A Novel Pathway for Enhanced Metabolic Capacities Underlies the Neuroprotective Actions of Teneurin C-Terminal Associated Peptide (TCAP)-1

by

Mei Xu

A thesis submitted in conformity with the requirements for the degree of Masters of Science in Cell and Systems Biology

> Department of Cell and Systems Biology University of Toronto

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Abstract

Teneurin C-terminal Associated Peptide (TCAP)-1 is postulated to play a critical role in cellular defense mechanisms as it is highly neuroprotective against alkalotic and hypoxic stress. Optimization of metabolic pathways is recognized as an essential survival tactic by alleviating energy deficits and meeting the demands to cope with the stressors. The aim of this research was to delineate the mechanism through which TCAP-1 confers protection. My findings show that TCAP-1 increases the overall expression of GLUT1 and enhances overall expression and membrane localization of GLUT3. With respect to metabolic parameters, chronic TCAP-1 application led to increased intracellular [ATP] with decreased intracellular [lactate], both in a dose-dependent manner, but did not alter tumourgenic glycolytic enzyme expression or mitochondrially associated apoptotic protein expression. Contrastingly, acute TCAP-1 led to decreased intracellular [ATP]. Indicative of increased cellular ATP production and physiological energy expenditure, TCAP-1 reduced serum insulin levels and subcutaneous adipocyte size *in vivo*.

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List of Abbreviations

2DG, 2-deoxyglucose 2PG, 2-phosphoglycerate 3PG, 3-phosphoglycerate ACE, angiotensin-converting enzyme ACTH, adrenocorticotrophic hormone ADAMs, disintegrin-type metalloproteinases ALDOB, aldolase B AMPK, AMP-activated protein kinase ANOVA, analysis of variance APAF-1, Apoptotic protease activating factor 1 APP, amyloid precursor protein ARC, arcuate nucleus ATP, adenosine triphosphate AVP, arginine vasopressin BCA, bicinchoninic acid assay BMI, body mass index CCF, corrected cell fluorescence cDNA, complementary deoxyribonucleic acid CNS, central nervous system CRF, corticotropin-releasing factor CTFC, corrected total cell fluorescence dATP, 2'-deoxyadenosine triphosphate DAPI, 4',6-diamidino-2-phenylindole DH, diuretic hormone dH2O, distilled water DHAP, dihydroxyacetone phosphate DNP, 2,4-dinitrophenol ECL, enhanced chemiluminescence EDTA, ethylenediaminetetraacetic acid EEG, electroencephalography ENOL, enolase EPO, erythropoietin ERK, extracellular signal-regulated kinases ETC, electron transport chain F2,6BP, fructose 2,6-bisphosphate FAD, flavin adenine dinucleotide FITC, fluorescein isothiocyanate G6P, glucose-6-phosphate

GA3P, glyceraldehyde-3-phosphate GAPD, glyceraldehyde-3-phosphate dehydrogenase GAS, General Adaptation Syndrome GPI, glucose-6-phosphate isomerase H₂O₂ hydrogen peroxide H&E, hematoxylin and eosin HB-EGF, heparin-binding epidermal growth factor HEPES, hydroxyethyl piperazineethanesulfonic acid HIF-1, hypoxia-inducible factor-1 HK, hexokinase HPA, hypothalamic-pituitary-adrenal HRP, horseradish peroxidase IL-8, Interleukin-8 iNOS, inducible NO synthase I.O.D., integrated optical density IRS, insulin receptor substrate K_m Michealis-Menten LDH, lactate dehydrogenase LUCA, Last Universal Common Ancestor MAP, mitogen-activated protein MMPs, matrix metalloproteinases mRNA, messenger ribonucleic acid NAD, nicotinamide adenine dinucleotide NPY, neuropeptide Y O_2^{-} , superoxide anion ODD, oxygen-dependent degradation domain p90RSK, p90 ribosomal S6 kinase PBS, phosphate buffer saline PBST, phosphate buffered saline with 0.2% Tween PFA, paraformaldehyde PI3K, phosphatidylinositol 3-kinases PK, pyruvate kinase PFK, phosphofructokinase PGK, phosphoglycerate kinase PGM, phosphoglycerate mutase PLC, phospholipase C POMC, proopiomelanocortin PVN, paraventricular nucleus of the hypothalamus shRNA, short hairpin RNA Raf, Ras associated factor

Ras, GTP binding protein ROS, reactive oxygen species SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis SEM, standard error of the mean SOD, superoxide dismutase TCAP, teneurin C-terminal associated peptide TGFα, transforming growth factor α TOM, translocase of the mitochondrial outer membrane TNF α , tumour-necrosis factor α TNFRI, TNF receptor-I TNFRII, TNF receptor-II TPI, triosephosphate isomerase Ucn, urocortin UCP, uncoupling proteins VDAC, voltage-dependent anion channel VEGF, vascular endothelial growth factor VHL, von Hippel-Lindau WHO, World Health Organization

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1 Chapter One: Introduction

1.1 Discovery and Biological Significance of the Teneurin C-terminal Associated Peptides (TCAP)

Peptide hormones appeared approximately 3.5 billion years ago in early life forms and represent some of the most primitive signalling systems (Chang and Hsu, 2004; Lovejoy et al., 2006). In 2006, Ranea et al., identified 140 ancestral protein domains that are present in almost all 114 prokaryotic and eukaryotic genomes, they are theorized to originate from the Last Universal Common Ancestor (LUCA). Furthermore, the earliest representatives of ancestral neuropeptides and protein hormones can be found in cnidarians – the lowest metazoan group with a nervous system (Grimmelikhuijzen et al., 2002). With the evolution of more complex organisms, gene duplication events allowed for the formation of complementary peptide families to fill new functional niches. The corticotropin releasing factor (CRF) family of peptides appeared early in metazoan evolution and consequently have a high probability of being exposed to gene duplication events (Figure 1.1), potentially resulting in large networks of paralogous genes (Lovejoy and Jahan, 2006). Four paralogous lineages are present in chordates – CRF, urocortin/urotensin-I, urocortin 2 and urocortin 3; ranging from 38-41 amino acids in length, their structure consists of amphiphilic alpha helices (Chang and Hsu, 2004; Lovejoy and Jahan, 2006). An orthologous diuretic hormone (DH) lineage, with distinct structural deviations from vertebrate CRF peptides, is present in insect; two related forms of the peptide have been identified to date (Coast et al., 2001) suggesting that the common ancestor of the insect and chordate peptides occurred before the bifurcation of the deuterostome-protostome split (Lovejov and Balment, 1999; Lovejoy and Jahan, 2006; Lovejoy and Barsyte-Lovejoy, 2010).

Efforts to identify additional paralogous genes to the CRF family led to the discovery of the Teneurin C-Terminal Associated Peptides (TCAP) in 2004 by Qian *et al.* A rainbow trout orthologue of teneurin-3 was identified during a low-stringency screen of rainbow trout hypothalamic cDNA using a hamster urocortin 1 probe. Located at the tip of each teneurin carboxy terminus, the 43-residue sequence for TCAP can be liberated and processed into a 40- or 41-amino acid peptide (Lovejoy *et al.*, 2006). The teneurin's are a family of four type II transmembrane proteins that are predominantly expressed in the developing vertebrate central

nervous system and are characterized by an extracellular-oriented carboxy-terminus (Lovejoy, *et al.*, 2006; Baumgartner *et al.*, 1994). In addition to their role as cell surface receptors, evidence shows that the teneurins may also function as transcriptional regulators through the release of their intracellular domain (Tucker and Chiquet-Ehrismann, 2006). These proteins are essential to normal neural development as gene mutations are embryonic lethal (Tucker and Chiquet-Ehrismann, 2006) and have been found to promote neurite growth, adhesion and axon guidance (Young and Leamey, 2009).



Four separately encoded TCAP variants have since been established using *in silico* analysis; TCAP-1, -2, -3 and -4 are derived from the extracellular C-terminal region of their corresponding teneurin 1-4 paralogues (Wang *et al.*, 2005). TCAP-1,-2, and -4 are 41 amino residues in length and TCAP-3 is 40 amino residues in length; despite similarities in size, the peptides possess less than 20% sequence identity to the CRF family of peptides (Lovejoy *et al.*, 2006). Furthermore, sequence analysis of the TCAP family of peptides revealed characteristics of a cleavable bioactive peptide – an amidation motif encoded on the carboxy terminus and a cleavage motif encoded on the amino terminus (Wang *et al.*, 2005). The secondary structure of TCAP-1 is also distinct from the CRF family of peptides; it is comprised of a single beta turn containing single parallel and anti-parallel strands, with an insignificant component of alpha helices (Nock *et al.*, manuscript in preparation).

Phylogenetic analysis shows that the TCAP family of peptides are ubiquitously expressed among all metazoan species (Lovejoy *et al.*, 2006; Tucker *et al.*, 2011). Two genes are present in invertebrate species, whereas all four are present in vertebrate species; the latter are highly conserved, with about 80% identity among all four paralogues (Lovejoy *et al.*, 2006; Lovejoy *et al.*, 2009). This high level of conservation and expression among divergent metazoan species denotes that this family of peptides evolved well before the protostome-deuterostome divergence, and further studies indicate that the teneurin may have evolved in the choanoflagellates – the protistan lineage that is thought to have given rise to the metazoan (Tucker *et al.*, 2011) and played a role with the ingestion of nutrients. Consequently, the TCAP gene is essential for survival of multicellular animals. Genes with this expression pattern are typically associated with fundamental biological processes.

1.2 Unicellular vs. Multicellular Homeostatic Challenges

All organisms are inextricably tied to their surroundings and, as such, face the universal challenge of maintaining a consistent internal equilibrium, despite external perturbations (Somero, 1986; Strange, 2004; Chondrogianni and Gonos, 2010). Introduced in 1935 by Walter Cannon, the term "homeostasis" was used to describe the rapid reaction of compensatory and anticipatory adjustments to preserve the internal environment, thereby enhancing survival. At the cellular level, transport of oxygen, carbon dioxide, nutrients and waste occurs through the processes of diffusion and osmosis - which depend on correct ion, energy and water balance

(Somero, 1986). Maintenance of such balance occurs through elaborate homeostatic signal transduction mechanisms and is of particular importance in ensuring optimal enzyme activity (Strange, 2004). Biological enzymes function as catalyzers to increase the rate of virtually all the reactions that occur within the cell (Somero, 1986; Wolfenden and Snider, 2001). By aiding in the formation of the transition state, a chemical species that is an intermediate of the reactant(s) and product(s), enzymes can accelerate reaction rates by over a million-fold. This was reaffirmed in a study by Doshi et al. (2012) on enzyme conformational dynamics in Cyclophilin A – a peptidyl-prolyl cis-trans isomerase that acts on preceding proline residues in proteins to catalyze the isomerization of the peptide bond. The uncatalyzed reaction had an activation barrier of 20kcal/mol and required hundreds of seconds; this was accelerated by 10^{5} - 10^{6} times with the addition of Cyclophilin catalysts and reduced the energy barrier by approximately 9.3kcal/mol. There are two main models for enzyme activity; 1) the lock-and-key model, in which the substrate fits directly into the active-site of the enzyme and 2) the induced-fit model, in which binding of the substrate causes a conformational change in the enzyme and substrate (Wolfenden and Snider, 2001). Both these mechanisms highlight the specificity of enzymes to the reactions they govern and the importance of their 3D conformation (Somero, 1986; Wolfenden and Snider, 2001). Furthermore, enzymes have become highly evolved to function best under specific temperature, pH and substrate conditions (Somero, 1986; Dmitriev, 2003); if the correct cellular environment is not maintained, loss of enzyme function would ultimately lead to cell death.

Free-living organisms are directly exposed and subjected to continuous environmental fluctuations (Gasch *et al.*, 2000). Despite having limited ability to control these external variables unicellular yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, are able to cope with a surprising variety of sudden and drastic environmental perturbations. Well-studied conditions include growth at temperatures above or below 25°C, mediums of high osmolarity, toxic chemical exposure, desiccation and nutrient starvation (Attfield, 1997). These conditions trigger a rapid change in their internal milieu through activation of a large generalized transcriptional response. Classified as the "environmental stress response" (ESR) (Gasch *et al.*, 2000; Causton *et al.*, 2001; Chen *et al.*, 2003), it encompasses 14% of the genome, with increased expression of over 300 genes and reduced expression of over 600 genes.

In multicellular organisms, the difficulties of maintaining homeostasis is compounded as it must occur at both the cellular and organismal level (Kirouac *et al.* 2010); a generalized stress response would be counterproductive in a multicellular organism whose cellular activities are interconnected. At the organismal level, a threat that could potentially be detrimental to distant cells, and even the organism as a whole, may be initially experienced or sensed by a particular population of cells. The challenge lies with communicating a warning signal from these cells to non-neighbouring cells. A study of the gene expression response to heat shock, endoplasmic reticulum stress, oxidative stress, and crowding on HeLa cells and primary human lung fibroblast found the greatest changes to occur in genes involved with cell-cell communication (Murray, 2004). This phenomenon is characteristic of multicellular cells following homeostatic threats (Boldrick *et al.*, 2002). Thus, the establishment of intercellular stress response signalling molecules is imperative for the survival of multicellular organisms. During the course of animal evolution this event would quickly follow the transition from a unicellular protozoan to a multicellular metazoan (Srivastava *et al.* 2010, Figure 1.2).



Figure 1.2: Phylogeny of holozoans, metazoans and eumetazoans. Phyla for representative genomes that were utilized for comparison are depicted. Tree topology is a consensus based on sequence analyses of orthologous genes from complete animal genomes. Dark bars represent periods of extensive evolution. Adapted from Degnan *et al.*, 2009 and Srivastava *et al.* 2010.

1.3 Stress, the HPA axis and Primitive Stress Pathways

Along with introducing the concept of homeostasis, Walter Cannon identified the noxious aspects of the environment that threaten a steady internal milieu as "stress". In 1936, Hans Selye introduced the General Adaptation Syndrome (GAS) in a *Nature* publication titled "A Syndrome produced by Diverse Nocuous Agents". He established stress as a response state, regardless of the stressor, that could result in long-term changes. Cannon's definition of homeostasis is based on the premise that there is an ideal set of conditions for the internal milieu, but failed to account for the elicitation of different responses from different stressors. The term "allostasis" was introduced by Sterling and Eyer in 1988 and recognizes that there is no single ideal set of steady-state conditions; instead, set points and other response criteria change continuously. Allostatic mechanisms comprise an active, adaptive process that restores homeostasis and anticipates future assaults (Goldstein and Kopin, 2007). Sustained or repeated activation of the system may create "allostatic load", leading to adverse consequences and highlighting the importance of regulatory feedback loops (Goldstein and McEwen, 2002). The modern concept of stress is defined as a condition that is activated in response to a perceived discrepancy in information about a monitored variable to reduce the discrepancy (Goldstein and Kopin, 2007).

In highly derived organisms, the brain is the main regulatory organ responsible for the stress response. Exposure to stressors can generate an acute response via the sympathoadrenomedullary axes, which prepares the body for the classic "fight or flight" response (Cannon, 1935; Ulrich-Lai and Herman, 2009). Activation of preganglionic sympathetic neurons in the spinal cord travels to pre- or paravertebral ganglia, leading to stimulation of end organs and chromaffin cells of the adrenal medulla (Ulrich-Lai and Herman, 2009; Goldstein, 2010). The result is secretion of catecholamines, epinephrine from the adrenal medulla and norepinephrine from sympathetic nerves, increased heart rate, peripheral vasoconstriction and energy mobilization (Goldstein, 2010). An increase in sympathetic tone is often accompanied by a reduction in parasympathetic tone – a system whose actions work in opposition to the sympathetic system (Ulrich-Lai and Herman, 2009).

With continued stressor exposure, activation of the hypothalamic-pituitary-adrenocortical (HPA) axis ensues. The HPA axis is a neuroendocrine feedback system that is activated when afferents

from the sensory system and brainstem signal the hypophysiotrophic neurons in the paraventricular nucleus (PVN) to secrete corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) hormones into the hypophyseal portal system at the median eminence (Tsigos and Chrousos, 2002; Ulrich-Lai and Herman, 2009). Upon reaching the anterior pituitary, CRF stimulates the corticotropes to secrete adrenocorticotropic hormone (ACTH) into the systemic circulation ((Tsigos and Chrousos, 2002; Denver, 2009). ACTH acts on the zona fasiculata of the inner adrenal cortex to promote the synthesis and release of glucocorticoids, such as cortisol (Denver, 2009; Ulrich-Lai and Herman, 2009). Circulating glucocorticoids induce a multitude of different responses which all act to promote energy mobilization; these include gluconeogenesis in the liver, liberation of amino acids, inhibition of glucose uptake into muscle and adipose tissue, increased lipolysis and suppression of immune and reproductive functions (Ulrich-Lai and Herman, 2009). Regulation of the system occurs through negative feedback of glucocorticoids at the hypothalamus and anterior pituitary, inhibiting the release of CRF and ACTH respectively; in addition, the action of glucocorticoids on neuronal inputs to the PVN restricts activation of the HPA axis (Tsigos and Chrousos, 2002; Denver, 2009; Figure 1.3).



Figure 1.3: The hypothalamic-pituitary-adrenal (HPA) axis. Stressors stimulate the release of corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) from the paraventricular nucleus of the hypothalamus (PVN) into the hypophyseal portal system. CRF and AVP signal for the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the systemic blood flow; ACTH acts on the adrenal cortex to promote the production and release of glucocorticoids. Glucocorticoids function to mobilize energy reserves and negatively feedback onto the brain, PVN and anterior pituitary, thereby limiting its release.

Although the CRF family of peptides play a crucial role in vertebrate stress regulation, absence of the peptides in taxa phylogenetically older than the protostome/deuterostome bifurcation ascertains the existence of an ancestral stress response (Lovejoy and Jahan, 2006; Lovejoy and Barsyte-Lovejoy, 2010). In fact, the first bioactive peptides are estimated to have appeared 2 billion years prior to their evolution and thus primitive stress response systems were present well before their existence (Chang and Hsu, 2004; Lovejoy *et al.*, 2006; Srivastava *et al.* 2010). The presence of CRF-like peptides in the chordate and insect lineages (refer to Figure 1) is indicative of a common ancestral proto-CRF peptide; comparison of the lineages suggests that the ancestral peptide would bear little resemblance to the chordate form (Coast *et al.*, 2001; Lovejoy and

Barsyte-Lovejoy, 2010). The evolutionarily ancient TCAP family of peptides is universally present in all metazoan species and may represent one such primitive stress signal; they differ significantly from the CRF family of peptides in both their sequencing and secondary structure. TCAP-1 has been found to be highly neuroprotective against high alkaline, high calcium and low oxygen stressors (Trubiani *et al.*, 2007). Moreover, cell viability in cultures of immortalized embryonic mouse neurons is significantly reduced in shRNA knockdowns of TCAP-3, the TCAP paralogue predominantly expressed in embryonic mammalian neurons (Al Chawaf *et al.*, 2007; Casatti et al., manuscript in preparation).

1.4 TCAP-1 Processing and Signaling

The advent of multicellularity would require one crucial innovation – signalling systems for communication, regulation and coordination of cellular activities within the organism. Communication between cells is accomplished by a variety of electrical and chemical signals; these include hormones, cytokines, ions, growth factors, small molecules, neurotransmitters, peptides, proteins, lipids and carbohydrates (Ben-Jonathan and Liu, 1992). These trigger a cascade of events, known as a signalling pathway, resulting in numerous cellular responses. Although the cell membrane acts as a boundary between the exterior and interior of a cell, certain populations of cells have their cytoplasm directly connected by protein bridges, known as gap junctions (Denef, 2008). Within the brain and heart, they form electrical synapses which allowed for the propagation of electrical impulses. In addition, gap junctions also allow for the bidirectional transport of metabolites, second messengers, ions and other molecules smaller than 1kD (Meier and Dermietzel, 2006). More widespread modes of intercellular communication fall under the category of receptor-mediated signalling and can be divided into four types: autocrine, juxtacrine, paracrine and endocrine signalling (Figure 1.4). Short-distance communication occurs through: (1) autocrine, with the sender and the receiver being the same cell; (2) paracrine, where a cell sends a signal which is received by neighbouring cells; and (3) juxtacrine signals, where one cell sends a signal to a physically adjacent cell (Ben-Jonathan and Liu, 1992; Abe, 2000; Blobel, 2005; Denef, 2008). These signals play a critical role in the local niche of a cell and likely represent more primitive forms of communication (Srivastava et al. 2010). Increased morphological complexity and tissue specialization would create a need, and provide the means,

for long-distance communication through endocrine signalling; this entails travel of a chemical signal through an extracellular medium to reach distant cells (Ben-Jonathan and Liu, 1992; Abe, 2000).



Figure 1.4: The four modes of receptor-mediated intercellular signalling. Autocrine signals are sent and received by the same cell. Juxtacrine signals are sent from one cell and are received by a physically adjacent cell. Paracrine signals are sent from one cell and are received by cells within the surrounding vicinity. Endocrine signals are sent from a cell, travel through an extracellular medium and are received by distant cells. A membrane-anchored signal may be cleaved and released through a process known as ectodomain shedding. TCAP is found primarily in the cytosol and postulated to function as a paracrine stress signal following release from nearby dying cells. Adapted from Abe, 2000; Blobel, 2005.

