

**INSULIN MODULATES THE ELECTRICAL ACTIVITY OF DISSOCIATED  
AND CULTURED SUBFORNICAL ORGAN (SFO) NEURONS IN MALE  
SPRAGUE DAWLEY RATS**

by

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Abstract:

The brain is protected by the blood brain barrier (BBB); areas lacking the BBB are termed circumventricular organs (CVOs). The SFO, a CVO is capable of detecting and responding to satiety signals that regulate energy balance. Insulin, a satiety signal, plays a role in energy balance and its actions at the SFO are unknown. The goal was to determine if cultured SFO neurons are electrophysiologically sensitive to insulin. Of 27 neurons tested 33% neurons hyperpolarized ( $-8.7 \pm 1.7$  mV), 37% neurons depolarized ( $10.5 \pm 2.8$  mV) and 30% neurons (8 out of 27) showed no change in membrane potential. Input resistance changes indicated the modulation of two ion channels. Pharmacological data suggests hyperpolarization arises from the opening of  $K_{ATP}$  channels and depolarization results from the opening of non-selective cationic channels. Thus insulin modulates the electrical activity of SFO neurons and supports that the SFO is a sensor for maintaining energy homeostasis.

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## List of abbreviations

ACSF	Artificial cerebral spinal fluid
ADP	Adenosine diphosphate
AGRP	Agouti-related protein
ANGII	Angiotensin II
AP	Area postrema
ARC	Arcuate nucleus
ATP	Adenosine tri-phosphate
AV3V	Anteroventral third ventricle
BMH	Basomedial hypothalamus
BMI	Body mass index
CART	Cocaine and amphetamine-regulated transcript
CCHS	Canadian community health survey
CNS	Central nervous system
CSF	Cerebral spinal fluid
CVO	Circumventricular organ
DMSO	Dimethyl sulfoxide
EGTA	Ethyleneglycol-bis(2-aminoethylether)- <i>N,N,N',N'</i> -tetraacetic acid
ERS	External recording solution
GABA	$\gamma$ -aminobutyric acid
HCN	Hyperpolarized cationic channel
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
I <sub>A</sub>	A-type potassium current
I <sub>k</sub>	Fast inactivating potassium current
IR	Insulin receptor
IRS-2	Insulin receptor substrate 2
K <sub>ATP</sub>	ATP- gated potassium channel
LH	Lateral hypothalamus
MC4R	Melanocortin 4 receptor
MNC	magnocellular neurosecretory
MnPO	Median preoptic nucleus
NPY	Neuropeptide Y
NSCC	Nonselective cation channel

NTS	Nucleus of the solitary tract
OVLTL	Organum vasculosum of the lamina terminalis
PB	Parabrachial nucleus
PI3K	Phosphate 3- kinase
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
POMC	Proprionmelanocorticon
PVN	Paraventricular nucleus
PYY	Peptide YY
RAAS	Renin-angiotensin-aldosterone system
RE	Nucleus reuniens of the thalamus
SCN	Suprachiasmatic nucleus
SD	Sprague Dawley
SFO	Subfornical organ
SON	Supraoptic nucleus
SUR	Sulfonylurea receptor
VMH	Ventral medial hypothalamus
VP	Vasopressin
$\alpha$ -MSH	Alpha melanocyte-stimulating hormone

## **Chapter 1 Introduction:**

This thesis will describe a series of experiments demonstrating insulin's role in modulating the electrical properties of subfornical organ (SFO) neurons. This work serves to provide additional evidence of the contribution of the SFO to the overall regulation of energy homeostasis by the central nervous system (CNS).

### **1.1 Obesity -What it is and why it is a health care crisis**

The World Health Organization (WHO) defines obesity as an abnormal accumulation of body fat which is commonly assessed by the body mass index (BMI – weight (kg)/height (m<sup>2</sup>)). This scale places individuals into specific categories; BMI under 18.5 (underweight), 18.5 – 24.9 (normal weight), 25.0-29.9 (overweight), 30.0-34.9 (obese class I), 35.0-39.9 (obese class II), and 40 or greater (obese class III). Currently, 300 million people worldwide have been diagnosed as obese (Zinn and Palmer 2010), with 24.1% of the adult Canadian population (4.2 million) contributing to the global pandemic (Statistics Canada, 2011), and the number of Canadians diagnosed continues to increase (Vanasse *et al.*, 2006). In Canada, chronic health conditions associated with obesity such as diabetes, hypertension, gallstone formation, and the development of colon cancer (Lau *et al.*, 2006) are also increasing, as is the cost of obesity treatment, which is currently estimated at \$4.3 billion (\$1.6 billion of direct costs and \$2.7 billion of indirect costs) (Birmingham *et al.*, 1999; Katzmarzyk and Janssen 2004). Moreover, the Canadian Community Health Survey (CCHS) in 2010 predicted an influx of patients into the health care system, further taxing the system.

### 1.1.1 How obesity arises

Energy balance, also known as energy homeostasis, refers to the delicate equilibrium between energy intake (food input) and energy expenditure (Lenard and Berthoud 2008). Previously, it was believed that energy homeostasis was regulated primarily by plasma levels of glucose (Mayers 1953; for review see Mobbs *et al.*, 2005) and thus fluctuations of blood glucose levels would either result in feeding or satiation. Subsequent to the discovery of metabolites and circulating peptide signals (Bray, 2000; Woods *et al.*, 1998), however, the glucostatic hypothesis of energy homeostasis has been considered flawed. Circulating peptide satiety signals, such as neuropeptide Y, galanin, leptin, insulin, orexin, bombesin, glucagon, somatostatin, adiponectin, and ghrelin (for review see Blevins *et al.*, 2002) are produced in a variety of different tissues, and their concentrations in the blood and organs fluctuate with food consumption, energy expenditure, age, and gender (Bray and Campfield, 1975; Lebowitz *et al.*, 1992). Furthermore, these peptides have been shown to act within the CNS, and thus it is believed that energy homeostasis is regulated through a constant dialogue between the periphery and CNS, while additional factors, such as genetics, environmental, social and psychological factors (for review see Levin, 2004; Berthoud 2007; Lenard *et al.*, 2008) provide input into this dialogue.

Currently, the most accepted representation of obesity is a state of excess body weight from additional calories stored in adipose tissue (Moller & Kaufman, 2005) which is thought to arise due to a dysregulation of energy homeostasis (Lenard and Berthoud 2008). Thus, understanding the physiological mechanisms by which the CNS

regulates energy balance may provide us with insight into ways of treating obesity more effectively.

### **1.1.2 Is obesity a treatable disease?**

Exercise and diet are the most effective strategies to control and maintain body weight (Galani *et al.*, 2007, Klem *et al.*, 1997, Lau *et al.*, 2006, for review see Wing and Hill 2001). Although, exercise is the most effective method to promote and maintain weight loss (Wing and Hill 2001), many individuals find it difficult to incorporate the amount of activity required for adequate weight management into their lifestyle (Levin 2004). For example, for obese individuals, sustained weight loss is dampened by a cascade of physiological events which decrease energy expenditure and result in the 90% recidivism rate for weight regain (Truong *et al.*, 2006). This suggests that body weight is tightly regulated (for review see Cummings and Schwartz 2003), and some theories suggest that the CNS is “hard-wired” to monitor individual set points related to weight and energy metabolism to restore the ‘balance’ (Leibel *et al.*, 1995 for more complete review see Levin 2007).

Currently there are two broad classes of pharmacological therapies available to help treat patients with extreme visceral fat accumulation: appetite suppressants (anorexiant) and drugs that decrease nutrient absorption (for review see Campbell and Mathys 2001). Some of these drugs act within the CNS to decrease weight, however, they have numerous side effects. While some therapies have been effective at causing weight loss, adverse effects have led to these drugs being pulled from the market. For example, Phen-Fen (composed of phentermine and fenfluramine), developed to increase serotonin

concentrations in the synaptic cleft to suppress appetite (Rothman *et al.*, 1998), has been banned from the market after clinical reports indicated that continued use of Phen-Fen resulted in severe side effects such as pulmonary hypertension, coronary artery disease and heart valve thickening causing death (Connolly *et al.*, 1997; Mark *et al.*, 1997; for review see Campbell and Mathys 2001). Sibutramine (trade name Meridia), another anorexiant which acts by blocking reuptake of serotonin and norepinephrine (for review see Stock 1997; Lean 2001), also manifests severe side effects, such as hypertension and congestive heart failure after long-term use (as reviewed Aronne 1997). Rimonabant (trade name Acomplia), is an appetite suppressor acting as an inverse agonist, which binds to the endocannabinoid CB<sub>1</sub> receptor and decreases the activation of the cannabinoid system, resulting in the suppression of appetite (Rinaldi-Carmona *et al.*, 1994, for review see Xie *et al.*, 2007). Side effects of Rimonabant may include severe depression and dyslipidaemia. Rimonabant has been off of the market since 2009 in European Union countries and Canada. Orlistat (trade name Xenical or Alli), the only drug currently available within the marketplace, decreases 30% of fat ingested by inhibiting pancreatic lipases as well inhibiting gastric and carboxyl-ester lipases (Davidson *et al.*, 1999; for review see Heck *et al.*, 2000). Although effective, Orlistat causes steatorrhea, and liver damage has been reported after long-term use (Cavaliere *et al.*, 2001; for review see Glazner 2000). Surgical treatment, the last option available for the morbidly obese (individuals those with a BMI > 40 kg/m<sup>2</sup>), is more invasive, and candidates for bariatric surgery are stringently screened before surgery as mortality rates are higher for older individuals suffering from additional health problems (for review see Bult *et al.*, 2008).

The use of many weight-loss drugs is limited because of their unknown actions within the periphery and CNS. Therefore, gaining a better understanding of how the CNS responds to challenges of energy homeostasis will enable us to develop better pharmacological approaches to combat the obesity epidemic.

## **1.2 Hypothalamic Control of Energy Regulation**

Research on nuclei within the CNS suggests that a greater understanding of these mechanisms may provide valuable insights into the solutions for obesity (Schwartz *et al.*, 2005). Early studies link a variety of hypothalamic nuclei within the CNS to regulation and maintenance of energy balance (Hetherington and Ranson 1940 for review see Elmquist *et al.*, 1999). For example, Boyle and Keesey (1975) reported lesioning of the lateral hypothalamus (LH) resulted in a decrease in food intake. Furthermore, cell-specific lesioning of the LH with kainic acid also produced similar results (Grossman *et al.*, 1978). Moreover, lesioning of the ventromedial hypothalamus (VMH), an area adjacent to the LH, resulted in increased food intake (Elmquist *et al.*, 1999). Thus, the VMH and LH were thought to be the centres responsible for intake and satiety, respectively. The paraventricular nucleus of the hypothalamus (PVN) has also been demonstrated to play a key role in the neural regulation of energy balance. PVN-vagal efferent connections to the gastrointestinal tract have long been proposed to play a major role in the autonomic regulation of feeding (Saper *et al.*, 1976). Moreover, electrical stimulation of the PVN decreases gastric motility (Sakaguchi and Ohtake 1985), an effect which is absent in vagotomized rats (Rogers and Hermann 1987). Also, lesions within the PVN have been reported to induce hyperphagia as well increases in body weight (Sims and Lorden 1986).

### **1.2.1 ARC is a key centre for the regulation of energy balance by leptin and insulin**

In recent years, the arcuate nucleus (ARC) has been thought of as the key site in the CNS for the regulation of energy balance. The ARC possesses two separate subpopulations of first order neurons. Propiomelanocorticon (POMC) neurons coexpress messenger ribonucleic acid (mRNA) encoding cocaine and amphetamine-regulated transcript (CART) and alpha melanocyte-stimulating hormone ( $\alpha$ -MSH), which produce anorexigenic neuropeptides, whereas the other subpopulation expresses orexigenic neuropeptides, neuropeptide Y (NPY) and agouti-related protein (AGRP). Both subpopulations of neurons project to the PVN and LH as well as the nucleus of the solitary tract (NTS) in the brain stem to regulate feeding (for review see Schwartz *et al.*, 2000; Cowley 2008; Konner *et al.*, 2009). The significance of the above neural circuitry present within the ARC in relation to energy homeostasis was brought into the forefront after the discovery of leptin and its actions within the ARC.

Leptin is a 16 kilodalton (kDa) adipostat that is secreted from white adipocytes in concentrations proportional to body fat content and energy balance (Zhange *et al.*, 1994; Halaas *et al.*, 1995; Pellymounter *et al.*, 1995; for review see Houseknecht *et al.*, 1998). Leptin acts as a satiety signal which targets POMC/CART neurons and increases mRNA synthesis of  $\alpha$ -MSH and CART while down-regulating the expression of NPY and AGRP neurons (Elias *et al.*, 1999; Ahima *et al.*, 1999; for review see Friedman 2002, for review see Schwartz *et al.*, 2005).  $\alpha$ -MSH and AGRP both bind melanocortin - 4 receptor (MC4R) which are found on second order hypothalamic neurons and have anatagonistic effects within the feeding circuit. Electrophysiological studies of leptin's actions within the ARC demonstrate that leptin simultaneously depolarizes POMC/CART neurons

through a non-selective cation channel and also decreases inhibitory  $\gamma$ -aminobutyric acid (GABA) input from local NPY and AGRP neurons which results in an increased action potential firing frequency of POMC/CART neurons. Leptin hyperpolarizes the membrane potential of NPY neurons by modulation of a ATP-gated  $K^+$  adenosine triphosphate channel ( $K_{ATP}$ ) which results in a decrease in action potential firing frequency (Cowley *et al.*, 2001; Mirshamsi *et al.*, 2004; Pinto *et al.*, 2004; Takahashi and Cone 2005 for review see Jobst *et al.*, 2004). Together, these actions of leptin result in the inhibition of feeding. Insulin, another adipostat signal, is a long-term regulator of energy balance. Below, the structure and function of insulin will be presented followed by a brief discussion of the electrophysiological actions of insulin within the ARC.

#### **1.2.1.1 Insulin-Structure and function**

Insulin is a 58 kDa anorexigenic peptide released from pancreatic  $\beta$ -cells and acts as a long-term anorectic signal within the CNS (for review see Woods *et al.*, 1985). Similar to leptin, the plasma concentrations of insulin are correlated with body mass and additionally to hepatic glucose production (Bagdade *et al.*, 1967; Polonsky *et al.*, 1988). Historically, insulin's prominent role has been associated with carbohydrate metabolism and the regulation of plasma glucose levels (Koch *et al.*, 2008), however, insulin's role within the CNS was largely neglected until it was demonstrated that direct injection of insulin into the third ventricle of baboons decreased food intake and body weight (Brief and Davis 1984; Florant *et al.*, 1991, Porte and Woods 1981, Woods *et al.*, 1979). The high levels of insulin receptors and insulin signalling molecules localized within a variety of CNS regions (for review see Schulingkamp *et al.*, 2000), including the hypothalamic ARC and VMH (Schwartz *et al.*, 2000; Strubbe and Mein 1977; for

review see Plum *et al.*, 2006), LH (Carr *et al.*, 2000) and ventral tegmental area (Figlewicz *et al.*, 1994; Sipols *et al.*, 2000) suggest a functional role for insulin within the CNS. Moreover, insulin's critical role in maintenance of body weight has been shown in neuronal insulin receptor knockout mice, which exhibit increased patterns of food intake, diet induced obesity, as well as higher circulating plasma insulin levels and develop insulin resistance (Bruning *et al.*, 2000). Additionally, the increase in insulin concentrations in cerebrospinal fluid (CSF) when plasma levels of insulin are high suggest the existence of a saturable transporter (Banks and Kastin 1996; for review see Banks *et al.*, 2004), to move insulin from circulation to the CNS.

### **1.2.3 Insulin's role in the maintaining energy balance via its actions in the ARC**

Insulin has been demonstrated to have a number of effects at the ARC which are analogous to leptin's actions, such as an alteration in expression of satiety neuropeptides NPY, POMC and alpha MSH (Wang and Leibowitz 1997; Benoit *et al.*, 2002; Schwartz *et al.*, 2000). Insulin has also been demonstrated to modulate the electrical activity of hypothalamic neurons via the opening of the  $K_{ATP}$  channel, which results in a hyperpolarization of the membrane potential and a decrease in the spontaneous firing rates of a subpopulation of neurons (for review see Jobst *et al.*, 1999; Plum *et al.*, 2005, Spanswick *et al.*, 1997; and 2000 Williams *et al.*, 2010). Furthermore, the similarity of electrophysiological actions of leptin and insulin within the CNS are suggested to arise because of the activation of commonly shared signaling pathway molecules such as phosphate 3- kinase (PI3K) (van den Top *et al.*, 2007). See Figure 1.1.

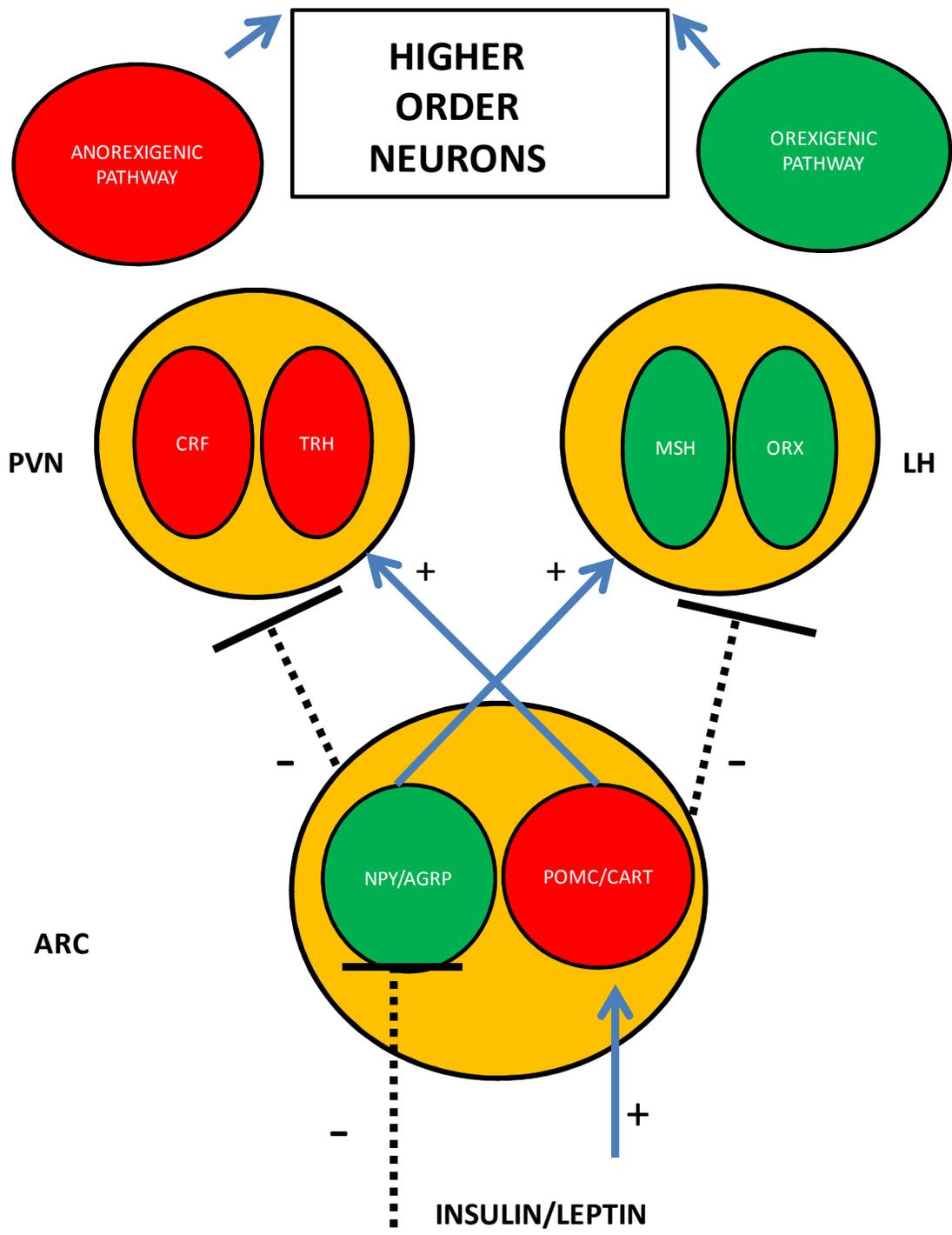
It is evident that insulin is able to influence hypothalamic nuclei, however, the requirement for transport across the BBB suggests that rapid detection of changing circulating insulin concentrations in these neurons may be somewhat impeded by the relatively slow process of receptor mediated transport. This leads to the belief that alternative neural signaling pathways may exist that allow for more rapid detection of insulin levels in the CNS (for review see Fry *et al.*, 2007; Price *et al.*, 2008; Smith and Ferguson, 2008; 2010). Below, a brief description of the BBB will be provided followed by an extensive discussion on circumventricular organs (CVOs), areas of the brain not regulated by the BBB, which thus have unhindered access to peripheral metabolic signals, followed by a specific focus on the SFO.

### **1.3 Blood Brain Barrier (BBB)**

The BBB is a specialized impermeable barrier composed of brain endothelial cells, astrocytic end-feet and pericytes (for review see Ballabh *et al.*, 2004), which collectively function to protect the brain from shifts in glucose, ion, hormonal concentration and pH changes. Lipophilic molecules, such as alcohol and oxygen, cross the BBB with ease, whereas molecules indicating energy status, such as glucose, amino acids and peptides, including leptin, insulin and ghrelin, require specialized transporters (Abbott *et al.*, 2006). This raises the question “How does information regarding energy status reach the hypothalamus?” Within the literature, there is controversy over the exact mechanism of transport of peptide satiety signals at the ARC. In particular some have proposed that the BBB is absent at the ARC (Bagnasco *et al.*, 2003) while others maintain that the BBB is compromised or weakened (Traebert *et al.*, 2002).

Figure 1.1 Actions of leptin and insulin in the arcuate nucleus of the hypothalamus.

A schematic depicting the organization of the POMC/CART and NYPY/AgRP neurons within the ARC nucleus and their projections to higher centers within the hypothalamus. The actions mediated by insulin binding to the insulin receptors are shown in the diagram. The plus signs indicate upregulation and the negative sign indicates a downregulation of mRNA such as CART or  $\alpha$ -MSH transcripts.



An alternative hypothesis suggests circulating satiety signals are in direct contact with neuronal perikarya at the circumventricular organs (CVOs); specialized areas of the brain not completely regulated by the BBB (Johnson and Gross 1993; McKinley *et al.*, 2003; Fry *et al.*, 2007).

#### **1.4 The Circumventricular Organs (CVOs) – “The Windows of the Brain”**

Permeation of neuronal structures by peripherally injected silver nitrate and trypan blue demonstrated the lack of a BBB (Wislock and Leduc 1952) at specialized midline structures, which Hofer (1958) termed CVOs (for review see McKinley, 2003). CVOs are highly fenestrated (Gross 1991) and permeable to molecules that are unable to cross the BBB (Gagnon 2000). There are eight CVOs in mammalian brains: the median eminence, pineal gland, subcommissural organ and choroid plexuses which are referred to as secretory CVOs because they are comprised of terminals, axons, glial and/or epithelial cells. Three CVOs are classified as sensory because they contain neuronal cell bodies which are capable of detecting and responding to circulating metabolites by transmitting information via their axons to other nuclei. These include subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT) and area postrema (AP) (Figure 1.2 for the location of the 3 CVOs).

