Teneurin C-terminal associated peptide (TCAP)-1 mediates Gnadotropin releasing hormone (GnRH)–independent testosterone production in male mice: Evidence from in vivo and in vitro models.

by

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A thesis submitted in conformity with the requirements for the degree of

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Department of Cell and Systems Biology
University of Toronto

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ABSTRACT

Teneurin C-terminal associated peptide (TCAP) is encoded by the terminal exon of each of four teneurin genes, the established endogenous ligands of the adhesion G-protein coupled receptor (GPCR), ADGRL. Although little is known about the teneurin/TCAP-ADGRL complex activity in non-neural tissues, the combined application of in vivo and in vitro methods indicated that TCAP-1 has the ability to regulate male gonadal testosterone production. Using my in vivo data, TCAP-1 was shown to increase male mouse fecal testosterone concentrations. Furthermore, my in vitro studies utilizing immortalized mouse Leydig (TM3) and Sertoli (TM4) cells suggested that TCAP-1 activates these cells differentially. In the TM3 cells, a significant increase in testosterone synthesis was observed upon TCAP-1 stimulation, indicating that this mechanism may be independent of Gonadotropin releasing hormone (GnRH) and gonadotropin action. Additionally, using siRNA transient transfection, I showed that this mechanism might be dependent upon binding and activation of the ADGRL receptor.
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ABBREVIATIONS

3βHSD. 3β-hydroxysteroid dehydrogenase

17KSR. 17-ketosteroid reductase

ACTH. Adrenocorticotropic hormone

ADGRL. Adhesion GPCR Receptor Latrophilin

BDNF. Brain derived neurotrophic factor

BM. Basement membrane

BTB. Blood-testis barrier

CGA. Congenital general anosmia

cAMP. Cyclic adenosine monophosphate

CNS. Central nervous system

CRH. Corticotropin releasing hormone

CTF. C-terminal fragment

DAG. Diacylglycerol

DAPC. Dystropin-associated protein complex

DAPI. 4’,6-diamidino-2-phenylindole

DG. Dystroglycan

DHT. Dihydrotestosterone

ECD. Extracellular domain

ECM. Extracellular matrix

ELISA. Enzyme-linked immunosorbent assay

ER. Endoplasmic reticulum
FITC. Fluoresceinisothiocyanate

FSH. Follicle stimulating hormone

GH. Growth Hormone

Gn11. Immortalized GnRH –secreting cell line

GnRH. Gonadotropin-releasing hormone

GPCR. G-protein coupled receptor

GPHR. Glycoprotein hormone receptor

HBD. Hormone binding domain

HPA. Hypothalamic-pituitary-adrenal

HPG. Hypothalamic-pituitary-gonadal

ICD. Intracellular domain

IP_3. Inositol triphosphate

ITT. Intratesticular

KD. Knockdown

LH. Luteinizing hormone

LHR. LH receptor

LPHN. Latrophilin

MTT. 4,5-dimethylthiazol-2-yl

NGS. Normal goat serum

NTF. N-terminal fragment

PCR. Polymerase Chain Reaction

PFA. Paraformaldehyde

PKA. Protein kinase A
PKC. Protein kinase C

PLC. Phospholipase C

SC-TCAP-1. Scrambled teneurin C-terminal associated peptide

SSA. Splice site A

ST. Seminiferous tubules

StAR. Steroidogenic acute regulatory

TCAP. Teneurin C-terminal associated peptide

TM3. Immortalized Leydig cells

TM4. Immortalized Sertoli cells

TMR. Transmembrane region
Chapter 1

INTRODUCTION

The teneurins are a highly conserved family of transmembrane proteins that play a role in cell-cell adhesion and signaling (Drabikowski et al., 2005; Tucker et al., 2001; Tucker and Chiquet-Ehrismann, 2006;). These proteins are complex in structure, and contain an amino acid sequence in their carboxy terminus called teneurin C-terminal associated peptide (TCAP) that acts as an independent bioactive peptide (Chand et al., 2012). TCAP is highly conserved across the Metazoa, which indicates functional importance (Chand et al., 2013a). The teneurins are known to associate with and function, in part, redundantly to dystroglycan, an extracellular matrix protein that is linked to the cytoskeleton (Hara and Campbell, 2015; Trzebiatowska et al., 2008). Dystroglycan is a multifunctional scaffold capable of interacting with components of the mitogen-activated protein (MAP) kinase kinase 2 (MEK2) and extracellular signal-regulated kinase (ERK) cascade (Chand et al., 2012). The MAPKs have been heavily implicated in the regulation of steroid synthesis (Poderoso et al., 2009). Recent work now indicates that TCAP stimulates testosterone production in male mice (Chand et al., 2014). The established cognate receptor of the teneurin/TCAP protein, adhesion G-protein coupled receptor Latrophilin (ADGRL) is capable of activating the PLC-PKC signaling cascade (Lovejoy and Pavlovic, 2015; Woelfle et al., 2015). As teneurin, TCAP, dystroglycan and ADGRL are all expressed in the testes, together they may form an intercellular adhesion complex capable of downstream activation of the MEK/ERK1/2 signaling cascade, and further activation of the testosterone steroidogenic pathway. This thesis addresses the possibility using a mouse model and immortalized mouse Sertoli and Leydig cells.

1.1 Teneurin C-terminal Associated Peptide (TCAP)

1.1.1 Discovery

In the search for putative paralogous corticotropin releasing hormone (CRH)-like sequences, teneurin C-terminal associated peptide (TCAP), a novel family of neuropeptides translated from the final exon of the teneurin genes, was discovered (Qian et al., 2004). Using a hamster urocortin
probe, a rainbow trout hypothalamic cDNA library was screened and the partial sequence of a putative vertebrate teneurin-3 homologue was isolated (Qian et al., 2004). Alignment with mouse, zebrafish and human teneurin-3 orthologues and comparisons with the human teneurin-1 gene uncovered TCAP, a 40-41 residue carboxy-terminal sequence possibly distantly related to the CRH family of peptides, and located in the final 3’ exon of teneurin (Qian et al., 2004).

1.1.2 Structure
In vertebrates, four isoforms of TCAP (TCAP-1, -2, -3 and -4) have been identified and are found on the last exon of the four corresponding teneurin genes. In terms of amino acid sequence, the four human paralogues share 73-88% identity, whereas mouse paralogues possess 71-87% identity relative to TCAP-1 (Chand et al., 2013a). Mouse and rat TCAPs are identical, although the sequence similarity between human and mouse orthologues is 95-100% (Qian et al., 2004).

However, TCAP shares the closest resemblance to the CRH and calcitonin peptide families (Chand et al., 2013a; Qian et al., 2004). Furthermore, conserved cleavage motifs at the amino and carboxy termini of this peptide sequence suggest it is cleaved and acts as an endogenous bioactive peptide. Studies have already indicated that TCAP could function to regulate the synthesis of the teneurin gene (Qian et al., 2004).

1.1.3 Expression
Little is known about the mechanisms of TCAP expression. Currently, it is unclear if TCAP exerts its effects as part of teneurin, or if it has an independent role, either as a splice variant or post-translational proteolytic cleavage product of teneurin (Chand et al., 2013b). The endogenous release of TCAP-1 in neuronal tissue may be stimulus-dependent and results indicate an independent function from teneurin-1 (Chand et al. 2013a). However, it is still unclear how TCAP is released from the cell. One possibility is that independent TCAP transcripts (1 and 3) lacking signal sequences follow the non-classical protein export, or ER/Golgi-independent secretion of peptides whereby release into the extracellular space is achieved through cell injury/death or leakage, and the formation of exosomes in a process known as membrane blebbing (Chand et al., 2013b). The mechanism and components of this pathway are currently unknown (Chand et al., 2013b). Alternatively, the TCAP peptide sequence containing a signal sequence may be liberated
by proteolytic cleavage by vesicle or plasma membrane bound peptidases and then secreted into the extracellular matrix (ECM) (Chand et al., 2013b).

Upon its release, TCAP could carry out a further paracrine function via associations with extracellular matrix (ECM) proteins, such as β-dystroglycan (DG) (a known activator of the MEK/ERK pathway), on nearby cells thereby stimulating cellular signaling. Studies have already indicated TCAP-1 regulates cytoskeletal dynamics in hippocampal neurons by stimulating ERK1/2-dependent phosphorylation of stathmin at serine-25 and filamin A at serine 2152 (Chand et al., 2012). In addition, TCAP-1 induces actin polymerization, causes a corresponding increase in filopodia formation and mean filopodia length in cultured hippocampal cells. (Chand et al., 2012). This is novel evidence of a functionally linked teneurin and dystroglycan system.

1.1.4 Function

A number of biological functions both in vitro and in vivo have been attributed to the TCAP family of peptides. In vitro, TCAP-3 was found to have a dose-dependent effect on cAMP levels, teneurin-1 gene expression levels, and cell proliferation as measured by a MTT assay in Gn11 cells, immortalized, Gonadotropin releasing hormone (GnRH) expressing mouse neurons (Qian et al., 2004). GnRH may be present in most, if not all invertebrates and vertebrates (Belsham and Lovejoy, 2005). Developing GnRH neurons migrate into the forebrain from outside the brain, originating in the olfactory placode (Belsham and Lovejoy, 2005). Recent work has indicated a role of teneurins with olfactory dysfunction, similar to Kallman’s syndrome whereby GnRH and olfaction are compromised (Alkelai et al., 2016). This suggests that teneurins may play a role in the migration of the GnRH neurons and reproductive success. Further in vitro work established multiple other effects. TCAP-1 was neuroprotective in hypothalamic neurons subjected to alkalotic and oxygen free radical stress (Trubiani et al., 2007). In unstressed hypothalamic cells and primary hippocampal neurons, TCAP-1 treatment increases expression of α-actinin-4, β-actin and β-tubulin, induces neurite outgrowth, dendritic arborization, and axon fasciculation (Al Chawaf et al., 2007a). TCAP-1 also induces a significant decline in brain derived neurotrophic factor (BDNF) transcription and protein labeling in immortalized mouse hypothalamic neurons and could act as a novel BDNF inhibitory factor (Ng et al., 2012).
**In vivo**, TCAP-1 has emerged as a novel candidate in the integration and modulation of a number of psychiatric disorders including stress, anxiety and addiction. (Kupferschmidt et al., 2011; Rotzinger et al., 2010; Tan et al., 2011b). Most recently, it has been shown to increase circulating testosterone levels in male mice, possibly independently from the hypothalamic-pituitary-gonadal axis (Chand et al., 2014).

### 1.2 Teneurin

#### 1.2.1 Discovery and Nomenclature

The teneurins comprise one family of type II transmembrane proteins highly conserved across metazoans (Tucker and Chiquet-Ehrismann, 2006). Originally discovered as a new *Drosophila* gene by two independent groups looking to identify novel vertebrate tenascin orthologues (Baumgartner et al., 1994), and tyrosine phosphorylated proteins (Levine et al., 1994), the teneurins were first referred to as tenascin-major (ten-m) due to their structural similarity to tenascin, and Odd Oz (odz), which reflected its function as a pair-rule gene. The first vertebrate ten-m homologue was discovered in mice (Wang et al., 1998), and later was described in the chicken (Mieda et al., 1999; Rubin et al., 1999; Tucker et al., 2000), zebrafish (Mieda et al., 1999), mouse (Oohashi et al., 1999), rat (Otaki & Firestein, 1999), human (Minet et al., 1999; Minet & Chiquet-Ehrismann, 2000), and *Caenorhabditis elegans* (*C. elegans*) (Drabikowski et al., 2005). This gene family became known as the “teneurins”, suggesting their preferential expression in the developing and adult nervous system (Ben-Zur et al., 2000; Oohashi et al., 1999; Minet et Chiquet-Ehrismann, 2000; Trzebiatowska et al., 2008). Four homologues, teneurin-1 to -4, were identified in vertebrates, whereas one or two only are currently found in most invertebrates – Ten-a and Ten-m/Odz in *Drosophila* and Ten-1 in *C. elegans* (Drabikowski et al., 2005; Rubin et al., 1999; Lovejoy et al., 2006).

#### 1.2.2 Structure and Function

The teneurin gene encodes a large 300kDa plasma membrane protein with a single transmembrane domain. Approximately 2800 amino acids in length, it contains three domains: an N-terminal
intracellular domain (ICD); a single span transmembrane domain; and a large highly conserved C-terminal extracellular domain (ECD; Rubin et al., 1999; Tucker and Chiquet-Ehrismann, 2006; Tucker et al, 2007). The N-terminal intracellular domain (ICD) is a stretch 400-amino acids long, and is suggested to play a role in transcriptional regulation (Schöler et al., 2015). The C-terminal extracellular domain (ECD) is 2400 amino acids in length and is divided into three domains, the first containing eight tenascin-type EGF-like repeats, followed by a region of conserved cysteine residues, and ending with a third region containing a unique stretch of 26 tyrosine-aspartate (YD)-repeats (Fig. 1.1; Minet and Chiquet-Ehrismann, 2000; Young and Leamey, 2009). Because the second and fifth EGF-like repeat contain an odd number of cysteines, it has been postulated that the unpaired cysteine residues form disulfide bridges with an adjacent teneurin molecule leading to homo- or heterodimer formation (Oohashi et al., 1999). Correct protein folding has been attributed to the cysteine region, which is highly conserved throughout family members across all species (Tucker et al., 2007). Interestingly, among all eukaryotes, the 26 YD repeats are found only in the teneurins (Feng et al., 2002). Fundamental to development both in neural and non-neural tissues, the teneurins play a vital role in intercellular adhesion and communication, as well as pattern formation.

1.2.3 Expression and Processing
All four vertebrate orthologues are expressed in the brain in the cortex, thalamus, hippocampus and the cerebellum (Tucker and Chiquet-Ehrismann, 2006; Zhou et al., 2003). In the chicken embryo, both teneurin-1 and teneurin-2 are prominently expressed in non-overlapping populations of neurons in the developing visual system (Minet et al., 1999; Rubin et al., 1999). Interestingly, Ten-m/Odz is also expressed in the developing optic system of the fly (Tucker and Chiquet-Ehrismann, 2006). The mouse teneurins are prominently expressed during the development of the nervous system, primarily in the hippocampus (pyramidal layer dentate gyrus) and cerebellar Purkinje cell layer (Ben-Zur et al., 2000; Ooashi et al., 1999; Zhou et al., 2003).

