# Glycemic State Regulates Brain-Derived Neurotrophic Factor Responsiveness of Neurons in the Paraventricular Nucleus of the Hypothalamus

by

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**Abstract**

Brain derived neurotrophic factor (BDNF) is a member of the family of neurotrophins and binds to the tropomyosin-related kinase B (TrkB) receptor. Like other neurotrophic factors, BDNF is involved in the development and differentiation of neurons. Recently, studies have suggested important roles for BDNF in the regulation of energy homeostasis. The paraventricular nucleus (PVN) is critical for normal energy balance contains high levels of both BDNF and TrkB mRNA. Studies have shown that microinjections of BDNF into the PVN increase energy expenditure, suggesting BDNF plays a role in energy homeostasis through direct actions in this hypothalamic nucleus.

We used male Sprague-Dawley rats to perform whole-cell current-clamp experiments from PVN neurons in slice preparation. BDNF was bath applied at a concentration of 2nM and caused depolarizations in 54% of neurons (n = 25; mean change in membrane potential: 8.9 ± 1.2 mV), hyperpolarizations in 23% (n = 11; mean change in membrane potential: -6.7 ± 1.4 mV), while the remaining cells tested were unaffected. Previous studies showing effects of BDNF on γ-aminobutyric acid type A ($GABA_A$) mediated neurotransmission in PVN led us to examine if these BDNF-mediated changes in membrane potential were maintained in the presence of tetrodotoxin (TTX) sodium channel blocker (N = 9; 56% depolarized, 22% hyperpolarized, 22% non-responders) and bicuculline ($GABA_A$ antagonist) (N = 12; 42% depolarized, 17% hyperpolarized, 41% non-responders), supporting the conclusion that these effects on membrane potential were postsynaptic.

We also evaluated the effects of BDNF on these neurons across varying physiologically relevant extracellular glucose concentrations. At 10 mM 23% (n = 11; mean: -6.7 ± 1.4 mV) of

PVN neurons hyperpolarized in response to BDNF treatment, whereas at 0.2 mM glucose, 71% showed hyperpolarizing effects (n = 12; mean: -6.3 ± 2.8 mV).

Our findings reveal that BDNF has direct impacts on PVN neurons and that these neurons are capable of integrating multiple sources of metabolically relevant input. Our analysis regarding glucose concentrations and their effects on these neurons' response to other metabolic signals emphasizes the importance of using physiologically relevant conditions for study of central pathways involved in the regulation of energy homeostasis.

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**List of Abbreviations**

α-MSH……Alpha-Melanocyte-Stimulating Hormone
aCSF.....Artificial Cerebrospinal Fluid
ACTH……..Adrenocorticotropic Hormone
AgRP…….Agouti-Related Peptide
ARC.....Arcuate Nucleus
ATP.....Adenosine Triphosphate
BBB.....Blood Brain Barrier
BDNF……Brain-Derived Neurotrophic Factor
CART……Cocaine- and Amphetamine-Regulated Transcript
CNS.....Central Nervous System
CREB……Cyclic AMP-Response Element Binding Protein
CRH…….Corticotrophin Releasing Hormone
CVO.....Circumventricular Organ
DAG……Diacylglycerol
DMH…….Dorsalmedial Hypothalamus
DMN……..Dorsal Motor Nucleus of the Vagus
$GABA_A$……..γ-aminobutyric acid type A
GE……Glucose Excited
GI……..Glucose Inhibited
GPCR……..G-protein-coupled receptor
$I_A$……Transient (A-type) Potassium Current
$I_H$…….Hyperpolarization-Gated Cation Non-Selective Channel Activation
ICV.....Intracerebroventricular
$IP_3$……..Inositol Trisphosphate
$K_{ATP}$.....ATP-sensitive Potassium Conductance
LH……...Lateral Hypothalamus
LTP.....Long Term Potentiation
LTS…….……Low Threshold Spike
mIPSC……Miniature Inhibitory Postsynaptic Current
MNC……...…..Magnocellular Neurons
mRNA.....Messenger Ribonucleic Acid
NE……..Parvocellular Neuroendocrine Neurons
NGF……Nerve Growth Factor
NPY……..Neuropeptide Y
NT-3……Neurotrophin-3
NT-4/5……Neurotrophin-4/5
NTS.....Nucleus Tractus Solitarius
OT……Oxytocin
OVLT.....Organum Vasculosum of the Lamina Terminalis
$p75^{NTR}$……Pan-Neurotrophin Receptor
PA……Parvocellular Preautonomic Neurons
POMC……Pro-opiomelancocortin
PLC-γ…….Phospholipase-C-γ
PVN.....Paraventricular Nucleus

Ras-MAP……..Mitogen-Activated Kinase Cascade
RVLM……Rostral Ventrolateral Medulla
SCN…….Suprachiasmatic Nucleus
SFO.....Subfornical Organ
SON..... Supraoptic Nucleus
T3…….3,3',5-Triodothronine
T4……...Thyroxine
TNFR……...……...Tumor Necrosis Factor
TRH……Thyrotropin-Releasing Hormone
TrkB……Tropomyosin-Related Kinase B
TSH……Thyroid-Stimulating Hormone
TTX.....Tetrodotoxin
VMH……Ventromedial Hypothalamus
VP…….Vasopressin
VTA……Ventral Tegmental Area
WAGR……..Wilms' Tumor, Aniridia, Genitourinary Anomalies and Retardation

## Chapter 1: Introduction

### *1.1 Energy Homeostasis and Obesity*

Despite recent advances in our knowledge of mechanisms responsible for regulating energy expenditure, obesity remains one of the most serious health concerns worldwide. Obesity is associated with a wide array of adverse health effects including insulin resistance, glucose intolerance, cardiovascular disease, dyslipidemia, and hypertension, collectively termed the "metabolic syndrome" (164). Although an effective pharmacological intervention for obesity remains to be developed, the secondary health risks suggest that any treatment for obesity would ultimately need to target all the associated symptoms. Prevalence estimates indicated that 15% of the adult population in Canada is obese and 33% are classified as overweight (19). As obesity rates continue to grow, there is constant strain imposed on the health system and pressure to better understand the mechanisms underlying obesity and energy homeostasis.

Obesity ensues when energy intake greatly exceeds energy expenditure and can be caused when environmental, social, psychological, and genetic factors aggregate to disturb this delicate balance. Despite the directness behind this concept, our understanding of the mechanisms involved is limited. Lesion studies in rodents have implicated the hypothalamus as a region that may play a major role in controlling energy homeostasis (112). More specific experiments have shown that lesioning the paraventricular nucleus (PVN) results in obesity and hyperphagia (69; 169). The role of the hypothalamus in the regulation of energy homeostasis is a result of the critical roles played by neurons in this nucleus in the regulation of neural, endocrine and metabolic signals that result in integrated physiological autonomic, endocrine, and behavioural responses (177).

### *1.2 Central Control of Energy Homeostasis*

The hypothalamus is a region of the brain that is critical for the regulation of feeding, reproduction, cardiovascular regulation and thermoregulation (54). Within the hypothalamus there are a number of distinct nuclei which have been suggested to play important roles in the regulation of food intake, such as the arcuate nucleus (ARC), the ventromedial hypothalamus (VMH), the dorsalmedial hypothalamus (DMH), the lateral hypothalamus (LH), and the PVN (13; 36; 64; 144; 165)

The ARC contains two recognizable neuronal populations: neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons and neurons that coexpress pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). NPY is the main neurotransmitter of the ARC that stimulates feeding and weight gain (178) and these NPY neurons project to the PVN (133). POMC/CART neurons produce alpha-melanocyte-stimulating hormone (α-MSH) which suppresses food intake (20; 46) and also project to the PVN (116). Thus, NPY and POMC neurons may be involved in regulating food intake through their efferent projects to the PVN.

The VMH is a hypothalamic nucleus involved in feeding and thermoregulation. Early studies have shown that lesions of this nucleus resulted in over eating in rats (53). Furthermore, when the VMH is stimulated electrically, a decrease in food intake is realized (179).Similar to the ARC, the VMH has been shown to project to the PVN (186). Importantly, VMH expresses high levels of brain derived neurotrophic factor (BDNF) mRNA (43).

The DMH is known as a critical site in the control of feeding and metabolic regulation that receives input from both the VMH and the LH and in turn sends projections to the PVN (22). Early electrolytic lesion studies have found that lesions of the DMH result in hypophagic and hypodipsic rats (23). This study showed that the lesioned rats had become hypophagic and

reduced percentage of lean body mass compared to pair-fed rats (21). These findings support that this hypothalamic locus plays an important role in energy homeostasis.

Finally, the LH is another crucial regulator of energy balance that is situated in the hypothalamus. Electrical stimulation of the LH promotes feeding and drinking behaviors as well as increased physical activity (51; 124). Interestingly, LH-lesioned rats appear to have the ability to eat and drink, but they lose all motivation to do so and this ultimately results in death from starvation (126). Overall, LH's regulation of motivation and its vast projections to many areas of the brain involved in motivation and feeding reveal its potential to contribute to energy balance.

*1.3 Neurotrophic Factors*

*1.3.1 Family of Neurotrophins*

Neurotrophins are a related family of growth factors that are crucial for the growth and survival of developing neurons in both the central and peripheral nervous system (50). Nerve growth factor (NGF) was the first of this family to be discovered in 1950 through its effects on sympathetic neurons, showing that fibers emerging from sympathetic ganglion cells in chick embryos were observed to grow directionally towards sarcomas that provide favorable medium for nerve fiber outgrowth at the base of limb buds (103). Since this time, other neurotrophic factors have been described, including neurotrophin-3 (NT-3) (109), neurotrophin-4/5 (NT-4/5)(67; 78) and BDNF (17).

*1.3.2Brain-Derived Neurotrophic Factor*

BDNF was the second member of the neurotrophic family to be discovered when it was shown to be paramount for the survival of dorsal root ganglion neurons (2; 17). BDNF is synthesized as pro-BDNF (32 kDa) and is then later cleaved into its mature form which dimerizes in order to bind receptors (87). Although mature BDNF is considered the active form, pro-BDNF

may be secreted and interact with pan-neurotrophin receptor $p75^{NTR}$ (210). $p75^{NTR}$ is a member of the tumor necrosis factor (TNFR) superfamily and has a glycosylated extracellular region that is important for ligand binding, a transmembrane region, and a short cytoplasmic sequence (39; 49). p75 is of particular interest because it has biological functions that are distinct from those of tropomyosin-related kinase (Trk) receptors; perhaps the most important of which to date is its demonstrated role in initiation of programmed cell death, also known as apoptosis (37; 49; 60; 159)

Mature BDNF binds to the TrkB receptor with high affinity (16), and to $p75^{NTR}$ with low affinity (39). In addition, it has been shown that $p75^{NTR}$ can increase TrkB's specificity for BDNF (25). Both BDNF and TrkB mRNA widely expressed throughout the central nervous system (CNS) (43; 115) and particular peripheral tissues including muscles (128), adipose (197), and liver (38). As a result, both proteins are widely distributed throughout the nervous system(43; 217) while, at the cellular level, BDNF and its receptors, TrkB and p75, can be located in both the axon terminals and the dendrites of neurons (195). When bound to its receptor, BDNF promotes neuronal survival (5; 65; 74), differentiation (5), and long-term potentiation (LTP) (88).

