Modulation of Subfornical Organ Neurons by Neurotensin

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Abstract

Neurotensin (NT) is a 13 amino-acid peptide which is both a hormone and neuromodulator. Through its 3-4 known receptors, NT is shown to influence several physiological responses including autonomic output, hydromineral balance, and cardiovascular regulation. Recent transcriptomic studies have revealed that the subfornical organ (SFO) expresses a high abundance of neurotensin receptors (NTSR), specifically NTSR2 and NTSR3, with levels comparable to other prominent neuromodulators that act in this region. The SFO is known for its involvement in cardiovascular regulation, sympathetic output, and hydromineral balance. As a circumventricular organ, it lacks the blood brain barrier, providing a direct interface with peptides in circulation, in addition to centrally released signals in cerebrospinal fluid. Both NT and the SFO have been found to influence satiety and water intake; thus, the SFO, through various peptides, and NT as a peptide have both been found to play a key role in hydromineral balance; which, in turn, plays a role in cardiovascular regulation.

Due to their intersecting areas of influence, and as the SFO is a major integration center for peripheral and central signals, we hypothesized that this neuropeptide modulates neurons in this region to elicit physiological responses. Whole-cell patch-clamp recordings from this study demonstrate that NT (1μM) modulates synaptic activity in 54% (34/63) of SFO neurons tested, in acutely prepared brain slices. Specifically, we found that NT significantly increased excitability of SFO neurons by depolarizing the resting membrane potential, and that NT decreased the amplitude of EPSCs, but not EPSC interevent interval. By also treating 35/63 cells previously tested for NT, with Ang II, we found that there appears to be a subpopulation of SFO neurons sensitive to both NT and Ang II. Most of these cells exhibited a decrease in EPSC amplitude to both peptides, suggesting that they may operate through this region of the CNS to affect the same homeostatic mechanisms.

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aCSF – Artificial cerebrospinal fluid

Ang II – Angiotensin II

AP – Area postrema

AT – Angiotensin receptor

BBB – Blood-brain barrier

CCK – Cholecystokinin

CNS – Central nervous system

CVLM – Caudal ventrolateral medulla

CVO – Circumventricular organ

DAG – Diacylglycerol

EPSC – Excitatory post synaptic current

ERK – Extracellular-signal-regulated-kinase

GPCR – G-protein coupled receptor

GTP – Guanosine triphosphate

I_k – Delayed-rectifier potassium current

I_{NSC} – Non-selective cation current

IL-B – Interleukin-B

IP – Inositol triphosphate

LHA – Lateral hypothalamic area

NT - Neurotensin

NTSR – Neurotensin receptor

OVLT – Organum vasculosum of the lamina terminalus

PVN – Paraventricular nucleus

SFO – Subfornical organ

SON – Supraoptic nucleus

TRP – Transient receptor potential

Introduction

1.1 The cardiovascular system is tightly regulated

Homeostatic mechanisms involve electrical, mechanical and chemical signaling pathways from both the central nervous system (CNS) and periphery (Dampney et al., 2002; Gordan et al., 2015). These include positive and negative feedback mechanisms to regulate a variety of biological systems. The cardiovascular system contributes to regulation and homeostasis by delivering oxygen and critical nutrients to tissues filtering waste through the kidney, liver, and lungs. To do this, the heart must rhythmically contract to supply blood that meet the metabolic demands of each tissue and organ system, through cardiac output, which involves feedback and feedforward mechanisms affecting autonomic, endocrine, immune, and other regulatory signaling (Brunton, 2005; Young, 2010).

1.1.2 Dysregulation of the cardiovascular system is linked to death and chronic disease

Hypertension, a cardiovascular disease (CVD), affects about 16 percent of the global population and is a major factor in premature death (World Health Organization, 2021). This condition, along with other hemodynamic abnormalities, can lead to loss of homeostasis in several body systems, likely accounting for the comorbidity of cardiovascular dysfunction with other ailments. Kidney disease, obesity, diabetes, non-alcoholic fatty liver disease, along with other diseases of the brain and periphery have been linked to hypertension and cardiovascular dysfunction (Alkhouri et al., 2010; Emdin et al., 2015; Kendir et al., 2018; Whelton et al., 1996; World Health Organization, 2019) and are thought to be pre-cursors for CVD, the leading cause of non-communicable deaths globally (World Health Organization, 2021). Studies suggest that exercise, regulation of salt intake, pharmaceuticals and other treatments affect hemodynamics and may reverse, prevent, or alleviate effects of comorbidities (Lavie et al., 2015; Lee et al.,

2012, 2014; Ogden et al., 2000; Stolar & Chilton, 2003; Zatz et al., 1986). This suggests complex mechanisms of interaction between the cardiovascular system, tissues and organ systems of the body of which the dysregulation of homeostasis in one may result in cyclical dysfunction of the other (Akoumianakis et al., 2017; Jiang et al., 2016; Rushton & Kadam, 2014). Understanding the neural mechanisms behind cardiovascular regulation and unraveling complex signalling may aid in the development of pharmaceuticals to mitigate dysfunction and possibly the prevention of associated diseases.

1.2 CNS regulation of the cardiovascular system

The CNS is involved in pathways that regulate cardiovascular homeostasis through two primary mechanisms: reflex and central command (Dampney, 2016). Select processing centres in the brain integrate information from various CNS and peripheral systems, driving changes that sustain the whole organism through feedback and feedforward mechanisms of regulation. Chemical, mechanical, and electrical signals are involved in the local and distant transmission of information (Gordan et al., 2015) pertaining to the cardiac and hematic state of the organism. The subfornical organ (SFO) plays a key role in peripheral signal detection and is thought to play a role in cardiovascular regulation through signaling to key coordination centres of the brain.

1.2.2 CNS integrates extrinsic and intrinsic information to the baroreceptor reflex circuitry

Several reflexes are involved in cardiac regulation and hemodynamics (for review see Dampney, 2016). Some reflexes involve signal integration in various homeostatic pathways and higher brain regions to regulate sympathetic, nociceptive, skeletal muscle and other activities (Cobos et al., 2003; Dampney, 2016; van der Kooy et al., 1984). The baroreceptor reflex is a feedback mechanism of which the CNS coordinates signals of the periphery to regulate autonomic outflow. This reflex is in constant operation and responds to small deviations from the

set-point range. The caudal ventrolateral medulla (CVLM) has been shown to mediate the baroreceptor response through modulation of neurons in the SFO, the region of interest to this study (Ciriello, 2013; Dampney, 2016).

1.2.3 Central command of cardiovascular regulation and integration centres

Mechanisms outside of the lower brain reflexes indirectly affect cardiovascular regulation. Autonomic activation can occur in response to conditioned and unconditioned psychological stimulation resulting in complex coordination, usually at the subcortical level. An example used by Dampney (2016) to highlight this higher control is the fight or flight response, which operates through the sympathetic nervous system. Input from the thalamus is sent to the midbrain, cortex, or amygdala to elicit sympathetic, respiratory, and motor responses. The amygdala plays a critical role in integrating input from the thalamus, cortex, and hippocampus and signaling, through the midbrain and hypothalamus to regions of the brainstem responsible for sympathetic, respiratory, and motor activity.

The perifornical and dorsomedial nuclei of the hypothalamus are involved in cardiovascular and respiratory regulation, satiety and glucose signalling, and have projections with the cortex and brainstem (Furlong et al., 2014; Pierret et al., 1994). Moreover, hypothalamic nuclei coordinate a variety of behaviours and homeostatic functions including sleep-wakefulness, feeding, sexual and aggressive behaviours, energy balance, autonomic reflexes, endocrine regulation, and thermoregulation, among others (Furlong et al., 2014; Pierret et al., 1994; Saper & Lowell, 2014). This region functions as major integration centre for CNS signals; however, neurons in the hypothalamus have also been shown to activate in response to circulating molecules to influence energy balance and homeostasis, and the cardiovascular system (de Wardener, 2001; Elmquist et al., 1997; Furlong et al., 2014; Namvar et al., 2016; Ortiga-

Carvalho et al., 2016). This is, in part, due to the many projections between these coordination centres with specialized structures called circumventricular organs (CVOs) (de Wardener, 2001; McKinley et al., 2003; Saper & Lowell, 2014; Silverman et al., 1981).

1.3 Circumventricular organs are specialised structures lacking the blood brain barrier

The blood brain barrier (BBB) protects the CNS from circulating substances of the periphery. Its specialized capillary endothelial cells are joined by tight junctions and surrounded by glial cells to limit the movement of pathogens, hormones, and other large molecules from entering the brain, maintaining the microenvironment required for proper function, yet restricting communication between the brain and periphery (Abbott et al., 2010). Distinctive midline structures known as CVOs lack the BBB and exist along the third and fourth ventricle, providing a direct interface between the circulatory system and CNS.

1.3.2 Secretory and sensory circumventricular organs

There are seven CVOs that play a role in homeostasis through CNS - periphery communication. These structures are categorized into two functional groups, secretory and sensory. Although each of these regions have distinguishing roles in regulation and homeostasis, it is important to note that axonal projections exist among them, promoting crosstalk and signal integration through synaptic terminals and projecting fibers.

The four secretory CVOs include the pineal gland, median eminence, subcommissural organ, and the neurohypophysis, which are known to secrete hormones, proteins and other signaling factors into the periphery and CNS. These structures are thought to play a role in chronobiology through melatonin synthesis; metabolism, reproduction and growth, stress responses, and integration of endocrine and neurohormones; neural development, cerebrospinal

fluid production, composition and circulation; and secretion of vasopressin and oxytocin, respectively (Kaur & Ling, 2017; Ufnal & Skrzypecki, 2014). Axons and terminals of neurosecretory projections from multiple sites pass through, or end in, secretory CVOs, aiding in signal integration and regulation (Kaur & Ling, 2017).

The SFO, area postrema (AP), and the organum vasculosum of the lamina terminalis (OVLT) make up the three sensory CVOs. These structures play a key role in detecting peripheral signals from the blood and cerebrospinal fluid due to their adjacency to the ventricular system. Neuronal cell bodies within the sensory CVOs extend axonal projections to regions of the brain that are involved in metabolism, satiety and thirst, pain, cardiovascular regulation and hemodynamics, homeostasis, and autonomic regulation (Fry et al., 2007; Fry & Ferguson, 2017; McKinley et al., 2003; Ufnal & Skrzypecki, 2014). Major coordination centres of the CNS such as the hypothalamus and amygdala recruit synaptic connections from sensory CVOs, likely contributing to their involvement in a wide range of biological functions. Of interest to this study is the SFO, therefore this review will not describe the AP or OVLT in detail.

1.4 The subfornical organ

1.4.1 Anatomy and localization of the subfornical organ

The SFO protrudes into the third ventricle along the ventral surface of the fornix. This convex structure appears translucent under the microscope, spanning approximately 600 μm horizontally across a coronal slice of the rat brain (Figure 1.1). Uniquely possessing three capillary subtypes, its complex vasculature allows for extended exposure to circulating hormones and other substances, promoting increased interaction with receptor-containing neurons (Gross, 1991). Several studies on characterizing the SFO have identified a variation of neuronal groups based on structure, subpopulation reactivity to substances, and overall heterogeneity of the

region (Gross, 1991). McKinley and colleagues (2003) simplified the SFO into two functional regions, dorsolateral shell (outer shell) and ventromedial core.

Outer shell ependymal cells of the SFO contain tight junctions, have few cilia, and are characteristically flat compared to those of surrounding regions (Gross, 1991; McKinley et al., 2003). Major efferent neuronal projections exist in this region and differ slightly from those prominent in the ventromedial core (Table 1.1). One significantly discriminating feature of outer shell neurons is the expression of calretinin (Figure 1.2B), a calcium binding protein absent within the core (McKinley et al., 2003). Electrophysiological evidence has shown that the shell expresses more tonic firing behaviour compared to ventromedial neurons (Huang et al., 2019).

The ventromedial core of the SFO contains fenestrated capillaries, a higher density of neurons and lack the cilia observed in the shell (Gross, 1991; McKinley et al., 2003). Unlike the outer shell, ventromedial core neurons express calbindin (Figure 1.2C) (McKinley et al., 2003), another Ca^{2+} buffer protein. Neurons in the ventromedial core seem to present characteristic bursting action, higher Sodium (Na^{+}) channel density, and a more depolarized resting membrane potential (Huang et al., 2019a) compared to those in the outer shell.

Neuronal populations existing in the outer shell and ventromedial core of the SFO differ in their response to varying neuropeptides and other molecules. This is due to factors such as the expression of receptors in the neuronal membrane, local synapses, and access to the specific signals. One aim of this study is to determine if a distinct subpopulation may be sensitive to both NT and Ang II which could suggest possible mechanisms of action of the endogenous peptide.

Table 1.1. The SFO has several afferent and efferent projections throughout the brain. The table below identifies many projections identified from literature along with their references.

Brain Region	SFO Region(s) (if identified)	Specified Projection	Reference
Amygdaloid nucleus		Efferent	7
Arcuate Nucleus		Efferent/Afferent	7, 16
Bed Nucleus of the Stria Terminalis	Core	Efferent/Afferent	7, 12, 13, 18
Caudal ventrolateral medulla	Shell	Afferent	4
Central medial thalamic nucleus		Efferent	7
Dorsomedial hypothalamic nucleus		Efferent/ Afferent	7
Infralimbic Area	Shell	Efferent	7, 17
Lamina Terminalis		Efferent	6
Lateral Hypothalamic Area	Shell/Rostral	Efferent/Afferent	7, 13, 14, 18
Lateral Parabrachial Nucleus		Efferent	15
Lateral Septal Nucleus		Efferent	7
Medial Septal Nucleus	Dorsal Stalk	Afferent/Efferent	7, 11, 14
Median Preoptic Nucleus	Shell/Rostral/Throughout	Efferent/Afferent	5, 7, 8, 11, 13, 14
Nucleus circularis	Rostral	Efferent	14
Nucleus of the Solitary Tract	Core/Caudal	Afferent	3, 7, 17
Nucleus Reunions		Afferent/Efferent	7
Organum Vasculosum of the Lamina Terminalis	Core/Rostral	Efferent/Afferent	7, 11, 12, 14
Paraventricular Nucleus	Shell/Rostral/Throughout	Efferent	2, 5, 7, 9, 10,11, 12, 14, 17,
Paraventricular thalamic nucleus		Efferent	7
Perifornical Area	Rostral	Efferent	14
Periventricular Area	Rostral	Efferent/Afferent	7, 11, 14
Raphe Nucleus	Throughout	Afferent/ Efferent	7, 11
Renal Nerves		Afferent	4
Substantia Innominata	Shell	Efferent	7, 18
Suprachiasmic Nucleus		Efferent	7, 11
Supraoptic Nucleus	Shell/Rostral	Efferent	1, 6, 7, 11, 13, 14
Zona Incerta	Shell	Efferent/ Afferent	7, 18

References

1. Boudaba et al., 1995; 2. Ciriello, 1997; 3. Ciriello & Zhang, 1997; 4. Ciriello, 2013; 5. Duan et al., 2008; 6. Freece et al., 2005; 7. Johnson & Gross, 1993; 8. Kawano, 2017; 9. Larsen & Mikkelsen, 1995; 10. Li & Ferguson, 1993; 11. Lind & Johnson, 1982; 12. McKinley et al., 1997; 13. McKinley et al., 2003; 14. Miselis, 1981; 15. Roncari et al., 2014; 16. Rosas-Arellano et al., 1996; 17. Shioya & Tanaka, 1989; 18. Swanson & Lind, 1986.