Although prominent in the nervous system, teneurin expression has also been found in non-neuronal tissue. In Drosophila, Ten-m/Odz is expressed in alternating stripes in early embryos and
Figure 1.1 Structural organization of teneurin/TCAP proteins. At the intracellular amino terminus, there are two EF hand-like Ca2+ binding sequences and two proline-rich sites. The large extracellular domain contains 8 epidermal growth factor (EGF)-like repeats, a cysteine-rich region, and 26 tyrosine-aspartate acid repeats (Tucker and Chiquet-Ehrismann, 2006). TCAP, 40-41 amino acids long, is found at the carboxy terminus of teneurin and is 40-41 amino acids long (Chand et al., 2013a). Adapted from Woelfle et al. (2015).

is required for normal patterning, in tracheal precursor cardiac cells and muscle tissue attachment (Baumgartner et al., 1994; Levine et al., 1994; Tucker and Chiquet-Ehrismann, 2006). The expression of Caenorhabditis elegans Ten-1 has been found in hypodermal cells, precursor cells of the pharynx, gut, and gonad during embryonic development (Drabikowski et al., 2005). In vertebrates, the teneurins are also expressed during pattern formation and morphogenesis (Tucker
et al., 2007). This is seen most clearly in the developing chicken limb with teneurin-2 and teneurin-4 (Tucker et al., 2000; Tucker et al., 2001). Teneurin-1 expression was never observed in non-neuronal chicken tissue (Tucker and Chiquet-Ehrismann, 2006). Teneurin-3 has been found in the pharyngeal arches of the zebrafish (Mieda et al., 1999), whereas teneurin-2 and teneurin-3 are transiently expressed in the notochords of birds (Tucker et al., 2001) and zebrafish (Mieda et al., 1999), respectively. Teneurin-1 expression has only been detected, by Northern blotting in the adult mouse kidney and testes (Oohashi et al., 1999). Teneurin-3 is also present in adult mouse testes, although at a much lower level than in the brain (Ben-Zur et al., 2000; Ooashi et al., 1999).

Considerable progress has been made in recent years regarding teneurin processing, which has brought forward the idea that teneurins could act, in part, as transcription factor regulators (Bagutti et al., 2003; Tucker et al., 2007). ICD cleavage near (or possibly in) the transmembrane domain is suspected and leads to nuclear localization of the ICD. This has been demonstrated using luciferase activity with the N-terminus of teneurin-2. It has also been suggested that teneurin-teneurin interactions may control teneurin-mediated transcriptional regulation (Tucker et al., 2007).

1.3 ADGRL, a Cognate Receptor of Teneurin/TCAP

1.3.1 Receptor for Potent Black Widow Spider Toxin

Latrophilin, now classified as the adhesion G-protein-coupled receptor (GPCR) ‘ADGRL’ (Meza-Aguilar and Boucard, 2014), is the Ca\(^{2+}\)-independent receptor for \(\alpha\)-latrotoxin (LTX), the potent neurotoxin secreted by the Black Widow spider. \(\alpha\)-LTX is one of 86 different components of BWS venom (Knipper et al., 1986). Implicated in neurotransmitter release and morphogenesis, it is now known as the established receptor of the teneurins (Holz et al., 1998; Matsushita et al., 1999; Meza-Aguilar et Boucard, 2014; Silva et al., 2011; Woelfle et al., 2015). ADGRL was first isolated in 1996 as a result of extensive efforts of a number of laboratories trying to identify the functional receptor(s) of \(\alpha\)-LTX (Silva & Ushkaryov, 2010). It has now become one of the first members of the adhesion family of GPCRs to be identified, and an invaluable model for understanding this peculiar family of GPCRs (Silva & Ushkaryov, 2010).
1.3.2 Structure and Function

Adhesion (a)GPCRs are heptahelical receptors thought to mediate cell-cell and cell-matrix interactions resulting in different cell guidance signals (Silva et al., 2009). In addition to characteristically large extracellular N-terminal domains, which resemble cell-adhesion receptors, aGPCRs also contain a C-terminal heptahelical domain that may couple to G-proteins (Silva et al., 2009). The extracellular domain (approximately 1000 residues) is comprised of an N-terminal lectin-binding domain, an olfactomedin binding domain, a hormone-binding domain that is highly homologous to that of the otherwise unrelated CRH receptor, and a GAIN domain that includes the GPCR proteolysis site (Figure 1.2)(Boucard et al., 2012; Bouchard et al, 2014).

The latrophilins are a relatively unexplored group of proteins and are considered the first aGPCR for which a site of intramolecular post-translational cleavage was identified, most likely autocatalytic (Lin et al., 2004; Silva et al., 2009; Silva & Ushkaryov, 2010; Wei et al., 2007). After synthesis, proteolytic cleavage occurs between the cell-adhesion and GPCR domains upstream of the first transmembrane domain, producing an N-terminal fragment (NTF) and a C-terminal fragment (CTF), and is carried out in the cell (Figure 1.3). In fact, full-size, non-cleaved aGPCRs are not normally found in tissues (Davletov et al., 1996; Gray et al., 1996; Krasnoperov et al., 1997; Silva et al., 2011) and may never be delivered to the cell surface (Volynski et al., 2004). Interestingly, the NTF remains associated with the cell surface membrane despite the lack of a transmembrane region (TMR). This was initially thought to be a result of non-covalent bond formation between the NTF and CTF, however now the two fragments have been found to behave as independent cell surface proteins (Silva et al., 2009; Silva et al., 2011). Although upon binding to the agonist, the two fragments will re-associate and induce cellular signaling (Silva et al., 2009). This organization has led to challenges in understanding receptor-ligand binding mechanisms in this class of receptors (Silva et al., 2009). Furthermore complementary fragments from distinct aGPCRs can cross-interact, forming functionally active cross-complexes. This unusual cross-assembly of aGPCR fragments could couple cell-surface interactions to multiple signaling pathways (Silva et al., 2009). Although the ligands of the aGPCRs are relatively unknown, these findings point to the multifunctional aspects of the receptors and may reflect some of the attributes of teneurin and TCAP binding and activation.
Figure 1.2 Structure of the adhesion G-protein coupled receptor, Latrophilin (ADGRL). ADGRL structure contains a large N-terminal extracellular stalk (cell adhesion domain), containing the lectin binding domain (BD), the olfactomedin BD, the hormone BD, as well as the GPCR proteolysis site (GPS)(Boucard et al., 2012; Boucard et al., 2014). Seven transmembrane regions span the plasma membrane, followed by the intracellular C-terminus (GPCR domain). Adapted from Martinez et al. (2011).

All aGPCRs within the same family now have been shown to undergo proteolysis at a strictly conserved position (Silva and Ushkaryov, 2010). Furthermore, ADGRL has become the first receptor of the aGPCR group for which specific binding to G proteins, namely $G_0$ and $G_{q/11}$, was demonstrated (Silva et Ushkaryov, 2010). Therefore, it could be responsible for activating the phospholipase C (PLC) - protein kinase C (PKC) signaling cascade (Hiramatsu et al, 2010; Liu et al, 2005; Marinissen and Gutkind, 2001; Martinez et al, 2010). Most of the alternative splicing of ADGRL affects its cytoplasmic tail except for splice site A (SSA) in its N-terminal sequence (Boucard et al., 2012).

The three known homologous proteins that make up the latrophilin family, ADGRL 1, 2 and 3 have similar architecture and demonstrate differential tissue distribution, suggesting distinct
functions among them (Matsushita et al, 1999). ADGRL 1 is abundant in brain, present in endocrine cells and the only homologue that binds α-LTX; ADGRL 3 is also brain specific, whereas ADGRL 2 is ubiquitous (Matsushita et al, 1999). Furthermore, according to the gene expression bank neXtProt, both ADGRL 1 and 2, but not ADGRL3 have been found in the testes.

1.3.3 Evidence for ADGRL as Cognate Receptors of Teneurins and TCAPs

A striking sequence similarity exists between the ADGRLs and receptors of CRH, growth hormone-releasing factor as well as secretin and parathyroid hormone (Matsushita et al, 1999). The latter are grouped together under the secretin family of GPCRs. On the other hand, TCAP-like

![Diagram](image)

**Figure 1.3 ADGRL is cleaved into the N-terminal and C-terminal fragments.** After synthesis, proteolytic cleavage at the GPS site occurs between the cell adhesion domain and GPCR domain, producing an N-terminal fragment (NTF) and a C-terminal fragment (CTF). The full-sized GPCR is not normally found (Silva et al., 2011).
sequences are found in most metazoans and possess sequence similarity to a diverse range of hormones associated with the secretin GPCR family of ligands in vertebrates, and toxins found in both invertebrates and vertebrates (Woelfle et al., 2015). Particularly conserved is the hormone binding domain (HBD), originally characterized in CRH receptors (Perrin et al., 1998). Given the structural similarity of TCAP with the secretin and CRH family of ligands, this provides further evidence that TCAP is one of the cognate ligands of the ADGRLs.

TCAP and teneurins are found in all tissues that express the ADGRLs (Arcos-Burgos et al., 2010; Silva et al., 2011). Furthermore, the highest sequence conservation in ADGRL proteins is found in the lectin-binding domain, suggesting that this region is important for receptor function (Matsushita et al., 1999). Teneurins bind to the lectin-binding domain at very high physiologically compatible affinities (Boucard et al., 2012; Silva et al., 2011). Therefore, it is highly plausible that teneurin/TCAP with ADGRL is an ancient and important association situated at the cellular surface (Figure 1.4), possibly even at the tunica propria in the testes, which could be regulating cell signaling cascades via the activation of downstream GPCR signaling cascades (Boucard et al., 2014; Matsushita et al., 1999; Meza-Aguilar & Boucard, 2014; Silva et al., 2011). It is important to note that the teneurin-ADGRL association is the only synaptic intermolecular pair that has been conserved between invertebrates and vertebrates.
1.4 Additional Proteins in the Teneurin/TCAP-ADGRL Intercellular Adhesion Complex

Dystroglycan, an extracellular matrix (ECM) protein, was first isolated from and characterized in skeletal muscle (Henry et al, 1998). As a core component of the dystropin-associated protein complex (DAPC), dystroglycan plays a vital role in maintaining muscle integrity, and prevents muscle dystrophy (Chamberlain, 1999; Spence et al., 2004). Expression of dystroglycan has also been found in the brain, retina, epithelial cells, as well as early embryonic tissue (Durbeej et al, 1998). It is proteolytically cleaved into two subunits, the extracellular α-dystroglycan, non-covalently associated with the transmembrane β-dystroglycan. β-dystroglycan serves as a scaffold for the mitogen-activated protein (MAP) kinase kinase 2 (MEK2) and extracellular signal-regulated kinase (ERK) signaling cascade which is involved in a vast number of cellular processes (Spence et al., 2004). What is very interesting is that dystroglycan and teneurin-1 have been shown to function redundantly in maintaining basement membrane integrity in the gonad of *C. elegans*. 

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Figure 1.4 Teneurin/TCAP-ADGRL intercellular adhesion complex. Teneurin is bound to the lectin and olfactomedin binding domains on the ADGRL extracellular stalk (Silva et al., 2011). The teneurins additionally are associated with the dystroglycans (DG), specifically the α-DG subunit, while TCAP is known to bind to the β-DG subunit as well as the hormone-binding domain on ADGRL.
(Trzebiatowska et al, 2008). Therefore, both dystroglycan and teneurin may possess a functional role in the mammalian testes, and quite possibly the mammalian ovaries as well.

Although very little is known about the relationship between the teneurins, TCAP and ADGRL in non-neuronal tissue, more research is currently underway in reproductive tissue. There is now increasing evidence that an intercellular adhesion complex comprised of the Teneurin/TCAP-ADGRL complex is situated in the mammalian testes, particularly in the male somatic cells that regulate cellular activity vital for fertility (Figure 1.4).

1.5 The Mammalian Testis
The testis is home to a dynamic and intricate network of cell-cell interactions that occur via hormones, paracrine factors and signaling molecules (Loss et al., 2007). It comprises a cellular network that includes the germ cells, Sertoli cells, Leydig cells, and peritubular cells. The two major functions of the testis are the production of: i) spermatozoa via spermatogenesis, and ii) testosterone via steroidogenesis. Both are regulated by the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) respectively.

Spermatogenesis takes place in the Sertoli cells within the seminiferous tubules (ST) whereas steroidogenesis takes place in the Leydig cells, located in the interstitial space (Sie & Cheng, 2004). Each ST is composed of the seminiferous epithelium and the seminiferous lumen at the centre, where mature spermatozoa detach from the epithelium, in the process known as spermiation (Griswold, 1998). The seminiferous epithelium of the testis is one of the most complex tissues and is strategically situated to receive, integrate and emit all signals required for spermatogenesis (Loss et al., 2007).

Considered the nurse cells, Sertoli cells provide a specialized, protected environment for developing germ cells, and can support as many as 50 germ cells at a time in the seminiferous epithelium. The number of Sertoli cells ultimately determines testicular size, germ cell numbers per testis and spermatozoa output (Orth et al., 1988; Walker & Cheng, 2005). Due to stimulation by FSH, Sertoli cells are a constant source and supply of growth factors and nutrients for germ cells (Griswold, 1998). They also secrete androgen-binding protein that concentrates intratesticular
testosterone to supraphysiological levels required for spermatogenesis (50-100 fold higher than serum levels) (Walker & Cheng, 2005).

Between the ST lies the interstitium that is filled with blood vessels and home to clusters of androgen-producing Leydig cells (Siu & Cheng, 2004). These two compartments are separated by the blood-testis barrier, which restricts passage of many factors from the interstitium into the ST (Saez et al., 1989). The Leydig cells were first discovered in 1850 by the German physician Franz Leydig, who is now considered a pioneer of, and responsible for helping to lay down the foundations of histology (Christensen, 2007). Many years of speculation followed regarding their function, and direct evidence of their endocrine function did not emerge until the 1950s (Christensen, 2007).

Testicular steroidogenesis is now known to have two active phases during development. In fetal and neonatal life the Leydig cells actively produce androgens for proper masculinization of the primary sex characteristics (Dufau, 1998; Rahman and Huhtaniemi, 2004). Cells then become quiescent until puberty, followed by the emergence of the adult Leydig cell population and the second active phase of testicular steroidogenesis, which is responsible for pubertal masculinization and Leydig cell function in adult age (Dufau, 1988; Rahman and Huhtaniemi, 2004). A unique aspect of fetal Leydig cell function is that it is pituitary independent (O’Shaughnessy et al., 1998). In vivo studies using hpg mice and mice with disruption of the common pituitary alpha (α)-subunit show a normal male phenotype at birth, which demonstrated that the fetal testis must release androgens in the absence of circulating gonadotropins (O’Shaughnessy et al., 1998). It remains to be shown whether Leydig cells are constitutively active during this time or whether paracrine factors serve to regulate androgen production. After birth however, gonadotropins become an essential component of the maintenance of normal testicular androgen levels, where Leydig cell control shifts to the pituitary (O’Shaughnessy et al., 1998).

The absolute dependence of Sertoli cells on Leydig cell function is now a well-established fact. Without testosterone, spermatogenesis fails. However, the concept that Sertoli cells may in turn also be regulating Leydig cell function is more recent (Saez et al., 1989). Increasing evidence indicates that one or more factors secreted by the Sertoli cells may modulate Leydig cell androgenic function, including transforming growth factor (TGF)-β and insulin-like growth factor.
(IGF)-I (Reventos et al., 1989; Saez et al., 1989). These factors could play a key role in the expression and maintenance of steroidogenic cell function (Saez et al., 1989). Therefore, the combined effort of both cell types is responsible for the steroidogenic responsiveness and activity of the steroidogenic pathway in Leydig cells (Reventos et al., 1989; Saez et al., 1989; Siu and Cheng, 2004). Cell lines have now been created to better understand specific aspects of Sertoli and Leydig cell function, including the regulation of gap junctions in the testis (Goldenberg et al., 2003; Pointis et al., 2005; Tarulli et al., 2008), lipid metabolism (Selva et al., 2004), the regulation of androgen/FSH receptor cascades that modulate Sertoli cell function and spermatogenesis (Zhang et al., 2012), as well as toxicity studies to improve the current understanding of the pathogenesis of disease (Ema et al., 2010; Singh et al., 2009).

