The role of the teneurin C-terminal associated peptide (TCAP) family in energy metabolism and skeletal muscle physiology in protochordates and chordates

by

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Abstract

Stress can be defined as any event that threatens homeostasis. Organisms have a finite source of energy to allocate towards stress-coping mechanisms, while maintaining enough energy for other key life aspects such as growth and reproduction. Thus, when stress demands a higher portion of the energy budget to overcome the stressor, it impacts the overall fitness of the organism. As stress and energy are intrinsically linked, mechanisms of enhancing energy production would provide the organism with a better capacity to deal with stress. Recently, a highly conserved stress-modulating peptide, teneurin C-terminal associated peptide (TCAP), has been described. As TCAP has roles in stress regulation, it led to question if the TCAP family could have roles in energy metabolism. Therefore, the primary objective of this work was to determine if the TCAP family plays a role in skeletal muscle metabolism and stress, in relation to muscle function, as skeletal muscle is a key metabolic regulator and is involved in the organismal stress response.

Due to its early evolutionary history, three model organisms were chosen across evolution to study TCAP actions. Using the protochordate, Ciona intestinalis, the cyprinid, Danio rerio, and the rodent, Rattus norvegicus, this research established that the TCAP peptides enhanced contractile function in skeletal muscle via increased metabolic activity, and that these effects are highly conserved. Importantly, the resulting enhanced ATP production provides an increased threshold for stress, as demonstrated by a decrease in stress-related responses with TCAP treatment under stressful conditions. In addition, this work is the first to demonstrate the expression and function of the teneurin/TCAP-ADGRL system in the skeletal muscle of all three organisms, revealing a novel mechanism of action for the TCAP family. Thus, this research provides critical insight into the dynamic regulation of skeletal muscle function and metabolism by the TCAP family.
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List of Abbreviations

1/2RT, half relaxation rate

$^{18}$F-DG, $^{18}$F-2-deoxyglucose

2-APB, 2-aminoethoxydiphenyl borate

2D, 2-dimensional

2-DG, 2-deoxyglucose

2-DG-6-P, 2-deoxyglucose-6-phosphate

3D, 3-dimensional

$^3$H-DG, $^3$H-deoxyglucose

AARC, Aquatic Animal Research Core

ACTH, adrenocorticotropic hormone

ADGRL, adhesion GPCR class L/latrophilin

ADHD, attention-deficit/hyperactivity disorder

AFU, arbitrary fluorescent unit

AICAR, AICA ribonucleotide

AMP, adenosine monophosphate

AMPK, AMP kinase

ANOVA, analysis of variance

ATP, adenosine triphosphate

BCA, bicinechonic acid

BOC, buccal opening contraction

btGLUT4, brown trout GLUT4
C2C12, murine skeletal muscle cell line
Ca\(^{2+}\), calcium
CAMK, calcium/calmodulin-dependent protein kinase
CAMKK, CAMK kinase
cAMP, cyclic AMP
CDLP, CRF/DH-like peptide
CIRL, calcium-independent receptor for latrotoxin
COC, cloacal opening contraction
CPM, counts per minute
CRF, corticotropin-releasing factor
CRFR, CRF receptor
CS, citrate synthase
cTCAP, Ciona TCAP
DAG, diacylglycerol
\(dCIRL^{\text{KO}}\), Drosophila CIRL knockout
ddH\(_2\)O, double distilled water
DGC, dystrophin-glycoprotein complex
DH, diuretic hormone
DHPR, dihydropyridine receptor
diH\(_2\)O, deionized water
DMC, dynamic muscle control
DMD, Duchenne muscular dystrophy
DMEM, Dulbecco’s Modified Eagle Medium
DMSO, dimethyl sulfoxide
Dpf, days post-fertilization
EC, excitation-contraction
EDL, extensor digitorum longus
EGF, epidermal growth factor
ELISA, enzyme-linked immunosorbent assay
ERK, extracellular signal-related kinase
ETC, electron transport chain
FBS, fetal bovine serum
FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FI, fluorescent intensity
FITC, fluorescein isothiocyanate
FLRT, fibronectin leucine-rich transmembrane
fPET, functional positron emission tomography
G6PDH, glucose-6-phosphate dehydrogenase
GABA, gamma-aminobutyric acid
GAIN, G-protein coupled receptor autoproteolysis-inducing
GFP, green fluorescent protein
GLUT, glucose transporter
Gn11, immortalized murine neuronal cell line
GnRH, gonadotropin-releasing hormone
GPCR, G-protein coupled receptor
GPS, GPCR proteolytic site
HDAC, histone deacetylase
HEK293, human embryonic kidney cell line
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGT, horizontal gene transfer
HPA, hypothalamic-pituitary-adrenal
HPG, hypothalamic-pituitary-gonadal
HPLC, high-performance liquid chromatography
Hr, hour
HRP, horse-radish peroxidase
HS, horse serum
IHC, immunohistochemistry
IMF, intermyofibrillar
IMS, inner mitochondrial space
IP3, inositol triphosphate
IP3R, IP3 receptor
IR, insulin receptor
IRS, IR substrate
KLH, keyhole limpet hemocyanin
LASSO, LPHN-1 associated synaptic surface organizer
LAT, lateral contraction
LD cycle, light-dark cycle
LDH, lactate dehydrogenase
LG, laminin G
LKB1, tumour suppressor kinase
LNS, laminin-neurexin-sex hormone-binding globulin
LONG, longitudinal contraction
LPHN, latrophilin
LRR, leucine-rich repeats
MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
MAPK, mitogen-activated protein kinase
Max dx/dt, contraction velocity
MCU, mitochondrial calcium uniporter
MEF, myocyte enhancer factor
MEK, MAPK-ERK Kinase
MES, 2-(N-morpholino)ethanesulfonic acid
MHC, myosin heavy chain
Min, minute
N38, immortalized murine hypothalamic cell line
NADH, nicotinamide adenine dinucleotide
NAFLD, non-alcoholic fatty liver disease
NBT, nitro blue tetrazolium chloride
NCX, sodium-calcium exchanger
NFAT, nuclear factor of activated T cells
NGS, normal goat serum
Odz, oddless
OFT, open field test
PBS, phosphate buffer solution

PBST, PBS with 0.2% Tween®20

PDK, protein-dependent kinase

PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α

PI3K, phosphatidylinositol 3-kinase

PIP3, phosphatidylinositol 3,4,5-triphosphate

PKB, protein kinase B

PKC, protein kinase C

PLC, phospholipase C

PMSF, phenylmethylsulfonyl fluoride

Rcf, relative centrifugal force

Rhodamine-123, R123

Rhodamine-2-AM, Rhod-2

RIPA, radioimmunoprecipitation assay

rM02, routine oxygen consumption rate

RNA, ribonucleic acid

ROI, region of interest

ROS, reactive oxygen species

RT, room temperature

rtMPC, rainbow trout myogenic precursor cell

RT-PCR, reverse transcription-polymerase chain reaction

RT-qPCR, quantitative RT-PCR

rtTCAP-3, rainbow trout TCAP-3
RyR, ryanodine receptor

Sc-TCAP-1, scrambled-TCAP-1

SDH, succinate dehydrogenase

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec, second

SEM, standard error of mean

SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase

SR, sarcoplasmic reticulum

SS, sub-sarcolemmal

TA, tibialis anterior

TCA, tricarboxylic acid

TCAP, teneurin C-terminal associated peptide

Ten-a, tenascin-like protein accessory

Ten-m, tenascin-like protein major

TMB, 3,3’,5,5’-tetramethylbenzidine

TRP, transient receptor potential

UACC, University animal care committee

VO₂, oxygen consumption rate

YD, tyrosine-aspartic acid

α-LTX, α-latrotoxin
Chapter 1

Introduction

1.1 Abstract

Stress, a term coined by Hans Selye in 1936, can be defined as any event that threatens homeostasis. Organisms have a finite source of energy to allocate towards stress-coping mechanisms, while maintaining enough energy for other key life aspects such as growth and reproduction. Thus, when stress demands a higher portion of our energy budget to overcome the stressor, it impacts the overall fitness of the organism. As stress and energy are intrinsically linked, mechanisms of enhancing energy production would provide the organism better capacity to deal with stress.

Recently, a novel stress-modulating peptide has been described. The teneurin C-terminal associated peptides (TCAP) are a family of bioactive peptides that have robust anxiolytic roles at cellular and organismal levels. TCAP is located on the extracellular carboxy terminus of each of the four teneurin type-II transmembrane proteins and have functional independence from their pro-proteins. The TCAP family of peptides is one of the oldest peptide families known, and can be found in early unicellular organisms, demonstrating a long evolutionary history (Chand et al., 2013; Lovejoy and de Lannoy, 2013). Recent studies have described a novel role of TCAP-1 in regulating glucose metabolism in the brain (Hogg et al., 2018), indicating that TCAP-1 may act to optimize and increase energy production, ultimately resulting in a greater ability to overcome stress. One key component to the stress response is the ‘fight or flight’ response as described by Walter Cannon (1932), which reflects an organism's decision to either challenge the stressor or flee from danger. Regardless of the decision, a heavy energetic demand is placed on muscular locomotion for survival. As skeletal muscle is a metabolic regulator, and is involved in the organismal stress response, it is an ideal tissue to investigate the roles of TCAP in stress and metabolism. Therefore, the primary objective of this work was to determine if the TCAP family of peptides play a role in skeletal muscle metabolism and stress in relation to muscle function.
1.2 Definition and History of the Understanding of Stress

In 1934, the French surgeon René Lerviche reported a clinical syndrome in his patients, where they would exhibit a specific array of symptoms that would only appear after surgical intervention, regardless of the surgery type. He called this syndrome ‘la maladie post-opératoire,’ which is now better known as ‘stress,’ as coined by Hans Selye in 1936. Stress can be defined as any event, real or perceived, that threatens homeostasis (Lovejoy and Barsyte, 2011).

There are systems within an organism that respond to either acute stress or chronic stress (Lovejoy and Barsyte, 2011). Under acute stress conditions, where the stressor must be dealt with immediately, there is the ‘fight or flight’ response, as described by Walter Cannon (1932). This response reflects an organism's decision to either challenge the stressor or flee from danger. The stress response under chronic stress conditions was a concept initially described by Hans Selye, which he called the ‘general adaptation syndrome’. This entails a 3-stage physiological response to a stressor. The first stage is the alarm reaction, which is the perception of the stressor and the initiation of the stress response. The second stage is resistance, which is characterized by the increase of tolerance to the stressor. The last stage, termed the stage of exhaustion, is the summation of the reactions of the body that developed during the long-term exposure to stress, which ultimately leads to the failure of the body to respond to stress any longer.

The stress response theories put forth by Cannon and Selye were later supported with the first description of a stress-modulating neuropeptide, corticotropin-releasing factor (CRF), by Vale and colleagues (1981). Since its discovery, it has been established as one of the key players in the neuroendocrine stress response, known as the hypothalamic-pituitary-adrenal (HPA) axis. In this response, CRF stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which initiates the release of cortisol from the adrenal glands. Moreover, evolutionary analyzes reveal that CRF is a highly conserved and phylogenetically older peptide, which indicates a long evolutionary history. This finding suggests that not only is the CRF-mediated stress axis well-conserved across evolution, but that other stress-regulating peptides are likely involved (Lovejoy and Barsyte, 2011; Nicolaides et al., 2015).
1.3 Discovery and description of teneurins

1.3.1 Discovery of teneurins

When the teneurins were first discovered, there was little indication that they played a role with stress homeostasis. However, this multifunctional protein family is now known to be a component of the organismal stress response.

The teneurin family of transmembrane glycoproteins were discovered in 1994 in *Drosophila melanogaster* by two independent groups. One group was searching for tenascin homologues in arthropods by screening a *Drosophila* library with a chicken tenascin probe (Baumgartner and Chiquet-Ehrismann, 1993; Baumgartner et al., 1994). They identified two proteins and named them tenascin-like protein major (ten-m) and tenascin-like protein accessory (ten-a). Concurrently, another group was screening for novel phosphotryosine-containing proteins in *Drosophila* (Levine et al., 1994). They identified a protein with homology to the tenascin family and named it Oddless (*Odz*) as the *odz* mutant embryos were missing every odd-numbered body segment. Since their initial discovery, these proteins were renamed as they were found to be structurally and functionally distinct from the tenascins (Tucker et al., 2006). Thus, the teneurin family emerged, taking a name that reflected their structural similarity to tenascin in *Drosophila*, as well as their high expression pattern in the nervous system (Minet et al., 1999). In 1998, the first vertebrate orthologue of teneurin was reported in mice (*Mus musculus*) while the authors were identifying genes that were upregulated under endoplasmic reticulum stress (Wang et al., 1998). The teneurins were then quickly identified in many organisms including *Caenorhabditis elegans* (Drabikowski et al., 2005), zebrafish (*Danio rerio*; Mieda et al., 1999), chickens (*G. gallus*; Mieda et al., 1999; Rubin et al., 1999) and humans (*Homo sapiens*; Minet et al., 1999). Teneurins have now been established to have one isoform in invertebrates (exception is two isoforms in *Drosophila*), and four isoforms in vertebrates, all of which show high structural conservation to each other (Tucker et al., 2007).
1.3.2 Structure and expression of teneurins

Teneurins are type-II glycoproteins, indicating that the N-terminus is intracellular, and the C-terminus is extracellular. They are about 2800 kDa in size (Tucker et al., 2007). Their N-terminus contains polyproline-rich sites and two EF-hand-like putative calcium binding sites. After a single transmembrane domain, they have a large extracellular domain. Their extracellular region is highly conserved and unique to this family, with eight tenascin-type epidermal growth factor (EGF)-like repeats, 17 cysteine residues, and 26 tyrosine-aspartic acid (YD) repeats (Figure 1.1). These YD repeats are usually only found in cell wall proteins of some prokaryotes, and not found in any other eukaryotic proteins (Tucker et al., 2007). The distal extracellular region contains a bioactive peptide sequence termed teneurin C-terminal associated peptide (TCAP).

**Figure 1.1. A schematic of the teneurin protein structure.** Teneurin is a type-II transmembrane protein, with an intracellular N-terminal domain containing EF-hand-like calcium (Ca^{2+}) binding motifs and polyproline sites. The extracellular domain of teneurin contains 8 EGF-like repeats, 26 tyrosine-aspartic acid (YD) repeats, and a bioactive peptide sequence at the distal tip of the C-terminal, termed teneurin C-terminal associated peptide (TCAP). Adapted from Woelfle et al., 2015.

Teneurins are expressed throughout the central nervous system and bind to each other in a homo- or hetero-dimeric manner to activate downstream signalling cascades (Baumgartner et al., 1994; Kenzelmann et al., 2007). They are also expressed in non-neuronal tissues, as demonstrated by Baumgartner and colleagues (1993; 1994) in *Drosophila*, where teneurin expression was shown in
cardiac cells, muscle attachment sites and tracheal systems. This expression pattern is consistent with vertebrates and other invertebrates (Drabikowski et al., 2005; Tucker et al., 2000).

1.3.3 Actions of teneurins

Teneurins are critical for development, as teneurin gene knockout results in an embryonic lethal phenotype in *Drosophila* (Baumgartner et al., 1994; Levine et al., 1994). Specifically, they are critical for normal development of visual, auditory and olfactory pathways (Hong et al., 2012; Young and Leamy, 2009). Differential expression patterns of the four teneurin isoforms suggest that they play different roles in these systems. For example, teneurin-1 is expressed in the neocortex and piriform cortex, which are olfactory-associated regions (O’Sullivan et al., 2014; Wang et al., 2005), whereas teneurin-3 plays a more predominant role in the visual system, as it is expressed in the ventral retina and dorsal lateral geniculate nucleus (Young and Leamey, 2009). Teneurins are also involved in development of systems in peripheral tissue. In *C. elegans*, *ten-1* knockout mutants have disorganized pharyngeal basement membranes, demonstrating that teneurin has functions in adhesion that is required for development (Trzebiatowska et al., 2008). Recently, novel functions of teneurins in axon guidance and synapse formation have been elucidated upon the discovery of a putative receptor, discussed further in section 1.5. However, further evidence has indicated that the teneurins possess a bioactive peptide on the distal tip of the extracellular region of this protein.

1.4 Discovery and description of TCAP

1.4.1 Discovery of TCAP

Given the importance of the CRF-associated peptides in the energetics of the stress response, it was postulated that other peptides related to CRF should be present in the genome (Lovejoy and Balment, 1999). Consequently, the TCAP peptides were discovered when Qian and colleagues (2004) were in search of CRF-related paralogues by screening the rainbow trout (*Oncorhyncus mykiss*) hypothalamic cDNA library with a hamster urocortin probe. They isolated and identified a clone that was the C-terminal region of rainbow trout teneurin-3, which they named teneurin C-
terminal associated peptide (TCAP). The TCAP portion is 40 amino acids in length and shares about 20% sequence similarity with CRF. Further evolutionary analyzes reveal that in addition to CRF, TCAP shares structural similarity with calcitonin, and therefore could be related to the Secretin family of ligands (discussed further in section 1.6.1).

Sequencing of this novel peptide revealed that it has a cleavage motif on the amino terminus, and an amidation motif on the carboxy terminus, which are characteristics of a bioactive peptide (Qian et al., 2004; Wang et al., 2005). Consistent with its pro-protein, teneurin, there is one TCAP paralogue in invertebrates (with the exception of two in Drosophila), and four paralogues in vertebrates (Wang et al., 2005). Protein sequence alignments of the four mouse paralogues show that these peptides share 71-87% sequence similarity (Figure 1.2A; Wang et al., 2005). Moreover, comparison of protein alignments of rainbow trout TCAP-3 (rtTCAP-3) to corresponding homologues in zebrafish, mice and human showed 88-90% sequence similarity (Qian et al., 2004), highlighting how highly conserved this peptide is across evolution (Figure 1.2B). Importantly, this provides the rationale for using synthesized rtTCAP-3 treatment in zebrafish for studies performed in Chapter 3. As TCAP sequences from rainbow trout and zebrafish are structurally similar, it can be expected that rtTCAP-3 would work in a similar manner to zebrafish TCAP-3 when treated in zebrafish.
Figure 1.2. TCAP protein sequence alignment demonstrating high sequence similarity between the four vertebrate paralogues in mice as well as among rainbow trout (O. mykiss), zebrafish (D. rerio), mouse (M. musculus), and human (H. sapiens). The four mouse TCAP paralogues share 71-87% sequence similarity relative to TCAP-1 (A). TCAP-3 orthologues share 88-90% sequence similarity among rainbow trout, zebrafish, mice and human sequences (B). Light grey boxes indicate amino acid substitutions. Accession numbers provided by and figure adapted from Qian et al., 2004 and Wang et al., 2005.

1.4.2 Expression and Processing of TCAP

*In situ* hybridization analyzes of the TCAP mRNA revealed its gene expression in brain regions such as the olfactory bulb, cerebellum and brainstem, including high levels of expression in the hippocampus, hypothalamus, and dentate gyrus in the rat brain (Wang et al., 2005). This finding was replicated in mouse brain using immunohistochemical techniques (Chand et al., 2013). TCAP shares similar expression pattern to teneurins, as their expression overlaps in the olfactory bulb and cerebellum, however their expression is distinct in some regions such as the limbic area (Chand et al., 2013; Wang et al., 2005; Zhou et al., 2003). These studies demonstrate that the teneurin gene can be differentially expressed.

In addition, teneurin and TCAP localization in the cell was investigated using immortalized murine hippocampal E14 cells. TCAP-1 shows a distinct expression pattern to teneurin-1, where TCAP-1 immunoreactivity is found exclusively in the cytosol while teneurin-1 immunoreactivity is found on the membrane (Chand et al., 2013). Teneurin-1 immunoreactivity localization is expected as
teneurin-1 is a transmembrane protein, however the localization of TCAP-1 immunoreactivity may indicate a potential alternative mechanism of TCAP processing.

Endogenous TCAP processing remains unclear, however there is evidence to suggest that TCAP can be either independently transcribed from the teneurin gene or is potentially cleavable from teneurin protein (Figure 1.3). First, 5’ RACE PCR analyzes demonstrate that of the four TCAP paralogues, TCAP-1 and TCAP-3 can be independently transcribed from their respective teneurin genes (Chand et al., 2013). Second, Northern blot analyzes revealed that the full-length teneurin-1 mRNA hybridizes with the TCAP-1 probe, which suggests that TCAP-1 can still be expressed as part of the full length teneurin and may be potentially cleavable (Chand et al., 2013). This is supported by a putative furin cleavage motif found upstream of the mature TCAP-1 peptide. Moreover, the orientation of teneurin as a type-II transmembrane protein allows the TCAP portion to be in the extracellular space, which would free TCAP to bind to other cells nearby upon cleavage from teneurin, suggesting TCAP may work in a paracrine manner (Chand et al., 2013). One hypothesis suggests that TCAP-1 may be released as a distress signal in necrotic cells, as it could be released after cell rupture and signal on nearby cells (Figure 1.3; Chand et al., 2013).

With its distinct localization and the ability to be independently transcribed and processed, TCAP-1 likely has actions independent from its pro-protein, teneurin-1; however, endogenous processing of TCAP-1 remains unknown (Chand et al., 2013). Despite this, numerous studies indicate that a synthetic version of TCAP-1 is bioactive (see following sections).
Figure 1.3. Proposed mechanisms of endogenous TCAP-1 processing in cells. (1) TCAP-1 is transcribed as part of the full-length teneurin mRNA or is individually transcribed. (2) The full length is translated as part of the secretory pathway (2, 3), and is packaged in vesicles (4). The vesicle fuses with the membrane (5), where TCAP-1 may be cleaved within the secretory vesicles or on the plasma membrane. Alternatively, the shorter TCAP-1 mRNA is translated by free ribosomes (6) and remains in the cytosol as part of the soluble protein translation endoplasmic reticulum/Golgi-independent pathway (7). Cell rupture upon cell death releases TCAP-1 into the extracellular space, where it may undergo further proteolytic cleavage. Cleavage indicated as lightening bolt symbol. Adapted from Chand et al., 2013.

1.4.3 Actions of TCAP

TCAP and CRF share structural similarity, therefore in initial studies CRF was used as a model to try to understand TCAP signalling and actions. The CRF receptors (CRFR; CRFR-1 and -2) are G protein-coupled receptor (GPCRs), indicating they transduce signalling pathways via associated Ga subunits. There are 3 main Ga subunits; Gaα, Gaβ, and Gaq11. Both CRFRs have been shown
to associate with high affinity to Gaα, which stimulates adenylyl cyclase activity to generate cyclic AMP (cAMP). Thus, initial studies done on TCAP were modelled after CRF pathways, as it was suspected that TCAP could also work via GPCR signalling. Immortalized neuronal cells lines (Gn11 and N38 cells) were utilized in these studies as teneurin and TCAP expression show high expression in the brain and these cell lines were well established neuronal models (Belsham et al., 2004; Tellam et al., 1998).

Rainbow trout TCAP-3 (rtTCAP-3) was the first synthetic form of TCAP that was tested for bioactivity (Qian et al., 2004). rtTCAP-3 treatment in Gn11 cells increased cell proliferation, as was visualized by MTT assays, and stimulated cAMP production after 15 minutes in a dose-dependent manner, where low TCAP doses stimulated cAMP, while high doses inhibited cAMP production. These findings were replicated using mouse TCAP-1, as it had a similar effect increasing cell proliferation and a dose-dependent cAMP response (Wang et al., 2005). The dose-dependent relationship with cAMP production may suggest that the receptor for TCAP may associate with multiple Ga units, which has been observed in many different GPCRs. In addition, TCAP-1 treatment to hippocampal cells showed enhanced cytoskeletal rearrangement and neurite filopodia growth. This was the result of increased β-tubulin and F-actin polymerization via stimulation of the MEK/ERK pathways (Figure 1.4; Chand et al., 2012; Chand et al., 2013; Tan et al., 2007). Moreover, TCAP-1 had a neuroprotective role in hypothalamic neurons that were subjected to chemical insult (Ng et al., 2010; Trubiani et al., 2007). As a marker of cellular damage, reactive oxygen species (ROS) levels were measured in the cell after chemical insult. TCAP-1 significantly lowered ROS levels in the cells via an upregulation of ROS scavengers, such as superoxide dismutase. This ultimately resulted in reduced ROS-induced damage and increased cell viability, thus demonstrating the roles of TCAP-1 in neuroprotection.

One of the primary goals of neuroprotection is metabolic optimization under stress, which aims to increase efficiency of resource utilization under stressed conditions. Glucose is the primary energy source in the brain, therefore maintaining an adequate glucose supply during times of stress is critical for neuronal integrity (Alberts et al., 2008). Thus, the roles of TCAP-1 regarding glucose metabolism were investigated. Recent findings demonstrate that TCAP-1 significantly increases
glucose uptake into hypothalamic cells after 30 minutes of treatment as visualized by titrated deoxyglucose (\(^3\)H-DG) uptake (Hogg et al., 2018). It was further shown that TCAP-1 induces glucose transporter (GLUT)-1 and -3 translocation to the membrane to facilitate glucose uptake, likely via phosphorylation of AKT and/or ERK, which were both shown to be significantly increased after 30 minutes of TCAP-1 treatment. Thus, TCAP-1 enhances cellular metabolism that ultimately leads to an increase in intracellular ATP levels (Figure 1.4; Hogg et al., 2018).

TCAP-1 has potent effects \textit{in vivo}. Due to its structural similarity with CRF, TCAP-1 was examined for its role in the organismal stress-response. Interestingly, TCAP-1 has antagonistic actions to CRF, as demonstrated by exogenous application of synthetic TCAP-1 resulting in anxiolytic behaviours in treated animals (Al Chawaf et al., 2007; Tan et al., 2011; Tan et al., 2009). Animals treated with TCAP-1 had reduced stress responses in acoustic startle paradigms and open field tests compared to vehicle-treated animals (Al Chawaf et al., 2007; Tan et al., 2011; Tan et al., 2009). Animals were also tested in the cocaine-addiction stress paradigm, which measures cocaine-reinstatement behaviour after establishing cocaine addiction (Kupferschmidt et al., 2011). When cocaine-addicted animals received CRF treatment, they showed a significant increase in cocaine-seeking behaviour. TCAP-1 pre-treatment to the animals blocked this CRF-induced cocaine-reinstatement behaviour; however, it did not block foot shock-induced or cocaine-induced cocaine-reinstatement behaviours. Further, it was demonstrated that TCAP-1 treatment blocks CRF-induced c-FOS expression in the limbic regions of the brain, while also increasing the spine density of neurons in the hippocampus (Tan et al., 2009; 2011). These studies provide strong evidence that TCAP-1 has modulating effects on the CRF pathway, and, therefore, plays a critical role in the CRF-mediated hypothalamic-pituitary-adrenal (HPA) stress axis.

In addition, TCAP-1 increases glucose uptake into the brain, as visualized in Wistar rats by functional positron emission tomography (fPET), with a concomitant decrease in blood glucose (Hogg et al., 2018). Importantly, TCAP-1-mediated glucose regulation was shown to be insulin-independent, as TCAP-1 treatment reduced blood glucose levels in hyperglycemic Goto-Kakazi rats that are insensitive to insulin (Hogg et al., 2018). Taken together, these data indicate TCAP-1 is a novel insulin-independent glucose regulator in the brain. Moreover, as previously described,
glucose regulation is critical for brain function, especially under stressful conditions (Alberts et al., 2008).

The actions of TCAP-1 are not limited to the CNS. TCAP-1 also has effects in non-neuronal tissue. TCAP-1 significantly increases serum testosterone levels after 9 days of treatment in male mice (Chand et al., 2014). Testosterone production is highly regulated via the hypothalamic-pituitary-gonadal (HPG) axis. Interestingly, TCAP-1-mediated increase in testosterone levels was shown to be independent of gonadotropin-releasing hormone (GnRH), thus suggesting TCAP is not likely modulating the HPG axis to transduce these effects (Chand et al., 2014). Overall, the increase in testosterone resulted in an increase in testes size and epididymis tubule short-diameter in TCAP-1-treated animals compared to vehicle-treated animals.

These in vitro and in vivo studies demonstrate the critical roles of TCAP-1 in glucose and stress regulation in the brain. TCAP-1 increases glucose uptake into the brain in an insulin-independent-manner, as seen in hypothalamic neurons in vitro where TCAP-1 had a different glucose uptake profile than insulin, as well as in an insulin-insensitive rat model in vivo that responded to TCAP-1-mediated glucose uptake. In addition, the roles of TCAP-1 in stress were clearly shown in hypothalamic cells and in rats. TCAP-1 increased cell communication and viability under stress by enhancing cytoskeletal arrangement and ATP production. In rats, TCAP-1 blocked CRF-mediated stress responses and decreased stress-related behaviours, while also promoting re-arrangement of cytoskeletal elements to increase spine density. Although TCAP-1 shares 20% sequence similarity with CRF, it does not bind to CRFR-1 or -2, or to any other member of the secretin family of GPCRs (Lovejoy et al., 2006; Lovejoy and de Lannoy, 2013; Woelfle et al., 2015). In an effort to identify the TCAP-1 receptor, gene array studies were performed. These studies led us to investigate dystroglycan, a protein that links the intracellular cytoskeleton to the extracellular matrix that is capable of transducing downstream signal cascades (Chand et al., 2012; 2014). Although it was found that TCAP-1 and dystroglycan are strongly co-localized, TCAP-1 signalling mechanisms were still unclear. Taken together, these studies provided the first insight into the receptor and mechanisms of TCAP-1 action. However, it was the recent discovery of the
putative receptor that provided a deeper understanding of these early studies and of TCAP-1 mechanisms (discussed in section 1.4).

Figure 1.4. Schematic of the elucidated actions of TCAP-1 in neurons. TCAP-1 treatment of immortalized hypothalamic neurons shows MEK/ERK-dependent increases in cytoskeletal rearrangement and glucose uptake. These actions ultimately result in a neuroprotective role of TCAP-1 in the brain. The question mark indicates an unknown receptor; however, TCAP-1 is shown to co-localize with dystroglycans. Adapted from Chand et al., 2012.
1.5 Discovery and description of the teneurin/TCAP receptor, ADGRL

1.5.1 Discovery of ADGRL

Although the earlier studies on TCAP bioactivity established the efficacy of the synthetic peptide, establishing a clear mechanism was problematic, because the cognate receptor was not known. As TCAP shares high structural similarity with CRF whose receptor is a GPCR, and that TCAP treatment demonstrated a dose-dependent relationship with cAMP in neurons, it suggested the putative receptor may be a GPCR. Recent studies shed critical insight on novel TCAP signalling pathways, as it established the adhesion GPCR class L/latrophilin (ADGRL) family of GPCRs to be a putative receptor for teneurins (Silva et al., 2011; discussed further in 1.5.1).

The ADGRL family of GPCRs were first identified from its association with \( \alpha \)-latrotoxin (\( \alpha \)-LTX), the principle toxic component in black widow spider venom (Frontali et al., 1976; Rosenthal et al., 1990). \( \alpha \)-LTX is able to form tetramers and insert itself into the cell membrane to create a pore that allows for external calcium flux, or bind to its calcium-dependent receptor, neurexin. However, it still had potent toxic ability in calcium-free conditions (Petrenko et al., 1990; Ushkaryov et al., 1992). In an effort to identify the calcium-independent mechanism of action for \( \alpha \)-LTX, Davletov and colleagues (1996) used detergent-solubilized bovine brain membranes in calcium-free conditions and demonstrated \( \alpha \)-LTX high affinity binding to a protein they later purified and termed calcium-independent receptor for latrotoxin (CIRL). Meanwhile, another laboratory engineered a PC12 cell line overexpressing this protein, which they termed latrophilin (LPHN; Krasnoperov et al., 1997). These LPHN-overexpressing cells demonstrated increased sensitivity to \( \alpha \)-LTX, thus further corroborating the results reported from Davletov and colleagues (1996). Since its discovery, CIRL and LPHN have been renamed as ADGRL to reflect its newly discovered functions and classification, which are further discussed in section 1.4.3 and 1.6.2, respectively (Hamann et al., 2015).
1.5.2 Structure and Expression of ADGRL

There are three vertebrate paralogues of ADGRL: ADGRL1-3. The ADGRL structure is highly conserved and consists of a long extracellular portion that is comprised of a lectin-like domain, an olfactomedin-like domain, a hormone-binding domain, and a G-protein coupled receptor autoproteolysis-inducing (GAIN) domain containing a GPCR proteolytic site (GPS). This is followed by the seven-transmembrane domain and finally a C-terminal intracellular tail (Figure 1.5; Krasnoperov et al., 1997; Lelianova et al., 1997). Invertebrate isoforms do not contain the olfactomedin-like domain, and it is thought to be the result of genomic domain shuffling process during vertebrate evolution (Fredriksson and Schioth, 2005; Strotmann et al., 2011). In addition, the hormone-binding domain found in ADGRL is a characteristic of the Secretin family of GPCRs, thus it was initially thought to be part of this family, however it has been recently reclassified (discussed further in 1.6.2). ADGRL1 and ADGRL3 are highly expressed in neural tissues, although low levels of expression have been shown in various peripheral tissues, such as spleen, kidneys and lungs (Sugita et al., 1998). Conversely, ADGRL2 is expressed predominantly in non-neuronal peripheral tissues with little expression found in the brain (Sudhof, 2001; Sugita et al., 1998).
Figure 1.5. A schematic of ADGRL protein structure. ADGRL is a G protein-coupled receptor (GPCR) that has a long extracellular N-terminal region in the extracellular (EC) region, which consists of a lectin-like domain (LEC), olfactomedin-like domain (OLF), hormone-binding domain (HBD), and a GPCR autoproteolysis-inducing (GAIN) domain containing a GPCR proteolytic site. This is followed by seven transmembrane-spanning domains and an intracellular (IC) C-terminus tail. Adapted from Woelfle et al., 2015.

1.5.3. Functions of ADGRL

As a receptor for α-LTX, ADGRL mediates presynaptic release of neurotransmitters caused by α-LTX-induced calcium surge. In vertebrates, α-LTX induces vesicular release of a variety of neurotransmitters including noradrenaline, acetylcholine, dopamine, glutamate, and gamma-aminobutyric acid (GABA; Linetska et al., 2004; Nicholls et al., 1982; Rahman et al., 1999; Rosenthal et al., 1990; Tzeng and Siekevitz, 1978). However, the endogenous functions of ADGRL were difficult to elucidate without the identification of its endogenous ligands.

Recently, the ADGRL family of GPCRs have shown to be critical in cell-cell communication by regulating synapse formation and cell-cell adhesion. ADGRL2 controls the number of synapses in
the hippocampus by acting as a post-synaptic target-recognition molecule (Anderson et al., 2017). Consistent with this, ADGRL3 was found to regulate the number of glutamatergic and dopaminergic synapses. In addition, ADGRL3 promotes cell-cell adhesion together with its association with fibronectin leucine-rich transmembrane (FLRT)-3 protein (discussed further in 1.5.2; Lange et al., 2012; O’Sullivan et al., 2012; Ranaivoson et al., 2015).

More recently, ADGRL demonstrated important roles in locomotion and mechanosensation in *Drosophila*. Scholz and colleagues (2015) developed a null mutant for the sole ADGRL isoform in *D. melanogaster*, which they termed *dCIRL* KO mutants. Although the larval mutants showed no obvious signs of delayed development or morphology, they exhibited significantly less locomotion than controls. Using a reporter allele tag, *dCIRL* was shown to be highly expressed in the larval chordotonal organs, which have stretch-receptors responsible for transmitting mechanosensation information (Kernan, 2007). *dCIRL* is thus necessary for the electrical mechanosensory response in these organs, and *dCIRL* KO larvae demonstrate impaired locomotion and startle responses (Scholz et al., 2015).

### 1.6 Interaction of Teneurin and TCAP with the ADGRL receptors

#### 1.6.1. Teneurin and TCAP as ADGRL ligands

The teneurin/TCAP and ADGRL bodies of work were done in parallel as both were considered orphan peptides and receptors, respectively. It was only until 2011, when Silva and colleagues were the first to bridge these fields by being the first show that teneurin-2 and ADGRL1 can bind with nanomolar affinity at the lectin-domain of ADGRL1. They further showed that a splice variant of C-terminal domain of teneurin-2, termed LPH1-associated synaptic surface organizer (Lasso), can also bind to ADGRL with high affinity. They generated constructs of Lasso with various truncations and tested them in pull-down assays with ADGRL. This demonstrated that ADGRL1 binding localized to the C-terminal globular domain of Lasso. In addition, they incubated the Lasso constructs with rat brain lysate and performed immunoprecipitation analyzes. By utilizing antibodies against each of the ADGRL homologues, they showed that ADGRL1 is the main ligand of Lasso. Collectively, these studies revealed that Lasso binds to ADGRL1 at its
C-terminal domain with high-affinity at the synapse. Later, it was determined that other teneurin paralogues, teneurin-1 and teneurin-4, can also bind ADGRL1 (Boucard et al., 2013; O'Sullivan et al., 2014). These studies further demonstrated that the olfactomedin-like and lectin-like domains of ADGRL1 are both critical for teneurin binding as truncated constructs of ADGRL1 were not bind to the teneurins as indicated by immunoprecipitation assays (Boucard et al., 2013; O'Sullivan et al., 2014).

