EVIDENCE FOR EFFECTS OF PHOENIXIN ON NEURONS OF THE PARAVENTRICULAR NUCLEUS

by

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Abstract

The paraventricular nucleus (PVN) of the hypothalamus has been implicated in autonomic regulation. Through projections to the pituitary gland, median eminence and hindbrain, the PVN plays a role in stress response and other autonomic functions. Recent findings have indicated that both phoenixin (PNX), a peptide involved in such responses, and its receptor, GPR173, are expressed throughout the PVN. PNX is thought to sensitize the pituitary gland to releasing hormones, as well as contribute to regulation of reproductive and stress hormone release. Recently, specific stress-related effects of this peptide in the nucleus of the solitary tract (NTS) have been demonstrated. In this study we investigated the effects of PNX on PVN neuronal activity using *in vitro* extracellular recordings. Recordings from a total of 824 neurons showed that 16% (n= 130) of these cells were activated by bath administration of PNX, while 14% (n= 117) were inhibited. Remaining cells tested showed no obvious changes in spike frequency. Furthermore, we observed that both these excitatory and inhibitory effects are still observed when recordings are obtained in low-Ca^{2+}/ high-Mg^{2+} artificial cerebral spinal fluid (to block synaptic release of neurotransmitters and thus synaptic transmission). A total of 187 neurons showed that 5% (n= 7) are excited and 22% (n= 43) are inhibited in the low calcium condition, supporting the conclusion that the effects of PNX on PVN neurons are both direct and indirect as both response types are observed in both conditions. These results implicate PNX in autonomic function, specifically in the central regulation of stress response, fluid and electrolyte balance, food intake, and reproductive function.

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List of Abbreviations

aCSF.....Artificial Cerebrospinal Fluid
ACTH.....Adrenocorticotropic Hormone
ANG II ..... Angiotensin II
AP .....Area Postrema
Arc.....Arcuate Nucleus
ANS..... Autonomic Nervous System
AVP.....Arginine Vasopressin
cAMP .....Cyclic-Adenosine Monophosphate
CART ..... Cocaine-amphetamine regulated transcript
CHF.....Congestive heart failure
CNS..... Central Nervous System
CREB ..... cAMP response element-binding protein
CRH .....Corticotropin-Releasing Hormone
DMNV ..... Dorsal Motor Nucleus of the Vagus
ER .....Estrogen Receptor
FSH .....Follicle-stimulating hormone
GABA ..... Gamma-Aminobutyric Adic
GI ..... Gastrointestinal
GHRH .....Growth hormone-releasing hormone
GLU .....Glutamate
GnIH .....Gonadotropin-Inhibitory Hormone
GnRH .....Gonadotropin-Releasing Hormone
GPCR .....G-Protein Coupled Receptor
HPA..... Hypothalamic-Pituitary-Adrenal
HPG.....Hypothalamic-Pituitary-Gonadal
I_A .....Transient K^+ current
ICV..... Intracerebroventricular
LH .....Luteinizing Hormone
ME..... Median Eminence

MEA ..... Multi-Electrode Array
MNC ..... Magnocellular
NANC ..... Non-adrenergic non-cholinergic
NE ..... Neuroendocrine
NTS ..... Nucleus of the Solitary Tract
NO ..... Nitric oxide
OXT ..... Oxytocin
OXTR ..... Oxytocin receptor
PA ..... Pre-Autonomic
PKA ..... Protein Kinase A
PNS ..... Parasympathetic Nervous System
PNX ..... Phoenixin
PVN ..... Paraventricular Nucleus
RT-PCR ..... Real-time polymerase chain reaction
RVLM ..... Rostral Ventrolateral Medulla
SCN ..... Suprachiasmatic nucleus
SFO ..... Subfornical Organ
SMIM20 ..... Small integral membrane protein 20
SNS ..... Sympathetic Nervous System
SON ..... Supraoptic Nucleus
TRH ..... Thyrotropin-releasing hormone
TSH ..... Thyroid-stimulating hormone
VIP ..... Vasoactive intestinal polypeptide
V_{1A}/ V_{1B}/ V_2 ..... Vasopressin receptor
$[Ca^{2+}]_o$ ..... Extracellular calcium

Chapter 1

Introduction

The autonomic nervous system (ANS) is comprised of two separate divisions that co-ordinate central and peripheral signals in order to maintain homeostatic balance of physiological systems (Ernsberger & Rohrer, 2018; Cannon, 1929). Control over autonomic function is maintained by the parasympathetic (PNS) and sympathetic nervous system (SNS), which are characterized by unique molecular and cellular properties (Koopman *et al.,* 2011). This regulation classically applies to cardiovascular, gastrointestinal and renal function, as well as to metabolic, stress, immune response and reproductive processes (Ferguson *et al.,* 2008). A variety of peripheral signals convey the status of these systems to several central regulatory regions, which act to integrate and relay the information through a complex co-ordination of efferent and afferent mechanisms (Benarroch, 1993; Ferguson *et al.,* 2008). Although distinct, a synchronized network is formed by regions including, but not limited to, the arcuate nucleus (Arc), nucleus of the solitary tract (NTS), lateral hypothalamus and paraventricular nucleus (PVN) (Benarroch, 1993; Ferguson *et al.,* 2008). Through cellular and molecular means, this network maintains an ongoing response to deviations from homeostasis to restore physiological balance.

1.1 Autonomic Nervous System and Paraventricular Nucleus

The PVN has been established as a prominent regulator of autonomic control, in addition to fulfilling critical roles in neuroendocrine function (Ferguson *et al.,* 2008). The PVN is a bilateral structure residing adjacent to the third ventricle, which receives extensive sensory information regarding overall autonomic state from the central nervous system (CNS) and the

periphery. The PVN receives direct projections from the subfornical organ (SFO), bed nucleus of the stria terminalis, and entire hypothalamus with the exception of the supraoptic (SO) and mammillary nuclei (Sawchenko & Swanson, 1982). Further, indirect projections are relayed by other hypothalamic regions from the hippocampal formation, amygdala and lateral septum (Sawchenko & Swanson, 1982). This complex system of innervation involves unique target distributions that correlate with the three distinct neuronal subpopulations characterizing PVN, namely the magnocellular (MNC) and two separate parvocellular divisions. While these inputs provide critical sensory information to the PVN, the critical roles of this nucleus in autonomic regulation are primarily dependent on the unique abilities of PVN efferents to regulate almost all components of the extended autonomic infrastructure. Projections of these subpopulations of PVN neurons include the posterior pituitary gland (MNC neurons), median eminence (ME) (parvocellular neuroendocrine neurons), as well as numerous ANS regions in the brainstem and spinal cord (parvocellular pre-autonomic neurons) (Sawchenko & Swanson, 1982). Functionally, the excitability of these neurons is regulated through not only afferent inputs but also through both excitatory and inhibitory inputs from local interneurons, a network that is critical for intra-nuclear integration (Boudaba *et al.,* 1996; Daftary *et al.,* 1998; Ferguson *et al.,* 2008). As a central site of integration for autonomic signaling, the PVN is uniquely equipped to regulate integrated autonomic state as a result of the complex cellular and molecular organization throughout this network.

1.2 Cellular and Molecular Structure of the PVN

Of the 20,000 neurons located throughout the PVN, approximately 16,000 are part of the parvocellular division (Nunn *et al.,* 2011; Rhodes *et al.,* 1981; Sawchenko & Swanson, 1981).

These neurons are further divided into spinally projecting pre-autonomic (PA) neurons and neuroendocrine (NE) neurons of the neurohypophysis based on projection and function (Prabha *et al.,* 2002; Sawchenko & Swanson, 1982). The remaining cell groups in the PVN include MNC neurons, characteristically linked to the posterior pituitary, as well as networks of interneurons, being both excitatory and inhibitory (Brownstein & Mezey, 1980; Sawchenko & Swanson, 1981).

1.2.1 Parvocellular Pre-Autonomic Neurons

PA neurons have been localized to five areas within the PVN (Armstrong *et al.,* 1980; Sawchenko & Swanson, 1982); grouped across periventricular, anterior, medial, dorsal and lateral aspects of the PVN (Sawchenko & Swanson, 1982). Despite being a heterogeneous population, PA neurons can be identified by various shared neurochemical, electrophysiological and physical properties (Armstrong *et al.,* 1980; Sawchenko & Swanson, 1982; Stern, 2001).

The principle method for distinguishing between the three types of PVN neurons is by characterizing electrophysiological fingerprints (Tasker & Dudek, 1991; Luther *et al.*, 2002). In response to hyperpolarizing current pulses, PA neurons uniquely display the activation of a T-type Ca^{2+} current, generating low-threshold calcium spikes and an inwardly rectifying current (Stern, 2001; Luther et al., 2002). PA neurons can also be characterized by their chemical phenotype as subpopulations contain vasopressin (AVP), oxytocin (OXT), corticotrophin-releasing hormone (CRH) or thyrotropin-releasing hormone (TRH) (Berecek & Swords, 1990; Li & Ferguson, 1996; Poulin *et al.,* 1994).

AVP, OXT, CRH and TRH are the principle peptides of the PVN and are released in response to complex afferent inputs. As identified through retrograde transport experiments, the

PA division receives input from pathways originating in the hypothalamic preoptic, suprachiasmatic (SCN), ventromedial, dorsomedial, pre-mammillary, lateral and posterior regions, as well as the SFO and bed nucleus of the stria terminalis (Sawchenko & Swanson, 1982). These inputs convey information on the thyroid axis, circadian rhythms, energy and fluid balance, blood pressure, as well as stress responses to the PVN, alongside comparable information from all other autonomic systems (Larsen & Mikkelsen, 1998; Ferguson *et al.,* 2008). Characteristically, the PA division projects directly to sympathetic pre-ganglia in the spinal cord, exerting direct affects over the SNS to drive a fight-or-flight response in the event of greater arousal and a corresponding need for increased cardiac output (Nunn *et al,* 2011; Silverman *et al.,* 1981).

Regulation of sympathetic outflow through PVN involves resting and bursting activity patterns of efferent projections (Kenney *et al.,* 2002). Alongside the previously discussed peptides, nitric oxide (NO), *γ*-aminobutyric acid (GABA) and angiotensin II (ANG II) have been shown to exert excitatory effects on these neurons (Kenney *et al.,* 2002). Microinjection studies have demonstrated that these neuromodulators act in PVN to enhance sympathetic nerve activity (Chen *et al.,* 2003; Kenney *et al.,* 2002), which is basally maintained by tonic glutamatergic input (Li & Pan, 2007).

1.2.2 Parvocellular Neuroendocrine Neurons

The remainders of the parvocellular neurons are classified as NE due to their neurosecretory properties and projections to the ME (Palkovits, 1986; Prabha *et al.,* 2002; Tasker & Dudek, 1981). NE neurons of the PVN are situated in the medial aspect of the nucleus, specifically in the periventricular and dorsal regions (Luther *et al.,* 2002; Sawchenko &

Swanson, 1982). NE cells send efferent projections to the ME where releasing factors are secreted into the adenohypophysial portal circulation, which when they reach the anterior pituitary control the secretion of anterior pituitary hormones (Alonso & Assenmacher, 1981; Armstrong, 2004; Prabha *et al.*, 2002; Tasker & Dudek, 1981). The PVN NE neurons have common neurochemical, physical and electrophysiological properties that are different to those described for PA neurons.

Specifically, NE neurons do not possess the unique electrophysiological properties of PA neurons described above (Stern, 2001; Luther *et al.*, 2002). Thus, this absence of an electrophysiological signature becomes characteristic itself for the NE division. NE neurons do have a molecular profile. As critical regulators of both the stress and thyroid axes, separate populations of NE neurons produce CRH, TRH, growth hormone-releasing hormone (GHRH) or somatostatin (Swanson & Sawchenko, 1980; Merchenthaler *et al.*, 1984), and in some instances co-expression of AVP and/ or OXT has been described (Sawchenko *et al.*, 1984).