1.6 Sertoli and Leydig Cell Culture

Cell cultures are indispensable in many types of endocrine research, such as signal transduction of cell membrane receptors, and have played an important role in furthering understanding of most aspects of hormone biosynthesis, action and target cell responses (Ascoli, 1981a,b; Dilworth, 1990; Rahman and Huhtaniemi, 2004). The testis is a complex organ consisting of several distinct cell types including the Sertoli and Leydig cells, which each have their own hormone requirements and responses (Mather, 1980). An in depth understanding of each of these cell types is difficult to achieve, without being able to study each cell type individually (Mather, 1980). Therefore, in order to understand certain aspects of testes function, different cell lines have been developed in order to study closer the physiology of Leydig and Sertoli cells. However, a major challenge in cell line development is to maintain as closely as possible normal functional properties upon prolonged culture (Rahman and Huhtaniemi, 2004). There are numerous origins of cell lines, such as: normal tissue (where occasional cells survive in culture and continue to grow); cells becoming spontaneously transformed; cells originating from spontaneous tumours; cells arising from fusion of normal cells with transformed cells; or via the introduction of foreign DNA into cells either \textit{in vivo} (transgenesis), or \textit{in vitro} (transfection) (Rahman and Huhtaniemi, 2004).

The use of primary Sertoli cell cultures has been vital for establishing their role in protein secretion and responsiveness to FSH stimulation (Skinner and Griswold, 1982; Dorrington and
Armstrong, 1979; Rahman and Huhtaniemi, 2004). However, the ability to respond to hormonal stimulation is diminished as number of days in culture increases, so the demand for the establishment of a well characterized Sertoli cell line was heightened in order to better understand its physiological role in germ cell proliferation and differentiation. Currently, cell lines have been created either via spontaneous immortalization, such as the TM4s (epithelial nontumourigenic), or from transfected somatic cell lines or targeted testicular tumours (Rahman and Huhtaniemi, 2004).

Regarding the Leydig cells, currently no immortalized fetal Leydig cell line exists. The creation of such a cell line would be useful for studying the unique and not-entirely understood features of this cell type, including their resistance to LH-induced LH receptor (R) downregulation and blockage of steroidogenesis (desensitization) (Rahman and Huhtaniemi, 2004). This is one of the most prominent differences between the fetal and adult Leydig cells (Rahman and Huhtaniemi, 2004). However, a number of adult Leydig cell lines have been created which have been vital for understanding cellular response to hormone stimulation and signalling cascades (Rahman and Huhtaniemi, 2003). One commonly used cell line, the TM3s (epithelial nontumourigenic) have also been established from spontaneous immortalization (Rahman and Huhtaniemi, 2004).

Both the TM3 and TM4 immortalized Leydig and Sertoli cell lines are produced using the same protocol, are epithelial in appearance and form contact-inhibited monolayers at confluence, but differ in appearance (Mather, 1980). TM3s have the larger surface area and tend to grow in connected ropes (Figure 1.5) rather than spreading out over the plate, such as the TM4s (Figure 1.6). Both lines grow with a doubling time of approximately 16 hours in stock medium (Mather, 1980). In serum-free media, various hormones including insulin, transferrin, epidermal growth factor (EGF), growth hormone (GH) and FSH stimulate TM4 growth. No response is observed for LH stimulation (Mather, 1980). The TM3s are also responsive to insulin, transferrin and EGF, as well as LH, but show no response to FSH (Mather, 1980; Mather et al., 1982).
Figure 1.5 TM3 clonal cell line after 2 years in culture. The cells were photographed 4 days after subculturing into medium supplemented with A) 5% fetal calf serum, or B) insulin, transferrin, and epidermal growth factor. Magnification 110X. (Mather, 1980).

Figure 1.6 TM4 clonal cell line after 2 years in culture. The cells were photographed 4 days after subculturing into medium supplemented with A) 5% fetal calf serum, or B) insulin, transferrin, epidermal growth factor, follicle stimulating hormone (FSH), and growth hormone (GH). Magnification 110X. (Mather, 1980).
1.7 The Hypothalamic-Pituitary-Gonadal (HPG) Axis and Steroidogenesis

In males and females, production of sex steroids is key to maintaining a functional reproduction system. The hypothalamic-pituitary-gonadal (HPG) axis is responsible for the control of hormone synthesis essential for proper progression through the different reproductive stages of life. Each one of the three main levels of the HPG axis results in unique hormone secretion, which regulate one another by using established feedback mechanisms (Maffucci and Gore, 2009; Pinilla et al, 2012). Beginning with the release of the decapeptide Gonadotropin-releasing hormone (GnRH) from neurons in the hypothalamus, GnRH travels to the anterior pituitary gland, via the portal capillary system, operating as the final output signal for hypothalamic regulation of the downstream elements of the HPG axis and master hormone regulator of reproduction (Pinilla et al, 2012; Tsutsumi et Webster, 2009). Here, it binds to receptors on the membrane surface of the gonadotropes, cell types responsible for secretion of two key gonadotropins and members of the glycoprotein hormone family, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH exert their effects on male and female gonads – the testes and ovaries – by binding and activating their respective receptors, thereby promoting the release of sex steroids (androgens, estrogens and progestins). These hormones play an essential role in maintenance of the reproductive cycle, by regulating vital gonadal functions including spermatogenesis, folliculogenesis and ovulation (Maffucci and Gore, 2009; Tsutsumi and Webster, 2009).

Sex steroids are hormones that interact with vertebrate androgen and estrogen receptors. In males, testosterone is the principle sex hormone essential for male sexual differentiation, maintenance of spermatogenesis and expression of male sexual characteristics (Payne and Youngblood, 1995). Production is carried out in the Leydig cells of the testes, located in the interstitial space, which are dependent on cell surface receptor stimulation by LH (Payne and Youngblood, 1995). The LH receptor (LHR) is a member of the GPCR superfamily, and belongs to the glycoprotein hormone receptor (GPHR) cluster within the rhodopsin family (Puett et al, 2007).

Optimal expression of the steroidogenic enzymes necessary for biosynthesis of testosterone from free cholesterol requires activation of adenylate cyclase, followed by increased production of intracellular cAMP (Payne and Youngblood, 1995). Both chronic and acute effects of LH (as well as FSH) are mediated by increases in cAMP, which activates expression of steroidogenic acute
regulatory (StAR) protein (Figure 1.7)(Evaul and Hammes, 2008; Payne and Youngblood, 1995). Considered to be the rate-limiting step during hormone production, the transport of free cholesterol across the mitochondrial membrane via StAR protein is one mechanism that is still not entirely clear (Evaul and Hammes, 2008; Payne and Youngblood, 1995).

Once in the mitochondria, C27 (free) cholesterol is converted to the C21 steroid, pregnenolone, in a step catalyzed by mitochondrial cholesterol sidechain cleavage cytochrome P450 (P450_{scc}) (Payne and Youngblood, 1995; Young et al, 2010). Next, pregnenolone diffuses back across the mitochondrial membrane into the cytosol, where it enters the testosterone synthesis pathway within the endoplasmic reticulum (ER), that is comprised of the enzymes: 3β-hydroxysteroid dehydrogenase (3βHSD), 17α-hydroxylase, C 17-20 lyase, and 17-ketosteroid reductase (17KSR), respectively (Payne and Youngblood, 1995; Young et al, 2010). The products of each step include: progesterone, 17α-hydroxyprogesterone, and androstenedione (the immediate precursor to testosterone), respectively. An additional and irreversible step is the aromatization of testosterone to 17β-estradiol via cytochrome P450arom (Carraeu et al, 2006; Payne and Youngblood, 1995). The principle sites of aromatase expression in premenopausal women are the ovarian Granulosa cells, and Leydig cells in men, while activity in Sertoli cells is still being debated (Carreau, 2001; Carreau et al, 2002; Nelson and Serdar, 2001).

The classical method by which androgens and other steroid hormones exert their effects begins with hormone diffusion through the plasma membrane and into the target cell. Steroid hormones bind with high affinity to their specific intracellular receptor present in the cytoplasm and/or nucleus (Tsai and O’Malley, 2004; Walker and Cheng, 2005). Upon steroid-receptor binding, a conformational change is triggered that results in the formation of a “transformed” or activated receptor that has high affinity for specific DNA-binding sites (Tsai and O’Malley, 2004). This newly formed ligand-receptor complex acts as a ligand-inducible transcription factor that is able to recruit co-activator proteins and stimulate gene transcription (Bagchi et al., 1992). Currently however, it is unclear how testosterone acts via gene regulatory mechanisms to support spermatogenesis (Walker and Cheng, 2005).

The MEK/ERK pathway has recently been implicated in steroidogenic gene expression and has been shown to play a key role in male and female fertility (Matzkin et al, 2013; Yamashita et al,
2011). Upon activation of the LHR, stimulation of this pathway ensues, but exact individual protein contribution is still unknown. In males, MEK/ERK knockout mouse models have been shown to develop Leydig cell hypoplasia, hypergonadotropic hypogonadism, as well as a loss in fertility with a decrease in testosterone production, even in the added presence of pregnenolone or androstenedione (Matzkin et al, 2013; Yamashita et al, 2011). Although the cause is still unclear, a decrease in testosterone levels may be attributed to a decrease in availability of free cholesterol and/or transport into the mitochondria, due to a possible inactivation of the StAR protein; gene expression levels of other proteins have yet to be analyzed (Matzkin et al, 2013).

Figure 1.7 Leydig cell steroidogenic pathway. LH receptor activity promotes intracellular cAMP-mediated increase in StAR protein expression, and the translocation of free cholesterol into the mitochondria. Here cholesterol is converted and transported back into the cytosol as Pregnenolone. In the ER, due to the action of a series of enzymes, testosterone is produced and shunted out of the ER, for either further conversion into Estradiol or tissue-stimulation (Evaul and Hammes, 2008; Payne and Youngblood, 1995; Young et al, 2010). Adapted from Payne and Youngblood (1995).
1.8 Role of Teneurins and TCAPs in Reproductive Physiology, and Evolutionary Significance

Besides prominent expression in the nervous system, teneurin has also been found in non-neuronal tissues, mainly at sites of pattern formation and cell migration (Drabikowski et al., 2005; Oohashi et al., 1999; Trzebiatowska et al., 2008; Tucker et al., 2007; Zhou et al., 2003). Invertebrate studies with C. elegans indicate that the ten-1 gene is essential for several aspects of cell adhesion, integrity, and morphogenesis. The gonads of ten-1 mutants were observed to burst early in development, resulting in germ cell-filled body cavities, and did not contain a properly maintained basement membrane (Drabikowski et al., 2005; Trzebiatowska et al., 2008). Some worms also developed tumours, as well as azoospermia and many ovulation defects (Drabikowski et al., 2005).

In vertebrate species, mouse teneurin-1 has similarly been observed in the testes (Oohashi et al., 1999; Chand et al., 2013). However, the presence of teneurin-2, -3, or -4 in vertebrate reproductive tissue has been undetermined (Ben-Zur et al., 2000; Drabikowski et al., 2005; Kenzelmann et al., 2008, Leamey et al., 2007, Leamey et al., 2008, Li et al., 2006, Lossie et al., 2005, Mieda et al., 1999, Minet et al., 1999, Oohashi et al., 1999, Rubin et al., 1999, Tucker et al., 2000, Tucker et al., 2001, Zhou et al., 2003).

In vitro studies have revealed the presence of both teneurins and TCAP in reproductive tissue, and have provided further evidence for independent function. In the adult mouse testis, immunoreactive studies using TCAP-specific antisera indicated that TCAP-1 was localized to spermatogonia and primary spermatocytes, and specifically co-localized with β-dystroglycan (Chand et al., 2014). In contrast, teneurin-1 was localized to the peritubular myoid cell layer of seminiferous tubules and tubules within the epididymis, and co-localized with α-DG and α-smooth muscle actin. TCAP-1 binding sites were identified in the germ cell layers and spermatid regions of the seminiferous tubules, and epithelial cells of the epididymis (Chand et al., 2014). This is novel evidence of TCAP-1 localization to the testes independent of teneurin-1, but integrated through an association with the dystroglycan complex.

In female mouse reproductive tissue, sites of TCAP-1-immunoreactivity were observed in ovarian surface epithelia, the cytosol of the oocyte and granulosa cells, as well as the columnar epithelial cells of the fallopian tubes. There have been no reports of neither teneurin-1 expression in the ovary (providing further evidence for TCAP’s independent role in cell adhesion), nor theca
cell TCAP expression (Chand and Lovejoy; unpublished data). FITC-TCAP-1 binding sites were identified in these same regions; only binding appeared strongest in the region of the granulosa cell closest to the oocyte (Chand and Lovejoy; unpublished data). As the oocyte and granulosa cells are connected by cytoplasmic bridges that rely heavily on the modulation of actin and tubulin-based cytoskeletal elements, along with the fact that TCAP has already been shown to contribute to microtubule dynamics and organization via interaction with dystroglycan and the MEK/ERK pathway, it is plausible that TCAP is essential for cell signaling cascades responsible for communication between these two cell types (Chand et al., 2012; Gutzeit, 1986). If TCAP does indeed drive microtubule organization, then it may be essential for microtubular activity that plays a vital role in nuclear polarization of the developing oocyte during meiosis, that is essential for function (Picton, 2001).

*In vivo*, repeated TCAP-1 administration to adult mice resulted in increased testicular size, seminiferous and epididymis tubule short-diameter and elevated testosterone levels (Chand et al., 2014). In addition, increased TCAP-1 immunoreactivity in the caput and corpa epididymis was observed, as well as a significant increase in testosterone production in mice treated with TCAP-1 (Chand et al, 2014).

GnRH, the master regulator of reproduction, is an example of an evolutionary conserved and fundamental gene (Belsham and Lovejoy, 2005). It is one of the phylogenetically oldest peptides known and is present in most invertebrates and vertebrates. Two genes (possibly 3 in some species) are found in mammals, GnRH-I and GnRH-II, where GnRH-II has been conserved for over 400 millions years (Belsham and Lovejoy, 2005). GnRH appears to be the only releasing factor that migrates into the forebrain from outside the CNS. Immunoreactivity studies indicate that GnRH neurons originate in the olfactory placode (Quanbeck et al., 1997). Interestingly, current work has indicated an overlap in immunoreactive teneurin-1 and GnRH neuron expression in the rat telencephalon (Chand and Lovejoy, unpublished work). GnRH also shares similar features with the Teneurins. The two GnRH genes found in mammals encode a signal peptide (Belsham and Lovejoy, 2005). The migration of the GnRH from the nasal placode to the rostral forebrain requires many delicate cues and signals ensuring the cells reach their final destination (Belsham and Lovejoy, 2005).
Studies now show that the teneurins may have a role to play in olfaction (Alkelai et al., 2016). The neurological disorder congenital general anosmia (CGA) has recently been linked to a rare X-linked missense mutation in the teneurin-1 gene (Alkelai et al., 2016). CGA often appears in conjunction with Kallman’s syndrome, characterized by the failure of onset of puberty due to abnormal migration of olfaction neurons, including GnRH (Rugarli et al., 1993). For the last decade, research has indicated that the gene deleted in this syndrome shares homology with neural cell adhesion and axonal path-finding molecules (Franco et al., 1991; Legouis et al., 1991). This indicates that the teneurins/TCAP system is an evolutionary conserved system that may have a functional role to play in reproduction and survival.