As both the full-length teneurins and Lasso showed high-affinity binding with ADGRL1, these studies suggested that TCAP could bind with the ADGRL family. Because the hormone-binding domain is the highest conserved region, this was our first candidate region to investigate. Immunoprecipitation experiments revealed that TCAP-1 was isolated using hormone-binding domain bait, thereby indicating an interaction between TCAP-1 and the hormone-binding domain (Husić et al., manuscript in preparation). Moreover, human embryonic kidney (HEK293) cells, which have minimal endogenous expression of ADGRL1, were transfected to overexpress ADGRL1. ADGRL1-expressing transfected cells had increased uptake of TCAP-1 compared to wild-type HEK293 cells, as visualized by FITC labelling. In addition, the transfected cells underwent significant cytoskeletal changes with TCAP-1 treatment whereas wild-type cells did not (Husić et al., manuscript in preparation). These studies are the first to provide evidence that free TCAP-1 can bind to ADGRL1, and that the putative TCAP-1 binding site is the hormone-binding domain, (Husić et al., manuscript in preparation). Taken together, these studies provided the first insight into the teneurin/TCAP-ADGRL ligand-receptor complex.

1.6.2 Stoichiometric description of the Teneurin-ADGRL ligand-receptor complex

The result of these studies, along with associated studies, led to a plausible understanding of the teneurin/TCAP-ADGRL complex stoichiometry with additional accessory proteins in neurons. The culmination of this work has resulted in the description of a complex system of scaffolding proteins that acts to organize and stabilize this trans-synaptic pair (Figure 1.6). The discovery of the teneurin/TCAP-ADGRL ligand-receptor complex, along with its accessory proteins, was a critical step in understanding the roles it plays in the cell. Consistent with the elucidated roles of
each of the proteins involved in the complex, this trans-synaptic complex is critical in cell-cell adhesion, with roles in axon guidance, synapse formation and plasticity, and brain development (Boucard et al., 2012; 2014; Li et al., 2018; O'Sullivan et al., 2012; Silva et al., 2011). Dystroglycans, FLRT proteins, and neurexins have all shown association with one or more members of the teneurin/TCAP-ADGRL ligand-receptor complex (Figure 1.6).

Figure 1.6. Schematic of the teneurin/TCAP-ADGRL ligand-receptor complex with neurexin, fibronectin leucine-rich transmembrane (FLRT) protein and dystroglycan in neurons.

Dystroglycans are scaffolding proteins that were originally discovered in skeletal muscle as a main component of the dystrophin-glycoprotein complex (DGC), which is a multi-protein complex that
acts to stabilize sarcolemma structure during muscular contractions (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992; Petrov et al., 1993). Dystroglycan is a single gene that is translated and cleaved into two subunits; α-dystroglycan, which links to the extracellular matrix proteins, and β-dystroglycan, which is sequestered in the membrane as a link to the intracellular cytoskeleton (Henry and Campbell, 1996). The actions of TCAP-1 in cytoskeletal rearrangement within neurons are induced through co-localization with and activation of β-dystroglycan, which stimulates MEK/ERK-dependent phosphorylation of stathmin (Chand et al., 2012). Moreover, previous studies have suggested that teneurins itself also has an interaction with the dystroglycans (Trzebiatowska et al., 2008). The stoichiometry of teneurin/TCAP and dystroglycan was eventually elucidated in testes tissue, which showed teneurin-1 co-localization with α-dystroglycan, and TCAP-1 co-localization with β-dystroglycan (Chand et al., 2014).

FLRTs are widely expressed proteins that are also associated with the teneurin/TCAP-ADGRL ligand-receptor complex. FLRTs are single transmembrane-spanning proteins that contain 10 leucine-rich repeats (LRR) and a fibronectin type-III repeat on their extracellular domain, and a C-terminal intracellular tail (Chen et al., 2006; Lacy et al., 1999). There are three vertebrate FLRT isoforms (FLRT1-3), which can bind in a homophilic manner to have roles in neurite outgrowth and synapse formation (Karaulanov et al., 2005). O’Sullivan and colleagues (2012; 2014) were the first to report the interaction of FLRT3 with both ADGRL1 and ADGRL3, demonstrating direct binding at the olfactomedin-like domain of ADGRL.

Lastly, neurexins are neuronal-specific type-I proteins that are highly involved in this complex, with multiple binding partners being identified. Neurexins have similar functions to ADGRL, as neurexins are also putative receptors for α-LTX and have been shown to have roles in cell adhesion and regulation (Petrenko et al., 1990; Ushkaryov et al., 1992). Although the neurexins are only encoded by three genes in vertebrates, they have several hundred alternatively-spliced isoforms (Missler and Sudhof, 2008; Ullrich et al., 1995). α-neurexin has a large extracellular domain that includes six laminin-neurexin-sex hormone-binding globulin (LNS)-laminin G (LG) domains, and three EGF-like sequences. The LNS-LG domain is a binding motif domain for dystroglycans and extracellular matrix proteins in non-neuronal tissue. Likewise, in neuronal cells, there is high
affinity binding of α-neurexin and α-dystroglycan at the LNS-LG domain, making neurexin the endogenous ligand for dystroglycan in neurons (Sugita et al., 2001). α-dystroglycan and α-LTX compete for binding with neurexin at this domain (Sudhof, 2001; Sugita et al., 2001). In addition, neurexins and ADGRL were shown to also have direct binding with each other, thus identifying another binding pair in this complex (Boucard et al., 2012; Tobaben et al., 2001).

Thus, the stoichiometry of teneurin/TCAP-ADGRL system with dystroglycans, FLRTs, and neurexins have been well established. This can be used as a model to understand the molecular interactions of the teneurin/TCAP-ADGRL complex and how it is associated with the transduction of the teneurin/TCAP-ADGRL signal.

1.6.3. Associated pathologies with teneurin/TCAP-ADGRL

As teneurins, TCAPs and ADGRLs are highly involved in neural development and synapse regulation, it is to be expected that their disruption would be linked with psychiatric disorders. Genetic analyzes revealed an association between ADGRL3 and attention-deficit/hyperactivity disorder (ADHD; Arcos-Burgos et al., 2010; Martinez et al., 2011). ADGRL-knockdown studies by Lange and colleagues (2012) in zebrafish aimed at identifying this etiology. In the teleost zebrafish, there are six isoforms of ADGRL due to a lineage-specific genome duplication. Knockdown of Lphn3.1, an orthologue of ADGRL3, resulted in the loss and disorganization of dopaminergic neurons in the posterior tuberculum. This neuro-developmental defect ultimately led to an ADHD phenotype as observed by locomotor hyperactivity and impulsivity (Lange et al., 2012). Importantly, they observed Lphn3.1 mutants with greater disruption of the dopaminergic neurons exhibited more hyperactive behaviour, thereby linking dopamine and the locomotor signature in ADHD. These results were further corroborated with the generation of ADGRL3-null mice and dCIRL-null Drosophila mutants that both demonstrated dopamine-related locomotor hyperactivity, consistent with ADHD phenotype (Wallis et al., 2013; van der Voet et al., 2016). Moreover, teneurins and TCAPs have also been shown to have important roles in brain development and maturation as they regulate synapse plasticity (described in 1.3.3). As TCAP-1 has potent anxiolytic roles in vivo (described in 1.3.3), this lends support to the roles of teneurins
and TCAPs in mood disorder etiology (Woelfle et al., 2016). Taken together, these studies indicate that the teneurin/TCAP-ADGRL complex has the potential to regulate a number of mood disorders, such as ADHD. This may be related to the early evolution of both the teneurin and latrophilin genes.

1.7. Evolution of the Teneurin/TCAP-ADGRL system

1.7.1. Teneurin and TCAP evolution

The teneurin/TCAP lineage predates the earliest metazoans and likely originated from a bacterial polymorphic toxin that was introduced into eukaryotes via lateral gene transfer and subsequently evolved to benefit metazoans (Figure 1.7; Chand et al., 2013; Tucker et al., 2013; Zhang et al., 2012). Evolutionary evidence suggests that the teneurins arose from a single ancestral gene; gene comparisons across Drosophila, C. elegans and humans reveal conserved intron and exon sequences (Minet and Chiquet-Ehrismann, 2000). Moreover, it is likely that the two isoforms found in Drosophila arose from an independent lineage-specific gene duplication, whereas the vertebrate teneurins duplicated before vertebrate radiation (Tucker et al., 2012; 2013).

It has been recently postulated that ancestral teneurin and TCAP proteins worked in early multicellular organisms as a latching mechanism to catch unicellular prey, where the teneurin protein would use its long extracellular domain to attach itself to a nearby cell (Chand et al., 2013; Zhang et al., 2012; unpublished observations). Once secured, it would release a toxic payload, the ancestral TCAP portion, into the cell. This was likely to promote the acquisition of nutritional sources by engulfing prokaryotes (Ni et al., 2012; Ramulu et al., 2012; Tucker et al., 2012; Tucker, 2013). TCAP shares sequence similarity, yet evolutionarily predates, the CRF/calcitonin/secretin peptide families, suggesting that TCAP was an ancestral progenitor peptide gene leading to the evolution of this family of peptide hormones (Chand et al., 2013; Sekar and Chow, 2013). Interestingly, these protein families all have established roles in energy metabolism and elements of the stress-response, thereby lending support to the ancestral roles of teneurin/TCAP.
1.7.2. ADGRL evolution

GPCRs have a long evolutionary history, as evidenced by their presence in plants, yeast, slime mold and protozoa (Bockaert and Pin, 1999). ADGRL expression has been found in early unicellular organisms, such as Monosiga brevicollis, the closest relative of metazoans, demonstrating it likely evolved before metazoan evolution and was acquired via lateral gene transfer (King et al., 2003, 2008; Strotmann et al., 2011; Zhang et al., 2012). ADGRL was first classified as part of the secretin family GPCRs as it contains a hormone-binding domain in its extracellular domain (Lelianova et al., 1997; Perrin et al., 1998). It has recently been reclassified as an Adhesion GPCR as it shares many signatures of this family, such as a long extracellular portion, a GAIN/GPS site, and its newly elucidated roles in adhesion. Evolutionary analyzes reveal that the Adhesion family of GPCRs was likely a progenitor to the Secretin family of GPCRs, which provides an explanation for ADGRL having structural characteristics of both families (Nordstrom et al., 2009; Strotmann et al., 2011).

Figure 1.7. Phylogenetic schematic of teneurin evolution. Evolutionary analyzes suggest teneurins underwent horizontal gene transfer (HGT) from prokaryotes to choanoflagellates. They subsequently evolved in metazoans but were lost in Diploblasta. Adapted from Chand et al., 2013.
1.7.3. The co-evolution of teneurin/TCAP and ADGRL for stress and energy metabolism

Some of the first known stressors of early unicellular organisms were osmoregulation and the acquisition and digestion of food and nutrients (Suescun-Bolivar and Thome, 2015). Early organisms had food requirements greater than what could be acquired in their immediate vicinity, thus establishing the need for locomotion (Lovejoy, 2005). Before the development of a nervous system, early mouth structures were responsible for digestion of food material. When mechanisms of opening and closing the mouth structure were eventually developed, they likely required arrangement of nerve cells around the aperture, thereby leading to the first formation of a nerve ring. Thus, this mechanism evolved for feeding was later used for the basis of the locomotory system, indicating a clear evolutionary relationship between feeding and locomotion.

Both TCAP and ADGRL have roles in the promotion of feeding, enhancing energy metabolism and stress-related locomotion. This further underlines their ancestral roles in the modulation of stress and survival. Consistent with this, they are the only trans-synaptic pair retained across evolution in both vertebrates and invertebrates (Tucker et al., 2006; Woelfle et al., 2015). Teneurin/TCAP and ADGRL both have a long evolutionary history and have been suggested to be the results of lateral gene transfer events. Recent studies indicate that Adhesion GPCRs and their ligands, specifically the TCAP family, may have been the progenitors to the Secretin families of receptors and ligands, respectively, all of which have roles in stress, diuresis, and energy metabolism (Nordstrom et al., 2009; Sekar and Chow, 2013). This further corroborates the conserved roles of the teneurin/TCAP-ADGRL system in energy regulation and locomotion under stress.

As these ancient feeding and locomotory systems led to the complex muscular system found today in modern organisms, skeletal muscle represents an ideal tissue to study the roles TCAP and ADGRL may play in energy metabolism and muscle function. Moreover, in vertebrates, one of the key components of a stress response is the ‘fight or flight’ response, which requires rapid movement to either confront the stressor or quickly flee. This places a high energetic demand on
skeletal muscle and demonstrates its involvement in the stress response, further making it the ideal tissue to investigate the roles of TCAP in stress as previously demonstrated in neurons.

1.8. Mechanisms of Energy Regulation in Skeletal Muscle

1.8.1. Glucose regulation in Skeletal Muscle

Glucose uptake into the muscle is critical to the organism as it provides the energy required to meet physical demands. Skeletal muscle is one of the most important sites of glucose metabolism, as it is responsible for 40% of glucose-associated energy requirements (Richter and Hargreaves, 2013) and 80% of glucose disposal under insulin-stimulated conditions (Santos et al., 2008). Glucose imported into muscle has multiple fates: the majority of glucose is stored as glycogen, whereas the rest is metabolized by either entering anaerobic (lactate) or aerobic (oxidative phosphorylation) pathways to produce the ATP required for skeletal muscle energy demands (Alberts et al., 2008). Glucose requires GLUTs to cross the plasma membrane by facilitated diffusion. The most prominent GLUT isoform in skeletal muscle is GLUT4, which is packaged in intracellular vesicles in the T-tubules until they are stimulated to fuse with the sarcolemma (Richter and Hargreaves, 2013; Santos et al., 2008). Once the vesicles have fused, GLUT4 becomes active and facilitates glucose uptake into the cell.

GLUT4 can be mobilized to the sarcolemma via insulin-stimulated or contraction-stimulated pathways (Figure 1.8; Santos et al., 2008). Insulin stimulates GLUT4 fusion by binding to its receptor on the cell surface to activate insulin receptor substrate family 1/2 (IRS1/2), which in turn activates phosphatidylinositol 3-kinase (PI3K). PI3K stimulates the production of phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 regulates the activity of protein-dependent kinase (PDK), which phosphorylates protein kinase B (PKB/AKT), that subsequently induces GLUT4 mobilization. Functional cytoskeleton dynamics are required for efficient insulin-mediated GLUT4 trafficking to the sarcolemma, as pathologies that result in a disrupted cytoskeleton, such as Duchenne muscular dystrophy (DMD), are associated with impaired glucose uptake (Raith et al., 2013). Additionally, insulin-mediated GLUT4 translocation is calcium-dependent. Specifically, insulin stimulates calcium release via the inositol triphosphate (IP3)
receptor (IP3R) on the sarcoplasmic reticulum (SR) to induce fusion of GLUT4 at the sarcolemma (Contreras-Ferrat et al., 2010).

Contraction-stimulated GLUT4 trafficking pathways are further divided into two mechanisms, one driven by AMP kinase (AMPK), and one driven by calcium (Figure 1.8; Jessen and Goodyear, 2005; Santos et al., 2008). AMPK is a heterotrimeric protein that is suggested to act as an energy-sensor in the cells, as it is phosphorylated (p-AMPK) via tumour suppressor kinase (LKB1) when ATP and phosphocreatine levels are low due to contraction stimulation (Jessen and Goodyear, 2005). Activators of AMPK, such as hypoxia and the pharmacological activator, AICA ribonucleotide (AICAR), are effective at importing glucose via GLUT4. The calcium-dependent mechanism of contraction-stimulated glucose uptake depends on calcium release initiated by contraction-mediated membrane depolarization. The main protein that activates GLUT4 mobilization under calcium stimulation is calcium/calmodulin-dependent protein kinase (CaMK) which is activated by CaMK kinase (CaMKK).
Figure 1.8. Schematic of insulin-mediated and contraction-mediated glucose uptake. (A) Upon insulin binding to its receptor (insulin receptor, IR), the activation of insulin receptor substrate (IRS1/2) stimulates phosphatidylinositol 3-kinase (PI3K-α) to increase phosphatidylinositol 3,4,5-triphosphate (PIP3) levels. This increase stimulates protein dependent kinase (PDK) to phosphorylate AKT, which induces GLUT4 mobilization to the sarcolemma. In addition, insulin stimulates phospholipase C (PLC) to produce inositol triphosphate (IP3). This stimulates calcium release from the IP3 receptor (IP3R) on the sarcoplasmic reticulum, which is required for GLUT4 mobilization. (B) Contractions initiate release of calcium, which activates the calcium/calmodulin-dependent protein kinase (CaMK) via CaMK kinase (CaM KK). Additionally, contractions lower the ATP/ADP ratio stimulating tumour suppressor kinase (LKB1). This phosphorylates AMP kinase (AMPK). Both CAMK and phosphorylated AMPK (p-AMPK) lead to GLUT4 mobilization to the sarcolemma. Once GLUT4 has translocated and fused to the sarcolemma, it facilitates glucose uptake into the cell.

1.8.2. Muscle energetics and contraction

Muscle function and metabolism are intrinsically linked, as evidenced by metabolic syndromes that result in poor muscle function and degradation. A key example of this is demonstrated in patients with type-II diabetes, whose muscle is insulin-insensitive which ultimately results in hyperglycemia. Diabetic patients have reduced skeletal muscle function in the grip force test compared to non-diabetic patients (Sayer et al., 2005). This is thought to be, at least in part, due to the inefficient ability of the muscle to switch between fatty acid oxidation to glucose oxidation in response to insulin, referred to as metabolic flexibility (Phielix and Mensink, 2008). Consistent with this, decreased mitochondrial oxidative capacity was found in muscle from diabetic patients.
Interestingly, researchers are still not in consensus whether this underlying metabolic defect in mitochondrial activity represents either the pathogenesis of insulin resistance or is another feature of insulin resistance. Regardless, the support of the interaction between muscle metabolism and function is strong.

The intricacies of this interaction between muscle metabolism and function is further supported when considering the energetic demands of skeletal muscle contractions. Muscle contractions convert chemical energy into mechanical energy, a requirement for excitation-contraction (EC) coupling. Briefly, action potentials, stimulated via acetylcholine release from the motor neuron axon terminal, cause depolarization of the T-tubules. This depolarization leads to a conformational change in the dihydropyridine receptor (DHPR), which stimulates calcium release from the sarcoplasmic reticulum via ryanodine receptors (RyR). This rapidly causes contraction, as the binding of calcium to troponin C and ATP hydrolysis both allow myosin-actin cross bridges to form. Reuptake of calcium via sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCAs) is then required to complete the calcium cycle and prepare the cell for the next contraction (Figure 1.9; Bellinger et al., 2008). Both ATP and calcium are necessary for the initiation and relaxation of a muscle contraction. However, during prolonged muscle stimulation such as exercise, this process can become uncoupled. This results in aberrant glucose and calcium regulation, which ultimately leads to decreased force production, also known as fatigue. Dysregulated glucose levels may result in lowered production of ATP, thus impacting contractile kinetics of ATP binding directly on the sarcomere. In addition, a decrease in ATP levels also indirectly impacts calcium re-uptake by not meeting the energy demands of the SERCA pumps. When sarcoplasmic reticulum-sarcomere calcium cycling becomes inefficient increased accumulation of cytoplasmic calcium occurs. Both of these factors lead to poor contraction strength and velocity (Allen et al., 2008; Bellinger et al., 2008). Therefore, methods of increasing or stabilizing glucose and/or calcium regulation during muscle stimulation stress would maintain muscle function longer without the decay of contractile kinetics, thus enhancing muscular performance (discussed further in Chapters 4 and 5).
Figure 1.9. Schematic of a muscle contraction. (1) An action potential is initiated by the motor neuron and is propagated across the sarcolemma. (2) This activates the voltage-gated channel, dihydropyridine receptor (DHPR), which undergoes a conformational change to stimulate the ryanodine receptor (RyR) on the sarcoplasmic reticulum (SR). (3) Activation of RyR induces a large calcium release which (4) binds to the sarcomeres to initiate muscular contraction. (5) Sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCAs) pump calcium back into the SR to initiate muscle relaxation and to prepare for the next contraction.

1.9. Hypothesis and Rationale

1.9.1. Rationale

The evolutionary history of the teneurins and ADGRLs indicate that this ligand-receptor system may play an essential role, in part, for the success of multicellular organisms. Its importance is further highlighted by its high conservation across evolution. The current model of teneurin and TCAP evolution suggests that it aided in the coordination and capture of prey in early metazoans. Given this, we would expect TCAP to be associated with the musculature of chordates with respect to feeding and movement. Moreover, the expression and actions of the teneurin/TCAP-ADGRL system have been well described in neuronal tissue, with respect to stress and metabolism; however, they have not yet been investigated in skeletal muscle. These studies suggest that TCAP
has the ability to regulate skeletal muscle function by enhancing glucose metabolism. Moreover, as skeletal muscle is a critical component in the stress response and a key metabolic regulator, this is an ideal tissue to study the roles of TCAP. Therefore, my Ph.D. thesis aimed at elucidating the roles of the TCAP family, with respect to stress and metabolism, in skeletal muscle of model organisms chosen across evolution.

1.9.2. Hypothesis

Based on the rationale above, I hypothesized that the teneurin/TCAP-ADGRL ligand-receptor complex is present and functional in skeletal muscle. Moreover, I hypothesized that the TCAP family plays a critical role in skeletal muscle metabolism and physiology, which in turn improves the organism's ability to respond to stress. Lastly, I hypothesized that these effects are conserved evolutionarily.

1.9.3. Objectives and Aims

Therefore, given the early evolutionary history of TCAP, my first objective was to investigate the evolutionarily conserved roles of the TCAP family in muscle. Thus, I have chosen model organisms from various metazoan phyla with varying muscle complexity. Ciona intestinalis is a protochordate, which represents the most phylogenetically recent sister lineage to the chordates. Moreover, their muscle wall shares similar characteristics with vertebrate skeletal muscle, as they have organized orientation of their muscle fibres and depend on acetylcholine for muscle contraction initiation. Zebrafish (D. rerio) is an actinopterygian species. Their skeletal muscle has many shared characteristics with rodent skeletal muscle; however, zebrafish have distinct populations of homogenous muscle fibre types, in contrast to rodent muscle, which has a heterogenous mixture of fibres. The varying levels of muscle complexity in C. intestinalis, zebrafish and rodents will allow for a deeper understanding of the conserved roles of the TCAP family.
This work was organized into four main objectives: to establish the metabolic and physiological role of TCAP in the musculature of 1) vase tunicates (*C. intestinalis*), 2) zebrafish (*D. rerio*), and 3) rats (*R. norvegicus*), and 4) to establish the mechanism of TCAP action by *in vitro* investigations in immortalized C2C12 murine skeletal muscle cells. These objectives, along with their respective aims, are outlined below.

**Objective 1: Establish that TCAP is functional in a chordate ancestor (*C. intestinalis*)**
- **Aim 1.1.** Determine cTCAP and CDLP gene and protein expression in *C. intestinalis* tissues using RT-PCR and western blot analyzes.
- **Aim 1.2.** Investigate cTCAP and CDLP actions on contractile behaviour *in vivo* using a novel behaviour assay.
- **Aim 1.3.** Analyze cTCAP and CDLP actions on stress-related behaviour *in vivo* using a food-stimulation assay.

**Objective 2: Establish that TCAP acts on an actinopterygian species (*D. rerio*)**
- **Aim 2.1.** Determine TCAP-3 expression in zebrafish skeletal muscle using RT-PCR.
- **Aim 2.2.** Develop a novel method of assessing metabolism in adult zebrafish *in vivo* using resazurin sodium salt.
- **Aim 2.3.** Utilize the resazurin assay to measure the metabolic effects of rtTCAP-3 in larval and adult zebrafish under basal and stress conditions.
- **Aim 2.4.** Corroborate rtTCAP-3 actions in metabolism in zebrafish adults using Loligo metabolic respirometry chambers under basal and stress conditions.
- **Aim 2.5.** Establish TCAP-3 expression in zebrafish skeletal muscle using RT-PCR.
- **Aim 2.6.** Assess roles of rtTCAP-3 in glucose uptake in primary muscle cell culture by 2-DG assay.

**Objective 3: Establish that TCAP acts on a tetrapod species (*R. norvegicus*)**
- **Aim 3.1.** Establish short-term TCAP-1 actions on glucose metabolism *in vivo* by analyzing fPET scans of 18F-deoxyglucose uptake in male Wistar rats.
• Aim 3.2. Analyze short-term TCAP-1 actions on skeletal muscle performance under metabolic fatigue by electrical stimulation of the muscle in male Sprague-Dawley rats.

• Aim 3.3. Determine long-term TCAP-1 actions on glucose metabolism and metabolic hormones by monitoring blood glucose in Sprague-Dawley rats over 3 months.

• Aim 3.4. Establish long-term TCAP-1 actions on skeletal muscle performance and physiology under metabolic fatigue by electrical stimulation of the muscle.

• Aim 3.5. Investigate long-term TCAP-1 actions on stress-related behaviours by assessing behaviours in the open field test.

**Objective 4: Establish mode of action of TCAP in skeletal muscle in vitro**

• Aim 4.1. Establish teneurin/TCAP-ADGRL expression in rodent skeletal muscle via PCR and Western blots and establish co-localization of the complex using immunohistochemistry.

• Aim 4.2. Elucidate TCAP-1 actions on the downstream components of ADGRL, IP3 and DAG, using ELISA kits.

• Aim 4.3. Determine if TCAP-1 plays roles in cytosolic calcium in C2C12 myotubules using Fluo-4 fluorescent live-cell calcium indicator and pharmacological inhibitors.

• Aim 4.4. Investigate the roles of TCAP-1 on mitochondria by measuring changes in mitochondrial membrane potential using the fluorescent live-cell indicator, Rhodamine-123.

• Aim 4.4. Investigate the mechanism of TCAP-mediated glucose uptake *in vitro* by assessing GLUT4 activation after treatment in C2C12 cells.

• Aim 4.5. Measure NADH and ATP as biomarkers of TCAP-1 actions on energy metabolism.
Chapter 2

Expression and actions of the teneurin C-terminal associated peptide (TCAP) in the vase tunicate, *Ciona intestinalis*: A novel regulator of contractile activity and stress-related behaviours.

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2.1 Abstract

Teneurin C-terminal associated peptide (TCAP) is a neuropeptide that bears some structural similarity to the corticotropin-releasing factor (CRF) family of peptides. TCAP and CRF are both implicated in the regulation of stress-related behaviours, as established in rodent models. However, in vertebrates, both TCAP and CRF possess three additional paralogous forms making vertebrate models difficult to assess with respect to TCAP-CRF interaction. As a urochordate, this species possesses single homologues of TCAP and of a CRF/Diuretic-like peptide (CDLP) in the genome, thereby establishing *C. intestinalis* as an excellent model organism to examine the interaction of these peptide systems. However, the lack of *C. intestinalis* synthetic peptides and specific antisera has complicated experimentation. In this work, synthetic versions of CDLP and *Ciona*-TCAP (cTCAP) were prepared to investigate their bioactivity in this species. To analyze stress-related behaviours, a novel behavioural assay was used to characterize different types of contraction-based behaviours, using buccal opening contractions, cloacal opening contractions, lateral contractions, longitudinal contractions and expulsions. Protein and mRNA expression data indicate that the mature versions of both peptides are present in a number of tissues. With respect to behavioural activity, both cTCAP- and CDLP-treated animals had distinct contraction profiles under ambient conditions. Moreover, food stimulation tests revealed that CDLP-treated animals displayed a strong expulsion behaviour in response to feeding, whereas cTCAP-treated animals did not show this effect. These actions are consistent with previous studies done in vertebrates.
2.2 Introduction

The teneurin C-terminal associated peptides (TCAPs) were initially identified on their basis of structural similarity to the corticotropin-releasing factor (CRF) family of peptides (discussed in section 1.3.1). CRF plays a fundamental role in the vertebrate stress response (Vale et al., 1981) which does not exist in invertebrates. Although invertebrates, notably insects, possess CRF orthologues, referred to as diuretic hormones (DH), the structure of CRF is significantly derived relative to the DHs. This is likely as a result of the significant role CRF plays in the hypothalamic-pituitary-adrenal (HPA) axis in vertebrates (Lovejoy and Balment 1999; Lovejoy and Jahan, 2006; Lovejoy, 2009; Lovejoy and de Lannoy, 2013). Previous studies indicate that CRF peptides evolved early in metazoan history suggesting that they may be evolutionarily associated with other peptide hormones as a result of subsequent gene and genomic expansion events (Lovejoy and Balment, 1999; Lovejoy and Jahan, 2006; Lovejoy and de Lannoy, 2013). While trying to elucidate potential distant paralogues of CRF, the teneurin C-terminal associated peptides (TCAPs) were identified in vertebrates (Qian et al., 2004; Wang et al., 2005). The TCAPs are encoded in the terminal exon of each of the four teneurin transmembrane proteins (Baumgartner et al., 1994; Levine et al., 1994) and were later found to be in most metazoans including C. intestinalis (Tucker et al., 2012).

Although the exact phylogenetic relationship between CRF and TCAP is unclear, numerous physiological studies in vertebrates indicate that the two peptides have antagonistic actions. In rats, TCAP-1 inhibits the CRF-mediated acoustic startle response (Tan et al., 2008) as well as CRF-induced cocaine addiction reinstatement (Kupferschmidt et al., 2011; Erb et al., 2013). Moreover, TCAP-1 modulates CRF-induced behaviour in the elevated plus-maze and open field tests in rats (Tan et al., 2008, Al Chawaf et al., 2007b). These behavioural studies are corroborated by additional findings indicating that TCAP-1 inhibits the CRF-induced c-Fos expression in rat brain (Tan et al., 2009) and affects stress-induced spine density in the hippocampus (Tan et al., 2011). Despite the clear interaction of TCAP and CRF, TCAP does not bind to or interact with the CRF receptors or binding proteins (Lovejoy and de Lannoy, 2013). Vertebrates possess four paralogues of CRF, that include in addition to CRF, urotensin I (urocortin, sau vagine), urocortin 2 and 3, and four paralogues of TCAP and teneurins (teneurins-1-4). Thus, to elucidate the complex interaction between the CRF and TCAP paralogues is a daunting task.
Tunicates, such as *C. intestinalis*, represent the most phylogenetically recent sister lineage to the chordates. A single CRF orthologue gene was identified in *Ciona intestinalis*, however, as the structure of the putative *Ciona* CRF peptides showed sequence similarity both with the vertebrate CRF family as well as the insect DH family, they have been termed ‘CRF/DH-like’ peptides (CDLP; Lovejoy and Barsyte-Lovejoy, 2010). Moreover, it has been recently established that *C. intestinalis* possesses a single *Ciona*-TCAP (cTCAP) gene as well (Colacci et al., 2015). Compared to the multiple forms of CRF and TCAP in vertebrates, *C. intestinalis* is an ideal model to understand how these two peptidergic systems interact with each other.

One limitation of using *C. intestinalis* as a model is that its behaviour is not well-characterized. They have simply arranged muscle units and contractile characteristics, with the only known behavioural responses reported being ‘squirting’, which is when they expel water out of their siphons, and ‘cowering’, when they contract their entire body downwards in response to agitation (Nevitt and Gilly, 1986). Interestingly however, recent studies showed that their contractile activity is a valid method of investigating responses to peptide treatment. Further, these studies demonstrated that synthetic mouse-TCAP-1 peptide treatment could increase contractile behaviour in *C. intestinalis* (Colacci et al., 2015).

In this current investigation, a number of specific antisera were raised to both *C. intestinalis* CDLP and cTCAP and show that both the mRNA and peptide is expressed in mature *C. intestinalis*. Further, the work presented here builds upon the foundation described by Colacci and colleagues (2015). To investigate the effects of CDLP and cTCAP peptides, behaviours were characterized using muscle contractile activity, which resulted in the identification of six novel behaviours. This novel, yet simple, behavioural assay demonstrated that CDLP and cTCAP treatment play a role in a number of contractile behaviours. Moreover, CDLP, like its insect and chordate orthologues, plays a role in the inhibition of the feeding response. Currently, *C. intestinalis* is posing a major threat to North American coastlines as an aquatic invasive species (Carver et al., 2003; Cayer et al., 1999; Sephton et al., 2011), thus this research may provide directions toward a practical application in the control of these aquatic pests.
2.3 Methods

2.3.1 Collection of Animals

Mature specimens of *Ciona intestinalis* ranging in size from 5 to 15 cm were collected from a commercial mussel farm near Indian Point in Mahone Bay, Nova Scotia, Canada. Upon collection, the animals were transferred to polymer containers filled with ambient seawater, then transported to the Bedford Institute of Oceanography (Bedford, Nova Scotia) where they were transferred to circulating filtered seawater aquaria at a temperature of 8°C with a light- dark cycle of 12 hrs.

2.3.2 Synthesis of peptides

All peptides used in this study were synthesized by Ambiopharm Inc. The cTCAP sequence in *C. intestinalis* was identified as described previously (Figure 2.1; Colacci et al., 2015) and the CDLP sequence was synthesized according to the *C. intestinalis* genomic sequence previously reported (Lovejoy and Barsyte, 2010). Both peptides were prepared as acetate salts using solid-phase methods and purified to >95%. The structure was confirmed by mass spectrometry and MALDI-TOF with a determined mass of 4761.0 for cTCAP and 5559.6 for CDLP. cTCAP was synthesized as a C-terminal free acid (-OH) form, whereas CDLP was amidated at the C-terminus as suggested by the respective gene sequences.

The cTCAP sequences used as haptens for antisera production were H-QEIRSRHRLSSYQLRPTWDPE-OH for the N-terminus and H-PERYPELADSGRNMRFVKVS-OH for the C-terminus. For the CDLP sequence, PALGRNPFLSDLVPLSFLSKLMEV-OH was used as the N-terminal fragment whereas KRREENKIAKIKSVHNSSLMKRI-NH2 was used as the C-terminal fragment. Peptides were prepared by solid-phase synthesis and purity was ascertained by HPLC and MALDI-TOF MS. Peptides were prepared 90 to 95% purity. Both cTCAP peptides and the N-terminal CDLP peptide was synthesized as a free acid at the C-terminus whereas, the C-terminal CDLP fragment was amidated at the C-terminus.
Figure 2.1. cTCAP and CDLP peptide structure. Alignment of hapten peptides, and similarity to other orthologues in metazoans. Schematic of the position of C-terminal and N-terminal cTCAP and CDLP haptens relative to the full-length Ciona peptides (A), alignment of the Ciona intestinalis cTCAP with other TCAP sequences (B) and the alignment of the Ciona intestinalis CDLP with other known CRF sequences (C).

2.3.3 Antisera production and characterization

The N and C terminal fragments of the CDLP and cTCAP peptides were used as haptens for the development of the antisera. Briefly, 2 mg solubilized in 450 μL of buffer (MES, pH 4.7) of each N-terminal cTCAP, C-terminal cTCAP, and C-terminal CDLP were conjugated with 2 mg keyhole limpet hemocyanin (KLH; Pierce Chemical Co.) by following the manufacturer’s protocol. N-terminal CRF at a 1 mg/ml (450 μg in 450 μL) concentration, was solubilized in borate buffer (pH
8.5). cTCAP and CDLP haptens were covalently bonded to KLH using the ‘Imject EDC Carrier Protein Spin Kit’, with EDC as the bifunctional crosslinking reagent. 2 mg of peptide were dissolved into 450 μl of ‘Imject EDC Conjugation Buffer’ and mixed with a 200-μL solution of 10 mg/ml mcKLH and 50 μL of the 10 mg/ml EDC buffer. This solution was incubated for 2h at RT and purified by desalting to remove the non-reacted cross-linker and sodium azide. Each of the four conjugated haptens were injected into three rabbits each to immunize for antisera production. The titer for each rabbit was determined each month over three months using an enzyme-linked immunosorbent assay (ELISA).

A ‘Direct ELISA’ (Thermo Fisher Scientific, Denmark) was used to test the antisera titer for each of the conjugated haptens. Briefly, after coating with 100 μL of C-terminal or N-terminal peptide at a concentration of 5 μg/mL in 50 mM carbonate buffer, and overnight incubation at RT in a humid chamber, the coating solution was removed, the plate rinsed 2X with 0.5% Tween PBS, and subsequently blocked with 200 μL of 0.1% BSA/0.5% Tween PBS solution for 2h at RT. After blocking, 100 μL of antiserum at various concentrations in 0.1% BSA/0.1% Tween PBS was added to the wells, incubated for 2h RT, washed 3X with 0.1% Tween PBS. Afterward, 100 μL of goat anti-rabbit horseradish peroxidase (HRP) conjugate at 1: 10,000 dilution was added and incubated for another 2h at RT. This was followed by treatment with 100 μL TMB for 10 mins and measured using a Spectramax Plus 384 spectrophotometer (Molecular Devices) set at 450 nm.