1.2.3 Magnocellular Neurons

The MNC subpopulation of the PVN is distinguished based on a larger soma diameter, 20-30 μm, primarily localized to the lateral region of the rostral PVN (Swanson & Sawchenko, 1983; Tasker & Dudek, 1991). MNC neurons send axonal projections to the posterior pituitary gland, where OXT and AVP are released from their nerve terminals into the general circulation (Swanson & Kuypers, 1980).

These larger PVN neurons are characterized by a transient K^+ current, I_A, corresponding to transient outward rectification. This electrophysiological fingerprint manifests as a delayed return to baseline and delayed onset of firing in response to a hyperpolarizing current pulse

(Tasker & Dudek, 1991; Li & Ferguson, 1996). MNC terminals possess dense-core vesicles that contain and release numerous peptides upon firing, primarily AVP and OXT (Glasgow *et al.*, 1999; Ludwig & Leng, 2006; Xi *et al.*, 1999). Given that MNC neurons may co-express these peptides, classification has recently been associated with relative expression levels of OXT and AVP (Glasgow *et al.*, 1999; Sawchenko &Swanson, 1984; Xi *et al.*, 1999).

Following release of these peptides into circulation from the posterior pituitary they play important physiological roles in the endocrine regulation of blood pressure, fluid balance, and reproductive function (Brown *et al.*, 1982; Ludwig & Leng, 2006; Stern &Armstrong, 1995).

1.3 Correlates of Chemical Phenotype with Physiological Function

AVP, the antidiuretic hormone, is synthesized in the SCN, PVN and bed nuclei of the stria terminalis. AVP exerts its effects on fluid balance and vasoconstriction after entering circulation from the posterior pituitary gland. Elevated plasma osmolality, decreased arterial blood pressure and decreased blood volume stimulate the release of AVP (Garrahy & Thompson, 2019). AVP-immunoreactive cells have been observed in the dorsomedial hypothalamus, locus coeruleus and medial amygdaloid nucleus (Caffé & Leeuwen, 1985). AVP from centrally-projecting neurons plays a role in cardiovascular function and stress response (Aguilera *et al.*, 1983), while AVP in the ventral septal area reduces fever (Disturnal *et al.*, 1986). AVP has also been implicated in pair-bonding as ICV injections increased social interaction, an effect that is thought to occur in humans as well (Cho *et al.*, 1999; Walum *et al.*, 2008).

Three G-protein coupled receptors (GPCRs) comprise the receptor subtypes to which AVP binds. AVP stimulates vasoconstriction in vascular smooth muscle, where the receptor V_{1A} is located (Holmes *et al.*, 2001). The anterior pituitary contains V_{1B}, the receptor which mediates

secretion of adrenocorticotropic hormone (ACTH) (Aguilera *et al.*, 1983). The third subtype, V_2, is located in the collecting duct in the kidney, coinciding with fluid balance maintenance (Gimpl & Fahrenholz, 2001; Manning *et al.*, 2008). Overall, AVP acts to promote reabsorption of water into the bloodstream, drive thirst and drinking behaviours, and homeostatically regulate plasma osmolality (Caffé & Leeuwen, 1985). These critical roles are carried out, in part, through the previously described neuronal populations in the PVN.

Similarly, OXT is a nonapeptide secreted by various neurons in the hypothalamus (Russell & Brunton, 2009). Principally known for its roles in childbirth and lactation, OXT also enters circulation following secretion in the posterior pituitary gland. This reproductive peptide is synthesized not only in the hypothalamus, but in limbic regions, brainstem, olfactory regions and cortex (Gimpl & Fahrenholz, 2001; Russell & Brunton, 2009). An individual receptor for OXT (OXTR) resides in both the CNS and periphery, namely the mammary and uterine tissues, as well as in the heart, kidneys and testes (Gimpl & Fahrenholz, 2001; Rozen *et al.*, 1995). These sites of action are indicative of additional functions including regulation of fluid balance, feeding and drinking, social behaviours, and cardiovascular control (Douglas *et al.*, 2001; Russell & Brunton, 2009; Petersson *et al.*, 1996). Due to similarities in structure, function and localization, these roles may be carried out in coordination with AVP secretion.

Coordination of responses to autonomic and neuroendocrine stimuli requires other neuropeptides, in addition to AVP and OXT. Releasing hormones CRH and TRH are the most relevant to the neuronal subpopulations of PVN. CRH has been referred to as the central hormone driving integrated stress response and is produced by the PVN neurons and released into the median eminence to drive ACTH secretion from the anterior pituitary gland (De Kloet *et al.*, 2005; Charmandari *et al.*, 2005). The effects of CRH are mediated through two GPCRs,

CRH-R1 and CRH-R2 (Raftogianni *et al.*, 2018). The CRH neurons of the PVN are integral to the hypothalamic-pituitary-adrenal (HPA) axis that drives responses to stress. In the event of a stressor, NE neurons release more CRH to the median eminence in order to facilitate ACTH output (Charmandari *et al.*, 2005). This role is crucial as the enhanced ACTH release from the anterior pituitary results, in turn, in increased levels of glucocorticoids that act to mediate demands associated with stress response through actions on glucose metabolism (Charmandari *et al.*, 2005; Oakley & Cidlowski, 2010; Owens & Nemeroff, 1991).

TRH, also recognized as a prominent releasing hormone, is located in the medial and periventricular areas of the PVN (Joseph-Bravo *et al.*, 2015). The thyroid axis is regulated by PA neurons of the PVN, more specifically NE neurons, which send projections throughout the CNS (Wittman *et al.*, 2009). The NE hypophysiotropic neurons extend afferent projections to convey relevant physiological demands (Lechan & Fekete, 2006). Mechanistically, NE neurons in the PVN produce and secrete TRH, which acts to stimulate release of thyroid-stimulating hormone (TSH) from the anterior pituitary. This interaction induces release of TRH into the hypophyseal portal system from the median eminence (Lechan *et al.*, 1980; Lechan & Fekete, 2006). Consequently, TSH acts upon the thyroid gland to regulate the set point of thyroid hormones in circulation (Koller *et al.*, 1987). Functionally, TRH secretion from the PVN results in decreased food consumption, increased thermogenesis, sympathetic activation, as well as the separate elevation of thyroid hormones in circulation (Suzuki *et al.*, 1982; Lechan & Fekete, 2006). As TRH expression is not mutually exclusive with CRH, NE neurons of the PVN exemplify the complex coordination of autonomic and neuroendocrine function that characterizes the PVN. Such complexity is not limited to the principle molecular phenotype of these neurons, but rather

extends to the co-expression of additional peptides that contributes to the phenotype of PVN subpopulations (Eaton *et al.,* 1996; Landgraf *et al.,* 1990).

Although the molecular makeup of PVN neurons emphasizes AVP, OXT, CRH and TRH, it has been established that all neurons in PVN express multiple other peptides that contribute to, and modulate, the responses of these principle signals. In addition to this, co-expression is prominent amongst the four principle peptides as well. For example, 50% of CRH neurons co-express AVP in their projections to the median eminence (Whitnall *et al.,* 1985). Additional examples include the co-expression of dynorphin in AVP dense core vesicles, as well as cocaine-amphetamine regulated transcript (CART) in neurons expressing TRH (Whitnall *et al.,* 1985; Raptis *et al.,* 2004). Presently, the functional relevance of such co-expression is not yet fully understood, but likely representative of the extensive integration that characterizes the PVN.

1.4 Physiological Regulation and the PVN

The PVN is recognized as one of the most crucial central sites of autonomic regulation, contributing to stress, reproduction, gastrointestinal function, fluid balance, immunity, growth and cardiovascular function (Ferguson *et al*,. 2008; Sawchenko & Swanson, 1982). Importantly, the PVN integrates information about each of these systems in order to maintain homeostasis throughout the ANS.

1.4.1 Cardiovascular Regulation and the PVN

Cardiovascular regulation involves continuous modulation of arterial pressure, venous pressure and cardiac output, the coordination of which forms a division of the ANS (Petersson *et*

al., 1996). Such constant regulation relies upon a steady communication of physiological state and responsiveness. In order to communicate physiological state relating to cardiovascular function, a complex integration of signals must be maintained and relayed to initiate effector responses (Porta *et al.*, 2009). The PVN is a key central site where such specialized modulation is carried out, for which it is recognized as one of the most important autonomic regulatory sites (Ferguson *et al.,* 2008; Pyner, 2014). Central regulation of cardiovascular function extensively considers physical, molecular and chemical information, such as baroreflexes, blood-oxygen levels, as well as sympathetic tone (Porta *et al.,* 2009). Signals that provide this information are relayed from other nuclei in the hypothalamus, basal forebrain and cerebral cortex, often to areas within the lower brain stem and, importantly, to the PVN (Loewy, 1991; Ferguson *et al.*, 2008).

The PVN is recognized as a crucial central site for modulation of sympathetic activity (Guyenet, 2006). Additionally, the RVLM and NTS both possess cardiovascular sensing neurons which provide afferent information to PA neurons in the PVN. These afferents either excite or inhibit the PVN neurons depending on the signal in relay, whether it originated at arterial baroreceptors, chemoreceptors, or venous receptors (Pyner, 2014). In particular, the PVN has been shown to influence the excitability of output signaling following the integration of the described afferents (Ferguson *et al.,* 2008). Following signal integration, the PVN exerts effects on other central sites and target organs, which ultimately result in integrated cardiovascular responses (Pyner, 2013; Coote, 2005).

Pathways in the PVN influence sympathetic activity through direct synapses on pre-ganglionic sympathetic neurons in the intermediolateral cell column of the spinal cord, innervating the heart, kidneys, adrenal medulla, and vasculature (Pyner, 2013). Sympathetic tone is subject to tonic inhibition by GABAergic synapses on PA neurons and nitric oxide which

facilitates them (Ferguson *et al.*, 2008; Pyner, 2013) and is increased through disruption of the tonic inhibition by various signaling molecules, such as glutamate (GLU), ANG II, OXT, AVP, and dopamine (Pyner, 2013).

1.4.2 Gastrointestinal Function and the PVN

The GI system involves the absorption of necessary fluids and nutrients, as well as the excretion of metabolites and other waste products. Stress, inflammation and other autonomic responses play a role in these processes, which are additionally under the regulation of the gut-brain axis (Greenwood-Van Meervald *et al.*, 2016). Such regulation, however, is not limited to autonomic processes but also extends to behavioural responses, including satiety signals (Browning & Travagli, 2011). Interestingly, central regulation of GI function is increasingly implicated in GI diseases and dysregulation in energy balance, despite the presence of a relatively autonomous regulatory network of intrinsic neural plexuses (Greenwood-Van Meervald *et al.*, 2016; Browning and Travagli, 2011). Central regulation of energy balance integrates adiposity signals, osmotic stimuli, and detailed information about the gut, such as composition of the microbiota (Qin *et al.*, 2018). Regulation of gastrointestinal function, and fluid and electrolyte balance, are among the classical roles for which the PVN is recognized (Ferguson *et al.*, 2008).

Through sympathetic pathways, the PVN is known to control both smooth and coordinated digestive processes in the esophagus, stomach, intestines, as well as GI blood flow (Browning & Travagli, 2011; Travagli *et al.*, 2006). Innervations of the stomach, small intestine and colon originate from the dorsal motor nucleus of the vagus (DMNV), a projection site of the PA neurons (Browning & Travagli, 2011). Neurons of the DMV give rise to two pathways for

central regulation of GI function, the excitatory cholinergic pathway and the inhibitory non-adrenergic non-cholinergic (NANC) pathway. The former drives contraction of smooth muscle through muscarinic cholinergic receptors, while the latter promotes smooth muscle relaxation through release of nitric oxide or vasoactive intestinal polypeptide (VIP) (Browning & Travagli, 2011). The PVN has been shown to modulate these pathways through direct projections, many of them associated with specific peptide secretion (e.g., CRH) (Browning & Travagli, 2011; Stengel and Taché, 2011). The CRH axis of the PVN likely initiates a corticotrophin-releasing factor (CRH) signaling cascade that drives a visceral response. Interestingly, this is one mechanism that may account for the impact of stress responses on GI function, implicating the role of stress responses in autonomic functions through the PVN (Bains *et al.,* 2015; Dunn & Berridge, 1990; Stengel and Taché, 2011; Taché *et al.,* 2001).