Reproductive capacity is critical to the survival of the species. It is a highly energetically costly function, and is therefore highly sensitive to disturbances in physiological homeostasis governing the optimal conditions for reproductive success (Belsham and Lovejoy, 2005). The stress-response axis – the hypothalamic-pituitary-adrenal (HPA) axis – is similar to the reproductive axis. Corticotropin releasing hormone (CRH) is responsible for control of this axis, mirroring GnRH function in the HPG axis. From the pituitary, CRH regulates the secretion of adrenocorticotropic hormone (ACTH) into the system blood stream, which goes on to stimulate the release of glucocorticoids from the adrenal gland (Lovejoy and Barsyte-Lovejoy, 2013).

The evolution of the organismal stress-response and fertility are considered two of the most important aspects driving reproductive success of a species (Lovejoy and Barsyte-Lovejoy, 2013). Recent studies indicate that both GnRH and CRH are each composed of at least two functionally discrete paralogous lineages. In phylogenetically older species, representatives of both GnRH and CRH family lineages have been characterized. Therefore, there is strong structural and functional conservation in these peptide systems in vertebrates (Lovejoy, 1996). Interestingly, both CRH and GnRH-1 are closely anatomically situated in the forebrain of vertebrates.

The teneurin C-terminal associated peptide (TCAP) peptide sequence remains one of the most highly conserved in the Metazoa. It shares the closest resemblance to the CRH family of peptides than with any other known peptide sequences (Chand et al., 2013; Qian et al., 2004). Further, mRNA expression of TCAP is prominent in limbic regions that regulate the stress response and has been shown to have neuroprotective properties. Additionally, TCAP-1 expression has been located
in the testes and it has now been shown to increase steroidogenesis, possibly via adhesion GPCR signaling. It is thought to bind to the hormone binding domain on its cognate receptor (ADGRL), which is a highly conserved region that was originally characterized in CRH receptors. In summary, the TCAP and teneurin system is a phylogenetically old system that appears to play a significant role in the regulation of stress and reproduction.

There is currently an abundance of evidence suggesting that Teneurin/TCAP, the ADGRLs and Dystroglycans form an intercellular adhesion complex that modulates cellular activity. TCAP-1 and teneurin-1 are known to associate and independently bind to dystroglycan and ADGRL. Further, the inverse dose-dependent effect of TCAP on cAMP levels shown previously (Qian et al., 2004) suggests that the TCAP receptor belongs to the G-protein coupled family, consistent with the other peptides it is structurally related to. Moreover, TCAP could be operating on two different selective receptors; a high affinity receptor may act to stimulate cAMP at low concentrations, whereas a lower affinity receptor could act to inhibit cAMP at higher concentrations of the peptides. ADGRLs have three homologous forms in vertebrates. However, due to autoproteolytic cleavage, many more isoforms could exist, with potentially different G-protein coupled reactions, which could therefore contribute to a variation in ligand-receptor kinetics.

Circumstances of LH-independent testosterone production have been demonstrated. In LH receptor knockout mice, spermatogenesis has been shown to complete up to the elongating spermatid phase of development (Zhang et al, 2003). As TCAP is known to stimulate testosterone production in male mice, there is sufficient evidence to indicate that TCAP-1 activates a gonadotropin-independent steroidogenic pathway that activates MEK/ERK signaling cascades and downstream steroidogenesis. This pathway can be theoretically activated by both dystroglycan via MEK and ADGRL by PKC, so together, teneurin/TCAP-ADGRL may form an intercellular adhesion complex responsible for activating cell signaling pathways, specifically the MEK/ERK and PLK-PKC pathways (Figure 1.8).
Figure 1.8 The teneurin/TCAP-ADGRL complex activates MEK/ERK signaling. The teneurin/TCAP-ADGRL intercellular adhesion complex is capable of activating the MEK/ERK pathway via the dystroglycans or ADGRL GPCR downstream secondary messengers, diacylglycerol (DAG) and inositol triphosphate (IP3) that are known to activate the MEK/ERK1/2 cascade of signaling events.

1.9 Objectives and Hypothesis

Given that a number of studies indicate a role of teneurin/TCAP and ADGRL in reproduction, I hypothesize that TCAP-1 activates steroidogenesis independently of the HPG axis by stimulating G-protein signaling cascades upstream of the MEK/ERK signaling pathway.

This hypothesis was tested by completing the following four objectives:

1) Validate *in vivo* effects of TCAP-1 on testosterone and luteinizing production, key hormones regulating male fertility
This animal study will serve to strengthen the current evidence of a steroidogenic effect of TCAP-1 in the mammalian gonad. It will also provide further insight into TCAP-1’s effect on the HPG-axis, as it is not understood if the observed increase in testosterone is due to stimulation of the gonads or the HPG axis.

2) Establish the presence of teneurin/TCAP and ADGRL in male gonadal somatic cells, the immortalized Leydig (TM3) and immortalized Sertoli (TM4) cells

As little is known about these proteins and their interactions in the mammalian testes, in order to prove that the teneurin/TCAP-ADGRL intercellular complex is responsible for the observed effects of TCAP in the mouse testes, it will be vital to first show that all three players do exist in male somatic cells, the TM3s and TM4s. These cells together regulate steroidogenesis in the male reproductive system. Further, these studies will help develop the TM3 and TM4 cell lines as an in vitro model for TCAP-1 action on the testes.

3) Provide effects of TCAP-1 on testosterone production and the ADGRL signaling pathway in vitro

By measuring testosterone levels secreted by immortalized Leydig cells (TM3s) post TCAP-1 treatment, this will further corroborate the in vivo findings. An increase in testosterone will also indicate that TCAP-1 is working independently from GnRH and the HPG axis. Therefore, this indicates a novel route for steroidogenesis. Also, by assaying for the downstream messengers of the ADGRL signaling pathway, including DAG and IP3, in TCAP-1 treated cells, this will provide evidence of TCAP and ADGRL interaction. Further, I can use the two cell systems to identify discrete differences of TCAP-1 action on these cells.

4) Confirm adhesion GPCR signaling is a vital component of the TCAP-1-mediated increase in steroidogenesis
By knocking down ADGRL in the TCAP-1-treated immortalized Leydig cells (TM3s), and analyzing for downstream messengers, this will further clarify the role of adhesion GPCR signaling in TCAP activity, specifically relating to steroidogenesis.
Chapter 2

MATERIALS AND METHODS

2.1 In Vivo Studies: Testosterone Modulation in Adult Male Mice

2.1.1 Animals
All procedures were approved by the local animal care committee and were in accordance with the Canadian Council on Animal Care. Adult male BALB/c mice (n=40, Charles River Laboratories, Montreal, Quebec, Canada) were housed in groups of four in 27L x 16W x 16H cm shoebox cages in a controlled and enriched environment with a 12 h light/dark cycle at a constant temperature of 21°C. Mice were provided with standard mouse chow (LabDiet, Ren’s Feed and Supply Ltd., Aberfoyle, ON) and water ad libitum and allowed to acclimatize to laboratory conditions for two weeks before injection. Following the acclimatization period, mice entered an 8-day study period. Upon completion of the study, the animal care staff euthanized animals with CO₂ followed by cervical dislocation.

2.1.2 Subcutaneous Injections
Lyophilized synthetic TCAP-1 peptide was solubilized in sterile saline (HouseBrand, University of Toronto Medstore) to a stock concentration of 2 x 10⁻⁶ M. To help increase solubility, pH was lowered by adding ammonium hydroxide vapour. 100μL synthetic TCAP-1 was administered at 1200 h, via subcutaneous injection (26G needle) at 25pmoles/kg (n=10) or 250pmoles/kg (n=10), on every odd day. Groups not receiving TCAP either received 100μL Scrambled TCAP (a non-functional control; n=10) (250pmoles/kg) or saline (n=10).

2.1.3 Blood Collection
Blood samples (100μL whole blood) were collected once from the saphenous vein at 0900 h on Days 1 (baseline conditions), 2, 4, 6 and 8 by an animal care staff member. A total of 20 mL of whole blood was collected, 500μL from each mouse. The blood collected was then centrifuged and serum was collected for steroid and gonadotropin assays.
2.1.4 Serum Analysis

2.1.4.1 Testosterone Assay

Serum levels of testosterone were measured by a solid-phase ELISA kit according to the manufacturer’s instructions (MyBioSource, San Diego, California, U.S.A.). All kit reagents were prepared and brought to room temperature along with serum (detection range 25 – 0.1 ng/mL). 10µL of standard and sample were added to the appropriate wells (pre-coated with testosterone antiserum) in the microELISA strip-plate provided. 100µL of Incubation buffer was added to each well. Next, 50µL enzyme conjugate was dispensed into each well.

After 1 hr incubation at 37°C, contents of the wells were discarded. Manual washing was repeated four times, using 300µL (1X) wash solution per well. 200µL of substrate solution was added to each well after blot drying the plate. The plate was then left to incubate at room temperature (21-25°C) in the dark for 30 minutes. 50µL of Stop solution was added to each well to stop the reaction.

Using spectrophotometry, absorbance was then measured at 450 nm in a microplate reader. A standard curve was plotted relating optical density to the concentration of standards and testosterone concentrations were then interpolated from the standard curve.

2.1.4.2 Luteinizing Hormone (LH) Assay

Serum LH levels were measured by a competitive ELISA kit according to the manufacturer’s instructions (MyBioSource, San Diego, California, U.S.A.). All kit reagents were prepared and brought to room temperature (21-25°C) along with serum (detection range 100 - 0 ng/mL). 100µL of standard and sample were added to the appropriate wells (pre-coated with anti-LH antibody) in the microelisa stripplate provided. Standards were run in duplicate. 100µL (1X) PBS was added to two blank control wells. 50µL of enzyme conjugate was then dispensed into each well, excluding the blank controls.

After 1 hr incubation at 37°C, contents of the wells were discarded. Manual washing was repeated five times, using 300µL (1X) Wash solution per well. 50µL of Substrate A and Substrate B were added to each well successively, including the blank controls, after blot drying the plate. The plate was then left to incubate at 37°C for 15 minutes. 50µL of Stop solution was added to
each well in order to stop the reaction.

Using spectrophotometry, absorbance was then measured at 450 nm in a microplate reader. A standard curve was plotted relating optical density to the concentration of standards and testosterone concentrations were then interpolated from the standard curve.

2.1.5 Fecal Collection

Fecal pellets were collected twice daily (8am/5pm) from cages only containing mice from a single treatment group (out of the ten cages, cages 3 and 8 contained mice from two different treatment groups as 4 is not a factor of 10), and were used for steroid quantification.

2.1.6 Fecal Analysis

2.1.6.1 Steroid Extraction

Fecal samples were oven-dried and large particulate matter was removed. A minimum of 0.2 g of crushed dried fecal matter (per sample) was placed in a 15 mL falcon tube and 1 mL of ethanol for every 0.1 g of solid was added. Tubes were placed on a shaker at high power for 30 minutes. After centrifugation at 5,000 rpm for 15 minutes, supernatant was added to a sterilized centrifuge tube and evaporated in a SpeedVac. Dried samples were stored at -20°C in desiccant. Extracted samples were dissolved with 100µL ethanol, followed by Assay Buffer (provided by Arbor Assay, Ann Arbor, MI, U.S.A.). Ethanol content was kept below 5%. Reconstituted samples were run immediately in the assay.

2.1.6.2 Testosterone Assay

Testosterone levels were measured using an ELISA kit according to the manufacturer’s instructions (Arbor Assay, Ann Arbor, MI, U.S.A.). All kit reagents were prepared and brought to room temperature (21-25°C)(detection range 10,000 – 40.96 pg/mL). 50µL of standard and sample were pipetted into the appropriate wells (pre-coated with anti-rabbit antibody). 75µL of assay buffer were added to two non-specific binding (NSB) wells, while 50µL of assay buffer were added to maximum binding wells. Then, 25µL DetectX® Testosterone Conjugate was pipetted into
each well, followed by the addition of 25µL DetectX® Testosterone Antibody into all wells excluding NSB wells.

After shaking for 2 hours, well contents were aspirated and manually washed four times with 300µL (1X) wash buffer. Once the plate was tap dried on absorbent paper towel, 100µL of TMB substrate was added to each. After incubation at room temperature for 30 minutes, 50µL of stop solution was dispensed into each well.

Using spectrophotometry, absorbance was then measured at 450 nm in a microplate reader. A standard curve was plotted relating optical density to the concentration of standards and testosterone concentrations were then interpolated from the standard curve.

2.1.7 Statistical Analysis
The data were analyzed by two-tailed, one-way or two-way ANOVA followed by Bonferroni’s post hoc test using GraphPad Prism Ver. 6. Mean values obtained were expressed as mean ± standard error of the mean (SEM). A priori hypothesis of p<0.05 was used for all analyses.

2.2 In Vitro Studies: TM3 and TM4 Cell Model for TCAP-1 Mediated Testosterone Production
2.2.1 Cell Culture
Immortalized TM3 and TM4 mouse cell lines (obtained from ATCC, Manassas, Virginia, U.S.A.) were grown in a standard medium (1:1 mixture of Ham’s F12 medium + DMEM medium; 5% horse serum; 2.5% fetal bovine serum; 50 unit/ml Penicillin; 50 mg/ml Streptomycin) in 100 mm petri dishes until 80% confluent (approximately 3 days) at 37°C and 5% CO2. The media was changed every 24 hours throughout the incubation period. Cells were passaged into a six-well plate for all further treatment.

2.2.2 FITC-TCAP-1 Binding and Uptake Studies
Immortalized TM3 and TM4 cells were plated on Poly D-lysine (Sigma-Aldrich, Canada)-coated cover slips (Sarstedt, Montreal, QB, Canada) and cultured to 80% confluence. Cells were serum-

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starved for 3 hours in serum-free media. Post starvation, well contents were aspirated, cells were rinsed with 1X phosphate buffer solution (PBS) 1X and incubated with either fluorescein isothiocyanate (FITC)-labeled TCAP-1 (diluted 400X in regular growth medium) or scrambled (SC)-TCAP (negative control, diluted 400X in regular growth medium) for 60, 30, and 15 minutes. Cells were then washed with PBS, fixed with 4% Paraformaldehyde (PFA) for 20 minutes, and permeabilized for 10 minutes with 0.3% Triton x-100 (Sigma-Aldrich, Canada). After blocking with 10% normal goat serum (NGS; New England Biolabs, Whitby, ON, Canada) for 1 h in the dark and at room temperature (21-25°C), cells were incubated with Latrophilin 3 (P17) goal polyclonal primary antibody (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.; diluted 500X in 1% NGS solution). The plate was then sealed tightly with parafilm and left overnight at 4°C. Cells were then washed four times for 5 minutes and incubated for 1 hour in the dark at room temperature (21-25°C) with secondary antibody (AlexaFluor® 594, donkey anti-goat, ThermoFisherScientific) diluted 500 X in 1% NGS. Following four 5-minutes PBS washes, cells were counterstained with 4’, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Canada) and mounted with clear nail polish (OPI). For controls, cells were incubated with primary antibody alone, secondary antibody alone, or neither primary nor secondary antibodies.