*1.3.3 TrkB Receptor*

TrkB is a tyrosine kinase which dimerizes upon activation by BDNF. Following kinase activation, TrkB transphosphorylates on multiple tyrosine residues and creates specific binding sites for intracellular target proteins. These target proteins can then bind the activated receptor via SH2 domains (16; 142)and initiate subsequent intracellular signaling cascades. These pathways include phospholipase C (PLC-γ), mitogen-activated kinase cascade (Ras-MAP) and phosphorylation of cyclic AMP-response element binding protein (CREB) (142). This receptor can exist in one full-length form and two truncated forms that do not possess tyrosine kinase

domain and are inactive (6), the latter of which have been shown to function as inhibitory modulators of neurotrophin responsiveness by forming heterodimers with the full-length form and inhibiting BDNF activity (4).

*1.3.4 BDNF and the Control of Energy Homeostasis*

Recently, BDNF has gained increasing recognition as a potential regulator of energy metabolism. It has been shown that chronic intraventricular (ICV) administration of BDNF in rats with fimbrial lesions attenuated the lesion-induced weight gain, suggesting BDNF has a role in the regulation of food intake through acting on central neurons. BDNF given introcerebroventricularly to rats for 14 days caused a notable and dose-dependent decrease in body weight and energy intake (143). In addition, adult rats implanted with osmotic micro-pumps in the lateral ventricle for delivery of BDNF displayed increased locomotor activity and body temperature, indicating a role for BDNF in the hypothalamic-pituitary-adrenal axis and its subsequent effects on locomotor rhythms (129).Using the cre-lox P recombination system to generate conditional mutants in which BDNF has been eliminated from the brain, studies have shown that homozygous and heterozygous knockout of BDNF in the brain results in an obese phenotype along with elevated serum insulin, cholesterol and glucose (152). Importantly, ICV administration of BDNF to heterozygous knockout mice can transiently reverse the previously observed abnormal eating behaviour (85). Finally, haplosufficiency of BDNF in humans with WAGR syndrome (Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation) is associated with obesity and hyperphagia, indicating BDNF may be critical for the regulation of food intake in humans (132).

Within the hypothalamus, BDNF has been shown to be synthesized within the VMH, PVN, DMH, and LH (43). Microarray data from our laboratory has shown that both the BDNF

protein and its associated receptor, TrkB, are expressed at high levels within the PVN (unpublished).

*1.4 The Paraventricular Nucleus of the Hypothalamus*

*1.4.1A Brief Overview*

The PVN is perhaps one of the most important hypothalamic integrative autonomic control nuclei in that it is now well established to play critical roles in the regulation of the immune system, cardiovascular regulation, the control of fluid balance and energy homeostasis (45; 58; 83; 185; 209). The PVN is bilaterally located adjacent to the dorsal portion of the third ventricle and it contains three primary output cell types which express distinct neurotransmitters and have varying projections. These different cell types are magnocellular (MNC) oxytocin (OT) and vasopressin (VP) secreting neurons which project to the posterior pituitary, the OT, VP, thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone(CRH) parvocellular preautonomic (PA) neurons which project to the medulla and the spinal cord, and the CRH and TRH neuroendocrine (NE) cells which project to the median eminence. In addition, anatomical studies have shown GABAergic interneurons dispersed throughout the nucleus as well as the surrounding "halo" zone (154).

*1.4.2 Afferent Inputs to the PVN*

PVN receives numerous and diverse afferent projections from various areas of the CNS including the hypothalamus, brainstem, and extra-hypothalamic sites (96). Retrograde transport experiments have described inputs to the PVN from the suprachiasmatic nucleus (SCN), the VMH, the dorsal medial nucleus, the medial preoptic area, areas of the LH (161), and the ARC, as well as from the forebrain sensory circumventricular organs including the subfornical organ (SFO) and the vascular organ of the lamina terminalis (OVLT) (96). Finally, the PVN also

receives projections from critical autonomic control nuclei in the medulla including the nucleus tractus solitarius (NTS), the ventrolateral medulla, and the dorsal motor nucleus of the vagus (DMN) and other various projections from areas such as the ventrolateral septum, locus coeruleus, noradrenergic neurons of the brainstem, the parastrial nucleus, the amygdala and the prefrontal cortex (70; 95; 96).

*1.4.3 Efferent Projections of the PVN*

PVN neurons have been described extensively and have been shown to project to 3 main areas. MNC neurons are located in the ventrolateral portion of the PVN and project to the posterior pituitary where they release VP or OT into the peripheral circulation. Axons from both the MNC neurons of the PVN and the supraoptic nucleus (SON) combined with the posterior pituitary gland form the neurohypohyseal system. PA neurons innervate the NTS (162) and DMN of the medulla and the intermediolateral cell column of the spinal cord to direct autonomic control related to energy balance and cardiovascular regulation (68; 91). Finally, NE neurons project to the median eminence to release hypophysial peptides into the pituitary portal system, which in turn play critical roles in the regulation of anterior pituitary hormone release (207).

*1.4.4 PVN Neuronal Subtypes*

Studies using intracellular recording, labeling, and immunohistochemistry collectively established the morphology and basic electrophysiological characteristics that allow for the distinction of PVN neurons into: MNC, PA, and NE in accordance with anatomical characteristics and electrophysiological fingerprints. These characteristics allow investigators to study each cell type and its function individually.

*1.4.5 Magnocellular Neurons (MNC)*

Magnocellular neurons, as the name suggests, are morphologically distinct when compared to neighboring cell types in the PVN in that they are relatively large (soma diameter 20 – 30 μM) bipolar or multipolar cells that typically have one or two large dendrites. Magnocellular neurons typically send projections above and below the fornix to the posterior pituitary (117), where they release peptides contained in large dense-core vesicles from their axon terminals into general circulation (187). MNC neurons synthesize three primary peptides: OT, VP, and enkephalin (18; 155; 163; 165; 201).

Interestingly, a study has shown that some MNC cells of the PVN are dye-coupled and possess gap junctions that electronically couple cells to one another (8). This assists in coordinating action potential firing patterns which results in a synchronously firing endocrine population and high concentrations of hormones being released into the posterior pituitary. This may help with the spike burst of OTergic MNC's before the milk let-down reflex or to help in the recruitment of VPergic MNC's into phasic firing patterns (8).

MNC neurons have a unique electrical phenotype that is easily identifiable and allows for the distinction from surrounding cells when performing electrophysiological experiments. MNC cells exhibited linear current-voltage relations to very hyperpolarized potentials as well as an evident transient outward rectification due to fast-inactivating A-type K+ current (190). These neurons also exhibit a delayed onset to spike firing caused by a hyperpolarizing 'indentation' in the membrane potential and a delayed return to baseline following hyperpolarizing pulses. Being relatively large cells, MNC neurons can be distinguished based on their physical characteristics. Whole-cell capacitance measurements, a direct measurement of membrane surface area, as well as perikarya staining can be used to determine cell size to characterize MNC cells (190).

*1.4.6 Pre-autonomic (PA) Parvocellular Neurons*

Collectively, the PA neurons are much smaller than magnocellular neurons and tend to have 2-3 primary dendrites (187). Although the PA neurons show common morphological and electrophysiological properties that are distinct when compared to magnocellular neurons, as a population they are a cellular mosaic (117). PA neurons are not confined to their respective subnuclei and can extend into the posterior magnocellular, periventricular, or the medial parvocellular areas within PVN (180). PA neurons send long descending projections to various places in the brainstem that are vital with respect to autonomic control. These areas include the DMN and the rostral ventrolateral medulla (RVLM) and the NTS (12; 184). At the brainstem, PA neurons of the PVN have been shown to secrete OT, VP,CRH, and TRH.PA neurons also project to the spinal cord, where VPergic PA cells have been found to have fibers with varicosities close to the cell bodies and dendrites of stellate ganglion (150) compared to OT spinally projecting neurons that appear to be sparsely distributed throughout the spinal cord (127). In addition, TRH and CRH neurons from the PVN directly project to the autonomic preganglionic neurons controlling autonomous responses to convey information to peripheral targets involved in thermogenesis, cardiovascular regulation, glucose and insulin regulation, and other forms of stress (9; 138).

PA neurons have unique electrical properties that distinguish them from neighbouring magnocellular neurons in that they exhibit low threshold spikes (180) attributed to a low-threshold T-type $Ca^{2+}$ current characterized by its hyperpolarized activation threshold (-60 mV) , fast rate of inactivation, and the fact that it can be blocked nickel chloride (108). Another characteristic that is shared amongst PA cells is the strong inward rectification in response to hyperpolarizing current pulses. It has been postulated that this may be due to $I_H$ (hyperpolarization-gated cation non-selective (HCN) channel activation) (113). In addition, PA

neurons show three different types of spontaneous action potential firing that include tonic regular, tonic irregular, and bursting. This suggests that the expression or properties of some ion channels differ between PA cells which further highlights the heterogeneity of this subgroup (14).

*1.4.7 Neuroendocrine (NE) Parvocellular Neurons*

NE parvocellular neurons are located in the medial portion of the PVN close to the 3$^{rd}$ ventricle, as shown by the observation that these cells are preferentially filled by circulating fluoro-gold retrograde tracers into rats (107). These neurons send their projections to the external lamina of the median eminence where they secrete CRH and TRH (117; 200).

Neuroendocrine cells cannot be identified any distinct electrophysiological feature, instead they can be pinpointed by the absence of any of the unique electrical characteristics that are demonstrated by the previous two cells types (190). Studies have employed retrograde tracer, fluoro-gold, to label neuroendocrine cells and compare the electrophysiological properties of the labeled neuroendocrine cells and unlabeled preautonomic neurons using whole-cell recordings in hypothalamic slices. A low threshold spike (LTS) was detected in most of the unlabeled preautonomic cells, however the neurosecretory parvocellular neurons did not express an LTS (107; 181). In addition, NE cells do not experience the delay to first spike that can be seen in magnocellular neurons due to their hyperpolarized-activated $I_A$ (190).

*1.5 PVN Cell Phenotypes and Physiological Relevance*

*1.5.1 Vasopressin (VP)*

VP (also known as anti-diuretic hormone) is a small 8 amino-acid peptide that is synthesized with its carrier protein neurophysin as part of a precursor or pre-prohormone in many areas of the brain, but most prominently expressed in the SON and PVN. This precursor is packaged into granules which are transported along the axon to the neurohypophysis where these

vesicles are released following depolarization, opening of voltage-gated channels, influx of calcium followed by exocytosis (26). Investigators have found that the VP expressing neurons are concentrated in the posteriodorsolateral area of the magnocellular region of PVN (187).

