Subfornical organ neurons integrate cardiovascular and metabolic signals

by

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Abstract

The subfornical organ (SFO) is a critical circumventricular organ involved in the control of cardiovascular and metabolic homeostasis. Despite the abundant literature clearly demonstrating the ability of SFO neurons to sense and respond to a plethora of circulating signals that influence various physiological systems, investigation of how simultaneously sensed signals interact and are integrated in the SFO is lacking. In this study, we use patch clamp techniques to investigate how the traditionally classified ‘cardiovascular’ hormone angiotensin II (ANG), ‘metabolic’ hormone cholecystokinin (CCK) and ‘metabolic’ signal glucose interact and are integrated in the SFO. Sequential bath-application of CCK (10nM) and ANG (10nM) onto dissociated SFO neurons revealed that: 63% of responsive SFO neurons depolarized to both CCK & ANG; 25% depolarized to ANG only; and 12% hyperpolarized to CCK only. We next investigated the effects of glucose by incubating and recording neurons in either hypo-, normo- or hyperglycemic conditions for a minimum of 24 hours and comparing the proportions of responses to ANG (n=55) or CCK (n=83) application in each condition. A hyperglycemic environment was associated with a larger proportion of depolarizing responses to ANG ($\chi^2$, p<0.05), and a smaller proportion of depolarizing responses along with a larger proportion of hyperpolarizing responses to CCK ($\chi^2$, p<0.01). These data demonstrate that SFO neurons excited by CCK are also excited by ANG, suggesting that CCK may influence fluid intake or blood pressure via the SFO, complementary to the well-understood actions of ANG at this site. Additionally, the demonstration that glucose environment affects the responsiveness of neurons to both these hormones highlights the ability of SFO neurons to integrate multiple metabolic and cardiovascular signals to affect transmission of information from the circulation to the brain, which has important implications for this structure’s critical role regulation of autonomic function.
Co-Authorship

Components of the introduction of this thesis have been borrowed, with permission, from Chapter 1.2.2. “Regulation of Nervous System Function by Circumventricular Organs” from Neuroimmune Pharmacology, 2nd edition submitted to Springer in the fall of 2015, which is currently in press, and from the review article “Neurohormonal Integration of Cardiovascular Function by the Lamina Terminalis” published in Current Hypertension Reports in December 2015. Both works were a collaboration between Nicole Cancelliere, Emily Black, and Dr. Alastair Ferguson.
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### List of Abbreviations

(a)CSF ............................................. (artificial) cerebral spinal fluid  
AGRP/NPY .............................................................. agouti-related peptide/neuropeptide Y producing neurons  
ANG ........................................................................... angiotensin II  
ANOVA ........................................................................ analysis of variance  
ARC ............................................................................. arcuate nucleus of the hypothalamus  
AT1R ............................................................... angiotensin 1 receptor  
ATP ............................................................................ adenosine triphosphate  
AV3V .......................................................... anteroventral third ventricle region  
BBB .......................................................................... blood-brain barrier  
BP ............................................................................. blood pressure  
CAMKII .................................................. calcium/calmodulin-dependent protein kinase II  
CCK ............................................................................. cholecystokinin  
CCK1R ............................................................. cholecystokinin receptor 1  
CCK2R ............................................................. cholecystokinin receptor 2  
CNS ................................................................. central nervous system  
CVO(s) ..................................................................... circumventricular organ(s)  
DAG ........................................................................... diacylglycerol  
DREADD .............................................. Designer Receptors Exclusively Activated by Designer Drugs  
Fig ............................................................................. figure  
GABA ........................................................................... gamma-aminobutyric acid  
GE ............................................................................ glucose excited neurons  
GI ............................................................................. glucose inhibited neurons
GLP-1 .......................................................... glucagon-like peptide- 1
hr(s) ........................................................... hour(s)
i.c.v. ............................................................... intracerebroventricular
i.d. .............................................................. intraduodenal
i.v. ............................................................... intravenous
IP3 ............................................................. inositol triphosphate
KO ............................................................... knock-out
LH ............................................................... lateral hypothalamus
LTS ............................................................... lamina terminalis
min(s) ........................................................... minute(s)
MnPO ........................................................... median preoptic nucleus
$V_m$ ............................................................ membrane potential(s)
$V_r$ ............................................................. resting membrane potential
NSCC ........................................................... non-selective cation channel
NT ............................................................... normotensive rats
NTS ............................................................. nucleus of the solitary tract
OVLT ........................................................... organum vasculosum of the lamina terminalis
PIP2 ........................................................... phosphatidylinositol bisphosphate
PVN ........................................................... paraventricular nucleus of the hypothalamus
mPVN ......................................................... magnocellular neurons of the paraventricular nucleus
pPVN ......................................................... parvocellular neurons of the paraventricular nucleus
PYY ........................................................... peptide tyrosine tyrosine (or YY)
qPCR ........................................................... quantitative polymerase chain reaction
RT-PCR ................................................. reverse transcription polymerase chain reaction
s.............................................................second(s)
SFO....................................................................................................................................subfornical organ
SHR ..........................................................................................................................spontaneously hypertensive rats
SNS ...............................................................................................................................sympathetic nervous system
SON............................................................................................................................supraoptic nucleus of the hypothalamus
TIID ....................................................................................................................................type II diabetes
Chapter 1

Introduction

1.1 Homeostasis

In order to maintain homeostasis, it is important that the brain is able to integrate the various signals circulating in the blood, which represent information about the “milieu interieur”. The central nervous system (CNS) requires this sensory feedback in order to initiate appropriate physiological responses to keep up with a constantly changing external and internal environment. Life itself is ultimately dependent on the ability of the organism to continually and simultaneously regulate these physiological processes, and if equilibrium is not effectively maintained, disease may ensue. For example, an animal that consumes food in excess of its metabolic needs may develop obesity (Wynne et al., 2005), or another that is exercising and unable to increase cardiac output can develop hypotension leading to a loss of consciousness (Dampney et al., 2002). Regulation of cardiovascular and metabolic homeostasis is critical for survival as these physiological processes ensure that tissues receive the necessary oxygen and nourishment required to maintain life. These processes include, but are not limited to, control of heart rate, blood pressure, glucose availability, fluid and food intake (Sherwood, 2008). Information regarding these autonomic systems come from a variety of sensors including thermoreceptors, baroreceptors, chemoreceptors, as well as those monitoring fluid volume and metabolic state (Dampney et al., 2002; Andersson et al., 1982; Dhill, 2007). Sensors convey information in the form of signaling molecules, such as amino acids, peptides, gases and larger macromolecules. These signals are
released from one cell and then influence the function of another. They are delivered to their site of action at target cells by the blood (hormones), synaptic terminals (neurotransmitters) or local mechanisms (paracrine) (Sherwood, 2008). Neuronal systems within the CNS that control autonomic function can detect these molecules and adjust physiological systems of the body accordingly in order to maintain the critical internal environment of a well-regulated homeostatic system. However, many of these signaling molecules that originate in the periphery and play important roles in the central regulation of cardiovascular and metabolic function are large and/or lipophobic and thus do not readily cross the blood brain barrier (BBB) (Ermisch et al., 1993). How then do circulating signals access the CNS to provide feedback information about the peripheral environment?

1.2 The Blood-Brain Barrier Conundrum

The BBB is a physiochemical barrier that separates the CNS from the peripheral circulation. A key feature that makes the BBB resistant to diffusion of polar solutes and macromolecules from the blood plasma to the brain extracellular fluid is the presence of tight junction proteins, such as zona-occludins 1, between cerebral capillary endothelial cells (Abbott et al., 2010). The endfeet of astrocytic glial cells are important for upregulating tight junction proteins and are therefore play a crucial role in the differentiation of the endothelium into the barrier during embryonic angiogenesis and in maintaining barrier properties later in life (Abbott et al., 2006). Barrier induction, maintenance and function are also supported by pericytes, microglia, and nerve terminals, which are also associated with the endothelium (Abbott et al., 2006).
The BBB therefore restricts the way in which circulating signals, which carry important information regarding physiological status, can access the CNS. Routes of transport across the BBB include passive diffusion and active efflux carriers (ie. lipid soluble non-polar molecules), passive or active carrier-mediated influx via solute carriers (ie. glucose and small peptides), receptor mediated transcytosis (ie. insulin and leptin), adsorptive-mediated transcytosis (ie. histone and select positively charged macromolecules), or via diapedesis through endothelial cells (ie. leukocytes) (Abbott et al., 2010). Another way circulating signals can be communicated to the CNS is via signaling at areas of the brain that lack a complete BBB, known as the sensory circumventricular organs (CVOs).
2.1 Circumventricular Organs

The sensory CVOs are highly vascularized midline structures characterized by a high expression of receptors for circulating signals, and include the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT) (which protrude from the rostral wall and sit in anterior wall of the third ventricle located in the forebrain, respectively), and area postrema (AP) (which protrudes into the fourth ventricle situated in the hindbrain adjacent to the nucleus of the solitary tract) (Ganong, 2000). Immunofluorescent studies visualizing occludin-associated protein distribution in the brain have shown that the sensory CVOs lack tight-junction proteins (claudin-5, occludin and zona occludins-1) (Morita et al., 2015; Petrov et al., 1994), which is what give them this unique feature of a “leaky” barrier. As demonstrated in Figure 1, lack of tight junction proteins leads to the formation of fenestrations between endothelial cells, which create Virchow-Robin Spaces (Broadwell & Sofroniew, 1993). These spaces allow circulating blood containing large lipophobic signals to enter and pool, facilitating free exchange between the blood and nervous tissue of the body.
Figure 1. Circumventricular Organs contain a leaky blood-brain barrier, unlike the rest of the brain (modified from Price et al., 2008)

Endothelial cells (blue) lack tight junction proteins (such as occludin), which lead to fenestrations that allow large lipophobic molecules circulating in the blood to access the neurons (green), expressing various signal receptors, within the CNS. Fenestrated capillaries allow for the formation of sinuses called Virchow-Robin spaces that slow the flow of blood passing by the CVOs and aid the diffusion of molecules from the blood to the nervous tissue.

Additional areas of the brain that lack a complete BBB also exist, and these are known as the secretory CVOs. These structures are in many ways contrasting to the sensory CVOs in that their apparent primary function is to release, rather than detect, signals at the blood-brain interface. The secretory CVOs include the neurohypophysis, median eminence, intermediate lobe, and pineal gland (Duvernoy & Risold, 2007). These structures play an important role in the release of centrally synthetized lipophobic hormones directly into the circulation or cerebrospinal fluid. The secreted products are synthesized in parts of the brain that are protected by the BBB such as the supraoptic, paraventricular and arcuate nuclei, and thus these specialized areas of fenestrated
capillaries permit the release of synthesized lipophobic products into the general circulation (Duvernoy & Risold, 2007). The neurohypophysis is the posterior lobe of the pituitary gland and secretes oxytocin and vasopressin, hormones involved in reproduction and fluid balance, directly into general circulation (Sofroniew, 1983; Sofroniew et al., 1981; Ooi et al., 2004). The intermediate lobe of the pituitary gland, which separates the anterior and posterior lobes of this endocrine gland, in humans, synthesizes and secretes melanocyte-stimulating hormone (MSH) (Ooi et al., 2004). The median eminence (ME), perhaps the most important of the secretory CVOs, consists of neuronal terminals which “synapse” on the hypophyseal portal capillaries, the entry point for hypothalamic trophic hormones controlling the extensive endocrine roles of the anterior pituitary. The hormones secreted at the ME include gonadotropin-releasing hormone, growth hormone-releasing hormone, dopamine, thyrotropin-releasing hormone, and corticotropic-releasing hormone (Ganong, 2000; Ooi et al., 2004). The pineal gland is the final secretory CVO which produces melatonin, a critical component in the regulation of circadian rhythms. It secretes this hormone not only into the circulation, but also into the cerebrospinal fluid (Cassone et al., 1993).