The experiment was replicated twice to examine expression of ADGRL 3 in the TM4s and TM3s, as well as FITC-TCAP-1 uptake.

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

Extracted mRNA from TM3 and TM4 immortalized cells was reverse transcribed to create DNA template for amplification. mRNA was mixed with reverse transcription reagents: oligo dT primers (Thermo Scientific, Canada), dNTPs (Thermo Scientific, Canada), RNA-free H2O (Sigma-Aldrich, Canada), first strand buffer (Thermo Scientific, Canada), DTT (Invitrogen, Canada), and Superscript II reverse transcriptase (Invitrogen, Canada) and heated to form complementary DNA (cDNA).

Sufficient master mix, now using gene specific primers, was prepared for multiple PCR reactions. The newly synthesized cDNA sample was mixed with deionized H2O, Taq buffer (Invitrogen, Canada), dNTPs, forward and reverse primers (see Table 2.1), and the thermostable
DNA 1 polymerase, *Taq* DNA polymerase (Thermo Scientific, Canada) required to convert the single strand cDNA into double-strand DNA.

PCR reaction tubes were placed in the Thermocycler at the following pre-programmed settings: 1) DNA template Initial denaturation at 95°C for 7 minutes; 2) 28 repeated amplification cycles (3 minutes, 5 seconds): denaturation at 94°C, primer annealing at varying temperatures (63°C - 69°C), and elongation at 72°C; 3) final elongation step at 72°C for 5 minutes.

2.2.4 Analysis of PCR Products
mRNA expression was analyzed by gel electrophoresis at 100V. 2% (w/v) agarose gel (pre-mixed with Ethidium bromide) was poured into the casting tray and allowed to solidify. 1X TBE running buffer was then added to the electrophoresis chamber and gel wells were individually loaded with DNA samples pre-mixed with loading dye. The first lane contained 1 kb DNA ladder. The gel was run until the front loading dye reached ¾ of the way down the gel (1hr). The gel was imaged using one of two BIORAD systems: Gel Doc™ EZ System or ChemiDoc™ Imaging systems.
Table 2.1 Primers used for reverse-transcription polymerase chain reaction (RT-PCR).

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence (5' to 3')</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>teneurin-1</td>
<td>F* GTGTCACCTGTAGGCACTCCCTCTAT</td>
<td>402</td>
</tr>
<tr>
<td></td>
<td>R* TCCTGGTGATGCACTCCGGCCAA</td>
<td></td>
</tr>
<tr>
<td>teneurin-2</td>
<td>F* ATCTCTGAGACCTGCTCCCTTA</td>
<td>405</td>
</tr>
<tr>
<td></td>
<td>R* TCTGGAAGCTGACTCGAGCACGC</td>
<td></td>
</tr>
<tr>
<td>teneurin-3</td>
<td>F* AGTGGAATCCCGCTGGAGAAGCA</td>
<td>427</td>
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<tr>
<td></td>
<td>R* GTGATGCTCCCTGCTGCAAGATG</td>
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<td>teneurin-4</td>
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<td></td>
<td>R* CATGTCGCTGAGAGAACG</td>
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</tr>
<tr>
<td>TCAP-1</td>
<td>F* AGTCAGTGGAACTGAGGACT</td>
<td>351</td>
</tr>
<tr>
<td></td>
<td>R* CTGCTGCTGCTGCTGCTGTTAC</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td></td>
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<td>R* GCTGTGTGAGAA</td>
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<td>R1* ATGTTGCTGCTGGTGGTTA</td>
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<td>LPHN-3</td>
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<td>327</td>
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<td>R2* ATGCTAGACCCGCTTACAGGA</td>
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<tr>
<td>beta-actin</td>
<td>F* CAGGTCATGCATGCTGGCAACGAG</td>
<td>357</td>
</tr>
<tr>
<td></td>
<td>R* CTCACTCGACTCTGCTTGGTAT</td>
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</tr>
</tbody>
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F* = Forward primer; R* = Reverse Primer

2.2.5 Enzyme-linked Immunosorbent Assays (ELISAs) of DAG, IP3 and Testosterone

2.2.5.1 Cell Treatment and Lysis

Immortalized mouse cell lines (TM3 and TM4) were cultured individually. Once 80% confluent, cells were serum starved for 3 hours, where complete growth media was aspirated and replaced with serum-free media. After starvation, cells were treated with 100nM TCAP-1 at different time points (0, 5, 15, 30, 60 minutes), with an n=5 per treatment time. Deionized water was used as the negative control.
Upon completion of treatment, media was aspirated and wells were rinsed with ice-cold PBS. PBS was then removed and 400μL RIPA cell lysis buffer (150mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0, New England Biolabs, Whitby, ON, Canada) was added to each well for 5 minutes. The well contents were scraped using a spatula, pipetted into sterilized centrifuge tubes and centrifugation was carried out for 10 minutes at 4°C at 14,000 RPM. Supernatant was pipetted into new centrifuge tubes and stored at -20°C. Protein content was later quantified and samples diluted accordingly in preparation for assays.

2.2.5.2 Diacylglycerol (DAG) Assay

TM3 and TM4 diacylglycerol (DAG) levels were measured by quantitative sandwich ELISA according to the manufacturer’s instructions (MyBioSource, San Diego, California, U.S.A.). All working reagents were prepared and brought to room temperature (21-25°C) along with samples to be tested for DAG concentration (detection range 0.625 - 20 μmol/L). 50μL of standard and sample were added to the appropriate wells (pre-coated with purified mouse DAG antibody) in the microelisa stripplate provided. All standards and samples were run in duplicate. 50μL of sample diluent was added to two blank control wells. Next, 100μL of HRP-conjugate reagent was added to each well, including the blank control. Plate was covered with an adhesive strip and incubated at 37°C for 60 minutes.

Incubation mixture was aspirated from the plate and manual washing was repeated four times, using (1X) wash solution. The plate was blot dried by hitting plate onto absorbent paper towel following the final wash. 50μL of Chromogen Solution A and Chromogen Solution B were added to each well successively, including the blank control, and the plate was incubated at 37°C for 15 minutes. 50μL of Stop solution were then added to each well.

Using spectrophotometry, absorbance was measured at 450 nm in a microplate reader. A standard curve was plotted relating optical density to known concentrations, and unknown DAG concentrations were then interpolated from the curve.
2.2.5.3 **Inositol Triphosphate (IP$_3$) Assay**

TM3 and TM4 inositol triphosphate (IP$_3$) levels were measured by competitive ELISA according to the manufacturer’s instructions (MyBioSource, San Diego, California, U.S.A.). All working reagents were prepared and brought to room temperature (21-25°C) along with samples to be tested for IP$_3$ concentration (detection range 0 - 50 ng/mL). 100µL of standard and sample were added to the appropriate wells (pre-coated with purified mouse anti-IP$_3$ antibody) in the microelisa stripplate provided. All standards and samples were run in duplicate. 100µL of PBS was added to two blank control wells. Next, 50µL of enzyme conjugate was added to each well, excluding the blank control. Contents of wells were mixed well by shaking the plate on the bench surface.

After incubation at 37°C for 60 minutes, well contents were aspirated and manual washing was repeated five times, using (1X) wash solution. Once the plate was blot dried by hitting plate onto absorbent paper towel, 50µL of Substrate A and Substrate B were added to each well successively, including the blank control wells. Following incubation at 37°C for 15 minutes, 50µL of Stop solution was added to each well.

Using spectrophotometry, absorbance was measured at 450 nm in a microplate reader. A standard curve was plotted relating optical density to known concentrations, and unknown IP$_3$ concentrations were then interpolated from the curve.

2.2.5.4 **Testosterone (TESTO) Assay**

TM3 testosterone levels were measured by competitive ELISA according to the manufacturer’s instructions (MyBioSource, San Diego, California, U.S.A.). All working reagents were prepared and brought to room temperature (21-25°C) along with samples to be tested (detection range 0-50 ng/mL). 50µL of standard and sample were added to the appropriate wells (pre-coated with monoclonal anti-TESTO antibody) in the microelisa stripplate provided, and 10µL of balance solution was dispensed into the sample wells. All standards and samples were run in duplicate. 50µL of PBS was added to two blank control wells. Next, 50µL of enzyme conjugate was added to each well, excluding the blank control. Contents of wells were mixed well by shaking the plate on the bench surface.

After incubation at 37°C for 60 minutes, well contents were aspirated and manual washing was
repeated five times, using wash solution (1X). Once the plate was blot dried, 50µL of Substrate A and Substrate B were added to each well successively, including the blank control wells. Following incubation at 37°C for 15 minutes, 50 uL of Stop solution was added to each well.

Using spectrophotometry, absorbance was measured at 450 nm in a microplate reader. A standard curve was plotted relating optical density to known concentrations, and unknown testosterone concentrations were then interpolated from the curve.

2.2.6 Statistical Analysis
Data were analyzed by a two-tailed, one-way or two-way ANOVA followed by Bonferroni’s post hoc test using GraphPad Prism Ver. 6. Mean values obtained were expressed as mean ± standard error of the mean (SEM). A priori hypothesis of p<0.05 was used.

2.3 TM3 Cell Transfection Studies: ADGRL 1 and 3 Knockdown
2.3.1 siRNA Resuspension
5nmol of ON-TARGETplus Mouse Adgrl1, Adgrl3 siRNA-SMARTpool and ON-TARGETplus non-targeting pool were supplied by GE Healthcare Bio-Sciences (Cat. No. L-061299-00-0005, L-040779-00-0005, and D-001810-10-05). Tubes containing the siRNA were briefly centrifuged to ensure the siRNA pellet collected at the bottom. Using the manufacturer’s resuspension buffer (Cat. No. B-002000-UB-100), siRNA was diluted to the final working concentration (10µM) by pipetting the solution up and down 3-5 times. Tubes were placed on an orbital mixer for 30 minutes at room temperature to allow for thorough mixing.

2.3.2 TransIT-X2 Dynamic Delivery System:siRNA Complex Formation
Before transfecting TM3 cells with siRNA, the TransIT-X2 (supplied by Dalia Barsyte-Lovejoy, Structural Genomics Consortium, University of Toronto,) was warmed to room temperature (21-25°C) and vortexed gently. 12 sterile tubes were filled with 250µL serum- and antibiotic-free media, and divided into two groups of 6, where each of the two groups represented a different
transfection time, 24 or 48 hours. Each group was further subdivided into two groups, Adgrl1 or Adgrl3, 3 tubes per group (representing a different TransIT-X2 volume). A total of 6.8μL of the 10μM stock siRNA (either Adgrl 1 or 3) were added to the appropriate tube. Next, one of three different working volumes of TransIT-X2 delivery system (5, 7.5, or 10μL) was added to tubes designated ADGR1l and ADGR13 in both 24 and 48 hour treatment groups. The TransIT-X2:siRNA solutions were then incubated at room temperature (21-25°C) for 30 minutes to allow sufficient time for complex formation.

2.3.3 siRNA Transient Transfection
TM3 cells were cultured in 100 mm dishes and passaged into six-well plates (refer to section 2.2.1 for cell culture conditions). Once cells were 80 % confluent, siRNA transient transfection began and the pre-made siRNA:delivery complex was added drop-wise to the appropriate wells. Cells were transfected for either 24 hours or 48 hours with three different volumes of TransIT-X2 delivery system, with either Adgrl1 or Adgrl3 siRNA.

2.3.4 Assessing for Knockdown
2.3.4.1 Reverse-Transcription Polymerase Chain Reaction (RT-PCR)
mRNA was extracted and mixed with reverse transcription reagents: oligo dT primers, dNTPs, RNA-free H₂O, first strand buffer, DTT and Superscript II reverse transcriptase, and heated to form complementary DNA (cDNA).

Sufficient master mix, now using gene specific primers, was prepared for multiple PCR reactions. The newly synthesized cDNA sample was mixed with deionized H₂O, Taq buffer, dNTPs, forward and reverse primers (ADGR1 1 and 3), and the thermostable DNA 1 polymerase, Taq polymerase required to convert the single strand cDNA into double-strand DNA.

PCR reaction tubes were placed in the Thermocycler at the following pre-programmed settings: 1) DNA template initial denaturation at 95°C for 7 minutes; 2) 35 repeated amplification cycles (3 minutes, 5 seconds): denaturation at 94°C, primer annealing at varying temperatures (63-69°C), and elongation at 72°C; 3) final elongation step at 72°C for 5 minutes.
2.3.4.2 Analysis of PCR Products

mRNA expression was analyzed by gel electrophoresis at 100V. 2% (w/v) agarose gel (pre-mixed with Ethidium bromide) was poured into the casting tray and allowed to solidify. 1x TBE running buffer was then added to the electrophoresis chamber and gel wells were individually loaded with DNA samples pre-mixed with loading dye. The first lane contained 1 kb DNA ladder. The gel was run until the front loading dye reached ¾ of the way down the gel (1hr). The gel was imaged using one of two BIORAD systems: Gel Doc™ EZ System or ChemiDoc™ Imaging systems.

2.3.5 Assessing the Effects of KD on TCAP-1 Modulation of the ADGRL-Pathway

Cells were transfected for 48 hours with ADGRL 1 and 3 siRNA, followed by 30 and 60 minute TCAP-1 incubation periods. Using RIPA lysis buffer, cells were then harvested for further analyses (as explained in section 2.2.5.1). Following lysis, cell lysate protein content was quantified using the BCA Assay (ThermoScientific), and equalized via dilution with RIPA buffer. Samples were then used in DAG and IP3 ELISAs (refer to sections 2.2.5.2 and 2.2.5.3).
Chapter 3

RESULTS

3.1 TCAP-1 affects testosterone and luteinizing hormone (LH) production in vivo: evidence for an HPG-axis-independent mode of activity

3.1.1 TCAP-1 increased circulating levels of testosterone

Evidence of a TCAP-1 mediated increase in testosterone levels was first observed in vivo, in adult male BALB/c mice, where TCAP-1 was administered on a daily basis throughout the study period (Chand et al., 2014). To provide additional evidence of a role of TCAP-1 in testosterone production, further investigation was performed on the same strain of male mice (BALB/c), with TCAP-1 injections administered every other day instead of every day to reduce the amount of exogenous TCAP. Both fecal and serum testosterone levels, and serum luteinizing hormone (LH) levels were analyzed over an eight-day period for control and treatment groups (Figures 3.1, 3.2 & 3.3).

Fecal samples collected from TCAP-1-treated mice showed significant increases in testosterone as compared to the control groups (Figure 3.1 A). Mice treated with 25 pmol TCAP-1 had significantly higher levels of testosterone on day 4 (5312 ± 1478 pg/mL, p<0.0332) and day 5 (9179 ± 872 pg/mL, p<0.0002) compared to day 4 (1631 ± 654 pg/mL) and day 5 (3652 ± 575 pg/mL) SC-TCAP-treated mice, and significantly higher levels on day 5 (p<0.0001) compared to the vehicle (2812 ± 1008 pg/mL).

