The Effect of Teneurin C-terminal Associated Peptide-1 (TCAP-1): Protection Against Hypoxic-stress and Regulation of Brain-derived Neurotrophic Factor (BDNF) in Immortalized Hypothalamic N38 Cells

By

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Abstract

Teneurin C-terminal associated peptide-1 (TCAP-1) is a recently characterized peptide that may act as one potential neuroprotective agent as it has been shown to regulate several stress-associated behaviours in rodents and possesses a number of protective actions on cells, however the mechanism remains unknown. Brain-derived neurotrophic factor (BDNF) is a neurotrophin recognized for mediating survival, differentiation, and proliferation. TCAP-1 may act, in part, via BDNF to provide neuroprotection via modulation of BDNF expression. The aim of this research was to further investigate the mechanism of TCAP's neuroprotective actions. I show that TCAP-1 is neuroprotective and a potent enhancer of cell numbers under varying levels of oxygen. I also establish that TCAP-1 is able to influence neuronal behaviour by differentially regulating neurite growth. In addition, I indicate that TCAP-1 is able to regulate BDNF expression in immortalized mouse hypothalamic N38 cells, which suggests that TCAP-1's neuroprotective mechanism may involve BDNF.

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Overall, getting my M.Sc. has been an exquisite experience that I wouldn't trade for the world.

Table of Contents

Abbreviations

6-OHDA, 6-hydroxydopamine

AD, Alzheimer's disease

ALS, amyotrophic lateral sclerosis

ATP, adenosine triphosphate

BDA, bicinchoninic acid

BDNF, brain-derived neurotrophic factor

BLAST, Basic Local Alignment Search Tool cAMP, cyclic adenosine monophosphate

bp, base-pairs

Ca^{2+}, calcium

CaMKII, Calcium/calmodulin dependent protein kinase II

CBP, CREB binding protein

cDNA, complementary deoxyribonucleic acid

Cl^-, chloride

CNS, central nervous system

CPE, carboxypeptidase E

CRE, cAMP response element

CREB, cAMP-response element binding

DNA, deoxyribonucleic acid

DTT, Dithiothreitol

ERK (MAPK), extracellular signal-regulated kinase

Gab1, Grb associated binder 1

GAPDH, glyceraldehydes 3-phosphate dehydrogenase

Grb2, growth factor receptor-bound protein 2

GSH, glutathione

HIF-1α, hypoxia-inducible factor-1α

ICV, intracerebroventricular

IRS1/2, insulin receptor substrates 1/2

IV, intravenous

DIC, differential interference contrast

HPA, hypothalamic-pitutary-adrenal

JNK, Jun N-terminal kinase

K^+, potassium

MEK, MAP/Erk kinase

MPP+, 1-methyl-4-phenylpyridinium

mRNA, messenger ribonucleic acid

mTOR, mammalian target of rapamycin

MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Na^+, sodium

NCBI, National Center for Biotechnology Information

NGF, nerve growth factor

NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells

NT-3, neurotrophin-3

NT-4/5, neurotrophin-4/5

NT-6, neurotrophin-6

PBST, phosphate buffered saline with 0.2% Tween®20

PCR, polymerase chain reaction

PD, Parkinson's disease

PI-3K, phosphotidylinositol-3 kinase

PLC- ɣ, phospholipase C-ɣ

pVHL, von Hippel-Lindau tumor suppressor protein

qPCR, quantitative polymerase chain reaction

Raf, Ras associated factor

Ras, GTP binding protein

RIP2, receptor interacting protein 2

ROS, reactive oxygen species

RNS, reactive nitrogen species

RLT, RNeasy Lysis Buffer (Qiagen)

RTPCR, reverse transcription polymerase chain reaction

Shc, src homology domain containing

SOS, son of sevenless

TBP, TATA-binding protein

ten-a, tenascin-like molecule accessory

ten-m, tenascin-like molecule major

TCAPs, teneurin C-terminal associated peptides

TCAP-1, teneurin C-terminal associated peptide-1

TGN, trans-Golgi network

TRAF4/6, tumour necrosis factor receptor associated factor 4/6

TrkA, tropomyosin-related kinase A

TrkB, tropomyosin-related kinase B

TrkC, tropomyosin-related kinase C

UTR, untranslated regions

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Chapter 1 : Introduction

1.1 Introduction to Neuroprotection

Neuroprotection refers to the mechanisms and strategies that prevent the death and/or limit the degeneration of vulnerable neurons in the nervous system. Under unstressed conditions, neurons utilize enormous amounts of energy to synthesize the proteins required for function and transmission. Under these circumstances, neurons are able to extend their neurites and develop stronger connections for communication with other neurons. However, when neurons are stressed, they exhibit changes in their growth parameters and often shunt their energy, in an act to preserve their cellular life. This may include inhibition of protein synthesis, an alteration in the balance between cell growth and cell death, and the retraction of neurites that allow for its function as a simpler cell.

In the vertebrate central nervous system (CNS), most neurons that are destroyed cannot be replaced or have limited regenerative capacity and often will result in severe and debilitating consequences. Understanding cell-death mechanisms and processes that inhibit or delay its onset will provide a basis for developing tools and agents aimed at preventing neural pathologies such as stroke, Parkinson's disease and Alzheimer's disease.

Although neuronal death plays a central role in the further progression of many neurological diseases, this process is important in shaping the central nervous system (CNS). During development, there is an overproduction of neurons (neurogenesis) followed by a period of programmed cell death. Immature neurons must divide, migrate and differentiate into the appropriate types that possess certain properties allowing communication between specific synapses. Neurons that fail to form synapses or innervate inappropriate targets are eliminated via

apoptosis (Yuan and Yankner, 2000). The surviving neuronal population then goes on to compete for neurotrophic factors released by the target tissue. Those neurons that come out victorious in the competition for survival factors innervate the target, while the defeated neurons undergo cell death in a process referred to as 'pruning' (O'Leary, 1992). In this way, neurons establish and fine tune connections to achieve a high degree of communication with appropriate targets and allows for the perception and integration of sensory information that form complex multi-cellular tissues such as the brain.

Regulated death of immature neuronal populations (apoptosis) ultimately results in the predominantly non-regenerative mature CNS (Yuan and Yankner, 2000). As a result, the adult CNS is more vulnerable than other tissues as it relies heavily on its ability to promote neuronal survival and reduce the decline in neuronal numbers that are associated with increasing age or injury. Therefore, it is imperative that research to develop new treatments and drugs that can promote neuronal survival are continued and prioritized. As such, the neuroprotective effects of a novel peptide, teneurin C-terminal associated peptide-1 (TCAP-1), and its ability to regulate brain-derived neurotrophic factor (BDNF) was investigated. TCAP-1 may, in part, be involved in the promotion of neuronal survival and proliferation through the BDNF mechanism.

1.2 Discovery of teneurins

Teneurins are the vertebrate orthologues of the *ten-m/odz* genes which were initially discovered using two separate methods in *Drosophila* (Baumgartner *et al.,* 1994; Levine *et al.,* 1994). Baumgartner *et al.,* (1994) discovered ten-a (tenascin-like molecule accessory) and ten-m (tenascin-like molecule major) after conducting a low-stringency screen for vertebrate tenascin-C in a Drosophila library. Alternatively, Levine *et al.* (1994) discovered *odd Oz* (or *Odz*) by

screening for tyrosine phosphorylation where the two dozen "YD" repeats near the carboxy terminal of *Odz/ten-m* was instrumental in its identification.

The teneurins are roughly 300 kDa and 2500 to 2800 amino acids in length. They display characteristics of a type II transmembrane protein with teneurin`s C-terminal located on the extracellular face of the plasma membrane, and its N-terminal in the cytosol. Structurally, teneurins possess a hydrophobic region of approximately 30 amino acids, an N-terminal cytoplasmic domain of about 300-400 amino acids and a C-terminal extracellular domain of about 2300 amino acid residues, depending on the teneurin paralogue (Lovejoy, *et al.,* 2006).

The teneurins are a highly conserved group making up four members known as teneurins1-4 in vertebrates. In vertebrates, these four teneurin paralogues are predominantly expressed in the developing central nervous system (Baumgartner *et al.,* 1994). Evidence shows that their functional role is two-fold: as cell surface class II transmembrane receptors, and as transcriptional regulators via the release of their intracellular domain (Tucker and Chiquet-Ehrismann, 2006). Most of teneurin expression occurs in the central nervous system and studies indicate that teneurins have profound influences on neurogenesis, morphogenesis and differentiation (Lovejoy *et al.,* 2006; Tucker and Chiquet-Ehrismann, 2006).

Although the functional role of the teneurin proteins remains to be fully elucidated, they are known to be essential for normal neural development as mutations to the teneurin gene are embryonic lethal (Lovejoy *et al.,* 2007) The teneurins have been implicated in synaptic plasticity, the neuronal stress response, and in processes such as cellular adhesion, filopodia and neurite outgrowth and transcriptional regulation (Wang *et al.,* 2005; Tucker and Chiquet-Ehrismann, 2006). Overall, a better understanding on the functionality of the teneurins may shed some light

on the role of recently discovered teneurin C-terminal associated peptides (TCAPs) in the central nervous system.

1.3 Discovery of teneurin C-terminal associated peptides (TCAPs)

Qian *et al.* (2004) discovered a novel family of bioactive peptides which they named TCAP. TCAP was discovered during a low stringency screen for additional CRF paralogues, using urocortin as a probe. This 3'- region of teneurin-3 was subsequently cloned and synthesized using solid phase synthesis as previously described by Qian *et al.* (2004). In total, there are four members processed from the corresponding teneurin1-4 paralogues labelled TCAP1 through to 4. TCAP-1,-2, and -4 are 41 amino acids long and TCAP-3 is 40 residues in length. Although TCAP is derived from the extracellular C-terminal of the teneurins, its exact processing mechanism is unknown.

Interestingly, TCAP bears characteristics of a cleavable bioactive peptide as reflected in a pro-hormone cleavage motif on the extracellular C-terminal and an amidation motif on its N-terminal domain. Sequence analysis suggests that TCAP's potential functional and/or processing domain may be located adjacent to the mature peptide on the C-terminal. Specifically, dibasic residue motifs found in TCAP-1, -2, and -4 and a single basic residue in TCAP-3 are proposed to be involved in TCAP's potential modulation of cellular signalling.

A potential signalling pathway that has been associated with TCAP is the stress-induced pathways. Notably, TCAP's structural similarity to the corticotrophin-releasing factor and calcitonin families of peptides suggests its involvement in regulating cellular stress (Lovejoy *et al.,* 2007). Observations from i*n situ* hybridization studies that localizes teneurin-1 mRNA

(including TCAP-1) to the forebrain and limbic regions involved in stress, emotions and anxiety, further highlights TCAP's role in the regulation of cellular signalling (Wang *et al.,* 2005).

1.4 Functional effects of TCAP on neuromodulation

Several studies have been conducted that present synthetic TCAP as a neuromodulatory peptide *in vitro*. In 2007(a), Al Chawaf *et al.* showed that treatment with TCAP-1 increased neurite length and axon formation implicating the peptide as a component involved in neuroplastic mechanisms. This was supported by TCAP-1's ability to influence the levels and distribution of key cytoskeletal proteins and genes associated with axon outgrowth in immortalized cell lines. In brief, the significantly increased neurite length following TCAP-1 treatment was complemented by western blot and real-time reverse-transcription polymerase chain reaction (RT-PCR) analyses showing increased β-tubulin protein and mRNA expression, respectively. TCAP-1 treatment was also able to increase the synthesis or translation of other cytoskeletal proteins such as α-actinin-4 and β-actin, respectively. These observations were paralleled by gene microarray analyses that indicated altered levels of various cytoskeletal genes and protein expression by TCAP-1 (Trubiani *et al.,* unpublished data).

Based on the findings that there is a high level of TCAP mRNA expression in the hippocampus (Wang *et al.,* 2005), Al Chawaf *et al.* (2007a) studied TCAP's neuromodulatory effect in primary rat hippocampal cultures and found similar results to those initially seen in immortalized cells. Primary hippocampal cells treated with TCAP-1 showed increased proliferation, neurite number, dendritic arborization, and axon fasciculation (Al Chawaf *et al.,* 2007a). Immunofluorescence analysis also depicted greater immunoreactivity and expression of β-tubulin.

TCAP's bioactive actions *in vitro* may be due to its ability to modulate cyclic AMP (cAMP). Synthetic TCAP-1 (Wang *et al.,* 2005) and TCAP-3 (Qian *et al.,* 2004) were both shown to increase cAMP in immortalized cell lines. Qian *et al.* (2004) found similar dose-dependent increases in cAMP and MTT proliferation profile patterns following rtTCAP-3 treatment in immortalized Gn11 hypothalamic cells. That is, both cAMP and proliferation were increased at low concentrations of TCAP and decreased at higher concentrations. Al Chawaf *et al.* (2007a) also found that TCAP-1 had actions on cAMP accumulation and supported a potential cAMP-dependent mechanism underlying observed increases in β-tubulin and α-actin immortalized N38 hypothalamic cell lines. Collectively, these studies suggest a potential role for TCAP in regulating intracellular signal transduction pathways at the cellular level that may ultimately regulate changes in behaviour.

Over the years, neuropeptide systems have gained greater recognition in their ability to modulate behaviour, especially as it pertains to mood disorders such as depression and anxiety. *In situ* hybridization studies by Wang *et al.* (2005) indicated that the highest level of teneurin-1 containing TCAP-1 mRNA expression occurred in the CA1, CA2 and CA3, major cell groups of the hippocampal formation, and dentate gyrus. Other limbic regions that clearly expressed TCAP include the piriform cortex, bed nucleus of the stria terminalis, and the central and basolateral nuclei of the amygdala (Wang *et al.,* 2005). The high expression in the forebrain and limbic regions of the rat brain, regions known to regulate emotions and the stress response, served as a basis for conducting studies supporting TCAP's function in modulating behaviour.

Several *in vivo* studies in rats have indicated a modulatory effect on emotionality and anxiety by TCAP-1. In 2005, Wang *et al.* conducted an acute effect study where synthetic TCAP-1 was injected into the basolateral nucleus of the amygdala over a course of weeks and a

longer termed chronic effect study with intracerebroventricular (ICV) TCAP-1 repeatedly administered into rat brains. In the acute study, TCAP-1 had a differential modulatory effect on the acoustic startle response (ASR) response depending on the baseline reactivity. Rats that were initially grouped as having low-anxiety experienced a greater sensitization of the ASR response, while the opposing effect was seen for rats initially grouped as having high-anxiety. In the chronic study, vehicle-treated rats showed a significantly increased sensitization of the ASR response while sensitization did not occur in TCAP-1 treated rats. This pointed to TCAP's ability to induce long-term neurological changes that modulated the behavioural response of rats in the ASR and suggests alterations in brain plasticity that have huge implications in the modulation of anxiety and depression.

Several studies further establish TCAP's long-lasting neuromodulatory actions and its ability to regulate corticotrophin-releasing factor (CRF) *in vivo*. Al Chawaf *et al.* (2007b) investigating anxiety-related behaviours in male rats following repeated intravenous (IV) TCAP-1 administration with either an acute intracerebroventricular (ICV) or IV CRF challenge. In this experiment, the mode of CRF injection was important and it was found that TCAP-1 reduced anxiety-behaviours on ICV CRF responses in the EPM while increasing anxiety on IV CRF-responses in the open field. Another study with repeated ICV injections of TCAP-1 for 5 days also had a significant effect on behaviour in rat models of anxiety that were administered an acute CRF challenge prior to testing (Tan *et al.,* 2008). Interestingly, TCAP-1 only presented an effect with a CRF challenge and TCAP-1 treated rats compared to saline-treated rats resulted in significant reduction in the ASR anxiety response. In contrast, using the same experimental design and treatment regime, Tan *et al.* (2008) showed that TCAP-1 treated rats displayed an enhanced anxiogenic effect of CRF in the open field and elevated plus maze (EPM). The

differing results may be attributed to the different brain areas used for each test. Although the mechanism by which TCAP-1 regulates CRF is unknown, recent studies by Tan *et al. (*2009) reported that acute TCAP-1 inhibited CRF-induced cfos expression in the brain. Such modulation of c-fos expression may explain the observed alterations in the behavioural responses of CRF. Overall, each of these studies point to fundamental roles for TCAPs in modulating neuronal function and behaviour.

1.5 TCAP's role in neuroprotection

Treatment with synthetic TCAP-1 has been shown to confer neuroprotection. Chronic TCAP-1 treatments to various neural-like N38 cell lines prevented pH-induced necrotic cell death (Trubiani *et al.,* 2007). From these experiments, TCAP-1 mediates its neuroprotective effects by inhibiting caspase-3 cleavage, promoting cell proliferation, attenuating cell degeneration, and upregulating superoxide dismutase (SOD1) and catalase (Trubiani *et al.,* 2007). Additionally, new preliminary studies indicate that TCAP-1 treatment of immortalized hypothalamic cells can increase the expression of BDNF mRNA and translation of the mature BDNF protein in cells that have undergone various stresses. Overall, these studies indicate that some of TCAP-1's neuroprotective effects may be mediated by BDNF. Together, the studies mentioned above support a critical role for TCAP in regulating emotional behaviour, neuromodulation and neuroprotection. Investigating the mechanism and identifying TCAPs action on BDNF may prove to have potential diagnostic and therapeutic value.

1.6 The role of neurotrophic factors in neuroprotection

Landmark studies by Levi-Montalcini and Hamburger (1953) spurred the genesis of growth factor research in the nervous system. Classically, growth factors refer to substances that can stimulate hyperplasia (cell division) or hypertrophy (increased cell size). Within this heterogeneous group is a subset of growth factors known as the neurotrophic factors including ciliary neurotrophic factor (CNF), fibroblast growth factors (FGF), insulin-like growth factors (IGF), epidermal growth factors (EGF), and transforming growth factors (TGF). These endogenously expressed proteins regulate a variety of processes including neuronal survival, maintenance, growth, morphological plasticity, and/or synthesis of proteins for differential functions in neural tissue (Fallon and Loughlin, 1993; Hefti, 1997). The actions of the neurotrophic factors begin with their synthesis by producer neurons and release to bind receptors on target responsive neurons. This is followed by either the retrograde transport of the internalized neurotrophic factor-receptor complex (or less commonly by anterograde transport) to the cell soma, or stimulation of signal transduction secondary messenger systems, both of which elicit multiple survival-promoting effects via control of gene expression (Fallon and Loughlin, 1993).

However it was the discovery of nerve growth factor by Levi-Montalcini and Hamburger in the early 1950's that initiated a plethora of studies making it the first in the family of neutrophins. Growing interest in NGF led to a better understanding of its neuroprotective and repair functions (reviewed in Sofroniew *et al.* 2001). The popularity of studying NGF led others to the subsequent discovery (Barde *et al.,* 1982) and cloning of BDNF (Leibrock *et al.,* 1989), the second neurotrophin member of particular importance to this study. Other members of the neurotrophin family that would later be included are neurotrophin-3 (NT-3) (Maisonpierre *et al.,*

1990), neurotrophin-4/5 (NT-4/5) (Hallbook *et al.,* 1991) and neutrotrophin-6 (NT-6) (Götz *et al.,* 1994). The effects of these neurotrophins are mediated by binding to protein kinase receptors of the *trk* (tropomyosine-related kinase) family. NGF primarily acts on TrkA with high-affinity, BDNF and NT-4/5 on TrkB, NT-6 on TrkA, while NT-3 interacts mainly with TrkC and at lower affinity with TrkA and TrkB receptors. These neurotrophins also all bind the low affinity neurotrophin receptor ($p75^{LNTR}$) (Chao and Hempstead, 1995).

At present, there is greater recognition that much of the cellular damage resulting from CNS insults such as stroke and degenerative disease may be caused by the limited number of endogenously generated neurotrophins. Rapidly emerging studies on this family and their versatile effects on different target neuronal populations involved in neurodegenerative diseases give this family a high therapeutic potential in the treatment of Alzheimer's and Parkinson's disease and amyotrophic lateral sclerosis (ALS) where there is selective degeneration of certain neuronal groups. For example, in Parkinson's disease, the vulnerable dopaminergic neurons of the substantia nigra degenerate while in ALS the motor neurons are affected. These neuronal populations were found to be responsive to BDNF (Hyman *et al.,* 1991) and NT-4/5 (Davies *et al.,* 1993). In addition, NT-3 is trophic for sensory and motor neurons involved in mediating proprioceptive sensation (Hory-Lee *et al.,* 1993; Ernfors *et al.,* 1994). In Alzheimer's disease, one population of neurons that degenerate are the central cholinergic neurons, which were found to respond to NGF administration (Fischer *et al.,* 1987; Hefti *et al.,* 1989). In animal stroke models, the neurotrophin family also plays a protective role. Several studies show that exogenous administration of BDNF or NT-4 reduced brain damage associated with cerebral ischemia (Tsukahara *et al.,* 1994; Chan *et al.,* 1996; Alexi *et al.,* 1997; Cheng *et al.,* 1997; Schäbitz *et al.,* 1997).

The neurotrophin family also has implications in stress-related neuropsychiatric disorders. These factors are able to act in concert with the hypothalamic-pitutary-adrenal (HPA) axis to modulate brain plasticity whereby dysregulation of factors such as BDNF, NT-3 and NGF have often been linked to morbidity states such as schizophrenia, mood disorders and depression (reviewed in Alleva and Francia, 2009). For example, several studies report low serum BDNF levels in schizophrenic patients and patients with depressive disorder (Karege *et al.,* 2002; Toyooka *et al.,* 2002*).* In addition, anti-depressant effects can be produced following exogenous administration of BDNF in specific brain regions of animal models (reviewed by Duman and Monteggia, 2006).

Based on the growing number of studies on neurotrophins, the idea of neurotrophic therapy is logical and has generated excitement among members of the scientific and medical communities for use in the slowing or arresting of various neurodegenerative diseases. In recent years, the biotechnology and pharmaceutical industries have attempted to replicate the findings in animal models and translate them for use in the clinic. In fact, many of these factors have already been tested in phase I, II and III clinical trials but only a few have shown moderate efficacy (see Apfel, 2001 for review).

From these clinical trials, the challenges and lessons learned must be addressed in future studies. This includes devising better ways of administrating polypeptide growth factors and ensuring they cross the blood-brain barrier. Thus far, genetic engineering of protein carriers for neurotrophic factors, implantation of genetically engineered cells, modifications including conjugation to drug targeting systems provides a solution for the delivery in animal models (Zuccato and Cattaneo, 2009; Zhang and Pardridge, 2001). However, in spite of well founded

clinical experiments stemming from the success of experimental work in animal models, the results have been disappointing.

Many neuropeptides have been shown to promote neurotrophic activity by means of modulating cell differentiation, survival, phenotypic expression, plasticity, cellular hypertrophy, and neurite extension (for review see Gozes and Brenneman, 1993; Hökfelt, 1991). Therefore, the focus of this thesis attempts to explore one such novel family of neuropeptides, known as teneurin C-terminal associated peptides (TCAPs) and its regulation of BDNF. Therefore, as an alternative to neurotrophic factor therapy, we focus on the use of peptides in this study.

1.7 Introduction to brain-derived neurotrophic factor (BDNF)

Brain-derived neurotrophic factor (BDNF) was first discovered in 1982 from studies showing a distinct but nerve growth factor (NGF)-like activity in the growth medium of cultured glioma cells. Subsequently, the purification of this molecule from pig brain confirmed similar and yet distinct properties of its predecessor, NGF. Substantial amount of excitement was generated when molecular cloning of BDNF in porcine, murine and humans finalized its relationship with NGF, which found both factors to be part of the same gene family known as the neurotrophins (Leibrock *et al.,* 1989; Hofer *et al.,* 1990; Rosenthal *et al.,* 1991). As the most abundant and widely distributed neurotrophin in the CNS, BDNF has a fundamental role in mediating survival, differentiation, and outgrowth of central neurons during development and in adulthood (reviewed by Bailey, 1996; Hashimoto, 2004; Tapia-Arancibia, 2004). Therefore, it is conceivable that many CNS neurons would respond to BDNF, particularly those neuronal populations associated with neurodegenerative diseases including, cholinergic neurons (Alderson

et al., 1990; Knüsel *et al.,* 1991; Nonomura and Hatanaka, 1992; Morse *et al.,*1993; da Penha Berzaghi *et al.,* 1993), dopaminergic neurons (Hyman *et al.,* 1991; Knüsel *et al.,* 1991; Beck *et al.,* 1993) and motor neurons (Oppenheim *et al.,* 1992; Henderson *et al.,* 1993; Kato and Lindsay, 1994). BDNF has also been implicated in regulating synaptic transmission and plasticity (see reviews McAllister *et al.,*1999 ; Yamada and Nabeshima, 2003; Bramham and Messaoudi, 2005; Cunha *et al.,* 2010) thereby linking it not only to learning and memory, but in the pathophysiology of mood disorders and stress (for review, see Calabrese *et al.,* 2009; Martinowich and Lu, 2008; Lipsky and Marini, 2007; Duman and Monteggia, 2006; Hashimoto *et al.,* 2004). Although extensive studies have provided substantial support for BDNF's pleiotropic effects on neuronal development, survival and synaptic plasticity, future studies need to investigate the mechanisms of BDNF's actions on complex behaviour and cognition so that its full therapeutic potential for the treatment of CNS human disease can be harnessed.

1.8 BDNF synthesis, processing, sorting, transport and signalling

Recent evidence by Aid *et al.* (2007) has reorganized the entire rodent *bdnf* gene structure to be much more complex than previously thought. This new organization is now comprised of eight 5'-non-coding exons and 1 common 3'-coding exon. Through the use of alternative promoters, splicing and polyadenylation sites, this model suggests the production of 18 to 24 different BDNF variant transcripts (Tabuchi, 2008; Greenberg *et al.,* 2009; Cunha *et al.,* 2010). The complex multi-level regulatory system of expression described above together with differential mRNA stability and subcellular localization of BDNF mRNA and protein has been thought necessary for the fine-tuning of BDNF function (Tabuchi, 2008; Greenberg *et al.,* 2009).

The transcriptional activation of BDNF gene expression is primarily regulated through activation of the transcription factor, cAMP-response element binding (CREB). CREB-induced transcription of BDNF can occur via growth factors, neurotransmitters, depolarization of ion channels and stressors (Sossin and Barker, 2007). Essentially, the aforementioned activators lead to the activation of downstream protein kinases that then translocate to the nucleus and phosphorylate CREB. Subsequently, phosphorylated CREB together with its coactivators, CREB binding protein (CBP) and p300, bind to the cAMP response element (CRE) located upstream of the BDNF gene to initiate BDNF transcription (Finkbeiner *et al.,* 1997).

There is also regulation at the protein level (see Figure 1.1). BDNF is translated in the rough endoplasmic reticulum as a preproBDNF protein. When the signal peptide is cleaved, the 32 kDa proBDNF protein is transported to the Golgi apparatus network. At the trans-Golgi network (TGN), proBDNF can be packaged into two kinds of secretory vesicles: 1) those of the constitutive secretory pathway that do not require stimuli and are transported to and fuse with the plasma membrane to be released into the extracellular space or 2) those of the regulated secretory pathway that are transported and accumulated at the plasma membrane in preparation to be released into the extracellular space by intracellular or extracellular signals (Kelly, 1985; Glombik and Gerdes, 2000). However, although neurotrophins can enter both pathways, proBDNF predominately binds the lipid-raft-associated sorting receptor carboxypeptidase E (CPE) that normally targets proteins for the regulated secretory pathway (Cool *et al.,* 1997; Lou *et al.,* 2005). ProBDNF can be proteolytically processed to the 14 kDa mature BDNF by members of the subtilisin-kexin family of endoproteases (e.g. furin) at the TGN or in the immature secretory granules by proprotein convertases (Mowla *et al.,* 1999). Alternatively, proBDNF may be secreted into the extracellular space and cleaved by plasmin or

metalloproteinases into the mature form (Lee *et al.,* 2002; Pang *et al.,* 2004). Both the pro- and mature BDNF proteins are active and known to bind to different transmembrane-receptor signalling systems: the high-affinity tropomyosin-related TrkB tyrosine kinase receptor and the low-affinity $p75^{LNTR}$ neurotrophin receptor (reviewed by Chao, 2003). From this, various downstream intracellular pathways are activated and can influence survival or neuronal death, respectively (Matsumoto *et al*, 2008).