VP is vital to the maintenance of fluid volume and osmolarity (47). When plasma osmolality increases to levels above normal physiological ranges, VP secretion in increased at the neurohypophysis. VP is then free to bind to receptors that reside in the kidney to decrease the excretion of water and return more water to the blood through increasing the expression of aquaporin-2 (79; 80; 119; 166).

VP has a wide range of actions that include vasoconstriction(94), water retention, stimulation of glycogenolysis, effects on lipids (73), regulation of adrenocorticotropic hormone (ACTH) release from the pituitary (89), and release of insulin and glucagon from the pancreas (160). Therefore, VP may be involved in the development of metabolic syndrome (160).VP acts on the pituitary to promote the release of ACTH under normal conditions (11). Interestingly, stress will amplify the actions of VP on V1b receptors in the pituitary to release ACTH which causes increased cortisol levels in the serum. High serum cortisol has been shown to cause obesity, hyperglycemia and insulin resistance (55; 141). These studies highlight the potential importance of VP from the PVN influencing metabolic homeostasis.

*1.5.2 Oxytocin (OT)*

Like VP, OT is highly expressed in the magnocellular neurons of the PVN and SON and is released from their axon terminals in the neurohypophysis in response to action potentials (149). OT is also synthesized as a pre-prohormone and is linked with a neurophysin that is cleaved in secretory granules and released at target sites where it acts on G-protein coupled receptors (GPCR) (62; 86). Neurons expressing OT can be found in both the parvocellular and

magnocellular regions of the PVN where they can be involved endocrine, autonomic, and energy homeostatic functions (187). Although OTergicneurons are found throughout PVN, those projecting spinally make up roughly 40% of neurons that project to that location and they reside in the lateral parvocellular subdivision (66).

OT is appropriately named the "quick birth" hormone as it is a major regulator of intimacy in many species. Knock-out experiments using mice have demonstrated that OT is necessary for successful milk ejection in response to suckling (131). OT is also important for stimulation of uterine smooth muscle contractions during labour (62). OT-expressing neurons in the PVN have direct projections to the dorsal brainstem which is a crucial region for cardiovascular regulation (173; 174). In addition, rats with reduced OT mRNA expression were shown to develop hypertension (199). Previous studies have shown that acute administration of synthetic OT increases glomerular filtration rates in the kidneys and reduces tubular $Na^+$ reabsorption (44), implicating OT in fluid homeostasis. Finally, OT released in the brain may protect the cardiovascular system from excessive work loading through reducing mean arterial pressureand reducing contraction force (62; 212).

Arletti et al. (1989) (10) eluded to the potential role of OT in controlling energy homeostasis by showing that ICV administration of OT reduced eating and administration of an OT receptor antagonist increased eating in rats. Since that preliminary investigation, OT has been implicated in the control eating behaviour in a number of studies that have shown that hypothalamic OT mRNA expression is reduced with fasting and restored upon feeding. In addition, eating activates OT neurons in rats, while reducing OT protein the PVN results in mice with an obese phenotype (92; 191). The receptor for OT is highly expressed in the NTS, a well

described site important for the regulation of feeding (110). This pathway is well known for regulating feeding, further supporting OT's potential role in regulating energy homeostasis.

*1.5.3 Corticotropin-Releasing Hormone (CRH)*

CRH is a 41-amino acid polypeptide which is the predominant chemical messenger by which the CNS controls the activity of the pituitary-adrenal axis (198). CRH exerts its biological effects through $CRF_1$, a GPCR that is expressed highly in the area postrema (AP), cerebellum, cortex, and amygdala (146; 147). Activation of $CRF_1$ in the pituitary stimulates the synthesis and secretion of ACTH which in turn increases glucocorticoid release from the adrenal glands downstream(198)important hormones for the stress response as well as lowering blood-glucose concentrations. In addition, CRH is important to the control of autonomic, immune and behavioural responses to stress (138).

In addition to controlling the stress response, CRH also regulates metabolic homeostasis (196). Glucocorticoids may have profound impacts on feeding habits through their regulation of classical anorexigenic and orexigenic peptides (93). When CRH is injected centrally, it has been shown to reduce food intake and reduce body weight in rats (203). When CRH is administered peripherally, it increases energy expenditure and increases fat oxidation (170). Studies have shown that leptin administration enhances CRH expression andmore importantly, pretreatment with a CRH antagonist attenuates the leptin-induced weight loss and reduced food intake (111). Additionally, CRH plays an important role in the regulation of autonomic function (138). Increasing sympathetic activation results in increased energy metabolism and reduction in feeding through increased oxygen consumption, blood glucose levels, glucagon, thermogenesis and locomotion (32; 138). Experiments employing microinjections of CRH into the lateral cerebral ventricles of rats have mimicked these effects (31).

*1.5.4 Thyrotropin-releasing hormone (TRH)*

TRH is a tripeptide that is synthesized in neuronal cell bodies of various regions of the brain from a larger precursor protein known as prepro-TRH (ppTRH). This protein is then processed in order to produce the active form of the peptide (29; 97). TRH is produced by many hypothalamic nuclei; however, it is the TRH-expressing neurons in the mid-caudal parvocellular region of the PVN (99) that control thyrotropin (TSH) producing cells of the anterior pituitary. The TRH neurons of the PVN project to the median eminence where TRH is released into the vessels of the pituitary portal system, subsequently reaching the anterior pituitary where TSH is released. TSH is then free to bind to its receptor in the thyroid where thyroid hormone can be released as thyroxine (T4) and 3,3',5-triiodothyronine (T3) which exert physiological actions and feedback (40; 48).

TRH neurons are critical components of the hypothalamic-pituitary-adrenal axis and its influence on energy homeostasis. Actions of TRH target multiple centers that are involved in energy expenditure and regulation. Studies have shown that fasting states, food restriction, or infection decrease serum levels of TH and TRH expression in TRH neurons. TRH neurons receive projections from MSH/CART and NPY/AgRP neurons from the ARC whose activity is highly influenced by leptin (167). This observed decrease in TRH expression may be due to reduced leptin levels that increase the inhibitory impact of NPY/AgRP on TRH neurons (56). TRH administered centrally has been shown to reduce feeding in fasted and normal fed animals. TRH also exerts various effects in the CNS that may contribute to the control of energy balance. Some of these effects include increased thermogenesis, locomotor activity and arousal, and regulation of digestion (98).

*1.6 Glucose-Sensing*

As highlighted earlier, the hypothalamus is critical to the maintenance of energy homeostasis and as a result, these neurons are able to respond to a variety of signals that indicate the energy state of the body. One of the most important signals that indicate energy state is glucose, both in the circulation and in the brain. Changes in glucose can result in changes in hormonal release, metabolic rate, energy expenditure and feeding behaviour (34; 105). Glucose-sensing neurons have been categorized as being either glucose excited (GE) or glucose inhibited (GI) in accordance to their responsiveness to extracellular glucose. GE neurons act similarly to β-cells in the manner that when there is high extracellular glucose, it is transported into the cell and transformed into ATP. ATP is then able to bind and close ATP-sensitive $K^+$ ($K_{ATP}$) channels leading to a depolarization and signaling for release of transmitter, or in the case of β-cells, insulin (105; 123; 158). GI cells respond in a different manner to increases in extracellular glucose. GI cells hyperpolarize in response to glucose and this is due to the involvement of either the $Na^+/K^+$ pump (137) or $Cl^-$ channels (175).

Glucose-sensing neurons are found in many brain regions including the brainstem (3) and many nuclei within the hypothalamus (157). More importantly, glucose-sensing neurons have been observed in hypothalamic nuclei that are involved in the regulation of energy expenditure and food intake. Some of these nuclei include the LH, ARC, and VMN hypothalamic regions (7; 136; 156; 158; 205). The PVN, as outlined above, is a critical nucleus involved in the control of energy homeostasis but may also be critical for the regulation of glucose and insulin concentration. This concept has been proposed by lesion experiments, chemical stimulation as well as viral tracing (100-102). More recently, investigators performed patch-clamp recordings to determine that PVN contains GE and GI cells that may be important for the regulation of autonomic functions and energy metabolism (114). The authors employed pharmacological

techniques to demonstrate that parvocellular neurons likely sense glucose independently of $K_{ATP}$ channels in contrast to other neurons in the hypothalamus.

*1.7 PVN as an Integrative Center*

The PVN and its surrounding hypothalamic nuclei form an interconnected network of pathways sensing and integrating orexigenic and anorexigenic signals. The neurons in the PVN are constantly subject to modulation by signals involved in energy homeostasis and other biologically active molecules. This positions the PVN as a potentially unique integrative center that is constantly monitoring multiple signals, integrating this information, and promoting the appropriate physiological response via its outputs. Integrating all of these signals creates a balance of orexigenic and anorexigenic signals to control energy homeostasis that can be shifted in either direction by subtle perturbations in the environment.

Recent studies have reported that injections of BDNF into the periphery result in hypoglycemia in obese hyperglycemic animals, suggesting an antidiabetic effect of this neurotrophic factor (130; 134). Additionally, BDNF mutant mice develop obesity but also show elevated insulin and glucose levels (152). Finally, other studies have suggested that BDNF may contribute to glucose metabolism and may play a role in the development of type 2 diabetes mellitus in humans (61).

Although studies have investigated the effects of signals such as glucose and BDNF on PVN neurons, many studies investigating this region evaluate the effects of only one input at a time and not exploiting the PVN's integrative nature. Furthermore, many of these studies that employ electrophysiological recordings conduct their experiments at only one supraphysiological glucose level that represents one physiological energy state of the animal. As a result, this present study was undertaken investigate the influence of BDNF on the excitability of all three PVN

neuronal subtypes as well as evaluate the ability of each subtype to integrate metabolically relevant signals by determining if the responsiveness of PVN neurons to BDNF is altered by changes in extracellular glucose concentrations.

In this study we sought to test three main hypotheses. The first is that BDNF influences the excitability of PVN neurons. The second is that BDNF has direct, postsynaptic effects on PVN neurons. Finally, we tested the hypothesis that PVN neurons' responsiveness to BDNF is dynamically altered in accordance with changing levels in local extracellular glucose concentrations.

## Chapter 2: Materials and Methods

All procedures were in accordance with the ethical criteria established by the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee.