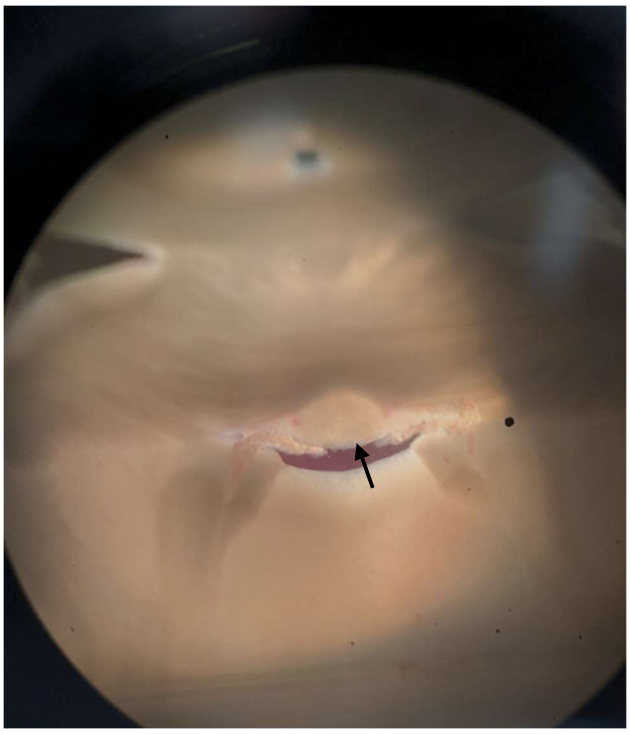

Figure 1.1. Coronal brain slices were observed under 40x magnification prior to beginning patch clamp experiments. The black arrow indicates the location of the SFO.

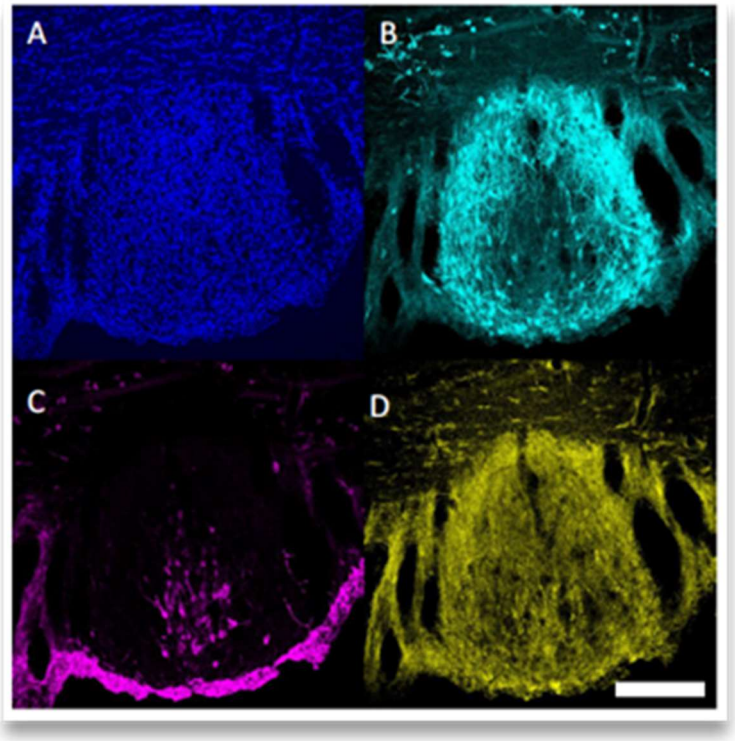

Figure 1.2. Montage of initial immunofluorescent staining of rat brain SFO (Fry, unpublished data). The image shows the expression of neuronal cell bodies (Panel A; DAPI staining), calretinin (Panel B); calbindin (Panel C); and neurotensin (D).

1.4.2 Receptor expression and molecules of physiological action within the subfornical organ

SFO neurons detect and responds to numerous signaling factors, such as peptides, steroids, metabolites, and ions, through activation of numerous receptors and modulation of ion channels. Studies show heterogeneous responses to hormones such as serotonin (Scrogin et al., 1998), noradrenaline (Takahashi & Tanaka, 2017), arginine vasopressin (Anthes et al., 1997), and leptin (Smith et al., 2009), among others, due to mediation through different receptor types and their varying mechanisms of action (Table 1.2). Desson and Ferguson (2003) demonstrated that the SFO may play a role in the immune response through stimulation by circulating interleukin-1β (IL-1β), producing heterogeneous responses through non-selective cation channels dependent on concentration. Extensive research has focused on the activity of angiotensin II (Ang II), a peripheral peptide and neurotransmitter we will review briefly in Section 1.4.5, in the SFO. Data suggests, circulating concentrations of Ang II primarily act on SFO outer shell neurons through AT_1 receptors (McKinley et al., 1995; Ono et al., 2001) to cause depolarization and increased action potential frequency (Li & Ferguson, 1993; Ono et al., 2001). Microarray and transcriptomic studies show significant expression of Ang II, IL-1β, and other molecules and receptors that are involved in metabolism and energy balance, cardiovascular regulation, and autonomic output (Fry & Ferguson, 2021; Hindmarch et al., 2008; Nunes & de Andrade Braga, 2011; Peterson et al., 2018), which we will briefly explore below.

Table 1.2. Several molecules and their receptors are found in or modulate the SFO. The following table provides a list of some of these molecules and literature references.

Molecule	Interaction	Reference(s)
Amylin	Modulate SFO neurons	7, 29, 30
Angiotensin II	Expressed in SFO, Modulate SFO neurons	6, 8, 9, 10, 13, 16, 19, 20, 21, 23, 28, 33, 38, 39, 40, 44
Apelin	Modulates SFO neurons	11
Calbindin	Expressed in SFO	15, 25
Calcitonin and related peptides	Modulate SFO neurons	30
Calretinin	Expressed in SFO	25
Cholecystokinin	Modulates SFO neurons	1, 6
Choline acetyltransferase	Expressed in SFO	32
Dopamine	Expressed in SFO	32
Ghrelin	Modulates SFO neurons	29
Glucose	Modulates SFO neurons	6,7, 26, 30
Histamine	Expressed in SFO	32
Hydrogen Sulfide (NaHS)	Precursor expressed, Modulates SFO neurons	17
Hypertonic Saline and aCSF (Na+)	Modulates SFO neurons	2, 31,38
Il-β	Expressed in SFO, Modulates SFO neurons	12, 41, 42
Leptin	Modulates SFO neurons	35, 37
Nesfatin-1	Expressed in SFO, Modulates SFO neurons	18, 27
Neurotropic Factor	Modulates SFO neurons	4
Norepinephrine	Expressed in SFO	32
NPY	Modulates SFO neurons	34
Nuclear factor kappa-light-chain-enhancer of activated B cells & Inhibitor	Modulate SFO neurons	42
Orexin-A	Modulate SFO neurons	36
Oxytocin	Expressed in SFO, Modulates SFO neurons	5, 14
Prostaglandin E_2 (PGE_2,OX-2)	Expressed in SFO	8, 42, 43
Relaxin	Expressed in SFO, Modulates SFO neurons	24
Renin	Expressed in SFO	19, 20
Serotonin	Expressed in SFO, Modulates SFO neurons	22, 32, 33
TNF-α	Expressed in SFO, Modulates SFO neurons	41, 42, 43
Tryptophan hydroxylase	Expressed in SFO	32
Tyrosine hydroxylase	Expressed in SFO	32
Vasopressin	Modulates SFO neurons	3
References		
1. Ahmed et al., 2013; 2. Anderson et al., 2000; 3Anthes et al., 1997; 4. Black et al., 2018; 5. Blackmore et al., 2018; 6. Cancelliere & Ferguson, 2016; 7. Medeiros et al., 2012; 8. Cao et al., 2012; 9. Ciriello, 1997; 10. Coble et al., 2014; 11. Dai et al., 2013 12. Desson & Ferguson, 2003; 13. Ferguson & Kasting, 1988; 14. Hosono et al., 1999; 15. Huang et al., 2019b; 16. Ku & Li, 2003; 17. Kuksis et al., 2014a; 18. Kuksis & Ferguson, 2014; 19. Lavoie et al., 2004; 20. Lavoie et al., 2004b; 21. Lind & Johnson, 1982; 22. Lind, 1986; 23. Mangiapane & Simpson, 1980; 24. McKinley et al., 1997; 25. McKinley et al., 2003; 26. Medeiros et al., 2012; 27. Moreau & Ciriello, 2013; 28. Ono et al., 2001; 29. Pulman et al., 2006; 30. Riediger et al., 1999); 31. Rohmeiss et al., 1995; 32. Saavedra et al., 1976; 33. Scrogin et al., 1998; 34. Shute, 2016; 35. Smith & Ferguson, 2012; 36. Smith et al., 2007; 37, Smith et al., 2009a; 38. Tiruneh et al., 2013; 39. van Houten et al., 1980; 40. Wang et al., 2016; 41. Wei et al., 2013; 42. Yu et al., 2017; 43. Yu et al., 2018; 44. Li & Ferguson, 1993.		

1.4.3 The subfornical organ is involved in energy balance

Circulating molecules involved in energy balance, such as glucose (Medeiros et al., 2012), adiponectin (Alim et al., 2010), ghrelin, and amylin (Pulman et al., 2006) are detected by neurons of the SFO. Electrophysiological evidence further shows that neuronal subpopulations in the SFO respond to these molecules through changes in action potential output and cellular excitability, depolarizing or hyperpolarizing in response to circulating glucose (Medeiros et al., 2012), as well as other peptides and hormones involved in energy homeostasis and feeding (Cancelliere & Ferguson, 2016) which we will expand upon.

The SFO influences hydro-mineral balance and satiety by modulating food and fluid intake. Electrical and optogenetic stimulation of SFO neurons increase both drinking and feeding behaviour in satiated rats (Oka et al., 2015; Smith et al., 2010; Smith & Ferguson, 2010); whereas lesions have been shown to reduce feeding and drinking behaviour (Baraboi et al., 2010; Freece et al., 2005; Thunhorst et al., 1999). Localization and electrophysiological data in this region show the action of hormones involved in feeding and drinking behaviour such as leptin (Smith et al., 2009, 2010), amylin (Riediger et al., 1999), ghrelin (Pulman et al., 2006), and orexin (Smith et al., 2007), among others (Table 1.2). These signaling molecules and their receptors have been studied to determine biological mechanisms of action in the SFO. For example, genetic ablation of insulin results in weight gain, increased adiposity in the liver, and increased triglyceride levels (Jeong et al., 2018, 2019), and further studies show insulin to directly modulate SFO neurons (Lakhi et al., 2013). Medeiros et al. (2011) demonstrated that the SFO exhibits both glucose-inhibited and glucose-excited subpopulations, which were shown to increase outward glucose current, likely through mediation of non-selective cation conductance, and increase inward glucose current, likely through mediation of K_{ATP} channels, respectively. Circulating leptin and ghrelin modulate sub-populations of SFO neurons to depolarize and

increase action potential frequency(Pulman et al., 2006; Smith et al., 2009). These studies indicate direct modulation of SFO subpopulations by these signals.

1.4.4 The subfornical organ is involved in hydro-mineral balance

Subfornical organ regulated energy balance and fluid homeostasis has an integratory role in whole-organism homeostasis; thus, specific attention should be paid to its role in Na^+ appetite and meal-associated drinking when studying cardiovascular regulation. Peripheral signals involved in fluid-mineral balance, thermoregulation, immune responses, and cardiovascular regulation are detected by the SFO. Interleukin-1β, for example, is a pro-inflammatory cytokine found in circulation that, when injected into the SFO of Na^+ depleted rats, results in dose-dependent inhibition of salt-intake, increased body temperature, and hypertension (Cerqueira et al., 2016). Evidence of this cytokine, and other SFO modulating signals involved in hydro-mineral balance, also eliciting cardiac, hematic, and other autonomic responses is notable (Dai et al., 2013; Fry & Ferguson, 2021; Hindmarch & Ferguson, 2016; Lazarus et al., 1977; Wei et al., 2013) and data suggests there may be integration of their signaling pathways. We will briefly review literature supporting this concept in the section below.

1.4.5 The subfornical organ's role in cardiovascular regulation

The SFO responds to information about cardiovascular and hematic state through central signaling pathways and circulating molecules, contributing to feedback and feedforward regulation (Dampney, 2016). Baroreceptor reflex mediation of the CVLM inhibits SFO neuron sub-populations through afferent projections (Ciriello, 2013). Neuronal inhibition through these projections, decrease the excitatory response of SFO neurons to circulating Ang II (Ciriello, 2013), a key regulator of water balance, blood volume and pressor activity. This response demonstrates integratory modulation of SFO neurons in feedforward and feedback

cardiovascular regulation. NTS fibers leading to SFO neurons that project to the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) are also involved in cardiovascular regulation via the baroreceptor reflex and fluid homeostasis (Dampney, 2016; Ferguson & Renaud, 1984; Miselis, 1981; Shioya & Tanaka, 1989). These fibers are suspected to also mediate autonomic response through regulation of vasopressin, and angiotensinergic neurons (Larsen & Mikkelsen, 1995; Li & Ferguson, 1993). More evidence has indicated that the autonomic pressor and depressor response is modulated through Ang II, Na^+, orexin-A and proinflammatory cytokine activation of the SFO (Fry & Ferguson, 2021; Larsen & Mikkelsen, 1995; Mangiapane & Simpson, 1980; Smith et al., 2007; Tiruneh et al., 2013; Wei et al., 2013), and further autonomic activity may be stimulated through oxytocinergic microglial populations within the SFO (Blackmore et al., 2018). These signals increase pressor activity and further activate regions of the brain involved in cardiovascular regulation and hemodynamics.

The renin-angiotensin-aldosterone system regulates hydro-mineral content and the cardiovascular system, and acts, in part, through the SFO to elicit physiological responses. Two key peptides involved in the physiology of this system, Ang II and renin, are found in the SFO (Lavoie et al., 2004a), and mediate physiological pressor responses and hydro-mineral balance (Coble et al., 2014). Extensive research shows that Ang II is not only a peripheral peptide detected by the SFO but is also a neurotransmitter used by its neurons (Li & Ferguson, 1993). Actions of Ang II have been investigated in detail, demonstrating that it influences activity of SFO neurons, eliciting release of oxytocin and vasopressin, increase dipsogenic behaviour; and affecting cardiovascular and hematic regulation (Ciriello, 2013; Collister & Hendel, 2005; Ferguson & Kasting, 1988; Fry & Ferguson, 2017, 2021; Mangiapane & Simpson, 1980; Roncari et al., 2014; Simpson & Routtenberg, 1973). Studies of the SFO transcriptome shows a high

abundance of Ang II receptors (AT) (Peterson et al., 2018), making it a key protein indicator of this region. For this reason, and based on the extensive electrophysiological literature investigating action of Ang II in the SFO, this study aims to determine if there is an overlap in neuronal populations responsive to both Ang II and NT.