Neurons within sensory CVOs are exposed to peripheral circulation constituents and are able to detect changes, respond, and relay information to other nuclei through their efferent projections (for review see McKinley *et al.*, 2003). Much of the research regarding energy balance has focused on the AP, however, recently the SFO has garnered attention after demonstrating responses to food consumption and feeding associated drinking (Riediger *et al.*, 2001; Pulman *et al.*, 2006; Smith *et al.*, 2010). The central focus

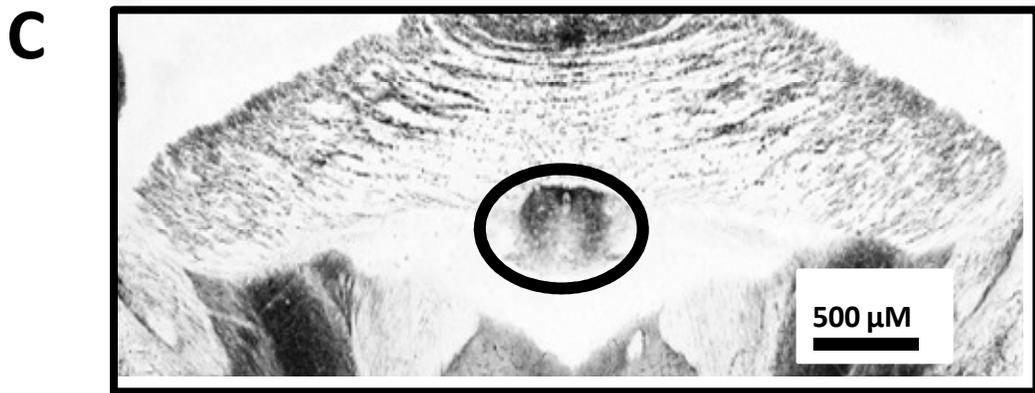
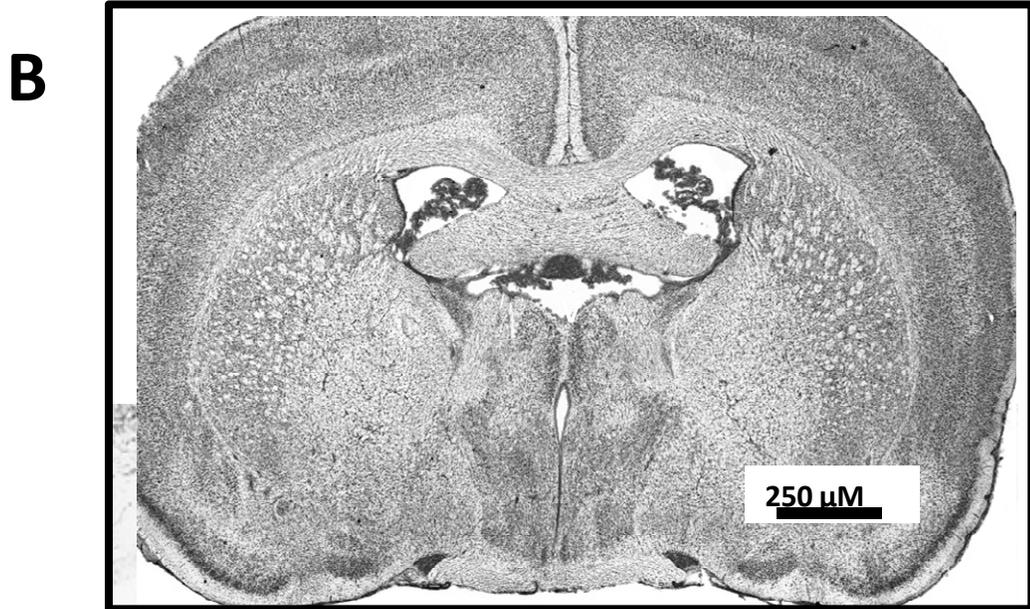
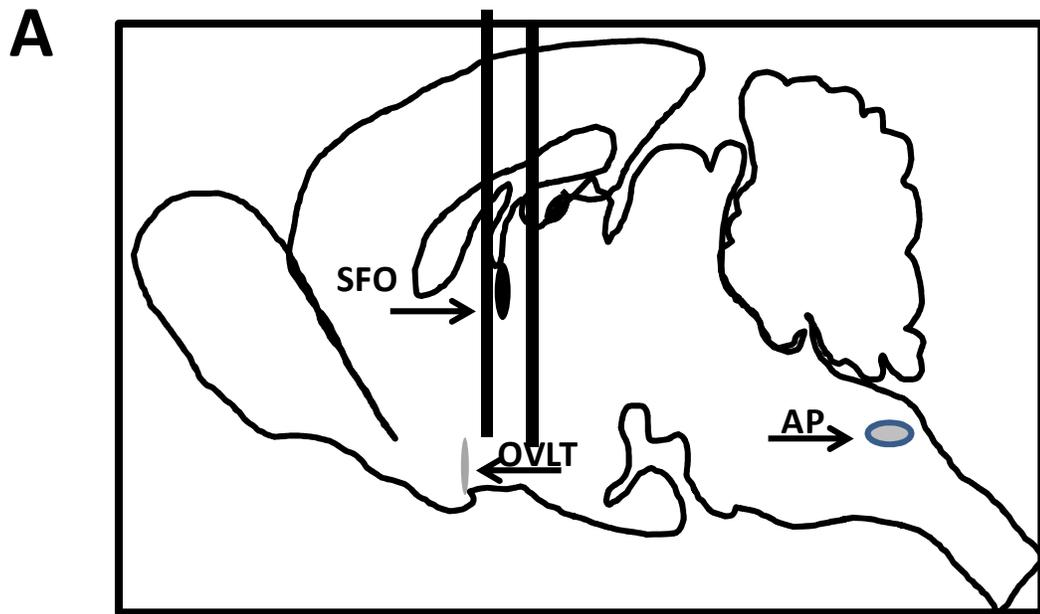
of this project is on the neurons of the SFO, and thus the morphology, anatomy and physiological functions of the SFO will be briefly discussed below.

#### **1.4.1 Anatomy and Morphology of the Subfornical Organ (SFO)**

The SFO is located at the intersection of the lateral ventricle with the third ventricle and appears to have a bulging appearance (Figure 1.2). The SFO is divided into two functional zones: the outer shell and the ventromedial core (for review see McKinley *et al.*, 2003). Blood flow through core of the SFO is complex and slow, as capillary density is suggested to be 4-5 times greater than in areas with an intact BBB (Fenstermacher *et al.*, 1988; Gross, 1992). It has been suggested that large pericapillary spaces (Virchow-Robin spaces) serve to pool and decrease the perfusion rate of blood, allowing neurons within the core to perform their sensory functions and relay information (Gross 1991). Three different cell types have been characterized within the SFO: neuronal cell bodies, glial cells and ependymal cells (Dellman and Simpson 1979; Dellman 1998). The core contains neurons capable of detecting a variety of peptidergic receptors such as angiotensin II (ANG) (Anderson *et al.*, 2001), endothelin (Wall and Ferguson, 1992) and vasopressin (Smith and Ferguson, 1997). Ependymal cells line the third ventricle, whereas glial cells provide support. Furthermore, architecturally ventral SFO neurons are in direct contact with circulating CSF and peripheral circulation thus allowing for the SFO to act as a sensor.

Figure 1.2 The location of the three sensory CVOs.

A midsagittal cross section through a rat brain showing the anatomical location of the three sensory circumventricular organs, the subfornical organ (SFO), the area postrema (AP), and organum vasculosum of the lamina terminalis (OVLT). The SFO is located dorsal to the anterior commissure. Note the diagram is not drawn to scale (A). A coronal section showing the position of the SFO in a slide (B). A detailed image highlighting the SFO. (C). All images are modified from Rat Brain (Paxinos and Watson 2009).



## **1.4.2 Anatomical Connectivity**

### ***1.4.2.1 Afferent projections:***

Afferent projections to the SFO originate from the midbrain raphe nuclei (RN) (Lind *et al.*, 1982), the LH (Lind *et al.*, 1982), the nucleus tract of the solitaratus (NTS) (Zardetto-Smith and Gray, 1987), the lateral divisions of the parabrachial nucleus (PB) (Gu and Ju, 1995), the nucleus reuniens of the thalamus (RE) (Lind *et al.*, 1982), the medial septum (Lewis and Shute, 1967) and median preoptic nucleus MnPO (Lind *et al.*, 1982). See Figure 1.3 A.

### ***1.4.2.2 Efferent projections:***

Radio-autoradiographic tracing methods and labelling with horse radish peroxidase have delineated extensive projection pathways from the SFO which can be subdivided into those projecting to either the anteroventral third ventricular region (AV3V) or neurosecretory regions of the hypothalamus (Miselis *et al.*, 1979; and Miselis 1981, Lind *et al.*, 1982). Projections from the AV3V region contain bundles of axonal fibres which traverse the ventral stalk of the MnPO and target the MnPO, medial septum and the medial preoptic area (Lind *et al.*, 1982). Fibres projecting to the MnPO further branch to innervate the OVLT, supraoptic nucleus (SON) and the suprachiasmatic nucleus (SCN) (See Figure 1.3 B).

SFO fibres also directly project to key autonomic regulating centres within the hypothalamus terminating within the SON or PVN. SFO projections to the magnocellular neurosecretory neurons (MNCs) within the SON and PVN have generated a lot of attention due to the MNCs' controlled release of vasopressin (VP) (Ferguson and Kasting

1986) and oxytocin (Ferguson and Kasting 1987). Furthermore, collateral inputs to other nuclei within the hypothalamus (Weiss and Hatton, 1990) suggest that the SFO is able to coordinate multiple nuclei within the hypothalamus such as the PNV and SON. Thus, the SFO is not only well positioned to receive and send information from systemic circulation to the central nervous system (CNS), but it is also capable of controlling a variety of autonomic activities.

### **1.5 Physiological functions of the SFO:**

A well established and extensively studied role of SFO is its role in the maintenance of hydro-mineral balance. However, the focus of this thesis is on the SFO's role in regulating and maintaining energy balance, which will be discussed briefly below.

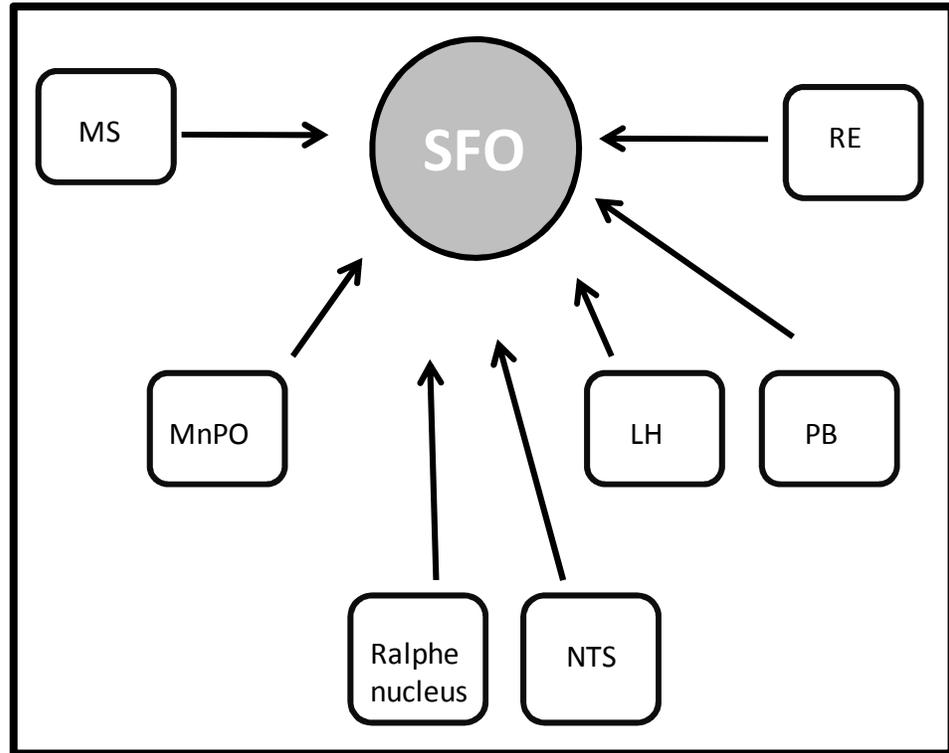
#### **1.5.1 Role in the detection of energy balance:**

Until recently, the AP was the sole sensory CVO associated with the regulation of energy homeostasis. Now, however, the SFO's role in detection of energy homeostasis is also well recognized. Anatomically, SFO projections to the hypothalamic nuclei linked to feeding suggest its roles in energy balance (McKinley *et al.*, 2003; Timofeeva *et al.*, 2005). Also, anorexia (McKinley *et al.*, 1996) and loss of fat (Takahashi *et al.*, 1997) have been reported following lesioning of SFO. Furthermore, the presence and expression of many receptors involved in food intake and satiety, such as adiponectin, amylin, calcitonin gene-related peptide, calcitonin, ghrelin, glucose, leptin, orexin, and

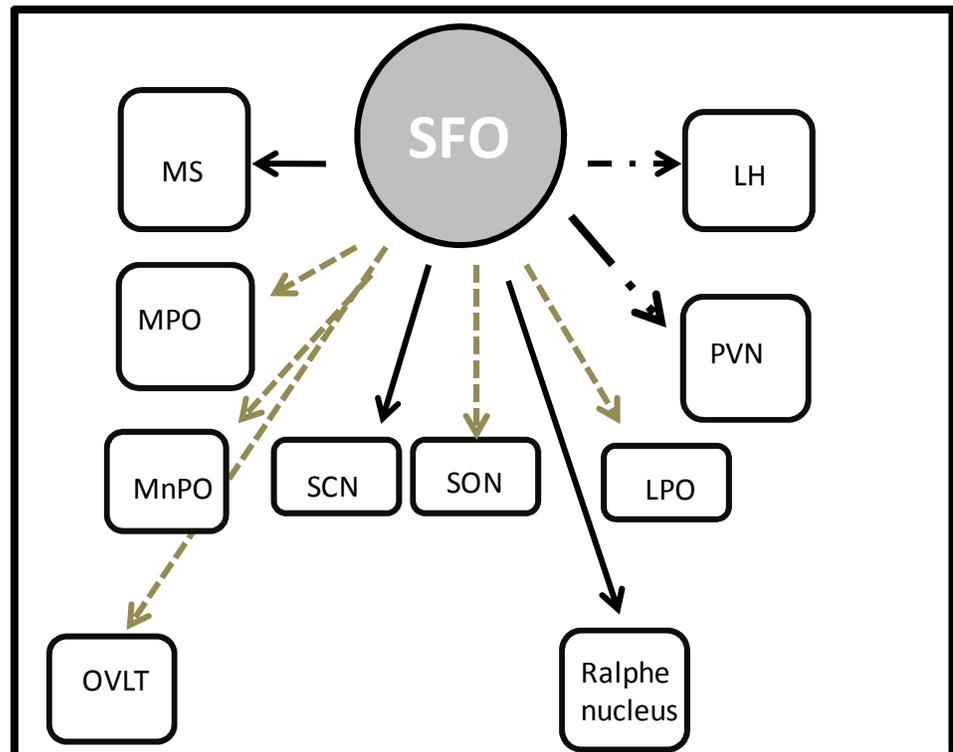
Figure 1.3 Afferent and efferent projections from the SFO.

Majority of input signals to the SFO come from CSF or circulation, however, there are a few CNS sites that project to the SFO and they include: median preoptic nucleus (MnPO), medial septum (MS), nucleus reunions of the thalamus (RE), lateral divisions of the parabrachial nucleus (PB), nucleus tractus solitaries (NTS), lateral hypothalamus (LH), and the raphe nuclei (RN) (**A**). A schematic depicting the efferent projections from the SFO. The SFO projects to multiple CNS sites including medial septum (MS), medial preoptic area (MPO), and median preoptic nucleus (MNPO). In the hypothalamus, the SFO projects to the periventricular nucleus (PVN), and the supraoptic nucleus (SON). Other SFO efferent sites include: suprachiasmatic nucleus (SCN), lateral preoptic area (LPO), lateral hypothalamus (LH), raphe nuclei and organum vasculosum of the lateral terminalis (OVLT). The grey dashed arrows represent key projections involved in fluid balance while the black dashed arrows represent projections to areas involved in energy homeostasis (**B**).

**A**



**B**



PeptideYY (PYY) (Fry *et al.*, 2006; Sexton *et al.*, 1994; Christopoulos *et al.*, 1995; Rouleau *et al.*, 1984; Pulman *et al.*, 2006; Smith *et al.*, 2009; Kunii *et al.*, 1999; Hindmarch *et al.*, 2007) have been localized to the SFO. It has also been demonstrated that activation of these peptide receptors affect the electrical activity of SFO neurons (Alim *et al.*, 2010; Pulman *et al.*, 2006; Smith *et al.*, 2009; Ono *et al.*, 2008; for review see Fry *et al.*, 2007). Furthermore, direct stimulation of SFO in water-satiated animals elicits a near immediate eating response (Smith *et al.*, 2010).

While insulin has been demonstrated to modulate the electrophysiological activity of hypothalamic neurons such as the ARC, insulin must be transported across the BBB. The SFO, however, has direct access to circulating insulin. Therefore, it is postulated that insulin is capable of modulating the electrical activity of SFO neurons. In support of this notion is the finding that insulin receptors have been localized within the SFO at a density even higher than areas classically characterized as integral to feeding behavior, such as AP and OVLT (van Houten *et al.*, 1979 ; van Houten *et al.*, 1983; Moss *et al.*, 1990). Furthermore, insulin receptor (IR) and insulin receptor substrate two (IRS-2), which are crucial signaling molecules in the insulin response cascade, are found within the SFO (Unger *et al.*, 1989), suggesting the presence of a functional insulin signaling pathway in the SFO. Moreover, at the AP, insulin has been demonstrated to cause an increase in the firing rate of neurons (Carpenter and Briggs 1986). Thus, it is logical to predict an electrophysiological action of insulin in SFO neurons.

Furthermore, also noted within the literature is stimulation of thirst in humans after an intravenous injection of insulin into the peripheral circulation which suggests that insulin-induced thirst is a precept to triggering a feeding response (Vijande *et al.*, 1990).

Considering the extensive volumes of literature demonstrating the SFO's role in the maintenance of hydro-mineral balance, a possible role of insulin within the SFO is the activation of the drinking reflex. This thesis focuses on the modulation of electrical activity of SFO neurons by exogenously applied insulin.

### **1.7 Aims of this study:**

Insulin, a vital hormone and metabolic signal, plays a key role in the regulation of energy balance (for review see Schwartz *et al.*, 2000). Moreover, it is evident that insulin receptors and signaling substrates are localized within the SFO (van Houten *et al.*, 1979; van Houten *et al.*, 1983; Moss *et al.*, 1990; Unger *et al.*, 1989). Recently the discovery of a wide variety of orexigenic and anorexigenic peptide receptors expressed within the SFO (Hindmarch *et al.*, 2007) further suggests that the role of the SFO is not limited to detection and maintenance of fluid balance. It has been demonstrated that SFO neurons are responsive to circulating glucose and ion concentrations, hormones, peptides, and satiety signals. Furthermore, electrical activity of SFO neurons is modulated in response to changes in the concentrations of circulating signals (Pulman *et al.*, 2006; Smith *et al.*, 2009; Alim *et al.*, 2010). However, electrophysiological responses of SFO neurons to insulin have not been previously demonstrated. The first goal of this study was to determine if SFO neurons are sensitive to insulin. To test if SFO neurons are sensitive to changes in insulin concentration, insulin was applied, and electrical properties of the neurons were measured using patch clamp electrophysiology.

This thesis will specifically address the following hypothesis:

- 1. Acute exposure to insulin results in a modulation of the electrical properties (membrane potential (MP), and action potential firing frequency) of SFO neurons.**

Once sensitivity of SFO neurons to insulin is established, the second goal of this study is to investigate the ionic mechanism(s) responsible for the modulation of the electrical properties of SFO neurons.

The second hypothesis is described below:

- 2. Insulin modulates the electrical excitability of SFO neurons through a modulation of the activity of a variety of ion channels.**

## **Chapter Two Materials and Methods**

### **2.1 Animals**

Male Sprague Dawley rats (150-200 grams) were used for experiments. Rats were housed on 12/12 light/dark cycle (light onset 07:00) with food and water provided *ad libitum*. All animal experiments were carried out in accordance with the Canadian Council on Animal Care and the University of Manitoba Animal Care Committee.

### **2.2 Preparation of Subfornical Organ Neurons and Cell Culture**

Briefly, for each set of cultures, 2-3 male Sprague-Dawley rats (150-200 grams) were decapitated, and the brains were placed into an ice-cold oxygenated artificial cerebrospinal fluid (ACSF) consisting of (in mM): 87 NaCl, 2.5 KCl, 7 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 0.5 CaCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 25 glucose and 75 sucrose. Brain slices were cut at 300-350 µm thickness on a VF-200 Microtome (Precisionary Instruments Inc. Greenville, NC) and transferred into Hibernate media (Brain Bits, Springfield, IL) supplemented with 1X B27 (Invitrogen, Burlington, ON). The SFO was microdissected away from the surrounding tissue and transferred into 5 ml of Hibernate media containing 10mg of papain (Worthington, Lakewood, NJ) and incubated at 30°C for 30 minutes. The tissue was washed in Hibernate/B27, titrated and centrifuged for 5 minutes at 240g. Neurons were resuspended in Neurobasal-A media supplemented with 1X B27/glutamax (Invitrogen, Burlington, ON), plated on 35mm glass bottom dishes (MatTek Ashland, MA) and incubated at 37°C in 5% CO<sub>2</sub>. Electrophysiological experiments were

performed on neurons within 1-4 days. Prior to recording, neurons were gently washed for 20 minutes in B27-free Neurobasal media.

### **2.3 Electrophysiological Techniques**

Whole-cell recordings were acquired using a HEKA EPC10 patch clamp amplifier running Patchmaster v2.53 software (HEKA, Mahone Bay, Nova Scotia, Canada). Data were filtered at a range of 5 to 100 kHz and acquired at 10 kHz. During recording, neurons were perfused with a physiological external recording solution containing (in mM): 140 NaCl, 4 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and 10 glucose, pH 7.3 with NaOH. Recording electrodes were fabricated from borosilicate glass using a P-97 Flaming/Brown Pipette Puller (Sutter Instruments, Novato, CA) and had resistances ranging from 2-5 MΩ when filled with an internal recording solution containing (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 5 ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, and 4 NaATP, pH 7.25 with KOH.

Using a digital micro-manipulator, (Sutter Instruments, Novato, CA) the patch electrode was positioned onto a cell until contact was made, and gentle suction was provided until a gigaohm (GΩ) seal formed. The whole-cell recording configuration was obtained by a brief pulse of negative pressure. Access resistance to the cell was monitored, and neurons were rejected if the access resistance was greater than 30 (MΩ). Whole-cell voltage clamp recordings were carried out on SFO neurons similar to that previously described (Pulman *et al.*, 2006; Fry *et al.*, 2009). Under voltage clamp,

neurons were identified by the presence of a voltage-gated  $\text{Na}^+$  current as well as the presence of one of two types of  $\text{K}^+$  currents: an outward transient current ( $I_A$ ) or a delayed rectifier ( $I_K$ ). In current-clamp, action potentials of at least 70 mV were identified. During most current-clamp recordings, neurons were held near -60 mV.