In particular, BDNF can bind both the TrkB and $p75^{LNTR}$ receptor but preferentially binds as a dimer to pre-synaptic and post-synaptic TrkB receptors (Figure 1.2). In contrast, proBDNF binds preferentially to the low-affinity $p75^{LNTR}$ receptor to affect distinct cellular pathways (e.g. pro-apoptotic) from BDNF (Teng *et al.,* 2005). The binding of BDNF to TrkB receptor leads to its dimerization and autophosphorylation that signals activation of many downstream intracellular pro-survival pathways including those mediated by extracellular signal-regulated kinase (ERK, MAPK), phosphotidylinositol-3 kinase (PI-3K), and phospholipase C-ɣ (PLC-ɣ). In contrast, binding of BDNF dimers to p75 neurotrophin receptors predominantly signals to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and Jun N-terminal kinase (JNK), which are factors related in cell death and inflammation, respectively (reviewed in Chao, 2003). Interestingly, BDNF binding can also initiate a positive pathway whereby its activation of downstream intracellular cascades can induce its own transcription via the CREB transcriptional factor (reviewed in West *et al.,* 2002).

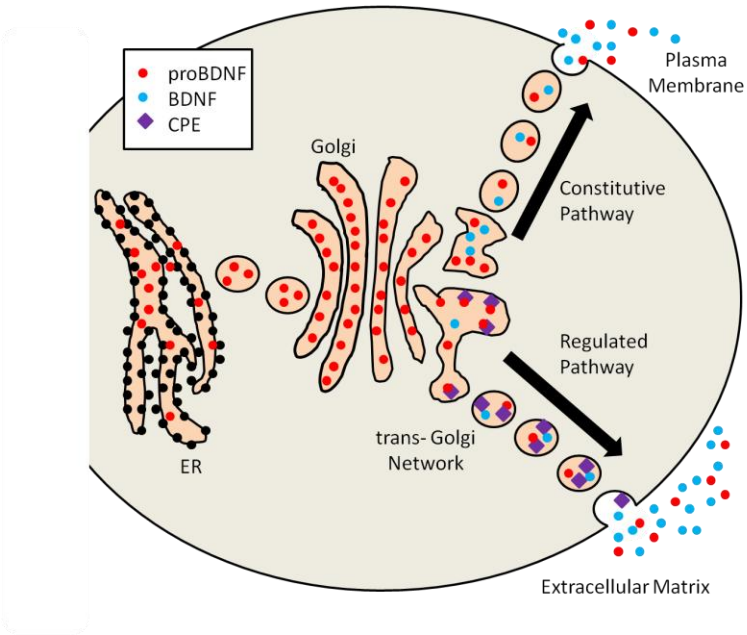

Figure 1.1: BDNF processing, packaging and secretion in neurons.

BDNF is translated in the rough endoplasmic reticulum and produces a 32 kDa proBDNF protein (red circle) that is transported to the Golgi apparatus network. At the trans-Golgi network, proBDNF can be pass down the following pathways: 1) the constitutive secretory pathway or 2) the regulated secretory pathway. ProBDNF predominately binds the sorting receptor carboxypeptidase E (CPE) that normally targets proteins for the regulated secretory pathway. ProBDNF can then be proteolytically processed to the 14 kDa mature BDNF by members of the subtilisin-kexin family of endoproteases (e.g. furin) at the TGN or in the immature secretory granules by proprotein convertases. Alternatively, proBDNF may be secreted into the extracellular space and cleaved by plasmin or metalloproteinases into the mature form. (*Figure adapted from Cunha et al.,* 2010).

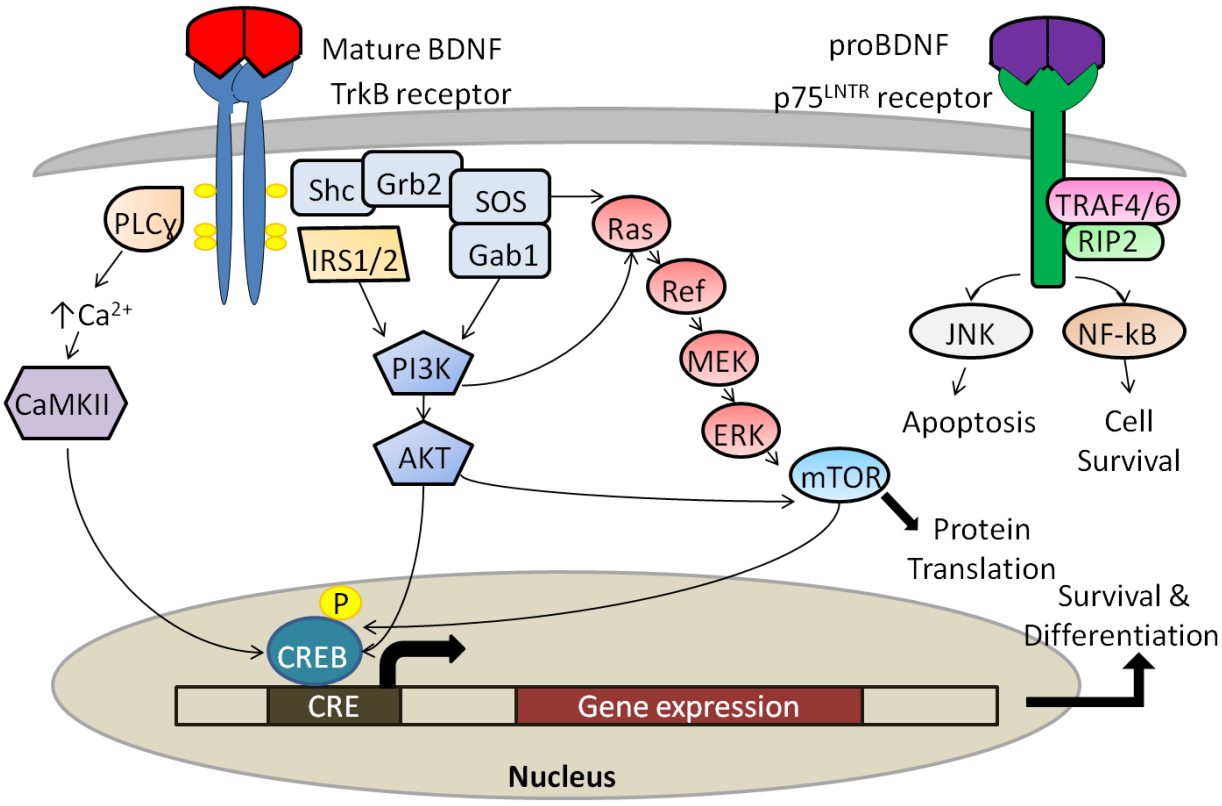

Figure 1.2: BDNF and proBDNF signalling transduction mechanisms.

Mature BDNF can bind both the TrkB and $p75^{LNTR}$ receptor but preferentially binds as a dimer with high-affinity to the TrkB receptor. In contrast, proBDNF binds preferentially to the low-affinity $p75^{LNTR}$ receptor. The binding of mature BDNF to the TrkB receptor leads to its dimerization and autophosphorylation of tyrosine residues (yellow circles) that signals activation of three intracellular pro-survival pathways: extracellular signal-regulated kinase (ERK), phosphotidylinositol-3 kinase (PI-3K), and phospholipase C-ɣ (PLC-ɣ) cascades. Ultimately, this activates the transcription factor cAMP-calcium response element binding protein (CREB), which mediates genes involved in neuronal survival and differentiation. In the PLC-ɣ pathway, intracellular Ca^{2+} levels are increased and act on CaMKII, which phosphorylates CREB. In the ERK pathway, phosphorylation of ERK directly phosphorylates CREB. In the last pathway, activation of PI3K occurs via the Shc/Grb2/SOS complex and Gab1 and IRS1/2 which produces lipid products that bind and activate protein kinase Akt ultimately phosphorylating CREB. Note that both Akt and ERK activate mTOR which is responsible for enhanced translation initiation. In contrast, binding of proBDNF dimers to the p75LNTR receptor predominantly signals to activate nuclear factor kB (NF-kB) and Jun N-terminal kinase (JNK), which are factors related to survival or cell death, respectively. *(Figure adapted from Cunha et al., 2010)*

Shc, src homology domain containing; Grb2, growth factor receptor-bound protein 2; SOS, son of sevenless; Gab1, Grb associated binder 1; IRS1/2, insulin receptor substrates 1/2; Ras, GTP binding protein; Raf, Ras associated factor; MEK, MAP/Erk kinase; mTOR, mammalian target of rapamycin; TRAF4/6, tumour necrosis factor receptor associated factor 4/6; RIP2, receptor interacting protein 2.

1.9 BDNF and neuroprotection

A major goal for neuroprotective strategies as it pertains to neurodegenerative diseases is to prevent or delay the slow and progressive loss of neurons in various areas of the central nervous system. BDNF has emerged as a critical factor with an essential role in the delayed progression of neurodegenerative and psychiatric diseases through its regulation of synaptic plasticity and support for neuronal survival. The levels of neurotrophins have been thought to play a fundamental role in determining the balance between cell survival and apoptosis during development. Indeed, mutant mice lacking neurotrophins die during the first few weeks after birth whereas heterozygous mice (BDNF +/-) that have decreased BDNF levels still remain viable with deficits in memory and prominent neuronal loss (Chao, 2003).

BDNF's intimate involvement with neuroprotection is supported by many studies that have implicated it in the protection of neurons following spinal cord injury, global or regional brain ischemia and stress in adult rats (Snider, 1994; Zhang *et al.,* 2001; Husson *et al.,* 2005). A study by Barde (1989) showed that BDNF promoted survival of dorsal root ganglion neurons in culture and *in vivo* if administered to embryos during the period of natural neuronal death. This was demonstrated by increased number of neurons and a reduction in the number of pyknotic cells. In addition, alterations in BDNF expression, distribution and activities have associated it with the pathogenesis of several neurodegenerative diseases (Zuccato and Cattaneo, 2009) including Alzheimer's (Phillips *et al.,* 1991; Murray *et al.,* 1994*;* Ferrer *et al.,* 1999; Fumagalli *et al.,* 2006; Tapia-Arancibia *et al.,* 2008), Parkinson's (Howells *et al.,* 2000) and mood disorders such as depression (Dunman and Monteggia, 2006). Thus, this section attempts to highlight some of BDNF's neuroprotective roles in neurodegenerative disease.

Alzheimer's disease (AD) is an age-related neurodegenerative disease that is characterized by progressive dementia and impairment of cognition due to the loss of synaptic connections and accumulation and formation of β-amyloid plaques and Tau/neurofibrillary tangles (Fumagalli *et al.,* 2006). The clinical and neuropathological manifestations of AD stem from the severe degeneration of the basal forebrain cholinergic system and failure of synaptic plasticity which have been linked with a depletion of neurotrophic factors (Whitehouse *et al.,* 1982; Fumagalli *et al.,* 2006). In several studies, BDNF has shown to promote the survival and differentiation of basal forebrain cholinergic neurons (Alderson *et al.,* 1990; Knüsel *et al.,* 1991; Fahnestock *et al.,* 2002). Interestingly, in 1999 immunoreactive studies by Murer *et al.* reported that neurons containing neurofibrillary tangles did not contain BDNF whereas neurons that didn't possess tangles had intense labelling with BDNF-specific antibodies. The association with BDNF and AD has also been supported by several behavioural studies. Heterozygous mice containing a deletion in one BDNF allele showed deficits in learning and memory (Linnarsson *et al.,* 1997). However, post-lesion gene transfer of BDNF in a rat dementia model with cognitive deficits was able to partially restore the deficits in learning capacity (Ando *et al.,* 2002). In addition, knockouts of TrkB, the high-affinity receptor of BDNF, specifically in the forebrain results in impaired learning (Minichiello *et al.* 1999). Together, these studies suggest that reductions in BDNF synthesis may enhance the deterioration of cellular homeostasis regulating neurons in the basal forebrain which will ultimately give rise to AD.

Another age-related central nervous system disorder is Parkinson's disease (PD) which is characterized primarily by the degeneration of dopaminergic neurons in the substantia nigra and the hallmark appearance of cytoplasmic inclusion bodies known as Lewy bodies (reviewed in Hindle, 2010). Clinical symptoms of PD include rest tremor, rigidity, impaired physical

movement, and postural instability (Jankovic, 2008; Hindle, 2010). Like AD, PD has also been linked to BDNF. Several studies demonstrate that PD-patients exhibit reductions in the levels of BDNF in the substantia nigra (Parain *et al.,* 1999; Mogi *et al.,* 1999) and indicate BDNF's survival effect on dopaminergic neurons (Hyman *et al.,* 1991; Knüsel *et al.,* 1991; Beck *et al.,* 1993). In PD, oxidative stress acts as one contributing factor to the pathogenesis of the disease and studies have shown that BDNF is able to support survival against oxidative stress. Spina *et al.* (1992) showed that dopamine neurons treated with the neurotoxin, 6-hydroxydopamine (6-OHDA), a common PD model that causes significant reduction in dopamine neurons, was able to counteract the effects of oxidative stress possibly by increasing levels of glutathione reductase. These findings were also consistent with in vivo studies where BDNF was able to protect dopaminergic neurons against neurotoxicity following 1-methyl-4-phenylpyridinium (MPP+) treatment (Frim *et al.,* 1994). Studies by Levivier *et al.* (1995) also found BDNF to be protective in a PD rat model. Intrastriatal grafts of BDNF-producing fibroblast cells were able to protect against a 6-OHDA-induced rat model of PD by partially reducing the loss of cell bodies and nerve terminals. Therefore, BDNF appears to mediate survival effects on vulnerable dopaminergic cells targeted in PD and thus, may serve as a putative therapy for PD.

In addition, it is no surprise that BDNF's role in activity-dependent mechanisms such as long-term potentiation and synaptic transmission (see reviews McAllister *et al.,*1999 ; Yamada and Nabeshima, 2003; Bramham and Messaoudi, 2005; Cunha *et al.,* 2010) implicate its involvement in mood disorders (Hashimoto *et al.,* 2004). Among mental disorders, mood disorders are by far the most prevalent, recurrent and disabling. Conditional deletions of BDNF in the brains of postnatal mice have led to hyperphagia, hyperactivity and higher levels of anxiety (Chao, 2003). Such behavioural abnormalities highlight a role for BDNF in normal

behaviour in the central nervous system. Of the many mood disorders, research on depression has gained interest amongst the scientific community, in part because of its complex aetiology. Despite the heterogeneity of the disease, recent basic and clinical work on BDNF has identified a single-nucleotide polymorphism in proBDNF (Val66Met) to exhibit aspects of depression or bipolar disease (Dunman and Monteggia, 2006). Additionally, depression has been linked to a decrease in BDNF expression. Together with stress, depression can exacerbate decreases in BDNF and CREB activity resulting to further neuronal atrophy and cell loss in key limbic brain regions, including the amygdala, prefrontal cortex and hippocampus regions implicated with mood and cognition (reviewed by Duman and Monteggia, 2006). Further emphasis of BDNF's involvement in depression and mood disorders comes from a recently described finding suggesting that BDNF expression may be a downstream target of antidepressant treatments and mood stabilizers (Hashimoto *et al*, 2004). Thus, BDNF plays a pivotal role in the pathophysiology of depression, and can confer therapeutic benefits through its increased expression (Duman and Monteggia, 2006).

The studies described above support BDNF's role in neuroprotection of a variety of diseases such as Alzheimer's, Parkinson's and depression. These disorders require pharmacological intervention to improve neuronal survival and preserve cognitive function and although BDNF is an attractive target to study for translational purposes, BDNF poises many challenges for effective therapeutic use. For example, BDNF has a short half live *in vivo* and a limited ability to mediate its effects across the blood-brain barrier. When BDNF is administered into the brain by minipump, the steep concentration gradient that results beginning at the site of infusion generates adverse effects (Zuccato and Cattaneo, 2009). Therefore, others modes of

delivering BDNF has been considered including gene therapy, BDNF releasing cell grafts, BDNF mimetics (small molecules that bind BDNF receptors at target sites), or drugs.

Thus, our present study aims to study the effects of a novel bioactive peptide on BDNF regulation. Previously, studies have shown that TCAP modulates emotionality and anxiety *in vivo* (Wang *et al.,* 2005; Al Chawaf *et al.,* 2007a; Tan *et al.,* 2008; 2009) and can be neuroprotective *in vitro* (Trubiani *et al.,* 2007). In this study, we investigate TCAP's neuroprotective mechanism which may, in part, involve BDNF.

1.10 Stroke and Ischemia

Stroke is the second most common cause of death, after ischemic heart disease, and a major cause of long-term disability worldwide (Murray and Lopez, 1997). This occurrence is expected to increase moving forward largely as a result of our burgeoning elderly population and the relatively low priority afforded to general stroke research. The rising prevalence of stroke worldwide and the escalating health-care costs associated with stroke and/or demands on social-care systems (American Heart Association, 1997; Dewey *et al.,* 2001) motivate scientists and clinicians to better understand the mechanisms underlying stroke and invent safer and more effective therapeutic tools to counteract this trend. Here, the underlying pathophysiology of stroke is discussed and the intertwined pathways that are the target of therapeutic intervention highlighted.

Stroke results from disruptions or interruptions in the blood supply to the brain. This condition leads to rapid loss of brain functions, which without immediate treatment may result in permanent neurological damage underlying clinical symptoms such as impaired movement, sensation, and/or cognition (Murphy and Corbett, 2009). This neurological condition is highly

heterogeneous and varies in severity from full recovery to severe disability or to death (Warlow *et al.,* 1996).

In broad terms, stroke can be categorized as either ischemic or haemorrhagic. Predominately, 80% of strokes arise due to cerebral ischemia (Thrift *et al.,* 2001) with only 15-20% attributable to intracerebral and subarachnoid haemorrhaging (Bamford *et al.,* 1990). For the purpose of this study only the former will be discussed as it has primarily been the focus of most drug trials (Rosamond *et al.,* 2007).

In general, brain injury from ischemic-stroke can result from thrombosis, embolisms or systemic hypo-perfusion. The resulting vascular occlusion restricting blood flow and thus the delivery of necessary substrates such as oxygen and glucose to the brain can lead to irreversible cellular injury and tissue necrosis via the initiation of the 'ischemic cascade' (Hossmann, 1994). The events of this cascade include excitotoxicity, acidotoxicity, ionic imbalance, peri-infarct depolarization, oxidative and nitrative stress, inflammation and apoptosis (reviewed by Doyle *et al.,* 2008).

To summarize, the high aerobic metabolism of the brain renders it helpless and incapable of energy production via oxidative phosphorylation during focal cerebral ischemia. The cascade begins with energy depletion and the dissolution of ionic gradients, which causes neuronal peri-infarct depolarizations. This activates voltage-dependent Ca^{2+}-channels and the accumulation of excitatory amino acids such as glutamate in the extracellular space. This glutamate-mediated excitotoxicity causes an influx of Na^{+} and Cl^{-} at a rate faster than the efflux of K^{+}. Water is then passively drawn into the cell giving the characteristic oedema of stroke patients observed after the first few hours. Contributing to the damage is the elevation of Ca^{2+} that activates proteolytic enzymes known to degrade cytoskeletal components, and intracellular secondary messenger

systems that increase hypoxia-induced gene expression (e.g. proinflammatory genes via hypoxia-inducible factor-1 (HIF-1)) (Ruscher *et al.,* 1998). This is followed by the production of free-radical species that stress endogenous scavenging systems and damage membranes. The mitochondrial membranes become disrupted and there is release of more reactive oxygen species and the apoptosis-inducing cytochrome c protein (see Dirnagl *et al.,* 1999 for a more detailed review).

The processes described above do not occur homogenously in the ischemic lesion and the concept of the ischemic penumbra becomes important in characterizing the severity of the injury. This region is located between the damaged necrotic core region of the affected tissue and the normal brain. The penumbra has been defined as ischemic tissue that can be rescued from further damage despite having reduced blood flow and partially preserved energy metabolism (Hakim, 1987). However, over time if left untreated, the penumbra can progress to infarction.

There are two types of ischemic stroke: global cerebral ischemia and focal cerebral ischemia. In global cerebral ischemia, the entire brain is involved and takes place either during cardiac arrest or severe systemic hypotension. In contrast, the more prevalent focal cerebral ischemia pertains to isolated brain regions that commonly result from cerebral vascular atherosclerosis (Iadecola, 1999). Currently, there has been much research on experimental stroke models of focal cerebral ischemia in the past decade. However, this has proven to be a daunting task with few new compounds and therapies demonstrating neuroprotective properties being introduced into clinical studies due to problems with toxicity and pharmacological effectiveness. One biologically effective treatment for reopening up occluded blood vessels in acute ischemic stroke is thrombolysis otherwise known as recombinant tissue plasminogen activator (The National Institute of Neurological Disorders, 1995). However, the challenge with utilizing this

option is the narrow therapeutic window of effectiveness and this makes the development for alternative new therapeutics an even greater priority.

Brain tissue survival can be improved to some extent if one or more of these processes in the ischemic cascade described above are inhibited. Previous studies reviewed by Doyle *et al.,* (2008) highlight some neuroprotective drugs that scavenge reactive oxygen species, inhibit apoptosis, or inhibit excitotoxic neurotransmitters to reduce tissue injury due to ischemia.

The expression of several growth factors also provides another avenue for therapeutic development following focal cerebral ischemia. Among the various growth factors that are involved in reducing ischemic injury or inhibiting components of the ischemic cascade is BDNF (Tsukahara *et al.,* 1994; Cheng et al, 1997; Larsson *et al.,*1999; Han *et al.,* 2000; Schäbitz *et al.,* 2000). Interestingly, previous studies show that TCAP-1 treatment is able to reduce ROS by upregulating antioxidant defence mechanisms via superoxide dismutase and catalase (Trubiani *et al.,* 2007). This study aims to highlight TCAP's ability to regulate BDNF and confer neuroprotection under hypoxic conditions *in vitro*. For this reason, TCAP may present itself as an attractive candidate in the treatment of stroke and ischemia.

1.11 Hypoxic Stress: HIF-1α, ROS and BDNF

In order to maintain oxygen homeostasis, the cells and tissues of higher organisms must be able to adapt and respond to changes in oxygen concentration via highly elaborate and regulated signal transduction mechanisms and oxygen-responsive gene transcription systems (for review see Wenger, 2000). For the purpose of this study, there will be a greater emphasis on hypoxia, which is a condition where there is lower than normal oxygen concentrations.

The brain is a complex oxygen-sensitive tissue that is highly metabolically active and therefore requires an abundant supply of oxygen and glucose to maintain function. As a result, the brain must possess and employ strategies that will be protective against damage by oxidative stress. When there is an imbalance in the brain's demands for and supply of essential substrates such as glucose and oxygen, then such adverse effects of hypoxic stress may result in cardiovascular and neurodegenerative diseases.

When oxygen is available, aerobic metabolism occurs and transforms nutrients such as glucose into adenosine triphosphate (ATP), a primary source of metabolic energy for aerobic organisms. Glycolysis in the cytoplasm converts glucose into pyruvate which proceeds to the tricarboxylic acid cycle and oxidative phosphorylation in the mitochondria. Ultimately, oxygen acts as the terminal electron acceptor. On the other hand, under hypoxic conditions, cells undergo less-efficient non-oxygen-dependent energy metabolism that converts pyruvate into lactic acid (reviewed in Weidemann and Johnson, 2008). Interestingly, this molecular hypoxic response mechanism is mediated by hypoxia-inducible factor-1α (HIF-1α).

HIF-1α is a transcription factor that is heterodimeric and composed of an unstable α-subunit and a common β-subunit. In normoxia, the HIF-1α subunit has a relatively short half-life. Following hydroxylation of its two proline residues (Pro402 and Pro564) on the oxygen-dependent degradation domain (ODD), hydroxylated HIF-1α interacts with the von Hippel-Lindau tumor suppressor protein (pVHL). This results in HIF-1α being ubiquitinated and degraded in the proteosomes to often undetectable levels (Weidemann and Johnson, 2008). In contrast, where there is limited oxygen availability, HIF-1α does not get hydroxylated and avoids degradation via the ubiquitin-proteosome pathway. Instead, it translocates into the nucleus and binds to a consensus DNA binding sequence on a hypoxia-response element promoter to mediate

oxygen-regulated gene expression (Wenger and Gassmann, 1997). HIF-1 target genes include those involved in oxygen transport, oxygen homeostasis, iron metabolism, and anaerobic energy production (Wenger, 2002). Therefore, at the cellular level, a reduction in ATP production in the mitochondria as would occur in hypoxic conditions is compensated by anaerobic glycolysis where HIF-1α upregulates glucose transporters and gluconeogenic and glycolytic enzymes. Since HIF-1α is implicated in a variety of hypoxia-dependent processes, it serves as a good indicator of intracellular hypoxia.

Interestingly, several studies show that moderate exposure of cells and tissue to hypoxia increases ROS generated from the mitochondria (reviewed by Chandel and Budinger, 2007). Hypoxia is also involved in the pathobiology of hypoxic-ischemic stroke that begins with neurons experiencing hypoxic stress followed by the depletion of oxygen and glucose stores that would normally be supplied given adequate blood supply. This proceeds to initiate the 'ischemic cascade' where activation of phospholipase A2 and cyclooxygenase generate ROS that threaten cell survival and add pressure to endogenous scavenging mechanisms (Dirnagl *et al.,* 1999). In particular, damage to the mitochondria by ROS can further compromise its antioxidant defence systems and thus exacerbate the imbalance between the production and removal of ROS (Lin and Beal, 2006).

Furthermore, the upregulation of HIF-1α under times of hypoxic stress and the generation of ROS observed in hypoxic ischemic-stroke can be linked to BDNF, which is sensitive to changes in oxygen availability. Indeed, the HIF-1α system has a positive effect on BDNF where activation of the BDNF-TrkB system increased HIF-1α in neuroblastoma cells (Nakamura *et al.,* 2006). In addition, BDNF provides a promising solution to hypoxic injury due to its survival-promoting effects. In models of ischemic-hypoxic brain damage, increased BDNF levels have

been protective against apoptotic-like neuronal death and thought to underlie the resistance of certain neuronal populations to hypoxia (Walton *et al.,* 1999; Han and Holtzman, 2000). Several studies have also shown that intraventricular or intrastriatal injections of BDNF resulted in a neuroprotective effect in response to focal ischemia (Andsberg *et al.,* 2002; Zhang *et al.,* 2001).

In relation to ROS, one study suggests a role for BDNF in oxidative stress metabolism whereby BDNF rescued neurons from H_2O_2-induced cell death (Onyango *et al.,* 2005). In this study, BDNF was shown to utilize distinct signalling cascades to increase intracellular glutathione (GSH). Another study by Wang *et al.* (2006) found that exposure of brain endothelial cells to intermittent hypoxia (IH) stimulated BDNF secretion. In addition, exercise induces BDNF upregulation thereby suggesting its role in combating the products of enhanced aerobic and/or anaerobic metabolism such as increased ROS formation (Mattson and Magnus, 2006).

The survival promoting effects of BDNF under oxidative stress and hypoxic conditions make it a promising target to study to protect against neuronal death and dysfunction. Ultimately, if TCAP-1 treatment can regulate BDNF expression, then it may serve as a future therapeutic agent against neurodegenerative and cardiovascular diseases. However, due to the challenges of BDNF delivery and the relatively low levels of endogenous antioxidants in the brain (Coyle and Puttfarcken, 1993), alternative strategies to combat such adverse effects of hypoxia and ROS are needed. TCAP serves as a potential therapeutic agent as demonstrated by its ability to upregulate superoxide dismutase and catalase and protect cells from pH-induced alkalotic necrosis (Trubiani *et al.,* 2007).