### 2.1 Slice Preparation

Coronal slices at the level of the PVN were prepared daily from male Sprague-Dawley rats (Charles River, Quebec, Canada) aged 21-28 days, allowed ad libitum access to food and water. Unanesthetized rats were decapitated and the brains were removed and submerged in ice-cold slicing solution composed of (in mM): 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.25 $NaH_2PO_4$, 25 glucose, 75 sucrose. This solution was bubbled with 95% $O_2$/5% $CO_2$.The region of the hypothalamus containing the PVN was isolated and 300 μm sections were cut using a vibratome (Leica, Nussloch, Germany). Slices were incubated at 32°C in artificial cerebrospinal fluid (aCSF) that had a pH of 7.2, measured 280-300 mOsm, and composed of (in mM): 124 NaCl, 2.5 KCl, 20 $NaHCO_3$, 2 CaCl2, 1.3 $MgSO_4$, 1.24 $KH_2PO_4$ bubbled with 95% $O_2$/5% $CO_2$. The glucose concentration of the aCSF was subject to change and was 10, 3, 1, or 0.2 mM. Slices were incubated for a minimum 1 hour before electrophysiological recordings were performed.

**2.2 Electrophysiology**

Slices were relocated to a recording chamber perfused with carbogenated (95% $O_2$/5% $CO_2$) aCSF heated to ~32°C at a flow rate of 1.5-2.5 ml/min. The baseline glucose concentration of the aCSF in the recording chamber was the same as that used during the incubation period following slice preparation. An upright differential interference contrast microscope at x40 was used to visualize neurons (Scientifica, East Sussex, United Kingdom). Boroscillicate glass electrodes (World Precision Instruments, Sarasota, FL) were pulled on a Sutter Instruments P97 micropipette puller and filled with an intracellular solution composed of (in mM) 125 potassium gluconate, 10 KCl, 2$MgCl_2$, 0.1 $CaCl_2$, 5.5 EGTA, 10 HEPES, 2 NaATP at a pH of 7.2 (KOH) and osmolarity of 280-300 mOsm. Electrodes were optimized to have a resistance of 2-5.5 MΩ when filled with the solution. After obtaining a high-resistance seal (minimum 1 GΩ), a brief period of negative pressure was employed to break the membrane and obtain whole cell access. Whole-cell recordings were made with a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) sampled at 10kHz, filtered at 2.4 kHz using a Micro 1401 interface. Data were collected with Spike 2 software for offline analysis (Cambridge Electronic Devices, Cambridge, UK). At all glucose concentrations used, neurons were required to have a minimum spike amplitude of 50 mV (range 50-100 mV) and a stable baseline membrane potential for a minimum baseline period of 100s to be included in our subsequent analysis. PVN neurons were characterized as MNC (delayed return to baseline in response to a hyperpolarizing pulse), PA (calcium spike in response to a hyperpolarizing pulse), or NE (the absence of the previous two characteristics) using a standard pulse protocol prior to the application of BDNF (190).

All solutions were applied to PVN slices via bath perfusion. Responses to BDNF were assessed by comparing the 100s baseline membrane potential of the neurons before and during

subsequent 100s periods following peptide application. A response was determined to be significant if the change in membrane potential after peptide application was at least twice the standard deviation of the baseline membrane potential 100s period before application of BDNF. The recorded membrane potential was adjusted to correct for the calculated junction potential (15 mV). Error values represent ± the standard error of the mean (SEM). All statistical analyses were performed using Graphpad Prism 6.01 (La Jolla, CA, USA). One-way analysis of variance (ANOVA), post-hoc Tukey test, and Chi-square test were applied. The statistical significance was set at $P < 0.05$.

### 2.3 Glucose Concentrations

10 mM glucose was employed initially in view of its widespread acceptance as the optimal concentration for electrophysiological recordings of neurons in slice preparation. 3 mM glucose was used to mimic central normoglycemia, 1 mM to mimic moderate central and 0.2 to mimic hypoglycemia as it is the lowest concentration observed in the brain in vivo (77; 114; 168).

### 2.4 Chemicals and drugs

Slicing solution, aCSF, bicuculline, and intracellular recording solution were made with salts obtained from Sigma Pharmaceuticals (Oakville, Ontario, Canada). BDNF was obtained from Phoenix Pharmaceuticals (Belmont, CA) and prepared as 2 nM solution in aCSF. Both higher and lower concentrations of BDNF were utilized, however 2 nM was determined to exert maximal effects with recovery. Tetrodotoxin (TTX) was obtained from Alamone Laboratories (Jerusalem, Isarael) and applied as a 1 μM solution.

## Chapter 3: Results

### 3.1 BDNF affects the membrane potential of PVN neurons

We employed whole-cell patch-clamp recordings in current-clamp configuration to assess the effects of BDNF on the membrane potential of PVN neurons. Neurons that were included in our analyses were able to fire action potentials greater than 50 mV (spontaneously or induced by a current pulse of up to 20 pA) and showed a stable baseline lasting at least 100 seconds. A total of 47 neurons met these requirements and were tested for responsiveness to bath application of 2 nM BDNF dimer. PVN neurons did not appear to show a clear response to the BDNF monomer.

The majority of these cells, 25 out of the 47 neurons (54%), showed a long lasting (mean: 1069 ± 259.4 s) depolarization (mean depolarization: 8.9 ± 1.2 mV) that varied in time to onset (mean: 158.5 ± 22.9 s) (See Figure 1A). Bath application of 2 nM BDNF also induced long lasting (mean: 1472 ± 640.8 s) hyperpolarizing responses (mean: -6.7 ± 1.4 mV) with varied times to onset (196.7 ± 33.7 s) in PVN neurons in 11 of 47 (23%) neurons tested a (Figure 1B). The remaining PVN neurons tested (11 of 47, 23%) showed no clear changes in membrane potential in response to bath application of BDNF. When recordings were maintained for long periods of time, individual neurons were tested with BDNF multiple times, although the effects observed in response to the first application were often not reproducible (data not shown) and as a result, only one neuron was tested per tissue slice thereafter. These data are further illustrated in Figures 2A that demonstrates the distribution of neurons that depolarized, hyperpolarized, or were unresponsive to BDNF treatment. In addition, Figure 2B further elucidates the mean responses of each response type.

**3.2 BDNF-induced effects on membrane potential are postsynaptic**

In order to determine whether the observed effects of BDNF were due to direct actions of this peptide on PVN neurons, additional current-clamp recordings were performed in the presence of tetrodotoxin (TTX), a voltage-gated sodium channel blocker, at a 1 μM concentration

which was confirmed to be effective by its ability to block both spontaneous and/or current-evoked action potentials. The responsiveness of PVN neurons to BDNF remained in the presence of TTX (n =9) as both depolarizing (n = 5; mean: 7.4 ± 2.5 mV) and hyperpolarizing (n = 2; mean: -5.9± 2.2 mV) effects were still observed in response to BDNF, with few non-responsive cells (n = 2) (as illustrated in Figure 3A and 3B respectively), data which support the conclusion that these effects on membrane potential likely result from postsynaptic action on PVN neurons.

**3.3 BDNF-induced effects on membrane potential are not mediated by $GABA_A$ receptors**

PVN neurons are constantly barraged by a tonic level of miniature inhibitory postsynaptic currents (mIPSCs) which are independent of the action potential driven-release of GABA onto neurons. It has been shown that BDNF can alter synaptic inhibition by decreasing the surface expression of $GABA_A$ receptors through activation of postsynaptic TrkB receptors (33; 81; 189). Recently, Hewitt and Bains (2006) demonstrated that BDNF decreases inhibitory GABAergic transmissions onto parvocellular NE cells in the PVN and that these effects were blocked by inhibiting dynamin, a critical component in the endocytosis of $GABA_A$ receptors (72). Thus, in order to test whether the effects of BDNF on membrane potential we were observing were a result of changes in the surface expression of the $GABA_A$ receptor, we blocked $GABA_A$ using 10μM bicuculline methiodide, a $GABA_A$ receptor antagonist.

We bath applied 2nM BDNF for 2 minutes and assessed changes in membrane potential for all three neuronal subtypes that reside in the PVN. We found that the effects of BDNF on PVN neurons remained in the presence of the $GABA_A$ antagonist and both depolarizing (n = 5; mean: 7.7 ± 0.99 mV) and hyperpolarizing (n = 2; mean: -5.7 ± 1.67 mV) responses were observed (Figure 4A and B). Figure 4C shows the summary of the responses of all neurons to BDNF and Figure 4D shows the distribution of responses which is very similar to that observed

in the absence of bicuculline. Of the neurons tested, 42% exhibited a depolarizing response to BDNF and 17% resulted in a hyperpolarization.

There was no significant difference in the amplitude of depolarizing responses between control neurons treated with BDNF and those given BDNF in the presence of bicuculline (unpaired t-test: $p = 0.628$) nor was there any difference in the amplitude of hyperpolarizing responses between groups (unpaired t-test: $p = 0.794$). In addition, there was no significant difference in the proportion of responses of bicuculline-treated neurons that responded compared to control neurons that responded to treated with BDNF ($\chi^2$,$p = 0.746$).

**3.4 BDNF differentially influences subpopulations of PVN neurons**

We also examined the effects of BDNF on PVN neurons classified as MNC ($n = 13$), NE ($n = 9$), or PA ($n = 25$) in accordance with their electrophysiological fingerprints. BDNF influenced the membrane potential of all three subgroups of neurons although neurosecretory cells demonstrated primarily depolarizing responses (Figure 5). In contrast, while a large proportion of PA cells were influenced (84%) by BDNF, these neurons show a more even split of depolarizing (48%) and hyperpolarizing (36%) effects with a small number of non-responders (16%).

