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THE ROLE OF BDNF-MEDIATED NEUROPLASTICITY IN CARDIOVASCULAR
REGULATION WITHIN THE HYPOTHALAMUS AND BRAINSTEM

A Dissertation Presented

by

Daniella Thorsdottir

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ABSTRACT

The paraventricular nucleus of the hypothalamus (PVN) is an important cardiovascular and autonomic regulatory center. Activation of PVN neurons projecting to the brainstem and spinal cord elevates sympathetic activity and blood pressure. Brain derived neurotrophic factor (BDNF) plays a key role in stress-induced cardiovascular responses within the PVN and is also known to be upregulated in the PVN in response to stress and hyperosmolality. PVN overexpression or acute injection of BDNF also elevates blood pressure chronically. However, the mechanism behind BDNF-mediated cardiovascular regulation is not fully understood. BDNF is known to modulate excitatory/inhibitory signaling by altering the expression and membrane trafficking of neurotransmitter receptors. Further, changes in the excitatory/inhibitory signaling in the PVN have been shown to elevate blood pressure and sympathetic activity chronically in various hypertensive models. Thus, we set out to examine the long-term effects of BDNF overexpression within the PVN on NMDA-, GABAA- and catecholaminergic receptor-mediated blood pressure mechanisms. We tested the hypothesis that BDNF increases blood pressure, in part by diminishing catecholaminergic inhibitory input from the nucleus of the solitary tract (NTS) to the PVN, while also enhancing NMDA and diminishing GABAA-signaling in the PVN. Sprague-Dawley (SD) rats received bilateral PVN injections of viral vectors expressing either green fluorescent protein (GFP) or BDNF and bilateral NTS injections of vehicle or anti-dopamine- β -hydroxylase-conjugated saporin (DSAP), which selectively lesions catecholaminergic neurons. BDNF overexpression in the PVN without NTS lesioning significantly increased mean arterial pressure in awake animals ($p < 0.001$). DSAP treatment also increased blood pressure in the GFP group but failed to affect blood pressure in the BDNF group. In addition, hypotensive responses to PVN injections of a β -adrenergic agonist were significantly attenuated by BDNF overexpression, while BDNF treatment also significantly reduced β 1-adrenergic receptor mRNA expression in the PVN ($p < 0.01$). Cardiovascular responses to PVN injections of NMDA and GABAA agonists and antagonists were also recorded in GFP and BDNF rats. NMDA inhibition led to greater decreases in blood pressure in the BDNF group ($p < 0.05$), while NMDA activation did not significantly alter blood pressure in the two groups ($p = 0.31$, n.s.). NMDA-signaling is elevated in the PVN in response to BDNF, which may be due to an increase in presynaptic release of glutamate or an increase in glutamatergic innervation. Meanwhile, GABAA inhibition led to greater increases in blood pressure in the GFP group, while GABAA activation led to a greater decrease in blood pressure in the GFP group. GABAergic signaling is reduced in the PVN in response to BDNF, potentially due to a decrease in both presynaptic and postsynaptic GABAA signaling mechanisms. Protein expression of NMDAR1 in the PVN was also significantly elevated in the BDNF group ($p < 0.05$) while GABAA-alpha1 expression in the PVN was diminished in the BDNF group ($p < 0.01$). In summary, increased BDNF expression in the PVN elevates blood pressure, in part by downregulating β -receptor signaling and diminishing hypotensive catecholaminergic input from the NTS to the PVN, while also promoting NMDA-mediated excitatory activity and diminishing GABAA-mediated inhibitory activity in the PVN.

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LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ACTH	Adrenocorticotropic Hormone
AGRP	Agouti-Related Protein
Alpha-MSH	Alpha Melanocyte-Stimulating Hormone
AMPA	α -Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
ANA-12	[N2-2-2-oxoazepan-3-yl amino] carbonyl phenyl benzo (b)thiophene-2-carboamide
Ang II	Angiotensin II
AT1R	Angiotensin Type 1 Receptor
AVP	Arginine Vasopressin
BDNF	Brain-Derived Neurotrophic Factor
BNST	Bed Nucleus of the Stria Terminalis
cAMP	Cyclic Adenosine Monophosphate
CNS	Central Nervous System
CPA	Caudal Pressor Area
CRH	Corticotropin Releasing Hormone
CRHR1	Corticotropin-Releasing Hormone Receptor 1
CRHR2	Corticotropin-Releasing Hormone Receptor 2
CVLM	Caudal Ventrolateral Medulla
DBH	Dopamine Beta Hydroxylase
DMH	Dorsomedial Nucleus of the Hypothalamus
DMV	Dorsal Motor Nucleus of the Vagus
DVC	Dorsal Vagal Complex
DSAP	DBH-Conjugated Saporin
EAAT	Excitatory Amino Acid Transporter
Erk	Extracellular Signal-related Kinase
GABA	γ -Aminobutyric Acid
GAD	Glutamic Acid Decarboxylase

GAT GABA Transporter
GPCR Guanine Nucleotide-Binding protein-Coupled Receptors
GR Glucocorticoid Receptor
HPA Hypothalamic-Pituitary-Adrenal Axis
HPT Hypothalamic-Pituitary-Thyroid Axis
ICV Intracerebroventricular
IML Intermediolateral Cell Column of the Thoraco Lumbar Spinal Cord
KCC2 K⁺- Cl⁻ cotransporter 2
KO Knockout
LH Lateral Hypothalamic Area
LFS Low Frequency Stimulation
LTD Long-Term Depression
LTP Long-Term Potentiation
MAP Mean Arterial Pressure
MC4r Type 4 Melanocortin Receptor
mEPSC Miniature Excitatory Postsynaptic Current
mIPSC Miniature Inhibitory Postsynaptic Current
MnPO Median Preoptic Nucleus
mGluR Metabotropic Glutamate Receptor
MAPK Mitogen-activated Protein Kinase
MR Mineralocorticoid Receptor
NADPH Nicotinamide Adenine Dinucleotide Phosphate
NGF Nerve Growth Factor
NKCC1 Sodium Potassium Chloride Co-Transporter
NMDA N-Methyl-D-Aspartate Receptor
NPY Neuropeptide Y
NT-3 Neurotrophin 3
NT-4/5 Neurotrophin 4/5

NTS Nucleus of the Solitary Tract
OVLТ Organum Vasculosum Lamina Terminalis
OXT Oxytocin
p75NTR Pan Neurotrophin Receptor 75
PAG Periaqueductal Gray
PI3K Phosphoinositide 3-Kinase
PLC-γ Phospholipase C-γ
PKC Protein kinase C
POMC Proopiomelanocortin
PVN Paraventricular Nucleus of the Hypothalamus
ROS Reactive Oxygen Species
RVLM Rostral Ventrolateral Medulla
SD Sprague Dawley
SFO Subfornical Organ
SHR Spontaneously Hypertensive Rat
SNP Single Nucleotide Polymorphism
STAT3 Signal Transduced and Activator of Transcription 3
TH Tyrosine Hydroxylase
TRH Thyroid Releasing Hormone
Trk Tyrosine Kinase Receptor
TRPC3 Transient Receptor Potential Cation Channel Subfamily C Member 3
VGLUT Vesicular Glutamate Transporter
VLM Ventrolateral Medulla
VTA Ventral Tegmental Area
VMN Ventromedial Nucleus of the Hypothalamus
WKY Wistar-Kyoto Rat

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction: Hypertension and Sympathetic Control of Blood Pressure

1.1.1. Hypertension Etiology and Prevalence

Hypertension is a chronic medical condition characterized by persistent long-term elevation of blood pressure in arteries measured on two or more occasions, where systolic pressure measured is at ≥ 130 mmHg and the diastolic pressure is measured at ≥ 80 mmHg (Muntner et al., 2018). Primary hypertension is a chronic elevation of blood pressure without a direct causal factor, and accounts for more than 90% of hypertension cases. Secondary hypertension occurs due to an identifiable cause, such as renal failure, pheochromocytoma or pregnancy (Carretero & Oparil, 2000; Lindheimer, Taler, & Cunningham, 2010; Oparil, Zaman, & Calhoun, 2003). Several factors have been implicated in the development of primary hypertension. Familial and twin studies have suggested that 30%-50% of blood pressure variance can be attributed to genetic heritability, while roughly 50-70% can be attributed to environmental factors (Butler, 2010; Jeunemaitre et al., 1992). Specific environmental factors that have been shown to increase the risk for the development of hypertension include high stress (Hamer, Molloy, & Stamatakis, 2008; McEwen & Gianaros, 2010), high caloric (Kannel, Brand, Skinner, Dawber, & McNamara, 1967) and high salt diets (Denton et al., 1995; F. J. He & MacGregor, 2002; "Intersalt: an international study of electrolyte excretion and blood pressure. Results for 24 hour urinary sodium and potassium excretion. Intersalt Cooperative Research Group," 1988; M. Zhang et al., 2015). A diagnosis of hypertension is also a risk factor for the development of other cardiovascular diseases (Flint et al., 2019;

Rapsomaniki et al., 2014; Whelton et al., 2018), such as stroke, myocardial infarction, coronary heart disease, congestive heart failure, atrial fibrillation and chronic kidney disease (Angeli, Reboldi, & Verdecchia, 2014; B. M. Egan, Zhao, Axon, Brzezinski, & Ferdinand, 2011; J. He & Whelton, 1997; Lawes, Vander Hoorn, & Rodgers, 2008; Persell, 2011; Sarafidis et al., 2012; Whelton, 1994). Additionally, over 80% of hypertensive patients present with various co-morbidities such as glucose intolerance, hyperinsulinemia, lipid disorders, and obesity (Kannel, 2000; Kannel et al., 1967).

Primary hypertension is estimated to affect approximately 31% of the world's adult population (Kearney et al., 2005; D. Lloyd-Jones et al., 2010; Mills et al., 2016), with roughly 66% of those cases occurring in developing countries (Kearney et al., 2005). Prevalence of hypertension is estimated to be highest among older adults, aged 70 and above (Buford, 2016; Muntner et al., 2018; Vasan et al., 2002), as well as among adults with diabetes and non-Hispanic black adults (Gillespie & Hurvitz, 2013). Within the United States the crude prevalence of hypertension among adults has been estimated at 45.6%, where antihypertensive medication was recommended for 36.2% and nonpharmacological treatment was advised for 9.4% of adults diagnosed with hypertension (Muntner et al., 2018). Studies have further indicated that hypertension is inadequately controlled in the United States (Burt et al., 1995; D. J. Hyman & Pavlik, 2000; D. M. Lloyd-Jones et al., 2000). Rates of blood pressure above treatment goal among US adults taking antihypertensive medications range from 53-56% (D. Lloyd-Jones et al., 2010; Muntner et al., 2018). A separate study reported that 14% of patients diagnosed with hypertension had resistance to common antihypertensive treatments (Carey, 2013).

Currently there are several antihypertensive therapies available and prescribed for hypertension treatment. These include diuretics, beta blockers, calcium channel blockers, angiotensin converting enzyme (ACE) inhibitors, alpha-1 antagonists, and angiotensin receptor blockers (Aronow, 2018). However, while these drugs do lower blood pressure significantly, they do not significantly lower sympathetic nerve activity (Q. Fu et al., 2005). Elevated sympathetic activity is an important factor in the development of most cases of primary hypertension (Oparil, 1986; Rahn, Barenbrock, & Hausberg, 1999; Schlaich, 2004; Wyss, 1993), and thus developing treatments that target sympathetic nerve activity could offer more effective hypertensive therapeutic options. One of the main drugs currently available that can lower sympathetic activity is the alpha-2 blocker, Clonidine, but the need for a greater number of pharmaceuticals that lower sympathetic activity long-term are still needed (Foote, Bloom, & Aston-Jones, 1983). Surgical interventions that lower sympathetic nerve activity include renal denervation, which has shown limited effectiveness in lowering blood pressure in clinical trials (Fengler et al., 2019; Kuhl, Frey, & Frank, 2016). Another is a catheter-based renal endovascular approach used to disrupt renal nerve activity by using radiofrequency, ultrasound or chemical agents which has shown some success in several clinical trials (Azizi et al., 2018; Desch et al., 2015; Mahfoud et al., 2017). Baroreflex activation therapy is a third option, which involves electrically stimulating the carotid baroreceptors, which help regulate blood pressure (Gordin et al., 2016; Kuhl et al., 2016). This method has been shown to reduce blood pressure in treatment resistant hypertensive patients in particular (de Leeuw et al., 2017; Wustmann et al., 2009). Both pharmacological and surgical interventions remain crucial

for the treatment of hypertension, but better understanding of the central control of blood pressure could lead to the development of novel, more targeted therapies.

1.1.2. Sympathetic and Neural Control of Blood Pressure Regulation

Increased sympathetic activity is a significant factor in the development of hypertension (Carmichael & Wainford, 2015; Mancia & Grassi, 2014), which can be exacerbated by pro-hypertensive stimuli such as high stress, high-caloric and high-salt diets (Chida & Steptoe, 2010; Lambert & Lambert, 2011; Steptoe & Kivimaki, 2012; Whelton, 1994). This increase in sympathetic activity has been observed in young, middle-aged, and elderly adults with hypertension, as well pregnant and obese individuals (Hart & Charkoudian, 2014; Mancia & Grassi, 2014). Sympathetic activation is thought to be mainly driven by the paraventricular nucleus of the hypothalamus (PVN), contributing to various disease conditions such as hypertension, heart failure and post myocardial infarction (Floras, 2009). Both increases in excitation and decreases in inhibitory mechanisms have been suggested to enhance sympathetic output from the PVN (D.-P. Li & H.-L. Pan, 2007; D. P. Li & Pan, 2006; K. Zhang, Mayhan, & Patel, 1997; Z. H. Zhang, Yu, Kang, Wei, & Felder, 2008). Presympathetic neurons within the PVN stimulate spinal sympathetic preganglionic neurons in the intermediolateral (IML) cell column directly or via the rostral ventrolateral medulla (RVLM) (R. A. Dampney, 1994; Stornetta, 2009). Sympathetic preganglionic neurons innervate the heart, blood vessels, kidneys, adrenal medulla and peripheral vasculature to elevate blood pressure, heart rate and sympathetic nervous system activity which in turn activates baroreceptors (Guyenet, 2006). These are mechanoreceptors located in the aortic arch and carotid sinus, capable of sensing blood

pressure changes via distention of the arterial wall. The baroreceptors activate sensory afferents via the vagus and glossopharyngeal nerves that project to the nucleus of the solitary tract (NTS), where they excite the caudal ventrolateral medulla (CVLM), which in turn inhibits the RVLM from increasing sympathetic output. This negative feedback loop between the NTS, CVLM and RVLM, is known as the baroreflex (**Fig. 1.**). This pathway serves an important role in processing sensory information, responding to blood pressure changes and regulating sympathetic nervous system activity (Agarwal, Gelsema, & Calaresu, 1990; Andresen & Kunze, 1994; Benarroch, 2008; Coote, 2005; R. A. Dampney, 1994; Thrasher, 2005). Alterations in excitatory and inhibitory signaling within this neural circuitry, particularly within the PVN, has been shown to contribute to elevated blood pressure and sympathetic activity (Fisher, Young, & Fadel, 2009; Guyenet, 2006; J. He & Whelton, 1997; D.-P. Li & H.-L. Pan, 2007; D. P. Li & Pan, 2006; Mark, 1996). However, our understanding of the complex neuroanatomical interactions governing the central regulation of sympathetic nervous system overactivity and its role in the pathophysiology of hypertension remains unclear.

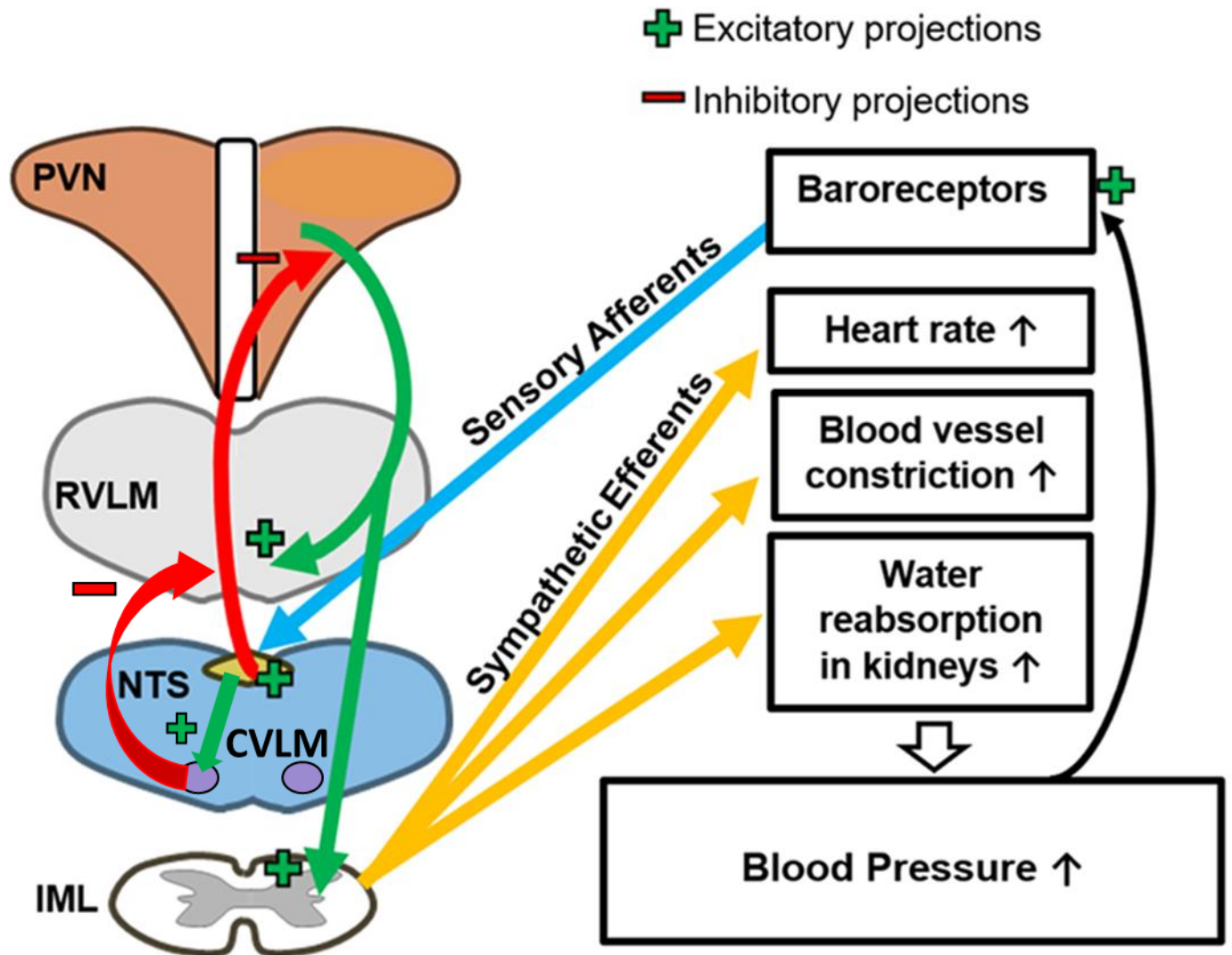


Figure 1. Presympathetic neurons within the PVN stimulate spinal sympathetic preganglionic neurons in the intermediolateral (IML) cell column directly or via the rostral ventrolateral medulla (RVLM). Sympathetic preganglionic neurons innervate the heart, blood vessels and kidneys to elevate blood pressure, heart rate and sympathetic nervous system activity which in turn activates baroreceptors. The baroreceptors activate sensory afferents via the vagus and glossopharyngeal nerves that project to the nucleus of the solitary tract (NTS), where they excite the caudal ventrolateral medulla (CVLM), which in turn inhibits the RVLM from increasing sympathetic output. This negative feedback loop between the NTS, CVLM and RVLM, is known as the baroreflex. The NTS also sends inhibitory catecholaminergic projections to the PVN to inhibit PVN neuron activity.

1.2. Brain Regions Involved in Sympathetic and Cardiovascular Regulation

1.2.1. Paraventricular Nucleus of the Hypothalamus (PVN)

The PVN is an important integration center involved in autonomic, neuroendocrine, behavioral and cardiovascular regulation (R. A. Dampney, 1994, 2005). The PVN integrates a variety of information from the cortex, brainstem, and hypothalamic areas. It receives interoceptive information from nociceptors, visceral receptors, and thermoreceptors carried by spinal afferents synapsing onto PVN neurons via the dorsal horn, the NTS, parabrachial nucleus, and the ventrolateral medulla (VLM) (Sawchenko, Li, & Ericsson, 2000). The PVN receives information related to blood osmolality levels, circulating steroids, and glucose levels from the circumventricular organs, mainly the median preoptic nucleus (MnPO), organum vasculosum lamina terminalis (OVLT) and the subfornical organ (SFO) (R. A. Dampney, Michelini, Li, & Pan, 2018; McKinley et al., 2001; Sawchenko et al., 2000). The PVN also receives limbic information from the amygdala and orbitomedial prefrontal cortex, related to emotional responses to stressors, relayed by the bed nucleus of the stria terminalis (BNST) and the dorsomedial nucleus of the hypothalamus (DMH) (R. A. Dampney et al., 2018; Sawchenko et al., 2000). The PVN receives information from the suprachiasmatic nucleus (SCN) about circadian control of endocrine and autonomic function. Finally, the PVN receives metabolism and food intake information from the arcuate nucleus and perifornical area (R. A. Dampney et al., 2018; A. V. Ferguson & Samson, 2003; Mastorakos & Zapanti, 2004).

The PVN sends efferent projections primarily to neuroendocrine and cardiovascular areas. The PVN sends neuroendocrine projections to the median eminence

and the posterior pituitary to activate the hypothalamic pituitary adrenal (HPA) axis via corticotrophin releasing hormone (CRH) neurons, and the hypothalamic pituitary thyroid (HPT) axis via thyroid releasing hormone (TRH) neurons. The PVN also regulates body fluid balance via arginine vasopressin (AVP) and oxytocin (OXT) neurons, regulates growth and development via somatostatin neurons, and transmits gastrointestinal and cardiovascular information via the NTS, RVLM, dorsal motor nucleus of the vagus (DMV), nucleus ambiguus, parabrachial region and midbrain periaqueductal grey (PAG) (Biag et al., 2012; R. A. Dampney et al., 2018; Alastair V. Ferguson, Latchford, & Samson, 2008; Pyner & Coote, 1999).

The functional and structural architecture of the PVN has been extensively studied via neuroanatomical tracing and immunohistochemistry, mostly in rats (Cechetto & Saper, 1988; Hallbeck, Larhammar, & Blomqvist, 2001; Jansen, Wessendorf, & Loewy, 1995; Sawchenko et al., 1996; Swanson, 1991). The PVN borders the third ventricle bilaterally and is broadly divided into a magnocellular and a parvocellular area. The parvocellular area is further subdivided into three sub-nuclei: dorsal, medial and ventrolateral parvocellular nuclei (Stocker, Cunningham, & Toney, 2004; Swanson & Kuypers, 1980). The magnocellular subdivision contains large neurons involved in the synthesis of AVP and OXT. These neurons project to the posterior pituitary and are involved in neuroendocrine regulation and the release of AVP and OXT into the blood stream (Benarroch, 2005; Engelmann, Landgraf, & Wotjak, 2004; Renaud & Bourque, 1991). Magnocellular PVN neurons also play a role in osmotically related sympathoexcitation and blood pressure elevation (Ribeiro, Panizza Hdo, Santos, Ferreira-Neto, & Antunes, 2015;

Son et al., 2013), whereby AVP is released from magnocellular neurons which increases PVN and RVLM neuronal activity, which can then be blocked by an AVP receptor antagonist in the PVN (Ribeiro et al., 2015; Son et al., 2013).

The medial parvocellular subdivision contains small neurons that synthesize CRH, somatostatin and other regulatory hormones. These neurons project to the median eminence to regulate hormonal secretion from the anterior pituitary (Benarroch, 2005; Sawchenko et al., 1996). Parvocellular CRH neurons also stimulate the production of adrenocorticotrophic hormone (ACTH) which activates the HPA axis (Sawchenko et al., 2000). The dorsal and ventrolateral parvocellular subdivisions of the PVN project to autonomic areas of the brainstem and spinal cord (Badoer, 2001; Coote, Yang, Pyner, & Deering, 1998). These neurons can be immunoreactive for a variety of neurotransmitters such as AVP, OXT, CRH, dopamine, tyrosine hydroxylase (TH), somatostatin, enkephalin, angiotensin and neurotensin (Cechetto & Saper, 1988; Hallbeck et al., 2001; Jansen et al., 1995). PVN neurons can also be divided up into three different categories based on their electrophysiological and neuroendocrine properties. Type I neurons are the magnocellular neurons involved in AVP and OXT secretion (Hoffman, Tasker, & Dudek, 1991), they also express a pronounced transient A-type K^+ current and project to the posterior pituitary. Type II neurons are neurosecretory parvocellular neurons that synthesize and secrete CRH or TRH, and project to the median eminence to control hormone release from the anterior pituitary and initiate the HPA and HPT axes (Hoffman et al., 1991; Lechan & Fekete, 2006; Luther & Tasker, 2000; Tasker & Dudek, 1991; Vale, Spiess, Rivier, & Rivier, 1981). Finally, type III neurons, also known as pre-autonomic, presympathetic or non-

neurosecretory parvocellular neurons, express a Ca^{2+} - dependent low-threshold spike (LTS) current. They project to the brainstem and spinal cord and are involved in the regulation of sympathetic activity (S. Lee et al., 2008; Luther et al., 2002; Stern, 2001).

Type III neurons project mainly to autonomic centers in the central nervous system (CNS), such as the spinal cord, NTS, DMV and the RVLM, where they play a critical role in the regulation of blood pressure and sympathetic activity (R. A. Dampney et al., 2018; Guyenet, 2006). This is supported by studies showing that bilateral inhibition of the PVN significantly decreases mean arterial pressure (MAP) and sympathetic nerve activity (Allen, 2002a; Zahner & Pan, 2005). Approximately 30% of spinally-projecting PVN neurons also project to the RVLM. PVN neurons can thus influence sympathetic activity via a PVN-IML pathway directly or indirectly via a PVN-RVLM-IML pathway (Badoer, 2001; Card et al., 2006; Coote et al., 1998; Cruz, Bonagamba, Machado, Biancardi, & Stern, 2008; Moon, Goodchild, & Pilowsky, 2002; Pyner & Coote, 1999; Ross, 1984; Z. Yang & Coote, 1998). Neuropeptide expression within the PVN is also known to be very plastic and can change in response to alterations in the hormonal environment (Benarroch, 2005; Swanson, 1991). PVN neurons are sensitive to both glutamate and γ -aminobutyric acid (GABA) and numerous studies have examined the functional roles of these transmitters within the PVN. There are several glutamate interneurons within the PVN, and extensive glutamate inputs to the PVN, mostly from other hypothalamic nuclei and forebrain nuclei (Alastair V. Ferguson et al., 2008; Ulrich-Lai, Jones, Ziegler, Cullinan, & Herman, 2011). GABA interneurons are extensively distributed around the PVN in an area sometimes referred to as the peri-PVN area, specifically known as the anterior

hypothalamic area and the perifornical regions. Within the PVN, there is also sparse GABA neuron expression (Alastair V. Ferguson et al., 2008). Finally, human studies examining the role of the PVN in hypertension have shown increased activity of neurosecretory cells within the PVN in patients with hypertension (Postnov Yu, Strakhov, Glukhovets, & Gorkova, 1974). However, the implication of this observation for specific PVN subpopulations remains to be determined.

1.2.2. Rostral Ventrolateral Medulla (RVLM)

The rostral ventrolateral medulla (RVLM) is a critical autonomic brainstem region involved in elevating sympathetic activity, associated with several cardiovascular diseases such as hypertension. The RVLM receives excitatory glutamatergic input from the PVN and inhibitory, largely GABAergic inputs, from the CVLM, NTS, the midbrain PAG, pontine reticular formation and the caudal pressor area (CPA) (Campos et al., 2008; Campos & McAllen, 1999; R. A. Dampney, 1994). Excitation of RVLM neurons via the application of glutamate, GABA antagonists or electrical stimulation, in both anesthetized and awake animals leads to an increase in sympathetic activity and blood pressure (de Paula & Machado, 2000; Moraes, Bonagamba, Zoccal, & Machado, 2011; Reis, Ross, Ruggiero, Granata, & Joh, 1984; Ross, Ruggiero, Joh, Park, & Reis, 1984; Sakima, Yamazato, Sesoko, Muratani, & Fukiyama, 2000; Willette, Barcas, Krieger, & Sapru, 1983). Meanwhile, microinjections of GABA agonists, tetrodotoxin application, or electrolytic lesions of the RVLM, lead to decreases in blood pressure (Benarroch, Granata, Ruggiero, Park, & Reis, 1986; R. A. Dampney & Moon, 1980; Reis et al., 1984; Willette et al., 1983)

RVLM glutamatergic neurons project directly to sympathetic preganglionic neurons in the IML in the thoracic spinal cord and provide tonic excitation (Amendt, Czachurski, Dembowski, & Seller, 1979; Ross et al., 1984). These sympathetic preganglionic neurons provide inputs to sympathetic postganglionic neurons, in the paravertebral sympathetic ganglia near the spinal cord or prevertebral sympathetic ganglia near the viscera. Postganglionic sympathetic neurons then provide inputs to glands and smooth muscle (Sugiyama, Suzuki, & Yates, 2011). Glutamatergic neurons are the largest neuronal population present in the RVLM, some of them also contain phenylethanolamine-N-methyl transferase (PNMT), which categorizes them as C1 epinephrine-synthesizing neurons (Ross, 1984; Ross et al., 1984). Photostimulation of these C1 neurons in the RVLM using a lentivirus that expresses channelrhodopsin-2 has been shown to increase blood pressure and sympathetic nerve activity in rats in vivo which further emphasizes their involvement in cardiovascular regulation (Abbott, Stornetta, Socolovsky, West, & Guyenet, 2009).

1.2.3. Caudal Ventrolateral Medulla (CVLM)

The CVLM is an important vasodepressor area in the brainstem, containing many cell populations involved in different aspects of cardiovascular regulation (Feldberg & Guertzenstein, 1976). Stimulation of the CVLM produces hypotension and bradycardia due to a decrease in sympathetic nerve activity, while inactivating or lesioning CVLM neurons leads to hypertension due to increases in sympathetic activity (Blessing & Reis, 1982, 1983; Cravo, Morrison, & Reis, 1991). CVLM neurons tonically inhibit RVLM sympathoexcitatory neurons by forming mostly inhibitory GABAergic synapses via the

baroreflex, contacting both adrenergic and non-adrenergic neurons in the RVLM (Aicher et al., 1996). The majority of CVLM projections contacting the RVLM are GABAergic but there is also some evidence of sparse catecholaminergic projections to the RVLM (Colombari et al., 2001; Granata, Numao, Kumada, & Reis, 1986; Milner, Pickel, Morrison, & Reis, 1989; Suzuki, Takayama, & Miura, 1997). The CVLM can be divided into a rostral portion, involved in control of sympathetic outflow and the baroreceptor reflex, and a caudal portion, involved in sympathetic outflow but importantly not involved in mediating the baroreceptor reflex (Cravo et al., 1991).

A1 noradrenergic neurons in the CVLM have been implicated in the regulation of cardiovascular homeostasis (Pedrino, Rosa, Korim, & Cravo, 2008). Neuroanatomical studies have shown that A1 neurons in the CVLM are mostly reciprocally connected to hypothalamic structures, involved in cardiovascular and neuroendocrine functions (Blessing & Reis, 1982). Approximately, 80% of the A1 noradrenergic neurons in the CVLM have been suggested to project to the PVN and the MnPO (D. C. Tucker, Saper, Ruggiero, & Reis, 1987). A recent study injecting dopamine-beta-hydroxylase (DBH) conjugated to saporin (DSAP) to lesion A1 neurons in the CVLM, showed an 81% decrease in A1 neurons in the CVLM and a 10% decrease in the number of C1 neurons in the RVLM (da Silva et al., 2013). However, these A1 noradrenergic projections may prove significant in blood pressure regulation and have not been fully examined yet.

1.2.4. Nucleus of the Solitary Tract (NTS)

The NTS is an important brainstem area involved in the integration of sensory afferent information from sensory afferents such as the arterial baroreceptors (Kline, King,

Austgen, Heesch, & Hasser, 2010). Projections from the NTS relay this information to various brain regions involved in autonomic regulation such as the PVN, CVLM and RVLM (Andresen & Kunze, 1994; Spyer, 1994). Ablating the NTS in normotensive rats by electrolytic lesioning elevates blood pressure (Doba & Reis, 1974; Duale et al., 2007; Talman, Snyder, & Reis, 1980), while electrically stimulating the NTS results in baroreceptor-like responses such as hypotension and bradycardia (Crill & Reis, 1968). Specifically, A2 noradrenergic neurons in the NTS have been broadly implicated in the chronic regulation of blood pressure (Affleck, Coote, & Pyner, 2012; Duale et al., 2007; McKellar & Loewy, 1981; Ricardo & Koh, 1978; Ter Horst, de Boer, Luiten, & van Willigen, 1989). Lesioning NTS catecholaminergic neurons in rats has led to contradictory results, either increasing (Daubert, McCowan, Erdos, & Scheuer, 2012; Duale et al., 2007; Itoh & Bunag, 1993) or having no effect on blood pressure (Itoh, Alper, & Bunag, 1992; Itoh & Bunag, 1992; Talman et al., 1980). Destruction of A2 noradrenergic neurons within the NTS using the neurotoxin 6-hydroxydopamine (6-OHDA), increases lability of blood pressure (Reis, Doba, Snyder, & Nathan, 1977; Talman et al., 1980) and decreases the baroreceptor reflex (Talman et al., 1980). A2 noradrenergic neurons are believed to tonically inhibit brain regions that generally increase blood pressure, such as the PVN (Cunningham & Sawchenko, 1988; R. A. Dampney, 1994; Loewy & McKellar, 1980; Sawchenko & Swanson, 1982b). The possible inhibitory role of NTS-PVN noradrenergic projections in blood pressure regulation has been shown but the mechanism behind the apparent reduction in inhibitory function under hypertensive conditions remains to be explored.