1.12 A potential relationship between BDNF, TCAP and ROS

Reactive oxygen species (ROS) are normal products of aerobic metabolism that can be produced during aerobic respiration by the incomplete reduction of molecular oxygen in the electron transport chain. Consequently, organisms have developed antioxidant defence mechanisms to protect themselves against the accumulation of reactive oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) that threaten cellular integrity and survival.

Previously, TCAP-1 has been shown to protect immortalized embryonic mouse hypothalamic N38 cells subjected to pH-induced alkalotic stress by reducing the number of cells undergoing necrosis, inhibiting the decline in cell proliferation, and upregulating the superoxide dismutase-catalase pathway (Trubiani *et al.,* 2007). *In vitro,* preliminary studies have indicated that TCAP-1 increases BDNF mRNA and protein expression in immortalized mouse hypothalamic and hippocampal cells subjected stress. However, the mechanism by which this occurs is unknown (Trubiani*,* 2008). Thus, I hypothesize that TCAP may confer neuroprotection by mediating BDNF activity that then proceeds to activate protective cellular signalling cascades during times of brain insult or stressful stimuli (Figure 1.3).

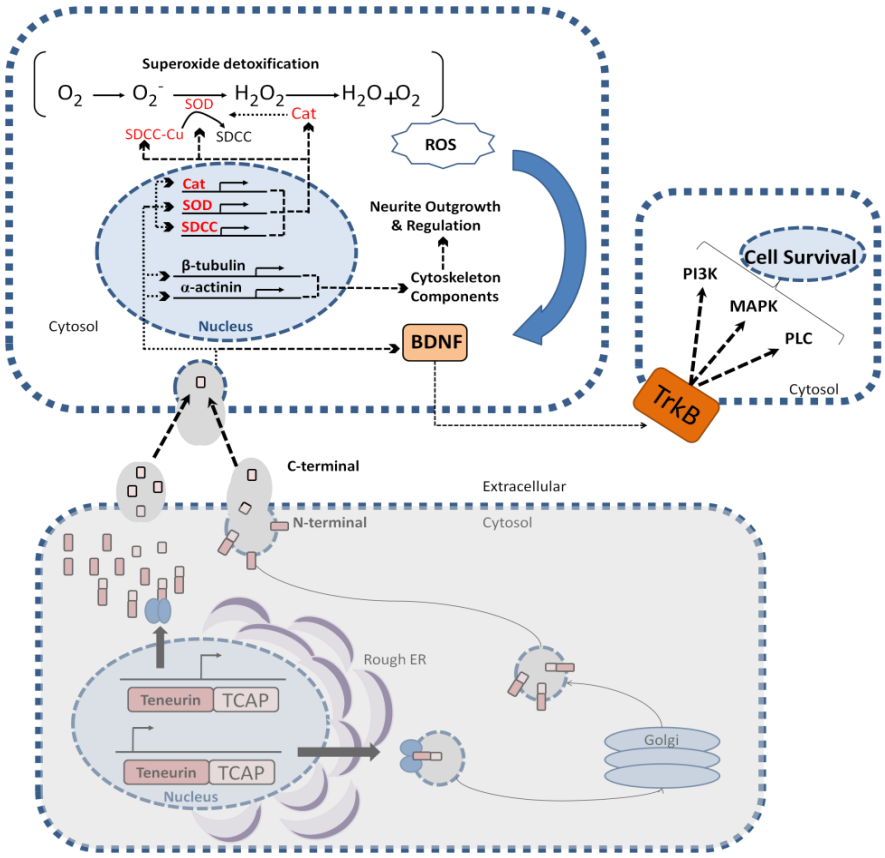

Figure 1.3: Potential mechanisms by which TCAP confers neuroprotection.

TCAP has been shown to upregulate antioxidant defense systems to counteract ROS following stress. TCAP may promote cell survival by directly upregulating BDNF release, allowing for the activation of the TrkB receptor and downstream signaling pathways (MAPK, PI3K, and PLC). I hypothesize that an interaction between TCAP, ROS and BDNF may exist, however the mechanism for this relationship is unknown.

1.13 Thesis rationale and experimental design

Previous findings in our laboratory prompted a new project that would further develop and investigate the potential neuroprotective effects of TCAP-1. Thus the objectives of my thesis are as follows:

1. Investigate the neuroprotective effect of acute and chronic TCAP-1 on immortalized embryonic mouse N38 cells under hypoxic stress (1% O_2). To do so, I will perform the following set of experiments:
   A. Western blot analysis indicating HIF-1α upregulation, which is characteristic of hypoxic conditions.
   B. Investigating cell growth parameters (number of live cell nuclei, number of neurites) of N38 cells following acute and chronic TCAP-1 treatment under 1% O_2 levels.
   C. Western blot analysis to determine effect of acute and chronic TCAP-1 treatment on BDNF translation under 1% hypoxic stress.

2. Establish immortalized embryonic mouse N38 cells as a suitable model for studying BDNF, TCAP and neuroprotection. To do so, I will show or perform the following set of experiments:
   A. Immunocytochemistry studies of FITC-[K8]-TCAP-1 binding and uptake with BDNF localization in N38 cells.
   B. Immunocytochemistry studies of binding and uptake of FITC-[K8]-TCAP-1 with BDNF localization in N38 cells and primary rat embryonic hippocampal cultures.

C. Western blot analysis to confirm the inducibility of N38 cells by forskolin in altering BDNF protein expression.

3. Investigate the effect of acute TCAP-1 on immortalized embryonic mouse N38 cells on BDNF gene and protein expression under basal conditions. To do so, I will perform the following set of experiments:
   A. N38 cell culture treatment with either acute treatment of TCAP-1 or vehicle over 0-8 hours under basal conditions.
   B. Western blot analysis to determine effect of acute TCAP-1 treatment on BDNF translation under basal conditions.
   C. Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis to determine effect of acute TCAP-1 treatment on BDNF transcription under basal conditions

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Chapter 2 : Actions of Teneurin C-terminal Associated Peptide (TCAP)-1 on cell growth as a function of varying oxygen partial pressures.

This chapter, along with additional material will be submitted for publication:

Tiffany Ng, Reuben De Almeida, Monica Pulec and David A. Lovejoy. Actions of Teneurin C-terminal Associated Peptide (TCAP)-1 on cell growth under stressed and non-stressed conditions. (For submission to Neuroscience)

2.1 Introduction

During embryogenesis, the brain emerges from the neural tube and undergoes rapid neuronal proliferation under low oxygen levels. Developing embryos require this hypoxic microenvironment for tissue morphogenesis and neuronal proliferation and differentiation (Morriss and New, 1979; Morrison *et al.,* 2000; Studer *et al.,* 2000). For most mammalian cells, physiological normoxic conditions refer to an oxygen concentration ranging from 2% to 9% (14.4-68.8 mmHg) in contrast to the normally cited 21% ambient oxygen concentration (Simon and Keith, 2008). Indeed the partial pressure of oxygen in various brain regions has been measured by microelectrodes and reported to range from 0.55% to 8%, a considerably lower level compared to ambient oxygen (Erecińska and Silver, 2001). In addition, the 'oxygen cascade' may contribute to the low oxygen environment at the level of the cell. Oxygen is transported in steps from the environment to the cell where along the way it is extracted at the lungs, circulation, tissue and finally by the cellular mitochondria (Treacher and Leach, 1998).

Currently, the mechanism and modifications allowing neural cells to function under very low oxygen levels are not known. However, the teneurin family of proteins may play a role. These proteins are ubiquitously expressed in all metazoans and appeared around the time when

the first metazoan nervous system was evolving (Lovejoy *et al.,* 2006). The teneurins are a family of four transmembrane proteins that are highly expressed in the central nervous system where they are involved in embryonic development of the brain and regulation of neuronal activity in adults (reviewed in Tucker *et al.,* 2007). In particular, the carboxy terminus contains a synthetic 41-mer peptide based on the expected cleavage product of the terminal 43 residues of the extracellular domain of the teneurin-1 protein that we term teneurin C-terminal associated peptide (TCAP)-1 (Lovejoy *et al.,* 2006). Previous studies have found TCAP-1 to be neuroprotective against pH-induced necrotic cell death via its upregulation of ROS scavenging systems and promotion of cell proliferation (Trubiani *et al.,* 2007).

Presently, no studies have been conducted on TCAP-1's role in oxygen metabolism and its effect on neuronal growth under varying oxygen tensions. Therefore, this study aims to examine the effect of TCAP-1 on growth parameters under several oxygen concentrations (21%), (4%) and (1%) in immortalized hypothalamic N38 cells.

2.2 Materials and Methods

2.2.1 Preparation of cell culture

All N38 immortalized hypothalamic embryonic mouse cells were grown in Dulbecco's Modified Eagle Medium containing 4 500 mg/L D-glucose, L-glutamine, and 25 mM HEPES buffer, but no sodium pyruvate and no phenol red (Gibco, Cat. No. 21063029) supplemented with 10% fetal bovine serum (Invitrogen, Cat. No. 16000-036) and including penicillin and streptomycin antibiotics. Immortalized mouse hypothalamic N38 cells were grown in 6-well

tissue culture treated plates (Corning Incorporated, Cat. No. 3516) at a density of 100 000 cells/9 cm^2 wells for 24 hours before treatment with either 10^{-8} M TCAP-1 or vehicle (NH_4^+ in ddH_2O).

2.2.2 Hypoxia treatment

Two Thermo Forma Series II model 3130 incubators (Thermo Fischer Scientific, cat no. 3130) were dedicated for the hypoxia study and set either at 1%, 4% or 21%. Prior to hypoxic exposure, the incubator was flushed for 1 hour at 37°C with 5% carbon dioxide and 95% nitrogen to reduce oxygen levels to 1% or 4%. During equilibration of the hypoxic incubator, the normoxic incubator was allowed 1 hour to equilibrate to 21% oxygen. Oxygen sensors provided real-time digital readouts of oxygen levels. Normoxia and hypoxia levels were checked using a Bacharach Fyrite® Gas Analyzers provided with the incubators. Western blot analysis on hypoxia-inducible factor 1α (HIF-1α) over a time course further confirmed hypoxia.

2.2.3 Acute TCAP hypoxic study

Cells were seeded into 6-well plates as described above and incubated at either 21%, 1% or 4% oxygen for 0 to 24 hours. Acute TCAP-1 (10^{-8} M) treatment began at the onset of hypoxic exposure (0 hours). A Zeiss AxioObserver Z1 inverted microscope (Germany) programmed to automatically take five different images per well for a total of six wells acquired 30 images per treatment group at the respective time point (Figure 2.1). Proliferation of immortalized N38 cells has been considerably studied by our laboratory. Based on our observations, healthy N38 cells spread out and remain attached to the bottom of the well, whereas dying cells appear rounded and lift off from the plate. Thus, quantification studies to determine the proliferation and cell

growth parameters were conducted. The number of live cell nuclei were counted and the mean determined for each of the images. The mean from each image was then averaged to find the mean live cell nuclei per well. Ultimately, a final mean of the entire treatment group (6 wells, n=6) was determined ± SEM. As another growth parameter studied, neurite numbers per cell were counted. Neurites were defined as distinct protrusions from the cell and only non-impinging neurites were included in the scoring. The number of neurites per cell were scored per image to produce a mean for the well and later the final mean for the treatment group. All final means were normalized to baseline (0 hours) to provide % relative changes where baseline represented 100%. Statistical analysis was done using an unpaired two-tailed t-test (*$p<0.05$, **$p<0.001$, ***$p<0.0001$; n=6). In addition, protein extractions were collected at the beginning of the experiment (0 hours) and at the end (24 hours) for Western blot analysis.

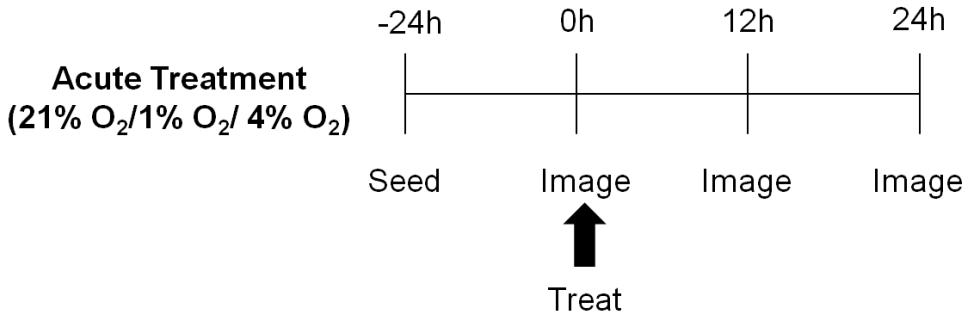

Figure 2.1: Acute TCAP treatment regime over 24 hours at ambient oxygen (21% O2) and low oxygen (1% O2) conditions.

Immortalized N38 cells were cultured under 21% or 1% O_2 and treated with either TCAP-1 or vehicle at baseline (0 hours). Five different images per well were automatically obtained by a Zeiss AxioObserver Z1 inverted microscope (Germany) giving a total of thirty images per 6-well plate treatment group at a specific time point.

2.2.4 Chronic TCAP hypoxia study

Cells were seeded into 6-well plates as described above and incubated at either 21% or 1% oxygen for 0 to 36 hours. TCAP-1 (10^{-8} M) treatment began at the 12 hours prior to the onset of hypoxic exposure (0 hours) and was given at each time point (0, 12, 24, 36 hours). A Zeiss AxioObserver Z1 inverted microscope (Germany) programmed to automatically take five different images per well for a total of six wells acquired a 30 images per treatment group at the respective time point (Figure 2.2). As in the acute TCAP study, quantification studies to determine the proliferation and cell growth parameters were conducted. The number of live cell nuclei were counted and the mean determined for each of the images. The mean from each image was then averaged to find the mean live cell nuclei per well. Ultimately, a final mean of the entire treatment group (6 wells, n=6) was determined ± SEM. As another growth parameter studied, neurite numbers per cell were counted. Neurites were defined as distinct protrusions from the cell and only non-impinging neurites were included in the scoring. The number of neurites per cell were scored per image to produce a mean for the well and later the final mean for the treatment group. All final means were normalized to baseline (0 hours) to provide % relative changes where baseline represented 100%. Statistical analysis was done using an unpaired two-tailed t-test (*$p<0.05$, **$p<0.001$, ***$p<0.0001$; n=6). In addition, protein extractions were collected at the beginning of the experiment (0 hours) and at the end (36 hours) for Western blot analysis.

Figure 2.2: Chronic TCAP treatment regime over 36 hours at ambient oxygen (21% O_2) and low oxygen (1% O_2) conditions.

Immortalized N38 cells were cultured under 21% or 1% O_2 and treated with either TCAP-1 or vehicle 12 hours before the start of the experiment, at baseline (0 hours), and every 12 hours after until termination at 36 hours. Five different images per well were automatically obtained by a Zeiss AxioObserver Z1 inverted microscope (Germany) giving a total of thirty images per 6-well plate treatment group at a specific time point.

2.2.5 Western blot analysis of BDNF and HIF-1 α

All immortalized mouse embryonic hypothalamic N38 cells were washed three times with sterile phosphate buffer saline (PBS) at pH 7.4. Cells were then scraped in 200 µl of cold 1% lysis buffer [1% Triton X-100, 1M Dithiothreitol (DTT), Protease Inhibitor Cocktail Set III (Calbiochem, Cat. No. 539134)]. The lysate was vortexed and incubated on ice for 5 to 10 minutes after which it was centrifuged at 14 000 rpm for 15 minutes at 4°C.The supernatant was aliquoted into fresh 1.5ml tubes and stored in -20°C for western blot analysis.

Protein extracts were quantified in accordance with the manufacturer's protocol (Thermo Fischer Scientific, Cat. No. 23225). In brief, 5 µl of each protein sample was aliquoted into 96-well plates in triplicates. 200 µl of bicinchoninic acid (BCA) Working Reagent was added to each sample. Diluted albumin standards were prepared in triplicates providing the assay with a working range from 20 to 2000 µg/ml. The 96-well plate was covered and incubated with shaking at 37°C for 30 minutes. An absorbance reading was subsequently obtained at 562 nm using Spectramax Plus 384 (Molecular Devices, cat no. 340PC 384).

Protein samples (12.5 μg) were then run on 4-20% Mini-Protean TGX gels (cat no. 456-1094) at 100V for approximately 1 hour. Proteins were subsequently electro-transferred to a Hybond-ECL-nitrocellulose membrane (VWR, Cat. No. RPN203D) for 2 hours at 100V. The membrane was washed with phosphate saline buffer (PBS) with shaking for 5 minutes and incubated with BDNF rabbit polyclonal primary antibody (Santa Cruz, cat no. sc-20981) diluted 1:500 or HIF-1α rabbit polyclonal antibody (Abcam, cat no. ab2185) diluted 1:250 in 7 ml of milk-PBST (PBS with 0.2% Tween®20) with gentle agitation overnight at 4°C. After 24 hours, the membrane was washed with fresh PBST three times with shaking for 5 minutes, 10 minutes, and 10 minutes, respectively. Following, the membrane was incubated with anti-rabbit horseradish peroxidise (HRP)-conjugated secondary antibody (VWR, Cat. No. RPN2135 or Amersham, cat no. NIF824) diluted 1:5000 in milk-PBST for 1 hour at room temperature with gentle agitation. After incubation, the membrane was washed three times with fresh PBST for 5 minutes, 10 minutes and 10 minutes, respectively, and exposed onto ECL Hyperfilm (VWR, Cat. No. 95017-653L) for 20 minutes. The integrated optical density (IOD) of the blots were quantified using LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.). GAPDH (Abcam, ab9485; 1:2500) was used as an additional loading control and remained unchanged for all protein samples.

2.3 Results

2.3.1 Hypoxia induced expression of HIF-1α

Various methods were used to ensure that the cell culture were hyposic. First, real-time digital readouts were checked constantly for stable readings throughout the experiment. Secondly, a Bacharach Fyrite® Gas Analyzer kit was used to check oxygen levels in the middle

of the experiment to ensure accurate O_2 levels within the incubator. As a further confirmation study for hypoxic exposure, N38 cells were incubated at either ambient (21% O_2) or hypoxic (1% O_2) conditions and cell lysates were harvested. Western blot analysis detected HIF-1α protein expression at 1% O_2 over a time period of 36 hours compared to 21% O_2 (Figure 2.3). HIF-1α protein expression increases over time and peaks at 5 hours following 24 hours of chronic hypoxic exposure onset. This is consistent with the range of 4-8 hours of hypoxia found in other studies (Wang *et al.,* 1995; Jiang *et al.,* 1996; Pascual *et al.,* 2001). Thus, these three methods confirm that the N38 cells were hypoxic under the conditions used.

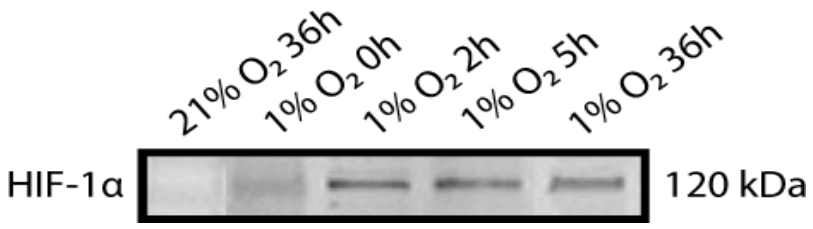

Figure 2.3: HIF-1α protein expression indicates hypoxic conditions in untreated immortalized N38 hypothalamic cells over time.

Whole cell lysates of immortalized N38 cells cultured in 21% at 36 hours and 1% O_2 for 0 to 36 hours were immunoblotted for HIF-1α protein expression (120 kDa).

2.3.2 The effect of acute TCAP-1 treatment (10^{-8}M) on cell proliferation under ambient oxygen levels (21% O_2) and reduced and low oxygen (4% O_2 and 1% O_2)

To study the effects of acute TCAP-1 treatment on an immortalized mouse hypothalamic cell line, N38 cells were cultured under varying levels of oxygen (21%, 4% and 1% O_2). Cell

proliferation was examined by counting live cell nuclei and normalized as a relative % to baseline (0 hours). Initially, the experiment was conducted in 4% O_2 over a 24-hour time period (Figure 2.4). I observed a significant increase in cell numbers in the acutely administered TCAP-1 group compared to vehicle-treated cells relative to baseline (0 hours) at 12 hours (160.5 ± 3.0% vs. 128.2 ± 3.3%; $p<0.001$) and 24 hours (385.9 ± 5.6% vs. 181.4 ± 3.6%; $p<0.001$) in 21% O_2 (Figure 2.5A). Similarly, acute TCAP-1 compared to vehicle-treated cells resulted in elevated cell numbers relative to baseline (0 hours) at 12 hours (173.1 ± 7.0% vs. 155.4 ± 3.0%; $p<0.05$) in 4% O_2 (Figure 2.5B).

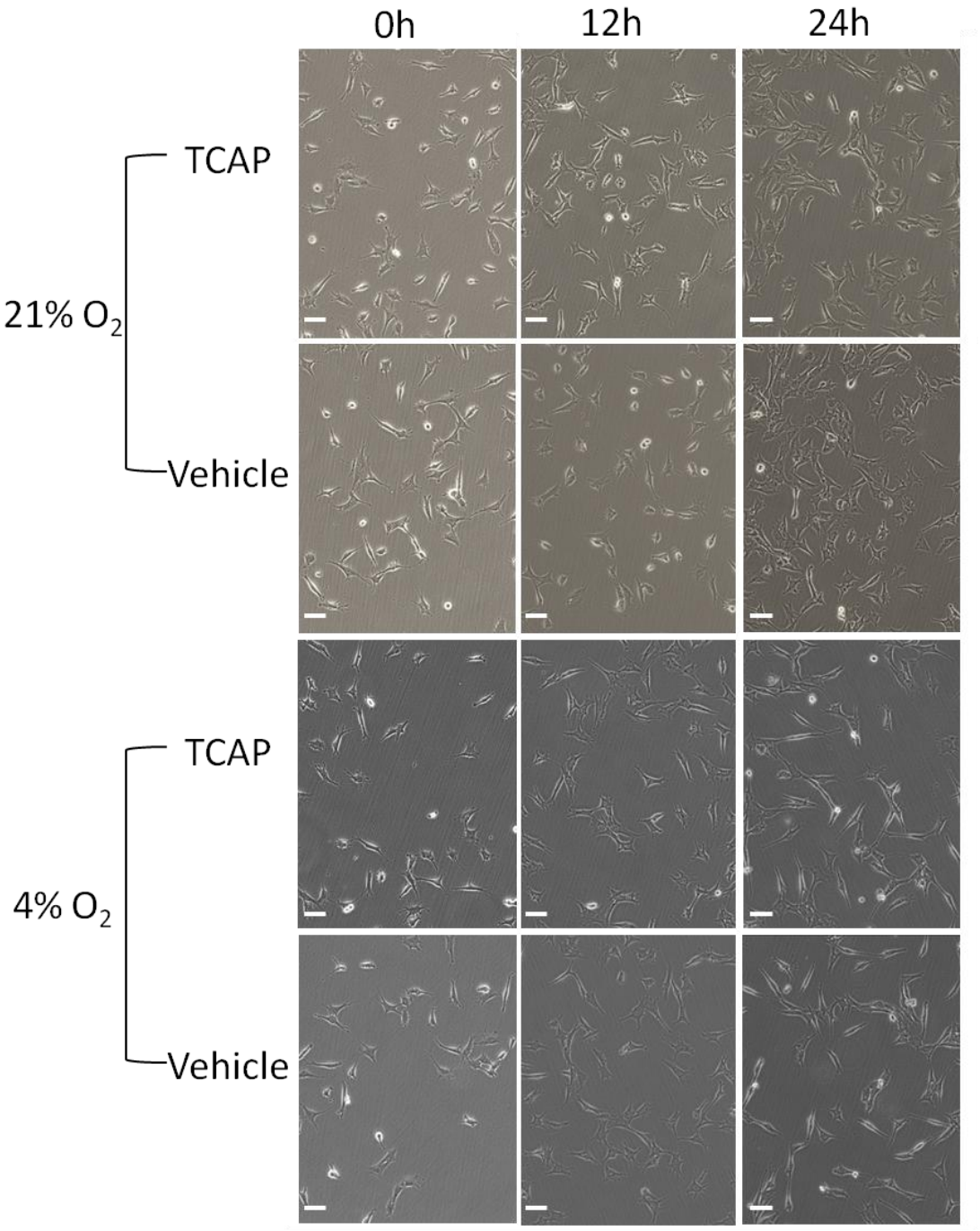

Figure 2.4: Representative images of immortalized N38 hypothalamic cells treated with acute TCAP or vehicle at ambient oxygen (21% O_2) or reduced oxygen (4% O_2) levels over an incubation duration of 24 hours.

Thirty **i**mages per 6-well cell culture plate were taken automatically using a Zeiss AxioObserver Z1 inverted microscope (Germany). Scale bar = 63.00 µm

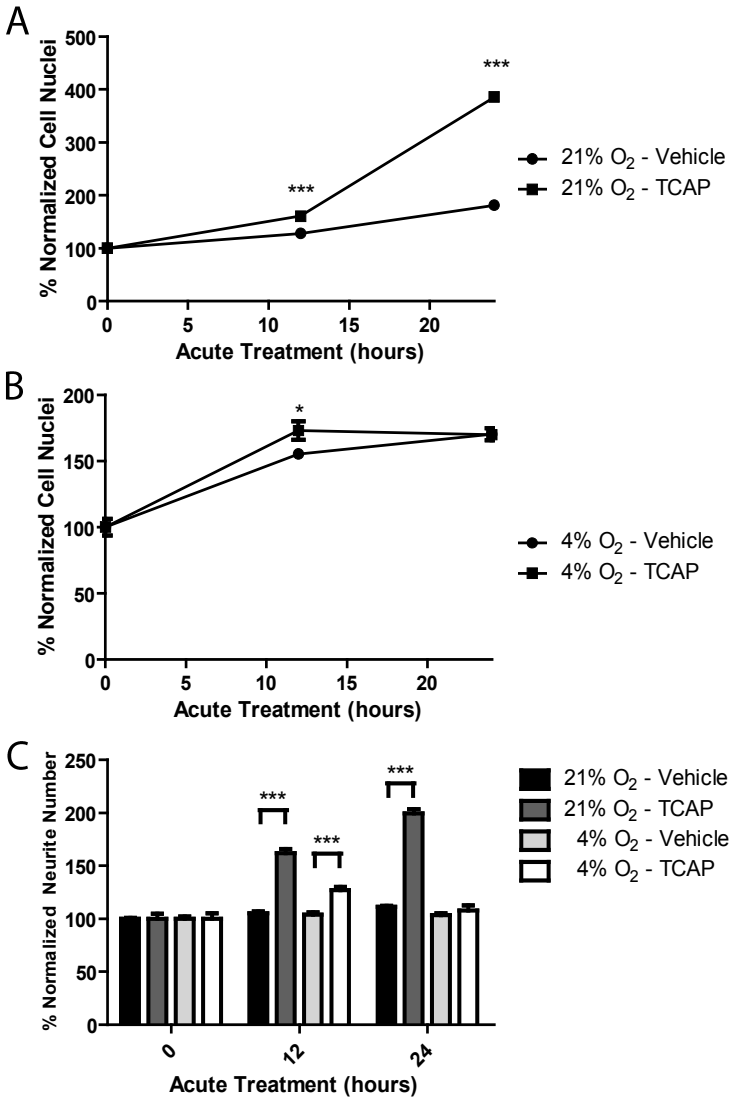

Figure 2.5: Changes in immortalized N38 hypothalamic growth parameters following treatment with either acutely administered 10^{-8} M TCAP or vehicle at ambient oxygen (21% O_2) or reduced oxygen (4% O_2) levels over an incubation period of 24 hours.