The current proposed model for TCAP processing constitutes two separate, but not necessarily exclusive, pathways: 1) as part of the complete teneurin mRNA, where it is cleaved in vesicles or at the plasma membrane or 2) as a shorter splice variant by free ribosomes, where it is released upon cell death (Figure 1.5). The first processing pathway, classified as protein ectodomain shedding, contributes to the processing of several functionally diverse molecules; cytokines and growth factors, including tumour-necrosis factor α (TNF α), transforming growth factor α (TGF α), heparin-binding epidermal growth factor (HB-EGF), which all require membrane cleavage to

reach their target cells (Blobel, 2005; Montes de OcaB, 2010). Ectodomain shedding also participates in TNF receptor-I (TNFRI), TNF receptor-II (TNFRII) and ErbB4 receptor activity and the processing of other molecules, including amyloid precursor protein (APP) and angiotensin-converting enzyme (ACE) (Blobel, 2005). Two large families of zinc-dependent proteinases, the matrix metalloproteinases (MMPs) and the disintegrin-type metalloproteinases (ADAMs), are responsible for membrane-proximal cleavage of the protein to release the soluble ectodomain (Montes de OcaB, 2010). Despite the prevalence of ectodomain shedding in the processing of many signalling molecules, TCAP-1 findings favour the second processing pathway. Absence of a signal peptide on the exon encoding the short transcript indicates that the peptide is soluble in the cytosol (Nock *et al.*, manuscript in preparation); this is characteristic of proteins that are synthesized on free ribosomes. Cytosolic processing of TCAP peptides is further substantiated by findings of extensive cytosolic labelling for immunoreactive TCAP-1 (Chand et al., 2010). Once released, TCAP stimulation of neighbouring cells may subsequently activate survival mechanisms. In vitro application of FITC-TCAP-1 is found to co-localize with βdystroglycan subunit of the dystroglycan complex at the plasma membrane, where it binds to activate intracellular signaling cascades (Chand et al., manuscript submitted). Cell culture studies show that TCAP-1 activates MEK1/2 and its downstream kinase ERK1/2, leading to the phosphorylation of stathmin at serine-25 and p90-ribosomal S6 kinase (p90RSK) at serine-380 (Chand *et al.*, manuscript submitted). Following β-dystroglycan stimulation, TCAP-1 may be internalized into the cell via receptor-mediated endocytosis. Although the functional significance of this is unknown, FITC-TCAP-1 was found to penetrate the cytosol at 15 minutes with strong nuclear localization at 60 minutes (Ng et al., 2011). It is theorized that TCAP trafficking toward the nucleus may activate additional cellular responses or be targeted for degradation (Ng et al., 2011).



Figure 1.5: Proposed Processing and Release Pathways for TCAP. (1) TCAP may be processed as part of the complete teneurin mRNA, where it is cleaved in vesicles or at the plasma membrane via ectodomain shedding. (2) TCAP processing may occur in the cytosol, where a shorter splice variant is processed on free ribosomes and released upon cell death. (Ng., 2010)

1.5 TCAP-1 Expression in regions sensitive to stress and/vital for organismal survival

In accordance with TCAP's protective effects, the release of the peptide following cell death may function to activate cellular survival pathways in the remaining viable population. Colocalization of FITC-labeled TCAP-1 and immunoreactive cytosolic TCAP-1 within hippocampal regions of the mouse brain is suggestive of a paracrine function (Chand *et al., 2010*). As a local paracrine modulator, TCAP release from discrete populations would induce localized changes. This mechanism is crucial for regions sensitive to stress and/or vital for survival of the organism; substantial expression of TCAP-1 throughout the brain and reproductive tissue supports this theory.

As the body's master regulator, the brain demands a continuous and enormous amount of oxygen and glucose to fuel the multitude of functions that it governs; it accounts for only 2% of body weight, yet utilizes nearly 20% of cardiac output (Ratan *et al.*, 2007). These high energetic demands render the brain susceptible to a variety of traumas such as hemorrhagic stroke, ischemic stroke and traumatic injuries. Hemorrhagic stroke is the consequence of bleeding in the brain; the build-up of blood simultaneously increases pressure on the brain while cutting off the

blood supply. Although it is the more difficult type of stoke to treat, it is responsible for only a small percentage of strokes (Bamford *et al.*, 1990). The majority of strokes occur as a consequence of ischemia – the process by which diminished tissue perfusion from vascular occlusion creates an energy deficit (Ratan *et al.*, 2007). Early manifestation of symptoms and rapid tissue decline highlight the gravity of this deficit. A reduction in hemispheric cerebral blood flow to 40-50% of baseline levels leads to EEG slowing, attenuation of evoked potentials, and reduction in the membrane potential in individual cortical neurons (Sharbrough *et al.*, 1973). If blood flow falls to 20-30% of baseline levels, permanent damage and death will quickly follow (Hossmann *et al.*, 1988). Distribution of TCAP-1 in the brain coincides with stress and anxiety associated regions; immunoreactivity and mRNA expression in the primate brain occurred in the cerebral cortex, cerebellum, hippocampus, amygdala, basal nucleus of Meynert and supraoptic nucleus (Bittencourt, unpublished findings). Similar mRNA distribution was found in the rat brain with additional expression in the ventromedial nucleus of the hypothalamus, subthalamic nucleus, olfactory bulb and brainstem (Wang *et al.*, 2005).

Rapid cellular proliferation is exhibited in reproductive tissues – consequently, these biologically valuable tissues are prone to hypoxia (Verratti et al., 2008) which may ultimately result in fertility problems arising from tissue damage (Gat et al., 2006). Low fertility observed in males living in high altitudes first alluded to the effects of hypoxia on fertility (Verratti *et al.*, 2008). Hypoxia disrupts the delicate process of spermatogenesis by affecting the onset of mitosis and spermiation, resulting in decreased sperm count and motility (Gat et al., 2006; Verratti et al., 2008). Restoration of normal sperm parameters, after a period of 6 months at sea level, indicate that the hypoxic environment associated with high altitude living is responsible for reduced male fertility (Verratti *et al.*, 2008). Hypoxic stress can also lead to irreversible infertility; for instance, hypoxic testicular microcirculation gives rise to germ cell aplasia (Gat *et al.*, 2006). This is a common occurrence in males suffering from Varicocele – a bilateral vascular disease (Gat et al., 2006). Evidence that oxygenation is also important in female reproductive tissues is exhibited by the upregulation of angiogenic growth factors, such as IL-8, in response to hypoxic stress within the ovaries (Yoshino *et al.*, 2003). Within the reproductive tissues, high expression of TCAP-1 is observed in both the female and male sex cells and additionally, the ovarian and follicular tube epithelia of females (Chand et al., 2010).

1.6 Neuroprotective Actions of TCAP-1

The cellular mechanism of TCAP-1 is not entirely understood but previous studies indicate that the peptide is active in stressed cells and may play a critical role in cellular defense mechanisms and survival pathways (Ng *et al.*, manuscript in preparation; Trubiani *et al.*, 2007). Early observations of increased survivability of TCAP-1 treated neuronal cultures under stressful conditions, such as high confluency, inspired a new direction of investigation into the possible neuroprotective actions of TCAP (Trubiani *et al.*, 2007).

Successful rescue from pH-induced stress, which is associated with hypoxia-ischemia and metabolic alkalosis, was demonstrated in immortalized embryonic mouse hypothalamic mHypoE-38 neurons by Trubiani *et al.* (2007) using chronic TCAP-1 treatments. Recovery of basal cellular proliferation levels was observed at pHs 8.0 and 8.4 following 100nM TCAP-1 treatments. Furthermore, 100nM TCAP-1 decreased the incidence of necrotic cells at pHs 6.8, 8.0 and 8.4; this was morphologically observed as a reduction in the number of small, crenated cells and confirmed by reductions in membrane permeability to the nucleic acid probe, ethidium homodimer III (EtD II). This neuroprotective phenomenon may be attributed to TCAP's ability to decrease reactive oxygen species (ROS) through upregulation of superoxide dismutase 1 (SOD1) protein and mRNA expression, catalase activity and SOD copper chaperone mRNA expression (Trubiani *et al.*, 2007).

In addition to oxidative stress protection, Ng (2010 and manuscript in preparation) established that TCAP-1 also protects mHypoE-38 cells from hypoxic stress. Under acute hypoxic stress of 4% O₂, TCAP-1 was found to increase neurite cell outgrowth. Chronic studies show that TCAP-1 increases neurite outgrowth under hypoxic conditions of 1% O₂ but decreases neurite outgrowth under normoxic conditions. One hallmark characteristic of hypoxic stress is the expression of hypoxia inducible factor-1 (HIF-1) (Wang *et al.*, 1993; Salceda *et al.*, 1997; Duffy *et al.* 2003). The transcription factor HIF-1 is composed of two subunits, a beta subunit that is constitutively expressed and an alpha subunit that is upregulated during hypoxia (Wang *et al.*, 1993; Duffy *et al.* 2003). Under normoxic conditions, hydroxylation of proline residues, Pro402 and Pro564, by the active prolyl-4-hydroxylase enzyme facilitates binding of the von Hippel-Lindau (VHL) protein to the oxygen-dependent degradation domain (ODD) on HIF-1 α (Salceda *et al.*, 1997; Duffy *et al.* 2003). Encoded for by the VHL tumour suppressor gene, the VHL protein is a subunit of the E3 ubiquitin ligase complex and leads to rapid degradation of HIF-1 α through the ubiquitin-proteasome system; this results in a normoxic cellular half-life of 5 minutes (Salceda *et al.*, 1997; Brahimi-Horn *et al.*, 2001). Inactivation of the VHL gene is associated with tumourgenesis and occurs in 70% of clear-cell renal carcinoma cases (Cronin *et al.*, 2010). Hypoxia stabilizes HIF-1 α through inactivation of the hydroxylase enzyme, allowing the HIF-1 α subunit to translocate into the nucleus where it dimerizes with HIF-1 β to form the active HIF-1 complex (Salceda *et al.*, 1997). Binding of HIF-1 to hypoxia response elements alters transcriptional activity of select genes involved in angiogenesis (VEGF), vasodilation (INOS), oxygen carrying capacity (EPO), and glycolytic metabolism (Duffy *et al.* 2003). Treatment with 100nM of TCAP-1 led to a significant reduction in HIF-1 α expression in mHypoE-38 cells experiencing hypoxic stress of 1% O₂, resulting in expression levels that were comparable to cells grown under normoxic conditions of 21% O₂ (Ng, 2010 and manuscript in preparation). These findings suggested that TCAP-1 exerts its effects, in part, by reducing the aerobic demand on cells.

1.7 The Warburg Effect of Tumourgenesis

Cancer is characterized by uncontrolled cellular proliferation; consequently, hypoxia is a common challenge for growing tumours (Koukourakis *et al.*, 2006). Oxygen tensions in tumour tissues are significantly lowered, ranging between 0-20 mmHg, compared to values of 24-66 mmHg in adjacent tissues (Cronin *et al.*, 2010). Chronic diffusion-limited hypoxia occurs in solid tumours 100µm from vessels and transient state hypoxia occurs during fluctuations in perfusion (Koukourakis *et al.*, 2006; Cronin *et al.*, 2010). Although seemingly counterintuitive, there is a positive correlation between hypoxia and tumour malignancy (Duffy *et al.* 2003; Knowles and Harris, 2010). The microenvironment plays an integral role in tumourgenesis at both the molecular and cellular level (Duffy *et al.* 2003). It is theorized that hypoxic stress leads to an adaptive response, which improves survival and proliferation of neoplastic lesions (Knowles and Harris, 2010). The compensatory mechanisms required for continued tumourgenesis under limited oxygen availabilities provided a possible mechanism for TCAP-1 mediated hypoxia protection.

The glycolytic addiction of hypoxic cancers is known as the Warburg Effect (Haldar *et al.*, 1994; Gupta *et al.*, 2000). While studying the process of oxygen consumption in living cells, Otto Heinrich Warburg observed that growing cancer cells exhibited reduced oxygen demands and abnormally high rates of glucose consumption through favouring glycolytic metabolism (Warburg, 1956; Kim and Dang, 2007). This resulted in increased production of the glycolytic by-product lactic acid and provided an explanation for the acidic pH observed in tumours (Warburg, 1956; Airley and Mobasheri, 2007; Kim and Dang, 2007). He published his findings in 1956 and theorized that cancer was the product of defective cellular metabolism. Although mutational accumulation is now known to be the cause of carcinogenesis, it is important to acknowledge the significant contribution of Warburg's research to understanding cancer cell metabolism. The metabolic shift that allows cancer cells to thrive under hypoxic conditions requires complex metabolic rearrangements (Semenza *et al.*, 1994; Hammerman *et al.*, 2004; Garber, 2006; Kim and Dang, 2007; Scatena *et al.*, 2008) and may provide insight on the changes in protein expression that occur during TCAP-1 mediated hypoxic stress protection.

1.8 Detrimental Consequences of Sustained Glycolysis

Maintenance of cellular metabolism is crucial to proper biological functioning and tissue energy demands need to be satisfied regardless of substrate availability (Boutilier, 2001; Ravij *et al.*, 2007). Failure to meet energy demands due to reduced oxygen availability renders cells stressed, eventually resulting in death (Boutilier, 2001; Jin *et al.*, 2007). Although glycolysis offers an immediate solution, the detrimental consequences associated with prolonged reliance render it unsustainable (Mazoor *et al.*, 2008). The process yields a net production of only 2 ATP molecules and generates the undesirable by-product of lactic acid (Kim and Dang, 2007; Mazoor *et al.*, 2008). Quickly multiplying cells surrounding a necrotic core is characteristic of solid tumours due to the non-uniform distribution of nutrient availability (Jin *et al.*, 2007; Mazoor *et al.*, 2008).

Cancer cells progressively evolve functions that promote growth, disable cell death mechanisms and evade immune detection; thereby releasing them from conventional regulations where cell growth is tightly regulated by metabolic processes. The process begins with activation of oncogenes or inactivation of tumour suppressor genes (Tabor, 1994; Dang *et al.*, 1997). Proto-oncogenes, such as the MYC gene, are normal genes involved in the regulation of controlled cell growth; mutations or abnormally high protein expression levels manifests as unregulated cell

growth and transformation (Dang et al., 1997). In contrast, tumour suppressor genes, such as p53, encode for proteins that function to repress cell cycling, repair damaged DNA or promote apoptosis; inactivation is a "two-hit" process requiring functional loss at both alleles (Tabor, 1994). As the tumour cells divide, they outgrow the surrounding vasculature. In response to reduced perfusion, pro-angiogenic signals, such as VEGF, are released in an attempt to restore nutrient balance (Duffy et al. 2003; Knowles and Harris, 2010). Yet the unregulated nature of tumours results in a poorly formed entanglement of vessels around the lesion (Koukourakis et al., 2006; Cronin et al., 2010). In addition, lack of oxygen availability leads to reliance on glycolytic metabolism and the generation of lactate (Warburg, 1956; Airley and Mobasheri, 2007). Normally, intracellular lactate accumulation exits the cell and enters the bloodstream to be cleared via a process known as the "lactate shuttle" hypothesis (Brooks, 1985). During strenuous exercise, skeletal muscle lactate is shuttled to areas of high cellular respiration where it is removed through oxidation; for instance, oxidation at the liver regenerates glucose. However, this has now been classified as the "cell-cell lactate shuttle" as discovery of lactate oxidation in isolated liver mitochondria has prompted the introduction of the "intracellular lactate shuttle" hypothesis (Brooks, 1988). In this hypothesis, actively respiring mitochondria are capable of gluconeogenesis due to the intra-mitochondrial localization of LDH (Brandt et al., 1987). Since hypoxic tumours lack both the vasculature to export lactate through the cell-cell lactate shuttle and the oxygen tensions required for the intracellular lactate shuttle, lactate accumulates in the tumour, leading to increasing acidity (Warburg, 1956; Manzoor et al., 2008). The end result is formidable metabolic and acidic stress.

Under normal circumstances, cells experiencing high levels of stress undergo apoptosis – a process of programmed cell death which minimizes tissue disruption, prevents inflammation and allows for reutilization of cellular components (Majno and Joris, 1995; Fink and Crookson, 2005; Jin *et al.*, 2007). Nuclear and cytoplasmic condensation of the dying cell is followed by fragmentation into membrane-bound apoptotic bodies, which can be easily engulfed by phagocytes to avoid damaging surrounding tissues (Majno and Joris, 1995; Emery *et al.*, 1998; Fink and Crookson, 2005). In contrast, necrosis is a form of unregulated cell death arising from impairment of homeostatic mechanism (Jin *et al.*, 2007; Majno and Joris, 1995; Fink and Crookson, 2005). Oncosis, from "onkos" meaning swelling, describes a particular necrotic pathway arising from severe lack of perfusion, and possibly by toxic agents, that interferes with

ATP generation or increases the permeability of the plasma membrane (Fink and Crookson, 2005). Failure of the ionic pumps at the plasma membrane leads to in an influx of water and extracellular ions, followed by organelle and cellular swelling, and results in rupturing of the cell membrane (Majno and Joris, 1995; Fink and Crookson, 2005). The ensuing release of toxic cytoplasmic components onto surrounding cells is often associated with extensive tissue damage and inflammation (Jin *et al.*, 2007; Majno and Joris, 1995; Fink and Crookson, 2005). Cancerous cells may defer apoptosis, but the toxicity of glycolytic by-products and ablation of available nutrient sources eventually causes them to succumb to necrosis (Jin *et al.*, 2007).

1.9 Metabolic Optimization – how efficient resource utilization and energy production alleviates cellular stressors

The expression of TCAP in biologically essential tissues warrants a more pragmatic approach for sustained energy production. Glucose oxidation allows for maximal energy rendering per glucose molecule - one mole of glucose yields roughly 34 mol of ATP (Klip et al., 2009). In addition, the use of oxygen as the final electron acceptor produces a harmless by-product, water (Jin et al., 2007; Mazoor et al., 2008). Recently, acknowledgement of the energetic costs and limiting factors associated with metabolic processes has overturned previous notions of a set ATP return per glucose molecule (Hammerman et al., 2004; Brand, 2005; Sanz et al., 2006). This has fostered interest in targeting cellular energy production for the treatment of neurological disorders, as a neuroprotective strategy against acute and chronic neurological diseases, and for the improvement of cognitive functioning (Liu et al., 2002; Baker and Tarnopolsky, 2003; Gold, 2005). Furthermore, defective cellular energetics is not limited to pathologies of the nervous system (Bains, 2008; Fragasso *et al.*, 2008). Cardiac metabolic substrate use shifts between fatty acids and glucose; manipulations that shift substrate utilization towards glucose, as glucose provides a greater efficiency in producing high energy products per oxygen consumed compared to fatty acids, have been found to improve cardiac function and slow the progression of heart failure (Fragasso et al., 2008; Beadle and Frenneaux, 2010). These studies offer key insights into the mechanisms by which TCAP rescues cells from a multitude of stressors. Reorganization and reallocation of cellular resources to optimize metabolic functions would lead to more economical use of substrates and allow for greater net ATP yield – a process deemed as metabolic optimization.

1.10 Target 1: Glucose Transporters

A shift in the rate limiting process of glucose transport is the first potential step in metabolic optimization and is mediated by facilitative, Na⁺-independent, glucose transporter proteins of the GLUT/SLC2A family (Airley and Mobasheri, 2007). These transporters are characterized by several conserved sequence motifs and the presence of 12 membrane spanning helices (reviewed by Joost and Thorens, 2001); their expression is tissue and cell specific, where they exhibit distinct kinetic properties, substrate specificities and regulatory mechanisms (Airley and Mobasheri, 2007; Table 1.1). Despite their differences, all transporters of this family utilize the diffusion gradient of glucose across plasma membranes for facilitative transport. In comparison, members of the SLC5A family are Na⁺-dependent glucose co-transporters that transport glucose across the luminal membranes of cells lining the small intestines and proximal tubules of the kidneys (Wood and Trayhurn, 2003). Glucose transport against its concentration gradient is fueled by the Na⁺-electrochemical gradient generated by the Na⁺ - K⁺ ATPase Pump (Wood and Trayhurn, 2003; Airley and Mobasheri, 2007). Since this type of transport functions primarily in digestive absorption, it bears no relevance to the process of metabolic optimization.

Isoform	Class	Tissue localization	Substrate	Insulin sensitive
GLUT1	Ι	Ubiquitous, all cells	glucose	no
GLUT2	Ι	Liver, intestines, pancreas and kidneys	Glucose (low affinity); fructose	no
GLUT3	Ι	Brain, cartilage	Glucose (high affinity)	no
GLUT4	Ι	Heart, muscle, adipose tissue, brain	Glucose (high affinity)	yes
GLUT5	II	Intestine, testes, kidney	Glucose (very low affinity); fructose	no
GLUT6	III	Brain, spleen, leukocytes	glucose	no
GLUT7	II	intestine	Glucose and fructose	no
GLUT8	III	Testes, brain and other tissues	glucose	yes (blastocyst)
GLUT9	II	Liver, kidney, placenta, cartilage	n.d.	n.d.
GLUT10	III	Liver, pancreas	glucose	no
GLUT11	II	Heart, muscle	Glucose (low affinity); fructose (long form)	no
GLUT12	III	Heart, muscle, prostate, small intestine, fat, cartilage	n.d.	yes
HMIT (GLUT1 3)	III	brain	H^+ myoinostitol	n.d.
GLUT14	Ι	testes	n.d.	n.d.

 Table 1.1: The GLUT/SLC2A family of facultative sugar and polyol transporters. Adapted from Airley and Mobasheri, 2007.

Sustained glucose supplies are of paramount importance to neuronal integrity as it is the principal metabolic substrate for neuronal tissue; the transporters GLUT1, 3, 4 and 8 are responsible for maintaining this supply (Duelli and Kuschinsky, 2001; Weisova *et al.*, 2009). Pharmacological inhibition of glucose transporters *in vitro* by phloretin diminished glucose transfer across neuronal membranes to <5% of control values, indicating that GLUT transporters mediate >95% of glucose transfer in the brain (Duelli and Kuschinsky, 2001). Modification of glucose transportation can occur through (Figure 1.6) : 1) translocation of cytosolic reserves of GLUT to the plasma membrane (Alquier *et al.*, 2006; Klip *et al.*, 2009; Weisova *et al.*, 2009); 2) *de novo* synthesis of new transporters (Duelli and Kuschinsky, 2001; Klip *et al.*, 2009); and 3) increased efficiency of existing membrane transporters through interactions with regulatory proteins (Duelli and Kuschinsky, 2001; Zaid *et al.*, 2009).