1.4.3 Reproductive Function and the PVN

A bidirectional relationship links the HPA and HPG axes, or stress and reproductive axes, whereby signaling events in one axis may alter those in the other. Activation of the HPA axis influences signals that compromise the HPG axis, such as sex hormone secretion and receptor expression (Toufexis *et al.,* 2014). Although many central sites are known to sense reproductive state and initiate reproductive responses, the PVN has been implicated in the integration and regulation of these pathways.

The PVN receives afferents from regions with possible reproductive roles, including the NTS, and has also been shown to express the estrogen receptor (ER) (Simerly, 1998; Simerly *et al.,* 1990; Estacio *et al.,* 1996). Further, central regulation of reproduction involves modulation of gonadotropin secretion, which is predominantly under the inhibitory control of GnRH

(Tsuitsui *et al.,* 2010). GnRH modulates release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GnRH II has been identified in the PVN (Bentley et al., 2006; Rivalland et al., 2006). It is known that CRH and AVP suppress the reproductive axis, and that GnRH expression is reduced in stressed animals (Rivalland *et al.,* 2006). Neurosecretory cells within the PVN may modulate the reproductive axis in the presence of stressors through this mechanism. Importantly, the reciprocal relationship of the HPA and HPG axes is dependent upon pathways which link the two together (Melón & Maguire, 2016).

Additional interactions occur between reproductive pathways and those relating to energy balance and cardiovascular control (López & Tena-Sempere, 2015). The independent activity and the bidirectional interactions of these axes are closely modulated by the PVN, dysregulation of which may contribute to alteration of behavioural and physiological responses (Toufexis et al., 2014; Ferguson et al., 2008). Previous studies have identified a decrease in secretion of LH in acutely fasted animals, proposing the effect to be mediated through noradrenergic inputs to the PVN and CRH release in light of fasting being a type of stressor (Estacio et al., 1996). ER mRNA expression is necessary for fasting-inhibited LH release pulses and was elevated in the PVN following acute fasting (Estacio et al., 1996). The effects of fasting on these select reproductive signals exemplify the pathways by which autonomic systems interact.

1.4.4 Stress Responses and the PVN

Intriguingly, stressors and other stimuli are thought to primarily influence reproduction via the brain, although interactions occur through other major organs (Brann & Mahesh, 1994; Tilbrook *et al.,* 2000). However, the reproductive axis is not the sole target, or initiator, of stress responses. The term stressor encapsulates numerous and diverse disruptions to homeostatic

function, which is restored through orchestrated responses at central regulatory sites (Tilbrook *et al.,* 2000). Stressors may manifest as environmental factors or internal physiological processes and influence the relationships between physiological systems (Killen *et al.,* 2013).

Stress response initiation activates the HPA axis, in turn, initiating the release of CRH and AVP at the median eminence and subsequent receptor binding of respective anterior pituitary receptors. ACTH is released from the anterior pituitary, exerts effects on the adrenal cortex and triggers the release of glucocorticoids into circulation (Herman *et al.,* 1996; Herman *et al.,* 2000; Tsigos *et al.,* 2002). Chronic activation of the HPA axis has been linked to the development of disease states, as such, glucocorticoid feedback mechanisms act on the axis to inhibit CRH and ACTH release. However, stress also serves an adaptive evolutionary purpose through activation of the SNS and corresponding flight-or-flight response. As previously mentioned, sympathetic activation satisfies the need for increased cardiac output during life-threatening events (Herman *et al.,* 2000; Nunn *et al,* 2011). It is critical that this pathway return to baseline once a stress response is no longer beneficial as when this does not occur, pathophysiological states can develop, and stress can become detrimental.

The PVN has been implicated in the regulation of both behavioural and physiological responses to stress as it drives, and is subject to, the central, endocrine and immune responses which comprise them (Smith & Vale, 2006). Recognized as the principle effector of stress responses, the PVN is responsible for the release of the principle regulator of stress responses, CRH (Herman & Tasker, 2016; Smith & Vale, 2006). Both the MNC and parvocellular divisions of the PVN are additionally responsible for secretion of AVP, which modulates the effects of CRH on release of ACTH after entering portal circulation (Herman & Tasker, 2016). Further, GABAergic neurons have been shown to regulate the integration of afferents involved in stress

signaling. Central regulatory sites of energy balance, immunity, cardiovascular regulation and respiratory function directly innervate the PVN, and are also target sites of PVN projections (Herman & Tasker, 2016; Smith & Vale, 2006).

Not only does chronic activation of this system result in certain pathologies, but insufficient activation may be as equally detrimental. Interestingly however, the PVN is especially important in adaptations to chronic stress. A unique level of cellular and molecular plasticity has been demonstrated in NE CRH neurons in the presence of repeated stress exposure (Herman & Tasker, 2016). Through lesioning studies, the CRH neurons have been implicated in both physiological and behavioural responses to stress exposure. For example, lesions of the subpopulation in rats decrease anxious behaviours, such as grooming, when in novel environments (Herman & Tasker, 2016). Additionally, these PVN neurons are upregulated and become increasingly excitable following restraint, foot-shock, predation exposure and chronic stress (Maxson *et al.,* 2009; Herman & Tasker, 2016; Smith & Vale, 2006).

Chronic stress exposure can become detrimental due to sensitization of the HPA axis or desensitization to inhibitory glucocorticoid feedback (Maxson *et al.,* 2009). The development of hypertension, congestive heart failure (CHF), obesity and other disease processes are common consequences of repeated and prolonged exposure to stress (Ferguson *et al.,* 2008). Alterations in regulation by PVN contribute to the development of these diseases, as well as that of autoimmune, reproductive health, renal, mood and respiratory diseases, (Abelson *et al.,* 2010; Benarroch, 1993; Ferguson *et al.*, 2008; Hall *et al.*, 2014; Watson & Mackin, 2006). Extensive understanding of a role for PVN in autonomic processes, but importantly in both adaptive and maladaptive stress response, has been developed and necessarily sparked investigation into possible molecular mechanisms underlying such effects.

1.5 Phoenixin

Phoenixin (PNX) is a novel peptide initially described for its potential roles in the regulation of reproductive functions, but which has more recently also been suggested to be involved in memory consolidation, anxiety and stress responses, feeding behaviours, gastrointestinal motility, visceral pain and hormonal cyclicity (Yuan *et al.,* 2017; Yosten *et al.,* 2013). Although the PNX receptor, G protein-coupled receptor 173 (GPR173), has been identified, underlying mechanisms of action need to be further investigated (Yuan *et al.,* 2017; Yosten *et al.,* 2013; Hazell *et al.,* 2012). It is accepted that PNX is highly conserved across species, meaning 0 and exhibits diverse expression throughout the body and CNS (McIlwraith *et al.,* 2018; Treen *et al.,* 2016). Current hypotheses suggest that PNX may act as a facilitator signal for reproduction when adequate nutritional requirements are met, as it is known to sense nutritional state as well as influence reproductive signals (McIlwraith *et al.,* 2018). Thus, this novel peptide may exert metabolically influenced control over reproduction, simultaneously mediating a homeostatic balance between other autonomic systems.

Prior to fulfilling these roles, PNX expression may be influenced by both physiological state and the presence or absence of other transmitters and peptides including palmitate, DHA, oleate and BPA (McIlwraith *et al.,* 2018). Initial cleavage of the peptide PNX is achieved with the small integral membrane protein 20 (SMIM20), which generates isoforms PNX-14 and PNX-20 (McIlwraith *et al.,* 2018). Expression varies between the two isoforms, with common immunoreactivity being greatest throughout the SON, AP, Arc, DMVS, ME, NTS, zona incerta, Erdinger-Westphal nucleus, and importantly, the PVN (Yosten *et al.,* 2013; Prinz *et al.,* 2017). PNX-14 is additionally localized to the rat spinal cord, spinal trigeminal tract, dorsal root ganglion cells and dorsal horn (Lyu *et al.,* 2013; Cowan *et al.,* 2015). Outside of the CNS, PNX

is also expressed within the heart, GI tract, kidneys, pancreas, ovaries, testis and lungs (McIlwraith *et al.,* 2018; Stein *et al.,* 2016). PNX expression is greatest in the hypothalamus, a central site by which it is now thought to modulate aspects of autonomic function, in addition to the reproductive pathways.

Both isoforms of PNX act on the hypothalamus-pituitary-genital (HPG) axis by modulating other peptides, such as kisspeptin, gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) (Treen *et al.,* 2016; Yuan *et al.,* 2017). Additionally, PNX modulates the GnRH receptor which results in regulation of gonadotropin release from the pituitary (Yosten *et al.,* 2013). A reduction in PNX as a hypothalamically-derived peptide has been shown to compromise the onset of oestrous, with a corresponding decrease in GnRH receptor expression levels throughout the pituitary (Matsumoto *et al.,* 2000; Stein *et al.,* 2016; Yosten *et al.,* 2013). PNX may therefore mechanistically explain the role of stress in compromised cyclicity and reproductive pathologies (Yosten *et al.,* 2012; McIlwraith *et al.,* 2018).

Recent studies have demonstrated a potential role for PNX in the central regulation of stress responses, specifically within the NTS, an area with vast reciprocated projections to the PVN. Together with the previously described effects on oestrous cycling, a clearer understanding of the central role of PNX may provide insight into mechanisms underlying stress-related infertility (Grover & Ferguson, 2018). The NTS not only shares these projections with the PVN but is physiologically relevant to its regulation of autonomic function (Kalia & Mesulam, 1980). Additionally, given the high expression levels of PNX throughout the PVN and the presence of receptor GPR173, the PVN represents a potentially important site at which PNX may act to elicit many of its physiological actions.

1.6 Aim of the Study

This study was undertaken to investigate the effects of PNX in the PVN. We hypothesized that PNX would stimulate changes in extracellular spike frequency in PVN neurons due to the substantial expression of PNX and GPR173, as well as previous depolarizing effects demonstrated on AVP neurons. To examine the effects of PNX, we used a multi-electrode array system and recorded extracellular spike frequency after bath application of the peptide. After observing responses in PVN neurons, we sought to determine whether these responses were due to direct or indirect actions of PNX. Therefore, the aim of the study was to establish the PVN as a site of action for PNX, and whether those actions were dependent on synaptic transmission. Our specific hypotheses to be tested were that PNX would 1) elicit changes in spike frequency in PVN neurons, and 2) exert both direct and indirect effects on these neurons.

Chapter 2

Materials and Methods

2.1 Animals

All experiments utilized juvenile (post-natal 21-28 days) male Sprague-Dawley rats that were purchased from Charles River Laboratories (Montreal, Quebec, Canada). Male rats were used to obtain data comparable to the current literature. Animals were provided with both food and water ad libitum while housed in a pathogen-free room maintained at 22^{o}C with a 12:12 hour light: dark cycle. Approval for protocol number 1735 was obtained for all experimental procedures using these animals from the Queen's University Animal Care Committee and was carried out in agreement with the Canadian Council on Animal Care Guidelines.

2.2 Slice Preparation

On each day of experimentation, coronal hypothalamic slices containing PVN were obtained from isoflurane-anesthetized rats. Following decapitation, brains were quickly removed and immersed in ice-cold (0–4°C) slicing solution containing the following (in mM): 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.25 NaH_2PO_4, 25 glucose, and 75 sucrose, as well as bubbled with 95% O_2/5% CO_2. Each brain was blocked, mounted and cut into 300 μm coronal slices, containing the PVN, using a vibratome (VT1000 S; Leica, Germany). The slices were then incubated at 32°C, for a period of at least 1 h, in artificial cerebrospinal fluid (aCSF) containing the following (in mM): 124 NaCl, 2.5 KCl, 20 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgSO_4$, 1.24 KH_2PO_4 and 10 glucose, pH 7.3-7.35, osmolarity 280-290 mOsm, and bubbled with 95% O_2/5% CO_2.