2.3.4 Western blot analyses of CDLP and cTCAP expression

*C. intestinalis* tissues were harvested and flash-frozen in liquid nitrogen and subsequently stored at -80 °C. Individual tissues were lysed by adding 300 μl radioimmunoprecipitation assay (RIPA) Buffer [1% Triton X-100 (Thermo Scientific), 50 mM Tris, pH 8.0 (Sigma Chemical Co.), 0.5% Sodium Deoxycholate (Sigma Chemical Co.), 0.1% SDS ( Bioshop)] per 5 mg of tissue and homogenized using a mortar and pestle. Additional RIPA buffer was used to dilute the samples to a concentration of 5 mg/ml. Tissue lysates then underwent constant agitation for 2 hrs at 4°C. Following this, the lysates were centrifuged at 20,000 relative centrifugal force (rcf) at 4°C for 15 mins. The supernatant was aspirated, divided into aliquots and stored at 20°C until used for
Western blot analysis. The pellet was discarded. Protein concentration in the extracts was quantified using the Pierce BCA protein assay in accordance with the manufacturer’s protocol (Thermo Fisher Scientific). In brief, 1.5 μL of each protein sample was transferred into 96-well plates in duplicate. An aliquot of 150 μL of ‘BCA Working Reagent’ was added and diluted albumin standards were prepared in duplicate providing the assay with a working range from 20 to 2,000 μg/ml. The 96-well plate was incubated with shaking at 37°C for 30 mins before the absorbance was measured at 562 nm using the SpectramaxPlus 384 spectrophotometer. Protein samples (15 μg) were run on a 16% agarose gel at 100V for 1.5 hrs. Proteins were subsequently electro-transferred to a Whatman-Protran nitrocellulose membrane (VWR) for 90 mins at 100 V. The membrane was washed in PBST (PBS with 0.2%Tween20; Sigma Chemical Co.) for 5 mins and incubated with either cTNR5 (Ciona TCAP N-terminal Region Rabbit 5) or cCCR8 (Ciona CDLP C-terminal Region Rabbit 8) primary antibody diluted 1:10000 in 5 ml of milk-PBST with agitation overnight at 4°C. After 24 hrs, the membrane was washed with fresh PBST for 30 mins. Then, the membrane was incubated with anti-rabbit HRP-conjugated secondary antibody (VWR) diluted 1:7500 in milk-PBST for 1h at RT with agitation. After incubation, the membrane was washed as described and exposed onto ECL Hyperfilm (VWR) for 15-60 sec.

2.3.5 Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

Ciona intestinalis tissues were harvested as described above. Manufacturer’s instructions were followed for TRIzol reagent to extract RNA from samples. Reverse transcription reactions contained 2 μL purified total RNA, 1 μl Random Primer Mix (Bio-Rad), 1 μL deoxynucleotide solution mix (New England Biolabs), and 8 μL water. Reactions were incubated in a Fisher Scientific Isotemp 125D Dry Bath Incubator for 5 mins at 16°C and then for 1 min at 4°C. Aliquots of 4 μL First Strand Buffer (Invitrogen), 2 μL 0.1 M DTT (Invitrogen), and 1 μL SuperScript II Reverse Transcriptase (Invitrogen) were added to the reaction mix. The 20 μL reactions were incubated for 10 min at 25°C, 50 min at 42°C, 15 min at 70°C, and then held at 4°C. The 29.5 μL PCR reaction mix included 5 μL cDNA, 2 μL Forward Primer and 2 μL Reverse Primer (Invitrogen), 14.2 μL water (Sigma), 3 μL 10x Taq Buffer with KCl (Thermo Scientific), 1.8 μL MgCl₂ (Thermo Scientific), 1 μL Deoxynucleotide Solution Mix (New England Biolabs), and 0.5 μL Taq DNA Polymerase (BioShop). The reactions were incubated in an Eppendorf Mastercycler Gradient Thermal Cycler for 7 min at 95°C; followed by 35 cycles of 60 sec at 95°C, 90 sec at
65°C, and 35 sec at 72°C; and then held at 4°C. DNA samples (14.4 μL) were then run on an agarose gel at 100 V for 1.5 hrs. The gel was visualized by a Bio-Rad ChemiDoc MP System with 0.5 sec exposure. For all RT-PCR analyzes, β-actin was used as a positive control, and mRNA without the addition of taq polymerase nor primer pairs was used as a negative control. Primer sequence information can be found in Table 2.1.

<table>
<thead>
<tr>
<th>CDLP</th>
<th>CAAGGAAGAATACGCAGCAC</th>
<th>AGTCTGTTCAACAATAGCAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>cTCAP</td>
<td>CGCCACCCTTTATCATCTTT</td>
<td>CTTAACAAAGCGCATTTTC</td>
</tr>
</tbody>
</table>

Table 2.1. Primer sequences for *Ciona intestinalis* CDLP and cTCAP RT-PCR analyzes.

### 2.3.6 Behavioural Stimulation Test

Behavioural stimulation tests were performed as described previously (Colacci et al., 2015). Briefly, each *C. intestinalis* was individually pinned by their tunic to 10 cm petri dishes coated in Sylgard 184 Elastomer (Kraydon Inc. Denver, CO, USA) and were allowed to acclimate overnight. The behaviour stimulation test began with a 10 μL injection of filtered sea water (sham control) into the buccal siphon, which was then recorded using Canon FS30A 41x Advanced Camcorder for the next 15 mins. Afterward within 1 min either, 10 μL of CDLP or cTCAP was injected into the buccal siphon and recorded for a further 15 mins. This protocol was repeated for each animal and for each of the various doses of cTCAP and CDLP treatment (0.5 μg, 1 μg, 5 μg, 10 μg). Dose-responses were constructed based on the type of contractions and duration of contraction length witnessed of all animals using GraphPad Prism V.6.

In order to analyze the *C. intestinalis* repertoire of behaviours, the recorded activity of all experiments was digitally analyzed by two ‘blind’ observers examining the time and number of events for each behaviour based on the identification of contractile activity previously reported (Colacci et al., 2015). The major behaviours consisted of buccal aperture closing (BOC), cloacal aperture closing (COC), lateral contractions leading to a slimming of the organism (LAT) and longitudinal contractions resulting in a vertical shortening of the animal (LONG). Expulsion events
were characterized by a rapid lateral contraction and/or longitudinal contraction resulting in the animal expelling its contents or causing a disruption of water above. The onset and duration of all behaviours were recorded as ethograms and compared to the sham controls for each behaviour. The co-incidence of each behaviour with other single or combinations of behaviours were examined statistically.

2.3.7 *Feeding Stimulation Test*

Animals were pinned and injected with treatments as described above. After either CDLP or cTCAP treatment, animals were given a 10 μL algae supplement of their typical food source by injection into the buccal siphon. They were then recorded for the subsequent 15 mins using the methods described above. Dose-responses were constructed based on the type of contractions and duration of contraction length determined digitally.

2.3.8 *Statistical Analyzes*

Comparison of the dose-response curves between cTCAP and CDLP were analyzed by a parametric two-tailed, two-way ANOVA for each of the BOC, COC and LAT behaviours. A sample size of n=4 was used for all analyses. In each case, the treatment effect was expressed relative to the sham control performed before that of the treatment. However, because previous studies on TCAP behaviour in rodents follow a quadratic function (i.e. U-shaped curve), a secondary analysis on the lowest dose was performed using a two-tailed students t-test. Analysis of cTCAP or CDLP dose was performed individually using a two-tailed, one-way ANOVA. All further analyzes of the behaviours were calculated with two-tailed, one-way ANOVAs. Bonferroni’s post-hoc tests were used whenever necessary to determine the comparisons between treatments. Comparison of cTCAP and CDLP for the feeding studies utilized two-tailed student’s t-tests. An *a priori* confidence level of *p*<0.05 was considered significant for all analyzes. All analyzes were performed using GraphPad Prism 6 software.
2.4 Results

2.4.1 Development of Antisera

A total of 6 polyclonal antisera were developed each for CDLP and cTCAP. Of the 6 developed, 3 were targeted for the C-terminus and 3 for the N-terminus of each peptide. The titre was initially determined by dot blot to determine that a robust immune response had occurred. Following this, each serum was tested using ELISA to establish its efficacy. As expected, there was considerable variability in the antigen-antibody reactions among the titre results of the various antisera of the individual rabbits (Figure 2.2). Despite this, a number of rabbits showed strong immunization reactions. For the cTCAP series, cTNR5 showed a rapid increase in the immune response within 3 weeks and remained high until 12 weeks when the blood was collected. As a result, this antiserum was used for further studies. With respect to CDLP, both the cCCR8 and cCCR9 antisera showed the strongest titer after 12 weeks. The N-terminal fragments of CDLP showed relatively weak immune responses. As a result, antiserum cCCR8 was used for future studies.
Figure 2.2. Antiserum production. Comparative binding activity of each antiserum using ELISA. The nomenclature for the antisera production is as follows: c refers to 
*Ciona*, in the second position T or C refers to cTCAP or CDLP, respectively, in the third position N or C refers to N-terminus and C-terminus, respectively, and the R# refers to the rabbit ID. Antisera cTNR5 and cCCR8 were selected.

### 2.4.2 Expression of Peptides in Tissues

Several tissues were excised to examine the expression of cTCAP and CDLP (Figure 2.3). Care was taken to remove the target tissues without contamination from surrounding tissues. Western blot studies of the *C. intestinalis* CDLP peptide revealed that immunoreactive bands were found in all tissues except ovary and testes (Figure 2.4A). The testis exists as a delicate diffuse network over the stomach, and thus sufficient tissue could not be obtained to get a clear response distinct from the stomach. In all cases, the CDLP size on the western blot was consistent with the size of the synthetic peptide. A similar profile was found with cTCAP with a clear signal in the gonads lacking. Again, the immunoreactive bands for cTCAP was the same size as the synthetic peptide, like the CDLP signal, indicating that the expressed form of the peptide is the same size as the theoretical size and synthetic peptide.
To identify tissues where cTCAP and CDLP mRNAs were expressed, RT-PCR analyzes were performed. CDLP expression was found in the brachial basket, testes, and CNS, however there was no clear expression of CDLP in the buccal siphon, cloacal siphon, stomach and ovaries despite using several primer pairs (Figure 2.4B). cTCAP mRNA expression rendered a slightly different picture. Strong signals were shown in the buccal siphon, central ganglion, testes, ovary and stomach. A weak signal was noted in the brachial basket (Figure 2.4B). Again, given the close association between the testes and stomach, it cannot be ascertained that the testes result is distinct from that of the stomach. However, taken together, these protein and mRNA expression studies indicate that CDLP and cTCAP is present in both neural and non-neural tissues, consistent with findings in chordates.

Figure 2.3. Anatomy of dissected tissues in *C. intestinalis*. Several organs and tissues were excised for cTCAP and CDLP protein and mRNA expression.
Figure 2.4. Expression of CDLP and cTCAP protein and mRNA in *C. intestinalis* tissues. Western blot showing protein expression of CDLP and cTCAP in various tissues (A). RT-PCR revealed cTCAP mRNA expression in the brachial basket, testes, CNS, buccal siphon, cloacal siphon, stomach and ovaries. RT-PCR revealed CDLP expression in brachial basket, testes, and CNS. CDLP was not expressed in buccal siphon, cloacal siphon, stomach, and ovaries. Negative control for RT-PCR is mRNA without the addition of taq polymerase nor primer pairs, and positive control is β-actin (B).

2.4.3 Identification of *C. intestinalis* behaviours

Previously, a number of common behaviours were identified in *C. intestinalis* (Colacci et al., 2015). In this study, the identification of more complex behaviours was achieved by constructing
a series of ethograms (Figure 2.5). Behaviours were initially examined with respect to the total number of behavioural bouts (any incidence of contractile behaviour), the total time of behavioural bouts (total time of all contractile behaviours). Secondarily, the behaviours identified and based on the ethograms were examined separately.

Overall, there were no significant differences with respect to total bouts or total time among the sham-treated animals indicating that the time of analysis or small temperature fluctuations in the ambient seawater had no effect on the overall activity of the animals. There were, however, considerable variations among individual *C. intestinalis* specimens, where some were highly active, and others showed little activity. Thus, despite their comparatively simple morphology and physiology, the animals displayed highly individualistic activity. This individual activity was reflected within the peptide treatment as well. Highly active animals observed in the sham treatment displayed similar activity in the peptide treatment, and low activity animals, likewise showed attenuated responses with respect to the peptide administration. For this reason, all analyses used the relative effect of peptide activity to sham activity in order to determine the specific activity of the peptide treatment.
Figure 2.5. Characterization of *C. intestinalis* behaviours. Images of *C. intestinalis* demonstrating the various characterized behaviours of relaxed (A), buccal opening contraction (BOC; B), cloacal opening contraction (COC; C), longitudinal contraction (LONG; D), lateral contraction (LAT; E). Representative ethograms for both CDLP-treated animal (F) and cTCAP-treated animal (G). In these ethograms, the black bars and corresponding numbers represent the duration of a certain behaviour displayed by the animal. Animals were observed for 15 mins, but the first 3 mins are not counted into the analyzes to allow the animal to acclimate from the withdrawal response caused by injection of treatment.
2.4.4 **Effect of peptides on contractile behaviour**

The total number of contractile bouts was tabulated for cTCAP and CDLP and compared (Figure 2.6A, B, C) for BOC, COC, and LAT. In all cases, cTCAP showed a significantly (p<0.05) greater increase in contractile activity at the lowest dose of 0.5 μg in comparison to that observed to CDLP, for the relative number of contractile bouts for BOC, COC and LAT behaviours. The effects on LONG behaviours are not shown, as the effect was not significant. The trend for BOC, COC and LAT behaviours was also not significant over increased doses of either cTCAP or CDLP. As a result, the data was examined using the time of each type of contraction. Using the relative change from the peptide action to the sham action on behaviour, cTCAP induced a significant (p<0.05) negative dose-response relationship for BOC, LAT and LONG contraction duration. Whereas a similar trend was apparent for COC, it was sub-significant (Figure 2.6D). In contrast, although CDLP treatment did not induce a significant effect on BOC at the lowest dose, it showed an increased effect of contraction duration at the highest dose of 10 μg (Figure 2.6E).

Once the individual behaviours were examined with respect to time, a clearer picture of the effects of peptides on *C. intestinalis* behaviour emerged (Figure 2.6D, E). In all four contractile behaviours, cTCAP induced a negative dose-response relationship. Thus, in all cases with the exception of COC, the amount of contractile duration was significantly reduced (p<0.05) at the highest concentration in comparison to the lowest concentration. However, in all cases, the reduction in contractile duration was not significantly different from the baseline effect, and therefore, cTCAP did not appear to reduce contractile behaviour below normal expression (Figure 2.6D). CDLP induced the opposite trend as the lowest contractions of 0.5 μg did not show a significant effect on contractile behaviour. All behaviours showed a mean increase in contractile duration at 10 μg relative to 0.5 μg although the effect on BOC and COC was not significant at p<0.05. However, in the case of LAT and LONG there was a significant increase (p<0.05) at the highest concentration (Figure 2.6E). Of the four behaviours examined among the sham treatments, BOC accounted for 34 ± 10% when compared to the active behaviours, and 11 ± 3 % of total behaviours across the 720-second (12 min) observation period. COC activity was similar with 35 ± 11 % of relative active behaviours or 13 ± 4% of total time, LAT showed 25 ± 8 % of active
behaviours and $8 \pm 2\%$ of total time and LONG behaviours accounted for $4\pm 3\%$ of active behaviours and less than $2\%$ of total time.

With respect to each type of behaviour, rarely were they expressed in isolation and were generally associated with other expressed behaviours (see Figure 2.5F and Figure 2.5G for example ethograms). BOC tended to occur most predominantly with COC contractile activity and also with COC and LAT activity combined. All combinations of behaviours were examined although, with the exception of those described above, all were particularly rare. With respect to cTCAP, there was a significant ($p<0.05$) reduction in the BOC/COC combination at 1 $\mu$g although other doses were similar to baseline levels (Figure 2.6F). There was a significant increase in combined BOC/COC/LAT combinations at 0.5 $\mu$g whereas all others were similar to baseline (Figure 2.6F). Again, the opposite trend occurred with CDLP treatment. Both BOC/COC and BOC/COC/LAT combinations showed an increase at the highest dose although only the BOC/COC combination was significantly different ($p<0.05$; Figure 2.6G).
Figure 2.6. Actions of CDLP and cTCAP on contractile behaviour. Briefly, *C. intestinalis* first received a sham (filtered sea water) injection into their buccal siphon and were observed for 15 minutes. Immediately following, animals were injected with either cTCAP (0.5 mg, 1 mg, 5 mg, 10 mg) or CDLP (0.5 mg, 1 mg, 5 mg, 10 mg) and observed for an additional 15 mins. Changes in the number of specific activity bouts for cTCAP (black) and CDLP (grey) were compared to their respective sham treatments for buccal opening contraction (BOC; A), cloacal opening contraction (COC; B), and lateral contraction (LAT; C). Changes in duration of specific behaviours as a function of treatment concentration compared to sham for BOC, COC, LAT and LONG for cTCAP (D) and CDLP (E) were also analyzed. Changes in duration of time of combined contraction events of BOC/COC together and BOC/COC/LAT all together were analyzed as a function of cTCAP (F) and CDLP (G) concentration. (Mean ± SEM; n=4; *p<0.05; two-way ANOVA).
2.4.5 Effect of CDLP and cTCAP on feeding behaviour

On a couple of occasions, a combination of LAT contractile activity with no buccal or cloacal constriction was noted (Figure 2.7A-C). In other situations, lateral contractions could occur with the buccal aperture closed (Figure 2.7D-F). This suggested that these contractile combinations could act to expel material from the brachial basket outward with respect to the first case and downward into the GI tract in the second case. As a result, a feeding experiment was performed where the animals were given a concentrated aliquot of their normal algae food source (Figure 2.7). Animals treated with the highest concentration of either peptide were used for the experiments. After introduction of the food source, LAT activity in the absence of any other contractile activity, was only observed in the CDLP-treated animals for both number of events (Figure 2.7G, p<0.0003, t-test) and time (Figure 2.7H, p=0.0011, t-test). There were no significant differences between LAT occurring with a closed buccal opening for either number of events (Figure 2.7I) or time (Figure 2.7J) between the cTCAP- and CDLP-treated animals.
Figure 2.7. Actions of CDLP and cTCAP on food-stimulation behaviour. Expulsion behaviour identification where there were lateral contractions without buccal or cloacal behaviours (A-C). Ingestion behaviour where lateral contractions occurred with a closed buccal aperture (D-F). CDLP treatment increased number of expulsion events (G) as well as increased total time (H) compared to cTCAP-treated animals. Other behaviours were not affected (I, J). (Mean ± SEM; n=4; **p<0.01, ***p<0.001; t-test).
2.5 Discussion

In this study, it was established that CDLP and cTCAP are expressed both as the protein and mRNA in a number of Ciona intestinalis tissues. Moreover, synthetic versions of the C. intestinalis CDLP and cTCAP are highly active at differentially regulating contractile behaviours under ambient conditions, where cTCAP treatment significantly increases contractile behaviour. Importantly, both peptides show distinct patterns of behaviour during feeding studies where CDLP acts to inhibit the feeding response. This study was performed using specific antisera to the endogenous C. intestinalis peptides, as well as using synthetic versions of C. intestinalis CDLP and cTCAP sequences. Further, a simple and novel behavioural assay for studying peptide action on C. intestinalis, and perhaps for tunicates in general, was developed. These studies build upon previously published studies on C. intestinalis (Colacci et al., 2015) and provide molecular tools and assays to study future peptide interactions in C. intestinalis.

The results of this study strongly corroborate previous research done in vertebrates, as the expression patterns of cTCAP and CDLP found in C. intestinalis are consistent with vertebrate expression patterns (Colacci et al., 2015; Bale and Vale, 2004). In order to perform these studies, a number of C. intestinalis specific polyclonal antisera to both CDLP and cTCAP were prepared. Three antisera were prepared for each of the amino- or carboxy-terminal peptide fragments from both C. intestinalis CDLP and cTCAP peptides. On the basis of their dot blot and competitive ELISA data, the best antisera were chosen to examine the immunoreactive profiles in dissected tissues. These studies indicated that both peptides were present in most tissues. The size of the CDLP and cTCAP immunoreactive profile on the Western blot was consistent with the expected size of the peptide. Although CRF has been well established to exist as a separate peptide in a number of species, TCAP has yet to be. In this study, the western blot profile of cTCAP was consistent with its expected peptide size suggesting that cTCAP can be liberated as a peptide separate from teneurin, which has been previously been suggested to be the case with murine-TCAP-1 (Chand et al., 2013).

cTCAP treatment significantly increased muscle contractile activity, consistent with previous studies performed with mouse-TCAP-1 in C. intestinalis that also exhibited increased contractile
activity. This clearly demonstrates that the TCAP family is a potent regulator of contractile activity. Moreover, the fact that cTCAP and CDLP had significantly different contraction profiles not only validates the efficacy of this novel behavioural assay, but also confirms that *C. intestinalis* is a suitable model organism to study the interaction of these two peptides. As *C. intestinalis* muscle is simply arranged in circular and longitudinal bands, behaviour characterization was limited to ‘squirting’ and ‘cowering’ behaviour until now (Nevitt and Gilly, 1986). Despite their simple morphology and organization, *C. intestinalis* displayed considerable individual differences with respect to behaviour. Some individuals were highly responsive to either peptide treatment whereas others showed a considerably attenuated response. In the past, when working with these peptides in rodents, individual reactivity was assessed by pre-experimental screening (Wang et al., 2005). However, with *C. intestinalis*, it was unclear how to assess their behaviour, thus all animals were grouped together for the purpose of these studies. However, because of these differences in individual behaviours, it was necessary to establish a normal response first by including a sham administration where only filtered seawater was injected into the buccal opening. Colacci et al. (2015) previously established this method as a viable method to compare the treatment effect to baseline effect in each animal. Moreover, these studies also indicated that a second sham injection was not significantly different from the first (Colacci et al. 2015). Therefore, in this current study, the behaviour of each animal was normalized by determining the relative change in activity upon treatment with cTCAP or CDLP compared to that after treatment with sham.

For each of the peptidergic treatments, the effects of different doses on *C. intestinalis* were investigated. For cTCAP-treated animals, the lowest dose (5 µg) significantly increased number of BOC, COC and LAT contractions, while other doses had no effect. Moreover, there was a significant negative cTCAP dose-dependent response on duration of contraction. Taken together, these results indicate cTCAP is increasing the contractility of the animals, inducing short and frequent contractions (see Figure 2.5 for representative ethogram). These data are consistent with a previous study done with mouse-TCAP-1 in *C. intestinalis* (Colacci et al., 2015). In contrast, CDLP-treated animals had no significant changes in the number of BOC, COC or LAT contractions and demonstrated a linear dose-response, where the duration of contraction increased as dose increased. This results in an overall decrease in contractility, which is consistent with studies done in vertebrates (Enck and Holtmann, 1992).
As part of this study, a feeding response component was incorporated to further characterize *C. intestinalis* stress-response and its corresponding behaviours. In previous unpublished studies, an apparent expulsion behaviour was observed when the animals were fed extracts of ground up *C. intestinalis* tissues. However, because this is unlikely to occur in their environment, these studies were repeated with a more physiologically relevant stimulant, using algae extracts. After administration of the highest dose of CDLP or cTCAP, each animal was fed 10 μL of a concentrated algae supplement of the same composition as what they were supplied with during culturing. This showed unequivocally that the expulsion behaviour only occurred in CDLP-treated animals, as it was never observed in the cTCAP-treated animals. A CRF-induced expulsion event can also be seen in vertebrate models (Enck and Holtmann, 1992), as this response is physiologically similar to emptying of stomach by purging. Moreover, CDLP-treated *C. intestinalis* showed a significant increase in cloacal aperture contractions. This is also consistent with vertebrate models where an increase in colonic motility and inhibition of food intake has been well described after increases in CRF (Taché et al., 2004; Zorilla et al., 2003). On some occasions, waste expulsion from the cloaca siphon was observed; however, this event was rare and could not be corroborated with peptide treatment. These behaviours are associated with high-anxiety or stress response (Coşkun et al., 1997; Taché et al., 2004). In contrast, the behaviours witnessed in cTCAP-treated animals were associated with a low-anxiety response. cTCAP-treated animals showed an increase in number of contractions as well as acceptance of food intake. cTCAP is suggested to be an ancient protein that is involved with energy regulation and metabolism (Woelfle et al., 2015), which would be consistent with the results shown here. Overall, the distinct actions of cTCAP and CDLP were clearly visualized in *C. intestinalis* with respect to stress-related behaviour.

The development of a novel behavioural assay has made *C. intestinalis* a model organism to study the roles of cTCAP and CDLP in contractile activity and stress-related behaviours. This study has provided strong evidence of the expression and actions of cTCAP and CDLP in urochordates, corroborating previous studies done in vertebrates. This research may have practical applications in controlling the invasion of *C. intestinalis* as they are highly invasive in many parts of the world and has a negative economic impact on a number of aquacultural activities due to its biofouling
activity. These findings that CDLP has an inhibitory effect on feeding may lead to the development of methods to control the spread of *C. intestinalis*.
Chapter 3
Characterization of the metabolic actions of rainbow trout teneurin C-terminal associated peptide (rtTCAP-3) in larval and adult zebrafish, *D. rerio*, under basal and stress conditions.

A portion of this chapter has been previously published in Comparative Biochemistry and Physiology: Part C; D’Aquila, A.L., Reid, R.M., Biga, P.R., 2017. The validation of a sensitive, non-toxic in vivo metabolic assay applicable across zebrafish life stages. Comp Biochem Physiol C Toxicol Pharmacol. DOI: 10.1016/j.cbpc.2017.11.004 [AD contribution: experimental design, performed some experiments (3/6) and analyzed all experiments (6/6 experiments), wrote large portion of manuscript.]

A portion of this chapter is being currently prepared as a manuscript for submission; Reid, R.M., D’Aquila, A.L., Friej, K., Requena, L.A., Lovejoy, D.A., Biga, P.R. Teneurin C-terminal associated peptide (TCAP)-3: A novel metabolic regulator in Zebrafish (*D. rerio*). [AD contribution: experimental design, performed (2/6) and analyzed experiments (5/6 experiments), wrote moderate portions of the manuscript.]

3.1 Abstract

The teneurin C-terminal associated peptides (TCAP) family has been shown to increase neuronal glucose metabolism in rats, however, its actions are not well described in other organisms. Zebrafish are a well-established model for metabolic studies as they share similar glucose regulatory systems as other vertebrates. Thus, this chapter was aimed at utilizing zebrafish to study the metabolic effects of rainbow trout TCAP-3 (rtTCAP-3) in basal and stressed conditions. In this work, the use of resazurin, a compound that is conformationally changed into fluorescent resorufin upon metabolic reduction by NADH, was validated as a metabolic assay for adult zebrafish. Subsequently, the use of this assay revealed that rtTCAP-3 significantly enhanced metabolic activity in larvae and adults after 48 hours of treatment. Moreover, rtTCAP-3-treated larvae maintained elevated metabolic activity even when exposed to low temperature stress, whereas it was depressed in vehicle-treated larvae. This finding was replicated in adults that were subjected to a metabolic stress test; demonstrating the potent role of rtTCAP-3 in zebrafish metabolism under stress. Lastly, rtTCAP-3 increased glucose uptake into rainbow trout myogenic precursor cells (rtMPCs) to a similar degree as insulin treatment. The results presented in this chapter demonstrates the roles of rtTCAP-3 in zebrafish metabolism and glucose regulation.
3.2 Introduction
Zebrafish have recently been established as an excellent model for translational studies on metabolic disease for several reasons. The first reason is that zebrafish have a similar metabolic profile to other vertebrates and have the key homologous organs required for metabolism. For example, they have conserved hypothalamic circuitry, a known regulator of energy balance in vertebrates, as well as conserved insulin-sensitive tissues such as liver, muscle, and white adipose tissue (Seth et al., 2013). Similar to rodents, insulin treatment induces glucose uptake in rainbow trout skeletal muscle via the GLUT4-homologue, brown trout GLUT4 (btGLUT4; Capilla et al., 2002; Diaz et al., 2007). Further, the development of the pancreas is also well established, with the primary islet of the pancreas being visible at 24 hours post-fertilization (Argenton et al., 1999), and the secondary islet at 5 days post-fertilization (Hesselson et al., 2009). Zebrafish pancreas islet composition of insulin-secreting beta-cells and glucagon-secreting alpha cells is highly conserved. The use of zebrafish as a model organism to study obesity, diabetes, non-alcoholic fatty liver disease (NAFLD), and atherosclerosis have all been validated, as zebrafish show similar phenotypes and pathogenic progression in these diseases as seen in humans (reviewed in Seth et al., 2013). Finally, zebrafish offer both technical and practical advantages over terrestrial models due to their rapid developmental life stages, ease of husbandry, full genome sequence availability (Howe et al., 2013), and vast applications via experimental designs and available strains; all of which serve to further the understanding of metabolic processes.

Given these advantages, zebrafish make for a useful model for studying both short and long-term effects on metabolic processes. However, methods for measuring metabolic rates are often complicated, time consuming and expensive, which causes stressful conditions for the organism tested. Thus, elucidating other accurate methods of metabolic markers that would be quick and non-invasive would be of great interest. Resazurin is a water-soluble, non-toxic sodium salt that has been well established as the main active ingredient in the AlamarBlue® cell viability assay (ThermoFisher Scientific, Waltham, MA). When resazurin is solubilized in water it creates a dark blue non-fluorescent coloured solution. Once the solution is added to the cells, it can permeabilize through the cell membrane where it becomes conformationally changed into resorufin under metabolic reduction. This reduction is a result of the oxidized blue resazurin solution accepting
electrons from metabolic enzymes i.e., NADH, which causes the solution to become reduced into the resorufin solution, which is pink and fluorescent (Gonzalez and Tarloff, 2001; O’Brien et al., 2000; Prabst et al., 2017; Rampersad, 2012). Therefore, higher metabolic rates can be directly measured fluorometrically via increases in fluorescent intensity of the media as it is released out of the organism into the external environment (Rampersad, 2012). In 2013, Renquist and colleagues (2013; Williams and Renquist, 2016) bridged that gap for resazurin assays by applying an in vitro method to their in vivo studies. They were the first to demonstrate that resazurin could be used for energy and metabolism in vivo studies, by showing its effectiveness in assaying the metabolism of zebrafish larvae after drug application and genetic manipulation. However, these experiments were conducted only during larval stages and as different metabolic phenotypes emerge throughout all life stages, it was of interest to investigate if this technique could be applied across all stages of zebrafish development to better optimize this assay for metabolic studies. In this work, the use of resazurin as a metabolic in vivo assay was validated in both larval and adult zebrafish.

Teneurin C-terminal associated peptides (TCAPs) are a family of bioactive peptides found on the distal end of the teneurin transmembrane protein. Although TCAP-1 has been well-established to have roles in stress-related behaviours, it has recently been demonstrated to have metabolic actions in rodents (Al Chawaf et al., 2007b; Hogg et al., 2018; Tan et al., 2009, 2011). However, the metabolic actions of TCAP-1 have not been well described in other species, thus the work in this chapter aims at characterizing the roles of rainbow trout TCAP-3 (rtTCAP-3) in zebrafish, as it is an ideal metabolic model. Utilization of the resazurin metabolic assay and the Loligo metabolic respirometry chambers allowed for measurement of metabolic activity during basal and stress conditions. These studies will provide critical insight into the conservation of the TCAP family in respect to roles in metabolic regulation.

3.3 Methods

3.3.1 Reverse transcriptase (RT)-polymerase chain reaction (PCR)

RNA was extracted from adult zebrafish skeletal muscle and brain tissue using TRIzol (Thermo Scientific, Waltham, MA, USA) following the manufacturer’s instructions. Reverse transcription
reactions contained 2 μL purified total RNA, 1 μl random primer Mix (Bio-Rad), 1 μL deoxynucleotide solution mix (New England Biolabs), and 8 μL water. Reactions were incubated in a Fisher Scientific Isotemp 125D Dry Bath Incubator for 5 mins at 16°C and then for 1 min at 4°C. Aliquots of 4 μL First Strand Buffer (Invitrogen), 2 μl 0.1 M DTT (Invitrogen), and 1 μL SuperScript II Reverse Transcriptase (Invitrogen) were added to the reaction mix. The reactions were incubated for 10 mins at 25°C, 50 mins at 42°C, 15 mins at 70°C, and then held at 4°C. The PCR reaction mix included 5 μL cDNA, 2 μL Forward Primer and 2 μL Reverse Primer (Invitrogen; Table 3.1), 14.2 μL water (Sigma, Oakville, ON), 3 μL 10x Taq Buffer with KCl (Thermo Scientific), 1.8 μL MgCl₂ (Thermo Scientific), 1 μL Deoxynucleotide Solution Mix (New England Biolabs), and 0.5 μL Taq DNA Polymerase (BioShop). The reactions were incubated in an Eppendorf Mastercycler Gradient Thermal Cycler for 7 mins at 95°C; followed by 35 cycles of 60 sec at 95°C, 90 sec at 67°C, and 35 sec at 72°C; and then held at 4°C. DNA samples were then electrophoresed on a 3% agarose gel at 100 V for 1.5 hours. Gels were visualized using a Bio-Rad ChemiDoc MP System with 0.5 second exposure.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Expected Band Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCAP-3</td>
<td>AAGGTGCTGGGTACGATGG</td>
<td>GACATTGTTGACTGTGG</td>
<td>78 bp</td>
</tr>
<tr>
<td>MaxD</td>
<td>GCCGAAGAAATGAGCGACAAC</td>
<td>TTAGCTATCCCTCAGGCGAT</td>
<td>465 bp</td>
</tr>
<tr>
<td>CRF</td>
<td>CGAGACATCCGTATGCAAA</td>
<td>GATGACAGTGTGCGCTTCT</td>
<td>465 bp</td>
</tr>
</tbody>
</table>

Table 3.1. Primers used for RT-PCR analyzes. Forward and reverse primer pairs for zebrafish TCAP-3, MaxD (muscle tissue control) and corticotropin-releasing factor (CRF; brain tissue control).

3.3.2 Ethical procedures

All in vivo experimentation involving larval and adult zebrafish was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and is consistent with the guidelines established by the Office of Laboratory Animal Welfare, National Institutes of Health of the U.S. Department of Health and Human Services.
3.3.3 Animals

Wild-type adult (>1 year of age) AB strain zebrafish (Danio rerio) were obtained from the Aquatic Animal Research Core (AARC) at the University of Alabama at Birmingham (UAB). Fish were maintained in a recirculating aquatic system (dechlorinated city water) at 25°C under a 14-hr light-10-hr dark photoperiod. Fish were fed once daily ad libitum with otohime (Pentair Aquatic Eco-System, Inc., Apopka, FL). Wild-type AB strain adult zebrafish were bred following the procedures of Westerfield and colleagues (1995). Once the eggs were collected, they were pipetted into a 10-cm petri dish with a stocking density of 100 -150 embryos. For the following days 1-5, embryos were maintained in a 28°C housing chamber with a 14-hr light-10-hr dark photoperiod. Any embryos that had died or were unfertilized were removed with a disposable pipette and water changes were performed daily with 4 mL of fresh tank water; ensuring not to disturb or pipette out the healthy embryos. Embryos were allowed to develop until 5 days post-fertilization for all larvae experiments. Day 5-larvae developmental stage was determined according to Kimmel and colleagues (Kimmel et al., 1995; Parichy et al., 2009).

3.3.4 Resazurin Stock Solution Preparation

A resazurin stock solution was prepared by mixing 0.5 g of resazurin sodium salt (Sigma-Aldrich Corp., St. Louis, MO) with 10 mL of distilled water and 10 µL of dimethyl sulfoxide (0.1% DMSO, Sigma-Aldrich Corp., St. Louis, MO) bringing the stock solution to a total concentration of 50 mg/mL.

3.3.5 Resazurin Dilution Preparation

1L of tank water was collected from the aquatic system and acclimated to room temperature (RT; 23°C) in the experimental room. Tank water was prepared by running municipal water first through an AquaFX RO filter, then through a micro UV, charcoal, and mechanical sterile filter (Pentair Aquatic Eco-System, Inc., Apopka, FL). The filtered H2O was then brought to a conductivity range of 800-1200 µS with instant ocean sea salt (Instant Ocean, Cincinnati, OH) and had a pH of 7.1-7.5. Resazurin stock solution was diluted with tank water according to Table 3.2.
<table>
<thead>
<tr>
<th>Working Stock</th>
<th>Volume of Resazurin Solution (mL)</th>
<th>Volume of Tank Water (mL)</th>
<th>Total Volume of Solution (mL)</th>
<th>Resazurin Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mL Stock</td>
<td>1 mL Stock (50mg/mL)</td>
<td>49 mL</td>
<td>50 mL</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>Dilution 1</td>
<td>5 mL (Working Stock)</td>
<td>45 mL</td>
<td>50 mL</td>
<td>0.1 mg/mL</td>
</tr>
<tr>
<td>Dilution 2</td>
<td>1 mL (Working Stock)</td>
<td>49 mL</td>
<td>50 mL</td>
<td>0.02 mg/mL</td>
</tr>
<tr>
<td>Dilution 3</td>
<td>0.5 mL (Working Stock)</td>
<td>49.5 mL</td>
<td>50 mL</td>
<td>0.01 mg/mL</td>
</tr>
<tr>
<td>Dilution 4</td>
<td>0.1 mL (Working Stock)</td>
<td>49.9 mL</td>
<td>50 mL</td>
<td>0.002 mg/mL</td>
</tr>
<tr>
<td>Dilution 5</td>
<td>0.05 mL (Working Stock)</td>
<td>49.95 mL</td>
<td>50 mL</td>
<td>0.001 mg/mL</td>
</tr>
</tbody>
</table>

Table 3.2. Preparation of resazurin sodium salt for optimization curve. Resazurin stock solution was diluted and prepared for use in optimization curve. This table shows the dilution steps to obtain the concentrations required for the curve.

3.3.6 Larvae and Diluent Assay Preparation

3.3.6.1 Diluent Preparation

To investigate if different diluents would have different effects on resazurin sensitivity, the resazurin stock was diluted to a final concentration of 0.02 mg/mL using either tank water, autoclaved tank water, deionized water, or autoclaved deionized water. 1 L of tank water was obtained from the aquatic system and acclimated to RT in the experimental room. 500 mL of the acclimated tank water was aliquoted into a 1 L autoclavable glass container and placed into the STERIS AMSCO Lab-205 autoclave (STERIS Corp., Mentor, OH) for 1 hr. Deionized water was obtained from the Millipore 30 L Storage Tank (MilliporeSigma, Burlington, MA) that was prepared by running municipal water through the Milli-Q Direct 8 Water Purification System (MilliporeSigma, Burlington, MA). Similar to tank water preparations, the 1 L of deionized water was acclimated to RT and 500 mL were aliquoted into a new 1 L autoclavable glass container,
followed by the autoclave protocol. Water samples were prepared as outlined in Table 1-Dilution 2. 2 mL of the resazurin diluent solutions (0.02 mg/mL) were pipetted into one of the wells of the 35 mm 6-well plate. Then, the 6-well plate was covered with the lid and the edges were taped with masking tape.