To examine if SFO neurons were electrically responsive to insulin, the following experiment was conducted. After the cell established a stable baseline, a change in the membrane potential of the cell or action potential firing frequency of the cell to insulin was measured by perfusing the dish with 100 nM insulin for three minutes at a rate of 1 millilitre per minute (ml/min).

Changes in input resistance and ionic conductance (reversal potential of the effect) were investigated using a series of hyperpolarizing and depolarizing current injections before and after application of insulin. Lastly, to confirm the identity of ion channels modulated by insulin, neurons were pre-treated with a pharmacological blocker: either 30  $\mu\text{M}$  glibenclamide or 3 mM cesium ( $\text{Cs}^+$ ) cell prior to application of insulin. Insulin was perfused over the cell in the presence of either glibenclamide or cesium to investigate the identity of the ion channels modulated by insulin.

## **2.4 Electrophysiological Properties Measured from SFO neurons**

A number of electrical parameters of SFO neurons were investigated, including the presence of  $I_A$  versus  $I_K$ , membrane potential, action potential firing frequency, input resistance, reversal potential of the effect and membrane capacitance.

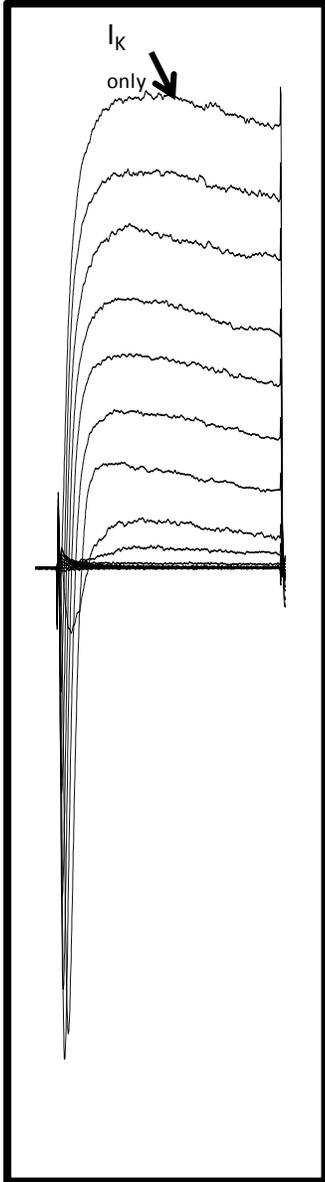
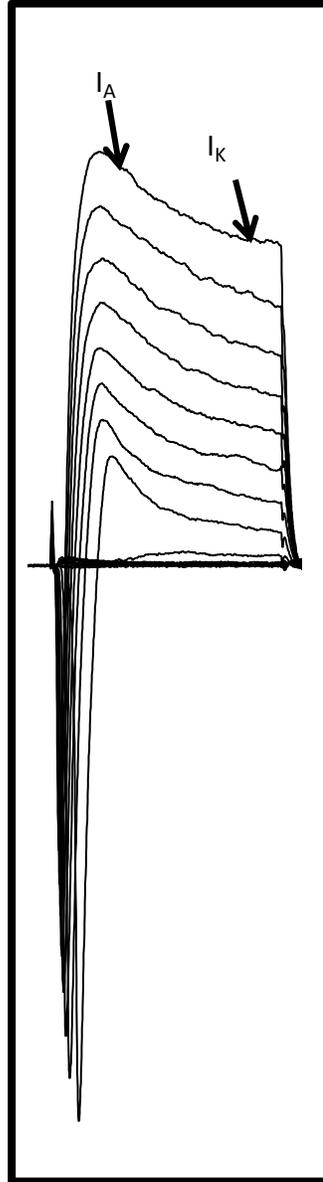
1.  $I_A$  versus  $I_K$ : SFO neurons which project to the PVN exhibit two types of potassium currents; an outward transient current ( $I_A$ ), and a delayed rectifier current ( $I_K$ ),

whereas SFO neurons which do not project to the PVN express only the  $I_K$  (Washburn *et al.*, 2001) This electrophysiological fingerprint helps support the existence of subpopulations of SFO neurons which have demonstrated varying electrical responses. Figure 2.1.

2. Membrane potential: Of a cell measured in millivolts, (mV) during whole-cell current clamp recording, is the voltage across the cell membrane when a constant amount of current is injected into the cell. When the membrane potential of the neuron becomes more negative after peptide or hormone application, the response is a hyperpolarization of the membrane potential. Likewise, if the membrane potential of neuron becomes more positive after a peptide or hormone application, a depolarization of the membrane potential results.
3. Action potential firing frequency: (Measured in Hertz) is a measure of how often the cell fires. This property is correlated with a change in membrane potential. Thus when a cell's membrane potential hyperpolarizes, the action potential firing frequency decreases, whereas when a cell's membrane potential depolarizes, the action potential firing frequency increases.

Figure 2.1 Whole-cell voltage-clamp recordings from SFO neurons demonstrating the presence of  $I_K$  and  $I_A$ .

Using whole-cell voltage clamp techniques identifying the presence or absence of  $I_A$  in patched SFO neurons can help suggest whether the SFO neuron projects to the PVN. The first recording trace is obtained from a patched SFO neuron which lacks the  $I_A$  current seen by the plateau nature of each recording sweep (**A**). The second trace is from a different SFO neuron and demonstrates the presence of a rapidly decaying  $I_A$  current suggesting that this neuron projects to the PVN (**B**).

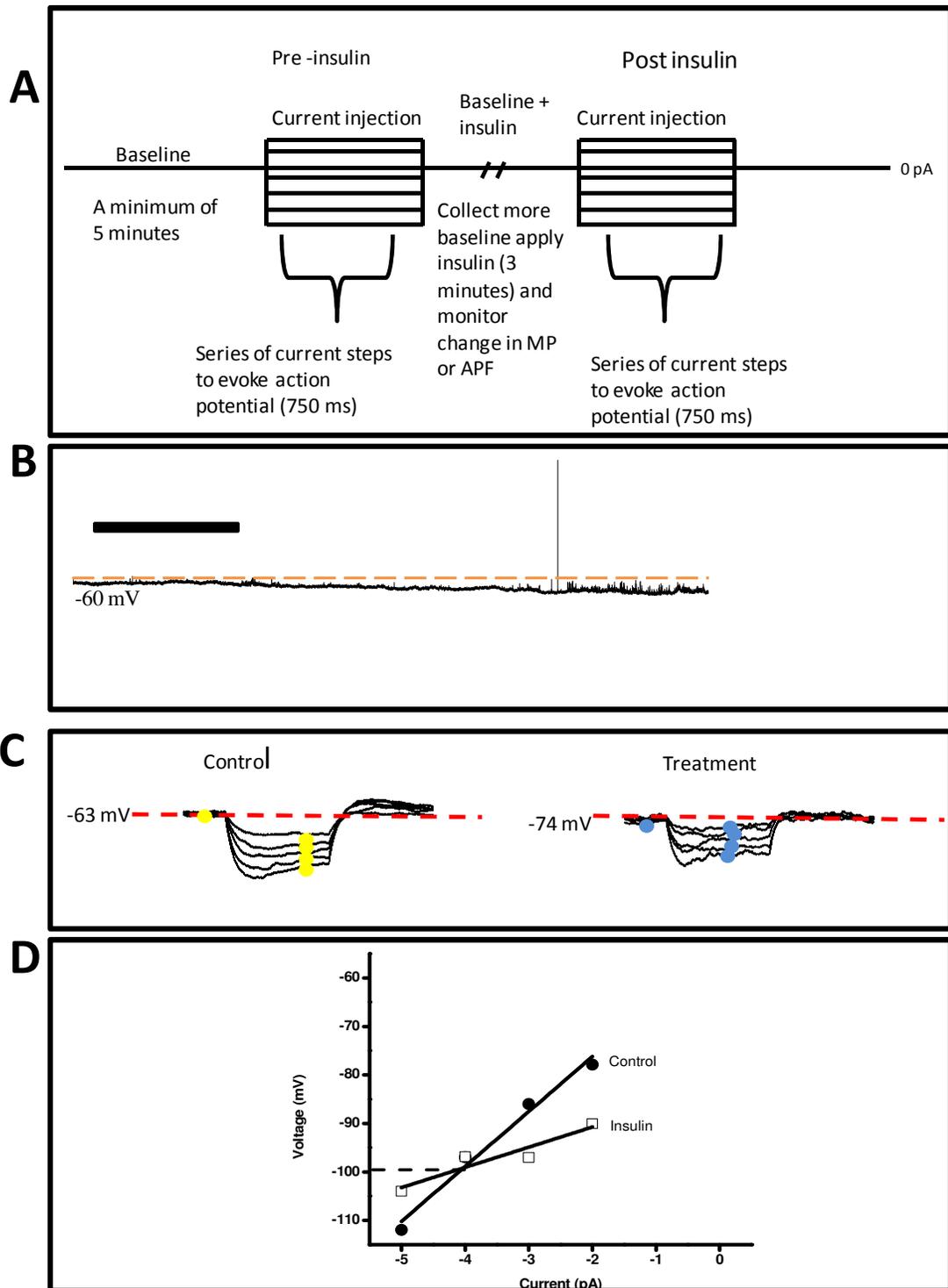
**A****B**

250 pA  
2 ms

4. Input resistance of cell: (Measured in the current-clamp configuration) is a measure of the overall ionic conductance (number of ion channels open or closed) across the cell membrane and is calculated using subthreshold current injections. See Figure 2.2 for an example of the protocol administered to determine input resistance.
5. Reversal potential of the effect: Changes in input resistance reflect changes in ion channel activity. Identity of ion channels affected can be determined by examining the reversal potential of the effect. The reversal potential of the effect can be obtained from the intersection of the voltage/current (V/I) curves (Results section 3.3). The intersection point indicates the point of electrochemical equilibrium of an ion which would suggest no net movement across an ion channel. This point can be calculated using the Nernst equation and the ion concentrations of the solutions used in the experiment.
6. Membrane capacitance: The cell membrane of a neuron behaves as a capacitor. Membrane capacitance is indicative of a cell's size and measured in picofarads (pF).

Figure 2.2 Current-clamp protocol designed to determine input resistance of all SFO neurons.

During whole-cell current clamp the membrane potential of a neuron was monitored until it established a stable baseline. At this point the neuron was subjected to a series of hyperpolarizing and depolarizing current injections, which lasted 750 milliseconds. The protocol was designed to return to the previous method of data acquisition once the series of current pulses was complete (**A**). An example of a sample current-clamp trace demonstrating responsiveness to perfusion of insulin. The trace was then interrupted with the series of current injections to obtain changes in input resistance. Note that the spike in the recording trace is a recording artefact and not an action potential (**B**). An example of a recording trace indicating the series of hyperpolarizing current pulses (control) before insulin application and (treatment) after insulin application. The circles indicate the points chosen to compare voltage deflection before and after insulin application (**C**). A scatter plot depicting a voltage/current (V/I) plot to compare changes in input resistance to identify if application of insulin would affect input resistance. Changes in slope of the lines of best fit indicate a decrease in input resistance. Also the point of intersection of the plotted lines indicates a reversal potential of -90 mV (close to the equilibrium potential of  $K^+$  as predicted by the Nernst equation) (**D**).



## 2.5 Peptides and Drugs

Insulin (Sigma, Oakville, Canada) was prepared daily from a 1  $\mu$ M stock to a concentration of 100 nM in external recording solution. This concentration is greater than the EC<sub>50</sub> value of insulin which is 3.3 nM as reported by Cortero and Routh (2009). However, other electrophysiology experiments use insulin concentrations ranging from 300 nM -500 nM (Williams *et al.*, 2010; and Spanswick 2000). 100 nM insulin was chosen to ensure that a maximal activation of insulin receptor occurred while trying to be as close to physiological levels as possible.

In section 3.4, a set of experiments was undertaken to identify the ion channels modulated by insulin. Glibenclamide (MP Biomedicals, Solon OH) was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mM and diluted daily to a working concentration of 30 $\mu$ M in external recording solution to ensure complete blockage of the K<sub>ATP</sub> channel (Schultz *et al.*, 1996) (see Results section 3.4 for more information).

1 M CsCl (Sigma, Oakville Canada) stock was prepared and diluted into external recording solution at a concentration of 3 mM to ensure a complete blockage of hyperpolarized activated cation channel (HCN) (Washburn and Ferguson 2001) (see Results section 3.5 for more information). All peptides and drugs were applied via bath perfusion.

## **2.6 The Bath Perfusion System**

A perfusion system to allow flow of solutions of pharmacological agents into the patch clamp recording dish was constructed. The perfusion system, made up of three 60 ml plastic syringes attached to silicon tubing (Cole-Palmer, Montreal Canada), was suspended from the Faraday cage. A solution inlet line was introduced into the recording dish alongside with a suction line to remove excess fluid (See Figure 2.3). The syringes contained either external recording solution or external recording solution containing pharmacological agents.

Control experiments were carried out to ensure that the perfusion system was functional. During the positive control experiments perfusion with a high  $K^+$  (15 mM instead of 4 mM) recording solution over a patch-clamped neuron resulted in a depolarization of the membrane potential and change in action potential firing frequency. Furthermore, negative control experiments where perfusion of external recording solution alone was performed to ensure that the perfusion system did not induce changes in membrane potential during recording.

## **2.7 Statistical Analysis**

### **2.7.1 Determining responsiveness of neurons to applied 100 nM insulin**

Neurons were patch clamped in the current-clamp configuration and perfused with standard external recording solution. After a stable baseline was established, external recording solution containing 100 nM insulin was perfused on the cell for 3 minutes at 1 ml/min. To determine if a cell was responsive, the recording trace was

segmented into 100-second time blocks up to a maximum of 1000 seconds after insulin application. Mean membrane potential was determined for each of those blocks and compared to baseline membrane potential.

A cell was considered responsive to insulin if the mean membrane potential changed by 4 mV or more. The rationale for this value of 4 mV is as follows: during a 100 second time period whole-cell current clamp recording, the standard deviation of membrane potential of many SFO neurons is 2 mV or less. A shift of more than two times the standard deviation of membrane potential represents a conservative cut-off threshold to identify neurons that exhibit a change in membrane potential from those that do not. This technique has been used in many electrophysiology studies (Pulman *et al.*, 2006, Fry *et al.*, 2007, Alim *et al.*, 2010, Hoyda and Ferguson 2010).

Based on the criterion presented above, neurons were categorized according to response: a hyperpolarization of the membrane potential, a depolarization of the membrane potential or no response.

### **2.7.2 Determination of statistical tests used**

Initially, the distribution of the 27 neurons was tested using the Kolmogorov-Smirnov test. Using this analysis it was determined that the data was not normally distributed, and thus applying a Student's *t-test* to analyze the data would be incorrect. Therefore, multiple Mann-Whitney non-parametric pairwise comparison tests with a Bonferroni correction were used to compare the three categories. The Bonferroni correction was used to ensure that the probability of making a type one error was reduced. Therefore, only

data that is statistically significant using the Bonferroni correction is denoted with an asterisk (\*) throughout this thesis. Moreover, for control cells which were perfused with regular external recording solution, a Wilcoxon's signed-ranks test was used to determine if a statistically significant change occurred in the interspike interval before and after turning the perfusion system on and off. Chi-square analysis was conducted to determine if the proportion of neurons influenced by insulin was significantly different between control conditions and application of glibenclamide or Cs.<sup>+</sup> For example, of the 27 neurons, 9 hyperpolarized, 10 depolarized and 8 showed no change in membrane potential. 16 neurons were pre-treated with glibenclamide (see Results section 3.4 for more detail). Of the 16 neurons, zero cells hyperpolarized, 7 depolarized and 9 showed no change in membrane potential. A 3 x 2 table was made to compare the proportion of neurons that responded to insulin application (expected) to those which did not (observed) using 2 degrees of freedom. Lastly, a cluster analysis was performed to determine if variables such as capacitance, presence or absences of  $I_{sag}$  etc. correlated with the membrane response (hyperpolarization, depolarization or non responsive). A EM algorithm with a V-fold validation were used to ensure stringency when reporting the number of clusters.

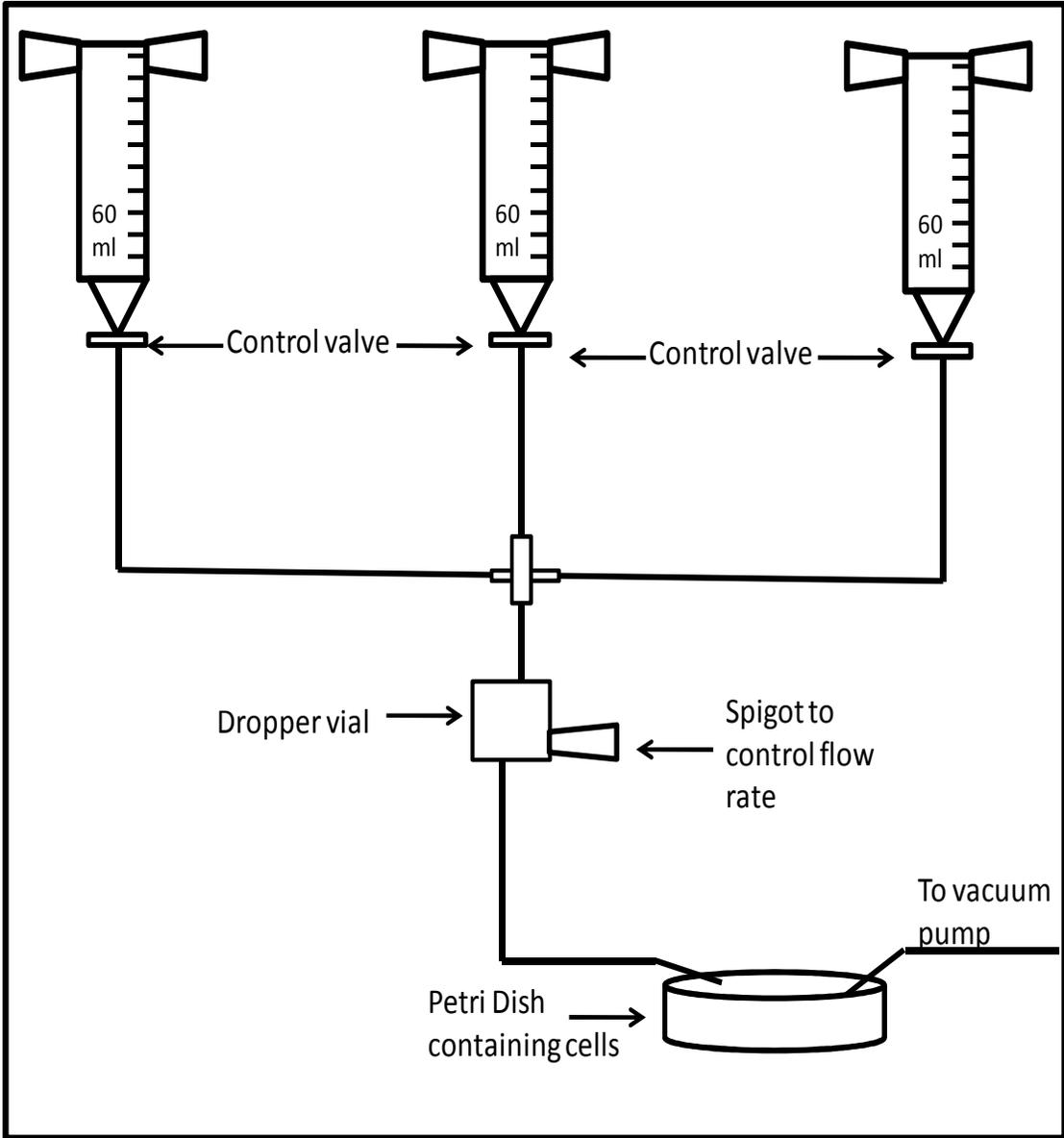
### **2.7.3 Software**

Electrophysiology data were acquired using Patchmaster version 2.53 (HEKA, Mahone Bay, Nova Scotia, Canada). Raw data files were imported into Spike 2 v5.0 (Cambridge Electronics Design, Cambridge, UK) software to measure membrane potential and action potential firing rates. Statistical comparisons were carried out using SPSS 17.1 (IBM,

Somers NY), Origin 8.1 (Microcal Software Northampton, MA) and Statistica 10 (Stat Soft. Inc, Tulsa OK).

Figure 2.3 The perfusion system.

A schematic diagram showing the 3 chamber perfusion system. Standard 60 mL syringes are attached to a dropper bottle which is then attached to a solution line made of silicon tubing. This line carries solution to the recording chamber. The flow rate can be adjusted by tightening or loosening of a spigot. On the other side of the recording dish is a solution outlet line which pulls solution out of the dish via a vacuum pump. Pharmacological agents are applied to cells by changing solution flow from one syringe to another via stopcocks.



## Chapter 3 Results

### 3.1 Validation of the perfusion system

The project aims to examine if electrical activity of SFO neurons maintained in culture is modulated by insulin. A series of control experiments were first carried out to confirm that the perfusion system was stable and that any changes in membrane potential and action potential firing frequency are the result of exogenously applied insulin. First, in order to determine that we could detect a change in membrane potential in current-clamped neurons, external recording solution with elevated  $K^+$  was perfused over a neuron after a baseline was established in control solution. In 6 out of 6 neurons, application of solution with elevated  $K^+$  resulted in a depolarization of the membrane potential. The mean depolarization was  $27.8 \pm 1.1$  mV, which is consistent with the shift in membrane potential predicted by the Nernst equation. It was also determined that there was a latency period of  $61.5 \pm 0.6$  seconds between switching on the flow of elevated  $K^+$  and when the membrane potential started to change. The latency represents the time taken for the elevated  $K^+$  external recording solution to flow through the perfusion system. A rapid washout of the elevated  $K^+$  solution followed, which occurred over a 300 second period (see Figure 3.1). All values reported are shown as mean  $\pm$  SEM.

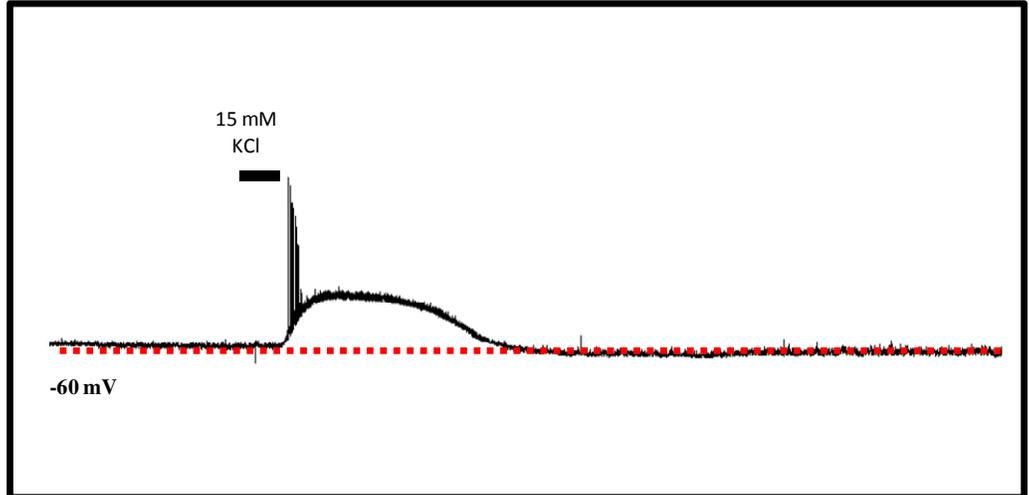
Figure 3.1 In a control experiment, perfusion of external recording solution with an elevated concentration of KCl (15 mM) causes a rapid depolarization of resting membrane potential in a patched SFO neuron.