2.3 Electrophysiology

The MED64 (AlphaMED Sciences, Japan), a system comprised of a 64-electrode array, was used to record *in vitro* extracellular potentials in the slices containing PVN. Recordings were obtained using an 8x8 planar multi-electrode array (MEA), covering a total area of $1mm^2$, with a spacing diameter of 150 μm between each electrode (P515A Probe, AlphaMED Sciences). Prior to use, each new probe underwent overnight treatment with aqueous 0.1% polyethyleneimine solution to decrease hydrophobicity of the platinum black microelectrodes, which were then submerged in distilled water and stored at 4°C for subsequent experimentation.

At the start of each day of recording, noise optimization was carried out with the positioned MEA probe and perfusion of carbogenated aCSF, maintained at a temperature of 37°C (MED64 ThermoBase, MED-CPB01). The slices containing PVN were quickly transferred to the MEA probe, following which excess solution was removed to allow for adequate contact between the slice and bottom of the probe. Once approximately positioned, a U-shaped weighted slice anchor with synthetic threading was placed in order to hold the slice down and maintain contact with the electrode array (Scientific Systems Design Inc., Mississauga, Ontario). Prior to final positioning of each slice, carbogenated aCSF was carefully dropped onto the slice, which was then visualized under a Nikon Eclipse TS100 10x magnification dissection microscope (New York, USA), and the slice optimally orientated using a small brush such that the electrode array was below PVN on each side of the 3rd ventricle **(Figure 1).** In order to anatomically verify each individual slice position relative to the probe, digital images were taken prior to each experiment using the Nikon microscope. Finally, the probe was then positioned within the plate and attached to the MED Connector (MED-C03, AlphaMED Sciences), and carbogenated aCSF heated to 37°C was perfused over the slice through a cap attachment (MED-KCAP01,

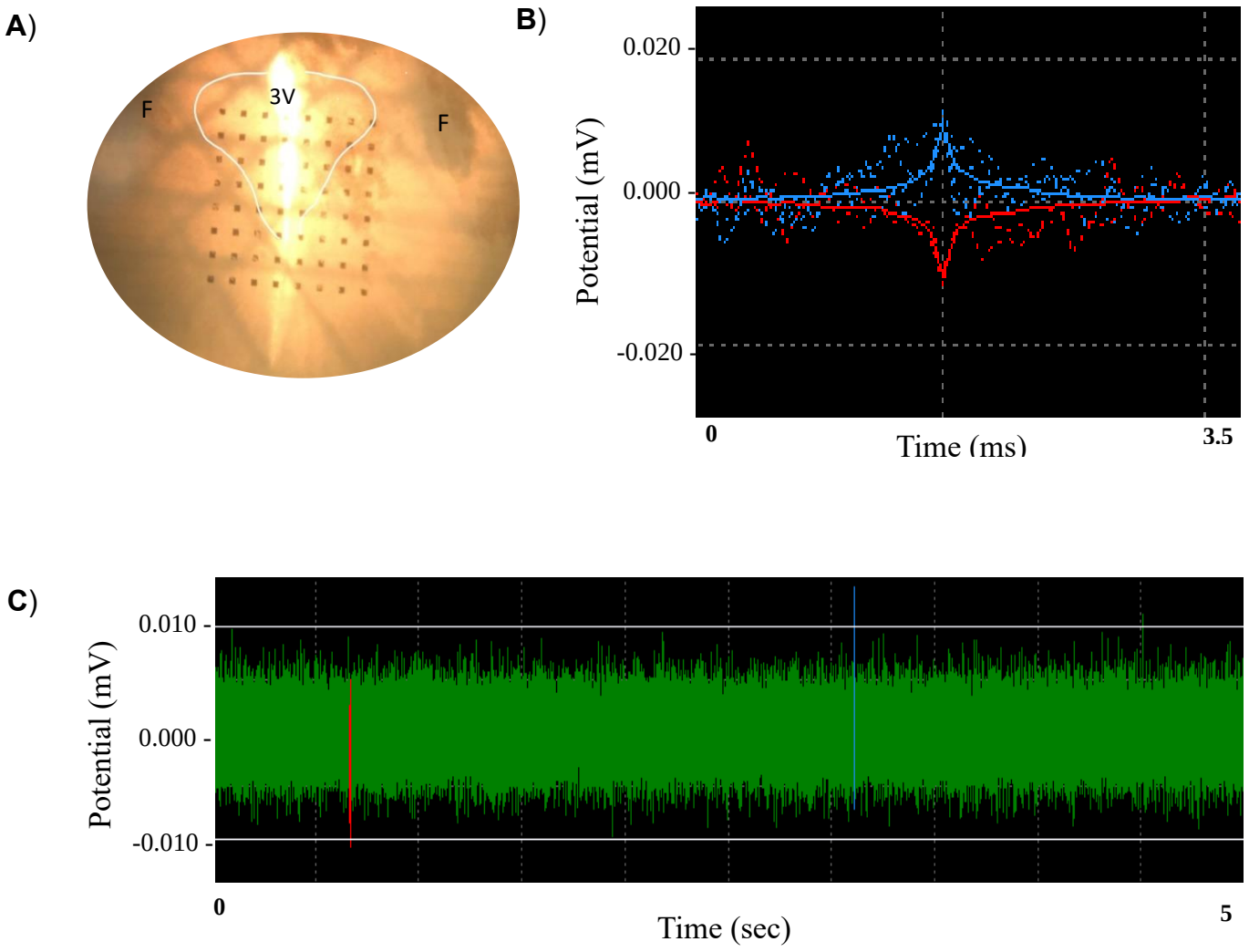

Figure 1. Extracellular spikes in PVN. **A)** Coronal slice containing PVN (white line) with MEA correctly positioned (black grid), yielding 18 electrodes selected for analyses in the Mobius software. Anatomical reference points include the fornix (F) and third ventricle (3V). **B)** Extracted spike centroids over a 2 ms period, demonstrating two distinct spike populations (red and blue traces) based on classification criteria: 50% similarity radius and ± 0.01 mV spike amplitude thresholds. Each centroid demonstrates the unique amplitude and temporal characteristics of distinct spike populations. The dotted lines for each centroid represent variability of the individual spikes within the respective centroid classification. **C)** Two individual extracellular spikes (red and blue lines) from two distinct spike populations. Each spike corresponds to the B) centroid classifications.

AlphaMED Sciences) at a rate of 1mL/min controlled by a matched intake/ output peristaltic pump Perfusion occurred at a rate of 1mL/ min via an intake/ outtake peristaltic pump (Gilson Minipuls 3, Wisconsin).

Prior to the onset of the spontaneous spike recording protocol, each slice was allowed to rest in the MEA probe for a period of at least 30 minutes. Signals were amplified 1000x and recorded at a sampling rate of 20 kHz with 16-bit resolution (MED-A64HE1S and A64MD1 amplifiers, AlphaMED Sciences). Baseline activity was recorded for a period of 10 minutes, following which the perfusion intake was paused for 5 seconds while intake tubing was transferred to beaker containing carbogenated aCSF with 100nM PNX, utilized so that effects would be observed in initial experiments if present. Perfusion was resumed and a 300 second period of application of PNX in aCSF was completed. Following application, the same protocol was followed in order to return the tubing to the regular aCSF. For control purposes, initial recordings involved subsequent application of ANG II, which was applied under the previously described protocol after completion of a minimum washout period of 30 minutes.

Offline analysis of recordings was completed using Mobius, AlphaMED Science software. Channels which corresponded to the area of slice containing PVN were selected for analysis using Mobius. Extracellular spikes were categorized into distinct populations, along with the generation of spike centroids based on a minimum 50% similarity radius to the recorded spike. The spike frequency (Hz) of each centroid was plotted for the total recording period, in bins of 30 sec. The following criteria for determining responsiveness was established and used for all analyses. Neurons were identified as responsive to PNX if the change in spike frequency during the application period was greater or less than 20% for excitations and inhibitions, respectively, of the baseline period immediately prior to application (270 sec). The arbitrary

determination of criteria for placing single neurons into each of these response groups precludes further statistical analysis.

2.4 Chemicals and Drugs

All compounds utilized for making slicing solution, aCSF and MEA polyethyleneimine solution were purchased from Sigma Pharmaceuticals (Oakville, Ontario, Canada). Isoflurane was obtained from Fresenius Kabi (Toronto, Canada). PNX was purchased from Phoenix Pharmaceuticals (California, USA). PNX at 4µM was diluted with distilled water to 100nM, and then stored in 10µL aliquots kept at -80°C. Immediately before application for each experiment, PNX was diluted in aCSF to a desired concentration of 100nM. ANG II was likewise purchased from Phoenixin Pharmaceuticals (California, USA), diluted with distilled water to 0.1mM and stored at -80°C in 10µL aliquots. Immediately before application for each experiment, ANGII was diluted in aCSF to a desired concentration of 100 nM.

2.5 Statistical Analyses

A Chi-Squared Test was completed for analysis of response proportions in regular aCSF and low-Ca^{2+}/ high-Mg^{2+} aCSF. To analyze the mean baseline firing frequency in neurons from the same groups, regular and low-Ca^{2+}/ high-Mg^{2+} aCSF, a Student's Unpaired T-Test was used.

Chapter 3

Results

3.1 PNX Modulates Spike Frequency of PVN Neurons

Initially, we aimed to determine if there were effects of PNX on the firing frequency of PVN neurons using a multi-electrode array (MEA). Extracellular recordings were carried out in regular aCSF from 25 slices taken from 15 rats, each yielding one to two slices containing the PVN. Following positioning on the MEA probe, maximizing the number of the available 64 electrodes over the PVN, each slice was allowed to stabilize for a period of 30 minutes with continuous perfusion of carbogenated aCSF throughout. Stabilization was completed prior to baseline recordings, during which the majority of selected channels exhibited visible spontaneous extracellular spiking activity. Between 12 and 18 channels from each slice were selected for analysis based on anatomical positioning within the PVN, as identified by adjacent structures including the third ventricle and bilateral fornix **(Figure 1A).** These methods generated a total of 824 PVN neuron recordings.

The built-in spike recognition tool was used to distinguish the spikes of individual neurons in Mobius software following recording sessions. Extracted spike centroids, or spike categories, are determined based on manually selected classification criteria of a 50% similarity radius **(Figure 1B)** and ± 0.01 mV spike amplitude thresholds **(Figure 1C)**, as well as a minimum of 100 spikes identified during the recording time. Each centroid represents the average physical (amplitude, shape) and temporal characteristics of the distinct spike populations. Thus, all spikes classified into one centroid are at least 50% similar to each other in the physical and temporal characteristics, and so representing spikes from an individual neuron.

On average, two to three distinct populations of spikes were identified for each individual electrode.

Following the stabilization period and baseline recording, the effects of PNX were investigated. Bath application of 100nM PNX for 300 seconds resulted in differential responses. Specifically, we observed three different types of responses in PVN neurons: increased spike frequency **(Figure 2A)**, decreased spike frequency **(Figure 2B)**, and elicited no change in spike frequency **(Figure 2C)** in PVN neurons.

In the total 824 neurons, PNX elicited an increase in spike frequency in 16% (n=130)**,** decreased in 14% (n= 117)**,** and elicited no effect on the spike frequency of the remaining 70% (n= 577) of neurons **(Figure 3).** Therefore, PNX modulates the spike frequency of PVN neurons, through both excitations and inhibitions, as observed through extracellular recordings.