3.3.6.2 Larvae Preparation
To analyze resazurin effectiveness in larval fish, 100 5 days post-fertilization (dpf) larvae were combined into a single 100 mL sterile beaker with 20 mL of fresh tank water that had been acclimated to RT (23°C) in the experimental room. The larvae were then rinsed 2-3 times with a disposable transfer pipette, ensuring that for every 10 mL of tank water removed, it was replaced with another 10 mL of fresh tank water. After the final rinse, 10 mL of the tank water was removed from the beaker leaving a final volume of 10 mL. With the same disposable transfer pipette either 10, 20, or 40 larvae were transferred through gentle suctions from the 100 mL beaker and placed into one of the wells of the 35 mm 6-well plate (refer to Figure 3.1A). One of the wells contained no larvae, serving as the “blank.” When 5 of the 6 wells had the same desired number of larvae per well (i.e., 10, 20, or 40), any extra tank water in the wells was aspirated out, without touching or aspirating the larvae. Following the aspiration, 2 mL of the Dilution 2: working stock resazurin (0.02 mg/mL) were added to all 6 wells. Then, the 6-well plate was covered with its lid and the edges were taped with masking tape. After the last plate was loaded with the larvae in the resazurin solution, larvae were fasted, as adding food to the solution could compromise the assay. In addition, the larvae were kept in a 14-hr light-10-hr dark photoperiod, which limited any circadian effects and were either placed into a chilled incubator (16°C) or kept at RT (23°C), to test the effects temperature had on metabolism. Moreover, if an experimental design had more than 6-time points, the amount of resazurin solution (0.02 mg/mL) per well was increased by 1 mL to compensate for the additional time points in the experimental design.

3.3.7 Adult Assay Preparation
Prior to the assay, 1 L of tank water had been collected and acclimated to RT (23°C) in the experimental room. The 1 L of tank water was used to make the working stock of resazurin at the Dilution 2 concentration (0.02 mg/mL) in Table 1. Next, 25 mL of the resazurin solution were
pipetted into a 50-mL conical tube. Adult zebrafish were collected with a small net and individually weighed (grams) prior to their placements into the 50-mL conical tube filled with the 25 mL of resazurin solution (0.02 mg/mL). For the metabolic assays, either 1, 2, or 3 fish were placed into one 50-mL conical tube filled with the 25 mL of resazurin solution (0.02 mg/mL; Figure 3.1B). One of the 50-mL conical tubes with the 25 mL resazurin solution (0.02 mg/mL) contained no adult zebrafish, serving as the “blank”. The lids of the 50-mL conical tubes were then placed back on the tubes, but not tightened to allow for oxygen flow. The conical tubes were then positioned at a 45° angle, which reduced the stress on the fish and spillage of the resazurin solution. Once the fish were in the resazurin solution, they were fasted, as adding food to the solution could compromise the assay. In addition, the fish were kept in a 14-hr light-10-hr dark photoperiod, which limited any circadian effects and were either placed into a chilled incubator (16°C) or kept at RT (23°C), to test the effects temperature had on metabolism. If an assay was designed to have more than 6-time points, the amount of resazurin solution (0.02 mg/mL) per conical tube was increased by 2.5-3 mL to compensate for the additional time points in the experimental design.
3.3.8 Larvae and Diluent Assay Sample Collection and Preparation

After the larval fish were placed in their appropriate wells (Figure 3.1A) and the resazurin diluents, the first samples were collected immediately, to represent the time point 0 (T₀). The resazurin diluents (0.02 mg/mL) and larval solutions were collected by pipetting 180 µL of the solution out of the wells with the larvae or diluent, making sure that the larvae in the wells were not disturbed or pipetted out of the well. The 180 µL sample was transferred into a new 1.5 mL eppendorf tube and sealed with parafilm. Samples were collected at time points 0, 3, 6, 12, 24, and 48 hours post initial T₀ collection (Figure 3.1C). Once all the samples for that time point had been collected, they were placed in a cool, dark environment i.e., a refrigerator at 4°C. The sample collection method
was repeated for all the designed time points and continued until the end of the experiment. 2 mL of resazurin solution (0.02 mg/mL) per well worked best with the 5-time points at 0, 3, 6, 12, 24, or 48 hours of sample collections. For the larvae assay, samples from the “blank” wells were collected in conjunction with the samples collected at the designed time points. If there were multiple plates with samples, then a “blank” was collected from each plate to avoid inter-plate variation. When all the 180 µL larvae and diluent samples were collected for their desired time points, a clear flat bottom 96-well plate was loaded with 85 µL of sample, loaded in duplicate. Once the 96-well plate(s) was loaded, the plate was then covered with its lid, wrapped in aluminum foil, protected from light, and temporarily stored (< 30 mins) in a cool place, until the analysis.

3.3.9 Adult Zebrafish Assay Sample Collection and Preparation

After the adults had been placed in their 50-mL conical tubes (Figure 3.1B), the first sample was collected and represented time point 0. The resazurin (0.02 mg/mL) and fish solution was collected by pipetting 300 µL of the solution out of the 50-mL conical tubes, making sure that the fish were not disturbed or touched in the tubes. The 300 µL sample was transferred into a new 1.5 mL Eppendorf tube and sealed with parafilm. Samples were collected for the increasing number of fish and temperature assays at time points: 0, 3, 6, 12, 24, and 48 hours post initial T0 collection (Figure 3.1C). A sample from the “blank” 50-mL conical tube was collected in conjunction with the other samples of the same time points. Once all the samples for each time point had been collected, they were placed in a cool, dark environment i.e., a refrigerator at 4°C, and temporarily stored (< 30 mins) until they were prepared for analysis.

3.3.10 rtTCAP-3 treatment in larvae and adult zebrafish

rtTCAP-3 was administered to larvae and adult zebrafish to investigate its metabolic effects. For larvae, vehicle (PBS) or rtTCAP-3 was treated to the water in the wells. For adult zebrafish, vehicle (PBS) or rtTCAP-3 (120 ng/g) was injected intraperitoneally after numbing the fish for 1 min with ice. This dose of rtTCAP-3 was selected based on rodent studies that demonstrated this dose was effective in regulating glucose (Hogg et al., 2018).
3.3.11 Assay Analysis

After the last sample was pipetted into the 96-well plate and wrapped with aluminum foil, additional plate(s) were removed from their temporary storage. A fluorescent plate reader was required to analyze the samples (BioTek Synergy 2, BioTek Instruments Inc., Winooski, VT). Using the BioTek Gen 5 Microplate Reader and Imager Software (BioTek Synergy 2, BioTek Instruments Inc., Winooski, VT), the 96-well plates were read with an excitation wavelength at 530 nm, followed by an emission wavelength at 590 nm to read the resorufin fluorescence intensity (FI).

Total resorufin FI was calculated by taking the average total resorufin FI values of the wells for the samples of interest at a specific time point and subtracting them from the averaged total resorufin FI of the blank wells at the same time point and this was reported as arbitrary fluorescence units (AFU). For adult zebrafish, the total resorufin values were normalized to the weight of the individual adult zebrafish, by dividing the average total resorufin FI by the weight of the fish in grams and was reported as arbitrary fluorescence units per grams (AFU/g). Conceptually, the samples at time point 0 and the blank were equivalent and total resorufin FI resulted in extremely close values for the AFU. Thus, time point 0 was set to 0 for all the samples (Time point 0-blank = 0). Any differences were negligible and were attributed to plate reading variability and sample loading errors.

\[
AFU = \bar{x}_{Sample \, Tx} - \bar{x}_{Blank \, Tx}
\]

- \( Tx = \) Time point X (X = 0, 3, 6, 12, 24, and 48 hours)
- \( \bar{x}_{Sample \, Tx} = \) Average Sample of Interest of total Resorufin FI at timepoint X
- \( \bar{x}_{Blank\, Tx} = \) Average Blank of total Resorufin FI at timepoint X

For studies that analyzed the effectiveness of resazurin to measure metabolic activity as number of organisms increased, resazurin response kinetics were calculated as described by Renquist et al. 2013. Briefly, the control number of organisms (for larvae = 10, adults = 1) was set to 1 and relative change in resorufin fluorescence as number of organisms increased was measured. Linear regression line was analyzed and denoted by \( R^2 \) value (closer to \( R^2 = 1 \) indicates linearity). \( R^2 \)
suggests the strength of the relationship between the expected model to the experimental model and was used in these experiments as a qualitative characteristic to visualize resorufin response kinetics.

3.3.12 Loligo Metabolic Respirometry Chamber

Loligo Metabolic Respirometers were obtained from the Aquatic Animal Research Core (AARC) at the University of Alabama at Birmingham (UAB) core. The chambers were setup following the instructions provided by Loligo Systems: AutoResp (Loligo Systems, Viborg, Denmark) Adult zebrafish were injected with either vehicle (PBS) or rtTCAP-3 (120 ng/g) and tested in Loligo metabolic respirometer chamber 24 hrs after injection. The protocol began with a 10-min acclimation period, followed by a 7-min basal period and 5-min stress period. The water was continuously recirculated and oxygen sensors recorded levels continuously. In between each period was a flush of fresh oxygenated water. The stress period began with a longer and more forceful flush, which forced the fish to swim faster against the current. Whole animal metabolic rates were calculated by measuring oxygen consumption (VO₂). The equation to calculate VO₂ was provided by Loligo Systems.

\[ VO_2 = \left( [O_2]_{t_0} - [O_2]_{t_1} \right) \cdot \frac{V}{t} \cdot \frac{1}{BW} \]

- VO₂ = oxygen consumption rate (mg O₂/ kg/hour).
- \([O_2]_{t_0}\) = oxygen concentration at time \(t_0\) (mg O₂/ liter)
- \([O_2]_{t_1}\) = oxygen concentration at time \(t_1\) (mg O₂/ liter)
- \(V\) = respirometer volume minus volume of the experimental animal (liter)
- \(t = t_1 - t_0\) (hour)
- \(BW\) = body weight of the animal

3.3.13 2-deoxyglucose (2-DG) uptake assay in primary rainbow trout myogenic precursor cells (rtMPCs)

rtMPCs extracted from the epaxial muscle were cultured and differentiated up until day 5 as described in Froelich et al., 2014. 2-deoxyglucose (2-DG) uptake was measured by the protocol as described in Yamamoto et al., 2006. Briefly, this assay uses resazurin sodium salt to measure the
oxidation of 2-DG into 2-deoxyglucose-6-phosphate (2-DG-6-P) by glucose-6-phosphate dehydrogenase (G6PDH). Thus, as the 2-DG-6-P accumulates in the cells, this oxidation produces NADPH, which is reduced to NADP⁺ by the resazurin solution. As a result, the reduction results in the fluorescent product, resorufin, that could be measured and quantified to directly measure the glucose uptake (Yamamoto et al., 2006). On differentiation day 5, when the myocytes had fully differentiated into myotubes, cells were washed with HEPES buffered saline, and serum starved with minimal media for 3 hours. After the 3 hours, cells were washed again with HEPES buffered saline and treated with 2-DG and either vehicle (PBS), rtTCAP-3 (100 nM), or insulin (10 nM) for 30 mins. Following another wash, the HEPES buffered saline was removed and the cells were collected in 500 µL 0.1N NaOH. Once collected, the cells underwent a freeze-thaw cycle in -80°C freezer for approximately 20 mins and then subsequently thawed at RT. After thawing, the cells were placed in a heat block at 85°C for 40 mins. The samples were then prepped for the assay by the addition of 100 µL of 0.1N HCl and 100 µL TEA buffer to each tube. The cells were mixed with the components and 50 µL of each sample was added to a 96-well plate in triplicate. Each well containing a sample had 150 µL of activated assay buffer (0.2% BSA, 0.8 mM MgSO₄, 0.1 mM NADP, 0.5 mM ATP, 0.2 U/mL diaphorase, 5 mM resazurin, 2 U/mL hexokinase, and 15 U/mL G6PDH) added to it. Finally, the 96-well plates were covered to be protected from the light by aluminum foil wrap, and subsequently analyzed. Samples were analyzed by a fluorescent plate reader (BioTek Gen 5 Microplate Reader and Imager Software) to read the resorufin fluorescence intensity (FI).

3.3.14 Statistical Analyzes

All data on graphs are represented as mean +/- SEM. All data were analyzed by t-test or one- or two-way ANOVA, unless otherwise stated. Tukey’s post-hoc test and Sidak’s post-hoc test was used to determine significance in one-way and two-way ANOVA analyzes, respectively. An a priori hypothesis states that the asterisks represent the following: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. GraphPad Prism 7 was used to analyze each statistical test and for all graphing.
3.4 Results

3.4.1 TCAP-3 is expressed in zebrafish skeletal muscle and brain

To determine if TCAP-3 is endogenously expressed in zebrafish, RNA was extracted from adult zebrafish skeletal muscle and brain tissues. The expression of teneurins in these tissues have been previously reported (Tucker et al., 2006), thus it suggested that TCAP-3 may be expressed as well. RT-PCR revealed TCAP-3 expression in both skeletal muscle and brain tissues (Figure 3.2). Expression of tissue-specific genes, MaxD and CRF, was used as a positive control for muscle and brain tissue, respectively.

![Figure 3.2. Zebrafish skeletal muscle and brain expresses TCAP-3.](image)

RNA from muscle and brain tissues were extracted and used for RT-PCR analyzes for TCAP-3. TCAP-3 is expressed in both the muscle and brain. Tissue-specific positive controls, MaxD and corticotropin-releasing factor (CRF), were used for muscle and brain, respectively. All band sizes were consistent with expected band sizes. (TCAP-3, 78 bp; MaxD, 465 bp; CRF, 465 bp).
3.4.2 Optimization of resazurin concentration in solution for in vivo experiments while maintaining sensitivity and non-toxicity

The first step in establishing the resazurin assay was to determine the optimal concentration of resazurin to ensure maximal sensitivity while also ensuring it is not toxic to the fish was determined. Individual adult zebrafish were placed in 50-mL conical tubes at 23°C containing the serial concentrations (refer to Table 3.1) and samples were collected and analyzed 24 hours later. This assay showed that 0.01 mg/mL concentration of resazurin was the most sensitive concentration (Figure 3.3A). This experiment was repeated at 16°C to determine if this range was still the most effective even under low temperature conditions (Figure 3.3B). This showed that although 0.01 mg/mL was the most sensitive, it also was the most variable. Lastly, we next ascertained if a 0.02mg/mL dose was toxic to the fish. Adult zebrafish were placed in 50-mL conical tubes containing the resazurin solution and measured for 5 days (Figure 3.3C). After 5 days, fish were still lively and showed no obvious signs of distress (visual observations). Thus, a resazurin concentration range from 0.01-0.02 mg/mL should be used for in vivo studies, however, for less variable data the concentration of 0.02 mg/mL is recommended.

Figure 3.3. Optimization of resazurin concentration for sensitivity and non-toxicity. Individual adult zebrafish were placed in conical tubes containing serial dilutions of resazurin stock solution to determine optimal resazurin concentration for sensitivity for in vivo studies at 23°C (A). Resazurin optimization experiment was repeated with individual adult zebrafish at 16°C (B). This dose is non-toxic to fish for at least 5 days (C). (Mean ± SEM; n=3-5).
3.4.3 *Tank water can be used as an appropriate diluent and blank solution for resazurin assay*

As described in Method 3.3.5 resazurin sodium salt was diluted using system tank water. The next aim was to investigate the differences in types of water as diluents, as well as a “blank” control. Samples of deionized water (diH₂O), autoclaved diH₂O, tank water, and autoclaved tank water were added to resazurin solution and measured over 48 hours (Figure 3.4A). diH₂O and tank water had a significantly different baseline resorufin FI (p<0.0001; Figure 3.4A). Second, sterilization of the diH₂O water by autoclaving had no effect on AFU (Figure 3.4A). However, autoclaving the tank water resulted in a significant decrease at 48 hours (p=0.0003) in total resorufin FI, when compared to non-sterile tank water (Figure 3.4A). More importantly, although the different types of water have some moderate variances between them as control blanks, these are insignificant changes when compared to changes induced by metabolic activity due to organism, such as in this case, larvae (Figure 3.4B). Only the wells containing larvae had significantly higher total AFU (p<0.0001; Figure 3.4B).
Figure 3.4. Tank water is an appropriate control for the ‘blank’ wells in the assay. Samples of deionized water (diH₂O), autoclaved diH₂O, tank water and autoclaved tank water were added to resazurin solution and measured over 48 hours (A). This assay demonstrated that diH₂O and tank water have inherently different baseline fluorescence (p<0.0001). There was no significant difference between diH₂O and autoclaved diH₂O, although there was a significant difference between tank water and autoclaved tank water at 48 hours (p=0.0003). Results of the control blank assay compared to wells with larvae, which had significantly higher AFU than blank wells (p<0.0001; B). (Mean ± SEM; n=3-4; ***p<0.001, ****<0.0001; two-way ANOVA).

3.4.4 Resazurin assay can measure increases in metabolic activity in both larval and adult zebrafish due to increasing number of organisms.

To demonstrate that the resazurin assay can measure increases in metabolic activity, the total resoruvin FI was measured with increasing numbers of both larvae and adult zebrafish. This is based on the principle that as number of organisms increase, there should be a resulting increase resazurin conversion to fluorescent resoruvin.

For the larval study, zebrafish larvae (5 days post-fertilization, dpf) were pooled in wells of a 6-well plate that contained resazurin solution. Wells contained either 10, 20 or 40 larvae, and solution
samples were collected from the wells over 48 hours. The first differences were seen at 24 hours between wells that contained 10 larvae compared to 40 larvae, with the latter having higher AFU (p=0.033; Figure 3.5A). Moreover, at 48 hours, wells that contained 40 larvae had significantly higher total resorufin FI compared to wells that contained 10 larvae and 20 larvae (p=0.0015 and p=0.02, respectively; Figure 3.5A). Additionally, by measuring the relative change in resorufin fluorescence of 10 larvae compared to 20 and 40 larvae, we were able to assess the larval response kinetics of resazurin. The response was shown to be linear, as defined by an R² value of 0.9998 of the slope (Figure 3.5B).

![Graph A](image1.png)

**Figure 3.5.** Increasing the number of pooled larvae zebrafish significantly increases the total resorufin fluorescence intensity (AFU) in a linear response. 6-well plate wells that contained resazurin solution housed either 10, 20 or 40 zebrafish larvae (5 dpf). Significant increases in total resorufin fluorescence intensity were seen at 24 hr (p=0.033) between 10 and 40 larvae. After 48 hr, 40 larvae had significantly higher total resorufin fluorescent intensity when compared to 10 larvae (p=0.0015) and 20 larvae (p=0.02; A). Larval response kinetics were analyzed by measuring the relative change in AFU/g of 20 and 40 larvae compared to 10 larvae, and it had an R² value of 0.9998 (B). (Mean ± SEM; n=4-5; *p<0.05, **p<0.01; two-way ANOVA).
The same assay design was performed with adult zebrafish. Conical tubes containing resazurin solution housed either 1, 2 or 3 fish and samples were collected over 24 hours. After 6 hours, the total resorufin FI of 3 fish was greater than 1 fish (p=0.04). At 12 hours, 2 fish and 3 fish had increased AFU compared to 1 fish (p=0.0006, 2 fish; p<0.0001, 3 fish). At 24 hours, all fish resorufin FI were different from each other (p<0.0001; Figure 3.6A). Resazurin response kinetics was also measured in adult zebrafish by comparing the relative change in AFU/g of 1 fish to 2 and 3 fish. The response was shown to be linear, as defined by an R^2 value of 0.9765 of the slope (Figure 3.6B).

Figure 3.6. Increasing the number of adult zebrafish significantly increases the total resorufin fluorescence intensity (AFU) in a linear response. 1, 2 or 3 adult zebrafish were added to resazurin solution in conical tubes and samples were collected over 24 hours. At 24 hours, the metabolic rates of the number of fish are all significantly different (p<0.0001), where the greater number of fish result in higher AFU (A). Adult response kinetics were analyzed by measuring the relative change in AFU of 3 and 2 fish compared to 1 fish, and it had an R^2 value of 0.9765 (B). (Mean ± SEM; n=4-5; *p<0.05, ***p<0.001, ****p<0.0001; two-way ANOVA).
3.4.5 Resazurin assay can measure decreases in metabolic activity in both larval and adult zebrafish due to low temperature exposure.

It is well established that low temperatures decrease metabolic rates in terrestrial and aquatic animals. To demonstrate that the resazurin assay can measure decreases in metabolism, larval and adult zebrafish were placed in both 23°C or 16-17°C conditions and measured total resazurin conversion to fluorescent resorufin.

Zebrafish larvae were pooled in 6-well plate well (10 larvae/well) containing resazurin solution and were exposed to either 23°C or 17°C for 48 hours. Larvae that were exposed to the low temperature had significantly lower AFU at 48 hours (p<0.0001; Figure 3.7).

**Figure 3.7.** Pooled larvae zebrafish had significantly lowered metabolism after exposure to low temperature for 48 hours. Larvae zebrafish that were pooled in 6-well plate contained in resazurin solution were exposed to either 23°C or 17°C temperature for 48 hours. Larvae had significantly (p<0.0001) lower metabolism at 48 hours as measured by total resorufin fluorescent intensity (AFU). (Mean ± SEM; n=3-4; ****p<0.0001; two-way ANOVA).
This experiment was repeated with zebrafish adults. Adults were placed in 50-mL conical tubes containing resazurin solution and exposed to the same temperature conditions as the larvae, either 23°C or 16°C for 24 hours. At 12 hours, animals exposed to the low temperatures exhibited lower total resorufin FI, which is continuously suppressed through 24 hours later (p=0.0002, 12 hours; p<0.0001, 24 hours; Figure 3.8).

![Graph showing resorufin AFU/g over time for 23°C and 16°C temperatures.](Image)

**Figure 3.8.** Adult zebrafish had significantly lowered metabolism after exposure to low temperature for 24 hours. Individual adult zebrafish contained in resazurin solution were exposed to either 23°C or 16°C temperature for 24 hours. At 12 hours, the animals had significantly lower metabolic rate (p=0.0002), which was maintained until 24 hours (p<0.0001), as measured by total resorufin fluorescent intensity (AFU). (Mean ± SEM; n=5-8; ***p<0.001; ****p<0.0001; two-way ANOVA).
3.4.6 rtTCAP-3 increases metabolic activity in larvae and adult zebrafish.

Zebrafish larvae were pooled in 6-well plate well (10 larvae/well) containing resazurin solution and were treated with either vehicle (PBS) or various doses of rtTCAP-3 (0.5 µM, 1.0 µM, 5.0 µM, 10.0 µM) for 48 hours. After 24 hours, 5.0 µM and 10 µM rtTCAP-3 significantly increased total resorufin FI compared to all other treatments (p<0.0001), which was maintained until the end of the experiment (Figure 3.9). After 48 hours, 10 µM rtTCAP-3 had significantly higher total resorufin FI compared to 5.0 µM treatment (p<0.05; Figure 3.9). These results indicate that rtTCAP-3 significantly enhances metabolic activity in larvae zebrafish as seen by increases in total resorufin FI.

Figure 3.9. rtTCAP-3 treatment enhances metabolic activity in larvae. Pooled larvae were treated with either vehicle (PBS) or various doses of rtTCAP-3 (0.5, 1, 5, 10 µM). Samples were collected over 48 hours. rtTCAP-3 at 5 µM and 10 µM significantly increased AFU at 24 and 48 hours compared to all other treatments. At 48 hours, 10 µM had increased AFU compared to 5 µM. (Mean ± SEM; n=4; *p<0.05; ****p<0.0001; two-way ANOVA).
Adult male zebrafish were intraperitoneally injected with either vehicle (PBS) or rtTCAP-3 (120 ng/g) and placed in 50 mL conical tubes containing resazurin solution. After 48 hours, rtTCAP-3 treated adults had significantly higher total resorufin FI (p<0.0001; Figure 3.10), thus demonstrating that rtTCAP-3 increases metabolic activity in adults. Taken together, these resazurin assay data indicate that rtTCAP-3 enhances organismal metabolism both in larvae and adult zebrafish.

![Figure 3.10. rtTCAP-3 enhances metabolic activity in adult zebrafish.](image)

**Figure 3.10. rtTCAP-3 enhances metabolic activity in adult zebrafish.** Adult zebrafish were intraperitoneally injected with either vehicle (PBS) or rtTCAP-3 (120 ng/g) and placed in 50 mL conical tubes containing resazurin solution. After 48 hours, rtTCAP-3 significantly increased total resorufin FI. (Mean ± SEM; n=4; ****p<0.0001; two-way ANOVA).
3.4.7 *rtTCAP-3 increases metabolic activity in larvae under low temperature stress.*

Zebrafish larvae were pooled (10 larvae/well) and treated with either vehicle (PBS) or rtTCAP-3 (5 µM). They were subsequently placed in either 23°C or 17°C conditions. These low temperature conditions cause a decrease in metabolic activity in vehicle-treated larvae after 48 hours (p<0.001; Figure 3.11A). However, larvae treated with rtTCAP-3 did not experience a decrease in metabolic activity, thus demonstrating the potent effect of rtTCAP-3 on metabolic regulation even under stress conditions (Figure 3.11B).

![Figure 3.11](image.png)

**Figure 3.11. rtTCAP-3 increases metabolism in larvae in low temperature stress.** Larvae were pooled (10 larvae/well) and treated with either vehicle (PBS) or rtTCAP-3 (5 µM) and placed in either 23°C or 17°C temperatures. Vehicle-treated larvae had reduced metabolic activity under low temperature stress, as seen by significant decrease in total resorufin FI (***p<0.001; A). rtTCAP-3-treated larvae maintained elevated metabolic activity under low temperature stress, as there was no significant difference between temperature conditions (B). (Mean ± SEM; n=4-5; ***p<0.001; two-way ANOVA).
3.4.8 Adult zebrafish treated with rtTCAP-3 have increased oxygen consumption under basal and stress conditions.

Adult male zebrafish were intraperitoneally injected with either vehicle (PBS) or rtTCAP-3 (120 ng/g) and placed in Loligo metabolic respirometry chambers 24 hours later. rtTCAP-3-treated zebrafish had significantly increased basal oxygen consumption rate (VO$_2$) compared to vehicle-treated zebrafish (p<0.01; Figure 3.12A, B). The zebrafish were subsequently tested in a stress protocol where the recirculating water flush rate was significantly increased within the chambers and forced the fish to swim faster against the current. rtTCAP-3-treated zebrafish had significantly increased VO$_2$ compared to vehicle-treated under stress conditions (p<0.01; Figure 3.12C, D). These data are consistent with the previous studies done with the resazurin assay, as both studies demonstrate the role of rtTCAP-3 in metabolic regulation under basal and stress conditions.
Figure 3.12. rtTCAP-3 increases oxygen consumption in adult zebrafish after 24 hours in basal and stress conditions. Adult male zebrafish were intraperitoneally injected with either vehicle (PBS) or rtTCAP-3 (120 ng/g) and tested in Loligo metabolic respirometry chambers 24 hours later. rtTCAP-3-treated zebrafish had significantly increased basal oxygen consumption rate (A, B). After basal rates were recorded, a stress protocol was initiated by a strong increase in flush force within the chambers, forcing the fish to swim faster against the current. rtTCAP-3-treated zebrafish had significantly increased oxygen consumption under stress conditions (C, D). (Mean ± SEM; n=10-11; **p<0.01; t-test).
3.4.9 *rtTCAP-3 increases glucose uptake into rainbow trout myogenic precursor cells (rtMPCs).*

Rainbow trout myogenic precursor cells (rtMPCs) were extracted from rainbow trout epaxial muscle and differentiated until day 5. rtMPCs were treated with either vehicle (PBS), rtTCAP-3 (100 nM), or insulin (10 nM) and glucose uptake was measured. After 30 minutes of treatment, rtTCAP-3 significantly increased 2-deoxyglucose (2-DG) uptake over 200% compared to vehicle (p<0.05, Figure 3.13). rtTCAP-3 and 10 nM insulin had similar responses and had no significant difference in glucose uptake (Figure 3.13).

![Figure 3.13. rtTCAP-3 increases glucose uptake into primary rainbow trout muscle cell culture.](image)

**Figure 3.13. rtTCAP-3 increases glucose uptake into primary rainbow trout muscle cell culture.** Rainbow trout myogenic precursor cells (rtMPCs) were extracted from rainbow trout epaxial muscle and differentiated until day 5. They were then treated with either vehicle (PBS), rtTCAP-3 (100 nM), or insulin (10 nM). After 30 minutes 2-deoxyglucose (2-DG) uptake was measured. rtTCAP-3 significantly increased 2-DG uptake over 200% compared to vehicle (p<0.01) but did not significantly differ from 10 nM insulin treatment. (Mean ± SEM; n=4; **p<0.01; one-way ANOVA).
3.5 Discussion

In this work, the effects of rtTCAP-3 were investigated by establishing a novel *in vivo* metabolic assay. First, it was validated that TCAP-3 is endogenously expressed in skeletal muscle. Next, resazurin was established to be effective in measuring metabolic output *in vivo* in zebrafish across all life stages. As total resorufin fluorescence intensity is proportional to the amount of NADH reduced in the organism, it gives evidence of changes in metabolism within the organism. This study builds upon previous reports from Renquist and colleagues (2013; Williams and Renquist, 2016) that demonstrate the effectiveness of resazurin as an *in vivo* zebrafish larvae metabolic detector, whereas, this work validates the use of resazurin in zebrafish adults. Moreover, Renquist and colleagues (2013) demonstrated that larvae adequately internalize resazurin by visualization of confocal image after 1 hour of exposure, indicating that changes seen in NADH are reflecting global consumption, and are able to sense changes in the external environment by reduced resorufin fluorescence intensity. This assay was then utilized to measure the metabolic effects of rtTCAP-3 treatment. rtTCAP-3 significantly increased metabolic activity under basal and stress conditions in both zebrafish larvae and adults. This is the first work to demonstrate the bioactive roles of rtTCAP-3 in metabolism.

First, optimal resazurin concentration was found to be 0.01-0.02 mg/mL for *in vivo* studies as it maximizes sensitivity as well as ensuring it is a non-toxic dose (Figure 3.3). The following studies were done at the dose of 0.02 mg/mL as it was less variable in its response and was non-toxic to the fish. For these studies, resazurin sodium salt was diluted in tank water. Thus, it was next investigated if different diluents would have different effects on resazurin sensitivity. Samples from resazurin diluted with either deionized water, autoclaved deionized water, tank water, or autoclaved tank water were compared (Figure 3.4A). Deionized water and tank water demonstrated significantly different baseline resorufin fluorescent intensities (Figure 3.4A), which may have to do with a difference in chemical composition of the tank water and its interaction with the resazurin compound itself. This indicates that although both can be used as diluents for resazurin, the blank solution must be kept consistent with whichever diluent used to properly control for difference in background fluorescence.
Although sterilization of the water via autoclaving had no effect on the fluorescence of deionized water, it did have a significant impact on tank water (Figure 3.4A). This may be because deionized water is already presumable sterile, whereas tank water has naturally occurring microbes. This could indicate that the resazurin assay is sensitive enough to detect very minor changes in metabolic output induced by these microorganisms, which would be lost upon sterilization. However, previous reports from Williams and Renquist (2016) suggest that any increased signal response from these microorganisms would be rare and by having a blank, controls for any effects microbiota have on total resorufin fluorescence. Importantly however, these differences in total resorufin fluorescence intensities between the types of water or sterility are negligible when in the presence of organisms (Figure 3.4B).

Next, it was determined that the resazurin assay could detect changes in metabolic output as the number of organisms increase within the well. This positive control test validates that resazurin is sensitive enough to detect changes in numbers of pooled larvae (Figure 3.5A) as well as in zebrafish adults (Figure 3.6A). This is consistent with reports that demonstrated resazurin was equally sensitive to measuring metabolic output increase as larvae number increase in 96-well plates as the BD Biosensor assay for oxygen consumption (Renquist et al., 2013). Second, to demonstrate that resazurin can measure decreases in metabolic output, larvae (Figure 3.7) and adults (Figure 3.8) zebrafish were exposed to low temperatures, which is a known metabolic suppressor (Vergauwen et al., 2010). As expected, the resazurin assay accurately demonstrated that organisms exposed to low temperatures had significantly lowered metabolism (Figure 3.6, Figure 3.8). The larvae results are corroborated by those presented in Renquist and colleagues (2013) which show low temperature decreases metabolic output. The results obtained from adult zebrafish are corroborated by those presented in Uliano and colleagues (2010), where they used a closed respirometry system to measure oxygen consumption rate in adult *Danio rerio* under acute temperature stress. They demonstrated that a 7°C degree decrease in experimental temperature (27°C to 20°C) was sufficient to induce a significant decrease in metabolism as measured by routine oxygen consumption rate (rM0₂). The results reported here showed a 2.5-fold decrease compared to their approximately 1.6-fold decrease, most likely due to the experimental temperature tested here was relatively lower into the zebrafish temperature tolerance range (23°C to 16°C). This demonstrates that the resazurin method of assessing low temperature stress is
corroborated by other standard methods of metabolism measurement. In addition, Renquist and colleagues (2013) showed dose-dependent increases in metabolic activity by the hormones, insulin and leptin, and that genetic manipulations affecting metabolism could also be measured by resazurin. As the results in adults have been consistent with the experiments performed in larvae, based on these reports we hypothesize that the same experimental concepts of hormonal and genetic manipulation can be performed in adult zebrafish. These works validate the use of the resazurin assay in adult zebrafish as a metabolic assay. By utilizing the natural properties of the resazurin compound conformational change into resorufin upon reduction, the relative changes in total resorufin fluorescent intensities provide insight into the metabolic output of the organisms.

Once the resazurin assay was established and validated, it was used to measure the potential roles of rtTCAP-3 in metabolism in zebrafish. rtTCAP-3 enhanced metabolic activity in larvae (Figure 3.9) and male adults (Figure 3.10) after 48 hours as seen by significant increases in resorufin FI, indicating a clear role in zebrafish metabolism. Further, rtTCAP-3-treated larvae maintained elevated metabolic rates while under metabolic low temperature stress, whereas vehicle-treated larvae had significantly lowered metabolism (Figure 3.11). Adult zebrafish were also subjected to metabolic stress using the Loligo metabolic respirometry chambers. After 24 hours of treatment, rtTCAP-3-treated animals had increased oxygen consumption rates (V02) in basal conditions compared to vehicle-treated animals (Figure 3.12). Metabolic stress was induced by forceful flushing of the recirculating chamber water, which thereby demanded the fish to swim faster against the current. rtTCAP-3-treated animals had significantly higher V02 under stress conditions compared to vehicle-treated. These studies are the first to demonstrate the potent actions of rtTCAP-3 in metabolism under stress conditions.

Lastly, as animals treated with rtTCAP-3 demonstrated enhanced metabolic activity, the roles of rtTCAP-3 on glucose regulation was investigated in vitro. rtMPCs were utilized to measure glucose uptake by 2-DG assay. rtTCAP-3 treatment in rtMPCs increased 2-DG uptake by over 200% after 30 minutes of treatment compared to vehicle treatment (Figure 3.13). Moreover, insulin treatment similarly caused approximately 230% increase in 2-DG uptake, consistent with previous reports that demonstrate that this time-point and dose is most effective for insulin response in
rtMPCs (Castillo et al., 2004; Diaz et al., 2007). This study shows that rtTCAP-3 enhances glucose uptake into the muscle, consistent with its roles in increasing metabolic activity. It further demonstrates that rtTCAP-3 promotes glucose uptake to a similar degree as insulin treatment. Although this study was performed in cells isolated from rainbow trout, these studies are still corroborative of the results seen in zebrafish as these sister-species have similar metabolic processes and glucose regulatory systems (Polakof et al., 2012). Zebrafish and rainbow trout do differ in their overall growth paradigm however, where they exhibit determinate and indeterminate growth, respectively, and thus respond differentially to growth hormone treatment (Biga and Goetz, 2006; Biga and Meyer, 2009). Despite this, the glucose regulatory systems of the cells should not be significantly different. Thus, as rtMPCs were previously established to have significant glucose uptake response to insulin treatment, they were used for this study (Castillo et al., 2004; Diaz et al., 2007).

rtTCAP-3 was originally discovered in the search for CRF paralogues by screening a rainbow trout cDNA library with a hamster urocortin probe (Qian et al., 2004). rtTCAP-3 treatment to murine neuronal Gn11 cells resulted in a significant increase in MTT activity after 24 hours, which is indicative of cell proliferation. This is consistent with increased metabolic activity that results in greater proliferative activity of the cells, supporting the roles observed in zebrafish. Further, it demonstrates that rtTCAP-3 treatment was effective when treated to a different species, in this case, in murine cells. This is likely due to the high level of conservation of the TCAP family, where mouse-TCAP-3 and rtTCAP-3 share about 88% protein sequence similarity (discussed in section 1.3.1; Qian et al, 2004; Wang et al., 2005).