A photo demonstrating a patch clamped SFO neuron (**A**). A recording obtained from a patched SFO neuron. After establishing a stable membrane potential in current-clamp external recording solution containing 15 mM KCl was perfused on the cell (thick black line). Analysis of the recording traces indicated a  $61.5 \pm 0.6$  second latency. Upon addition of the 15 mM KCl, the resting membrane potential of the cell depolarized, which is consistent with the Nernst equation (**B**). A current-clamp recording of membrane potential from an SFO neuron firing spontaneous action potentials. External recording solution was switched from one reservoir to another (thick black line). Upon addition of the external recording solution the SFO neuron shows no change in its baseline membrane potential or action potential firing frequency. The cell was held at -62 mV.

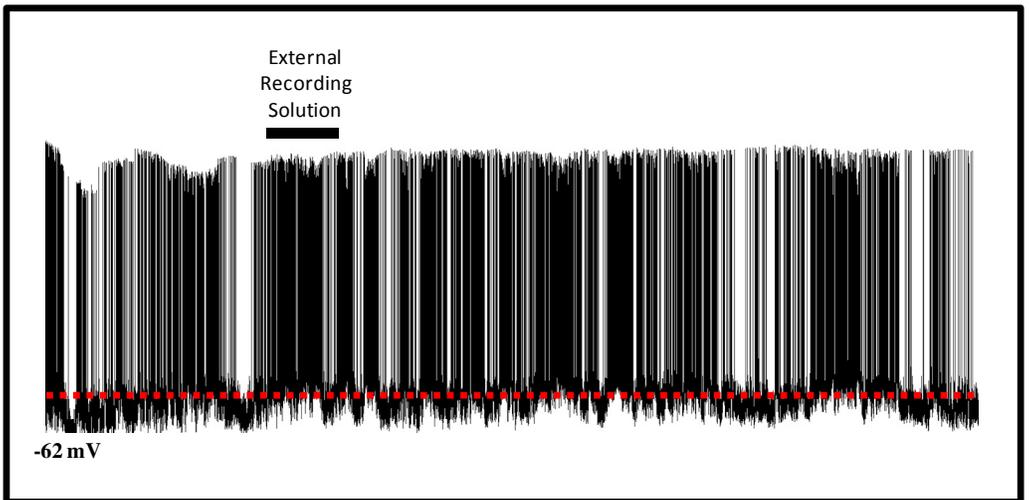
**A**



**B**



**C**



20 mV  
└─┬─┘  
100 ms

Next, to ensure that the act of switching solutions did not perturb the membrane potential of neurons, external recording solution (solution identical to the solution in the recording chamber) was perfused over 6 SFO neurons. The membrane potential and action potential firing frequency before and after perfusion were compared using the nonparametric Wilcoxon signed-rank test. It was observed that bath perfusion of external recording solution alone did not cause a significant change in resting membrane potential ( $n = 6$ ,  $1.1 \pm 0.5$  mV  $p = 0.63$ ) or action potential firing rates (assessed by comparing the interspike interval,  $n=6$ ,  $2.2 \pm 0.6$  seconds  $p = 0.76$ ; Figure 3.1 C).

### **3.2 Insulin influences the excitability of SFO neurons**

#### **3.2.1 Bath application of insulin affects the membrane potential of SFO neurons**

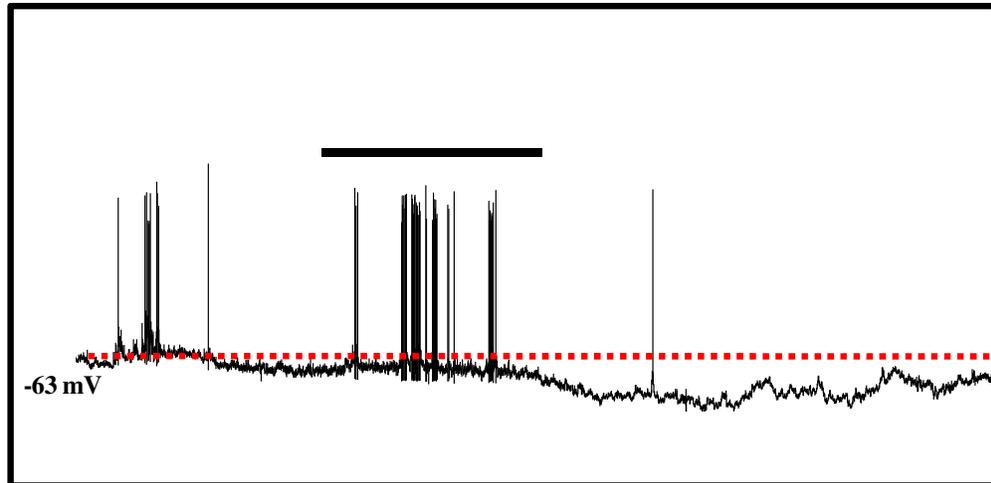
In order to examine the effect of insulin on the electrical properties of SFO neurons, whole-cell current-clamp recordings from SFO neurons maintained in culture for 1-4 days were obtained and changes in membrane potential and action potential firing rates were measured. SFO neurons maintained in culture were phase bright, 5-9  $\mu\text{m}$  in diameter, had a mean resting membrane potential of  $-49 \pm 0.6$  mV, and had a mean input resistance of  $2.6 \pm 4.1$  G $\Omega$  ( $n=27$ ), similar to previous reports (Anderson *et al.*, 2001; Washburn *et al.*, 2001; Pulman *et al.*, 2006). Forty-eight percent (13/27) of these neurons displayed spontaneous activity with a mean frequency of  $1.6 \pm 0.6$  Hz. (Figure 3.4 A). In 63% (17/27) of SFO neurons, an easily discernable  $I_A$  current was present. Whole-cell current clamp recordings of membrane potential were obtained from 27 dissociated SFO neurons. A minimum of at least 5 minutes stable membrane potential was obtained before bath application of 100 nM insulin for 3 minutes at a rate of 1 ml/min. Insulin acts

through a second messenger signal cascade (van Obberghen *et al.*, 2001) which can take seconds to minutes to act (Ozes *et al.*, 2001) and cause a change in the membrane potential of a cell. The membrane potential was monitored for a maximum of 1000 seconds after returning to external recording solution perfusion. Of the 27 SFO neurons tested, bath application of 100 nM insulin influenced the membrane potential and/or action potential firing frequency of 70% (19 out of 27 neurons). Three distinct response groups were observed after application of insulin. In 33% (9 of 27) of the neurons tested, a hyperpolarization of the membrane potential was seen ( $-8.7 \pm 1.7$  mV; Figure 3.4A). In 37% (10 out of 27) of the neurons, a depolarization of membrane potential was seen ( $10.4 \pm 2.2$  mV; Figure 3.4B), and 30% (8 out of 27) of neurons showed no change in the membrane potential after application of insulin ( $1.1 \pm 0.8$  mV; Figure 3.4C). The mean changes in membrane potential were significantly different across all three responses (hyperpolarization, depolarization and non-responders;  $p < 0.05$ , Mann-Whitney with Bonferroni correction). These findings are summarized in Figure 3.5 A.

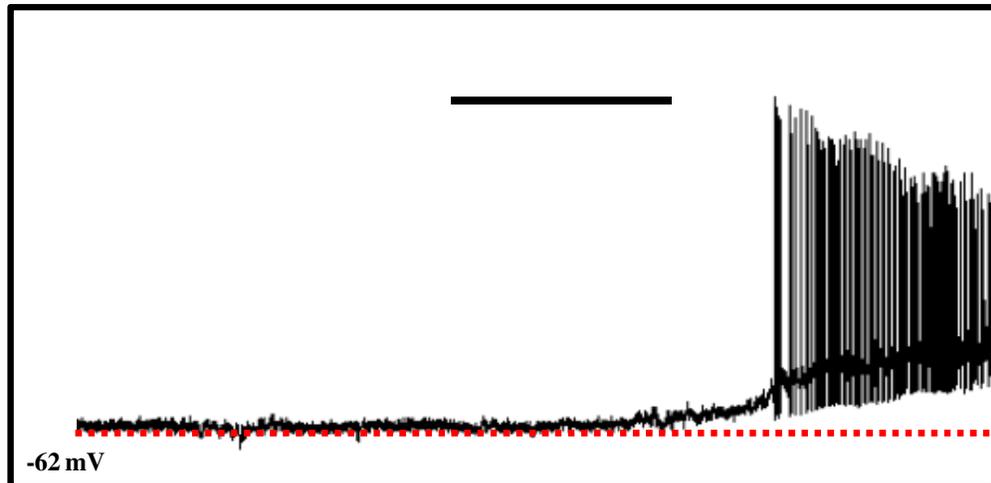
Figure 3.4 Insulin modulates the electrical activity of SFO neurons.

Representative current-clamp recordings demonstrating the responses observed in 3 separate dissociated SFO neurons after application of 100 nM insulin (black bar) demonstrating hyperpolarization (**A**), depolarization (**B**) or unaffected (**C**). Mean changes from membrane potential are indicated by the dashed line. A bar graph summarizing the effects of insulin on the membrane potential of 27 dissociated SFO neurons.

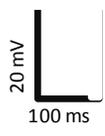
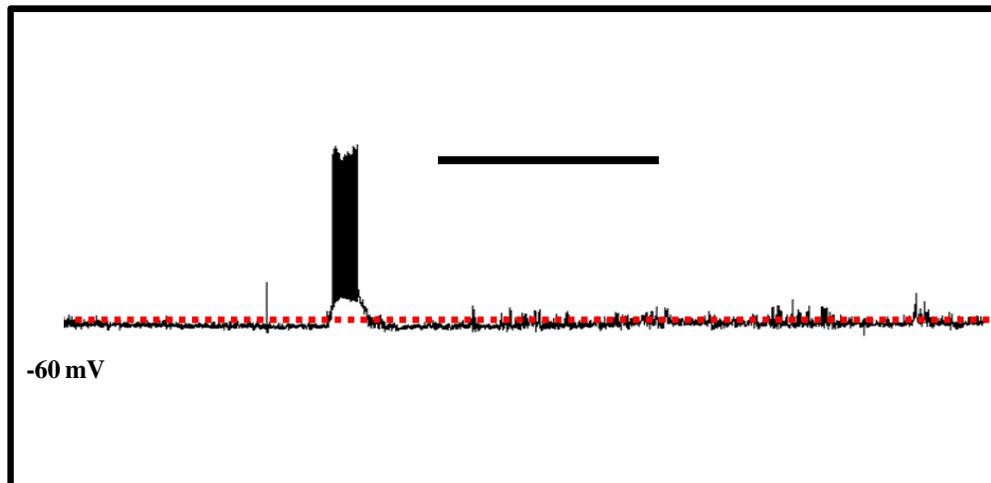
**A**



**B**



**C**

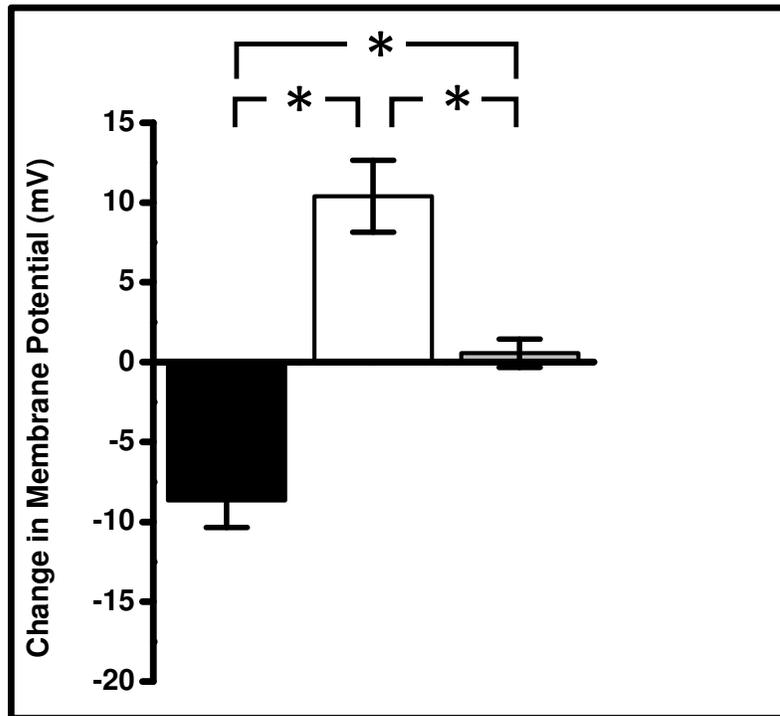
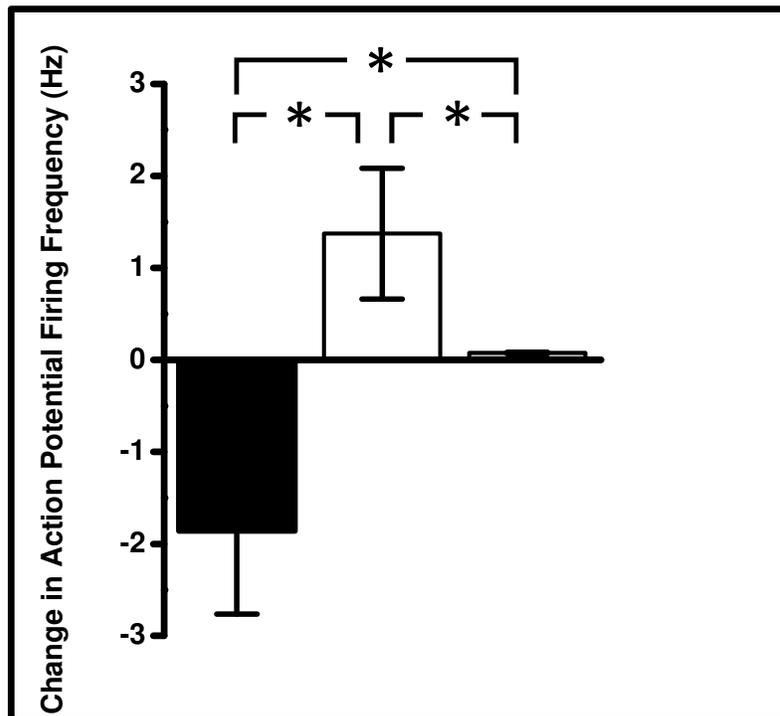


### **3.2.2 Bath application of 100 nM insulin influences the action potential firing frequency**

As indicated from the recording traces in Figure 3.4 A, B, C, bath application of 100 nM insulin also affected action potential firing frequency in some neurons. Insulin-induced hyperpolarizations were accompanied with a mean decrease in firing frequency of  $1.9 \pm 0.9$  action potentials/second, whereas, insulin-induced depolarization increased mean firing frequency by  $1.4 \pm 0.8$  action potentials/second. Neurons that did not exhibit a change in membrane potential did not exhibit a significant change in action potential firing frequency ( $0.05 \pm 0.01$  action potentials/second). The mean changes in action potential firing frequency were significantly different across all three response categories (hyperpolarization, depolarization and non-responders; Mann-Whitney with Bonferroni correction; \*  $p < 0.05$ , Figure 3.5 B).

Figure 3.5 Summary of the effects of insulin on membrane potential and action potential firing frequency.

A bar graph summarizing the effects of insulin on the membrane potential of 27 dissociated SFO neurons (**A**). Black bar denotes hyperpolarized (mean change  $-8.7 \pm 1.7$  mV); white bar denotes depolarized (mean change  $10.4 \pm 2.2$  mV); grey bar denotes insensitive (mean change  $1.1 \pm 0.8$  mV). A bar graph summarizing the effects of insulin on the action potential firing frequency of SFO neurons (**B**). Black bar denotes hyperpolarized neurons with a decrease in spike frequency (mean change  $1.9 \pm 0.9$  action potentials/ second); white bar denotes depolarized neurons with an increase in spike frequency (mean change  $1.4 \pm 0.8$  action potentials/ second); grey bars denotes insensitive neurons which did not change spike frequency (mean change  $0.05 \pm 0.07$  action potentials/second). The means changes in membrane potential and action potential firing frequency were significantly different between all groups using the Mann-Whitney non-parametric pairwise comparison test with a Bonferroni correction ( \*  $p < 0.05$ ).

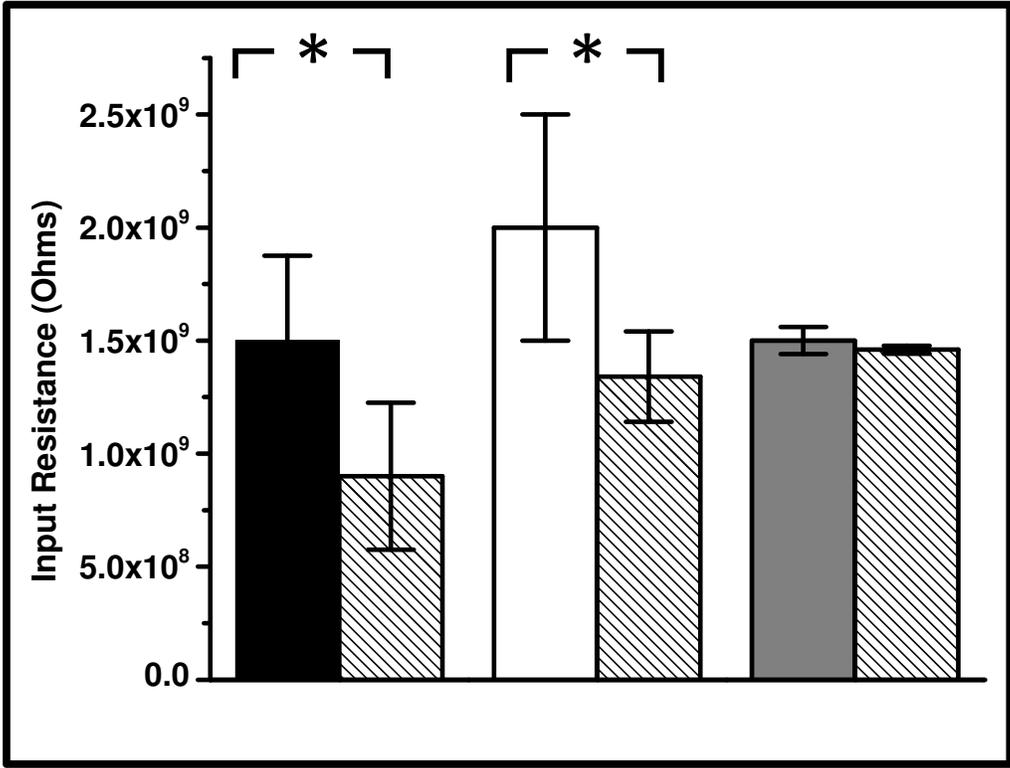
**A****B**

### 3.3 Insulin affects the input resistance of SFO neurons

In addition to measuring changes in membrane potential and action potential firing frequency, changes in the cells' input resistance were measured. To investigate the changes in input resistance (the difference in channels either opening or closing) after bath application of insulin, a series of hyperpolarizing current pulses were applied to the 27 neurons before and after insulin application (see Figure 2.1 in the Methods section for the experimental protocol). The voltage deflection (measured in mV) was plotted against the current injected (measured in pA) and fitted mathematically with a straight line to obtain the input resistance. Graphically, the construction of a voltage-current (V/I) curve represents the input resistance of a cell. Treatments that alter ionic conductance (and thus input resistance) will also alter the slope of the V/I curve. An increase in the slope of the V/I curve indicates increased input resistance (more channels closing comparatively), whereas a shallower slope indicates a decreased input resistance (more channels opening). A decrease in input resistance can reflect opening of channels that can cause depolarization or hyperpolarization of the membrane potential. Of the 27 neurons examined, 9 showed a hyperpolarization of membrane potential, and 10 showed a depolarization, whereas 8 neurons showed no change in membrane potential. The hyperpolarizing neurons exhibited a significant mean decrease in input resistance of 40.3%, whereas the depolarizing neurons exhibited a significant mean decrease in input resistance of 32.6%, and the non-responsive neurons exhibited a non-significant change of 2.5% ( $n=27$ , \*  $p < 0.05$ , Mann-Whitney with Bonferroni correction; Figure 3.6).

Figure 3.6 Insulin affects the input resistance of SFO neurons.

A bar graph depicting changes in input resistance following insulin treatment. The solid bars indicate the input resistance before insulin treatment and the adjacent shaded bars indicate the change in input resistance following insulin treatment. The solid black bar denotes neurons whose membrane potential hyperpolarizes. The solid white bar denotes depolarized neurons and the solid grey bar denotes neurons which did not respond to insulin application. The means changes in membrane potential were significantly different between all groups using the Mann-Whitney non parametric pairwise comparison test. \*  $p < 0.05$ .



The observed changes in input resistance suggested that ion channels were being modulated. The data obtained from the voltage/current (V/I) plots to determine input resistance can also help to determine the identity of the ion channel(s) being modulated. On the V/I plot, the intersection of the two lines of best fit (before and after application of insulin) indicates the equilibrium potential of ionic flow through the channel being modulated –the so-called reversal potential of the effect.

In 9 neurons which hyperpolarized, the mean reversal potential of the effect was  $-100.0 \pm 5.8$  mV, a value very close to the calculated equilibrium potential for  $K^+$  (-100 mV) (see Figure 3.7A). Together with the observed decrease in input resistance, these data suggest that the hyperpolarization of the membrane potential was due to activation of  $K^+$  channels. This possibility is addressed in section 3.4.

Conversely, V/I plots constructed for neurons which depolarized after application of insulin indicated a convergence point  $-55.3 \pm 1.7$  mV ( $n = 10$ ). Together with the decrease in input resistance, these data suggest activation of a depolarizing channel, such as a non-specific cation channel (NSCC); (Figure 3.7B). These results are summarized in a bar graph which can be seen in Figure 3.8.

Figure 3.7 Insulin induces a change in the input resistance suggest modulation of  $K^+$  channels and nonspecific cation channels.

(V/I) plot showing the change in input resistance after a 100 nM insulin application. The intersection point indicates the reversal potential of the effect at (-100 mV) which is close to the reversal potential for potassium channels. Inset: current-clamp recording shows an insulin-induced hyperpolarization response in an SFO neuron. The closed circles represent input resistance before insulin application and open squares represent input resistance after insulin application. **(A)**. (V/I) plot showing the change in input resistance in neurons which depolarized after a 100 nM insulin application. The intersection point indicates the reversal potential of the effect near -50 mV which is close to the reversal potential for non-selective cation channels (NSCC). Inset current-clamp recording shows an insulin-induced depolarization response in an SFO neuron. The closed circles represent input resistance before insulin application and hollow squares represent input resistance after insulin application **(B)**.