**3.5 Extracellular glucose modifies responsiveness of PVN neurons to BDNF**

Normal extracellular glucose concentrations in the rat brain range from 2.5 – 3.5 mM(168), while concentrations of 5 mM, 1 mM, and 0.2 mM have been characterized as hyperglycemia and hypoglycemia and severe hypoglycemia, respectively (77; 114; 168). However, to date, the majority of brain slice recordings are obtained with extracellular glucose concentrations in the hyperglycemic range (10 mM). More importantly, current studies examining the role of signaling molecules critical to the regulation of energy homeostasis employ

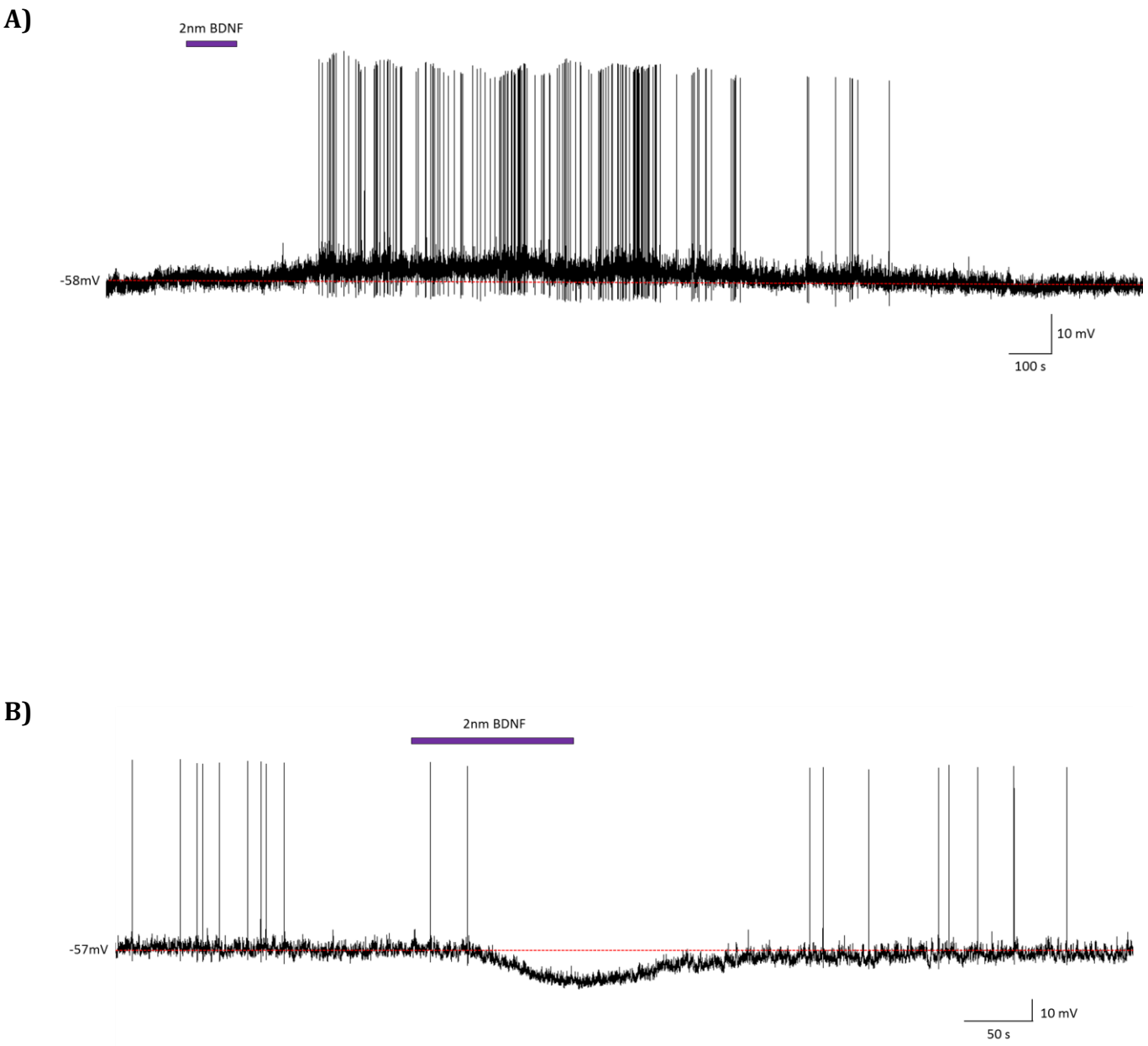
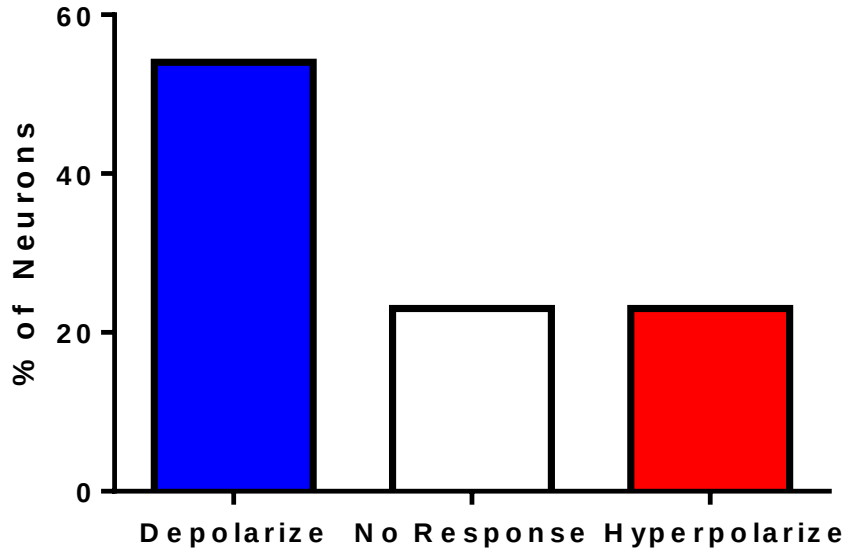



**Figure 1 – BDNF influences the membrane potential PVN neurons.** Current-clamp recordings from two different PVN neurons in different hypothalamic slices. Upper panel (A) shows bath application (purple bar) of 2nM BDNF caused a depolarization of the membrane potential while a different PVN neuron (B) showed a hyperpolarizing response to 2nM BDNF. Both neurons showed a return to baseline membrane potential following washout of BDNF

A)




B)

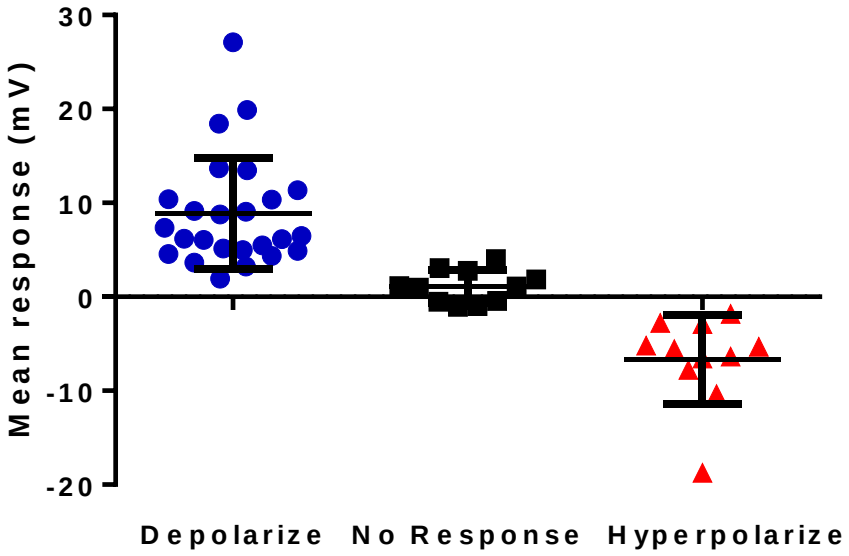



**Figure 2 – Summary of BDNF effects on PVN neurons.** The bar graph (A) shows the proportion of neurons that depolarize, do not respond, or hyperpolarize in response to bath application of 2 nM BDNF. The scatter plot (B) illustrates the changes in membrane potential of each individual neuron tested in response to bath application of 2nM BDNF. The horizontal bar represents the mean and the error bars represent the standard error of the mean. Mean depolarization: 8.9 ± 1.2 mV. Mean hyperpolarization: -6.7 ± 1.4 mV.

supraphysiological concentrations of glucose (10 mM) not likely to be encountered by the CNS neurons *in vivo*.

As a result, we sought to investigate the impacts of different glucose concentrations (10 mM, 3mM, 1 mM, and 0.2 mM) on PVN neurons responding to BDNF. We evaluated whether the effects of BDNF on the excitability of PVN neurons may be altered as extracellular glucose concentration were modified. We maintained rat brain slices containing the PVN at 10, 3, 1, or 0.2 mM glucose and subsequently perfused 2 nM BDNF to evaluate the effects of the peptide on the membrane potential of PVN cells. Our findings are summarized in Figure 6, which shows the changes in membrane potential (Figure 6A) and the distribution of responses (Figure 6B) of PVN neurons at each glucose concentration. From the results shown previously, slices maintained at 10 mM showed effects of BDNF (2 nM) on 77% of PVN neurons, 54% of which depolarized (n = 25; mean: 8.9 ± 1.2 mV), 23% of which hyperpolarized (n = 11; mean: -6.7 ± 1.4 mV), and the remaining 23% of which did not respond to BDNF (n = 11). At 3 mM extracellular glucose, 70% of PVN neurons (n = 20) responded to BDNF treatment, 35% of which depolarized (n = 7; mean: 8.7 ± 1.5 mV), 35% of which hyperpolarized (n = 7; mean: -8.9 ± 1.4 mV), and the remaining 30% (n = 6) did not respond to BDNF. When extracellular glucose concentrations were then reduced to 1 mM, 80% of PVN neurons (n = 20) responded to BDNF treatment, 38% with a depolarization (n = 9; mean: 9.7 ± 2.3 mV), 42% with a hyperpolarization (n = 10; mean: -10.5 ± 1.7 mV), and the remaining 20% did not respond. Finally, at 0.2 mM extracellular glucose, 77% of PVN neurons (n = 17) responded to BDNF administration, 6% with a depolarization (n = 1; mean: 4.8 ± 0 mV), 71% with a hyperpolarization (n = 12; mean: -6.3 ± 2.8 mV), and the remaining 23 % (n = 4) did not respond.

Thus there was no difference in the amplitude of the depolarizations or hyperpolarizations