Contrary to the secretory CVOs, the three sensory CVOs contain minimal afferent input relative to their extensive neural outputs. Additionally, most of the afferent input received by the sensory CVOs seems to come from the very structures onto which they project, indicating the probability of reciprocal communication between the sensory CVOs and the other structures (Cottrell & Ferguson, 2004). The information received by these structures is communicated via efferent neurons to areas in the brain protected by the BBB, many of which utilize the secretory CVOs to secrete hormones to affect physiological function (Boudaba et al., 1995). The next section
of this thesis will review the current understanding of the anatomy, circuitry and functions of the SFO, the sensory CVO of interest for my master’s thesis.

2.2 The Subfornical Organ

2.2.1 Neuroanatomy

Understanding the anatomy and connectivity of any region of the brain is important as it provides valuable insight into its role in the regulation of nervous system function. Thus, this section will provide a more detailed description of anatomical location and outline the neuronal connections associated with the SFO in order to provide a framework for the understanding of its central roles in the regulation of autonomic state.

The SFO is a highly vascularized, translucent midline structure protruding from the rostral wall of the third ventricle in the dorsal region of the lamina terminalis (Duvernoy & Risold, 2007). It lies between the columns of the fornix and its dorsal end is attached to the hippocampal commissure. It consists of a ventral stalk and dorsal crest, which connects to the median preoptic nucleus and the tela choroidea of the third ventricle, respectively (Oldfield & McKinley, 1985) (see review McKinley et al., 2003). The rich blood supply that surrounds this structure is formed by an anastomosis between branches of the anterior cerebral artery and the posterior choroidal artery (Polzovic et al., 1994). The vascularization is so dense that it must be peeled away during microdissection in order to see the SFO. The SFO can be subdivided into two major regions based on histological and functional assessment- the ventromedial core and the outer shell (McKinley et al., 2003;Morita et al., 2015). The core region is the largest, and is exclusively composed of
neuronal cell bodies and glial cells, and the rostral and caudal regions surround the core and contain very few neurons and glial cells, consisting mainly of nerve fibers (Dellmann & Simpson, 1979).

The major contributions to the current understanding of the SFO’s neurocircuitry can be attributed to the comprehensive studies by the labs of Hernesniemi (1972), Miselis (1981 and 1982), and Lind (1982). The earlier study used lesion techniques and Golgi staining methods (Hernesniemi et al., 1972), and the latter investigations used horseradish peroxidase injections to follow anterograde and retrograde transport of labeled proteins through axons (Miselis, 1982; Miselis, 1981; Lind et al., 1982). Evidence for these connections are supported in more recent studies using electrophysiological stimulation (Ferguson & Bains, 1996; Bains & Ferguson, 1995).

A summary of the major and minor afferent and efferent neuronal connections to and from the SFO are outlined in Figure 2.
Figure 2. The major afferent and efferent connections of the SFO

OVLT organum vasculosum of the lamina terminalis; *Hypothalamic structures*: MnPO median preoptic nucleus, SON supraoptic nucleus, PVN paraventricular nucleus, LH lateral hypothalamus, Arc arcuate nucleus; *Hindbrain*: LPBN lateral parabrachial nucleus, NTS nucleus of the solitary tract. (See text for references)
The major efferent connections can be grouped into two general areas:

1. *The neuroendocrine and autonomic control centers of the hypothalamus*

   Direct (monosynaptic) and indirect (polysynaptic) efferent projections terminate in the anterior and tuberal supraoptic nuclei (SON), and the paraventricular nucleus (PVN) including its rostral accessory cluster, respectively (Miselis, 1981). Electrophysiology studies have demonstrated excitatory SFO projections to vasopressin- and oxytocin-secreting magnocellular neurons in the SON and PVN, as well as in parvocellular areas of the PVN, which, in turn, project either to the median eminence, the medulla, or the spinal cord (Ferguson & Bains, 1996). Many efferent fibers to the hypothalamus emerge from the rostral SFO and enter the columns of the fornix, diverge with the ventral stria medullari to disperse medially and laterally over the columns of the fornix and along their dorsal border at the anterior dorsal level of the columns trajectory through the hypothalamus (Miselis, 1981).

2. *The anteroventral third ventricular (AV3V) area*

   This includes the median preoptic nucleus (MnPO), the anterior periventricular (Pe) area of the hypothalamus, and the organum vasculosum of the lamina terminalis (OVLT). Many efferent fibers to this area emerge from the rostral SFO, pass anteriorly over the anterior commissure in the midline and either descend along the anterior border of the MnPO or enter the Pe dorsally just beneath the anterior commissure (Miselis, 1981).

   It is important to note that dendritic trees of SFO neurons are relatively compact and the extent of afferent connectivity is not nearly as elaborate as the efferent (Dellmann & Simpson, 1979). These neuroanatomical findings suggest that there is reciprocal communication occurring
between these brain regions, and that perhaps the SFO’s primary afferent information is received from circulating signals in the peripheral circulation as opposed to signals from other brain regions.

Now that a foundation has been laid for understanding the circuits in which the SFO exists, the following sections will explore how activation of the SFO and its connections to these various autonomic regulating nuclei influence cardiovascular and metabolic homeostasis.

### 2.2.2 Role in Regulation of Cardiovascular Homeostasis

Understanding the regulation of blood pressure is classically focused primarily on the mechanisms controlling heart rate and blood vessel contractility. However, regulation of fluid balance and sodium homeostasis also exert profound effects especially on the long-term integrated control of the cardiovascular system. The ways in which the SFO integrates and then regulates information from these three complementary physiological processes will be the primary focus of this section.

Our understanding of the role of the SFO in cardiovascular regulation initially developed from studies describing its unique ability to detect peripheral vasoactive peptides and, consequentially, influence neuronal activity in central pathways (neuroendocrine and autonomic) known to play critical roles in the regulation of blood pressure, which include the lamina terminalis (LT- includes the SFO, MnPO and OVLT) and the brainstem (NTS) (Figure 3). Much of this information grew out of the demonstration in the 1980’s, reviewed below, that the SFO was the primary CNS site at which circulating angiotensin II (ANG) acted to influence cardiovascular function (Mangiapan & Simpson, 1980b; Gutman et al., 1988).
Figure 3. Neuronal pathways in the lamina terminalis that influence cardiovascular homeostasis (modified from Cancelliere et al., 2015)

Circulating signals in the periphery are sensed by osmosensitive neurons (yellow arrows) in the circumventricular organs of the lamina terminalis (pink circles) and communicated to:

i) vasopressin- and oxytocin-producing magnocellular neurons (solid green arrows) in hypothalamic neuroendocrine effector nuclei (blue circles) directly, or via the median preoptic nucleus (green circle). These hormones are released from the posterior pituitary gland into the circulation (dotted green arrows) and cause vasoconstriction in blood vessels, and antidiuresis or natriuresis in the kidneys to influence blood pressure; or

ii) central pre-autonomic neurons (purple arrows) in the paraventricular nucleus of the hypothalamus that project to the nucleus of the solitary tract in the hindbrain (an integrative structure involved in autonomic regulation), or the intermediolateral cell column of the spinal cord, which ultimately stimulates the sympathetic nervous system to influence blood pressure.

SFO subfornical organ; MnPO median preoptic nucleus; OVLT organum vasculosum of the lamina terminalis; PVN paraventricular nucleus; SON supraoptic nucleus; PP posterior pituitary; NTS nucleus of the solitary tract; VP vasopressin; OXY oxytocin; IML intermediolateral cell column.
Angiotensin

ANG is a product of the renin-angiotensin system and is produced when plasma sodium levels or renal blood flow are low (Fitzsimons, 1998). Experiments utilizing high performance liquid chromatography and radioimmunoassay techniques demonstrated that plasma angiotensin II levels in normal male pentobarbital-anesthetized rats were between 53.0 and 141.6 pg/ml (mean: 103.9 +/- 29.7 pg/ml) or about 100pM (Huang et al., 1989). This study also showed that ANG plasma levels reached an average high of about 300pM (300.0 +/- 100.6 pg/ml) and a low of 35pM (35.7 +/- 28.0 pg/ml) in rats fed a sodium deficient diet or oral enalapril (angiotensin converting enzyme inhibitor) for 1 week, respectively. Levels fell below 3pM in bilaterally nephrectomized rats 48 hours post-surgery (Huang et al., 1989). Aside from ANG’s well-described pressor roles in the body, including constriction of blood vessels and sodium reabsorption in the kidney (Ramkumar & Kohan, 2016)), ANG has also been shown to regulate cardiovascular homeostasis via signaling in the brain. However, due to this peptide hormone’s hydrophilic properties, it is unable to cross the BBB, and instead is understood to trigger changes in the brain via activation of angiotensin 1 receptors (AT1Rs) at the CVOs (Lenkei et al., 1995; Ferguson & Bains, 1997).

Since the early 1980’s, it has been repeatedly shown that circulating ANG acts at the SFO to initiate a pressor response in the rat (Mangiapanese & Simpson, 1980b; Gutman et al., 1988), a response which can be reduced by lesioning the SFO (Mangiapanese & Simpson, 1980a). Mangiapanese and Simpson demonstrated, in both conscious and anaesthetized rats, increased blood pressure in response to ANG administered into the SFO that was blocked by administration of the ANG antagonist saralasin (Mangiapanese & Simpson, 1980b), identifying the SFO as the specific CNS site for these actions and also confirming them to be ANG receptor-mediated. Using in vivo electrophysiological recordings, Gutman et al. showed that peripheral administration of ANG
caused increased firing rate in about half of the SFO neurons that were recorded (Gutman et al., 1988). Interestingly, of those that responded, 38% projected to the PVN and 36% to the SON. Electrical stimulation of the SFO has been shown to increase blood pressure (Ferguson & Renaud, 1984), actions which are a consequence of sympathetic activation and vasopressin release (Ferguson & Kasting, 1986), causing vasoconstriction and water retention by the kidneys.

Implication of a role for ANG and the SFO in the disease state of hypertension has been recognized for quite some time. In the late 1980’s, radioactive ANG antagonists were used to autoradiographically localize and compare ANG receptor binding sites across the central nervous system of spontaneously hypertensive (SHR) and normotensive (NT) rats (Gutkind et al., 1988). Gutkind et al. report that both young and old SHR had significantly higher ANG binding site concentrations in the SFO, MnPO, PVN, and NTS when compared to age matched NT animals. Interestingly, the number of ANG receptors in the SFO of SHR can be decreased via systemic inhibition of angiotensin converting enzyme with enalapril (Nazarali et al., 1990). In vivo electrophysiological data show that SFO neurons in SHR that project to the PVN have a significantly higher spontaneous firing rate and a greater excitatory response to ANG injection into the carotid artery when compared to NT animals (Miyakubo et al., 2002). Additionally, deoxycorticosterone acetate (DOCA)-salt-induced hypertension is blunted by the deletion of angiotensin type 1a receptors (AT1aR) within the subfornical organ (Hilzendeger et al., 2013). These data implicate ANG receptor expression in the SFO and the neural connection between the SFO and PVN in the pathology of hypertension. Interestingly, not only can the SFO sense circulating ANG, but there is evidence to suggest that ANG is produced by the SFO and functions as a neurotransmitter in the central angiotensinergic pressor response circuit (Sinnayah et al., 2006) as well.
We have selected ANG as our ‘cardiovascular hormone’ based on this rich understanding of its mechanism of actions for regulating cardiovascular homeostasis at the SFO.