Figure 1.6: Mechanisms for increased glucose acquisition. (1) Translocation of cytosolic reserves of GLUT to the plasma membrane (2) *De novo* synthesis of new GLUT transporters (3) Increased efficiency of existing membrane transporters through interactions with regulatory proteins.

In the mammalian body, the largest consumption of glucose occurs at the skeletal muscles and is mainly mediated by GLUT4 translocation (Joost and Thorens, 2001; Klip *et al.*, 2009). Under resting conditions, GLUT4 is primarily retained in intracellular depots to the plasma membrane, with a small fraction cycling to and from the plasma membrane. This dynamic cycling of transporters from intracellular stores to the plasma membrane is highly responsive to signalling from insulin and other stimuli, resulting in a 2-10 fold increase in surface expression (Klip, 2009). Insulin is primarily responsible for shuttling GLUT4 reserves to the membrane via the insulin receptor substrate-1 (IRS) and phosphatidylinositol 3-kinase (PI3K) (Alquier *et al.*, 2006; Klip *et al.*, 2009). Downstream targets include an Akt-AS160-Rab axis, which promotes vesicle traffic upon stabilization of target Rab proteins by Akt phosphorylation of AS160, and a Racactin- α -actinin-4 axis, which assists in tethering, docking and fusion to the cell membrane (reviewed by Klip, 2009). Inhibition of GLUT4 endocytosis also contributes to increased GLUT4 densities; this is governed by AMP-activated protein kinase (AMPK) and Ca²⁺dependent signals in response to energy deprivation from muscle contraction and membrane depolarization (Klip et al., 2009). Energy deprivation would render ADP from falling ATP levels, leading to myokinase derived AMP from ADP, and subsequent activation of AMPK (Klip et al., 2009). The presence of translocable glucose transporters has recently been discovered in the brain; GLUT4 and GLUT8 are exclusively neuronal (Alquier et al., 2006). GLUT8 has several similarities to GLUT4, including a dileucine internalization motif in the cytoplasmic tail and comparable K_m of 2.4 mmol/l (Corvera et al., 1994). GLUT8 expression is highest in the hippocampus, amygdala, hypothalamus and cerebellum (Ibberson et al., 2002); in addition to these areas, GLUT4 expression is more diverse and exhibited in the thalamus, brainstem and olfactory bulbs, with the highest expression in the cortex and cerebellum (El Messari et al., 1998). Insulin-induced translocation of GLUT4 to the plasma membrane and associated enhancement of glucose uptake was observed in human neuroblastoma cells; this response was abolished by application of a PI3K inhibitor (Benomar et al., 2006). Studies on insulin-induced GLUT8 translocation have yielded contradictory findings: translocation *in vitro* was only observed in blastocysts (Alquier et al., 2006); however, endogenous insulin rises from glucose administration in vivo stimulated translocation in hippocampal neurons (Piroli et al., 2002). It has also been suggested that brain insulin may modulate the sensing mechanisms of hypothalamic glucosensing neurons (Alquier et al., 2006).

Synthesis of new transporters is a slower and more sustained method of increasing glucose transport. GLUT1 is universally expressed in all cells and responsible for the maintenance of basal glucose transport (Airley and Mobasheri, 2007). Its expression can be regulated through transcriptional, posttranscriptional and posttranslational activities; for instance, GLUT1 mRNA expression increases following hypoxic stress and mRNA translation is upregulated by insulin exposure (Duelli and Kuschinsky, 2001; Airley and Mobasheri, 2007). Within the brain, GLUT1 and GLUT3 are the major isoforms for maintaining glucose transport; their densities have been found to increase in parallel to elevated local cerebral glucose utilization – a 15% increase in glucose utilization induced a 19% increase in transporter densities (Duelli and Kuschinsky, 2001). The low Michealis-Menten (K_m) value of GLUT3 makes it particularly important to neurons as it can facilitate continuous glucose intake under low interstitial glucose concentrations (Wood and Trayhurn, 2003).
In skeletal muscles, insulin-dependent reciprocal binding of GAPDH and hexokinase II regulate the intrinsic activity of GLUT4 transporters (Klip, 2009; Zaid *et al.*, 2009). Insulin causes HKII to dissociate from GLUT4 and associate with the outer mitochondrial membrane; this binding is theorized to enhance the ability of HKII to phosphorylate glucose into glucose-6-phosphate, thereby generating more ATP (Mathupala *et al.*, 2006; Zaid *et al.*, 2009). The increasing ATP levels favour GAPDH binding to the, now unoccupied, GLUT4 transporters where it is theorized to facilitate GLUT4 transporter activity by increasing the exofacial availability of the glucosebinding site (Klip, 2009; Zaid *et al.*, 2009). A mechanism independent of transcription and translation has also been identified for GLUT3 in neurons. In response to increased glucose demand, the half-life of GLUT3 is extended through stabilization of the protein (Duelli and Kuschinsky, 2001). This results in an increase in overall GLUT3 expression, irrespective of synthesis rate.

1.11 Target 2: Glycolytic Enzymes

Once glucose enters the cell, it undergoes glycolysis (Figure 1.7); this process is catalyzed by enzymes and results in the production of 2 pyruvate molecules per glucose (Kim and Dang, 2005; Scatena *et al.*, 2008). Thus, glycolytic production of pyruvate represents a rate limiting step in cellular metabolism. The complex metabolic rearrangements which allow cancerous cells to maintain glycolytic ATP production under hypoxic conditions, such as upregulation of key glycolytic enzymes and expression of favourable isoforms, could potentially aid in TCAP-1 mediated hypoxic stress protection. However, this mechanism would focus on glycolytic production of pyruvate rather than glycolytic generation of ATP.

Hexokinase catalyzes the first step in glycolysis by phosphorylating a six-carbon sugar (usually glucose) to a hexose phosphate (glucose-6-phosphate) (Robey and Hay, 2006); this is part of the priming phase and traps glucose in the cytoplasm (Mathulpala *et al.*, 2006). The association of hexokinase II with mitochondria though binding to a specific porin allows it direct access to mitochondrial ATP, thereby resulting in efficient glucose phosphorylation (Mathulpala *et al.*, 2006; Robey and Hay, 2006). It is elevated 200 fold in rapidly growing carcinomas and can also inhibit apoptotic pathways through Akt and Bcl-2 (Robey and Hay, 2006).



Figure 1.7: Glycolytic enzymatic reactions. A series of enzymatic steps catalyze the conversion of glucose into pyruvate, yielding a net production of 2 ATP molecules. Pyruvate can subsequently proceed to undergo oxidative phosphorylation at the mitochondria or converted into lactate to regenerate NAD+.

The reversible isomerization of glucose-6-phosphate into fructose-6-phosphate by glucose-6-phosphate isomerase (GPI) is followed by phosphorylation of fructose 6-phosphate into fructose 1,6-bisphosphate by phosphofructokinase (PFK) (Scatena *et al.*, 2008). This highly regulated allosteric enzyme is composed of four subunits and considered the most important glycolytic regulator (Scatena *et al.*, 2008; Bando *et al.*, 2005). PFK activity is regulated by numerous different activators, such as AMP, ADP and inorganic phosphates, and inhibitors, including ATP, citrate and fatty acids (Bando *et al.*, 2005). As a pacemaker of carbohydrate metabolism, PFK may seem counterproductive to increased energy production. However, it has been found Akt may phosphorylate and activate PFK, releasing the enzyme from ATP inhibition (Pelicano *et al.*, 2006). Additionally, certain cancer cells have been shown to induce the synthesis of a potent activator for PFK, fructose-2,6-bisphosphate (F2,6BP) (Bando *et al.*, 2005; Chesney, 2006).

In the next step of glycolysis, aldolase (ALDOB) splits fructose 1,6-bisphosphate into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GA3P) (Kim and Dang, 2005). The three different isozymes, A, B and C, are encoded by different genes and differentially expressed during development. Aberrant expression of ALDOB is commonly found in cancer cells undergoing high rates of glycolysis (Scatena *et al.*, 2008).

Dihydroxyacetone phosphate is reversibly converted into glyceraldehyde-3-phosphate by triosephosphate isomerase (TPI), a subsequent redox reaction by glyceraldehyde-3-phosphate dehydrogenase (GAPD) reduces NAD+ to NADH and yields 1,3-bisphosphoglycerate (Kim and Dang, 2005; Scatena *et al.*, 2008). 1,3-bisphosphoglycerate then undergoes substrate level phosphorylation; transfer of a phosphoryl group is catalyzed by phosphoglycerate Kinase (PGK) and leads to the formation of ATP and 3-phosphoglycerate (Scatena *et al.*, 2008). Multi-drug resistance is associated with overexpression of the PGK-1 isoform (Duan *et al.*, 2002).

The next enzyme, phosphoglycerate mutase (PGM), converts 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) by transferring a phosphate group from C-3 to C-2 (Kim and Dang, 2005; Scatena *et al.*, 2008); upregulation of the enzyme has been found in cancers of the lung, liver, colon and breast (Duan *et al.*, 2002). Furthermore, deficiencies in PGM enzyme functioning manifests in humans as muscular dystrophy (Scatena *et al.*, 2008).

Subsequently, enolase (ENOL) produces phosphoenolpyruvate through catalyzing the reversible dehydration of 2-phosphoglycerate (Scatena *et al.*, 2008). It is a multifunctional enzyme that influences glycolysis, growth, hypoxia tolerance and allergic responses (Pancholi, 2001; Kim and Dang, 2005). Adult human cells express all three isozymes: enolase-1,2, and 3; tumours of neuroendocrine origin exhibit increased serum levels of ENOL2 (Pancholi, 2001).

The final step of glycolysis is catalyzed by pyruvate kinase (PK); it produces pyruvate and ATP by catalyzing the irreversible transfer of a phosphoenolpyruvate phosphate group to ADP (Scatena *et al.*, 2008). The M2 isoenzyme is highly expressed in tumour cells and, when in the inactive dimeric form, is responsible for channeling of glucose carbons towards synthetic processes or, when in the highly active tetrameric form, for glycolytic energy production. Switching between the two forms facilitates nutrient adaptation (Mazurek *et al.*, 2005).

One crucial difference between the mechanisms of tumourgenic metabolic adaptation and TCAP-mediated metabolic enhancement exists in the final step of glycolysis. The glycolytic shift of cancer cells requires the regeneration of NAD⁺ from NADH, a process that usually occurs at the mitochondrial electron transport chain (Airley and Mobasheri, 2007; Kim and Dang, 2007). Lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate to lactate, which also regenerates NAD+ from NADH (Kolev *et al.*, 2008). Thus, LDH plays a fundamental role in the maintenance of growing tumours. However, the metabolic changes activated by TCAP-1 circumvent this step and pyruvate enters the mitochondria to fuel oxidative phosphorylation.

1.12 Target 3: Mitochondria and the Electron Transport Chain

The Electron Transport Chain (ETC) is located along the inner membrane of the mitochondria and governs the series of redox reactions which couples substrate oxidation with ATP formation (reviewed by Wallace, 2005). High-energy electron carriers, NADH and FADH₂, are created during glycolysis and the citric acid cycle (Kim and Dang, 2005; Schrauwen and Hesselink, 2002). At complex I, oxidation of NADH results in the transfer of high-energy electrons and regeneration of NAD⁺; FADH₂ is oxidized at complex II into FADH. Upon entering the transport chain, high-energy electrons will cause protons from the inner matrix to be transported to the cytosolic side of the inner mitochondrial membrane as they move along from Complex I through to Complex IV – where water is the final electron acceptor (Wallace, 2005). Accumulation of positive hydrogen ions creates a proton gradient across the membrane, known as the proton motive force (Schrauwen and Hesselink, 2002; Wallace, 2005). The implications of this force were first noted by Peter D. Mitchell (Mitchell, 1966) in his Nobel-prize-winning chemiosmotic hypothesis. He theorized that the flow of protons, through a F_0F_1 -complex, back across the inner mitochondrial membrane would provide the necessary energy to catalyse the phosphorylation of ADP into ATP.

Despite being responsible for generating the majority of cellular energy, coupling of substrate oxidation to ATP formation is not 100% efficient. Energetically significant proton leaks across the membrane dissipate energy as heat, thereby reducing the efficiency of substrate oxidation; in rats, this encompasses approximately 20% of the standard metabolism (Stuart *et al.*, 1999; Brand, 2005). There are two classes of proton leaks: basal and inducible. Basal proton leaks are universal to all mitochondria, arising from the presence of the adenine nucleotide translocase the protein responsible for exporting ATP synthesized by the mitochondria (Stuart *et al.*, 1999; Rousset et al., 2004). Inducible proton leaks (Figure 1.8) are catalyzed by the uncoupling proteins (UCP), UCP1, 2 and 3, and the adenine nucleotide translocase (Stuart *et al.*, 1999; Schrauwen and Hesselink, 2002; Rousset et al., 2004 Echtay, 2007). In brown adipose tissue, UCP1 mediates adaptive thermogenesis for the maintenance of body temperature in small rodents and infants (Rousset et al., 2004). UCP2 is universally expressed, with high levels in the immune system and pancreatic islets - where it is postulated to regulate insulin secretion (Brand, 2005; Echtay, 2007). UCP3 expression is primarily in skeletal muscles and associated with fatty acid metabolism (Schrauwen and Hesselink, 2002). The uncoupling proteins are also implicated in cellular protection from reactive oxygen species (ROS); under limited ADP availability, mild uncoupling could reduce the generation of ROS from high proton gradients (Brand, 2005). Within the brain, UCP2-mediated shifts in ROS release have been implicated as neuroprotective by inhibiting the release of apoptogenic proteins (Mattiasson et al., 2003).



Figure 1.8: Uncoupling of substrate oxidation to ATP formation in the mitochondria. Pumping of protons into the intermembrane space generates the proton motive force, which subsequently fuels ATP synthase activity. Uncoupling proteins reduce this force by allowing protons to leak back across the inner membrane.

The process of mitochondrial oxidative phosphorylation is the predominant method by which ATP is formed and is responsible for generating over 90% of cellular energy (Wallace, 2005). Consequently, energy production can be greatly enhanced by refinement of mitochondrial functioning. Increased oxidative capacity can also occur through mitochondrial biogenesis and is commonly observed in long-term exercise training due to chronic energy deprivation. AMPK has been implicated as a critical mediator of this signaling pathway; in 2002 Zhong *et al.*, demonstrated that AMPK activation is required for muscle mitochondrial biogenesis and regulates the expression of master regulators of mitochondrial biogenesis: peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) and calcium/calmodulin-dependent protein kinase IV (CaMK IV) (Zhong *et al.*, 2002). In addition, AMPK is important in the process of mitophagy – the recycling of mitochondria that are either dysfunctional or exceeds the current cellular requirements (Hardie *et al.*, 2012). Generation of ROS at the mitochondria make it particularly susceptible to oxidative damage and thus removal of defective mitochondria may be of equal importance to enhancing overall cellular ATP production.

Preservation of limited substrates for energy production through alterations of metabolic pathways may account for the increased survivability of TCAP treated cultures under hypoxic and high density stress. The creation of an energy surplus would also fuel survival mechanisms against additional stressors and prime the cell against future insults. In the absence of homeostatic threats, optimized energy production could be utilized for cellular growth and proliferation. Administration of TCAP-1 on unstressed neuronal cultures has been found to stimulate ERK1/2-dependent phosphorylation of the cytoskeletal regulatory proteins stathmin and filamin a, resulting in the upregulation of cytoskeletal proteins such as α -actinin-4 and β tubulin (Al Chawaf *et al.*, 2007; Chand *et al.*,2012). These rearrangements correspond with observations of increased neurite outgrowth and axon fasciculation demonstrated in primary hippocampal cultures following TCAP-1 treatment (Al Chawaf *et al.*, 2007).

1.13 Activation of Anti-apoptotic Pathways

In addition to metabolic adaptation, rescue from cellular stressors can occur through inhibition of apoptotic signalling cascades. Under severe hypoxic stress, binding of HIF-1 to hypoxia response elements alters transcriptional activity of select genes, one notable gene being vascular endothelial growth factor (VEGF) (Brahimi-Horn et al., 2001). Recent studies indicate that induction of VEGF can inhibit pro-apoptotic pathways during serum deprivation of endothelial cells and tumour cells (Baek et al., 2000; Gupta et al., 2000). VEGF is an angiogenic growth factor belonging to the platelet-derived growth factor family (Ferrara, 1999). VEGF-A is a dimeric cytokine that acts on endothelial cells through specific transmembrane tyrosine kinase receptors, VEGFR-1 and VEGFR-2 (Ferrara, 1999; Dvorak, 2002). Binding of the ligand induces receptor dimerization, autophosphorylation and subsequent activation of multiple signalling pathways such as MAPK, PI3-kinase, Ras, and phospholipase C (PLC) cascades (Dvorak, 2002). The downstream effects of VEGF signalling include stimulation of endothelial cell proliferation, migration, gene transcription and increased microvessel permeability (Dvorak, 2002; Knowles and Harris, 2010). Interestingly, VEGF also inhibits apoptosis and certain tumour cells have been found to express VEFG receptors (Baek et al., 2000; Gupta et al., 2000). Specifically, there was a reduction in the cellular Bax:Bcl-2 ratio, cytochrome c release and caspase 3 activity (Haldar et al., 1994; Baek et al., 2000). In addition, neutralization of VEGF, using anti-VEGF antibodies or inhibition of receptor tyrosine kinase signalling, rescues apoptotic responses to hypoxia (Baek et

al., 2000). Since TCAP-1 reduces the expression of HIF-1 in cells, this indicates that cell survival by TCAP-1 induction is not likely attributed to this anti-apoptotic pathway. However, full exclusion of this pathway warrants further investigation.

The Bcl family of proteins are crucial regulators of mitochondrial apoptotic pathways (Galluzzi *et al.*, 2009; Tait and Green, 2010). Bax/Bak mediate the formation of a mitochondrial pore through which cytochrome c is released (Galluzzi *et al.*, 2009; Shen *et al.*, 2010; Tait and Green, 2010). Binding of cytochrome c to the Apoptotic protease activating factor-1 (APAF-1) and procaspase-9 in the presence of dATP forms the apoptosome complex; activation of apoptosis ensues upon cleavage of caspase-3 by the apoptosome complex (Galluzzi *et al.*, 2009; Tait and Green, 2010). Consequently, cytochrome c release is considered the commitment step for apoptosis – Bcl-2 and Bcl-XL inhibit this release (Galluzzi *et al.*, 2009; Shen *et al.*, 2010). Inhibition of these anti-apoptotic proteins occurs through the formation of a heterodimer with Bad (Shen *et al.*, 2010). Interestingly, inactivation of Bad, and release of Bcl-2 and Bcl-XL, can occur through phospho-p90RSK phosphorylation of Bad (Shen *et al.*, 2010); this anti-apoptotic response has been observed with TCAP-1 treatments *in vitro* (Chand *et al.*, 2012). Although the protective mechanism of TCAP-1 may be partially attributed to inhibition of apoptosis (Trubiani et al., 2007), it is important to acknowledge the necessity of enhanced metabolic capacities to prevent the detrimental consequence of necrosis.



Figure 1.9: p90RSK inhibition of apoptosis. TCAP-1 phosphorylation of p90RSK and subsequent phosphorylation of BAD could inhibit apoptosis by releasing the anti-apoptotic factors, Bcl-2 and Bcl-XL.

1.14 Evidence for the Synergistic Actions of Metabolic Optimization and Anti- apoptotic Pathways at the Mitochondria

The distinction between metabolic and anti-apoptotic pathways has been blurred by recent findings of novel functions for proteins previously considered exclusive to one particular pathway (Hammerman *et al.*, 2004; Mathulpala *et al.*, 2006; Robey and Hay, 2006; Alavian *et al.*, 2011). Convergence of these pathways occurs at the mitochondria; the gravity of maintaining mitochondrial integrity, particular during bouts of stress, is implicit in its dual role of sequestering the apoptotic protein cytochrome c and execution of oxidative phosphorylation. The elegant synergism between these two pathways highlights the need to account for both processes when delineating TCAP's neuroprotective actions.

Hexokinase is the glycolytic enzyme responsible for catalyzing the phosphorylation of glucose into glucose-6-phosphate; this conversion is the first priming step in glycolysis and prevents glucose from exiting the cell (Mathulpala et al., 2006; Robey and Hay, 2006). Current theories implicate the enzyme as an anti-apoptotic protein due to its ability to bind with high affinity to the mitochondrial voltage-dependent anion channel (VDAC) (Hammerman et al., 2004; Mathulpala et al., 2006; Robey and Hay, 2006). The mitochondrial VDAC are located on the outer mitochondrial membrane where they play an integral role in maintenance of mitochondrial homeostasis, release of apoptogenic factors and ATP production; in response to various stressors, changes in the permeability of the mitochondrial outer membrane can trigger apoptotic pathways (Hammerman et al., 2004). Hexokinase interaction with the mitochondrial VDAC is capable of preventing cytochrome c release through direct modification of VDAC conductance properties and by inhibition of VDAC interactions with pro-apoptotic proteins (Mathulpala et al., 2006; Robey and Hay, 2006). There are multiple mammalian isoforms: hexokinase I, II, III and IV (Scatena et al., 2008). Hexokinase I, II and III are associated with the outer mitochondrial surface and directly couples glycolysis to oxidative phosphorylation, allowing for efficient access to mitochondrial ATP (Mathulpala et al., 2006; Robey and Hay, 2006). Various factors influence the mitochondrial association of hexokinase. Most notably, intracellular glucose-6phosphate promotes the dissociation of hexokinase from the mitochondria and is a form of apoptotic sensitization (Robey and Hay, 2006). Metabolic optimization would allow for efficient utilization of glucose-6-phosphate, thereby preventing accumulation.