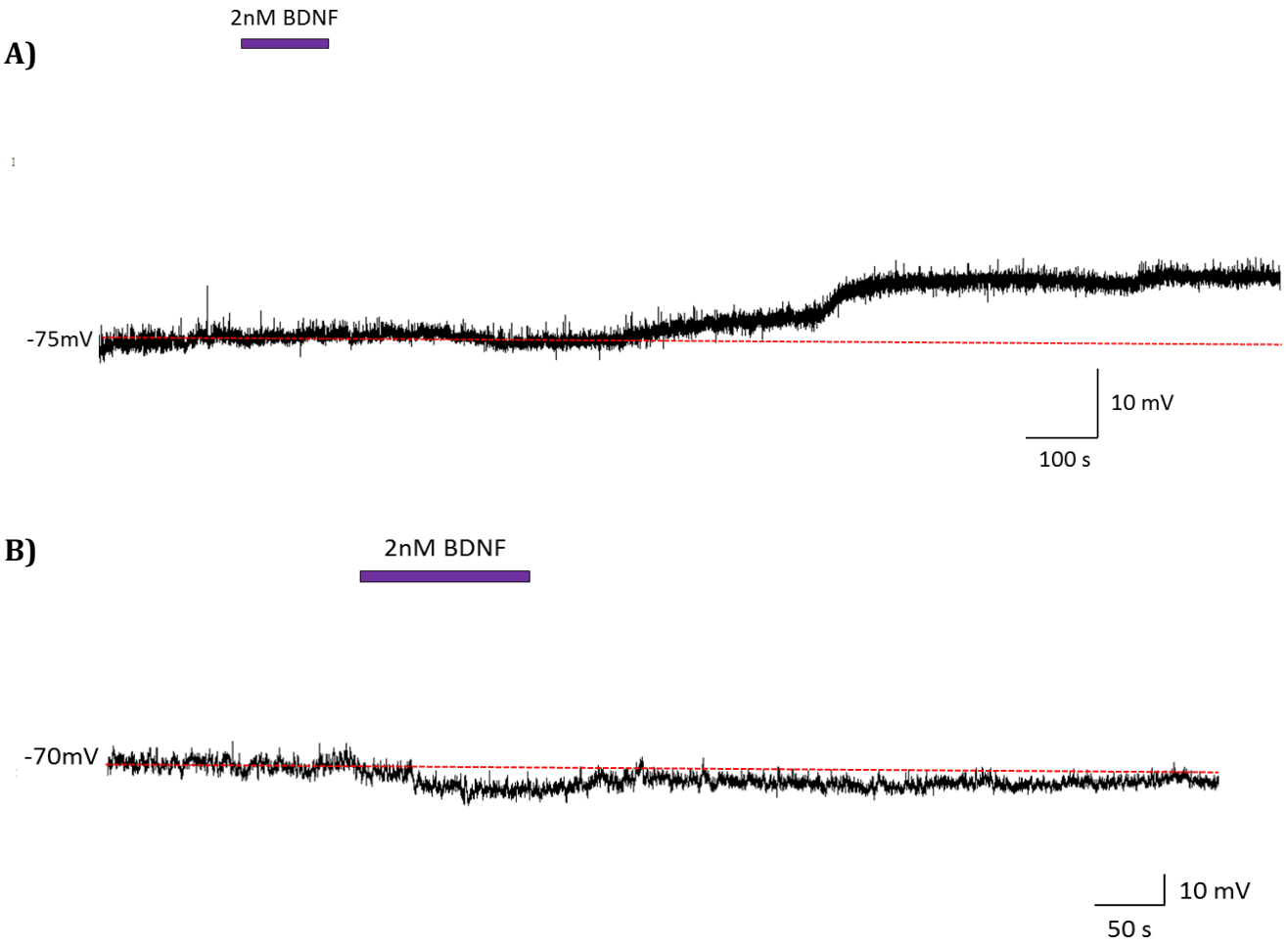



**Figure 3 – BDNF actions on PVN neurons persist in the presence of TTX**. Current-clamp recordings from two PVN neurons pretreated with 1µM TTX. Depolarizing (A) and hyperpolarizing (B) responses to 2 nM BDNF are still observed in the presence of TTX (N=9).The scatterplot (C) shows the changes in membrane potentialand the bar graph (D) shows the proportion of neurons that depolarize, do not respond, or hyperpolarize. Mean depolarization: 7.4 ± 2.5 mV. Mean hyperpolarization-5.9 ± 2.2 mV.

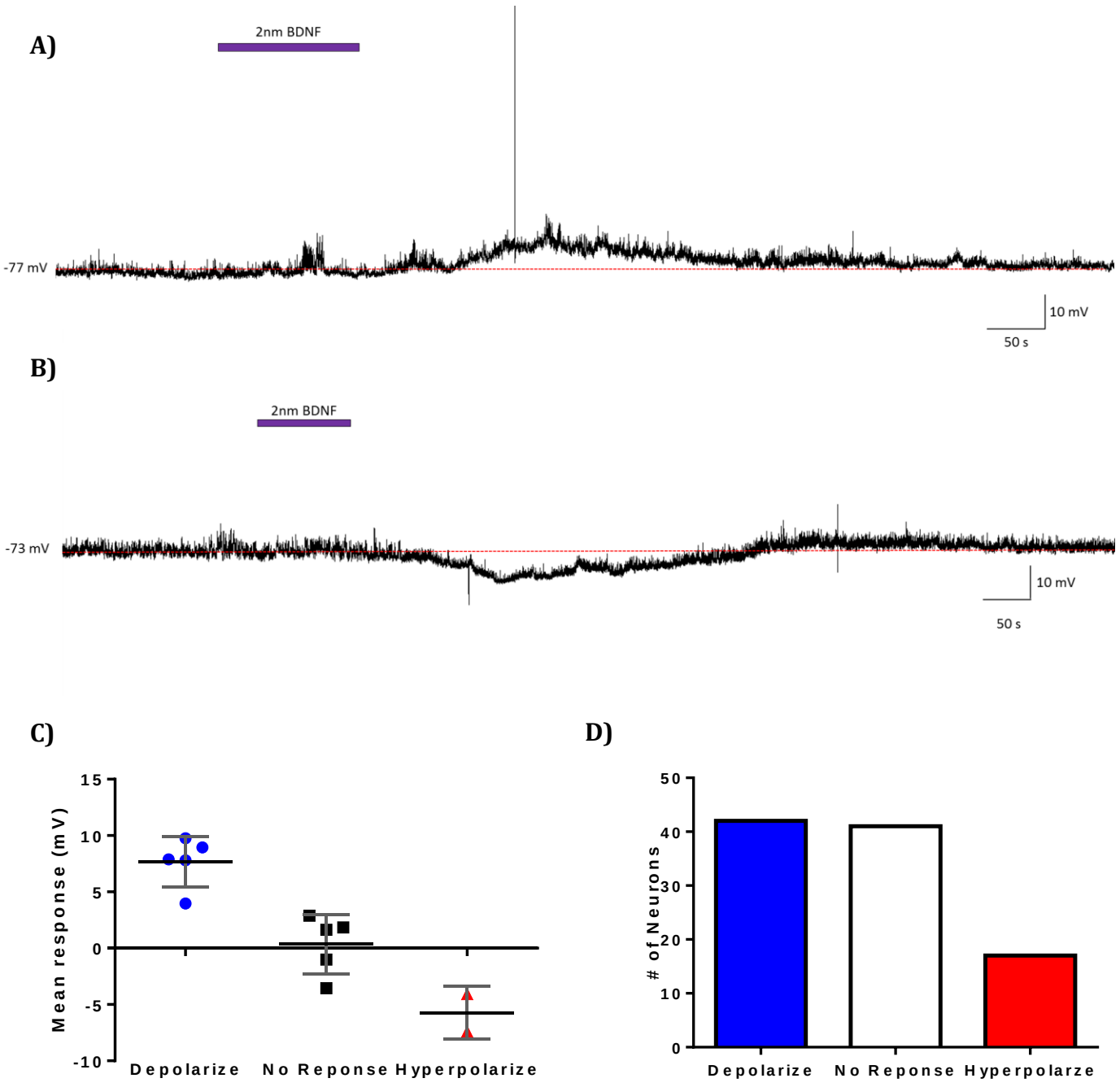



**Figure 4 – BDNF's actions on PVN neurons are not mediated by $GABA_A$ receptors.**Previous studies have shown that BDNF decreases the surface availability of $GABA_A$ receptors to decrease inhibitory inputs. As a result, we conducted current-clamp recordings from two different PVN neurons in slice preparation pretreated with 10 µM bicuculline. Both the depolarizing (A) and hyperpolarizing (B) effects of BDNF were observed in the presence of bicuculline. The scatterplot (C) shows the changes in membrane potentialand the bar graph (D) shows the proportion of neurons that depolarize, do not respond, or hyperpolarize in response to bath application of 2nM in the presence of bicuculline (N=12). Mean depolarization: 7.7 ± 0.99 mV. Mean hyperpolarization: -5.7 ± 1.67.

observed at all extracellular glucose concentrations employed (one-way ANOVA with Tukey's multiple comparison test; $p = 0.76$ and $p = 0.12$ for depolarizations and hyperpolarizations, respectively; Figure 6A).

Despite no changes in the amplitudes of responses across varying extracellular glucose concentrations, our findings do indicate that PVN neurons show significantly more hyperpolarizing responses to BDNF than depolarizing when glucose is decreased from 10 mM to 3 mM ($\chi^2$, $p = 0.003$). When glucose is decreased from 3 mM to 1 mM there was no significant change in the proportion of responses in PVN neurons to BDNF administration ($\chi^2$, $p = 0.08$). Finally, further decreasing the extracellular glucose concentration to 0.2 mM resulted in significantly more hyperpolarizations than depolarizations in response to BDNF treatment ($\chi^2$, $p < 0.0001$).

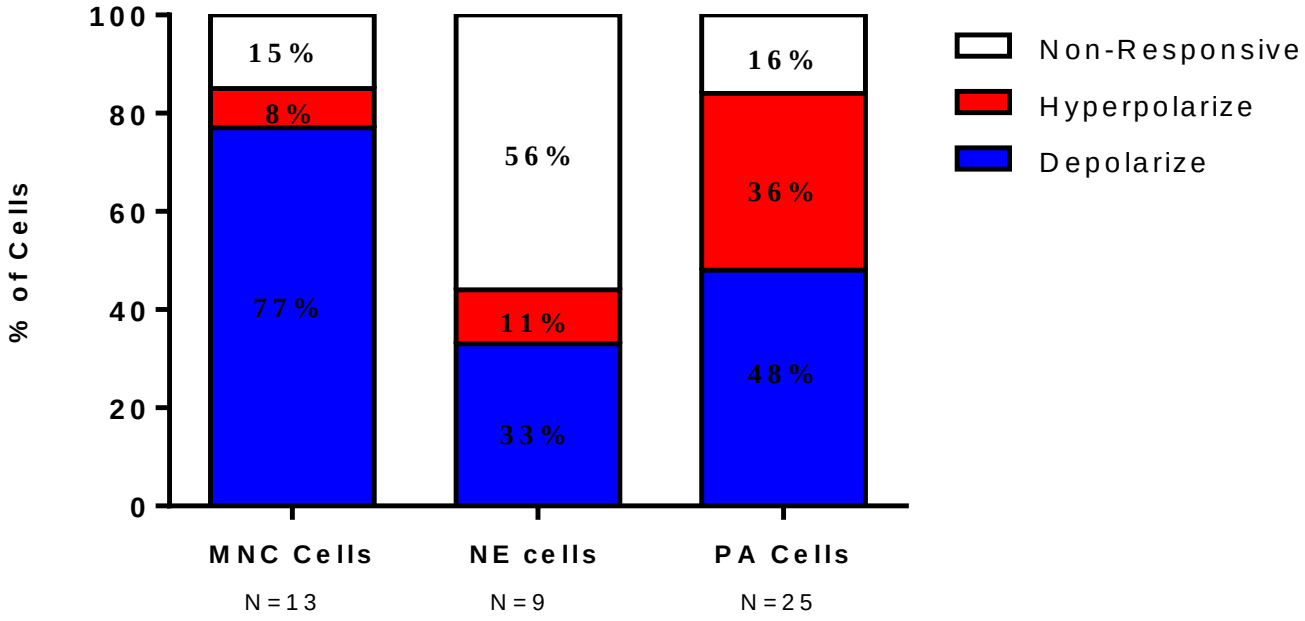



**Figure 5 – BDNF differentially influences subpopulations of PVN neurons.** This bar graph illustrates the proportion of magnocellular (MNC), neuroendocrine (NE), and preautonomic neurons (PA) that depolarize or hyperpolarize in response to bath application of 2 nM BDNF.