**Fluid Balance**

Various physiological processes are responsible for the regulation of fluid balance, including thirst that leads to water consumption, and vasopressin release that leads to antidiuresis. Numerous studies over the past few decades have used lesion and stimulation techniques to enhance our understanding of the roles of the SFO in the regulation of fluid balance.

Early studies showed that lesions of the AV3V region (which includes efferent projection fibers from the SFO and major components of the LT) produced severe adipsia (Buggy & Johnson, 1977; Lind & Johnson, 1983). In conscious rats, the immediate early gene c-Fos was expressed in the SFO, MnPO and OVLT in response to thirst inducing doses of hypertonic solutions or ANG administered intravenously, effects which were abolished after lesioning the SFO and OVLT (Oldfield *et al.*, 1991; McKinley *et al.*, 1992).

It has been known for quite some time that electrical stimulation of the SFO induces drinking behaviour in rats (Smith *et al.*, 1995), observations confirmed and expanded in recent *in vivo* studies utilizing optogenetic and Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology (Oka *et al.*, 2015; Nation *et al.*, 2016). Oka et al., 2015 used optogenetics to stimulate calcium/calmodulin-dependent protein kinase II (CAMKII)-positive or vesicular GABA transporter-positive SFO neurons and showed that water intake by these animals could be elicited and inhibited, respectively (Oka *et al.*, 2015). The excitatory neuronal projections were shown to express a high level of AT1 receptors and had nerve terminals projecting to the

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MnPO and PVN (Oka et al., 2015). Nation et al., 2016 showed that activation of CAMKII-positive SFO neurons using an excitatory DREADD caused robust increases in water intake in animals (Nation et al., 2016).

**Sodium Homeostasis**

The body has a number of mechanisms it can use to ensure that plasma sodium levels are properly regulated in order to maintain healthy cardiovascular status. During dehydration, preference for salt is reduced. Also, the kidneys are able to regulate plasma sodium levels via sodium excretion, renal sympathetic nerve activity, and secretion of renin.

Interestingly, lesioning the ventral area of the LT has been shown to inhibit salt intake in rats (Chiaraviglio & Perez Guaita, 1984). Ablation of the AV3V area in sheep abolished dehydration-induced natriuresis and caused a severe increase in serum sodium concentration and osmolality (McKinley et al., 1983). A study by Park et al. (1989) indicated that dehydration-induced natriuresis was not affected despite renal denervation, suggesting that the signal from the brain to the kidneys is hormonal as opposed to electrical (Park et al., 1989). There is evidence to suggest that this signal is oxytocin in rats (Huang et al., 1996), but not in humans (Rasmussen et al., 2004). This hormonal signal in humans is still under investigation.

Renal vasodilation and renin excretion are both influenced by renal sympathetic nerve activity. Using injections of the pseudorabies virus into the rat kidney, retrograde infection unveiled polysynaptic connections between the SFO, OVLT, MnPO and the kidney (Sly et al., 1999). They then showed that these sympathetic nerves could be stimulated via systemic infusion of hypertonic saline, ANG, and dehydration (Sly et al., 2001); a result that could be prevented via
ablation of the LT (May et al., 2000). Neurons in the SFO and OVLT are able to sense the increased levels of peripheral sodium, whether due to injection of hypertonic saline or dehydration, as a consequence of the sodium sensing ability of Na\textsubscript{x} channels which are highly and specifically expressed in these regions (Noda, 2006). Interestingly, unlike healthy controls, Na\textsubscript{x} knock-out (KO) mice do not stop ingesting salt when dehydrated and do not show an aversion to salt intake after microinjection of hypertonic NaCl into the cerebral ventricle. This abnormal salt-intake behaviour of Na\textsubscript{x} KOs was rescued by transduction of the Na\textsubscript{x} gene into the SFO, suggesting that Na\textsubscript{x} channel expression in the SFO is imperative to maintaining healthy sodium homeostasis (Noda, 2006).

2.2.3 Role in Regulation of Metabolic Homeostasis

Before, after, and between meals exists a complex system of hormonal signaling which aids in the digestion and regulation of food intake and metabolism. Production and secretion of various blood-borne peptides are stimulated by specific physiological events or conditions, each of which has the potential to be influenced by physiological state. For example, ghrelin is a peptide produced by ghrelinergic cells in the gastrointestinal (GI) tract when the stomach is empty and increases gastric acid secretion and GI motility to prepare the body for food intake (Al et al., 2014). During meal consumption, the introduction of chyme (containing fatty acids, certain amino acids, and nutrients) from the stomach into the small intestine stimulates the I- and L-cells in the mucosa of the gastrointestinal tract to release various gut hormones such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and peptide YY (PYY) (Dhillon, 2007). These peptides then enter the bloodstream and travel to effector organs to perform a multitude of functions including
signaling the release of various digestive enzymes from the pancreas and bile from the gallbladder (role of CCK), stimulating the release of peptides hormones such as insulin or glucagon from the pancreas (role of GLP-1), or inhibiting gastric motility (role of PYY) (Murphy & Bloom, 2006). Insulin and amylin are co-secreted from pancreatic beta cells in response to rising glucose levels and function to prevent post-prandial spikes in blood glucose levels. Between meals, adipocytes, or fat cells, release the protein hormones adiponectin and leptin that are responsible for regulating metabolic processes involved in energy homeostasis such as glucose regulation and fatty acid oxidation (Ahima, 2006).

Interestingly, in addition to their unique roles in digestion and energy metabolism, these blood borne peptides all possess the ability to act as hunger or satiety signals in the central nervous system (Murphy & Bloom, 2006; Murphy & Bloom, 2004). They can be simplistically categorized into two types: orexigenic- causing a sensation of hunger and promoting food intake (only known GI peptide to date is ghrelin), or anorexigenic- causing a sensation of satiety and inhibiting food intake (ie. CCK, GLP-1, PYY, adiponectin, and leptin). However, due to the large size or lipophobic nature of these peripheral signals, they are unable to cross the BBB and therefore alternative methods through which they communicate with the brain have been proposed, one being by actions in the sensory CVOs.

**Energy Balance**

Over the past few decades there has been growing evidence to support the idea that sensory CVOs, initially the AP and more recently the SFO, may be involved in the neural circuitry underlying hunger and satiation, specifically by acting as an integrative ‘gate keeper’ which senses
various circulating hormones and transmits the information to areas of the hypothalamus and brainstem. The presence of receptors in the SFO for these various gastrointestinal hormones involved in energy homeostasis have been confirmed using various scientific approaches including immunostaining, in situ hybridization, as well as pharmacological approaches. Many of these peptide hormones cause increased c-Fos expression (an indirect marker of neuronal activity) in the SFO when injected intraperitoneally, and/or have been shown to cause changes in the excitability of neurons in dissociated or slice preparations. The SFO can sense and respond to various metabolic signals including glucose (Medeiros et al., 2012), ghrelin (Takayama et al., 2007; Pulman et al., 2006), leptin (Smith et al., 2009), amylin (Riediger et al., 1999), adiponectin (Alim et al., 2010), PYY (Baraboi et al., 2010a) and cholecystokinin (Ahmed et al., 2014) (see reviews Cottrell et al., 2004; Hoyda et al., 2009; Smith & Ferguson, 2010). These data are important as they show that the receptors expressed by the SFO are in fact able to continuously monitor these circulating signals involved in energy homeostasis.

When examining the role of the SFO in energy balance on a behavioural level, stimulation of the SFO has been shown to cause increased food consumption in rats (Smith et al., 2010). Additionally, combined lesion of the SFO and AP causes significantly less daily food intake and decreased body weight for up to one month compared to sham animals and animals with only one of the two regions lesioned (Baraboi et al., 2010b). More recent literature, however, has suggested that the SFO’s role in food intake is not as clear as its effects on drinking. Oka et al., 2015 used optogenetics to stimulate what they defined as excitatory and inhibitory SFO neurons and showed that water intake by these animals could be elicited and inhibited, respectively, without effects on food intake (Oka et al., 2015). Conversely, Nation et al., 2016 showed that DREADD activation of CAMKII-positive SFO neurons caused robust increases in water intake accompanied by a small
increase in body weight after chronic stimulation using a synthetic ligand (Nation et al., 2016). Interestingly, when looking at how the SFO’s transcriptome is modified in both dehydrated and fasted rats compared to controls, it was found that the number of gene transcripts changed in animals that were deprived of food were 15x higher than those that were water-deprived (Hindmarch et al., 2008). Together these studies provide clear evidence of a critical role for the SFO in regulation of the cardiovascular system and an influential role in energy metabolism and provide conflicting evidence for whether or not these systems are controlled by interconnected or two separate circuits involving the SFO. The focus of my thesis research was to investigate the integration of cardiovascular and metabolic signals in the SFO in hopes of providing further insight into how these circuits interact.

**Cholecystokinin**

The ‘metabolic hormone’ signal we have chosen to investigate in our experiments is CCK. As previously described, this peptide hormone is released by the small intestine postprandially and has been shown to decrease meal size in rats (Peikin, 1989) and humans (Kissileff et al., 1981). Consequently, rats with a spontaneous CCK1R KO, known as Otsuka–Long–Evans–Tokushima Fatty (OLETF) rats, and CCK2R KO (CCK2R−/−) mice are both hyperphagic and develop obesity (Moran & Bi, 2006; Clerc et al., 2007). Obese CCK2R−/− mice also demonstrated hyperglycemia, hyperinsulinemia, impaired glucose tolerance and hepatic insulin resistance (Clerc et al., 2007). Lewis and Williams (1990) used duodenal perfusion of various foods and arterial blood sampling from unrestrained male Wistar rats to assess levels of endogenous CCK secretion. Their results showed that intact protein (casein- found in milk) and fatty acids (oleate-naturally occurring)
increased plasma CCK from a fasting level of 0.5 ± 0.1, to 3.8 ± 0.4 pM and 3.7 ± 0.6 pM, respectively, whereas glucose and calcium did not change plasma CCK levels significantly (Lewis & Williams, 1990). A similar experiment was conducted in humans using intraduodenal (i.d.) administration of fat, protein and starch and showed that similar to the rat, fat and protein significantly increased human CCK levels from baseline concentrations between 0.6 pM-2.1 pM, to peak concentrations of 4.8 ± 0.9 pM and 3.5 ± 0.5 pM, respectively, between 15-45 min post i.d. administration (Hopman et al., 1985). Studies have shown that circulating CCK elicits its anorexigenic effects by activating CCK₄ receptors located on the gastric branch of the vagus nerve (Edwards et al., 1986; Joyner et al., 1993; Reidelberger et al., 2003) and the area postrema (Edwards et al., 1986; Sun & Ferguson, 1997), and by indirect activation of CART neurons in the PVN of the hypothalamus (Peter et al., 2010) and the medial and commissural subnuclei regions of the NTS (Edwards et al., 1986; Cano et al., 2003). There is, however, a growing literature to suggest that the anorectic effects of CCK are not completely vagal afferent nerve-mediated as the inhibitory effects of CCK on food intake have been shown to be only partially attenuated via bilateral vagotomy (Joyner et al., 1993) and CCK₁ antagonists that cross the BBB are able to stimulate food intake in vagotomized animals (Reidelberger, 1992; Reidelberger et al., 2004), which suggests a centrally mediated role for CCK in food intake as well (Reidelberger, 1994). Radiolabeled CCK ([¹²⁵I]-CCK) injected into the rat common carotid artery and intravenously in the rabbit found no significant levels of radiolabeled CCK in the CSF of the brain, suggesting that this peptide does not cross the BBB (Oldendorf, 1981; Passaro et al., 1982). Interestingly, the latter study found that [¹²⁵I]-CCK injected into the lateral ventricle of the rabbit was found in the peripheral blood, suggesting a unidirectional non-carrier-mediated rapid diffusion of CCK from the CSF to the blood (Passaro et al., 1982). As previously discussed, another way in which
circulating signals that do not cross the BBB can communicate information to the brain is via the CVOs.