Conversely, the anti-apoptotic bcl family of proteins have been implicated in increasing the efficiency of energy metabolism. A new metabolic role for Bcl-XL has recently been identified in a study by Alavain *et al.* (2011) on hippocampal neurons. Interaction of Bcl-XL with the β -subunit of the F₁F₀ ATP synthase enhanced mitochondrial performance through: 1) reducing the membrane leak conductance across the inner membrane, allowing for increased net transport of hydrogen ions to create a stronger proton motive force; 2) directly increasing the level of ATPase enzymatic activity (Alavain *et al*, 2011). The authors postulate that this improved metabolic functioning is responsible for the enhanced synaptic efficacy observed in neurons expressing Bcl-XL. Although the detrimental impact of severe metabolic impairment on cognitive functioning has been widely established, emerging studies are now focusing on the impacts of improved metabolic functioning on learning, memory and behavior (Liu *et al.*, 2002; Baker and

Tarnopolsky, 2003; Gold, 2005). Alterations in neuronal metabolism may contribute to the behavioral changes associated with *in vivo* administration of TCAP-1.

1.15 Translation into Whole Body Energy Dynamics Resulting in Reduced Adiposity

The *in vitro* metabolic changes observed at the cellular level will manifest at the organismal level in response to *in vivo* TCAP-1 administration and the greatest changes will be observed in metabolically sensitive tissues and hormones. TCAP is expressed at low levels throughout the body, with peripheral concentrations of less than 5pmol, as measured by ELIZA (Tan *et al.*, manuscript in preparation) and its receptor, the β -dystroglycan subunit, is also ubiquitously expressed, with high expression occurring in muscle fibers (Chand *et al.*, manuscript submitted). Therefore, synthetic TCAP-1 should elicit overall physiological changes in metabolism with a notably strong response in skeletal muscle. Fueling of skeletal muscle tissue accounts for a substantial amount of energy expenditure (Westerblad *et al.*, 2010); initial *in vivo* observations suggest that TCAP-1 may induce skeletal muscle hypertrophy and promote increased activity levels. Increased energy utilization induced by TCAP-1, especially at the muscles, will ultimately translate into a shift in the relationship between energy expenditure and storage. The long-term impact of this shift is reflected in the distribution of white adipose tissue; it will likely cause a depletion of existing nutrient stores as they become liberated to fuel ATP production in energetically demanding tissues (Pénicaud *et al.*, 2000; Jo *et al.*, 2009).

There are two main types of adipose tissue: (1) brown adipose tissue, which functions to generate larger amounts of heat from dense mitochondrial reserves, is predominantly found in newborns and hibernating animals; (2) white adipose tissue, which functions to store energy in the form of triglycerides and insulate body heat, is distributed throughout the body (Pénicaud *et al.*, 2000). White adipose tissue consists of a loose association of lipid-filled cells known as adipocytes with a variety of stromal vascular cells, held in a matrix of collagen (Johnson and Albert, 1985) and may be impacted as a secondary response to increased energy expenditure by exogenous TCAP-1 administration. Growth of adipose tissue occurs through two mechanisms: hypertrophy – an increase in cell size and hyperplasia – an increase in cell number (Jo *et al.*, 2009). Adipocyte hypertrophy occurs when energy storage exceeds energy expenditure and proceeds via two pathways: 1) direct uptake and subsequent hydrolyzation of triacylglycerols by lipoprotein lipase into non-esterified free fatty acids and 2) the lipogenic pathway of glucose transport through the

insulin-sensitive isoform, GLUT4, followed by synthesis of long-chain fatty acids (Pénicaud *et al.*, 2000). As lipids continue to accumulate, eventually a maximum fat cell size is reached and this triggers recruitment of new fat cells through hyperplasia (Jo *et al.*, 2009); preadipocytes, derived from mesenchymal stem cells, are stimulated to proliferate through clonal expansion and then commit to differentiate into mature adipocytes (Johnson and Albert, 1985; Pénicaud *et al.*, 2000). Conversely, when energy output exceeds energy storage, adipocytes shrink in size; studies of severe food deprivation resulted in delipidation of adipocytes, without disruption of the total number of differentiate fat cells (Johnson and Albert, 1985; Galic *et al.*, 2010). Albeit some mature adipocytes have been found to undergo apoptotic cell death, this is a rare occurrence (Pénicaud *et al.*, 2000).

Although adipose tissue has long been recognized as the main storage center for energy, recent advances have resulted in emerging perspectives on the important endocrine function of adipose tissue in modulation of metabolism, immunity and satiety suggest that fat distribution may underlie certain neurological disorders (Pénicaud *et al.*, 2000; Niswender and Magnuson, 2007, Galic *et al.*, 2010). One of the main hormones secreted from adipose tissue is leptin; high expression of its receptors occurs in areas of the brain that regulate feeding, such as the arcuate, dorsomedial and ventromedial hypothalamic nuclei (Galic *et al.*, 2010). Signal transduction of leptin signalling in the hypothalamus elicits specific responses that supress orexigenic (appetite stimulating) gene expression (Pénicaud *et al.*, 2000; Galic *et al.*, 2010). A positive correlation between circulating serum leptin levels and total body fat mass can be explained by increased leptin secretion from larger cells; therefore, fat loss resulting in smaller cells will be accompanied by reduced leptin secretion (Lönnqvist *et al.*, 1997). Interestingly, the actions of leptin and insulin both converge in an endocrine feedback loop that signals to the hypothalamus to regulate energy homeostasis (Figure 1.10; Niswender and Magnuson, 2007; Galic *et al.*, 2010).



Figure 1.10: Feedback regulation of energy homeostasis by leptin and insulin. Food intake stimulates pancreatic β cells to release insulin, which triggers the lipogenic pathway of adipocyte growth and acts on the central nervous system (CNS) to reduce nutrient intake. Increasing energy storage causes adipocytes to swell and divide, thereby increasing leptin secretion. Leptin has an inhibitory impact on β cells and supresses orexigenic pathways in the CNS. It is theorized that these pathways converge in the arcuate nucleus of the hypothalamus to inhibit appetite. The reduction in food intake leads to shrinking of adipocyte size and decreased leptin secretion. Adapted from Niswender and Magnuson, 2007.

1.16 Thesis rationale and experimental design

Protective mechanisms that alleviate energy deficits and prevent premature apoptosis, during bouts of cellular stress, are essential for the survival of multicellular organisms - these mechanisms are evolutionarily ancient, universally present, and highly conserved. The Teneurin C-terminal Associated Peptide (TCAP) family of peptides possess characteristics of a cleavable bioactive peptide – an amidation motif encoded on the carboxy terminus and a cleavage motif encoded on the amino terminus, are profoundly conserved and ubiquitously expressed throughout divergent metazoan species.

TCAP-1 is highly neuroprotective against alkalotic and hypoxic stress. Protection from alkaline stress is attributed, in part, to the upregulation of ROS scavenging systems. In addition, rescue of cells from hypoxic stress was demonstrated through increased viability and a reduction in the expression of HIF-1 α , a characteristic marker of hypoxic stress. Although the cellular mechanism is not entirely understood, studies indicate that the peptide is active in stressed cells and may play a critical role in cellular defence mechanisms and survival pathways.

Therefore, I hypothesize that TCAP-1 signalling could alleviate cellular stress through optimization of metabolic capacities; these actions would confer protection from cellular death by meeting the energy demands necessary to cope with the stressor. Additionally, this may result in increased mitochondrial integrity, thereby reducing apoptosis. A shift in the rate limiting process of glucose transport is the first potential step in metabolic optimization; modifications in transporter capacity can occur through: 1) translocation of cytosolic reserves of GLUT to the plasma membrane; 2) de novo synthesis of new transporters; 3) increased efficiency of existing membrane transporters. Once glucose enters the cell, it undergoes glycolysis. Optimization of glycolytic processes could be attributed to: 1) upregulation of key glycolytic enzymes; 2) activation of favourable enzyme isoforms; 3) coupling of glycolysis with oxidative phosphorylation from hexokinase reallocation. The subsequent process of oxidative phosphorylation is responsible for generating over 90% of cellular energy. Consequently, energy production can also be greatly enhanced by refinement of mitochondrial functioning. If TCAP-1 induces a state of heighted cellular metabolism, exogenous administration would theoretically cause a depletion of existing fat reserves and inhibit the storage of new adipose tissues as a

secondary response to increased energy expenditure; this would occur in tandem with alterations in associated endocrine responses.

Thus the objectives of my thesis are as follows:

Objective 1: To determine if TCAP-1 enhances glucose transporter capacity to increase glucose transport in immortalized mouse embryonic hypothalamic mHypoE-38 neurons.

Objective 2: To identify the metabolic parameters associated with the intracellular actions of TCAP-1 using immortalized mouse embryonic hypothalamic mHypoE-38 neurons.

Objective 3: To investigate the *in vivo* manifestation of TCAP-1 treatments on adiposity and associated leptin and insulin endocrine responses using a mouse model.

1.17 References

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2 Chapter Two: Materials and Methods

2.1 Objective #1: Enhancement of glucose transporter capacity by Teneurin C-terminal Associated Peptide (TCAP) -1 in mHypoE-38 immortalized embryonic hypothalamic neurons.

2.1.1 Preparation of cell cultures

Cell cultures of mHypoE-38 immortalized hypothalamic embryonic mouse neurons (gift from Dr. Denise Belsham, University of Toronto) were grown in Dulbecco's Modified Eagle Medium containing 4 500 mg/L D-glucose, L-glutamine, and 25 mM HEPES buffer, without sodium pyruvate or phenol red (Gibco, Cat. No. 21063029), supplemented with 10% fetal bovine serum (Invitrogen, Cat. No. 16000-036) and including penicillin and streptomycin antibiotics. For glucose and glucose-6-phosphate (G6P) assays, mHypoE-38 hypothalamic cells were grown in 10cm tissue culture treated polystyrene plates (Corning Incorporated, Cat. No. 430293) at a seeding density of 400 000 cells/55cm² dish in a 37°C normoxic incubator. Cultures were grown for 32 hours to ~80% confluence and subsequently synchronized for 16 hours, in serum free media with penicillin and streptomycin antibiotics, prior to chronic TCAP-1 treatments. For immunocytochemistry, mHypoE-38 hypothalamic cells were grown in 6-well tissue culture dishes (Corning Incorporated, Cat. No. 3516) at a seeding density of 200 000 cells/9.5cm² well in a 37°C normoxic incubator. Cultures were grown for 32 hours to ~60% confluence and subsequently synchronized for 16 hours, in serum free media with penicillin and streptomycin antibiotics, prior to ~60% confluence and subsequently synchronized for 16 hours in a 37°C normoxic incubator. Cultures were grown for 32 hours to ~60% confluence and subsequently synchronized for 16 hours, in serum free media with penicillin and streptomycin antibiotics, prior to 7CAP-1 treatments.

2.1.2 Chronic TCAP-1 studies

Cells were grown to ~80% confluence and synchronized in 10cm culture dishes, as previously described. Afterwards, fresh media, containing 10% fetal bovine serum with penicillin and streptomycin antibiotics, was added and marked t=0. Treatments of either TCAP-1 of 1nM, 10nM and100nM or vehicle ($H_2O + NH_3$; 0nM) were applied at t=0h, 3h, 6h and 9h (Figure 2.1). At t=12h, cells were washed three times with ice cold sterile phosphate buffer saline (PBS) at pH 7.4 and lysed.



Figure 2.1: Glucose Experimental Paradigm for Chronic TCAP-1 Treatments on mHypoE-38 Immortalized Embryonic Hypothalamic Neurons. Cells were grown to ~80% confluence and serum deprived for 16h to synchronize. TCAP-1 treatments of 0nM (vehicle), 1nM, 10nM or 100nM were administered at t=0, 3, 6, and 9h and cultures lysed at 12h. Colorimetric assays were subsequently conducted.

2.1.3 Glucose and G6P colorimetric assays

Cultures of mHypoE-38 neurons were lysed with either 350µl of glucose assay buffer (Biovision, Cat. No. K606-100-1) or G6P assay buffer (Biovision, Cat. No. K657-100-1), each containing a proprietary protease inhibitor cocktail. Cells were harvested using a cell scraper and centrifuged at 20 817 g at 4°C for 20 minutes. The pellet was discarded and supernatant aliquoted into two tubes, 30µl for protein quantification and the remainder ~300 µl for the appropriate assay kit, and stored in -80°C. A Pierce® BCA Protein Assay (Thermo Fischer Scientific, Cat. No. 23225) was performed to quantify protein concentrations for standardizing dilutions of respective supernatant samples. Diluted samples were then deproteinized using disposable ultrafiltration 10Kd spin columns (Biovision, Cat. No 1997-25) at 10 000 g for 10 minutes at 4°C.

The colorimetric protocol for the Glucose Assay Kit (Biovision, Cat. No. K606-100) or the G6P Assay Kit (Biovision, Cat. No. K657-100) was followed, in which glucose or G6P is oxidized by an enzyme mix and then reacted with a dye to generate a color. In short, 50µl of each deproteinized sample was added in triplicates to a 96-well plate. Glucose or G6P standards of 0, 2, 4, 6, 8, and 10nmol/well were prepared, as per kit instructions, and added in 50µl triplicates to generate a standard curve. A reaction mix for glucose or G6P, containing 46µl of corresponding assay buffer, 2µl of corresponding probe and 2µl of corresponding enzyme mix, was prepared and 50µl mixed to each sample or standard for a total volume of 100µl/well. The reaction was

incubated for 30 minutes, at 37°C for glucose or room temperature for G6P, protected from light. An absorbance reading was obtained at λ =570nm for glucose or λ =450nm for G6P using a Spectramax Plus 384 Absorbance Microplate Reader (Molecular Devices, USA).

2.1.4 *GLUT immunocytochemistry*

Cells were grown to ~60% confluence and synchronized on 22x22mm glass cover slips (VWR®, Cat. No. 48366-067) in 6-well culture dishes, as previously described. Afterwards, fresh media, containing 10% fetal bovine serum with penicillin and streptomycin antibiotics, was added and marked t=0. Treatments of either 100nM TCAP-1 or vehicle ($H_2O + NH_3$; 0h TCAP-1 incubation) were applied at t=1h and 100nM TCAP-1 applied at t=3h (Figure 2.2). At t=4h, cells were washed three times with ice cold sterile PBS at pH 7.4.



Figure 2.2: Experimental Paradigm for Immunocytochemistry Studies of TCAP-1 Treatments on mHypoE-38 Immortalized Embryonic Hypothalamic Neurons. Cells were grown to ~60% confluence and serum deprived for 16h to synchronize. Treatments of 100nM TCAP-1 or vehicle $(H_2O + NH_3)$ were applied at t=1h and 100nM TCAP-1 applied at t=3h. At t=4h, cells were fixed for immunocytochemistry.

Cultures of mHypoE-38 neurons were then fixed with filter sterilized 4% paraformaldehyde (PFA) for 20 minutes and underwent three 5-minute 0.01M PBS washes. Selective plasma membrane labeling was achieved with a 5µg/mL wheat germ agglutinin, Alexa Fluor 594 conjugate (Invitrogen, Cat. No. W11262), labeling solution incubated for 10 minutes at room temperature. After labeling, cells were washed three times with PBS and permeabilized using 0.3% Triton X-100 (Fisher Scientific, Cat. No. BP151) diluted in PBS for 10 minutes. The cells were then given three 5-minute washes in PBS and blocked for 1 hour with 10% normal goat serum (Vector Labs, Cat. No. S-100) at room temperature. Afterwards, cells were incubated

overnight at 4°C with a primary rabbit antibody solution diluted in 1% normal goat serum. The primary antibody solutions used were: rabbit polyclonal GLUT1 1:1000 dilution (Abcam, Cat. No. ab652), rabbit polyclonal GLUT3 1:250 dilution (Abcam, Cat. No. ab652) and rabbit polyclonal GLUT4 1:750 dilution (Abcam, Cat. No. ab654). Primary antibodies were washed away with three 5-minute PBS washes and incubated for 1 hour at room temperature with a 1:500 dilution of Alexa Fluor 488 green goat anti-rabbit IgG (Invitrogen, Cat. No. A11008) in 1% normal goat serum. Coverslips were mounted onto slides (VWR, Cat. No. 48311-703) using a hard-set VECTASHIELD® Mounting Medium with DAPI (Vector Labs, Cat. No. H-1500) and stored at 4°C. A WaveFX spinning-disk confocal microscope (Quorum Technologies, Canada) was used to acquire images. Cell fluorescence was measured with ImageJ (v10.2) software and corrected cell fluorescence (CCF) calculated using the following equation: CCF = Integrated Density – (Area of selected region x mean fluorescence of background readings). Blinded measurements for whole cell, perinuclear and membrane localization were obtained (Refer to Figure 2.3). Controls included: primary antibody only, secondary antibody only and no antibodies.



Figure 2.3: Measuring Cell Fluorescence using ImageJ. Cell images containing only the green fluorescence (GLUT) of interest were uploaded to ImageJ (**B**,**C** and **D**). Using the original image containing the membrane marker and DAPI as a guide (**A**), the entire cell was traced and a measurement made for the whole cell (**B**). Afterwards, a grid was laid over the cell and a measurement for a 1 unit ring around the nucleus was made for the perinuclear region (**C**), followed by a measurement for the nucleus (**D**). The membrane region was determined by subtracting the nuclear and perinuclear measurements from the total cell. For a background reading, 3 regions around the cell containing no fluorescence were selected and measured. Fluorescence for whole cell, nuclear and membrane regions were calculated using the following equation: Corrected Cell Fluorescence (CCF) = Integrated Density – (Area of selected region x Mean fluorescence of background readings).

2.2 Objective #2: Identification of the metabolic parameters associated with the intracellular actions of Teneurin C-terminal Associated Peptide (TCAP) -1 in mHypoE-38 immortalized embryonic hypothalamic cells.

2.2.1 Preparation of cell cultures

Cell cultures of mHypoE-38 immortalized hypothalamic embryonic mouse neurons were grown in 10cm tissue culture treated polystyrene plates (Corning Incorporated, Cat. No. 430293) at a seeding density of 400 000 cells/55cm² dish in a 37°C normoxic incubator. The medium used was Dulbecco's Modified Eagle Medium containing 4 500 mg/L D-glucose, L-glutamine, and 25 mM HEPES buffer, without sodium pyruvate or phenol red (Gibco, Cat. No. 21063029), supplemented with 10% fetal bovine serum (Invitrogen, Cat. No. 16000-036) and including penicillin and streptomycin antibiotics. Cultures were grown for 32 hours to ~80% confluence and subsequently synchronized for 16h, in serum free media with penicillin and streptomycin antibiotics, prior to TCAP-1 or scrambled TCAP-1 treatments.

2.2.2 Chronic TCAP-1 Studies

Cells were grown to ~80% confluence and synchronized in 10cm culture dishes, as previously described. Afterwards, fresh media, containing 10% fetal bovine serum with penicillin and streptomycin antibiotics, was added and marked t=0. TCAP-1 or Scrambled TCAP-1 treatments of 1nM, 10nM and100nM or vehicle treatments ($H_2O + NH_3$; 0nM) were applied at t=0h, 3h, 6h and 9h. At t=12h, cells were washed three times with ice cold sterile PBS at pH 7.4 and lysed. Chronic TCAP-1 application occurred over a period of 12 hours to simulate conditions of sustained stress and treatments were given every 3 hours due to the uptake of FITC-TCAP-1, which localized to the cell membrane at 15 minutes and was subsequently internalized into the cytosol at 30 minutes and trafficked towards the nucleus at 60-90 minutes (Ng *et al.*, 2011).



Figure 2.4: Experimental Paradigm for Chronic TCAP-1 Treatments on mHypoE-38 Immortalized Embryonic Hypothalamic Neurons. Cells were grown to ~80% confluence and serum deprived for 16h to synchronize. TCAP-1 treatments of 0nM (vehicle), 1nM, 10nM or 100nM were administered at t=0, 3, 6, and 9h and cultures lysed at 12h. Colorimetric assays and western blot analysis were subsequently performed.

2.2.3 Acute TCAP-1 Studies

Cells were grown to ~80% confluence and synchronized in 10cm culture dishes, as previously described. Afterwards, fresh media, containing 10% fetal bovine serum with penicillin and streptomycin antibiotics, was added and marked t=0. An acute treatment of vehicle (H₂O + NH₃; 0nM), 1nM, 10nM or 100nM of TCAP-1 was applied at t=1h. At t=2h (1 hour following treatment), cells were washed three times with ice cold sterile PBS at pH 7.4 and lysed. A single acute TCAP-1 application was given to simulate an isolated incident of stress. Analysis at 1 hour was performed to examine the immediate impact of TCAP-1 on metabolic parameters as previous studies show strong responses at 60 minutes following an acute TCAP-1 dose; such as increased labeling of long β -actin polymers, α -tubulin (B) and β -tubulin (Chand *et al.,* manuscript submitted).



Figure 2.5: Experimental Paradigm for Acute TCAP-1 Treatment on mHypoE-38 Immortalized Embryonic Hypothalamic Neurons. Cells were grown to ~80% confluence and serum deprived for 16h to synchronize. A TCAP-1 treatment of 0nM (vehicle), 1nM, 10nM or 100nM was administered at t=1h and cultures lysed at t=2h. Colorimetric assays were subsequently conducted.