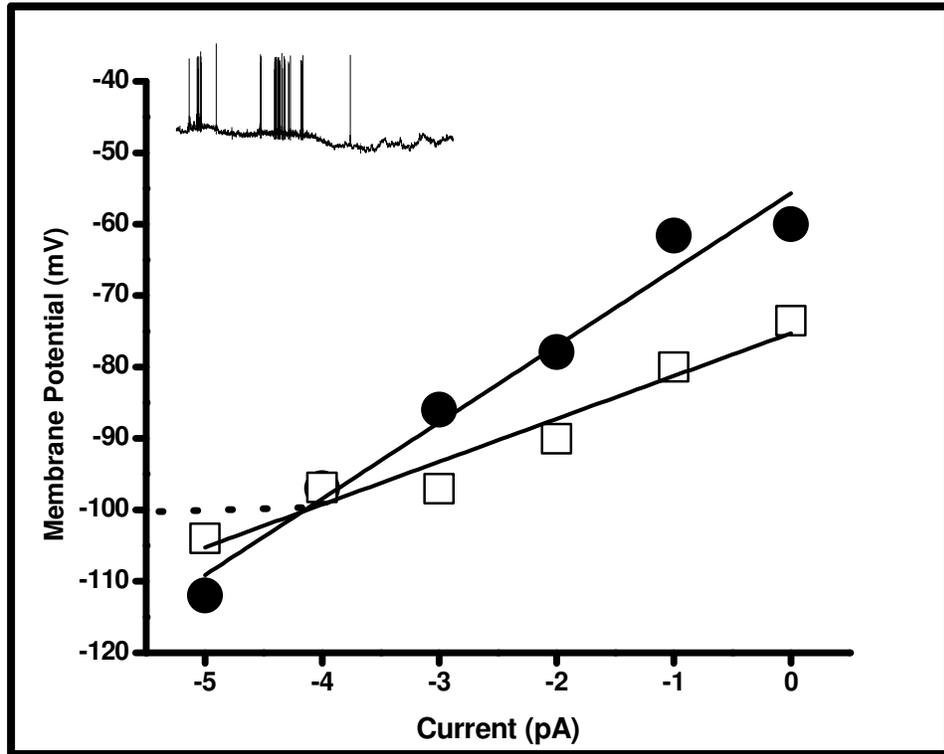
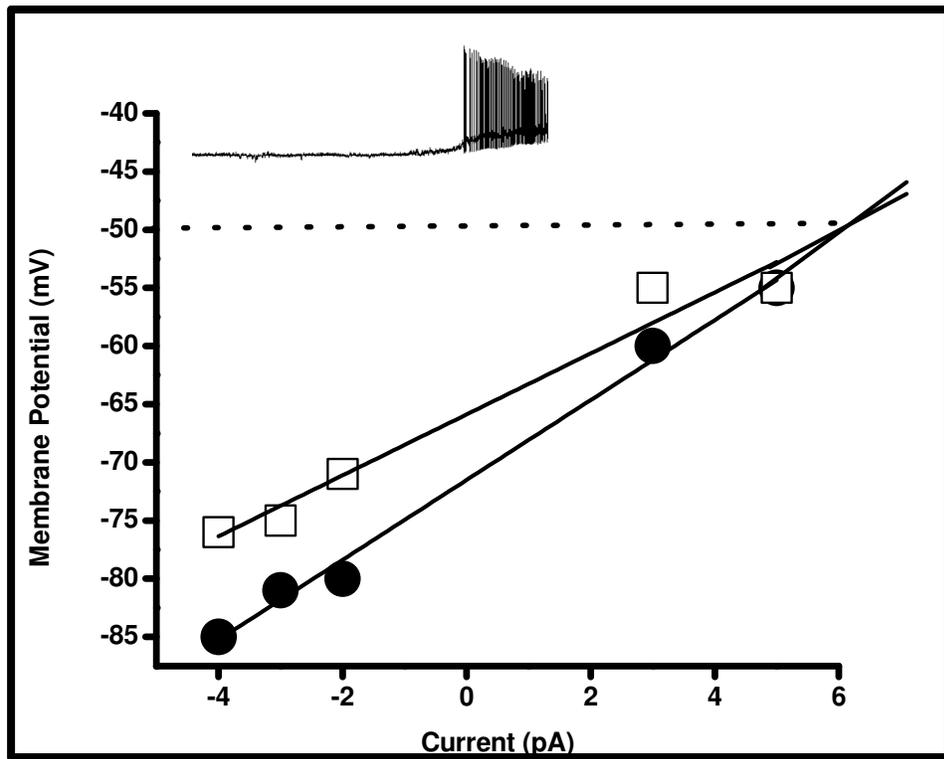
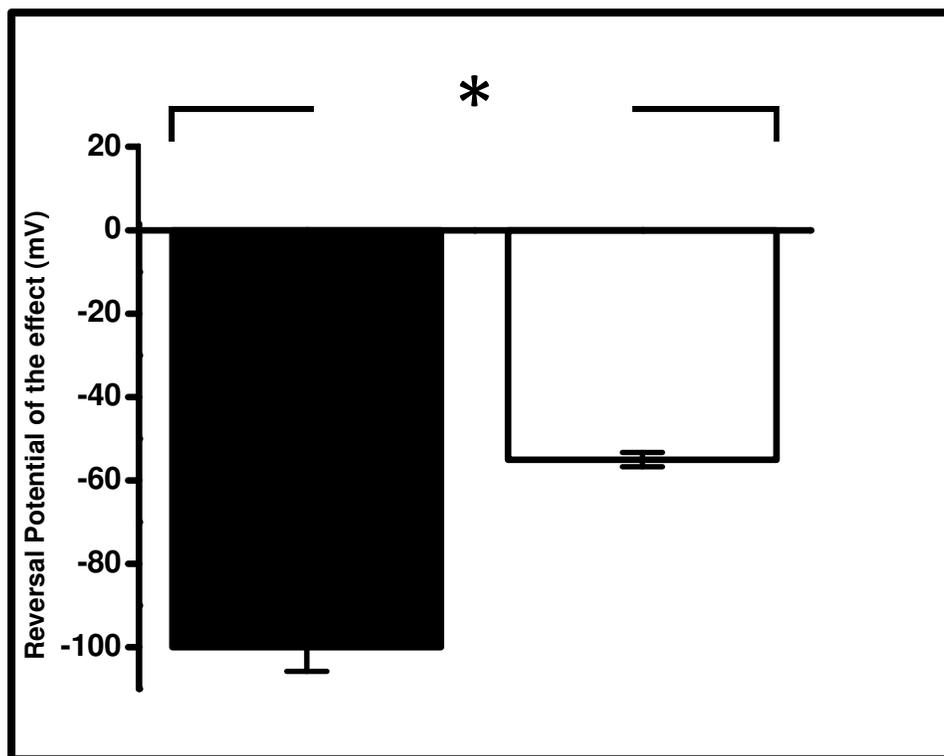
**A****B**

Figure 3.8 Insulin induces a change in the input resistance suggest modulation of K<sup>+</sup> channels and nonspecific cation channels.

A bar graph summarizing the response of the membrane potential and reversal potential of the effect after insulin application. The black bar denotes cells which hyperpolarized and have a reversal potential of the effect near -100 mV. The white bar denotes cells which depolarized and have a reversal potential near -55 mV. The mean changes in membrane potential were significantly different between the two groups using the Mann-Whitney non-parametric pairwise comparison test. \*  $p < 0.05$ .

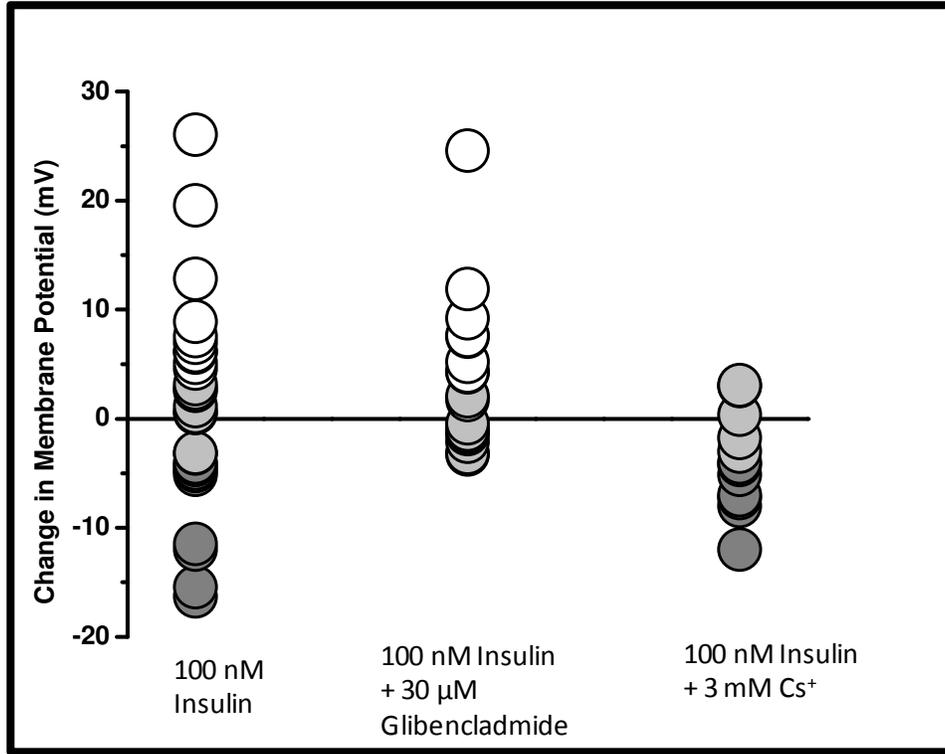


### 3.4 Insulin-induced hyperpolarization of membrane potential abolished in the presence of glibenclamide

Insulin-induced hyperpolarization of neurons within the ARC and the PVN has been extensively investigated (Spanswick *et al.*, 1997; Spanswick *et al.*, 2000; van den Top *et al.*, 2007). Insulin-induced hyperpolarization of membrane potential of these neurons has been suggested to be mediated by the opening of the  $K_{ATP}$  channel at the cellular level through activation of phosphatidylinositol 3-kinase (PI3K) (van den Top *et al.*, 2007; for review see Plum *et al.*, 2005). To determine if insulin-induced hyperpolarization of SFO neurons observed in the present study was also due to the activation of the  $K_{ATP}$  channel, an additional set of experiments was carried out. Whole-cell current clamp recordings were obtained from 16 SFO neurons pretreated with 30  $\mu$ M glibenclamide, a  $K_{ATP}$  channel antagonist. In these experiments, pretreatment with glibenclamide abolished the hyperpolarizing effect of insulin, however the depolarizing effect remained intact. Of the 16 neurons tested in the presence of glibenclamide, 7 exhibited a depolarization of membrane potential (mean change of  $10.0 \pm 2.6$  mV), whereas 9 showed no change in membrane potential (mean change of  $0.7 \pm 0.7$  mV; Figure 3.9). Specifically, the proportion of neurons influenced by insulin in the presence of glibenclamide was significantly different from that under control conditions, as determined by chi-square analysis ( $\chi^2 = 9.2$ , DF=2,  $P = 0.01$ ). These data indicate that the hyperpolarization of the membrane potential and overall decrease in action potential firing frequency of some neurons was mediated via activation of the  $K_{ATP}$  channel.

Figure 3.9 Pharmacological blockers suggest the identity of ion channels responsible for the modulation of the electrical properties of SFO neurons following insulin application.

A scatter plot depicting the mean change in membrane potential of each SFO neuron tested. Application of insulin to SFO neurons causes hyperpolarization, depolarization or no effect on the membrane potential (grey, white, light grey). Hyperpolarization of the membrane potential was abolished in cells pretreated with 30  $\mu$ M glibenclamide, a  $K_{ATP}$  channel blocker. Pretreatment with 3 mM  $Cs^+$  an HCN channel blocker, abolished the depolarization of the membrane potential.



### **3.5 Insulin-induced depolarization of the membrane potential abolished in the presence of cesium**

Whereas 30  $\mu\text{M}$  glibenclamide abolished the insulin-induced hyperpolarization in 16 SFO neurons tested, 7 neurons continued to exhibit a depolarization of the membrane potential suggesting that modulation of  $\text{K}_{\text{ATP}}$  channel did not play a role in the depolarization of the membrane potential. Furthermore, it was also noticed that while subjecting neurons to a series of hyperpolarizing current injections some neurons (7 of 27 neurons) developed a characteristic  $I_{\text{sag}}$  current, which is produced by activation of hyperpolarization activated cation channels (HCN). HCN channels are selective for either  $\text{Na}^+$  and/or  $\text{K}^+$  (Biel *et al.*, 1999).  $I_{\text{sag}}$  currents have previously been reported in the pacemaker neurons of the heart as well as in the brain (for review see Gauss and Seifert 2000). Moreover, the presence of a cesium -sensitive  $I_{\text{sag}}$  has been reported within SFO neurons, which is suggested to contribute to the membrane depolarization and action potential firing pattern observed within SFO neurons (Washburn *et al.*, 2000).

In order to specifically test the hypothesis that activation of  $I_{\text{sag}}$  was responsible for the observed insulin-induced depolarization of SFO neurons, current-clamped neurons were subjected to pretreatment with 3 mM cesium to block the HCN before application of insulin. In these experiments, pretreatment with  $\text{Cs}^+$  abolished the depolarizing effect of insulin; however the hyperpolarizing effect remained intact. Specifically, the proportion of neurons influenced by insulin was significantly different from that under control conditions: of the 13 neurons tested in the presence of  $\text{Cs}^+$ , 8 neurons exhibited a hyperpolarization of membrane potential (mean change of  $-5.7 \pm 1.4$  mV), whereas 5 neurons showed no change in membrane potential (mean change of  $0.4 \pm 1.2$  mV), and

no neurons exhibited a depolarization of the membrane potential ( $\chi^2 = 8.3$ ,  $DF = 2$   $P = 0.01$ ; Figure 3.9). These data indicate that the depolarization of the membrane potential and increase in action potential firing frequency is mediated via activation of the HCN channel which produces an  $I_{\text{sag}}$  current.

### **3.6 Insulin activates an $I_{\text{sag}}$ current in both depolarizing and hyperpolarizing SFO neurons**

Closer examination of the current-clamp experiments described in section 3.2 in which neurons were subjected to a series of hyperpolarizing current injections revealed that 44% of neurons ( $n=12/27$ ) exhibited  $I_{\text{sag}}$ . In all of the 12 neurons (3 neurons which hyperpolarized, 6 neurons depolarized, and 3 neurons had no change in membrane potential), application of insulin caused a qualitative increase in  $I_{\text{sag}}$ . Furthermore, 7 of the remaining 15 neurons developed an  $I_{\text{sag}}$  current following insulin application. Of these 7 neurons, 2 neurons hyperpolarized, 4 neurons depolarized and 1 neuron had no change in membrane potential. Of the 8 neurons that showed no activation of  $I_{\text{sag}}$  it was easily discernable (See Figure 3.10). These data do not suggest that only  $K_{\text{ATP}}$  channels are activated only in the population of neurons that exhibit hyperpolarization whereas HCN is activated only in the population of neurons that depolarize. Instead, these data suggest that following application of insulin, there is a simultaneous activation of the  $K_{\text{ATP}}$  channels and the HCN channel and that the net effect of change on membrane potential may be due to the relative difference in absolute magnitude of the two currents possessed by a single neuron. Figure 3.11 depicts two different neurons, a hyperpolarizer and a depolarizer respectively. Both neurons developed  $I_{\text{sag}}$  after application of insulin and decreased their input resistance.

Figure 3.10 A flowchart demonstrating the effects of insulin on  $I_{sag}$  current present from the 27 recorded SFO neurons.

A flow chart to demonstrate that of the 27 cells observed, 12 cells previously demonstrated the presence of  $I_{sag}$ . Furthermore seven additional cells developed  $I_{sag}$  following insulin application. However, there was no consistency between the development of  $I_{sag}$ , response of membrane potential, and input resistance.

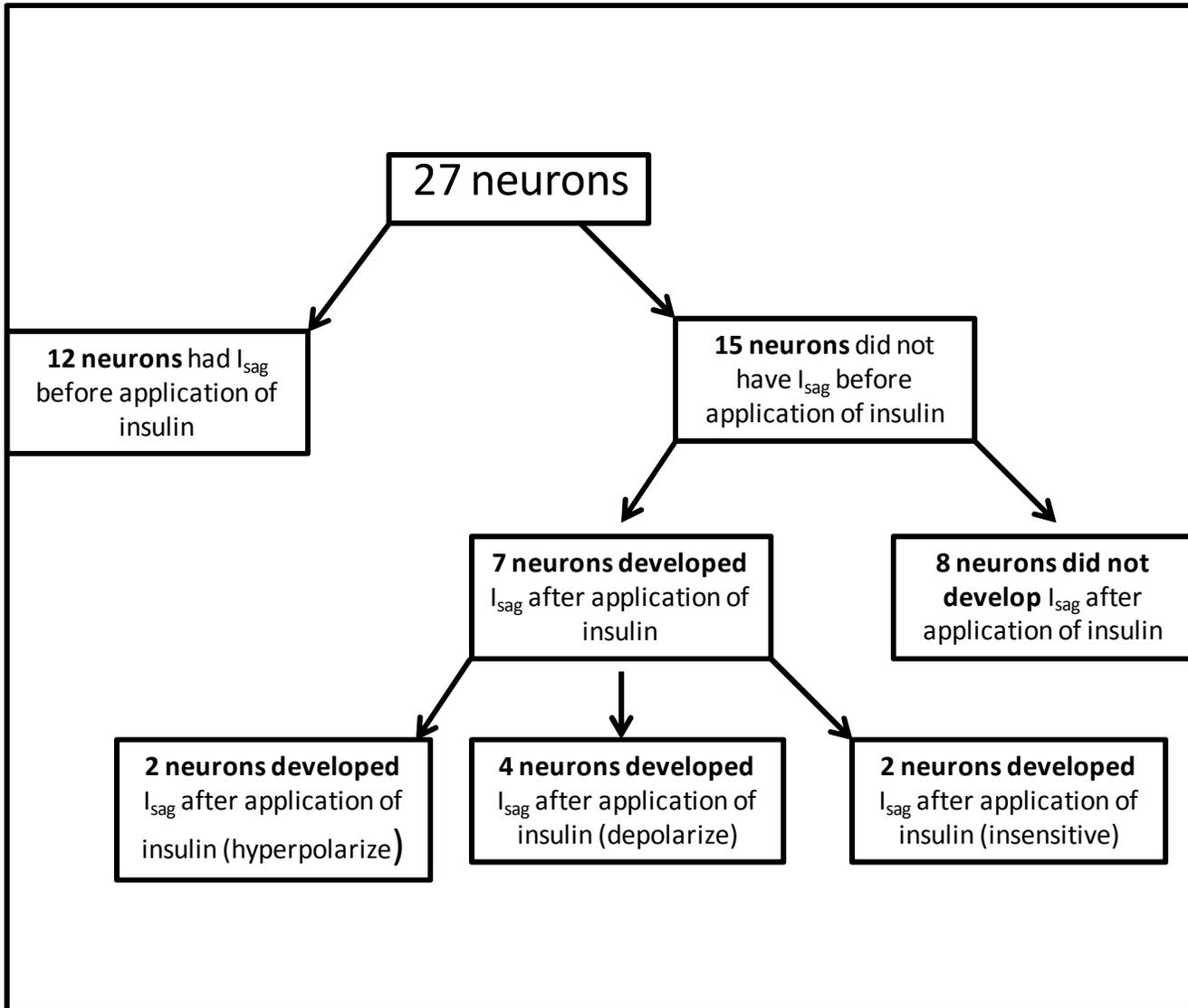
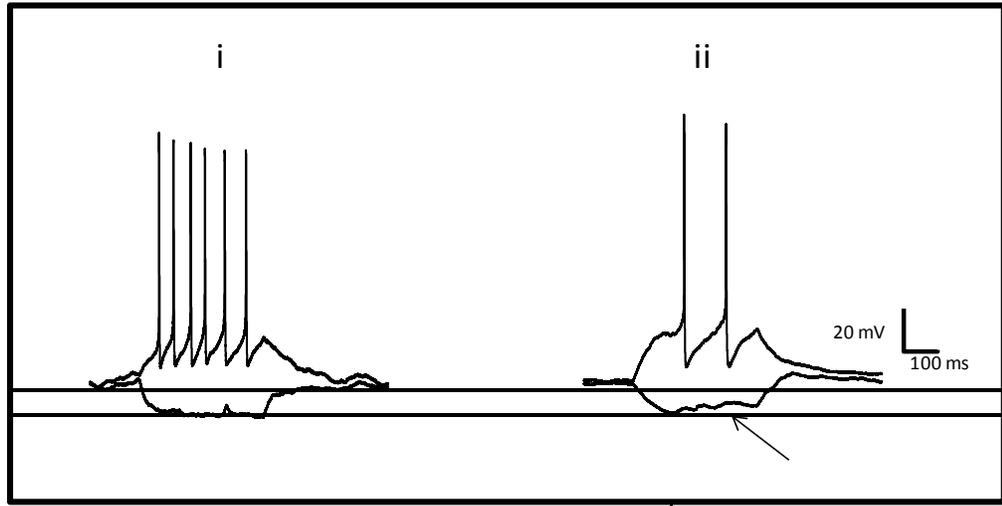


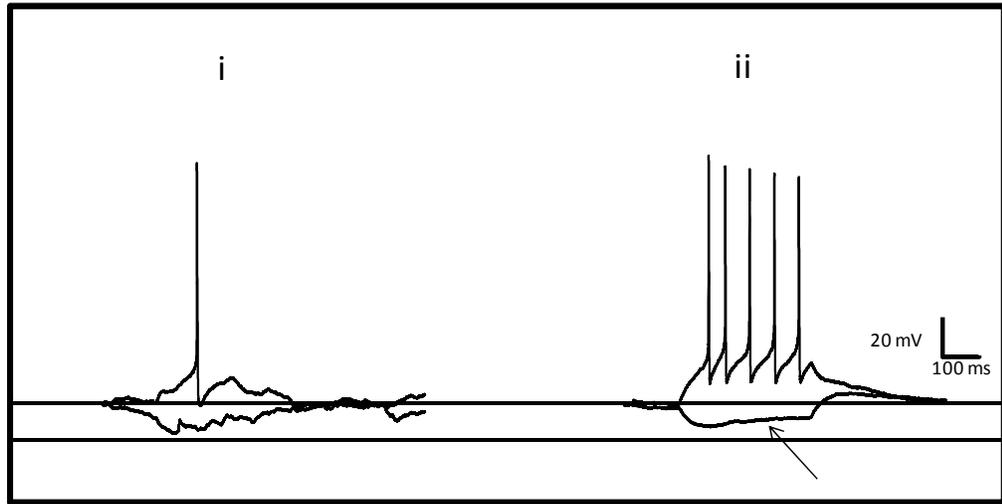
Figure 3.11 Insulin activates an  $I_{\text{sag}}$  current in some SFO neurons

Current-clamp recording traces of membrane potential from SFO neuron subjected to a series of current injections. An example of a neuron whose membrane potential hyperpolarized in response to insulin (i = before insulin) and developed an  $I_{\text{sag}}$  current (ii = after insulin). (**A**). A neuron which depolarized in response to insulin (i = before insulin) also develops an  $I_{\text{sag}}$  current (ii = after insulin) (**B**). The grey arrows indicate the development of  $I_{\text{sag}}$ . These observations suggest that insulin activates other ion channels in addition to HCN channels responsible for  $I_{\text{sag}}$ .