In addition, rtTCAP-3 treatment to murine neuronal Gn11 cells resulted in a significant increase in cyclic AMP (cAMP) after 15 minutes, which suggested rtTCAP-3 was a ligand of a G protein-coupled receptor (GPCR). At the time, the putative receptor had not yet identified, however now it has been established that the receptor is adhesion G protein-coupled receptor class L/latrophilin (ADGRL; discussed in section 1.5; Boucard et al., 2014; Husic et al., in prep; Silva et al., 2011; Woelfle et al., 2015). In most vertebrates, there are three isoforms of ADGRL, however, due to a lineage-specific gene duplication, zebrafish have six isoforms of ADGRL (Lange et al., 2012).
Although the specific differences among the zebrafish ADGRL isoforms have not yet been fully determined, there is recent evidence that shows functional similarity to its murine orthologues. Knockout of *Lphn3.1* in zebrafish larvae results in an attention-deficit/hyperactivity disorder (ADHD) phenotype, consistent with phenotypes observed in *ADGRL3* knockout mice (Lange et al., 2012; Wallis et al., 2013). Thus, it can be postulated that rtTCAP-3 transduces its effects via ADGRL in zebrafish in a manner similar to other vertebrates.

Taken together, this work provides the first insight into the roles of rtTCAP-3 in metabolism and glucose regulation in zebrafish. Moreover, rtTCAP-3 upregulates metabolic activity under stress conditions as well, demonstrated in both larval and adult studies. Future studies will aim at elucidating the underlying mechanism of action of rtTCAP-3 via ADGRL.
Chapter 4

A portion of this chapter is being currently prepared as a manuscript: D’Aquila, A.L., Reid, M.L., Hogg, D.W., Dodsworth, T., Husic, M., Chen., Y., Locke, M., Slee, A., Biga, P.R., Lovejoy, D.A. The C-terminal domain of teneurin as a novel regulator of glucose metabolism and muscle function. [AD contribution: experimental design, performed majority of experiments (9/10) and analyzed all experiments (10/10 experiments), wrote majority of manuscript.]

4.1 Abstract

TCAP-1, a bioactive peptide found on the C-terminal domain of the teneurin-1 protein, is a novel glucose regulator. TCAP-1 actions have been well-established in the brain, where it upregulates neuronal glucose metabolism and lowers stress-related behaviours in vivo. However, the roles of TCAP-1 have not been characterized in other tissues. As skeletal muscle is a major target of glucose uptake, TCAP-1 effects with respect to energy metabolism was investigated in this tissue. Moreover, as muscle metabolism and function are intrinsically linked, the roles of TCAP-1 in muscle contractile kinetics were measured. Short-term application of TCAP-1 upregulates glucose uptake into skeletal muscle and increases NADH turnover rate, visualized in functional positron emission tomography (fPET) and histological analyzes, respectively. Muscle function of the tibialis anterior was enhanced after TCAP-1 treatment, as seen by increased peak twitch force, slower contraction velocity and faster half-relaxation (1/2RT) rate. Moreover, TCAP-1-treated rats maintained efficient contractile function during fatigue stress, which was lost in vehicle-treated rats. Long-term TCAP-1 treatment lowered serum glucose over 12 weeks, and had enhanced muscle function, consistent with the findings with short-term application. In addition, long-term TCAP-1-treated animals were tested in the stress paradigm open field test, where they exhibited less stress-related behaviours compared to vehicle-treated animals, consistent with previous work. In conclusion, the work in this chapter demonstrates the potent role of TCAP-1 in glucose regulation and muscle function in rodents with both short-term and long-term dose regimens.
4.2 Introduction

Muscle contractions depend on the chemical energy provided by the cell to induced excitation-contraction coupling (reviewed in section 1.7.2). Briefly, a skeletal muscle contraction is initiated by depolarization of the sarcolemma by an action potential signalled from a motoneuron. This signal results in the conformational change of the dihydropyridine receptor (DHPR), which activates the ryanodine receptor (RyR) on the sarcoplasmic reticulum (SR). This elicits a large surge in calcium release out of the SR. Calcium subsequently binds to troponin C, and along with ATP hydrolysis, allows for myosin and actin to form cross-bridges such that they may slide past each other. This process results in the contraction of the sarcomere and leads to force production of the muscle. To relax the muscle, ATP binds to the myosin heads to detach the cross-bridges, calcium dissociates from troponin C and is actively transported into the SR via sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA) pumps. This prepares the muscle for the next contraction. Different contractile characteristics can be measured, such as peak twitch force, contraction velocity, and half-relaxation rate (1/2RT). Peak twitch force is defined as the maximal force that is produced during a muscle twitch contraction. Contraction velocity is the speed at which the contraction occurs. 1/2RT is the rate of which the muscle is relaxed to half the force elicited, which predominantly depends on calcium cycling to relax the muscle.

Many factors can influence these parameters, such as fibre-type and metabolic signalling. Fibre-types are categorized by contractile kinetics and ATP-derived source. Type I fibres are small, slow-twitch fibres that depend highly on oxidative phosphorylation for ATP production. These fibres are more resistant to fatigue than type II fibres. Type II fibres are large, fast-twitch fibres that are sub-divided into type IIa/x fibres and type IIb fibres. Type IIb fibres are highly glycolytic, whereas type IIa/x fibres are often referred to as intermediary fibres as they have the capacity to be influenced to be either more glycolytic or oxidative in nature. Thus, different compositions of fibre types can impact contractile kinetics of the muscle. Prolonged stimulation, such as exercise, can also affect these contractile parameters as it impairs key metabolic signalling, such as calcium and ATP. Prolonged stimulation leads to aberrant cycling of calcium between the SR and sarcomere, and heavily diminishes ATP, with the myosin ATPases and SERCAs consuming large fractions of ATP rapidly (Bellinger et al., 2008). Ultimately, this results in high cytoplasmic calcium which
lowers the ability of the muscle to produce contractile force and reduces contraction velocity and 1/2RT. Thus, methods of stabilizing calcium cycling and ATP production during muscle stimulation could potentially rescue fatigue-induced decay of muscle function.

Teneurin C-terminal associated peptide (TCAP)-1, is a novel bioactive peptide that has a role in neuronal glucose regulation, where it increases glucose uptake into the brain with a concomitant decrease in serum glucose (Hogg et al., 2018). As skeletal muscle is a major target of glucose uptake, elucidating the roles of TCAP-1 in glucose regulation in the muscle was of interest. Moreover, as muscle metabolism and muscle function are intrinsically linked by the demand of ATP during muscle contraction, the effects of TCAP-1 on contractile kinetics of the tibialis anterior muscle was investigated. The tibialis anterior muscle is comprised of 95% type II fibres, which are easily fatigable muscle fibres, and thus is an ideal muscle to study the effects of muscle stress, such as fatigue.

In addition, two different TCAP-1 dose regimens were utilized in these studies. The first regimen was a short-term treatment spanning the course of a week, which was consistent with previous studies that validated this dose regimen with respect to TCAP-1 effects on neuronal glucose regulation (Hogg et al., 2018). The second dose regimen was a long-term TCAP-1 treatment over the course of 3 months. This regimen was developed to investigate any possible effects of TCAP-1 on muscle adaptation. Lastly, these long-term TCAP-1-treated animals were tested in stress paradigms in order to corroborate TCAP-1 efficacy with previously reported TCAP-1 actions on stress-related behaviour (Al Chawaf et al., 2007b; Tan et al., 2011).

4.3 Methods

4.3.1 Animals

All experimental protocols involving animals were approved by the University Animal Care Committee (UACC). Male adult Sprague-Dawley rats (~350 g) were used for the short-term and long-term muscle function studies. Male adult Wistar rats (~250 g) were used for the functional
positron emission tomography tests. Animals were weighed once per week and monitored for any signs of distress or illness (loss of hair, extreme weight loss, etc.).

4.3.2 Regimen of short-term application of TCAP-1 on male Sprague-Dawley rats
Sixteen male adult Sprague-Dawley rats (250g) were allowed to acclimate for 1 week on a 12:12 light-dark (LD) cycle. For 5 days daily, the animals were treated with either vehicle (0.9% saline) or mouse TCAP-1 (10 nmoles/kg) by subcutaneous injection in the intrascapular region. Animals were tested for muscle function and integrity by electrical muscle stimulation (Method 4.3.4) three days post-last injection of treatment. Animals were immediately euthanized after protocol.

4.3.3 Functional Positron Emission Tomography (fPET)
To visualize glucose uptake at the whole organism level, functional positron emission tomography (fPET) scans were used. Male Wistar rats were treated with either vehicle (0.9% saline) or TCAP-1 (10 nmoles/kg) by subcutaneous injection in the intrascapular region. Three days after treatment, fPET scans were performed using a Siemens Inveon microPET small animal PET scanner, and [18F]-2-deoxyglucose ([18F-DG) radiotracer (IBA Molecular). Protocol was followed as previously described (Hogg et al., 2018). Briefly, animals were injected intravenously with approximately 1mCi of [18F-DG. The [18F-DG uptake occurred under anesthesia for 90 mins. Body temperature was maintained with a thermostat-regulated recirculating water heated pad. Static emission data was acquired for 20 minutes. The PET list mode data was converted to 2-dimensional (2D) sinograms, corrected for random coincidences, and normalized for scanner uniformity. PET image analysis was performed using the Amira 5.5.0 analysis software package. For whole body ROIs, a low threshold was set to delineate specific signals in the whole body while eliminating background. The total PET counts were calculated from all voxels within the segmented volumes of interest. These images were then compiled into 3-dimensional (3D) projections, thus allowing for accurate analyzes of muscle tissue. Fluorescence of the mean pixel was calibrated to volume of muscle being analyzed (mean pixel fluorescence/mm³).
4.3.4 **NADH staining and analysis**

Tibialis anterior muscles from the treated Sprague-Dawley rats were flash-frozen in liquid-nitrogen-cooled isopentane and subsequently cryosectioned at 10 μm thickness. Cryosections were then washed 2x with PBS, and then 0.2% NBT solution in PBS (Sigma, Oakville, ON) containing 0.1% NADH (Sigma, Oakville, ON) was added and allowed to incubate for 30 mins at 37°C. Slides were washed 2x in PBS before mounted and were imaged with a Canon camera attached to a Leica bright-field microscope at 100x magnification. Images were analyzed on Image J software for pixel density, where the darker the pixel represents higher expression of NADH. Expression of NADH was analyzed based on a minimum of 100 fibres per tissue, with a minimum of three tissues analyzed for each group.

4.3.5 **Muscle function and integrity testing by electrical muscle stimulation**

Electrical muscle stimulation protocol was followed as described by Holwerda and Locke (2014) with minor modifications. Briefly, animals were anesthetized with 5% isoflurane in 1L/min O₂, and subsequently positioned into testing apparatus. A 25g needle was inserted through the soft tissue of the knee in order to ensure a stable position. The foot was placed on the lever attached to a servomotor and taped in position. Electrodes were placed below the skin but adjacent to the tibialis anterior (TA) muscle. Dynamic Muscle Control (DMC; Version 5; Aurora Scientific) software was used for electrical stimulation and analyzes. The correct voltages for peak tetanic tension was established by increasing voltage by 1V increments until optimal tetanus twitch was achieved. The test began with a single tetanus and single twitch protocol to establish baseline. The 6-min fatigue protocol (8V, 200 Hz, 300 ms) was started. Immediately following the termination of the protocol, tetanic and twitch tensions were recorded at 0, 1, and 5 mins. Animals were immediately euthanized after recovery measurements were recorded. Importantly, by using electrical stimulation of the muscle, the neurological system of muscle control at the neuromuscular junction is completely bypassed. This removes variables such as motivation, which is necessary as previous studies have shown that TCAP-1 modulates behaviour (Al Chawaf et al., 2007b; Tan et al., 2011; Kupferschmidt et al., 2011; Chen et al., 2013).
4.3.6 Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

RNA was extracted from tibialis anterior muscles using TRIzol (Thermo Scientific, Waltham, MA, USA) following the manufacturer’s instructions. Reverse transcription reactions contained 2 μL purified total RNA, 1 μl random primer Mix (Bio-Rad), 1 μL deoxynucleotide solution mix (New England Biolabs), and 8 μL water. Reactions were incubated in a Fisher Scientific Isotemp 125D Dry Bath Incubator for 5 mins at 16°C and then for 1 min at 4°C. Aliquots of 4 μL First Strand Buffer (Invitrogen), 2 μL 0.1 M DTT (Invitrogen), and 1 μL SuperScript II Reverse Transcriptase (Invitrogen) were added to the reaction mix. The reactions were incubated for 10 mins at 25°C, 50 min at 42°C, 15 mins at 70°C, and then held at 4°C. The PCR reaction mix included 5 μL cDNA, 2 μL Forward Primer and 2 μL Reverse Primer (Invitrogen; Table 4.1), 14.2 μL water (Sigma, Oakville, ON), 3 μL 10x Taq Buffer with KCl (Thermo Scientific), 1.8 μL MgCl2 (Thermo Scientific), 1 μL Deoxynucleotide Solution Mix (New England Biolabs), and 0.5 μL Taq DNA Polymerase (Bioshop). cDNA from all samples were used to make cDNA pools to establish standard curves of each gene. cDNA pool and cDNA samples were mixed with MasterMix containing SYBR select. The reactions were loaded in a 384-well PCR plate and run in a BioRAD qRT thermal cycler for 2 mins at 50°C, 7 mins at 95°C; followed by 39 cycles of 60 sec at 95°C, 90 sec at 67°C, and 35 sec at 72°C. Melting curves were established by a step-wise gradient from 60-90°C.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHCI</td>
<td>GAA TGG CAA GAC GGT GAC TGT</td>
<td>GGA AGC GTA CCT CTC CTT GAG A</td>
</tr>
<tr>
<td>MHCIIa</td>
<td>ATG ACA ACT CCT CTC GCT TTG</td>
<td>TTA AGC TGG AAA GTG ACC CGG</td>
</tr>
<tr>
<td>MHCIIb</td>
<td>GAA CAC GAA GCG TGT CAT CCA</td>
<td>AGG TTT CGA TAT CTG CGG AGG</td>
</tr>
<tr>
<td>MHCIIx</td>
<td>CCA ATG AGA CTA AGA CGC CTG G</td>
<td>GCT ATC GAT GAA TTG TCC CTC G</td>
</tr>
<tr>
<td>β-actin</td>
<td>AGCCATGTACGTAAGCCA</td>
<td>CTCTCAGCTGTTGGTGTTGAA</td>
</tr>
</tbody>
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Table 4.1. Primers used for qRT-PCR. Forward and reverse primer pairs for the four rat MHC isoforms and β-actin control (as described in Van Daele, 2010).
4.3.7 Regimen of long-term application of TCAP-1 on male Sprague-Dawley rats.
Twenty male adult Sprague-Dawley rats (350g) were allowed to acclimate for 1 week on a 12:12 LD cycle. Animals were treated for 3 months with either vehicle (0.9% saline) or mouse TCAP-1 (25 nmoles/kg) for 3 months (1 injection/week) by subcutaneous injection in the intrascapular region. Animals were tested in the open field test (Method 4.3.9) one-week post-last injection. Muscle function and integrity by electrical muscle stimulation (Method 4.3.4) was tested two weeks post-last injection of treatment. Animals were immediately euthanized after electrical muscle stimulation protocol.

4.3.8 Measuring glucose levels over long-term application of TCAP-1
Glucose levels were measured three times per week; once before injection, once the day after injection, and once three days after injection. Glucose measurements were taken from a blood droplet acquired from needle prick at the base of the tail and immediately measured on One Touch© glucose monitor device.

4.3.9 Open field Test
The open field test is a stress-paradigm that consists of a large square plexiglass box apparatus with a designated “centre zone”. Tracking software (ANYmaze) records animals for 5 minutes and tracks the entries into and time spent in centre zone. Less anxious animals will explore the testing apparatus, thus entering the centre zone more frequently. In addition to centre zone recordings, behaviours were recorded and observed manually. The three main behaviours witnessed were peering, grooming and rearing. Peering is defined as when the animal looks over the side of the testing apparatus. Grooming is defined as when an animal stops locomotion to groom hands and face. Rearing is defined as when an animal gets on its hind limbs in exploration.

4.3.10 Statistical Analyzes
All data on graphs are represented as mean +/- SEM. All data were analyzed by student’s t-test or one or two-way ANOVA, as described within each figure caption. Tukey’s post-hoc test and
Sidak’s post-hoc test was used to determine significance in one-way and two-way ANOVA analyzes, respectively. An _a priori_ hypothesis states that the asterisks represent the following: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. GraphPad Prism 7 was used to analyze each statistical test and for all graphing.

4.4 Results

4.4.1 _Short-term TCAP-1 treatment did not alter body or muscle weight._

Adult male Sprague-Dawley rats were treated with either vehicle (0.9% saline) or TCAP-1 (10 nmoles/kg) subcutaneously for 5 days, daily. Animals were weighed one week after last treatment and muscle was excised at the end of experiments. Short-term treatment with TCAP-1 did not have any effect on overall body weight (Figure 4.1A) or on muscle weights of the tibialis anterior (TA) or extensor digitorum longus (EDL; Figure 4.1B).

![Figure 4.1. Short-term treatment of TCAP-1 does not alter body or muscle weight.](image)

Male Sprague-Dawley rats were treated for 5 days daily with either vehicle (0.9% saline; black) or TCAP-1 (10 nmoles/kg; grey). Animals were weighed one week after treatment. Body weights (A) and muscle weights (B) of tibialis anterior (TA) and extensor digitorum longus (EDL) are comparable between vehicle- and TCAP-1-treated groups. (Mean ± SEM; n=9-10; t-test).
4.4.2 Short-term TCAP-1 treatment significantly increases glucose uptake in skeletal muscle in vivo.

TCAP-1 mediated glucose uptake was investigated in male Wistar rats by injecting the animals with radioactively-labeled deoxyglucose (\(^{18}\text{F-DG}\)) and visualizing the fate of this glucose by using functional positron emission tomography (fPET). These scans were then constructed into 3D-projections, allowing for very accurate depiction of glucose uptake in the muscle specifically (Figure 4.2A, B). A single subcutaneous administration of TCAP-1 (10 nmoles/kg) resulted in a significant increase \(^{18}\text{F-DG}\) uptake in the hind limb muscles 3 days later as seen by increases in fPET fluorescence compared to vehicle treatment (p<0.05; Figure 4.2C).

![Figure 4.2. Short-term TCAP-1 treatment significantly increases glucose uptake in skeletal muscle in vivo.](image)

Single subcutaneous administration of TCAP-1 in Wistar rats results in significant increase of \(^{18}\text{F-deoxyglucose}\) uptake 3 days later in the hind limb muscle by visualization of 3D functional positron emission tomography (fPET). Representative sagittal view images of 3D fPET scans of hind limb muscles of a vehicle-treated animal (A) and a TCAP-treated animal (B). Quantification of fPET fluorescence (C). (Mean ± SEM; n=5; *p<0.05, t-test).
4.4.3 Short-term TCAP-1 treatment increases NADH production in skeletal muscle tissue.

The tibialis anterior is a muscle that is comprised primarily of type II fibres, thus most of the fibres are specialized for glycolysis. TCAP-1-mediated increases in glucose uptake into skeletal muscle may lead to an increase in energy metabolism which would be visualized by increases in NADH turnover, a product of glycolysis as well as Kreb’s cycle. Tibialis anterior muscles were analyzed for morphology (Figure 4.3A, C, E, G) and NADH staining (Figure 4.3B, D, F, H), where darker blue colour stain represents higher NADH production rate. Muscles were taken from the contralateral control (unstimulated) limb as well as the exercised (stimulated) limb. TCAP-1-treated muscles from control limbs showed a significant increase in frequency of stronger NADH-positive staining when compared to vehicle-treated muscles (p<0.05; Figure 4.3I), thus suggesting TCAP-1 increases baseline energy metabolism in ambient conditions. After electrical stimulation of the muscle, TCAP-1-treated muscle had a trend towards increased NADH-positive fibre frequency compared to vehicle-treated muscle, however it was sub-significant (p=0.1; Figure 4.3J).
4.4.4 Short-term TCAP-1 treatment significantly increases skeletal muscle function via increases in twitch kinetics.

The link between metabolism and muscle function has been well established. Thus, because TCAP-1 enhances muscle metabolism, it was subsequently investigated if TCAP-1-mediated glucose uptake may translate into physiological changes in muscle contractile kinetics. Male Sprague-Dawley rats were treated for 5 days with either vehicle (0.9% saline) or TCAP-1 (10 nmoles/kg) and were tested in a 6-minute metabolic fatigue protocol by use of electrical stimulation of the tibialis anterior. Animals were tested for twitch contractile kinetics before the
start of the fatigue protocol, and 0, 1 and 5 minutes afterwards. As the servomotor is connected to Dynamic Muscle Control (DMC, Version 5, Aurora Scientific) software, contractile parameters of peak twitch force, contraction velocity and contraction relaxation rate can be measured. Representative twitch baseline (pre-test) traces are shown in Figure 4.4A. Animals treated with TCAP-1 had significantly higher twitch peak force at baseline compared to vehicle-treated animals (p<0.05; Figure 4.4B). In addition, the contraction velocity was significantly slower, and the contraction relaxation rate was increased in TCAP-1-treated animals (Figure 4.4C, D). After the 6-minute fatigue protocol, TCAP-1-treated animals had a significantly better recovery as; the force generated was increased (Figure 4.4E), the contraction velocity and relaxation of the contractions (max dx/dt and 1/2RT, respectively) was maintained throughout the protocol whereas the vehicle-treated group showed a steady decline in both contractile parameters (p<0.05; Figure 4.4F, G). Importantly, tibialis anterior muscles from both groups showed no difference in muscle mass (Figure 4.4H) and responded comparably in tetanic force production and fatigue curves (Figure 4.4I, J). Taken together, these results suggest TCAP-1 enhances the quality of the muscle contraction, rather than increasing muscle mass, in order to enhance muscle function. These data demonstrate that TCAP-1 maintains contraction cycling efficiency during fatigue, which is lost in vehicle-treated muscles.
Figure 4.4. Short-term TCAP-1 administration significantly increases skeletal muscle function via increases in twitch kinetics. Male Sprague-Dawley rats were administered either vehicle (0.9% saline; black) or TCAP-1 (10 nmoles/kg; grey) subcutaneously for 5 consecutive days. Muscle function was tested in vivo 3 days post last injection by electrical stimulation tests of the tibialis anterior muscle. Baseline muscle contractile kinetics of contraction force, contraction velocity (max dx/dt), and contraction relaxation rate (1/2RT) were tested first. Subsequently, a 6-minute fatigue protocol was electrically induced in the muscle. Muscle contractile kinetics were then again analyzed at 0, 1, and 5 mins after the fatigue protocol, demonstrating recovery period. Representative twitch traces shown in A. TCAP-1-treated animals had enhanced baseline contraction kinetics, where it demonstrated significantly increased peak twitch force (B), slower contraction velocity (C), and faster relaxation rate (D) compared to vehicle. TCAP-1-treated animals also demonstrated better recovery, though it did not have an effect on peak twitch force (E), it significantly maintained twitch max dx/dt (F) and 1/2RT (G) over the fatigue protocol which was lost in vehicle-treated animals. All data was normalized to muscle weight. Treatment did not affect muscle weight (H), tetanic force (I) or fatigue force curve (J). (Mean ± SEM; n=7-8; *p<0.05; two-way ANOVA).
4.4.5 *Short-term TCAP-1 treatment modulates myosin heavy chain expression in the muscle.*

As TCAP-1-treated muscle demonstrated significant changes in function *in vivo*, the next step was to investigate a possible underlying mechanism. Many factors can influence contractile characteristics, such as fibre-type and metabolic signalling. Myosin heavy chain (MHC) expression was thus investigated to observe any changes in fibre-type in the tibialis anterior muscle. TCAP-1-treated muscle demonstrated a significant increase in MHCI expression by approximately 280% compared to vehicle-treated muscle (Figure 4.5). As well, TCAP-1-treated muscle exhibited an associated significant decrease in MHCIIa, MHCIIx and MHCIIb expression (Figure 4.5). This data indicates that short-term TCAP-1 treatment modulates MHC expression towards slow-twitch oxidative fibre types.
Figure 4.5. Short-term TCAP-1 treatment modulates myosin heavy chain (MHC) expression. Tibialis anterior muscles excised from animals treated with vehicle (0.9% saline) or TCAP-1 (10 nmoles/kg) for 5 days daily were analyzed for MHC expression by qRT-PCR. TCAP-1 treatment increased MHCI expression, whereas it decreased MHCIIa, MHCIIx and MHCIIb expression. All genes were normalized to housekeeping gene, β-actin. (Mean ± SEM; n=7; **p<0.01, *p<0.05; t-test per MHC).

4.4.6 Long-term TCAP-1 treatment did not alter body or muscle weight.

Adult male Sprague-Dawley rats were treated with either vehicle (0.9% saline) or TCAP-1 (25 nmoles/kg) subcutaneously for 3 months (1 injection/week). Animals were weighed once a month and muscles were excised at the end of the experiment. Long-term treatment with TCAP-1 did not alter overall body weight (Figure 4.6A) or muscle weights of the tibialis anterior (TA) or extensor digitorum longus (EDL; Figure 4.6B).
Figure 4.6. Long-term treatment of TCAP-1 does not alter body or muscle weight. Male Sprague-Dawley rats were treated for 3 months with either vehicle (0.9% saline; black) or mouse-TCAP-1 (25 nmoles/kg; grey). Animals were weighed once a month. Body weights (A) and muscle weights (B) of tibialis anterior (TA) and extensor digitorum longus (EDL) are comparable between vehicle- and TCAP-1-treated groups. (Mean ± SEM; n=9-10; two-way ANOVA).

4.4.7 Long-term TCAP-1 treatment decreases blood glucose levels in rats.

Blood glucose levels were closely monitored over 3 months of treatment. In an effort to remove experimental noise, measurements were taken at the same time in the day and animals were fasted for 3 hours before blood glucose was recorded (Figure 4.7A). TCAP-1 treatment significantly reduced blood glucose levels after 3 months of treatment (Figure 4.7B). Importantly, glucose levels stayed within normal ranges throughout the course of the experiment. This suggests that TCAP-1 could be decreasing serum glucose by promoting glucose uptake into tissue, such as skeletal muscle, as seen after short-term TCAP-1 treatment.
4.4.8 Long-term TCAP-1 treatment modifies baseline contractile twitch kinetics, however did not enhance overall skeletal muscle function during fatigue.

Two weeks post-last treatment, muscle function was tested in vivo by electrical muscle stimulation tests. First, baseline muscle contractile kinetics were tested for peak twitch force, contraction velocity and 1/2RT rate. TCAP-1-treated animals elicited a comparable peak twitch force to vehicle-treated animals (Figure 4.8A), however had significantly slower contraction velocity (Figure 4.8B) and faster relaxation rate (Figure 4.8C). When the animals were tested in a 6-minute fatigue protocol, both groups responded similarly in regard to peak twitch force, contraction velocity, and 1/2RT over time (Figure 4.8D, E, F).
Figure 4.8. Long-term TCAP-1 administration significantly increases skeletal muscle function via increases in twitch kinetics. Male Sprague-Dawley rats were administered either vehicle (0.9% saline; black) or TCAP-1 (25 nmoles/kg; grey) subcutaneously for 3 months (1 injection/week). Muscle function was tested in vivo 2 weeks post last injection by electrical stimulation tests of the tibialis anterior muscle. Baseline muscle contractile kinetics of contraction force, contraction velocity (max dx/dt), and contraction relaxation rate (1/2RT) were tested first. Subsequently, a 6-minute fatigue protocol was electrically induced in the muscle. Muscle contractile kinetics were then again analyzed at 0, 1, and 5 minutes after the fatigue protocol. TCAP-1-treated animals had modified contraction kinetics, where although it did not have different peak twitch force (B), it showed significantly slower contraction max dx/dt (C), and faster 1/2RT rate (D) compared to vehicle-treated animals. (Mean ± SEM; n=7-8; *p<0.05; t-test). Both groups of animals showed comparable responses to the fatigue protocol. All data was normalized to muscle weight. (Mean ± SEM; n=7-8; two-way ANOVA).
4.4.9 *Long-term TCAP-1 treatment modulates myosin heavy chain expression.*

Tibialis anterior muscles were excised from long-term-treated animals and analyzed for MHC expression by qRT-PCR. TCAP-1-treated muscles had significantly higher expression of MHCI, approximately 350% compared to vehicle-treated muscle (Figure 4.9). In association, TCAP-1-treated muscle had significantly decreased MHCIIb expression (Figure 4.9). Expression of MHCIIa and MHCIIx were not affected (Figure 4.9). This data is consistent with the short-term results that indicate TCAP-1 treatment induces a fibre-type switch from fast-twitch to slow-twitch.

![Figure 4.9. Long-term TCAP-1 treatment modulates myosin heavy chain (MHC) expression.](image)

*Tibialis anterior muscles excised from animals treated with vehicle (0.9% saline) or TCAP-1 (25 nmoles/kg) for 3 months were analyzed for MHC expression by qRT-PCR. TCAP-1-treated muscles had significantly higher expression of MHCI, approximately 350% compared to vehicle-treated muscle. In association, TCAP-1-treated muscle had significantly decreased MHCIIb expression. Expression of MHCIIa and MHCIIx were not affected. All genes were normalized to housekeeper gene, β-actin. (Mean ± SEM; n=9; **p<0.01; t-test per MHC).*
4.4.10 Long-term TCAP-1 treatment reduces anxiety and stress-related behaviours.

Sprague-Dawley rats were treated with either vehicle (0.9% saline) or TCAP-1 (25 nmoles/kg) for 3 months. One week after the last injection of treatment, animals were tested in an open field test, a test used to assess anxiety and stress-related behaviours in rodents. In the open field test, an animal was placed into a large plexiglass box and recorded for 5 minutes to analyze behaviour. Animals that are experiencing high stress will not explore the open testing apparatus space and will tend to stay to the outside rim of the box. Conversely, animals that are experiencing little stress are typically more explorative and move freely throughout the box. This behaviour can be quantified as the tracking software (ANYmaze) can determine the amount of time and distance is travelled in the “centre zone”, a region that represents the centre of the box (Figure 4.10A). Representative track plots of animals are shown in Figure 4.10B, C. Although both groups travelled the same amount of total distance in the box (Figure 4.10D), animals treated with TCAP-1 entered the centre zone more times than vehicle-treated (p<0.05; Figure 4.10E) and travelled more distance in the centre zone (p<0.05; Figure 4.10F). TCAP-1 treatment also significantly increased number of rearing events (Figure 4.11A), which is a well-established marker of low anxiety. However, TCAP-1 treatment did not affect other types of behaviour such as grooming or peering (Figure 4.11B, C). As both TCAP-1-treated animals and vehicle-treated animals travelled similar distances within the test, this suggests that TCAP is not affecting locomotory circuits, but rather that TCAP-1 specifically reduces stress-related and anxiety-related behaviours.
Figure 4.10. Long-term TCAP-1 treatment significantly reduced stress-related behaviour as seen in open field test. Sprague-Dawley rats were treated with either vehicle (0.9% saline) or TCAP-1 (25 nmoles/kg) for 3 months. Animals were tested in the Open field Test behaviour paradigm. This apparatus is a large square plexiglass box (A) that is linked with tracking software (ANYmaze) to observe the animals’ movements for 5 mins. Representative track plots for vehicle-treated (B) and TCAP-1-treated (C) animals are shown. Animals did not differ in total distance travelled (D), however, TCAP-1-treated animals had a significant increase in number of times entered the centre zone and travelled more distance inside the zone compared to vehicle-treated animals (E). (Mean ± SEM; n=10; *p<0.05; t-test).
Figure 4.11. Long-term TCAP-1 treatment significantly increases explorative behaviour in open field test, while not affecting other types of behaviour. Sprague-Dawley rats were treated with either vehicle (0.9% saline) or TCAP-1 (25 nmoles/kg) for 3 months. TCAP-1-treated animals demonstrated a significant increase in rearing behaviour, a well-established explorative behaviour, compared to vehicle-treated animals. Both groups of animals showed comparable responses in peering and grooming behaviour. (Mean ± SEM; n=10; *p<0.05; t-test)

4.5 Discussion

In this work, the physiological roles of TCAP-1 in rodent skeletal muscle both in short-term and long-term dose regimens were determined, specifically with respect to glucose regulation and muscle function. Short-term TCAP-1 treatment enhanced glucose metabolism as indicated by increased glucose uptake into the muscle and increased turnover rate of NADH. As muscle metabolism and function are intrinsically linked, the effects of TCAP-1 on skeletal muscle function were tested. Short-term TCAP-1 treatment enhanced baseline contractile kinetics under basal and fatigue conditions. Long-term TCAP-1 treatment showed similar results as it also enhanced glucose metabolism and muscle function. Moreover, long-term TCAP-1 treatment decreased stress-related behaviours in rats that were tested in the open field test. These data demonstrate that TCAP-1 is a potent metabolic regulator in muscle, and influences muscle contractile dynamics.
Previous work has shown that TCAP-1 increases glucose uptake into the brain, with a concomitant decrease in serum glucose levels (Hogg et al., 2018). Consistent with this, TCAP-1 treatment significantly upregulated glucose uptake into the hindlimb muscles of the rats compared to vehicle treatment (Figure 4.2A-C). In addition, NADH levels were measured in tibialis anterior muscles of animals that were administered TCAP-1 short-term. As NADH is a by-product of glycolysis and oxidative phosphorylation, it is a marker of cellular metabolism. TCAP-1-treated muscles had significantly higher expression of NADH-positive fibres in control conditions (Figure 4.3). These results highly corroborate the roles of TCAP-1 in glucose regulation and metabolism, as previously demonstrated in the brain (Hogg et al., 2018).

Calcium and ATP are required for proper muscle contraction initiation and relaxation. As glucose provides the main energy source to skeletal muscle function, an upregulation of glucose metabolism could impact skeletal muscle function. TCAP-1-treated muscles had significantly enhanced baseline muscle function, demonstrating a significantly higher peak twitch force (Figure 4.4B), slower contraction velocity (Figure 4.4C), and faster 1/2RT rate (Figure 4.4D). Further, after the fatigue stress on the muscle, the TCAP-1-treated muscles had a higher capacity to maintain contraction velocity (Figure 4.4F) and 1/2RT rate (Figure 4.4G), which is considerably reduced in vehicle-treated animals. Taken together, the influence of TCAP-1 on these contractile kinetic parameters indicates that TCAP-1 is modulating calcium levels to enhance SR-sarcomere coupling. During fatigue, cytosolic calcium levels accumulate due to inefficient SR-sarcomere coupling, thereby reducing muscle function as can be seen in contractile kinetic parameters such as peak twitch force and 1/2RT. TCAP-1 treatment significantly increased both of these parameters, indicating a clear role in calcium modulation. Moreover, these results also suggest that TCAP-1 increases ATP production rate to meet the energetic demands of the muscle during fatigue. After the contraction, calcium must be cleared from the cytosol and be re-uptaken into the SR via SERCA pumps. SERCA pumps are high energy-consuming channels that account for 20-50% of the energy turnover in a single contraction cycle (Leijendekker et al., 1987; Bellinger et al., 2008). Prolonged stimulation results in the rapid depletion of ATP, thus reducing SERCA activity, ultimately leading to the accumulation of cytosolic calcium. However, as TCAP-1 increases glucose uptake into the muscle, it may provide additional substrate for energy
metabolism which could maintain SERCA activity during fatigue. This is supported by the finding that TCAP-1 had significantly faster 1/2RT during fatigue compared to vehicle treatment. In summation, these results suggest TCAP-1 modulates contractile kinetics, likely via calcium dynamics and ATP production, which are potent regulators of muscle contractions.

Another factor that regulates contractile kinetics is fibre-type characteristics. The tibialis anterior muscle is comprised of 95% type II fibres, which is broken down into two further subtypes, type IIa/x and type IIb. Type IIb are predominantly glycolytic fibres, while type IIa/x are intermediary fibres that can be influenced to be more glycolytic or oxidative in nature. TCAP-1 may be influencing the type IIa/x fibres to rely more on oxidative phosphorylation by switching to a type I phenotype, which is reflected in the contractile characteristics of TCAP-1-treated muscle. Thus, to determine effects on fibre-type, MHC expression was investigated using qRT-PCR. TCAP-1-treated muscle had increased expression of MHC-I with an associated decrease in MHC-IIa/x (Figure 4.5), which corroborates fibre-type switching towards slow-twitch fibre phenotype. In support of this, short-term TCAP-1 treatment enhanced NADH enzymatic activity along the periphery of the muscle fibre wall. As sub-sarcolemmal (SS) mitochondria are localized to the peripheral wall of the muscle fibre, it thus may reflect mitochondrial NADH, suggesting TCAP-1 may be either increasing mitochondrial content or enhancing TCA cycle metabolism, consistent with type I fibre phenotype (Lin et al., 2002). Taken together, these data suggest that TCAP-1 is influencing fibre-type switch towards type I slow oxidative fibres.

However, the short-term application of TCAP-1 renders a less resolved understanding of TCAP-1 actions in muscle, with some authors reporting it could take up to 10 weeks before seeing changes (Farup et al., 2014). Therefore, long-term application of TCAP-1 was subsequently investigated to analyze changes in muscle adaptation over time. In addition, it was used to test the efficacy of long-term TCAP-1 administration in stress-related behaviours as previously shown with short-term application. For these studies, TCAP-1 was administered for 3 months, with one injection of 25 nmoles/kg per week. Previous short-term studies administered 10 nmoles/kg of TCAP-1 via subcutaneous injection daily, for up to 10 days. In order to limit the injection stress of the animals over 3 months, these studies administered one higher concentration of TCAP-1 per week.
As short-term studies demonstrated the potent effect of TCAP-1 in glucose regulation, this was tested under long-term TCAP-1 treatment. Blood glucose was closely monitored and showed that TCAP-1 decreases blood glucose over the course of the experiment (Figure 4.7). This suggests that TCAP-1 decreases serum glucose by promoting glucose uptake into tissues, such as skeletal muscle. Importantly, the blood glucose levels of TCAP-1-treated animals never reached hypoglycemic levels, which indicates TCAP-1 and other glucose-regulating hormones, such as glucagon and insulin, work together to maintain healthy homeostatic levels.

