Teneurin C-terminal Associated Peptides (TCAPs): Evolutionary Origins and the Role of TCAP-1 on Calcium Flux in Astrocyte Monocultures and in Astrocytes Co-cultured with Neurons

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Teneurin C-terminal associated peptides (TCAPs) are a family of 40-41 amino acid peptides located at the C-terminus of each of the four teneurin type II transmembrane proteins. The teneurin-TCAP system is ancient and likely arose as a result of a horizontal gene transfer event. TCAPs share sequence similarity to the Secretin superfamily, a less evolutionarily ancient family that includes corticotropin-releasing factor (CRF). The phylogenetic position of TCAP places it as a putative ancestor of the Secretin superfamily. Calcium ion signaling is integral to all living organisms and astrocytes are integral to the development of the central nervous system in chordates. It is likely that TCAP played a role in these mechanisms. TCAP-1 stimulated calcium signaling in astrocyte monocultures but not in astrocytes co-cultured with neurons. This effect is mediated, in part, by the glutamatergic system. Therefore, the ancient peptide TCAP-1 plays a role in regulating calcium flux in astrocytes.
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# Table of Contents

Acknowledgements ........................................................................................................... iii

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

List of Figures and Tables ................................................................................................. vi

List of Abbreviations ......................................................................................................... x

List of Appendices ............................................................................................................. xiv

Chapter 1 Introduction ........................................................................................................ 1

1 Abstract ............................................................................................................................. 1

1.1 Discovery and function of the teneurin-TCAP system .................................................. 1

1.1.1 Teneurins .................................................................................................................. 1

1.1.2 Teneurin C-terminal associated peptides ................................................................. 2

1.2 TCAP shares sequence similarity to Secretin superfamily members ......................... 2

1.3 Structural similarity of ADGRL to Secretin GPCRs ..................................................... 5

1.4 The teneurin-ADGRL complex is ancestral to Secretin GPCRs and their ligands .......... 6

1.5 The effect of an ancestral peptide on calcium ion regulation ........................................ 7

1.6 Astrocytes: Function and evolution ............................................................................... 7

1.7 Intracellular calcium signaling in astrocytes: A form of excitability ............................... 8

1.8 Intercellular calcium waves: Communication between astrocytes ............................... 9

1.9 Bi-directional communication: The tripartite synapse complex ................................. 9

1.10 Hypothesis and experimental objectives .................................................................... 11

Chapter 2 Analysis of newly identified corticotropin releasing factor (CRF) family

*Petromyzon marinus* sequences: refining the current model of CRF evolution ................. 13

2 Abstract ............................................................................................................................. 13

2.1 Introduction .................................................................................................................... 13

2.2 Materials and Methods ............................................................................................... 16

2.3 Results .......................................................................................................................... 18

2.4 Discussion ..................................................................................................................... 22
Chapter 3 Teneurin C-terminal associated peptide (TCAP) family: A putative progenitor for Secretin superfamily ligands ................................................................. 25

Abstract .................................................................................................................. 25

3.1 Introduction ........................................................................................................... 25
3.2 Materials and Methods ....................................................................................... 28
3.3 Results .................................................................................................................. 30
3.4 Discussion ............................................................................................................ 40

Chapter 4 Stimulation of a glutamate-associated calcium-mediated astrocyte syncytium in immortalized cell lines: the effects of TCAP-1 on astrocyte monocultures and in astrocytes co-cultured with neurons ................................................................. 44

Abstract .................................................................................................................. 44

4.1 Introduction ........................................................................................................... 44
4.2 Materials and Methods ....................................................................................... 47
4.3 Results .................................................................................................................. 55
4.4 Discussion ............................................................................................................ 79

Chapter 5 Final Discussion and Concluding Remarks ............................................. 95

Abstract .................................................................................................................. 95

5.1 Introduction ........................................................................................................... 95
5.2 Evolution of the teneurin-TCAP system ............................................................. 95
5.3 Evolution of astrocytes and calcium signaling systems ..................................... 98
5.4 Concluding Remarks ......................................................................................... 99

References ............................................................................................................... 100

Appendix I: List of Accession Numbers ................................................................. 110
List of Figures and Tables

Figure 1.1: The tripartite synapse complex.................................................................11

Figure 2.1: Mature peptide sequence alignment of CRF, UI and Ucn3 peptides in *Petromyzon marinus* with respective orthologues.................................................................18

Figure 2.2: Evolutionary relationships of *Petromyzon marinus* CRF family member pre-propeptides.........................................................................................................................19

Figure 2.3: Evolutionary relationships of the *Petromyzon marinus* CRF family member mature peptides.........................................................................................................................19

Figure 2.4: Evolutionary relationships of the *Petromyzon marinus* CRF receptors.........................20

Figure 2.5: Evolutionary relationships of the *Petromyzon marinus* CRF receptor hormone binding domains.........................................................................................................................20

Figure 2.6: Evolutionary relationships of the *Petromyzon marinus* CRFBP............................................21

Figure 2.7: Proposed model for the refined evolutionary scheme of CRF peptides, receptors and binding protein.........................................................................................................................24

Figure 3.1: Mature peptide sequences of TCAP paralogues in mouse..............................................30

Figure 3.2: Mature peptide sequences of TCAP orthologues in vertebrates.................................30

Figure 3.3: Phylogenetic analysis of CRF, calcitonin, insulin and secretin family pre-propeptides with TCAP propeptides (unrooted)......................................................................................32

Figure 3.4: Phylogenetic analysis of CRF, calcitonin, insulin and secretin pre-propeptides with TCAP propeptides (rooted to TCAP).....................................................................................................33

Figure 3.5: Phylogenetic analysis of insulin and calcitonin mature peptides (unrooted).................34

Figure 3.6: Phylogenetic analysis of insulin, calcitonin and TCAP mature peptides.................35
Figure 3.7: Phylogenetic analysis of insulin, calcitonin, CRF and TCAP mature peptides

Figure 3.8: Phylogenetic analysis of insulin, calcitonin, CRF, secretin and TCAP mature peptides

Table 4.1: List of Primers used for PCR

Table 4.2: List of Antibodies used for Western Blotting

Figure 4.1: Characterization of C8D1A mouse cerebellar astrocytes

Figure 4.2: C8D1A astrocytes treated with TCAP-1 demonstrate an oscillatory increase in intracellular calcium

Figure 4.3: Average amplitude and peak frequency of C8D1A astrocytes treated with TCAP-1 was significantly greater than with vehicle treatment

Figure 4.4: C8D1A astrocytes treated with TCAP-1 for 10 minutes demonstrated significant increases in intracellular calcium at 3.5-6 and 6.5-9 minute time points compared to vehicle

Figure 4.5: C8D1A astrocytes that are close in proximity exhibit increases in intracellular calcium at similar time points when treated with TCAP-1

Figure 4.6: C8D1A astrocytes transfected with mCherry demonstrate an increase in intracellular calcium during TCAP-1 treatment

Figure 4.7: N38 neurons treated with TCAP-1 demonstrate a decrease in intracellular calcium

Figure 4.8: N38 neurons treated with TCAP-1 for 10 minutes demonstrated a significant decreases in intracellular calcium at 0.5-3, 3.5-6 and 6.5-9 minute time points compared to vehicle

Figure 4.9: N38 neurons transfected with BFP demonstrate unstable calcium levels when treated with vehicle and TCAP-1
Figure 4.10: C8D1A-mCherry astrocytes and N38-BFP neurons exhibit approximately a 1:1 ratio with the established co-culture conditions……………………………………………………………………………………………..64

Figure 4.11. C8D1A astrocytes and N38 neurons in co-culture exhibit characteristically different morphologies and significantly different basal calcium/fluo-4 fluorescence………………………………………65

Figure 4.12: C8D1A astrocytes and N38 neurons in co-culture exhibit characteristically different calcium responses when treated with vehicle………………………………………………………………………………..66

Figure 4.13: C8D1A astrocytes has a significantly greater average amplitude and average peak frequency compared to N38 neurons in co-culture…………………………………………………………………………………..66

Figure 4.14: C8D1A astrocytes in co-culture with N38 neurons respond differently when treated with TCAP-1 than when treated with vehicle…………………………………………………………………………………………..68

Figure 4.15: C8D1A astrocytes in co-culture with N38 neurons demonstrated a significant decrease in average amplitude during TCAP-1 treatment relative to before but no significant difference for average peak frequency……………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………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Figure 4.21: NMDAR, AMPAR and mGluR5 antagonist administration (AP5, CNQX and MPEP) significantly decreased intracellular calcium levels compared to glutamate and vehicle administration in C8D1A astrocytes.................................................................74

Figure 4.22: C8D1A astrocytes treated with TCAP-1 and NMDAR, AMPAR and mGluR5 antagonists (AP5, CNQX and MPEP) did not demonstrate and changes in intracellular calcium........................................................................................................75

Figure 4.23: NMDAR, AMPAR and mGluR5 antagonist administration (AP5, CNQX and MPEP) significantly decreased intracellular calcium levels compared to TCAP-1 and vehicle administration in C8D1A astrocytes.........................................................................................76

Figure 4.24: Average amplitude and average peak frequency of C8D1A astrocytes treated with TCAP-1 and glutamate were not significantly different.................................................................77

Figure 4.25: Raw traces of TCAP-1 and glutamate treated C8D1A astrocytes can be classified into two different responses, an initial peak and continuous oscillations.................................78

Figure 4.26: Suggested areas of future research pertaining to metabotropic and ionotropic pathways that contribute to intracellular and intercellular calcium signaling in astrocytes.........................................................................................94

Figure 5.1: Two possible evolutionary schemes of TCAP in relation to the Secretin superfamily of peptides...............................................................................................................................97
List of Abbreviations

aCSF, artificial cerebral spinal fluid
ACTH, adrenocorticotropic hormone
ADGRL, adhesion G-protein coupled receptor subfamily L/latrophilins
ADM, adrenomedullin
ADM2, adrenomedullin 2
AIC, Akaike Information Criterion
AM, amylin
AM, acetomethyl
AM, acetomethyl
AMPAR, α-amino-3-hydroxy-5-methy-4-isoxazolepropinoic acid receptor
ANOVA, analysis of variance
AP5, D(-)-2-Amino-5-phosphonopentanoic acid
BCA, bicinehoninic acid
BIC, Bayesian Information Criterion
BFP, blue fluorescent protein
BME, β-mercaptoethanol
Bps, base pairs
BSA, bovine serum albumin
C, cysteine
CALC, Calcitonin
CALCR, calcitonin receptor
CALCRL, calcitonin receptor-like
CDLP, CRF/DH-like peptide
cDNA, complimentary DNA
CGRP, calcitonin gene-related peptide
CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione
CNS, central nervous system
CRF/CRH, corticotropin-releasing factor/corticotropin-releasing hormone
CRFBP, corticotropin-releasing factor binding protein
CRFR1, corticotropin releasing factor receptor 1
CRFR2, corticotropin-releasing factor receptor 2
ddH₂O, double distilled water
DEPC, diethyl pyrocarbonate
DH, diuretic hormone
DIC, differential interference contrast
DMEM, Dulbecco’s Modified Eagle’s Medium
DMSO, dimethyl sulfoxide
dNTP, deoxynucleotide triphosphate
EGF, epidermal growth factor
ER, endoplasmic reticulum
GCG, glucagon
GHH, glycine histidine histidine
GHRH, growth hormone releasing hormone
GIP, gastric inhibitory peptide
GPCR, G-protein coupled receptor
HBD, hormone-binding domain
HCl, hydrochloric acid
HGT, horizontal gene transfer
HNH, histidine asparagine histidine
HPA, hypothalamic pituitary adrenal
IP₃, inositol 1,4,5-triphosphate
JTT, James-Taylor-Thorton
K, lysine
kDa, kilodalton
L, leucine
LG, Le Gascuel
lnL, log likelihood
MEGA, Molecular Evolutionary Genetics Analysis
mGluR, metabotropic glutamate receptor
ML, maximum likelihood
MPEP, 6-Methyl-2-(phenylethynyl)pyridine hydrochloride
MUSCLE, Multiple Sequence Comparison by Log Expectation
NCBI, National Center for Biotechnology Information
N, asparagine
NJ, Neighbor-Joining
NNI, Nearest-Neighbor Interchange
NMDAR, N-methyl-D-aspartate receptor
NPY, neuropeptide Y
odz, Odd oz
PACAP, pituitary adenylate cyclase-activating peptide
PBS, phosphate buffered saline
PLC, phospholipase C
PMSF, phenylmethylsulfonyl fluoride
PPT, proteinaceous polymorphic toxin
R, arginine
RHS, rearrangement hot spot
RIPA, radioimmunoprecipitation assay
RT-PCR, reverse transcription-polymerase chain reaction
S, serine
SCT, secretin
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM, standard error of the mean
Svg, sauvagine
TCAP, teneurin C-terminal associated peptide
TCN, teleocortin
ten-a, tenascin-like molecule accessory
ten-m, tenascin major
Ucn, urocortin
Ucn2, urocortin 2
Ucn3, urocortin 3
UI, urotensin
VIP, vasoactive intestinal peptide
W, tryptophan
YD, tyrosine aspartic acid
5’RACE PCR, 5’ rapid amplification of cDNA ends
List of Appendices

Appendix I: List of Accession Numbers.................................................................110
Chapter 1
Introduction

1 Abstract

This chapter is intended to provide the reader with an overview of fundamental information with respect to the findings covered in subsequent chapters. It provides an overview with respect to the discovery, function and ancient evolutionary origins of the teneurin C-terminal associated peptide (TCAP) system, the similarity that TCAP shares with members of the Secretin superfamily of peptides, including corticotropin releasing factor (CRF), with an overview of the CRF family, including the discovery and function. Moreover, a brief overview of the importance of calcium signaling early in evolution and the function and evolution of astrocytes is presented. Finally, both intracellular and intercellular calcium signaling in astrocyte networks as well as communication between astrocytes and neurons are reviewed.

1.1 Discovery and function of the teneurin-TCAP system

1.1.1 Teneurins

Teneurins 1-4 are a family of type II trans membrane proteins that are about 2800 amino acids in length and possess a highly conserved structure (Tan et al., 2012; Tucker et al., 2007). The first teneurin gene was discovered in 1993 as tenascin-like molecule accessory (ten-a) in Drosophila (Baumgartner & Chiquet-Ehrismann, 1993). A second homologue was discovered in 1994 by two independent teams (Baumgartner et al., 1994; Levine et al., 1994). Baumgartner and colleagues characterized tenascin major (ten-m), whereas Levine and colleagues characterized Odd oz (odz) and found that both Drosophila genes were structurally related to tenascin, a multifunctional transmembrane protein. Mutations of this ten-m/odz gene result in embryonic lethality, indicating that the teneurins were crucial in development (Baumgartner et al., 1994; Levine et al., 1994). In 1999, the teneurins were discovered in vertebrates when Minet and colleagues identified a teneurin homologue in chicken. Subsequently, various other homologues were discovered using database searches in mouse and they were termed the teneurins to reflect their association with ten-m as well as their expression in the nervous system (Ben-Zur et al., 2000; Minet & Chiquet-Ehrismann, 2000). Later, Oohashi and colleagues (1999) established that...
teneurin is a type-II trans membrane protein, meaning that the carboxy-terminus is found in the extracellular domain.

1.1.2 Teneurin C-terminal associated peptides

TCAPs are a family of four bioactive peptides that are 40-41 amino acids in length and are located on the C-terminus of each of the four teneurins (Qian et al., 2004). Qian and colleagues (2004) discovered TCAP while screening the rainbow trout hypothalamic cDNA library for CRF-related homologues, using a hamster urocortin probe. They identified a clone that represented the rainbow trout orthologue of teneurin-3 and discovered a neuropeptide-like sequence encoded at the carboxy-terminus in the last exon of teneurin-3, which was termed TCAP-3. Subsequently, TCAP-1, 2 and 4 were discovered after further analyses of teneurins 1, 2 and 4 (Wang et al., 2005).

The TCAPs are classified as cleavable bioactive peptides that can be cleaved from the teneurin protein post-translationally because they possess a cleavage motif at the N-terminus and an amidation motif at the C-terminus (Chand et al., 2013; Wang et al., 2005). However, evidence from northern blot analysis and 5’RACE PCR suggests that TCAPs can also be independently transcribed from the teneurins (Chand et al., 2013; deLannoy, 2013). Two separate transcripts, one representative of the full-length teneurin and the second consistent with the length of the terminal exon where TCAP-1 is expressed, were identified using northern blot analysis (Chand et al., 2013). Further, 5’RACE PCR that targeted the region upstream of the TCAP-1 sequence identified an mRNA sequence that was 402 base pairs (bps) long, indicative of TCAP-1 mRNA (deLannoy, 2013). Therefore, given these findings, TCAP-1 can be cleaved post-translationally from the full-length teneurin protein or it can be independently transcribed as a separate transcript.

1.2 TCAP shares sequence similarity to Secretin superfamily members

The Secretin superfamily of peptides is a diverse assemblage of peptide lineages with a number of overlapping biological functions that bind structurally-related Secretin GPCRs. This family consists of CRF and calcitonin families, secretin, glucagon, growth hormone releasing hormone
(GHRH), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP) and gastric inhibitory peptide (GIP) (Fredriksson et al., 2003).

Previous studies indicate that the TCAP family may possess sequence similarity to both the CRF and calcitonin families of peptides, members of the Secretin superfamily (Lovejoy et al., 2006). The CRF family is involved in regulating the stress response as well as energy metabolism in both vertebrates and invertebrates. Among vertebrates, the CRF family consists of four paralogues. These peptides are the result of two rounds of genome duplication (2R hypothesis) that occurred early in evolution (Holland et al., 1994; Lovejoy & Balment, 1999; Lovejoy & Jahan, 2006; Lundin, 1993). Evidence suggests that the first genome duplication event likely occurred sometime after the emergence of the protochordates but before the agnathans (Dehal & Boore, 2005; Lundin 1993). This gave rise to two paralogous lineages: a CRF-urocortin (Ucn) lineage and a urocortin 2 (Ucn2)-urocortin 3 (Ucn3) lineage (Chang & Hsu, 2004; Lovejoy & Balment, 1999; Lovejoy & Jahan, 2006). The second genome duplication occurred later in metazoan phylogeny, likely before the actinopterygian-sarcopterygian split and resulted in the four paralogous peptides that are currently found in vertebrates: CRF, Ucn/urotensin (UI)/sauvagine (Svg), Ucn2 and Ucn3 (Chang & Hsu, 2004; Lovejoy & Balment, 1999; Lovejoy & Jahan, 2006). The second genome duplication resulted in the four paralogous peptides that are currently found in vertebrates: CRF, Ucn/urotensin (UI)/sauvagine (Svg), Ucn2 and Ucn3 (Chang & Hsu, 2004; Lovejoy & Balment, 1999; Lovejoy & Jahan, 2006).

The CRF family of peptides exerts its effects by binding to CRF receptor (CRFR) 1, mainly involved in the stress component, and CRFR 2 involved in energy regulation and metabolism, which are classified as Secretin GPCRs (Fredriksson et al., 2003; Kuperman & Chen, 2008). CRF peptides can also bind the CRFBP, a glycoprotein that regulates activity and can inhibit the downstream signaling effects of CRF, such as the release of adenocorticotropic hormone (ACTH) from the pituitary (Westphal & Seasholtz, 2006).

CRF is a 41-amino acid peptide that plays a fundamental role in coordinating the stress response through the hypothalamic-pituitary-adrenal (HPA) axis (De Souza, 1995). Discovered in 1955, it was simultaneously purified by two different teams, Schally and Saffran as well as Guillemin and Rosenberg (Guillemin & Rosenberg, 1955; Schally & Saffran, 1955). However, because of the technical difficulties in purification, it was not until 1981 that Vale and colleagues extracted CRF from sheep and determined that it was largely involved in mediating the stress response among vertebrates (Vale et al., 1981). Svg is a 40-amino acid peptide that was discovered by
Montecucchi and colleagues in 1980 after being extracted from the skin of *Phyllomedusa sauvagei*, the peptide was classified as a paralogue of the CRF family (Montecucchi, 1980). UI is a 41-amino acid peptide that was discovered by Lederis and colleagues in 1982. It was first identified in the urophysis, the caudal neurosecretory organ in *Catostomus commersonii*, the white sucker (Lederis et al., 1982). Subsequently, Ucn, a 40-amino acid peptide, was identified in 1995 because of a homologous relationship to UI (Vaughan et al., 1995). The high sequence conservation of the CRF family of peptides among vertebrates suggests that it has played a fundamental role in regulating the stress response throughout evolutionary time.

Among invertebrates, this family of peptides is orthologous to the diuretic hormones (DHs) in arthropods and the CRF/DH-like peptides (CDLPs) among tunicates (Lovejoy & Balment, 1999; Lovejoy & Jahan, 2006). Kataoka and colleagues discovered the first diuretic hormone, Manse-DH in 1989 in the tobacco hornworm, *Manduca sexta* (Kataoka et al., 1989). It was 41 amino acids in length and shared large sequence similarity with Svg, suggesting that it could be related to the CRF family of peptides among vertebrates. Subsequently, a second diuretic peptide termed MasDP-II was discovered in 1991 (Blackburn et al., 1991). This peptide was 30 amino acids in length and shared sequence homology with Manse-DH as well as the vertebrate CRF family of peptides (Blackburn et al., 1991). More diuretic peptides bearing sequence similarity to the CRF family of peptides continued to be discovered in insects and they were involved in osmoregulation and diuresis (Coast, 1998).

Moreover, the DHs possess sequence similarity to the calcitonin family of peptides. This family of peptides includes calcitonin-gene related peptide (CGRP) 1, CGRP 2, amylin, adrenomedullin, adrenomedullin 2 (Naot & Cornish, 2008). Calcitonin decreases blood calcium levels by inhibiting bone resorption and other members of this peptide family have differing functions, however they all commonly target bone (Naot & Cornish, 2008). Members of this family bind to the calcitonin receptor (CALCR) and calcitonin receptor-like (CALCRL), classified as Secretin GPCRs (Fredriksson et al., 2003). The discovery of a 31-amino acid peptide in *Schistocerca americana*, the American grasshopper, as well as a DH31 in cockroaches, which shared high sequence similarity with calcitonin, demonstrated that the DHs share structural similarity with CRF, but might be more closely related to the calcitonin family of peptides (Coast et al., 2002;
Furuya et al., 2000). Evidence suggests that the DHs are more closely related to the calcitonin family of peptides so it is possible that the CRF and calcitonin families share a common origin (Coast et al., 2001; Furuya et al., 2000; Lovejoy & de Lannoy, 2013; Lovejoy & Jahan, 2006).