2.2.4 Western blot analysis

Following chronic TCAP-1 treatments, mHypoE-38 immortalized hypothalamic neurons were lysed with 500µl of radio-immunoprecipitation assay buffer (RIPA) buffer, containing 1%Triton X-100, 50mM TRIS-HCl (pH 7.4), 150mM NaCl, 0.1%SDS, 0.5% Sodium De-oxycholate, 1mM EDTA, 1% Protease Inhibitor Cocktail Set III (Calbiochem, Cat. No. 539134), and 25mM DTT. Cells were harvested using a cell scraper and centrifuged at 20 817 g for 20 minutes at 4°C. The pellet was discarded and supernatant aliquoted into two tubes, 30µl for protein quantification and the remainder ~450µl for western blot analysis, and stored in -20°C. A Pierce® BCA Protein Assay (Thermo Fischer Scientific, Cat. No. 23225) was performed to quantify protein concentrations for standardizing dilutions of respective supernatant samples. Samples (15ug) were resuspended in Laemmeli sample buffer and size fractioned by SDS-PAGE (10%) at 100V for approximately 1 hour. Proteins were then electro-transferred to Hybond-ECL-nitrocellulose membranes (Amersham, Cat. No. RPN303D) for approximately 2 hours at 100V. Membranes were washed with PBS and blocked in 5% milk-PBST (5% w/v non-fat Carnation milk powder in PBS with 0.2% Tween 20) at room temperature for 1 hour on a shaker. Afterwards, membranes were incubated with rabbit primary antibodies in 1% milk-PBST overnight at 4 °C with gentle agitation (Refer to Table 2.1 and Table 2.2 for dilutions of antibodies used). Following 24 hours, the membranes were given three 5-minute washes in fresh PBST at room temperature and incubated with anti-rabbit horseradish peroxidase (HRP) - conjugated secondary antibody (VWR, Cat. No. RPN2135 or Amersham, Cat. No. NIF824) diluted to 1:5000 in 1% milk-PBST for 1 hour at room temperature with gentle agitation. The membranes underwent three more 5-minute washes in fresh PBST at room temperature. Subsequently, proteins were detected by adding chemiluminescence detection reagent (ECL Amersham, Cat. No. RPN2232) to the membranes and exposing onto ECL Hyperfilm (VWR, Cat. No. 95017-653L) for 10-60 minutes. The integrated optical density (I.O.D.) of the blots were quantified using LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd. Bio-imaging systems v4.0.0.8). As a loading control, GAPDH (Abcam, Cat. No. ab9485; 1:2500) was used and remained constant for all protein samples.

2.2.5 *Antibody optimization for glycolytic enzymes*

Antibody	Dilution	Predicted	Experimental Band	Special notes
		Band		
LDH5	1:1000	36kDa	37kDa, faint band at 75kDa	Clean antibody that yields
(Abcam; ab53010)				consistent results
PGM1	0.5ug/ml	61kDa	75kDa, faint banding at 130kDa and	Loading less protein yields
(Abcam; ab94601)			23kDa.	cleaner results.
PFKL	1:200	85kDa	80kDa, 60kDa, faint banding at	Loading less protein yields
(Abcam; ab37583)			70kDa.	cleaner results.
ALDOB	1:500	39kDa	160kDa (tetramer)	Clean antibody that yields
(Abcam; ab75751)				consistent results
PGK1	1:200	44kDa	50kDa	Loading less protein yields
(Abcam; ab38007)				cleaner results.
Enolase –2	1:1000	47kDa	47kDa	Clean antibody but difficult to
(Cell Sig.; #9536)				detect.
Hexokinase II	1:1000	102kDa	102kDa	102kDa band is always strong
(Cell Sig.;#2867)				and consistent.

Table 2.1: Western Blot Optimization Results for Glycolytic Proteins

2.2.6 *Antibody optimization for mitochondrial apoptotic enzymes*

Table 2.2: Western Blot Optimization Results for Apoptotic Proteins

Antibody	Dilution	Predicted	Experimental	Special notes
		Band(s)	Band	•
Bcl-2	1:1000	26 kDa	faint at 26kDa,	The strong banding at 90kDa is displayed
(Cell Sig.;#3498)			strong at 90kDa	prominently at high loading
			-	concentrations.
Bax	1:1000	20 kDa	20kDa, faint	Loading less protein yields cleaner results.
(Cell Sig.;#2772)			bands at 23kDa,	
			24kDa and 26kDa	
Cytochrome c	1:1000	14kDa	15kDa	Loading less protein yields cleaner results,
(Cell Sig.; #4272)				but makes detection difficult.
Caspase-3	1:1000	17kDa,	35kDa, faint at	The band at 35kDa is the uncleaved
(Cell Sig.;#9662)		19kDa, 35kDa	19kDa	protein and has the strongest signal.

2.2.7 Lactate assays

Cultures of mHypoE-38 neurons were lysed with 350µl of Lactate assay buffer (Biovision, Cat. No. K607-100-1), containing a proprietary protease inhibitor cocktail. Cells were harvested using a cell scraper and centrifuged at 20 817 g for 20 minutes at 4°C. The pellet was discarded and supernatant aliquoted into two tubes, 30µl for protein quantification and the remainder \sim 300 µl for the lactate assay kit, and stored in -80°C. A Pierce® BCA Protein Assay (Thermo Fischer Scientific, Cat. No. 23225) was performed to quantify protein concentrations for standardizing dilutions of respective supernatant samples. Diluted samples were deproteinized using disposable ultrafiltration 10Kd spin columns (Biovision, Cat. No 1997-25) at 10 000 g for 10 minutes at 4°C.

The colorimetric protocol for the Lactate Assay Kit (Biovision, Cat. No. K607-100) was followed, in which an enzyme mix reacts with lactate and the product then interacts with a probe to generate a measurable color. Briefly, 50µl of each deproteinized sample was added in triplicates to a 96-well plate. Lactate standards of 0, 2, 4, 6, 8, and 10nmol/well were prepared, as per kit instructions, and added in 50µl triplicates to generate a standard curve. A lactate reaction mix, containing 46µl of the assay buffer, 2µl of the probe and 2µl of the enzyme mix, was prepared and 50µl mixed to each sample or standard for a total volume of 100µl/well. The reaction was incubated for 30 minutes at room temperature, protected from light. An absorbance reading was obtained at λ =570nm using a Spectramax Plus 384 (Molecular Devices, USA).

2.2.8 ATP assays

The previous protocol was also utilized for the colorimetric ATP Assay Kit (Biovision, Cat. No. K354-100), in which ATP phosphorylation of glycerol interacts with a probe to generate a color measurable at λ =570nm, with a few modifications. An ATP assay buffer (Biovision, Cat. No. K354-100-1), containing a proprietary protease inhibitor cocktail, was used. Additionally, the ATP reaction mix contained 44 µl of the assay buffer, 2 µl of the probe, 2µl of the converter and 2µl of a developer mix.
2.3 Objective #3: *In vivo* physiological manifestation of Teneurin C-terminal Associated Peptide (TCAP)-1 administration on adiposity and associated leptin and insulin endocrine responses in male BALB/C mice.

2.3.1 in vivo TCAP-1 administration

Male BALB/c mice were obtained from Charles River Laboratories (n=31; 6-8 weeks old) and were divided into three treatment groups: saline (n=10), 25pmol/kg TCAP-1 (n=11) and 250pmol/kg TCAP-1 (n=10). Mice were housed in Plexiglass shoebox cages under standard laboratory conditions (12:12 h light:dark cycle, lights on at 0700 h, temperature 21 + 1 °C) with unrestricted access to food and water. Upon arrival, the mice underwent two weeks of handling and then given daily subcutaneous injections of 300μ l for nine days, using a 26 1/2G needle (Figure 2.6). On the tenth day, the mice were put down using isoflurane, after which serum and tissue samples were immediately extracted. All procedures were approved by the University of Toronto Animal Care Committee in accordance with the Canadian Council on Animal Care.



Figure 2.6: Experimental Paradigm for *in vivo* **TCAP-1 administration on male BALB/c mice.** Upon arrival, male BALB/c mice underwent two weeks of handling before receiving subcutaneous injections of saline, 25pmol/kg or 250pmol/kg of TCAP-1 for 9 consecutive days. On the 10th day, they were put down using isoflourane. Subcutaneous adipose tissue was harvested and stained with Heamatoxylin and Eosin for morphological analysis. In addition, serum was collected to generate a metabolic marker map for serum insulin and leptin levels.

2.3.2 Serum processing and analysis

Whole blood samples were collected, from which the serum was separated from the cells and fibrin by centrifuging the collection tubes for five minutes at 7000 g at 25°C. Afterwards, the serum was aspirated by pipette into plastic microcentrifuge tubes, a minimum sample volume of 70µl was required for the Mouse MetabolicMarker® Map (Myriad RBM, Inc). Immediately

following collection and processing, the samples were stored and maintained at a freezing temperature of -20°C or less. The samples were then submitted, according to company instructions, to Myriad RBM, Inc. for analysis and generation of a Mouse MetabolicMAP®.

2.3.3 Adipose tissue processing

Subcutaneous adipose tissue samples were immediately extracted following cessation of life. The adipose tissue was fixed with 4% paraformaldehyde in 0.1M PBS, pH 7.4, at 4°C overnight; extra care was taken to ensure that the adipose tissue was completely submerged. The adipose samples were then washed with PBS, pH 7.4, and dehydrated with increasing sucrose solutions of 10%, 20% and 30% dissolved in 0.1M PBS, respectively. Afterwards, the adipose samples were tightly sealed in aluminum foil before being flash frozen in isopentane and stored at -80°C.

The frozen adipose samples were mounted onto cryostat chucks using O.C.T. compound and sliced using a cryostat to a thickness of 10μ m; maintenance of a chamber temperature between - 30° C and - 40° C was crucial to prevent melting or cracking of the adipose tissue. Slices were mounted onto Superfrost Plus Micro Slides (VWR, Cat. No. 48311-703) and stored in slide boxes at - 80° C.

2.3.4 Hematoxylin and eosin (H&E) staining and analysis of adipose samples Slides with adipose tissue slices were placed in glass Coplin jars (Sigma, Cat. No. S5516), allowed to thaw from -80°C to room temperature for 5 minutes and washed three times in PBS. The PBS was rinsed away with distilled water and tissues were stained with hematoxylin (Vector Labs, Cat. No.H-3404) for 30 seconds before being quickly rinsed away under distilled water. Subsequently, slides were immersed in 95% ethanol for 30 seconds and a counterstain for eosin (Sigma-Aldrich, Cat. No. HT110316) was applied for 60 seconds. Slides were then equilibrated in 95% ethanol for two 5-minute incubations, followed by 100% ethanol for two 5-minute incubations and finally two 5-minute xylene (Sigma-Aldrich, Cat. No. 534056) incubations. A glass coverslip was mounted overtop tissue samples using a hard-set VECTASHIELD® Mounting Medium (Vector Labs, Cat. No. H-1400). From each sample, three images at 200X magnification were taken with a Nikon Digital Sight DS-U1 microscope camera (Nikon Instruments Inc., USA) and blinded measurements for the number of adipocytes per image were obtained using NIS-Elements BR 2.30 software (Nikon Instruments Inc., USA).

2.4 Statistical Analysis

All results are expressed as mean \pm SEM. Statistical significance was assessed by 1-ANOVA, a *p* value less than 0.05 was considered to be statistically significant.

3 Chapter **3**: Results

- **3.1** Objective #1: Enhancement of glucose transporter capacity by Teneurin C-terminal Associated Peptide (TCAP) -1 in mHypoE-38 immortalized embryonic hypothalamic neurons.
- 3.1.1 The effect of chronic TCAP-1 treatments of 0nM (vehicle), 1nM, 10nM and 100nM on intracellular glucose concentrations.

Glucose is a key metabolic substrate whose transport into the cell represents a limiting constraint for ATP production (Airley and Mobasheri, 2007). An increase in intracellular glucose concentration could be a good indicator of elevated transport concentration at the membrane or efficiency. Synchronized mHypoE-38 cell cultures were given chronic TCAP-1 treatments of 0nM, 1nM, 10nM and 100nM at t=0, 3, 6, and 9h and lysed at 12h; the intracellular glucose concentrations were then measured using a colorimetric glucose assay kit. Initial experimental trials resulted in highly variable results which showed no statistical difference between control and TCAP-1 treatments (Figure 3.1). This may be due to the high glucose concentration of the culturing media; some residual glucose on the cell membrane may confound the findings, as per the troubleshooting manual (Biovision Cat. No. K606-100-1).



Figure 3.1: Colorimetric glucose assay following chronic TCAP-1 treatments. Synchronized mHypoE-38 immortalized hypothalamic neurons were given chronic treatments of 0nM, 1nM, 10nM and 100nM TCAP-1. Colorimetric glucose assays yielded highly variable results. (Mean ± SEM; n=4; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.1.2 The effect of chronic TCAP-1 treatments of 0nM (vehicle), 1nM, 10nM and 100nM on intracellular glucose-6-phosphate concentrations.

After entering the cell, glucose is quickly phosphorylated by hexokinase into glucose-6-phosphate (G6P); this essential step inhibits the substrate from leaving the cell (Robey and Hay, 2006). Measurement of G6P represented a more favourable option to direct glucose assays, as intracellular G6P concentrations correlate with glucose uptake, while possibly reducing the problems associated with high culture media glucose concentrations. The same procedure for glucose was performed except with the use of a colorimetric G6P assay kit. The previously mentioned issues, with respect to high media glucose concentrations, were also experienced in this assay. Release of hexokinase upon lysis would result in production of G6P from unwashed glucose, as per kit troubleshooting (Biovision, Cat. No. K657-100-1). Although some individual samples contained elevated G6P levels with TCAP-1 treatments, the overall experimental findings showed no statistical significance between control and treated cultures.



Figure 3.2: Glucose-6-phosphate assay following chronic TCAP-1 treatments. Synchronized mHypoE-38 immortalized hypothalamic neurons were given chronic treatments of 0nM, 1nM, 10nM and 100nM TCAP-1. Colorimetric G6P assays yielded highly variable results. (Mean \pm SEM; n=4; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.1.3 The effect of TCAP-1 on GLUT1 expression and cellular localization

TCAP-1 may increase intracellular glucose concentration by activating transcription and translation of *de novo* glucose transporters (Duelli and Kuschinsky, 2001). Aside from de novo synthesis of glucose transporters, the peptide may also up regulate glucose transport in an insulin-like manner through translocation from vesicles to the cell membrane or increasing the activity of transporters at the membrane (Klip *et al.*, 2009). GLUT1 transporters are responsible for maintaining basal glucose transport in all cells and are frequently upregulated in response to cellular metabolic stress (Duelli and Kuschinsky, 2001). Synchronized mHypoE-38 cell cultures were treated with vehicle (0h TCAP-1) at t=1h or 100nM TCAP-1 at t=1h or 3h; immunocytochemical analysis for GLUT1 was subsequently performed. Immunofluorescence images show what appear to be both an increase in overall GLUT1 expression at 1 hour and an increase in the membrane localization of GLUT1 (Figure 3.3).



Figure 3.3: Representative immunocytochemical images for GLUT1 in mHypoE-38 immortalized embryonic hypothalamic neurons following 1 or 3 hours of 100nM TCAP-1 treatment. Top row: corresponding DIC images (A,B and C). Middle row: GLUT1 fluorescence at 0 hours (control; D), 1 hour (E) and 3 hours (F) following 100nM TCAP-1 treatment. Bottom row: Merge of DAPI (nucleus), GLUT1 (glucose transporter) and wheat germ agglutinin (cell membrane) fluorescence (G, H and I). Images were obtained using a WaveFX spinning-disk confocal microscope (Quorum Technologies, Canada).Magnification of 630x, 15µm scale bar.

An increase in the overall fluorescence would be indicative of increased GLUT1 synthesis and an increase in the membrane to perinuclear region ratio would be indicative of GLUT1 translocation towards the cell membrane. 100nM TCAP-1 treatment led to a significant increase in the Corrected Total Cell Fluorescence (CTFC) 1 hour after treatment of GLUT1 compared to control (3448656 ± 283651 vs. 2383102 ± 107246 ; p< 0.05; Figure 3.4A); but did not lead to a significant change in the CTFC after 3 hours of treatment compared to control (3.099440 ± 351365 vs. 2383102 ± 107246 ; Figure 3.4A). Compared to control, although there appeared to be an increase in GLUT1 membrane localization, statistical analysis using a 1-way ANOVA and Bonferonni's post hoc test revealed no significant changes (1.449 ± 0.1902 vs. 2.126 ± 0.1630 and 1.449 ± 0.1902 vs. 2.298 ± 0.3192 ; Figure 3.4B).



Figure 3.4: Immunocytochemical analyses for GLUT1 in mHypoE-38 immortalized embryonic hypothalamic neurons following 1 or 3 hours of 100nM TCAP-1 treatment. Images were analyzed using ImageJ (v10.2) software for (A) Corrected Total Cell GLUT1 Fluorescence (*de novo* synthesis) and (B) membrane/perinuclear GLUT1 ratio (translocation). A significant increase in total cell GLUT1 fluorescence was found following 1 hour of TCAP-1 treatment. No statistical changes were found for membrane/perinuclear GLUT1 ratio following TCAP-1 treatment. (Mean \pm SEM; n=6; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.1.4 The effect of TCAP-1 on GLUT3 expression and cellular localization

GLUT3 is the main neuronal transporter whose expression level is tightly correlated with regional cerebral glucose utilization. The transporter's high glucose affinity ($K_m \sim 1.5 \text{ mM}$) and highest calculated turnover rate make this isoform of particular importance in neuronal nutrient acquisition under stressed conditions. Synchronized mHypoE-38 cell cultures were treated with vehicle (0h TCAP-1) at t=1h or 100nM TCAP-1 at t=1h or 3h; immunocytochemical analysis for GLUT3 was subsequently performed. Immunofluorescence images indicate an increase in total cell GLUT3 expression, seen by the overall gain in fluorescence at 3 hours (Figure 3.5D vs. F).

An increase in the membrane localization of GLUT3 was also observed; at 0 hours the fluorescence is concentrated around the nucleus, 1 hour following treatment the fluorescence is more diffuse, moving away from the nucleus, and continues to spread throughout the cell at 3 hours (Figure 3.5). These observations are consistent with the important role of GLUT3 transporters in sustaining neuronal glucose uptake.



Figure 3.5: Representative immunocytochemical images for GLUT3 in mHypoE-38 immortalized embryonic hypothalamic neurons following 1 or 3 hours of 100nM TCAP-1 treatment. Top row: corresponding DIC images (A,B and C). Middle row: GLUT3 fluorescence at 0 hours (control; D), 1 hour (E) and 3 hours (F) following 100nM TCAP-1 treatment. Bottom row: Merge of DAPI (nucleus), GLUT3 (glucose transporter) and wheat germ agglutinin (cell membrane) fluorescence (G, H and I). Images were obtained using a WaveFX spinning-disk confocal microscope (Quorum Technologies, Canada).Magnification of 630x, 15µm scale bar.

Treatment with 100nM TCAP-1 resulted in a significant increase in the Corrected Total Cell Fluorescence after 3 hours compared to control (3467517 ± 221898 vs. 2302632 ± 168244 ; p< 0.0001 ; Figure 3.6A) and may be indicative of increased GLUT3 synthesis or stabilization. Furthermore, TCAP-1 induced a statistically significant movement in GLUT3 from the perinuclear region to the cell membrane region at 1 hour and 3 hours following treatment compared to control (2.734 ± 0.1961 and 2.824 ± 0.2244 vs. 1.988 ± 0.1018 ; p< 0.05; Figure 3.6B), and is suggestive of GLUT3 translocation towards the membrane.



Figure 3.6: Immunocytochemical analyses for GLUT3 in mHypoE-38 immortalized embryonic hypothalamic neurons following 1 or 3 hours of 100nM TCAP-1 treatment. Images were analyzed using ImageJ (v10.2) software for (A) Corrected Total Cell GLUT3 Fluorescence (*de novo* synthesis or stabilization) and (B) membrane/perinuclear GLUT3 ratio (translocation). A significant increase in total cell GLUT3 fluorescence was found following 3 hours of TCAP-1 treatment. GLUT3 translocation towards the membrane was discovered at both 1 and 3 hours following treatment, as determined from the membrane/perinuclear GLUT3 ratio. (Mean \pm SEM; n=6; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.1.5 The effect of TCAP-1 on GLUT4 expression and cellular localization

The GLUT4 isoform is constantly cycled between the cell membrane and intracellular reserves; it is highly responsive to insulin, which triggers rapid translocation of GLUT4 towards the cell membrane (Klip *et al.*, 2009). It is mainly expressed in skeletal and muscle tissues but has recently been implicated in the sensing mechanisms of hypothalamic glucosensing neurons in response to brain insulin (Alquier *et al.*, 2006). Synchronized mHypoE-38 cell cultures were

treated with vehicle (0h TCAP-1) at t=1h or 100nM TCAP-1 at t=1h or 3h; immunocytochemical analysis for GLUT4 was subsequently performed. Following TCAP-1 treatment, there were no observable changes in GLUT4 membrane localization and there appeared to be a slight decrease in total GLUT4 at 1 hour and only a slight increase in total cell GLUT4 at 3 hours, seen as a small gain in fluorescence at 3 hours (Figure 3.7).



Figure 3.7: Representative immunocytochemical images for GLUT4 in mHypoE-38 immortalized embryonic hypothalamic neurons following 1 or 3 hours of 100nM TCAP-1 treatment. Top row: corresponding DIC images (**A,B and C**). Middle row: GLUT4 fluorescence at 0 hours (control; D), 1 hour (**E**) and 3 hours (**F**) following 100nM TCAP-1 treatment. Bottom row: Merge of DAPI (nucleus), GLUT4 (glucose transporter) and wheat germ agglutinin (cell membrane) fluorescence (**G, H and I**). Images were obtained using a WaveFX spinning-disk confocal microscope (Quorum Technologies, Canada).Magnification of 630x, 15µm scale bar.