1.2.5. Baroreflex

The arterial baroreflex acts as a negative feedback system that senses and responds to blood pressure changes that occur during changes in behavior, emotion, posture, exercise, and other conditions (Benarroch, 2008; R. A. Dampney et al., 2018; R. A. L. Dampney, 2017; Veerman, Imholz, Wieling, Wesseling, & van Montfrans, 1995). The baroreflex depends on a feedforward mechanism that serves as a general adaptive response to stress and exercise, and a second feedback mechanism which allows for moment-to-moment regulation of blood pressure in response to peripheral cardiovascular, respiratory, and vestibular receptor information (Benarroch, 2008; Gordan, Gwathmey, & Xie, 2015). Activation of baroreceptors due to an increase in blood pressure results in a decrease of sympathetic nerve activity innervating the heart, arterioles, and veins, while also increasing the vagal output of the heart. This results in a decrease of total peripheral resistance, venous return, heart rate and cardiac output. However, in response to a decrease in blood pressure, reduced baroreceptor activity leads to sympathoexcitation and inhibition of cardiovagal output, leading to an increase in total peripheral resistance and tachycardia (Benarroch, 2008; Gordan et al., 2015).

Blood pressure changes are sensed by changes in blood vessel stretch by mechanosensitive arterial baroreceptors located in the carotid sinus, innervated by the glossopharyngeal nerve, and aortic arch, innervated by the vagus nerve. Information is then conveyed via glutamatergic afferents which synapse onto excitatory glutamatergic neurons in the NTS. The NTS then sends excitatory glutamatergic neurons to the CVLM to synapse with GABAergic inhibitory neurons which then proceed to inhibit sympathetic premotor

neurons in the RVLM. These sympathetic premotor neurons in the RVLM then project to sympathetic preganglionic neurons within the IML of the spinal cord where they synapse onto sympathetic preganglionic neurons which then contact organs in the periphery (Benarroch, 2008; Guyenet, 2006; Swenne, 2013). Both baroreceptor sensitivity and function can be dysregulated by chronic increases in blood pressure, as seen in hypertension (Chapleau, Li, Meyrelles, Ma, & Abboud, 2001).

1.3. Neurotransmitter and Neuromodulator Signaling Mechanisms Involved in the Regulation of Sympathetic, Cardiovascular and Neural activity in the PVN

1.3.1. Glutamatergic signaling in the PVN

Glutamate and GABA are the main excitatory and inhibitory neurotransmitters in the brain, and the firing activity of PVN neurons is known to be affected by the signaling of these two transmitters (D. P. Li, Yang, Pan, & Pan, 2008a, 2008b). The PVN has a dense concentration of excitatory glutamatergic nerve terminals and receptors (Boudaba, Schrader, & Tasker, 1997; Herman, Eyigor, Ziegler, & Jennes, 2000; van den Pol, 1991). Glutamate receptors in the PVN include N-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) receptors, and kainite receptors which are ionotropic receptors, and the PVN also expresses metabotropic glutamate receptors (mGluRs) (R. A. Dampney et al., 2018). The PVN receives glutamatergic inputs mainly from the DMH and the perifornical region (Boudaba et al., 1997), as well as glutamatergic interneurons within the PVN and from telencephalic regions (D.-P. Li & H.-L. Pan, 2007). The PVN sends glutamatergic projections, mainly to the RVLM and the IML of the spinal cord (Xu, Zheng, & Patel, 2012).

Glutamate can alter neuronal signaling and plasticity by acting at both ionotropic and metabotropic glutamate receptors (Evanson & Herman, 2015). In terms of blood pressure regulation, the NMDA receptor is particularly important since the PVN is populated by neurons that express NMDA receptor subunit genes and proteins (Eyigor, Centers, & Jennes, 2001; Herman et al., 2000; Petralia, Yokotani, & Wenthold, 1994), and the expression of NMDA receptor subunits in the PVN can be modified by changes in blood pressure (D.-P. Li & H.-L. Pan, 2007; Y. F. Li, Cornish, & Patel, 2003). A microinjection of glutamate or NMDA into the PVN has been found to increase blood pressure, and sympathetic nerve activity in both anesthetized and conscious rats (Busnardo, Crestani, Tavares, Resstel, & Correa, 2010; Kannan, Hayashida, & Yamashita, 1989; D. P. Li et al., 2008b). Meanwhile, microinjections of glutamate receptor antagonists, such as the NMDA receptor antagonist AP5, has little effect on sympathetic activity and blood pressure in normotensive animals (Q. H. Chen, Haywood, & Toney, 2003; D.-P. Li & H.-L. Pan, 2007; D. P. Li et al., 2008b; Zahner & Pan, 2005). This suggests that under normotensive conditions, glutamatergic input in the PVN is not crucially involved in tonic control of sympathetic tone.

In contrast, when the NMDA receptor antagonist AP5 is injected into a spontaneously hypertensive rat (SHR), sympathetic nervous system activity and blood pressure is significantly decreased (D.-P. Li & H.-L. Pan, 2007; D. P. Li et al., 2008b). Microinjections of NMDA receptor agonists in the PVN also increases neuronal activity in the RVLM, which is reduced by the application of glutamate receptor inhibitors in the RVLM (Z. Yang & Coote, 1998). This suggests that sympathoexcitatory glutamatergic

PVN-RVLM projections are crucially involved in the cardiovascular response to increased glutamate release in the PVN. SHRs also have a higher excitability of PVN presympathetic neurons, which contributes to increased sympathetic outflow (Allen, 2002b; D. P. Li et al., 2008b; Ye, Li, Li, & Pan, 2011), while also showing elevated glutamatergic inputs to PVN presympathetic neurons. This is believed to be maintained by an upregulation of postsynaptic NMDA receptors and an increase in presynaptic glutamate release (D.-P. Li & H.-L. Pan, 2007; D. P. Li et al., 2008b).

1.3.2. GABAergic signaling in the PVN

GABAergic inhibition plays a major role in regulating the activity of the PVN. GABA, acting mostly on ionotropic GABAA receptors but also partly on metabotropic GABAB receptors, tonically restrains PVN neuronal activity and autonomic outflow (Biancardi, Campos, & Stern, 2010; Q. H. Chen et al., 2003; Decavel & Van den Pol, 1990; D. P. Li & Pan, 2006; Y. F. Li & Patel, 2003; Tasker & Dudek, 1993; K. Zhang, Li, & Patel, 2002). Tonic inhibition is largely believed to be controlled by delta-containing GABAA receptors, due to their high sensitivity for GABA (Zheleznova, Sedelnikova, & Weiss, 2009). The immediate surround of the PVN, also referred to as the peri-PVN area, appears to consist of intermixed populations of glutamate and GABA neurons that send local excitatory and inhibitory projections to PVN neurons, including PVN parvocellular neurons (Cullinan, 2000). GABAA receptor subunit mRNAs for alpha1–2, beta1–3 and gamma1–2 and delta are expressed within CRH neurons and in parvocellular PVN neurons broadly (Kiss, 1988). In addition to GABAA receptors, recent studies have also revealed that GABAB receptors are expressed in the parvocellular PVN (Margeta-Mitrovic,

Mitrovic, Riley, Jan, & Basbaum, 1999). The role of these receptors in stress regulation has yet to be extensively investigated.

Altered PVN GABAergic function has been associated with increased sympathetic activity during hypertension (D. P. Li & Pan, 2006; D. S. Martin & Haywood, 1998) and heart failure (K. Zhang et al., 2002). PVN-RVLM neurons and sympathetic outflow are also tonically inhibited by GABA (Q. H. Chen et al., 2003; R. A. Dampney et al., 2018; D. P. Li & Pan, 2005; Y. F. Li & Patel, 2003; K. Zhang & Patel, 1998). In the SHR, GABAergic activity is altered, resulting in a decrease in GABAA receptor function and an increase in GABAB receptor function. This alteration may contribute to the elevated sympathetic outflow, as seen in hypertension (Cork, Chazot, & Pyner, 2016; Y. F. Li, Jackson, Stern, Rabeler, & Patel, 2006). Microinjection of the GABAA antagonist bicuculline into the PVN attenuate increases in sympathetic nerve activity, blood pressure and heart rate in renal-wrapped hypertensive rats (D. S. Martin & Haywood, 1998). Microinjections of the GABAA antagonist, gabazine, into the PVN of both WKY and SHR rats has also been found to increase sympathetic activity and blood pressure. However, this effect was found to be significantly reduced in SHRs compared to WKY rats (D. P. Li & Pan, 2005; D. P. Li & H. L. Pan, 2007). The GABAA receptor agonist isoguvacine and muscimol have also been tested in the PVN and been found to reduce blood pressure significantly more in normotensive rats compared to SHRs (Ding et al., 2015; D. P. Li & H. L. Pan, 2007). Finally, a microinjection of baclofen, a GABAB receptor agonist, into the PVN produces a greater inhibitory effect on sympathetic outflow in SHR than in normotensive WKY rats (D. P. Li & H. L. Pan, 2007).

Another mediator of GABAergic signaling are GABA transporters (GATs). GATs have been shown to play a critical role in modulating tonic GABA_A inhibition (Dalby, 2003). GAT1 is the most abundantly expressed form of GAT, it is mainly expressed in presynaptic neuronal terminals (Gadea & López-Colomé, 2001; Schousboe, 2000). Other notable GATs include GAT3 and GAT4 transporters, which are mostly expressed in glial cells (Minelli, Alonso-Nanclares, Edwards, DeFelipe, & Conti, 2003; Minelli, DeBiasi, Brecha, Zuccarello, & Conti, 1996; Ribak, Tong, & Brecha, 1996). PVN injections of the GAT inhibitor, nipecotic acid, has been shown to decrease renal sympathetic nervous system activity and heart rate but have no effect on blood pressure in normotensive rats. The study also found greater immunoreactivity of GAT3 over GAT1 in the PVN of these rats, which tended to display glial like staining and partially colocalize with glial fibrillary acidic protein (J. B. Park, Jo, Zheng, Patel, & Stern, 2009). Thus, GATs may play a significant role in modulating GABAergic signaling, especially under hypertensive conditions considering that GABA_A activity in the PVN is diminished in SHRs. Finally, glutamic acid decarboxylase 65 (GAD65) and 67 (GAD67) are enzymes that catalyze the conversion of glutamate to GABA and CO₂. They are also often used as markers of GABAergic activity and the expression of GAD67 has been shown to be elevated in renovascular hypertensive rats compared to normotensive control animals (Biancardi et al., 2010). This may suggest that alterations in the level of expression of GAD67 in the PVN reflect changes in GABAergic signaling in hypertension.

1.3.3. Catecholaminergic signaling in the PVN

A few key brain regions in the brainstem are known to synthesize catecholamines, all of which help regulate sympathetic activity. These include the A2 noradrenergic and C2 adrenergic neurons in the NTS, A5 noradrenergic neurons in the ventrolateral pons, and A1 noradrenergic and C1 adrenergic neurons in the VLM (Cunningham & Sawchenko, 1988; Sawchenko & Swanson, 1982a). The majority of catecholaminergic projections into the PVN come from A2 noradrenergic neurons in the NTS, which tonically inhibit PVN presympathetic neurons (Cunningham & Sawchenko, 1988; R. A. Dampney, 1994; Loewy & McKellar, 1980; Sawchenko & Swanson, 1982b). Ablating the NTS in normotensive rats by electrolytic lesioning results in hypertension (Doba & Reis, 1974; Duale et al., 2007; Talman et al., 1980), while electrically stimulating the NTS results in hypotension and lower heart rate (Crill & Reis, 1968). Specifically, lesioning NTS A2 noradrenergic neurons in rats has led to contradictory results, either increasing (Daubert et al., 2012; Duale et al., 2007) or having no effect on blood pressure (Itoh et al., 1992; Itoh & Bunag, 1992; Talman et al., 1980). Studies that claim that NTS noradrenergic projections are excitatory are mostly stress studies, which may indicate that these neurons inhibit PVN neurons under baseline conditions but exert the opposite effect under stress conditions. Additionally, the baroreceptor reflex has been reported to decrease after noradrenergic and dopaminergic neuron destruction using the neurotoxin 6-Hydroxydopamine (Talman et al., 1980).

In terms of receptor expression, adrenergic alpha-1a, alpha-1b, alpha-2, and beta adrenergic receptors have all been identified in parvocellular neurons within the PVN

(Cummings & Seybold, 1988; Little, Duncan, Breese, & Stumpf, 1992). Alpha 1a and alpha-1b adrenergic receptors are highly expressed in both the PVN and NTS (Day, Campeau, Watson, & Akil, 1997). Stimulation of alpha1 adrenergic receptors in the PVN leads to increased blood pressure and sympathetic outflow (Boudaba et al., 1997; Q. Chen, Li, & Pan, 2006; Daftary, Boudaba, Szabó, & Tasker, 1998; Daftary, Boudaba, & Tasker, 2000). Meanwhile, alpha-2 adrenergic receptors have been shown to exert a hypotensive effect throughout the CNS, reducing sympathetic nerve activity and blood pressure, mostly in the forebrain (Wyss & Carlson, 1999). The role of alpha-2 adrenergic receptors in the PVN is unclear, with one study showing limited effect on blood pressure regulation when the alpha-2 adrenergic receptor antagonist yohimbine is administered to the PVN, which does not significantly reduce the increased MAP response to histamine administration (Bealer & Abell, 1995). Another publication has implicated alpha-2 receptors in the PVN in the modulation of synaptic GABA release and regulation of sympathetic outflow, suggesting a hypotensive effect of alpha-2 activation (D. P. Li & Pan, 2005).

Beta 1 and beta 2 adrenergic receptors in the PVN have been shown to exert an inhibitory action on AVP secretion (Veltmar, Culman, Qadri, Rascher, & Unger, 1992), suggesting that they may inhibit AVP-mediated increases in blood pressure. Further, microinjections of the beta-adrenergic receptor antagonist propranolol into the PVN have been found to increase spontaneous electrical activity of catecholaminergic neurons, preventing the inhibitory actions of NTS noradrenergic projections to the PVN (Saphier & Feldman, 1991). Propranolol is also known to significantly decrease the gain of reflex bradycardia, while isoprenaline, a general beta-adrenergic agonist, significantly increases

the gain of reflex bradycardia and increases baroreflex sensitivity (D. Wang et al., 2013). Finally, the beta-2 adrenergic agonist fenoterol has been shown to reduce blood pressure in rats, when injected into the PVN (Tsushima, Fujimoto, & Matsuda, 1994). This suggests that beta-adrenergic signaling in the PVN may play a part in cardiovascular and baroreflex regulation.

1.3.4. Angiotensinergic signaling in the PVN

Several cardiovascular nuclei in the hypothalamus and brainstem contain angiotensin type I and II (Ang I and II) cell bodies and nerve terminals, as well as their respective receptors angiotensin type I and type 2 receptors (AT1R and AT2R). These nuclei include the RVLM, SFO, SON and the PVN (Lind, Swanson, & Ganten, 1985; J. M. Wang, Veerasingham, Tan, & Leenen, 2003). Ang II stimulates a G-protein signaling pathway associated with protein kinase activation which leads to an increase in reactive oxygen species (ROS) (G. Wang et al., 2006; Zimmerman, Lazartigues, Sharma, & Davisson, 2004), which can inhibit voltage-gated potassium channels to increase neuronal activity (Zimmerman et al., 2004). Ang II has been shown to activate presympathetic neurons in the PVN either directly or via their presynaptic terminals (Cato & Toney, 2005), and can also affect the release of neurotransmitters such as glutamate and GABA (Tsuda, 2012). In the PVN, activation of AT1R on GABAergic interneurons stimulates G-protein coupled signaling, activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit Nox2, which increases ROS and inhibits GABA release onto presympathetic neurons projecting to the RVLM or IML (Q. Chen & Pan, 2007; D. P. Li & Pan, 2005). Ang II can also increase the release of glutamate from glutamate

interneurons in the PVN to activate magnocellular neurons (Latchford & Ferguson, 2004), similar to what occurs in the RVLM (Nishihara, Hirooka, Matsukawa, Kishi, & Sunagawa, 2012; Zhu, Moriguchi, Mikami, Higaki, & Ogihara, 1998).

Studies have found consistent evidence for a sympathoexcitatory role for the angiotensinergic system in the PVN, RVLM and IML of the spinal cord. Regions of the SFO, OVLT and MnPO that express high levels of Ang II, are known to project to the parvocellular region of the PVN (Lind et al., 1985; McKinley, Allen, Burns, Colvill, & Oldfield, 1998; Oldfield, Ganten, & McKinley, 1989; Sunn, McKinley, & Oldfield, 2001; Westerhaus & Loewy, 1999). Projections between the SFO and the PVN use Ang II as a neurotransmitter, since activating SFO neurons by applying either Ang II or glutamic acid increases the release of Ang II in the PVN (Wright, Roberts, Stublely, Hanesworth, & Harding, 1993). SFO microinjections of Ang II also increase blood pressure, which is prevented by applying an AT1R blocker in the PVN (Ku, Jia, & Chang, 1999). Additionally, in vitro application of Ang II on PVN neurons excites PVN-RVLM neurons (Cato & Toney, 2005). Applying the GABAA receptor antagonist bicuculline in the PVN increases renal sympathetic nerve activity and blood pressure, but these effects are attenuated by 40-50% by using an AT1R blocker in the RVLM (Tagawa & Dampney, 1999). Finally, microinjecting Ang II into the PVN increases blood pressure which can be blocked by using an AT1R blocker in the RVLM (Ku et al., 1999). Additionally, Ang II in the RVLM increases local glutamate release, blood pressure and heart rate (Zhu et al., 1998). A decrease in GABAA inhibition of the PVN and an increase in Ang II signaling in

the PVN-RVLM pathway leads to activation of the AT1R in the RVLM, thereby increasing blood pressure, sympathetic activity and glutamate release (Gabor & Leenen, 2012b).

Angiotensinergic signaling in the CNS is also broadly known to contribute to elevated sympathetic activity and blood pressure in cold exposure (Sun, Cade, & Morales, 2002), DOCA-salt models (C. G. Park & Leenen, 2001), during increased plasma aldosterone (Xue et al., 2011), and in high salt diets in Dahl S rats and SHR (B. S. Huang & Leenen, 1996, 1998). Elevated AT1R activity may be explained by elevated synthesis of Ang II or an increased responsiveness to Ang II, but both may act in combination since ACE and AT1R are increased in the PVN of Dahl S rats on a high salt diet (J. M. Wang et al., 2003). These studies underline the importance of the role of the angiotensinergic system in the PVN and RVLM in central cardiovascular regulation.

1.3.5. Vasopressinergic, Oxytocinergic and Corticotrophin Signaling in the PVN

The neuropeptides AVP and OXT are synthesized, transported and secreted by magnocellular neurons in both the PVN and the SON (Burbach, Luckman, Murphy, & Gainer, 2001; Hatton, 1990; Japundžić-Žigon, 2013), and also partly by parvocellular neurons in the PVN and the SCN (DeVries, Buijs, Van Leeuwen, Caffé, & Swaab, 1985). The magnocellular PVN and SON neurons project to the neurohypophysis where AVP and OXT are released into the general circulation as hormones affecting distant organs and participating in the regulation of osmotic homeostasis and reproduction (Stoop, 2012). The parvocellular PVN projects to the median eminence to release AVP and OXT into the portal circulation, modulating the release of ACTH during the stress response. AVP and OXT are also known to act in a paracrine or autocrine manner to reduce or enhance

neuronal activity throughout the CNS (Geerling, Shin, Chimenti, & Loewy, 2010; M. Ludwig & Leng, 2006). AVP and OXT are both cyclic nonapeptides and differ only in two amino acids (De Bree et al., 2003). They both bind to V1a, V1b, V2 and one OXT receptor, which are all a part of a family of heptahelical guanine nucleotide-binding protein-coupled receptors (GPCRs), which are located widely in the brain and periphery (Kimura, Tanizawa, Mori, Brownstein, & Okayama, 1992; Koshimizu et al., 2012).

Application of selective antagonists, peripherally and centrally, has shown that physiological concentrations of AVP and OXT do not modulate or maintain blood pressure under baseline physiological conditions (Japundzic-Zigon, 2001; Milutinovic, Murphy, & Japundzic-Zigon, 2006). However, V1a receptor knockout (KO) mice show a lower baseline blood pressure compared to wildtype control animals (Koshimizu et al., 2012), and microinjections of AVP into the PVN of euhydrated rats has been shown to increase blood pressures and sympathetic nerve activity. Meanwhile, pretreatment with a V1a receptor antagonist in the PVN decreases sympathetic nerve activity and blood pressure in dehydrated rats compared with euhydrated rats. Thus, AVP in the PVN seems to modulate salt-intake related blood pressure elevations, via activation of V1a receptors (Ribeiro et al., 2015). On the other hand, OXT KO mice show a greater baseline blood pressure and heart rate compared to control animals (Bernatova, Rigatto, Key, & Morris, 2004), and further chronic PVN OXT neuron activation in vivo has been shown to prevent hypertension that occurs in rats exposed to three weeks of chronic intermittent hypoxia-hypercapnia (Jameson et al., 2016). These findings indicate that both AVP and OXT signaling play a vital role within the PVN in regulating blood pressure in hypertension.

Another important signaling mechanism in the PVN involved in blood pressure regulation and the initiation of the HPA axis is CRH which binds to CRH type 1 (CRHR1) and CRH type 2 (CRHR2) receptors (Dautzenberg, Dietrich, Palchadhuri, & Spiess, 1997). The release of CRH from the PVN leads to the release of ACTH (Vale et al., 1981), which then in turn acts on adrenal cortical cells to elevate corticosteroid production, the primary effector hormone of the HPA axis. Elevated corticosteroids activate glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). Activation of these receptors can modify gene expression via binding to specific DNA sequences and recruitment of transcriptional complexes (Arriza et al., 1987; de Kloet, 2000; Joels & de Kloet, 1992; Weinberger et al., 1985; Whirledge & DeFranco, 2018), and they further activate cognitive and metabolic resources to respond to current and future stressors and threats (Dallman & Yates, 1967; McEwen & Sapolsky, 1995; Swanson, Sawchenko, Rivier, & Vale, 1983; Ulrich-Lai & Herman, 2009; Vale et al., 1981).

Dysregulation of the HPA axis is widely associated with stress and hypertension (Burford, Webster, & Cruz-Topete, 2017; Whitworth, Mangos, & Kelly, 2000). CRH neurons in the PVN are known to project to the RVLM (Coldren, Li, Kline, Hasser, & Heesch, 2017; Milner, Reis, Pickel, Aicher, & Giuliano, 1993) and the NTS (Sawchenko, 1987), where they regulate sympathetic activity and blood pressure (Bardgett, Sharpe, & Toney, 2014; Milner et al., 1993; T. Nakamura, Kawabe, & Sapru, 2009; T. Nakamura & Sapru, 2009; Yamazaki et al., 2008). In an unstressed SHR, CRH mRNA was found to be elevated in the PVN compared with normotensive WKY rats. In addition, stress-induced increase in CRH mRNA levels was found to be much higher in the PVN of the SHR

(Krukoff, MacTavish, & Jhamandas, 1999). ICV injection of CRH, which increases the CRH gene in the PVN (Mansi, Rivest, & Drolet, 1996; Parkes, Rivest, Lee, Rivier, & Vale, 1993), also activates the HPA axis, and leads to an increase in blood pressure and heart rate (Brown, Hauger, & Fisher, 1988; Kalin, Shelton, Kraemer, & McKinney, 1983; Saunders & Thornhill, 1986). ICV administration of CRH additionally enhances activity of neurons in brain regions involved in cardiovascular regulation (Cummings, Elde, Ells, & Lindall, 1983; De Souza et al., 1985; Swanson, Sawchenko, & Lind, 1986; Swanson, Sawchenko, Lind, & Rho, 1987; Valentino & Foote, 1988; Valentino, Foote, & Aston-Jones, 1983). These findings suggest that CRH signaling in the PVN plays an important role in activation of the HPA axis, and regulation of cardiovascular responses in the hypothalamus and brainstem.

1.4. BDNF Signaling in the Central Nervous System

1.4.1. BDNF Synthesis, Transport and Secretion

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors which also includes nerve growth factor (NGF), neurotrophin (NT) 3, and NT4/5 (Barbacid, 1995; Berkemeier et al., 1991; Ernfors, Ibanez, Ebendal, Olson, & Persson, 1990; Gotz et al., 1994; Leibrock et al., 1989; Levi-Montalcini, 1987; Maisonpierre et al., 1990). BDNF is encoded by the BDNF gene, and is first synthesized as the precursor molecule pro-BDNF (32 kDa), which is later cleaved by the extracellular protease plasmin into the mature and biologically active form of BDNF (~13 kDa) or a BDNF pro-peptide (~17 kDa) (Z. Y. Chen et al., 2004; Negro, Tavella, Grandi, & Skaper, 1994; Pang et al., 2004; Timmusk et al., 1993; B. Yang, Ren, Zhang, Chen, & Hashimoto,

2017). NGF binds to the tyrosine kinase A (TrkA) receptor, NT-3 binds to the tyrosine kinase C (TrkC) receptor, and NT-4/5 binds to the tyrosine kinase B (TrkB) receptor. The mature form of BDNF also binds with high affinity to the TrkB receptor (Dawbarn & Allen, 2003; R. Klein et al., 1991) and binds with low affinity to the pan neurotrophin receptor 75 (p75NTR) (Chao et al., 1986; Teng et al., 2005), while pro-BDNF binds to p75NTR (Goodman et al., 1996; B. Yang et al., 2017). Binding of BDNF to TrkB receptors activates intracellular signaling cascades involving Ras and extracellular signal-related kinase (Erk) 1 and 2, phospholipase C- γ (PLC- γ), phosphatidylinositol 3-kinase (PI3K), and protein kinase C (PKC) (Nakagawara, Azar, Scavarda, & Brodeur, 1994; Zirrgiebel et al., 1995). Induction of several transcription factors follows this, including c-Jun, c-Fos, and early growth response 1 (Egr-1). Finally, activation of cyclic adenosine 3',5'-monophosphate (cAMP) response element binding protein occurs (Finkbeiner et al., 1997; Gaiddon, Loeffler, & Larmet, 1996; Nakagawara et al., 1994).

BDNF and the TrkB receptor are present in postsynaptic dendrites and presynaptic axon terminals, where they are capable of bidirectional release and activity (Tyler, Alonso, Bramham, & Pozzo-Miller, 2002). BDNF signaling stimulates neuron differentiation and development (Alderson, Alterman, Barde, & Lindsay, 1990; Knusel & Hefti, 1991), promotes neuron survival (Grothe & Unsicker, 1987; Hofer & Barde, 1988; Kalcheim & Gendreau, 1988) and long-term potentiation (LTP) (Korte et al., 1995; Korte, Staiger, Griesbeck, Thoenen, & Bonhoeffer, 1996; Patterson et al., 1996). BDNF is expressed throughout the developing and mature CNS (Bishop, Mueller, & Mouradian, 1994; Leibrock et al., 1989; Timmusk et al., 1993) and in peripheral tissues such as muscle, liver,

kidney, lung and adipose tissue (Cassiman, Deneff, Desmet, & Roskams, 2001; Lommatzsch et al., 1999; Mousavi & Jasmin, 2006; Ukropec, Ukropcova, Kurdiova, Gasperikova, & Klimes, 2008). Regional differences have been reported between BDNF mRNA levels and protein concentrations in the CNS (Altar et al., 1997; Conner, Lauterborn, Yan, Gall, & Varon, 1997; Narisawa-Saito, Wakabayashi, Tsuji, Takahashi, & Nawa, 1996; Nawa, Carnahan, & Gall, 1995), which may be due to BDNF anterograde transport (Altar et al., 1997) and mRNA decay (Malter, 2001). BDNF is synthesized in several areas of the hypothalamus, such as the PVN, the lateral hypothalamic area (LH), and the ventromedial hypothalamic nucleus (VMN) (Conner et al., 1997). BDNF is also expressed in areas of the thalamus and hindbrain, in the arcuate nucleus, hippocampus, amygdala, ventral tegmental area (VTA), and the dorsal vagal complex (DVC) (Bariohay, Lebrun, Moyse, & Jean, 2005; Conner et al., 1997).

BDNF is transported to secretion sites by motor protein complexes of neurons, as has been observed in axons and dendrites of cultured cortical and hippocampal neurons (N. Adachi, Kohara, & Tsumoto, 2005; Naoki Adachi, Numakawa, Richards, Nakajima, & Kunugi, 2014; Jakawich et al., 2010; Matsuda et al., 2009), and in knock-in mice expressing myc-tagged BDNF in the hippocampus at presynaptic terminals of neurons (Dieni et al., 2012). BDNF is transported both retrogradely and anterogradely in the CNS (N. Adachi et al., 2005; Altar & DiStefano, 1998; Caleo, Menna, Chierzi, Cenni, & Maffei, 2000; Rind, Butowt, & von Bartheld, 2005; Spalding, Tan, Hendry, & Harvey, 2002). Retrograde transport has been shown in motor neurons, where BDNF is released from postsynaptic sites and internalized by afferent presynaptic terminals (Rind et al., 2005).

Anterograde transport of BDNF involves motor protein kinesin and coordinator dynactin (Kwinter, Lo, Mafi, & Silverman, 2009), and has been shown to occur in the visual system (Caleo et al., 2000; Spalding et al., 2002). Activity-dependent local BDNF secretion occurs in the axon, soma, and dendrites of neurons (Edelmann, Lessmann, & Brigadski, 2014; Horton & Ehlers, 2003; Horton et al., 2005; Lessmann & Brigadski, 2009), and involves Ca^{2+} influx via inotropic glutamate receptors or L-type voltage gated calcium channels. This is then followed by signal amplification through Ca^{2+} -induced Ca^{2+} release from the ryanodine receptors (Lessmann & Brigadski, 2009). Activation of both CaMKII and PKA is required for BDNF secretion (Edelmann et al., 2014; Kolarow, Brigadski, & Lessmann, 2007).

The human BDNF gene contains 10 exons coding for the 5' untranslated region and are alternatively spliced to a common 3' coding exon, resulting in 34 possible mRNA transcripts (Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007). The rodent BDNF contains 9 exons, encoding 24 different mRNA transcripts, each of them translating into an identical mature BDNF (Cunha, Brambilla, & Thomas, 2010). Transcription of the BDNF gene occurs principally via exons I and IV, with the promoter on exon IV being particularly sensitive to increases in Ca^{2+} levels (Tao, West, Chen, Corfas, & Greenberg, 2002; Zheng et al., 2011; Zheng, Zhou, Moon, & Wang, 2012). The BDNF gene has multiple promoters regulated in specific ways under specific conditions to produce different mRNAs in certain tissues and in response to certain environmental stimuli (Pruunsild et al., 2007; Timmusk et al., 1995; Timmusk et al., 1993). Environmental factors such as stress can alter transcript expression of BDNF (Fuchikami, Morinobu, Kurata,

Yamamoto, & Yamawaki, 2009; Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003; Tognoli et al., 2010), which causes alterations in the ratio between pro-BDNF and total BDNF (Tognoli et al., 2010). Defeat stress significantly decreases BDNF mRNA by downregulating BDNF transcripts from promoters IV and VI (Tsankova et al., 2006). Meanwhile chronic stress in rats increases BDNF mRNA expression in the BNST (Hammack et al., 2009), while acute and repeated immobilization stress in rats increases BDNF mRNA expression in the parvocellular PVN, the lateral hypothalamus, and the pituitary gland (Smith, Makino, Kim, & Kvetnansky, 1995). Other pro-hypertensive stimuli such as high salt intake increase BDNF mRNA expression in the SON (Choe et al., 2015).

1.4.2. BDNF and Catecholaminergic Signaling

BDNF is a known modulator of catecholaminergic neurotransmission in the CNS (Castren, Thoenen, & Lindholm, 1995; M. J. Chen, Nguyen, Pike, & Russo-Neustadt, 2007; Scott, Zhang, & Nurse, 2015), and has been shown to effect the survival and regulation of catecholaminergic neurons. BDNF treatment of cultured neurons has been shown to lead to a dose-dependent increase in the number of TH-positive neurons, dopamine content, and dopamine uptake activity (C. Hyman et al., 1994). Further, BDNF-TrkB signaling increases the release of dopamine via stimulation of PI3K and mitogen-activated protein kinase (MAPK) in brain striatal slices (Goggi, Pullar, Carney, & Bradford, 2003). Within the hypothalamus BDNF has been shown to increase norepinephrine uptake and decrease its stimulated release (Rodriguez Fermepin, Trincherro, Minetto, Beltran, & Fernandez, 2009), while homozygous BDNF mutations lead to a

significant reduction of norepinephrine signaling (Dluzen, Story, Xu, Kucera, & Walro, 1999). The PVN receives substantial catecholaminergic input from NTS A2 and C2 neurons (Cunningham & Sawchenko, 1988; Pacak, Palkovits, Kopin, & Goldstein, 1995; Saphier & Feldman, 1991; Sawchenko & Swanson, 1982b), and although the role of these projections is not completely understood, prior studies have indicated that these neurons exert a hypotensive effect at baseline (Daubert et al., 2012; Duale et al., 2007; Itoh & Bunag, 1993). In addition, NTS catecholaminergic neurons are thought to be activated during both acute and chronic stress (Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Teppema et al., 1997; R. Zhang et al., 2010) and to play a role in the HPA axis response to systemic stressors (Rinaman & Dzmura, 2007; Ulrich-Lai & Herman, 2009). However, the effect of BDNF on catecholaminergic signaling in the PVN and its relation to altered cardiovascular function has not yet been studied

1.4.3. BDNF and Glutamatergic Signaling

BDNF has been shown to increase excitatory synaptic transmission in the CNS through both pre- and postsynaptic mechanisms (Minichiello, 2009; Waterhouse & Xu, 2009). Post-synaptically, BDNF enhances excitatory synaptic efficacy by modulating NMDA receptor function (Lin et al., 1998; Suen et al., 1997), Na⁺ channels (Lesser, Sherwood, & Lo, 1997), Kv1.3 (K. Tucker & Fadool, 2002) and transient receptor potential cation subfamily C member 3 (TRPC3) channels (H. S. Li, Xu, & Montell, 1999). BDNF also promotes the expression of NMDA (Caldeira et al., 2007) and AMPA (Itami et al., 2003) receptors in postsynaptic sites. However, BDNF has only been shown to selectively enhance postsynaptic responses to exogenously applied NMDA, but not AMPA (Crozier,

Black, & Plummer, 1999; Levine, Crozier, Black, & Plummer, 1998), while also increasing NMDA single-channel open probability (Levine et al., 1998; Levine & Kolb, 2000). The mechanism behind this may involve BDNF enhancement of the phosphorylation of NMDA receptor subunits (Lin et al., 1998; Suen et al., 1997). Pre-synaptically, BDNF enhances glutamate release in synaptosomes (Pascual, Climent, & Guerri, 2001; Sala et al., 1998) and increases the frequency of miniature excitatory post-synaptic currents (mEPSCs) in the cultured hippocampal and visual cortex neurons (Carmignoto, Pizzorusso, Tia, & Vicini, 1997; Lessmann & Heumann, 1998; Y. X. Li, Zhang, Lester, Schuman, & Davidson, 1998; Schinder, Berninger, & Poo, 2000; Tyler & Pozzo-Miller, 2001). BDNF has also been reported to enhance NMDA signaling by increasing the expression and membrane trafficking of NMDA receptor 1 (NMDAR1) and 2 subunits via a TrkB and MAPK-pathway (Caldeira et al., 2007; Kim et al., 2012). BDNF can also phosphorylate NMDA receptor subunits to increase the open probability of the NMDA receptor, thereby enhancing synaptic transmission (Black, 1999). Finally, acute exposure to BDNF enhances the magnitude of NMDA receptor mediated synaptic currents, increasing the activity of NMDA receptors containing NMDAR2 (Kolb, Trettel, & Levine, 2005). BDNF has also been shown to regulate vesicular glutamate transporters (VGLUT), which mediate L-glutamate uptake into synaptic vesicles [332]. BDNF upregulates VGLUT1 and 2 gene and protein expressions in cultured hippocampal neurons, through activation of the PLC γ signaling pathway [333].