Moreover, the TCAP sequences share amino acid motifs with both CRF and calcitonin families of peptides (Lovejoy et al., 2006). Most recent sequence comparisons between the TCAPs and the CRF family of peptides show about 20% sequence similarity, though TCAPs are the same size as CRF and UI (Lovejoy, 2009; Lovejoy et al., 2006; Tan et al., 2012). The level of sequence similarity as well as their similar size alludes to a common evolutionary origin of the TCAPs with the CRF family of peptides and because the calcitonin family has structural similarity with the CRF family members, this suggests that these three peptide families are closely related. However, the evolutionary relationships between the TCAP lineages and other peptide families are not well understood and it is not known at what point in time they possessed a common ancestor (Chand et al., 2013; Lovejoy & de Lannoy, 2013; Tan et al., 2012).

1.3 Structural similarity of ADGRL to Secretin GPCRs

Discovered in 1997, adhesion G-protein coupled receptor latrophilin (ADGRL), then termed latrophilin, was identified as the receptor for α-latrotoxin, the black widow spider toxin (Lelianova et al., 1997). ADGRL was first classified as a Secretin family GPCR because it possessed a unique hormone-binding domain (HBD) that is also present among Secretin GPCRs (Fredriksson et al., 2003; Nordström et al., 2009; Schiöth et al., 2010). ADGRL was misclassified due to high sequence similarity and as a result of similar structural motifs to the Secretin GPCRs, but more recent analyses have categorized ADGRL as an Adhesion GPCR and have established that teneurin binds to this receptor (Fredriksson et al., 2003; Silva et al., 2011). Also, findings from our laboratory demonstrate that TCAP-1 binds to ADGRL (Husic, 2016). Moreover, the Secretin family of GPCRs contains many highly conserved sequence motifs that are also present among the Adhesion GPCRs, suggesting that the Secretin GPCRs evolved from the more evolutionarily ancient Adhesion GPCRs (Nordström et al., 2009; Schiöth et al., 2010).
1.4 The teneurin-ADGRL complex is ancestral to Secretin GPCRs and their ligands

The teneurin-TCAP system likely evolved from a prokaryotic proteinaceous polymorphic toxin (PPT) gene by means of a horizontal gene transfer (HGT), a mechanism by which genes may be introduced into an organism through bacterial infection or symbiosis (Zhang et al., 2012). As prokaryotes were likely the major food source of early eukaryotes, a choanoflagellate (a primitive unicellular organism) may have engulfed a prokaryote containing the PPT gene, which was integrated into the choanoflagellate genome and lost its toxic role overtime (Tucker, 2012; Zhang et al., 2012). In support of this, the teneurin gene has been identified in one species of choanoflagellate, *Monosiga brevicollis* (Tucker et al., 2012). Additionally, the type II transmembrane orientation of both PPTs and teneurins as well as the presence of rearrangement hot spot (RHS) domains and the similarity between the C-terminal domain of the teneurin to the histidine asparagine histidine (HNH) bacterial toxin of the glycine histidine histidine (GHH) clade, suggests that the teneurin gene is of bacterial origin as these are characteristics of PPTs (Minet et al., 1999; Zhang et al., 2012). The GHH domain, a putative ancestor of TCAP, may have lost its toxic role and instead functioned as an intracellular signaling molecule (Zhang et al., 2012). Moreover, the C-terminal region of the *M. brevicollis* teneurin protein contains tyrosine aspartic acid (YD) repeats characteristic of proteobacteria and most of the extracellular domain is encoded on one large 6829 base-pair exon, characteristic of prokaryotic genomes and of HGT (Tucker et al., 2012). The N-terminal region of the teneurin, however, contains EGF repeats that are unique to eukaryotes, suggesting that the teneurin gene became associated with another eukaryotic gene throughout time (Tucker et al., 2012). As the extracellular domain of teneurin is involved in mediating cell adhesion, the C-terminal region may have been used to ‘fish’ for prokaryotic organisms, increasing its ability to acquire food (Tucker et al., 2012). This could have facilitated the development of multicellularity, stimulating the gene to become ensconced into the metazoan genome and passed into subsequent speciation events throughout evolutionary time (Chand et al., 2013; Tucker et al., 2012; Tucker, 2013).

Moreover, evidence suggests that Secretin GPCRs evolved from Adhesion GPCRs. Adhesion GPCR genes have been identified in the genome of amphioxus, *Branchiostoma*...
_floridiae_, the choanoflagellate, _M. brevicollis_ and the sea anemone, _Nematostella vectensis_, however the genomes of these species do not contain Secretin GPCR genes, suggesting Adhesion GPCRs evolved prior to Secretin GPCRs (Nordström et al., 2008; 2009). Also, Adhesion Group V sequences in the _N. vectensis_ genome have the same splice sites and a highly-conserved motif that is also found in Secretin GPCRs, suggesting that Adhesion Group V is the most closely related to the Secretin GPCRs and that Secretin GPCRs may be derived from Adhesion GPCRs (Nordström et al., 2009; Schiöth et al., 2010). This data, as well as the previously mentioned high sequence conservation between Secretin and Adhesion receptors and their ligands, suggest that the TCAP receptor system evolved earlier than the Secretin superfamily of peptides.

### 1.5 The effect of an ancestral peptide on calcium ion regulation

Maintaining ion homeostasis was a stressor imposed on early cells. Charged ions crossed cell membranes by mechanisms of passive diffusion so regulating ion flux was crucial to ensure survival of organisms (Lovejoy, 2005). Calcium likely played a major role in the molecular mechanisms of the earliest organisms, as it is highly toxic in large amounts and therefore, needed to be utilized readily or it could impair their ability to survive (Lovejoy, 2005). Evidence of calcium signaling in protists, fungi and _M. brevicollis_, which contain multiple channels and the machinery needed to remove calcium as well as calcium ion signaling pathways, demonstrate that calcium ion signaling evolved prior to the emergence of the Metazoa (Cai, 2008; Cai & Clapham, 2012). Since calcium ion regulation has played a major role in the evolution of organisms and given the importance of calcium as a mediator of biological responses in the central nervous system (CNS), elucidating the effects of TCAP as an ancestral peptide on ion regulation, will better our understanding of its functional role.

### 1.6 Astrocytes: Function and evolution

Astrocytes are one of the most abundant glial cells in the mammalian CNS (Zhang & Barres, 2010). These multifunctional cells provide structural support, maintain ion homeostasis in the interstitial fluid, provide a stable environment for neurons and help clear the synaptic cleft of neurotransmitters (Sofroniew & Vinters, 2010). Calcium ion regulation plays a very important role in this cell type as astrocytes use it as a form of communication with one another but also
with the network they form with other cell types including neurons; a single astrocyte surrounds about 140,000 synapses (Agulhon et al., 2008; Bushong et al., 2002).

In terms of their evolutionary origin, astrocytes likely arose around the time when neural cells began to assemble into networks. This is supported by the identification of proto-astrocyte cells that share similar mechanisms of calcium regulation in the nematode, *Caenorhabditis elegans* (Oikonomou & Shaham, 2011). Proto-astrocyte cells are also found among protochordates but the earliest evidence of astrocyte-like cells among chordates is found in hagfish (Appel, 2004; Wicht et al., 1994). Due to their presence in some chordate lineages but not others, astrocytes are thought to have arisen independently numerous types from their proto-astrocyte ancestor (Appel, 2004). The evolution of astrocytes at the base of chordate evolution likely played a significant role in the development of the complex CNS found in chordates. Therefore, given their high distribution, functional importance in the CNS, association with calcium signaling (see Section 1.7 below) and early evolutionary origin, it is likely that astrocytes were regulated by a number of ancestral signaling systems, such as TCAP. Thus, these cells may make a good model for understanding the functional role of TCAP in the CNS.

### 1.7 Intracellular calcium signaling in astrocytes: A form of excitability

Although astrocytes are not electrically excitable cells like neurons, which propagate action potentials as a form of signaling, they exhibit a form of excitability manifested by increases in intracellular calcium (Charles et al., 1991; Cornell-Bell et al., 1990). Intracellular calcium increases can occur in astrocytes alone, termed intrinsic astrocyte excitability, that is dependent on secondary calcium messengers (Nett et al., 2002; Parri et al., 2001) or they can be evoked by external stimuli from other cell types, such as neurotransmitters released from neurons (Charles et al., 1991; Cornell-Bell et al., 1990; Pasti et al., 1997; Porter & McCarthy, 1996). The endoplasmic reticulum (ER) is the main source of calcium underlying this signal (Finkbeiner, 1993; Scemes, 2000; Sheppard et al., 1997; Verkhratsy et al., 2012). Stimulation of the release of calcium stores from the ER is dependent on the activation of GPCRs located on the astrocyte cell membrane (those linked to a $G_q$ subunit) which stimulates the phospholipase C/inositol 1,4,5-triphosphate (PLC/IP$_3$) pathway, resulting in the mobilization of intracellular calcium from the
ER through IP3 receptors (Agulhon et al., 2008; Golovina & Blaustein, 2000; Parri & Crunelli, 2003; Scemes, 2000; Sheppard et al., 1997). Therefore, astrocytes undergo intracellular calcium increases, as a result of the mobilization of calcium stores from the ER. These calcium increases can occur as an intrinsic property in one cell or they can also be passed on to neighbouring cells as intercellular calcium waves, allowing for astrocytes to communicate in vast networks.

1.8 Intercellular calcium waves: Communication between astrocytes

Astrocytes exhibit increases in intracellular calcium which can propagate to other cells in a wave-like fashion, thought to be involved in both astrocyte-astrocyte and astrocyte-neuron communication (Cornell-Bell et al., 1990; Konietzko & Muller, 1994). Since astrocyte cell networks are connected to one another by gap junctions, their cytosols form a syncytium where intercellular calcium waves function as a signal that can be transmitted to neighbouring cells, synchronizing their activity and allowing them to modulate neuronal activity in vast areas of the CNS (Araque et al., 2014; Cornell-Bell et al., 1990; Covello & Araque, 2016; Finkbeiner, 1992; Glaum et al., 1990; Gundersen et al., 2015). There are two main ways that calcium waves can be passed on to neighbouring astrocytes and they are thought to occur simultaneously in astrocyte networks. The first involves the transfer of calcium and second messengers, like IP3 from cell to cell through gap junctions, which continuously stimulate the release of calcium in neighboring cells. The second is an extracellular pathway that takes place when the activation of receptors on the astrocytic membrane generates these messengers, such as what occurs with the PLC/IP3 pathway upon the activation of metabotropic glutamate receptors (mGluRs). For example, glutamate is a molecule that induces intercellular calcium waves in astrocyte networks (Cornell-Bell et al., 1990; Finkbeiner, 1992).

1.9 Bi-directional communication: The tripartite synapse complex

Astrocytes respond to neuronal activity, suggesting that neurons are capable of communicating with astrocytes. Neurotransmitters, such as glutamate, released from pre-synaptic terminals of neurons can induce increases in intracellular calcium in astrocytes (Cornell-Bell et al., 1990; Cornell-Bell & Finkbeiner, 1991; Kim et al., 1994; Navarrete et al., 2013; Perea & Araque, 2005; Porter & McCarthy, 1996). Glutamate induces its effects on astrocytes by functioning through
ionotropic receptors, such as N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, causing an influx in calcium and sodium ions as well as mGluRs, such as mGluR5, inducing the PLC/IP3 pathway (Cornell-Bell et al., 1990; Cornell-Bell & Finkbeiner, 1991; Kim et al., 1994). On the other hand, neurons respond to astrocyte activity, suggesting that astrocytes communicate with neurons. Glutamate can also function as a gliotransmitter that is released from astrocytes upon release of calcium from the ER and as a result, an increase in intracellular calcium levels (Innocenti et al., 2000; Parpura et al., 2011). Glutamate that is released from astrocytes can trigger and modulate neuronal activity (Angulo et al., 2004; Fellin et al., 2004; Navarrete et al., 2013; Parpura et al., 1994; Perea & Araque, 2005).

The ability of astrocytes and neurons to respond to signals from one another indicates bi-directional communication between the two cell types, as is described by the tripartite synapse complex (Fig. 1.1) (Araque et al., 1998; Perea et al., 2009). This concept encompasses the activation of glutamate receptors on the astrocyte membrane by glutamate released from neuronal pre-synaptic terminals as well as the activation of glutamate receptors on post-synaptic terminals by glutamate that is released from astrocytes, which modulates neuronal activity. Therefore, astrocytes are capable of calcium signaling with other astrocytes and are involved in bi-directional communication with neurons.
1.10 Hypothesis and experimental objectives

As was outlined in the previous section, the model that Secretin GPCRs derived from Adhesion GPCRs has previously been established. However, this has not been done with respect to the Secretin superfamily, ligands that bind to these receptors. Based on the sequence similarity between TCAP, CRF and calcitonin families, members of the Secretin superfamily of peptides, as well as the similarity of ADGRL to Secretin GPCRs, I postulate that the teneurin-TCAP system is ancestral to and a putative progenitor of the Secretin superfamily. Moreover, given the importance of tight calcium ion regulation early in evolution and that calcium signaling is a form of astrocyte excitability, I will examine the effect of TCAP-1 on calcium signaling in astrocytes, elucidating the teneurin-TCAP system in astrocytes in vitro for the first time. Given the ancient evolutionary origins of TCAP, I predict that TCAP-1 will regulate calcium signaling in...
astrocytes. These hypotheses were examined and the results follow in three subsequent chapters based on the objectives outlined below.

**Chapter 2: Analysis of newly identified corticotropin-releasing factor (CRF) family Petromyzon marinus sequences: refining the current model of CRF evolution**

**Objective:** Analyze newly identified sequences of CRF family members in *Petromyzon marinus*, to contribute to the current model of CRF evolution. Formulating a more accurate model of CRF evolution will allow for a better understanding of the evolution of ancestral peptide systems like TCAP, and these sequences can be used for analysis in Chapter 3.

**Chapter 3: Teneurin C-terminal associated peptide (TCAP) family: A putative progenitor for Secretin superfamily ligands**

**Objective:** Establish the teneurin-TCAP system as evolutionarily ancient and as a possible progenitor of the Secretin superfamily. Determine the phylogenetic relationships among Secretin superfamily members in relation to one another using TCAP as an outgroup.

**Chapter 4: Stimulation of a glutamate-associated calcium-mediated astrocyte syncytium in immortalized cell lines: the effects of TCAP-1 on astrocyte monocultures and in astrocytes co-cultured with neurons**

**Objective:** Determine the effects of TCAP-1 on the ancient calcium signaling system in astrocytes, *in vitro*.

a. Determine whether teneurin 1-4, TCAP 1-4 and ADGRL 1-3 are endogenously expressed in cultured astrocytes.

b. Determine whether TCAP-1 regulates calcium ion flux in astrocyte monoculture and astrocytes co-cultured with neurons.

c. Provide an understanding of the mechanism through which TCAP-1 is modulating calcium ion flux in cultured astrocytes.
Chapter 2
Analysis of newly identified corticotropin releasing factor (CRF) family *Petromyzon marinus* sequences: refining the current model of CRF evolution

*A version of this chapter has already been published. Please note that the published manuscript referred to CRF as corticotropin-releasing hormone (CRH), so the figures use this abbreviation (Endsin et al., 2017).*

2 Abstract

The purpose of this chapter was to examine novel CRF family sequences in the sea lamprey, *Petromyzon marinus*. As little is known with respect to the evolution of the CRF family in early chordates, the phylogenetic position of the lamprey, an ancestral vertebrate, provides important information. CRF peptide, receptor and BP sequences were analyzed and provided evidence of when CRF family members evolved, allowing for the refinement of the current model of CRF evolution. Results demonstrated that the CRF-UI lineage diverged prior to the emergence of the Agnatha, consistent with the current model. However, the distinct forms of Ucn2 and Ucn3 arose much later than was previously thought. Finally, with respect to the receptors and BP, some lineages were likely lost and then underwent duplications that resulted in forms that are present among vertebrates.

2.1 Introduction

TCAPs were initially discovered while looking for CRF-related homologues in a gene library (Qian et al., 2004). Further *in silico* studies established that the TCAPs possessed sequence identity to the CRF family of peptides (Lovejoy et al., 2006). It was not clear at the time however, how the structure of the TCAP family was related to vertebrate CRF. Although numerous CRF peptides have been discovered in vertebrates (Lovejoy and Balment, 1999; Lovejoy et al., 2006), CRF from the most ancestral clades of vertebrates, including hagfish and lamprey, had yet to be elucidated and as a result, the evolution of extant vertebrate CRFs could not be reconciled with invertebrate CRF homologues. As there is more sequence information available for extant vertebrate species, understanding the key evolutionary changes of these peptides is necessary in order to determine how this peptide family evolved.
CRF, itself, is associated with the hypothalamic-pituitary-adrenal (HPA) axis in vertebrates, integrating the perception of stressful stimuli by the CNS with glucocorticoid regulation. The CRF family is fundamental in regulating the stress response and energy metabolism in both vertebrates and invertebrates. This family includes four paralogous peptides in vertebrates, orthologous peptides in invertebrates, two receptors and a BP, all important components in integrating the stress response.

Found among both vertebrates and invertebrates, this peptide family is highly conserved underlining its functional importance throughout evolution. Among invertebrates, the CRF family is orthologous to CDLP in tunicates and DHs in arthropods (Lovejoy & Balment, 1999; Lovejoy & Basyte-Lovejoy, 2010; Lovejoy & Jahan, 2006). The presence of one CRF orthologue in tunicates that shares sequence motifs to CRF and UI as well as the DHs is consistent with the current model of CRF evolution in that one proto-CRF ancestor existed before the emergence of vertebrates. According to this model, termed the 2R Hypothesis, the ancestral CRF gene evolved about 500 million years before the CRF peptides became associated with glucocorticoid regulation and in vertebrates, it underwent two rounds of genome duplication that resulted in four paralogous peptides that are present among extant vertebrates (Campbell et al., 2004; Holland et al., 1994; Lovejoy & Balment, 1999; Lovejoy & Jahan, 2006; Lundin, 1993). The first genome duplication event occurred some time after the emergence of the protochordates but before the agnathans and resulted in two paralogous lineages: a CRF-UI (or Ucn in mammals) lineage and an Ucn2-Ucn3 lineage. The second genome duplication occurred before the actinopterygian-sarcopterygian split but this may have been either before or after the agnathans diverged from the gnathostomes. It resulted in four paralogous peptides among vertebrates: CRF, UI (or Ucn in mammals and Sql in amphibians), Ucn2 and Ucn3 (Chang & Hsu, 2004; Lovejoy & Balment, 1999; Lovejoy & Jahan, 2006). However, the evolutionary relationships among these four paralogues are not entirely clear.

In vertebrates, the CRF peptides exert their effects by binding to CRFR1, mainly involved in mediating the stress response through the HPA axis and CRFR2 involved in energy regulation and metabolism, responses needed to cope with the stressor (Fredriksson et al., 2003; Kuperman & Chen, 2008). CRF peptides can also bind the CRFBP, a glycoprotein that regulates activity
and can inhibit the downstream signaling effects of CRF, such as the release of ACTH from the pituitary (Westphal & Seasholtz, 2006). The evolutionary scheme of the receptors and BP is not as clearly established as for the CRF peptides. In terms of the receptors, there is evidence of one CRF-like receptor in non-chordates and two receptors are consistently present among chordates (Lovejoy et al., 2014). The hypothesized evolutionary scheme for both the receptors and binding protein is not consistent with the 2R Hypothesis. This suggests that either the entire CRF system did not evolve as a result of two separate events, but due to smaller sub-genomic duplications or that some of the receptor and BP genes were lost throughout evolutionary time. To conclude, the current hypothesis suggests that only one CRF-like ligand, receptor and BP were present in non-chordates, they became inherited by chordates and developed into a more complex, specialized system that is now present among vertebrates.

This is our current understanding of CRF family evolution given existing data. However, there is little available information on this peptide family in early chordates. *Petromyzon marinus*, the sea lamprey, is a jawless fish that holds an important phylogenetic position as it is classified as an agnathan, considered to be the most basal lineage of vertebrates. The class Agnatha encompasses the lampreys (Petromyzontiformes) and hagfish (Myxiniformes) and is considered a monophyletic group in most recent literature (Oisi et al., 2013; Schwarze et al., 2014; Takezaki et al., 2003). Therefore, adding the newly identified CRF family sequences from one of the earliest evolving vertebrates *P. marinus*, is an important contribution towards strengthening the current model of CRF evolution.
2.2 Materials and Methods

2.2.1 Sequence Analysis

Collection of Sequences

As part of a collaboration with Dr. Richard Manzon at the University of Regina, CRF peptide, receptor and binding protein peptide sequences for a variety of fish species, as well as *Petromyzon marinus*, were obtained. The list of sequences and their accession numbers can be found in the manuscript (Endsin et al., 2017).

Sequence Alignments

Sequences were aligned using the Clustal Omega multiple sequence alignment program (Goujon et al., 2010; Sievers et al., 2011). HBD sequences were determined using the Graphics option of the NCBI website and if not available, the sequences were aligned to previously known HBD sequences using Clustal Omega and determined based on this alignment.

2.2.2 Phylogenetic Analysis

Phylogenetic tree construction and statistical analyses were carried out in MEGA 6.0 (Tamura et al., 2013).

Neighbour-Joining (NJ) Method

The amino-acid substitution model was set to p-distance to represent evolutionary distances as the proportion of amino acid differences among sites. The rate variation among sites was uniform with a homogenous pattern among lineages. A complete deletion was applied so all sites that included a gap were deleted. The trees were not rooted. Reliability of the tree was tested using 1000 bootstrap replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed.
For analysis of CRF pre-propeptides and mature peptides, a total of 42 amino acid sequences were used, with a total of 67 positions for pre-propeptides and 12 positions for mature peptides in the final data set after complete deletion was applied.

For analysis of CRF receptors, a total of 27 amino acid sequences were used, with a total of 366 positions in the final dataset of complete receptor sequences and a total of 67 positions of the HBD sequence, after complete deletion was applied.

Finally, for BP analysis, a total of 20 amino acid sequences were used with a total of 220 positions in the final dataset after complete deletion was applied.
2.3 Results

The newly identified *Petromyzon marinus* sequences (three peptide sequences, two receptor sequences and one BP sequence) were obtained by Dr. Richard Manzon at the University of Regina and were subject to sequence and phylogenetic analysis.