(A) Proliferation as measured by live cell nuclei normalized to 0h following acute treatment with 10^{-8} M TCAP or vehicle at 21% O_2. (B) Proliferation measured by live cell nuclei normalized to 0 hours following acute treatment with 10^{-8} M TCAP or vehicle at 4% O_2. (C) Neurite number per cell normalized to 0 hours following acute treatment with 10^{-8} M TCAP or vehicle at 21% or 4% O_2. Statistical analysis was conducted using two-tailed unpaired t-tests comparing TCAP and vehicle treated groups at either ambient oxygen or reduced oxygen conditions (n=6, *P<0.05, **p<0.01, *** p<0.001)

When 4% O_2 was insufficient to cause reduced cell proliferation relative to baseline, the experiment was completed over a 0 to 8 hour time course under a more reduced oxygen condition (1% O_2) (Figure 2.6). Acute TCAP-1 compared to vehicle-treated cells resulted in a marked increase in cell proliferation when normalized to baseline (0 hours) after 4 hours (145.7 ± 4.9% vs. 86.7 ± 4.0%; p<0.001) and 8 hours (140.1 ± 3.8% vs. 84.8 ± 14.2%; p<0.01) under 21% O_2 conditions (Figure 2.7A). In the lowest oxygen condition studied (1% O_2), acute TCAP-1 compared to vehicle-treated cells significantly increased cell numbers when normalized to

baseline (0 hours) after 4 hours (183.4 ± 5.5% vs. 100.9 ± 4.0%; p<0.001) and 8 hours (204.6 ± 9.1% vs. 93.6 ± 6.0%; p<0.001) (Figure 2.7B).

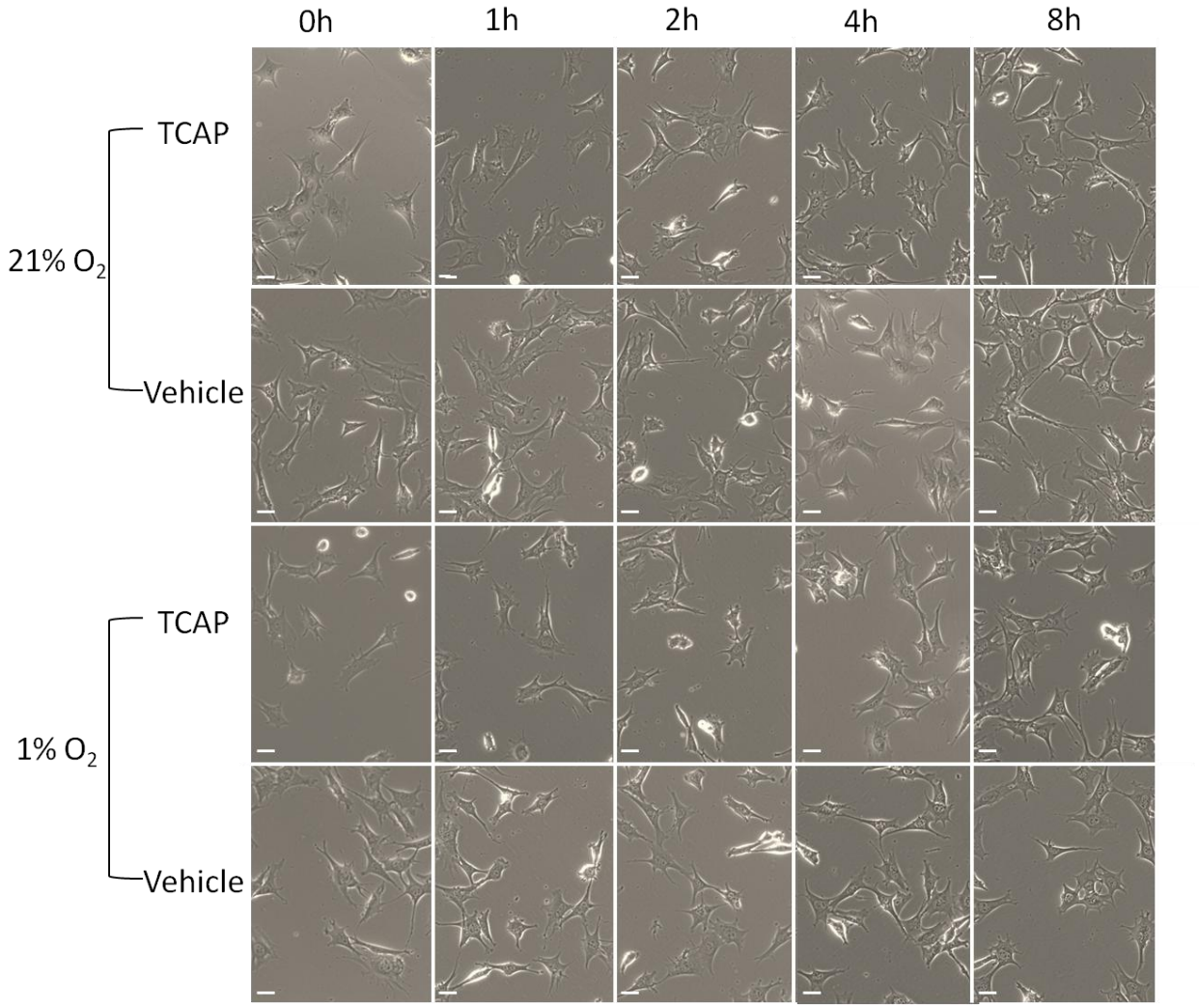

Figure 2.6: Representative images of immortalized N38 hypothalamic cells treated with acutely administered TCAP or vehicle at ambient oxygen (21% O_2) or low oxygen (1% O_2) levels over an incubation duration of 8 hours.

Thirty images per 6-well cell culture plate were taken automatically using a Zeiss AxioObserver Z1 inverted microscope (Germany). Scale bar = 32.00 µm.

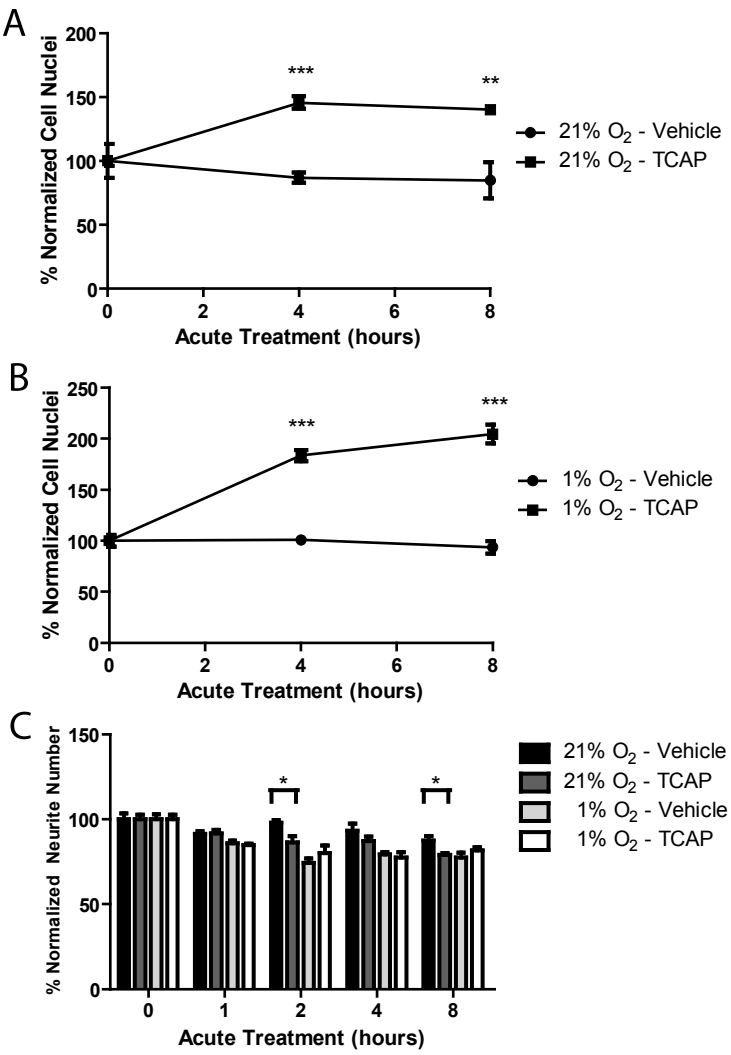

Figure 2.7: Changes in immortalized N38 hypothalamic growth parameters following treatment with either acutely administered 10^{-8} M TCAP or vehicle at ambient oxygen (21% O_2) or low oxygen (1% O_2) levels over a duration of 8 hours.

(A) Proliferation as measured by live cell nuclei normalized to 0h following acute treatment with 10^{-8} M TCAP or vehicle at 21% O_2. (B) Proliferation measured by number of live cell nuclei following acute treatment with 10^{-8} M TCAP or vehicle at 1% O_2. (C) Neurite number per cell normalized to 0 hours following acute treatment with 10^{-8} M TCAP or vehicle at 21% or 1% O_2 levels. Statistical analysis was conducted using two-tailed unpaired t-tests comparing TCAP and vehicle treated groups in either ambient oxygen or low oxygen (n=6, *P<0.05, **p<0.01, *** p<0.001).

Since cell death was not achieved after 8 hours, the experiment was repeated again with an extended incubation period of 24 hours for both 21% and 1% O_2 to examine whether TCAP-1 was able to have a neuroprotective effect (Figure 2.8). In this experiment, acute TCAP-1 compared to vehicle-treated cells increased cell proliferation significantly relative to baseline (0 hours) at 12 hours (164.7 ± 2.1% vs. 149.5 ± 5.0%; $p<0.05$) and 24 hours (427.3 ± 4.8% vs. 231.4 ± 3.8%; $p<0.001$) in 21% O_2 (Figure 2.9A). Cells that were cultured in 1% O_2 and received acute TCAP-1, resulted in greater cell proliferation relative to baseline (0 hours) and compared to vehicle-treatment only at 24 hours (203.3 ± 2.6% vs. 75.0 ± 7.3%; $p<0.001$) (Figure 2.9C).

Therefore, acute TCAP-1 treatment had a stimulatory effect on cell proliferation at both high and low oxygen levels.

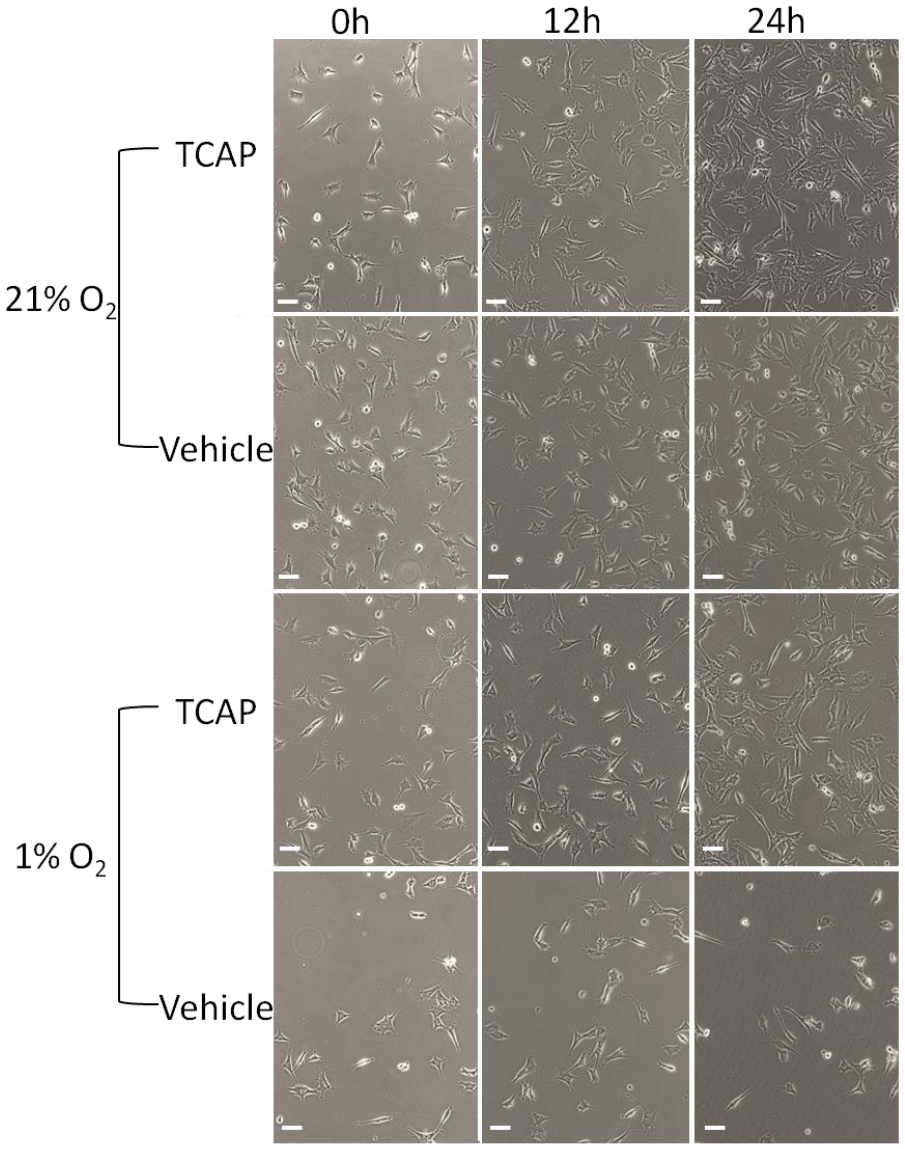

Figure 2.8: Representative images of immortalized N38 hypothalamic cells treated with acutely administered TCAP or vehicle at ambient oxygen (21% O_2) or low oxygen (1% O_2) conditions over an incubation period of 24 hours.

Thirty **i**mages per 6-well cell culture plate were taken automatically using a Zeiss AxioObserver Z1 inverted microscope (Germany). Scale bar = 63.00 µm

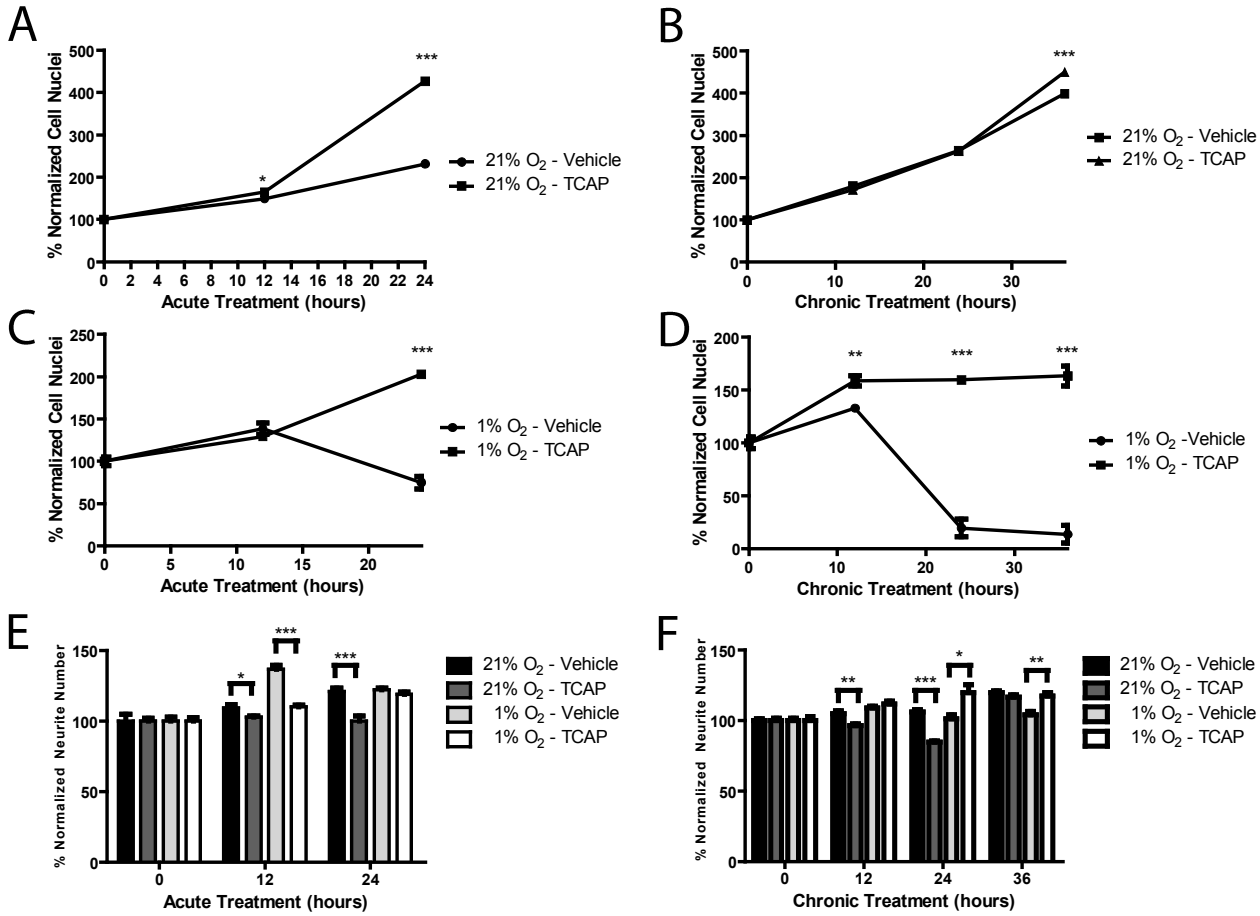

Figure 2.9: Changes in immortalized N38 hypothalamic growth parameters following acute or chronic treatment of 10^{-8} M TCAP or vehicle in ambient oxygen (21% O_2) or low oxygen (1% O_2) over a time course.

Proliferation as measured by live cell nuclei normalized to 0 hours following acute (A) or chronic (B) treatment with 10^{-8} M TCAP or vehicle in normoxia. Proliferation measured by live cell nuclei normalized to 0 hours following acute (C) or chronic (D) treatment with 10^{-8} M TCAP or vehicle in low oxygen conditions. Neurite number per cell normalized to 0 hours following acute (E) or chronic (F) treatment with 10^{-8} M TCAP or vehicle at 21% and 1% O_2. Statistical analysis was conducted using two-tailed unpaired t-tests comparing TCAP and vehicle treated groups in either ambient or low oxygen levels (n=6, *P<0.05, **p<0.01, *** p<0.001).

2.3.3 The effect of chronic TCAP-1 treatment (10^{-8} M) on cell proliferation under ambient oxygen (21% O_2) and low oxygen conditions (1% O_2)

To investigate whether changing the treatment regime and extending the duration of chronic oxygen would alter TCAP-1's enhanced effect on cell proliferation, cells were cultured

similarly to the acute experiment but with either TCAP-1 or vehicle treatment given 12 hours before baseline (0 hours), at baseline and every 12 hours after up to 36 hours under 21% or 1% O_2 (Figure 2.10). In this experiment, chronically administered TCAP-1 compared to vehicle-treated cells significantly increased proliferation relative to baseline (0 hours) at 36 hours (449.8 ± 4.1% vs. 398.4 ± 2.0%; $p<0.001$) in 21% O_2 (Figure 2.9B). In contrast, chronic TCAP-1 treatment prevented the steep decline in cell numbers that occurred in vehicle-treated cells and showed a significant increase in cell numbers relative to baseline (0 hours) at 12 hours (158.8 ± 5.0% vs. 132.8 ± 3.8%; $p<0.01$), 24 hours (159.6 ± 2.8% vs. 19.6 ± 8.2%; $p<0.001$) and 36 hours (163.3 ± 9.2% vs. 13.5 ± 8.5%; $p<0.001$) under low oxygen (1% O_2) (Figure 2.9D). Therefore, in 1% O_2, chronic TCAP-1 exerted a neuroprotective effect on N38 cells and promoted their survival and proliferation compared to vehicle-treatment alone.

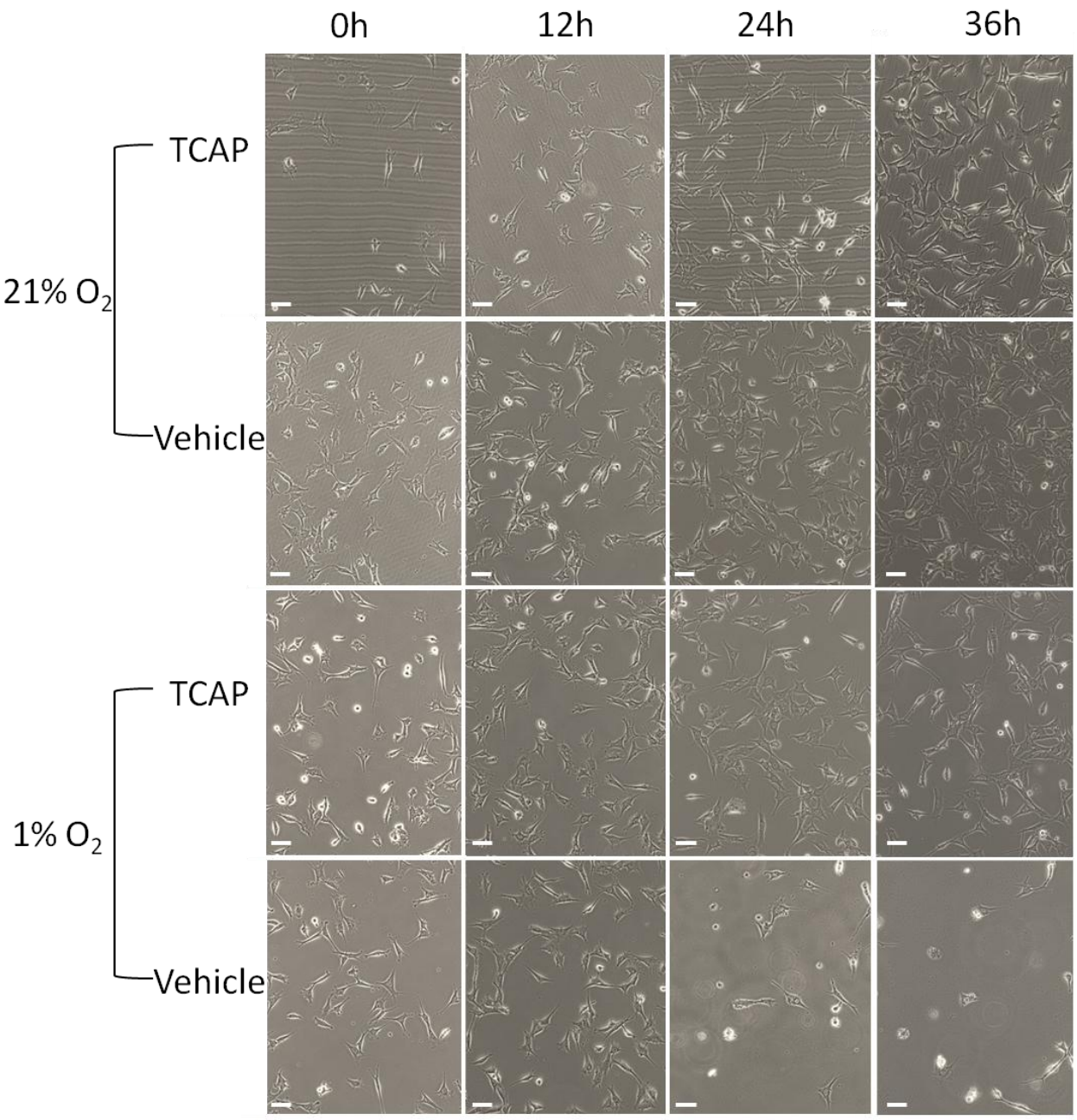

Figure 2.10: Representative images of immortalized N38 hypothalamic cells treated with chronically administered TCAP (10^{-8} M) or vehicle at ambient oxygen (21% O_2) or low oxygen (1% O_2) levels over an incubation period of 36 hours.

Thirty images per 6-well cell culture plate were taken automatically using a Zeiss AxioObserver Z1 inverted microscope (Germany). Scale bar = 63.00 μm

Overall, TCAP-1 has a proliferative effect on cell growth and appears to consistently elevate live cell numbers in all levels of oxygen studied (21% O_2 vs. 4% O_2 vs. 1% O_2). In addition, the treatment regime (acute vs. chronic) did not influence TCAP's promotion of proliferation (see Table 2.1).

Table 2.1: Summary results of acute and chronic experiments

Experiment	Oxygen	Live Cell Nuclei	Neurite Number
Acute *(0-8h)*			
TCAP vs. Vehicle	21%	↑ (***4h, **8h)	↓ (*2h, *8h)
	1%	↑ (***4h, ***8h)	ns
Acute *(0-24h)*			
TCAP vs. Vehicle	21%	↑ (*12h, ***24h)	↓ (*12h, ***24h)
	1%	↑ (***24h)	↓ (***12h)
Acute *(0-24h)*			
TCAP vs. Vehicle	21%	↑ (***12h, ***24h)	↑ (***12h, ***24h)
	4%	↑ (*12h)	↑ (***12h)
Chronic *(0-36h)*			
TCAP vs. Vehicle	21%	↑ (***36h)	↓ (**12h, ***24h)
	1%	↑ (**12h, ***24h, ***36h)	↑ (*24h, **36h)

*p<0.05, **p<0.01, ***p<0.001, ns (non- significant)

2.3.4 The effect of acute TCAP-1 treatment (10^{-8} M) on neurite growth parameters under ambient oxygen (21% O_2) and reduced and low oxygen levels (4% O_2 and 1% O_2)

Since TCAP-1 displayed consistent proliferative effects on cells subjected to varying oxygen levels from 21% to 1% O_2, the effects of TCAP-1 were explored on another cell growth parameter. Neurite numbers per cell were counted and normalized to baseline (0 hours) as a relative %. Initially, cells incubated in 21% O_2 and with acutely administered TCAP-1 treatment displayed a significant increase in neurite numbers compared to vehicle-treated cells relative to baseline (0 hours) at 12 hours (162.3 ± 3.9% vs. 105.6 ± 1.3%; $p<0.001$) and 24 hours (199.5 ± 4.0% vs. 111.5 ± 1.0%; $p<0.001$) (Figure 2.5C). Similarly, acute TCAP-1 compared to vehicle-treated cells showed elevated neurite numbers at 12 hours (126.9 ± 3.5% vs. 103.9 ± 2.5%; $p<0.001$) in 4% O_2 (Figure 2.5C).

In the second experiment over an 8-hour period (Figure 2.6), 21% O_2 conditions with acutely administered TCAP-1 treatment had differing results from initial observations. Instead, a significant reduction relative to baseline (0 hours) in neurite numbers at 2 hours (86.4 ± 3.7% vs. 98.1 ± 1.5%; $p<0.05$) and 8 hours (79.0 ± 1.3% vs. 87.6 ± 2.6%; $p<0.05$) compared to vehicle-treated cells was observed (Figure 7C). At 1% O_2 no significant difference in neurite numbers by TCAP-1 compared to cells that received only vehicle were found (Figure 2.7C).

When the time period was extended to 24 hours, similar results were observed as the 8 hour experiment with acutely administered TCAP-1 (Figure 2.8). Acute treatment of TCAP-1 significantly lowered neurite numbers relative to baseline (0 hours) compared to vehicle-treated cells at 12 hours (102.6 ± 1.0% vs. 109.3 ± 2.3%; $p<0.05$) and 24 hours (99.9 ± 3.7% vs. 120.8 ± 2.3%; $p<0.001$) in 21% O_2 (Figure 2.9E). Again, consistent results with the 8-hour experiment were observed when cells were incubated in 1% O_2. Acutely treated cells with TCAP-1 had a marked reduction in neurite numbers relative to baseline (0 hours) at 12 hours (110.0 ± 1.1% vs.

136.7 ± 2.9%; $p<0.001$) compared to the vehicle-treated group (Figure 2.9E). Based on these experiments, TCAP-1 treatment had differential effects on neurite growth depending on the level of oxygen and conflicting observations at 21% O_2 between initial experiment and subsequent experiments were found.