Recently, CCK has been shown to activate SFO neurons. Using whole tissue RT-PCR, Ahmed et al., 2014 demonstrated that the SFO expresses both CCK receptors (CCK₁ & CCK₂) (Figure 4A). This study also showed that SFO neurons are activated in vivo and in vitro using the functional activation markers c-Fos (Figure 4B&C) and p-ERK, and electrophysiological techniques, respectively (Ahmed et al., 2014). Although this study provides clear evidence that the SFO is a site of action of circulating CCK, at the current time, the physiological effects of CCK acting at the SFO are unknown. As the SFO is implicated in the regulation of both cardiovascular and metabolic homeostasis, it is possible that CCK could affect either of these processes.
Figure 4. The SFO expresses both CCK receptors and is activated by i.p. CCK administration (modified from Ahmed et al., 2014)

A: Ethidium bromide-stained agarose gel of the RT-PCR products generated with the CCK1R and CCK2R primers. Lane 1, DNA marker; lane 2, CCK1R receptor (301 bp); lane 3, no template control (NTC); lane 4, CCK2R (270 bp); lane 5, NTC. B&C: CCK-8s induces c-Fos activation in the SFO. B: Bar graphs comparing the number of c-Fos immunoreactive cells in vehicle treated (while bars) versus CCK-treated animals in the SFO. Data are presented as means ± SE (n = 9), **P < 0.01 represents a significant difference from the respective control. C: Representative immunofluorescence micrographs are shown. c-Fos immunoreactivity was very low in animals injected intraperitoneally with vehicle (CTL). Note that c-Fos immunoreactivity was observed in the SFO in the CCK-treated animals (bregma, −1.3). Scale bar = 100 μm.
Glucose

The third signal we have chosen to investigate the integrative capacity of the SFO with, is glucose. It is the body’s key source for energy as it is the substrate for aerobic respiration, anaerobic respiration and fermentation- the body’s metabolic processes that produce adenosine triphosphate (ATP) (Bonora et al., 2012). ATP is required to perform almost all cellular functions, including protein and membrane synthesis, cellular division, active transport and muscle contraction (Bonora et al., 2012), therefore, a change in glucose concentration is arguably one of the most important signals for communicating metabolic needs. Despite only comprising 2% of total body mass, the human brain consumes 20% of resting metabolic energy (Clarke & Sokoloff, 1999). The various processes performed by neurons, in order of energy consumption, include EPSPs evoked at postsynaptic sites, propagation of the signal along the axon, neurotransmitter release and glutamate recycling (Lennie, 2003). Glucose crosses the BBB via glucose transporters, the most abundant being the sodium independent facilitative transporter GLUT1 (Patching, 2016).

Blood glucose levels are closely regulated by the body as part of maintaining healthy metabolic homeostasis. High circulating glucose concentrations, or hyperglycemia, can occur due to leptin deficiency or leptin resistance. Leptin resistance often develops as a result of long-term obesity and lack of physical exercise, and if untreated can lead to the metabolic disease, type II diabetes (TIID). Hyperglycemia affects both metabolic and cardiovascular homeostasis, causing polyphagia, polydipsia and polyuria (known as the ‘hyperglycemic triad’), which make glucose a suitable signal to use, in addition to CCK and ANG, to investigate the integrative capacity of the SFO; an area of the brain implicated in cardiovascular and metabolic control.

Not only do neurons metabolize glucose for energy, but some have the ability to sense glucose; two different processes that should not be confused. Various area of the brain implicated
in control of energy homeostasis have been shown to be glucose-sensing, including the LH, ARC and VMN of the hypothalamus (Burdakov et al., 2005b), the NTS (Adachi et al., 1995) and the AP (Adachi et al., 1995) in the hindbrain, and the SFO (Medeiros et al., 2012). Neurons are classified as “glucose excited” (GE) if they depolarize to an increase in glucose or hyperpolarize to a decrease in glucose concentration, and “glucose inhibited” (GI) if they hyperpolarize to an increase in glucose or depolarize to a decrease in glucose concentration. Previous work in our laboratory showed that about half of SFO neurons are glucose-responsive, and of those, half are GE and the other half GI (Figure 5) (Medeiros et al., 2012). Using voltage-clamp experiments to find the reversal potentials of the channels mediating each response, this study showed that GE neurons were depolarized via activation of a channel with a reversal potential of -24mV, possibly a non-selective cationic channel, and that GI neurons were hyperpolarized by activation of ATP-sensitive potassium channel (K\textsubscript{ATP}) with a mean reversal potential of −78 ± 1.2 mV, as GI responses were not observed in the presence of the K\textsubscript{ATP}-channel blocker glibenclamide (Medeiros et al., 2012).
Figure 5. SFO neurons are glucose excitatory and inhibitory (modified from Medeiros et al., 2012)

A: Current-clamp recordings showing a depolarization with no return to baseline in response to increasing glucose from 1 to 10 mM, representing a GE neuron (left). B: Current-clamp recording from a separate SFO neuron showing a hyperpolarization with a return to baseline in response to increasing glucose from 1 to 10 mM, representing a GI neuron (right). C: Current-clamp recordings showing a hyperpolarization with recovery in response to decreasing glucose from 10 to 5 mM, representing a GE neuron. D: Current-clamp recordings showing a depolarization with return to baseline in response to decreasing glucose from 10 to 5 mM, representing a GI neuron. The red dashed lines represent the control baseline membrane potential.
2.2.4 Summary

In conclusion, cardiovascular regulation is irrevocably tied to the regulation of blood volume and osmolarity, as well as sodium concentration. These parameters are all regulated by various physiological structures and molecular signals and, as outlined above, a clear literature now supports the conclusion that the SFO plays a critical role in this integrated regulation of sodium, osmolarity, volume and blood pressure. Such integration occurs as a consequence of the complex sensory abilities of neurons within these structures, the interconnectivity of the SFO with the LT, hypothalamus and brainstem, and of course the ability of neural efferents to modulate diverse autonomic outputs in a physiologically integrated manner, as was summarized in Figure 3. Perhaps more important than this is an emerging perspective that the SFO, so well understood for its important role in fluid balance and cardiovascular regulation, also plays an important role in sensing metabolic signals. It is becoming clear that outputs from the SFO can influence the control of energy metabolism. Hence, there is a call for investigation of how cardiovascular and metabolic signals are interact and are integrated in the SFO.

2.3 The SFO as an Integrative Centre

Based on the data presented from the current literature, it is clear that the SFO is continuously sensing a plethora of circulating signals that influence a number of different physiological systems, leading to the obvious suggestion that single SFO neurons will sense and integrate information from multiple signals before relaying it to the rest of the brain. Despite the numerous studies showing the clear effects of individual signals on neurons, data examining the interaction and integration of these signals in single SFO neurons are limited. Previous
electrophysiology studies have shown that a subpopulation of angiotensin-responsive neurons in the SFO can also respond to vasopressin (Anthes et al., 1997), another cardiovascular hormone, and another ANG-responsive group also responds to oxytocin (Hosono et al., 1999), a traditionally classified reproductive hormone. When investigating the integration of two different satiety hormones, amylin and leptin were shown to both influence the same subpopulation of SFO neurons (Smith et al., 2009), whereas ghrelin and amylin affected different neurons (Pulman et al., 2006).

There is only one study to our knowledge that shows an interaction between a traditionally classified cardiovascular and a metabolic signal in single SFO neurons. This study published last year by Young et al. showed that selective deletion of angiotensin type 1a (AT1a) receptors in the SFO attenuated leptin-induced weight loss in mice by abolishing leptin-induced sympathetic activation of brown adipose tissue thermogenesis (Young et al., 2015). Collectively these data suggest that circulating signals are integrated in the SFO and may ultimately influence physiological function; emphasizing the need for further investigation of its integrative capacity.

2.3.1 Study Aims

Thus, the present studies were undertaken to investigate the interaction between select cardiovascular and metabolic signals that have been shown to influence SFO neurons, in order to gain more insight into how the SFO integrates signals circulating in the blood stream and communicates them to the CNS. Based on the fact that the SFO is a structure implicated in the control of both metabolic and cardiovascular homeostasis and is able to sense numerous signals implicated in each of these systems, the two hypotheses tested for this thesis are:
1. The majority of SFO neurons that respond to CCK will not respond to ANG, with some neurons responding to both.

2. Alterations in extracellular glucose concentrations will modify the responsiveness of SFO neurons to both ANG and CCK.

To do this, recordings from dissociated SFO neurons were carried out using perforated patch-clamp techniques and CCK and ANG were sequentially bath applied to neurons to examine if the same neuron can respond to both signals. In our second set of experiments, dissociated SFO neurons were incubated (for a minimum of 24 hours) in either hypoglycemic (1mM), normoglycemic (5mM) or hyperglycemic (10mM) conditions and responsiveness of neurons to each peptide was compared across glycemic conditions. The following chapter will explain our methodology for these experiments in further detail.
Chapter 3

Materials and Methods

3.1 Ethics Approval & Animals

All experimental procedures in this study were carried out in accordance with the ethical criteria established by the Canadian Council on Animal Care, after approval from the Animal Care Committee at Queen’s University (Kingston, ON); Protocol #2013-032. Dissociated SFO neurons were prepared from male Sprague-Dawley rats (100-150g) purchased from Charles River Laboratories (Montreal, QB). Upon arrival to the Queen’s Animal Care facility, rats were housed in pathogen-free conditions on a reversed 12-h light-dark cycle with free access to food and water for a minimum of 4 days before use.

3.2 Subfornical Organ Neuron Dissociation

The protocol used for dissociation of these cells was adapted from those previously described (Ferguson et al., 1997). Rats were decapitated and the brains were removed and immediately placed in an oxygenated (95% O₂ & 5% CO₂), ice-cold artificial cerebral spinal fluid (aCSF) solution containing (in mM): 124 NaCl, 2.5 KCl, 1.3 MgSO₄ hexahydrate, 1.24 KH₂PO₄, 20 NaHCO₃, 10 glucose, 2.27 CaCl₂. First, a block of tissue containing the SFO hanging below the hippocampal commissure was removed from the brain. Then the SFO, which is readily distinguished from the adjacent tissue, was microdissected and placed in a drop of Hibernate-A media (Thermo Fisher Scientific) supplemented with B-27 (Gibco). After extracting the SFOs
from 3 rats, all were placed in a solution, containing 10-16mg of papain (lyophilized; Worthington Biochemical, Lakewood, NJ) dissolved in 5mL of Hibernate-A, for 30 min at 31°C to break down the connective tissues. They were then rinsed twice with the Hibernate-A/B27 solution to remove the papain. The SFOs then underwent three cycles of trituration, which requires using a 1mL pipette and turbulent flow to break neurons apart. The tube with the solution now containing single dissociated neurons was centrifuged for 8 min at 100g to form a pellet of neurons at the bottom of the tube. The supernatant was then carefully removed and the pelleted cells were resuspended in a Neurobasal-A solution (Thermo Fisher Scientific). This solution was ordered without D-glucose or sodium pyruvate, and then supplemented with B-27, 227μM sodium pyruvate (Thermo Fisher Scientific), 100U/ml penicillin-streptomycin (Thermo Fisher Scientific), 0.4mM L-glutamine (Thermo Fisher Scientific) and glucose (final concentration either 1, 5 or 10mM glucose depending on the experiment). The solution was then aliquoted onto 35-mm tissue-culture treated plastic-bottom Corning dishes (Sigma) at a low density to avoid development of synaptic connections. Plates were incubated for 2.5 hrs to allow for the dissociated neurons to sink and adhere to the bottom of the plate. The incubator (Forma Scientific) was maintained at 37°C and 5% CO₂ balanced with ambient air. Then, about 2mL of the previously described supplemented Neurobasal-A solution (containing either 1, 5 or 10mM glucose) was added to each plate. Plates were maintained in culture for a minimum of 24-hrs before use, and all recordings were performed within 4 days of the dissociation process.