3.2 PNX Exerts both Direct and Indirect Effects on PVN Neurons

Subsequent investigations sought to determine if PNX modulates PVN neurons through direct or indirect actions. Specifically, we investigated if the responses were due to modulation of synaptic transmission, modulation of intrinsic neuronal excitability, or a combination of the two mechanisms. In order to block synaptic transmission and isolate the potential direct effects of PVN, recordings were carried out in low-Ca^{2+}/ high-Mg^{2+} aCSF (Hackett, 1976; Dingledine & Somjen, 1981; Sun & Ferguson, 1997). These experiments demonstrated that the effects of PNX are maintained in low-Ca^{2+}/ high-Mg^{2+} aCSF. A total of 10 slices from 8 animals provided a total of 187 cells; of which, increased spike frequency **(Figure 4A)** was observed in 5% of PVN neurons (n= 7), decreased spike frequency **(Figure 4B)** in 22% (n= 43), and no elicited response was observed in the remaining 73% (n= 137) of neurons.

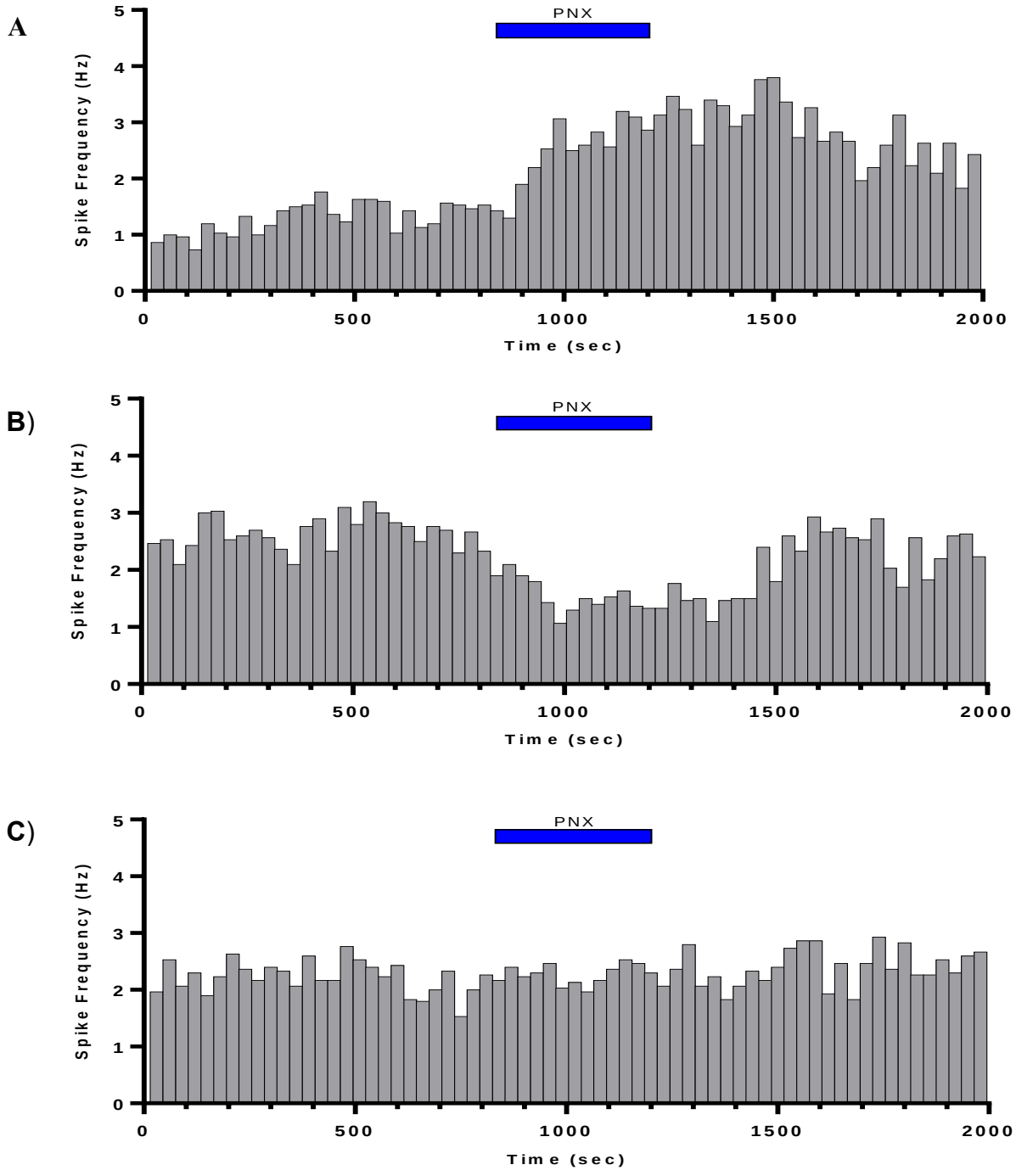

Figure 2. Extracellular effects of 100nM PNX (blue bar, 300 seconds) on PVN neurons. Each record is an individual example (n=1) from each response type. Recording is calculated in 30-second bins over the recording period for an individual slice, and represents the extracellular spike frequency (Hz) of one cell taken from one channel of the MEA probe. **A)** Example ratemeter record for one excited cell, from a total of n =130. **B)** Example ratemeter record for one inhibited cell, from a total of n = 117. **C)** Example ratemeter record for one non-responsive cell, from a total of n = 577.

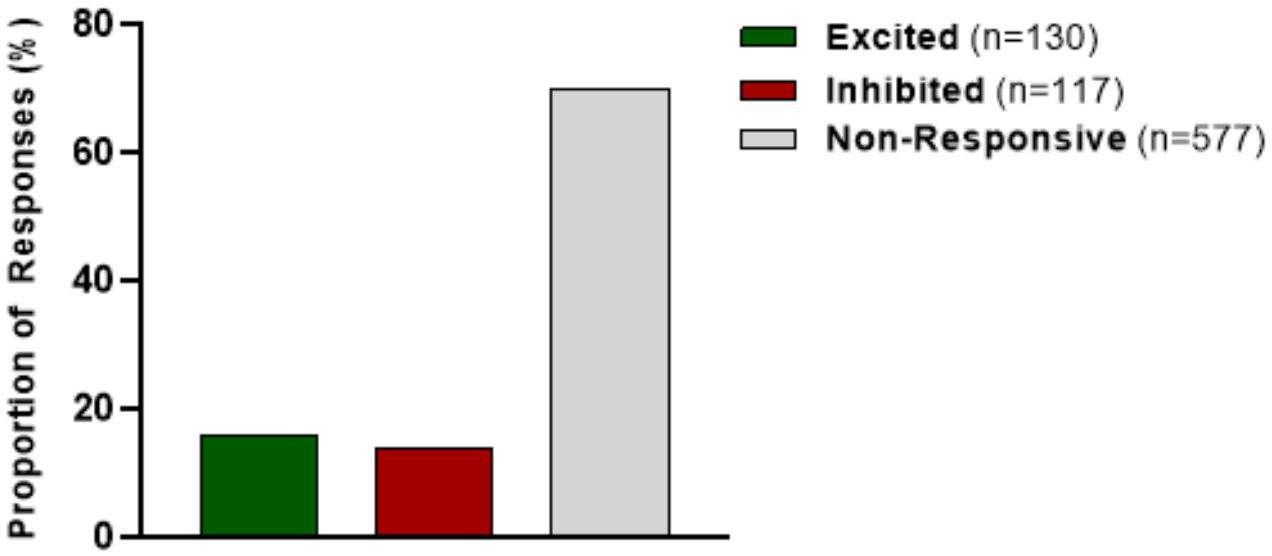

Figure 3. Proportion summary of response types to PNX in PVN neurons recorded from individual slices in regular aCSF. A total of 824 cells were selected for analyses, with 16% (n=130) being excited, 14% (n=117) inhibited and 70% (n=577) non-responsive. Current literature has identified larger proportions of excitations than observed in our present data. This may be explained by our different methods for selecting cells for analysis.

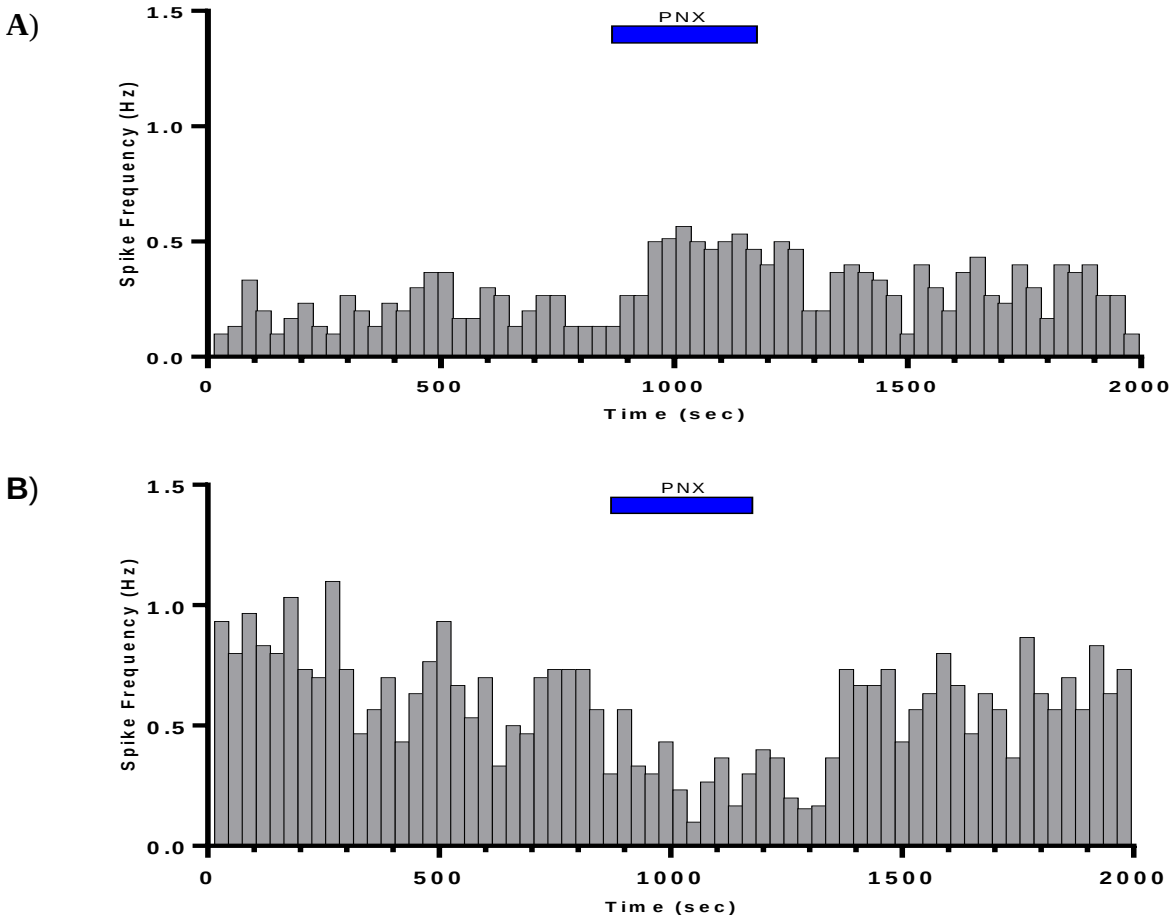

Figure 4. Extracellular effects of 100nM PNX (blue bar, 300 seconds) on PVN neurons from slices in low-Ca^{2+}/ high-Mg^{2+} aCSF. . Each record is an individual example (n=1) from each response type. Recording is calculated in 30-second bins over the recording period for an individual slice, and represents the extracellular spike frequency (Hz) of one cell taken from one channel of the MEA probe. **A)** Example ratemeter record for one excited cell, from a total of n = 7. **B)** Example ratemeter record for one inhibited cell, from a total of n = 137.