Many SFO neurons are stimulated, or inhibited, through circulating factors to alter several physiological indicators of the cardiovascular and hematic state, such as blood pressure, blood and plasma osmolality, heart rate, and overall homeostasis (Anderson et al., 2000; Antunes-Rodrigues et al., 2004; Ciriello, 2013; Ciriello & Gutman, 1991; Dampney, 2016; Fry & Ferguson, 2017; Kuksis et al., 2014b). Insulin receptors, for example, modulate mean arterial pressure by affecting diastolic blood pressure through this CVO (Jeong et al., 2019). Smith and Ferguson (2012) demonstrated that the anorexigenic peptide leptin mediates SFO neurons in non-obese rats to regulate blood pressure through autonomic pathways. Cancelliere and Ferguson (2017) used patch-clamp electrophysiological techniques to investigate the influence of hyperglycemia on Ang II and cholecystokinin (CCK), classic cardiovascular and metabolic peptides. Subpopulations of SFO neurons depolarized when exposed to both CCK and Ang II (63%), depolarized only in response to Ang II (25%), or hyperpolarized only in response to CCK (12%) (Cancelliere & Ferguson, 2016). These excitatory responses to Ang II, and CCK inhibitory responses were attenuated in hyperglycemic environments (Cancelliere & Ferguson, 2016). Through detection of these peripheral factors and resulting modulation through their receptor populations, cardiovascular and hematic regulation are influenced by fluid homeostasis, suggesting the SFO is a key site for metabolic and cardiovascular integration.

Recent transcriptomic data suggests neurotensin receptors (NTSRs) are strongly expressed within the SFO, some with a greater abundance than AT_{1A} receptors (Peterson et al.,

2018), a known and prominent SFO receptor. Recent electrophysiological studies show that NT modulates electrical activity by depolarizing and increasing action potential frequency of dissociated SFO neurons (Peterson, 2019). This study will focus on the regulation of SFO neurons, in acutely prepared brain slices, by NT, a neuropeptide described in the section below.

1.5 Neurotensin

The endogenous peptide, neurotensin (NT), was serendipitously discovered by Caraway and Leeman (1973) during the isolation of a novel sialogenic peptide in the bovine hypothalamus. Since its discovery, NT has been known for its role in enteric motility and variable effects on blood pressure (Carraway & Leeman, 1973), working through both peripheral and central locations to elicit physiological responses. Further studies have shown NT to modify hormone release and other peptides involved in the pituitary, enteroendocrine, immune, cardiovascular and sympathetic systems (Allen & Cechetto, 1995; Grunddal et al., 2016; Kalafatakis & Triantafyllou, 2011; Rowe et al., 1997; Stolakis et al., 2010). NT and its receptors have also been implicated in nociception, pain, and dopamine pathways (Binder et al., 2001; Dobner, 2006; Kalafatakis & Triantafyllou, 2011; Smits et al., 2004), providing further evidence of its widespread influence, acting as both neuromodulator and hormone.

Localization studies on the rat brain indicated this neuropeptide to be prominently localized in the hypothalamus (35% of CNS NT) and brain stem (35% of CNS NT) in the CNS (10% of total NT); however, the digestive system harbours the highest concentration (~90% of total NT) (Atoji et al., 1995; Carraway & Leeman, 1976; Kanba et al., 1986; Smits et al., 2004; Uhl, 1982). Due to its presence throughout the CNS and periphery, and its cross-phyla distribution (Carraway et al., 1982), NT, and related peptides (see Friry et al., 2002 for additional

information), are biologically important peptides involved in several different functions which will be briefly explored below.

1.5.2 Neurotensin is a 13 amino-acid molecule that acts primarily through three known receptors

Evidence shows NT in the CNS is primarily released from the hypothalamus and in the periphery from intestinal endocrine cells (N cells) of the small intestine (Allen & Cechetto, 1995; Kitabgi, 2006; Kitabgi et al., 1990, 1992). Biosynthesis of NT varies based on location and involves the cleavage of an inactive pre-cursor complex via members of the pro-protein convertase family (Kitabgi, 2006). This processed peptide consists of 13 amino-acids, of which its C-terminal has been identified as the most biologically active site and the most conserved region of the translated pre-cursors sequence (Carraway et al., 1982; Kitabgi, 2006).

There are currently three known NTSRs; NTSR1-3 (Kalafatakis & Triantafyllou, 2011; Vincent et al., 1999). Two of these receptors, NTSR1 and NTSR2, belong to the G-protein coupled family; each with seven trans-membrane domains with a binding site localized between the sixth and seventh membrane spanning region at the third extracellular loop (Vincent et al., 1999). NTSR2 has a shorter N-terminal than NTSR1, a shorter intracellular loop, and is less sensitive to Na^{+} and Guanosine triphosphate (GTP), likely accounting for different binding interactions of each receptor type, with NTSR1 having stronger binding affinity than NTSR2. These G-protein coupled receptors (GPCRs) take action through mobilization of G-proteins such as Gαi1, Gαo, $G\alpha_{q}$, and $G_{\alpha 13}$, among others, to depolarize and hyperpolarize neurons, mobilize Ca^{2+}, increase extracellular-signal-regulated kinase (ERK) signaling, activate and suppress cAMP signaling, increase inositol triphosphate (IP), synthesize cellular proteins, and additional cellular effects (Ayala-Sarmiento et al., 2015; Besserer-Offroy et al., 2017; Hwang et al., 2010).

1-10μM of NT both increased (50%) and decreased (10%) firing rate of cells tested within the suprachiasmatic nucleus via NTSR1 and NTSR2 (Coogan et al., 2001), suggesting NT can produce differential effects within the same region of the CNS based on differential activation of receptor types. Hwang and colleagues (2010) demonstrated that NTSR2 may regulate NTSR1 activities and expression, contributing to the variety of effects elicited by their activation. The NTSR3 protein, also known as Sortilin, belongs to the vacuolar-protein-sorting-10 protein (VPS10P) family. This ligand-gated membrane receptor's extracellular VPS10P domain forms a bladed β-propeller in which its inner tube is thought to be the binding site for NT (Nykjaer & Willnow, 2012). Sortilin binds other neuropeptides and neurotrophic factors and acts with other channels and receptors in a variety of biological functions. Mechanisms of interaction with NT are not well understood; however, the VPS10P protein sorting function is inhibited when NT is bound to this receptor. Studies have demonstrated differential effects of NT through this protein, including the internalization of NTSR1 leading to inhibition of MAP kinase ERK and, in the absence of NTSR1 and NTSR2, its increase (Nykjaer & Willnow, 2012). The human sorting protein-related-receptor (SorLA), LR11, has been proposed as a fourth NTSR due to the peptide's binding affinity to this the receptor; however, it's binding may be due it's similarity to its VPS10P relative, Sortilin (Jacobsen et al., 2001; Tschumi & Beckstead, 2019).

Neurotensin receptors are found throughout the CNS and have a peripheral distribution which includes the gastrointestinal tract, heart, autonomic nervous system, enteric nervous system, peripheral nervous system, carotid body, pancreas, adipose tissue, skeletal muscle, thyroid gland, testes, placenta, crypt cells of the colon and cells of the immune system (Bloom & Polak, 1982; Grunddal et al., 2016; Kalafatakis & Triantafyllou, 2011; Lazarus et al., 1977). Compared to NTSR1 and NTSR3, NTSR2 seems to be most highly represented in the CNS; although, functional confirmation via antagonist and agonist application are limited. Data from

Peterson et al. (2018) show NTSR2 and NTSR3 to be strongly expressed within the SFO, suggesting NT may play an important modulatory role on neurons in this region. This study primarily aims to further characterize the electrophysiological effects of NT on SFO neurons.

1.5.3 Electrophysiological actions of neurotensin on selected neurons

Like other peptide neurotransmitters, NT release is Ca^{++} dependent. Several studies indicate that its release is inhibited in stimulated hypothalamic cells when superfused in Ca^{++} free solution (Iversen et al., 1978; Kitabgi et al., 1990). Interestingly, NT seems to mobilize Ca^{++}, both intracellular stores and extracellular concentrations, and affect other ion channels. A study conducted on NT induced mast cell histamine release suggests that intracellular calcium stores, more than extracellular, and cAMP, are involved in the mechanism of NT stimulation (Rossie & Miller, 1982). The role of Ca^{++} in NT release is further supported within dopaminergic cells; however, these studies suggest extracellular calcium to be a stronger mechanism of influence than to intracellular stores (St-Gelais et al., 2004). Furutani et al. (2013) demonstrated that the total inward current is potentiated in orexigenic cells of the hypothalamus following the application of NT, in the absence of extracellular calcium. This was likely due to the involvement of non-selective cation channels such as the transient receptor potential (TRP) channel (St-Gelais et al., 2004). Neurotensin effects on the TRP non-selective cation family has been postulated in several other studies due to the IP_3 and diacylglycerol (DAG) second messenger pathways activated by GPCRs.

Direct effects of NT on individual neurons suggest ionotropic mechanisms of action, specifically cation mobilization. Using outside-out patch-clamp electrophysiology in the ventral tegmental area, dopaminergic cell non-specific cations are shown to be mediated by NT (Chien et al., 1996). Particularly, this single channel study by Chien et al. (1996) suggests that NT

affects whole cell current via movement of Na^+ and Cs^+ (used in lieu of K^+) to excite neurons. Other studies in the ventral tegmental area show reduction in K^+ current in addition to an increase in Na^+ conductance often accompanied by increased resistance (Jiang et al., 1994). Peterson (2019), using current-clamp electrophysiology in whole cell configuration, found NT to depolarize cells and increase spontaneous potentials of dissociated neurons within the SFO. Data from voltage-clamp experiments suggest that these physiological effects are likely mediated through non-selective cation currents and delayed-rectifier K^+ (I_k) currents (Peterson, 2019). That study also demonstrated both increased and decreased action potential firing in response to NT (Peterson, 2019), suggesting multiple pathways of which NT modulates dissociated cells within the SFO. This study will examine if NT modulates synaptic activity in acutely prepared brain slices, further characterising synaptic activity in SFO neurons exposed to extracellular NT.

1.5.4 Neurotensin influences excitatory and inhibitory synaptic activity in the central nervous system

Numerous studies have demonstrated NT modulates the membrane potential and synaptic activity of neurons. For example, NT is thought to influence NMDA-glutamate transmission in the cortex, likely acting through NTSR1-NMDA coupling (Ferraro et al., 2011). Periaqueductal grey neurons are depolarized by NT to inhibit GABA release, (Mitchell et al., 2009); whereas cells in the substantia nigra respond to NT with heterogeneous effects, acting pre-synaptically on NTSR1s to increase $GABA_A$ currents, and post-synaptically through NTSR2s to decrease $GABA_B$ currents (Tschumi & Beckstead, 2019). NT application to amygdala neurons show it plays a role in modulation of evoked GABA IPSCs, influencing signaling to regions involved in feeding behaviours and chronic stress (Normandeau et al., 2018). Its influence on these major

neurotransmitters of the CNS suggest NT may modulate several regulatory pathways through central modes of action.

1.5.5 Neurotensin mediates several regulatory functions

NT is involved in a variety of homeostatic processes. For example, studies suggest NT has a modulatory role in the dopaminergic-serotonin system that suggest its involvement in the circadian regulatory processes, motor control, reward and learning behaviour and other physiological behaviours influenced by central dopaminergic systems (Govoni et al., 1980; Smits et al., 2004; Tschumi & Beckstead, 2019; Uhl & Kuhar, 1984). Literature shows that, NT released from neurons of the arcuate nucleus, paraventricular nucleus, medial preoptic area, median eminence and other hypothalamic regions, modulates pituitary hormone release to influence reproduction, development, and regulation (Stolakis et al., 2010), prolactin release, and thyroid-stimulating hormone release (Maeda & Frohman, 1978; Stolakis et al., 2010; Vijayan et al., 1988). Abnormal concentrations of NT and its precursor molecules (both elevated and lowered concentrations respectively) have been linked to inflammation of adipose tissue via increased expression of immune factors (Barchetta et al., 2018) and are thought to be associated with type 2 diabetes, non-alcoholic fatty liver disease (Auguet et al., 2018); although, its role is not yet understood.

Neurotensin effects on hyperglycemia, glucagon release and impairment of insulin response to hyperglycemia, and antinociceptive effects are completely or partially blocked by histamine in immune response and associated diseases(Nagai & Frohman, 1978). Neurotensin has been found in primary afferents associated with nociceptive pathways and is upregulated in regions associated with neuropathic pain to mediate the effects of opioids (Difiglia et al., 1984; Dobner, 2006; Vachon et al., 2004). Analgesic effects of NT independent of opioids are thought

to be mediated by NT and NTSR2, in descending pain pathways (Wang et al., 2014). Additionally, NTSR1 and NTSR2 have been linked to antinociception in hot-plate and acid-induced writhing tests (Dobner, 2006). The presence of NT in fibers involved in nociceptive signaling, NTSR localization within nociceptive pathways, and behavioural responses observed in the absence or presence of these receptors, or the peptide itself, suggest that NT plays a role in signaling pain. Well-described roles of NT in energy balance and cardiovascular regulation are important to this study due intersection with known roles of the SFO; thus, they will be outlined in more detail in the following sections.

1.5.6 Neurotensin interacts with pathways mediating energy balance and metabolism

The focus of this study is NT in the central nervous system; however, peripheral NT has been extensively studied, and is an important signaling peptide which will be briefly mentioned in this section. The application of NT has been shown to result in physiological changes in the digestive system including ileum contraction (guinea pig), relaxation of the duodenum (rat), inhibition of gastric secretion, mild stimulation of pancreatic bicarbonate, stimulation of fatty acid absorption and decreases in AMP activated protein kinase in enteric cells (Bloom & Polak, 1982; Carraway & Leeman, 1973; Grunddal et al., 2016; Li et al., 2016). Release of NT from the pancreas is thought to be insulin responsive; thus, may have a physiological role in pancreatic hormone regulation through islet and beta-cell secretion. In a study conducted by Berelowitz and Frohman (1982), insulin deficient mice, but not insulin resistant mice, were found to have increased pancreatic NT. Further studies indicated that pancreatic NT (0.1μM) stimulates insulin secretion in at low glucose levels (2mM) and inhibits its release in high glucose levels (20mM) in both beta and islet cells. This activity is thought to be mediated by NTSR2 and 3 influence on

calcium influx via protein Kinase A and C pathways in pancreatic beta-cells (Béraud-Dufour et al., 2010).

Literature suggests that NT is co-expressed with central (Brown et al., 2018; Furutani et al., 2013) and peripheral peptides (Grunddal et al., 2016) involved in feeding and digestion. As levels of peripheral NT rise following feeding (Bloom & Polak, 1982), co-expression of NT with glucagon-like peptide- 1, peptide YY, and other enteroendocrine hormones can result in synergistic effects, including an enhanced decrease in feeding behaviour and potentially reversing obesity (Grunddal et al., 2016; Ratner et al., 2019). The release of peripheral NT is thought to occur following the hydrolysis of fat where the resulting long-chain fatty acids (C18), but not medium (C8), triggers its release into circulation (Drewe et al., 2008; Rosell, 1982).

Several studies have shown neurotensinergic neurons expressing leptin-receptors negate lateral hypothalamic orexin neurons (Brown et al., 2018; Leinninger et al., 2011). Anorexigenic effects of the leptin hormone are heightened in NTSR1 knockout mice, suggesting that NT contributes to the regulation of energy balance and homeostasis, countering effects of leptin signaling (Brown et al., 2018; Izaguirre et al., 2016; Opland et al., 2013). Furutani et al. (2013) used immunostaining to demonstrate co-expression of NT and orexin in lateral hypothalamic neurons and demonstrated that there are connections from other NT and orexin releasing neurons within the lateral hypothalamic area (LHA). This suggests that NT may play a role in autoregulation of itself and orexin to regulate feeding.