1.4.4. BDNF and GABAergic Signaling

BDNF is a crucial regulator of synapse development and function in the CNS (Cohen-Cory, Kidane, Shirkey, & Marshak, 2010; B. Lu, Pang, & Woo, 2005), and GABAergic neuronal development is known to rely on BDNF (Gottmann, Mittmann, & Lessmann, 2009; Hong, McCord, & Greenberg, 2008; Kuzirian & Paradis, 2011; Sakata et al., 2009). Acute and chronic BDNF administration can suppress presynaptic GABA transmission in hippocampal slices (Bolton, Pittman, & Lo, 2000; Frerking, Malenka, & Nicoll, 1998), but alternately potentiate GABA transmission in cultured hippocampal neurons (Baldelli, Novara, Carabelli, Hernandez-Guijo, & Carbone, 2002). BDNF potentiation (Jovanovic, Thomas, Kittler, Smart, & Moss, 2004) and suppression (T. Tanaka, Saito, & Matsuki, 1997) has also been demonstrated in postsynaptic GABAergic currents. BDNF is known to modulate the efficacy of synaptic responses via TrkB and p75NTR (B. Lu et al., 2005), and alter the development of neuronal circuits and construction of inhibitory connections (Gottmann et al., 2009; Gubellini, Ben-Ari, & Gaiarsa, 2005; Kovalchuk, Holthoff, & Konnerth, 2004). BDNF binds to TrkB, which couples to the PLC- γ /PKC- δ and ERK/ MAPK pathway. Activated PLC- γ can induce PI3K activation which increases intracellular Ca²⁺ concentration (M. Yamada, Mizuguchi, Rhee, & Kim, 1991) consequently, BDNF disrupts GABA_A receptor function through elevated intracellular Ca²⁺ concentration (T. Tanaka et al., 1997).

BDNF-TrkB signaling regulates the expression and trafficking of various GABA_A receptor subunits (Bell-Horner, Dohi, Nguyen, Dillon, & Singh, 2006; Y. Nakamura, Darnieder, Deeb, & Moss, 2015), GAD65 (Hanno-Iijima, Tanaka, & Iijima, 2015;

Sanchez-Huertas & Rico, 2011), GAD67 (Hanno-Iijima et al., 2015) and GATs (Vaz et al., 2011), through the recruitment of the ERK-MAP kinase cascade, which activates the CREB protein (Yoshii & Constantine-Paton, 2010). In primary cultured hippocampal neurons and cerebellar granule cells, BDNF decreases the plasma membrane expression of GABAA receptors (Brunig, Penschuck, Berninger, Benson, & Fritschy, 2001; Q. Cheng & Yeh, 2003) and reduces GABAergic miniature inhibitory postsynaptic currents (mIPSC) (Brunig et al., 2001). BDNF can also increase the expression of the GABAA-alpha4 subunit and decrease the alpha1 subunit in hippocampal neurons, suggesting that BDNF has a potential role in differentially regulating the expression of extrasynaptic and synaptic GABAA receptors (Roberts, Hu, Lund, Brooks-Kayal, & Russek, 2006). Within the hypothalamus, BDNF has also been shown to alter GABAergic neurotransmission, by reducing the expression of GABAA receptors, membrane density and function in hypothalamic neurons (Hewitt & Bains, 2006; Lund et al., 2008).

Indirect modulation of GABAergic neurotransmission by BDNF can occur through the regulation of Cl^- transport (Wardle & Poo, 2003). Previous studies suggest that downregulation of the K^+ - Cl^- cotransporter 2 (KCC2) may decrease the efficacy of inhibitory transmission (C. Rivera et al., 1999). In developing neurons, BDNF increases KCC2 mRNA transcription through activation of the Shc pathway (Aguado et al., 2003; A. Ludwig et al., 2011; Claudio Rivera et al., 2004), but in adult neurons BDNF decreases both mRNA and protein KCC2 via activation of both Shc and PLC γ cascades (Boulenguez et al., 2010; Claudio Rivera et al., 2002; Claudio Rivera et al., 2004; Shulga et al., 2008; Wake et al., 2007). KCC2 expression is also significantly decreased in TrkB double KO

mice hippocampi (Carmona et al., 2006). Similarly, neurons in the dorsal horn of the spinal cord treated with BDNF show a depolarizing shift of the GABA reversal potential (Coull et al., 2005; Coull et al., 2003).

1.4.5. BDNF-mediated Neuroplasticity

Neurotrophins are important mediators of neuronal plasticity in the developing and mature CNS. They serve to modulate axonal and dendritic growth, neuronal survival, neuronal migration, membrane receptor trafficking, neurotransmitter signaling and release, and synapse formation (B. Lu et al., 2005; Poo, 2001). BDNF is an important mediator of neuronal plasticity, due to its high expression in brain regions relevant for plasticity and because of its function as an activity-dependent modulator of neuronal structure, function and signaling (Bramham & Messaoudi, 2005). BDNF has been shown to perform excitatory neurotransmitter and neurotrophic functions to facilitate long-term alterations within the CNS (Blum, Kafitz, & Konnerth, 2002; Matsumoto et al., 2008; H. Park & Poo, 2013). These include increasing axonal growth (Baldelli et al., 2002; P. L. Cheng et al., 2011; Cohen-Cory & Fraser, 1995; Shelly et al., 2011; Song, Ming, & Poo, 1997; Yoshimura et al., 2005), altering the structure of synapses and neurons (Gottmann et al., 2009; H. Park & Poo, 2013; Zagrebelsky & Korte, 2014), and increasing dendritic spine density, and dendritic arbor of neurons (N. Adachi et al., 2005; Gorski, Zeiler, Tamowski, & Jones, 2003; Ji et al., 2010; Ji, Pang, Feng, & Lu, 2005; Kellner et al., 2014; Yoshii & Constantine-Paton, 2007).

In hippocampal neurons, BDNF increases axon-growth via a TrkB-dependent local elevation and stabilization of cAMP/PKA activity (P. L. Cheng et al., 2011; Shelly et al.,

2011), and plays a role in microtubule formation during axon growth via activation of TrkB receptors which phosphorylate Akt and reduce glycogen synthase kinase 3 activation, leading to a decrease in active collapsing response mediator protein-2 formation (Baldelli et al., 2002; Song et al., 1997; Yoshimura et al., 2005). Additionally, in cultured xenopus spinal neurons, BDNF concentration gradients produced by BDNF-containing micropipettes can cause axonal growth cones to turn towards a higher BDNF concentration in a cAMP-dependent way (Cohen-Cory & Fraser, 1995; Song et al., 1997)

Dendritic arborization is also affected by BDNF, where it has been shown to increasing the length and number of apical dendrites of pyramidal cells in rats (Wirth, Brun, Grabert, Patz, & Wahle, 2003), which is mediated by MAP kinase and PI3 kinase activation (Dijkhuizen & Ghosh, 2005; Miller & Kaplan, 2003). BDNF also promotes the growth of cultured striatal and hippocampal neurons and their dendrites (Kwon, Fernández, Zegarek, Lo, & Firestein, 2011; Moya-Alvarado, Gonzalez, Stuardo, & Bronfman, 2018; Rauskolb et al., 2010), as well as increasing the dendritic spine density of cortical pyramidal neurons (Horch & Katz, 2002) and hippocampal neurons (Alonso, Medina, & Pozzo-Miller, 2004). Finally, BDNF is known to increase the soma size of neurons in the striatum (Yu et al., 2018), in the hippocampus and cortex (Rutherford, Nelson, & Turrigiano, 1998), as well as GABAergic neurons in the hippocampus (M. K. Yamada et al., 2002).

BDNF is also a key regulator of synapse formation and stabilization (Vicario-Abejon, Owens, McKay, & Segal, 2002), particularly in inhibitory GABAergic neuronal connections in the visual cortex (Z. J. Huang et al., 1999), cerebellum (Rico, Xu, & Reichardt, 2002) and hippocampus (Marty, Wehrle, & Sotelo, 2000). Exogenous

application of BDNF onto rat hippocampal neurons promotes both excitatory and inhibitory synapse formation, as well as increasing excitatory synapse numbers (Tyler & Pozzo-Miller, 2001; Vicario-Abejon, Collin, McKay, & Segal, 1998). Finally, selectively knocking out TrkB in the cerebellum leads to a reduction in inhibitory synapses, and a significant decline in synapses in the hippocampus (Martinez et al., 1998).

BDNF is also a key facilitator of LTP at excitatory glutamatergic synapses (Bliss & Collingridge, 1993; Y. Lu et al., 2011; Panja & Bramham, 2014; Patterson et al., 1996). LTP is a process of strengthening synaptic connections between neurons with frequent activation, essential for memory formation and long-term memory consolidation. BDNF mRNA levels have been shown to increase following LTP in the hippocampal CA1 region and dentate gyrus (Castrén et al., 1993; Dragunow et al., 1993; Morimoto, Sato, Sato, Yamada, & Hayabara, 1998; Pang & Lu, 2004; Patterson, Grover, Schwartzkroin, & Bothwell, 1992). Meanwhile BDNF KO mice show impaired LTP (Minichiello et al., 1999), which is reversed following BDNF application (Patterson et al., 1996; Pozzo-Miller et al., 1999). Deficient LTP in BDNF KO mice also leads to behavioral impairments such as reduced conditioned fear extinction, reduced novel object recognition, and reduced spatial learning in the Morris water maze. (Heldt, Stanek, Chhatwal, & Ressler, 2007). Heterozygous BDNF KO mice also show a significant deficit in LTP induced by theta burst stimulation or forskolin application (Korte et al., 1995; Pang et al., 2004; Patterson et al., 2001). Further, applying the BDNF scavenger TrkB-Fc or antibodies against BDNF or TrkB also inhibits LTP in hippocampal slices (Kang, Welcher, Shelton, & Schuman, 1997).

The importance of BDNF signaling in learning and memory formation has been well-demonstrated in the literature (Croll, Ip, Lindsay, & Wiegand, 1998; Tokuyama, Okuno, Hashimoto, Xin Li, & Miyashita, 2000), particularly in the conversion of short-term to long-term memories (Alonso et al., 2002; Y. Lu et al., 2011; Radiske et al., 2015). Disrupting BDNF expression impairs spatial memory and learning, which has been shown using antisense BDNF KO mice (Linnarsson, Bjorklund, & Ernfors, 1997), anti-BDNF antibodies (Mu, Li, Yao, & Zhou, 1999), and oligonucleotides (Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000). BDNF could prove important for LTP because of its promotion of excitatory neurotransmitter activity. To this end, nanomolar concentrations of BDNF in the hippocampus, cortex, and cerebellum all elevate neuronal excitation (Kafitz, Rose, Thoenen, & Konnerth, 1999), via promoting glutamate activity through the NMDA receptor (Blum et al., 2002), and GABA receptors during development when GABA is excitatory (Brunig et al., 2001; T. Tanaka et al., 1997). Meanwhile pro-BDNF primarily promotes long-term depression (LTD) following p75NTR activation in the hippocampus (Rösch, Schweigreiter, Bonhoeffer, Barde, & Korte, 2005; Woo et al., 2005). This occurs independent of NMDA or AMPA receptors, since p75NTR KO mice have been shown to have NMDA receptor dependent LTP and LTD which were not affected (Woo et al., 2005). Pro-BDNF also has the opposite effect on neuronal morphology and synaptic plasticity compared to BDNF, where it reduces dendrite morphology and spine density in pyramidal neurons in the hippocampus (Zagrebelsky et al., 2005).

1.5. BDNF Signaling in the Hypothalamus

1.5.1. BDNF-mediated Cardiovascular and Sympathetic Regulation

In the hypothalamus, BDNF modulates several homeostatic mechanisms, such as regulation of food intake and body weight (Lebrun, Bariohay, Moyse, & Jean, 2006; Pellemounter, Cullen, & Wellman, 1995; Toriya et al., 2010), as well as the activation of the HPA axis, which is responsible for the neuroendocrine-related aspects of the stress response (Givalois et al., 2004; Jeanneteau et al., 2012). The importance of BDNF in cardiovascular regulation is supported by findings that BDNF mRNA and protein expression is increased in the PVN in response to hypertensive stimuli such as chronic and acute stress, hyperosmolality, and repeated amphetamine administration (Aliaga, Arancibia, Givalois, & Tapia-Arancibia, 2002; Hammack et al., 2009; Meredith, Callen, & Scheuer, 2002; Rage, Givalois, Marmigere, Tapia-Arancibia, & Arancibia, 2002; Smith, Makino, Kim, et al., 1995). This indicates that BDNF may serve as a stress-responsive intracellular messenger and could be a key mediator of the stress response (Castren et al., 1995; Hammack et al., 2009; Rage et al., 2002; Smith, Makino, Kim, et al., 1995). Using *in situ* hybridization, BDNF mRNA was shown to be present in the PVN, and acute or repeated immobilization stress was found to significantly increase its expression there (Smith, Makino, Kim, et al., 1995). Rats exposed to a 7-day chronic stress procedure have also showed a significant increase in BDNF levels in the PVN (Hammack et al., 2009). Finally, hypertonic saline injections also increase BDNF mRNA expression in both the magnocellular and parvocellular PVN (Castren et al., 1995). The mechanism behind how

stress and other hypertensive stimuli elevate BDNF expression in the PVN is not fully understood.

Recent studies have suggested that BDNF may play a critical role in the development of hypertension. We have recently shown that both long-term overexpression and acute microinjection of exogenous BDNF into the PVN leads to significant increases in blood pressure and heart rate under baseline conditions (Erdos, Backes, McCowan, Hayward, & Scheuer, 2015; Chris L. Schaich, Wellman, Koi, & Erdos, 2016). BDNF has also been shown to modulate cardiovascular responses to acute water and acute restraint stress, where increases in blood pressure due to stress were significantly reduced in BDNF rats compared to control GFP rats, but heart rate responses to acute stress were unaffected by BDNF (Erdos, Backes, et al., 2015). Additionally, we have shown that inhibiting the TrkB receptor in the PVN lowers acute stress-induced pressor responses in SD rats, while resting blood pressure, heart rate, food intake and body weight were unaffected (C. L. Schaich, Wellman, Einwag, Dutko, & Erdos, 2018).

The elevations in blood pressure and heart rate caused by BDNF overexpression in the PVN are mediated partly by angiotensin signaling. BDNF promotes angiotensin II signaling in the PVN and elevates the expression of AT1R in the PVN, which is associated with increased blood pressure (Becker, Wang, Tian, & Zucker, 2015; Erdos, Backes, et al., 2015; Erdos, Clifton, et al., 2015; Chris L. Schaich et al., 2016). Inhibiting AT1Rs with losartan in the PVN has also been shown to cause a significant reduction in MAP and heart rate (Erdos, Backes, et al., 2015). Furthermore, infusion or pretreatment with the AT1R antagonist losartan in the PVN of conscious and anesthetized rats has been shown to

attenuate BDNF-induced increases in MAP (Chris L. Schaich et al., 2016). Additionally, peripheral blockade using hexamethonium was found to diminish average and peak elevations in MAP, similar to rats pretreated with losartan while also diminishing the heart rate response, and decreasing baseline MAP (Chris L. Schaich et al., 2016).

These results suggest that angiotensin signaling is a potential mediator of the acute blood pressure response to BDNF within the PVN (Chris L. Schaich et al., 2016). Another potential mediator of BDNFs effects on cardiovascular parameters in the PVN is AVP. BDNF is known to stimulate the synthesis of AVP in the PVN (Aliaga et al., 2002; Givalois et al., 2004), which may promote sympathetic activity acting centrally within the PVN (El-Werfali, Toomasian, Maliszewska-Scislo, Li, & Rossi, 2015) or elevate blood pressure through peripheral mechanisms (Henderson & Byron, 2007). Other mechanisms may be involved, and further research is needed to elucidate the specific signaling and structural effects of BDNF within the PVN.

1.5.2. BDNF-mediated Regulation of the Hypothalamic-Pituitary-Adrenal (HPA)

Axis

The HPA axis involves a variety of feedback mechanisms between the hypothalamus, the pituitary gland, and the adrenal gland. The PVN is a key regulator and initiator of HPA axis activity which mediates the neuroendocrine stress response and adaptation to future stressors (Bartanusz, Aubry, Jezova, Baffi, & Kiss, 1993; Bartanusz, Jezova, et al., 1993; Givalois et al., 2004). The pathway is initiated by the release of CRH and AVP from neuroendocrine neurons in the medial parvocellular region of the PVN, which then travel to the median eminence (Antoni, 1993; Herman & Tasker, 2016;

Whitnall, Mezey, & Gainer, 1985). From there they are released into portal circulation (Plotsky, 1987), leading to ACTH secretion from the anterior pituitary (Antoni, 1986; Gillies, Linton, & Lowry, 1982; Herman & Tasker, 2016; Merchenthaler, Vigh, Petrusz, & Schally, 1982), which triggers the release of glucocorticoids from the adrenal cortex. Glucocorticoids are important for stress adaptation, and their receptors are involved in the negative feedback regulation of the HPA axis during stress (Reul & de Kloet, 1985). Glucocorticoids are also colocalized with CRH and highly expressed in the PVN (Liposits et al., 1987; Uht, McKelvy, Harrison, & Bohn, 1988). CRH neurons are important for adaptation to chronic (Herman, 1995; Herman, Adams, & Prewitt, 1995) and acute stress responses (Whitnall, 1989), and are the main initiators of the HPA axis pathway. However, AVP is also co-expressed in about 50% of CRH neurons (Aguilera, 1998) and can significantly potentiate the effect of CRH to stimulate ACTH release (Gillies et al., 1982).

CRH neurons are under excitatory control from catecholaminergic neurons in the brain stem and glutamatergic interneurons in the periventricular area (Herman, Prewitt, & Cullinan, 1996). Indirect projections from the forebrain via the BNST and amygdala can also stimulate CRH neurons by activating glutamatergic or inhibiting GABAergic interneurons (Herman et al., 1996). Deletion of the CRH gene blocks baseline and stress-induced release of ACTH (Muglia, Bethin, Jacobson, Vogt, & Majzoub, 2000), which shows the necessity of CRH signaling in HPA axis activation. CRH and AVP both trigger the secretion of ACTH by binding to their respective receptors, CRH binds to CRHR1 (Aguilera, 1994), and AVP binds to AVP 1B receptors to assist in CRH-mediated ACTH release (Rabadan-Diehl, Kiss, Camacho, & Aguilera, 1996). In addition to activating the

HPA axis, binding of CRH to CRHR1 coordinates stress responses and stress-related behaviors, such as sympathetic activation, central suppression of the immune system, and arousal (Bale & Vale, 2004). Parvocellular CRH neurons also express a variety of neuropeptides such as glutamate, Ang II, cholecystokinin, and neurotensin (Hrabovszky, Wittmann, Turi, Liposits, & Fekete, 2005; Swanson et al., 1987), which may indicate that CRH neurons can effect various different cell types. This could lead to stimulatory effects of not only CRH and AVP on ACTH secretion, but cholecystokinin and angiotensin also (Mezey, Reisine, Skirboll, Beinfeld, & Kiss, 1986; Swanson et al., 1986).

Inactivation of the HPA axis is important for minimizing the deleterious effects of excessive CRH production. This involves changes in excitatory and inhibitory neurocircuitry and negative feedback by glucocorticoids. Glucocorticoids inhibit pituitary ACTH secretion and POMC transcription (Autelitano, Lundblad, Blum, & Roberts, 1989; Dallman et al., 1987), while administration of glucocorticoids inhibits CRH transcription and desensitizes the HPA axis response to stress (Dallman et al., 1994; Lightman & Young, 1989; Ma & Aguilera, 1999). Removal of glucocorticoids results in the opposite effect (Ma & Aguilera, 1999; Makino et al., 1995). Glucocorticoids also have an indirect inhibitory effect on CRH neurons, through decreasing alpha-adrenergic receptors in the PVN and inhibiting stress-induced release of norepinephrine from the PVN (Day, Campeau, Watson, & Akil, 1999; Pacak, Palkovits, Kvetnansky, et al., 1995). Glucocorticoids can also signal via the mineralocorticoid receptors (MR) since MR receptors are known to be expressed in the PVN and occupation of the MR receptor is required during certain kinds of stress

(Arriza, Simerly, Swanson, & Evans, 1988; Cole et al., 2000). However, the specific role of MR receptors in regulation of the HPA axis is currently unknown.

BDNF mRNA expression in the PVN is elevated in response to acute and chronic stress (Givalois et al., 2004; Hammack et al., 2009; Smith & Cizza, 1996; Smith, Makino, Kim, et al., 1995). Immobilization stress significantly increases BDNF mRNA expression in both the pituitary and hypothalamus (Givalois et al., 2004; Rage et al., 2002). BDNF is also involved in the activation of the HPA axis via the regulation of CRH and AVP (Givalois et al., 2001; Jeanneteau et al., 2012). ICV BDNF injections increase CRH mRNA and, decrease AVP mRNA expression in the parvocellular and magnocellular PVN (Givalois et al., 2004). In situ hybridization shows additionally that BDNF co-localizes with both CRH and AVP in the parvocellular and magnocellular areas of the PVN after immobilization stress (Aliaga et al., 2002; Givalois et al., 2001; Smith, Makino, Kvetnansky, & Post, 1995), and CRH co-localizes with AVP (Whitnall, 1993). Deletion of the glucocorticoid receptor in the PVN also upregulates CRH and BDNF expression while also disinhibiting the HPA axis (Jeanneteau et al., 2012). Finally, BDNF may play a part in replenishing neurohormone stock by stimulating the synthesis of neuropeptides in cultured hypothalamic neurons (Loudes, Petit, Kordon, & Faivre-Bauman, 1999, 2000).

1.5.3. BDNF-mediated AVP Secretion and Osmoregulation

Osmoregulation refers to the process of maintaining water and salt balance throughout the body and involves various signaling mechanisms that report body fluids and distribution, as well as integration of that information leading to autonomic, endocrine, and behavioral effects (Johnson, 2009). Secretion of AVP is known to be influenced by

changes in both blood osmolality and volume (Robertson & Athar, 1976). AVP is synthesized and secreted from magnocellular neurons in the PVN (Lechan & Toni, 2000), where AVP increases plasma volume leading to the induction of cAMP and the translocation of aquaporin-2 water channels to the plasma membrane of tubular epithelial cells, allowing for water reabsorption (Shaw & Marples, 2002; Valenti, Procino, Tamma, Carmosino, & Svelto, 2005). When plasma osmolality increases, thirst is increased shortly following the release of AVP (Lechan & Toni, 2000).

Osmoregulatory input to the PVN comes from projections from the OVLT and the MnPO nucleus, both of which promote AVP secretion. Their role in the thirst response to hyperosmolarity has also been demonstrated in lesion studies (Bourque, Oliet, & Richard, 1994; McKinley, 2001). The PVN itself is also known to be osmosensitive to some extent (Honda, Negoro, Higuchi, & Tadokoro, 1987; Osaka, Kannan, Kasai, Inenaga, & Yamashita, 1990). Another important brain region involved in blood volume regulation is the SFO, which sends projections to the PVN and the OVLT, and contribute to AVP secretion in response to hyperosmolarity using Ang II as a mediator (Bourque et al., 1994; A. V. Ferguson & Renaud, 1986; Krause et al., 2008; J. Tanaka, Nojima, Yamamuro, Saito, & Nomura, 1993). The NTS carries information involved in AVP secretion as well, such as baroregulatory information (Bourque et al., 1994; Lechan & Toni, 2000), which is relayed to the NTS from baroreceptors in the aortic arch and carotid sinus via the vagus and glossopharyngeal nerves. This information then travels to the PVN via catecholaminergic neurons (Lechan & Toni, 2000).

High salt intake is known to upregulate the expression of BDNF (Carreno, Walch, Dutta, Nedungadi, & Cunningham, 2011; Choe et al., 2015; Choe, Trudel, & Bourque, 2016; Marosi & Mattson, 2015) and BDNF can also stimulate the secretion of AVP in response to high salt and hyperosmotic stimuli (Aliaga et al., 2002; Castren et al., 1995). BDNF mRNA expression precedes AVP increases in the SON and PVN following i.p. injections of hypertonic saline. This may suggest that BDNF can function as an autocrine or paracrine regulator of AVP secretion (Aliaga et al., 2002). Increases in BDNF expression also reverse the GABAA mediated inhibition of AVP neurons by increasing intracellular chloride. This is mediated by BDNF-TrkB signaling and the downregulation of KCC2, leading to a sustained release of AVP and an increase in blood pressure (Choe et al., 2015; Choe et al., 2016; Marosi & Mattson, 2015). Acute immobilization stress also significantly increases both AVP mRNA and hypothalamic BDNF mRNA (Givalois et al., 2004). BDNF additionally positively modulates AVP release in studies of isolated hypothalamic cell cultures (Moreno, Piermaria, Gaillard, & Spinedi, 2011). Finally, osmotic stress can lead to morphological changes in the PVN and SON (Hatton, 1997), which could indicate that elevations in BDNF mRNA expression may prepare neurons for osmolarity associated alterations in metabolic demand (McAllister, Katz, & Lo, 1999).

1.5.4. BDNF-mediated Regulation of Metabolism and Food Intake

The hypothalamus has been shown to play a significant role in metabolism and food intake since lesioning the hypothalamus results in obesity (Brobeck, 1946). Conversely, lesioning the lateral hypothalamus has been shown to lead to anorexia and weight loss (D. J. Lee, Elias, & Lozano, 2018). Leptin serves as a critical regulator of metabolism and food

intake, broadly acting as a satiety signal, responsible for inhibiting hunger. Plasma concentrations of leptin can also serve as an important indicator of body fat stores (Ahima, Saper, Flier, & Elmquist, 2000; Barsh & Schwartz, 2002; Gautron & Elmquist, 2011; Kalra et al., 1999; Ollmann et al., 1997; Schwartz, Woods, Porte, Seeley, & Baskin, 2000). An increase in nutrient consumption increases leptin secretion thereby decreasing appetite, while nutrient insufficiency decreases leptin secretion thereby increasing appetite and consumption. Leptin acts primarily in the arcuate nucleus and exerts its effects depending on its concentration level.

High levels of leptin are associated with increased expression of weight loss promoting genes such as elevated POMC neuron activity and the production of several hormones involved in feeding behavior, including alpha melanocyte-stimulating hormone (alpha-MSH), ACTH, and b-endorphins (Millington, 2007). Mutations interfering with the functioning of type 4 melanocortin receptor (MC4r) or MC43 KO in animals leads to the development of severe obesity syndrome (Farooqi et al., 2000; Huszar et al., 1997; Jackson et al., 1997; Krude et al., 1998). Additionally, weight gain associated with aging can be attributed to the reduction of melanocortin signaling (Gruenewald & Matsumoto, 1991; Mobbs et al., 2001). Melanocortin signaling may be a critical regulatory system involved in appetite and satiety (Lechan & Toni, 2000). Similarly, there is also evidence that cancer cachexia, a wasting syndrome characterized by weight loss, may be prevented by the melanocortin receptor antagonist administration (Marks, Ling, & Cone, 2001; Wisse, Frayo, Schwartz, & Cummings, 2001).

Low levels of leptin lead to increased expression of genes that promote weight gain and reduce energy expenditure such as agouti-related protein (AGRP) and neuropeptide Y (NPY) (Decourt, Tillet, Caraty, Franceschini, & Briant, 2008). These neurons act via the PI3K signaling pathway and by phosphorylating signal transducer and activator of transcription 3 (STAT3) (Bates et al., 2003; Lechan & Toni, 2000; Zhao, Huan, Gupta, Pal, & Sahu, 2002). NPY and AGRP neurons send projections to similar regions in the hypothalamus as POMC neurons, before being relayed to other brain regions associated with feeding behavior and energy expenditure (Ahima et al., 2000; Barsh & Schwartz, 2002; Kalra et al., 1999; Schwartz et al., 2000). Reciprocal connections also exist between the arcuate nucleus NPY/AGRP neurons and the alpha-MSH neurons (Cowley et al., 2001). Injecting AGRP or NPY into the brain causes an increase in food intake (Aponte, Atasoy, & Sternson, 2011), and AGRP also antagonizes melanocortin receptors, leading to lowered melanocortin and leptin due to a rise in AGRP (Nijenhuis, Oosterom, & Adan, 2001; Ollmann et al., 1997).

TRH neurons in the medial parvocellular PVN receive direct projections from NPY/AGRP and alpha-MSH/CART neurons, which serve to alter the range at which circulating thyroid hormone inhibits TRH (Fekete et al., 2001; Fekete, Legradi, et al., 2000; Fekete, Mihaly, et al., 2000; Fekete et al., 2002). The anterior and ventral parvocellular PVN also receive a significant amount of input from alpha-MSH and AGRP axons which are thought to regulate various feeding behaviors (Hahn, Breininger, Baskin, & Schwartz, 1998; Spiegelman & Flier, 2001). The anterior parvocellular PVN sends projections to the limbic system which may regulate behavioral aspects of feeding (Luiten, ter Horst, Karst,

& Steffens, 1985; Roeling, Veening, Peters, Vermelis, & Nieuwenhuys, 1993). Meanwhile, ventral parvocellular PVN neurons send projections to the brainstem and spinal cord which may regulate energy disposal (Roeling et al., 1993; Sawchenko & Swanson, 1982a; Swanson & Kuypers, 1980).

BDNF signaling has been linked to body weight and food intake regulation (Lebrun et al., 2006). Central and peripheral administration of BDNF induce appetite suppression and weight loss (Pelleymounter et al., 1995; C. Wang, Bomberg, Billington, Levine, & Kotz, 2007b; C. Wang, Bomberg, Levine, Billington, & Kotz, 2007; C. Wang, Godar, Billington, & Kotz, 2010), increases locomotor activity (Naert, Ixart, Tapia-Arancibia, & Givalois, 2006), lowers resting metabolic rate (C. Wang, Bomberg, Billington, Levine, & Kotz, 2007a; C. Wang, Bomberg, Levine, et al., 2007), lowers blood glucose and increases energy expenditure in type 2 diabetes animal models (Nakagawa et al., 2000). Chronic ICV administration of BDNF also prevents weight gain (Lapchak & Hefti, 1992), whereas an obese phenotype is observed in BDNF conditional KO mice (Rios et al., 2001). BDNF is also known to have peripheral actions that affect glucose metabolism (Yamanaka, Itakura, Ono-Kishino, et al., 2008; Yamanaka, Itakura, Tsuchida, Nakagawa, & Taiji, 2008; Yamanaka, Tsuchida, et al., 2007), energy expenditure (Yamanaka, Itakura, et al., 2007; Yamanaka, Tsuchida, et al., 2007), and food intake (Yamanaka, Itakura, Ono-Kishino, et al., 2008). These effects of BDNF are also clear from conditions where BDNF is globally reduced, such as in rodents and humans with haploinsufficiency for the BDNF gene, which results in obesity and hyperphagia (Gray et al., 2006; Han et al., 2008; Kernie, Liebl, & Parada, 2000). BDNF also interacts with leptin signaling since leptin injections into the

VMN of the hypothalamus increase BDNF mRNA expression, which is also regulated by nutritional state and MC4r signaling (Komori, Morikawa, Nanjo, & Senba, 2006; Baoji Xu et al., 2003). Finally, BDNF also interacts with CRH signaling to decrease body weight and food intake through upregulating CRH and activating CRHR2 in the PVN (Toriya et al., 2010).

We have previously shown that increased BDNF expression in the PVN significantly increases nighttime physical activity and significantly reduces body weight compared to pre-injection body weight and compared to control GFP treated rats (Erdos, Backes, et al., 2015). Losartan treatment prevents BDNF-induced weight loss, however in a combined treatment of BDNF and losartan, body weight was still significantly lower compared to control animals (Erdos, Backes, et al., 2015). These results suggest that angiotensin signaling mediates some of the BDNF-induced reductions in body weight (Erdos, Backes, et al., 2015; Chris L. Schaich et al., 2016). Mutations in the TrkB receptor which affect its ability to autophosphorylate have also been associated with obesity and hyperphagia (Yeo et al., 2004). Wild type mice that express the truncated form of the TrkB receptor, which inhibits the activity of BDNF, are obese (Xu et al., 2000; B. Xu et al., 2003). However, when the TrkB ligand NT4 and a TrkB-specific antibody are administered to the hypothalamus of mice, a reduction in food intake and resistance to diet-induced obesity is seen (Tsao et al., 2008).