Although both affects were maintained, we observed that blocking synaptic transmission reduced the proportion of excitations and increased the proportion of inhibitions. Therefore, PNX may excite PVN neurons both directly and indirectly. The later may occur through inhibitory effects on inhibitory GABAergic interneurons to indirectly modulate PVN neurons. These observations support the hypothesis that actions of PNX on PVN neurons are both direct and indirect **(Figure 5).** The observed increase in the proportions of inhibitions may be explained as a mathematical artifact resulting from the different number of total recordings obtained in the low-Ca^{2+}/ high-Mg^{2+} aCSF data set, as data is interpreted as a percentage. Further, these conditions are known to decrease spontaneous activity in neurons and, as such, may influence the number of recordings obtained from certain populations of neurons as channels without spontaneous activity are not selected for analysis.

3.3 PNX Response Characteristics

The mean spike frequencies for all excited, inhibited and non-responsive neurons demonstrate clear effects of the peptide PNX on PVN neurons **(Figure 6).** In regular aCSF, PNX elicited a peak increase in spike frequency for all excited neurons (n= 130) of 0.44 ± 0.275 Hz, and a mean peak reduction in spike frequency of 0.19 ± 0.074 Hz across all inhibited neurons (n= 117). The onset of response was delayed in excitations, with an average duration of 37 seconds, compared to the shorter average onset duration of 17 seconds that was observed across the inhibited neurons. Complete recovery was not observed in either group of neurons, which is likely a result of the time course of washout, as determined by slice thickness and the varying location of cells throughout.

Additionally, in a subgroup of excited neurons demonstrating increases in spike frequency of greater than 100% (n= 25) **(Figure 7)**, both latency to and duration of response appeared similar to those with a greater than 20% increase; thus, suggesting that these cells truly represent differential responsiveness of subgroups of PVN neurons. PNX elicited a peak increase in spike frequency of 2 ± 0.041 Hz for this group of neurons, compared to the 0.44 ± 0.275 Hz seen in all other excited neurons. No neurons exhibited decreases in spike frequency by greater than 100%. These findings provide additional support for the hypothesis that PNX differentially modulates subpopulations of PVN neurons.

It was observed that the neurons examined in low-Ca^{2+}/ high-Mg^{2+} aCSF had a lower mean spike frequency (0.81 ± 0.018, 0.63 ± 0.010, 1.13 ± 0.014) than those in regular aCSF (0.22 ± 0.006, 0.25 ± 0.005, 0.71 ± 0.007), for excited, inhibited and non-responsive neurons, respectively **(Table 1).** Baseline activity represents neuronal spontaneous activity. The average baseline spike frequency was significantly lower in neurons in low-Ca^{2+}/ high-Mg^{2+} aCSF than those in regular aCSF, as indicated by a Student's Unpaired T-Test ($P < 0.001$). This impact on spontaneous activity is expected as calcium current is being eliminated from the observed spikes.

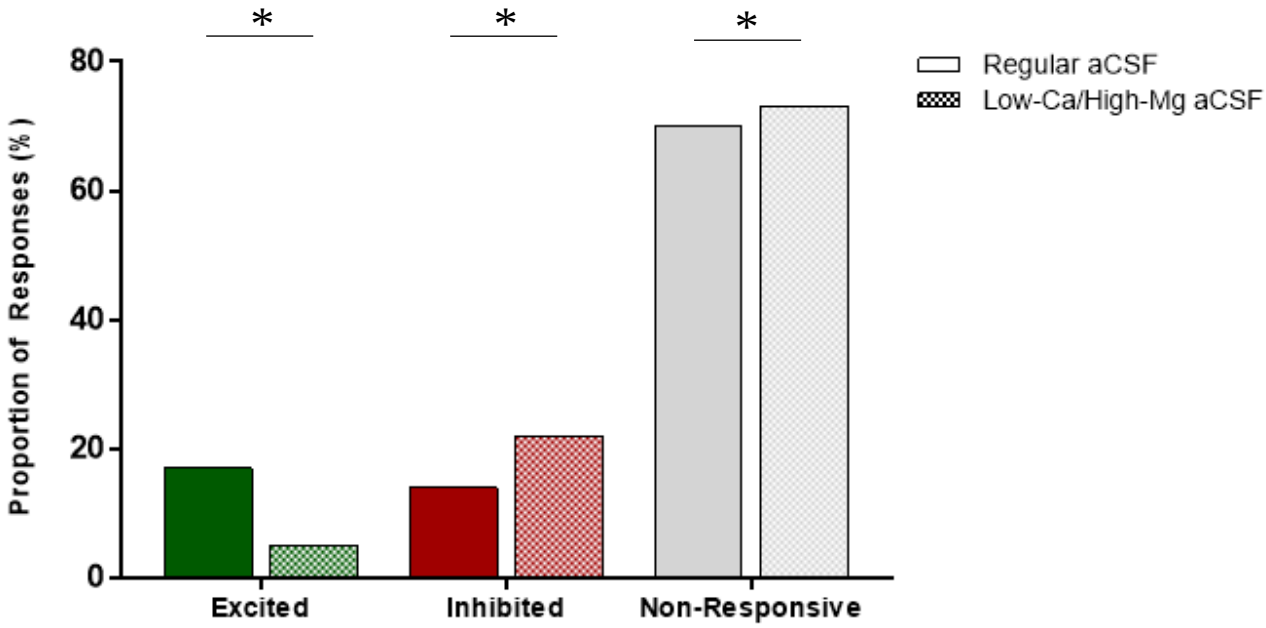

Figure 5. Summary of proportions of response types to PNX of PVN neurons from slices in regular and low-Ca^{2+}/ high-Mg aCSF. A total of 187 neurons were recorded from in low-Ca^{2+}/ high-Mg aCSF, with 5% being excitatory (n=7), 22% inhibited (n=43) and 73% non-responsive (n=137). In regular aCSF, a total of 824 neurons were recorded from with 70% non-responsive (n=577), 16% (n= 130) were activated, while 14% (n= 117) were inhibited. As determined by a Chi-Squared Analysis, the observed values in regular aCSF are statistically different than the values obtained in low-Ca^{2+}/ high-Mg^{2+} aCSF (P = < 0.05).

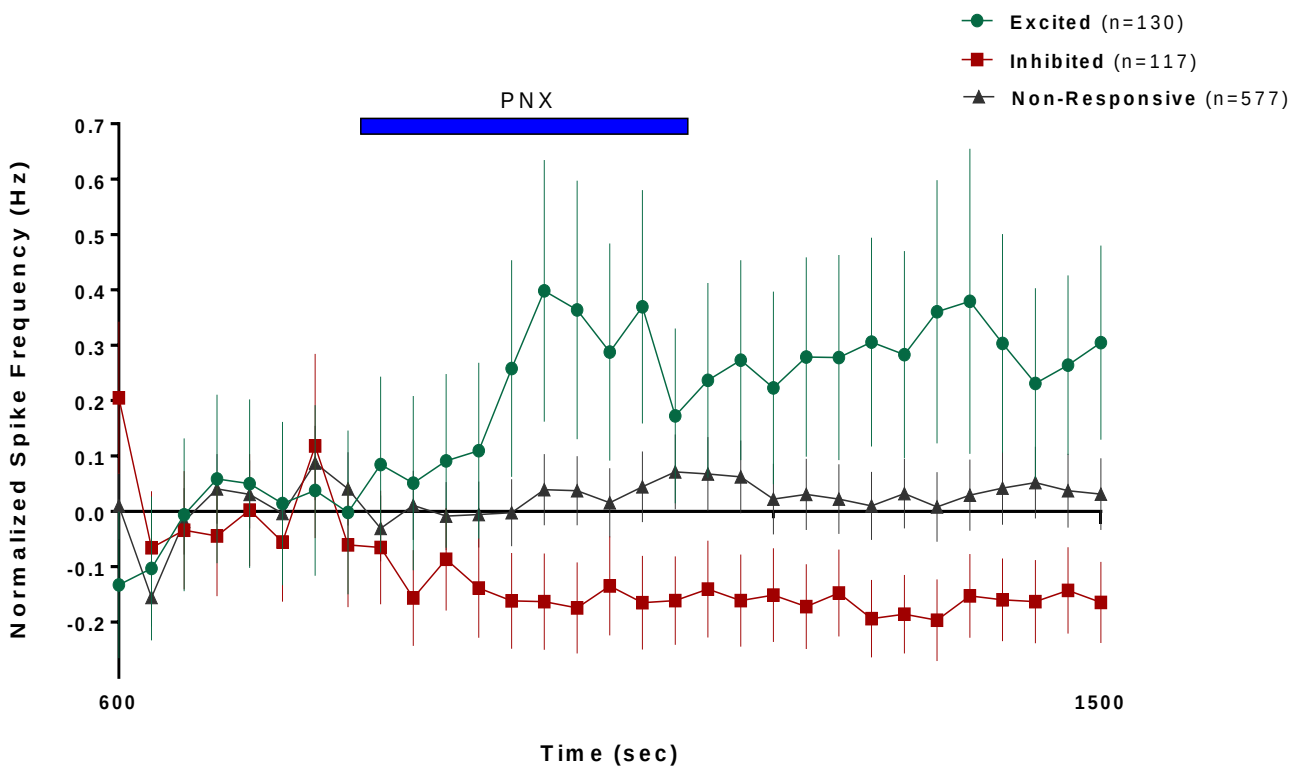

Figure 6. The mean spike frequency for recordings from a total of 824 neurons, with 16% (n= 130) of cells being activated by bath administration of 100nM PNX (blue bar), while 14% (n= 117) were inhibited. Remaining cells tested, 70%, were classified as non-responsive (n= 577). A peak firing frequency of 0.44 Hz (SEM= 0.275) was observed in excited cells, as well as a reduction in firing frequency of 0.19 Hz (SEM= 0.074) in cells that were inhibited. Remaining cells tested were classified as non-responsive. Error bars represent SEM. Results are normalized to the baseline period corresponding with onset of PNX application preparation to the onset of bath-application.