Neurotensin is thought to regulate other mechanisms of feeding behaviour. For example, animals treated with NT show decreased motivated feeding (centrally administered) and increased drinking (systemically administered) (Stanley et al., 1983). NTSR1 ablation and knockout studies suggest alteration of NT signaling may promote increased selectivity to alcohol

consumption, over water and over palatable foods (Lee et al., 2011; Torruella-Suárez & McElligott, 2020).

1.5.7 Neurotensin's role in cardiovascular regulation

There is significant evidence of NTs role in cardiovascular and sympathetic regulation. Studies demonstrate that plasma NT levels correlate with hypertension, ventricular hypertrophy and CVD related dysfunction; specifically, lower levels of NT are found in individuals with hypertension, with concentrations found to be inversely correlated with systolic and diastolic blood pressure (Bolat et al., 2020; Fawad et al., 2018; Guo et al., 2005; Osadchii, 2015). Interestingly studies report conflicting results following acute administration of NT. Early studies using the rat demonstrated a fall in blood pressure accompanied by vasodilation and cyanosis following the vascular administration of NT (Carraway & Leeman, 1973). Further experiments have demonstrated that NT has hypertensive effects in the guinea-pig (Bachelard et al., 1987) and more recent studies using the rat reported bi- or tri- phasic effects on blood pressure in response to systemic NT (Kaczyńska & Szereda-Przestaszewska, 2012; Oishi et al., 1981; Rioux et al., 1982). These cardiovascular effects are partially due to mediation of catecholamine and histamine release by NT (Oishi et al., 1981; Rioux et al., 1982). In addition to various effects on blood pressure, systemic NT has been shown to increase heart rate; effect myocardial contractility; increase vasocontraction in the rat portal vein and human umbilical vein; increase coronary vascular constriction in rats and dilation in guinea-pigs; reduce blood flow of gastric mucosa in humans and increases intestinal blood flow in the rat, cat, and dog; and following feeding, NT increase results in vasoconstriction within adipose tissue (Rosell, 1982), among other cardiovascular and hematic effects (for additional review, see Osadchii, 2015). The

homeostatic effects influencing hemodynamics and the cardiovascular system in these studies demonstrate that peripheral NT modulates cardiovascular activity and sympathetic output.

NT is involved in major cardiovascular regulatory pathways of the CNS. Hypothalamic NT, stimulated via the insular cortex, enhances excitatory response and increases spontaneous firing of lateral hypothalamic cells, projecting to pressor/depressor cells, leading to decreased blood pressure and sympathetic nerve response in rats (Allen & Cechetto, 1995; Kalafatakis & Triantafyllou, 2011). Spinal injections and microinjections of NT to the nucleus of the solitary tract have been shown modulate and increase sensitivity to the sympathetic baroreceptor reflex, (Kubo & Kihara, 1990; Osadchii, 2015). These findings are in-line with other studies indicating the role of central NT in cardiovascular regulation.

1.6 Intersection of neurotensin and the subfornical organ in homeostasis

Mapping of NT expressing neurons show afferent and efferent projections (Table 1.3) with control and coordination centres of the CNS (Allen & Cechetto, 1995; Difiglia et al., 1984; Dobner, 2006; Izaguirre et al., 2016; Opland et al., 2013; Wang et al., 2014), many of which were indicated when characterizing the SFO. Evidence from mapping NT and its receptors show this peptide to be present in regions suspected to project to the SFO (Table 1.1). Reflecting on the above literature, both NT and the SFO are involved in cardiovascular and hematic regulation, ingestive behaviours and hydromineral balance, among other homeostatic functions. For example, the SFO receives afferent projections from the nucleus of the solitary tract, thought to be involved in relay of information from the baroreceptor reflex (Dampney, 2016; Shioya & Tanaka, 1989). Interestingly, using radioligand experiments and immunohistochemistry, the nucleus of the solidary tract has been found to contain binding sites for NT and is immunoreactive to this peptide (Higgins et al., 1984; Jennes et al., 1982; Quirion et al., 1982).

Further investigation using microinjection into the rat nucleus of the solitary tract have demonstrated that NT contributes to vasodepressor response and bradycardia response, via vagal excitation and sympatho-inhibition, in the caudal nucleus of the solitary tract; providing evidence that the SFO and NT are involved in the baroreceptor response through the nucleus of the solidary tract (Ciriello & Zhang, 1997). SFO neurons project axons to the hypothalamic SON and PVN, increasing sympathetic activity and modulating the pituitary through vasopressin release (Rowe et al., 1997). Neurotensin in the SON increases the firing rate of oxytocin and vasopressin neurons in vitro and is also implicated in modulation of the pituitary. Immunohistochemical studies demonstrated NT or fibers containing NT exist in the SON, PVN as well as several regions of the brain with afferent projections to the SFO, including regions of the lamina terminalis, NTS, bed nucleus of the stria terminalis, and the medial preoptic area. A study conducted by Rosas-Arellano et al. (1996) demonstrated projections from the arcuate nucleus displaying immunoreactivity for NT projected to regions of the SFO, suggesting that, in addition to evidence of physiological overlap, NT potentially interacts directly with the SFO.

1.6.1 Aims of this thesis

The physiological overlap of NT and SFO roles in homeostasis, presence of circulating NT which could potentially interact with the SFO, and evidence of NT projections directly to the SFO suggests that NT may modulate activity of SFO neurons. Initial studies, as mentioned, demonstrated that NTSRs are present within the SFO, and NT elicits physiological responses in dissociated neurons in the SFO. In this study, we aimed to characterise mechanisms in which NT modulates SFO neurons from coronal brain slices that maintain some local synaptic connectivity. The overall objective is to further define signaling pathways and ionic mechanisms of

neurotensin regulation of SFO neurons. To achieve this, the following two hypotheses will be addressed:

Hypothesis 1: A subpopulation of SFO neurons directly respond to neurotensin.

Fibers from the arcuate nucleus that are immunoreactive for NT project to the SFO, suggesting a pathway for endogenous NT to directly interact with the SFO (Rosas-Arellano et al., 1996). Due to this information and more recent evidence that dissociated SFO neurons respond to NT (Peterson, 2019); we hypothesize that NT applied to SFO neurons in slice preparation will elicit physiological responses directly on those neurons. Specifically, we expect that application of NT will show both an increase and decrease in neuronal excitability, both depolarizing and hyperpolarizing the membrane of SFO neurons in tissue. Voltage-clamp protocols will be used to confirm previous findings, that NT modulates excitability of SFO neurons, and to provide information about electrical behaviour before and after NT, including input resistance, which will reveal channel activity. Using both voltage clamp and current clamp experiments, Ang II will be applied following NT wash-out to determine if NT and Ang II modulate the same neuronal populations within the SFO and provide insight as to possible NT pathways of effect. Significant electrophysiological changes ($p \leq 0.05$) observed after the application of NT and not Ang II, or after both NT and Ang II, we can reject the null hypothesis that there are no subpopulations of SFO neurons that respond to NT.

Hypothesis 2: Neurotensin regulates synaptic inputs on SFO neurons.

Previous studies from other regions of the brain show that synaptic activity of NT involves modulation of post synaptic current amplitude (Ogawa et al., 2005; Tschumi & Beckstead, 2019) and frequency(Ogawa et al., 2005). Recent work has identified the potential of the SFO to detect and respond to neurotensin (Peterson, 2019). To investigate possible synaptic

modulation of SFO neurons, NT and a neurotensin receptor antagonist will be applied to patch clamped SFO neurons in acute brain slice preparations. Voltage-clamp protocols will be used to measure excitatory post-synaptic currents (EPSCs), examine the complexity of NT signaling, and possibly reveal neuromodulation not previously observed in studies on dissociated neurons (Peterson, 2019). Neurotensin is reported to increase both GABA and glutamate release (Tschumi & Beckstead, 2019; Zhang et al., 2015); therefore, it is expected that we will observe both increase and decrease in excitatory currents. Significant changes ($p \leq 0.05$) in frequency, amplitude, and shape will provide evidence to reject the null hypothesis, that there is no difference in EPSCs before and after the application of NT.

Table 1.3. Neurotensin has been found in neurons and fibers throughout the central nervous system.

Location	Study type & methods used	Neuron, Fiber, Unspecified	Model
Perifornical area of the hypothalamus	immunohistochemistry[1,2] retrograde labeling from LHA[1]	N,	Rats[1], Dog[2]
Central amygdaloid nuclei	immunohistochemistry[1,2] retrograde labeling from LHA[1]	N, F, U	Rats[1,]
Cortical amygdaloid nuclei	immunohistochemistry2,	N, F	Dog[2]
Medial amygdaloid nuclei	immunohistochemistry[2]	N, F	Dog[2]
Bed nucleus of the stria terminalis	immunohistochemistry[1,2] retrograde labeling from LHA[1] fluorescent in-situ hybridization[13]	N, F	Rats[1], Dog[2,] Mice[13]
Medial preoptic area	immunohistochemistry[1,2] retrograde labeling from LHA[1]	N, F	Rats[1], Dog[2]
Paraventricular nucleus	immunohistochemistry[1,2] retrograde labeling from LHA[1]	N, F	Rats[1], Dog[2]
Periventricular nucleus	immunohistochemistry[1]	N,	Rats[1],
Raphe nuclei	immunohistochemistry[1,2]	N, F	Rats[1],
Nucleus of the solitary tract	immunohistochemistry[1,2] retrograde labeling from LHA[1]	N,	Rats[1], Dog[2]
Nucleus accumbens	immunohistochemistry[1,2] radioimmunoassay[11]	N, F, U	Rats[1, 11],
Lateral septal nucleus	immunohistochemistry[1,2] retrograde labeling from LHA[1]	N, F	Rats[1], Dog[2]
Olfactory Bulb	immunohistochemistry[1,2] retrograde labeling from LHA[1]	N, F	Rats[1], Dog[2]
Olfactory nucleus	immunohistochemistry[2]	N, F	Dog[2]
Olfactory tubercle	immunohistochemistry[2]	N, F	Dog[2]
Diagonal band of Broca	immunohistochemistry[2] retrograde labeling from LHA[12]	N, F	Dog2, Mice[12]
Medial septal nucleus	immunohistochemistry[2]	N, F	Dog[2]

Lateral preoptic nucleus	immunohistochemistry[2] retrograde labeling from LHA[12]	N, F	Dog2, Mice[12]
Piriform lobe	immunohistochemistry[2]	N, F	Dog[2]
Entorhinal cortex	immunohistochemistry[2]	N,	Dog[2]
Anterior commissure	immunohistochemistry[2]	N, F	Dog[2]
Alveus	immunohistochemistry[2]	F	Dog[2]
Fimbria	immunohistochemistry[2]	F	Dog[2]
Subiculum	immunohistochemistry[2]	N,	Dog[2]
Substantia innominata	immunohistochemistry[2] retrograde labeling from LHA[12]	N, F	Dog2, Mice[12]
Globus Pallidus	immunohistochemistry[2] radioimmunoassay[11]	N, F, U	Dog[2], Rat[11]
Caudate nucleus	immunohistochemistry[2]	N, F	Dog[2]
Zona Incerta	immunohistochemistry[2]	N, F	Dog[2]
Thalamic tubercle	immunohistochemistry[2]	N,	Dog[2]
Median eminence	immunohistochemistry[2]	F,	Dog[2]
Nucleus reuniens	immunohistochemistry[2]	N,	Dog[2]
Nuclei of the pulvinar	immunohistochemistry[2]	N,	Dog[2]
Lateral dorsal nucleus	immunohistochemistry[2]	N, F	Dog[2]
Supraoptic nucleus	immunohistochemistry[2]	N, F	Dog[2]
Hypothalamic dorsomedial nucleus	immunohistochemistry[2]	N, F	Dog[2]
Ventromedial nucleus of the hypothalamus	immunohistochemistry[2]	N,	Dog[2]
Lateral Hypothalamic Area	immunohistochemistry[1,2,5,6,8,12] retrograde labeling from LHA[1,6,7]	N, F	Rats[1,], Dog[2], Mice[5,6,7,8,12]
Periventricular nucleus	immunohistochemistry[1,2] retrograde labeling from LHA[1]	N, F	Rats[1], Dog[2]
Posterior hypothalamic area	immunohistochemistry[2]	F	Dog[2]
Anterior hypothalamic area	immunohistochemistry[2]	N,	Dog[2]

Arcuate nucleus	immunohistochemistry[1,2,6] retrograde labeling from LHA[1] immunofluorescence[6]	N,	Rats[1], Dog[2,], Mice[6,]
Supramammillary nucleus	immunohistochemistry[2]	N,	Dog[2]
Lateral habenular nucleus	immunohistochemistry[2]	N, F	Dog[2]
Subthalamic nucleus	immunohistochemistry[2] retrograde labeling from LHA[12]	F	Dog[2], Mice[12]
Ventral tegmental decussation	immunohistochemistry[2]	N, F	Dog[2]
Caudal colliculus	immunohistochemistry[2]	N,	Dog[2]
Superior colliculus	immunohistochemistry[2]	F,	Dog[2]
Rostral colliculus	immunohistochemistry[2]	N,	Dog[2]
Periaqueductal gray	immunohistochemistry[2] retrograde labeling from LHA[12]	N, F	Dog2, Mice[12]
Ventral Tegmental Area	immunohistochemistry[2] retrograde labeling from LHA[4,6,7] immunofluorescence[6,7] radioimmunoassay[11]	N, F, U	Dog[2], Mice[4,6,7], Rat [11]
Interpeduncular nucleus	immunohistochemistry[2]	N, F	Dog[2]
Cuneiform nucleus	immunohistochemistry[2]	N, F	Dog[2]
Locus coeruleus	immunohistochemistry[2] retrograde labeling from LHA[12]	N, F	Dog[2,] Mice[12]
Parabrachial nuclei	immunohistochemistry[2] retrograde labeling from LHA[12]	N, F	Dog[2], Mice[12]
Reticular formation	immunohistochemistry[2]	F,	Dog[2]
Nucleus profundus mesencephali	immunohistochemistry[2]	N,	Dog[2]
Substantia grisea centralis pontis	immunohistochemistry[2]	F,	Dog[2]
Gigantocellular reticular nucleus of the medulla	immunohistochemistry[2]	N, F	Dog[2]
Nucleus reticularis pontis caudalis	immunohistochemistry[2]	N, F	Dog[2]
Nucleus reticularis parvocellularis	immunohistochemistry[2]	N, F	Dog[2]