Finally, BDNF signaling can be altered through the Val66Met, single nucleotide polymorphism (SNP), which substitutes a valine for methionine residue at position 66. This substitution alters the intracellular packaging of pro-BDNF and may affect the signaling

activity of mature BDNF (Z. Y. Chen et al., 2004; M. F. Egan et al., 2003). This SNP strongly correlates with anorexia nervosa in humans and is further associated with a low body mass index (Ribases et al., 2003). However, there are conflicting studies regarding this gene variant, some studies indicate that the Val66Met polymorphism is associated with an increased risk of eating disorders (Gratacos et al., 2007), while others have found no association between eating disorders and Val66Met (Arija, Ferrer-Barcala, Aranda, & Canals, 2010; Dardennes et al., 2007), and still others report a link between Val66Met and obesity in females (Beckers et al., 2008). This dissertation will attempt to show how BDNF might be altering catecholaminergic, glutamatergic and GABAergic signaling mechanisms in the PVN to elevate blood pressure by looking at the effect of BDNF overexpression in the PVN on the expression of catecholaminergic, glutamatergic and GABAergic signaling components within the PVN and the NTS.

CHAPTER 2: BDNF Downregulates Beta-Adrenergic Receptor-Mediated Hypotensive Mechanisms in the Paraventricular Nucleus of the Hypothalamus

2.1. Introduction

Increased sympathetic activity is a significant factor in the development of hypertension and a major contributor to cardiovascular risk factors such as stress, high fat and high salt diets (Chida & Steptoe, 2010; Guyenet & Stornetta, 2004; Lambert & Lambert, 2011; Steptoe & Kivimaki, 2012; Whelton, 1994). Neural control of blood pressure is mediated by a core network of hypothalamic and brainstem nuclei. Presympathetic neurons within the PVN stimulate spinal sympathetic preganglionic neurons directly or via the RVLM (R. A. Dampney, 1994; Stornetta, 2009), while the

nucleus of the NTS plays an important role in processing sensory information, responding to blood pressure changes and regulating sympathetic nervous system activity through the baroreflex (Andresen & Kunze, 1994; R. A. Dampney, 1994). The PVN plays a critical role in integrating autonomic, neuroendocrine and cardiovascular responses to stressful stimuli (Busnardo, Tavares, & Correa, 2014; R. A. Dampney, 2005; Wamsteeker & Bains, 2010), and our recent findings indicate that BDNF, acting in the PVN through its high-affinity TrkB receptor, may be a novel regulator of stress-induced elevations in sympathetic activity and blood pressure (Erdos, Backes, et al., 2015; C. L. Schaich et al., 2018; Chris L. Schaich et al., 2016).

BDNF is a member of the neurotrophin family, capable of excitatory neurotransmitter and neurotrophic functions to induce both short- and long-term adaptive alterations throughout the CNS (Blum et al., 2002; Matsumoto et al., 2008; H. Park & Poo, 2013). In the hypothalamus, BDNF modulates several homeostatic mechanisms such as regulation of food intake and body weight (Lebrun et al., 2006; Pellemounter et al., 1995; Toriya et al., 2010) and activation of the hypothalamic pituitary adrenal (HPA) axis, which is responsible for the neuroendocrine aspects of the stress response (Givalois et al., 2004; Jeanneteau et al., 2012). In addition, BDNF mRNA and protein levels in the PVN are elevated in response to several hypertensive stimuli such as chronic and acute stress, hyperosmolality and repeated amphetamine administration (Aliaga et al., 2002; Hammack et al., 2009; Meredith et al., 2002; Rage et al., 2002; Smith, Makino, Kim, et al., 1995), and we have recently shown that both long-term overexpression and acute microinjection of exogenous BDNF into the PVN lead to significant increases in sympathetic nervous

system activity, blood pressure and heart rate (Erdos, Backes, et al., 2015; Chris L. Schaich et al., 2016). Furthermore, we have found that inhibition of BDNF signaling by blocking its high affinity receptor TrkB in the PVN reduces acute stress-induced blood pressure elevations without affecting baseline cardiovascular function (C. L. Schaich et al., 2018).

BDNF has been shown to promote angiotensin II signaling and elevate the expression of AT1R in the PVN which is associated with elevated blood pressure (Becker et al., 2015; Erdos, Backes, et al., 2015; Erdos, Clifton, et al., 2015; Chris L. Schaich et al., 2016), and BDNF is also known to stimulate the synthesis of arginine vasopressin (AVP) in the PVN (Aliaga et al., 2002; Givalois et al., 2004) which may promote sympathetic activity acting centrally within the PVN (El-Werfali et al., 2015) or elevate blood pressure through peripheral actions (Henderson & Byron, 2007). However, BDNF is also a known modulator of catecholaminergic neurotransmission (Castren et al., 1995; M. J. Chen et al., 2007; Scott et al., 2015), and within the hypothalamus, BDNF has been shown to increase norepinephrine uptake and decrease its evoked release (Rodriguez Fermepin et al., 2009). The PVN receives substantial catecholaminergic input from NTS A2 and C2 neurons (Cunningham & Sawchenko, 1988; Pacak & Palkovits, 2001; Saphier & Feldman, 1991; Sawchenko & Swanson, 1982b), and while the role of these projections is not completely understood, several studies have indicated that these neurons exert a hypotensive action at baseline (Daubert et al., 2012; Duale et al., 2007; Itoh & Bunag, 1993). In addition, NTS catecholaminergic neurons are thought to be activated during both acute and chronic stress (Cullinan et al., 1995; Teppema et al., 1997; R. Zhang et al., 2010) and to play a role in the HPA axis response to systemic stressors (Rinaman & Dzmura, 2007; Ulrich-Lai &

Herman, 2009). Thus, an increase in BDNF expression in the PVN in response to hypertensive stimuli may have a significant impact on cardiovascular regulation by interfering with catecholaminergic signaling.

Here, we set out to test the hypothesis that upregulation of BDNF in the PVN elevates blood pressure either by diminishing catecholaminergic β receptor-mediated inhibitory input from the NTS or by augmenting α receptor-mediated excitatory mechanisms. We used our previously published model (Erdos, Backes, et al., 2015) of vector-mediated upregulation of BDNF in the PVN to induce hypertension and investigated the interaction of BDNF and catecholaminergic signaling by selectively lesioning NTS catecholaminergic neurons using the neurotoxin anti-dopamine- β -hydroxylase-conjugated saporin (DSAP) (Madden, Ito, Rinaman, Wiley, & Sved, 1999). In addition, cardiovascular responses to PVN microinjections of adrenergic receptor agonists and mRNA expression of adrenergic receptors were analyzed following overexpression of BDNF in the PVN. Better understanding of BDNF-mediated cardiovascular regulatory mechanisms within the PVN is crucial for elucidating the central circuitry involved in the development of hypertension and could eventually lead to the identification of novel therapeutic targets.

2.2. Methods

All animal housing, handling, surgical and experimental procedures were conducted within an Association for the Assessment and Accreditation of Laboratory Care International-accredited animal care facility at the University of Vermont, in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory

Animals. Experiments were performed in male Sprague-Dawley (SD) rats obtained from Charles River (Saint-Constant, QC, Canada). Rats were housed individually with a 12:12-h light-dark cycle (lights on at 6:00 AM), with free access to food (standard chow) and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

2.2.1. Experimental Design

Experiment 1. The aim of this experiment was to determine how increased BDNF expression in the PVN affects catecholaminergic input from the NTS. Radiotelemetric transmitters were implanted for blood pressure measurement in 8-week-old male SD rats, and animals were allowed to recover for two weeks. Baseline cardiovascular parameters were recorded for 6 days, then, adeno-associated viral vectors (AAV2, 10^{12} vps/mL; 200 nL/side) expressing GFP or myc epitope-tagged BDNF fusion protein (BDNFmyc) were injected bilaterally into the PVN. In addition, PBS (100 nL) or DSAP (22 ng in 100 nL PBS), was injected bilaterally into the NTS as described previously (Daubert et al., 2012). After a week of recovery, cardiovascular parameters were recorded for 3 weeks. During this period, rats were subjected to an acute restraint stress procedure 3 weeks after brain injections and to an acute water stress procedure 4 weeks after brain injections (**Fig. 2A**). At the end of the experiment, animals were deeply anesthetized with isoflurane and perfused transcardially with ice-cold PBS and 4% paraformaldehyde. PVN expressions of GFP and BDNFmyc as well as effects of DSAP lesioning in the NTS were verified with immunofluorescence (**Fig. 2B – H**). In addition, percent volumetric density of D β H-

positive vesicles, an indicator of catecholaminergic projections into the PVN, was determined within the PVN using immunofluorescence and confocal microscopy.

Experiment 2. The goal of this experiment was to determine the effect of BDNF on adrenergic receptor signaling in the PVN. 7-week-old male SD rats received bilateral PVN injections of AAV2 viral vectors (10^{12} vps/mL; 200 nL/side) expressing GFP or BDNFmyc. Three weeks later, blood pressure and heart rate responses were recorded following unilateral PVN injections of isoprenaline (125 μ M or 250 μ M, 200 nL/side) a β -adrenergic agonist under α -chloralose-urethane anesthesia. In addition, responses to unilateral PVN injections of phenylephrine (200 μ M, 200 nL), an α 1 agonist or vehicle (aCSF, 200 nL/side) were also recorded. At the end of the experiment, animals were deeply anesthetized and perfused transcardially with PBS and 4% paraformaldehyde. PVN expressions of GFP, BDNFmyc and drug injection sites of isoprenaline, phenylephrine and aCSF indicated by a red fluorescent microbead solution were verified with immunofluorescence and fluorescent microscopy in coronal sections of the PVN (**Fig. 3A – C**).

Experiment 3. The goal of this experiment was to determine the effect of BDNF on adrenergic receptor expression in the PVN. 7-week-old male SD rats received bilateral PVN injections of AAV2 viral vectors (10^{12} vps/mL; 200 nL/side) expressing GFP or BDNFmyc. Three weeks later, animals were euthanized, and the brains were quickly removed and snap frozen. PVN and NTS tissue samples were isolated and mRNA levels of BDNF, corticotrophin releasing hormone (CRH), α 1a, α 1b, α 2a, β 1 and β 2 were

determined in the PVN and mRNA levels of tyrosine hydroxylase (TH) and D β H were determined in the NTS with quantitative real-time RT-PCR.

2.2.2. Surgical Procedures

Surgeries were performed using aseptic techniques under continuous isoflurane anesthesia (5% induction, 2-3% maintenance) delivered in oxygen. Depth of anesthesia was assured by lack of a reflex response to pinch of the hind paw. Carprofen (5 mg·kg⁻¹·day⁻¹ sc) was used for postsurgical analgesia administered at the beginning of surgery and for 2 days after surgery.

2.2.2.1. Telemetry Transmitter Implantation

In the first experiment, radiotelemetric transducers (model HD-S10; Data Sciences International, St. Paul, MN) were implanted into the descending aorta via a midline abdominal incision. The aorta was isolated and briefly occluded, and the tip of the catheter was inserted using a 21-gauge needle. Surgical glue (3M Vetbond Tissue Adhesive) and a nitrocellulose patch were applied to secure the catheter in place. The transducer was sutured to the abdominal muscle, and the incision closed in layers.

2.2.2.2. Stereotaxic Viral Vector and DSAP Injections

Rats were put under isoflurane anesthesia and placed in a stereotaxic frame, BDNFmyc and GFP viral vectors (10¹² viral particles/ml; 200 nL/side) were injected bilaterally into the PVN using pipettes pulled from thin walled borosilicate glass capillary tubes (OD, 1 mm; ID, 0.58 mm; tip diameter: ~25 μ m; World Precision Instruments Inc., Sarasota, FL, USA) at the following stereotactic coordinates: 1.80 mm posterior to bregma; 1.70 mm lateral to the midline; and 7.65 mm ventral from the dorsal surface of the brain,

with the micropipette tilted 10° laterally toward the midline. Virus stocks were injected over 5 min using a pneumatic pico pump (World Precision Instruments, Sarasota, FL). The pipette was left in place for an additional 3 min before being withdrawn.

In experiment 1, PVN injections were combined with bilateral NTS injections of sterile PBS (for control) or DSAP (Advanced Targeting Systems, San Diego, CA, USA), an immunotoxin that selectively lesions catecholaminergic neurons (Madden et al., 1999). The head was ventro-flexed at an angle of approximately 30° to allow for surgical exposure of the dorsal surface of the hindbrain. The micropipette tip was lowered at the calamus scriptorius (rostral–caudal), 0.3 mm lateral from midline and 0.5 mm ventral to the surface of the medulla and 100 nL of PBS or DSAP diluted to a concentration of 0.22 mg·ml⁻¹ in sterile PBS was injected over 5 min using a pneumatic pico pump. This concentration of DSAP was chosen since it has been used previously to lesion NTS catecholaminergic neurons without any significant impact on other catecholaminergic nuclei (Daubert et al., 2012). The pipette was left in place for an additional 3 min before being withdrawn.

2.2.2.3. Assessment of Cardiovascular Responses to Adrenergic Receptor Agonists

In experiment 2, the left femoral artery and vein were catheterized under isoflurane anesthesia 3 weeks after bilateral PVN injections of AAV2-GFP or AAV2-BDNFmyc. Rats were then placed in a stereotaxic frame, and isoflurane anesthesia was gradually switched to intravenous α -chloralose (60 mg kg⁻¹ hr⁻¹) and urethane (800 mg kg⁻¹ hr⁻¹) anesthesia, administered through the femoral vein catheter over a 30-min period, during which, isoflurane was gradually reduced from 2.5% to 0%. Blood pressure, heart rate, toe-pinch and eye-blink reflexes were monitored closely to ensure the animal remained

anesthetized. After complete withdrawal of isoflurane, anesthesia was maintained by intravenous α -chloralose ($15 \text{ mg kg}^{-1} \text{ hr}^{-1}$) and urethane ($200 \text{ mg kg}^{-1} \text{ hr}^{-1}$) infusion for the remainder of the experiment. After establishing steady baseline blood pressure and heart rate for a minimum of 30 min, rats received unilateral PVN injections of isoprenaline hydrochloride ($125 \text{ }\mu\text{M}$ or $250 \text{ }\mu\text{M}$, 200 nL/side ; Acros Organics, AC437210050), phenylephrine ($200 \text{ }\mu\text{M}$, 200 nL/side ; Fisher Scientific, AC207240100) or aCSF (200 nL/side). Blood pressure was monitored via the catheter placed in the left femoral artery, and heart rate was extracted from the pulsatile pressure wave using Lab Chart Pro 8 (ADInstruments). To verify locations of the isoprenaline, phenylephrine and aCSF injections in the PVN with fluorescent microscopy, 10% rhodamine-labelled fluorescent microspheres ($0.04 \text{ }\mu\text{m}$; Molecular Probes) were mixed into the injection solution.

2.2.3. Viral Vector-mediated Gene Transfer into the PVN and DSAP Lesioning of NTS Neurons

AAV2 viral vectors were used to elicit the expression of enhanced GFP and BDNF_{myc}, derived from rat *bdnf*, constructed and packaged by Vector Biolabs (Philadelphia, PA). The expression of GFP and BDNF_{myc} was driven by a chicken- β -actin promoter with human cytomegalovirus enhancer, and a woodchuck post-transcriptional regulatory element, which enhanced the expression of transgenes, present downstream of GFP and BDNF_{myc}. The BDNF_{myc} plasmid was a generous gift from Dr. Ronald Klein (LSU Health Sciences Center Shreveport) and was used previously to protect retinal ganglion cells in a rat glaucoma model (K. R. Martin et al., 2003), and to study cardiovascular effects of BDNF in the PVN (Erdos, Backes, et al., 2015). In addition, full

efficacy of BDNFmyc expression driven by the rat neuron-specific enolase promoter was confirmed previously both *in vitro* and *in vivo* (R. L. Klein et al., 1999). PVN injections of AAV2-GFP and AAV2-BDNFmyc resulted in marked expressions of GFP and BDNFmyc in the PVN as confirmed by fluorescent imaging and an antibody against the myc tag (**Fig. 2B – C**). Only animals with bilateral GFP or BDNF expression in the PVN were included in the study, and analysis of the number of GFP or myc positive cells in the subnuclei of the PVN indicated that DSAP treatment in the NTS had no effect on GFP or BDNFmyc expression in the PVN (**Fig. 2F – G**). In addition, analysis of D β H-positive neurons in the NTS indicated a significant DSAP-mediated reduction of catecholaminergic neurons in the NTS in both the GFP and BDNF group, and vector treatment had no effect on the efficacy of DSAP lesioning (**Fig. 2H**).

2.2.4. Analysis of Resting Radiotelemetric Cardiovascular Data

Blood pressure, heart rate, and physical activity of the animals were analyzed with Dataquest A.R.T. Analysis software (Data Sciences International). Data were recorded every 10 min for 15 seconds and averaged between 8:00 AM and 4:00 PM to calculate daytime values and between 8:00 PM and 4:00 AM to calculate nighttime values for each animal. Spontaneous baroreflex sensitivity was calculated with the sequence technique with the freely available HemoLab software (<http://www.haraldstauss.com/HaraldStaussScientific/hemolab/>), as previously described (Bhatia, Rarick, & Stauss, 2010). Blood pressure was recorded continuously at a sampling rate of 500 Hz for a minimum of 2 hours between 8:00 AM and 10:00 PM before water stress experiments (3 weeks after vector injections). Sampling rate of the data sets was first

increased to 1,500 Hz with spline interpolation. Then, sequences defined as a minimum of three consecutive (beat by beat) increases or decreases in systolic blood pressure accompanied by likewise increases or decreases in the pulse interval, were identified. Sequences with increases in systolic blood pressure (“up sequences”) and decreases in systolic blood pressure (“down sequences”) were pooled. No time delay and no thresholds for changes in systolic blood pressure or pulse interval were used. However, only sequences with a correlation coefficient (R) for the linear correlation between systolic blood pressure and pulse interval of >0.8 were included in the analysis, and the slope of the linear correlation was taken as the gain of the baroreceptor-pulse interval reflex (Bhatia et al., 2010; Laude, Baudrie, & Elghozi, 2008).

2.2.5. Acute Stress Procedures

Acute stress procedures were performed between 10:00 AM and 12:00 PM and were started after obtaining baseline blood pressure and heart rate recordings for a minimum of 30 min. For water stress, rats were placed in standard rat cages filled with 1 cm deep water (room temperature, $\sim 25^{\circ}\text{C}$) for 15 min. Restraint stress was performed by placing animals in cylindrical plastic restrainers for 60 min. After animals were returned to their home cages, blood pressure and heart rate were recorded for an additional 60-min recovery period. Blood pressure and heart rate data were exported with 3-minute moving average from continuously recorded blood pressure data, and baseline values were calculated by averaging the baseline period after physical activity-related peaks from blood pressure and heart rate datasets were removed. Average blood pressure and heart rate

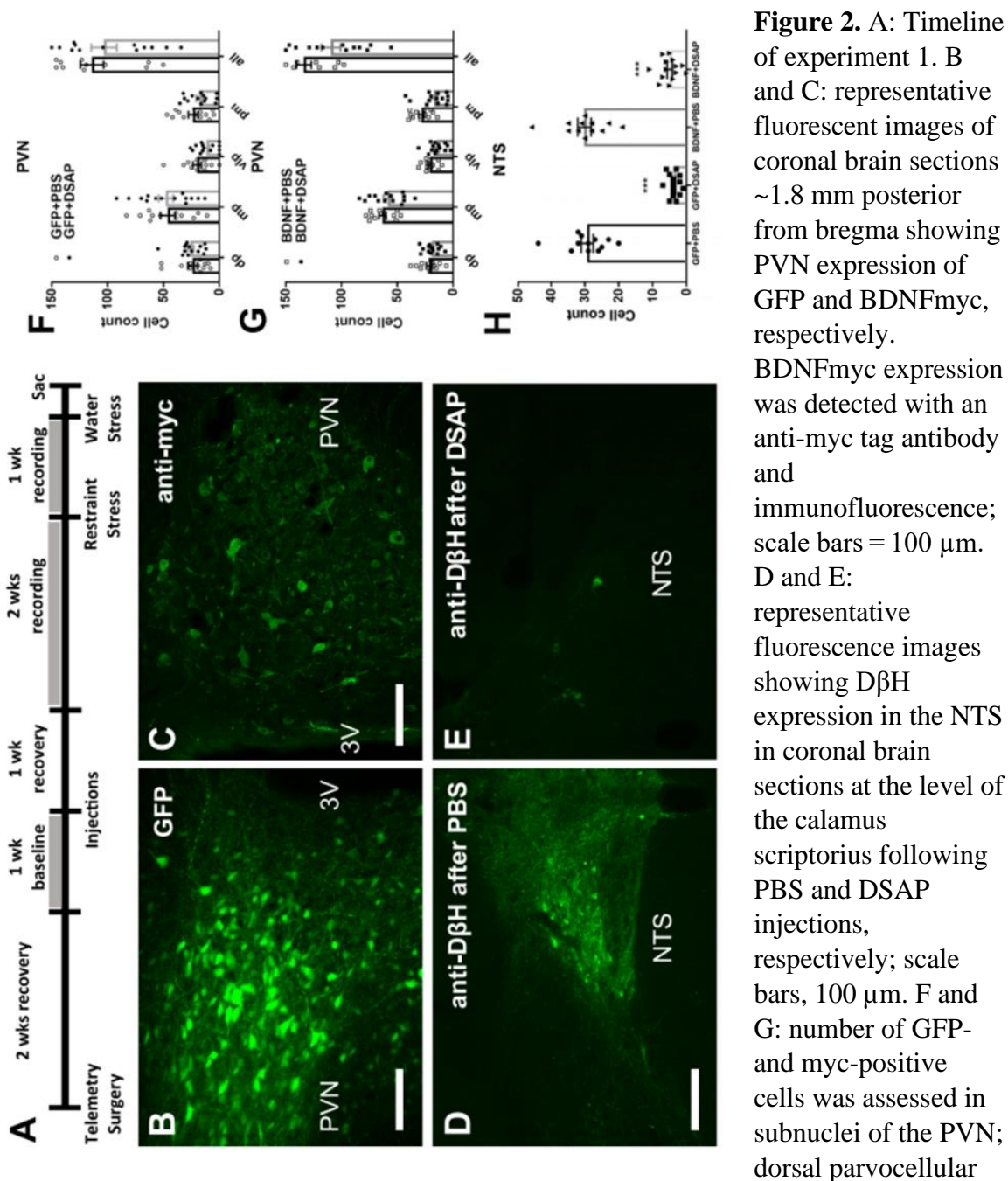
changes from baseline (during stress and recovery periods), as well as amplitude and time delay of peak responses, were calculated.

2.2.6. Analysis of Cardiovascular Data from Anesthetized Animals

Blood pressure and heart rate were recorded using LabChart software version 8.0.7 (ADInstruments, Dunedin, New Zealand) at a 1000 Hz sampling rate and condensed to 10-second moving averages for data analysis and presentation. Maximum and average changes in MAP and heart rate following aCSF, isoprenaline and phenylephrine microinjections were evaluated.

2.2.7. Immunofluorescence

Following experiment 1 and 2, animals were perfused with 400 mL of ice-cold PBS followed by 400 mL of ice-cold 4% paraformaldehyde in PBS. Brains were removed and post-fixed for 2 hours in 4% paraformaldehyde and then equilibrated in 30% sucrose solution at 4°C. Coronal sections (40 µm) were cut on a microtome (Leica SM2000R) and mounted on Fisher Superfrost Plus slides. BDNFmyc and DβH were detected using the following primary antibodies: anti-c-Myc (Santa Cruz-9E10, 1:200, overnight incubation at 4°C) and anti-DβH primary antibody (Millipore, Billerica, MA, USA, 1:1000, overnight incubation at 4°C). Secondary antibodies were donkey anti-mouse AF546 (Invitrogen, 1:200, 2-h incubation at room temperature) and donkey anti-rabbit A555 (Invitrogen, 1:200, 2-h incubation at room temperature). GFP, BDNFmyc and DβH immunofluorescence in the PVN and NTS were detected with a fluorescent microscope (Nikon Eclipse 50i).



nuclei (dp), medial parvocellular nuclei (mp), ventrolateral parvocellular nuclei (vlp), and posterior magnocellular nuclei (pm) following AAV2-GFP or AAV2-BDNFmyc injections in the PVN and PBS or DSAP injections in the NTS. DSAP had no significant effect on vector mediated gene transduction in GFP or BDNF rats. GFP+PBS (n = 12), GFP+DSAP (n = 14), BDNF+PBS (n = 12), BDNF+DSAP (n = 14); n refers to number of PVN sides. **H:** number of D β H-positive neurons in the NTS following AAV2-GFP or

AAV2-BDNFmyc injections in the PVN and PBS or DSAP injections in the NTS. DSAP significantly reduced the number of D β H-positive neurons in both the GFP and BDNF groups. 3V, third ventricle; PVN, paraventricular nucleus; NTS, nucleus of the solitary tract; GFP, green fluorescent protein; PBS, phosphate-buffered saline; BDNF, brain-derived neurotrophic factor; BDNFmyc, myc epitope-tagged BDNF; D β H, dopamine β -hydroxylase; DSAP, anti-dopamine- β -hydroxylase-conjugated saporin; AAV2, adeno-associated viral vector 2. ***P < 0.001 for DSAP (1-way ANOVA).

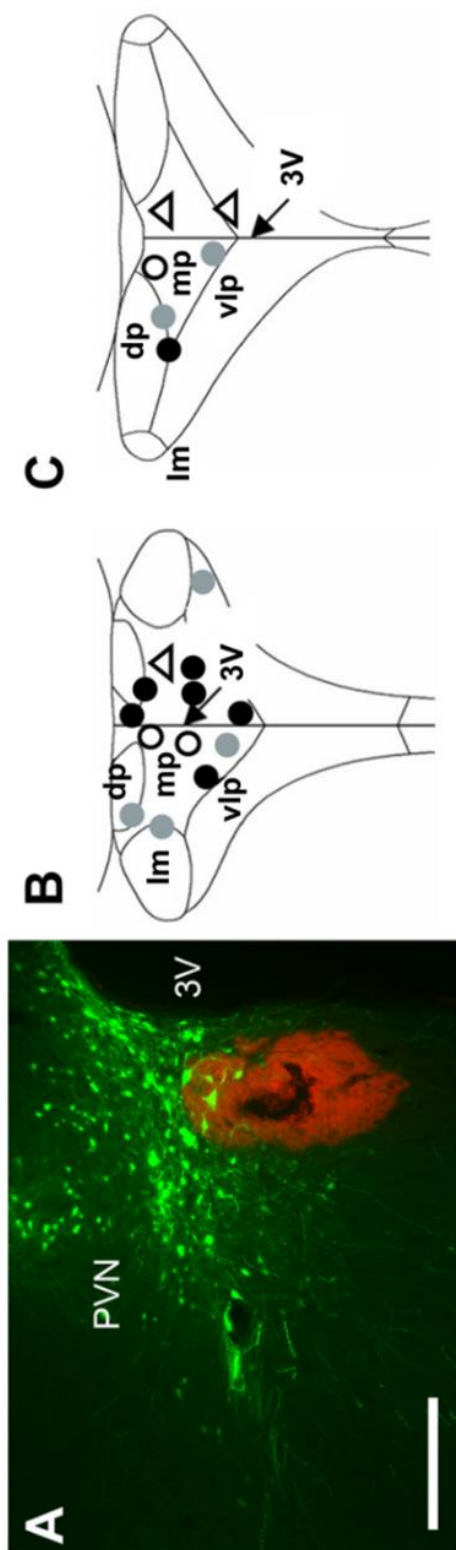


Figure 3. A: Representative fluorescent image of a coronal brain section ~1.8 mm posterior to bregma showing PVN expression of GFP and the red fluorescent microbead solution that was mixed with injected drug solution to verify injection sites; scale bars, 250 μ m. B and C: diagrams of the PVN ~1.8 mm and ~1.9 mm posterior to bregma showing locations of isoprenaline injections in rats previously injected with AAV2-GFP (gray circles) or AAV2-BDNFmyc (filled circles), and phenylephrine (open circles) and aCSF (open triangles) injections in untreated rats. 3V, third ventricle; lm, lateral magnocellular nuclei; dp, dorsal parvocellular nuclei; mp, medial parvocellular nuclei; vlp, ventrolateral parvocellular nuclei; PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; BDNFmyc, myc epitope-tagged BDNF; AAV2, adeno-associated viral vector 2; aCSF, artificial cerebrospinal fluid.

2.2.8. Confocal Microscopy and Image Analysis

PVN brain sections from experiment 1 were used to determine the effect of BDNF overexpression on volumetric density of D β H-positive vesicles located on axon terminals in the PVN. Z-stack images of PVN brain slices labeled with immunofluorescence as described above were taken using a Zeiss LSM 510 Meta confocal laser scanning imaging system. The z-stacks were then deconvoluted using the deconvolution software AutoQuant X3 (Media Cybernetics, Inc.). Volumetric density of D β H-positive vesicles within the PVN was then calculated with the custom-written software Volumetry G7 (Grant Hennig; Dept. of Pharmacology UVM). D β H immunoreactivity is limited to vesicles in the PVN, which appear as distinct points in the image. To precisely capture those points in the PVN, each image was thresholded such that all vesicles were captured, and any background fluorescence was removed. D β H percent density was quantified in each slice of the z-stack of each side of the PVN. Then, the z-stack slice with the highest percent density was selected on each side of the PVN in each group and the mean percent density calculated for each experimental group.

2.2.9. Real-Time RT-PCR

Frozen forebrains and hindbrains were mounted on a cryostat, and rostral coronal sections were cut until reaching the level ~1.5 mm posterior of the bregma for the PVN and ~14.1 mm posterior of the bregma for the NTS. Then, tissue samples 0.75 mm in diameter and 0.5 mm deep were punched bilaterally from the region of the PVN or NTS under microscope with the third ventricle used as reference for the PVN and the midline and dorsal brainstem surface for the NTS. The depth of the punches was limited by a spacer

glued on the outside of the punch tool. Total RNA was extracted from brain punches with Qiashredder columns (Qiagen, Valencia, CA) and the RNeasy Micro Kit (Qiagen). Samples were treated with an RNase-free DNase Set (Qiagen) on column to remove genomic DNA. RNA was quantified with a Qubit Fluorometer, and cDNA was synthesized with a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to manufacturer's instructions and stored at -20°C . All quantitative RT-PCR reactions were run in duplicate in eight-well optical-grade strips in a Prism 7000 Sequence Detection System (Applied Biosystems) and quantified with the cycle threshold (CT) method. The mRNA levels of BDNF and CRH, which is known to be elevated by BDNF (Jeanneteau et al., 2012), adrenergic receptors and the reference genes beta actin (ActB) and 18S were analyzed with quantitative real-time RT-PCR using specific oligonucleotide primers and TaqMan probes (Applied Biosystems, Foster City, CA; BDNF: Rn02531967_s1; CRH: Rn01462137_m1; ActB: Rn00667869_m1; α 1a: RN00567876_m1; α 1b: RN01471343_m1; α 2a: RN00562488_s1; β 1: RN008244536_s1 and β 2: RN00560650_s1; 18S: Hs03003631_g1; D β H: Rn00565819_m1; TH: Rn00562500_m1). Control reactions containing no template were run for each plate, and results were analyzed with the $2^{-\Delta\Delta\text{CT}}$ method.

2.2.10. Statistics

Baseline (day/night) telemetry data was analyzed using two-way repeated measures ANOVA with Tukey's post-hoc test. Peak and average changes in MAP and heart rate during acute stress and microinjection experiments, as well as mean D β H percent volumetric density were analyzed by one-way ANOVA with Bonferroni's multiple

comparisons test. RT-PCR data was analyzed by unpaired t-test. Statistical tests were performed using Prism 7.0 software (GraphPad, San Diego, CA). Results are expressed as means \pm SE, and the criterion for statistical significance was $P < 0.05$.

2.3. Results

2.3.1. Effects of BDNF and DSAP Treatments

Results from our first experiment confirmed our previous findings (Erdos, Backes, et al., 2015) and showed that BDNF overexpression in the PVN without DSAP lesioning in the NTS significantly increased MAP and heart rate during both day- and nighttime compared with the GFP group (**Fig. 4** and **Table 1**). In addition, we found that in GFP animals, DSAP lesioning of NTS catecholaminergic neurons significantly increased MAP both during day- and nighttime, whereas DSAP lesioning in BDNF animals had no further effect on blood pressure (**Fig. 4** and **Table 1**). Heart rate was significantly elevated by DSAP treatment in the GFP group during week 3 and 4 both day- and nighttime, and while it tended to increase heart rate in the BDNF animals, the difference between BDNF+PBS and BDNF+DSAP groups was not statistically significant (**Fig. 4** and **Table 1**). Furthermore, BDNF overexpression reduced spontaneous baroreflex sensitivity in rats receiving PBS injections into the NTS ($p < 0.05$), and DSAP reduced baroreflex sensitivity in GFP rats ($p < 0.05$). However, DSAP failed to have an effect in the BDNF group ($p = 0.99$, **Table 1**).

In addition to its effects on cardiovascular function, BDNF overexpression also significantly reduced body weight gain in agreement with our previous study (Erdos, Backes, et al., 2015). At baseline, the body weight of the GFP+PBS group was 428 ± 18 g,

and the BDNF+PBS group was 414 ± 11 g. At the end of the experiment the GFP+PBS group was 554 ± 26 g and the BDNF+PBS group was 445 ± 17 g ($p < 0.05$). In contrast, DSAP lesioning of NTS catecholaminergic neurons had no effect on body weight gain in either GFP or BDNF rats. At baseline, the body weight of the GFP+DSAP group was 420 ± 12 g, and final body weight was 566 ± 12 g, compared to the BDNF+DSAP group whose body weight at baseline was 411 ± 10 g, and final body weight was 448 ± 15 g. BDNF overexpression was also found to increase daytime locomotor activity compared to the GFP group ($p < 0.01$), at the end of week 3 (**Table 1**).