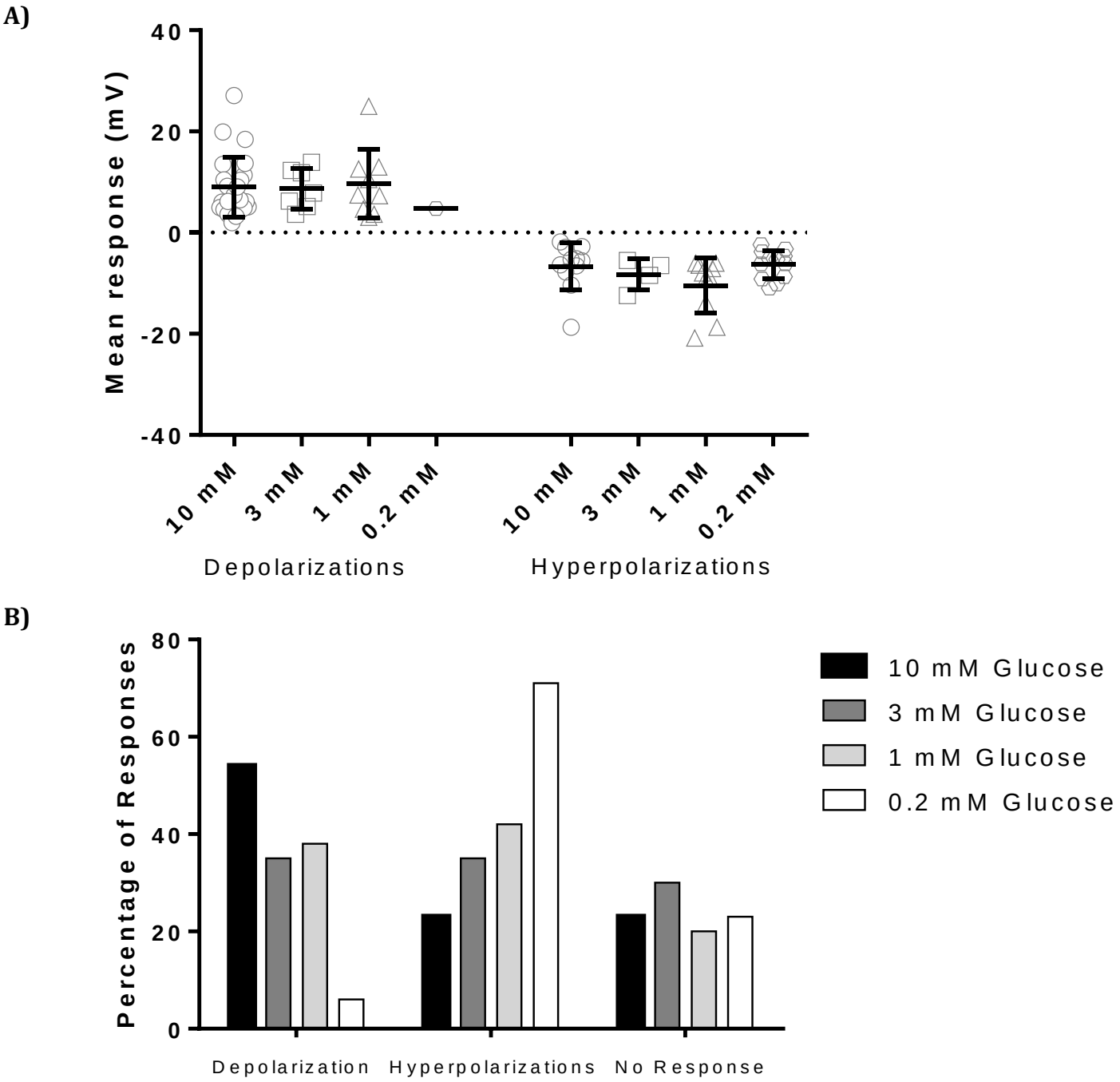



**Figure 6 – Amplitude of BDNF effects are consistent across glucose concentrations, but as glucose lowers, more hyperpolarizations are observed.** A: Scatterplot depicting the range of responses elicited by 2 nM BDNF at varying extracellular glucose concentrations. Mean depolarization at 10 mM was 8.9 ± 1.2 mV (n = 25), 3 mM, 8.7 ± 1.5 mV (n = 7), 1 mM, 9.7 ± 2.3 mV (n = 9), and 0.2 mM, 4.8 ± 0 mV (n = 1). Mean hyperpolarization at 10 mM was -6.7 ± 1.4 mV (n = 11), 3 mM, -8.9 ± 1.4 mV (n = 7), 1 mM, -10.5 ± 1.7 mV (n = 10), and 0.2 mM, -6.3 ± 2.8 mV (n = 12). B: Bar graph showing the percentage of PVN neurons that respond to BDNF with a depolarization, hyperpolarization, or no response across all glucose concentrations used. There is no difference in the amplitude of BDNF response across groups (ANOVA with Tukey's multiple-comparison test; P = 0.76 and P = 0.12 for depolarizations and hyperpolarizations, respectively). The distribution of BDNF responses across groups differed between 10 mM and 3 mM ($\chi^2$, P = 0.003) and 1 mM to 0.2 mM ($\chi^2$, P = < 0.0001), but not from 3 mM to 1 mM ($\chi^2$, P = 0.08).

## Chapter 4: Summary

The developing obesity epidemic has been shown to be linked to dysfunction in the regulation of energy balance at the central level (104).Over the past decade many studies have contributed to enhancing our understanding of the critical roles of the hypothalamus and specific nuclei within this region in the central regulation of appetite and energy homeostasis (42; 151; 178; 202; 211). There are numerous signals that are involved in regulating the energy state of an organism but extracellular glucose is perhaps one of the most critical. Neurons in many critical CNS nuclei involved in the regulation of energy homeostasis have been shown to be glucose sensitive (3; 136; 156; 205), and recent studies have suggested that extracellular glucose concentrations in these areas may in fact modify the responsiveness of individual neurons to other signaling molecules involved in energy regulation (71; 118). Understanding such plasticity in the neural circuitry involved in the regulation of food intake and energy homeostasis represents an important step to the elucidation of the complex interactions between the many signals which contribute to the regulations of metabolic homeostasis.

Traditionally known as an important member of the neurotrophin family, BDNF promotes neuronal differentiation, survival during development, neurogenesis and neural plasticity (5; 65; 88). While BDNF has been shown to modify circuits important to food intake and energy expenditure (82; 84; 183), the cellular mechanisms underlying these effects remain poorly understood. This is the first study to show that the neurotrophin BDNF has direct effects on the membrane potential of PVN neurons that are not a consequence of the modulation GABA transmission. Due to the heterogeneous nature of the PVN, we subsequently directed our focus to understanding how each neuronal subtype is affected by BDNF administration. Using patch-clamp electrophysiology, we investigated PVN sensitivity to BDNF under different glucose

concentrations in an attempt to mimic different feeding states, thus examining if single PVN neurons sense and integrate multiple feeding signals. This study shows that the responses of PVN neurons to BDNF are dynamically altered in accordance with the glucose concentrations in the CNS and by extension, the metabolic status of the organism.

*4.1BDNF directly affects membrane potential of PVN neurons*

Our initial experiments were designed to establish whether PVN neurons respond to BDNF with changes in membrane potential. Previous studies utilizing whole-cell patch-clamp recordings from PVN neurons in coronal slices have shown that BDNF selectively inhibits GABA synapses through a dynamin-mediated endocytosis of postsynaptic $GABA_A$ receptors onto parvocellular neuroendocrine neurons (72). However, this study examined effects on mIPSCs on a single subtype of PVN neurons (NE) and demonstrated TrkB-mediated inhibition of GABA signaling. These observations were consistent with previous work in hippocampal slices (33), although it should be noted that others have reported that BDNF can also potentiate $GABA_A$ receptor function (15). However, none of these previous studies reported effects of BDNF on the membrane potential of recorded neurons which we observed in our patch-clamp recordings from PVN neurons. As described above these effects were quite rapid in onset and often completely reversible within the time frame of our recordings. Importantly, effects on neuronal excitability were maintained in TTX, supporting the conclusion that the effects were not the result of effects on other neuronal soma in our slice preparation. In addition, these effects were maintained in bicuculline suggesting these actions are not related to the modulation of GABAergic neurotransmission. Collectively, these observations suggest that BDNF exerts direct post-synaptic actions on PVN neurons to depolarize or hyperpolarize the membrane potential of these critical autonomic cell groups.

Our observations suggest that BDNF may be influencing PVN neurons in a manner independent of the previously suggested endocytosis of $GABA_A$ receptors. Other studies have shown BDNF has a multitude of effects on many different channels of neurons, one of which is calcium. (24). In support of this, another study detected BDNF-evoked $Ca^{2+}$ transients in neuronal cell bodies in the absence of extracellular $Ca^{2+}$ (35). Binding of BDNF to TrkB activates various intracellular signaling cascades which lead to increased calcium(188). Once activated the TrkB receptor autophosphorylates and recruits intracellular proteins that trigger the phospholipase C-γ (PLC) pathway resulting in the formation of the second messengers diacyl-glycerol (DAG) and inositol 1, 4, 5-triphosphate ($IP_3$).This ultimately induces calcium release from intracellular stores which can occur within 20 seconds and lasts up to 20 minutes after addition of BDNF (208). Such changes correlate quite well with the time course of action of BDNF observed in our PVN recordings.

Protein kinases activated by TrkB phosphorylation may modulate ion channels to alter the excitability of PVN (28; 90). Importantly, several subtypes of potassium channels are subject to tyrosine phosphorylation after application of BDNF. Acute BDNF activation of TrkB receptors in the olfactory bulb increased tyrosine phosphorylation of $K_v1.3$ within 15 minutes (194). Additionally, BDNF can alter the activity of G-protein-activated inward rectifying potassium channels (Kir3) (153). PLC-dependent calcium elevations have been demonstrated to induce calcium-activated potassium currents that are expressed in most neuronal cells where they are involved in spike repolarization and after-hyperpolarization (206). A study involving neurons from the visual cortex showed that BDNF administration caused both a long-lasting increase in intracellular calcium and an outward current that was identified as calcium-activated potassium current (122). This is another possible mechanism through which BDNF may exert its effects on

PVN neurons. However, determining the channels involved in BDNF's effect on PVN neurons was beyond the scope of this study and, consequently, further experiments involving voltage-clamp analysis would represent an important extension of our studies.

Our results show that BDNF induces both depolarizing and hyperpolarizing responses in neurons that reside in PVN. Hypothalamic PVN neurons are a heterogeneous mixture of various cell types that express a wide array of neurotransmitters and projection sites, this may explain why BDNF induces both depolarizing and hyperpolarizing responses in neurons from this region. As a result, we sought to investigate whether BDNF impacted PVN neuronal subtypes in different ways.