Nucleus reticularis ventralis	immunohistochemistry[2]	N, F	Dog[2]
Pars caudalis	immunohistochemistry[2]	N, F	Dog[2]
Pars oralis	immunohistochemistry[2]	F,	Dog[2]
Pars interpolaris	immunohistochemistry[2]	F,	Dog[2]
Dorsal nucleus of the vagus nerve	immunohistochemistry[2]	N, F	Dog[2]
Nucleus olivaris	immunohistochemistry[2]	F	Dog[2]
Hypothalamus unspecified	radioimmunoassay[3,9, 11] chromatography[10] electrophoresis[10]	U	Rat[3,9,11], Bovine[10]
Substantia Nigra	immunohistochemistry [6,7] retrograde labeling from LHA[4,7] radioimmunoassay [11]	N, F	Mice[4,6,7], Rat[11]
Pituitary unspecified	radioimmunoassay[9]	U	Rat[9]
Thalamus unspecified	radioimmunoassay[9]	U	Rat[9]
Brains stem unspecified	radioimmunoassay[9]	U	Rat[9]
Cortex unspecified	radioimmunoassay[9,11]	U	Rat[9, 11]
Cerebellum unspecified	radioimmunoassay[9]	U	Rat[9]
Striatum	radioimmunoassay[11]	U	Rat[11]
Septum	radioimmunoassay[11]	U	Rat[11]
Mammillary body	radioimmunoassay[11]	U	Rat[11]
Amygdala unspecified	radioimmunoassay[11]	U	Rat[11]
Hippocampus unspecified	radioimmunoassay[11]	U	Rat[11]
Retrorubral region	retrograde labeling from LHA[12]	F	Mice[12]
Ventral pallidum	retrograde labeling from LHA[12]	F	Mice[12]
Raphae pallidus	retrograde labeling from LHA[12]	F	Mice[12]
Parapyramidal region	retrograde labeling from LHA[12]	F	Mice[12]
Authors			
1. Allen & Cechetto, 1995; 2. Atoji et al., 2995; 3. Berelowitz & Frohman, 1982; 4. Brown et al., 2019; 5. Brown et al., 2018; 6. Leinninger et al., 2011; 7. Opland et al., 2013; 8. Brown et al., 2017; 9. Carraway & Leeman, 1976; 10. Carraway & Leeman, 1973; 11. Kitabgi et al., 1991; 12. Naganuma et al., 2019; 13. Normandeau et al., 2018			

Methods

2.1. Animals and dissection

Animals and protocols were used in accordance with the guidelines from the Canadian Council for Animal Care and approved by the University of Manitoba Central Animal Care Committee, prior to commencing experiments. Male Sprague Dawley Rats ~200g were sacrificed by decapitation, their brains extracted and placed in a cold bath of oxygenated, artificial cerebrospinal fluid (aCSF) (composition in mM: 126 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, and 11 D-glucose, pH 7.3-7.4, 295 mOsm) (Kinsman et al., 2020). All salts were obtained from Sigma-Aldrich or Thermo Fisher Scientific (Oakville, On). After two minutes in the cold bath, the brain was placed in a rodent brain matrix and a 3-4mm coronal section was removed between the optic chiasm and mammillary body (Figure 2.1). This 3mm section was glued to the specimen disk of a Lecia VT1000S vibratome (Concord, On), anterior side of the slice facing upward and held in-place with three blocks of agarose. Ice cold oxygenated aCSF was then placed into the buffer tray on the vibratome, and the specimen disk inserted into the tray and locked in place. A series of 300µm coronal brain slices were cut using disposable Derby Premium stainless-steel blades, transferring each slice into oxygenated Krebs aCSF at 37 ˚C for at least 90 minutes.

2.2 Electrophysiology

All electrophysiological recordings were performed within eight hours of the tissue dissection. Room temperature, oxygenated Krebs buffer aCSF, as mentioned above, was used as external recording solution and coronal slices were held in-place using a harp slice grid. Extracellular recording solution and treatments were flowed through the recording chamber at 2mL/min using a peristaltic pump. Standard Intracellular Solution was composed of (in mM):

128 K-gluconate, 10 KCl, 0.3 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 1.0 EGTA, 4 MgATP, 4 Na_2 phosphocreatine, 0.3 NaGTP (Kinsman et al., 2020) was used for whole-cell recordings. Patch electrodes were fabricated from thick wall borosilicate capillary tubes (BF-150-110-10HP; Sutter Instruments) to an initial resistance between 4-6MΩ. Data was collected using a HEKA EPC10 patch clamp amplifier and Patchmaster 2.53 software (Mahone Bay, Nova Scotia, Canada) and cells observed with the DAGE-MIT IR-1000 camera attached to a fixed stage microscope at 400x magnification (BX51WI, Olympus), equipped with IR-DIC (Figure 2.2).

2.2.1 Changes in membrane potential and interevent interval were recorded in current clamp

Baseline current clamp recordings from cells that formed a seal above 1GΩ and achieved whole-cell configuration were obtained while the cell membrane stabilized. Neurons were held at a current that maintained the membrane potential near physiological conditions between -60 to -70mV. A 200-millisecond hyperpolarizing current (-5 or -10pA), followed by a 200-millisecond depolarizing current (5 or 10pA), was elicited every 20-seconds during recording. Test solution was added after at least 3-minutes of baseline recordings. The test solution (1μM NT; Tocris, 1909) was washed over the tissue (~2min), and membrane potential was recorded. For cells also tested for Ang II sensitivity, at least 10 minutes of washout lapsed prior to repeating the protocol as outlined above with an Ang II (100nM; Tocris, 1158) test solution.

Reversal potential of effect and input resistance were calculated to identify possible ion channels involved in observed electrical effects. Membrane activity was observed using 5-10pA current steps from before (control) and after treatment. A voltage-current plot showing the relationship between the voltage and current was used to determine the input resistance (slope) and reversal potential (intersection point). Cells were considered responsive if changed by 5mV when comparing control (aCSF) to treatment.

2.2.2 Properties of spontaneous excitatory post synaptic currents were obtained in voltage clamp configuration

SFO neurons were identified, and patch clamped. Only cells with stable membrane potentials were used for analysis. Neurons were held at -60mV holding potential during recordings. To determine synaptic effects of NT on SFO neurons, excitatory post synaptic current (EPSC) amplitude and interevent interval were recorded from cells during control (aCSF) conditions and after treatment (1μM NT). After 4-minutes of baseline recordings, test solution (1μM NT) was administered into the bath for 2-minutes. A minimum of 10-minutes of washout was again provided for cells that were also tested for Ang II sensitivity.

The NTSR1 and NTSR2 non-selective antagonist, SR142948 (Tocris; 2309), was used to determine if the observed effects were due to an interaction with a NT receptor. Cells were again held at -60 mV throughout. To determine any effects elicited by the SR142948, at least 4-minutes of baseline EPSCs were recorded prior introducing the antagonist solution (1μM SR142948). Saturation of the antagonist occurred for at least 4-minutes prior to adding equimolar concentrations of NT to the bath (1μM SR142948:1μM NT). In all experiments, control (aCSF), antagonist (1μM SR 142948), and treatment (1μM NT and SR142948) were compared.

2.2.3 Statistics

OriginLab 2018 software was used to conduct all statistical analysis. Mean membrane potentials were compared from current clamp experiments from the control and after treatment. Cells were considered responsive if they displayed a change in resting membrane potential by 5mV from the baseline mean following treatment (1μM NT or 100nM Ang II). A Wilcoxon Signed Ranks Test ($p \leq 0.05$) was used to determine significance between control and treated

groups. Post-hoc analysis using a Kruskal-Wallis ANOVA ($p \leq 0.05$) were used to test the difference among different groups based on affects observed. Input resistance was calculated from the slope of the voltage-current plot in which the control and treatments were compared. Changes to membrane potential and input resistance were plotted and a Linear Fit test ($p \leq 0.05$) was conducted to determine the reversal potential of effect.

EPSC amplitude and interevent interval were analyzed from voltage clamp experiments using the Kolmogorov-Smirnov non-parametric test to determine if there were significant changes ($p \leq 0.05$) in parameters (EPSC interevent interval, amplitude) of individual cells during control (aCSF) and after treatment (1µM NT or 100nM Ang II). Event distributions were visualized with cumulative histograms comparing control and treatment (NT). A Wilcoxon Signed Ranks Test ($p \leq 0.05$) was conducted to determine significant changes in population EPSC amplitude and interevent interval from control (aCSF) and treatment (NT).

A Chi Square test for significance was used to measure the difference in proportion of neurons responsive to NT in the presence and absence of antagonist. Differences between the treatment (1µM NT) and control (aCSF) were compared using the Kolmogorov-Smirnov non-parametric test both with and without the NTSR antagonist SR142948.

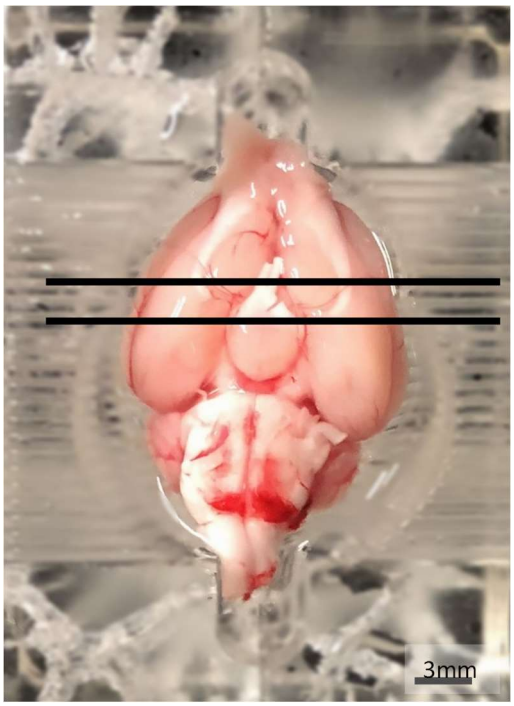

Figure 2.1. Representative Sprague Dawley rat brain, seated with ventral side up, in a brain matrix. Two vertical lines on this image identify the section cut to obtain a 3-4mm coronal brain slice between the Optic Chiasm and Mammillary body. These sections were further cut into 300µm slices to conduct patch-clamp electrophysiology of the SFO.

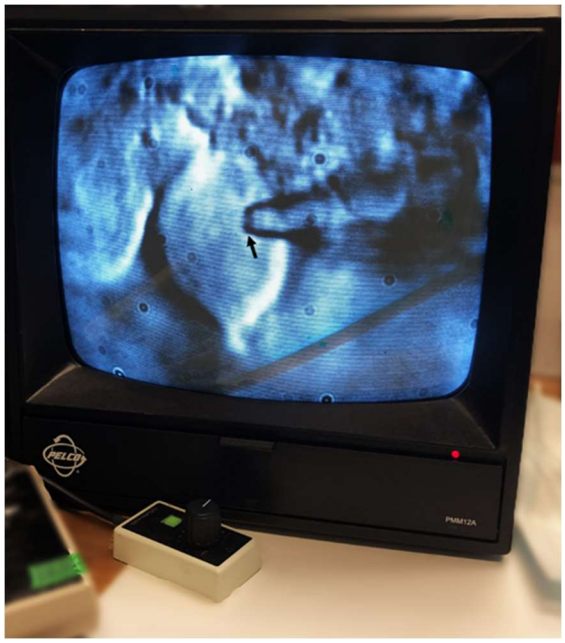

Figure 2.2. SFO neurons were observed using 400x magnification. Each cell was projected through a microscopy camera projected to a monochrome closed-circuit video monitor. The representative image above shows a microelectrode (arrow) approaching a SFO neuron prior to achieving whole-cell patch clamp for recordings.

Results

In order to determine if NT modulated activity of neurons in acutely prepared brain slices, whole-cell patch clamp physiology was used to determine direct and synaptic effects of the peptide using current clamp and voltage clamp techniques, respectively. As Ang II shows consistent modulatory effects on SFO neurons (Ferguson & Li, 1996; Li & Ferguson, 1993), we also conducted initial investigations into any possible correlation in the sensitivity of cells to both peptides.

3.1 Neurotensin directly affects electrical activity of subfornical organ neurons

We first carried out recording in current clamp configuration to determine if NT modulated electrical activity of SFO neurons. Resting membrane potential and action potential activity was observed before and after peptides were washed over the tissue for 60-seconds to allow saturation. This time was determined based on initial experiments with a high potassium (+5mM) solution which we calculated latency of effect between the time we observed the treatment solution entered to the bath and when we first observed an effect on the membrane potential (Figure 3.1). In this preliminary experiment, we observed a 40-second latency of effect after the test solution (7.5mM KCl) entered the bath, suggesting that at least 40-seconds would be required for NT to affect the neuron in a way that made a physiologically detectable change.

Consistent with previous studies (Peterson, 2019; 54.2%), 48.5% of cells (16/33) responded to NT with a change in mean resting membrane potential. Cells responded in three ways when comparing aCSF controls and treatments with NT (Figure 3.2). The resting membrane potential of tested cells either depolarized (n=12), hyperpolarized (n=4), or had no significant change (n=17) from baseline conditions, following treatment (Figure 3.3). This data shows that NT significantly changes the resting membrane potential in SFO neurons from

control (Wilcoxon Signed Ranks Test; p =0.04). Specifically, there is a significant difference between the population of neurons that hyperpolarized and depolarized (Kruskal-Wallis ANOVA; p=<0.0001) and the population that depolarized and showed no change from baseline (Kruskal-Wallis ANOVA; $p=2.09x10^{-4}$); however, there was no significant difference between the cells which hyperpolarized and showed no change from baseline (Kruskal-Wallis ANOVA; p=0.15).

Having confirmed SFO neuron sensitivity to NT, further analysis was conducted to gain insight as to possible ionic mechanisms of response. Voltage-current plots were used to determine the input resistance from the slope of the best fit line of each cell under control and treatment conditions. The reversal potential of effect was determined by the intersect of the two fit lines for each individual cell (Figure 3.4). Together, this information on the voltage-current relationship and reversal potential of effect can assist in indicating the identity of channels modulated, which lead to the change in input resistance.

As previously reported (Peterson, 2019), cells either exhibited no significant change (n=17) or responded with an increase (n=9) or decrease (n=7) in input resistance after being treated with NT. At the population level, we observed no significant change in input resistance, and there appeared to be no significant differences in the input resistance between the groups of cells which hyperpolarized, exhibited no change, or depolarized in response to NT (Kruskal-Wallis ANOVA; p≤5). By plotting the change in mean input resistance against change in mean membrane potential and observing the best fit line, we observed no significant linear correlation between the two parameters (Pearson Correlation; r=-0.33, p=0.05882) (Figure 3.4 C). Interestingly, when observing change in input resistance against types of responses we found that cells which hyperpolarized had an increase in median input resistance (Q1=0.09, Q3=0.56) and

the mean reversal potential of effect of these cells either averaged at -68.5mV (n=2) or -43.5mV (n=2); suggesting possible closure of potassium channels or cation leakage channels respectively. Of the cells which depolarized, the mean reversal potential of effect for cells which decreased or increased in input resistance was near that of potassium (-87.9mV; n=5) or within the range observed with non-selective cation channels (-51.1mV; n=5). The broad variance in input resistance suggests that there may be both direct and synaptic mechanisms of NT modulation on SFO neurons.

In order to determine if neurons that were sensitive to NT were also sensitive to Ang II, a key hormone for the regulation of water and salt balance, vascular tone, and sympathetic output, we first tested cell sensitivity to NT. Fifteen SFO neurons that were tested for NT sensitivity were also tested for sensitivity to Ang II in a similar manner. Eight neurons of fifteen tested showed no significant change in membrane potential to either peptide, whereas others responded to both (n=2), NT only (n=3), or Ang II only (n=2) (Table 3.1). Thes data indicate that while there is some overlap of cells, in that they respond to both NT and Ang II, they are not mutually inclusive or exclusive subpopulations.