**A**



**B**



### **3.7 Cluster analysis suggests neurons which depolarize after insulin application are likely to have higher capacitance, possess $I_{\text{sag}}$ and $I_{\text{A}}$**

A single SFO cell does not belong to a homogeneously distributed population of neurons. In fact, subpopulations of SFO neurons exist which can express different receptors and have varied electrophysiological responses to stimuli (Washburn *et al.*, 2001; Gutman *et al.*, 1988.). Thus, observed properties of SFO neurons in the current study, such as membrane response to insulin, capacitance (a product of size), presence of  $I_{\text{K}}$  and/or  $I_{\text{A}}$ , versus  $I_{\text{K}}$  alone, the ratio of  $I_{\text{A}}$  to  $I_{\text{K}}$ , the presence of  $I_{\text{sag}}$  and/or the development of  $I_{\text{sag}}$  following insulin treatment were compared to each other using cluster analysis to determine if neurons clustered together based on similar properties and responses. For the 27 neurons tested, categorical variables such as the presence or absence of  $I_{\text{sag}}$  before and after application of insulin were clustered with continuous variables such as the ratio of early  $\text{K}^+$  current to late potassium current, change in membrane potential (mV), and cell capacitance. Using an EM algorithm with a V-fold validation, two cluster groups were observed. One cluster predominately grouped neurons which were larger in size (capacitance), depolarized after insulin, developed  $I_{\text{sag}}$  after application of insulin and possessed an  $I_{\text{A}}$  current. Therefore, it is plausible to predict that neurons which are larger may develop  $I_{\text{sag}}$  after application of insulin and may possess  $I_{\text{sag}}$ . Capacitance specifically reflects the surface area of a cell membrane and can be a way to identify subpopulations of neurons. Dorsal root ganglion neurons and motor neurons are well known examples of neurons that can be sorted this way. Moreover, there is a statistically significant relationship between cell capacitance (cell size) and the membrane response after insulin application between cells which hyperpolarized and

depolarized using a Mann-Whitney comparison test ( $p < 0.05$ ) between cells that hyperpolarized and depolarized. Figure 3.12 demonstrates the relationship between cell capacitance (size) and response. This result suggests an association between capacitance and membrane response after insulin but is not a conclusive test.

## **Chapter 4 Discussion**

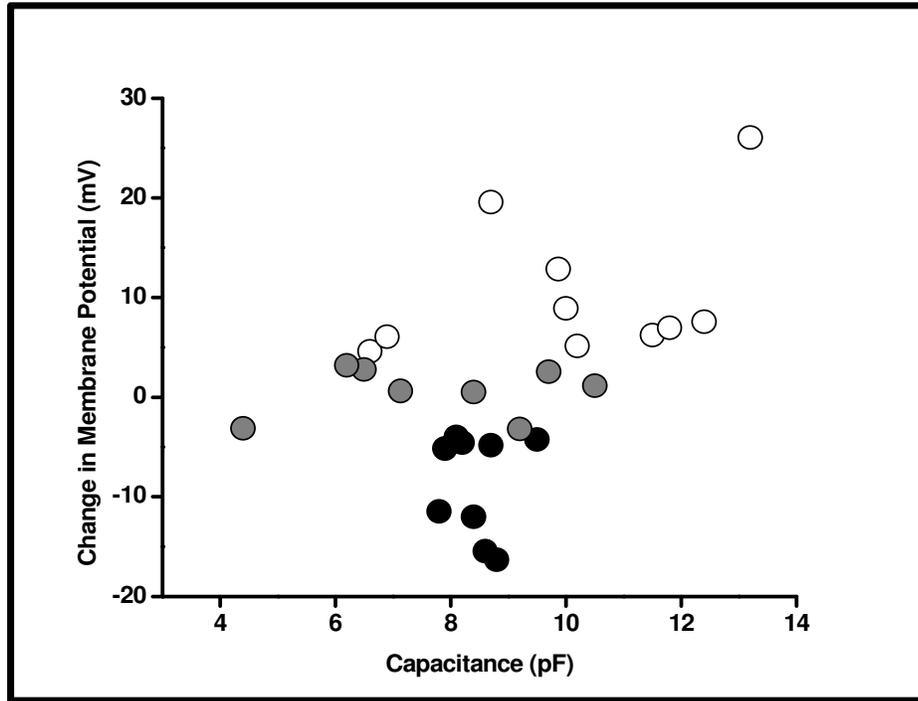
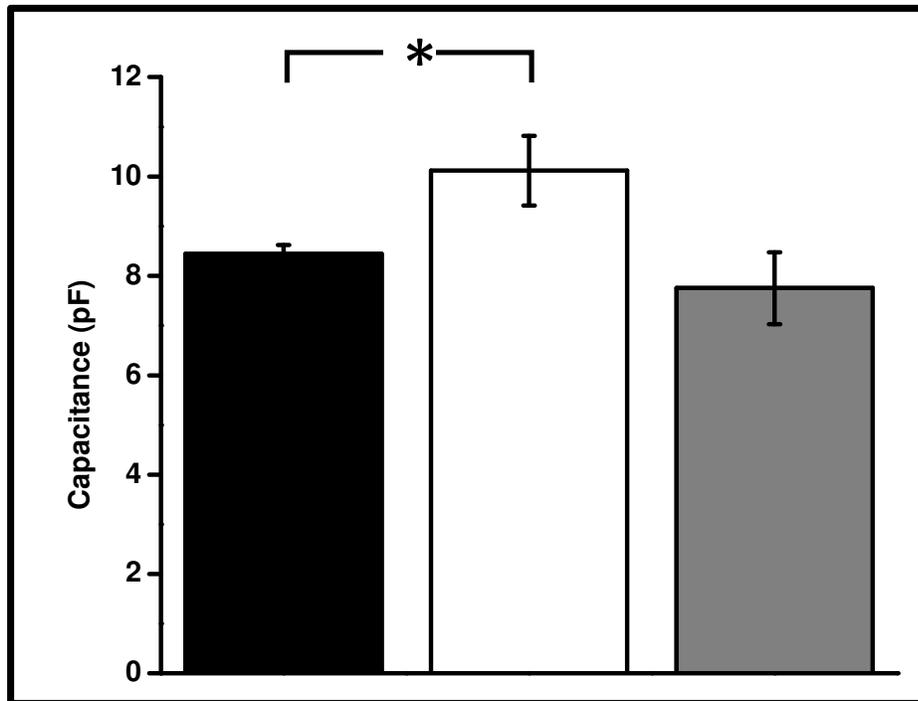
This is the first study to demonstrate that SFO neurons are sensitive to insulin and can respond with changes in electrical signal properties after exposure to insulin. Whole-cell current clamp experiments measuring changes in membrane potential after application of insulin revealed 70% of the tested neurons responded to insulin with a change in membrane potential of action potential firing frequency, whereas 30% of the neurons tested were insensitive to the hormone. Approximately one-third of neurons tested exhibited a hyperpolarization of membrane potential, one-third depolarized, and the remaining one-third did not show a change in membrane potential. These findings further support the notion that the SFO is a key site within the CNS that acts as a sensor for detecting peripheral satiety energy signals and may play a key role in regulating energy balance.

### **4.1 The experimental procedure and insulin concentration used in the present study**

Primary cultures of SFO neurons were prepared and maintained in culture for up to 4 days. B27 is a key supplement that is added to the cell culture media (NB) to promote growth and maintenance of neural neurons (Brewer *et al.*, 1993). The composition of this supplement is defined and a better alternative to previously used

Figure 3.12 Cluster analysis reveals a possible relationship between neuron capacitance, and response.

Using cluster analysis, grouping of variables such as a cell capacitance, and response were seen. A scatter plot demonstrating the relationship between cell capacitance and response. Black circles indicate hyperpolarizers, white circles are those which depolarized, grey circles are non-responders (**A**). A bar graph demonstrating a non significant difference between cell capacitance and response of neurons. The black bar indicates hyperpolarizers, white bar indicates depolarizers and grey bar indicates non responders (**B**). The means changes in membrane potential were significantly different between hyperpolarizers and depolarizers only using the Mann-Whitney non parametric pairwise comparison test. \*  $p < 0.05$ .

**A****B**

fetal bovine serum (Brewer 1995), however, the supplement also contains insulin at a concentration of 70 nM (Paek *et al.*, 2004). Therefore, in order to ensure that the electrical activity of SFO neurons was not affected by this concentration of insulin prior to recording, SFO neurons were washed with insulin-free cell culture media and allowed to recover for 30 minutes before recording. The external recording solution was also insulin-free, therefore the changes in the electrical properties of SFO neurons were due to the acute application of the exogenously applied insulin at a concentration of 100 nM.

Cotero and Routh (2009) have indicated that the EC<sub>50</sub> value of insulin in the rat brain is 3.3 nM, with 100 nM being the lowest concentration that gets the maximal effect. Other electrophysiology experiments have used concentration in the range of 300 nM - 500 nM (Williams *et al.*, 2010; and Spanswick 2000). We chose to use 100 nM insulin to ensure that we achieved maximal activation of insulin receptor while remaining as close to physiological levels as possible. While plasma concentration of circulating insulin is approximately 7 nM and varies from 1-10 nM (Suzuki and Kono 1980), insulin must be transported across the BBB via a saturable transporter (Banks 2004). Thus, SFO neurons, which are not guarded by the BBB, are proposed to see a higher concentration of circulating insulin (Fry *et al.*, 2007). While the concentration of insulin used in this study is not physiological, it is used to demonstrate that SFO neurons are capable of detecting insulin and responding by modulating ion channels.

#### **4.2 The dissociated cell model for electrophysiological studies**

The dissociated cell preparation technique allows for accurate observation of the effects of exogenously applied hormones and peptides on individual neurons. In the

present study, this technique allowed for the direct observation of the effects of insulin on the intrinsic electrical properties of SFO neurons without being concerned with additional factors, such as cell-to-cell communication, or recording artefacts, including space clamp errors (both which are concerns present when utilizing neurons in brain slices). Moreover, in culture, dissociated SFO neurons maintain their electrophysiological properties such as resting membrane potential, oscillation of membrane potential (Fry and Ferguson 2007) and a bursting action potential discharge (Wasburn *et al.*, 1999), for up to 5 days (Ferguson and Bains 1996). The properties of SFO neurons recorded in this study are in accordance with those reported in previous studies using dissociated SFO neurons (Ferguson and Bains, 1997; Pulman *et al.*, 2008; and Smith *et al.*, 2009). Therefore, the electrical effects of insulin on SFO neurons are direct.

#### **4.3 Statistical stringency may limit the ability to detect small changes in membrane potential in some SFO neurons**

Changes in membrane potential were observed after application of insulin and deemed a response if the mean change in membrane potential was two times the standard deviation of the baseline obtained from the cell prior to application of insulin. While this method permits a quantitative categorization of insulin-induced responses from intrinsic membrane potential fluctuations, this criterion ignores neurons which had a smaller change in membrane potential after application to insulin, possibly resulting in under representation of the number of SFO neurons responsive to insulin.

#### **4.4 Insulin-induces responses in SFO neurons**

Insulin-induced hyperpolarization of membrane potential:

Insulin induced a hyperpolarization of the membrane potential in 33% of SFO neurons in the present study. This change in membrane potential was accompanied by a concomitant decrease in the action potential firing frequency. Thus, to ensure that the insulin-induced hyperpolarization of the membrane potential was consistent with previous reports, changes in input resistance were measured to determine which ion channel was responsible for the observed hyperpolarization. Not only was there a decrease in input resistance, suggesting an opening of ion channels, the reversal potential of the effect was determined to be about -100 mV, which is similar to the reversal potential of  $K^+$  (calculated to be -91mV). The discrepancy between the value of the reversal potential of the effect documented in this experiment and the  $K^+$  reversal potential is due to the voltage offset, which is approximately -12-15 mV in this experiment). This finding suggests that the observed insulin-induced hyperpolarization is due to the activation of a  $K^+$  channel. To determine if the  $K^+$  channel was the  $K_{ATP}$  implicated in several studies, SFO neurons were pre-treated with glibenclamide, which binds to the  $K_{ATP}$  channel and prevents the channels from opening. In the present study the hyperpolarization of the membrane potential of SFO neurons was abolished following application of glibenclamide, thus confirming the role of the  $K_{ATP}$  in insulin-induced hyperpolarization of the membrane potential. This data is consistent with previous electrophysiological studies which have demonstrated responsiveness of hypothalamic neurons to insulin (van Topp 2007, Cotero and Routh 2008; Williams *et al.*, 2010 for review see for review see Plum *et al.*, 2005 and Jobst *et al.*, 2004). Previous reports attribute insulin-induced hyperpolarization to the activation of phosphatidylinositol 3-kinase (PI3 kinase), which in turn activates a downstream signaling molecule phosphatidylinositol 3,4,5-trisphosphate

(PIP<sub>3</sub>) (Niswender *et al.*, 2003; Mirshamsi *et al.*, 2004; for review see Shepherd *et al.*, 1998). PIP<sub>3</sub> modulates the activity of K<sub>ATP</sub> channels by allowing K<sup>+</sup> efflux, resulting in a loss of positive charge and hyperpolarization of the membrane potential, whereas application of wortmannin, a PIP<sub>3</sub> inhibitor, results in an abolishment of insulin-induced hyperpolarization (Shepherd *et al.*, 1998; Spanswick *et al.*, 2000; Cotero and Routh 2009).

#### Insulin-induced depolarization of membrane potential:

While the insulin-induced hyperpolarization of the membrane potential of hypothalamic neurons is consistent with responses previously reported in the literature, a depolarization of the neuronal membrane potential has only been reported when the concentration of glucose is 0.1 mM (Wang *et al.*, 2004). This study was the first to demonstrate that application of insulin on neurons resulted in a depolarization of the membrane potential, at a glucose concentration of 10 mM, which was seen in 37% of the SFO neurons tested. Although the concentration of glucose in the external recording solution is higher than reported in Wang *et al.*, (2004) the change in glucose concentration during the washing step when cell culture media is exchanged with external recording solution (a change in glucose concentration from 25 mM to 10 mM) may add to the non-physiological nature of the experiment. A sudden decrease in glucose concentration has been suggested to drive the K<sub>ATP</sub> channel towards an open state and thus the channel has less of an effect on the overall neuronal activity (Wang *et al.*, 2004). The configuration of the K<sub>ATP</sub> channels is also determined by the relative ratio of ATP/ADP and a decrease in glucose concentration may signal a decrease in ATP production within a neuron and thus indicate higher levels of glucose transport across a

neuron. Kang *et al.*, (2004) have suggested that the excitatory effects of insulin may be due to the increased glucose transport in glucose-excited cells. SFO neurons have also been demonstrated to be sensitive to changes in glucose concentrations (Medeiros *et al.*, 2011) and although changing the glucose concentration of the external recording solution was not a part of the present experimental design, it may be advantageous to conduct another set of experiments in which glucose concentrations are varied in the presence of insulin because the interaction between glucose and insulin within the brain may affect the electrical activity of SFO neurons. However, a change in membrane potential following insulin application was further accompanied by a decrease in input resistance, suggesting the opening of ion channel(s). Using the V/I plots the identity of the ion channel indicates a non-selective cation channel (NSCC) with a reversal potential around approximately -55 mV. Therefore the depolarization of the membrane potential in patched SFO neurons is suggested to arise from the modulation of a NSCC channels and not from lack of  $K_{ATP}$  channel modulation. Lastly, it was observed that in neurons that possessed an  $I_{sag}$  current, the size of  $I_{sag}$  current increased after insulin application. Activation of the HCN channels produce the observed  $I_{sag}$  which is a phenomenon seen in a portion of SFO neurons (Washburn *et al.*, 2000). HCN channels can be activated by hyperpolarization of the membrane potential and have a conductance for both  $Na^+$  and  $K^+$  (Santoro and Tibbs 1999), and activation of the  $I_{sag}$  current is slow and inward (Figure 3.11). Shifting the activation kinetics by phosphorylation can lead to opening at more depolarized potentials leading to generation of action potentials (Hille 2003). Thus to investigate if the insulin-induced depolarization of the membrane potential of SFO neurons was due to the modulation of  $I_{sag}$ , neurons were pre-treated with  $Cs^+$ , a known

blocker for  $I_{\text{sag}}$ . As a result, the insulin-induced depolarization of the membrane potential was abolished. Moreover, another interesting observation was the development of  $I_{\text{sag}}$  in a 7 neurons after application of insulin. A plausible explanation of this data is that there are a number of different ion channels responsible for a change in the membrane potential, and at a given time, the individual currents generated by each ion channel in a cell may potentiate a given response. For example, both HCN and  $K_{\text{ATP}}$  channels may coexist in the same SFO neuron and both channel types may be modulated simultaneously. Finally, 30% of the neurons showed no change in the membrane potential or action potential firing frequency as well as no change in input resistance suggesting that a subpopulation of SFO neurons is insensitive to insulin changes.

SFO neurons are a heterogeneous population of neurons, and various electrophysiological studies have demonstrated the presence of subpopulations of SFO neurons. Insulin's actions on separate subpopulations of neurons of the SFO are similar to published reports demonstrating that circulating satiety signals such as ghrelin, amylin (Pulman *et al.*, 2006), leptin (Smith *et al.*, 2009), and adiponectin (Alim *et al.*, 2010) can modulate the electrical activity of separate subpopulations of neurons within the SFO by a variety of ion channels (for review see Fry *et al.*, 2007). Electrical responsiveness of separate subpopulations of SFO neurons is suggested to be correlated with the different axonal projections transmitting signals to different areas within the anteroventral third ventricle (AV3V) and the hypothalamus (Miselis *et al.*, 1979; and Miselis 1981; Lind *et al.*, 1982). Hypothalamic projections which terminate in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) (Miselis 1982) suggest an autonomic role of SFO neurons in the regulation of oxytocin and vasopression secretion. Furthermore,

antidromic identification of SFO neurons projecting to the PVN show them to be more responsive to ANGII than those neurons which project to the SON (Gutman *et al.*, 1988; for review see Ferguson and Bains 1996). Electrophysiological studies have also demonstrated that SFO neurons which are more sensitive to ANGII are located within the core of the SFO (Ono *et al.*, 2001). Therefore, it is not unusual for other circulating hormones to have differential responses within different populations of SFO neurons.

Currently, a weakness of the dissociated method is the inability to predict afferent and/or efferent projections from the cell under examination. Therefore, it becomes difficult to identify the SFO neurons in the present study and accurately predict physiological relevance. However, while the dissociation process removes dendritic processes and the axon, a novel electrophysiology study was able to identify an electrophysiological fingerprint of SFO neurons which project to the PVN (Washburn *et al.*, 2001). SFO neurons which project to the PVN exhibit an electrophysiological signature, the presence of an  $I_A$  current, a large transient outward potassium current which is activated at hyperpolarized potentials. In our study, 67% of the neurons which hyperpolarized exhibited a strong  $I_A$  current, whereas 60% of the neurons which depolarized also had an  $I_A$ . These data suggest that many of the above neurons project to the PVN. However, there was no correlation between the type of response and presence or absence of  $I_A$ , suggesting that insulin affects the electrical activity of SFO neurons which project to several areas. The PVN is a key hypothalamic nucleus that regulates autonomic function, such as fluid balance as well as energy homeostasis, and the presence or absence of an  $I_A$  current may suggest that the recorded SFO neuron is

projecting to a major nucleus involved in regulating autonomic processes and energy homeostasis.

Furthermore in this present study, cluster analysis was used to attempt to categorize SFO neurons into groups according to their intrinsic properties and responses to insulin. This analysis is not hypothesis driven and is not conducted under *a priori* conditions. The outcome of the cluster analysis did suggest that neurons whose membrane potential depolarized in response to insulin application had a greater mean cell capacitance than those neurons that either hyperpolarized or were insensitive to insulin. These neurons also had an observable  $I_A$  current and developed an  $I_{sag}$  current after application of insulin. Due to the smaller sample size, it is difficult to cluster the neurons into definitive groups, and thus a larger sample size may be needed to differentiate the neurons into tighter clusters. This finding may prove useful for later studies which could compare capacitance and response with relative levels of ion channel expression (i.e. HCN channel and  $K_{ATP}$  channels using single cell RT-PCR) which could help predict the response of a SFO neuron to insulin.

#### **4.5 Physiological Relevance of insulin's effects on SFO**

##### **4.5.1.1 Insulin's role in regulating energy balance**

This was the first study to directly demonstrate that SFO neurons are electrophysiologically sensitive to insulin. Insulin receptors have been described within other parts of the brain (Schulingkamp *et al.*, 2000), and localization of a dense population of insulin receptors within the SFO suggests a functional mechanism which may have neuro-humoral modulating effects. Miselis *et al.*, (1987) suggested that the

SFO also plays a role in food intake, and many electrophysiological studies have demonstrated that the SFO is sensitive to blood-borne hormones and satiety signals involved in energy regulation (Reidger *et al.*, 1999; Pulman *et al.*, 2006; Smith *et al.*, 2009; Alim *et al.*, 2010). Interestingly, amylin, another pancreatic hormone, results exclusively in a depolarization of SFO neurons (Pulman *et al.*, 2006), whereas insulin can induce either a depolarization or hyperpolarization of the membrane potential in separate subpopulations of SFO neurons. Hyperpolarization of the membrane potential is consistent with other published literature concerning insulin's actions within the CNS and further strengthens the notion that the SFO is also a crucial site for the regulation of energy balance. The opposing responses to insulin which were observed suggest that different subpopulations of neurons not only express different types of ion channels, but also that these neurons may project to different targets. This could be examined using a voltage clamp experiment which is further described in Section 4.7. Moreover, differential responses of SFO neurons have been described previously with respect to exogenous application of circulating peptides and hormones such as adiponectin, ANGII, and interleukin-1 $\beta$  (Alim *et al.*, 2010; Ferguson and Bains, 1997; Desson and Ferguson, 2003). Also, Pulman *et al.*, (2006) have demonstrated that SFO neurons which are sensitive to amylin are not sensitive to ghrelin, an orexigenic hormone released from the fundus of the stomach which stimulates feeding. Therefore, it is plausible that differential sensitivity may be reflective of efferent projections as well as differential modulation of ion channels.