2.3.1 Analysis of *Petromyzon marinus* CRF peptides

Analysis of the three novel peptide sequences revealed that two of the peptides are more closely related to the CRH-UI lineage, one an orthologue of CRF and the other of UI, whereas the third peptide sequence was identified as an orthologue of Ucn3. An orthologue of Ucn2 was not identified. The *P. marinus* mature peptide sequences contained characteristic peptide motifs that identified them in relation to their respective orthologue. The *P. marinus* CRF sequences contained residues characteristic of CRF but not UI (V18, A28, Q29, H32) whereas *P. marinus* UI shared residue characteristics with both CRH and UI peptides (Fig. 2.1). *P. marinus* Ucn3 contained a ‘PTN’ motif specifically characteristic of Ucn3 orthologues as it is not found in Ucn2 (Fig. 1).

| >Manduca sexta DH | -RSFEEHLQDPSV|EISEAKKKMKFAKSFLL| |
| >Solea senegalensis CRH | R2DFDEILLTRRERQMNDDRANKGKAAAQKDDAYKLYKFKYELVR | |
| >Sparus aurata CRH | DTDKIDKLTLTTSSEMMMYRANKGKAAAQKDDAYKLYKFKYELVR | |
| >Bos taurus CRH | SQ2R11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Ovis aries CRH | S2EQ11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Xenopus laevis CRF | A57B11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Homo sapiens CRH | B3BP11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Sus scrofa CRH | SP7P11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Rattus norvegicus CRH | SQ2P11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Catostomus commersonii CRH2 | SQ2Z11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Catostomus commersonii CRH1 | ST7B11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Cyprinus carpio CRH2 | SQ0L11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Danio rerio CRH | SQ0L11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Cyprinus carpio CRH1 | ST7B11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Petromyzon marinus CRH | SQ7A11DLTTTLRRELTYADQEAQRKNSLDD-LA | |
| >Petromyzon marinus Ucn | AE2P11DLTTTLRRELQARVEKLD-QNAFAKQMVENV- |
| >Petromyzon marinus Ucn3 | D3PD11DLTTTLRRELQARVEKLD-QNAFAKQMVENV- |
| >Homo sapiens Ucn | -DNHRI1DLTTTLRRELQARVEKLD-QNAFAKQMVENV- |
| >Rattus norvegicus Ucn | -DDPE11DLTTTLRRELQARVEKLD-QNAFAKQMVENV- |
| >Cyprinus carpio UI | -DDPE11DLTTTLRRELQARVEKLD-QNAFAKQMVENV- |
| >Carassius auratus UI | -DNDB11DLTTTLRRELQARVEKLD-QNAFAKQMVENV- |
| >Oncorhynchus mykiss UI | -DNDB11DLTTTLRRELQARVEKLD-QNAFAKQMVENV- |
| >Oryzias latipes UT | -DNDB11DLTTTLRRELQARVEKLD-QNAFAKQMVENV- |
| >Oryzias latipes Ucn3 | -DNDB11DLTTTLRRELQARVEKLD-QNAFAKQMVENV- |
| >Mus musculus Ucn2 | ---IVIDLPVPGQILFLQAARAAKAAQCOPTMAIRALAV- |
| >Mus musculus UI | ---IVIDLPVPGQILFLQAARAAKAAQCOPTMAIRALAV- |
| >Rattus norvegicus Ucn2 | ---IVIDLPVPGQILFLQAARAAKAAQCOPTMAIRALAV- |
| >Oryzias latipes Ucn2 | ---IVIDLPVPGQILFLQAARAAKAAQCOPTMAIRALAV- |
| >Oryzias latipes Ucn3 | ---IVIDLPVPGQILFLQAARAAKAAQCOPTMAIRALAV- |

*Figure 2.1. Mature peptide sequence alignment of CRF, UI and Ucn3 peptides in Petromyzon marinus with respective orthologues (derived from Endsin et al., 2017). The alignment was made using Clustal Omega multiple sequence alignment program and modified to ensure that the characteristic residue motifs were conserved.*
Phylogenetic analysis of pre-propeptides of the novel lamprey orthologues indicated that *P. marinus* CRF clustered closer to the other CRF orthologues and that UI clustered closer to UII orthologues, however they appear to be intermediate forms, whereas Ucn3 was part of the Ucn3 clade (Fig. 2.2). Phylogenetic analysis of the mature *P. marinus* sequences demonstrated the same relationship as with the pre-propeptides, corroborating the classification of each homologue into CRF, UI and Ucn3 orthologues in *P. marinus* (Fig. 2.3).

**Figure 2.2.** Evolutionary relationships of the *Petromyzon marinus* CRF family member pre-propeptides (derived from Endsin et al., 2017). Analysis was conducted using the neighbor joining method with evolutionary distances computed using the p-distance method, presented as the number of amino acid differences per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

**Figure 2.3.** Evolutionary relationships of the *Petromyzon marinus* CRF family member mature peptides (derived from Endsin et al., 2017). Analysis was conducted using the neighbor-joining method with evolutionary distances computed using the p-distance method, presented as the number of amino acid differences per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.
2.3.2 Analysis of *Petromyzon marinus* CRF receptors

Phylogenetic analysis of the two *P. marinus* CRF receptor sequences, termed CRFRα and CRFRβ, revealed that the lamprey receptors formed a distinct clade from other CRFRs (Fig. 2.4). Analysis of the HBD region, a highly conserved domain characteristic of the Secretin GPCR family, demonstrated that the *P. marinus* HBD sequence was part of a clade with both CRFR1 and CRFR2 HBD vertebrate sequences that was distinct from the DH receptors (Fig. 2.5).

![Figure 2.4. Evolutionary relationships of the *Petromyzon marinus* CRF receptors (derived from Endsin et al., 2017). Analysis was conducted using the neighbor joining method with evolutionary distances computed using the p-distance method, presented as the number of amino acid differences per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.](image1)

![Figure 2.5. Evolutionary relationships of the *Petromyzon marinus* CRF receptor hormone binding domains (derived from Endsin et al., 2017). Analysis was conducted using the neighbor joining method with evolutionary distances computed using the p-distance method, presented as the number of amino acid differences per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.](image2)
2.3.3 Analysis of *Petromyzon marinus* CRF binding proteins

Phylogenetic analysis of the *P. marinus* CRF binding proteins (CRFBPs) revealed that it formed a sister lineage to vertebrate CRHBPs that was separate from invertebrate CRHBPs (Fig. 2.6).

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![Figure 2.6. Evolutionary relationships of the *Petromyzon marinus* CRF binding proteins](image)

Figure 2.6. Evolutionary relationships of the *Petromyzon marinus* CRF binding proteins (derived from Endsin et al., 2017). Analysis was conducted using the neighbor joining method with evolutionary distances computed using the p-distance method, presented as the number of amino acid differences per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.
2.4 Discussion

The identification of CRF family members in *Petromyzon marinus* is an important contribution to our current understanding of CRF phylogeny as this is the first time a functional CRF system has been classified in an agnathan. The current model of CRF evolution suggests that a proto-CRF ancestor underwent two rounds of whole genome duplication, resulting in four paralogous genes currently found in vertebrates (2R Hypothesis). The timing of the first genome duplication occurred after the emergence of protochordates but before the agnathans and it gave rise to two paralogous lineages (CRF-UI and Ucn2-Ucn3). The timing of the second genome duplication and at what point the peptides diverged into four paralogues is less certain. Therefore, the identification of CRF family members in *P. marinus* allows for a contribution towards refining the current hypothesis. In making the argument and adding to the current model of CRF family evolution, the Agnatha will be considered a monophyletic group based on most recently established literature, as was mentioned earlier. Importantly, these data provide a much clearer relationship among the invertebrate and vertebrate CRF family homologues and can provide a more accurate understanding of ancestral peptides, including TCAP.

2.4.1 Analysis of *Petromyzon marinus* CRF peptides

Phylogenetic analysis as well as careful sequence inspection of CRF peptides in *P. marinus* suggest that some of the genes are not fully differentiated into their distinct functional entities as they are in more derived vertebrates. This is observed with respect to CRF and UI, as they show a much closer sequence similarity to one another than what is observed among less evolutionarily ancient species. This finding is consistent with the previously established timing of the first genome duplication, as the CRF-UI lineage was clearly present prior to the emergence of the agnathans. Therefore, given the identification of distinct CRF and UI orthologues in *P. marinus*, it is likely that a partial sub-genomic duplication of the CRF-UI gene occurred prior to the agnathans and gave rise to the CRF and UI lineages (Fig. 2.7).

Moreover, Ucn2 was not found in *P. marinus*, however Ucn3 was identified. This suggests that Ucn2 may have begun to differentiate as an independently functional gene after Ucn3. Given evidence that Ucn2 has not been found to exist among Chondrichthyes (Lovejoy & de Lannoy,
2013; Nock et al., 2011), two plausible scenarios emerge. The first, Ucn2 existed as a pseudogene and only became functional after the Chondrichthyes, while Ucn3 was functional. The second, the Ucn3 gene may have undergone a second round of partial genome duplication sometime after the emergence of the Chondrichthyes, giving rise to Ucn2. The second scenario is more likely as Ucn2 and Ucn3 share high similarity and it would be unlikely that this would be the case 100 million years after the existence of a non-functional gene. Evidence of an Ucn3 orthologue in *P. marinus*, but not Ucn2 suggests that the Ucn2-Ucn3-like lineage did undergo the first genome duplication but did not undergo the same partial sub-genomic duplication prior to the emergence of the agnathans that the CRF-UI-like lineage did. Therefore, as there is no evidence of an Ucn2 gene prior to the Chondrichthyes, it is possible that the Ucn3 gene underwent a separate genome duplication that gave rise to Ucn2 and Ucn3 after the emergence of the Chondrichthyes (Fig. 2.7). These findings propose a different model for the evolution of Ucn2 and Ucn3, than what has been proposed in the past, which stated that the four paralogues arose as a result of two complete rounds of genome duplication. Therefore, the *P. marinus* sequences have contributed new information towards the refinement of the current model.

### 2.4.2 Analysis of *Petromyzon marinus* CRF receptors and binding protein

Like the CRF and UI peptides in *P. marinus*, the CRF receptors also resemble forms that have not fully been differentiated into their respective subtypes. Interestingly, this is also the case with the CRF receptors identified in the tunicate, a protochordate (Campbell et al., 2004; Sherwood et al., 2006). Considering the hypothesis of CRF receptor evolution, in that only one CRFR gene was inherited by early chordates, it is possible that as a result of local gene duplication in the protochordates, two forms were present but one of the forms was an inactive receptor gene. Subsequently, after the first genome duplication prior to the agnathans, one receptor lineage was lost and the second lineage split into two closely related genes that eventually evolved in to the CRFR1 and CRFR2 forms found in chordates (Fig. 2.7). It is possible that a similar event occurred with respect to the binding protein, however one of the genes may have been lost prior to the emergence of the agnathans, which resulted in only one extant CRFBP gene found in chordates (Fig. 2.7).
2.4.3 Concluding remarks

Taken together, these findings indicate that the evolution of CRF peptides occurred in three phases. First, the CRF-UI-like and Ucn2-Ucn3-like paralogous lineages arose as a result a whole genome duplication event prior to the agnathans. Second, the CRF-UI lineage underwent a partial sub-genomic duplication event prior to the agnathans, resulting in the emergence of distinct CRF and UI genes. The Ucn2-Ucn3 lineage did not undergo this sub-genomic duplication. Third, the distinct Ucn2 and Ucn3 forms arose as a result of a genome duplication event after the Chondrichthyes. In terms of the receptors, one receptor lineage was likely lost and the remaining form gave rise to two receptor subtypes as a result of genome duplication prior to the agnathans. Similarly, in terms of the BP, one lineage was likely lost, the remaining gene underwent a genome duplication event prior to the agnathans after which one paralogous lineage was lost again and this resulted in one remaining CRFBP gene among chordates. Therefore, the newly identified *P. marinus* CRF family sequences have contributed towards refining the current model of CRF family evolution and allowed for the formation of a more accurate picture of the ancestral peptide of CRF and related families. These new sequences can now be used to help determine the ancestral relationships among TCAP, CRF and related peptides (See Chapter 3).
Chapter 3
Teneurin C-terminal associated peptide (TCAP) family: A putative progenitor for Secretin superfamily ligands

3  Abstract

Given the ancient origins of the teneurin-TCAP system and using the up-to-date information for CRF, calcitonin and secretin evolution, peptides were analyzed with respect to TCAP to establish phylogenetic relationships among the families. Results demonstrated that calcitonin and insulin are sister lineages and that they are more related than was previously thought. Also, the calcitonin and insulin lineage appears to be a sister lineage to CRF and together, calcitonin, insulin and CRF form a sister lineage to secretin. Therefore, by placing TCAP as a putative progenitor to the Secretin superfamily, relationships among CRF, calcitonin and secretin lineages were examined.

3.1 Introduction

The Secretin superfamily of peptides is a diverse assemblage of peptide lineages with overlapping functions and structurally related receptors. These peptides all bind to Secretin GPCRs and among this family are CRF, calcitonin, secretin, glucagon, GHRH, VIP, PACAP, among others (see Appendix I for full list) (Fredriksson et al., 2003). These peptide lineages have expanded and radiated before the bifurcation of the deuterostomes and protostomes, however, the progenitor peptide and receptor of this diverse assemblage has not been established. Recent studies suggest that this assemblage originated as a result of a lateral gene transfer event from the prokaryotes to a choanoflagellate ancestor of the Metazoa. Here, TCAP is presented as an ancestor of the Secretin superfamily.

The Secretin superfamily of peptides is one of the five main families of ligands that bind to GPCRs. The GPCRs have most recently been classified into five main families using the GRAFS system, Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled/Taste2 (F) and Secretin (S) (Fredriksson et al., 2003). Among these, Adhesion and Secretin GPCRs are the most evolutionarily ancient (Nordström et al., 2009). Adhesion GPCRs have a characteristically long N-terminus that is rich in serine and threonine residues while Secretin GPCRs have a characteristic HBD in their N-terminus, the portion of the receptor that binds the ligand
The evolutionary scheme of these two families is well established and there is considerable evidence that the Secretin-related receptors form a single monophyletic clade that derived from the Adhesion GPCRs (Schiöth et al., 2010). Adhesion GPCR genes have been identified in amphioxus, choanoflagellate and sea anemone genomes but Secretin GPCR genes have not suggesting that Adhesion GPCRs are ancestral to Secretin GPCRs (Nordström et al., 2009). Also, more derived Adhesion GPCR members possess a HBD, highly conserved motifs and the same splice site setup as Secretin GPCRs, further confirming the close relationship between these two families (Nordström et al., 2009). Therefore, the phylogeny of these receptors is well established, however evidence of a similar relationship of the peptides has not been elucidated.

One such Adhesion GPCR that does possess a HBD and similar structural motifs to Secretin GPCRs is ADGRL. It was originally misclassified as a Secretin GPCR, due to its characteristic HBD, but has now been re-classified as an Adhesion GPCR (Fredriksson et al., 2003). The first identified ligand for ADGRL was α-latrotoxin, the major component of black widow spider toxin venom, however this is an exogenous ligand (Lelianova et al., 1997). Our laboratory has recently identified that TCAP-1 is an endogenous ligand that interacts with the HBD of ADGRL (Husic, 2016). The TCAPs are a family of four bioactive peptides that are 40-41 amino acids in length and are located at the C-terminus of each of the teneurin transmembrane proteins (Qian et al., 2004). Qian and colleagues discovered TCAP while screening the rainbow trout hypothalamic cDNA library for CRF-related homologues, using a hamster urocortin probe. They identified a clone that represented the rainbow trout orthologue of teneurin 3 and discovered a neuropeptide-like sequence encoded at the carboxy-terminus in the last exon of teneurin 3, termed TCAP-3. Subsequently, TCAP-1, -2 and -4 were discovered after further analysis of teneurins 1, 2 and 4 (Wang et al., 2005). TCAPs are classified as cleavable bioactive peptides because they possess a cleavage motif at the N-terminus and an amidation motif at the C-terminus (Lovejoy et al., 2006). However, evidence also suggests that the TCAPs are independent of the teneurins, as mouse TCAP-1 is expressed as a separate transcript from the teneurin gene and is independently transcribed (Chand et al., 2013). Interestingly, TCAP shares about 20% sequence similarity with α-latrotoxin and α-latrotoxin shares sequence similarity with other Secretin family ligands (Holz
Moreover, as our laboratory previously established, TCAP shares about 20% sequence similarity with CRF and calcitonin, members of the Secretin superfamily of ligands, suggesting a common evolutionary origin (Lovejoy et al., 2006).

Therefore, as TCAP binds to an Adhesion GPCR and shares sequence similarity to CRF and calcitonin, ligands that bind to Secretin GPCR receptors and are classified as being most closely related to ancestral Adhesion GPCRs, this prompted the investigation of TCAP as a progenitor of the Secretin superfamily. Evidence of TCAP being ancestral peptide that arose as a result of a HGT event from a prokaryote to a choanoflagellate ancestor, has been previously suggested (Tucker et al., 2012; Zhang et al., 2012). Results from phylogenetic analysis suggest that TCAP is a highly conserved peptide that forms a distinct clade from the Secretin superfamily, allowing for the elucidation of relationships among members of this peptide superfamily.
3.2 Materials and Methods

3.2.1 Sequence Analysis

Collection of Sequences

Peptide sequences of Secretin GPCR ligands, including CRF, calcitonin and secretin families and Adhesion GPCR ligands, including TCAP 1-4, as well as reference groups including NPY and insulin were collected among a range of extant protostomes and deuterostomes, using the GenBank genome sequence analysis program on the NCBI website. The hormones were organized by organism, phylum, class and order and were tabulated and their accession numbers were recorded (see Appendix I for full list). Sequences were divided into pre-propeptides (or propeptides for TCAP) and mature peptides, after which were imported to MEGA 6.0 for analysis (Tamura et al., 2013). Downloaded from http://www.megasoftware.net/.

Sequence Alignments

Peptide sequences were aligned using the MUSCLE algorithm (Edgar, 2004). The alignment was examined, reviewed for duplicate sequences using pairwise distances (d= 0.0 was identical) and excess sequence was cut at both 5’ and 3’ ends, as these fragments did not contribute to the alignment. Modifications to the alignment were made to ensure that the characteristic residue motifs were conserved. This included highly conserved cysteine (C), tryptophan (W), arginine (R) and lysine (K) residues throughout as well as motifs characteristic of each family. For the CRF family this was the 5’ leucine (L), serine (S) and the 3’ asparagine (N) motif that is conserved throughout the entire family, the ‘TCV’ or ‘TCXV’ motif that is conserved among the calcitonin family and the ‘PELAD’ motif that is conserved among the TCAP family.

3.2.2 Phylogenetic Analysis

Phylogenetic tree construction and statistical analyses were carried out in MEGA 6.0 (Tamura et al., 2013). Downloaded from: http://www.megasoftware.net/.
Maximum Likelihood (ML) Method

The amino acid substitution model and the rate among sites were both chosen based on the model that resulted in the greatest log likelihood, the lowest Akaike Information Criterion (AIC) and the lowest Bayesian Information Criterion (BIC), parameters calculated by MEGA 6.0. To ensure the most accurate analysis, these parameters were calculated for each constructed tree. The model that maximized the log likelihood was used for analysis. A partial deletion of sequences with too many gaps/missing data was applied with a cutoff of 95%, so sites that were not found in at least 95% of sequences were not used towards the analysis. The applied heuristic method was Nearest-Neighbour Interchange (NNI), so the initial trees were obtained using the NJ method to a matrix of pairwise distances estimated using a JTT model. Reliability of the tree was tested using 1000 bootstrap replicates.

Two sets of analyses were performed. The first involved Secretin superfamily pre-propeptides, which are composed of a signal, cryptic and mature peptide and TCAP propeptides, as TCAP does not possess a signal peptide. Given the functional importance and bioactivity of mature peptides throughout evolution, a second separate analysis was performed on mature peptides of both Secretin superfamily and TCAP family members.

For analysis involving Secretin superfamily pre-propeptides and TCAP family propeptides, a total of 181 amino acid sequences were used, with a total of 44 positions in the final dataset after all positions with less than 95% site coverage were eliminated.