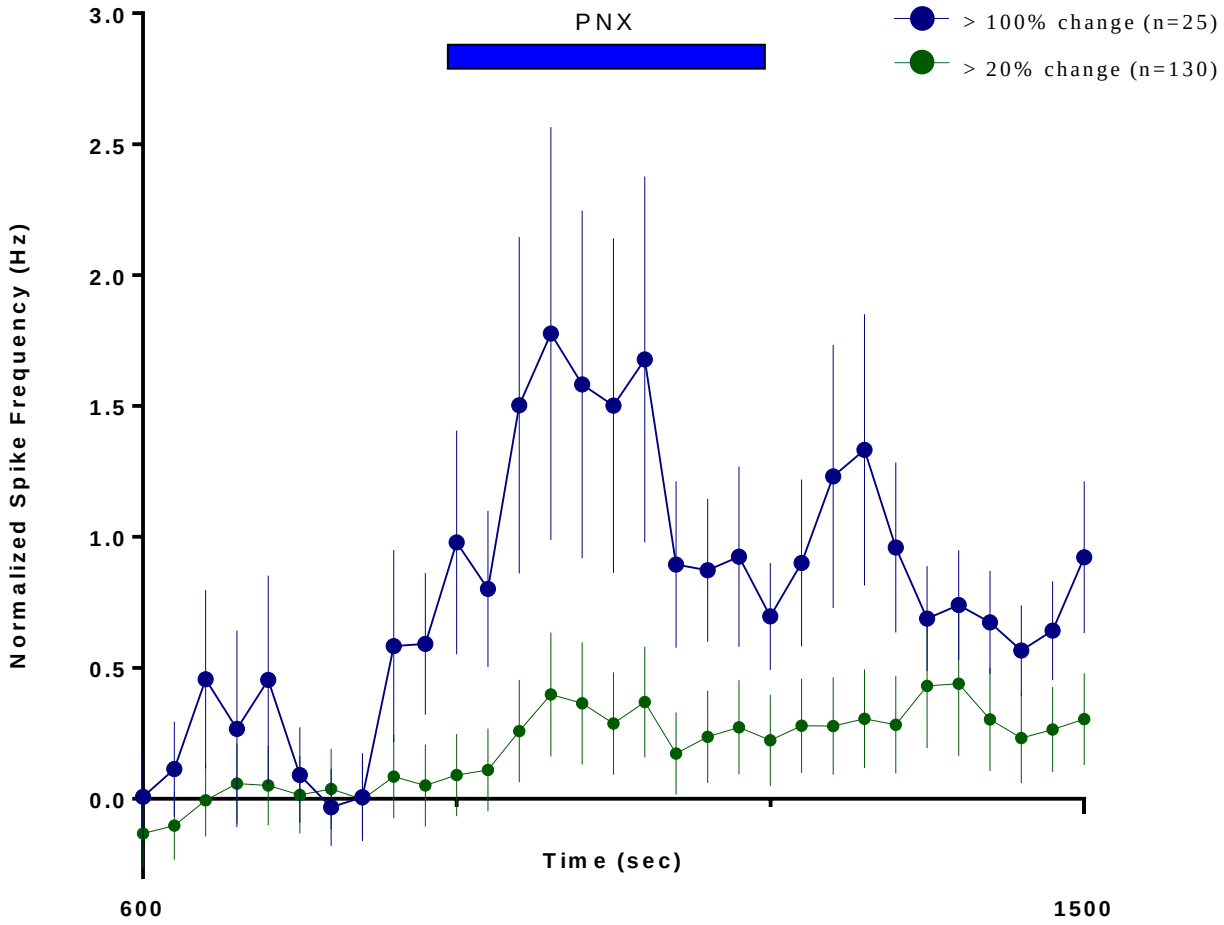

Figure 7. Extracellular effects of 100nM PNX (blue bar, 300 seconds) on PVN neurons from slices in regular aCSF. Ratemeter record of extracellular spike frequency (Hz) for excited cells that responded to PNX with at least a 100% increase in spike frequency (n= 25). Excited cells that responded to PNX with at least a 20% increase in spike frequency (n=130), as shown in Figure 6. Recordings are calculated in 30-second bins over the recording period for an individual slice. Error bars represent SEM. The demonstrated difference in these response amplitudes may represent differentially affected populations of neurons or differential expression of GPR 173.

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Response Type	Regular aCSF	Low-Ca^{2+}/ High Mg^{2+} aCSF
Excited	0.81 ± 0.018	0.22 ± 0.06
Inhibited	0.63 ±0.010	0.25 ± 0.005
Non-Responsive	1.13 ± 0.014	0.71 ± 0.007

Table 1. The mean baseline firing frequency for all neurons classified in each of the three response types of PVN neurons, in regular aCSF and in low-Ca^{2+}/ high-Mg^{2+} aCSF. As determined by a Student's Unpaired T-Test, baseline values were significantly lower in neurons in low-Ca^{2+}/ high-Mg^{2+} aCSF than those in regular aCSF ($P < 0.001$).

Chapter 4

Discussion

This study has identified modulatory effects of the novel peptide, PNX, on neuronal spike frequency in the neurons of the hypothalamic PVN. Additionally, we provide evidence for both the direct and indirect effects of PNX in on PVN neuronal excitability. Together, these findings suggest that the PVN is a potential site of action for central PNX, which may influence the excitability of PA, NE, MNC or interneuron populations that comprise this hypothalamic region.

4.1 PNX Modulates Spike Frequency of PVN Neurons

GPR173 has been reported throughout the PVN. Thus, our study sought to confirm and investigate further actions of PNX on the extracellular spike frequency of PVN neurons, based on this expression of the peptide and its receptor, GPR173 in the PVN (Yuan *et al.,* 2017; Yosten *et al.,* 2013; Gasparini *et al.,* 2018). In addition, it has been shown that PNX depolarizes PVN neurons, stimulating AVP release in hypothalamo-neurohypophysial explants (Gasparini *et al.,* 2018). However, no prior evidence for the effects of PNX on the extracellular firing properties of PVN neurons has been presented. As such, we tested the hypothesis that exogenous application of PNX acts on PVN neurons and will change extracellular spike frequency, an effect that can be recorded using the multi-electrode array system (MED64). Our observations confirmed our hypothesis as changes in extracellular spike frequency were seen in response to PNX.

4.2 PNX Exerts both Direct and Indirect Actions on PVN Neurons

We also attempted to determine whether these effects of PNX on PVN neurons were direct or indirect, or a combination of both. Experiments were conducted in low-Ca^{2+}/ high-Mg^{2+} aCSF to determine if actions of PNX in the PVN are dependent on synaptic transmission, which is dependent on Ca^{2+} and a function of external calcium concentration ($[Ca^{2+}]_o$) (Hackett, 1976; Dingledine & Somjen, 1981). The low-Ca^{2+} condition is accepted to reduce synaptic transmission. Specifically, reducing $[Ca^{2+}]_o$ below the baseline concentration of 1.2mM has been shown to prevent synaptic transmission in cerebellar slices from frogs (Hackett, 1976). In our present study, we used external concentrations of 0.25mM Ca^{2+} and 9mM Mg^{2+} as they have previously been tested, given that Mg^{2+} maintains osmolality of the solution without substituting or restoring the function of Ca^{2+} (Sun & Ferguson, 1997).

Our data suggests that the effects of PNX are both direct, as responses to PNX were maintained in the low-Ca^{2+}/ high-Mg^{2+} aCSF, as well as indirect, where the proportions of responses substantially decreased for excitations and increased for inhibitions when transmission was blocked. PNX may exert direct effects by binding to GPR173 to initiate second messenger signaling, in turn, modulating ion channel function. PNX binding to GPR173 activates cAMP/ PKA intracellular pathways to drive phosphorylation of CREB. Alternatively, PNX may bind GPR173 to exert indirect effects by modulating synaptic transmission (Nguyen *et al.,* 2019). We hypothesize that PNX exerts direct excitatory effects on MNC neurons, and likely further excites the PVN by inhibiting inhibitory GABAergic interneurons, given that a portion of excitations are not maintained in low-Ca^{2+}/ high-Mg^{2+} aCSF. This hypothesis also accounts for the portion of inhibitions that are maintained in low-Ca^{2+}/ high-Mg^{2+} aCSF **(Figure 8).** An increase in the proportion of inhibitions was observed in our present data, which we hypothesize to likely be a

result of the decreased spike frequency that occurs in low-Ca^{2+}/ high-Mg^{2+} aCSF. Decreased spike frequency alters the proportions of neurons which are recorded from and may therefore influence the proportion of excitations and inhibitions as a result.

4.3 Limitations

Conclusions that may be drawn from data presented here are subject to a number of limitations, primarily those associated with the use of extracellular recording techniques, and the unbalanced and exclusive use of male animals in the present study.

4.3.1 Extracellular Recording

All data collected were the extracellular spike frequency of PVN neurons, recorded with a multi-electrode array system. Extracellular recordings are advantageous in that numerous single unit recordings can be gathered from one experimental setup. However, limitations arise with this technique as data only consists of extracellular spike recordings, although this does allow for substantial data to be gathered from individual slices despite the unimodal nature. Additionally, given that spike frequency changes could only be analyzed in neurons that were large enough and/ or close enough to one of the 64 electrodes in our array, smaller neurons may not have been as well represented in our data. In future, replicating experiments in a set of patch-clamp recordings of PVN neurons could better characterize the proportions observed in our present findings, identifying the sensitivity of different PVN neuron subpopulations to PNX. Additionally, these experiments would better characterize the PNX sensitivity of neurons with no spontaneous activity, as well as those exhibiting very small changes in membrane potential because this data cannot be obtained from extracellular recordings.

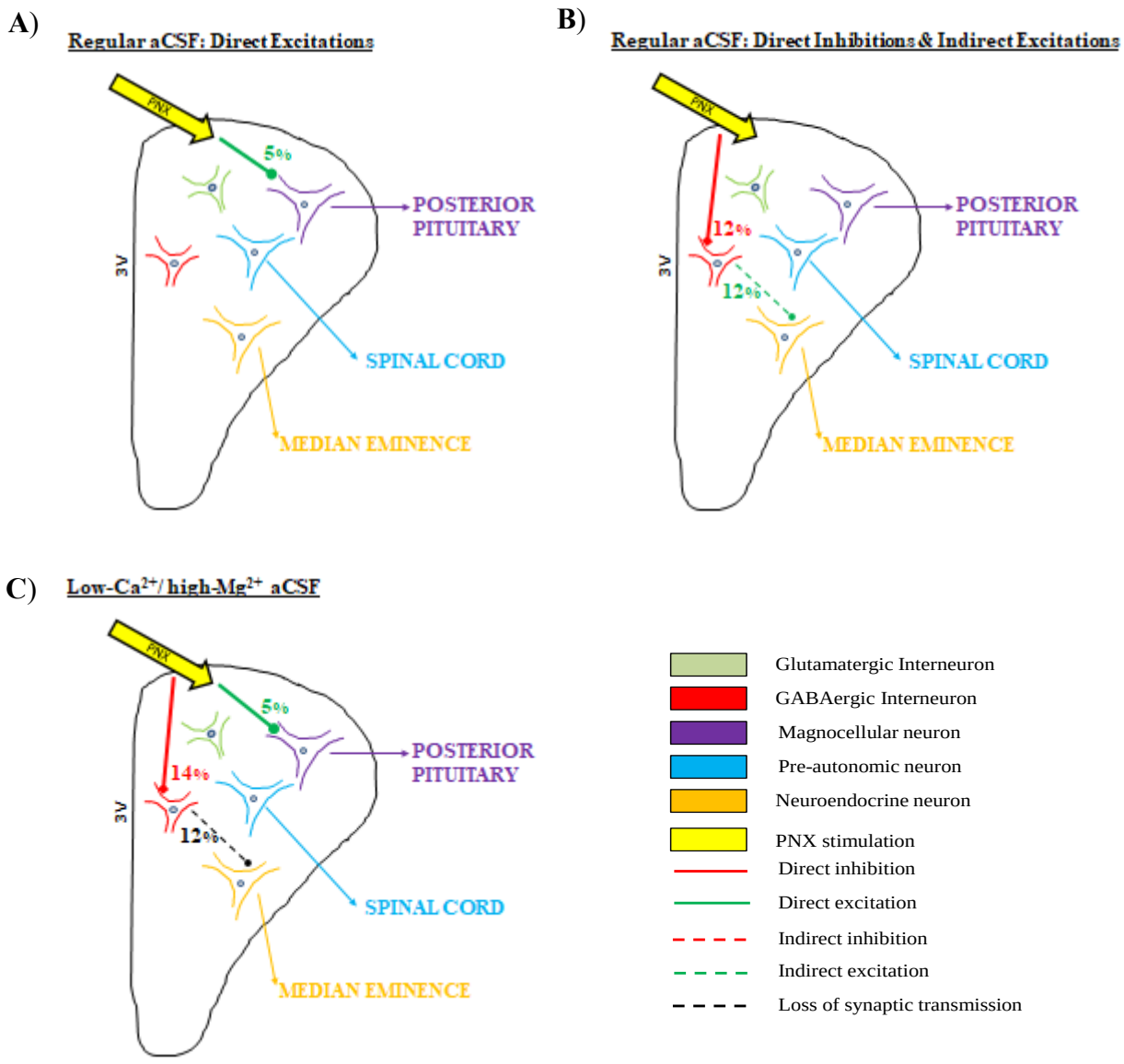

Figure 8. Schematic diagram hypotheses of the bilateral PVN. **A)** In regular aCSF, PNX exerts direct excitatory effects on MNC neurons **B)** In regular aCSF, PNX exerts direct inhibitory effects on GABAergic interneurons, resulting in indirect excitations on other neurons. **C)** In low-$Ca^{2+/}$ high-Mg^{2+} aCSF, the direct excitations and inhibitions are maintained, while the indirect excitations are lost due to lack of synaptic transmission.