2.3.5 The effect of chronic TCAP-1 treatment (10^{-8} M) on neurite growth parameters under ambient oxygen levels (21% O_2) and low oxygen conditions (1% O_2)

In the chronic regime (Figure 2.2), chronic TCAP-1 mediated neurite growth differently from the acute regime (Figure 2.1). We found that cells treated with chronic TCAP-1 compared to vehicle in ambient oxygen conditions (21% O_2) showed reduced neurites relative to baseline (0 hours) at 12 hours (96.5 ± 1.2% vs. 105.0 ± 1.9%; $p<0.01$) and 24 hours (84.5 ± 1.1% vs. 106.3 ± 1.3%; $p<0.001$) (Figure 2.9F). In contrast to the findings in the acute regime, under low oxygen levels (1% O_2) chronic TCAP-1 treatment caused N38 cells to increase neurites number compared to vehicle relative to baseline (0 hours) at 24 hours (119.9 ± 1.1% ± 5.7% vs. 101.4 ± 1.1% ± 2.7%; $p<0.05$) and 36 hours (117.3 ± 2.6% vs. 104.1 ± 2.4%; $p<0.01$) (Figure 2.9F).

2.3.6 The effect of acute TCAP-1 treatment (10-8 M) on HIF-1α and BDNF expression under ambient oxygen levels (21% O_2) and reduced and low oxygen (4% O_2 and 1% O_2) conditions

To investigate the mechanism by which TCAP-1 imparts its neuroprotective effect, we looked at the expression of brain-derived neurotrophic factor, which has been associated with neurite outgrowth and proliferation (Bailey, 1996; Cunha *et al.,* 2010). Protein lysate from N38 cells collected at the beginning of the acute experiment (0 hours) and at the very end were

immunoblotted for BDNF, HIF1 and GAPDH (Figure 2.11). Preliminary blots showed that acute TCAP-1 treatment showed no observable change in brain-derived neurotrophic factor (BDNF) compared to vehicle, however there appeared to be a decrease in HIF-1α expression after 24 hours at 1% O_2. As expected at ambient oxygen (21% O_2), there was minimal if any HIF-1α expression detected and prominent expression at lowered O_2 levels. Interestingly, at ambient oxygen levels (21% O_2) BDNF appears to be decreased in TCAP-1 compared to vehicle-treated cells.

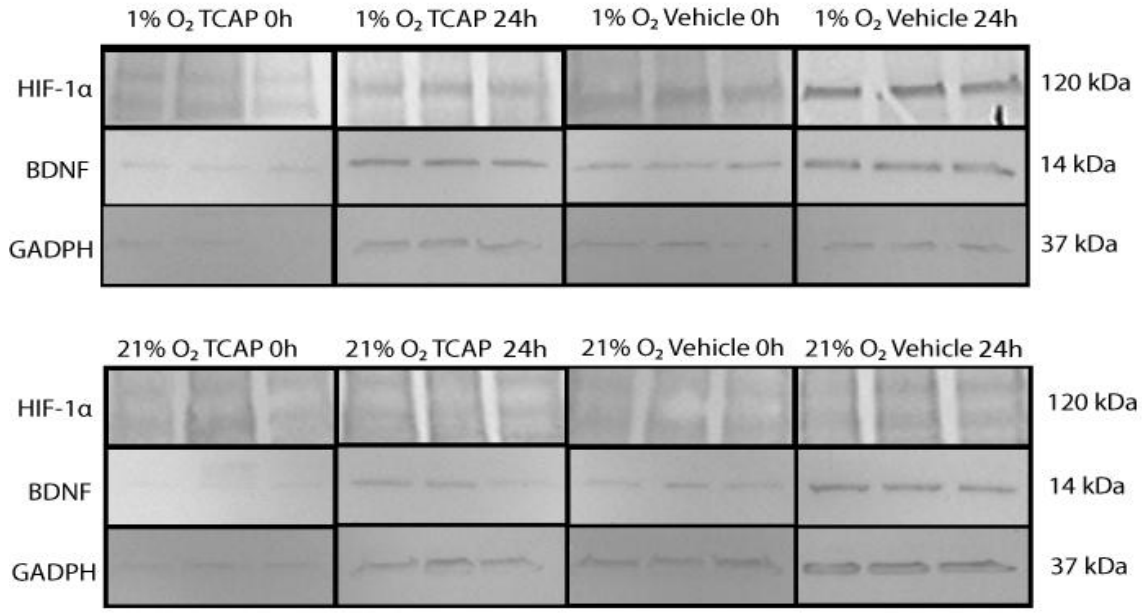

Figure 2.11: HIF-1α, BDNF and GAPDH protein expression for acute TCAP treatment hypoxic study.

Whole cell lysates of immortalized N38 cells cultured in 21% and 1% O_2 for 36h were immunoblotted for HIF-1α (120 kDa), BDNF (14 kDa) and GAPDH (37 kDa) protein expression.

2.4 Discussion

In development, processes governing proliferation, differentiation and synaptogenesis act synergistically in a temporally and spatially-specific way. During this critical time, perturbations in the oxygen microenvironment can interfere with the normal development of the central nervous system and affect behaviour. For example, early hypoxia has been linked with the development of schizophrenia in humans (Davies *et al.,* 1998; Boksa and El-Khodor, 2003), hypoxic-ischemic brain lesions in neonates (Berger and Garnier 1999) and contributes to behavioural hypersensitivity to stress in adulthood (El-Khodor and Boksa, 2000).

The adult mammalian brain is a highly metabolic organ that represents approximately 2% of body weight and utilizes 20% of total body oxygen content (Magistretti and Pellerin, 2000). This renders the brain very sensitive to changes in oxygen and deviations from physiological oxygen levels can be damaging. Such oxygen-sensitive neurological dysfunction is characteristic of ischemic-stroke where there is inadequate oxygen supplied to tissues.

The majority of neuronal cell culture studies are routinely conducted at 37°C and in 5% CO_2 and 95% air. Although 37°C represents a physiological mammalian core temperature and 5% CO_2 reflects venous PCO_2 concentrations, culturing at ambient oxygen conditions (21% O_2) is not representative of physiologically relevant oxygen concentrations in neural tissues. Indeed, several cell types have responded differently when placed in their physiological microenvironment. In particular, various cell types including liver, bone marrow, and neural stem cells differ in behavior from that observed under ambient oxygen levels (Studer *et al.,* 2000; Chen *et al.,* 2001; Zhu *et al.,* 2005; Potier *et al.,* 2008).

In this study, the impact of reduced O_2 in the neuron-like immortalized embryonic mouse hypothalamic N38 cell line and the effect of TCAP-1 on neural behaviour under the varying

levels of oxygen concentrations were studied. Validation that N38 cells were hypoxic under the conditions used were conducted and showed that this particular cell line was responsive to changes in O_2 levels. In the N38 cells, TCAP-1 provided enhanced cell proliferation across all oxygen levels studied (21%, 4% and 1%) as early as 4 hours after treatment (Figure 2.7). Acute and chronic treatment regiments did not change the generally increasing trend of TCAP-1's proliferative effect. Therefore treatment with TCAP-1 may aid N38 cells in dealing with oxygen-related stress (enhance hypoxic tolerance) and allow for the maintenance of oxygen homeostasis at varying levels of reduced oxygen.

Although the mean levels of oxygen in the rodent brain have been reported as 1.6% and physiological oxygen concentrations in the hypothalamus range from 1.4 to 2.1% (Erecińska and Silver, 2001), the immortalized cell line were generated and selected to survive under ambient oxygen conditions (21% O_2) (Belsham *et al.,* 2004). As such, the N38 cells may be adapted to live under higher than physiological oxygen levels and could be an explanation for the cell death observed following chronic incubation at 1% O_2. Interestingly, 4% O_2 did not have a marked increase in cell death as cell numbers did not fall below baseline (100% at 0 hours) and may reflect an oxygen level that is within an intermediary range of hypoxic stress tolerance in N38 cells. Thus, TCAP likely acts as a neuroprotective factor that is able to prevent the decline in cell number, promote survival and stimulate general cell proliferation at 21%, 4% and 1% O_2.

The present data is consistent with previous studies showing that TCAP-1 has a proliferative effect on N38 cells under basal conditions and following pH-induced stress (Wang *et al.,* 2005; Trubiani *et al.,* 2007). In fact, the results are complimentary and in line with the neuromodulatory effect by TCAP-1 on this N38 cell model, which has also been implicated in

the scavenging of reactive oxygen species (Wang *et al.,* 2005; Al Chawaf *et al.,*2007a; Trubiani *et al.,*2007).

After it was established that TCAP-1 promotes proliferation, the peptide was examined to confirm whether it could also modulate neurite growth under varying levels of oxygen. The importance of neurite growth and synaptogenesis has been well established for its role in target recognition, neuronal survival and synaptic plasticity (Kuhl, 1999; Song and Poo, 2001; Kiryushko *et al.,* 2004). As well, a recent study by Al Chawaf *et al.* (2007a) found that TCAP-1 treatment increased neurite length and axon formation by influencing the levels and distribution of key cytoskeletal proteins and genes associated with axon outgrowth in N38 cells. Although the exact mechanism is unknown, experiments on cAMP following TCAP-1 treatment suggest a potential cAMP-dependent mechanism underlying the observed increases in β-tubulin and α-actin (Al Chawaf *et al.,* 2007a).

It is important to note that there was a discrepancy in neurite number. At ambient oxygen levels (21% O_2), there was an increase in neurite numbers initially (in the first experiment analyzing 21% vs. 4% O_2 cell growth parameters) but a decrease in the number of neurites in subsequent experiments under both the acute and chronic regiment (when analyzing 21% vs. 1% O_2) after TCAP-1 treatment. This may also be a result of variations between cell culture batches despite every attempt to maintain consistency or represent the real effect by TCAP-1 on neurite outgrowth. Since this is the first study investigating the response of N38 cells to TCAP-1 after hypoxic stress, the actual 'correct' and expected response remains to be determined. Based on the initial study, TCAP-1 acts to increase neurite outgrowth at both 21% and 4% O_2. It is conceivable that neurite growth would occur at 4% O_2 and is in line with the study by Genetos *et al.,* (2010) on PC12 cells where 4% O_2 maximally increased neurite growth via a mechanism that

may be mediated by adenosine A2A receptors (O'Driscoll and Gorman, 2005). Therefore, if the results obtained at 21% are true and if immortalized cells are able to tolerate growth under 4% O_2 as suggested by the proliferation data, then perhaps TCAP-1 is stabilizing the N38 cells to continue growing as they would in the oxygen concentration they were selected and adapted to survive in. In this situation, TCAP-1 acts as a signal that reduces the neurite growth characteristic of cells perceiving and responding to stress and thereby enhances neurite outgrowth.

For the short acutely administered TCAP-1 study, the results were found to be a preliminary step in establishing that a longer duration was necessary to yield effects expected for hypoxic stress to cells perceivably optimized for growth at 21% O_2. Since the hypoxic incubators had to be opened up at time points that were close together, the time required for the incubator to re-equilibrate to the desired oxygen concentration may have confounded the results. This may be implied by the lack of effect on neurite growth seen in TCAP-1 treated N38 cells incubated at 1% O_2 (Figure 2.11).

Under the acute treatment regime both experiments with cells chronically incubated for 24 hours at 21%, 1% or 4% O_2 exhibited a similar trend when comparing measurements of lowered oxygen (1% or 4% O_2) to those measured at 21% O_2. It may be that TCAP-1 is acting to re-establish the 'normal' response to TCAP-1 at 21% O_2. In other words, TCAP-1 is preventing the stress response from lowered oxygen concentrations and is able to trick the cell into believing it is not experiencing oxygen-related stress. This is consistent with qualitative western blot data (Figure 2.11) showing that TCAP-1 treatment, compared to vehicle, reduces HIF-1α expression that would normally be maximally upregulated during hypoxic stress (Weidemann and Johnson, 2008).

As mentioned above and in contrast to the initial study, both the acute and chronic TCAP-1 regiments consistently show decreased neurite growth at 21% O_2. If this is considered the true result, then in the 24-hour acute TCAP-1 study, the reduction in neurite numbers of TCAP-1 treated N38 cells at 21% or 1% O_2 indicates a conservative effect by TCAP-1. This is implied by the retraction of neurites at 12 hours. Since neurite outgrowth is metabolically costly for cells, TCAP-1 may be priming the cell in readiness for a pre-emptive or more potent stress under both 21% and 1% O_2 levels and hence acts as a protective energy-conserving factor.

On the other hand, the chronic TCAP-1 study has different effects on neurite growth that may be attributable to the altered TCAP-1 administration (chronic vs. acute). Therefore, it is possible that prolonged chronic TCAP-1 administration initially primes cells which then become desensitized to TCAP-1's message of caution and thereby better adapts to hypoxic conditions and continues neurite growth. Chronic TCAP-1 treatment may stimulate cells to form neurites and establish synaptic connections with their neighbours at least during stressful stimuli lasting 24 hours and longer (Figure 2.9F).

Thus far, the study highlights TCAP-1's modulatory effect on proliferation and neurite behaviour. To link the proliferation studies with the neurite data, an interesting point must be considered. Cells numbers are rapidly reduced in vehicle-treated cells under 1% O_2 in both acute and chronic TCAP-1 treatment regiments. This indicates that surviving cells are not likely to be in close proximity to easily establish synaptic connections. As such, the data indicates that there is an increasing trend in neurites of vehicle-treated cells compared to baseline (0 hours) under the acute treatment regime at 1% O_2 (Figure 2.9E). This implies that surviving N38 cells must actively extend their neurites to communicate with neighbouring cells for which survival may depend on. In the chronic regime, vehicle-treated cells display an initial increase in neurite

numbers that tends back towards baseline (0 hours) at later time points in 1% O_2. Again this may indicate that surviving cells are trying to make connections with surrounding cells and survive (Figure 2.9F).

There also appears to be an increasing trend in neurite numbers for N38 cells acutely treated with TCAP-1 over time at 1% O_2, but not as observable as the increase seen in vehicle-treated cells. At 21% O_2, TCAP-1 primed cells appeared to have an energy-conserving effect over time in contrast to the increasing neurite growth trend observed in vehicle-treated cells. Under the chronic regiment in 1% O_2, the data points at an increasing trend at all time points studied in the TCAP-1 treated cells that are significantly increased compared to vehicle-treated cells at 24 hours and 36 hours. At 21% O_2, TCAP-1 treated cells had a trend of decreasing neurite numbers that appeared to reverse after 24 hours. This is consistent with the idea that the cell is becoming desensitized to TCAP-1 and resuming growth.

A potential mechanism by which TCAP-1 could impart its neuromodulatory actions on N38 cells under varying levels of oxygen via brain-derived neurotrophic factor (BDNF) was investigated. Although BDNF has wide implicated in a variety of survival promoting processes that include enhanced proliferation and neurite outgrowth (Bailey, 1996; Kiryushko, 2004), qualitative western blot analysis show that TCAP-1 does not have an apparent affect on BDNF protein expression in cells incubated at low oxygen levels. Thus, although it was initially thought that TCAP-1 may be acting through BDNF the present data shows no clear effect on BDNF under low oxygen conditions. However, TCAP-1 did appear to modulate BDNF under basal conditions. Therefore, the next experiment aims to study the effect of TCAP-1 on BDNF expression under 21% O_2. This would provide a basis that defines basal conditions and may shed light onto TCAP-1's neuroprotective mechanism.

However, it should be noted that the data from this study does corroborate with gene array analysis of TCAP-1 treated N38 cells. Preliminary gene array analysis on N38 cells treated with by TCAP-1 showed a significant regulation of cell adhesion molecules (CAM) and extracellular matrix proteins (ECM) (Trubiani *et al.,* 2008; unpublished). This suggests that TCAP-1 may act to impact neurite growth by modulating the homophillic and heterophillic interactions between neurites and CAM and ECM proteins. Further experiments must be conducted to determine whether the exact mechanism of TCAP-1's action involves an interaction with CAM and ECM proteins that contribute to the observed neuroprotective effects under low oxygen conditions.

It is not surprising that TCAP-1 impacts neurite outgrowth. TCAP-1 was initially discovered by Qian *et al.* (2004) and shown to be derived from the extracellular C-terminal end of the teneurins, a type II transmembrane protein. In addition, the discovery of teneurins was conducted by Baumgartner *et al.,* (1994) using a low-stringency screen for vertebrate tenascin-C in a Drosophila library. Similar to TCAP-1, both teneurins and tenascin have previously been implicated in promoting neurite growth (Rigato *et al.,* 2002; Al Chawaf *et al.,* 2007a; Kenzelmann *et al.,* 2007). This relationship may reflect a group of similar molecules that may act together or independently to mediate proper development of the vertebrate nervous system.

In fact, this study supports other previous studies highlighting a potential role for TCAP in regulating intracellular signal transduction pathways to possibly mediate changes involved in synaptic plasticity and the enhancement of cell survival. Therefore this study provides further evidence for TCAP's role in neuromodulation and support its effect in behavioral *in vivo* studies (Wang *et al.,* 2005; Al Chawaf *et al.,* 2007b; Tan *et al.,* 2008, 2009).

Overall, these findings demonstrate that the oxygen levels are able to regulate behaviour of immortalized N38 cells and that it can be modulated by TCAP-1. TCAP-1 has a generally enhancing proliferative effect and differentially regulates neurite numbers in N38 cells based on the level of oxygen (21% O_2 vs. 4% O_2 vs. 1% O_2) and the treatment regiment (acute vs. chronic) (see TABLE 2.1 for a summary). In the acute study, TCAP-1 increased neurite outgrowth with 4% O_2 but decreased it with 1% O_2. In the chronic TCAP-1 study, neurite numbers were differentially regulated based on oxygen levels by TCAP-1 with a reduction in neurite outgrowth under ambient conditions while producing an enhancement at 1% O_2. Again it should be noted that there was a discrepancy at ambient 21% O_2 levels. The initial acute experiment which compared ambient with 4% oxygen levels found an increase in neurite growth at 21% O_2 whereas experiments conducted after showed a consistent decrease 1% O_2. Therefore, I propose that acute TCAP-1 treatment primes N38 cells and conserves their metabolic energy for use when an unexpected stress occurs and that chronic TCAP-1 leads to cells becoming desensitized to the TCAP-1 response, and thereby resumes growth.

These findings have implications for the use of TCAP-1 as a therapeutic agent in the treatment of ischemic stroke. Ischemia-stroke results from disruptions or interruptions in the blood supply to the brain. This condition leads to rapid loss of brain functions, which without immediate treatment may result in permanent neurological damage underlying clinical symptoms such as impaired movement, sensation, and/or cognition (Murphy and Corbett, 2009). Often an ischemic cascade is elicited and is associated with increased energy deprivation, an accumulation of intracellular calcium that leads to glutamate excitotoxcity and to the disruption of membranes and that ultimately ends with neuronal death (Dirnagl *et al.,* 1999). Therefore, TCAP-1 has a

neuromodulatory role that enhances proliferation and modulates neurite outgrowth for the overall protection of cells stressed by hypoxia.

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Chapter 3 : Regulation of Brain Derived Neurotrophic Factor (BDNF) of Teneurin C-terminal Associated Peptide (TCAP)-1 in immortalized embryonic hypothalamic cells

This chapter, along with additional material will be submitted for publication:

Tiffany Ng, Dhan Chand, Lifang Song, John Watson, Paul Boutros, Denise Belsham and David A. Lovejoy. Regulation of Brain Derived Neurotrophic Factor (BDNF) of Teneurin C-terminal Associated Peptide (TCAP)-1 in immortalized embryonic hypothalamic cells (Submitted to Neuroscience)

3.1 Introduction

The teneurins are a family of four large transmembrane proteins that are highly expressed in the central nervous systems where they have been implicated in development and neuron functions (for recent reviews see Young and Leamey, 2009; Kenzelmann *et al.,* 2007*;* Tucker *et al.,* 2007; Tucker and Chiquet-Ehrismann, 2006). Previous studies indicate that they play a role with neurite and process development (Leamey *et al.,* 2007; Minet *et al.,* 1999; Rubin *et al.,* 1999), and interact with elements of the extracellular matrix (Leamey *et al.,* 2008; Rubin *et al.,* 2002). The structure of the teneurins is complex, consisting of a number of subdomains in both intracellular and extracellular components (Kenzelmann *et al.,* 2008; Nunes *et al.,* 2005; Bagutti *et al.,* 2003; Feng *et al.,* 2002; Minet and Chiquet-Ehrismann, 2000; Minet *et al.,* 1999; Oohashi *et al.,* 1999). As a type II transmembrane proteins, the carboxy terminal region of the proteins is oriented in the extracellular part of the protein. At the tip of the carboy terminus lies a 43-amino acid sequence, that when processed, could liberate an amidated 41-mer peptide. We have termed this region the teneurin C-terminal associated peptide (TCAP). There is a highly conserved TCAP region on each of the four teneurin proteins (Lovejoy *et al.,* 2006)

A synthetic version of TCAP-1 has a number of bioactive effects. *In vivo*, administration of this peptide regulates stress-induced behaviours in rats (Wang *et al.,* 2005; Al Chawaf *et al.,* 2007a; Tan et al 2008; 2009), inhibits coroticotropin releasing factor (CRF)-induced c-fos expression in the brain (Tan *et al.,* 2009), inhibits CRF-induced increases in cocaine-seeking (Kupferschmidt *et al.,* unpublished) and causes dendritic remodelling of CA3 neurons in the hippocampus (Chand *et al.,* unpublished). *In vitro*, TCAP-1 regulates cytoskeletal elements (Al Chawaf *et al.,* 2007b), and reduced reactive oxygen species (ROS) by upregulation of superoxide dismutase and catalase (Trubiani et al 2007).

However, it is not clear whether TCAP-1 exerts its effects directly on these cellular systems or whether it does so by acting upon another system. One such candidate for this latter interaction is brain-derived neurotrophic factor (BDNF). BDNF has been shown to not only have a number of neurotrophic and neuroprotective effects on various brain cells (Lindholm *et al.,* 1993; Burke *et al.,* 1994; Ghosh *et al.,* 1994; Nakao *et al.,* 1995; Cheng *et al.,* 1997; Marini *et al.* 1998; Bemelmans *et al.,* 1999; Tremblay *et al.,* 1999; Walton *et al.,* 1999; Han *et al.,* 2000; Han and Holtzman, 2000; Schäbitz *et al.,* 2000; Husson *et al.,* 2005), but also plays a role in synaptic plasticity (see reviews McAllister *et al.,*1999 ; Yamada and Nabeshima, 2003; Bramham and Messaoudi, 2005; Cunha *et al.,* 2010) and, therefore, is associated with anxiety behaviours and mood disorders such as depression (for review, see Calabrese *et al.,* 2009; Martinowich and Lu, 2008; Lipsky and Marini, 2007; Duman and Monteggia, 2006; Hashimoto *et al.,* 2004).

In order to investigate the hypothesis that TCAP-1 has a direct effect in BDNF regulation, we examined the relationship between BDNF expression and TCAP-1 action in a recently developed immortalized embryonic mouse hypothalamic cell line (Belsham et al, 2006). We have previously shown that TCAP-1 has a number of actions on this cell line although the

mechanism by which it exerts its effects in not clear (Wang *et al.,* 2005; Al Chawaf *et al.,* 2007b; Trubiani *et al.,* 2007). We now show that TCAP -1 binds to these cells where it is internalized and enters the nucleus. TCAP-1 significantly regulates the expression and intracellular concentration of BDNF.

3.2 Materials and Methods

3.2.1 Peptide synthesis

Mouse TCAP-1 was prepared by solid-phase synthesis by American Peptide Company (Sunnyvale, USA) and solubilized in phosphate-buffered saline (PBS) to a stock concentration of $2x10^{-5}$M. Stock mouse TCAP-1 was then diluted in medium to a final concentration of 10^{-8} M. The inactive peptide, $TCAP_{9-41}$, was diluted in medium to a final concentration of 10^{-8}M and used as a negative control. Since both mouse TCAP-1 and $TCAP_{9-41}$ were dissolved in ddH2O and two puffs of NH_4^+ vapour, as an additional negative control, NH4+ and ddH_2O control treatment was prepared by dissolving two puffs of NH_4^+ vapour in 1.5 ml ddH_2O. Forskolin stock (10^{-2} M; Sigma, Cat. No. F3917) dissolved in DMSO was prepared and diluted to a final concentration of 10-5M for treatment.

3.2.2 Preparation of fluoresceinisothiocyanate (FITC)-labelled TCAP-1

[K8] -TCAP-1 was labelled with FITC as previously reported (Al Chawaf *et al.,* 2007b). Briefly, the lyophilized peptide was solubilized in 50 mM borate buffer (pH 8.5) to a final concentration of 1 mg/ml. TCAP-1 peptide was labelled with FITC (EZ-Label FITC Protein

labelling kit; Pierce). FITC was dissolved in dimethylformamide, mixed with the TCAP-1 at a 24:1 excess molar ratio and incubated for 2 hours at RT in the dark. FITC-TCAP-1 was purified using a dextran column. The 4 fractions with the highest protein absorbance (280nm) readings were combined and sterilize-filtered using an Acrodisc Syringe 0.2 mm Super low protein binding filter (Pall Life Sciences). The filtrate was concentrated using a Microsep 1K omega centrifugal device (Pall Life Sciences) and stored at 4-8°C.

3.2.3 Primary embryonic hippocampal cell cultures

All procedures were approved by the University of Toronto Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care. Low-density cultures of dissociated embryonic rat hippocampal neurons were prepared as previously described (Woodin *et al.,* 2003). Briefly, E18 pregnant Sprague Dawley rats were exposed to carbon dioxide for several minutes and cervically dislocated. The E18 rat embryonic hippocampi were isolated and treated with trypsin for 15 minutes at 37°C. Cells were dissociated by gentle trituration and plated at a density of 50 000 cells/mL on poly-D-lysine-coated 25-mm glass coverslips in 35-mm petri dishes. Cells were plated in Neurobasal medium (Invitrogen, Carlsbad, California, USA), supplemented with 2% B-27 (Invitrogen). Every three days, one-third of the medium was replaced with fresh Neurobasal medium supplemented with B-27.

3.2.4 Immunoflorescence confocal microscopy

Immortalized mouse embryonic hypothalamic N38 cells (gift from Dr. Denise Belsham, University of Toronto) were grown on 25mm coverglass (Warner Instruments, Cat. No. 64-0715)

in 6-well tissue culture treated plates (for 24 hours. When the cells were approximately 50% confluent, cells were treated with 10^{-8} M TCAP-1, inactive 10^{-8} M $TCAP_{9-41}$, or vehicle NH_4^+ in distilled deionized H_2O (ddH_2O) for 0, 1, 2, 4 and 8 hours. Immunocytochemistry was conducted on immortalized N38 cells incubated with FITC-[K8]-TCAP-1 for 90 minutes. Rabbit polyclonal BDNF primary antibody (Santa Cruz, sc-20981; 1:500) and anti-rabbit Texas red TM-conjugated secondary antibody (Abcam, ab7088; 1:200) were used and images were acquired with a Zeiss AxioObserver (Zeiss, Germany) inverted microscope equipped with a confocal light path (WaveFx; Quorum, Canada) based on a modified Yokogawa CSU-10 head (Yokogawa Electric Corporation, Japan).

3.2.5 Forskolin treatment

A freshly prepared stock of 10^{-2} M forskolin (Sigma, Cat. No. F3917) was prepared and added to cell media to give a final concentration of 10^{-5} M. Media was removed and treatment groups were given fresh DMEM supplemented with 10% FBS and penicillin and streptomycin with forskolin. In contrast, for control groups, media was removed and fresh DMEM supplemented with 10% FBS and penicillin and streptomycin with sterile dimethyl sulfoxide (DMSO; Sigma, Cat. No. D8418) was applied to N38 cells.