For analysis involving Secretin superfamily mature peptides and TCAP mature peptides, a multistep analysis was undertaken in order to elucidate the relationships of each family with respect to one another and TCAP. For insulin and the calcitonin family, analysis involved 72 amino acid sequences, with a total of 14 positions in the final dataset. For insulin, calcitonin and TCAP, analysis involved 95 amino acid sequences, with a total of 14 positions in the data set. For insulin, calcitonin, CRF and TCAP families, analysis involved 135 amino acid sequences leaving 12 positions in the final data set. Lastly, for insulin, calcitonin, CRF, secretin and TCAP families, analysis included 179 amino acid sequences leaving 15 positions in the final dataset.
3.3 Results

3.3.1 Sequence analysis of TCAP paralogues and orthologues

TCAP paralogues, that diverged as a result of a genome duplication event (Fig. 3.1), and orthologues, that arose as a result of a species divergence (Fig. 3.2), demonstrate a high degree of conservation among vertebrates. This family of peptides contains a characteristic ‘PELAD’ motif located at residues 24-28 from the N-terminus.

| Mouse TCAP4 | QQILNTRVQGYGFVLSVEQYPESPNHQKQSEI |
| Mouse TCAP3 | QQILNTRVQGYGFVLSVEQYPESPNHQKQSEI |
| Mouse TCAP2 | QQILNTRVQGYGFVLSVEQYPESPNHQKQSEI |
| Mouse TCAP1 | QQILNTRVQGYGFVLSVEQYPESPNHQKQSEI |

**Figure 3.1. Mature peptide sequences of TCAP paralogues in mouse.** Aligned using MUSCLE. Dark grey boxes indicate amino acid identity and light grey boxes indicate a functional replacement.

| A) MouseTCAP1 | QQILGTRVQGYDQYVSVEQYPESPNHQKQSEI |
| HumanTCAP1 | QQILGTRVQGYDQYVSVEQYPESPNHQKQSEI |
| ChickenTCAP1 | QQILGTRVQGYDQYVSVEQYPESPNHQKQSEI |
| ClawedfrogTCAP1 | QQILGTRVQGYDQYVSVEQYPESPNHQKQSEI |
| Eleph.sharkTCAP1 | QQILGTRVQGYDQYVSVEQYPESPNHQKQSEI |
| ZebrafishTCAP1 | QQILGTRVQGYDQYVSVEQYPESPNHQKQSEI |

| B) MouseTCAP2 | QQILNTRVQGYGYYLPEQYPESPNHQKQSEI |
| HumanTCAP2 | QQILNTRVQGYGYYLPEQYPESPNHQKQSEI |
| ChickenTCAP2 | QQILNTRVQGYGYYLPEQYPESPNHQKQSEI |
| ClawedfrogTCAP2 | QQILNTRVQGYGYYLPEQYPESPNHQKQSEI |
| Eleph.sharkTCAP2 | QQILNTRVQGYGYYLPEQYPESPNHQKQSEI |
| ZebrafishTCAP2A | QQILNTRVQGYGYYLPEQYPESPNHQKQSEI |
| ZebrafishTCAP2B | QQILNTRVQGYGYYLPEQYPESPNHQKQSEI |

| C) MouseTCAP3 | QLLSAARVQGYDQYVSVEQYPESPNHQKQSEI |
| HumanTCAP3 | QLLSAARVQGYDQYVSVEQYPESPNHQKQSEI |
| ChickenTCAP3 | QLLSAARVQGYDQYVSVEQYPESPNHQKQSEI |
| ClawedfrogTCAP3 | QLLSAARVQGYDQYVSVEQYPESPNHQKQSEI |
| Eleph.sharkTCAP3 | QLLSAARVQGYDQYVSVEQYPESPNHQKQSEI |
| ZebrafishTCAP3 | QLLSAARVQGYDQYVSVEQYPESPNHQKQSEI |

| D) MouseTCAP4 | QQLNGTRVQGYGFVLSVEQYPESPNHQKQSEI |
| HumanTCAP4 | QQLNGTRVQGYGFVLSVEQYPESPNHQKQSEI |
| ChickenTCAP4 | QQLNGTRVQGYGFVLSVEQYPESPNHQKQSEI |
| ClawedfrogTCAP4 | QQLNGTRVQGYGFVLSVEQYPESPNHQKQSEI |
| Eleph.sharkTCAP4 | QQLNGTRVQGYGFVLSVEQYPESPNHQKQSEI |
| ZebrafishTCAP4 | QQLNGTRVQGYGFVLSVEQYPESPNHQKQSEI |

**Figure 3.2. Mature peptide sequences of TCAP orthologues in vertebrates.** Aligned using MUSCLE. Dark grey boxes indicate amino acid identity and light grey boxes indicate a functional replacement.
3.3.2 Evolutionary analysis of Secretin superfamily and TCAP pre-propeptides

Phylogenetic analysis of CRF, calcitonin, insulin, secretin pre-propeptides and TCAP family propeptides revealed that each family formed a distinct group: TCAP, CRF and secretin appear as distinct clades and insulin appears to form a sister group with calcitonin (Fig 3.3; 3.4). NPY, a reference group, clustered with the secretin family (Fig. 3.3; 3.4). Given the assumption that TCAP-1 is an ancestral peptide, an analysis of how other peptide families would cluster upon rooting the tree to TCAP was undertaken (Fig. 3.4).
Figure 3.3. Phylogenetic analysis of CRF, calcitonin, insulin and secretin family pre-propeptides with TCAP propeptides (unrooted). Each family is highlighted with a different colour: CRF (red), calcitonin (orange), secretin (purple), TCAP (blue). Analysis was conducted using the maximum likelihood method based on the JTT+G matrix-based model (lnL=-11224.5064; +G, parameter=1.3976) (Jones et al., 1992). Initial trees for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using a JTT model. Branch lengths represent the number of substitutions per site, with the tree shown to scale. Bootstrap analysis involved 1000 replicates. Abbreviations: **CRF family**: corticotropin-releasing factor (CRF); telecortin (TCN); urocortin (UCN); urocortin 2 (UCN2); urocortin 3 (UCN3); urotensin (UI); sauvgaine (SVG); diuretic hormone (DH); **Calcitonin family**: calcitonin (CALC); calcitonin-gene-related peptide 1 (CGRP1); calcitonin-gene-related peptide 2 (CGRP2); amylin (AM); adrenomedullin (ADM); adrenomedullin 2 (ADM2); **Secretin family**: secretin (SCT); growth hormone releasing hormone (GHRH); gastric inhibitory peptide (GIP); glucagon (GCG); pituitary adenylate cyclase-activating peptide (PACAP); vasoactive intestinal peptide (VIP); **Reference groups**: teneurin C-terminal associated peptide (TCAP); neuropeptide Y (NPY) and insulin (INS).
Figure 3.4. Phylogenetic analysis of CRF, calcitonin, insulin and secretin family pre-propeptides with TCAP propeptides (rooted to TCAP). Each family is highlighted with a different colour: CRF (red), calcitonin (orange), secretin (purple), TCAP (blue). Analysis was conducted using the maximum likelihood method based on the JTT+G matrix-based model (lnL=-11224.5064; +G, parameter=1.3976) (Jones et al., 1992). Initial trees for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using a JTT model. Branch lengths represent the number of substitutions per site, with the tree shown to scale. Bootstrap analysis involved 1000 replicates. Abbreviations: **CRF family:** corticotropin-releasing factor (CRF); teleocortin (TCN); urocrortin (UCN); urocrortin 2 (UCN2); urocrortin 3 (UCN3); urotensin (UI); sauvgaine (SVG); diuretic hormone (DH); **Calcitonin family:** calcitonin (CALC); calcitonin-gene-related peptide 1 (CGRP1); calcitonin-gene-related peptide 2 (CGRP2); amlyin (AM); adrenomedullin (ADM); adrenomedullin 2 (ADM2); **Secretin family:** secretin (SCT); growth hormone releasing hormone (GHRH); gastric inhibitory peptide (GIP); glucagon (GCG); pituitary adenylate cyclase-activating peptide (PACAP); vasoactive intestinal peptide (VIP); **Reference groups:** neuropeptide Y (NPY) and insulin (INS); **Outgroup:** teneurin C-terminal associated peptide (TCAP).
3.3.2 Evolutionary analysis of Secretin superfamily and TCAP mature peptides

A separate analysis was performed with mature peptide sequences of the Secretin superfamily and TCAP mature peptides. A multi-step approach was undertaken in order to understand the relationship of each family relative to TCAP prior to conducting a comprehensive analysis of all of the families. First, phylogenetic analysis of calcitonin mature peptides and insulin A and B mature chains revealed a close relationship between the two families (Fig. 3.5). Insulin A and B chains appeared to be equidistant relative to the calcitonin family of peptides.

![Figure 3.5. Phylogenetic analysis of insulin and calcitonin mature peptides (unrooted).](image)

Each family is highlighted with a different colour: calcitonin (orange), insulin (green). Analysis was conducted using the maximum likelihood method based on the JTT matrix-based model (lnL=−677.0993; +G, parameter=2.9043) (Jones et al., 1992). Initial trees for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using a JTT model. Branch lengths represent the number of substitutions per site, with the tree shown to scale. Bootstrap analysis involved 1000 replicates. Abbreviations: **Calcitonin family:** calcitonin (CALC); calcitonin-gene-related peptide 1 (CGRP1); calcitonin-gene-related peptide 2 (CGRP2); amylin (AM); adrenomedullin (ADM); adrenomedullin 2 (ADM2); **Insulin:** insulin A chain (INSa); insulin B chain (INSb).
Second, phylogenetic analysis of calcitonin mature peptides, insulin A and B mature chains and TCAP demonstrated that calcitonin and insulin families formed sister groups (Fig. 3.6). Insulin A appeared to be more closely related to the calcitonin family than insulin B when the tree was rooted to TCAP (Fig. 3.6b).

**Figure 3.6. Phylogenetic analysis of insulin, calcitonin and TCAP mature peptides.** Each family is highlighted with a different colour: calcitonin (orange), insulin (green), TCAP (blue). The trees are represented as A) unrooted and B) rooted to TCAP. Analysis was conducted using the maximum likelihood method based on the JTT matrix-based model (lnL=−919.2846; +G, parameter=6.6766) (Jones et al., 1992) Initial trees for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using a JTT model. Branch lengths represent the number of substitutions per site, with the tree shown to scale. Bootstrap analysis involved 1000 replicates. Abbreviations: **Calcitonin family:** calcitonin (CALC); calcitonin-gene-related peptide 1 (CGRP1); calcitonin-gene-related peptide 2 (CGRP2); amylin (AM); adrenomedullin (ADM); adrenomedullin 2 (ADM2); **Insulin:** insulin A chain (INSa); insulin B chain (INSb); **Outgroup:** teneurin C-terminal associated peptide (TCAP).
Phylogenetic analysis of calcitonin, insulin A and B chains, CRF and TCAP mature peptides revealed that calcitonin and insulin families were sister groups and that CRF formed a separate clade to these two families (Fig. 3.7).
Figure 3.7. Phylogenetic analysis of insulin, calcitonin, CRF and TCAP mature peptides. The trees are represented as A) original tree with the appropriate scale B) magnified and unrooted, C) magnified and rooted to TCAP. Each family is highlighted with a different colour: CRF (red), calcitonin (orange), insulin (green), TCAP (blue). Analysis was conducted using the maximum likelihood method based on the Dayhoff matrix-based model (lnL=-1019.5552; +G, parameter=6.6766) (Jones et al., 1992). Initial trees for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using a JTT model. Branch lengths represent the number of substitutions per site, with the tree shown to scale. Bootstrap analysis involved 1000 replicates. Abbreviations: Calcitonin family: calcitonin (CALC); calcitonin-gene-related peptide 1 (CGRP1); calcitonin-gene-related peptide 2 (CGRP2); amylin (AM); adrenomedullin (ADM); adrenomedullin 2 (ADM2); Insulin: insulin A chain (INSa); insulin B chain (INSb); CRF family: corticotropin-releasing factor (CRF); teleocortin (TCN); urocortin (UCN); urocortin 2 (UCN2); urocortin 3 (UCN3); urotensin (UI); sauvagine (SVG); diuretic hormone (DH); Outgroup: teneurin C-terminal associated peptide (TCAP).
Phylogenetic analysis of calcitonin, insulin A and B chains, CRF, secretin and TCAP mature peptides revealed that calcitonin and insulin families were sister groups and that both CRF and secretin formed separate groups from these two families (Fig. 3.8). TCAP appeared to form a distinct clade from Secretin superfamily members.
Figure 3.8. Phylogenetic analysis of insulin, calcitonin, CRF, secretin and TCAP mature peptides. The trees are represented as A) unrooted and B) rooted to TCAP. Each family is highlighted with a different colour: CRF (red), calcitonin (orange), insulin (green), secretin (purple), TCAP (blue). Analysis was conducted using the maximum likelihood method based on the Whelan and Goldman model (lnL=-1781.0007; +G, parameter=23.5912) (Jones et al., 1992). Initial trees for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using a JTT model. Branch lengths represent the number of substitutions per site, with the tree shown to scale. Bootstrap analysis involved 1000 replicates. Abbreviations: Calcitonin family: calcitonin (CALC); calcitonin-gene-related peptide 1 (CGRP1); calcitonin-gene-related peptide 2 (CGRP2); amylin (AM); adrenomedullin (ADM); adrenomedullin 2 (ADM2); insulin A chain (INSa); insulin B chain (INSb); CRF family: corticotropin-releasing factor (CRF); teleocortin (TCN); urocortin (UCN); urocortin 2 (UCN2); urocortin 3 (UCN3); urotensin (UI); sauvagine (SVG); diuretic hormone (DH); Secretin family: secretin (SCT); growth hormone releasing hormone (GHRH); gastric inhibitory peptide (GIP); glucagon (GCG); pituitary adenylate cyclase-activating peptide (PACAP); vasoactive intestinal peptide (VIP); Outgroup: teneurin C-terminal associated peptide (TCAP).
3.4 Discussion

In light of the considerable amount of evidence to suggest that the teneurin-TCAP system evolved prior to the emergence of the Metazoa (see Chapter 1 for discussion), the previously established relationship that Secretin GPCRs derived from Adhesion GPCRs (Schiöth et al., 2010), evidence that TCAP binds to ADGRL, an Adhesion GPCR with a HBD characteristic of Secretin GPCRs (Fredriksson et al., 2003) and given the sequence similarity that TCAP shares with Secretin superfamily members, CRF and calcitonin (Lovejoy et al., 2006), an investigation using TCAP as a putative progenitor of the Secretin superfamily was undertaken. A putative progenitor of the Secretin superfamily of ligands has not been previously established. Sequence analysis of TCAP family members demonstrated a highly conserved peptide and phylogenetic analysis of the Secretin superfamily in relation to TCAP as a putative progenitor revealed a set of relationships among Secretin superfamily members. Calcitonin and insulin families appear to be sister lineages and they are much more closely related to one another than was previously thought. Also, calcitonin and insulin are sister lineages that appear to be distinct lineages to CRF and secretin families. Therefore, placing TCAP as an ancestor of the Secretin superfamily is an insightful way to elucidate the evolutionary relationships among Secretin family members.

3.4.1 Sequence analysis of TCAP paralogues and orthologues

Sequence analysis of both TCAP paralogues and orthologues revealed that this family of peptides is very highly conserved. The presence of a highly conserved ‘PELAD’ motif among TCAP orthologues and paralogues, suggests that it may possess a functional attribute, such as a receptor-binding or activation site (Lovejoy, 2005). A peptide system with such a high amount of conservation is indicative of great functional importance that may have been selected for. This is also the case with NPY, which has been suggested to be one of the most highly conserved neuropeptides and has also been suggested to be evolutionarily ancient (Blomqvist et al., 1992). Therefore, the high sequence conservation among TCAP orthologues and paralogues suggests that this peptide system is evolutionarily ancient and may have been strongly selected for throughout evolutionary time.
3.4.2 Evolutionary analysis of Secretin superfamily and TCAP pre-propeptides

Phylogenetic analysis of Secretin superfamily pre-propeptides and TCAP family pro-peptides was undertaken in order to elucidate the structural relationships among these peptides. Pre-propeptides are composed of a signal peptide, for secretion, a cryptic peptide, the function of which is unknown and a bioactive mature peptide (Lau et al., 1993). The TCAP family has not been found to possess a signal peptide (Chand et al., 2013) so the TCAP propeptides were examined in relation to other family pre-propeptides. Analysis revealed that calcitonin, CRF, secretin and TCAP families formed distinct groups. Despite being chosen to serve as a reference group because it binds to a tyrosine kinase receptor and not a GPCR, insulin formed a group with calcitonin, suggesting that they may be sister lineages (Fig. 3.4). The close relationship between calcitonin and insulin has previously been explored where Wimalawansa and colleagues (1997) suggested that insulin and calcitonin families are closely related. This is supported by phylogenetic analysis of the pre-propeptides and suggests that insulin and calcitonin are sister lineages. When the tree was rooted to TCAP (Fig. 3.4), to establish the assumption that TCAP is more distantly related to the rest of the sequences than these sequences are to one another, CRF, calcitonin and secretin families formed distinct groups, however CRF and calcitonin families and CRF and secretin families appeared to be more closely related to one another. This is consistent with what has been observed with respect to Secretin GPCR evolution, where CRF and calcitonin receptors share the greatest amount of sequence similarity among Secretin GPCRs (Fredriksson et al., 2003). Therefore, it is possible that a similar evolutionary scheme occurred with respect to the ligands. This evolutionary analysis also suggests that the secretin family forms a separate clade that is a sister to CRF and calcitonin families, which, in turn, are sisters to one another. Therefore, analysis of Secretin superfamily pre-propeptides with TCAP propeptides suggests that insulin and calcitonin are closely related lineages that form a group, that calcitonin and CRF lineages are closely related and that calcitonin and CRF may form a distinct sister lineage to the secretin family.
3.4.3 Evolutionary analysis of Secretin superfamily and TCAP mature peptides

The mature peptide sequences of Secretin superfamily members are largely available and due to their functional importance and bioactivity, have been highly conserved throughout evolution. As a result, a separate analysis was performed with the mature peptide sequences of the Secretin superfamily and the TCAP family. Due to the high sequence conservation of NPY that may have resulted in the odd placement of NPY in the pre-propeptide analysis and given that the NPY mature peptide is even so more highly conserved, it was not included as an outgroup in the mature peptide analysis.

First, a phylogenetic analysis was conducted with calcitonin and insulin A and B chains (Fig. 3.5). As insulin has a tertiary structure where the peptide folds and the two mature chains are connected by sets of disulfide bonds from the cysteine residues (Weiss et al., 2014), the mature peptide had to be divided into A and B chains for the purpose of this analysis. Analysis revealed that insulin A and B chains had an equidistant relationship to calcitonin. This is different from what was previously suggested by Wimalawansa and colleagues (1997), who noted greater sequence similarity between insulin B chain and calcitonin than with insulin A. Therefore, further analysis was needed to understand the evolutionary relationship of insulin and calcitonin.

Subsequently, TCAP family mature peptide sequences were added to the calcitonin and insulin mature sequences (Fig. 3.6). The insulin A sequences clustered with calcitonin sequences, suggesting that it was actually the insulin A chain that may be more closely related to the calcitonin family, different from what was previously suggested (Wimalawansa et al., 1997).

Next, CRF was added to the calcitonin, insulin and TCAP mature sequences (Fig. 3.7). This analysis revealed that the insulin A chain did indeed appear to be more closely related to the calcitonin family than the insulin B chain, whereas the CRF mature peptides were more closely related to the insulin A chain. Taken together, it appears that insulin and calcitonin are forming closely related sister groups that form a separate sister group with the CRF peptides.

To confirm this hypothesis, mature secretin peptide sequences were added to the analysis along with calcitonin, insulin, CRF and TCAP mature peptides (Fig. 3.8). Placing all of the Secretin
superfamily members with TCAP as an outgroup, confirmed that insulin A chain is more closely related to calcitonin mature peptides than insulin B chain. Moreover, it also confirmed that insulin and calcitonin formed closely related sister groups, which in turn formed a sister clade with CRF. The calcitonin-insulin and CRF groups appear to be sisters to the secretin mature peptides that are yet again, distinct groups from the TCAP peptides, as well.

3.4.4 Concluding remarks

Taken together, phylogenetic analysis of members of the Secretin superfamily when rooted to TCAP demonstrated relationships among Secretin superfamily members. First, calcitonin forms a closely related sister lineage to insulin, particularly the insulin A chain with respect to mature peptides, but this has also been observed with the pre-propeptides. Also, the calcitonin-insulin and CRF families appear to be more closely related to one another than they are to secretin or TCAP, which is supported by the evolutionary scheme of their receptors, which also appear to be very closely related. Finally, secretin appears to form a sister lineage to a lineage that comprises both calcitonin-insulin and CRF. Therefore, given the assumption that the teneurin-TCAP system arose as a result of a HGT event prior to the emergence of the Metazoa, as well as the previously established structural similarity of TCAP to calcitonin and CRF, members of the Secretin superfamily, the phylogenetic analysis undertaken here allowed for the elucidation of relationships among members of the Secretin superfamily. However, further analysis should be undertaken in order to suggest that TCAP is a progenitor of the Secretin superfamily.
Chapter 4
Stimulation of a glutamate-associated calcium-mediated astrocyte syncytium in immortalized cell lines: the effects of TCAP-1 on astrocyte monocultures and in astrocytes co-cultured with neurons

4 Abstract
In light of the importance of calcium signaling early in evolution and previous findings that TCAP-1 modulates calcium, the effects of TCAP-1 were examined with respect to calcium flux in astrocytes, for the first time. Members of a functional teneurin, TCAP and ADGRL system were identified in astrocytes in vitro. Also, administering TCAP-1 to astrocytes demonstrated that it was stimulating calcium signaling in these cells. This effect was not observed in astrocytes co-cultured with neurons. Finally, the administration of glutamate to astrocytes demonstrated a similar response to that observed with TCAP-1 treatment and the application of glutamate receptor antagonists ablated calcium signaling in both astrocytes treated with glutamate and TCAP-1. Therefore, these findings suggest that TCAP-1 plays a role in modulating a TCAP-calcium-glutamate mechanism that had not been previously examined.

4.1 Introduction
Calcium ion regulation likely played a major role in the molecular mechanisms of the earliest organisms (see Chapter 1). Given the importance of calcium as a mediator of biological responses in the CNS, elucidating the effects of TCAP-1, an ancient peptide (see Chapter 3) on calcium ion regulation will better our understanding of its functional role. Astrocytes are one of the most abundant glial cells in the mammalian CNS and they are important calcium ion regulators, as they can use it as a means of communication, making them a particularly interesting model to examine the effects of TCAP-1. This is the first time that the effects of TCAP-1 with respect to calcium ion regulation have been examined in astrocytes in vitro.

TCAPs are a family of four bioactive peptides that are 40-41 amino acids in length and are located on the C-terminus of each of the four teneurins (Qian et al., 2004). The TCAPs are classified as cleavable bioactive peptides because they possess a cleavage motif at the N-
terminus and an amidation motif on the C-terminus (Lovejoy et al., 2006). However, there is also evidence to suggest that the TCAPs are independent of the teneurins, as mouse TCAP-1 is expressed as a separate transcript from the teneurin gene and is independently transcribed (Chand et al., 2013). Studies in vitro have demonstrated that a C-terminal fragment of teneurin 2, which contained the TCAP sequence, induced increases in intracellular calcium in neurons (Silva et al., 2011). Astrocytes are multifunctional cells that provide structural support, maintain ion homeostasis in interstitial fluid, provide a stable environment for neurons and help clear the synaptic cleft of neurotransmitters (Sofroniew & Vinters, 2010). Although astrocytes are not electrically excitable cells like neurons, which propagate action potentials as a form of signaling, they exhibit a form of excitability manifested by increases in intracellular calcium (Charles et al., 1991; Cornell-Bell et al., 1990). As a result, examining the effect of TCAP-1 on calcium dynamics in astrocytes is of particular interest.