Long-term TCAP-1 actions in glucose regulation were similar to short-term application, thus muscle contractile kinetics were also tested in the long-term TCAP-1-treated animals. TCAP-1-treated muscle baseline kinetics had slower contraction velocity and faster 1/2RT compared to vehicle-treated muscle, with no effect on peak twitch force (Figure 4.8A-C). Both groups responded similar under fatigue conditions in all contractile parameters (Figure 4.8D-F). These results were in part, consistent with the short-term TCAP-1 treatment results. Both baseline kinetics had slower contraction velocities and faster 1/2RT rates. This data may corroborate the notion that TCAP-1 can influence fibres to switch to type I fibres over time. Thus, to investigate this possibility, MHC expression was next analyzed. qRT-PCR analyzes revealed that long-term TCAP-1-treated muscle expressed MHCI approximately 350% more than vehicle-treated muscle, indicating a marked increase in type I fibres (Figure 4.9). Consistent with an increase in MHCI, there was a concomitant decrease in MHCIIb expression in TCAP-1-treated muscle. These studies demonstrate that the changes observed in muscle function in vivo are likely a result of MHC expression changes. Moreover, this data corroborates the MHC expression shift towards MHCI observed in short-term TCAP-1 application. While the effect of long-term TCAP-1 application on fibre-type change was greater than short-term application, this was expected as authors have reported changes in fibre-type is usually best observed after 10 weeks of muscle adaptation or treatment (Farup et al., 2014).
In order to induce fibre-type changes at the genetic level, TCAP-1 actions must lead to transcriptional changes in the muscle. There are few transcription factors that have been established to switch type II fast glycolytic fibres to type I slow oxidative fibres. Myocyte enhancer factor 2 (MEF2) is a key transcription factor that has potent ability to induce fibre type switch towards type I (Potthoff et al., 2007). It has many different regulatory partners, such as calcineurin, calcium/calmodulin-dependent protein kinase (CaMK), nuclear factor of activated T cells (NFAT), and PGC-1α (Czubryt et al., 2003; Lin et al., 2002; Naya et al., 2000; Potthoff et al., 2007). While the exact interactions amongst these partners are not well defined, it is postulated that intracellular calcium signaling is a major component to the activation of MEF2. Thus, TCAP-1 may lead to the activation of MEF2 to induce fibre type switch to type I fibres (discussed further in section 6.5.3.).

Although there were many similarities in the muscle function results between short- and long-term TCAP-1 treatment, short-term TCAP-1-treated muscle had enhanced peak twitch force and maintained elevated contractile kinetic function during fatigue, which was not seen in long-term TCAP-1-treated muscle. One of the differences between these two dose regimens was when the muscle stimulation test was performed. In the short-term application, muscle function was tested 3 days post-last injection, whereas muscle function was tested 2 weeks post-last injection. One explanation is that the metabolic enhancement induced by TCAP-1 is relatively short-lived. However, long-term application of TCAP-1 still resulted in dynamic changes in the contractile kinetics, due to an influence on fibre-type characteristics. Collectively this data suggests that TCAP-1 has both immediate and long-term actions, in metabolism and fibre-type changes, respectively. These types of immediate and long-term actions have been seen in other hormones. Insulin, for example, acutely stimulates glucose uptake into the cell, but also stimulates genetic transcription via MEK/ERK (Santos et al., 2008).

In addition to investigating the roles of TCAP-1 with respect to glucose regulation and muscle function, long-term treated animals were also tested in a stress behaviour model to investigate the efficacy of TCAP-1 treatment over time. This was done to corroborate earlier TCAP treatments on behaviour with the present glucose and muscle kinetic studies. Previous studies showed that short-term TCAP-1 treatment resulted in anxiolytic behaviours in rats (Al Chawaf et al., 2007b;
Tan et al., 2011; Tan et al., 2009). The results of the long-term application of TCAP-1 were consistent with previous studies and acted not only as a positive control, but also provided new information on long-term actions of TCAP-1. Animals were tested in an open field test, which suggests that the more time an animal spends in the designated centre zone, the less stress-related behaviour the animal is exhibiting (Figure 4.10A-C). Although both groups of vehicle- and TCAP-1-treated animals travelled the same distance within the test (Figure 4.10D), TCAP-1-treated animals had more entries into the centre zone (Figure 4.10E) and spent more time in the centre zone (Figure 4.10F). Additionally, TCAP-1-treated animals exhibited a more explorative behaviour in the test, termed rearing (Figure 4.11A), while not affecting other behaviours such as grooming or peering (Figure 4.11B, C). These data suggest that TCAP-1-treated animals demonstrated less stress-related behaviours, consistent with previous work in rodents.

In conclusion, this work demonstrates the significant role of TCAP-1 in muscle metabolism and function, with potent actions on glucose regulation and contractile kinetics of the muscle. Both short-term and long-term applications of TCAP-1 significantly increased glucose uptake into the muscle. Moreover, these data clearly demonstrate that TCAP-1 enhances skeletal muscle contractile kinetics both with short-term and long-term application, with possible time-dependent actions. In addition, long-term application of TCAP-1 resulted in less stress-related behaviours compared to vehicle-treated animals, consistent with previous studies.
Chapter 5
Elucidation of the mechanism of action for teneurin C-terminal associated peptide (TCAP)-1 in calcium and glucose regulation; an in vitro investigation.

A portion of this chapter is being currently prepared as a manuscript: D’Aquila, A.L., Reid, M.L., Hogg, D.W., Dodsworth, T., Husic, M., Chen., Y., Locke, M., Slee, A., Biga, P.R., Lovejoy, D.A. The C-terminal domain of teneurin as a novel regulator of glucose metabolism and skeletal muscle function. [AD contribution: experimental design, performed and analyzed majority of experiments (11/13), wrote majority of manuscript.]

5.1 Abstract

The results presented in Chapter 4 indicate that TCAP-1 enhances glucose metabolism in vivo as well as enhances skeletal muscle function during fatigue, however, the molecular mechanism of action remains unknown. Recently, the putative receptor for TCAP-1, ADGRL, has been established in neurons. Thus, it is hypothesized that the teneurin/TCAP-ADGRL complex is present and functional in skeletal muscle. Moreover, this work investigates the molecular mechanism of TCAP-1 action in energy metabolism in vitro using the C2C12 murine skeletal muscle cell line. These data demonstrate the first evidence of the teneurin/TCAP-ADGRL complex in skeletal muscle using RT-PCR and Western blot techniques. Next, as ADGRL is associated with calcium regulation, and in vivo effects of TCAP-1 suggest a role in calcium regulation, changes in cytosolic calcium was measured. TCAP-1 induced a rapid release of calcium out of the sarcoplasmic reticulum that corresponds in a similar timeline to increase in mitochondrial calcium levels, suggesting calcium importation into the mitochondria. When mitochondrial metabolism is enhanced with calcium importation, there is typically a corresponding increase in glucose uptake. TCAP-1 administration increased $^3$H-deoxyglucose uptake by 300%, which was associated with a concomitant increase in GLUT4. Consistent with this, ATP, NADH and succinate dehydrogenase levels increased with TCAP-1 treatment, demonstrating enhanced mitochondrial activity. Thus, this work demonstrates that the teneurin/TCAP-ADGRL complex is expressed in muscle and provides a possible explanation of the underlying mechanism of TCAP-1 actions on metabolism.
5.2 Introduction

Muscle function and metabolism are intrinsically linked as evidenced by metabolic syndromes, such as diabetes, that result in poor muscle function or even its degradation (discussed in section 1.7.2; Sayer et al. 2005). Skeletal muscle is one of the most important sites of glucose metabolism considering it is responsible for 40% of glucose-associated energy requirements (Richter & Hargreaves, 2013), and responsible for 80% of glucose disposal under insulin-stimulated conditions (Santos et al., 2008). Glucose provides the required energy to meet the demands of muscle contractions by producing ATP via either anaerobic or aerobic pathways. Muscle contractions convert chemical energy into mechanical energy, a requirement for excitation-contraction (EC) coupling. Both ATP and calcium are necessary for the initiation and relaxation of a contraction. However, during prolonged muscle stimulation, such as exercise, this process can become uncoupled resulting in aberrant glucose and calcium regulation which ultimately leads to decreased force production, known as fatigue. Dysregulated glucose may result in a lowered production of ATP, thus impacting contractile kinetics directly on the sarcomere, as well as indirectly via ATPases not meeting energy demands. With respect to calcium, when sarcoplasmic reticulum (SR)-sarcomere cycling becomes inefficient, it leads to increased accumulation of cytoplasmic calcium. Both factors contribute to poor contraction strength and velocity (Allen et al., 2008; Bellinger et al., 2008). Thus, mechanisms that can either increase ATP and/or modulate cytosolic calcium levels during stress in the muscle can enhance muscle function throughout stressful conditions.

Previous studies have demonstrated that TCAP-1, a novel bioactive peptide, enhances skeletal muscle glucose metabolism and function during fatigue (refer to Chapter 4). To understand the roles that TCAP-1 plays at the physiological level, the cellular mechanism of action must be elucidated. Although in vitro cell models do not fully recapitulate organismal metabolism complexity, it does allow for deeper investigation of possible mechanisms of TCAP-1 actions. For these studies, the C2C12 cell line developed by Yaffe and Saxel (1977) will be utilized to study skeletal muscle in vitro. C2C12 cells are an immortalized murine myoblast cell line that were originally cultured from C3H mice after crush injury. These cells readily differentiate into multi-
nucleated myotubules and provide a foundation to explore TCAP-1 actions on calcium and glucose in skeletal muscle.

5.3 Methods

5.3.1. Culture of C2C12 cell line
Immortalized murine skeletal cell line C2C12 cells were used for all in vitro studies. Cells were maintained at 60-70% confluency with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and penicillin/streptomycin antibiotic combination (Invitrogen, Burlington, ON, Canada). To induce differentiation, the media was changed to DMEM supplemented with 10% horse serum (HS) and penicillin/streptomycin antibiotic combination, and cells were allowed to differentiate for 6 days (media replaced every 24 hours). For treatment, cells were serum starved for 3 hours and then treated with either vehicle (ddH2O) or TCAP-1 (100 nM).

5.3.2. Reverse Transcription (RT)- Polymerase Chain Reaction (PCR)
RNA was extracted from differentiated mouse C2C12 skeletal muscle cells and homogenized mouse hind limb muscles using TRIzol (Thermo Scientific, Waltham, MA, USA) following the manufacturer’s instructions. Reverse transcription reactions contained 2 μL purified total RNA, 1 μl random primer Mix (Bio-Rad), 1 μL deoxynucleotide solution mix (New England Biolabs), and 8 μL water. Reactions were incubated in a Fisher Scientific Isotemp 125D Dry Bath Incubator for 5 mins at 16°C and then for 1 min at 4°C. Aliquots of 4 μL First Strand Buffer (Invitrogen), 2 μL 0.1 M DTT (Invitrogen), and 1 μL SuperScript II Reverse Transcriptase (Invitrogen) were added to the reaction mix. The reactions were incubated for 10 mins at 25°C, 50 min at 42°C, 15 mins at 70°C, and then held at 4°C. The PCR reaction mix included 5 μL cDNA, 2 μL Forward Primer and 2 μL Reverse Primer (Invitrogen; Table 5.1), 14.2 μL water (Sigma, Oakville, ON), 3 μL 10x Taq Buffer with KCl (Thermo Scientific), 1.8 μL MgCl2 (Thermo Scientific), 1 μL Deoxynucleotide Solution Mix (New England Biolabs), and 0.5 μL Taq DNA Polymerase (Bioshop). The reactions were incubated in an Eppendorf Mastercycler Gradient Thermal Cycler for 7 mins at 95°C; followed by 35 cycles of 60 sec at 95°C, 90 sec at 67°C, and 35 sec at 72°C;
and then held at 4°C. DNA samples were then electrophoresed on a 3% agarose gel at 100 V for 1.5 hrs. Gels were visualized using a Bio-Rad ChemiDoc MP System with 0.5 sec exposure.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Expected Band Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teneurin-1</td>
<td>CACAGTCAGCGCGTTACATCTTTGAG</td>
<td>GAATCCGTATGCACTGGGGAAGCAGT</td>
<td>342 bp</td>
</tr>
<tr>
<td>Teneurin-2</td>
<td>ATTCCTGAACTCGCGCTCCTCTTTA</td>
<td>CTCCAGGTTGTAGGGAACGCACG</td>
<td>405 bp</td>
</tr>
<tr>
<td>Teneurin-3</td>
<td>GTGAATGCTGGTTAGTCAAAGATG</td>
<td>AGTGAATACCCGCGGGAACGCAC</td>
<td>427 bp</td>
</tr>
<tr>
<td>Teneurin-4</td>
<td>ATCGACCAATTCCTGCTGAGGCAAG</td>
<td>CCAGAGAGGCACTCCCGGTAGGTC</td>
<td>567 bp</td>
</tr>
<tr>
<td>TCAP-1</td>
<td>AGTGTCAGTGAAATGGGAGGACTA</td>
<td>CCTCTGCTATTTTACTCTGCTCAT</td>
<td>351 bp</td>
</tr>
<tr>
<td>TCAP-2</td>
<td>GACAGATGCACTACAGCATCGAG</td>
<td>CCATCTCATCTGTCTTTAGAAGC</td>
<td>496 bp</td>
</tr>
<tr>
<td>TCAP-3</td>
<td>CAACAAAGCGCTTCTACCTGGAAAC</td>
<td>CGATCTCATTGTGGCAAGAAT</td>
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</tr>
<tr>
<td>TCAP-4</td>
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<td>TGATATTTGTGGCGCTGTGCTGAC</td>
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</tr>
<tr>
<td>ADGRL1</td>
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<td>TTCTAGGCTCACAGCTACAT</td>
<td>249 bp</td>
</tr>
<tr>
<td>ADGRL2</td>
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<tr>
<td>ADGRL3</td>
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<td>TAACCACCAGCCACACCAT</td>
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</tr>
<tr>
<td>β-actin</td>
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<td>ATGTACAGCAGATTGATTCCTT</td>
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</tbody>
</table>

Table 5.1. Primers used for RT-PCR analyzes. Forward and reverse primer pairs for the four teneurins, TCAPs, three ADGRLs, and β-actin control.

5.3.3. Western blot of ADGRL isoforms in C2C12 cells.
Following TCAP-1 treatments, C2C12 cells were lysed with 500 μL of RIPA buffer supplemented with protease inhibitor, PMSF (Cell Signalling). Cells were harvested and centrifuged at 20,000 rcf for 20 mins at 4°C. A Pierce BCA protein assay (Thermo Fischer Scientific) was performed to quantify protein concentrations for standardizing dilutions of respective supernatant samples. Samples (15 μg) were re-suspended in sample buffer and size fractioned by SDS-PAGE (10%) at 100V for 1 hr. Proteins were then electrotransferred to Hybond-ECL nitrocellulose membranes (Amersham) for 2 hrs at 100 V. Membranes were washed with phosphate buffer solution (PBS) and blocked in 5% milk-PBST (5% w/v non-fat milk powder in PBS with 0.2% Tween®20) at RT for 1 hr under agitation. Afterwards, membranes with incubated with primary antibodies (Table 5.2) in 1% milk-PBST overnight at 4°C with gentle agitation. Following 24 hrs, the membranes were given 3x 5-minute washes in fresh PBST at RT and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Table 5.2; VWR, Amersham) diluted to 1:7500 in 1%
milk-PBST for 1 hr at RT with gentle agitation. The membranes underwent 3x 5-min washes in fresh PBST at RT. Subsequently, proteins were detected by adding chemiluminescence detection reagent (ECL Amersham) to the membranes and exposing onto ECL Hyperfilm (VWR) for 10-60 mins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Expected band size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADGRL1</td>
<td>ADGRL1 Goat polyclonal IgG</td>
<td>Donkey anti-goat HRP-linked</td>
<td>130 kDa</td>
</tr>
<tr>
<td>ADGRL2</td>
<td>ADGRL2 Goat polyclonal IgG</td>
<td>Donkey anti-goat HRP-linked</td>
<td>163 kDa</td>
</tr>
<tr>
<td>ADGRL3</td>
<td>ADGRL3 Rabbit polyclonal IgG</td>
<td>Donkey anti-rabbit HRP-linked</td>
<td>162 kDa</td>
</tr>
<tr>
<td>SDH-A; β-actin</td>
<td>Mitobiogenesis Western Blot Cocktail (Abcam123545)- Mouse monoclonal</td>
<td>Rabbit anti-mouse HRP linked</td>
<td>70 kDa, SDH-A; 42 kDa, β-actin.</td>
</tr>
</tbody>
</table>

Table 5.2. Primary and secondary antibodies used in western blot analyzes.

5.3.4. ADGRL expression and quantification by immunohistochemistry

The tibialis anterior (TA) muscle was excised from young adult male Sprague-Dawley rats and flash-frozen in liquid nitrogen cooled-isopentane, where it was stored at -80°C until use. The tissue was sectioned at 10 μm using a cryostat at -20°C and transferred to coverslides. The sections were fixed using ice-cold methanol. Sections were then blocked for 1 hour using 10% normal goat serum (NGS, Cell Signalling). Primary antibody diluted in 1% NGS was then added and allowed to incubate overnight at 4°C. The following morning after 3x PBS washes, the secondary antibody was added and allowed to incubate for 1 hr at RT in the dark. Coverslips were attached, and the tissue imaged using confocal microscopy (Leica TCS-SP8) at 400x magnification. For fluorescence analyzes of protein expression, Image J software was used to measure arbitrary fluorescent units (AFU), where an increase of AFU represents an increase in protein expression (n= 5 fibres, 8 ROIs measured per fibre).
5.3.5. *Mouse diacylglycerol (DAG) and mouse inositol triphosphate (IP3) ELISA assays*

The protocols provided by commercial DAG and IP3 assays (CAT#MBS2607255 and MBS705160, respectively; MyBiosource, San Diego, California, USA) were followed. Briefly, immortalized mouse C2C12 cells were prepared using the TCAP-treatment protocol described in method 5.3.1. Cell lysates were added to a microELISA plate coated with purified mouse DAG or IP3 antibodies. Subsequently, 3,3′,5,5′-tetramethylbenzidine (TMB) solution was added to detect the HRP-conjugates as the colour changes. Finally, a sulphuric acid solution was added to terminate the reaction. The absorbance change was measured at a wavelength of 450 nm using a spectrophotometer (SpectraMax Plus, Hampton, New Hampshire, USA).

5.3.6. *Live-cell calcium imaging in C2C12 myotubules.*

For live-cell fluorescence experiments, immortalized murine C2C12 skeletal muscle cells were grown and differentiated on poly-D-lysine coated 25 mm round No. 1 glass coverslips (Warner Instruments, Hamden, CT, USA). Changes in intracellular calcium were assessed using the membrane-permeable calcium sensitive fluorescent indicator fluo-4, AM (Invitrogen, Burlington, ON, Canada). Cells were loaded with fluo-4 by incubating a single coverslip in DMEM containing 10 μM fluo-4 (from a 1 mM stock solution in DMSO) for 30 mins (37°C) followed by a 15 min wash in Locke’s Buffer containing (in mM): 154 mM NaCl, 4 mM NaHCO3, 5 mM KCl, 2.3 mM CaCl2, 1 mM MgCl2, 5 mM glucose and 10 mM HEPES (pH 7.4); osmolarity 305-310 mOsmol at 22°C). During loading, the acetate groups on fluo-4 are removed by intracellular esterases thereby sequestering the dye within the cytosol. In experiments assessing changes in intracellular calcium, coverslips were placed in a flow-through bath chamber (RC-40HP, Warner Instruments, Hamden, CT, USA) of an inverted microscope (Axio Observer Z1, Zeiss, Toronto, ON, Canada) equipped with a 40× oil immersion objective. Cells were continuously bulk perfused with Locke’s buffer via a gravity drip perfusion system at a rate of 2–3 ml min⁻¹ at RT. Changes in fluo-4 fluorescence was imaged using a green fluorescent protein (GFP) filter set (Semrock, Rochester, NY, USA) and a X-Cite 120 fluorescence illumination system (Excelitas Technologies, Mississauga, ON, Canada), controlled by Volocity 4.0 imaging software (Quorum Technologies Inc., Guelph, ON, Canada). Fluorescence emissions were detected with an Orca-ER Hamamatsu
B/W CCD digital camera (Hamamatsu, Middlesex, NJ, USA). Fluo-4 was excited with a wavelength of 480 nm for 100 ms every 3-5 seconds and fluorescence emission was measured at wavelength of 516 nm.

For the caffeine stimulation experiments, caffeine (4 mM; Sigma Aldrich, Oakville, ON) was applied to C2C12 myotubes to stimulate calcium release from the sarcoplasmic reticulum. Cells were either pre-treated with TCAP-1 (100 nM) for 1 hr before stimulation with caffeine. Using velocity 4.0 imaging software, multiple regions of interests (ROIs) were taken from cytosolic regions in the myotubules (n= 4 coverslips, 4-5 ROIs per coverslip).

For the IP3R inhibitor 2-aminoethoxydiphenyl borate (2-APB; Sigma Aldrich, Oakville, ON) experiments, 2-APB (100 µM) was applied for 4 mins before the start of treatment with either sham (Locke’s buffer) or TCAP-1 (100 nM), which also contained 2-APB in the drug application bottles for continuous blocking of IP3R. Unfortunately, some authors have reported that 2-APB can have unspecific effects leading to the inhibition of other calcium channels (Bootman et al., 2002). To account for this, a low dose of 2-APB was used to minimize unspecific effects.

5.3.7. Measuring mitochondrial calcium levels in C2C12 myotubules.
Changes in mitochondrial calcium levels were assessed using fluorescent indicator Rhodamine-2-AM (Rhod-2). C2C12 myotubules were loaded with Rhod-2 by incubating a single coverslip in DMEM containing 4uM Rhod-2 (from a 1mM stock solution in DMSO with 20% pluronic; Invitrogen - Pluronic™ F-127) for 30 min at 22°C. It was subsequently washed for 30 minutes at 37°C in Locke’s Buffer containing (in mM): 154 mM NaCl, 4 mM NaHCO3, 5 mM KCl, 2.3 mM CaCl2, 1 mM MgCl2, 5 mM glucose and 10 mM HEPES (pH 7.4); osmolarity 305-310 mOsmol at 22°C). Cells were then allowed 10-15 mins to acclimate to 22°C before beginning experiment. In experiments assessing changes in mitochondrial calcium levels, coverslips were placed in a flow-through bath chamber (RC-40HP, Warner Instruments, Hamden, CT, USA) of an inverted microscope (Axio Observer Z1, Zeiss, Toronto, ON, Canada) equipped with a 40× oil immersion objective. Cells were continuously bulk perfused with Locke’s buffer via a gravity drip perfusion system at a rate of 2–3 ml min⁻¹ at RT. Changes in Rhod-2 fluorescence was imaged using a
TRITC filter set (Semrock, Rochester, NY, USA) and a X-Cite 120 fluorescence illumination system (Excelitas Technologies, Mississauga, ON, Canada), controlled by Volocity 4.0 imaging software (Quorum Technologies Inc., Guelph, ON, Canada). Fluorescence emissions were detected with an Orca-ER Hamamatsu B/W CCD digital camera (Hamamatsu, Middlesex, NJ, USA). Rhod-2 was excited with a wavelength of 552 nm for 100 ms every 5 secs and fluorescence emission was measured at wavelength of 577 nm. Using velocity 4.0 imaging software, multiple ROIs were taken from nuclear regions of the myotubules (n= 5 coverslips, 5-7 ROIs per coverslip).

5.3.8. Measuring mitochondrial membrane potential in C2C12 myotubules.

Changes in mitochondrial membrane potential were assessed using fluorescent indicator Rhodamine-123 (R123). C2C12 myotubules were loaded with R123 by incubating a single coverslip in DMEM containing 5 μM R123 (from a 1 mM stock solution in DMSO) for 30 minutes (37°C) followed by a 15-minute wash in Locke’s Buffer containing (in mM): 154 mM NaCl, 4 mM NaHCO3, 5 mM KCl, 2.3 mM CaCl2, 1 mM MgCl2, 5 mM glucose and 10 mM HEPES (pH 7.4); osmolarity 305-310 mOsmol at 22°C). In experiments assessing changes in mitochondrial membrane potential, coverslips were placed in a flow-through bath chamber (RC-40HP, Warner Instruments, Hamden, CT, USA) of an inverted microscope (Axio Observer Z1, Zeiss, Toronto, ON, Canada) equipped with a 40× oil immersion objective. Cells were continuously bulk perfused with Locke’s buffer via a gravity drip perfusion system at a rate of 2–3 ml min−1 at RT. Changes in R123 fluorescence was imaged using a green fluorescent protein (GFP) filter set (Semrock, Rochester, NY, USA) and a X-Cite 120 fluorescence illumination system (Excelitas Technologies, Mississauga, ON, Canada), controlled by Volocity 4.0 imaging software (Quorum Technologies Inc., Guelph, ON, Canada). Fluorescence emissions were detected with an Orca-ER Hamamatsu B/W CCD digital camera (Hamamatsu, Middlesex, NJ, USA). R123 was excited with a wavelength of 480 nm for 100 ms every 5 secs and fluorescence emission was measured at wavelength of 516 nm. Caffeine (4 mM; Sigma Aldrich, Oakville, ON) was applied to C2C12 myotubes to stimulate calcium release from the sarcoplasmic reticulum. Cells were either pre-treated with TCAP-1 (100 nM) for 1 hr before stimulation with caffeine. Using velocity 4.0 imaging software, multiple ROIs were taken from nuclear regions of the myotubules (n= 3 coverslips, 5-7 ROIs per coverslip).
5.3.9. **Radioactive glucose uptake** in vitro

Radioactive glucose uptake protocol was followed as previously described with minor modifications (Maher, 1995; Uemura and Greenlee, 2006). At day-6 post-plating, C2C12 myotubules were washed twice with Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl2, 3.6 mM NaHCO3, 5 mM HEPES) without serum and glucose. The culture was incubated in the Locke’s buffer for 1 hr at 37°C followed by exposure to 100 nM insulin, 100 nM TCAP-1, 100 nM SC-TCAP-1, or saline. $^3$H-2-deoxyglucose (0.5 μCi/ml) was added to culture 5 mins before termination of treatment exposure. Uptake of $^3$H-2-deoxyglucose was stopped immediately after 5 mins with 3x washes of ice-cold 0.9% NaCl solution. The cells were digested with 1 mL of 0.05 N NaOH at 0, 30, 45, and 60 mins after treatment. Cell lysates were used for determination of radioactivity by a beta liquid scintillation counter (Beckman Coulter), represented in counts per minute (CPM).

5.3.10. **GLUT4 Translocation studies**

C2C12 skeletal muscle cells were differentiated on poly-D-lysine coated coverslips. After 3 hrs of serum starvation, myotubules were treated either with vehicle (ddH₂O), TCAP-1 (100 nM) or insulin (100 nM) for 15 or 30 mins. Cells were then fixed using 4% paraformaldehyde and subsequently blocked with 10% NGS for 1 hour at room temperature. GLUT4 primary antibody diluted in 1% NGS was added to the cells and was incubated at 4°C overnight. The following morning, after four PBS washes, the secondary antibody (also diluted in 1% NGS) was added and kept for 1 hr at RT. The coverslips were mounted on slides using Vectashield (containing DAPI), and subsequently sealed with nail polish once dry.Slides were imaged on a confocal microscope at 40x oil objective. As myotubules were not permeabilized, GLUT4 antibody binding resulted in labelling of only translocated GLUT4 at the plasma membrane. Images were analyzed using Image J, where myotubules were selected as ROIs and were analyzed for red pixel intensity values, representing GLUT4 levels, and normalized to area size (n= 3 coverslips per treatment, 7-8 myotubules per coverslip).
5.3.11. Intracellular ATP live-cell assay
ATP assay kits were purchased from Promega (Cat#: G7570; Wisconsin, USA) and manufacturer’s instructions were followed. Briefly, C2C12 cells were seeded at 10,000/well in a 96-well plate. The following day, cells were either treated with vehicle (ddH2O) or TCAP-1 (100 nM) and lysed at 0, 15, 30 and 60 minutes after treatment. Ultra-Glo™ recombinant luciferase was added to the media to determine ATP levels. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of magnesium, ATP and molecular oxygen. Thus, increases in luciferin fluorescence directly correlates to an increase in ATP levels. Fluorescence from blank wells were subtracted from all samples to account for any background noise. As the fluorescence signal naturally decays over the course of the experiment, TCAP-1-treated cells were compared relatively to the vehicle-treated cells for each time point (n=8).

5.3.12. Resazurin NADH assay
C2C12 cells were seeded at 10,000/well in a 96-well plate. Resazurin assay was started the following day, by adding the resazurin solution (525 nM, Sigma) to all the wells. Cells were treated with either vehicle (ddH2O) or TCAP-1 (100 nM). Fluorescent readings were measured every 5 mins over an hour, with excitation at 530 nm and emission read at 590 nm. Measurements of blank wells that contained no cells were subtracted from all readings.

5.3.13. Statistical Analyzes
Data on graphs are represented as mean +/- SEM. All data were analyzed by student’s t-test or one or two-way ANOVA, as described within each figure caption. Tukey’s post-hoc test and Sidak’s post-hoc test was used to determine significance in one-way and two-way ANOVA analyzes, respectively. An a priori hypothesis states that the asterisks represent the following: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. GraphPad Prism 7 was used to analyze each statistical test and for all graphing.
5.4. Results

5.4.1. Skeletal muscle expresses teneurins, TCAPs, and ADGRLs

In order to determine genetic expression of the molecular components associated with the teneurin/TCAP-ADGRL complex, reverse transcription (RT)-PCR analyzes using RNA extracts from both C2C12 murine skeletal cells and mouse hind limb muscle were used for all of the four teneurins and TCAPs, and the three ADGRL isoforms. In the C2C12 cells, of the four teneurins, only teneurin-3 was expressed, whereas all four of the TCAP peptides were expressed. ADGRL1 and ADGRL3 were both expressed, but no expression of ADGRL2 was observed (Figure 5.1A-C). Mouse hind limb skeletal muscle extracted and homogenized showed similar expression to the in vitro cell line. It showed expression of teneurin-3 as well as teneurin-4. Similar to cell line results, the muscle extract showed expression of all of the four TCAP peptides as well as ADGRL1 and ADGRL3, with no expression of ADGRL2 (Figure 5.1D-F). Moreover, protein level expression shown by western blot analyzes of C2C12 cell lysates corroborated the genetic expression pattern established by RT-PCR (Figure 5.1G). After establishing that the necessary molecular components were expressed in vitro and in vivo, it was next examined if these proteins co-localized together to demonstrate they could potentially form the teneurin/TCAP-ADGRL complex (reviewed in Woelfle et al., 2015). Immunohistochemistry on rat tibialis anterior muscle sections was used to visualize teneurin-3 (Figure 5.1H, blue), ADGRL1 (Figure 5.1H, red), and β-dystroglycan (Figure 5.1H, green) at the plasma membrane. There was strong co-localization of all three proteins (Figure 5.1H, co-localization represented in purple), consistent with previous literature of this complex observed in different tissues (Boucard et al., 2014; Chand et al., 2012; 2014). Further analyzes of ADGRL1, the putative receptor for TCAP-1, were analyzed for muscle fibre patterning. The tibialis anterior muscle is comprised mainly of type II fibres, which are broken down into two further types; type Ila/x fibres and type IIb fibres. Type Ia/x fibres are generally much smaller in diameter compared to type IIb fibres, thus, size of fibres was used as a proxy for fibre type. Immunofluorescent analyses showed that ADGRL1 expression was significantly higher on small- and moderate-sized fibres compared to large-sized fibres (p<0.0001; Figure 5.1I-J), suggesting ADGRL1 is more abundant on type Ila/x fibres. Thus, the validation of genetic and protein expression of the teneurins, TCAPs, and ADGRLs in skeletal muscle is consistent with the ligand-receptor model established in neurons.
Figure 5.1. Skeletal muscle contains the molecular components required for the teneurin/TCAP-ADGRL interaction. RT-PCR mRNA expression analyzes of murine C2C12 skeletal muscle cells (A-C) and mouse hind limb muscle extract (D-F) were performed for all four teneurin, four TCAP and three ADGRL isoforms. β-actin was used as positive control. mRNA expression shows skeletal muscle both in vitro and in vivo expresses teneurins, TCAPs and ADGRLs. Western blot analyzes of C2C12 cell lysates corroborate these findings at the protein level (G). Rat tibialis anterior muscle sections demonstrate β-dystroglycan (β-DG, green), teneurin-3 (TEN3, blue), and ADGR1 (red) show strong co-localization of this complex in muscle, represented by the colour purple (white arrows; H). ADGRL1 shows specific fibre pattern expression (I), as it is significantly expressed higher in smaller sized fibres (black arrows) compared to larger fibres (grey arrows; J). (Mean ± SEM; n=5; ****p<0.0001; t-test).
5.4.2. TCAP-1 can modulate downstream targets of ADGRL1 signaling, diacylglycerol (DAG) and inositol triphosphate (IP3).

ADGRL downstream signaling has been associated with phospholipase C (PLC)-inositol triphosphate (IP3)/diacylglycerol (DAG) pathway as shown by stimulation with its exogenous ligand, α-latrotoxin (α-LTX; Davletov et al., 1998; Rahman et al., 1999), thus the effect of TCAP-1 on these downstream molecular signals was investigated in the C2C12 skeletal muscle cell model. TCAP-1 significantly increases DAG levels at 5 minutes and 15 minutes (p<0.0001 and p=0.004, respectively), followed by significant reduction at 60 minutes (p=0.0009) compared to vehicle treatment (Figure 5.2A). Moreover, TCAP-1 significantly increases IP3 levels at 1, 5 and 15 minutes of treatment compared to vehicle treatment (p<0.0001; Figure 5.2B). These results indicate TCAP-1 can modulate downstream signaling of ADGRL1, thereby corroborating the functionality of the teneurin/TCAP-ADGRL complex in skeletal muscle.

Figure 5.2. TCAP-1 significantly increases intracellular diacylglycerol (DAG) and inositol triphosphate (IP3) levels in C2C12 skeletal cell line. C2C12 cells were treated with either vehicle (ddH2O) or TCAP-1 (100 nM) and measured for DAG and IP3 levels. TCAP-1 significantly increases DAG levels at 5 and 15 minutes (p<0.0001, p=0.004, respectively), followed by significant reduction at 60 minutes (p=0.0009) compared to vehicle treatment (A). TCAP-1 significantly increases IP3 levels at 1, 5 and 15 minutes of treatment compared to vehicle treatment (p<0.0001; B). (Mean ± SEM; n=4-5; **p<0.01, ***p<0.001, ****p<0.0001; two-way ANOVA).
5.4.3. TCAP-1 induces a biphasic calcium response in C2C12 myotubules.

The exogenous ligand of ADGRL, α-LTX, stimulates a large release of calcium via IP3 and DAG production after binding ADGRL, that ultimately leads to neurotransmitter release. Thus, as ADGRL and IP3 have roles in calcium, the roles of TCAP-1 in calcium modulation were investigated. C2C12 cells were differentiated and pre-loaded with the cytosolic calcium fluorescent indicator, fluo-4-AM, where changes in fluorescence are directly proportional to changes in cytosolic calcium levels. Myotubules were bath-perfused with either sham (Locke’s buffer) or TCAP-1 (100 nM; Figure 5.3A). TCAP-1 treatment induced two significant peaks in cytosolic calcium; one occurred within 20 seconds (p<0.05, Figure 5.3B) and the other after 3 minutes (p<0.05, Figure 5.3C) of application. At the end of the experiment, caffeine (4 mM) was applied as a positive control for calcium release. Both groups responded comparably in response to caffeine treatment as there was no significant difference between the treatments (Figure 5.3D).
Figure 5.3. TCAP-1 induces a biphasic calcium response in C2C12 myotubules. Fully differentiated C2C12 myotubules were pre-loaded with the cytosolic calcium fluorescent indicator, fluo-4-AM. Cells were bath perfused with either TCAP-1 (100 nM; green) or sham (Locke’s buffer; black) treatment (A). Two peaks of cytosolic calcium were induced occurring within 20 seconds (B) and 3 minutes (C) of TCAP-1 application. Both groups responded comparably to caffeine (4 mM) stimulation (D). (Mean ± SEM; n=7-8; *p<0.05; t-test).
5.4.4. TCAP-1 induces calcium release from the SR in C2C12 myotubes, as evidenced by decreased caffeine response.