4.3.2 Male Rats

The peptide PNX is known to play a role in both stress pathways and reproductive function, such as oestrous cycling in female rats. In the present study, all experiments were carried out using male rats and so are limited in the conclusions that may be drawn from them. Given the potential of stress to affect reproductive function, the likely role of PNX in this system, as well as the established involvement in estrus cycling, it is obviously also important to investigate the effects of this peptide in female rats. Male rats were used in our present study in order to first establish that PNX affects PVN neurons under basal conditions, and to obtain data that is comparable to the current literature.

Previously, studies of PNX expression in both male and female rats have suggested that PNX plays diverse roles despite sex of the animal (Yosten *et al*., 2013). Studies investigating effects of PNX on the NTS of female rats, in comparison to those observed in male rats, found no significant difference in responses (N Ferguson & AV Ferguson, 2019). However, these experiments were carried out only in juvenile female rats that had not reached sexual maturity. Together, these findings emphasize the need for future experiments to question whether PNX exerts sexually dimorphic actions in adult female rats. Mature female rats have reached reproductive maturation and the onset of estrous cycling, during which time there may be changes at the cellular level that influence the effects of PNX; however, this is unknown.

The PVN is known to have sexually dimorphic responses to other peptides such as AVP and OXT, and projects to sexually dimorphic regions involved in autonomic regulation (Wagner & Clemens, 1991). The dimorphic peptides are implicated in reproduction, stress response, cardiovascular control and GI function, systems to which PNX has also recently been linked (Rose *et al.,* 2014; Loewen *et al.,* 2017). Specifically, the regulation of CRH expression in PVN also

varies between sexes and has implications for the sexually dimorphic nature of stress response (Vamvakopoulos & Chrousos, 1993; Senst *et al.,* 2016). Interestingly, the properties of PVN NE neurons expressing CRH have been found to undergo sexually dimorphic changes under stress conditions, such as social isolation (Senst *et al.,* 2016). Such sex-specific cellular properties may increase the likelihood that similar sex-related responses to PNX be seen in the PVN. Therefore, future investigations should determine if there are sexually dimorphic responses to PNX in PVN, across each phase of the estrous cycle. These experiments were not presently undertaken due to time restrictions in obtaining sufficient data as a minimum of ten weeks would be required to test female rats during each phase of oestrous.

4.4 Future Directions

Our present data provide three critical directions for future experimentation with PNX and the PVN. Presently, we are unable to determine if there is a differential sensitivity of PVN subpopulations to PNX. Our data suggest that PNX does exert unique effects on the three subpopulations of PVN neurons, as well as on the interneuron populations throughout. Based on our current understanding of PNX and the PVN, there may also be sex-dependent differences in responses to this peptide in sexually mature animals.

4.4.1 Patch-Clamp Recordings and Single Cell RT-PCR

The present data have confirmed that PNX modulates spike frequency of PVN neurons, but additional experiments would be required to confirm if similar effects are exerted on the membrane potential of these cells. Application of PNX may similarly influence the membrane potential of PVN neurons as seen with extracellular spike frequency. Obtaining data on

membrane potential would also identify the type of neuron being recorded from, therefore revealing the response of each specific PVN subpopulations of MNC, PA or NE neurons. This hypothesis was tested through a set of current-clamp experiments, which identified depolarizing effects of PNX on MNC neurons in the PVN (Gasparini *et al.,* 2018). These findings are consistent with our present data as we observed direct excitations. It was additionally observed that increases in spike frequency often occurred with depolarization; thus, PNX mediated membrane potential and action potential firing in these neurons, effects which were reversible in most cells (Gasparini *et al.,* 2018). Our data may represent responses in all or some of the subgroups of PVN neurons, which should be further investigated through similar experiments. The combination of patch-clamp recording with single-cell RT-PCR, through which the molecular phenotype of cells can be determined, would clearly allow a more complete evaluation of the effects of PNX on separate subpopulations of MNC, PA and NE neurons within the PVN.

4.4.2 Female Rats

Finally, while previous studies have found no sex-specific differences in the responses to or expression of PNX in juvenile male and female rats, it has yet to be determined whether such sex differences exist across adult rats. It should first be confirmed that responses to PNX in the PVN of juvenile male and female rats do not differ, as it is unknown whether this differs for the PVN. If the proportion of responses to PNX in juvenile male and female rats is comparable, investigation into the responses to PNX during each phase of the estrous cycle in adult mice follows. As previously discussed, sex-dependent differences in peptide response have been observed in the PVN; specifically, in AVP and OXT cell types that are believed to be sensitive to PNX (Yamamoto *et al.,* 2004; Wagner & Clemens, 1991). Therefore, examination of sexually

dimorphic patterns in PNX responses in the PVN might reveal very different effects of this signaling molecule at different stages of the oestrous cycle in sexually mature animals.

4.5 Physiological Relevance of PNX Signaling in the PVN

Our data show responsiveness in PVN neurons to PNX, which suggests distinct populations of neurons have differential PNX sensitivity. Excitatory effects of PNX on MNC neurons have been previously demonstrated (Gasparini *et al.,* 2018) and our present data shows inhibitory effects on a different, unidentified population of PVN neurons. We hypothesize that these effects are occurring on another population of cells as no inhibitions were observed in the previous study (Gasparini *et al.,* 2018). Unique responses of PVN neurons to PNX suggest that PNX plays multiple important roles in this region. These roles may include fluid and electrolyte balance, as well as reproductive function and stress pathways (Ferguson *et al*., 2008).

4.5.1 Magnocellular Neurons and Fluid and Electrolyte Balance

Application of PNX caused AVP release in HNS explants and depolarized PVN neurons in a set of patch-clamp experiments (Gasparini *et al.*, 2018). The depolarized populations of PVN cells were electrophysiologically characterized as MNC neurons, which also often exhibit an increase in spike frequency following depolarization (Gasparini *et al.,* 2018). Based on our current understanding of the literature, it is likely that the observed AVP release in HNS explants was a result of PNX stimulation on AVP-synthesizing MNC neurons that send axons to the posterior pituitary (Watson *et al.,* 1982; Gasparini *et al.,* 2018).

The physiological relevance of these actions likely relates to fluid and electrolyte balance, a system that is regulated by central regions through control of peripheral systems, such as the kidneys. The PVN is well established as one such regulatory region, where the synthesis and release of AVP is the primary central mechanism for maintaining fluid balance (Ferguson *et al.,* 2008; Ferguson *et al.,* 1984; Haselton *et al.,* 1994). Maintaining equilibrium in this system is critical because disruption to the osmolality of extracellular fluid (ECF) disrupts cell volume and integrity (Steenbergen *et al.,* 1985; Verney, 1947). However, ECF osmolality is influenced by other physiological systems, thus variations are sensed by responsive central and peripheral osmoreceptors (Verney, 1947). Signals conveying osmolality changes stimulate AVP release, for example, increases in plasma osmolality and decreased plasma volume (Dunn *et al.,* 1973; Baylis, 1987). Considering the current literature and present data, potential roles for PNX in autonomic function may now extend to fluid and electrolyte balance. Specifically, it is possible that PNX is an additional signal by which changes in osmolality are conveyed to the PVN to stimulate AVP release to drive

Our present data demonstrate excitatory and inhibitory effects of PNX on spike frequency of PVN neurons. We hypothesize that these observed excitations represent the MNC neuron populations in which depolarizations were previously reported. Interestingly, Gasparini and colleagues (2018) identified effects of PNX in 69% of neurons, which were entirely excitatory. This brings into question the physiological relevance of inhibitions observed in our present data and potential for distinct PNX sensitivities of other PVN subpopulations. PNX may directly inhibit PA or NE neurons, or exert indirect effects through inhibitions on interneurons.

4.5.2 Neuroendocrine Neurons: Food Intake or Stress

Analyses of mRNA expression for GPR173 throughout the brain identified dense expression throughout the entire PVN region, along with PNX immunoreactivity (Schalla & Stengel, 2018). PNX may directly bind this receptor on NE neurons as well, initiating the canonical G protein-coupled receptor mechanism to induce downstream physiologically relevant pathways.

Interestingly, PNX has been associated with orexigenic effects following the stimulation of food intake by intracerebroventricular (ICV) injections of the peptide (Gasparini *et al.*, 2018; Schalla & Stengel, 2018). Although water intake was not stimulated in these experiments, the effects on food intake were specific to the sleep- or light-phase; in addition, a decrease in body temperature was observed (Schalla & Stengel, 2018). NE neurons of the PVN synthesize and release TRH in the median eminence, carrying out roles in regulation of energy homeostasis, feeding behaviour and thermoregulation (Nikodémová & Strbák, 1995; Lechan & Fekete, 2006). Specifically, TRH secretion in the PVN has been shown to decrease food intake and thermogenesis (Suzuki *et al.*, 1982; Lechan & Fekete, 2006). Aside from directly inhibiting either PA or NE neurons, one additional explanation for the inhibitions observed in our present data is that PNX binds inhibitory interneurons that inhibit NE neurons. In doing so, stimulating TRH secretion to decrease thermogenesis. Although this mechanism conflicts with previously observed orexigenic effects of PNX, it may be that increased food intake occurs secondary to decreased body temperature (Schalla & Stengel, 2018). Therefore, interactions between these effects may induce secondary responses, likely implicating the actions of PNX on PVN neurons in several of these autonomic systems.

4.5.3 Neuroendocrine Neurons: Stress and Reproduction

Both stress and reproduction have been proposed as autonomic systems subject to mediation by PNX. In agreement with such implications, GPR173 has been independently implicated in reproduction (Schalla & Stengel, 2018). The PVN has been proposed to modulate GnRH secretion in the presence of stressful stimuli (Ghuman *et al.*, 2010). A subset of NE neurons synthesizes and releases CRH, the hormone at the apex of stress response. Previous findings suggest that the ME is a region for direct regulation of GnRH release via CRH terminals from the PVN (Ghuman *et al.*, 2010). PNX increased GnRH, the respective receptor and mRNA expression in cell models, modulation which may involve actions of PNX on these NE neuron subpopulations (Treen *et al.*, 2016).

An additional explanation for the presently observed inhibitions may again reside in the inhibitory interneurons that are known to regulate these NE neurons (Boudaba *et al.*, 1996; Daftary *et al.*, 1998; Ferguson *et al.*, 2008; Herman *et al.*, 2002). In the presence of stressful stimuli, the NE neurons release CRH (Swanson & Sawchenko, 1980; Merchenthaler *et al.*, 1984). PNX may excite these neurons directly or inhibit the interneurons, to achieve indirect excitation of NE neurons.

PNX is known to stimulate LH in a GnRH-dependent manner. Inhibiting expression of PNX prevented expression of the GnRH receptor in the anterior pituitary, in turn, disrupting estrus cycling by approximately two days (Yosten *et al.*, 2013; Schalla & Stengel, 2018). Recent studies have suggested that PNX may be involved in the central regulation of stress response as extracellular effects of PNX on the spike frequency of NTS neurons were abolished in animals subject to stress conditions (Grover & Ferguson, 2018). Together, this represents stimulation of the HPG axis by PNX and stress-related effects in hypothalamic regulatory centers (Yosten *et*

al., 2013; Gasparini *et al.,* 2018; Schalla & Stengel, 2018; Grover & Ferguson, 2018). Thus, PNX potentially mechanistically explains the role of stress in compromised cycling and reproductive pathologies (Yosten *et al.,* 2013; McIlwraith *et al.,* 2018). Our extensive understanding of the regulation of these autonomic pathways by the PVN likely implicates this region as a central site by which PNX exerts these effects.

4.6 Conclusions

In conclusion, we have observed that PNX exerts effects on the extracellular spike frequency of PVN neurons, and that these effects are present in low-Ca^{2+}/ high-Mg^{2+} aCSF, indicating both direct and indirect mechanisms of action as proportions of the responses are altered. Following from our present knowledge of autonomic regulation and the PVN, PNX may represent another important signaling molecule which plays important roles in the integrated regulation of activity in these pathways. Future investigations will need to address whether PNX exerts sexually dimorphic effects in the PVN, and to more clearly understand the various roles of PNX in the integration of the complex autonomic outputs of the PVN.

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