Intracellular calcium increases can occur in astrocytes alone, termed intrinsic astrocyte excitability, that is dependent on secondary calcium messengers, such as IP$_3$ (Nett et al., 2002; Parri et al., 2001) or they can be evoked by external stimuli from other cell types, such as neurotransmitters released from neurons (Cornell-Bell et al., 1990; Charles et al., 1991; Pasti et al., 1997; Porter & McCarthy, 1996). The ER is the main source of calcium underlying this signal (Finkbeiner, 1993; Scemes, 2000; Sheppard et al., 1997; Verkhratsy et al., 2012). Stimulation of the release of calcium stores from the ER is dependent on the activation of GPCRs located on the astrocyte cell membrane (those linked to a $G_q$ subunit) which stimulates the PLC/IP$_3$ pathway, resulting in the mobilization of intracellular calcium from the ER through IP$_3$ receptors (Golovina & Blaustein, 2000; Parri & Crunelli, 2003; Scemes, 2000; Sheppard et al., 1997; Agulhon et al., 2008). Apart from being able to undergo increases in intracellular calcium within each cell, calcium can also be passed on to neighbouring cells as intercellular calcium waves, allowing for astrocytes to communicate in vast networks (Cornell-Bell et al., 1990; Konietzko & Muller, 1994). Astrocyte cell networks are connected to one another by gap junctions so their cytosols form a syncytium where intercellular calcium waves function as a signal that can be transmitted to neighbouring cells, synchronizing their activity and allowing them to modulate neuronal activity in vast areas of the CNS (Araque et al., 2014; Cornell-Bell et
al., 1990; Covello & Araque, 2016; Finkbeiner, 1992; Glaum et al., 1990; Gundersen et al., 2015). There are two main ways that calcium waves can be passed on to neighbouring astrocytes and they are thought to occur simultaneously in astrocyte networks. The first involves the transfer of calcium and second messengers, like IP$_3$ from cell to cell through gap junctions, which stimulate the release of calcium in neighbouring cells. The second is an extracellular pathway that takes place when the activation of receptors on the astrocytic membrane generate these messengers, such as what occurs with the PLC/IP$_3$ pathway upon the activation of metabotropic glutamate receptors.

Another ancient signaling molecule that has been found to induce both intracellular and intercellular calcium waves in astrocyte networks is glutamate (Cornell-Bell et al., 1990; Finkbeiner, 1992). Glutamate functions through ionotropic receptors, such as NMDAR and AMPAR, causing an influx in calcium and sodium ions as well as metabotropic glutamate receptors, such as mGluR5, inducing the PLC/IP$_3$ pathway which stimulates the release of calcium from the ER (Cornell-Bell et al., 1990; Cornell-Bell & Finkbeiner, 1991; Kim et al., 1994). Glutamate can also be released from astrocytes as a gliotransmitter, as a result of increases in intracellular calcium levels, released from ER stores (Innocenti et al., 2000; Malarkey & Parpura, 2009; Parpura et al., 2011). The release of glutamate from astrocytes can trigger and modulate neuronal activity (Angulo et al., 2004; Fellin et al., 2004; Navarrete et al., 2013; Parpura et al., 1994; Perea & Araque, 2005).

Therefore, determining the effects of TCAP, an ancient peptide, on calcium ion regulation in astrocytes is a significant contribution to our understanding of its functional role. These findings examine the effects of TCAP-1 in astrocytes in vitro for the first time.
4.2 Materials and Methods

4.2.1 Cell culture

*Immortalized cell lines (C8D1A, N38)*

C8D1A mouse cerebellar immortalized astrocytes (ATCC CRL-2541) and N38 mouse hypothalamic immortalized neurons (mHypoE-N38) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 4500 mg/L glucose content, 4mM L-glutamine, 1mM sodium pyruvate, 1500 mg/L sodium bicarbonate with 10% FBS, 100µg/mL penicillin and 100µg/mL streptomycin added to the medium. Both cell types were incubated at 37°C, 5% CO₂ and maintained at 70-80% confluency.

As the astrocytes needed to establish connections to neighboring cells to grow well, cell count was optimized to each culture dish size. Cells were counted using a hemocytometer and approximately 2,780,000 cells were plated in a 100mm petri dish (culture area=55cm²) and 718,000 cells were plated in a 3.5 cm 6-well plate (culture area=9.5cm²) to achieve 70-80% confluency in 48 hours. As the neurons had a much faster growth rate, they did not need to be seeded as precisely.

For co-cultures, both C8D1A astrocytes and N38 neurons were seeded at 500,000 cells per coverslip and grown a 6-well plate 48 hours prior to the experiment. This allowed for approximately a 1:1 astrocyte-to-neuron ratio, as was established using the transfected C8D1A-mCherry astrocytes and N38-BFP neurons.

*Transfected immortalized cell lines (C8D1A-mCherry, N38-BFP)*

Both transfection of C8D1A astrocytes and N38 neurons were performed at the Structural Genomics Consortium at the MaRS Discovery District in collaboration with Dr. Dalia Barsyte-Lovejoy. For astrocytes, the mCherry was subcloned to a derivative of the pRRL lentiviral transfer vector, with the E2F promoter driving the expression of the Cherry fluorescent protein. Likewise for neurons, BFP was subcloned to the same vector, with the E2F promoter during the
expression of the BFP protein. The cells were grown in the same medium and culture conditions as their wild type counterparts.

4.2.2 Reverse-transcription polymerase chain reaction

RNA Extraction

Once at 70-80% confluency in a 6-well plate, C8D1A astrocytes were serum-starved for 3 hours. 1mL Trizol reagent was added to each well to extract RNA from the cells after which they were incubated for 2 minutes at room temperature. Lysates were collected and 200µL of chloroform was added. The solution was thoroughly mixed and incubated at room temperature for 3 minutes. Subsequently, the samples were centrifuged at 12,000 RCF for 15min at 4°C. The RNA-containing supernatant was transferred to a new tube and 500µL of isopropanol was added. The solution was incubated at room temperature for 10 minutes and then centrifuged at 14,000 RCF for 10 minutes and 4°C. After centrifugation, the supernatant was discarded and the pellet was washed using 75% EtOH and centrifuged at 7400 RCF for 5 minutes. After two rounds of EtOH washes and centrifugation, the EtOH was removed and the pellet was re-suspended in 20µL diethyl pyrocarbonate (DEPC)-water. RNA sample absorbance was determined using the NanoDrop 2000 spectrophotometer at 260nm and 280nm wavelengths (Thermo fisher scientific, v.1.4.2) from which the ratio of A260/A280 was used to determine the concentration of the sample.

Reverse Transcription and Polymerase Chain Reaction

The RNA extracted from the C8D1A immortalized cell line was reverse transcribed to complimentary DNA (cDNA). RNA-free H₂O, random primers and deoxynucleotide triphosphates (dNTPs) were added to the sample RNA and the sample was heated to 65°C for 5 minutes. The sample was left on ice for 1 minute, after which 5X first strand buffer and 0.1M DTT were added, mixed and left at room temperature for 2 minutes. Subsequently, superscript II RT was added, after which the sample was at room temperature for 10 minutes, heated to 42°C for 50 minutes and then heated to 70°C for 15 minutes.
To perform PCR, a mastermix containing ddH$_2$O, Taq buffer, MgCl$_2$ and dNTP was prepared and the appropriate primer pairs were added (see Table 1) along with the cDNA template and Taq polymerase. The reaction tubes were then placed in the thermal cycler for the first cycle of denaturing for 7 minutes at 95°C, subsequently for 35 repeated cycles of denaturing for 1 minute at 95°C, annealing for 1 minute 30 seconds at 60-65°C and elongation for 35 seconds at 72°C and then for one final cycle of elongation for 5 minutes at 72°C. The DNA samples were stored at 4°C until gel electrophoresis was performed and they were run through a 3% agarose gel and imaged using the Image-Lab software (Bio-Rad v.4.1).

**Table 4.1. List of Primers used for PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Pair</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Exon</th>
<th>Product size (bp)</th>
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<td>BetaActinmF1</td>
<td>5’CAGGTCATCATCATTGGCAACGAG3’</td>
<td>4-6</td>
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<tr>
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<td>BetaActinmR1</td>
<td>5’CTCATCGTACCTGCTTGCTGAT3’</td>
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<td>Teneurin 1</td>
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<td>mTen2e23RVS</td>
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<td></td>
<td>mTen3e22RVS</td>
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<tr>
<td>TCAP 1</td>
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<tr>
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<td>mTCAP1e32RVS</td>
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<td>TCAP 2</td>
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<tr>
<td>TCAP 3</td>
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<td></td>
<td>mTCAP3eRVS</td>
<td>5’CGATCTCAGCCTTGGCAACGACT3’</td>
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</table>
4.2.3 Western Blotting

Collection of cell lysates

C8D1A astrocytes were grown to 70-80% confluency in a 6-well plate and then lysed in cold radioimmunoprecipitation assay (RIPA) buffer and phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor. Cells were scraped off the bottom of each well using a cell scraper and then centrifuged at 14,000 rpm for 20 minutes at 4ºC to remove any debris. The pellet was discarded and the supernatant collected and aliquoted into eppendorf tubes which were stored at -20ºC.

Bicinchoninic acid (BCA) protein assay

BCA protein assay was performed according to the manufacturer’s instructions, in order to determine the total protein concentration of cell lysate samples (Thermo fisher scientific). This allowed for lysates to be standardized relative to one another. Standards that contained known concentrations of bovine serum albumin (BSA) from 0 µg/mL to 2000µg/mL were prepared. Subsequently, 25µL of each standard and the collected cell lysates along with 200µL of working reagent were added per well in a 96-well plate. The plate was then put on a shaker for 30 seconds to mix the samples and reagents after which it was incubated for 30 minutes at 37ºC. Once the plate cooled to room temperature, the Spectramax Plus Microplate Reader spectrophotometer (Molecular Devices, USA) was used to measure the absorbance levels of standards and samples.
at 562nm. These absorbance values were used to interpolate the protein concentrations of cell lysates. The lysates were standardized accordingly to obtain an equal protein concentration for all samples and stored at -20°C.

**Western Blot**

Standardized lysate sample was combined with sample loading buffer and 2% v/v β-mercaptoethanol (BME) to achieve a 1:2 ratio of sample to tricine buffer. Samples were heated at 95°C for five minutes to denature any higher-level structures within the protein. Samples were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100V for approximately 1 hour in 1X reservoir running buffer. The resolved proteins were then transferred onto a Hybond ECL nitrocellulose membrane at 100V for 1 hour 15 minutes in 1X transfer buffer. Subsequently, the membranes were washed in phosphate buffered saline (PBS) for 10 minutes, three times. The membranes were then blocked in a 5% BSA-PBST solution (5% w/v dissolved in PBS with 0.2% Tween 20) on a shaker at room temperature for 1 hour. Finally, they were incubated in 1% BSA-PBST with the corresponding protein primary antibody at 4°C overnight on a rotator (as listed in Table 4.2). The next day, the membranes were washed for 10 minutes in PBST, three times after which they were incubated in 1%milk PBST (1% dehydrated milk w/v dissolved in PBST) containing the appropriate secondary antibody for 1 hour at room temperature (as listed in Table 4.2). The membranes were then washed for 10 minutes with 0.2% PBST 3 more times. After these washes, the membranes were incubated in a chemiluminescence detection reagent and were either exposed on ECL Hyperfilm for 1-10 minutes or were imaged using the BioRad ChemiDoc XRS+ imaging system (BioRad, USA).
Table 4.2. List of Antibodies used for Western Blotting

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary (1º) Antibody</th>
<th>1º Dilution</th>
<th>Secondary (2º) Antibody</th>
<th>2º Dilution</th>
<th>Weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK1/2</td>
<td>P42/44 MAPK (ERK1/2) Rabbit Ab</td>
<td>1:1000</td>
<td>Donkey anti-rabbit IgG linked</td>
<td>1:7500</td>
<td>42</td>
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<tr>
<td>Teneurin 1</td>
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<td>Goat anti-mouse HRP-linked</td>
<td>1:5000</td>
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<tr>
<td>Teneurin 3</td>
<td>Teneurin-3 rabbit polyclonal</td>
<td>1:100</td>
<td>Donkey anti-rabbit HRP-linked</td>
<td>1:2000</td>
<td>301</td>
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<tr>
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<td>Latrophilin-1 Goat polyclonal IgG</td>
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<td>Donkey anti-goat HRP-linked</td>
<td>1:7500</td>
<td>130</td>
</tr>
<tr>
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<td>Donkey anti-goat HRP-linked</td>
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<td>163</td>
</tr>
<tr>
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<td>Latrophilin-3 Rabbit polyclonal IgG</td>
<td>1:1000</td>
<td>Donkey anti-rabbit HRP-linked</td>
<td>1:7500</td>
<td>162</td>
</tr>
</tbody>
</table>

4.2.4 Intracellular Calcium Fluorescence Microscopy

\[ \text{[Ca}^{2+}]_i \text{ fluorescence measurements using fluo-4} \]

C8D1A astrocytes were plated at a cell number of approximately 500,000 cells per well and grown on coverslips coated with poly-D-lysine in a 6-well plate 48 hours prior to the experiment to reach 60-70% confluency. N38 neurons were plated at a cell number of approximately 200,000 cells per well onto coverslips to reach 60-70% confluency in 48 hours. For co-culture experiments, C8D1A astrocytes and N38 neurons were seeded at 250,000 cells each onto a coverslip in a 6-well plate 48 hours prior to the experiment.

Before the fluorescent measurements, each coverslip was incubated for 30 minutes at 37°C in 3.6μM fluo-4 acetomethyl (AM) ester (1μg/μL dissolved in DMSO; Thermofischer Scientific) in culture medium. Subsequently, the cells were washed with artificial cerebrospinal fluid (aCSF) (135mM NaCl, 4.5mM KCl, 2mM MgCl₂·6H₂O mM, 10mM HEPES, 10mM glucose, 2mM
CaCl$_2$•2H$_2$O) and the coverslip remained in aCSF until it was mounted to the stage of an inverted fluorescence microscope (Zeiss Axio Observer.Z1, Carl Zeiss Microimaging, Germany) for perfusion. The microscope was equipped with a 40X oil immersion lens (1.4 NA, Carl Zeiss Microimaging, Germany) and a digital CCD camera (C4742-80, Hamamatsu Photonics, Hamamatsu, Japan) to capture fluorescent images. Volocity cellular imaging software (Improvision, version 4.3.2) was used to obtain fluorescent images and emitted light was filtered and collected through an excitation filter, respective to each fluorophor. The excitation/emission wavelengths are 494/506nm, 587/610nm and 382/448nm for fluo-4, mCherry and BFP, respectively.

**Solution administration**

Both TCAP-1 and glutamate solutions were administered using a perfusion system. Mouse TCAP-1 was solubilized in ddH$_2$O and glutamate (Sigma Aldrich, MO, USA) was solubilized in 1mg/mL 1.0M HCl. Both solutions were diluted in aCSF to a 100nM TCAP-1 and 300µM glutamate concentration.

**Antagonist administration**

Antagonists of NMDAR, AMPAR and mGluR5 were administered to C8D1A astrocytes. NMDAR antagonist, D(-)-2-Amino-5-phosphonopentanoic acid (AP5) (Sigma Aldrich, MO, USA) was solubilized in ddH$_2$O to 9mg/mL and then diluted in aCSF to a 25µM concentration. AMPAR antagonist, 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) disodium salt hydrate (Sigma Aldrich, MO, USA) was solubilized in ddH$_2$O to 5mg/mL and then dissolved in aCSF to a 25µM concentration. mGluR5 antagonist, 6-Methyl-2-(phenylethynyl)pyridine hydrochloride (MPEP) (Sigma Aldrich, MO, USA) was solubilized in DMSO to 10mg/mL and then diluted in aCSF to a 30 µM concentration.

**Co-culture Analysis**

Three criteria needed to be met in order to identify each cell as an astrocyte or neuron in co-culture. The first involved basal fluo-4 fluorescence, which was characteristically different in
astrocytes compared to neurons. Astrocytes appeared much brighter in images and neurons were noticeably less fluorescent, but the raw traces of neurons also had characteristically lower basal calcium levels than astrocytes. The second involved characteristic calcium responses, as only astrocytes demonstrated calcium oscillations and neurons did not. The third involved differentiating between astrocyte and neuron morphology in images. Astrocytes had characteristically longer projections and often grew in closely intertwined networks where as neurons had shorter projections. Cells had to meet these three criteria in order to be classified as either an astrocyte or a neuron; if uncertain, the cells were not used towards analysis. Note that all raw traces are represented as a $\Delta F$ (F-background fluorescence).

**Statistical Analysis**

Data was normalized to the fluorescence ratio baseline ($\Delta F/F_0$) of each cell and presented as standard error of the mean (SEM). Comparisons of two factors on one variable were carried out using two-way ANOVA with a Bonferroni post-test. Comparison of two variables was carried out using non-parametric t-test with a Mann Whitney post-test or an unpaired t-test with Welch’s correction. Comparisons of three or more variables were carried out using one-way ANOVA with a Bonferroni post-test. p<0.05 was considered statistically significant. GraphPad Prism (v.5) was used for statistical analysis.
4.3 Results

4.3.1 Characterizing C8D1A astrocytes for teneurin, TCAP and ADRGL

Teneurin, TCAP and ADGRL expression has not been previously established in astrocytes. Therefore, C8D1A immortalized mouse cerebellar astrocytes were characterized for teneurin 1-4, TCAP 1-4 and ADGRL 1-3. Gene expression analysis, using RT-PCR, revealed a band of approximately 400 base pairs (bps) for teneurin 1, 430bps for teneurin 3 and 370bps for teneurin 4, with expected band sizes of 402bps, 427bps and 369bps, respectively (Fig. 4.1a). A band for teneurin 2 was not present, with the expected band size being 405bps (Fig. 4.1a). Therefore, teneurins 1, 3 and 4 were expressed in the C8D1A cell line, but teneurin 2 was not. With respect to the gene expression of TCAP 1-4, bands were detected for all paralogues. Bands of approximately 350bps for TCAP-1, 500bps for TCAP-2, 500bps for TCAP-3 and 600bps for TCAP-4 were present, with expected band sizes of 351bps, 496bps, 506bps and 602bps, respectively (Fig. 4.1a). Therefore, TCAPs 1-4 were expressed in the C8D1A cell line. Finally, with respect to ADGRL 1-3, a band was detected for ADGRL 1 at approximately 250bps and ADGRL 3 at 300bps, with expected band sizes of 249bps and 327bps, respectively (Fig. 4.1a). However, gene expression of ADGRL 2 was not detected, with an expected band size of 203bps (Fig. 4.1a). Therefore, ADGRL 1 and 3 were expressed but ADGRL 2 was not.

These findings were corroborated with protein expression using western blotting. Protein expression analysis revealed a single band of approximately 45kDa for the loading control, ERK1/2, which was the expected band size (Fig. 4.1b). Multiple bands were present for teneurin 1 and 3, with major bands at approximately 23, 30, 45, 50 and 90kDa for teneurin 1 and 23, 45 and 75kDa for teneurin 3 (Fig. 4.1b). The expected band sizes were 305kDa for teneurin 1 and 301kDa for teneurin 3. Therefore, protein expression of teneurin 1 and 3 was detected. Further, with respect to ADGRL 1-3, a single band was present at approximately 130kDa for ADGRI 1 and 170kDa for ADGRL 3, with expected band sizes of 130kDa and 162kDa, respectively (Fig. 4.1b). A band for ADGRL 2 was not detected, with an expected size of 163kDa (Fig. 4.1b). Therefore, protein expression of ADGRL 1 and 3 was detected but that of ADGRL 2 was not.
Figure 4.1. Characterization of C8D1A mouse cerebellar astrocytes. A) Gene expression was determined using reverse transcription-polymerase chain reaction (RT-PCR). Teneurin 1, 3 and 4 were expressed (n=4), but teneurin 2 was not (n=4). TCAPs 1-4 were expressed (TCAP-1 (n=3), TCAP-2 (n=5), TCAP-3 (n=3), TCAP-4 (n=3)). ADGRL 1 and 3 were expressed (n=3), but ADGRL 2 was not (n=3). β-actin served as the positive control (n=5). B) Protein expression was determined using western blotting techniques. Teneurin 1 and 3 were expressed (Ten-1 n=6, Ten-3 n=4) and ADGRL 1 and 3 were expressed (n=4), but ADGRL 2 was not (n=4). ERK1/2 was used as the sample control (n=6). Primers and antibodies are listed in Table 4.1 and 4.2, respectively.
4.3.2 The effect of TCAP-1 administration on C8D1A astrocytes

Administration of TCAP-1 to C8D1A astrocytes resulted in increases in intracellular fluo-4 fluorescence, indicating increases in intracellular calcium, as fluorescence is dependent on calcium binding the fluo-4 molecule, in an oscillatory manner that was not observed with vehicle treatment (Fig. 4.2). These intracellular calcium oscillations had an average amplitude that was approximately 50 arbitrary fluorescence units (AFU) greater than vehicle (p<0.01) and an average peak frequency that was about 0.5 peaks per minute greater than vehicle treatment (p<0.0001) (Fig. 4.3). Also, astrocytes exhibited about a 40% increase in intracellular calcium at 3.5-6 minutes (p<0.001) and about a 20% increase at 6.5-9 minutes (p<0.001), during 10 minute TCAP-1 treatment relative to vehicle (Fig. 4.4).

Figure 4.2. C8D1A astrocytes treated with TCAP-1 demonstrate an oscillatory increase in intracellular calcium. A) Differential interference contrast (DIC) image of astrocytes. B) Fluo-4 fluorescence shown prior to 100nM TCAP-1 administration and C) 3 minutes after 100nM TCAP-1 administration. D) Sample traces after treatment with vehicle (aCSF) and TCAP-1 (100nM). E) Normalized fluo-4 fluorescence comparing vehicle (n=5) and 10 min TCAP-1 (n=5) treatments. Each n is an average of 5 cells per coverslip.
Figure 4.3. Average amplitude and peak frequency of C8D1A astrocytes treated with TCAP-1 was significantly greater than with vehicle treatment. A) Average amplitude was significantly greater in C8D1A astrocytes treated with 100nM TCAP-1 relative to vehicle (aCSF) treatment. B) Average peak frequency, calculated as the number of peaks observed per minute, was significantly greater in C8D1A astrocytes treated with 100nM TCAP-1 relative to vehicle treatment. For A) and B), n=5 for each treatment, where n is an average of 5 cells per coverslip. Statistical analysis: unpaired t-test with Welch’s correction (mean ±SEM; **p<0.01, ***p<0.0001).

Figure 4.4. C8D1A astrocytes treated with TCAP-1 for 10 minutes demonstrated significant increases in intracellular calcium at 3.5-6 and 6.5-9 minute time points compared to vehicle. Normalized fluo-4 fluorescence represented at binned time points comparing 100nM TCAP-1 and vehicle (aCSF) treated C8D1A astrocytes. n=5 for each treatment, where n is an average of 5 cells per coverslip. Statistical analysis: two-way ANOVA and Bonferroni post-test (mean ±SEM; ***p<0.001).
A spatial-temporal analysis indicated that when treated with TCAP-1, C8D1A astrocytes located close to one another in culture demonstrated oscillatory increases in intracellular calcium at similar time points (Fig. 4.5). TCAP-1 treatment induced increases in intracellular calcium that appeared to propagate to many astrocytes within the network (see Supplementary Fig. 1). Increases in intracellular calcium were not observed in astrocytes treated with vehicle (as demonstrated in Fig. 4.2) so this data is not shown with respect to vehicle treatment.