Despite there being a slight observable increase in total cell GLUT4 at 3 hours, statistical analysis revealed no significant changes in the Corrected Total Cell Fluorescence following 1 and 3 hours of TCAP-1 treatment from control levels (1347152 ± 127807 and 1509487 ± 110129 vs. 1448977 ± 189374 ; Figure 3.8A). Translocation of GLUT4, which is highly sensitive to insulin signalling, was not detected after 1 and 3 hours of treatment compared to control (2.473 ± 0.2451 and 1.817 ± 0.1926 vs. 2.083 ± 0.1621 ; Figure 3.8B); this suggests that TCAP-1 does not increase nutrient acquisition in an insulin-like manner.



Figure 3.8: Immunocytochemical analyses for GLUT4 in mHypoE-38 immortalized embryonic hypothalamic neurons following 1 or 3 hours of 100nM TCAP-1 treatment. Images were analyzed using ImageJ (v10.2) software for (A) Corrected Total Cell GLUT4 Fluorescence (*de novo* synthesis or stabilization) and (B) membrane/perinuclear GLUT4 ratio (translocation). Statistical analysis determined no significant changes in total cell GLUT4 fluorescence and GLUT4 translocation following TCAP-1 treatments, irrespective of the time elapsed. (Mean \pm SEM; n=6; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.2 Objective #2: Identification of the metabolic parameters associated with the intracellular actions of Teneurin C-terminal Associated Peptide (TCAP) -1 in mHypoE-38 immortalized embryonic hypothalamic cells.

3.2.1 The effect of chronic TCAP-1 treatments of 0nM (vehicle), 1nM, 10nM and 100nM on tumourgenic glycolytic protein expression.

Increased glycolytic capacities can occur through up regulation of glycolytic enzymes and a transition towards more favourable enzyme isoforms (Hammerman *et al.*, 2004). The Warburg effect describes the metabolic adaptation of growing tumour cells towards glycolytic energy

production to satisfy nutrient deficits; unfortunately this mechanism offers a short-term solution and the detrimental consequences can lead to extensive tissue damage (Mazoor *et al.*, 2008). Synchronized mHypoE-38 cell cultures were given chronic TCAP-1 treatments of 0nM, 1nM, 10nM and 100nM at t=0, 3, 6, and 9h and lysed at 12h. Western Blot analyses were subsequently performed for the following tumourgenic glycolytic enzymes: LDH5, Enolase-2, Hexokinase, PFKL, ALDOB, PGK1, PGM1, Pyruvate Kinase and GAPDH (housekeeping protein). Chronic TCAP-1 treatments yielded no significant changes in immunoreactivity for the glycolytic proteins analyzed (Figure 3.9). Lack of a response in Warburg glycolytic enzymes supports the notion that TCAP-1 mediated protection occurs through a sustainable mechanism of metabolic optimization, as opposed to sole reliance on glycolytic energy production.



Figure 3.9: Chronic TCAP-1 treatments do not alter tumourgenic glycolytic enzyme expression in mHypoE-38 neurons. Immunoreactivity for the glycolytic enzyme isoforms LDH5, Enolase-2, Hexokinase II, PFKL, ALDOB, PGK1, PGM1, which are upregulated during tumourgenesis, and GAPDH (housekeeping protein) was examined using western blot analysis. No significant changes in immunoreactivity were observed at 12 hours following chronicTCAP-1 treatments. (A) Representative western blots for ALDOB, Hexokinase II and LDH5 (B) Representative statistical analysis for Hexokinase/GAPDH I.O.D. ratio. (Mean \pm SEM; n=4; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.2.2 The effect of chronic TCAP-1 treatments of 0nM (vehicle), 1nM, 10nM and 100nM on intracellular lactate concentrations.

Production of lactate is an inevitable by-product of sustained glycolysis as it is required to regenerate NAD+ from NADH (Kolev *et al.*, 2008). Thus, an increase in lactate concentrations would be indicative of a metabolic shift favouring glycolytic metabolism. Synchronized mHypoE-38 cell cultures underwent chronic TCAP-1 treatments of 0nM, 1nM, 10nM and 100nM at t=0, 3, 6, and 9h and lysed at 12h. Intracellular lactate concentrations were measured using a colorimetric lactate assay kit. The study was repeated with a scrambled version of TCAP-1 to rule out the possibility of an unspecific cellular response. Lactic acid concentrations were significantly reduced from control concentrations following chronic TCAP-1 treatments of 1nM , 10nM and 100nM in a dose-dependent manner (1.224 ± 0.01755 nmol/well vs. 1.046 ± 0.03518 nmol/well p<0.05, 0.9084 ± 0.01845 nmol/well p<0.001, 0.6954 ± 0.05810 nmol/well p<0.001, respectively; Figure 3.10A), indicating that TCAP-1 does not favour a glycolytic shift in metabolism. No significant changes were observed for treatments with scrambled TCAP-1 (Figure 3.10B).



Figure 3.10: Chronic TCAP-1 treatments significantly reduce lactate production in mHypoE-38 immortalized embryonic hypothalamic neurons. Lactate concentrations, in response to chronic treatments of (A) TCAP-1 and (B) Scrambled TCAP-1, were examined using a colorimetric lactate assay. There was a dose-dependent decrease in intracellular lactate concentration at 12 hours following chronic TCAP-1 treatments. (Mean \pm SEM; n=4; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.2.3 The effect of chronic TCAP-1 treatments of 0nM (vehicle), 1nM, 10nM and 100nM on intracellular ATP concentrations.

An increase in energy availability is the underlying fundamental process by which TCAP-1 is postulated to alleviate cellular stress. This process of metabolic optimization could enhance survivability by either directly by reducing energetic deficits of indirectly by fueling other protective mechanisms (Hammerman *et al.*, 2004; Brand, 2005). Synchronized mHypoE-38 cell cultures underwent given chronic TCAP-1 treatments of 0nM, 1nM, 10nM and 100nM at t=0, 3, 6, and 9h and lysed at 12h. Intracellular ATP concentrations were measured using a colorimetric ATP assay kit and the study was repeated with a scrambled version of the peptide to confirm the specificity of the response to TCAP-1. Chronic TCAP-1 treatments of 1nM, 10nM and 100nM resulted in a significant dose-dependent increase in intracellular ATP concentrations compared to control (1.414 ± 0.02712 nmol/well p<0.01,1.529 ± 0.03238 nmol/well p<0.001, 1.730 ± 0.03791 nmol/well p<0.001, respectively, vs. 1.175 ± 0.05746 nmol/well; Figure 3.11A); that were not observed with scrambled TCAP-1 treatments (Figure 3.11B).



Figure 3.11: Chronic TCAP-1 treatments significantly increase intracellular [ATP] in mHypoE-38 immortalized embryonic hypothalamic neurons. ATP concentrations, in response to chronic treatments of (A) TCAP-1 and (B) Scrambled TCAP-1, were examined using a colorimetric ATP assay. There was a dose-dependent increase in intracellular ATP concentration at 12 hours following chronic TCAP-1 treatments. (Mean \pm SEM; n=4; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.2.4 The effect of acute TCAP-1 treatments of 0nM (vehicle), 1nM, 10nM and 100nM on intracellular ATP concentrations.

Activation of stress pathways likely induces immediate cellular responses; the impact of this response on cellular ATP availability was assessed through acute TCAP-1 treatments of 0nM, 1nM, 10nM and 100nM on neurons at t=0 and lysed after 1h. In contrast to chronic treatments, there was a significant decrease in ATP concentration following acute TCAP-1 treatments of 10nM and 100nM compared to control (0.6380 ± 0.01274 nmol/well p<0.05 and 0.5851 ± 0.01767 nmol/well p<0.001, respectively, vs. 0.7574 ± 0.03177 nmol/well; Figure 3.12), with the largest concentration yielding the greatest deviations from control values. This suggests that intracellular ATP concentrations exhibit both a dose-dependent response and a time-dependent response to TCAP-1.



Figure 3.12: Acute TCAP-1 treatments significantly decrease intracellular [ATP] in mHypoE-38 immortalized embryonic hypothalamic neurons. ATP concentrations, in response to an acute TCAP-1 treatment, were examined using a colorimetric ATP assay. There was a dose-dependent decrease in intracellular ATP concentrations at 1 hour following an acute TCAP-1 treatment of 10 and 100nM. (Mean \pm SEM; n=4; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.2.5 The effect of chronic TCAP-1 treatments of 0nM (vehicle), 1nM, 10nM and 100nM on mitochondrial apoptotic protein expression.

The interplay between metabolic adaptation and anti-apoptotic pathways in maintaining mitochondrial integrity could influence the expression of mitochondrially associated apoptotic proteins (Hammerman *et al.*, 2004; Mathulpala *et al.*, 2006). Under stressful conditions, this

may translate into reduced susceptibility towards mitochondrial membrane permeabilization. Synchronized mHypoE-38 cell cultures were given chronic TCAP-1 treatments of 0nM, 1nM, 10nM and 100nM at t=0, 3, 6, and 9h and lysed at 12h. Western Blot analysis for the apoptotic proteins Bcl-2, Bax, Cytochrome c, and Caspase-3 was performed; GAPDH was utilized as a housekeeping protein. Chronic treatments of TCAP-1 did not induce any significant changes in immunoreactivity for the mitochondrial apoptotic proteins Bcl-2, Bax and Caspase-3 (Figure 3.13B). Although indication of a slight decrease in cytochrome c was observed, it was not statistically significant (Figure 3.13A). In addition, there was no evidence of caspase-3 cleavage; this would be predicted since caspase-3 activity should only occur under conditions of cellular stress (Figure 3.13A).



Figure 3.13: Chronic TCAP-1 treatments do not alter mitochondrial apoptotic protein expression under unstressed conditions in mHypoE-38 neurons. Immunoreactivity for the apoptotic proteins Bcl-2, Bax, Cytochrome c, Caspase-3 and GAPDH (housekeeping protein) was examined using western blot analysis. No significant changes in immunoreactivity were observed at 12 hours following chronic TCAP-1 treatments. (A) Representative western blots for Bcl-2, Bax, Cytochrome c and Caspase-3. Absence of Caspase-3 cleavage confirms unstressed conditions. (B) Representative statistical analysis for Bax/GAPDH I.O.D. ratio. (Mean \pm SEM; n=4; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.3 Objective #3: *In vivo* physiological manifestation of Teneurin C-terminal Associated Peptide (TCAP)-1 administration on adiposity and associated leptin and insulin endocrine responses in male BALB/C mice.

3.3.1 The in vivo effect of TCAP-1 administration on subcutaneous adipose tissue.

If TCAP-1 induces a state of heighted cellular metabolism, exogenous administration would theoretically cause a depletion of exiting fat reserves and inhibit the storage of new adipose tissues, as energy stores are liberated to fuel energetically demanding tissues. A preliminary in vivo trial was performed to examine the impact of TCAP-1 administration on normal metabolic parameters. Male BALB/c mice were treated with saline (n=10), 25 pmol/kg TCAP (n=11) and 250 pmol/kg TCAP (n=10) daily for 9 days and sacrificed on day 10, upon which subcutaneous adipose was collected and fixed in paraformaldehyde. Haematoxylin & Eosin (H&E) staining was performed and the size and density of adipocytes examined. A reduction in the overall size and increase in the density of adipocytes is characteristic of weight loss (Johnson and Albert, 1985; Galic et al., 2010). Blinded measurements were taken for the number of adipocytes/200x magnification square - a measure of adipocyte density, and a significant augmentation in adipocyte density was found in mice given 25 pmol/kg of TCAP-1 compared to saline (93.00 \pm 6.688 adipocytes/square vs. 69.76 ± 2.785 adipocytes/square; p<0.05; Figure 3.14A). Saline treated mice had an adipose tissue composition of mainly large oval adipocytes (Figure 3.14B). In contrast, mice treated with TCAP-1 exhibited an observable reduction in adipocyte size, resulting in numerous small and medium sized circular adipocytes surrounding a few large oval adipocytes; this was most prominent at 25 pmol/kg (Figure 3.14B and 3.14C).



Figure 3.14: TCAP-1 administration *in vivo* reduces subcutaneous adipocyte density and size at 25 pmol/kg. H&E staining was used to determine subcutaneous adipocyte density of male BALB/c mice treated with saline (**B**; n=10), 25pmol/kg TCAP (**C**; n=11) and 250pmol/kg TCAP (**D**; n=10). Statistical analysis (**A**) revealed a significant increase in adipocyte density at 25 pmol/kg TCAP-1 treatment. (Mean \pm SEM; n=10/11; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.3.2 Response of metabolic hormones, insulin and leptin, to TCAP-1 administration in vivo.

Exogenous treatments of TCAP-1 may alter body energetics through enhanced metabolic capacities; the ensuing endocrine responses would be transported through the blood. Serum samples were collected from the previously described mice, from which a Mouse MetabolicMAP® was generated. Insulin is an important regulator of energy homeostasis that is released from pancreatic β cells in response to increased blood glucose levels and triggers the lipogenic pathway of adipocyte growth (Niswender and Magnuson, 2007). A significant reduction in serum insulin levels was detected at 250 pmol/kg TCAP-1 compared to saline (1.400 ± 0.06547 ulU/mL vs. 1.988 ± 0.1060 ulU/mL; p<0.05; Figure 3.15A). Leptin is a satiety signal, secreted from adipose tissue, whose circulating serum levels have a positive correlation

with adipocyte size (Lönnqvist *et al.*, 1997; Galic *et al.*, 2010). Although some mice exhibited decreased serum leptin levels following TCAP-1 administration, no statistically significant changes were found for serum leptin levels following TCAP-1 treatment compared to saline controls (Figure 3.15B).



Figure 3.15: TCAP-1 *in vivo* may cause a shift towards enhanced energy production, resulting in alterations of metabolically related hormone secretion. A Mouse MetabolicMAP® was generated using serum collected from male BALB/c mice treated with saline (n=10), 25pmol/kg TCAP (n=11) and 250pmol/kg TCAP (n=10). (A) A significant reduction in serum insulin levels was observed at 250pmol/kg TCAP-1 treatment. (B) No statistically significant changes were observed in serum leptin levels following TCAP-1 administration. (Mean \pm SEM; n=10/11; *p<0.05, **p<0.01, ***p<0.001; 1-way ANOVA and Bonferonni's post hoc test).

3.4 References

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4 Chapter Four: Discussion

4.1 Summary of findings

The purpose of this study was to examine the fundamental mechanism through which TCAP-1 confers neuroprotection *in vitro* in mHypoE-38 neurons and understand the implications of exogenous applications in vivo. Glucose and G6P assays yielded highly variable results with no statistical significance, necessitating a different experimental approach. Owing to the direct positive correlation between membrane GLUT density and glucose uptake (Duelli and Kuschinsky, 2001; Alquier et al., 2006; Airley and Mobasheri, 2007; Klip et al., 2009; Zaid et al., 2009; Weisova et al., 2009), immunocytochemical analysis of the corrected total cell fluorescence (CTFC) and the membrane/perinuclear fluorescence ratio (Figure 2.3) for the glucose transporter isoforms GLUT1, GLUT3 and GLUT4, would examine the influence of TCAP-1 in vitro in glucose transporter capacity. Treatment with 100nM TCAP-1 produced a significant increase in CTFC for GLUT1 after 1 hour only and in GLUT3 after 3 hours only; membrane localization for GLUT3 was significantly augmented after 1 hour and sustained after 3 hours. No statistically significant changes were found for the GLUT4 isoform. In order for this to be of value to the cell, the glucose must be metabolized into useful energy in the form of ATP with mitochondrial respiration representing the most efficient and sustainable option (Stuart et al., 1999; Brand, 2005; Klip et al., 2009). Thus the effect of treatment concentration and duration of TCAP-1 on metabolic parameters and associated apoptotic responses was examined in vitro. Western blot analysis showed no response in tumourgenic glycolytic enzymes and a dosedependent reduction in intracellular lactate concentrations with chronic TCAP-1 treatments. A dose-dependent and time-dependent response in intracellular ATP concentration was found; a significant dose-dependent increase was revealed for chronic TCAP-1 treatments whereas a significant dose-dependent decrease was revealed for an acute TCAP-1 treatment. Immunoreactivity remained constant for mitochondrial associated apoptotic proteins as the cells were cultured under unstressed conditions; this was confirmed by the absence of caspase-3 cleavage. Although the in vitro studies for TCAP-1 were performed on mHypoE-38 hypothalamic neurons, phylogenetic analysis (Lovejoy et al., 2006; Tucker et al., 2011) and high expression in vital tissues (Wang et al., 2005; Bittencourt, unpublished findings; Chand et al., 2010) suggest that TCAP-1 signaling is fundamental and applicable to a multitude of cell-types.

Furthermore, TCAP is expressed in low concentrations throughout the periphery in tandem with its receptor, the β -dystroglycan subunit (Tan *et al.*, manuscript in preparation, Chand *et al.*, manuscript submitted). Results for the *in vivo* response of increased subcutaneous adipocyte density at 25 pmol/kg TCAP-1, signifying loss of white adipose tissue (Johnson and Albert, 1985; Galic *et al.*, 2010), are in agreement with this concept. In addition, these preliminary *in vivo* studies found a significant reduction in serum insulin at treatments if 250 pmol/kg TCAP-1, but no significant changes were found in serum leptin levels.

4.2 Objective #1: Enhancement of glucose transporter capacity by Teneurin C-terminal Associated Peptide (TCAP) -1 in mHypoE-38 immortalized embryonic hypothalamic neurons.

The neuroprotective actions of TCAP-1 against hypoxic and oxidative stress have been found to recruit a diverse array of survival responses, from phosphorylation of p90RSK (Chand et al., 2012) to upregulation of endogenous antioxidant production (Trubiani et al., 2007); irrespective of their differences, all these mechanisms are unified under one fundamental criterion -a need for enhanced energy production through metabolic optimization. This could provide a sustainable solution to alleviate hypoxic oxygen deficits and fuel survival mechanisms against a range of homeostatic insults; the first target for metabolic optimization is enhanced nutrient acquisition. In the periphery, glucose utilization is increased in response to stress as it generates more ATP per unit of oxygen consumed compared to fatty acids (Fragasso *et al.*, 2008). Enhancement of glucose uptake is of considerable importance to the CNS as neuronal metabolism is almost entirely reliant on glucose (Baker and Tarnopolsky, 2003; Duelli and Kuschinsky, 2001). The data obtained from colorimetric assays, for intracellular glucose and G6P concentrations following chronic TCAP-1 treatments, yielded highly variable results that were not statistically significant. Reaction of residual glucose to the probe could account for the variability observed, as per kit troubleshooting (Biovision, Cat. No. K606-100-1; Biovision, Cat. No. K657-100-1). To rectify this issue, some techniques reduce the high glucose concentration of the media to normal physiological levels of \sim 3mM and this may reduce the variability observed. However, since the neuroprotective actions of TCAP-1 in mHypoE-38 neurons were observed when cells were cultured with high extracellular glucose concentrations of ~25mM (Trubiani et al., 2007; Ng, 2010), which is a commonly employed neuronal culture media (Kleman et al., 2008), the glucose concentration of the media was not altered as it represents another variable.

The implications this issue will be further examined and highlights the need for more reliable techniques to reduce the variability (Discussion section 4.5).

Glucose transporters of the GLUT/SLC2A family utilize the diffusion gradient of glucose across plasma membranes to mediate facilitative transport of glucose into the cell (Joost and Thorens, 2001; Airley and Mobasheri, 2007). Consequently, glucose concentration gradient (Duelli and Kuschinsky, 2001; Joost and Thorens, 2001; Zaid et al., 2009), heterogeneous isoform composition (Duelli and Kuschinsky, 2001; Wood and Trayhurn, 2003) and overall density of GLUT transporters (Alquier et al., 2006; Airley and Mobasheri, 2007; Klip et al., 2009; Weisova et al., 2009) at the cell membrane directly relate to glucose uptake. Consequently, changes in glucose transporter capacity can be observed and measured by immunocytochemical analysis for corrected total cell fluorescence and ratio of membrane to perinuclear GLUT fluorescence. Fluorescence at 1 and 3 hours following TCAP-1 treatment were analyzed based on previous studies in which translocation was induced 30-60 minutes after stimulus and overall transporter accumulation at the cell membrane observed at 1 to several hours after stimulus (Duelli and Kuschinsky, 2001; Airley and Mobasheri, 2007; Klip et al., 2009; Weisova et al., 2009; Zaid et al., 2009). A prolonged experimental time-line could result in further increases in glucose transporter capacity; this would require repeated TCAP-1 stimulation as the internalization of the peptide is observed at 15 minutes, where it is theorized to activate additional responses before undergoing degradation (Ng et al., 2011).