3.3 Electrophysiology

3.3.1 Perforated Patch Recording
Perforated-cell current-clamp recordings from dissociated SFO neurons were collected and filtered at 2 kHz using a Multiclamp 700B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Analog signals of membrane potential ($V_m$) and whole-cell current data from the amplifier were digitized at 10 kHz using a Cambridge Electronics Design (CED, Cambridge, UK) Micro1401 interface. The software used to control recording parameters and display the current-and voltage-clamp data were Spike2 (Version 8.01) (CED) and Signal (Version 6.01) (CED). The perforating agent used for all parts of this study was Amphotericin B (AMPHB) (Sigma-Aldrich; catalogue # A4888), an antifungal from *Streptomyces* species. A stock of AMPHB was made based on previously described instructions (Linley, 2013) by adding 20μL of DMSO per mg of AMPHB and vortexing for 2 minutes. This solution was aliquoted and stored in the -20°C freezer for a maximum of 48 hours. Immediately before recording, ~6-8μL of this stock was defrosted and added to the internal pipette solution, vortexed for 30 sec and sonicated for 2-5 sec (final concentration 400μg/mL). This AMPHB pipette solution is made routinely every 2-3 hours.

### 3.3.2 Solutions and Electrodes

The internal pipette solution used for all recordings contained (in mM): 125 potassium gluconate ($C_6H_{11}KO_7$), 10 KCl, 2 MgCl$_2$ hexahydrate, 10 HEPES, 1 EGTA, 0.3 CaCl$_2$ dihydrate & 2 NaATP. KOH was added until the solution reached a pH of 7.3. The solution was then 0.22 micron filter sterilized and the osmolarity was checked (accepted range= 280-300 mOsm). The internal solution was then aliquoted and stored at -80°C for a maximum of 3 months. Recording electrodes made from borosilicate glass (World Precision Instruments, Sarasota, FL) were shaped using a Flaming Brown micropipette puller (P97; Sutter Instruments, Novato, CA) and had a resistance between 3-4 MΩ after being filled with the internal solution and placed in the bath. The
extracellular bath solution used for recordings contained (in mM): 140 NaCl, 5 KCl, 2 CaCl₂ dihydrate, 1 MgCl₂ hexahydrate, 10 HEPES. NaOH was added until the solution reached a pH of 7.3. The glucose concentration of the external was matched to the glucose concentration of the Neaurobasal-A solution that cells were incubated in, of either 1, 5 or 10mM glucose. For the 1 and 5mM glucose external solutions, mannitol was added to compensate for the difference in osmolarity (9mM and 5mM respectively). The acceptable osmotic range of the external solution was between 280-300 mOsm. Dissociated SFO neurons were perfused with the external bath solution (heated to 37°C) at an average rate of 1.0 mL/min using a gravity perfusion system, and fluid was removed from the dish via a vacuum pump. The electrode was dipped into the internal solution for half a second and then back-filled with 3 μL of the AMPHB pipette solution. The pipette tip was lowered onto the selected cell using an MP-225 micromanipulator (Sutter Instruments). Once contact with the cell membrane was made, slight negative pressure was applied to form a GΩ seal. Time was then allowed for AMPHB to form pores in the membrane until the series resistance stabilized (approximately 15-20 mins). Only those cells which reached a series resistance of <20MΩ were used. Cells were identified as neurons if fast activating and inactivating inward sodium currents could be evoked by a series of voltage steps in the voltage-clamp configuration (using the program, Signal), and only selected for experimentation if the cell could fire action potentials >60mV in the current-clamp configuration (either spontaneously or evoked by an injection of current). A calculated liquid junction potential of -15.7mV was added to the reported $V_m$ of all neurons recorded.

3.3.3 Peptide Hormones
After a stable baseline mp was obtained for a minimum of 5 minutes, 2-3 mL of 10nM cholecystokinin (sulfated octapeptide; Phoenix Pharmaceuticals; catalogue # 069-03), dissolved in the external recording solution, was bath applied to cells. Sulfated CCK-8 was selected as it binds to both CCK₁ and CCK₂ receptors. Following washout of CCK and a return to baseline if the cell responded, 2-3 mL of 10nM angiotensin II (Phoenix Pharmaceuticals; catalogue # 002-12) was applied to the same cell. A concentration of 10nM (10⁻⁸M) was selected for both peptides as it falls within the effective concentration ranges previously described (Ahmed et al., 2014; Okuya et al., 1987). For the first set of experiments, peptides were applied in a bath solution containing 5mM glucose. For the rest of the experiments, the glucose concentrations were either 1, 5 or 10mM glucose.

3.3.4 ‘Response’ Characterization

Responsiveness of SFO neurons was determined by analyzing the average mp before, during and after peptide application in 100s bins (100 x 10K = one million data points to calculate each mean), using a script in Spike2. The neuron was considered responsive if the change in mp during or after the application exceeded 2x the standard deviation (SD) of 100s of control baseline mp before the peptide hit the bath, and if it was followed by a return toward baseline mp following washout of the peptide. In the rare cases where the SD of the baseline mp was exceedingly large as a result of high frequency action potential firing in control conditions, a change in firing frequency >75% from the baseline firing frequency was used to classify the neuron as responding to the hormone. The values reported are the changes in mp representing the largest 100s change during or after peptide application. After classification of responses based on these criterion, group response averages are expressed as mean ± SEM.
3.4 Data Analysis

Chi square analysis of contingency tables were used to determine whether proportions of responses differed significantly between SFO neurons incubated and recorded in different glucose concentrations. One-way analysis of variance (ANOVA) tests were used to compare magnitudes of responses or resting mps of neurons before peptide application between the 10, 5 and 1mM glucose conditions. All analyses were performed using GraphPad Prism 6 software and used a 95% confidence interval.
Chapter 4

Results

4.1 Subfornical Organ Neurons Integrate Metabolic and Cardiovascular Hormones

Recent electrophysiology studies have shown that significant proportions of SFO neurons respond to the ‘metabolic’ signal, CCK (Ahmed et al., 2014), or the ‘cardiovascular’ signal, angiotensin (Li & Ferguson, 1993). However, whether these peptides affect different neuronal subpopulations or not has yet been elucidated. In order to answer this question, we performed perforated-cell current-clamp recordings from dissociated SFO neurons incubated and recorded in 5mM glucose. After establishing a minimum 100s stable baseline mp, bath application of CCK (10nM) was applied via a gravity perfusion system. If the neuron was classified as responsive to CCK (see ‘Response’ Characterization and Data analysis in Methods), sufficient time was allowed for recovery of the mp, and then ANG-II (10nM) was applied to the same dissociated SFO neuron. Recordings were obtained from 82 dissociated SFO neurons, of which sequential application of both peptides was achieved in a total of 30 cells. The average resting mp of neurons before hormone application was -66.6 ± 1.0 mV. All neurons showed a return to baseline mp. In some cases, however, a full recovery after ANG application was not observed, as some of the responses to CCK and/or ANG could each last more than 45 minutes, and recordings were not maintained long enough to see a full recovery to both.
4.1.1 A subpopulation of SFO neurons respond to both CCK & ANG, while two other groups of neurons respond to either hormone exclusively

Consistent with previous studies, we found that bath application of CCK on dissociated SFO neurons caused both depolarizing and hyperpolarizing responses (Ahmed et al., 2014), whereas ANG caused depolarizing responses only (Ferguson et al., 1997; Ono et al., 2001). A total of 16/30 neurons tested were classified as responsive, of which 63% depolarized to both CCK & ANG (n=10/16; CCK mean mp Δ = 7.2 ± 1.1 mV; ANG mean mp Δ = 11.0 ± 2.1 mV), as seen in example traces shown in Fig 6A and summarized in Fig 6D.
Figure 6. Three distinct neuronal subpopulations of the SFO respond differently to sequential application of the metabolic hormone, cholecystokinin (CCK), and the cardiovascular hormone, angiotensin II (ANG)

A: representative current-clamp recordings from two separate dissociated SFO neurons demonstrate one group of neurons that depolarize and increase firing frequency to bath application of both 10nM CCK (blue bar) and 10nM ANG (red bar). The first trace shows a neuron with a short reversible depolarization to both CCK and ANG, contrasted with the second
example that shows a longer response duration to both hormones, with the response to ANG lasting > 43 min. B: represents two separate neurons from another group of SFO neurons that do not respond to CCK and depolarize to ANG. The second trace compared to the first once again demonstrates that some neurons have a longer response duration to ANG than others. C: representative trace of the last group of SFO neurons that hyperpolarize to bath application of CCK and do not respond to ANG. Break represents a 5 min lapse of time in the y axis. D: Venn diagram represents the proportion of neurons in each group of responding SFO neurons. Of the 30 neurons tested, 16 were classified as responsive to either or both peptides. Of the responsive neurons: 63% depolarized to both CCK and ANG (n= 10/16), 25% depolarized to ANG only (n=4/16), and 12% hyperpolarized to CCK only (n= 2/16). Note that all neurons that depolarized to CCK also depolarized to ANG, and neurons that hyperpolarized to CCK did not respond to ANG. Scale bars= 100s x 10mV.

Interestingly, all of the neurons that depolarized to CCK also depolarized to ANG, although in contrast, a small proportion of the ANG-responsive neurons did not respond to CCK (Fig 6B). This ANG-only responsive group of neurons made up 25% of total responding neurons (Fig 6D) (n=4/16; mean mp Δ = 10.4 ± 2.6 mV). In contrast, the neurons that hyperpolarized to CCK (12%) did not respond to ANG (Fig 6C&D) (n=2/16; mean mp Δ = -7.2 ± 0.1 mV). There was no significant difference between the average resting mps of cells that depolarized or hyperpolarized to CCK (-68.0 ± 2.3 mV vs. -66.4 ± 0.5 mV; paired t-test, p= 0.77). These data suggest three separate subpopulations of SFO neurons exist: one that responds to CCK and ANG, one that responds only to ANG and another that responds only to CCK (Fig 6D).

4.2 Subfornical Organ Neurons Integrate Glucose with Cardiovascular and Metabolic Hormones

These initial recordings demonstrated that in 5mM glucose <50% of the 30 SFO neurons tested responded to bath application of angiotensin II (n=14/30); a significantly smaller proportion of cells when compared to previous findings in the literature that consistently report a responsiveness between 61%-72%: (Li & Ferguson, 1993)- 61%; (Ferguson et al., 1997)- 63%; (Okuya et al., 1987)- 66%; (Ono et al., 2001)- 72%. A major difference between these studies and
ours is that, traditionally, these experiments were performed at 10mM glucose, whereas the neurons in our experiment were recorded at 5mM glucose. In these experiments the use of 5mM glucose represented an attempt to mimic the normoglycemic circulating environment that the SFO is exposed to in these animals (Wang et al., 2010), but evolved to set the stage for the second set of our experiments which sought to investigate whether glycemic condition could affect the responsiveness of these cells to either a cardiovascular and/or metabolic signal such as ANG and CCK.