Mice treated with 250 pmol TCAP-1 had significantly higher levels of testosterone on day 5 (6568 ± 1968 pg/mL, p<0.0332) and on day 8 (7251 ± 1071 pg/mL, p<0.0002) as compared to day 5 (2812 ± 1008 pg/mL) and day 8 (1703 2812 ± 964 pg/mL) vehicle levels.

No significant difference was observed for fecal testosterone levels between the vehicle and scrambled-TCAP (SC-TCAP)-1 groups (p>0.999). Overall, rise in testosterone levels was most significant in groups receiving the 25 pmol dose of TCAP-1 vs. those receiving the 250 pmol dose.
Figure 3.1 TCAP-1 significantly increased fecal testosterone levels in adult male BALB/c mice. Mice treated with 25 pmol TCAP-1 had significantly higher levels of testosterone on day 4 (5312 \pm 1478 pg/mL, p<0.0332) and day 5 (9179 \pm 872 pg/mL, p<0.0002) compared to SC-TCAP-treated mice (1631 \pm 654 pg/mL and 3652 \pm 575 pg/mL), and significantly higher levels on day 5 (p<0.0001) compared to the vehicle (2812 \pm 1008 pg/mL). Mice treated with 250 pmol TCAP-1 had significantly higher levels of testosterone on day 5 (6568 \pm 1968 pg/mL, p<0.0332) and on day 8 (7251 \pm 1071 pg/mL, p<0.0002) as compared to the vehicle (2812 \pm 1008 pg/mL and 1703 2812 \pm 964 pg/mL). All values are the mean \pm SEM, *p<0.0332, ***p<0.0002, ****p<0.0001, two-way ANOVA with a Bonferroni’s post hoc test.

Serum testosterone levels were analyzed to provide further validation for the observed TCAP-1 mediated rise in fecal testosterone levels (Figure 3.2). Previous studies have indicated that TCAP-1 action is dependent upon the administered concentration (Qian et al., 2004; Wang et al., 2005), thus the testosterone data was examined separately for each of the four treatment groups. Mice treated with 25 pmol/kg TCAP-1 had significantly higher testosterone levels on day 4 (17.011 \pm 6.558 ng/mL, p=0.0259) compared to: day 1 (2.714 \pm 1.775 ng/ml), and day 2 (2.066 \pm 0.818 ng/mL). For mice treated with 250 pmol/kg TCAP-1, a significant increase in testosterone levels was also observed on day 4 (13.716 \pm 7.041 ng/mL, p=0.0438) compared to day 1 (0.297 \pm 0.103 ng/mL). On day 4 only, the TCAP-1 25 pmol/kg-treated mice had significantly higher testosterone levels (p=0.0474) compared to Vehicle (3.731 \pm 1.544 ng/mL), while no significance was observed for either dose compared to SC-TCAP. No significant changes were observed within the individual
vehicle and SC-TCAP groups, and the lower TCAP-1 dose resulted in the most significant increase in testosterone levels.

**Figure 3.2 TCAP-1 significantly increased serum testosterone levels in adult male BALB/c mice.** Mice treated with 25 pmol/kg TCAP-1 had significantly higher testosterone levels on day 4 (17.011 ± 6.558 ng/mL, p=0.0259) compared to: day 1 (2.714 ± 1.775 ng/ml), and day 2 (2.066 ± 0.818 ng/mL). 250 pmol/kg TCAP-1-treated mice showed a significant increase in testosterone levels on day 4 (13.716 ± 7.041 ng/mL, p=0.0438) compared to day 1 (0.297 ± 0.103 ng/mL). TCAP-1 25 pmol/kg-treated mice only had significantly higher testosterone levels (p=0.0474) compared to Vehicle (3.731 ± 1.544 ng/mL) on day 4 only, while no significance was observed for either dose compared to SC-TCAP. No significant changes observed within the individual vehicle and SC-TCAP groups. All values are the mean ± SEM, *p<0.0332, two-way ANOVA with a Bonferroni’s post hoc test.

Additionally, in order to determine if TCAP-1 is acting through the HPG-axis to increase steroid production, serum luteinizing hormone levels were measured using samples from day 4 of the treatment period, which corresponded to the highest rise observed in serum testosterone levels (Figure 3.3). Following analysis, no significant changes were observed within each treatment group (p>0.0918).
Figure 3.3 TCAP-1 did not significantly increase luteinizing hormone (LH) serum levels in adult male BALB/c mice. All values (7.609 ± 0.831 ng/mL, 5.878 ± 0.7684 ng/mL, 6.877 ± 0.993 ng/mL, 9.784 ± 1.561 ng/mL) are the mean ± SEM, one-way ANOVA with a Bonferroni’s post hoc test.

3.2 TM3 and TM4 immortalized cell lines were characterized to investigate TCAP-1 action on testosterone production: in vitro model for TCAP-1 activity in the testes

3.2.1 Teneurin, TCAP and ADGRL mRNA expression confirmed in TM3 and TM4 cells

TM3 and TM4 cells have never been characterized with respect to the teneurins, TCAP or their receptors. Therefore, it was necessary to perform mRNA expression studies. Reverse transcription polymerase chain reaction (PCR) was performed to analyze for TCAP, teneurin and ADGRL mRNA expression in both TM3 and TM4 immortalized cell lines (Figure 3.4 and 3.5).

In the TM4s, amplicons for Teneurin-1 (402bp), -3 (427bp) and -4 (369bp) were observed. No amplicon was seen for Teneurin-2 (405bp)(Figure 3.4 A). In TM3s, only the teneurin-3 amplicon was observed (Figure 3.5 A). Clear bands for TCAP-1 (351bp), -2 (496bp) and -4 (602bp) were seen for the TM4s, as well as multiple bands for TCAP-3 where the top-most band (506bp) corresponded to the expected product size (Figure 3.4 B). Only TCAP-1 and -3 were observed in the TM3s (3.5 B). ADGRL 1 amplicons (249bp; 398bp) and ADGRL 3 amplicons (268bp; 327bp) were observed in the TM3s and TM4s, although ADGRL 3 (327bp) was not expressed in the
Although the bands for ADGRL1 showed greater intensity than for ADGRL3 in both cell lines, this does not indicate a quantitative difference.

### 3.2.2 Teneurin-1 immunoreactivity and TCAP-1 uptake confirmed in TM3 and TM4 cells.

To investigate the location and internalization of TCAP-1 and Teneurin-1 in the TM3s and TM4s, as well as the occurrence of co-localization events between TCAP-1 and ADGRL3, cells were incubated with fluorescently labelled TCAP-1 (FITC-TCAP-1) and treated with either Teneurin-1 antibody (Alexa594 donkey anti-mouse) or ADGRL3 antibody (Alexa594 donkey anti-goat antiserum).

Fluoresceinisothiocyanate (FITC)-labelled TCAP-1 uptake studies were performed for both the TM3s and TM4s using fluorescence microscopy (Figure 3.6 and 3.7). Cells were incubated with FITC-TCAP-1 for 15, 30 and 60 minutes. In the TM3s, by 15 minutes FITC-TCAP-1 could be detected close to the plasma membrane, and in the cytosolic regions (Figure 3.6 C). At 30 minutes (Figure 3.6 F) and 60 minutes (Figure 3.6 I), FITC-TCAP-1 was predominantly detected in the cytosol, indicating internalization. By 60 minutes, a signal around the nucleus was also detected. In the TM4s, FITC-TCAP-1 was more strongly detected at the plasma membrane (Figure 3.7). At 15 minutes, detection was strongest at the plasma membrane (Figure 3.7 C), while internalization was evident after 30 minutes (Figure 3.7 F) and 60 minutes (Figure 3.7 I) from punctate-like cytosolic detection.

TCAP-1 and Teneurin-1 immunoreactivity was also investigated in the TM3s and TM4s. In the TM3s (Figure 3.8 A-D), Teneurin-1 was localized primarily at the plasma membrane (Figure 3.8 B). TCAP-1 was observed in the cytosolic regions, most prominently in regions of connectivity between neighbouring cells (Figure 3.8 C). As for the TM4s (Figure 3.8 E-H), Teneurin-1 was highly concentrated around the plasma membrane regions of the cells (Figure 3.8 F). TCAP-1 immunoreactivity was seen in the cell cytosol, also noticeably more concentrated in regions of higher connectivity as with the TM3s (Figure 3.8 G).

FITC-TCAP-1 co-localization with ADGRL3 was investigated in both the TM4s (Figure 3.9 A-E) and TM3s (Figure 3.9 F-J). FITC-TCAP-1 was localized to the cytosolic regions of
Figure 3.4 PCR analysis for teneurins, TCAPs and ADGRLs in immortalized mouse Leydig (TM3) cells. Band expression was observed corresponding to teneurin-3 (427bp; Ten3); and (B) strong bands were seen for TCAP-1 (351bp; T1) and TCAP-3 (506bp; T3); (C) Bands were observed corresponding to the product size of ADGRL 1 (A1.1 = 249bp; A1.2 = 398bp) and ADGRL 3 (A3.1 = 268bp; A3.2 = 327bp), where expression was stronger for ADGRL 1 than ADGRL 3. Beta-actin (Act) was used as a loading control.
both TM4s and TM3s (Figure 3.9 C and H, respectively). Prominent expression of ADGRL 3 was observed at the plasma membrane and regions of connectivity between neighbouring cells (around the membrane) of the TM4s (Figure 3.9 D). However, ADGRL 3 was not observed in the TM3s (Figure 3.9 I). Furthermore, what appeared as faint sites of co-localization between FITC-TCAP and ADGRL 3 could be seen around the plasma membrane in the TM4s (Figure 3.9 E). Further investigations would be required to validate the accuracy of these results and assess whether or not co-localization was due to tandem probability.
Figure 3.6 FITC-TCAP-1 uptake in immortalized mouse Leydig (TM3) cells. Cells were incubated with fluoresceinisothiocyanate (FITC)-labeled TCAP-1 for 15 min (A-C), 30 min (D-F), and 60 min (G-I). Uptake into the cytosol was observed after 15, 30 and 60 min of treatment, with signal around the nucleus appearing after 60 min. For each fluorescence image, the corresponding DIC image is represented on the left (A, D, and G). All sections were counterstained with DAPI (B, E, and H). Magnification, 40X. Scale bars, 250µm.
Figure 3.7 FITC-TCAP-1 uptake in immortalized mouse Sertoli (TM4) cells. Cells were incubated with fluorescein isothiocyanate (FITC)-labeled TCAP-1 for 15 min (A-C), 30 min (D-F), and 60 min (G-I). Detection at the plasma membrane was apparent after 15 min (C), while intracellular accumulation was most prominent after 30 min (F) and 60 min (I). For each fluorescence image, the corresponding DIC image is represented on the left (A, D, and G). All sections were counterstained with DAPI (B, E, and H). Magnification, 40X. Scale bars, 250 µm.
Figure 3.8 TCAP-1 and teneurin-1 immunoreactivity in immortalized mouse Leydig (TM3) and immortalized mouse Sertoli (TM4) cells. In TM3s (A-D) and TM4s (E-H), Teneurin-1 immunoreactivity was detected, using mouse monoclonal teneurin-1 antiserum, primarily at the plasma membrane (B and F). TCAP-1 immunoreactivity was observed, using mouse monoclonal TCAP-1 antiserum in the cytosolic regions for both TM3s (C) and TM4s (G), and was most prominent in regions of connectivity between neighbouring cells. Sections were counterstained with DAPI (A and E). Overlay represents all 3 channels. Magnification, 40X. Scale bars, 100µm.
Figure 3.9 Co-localization between FITC-TCAP-1 and ADGRL 3 in immortalized mouse Sertoli (TM4) and immortalized mouse Leydig (TM3) cells. FITC-TCAP-1 was localized to the cytosolic regions of both TM4s (C) and TM3s (H). Prominent expression of ADGRL 3 was observed at the plasma membrane and regions of connectivity between neighbouring cells (around the membrane) of the TM4s (D). ADGRL 3 was not observed in the TM3s (I). At least 4 possible sites of co-localization between FITC-TCAP and ADGRL 3 were seen around the plasma membrane (arrows representing localization events) in the TM4s (E). TM4 data represented in A-E, and TM3 data represented in F-J. For each fluorescence image, the corresponding DIC image is represented at the top of each column (A and F). All sections were counterstained with DAPI (B and G). Overlay represents all 4 channels. Magnification, 40X; Scale bars, 250µm.
3.3 TCAP-1 affects testosterone production and activity of downstream messengers of the ADGRL pathway

3.3.1 TCAP-1 significantly increased *in vitro* testosterone levels in TM3 cells

To further corroborate my observations for *in vivo* TCAP-1 activated steroidogenesis, the TM3s were analyzed for testosterone production post-TCAP-1 treatment (Figure 3.10). Results were compared between 100nM TCAP-1-treated cells and vehicle-treated cells at each time point and between time points. Values at 0 minutes, 5 minutes, 15 minutes, 30 minutes, and 60 minutes for both TCAP-1 and vehicle-treated samples were transformed relative to time 0 within each group.

Post TCAP-1 treatment, a significant increase in testosterone was observed at 15 minutes (170 ± 12 %; *p*<0.0002), 30 minutes (166 ± 32 %; *p*<0.0021), and 60 minutes (217 ± 13 %; *p*<0.0001), compared to 15, 30 and 60 minute vehicle values (61 ± 13 %, 73 ± 17 % and 55 ± 14 %).

![Graph showing testosterone levels](image)

**Figure 3.10 TCAP-1 increased testosterone in immortalized mouse Leydig (TM3) cells.** A significant increase in testosterone was seen in TCAP-1 treated TM3s compared to 0 minutes. Change was observed at 15 min (170 ± 12 %; *p*<0.0002), 30 min (166 ± 32 %; *p*<0.0021) and 60 min (217 ± 13 %; *p*<0.0001) compared to vehicle (61 ± 13 %, 73 ± 17 % and 55 ± 14 %). All values are the mean ± SEM, **p*<0.0021, ***p*<0.0002, ****p*<0.0001, one-way ANOVA with a Bonferroni’s post hoc test compared to time 0.
3.3.2 TCAP-1 significantly increased diacylglycerol (DAG) and inositol triphosphate (IP₃) levels in TM3 and TM4 cells, respectively

The significant *in vivo* (fecal) and *in vitro* increases seen in testosterone production suggests that TCAP-1 may be activating G-protein coupled reactions upstream of steroidogenesis, possibly the MEK/ERK1/2 pathway, via its cognate receptor ADGRL. Therefore, to further elucidate the mechanism driving TCAP activity, DAG and IP₃ assays were performed using ELISA for both the TM3s and TM4s (Figure 3.11 A-D).

TM3s and TM4s were analyzed post TCAP-1 or vehicle treatment. Similar to the testosterone assay, results between 100nM TCAP-1 treatment vs. vehicle treatment were compared at each time point and between time points. Values for 0, 5, 15, 30 and 60 minutes were transformed relative to 0 minutes within each group.