3.2.6 Western blot analysis of BDNF

All immortalized mouse embryonic hypothalamic N38 cells were grown in Dulbecco's Modified Eagle Medium containing 4 500 mg/L D-glucose, L-glutamine, and 25 mM HEPES buffer, but no sodium pyruvate (Invitrogen, Cat. No. 12430-054) supplemented with 10% fetal

bovine serum (Invitrogen, Cat. No. 16000-036) and including penicillin and streptomycin antibiotics. Immortalized N38 cells were grown in 6-well tissue culture treated plates (Corning Incorporated, Cat. No. 3516) at a density of 400 000 cells/9 cm^2 wells for 24 hours. With approximately 70-80% cell confluence at 0, 1, 2, 4 and 8 hours after treatment with either 10^{-8} M TCAP-1, inactive 10^{-8} M $TCAP_{9-41}$, vehicle NH4+ in ddH_2O or 10^{-5}M forskolin, cells were washed three times with sterile phosphate buffer saline (PBS) at pH 7.4 and harvested in 1 ml sterile PBS using a plastic cell scraper. Cells were then centrifuged at 2 000 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 200 µl of cold 1% lysis buffer [1% Triton X-100, 1M DTT, Protease Inhibitor Cocktail Set III (Calbiochem, Cat. No. 539134)]. The resuspended pellet was vortexed and incubated on ice for 5 to 10 minutes after which it was centrifuged at 14 000 rpm for 20 minutes at 4°C.The supernatant was aliquoted into fresh 1.5ml tubes and stored in -20°C for western blot analysis.

Protein extracts were quantified in accordance with the manufacturer's protocol (Thermo Fischer Scientific, Cat. No. 23225). In brief, 5 µl of each protein sample was aliquoted into 96 well plates in triplicates. 200 µl of BCA Working Reagent was added to each sample. Diluted albumin standards were prepared in triplicates providing the assay with a working range from 20 to 2000 µg/ml. The 96-well plate was covered and incubated with shaking at 37°C for 30 minutes. An absorbance reading was subsequently obtained at 562 nm using Spectramax Plus 384 (Molecular Devices).

Protein samples (15µg) were then run on a 4-20% Ready Tris-HCl Precast Gel (BioRad, Cat. No. 161-1159) at 200V for approximately 35 minutes. Proteins were subsequently electro-transferred to a Hybond-ECL-nitrocellulose membrane (VWR, Cat. No. RPN203D) for 90 minutes at 100V. The membrane was washed with phosphate saline buffer (PBS) with shaking

for 5 minutes and incubated with BDNF rabbit polyclonal primary antibody (Santa Cruz, sc-20981) diluted 1:500 in 7 ml of milk-PBST (PBS with 0.2% Tween®20) with gentle agitation overnight at 4°C. After 24 hours, the membrane was washed with fresh PBST three times with shaking for 5 minutes, 10 minutes, and 10 minutes, respectively. Following, the membrane was incubated with anti-rabbit horseradish peroxidise (HRP)-conjugated secondary antibody (VWR, Cat. No. RPN2135) diluted 1:5000 in milk-PBST for 1 hour at room temperature with gentle agitation. After incubation, the membrane was washed three times with fresh PBST for 5 minutes, 10 minutes and 10 minutes, respectively, and exposed onto ECL Hyperfilm (VWR, Cat. No. 95017-653L) for 10 seconds. The integrated optical density (IOD) of the blots were quantified using LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.). GAPDH (Abcam, ab9485; 1:2500) was used as an additional loading control and remained unchanged for all protein samples.

3.2.7 RNA extraction and quantitation

Immortalized mouse embryonic hypothalamic N38 cells were grown in 6-well tissue culture treated plates for 24 hours. When the cells were approximately 70-80% confluent, cells were treated with 10^{-8} M TCAP-1, inactive 10^{-8} M $TCAP_{9-41}$, or vehicle NH_4^+ in distilled deionized H_2O (ddH_20) for 0, 1, 2, 4 and 8 hours. Total RNA was extracted with the RNeasy Mini kit (Qiagen Inc. Mississauga, Ontario Canada) following manufacturer's instructions. The integrity of the total RNA was verified using the Agilent RNA 6000 Nano Assay and analyzed using the Bioanalyzer 2100 (Agilent Technologies Inc., Mississauga, Ontario Canada). Quantification of RNA was carried out using a Nanodrop 1000 spectrophotometer (Fisher Scientific, Ottawa, Canada). RNA was also extracted from approximately 70-80 mg of flash-

frozen whole mouse brain. The tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle, and homogenized in homogenizer solution (2.5 ml RLT buffer and 25µl β-mercaptoethanol) using a Brinkmann Polytron Homogenizer PT1600E with a PT-DA 1607 generator (Fisher Scientific, Ottawa, Ontario Canada). Total RNA was extracted with the RNeasy Mini kit (Qiagen) according to manufacturer's instructions. The total RNA was quantified and verified as described above.

3.2.8 Polymerase Chain Reaction assay design.

Primer and probe sequences for BDNF variants I-VI, VIII and IXA were designed (Figure 3.1) using the splice variant sequences reported by Aid *et al.* (2007), using the RealTime PCR Assay Design Tool (http://www.idtdna.com/catalog/primetime/primetime.aspx), as follows. The sequences reported by Aid *et al.* were identified in GenBank (http://www.ncbi.nlm.nih.gov/nucleotide/, Table 3.1) and the sequence of the region of interest was entered into the appropriate box of the Assay Design Page. The location of the intron was identified by entering "nnnnnn" in the appropriate position. This allowed the program to design the primers and/or probe to reside on opposite sides of the intron. This was desirable to reduce the likelihood of detecting genomic DNA contamination. The predesigned Primetime qPCR assays were supplied by Integrated DNA Technologies (IDT, Coralville Iowa, U.S.A.). Where possible, primer and probe design parameters were used with the amplicon size ranging from 70 to 150 base pairs (bp). BDNF variant VII primers and probes were designed separately to meet the above parameters using PrimerQuest Custom Design Tool (http://www.idtdna.com/Scitools/Applications/Primerquest/) and supplied by Integrated DNA

Technologies. Primers were checked to ensure variant specificity using Primer-Basic Local Alignment Search Tool (Primer-BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and probe sequences were checked using Nucleotide-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) (BLAST reference from the NCBI page). Sequences were compared to the National Center for Biotechnology Information (NCBI) nonredundant sequence database with the organism set to *Mus musculus* (taxid: 10090). As house-keeping controls, specific primers and probe pairs were also designed for TATA-binding protein and α–microglobulin and validated as above (Berggard *et al.,* 1998; Watson *et al.,* 2007). To determine the size of products in the PCR reactions, PCR product from whole mouse brain cDNA were diluted 1:10 and 1 µl was electrophoresed on a High Sensitivity DNA Chip (Agilent Technologies, Santa Clara, CA, U.S.A.). All probes were 5'-labeled with a 6-carboxyfluorescein reporter and 3'-labeled with an Iowa black FQ dark quencher. The primers and probes used are listed in Table 3.2. Primetime assays were resuspended in 500 µl RNAse-free water (Sigma-Aldrich, Oakville, Ontario Canada) to a 20x working concentration (1.0 fM (5nmol in 500µl) of each primer and 0.5fM (2.5nmol in 500µl) probe) as recommended by the manufacturer.

Table 3.1: GenBank Accession No. and BDNF Transcript

GenBank Accession No.	BDNF Transcript
EF125669.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant I (Bdnf) mRNA, complete cds, alternatively spliced
EF125670.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant IIA (Bdnf) mRNA, complete cds, alternatively spliced
EF125671.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant IIB (Bdnf) mRNA, complete cds, alternatively spliced
EF125672.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant IIC (Bdnf) mRNA, complete cds, alternatively spliced
EF125681.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant III (Bdnf) mRNA, complete cds, alternatively spliced
EF125673.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant IV (Bdnf) mRNA, complete cds, alternatively spliced
EF125682.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant V (Bdnf) mRNA, complete cds, alternatively spliced
EF125674.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant VI (Bdnf) mRNA, complete cds, alternatively spliced
EF125683.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant VII (Bdnf) mRNA, complete cds, alternatively spliced
EF125684.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant VIII (Bdnf) mRNA, complete cds, alternatively spliced
EF125685.1	Mus musculus brain-derived neurotrophic factor precursor transcript variant IXA (Bdnf) mRNA, complete cds, alternatively spliced

Table 3.2: Primers and probes used for quantitative real-time RTPCR

Target cDNA	Forward Primers (5'-->3')	Reverse Primers (5'-->3')	Probes	Amplicon Size (bp)
BDNF I	TTACCTTCCTGCATCTGTTGG	GTCATCACTCTTCTCACCTGG	56-FAM-AACATTGTGGCTTTGCTGTCCTGG-3IABLFQ	97
BDNF II	GAGAGCAGAGTCCATTCAGC	CCT TCATGCAACCGAAGTATG	56-FAM-CCTTGGACAGAGCCAGCGGATTT-3IABLFQ	143, 355, 439
BDNF IIB	AGAGTCAGATTTTGGAGCGG	GTCATCACTCTTCTCACCTGG	56-FAM-AGA CTC TTG GCA AGC TCC GGT TC-3IABLFQ	145, 229
BDNF IIC	GGCTGGAATAGACTCTTGGC	GTCATCACTCTTCTCACCTGG	56-FAM-CGGGTTGGTATACTGGGTTAACTTTGGG-3IABLFQ	139
BDNF III	CTTCCTTGAGCCCAGTTCC	AGTTGCCTTGTCCGTGG	56-FAM-CCGCCTTCATGCAACCGAAGT-3IABLFQ	130
BDNF IV	AGCTGCCTTGATGTTTACTTTG	CGTTTACTTCTTTCATGGGCG	56-FAM-AGGATGGTCATCACTCTTCTCACCTGG-3IABLFQ	139
BDNF V	AACCATAACCCCGCACAC	ATGCAACCGAAGTATGAAATAACC	56-FAM-ACCTTCCCGCACCACAAAGCTA-3IABLFQ	148
BDNF VI	GGACCAGAAGCGTGACAAC	ATGCAACCGAAGTATGAAATAACC	56-FAM-ACCAGGTGAGAAGAGTGATGACCATCC-3IABLFQ	131
BDNF VII	ACCCTCTGCCATCCTGC	ATGCAACCGAAGTATGAAATAACC	56-FAM-ACTTTCATCCGGGATTCCACCAGGTGAG-3IABLFQ	142
BDNF VIII	ATAACCCGAAGGACAACTGG	ATGCAACCGAAGTATGAAATAACC	56-FAM-AGCCTGAGCACATGGAGAAAACTGG-3IABLFQ	311
BDNF IXA	AGTTCTAACCTGTTCTGTGTCTG	CGTTTACTTCTTTCATGGGCG	56-FAM-AGGATGGTCATCACTCTTCTCACCTGG-3IABLFQ	140
TBP	GCCTTCCACCTTATGCTCAG	AGACTGTTGGTGTTCTGAATAGG	56-FAM-CCTTACGGCACAGGACTTACTCCAC-3IABLFQ	130

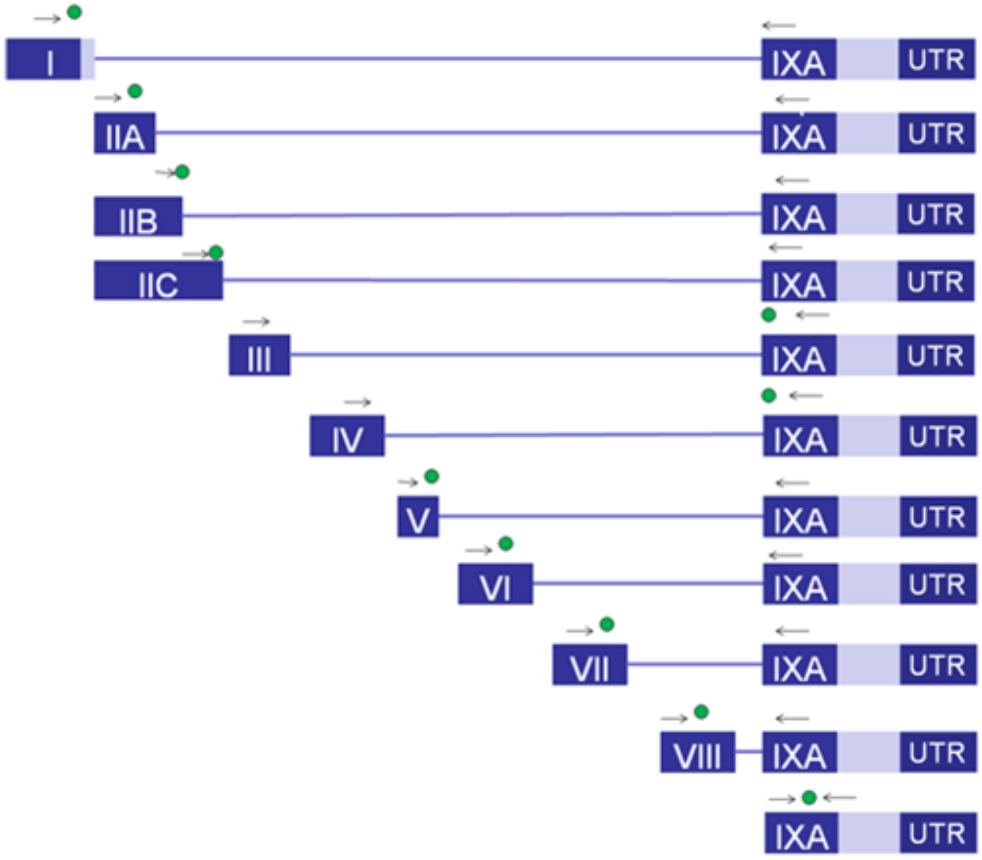

Figure 3.1: Primer and probe pair design for BDNF variants I-IXA used in real-time reverse transcription polymerase chain reaction (RTPCR).

Alternative transcripts of mouse BDNF genes are illustrated with exons shown as boxes and introns shown as lines. Light purple boxes are protein coding regions whereas dark purple boxes are untranslated regions. Each of the eight 5'untranslated regions are spliced to the common 3' IXA protein coding exon. Left-pointing arrows represent the forward primers and right-pointing arrows represent reverse primers. Probes with 5'-6-FAM florescent dye and 3'quencher molecule are represented in between as a green circle.

3.2.9 High throughput quantitative real-time reverse transcription linked polymerase chain reaction (RT-PCR)

Real-time reverse transcription linked polymerase chain reaction performed in laboratory of Dr. Paul Boutros with the assistance of Dr. John Watson. Total RNA from immortalized mouse embryonic hypothalamic N38 cells and used to synthesize cDNA using High Capacity RNA-to-cDNA Master Mix following the manufacturer's protocol (Applied Biosystems). Briefly, total RNA (1 µg), RNAse-free water and 4 µL High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Streetsville, Ontario Canada) was used to prepare a 20 µL final volume reverse transcription reaction carried out at 25°C for 5 min, then 42°C for 30 minutes, and finally 85°C for 5 minutes, in a Veriti® Thermal Cycler (Applied Biosystems). Parallel control reactions were carried out using the same conditions and reagents without reverse transcriptase. These control reactions were used to determine if the specific PCR reaction signal was caused by genomic DNA contamination. Resulting cDNA samples (1 µg) were diluted to a final concentration of 5 ng/µL by the addition of ddH_20. A 10 µL real-time RTPCR reaction containing [4 µL (20 ng) cDNA, 1 µL of Primetime Assay mix (forward primer, reverse primer and probe), and 5µl of the TaqMan® Gene Expression Master Mix (Applied Biosystems) was run in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The parameters were set as follows: temperature of 50°C for 2 min, then 95°C for 10 min, followed by 45 cycles of 95°C for 15s and 60°C for 1 min. Primetime assays were validated using 10-fold (10ng, 1ng, 0.1ng) serial dilutions of cDNA from N38 cells or mouse brain in PCR reactions. These reactions also determined the optimal amount of cDNA for detection.

3.3 Results

3.3.1 Binding activity of TCAP-1

The initial goal was to establish that FITC-labelled TCAP-1 could bind to N38 cells. The affinity of TCAP-1 for binding sites in cell extracts was measured by displacing FITC-labelled TCAP-1 with mouse TCAP-1 from N38 cells. A concentration-dependent displacement of FITC-TCAP-1 binding was observed with an apparent K_d of 2 x 10^{-7} M (Figure 3.2A). Moreover, after resolution on SDS-PAGE electrophoresis, the labelled TCAP bound to a protein about 35-50 kDa (Figure 3.2B).

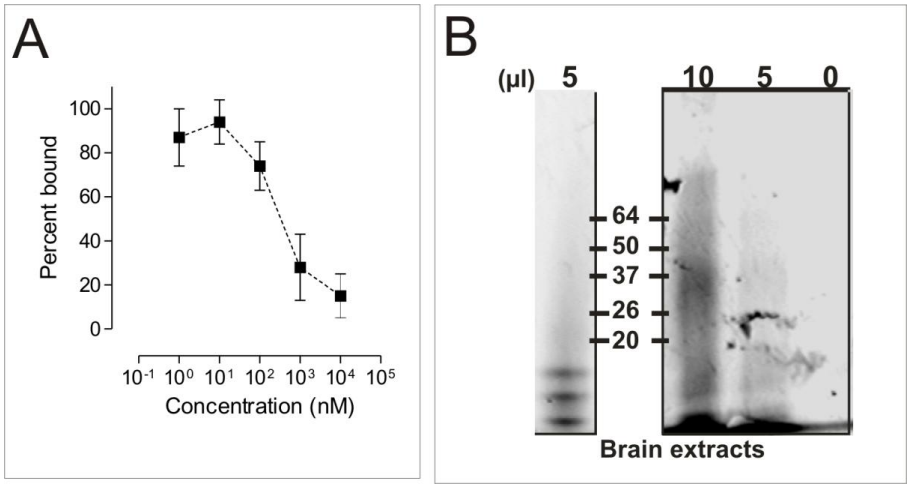

Figure 3.2: TCAP-1 binding to N38 cells.

(A) Displacement of FITC-K8-TCAP-1 by unlabeled TCAP-1 in cell preparations (Andrew and Lovejoy, unpublished). (B) SDS-PAGE image of FITC-K8-TCAP-1 binding to protein in N38 cell lysates (Al Chawaf, 2007c).

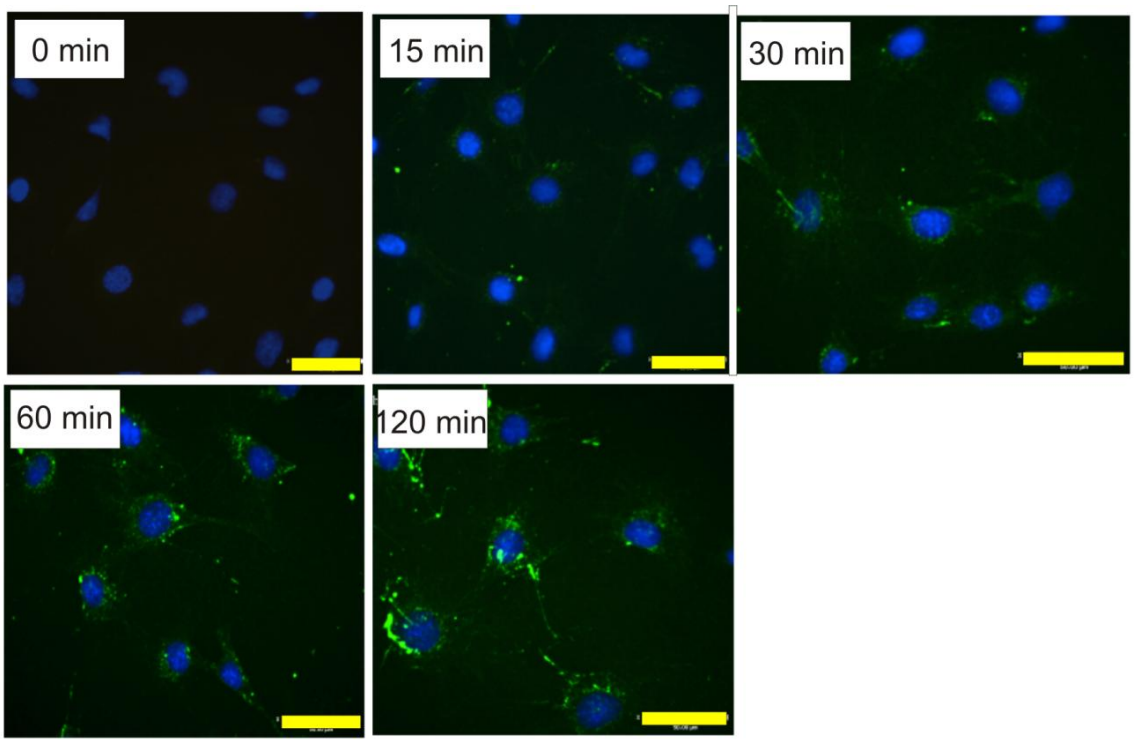

Figure 3.3: Time course of FITC-[K8]-TCAP-1 uptake into cytosol.
Green indicates the FITC-TCAP variant. The yellow bar at bottom represents 50 µm (courtesy of Dhan Chand and Lifang Song).

After verifying TCAP-1's ability to bind N38 cells, we proceeded to investigate whether TCAP-1 could be taken up into the cell. To examine the rate of uptake and determine the time course of this uptake with the goal of establishing a correlation between gene transcription events and uptake, the labelled TCAP was incubated with the N38 cells (Figure 3.3). By 15 minutes TCAP-1 could be detected at cell membranes with some penetration into the cytosol. By 30 minutes, entry into the cytosol was well depicted and by 60 minutes a strong signal around the nucleus could be detected.

Once we established that the N38 cells could internalize TCAP-1, the presence of immunoreactive BDNF was examined in these cells. A western blot analysis using BDNF

antisera showed a positive signal corresponding to the expected size of the mature BDNF protein (Figure 3.5). Moreover, this signal was inducible when the cells were treated with forskolin (Figure 3.5).

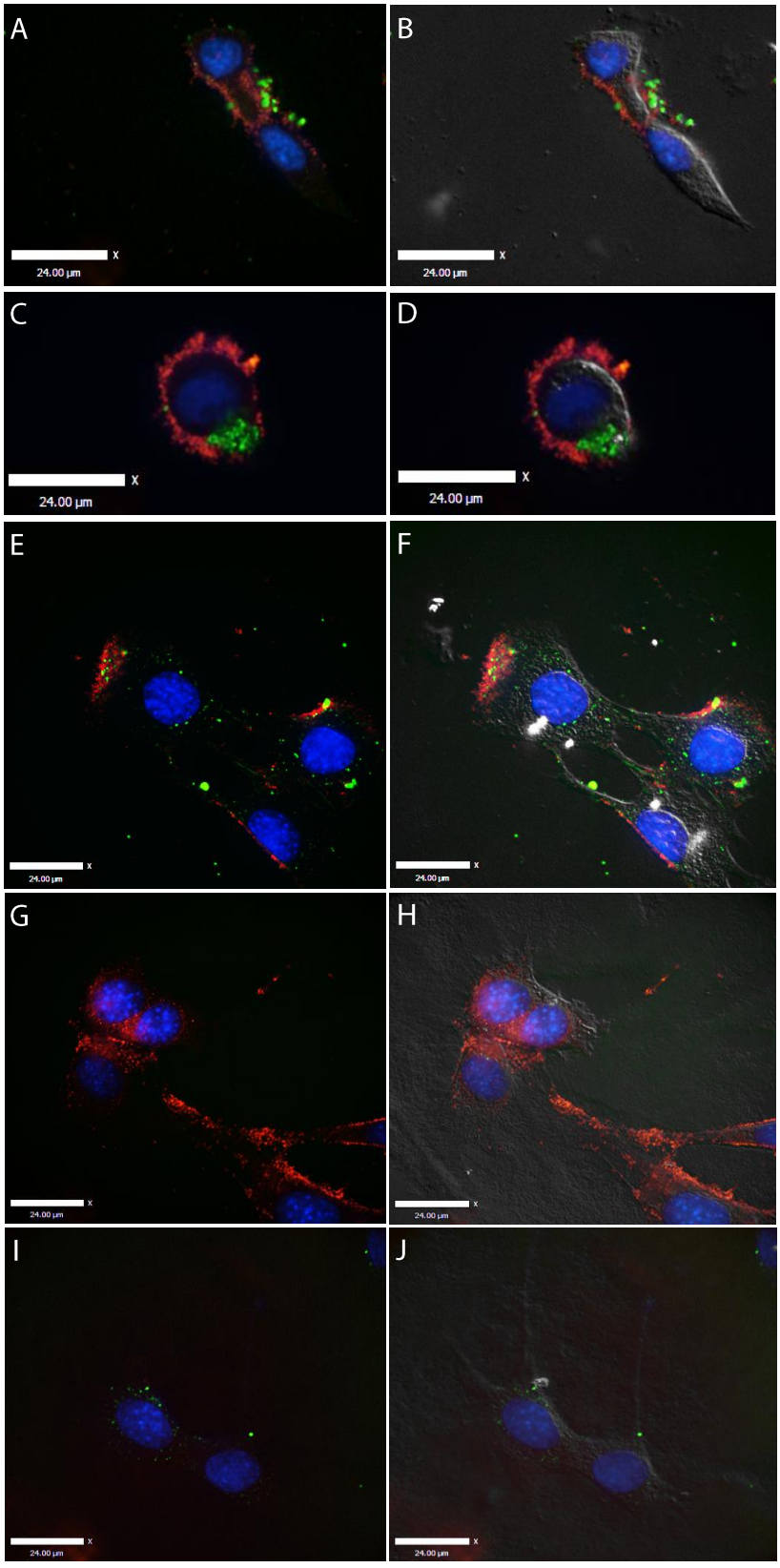

Figure 3.4: Expression of BDNF with FITC-TCAP binding, internalization and uptake into cytosol of immortalized N38 cells.

(A) Cells incubated with FITC-[K8]-TCAP for 90 min and co-labelled with BDNF (red) and nuclei stained with DAPI (blue). BDNF localizes to periphery of cell following binding of FITC-[K8]-TCAP to the plasma membrane. (C) FITC-[K8]-TCAP shown to be taken up into the cytosol as distinct puncta. BDNF appears localized to the periphery and released into extracellular space. (E) FITC-[K8]-TCAP fully internalized and dispersed in cytosol with BDNF primarily localized and released into the extracellular space. (G) Control showing cells minimal background florescence from anti-rabbit Texas red TM-conjugated secondary antibody (Abcam, 1:500) only and nuclei stained with DAPI (blue). (I) Control showing cells incubated with FITC only for 90 min and nuclei stained with DAPI. Image confirms florescence is specific for FITC-[K8]-TCAP. (B,D,F, H, and J) Same conditions as in (A, C, E, G, and I) but with DIC overlay, respectively. Scale bars = 24.00µm. Images were obtained by WaveFX spinning-disk confocal microscope (Germany).

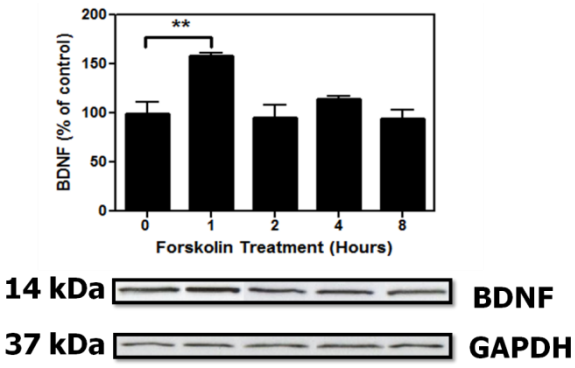

Figure 3.5: N38 immortalized cells are BDNF- inducible by forskolin treatment (10-5 M).

(A) BDNF expression significantly increased following 1h 10^{-5} M forskolin treatment as determined by Western blot analysis (n=3; **P<0.01, one-way analysis of variance (ANOVA) and a Bonferroni's Multiple Comparison post hoc test).

Following verification that the N38 cells bind and internalize TCAP-1 and that a positive and inducible form of immunoreactive BDNF was present, experiments were performed to firmly establish that the FITC labelled TCAP-1 and BDNF immunoreactivity could be shown in the same cells. Because developing neurons can express different biomarkers at various stages of development and growth conditions, it was important to show that both were colocalized (Figure 3.4).