Insulin has widespread physiological interactions within the body (i.e. satiation and blood glucose control), and it becomes difficult to attribute insulin's sole actions on a

single neuron without being able to observe a multitude of factors. However, insulin's direct effects within the CNS have demonstrated not only a decrease in food intake but also a decrease in body weight (Porter and Woods 1979). This is also seen in other animals such as dogs and hamsters as well (Porter and Woods 1980; Air *et al.*, 1983). Moreover, it has been identified that activation of the vagal efferents stimulate insulin release which decreases food intake (for review see Woods *et al.*, 1985), and intravenous injection of fructose following insulin treatment prevents food intake, a response which is negated in vagotomised rats (Friedman and Granneman 1983). While direct insulin injections within the brain result in reduced food intake, it is not correct to solely attribute satiety to insulin's actions within the brain. Satiety results from a multitude of compounding factors such as glucose concentrations, ion concentrations, and other peptide concentrations such as leptin, and while SFO neurons are sensitive to insulin and can modulate their electrical activity to respond, there are other interactions occurring that can contribute to reaching satiety.

#### **4.5.1.2 Insulin's role in regulating water balance at the SFO**

Maintenance of fluid balance is a well-established and accepted role of SFO neurons. The SFO's role in maintaining osmotic homeostasis was demonstrated in a classical experiment in which volumetric changes in SFO neurons were observed after perturbations to water and salt concentrations (Palkovits 1966). Furthermore, a high density of ANG II receptors have been localized within the SFO's core (Mendelson *et al.*, 1984 and Song *et al.*, 1992), and peripherally administered ANG II stimulates an increase in the thirst response (Simpson and Routtenberg, 1975; Simpson *et al.*, 1978). Lastly, low intensity acute electrical stimulation of SFO neurons induces drinking behavior in water–

satiated rats (Smith *et al.*, 2010), and lesions of SFO result in an abolishment of water ingestion (Starbuck *et al.*, 1997). However, the lines between hydro-mineral balance and food intake are not always clear cut, and an increase in water intake before a meal is believed to be a conditioned response (Fitzsimons 1979). Furthermore, the satiety signal amylin has been shown to increase water consumption when injected into the SFO (Riediger *et al.*, 1999), whereas intercranial injections of ghrelin have decreased water intake (Kozaka *et al.*, 2003), which has been proposed to be a result of the antagonistic effect of these peptides on separate subpopulations of SFO neurons (Pulman *et al.*, 2006). Therefore it is not surprising that insulin would play a role in water intake as well (Brime *et al.*, 1990; Vijande *et al.*, 1990). An intriguing possibility suggested by the present study is that the bifunctional nature of insulin in food intake and water intake may be localized to its site of action at the SFO.

#### **4.6 Conclusion and Perspectives**

The present study demonstrated that the electrical activity of dissociated SFO neurons is directly modulated by insulin. This effect remains to be tested in brain slices which would represent more *in vivo* like conditions (i.e. lack of glucose changes from cell culture media to recording media). The results of this study demonstrate that insulin modulates the electrical activity of SFO neurons and provides additional support for the role of the SFO as an important sensor for regulating energy homeostasis. Moreover, this research contributes to a better understanding of the complex physiological actions of insulin acting on the homeostatic control centres of the CNS. Furthermore, this research may aid in the development of future strategies to help prevent and treat obesity and obesity induced diseases. For example identifying CNS pathways involved in

maintaining energy homeostasis are crucial for the development of effective pharmaceutical therapies that can target such pathways.

#### **4.7 Future Directions:**

Cluster analysis revealed that some properties of SFO neurons clustered together, such as cell size and membrane response. To further investigate this finding, a set of voltage-clamp experiments to specifically isolate  $I_{\text{sag}}$  and  $K_{\text{ATP}}$  current using appropriate solutions to block all other ion channels would be warranted.

Another experiment which could further complement this study would be single cell reverse transcriptase polymerase chain reactions following insulin application. Obtaining mRNA from patched SFO neurons would provide greater identification of the channels and receptors expressed within single SFO neurons and thus allow for more detailed characterization of a group of heterogeneous neurons.

This study examined the electrophysiological properties of SFO neurons in isolation. Examining the electrophysiological properties of SFO neurons before and after insulin application in brain slices would confirm both the sensitivity and the mechanism of ion channel modulation and provide additional confirmation of the ability of SFO neurons to detect and respond to insulin in more *in vivo* like conditions.

Wang *et al.*,(2004) have suggested that glucose concentrations affect the configuration of  $K_{\text{ATP}}$  channels. Thus bathing the cells in a high glucose medium for 1-3 days (to stimulate hyperinsulinemia) would affect the configuration of the  $K_{\text{ATP}}$  channel which may influence the effects of insulin on SFO neurons. This would provide more insight into how the SFO modulates electrical responses of its neurons in a diseased state.

## References:

- Abbott, N. J., Patabendige, A. A., Dolman, D. E., Yusof, S. R., & Begley, D. J. (2010). Structure and function of the blood-brain barrier. *Neurobiology of Disease*, 37(1), 13-25.
- Adachi, A., Kobashi, M., & Funahashi, M. (1995). Glucose-responsive neurons in the brainstem. *Obesity Research*, 3 Suppl 5, 735S-740S.
- Ahima, R. S., Kelly, J., Elmquist, J. K., & Flier, J. S. (1999). Distinct physiologic and neuronal responses to decreased leptin and mild hyperleptinemia. *Endocrinology*, 140(11), 4923-4931.
- Air, E. L., Benoit, S. C., Clegg, D. J., Seeley, R. J., & Woods, S. C. (2002). Insulin and leptin combine additively to reduce food intake and body weight in rats. *Endocrinology*, 143(6), 2449-2452.
- Air, E. L., Strowski, M. Z., Benoit, S. C., Conarello, S. L., Salituro, G. M., Guan, X. M., et al. (2002). Small molecule insulin mimetics reduce food intake and body weight and prevent development of obesity. *Nature Medicine*, 8(2), 179-183.
- Alim, I., Fry, W. M., Walsh, M. H., & Ferguson, A. V. (2010). Actions of adiponectin on the excitability of subfornical organ neurons are altered by food deprivation. *Brain Research*, 1330, 72-82.
- Anderson, J. W., Smith, P. M., & Ferguson, A. V. (2001). Subfornical organ neurons projecting to paraventricular nucleus: Whole-cell properties. *Brain Research*, 921(1-2), 78-85.
- Babenko, A. P., Aguilar-Bryan, L., & Bryan, J. (1998). A view of sur/KIR6.X, KATP channels. *Annual Review of Physiology*, 60, 667-687.
- Bagdade, J. D., Bierman, E. L., & Porte, D., Jr. (1967). The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and nondiabetic subjects. *The Journal of Clinical Investigation*, 46(10), 1549-1557.
- Bagnasco, M., Tulipano, G., Melis, M. R., Argiolas, A., Cocchi, D., & Muller, E. E. (2003). Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. *Regulatory Peptides*, 111(1-3), 161-167.
- Ballabh, P., Braun, A., & Nedergaard, M. (2004). The blood-brain barrier: An overview: Structure, regulation, and clinical implications. *Neurobiology of Disease*, 16(1), 1-13.

- Banks, W. A. (2004). The source of cerebral insulin. *European Journal of Pharmacology*, 490(1-3), 5-12.
- Banks, W. A., & Kastin, A. J. (1996). Passage of peptides across the blood-brain barrier: Pathophysiological perspectives. *Life Sciences*, 59(23), 1923-1943.
- Benoit, S. C., Air, E. L., Coolen, L. M., Strauss, R., Jackman, A., Clegg, D. J., et al. (2002). The catabolic action of insulin in the brain is mediated by melanocortins. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 22(20), 9048-9052.
- Berthoud, H. R. (2006). Homeostatic and non-homeostatic pathways involved in the control of food intake and energy balance. *Obesity (Silver Spring, Md.)*, 14 Suppl 5, 197S-200S.
- Biel, M., Ludwig, A., Zong, X., & Hofmann, F. (1999). Hyperpolarization-activated cation channels: A multi-gene family. *Reviews of Physiology, Biochemistry and Pharmacology*, 136, 165-181.
- Blevins, J. E., Schwartz, M. W., & Baskin, D. G. (2002). Peptide signals regulating food intake and energy homeostasis. *Canadian Journal of Physiology and Pharmacology*, 80(5), 396-406.
- Boyle, P. C., & Keeseey, R. E. (1975). Chronically reduced body weight in rats sustaining lesions of the lateral hypothalamus and maintained on palatable diets and drinking solutions. *Journal of Comparative and Physiological Psychology*, 88(1), 218-223.
- Brands, M. W., Hall, J. E., Van Vliet, B. N., Alonso-Galicia, M., Herrera, G. A., & Zappe, D. (1995). Obesity and hypertension: Roles of hyperinsulinemia, sympathetic nervous system and intrarenal mechanisms. *The Journal of Nutrition*, 125(6 Suppl), 1725S-1731S.
- Bray, G. A. (2000). Afferent signals regulating food intake. *The Proceedings of the Nutrition Society*, 59(3), 373-384.
- Bray, G. A. (2000). Afferent signals regulating food intake. *The Proceedings of the Nutrition Society*, 59(3), 373-384.
- Bray, G. A., & Campfield, L. A. (1975). Metabolic factors in the control of energy stores. *Metabolism: Clinical and Experimental*, 24(1), 99-117.
- Brewer, G. J., Torricelli, J. R., Evege, E. K., & Price, P. J. (1993). Optimized survival of hippocampal neurons in B27-supplemented neurobasal, a new serum-free medium combination. *Journal of Neuroscience Research*, 35(5), 567-576.

- Brewer, G. J. (1995). Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. *Journal of Neuroscience Research*, 42(5), 674-683.
- Brief, D. J., & Davis, J. D. (1984). Reduction of food intake and body weight by chronic intraventricular insulin infusion. *Brain Research Bulletin*, 12(5), 571-575.
- Brime, J. I., Lopez-Sela, P., Bernardo, R., Costales, M., Diaz, F., Marin, B., et al. (1991). Psychological aspects of insulin-induced thirst. *Physiology & Behavior*, 49(1), 153-154.
- Bruning, J. C., Gautam, D., Burks, D. J., Gillette, J., Schubert, M., Orban, P. C., et al. (2000). Role of brain insulin receptor in control of body weight and reproduction. *Science (New York, N.Y.)*, 289(5487), 2122-2125.
- Bult, M. J., van Dalen, T., & Muller, A. F. (2008). Surgical treatment of obesity. *European Journal of Endocrinology / European Federation of Endocrine Societies*, 158(2), 135-145.
- Campbell, M. L., & Mathys, M. L. (2001). Pharmacologic options for the treatment of obesity. *American Journal of Health-System Pharmacy : AJHP : Official Journal of the American Society of Health-System Pharmacists*, 58(14), 1301-1308.
- Carpenter, D. O., & Briggs, D. B. (1986). Insulin excites neurons of the area postrema and causes emesis. *Neuroscience Letters*, 68(1), 85-89.
- Carr, K. D., Kim, G., & Cabeza de Vaca, S. (2000). Hypoinsulinemia may mediate the lowering of self-stimulation thresholds by food restriction and streptozotocin-induced diabetes. *Brain Research*, 863(1-2), 160-168.
- Cavaliere, H., Floriano, I., & Medeiros-Neto, G. (2001). Gastrointestinal side effects of orlistat may be prevented by concomitant prescription of natural fibers (psyllium mucilloid). *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity*, 25(7), 1095-1099.
- Christopoulos, G., Paxinos, G., Huang, X. F., Beaumont, K., Toga, A. W., & Sexton, P. M. (1995). Comparative distribution of receptors for amylin and the related peptides calcitonin gene related peptide and calcitonin in rat and monkey brain. *Canadian Journal of Physiology and Pharmacology*, 73(7), 1037-1041.
- Clark, M. G., Colquhoun, E. Q., Rattigan, S., Dora, K. A., Eldershaw, T. P., Hall, J. L., et al. (1995). Vascular and endocrine control of muscle metabolism. *The American Journal of Physiology*, 268(5 Pt 1), E797-812.

- Connolly, H. M., Crary, J. L., McGoon, M. D., Hensrud, D. D., Edwards, B. S., Edwards, W. D., et al. (1997). Valvular heart disease associated with fenfluramine-phentermine. *The New England Journal of Medicine*, *337*(9), 581-588.
- Cotero, V. E., & Routh, V. H. (2009). Insulin blunts the response of glucose-excited neurons in the ventrolateral-ventromedial hypothalamic nucleus to decreased glucose. *American Journal of Physiology. Endocrinology and Metabolism*, *296*(5), E1101-9.
- Cottrell, G. T., & Ferguson, A. V. (2004). Sensory circumventricular organs: Central roles in integrated autonomic regulation. *Regulatory Peptides*, *117*(1), 11-23.
- Cowley, M. A., Smart, J. L., Rubinstein, M., Cerdan, M. G., Diano, S., Horvath, T. L., et al. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature*, *411*(6836), 480-484.
- Crowley, V. E. (2008). Overview of human obesity and central mechanisms regulating energy homeostasis. *Annals of Clinical Biochemistry*, *45*(Pt 3), 245-255.
- Cummings, D. E., & Schwartz, M. W. (2003). Genetics and pathophysiology of human obesity. *Annual Review of Medicine*, *54*, 453-471.
- Davidson, M. H., Hauptman, J., DiGirolamo, M., Foreyt, J. P., Halsted, C. H., Heber, D., et al. (1999). Weight control and risk factor reduction in obese subjects treated for 2 years with orlistat: A randomized controlled trial. *JAMA : The Journal of the American Medical Association*, *281*(3), 235-242.
- Dellmann, H. D., & Linner, J. G. (1979). Ultrastructure of the subfornical organ of the chicken (*Gallus domesticus*). *Cell and Tissue Research*, *197*(1), 137-153.
- Dellmann, H. D., & Simpson, J. B. (1976). Regional differences in the morphology of the rat subfornical organ. *Brain Research*, *116*(3), 389-400.
- Desson, S. E., & Ferguson, A. V. (2003). Interleukin 1beta modulates rat subfornical organ neurons as a result of activation of a non-selective cationic conductance. *The Journal of Physiology*, *550*(Pt 1), 113-122.
- Dunn-Meynell, A. A., Rawson, N. E., & Levin, B. E. (1998). Distribution and phenotype of neurons containing the ATP-sensitive K<sup>+</sup> channel in rat brain. *Brain Research*, *814*(1-2), 41-54.
- Elias, C. F., Aschkenasi, C., Lee, C., Kelly, J., Ahima, R. S., Bjorbaek, C., et al. (1999). Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron*, *23*(4), 775-786.

- Elmquist, J. K., Elias, C. F., & Saper, C. B. (1999). From lesions to leptin: Hypothalamic control of food intake and body weight. *Neuron*, 22(2), 221-232.
- Fenstermacher, J., Gross, P., Sposito, N., Acuff, V., Pettersen, S., & Gruber, K. (1988). Structural and functional variations in capillary systems within the brain. *Annals of the New York Academy of Sciences*, 529, 21-30.
- Ferguson, A. V., & Bains, J. S. (1996). Electrophysiology of the circumventricular organs. *Frontiers in Neuroendocrinology*, 17(4), 440-475.
- Ferguson, A. V., Bicknell, R. J., Carew, M. A., & Mason, W. T. (1997). Dissociated adult rat subfornical organ neurons maintain membrane properties and angiotensin responsiveness for up to 6 days. *Neuroendocrinology*, 66(6), 409-415.
- Ferguson, A. V., & Kasting, N. W. (1986). Electrical stimulation in subfornical organ increases plasma vasopressin concentrations in the conscious rat. *The American Journal of Physiology*, 251(2 Pt 2), R425-8.
- Ferguson, A. V., & Li, Z. (1996). Whole cell patch recordings from forebrain slices demonstrate angiotensin II inhibits potassium currents in subfornical organ neurons. *Regulatory Peptides*, 66(1-2), 55-58.
- Fitzsimons, J. T. (1998). Angiotensin, thirst, and sodium appetite. *Physiological Reviews*, 78(3), 583-686.
- Florant, G. L., Singer, L., Scheurink, A. J., Park, C. R., Richardson, R. D., & Woods, S. C. (1991). Intraventricular insulin reduces food intake and body weight of marmosets during the summer feeding period. *Physiology & Behavior*, 49(2), 335-338.
- Flynn, F. W., & Grill, H. J. (1983). Insulin elicits ingestion in decerebrate rats. *Science (New York, N.Y.)*, 221(4606), 188-190.
- Friedman, J. M. (2002). The function of leptin in nutrition, weight, and physiology. *Nutrition Reviews*, 60(10 Pt 2), S1-14; discussion S68-84, 85-7.
- Friedman, M. I., & Granneman, J. (1983). Food intake and peripheral factors after recovery from insulin-induced hypoglycemia. *The American Journal of Physiology*, 244(3), R374-82.
- Fry, M., & Ferguson, A. V. (2007). The sensory circumventricular organs: Brain targets for circulating signals controlling ingestive behavior. *Physiology & Behavior*, 91(4), 413-423.
- Fry, M., Hoyda, T. D., & Ferguson, A. V. (2007). Making sense of it: Roles of the sensory circumventricular organs in feeding and regulation of energy homeostasis. *Experimental Biology and Medicine (Maywood, N.J.)*, 232(1), 14-26.

- Fry, M., Smith, P. M., Hoyda, T. D., Duncan, M., Ahima, R. S., Sharkey, K. A., et al. (2006). Area postrema neurons are modulated by the adipocyte hormone adiponectin. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 26(38), 9695-9702.
- Funahashi, M., & Adachi, A. (1993). Glucose-responsive neurons exist within the area postrema of the rat: In vitro study on the isolated slice preparation. *Brain Research Bulletin*, 32(5), 531-535.
- Galani, C., Al, M., Schneider, H., & Rutten, F. F. (2008). Uncertainty in decision-making: Value of additional information in the cost-effectiveness of lifestyle intervention in overweight and obese people. *Value in Health : The Journal of the International Society for Pharmacoeconomics and Outcomes Research*, 11(3), 424-434.
- Ganong, W. F. (2000). Circumventricular organs: Definition and role in the regulation of endocrine and autonomic function. *Clinical and Experimental Pharmacology & Physiology*, 27(5-6), 422-427.
- Gauss, R., & Seifert, R. (2000). Pacemaker oscillations in heart and brain: A key role for hyperpolarization-activated cation channels. *Chronobiology International*, 17(4), 453-469.
- Glazer, G. (2001). Long-term pharmacotherapy of obesity 2000: A review of efficacy and safety. *Archives of Internal Medicine*, 161(15), 1814-1824.
- Gross, P. M. (1985). The subfornical organ as a model of neurohumoral integration. *Brain Research Bulletin*, 15(1), 65-70.
- Gross, P. M. (1991). Morphology and physiology of capillary systems in subregions of the subfornical organ and area postrema. *Canadian Journal of Physiology and Pharmacology*, 69(7), 1010-1025.
- Gross, P. M., & Weindl, A. (1987). Peering through the windows of the brain. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, 7(6), 663-672.
- Grossman, S. P., Dacey, D., Halaris, A. E., Collier, T., & Routtenberg, A. (1978). Aphagia and adipsia after preferential destruction of nerve cell bodies in hypothalamus. *Science (New York, N.Y.)*, 202(4367), 537-539.
- Gu, G. B., & Ju, G. (1995). The parabrachio-subfornical organ projection in the rat. *Brain Research Bulletin*, 38(1), 41-47.

- Gutman, M. B., Ciriello, J., & Mogenson, G. J. (1988). Effects of plasma angiotensin II and hypernatremia on subfornical organ neurons. *The American Journal of Physiology*, 254(5 Pt 2), R746-54.
- Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., et al. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. *Science (New York, N.Y.)*, 269(5223), 543-546.
- Hall, J. E., Brands, M. W., Zappe, D. H., & Alonso Galicia, M. (1995). Insulin resistance, hyperinsulinemia, and hypertension: Causes, consequences, or merely correlations? *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, 208(4), 317-329.
- Hall, J. E., Brands, M. W., Zappe, D. H., & Alonso Galicia, M. (1995). Insulin resistance, hyperinsulinemia, and hypertension: Causes, consequences, or merely correlations? *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, 208(4), 317-329.
- Hall, J. E., Brands, M. W., Zappe, D. H., & Alonso-Galicia, M. (1995). Cardiovascular actions of insulin: Are they important in long-term blood pressure regulation? *Clinical and Experimental Pharmacology & Physiology*, 22(10), 689-700.
- Hall, J. E., Brands, M. W., Zappe, D. H., Dixon, W. N., Mizelle, H. L., Reinhart, G. A., et al. (1995). Hemodynamic and renal responses to chronic hyperinsulinemia in obese, insulin-resistant dogs. *Hypertension*, 25(5), 994-1002.
- Hall, J. E., Summers, R. L., Brands, M. W., Keen, H., & Alonso-Galicia, M. (1994). Resistance to metabolic actions of insulin and its role in hypertension. *American Journal of Hypertension*, 7(8), 772-788.
- Heck, A. M., Yanovski, J. A., & Calis, K. A. (2000). Orlistat, a new lipase inhibitor for the management of obesity. *Pharmacotherapy*, 20(3), 270-279.
- Hindmarch, C., Fry, M., Yao, S. T., Smith, P. M., Murphy, D., & Ferguson, A. V. (2008). Microarray analysis of the transcriptome of the subfornical organ in the rat: Regulation by fluid and food deprivation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 295(6), R1914-20.
- Houseknecht, K. L., Baile, C. A., Matteri, R. L., & Spurlock, M. E. (1998). The biology of leptin: A review. *Journal of Animal Science*, 76(5), 1405-1420.
- Hoyda, T. D., & Ferguson, A. V. (2010). Adiponectin modulates excitability of rat paraventricular nucleus neurons by differential modulation of potassium currents. *Endocrinology*, 151(7), 3154-3162.

- Jobst, E. E., Enriori, P. J., & Cowley, M. A. (2004). The electrophysiology of feeding circuits. *Trends in Endocrinology and Metabolism: TEM*, 15(10), 488-499.
- Johnson, A. K., & Gross, P. M. (1993). Sensory circumventricular organs and brain homeostatic pathways. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, 7(8), 678-686.
- Kang, L., Routh, V. H., Kuzhikandathil, E. V., Gaspers, L. D., & Levin, B. E. (2004). Physiological and molecular characteristics of rat hypothalamic ventromedial nucleus glucosensing neurons. *Diabetes*, 53(3), 549-559.
- Katzmarzyk, P. T., & Janssen, I. (2004). The economic costs associated with physical inactivity and obesity in Canada: An update. *Canadian Journal of Applied Physiology = Revue Canadienne De Physiologie Appliquee*, 29(1), 90-115.
- Klem, M. L., Wing, R. R., McGuire, M. T., Seagle, H. M., & Hill, J. O. (1997). A descriptive study of individuals successful at long-term maintenance of substantial weight loss. *The American Journal of Clinical Nutrition*, 66(2), 239-246.
- Koch, L., Wunderlich, F. T., Seibler, J., Konner, A. C., Hampel, B., Irlenbusch, S., et al. (2008). Central insulin action regulates peripheral glucose and fat metabolism in mice. *The Journal of Clinical Investigation*, 118(6), 2132-2147.
- Konner, A. C., Klockener, T., & Bruning, J. C. (2009). Control of energy homeostasis by insulin and leptin: Targeting the arcuate nucleus and beyond. *Physiology & Behavior*, 97(5), 632-638.
- Konner, A. C., Klockener, T., & Bruning, J. C. (2009). Control of energy homeostasis by insulin and leptin: Targeting the arcuate nucleus and beyond. *Physiology & Behavior*, 97(5), 632-638.
- Kott, J. N., Kenney, N. J., Bhatia, A. J., & Bhatia, A. M. (1989). Response to chronic insulin administration: Effect of area postrema ablation. *Physiology & Behavior*, 46(6), 971-976.
- Kozaka, T., Fujii, Y., & Ando, M. (2003). Central effects of various ligands on drinking behavior in eels acclimated to seawater. *The Journal of Experimental Biology*, 206(Pt 4), 687-692.
- Kunii, K., Yamanaka, A., Nambu, T., Matsuzaki, I., Goto, K., & Sakurai, T. (1999). Orexins/hypocretins regulate drinking behaviour. *Brain Research*, 842(1), 256-261.
- Latchford, K. J., & Ferguson, A. V. (2005). Angiotensin depolarizes parvocellular neurons in paraventricular nucleus through modulation of putative nonselective cationic and potassium conductances. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 289(1), R52-8.