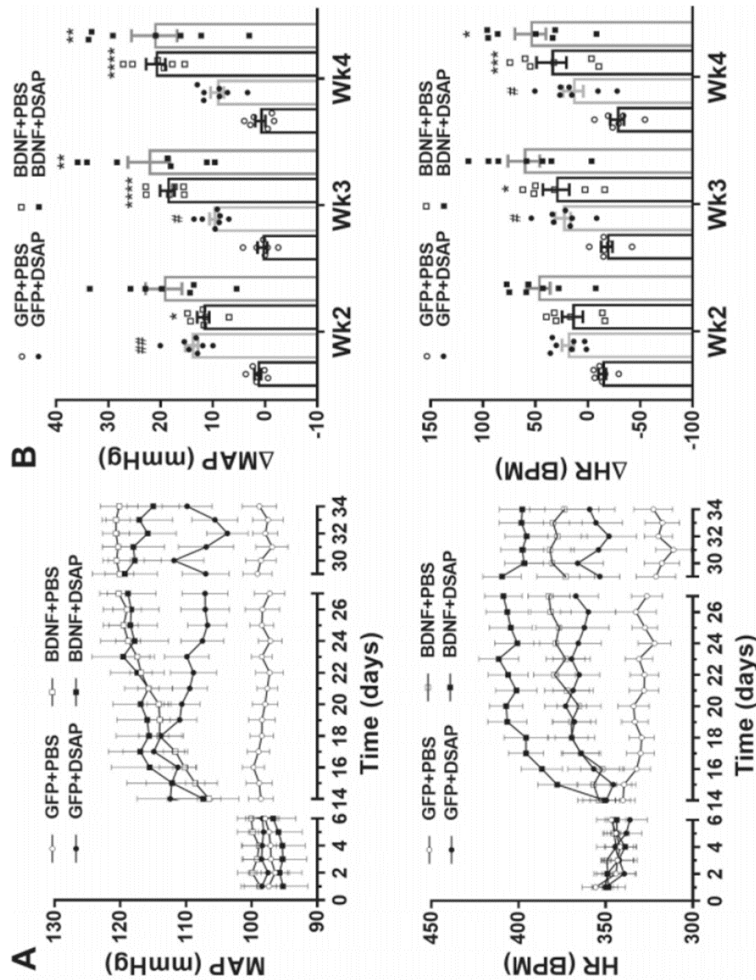


Figure 4. Radiotelemetric recordings of daytime mean arterial pressure (MAP) and heart rate [HR, in beats/min (bpm)]. Radiotelemetric parameters were recorded for 6 days before brain injections and during weeks 2–4 after brain injections. Recording was turned off during the postsurgical recovery phase (between days 6 and 14), and data from day 28 are omitted because animals were subjected to water stress on that day. Since viral vector and DSAP treatments had similar effects on daytime and nighttime parameters, only daytime parameters are shown (nighttime data are summarized in Table 1). A: daytime MAP (top) and HR

(bottom) in GFP+PBS (n = 6), GFP+DSAP (n = 7), BDNF+PBS (n = 6), and BDNF+DSAP (n = 7) rats. B: average changes in daytime MAP (top) and HR (bottom) during weeks 2, 3, and 4 from pretreatment baseline period. PVN, paraventricular nucleus; NTS, nucleus of the solitary tract; GFP, green fluorescent protein; PBS, phosphate-buffered saline; BDNF, brain-derived neurotrophic factor; DSAP, anti-dopamine- β -hydroxylase-conjugated saporin. Results represent means \pm SE. Two-way repeated-measures ANOVA on weekly averages indicated significant treatment effect for MAP and HR ($P < 0.001$), significant time effect for HR ($P < 0.05$), and significant treatment \times time interaction for both MAP and HR ($P < 0.01$). Post hoc analysis indicated * $P < 0.05$ for GFP+PBS vs. BDNF+PBS, ** $P < 0.01$ for GFP+DSAP vs. BDNF+DSAP, *** $P < 0.001$ for GFP+PBS vs. BDNF+PBS, **** $P < 0.0001$ for GFP+PBS vs. BDNF+PBS; # $P < 0.05$ for GFP+PBS vs. GFP+DSAP, ## $P < 0.01$ for GFP+PBS vs. GFP+DSAP.

	MAP, mmHg	HR, beats/min	DIA, mmHg	SYS, mmHg	ACT, AU	sBRS, ms/mmHg
GFP+PBS-D-Wk0	97.2 ± 1.8	346 ± 8.6	79.2 ± 1.0	120.1 ± 2.9	1.01 ± 0.14	
GFP+DSAP-D-Wk0	98.3 ± 2.8	342 ± 8.8	80.7 ± 2.5	120.9 ± 3.3	0.88 ± 0.10	
BDNF+PBS-D-Wk0	99.5 ± 2.3	347 ± 3.2	81.7 ± 1.7	122.0 ± 3.1	0.90 ± 0.09	
BDNF+DSAP-D-Wk0	95.7 ± 3.5	344 ± 7.4	78.1 ± 3.2	117.9 ± 3.8	0.93 ± 0.06	
GFP+PBS-N-Wk0	103.8 ± 1.4	400 ± 8.5	85.7 ± 0.9	127.0 ± 2.5	3.74 ± 0.61	
GFP+DSAP-N-Wk0	104.8 ± 3.0	398 ± 8.6	87.6 ± 2.6	127.6 ± 3.7	3.67 ± 0.38	
BDNF+PBS-N-Wk0	105.0 ± 2.6	411 ± 4.7	87.6 ± 2.0	127.2 ± 3.5	3.12 ± 0.24	
BDNF+DSAP-N-Wk0	101.2 ± 3.5	396 ± 7.6	83.7 ± 3.3	123.8 ± 3.6	3.39 ± 0.20	
GFP+PBS-D-Wk3	97.8 ± 1.9	328 ± 8.6	80.1 ± 1.5	121.2 ± 2.7	0.77 ± 0.12	1.63 ± 0.15
GFP+DSAP-D-Wk3	108.1 ± 3.2†	366 ± 12†	87.2 ± 2.8	134.7 ± 3.9	0.93 ± 0.08	1.16 ± 0.08†
BDNF+PBS-D-Wk3	118.2 ± 1.8***	378 ± 12*	97.5 ± 1.6	145.9 ± 2.2	1.24 ± 0.15**	1.12 ± 0.14*
BDNF+DSAP-D-Wk3	118.4 ± 4.3	406 ± 11	96.4 ± 4.3	145.2 ± 4.2	1.46 ± 0.12	1.06 ± 0.11
GFP+PBS-N-Wk3	105.3 ± 1.4	383 ± 7.9	87.0 ± 1.5	129.4 ± 2.0	3.21 ± 0.50	
GFP+DSAP-N-Wk3	116.2 ± 3.3†	433 ± 13†	95.0 ± 3.0	143.9 ± 4.0	3.30 ± 0.13	
BDNF+PBS-N-Wk3	124.6 ± 1.4***	450 ± 13**	103.0 ± 1.2	153.2 ± 2.0	3.56 ± 0.28	
BDNF+DSAP-N-Wk3	125.2 ± 5.3	476 ± 13	102.5 ± 5.2	153.9 ± 5.4	3.82 ± 0.32	

Table 1. Radiotelemetric parameters. Values are means ± SE. HR, heart rate; DIA, diastolic pressure; SYS, systolic pressure; ACT, activity; sBRS, spontaneous baroreflex sensitivity; AU, arbitrary units; BDNF, brain-derived neurotrophic factor; DSAP, anti-dopamine-β-hydroxylase-conjugated saporin; GFP, green fluorescent protein; PBS, phosphate-buffered saline; D, daytime; N, nighttime; week 0 values represent average of parameters measured during the week preceding vector injections; week 3 values represent average of parameters measured during week 3 after vector injections. GFP+PBS, n = 6; GFP+DSAP, n = 7; BDNF+PBS, n = 6; BDNF+DSAP, n = 7. †P < 0.05 GFP+PBS vs. GFP+DSAP; *P < 0.05 GFP+PBS vs. BDNF+PBS; **P < 0.01 GFP+PBS vs. BDNF+PBS; ***P < 0.001 GFP+PBS vs. BDNF+PBS (2-way repeated-measures ANOVA for MAP and HR, and 1-way ANOVA for sBRS).

Relative volumetric density of D β H-positive vesicles was quantified in PVN brain sections as an indicator of catecholaminergic projections to the PVN. DSAP treatment in GFP rats tended to reduce D β H-positive vesicle density in the PVN compared to the GFP+PBS group ($p=0.06$), and significantly reduced D β H-positive vesicle density compared to the BDNF+DSAP group ($p<0.05$). No significant difference was seen in the BDNF+DSAP group compared to the BDNF+PBS group. Additionally, BDNF alone did not significantly alter D β H-positive vesicle density in the PVN compared to the GFP+PBS group (**Fig. 5**).

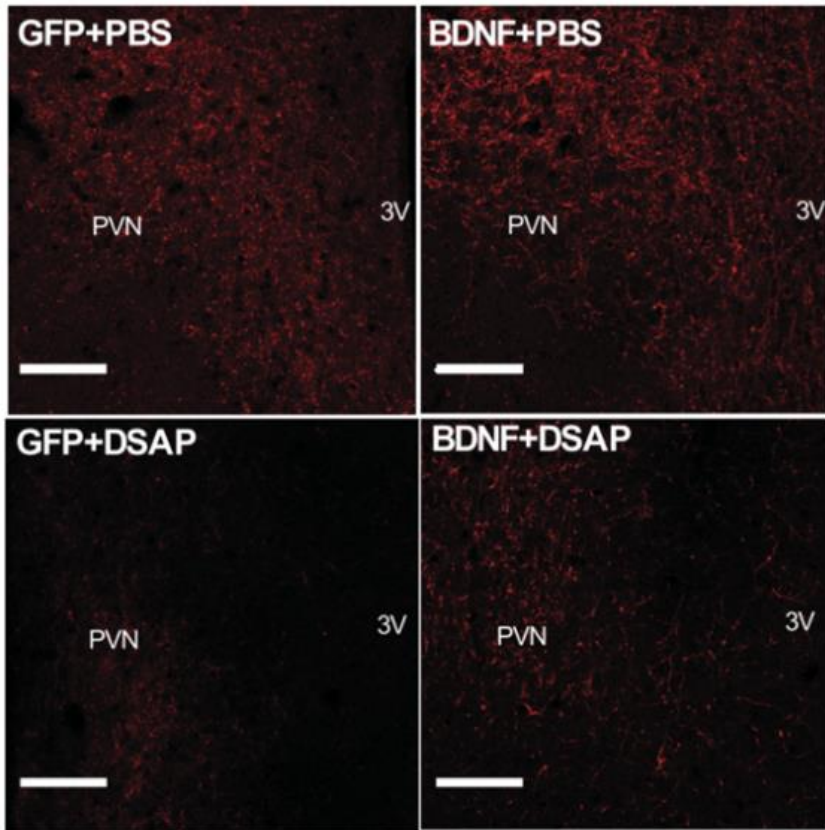
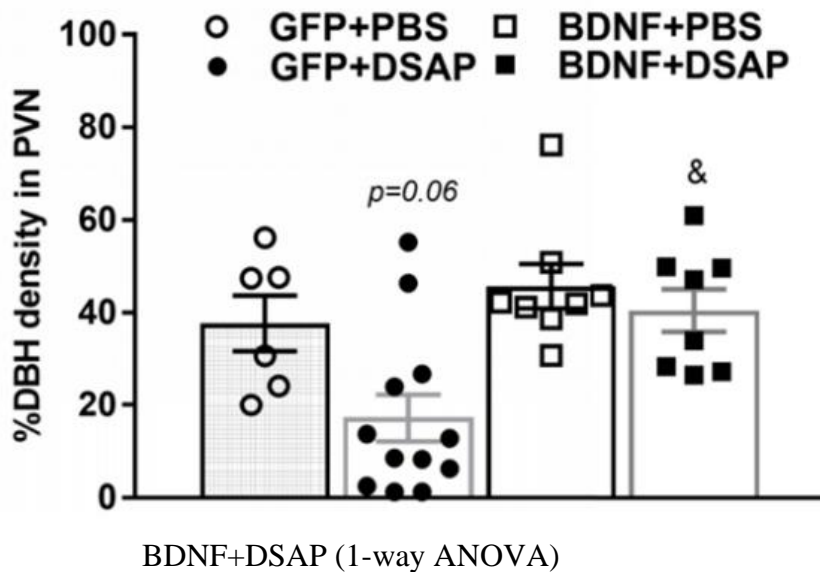


Figure 5. D β H-positive vesicles in the PVN of rats previously injected with AAV2-GFP or AAV2-BDNFmyc in the PVN and with PBS or DSAP in the NTS. Top: representative confocal images of D β H expression within the PVN in all experimental groups as detected with an anti-D β H antibody and immunofluorescence; scale bars, 100 μ m. Bottom: maximum percent volumetric density of D β H-positive vesicles within the PVN, averaged for each experimental group; n refers to number of PVN sides [GFP+PBS (n = 6), GFP+DSAP (n = 12), BDNF+PBS (n = 8), BDNF+DSAP (n = 8)]. 3V, third ventricle; PVN, paraventricular nucleus; NTS, nucleus of the solitary tract; GFP, green fluorescent protein; PBS, phosphate-buffered saline; BDNF, brain-derived neurotrophic factor; DBH, dopamine β -hydroxylase; DSAP, anti-dopamine- β -hydroxylase-conjugated saporin; AAV2, adeno-associated viral vector 2. Results represent means \pm SE. $P = 0.06$ for GFP+PBS vs. GFP+DSAP; & $P < 0.05$ for GFP+DSAP vs.



2.3.2. Acute Water and Restraint Stress

Water stress-induced peak pressor responses and average MAP increase during stress were significantly reduced in the BDNF+PBS group compared to the GFP+PBS group ($p < 0.01$), while amplitude of heart rate increases to water stress were unaffected by BDNF overexpression. DSAP treatment did not significantly alter the amplitude of pressor and tachycardic responses in either GFP or BDNF rats, and timing of peak MAP and heart rate responses were also similar in all four experimental groups (**Fig. 6**).

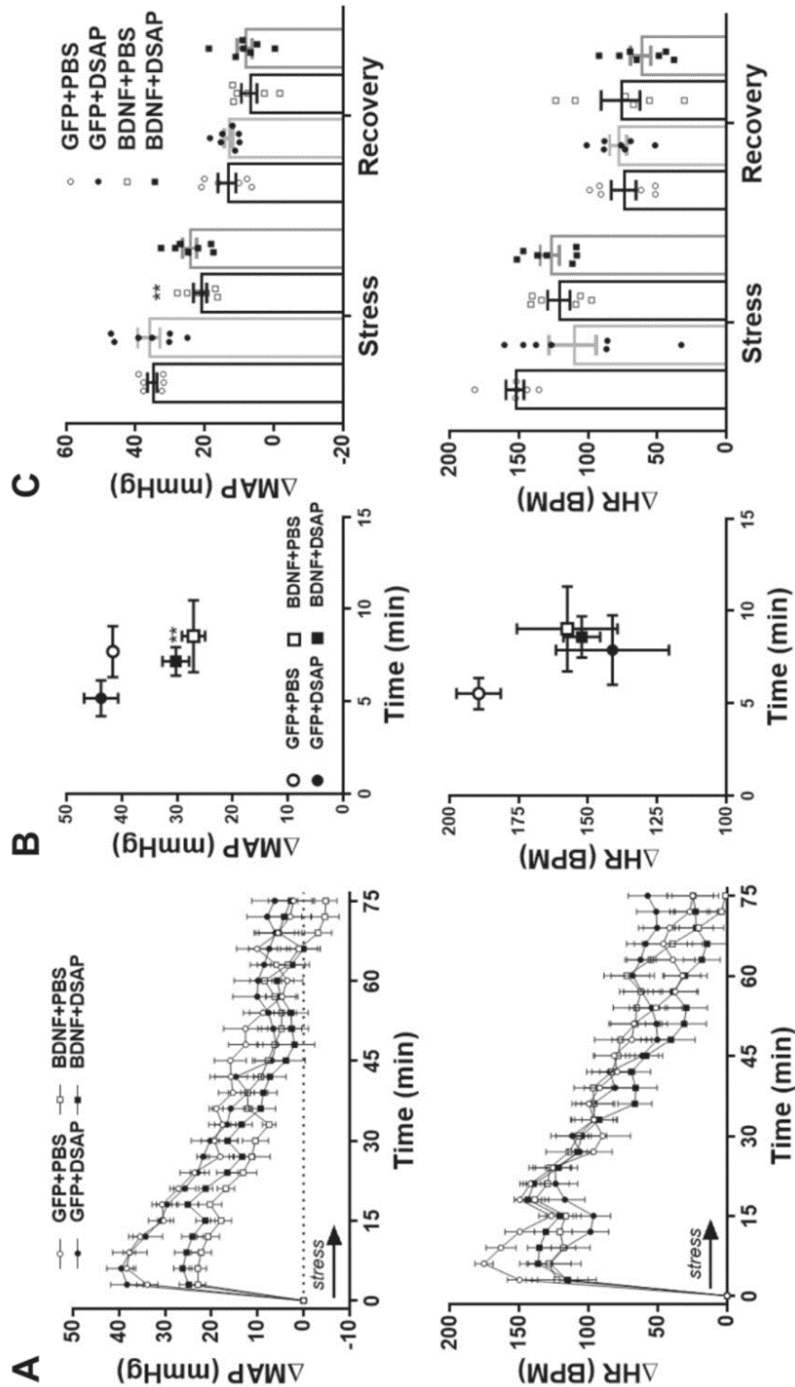


Figure 6. Radiotelemetric recordings of mean arterial pressure (MAP) and heart rate (HR) during acute water stress and poststress recovery in GFP+PBS (n = 6), GFP+DSAP (n = 7), BDNF+PBS (n = 6) and BDNF+DSAP (n = 7) rats. A: MAP (top) and HR (bottom) traces with 3-min moving average during stress (15 min, indicated by arrow) and during poststress recovery (60 min). B: amplitude and delay of peak MAP and HR responses. C: MAP and HR increases averaged during stress and poststress recovery. Prestress MAP and HR were 94 ± 3 mmHg and 308 ± 11 beats/min (bpm) in the GFP+PBS group, 101 ± 4 mmHg and 327 ± 9 beats/min in the GFP+DSAP group, 121 ± 4 mmHg and 366 ± 19 beats/min in the BDNF+PBS group, and 113 ± 5 mmHg and 378 ± 11 beats/min in the BDNF+DSAP group. GFP, green fluorescent protein; PBS, phosphate-buffered saline; BDNF, brain-derived neurotrophic factor; DSAP, anti-dopamine- β -hydroxylase-conjugated saporin. Results represent means \pm SE. **P < 0.01 for GFP+PBS vs. BDNF+PBS (1-way ANOVA).

Restraint stress-induced peak pressor responses were significantly reduced in the BDNF+PBS group compared to the GFP+PBS group ($p < 0.05$), while amplitude of heart rate increases to restraint stress were unaffected by BDNF overexpression. In contrast, DSAP treatment significantly reduced maximum heart rate increase in the GFP group ($p < 0.01$), but not in the BDNF group. DSAP treatment did not alter the amplitude of pressor responses in either GFP or BDNF rats, and timing of peak MAP and heart rate responses was also similar in all four experimental groups (**Fig. 7**).

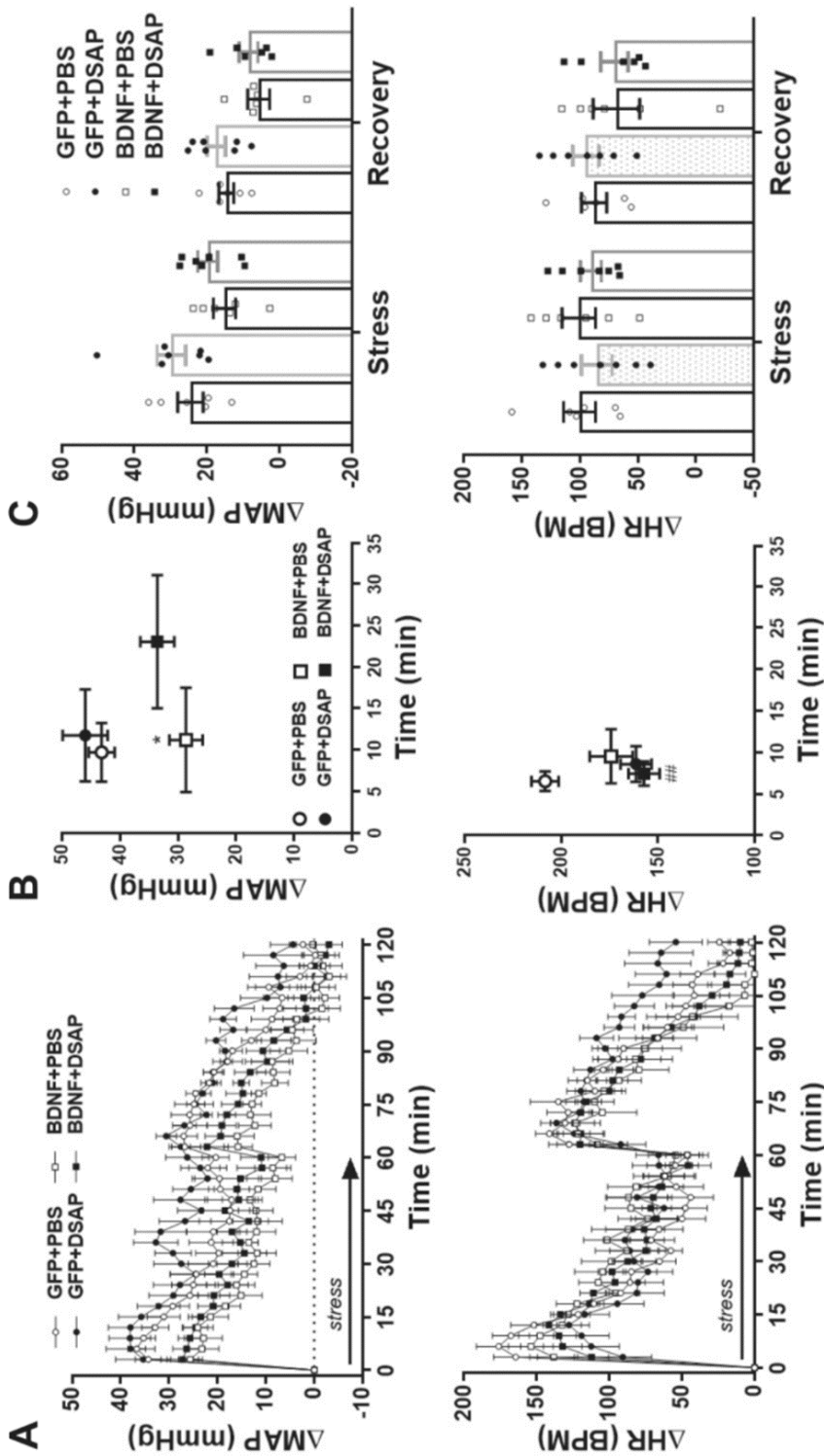


Figure 7. Radiotelemetric recordings of mean arterial pressure (MAP) and heart rate (HR) during acute restraint stress and poststress recovery in GFP+PBS (n = 6), GFP+DSAP (n = 7), BDNF+PBS (n = 6), and BDNF+DSAP (n = 7) rats. A: MAP (top) and HR (bottom) traces with 3-min moving average during stress (60 min, indicated by arrow) and during poststress recovery (60 min). B: amplitude and delay of peak MAP and HR responses. C: MAP and HR increases averaged during stress and poststress recovery. Prestress MAP and HR were 92 ± 3 mmHg and 311 ± 11 beats/min (bpm) in the GFP+PBS group, 101 ± 4 mmHg and 351 ± 14 beats/min in the GFP+DSAP group, 120 ± 2 mmHg and 372 ± 18 beats/min in the BDNF+PBS group, and 114 ± 3 mmHg and 380 ± 13 beats/min in the BDNF+DSAP group. GFP, green fluorescent protein; PBS, phosphate-buffered saline; BDNF, brain-derived neurotrophic factor; DSAP, anti-dopamine- β -hydroxylase-conjugated saporin. Results are represented as means \pm SE. * $P < 0.05$, for GFP+PBS vs. BDNF+PBS, ## $P < 0.01$, GFP+PBS vs. GFP+DSAP (1-way ANOVA).

2.3.3. Effect of BDNF on Adrenergic Receptor Signaling in the PVN

In our second experiment, we tested whether BDNF affects PVN adrenergic receptor mediated cardiovascular responses. We found that stimulation of β -adrenergic receptors with isoprenaline dose-dependently lowered MAP in GFP rats and that BDNF overexpression in the PVN almost completely abolished these hypotensive responses to β -adrenergic receptor stimulation ($p < 0.05$, **Fig. 8**). Heart rate tended to decline in the GFP group in response to isoprenaline, but its effects were not statistically significant in any of the experimental groups (GFP+ISO high dose vs aCSF, $p = 0.24$; GFP+ISO low dose vs aCSF, $p = 0.32$). In contrast with isoprenaline, activation of adrenergic α -receptors in the PVN by phenylephrine injections did not significantly alter either MAP ($p = 0.99$) or heart rate ($p = 0.99$) and injection of aCSF had no effect on cardiovascular parameters either (**Fig. 8**).

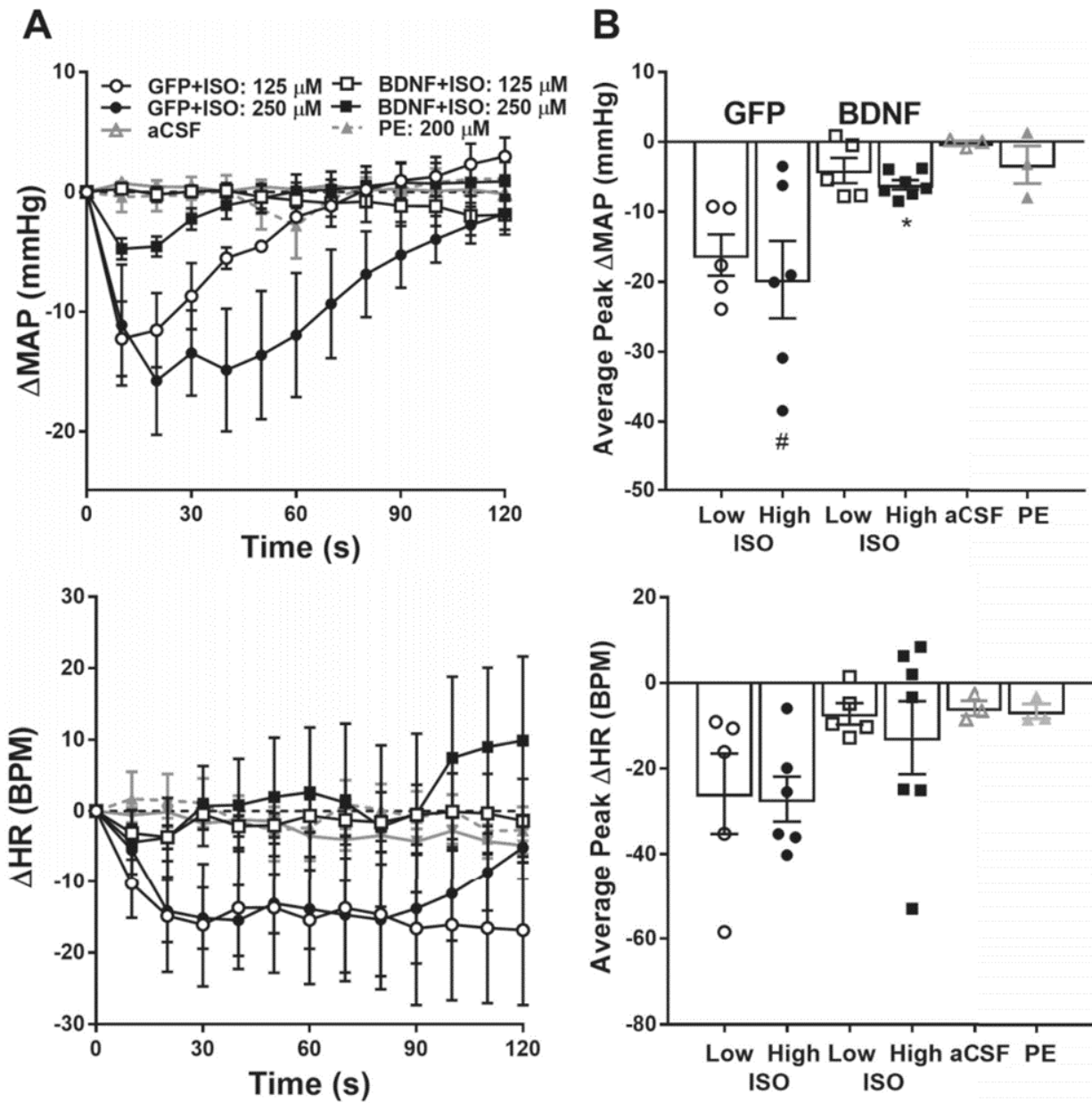


Figure 8. Changes in mean arterial pressure (MAP; top) and heart rate (HR; bottom) in response to PVN injections of adrenergic receptor agonists and vehicle in anesthetized rats previously treated with AAV2-GFP ($n = 6$) or AAV2-BDNFmyc ($n = 7$). A: MAP and HR obtained with 10-s moving average over 120 s following isoprenaline (ISO) injection in GFP and BDNFmyc treated rats, and phenylephrine (PE; $n = 3$) or aCSF, artificial cerebrospinal fluid (aCSF; $n = 3$) injections in untreated rats. B: average peak MAP and HR responses to ISO, PE, and aCSF injections. Baseline MAP and HR were 89 ± 6 mmHg and 351 ± 15 beats/min (bpm) in GFP and 108 ± 4 mmHg and 398 ± 15

beats/min in BDNF rats, and 105 ± 3 mmHg and 353 ± 7 beats/min in untreated rats. PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; BDNFmyc, myc epitope-tagged BDNF; AAV2, adeno-associated viral vector 2. Results represent means \pm SE. Statistical analysis was done on peak changes in MAP and HR. #P < 0.05 for 250 μ M ISO vs. aCSF, *P < 0.05, for GFP vs. BDNF (1-way ANOVA)

2.3.4. Expression of Components of Catecholaminergic Signaling

In our third experiment we tested the effect of BDNF upregulation in the PVN on expressions of catecholamine biosynthesizing enzymes in the NTS and adrenergic receptors in the PVN. To verify vector mediated BDNF transduction and activation of BDNF signaling pathways in the PVN, BDNF and CRH mRNA expressions were determined, and both were found to be significantly elevated in BDNF rats compared with GFP rats ($p < 0.01$, **Fig. 9A**). Analysis of adrenergic receptor expression showed that $\beta 1$ adrenergic receptor mRNA was significantly downregulated in the PVN of BDNF rats compared with GFP ($p < 0.01$), whereas expressions of $\alpha 1a$, $\alpha 1b$, $\alpha 2a$ and $\beta 2$ adrenergic receptors were unaffected by BDNF (**Fig. 9B**). Additionally, BDNF overexpression in the PVN also significantly upregulated TH ($p < 0.01$) and D β H ($p < 0.01$) mRNA expression in the NTS (**Fig. 9C**).

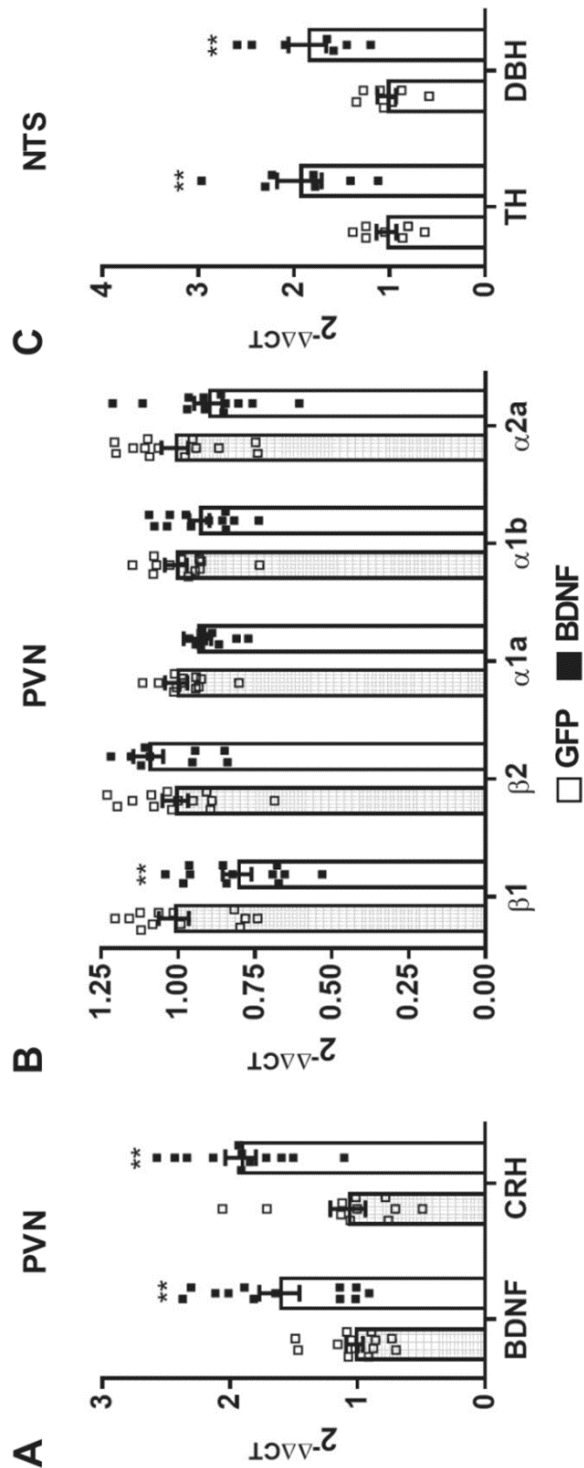


Figure 9. Expression of BDNF, CRH, and adrenergic receptor mRNA in the PVN, and TH and DβH mRNA in the NTS of rats previously injected with AAV2-GFP (n = 6) or AAV2-BDNFmyc (n = 6) in the PVN. A: significant increases in BDNF and CRH mRNA expressions verify successful vector mediated BDNF transduction and activation of BDNF-mediated signaling mechanisms. B: expressions of α1a-, α1b-, and α2a- and β1- and β2-adrenergic receptor mRNAs in GFP and BDNF rats. C: expressions of TH and DβH mRNAs in the NTS of AAV2-GFP (n = 7) and AAV2-BDNFmyc (n = 7) rats. PVN, paraventricular nucleus; NTS, nucleus of the solitary tract; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; BDNFmyc, myc epitope-tagged BDNF; TH, tyrosine hydroxylase; DBH, dopamine β-hydroxylase; AAV2, adeno-associated viral vector 2; CRH, corticotropin-releasing hormone. Results are expressed as 2-ΔΔCT (where CT is threshold cycle) normalized to the control group and presented as means ± SE. **P < 0.01 GFP vs. BDNF (unpaired t test).