In order to investigate this, neurons were dissociated in the same way as described above, but instead were incubated at either 1mM, 5mM or 10mM glucose for a minimum 24 hour period, up to 4 days. The glucose concentration in the external recording solution was also matched to the concentration the cells were incubated at. The 5mM ‘normoglycemic’ condition we have chosen is intended to mimic average circulating glucose concentrations of healthy rats, which is between 3.95mM (fasted) and 5.65mM (postprandial) (Wang et al., 2010). This is a very similar range to that present in normal infants, children, and adults (3.5-5.5mM) (Guemes et al., 2016). There is no set value to define the threshold for hypo- or hyperglycemia, rather, each is defined as when blood glucose becomes too low or high, exceeding the aforementioned normal range such that a variety of symptoms ensue. Hypoglycemic episodes may be caused by too much blood insulin or anorexia and include feelings of weakness and light-headedness, whereas hyperglycemia may result from lack of insulin or insulin resistance and can cause blurry vision and headaches. Interestingly, hyperglycemia can be used as an indicator of type II diabetes (TIID), a metabolic disorder that occurs as a result of obesity and insulin resistance (Alberti & Zimmet, 1998). As mentioned previously, we have chosen to use 10mM glucose for our hyperglycemic condition as it is what has been used for recordings in the past. However, in addition to that, 10mM glucose is also a
concentration suggested to model TII diabetes as it is beyond the 95% upper limits for fasted and fed states, which as Wang et al., 2010 define it is the threshold for the onset of diabetes in rats. We have chosen to mimic circulating glucose concentrations as opposed to brain concentrations, which are significantly lower, based on the fact that the SFO lacks a blood-brain barrier and is therefore consistently bathed in circulating glucose concentrations. Additionally, the SFO has previously been shown to respond electrophysiologically to changes in glucose concentrations interchanging between 10, 5 and 1mM glucose (Medeiros et al., 2012). Altogether, these data justified our use of these three glucose concentrations to represent hypo-, normo- and hyperglycemia.

4.2.1 An increasing glycemic environment increases the responsiveness of SFO neurons to ANG

We therefore next examined the effects of 10nM ANG on SFO neurons incubated and recorded in a hyperglycemic environment (10mM glucose) and found that under these conditions 73% of neurons tested depolarized (n=8/11; mean mp Δ = 6.5 ± 0.9 mV), as demonstrated in the example trace Fig 7A and summarized in Fig 7D. This proportion is consistent with the aforementioned studies investigating the effects of ANG on SFO neurons recorded at 10mM glucose (61%-72% ANG-responsive SFO neurons) (Okuya et al., 1987;Li & Ferguson, 1993;Ferguson et al., 1997;Ono et al., 2001). As previously mentioned, neurons incubated and recorded in 5mM glucose caused 50% of neurons to depolarize in response to bath application of ANG (Fig 7B&D) (n=16/32; mean mp Δ = 10.6 ± 1.4 mV), and after lowering the glucose to 1mM only 33% depolarized (Fig 7C&D) (n=4/12; mean mp Δ = 6.2 ± 2.2 mV). As demonstrated in Fig 7D, increasing the glycemic environment of neurons, from 1 -> 5 -> 10mM glucose was associated
with an increase in the proportion of depolarizing neurons, from 33% -> 50% -> 73%, to bath application of 10nM ANG.

**Figure 7.** Increasing glycemic environment is associated with an increase in the percentage of responding SFO neurons to ANG

A-C: representative current-clamp traces from three separate dissociated SFO neurons incubated (min. 24 hours) and recorded in (A) 10mM (hyperglycemic), (B) 5mM (normoglycemic) or (C) 1mM (hypoglycemic) glucose demonstrate depolarizing responses, accompanied by an increase in firing frequency, to bath application of 10nM ANG (red bar). Scale bars = 100s x 10mV. D: bar graph summarizes the proportion of neurons that depolarize to ANG (blue bars) at 10, 5 and 1mM glucose: 10mM= 73% (8/11); 5mM= 50% (16/32); 1mM= 33% (4/12). Note the significant trend between increasing glucose and increasing number of responsive SFO neurons to ANG ($\chi^2$, p < 0.05). Total number of neurons tested= 55. The remaining proportion of neurons did not respond (white bars).
Statistical analysis of the proportions of neurons responding to ANG at 1, 5 and 10mM glucose (including the population previously tested in our laboratory at 10mM glucose (Ferguson et al., 1997)) using χ² analysis of contingency tables showed a significant correlation between glucose concentrations and response profiles of SFO neurons (p < 0.05). A one-way ANOVA was conducted to compare the mean depolarization magnitudes to ANG in each of the glycemic conditions and determined that glucose did not have an effect on response magnitude, p=0.10. Also, there was no significant difference between the average resting mps of cells before ANG application in the 10mM (-66.3 ± 2.7 mV), 5mM (-66.5 ± 1.4 mV) or 1mM glucose (-64.2 ± 2.3 mV) condition; one-way ANOVA, p=0.71. Altogether, these data provide evidence for a positive relationship between increasing glucose concentration and the percentage of responding neurons to ANG.

4.2.2 A hyperglycemic environment increases the proportion of hyperpolarizing SFO neurons to CCK and decreases the proportion of depolarizing neurons

Almost identical to the results demonstrated in Ahmed et al., 2014, which is the only other electrophysiology experiment investigating the effects of CCK on SFO neurons, our data show that 38% of neurons recorded at 5mM glucose depolarized in response to bath application of 10nM CCK (Fig 8C&G) (n= 17/45; mean mp Δ = 8.2 ± 0.9 mV), and that 11% hyperpolarized (Fig 8D&G) (n=5/45; mean mp Δ = -6.3 ± 0.6 mV), with about half of the neurons not responding to CCK application (Fig 8G) (n=23/45 or 51%; mean mp Δ = 0.7 ± 0.5 mV). Increasing the concentration of glucose that cells were incubated and recorded in to 10mM glucose resulted in 19% of neurons to depolarize to bath application of CCK (Fig 8A&G) (n= 4/21; mean mp Δ = 8.4
± 2.5 mV), 29% of neurons to hyperpolarize (Fig 8B&G) (n= 6/21; mean mp Δ = -7.3 ± 1.3 mV), and 52% of neurons did not respond (Fig 8G) (n=11/21; mean mp Δ = -0.3 ± 0.7 mV). After lowering the glucose to 1mM, 35% of neurons depolarized (Fig 8E&G) (n= 6/17, mean mp Δ = 7.5 ± 0.9 mV), 6% hyperpolarized (Fig 8F&G) (n= 1/17, mp Δ = -13.5 mV), and 59% did not respond (Fig 8G) (n= 10/17, mean mp Δ = -1.0 ± 0.8 mV). As demonstrated in Fig 8G, increasing the glycemic environment of neurons, from 1 -> 5 -> 10mM glucose was associated with a decrease in the proportion of depolarizing neurons, from (35% -> 38% -> 19%), and increase in the proportion of hyperpolarizing neurons (6% -> 11% -> 29%) to bath application of 10nM CCK, without changing the proportion of non-responding neurons (59% -> 51% -> 52%).
Figure 8. Hyperglycemic environment is associated with an increase in the percentage of hyperpolarizing and decrease in the percentage of depolarizing SFO neurons to CCK

A-F: representative current-clamp traces from six separate dissociated SFO neurons incubated (min. 24 hours) and recorded in (A&B) 10mM (hyperglycemic), (C&D) 5mM (normoglycemic) or (E&F) 1 mM (hypoglycemic) glucose demonstrate depolarizing responses accompanied by an increase in firing frequency (A, C, E), and hyperpolarizing responses accompanied by a decrease in firing frequency (B, D, F) to bath application of 10nM CCK (blue bar). Scale bars= 100s x 10mV. D: bar graph summarizes the proportion of neurons that depolarize (blue bars) or hyperpolarize (stacked red bars) to CCK at 10, 5 and 1mM glucose: 10mM= 19% depolarizations (n= 4/21) & 29% hyperpolarizations (n= 6/21); 5mM= 38% depolarizations (n= 17/45) & 11% hyperpolarizations (n=5/45); and 1mM= 35% depolarizations (n= 6/17) & 6% hyperpolarizations (n= 1/17). Note the switch to predominately hyperpolarizing responses to CCK in hyperglycemic conditions, as opposed to the majority of depolarizing responses demonstrated in the normoglycemic and hypoglycemic conditions ($\chi^2$, p < 0.01). Total number of neurons tested= 83. The remaining proportion of neurons did not respond (white bars).
Statistical analysis of the proportions of neurons responding to CCK at these different glucose concentrations (including the population previously tested in our laboratory at 5mM glucose (Ahmed et al., 2014)) using \( \chi^2 \) analysis of contingency tables showed a significant correlation between glucose concentrations and response profiles of SFO neurons (p < 0.01). One-way ANOVAs were conducted to compare the mean depolarization and hyperpolarization magnitudes to CCK in the 1, 5 and 10mM glucose conditions and determined that glucose did not have a significant effect on either response magnitude (depolarizations: p=0.91; hyperpolarizations: p= 0.08). Also, there was no significant difference between the average resting mps of cells before CCK application in the 10mM (-65.3 ± 1.7 mV), 5mM (-67.9 ± 1.0 mV) or 1mM glucose (-64.1 ± 2.3 mV) condition; one-way ANOVA, p=0.16. Altogether, these data suggest that a hyperglycemic environment modifies the responsiveness of SFO neurons to CCK.

Interestingly, a hyperglycemic environment appears to have an opposite effect on these cardiovascular and metabolic regulating hormones, by increasing the proportion of depolarizing neurons to ANG and decreasing the proportion of depolarizing neurons to CCK, while increasing the proportion of hyperpolarizing neurons to CCK (Fig 9).
Figure 9. Glycemic condition has an opposite effect on the responsiveness of SFO neurons to the cardiovascular hormone, ANG, and the metabolic hormone, CCK

This graph demonstrates the positive relationship between increasing glucose concentration and the proportion of depolarizing neurons to ANG (33% -> 50% -> 73%) and hyperpolarizing neurons to CCK (6% -> 11% -> 29%), and an inverse relationship between increasing glucose concentration and the proportion of depolarizing neurons to CCK (35% -> 38% -> 19%).
Chapter 5

Discussion

The present study provides compelling evidence for the integrative capacity of single SFO neurons. Our results demonstrate that the population of SFO neurons that depolarize in response to application of the metabolic signal, CCK, also depolarize to the cardiovascular signal, ANG. In addition, the responses of neurons to both of these signals are altered in hyperglycemic states. To our knowledge, this study is the first to demonstrate direct excitation of the same SFO neuron by both a cardiovascular and metabolic hormone, as well as the ability of circulating glucose concentrations to modulate the way SFO neurons responds to hormonal stimuli.

