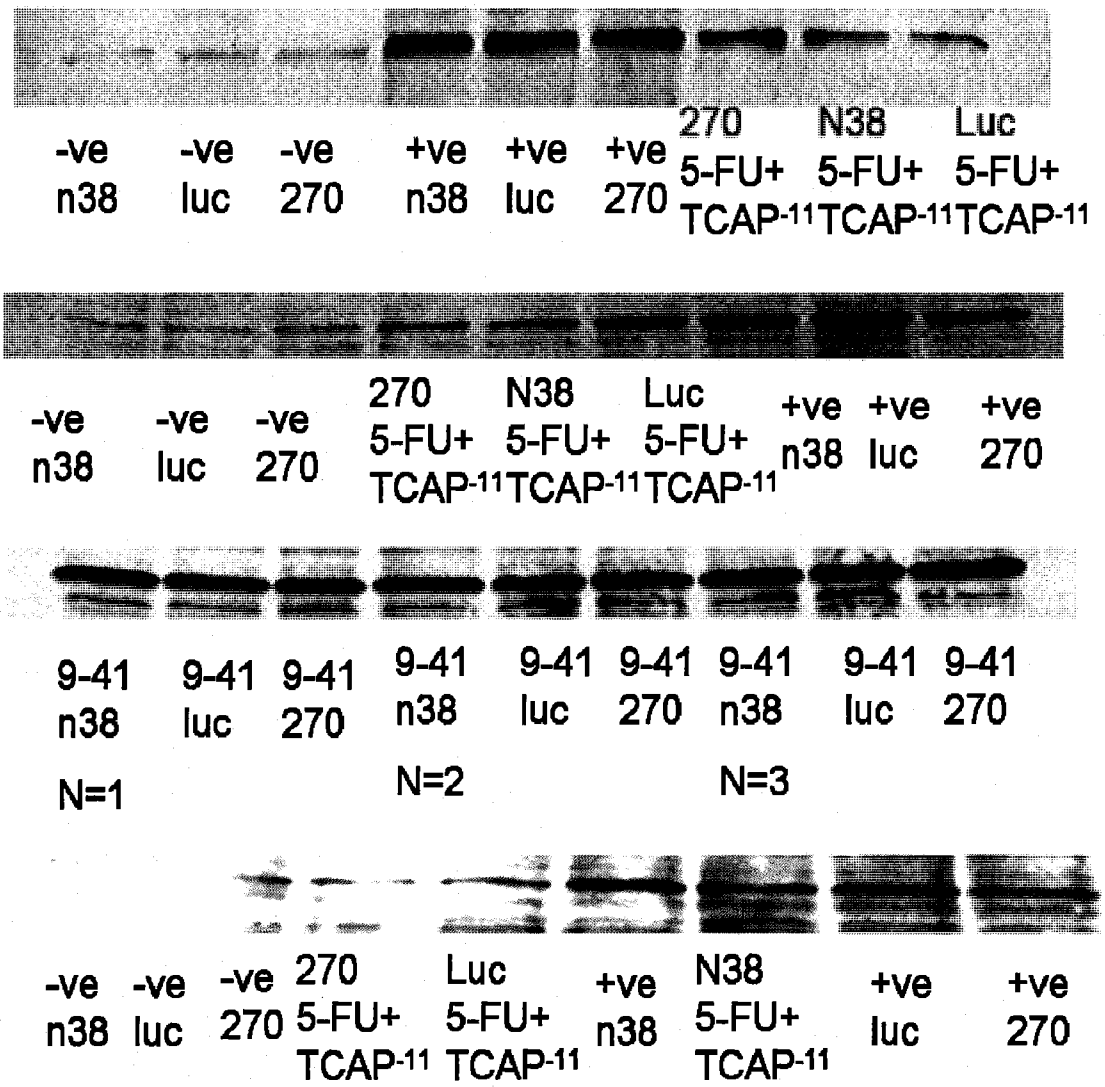
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Appendix

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