2.4. Discussion

Mounting evidence suggest that BDNF, acting in the PVN, is a key regulator of blood pressure and plays a major role in eliciting cardiovascular responses to stress and other hypertensive stimuli (Aliaga et al., 2002; Erdos, Backes, et al., 2015; Hammack et al., 2009; Rage et al., 2002; C. L. Schaich et al., 2018; Chris L. Schaich et al., 2016; Smith, Makino, Kim, et al., 1995). Results from this study further emphasize the hypertensive actions of BDNF within the PVN and demonstrate that a potential mechanism mediating these effects is a downregulation of inhibitory/hypotensive input from NTS catecholaminergic neurons via adrenergic β -receptor signaling. This is supported by our findings that selective lesioning of NTS catecholaminergic neurons increased blood pressure in control (AAV2-GFP-treated) animals but failed to exert a hypertensive effect in animals that were subject to BDNF overexpression in the PVN. Additionally, activating β -adrenergic receptors in the PVN markedly decreased blood pressure in control, AAV2-GFP treated rats, whereas β -adrenergic receptor stimulation had a significantly reduced hypotensive effect after BDNF overexpression in the PVN. Diminished β -adrenergic receptor signaling was at least partially due to a downregulation of adrenergic β 1 receptor mRNA expression in the PVN, whereas expression of other adrenergic receptors was unaffected by BDNF. Interestingly, we observed a BDNF-induced upregulation of catecholamine biosynthetic enzymes in the NTS and recruitment of catecholaminergic projections to the PVN in DSAP-treated animals, but it seems these compensatory mechanisms were unable to overcome reduced responsiveness of the PVN to these inputs.

BDNF, a member of the neurotrophin family, is a widely recognized regulator of neuronal function throughout the CNS (E. J. Huang & Reichardt, 2001; Matsuda et al., 2009; H. Park & Poo, 2013), and previous reports have indicated that BDNF is involved in blood pressure control in several cardiovascular regulatory nuclei. Our previous studies have shown that both acute injection and chronic overexpression of BDNF in the PVN elevates blood pressure, heart rate and sympathetic activity (Erdos, Backes, et al., 2015; Chris L. Schaich et al., 2016), and BDNF expression has been found to be significantly elevated in the PVN in response to several hypertensive stimuli such as acute and chronic stress (Givalois et al., 2004; Hammack et al., 2009; Rage et al., 2002; Smith, Makino, Kim, et al., 1995), hyperosmolality (Aliaga et al., 2002) and repeated amphetamine administration (Meredith et al., 2002). In addition, BDNF is an important modulator of stress-related neuroendocrine mechanisms such as CRH synthesis and activation of the HPA axis (Givalois et al., 2004; Jeanneteau et al., 2012). BDNF signaling within the supraoptic nucleus has also been shown to reduce GABAA-mediated inhibition of AVP neurons and increase blood pressure in response to high salt-intake in rats (Choe et al., 2015). In addition, BDNF microinjections into the RVLM and the medial NTS have also been found to elevate blood pressure (Clark, Hasser, Kunze, Katz, & Kline, 2011; H. Wang & Zhou, 2002), and BDNF modulates baroreflex sensitivity in the NTS (Becker, Tian, Zucker, & Wang, 2016). Thus, BDNF seems to be a key regulator of many different aspects of cardiovascular regulation both within the hypothalamus and brainstem. However, the downstream mechanisms mediating these cardiovascular actions of BDNF are not fully understood.

In this study, we investigated the potential modulatory role of BDNF on NTS – PVN catecholaminergic projections since these neurons play a significant role in blood pressure regulation (Daubert et al., 2012; Duale et al., 2007; Itoh & Bunag, 1993) and because BDNF is known to influence catecholaminergic signaling throughout the CNS (M. J. Chen et al., 2007; Rodriguez Fermepin et al., 2009; Scott et al., 2015). NTS A2 and C2 neurons provide the primary catecholaminergic input to the PVN (Cunningham & Sawchenko, 1988; Sawchenko & Swanson, 1982b). However, the role of these NTS – PVN projections in cardiovascular regulation is not clear. For example, selective lesioning of NTS catecholaminergic neurons in rats led to an increase in blood pressure in some cases (Daubert et al., 2012; Duale et al., 2007; Itoh & Bunag, 1993) indicating that these neurons exert an inhibitory / hypotensive role, but other studies indicated no effect on baseline blood pressure (Itoh et al., 1992; Itoh & Bunag, 1992; Talman et al., 1980). Stimulation of adrenergic receptors in the PVN has also led to conflicting results. For example, PVN microinjections of the β -adrenergic receptor agonist fenoterol were shown to reduce blood pressure in WKY rats and SHRs but had no effect in Wistar rats (Tsushima et al., 1994). However, other studies found that microinjections of the β -adrenergic receptor agonist isoprenaline in Wistar and SD rats had no effect on blood pressure (Mendonça et al., 2018; D. Wang et al., 2013), but did increase baroreflex sensitivity in SD rats (D. Wang et al., 2013). On the other hand, PVN injections of the α 1-adrenergic agonist phenylephrine or the non-selective α -adrenergic receptor antagonist phentolamine significantly increased renal sympathetic nerve activity and heart rate without an effect on blood pressure (Q. Chen et al., 2006; Mendonça et al., 2018). Our experiments demonstrated that in control GFP animals, selective catecholaminergic lesioning in the NTS induced a marked increase in

blood pressure, whereas stimulation of β -adrenergic receptors in the PVN resulted in significant hypotensive responses. These findings support the idea that under baseline conditions in normotensive animals, these catecholaminergic neurons exert an inhibitory role on hypertensive mechanisms and these actions are mediated at least in part by β -receptor signaling in the PVN. However, in contrast with control GFP rats, DSAP lesioning in the NTS of BDNF rats did not significantly affect blood pressure. This finding is especially surprising since catecholamine biosynthesizing enzymes, TH and D β H, were markedly upregulated in the NTS of BDNF rats indicating an elevated activity of these catecholaminergic neurons. We propose that a plausible explanation for these results is that elevated BDNF levels in the PVN diminished sensitivity of PVN neurons to β -adrenergic receptor-mediated hypotensive input from the NTS. Thus, despite a compensatory increase in catecholamine biosynthesis in NTS neurons, DSAP lesioning had no further effect on the already elevated baseline blood pressure. This hypothesis is also supported by our findings that hypotensive responses to PVN injections of isoprenaline were significantly diminished in BDNF rats compared to GFP controls.

Reduced β -adrenergic signaling following overexpression of BDNF in the PVN can be partially explained by downregulation of adrenergic β 1 receptor mRNA expression in the PVN as indicated by our results, but other mechanisms may also be involved. For example, BDNF increases the expression and release of AVP in the hypothalamus (Aliaga et al., 2002; Choe et al., 2015; Veltmar et al., 1992), and AVP can stimulate the recruitment of β -arrestins, which can inhibit adrenergic β -receptor signaling and enhance endocytosis of β -receptors (U. Klein, Muller, Chu, Birnbaumer, & von Zastrow, 2001; Oakley, Laporte,

Holt, Barak, & Caron, 1999). Another potential mechanism of BDNF-mediated downregulation of β -receptor signaling may involve inhibition of G-protein-coupled inwardly rectifying potassium (GIRK) channels. GIRK1-4 have been shown to be expressed in the PVN (Karschin, Dissmann, Stuhmer, & Karschin, 1996; Saenz del Burgo et al., 2008), and a subset of PVN parvocellular neurons respond to β -adrenergic activation by GIRK channel-mediated hyperpolarization and inhibition (Daftary et al., 2000). BDNF, on the other hand, has been shown to strongly inhibit the activity of GIRK1 and GIRK4 via activation of TrkB receptors (Rogalski, Appleyard, Pattillo, Terman, & Chavkin, 2000), thus, BDNF may oppose β -adrenergic inhibition of PVN neurons by blocking β -receptor-mediated stimulation of GIRK channels. However, future studies are needed to address specifically how elevated BDNF expression alters β -receptor signaling in the PVN.

In addition to diminishing β -adrenergic signaling in the PVN, BDNF overexpression also increased the expression of catecholamine biosynthetic enzymes in the NTS and prevented DSAP-induced reductions in the density of D β H-positive vesicles in the PVN. The upregulation of TH and D β H in the NTS of BDNF animals may be an indirect response caused by increased blood pressure and baroreflex-mediated activation of NTS neurons, but it could also be caused by retrograde actions of BDNF on NTS – PVN neurons. Retrograde actions of BDNF are well documented in the CNS (Sasi, Vignoli, Canossa, & Blum, 2017; Sobreviela, Pagcatipunan, Kroin, & Mufson, 1996), and BDNF is known to localize to noradrenergic nerve fibers and terminals (Fawcett et al., 1998), regulate the development of noradrenergic neurons (Holm et al., 2003) and stimulate TH gene expression (Fukuchi et al., 2010; Zhou, Bradford, & Stern, 1994). Thus, BDNF released

from PVN neurons could retrogradely enhance catecholamine biosynthesis in NTS neurons. Retrograde BDNF-mediated signaling may also be responsible for recruiting catecholaminergic projections from other nuclei after lesioning NTS A2 / C2 neurons with DSAP. Such a mechanism could explain why D β H vesicle density in the PVN remained unaffected by DSAP lesioning of the NTS in BDNF rats but decreased in the GFP group despite similar reductions in NTS D β H-positive neuron number in both groups. However, the current study only demonstrated an increase in TH and D β H mRNA in the NTS and D β H percent volumetric vesicle density in the PVN, but changes in NTS neuronal activity or NTS TH, D β H protein expression were not measured. Future studies will be needed to determine the existence and function of these compensatory mechanisms.

BDNF and catecholaminergic signaling both contribute to neuroendocrine stress responses and baroreflex regulation (Becker et al., 2016; Givalois et al., 2004; Jeanneteau et al., 2012; D. Wang et al., 2013). Here, in correlation with our previous study (Erdos, Backes, et al., 2015), we found that BDNF overexpression in the PVN reduced MAP increases to both acute water and restraint stress without an effect on heart rate responses. We have also shown previously that selective inhibition of TrkB signaling in the PVN reduces MAP elevations to acute stressors (C. L. Schaich et al., 2018). These findings combined clearly demonstrate that upregulation of BDNF expression in the PVN (Erdos, Backes, et al., 2015; C. L. Schaich et al., 2018; Chris L. Schaich et al., 2016) and subsequent activation of TrkB signaling cascades are required for eliciting blood pressure elevations to stress. Thus, both inhibition of TrkB receptors (C. L. Schaich et al., 2018) and masking the stress-induced BDNF upregulation in the PVN by vector-mediated

constitutive BDNF overexpression diminish stress-induced hypertensive responses. However, while TrkB inhibition fails to affect baseline blood pressure (C. L. Schaich et al., 2018), vector-mediated BDNF overexpression increases it. One important mechanism mediating these actions of BDNF could be its ability to interact with angiotensin II signaling. Angiotensin II is known to play a role in mediating cardiovascular stress responses (Busnardo et al., 2014), and overexpression of BDNF in the PVN has been shown to upregulate the expression of AT1R (Erdos, Backes, et al., 2015). In addition, BDNF-mediated blood pressure increases can be partially attenuated by AT1R antagonists or by an angiotensin converting enzyme inhibitor (Erdos, Backes, et al., 2015; Chris L. Schaich et al., 2016). On the other hand, angiotensin II can also upregulate BDNF and TrkB receptor expression in cultured catecholaminergic cells leading to a reduction in voltage-gated potassium currents and increases in neuronal excitability (Becker et al., 2015). Similarly, in chronic heart failure, elevated angiotensin II contributes to increased sympathoexcitation (I. H. Zucker, Schultz, Patel, Wang, & Gao, 2009) and can increase neuronal excitability in part by reducing voltage-gated potassium currents via BDNF signaling (Sumners & Gelband, 1998; Sumners, Zhu, Gelband, & Posner, 1996; Irving H. Zucker, Xiao, & Haack, 2014).

NTS catecholaminergic neurons are also involved in the regulation of acute and chronic stress responses. They can be activated in response to physiological and psychological stressors (Dayas, Buller, Crane, Xu, & Day, 2001; Pacak, Palkovits, Kopin, et al., 1995), and may play a role in altering cardiovascular responses to stress via projections to the PVN or other forebrain regions (Cunningham & Sawchenko, 1988;

Riche, De Pommery, & Menetrey, 1990). In the current study, DSAP lesioning of the NTS in the GFP group was found to reduce heart rate responses to restraint stress but not to water stress and failed to affect blood pressure responses to either stressor. Furthermore, DSAP treatment had no effect on heart rate and blood pressure in the BDNF group during either stress paradigm. Thus, our results suggest a limited role of catecholaminergic NTS neurons in the regulation of acute cardiovascular stress responses regardless of the level of BDNF expression in the PVN.

The PVN is known to modulate baroreflex-regulated sympathetic outflow (Patel & Schmid, 1988; Z. Yang & Coote, 1999), and neurons in the PVN have been shown to be activated by baroreflex challenges (Q. H. Chen & Toney, 2003; Polson, Mrljak, Potts, & Dampney, 2002). However, while BDNF has been shown to have a significant impact on baroreflex sensitivity in the NTS (Becker et al., 2016; Clark et al., 2011), its actions on the baroreflex within the PVN have never been examined. Interestingly, our studies demonstrated that both inhibition of TrkB signaling (C. L. Schaich et al., 2018) and upregulation of BDNF expression in the PVN (**Table 1**) reduced spontaneous baroreflex sensitivity. Further studies are needed to determine the underlying mechanisms, but trophic retrograde actions of BDNF on PVN-projecting NTS neurons could potentially be involved. Additionally, β -receptor signaling has been shown to modulate baroreflex sensitivity in the PVN (D. Wang et al., 2013); thus, BDNF may also alter baroreflex sensitivity by diminishing β -receptor signaling in PVN neurons.

Within the NTS, both catecholaminergic neurons and BDNF have been implicated in the regulation of baroreflex sensitivity. Baroreflex sensitivity was diminished both by

lesioning NTS catecholaminergic neurons (Itoh et al., 1992; Talman et al., 1980) and by antagonizing TrkB receptors in the NTS (Becker et al., 2016). Further, microinjections of function-blocking BDNF antibodies in the NTS have led to depressor responses and sympathoinhibition (Clark et al., 2011). Similarly, in this study, DSAP lesioning in the NTS was found to significantly reduce spontaneous baroreflex sensitivity in the GFP group but the same treatment had no further effect on the already reduced baroreceptor reflex response seen in the BDNF group. Therefore, the effect of DSAP lesioning on baroreflex sensitivity was similar to its effects on MAP with significant differences in the GFP, but not in the BDNF group.

2.5. Conclusion

In summary, our studies demonstrate that disruption of catecholaminergic signaling between the NTS and PVN contributes to the hypertensive effects of BDNF in the PVN. Lesioning NTS catecholaminergic neurons was found to increase blood pressure in GFP control rats but did not further elevate blood pressure following BDNF overexpression in the PVN. In addition, activating β -adrenergic receptors in the PVN significantly decreased blood pressure in the GFP control group but did not lower blood pressure in the BDNF group, and BDNF overexpression in the PVN also significantly reduced mRNA levels of adrenergic β 1 receptors in the PVN. These BDNF-mediated mechanisms may significantly contribute to stress-induced elevations in blood pressure and the development of stress-related cardiovascular diseases.

CHAPTER 3: BDNF Augments NMDA-signaling and Reduces GABAA-signaling in the Paraventricular Nucleus of the Hypothalamus (PVN) to Elevate Blood Pressure

3.1. Introduction

The paraventricular nucleus (PVN) of the hypothalamus is an important area involved in the regulation of autonomic, neuroendocrine, and cardiovascular responses (Martins-Pinge, Mueller, Foley, Heesch, & Hasser, 2013; Swanson & Sawchenko, 1983). The PVN plays a specific role in cardiovascular regulation via presympathetic neurons projecting to the rostral ventrolateral medulla (RVLM) or directly to spinal sympathetic preganglionic neurons in the intermediolateral (IML) cell column of the spinal cord (Badoer, 2001; R. A. Dampney, 1994; Hardy, 2001; Pyner & Coote, 2000). Increased activity of PVN presympathetic neurons contributes to elevated sympathetic activity and blood pressure in hypertension (R. A. Dampney et al., 2018; Guyenet, 2006; Ye, Li, & Pan, 2013), which can increase the risk of developing other cardiovascular, renal, and cerebrovascular disorders. (Floras, 2009; Judy et al., 1976). Glutamate and γ -aminobutyric acid (GABA) are the main excitatory and inhibitory neurotransmitters within the PVN and have been shown to alter the firing activity of PVN neurons (D. P. Li et al., 2008a, 2008b). Increases in excitatory and decreases in inhibitory mechanisms within the PVN are thought to contribute in part to enhanced sympathetic output in hypertension (Boudaba et al., 1997; Q. H. Chen et al., 2003; Y. F. Li et al., 2006).

Glutamate or NMDA PVN microinjections have been found to increase blood pressure, and sympathetic nerve activity in both anesthetized and conscious normotensive rats (Busnardo et al., 2010; Kannan et al., 1989; Y. F. Li et al., 2006; Y. F. Li, Mayhan,

& Patel, 2001). Meanwhile, NMDA receptor antagonist, AP5, PVN microinjections have been shown to have a larger inhibitory effect on cardiovascular parameters in the spontaneously hypertensive rat (SHR) compared to the normotensive Wistar Kyoto (WKY) rats (D.-P. Li & H.-L. Pan, 2007; Y. F. Li et al., 2006). The PVN is also known to be under immense tonic GABAA receptor-mediated inhibition under normotensive conditions (Decavel & Van den Pol, 1990; Tasker & Dudek, 1993), which is reduced during elevated sympathetic activity in hypertension and heart failure (D. P. Li & Pan, 2006; D. S. Martin & Haywood, 1998; K. Zhang et al., 2002). GABAA receptor agonists isoguvacine or muscimol PVN microinjections have been found to reduce blood pressure more significantly in WKY and Sprague Dawley (SD) rats compared to SHRs (Ding et al., 2015; D. P. Li & H. L. Pan, 2007). Meanwhile GABAA antagonist, gabazine or bicuculline, PVN microinjections have been found to increase sympathetic activity and blood pressure more significantly in WKY and Sprague Dawley (SD) rats compared to SHRs (Ding et al., 2015; D. P. Li & Pan, 2006; D. P. Li & H. L. Pan, 2007; Y. F. Li et al., 2006).

We have recently identified a key modulator of blood pressure, brain derived neurotrophic factor (BDNF), which has been shown to be elevated in the PVN in response to various hypertensive stimuli, such as chronic and acute stress, hyperosmolality, and repeated amphetamine administration (Aliaga et al., 2002; Hammack et al., 2009; Meredith et al., 2002; Rage et al., 2002; Smith, Makino, Kim, et al., 1995). We have also shown that long-term overexpression and acute microinjection of exogenous BDNF into the PVN leads to significant increases in blood pressure and

heart rate (Erdos, Backes, et al., 2015; Chris L. Schaich et al., 2016), and we have recently shown that BDNF can modulate catecholaminergic signaling in the PVN to elevate blood pressure (Thorsdottir, Cruickshank, Einwag, Hennig, & Erdos, 2019). BDNF is also a known modulator of glutamatergic and GABAergic signaling mechanisms in the central nervous system (CNS). BDNF can enhance NMDA-mediated signaling by activating the expression and membrane trafficking of NMDA receptors (Caldeira et al., 2007; Kim et al., 2012; Kolb et al., 2005), while also reducing the expression of GABAA receptors, membrane density and function in hypothalamic neurons (Carreno et al., 2011; Hewitt & Bains, 2006; Lund et al., 2008). Thus, BDNF may represent a novel mediator of glutamatergic and GABAergic signaling in cardiovascular regulation in the PVN.

BDNF can also alter the expression of other excitatory and inhibitory signaling components in the CNS, such as glutamic acid decarboxylase (GAD) 67, K⁺- Cl⁻ cotransporter 2 (KCC2), and synapsin 1a/b. GAD67 has been found to be elevated in renovascular hypertensive rats compared to normotensive control animals (Biancardi et al., 2010), while BDNF release has been shown to upregulate GAD67 (Hanno-Iijima et al., 2015). Further, GAD expression is reduced by application of the TrkB inhibitor, K252a (Hanno-Iijima et al., 2015). BDNF is also known to alter the expression and signaling of KCC2, which serves to establish the chloride ion gradient in neurons via the maintenance of low intracellular chloride concentration (Tang, 2020). The expression of KCC2 is significantly decreased in TrkB double KO mice hippocampi (Carmona et al., 2006), and in adult neurons BDNF has been found to decrease both mRNA and protein

KCC2 (Boulenguez et al., 2010; Claudio Rivera et al., 2002; Claudio Rivera et al., 2004; Shulga et al., 2008; Wake et al., 2007), while increasing KCC2 expression in immature neurons (Aguado et al., 2003; Boulenguez et al., 2010; A. Ludwig et al., 2011; Claudio Rivera et al., 2002; Claudio Rivera et al., 2004; Shulga et al., 2008; Wake et al., 2007). BDNF-TrkB-KCC2-mediated signaling is also implicated in salt-sensitive hypertension whereby this mechanism weakens baroreceptor inhibition of vasopressin magnocellular cells in the SON through a downregulation of KCC2 by BDNF (Choe et al., 2015). Finally, another important component of neurotransmitter signaling in the CNS involves synapsins which are phosphoproteins found at most synaptic terminals. They regulate the maintenance of the reserve vesicle pool, while also recruiting to the vesicle pool and increasing the release probability of synaptic vesicles to the readily releasable pool (Khvotchev & Sun, 2009). Deletion of synapsin genes has been shown to reduce the presynaptic response to BDNF, which may indicate that synapsins play a role in BDNF-mediated increase of glutamate release (Jovanovic, Czernik, Fienberg, Greengard, & Sihra, 2000; Kao et al., 2017). A link between chronic salt loading and increases in synapsin IIa and IIb transcripts in the magnocellular division of the PVN compared to control animals (Nomura et al., 2000) has also been observed.

Here, we set out to test the hypothesis that upregulation of BDNF in the PVN elevates blood pressure by altering the excitatory/inhibitory balance in the PVN by diminishing GABAA-mediated inhibitory mechanisms and augmenting NMDA-mediated excitatory mechanisms. We used our previously published model (Erdos, Backes, et al., 2015) of vector-mediated upregulation of BDNF in the PVN to induce hypertension and

investigated the interaction of BDNF with glutamatergic and GABAergic signaling by microinjecting NMDA and GABAA receptor agonists and antagonists into the PVN and measuring changes in cardiovascular responses, while also analyzing protein and mRNA expression of excitatory and inhibitory signaling components in the PVN. Understanding the mechanism behind BDNF-mediated alteration of excitatory and inhibitory signaling in the PVN will be important for a better understanding of the central blood pressure neurocircuitry which could aid in the development of novel therapies for the treatment of hypertension.

3.2. Methods

All animal housing, handling, and surgical and experimental procedures were conducted within an Association for the Assessment and Accreditation of Laboratory Care International-accredited animal care facility at the University of Vermont, in accordance with the National Institutes of Health (NIH) Policy on Humane Care and Use of Laboratory Animals and the NIH *Guide for the Care and Use of Laboratory Animals*. Experiments were performed in male Sprague-Dawley (SD) rats obtained from Charles River (Saint-Constant, QC, Canada). Rats were housed individually with a 12:12-h light-dark cycle (lights on at 6:00 AM), with free access to food (standard chow) and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

3.2.1. Experimental Design

Experiment 1. The aim of the first experiment was to determine the effect of BDNF on NMDA-mediated glutamatergic and GABAA-mediated GABAergic receptor signaling in

the PVN. Seven-week-old male SD rats received bilateral injections of adeno-associated viral vectors (AAV2, 10^{12} vps/mL, 200 nL/side) expressing GFP or myc epitope-tagged BDNF fusion protein (BDNFmyc) into the PVN. Three weeks later, blood pressure and heart rate responses were recorded following PVN injections of the NMDA agonist NMDA (100 μ M) (Y. F. Li et al., 2001), the NMDA antagonist AP5 (10 mM) (Q. H. Chen et al., 2003; D.-P. Li & H.-L. Pan, 2007), the GABAA agonist muscimol (10 mM) (D. P. Li & H. L. Pan, 2007), and the GABAA antagonist gabazine (2 mM) (D.-P. Li & H.-L. Pan, 2007) under alpha-chloralose-urethane anesthesia. At the end of the experiment, animals were deeply anesthetized and perfused transcardially with phosphate buffered saline (PBS) and 4% paraformaldehyde. GFP, BDNFmyc and drug injection sites of glutamatergic and GABAergic agonists and antagonists into the PVN were indicated by a red fluorescent microbead solution, were verified with immunofluorescence and fluorescent microscopy in coronal sections of the PVN (**Fig. 10, A–C.**).

Experiment 2. The goal of the second experiment was to determine the effect of BDNF on various components of inhibitory and excitatory signaling mechanisms in the PVN. Seven-week-old male SD rats received AAV2 viral vector (10^{12} vps/mL, 200 nL/side) expressing bilateral injections of GFP or BDNFmyc into the PVN. Three weeks later, the animals were euthanized, and the brains were quickly removed and snap-frozen. PVN tissue samples were isolated, mRNA levels of NMDAR1 and GABAA-delta subunit were determined in the PVN with quantitative real-time RT-PCR. A second group of animals also received bilateral PVN injections of AAV2 viral vectors (10^{12} vps/mL, 200 nL/side) expressing GFP or BDNFmyc. Three weeks later the animals were deeply anesthetized and perfused transcardially with PBS and 4% paraformaldehyde. NMDAR1, GABAA-alpha1 subunit,

GAD67, KCC2 and synapsin 1a/b expression in the PVN were assessed using immunofluorescence, confocal microscopy, and image analysis. Finally, the average area of GFP and BDNFmyc cells was also measured in GFP and BDNF groups.

3.2.2. Surgical Procedures

Surgeries were performed using aseptic techniques under continuous isoflurane anesthesia (5% induction, 2–3% maintenance) delivered in oxygen. Depth of anesthesia was ensured by lack of a reflex response to pinch of the hindpaw. Carprofen ($5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ sc) was used for postsurgical analgesia administered at the beginning of surgery and for 2 days after surgery. Anesthetized rats were placed in a stereotaxic frame, BDNFmyc and GFP viral vectors (10^{12} viral particles/mL, 200 nL/side) were injected bilaterally into the PVN using pipettes pulled from thin walled borosilicate glass capillary tubes (OD, 1 mm; ID, 0.58 mm; tip diameter: $\sim 25 \mu\text{m}$; World Precision Instruments Inc., Sarasota, FL) at the following stereotactic coordinates: 1.80 mm posterior to bregma, 1.70 mm lateral to the midline, and 7.65 mm ventral from the dorsal surface of the brain, with the micropipette tilted 10° laterally toward the midline. Virus stocks were injected over 5 min using a pneumatic pico pump (World Precision Instruments). The pipette was left in place for an additional 3 min before being withdrawn.

3.2.3. Assessment of cardiovascular responses to NMDA and GABAA agonists and antagonists

The left femoral artery and vein were catheterized under isoflurane anesthesia 3 weeks after bilateral PVN injections of AAV2-GFP or AAV2-BDNFmyc. Rats were then placed in a stereotaxic frame, and isoflurane anesthesia was gradually switched to

intravenous alpha-chloralose ($60 \text{ mg kg}^{-1} \text{ h}^{-1}$) and urethane ($800 \text{ mg kg}^{-1} \text{ h}^{-1}$) anesthesia administered through the femoral vein catheter over a 30-min period, during which, isoflurane was gradually reduced from 2.5% to 0%. Blood pressure, heart rate, toe-pinch, and eye blink reflexes were monitored closely to ensure the animal remained anesthetized. After complete withdrawal of isoflurane, anesthesia was maintained by intravenous alpha-chloralose ($15 \text{ mg kg}^{-1} \text{ h}^{-1}$) and urethane ($200 \text{ mg kg}^{-1} \text{ h}^{-1}$) infusion for the remainder of the experiment. After establishment of steady baseline blood pressure and heart rate for a minimum of 30 min, rats received NMDA ($100 \text{ }\mu\text{M}$, 200 nL/side , Sigma-Aldrich, M3262), AP5 (10 mM , 200 nL/side ; Tocris, 0106), muscimol (10 mM , 200 nL/side , Sigma-Aldrich, G019), and gabazine (2 mM , 200 nL/side , Tocris, 1262) injections into the PVN. Blood pressure was monitored via the catheter placed in the left femoral artery, and heart rate was extracted from the pulsatile pressure wave by using Laboratory Chart Pro 8 (ADInstruments). To verify locations of the drug injections in the PVN with fluorescent microscopy, 10% rhodamine-labeled fluorescent microspheres ($0.04 \text{ }\mu\text{m}$; Molecular Probes) were mixed into the injection solution.

3.2.4. Viral Vector-Mediated Gene Transfer into the PVN

AAV2 viral vectors were used to elicit the expression of enhanced GFP and BDNFmyc, derived from rat Bdnf, constructed and packaged by Vector Biolabs (Philadelphia, PA). The expression of GFP and BDNFmyc was driven by a chicken beta-actin promoter with human cytomegalovirus enhancer and a woodchuck posttranscriptional regulatory element, which enhanced the expression of transgenes present downstream of GFP and BDNFmyc. The BDNFmyc plasmid was a generous gift

from Dr. Ronald Klein (LSU Health Sciences Center Shreveport, LA) and was used previously to protect retinal ganglion cells in a rat glaucoma model (K. R. Martin et al., 2003), and to study cardiovascular effects of BDNF in the PVN (Erdos, Backes, et al., 2015). In addition, full efficacy of BDNFmyc expression driven by the rat neuron-specific enolase promoter was confirmed previously both in vitro and in vivo (R. L. Klein et al., 1999).

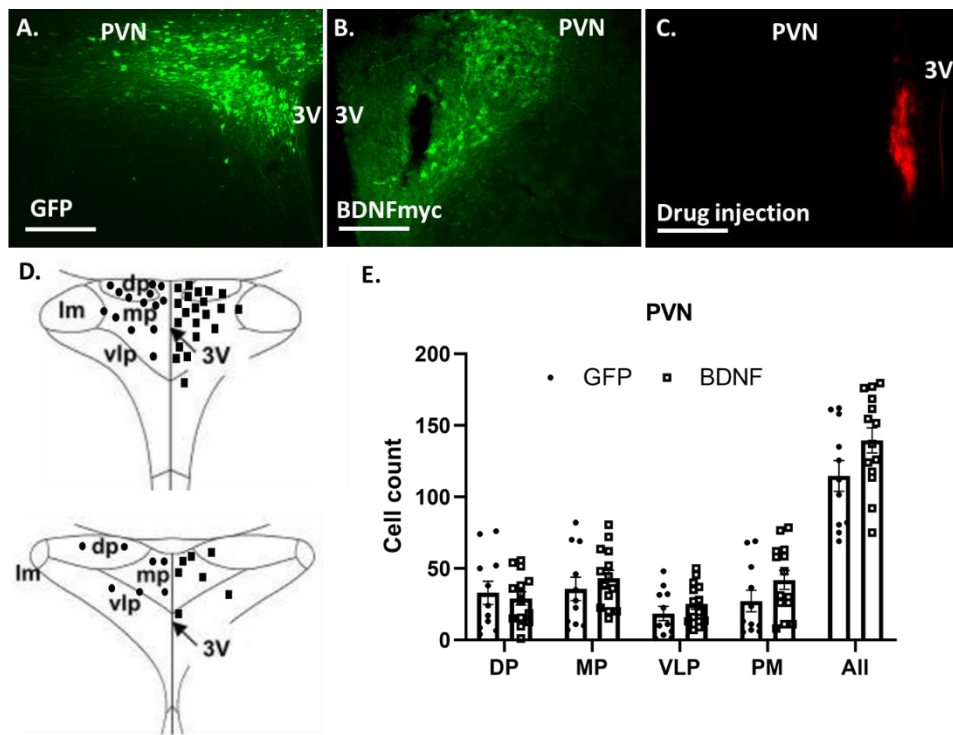


Figure 10. Representative fluorescent image of a coronal brain section ~1.8 mm posterior to bregma showing PVN expression of AAV2-GFP (A) or AAV2-BDNFmyc (B) and the red fluorescent microbead solution that was mixed with injected drug solution to verify injection sites (C); scale bars, 250 μ m. D. diagrams of the PVN ~1.8 mm and ~1.9 mm posterior to bregma showing locations of drug injections in rats previously injected with AAV2-GFP (circle, shown aggregated on the left side of the PVN) or AAV2-BDNFmyc (rectangle, shown aggregated on the right side of the PVN). E. number of GFP- and myc-positive cells was assessed in subnuclei of the PVN; dorsal parvocellular nuclei (DP), medial parvocellular nuclei (MP), ventrolateral parvocellular nuclei (VLP), and posterior magnocellular nuclei (PM) following AAV2-GFP or AAV2-BDNFmyc injections in the

PVN, 3V, third ventricle; PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; BDNFmyc, myc epitope-tagged BDNF; AAV2, adeno-associated viral vector 2.

3.2.5. Analysis of Cardiovascular Data from Anesthetized Animals

Blood pressure and heart rate were recorded using LabChart software version 8.0.7 (ADInstruments, Dunedin, NZ) at a 1,000-Hz sampling rate and condensed to 10 or 20 second moving averages for data analysis and presentation. Maximum and average changes in mean arterial pressure (MAP) and heart rate following drug microinjections were evaluated.

3.2.6. Immunofluorescence

After experiments 1 and 2, animals were perfused with 400 mL of ice-cold PBS followed by 400 mL of ice-cold 4% paraformaldehyde in PBS. Brains were removed and post-fixed for 2 h in 4% paraformaldehyde and then equilibrated in 30% sucrose solution at 4°C. Coronal sections (40 µm) were cut on a microtome (Leica SM2000R) and mounted on Fisher Superfrost Plus slides. BDNFmyc, NMDAR1, GABAA-alpha1, GAD67, synapsin 1a/b and KCC2 were detected using the following primary antibodies (1:200, overnight incubation at 4°C): anti-c-Myc (Santa Cruz Biotechnology; 9E10), anti-c-Myc (Abcam; ab9106) anti-NMDAR1 (Novus Biologicals, R1JHL), anti-GABAA-alpha1 (Abcam, ab33299), anti-GAD67 (Sigma-Aldrich, MAB5406; 1:400), anti-KCC2 (Sigma-Aldrich, MABN88), and anti-synapsin 1a/b (Santa Cruz Biotechnology, sc-7379). Secondary antibodies were (1:200, 2-h incubation at room temperature): donkey anti-

mouse A546 (Invitrogen), donkey anti-mouse A555 (A31570), donkey anti-rabbit A555 (Invitrogen), donkey anti-rabbit A488 (A21206), and donkey anti-goat A555 (ab150130). GFP, BDNFmyc, NMDAR1, GABAA-alpha1, GAD67, KCC2 and synapsin 1a/b immunofluorescence in the PVN were detected with a fluorescent microscope (Nikon Eclipse 50i) and a confocal microscope (Nikon A1R).