5.1 The Integration of Cardiovascular and Metabolic Hormones by the SFO

We first investigated whether neurons that responded to the metabolic signal, CCK, could also respond to the cardiovascular signal, ANG. This was based on previous studies showing that the SFO expresses receptors for, and single neurons respond to, a number of metabolic and cardiovascular signals (Mimee et al., 2013), including these two (Okuya et al., 1987; Ahmed et al., 2014). As stimulation of the SFO has been shown to increase both fluid (Oka et al., 2015; Smith et al., 1995) and food (Smith et al., 2010) intake, we hypothesized that the neurons that responded to these signals may represent separate subpopulations within the SFO with distinct electrical circuits controlling these physiological behaviours. Interestingly, only 25% of the total number of responding neurons depolarized to ANG exclusively, and only 12% exclusively responded to
CCK, these neurons being hyperpolarized by this peptide. To our surprise, all of neurons that underwent a depolarization of the mp in response to bath application of CCK, were also depolarized by ANG. This group of neurons made up 63% of the total responding neurons. These results were consistent with previous electrophysiology reports showing that CCK causes both depolarizing and hyperpolarizing responses in the SFO, the majority being depolarizing responses (Ahmed et al., 2014), as opposed to ANG which consistently causes a depolarization of SFO neurons (Okuya et al., 1987; Li & Ferguson, 1993; Ferguson et al., 1997; Ono et al., 2001).

5.1.1 Physiological Implications of Demonstrated SFO Response Profiles to Sequential Application of CCK and ANG

Unlike CCK, the actions of ANG at the SFO have been thoroughly investigated in vivo. Microinjection of ANG into the SFO elicits drinking (Smith et al., 1995; Oka et al., 2015) and pressor responses in rats (Mangiapan & Simpson, 1980b; Gutman et al., 1988) (see review Ferguson & Wall, 1992). Despite CCK’s traditional role in controlling food intake and digestion, the extremely large overlap of neurons that depolarize to both CCK and ANG demonstrated in our data suggests that stimulation of the SFO by CCK may elicit fluid intake or cause an increase in blood pressure. Interestingly, in accordance with this theory, intravenous (i.v.) administration of CCK in rats has been shown to increase systolic and diastolic blood pressure (Wisniewska, 1997). A more recent study showed that i.v. administration of CCK in anesthetized rats caused a tri-phasic response of blood pressure characterized by an immediate fall, then brief increase after 15s, followed by a maximum decrease at 3 minutes (Kaczynska & Szereda-Przestaszewska, 2015). The authors suggested that the decreases in blood pressure were mediated via CCK₁R activation on vagal afferents, whereas the pressor responses were likely mediated by CCK₁R activation in the
central nervous system (Kaczynska & Szereda-Przestaszewska, 2015). Our data showing that CCK depolarizes the majority of ANG-responsive neurons in the SFO, which have well-described cardiovascular effects when activated, may suggest a neuronal mechanism through which i.v. administration of CCK elicits its pressor effects.

5.1.2 Possible Mechanism of Action

One possible explanation for why the SFO exhibits heterogeneous responses to CCK application is differences in the $V_r$ of cells before CCK application. Neurons that depolarize to CCK could be below the reversal potential of the downstream channel activated by the CCK GPCR, or the rmp could be above the channel’s reversal potential for those that hyperpolarize. However, resting mps before peptide application were not significantly different between depolarizations and hyperpolarizations in our experiment and therefore does not likely explain the heterogeneity of responses to CCK.

A more likely explanation may be explained by that fact that the SFO expresses both CCK$_1$ and CCK$_2$ receptors (Ahmed et al., 2014). As these are both g-protein-coupled receptors, each receptor could be mediating one type of response by affecting different ion channels in the membrane via second messenger signaling pathways. Compilation of the numerous studies that used cells from the pancreas and GI tract to investigate the signal transduction pathways activated by CCK receptors have demonstrated that both the CCK1 and CCK2 receptors can activate phospholipase C (PLC)-β isoforms, likely through heterotrimeric G proteins of the G$_q$ family (Murthy & Makhlouf, 1995; Piiper et al., 1997) (see review Dufresne et al., 2006). PLC is a class of membrane-associated enzymes that hydrolyze phosphatidylinositol bisphosphate (PIP$_2$) to generate inositol triphosphate (IP$_3$) and diacylglycerol (DAG), which respectively induce calcium
mobilization from intracellular stores and stimulate several protein kinase C (PKC) isoforms. There are two studies, to our knowledge, that attempt to elucidate the mechanism between CCK receptor activation and $V_m$ changes in cells. Zhao and Simasko used calcium imaging techniques in conjunction with host of blockers for various voltage-gated calcium and non-selective cation channels to show that CCK$_1$ receptors expressed on vagal afferent neurons likely activate TRPV channels to depolarize these neurons (Zhao & Simasko, 2010). Another group used single channel recordings from HEK 293 cells co-transfected with CCK$_2$ and TRP5 receptors to show that CCK$_2$ receptors potentiate the function of TRPC5 channels, a process dependent on the function of $G_q$ proteins, PLC and extracellular calcium (Grisanti et al., 2012). Further experiments using blockers and/or voltage clamp experiments would be useful to delineate the pathways of CCK induced depolarizations and hyperpolarizations in SFO neurons. Future experiments could also use CCK agonists specific for either receptor to deduce the response each receptor mediates in the SFO, such as SR-146,131 (CCK$_1$R selective agonist) (Bignon et al., 1999) and BBL-454 (CCK$_2$R selective agonist) (Bellier et al., 2004).

As far as why certain neurons respond to both peptides or only one and not the other, it is possible that these neurons project to different areas of the brain involved with regulating different physiological functions. A plausible circuitry is explored in the following section.

### 5.2 Proposed Connectivity & Physiological Model

Based on the responses of SFO neurons to consecutive bath application of CCK and ANG, our results suggest the possible existence of three distinct subpopulations of neurons in the SFO that could be involved in different circuits affecting various physiological functions. A proposed model for these circuits is represented in Figure 10, and is based on the current understanding of
the SFO’s neuronal circuitry that was discussed in Chapter 2.2 of this thesis. SFO neurons that depolarize to both ANG and CCK may influence autonomic regulation of the cardiovascular system through connections to magnocellular neurons in the paraventricular nucleus (mPVN) or supraoptic nucleus (SON) of the hypothalamus, or to areas of the lamina terminalis, such as the organum vasculosum of the lamina terminalis (OVLT) and median preoptic nucleus (MnPO), which then project PVN and SON (Miselis, 1982;Misulis, 1981;Lind et al., 1982). Magnocellular neurons in the PVN and SON produce and release vasopressin (VP) and oxytocin (OXY) from the posterior pituitary (PP) into the circulation where they can influence the kidneys to cause antidiuresis and directly affect blood vessels to cause vasoconstriction to ultimately increase blood pressure (Ferguson & Kasting, 1986). Activation of this circuit has also been shown to influence cardiovascular homeostasis by stimulating fluid intake (McKinley et al., 2001). Neurons that exclusively respond to ANG may synapse onto parvocellular neurons in the PVN (pPVN) that project to sympathetic preganglionic neurons in the intermediolateral nucleus (IML) of the spinal cord (Bains & Ferguson, 1995;Sawchenko & Swanson, 1982;Sly et al., 1999). Activation of sympathetic nerve activity via this circuit can cause direct constriction of blood vessels, increased sympathetic tone to the sinoatrial node of the heart causing increased heart rate, and increased renal nerve activity that causes increased sodium and water reabsorption in the kidneys (Porter & Brody, 1985;Stauss et al., 1997;Haselton & Vari, 1998) - all physiological effects that can ultimately lead to an increase in systemic blood pressure (Nunn et al., 2011). The last group of neurons that only hyperpolarize to CCK may be projecting to areas of the brain associated with the regulation of food intake, such as the lateral hypothalamus or AGRP/NPY expressing neurons in the arcuate nucleus (Tanaka & Seto, 1988;Gruber et al., 1987). Decreased stimulation to these areas, which normally stimulate food intake, would result in decreased food intake.
This connectivity model provides a good foundation for interpretation of the results of the next studies of this thesis.
Figure 10. Hypothesized model of projection sites for three SFO subpopulations to areas of the hypothalamus and lamina terminalis involved in the regulation of cardiovascular and energy homeostasis

Angiotensin responsive neurons are represented in the red circle of the Venn diagram, and CCK responsive neurons are represented in the blue circle of the Venn diagram. SFO neurons that 1. Depolarize to both ANG & CCK are represented in the overlaid purple center of Venn diagram and may project to areas of the LT and hypothalamus involved in fluid balance; 2. Depolarize to ANG only are represented in the left red portion of the diagram and may project to the IML of the spinal cord, implicated in cardiovascular regulation via sympathetic nervous system activation; or 3. Hyperpolarize to CCK only are represented in the right blue portion of the diagram and may project to hypothalamic feeding centers.
See text for explanations and references.
5.3 The Integration of Glucose with Cardiovascular and Metabolic Hormones by the SFO

The next set of our experiments evolved from the observation that <50% of SFO neurons responded to bath application of ANG, compared to the literature which reproducibly reports a significantly higher responsiveness, between 61-72%, in both slice and dissociated neurons (Okuya et al., 1987; Li & Ferguson, 1993; Ferguson et al., 1997; Ono et al., 2001). One major difference between our protocol and those previously published was that we used 5mM glucose during incubation and recording of neurons, as opposed to the traditional 10mM glucose that has been used for decades. Thus, our second set of experiments sought to establish whether circulating glucose concentration could affect the responsiveness of SFO neurons to ANG and/or CCK in vitro; two hormones traditionally involved in the regulation of the cardiovascular system and energy metabolism, respectively.

5.3.1 Implications of Increased Responsiveness of SFO neurons to ANG in Higher Glucose Environments

Our study revealed a positive correlation between responsiveness of SFO neurons to ANG and increasing glucose concentration, where the percentage of depolarizing responses increased from 33% -> 50% -> 73% when glucose was increased from 1mM -> 5mM -> 10mM. If we compare our in vitro findings to what has been found in vivo, it appears as though the proportion of ANG-responding neurons in the normoglycemic condition of our experiment is still larger than that seen in vivo. Among the various single unit recordings from SFO neurons in vivo (Ciriello, 1997; Gutman et al., 1988; Rosas-Arellano et al., 1995), there is only one study that did not contain
a selection bias for their neurons (ie. other studies only reported results from SFO neurons that projected to a particular area, such as the PVN or SON) and that reported the number of nonresponsive neurons to allow for calculation of a percent (Rosas-Arellano et al., 1995). Rosas-Arellano et al., 1995 demonstrated that of the 67 spontaneously active neurons recorded from male Wistar rats, 21 were excited by ANG II (500ng in 100microL saline) injected into femoral vein, which means 31.3% of the neurons recorded in vivo were responsive to ANG (Rosas-Arellano et al., 1995). Interestingly, this is significantly lower than the percentage of responses we report in our 5mM glucose condition (50%). Admittedly, this discrepancy between the 5mM glucose group and in vivo findings is not surprising as not only are we unsure of the exact concentration of ANG that reached the SFO, but also there are a number of different signals in a living organism that provide the SFO with information about the circulating environment at all times that we must also consider. These other signals, such as osmolarity, oxygen, carbon dioxide, or even other hormones, could also affect the responsiveness of SFO neurons to ANG, as was demonstrated with glucose in our experiments. These signals may exert their effects in a number of different ways, including modulating receptor expression, changing the resting mp of the cell, or activating/inactivating various associated second messenger pathways or ion channels, to name a few examples. When these other signals are integrated with the effects of glucose they may change the responsiveness from what was seen in our in vitro experiments, which only manipulated one variable. Discrepancies such as this one emphasize how in vitro experiments, such as ours, can be extremely useful as they allow us to find answers to specific questions. When sewn together, answers to various specific questions enable us to understand the underpinnings of sometimes inexplicable results seen in vivo that contain multiple variables that are difficult to control for. By extrapolating our results and this concept to the human condition, one could theorize that the increased
responsiveness of SFO neurons to ANG seen at the high glucose condition in our experiment could explain an underlying neuronal mechanism responsible for the comorbidities of hyperglycemia and hypertension experienced by individuals suffering from obesity.