Basal glucose transport in almost all mammalian cells is maintained by GLUT1. Under normal conditions, distribution of the GLUT1 isoform density within the brain correlates with capillary density. Induction of increased local cerebral glucose utilization by nicotine infusion (Duelli and Kuschinsky, 2001) or water deprivation (Duelli *et al.*, 1999) resulted in paralleled increases in local GLUT1 densities of +15% and +26%, respectively. Kinni *et al.* (2011) observed this phenomenon in rats following forced and voluntary exercise; most notably, forced exercise generated a significant increase in GLUT1 mRNA and protein expression compared to both control and voluntary groups (p<0.01). The significant increase in immunocytochemical expression of GLUT1 after 1 hour of TCAP-1 treatment (p<0.05) indicates that TCAP-1 treatment might enhance the metabolic utilization of glucose, which is reflected in augmented transporters densities. Lack of GLUT1 membrane localization is consistent with prevailing

models for the regulation of GLUT1 expression at transcriptional, posttranscriptional, and posttranslational levels. The GLUT1 promoter region consists of several elements under transcriptional control; in response to hypoxic stress, translocation of HIF-1 to the nucleus of tumourgenic cells increases GLUT1 mRNA expression (Airley and Mobasheri, 2007). Oncotic transporter upregulation can also occur through a post-transcriptional mechanism, where elements in the 3'-untranslated region of the GLUT1 mRNA are recognized by mRNA binding proteins that stabilize the transcripts from decay (Qi and Pekala, 1999; Griffin *et al.*, 2004). Lastly, GLUT1 translation can be induced by insulin (Duelli and Kuschinsky, 2001). The decline in GLUT1 expression at 3 hours coincides with previous findings of HIF-1 α reduction following 24 hours of TCAP-1 treatment (Ng, 2010). Neuronal reliance on GLUT1 transport may be reduced due to the isoform's moderate affinity for glucose, demonstrated by a K_m= 5-10mM (Duelli and Kuschinsky, 2001), in favour of a more efficient isoform.

As the main neuronal isoform, GLUT3 has a low Michealis-Menten constant of K_m=1-5mM and is therefore able to facilitate continuous glucose transport even under low interstitial glucose concentrations (reviewed by Simpson et al., 2008). Similar to GLUT1, GLUT3 densities have also been found to tightly correlate local cerebral glucose utilization in the previously mentioned studies, with a larger response of +39% to nicotine infusion (Duelli and Kuschinsky, 2001). The high affinity of this transporter for glucose designates it as the ideal isoform for TCAP-1 mediated protection. Immunocytochemical analysis indicates that TCAP-1 may preferentially act on GLUT3 as significant changes were observed for both overall cell fluorescence and membrane localization. Significant augmentation in total cell GLUT3 fluorescence was found following 3 hours of TCAP-1 treatment, coinciding with the decrease in GLUT1 expression. In addition, accumulation of GLUT3 at the cell membrane was discovered at both 1 and 3 hours; this could be attributed to translocation and/or stabilization of the protein at the membrane. A study by Weisova et al. (2009) observed a significant increase in surface GLUT3 expression 30 minutes, following glutamate excitation, which persisted for up to four hours. They found that activation of AMPK, in response to excitatory alterations in the AMP:ATP ratio, inhibited endocytosis of GLUT3 from the cell membrane; a similar mechanism may account for the initial increase in GLUT3 membrane expression at 1 hour post TCAP-1 treatment. Although neuronal GLUT3 has been observed in distinct vesicles (Thoidis et al., 1999) and glutamate excitation parallels AMPK regulation of GLUT4 translocation (Weisova et al., 2009), there is a lack of

supporting evidence for insulin-induced translocation of GLUT3 to the membrane in neurons. One study by Uemura and Greenlee in 2006 found that insulin promoted translocation of GLUT3 vesicles to the membrane, but did not increase neuronal glucose uptake as the vesicles failed to fuse with the plasma membrane. An insulin-independent mechanism was exhibited during DNP-induced metabolic stress and could contribute to maintenance of GLUT3 membrane expression at 3 hours post TCAP-1 treatment; elevated membrane GLUT3 was achieved by transporter protein stabilization which prolonged the half-life (Khayat *et al.*, 1998). Interestingly, GLUT3 expression in reproductive tissues coincides with TCAP-1 distribution; the transporter plays a critical role in murine sperm function and is highly concentrated in the plasma membrane of the flagellum (reviewed by Simpson *et al.*, 2008).

Insulin-mediated regulation occurs primarily in skeletal muscles and adipose tissue for the translocable GLUT4 isoform (Klip, 2009). Within the brain, translocable glucose transporters GLUT4 and GLUT8 have been found to be insulin-responsive (Alquier *et al.*, 2006). Benomar *et al.* (2006) discovered a PI3K-dependent mechanism for insulin induced translocation of GLUT4 to the plasma membrane in human neuroblastoma cells. TCAP-1 treatments yielded no significant changes in total cell GLUT4 fluorescence and GLUT4 translocation, irrespective of the time elapsed. Since neuroblastoma cells have undergone tumourgenic metabolic changes, these findings are in agreement with the TCAP-1 model of neuroprotection which involves a different pathway for metabolic optimization.

It is important to consider the specific functional role of GLUT4 transporters to the mHypoE-38 hypothalamic neurons, which are derived from the arcuate nucleus (ARC) (Fick and Belsham, 2010). The hypothalamus is responsible for regulating energy homeostasis and localization of insulin-sensitive GLUT4 may be responsible for the glucosensing mechanisms of these neurons. Electrophysiological studies show that insulin modulation of arcuate nucleus cells is glucose dependent; activation of glucose-excited ARC neurons occurred at low concentrations of 0.1mmol/l glucose but was abolished at 2.5 mmol/l of glucose (Wang *et al.*, 2004). The absence of insulin stimulation and irrelevance of GLUT4 glucosesing mechanisms in neuroprotection support the exclusion of a GLUT4 response following TCAP-1 administration *in vitro*. However, TCAP-1 may have potential applications in regulating energy homeostasis *in vivo* with respect to insulin resistance – a condition characterized by loss of insulin responsiveness despite increased

insulin stimulation (Fick and Belsham, 2010). Increased adiposity is theorized to induce insulin resistance through: 1) secretion of cytokines, including TNF- α , IL-6, IL-1 β , and 2) increased circulating free fatty acids which shift metabolism of the muscle, liver and other tissues away from gluconeogenesis and glycolysis towards lipid deposition and oxidation (Koch *et al.*, 2008). To compensate, insulin secretion is augmented but futile as there is a corresponding reduction in insulin-receptor expression, from internalization and degradation, and reduction in signal transduction upon insulin receptor binding (Koch *et al.*, 2008, Fick and Belsham, 2010). The dependence of these mechanisms on insulin signaling, and the possible independence of TCAP-1 mediated glucose uptake from insulin, highlights a potential for TCAP-1 in maintaining glucose homeostasis.

Furthermore, enhanced glucose uptake has also been suggested to improve memory and cognition (reviewed by Messier, 2004; McNay and Gold; 2002). In 2002, McNay and Gold illustrated how increased glucose uptake by highly active neurons could significantly reduce the extracellular glucose concentration, thereby starving surrounding neurons of this crucial substrate and resulting in reduced performance. Intraperitoneal glucose injections were found to rescue hippocampal extracellular glucose levels from a 30% decline, induced by a four-arm alternation task, and translated into an increased number of alternations. Glucose is also vital for the direct synthesis of neurotransmitters, serving as the precursor for glutamate, GABA and acetylcholine synthesis (Messier, 2004). The augmentation of total cell and membrane GLUT3 fluorescence following TCAP-1 treatment could be useful in developing therapeutics for improving memory and is particularly useful as GLUT3 can function effectively under low glucose concentrations.

Objective #2: Identification of the metabolic parameters associated with the intracellular actions of Teneurin C-terminal Associated Peptide (TCAP) -1 in mHypoE-38 immortalized embryonic hypothalamic cells.

Immediately following transport, through glucose transporters, glucose is phosphorylated by hexokinase to maintain the concentration gradient and prevent substrate backflow. This is the first step in the ancient biochemical pathway of glycolysis, which originally supplied energy to anaerobic life forms before the existence of atmospheric oxygen (Kim and Dang, 2005). A

cardinal feature of many tumours is evasion of hypoxic stress through preferential glycolytic metabolism mediated by upregulation and transition towards favourable enzyme isoforms (Semenza et al., 1994; Hammerman et al., 2004; Kim and Dang, 2007; Scatena et al., 2008). However, high expression of TCAP-1 in the brain (Wang et al., 2005) and reproductive tissues (Chand et al., 2010) dictates the need for a sustainable stress response, likely through enhanced glucose oxidation. The peptides close association with metazoan multicellular evolution (Lovejoy et al., 2006) also dismisses a glycolytic shift since the metabolic by-product of lactic acid would be counterproductive to maintaining cooperation within a multicellular unit. Chronic TCAP-1 treatments yielded no significant changes in immunoreactivity for the tumourgenic glycolytic enzymes LDH5, Enolase-2, Hexokinase II, PFKL, ALDOB, PGK1, PGM1 and Pyruvate Kinase. The ability of TCAP-1 to reduce HIF-1α expression (Ng, 2010), whose downstream targets include upregulation of Warburg glycolytic metabolism (Airley and Mobasheri, 2007), is in agreement with these findings. These findings suggest that TCAP-1 does not upregulate enzyme isoforms for the support of continued glycolytic metabolism. However, since these glycolytic enzyme isoforms are specific to tumourgenic metabolism, they may prematurely exclude TCAP's role in upregulating glycolytic processes. Upregulation of glycolytic genes conducive to overall metabolic functioning may be involved in TCAP-1 functioning and enhance production of pyruvate for mitochondrial utilization. In addition, under stressed conditions of hypoxia, glycolytic gene expression may be upregulated and TCAP-1 may act to restore basal expression levels; however, this was not observed as neurons were cultured under unstressed conditions. Adding a positive control of a stressor, such as hypoxia, may provide useful insights into the intracellular mechanism of TCAP-1.

Exclusion of the Warburg effect in mediating TCAP-1 protection is further substantiated by lactate assays following chronic TCAP-1 treatments. Continued glycolysis requires the regeneration of NAD⁺ from NADH and is mediated by LDH conversion of pyruvate to lactate (Airley and Mobasheri, 2007; Kolev *et al.*, 2008). There was a significant dose-dependent decrease in intracellular lactate concentrations following chronic TCAP-1 treatments, with significant reductions occurring at even 1nM TCAP-1; this effect was not found with the scrambled peptide.

The ultimate purpose for the postulated neuroprotective mechanism of TCAP-1 is to enhance cellular energy production. As the universal currency of energy in the cell, ATP concentrations should be augmented following metabolic optimization (Hammerman et al., 2004; Brand, 2005; Sanz et al., 2006). This phenomenon was established following chronic TCAP-1 treatments in *vitro*, which resulted in a significant dose-dependent increase in intracellular ATP concentrations following chronic TCAP-1 treatments, beginning at 1nM TCAP-1. These results did not occur with application of the scrambled peptide and confirms the specificity of the response to TCAP-1. The rise in intracellular ATP concentrations following chronic TCAP-1 stimulation can occur through two methods: 1) increased ATP production or 2) decreased ATP utilization. However, other studies on the in vitro response to TCAP-1 treatments demonstrate an increase in cellular activities, such as upregulation of cytoskeletal elements (Al Chawaf et al., 2007; Chand et al., 2012), resulting in increased neurite outgrowth and axon fasciculation (Al Chawaf et al., 2007). Additionally, an energetically expensive increase in endogenous antioxidant production was observed by Trubiani et al. in 2007. These findings indicate that TCAP-1 activates cellular activities and the increase in intracellular ATP concentration is likely due to increased ATP production.

Cellular energy homeostasis is tightly regulated and requires a balance between ATP generation and utilization; this is achieved through numerous regulatory proteins that are sensitive to ATP and ADP levels. TCAP-1 augmentation of intracellular ATP concentrations may seem counterintuitive to the maintenance of enhanced cellular metabolism as high intracellular ATP could potentially inhibit metabolic processes. However, the rise in intracellular ATP, as a consequence of optimized metabolism, has been documented in numerous studies (Rathmell *et al*, 2003; Bando *et al.*, 2005; Pelicano *et al.*, 2006; Weisova *et al.*, 2009; Alavian *et al.*, 2012); these findings highlight the dynamic nature of metabolic regulation and object the notion of a set threshold for ATP. Tight control of the allosteric enzyme PFK - deemed the most important glycolytic regulator, is exhibited by inhibition from high levels of ATP. Several studies have shown that phosphorylation of PFK by Akt may activate the enzyme and release it from ATP inhibition (Rathmell *et al*, 2003; Pelicano *et al.*, 2006). A well-established potent activator of PFK activity is F2,6BP; in 2005, Bando *et al.* illustrated how upregulation of F2,6BP can diminish the inhibitory effects of ATP. AMPK is the principal energy sensor of eukaryotic cells that is activated by increased AMP/ATP and ADP/ATP ratios. The reversible reaction of 2ADP \leftrightarrow ATP + AMP is maintained close to equilibrium by adenylate kinase, falling energy levels cause the reaction to be displaced to the left, thereby activating AMPK (reviewed by Hardie *et al.*, 2012). Under opposing conditions of increased energy levels, AMPK would be inhibited. However, the increased catabolic activity induced by AMPK following cellular stress have been found to be maintained for several hours after metabolic stress and intracellular ATP increases of ~30% have been observed (Weisova *et al.*, 2009). Although the threshold for intracellular ATP concentration can be altered, there is undoubtedly a maximum threshold above which energy homeostasis can no longer be regulated, leading to catastrophic consequences. The increased intracellular ATP concentrations exhibited by TCAP-1 treatment, of ~20% at 1nM up to a maximum of ~47% at 100nM, fall within the documented range of other studies; for instance, experimental manipulations for Bcl-XL over-expression by Alavian *et al.* (2012) yielded an increase of ~80% in ATP levels, as measured by firefly luciferin; luciferase luminescence.

ATP can be generated through either substrate phosphorylation during glycolysis or oxidative phosphorylation at the mitochondria (Kim and Dang, 2005). Dose-dependent increases in ATP concentrations, in conjunction with dose-dependent decreases lactic acid production following chronic TCAP-1 administration allude to the possibility that TCAP-1 mediated protection involves upregulation of aerobic oxidation at the mitochondria. In addition, initial immunocytochemical studies show an increase in TOM20 fluorescence following TCAP-1 treatment (Xu et al., unpublished findings). Mitochondrial proteins are mainly encoded in the nucleus and need to be imported into the organelle. TOM20 is a mitochondrial importer receptor subunit that plays a crucial role in increasing import efficiency through recognition of the presequence targeting signal and tethering of the presequence to the TOM40 complex (Eliyahu et al., 2010). However, further investigation is required to establish the ability of TCAP-1 to enhance mitochondrial functioning. If TCAP-1 is found to enhance mitochondrial functioning, it could greatly enhance cellular ATP production through refinement of oxidative phosphorylation. Although mitochondrial respiration accounts for over 90% of cellular energy production, significant proton leaks account for the dissipation of approximately 20% of metabolic in the form of heat (Stuart et al., 1999; Brand, 2005). A reduction in inducible proton leaks, through inhibition of the mitochondrial UCP (Stuart et al., 1999), could be a plausible and efficient mechanism for TCAP-1 mediated enhancement of oxidative phosphorylation.

Metabolic adaptation to increase the overall efficiency of cellular respiration would account for the seemingly contradictory findings of TCAP-1 rescue from hypoxic and oxidative stress. Enhanced mitochondrial functioning could theoretically generate higher energy returns per unit of oxygen, which would alleviate the stress of low oxygen tensions, effectively keeping the cell below threshold for hypoxic expression of HIF-1α (Ng., 2010). Furthermore, increased mitochondrial activity would upregulate the capacity of the cell to cope against oxidative stressors by fueling the production of endogenous antioxidants (Figure 4.1). Mitochondrial production of reactive oxygen species, including superoxide (O_2) , hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH-), occurs from incomplete reduction of molecular oxygen in the electron transport chain and is an inevitable consequence of aerobic metabolism (Turrens, 2003, Wen et al., 2011). In response, organisms have evolved antioxidant defence mechanisms to protect against the detrimental impacts of free radical accumulation (Murphy, 2009). An increase in mitochondrial activity triggers the endogenous production of free racial scavenging systems (Murphy, 2009; Wen et al., 2011). Therefore, TCAP-1 upregulation of superoxide dismutase (SOD), catalase and the SOD copper chaperone (Trubiani et al., 2007) may be reflective of increased mitochondrial activity and would possibly alleviate the need for mitochondrial UCP protection from ROS production.



Figure 4.1: The enhanced mitochondrial ATP production during metabolic optimization may simultaneously stimulate and fuel endogenous antioxidant production. Inhibition of mitochondrial UCP leaks and increased oxidative phosphorylation would generate an accumulation of the ROS, superoxide anion $O_2^{-\bullet}$, from the ETC. In response to this, endogenous antioxidant production in stimulated and subsequently fueled by the increased ATP availability. Superoxide dismutase converts $O_2^{-\bullet}$ to hydrogen peroxide (H₂O₂), which is then converted into water and oxygen by catalase enzymes. (Adapted from Turrens, 2003).

In addition to improved coupling efficiency through inhibition of mitochondrial UCP, TCAP-1 may also activate other mechanisms to enhance mitochondrial efficiency. For instance, the use of an alternative electron carrier has been found to improve mitochondrial respiration and inhibit glycolysis. Methylene blue was demonstrated by Wen *et al.* (2011) to bypass complex I/III blockage by rerouting electrons from NADH directly to cytochrome c and function as a direct ROS scavenger, resulting in a pronounced increase in intracellular ATP concentration of over 200% from control at doses of 1ng/ml and up. The phosphorylation of p90RSK and Bad following TCAP-1 treatment (Chand, 2012) indicate that the peptide could increase mitochondrial performance through the bcl-family of apoptotic proteins. A member of this family, Bcl-XL, has recently been found to interact with the β -subunit of the F₁F₀ ATP synthase to reduce membrane leak conductance and increase ATPase enzymatic activity (Alavian *et al.*, 2012).

Proper mitochondrial functioning is paramount to maintaining energy homeostasis; the consequences of mitochondrial energy dysregulation and oxidative stress have been implicated as key contributors to the pathophysiology of a variety of neurological disorders (Baker and Tarnopolsky, 2003). In addition to generating the majority of cellular ATP, the mitochondria also play key roles in apoptosis, cytosolic calcium buffering, excitotoxicity and free radical generation (Murphy, 2009; Wen *et al.*, 2011). Deviations in these processes have been linked to neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis and Huntington's disease (Mattson *et al.*, 1999; Baker and Tarnopolsky, 2003; Wen *et al.*, 2011). For instance, analysis of post mortem Parkinson's disease and Alzheimer's disease brain tissue revealed impaired glucose uptake, mitochondrial dysfunction and oxidative stress (Mattson *et al.*, 1999). The ability of TCAP-1 to enhance intracellular ATP concentrations, upregulate endogenous antioxidant capacities (Trubiani *et al.*, 2007) and prevent cellular death under conditions of stress (Trubiani *et al.*, 2007; Ng., 2010), may hold promise in the development of neurodegenerative therapeutics.

The complex process of metabolic adaptation inherently requires a large initial energetic cost and would result in the decline in ATP concentration following acute TCAP-1 administration. Contributing costs associated with this process could include translocation of GLUT to the plasma membrane, synthesis of enzymes and reallocation of crucial proteins (Klip *et al.*, 2009). Translocation of GLUT4 receptors to the membrane comprises three major ATP-consuming steps: 1) movement of vesicles along microtubules and/or microfilaments, powered by molecular motors (Zaid et al., 2008); 2) concentration of vesicles near the plasma membrane through remodeling of actin filaments (Randhawa et al., 2008); 3) docking and fusion of vesicles to the plasma membrane (Williams and Pessin, 2008). Induction of cytoskeletal reorganization by TCAP-1 has been found to correspond with increased neurite outgrowth and axon fasciculation (Al Chawaf et al., 2007). These changes, which include phosphorylation of the cytoskeletal regulatory proteins stathmin and filamin a, resulting in the upregulation of cytoskeletal proteins such as α -actinin-4 and β -tubulin (Chand *et al.*, 2012), could be recruited during metabolic optimization to reallocate glucose transports. In the absence of stressors, the initial investment cost for enhanced metabolism would be quickly recovered and utilized for neurite growth and axon formation (Al Chawaf et al., 2007).

Protection from ROS generation and increased mitochondrial efficiency may translate into reduced susceptibility to mitochondrial membrane permeabilization by TCAP-1. Chronic treatments of TCAP-1 did not induce any significant changes in immunoreactivity for the mitochondrial apoptotic proteins Bcl-2, Bax and Caspase-3. Since the neurons were grown under unstressed conditions, there would be no apoptotic signals for TCAP-1 to inhibit and therefore no changes would be predicted. In accordance, there was no evidence of caspase-3 cleavage, which is mediated by the apoptosome complex prior to apoptosis (Galluzzi et al., 2009). However, indication of a slight decrease in cytochrome c was observed, but not found to be statistically significant. There are two different pools for cytochrome c within the mitochondria: loosely and tightly bound. In order for apoptotic processes to occur, cytochrome c must first be solubilized, which involves disruption of electrostatic and/or hydrophobic interactions (Ott et al., 2002). Afterwards, permeabilization of the mitochondria will allow for the release of cytochrome c (Galluzzi et al., 2009; Shen et al., 2010). Any metabolic perturbations at the mitochondrial level could result in strengthening or disruption of such interactions (Ott et al., 2002). Therefore, if TCAP-1 increases the efficiency of mitochondrial energy production, then this could result in strengthening of the association of cytochrome c to the mitochondria. This would manifest as a reduction in cytochrome c levels in the western blot analysis as more cytochrome c may be tightly bound to the mitochondria and pelleted out. Furthermore, if TCAP-1 increases mitochondrial integrity, thereby reducing the susceptibility of cells to mitochondrial membrane permeabilization, it may serve as a feasible safeguard against traumatic brain injuries, ischemic stroke, hemorrhagic stroke and myocardial infarctions.

4.3 Objective #3: *In vivo* physiological manifestation of Teneurin C-terminal Associated Peptide (TCAP)-1 administration on adiposity and associated leptin and insulin endocrine responses in male BALB/C mice.