3.2.7. Confocal Microscopy and Image Analysis

Images of GFP and BDNF PVN brain slices labeled with immunofluorescence as described above were taken using a Nikon A1R confocal laser-scanning imaging system. The z-stacks were then deconvoluted using the deconvolution software AutoQuant X3 (Media Cybernetics, Inc.). Then the green channel of the z-stack, expressing GFP or BDNFmyc, was opened in ImageJ and thresholded using the Phansalkar thresholding technique which is a modification on the Sauvola method, a variant of Niblack's method (Adler & Parmryd, 2014). It uses a combination of local mean, local standard deviation and normalization to set a threshold below the local mean to select the foreground, reject the background and boost the threshold at lower intensities (Neerad, Sumit, Ashish, & Madhuri, 2011). The function Remove Outliers in ImageJ was also used, which replaces a pixel if it deviates from the median by more than the threshold. The parameters were set for a 10-pixel radius, which represents the area that is used for calculating the median, at a 50 AU threshold, which determines by how much a pixel must diverge from the median to be replaced. Pixels that were brighter than the median were replaced. Finally, the Analyze Particles function was used to measure the area of the cells, set at a size of $50 \mu\text{m}^2$ – infinity.

Following this, region of interest (ROI) mean fluorescence intensity (ROI mean), and ROI maximum fluorescence intensity (ROI max) were measured in the red channel in the z-stack, expressing NMDAR1, GABAA-alpha1, GAD67, KCC2 or synapsin 1a/b, using the ROIs from the green channel representing the cell bodies. This analysis was conducted separately for each side of the PVN, and the mean calculated for each experimental group. Additionally, 1 slice from the red channel z-stack was selected to measure mean fluorescence intensity within the PVN (PVN mean) by drawing a border along the PVN. This analysis was also conducted separately for each side of the PVN, and the mean calculated within each experimental group. Finally, for the GABAA-alpha1 subunit expression, mean fluorescence intensity (Area mean) was measured in a defined area of the peri-PVN (186x186 μM) compared to an area within the medial parvocellular nucleus of the PVN of the same size.

3.2.8. Real-Time RT-PCR

Frozen forebrains were mounted on a cryostat, and rostral coronal sections were cut until reaching the level ~1.5 mm posterior of bregma for the PVN. Then, tissue samples 0.75 mm in diameter and 0.5 mm deep were punched bilaterally from the region of the PVN, with the third ventricle used as reference for the PVN. The depth of the punches was limited by a spacer glued onto the outside of the punch tool. Total RNA was extracted from brain punches with Qiashredder columns (Qiagen, Valencia, CA) and the RNeasy Micro Kit (Qiagen). Samples were treated with an RNase-free DNase Set (Qiagen) on the column to remove genomic DNA. RNA was quantified with a Qubit Fluorometer, and cDNA was synthesized with a high-capacity cDNA reverse transcription kit (Applied Biosystems)

according to manufacturer's instructions and stored at -20°C . All quantitative RT-PCR reactions were run in duplicate in eight-well optical-grade strips in a Prism 7000 Sequence Detection System (Applied Biosystems) and quantified with the cycle threshold (CT) method. The mRNA levels of NMDAR1 and GABAA-delta, and the reference gene beta-actin (ActB) were analyzed with quantitative real time RT-PCR using specific oligonucleotide primers and TaqMan probes (Applied Biosystems, Foster City, CA): NMDAR1, Rn01436034_m1 (reference sequence: NM_001270602.1); GABAA-delta, Rn01517017_g1 (reference sequence: NM_017289.1). Control reactions containing no template were run for each plate, and results were analyzed with the $2^{-\Delta\Delta\text{CT}}$.

3.2.9. Statistics

Maximum decreases or increases in MAP and heart rate during microinjection experiments were measured using an unpaired t-test. NMDAR1, GABAA-alpha1, GAD67, KCC2 and synapsin 1a/b ROI max, ROI mean, as well as PVN mean and Area mean in the peri-PVN and parvocellular PVN were analyzed using an unpaired t-test. RT-PCR data was also analyzed by unpaired t-test. Statistical tests were performed using Prism 8.2 software (GraphPad, San Diego, CA). Results are expressed as means \pm SE, and the criterion for statistical significance was $P < 0.05$.

3.3. Results

3.3.1. Effect of BDNF on NMDA-mediated signaling in the PVN

In our first experiment, we tested whether BDNF affects PVN NMDA-mediated cardiovascular responses. We found that inhibition of NMDA-receptors with AP5 significantly lowered MAP and heart rate in BDNF rats compared to the control group.

Specifically, the results from our first experiment showed that in the BDNF group, max average decreases in blood pressure and heart rate in response to AP5 were -17 ± 5 mmHg and -61 ± 18 BPM, compared with -2 ± 7 mmHg ($p<0.05$) and -6 ± 1 BPM ($p<0.05$) in the GFP group (**Fig. 11. A-B.**). We also found that activation of NMDA-receptors did not have a significant effect on MAP but did significantly lower heart rate responses in the BDNF group compared to the GFP group ($p<0.05$). In response to NMDA, max average increases in blood pressure and max average decreases in heart rate in the BDNF group were 5 ± 1 mmHg and -94 ± 23 BPM, compared with 8 ± 2 mmHg ($p=0.31$, n.s.) and -40 ± 13 BPM ($p<0.05$) in the GFP group. Max average decreases in blood pressure in the BDNF group was -3.9 ± 2 mmHg and -12.3 ± 4 mmHg in the GFP group ($p=0.13$, n.s.) (**Fig. 11. C-D.**).

The effect of BDNF overexpression in the PVN on NMDAR1 and synapsin 1a/b expression were also quantified (**Fig. 12-15.**). NMDAR1 ROI mean was found to be significantly elevated in the BDNF group, compared to the GFP group ($p<0.001$) (**Fig. 12C.**). NMDAR1 ROI max was also significantly higher in the BDNF group compared to the GFP group ($p<0.05$) (**Fig. 12D.**). Finally, PVN mean was significantly elevated in the BDNF group compared to the GFP group ($p<0.05$) (**Fig. 12E.**). Synapsin 1a/b ROI mean and ROI max was not significantly different between the GFP and BDNF groups ($p=0.48$, n.s., $p=0.29$, n.s.) (**Fig. 14C-D.**). PVN mean was also not significantly different between the GFP and BDNF groups ($p=0.79$, n.s.) (**Fig. 14E.**).

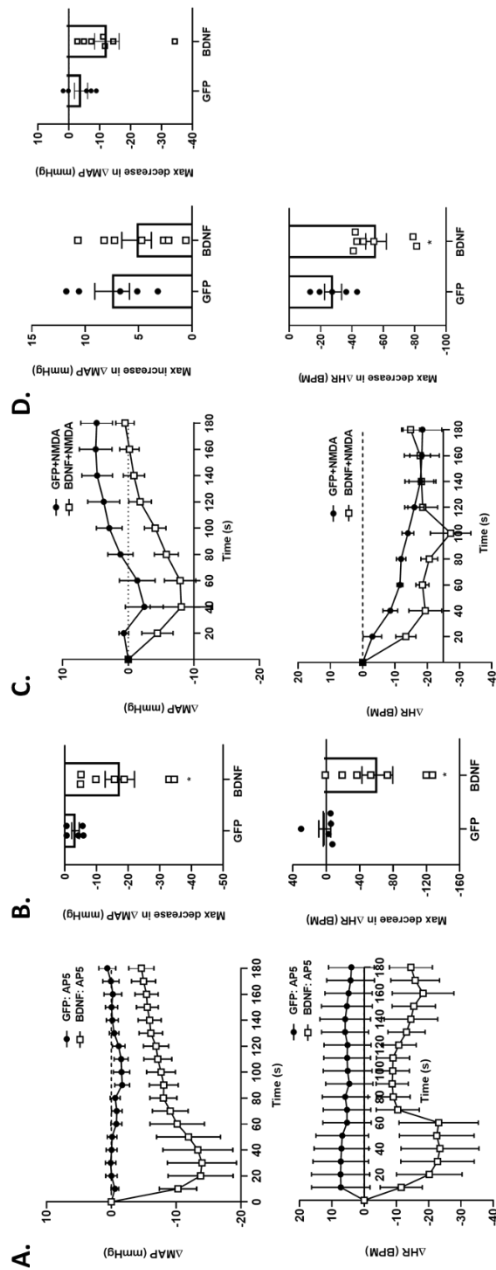


Figure 11. Changes in mean arterial pressure (MAP; top) and heart rate (HR; bottom) in response to PVN injections of the NMDA receptor antagonist AP5 (A, B) and NMDA-receptor agonist NMDA (C, D) in anesthetized rats previously treated with AAV2-GFP or AAV2-BDNFmyc. A: MAP and HR obtained with 10-s moving average over 180 seconds following AP5 injection in GFP (n=5) and BDNFmyc (n=7) treated rats. B: Average max decrease in MAP and heart rate responses to AP5 injections. C: MAP and HR obtained with 20-s moving average over 180 seconds following NMDA injection in GFP (n=5) and BDNFmyc (n=7) treated rats. D: Average max decrease and increase in MAP and average max decrease in heart rate responses to NMDA injections. Statistical analysis was done on max changes in MAP

and heart rate. PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; BDNFmyc, myc epitope-tagged BDNF; AAV2, adeno-associated viral vector 2. Results represent means \pm SE. Statistical analysis was done on peak changes in MAP and HR. *P < 0.05 for GFP vs. BDNF (unpaired t-test).

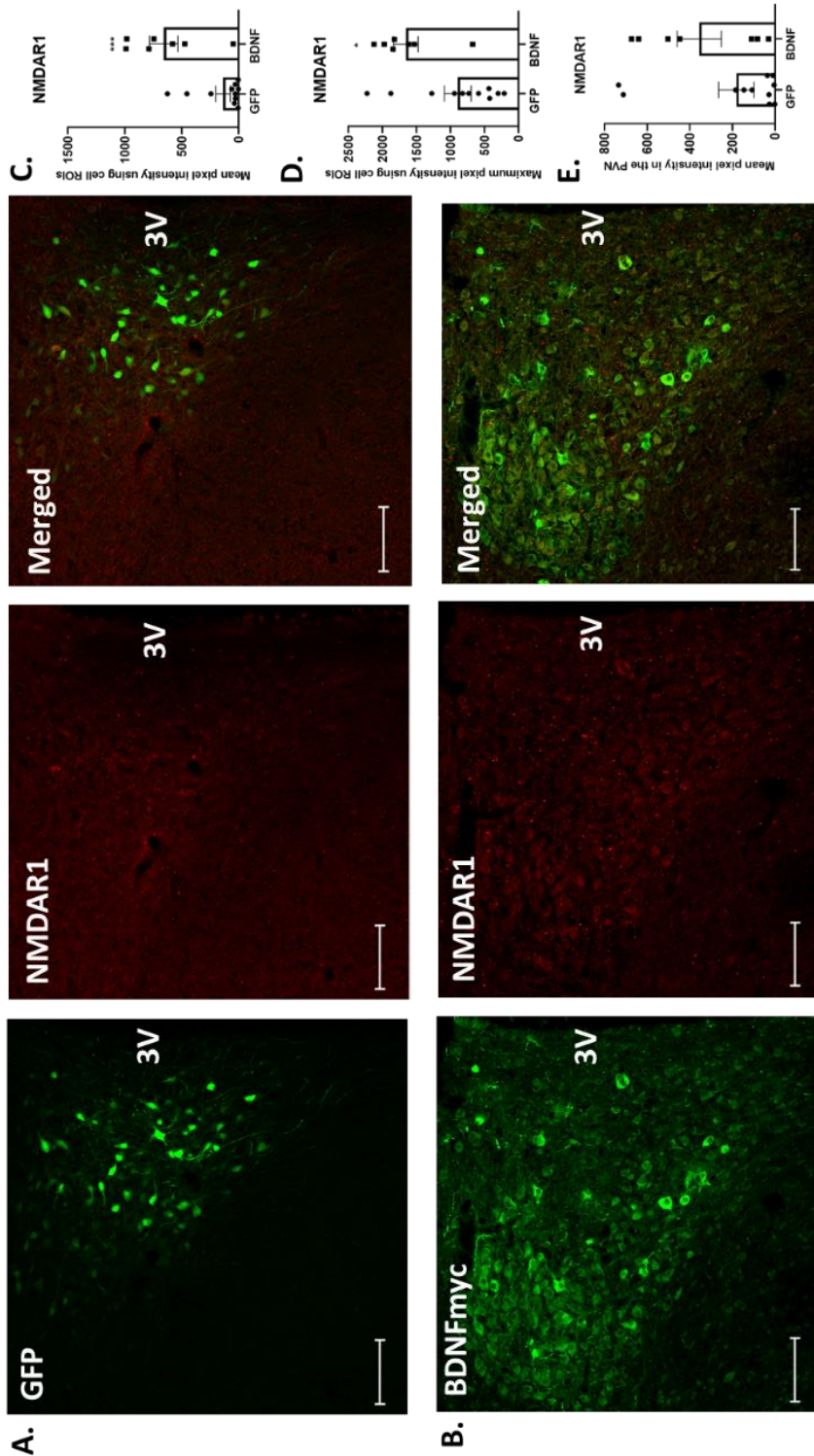


Figure 12. NMDAR1 fluorescent expression in the PVN of rats previously injected with AAV2-GFP (A) or AAV2-BDNFmyc (B) in the PVN. Representative confocal images of NMDAR1 expression within the PVN in GFP (A) and BDNFmyc (B) groups as detected with an NMDAR1 antibody and immunofluorescence; scale bars, 100 μ m. C-D. ROI mean (C) and ROI max (D) NMDAR1 in the PVN, averaged for each experimental group; n refers to number of PVN sides [GFP (n = 11) and BDNFmyc (n = 7)]. E: PVN mean NMDAR1 expression [GFP (n = 11) and BDNFmyc (n = 7)]. 3V, third ventricle; PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; AAV2, adeno-associated viral vector 2. Results represent means \pm SE. *P < 0.05 for GFP vs. BDNF; ***P < 0.001 for GFP vs. BDNF (unpaired t test).

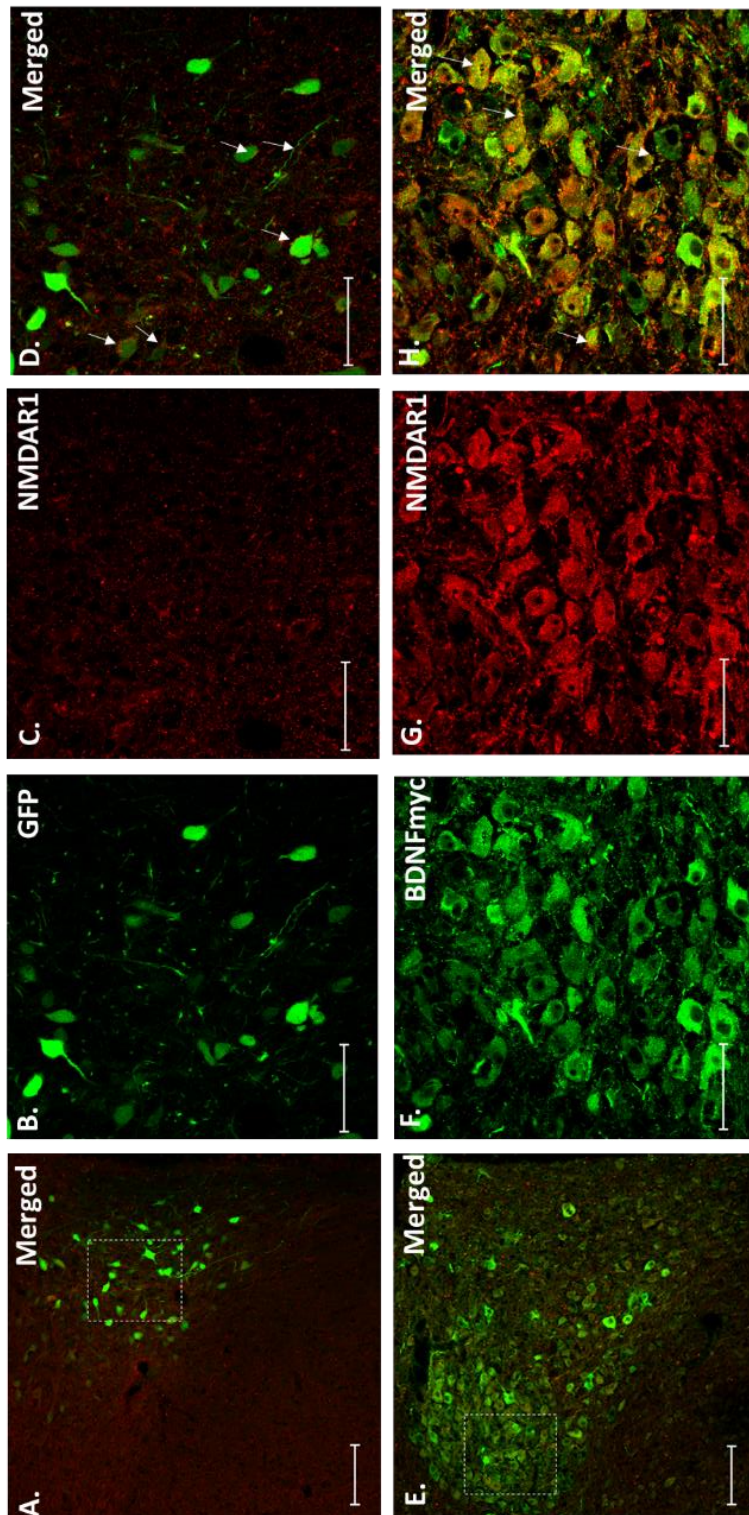


Figure 13. A: NMDAR1 fluorescent expression in the PVN of rats previously injected with AAV2-GFP, showing a rectangular area which is magnified in B-D, scale bar 100 μ M. B-D: Representative 60X magnification confocal images showing NMDAR1 and GFP expression in the PVN. Arrows show overlap of NMDAR1 staining with GFP infected cell bodies and fibers, scale bar 50 μ M. E: NMDAR1 fluorescent expression in the PVN of rats previously injected with AAV2-BDNFmyc, showing a rectangular area which is magnified in F-H, scale bar 100 μ M. F-H: Representative 60X magnification confocal images showing NMDAR1 and BDNFmyc expression in the PVN. Arrows show overlap of NMDAR1 staining with BDNFmyc infected cell bodies and fibers, scale bar 50 μ M.

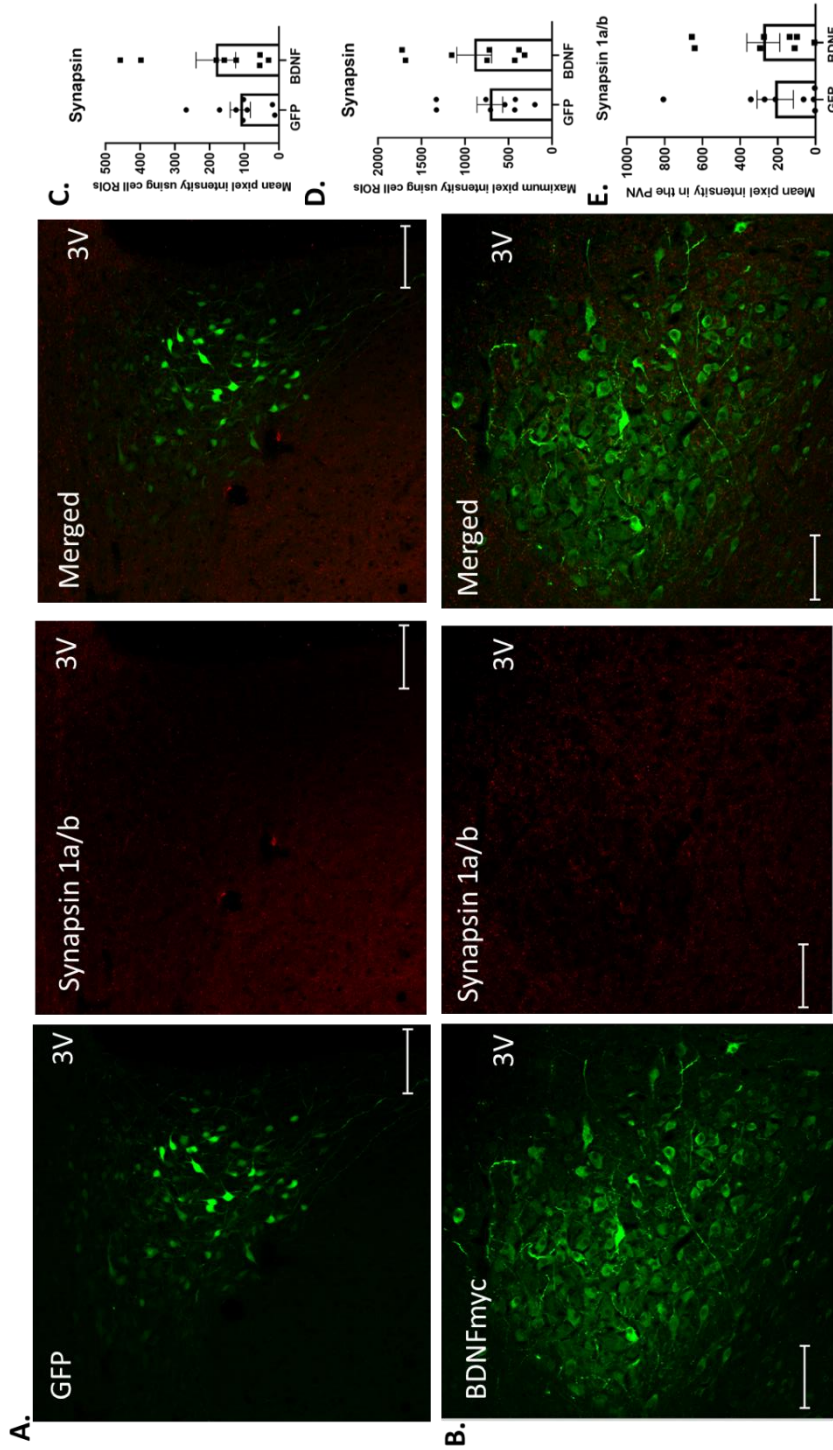


Figure 14. Synapsin 1a/b fluorescent expression in the PVN of rats previously injected with AAV2-GFP or AAV2-BDNFmyc in the PVN. A-B. Representative confocal images of NMDAR1 expression within the PVN in GFP (A) and BDNFmyc (B) groups as detected with a synapsin 1a/b antibody and immunofluorescence; scale bars, 100 μ m. C-D. ROI mean (C) and ROI max (D) synapsin 1a/b expression in the PVN, averaged for each experimental group; n refers to number of PVN sides [GFP (n = 8) and BDNFmyc (n = 8)]. E: PVN mean synapsin 1a/b expression [GFP (n = 8) and BDNFmyc (n = 8)]. 3V, third ventricle; PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; AAV2, adeno-associated viral vector 2. Results represent means \pm SE.

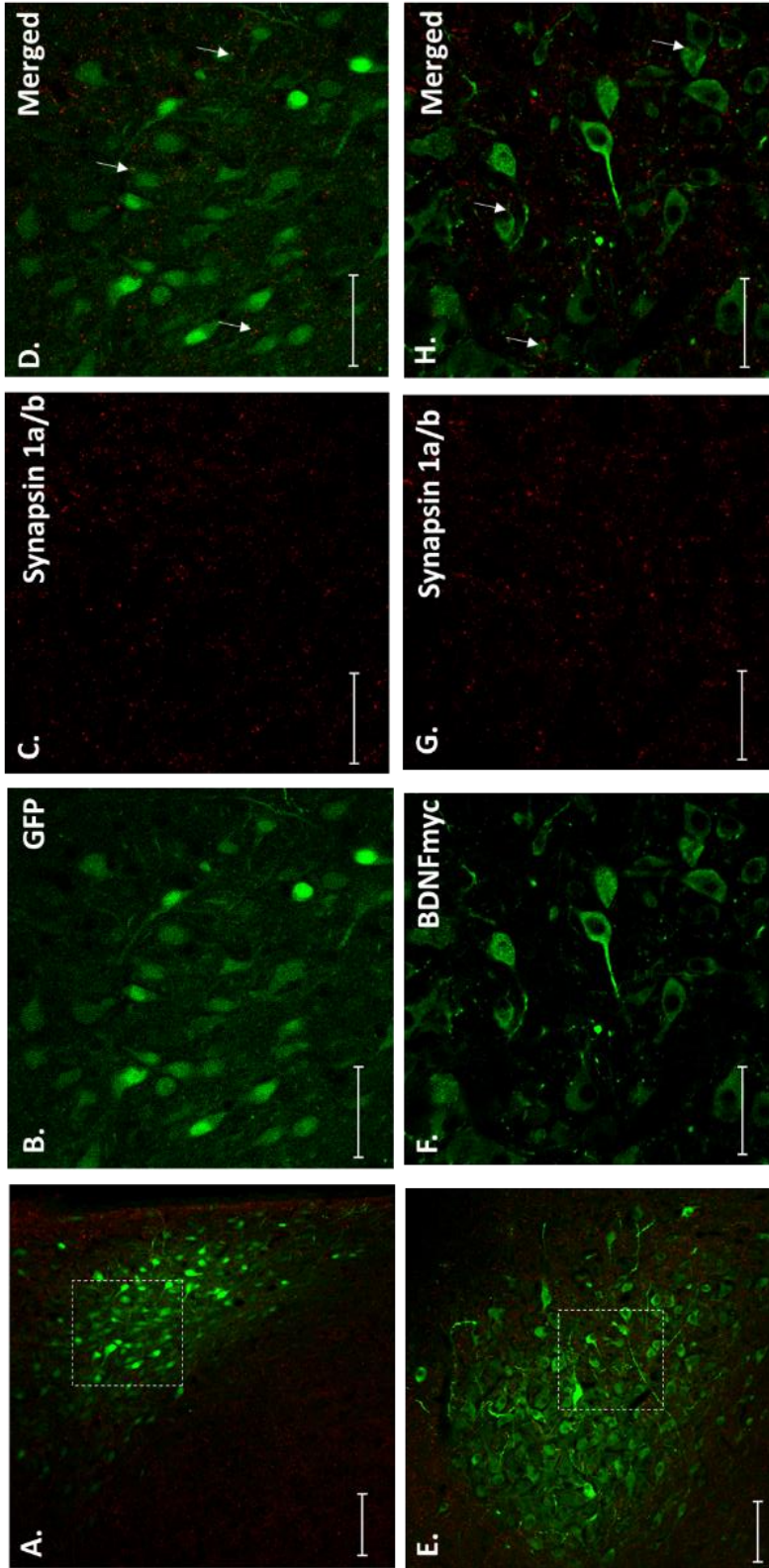


Figure 15. A: Synapsin 1a/b fluorescent expression in the PVN of rats previously injected with AAV2-GFP, showing a rectangular area which is magnified in B-D, scale bar 100 μ M. B-D: Representative 60X magnification confocal images showing synapsin 1a/b and GFP expression in the PVN. Arrows show overlap of synapsin 1a/b staining with GFP infected cell bodies and fibers, scale bar 50 μ M. E: Synapsin 1a/b fluorescent expression in the PVN of rats previously injected with AAV2-BDNFmyc, showing a rectangular area which is magnified in F-H, scale bar 100 μ M. F-H: Representative 60X magnification confocal images showing synapsin 1a/b and BDNFmyc expression in the PVN. Arrows show overlap of synapsin 1a/b staining with BDNFmyc infected cell bodies and fibers, scale bar 50 μ M.

3.3.2. Effect of BDNF on GABAA-mediated signaling in the PVN

In our first experiment we also tested whether BDNF affects PVN GABAA-mediated cardiovascular responses. We found that inhibition of GABAA receptors with gabazine significantly elevated MAP in the GFP group compared to the BDNF overexpressed group. In response to gabazine, average max increases in blood pressure and heart rate in the BDNF group were 34 ± 7 mmHg and 97 ± 26 BPM, compared with 70 ± 12 mmHg ($p<0.05$) and 155 ± 22 BPM ($p=0.12$, n.s.) in the GFP group (**Fig. 16A-B.**). We also found that activation of GABAA receptors with muscimol significantly decreased MAP in the GFP group compared to the BDNF overexpressed group. In response to muscimol, average max decreases in blood pressure and heart rate in the BDNF group were -15 ± 2 mmHg and -27 ± 8 BPM, compared with -26 ± 5 mmHg ($p<0.05$) and -48 ± 9 BPM ($p=0.10$, n.s.) in the GFP group (**Fig. 16C-D.**).

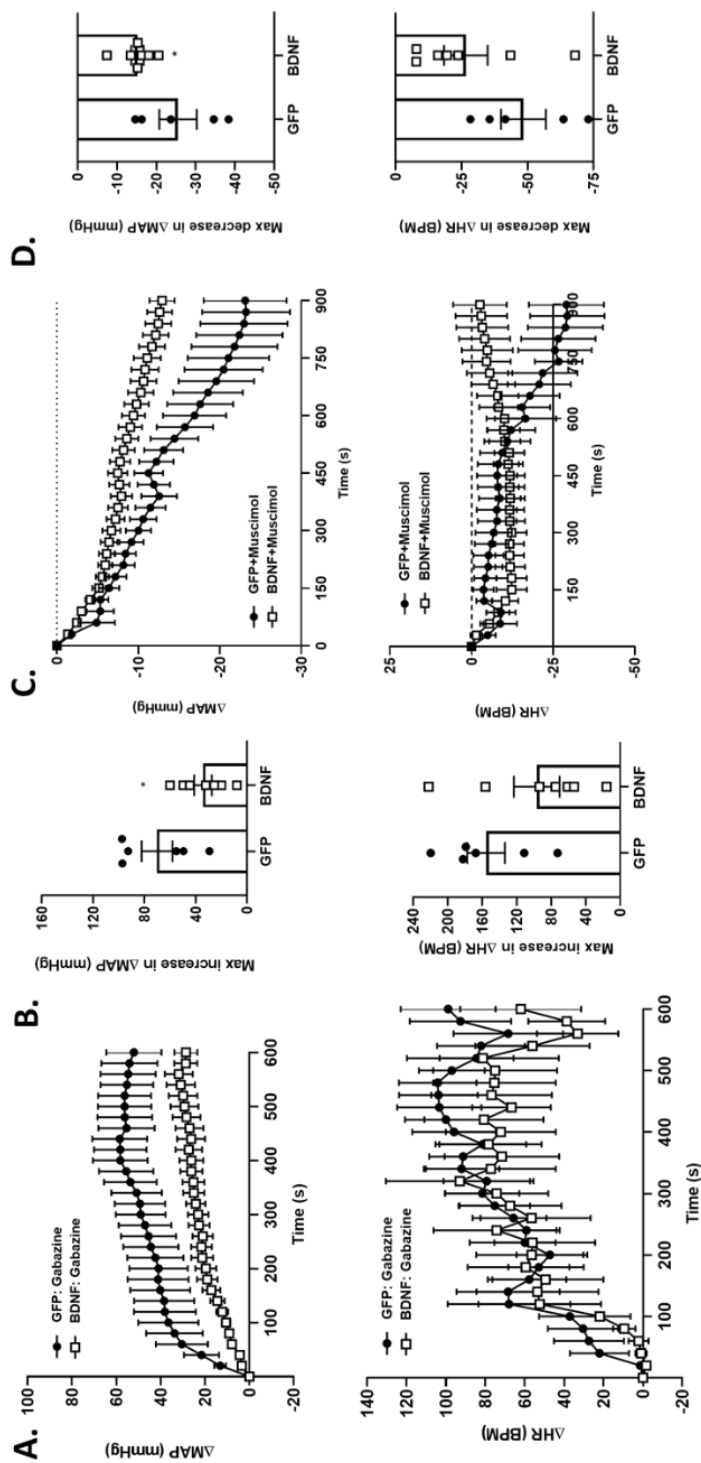


Figure 16. Changes in mean arterial pressure (MAP; top) and heart rate (HR; bottom) in response to PVN injections of the GABAA receptor antagonist gabazine (A, B) and GABAA-receptor agonist muscimol (C, D) in anesthetized rats previously treated with AAV2-GFP or AAV2-BDNFmyc. A: MAP and HR obtained with 20-s moving average over 600 seconds following gabazine injection in GFP (n=6) and BDNFmyc (n=7) treated rats. B: Average max increase in MAP and HR responses to gabazine injections. C: MAP and HR obtained with 20-s moving average over 900 seconds following muscimol injection in GFP (n=5) and BDNFmyc (n=7) treated rats. D: Average max decrease in MAP and HR responses to muscimol injections. Statistical analysis was done on average max changes in MAP and HR. PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; BDNFmyc, myc epitope-tagged BDNF; AAV2, adeno-associated viral vector 2. Results represent means \pm SE. Statistical analysis was done on peak changes in MAP and HR. *P < 0.05 for GFP vs. BDNF (unpaired t test).

The effect of BDNF overexpression in the PVN on GABAA-alpha1 was also quantified (**Fig. 17-18.**) GABAA-alpha1 ROI mean was not significantly different between the two groups ($p=0.56$. n.s.), but ROI max was significantly reduced in the BDNF group compared to GFP ($p<0.01$) (**Fig. 17C-D.**). PVN mean was not significantly different between the GFP and BDNF groups ($p=0.053$, n.s.) (**Fig. 17E.**). Area mean within the PVN in the BDNF group compared to the peri-PVN was significantly lower ($p<0.01$), additionally Area mean in the peri-PVN of the BDNF group was significantly lower compared to the peri-PVN of the GFP group ($p<0.05$) (**Fig. 17F.**).