Figure 4.5. C8D1A astrocytes that are close in proximity exhibit increases in intracellular calcium at similar time points when treated with TCAP-1. A) A cell identification map that describes the location of each astrocyte. B) Raw traces of the 7 astrocytes depicted in A). C) Fluo-4 fluorescence image taken at 9.5 minutes demonstrating firing astrocytes treated with TCAP-1. D) Raw traces of the astrocytes that underwent increases in intracellular calcium at 9.5 minutes, as are labeled by a single asterix (*) in C). E) Fluo-4 fluorescence image taken at 11 minutes demonstrating firing astrocytes in co-culture. F) Raw traces of the astrocytes that underwent increases in intracellular calcium at 11 minutes, as are labeled by a double asterix (**) in E).
4.3.3 Establishing C8D1A astrocyte and N38 neuron co-cultures

Determining the effect of TCAP-1 on astrocyte responses when grown with neurons required a method to co-culture the C8D1A and N38 cell lines. As this was the first time immortalized astrocyte-neuron co-cultures were attempted in our laboratory, many different approaches were undertaken in order to optimize the co-culture method, the results for which follow.

4.3.3.1 Examining a fluorescently labeled C8D1A cell line to identify astrocytes in co-culture

C8D1A astrocytes were transfected to express mCherry to allow for the identification of this cell type in co-culture. C8D1A-mCherry astrocytes displayed increases in intracellular calcium relative to vehicle treatment (Fig. 4.6). However, these increases were greatly reduced and did not display the same oscillatory response relative to those observed with wild type C8D1A astrocytes (Fig. 4.2). As a result, these cells were not used in co-culture, but instead were used to establish the morphology and ratio of the two cell types (see Section 4.3.3.4).

Figure 4.6. C8D1A astrocytes transfected with mCherry demonstrate an increase in intracellular calcium during TCAP-1 treatment. A) Differential interference contrast (DIC) image of astrocytes. B) Fluo-4 fluorescence image of astrocytes taken prior to TCAP-1 administration. B) Fluo-4 fluorescence shown prior to 100nM TCAP-1 administration and C) 1.5 minutes after 100nM TCAP-1 administration. D) mCherry fluorescence image of astrocytes. E) Sample traces after treatment with vehicle (aCSF) and TCAP-1 (100nM). F) Normalized fluo-4 fluorescence comparing vehicle (n=3) and 10 min TCAP-1 (n=3) treatments. Each n is an average of 5 cells per coverslip.
4.3.3.2 The effect of TCAP-1 administration on N38 neurons

Administration of TCAP-1 to N38 neurons resulted in decreases in intracellular calcium, which was not observed with vehicle treatment (Fig. 4.7). Also, neurons exhibited about a 15% decrease in intracellular calcium at 0.5-3 and 6.5-9 minutes (p<0.001) and about a 25% decrease at 3.5-6 minutes (p<0.001) during 10 minute TCAP-1 treatment relative to vehicle (Fig. 4.8).

![Image of neurons with DIC, Fluo-4 fluorescence, and normalized fluo-4 fluorescence traces]

Figure 4.7. N38 neurons treated with TCAP-1 demonstrate a decrease in intracellular calcium. A) Differential interference contrast (DIC) image of neurons. B) Fluo-4 fluorescence image of neurons taken prior to 100nM TCAP-1 administration and C) 2 minutes after 100nM TCAP-1 administration. D) Sample traces after treatment with vehicle (aCSF) and TCAP-1 (100nM). E) Normalized fluo-4 fluorescence comparing vehicle (n=5) and 10 min TCAP-1 (n=5) treatments. Each n is an average of 5 cells per coverslip.
Examine a fluorescently labeled N38 cell line to identify neurons in co-culture

N38 neurons were transfected to express blue fluorescent protein (BFP) to allow for the identification of this cell type in co-culture. N38-BFP neurons displayed unstable intracellular calcium dynamics when treated with both vehicle and TCAP-1 (Fig. 4.9). As a result, these cells were not used in co-culture.

Figure 4.8. N38 neurons treated with TCAP-1 for 10 minutes demonstrated significant decreases in intracellular calcium at 0.5-3, 3.5-6 and 6.5-9 minute time points compared to vehicle. Normalized fluo-4 fluorescence is represented at binned time points comparing 100nM TCAP-1 and vehicle (aCSF) treated N38 neurons. n=5 for each treatment where each n is an average of 5 cells per coverslip. Statistical analysis: two-way ANOVA and Bonferroni post-test (mean±SEM; ***p<0.001).
Figure 4.9. **N38 neurons transfected with BFP demonstrate unstable calcium levels when treated with vehicle and TCAP-1.** A) Differential interference contrast (DIC) image of blue fluorescent protein (BFP) neurons. B) Fluo-4 fluorescence image of BFP neurons taken prior to 100nM TCAP-1 administration and C) 4 minutes after 100nM TCAP-1 administration. D) BFP fluorescence image of neurons. E) Sample raw traces after treatment with vehicle (aCSF) and TCAP-1 (100nM). F) Raw traces of 5 cells on a coverslip treated with vehicle and 5 cells on a separate coverslip treated for 10 minutes with 100nM TCAP-1.
4.3.3.4 Establishing an appropriate astrocyte-to-neuron ratio for co-culture

Even though transfected cell lines were not used in co-culture, they did serve as a useful tool for establishing an appropriate astrocyte-to-neuron ratio. A visual representation of C8D1A-mCherry astrocytes and N38-BFP neurons in co-culture allowed for an approximation that the seeding ratios were appropriate for maintaining a 1:1 astrocyte: neuron ratio (Fig. 4.10).

Figure 4.10. C8D1A-mCherry astrocytes and N38-BFP neurons exhibit approximately a 1:1 ratio with the established co-culture conditions. A) Differential interference contrast (DIC) image of C8D1A-mCherry and N38-BFP in co-culture. B) mCherry fluorescence image of C8D1A astrocytes in co-culture with BFP neurons. C) BFP fluorescence image of N38 neurons in co-culture with C8D1A astrocytes.
4.3.3.5 Differentiating between wild type C8D1A astrocytes and N38 neurons in co-culture

In order to develop a co-culture model using wild type C8D1A astrocytes and N38 neurons, a method to differentiate between the two cell types was established. Three criteria were determined to differentiate between astrocytes and neurons in co-culture. Analysis of basal fluo-4 fluorescence in both C8D1A astrocytes and N38 neurons revealed that astrocytes had a basal level of calcium that was approximately two times greater than that in neurons (p<0.05) (Fig. 4.11b). Therefore, the first criterion was based on differing basal fluo-4/calcium levels between the two cell types. The second criterion was based on the differing calcium responses observed between the two cell types, as astrocytes displayed characteristic calcium oscillations of greater average amplitude by about 50 AFU (p<0.05) and average peak frequency of about 0.7 peaks per minute (p<0.0001) than neurons, which did not (Fig. 4.2; 4.3; 4.12; 4.13). The third criterion was based on the morphology of the cell types as astrocytes had characteristically long projections, were noticeably brighter as a result of higher basal fluorescence and often grew in closely intertwined networks whereas neurons had much shorter projections and were less fluorescent (Fig. 4.11a). This was corroborated by the observed morphology of both cell types in C8D1A-mCherry and N38-BFP co-cultures (Fig. 4.10).

Figure 4.11. C8D1A astrocytes and N38 neurons in co-culture exhibit characteristically different morphologies and significantly different basal calcium/fluorescein-4 fluorescence. A) Fluorescein-4 image of C8D1A astrocytes and N38 neurons in co-culture. Cells identified as astrocytes are labeled with a double asterix (**) and cells identified as neurons are labeled with a single asterix (*). B) Basal fluorescein-4 fluorescence in C8D1A astrocytes was significantly greater compared to N38 neurons for cells treated with vehicle (aCSF). n=25 for each cell type. Statistical analysis: t-test; non-parametric Mann Whitney (mean± SEM; **p<0.05)
Figure 4.12. C8D1A astrocytes and N38 neurons in co-culture exhibit characteristically different calcium responses when treated with vehicle. A) Raw traces of C8D1A astrocytes in co-culture when treated with vehicle (aCSF) demonstrated as arbitrary fluorescence units (AFU). Images are represented from 0-18 minutes, every 2 minutes. B) Raw traces of N38 neurons in co-culture when treated with vehicle demonstrated in AFU. Images are represented from 0-18 minutes, every 2 minutes. Cells in A) and B) were part of the same experiment with a total of 14 represented cell traces, including 7 astrocytes and 7 neurons. Solid line traces correspond to the cell depicted in the image.

Figure 4.13. C8D1A astrocytes had a significantly greater average amplitude and average peak frequency compared to N38 neurons in co-culture. A) Average amplitude in C8D1A astrocytes was significantly greater than N38 neurons. B) Average peak frequency in C8D1A astrocytes, was significantly greater than in N38 neurons. For A) and B), n=6 for each cell type where each n includes 5 measured cells. Statistical analysis: unpaired t-test with Welch’s correction (mean± SEM; *p<0.05; ***p<0.0001).
4.3.4 The effect of TCAP-1 on C8D1A astrocytes when in co-culture with N38 neurons

In order to understand the effects of TCAP-1 on astrocytes when grown with neurons and to examine how TCAP-1 may be affecting astrocyte-neuron communication, co-cultures were treated with TCAP-1. Astrocytes displayed oscillatory increases in intracellular calcium upon vehicle treatment when in co-culture with N38 neurons, whereas neurons did not (Fig. 4.14a,b). On the other hand, when treated with TCAP-1, astrocytes did not display intracellular calcium oscillations when co-cultured with N38 neurons (Fig. 4.14c,d). Average amplitude and average peak frequency of intracellular calcium oscillations in C8D1A astrocytes in co-culture with N38 neurons were not significantly different upon vehicle treatment (Fig. 4.15a,c). However, average amplitude was significantly greater by approximately 40 AFU (p<0.0001) prior to TCAP-1 treatment relative to during TCAP-1 treatment in C8D1A astrocytes co-cultured with N38 neurons (Fig. 4.15b). The average peak frequency was also greater by about 0.8 peaks per minute, but not significantly different (Fig. 4.15d). Neurons did not demonstrate intracellular calcium oscillations, so average amplitude and average peak frequency data is not shown.
Figure 4.14. C8D1A astrocytes in co-culture with N38 neurons respond differently when treated with TCAP-1 than when treated with vehicle. A) Raw traces of C8D1A astrocytes in co-culture treated with vehicle (aCSF) displayed oscillatory increases in intracellular calcium throughout an 18-minute treatment. Images are represented from 0-18 minutes and correspond to major calcium peaks. B) Raw traces of N38 neurons in co-culture when treated with vehicle did not demonstrate calcium increases. Images are represented from 0-18 minutes, every 2 minutes. Cells in A) and B) were part of the same experiment with a total of 7 represented cell traces, including 5 astrocytes and 2 neurons. C) Raw traces of C8D1A astrocytes in co-culture treated with vehicle and then TCAP-1 (100nM) did not display oscillatory increases in intracellular calcium during TCAP-1 treatment. Images are represented from 0-18 minutes and correspond to major calcium peaks. D) Raw traces of N38 neurons in co-culture when treated with TCAP-1 (100nM) did not demonstrate calcium increases. Images are represented from 0-18 minutes, every 2 minutes. Cells in C) and D) were part of the same experiment with a total of 8 represented cell traces, including 5 astrocytes and 3 neurons. Solid line traces for A)-D) correspond to the cells depicted in the image.
Figure 4.15. C8D1A astrocytes in co-culture with N38 neurons demonstrated a significant decrease in average amplitude during TCAP-1 treatment relative to before but no significant difference for average peak frequency. Significant differences were not observed for both parameters throughout vehicle treatment. Throughout vehicle treatment, cells in co-culture were treated with vehicle (aCSF) the entire time, where as for TCAP treatment cells in co-culture were treated with vehicle prior to 100nM TCAP-1 treatment for 10 minutes. A) Average amplitude was not significantly different throughout vehicle treatment. B) Average amplitude was significantly greater during pre-treatment with vehicle than during 100nM TCAP-1 administration. C) Average peak frequency was not significantly different throughout vehicle treatment. D) Average peak frequency was not significantly different during pre-treatment with vehicle compared to during 100nM TCAP-1 administration. For A) to D), for each cell type, n=6 where each n includes 5 measured cells. Statistical analysis for A) to D): unpaired t-test with Welch’s correction (mean± SEM; ***p<0.0001)
A spatial-temporal analysis of C8D1A astrocyte responses when co-cultured with N38 neurons revealed that astrocytes closer in proximity demonstrated oscillatory increases in intracellular calcium at similar time points when treated with vehicle (Fig. 4.16). Oscillatory increases in intracellular calcium were not observed in astrocytes treated with TCAP-1 (as demonstrated in Fig. 4.14) so this data is not shown with respect to TCAP-1 treatment.

Figure 4.16. C8D1A astrocytes that are close in proximity exhibit increases in intracellular calcium at similar time points when in co-culture with N38 neurons. A) A cell identification map that describes the location of each astrocyte in co-culture. B) Raw traces of the 8 astrocytes depicted in A). C) Fluo-4 fluorescence image taken at 4 minutes demonstrating firing astrocytes in co-culture. D) Raw traces of the astrocytes that underwent increases in intracellular calcium at 4 minutes, as are labeled by a single asterix (*) in C). E) Fluo-4 fluorescence image taken at 4.5 minutes demonstrating firing astrocytes in co-culture. F) Raw traces of the astrocytes that underwent increases in intracellular calcium at 4.5 minutes, as are labeled by a double asterix (**) in E).
4.3.5 Elucidating whether TCAP-1 is affecting glutamatergic systems

Given that glutamate is an extracellular signaling molecule that induces increases in intracellular calcium in astrocytes similar to those observed with TCAP-1 administration, it was of interest to examine whether TCAP-1 may be affecting glutamatergic systems.

4.3.5.1 The effect of glutamate administration on C8D1A astrocytes

C8D1A astrocytes treated with glutamate, demonstrated increases in intracellular calcium, in an oscillatory manner that was not observed with vehicle treatment (Fig. 4.17). These intracellular calcium oscillations demonstrated a greater average amplitude of approximately 50 AFU (p<0.0001) compared to vehicle (Fig. 4.18a). Average peak frequency was also about 0.7 peaks/minute greater compared to vehicle, though not significantly different (Fig. 4.18). Average intracellular fluorescence of astrocytes treated with glutamate was about 20% greater than those treated with vehicle (p<0.001) (Fig. 4.21). Also, astrocytes exhibited about a 40% increase in intracellular calcium at 0.5-3 minutes (p<0.001) and about a 15% increase at 3.5-6 and 6.5-9 minutes, not significant, during 10 minute glutamate treatment relative to vehicle (Fig. 4.19).

![DIC image of astrocytes](image1)

**Figure 4.17.** C8D1A astrocytes treated with glutamate demonstrate an oscillatory increase in intracellular calcium. A) Differential interference contrast (DIC) image of astrocytes. B) Fluo-4 fluorescence image of astrocytes shown prior to 300μM glutamate administration and C) 1.5 minutes after 300μM glutamate administration. D) Sample traces after treatment with vehicle (aCSF) and glutamate (300μM). E) Normalized fluo-4 fluorescence comparing vehicle (n=4) and 10 minute glutamate (n=4) treatments. Each n is an average of 5 cells per coverslip. Note: 4/20 randomly selected cells did not appear to respond to glutamate treatment and they were not included in analysis.
Figure 4.18. Average amplitude of C8D1A astrocytes treated with glutamate was significantly greater compared to vehicle treatment, however there was no significant difference in average amplitude frequency. A) Average amplitude was significantly greater in C8D1A astrocytes treated with 300µM glutamate relative to vehicle (aCSF) treatment. B) Average peak frequency, was not significantly different in C8D1A astrocytes treated with 300µM glutamate relative to vehicle treatment. For A) and B), n=4 for each treatment, where n is an average of 5 cells per coverslip. Statistical analysis: unpaired t-test with Welch’s correction (mean ±SEM; ***p<0.0001).

Figure 4.19. C8D1A astrocytes treated with glutamate for 10 minutes demonstrated a significant increase in intracellular calcium at the 0.5-3 minute time point. Normalized fluo-4 fluorescence is represented at binned time points comparing 300µM glutamate and vehicle (aCSF) treated C8D1A astrocytes. n=4 for each treatment where each n is an average of 5 cells per coverslip. Statistical analysis: two-way ANOVA and Bonferroni post-test (mean±SEM; ***p<0.001).
4.3.5.2 The effect of glutamate receptor antagonist administration on C8D1A astrocytes treated with glutamate

C8D1A astrocytes, treated with NMDAR, AMPAR and mGluR5 antagonists did not demonstrate increases in intracellular calcium in response to glutamate treatment (Fig. 4.20). Levels of intracellular calcium in astrocytes treated with glutamate receptor antagonists decreased by approximately 20% (p<0.001) relative to those treated with glutamate (Fig. 4.21). This confirmed that the glutamate receptor antagonists were functional.

Figure 4.20. C8D1A astrocytes treated with glutamate and NMDAR, AMPAR and mGluR5 antagonists (AP5, CNQX, MPEP) did not demonstrate any changes in intracellular calcium. A) Differential interference contrast (DIC) image of astrocytes. B) Fluo-4 fluorescence image of astrocytes shown prior to 300µM glutamate administration and C) 1.5 minutes after 300µM glutamate administration. D) Sample traces after treatment with vehicle (aCSF+antagonists:25µM AP5, 25µM CNQX, 30µM MPEP) and after glutamate (300µM) treatment with antagonists (aCSF+25µM AP5, 25µM CNQX, 30µM MPEP). E) Normalized fluo-4 fluorescence comparing vehicle (n=4) and 10 min glutamate (n=4) treatments. Each n is an average of 5 cells per coverslip. Note: 2/20 randomly selected cells demonstrated intracellular calcium increases and they were not included in the analysis.
Figure 4.21. NMDAR, AMPAR and mGluR5 antagonist administration (AP5, CNQX and MPEP) significantly decreased intracellular calcium levels compared to glutamate and vehicle administration in C8D1A astrocytes. The means of vehicle (aCSF+ antagonists: 25µM AP5, 25µM CNQX, 30µM MPEP), 300µM glutamate and glutamate receptor antagonists (aCSF+25µM AP5, 25µM CNQX, 30µM MPEP) are represented for a 10 minute treatment. n=4 for each treatment where each n is an average of 5 cells per coverslip. Statistical analysis: one-way ANOVA and Bonferroni post-test comparing all pairs of columns (mean±SEM; ***p<0.001).
4.3.5.3 The effect of glutamate receptor antagonist administration on C8D1A astrocytes treated with TCAP-1

C8D1A astrocytes, treated with NMDAR, AMPAR and mGluR5 antagonists did not demonstrate increases in intracellular calcium in response to TCAP-1 treatment (Fig. 4.22). Levels of intracellular calcium in astrocytes treated with glutamate receptor antagonists decreased by approximately 20% relative to those treated with TCAP-1 (p<0.001) (Fig. 4.23). This demonstrated that the glutamate receptor antagonists were working to ablate intracellular calcium oscillations induced by TCAP-1 treatment.

![Figure 4.22. C8D1A astrocytes treated with TCAP-1 and NMDAR, AMPAR and mGluR5 antagonists (AP5, CNQX, MPEP) did not demonstrate any changes in intracellular calcium. A) Differential interference contrast (DIC) image of astrocytes. B) Fluo-4 fluorescence image of astrocytes shown prior to 100nM TCAP-1 administration and C) 1.5 minutes after 100nM TCAP-1 administration. D) Sample traces after treatment with vehicle (aCSF+antagonists: 25µM AP5, 25µM CNQX, 30µM MPEP) and after TCAP-1 (100nM) treatment with antagonists (aCSF+25µM AP5, 25µM CNQX, 30µM MPEP). E) Normalized fluo-4 fluorescence comparing vehicle (n=4) and 10 min TCAP-1 (n=4) treatments. Each n is an average of 5 cells per coverslip.](image-url)
Figure 4.23. NMDAR, AMPAR and mGluR5 antagonist administration (AP5, CNQX and MPEP) significantly decreased intracellular calcium levels compared to TCAP-1 and vehicle administration in C8D1A astrocytes. The means of vehicle (aCSF+ antagonists: 25µM AP5, 25µM CNQX, 30µM MPEP), 100nM TCAP-1 and glutamate receptor antagonists (aCSF+25µM AP5, 25µM CNQX, 30µM MPEP) are represented for a 10 minute treatment. n=4 for each treatment where each n is an average of 5 cells per coverslip. Statistical analysis: one-way ANOVA and Bonferroni post-test comparing all pairs of columns (mean±SEM; ***p<0.001).
4.3.5.4 Similar effects of TCAP-1 and glutamate responses in C8D1A astrocytes

Similar responses were observed when TCAP-1 and glutamate were administered in C8D1A astrocytes. Intracellular calcium oscillations that were observed upon TCAP-1 and glutamate treatments demonstrated no significant differences with respect to average amplitude (Fig. 4.24a). The average peak frequency was approximately 0.1 peaks/minute greater upon glutamate treatment relative to TCAP-1, but this was not significant (Fig. 4.24b). Also, the raw traces of both TCAP-1 and glutamate treated C8D1A astrocytes were classified into two different responses, an initial peak and continuous calcium oscillations. When treated with TCAP-1, 15/25 cells demonstrated an initial peak (Fig. 4.25a) and 10/25 cells demonstrated continuous calcium oscillations (Fig. 4.25b). When treated with glutamate, 12/20 cells demonstrated an initial peak (Fig. 4.25c) and 4/20 cells demonstrated continuous calcium oscillations (Fig. 4.25d).