As a local paracrine signal (Chand *et al.*, 2010), the primary function of TCAP-1 release is theorized to serve as a warning signal to neighbouring cells by activating survival pathways for enhanced cellular metabolism in anticipation of impending threats. Therefore, an increase in metabolism would be observed at the localized tissue level following endogenous TCAP-1 release. Exogenous administration of synthetic TCAP-1 should theoretically upregulate overall metabolism, as both the peptide and β -dystroglycan receptor are expressed throughout the periphery (Tan *et al.*, manuscript in preparation), resulting in a shift in the balance between energy expenditure and storage towards net energy expenditure. Due to high expression of the βdystroglycan subunit receptor for TCAP-1 in muscle fibers (Chand *et al.*, manuscript submitted), skeletal muscles would likely exhibit a large response; this is supported by initial in vivo observations which that TCAP-1 may induce skeletal muscle hypertrophy and promote increased activity levels. The manifestation of prolonged exposure would consequently decrease white adipose mass, as a secondary response to the increased metabolic production of ATP in energetically demanding tissues, and was exhibited in vivo by preliminary studies on the impact of exogenous TCAP-1 administration. A significant reduction in the size and increase in the density of adipocytes at 25 pmol/kg TCAP-1 is indicative of fat loss as delipidation of adipocytes does not disrupt the total number of pre-existing adipocytes. At 250 pmol/kg TCAP-1 there was no significant reduction in adiposity; this biphasic response to TCAP-1 in vivo is a common occurrence and likely reflects the interaction of other homeostatic mechanisms (Tan, 2011). Another possibility is that naturally occurring variations in adipocytes, coupled with a limited sample size, could account for these results. Leptin is one of the main adipokine secreted from growing fat cells and serves as a satiety signal (Lönnqvist *et al.*, 1997); consequently, as adipocyte size shrinks, so will leptin secretion. There were no statistically significant reductions in serum leptin levels following TCAP-1 administration; this highlights the need for repetition of this study as some samples yielded results below the standard curve. This is likely due to detection issues arising from adapting a Rat MetabolicMAP® for use on mouse serum; resolution of this issue may require the use of a rat model instead. It would be expected that decreased adipocyte size with TCAP-1 treatment would occur in tandem with an associated reduction in serum leptin. Another possible explanation for this discrepancy is the short duration of 9 days for this preliminary study, which may not have allowed for sufficient decreases in adipocyte size to impact serum leptin secretion. Lin et al. (2000) examined diet induced obesity mouse models and found that obesity progression occurs through three stages over the course of 19 weeks, highlighting how *in vivo* physiological metabolic responses require an extended experimental timeline.

In addition to leptin, insulin is another key metabolic regulator whose main purpose is regulation of glucose homeostasis (Niswender and Magnuson, 2007). The significant reduction in serum insulin observed at 250 pmol/kg TCAP-1 *in vivo* is in accordance with the overlapping functions

of TCAP-1 and insulin on increasing cellular glucose transportation. Since insulin is released from pancreatic β cells in response to high blood glucose (Niswender and Magnuson, 2007), a possible TCAP-mediated mechanism for insulin-independent cellular glucose uptake would keep blood glucose levels sufficiently below the threshold for insulin release and decrease serum insulin levels. These findings, coupled with lack GLUT4 responsiveness to TCAP-1 *in vitro*, suggest that TCAP-1 does not directly antagonize insulin but rather reduction of insulin arises as a secondary effect from increased glucose uptake.

Insulin and leptin signaling both converge at the hypothalamus – a critical regulator of energy homeostasis (McNay et al., 2012). Ventromedial nucleus or arcuate nucleus lesions result in obesity (Olney, 1969), while lateral hypothalamic lesions are associated with weight loss (Anand Brobeck; 1951); understandably, integration of energy signals occurs at the arcuate nucleus and ventromedial hypothalamus. Leptin stimulates anorexigenic, appetite reducing and energy expending, proopiomelanocortin (POMC) neurons and inhibits or exigenic, appetite inducing and energy conserving, neuropeptide Y (NPY) neurons in the hypothalamus (Belsham and Flick, 2010; McNay et al., 2012). Similarly, insulin stimulation at terminals in the arcuate nucleus and median eminence elicit anorexigenic responses in the hypothalamus (Benomar et al., 2006; Belsham and Flick, 2010). The reduction in serum insulin levels following in vivo TCAP-1 administration could possibly activate the orexigenic neuropeptide Y neurons; this is supported by findings of significantly elevated sucrose intake observed in TCAP-1 treated rats (Lovejoy et al., unpublished findings). Although energy conservation also comprises the orexigenic pathway, the primary actions of TCAP-1 at the cellular level to enhance metabolic functioning and cellular activity likely preclude this response; however, further investigation into this mechanism is necessary.

The *in vivo* response to TCAP-1 administration holds promising potential for physiological diseases associated with energy dysregulation and may be of particular significance in light of the increasing obesity epidemic. Risks of developing type 2 diabetes, insulin resistance, hypertension, coronary artery disease and atherosclerosis significantly increase at a body mass index (BMI) of over 30 kg/m², the World Health Organization's (WHO) classification for obesity (Donohoe *et al.*, 2011). In 2012, a study by Adam *et al.*, found that modest weight loss
could reverse peripheral insulin insensitivity. Additionally, adiposity has also been linked with tumourgenesis and metabolic or bariatric surgery has been found to reduce the incidence of cancer, particularly in female patients (Ashrafian *et al.*, 2011). TCAP-1 offers a non-invasive alternative over surgeries; however, further investigation into the impact of TCAP-1 on adiposity and the associated endocrine responses is necessary (Discussion 4.4).

4.4 Models Used

The *in vitro* experiments of this study employed an immortalized embryonic mouse hypothalamic cell model to examine the mechanism by which TCAP-1 confers neuroprotection on account of an already established neuroprotective response in the mHypoE-38 cell line. Successful rescue from pH-induced stress was demonstrated in mHypoE-38 neurons following chronic TCAP-1 treatments and was attributed to upregulation of endogenous antioxidant capacity (Trubiani et al., 2007). TCAP-1 alleviation from hypoxic stress in mHypoE-38 neurons was exhibited by increased neurite outgrowth and restoration of basal HIF-1α expression (Ng, 2010). The immortalized neurons used in this study were generated by Belsham et al. (2004) to overcome the heterogeneity and complexity associated with the hypothalamus, thereby allowing for the mechanisms of individual hypothalamic neurons to be assessed. Primary mouse embryonic hypothalamic cultures were transformed with the potent oncogenic SV40 T antigen to generate immortalized neuronal cultures, from which a total of 38 cell lines were generated through further subcloning – one being the mHypoE-38 cell line. Despite being removed from their native environment, extensive profiling by Dalvi et al. (2011), have affirmed that these models express key neuronal markers and characteristic ultrastructural properties of mature endogenous neurons; these expression profiles are consistent for up to 30 passages. All the data obtained on the in vitro effects of TCAP-1 were performed on cells with a maximum of 5 passages. With respect to the phenotypic alterations that may occur as a consequence of transformation, Dalvi et al. (2011) found that SV40 T antigen enhancement of phosphoprotein activity was returned to basal levels following serum deprivation of the neurons. All mHypoE-38 cultures underwent serum deprivation for 16 hours prior to treatment, this procedure minimized confounding factors associated with cell cycling and restored any phenotypic alterations to basal levels. Therefore, provided that the correct experimental parameters are utilized, mHypoE-38

cells provide a useful and consistent model for the investigation of TCAP-1 neuroprotection *in vitro*.

The physiological distribution of the peptide and receptor, along with its evolutionary ancient phylogeny, indicate that the protective actions of TCAP-1 extend beyond neuronal tissue. Examination of the *in vitro* effect of TCAP-1 on various energetically demanding or sensitive tissues, such as the L6 myotube cell line developed by Dr. Amira Klip and Dr. Yosuke Ebina, represent important future studies to further define the metabolic parameters at the cellular level following TCAP-1 treatment. These L6 myotubes could be differentiated and transfected with myc-tagged Slc2a4 (L6-GLUT4myc), Slc2a3 (L6-GLUT3myc) and Slc2a1 (L6-GLUT1myc), thereby allowing for the study of glucose uptake and GLUT1, 3 and 4 translocation without the need to permeabilize or fractionate the cells through optical detection at the surface (Teixeira *et al.*, 2012). Application of insulin as a positive control for glucose translocation could be utilized and compared with TCAP-1 treated effects.

For the *in vivo* studies, male BALB/c mice were selected as a model to study the behavioural and physiological responses to exogenous TCAP-1 treatment. BALB/c mice have been found to exhibit greater behavioural reactivity across a range of tests, over other strains such as C57BL/6, and this corresponded to pronounced elevations in plasma corticosterone following stressors (Anisman et al., 2001). Similarities between TCAP-1 and the CRF family of peptides (Lovejoy et al., 2006), coupled with high expression of the peptide in the hippocampus and amygdala (Wang et al., 2005), indicate that TCAP-1 may play a role in vertebrate regulation of stress and anxiety. The synthetic peptide has been found to elicit numerous bioactive effects in vivo including attenuation of CRF-induced c-fos expression in the brain (Tan et al., 2009), inhibition of CRF-induced cocaine reinstatement (Kupferschmidt et al. 2011), modulation of behavioural stress responses in rats (Wang et al., 2005; Tan et al., 2011) and remodeling of dendritic morphology in the hippocampus (Tan et al., 2011). Since no previous studies were conducted on the *in vivo* metabolic responses to exogenous TCAP-1, it was crucial to start out using a mouse model with normal metabolic parameters. Therefore, the susceptibility of the BALB/c strain to stress makes it an ideal behavioural model and absence of metabolic aberrations makes it an ideal physiological model for preliminary studies. The initial preliminary findings show interesting avenues for future studies and the detection issues with the metabolic marker map

suggest that these studies may need to be repeated to confirm the impact of TCAP on normal metabolic parameters.

The significant reductions in subcutaneous adiposity and serum insulin levels prompt the examination of abnormal metabolic parameters, particularly obesity. There are various models that have been generated for the study of obesity, leptin resistance and insulin resistance; the most commonly employed are diet induced obesity mouse models. Using a high fat diet, Lin at al. (2000) induced both obesity and leptin resistance in C57Bl/6J mice. Compared to control mice, the high-fat diet fed mice exhibited a gradual increase in body weight, fat storage and plasma leptin starting at 1 week which progressively increased until the experiment end at 19 weeks. Additionally, the high-fat diet mice underwent three stages of leptin responsiveness: 1) an initial stage, sensitive to exogenous leptin; 2) a middle stage with reduced food intake in response to high endogenous leptin production; 3) a final stage where food intake was increased regardless of elevated leptin levels due to central insensitivity (Lin et al., 2000). Exogenous application of TCAP-1 on high-fat diet mice would likely result in augmented and clearly identifiable responses in adiposity distribution, serum insulin and serum leptin levels. In addition, these findings could be compared to values obtained from mice on low-fat diets to examine the therapeutic effectiveness of TCAP-1. The diet induced obesity HsdBlu:LE rat model, which is characterized by hyperinsulinemia, hyperleptinemia and insulin resistance (Woods et al., 2003), may be an appropriate rat model to ameliorate the detection values associated with adapting a Rat MetabolicMAP® for use on mouse serum.

4.5 **Experimental Techniques**

For all *in vitro* studies, mHypoE-38 neurons were cultured using the same media previously employed by Trubiani *et al.* (2007) and Ng (2010), under which the neuroprotective actions of TCAP-1 were observed; this was done to minimize the introduction of additional variables. The Dulbecco's Modified Eagle Medium contains 4 500 mg/L D-glucose, L-glutamine, and 25 mM HEPES buffer, without sodium pyruvate or phenol red (Gibco, Cat. No. 21063029), supplemented with 10% fetal bovine serum (Invitrogen, Cat. No. 16000-036) and including penicillin and streptomycin antibiotics. Although this culturing media contains a high glucose concentration of 25 mM, it is commonly employed as a growth medium for culturing neurons. However, a study by Kleman *et al.*, in 2008 illustrated the importance of physiological glucose to optimize neuronal viability and AMPK responsiveness *in vitro*. Physiological plasma glucose concentrations range from 5.5 to 7.8 mM while brain glucose concentration ranges from 0.82 to 2.4 mM (Kleman *et al.*, 2008); all these values fall below the 25 mM culturing concentration utilized. The authors compared neurons cultured under physiological glucose conditions of 3mM to non-physiological media conditions of 25 mM. Activation of AMPK was examined in cortical neurons following 1.5 mM treatment with AICAR by western blot analysis for AMPK and pAMPK. Neurons grown under physiologic glucose conditions exhibited a time-dependent increase in pAMPK; this was not observed with neurons cultured in non-physiological glucose conditions of 25 mM. These findings suggest that culturing of neurons under physiological glucose conditions of TCAP-1 were established under high glucose media concentrations, it would be important to re-establish these responses under physiological glucose media concentrations and would provide a positive control for the previously analyzed metabolic parameters.

4.6 References

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5 Chapter Five: Conclusion and Future Directions

5.1 Overview

The findings of this study show that the cellular mechanism by which TCAP-1 mediates cellular protection in vitro functions to increase intracellular ATP availability and reveal the physiological manifestation these effects in vivo (Figure 5.1). TCAP-1 initially enhances transporter capacity by increasing total GLUT1 expression and cell membrane GLUT3 localization; this is followed by upregulation of total GLUT3 expression and continued augmentation of the isoform at the membrane. Chronic TCAP-1 treatments yielded a dosedependent increase in intracellular ATP concentrations and a dose-dependent decrease in intracellular lactate concentrations; in comparison, an acute administration of TCAP-1 induced a dose-dependent reduction in intracellular ATP levels. In addition to a dose-dependent response, there appears to be a time-dependent response to TCAP-1. Increased ATP, in conjunction with decreased lactic acid, suggests that TCAP-1 mediated protection likely involves enhanced efficiency of aerobic oxidation but further studies are required to confirm this theory. If TCAP-1 does enhance mitochondrial functioning, this could account for the seemingly contradictory findings of TCAP-1 rescue from hypoxic (Ng, 2010) and oxidative stress (Trubiani et al., 2007). Higher energy returns per unit of oxygen would alleviate the stress of hypoxia while increased mitochondrial activity would upregulate endogenous antioxidant production. The creation of an energy surplus would fuel survival mechanisms and prime the cell against future insults. The inherently large energetic cost of metabolic adaptation could account for the acute decline in intracellular ATP concentrations (Klip et al., 2009); contributing costs may include de novo synthesis and translocation of GLUT transporters to the plasma membrane. Elevated nutrient acquisition at the cellular level would result in a physiological shift towards energy expenditure. As a secondary response to enhanced ATP production in energetically demanding tissues, a significant reduction in subcutaneous adiposity would be expected. TCAP-1 treatments led to a significant reduction in subcutaneous adipocyte cell size at 25 pmol/kg. Additionally, a reduction in serum insulin levels was observed at 250 pmol/kg TCAP-1 administration but no significant changes in serum leptin were found.



Figure 5.1: Overview of experimental findings. *In vitro*, TCAP-1 increasing total GLUT1 expression and membrane localization of GLUT3; this is followed by upregulation of total GLUT3 expression. Chronic TCAP-1 does not influence glycolytic enzyme expression and induces significant dose-dependent reductions in intracellular lactate concentrations. Chronic TCAP-1 leads to a dose-dependent increase in intracellular ATP concentrations whereas acute TCAP-1 causes a significant decline in intracellular ATP concentrations. There were no changes in apoptotic protein expression with chronic TCAP-1 treatment. *In vivo*, TCAP-1 led to significant reductions in subcutaneous adipocyte size. There was also a significant reduction in serum insulin levels but no changes in serum leptin levels.

5.2 Future directions

This thesis has demonstrated how TCAP-1 *in vitro* enhances glucose transporter capacity and acts on various metabolic parameters to increase intracellular ATP concentrations to protect the cell against present and impending stressors. Additionally, the *in vivo* findings demonstrate the impact of TCAP-1 on adiposity and related endocrine signals. However, the mechanism by which TCAP-1 mediates neuroprotection is a complex and intricate pathway; leaving many questions and highlighting key areas of future directions. The physiological impact of TCAP-1 *in vivo* comprises many tissue and organ systems; investigation of their responses is crucial in enhancing the understanding of potential therapeutic applications for TCAP-1. Some examples for future studies include:

5.2.1 Determine the actual rate of glucose uptake following TCAP-1 treatments

Since glucose is a substrate in metabolism, the results from the enzyme assays will be affected by downstream metabolic processes. 2-deoxyglucose (2-DG) is a glucose analog that enters through glucose transporters and is phosphorylated by hexokinase (Kim and Dang, 2007; Scatena *et al.*, 2008). However, it is not recognized by the subsequent metabolic enzyme, phosphofructokinase (PFK) (Scatena *et al.*, 2008). Therefore, it represents an ideal GLUT substrate that is truly representative of the actual rate of glucose uptake. Through utilization of radiolabelled 2-DG and quantification by liquid scintillation counting, a time course for glucose uptake can be established and used to determine if increased glucose uptake occurs through translocation of pre-existing transporters or production of new transporters. This technique has been commonly employed for measuring the impact of various exogenous treatments on cellular glucose acquisition (Duelli and Kuschinsky, 2001; Benomar *et al.*, 2006; Klip *et al.*, 2009; Weisova *et al.*, 2009; Zaid *et al.*, 2009). Furthermore, these findings could be compiled to determine how TCAP-1 application influences the kinetics of glucose uptake.

5.2.2 Analyze the mechanisms through which TCAP-1 increases glucose transporter densities at the membrane

In this study, TCAP-1 was found to significantly increase the CTCF of GLUT1 at 1 hour and GLUT3 at 3 hours post-treatment; in addition, the membrane localization of GLUT3 significantly increased after 1 hour and was maintained at 3 hours. However, the mechanism through which TCAP-1 induces these changes is not known. One potential pathway is through AMPK inhibition of GLUT3 endocytosis from the cell membrane (Klip *et al.*, 2009; Weisova *et al.*, 2009). The initial energetic cost of metabolic optimization, including GLUT1 *de novo* synthesis, could cause the reduction in intracellular ATP following 1 hour of acute TCAP-1 treatment. In a similar mechanism to one observed by Weisova *et al.* (2009) falling ATP levels would alter the AMP: ATP ratio and activate AMPK; this could account for the increase in GLUT3 membrane expression.

5.2.3 Examine if TCAP-1 synergistically acts on metabolic and apoptotic pathways to enhance survival

Reallocation of glycolytic enzymes has been shown to significantly alter cellular survival (Hammerman *et al*, 2004; Mathupala *et al.*, 2006; Kim and Dang, 2007). Specifically,

mitochondrial association of hexokinase II enhances metabolic efficiency by coupling glycolysis with oxidative phosphorylation and inhibits apoptosis (Mathupala *et al.*, 2006; Kim and Dang, 2007). Although the overall expression of hexokinase was not altered, hexokinase may still play a role in TCAP-1 mediated protection through preventing cytochrome c release through the mitochondrial VDAC. The ability of increased intracellular glucose-6-phosphate to promote dissociation of hexokinase form the mitochondria (Robey and Hay, 2006) supports the notion of improved VDAC binding in response to TCAP-1; upregulated oxidative phosphorylation would cause G6P to be quickly metabolized, keeping intracellular concentrations low. Immunocytochemical analysis for colocalization of hexokinase II with the mitochondrial membrane will confirm or refute this explanation.

5.2.4 Deduce the in vivo response of skeletal muscle tissue to TCAP-1

Energy production in the muscles is particularly sensitive to metabolic perturbations and is reflective of energy availability and expenditure at the organismal level. Initial *in vivo* studies indicate that TCAP-1 may induce skeletal muscle hypertrophy and promote increased activity levels. Despite a relatively low ATP storage capacity of <10mM, skeletal muscle ATP concentrations are rarely depleted within muscles ((Westerblad *et al.*, 2010). The energetic demands are satisfied by three metabolic energy sources: the phosphagen system, the glycogenlactic acid system and the aerobic system (Westerblad *et al.*, 2010). TCAP-1 administration may result in increased glycogen concentrations from elevated storage capacities and can be examined using an appropriate assay. Furthermore, the high expression of UCP3 in skeletal muscles provides a plausible target for increased efficiency of oxidative phosphorylation (Schrauwen and Hesselink, 2002; Echtay, 2007). These intracellular changes in skeletal muscle metabolism could have important ramifications in skeletal muscle morphology and should be further examined.

5.3 Concluding remarks

Vital to the evolution of multicellular metazoans is the establishment of protective intercellular warning mechanisms (Srivastava *et al.*, 2010); the ubiquitous expression and high conservation of the TCAP family of peptides are indicative of their fundamental biological role among all metazoan species (Lovejoy *et al.*, 2006). The neuroprotective actions of TCAP-1 against hypoxic

(Ng, 2010) and oxidative stress (Trubiani *et al.*, 2007) may converge on a single mechanism. This study illustrates how TCAP-1 *in vitro* acts on GLUT1 and GLUT3 to increase transporter capacity and subsequently impacts metabolic parameters to increase intracellular ATP availability, which can fuel cellular defense mechanisms and primes the cell against future insults. In addition, the activation of anti-apoptotic pathways, through TCAP-1 mediated signaling cascades, could work synergistically with metabolic adaptation to increase cell viability. Furthermore, my preliminary studies on the *in vivo* manifestation of TCAP-1 administration found a significant reduction in subcutaneous adipocyte size, indicative of fat loss, and significant reduction in serum insulin levels. The novel pathway for enhanced metabolic capacities mediated by TCAP-1 could lead to the development of targeted therapeutics for metabolic and apoptotic pathologies. Applications for TCAP-1 at the localized level for neurodegenerative diseases, ischemic-stroke infertility and neoplastic malignancies, combined with applications at the organismal level for treatment of insulin resistance and obesity, warrants its continued investigation.

5.4 References

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