TM3 cells incubated with TCAP-1 had significantly higher DAG levels (Figure 3.11 A) at 30 minutes (123 ± 12 %; p<0.0021) and 60 minutes (132 ± 3 %; p<0.0001) compared to 30 and 60 minute vehicle values (85 ± 3 % and 81 ± 7 %). IP₃ levels did not change (Figure 3.11 B). As for the TM4s, no change was observed for DAG levels post TCAP-1 incubation compared to vehicle (Figure 3.11 C), while a significant increase in IP₃ levels (Figure 3.11 D) at 5 minutes (153 ± 13 %; p<0.0001), 15 minutes (141 ± 7 %; p<0.0238), 30 minutes (153 ± 13 %; p<0.0001), and 60 minutes (140 ± 5 %; p<0.0051) was observed relative to vehicle (77 ± 3 %, 105 ± 5 %, 78 ± 6 % and 97 ± 14 %).
Figure 3.11 Diacylglycerol (DAG) and inositol triphosphate (IP$_3$) levels in immortalized mouse Leydig (TM3) and immortalized Sertoli (TM4) cells post TCAP-1 treatment (100nM) vs. Vehicle (ddH$_2$O). For the TM3s (A-B), a significant increase in DAG levels (A) was detected at 30 min (123% ± 12; p<0.0021) and 60 min (132 ± 3 %; p<0.0001) compared to 30 and 60 minute vehicle values (85 ± 3 % and 81 ± 7 %). No change was observed in IP$_3$ levels (B). For the TM4s (C-D), no change was seen in DAG levels (C) while a significant increase was observed for IP$_3$ (D) at 5 min (153 ± 13 %; p<0.0001), 15 min (141 ± 7 %; p<0.0238), 30 (153 ± 13 %; p<0.0001) and 60 min (140 ± 5 %; p<0.0051) relative to vehicle (77 ± 3 %, 105 ± 5 %, 78 ± 6 % and 97 ± 14 %). All values are the mean ± SEM, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001, one-way ANOVA with a Bonferroni’s post hoc test compared to time 0.
3.4 ADGRL knockdown blocks TCAP-1 effects on adhesion GPCR signaling

3.4.1 siRNA transient transfection knocked down ADGRL 1 and 3 in 48 hours

In order to further elucidate the role of ADGRL and adhesion GPCR signaling in this novel route of steroidogenesis triggered by exogenous TCAP-1, TM3 cells were transfected for either 24 or 48 hours with siRNA specific for both ADGRL 1 and 3. Transfection success was assessed by mRNA expression analysis via PCR (Figure 3.12). After 24 hours, faint bands (in comparison to controls treated with non-coding siRNA, lanes C1-3) appeared with corresponding product size to ADRL 1 and ADGRL 3 (A1-3; refer to Table 2.1 for primer sequences). However, after a full 48 hours, the three bands were not visible in the gel (B1-3).

![Figure 3.12 PCR analysis for knockdown of ADGRL 1 and 3 in transfected immortalized mouse Leydig (TM3) cells. Cells were treated with deliveryreagent:siRNA complex (for either 24 hours (A1-3) or 48 hours (B1-3)) specific for either ADGRL 1 (A/B 1 = 249bp; A/B 2 = 398bp) and ADGRL 3 (A/B 3 = 268bp). 24 hour treatment resulted in faint band expression, in comparison to controls (C1-3, non-coding siRNA treatment for 24 hours). After 48 hours, no bands were observed for either ADGRL 1 (B1-2) or ADGRL 3 (B3) compared to controls. Beta-actin (Act) was used as a loading control. n=3.](image-url)
3.4.2 siRNA knockdown of ADGRL 1 and ADGRL 3 blocked TCAP-1 mediated increase in adhesion GPCR messengers, diacylglycerol (DAG) and inositol triphosphate (IP3)

To prove that the ADGRL pathway is stimulated by TCAP-1 and the signaling system responsible for the rise observed in testosterone levels, transfected TCAP-1 treated TM3 cells were assayed for DAG and IP3. Time points were chosen for which TCAP-1 treatment was previously shown to cause significant change. TM3s transfected with ADGRL 1 siRNA were treated with TCAP-1 for either 30 or 60 minutes. Furthermore, cells transfected with ADGRL 3 siRNA were treated with TCAP-1 for 60 minutes.

No significant change in DAG levels was seen for cells treated with ADGRL 1 siRNA post 30 minute (2.909 ± 0.047 umol/L) and 60 minute (3.072 ± 0.156 umol/L) TCAP-1 treatment relative to 30 and 60 minute vehicle values (2.722 ± 0.153 umol/L and 2.894 ± 0.021 umol/L)(Figure 3.13 A). However, after 60 minutes of TCAP-1 treatment, a significant decrease was observed in cells transfected with ADGRL 3 siRNA (2.495 ± 0.06543 umol/L; p<0.0017) relative to vehicle (2.872 ± 0.026 umol/L)(Figure 3.13 B).

The same study was performed to measure IP3 levels (Figure 3.14). No significant change in IP3 levels was seen for cells treated with ADGRL 1 siRNA post 30 minute (7.595 ± 0.567 ng/mL) and 60 minute (7.383 ± 0.575 ng/mL) treatment compared to 30 and 60 minute vehicle values (5.859 ± 0.766 ng/mL and 8.259 ± 0.453 ng/mL)(Figure 3.14 A). Similarly, no significant change was seen in the ADGRL 3 KD cells post 60 minute (7.885 ± 0.424 ng/mL) TCAP-1 treatment vs. vehicle (8.259 ± 0.453 ng/mL)(Figure 3.14 B).
Figure 3.13 Diacylglycerol (DAG) levels in transfected immortalized Leydig (TM3) cells post TCAP-1 treatment (100nM). In ADGRL 1 knock down (KD) cells, no significant increase in DAG levels was observed at 30 minutes and 60 minutes for TCAP-1 treated cells vs. vehicle (A), one-way ANOVA with a Bonferroni’s post hoc test compared to vehicle. In ADGRL 3 KD cells treated with TCAP-1 for 60 minutes, a significant decrease in DAG levels was seen (2.495 ± 0.06543 umol/L; p<0.0017) compared to vehicle (2.872 ± 0.026 umol/L). Un-paired two-tailed t-test compared to vehicle. All values are the mean ± SEM, **p<0.0021.

Figure 3.14 Inositol triphosphate (IP₃) levels in transfected immortalized Leydig (TM3) cells post TCAP-1 treatment. In ADGRL 1 knock down (KD) cells, no significant increase in DAG levels was observed at 30 minutes (7.595 ± 0.567 ng/mL) and 60 minutes (7.383 ± 0.575 ng/mL) for TCAP-1 treated cells vs. vehicle (A)(5.859 ± 0.766 ng/mL and 8.259 ± 0.453 ng/mL), one-way ANOVA with a Bonferroni’s post hoc test compared to vehicle. In ADGRL 3 KD cells treated with TCAP-1 for 60 minutes (7.885 ± 0.424 ng/mL), no change was observed compared to vehicle (8.259 ± 0.453 ng/mL), un-paired two-tailed t-test compared to vehicle. All values are the mean ± SEM.
4.1 *In Vivo* Stimulation of Testosterone Production by TCAP-1

The first objective of my study was to provide evidence of the effects of TCAP-1 on the production of key hormones regulating reproduction, *in vivo*. After analyzing for testosterone production, as well as luteinizing hormone (LH) levels in male mice administered with TCAP-1, a significant increase was seen in fecal testosterone levels, as well as serum testosterone in an apparent dose-dependent manner. Although no significant change was observed in LH levels, this study confirmed the previous work that TCAP-1 increases male mouse testosterone production (Chand et al., 2014), and also provided evidence of an inverse relationship between TCAP-1 concentration and hormone production. Despite this, the mechanism by which this occurs is not entirely clear.

A significant increase in fecal, and serum testosterone levels was observed for both treatment groups individually, and compared to controls. These data served as further validation for a TCAP-1 mediated rise in testosterone levels seen previously (Chand et al., 2014). As seen in Figure 3.1 and 3.2, the highest increase in testosterone occurred in mice receiving 25pmol/kg dose of TCAP, the smaller of the two doses. A similar effect has been seen previously in Gn11 cells, an immortalized GnRH-secreting cell line, in which an inverse relationship was observed between TCAP-1 dose and resulting cAMP levels (Qian et al., 2004). Although this may seem counterintuitive, as it is tempting to assume that a higher dose would result in more product, this is not always the case as the outcome is dependent on the affinity of the receptor for its ligand. As a result of the Gn11 actions TCAP-1 has been hypothesized to operate on two different selective receptors, a high affinity and low affinity receptor, each coupled to differing G-proteins (Qian et al., 2004). Thus, one possibility is that lower concentrations activate a high affinity receptor to increase testosterone concentrations. Then, as TCAP-1 concentrations become higher, it activates a lower affinity receptor, which acts to inhibit the TCAP-1 action thus leading to the reduction in testosterone.
It is important to take into consideration the effects of individual variability on response. As is seen quite often in animal studies, certain animals respond to treatment, while others remain nonresponsive. This has been the outcome for a number of previous neurological *in vivo* studies performed on rodents administered with TCAP-1 (Kupferschmidt et al., 2011; Tan et al., 2008; Tan et al., 2009). The rate of steroid metabolism and excretion differs between steroids and species (Schwarzenberger, 2007; Whitten et al., 1998). Also, when in circulation, sex hormones, including testosterone, are bound by certain proteins, including albumin and androgen-binding protein (Anderson, 1974). However, only in the unbound state are hormones considered biologically active; only around 5% of total steroid is found unbound (Anderson, 1974). All these factors could be contributing to the outcome.

As both fecal and blood analyses have their advantages and disadvantages over one another, I concluded that the use of both techniques was pertinent to my study. Fecal steroid analysis techniques have several advantages over the traditional blood analysis. The most obvious one is the technique is non-invasive, where the investigator does not need to come into direct contact with the subject (Schwarzenberger, 2007). This reduces subject stress levels, which is reflected in their steroid profile. Fecal analyses can be applied to species of all sizes, from mice to whales, and in longitudinal studies, as well as in conjunction with other parameters, such as behaviour and reproduction (Schwarzenberger, 2007). Over the past two decades, many different techniques for fecal reproductive steroid hormone (including testosterone) analysis have been developed that can be applied to various research questions regarding both captive and free-ranging wildlife (Schwarzenberger, 2007). Due to species-specific differences in steroid metabolism, careful validation of each assay method has been performed (Schwarzenberger, 2007).

Before fecal steroids can be analysed however, steroid content of the samples must first be extracted and samples must be stored at adequate conditions (Schwarzenberger, 2007). Therefore, there are many steps during which steroid content may be lost if investigators are not careful or inexperienced. This is one particular disadvantage of fecal steroid analysis (Schwarzenberger, 2007). With blood analysis, samples can be immediately used once serum and plasma are separated via simple centrifugation. Another disadvantage is that fecal output and quality is heavily impacted by the diet of the individual subjects (Schwarzenberger, 2007). This was not a problem in my study, as the rodent chow had a managed formulation.
As for luteinizing hormone (LH) production, no significant change was observed between the different groups. Final response levels seen may be due to similar factors affecting the fecal and serum testosterone results, but it is difficult to say as no similar tests have been performed. Further, although insignificant, the TCAP-1 250 pmol/kg group appeared to have the highest circulating levels. Although this contradicts the findings for both fecal and serum testosterone levels, it is hard to say for certain that this would remain the case if the number of replicates (n) was increased.

Theoretically, if testosterone levels were to increase, then due to the negative feedback mechanisms in place, a resultant decrease in LH over time would be expected. More days worth of serum sample from this study would have had to be analyzed for LH in order to fully understand if LH production is being regulated by TCAP-1 or the resultant testosterone production. However, as seen in Figure 3.1, testosterone production in all groups follows a similar cyclical phase of increase and decrease, which suggests that hormone production could still be under the influence of the HPG-axis. However, it is unclear if the effect of exogenous TCAP-1 on testosterone production is due to stimulation of the gonads via interaction with the extracellular matrix at the tunica propria, independent from the hypothalamic-pituitary-gonadal (HPG) axis, or if it is regulating the HPG-axis at the CNS level. Thus, I was unable to satisfy the second part of my first objective.

An effect of TCAP on GnRH secretion cannot be entirely discounted. Recent evidence has indicated the implications of both GnRH and the teneurins in olfactory disorders, as well as Kallmann’s syndrome (Alkelai et al., 2016). Therefore it is possible that the teneurins are directly involved in the migration of the GnRH neurons.

4.2 Characterization of Cell Lines to Investigate TCAP-1 Action on Testosterone Production

My second objective was to establish the presence of Teneurin/TCAP and its putative receptor, ADGRL, in male somatic cells, the Sertoli and Leydig cells, in order to determine whether or not TCAP-1 action could have a direct effect on the testes. Immortalized Leydig (TM3) and Sertoli (TM4) cell lines were utilized, as they are among the most common cell model used to understand
aspects of testes function, although my study is the first to examine their use for TCAP-1 actions. The study by Chand et al., (2014) did not consider if TCAP-1 had a direct action on the testes, although the authors showed that both TCAP-1 and teneurins were present in several regions of the testes. Because of this, I wanted to examine how TCAP-1 could act directly on testicular cells.

The TM4 cells helped to give further perspective to this complex signaling system. These two cell types are in constant communication mode with one another via anchoring proteins at the basement membrane, and only together act to modulate the steroidogenic responsiveness and activity of the steroidogenic pathway in Leydig cells (Reventos et al., 1989; Saez et al., 1989; Siu and Cheng, 2004).

Using PCR analysis, it was confirmed for the first time that ADGRL, as well as the teneurins and TCAPs, are present in these cell types. For both the TM3s and TM4s, mRNA expression was seen for ADGRL 1 and 3 only, where ADGRL 1 expression was more prominent than for ADGRL 3. Up until now, both forms were established to be brain-specific, so these new results contradict those previous findings. For the TM3s, teneurin-3 as well as TCAP-1 and TCAP-3 mRNA expression was confirmed to be present, which is a novel finding for these cell types. This provided evidence that TCAP-1 can be independently transcribed from teneurin-1 in a non-neural tissue. As for the TM4s, teneurin-1, -3 and -4 mRNA expression was seen, as well as strong expression for TCAP-1, -2 and -4. It is very interesting that TCAP-2 was expressed in the absence of teneurin-2, as TCAP-2 and -4 independent expression from the teneurins has not been demonstrated previously in neural cell lines (there is little current understanding of TCAP and teneurin processing outside nervous tissue). Multiple bands were seen for TCAP-3, where the top-most band corresponded to the expected product size. It also seemed that the TM4s expressed more TCAP and teneurin homologues than the TM3s, which suggests that the structure of the teneurin/TCAP-ADGRL intercellular complex may vary between the two cell types, which could translate to cell-specific signaling activity. Further quantitative studies will need to performed to ascertain the differences in mRNA expression between the two cell types.