Interestingly, similar observations were found in a primary culture of rat hippocampal cells. FITC-[K8]-TCAP-1 was able to bind to the cell membrane (Figure 3.6A, B), be internalized into the cytosol as distinct puncta (Figure 3.6C, D), aggregate around the nucleus (Figure 3.6F, G) and finally enter the nucleus to effect gene transcription (Figure 3.6E).

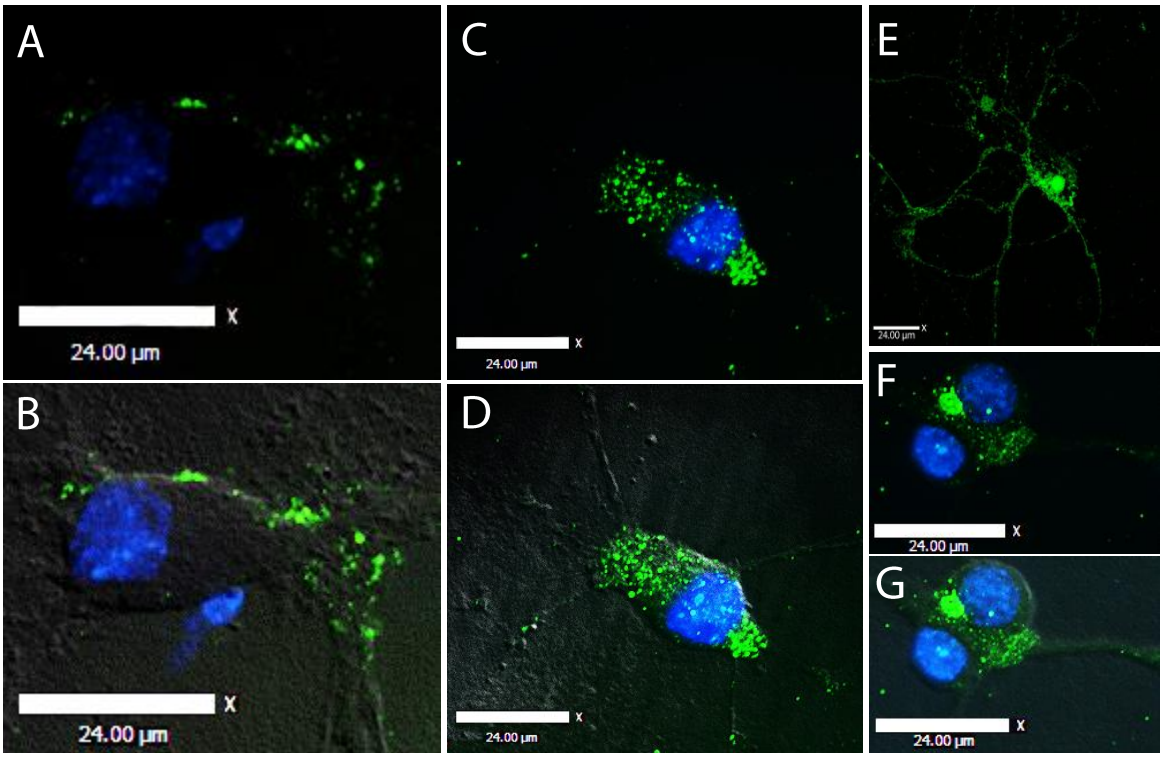

Figure 3.6: Similar observations to N38 immortalized cells of FITC-[K8]-TCAP binding, uptake into the cytosol, and transport into nucleus of primary rat hippocampal cells.

(A) Cells incubated with FITC-[K8]-TCAP for 30-90 min, and nuclei stained with DAPI (blue). Image shows FITC-[K8]-TCAP binding to membrane (white arrow). (B) Same conditions as in (A) but with DIC overlay. (C). FITC-[K8]-TCAP binding to membrane taken up into cytosol as distinct puncta. (D) Same conditions as (C) but with DIC overlay. (E) Overall image of FITC-[K8]-TCAP dispersed throughout neuron as distinct puncta that are transported into nucleus. (F) FITC-[K8]-TCAP localized around periphery of nuclear membrane. (G) Same conditions as in (F) but with DIC overlay. Scale bars = 24.00µm. Images were obtained by WaveFX spinning-disk confocal microscope (Germany).

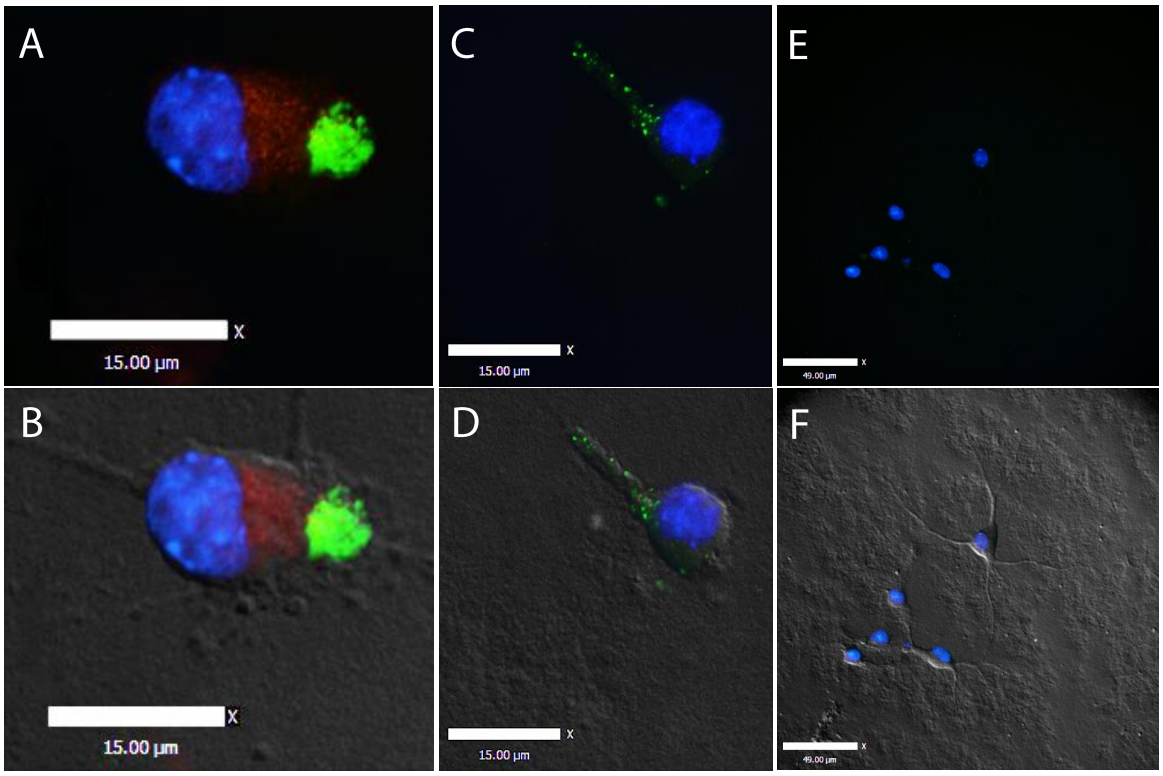

Figure 3.7: Positive BDNF immunoreactivity and FITC-[K8]-TCAP uptake in primary E18 rat hippocampal culture.

(A) Cells incubated with FITC-[K8]-TCAP for 90min, co-labelled with BDNF (red) and nuclei stained with DAPI (blue). Image shows FITC-[K8]-TCAP bolus taken up into cytosol. (B) Same conditions as in (A) but with DIC overlay. (C) Control showing cells minimal background florescence from anti-rabbit Texas red TM-conjugated secondary antibody (Abcam, 1:500) only and nuclei stained with DAPI (blue). (D) Same conditions as (C) but with DIC overlay. (E) Control showing cells incubated with FITC only for 90 min and nuclei stained with DAPI. Image confirms fluorescence is specific for FITC-[K8]-TCAP. (F) Same conditions as in (E) but with DIC overlay. Scale bars = 15.00µm for A-D and 45.00µm for E-F. Images were obtained by WaveFX spinning-disk confocal microscope (Germany).

Immunofluorescence confocal microscopy illustrated FITC-labelled TCAP binding to a number of N38 cells that were immunoreactive for BDNF (Fig 3.4). Primary rat hippocampal cultures, although BDNF-immunopositive, localized in the cytosol in comparison to the periphery as observed for immortalized N38 cells (Figure 3.7A, B). As controls, cells incubated with the either the FITC molecules or the secondary texas red-conjugated secondary antibody alone showed minimal background florescence thereby indicating that florescence was due to expression of FITC conjugated to TCAP-1 and BDNF (Figure 3.7C, D, E, F). Therefore, both the immortalized N38 cell line and primary hippocampal culture were able to bind and internalize TCAP-1 and co-express BDNF.

Confirming TCAP-1 binding to BDNF immunoreactive N38 cells made it then necessary to determine whether TCAP-1 could regulate BDNF immunoreactivity levels in these cells. This was established by administering TCAP-1 to N38 cells and examining immunoreactive levels by western blot analysis (Fig. 3.8). TCAP-1 induced a decrease in immuoreactive BDNF levels over an 8-hour period. At 8 hours, only about 25% of the initial BDNF levels could be determined ($p<0.05$). However, such a decrease could be attributed to a reduction in the transcription of BDNF and hence the resultant decrease in translation, or may be due to a release of BDNF as a result of exocytosis. To address this latter possibility immunoreactive TCAP-1 in N38 cells was examined by determining the level and pattern of expression in the cytosol (Figure 3.4).

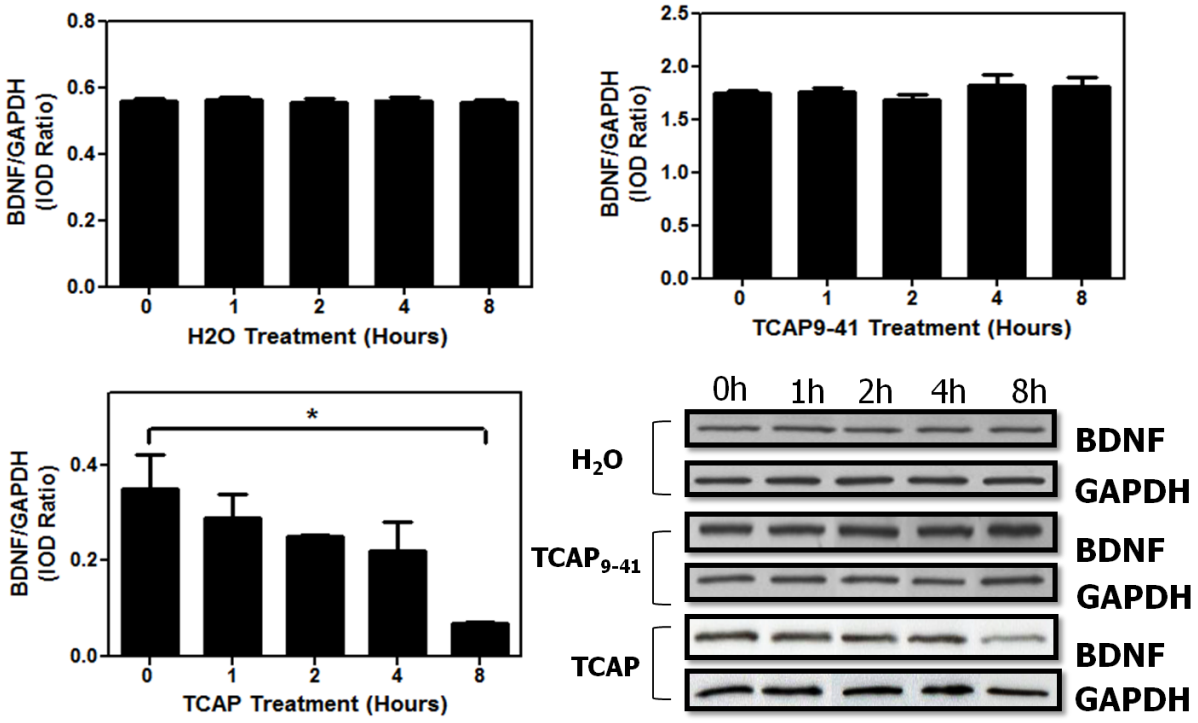

Figure 3.8: TCAP-1 treatment regulates BDNF protein expression as determined by Western blot analysis.

Treatment with vehicle (A), and inactive $TCAP_{9-41}$(10^{-8} M; B) in immortalized embryonic hypothalamic N38 cells showed no significant difference**.** However there was a significant difference in BDNF expression between 0h and 8h after acute treatment with TCAP-1 (10^{-8} M; C). (D) Representative Western blot bands for each time point and treatment where BDNF (14 kDa) was normalized to GAPDH (37 kDa). The level of significance was determined using a one-way ANOVA and Dunnett's Multiple Comparison post hoc test (n=3;*p<0.05).

These studies (Figure 3.4) indicated that upon binding of the labelled TCAP-1, immunoreactive BDNF could be found in the periphery of the soma, instead of well within the soma established previously. These studies suggest that TCAP-1 was inducing the release of BDNF from the cell. Examining BDNF in the cell media was unsuccessful as it was not possible to concentrate the protein levels to a sufficient degree to be measured by ELISA (data not shown).

Positive BDNF-immunoreactive immortalized N38 cells that expressed BDNF along the periphery of the cell suggested again that TCAP-1 may be able to regulate BDNF expression. As controls, cells incubated with either the FITC molecules or the secondary texas red-conjugated secondary antibody alone showed minimal background florescence thereby indicating that florescence was due to expression of FITC conjugated to TCAP-1 and BDNF (Figure 3.4G, H, I, J).

3.3.2 BDNF expression studies

The expression of BDNF splice variants is complex and several different variants may be expressed as a function of any given ligand. To examine the role of TCAP-1 on BDNF, 11 different splice variants were examined (Figure 3.1). Before establishing the regulation of these splice variants, the integrity of the primer-probe pairs in relationship to the splice variants they represented was first determined. The primer-probe pair's selected amplified products in whole mouse brain mRNA extracts (Fig. 3.9).

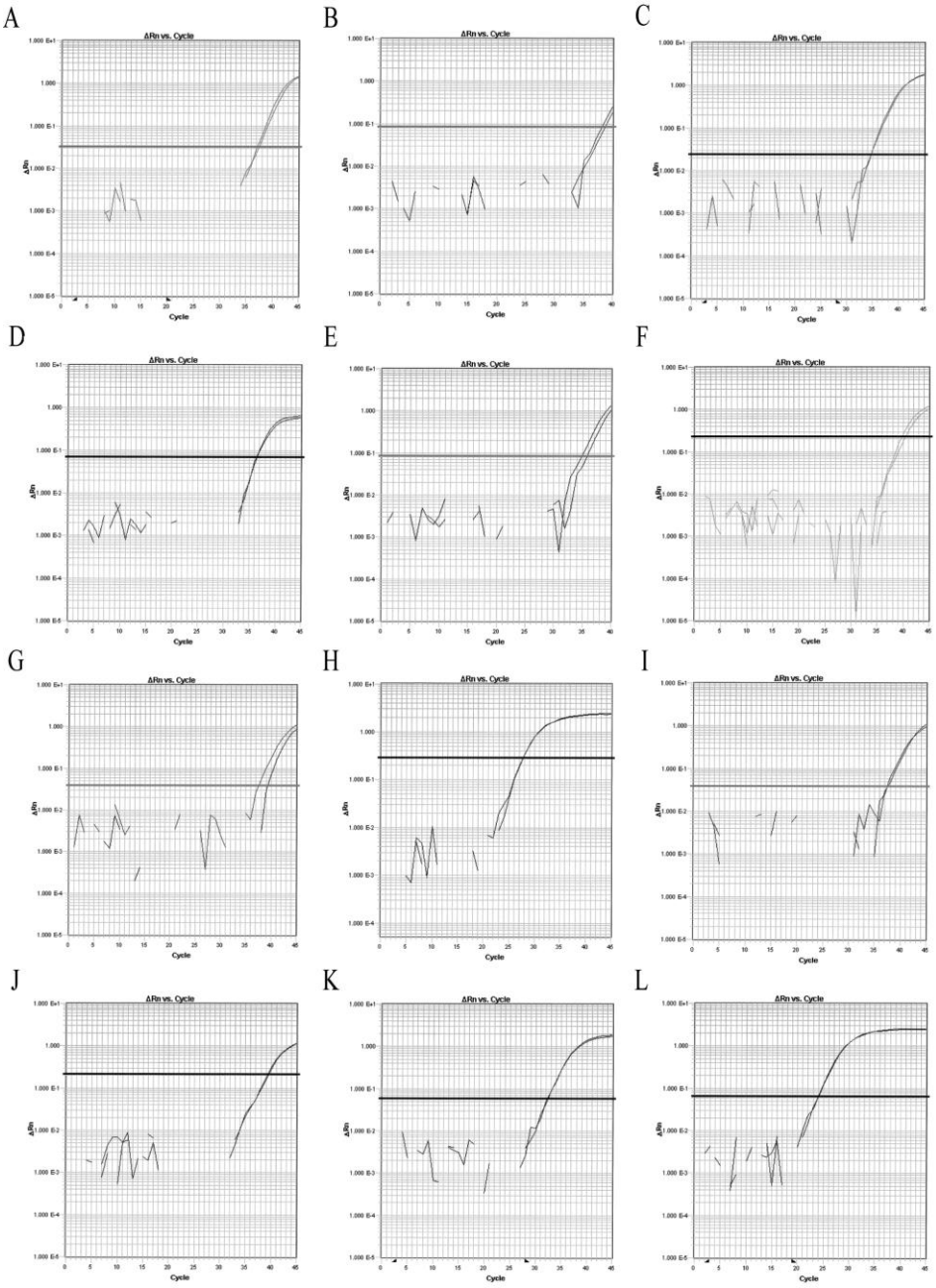

Figure 3.9: Representative real-time reverse-transcription polymerase chain reaction (RT-PCR) amplification plots for RNA positive control extracted from whole mouse brain tissue homogenate.

Representative ΔRn vs. cycle # amplification plots of replicates. All primers-probe pairs amplify as depicted for BDNF variants I, II, IIB, IIC, III, IV, V, VI, VII, VIII, IXA (A-K), respectively. The housekeeping gene control, TATA-binding protein (TBP), primer-probe pair also amplifies as expected (L).

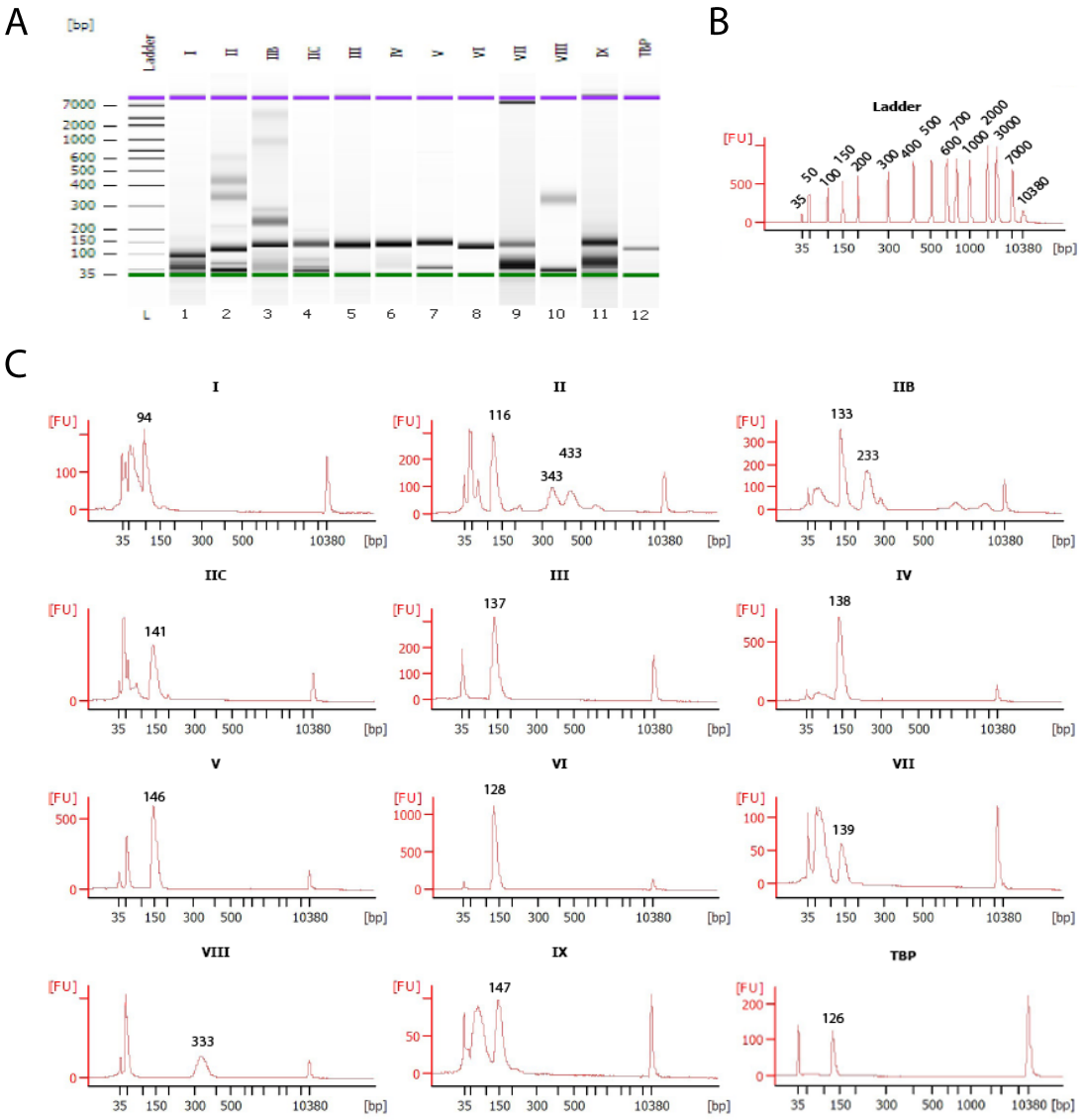

Figure 3.10: Confirmation of BDNF and TBP primer-probe pair specificity for expected amplicon size.

(A) PCR products generated from mouse brain cDNA were analyzed using a high sensitivity DNA kit and 2100 Agilent Bioanalyzer. Image depicts "electrophoresis" gel summary of amplicon bands for each BDNF variant or TBP primer-probe pair. (B) Electropherogram of DNA sizing ladder. The y-axis represents the fluorescence and the x axis represents size of base pairs. (C) Electropherogram of PCR products for BDNF variants I-IXA with detected amplicons size labelled .The the expected PCR fragments were identified on the electropherograms in all cases.

Each of these primer-probe pairs were then analysed and established that the amplified product was the same as the expected size of the *in silico* determined BDNF products (Fig. 9). As a positive control, cDNA from whole mouse brain tissue homogenate was amplified using real-time PCR. All primer-probe pairs for BDNF variants I, II, IIB, IIC, III, IV, V, VI, VII, VIII, IXA and housekeeping gene control, TATA-binding protein (TBP) were amplified as expected (Fig. 3.9). To confirm BDNF and TBP primer-probe specificity, PCR products from mouse brain cDNA were checked for accurate amplicon size (Table 3.2). In all cases, the expected PCR fragments were identified on the electropherograms (3.10). However, splicing within exon II yields three isoforms that were expressed, including IIB and IIC, and therefore detection of fragment sizes such as 116bp, 343bp and 433bp were observed. Primers for IIB amplified both IIB and IIC and detected amplicon sizes of 133bp and 233bp. The IIC primers only amplified the 139bp IIC fragment.

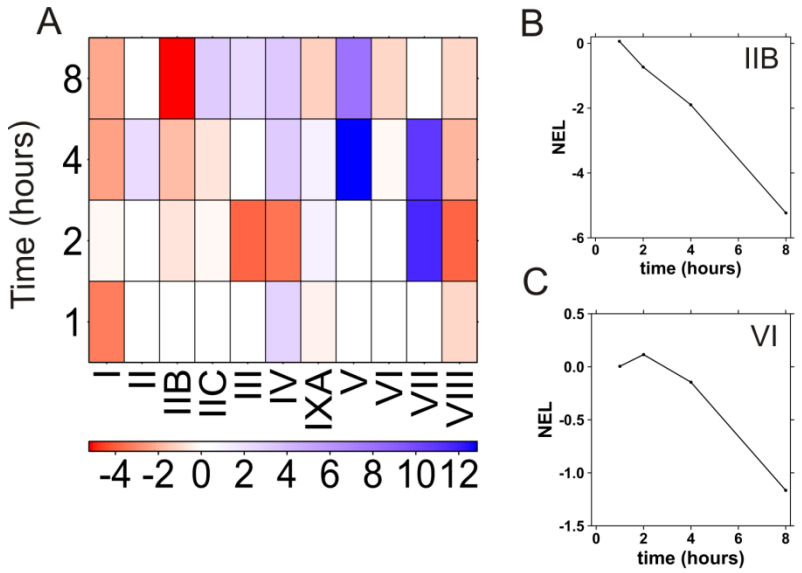

Figure 3.11. Regulation of BDNF splice variants by TCAP-1 as determined by reverse-transcriptase real-time PCR.

A. Heat map showing the change in splice variant expression levels over 8 hours. The splice variant is indicated by the Roman numeral at the bottom. A scale bar below the map shows the relationship of colour intensity to fold expression change. Red indicates a decrease in levels, whereas blue indicates and increase in levels. Significance of change was assessed by one-way ANOVA for each splice variant. The trends over 8 hours for splice variants that showed significance as a function of a post-hoc t-test are shown in B (splice variant IIB) and C (splice variant VI).

There was considerable variation among several of the splice variants, as would be expected (Fig 3.11A), however, three (III, IIB and VI) showed a significant effect of TCAP-1 treatment as determined by one-way ANOVA. Variant III ($p=0.0425$: ANOVA) at 2 hours followed by a slight increase by 8 hours. Both variants IIB ($p=0.0022$: ANOVA) and VI ($p<0.0001$; ANOVA) showed a significant effect of treatment by TCAP-1 when a significant decrease in expression levels occurred at 8 hours (IIB, $p=0.0318$; VI, $p=0.0031$: post-hoc t-test; Figs 3.11B and C). However among all splice variants a general decreasing trend was noted with the exception of V and VII which had large increases that were apparent among some treatment groups (Fig 10A).

3.4 Discussion

The data obtained in this study indicates that TCAP-1 has a direct effect on BDNF regulation in certain cell types. In 2004, Belsham *et al.* generated immortalized cell models from primary cultures of mouse embryonic hypothalamic cells that were transformed with the potent oncogene SV40 large T-Ag. The immortalized N38 cell line was used due to its initial characterizations that include having a neuronal phenotype, neurosecretory properties and long-term growth stability. Although the hypothalamus contains a heterogeneous mix of neuronal populations, the N38 cell line provided a distinct hypothalamic population to study the effects of TCAP-1 in isolation. This allowed for more focused analyses in future *in vivo* studies.

Previous studies indicate that TCAP-1 has a number of bioactive effects on these immortalized cells. The first TCAP-1 study on N38 cells described dose-dependent regulation of cAMP and cell proliferation (Wang *et al.,* 2005). In 2007(a), Al Chawaf *et al.* reported that TCAP-1 treatment increased neurite length and axon formation by influencing the levels and distribution of key cytoskeletal proteins and genes associated with axon outgrowth in N38 cells. Recently, TCAP-1 has been shown to confer neuroprotection and promote survival in N38 cells subjected to pH-induced cellular stress. TCAP-1 significantly enhanced cell proliferation, reduced the number of cells undergoing necrosis and combated superoxide radicals by stimulating the upregulation of superoxide dismutase and catalase (Trubiani *et al.,* 2007).