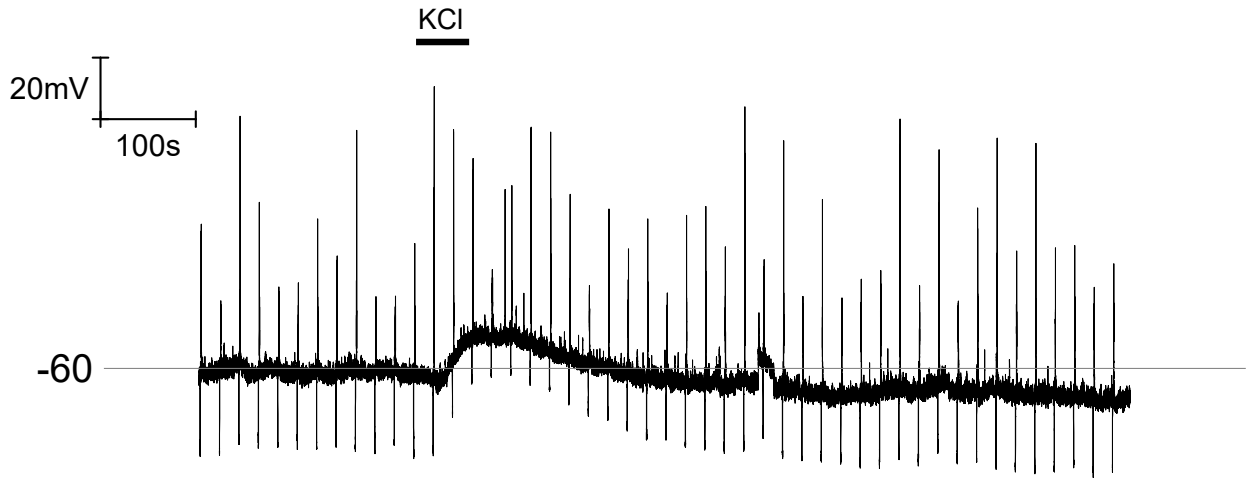

Figure 3.1. Latency to effect was calculated for experiments using KCl. A 7.5mM KCl solution, at the time indicated (black bar), was added to the bath and allowed to permeate the tissue. From the representative SFO recording, the KCl solution entered the bath at 240s (as determined by the movement of an air bubble). The effect was observed at 260s. Mean latency for KCl experiments was 40s; therefore, analysis of subsequent experiments were based on this.

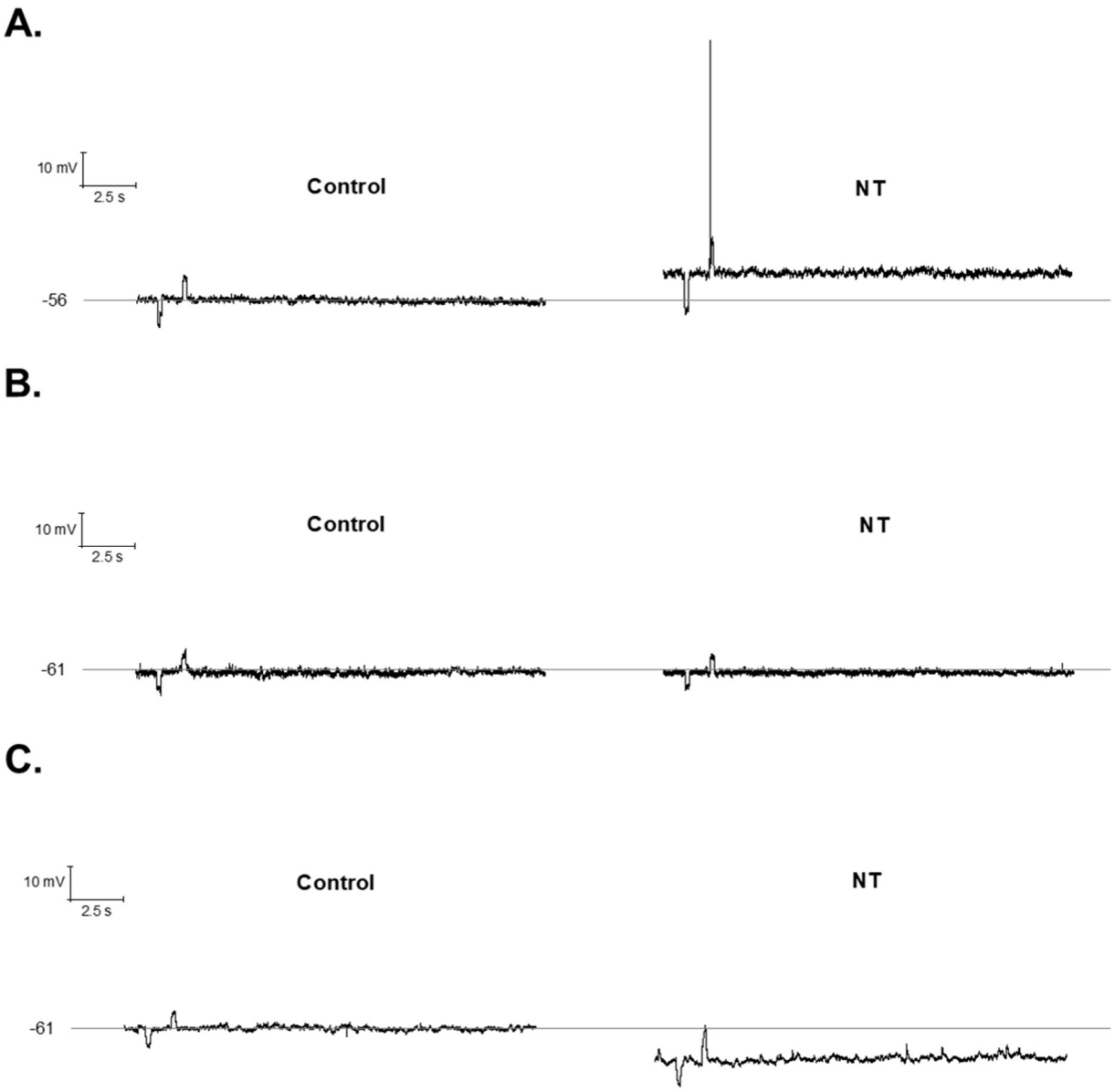

Figure 3.2. The electrical activity of SFO neurons is modulated by 1 μM NT. Representative recordings above from SFO neurons show that the application of 1μM NT (3mL) either resulted in a depolarization (A), no change (B), or hyperpolarization (C) of the cell membrane.

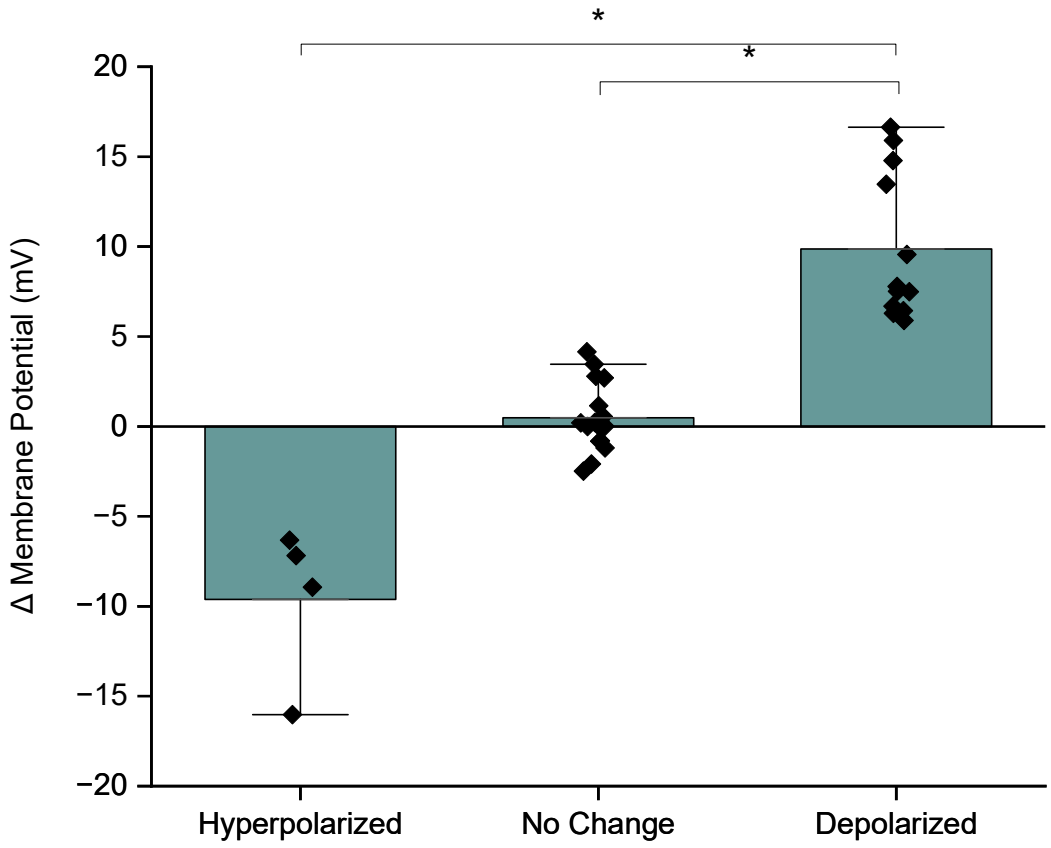

Figure 3.3. The membrane potential of SFO neurons, in coronal brain slices, is modulated by NT. Individual cells either hyperpolarized (n=4), had no change (n=17), or depolarized (n=12) following the application of 1uM NT (Kolmogorov-Smirnov Test; $p \leq 0.05$). Median membrane potential changes were significantly different between responders and cells showing no change (Wilcoxon Signed Ranks Test, $p \leq 0.05$). Significant change in median membrane potential was observed between groups that hyperpolarized and depolarized and between cells that depolarized and showed no change, but not between the group that hyperpolarized and those that showed no change (Kruskal-Wallis; $p \leq 0.05$).

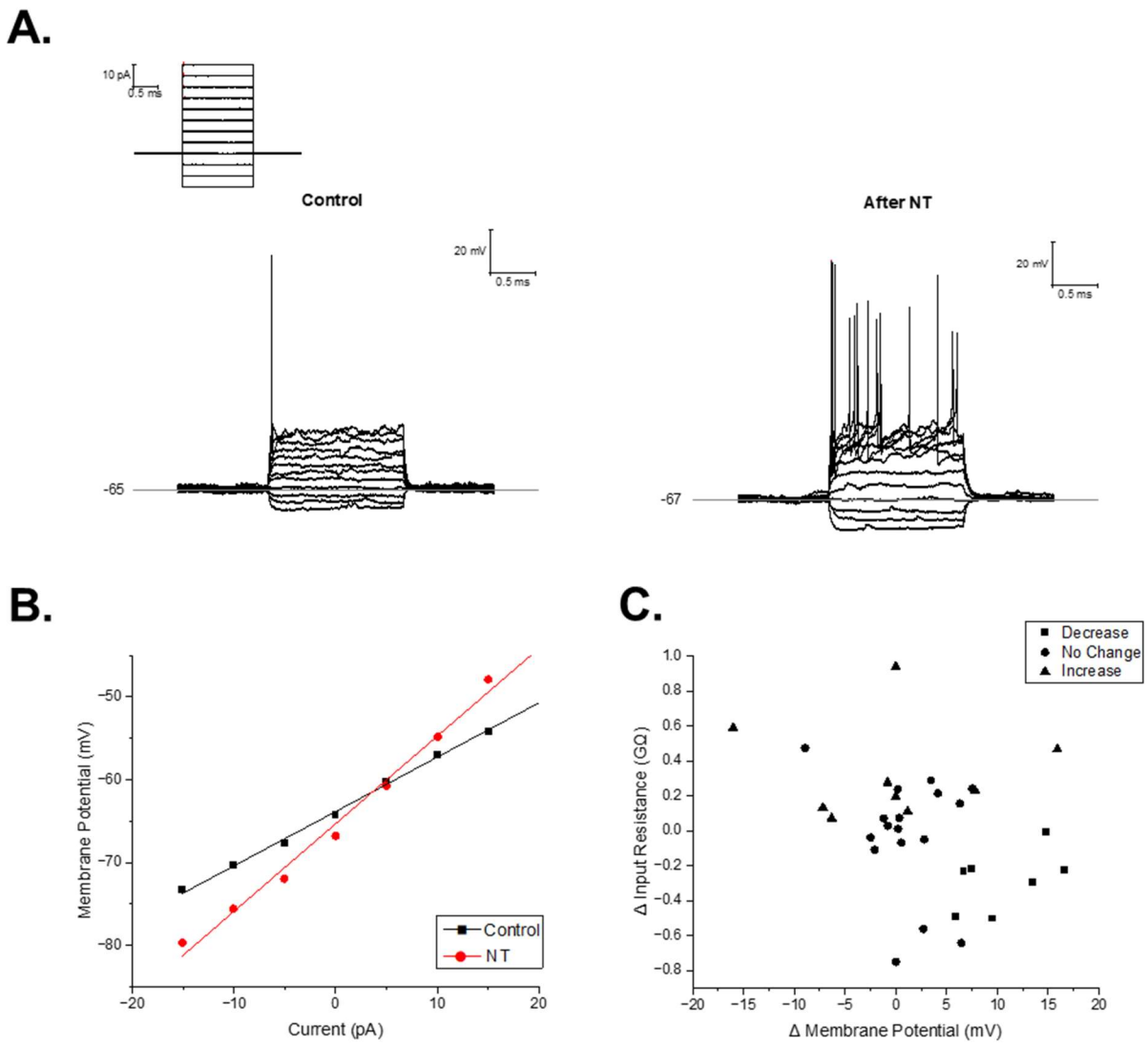

Figure 3.4. Analysis of changes in input resistance and reversal potentials of effect did not reveal a consistent ionic mechanism for changes in membrane potential. The first panel (A) shows current clamp recordings from a representative SFO neuron, in tissue, during control and after treatment (1µM NT). The input resistance from individual cells were calculated (n=33) from the slope of the VI-plot (B) from control (black squares) and treatment (red circles). By plotting the change in input resistance against the change in membrane potential (C) we observe no strong linear-correlation in the two parameters (ANOVA; $p = 0.058$).