Next, to investigate if the source of the calcium was from the SR, C2C12 myotubules were stimulated with caffeine. Caffeine is an agonist to the ryanodine receptor (RyR) on the SR which induces a potent calcium release, however, the diffusion efficacy of calcium release is dependent on calcium concentrations within the SR. When caffeine (4 mM) was applied, the C2C12 myotubes exhibited a significant peak in cytosolic calcium (p<0.0001), with gradual decrease over 5 minutes as expected (Figure 5.4A). To visualize the effects of TCAP-1, cells were pre-treated with TCAP-1 (100 nM) for 1 hour before stimulating with caffeine (Figure 5.4A). TCAP-1 pre-treatment decreased caffeine-induced calcium peak in the myotubules (p=0.08; Figure 5.4B). Upon further analysis, TCAP-1 significantly decreased the rate of caffeine-induced calcium release (p=0.0064; Figure 5.4C) but had no effect on rate of calcium re-uptake (Figure 5.4D). Sham-switch experiments show no changes in baseline calcium levels, demonstrating there is no false positive fluorescence from switching between drug perfusion bottles (Figure 5.4A). These data indicate that TCAP-1 likely stimulates calcium release from the SR.
Figure 5.4. TCAP-1 induces calcium release from the SR in C2C12 myotubes, as evidenced by decreased caffeine response. Fully differentiated C2C12 myotubes were pre-loaded with the cytosolic calcium fluorescent indicator, fluo-4-AM. Caffeine was used as stimulant for calcium release into the cytosol (red). C2C12 cells were then pre-treated with TCAP-1 (100 nM) for 1 hour and stimulated with caffeine (A). TCAP-1-treated cells decreased peak calcium signal (B). TCAP-1 significantly lowers rate of caffeine-induced calcium release (p=0.0064, C), but has no effect on rate of calcium reuptake (D). (Mean ± SEM; n=4; **p<0.01; t-test).
5.4.5. TCAP-1-induced calcium responses are blocked by 2-APB, an IP3R inhibitor.

To further interrogate the mechanism of TCAP-1 action on SR-mediated calcium, 2-aminoethoxydiphenyl borate (2-APB), an IP3R inhibitor, was used. 2-APB was pre-treated to C2C12 myotubules for 4 minutes and significantly reduced baseline cytosolic calcium levels by approximately 40% (p<0.0001; Appendix I). Subsequently, sham (Locke’s buffer) or TCAP-1 (100 nM) was applied. 2-APB blocked the TCAP-1-mediated calcium response, as shown by the loss of both calcium peaks (Figure 5.5A-C). These data indicate that IP3R is critical for TCAP-1-mediated calcium release. Moreover, both groups did not respond to caffeine stimulation (Figure 5.5D), which is further evidence of the potent actions of the inhibitor, as some authors have reported it has non-specific effects on other calcium pathways (Bootman et al., 2002).
Figure 5.5. Application of 2-APB, an IP3R inhibitor, blocks TCAP-1 mediated calcium response. C2C12 myotubules were pre-treated with 2-APB (100 µM), an IP3R inhibitor, for 4 minutes which resulted in about a 40% decrease in calcium baseline levels, demonstrating the efficacy of the inhibitor (Appendix I). Myotubules were then treated with either sham (Locke’s buffer) or TCAP-1 (100 nM) for 5 minutes, and subsequently treated with caffeine (4 mM) for 2 minutes at the end of the experiment (A). 2-APB inhibited both the first peak (B) and second peak (C) elicited by TCAP-1. Moreover, 2-APB also blocked caffeine-induced calcium response in both groups (D). (Mean ± SEM; n=4; a and b represent statistically different values; one-way ANOVA).
5.4.6. TCAP-1 increases calcium importation into the mitochondria in C2C12 myotubules.

The next question was to investigate the effects of TCAP-1-mediated calcium release. One candidate organelle for this was the mitochondria, considering that mitochondria are one of the largest calcium sinks within the cell, and that calcium has stimulatory roles upon enzymes in the TCA cycle. Moreover, TCAP-1 has roles in energy and glucose regulation in neurons and skeletal muscle (Hogg et al., 2018; Chapter 4), which may suggest TCAP-1 stimulates mitochondrial activation. Rhodamine-2-AM (Rhod-2) is a fluorescent indicator of mitochondrial calcium levels, where an increase in fluorescence indicates an increase in mitochondria calcium levels. C2C12 myotubules were pre-loaded with Rhod-2 and treated with either sham (Locke’s buffer) or TCAP-1 (100 nM). C2C12 myotubules treated with TCAP-1 had significantly increased mitochondrial calcium levels within 4 minutes of treatment (p<0.0001; Figure 5.6A, B). This data indicates that TCAP-1 increases calcium importation into the mitochondria.

![Figure 5.6. TCAP-1 increases mitochondrial calcium levels in C2C12 myotubules. C2C12 myotubules were pre-loaded with the mitochondrial calcium indicator, Rhodamine-2 (Rhod-2). C2C12 myotubes were treated with either sham (Locke’s buffer) or TCAP-1 (100 nM; A). TCAP-1-treated cells had significantly increased mitochondrial calcium levels compared to sham after treatment (B). (Mean ± SEM; n=5; ****p<0.0001; t-test).](image)
5.4.7. **TCAP-1 hyperpolarizes the mitochondrial membrane in C2C12 myotubules.**

As TCAP-1 increases calcium shuttling into the mitochondria, the mitochondrial membrane potential would be affected as result. For these experiments, Rhodamine-123 (R123), a self-quenching dye, was used to measure mitochondrial membrane potential, where an increase in fluorescence indicates mitochondrial membrane depolarization. C2C12 myotubules were pre-loaded with R123 and were treated with either sham (Locke’s buffer) or TCAP-1 (100 nM). TCAP-1-treated cells underwent a significant mitochondrial membrane hyperpolarization compared to sham after approximately 3 minutes of treatment (p<0.0001; Figure 5.7A-B). In addition, FCCP, a protonophore used as a positive control for membrane depolarization, was administered in the last 2 minutes of the experiment. TCAP-1-treated cells had a slightly higher sensitivity to FCCP actions, as demonstrated by an increase in mitochondrial membrane depolarization rate compared to sham (Figure 5.7C).
Figure 5.7. TCAP-1 hyperpolarizes the mitochondrial membrane in C2C12 myotubules. Fully differentiated C2C12 myotubules were pre-loaded with the mitochondrial membrane potential indicator, Rhodamine-123 (R123). C2C12 myotubes were treated with either sham (Locke’s buffer) or TCAP-1 (100 nM), with a positive control for mitochondrial depolarization by FCCP was administered in last 2 mins of experiment (A). TCAP-1-treated cells underwent a significant mitochondrial membrane hyperpolarization compared to sham (B). TCAP-1-treated cells had a slight increase in mitochondrial membrane depolarization rate compared to sham (C). (Mean ± SEM; n=7; ****p<0.0001; t-test).
5.4.8. TCAP-1 hyperpolarizes the mitochondrial membrane potential in C2C12 myotubules under stimulation conditions.

C2C12 myotubules were pre-treated with TCAP-1 (100 nM) for 1 hour before caffeine stimulation (Figure 5.8A). When caffeine was solely applied to the C2C12 myotubes, it resulted in a slight mitochondrial membrane depolarization at initial application, but otherwise had no effect on membrane potential. When the cells were pre-treated with TCAP-1 (100 nM) for 1 hour before caffeine stimulation, it resulted in a significant mitochondrial membrane hyperpolarization compared to caffeine stimulation alone and sham (p<0.05, Figure 5.7B). In addition, TCAP-1 pre-treatment resulted in a significantly higher rate of mitochondrial membrane depolarization by FCCP administration than caffeine or sham treatments (p<0.05, Figure 5.8C).
Figure 5.8. TCAP-1 hyperpolarizes the mitochondrial membrane in C2C12 myotubules under caffeine stimulation and enhances FCCP-induced mitochondrial depolarization rate. C2C12 myotubules were pre-loaded with the mitochondrial membrane potential indicator, Rhodamine-123 (R123). C2C12 myotubes were stimulated with caffeine (4 mM) or pre-treated with TCAP-1 (100 nM) for 1 hr before caffeine stimulation and positive control for mitochondrial depolarization by FCCP was administered in last 2 mins of experiment (A). TCAP-1-treated cells underwent a significant mitochondrial membrane hyperpolarization compared to sham and caffeine stimulation alone (B; mean ± SEM; n=7; *p<0.05; t-test). TCAP-1-treated cells had significantly higher mitochondrial membrane depolarization rate under FCCP administration compared to sham and caffeine stimulation alone (C). (Mean ± SEM; n=7; *p<0.05; one-way ANOVA).
5.4.9. TCAP-1 treatment significantly increased glucose uptake in C2C12 skeletal muscle cells via upregulation of GLUT4 translocation.

Glucose uptake was measured *in vitro* to investigate TCAP-1 actions in glucose regulation in skeletal muscle, as this action has been well established in neurons (Hogg et al., 2018). First, radioactively-labeled deoxy-D-glucose (³H-DG) was added to C2C12 myotubules treated with either vehicle (ddH₂O), TCAP-1 (100 nM), insulin (100 nM; positive control) or scrambled-TCAP-1 (Sc-TCAP-1; 100 nM; negative control). Deoxyglucose cannot be further metabolized in the glycolysis pathway once it enters the cell, thus it is a better representation of glucose uptake into the muscle cells. TCAP-1 treatment significantly increased ³H-DG uptake into C2C12 myotubules at 30 and 45 minutes (p<0.0001, p<0.001, respectively), peaking at 30 minutes with a 300% increase in glucose uptake compared to vehicle (Figure 5.9A). As expected, insulin treatment induced a significant increase in glucose uptake over 60 minutes, where scrambled-TCAP-1 had no effect (Figure 5.9A). Glucose must be facilitated into the cell via glucose transporters (GLUTs). GLUT4 is the most predominant GLUT isoform in skeletal muscle and is insulin-responsive, thus the ability of TCAP-1 to induce GLUT4 translocation was investigated. As GLUT expression precedes glucose uptake by several minutes, GLUT4 expression was measured at 15 and 30 minutes with exofacial antibodies in non-permeabilized C2C12 myotubules. This method allows the visualization of only active GLUT4 as it is docked to the sarcolemma. TCAP-1 significantly increased GLUT4 translocation at 30 minutes compared to vehicle, increasing GLUT4 plasma membrane expression by 55% (p=0.0007), similar to insulin actions (Figure 5.9B, C).
Figure 5.9. TCAP-1 significantly increases glucose uptake in C2C12 skeletal muscle cells in vitro via GLUT4 translocation. C2C12 myotubules treated with either vehicle (ddH$_2$O), TCAP-1 (100 nM), insulin (100 nM; positive control), or scrambled-TCAP-1 (Sc-TCAP-1; 100 nM; negative peptide control). TCAP-1 treatment significantly increased $^3$H-deoxy-D-glucose uptake at 30 and 45 mins compared to vehicle treatment, peaking at 330% increase at 30 mins (A). C2C12 myotubules were treated with either vehicle (ddH$_2$O), TCAP-1 (100 nM), or insulin (100 nM) and assayed for active GLUT4 after 15 and 30 mins. TCAP-1 significantly increases GLUT4 plasma membrane expression at 30 mins compared to vehicle, similar to insulin (B). Representative images of active GLUT4 shown in C, with white arrows pointing to areas of high GLUT4 activity after treatment. (Mean ± SEM; n=4; ***p<0.001, ****p<0.0001; two-way ANOVA).
5.4.10. **TCAP-1 increases ATP levels after 30 minutes.**

Based on these findings that TCAP-1 is upregulating glucose uptake in the muscle and is likely stimulating the mitochondria, ATP levels were measured. Enhanced mitochondrial activation results in increased ATP production, thus it would be expected that intracellular ATP levels are also affected under TCAP-1 administration. C2C12 cells were treated with either vehicle (ddH$_2$O) or TCAP-1 (100 nM) for 1 hour, and cells were lysed at 0, 15, 30 and 60 minutes. ATP is rapidly utilized in live cells, thus the lysing of the cells allowed for the measurement of ATP levels. TCAP-1-treated cells had significantly increased ATP levels compared to vehicle-treated cells after 30 minutes (p<0.0001; Figure 5.10).

![Graph showing ATP levels over time](image)

**Figure 5.10. TCAP-1 treatment increases intracellular ATP levels after 30 minutes of treatment.** C2C12 cells were treated with either vehicle (ddH$_2$O) or TCAP-1 (100 nM) and ATP levels were measured after 0, 15, 30 and 60 minutes of treatment. TCAP-1-treated cells had significantly increased ATP levels compared to vehicle-treated cells after 30 minutes of treatment (Mean ± SEM; n=8; ****p<0.0001; two-way ANOVA).
5.4.11. TCAP-1 increases NADH production rate in C2C12 skeletal muscle cells.

It would be expected that enhanced ATP levels would be accompanied with increases in other biomarkers of cellular metabolism, such as NADH. NADH is produced during glycolysis and oxidative phosphorylation, thus, increases in NADH turnover rate indicates an enhanced metabolic rate. The resazurin assay is a well established *in vitro* live-cell assay for measuring NADH. Upon reduction by NADH, resazurin undergoes an irreversible conformational change into resorufin and becomes fluorescent, thus an increase in fluorescence intensity is directly proportional to an increase in NADH production. To investigate the roles of TCAP-1 on glucose metabolism, C2C12 cells were seeded in a 96-well plate in resazurin solution. The following day, cells were treated with vehicle (ddH₂O) or TCAP-1 (100 nM) and measured over an hour. TCAP-1 treatment significantly increased NADH production rate at 15 minutes and remained elevated until the end of the experiment (p<0.0001, Figure 5.11). This analysis is consistent with previous experiments and strengthens the evidence for the roles of TCAP-1 in glucose metabolism.

![Figure 5.11. TCAP-1 treatment increases NADH production rate in C2C12 cells.](image)

C2C12 cells were seeded at 10,000/well in a 96-well plate. Resazurin solution was added to each well and fluorescence was measured at 590 nm, which is directly proportional to NADH turnover. TCAP-1 treatment significantly increased NADH production rate after 15 mins and remained elevated until end of experiment at 60 minutes. (Mean ± SEM; n=24; **p<0.01, ***p<0.001, ****p<0.0001; two-way ANOVA).
5.4.12. *TCAP-1 increases succinate dehydrogenase (SDH) protein levels.*

ATP and NADH are both products of the TCA cycle and glycolysis, thus to further investigate if this increase in cellular metabolism is mediated by the mitochondria, the enzyme succinate dehydrogenase (SDH) was also measured. SDH participates both in the TCA cycle and the electron transport chain (ETC), thus increases in SDH represent an increase in mitochondrial activity (Cimen et al., 2010). C2C12 myotubules were treated with either vehicle (ddH2O) or TCAP-1 (100 nM) for 1 hour. Western blot analyzes revealed that TCAP-1-treated cells had significantly increased SDH catalytic subunit-A (SDH-A) levels compared to vehicle-treated cells (p<0.05; Figure 5.12).

![Figure 5.12. TCAP-1 treatment increases SHD-A protein levels.](image)

*C2C12 cells were treated with either vehicle (ddH2O) or TCAP-1 (100 nM) for 1 hr. TCAP-1 treatment significantly increased SDH-A protein levels compared to vehicle treatment (A). Representative blots shown in B. (Mean ± SEM; n=4; *p<0.05; t-test).*
5.5. Discussion

In this work, the molecular mechanism of action of teneurin C-terminal associated peptide (TCAP)-1 was investigated in skeletal muscle cell culture. It was first shown that skeletal muscle expresses teneurin, TCAP, and ADGRL isoforms, and that they co-localize predominately on small- and moderate-sized fibres. Next, as ADGRL is associated with the PLC/IP3 pathway, the effects of TCAP-1 were investigated on these downstream targets. TCAP-1 treatment increased DAG and IP3 levels. ADGRL stimulates calcium release via IP3R after α-latrotoxin (α-LTX) treatment, thus changes in cytosolic calcium were measured under TCAP-1 application. TCAP-1 treatment induced a biphasic calcium release out of the SR, which was inhibited by the IP3R inhibitor, 2-APB. Moreover, it was found that TCAP-1 increases calcium importation into the mitochondria that is corroborated by TCAP-1-induced hyperpolarization of the mitochondrial membrane. As calcium has stimulatory effects on mitochondrial enzymes (Denton, 2009; Hansford, 1985; Wan et al., 1989), this would increase mitochondrial activity. Consistent with this, TCAP-1 increased glucose uptake, ATP and NADH turnover rate, indicating enhanced cellular metabolism. Lastly, TCAP-1 increased SDH-A levels, suggesting that TCAP-1-mediated cellular metabolism works via mitochondrial pathways, and may stimulate mitochondrial biogenesis. This work is the first to demonstrate the potent roles of TCAP-1 in calcium and glucose regulation in skeletal muscle and provides critical insight into the molecular mechanism of action of TCAP-1.

The first step was to determine that skeletal muscle contains the required molecular components to induce teneurin/TCAP-ADGRL actions. In recent years, the binding of teneurin to ADGRL has been described in the brain (Boucard et al., 2014; Silva et al., 2011; Woelfle et al., 2015); however, this complex interaction has not been well described in other tissues. Additionally, previous studies have shown that teneurin and TCAP co-localize with the dystroglycan complex in neuronal (Chand et al., 2012) and peripheral tissue (Chand et al., 2014), thus it was also investigated if this is conserved in muscle. ADGRL1-3 expression has been previously identified in rat skeletal muscle (GenBank gene ID: 65096, 171447, 170641, respectively), thus this work focused on elucidating its expression in C2C12 skeletal muscle cells as well as mouse skeletal muscle. The presence of the ligand-receptor complex of TCAP, teneurin and ADGRL was successfully established in
skeletal muscle via genetic expression (Figure 5.1A-F) and protein expression (Figure 5.1G). Moreover, this ligand-receptor complex was also found to be strongly co-localized in skeletal muscle tissue, along with β-dystroglycan, consistent with previous literature (Figure 5.1H; Chand et al., 2012, 2014). The expression of ADGRL1 was further investigated as it was presenting a strong pattern. ADGRL1 was significantly more expressed on smaller or moderately sized muscle fibres than on larger ones (Figure 5.1I). The tibialis anterior is comprised of approximately 95% large type II fibres, where type I and IIa/x fibres are generally smaller in size when compared to type IIb fibres. Thus, if this is used as a proxy for identification of fibres, it may suggest that ADGRL1 is more predominately expressed on type I or IIa/x fibres. ADGRL1 expression in skeletal muscle has not been well described, thus it is not known if it is muscle fibre-type specific.

To further show the association with this complex, downstream signaling cascades associated with ADGRL, such as the PLC-IP3 pathway, were investigated (Davletov et al., 1998; Rahman et al., 1999). α-LTX, the toxic component of black widow spider venom, is another known ligand to ADGRL, which stimulates IP3 and DAG production to elicit large calcium release from the SR via IP3R (Davletov et al., 1998; Rahman et al., 1999). TCAP-1 treatment in immortalized murine C2C12 skeletal muscle cells resulted in a significant increase in IP3 and DAG levels (Figure 5.2). The finding that TCAP-1 can modulate these downstream signaling molecules of ADGRL similar to other known ligands of ADGRL strongly suggests that TCAP-1 is working through the teneurin/TCAP-ADGRL complex, similar to its role in neurons.

Moreover, as ADGRL and IP3 are associated with calcium, the role of TCAP-1 in calcium modulation was investigated. TCAP-1 induced a rapid biphasic response, where there were two significant peaks of cytosolic calcium compared to sham treatment in C2C12 myotubules (Figure 5.3A-C). Both groups responded comparably to the positive control, caffeine (Figure 5.3D), indicating normal and healthy function. These data suggest that TCAP-1 induces calcium release, likely via IP3 production stimulating IP3R on the SR. To show that the calcium release is coming from the SR, C2C12 myotubules were pre-treated with TCAP-1 for 1 hour, and then stimulated with caffeine (Figure 5.4A). These cells showed less sensitivity to caffeine to evoke a calcium signal compared to cells that were not pre-treated with TCAP-1 (Figure 5.4B). Further analysis
showed it was due to a significantly lowered rate of calcium release under caffeine application (Figure 5.4C), while rate of calcium re-uptake was unaffected (Figure 5.4D). There are a few factors that can impact caffeine-induced SR calcium release. One of the largest driving factors being calcium concentration in the SR. If TCAP-1 lowered calcium concentration in the SR before caffeine stimulation, this would result in lowered caffeine-induced calcium release from the SR. One potential mechanism of this is via activation of IP3R-mediated calcium release. This would be stimulated by the increased IP3 levels produced by TCAP-1 treatment. To further interrogate this mechanism, 2-aminoethoxydiphenyl borate (2-APB), an IP3R inhibitor, was utilized. C2C12 myotubules were pre-treated with the inhibitor for 4 minutes before application of TCAP-1 (Appendix I). Under these conditions, TCAP-1 did not induce any changes in cytosolic calcium, indicating that the IP3R is critical for TCAP-1 mechanism of action (Figure 5.5A-C). It is important to note, however, that 2-APB has been shown to block other non-specific calcium channels such as the ryanodine receptor (RyR) and transient receptor potential (TRP) channels, which explains why the cells treated with 2-APB could not respond to caffeine stimulation (Figure 5.5D; Bootman et al., 2002). Taken together with the observed increase in IP3 production, this data clearly demonstrates a novel role of TCAP-1 in calcium modulation, likely via the IP3R on the SR.

Next, the role of the TCAP-1-mediated calcium release was investigated. As TCAP-1 has roles in calcium modulation and energy regulation, the prime candidate to investigate was the mitochondria. Mitochondria in skeletal muscle are important to produce the energy required for muscle function and metabolism, and as well are major calcium sinks. Investigation into mitochondrial calcium levels by fluorescent indicator Rhodamine-2-AM (Rhod-2) demonstrated that TCAP-1 treatment significantly increases mitochondrial calcium levels compared to sham in C2C12 myotubules (Figure 5.6), suggesting that TCAP-1 shuttles calcium from the SR to the mitochondria. When calcium is imported into the mitochondria via the mitochondrial calcium uniporter (MCU), it stimulates enzymes in the TCA cycle, specifically glycerol phosphate dehydrogenase, pyruvate dehydrogenase phosphatase, isocitrate dehydrogenase and oxoglutarate dehydrogenase (Denton, 2009; Wan et al., 1989). As a result of enhanced enzymatic activity pumping protons into the inner mitochondrial space (IMS), there are changes in mitochondrial membrane potential. Thus, to investigate mitochondrial activation, C2C12 myotubules were
administered Rhodamine-123 (R123), a live-cell fluorescent indicator of mitochondrial membrane potential, where an increase in fluorescence indicates mitochondrial depolarization. TCAP-1 treatment caused significant hyperpolarization of the mitochondrial membrane over 5 minutes of treatment (Figure 5.7A, B). FCCP, a protonophore that depolarizes the mitochondrial membrane, was applied during the last two minutes of the experiment as a control. Interestingly, TCAP-1-treated cells had an increased rate of depolarization under FCCP conditions than sham alone (Figure 5.7C). Hyperpolarization likely indicates the proton gradient in the IMS is affected, where there is more positive charge in the space. This suggests that the TCAP-1-mediated calcium importation into the mitochondria stimulates the ETC to pump more protons into the IMS, resulting in an overall hyperpolarization of the membrane. This would also provide an explanation for greater FCCP influence under TCAP-1 treatment. As FCCP proton driving force is dependent on the proton gradient in the IMS, a higher proton gradient would result in a greater depolarization event under FCCP stimulation. This finding was replicated under stimulation conditions. Myotubules were pre-treated with TCAP-1 for 1 hour before caffeine stimulation (Figure 5.8A). These myotubules underwent significant hyperpolarization even when treated with caffeine compared to when caffeine was treated alone (Figure 5.8B), as well as had significantly increased depolarization rate under FCCP administration (Figure 5.8C). This data suggests that TCAP-1 activates the mitochondria by enhancing ETC proton pumping with increased calcium import and thus may explain enhanced metabolism and function results under TCAP-1 treatment.

With enhanced mitochondrial activity, specifically increased TCA cycling, there is typically a corresponding increase in glucose uptake, thus the next step was to investigate the role of TCAP-1 in glucose regulation in skeletal muscle. In neurons, TCAP-1 significantly increases glucose uptake via upregulation of glucose transporter, GLUT-3, to the plasma membrane in order to facilitate diffusion of glucose into the cell. This ultimately leads to increased ATP production via aerobic pathways, as suggested by decreases in lactate levels (Hogg et al., 2018). Muscle function requires high energy demands, thus the role of TCAP-1 in skeletal muscle metabolism was investigated. First, radioactive $^3$H-deoxyglucose uptake was assessed in C2C12 myotubules after TCAP-1 administration. TCAP-1 induced a significant increase in glucose uptake, peaking at 30 minutes, which lasted until 45 minutes (Figure 5.9A), similar to its glucose uptake profile in neurons. TCAP-1 did not have a similar glucose uptake profile to insulin, which had increased...
glucose uptake over 60 minutes, peaking at 45 minutes (Figure 5.9A). As the predominant GLUT isoform in skeletal muscle is GLUT4, its translocation was measured by antibody labelling in non-permeabilized cells, thereby only showing active GLUT4 expression. Both TCAP-1 and insulin demonstrated a significant increase in GLUT4 activation at the sarcolemma (Figure 5.9B, C), thus revealing the mechanism of TCAP-mediated glucose uptake in skeletal muscle. In summation, these data indicate that TCAP-1 is a highly potent insulin-independent glucose regulator. Further, intracellular ATP levels were significantly increased in TCAP-1-treated cells compared to vehicle-treated cells (Figure 5.10), thus supporting these data that indicate TCAP-1 increases cellular metabolism. Consistent with this, NADH production rate was also found to be increased under TCAP-1 administration (Figure 5.11).

ATP and NADH are products of both glycolysis and the TCA cycle. Thus, to further investigate if the TCAP-1-mediated ATP production was derived from the mitochondria, succinate dehydrogenase (SDH) protein levels were measured in C2C12 cells. SDH participates in both the TCA cycle and the ETC by greatly contributing to the building up of the proton gradient (Van Vranken et al., 2014). Thus, SDH is an ideal target to demonstrate mitochondrial actions (Cimen et al., 2010) TCAP-1-treated cells had significantly increased SDH-subunit-A (SDH-A) protein levels compared to vehicle-treated cells after 1 hour of treatment (Figure 5.12). This finding was consistent with TCAP-1 stimulating mitochondrial activation via calcium importation. However, this data may also suggest that TCAP-1 is increasing mitochondrial biogenesis, as that would also result in an increase in SDH-A levels (Cimen et al., 2010).

Taken together, these data provide the foundation for a proposed mechanism of TCAP-1 action in skeletal muscle. When TCAP-1 is administered to the cells, TCAP-1 binds with ADGRL1 and stimulates the activation of G-protein coupled PLC (Figure 5.13). This leads to the increased conversion of PIP3 into IP3 and DAG. Increased IP3 levels stimulates the IP3R on the SR, which results in a calcium release, causing a rise in cytosolic calcium levels. This calcium is then imported into the mitochondria via the MCU which stimulates the TCA cycle and ETC via increases in enzymatic activity (Figure 5.14). Enhanced ETC activity results in increased proton levels in the IMS, causing hyperpolarization of the mitochondrial membrane. This ultimately
results in increased ATP and NADH production, as well as increased SDH-A levels. Calcium is subsequently pumped out of the mitochondria via sodium-calcium exchangers (NCX), thus restoring homeostatic levels of calcium.

This proposed mechanism explains why TCAP-1 lowered caffeine-induced calcium release as this release is dependent on calcium concentrations in the SR. As TCAP-1 stimulates calcium release via IP3R, as shown by 2-APB, an IP3R inhibitor, this lowers the concentration thus lowers the rate of release. Moreover, it also explains why FCCP actions would be enhanced under TCAP-1 treatment. As TCAP-1 increases the number of protons in the IMS as evidenced by hyperpolarization, there would be higher proton drive under FCCP conditions resulting in an enhanced depolarization event. In terms of glucose regulation, enhanced mitochondrial activity promotes glucose uptake into the cell. TCAP-1 upregulates GLUT4 translocation to facilitate glucose uptake in a similar manner, yet independent of, insulin. Importantly, insulin has been shown to require IP3R-mediated calcium release to stimulate GLUT4 translocation (Contreras-Ferrat et al., 2010), which is consistent with TCAP-1 actions. Lastly, the role of TCAP-1 in stimulating mitochondrial activity is evident, as seen by increases in ATP and NADH production.

Thus, this work for the first time demonstrates that the teneurin/TCAP-ADGRL complex is expressed in skeletal muscle and has a functional role in energy metabolism and skeletal muscle function. Moreover, this work indicates TCAP-1 enhances mitochondrial activation via calcium and glucose regulation.
Figure 5.13. **Schematic of proposed TCAP-1 mechanism of action.** The first phase of the TCAP-1 response begins with the binding of TCAP-1 to ADGRL1, which activates IP3 production via PLC. This stimulates potent calcium release. The second phase begins when the calcium is imported into the mitochondria, stimulating enzymatic activity of the ETC which ultimately leads to hyperpolarization of the mitochondrial membrane. Moreover, the calcium also leads to GLUT4 translocation which facilitates glucose uptake into the cell. Thus, this schematic represents a possible mechanism of action of the teneurin/TCAP-ADGRL complex on glucose metabolism regulation via calcium modulation. Abbreviations: IP3, inositol triphosphate; PLC, phospholipase C; GLUT; glucose transporter.
Figure 5.14. A schematic of mitochondrial metabolism after TCAP-1 treatment. (1) TCAP-1 mediates calcium import into the mitochondria via the mitochondrial calcium uniporter (MCU). (2) Calcium stimulates enzymes in the TCA cycle. (3) This results in the increased production of NADH and FADH$_2$. (4) Increased NADH and FADH$_2$ stimulates higher respiration rate of the electron transport chain, thus resulting in an increase of protons in the IMS, which causes mitochondrial hyperpolarization ($\Psi$mit). (5) Higher proton gradient stimulates an increased rate of ATP production, thus increasing ATP levels in the cell. Calcium is subsequently pumped out of the mitochondria via NCX exchanger. Arrows indicate movement of electrons across ETC. Abbreviations: IMM, inner mitochondrial membrane; IMS, inner membrane space; I, complex I; II, complex II; III, complex III; IV, complex IV; V, ATP synthase. Q, coenzyme Q; CytC, cytochrome C; NCX, sodium-calcium exchanger.
Chapter 6
Final Discussion and Concluding Remarks

6.1 Abstract

The aim of this research was to investigate the roles of the TCAP family with respect to their skeletal muscle function under normal and stress-induced conditions. Because the role of TCAP in muscle physiology has not been examined in the past, and that the gene is conserved throughout chordates, three models of TCAP action were utilized. Using the protochordate, vase tunicate, *Ciona intestinalis*, the cyprinid zebrafish *Danio rerio*, and the rodent, *Rattus norvegicus*, the actions of TCAPs were examined in all animals. This research established that the TCAP peptides, notably TCAP-1 and -3, are active in muscle physiology and metabolism in these three distinct models. This investigation in tunicates, zebrafish and rodents established that the TCAP family has conserved roles in glucose regulation and contractile function in skeletal muscle (Table 6.1). Moreover, the observed enhanced ATP production results in an increased threshold for stress, as demonstrated by a decrease in stress-related responses with TCAP treatment. This work is also the first to demonstrate the expression and function of the teneurin/TCAP-ADGRL system in skeletal muscle, revealing a novel potential mechanism of action for the TCAP family. Thus, this research provides new insight into the dynamic regulation of skeletal muscle function by TCAP and may represent a significant contribution to the field of muscle metabolism and function. Moreover, these data indicate the role of TCAP in metabolism and muscle physiology was established before the evolution of the chordates. In addition, this work may provide a novel understanding of the mechanisms associated with skeletal muscle pathology.

6.2 Significance of the Teneurin-Latrophilin Mechanism

Stress and energy metabolism are intrinsically linked, as stress demands a higher energy budget for an organism to overcome a stressor. This would have likely been a major challenge in the first metazoans. Recently, TCAP-1, a novel bioactive peptide, was shown to enhance glucose metabolism in the brain. This led to question what roles TCAP-1 may play in skeletal muscle, as
muscle is heavily involved in both organismal metabolism and the stress response. Moreover, as the TCAPs are one of the most highly conserved peptide families, the conservation of these roles in metabolism were also examined across species. For these reasons, three distinct species that included basal chordates, and the two main branches of vertebrate phylogeny, the Actinopterygii (bony fishes) and the Sarcopterygii (lobe-finned fishes and tetrapods), were utilized.

6.2.1 Studies on the Protochordate, Ciona intestinalis

The studies in Chapter 2 were performed on *C. intestinalis*, a protochordate that represents the phylogenetic sister lineage to the chordates. *C. intestinalis* only has one homologue of TCAP, termed cTCAP (Colacci et al., 2015), and one isoform of a CRF/DH-like peptide, termed CDLP (Lovejoy and Barsyte-Lovejoy, 2010). In addition, the muscle wall of *C. intestinalis* is simple in organization, yet shares many similarities with vertebrate muscle (Nevitt and Gilly, 1986), making this an ideal organism for the study of these peptide systems in muscle function. RT-PCR and Western blot analysis revealed cTCAP and CDLP expression in various *C. intestinalis* tissues, including the brachial basket, buccal siphons and cloacal siphons, which represent the muscle walls of *C. intestinalis* (Figure 2.4). cTCAP-treated *C. intestinalis* also exhibited an increased number of buccal opening contractions (BOC), cloacal opening contractions (COC) and lateral contractions (LAT) compared to CDLP-treated animals, demonstrating an overall increase in specific contractile activity (Figure 2.5; Figure 2.6).

Additionally, as part of this study, a feeding response component was incorporated to further characterize *C. intestinalis* stress-response and its corresponding behaviours. CDLP-treated animals underwent significant expulsion response to feeding stimulation, which was not observed in cTCAP-treated animals (Figure 2.7). In contrast, cTCAP-treated animals demonstrated behaviour that promoted food intake (Figure 2.7). A CRF-induced expulsion event can also be seen in vertebrate models (Enck and Holtmann, 1992), as this response is physiologically similar to emptying of stomach by purging. Moreover, CDLP-treated *C. intestinalis* showed a significant increase in cloacal aperture contractions. This is also consistent with vertebrate models where an increase in colonic motility and inhibition of food intake has been well described after increases
in CRF (Taché et al., 2004; Zorilla et al., 2003). In contrast, the behaviours witnessed in cTCAP-treated animals were associated with the acceptance of food intake. cTCAP is suggested to be an ancient protein that is involved with energy regulation and metabolism (Woelfle et al., 2015), which would be consistent with the results shown here. Overall, the distinct actions of cTCAP and CDLP were clearly visualized in *C. intestinalis* with respect to stress-related behaviour.

Lastly, cTCAP treatment resulted in an increase in number of contractions compared to sham and CDLP treatments, indicating that cTCAP increases contractile activity. The siphons are lined with muscle units that transduces these contractions, similar to those found in vertebrates (further discussed in section 6.5.1; Nevitt and Gilly, 1986; Oshiro et al., 2010). Although these studies established a potential role of cTCAP in muscle contractile activity, it also suggests a role in energy regulation as cTCAP-treated animals demonstrated behaviours that promote food intake, consistent with earlier postulations on the role of teneurins.

The methodology of administering peptides directly into the buccal opening of the tunicate does not appear to have been reported in the literature previously. During this study, other methods of peptide administration were considered such as injecting directly into the body cavity, or one of the organs such as the heart or stomach. However, given the small size of the target and the lack of rigidity of these organs, it was considered to be too invasive. Therefore, a pilot study was used to determine if peptides, solubilized in seawater, administered directly into the buccal opening could induce an effect. These studies indicated that this was a viable way of introducing novel peptide hormones (Colacci et al., 2015). The method of uptake into the vasculature, however, is not clear. This method should allow the peptides to be taken into the brachial basket direct. From here, cilia-lined cells could direct incoming currents into the gastrointestinal tract (Armsworthy et al., 2001). Theoretically, peptide transporter mechanisms in the gastrointestinal tract may then introduce the peptides into the vasculature, where they have actions on presumably the nervous system or directly upon skeletal muscle (Kawada et al., 2010). In any case, the administration protocol appears to have merit and could be used to study the actions of other peptide hormones in tunicates for future studies. These studies indicated that TCAP could potentially regulate both metabolism and muscle activity, but unfortunately, little is known about the physiology of either
system. For these reasons, a zebrafish model was employed to investigate the aspects of organismal metabolism to determine if these metabolic actions of TCAP are present in a vertebrate species.

6.2.2 Studies on the actinopterygian, Danio rerio.

The zebrafish, (Danio rerio) is a member of the Cypriniforms order of teleost fishes which belongs to a relatively basal order of teleosts. Because of its phylogenetic position, and that it has been utilized as a model species for a number of years, it is a particularly useful model to study metabolism (Seth et al., 2013). Also, because of its small size it can be easily manipulated for various experimental procedures. Thus, the goal of the research indicated in Chapter 3 was aimed at investigating the roles of rtTCAP-3 in organismal and skeletal muscle metabolism in this species.