*4.2 BDNF differentially influences subpopulations of PVN neurons*

Our experiments show for the first time that the majority (77%) of PVN neurons respond to BDNF. However, distinct neuronal subpopulations appear to respond differentially to BDNF application. MNC neurons exhibit primarily depolarizations (77%) with only one hyperpolarization (8%) in response to BDNF at standard electrophysiological concentrations of glucose (10 mM), which may indicate that MNC neurons projecting to the posterior pituitary are influenced. This possibility is supported by a study that employed ICV administration of BDNF and recorded VP mRNA signals from before and after BDNF injection using in situ hybridization. These investigators found that BDNF induced a decrease in VP mRNA expression while the hypothalamic hormone content increased (63). In addition, another group performed experiments on isolated hypothalamic cells from rats that showed that when BDNF is present in the culture medium, VP secretion increases significantly while OT secretion remains unchanged (125).Within the context of these observations, our data suggest that depolarizing effects of BDNF on MNCs may represent primary action on VP neurons.

PVN NE cells show mainly depolarizations (33%) or no response (56%) and again, only one hyperpolarization (11%) in response to BDNF. A possible explanation for this observation is that BDNF is influencing the neurons expressing CRH rather than those expressing TRH, leading to the observed division of responses. This theory would align well with previous studies that have shown that ICV BDNF upregulated CRH mRNA expression in the PVN (192). These results suggest that BDNF's effects on NE neurons may be directly through the CRH-urocortin pathway in the PVN and may cause a depression in feeding downstream. The PA cells show varied responses indicative of distinct cell types responding to BDNF. It is possible that BDNF is depolarizing the OT-expressing PA cells projecting to the medulla which have been previously implicated in having anorexigenic effects (27; 76).

Although electrophysiological fingerprints of PVN neurons offer a means of cellular subtype identification, they are not beyond compare. Further studies using single-cell reverse transcription-polymerase chain reaction (RT-PCR) along with slice patch-clamp recordings may be beneficial in determination of the precise cell type and their associated mRNA expression profiles in order to further elucidate how BDNF is impacting distinct cell type in the PVN.

*4.3 Glycemic state influences the proportion of responses amongst PVN neurons*

In this study, we initially chose to maintain PVN slices in 10 mM glucose, as this is the industry standard with regards to electrophysiological recordings in brain slices. Interestingly, this level of glucose is supraphysiological and does not represent a concentration that is normally experienced by the CNS but may be used in order to maintain the integrity of the brain slices for recording purposes. This concentration, that is widely used when performing electrophysiological recordings, is greatly contrasted by physiological brain glucose levels which are approximately 2.5 mM (158). Studies that have simultaneously measured circulating blood glucose levels in

relation to central extracellular glucose levels in rats have found extracellular glucose levels in the brain to range from 0.16 ± 0.03 during hypoglycemia to 4.5 ± 0.4 during hyperglycemia (168). Our chosen concentrations of 0.2, 1, and 3 mM extracellular glucose provide a closer mimic of the range of concentrations that may be encountered by PVN neurons *in vivo* and can be compared to 10 mM concentrations that previous whole-cell patch-clamp studies in that region utilize. It is evident from our results that the proportion PVN neurons that respond to BDNF with a depolarization appears to decline as extracellular glucose levels decrease. Conversely, there is a clear trend toward higher proportions of hyperpolarizing responses as extracellular glucose levels decrease. It is interesting to note that the largest change in the proportion of responses occurs when extracellular glucose levels change to 0.2 mM, at which point hyperpolarizations become the dominant response. Previous studies investigating PA neurons' responses to changes in extracellular glucose found that these neurons showed minimal changes when the concentration was changed from 10 mM to 2.5 mM, but from 10 mM to 0.2 mM 67.8% of PA neurons responded to low glucose (114). This may provide an explanation for the more drastic changes in response to BDNF observed at 0.2 mM compared to other glycemic states. Perhaps, this drastic hypoglycemia is required to provoke PVN neurons to alter their response to BDNF and ultimately promote changes in energy balance of the body in order to combat the perturbation. Our study demonstrates that although using supraphysiological concentrations of glucose may benefit the integrity of the PVN slices, it may produce different proportions of responses to BDNF than more physiologically relevant values. From our results, it is evident that the proportion of responses differs significantly between groups, but more importantly between the supraphysiological concentration of 10 mM and the more physiologically relevant value of 3 mM. It is important to note, that the proportion of non-responsive PVN neurons does not appear

to differ greatly between glycemic states, illustrating that perhaps if TrkB is not present, glycemic state has no impact on the responsiveness of those neurons to BDNF.

From this study, it appears that PVN neurons have a comparable sensitivity to glucose, in that the influence of BDNF is most effectively altered at low rather than high levels of glucose. Considering the nature of each PVN neuronal subtype, it seems intuitive that if extracellular glucose is low (indicating an energy-deprived state), reducing the anorexigenic signal that BDNF would promote by activating CRH, TRH, OT, and VP neurons in the PVN would be beneficial. As a result, at low glucose levels, these neurons become more inclined to hyperpolarize in order to oppose the low energy state of the brain reducing anorexigenic signaling. A study using acute injections of BDNF into the PVN showed that high fat diet induced obesity and metabolic syndrome-like measures (such as increased glucose) increased PVN's responsiveness to BDNF injection in those obese animals compared to rats with less body fat (204). This evidence also suggests that the fed state of the organism impacts the way in which the PVN responds to BDNF.

*4.4 BDNF's widespread actions*

It is important to note that mature BDNF (13.6 kDa) does not cross the blood–brain barrier (139; 140) and as such, may represent a distinct mechanism for the control of energy homeostasis. Subcutaneous BDNF increases muscle use of glucose and brown adipose tissue of *db/db* mice (216). In addition, BDNF injected subcutaneously reduces body weight in *db/db* mice and a single injection of BDNF has significant and long-lasting hypoglycemic effects (213-215). Thus, BDNF may act in the periphery to maintain proper blood glucose levels in mice.

BDNF acts on TrkB receptors in both the CNS and the periphery to reduce food intake and ultimately obesity in rodents, but this action is not paralleled in higher mammals. Monkeys demonstrate the anorectic effects of central administration of BDNF much like that which is seen

in rodents, however, the response to peripheral administration is orexigenic (106).It is interesting that this particular neurotrophic factor has opposing functions when administered in different areas of the body. However, despite not being able to cross the blood-brain barrier, perhaps peripheral information provided by BDNF may communicate with the CNS by other means. The subfornical organ (SFO) is one of a group of specialized structures which lack the normal blood-brain barrier, known as circumventricular organs (CVO's), that allow passage into the CNS for molecules whose information may otherwise be trapped in the periphery. The SFO is situated on the floor of the third ventricle and is known to be involved in cardiovascular (57) and metabolic (171; 172; 193) control. The SFO cerebral vasculature contains fenestrations between endothelial cells which make it ideal for detecting and regulating molecules in the periphery. The SFO is connected to many hypothalamic autonomic control centers, including the PVN, to which it can communicate the information it has gathered from the periphery (120; 121). In addition, microarray data from our laboratory has shown that the SFO has a high density of TrkB receptors. With this information, we administered BDNF to SFO neurons in slice preparation to investigate the effects BDNF on the excitability of this population of neurons. Our current-clamp recordings (data not shown) from SFO neurons show that BDNF effects the excitability of these neurons, resulting in 67% (n = 4) neurons responding to BDNF application with a large depolarization (Mean = 18.2 ± 7.6 mV). These results demonstrate that BDNF has widespread impacts with regards to energy metabolism, as well as illustrate the coordination between the periphery and CNS required to monitor and respond to any perturbations.

*4.5 Neurotrophic effects of other satiety signals*

Traditionally, BDNF has been seen as a well-documented regulator of plasticity, synaptic strength, neuron survival, and neuron differentiation (5; 65; 74) and because of this, one view is

that rather than having direct actions on anorexigenic or orexigenic pathways, BDNF or TrkB insufficiencies alter basic components of neuronal function. However, it is important to note that the satiety hormones leptin and ghrelin have profound effects on neuronal development and neurotransmission in addition to their impacts on energy homeostasis. For example, *ob/ob* mice have shown significant modifications in their neural projections from the ARC that cannot be rescued with leptin treatment in adulthood (30).Thus, indicating leptin's role in facilitating α-MSH and AGRP neuron fibers directional growth to their targets in the PVN. This study suggests that leptin acts as a neurotrophic signal during the development of the hypothalamus that precedes leptin's regulation of food intake in adulthood (30). Furthermore, leptin has also been shown to regulate ventral tegmental area (VTA) neuron excitability by activation and trafficking of potassium channels (75; 176). Finally, studies have demonstrated that leptin administration to hippocampal neuronal progenitor cells causes proliferation (145).

Ghrelin is an orexigenic hormone that has acts in the central nervous system and has exhibited some neurotrophic effects similar to those of BDNF's. Ghrelin regulates the excitability of neurons, neurotransmitter release, neuron proliferation, and neuron survival (59). Like BDNF, ghrelin has antiapoptotic effects, more specifically, through the protection of hypothalamic neurons experiencing oxygen-glucose deprivation by inhibiting reactive oxygen species production and inhibiting caspase 3 (41).

Both of these satiety signals are involved in synaptic remodeling processes that contribute to the control of food intake (148; 182). *Ob/ob* mice have shown differences in the number of excitatory and inhibitory synapses onto NPY and POMC cells in the ARC compared to wildtype mice, changes are expected to promote feeding (148). Alternatively, ghrelin demonstrates the

opposite effects to those of leptin in the ARC and increases excitatory input onto dopamine neurons in the VTA (1; 148).

Another similarity that these feeding signals share with neurotrophic factors is their antidepressant effects and influences on learning and memory (52; 135). Leptin impacts hippocampal synaptic plasticity by facilitating CA1 LTP and calmodulin protein kinase II activity (135). Similarly, ghrelin works in the hippocampus to promote LTP and dendritic spine synapse formation (52). These data suggest that BDNF's known impacts on neurotransmission and plasticity do not exclude it from having anorexigenic effects and a direct role in metabolic homeostasis.

*4.6 Conclusion*

In conclusion, our data show that BDNF has postsynaptic effects on the majority of PVN neurons and that these effects are not caused by modulation of GABA signaling. Despite many PVN neurons being affected by BDNF, distinct neuronal subpopulations appear to respond differentially. In addition, these responses to BDNF are dynamically altered in accordance with changes in local glucose levels. This study provides evidence of the integrative capacity of single PVN neurons through their ability to alter BDNF signals in changing extracellular glucose. Our data has important implications for future electrophysiological studies regarding the use of physiologically relevant glucose concentrations when evaluating the effects of feeding-related peptides in the PVN.

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