5.3.2 Possible Mechanism of Action for Interaction between Glucose and ANG

It is possible that the high glucose environment during the minimum 24 hour incubation time caused upregulation of angiotensin 1a receptor (AT1R) expression- the most abundant angiotensin receptor in the SFO which has been shown to mediate ANG’s cardiovascular effects at this site (Lenkei et al., 1995). Increased transcription and translation of AT1Rs could increase the total percentage of responding neurons by recruiting neurons that normally would not express enough AT1Rs to elicit a response in normal glucose conditions, into the ANG responsive population of SFO neurons. Wang et al., 2013 demonstrated using quantitative RT-PCR (qPCR) techniques that incubation of rat glomerular mesangial cells in a high glucose medium increased the expression of AT1R mRNA by 1.5x when compared to the normal glucose condition, in as little as 24 hours (Wang et al., 2013). Based on their results from various qPCR and western blot experiments paired with the use of blockers, it was suggested that the hyperglycemic environment caused overproduction of reactive oxygen species that stimulated p102 synthesis, a transcription activator, which in turn up-regulated AT1R expression in these cells (Wang et al., 2013). Similar studies using the same techniques also showed a dose-dependent positive correlation between increasing glucose concentrations and AT1R gene and protein expression in an insulin-producing pancreatic beta-cell line (Leung & Leung, 2008) and an immortalized proximal tubule epithelial cell line from rabbit kidneys (Becker et al., 1997). In order to determine whether upregulation of AT1Rs mediated the increased percentage of responding cells
in our data, a similar qPCR and/or western blot analysis of SFO neurons incubated in various glycemic environments would be important.

It is unlikely that the differences in responsiveness across glucose concentrations was due to $V_m$ changes as there was no significant difference between $V_r$ before ANG application.

5.3.3 Implications of Altered Responses of SFO neurons to CCK Application in a Hyperglycemic Environment

As glucose concentration represents energy status in an organism, ie. a fasted, fed, or obese state, it seemed logical that it could influence the actions of a metabolic hormone in a structure that has been shown to influence food intake. Investigation of the effects of glycemic condition on CCK responsiveness in our study revealed that, in contrast to what was observed with ANG, a negative correlation between depolarization of SFO neurons to CCK and increasing glucose concentration was seen (5mM - 38% -> 10mM – 19%). This was accompanied by an increase in the percentage of hyperpolarizing responses by more than two-fold from the normal to hyperglycemic condition, from 11% at 5mM glucose -> 29% at 10mM glucose. If we consider our hypothesized model from the first set of experiments (Fig 10), it was theorized that those neurons that hyperpolarize to CCK may be projecting to areas of the hypothalamus involved in the control of food intake, such as the LH and AgRP/NPY producing neurons in the arcuate. Building upon this, it is possible that in a hyperglycemic state, where there is already an abundance of energy available in the form of glucose, this glucose may modify SFO neurons to allow CCK to have more potent anorexigenic effects. CCK’s increased inhibition to the aforementioned hypothalamic areas involved in stimulation of food intake, via the SFO, could therefore decrease this desire to eat, producing a satiating effect.
5.3.4 Possible Mechanism of Action for Interaction between Glucose and CCK

It is unlikely that this shift from depolarizing to hyperpolarizing responses to CCK applied in hyperglycemic conditions is attributable to glucose causing changes in the resting mp, which has been shown in the canonical β-cell model (Burdakov et al., 2005b), as there was no significant difference between the baseline resting mps of neurons in each glucose concentration in our experiment, nor between neurons that depolarized or hyperpolarized within glucose groups. What may explain our findings, similar to what was previously discussed with ANG, is that glucose may modify expression of the CCK receptors. Ahmed et al., 2014 used CCK₁ and CCK₂ receptor blockers with peripheral administration of CCK and measured expression of c-Fos and p-ERK in the SFO to determine that CCK₂ receptors are the dominant excitatory receptor in the rat SFO (Ahmed et al., 2014), and also used PCR to show that the SFO expresses both CCK₁ and CCK₂ receptors. These data suggest that the CCK₂ receptors may mediate the depolarizing responses seen electrophysiologically while CCK₁ receptors may mediate the hyperpolarizing responses, although additional studies using specific blockers will be necessary to examine such a possibility. To our knowledge, studies investigating CCK receptor expression changes after exposure to varying levels of glucose, even in non-neuronal tissues of the body, have not been performed. What has been studied, though, is the change in the expression of various metabolic and cardiovascular hormone receptors in the SFO after fluid and food deprivation (Hindmarch et al., 2008). This study provides evidence for the plasticity of metabolic and cardiovascular receptors in the SFO, maintaining the ability of these receptors to adapt to changes in cardiovascular and metabolic status. As glucose is a signal representing metabolic state, this study warrants investigation of relative CCK₁ and CCK₂R expression in single SFO neurons subjected to hypo-, normo- and hyperglycemic states. The results of this proposed study could help elucidate the
mechanism for our observed increase in hyperpolarizations and decrease in depolarizations to CCK demonstrated in the hyperglycemic condition of our study.

5.4 Implications for Use of Appropriate Glucose Concentrations in Future Studies

Our results show that varying levels of glucose can have drastic effects on the responsiveness of SFO neurons to both ANG and CCK application. Yet at the current time, the standard glucose concentration prepared in solutions for incubation of cultured neurons, such as Neurobasal-A, is 25mM, and the standard artificial cerebral spinal fluid preparation for both cultured neuron and slice recording solutions is 10mM (Ferguson et al., 1997); concentrations much higher than the healthy physiological range (Wang et al., 2010; Guemes et al., 2016). The congruous misconception is that these solutions should provide a surplus of energy to ensure neuronal viability, when in fact our experiments, in addition to a number of experiments performed by our lab in various parts of the brain, show that this is simply not necessary. Experiments performed in the SFO (Ahmed et al., 2014), NTS (Mimee & Ferguson, 2015), and PVN (McIsaac & Ferguson, 2016 unpublished) repeatedly demonstrate that neurons bathed in glucose levels as low as 1mM and 0.2mM glucose are fully capable of firing action potentials >60mV, maintaining stable baseline mps, being held for long periods of recording, and surviving for multiple hours (slice preparations) to days (cultured SFO neurons) in vitro. In fact, chronic exposure to high levels of glucose has been shown to be toxic to a number of cell types including neurons, pancreatic beta cells and vascular endothelial cells (Campos, 2012). Perhaps more concerning than this, though, is the potential for altered glucose conditions to affect experimental outcomes. As previously discussed, high glucose levels can affect neuronal function in a number of different ways,
including modification of neuronal excitability (Burdakov et al., 2005a), modulation of ATP-sensitive channels, such as inactivation of ATP-sensitive K+ channels leading to V_m changes (Burdakov et al., 2005b), and receptor expression changes (Becker et al., 1997; Leung & Leung, 2008; Wang et al., 2013), which can modify the way neurons interpret and communicate signals, as demonstrated in our results. Therefore our study encourages the use of appropriate physiological glucose concentrations in future studies in order to obtain the most accurate and translational results from in vitro studies.

5.5 Traditional Classifications of Signaling Molecules and the Limitations

As discussed, the observation that 100% of neurons that depolarize to CCK also depolarize to ANG from our data suggests that CCK may affect cardiovascular regulation at the SFO. At first, this suggestion may seem nonsensible when considering the overwhelming literature that has established a clear role for CCK in regulating metabolic homeostasis. However, this is not the first time that peptides traditionally thought to be primarily involved in metabolic regulation have also been linked to hydration. When one considers that digestion relies on adequate fluid consumption (Watts, 1999; Watts & Boyle, 2010), it seems almost intuitive that many molecules classically considered to be involved in energy homeostasis should also influence cardiovascular regulation through their effect on fluid volume and sodium consumption.

Ghrelin, primarily known as the only gut-derived hormone to induce hunger (Nakazato et al., 2001; Yoshihara et al., 2002), has recently been shown to not only produce excitatory effects on vasopressin neurons (Haam et al., 2014), but to also have cardioprotective effects in rats suffering from conditions such as heart failure and myocardial infarction (Iadecola et al., 2001; Soeki et al., 2008). Additionally, ghrelin has a depolarizing effect on SFO neurons (Pulman
et al., 2006). Leptin, an adipokine of which circulating levels are an indication of energy availability (Maffei et al., 1995), also influences the excitability of SFO neurons (Smith et al., 2009), while microinjection of leptin into the SFO reduces BP, an effect that is not seen in obese, leptin-resistant rats (Smith & Ferguson, 2012). Interestingly plasma leptin also appears to be increased during cardiovascular disease (Alsmadi et al., 2014; Ghantous et al., 2015), and deletion of AT1aR in the SFO attenuates leptin-mediated weight loss (Young et al., 2015). Adiponectin is a second adipokine, serum levels of which can predict a subject’s risk of hypertension (Jung et al., 2014), which has also been shown to stimulate nitric oxide (NO) production in the vasculature (Chen et al., 2003), and have protective actions on the cardiovascular system (Lei et al., 2013). Adiponectin receptors (AdipoR1 and AdipoR2) are expressed in the SFO, and adiponectin has been shown to influence the excitability of SFO neurons (Alim et al., 2010), identifying the SFO as a potential site at which the possible cardiovascular actions could occur. Finally, insulin, which is of course primarily associated with the uptake of glucose (Muniyappa & Yavuz, 2013), has also been shown to increase total body fluid in Type 2 Diabetes patients (Mudaliar et al., 2010). Once again, the SFO is a potential site at which insulin actions on fluid balance occur, as it influences the excitability of these neurons (Lakhi et al., 2013).

Thus, the risk of categorizing anything (structure, molecule or pathway) as being specifically “metabolic” or “cardiovascular” (among others) becomes clear. The gamble lies in excluding possible functions when studying a structure or ligand and only observing parameters related to a particular pathway, simply because it has traditionally been classified under that pathway. The data are perhaps starting to point us in a different direction, namely that these autonomic control centres, molecules, and pathways are all, to some extent, pluripotent in that they
contribute to an appropriately maintained autonomic state that is so critical to good health and well-being of the organism.

5.6 Perspectives and Significance

In conclusion, our findings demonstrate that a subpopulation of SFO neurons respond to both ANG and CCK, hormones traditionally considered to influence cardiovascular and metabolic regulation, respectively, and that glycemic state influences the responsiveness of SFO neurons to both these signals. As the physiological role of CCK in the SFO has not yet been investigated, our demonstration that a large percentage of SFO neurons depolarize to both CCK and ANG may suggest a role for CCK in increasing fluid intake and/or blood pressure via activation of the SFO, complementary to the well-understood actions of ANG. Additionally, our results show that increasing glucose concentrations from 1 to 5 and 10mM are associated with increased responsiveness of SFO neurons to ANG, potentially revealing a neuronal mechanism contributing to the comorbidities of hyperglycemia and hypertension presented in obesity. In contrast, a hyperglycemic environment causes an increase in the proportion of SFO neurons that hyperpolarize to CCK, which may demonstrate an attempt to decrease food intake in an energy fulfilled state. These results provide clear evidence for the ability of a metabolic signal representing metabolic state, glucose, to modify the way SFO neurons respond to both a traditionally classified metabolic and a cardiovascular hormone, emphasizing the importance of using physiologically relevant glucose concentrations when designing in vitro experiments. Future studies investigating single-cell expression of receptors for circulating signals in different glucose conditions may provide further insight into the mechanisms underlying how SFO neurons integrate these various signals. Altogether, our findings highlight the SFO as an integrative site where various metabolic
and cardiovascular signals may interact to influence autonomic functions dissimilar to their initial classifications.