Figure 4.24. Average amplitude and average peak frequency of C8D1A astrocytes treated with TCAP-1 and glutamate were not significantly different. A) Average amplitude was not significantly greater in C8D1A astrocytes treated with 100nM TCAP-1 relative to 300µM glutamate. B) Average peak frequency was not significantly different in C8D1A astrocytes treated with 100nM TCAP-1 relative to 300µM glutamate. For A) and B), n=4 for each treatment, where n is an average of 5 cells per coverslip. Statistical analysis: unpaired t-test with Welch’s correction (mean ±SEM; ns).
Figure 4.25. Raw traces of TCAP-1 and glutamate treated C8D1A astrocytes can be classified into two different responses, an initial peak and continuous oscillations. A) Three representative raw traces that demonstrated an initial peak in calcium when treated with 100nM TCAP-1. 15/25 cells demonstrated this response. B) Three representative raw traces that demonstrate continuous calcium oscillations when treated with 100nM TCAP-1. 10/25 cells demonstrated this response. C) Three representative raw traces that demonstrated an initial peak in calcium treated with 300uM glutamate. 12/20 cells that were treated with 300µM glutamate demonstrated this response. B) Three representative raw traces that demonstrate continuous calcium oscillations upon 300µM glutamate treatment. 4/20 cells demonstrated this response. 4/20 cells did not demonstrate calcium peaks (data not shown).
4.4 Discussion

My findings demonstrated that teneurin, TCAP and ADGRL are endogenously expressed in astrocytes. Administration of TCAP-1 to astrocyte monocultures induced calcium signaling, an effect not observed when astrocytes were co-cultured with neurons. Also, glutamate administration to astrocyte monocultures induced calcium signaling, similar to that observed with TCAP-1. Finally, administration of glutamate antagonists that ablated the intracellular calcium increases upon TCAP-1 administration demonstrated that TCAP-1 is, in part, modulating glutamatergic systems.

4.4.1 Characterizing C8D1A astrocytes for teneurin, TCAP and ADRGL

Characterizing the C8D1A astrocyte mouse cerebellar immortalized cell line was important in order to ascertain the endogenous expression of teneurin, TCAP and ADGRL. This is the first time that a teneurin, TCAP and ADGRL system has been identified in astrocytes in vitro. Gene expression analysis of these homologues revealed the expression of teneurin 1, 3 and 4, but not teneurin 2 and TCAP 1-4 (Fig. 4.1a). For the bands that were present, the bands sizes were consistent with the expected size. The presence of TCAP-2 gene expression, but not teneurin 2 suggests that the teneurin 2 gene is not present in this cell line. It is important to mention that while conducting RT-PCR, care was taken to optimize the annealing temperature for each primer pair by conducting temperature gradients. Only after the optimal annealing temperature was determined, were results replicated from different samples. This careful experimental approach was intended to reduce the possibility of missing gene expression. Despite careful optimization for each primer pair, teneurin 2 gene expression was not observed in C8D1A astrocytes, suggesting that this cell line does not express the teneurin 2 gene. In support of this, Dr. Claudio Casatti, a collaborator at Sao Paulo State University in Brazil, found teneurin 2 gene expression in astrocytes in vivo, using the same primer pair (Tessarin et al., submitted manuscript). However, it is also possible that C8D1A astrocytes express a different splice variant of the teneurin 2 gene than what was targeted by the primer pair used. Protein expression of teneurin 2 should have been examined in order to confirm this result however due to the limited commercial availability of the antibody, western blot analysis was not performed. If in fact,
C8D1A astrocytes do express TCAP-2 but not teneurin 2, it is possible that TCAP-2 may be expressed as a separate transcript from the teneurin gene, as identified for TCAP-1 (Chand et al., 2013). However, given the gene expression data of our colleague who did find evidence of teneurin 2 gene expression in vivo, this is less likely to be the case. Moreover, gene expression of ADGRL 1 and 3 was identified but ADGRL 2 was not (Fig. 4.1a). This finding is supported by studies of Matsushita and colleagues (1999), who demonstrated that ADGRL 1 and 3 are predominantly expressed in the brain where as ADGRL 2 is ubiquitously expressed and is more prevalent in peripheral tissues (Matsushita et al., 1999). The band sizes of ADGRL 1 and 3 were consistent with the expected size. Therefore, it is likely that ADGRL 2 is not expressed in the C8D1A astrocyte cell line but ADGRL 1, 3 are.

Subsequently, protein expression was examined to corroborate findings pertaining to gene expression of teneurin 1 and 3 as well as ADGRL 1-3 (Fig. 4.1b). Due to limited commercial availability of teneurin and TCAP antibodies, protein expression analysis could not be undertaken for all members but future analysis should be undertaken. Protein expression was identified for teneurin 1 and 3 as well as ADGRL 1 and 3, but not ADGRL 2 which directly corroborated the results observed with respect to gene expression analysis. Despite both gene and protein expression data showing that ADGRL 2 was not expressed in C8D1A astrocytes, it should be noted that ADGRLs have numerous alternative splice sites (Matsushita et al., 1999) and therefore, the possibility that the primers and antibodies may have targeted an alternative splice variant that was simply not detected in this cell line, cannot be discounted. Interestingly, teneurin 1 and 3 western blot results demonstrated multiple bands, as opposed to the expected 305kDa and 301kDa bands, respectively (Fig. 4.1b). The presence of multiple bands was previously demonstrated by colleagues in the laboratory as well as other research teams who have suggested that the teneurins may undergo alternative splicing or post-translational processing (Chand et al., 2013; Husic, 2016; Kenzelmann et al., 2008). Therefore, it is possible that teneurin 1 and 3 do not display full-length western blot bands as a result of alternative splicing or post-translational processing. These results demonstrate that C8D1A astrocytes endogenously express teneurin 1,3,4, TCAP 1-4 and ADGRL 1 and 3 in vitro, for the first time.
4.4.2 The effect of TCAP-1 administration on C8D1A astrocytes

Given the importance of calcium ion regulation in ancestral peptide systems and the intrinsic property of astrocytes to undergo a form of calcium excitability, elucidating the effects of TCAP-1 in astrocytes was of particular interest. The actions of TCAP-1 on astrocyte calcium dynamics have not been previously examined. TCAP-1 administration to C8D1A astrocytes resulted in oscillatory increases in intracellular calcium, that had a significantly greater average amplitude and average peak frequency compared to vehicle treatment (Fig. 4.3). Also, astrocytes close in proximity were found to undergo increases in intracellular calcium at similar time points and these calcium increases were observed to propagate to other cells in the astrocyte network (Fig. 4.5; Supplementary Fig.1). This suggests that TCAP-1 was able to induce calcium signaling in astrocytes.

There are many plausible hypotheses for the mechanisms that may be working to stimulate this response in astrocytes. However, only generalized conclusions can be made until further investigation involving downstream signaling molecules, such as IP₃, is undertaken. Astrocytes express many GPCRs (particularly those with a Gq subunit) that are linked to the release of calcium from intracellular stores upon activation (Agulhon et al., 2008). As TCAP-1 exerts its effects by activating ADGRL, it is possible that the downstream signaling cascade is activating the IP₃/PLC pathway, resulting in the release of calcium from the ER. In fact, Parpura and colleagues (1994) found that the administration of bradykinin, a 9 amino acid peptide that binds to a Rhodopsin GPCR, induced oscillatory increases in intracellular calcium levels in cultured astrocytes. They also found that bradykinin stimulated the release of the gliotransmitter glutamate from astrocytes, as a result of these increases in intracellular calcium (Parpura et al., 1994). The ability of intracellular calcium increases in astrocytes to induce glutamate release is well established in literature (Innocenti et al., 2000; Parpura et al., 2011). In light of this, it is possible that TCAP-1 is also causing the release of glutamate from astrocytes. Future studies should be undertaken to determine whether TCAP-1 is inducing glutamate release from astrocytes and whether TCAP-1 administration has an effect on IP₃ levels in astrocytes. Therefore, considering these findings, it is possible that TCAP-1 is inducing signaling in
astrocytes through its receptor ADGRL that may be stimulating the IP$_3$/PLC pathway to release intracellular calcium stores from the ER.

4.3.3 Establishing C8D1A astrocyte and N38 neuron co-cultures

Many different approaches were undertaken in order to establish the optimal method for astrocyte-neuron co-culture using C8D1A astrocyte and N38 neuron immortalized cell lines. C8D1A cell lines and N38 neurons were transfected to allow for their identification in co-culture. Results pertaining to the optimization of the co-culture method are discussed below.

4.3.3.1 Examining a fluorescently labeled C8D1A cell line to identify astrocytes in co-culture

First, C8D1A astrocytes were transfected to express mCherry to allow for the identification of this cell type when in co-culture with wild type N38 neurons. To confirm the calcium responses of transfected astrocytes to TCAP-1 treatment prior to the commencement of their growth in co-culture, experiments were performed where C8D1A-mCherry astrocytes were treated with TCAP-1 (Figure 4.6). C8D1A-mCherry astrocytes demonstrated diminished calcium oscillations relative to wild type C8D1A astrocyte to TCAP-1 treatment (Figure 4.2), suggesting that the transfection may have affected the calcium dynamics of these cells. Indeed, a large amount of cell lysis was observed when C8D1A-mCherry cells were cultured, relative to that observed in wild type astrocytes. Also, transfected astrocytes appeared to be less adhesive to coverslips they were grown on prior to fluorescence experiments, making them less likely to withstand the perfusion system used at the fluorescence microscope. In fact, transfection has been found to affect cell viability in the past (Godbey et al., 2001). Astrocytes have a delicate morphology and it was observed that they require close associations in order for them to be able to grow, it is possible that the large amount of cell lysis as a result of transfection impeded their ability to establish close of connections and therefore altered their calcium dynamics. Efforts to co-culture C8D1A-mCherry astrocytes with wild type N38 neurons were also undertaken, as the co-culture of different cell types together often improves cell viability as a result of a release of extrinsic factors such as cytokines or growth factors (Goldring & Goldring, 1991; O’Malley et
al., 1994) and astrocytes and neurons have previously been found to grow better when cultured together (O’Malley et al., 1992). However, co-culture did not appear to have an impact on cell growth with the transfected cells. Therefore, the calcium dynamics of C8D1A-mCherry astrocytes were likely affected as a result of the transfection so this cell type was not used towards co-culture experiments.

4.3.3.2 The effect of TCAP-1 administration on N38 neurons

In order to understand the effect of TCAP-1 treatment on wild type N38 neurons grown under representative co-culture conditions prior to commencing experiments, N38 neurons were treated with TCAP-1. TCAP-1 administration on N38 neurons resulted in significant decreases in intracellular calcium that were statistically significant at 0.5-3, 3.5-6 and 6.5-9 minute time points relative to vehicle treatment (Fig. 4.7, 4.8). This is supported by previous findings in the laboratory where TCAP-1 was shown to induce decreases in intracellular calcium as well as depolarize mitochondrial membrane potential in neurons (Hogg, unpublished observations). Given this finding, it is possible that TCAP-1 is stimulating the uptake of calcium into mitochondria, however further experiments need to be undertaken to confirm this. Therefore, further studies with respect to how TCAP-1 affects the mitochondria in neurons as well as what its effects are on secondary calcium messengers in N38 neurons should be undertaken.

4.3.3.3 Examining a fluorescently labeled N38 cell line to identify neurons in co-culture

Since C8D1A-mCherry astrocytes were not determined as a viable model for labeling astrocytes in co-culture, N38 neurons transfected to express BFP were examined with respect to the effects of TCAP-1 administration on intracellular calcium levels. N38 neurons were observed to be a more resilient cell type and as a result, it was possible that the N38 immortalized cell line may be a better candidate for transfection to be able to identify between the cell types in co-culture. However, N38-BFP neurons demonstrated unstable calcium dynamics when treated with both vehicle and TCAP-1 (Figure 4.9), which was not observed in wild type N38 neurons (Figure 4.7). Therefore, as was the case for transfected astrocytes, the calcium dynamics of N38-BFP neurons were altered post-transfection. This posed a clear challenge from a cell viability
perspective, as transfected neurons did not demonstrate representative responses to both vehicle and TCAP-1 treatments. Also, from a logistical standpoint, this also caused a problem as in order to be able to compare the effects in many cells undergoing the same treatment, the fluorescence has to be normalized and to be able to do this, a steady state must be established, which was not possible for this cell type. As a result, the N38-BFP neurons were not used in co-culture with astrocytes.

4.3.3.4 Establishing an appropriate astrocyte-to-neuron ratio for co-culture

Despite the altered calcium dynamics of the transfected cell lines, C8D1A-mCherry astrocytes and N38-BFP neurons did serve as a useful tool in establishing an appropriate astrocyte-to-neuron ratio under the established co-culture conditions. The use of these cell lines allowed for an approximation that the seeding ratios were appropriate for maintaining a 1:1 C8D1A-to-N38 ratio (Figure 4.10). In contrast to the common statement that glia outnumber neurons 10-fold in the brain, a more recent understanding of the glia-neuron ratio in the brain demonstrates that it is actually much closer to a 1:1 relationship (Herculano-Houzel, 2014; Hilgetag & Barbas, 2009). For the purposes of establishing a co-culture between these two cell types and given that the immortalized C8D1A astrocyte cell line is derived from the cerebellum where as the immortalized N38 neurons are derived from the hypothalamus, a 1:1 ratio seemed appropriate. Therefore, by utilizing transfected astrocyte and neuron cell lines grown together in culture, it was possible to approximate a 1:1 ratio. Additionally, the transfected cell lines allowed for a better understanding of the visual morphological differences between these two cell types, where astrocytes were observed to have longer projections and grow in tight networks where as neurons did not, making it easier to differentiate between wild type astrocytes and neurons in co-culture.

4.3.3.5 Differentiating between wild type C8D1A astrocytes and N38 neurons in co-culture

Given the altered calcium responses of both C8D1A astrocytes and N38 neurons post-transfection, as discussed above, wild type cells lines for both astrocytes and neurons were used to establish co-cultures. Therefore, a method to differentiate between the two cell types was
required and as a result, three criteria for identification were established. First, astrocytes had a basal level of calcium that was approximately twice the amount of neurons (Fig. 4.11b). To my knowledge, this is the first time that a difference between basal calcium levels of astrocytes and neurons has been examined. Even though the concentration of fluo-4 was kept constant throughout experiments with all cell types, the possibility that astrocytes are able to uptake fluo-4 better than neurons, rather than having greater intracellular calcium levels, cannot be discounted. However, considering the tight regulation of calcium in neurons, as large amounts can lead to excitotoxicity and that astrocytes use calcium as their main form of excitability, it is highly likely that astrocytes have greater amounts of intracellular calcium than neurons (Cornell-Bell et al., 1990; Sofroniew & Vinters, 2010). The second criterion used to differentiate between the two cell types, was the presence of intracellular calcium oscillations in astrocytes that were not observed in neurons (Fig. 4.12, 4.13). This is an intrinsic property of astrocytes as they are non-excitable and use calcium as their primary form of signal transduction (Charles et al., 1991; Cornell-Bell et al., 1990). Finally, the third criterion involved the morphological differences as astrocytes have characteristically long projections that give them a ‘star-like’ appearance whereas neurons do not display this kind of morphology (Agulhon et al., 2008). Therefore, given that the transfected cell lines had altered calcium dynamics, using both wild type astrocyte and neuronal cell lines and establishing a way to identify between the two cell types was the most viable model for co-culture.

4.3.4 The effect of TCAP-1 on C8D1A astrocytes when in co-culture with N38 neurons

The effects of TCAP-1 treatment on astrocyte responses when in co-culture with neurons have not been previously established. These experiments were undertaken in order to enhance our understanding of how TCAP-1 may affect astrocyte-neuron communication. Astrocytes in co-culture with neurons did not undergo oscillatory increases in intracellular calcium when treated with TCAP-1, but did undergo increases when treated with vehicle (Fig. 4.14). The average amplitude of intracellular calcium oscillations decreased significantly during TCAP treatment relative to vehicle pre-treatment (Fig. 4.15b) but was not significantly different throughout vehicle treatment (Fig. 4.15a). However, the average peak frequency of intracellular calcium oscillations was not significantly different during TCAP-1 treatment relative to vehicle
pre-treatment (Fig. 4.15d) and was not significantly different throughout vehicle treatment (Fig. 4.15c). This suggests that TCAP-1 is decreasing the potency of intracellular calcium oscillations but is not having an effect on the number of intracellular calcium oscillations on C8D1A astrocytes in co-culture with N38 neurons.

These findings are different from what was observed in astrocyte monocultures, where TCAP-1 administration induced intracellular calcium oscillations and vehicle treatment did not (Fig. 4.2). This suggests that the presence of neurons may have had an effect on the way that the astrocytes responded to TCAP-1 treatment. As previously mentioned, Parpura and colleagues (1994) found that bradykinin induced glutamate release from astrocytes as a result of the intracellular calcium oscillations it evoked. This team also found that bradykinin induced intracellular calcium increases in both astrocytes and in neurons, but not in neuronal monocultures (Parpura et al., 1994). This finding indicated that the glutamate released from astrocytes was inducing these intracellular calcium increases in neurons. Considering this set of findings, as well as the fact that TCAP-1 did not induce increases in calcium in neuron monocultures (Fig. 4.7), it is unlikely that TCAP-1 is inducing the release of glutamate from astrocytes as neurons did not undergo increases in calcium when in co-culture. The effect of TCAP-1 on glutamate released from astrocytes should be quantified to confirm this. However, as these studies were conducted in vitro, it is also possible that the astrocyte-neuron co-culture is simply a more representative model of the effects of TCAP-1 in astrocytes.

In order to examine whether TCAP-1 has an effect on intercellular calcium wave propagation in co-culture, a spatial-temporal analysis of C8D1A astrocyte responses when in co-culture with N38 neurons was conducted. This analysis revealed that when treated with vehicle, astrocytes in close proximity displayed increases in intracellular calcium at similar time points. This suggests that astrocytes that are close to one another are shunting calcium to nearby astrocytes through gap junctions. Kim and colleagues (1994) suggested that intercellular calcium waves among astrocytes occur as a result of ionotropic receptor activation and calcium influx through the plasma membrane, rather than the release of calcium from intracellular stores. Therefore, it is possible that upon vehicle treatment, astrocytes are sending calcium signals to neighbouring cells. Additionally, Kim and colleagues (1993) investigated the role of the
Na⁺/Ca²⁺ exchanger, an antiporter that can either remove or import calcium from/into the cytosol depending on the sodium ion concentration, with respect to intercellular calcium signaling. They found that it was involved in the generation of intercellular waves, as when it was inhibited, intercellular waves were greatly reduced (Kim et al., 1993). Both of these findings suggest that ionotropic pathways as well as the Na⁺/Ca²⁺ exchanger are involved in intercellular wave propagation upon vehicle treatment. As increases in intracellular calcium were not observed in astrocytes upon TCAP-1 treatment (Fig. 4.14, 4.15), intercellular calcium waves were not observed upon TCAP-1 treatment either (data not shown). This suggests that TCAP-1 may be affecting ionotropic receptor pathways or the Na⁺/Ca²⁺ exchanger, thus reducing intercellular calcium wave propagation. Future experiments examining ionotropic pathways or inhibiting the Na⁺/Ca²⁺ exchanger with the application of an antagonist, should be undertaken.

4.3.5 Elucidating whether TCAP-1 is affecting glutamatergic systems

In light of the effects observed with TCAP-1 administration in astrocyte monocultures with respect to intracellular calcium oscillations, it was of interest to examine the effects of another excitatory molecule that has also been demonstrated to induce such calcium oscillations in astrocytes, glutamate. Therefore, an analysis was undertaken to better understand the mechanisms through which TCAP-1 may be functioning to induce the increases in intracellular calcium in astrocytes.

4.3.5.1 The effect of glutamate administration on C8D1A astrocytes

The administration of glutamate, an excitatory neurotransmitter, to C8D1A astrocytes resulted in oscillatory increases in intracellular calcium (Fig. 4.17) as demonstrated by the significant increase in average amplitude when compared to vehicle treatment (Fig. 4.18) and the significant increase in intracellular calcium at 0.5-3 minutes (Fig.4.19). This effect has been studied in the past and the observed response is consistent with previous findings (Cornell-Bell & Finkbeiner, 1991; Kim et al., 1994). Despite this being a well-characterized response in astrocytes, this set of experiments was undertaken to confirm that the C8D1A cell line would demonstrate a response to glutamate. Even though the C8D1A immortalized cell line has not
been characterized for the expression of these particular receptors, the fact that glutamate did invoke a characteristic response suggests that these receptors are present. Additionally, there is an extensive amount of supporting data \textit{in vivo} and \textit{in situ} regarding glutamate receptor expression in astrocytes, including NMDAR, AMPAR and mGluR5 (Porter & McCarthy, 1997; 1995). However, gene and protein expression of these receptors should be confirmed in the future.

\textbf{4.3.5.2 The effect of glutamate receptor antagonist administration on C8D1A astrocytes treated with glutamate}

Subsequently, glutamate receptor antagonists were applied to astrocytes with the aim to observe responses upon treatment with glutamate and TCAP-1. The first experiment involved treating astrocytes with vehicle (aCSF with NMDAR, AMPAR and mGluR5 antagonists) while administering glutamate. Results demonstrated that the application of glutamate antagonists was successfully able to abolish intracellular calcium increases (Fig. 4.20), as average fluo-4 fluorescence decreased significantly with the administration of glutamate antagonists compared to compared to glutamate treatment on its own (Fig. 4.21).

The antagonists were selected for these particular receptors because of their involvement in two pathways that have been elucidated in terms of the mechanisms underlying changes in intracellular calcium levels in astrocytes. The first, involves the activation of mGluR, which stimulates the PLC/IP$_3$ pathway, mediating calcium release from the ER. Even though 8 different subtypes of mGluRs exist, only mGluR1 and mGluR5 are coupled to the release of calcium (Pin & Duvoisin, 1995) and mGluR5 has been most extensively studied with respect to astrocyte calcium dynamics (Balazs et al., 1997; D’Ascenzo et al., 2006; Nakahara et al., 1997), which is why this subtype was targeted specifically in experiments. The second involves glutamate binding to ionotropic glutamate receptors, such as AMPAR and NMDAR, which leads to an influx of sodium and calcium ions into the cell and depolarizes the membrane. This depolarization has been demonstrated to cause increases in intracellular calcium as a result of calcium influx through voltage-dependent calcium channels located on the plasma membrane (Finkbeiner, 1993). As a result, by simultaneously administering metabotropic and ionotropic
receptor antagonists that are involved in these two pathways, it is possible to deduce that glutamate is involved in inducing increases in intracellular calcium in the C8D1A cell line.