Considering that TCAP-3 had not yet been studied in zebrafish, its endogenous expression was unknown. RT-PCR analyzes revealed that TCAP-3 mRNA was expressed in both zebrafish skeletal muscle and brain tissue (Figure 3.2). The metabolic pathways and muscular architecture in zebrafish are similar to those in rodents and humans, making this an ideal organism to study. The first step was to develop a novel method of studying metabolism in zebrafish across all life stages. The use of resazurin to measure increases and decreases in metabolic activity under experimental conditions was established both in zebrafish larvae and adults (Figure 3.5-3.8). Validation of the use of resazurin in vivo thus allowed for the use of this novel assay to investigate the possible roles of rtTCAP-3 in metabolism. After 24 hours of treatment, 5 µM and 10 µM doses of rtTCAP-3 significantly increased total resorufin FI in zebrafish larvae compared to all other treatments, thereby showing an increase in metabolic activity. At 48 hours post-treatment, both doses maintained elevated metabolism compared to other treatments; however, animals treated with 10 µM rtTCAP-3 also had an increased metabolic rate compared to 5 (Figure 3.9). Similarly, rtTCAP-3-treated adult male zebrafish exhibited increased total resorufin FI compared to vehicle-treated animals at 48 hours (Figure 3.10). The metabolic actions of rtTCAP-3 were then tested under low temperature stress conditions. rtTCAP-3-treated larvae tested under 48 hours of low temperature stress showed no signs of diminished metabolism, while vehicle-treated animals had
lowered metabolism (Figure 3.11), indicating that rtTCAP-3 is able to maintain elevated metabolism under stress. This was corroborated by studies that tested zebrafish adults in stressful conditions in a Loligo metabolic respirometry chamber. Recordings in basal conditions revealed that rtTCAP-3-treated adult zebrafish had significantly higher oxygen consumption rates compared to vehicle-treated adults. When the stress protocol was initiated by an increase in flush force of the chamber, rtTCAP-3-treated animals maintained elevated oxygen consumption rate and responded better to the stress than vehicle-treated animals (Figure 3.12). These studies demonstrate that rtTCAP-3 is a potent metabolic regulator in zebrafish larvae and adults in both basal and stress conditions. Lastly, the effect of rtTCAP-3 was investigated in vitro, utilizing primary muscle cells from rainbow trout (O. mykiss) termed rainbow trout myogenic precursor cells (rtMPCs). In this study, rtTCAP-3 significantly increased 2-DG uptake in rtMPCs by approximately 300% compared to vehicle treatment. rtTCAP-3 and insulin treatment resulted in comparable glucose uptake (Figure 3.13). Taken together, this chapter established both a novel method of measuring metabolism in zebrafish in vivo, and also established the presence and of TCAP-3 in metabolism and glucose regulation in the muscle of zebrafish.

6.2.3 Studies on the rodent, Rattus norvegicus

Although the findings in Chapter 3 on the zebrafish provided a new understanding of the role of TCAP on metabolism, the use of this animal model with respect to skeletal muscle metabolism is less understood than previous studies on rodents. Thus, the work in Chapter 4 was aimed at determining the physiological roles of TCAP-1 in rodent skeletal muscle both in short-term and long-term dose regimens, specifically in regard to glucose regulation and muscle function. The roles of TCAP-1 in stress-modulation has been extensively studied in rats, thus making this an ideal model for these studies as there are known biological actions of TCAP-1 already elucidated. In this work, the short-term regimen established in past studies was utilized. In addition, long-term application of TCAP-1 was also investigated to analyze changes in muscle adaptation over time. As some studies reported that it takes up to 10 weeks to see changes in skeletal muscle (Farup et al., 2014), a 12-week dose regimen was selected. Thus, this work was the first to study the roles of TCAP-1 in glucose regulation and muscle function, using both an established dose regimen and a new dose regimen, thus providing novel and critical insight into TCAP-1 actions in vivo.
In both short- and long-term TCAP-1-treated animals, there were a number of significant findings with respect to biomarkers of energy function. This was particularly evident in animals receiving TCAP-1 for the short duration. Functional positron emission tomography (fPET) scans were used to observe glucose uptake in specific tissues upon vehicle or TCAP-1 treatment. TCAP-1 significantly upregulated radioactively-labelled deoxyglucose uptake into the hindlimb muscles of the rats compared to vehicle treatment (Figure 4.2A-C). In animals given a short-term TCAP treatment, NADH levels were measured in tibialis anterior muscles of as a marker of cellular metabolism. TCAP-1-treated muscles had significantly higher expression of NADH-positive fibres in both control and stimulated conditions (Figure 4.3). This corroborates the roles of TCAP-1 in glucose regulation and metabolism. As glucose provides the main energy source for skeletal muscle function, an upregulation of glucose metabolism could impact skeletal muscle function. TCAP-1-treated muscles had enhanced baseline muscle function, demonstrating a significantly higher peak twitch force (Figure 4.4B), slower contraction velocity (Figure 4.4C), and faster 1/2RT rate (Figure 4.4D), with no increase in muscle mass (Figure 4.4H). After fatigue stress of the muscle, TCAP-1-treated muscles also had a higher capacity to maintain contraction velocity (Figure 4.4F) and 1/2RT rate (Figure 4.4G), which was lost in vehicle-treated animals. It is important to note that the animals treated with TCAP-1 short-term showed no difference in body weight or muscle weight over time compared to vehicle-treated animals (Figure 4.1A, B) indicating that any differences seen in muscle function are the result of enhanced cellular metabolism increasing the quality of the contractions, rather than increases in muscle mass. This was corroborated by the finding that TCAP-1 administration influenced muscle fibre-type by modulating myosin heavy chain (MHC) expression towards slow-twitch fibers. These studies provide strong evidence for the role of TCAP-1 in muscle metabolism and function when applied short-term.

Long-term application of TCAP-1 was subsequently investigated to analyze potential roles in muscle adaptation. Over 3 months of treatment, the male Sprague-Dawley rats also did not differ in body weight or muscle weight (Figure 4.5), consistent with short-term application of TCAP-1. Blood glucose measurements showed that TCAP-1 decreased blood glucose in the third month the
experiment compared to vehicle treatment (Figure 4.6). Moreover, TCAP-1-treated muscle baseline kinetics had slower contraction velocity and faster 1/2RT compared to vehicle treatment, with no affect on peak twitch force (Figure 4.8A-C). Both vehicle- and TCAP-1-treated groups also responded similar under fatigue conditions in all contractile parameters (Figure 4.8D-F). Similar to short-term application results, long-term TCAP-1 treatment resulted in a fibre-type switch towards type I slow oxidative fibers as seen by MHC expression, demonstrating a potent role of TCAP-1 in muscle function and physiology. Long-term TCAP-1-treated animals were also tested in stress paradigms to investigate the efficacy of TCAP-1 in stress-related behaviours over time. Although both groups of vehicle- and TCAP-1-treated animals travelled the same distance during an open field test (OFT), TCAP-1-treated animals had more entries into the centre zone and spent more time in the centre zone (Figure 4.9D-F). Additionally, TCAP-1 treatment increased explorative rearing behaviour (Figure 4.10A), while not affecting other behaviours such as grooming or peering (Figure 4.10B, C). These data suggest that TCAP-1-treated animals demonstrate less stress-related behaviours.

Although the effects between short- and long-term application were similar in many aspects, there were important differences seen in the regulation of contractile function. Short-term TCAP-1-treated muscle had enhanced peak twitch force and maintained elevated contractile kinetic function during fatigue, which was not seen in long-term TCAP-1-treated muscle. One of the differences between these two dose regimens was when the muscle stimulation test was performed. In the short-term application, muscle function was tested 3 days post-last injection, whereas muscle function was tested 2 weeks post-last injection. One explanation is that the metabolic enhancement induced by TCAP-1 is relatively short-lived. However, long-term application of TCAP-1 still resulted in dynamic changes in the contractile kinetics, likely due to its influence on fibre-type characteristics. Collectively this data suggests that TCAP-1 has both immediate and long-term actions, in metabolism and fibre-type changes, respectively. These types of immediate and long-term actions have been seen in other hormones. Insulin, for example, acutely stimulates glucose uptake into the cell, but also stimulates genetic transcription via MEK/ERK (Santos et al., 2008).
In conclusion, this work demonstrates the significant role of TCAP-1 in muscle metabolism and function, with potent actions on glucose regulation and contractile kinetics of the muscle. Both short-term and long-term applications of TCAP-1 had significant effects on glucose uptake into the muscle, which translated into changes in muscle contractile kinetics and muscle fibre phenotype. Moreover, long-term application of TCAP-1 resulted in a reduction of stress-related behaviours, consistent with previous studies.

6.2.4 Studies on the immortalized murine C2C12 skeletal muscle model

Although the *in vivo* data presented in Chapter 4 provides strong evidence of the role of TCAP-1 with respect to energy metabolism, these data led to a number of questions as to what the cellular mechanism of TCAP-1 is on skeletal muscle cells. Therefore, the focus of Chapter 5 was to elucidate the underlying mechanism of action of TCAP-1 using the C2C12 murine skeletal muscle cell line. The C2C12 cell line is a widely-used and well-established model for skeletal muscle as they readily differentiate into multi-nucleated myotubules (Burattini et al., 2004; Nedachi et al., 2008; Nozaki et al., 2016; Yaffe and Saxel, 1977).

The presence of the teneurin/TCAP-ADGR ligand-receptor complex was successfully established in skeletal muscle via genetic and protein expression (Figure 5.1A-G). Moreover, this ligand-receptor complex was also strongly co-localized in skeletal muscle tissue along with β-dystroglycan, consistent with previous reports (Figure 5.1H; Chand et al., 2012). Additionally, ADGR1 had significantly higher expression on tibialis anterior muscle fibres with a type I or IIA/x morphology compared to those with type IIb morphology (Figure 5.1I). To further show the association of TCAP with ADGR1, downstream signaling cascades associated with ADGR1, such as the phospholipase C (PLC)-inositol triphosphate (IP3) pathway, were investigated (Davletov et al., 1998; Rahman et al., 1999). TCAP-1 treatment in C2C12 skeletal muscle cells resulted in a significant increase in IP3 and DAG levels compared to vehicle treatment at 1, 5 and 15 minutes (Figure 5.2). Moreover, as IP3 is associated with calcium, the role of TCAP-1 in calcium modulation was investigated. TCAP-1 induced a biphasic response, where there were two significant peaks of cytosolic calcium compared to sham treatment in C2C12 myotubules (Figure
5.3A-C). Both groups responded comparably to the positive control, caffeine (Figure 5.3D), indicating normal function. This suggests that TCAP-1 induces calcium release, likely via IP3 production stimulating IP3 receptor (IP3R) on the sarcoplasmic reticulum (SR). To show that the calcium release originates in the SR, C2C12 myotubules were pre-treated with TCAP-1 for 1 hour, and then stimulated with caffeine (Figure 5.4A). These cells showed less capacity for a calcium signal compared to cells that were not pre-treated with TCAP-1 (Figure 5.4B). This was due to a significantly lowered rate of calcium release under caffeine application (Figure 5.4C), while rate of calcium re-uptake was unaffected (Figure 5.4D). To further delineate this mechanism, 2-aminoethoxydiphenyl borate (2-APB), an IP3R inhibitor, was pre-treated on C2C12 myotubules (Appendix I). Under these conditions, TCAP-1 could not induce any changes in cytosolic calcium, indicating that the IP3R is critical for the TCAP-1 mechanism of action (Figure 5.5A-C). Taken together, this data clearly demonstrates a novel role of TCAP-1 in calcium modulation via the IP3R on the SR.

Based on these findings, the role of the TCAP-1-mediated calcium release on mitochondrial activation was investigated as it is involved in energy metabolism and are major calcium sinks within the cell. TCAP-1 treatment caused significant calcium importation into the mitochondria which resulted in the hyperpolarization of the mitochondrial membrane (Figure 5.6; Figure 5.7). Moreover, TCAP-1-treated cells had an increased rate of depolarization under FCCP conditions compared to sham alone (Figure 5.7C). This data suggests that TCAP-1-mediated calcium importation into the mitochondria stimulates the electron transport chain (ETC) to pump more protons into the inner membrane space (IMS), resulting in an overall hyperpolarization of the membrane.

Enhanced mitochondrial activity, specifically the TCA cycle, typically results in an increase in glucose uptake to provide additional substrate for oxidative phosphorylation, thus, glucose uptake levels were measured. TCAP-1 induced a significant increase in radioactive 3H-deoxyglucose uptake (3H-DG), peaking at 30 minutes, which lasted until 45 minutes (Figure 5.9A), similar to its role in neurons. Interestingly, TCAP-1 had a different glucose uptake profile from insulin, which had increased glucose uptake over 60 minutes, peaking at 45 minutes (Figure 5.9A). Both TCAP-
1 and insulin demonstrated a significant increase in GLUT4 activation at the sarcolemma (Figure 5.9B, C), thus revealing the mechanism of TCAP-1-mediated glucose uptake in skeletal muscle. Consistent with this, TCAP-1 increased ATP and NADH production after treatment (Figure 5.10, Figure 5.11).

Thus, this work demonstrates, for the first time, that the teneurin/TCAP-ADGRL complex is expressed in skeletal muscle and has a functional role in energy metabolism and skeletal muscle function via calcium and glucose regulation.
<table>
<thead>
<tr>
<th>TCAP Expression in Muscle</th>
<th>Chapter 2: <em>C. intestinalis</em></th>
<th>Chapter 3: <em>D. rerio</em></th>
<th>Chapter 4/5: <em>R. norvegicus</em></th>
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<tr>
<td>• cTCAP was expressed in the muscle walls of the brachial basket, buccal siphon and cloacal siphon.</td>
<td>• TCAP-3 was expressed in zebrafish skeletal muscle tissue.</td>
<td>• All four TCAP isoforms were expressed in C2C12 cells and mouse hindlimb skeletal muscle.</td>
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<th>Energy Regulation</th>
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<td>• cTCAP-treated animals demonstrated behaviours that promote food intake</td>
<td>• rtTCAP-3 increased 2-DG uptake by over 200% after 30 minutes of treatment in rtMPCs, similar to insulin treatment.</td>
<td>• TCAP-1 increased $^3$H-DG by 300% after 30 minutes of treatment in C2C12 cells by upregulation of active GLUT4, similar to insulin treatment.</td>
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<tr>
<td>• rtTCAP-3 increased metabolic activity in larvae and adult zebrafish as shown by resazurin assays and metabolic respirometry chamber.</td>
<td>• TCAP-1 increased glucose uptake in rats after 3 days.</td>
<td>• TCAP-1 increased ATP and NADH production in C2C12 cells</td>
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<tr>
<th>Skeletal Muscle Function</th>
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<tr>
<td>• cTCAP increased contractile activity of the buccal and cloacal siphons compared to CDLP treatment.</td>
<td>• Not applicable.</td>
<td>• TCAP-1 increased baseline contractile kinetics in vivo; increased peak twitch force, slowed contraction velocity, and increased 1/2RT.</td>
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<tr>
<td>• cTCAP treatment had antagonistic responses compared to CDLP treatment in the food-stimulation test</td>
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<td>• TCAP-1 promotes switch to type I myofibres.</td>
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<th>Stress Response</th>
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<td>• cTCAP treatment had antagonistic responses compared to CDLP treatment in the food-stimulation test</td>
<td>• rtTCAP-3 enhanced metabolic activity in zebrafish larvae under low temperature stress conditions, which was lowered in vehicle-treated larvae.</td>
<td>• TCAP-1 enhanced contractile function during fatigue stress on the muscle.</td>
</tr>
<tr>
<td>• rtTCAP-3 increased oxygen consumption during stress conditions in metabolic respirometry chamber.</td>
<td>• rtTCAP-3 increased oxygen consumption during stress conditions in metabolic respirometry chamber.</td>
<td>• Long-term application of TCAP-1 lowered stress-related behaviours in the OFT stress paradigm.</td>
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Table 6.1. Overview summary of chapter results for *C. intestinalis, D. rerio, R. norvegicus.*
6.3 ADGRL expression across evolution

Importantly, ADGRL, the putative receptor for TCAP, has been identified in all three organisms studied during the course of this research. Rodents and *C. intestinalis* have three ADGRL isoforms, whereas zebrafish have six due to lineage specific gene duplication (Lange et al., 2012). Protein sequence alignments of ADGRL1 from rat, *C. intestinalis*, and zebrafish revealed a relatively high conservation. Rat and zebrafish ADGRL1 proteins share 70% sequence similarity, and both share about 25% sequence similarity with *C. intestinalis*. ADGRL1 in *C. intestinalis* is truncated and, unlike vertebrate ADGRL, it does not appear to have the long extracellular domain containing the hormone-binding domain that was recently shown to be critical for TCAP-1 binding (discussed in Section 1.5.1).

Interestingly, α-LTX, another known ligand of ADGRL, also binds to the hormone-binding region of ADGRL (Ushkaryov et al., 2004). Protein sequence alignments of TCAP-1 and α-LTX revealed that they share 20% sequence similarity (D’Aquila et al., in preparation). This is a surprisingly high similarity for sequences taken from mouse and black widow spider genomes, as even amongst the four sequenced latrotoxin paralogues the sequence similarity is 30-60% (Ushkaryov et al., 2004). α-LTX is a much larger protein than TCAP, having approximately 1200 amino acids compared to 41, respectively. Importantly, the 20% sequence similarity between TCAP and α-LTX occurs almost exclusively within the ‘wing’ portion of the α-LTX monomers, which is suggested to be the receptor-binding domain of the toxin (Garb and Hayashi, 2013; Orlova et al., 2000; Ushkaryov et al., 2004). Moreover, evolutionary analyses demonstrate that the wing domain is the highest conserved region of all α-LTX domains, underlining its ancestral importance (Garb and Hayashi, 2013). Toxins, such as α-LTX, depend on molecular mimicry to exploit receptors in other organisms (Garb and Hayashi, 2013; Holz and Habener, 1998). It is thus very likely that the similarity between these peptides is due to molecular mimicry of the endogenous ligand of ADGRL, TCAP (D’Aquila et al., in preparation; Garb and Hayashi, 2013).
6.4 The conserved role of the TCAP family in glucose regulation

Although organismal complexity varies across *C. intestinalis*, zebrafish and rats, their metabolic mechanisms are generally retained. In rodents, insulin treatment induces GLUT4 translocation to the membrane to facilitate glucose uptake into the muscle. Similarly, insulin treatment also induces glucose uptake in rainbow trout skeletal muscle via the GLUT4 homologue brown trout GLUT4 (btGLUT4; Capilla et al., 2002; Diaz et al., 2007). Thus, insulin was used as a positive control in the 2-DG uptake assays performed in murine C2C12 skeletal muscle cells and primary rainbow trout myogenic precursor cells (rtMPCs). TCAP-1 treatment in C2C12 cells induced a 300% increase in 2-DG uptake compared to vehicle, while insulin increased uptake by approximately 270% at 30 minutes, as expected. rtTCAP-3 treatment in rtMPCs increased 2-DG uptake by over 200% after 30 minutes of treatment compared to vehicle treatment. Moreover, insulin treatment similarly caused an approximately 230% increase in 2-DG uptake, consistent with previous reports (Castillo et al., 2004; Diaz et al., 2007). These studies demonstrate the conserved roles of the TCAP family in glucose regulation in rodents and fish. Interestingly, the rtMPCs showed a moderate decrease in insulin-stimulated glucose uptake compared to C2C12 cells. This is consistent with literature showing that fish have a lower capacity to respond to insulin, in contrast to mammals, which are highly sensitive to insulin effects (Polakof et al., 2012). This is may be due, in part, to insulin-like growth factor (IGF)-1 having a more dominant role in glucose regulation in fish than it does in mammals (Castillo et al., 2004; Polakof et al., 2012). Further, similar to insulin, rtTCAP-3 treatment to the rtMPCs also had a decreased glucose uptake response compared to C2C12 cells. This suggests that, in fish and rodents, the TCAP family may facilitate glucose uptake in a manner similar to insulin. Though TCAP and insulin pathways may converge, its actions have been shown to be insulin-independent, as studies in Goto-Kakazi rats, an insulin-insensitive model, respond to TCAP-1-mediated glucose regulation (Hogg et al., 2018).

Like fish and rodents, *C. intestinalis* also has insulin-like peptides and an insulin-like receptor expressed in its stomach, intestine, neural ganglion and muscle, indicating that they possess the required components for glucose metabolism by insulin-mediated pathways as well (Davidson et al., 1971; Olinkski et al., 2006; O’Neil et al., 1986; Satou et al., 2001; 2003). In vertebrates, insulin is responsible for clearing serum glucose whereas glucagon promotes glycogenolysis to increase
circulating serum glucose. Interestingly, no peptide resembling glucagon has been identified in *C. intestinalis*, indicating it does not have the same glucose regulatory systems as fish and rodents. This also suggests the insulin-like peptides in *C. intestinalis* may act more in growth regulation rather than in glucose metabolism (Satou et al., 2001; 2003). Although glucose regulation was not directly measured in *C. intestinalis*, it was observed that cTCAP treatment promoted ingestion behaviour when confronted in the food-stimulation test, possibly suggesting that it may still play a role in energy production and metabolism in *C. intestinalis*.

Taken together, these studies establish the conserved role of the TCAP family of peptides in glucose regulation. These roles were clearly demonstrated across multiple species across evolution. This data suggests that a basal role of the TCAP family is in energy production and metabolism. The high level of conservation in these roles underline its ancestral importance in survival.

6.5 The conserved role of the TCAP family in skeletal muscle function regulation

6.5.1 Muscle contractile kinetics are enhanced by TCAP treatment

Mechanisms of skeletal muscle contraction are highly conserved across evolution. Although *C. intestinalis*, zebrafish and rodents have varying muscle complexity, their muscles use the same dynamic actin-myosin based sarcomeres to induce contractions (Allen et al., 2008; Nevitt and Gilly, 1986; Oshiro et al., 2010). Further, *C. intestinalis* muscle is similar to that of vertebrates in that they both contain organized muscle bundles, excitation-contraction coupling machinery and cholinergic innervation. Between zebrafish and rodents, there are few differences. Zebrafish muscles exist almost as exclusively homogenous fibre-type compositions, resulting in pure white and red muscles, whereas rodent muscle has heterogenous fibre-type mixtures (Hernandez et al., 2005). Nonetheless, these three phylogenetically different species have similar skeletal muscle contraction mechanisms, which allows for a simple and elegant comparative model to compare the actions of TCAP treatment.
Skeletal muscle contractile function was tested in rats and *C. intestinalis* using different methods, yet a similar effect of TCAP treatment can be interpreted. TCAP treatment enhanced contractile function in rats and *C. intestinalis*. In rodents, TCAP-1 treatment enhanced contractile kinetics by increasing peak twitch force and half relaxation rate, as well as maintaining more efficient contractile function during fatigue. *C. intestinalis* treated with cTCAP for 15 minutes exhibited a significant increase in contractile activity, where they demonstrated an increase in short, frequent contractions. Although the cellular mechanism of TCAP on *C. intestinalis* muscle was not determined specifically, these findings do suggest a similar mechanism. Overall, these studies support previous suppositions that the TCAP family has conserved roles in regulating skeletal muscle function. In addition, although direct functional analyses on zebrafish muscle were not performed in this work, the high level of conservation of TCAP effects and skeletal muscle function suggests that zebrafish would likely have enhanced skeletal muscle function under rtTCAP-3 treatment as well. Muscle contractile kinetics can be influenced by fibre-type composition and metabolic signalling, thus the underlying mechanism by which TCAP alters muscle function was further explored (discussed in section 6.5.2 and 6.5.3).

6.5.2 Linking the in vivo contractile kinetics with in vitro mechanism of action

Evidence from the *in vitro* investigation into the mechanism of TCAP-1 has shed insight into the TCAP-1 effects observed in rats and *C. intestinalis*. TCAP-1 treatment of C2C12 myotubules resulted in a significant biphasic release in cytoplasmic reticulum via stimulation of the IP3R on the SR, as well as enhanced cellular metabolism. Thus, these data have provided a few possible explanations for the TCAP effects seen *in vivo*.

In rats, TCAP-1 treatment induced significant changes in contractile kinetics of the tibialis muscle. First, TCAP-1-treated muscle had significantly increased peak twitch force. Various biochemical events can influence the force production of a muscle, including calcium concentrations, intracellular pH, inorganic phosphate levels and reactive oxygen species (ROS) levels (Gardiner, 2010). Evidence from *in vitro* data demonstrates that TCAP-1 induces rises in cytosolic calcium from the SR in C2C12 cells (Figure 6.1A). Increased calcium release from the SR allows for more
actomyosin cross-bridges to form on the sarcomere, thus producing more force (Gardiner, 2010). This may provide an explanation for the increased force production observed *in vivo* under TCAP-1 treatment. Moreover, TCAP-1-treated muscle exhibits increased relaxation rates *in vivo*, suggesting TCAP-1 mediates increased calcium re-uptake. SERCAs are the rate-limiting step in cytosolic calcium re-uptake, and account for 20-50% of the energy turnover in a single contraction cycle (Bellinger et al., 2008; Leijendekker et al., 1987). To mediate this effect, TCAP-1 may increase ATP production to maintain SERCA activity (Figure 6.1B) and/or increase SERCA expression to enhance calcium re-uptake rate (Figure 6.1C). As TCAP-1 has been shown to increase intracellular ATP levels in C2C12 cells, the first scenario is the more likely contributor of enhanced calcium re-uptake under TCAP-1 treatment.
Figure 6.1. Schematic of proposed mechanism of TCAP-1 in skeletal muscle. In the muscle, TCAP-1 treatment increases contractile force, lowers contraction velocity, and increases half relaxation rate (1/2RT). This may be due to a few mechanisms: (A) TCAP-1 increases calcium release from the sarcoplasmic reticulum (SR) and allows for more actin-myosin cross bridges to form, thus producing more contractile force; (B) TCAP-1 increases ATP production via mitochondrial activation and thus enhances SERCA activity on the SR; (C) TCAP-1 may increase SERCA expression on the SR to facilitate enhanced calcium re-uptake. Abbreviations: PLC, phospholipase C; IP3, inositol triphosphate; IP3R, IP3 receptor; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPases; EC, extracellular; IC, intracellular.
An increase in contractile bouts were observed in *C. intestinalis*, as there was an increase in short and frequent contractions with cTCAP treatment. Although *C. intestinalis* muscle is similar to that of vertebrates, the *C. intestinalis* muscle has a unique troponin-tropomyosin regulatory system of contractions (Nevitt and Gilly, 1986; Ohshiro et al., 2010). Whereas vertebrate muscles have strong inhibition of troponin-tropomyosin interaction, ascidian muscles have little such inhibition, allowing for a significant increase in actomyosin \( \text{Mg}^{2+}-\text{ATPase} \) activity in the presence of calcium (Ohshiro et al., 2010). As calcium is the major component in contraction initiation, this suggests that *C. intestinalis* muscle has a lower threshold of calcium required to induce contractions. Thus, if cTCAP treatment increases intracellular calcium in *C. intestinalis* as TCAP-1 treatment does in rodents, this may provide an explanation for the increase in contractile activity in *C. intestinalis* treated with cTCAP. Moreover, *C. intestinalis* possesses many ion-gated channels also found in vertebrates, including TRP channels, voltage-gated calcium channels, and acetylcholine receptors (Okamura et al., 2005). Importantly, *C. intestinalis* muscle has shown evidence of a functional sarcoplasmic reticulum with many of the key receptors identified, such as DHPR, and RyR (Nevitt and Gilly, 1986; Oliphant and Cloney, 1972; Tsutsui, 1999). Adult *C. intestinalis* muscle responds to caffeine stimulation as seen by cytosolic calcium surge, corroborating these findings (Nevitt and Gilly, 1986). Evidence of a single IP3R isoform has been identified in *C. intestinalis*, with active roles in egg development (Arnoult et al., 1997; Okamura et al., 2005; Yoshida et al., 1998); however, its roles in ascidian muscle remain unknown. *In vitro* data suggests that TCAP-1 increases calcium via IP3R, thus it would be of interest to investigate if cTCAP works in a similar manner in *C. intestinalis* to induce an increase in contractile activity.

These data collectively demonstrate the highly conserved role of the TCAP family in skeletal muscle function across multiple species. As previously described, calcium and glucose are two of the most predominant metabolites that regulate skeletal muscle function. This work is supported by *in vitro* investigations utilizing C2C12 skeletal muscle cell culture that demonstrated the potent roles of TCAP-1 in calcium and glucose regulation. Thus, linking the *in vivo* and *in vitro* data have allowed for a more in-depth picture of the roles of the TCAP family in muscle metabolism and function.
6.5.3 Evidence of TCAP influencing fibre-type characteristics

The roles of TCAP in glucose regulation and skeletal muscle function have been clearly demonstrated in vitro and in vivo, however, the data also suggests that TCAP-1 influences fibre-type characteristics to induce its effects. The tibialis anterior, the muscle used in these studies, is comprised of mostly type II fibres. Type II fibres is further subdivided into type IIb, which are large glycolytic fibres, and type IIa/x, which are smaller in size and can use oxidative phosphorylation or glycolysis for ATP production. First, in vitro analysis of ADGRL expression in the rodent tibialis anterior muscle demonstrated a fibre-specific pattern, where it was predominantly found on small- and moderate-sized fibres, which are likely to be either type IIa/x or type I. In addition, when analyzing the muscle for NADH-positive fibres after TCAP-1 treatment, the small- and moderate-sized fibres demonstrated the largest increase in NADH production. Collectively, the data presented here suggests that TCAP-1 may have a greater effect on type I and/or type IIa/x fibres as a result of ADGRL expression being more predominant, as seen by the increase in NADH (Figure 6.2).
Various hormones can influence fibre-type characteristics by either changing metabolic phenotype of the muscle or by changing the expression of the MHC isoforms to transition to a different fibre-type. For example, chronic treatment with leptin, an adipocyte-derived hormone, decreases lactate dehydrogenase (LDH): citrate synthase (CS) ratio, resulting in an overall increase in oxidative capacity in the tibialis anterior muscle. Leptin treatment did not, however, change underlying MHC expression (Masuda et al., 2014). In contrast, thyroid hormone has potent ability to modify MHC expression, where hyperthyroidism induces a shift of slow-twitch muscle towards fast-twitch (Caiozzo et al., 1992).

In this work, qRT-analyses revealed that TCAP-1 modulates MHC expression by inducing a switch from type II fast glycolytic towards type I slow oxidative fibres. Although statistically sub-
significant, the tibialis anterior muscle is highly glycolytic muscle, thus the switch towards oxidative fibres observed is biologically relevant. This switch towards type I phenotype is supported by both in vivo and in vitro data. First, TCAP-1-treated muscle exhibited significantly decreased baseline contractile velocity. Contractile velocity is known to correlate strongly with MHC expression, such that an increase in MHCI expression reduces contractile velocity (Pette and Staron, 2001). Second, TCAP-1-treated muscle had significantly faster 1/2RT, which has been established as an indicator of calcium re-uptake during muscle relaxation. This supports the change in fibre-type as changes in SERCA isoforms would also be a result of fibre-type change, leading to enhanced calcium clearance (Pette and Staron, 2001). Lastly, histological analyses revealed strong NADH enzymatic activity at the periphery of the muscle walls. This likely represents the sub-sarcolemmal (SS) population of mitochondria, which would suggest TCAP-1 is increasing mitochondrial content. Moreover, SS mitochondria have been shown to be more adaptive to changes in fibre-type switch compared to the intermyofibrillar (IMF) population of mitochondria. These analyses suggest that TCAP-1 increased mitochondrial biogenesis, which is consistent with the phenotype of type I fibres.

The fibre-type switch towards type I slow oxidative fibres is also corroborated with in vitro data. C2C12 myotubules treated with TCAP-1 have increased TCA cycling as seen by increases in NADH turnover rate and intracellular ATP levels, consistent with type I fibre phenotype. In addition, TCAP-1 treatment significantly increased protein levels of succinate dehydrogenase (SDH), a component of the electron transport chain (ETC). This suggests that in addition to stimulating mitochondrial activity, TCAP-1 could be stimulating mitochondrial biogenesis. Increased mitochondrial content is a dominant characteristic of type I fibres, thus further supporting that TCAP-1 induces fibre-type change towards type I fibres.

In order to induce fibre-type changes at the genetic level, TCAP-1 actions must lead to transcriptional changes in the muscle. There are few transcription factors that have been established to switch type II fast glycolytic fibres to type I slow oxidative fibres. Myocyte enhancer factor 2 (MEF2) is a key transcription factor that has potent ability to induce fibre-type switch towards type I (Potthoff et al., 2007). Class II histone deacetylases (HDACs) regulate the
transcriptional activity of MEF2, where it represses MEF2 activity unless it is degraded or exported out of the nucleus (Czubryt et al., 2003). Thus, mechanisms that remove HDAC relieve the repression on MEF2 and promote the formation of slow type I myofibres. A small number of proteins have shown interaction with the MEF2/HDAC pathway to regulate fibre-type expression. Calcineurin and calcium/calmodulin-dependent protein kinase (CaMK) work synergistically to stimulate MEF2 activity (Figure 6.3). Upon activation by changes in intracellular calcium levels, calcineurin dephosphorylates nuclear factor of activated T-cells (NFAT), which causes NFAT translocation to the nucleus. Once within the nucleus, NFAT interacts with MEF2, resulting in MEF2 transcriptional activity, however, the exact mechanism by which it does this is unknown. Concurrently, CaMK is also activated by changes in intracellular calcium, where it phosphorylates HDAC5 thus causing nuclear export of HDAC5. Once MEF2 is unrestricted by HDAC, it can respond to the signals from calcineurin-dependent NFAT activation and promote gene expression of slow type I fibre proteins. Further, MEF2 activates peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α), a known transcription factor that stimulates mitochondrial biogenesis. Overexpression of PGC-1α in transgenic mice resulted in a marked increase in type I fibres compared to wild-type littermates (Lin et al., 2002). It was later found that the PGC-1α-mediated fibre-type switch was MEF2-dependent, as the PGC-1α gene has two MEF2-binding sites that can regulate its activity (Figure 6.3; Czubryt et al., 2003). Thus, as TCAP-1 increases cytosolic calcium levels it may be stimulating the calcineurin/CaMK pathway that leads to MEF2 transcriptional activity, leading to type I fibre-type switch and PGC-1α activation (Figure 6.3).
Figure 6.3. Schematic of the proposed mechanism of TCAP-1-mediated fibre-type change. TCAP-1 induces rise in cytosolic calcium via IP3R activation which stimulates calcineurin and calcium/calmodulin-dependent protein kinase (CaMK). (1) Calcineurin activates nuclear factor of activated T-cell (NFAT) which subsequently translocates to the nucleus. (2) CaMK phosphorylates histone deacetylase (HDAC) which causes HDAC to be exported out of the nucleus, thereby relieving myocyte enhancer factor 2 (MEF2) from its repression. (3) MEF2 becomes active once it is associated with NFAT and uninhibited from HDAC. This leads to stimulation of (4) PGC-1α and (5) transcription of slow and oxidative fibre-specific genes. Modified from Potthoff et al., 2007.

6.6 The conserved role of TCAP family in stress responses

In this work, to analyze the effects of TCAP in stress of the muscle, stress was investigated in the form of metabolic challenge. In zebrafish, rtTCAP-3 treatment to larvae under low temperature stress, a known metabolic depressor, can maintain increased metabolic activity compared to vehicle treatment. In addition, rtTCAP-3 treatment increased oxygen consumption rate in the forced-flush stress conditions in the metabolic respirometry chambers. This suggests that the rtTCAP-3-treated fish had higher metabolic activity to overcome the fatigue on their muscles with increased swim speed. A similar response was also observed in rodents, where TCAP-1 treatment allowed for sustained activity of the muscle through the fatigue protocol, as demonstrated by less
decay of contractile parameters compared to vehicle-treated animals. These studies clearly demonstrate that animals treated with TCAP have a greater ability to respond to metabolic stress.

The anxiolytic roles of TCAP-1 in stress are well established in rodents (Al Chawaf, et al., 2007b; Tan et al., 2009, 2011). TCAP has antagonistic roles to CRF in the stress response. Thus, in addition, this work investigated the roles of cTCAP and the CRF-orthologue in *C. intestinalis*, CDLP, in a food-stimulation test. Whereas CDLP-treated animals exhibited a strong expulsion response to the algae concentrate, cTCAP-treated animals showed a behaviour that promoted food intake. Although the antagonistic roles between TCAP and CRF is clear, the molecular mechanism remains unclear (Chen et al., 2013).

Taken together, this research show that all organisms had enhanced responses towards stress challenges when treated with TCAP, thus underlining its high conservation in this role. There is a finite source of energy in the organism to allocate towards dealing with stress, while maintaining enough energy for other key life aspects such as growth and reproduction. Thus, when stress demands a higher portion of our energy budget to overcome the stressor, it impacts the overall fitness of the organism. The TCAP family has now been established in enhancing metabolism and lowering stress-related responses. Thus, TCAP-1-mediated stress-modulation actions may be induced, in part, by increasing the ability of the organism to respond to stress as it has additional energy to overcome the stressor. Due to the high conservation of the roles of TCAP-1 in the stress response, it would be of interest to investigate the interaction of TCAP-1 and epinephrine under acute stress challenge. Epinephrine production is stimulated during acute stress and results in the “fight or flight” reaction of the organism. Thus, future directions should explore possible interactions of TCAP-1 with epinephrine as both have roles in glucose mobilization and stress response.
6.7 Final Remarks

This Ph.D. research entailed a three-part hypothesis: first, that the teneurin/TCAP-ADGRL complex is present and functional in skeletal muscle; second, that the TCAP family of bioactive peptides plays a critical role in skeletal muscle metabolism and physiology, which in turn improves the organism’s ability to respond to stress; and third, that these effects are evolutionarily conserved. The work completed in this thesis fully supports all three aspects of my hypothesis. Moreover, this work describes, for the first time, how skeletal muscle function and metabolism can be regulated by TCAP, thus providing novel insight into the regulatory functions of teneurin/TCAP-ADGRL complex and into contractile dynamics of skeletal muscle.
References


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Appendix I: IP3R inhibitor, 2-APB, significantly reduces cytosolic calcium baseline levels. After 4 minutes of treatment with 2-APB, a potent IP3R inhibitor, cytosolic calcium levels were reduced by 40%, demonstrating the efficacy of the pharmacological inhibitor. (Mean ± SEM; n=4; ****p<0.0001; t-test).