My results together with the aforementioned studies describe a TCAP-1 responsive cell line. I also establish the N38 cells as an appropriate model to study BDNF as indicated by the immunocytochemical studies illustrating positive immunoreactivity along with western blot analysis indicating forskolin-inducibility. In particular, N38 cells are believed to be derived from

the hypothalamic arcuate nucleus (Belsham, personal communication), a region rich in neuropeptides, such as neuropeptide Y, and intimately involved in energy regulation. In the hypothalamus, melanocortin neuropeptides inhibit food intake and control body fat via the melanocortin pathway (Wisse and Schwartz, 2003). Several studies have linked BDNF to energy homeostasis and obesity (Ono *et al.,* 1997; Rios *et al.,* 2001; Xu *et al.,* 2003) and suggest that BDNF-expressing hypothalamic neurons can inhibit food intake (Wisse and Schwartz, 2003). Therefore, the use of immortalized N38 cells as a model to study BDNF has implications in energy metabolism and in the treatment of obesity.

BDNF has attracted considerable interest due to its broad distribution in the central nervous system (Ernfors *et al.,* 1990; Phillips *et al.,* 1990; Ceccatelli *et al.,*1991; Friedman *et al.,* 1991; Guthrie and Gall, 1991; Gall *et al.,*1992; Castrén *et al.,* 1995). As a member of the neurotrophin family, BDNF has fundamental roles in mediating survival, differentiation, and outgrowth of central neurons during development and in adulthood (reviewed by Bailey, 1996; Hashimoto, 2004; Tapia-Arancibia, 2004). Therefore, it is conceivable that BDNF must possess a complex multi-level regulatory system of expression. Indeed, the rodent *bdnf* gene is comprised of eight 5'-non-coding exons and 1 common 3'-coding exon that produces an identical protein (Aid *et al.,* 2007). Presently, the use of alternative promoters, splicing and polyadenylation sites suggests the existence of 18 to 24 different BDNF variant transcripts (Timmusk *et al.,* 1993; Aid *et al.,* 2007; Tabuchi, 2008; Greenberg *et al.,* 2009; Cunha *et al.,* 2010). Several studies point at this complex regulation whereby BDNF function can be mediated by the use of different individual promoters that direct tissue-specific and activity-dependent expression of the various BDNF transcripts (Timmusk *et al.,* 1993; Aid *et al.,* 2007). The differential regulation of specific exon-containing transcripts by a variety of stimuli, mRNA

stability and subcellular localization of BDNF mRNA and protein has been thought to underlie the fine-tuning of BDNF function (Bailey, 1996; Tabuchi, 2008; Greenberg *et al.,* 2009).

In this study, immortalized N38 cells were established as a suitable model to study the effect of TCAP-1 on BDNF expression. N38 cells possess the ability to produce BDNF following treatment with forskolin and express components that enable TCAP to bind, be taken up into the cytosol and, under certain situations, enter the nucleus where it can effect gene transcription and elicit biological responses. The resulting reduction in intracellular BDNF protein corroborates with the marked shift of intracellular BDNF immunoreactivity to the cell periphery following acute TCAP-1 treatment and suggests that TCAP-1 is acting as a factor signalling the immediate release of BDNF into the extracellular matrix.

Several studies have shown alterations in the expression of BDNF mRNA following insult (Lindvall *et al.,* 1992) and during development (Ernfors *et al.,* 1992). In addition, BDNF transcripts are differentially regulated after kainic acid-induced seizures (Aid *et al.,* 2007). To date, expression studies in the central nervous system and dorsal root ganglion have been well studied (Timmusk *et al.,* 1993; Aid *et al.,* 2007; Matsuoka *et al.,* 2007). Therefore, due to the complex BDNF genomic control mechanisms governing BDNF transcripts, we specifically examine the effect of TCAP-1 on individual splice variants as reported by Aid *et al.* (2007). In this study, TCAP-1's ability to differentially regulate these BDNF transcripts suggests that it may be acting through specific BDNF transcripts to exert its neuroportective effects. Although only significant downregulation for the BDNF transcripts IIB and VI were observed, it is likely that any significant regulation of other transcripts could be masked by the considerable variation of TCAP's effect on all other splice variants studied. Interestingly, the upregulation of variant IIB and VI are involved in trafficking BDNF mRNA for local synthesis at the dendrites of

hippocampal neurons that favour epileptogenesis (Chiaruttini *et al.,* 2008). Regional expression of exon II mRNA cryptic splice variants A, B, and C and similarly exon III mRNA are differential and brain-specific with little expression in the nonneural tissues of rodents (Aid *et al.,* 2007). In contrast, exon VI is widely expressed in nonneural tissues. Exon II, III and VI containing transcripts are driven by separate promoters and the expression of exon III can also be regulated by histone modification and DNA methylation (Aid *et al.,* 2007). Essentially, it is not hard to conceive that such complex and differential regulation of BDNF splice variants may highlight distinct expression patterns that may be evident in neurodegenerative diseases, mood disorders and other pathologies where BDNF levels are altered.

Thus, we hypothesize that TCAP-1 provides the signal to release intracellular stores of BDNF, as found with the reduced protein expression and localization of BDNF at the cell periphery. Concomitantly, TCAP-1 acts to inhibit cell metabolism in order to conserve energy as reflected by the downregulation of energy-consuming cellular processes such as transcription. Ultimately, the release of BDNF following TCAP-1 treatment allows BDNF to act locally on neighbouring cells as a paracrine and/or autocrine factor. Binding of BDNF to its high-affinity receptor, TrkB, initiates downstream signal transduction pathways including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) or phospholipase Cɣ (PLC ɣ) that will promote survival (Yoshii and Constantine-Paton, 2009).

Despite having salutary neuroprotective actions, BDNF possess limitations that prevent its use as a pharmacological agent in treating diseases. Such challenges include a short plasma half-life *in vivo* and limited ability in exerting its effects across the blood-brain barrier (Kishino *et al.,* 2001). In spite of well founded clinical experiments stemming from the success of experimental work in animal models, the results have been disappointing. Various modes of

delivering BDNF have been considered and studied including gene therapy, BDNF releasing cell grafts, BDNF mimetics (small molecules that bind BDNF receptors at target sites), and drugs, however none have been approved due to a variety adverse effects (Zuccato and Cattaneo, 2009).

Interestingly, TCAP-1 has been shown to cross the blood-brain barrier (Al Chawaf, 2007c) and based on our present findings can regulate BDNF expression, to possibly mediate its neuroprotective effects. The results of this study are in line with our previous studies mentioned above whereby TCAP-1 is able to increase neurite outgrowth and proliferation and upregulate antioxidant defence mechanisms during pH-induced stress (Al Chawaf *et al.,* 2007a; Trubiani *et al.,* 2007). Unsurprisingly, BDNF has been implicated in neurite outgrowth and proliferation (Bailey, 1996; Cohen-Cory *et al.,* 2010) and has similarly been shown to combat reactive oxygen species via the upregulation of superoxide (Mattson *et al.,* 1995). Thus, TCAP-1 may serve as a potential therapeutic agent that exerts its neuroprotective effects via regulation of BDNF and presents as an alternative option to neurotrophic factor therapy.

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Chapter 4 : Conclusions

4.1 Summary

The findings of this study provide insight on the mechanism by which TCAP-1 has neuroprotective effects on cells *in vitro*. This study shows that TCAP-1 is able to enhance the number of immortalized mouse hypothalamic N38 cells under ambient (21% O_2) and lowered oxygen levels (5% and 1% O_2). TCAP-1 was able to influence neuronal behaviour of immortalized N38 cells by differentially regulating neurite outgrowth under varying levels of oxygen tension. It was found that treatment regiment (acute vs. chronic) and oxygen level played a role in the outgrowth of neurons. When the mechanism of TCAP-1s actions were investigated, it appeared that BDNF did not play a role. However, basal ambient conditions suggested that TCAP-1 influenced BDNF protein expression. Therefore, another aim in this study was to define the basal conditions of TCAP-1's neuromodulatory mechanism that may be involved in neuroprotection. I show that TCAP-1 downregulated BDNF protein and differentially regulated BDNF mRNA expression in N38 cells with an overall decreasing trend. I propose that TCAP-1 may act as a paracrine factor that signals the immediate release of BDNF followed by actions that inhibit energy-consuming processes such as transcription.

4.2 Overview

In chapter 2, TCAP-1's neuroprotective effect in immortalized embryonic mouse hypothalamic N38 cells under varying levels of oxygen tension was established. N38 cells were able to experience hypoxia and be responsive to changes in O_2 levels. Acute and chronic treatment with TCAP-1 was able to enhance N38 cell proliferation across all oxygen levels studied (21%, 4% and 1%) as early as 4 hours after treatment. The fact that 4% O_2 did not have a marked increase in cell death as cell numbers did not fall below baseline (100% at 0 hours)

reflects an oxygen level that is within an intermediary range of hypoxic stress tolerance in N38 cells. In addition, it is not clear whether cell proliferation (e.g. cell cycle) or cell death contribute to the increased number of live cell nuclei observed. Previous observations in the laboratory have not shown changes in cell cycle by TCAP-1, however, TCAP-1 has been able to reduce necrosis in N38 cells (Trubiani *et al.,* 2007).

Quantification of neurite numbers per cell made evident a discrepancy in the initial study at 21% O_2 that may be due to variations between cell culture batches despite every attempt to maintain consistency or represent the real effect by TCAP-1 on neurite outgrowth. I also observed a similar trend following acute TCAP-1 when comparing measurements of lowered oxygen (1% or 4% O_2) to those measured at 21% O_2 over 24 hours.

In the first experiment, if the results obtained at 21% are true and if immortalized cells are able to tolerate growth under 4% O_2 as suggested by my proliferation studies, then I hypothesize that TCAP-1 is stabilizing the N38 cells to continue growing as they would in the oxygen level they were selected and acclimated to survive in (21% O_2). In contrast both the acute and chronic TCAP-1 regiments consistently showed a decrease in neurite growth at 21% O_2. If this is the true result, then in the 24 hour study at 1% O_2, acutely administered TCAP-1 had a conservative effect by reducing neurite numbers while chronically administered TCAP-1 stimulates growth by elevating neurite numbers. However, since this is the first study to examine the response of N38 cells to TCAP-1 after the cells were subjected to hypoxic stress, the actual 'correct' and expected response still needs to be defined.

It should be mentioned that the 8 hour preliminary study was used to establish that a longer duration was necessary to yield effects expected for hypoxic stress to cells perceivably optimized for growth at 21% O_2. I associate the lack of effect on neurite growth seen in TCAP-1

treated N38 cells incubated at 1% O_2 with possible confounding variables not present in the longer study. I have also attempted to link my proliferation studies with my neurite data. The rapid decline in cells numbers in vehicle-treated cells under 1% O_2 in both acute and chronic TCAP-1 treatment regiments indicates that surviving cells are not likely to be in close proximity to easily establish synaptic connections. This supports the increasing trend in neurites of vehicle-treated cells compared to baseline (0 hours) under the acute treatment regime at 1% O_2. This implies that surviving N38 cells must actively extend their neurites to communicate with neighbouring cells for which survival may depend on. In the chronic regime vehicle-treated cells appear to display an initial increase in neurite numbers that tends back towards baseline (0 hours) at later time points in 1% O_2. Again this may indicate that surviving cells are trying to make connections with surrounding cells and survive.

Interestingly, I observed an increasing trend in neurite numbers for N38 cells acutely treated with TCAP-1 over time at 1% O_2 but not as observable as the increase seen in vehicle-treated cells. At 21% O_2, acute TCAP-1 primed cells appeared to have an energy-conserving effect over time in contrast to the increasing neurite growth trend observed in vehicle-treated cells. Under the chronic regiment in 1% O_2, TCAP-1 treated cells indicate an increasing trend at all time points studied that are significantly elevated compared to vehicle-treated cells at 24 hours and 36 hours. At 21% O_2, TCAP-1 treated cells had a decreasing neurite numbers trend that appeared to reverse after 24 hours which is consistent with the idea that the cell is becoming desensitized to TCAP-1 and resuming growth.

Lastly, I attempted to investigate the mechanism of TCAP's action in this study and thought to correlate it with brain-derived neurotrophic factor (BDNF), a neurotrophic factor that has been implicated in proliferation and neurite outgrowth (Bailey, 1996; Kiryushko, 2004).

However, my findings indicate that TCAP-1 does not have a clear effect on BDNF under low oxygen conditions. Of interest though is TCAP-1's ability to modulate BDNF under basal conditions which I investigate in chapter 3 to establish a basis that defines basal conditions a possible role for BDNF in TCAP-1's neuroprotective mechanism.

Therefore based on findings of chapter 2, I suggest that TCAP-1 may aid N38 cells in dealing with oxygen-related stress (enhance hypoxic tolerance) and allow for the maintenance of oxygen homeostasis at varying levels of reduced oxygen. Since physiological oxygen levels are much lower than traditional cell culturing concentrations and immortalized N38 cells were generated and selected to survive under ambient oxygen conditions (21% O_2), it is possible that N38 cells may have adapted to live under higher than physiological oxygen levels. This could explain the cell death observed following chronic incubation at 1% O_2. Therefore, I propose that TCAP acts as neuroprotective factor that prevents the decline in cell number, promotes survival and stimulates general cell proliferation at 21%, 4% and 1% O_2. At 4% O_2, TCAP-1 would act as a signal that reduces the neurite growth characteristic of cells perceiving and responding to stress and thereby enhances neurite outgrowth. The data suggests that in 1% or 4% O_2 conditions, acute TCAP-1 may be acting to re-establish its 'normal' response to cells treated with TCAP-1 at 21% O_2. The reduced HIF-1α protein expression following TCAP-1 treatment supports this notion that TCAP-1 is preventing the stress response from lowered oxygen concentrations and is able to trick the cell into believing it is not experiencing oxygen-related stress. The differences observed in neurite growth between acute and chronic TCAP-1 studies could be attributable to the altered TCAP-1 administration (chronic vs. acute). Since the metabolic cost of neurite outgrowth for cells is high under some circumstances, acute TCAP-1 may help prime the cell in readiness for a pre-emptive or more potent stress under both 21% and 1% O_2 levels and hence acts as a

protective energy-conserving factor. In contrast, prolonged chronic TCAP administration may initially prime cells which then later become desensitized to TCAP's message of caution and thereby better adapts to hypoxic conditions and continues neurite growth. Thus, chronic TCAP treatment may stimulate cells to form neurites and establish synaptic connections with their neighbours at least during stressful stimuli lasting 24 hours and longer.

Overall I present TCAP-1's modulatory effect on proliferation and neurite behaviour in immortalized N38 cells under varying oxygen levels. Although the exact mechanism of TCAP-1's actions is unknown, a possible interaction with cell adhesion molecules and extracellular matrix proteins may exist, however further experiments must be conducted. These findings have implications for the use of TCAP-1 as a therapeutic agent in the treatment of ischemic stroke that is characterized by interruptions in the blood supply to the brain. The lack of oxygen reaching neurons leads to rapid loss of brain functions that ultimately ends with neuronal death (Dirnagl *et al.,* 1999). Therefore, TCAP-1 may be a candidate treatment option to enhance proliferation, modulate neurite outgrowth, and increase hypoxic tolerance that mediates neuronal protection in stroke.

In chapter 3, the immortalized embryonic mouse hypothalamic N38 cells were established as a model for studying TCAP-1 and BDNF. The data indicates that TCAP-1 was able to bind, be internalized and sequestered in the nucleus of N38 cells. To verify the suitability of using N38 cells to study BDNF expression, Western blot analysis of N38 whole cell lysate was conducted and shown to detect the expected protein size for intracellular mature BDNF protein (14 kDa). Upregulation of BDNF after 1 hour of forskolin treatment confirms that N38 cells are BDNF-inducible. Immunoflorescence confocal microscopy further confirmed BDNF expression in N38 cells where it was localized in the cytosol. Similarly, primary rat hippocampal

cells were also able to bind and uptake TCAP-1 and co-express BDNF albeit with a difference that BDNF was localized in the cytosol following TCAP-1 treatment.

Having established N38 cells as a cell model that is responsive to TCAP-1 and exhibits the components for BDNF regulation, the effect of acute TCAP-1 treatment on BDNF expression was investigated. Western blot analysis found reduced BDNF protein, which could be due to a decrease in BDNF gene transcription or that it is being released from the cell. Following TCAP-1 administration, immunocytochemical studies depicted a clear shift in BDNF expression that was evident initially in the cytosol to the cell periphery. Indeed, real-time reverse transcription polymerase chain reaction found TCAP-1 to exert differential regulation of the 11 BDNF mRNA splice transcripts studied. In particular, BDNF IIB, III and VI were found to have a significant effect by TCAP-1 treatment. Overall, there was considerable variation among the variants some of which could have masked other significantly affected transcripts. A generally decreasing trend was observed however there were some exceptions including the BDNF variants V and VII which displayed increased levels at certain time points.

The immunocytochemical, Western blot and real-time RTPCR data suggests that TCAP-1 is signalling the immediate release of BDNF into the extracellular matrix and regulating BDNF mRNA variant expression, while concomitantly promoting a protective energy-conserving state implied by the reduced BDNF gene transcription. Ultimately, the immediate release of BDNF following TCAP-1 treatment has wide implications in the treatment of disease. Binding of BDNF to its high-affinity receptor, TrkB, initiates survival-promoting signal transduction pathways that could be beneficial in establishing TCAP-1 as a potential therapeutic drug for neurodegenerative diseases and obesity.

4.3 Future Direction

In the first study, I demonstrated that TCAP-1 has a neuroprotective effect under low oxygen conditions that was likely not mediated by BDNF. However, I did observe that TCAP-1 was able to regulate BDNF protein expression at ambient oxygen levels. In the second study, I aimed to define the basal conditions of TCAP-1's action on BDNF. Indeed, I confirmed that TCAP-1 regulates BDNF protein and mRNA. I propose several theories that could explain the downregulation of BDNF in immortalized N38 cells by acute TCAP-1 under the given basal conditions described above: 1) TCAP-1 could simply downregulate BDNF; 2) TCAP-1 causes the rapid release of BDNF with a concomitant downregulation; or 3) TCAP-1 causes the immediate release of BDNF as a signal for stress and proceeds to establish a protective and energy-conserving state in the cells that leads to the inhibition of a number of exocytotic mechanisms including BDNF.

It is important to note that the second study was done to establish the basal effect of acute TCAP-1 on BDNF. In the first study, when two different regiments (acute and chronic) of TCAP-1 administration were applied and yielded a difference in neurite numbers of N38 cells subjected to 1% O_2. This suggests that the mode of TCAP-1 delivery may be important in producing different effects under various conditions. Indeed, previous *in vitro* studies have exhibited neuroprotective effects by TCAP-1 using both chronic and acute modes of administration. Chronic TCAP-1 was able to confer neuroprotection to N38 cells by preventing pH-induced necrotic cell death (Trubiani *et al.,* 2007). In line with these studies was TCAP-1's ability to induce proliferation and neurite growth in N38 cells under an acute regiment (Wang *et al.,* 2005; Al Chawaf *et al.,* 2007a).

Behavioural studies *in vivo* have also shown different effects based on the mode of TCAP-1 injection into rats (Al Chawaf *et al.,* 2007b, Tan *et al.,*2008, 2009). One study that elegantly illustrates this point was conducted by Wang *et al.* (2005) who showed that acute injection of TCAP-1 into the basolateral nucleus of the amygdala resulted in a differential modulatory effect on the acoustic startle response (ASR) response that was dependent on the baseline reactivity. In contrast, when TCAP-1 was repeatedly administered intracerebroventricular (ICV) into rat brains, sensitization of the ASR did not occur. This highlights that TCAP-1 is able to differentially induce differential long-term neurological changes that can modulate the behavioural response of rats in the ASR based on the location and method of administration. Thus, both *in vitro* and *in vivo* aforementioned studies make it conceivable that the mode of TCAP-1 treatment (acute or chronic) plays a fundamental role in determining the outcome of TCAP-1's effects. To address this issue, the effect of TCAP-1 on BDNF regulation should be repeated using a chronic TCAP-1 regiment. This would define and establish the baseline conditions of TCAP-1's effect on BDNF under both acute and chronic delivery methods.

One other variation that may account for differences in the effect of TCAP is the concentration. The current study uses a concentration of 10^{-8} M TCAP-1. However, similar affects on neurite growth and proliferation by TCAP-1 using different concentrations have been observed. For example, TCAP-1 showed a significant effect in neurite length and neurite numbers per cell using 10^{-7} M TCAP-1 but not 10^{-9} M (Al Chawaf *et al.,* 2007a). In the study by Trubini *et al.* (2007), 10^{-7} M TCAP-1 enhanced cell proliferation under pH stress whereas Wang *et al.* (2005) indicated a dose-dependent effect on cell proliferation with a significant increase in activity at 10^{-9} M concentration and a decrease at 10^{-7} M concentrations at basal conditions.

Examining the various effects of TCAP-1 over a range of doses is important as TCAP-1's receptor has yet to be identified.

Based on the cAMP accumulation studies by Qian *et al.,* (2004) one hypothesis suggests that TCAP binds to two different G-protein coupled-receptors: a low-affinity receptor coupled to a G_i protein and a high-affinity receptor coupled to a G_s protein. This system would allow the high-affinity receptor to provide negative feedback on the TCAP precursor while the low-affinity receptor employs a positive feedback mechanism. Understanding TCAP's processing and receptor-mechanisms and its dose-dependent action will shed light on how TCAP acts independently of the teneurins in employing neuromodulation and neuroprotection at the cellular level and on behavior. Thus I propose producing dose-dependent curves when studying the effect of TCAP-1 under a certain model or stress paradigm.

Currently several studies have implicated TCAP in neuromodulation and neuroprotection and interestingly, BDNF may be involved in the mechanism of TCAP-1's actions. For example, Trubiani *et al.* (2007) showed that TCAP-1 was able to protect cells against damage by reactive oxygen species via the upregulation of superoxide dismutase and catalase. Similarly, BDNF has a fundamental role in oxidative stress metabolism whereby it was able to rescue neurons from H_2O_2-induced cell death (Onyango *et al.,* 2005). In another study, BDNF upregulates superoxide dismutase and this parallels the actions of TCAP-1 (Mattson *et al.,* 1995). To further corroborate a relationship between TCAP-1 and BDNF, these studies along with previous studies have implicated TCAP's role in proliferation and neurite growth and unsurprisingly, BDNF is recognized for its promotion of neurite outgrowth and proliferation (Bailey, 1996; Cohen-Cory *et al.,* 2010). Therefore, by establishing the basal conditions under a chronic regime, I may be better able to understand the mechanism by which both acute and chronic TCAP-1 regulates

BDNF mRNA and protein expression in N38 cells subjected to other stressful stimuli such as 5-fluorouracil-induced (apoptotic) cell death or glutamate-mediated excitotoxicity.

In the present study, I used an immortalized embryonic mouse cell line as a model due to the benefits of having stable cell growth and the opportunity to study an isolated population in the hypothalamus, a region known for its heterogeneous mix of neuronal populations. By using the N38 cells as a simpler model to investigate TCAP-1's effects, problems with primary hypothalamic cultures were avoided and include a short life span and the presence of glial populations. However, several disadvantages exist that must be addressed in future studies. Firstly, these cells were cultured under 21% O_2 (Belsham *et al.,* 2004), and based on this study, grows stably under these ambient conditions. Since oxygen levels typically range from 1.4 to 2.1% in the hypothalamus (Erecińska and Silver, 2001), the N38 cells may not produce the physiological response that would naturally occur *in vivo.* Secondly, although derived from primary cultures, the N38 cells contains the retrovirally transferred SV40 T-Ag immortalization factor (Belsham *et al* 2004) that offers a controlled variable which likely makes for a more robust cell line that may be more tolerant to stress. Thirdly, due to technical necessity, the N38 cells were derived from embryonic mouse cultures and thus are not representative of the adult hypothalamic neuron. In fact, the N38 cells express various neuropeptides that have not yet been reported in adult hypothalamic neurons (Belsham *et al* 2004). This is an important consideration as all *in vivo* TCAP studies to date have used behavioural models and postnatal rats. In this study, I point to TCAP-1's possible therapeutic benefits for the treatment of age-related neurodegeneration and stroke, which are pathologies that often occur later in adulthood. Therefore, due to the limitations of this cell line and the inherent problems with using primary hypothalamic cell cultures, I propose that *in vivo* experiments be conducted. For example,

immunohistochemistry studies on the brain slices of TCAP-1 treated rats for BDNF that may overlap at key areas of TCAP-1 expression. By identifying these BDNF-expressing and TCAP-1 responsive brain regions, a better understanding of what and where TCAP-1's role in modulating and contributing to the many BDNF-mediated pathways is. In the future, this will allow for a more focused study on specific BDNF pathways that promote survival, differentiation, synaptic plasticity and outgrowth of central neurons during adulthood (reviewed by Bailey, 1996; Hashimoto, 2004; Tapia-Arancibia, 2004).

Other *in vitro* studies that may strengthen the relationship between TCAP and BDNF include overexpressing TCAP-1 in N38 cells, and examining whether there is an alteration in basal BDNF expression levels. Alternatively, N38 cells induced by forskolin could be used to produce BDNF as done in the present study with the additional step in using acute and chronic TCAP-1 treatment that may possibly result in an enhancement or inhibition of BDNF mRNA and protein expression.

As mentioned above, many studies have implicated TCAP-1 to have a neuromodulatory effect (Qian *et al.,* 2004; Wang *et al.,* 2005; Al Chawaf *et al.,* 2007a, b; Tan *et al.,* 2008, 2009) which implies a role for TCAP in synaptic plasticity. Since BDNF has a recognized role in learning and memory and energy metabolism (Cunha *et al.,* 2010; Wisse and Schwartz, 2003), it is plausible that TCAP-1 may also be involved. Therefore, *in vivo* behavioral studies investigating TCAP-1's role in learning and memory and obesity would be interesting to further corroborate my *in vitro* studies. For example, TCAP-1 treatment could be administered to rats and their response tested in well established learning paradigms such as the Morris Water Maze. Alternatively, TCAP-1 may be tested on obese mice that may elucidate the peptide's potential role in the pathobiology of diabetes.

The use of *in vitro* and *in vivo* models to study neuroprotection differs fundamentally and poses different questions. This current study utilizes an *in vitro* model in order to breakdown complex biological processes into individual component parts in an attempt to better understand the cellular and molecular mechanisms of TCAP's actions. Moving forward, although my studies have benefitted from the use of immortalized cell types I recognize that it is also limited by the lack of complex synaptic connectivity and cell-cell interactions that normally occur in an intact brain.

Although it is accepted that *in vivo* models can be confounded by many variables, the next logical step would be to address the limitations of cell culture studies mentioned earlier. For example TCAP-1's neuroprotective effect and involvement in oxygen metabolism can be examined *in vivo*. TCAP-1 can be implicated in disease models and future experiments may utilize one or more of the several established focal ischemic stroke animal models: intraluminal suture, proximal or distal middle cerebral artery occlusion, middle cerebral artery embolism, endothelin 1 vasoconstriction, photothrombosis (reviewed by Murphy and Corbett, 2009). In addition how TCAP-1 may regulate BDNF in these models to confer neuroprotection should be examined. Collectively, both *in vitro* and *in vivo* studies would lead to a better understanding of the mechanism by which TCAP-1 treatment prevents neuronal death (e.g. due to ischemic stroke or chronic neurodegenerative diseases) as seen in my neuroprotective study.

4.4 Concluding Remarks

The teneurin's associations with neurite outgrowth, neurogenesis, axonal bundling and synaptic plasticity together with the aforementioned studies suggests TCAP may act as the functional C-terminal component of the teneurins. The present study points at the independent

actions of TCAP-1 from the rest of the teneurin protein in conferring neuroprotection and neuromodulation *in vitro.* Thus further *in vivo* studies and a greater understanding of TCAP's cellular mechanisms may lead to the use of TCAP-1 as a potential therapeutic agent in the treatment of neurodegenerative diseases, ischemic-stroke and obesity.

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