4.3.5.3 The effect of glutamate receptor antagonist administration on C8D1A astrocytes treated with TCAP-1

Subsequently, an investigation was prompted regarding how the administration of TCAP-1 to astrocytes treated with glutamate receptor antagonists would affect intracellular calcium oscillations in astrocytes. Therefore, the second experiment involved treating astrocytes with vehicle (aCSF with NMDAR, AMPAR and mGluR5 antagonists) while administering TCAP-1. Upon TCAP-1 and glutamate receptor antagonist treatment, intracellular calcium oscillations were not observed (Fig. 4.22) and average normalized fluorescence decreased significantly during the application of glutamate receptor antagonists compared to glutamate treatment (Fig. 4.23). This suggests that TCAP-1 may be affecting the signal transduction pathway that is associated with glutamate receptors or that it may be regulating a mechanism similar to that of glutamate (see 4.3.5.4 for full discussion).

4.3.5.4 Similar effects of TCAP-1 and glutamate responses in C8D1A astrocytes

Similar calcium responses were observed when C8D1A astrocytes were treated with TCAP-1 and glutamate. The average amplitude and average peak frequency of TCAP-1 and glutamate treated astrocytes were not significantly different relative to one another (Fig. 4.24). Both agonists demonstrated increases in intracellular calcium in an oscillatory manner where the average amplitude was significantly greater compared to vehicle treatment (Fig. 4.3a; 4.18a). This demonstrated that both were inducing intracellular calcium increases. However, TCAP-1 demonstrated a significant increase in the average peak frequency whereas glutamate did not, suggesting that TCAP-1 induced a sustained response in the intracellular calcium oscillations (Fig. 4.3b; 4.18b). Moreover, the temporal responses differed between the two treatments. TCAP-1 had a prolonged effect on the increases in intracellular calcium relative to vehicle treatment at 3.5-6 and 6.5-9 minute time points (Fig. 4.4) whereas glutamate exhibited a significant increase in intracellular calcium levels earlier on, at 0.5-3 minute time points (Fig.
This prolonged effect in intracellular calcium increases during TCAP-1 treatment corroborates with the significant increase in average peak frequency with TCAP-1 administration, suggesting that TCAP-1 is acting to induce increases in intracellular calcium that present as sustained oscillations. On the other hand, glutamate may be acting to induce an initial peak that is followed by a sustained calcium elevation, as is supported by the earlier temporal response observed with glutamate treatment and the fact that the average peak frequency was not significantly different from vehicle treatment.

In light of this, an investigation of the raw traces was prompted for astrocytes treated with glutamate or TCAP-1 as this can shed insight with respect to the underlying mechanisms involved in these responses. Kim and colleagues (1994) were able to characterize specific calcium responses with respect to the mechanisms underlying increases in intracellular calcium in astrocytes. They found that stimulation of the mGluR pathway with an agonist induced an initial spike and oscillatory intracellular waves whereas stimulation of the ionotropic pathway evoked a sustained calcium influx and intercellular calcium waves that were able to propagate to neighbouring cells (Kim et al., 1994). Given this, it may be possible to suggest what intracellular mechanisms are playing a role with the effects that TCAP-1 or glutamate treatment are inducing in these cells. Raw traces for TCAP-1 and glutamate suggest that both molecules are able to induce an initial spike as well as oscillatory increases in intracellular calcium (Fig. 4.25). Based on findings by Kim and colleagues (1994), this suggests that the signaling for both TCAP-1 and glutamate is dependent on the metabotropic receptor pathway in astrocyte monoculture, meaning that they are dependent on the release of IP₃ from the ER. Despite these similarities, there are some differences among the cellular responses to both treatments. TCAP-1 induced an initial peak and sustained response for 15/25 cells and oscillations for 10/25 treated cells. On the other hand, the initial peak and sustained response appeared to be more frequent with glutamate treatment as 12/20 cells demonstrated an initial peak and 4/20 cells demonstrated oscillations, 4/20 cells did not demonstrated any calcium spikes. It appears that glutamate administration predominantly underwent the initial peak and sustained response and again, glutamate appeared to be inducing its effects much faster and this may be because it is a smaller molecule than TCAP-1 or because it induces its effects through many more receptors where as TCAP-1 is only
known to function through ADGRL. Taken together, this suggests that TCAP-1 may be affecting the intracellular levels of secondary calcium messengers such as IP₃, similar to glutamate. However, more experiments would need to be undertaken in order to be able to ascertain this. Also, it is important to keep in mind that there are many intracellular mechanisms underlying the observed oscillatory increases in calcium in response to glutamate or TCAP-1 treatment and they can all occur in the same cell simultaneously. This makes it difficult to isolate individual responses and pinpoint the exact intracellular signaling mechanism that may be underlying these calcium dynamics.

4.5 Integrating discussion and concluding remarks

Taken together, these data suggest that TCAP-1 is inducing both intracellular and intercellular calcium increases in astrocyte monocultures, similar to the effects observed with glutamate. These are novel findings that have been investigated in the field for the first time so further investigation of different aspects is required before conclusions can be made. However, considering the parallels that were observed with TCAP-1 and glutamate in astrocyte monoculture as well as the effects that TCAP-1 had on astrocytes when co-cultured with neurons, possible pathways that TCAP-1 may be affecting and that should be investigated in the future are discussed here.

As was discussed earlier, intracellular calcium increases in astrocytes are linked to the activation of the PLC/IP₃ pathway and the IP₃-mediated release of calcium from ER stores (Kim et al., 1994). With respect to glutamate, this involves the activation of mGluRs (mainly subtypes 1 and 5) located on the astrocyte membrane, which are coupled to the release of calcium (Fig. 4.26a) (Pin and Duvoisin, 1995; Porter & McCarthy, 1997). In light of this, TCAP-1 modulates downstream effects upon binding to ADGRL, a GPCR linked to a Gₛ subunit that upon activation, modulates downstream effects. The activation of ADGRL has been shown to stimulate the PLC/IP₃ pathway, resulting in the release of calcium stores from the ER, as was demonstrated up the binding of α-latrotoxin to ADGRL, its exogenous ligand in neurons (Lelianova et al., 1997). Even though this pathway has not been elucidated in astrocytes (Fig. 4.26b), it can be hypothesized that TCAP-1 induces increases in intracellular calcium in
astrocytes upon binding to ADGRL. This is supported by findings that the C8D1A cell line expresses ADGRL homologues as well as by raw traces of both TCAP-1 and glutamate that displayed an initial peak and continuous calcium oscillations, determined by Kim and colleagues (1994) to be predominantly evoked by the metabotropic pathway. Given that these are speculations based on novel preliminary findings, further investigation should examine the effects of TCAP-1 on downstream messengers in this pathway, such as IP₃, in astrocytes.

Additionally, the mitochondrion is an important organelle for maintaining calcium ion homeostasis and it can sequester or release calcium (Fig. 4.26a). This has an effect on intracellular calcium levels in astrocytes as was demonstrated by Reyes and Parpura (2008). Inhibition of the mitochondrial calcium uniporter, through which calcium enters mitochondria, increased intracellular calcium increases in astrocytes whereas inhibition of the mitochondrial Na⁺/Ca²⁺ exchanger, which expels calcium from mitochondria, reduced intracellular calcium increases (Reyes & Parpura, 2008). The release of calcium from mitochondrial stores was also demonstrated to modulate the release of the gliotransmitter glutamate from astrocytes as blockade of the mitochondrial Na⁺/Ca²⁺ exchanger decreased glutamate release from astrocytes (Reyes & Parpura, 2008). Therefore, mitochondrial calcium stores contribute to intracellular calcium increases as well as glutamate release in astrocytes. In light of these findings by Reyes and Parpura (2008) and as our laboratory has determined that TCAP-1 induces mitochondrial depolarization in neurons, which suggests that calcium is being sequestered into mitochondria, the role of TCAP-1 with respect to activity of the calcium uniporter or the mitochondrial Na⁺/Ca²⁺ exchanger should be examined. The effect of TCAP-1 on mitochondrial depolarization should be confirmed in astrocytes. Also, given that the release of glutamate from astrocytes is dependent on the release of calcium stores from mitochondria in which the mitochondrial Na⁺/Ca²⁺ exchanger plays a role, TCAP-1 may not be stimulating the release of glutamate from astrocytes if it is causing calcium to sequester in mitochondria, as was observed by N38 neurons not responding to C8D1A astrocytes when in co-culture upon TCAP-1 treatment. Therefore, calcium sequestration and release to and from mitochondria should be examined upon TCAP-1 treatment in astrocytes.
Finally, as was previously discussed, Kim and colleagues (1994) suggested that intercellular calcium increases in astrocytes are linked to the activation of the ionotropic pathway (Fig. 4.26a). Glutamate binds to ionotropic receptors, such as NMDAR and AMPAR, and the activation of these receptors results in an influx of Ca\(^{2+}\) and Na\(^{+}\) ions into the cytosol in astrocytes (Kirischuk et al., 1997). When Na\(^{+}\) enters the astrocytes, the cell depolarizes. This activates the Na\(^{+}\)/Ca\(^{2+}\) exchanger on the plasma membrane, which at resting membrane potential expels calcium from the cell, but under depolarized conditions reverses the exchanger to expel sodium and bring calcium into the astrocyte (Kirischuk et al., 1997). This influx of calcium into the astrocytes through activation of the ionotropic pathway contributes to the propagation of intercellular calcium increases in astrocytes, as suggested by Kim and colleagues (1994). In light of this, though indirect evidence, results demonstrate that TCAP-1 may be inducing intercellular calcium signaling in astrocyte monocultures, which similar to glutamate, may be involved in the activation of ionotropic pathways. This is supported by findings that the administration of glutamate receptor antagonists, which included inhibitors for NMDAR and AMPAR, ablated the increases in intracellular calcium observed upon TCAP-1 treatment. Further, in support of the involvement of the Na\(^{+}\)/Ca\(^{2+}\) exchanger in regulating calcium levels, TCAP-1 treatment decreased intracellular calcium levels in neurons, suggesting that as calcium is being sequestered into mitochondria, the Na\(^{+}\)/Ca\(^{2+}\) exchanger is active in its forward confirmation, expelling calcium ions from the cytosol. Also, a colleague in the laboratory has demonstrated that inhibition of the Na\(^{+}\)/Ca\(^{2+}\) exchanger does not elicit decreases in intracellular calcium in neurons (Hogg, unpublished observations). Therefore, these findings point towards activation of the ionotropic glutamate pathway and future studies should target this pathway with respect to the effects of TCAP-1 in astrocytes.

In conclusion, these novel findings have laid a foundation to further explore the effects of TCAP-1 in astrocytes in vitro. I have demonstrated the presence of a functional teneurin, TCAP, ADGRL system in C8D1A astrocytes, for the first time. Also, TCAP-1 induced calcium signaling in astrocytes, an effect that was not observed when astrocytes were co-cultured with neurons. Finally, the administration of glutamate to astrocytes demonstrated a similar response to that observed with TCAP-1 treatment and the application of glutamate receptor antagonists
ablated calcium signaling in both astrocytes treated with glutamate and TCAP-1. This suggests that TCAP plays a role in modulating a TCAP-calcium-glutamate mechanism, which has not been previously established. Future studies should target both ionotropic and metabotropic pathways with respect to TCAP-1 administration in monoculture.

Fig. 4.26. Suggested areas of future research pertaining to metabotropic and ionotropic pathways that contribute to intracellular and intercellular calcium signaling in astrocytes. A) Three different pathways by which glutamate contributes to intracellular and intercellular calcium increases in astrocytes. 1) Activation of metabotropic receptors and the PLC/IP₃ leads to the release of stores from the ER and contributes to increases in intracellular calcium in astrocytes. 2) Calcium can be released from mitochondria, through the Na⁺/Ca²⁺ exchanger, or sequestered by entering through the uniporter to modulate intracellular calcium levels in astrocytes. 3) Activation of the ionotropic pathway leads to depolarization of the astrocyte, as a result of incoming Na⁺ and Ca²⁺, reversing the Na⁺/Ca²⁺ exchanger and bringing calcium into the cytosol. B) Areas that require future investigation with respect to the actions of TCAP-1 in astrocytes based on similar results observed with TCAP-1 and glutamate administration. 1) The hypothesized pathway through which TCAP-1 induces its effects. It binds to its receptor, ADGRL, stimulating the PLC/IP₃ pathway and leads to increases in intracellular calcium in astrocytes.
Chapter 5
Final Discussion and Concluding Remarks

5 Abstract

This final chapter aims to discuss the evolution of TCAP, astrocytes, calcium ion regulation and glutamate to integrate these ancestral systems. The evolutionary origins of each of these systems will be discussed as well as how my findings have contributed to our understanding of the functional role of TCAP as an ancestral peptide.

5.1 Introduction

TCAP is an ancestral peptide that affects calcium dynamics in astrocytes. The findings presented in this thesis are novel with respect to its phylogeny and actions on astrocytes. With respect to the evolution, this is the first time that the evolutionary history of TCAP has been attempted in relation to Secretin superfamily members using phylogenetic analysis and moreover, corroborates with previous phylogenetic studies performed on the associated receptors. Furthermore, this is the first time that TCAP, teneurin and ADGRL members have been identified in astrocytes in vitro. The effect of TCAP-1 with respect to calcium dynamics in astrocytes and neurons has also not been previously established. Also, this is the first time that astrocyte-neuron co-cultures have been attempted with the goal of understanding TCAP actions. The purpose of this chapter is to present an integrative discussion regarding how these findings have contributed to understanding the evolutionary scheme of TCAP and how this relates to astrocytes and calcium signaling.

5.2 Evolution of the teneurin-TCAP system

The ancestral origins of the teneurin-TCAP system have been reported in the past. As proposed by Zhang and colleagues (2012), this is an ancient system that likely evolved from a PPT gene that arose as a result of a HGT event from a prokaryote to a choanoflagellate, a primitive unicellular organism. The teneurin gene has been identified in the choanoflagellate, Monosiga brevicollis (Tucker et al., 2012). Choanoflagellates are thought to be a progenitor to the
Metazoans (Lang et al., 2002). This supports the hypothesis that a choanoflagellate may have engulfed a prokaryote containing the PPT gene, which became integrated into its genome and lost its toxic role overtime (Tucker, 2012; Zhang, 2012). With respect to structural evidence, the teneurins share characteristics of PPTs: the same type II orientation, RHS domains and close similarity to the C-terminal domain to the HNH bacterial toxin of the GHH clade (Minet et al., 1999; Zhang et al., 2012). The GHH domain may be an ancestor of TCAP that lost its toxic role and functioned as an intracellular signaling molecule (Zhang et al., 2012). Additionally, the C-terminal region of the *M. brevicollis* teneurin protein contains YD repeats characteristic of proteobacteria and most of the extracellular domain is encoded on one large 6829 base-pair exon, characteristic of prokaryotic genomes and of HGT (Tucker et al., 2012). This is the most direct evidence in support of this hypothesis (for more, see Chapter 1). Therefore, as mentioned earlier, the teneurin-TCAP system has previously been presented as evolutionarily ancient.

Moreover, with respect to my findings in Chapter 3, I presented TCAP as ancestral to the Secretin superfamily for the first time and the receptors of these ligands have previously been established as ancestral to one another. Nordström and colleagues (2009) demonstrated that Adhesion GPCRs are more evolutionarily ancient than Secretin GPCRs. Adhesion GPCR genes have been identified in the genome of amphioxus, *Branchiostoma floridae*, the choanoflagellate, *M. brevicollis* and the sea anemone, *Nematostella vectensis*, however the genomes of these species do not contain Secretin GPCR genes (Nordström et al., 2008; 2009). This suggests that Adhesion GPCRs were present prior to the protostome-deuterostome divergence, whereas Secretin GPCRs were not. The presence of a Secretin GPCR characteristic, the HBD, in ADGRL, an Adhesion GPCR and the receptor of TCAP, prompted the investigation in Chapter 3. As was presented by my findings, TCAP peptides appear to be evolutionarily ancient to the Secretin superfamily of ligands, which supports previously presented model of receptor evolution. Also, my findings in Chapter 2 added to the current model of CRF evolution, a Secretin superfamily member that shares sequence similarity with TCAP, further contributing to our understanding of the evolutionary scheme of these peptides. Therefore, the findings presented in Chapter 3 added to the current evidence that TCAP is an ancient peptide that evolved prior to the Secretin superfamily.
Considering the evidence with respect to the ancestral origin of the teneurin-TCAP system and in light of my findings in Chapters 2 and 3, which have contributed to our understanding of TCAP in relation to the Secretin superfamily, it is possible to present two hypotheses for the evolutionary scheme of these peptides. The first suggests that an ancient TCAP-like peptide may have been the ancestor of the Secretin superfamily and that it evolved prior to the emergence of CRF, calcitonin and secretin families (Fig. 5.1a). This is supported by the identification of TCAP in organisms prior to the protostome-deuterostome divergence, where as members of the Secretin superfamily have not been identified this early in evolution (Tucker et al., 2012). A second hypothesis, which suggests that the Secretin superfamily forms a parallel lineage to extant TCAP and that these two lineages evolved from a proto-CRF-calcitonin-secretin-TCAP ancestor that was related to all of these families, cannot be discounted (Fig 5.1b). Due to sequence availability, phylogenetic analysis was performed using extant Secretin superfamily and TCAP sequences. As a result, both of these hypotheses are plausible with respect to the relationship of TCAP to Secretin superfamily members. A separate analysis where extant TCAP sequences could be traced back to ancestral sequences based on highly conserved motifs, could be undertaken in the future. Also, an analysis including peptides that are more closely related to TCAPs or other ligands that bind Adhesion GPCRs could be undertaken to resolve the presented hypotheses.

Figure 5.1. Two possible evolutionary schemes of TCAP in relation to the Secretin superfamily of peptides. A) An ancient TCAP-like peptide was an ancestor of the Secretin superfamily. B) Extant TCAP evolved as a lineage parallel to the Secretin superfamily from a proto-CRF-calcitonin-secretin-TCAP ancestor.
5.3 Evolution of astrocytes and calcium signaling systems

With respect to astrocyte evolution, proto-astrocyte glia have been identified in organisms prior to the protostome-deuterostome divergence such as *C. elegans* (Reichenbach & Pannicke, 2008) and *D. melanogaster* (Awasaki et al., 2008). Proto-astrocyte glia are also found among protochordates, but the earliest evidence of astrocyte-like cells among chordates is in hagfish (Appel, 2004; Wicht et al., 1994), suggesting that astrocytes arose at the base of chordate evolution. Given that astrocytes are one of the most prominent glial cell types that are integral to the development of the CNS and that teneurins and TCAPs play a role in the development of the CNS, it was interesting to determine whether teneurin, TCAP and ADGRL were endogenously expressed in this astrocyte cell line. As presented in Chapter 4, my findings did demonstrate that this system is present in cultured astrocytes.

Moreover, previous work in our laboratory demonstrated that TCAP-1 affected calcium dynamics in neurons and calcium signaling is an important form of communication in astrocytes. So, as presented by my findings in Chapter 4, I investigated the effect of TCAP-1 on calcium signaling in astrocytes and in astrocytes co-cultured with neurons. Interestingly, calcium is an ion that played a major role in the molecular mechanisms of the earliest organisms. Early in evolution, calcium needed to be utilized readily by organisms as it is highly toxic in large amounts and incurs damage to cells (Case et al., 2007; Lovejoy, 2005). As a result, cells had to integrate it into their signaling machinery to be able to control and regulate the levels of calcium within the cytoplasm (Case et al., 2007; Verkhratsky et al., 2012). Evidence of calcium signaling in prokaryotes, protists, fungi and the choanoflagellate *M. brevicollis*, which contain multiple channels and the machinery needed to remove calcium as well as calcium ion signaling pathways, demonstrate that calcium ion signaling evolved prior to the emergence of the Metazoa (Cai, 2008; Cai & Clapham, 2012; Kippert et al., 1987). Interestingly, with respect to the discussion in Chapter 4, IP$_3$ receptors have been characterized in protists and both IP$_3$ receptors and Na$^+$/Ca$^{2+}$ exchangers have been characterized in *M. brevicollis*, indicating that this calcium machinery was present among organisms early in evolution (Cai, 2008; Cai & Clapham, 2012). The presence of this calcium machinery so early in evolutionary time, suggests that TCAP may have evolved to regulate such mechanisms.
Among other ancient signaling systems is the amino acid glutamate. As I demonstrated by my findings in Chapter 4, like TCAP-1, glutamate stimulates calcium signaling in astrocytes. Glutamate is thought to be an evolutionarily ancient molecule that has origins in the prebiotic earth as it has been found in meteorites (Lovejoy, 2005). Therefore, this amino acid transmitter was likely incorporated in the earliest cell and became ensconsed in the first neurological systems. Similarly, ligand-gated channel receptors, such as NMDAR and AMPAR that bind to glutamate, are postulated to be one of the oldest classes of receptors (Lovejoy, 2005) and they have been identified in the nematode, *C. elegans* and the invertebrate, *Drosophila melanogaster* (Maricq et al., 1995; Schuster et al., 1991). Given the ancestral origins of TCAP and glutamate, I was interested to determine whether TCAP-1 may be regulating glutamatergic systems. My findings demonstrated, in part, that the effects of TCAP-1 were mediated by the glutamatergic system. Therefore, given the ability of TCAP-1 and glutamate to stimulate calcium ion signaling in astrocytes and considering that TCAP, glutamate, calcium ion signaling and astrocytes are of ancient evolutionary origin, it is plausible that a TCAP-calcium-glutamate regulatory mechanism is retained in astrocytes.

5.4 Concluding Remarks

The results presented throughout my thesis have contributed to our understanding of TCAP as an ancestral peptide relative to other peptide systems, like CRF, and have examined its role in astrocytes in vitro, for the first time. Briefly, in Chapter 2 I analyzed newly identified CRF *Petromyzon marinus* sequences to contribute to the current model of CRF evolution, in order to use these sequences to better understand the relationship of TCAP to this peptide family. In Chapter 3, I identified relationships between CRF, calcitonin and secretin families, for the first time, using TCAP as a putative progenitor of the Secretin superfamily of peptides. Finally, in Chapter 4, I determined that TCAP-1 was inducing calcium signaling in astrocytes, providing evidence of the functional effects of the TCAP system in astrocytes in vitro for the first time. Therefore, my findings have laid the foundation to consider TCAP as an ancient peptide that plays a role in modulating calcium ion flux in astrocytes, which has never been examined before.
References


Qian, X., Barsyte-Lovejoy, D., Wang, L., Chewpoy, B., Gautam, N., Al Chawaf, A., Lovejoy, D.


## Appendix I: List of Accession Numbers

### CRF Family

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