- Lau, D. C., Douketis, J. D., Morrison, K. M., Hramiak, I. M., Sharma, A. M., Ur, E., et al. (2007). 2006 canadian clinical practice guidelines on the management and prevention of obesity in adults and children [summary. *CMAJ : Canadian Medical Association Journal = Journal De l'Association Medicale Canadienne*, 176(8), S1-13.
- Lean, M. E. (2001). How does sibutramine work? *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity*, 25 Suppl 4, S8-11.
- Lebowitz, M. R., Moses, A. M., & Scheinman, S. J. (1992). Effects of atrial natriuretic peptides on metabolism of arginine vasopressin by isolated perfused rat kidney. *The American Journal of Physiology*, 263(2 Pt 2), R273-8.
- Leibel, R. L., Rosenbaum, M., & Hirsch, J. (1995). Changes in energy expenditure resulting from altered body weight. *The New England Journal of Medicine*, 332(10), 621-628.
- Lenard, N. R., & Berthoud, H. R. (2008). Central and peripheral regulation of food intake and physical activity: Pathways and genes. *Obesity (Silver Spring, Md.)*, 16 Suppl 3, S11-22.
- Lepetit, P., Fevre-Montange, M., Gay, N., Belin, M. F., & Bobillier, P. (1993). Vasopressin mRNA in the cerebellum and circumventricular organs: A quantitative in situ hybridization study. *Neuroscience Letters*, 159(1-2), 171-174.
- Levin, B. E. (2007). Why some of us get fat and what we can do about it. *The Journal of Physiology*, 583(Pt 2), 425-430.
- Lewis, P. R., & Shute, C. C. (1967). The cholinergic limbic system: Projections to hippocampal formation, medial cortex, nuclei of the ascending cholinergic reticular system, and the subfornical organ and supra-optic crest. *Brain : A Journal of Neurology*, 90(3), 521-540.
- Lind, R. W., Swanson, L. W., & Sawchenko, P. E. (1985). Anatomical evidence that neural circuits related to the subfornical organ contain angiotensin II. *Brain Research Bulletin*, 15(1), 79-82.
- Lind, R. W., Thunhorst, R. L., & Johnson, A. K. (1984). The subfornical organ and the integration of multiple factors in thirst. *Physiology & Behavior*, 32(1), 69-74.
- Lind, R. W., Van Hoesen, G. W., & Johnson, A. K. (1982). An HRP study of the connections of the subfornical organ of the rat. *The Journal of Comparative Neurology*, 210(3), 265-277.

- Lopez-Sela, P., Brime, J. I., Diaz, F., Marin, B., Costales, M., & Vijande, M. (1989). Effects of inhibitors of the renin-angiotensin system on water intake after insulin administration. *Appetite*, *13*(2), 143-154.
- Mangiapane, M. L., & Simpson, J. B. (1980). Subfornical organ lesions reduce the pressor effect of systemic angiotensin II. *Neuroendocrinology*, *31*(6), 380-384.
- Mark, E. J., Patalas, E. D., Chang, H. T., Evans, R. J., & Kessler, S. C. (1997). Fatal pulmonary hypertension associated with short-term use of fenfluramine and phentermine. *The New England Journal of Medicine*, *337*(9), 602-606.
- Mayer, J. (1953). Glucostatic mechanism of regulation of food intake. *The New England Journal of Medicine*, *249*(1), 13-16.
- McKinley, M. J., Badoer, E., & Oldfield, B. J. (1992). Intravenous angiotensin II induces fos-immunoreactivity in circumventricular organs of the lamina terminalis. *Brain Research*, *594*(2), 295-300.
- McKinley, M. J., McAllen, R. M., Davern, P., Giles, M. E., Penschow, J., Sunn, N., et al. (2003). The sensory circumventricular organs of the mammalian brain. *Advances in Anatomy, Embryology, and Cell Biology*, *172*, III-XII, 1-122, back cover.
- McKinley, M. J., Pennington, G. L., & Oldfield, B. J. (1996). Anteroventral wall of the third ventricle and dorsal lamina terminalis: Headquarters for control of body fluid homeostasis? *Clinical and Experimental Pharmacology & Physiology*, *23*(4), 271-281.
- Medeiros, N., Dai, L., & Ferguson, A. V. (2011). Glucose-responsive neurons in the subfornical organ of the rat—a novel site for direct CNS monitoring of circulating glucose. *Neuroscience*,
- Mendelsohn, F. A., Quirion, R., Saavedra, J. M., Aguilera, G., & Catt, K. J. (1984). Autoradiographic localization of angiotensin II receptors in rat brain. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(5), 1575-1579.
- Miki, T., Liss, B., Minami, K., Shiuchi, T., Saraya, A., Kashima, Y., et al. (2001). ATP-sensitive K<sup>+</sup> channels in the hypothalamus are essential for the maintenance of glucose homeostasis. *Nature Neuroscience*, *4*(5), 507-512.
- Mirshamsi, S., Laidlaw, H. A., Ning, K., Anderson, E., Burgess, L. A., Gray, A., et al. (2004). Leptin and insulin stimulation of signalling pathways in arcuate nucleus neurones: PI3K dependent actin reorganization and KATP channel activation. *BMC Neuroscience*, *5*, 54.

- Miselis, R. R. (1981). The efferent projections of the subfornical organ of the rat: A circumventricular organ within a neural network subserving water balance. *Brain Research*, 230(1-2), 1-23.
- Mobbs, C. V., Isoda, F., Makimura, H., Mastaitis, J., Mizuno, T., Shu, I. W., et al. (2005). Impaired glucose signaling as a cause of obesity and the metabolic syndrome: The glucoadipostatic hypothesis. *Physiology & Behavior*, 85(1), 3-23.
- Moller, D. E., & Kaufman, K. D. (2005). Metabolic syndrome: A clinical and molecular perspective. *Annual Review of Medicine*, 56, 45-62.
- Moss, A. M., Unger, J. W., Moxley, R. T., & Livingston, J. N. (1990). Location of phosphotyrosine-containing proteins by immunocytochemistry in the rat forebrain corresponds to the distribution of the insulin receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 87(12), 4453-4457.
- Niswender, K. D., Gallis, B., Blevins, J. E., Corson, M. A., Schwartz, M. W., & Baskin, D. G. (2003). Immunocytochemical detection of phosphatidylinositol 3-kinase activation by insulin and leptin. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society*, 51(3), 275-283.
- Niswender, K. D., Morrison, C. D., Clegg, D. J., Olson, R., Baskin, D. G., Myers, M. G., Jr, et al. (2003). Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: A key mediator of insulin-induced anorexia. *Diabetes*, 52(2), 227-231.
- Nutrition classics. the anatomical record, volume 78, 1940: Hypothalamic lesions and adiposity in the rat.(1983). *Nutrition Reviews*, 41(4), 124-127.
- Ono, K., Honda, E., & Inenaga, K. (2001). Angiotensin II induces inward currents in subfornical organ neurones of rats. *Journal of Neuroendocrinology*, 13(6), 517-523.
- Ono, K., Miyahara, N., & Inenaga, K. (2008). Cell subpopulations of nicotine-sensitive subfornical organ neurons in rat. *Neuroscience Letters*, 442(1), 74-76.
- Ozes, O. N., Akca, H., Mayo, L. D., Gustin, J. A., Maehama, T., Dixon, J. E., et al. (2001). A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. *Proceedings of the National Academy of Sciences of the United States of America*, 98(8), 4640-4645.
- Palkovits, M. (1966). The role of the subfornical organ in the salt and water balance. *Die Naturwissenschaften*, 53(13), 336.

- Pardini, A. W., Nguyen, H. T., Figlewicz, D. P., Baskin, D. G., Williams, D. L., Kim, F., et al. (2006). Distribution of insulin receptor substrate-2 in brain areas involved in energy homeostasis. *Brain Research*, *1112*(1), 169-178.
- Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., et al. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science (New York, N.Y.)*, *269*(5223), 540-543.
- Pinto, S., Roseberry, A. G., Liu, H., Diano, S., Shanabrough, M., Cai, X., et al. (2004). Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science (New York, N.Y.)*, *304*(5667), 110-115.
- Plata-Salaman, C. R., Oomura, Y., & Shimizu, N. (1986). Dependence of food intake on acute and chronic ventricular administration of insulin. *Physiology & Behavior*, *37*(5), 717-734.
- Plum, L., Belgardt, B. F., & Bruning, J. C. (2006). Central insulin action in energy and glucose homeostasis. *The Journal of Clinical Investigation*, *116*(7), 1761-1766.
- Plum, L., Schubert, M., & Bruning, J. C. (2005). The role of insulin receptor signaling in the brain. *Trends in Endocrinology and Metabolism: TEM*, *16*(2), 59-65.
- Polonsky, K. S., Given, B. D., Hirsch, L., Shapiro, E. T., Tillil, H., Beebe, C., et al. (1988). Quantitative study of insulin secretion and clearance in normal and obese subjects. *The Journal of Clinical Investigation*, *81*(2), 435-441.
- Porte, D., Jr, & Woods, S. C. (1981). Regulation of food intake and body weight in insulin. *Diabetologia*, *20 Suppl*, 274-280.
- Price, C. J., Samson, W. K., & Ferguson, A. V. (2008). Nesfatin-1 inhibits NPY neurons in the arcuate nucleus. *Brain Research*, *1230*, 99-106.
- Pulman, K. J., Fry, W. M., Cottrell, G. T., & Ferguson, A. V. (2006). The subfornical organ: A central target for circulating feeding signals. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *26*(7), 2022-2030.
- Reaven, G. M. (1995). Pathophysiology of insulin resistance in human disease. *Physiological Reviews*, *75*(3), 473-486.
- Riediger, T., Rauch, M., & Schmid, H. A. (1999). Actions of amylin on subfornical organ neurons and on drinking behavior in rats. *The American Journal of Physiology*, *276*(2 Pt 2), R514-21.
- Rinaldi-Carmona, M., Barth, F., Heaulme, M., Shire, D., Calandra, B., Congy, C., et al. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Letters*, *350*(2-3), 240-244.

- Rogers, R. C., & Hermann, G. E. (1987). Oxytocin, oxytocin antagonist, TRH, and hypothalamic paraventricular nucleus stimulation effects on gastric motility. *Peptides*, 8(3), 505-513.
- Rothman, R. B., Ayestas, M. A., Dersch, C. M., & Baumann, M. H. (1999). Aminorex, fenfluramine, and chlorphentermine are serotonin transporter substrates. implications for primary pulmonary hypertension. *Circulation*, 100(8), 869-875.
- Rouleau, M. F., Warshawsky, H., & Goltzman, D. (1984). Specific receptors for calcitonin in the subfornical organ of the brain. *Brain : A Journal of Neurology*, 107 ( Pt 1)(Pt 1), 107-114.
- Sakaguchi, T., & Ohtake, M. (1985). Inhibition of gastric motility induced by activation of the hypothalamic paraventricular nucleus. *Brain Research*, 335(2), 365-367.
- Santoro, B., & Tibbs, G. R. (1999). The HCN gene family: Molecular basis of the hyperpolarization-activated pacemaker channels. *Annals of the New York Academy of Sciences*, 868, 741-764.
- Saper, C. B., Loewy, A. D., Swanson, L. W., & Cowan, W. M. (1976). Direct hypothalamo-autonomic connections. *Brain Research*, 117(2), 305-312.
- Schulinkamp, R. J., Pagano, T. C., Hung, D., & Raffa, R. B. (2000). Insulin receptors and insulin action in the brain: Review and clinical implications. *Neuroscience and Biobehavioral Reviews*, 24(8), 855-872.
- Schwartz, M. W., & Porte, D., Jr. (2005). Diabetes, obesity, and the brain. *Science (New York, N.Y.)*, 307(5708), 375-379.
- Schwartz, M. W., Woods, S. C., Porte, D., Jr, Seeley, R. J., & Baskin, D. G. (2000). Central nervous system control of food intake. *Nature*, 404(6778), 661-671.
- Sexton, P. M., Paxinos, G., Kenney, M. A., Wookey, P. J., & Beaumont, K. (1994). In vitro autoradiographic localization of amylin binding sites in rat brain. *Neuroscience*, 62(2), 553-567.
- Shepherd, P. R., Withers, D. J., & Siddle, K. (1998). Phosphoinositide 3-kinase: The key switch mechanism in insulin signalling. *The Biochemical Journal*, 333 ( Pt 3)(Pt 3), 471-490.
- Shiraishi, J., Yanagita, K., Fujita, M., & Bungo, T. (2008). Central insulin suppresses feeding behavior via melanocortins in chicks. *Domestic Animal Endocrinology*, 34(3), 223-228.
- Simpson, J. B., & Routtenberg, A. (1973). Subfornical organ: Site of drinking elicitation by angiotensin II. *Science (New York, N.Y.)*, 181(4105), 1172-1175.

- Simpson, J. B., & Routtenberg, A. (1975). Subfornical organ lesions reduce intravenous angiotensin-induced drinking. *Brain Research*, 88(1), 154-161.
- Sims, J. S., & Lorden, J. F. (1986). Effect of paraventricular nucleus lesions on body weight, food intake and insulin levels. *Behavioural Brain Research*, 22(3), 265-281.
- Small, C. J., Morgan, D. G., Meeran, K., Heath, M. M., Gunn, I., Edwards, C. M., et al. (1997). Peptide analogue studies of the hypothalamic neuropeptide Y receptor mediating pituitary adrenocorticotrophic hormone release. *Proceedings of the National Academy of Sciences of the United States of America*, 94(21), 11686-11691.
- Smith, P. M., & Ferguson, A. V. (1997). Vasopressin acts in the subfornical organ to decrease blood pressure. *Neuroendocrinology*, 66(2), 130-135.
- Smith, P. M., Chambers, A. P., Price, C. J., Ho, W., Hopf, C., Sharkey, K. A., et al. (2009). The subfornical organ: A central nervous system site for actions of circulating leptin. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 296(3), R512-20.
- Smith, P. M., Rozanski, G., & Ferguson, A. V. (2010). Acute electrical stimulation of the subfornical organ induces feeding in satiated rats. *Physiology & Behavior*, 99(4), 534-537.
- Song, K., Allen, A. M., Paxinos, G., & Mendelsohn, F. A. (1992). Mapping of angiotensin II receptor subtype heterogeneity in rat brain. *The Journal of Comparative Neurology*, 316(4), 467-484.
- Spanswick, D., Smith, M. A., Groppi, V. E., Logan, S. D., & Ashford, M. L. (1997). Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. *Nature*, 390(6659), 521-525.
- Spanswick, D., Smith, M. A., Mirshamsi, S., Routh, V. H., & Ashford, M. L. (2000). Insulin activates ATP-sensitive K<sup>+</sup> channels in hypothalamic neurons of lean, but not obese rats. *Nature Neuroscience*, 3(8), 757-758.
- Starbuck, E. M., Lane, J. R., & Fitts, D. A. (1997). Interaction of hydration and subfornical organ lesions in sodium-depletion induced salt appetite. *Behavioral Neuroscience*, 111(1), 206-213.
- Stock, M. J. (1997). Sibutramine: A review of the pharmacology of a novel anti-obesity agent. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity*, 21 Suppl 1, S25-9.
- Strubbe, J. H., & Mein, C. G. (1977). Increased feeding in response to bilateral injection of insulin antibodies in the VMH. *Physiology & Behavior*, 19(2), 309-313.

- Suzuki, K., & Kono, T. (1980). Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proceedings of the National Academy of Sciences of the United States of America*, 77(5), 2542-2545.
- Takahashi, K. A., & Cone, R. D. (2005). Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons. *Endocrinology*, 146(3), 1043-1047.
- Takahashi, Y., Smith, P., Ferguson, A., & Pittman, Q. J. (1997). Circumventricular organs and fever. *The American Journal of Physiology*, 273(5 Pt 2), R1690-5.
- Thomzig, A., Laube, G., Pruss, H., & Veh, R. W. (2005). Pore-forming subunits of K-ATP channels, Kir6.1 and Kir6.2, display prominent differences in regional and cellular distribution in the rat brain. *The Journal of Comparative Neurology*, 484(3), 313-330.
- Thorpe, K. E., Florence, C. S., Howard, D. H., & Joski, P. (2004). The impact of obesity on rising medical spending. *Health Affairs (Project Hope), Suppl Web Exclusives*, W4-480-6.
- Timofeeva, E., Baraboi, E. D., & Richard, D. (2005). Contribution of the vagus nerve and lamina terminalis to brain activation induced by refeeding. *The European Journal of Neuroscience*, 22(6), 1489-1501.
- Traebert, M., Riediger, T., Whitebread, S., Scharrer, E., & Schmid, H. A. (2002). Ghrelin acts on leptin-responsive neurones in the rat arcuate nucleus. *Journal of Neuroendocrinology*, 14(7), 580-586.
- Truong, K. D., & Sturm, R. (2005). Weight gain trends across sociodemographic groups in the united states. *American Journal of Public Health*, 95(9), 1602-1606.
- Unger, J., McNeill, T. H., Moxley, R. T., 3rd, White, M., Moss, A., & Livingston, J. N. (1989). Distribution of insulin receptor-like immunoreactivity in the rat forebrain. *Neuroscience*, 31(1), 143-157.
- van den Top, M., Lyons, D. J., Lee, K., Coderre, E., Renaud, L. P., & Spanswick, D. (2007). Pharmacological and molecular characterization of ATP-sensitive K(+) conductances in CART and NPY/AgRP expressing neurons of the hypothalamic arcuate nucleus. *Neuroscience*, 144(3), 815-824.
- Van Houten, M., & Posner, B. I. (1983). Circumventricular organs: Receptors and mediators of direct peptide hormone action on brain. *Advances in Metabolic Disorders*, 10, 269-289.

- van Houten, M., Posner, B. I., Kopriwa, B. M., & Brawer, J. R. (1979). Insulin-binding sites in the rat brain: In vivo localization to the circumventricular organs by quantitative radioautography. *Endocrinology*, *105*(3), 666-673.
- Van Obberghen, E., Baron, V., Delahaye, L., Emanuelli, B., Filippa, N., Giorgetti-Peraldi, S., et al. (2001). Surfing the insulin signaling web. *European Journal of Clinical Investigation*, *31*(11), 966-977.
- Vanasse, A., Demers, M., Hemiari, A., & Courteau, J. (2006). Obesity in canada: Where and how many? *International Journal of Obesity (2005)*, *30*(4), 677-683.
- Vijande, M., Lopez-Sela, P., Brime, J. I., Bernardo, R., Diaz, F., Costales, M., et al. (1990). Insulin stimulation of water intake in humans. *Appetite*, *15*(2), 81-87.
- Wall, K. M., & Ferguson, A. V. (1992). Endothelin acts at the subfornical organ to influence the activity of putative vasopressin and oxytocin-secreting neurons. *Brain Research*, *586*(1), 111-116.
- Wang, J., & Leibowitz, K. L. (1997). Central insulin inhibits hypothalamic galanin and neuropeptide Y gene expression and peptide release in intact rats. *Brain Research*, *777*(1-2), 231-236.
- Wang, R., Liu, X., Hentges, S. T., Dunn-Meynell, A. A., Levin, B. E., Wang, W., et al. (2004). The regulation of glucose-excited neurons in the hypothalamic arcuate nucleus by glucose and feeding-relevant peptides. *Diabetes*, *53*(8), 1959-1965.
- Washburn, D. L., Anderson, J. W., & Ferguson, A. V. (2000). The calcium receptor modulates the hyperpolarization-activated current in subfornical organ neurons. *Neuroreport*, *11*(14), 3231-3235.
- Washburn, D. L., Anderson, J. W., & Ferguson, A. V. (2000). A subthreshold persistent sodium current mediates bursting in rat subfornical organ neurons. *The Journal of Physiology*, *529 Pt 2*, 359-371.
- Washburn, D. L., Smith, P. M., & Ferguson, A. V. (1999). Control of neuronal excitability by an ion-sensing receptor (correction of anion-sensing). *The European Journal of Neuroscience*, *11*(6), 1947-1954.
- Weiss, M. L., & Hatton, G. I. (1990). Collateral input to the paraventricular and supraoptic nuclei in rat. I. afferents from the subfornical organ and the anteroventral third ventricle region. *Brain Research Bulletin*, *24*(2), 231-238.
- Williams, K. W., Margatho, L. O., Lee, C. E., Choi, M., Lee, S., Scott, M. M., et al. (2010). Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *30*(7), 2472-2479.

- Wing, R. R., & Hill, J. O. (2001). Successful weight loss maintenance. *Annual Review of Nutrition, 21*, 323-341.
- Wislocki, G. B., & Leduc, E. H. (1952). Vital staining of the hematoencephalic barrier by silver nitrate and trypan blue, and cytological comparisons of the neurohypophysis, pineal body, area postrema, intercolumnar tubercle and supraoptic crest. *The Journal of Comparative Neurology, 96*(3), 371-413.
- Woods, S. C., Lotter, E. C., McKay, L. D., & Porte, D., Jr. (1979). Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature, 282*(5738), 503-505.
- Woods, S. C., Porte, D., Jr, Bobbioni, E., Ionescu, E., Sauter, J. F., Rohner-Jeanrenaud, F., et al. (1985). Insulin: Its relationship to the central nervous system and to the control of food intake and body weight. *The American Journal of Clinical Nutrition, 42*(5 Suppl), 1063-1071.
- Woods, S. C., Seeley, R. J., Porte, D., Jr, & Schwartz, M. W. (1998). Signals that regulate food intake and energy homeostasis. *Science (New York, N.Y.), 280*(5368), 1378-1383.
- Xie, S., Furjanic, M. A., Ferrara, J. J., McAndrew, N. R., Ardino, E. L., Ngondara, A., et al. (2007). The endocannabinoid system and rimonabant: A new drug with a novel mechanism of action involving cannabinoid CB1 receptor antagonism--or inverse agonism--as potential obesity treatment and other therapeutic use. *Journal of Clinical Pharmacy and Therapeutics, 32*(3), 209-231.
- Zardetto-Smith, A. M., & Gray, T. S. (1987). A direct neural projection from the nucleus of the solitary tract to the subfornical organ in the rat. *Neuroscience Letters, 80*(2), 163-166.
- Zinn, A. R. (2010). Unconventional wisdom about the obesity epidemic. *The American Journal of the Medical Sciences, 340*(6), 481-491.