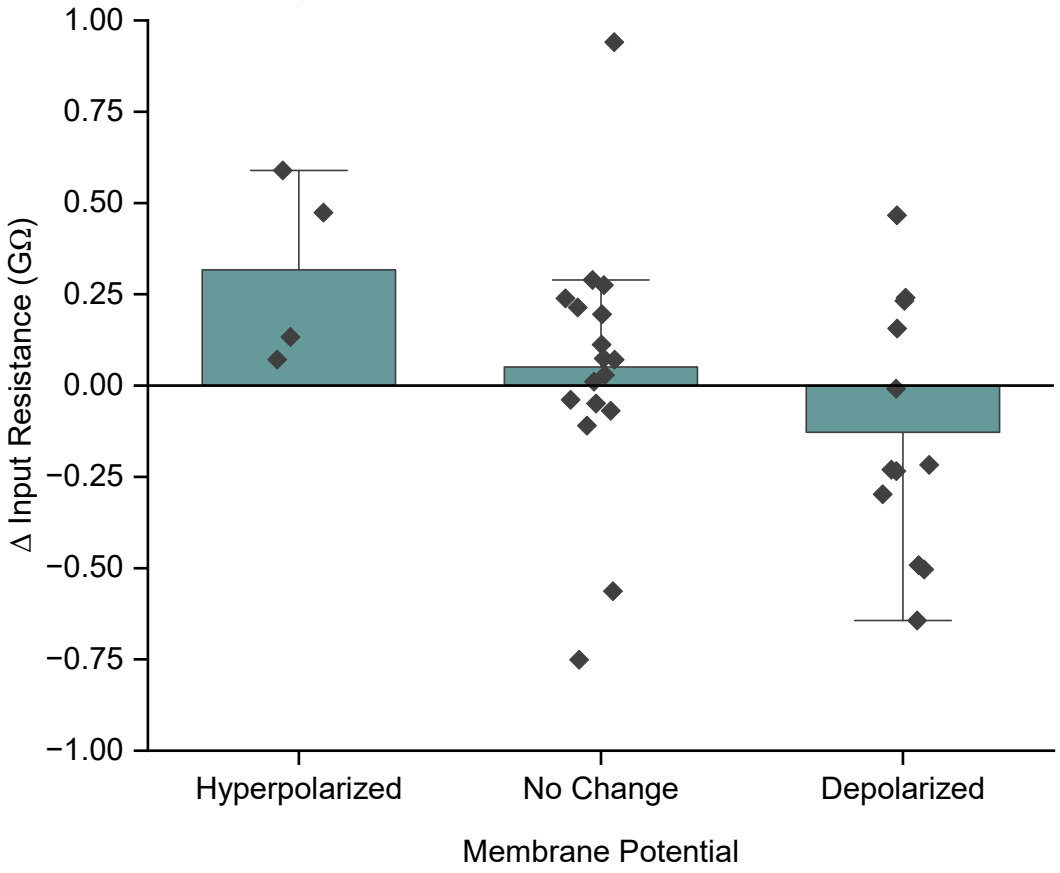

Fig 3.5. The application of 1µM NT did not result in consistent change in input resistance of SFO neurons. The bar graph above shows the change in input resistance of SFO neurons classified based on their response to 1µM NT (Hyperpolarized, No Change, Depolarized). Cells which hyperpolarized showed little variance in change of input resistance. Most cells with no significant change in membrane potential showed little variance in change. The distribution of change of mean input resistance varied the most in those cells which depolarized. No significant changes were observed between group median input resistance (Kruskal Wallis; $p \leq 0.05$).

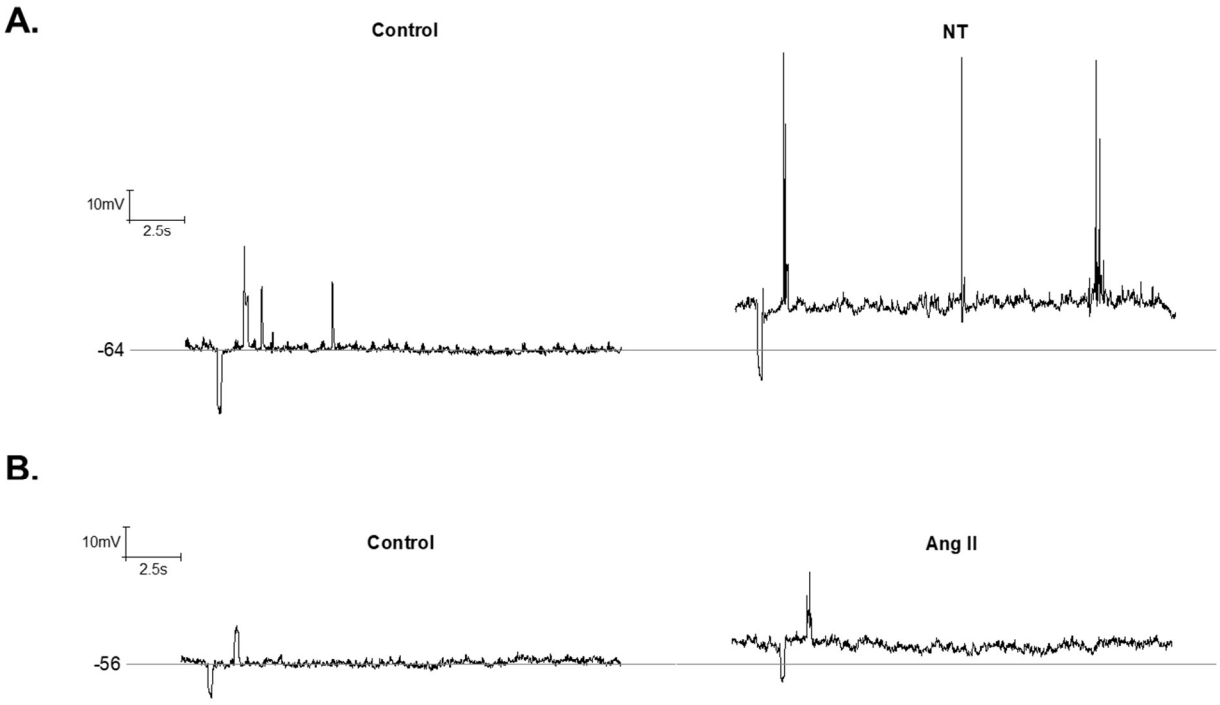

Figure 3.6. Neurons were tested for sensitivity to both 1μM NT and 100nM of Ang II. The representative SFO neuron was depolarized by both NT and Ang II.

Table 3.1. Summary of 15 neurons tested for sensitivity of membrane potential to both NT and Ang II. Individual cells tested for sensitivity to NT (1μM) were later tested with Ang II (100nM).

Observation	N
No response to either peptide	8
Responded to both peptides	2
Responded to NT only	3
Responded to Ang II only	2

3.2 Neurotensin modulates synaptic activity of subfornical organ neurons

Based on experiments examining effects of NT on membrane potential and input resistance, there was not a clear association between the change in membrane potential and change in input resistance, in that depolarized neurons did not always exhibit a decrease in input resistance with a given reversal potential of effect. We hypothesized that NT may also act indirectly via changing synaptic activity. To further examine this, voltage clamp technique was used to record EPSC amplitude and interevent intervals from SFO neurons under control conditions (aCSF) followed by treatment (1μM NT) (Figure 3.7). Mean EPSC data was examined from an individual cell in Figure 3.8, but this data was captured from a total of 30. Over half (60%; 18/30) of the cells responded by either a change only in mean EPSC amplitude (n=8), only in mean interevent interval (n=5), or a change in both (n=5) (Kolmogorov-Smirnov non-parametric test; $p \leq 0.05$). Comparing EPSCs during control and after treatment with NT (Figure 3.9), we determined that NT significantly modulates mean EPSC amplitude, by decreasing outward current, in SFO neurons (Wilcoxon Signed Ranks Test; $p=1.37 \times 10^{-4}$). However, there was no significant change observed in mean EPSC interevent interval (Wilcoxon Signed Ranks Test; p=0.14) among the population tested.

The GPCR NTSR2 is highly expressed within the SFO (Peterson et al., 2018); therefore, it is reasonable to consider that some modulatory effects observed may be through this receptor subtype. SR142948 non-selective NTSR1/2 antagonist was used in equimolar concentrations to NT (1μM), as in previous studies (Peterson, 2019), to determine if the effect on synaptic activity could be blocked by preincubation (Table 3.2). Baseline recordings using aCSF were taken from all cells prior to preincubation with the antagonist. After 10-minutes of pre-incubation to ensure successful block of the receptor, the test solution was added (antagonist + NT). SR142948 (1μM)

successfully blocked the effect of NT (1μM) on EPSC amplitude in all cells tested (n=8) when compared to the antagonist baseline; and blocked the effect of NT on interevent intervals in 7/8 cell (2x2 Contingency Chi-square; χ^2=3.96; p=0.04). Notably, when comparing baseline aCSF recordings to those following SR142948 incubation (no NT), 7/8 cells displayed a change to EPSC amplitude (5/8) or interevent interval (6/8) in the absence of NT.

To examine the possibility of overlapping synaptic effects with Ang II, 20 SFO neurons treated with NT were later treated with Ang II in voltage-clamp configuration. Of the 20 neurons tested, 50% (10/20) responded to both peptides, 6/20 responded to Ang II only, 2/20 responded to NT only, and 2/20 displayed no change in response to either peptide (Table 3.3). With a significant overlap in cell sensitivity to NT and Ang II, a Pearson's Correlation was used to determine if any statistically significant correlation exists between their responses. Evidence suggests a correlation between changes in mean EPSC amplitude (Pearson Correlation; r=0.78, p= 0.002), but not mean interevent interval (Pearson Correlation; r=0.064; p=0.78), of cells tested with the two peptides. This suggests that NT and Ang II could mediate the same biological effects through modulation of SFO neurons.

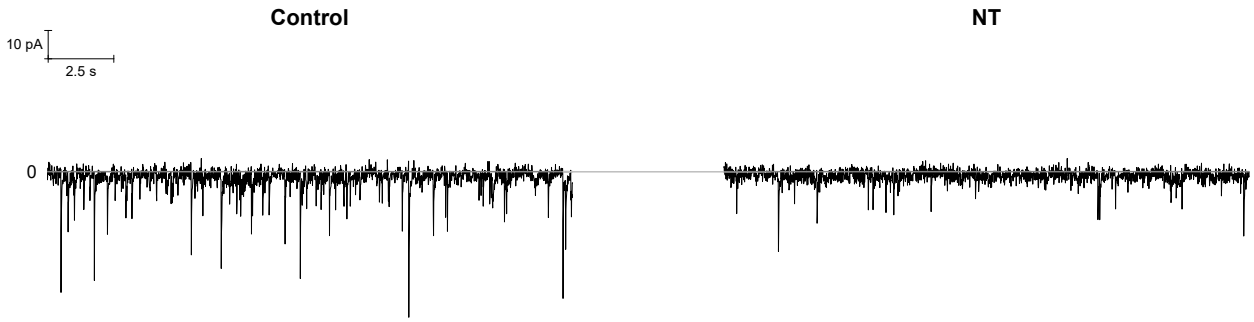

Figure 3.7. NT (1 μM) modulates synaptic activity of SFO neurons. Representative voltage-clamp recordings above from an SFO neuron showing that the application of 1μM NT results in a change in excitatory post synaptic currents.

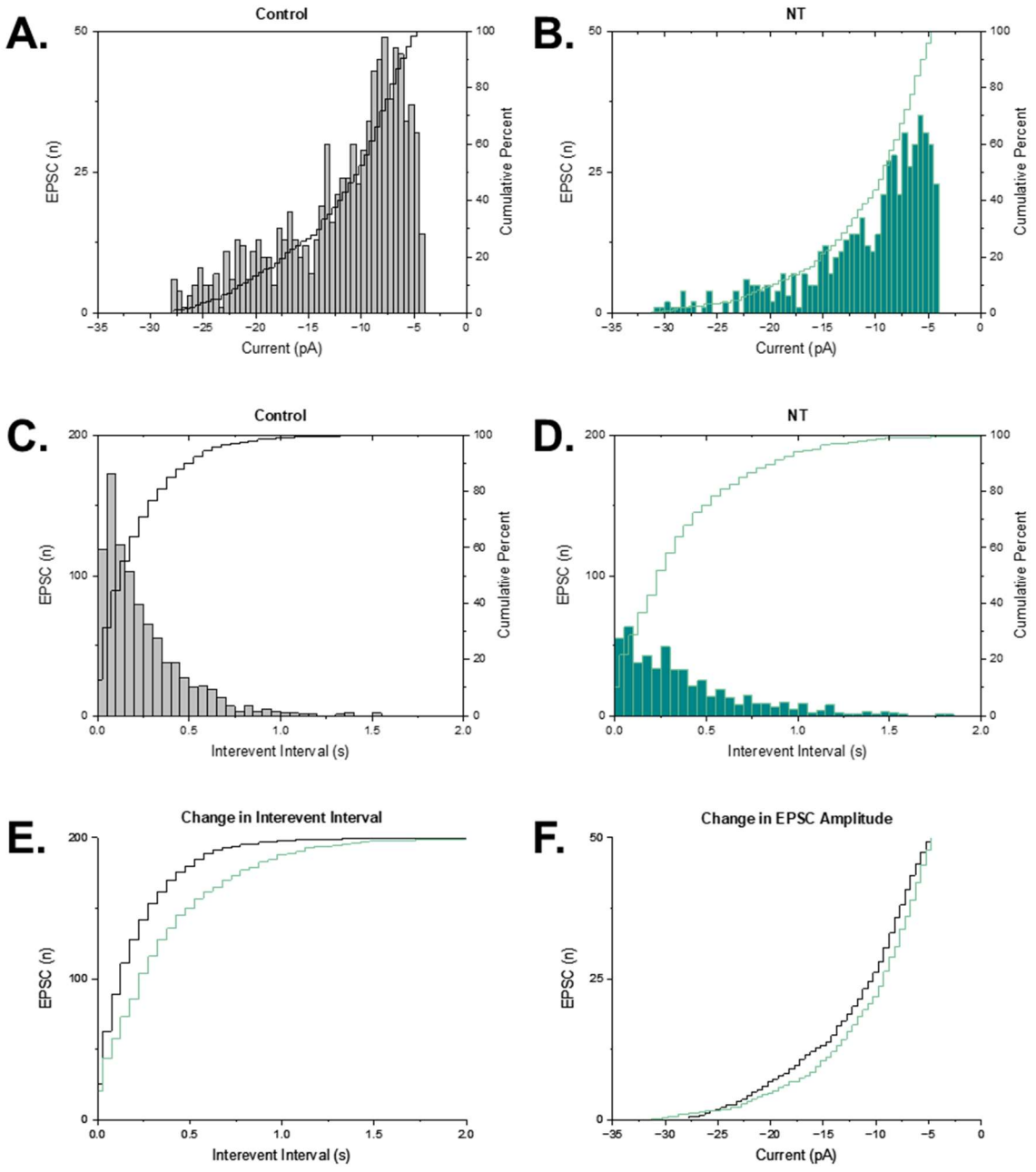

Figure 3.8. 1µM NT modulates EPSCs of SFO neurons. EPSC amplitude and interevent intervals were analyzed from control (A and C) and treatment (B and D) with NT. The bottom two panels (E and F) provide a comparison of the data for EPSC amplitude (E) and interevent interval (F). The representative cell above demonstrates a significant change to both EPSC amplitude (Kolmogorov-Smirnov non-parametric test; p=7.20x10-5) and interevent interval (Kolmogorov-Smirnov non-parametric test; p=5.07x10-14).

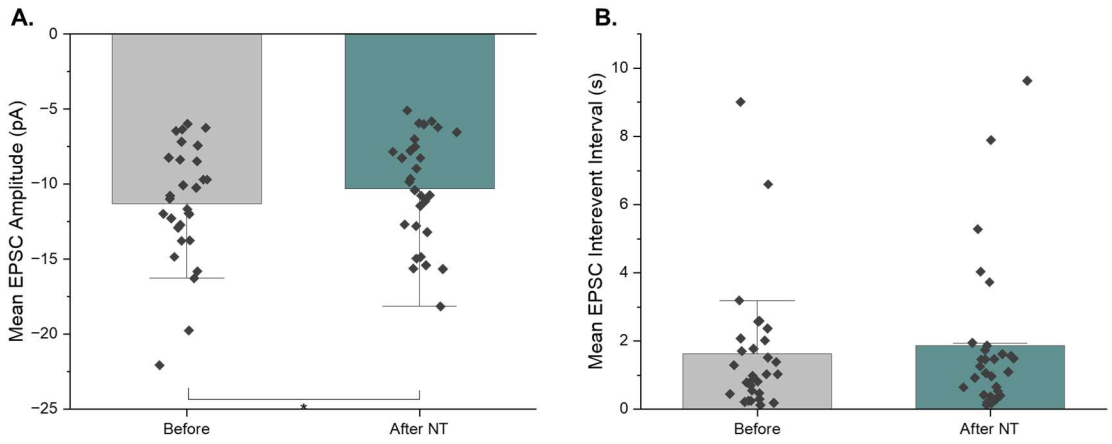

Figure 3.9. NT modulates synaptic activity in local SFO circuits. Bar graphs above show the mean data from the population of SFO neurons (n=30) under control (grey) and treatment (green) with NT in voltage-clamp. Data suggests that NT (1μM) modulates the mean EPSC amplitude (A) (Wilcoxon Signed Ranks Test; p=1.37x10-4), but not mean interevent interval (B) (Wilcoxon Signed Ranks Test; p=0.14) of SFO neurons in coronal brain slices.