The immunofluorescent studies provided further insight into the expression of the actual proteins and the potential organization of the teneurin/TCAP-ADGRL adhesion complex. Using fluoresceinisothiocyanate (FITC)-tagged TCAP-1, TCAP-1 uptake into both the TM3s and TM4s
was observed over time. This is suggestive of an active cellular transport mechanism. TCAP-1 internalization has been previously shown in hippocampal neurons (Chand et al., 2013b) indicating that a similar mechanism occurs in testicular cells. Strong co-localization between FITC-labeled TCAP-1 and caveolin-1 was observed near the plasma membrane of E14 hippocampal cells, which provided confirmation that TCAP-1 receptor internalization at the plasma membrane is dependent on caveolae-mediated endocytosis (Chand et al., 2013b). Interestingly ADGRL, the putative receptor of Teneurin/TCAP was expressed in both the TM3s and TM4s, the same transport system as described in neurons above could be present in the reproductive tissue. Further, Teneurin-1 antiserum as well TCAP-1 antiserum established teneurin-1 and TCAP-1 immunoreactivity for both the TM3s and TM4s. Teneurin-1 was prominently seen at the plasma membrane, while TCAP-1 signal was observed throughout the cytosolic regions, evenly distributed. These findings are consistent with previous findings that indicated independent localization of teneurin-1 and TCAP-1 in the testes (Chand et al., 2014). This is further supported by the PCR results, where TCAP-3 mRNA expression was found in the TM3s in the absence of teneurin-3 mRNA expression. Similarly, TCAP-2 mRNA expression was found in the TM4s in the absence of teneurin-2 mRNA expression. Taken together, these studies indicate that the molecular components of the teneurin/TCAP and receptor system is present in the TM3 and TM4 cells and may provide a model for TCAP-1 action in the testes.

In order to compare further the cellular expression and actions of TCAP-1 and ADGRL, immunofluorescent studies were conducted to identify co-localization sites between TCAP-1 and ADGRL 3. In the TM4s, TCAP-1 was localized to the cytoplasmic cellular regions, concentrated in regions of connectivity between neighbouring cells. ADGRL 3 immunoreactivity was prominent at the plasma membrane. Minor co-localization events were detected either right at or within close proximity to the plasma membrane, although further work and replication are required to confirm the validity of these results. As for the TM3s, ADGRL 3 immunoreactivity was not found, either at or near the plasma membrane, or within the cytosol. On the other hand, as ADGRL 3 mRNA expression was already seen in the TM3s, there is still evidence that ADGRL 3 is found in this cell line. Therefore antibody non-specificity may have been an issue in this particular study. The TM4 fluorescence results do however provide further evidence of an interaction between ADGRL and teneurin/TCAP, as both teneurin-1 and ADGRL 3 were detected at the plasma membrane, which could be responsible for the uptake and internalization of TCAP-1.
Previous work done by Chand et al. (2014) identified teneurin-1 and TCAP-1 localization sites in the mouse testis. Immunofluorescent labeling studies indicated that teneurin-1 is found exclusively at the tunica propria of the seminiferous tubule and the surrounding interstitial cells, while intense TCAP-1 immunoreactivity was observed specifically in the germ cells adjacent to the basement membrane, uniformly distributed in the cytoplasm only. This was the first line of evidence for TCAP-1 immunoreactivity and action outside the central nervous system. Lastly, TCAP-1 was observed to increase testes size and testosterone production. The newly gathered data for the TM3s and TM4s from my study have provided new insight into the stoichiometry of this teneurin/TCAP-ADGRL intercellular adhesion complex. Therefore, the TM3 and TM4 cells may provide an excellent model to understand TCAP-1 function in the testes.

4.3 TCAP-1 Action on Testosterone Production in TM3 Cells

My data indicates that TCAP-1 induces testosterone synthesis directly in an immortalized Leydig (TM3) cell model, suggesting that TCAP-1 can act directly on the testes to regulate testosterone synthesis, independent of GnRH and gonadotropins. As no similar studies have been performed to date, there are no corroborating works in these cell types on this type of action. However, findings from one study, using LH receptor (R) knock out mice indicated that LHR signaling may not be essential to maintain sufficient intratesticular testosterone levels for the completion and maintenance of spermatogenesis (Zhang et al., 2003). Therefore, there may be additional mechanisms in place, possibly independent of the HPG-axis, controlling steroidogenesis. The actions of TCAP-1 on testosterone synthesis in the TM3 cells may be one such mechanism. Currently there are no models for understanding the direct action of TCAP-1 on steroidogenesis. However, recent findings in our laboratory indicate that one of the primary actions of TCAP-1 may be to stimulate mitochondrial activity (D’Aquila, Hogg and Lovejoy, unpublished data). If so then TCAP-1 may act to increase the mitochondrial-dependent regulation of the early stages of steroid synthesis, leading to an increased production of pregnenalone, that can be utilized as a precursor for further cytosolic steroid synthesis. If so, this could lead to increased testosterone production within the Leydig cells. Such studies will have to be considered in the future, where findings could have important implications into the development of hormonal contraceptive methods for men.
(Zhang et al., 2003).

### 4.4 TCAP-1 Acts Through ADGRL Receptors

TCAP-1 is a relatively unexplored hormone with respect to its actions on its receptors and subsequent secondary signal transduction cascade. The third objective of this study was to validate the *in vivo* results by providing evidence of direct *in vitro* positive effects of TCAP-1 on steroidogenesis, as well as adhesion (a)GPCR signaling. The first part of this goal was satisfied with the observed significant increase in testosterone in the TM3 cells. In order to then connect this increase mechanistically to the ADGRL receptor pathway, both TM3s and TM4s were assayed for DAG and IP$_3$ levels. Teneurin, TCAP and ADGRL mRNA expression profiles have now been identified in the TM3s and TM4s. Hence the molecular machinery that could theoretically activate steroidogenesis via the secondary messengers, DAG and IP$_3$, is present in these cell types. ADGRL is an aGPCR that is known to activate the Ga$_{q11}$ signaling pathway resulting in the production of DAG and IP$_3$.

Both TM3s and TM4s were assessed for both DAG and IP$_3$ levels using enzyme-linked immunosorbent assays (ELISA) post TCAP-1 treatment. This was a novel study as nothing is currently known about ADGRL signaling in the TM3s and TM4s. My study indicates that TCAP-1 can regulate diacylglycerol (DAG) and inositol trisphosphate (IP$_3$) signaling, key secondary messenger systems stimulated in response to ADGRL activation. As can be seen in Figure 3.11, TCAP-1 triggered different effects between the TM3s and TM4s. A significant increase was observed in DAG levels at 30 minute and 60 minute time points in the TM3s, whereas no change was seen in IP$_3$ levels. As for the TM4s, no change was observed in DAG levels, but there was a significant increase in IP$_3$ levels across all time points. This is a very interesting finding as PIP$_2$ hydrolysis and the resultant DAG and IP$_3$ production were generally believed to occur together. However, now recent studies have brought about the new assumption that several features of G$_q$PCR signaling dissociate these three basic events from one another, where DAG and IP$_3$ production are not always equal, and the consequences of PIP$_2$ hydrolysis do not always occur together (Dickson et al., 2013; Falkenburger et al., 2013). Which branch of G$_q$PCR signalling, IP$_3$/calcium, DAG/PKC or PIP$_2$ is responsible for a given downstream effect is difficult to
determine, however (Falkenburger et al., 2013). It is important to note that different signalling endpoints require different intensities of stimulation of receptors and PLC (Dickson et al., 2013). Further, although the general consensus holds that PIP2 is the major substrate of PLC, DAG may also be produced directly from P1(4)P (Falkenburger et al., 2013).

This study provided evidence for the first time of a ligand-receptor relationship between TCAP and ADGRL. It also further corroborates that TCAP-1 may be acting through two different receptors, which could be cell-specific. The only other known ligand of ADGRL, α-latrotoxin (LTX) is known to activate both the PLC-IP3 pathways and PLC-DAG-PKC pathways, so ADGRL is capable of activating both pathways. Furthermore, the fact that IP3 levels were already elevated after 5 minutes in the TM4s while DAG levels in the TM3s did not change until 30 minutes post-treatment indicates that the ADGRL in the TM4s may have a higher affinity for TCAP-1.

Although testicular testosterone is only produced in the Leydig cells, it was important to analyse the Sertoli cells in this study as at the whole testis level, they are constantly in direct contact with the Leydig cells. Both cell types are in a dynamic relationship and highly dependent on one another for function. Also, as they are in contact at the tunica propria, it is possible that they are communicating via the TCAP-ADGRL intercellular complex. Therefore, to expand upon the current knowledge of this system, both cell types were analyzed. Without the data from the TM4, it would not have been apparent that TCAP-1 could activate two different signaling pathways via two different receptors in these cell types.

Further, the aGPCR-linked activity of TCAP-1 was confirmed by knocking down ADGRL 1 and ADGRL 3 in the TM3 cells. No significant change was observed for either DAG or IP3 levels in cells transfected with ADGRL 1 siRNA and treated with the same dose of TCAP-1 as in the previous DAG and IP3 assays. Alternatively, in the ADGRL 3 knockdown (KD) cells, although small, a significant decrease was observed in DAG levels, compared to controls. No change was again observed for the IP3 levels.

These findings indicate three key points: 1) TCAP-1 activity may be mediated through ADGRL; 2) ADGRL 3 may be the dominant receptor responsible for activating the DAG-PKC pathway; and 3) TCAP-1 action can be mediated by two different receptors. The first point can be concluded due to the fact that the opposite outcome was observed in this study. Previously an
increase in DAG levels was observed in the TM3s. However, no increase compared to vehicle was observed in this new study. Secondly, the ADGRL 3 KD caused a significant decrease in DAG levels, bringing them down below baseline. Although the observed decrease was small in comparison to the increase seen in DAG levels in the non-transfected TCAP-1 treated TM3s, it does still suggest functional importance. Considering that ADGRL 1 was still present in the ADGRL 3 KD TM3s, a severe drop in DAG levels could not expected. Nonetheless, TCAP-1 may be acting partially through ADGRL 1, as no increase was seen in DAG levels in the ADGRL 1 KD, however not to the same extent apparently. Lastly, the fact that a change was only observed in DAG levels once again further indicates that TCAP-1 has tissue specific effects due to tissue-specific receptor activity.

4.5 Conclusion

4.5.1 Summary of Findings

Several novel findings were found as a result of this research project. Importantly this study cooroborates previous work that showed TCAP-1 stimulates an increase in fecal and serum testosterone in male mice. A different TCAP-1 administration regime was utilized in my study however, where mice were treated less frequently, yet similar findings were achieved. To determine whether the observed TCAP-1 action has a direct action on the testes, I utilized immortalized mouse Leydig (TM3) and Sertoli (TM4) cell lines. Because these cells have not been characterized with respect to the presence of teneurins, TCAPs and their putative receptors (ADGRLs), I examined the presence of these molecular components using immunostaining and PCR-based techniques. This work represents the first time that immortalized mouse testes somatic cell lines have been characterized and developed as a model to understand TCAP-1 function in the testes. Functionally, I have shown that TCAP-1 induces isolated testosterone synthesis in these cells suggesting that TCAP-1 can act on the testes to regulate testosterone synthesis, independent of hypothalamic gonadotropin-releasing factor (GnRH) and the pituitary gonadotropins (luteinizing hormone, LH; follicle stimulating hormone, FSH). Finally I have shown that TCAP-1 modulates the activity of two key secondary messengers (diacylglycerol, DAG; inositol triphosphate, IP₃) associated with its putative receptor, ADGRL. This action was confirmed by knocking down the
dominant receptor isoforms, ADGRL 1 and 3, via transient transfection, in the TM3s. Identical 
TCAP-1 treatment was applied to the newly transfected cells and no increase in DAG or IP$_3$ was 
detected in the ADGRL 1 knockdown cells. Further, a significant decrease was observed for DAG 
levels in ADGRL 3 knockdown cells, while no change was observed for IP$_3$. This suggests that 
TCAP-1 is acting through two different receptors, which has been shown previously in 
hypothalamic neuronal cell lines. Taken together, these studies indicate that TCAP-1 may be a 
significant regulatory agent with respect to not only gonadal testosterone synthesis, but also 
reproduction in general. From a practical perspective, TCAP-1 may be useful to treat some forms 
of infertility in medical and veterinary situations.

4.5.2 Application of Findings

Currently very little is known about the adhesion GPCRs, as they are a relatively newly discovered 
family of receptors. To date, all ADGRL research has focused on its neuronal activity, and no 
investigations have been made into a possible involvement in reproductive signaling systems. 
Therefore, the purpose of this study was to explore the relationship between teneurin/TCAP and 
ADGRL in male reproductive tissue in order to further elucidate the signaling mechanism 
responsible for TCAP-1 activated increase in testosterone levels.

These results indicate that TCAP-1 is capable of stimulating steroid synthesis, with no observed 
side effects in vivo. Therefore, there are many possible applications in reproductive medicine as 
well as age-related disorders. It can be incorporated into a treatment regime for hypogonadism, 
especially in aging men. It could also be relevant to female aging, including forms of menopausal 
hormone therapy.

4.5.3 Future Studies

There is a lot still to be uncovered about the interplay of the teneurins and adhesion GPCR 
signaling, however it is evident that this is a phylogenetically conserved complex essential for 
reproductive function. The Teneurin/TCAP-ADGRL intercellular adhesion complex is now known 
to exist in the mammalian testis. This is novel evidence of the interaction of these three proteins 
outside of the CNS. It is capable of activating steroidogenesis independently of the HPG-axis in
As all the players in this adhesion complex have been found in the testis, it is therefore possible that TCAP-1 could be acting on the testes to stimulate testosterone production. However, future work still needs to be done in order to confirm a direct action of TCAP-1 on the gonads.

One study could focus on quantifying steroidogenic enzyme activity in TCAP-1-treated cells. For instance, both mRNA and protein expression levels of proteins involved in the steroidogenic pathway, including 17α-Hydroxylase, C17, 20 Lyase, Aromatase and 17 KSR isolated from TCAP-1 treated immortalized cells, such as the TM3s. The TM3s could also be co-cultured with the immortalized Sertoli cell line (TM4) in order to better understand the interplay between these two cell types.

New studies indicate a possible overlap in function between the teneurins and GnRH, and thus the HPG-axis. However very little is still known about the direct relationship. The in vitro results from this study indicate that TCAP-1 is capable of stimulating steroidogenesis independent of the HPG-axis. However, in vivo it is unknown if TCAP-1 acts directly on the gonads to stimulate steroidogenesis. It is possible that it could be stimulating GnRH activity by interacting with the GnRH receptor or possibly the Kisspeptins. A FITC-TCAP-1 uptake study could therefore be performed on either primary or immortalized hypothalamic cells to identify co-localization between either Kisspeptin or GnRH neurons with TCAP-1.

Two different mouse studies could be performed in order to better understand the direct actions of TCAP-1 in vivo. One might involve administering TCAP-1 subcutaneously to hypogonadal mice. Subsequent analysis of germ cell number would indicate if TCAP-1 is able to re-establish spermatogenesis by elevating intratesticular testosterone (ITT) levels. Alternatively, mice could be treated with LH agonists, thus eliminating the HPG-axis component to testosterone production and administered TCAP-1 in similar fashion. Results would indicate if TCAP-1 actions are independent of the HPG-axis.

Similar studies could be performed on female somatic cell lines, including the theca cells as well as the ovarian Granulosa cells. Theca cells are androgen-producing cells in the female reproductive system and therefore could be analysed similarly as the immortalized Leydig (TM3) cells. Granulosa cells have the capability to convert androgen to estradiol and function similarly as the male Sertoli cells. Co-culture studies could also be useful as this would be more
physiologically similar to natural conditions in the whole body level. Also, the fallopian tubes are known to show significant morphological changes during the different stages of the menstrual cycle (Crow et al., 1994). Therefore, it would be interesting to investigate teneurin/TCAP and dystroglycan activity in maintenance of the epithelial integrity in the fallopian tube.
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