Table 3.2. Summary of experiments using a NTSR antagonist. The non-selective NTSR antagonist, SR142945 (1μM), blocked the effect of NT (1μM) on SFO neurons (2x2 Contingency Chi-square; χ^2=3.96; p=0.046) when compared to the antagonist baseline recordings, and may have modulated synaptic activity after incubation, in the absence of NT, when compared to the aCSF control.

Solution	**Control (aCSF)**	**Antagonist (Control + SR142948)**	**Treatment (Antagonist + NT)**
No Observable effect	8	1	7
Change in amplitude and interevent interval	0	4	1
Change EPSC amplitude	0	1	0
Change EPSC interevent interval	0	2	0

Table 3.3. Observation of EPSC amplitude and interevent intervals show that 50% of cells respond to 1μM NT or 100nM Ang II. Most cells decreased outward current and number of interevent intervals following the application of both peptides.

Activity/Observation	**N**	**Δ Interevent Interval**	**Δ Current Amplitude**
Total cells tested for NT and Ang II	20	18	13
No changes observed	2	0	0
Responded to NT, not Ang II	2	2	0
Responded to Ang II, not NT	6	6	3
Responded to both	10	3	7
NT change		6	8
Ang II change		7	9

Discussion

Experiments in this thesis were aimed at determining if SFO neurons in coronal brain slices are modulated by NT and, if so, are the effects likely due to direct or synaptic interaction. Observing resting membrane potential of SFO neurons, we observed that 14/33 responded by either depolarizing or hyperpolarizing after the application of NT, from baseline membrane potentials. Using voltage-current data, we found that EPSC amplitude was significantly modulated after the application of NT in 43% of cells. Interevent interval significantly changed from control and treatment in 10/30 cells tested; however, this was not consistently significant in the population data. Taken together, this suggests that observed effects are mediated directly, through modulation of the membrane, and likely through synaptic activity, due to modulation of EPSCs. Ang II was applied to 15/33 neurons after they were tested for NT sensitivity in current-clamp configuration. These results showed that 50% of the neurons that were sensitive to Ang II were also sensitive to NT, and that 40% of the neurons that were sensitive to NT were also sensitive to Ang II. Voltage-clamp was used to observe any overlap with synaptic effects. We found that, of 20 cells tested, 18 responded to both peptides. To confirm the NTSRs were involved in the observed interaction, tissue was incubated with the non-selective NRST1/2 antagonist, SR142948. This NTSR antagonist blocked changes in EPSC amplitude and interevent interval, which we previously observed in individual SFO neurons following treatment of NT, suggesting that the observed effects were likely mediated by NT acting through NTSRs.

Acutely prepared brain slices were used for this study to confirm previously reported electrophysiological findings, that NT directly modulates SFO neurons (Peterson, 2019), in a more physiologically intact environment. Secondly, the use of tissue allowed us to maintain some local circuits and identify if observed effects are due to synaptic modulation. Ang II was

also used in this study to observe activity of a known peptide in the SFO neurons and determine if the subset of neurons sensitive to Ang II were also sensitive to NT.

A 100nM concentration of Ang II was used as it was previously observed at this concentration in other studies (Kai et al., 2006). The concentration of 1μM NT was chosen in this study because previous studies using NT in other regions of the brain identified a response between 1-10μM. Although concentrations between 1-100nM were observed to elicit a response by Peterson (2018) in dissociated SFO neurons, a higher concentration was decided upon to ensure effective permeation through the tissue.

4.1. Neurotensin directly effects electrical activity of subfornical organ neurons

Consistent with previous studies (Peterson, 2019), 48.5% of cells responded with a change in mean membrane potential. Both increase and decrease in neuronal input resistance were observed in individual cells, but there was no significant linear-correlation between the change in input resistance and membrane potential. There was, however, a tendency for cells that depolarized to show a decrease input resistance, and all cells which hyperpolarized seemed to have respond with a slight increase in input resistance. Contrary to findings from Peterson (2019), who demonstrated that NT depolarizes and increases action potential frequency in 54% SFO neurons while the remaining 46% showed no response, we found that 25% of NT-responsive cells hyperpolarized, while 75% of responsive cells depolarized. Duality in response to NT is also observed in other regions of the brain (Coogan et al., 2001; Tschumi & Beckstead, 2019). For example, excitability of suprachiasmatic nucleus neurons is both increased and decreased in response to 1-10μM of NT. The SFO itself, has displayed heterogeneous electrical response to other peptides such as insulin, leptin, adiponectin, and galanin (Alim et al., 2010; Kai et al., 2006; Lakhi et al., 2013; Smith et al., 2009a). Patch-clamped SFO neurons which were

tested for sensitivity to galanin, for example, exhibited inhibitory effects in 67% of units tested and 5% of cells tested excitatory (Kai et al., 2006). Discrepancy our study and that of Peterson (2019) may be due to the additional complexities of signalling under non-isolated conditions and possible confounding synaptic effects; therefore, we sought to determine any additional synaptic mechanisms of modulation contributing to these effects.

4.2. Synaptic activity of subfornical organ neurons may be modulated by neurotensin

In our current study, the unclear association between change in membrane potential and change in input resistance, and the variation of reversal potentials of effect, seem to indicate that there may also be an indirect modulation of SFO neurons by changing synaptic outputs. This was examined by observing EPSC amplitude and interevent interval of SFO neurons in voltage-clamp configuration. In this experiment, we demonstrated that NT significantly modulates EPSC amplitude of SFO neurons, but not the interevent interval. EPSC amplitude decreased in 77.8% of responsive cells and decreased in 22.2%, as expected from previous studies which showed heterogeneous changes in synaptic activity in response to NT (Tschumi & Beckstead, 2019). There was a change in interevent interval for 33.3% (10/30) of the individual cells tested. EPSC interevent intervals of these NT sensitive neurons were observed to have increased (60%; 6/10) and decreased (40%; 4/10), suggesting that there could be some pre-synaptic modulation by NT in the SFO. This information, taken with the broad variation of change to input resistance we observed previously, could indicate integration of pre- and post-synaptic activity which contributes to the overall NT net electrical effect (Tschumi & Beckstead, 2019).

Our results, concurrent with previous studies in other regions of the CNS, show variability of synaptic response to NT (Tschumi & Beckstead, 2019). EPSCs in tegmental area

glutamatergic neurons, for example, have shown increased (Bose et al., 2015) or decreased (Kortleven et al., 2012) amplitude in response to NT. Nucleus of the solitary tract neurons increased excitability in response to NT and, in the same study, were shown to increase both inhibitory and excitatory post synaptic currents (Ogawa et al., 2005); also demonstrating both direct and synaptic modulation by the peptide, as in our current research. It may be that we need to have ways to determine specific neuronal subtypes in order to see more consistent responses (see future research).

The non-selective NTSR1 and NTSR2 antagonist, SR142948, was used in our study to effectively block effects on EPSC amplitude and interevent interval, confirming NTSR involvement in observed SFO modulation. The effects of NT were successfully blocked in 7/8 cells tested; however, 1/8 cells exhibited some response after treated with NT which could suggest that some effects could be mediated through another NTSR (Bose et al., 2015; Coogan et al., 2001). Recall that NTSR2 and NTSR3, but not NTSR1, are highly expressed in the SFO (Fassio et al., 2000; Peterson et al., 2018). NTSR3 is a member of the VPS10P family and is thought to act with other channels and receptors, such as NTSR 1, to regulate neuronal function (Nykjaer & Willnow, 2012). To our knowledge, there is currently no known effective antagonist for this NTSR. NTSR3 has been demonstrated to affect signaling through modulation of NTSR1 (Martin et al., 2002; Nykjaer & Willnow, 2012); however, no evidence supports a similar effect on NTSR2. With this information, we can deduce that our observed effects are mediated through the G-protein coupled receptor, NTSR2.

4.3. A subtype of subfornical organ neurons seem to be sensitive to both neurotensin and angiotensin II

Additional experiments were conducted during our study to identify possible subtypes of NT sensitive neurons which were also sensitive to the peptide Ang II. Using current-clamp configuration, fifteen of the SFO neurons tested for NT sensitivity were then treated with Ang II. Comparing the resting membrane potential of the control to after treatment with Ang II, we observed that 50% of cells sensitive for Ang II were also sensitive to NT, and 40% of cells that responded for NT were sensitive to Ang, all depolarizing after the application of each peptide. We later observed EPSC amplitude and interevent interval voltage-clamp configuration to determine possible modulation of synaptic activity of SFO neurons to both peptides. Of the 20 cells tested, we found 50% overlap in SFO neurons sensitive to both NT and Ang II with 62.5% of Ang II-sensitive neurons being sensitive to NT, and 83.3% of NT-sensitive neurons being sensitive to Ang II. Li and Ferguson showed that 60% of SFO neurons directly respond to Ang II and, when studying synaptic activity, all cells tested in the SFO responded with a reduction in outward transient currents (Ferguson & Li, 1996; Li & Ferguson, 1993). Our experiments on the SFO show that only 26% in current clamp and 80% in voltage clamp were responsive to Ang II. Overlap clearly exists, but it appears that sensitivity to these peptides is not exclusively linked; however, observing modulation of EPSCs in our experiments, most cells that showed synaptic modulation to both peptides did so by decreased EPSC amplitude, which could suggest that the two peptides are involved in regulation of similar biological pathways.

Discrepancy between results in our study compared to previous studies involving the effects of Ang II within the SFO could be due to a several factors. For one, Li and Fergusons experiment (1993) showing excitatory effects on membrane potential and action potential used

extracellular recordings whereas we used intracellular/whole cell configuration. The difficulty to maintain cells following the application of Ang II in voltage-clamp configuration (>15 minutes) was mentioned by Ferguson and Li (1996) during one of the first studies recording synaptic activity of SFO neurons. In this experiment, they sought to characterise synaptic activity of SFO neurons, determining that both rapid transient and a sustained outward current characterized synaptic activity of these neurons. During this study they also used these neurons observe the synaptic effects of Ang II on SFO neurons; of which they determined that at least 60 minutes of baseline recordings were required prior to the application of Ang II (Ferguson & Li, 1996). This resulted in only 4 cells being tested for modulation of synaptic effects to Ang II, all showing a decreased amplitude in transient current. During our observations of synaptic activity if SFO neurons in response to two peptides, we utilized more cells (n=20) but a shorter duration than in previous studies (Li & Ferguson, 1993). We applied NT prior to the application of Ang II which, although over 10 minutes of washout was conducted for recovery, may not eliminate inherent changes to channels. In fact, Ogawa et al. (2005) reported the requirement for an extended period of washout for NT for any recovery (at least 10 minutes) and possibly tachyphylaxis or decrease in response. It is possible that, by applying NT first, we may have altered the Ang II response, though no evidence exists for or against this.

4.4 Conclusion

Integration of modulatory affects through different NTSRs resulting in a duality of net effect have been observed when studying NT in other areas of the CNS, revealing differences of effect based on concentration and even single cell activity (Coogan et al., 2001; Tschumi & Beckstead, 2019). Heterogeneity of receptor subtype and coupled G-proteins could initiate different second messenger pathways to evoke inherent cellular changes (Besserer-Offroy et al.,

2017; Coogan et al., 2001; Tschumi & Beckstead, 2019) and possibly, electrical responses. The NT net effect on substantia nigra dopamine neurons, for example, can either result in a net excitatory or inhibitory output through modulation of non-selective cation currents, K^+ currents, GABA currents, among other channels (Tschumi & Beckstead, 2019; Wu et al., 1995; Wu & Wang, 1995). Peterson (2019) showed that potassium currents ($I_{K)}$ and non-selective cation currents (I_{NSC}) are affected in dissociated SFO neurons are modulated by NT. Based changes in input resistance observed in cells which depolarized in response to NT, our data suggests that modulation of both potassium and non-selective cation channels are likely. Interestingly, during our experiments with SR142948, we observed that the application of SR142948 alone resulted in a change in EPSC amplitude and interevent interval, from baseline recordings, suggesting there may be an underlying NT tone within the SFO.

Central regions that are involved with cardiovascular regulation and fluid mineral balance such as the lateral hypothalamic area, median eminence, and medulla, have known projections to the SFO which have been found to have neurotensinergic cells (Table 3.3). NT expressing fibers have been found to project to the SFO from the arcuate nucleus suggesting this could be one pathway for NT to modulate SFO neurons (Fry unpublished data, Fig 1.2; Rosas-Arellano et al., 1996). Sapru (2013) suggests that cardiovascular regulation of pressor-depressor response by the arcuate nucleus may be mediated through SFO projections which are activated by circulating angiotensin. It is possible that feedback mechanisms exist between these circuits that utilize both NT and Ang II to regulate this response. Additional studies that investigate distribution of NT and NTSRs throughout the SFO, and possible retrograde tracing of NT fibers; and the integration of Ang II and NT signalling in this CVO are recommended to aid in further understanding of neural mechanisms behind cardiovascular regulation, hydromineral balance and homeostasis.

4.5 Future Research

Preliminary studies were conducted in collaboration with the Fry lab to determine distribution of NT (Fry unpublished data, Figure 1.2) and NTSRs within the SFO. Continuation of this research will identify the distribution of neurotensinergic inputs and receptors relative to SFO anatomy. The next step from this immunohistochemical study would be to determine subpopulations of SFO neurons which express NT or NTSRs. Colocalization studies, for example, could provide insight as to the possible system level regulation by NT.

Previous research has demonstrated that dissociated SFO neurons respond to NT (Peterson, 2019). Our research provides evidence that this response exists under more physiological conditions, with some intact local circuits. Our data suggests both direct and synaptic activity mediating the response; however, there is variability in observed response. The use of Ang II in our studies suggests different subtypes of SFO neurons respond to NT; those which are also sensitive to Ang II, and those which are not. Knowing which subtype of neuron is being tested could produce more consistent results. Future studies could aim to identify neurons expressing Ang II that are also modulated by NT. This could be done through application of adeno-associated virus vectors to identify angiotensin/receptor-expressing neurons within the SFO of transgenic rats and conducting a series of similar electrophysiological experiments. This experiment could also determine the signal integration between the two peptides. As mentioned previously, NT projections exist from the arcuate nucleus (Rosas-Arellano et al., 1996) to the SFO and is suggested to mediate cardiovascular regulation (Sapru, 2013), where Ang II has also been found to regulate cardiovascular activity through modulation of the SFO (Coble et al., 2014). Comparing data from electrical activity from the subpopulation of cells sensitive to both

peptides under multiple treatment conditions (i.e.. Control, NT only, NT and Ang II) could provide detail to support additive, competitive, or opposing effects.

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