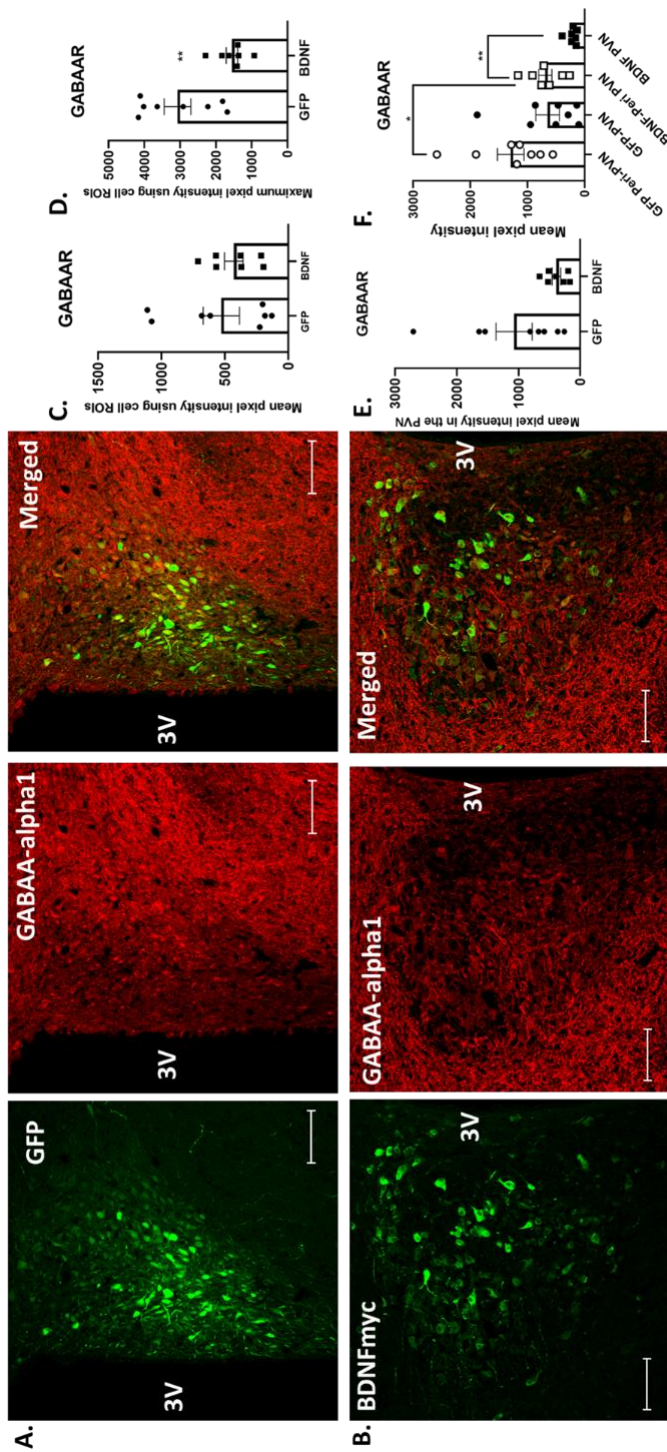


Figure 17. GABA α 1 fluorescent expression in the PVN of rats previously injected with AAV2-GFP or AAV2-BDNFmyc in the PVN. A-B. Representative confocal images of GABA α 1 expression within the PVN in GFP (A) and BDNFmyc (B) groups as detected with an GABA α -alpha1 antibody and immunofluorescence; scale bars, 100 μ m. C-D. ROI mean (C) and ROI max (D) GABA α -alpha1 expression in the PVN, averaged for each experimental group; n refers to number of PVN sides [GFP (n = 8) and BDNFmyc (n = 7)]. E: PVN mean GABA α -alpha1 expression [GFP (n = 8) and BDNFmyc (n = 7)]. F: Area mean of GABA α -alpha1 within the peri-PVN and the parvocellular PVN [GFP (n = 8) and BDNFmyc (n = 7)]. 3V, third ventricle; PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; AAV2, adeno-associated viral vector 2. Results represent means \pm SE. *P < 0.05 for GFP Peri-PVN vs. BDNFmyc Peri-PVN; **P < 0.05 for BDNF PVN vs. BDNF Peri-PVN; ***P < 0.01 for GFP vs. BDNF (unpaired t test).

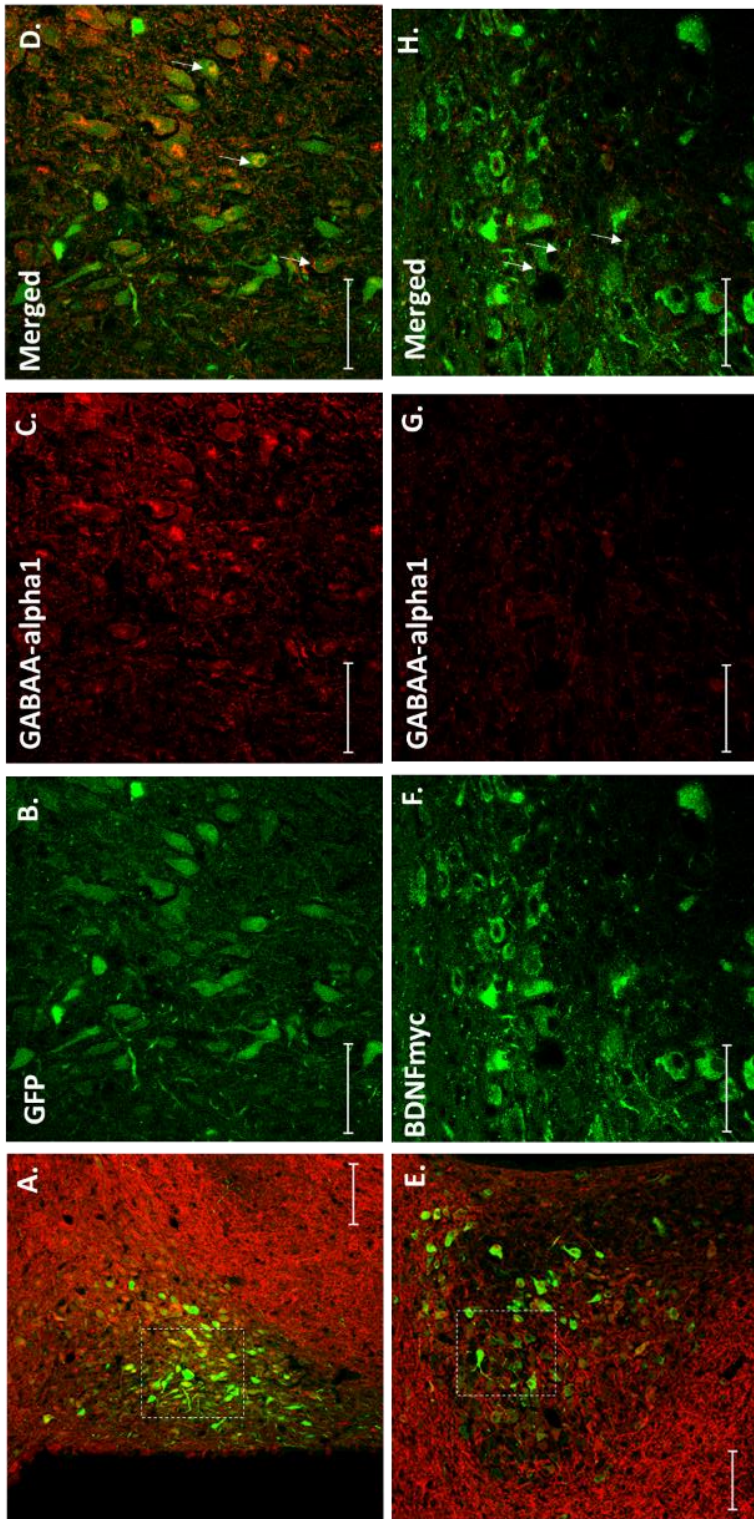


Figure 18. A: GABAA-alpha1 fluorescent expression in the PVN of rats previously injected with AAV2-GFP, showing a rectangular area which is magnified in B-D, scale bar 100 μM. B-D: Representative 60X magnification confocal images showing GABAA-alpha1 and GFP expression in the PVN. Arrows show overlap of GABAA-alpha1 staining with GFP infected cell bodies and fibers, scale bar 50 μM. E: GABAA-alpha1 fluorescent expression in the PVN of rats previously injected with AAV2-BDNFmyc, showing a rectangular area which is magnified in F-H, scale bar 100 μM. F-H: Representative 60X magnification confocal images showing GABAA-alpha1 and BDNFmyc expression in the PVN. Arrows show overlap of GABAA-alpha1 staining with BDNFmyc infected cell bodies and fibers, scale bar 50 μM.

GAD67 and KCC2 were also assessed in the PVN (**Fig. 19-22.**). GAD67 ROI max and ROI mean was not significantly different between the BDNF and GFP groups ($p=0.96$, n.s., $p=0.15$, n.s.) (**Fig. 19C-D.**). PVN mean was also not significantly different between the GFP and BDNF groups ($p=0.34$, n.s.) (**Fig. 19E.**). KCC2 ROI max and ROI mean were significantly elevated in the BDNF group compared with the GFP group ($p<0.001$, $p<0.05$) (**Fig. 21C-D.**). However, PVN mean was not significantly different between the two groups ($p=0.15$, n.s.) (**Fig. 21E.**). We also tested the effect of BDNF upregulation in the PVN on expression of NMDAR1 and GABAA-delta subunit mRNA. BDNF overexpression in the PVN did not significantly alter NMDAR1 ($p=0.58$, n.s.) or GABAA-delta subunit ($p=0.73$, n.s.) mRNA expression in the PVN (**Fig. 23.**). Finally, the average area of GFP and BDNFmyc cells was also measured, the average area of a GFP cell was $225.7\pm 15 \mu\text{m}^2$ and the average area of a BDNFmyc cell was $253.1\pm 22 \mu\text{m}^2$ ($p=0.30$, n.s.) (**Fig. 24.**).

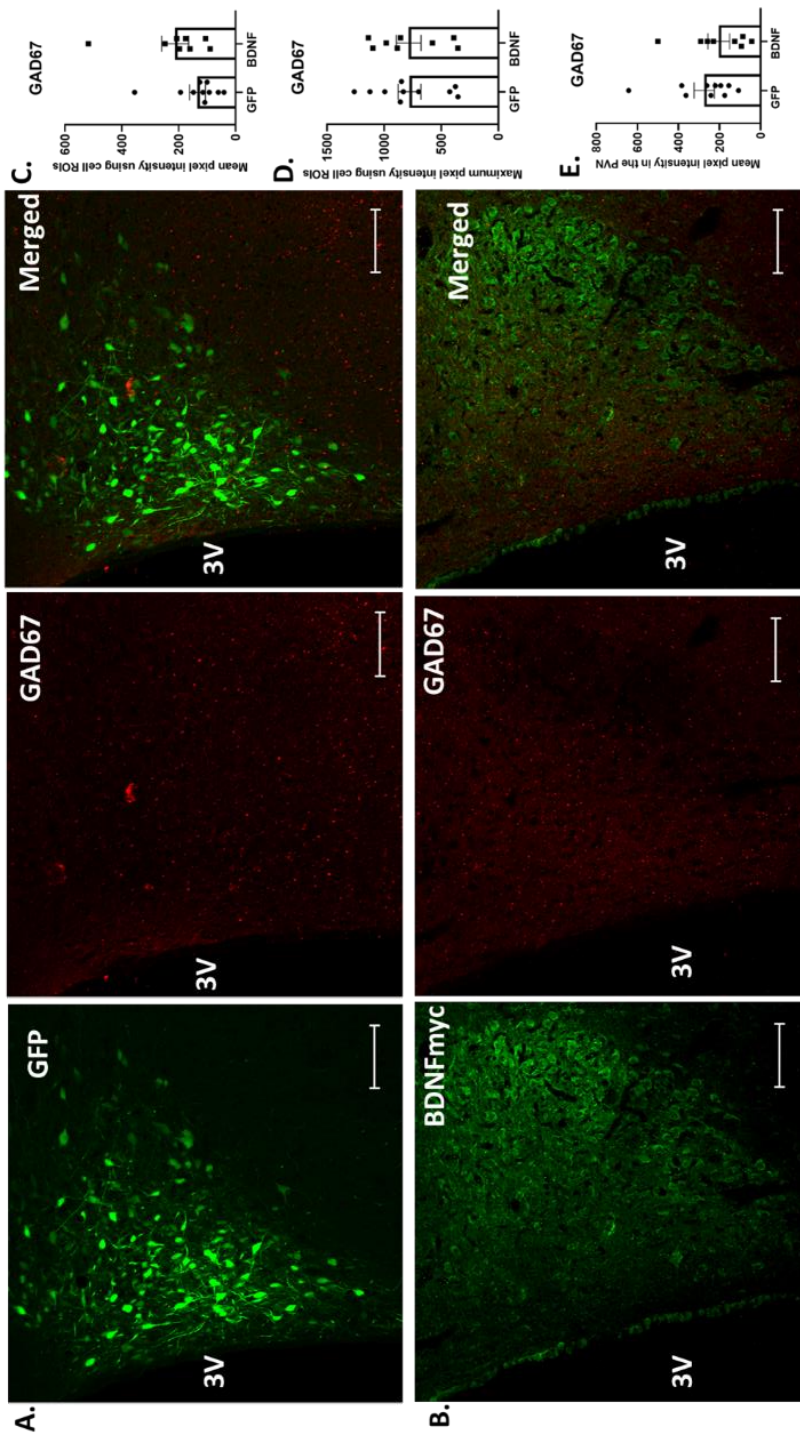


Figure 19. GAD67 fluorescent expression in the PVN of rats previously injected with AAV2-GFP or AAV2-BDNFmyc in the PVN. A-B.

Representative confocal images of GAD67 expression within the PVN in GFP (A) and BDNFmyc (B) groups as detected with a GAD67 antibody and immunofluorescence; scale bars, 100 μ m. C-D. ROI mean (C) and ROI max (D) GAD67 in the PVN, averaged for each experimental group; n refers to number of PVN sides [GFP (n = 9) and BDNFmyc (n = 8)]. E: PVN mean GAD67 expression [GFP (n = 9) and BDNFmyc (n = 8)]. 3V, third ventricle; PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; AAV2, adeno-associated viral vector 2. Results represent means \pm SE.

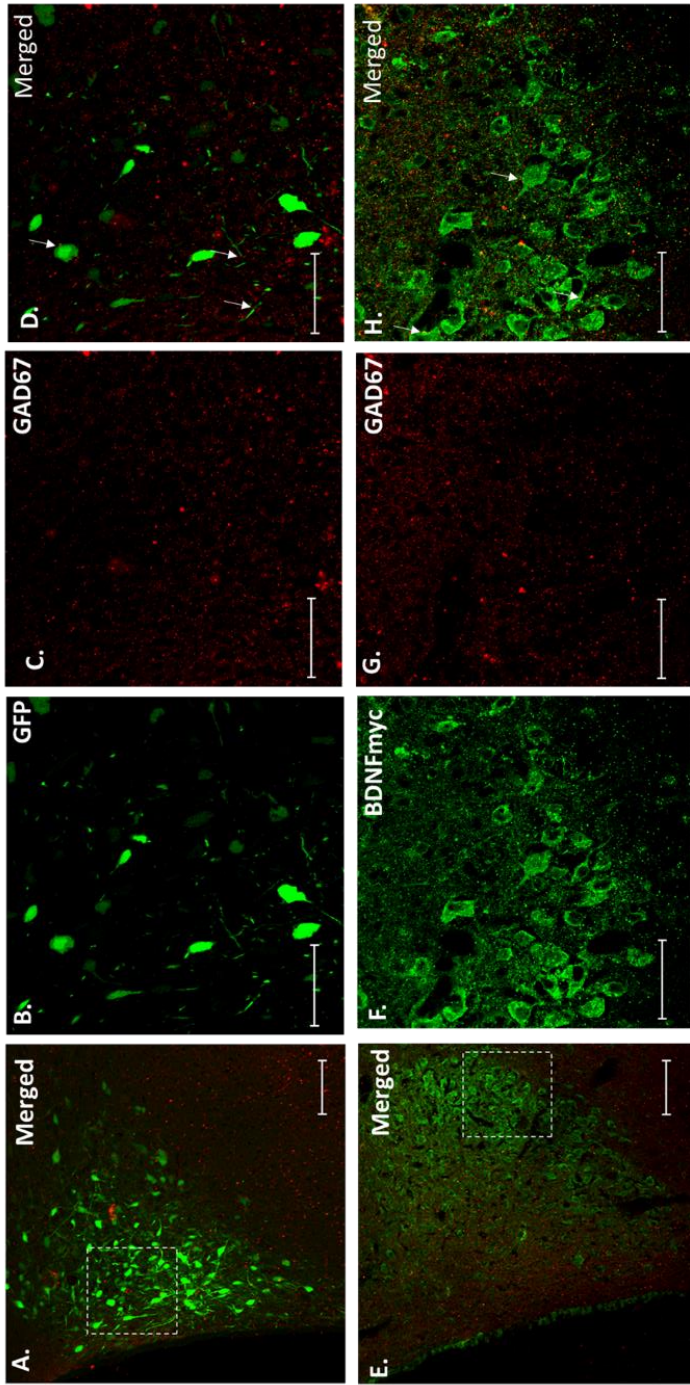


Figure 20. A: GAD67 fluorescent expression in the PVN of rats previously injected with AAV2-GFP, showing a rectangular area which is magnified in B-D, scale bar 100 μ M. B-D: Representative 60X magnification confocal images showing GAD67 and GFP expression in the PVN. Arrows show overlap of GAD67 staining with GFP infected cell bodies and fibers, scale bar 50 μ M. E: GAD67 fluorescent expression in the PVN of rats previously injected with AAV2-BDNFmyc, showing a rectangular area which is magnified in F-H, scale bar 100 μ M. F-H: Representative 60X magnification confocal images showing GAD67 and BDNFmyc expression in the PVN. Arrows show overlap of GAD67 staining with BDNFmyc infected cell bodies and fibers, scale bar 50 μ M.

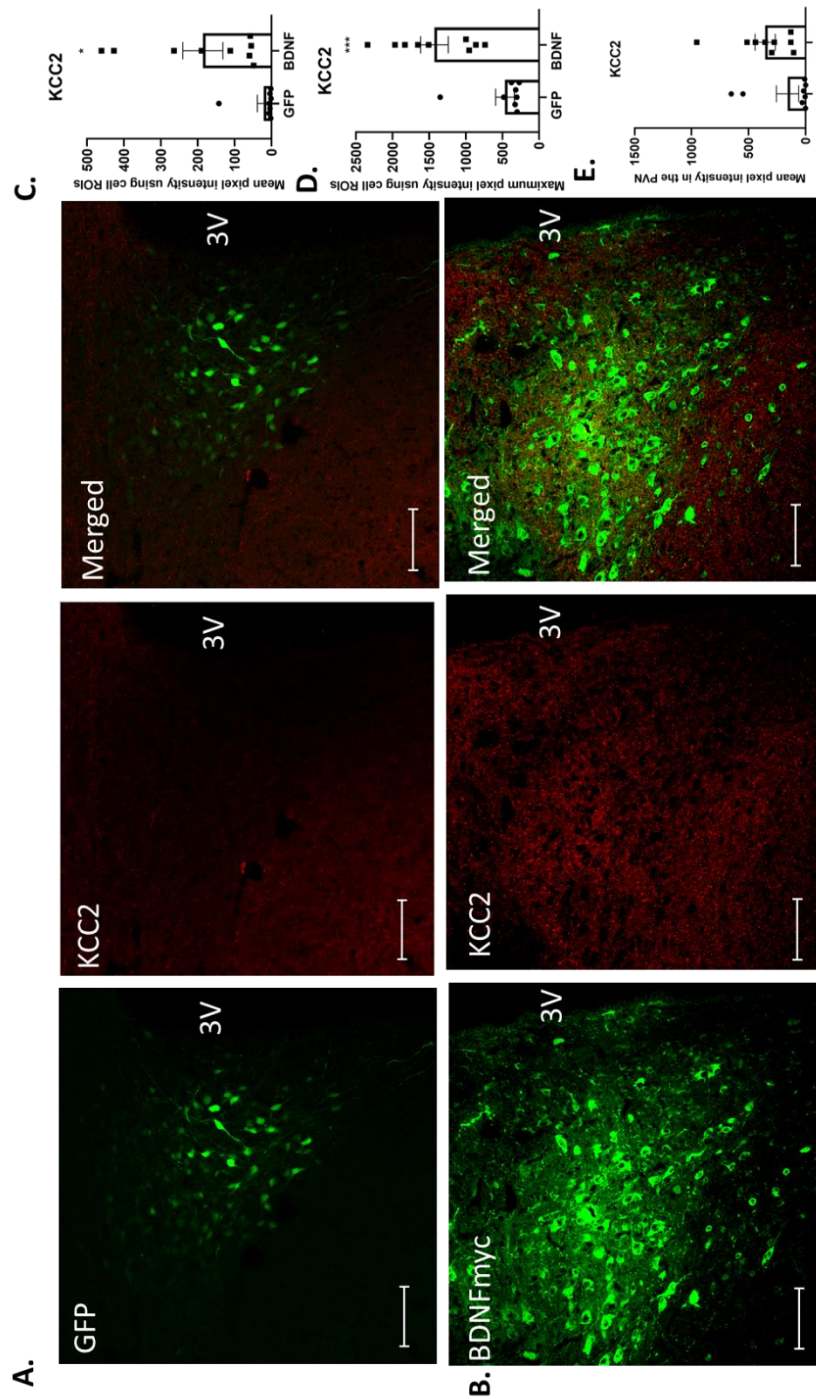


Figure 21. KCC2 fluorescent expression in the PVN of rats previously injected with AAV2-GFP or AAV2-BDNFmyc in the PVN. A-B. Representative confocal images of KCC2 expression within the PVN in GFP (A) and BDNFmyc (B) groups as detected with a KCC2 antibody and immunofluorescence; scale bars, 100 μm. C-D. ROI mean (C) and ROI max (D) KCC2 in the PVN, averaged for each experimental group; n refers to number of PVN sides [GFP (n = 8) and BDNFmyc (n = 9)]. E: PVN mean KCC2 expression [GFP (n = 8) and BDNFmyc (n = 9)]. 3V, third ventricle; PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; AAV2, adeno-associated viral vector 2. Results represent means ± SE. *P < 0.05 for GFP vs. BDNFmyc; ***P < 0.001 for GFP vs. BDNFmyc (unpaired t test).

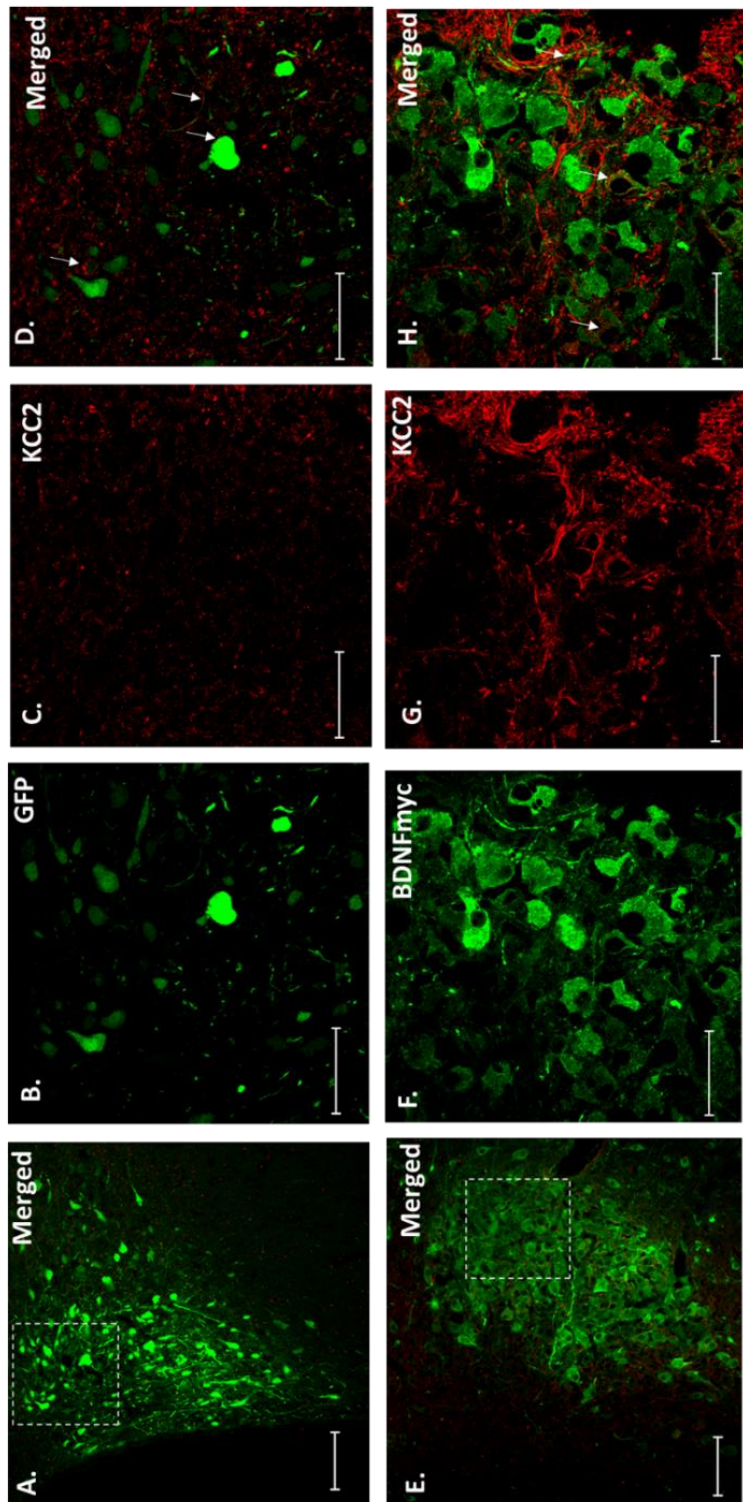


Figure 22. A: KCC2 fluorescent expression in the PVN of rats previously injected with AAV2-GFP, showing a rectangular area which is magnified in B-D, scale bar 100 μM. B-D: Representative 60X magnification confocal images showing KCC2 and GFP expression in the PVN. Arrows show overlap of KCC2 staining with GFP infected cell bodies and fibers, scale bar 50 μM. E: KCC2 fluorescent expression in the PVN of rats previously injected with AAV2-BDNFmyc, showing a rectangular area which is magnified in F-H, scale bar 100 μM. F-H: Representative 60X magnification confocal images showing KCC2 and BDNFmyc expression in the PVN. Arrows show overlap of KCC2 staining with BDNFmyc infected cell bodies and fibers, scale bar 50 μM.

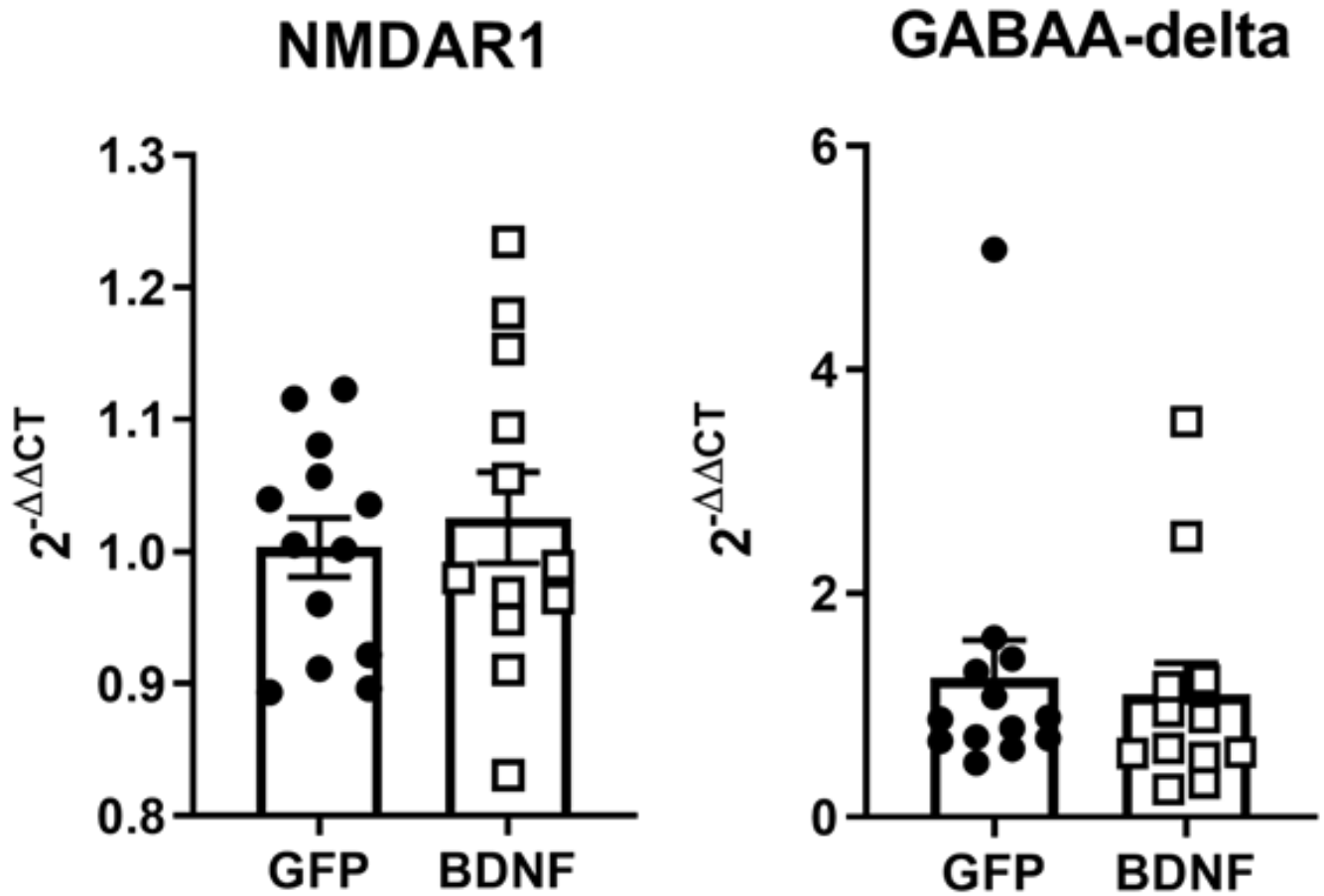


Figure 23. Expression of NMDAR1 and GABAA-delta receptor mRNA in the PVN of rats previously injected with AAV2-GFP (n = 6) or AAV2-BDNFmyc (n = 7) in the PVN. A: Expression of NMDAR1 mRNA in the PVN of AAV2-GFP (n = 12 sides of PVN) and AAV2-BDNFmyc (n = 13 sides of PVN) rats. B: Expression of GABAA-delta receptor mRNAs in the PVN of AAV2-GFP (n = 13 sides of PVN) and AAV2-BDNFmyc (n = 12 sides of PVN) rats. PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; BDNFmyc, myc epitope-tagged BDNF; AAV2, adeno-associated viral vector 2. Results are expressed as $2^{-\Delta\Delta CT}$ (where CT is threshold cycle) normalized to the control group and presented as means \pm SE.

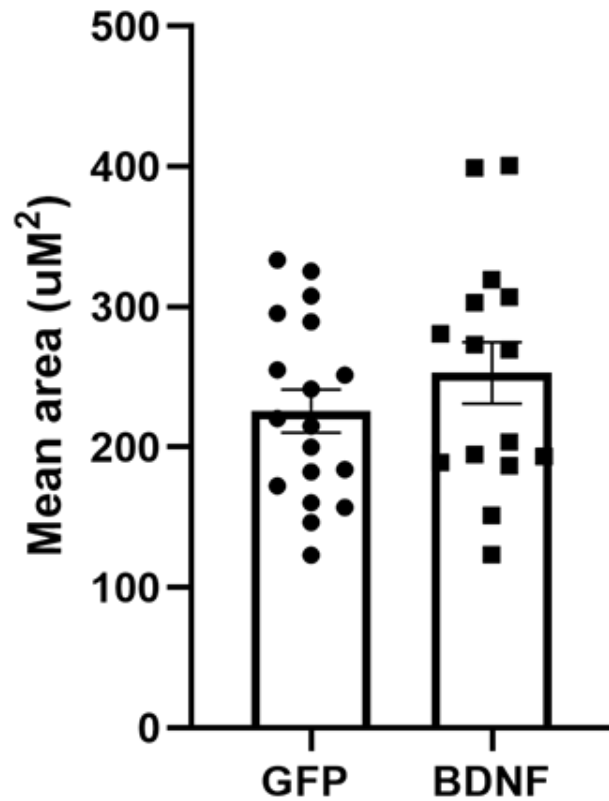


Figure 24. Mean area of neuronal soma of AAV2-GFP (n=18 sides of PVN) and AAV2-BDNFmyc (n=15 sides of PVN) infected cells in the PVN. PVN, paraventricular nucleus; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor; BDNFmyc, myc epitope-tagged BDNF; AAV2, adeno-associated viral vector 2.

3.4. Discussion

BDNF is upregulated in the PVN in response to hypertensive stimuli such as chronic and acute stress, hyperosmolality, and repeated amphetamine administration (Aliaga et al., 2002; Hammack et al., 2009; Meredith et al., 2002; Rage et al., 2002; Smith, Makino, Kim, et al., 1995). When acutely injected or overexpressed in the PVN, BDNF also significantly increases blood pressure and heart rate (Erdos, Backes, et al., 2015; C. L. Schaich et al., 2018; Chris L. Schaich et al., 2016). BDNF has been shown to alter both excitatory and inhibitory neurotransmitter signaling in the CNS (Bolton et al., 2000; Cohen-Cory et al., 2010; Frerking et al., 1998; B. Lu et al., 2005; Minichiello, 2009; Waterhouse & Xu, 2009). Similar cellular mechanisms may contribute to increasing blood pressure by shifting the balance of excitatory and inhibitory synaptic balance in the PVN. A shift in PVN excitatory/inhibitory balance towards excitation has been demonstrated to elevate blood pressure and sympathetic activity in various hypertensive models including in rats subjected to ICV infusion of Ang II (Sharma et al., 2021), in SHR_s (D.-P. Li & H.-L. Pan, 2007; D. P. Li & Pan, 2006), and in deoxycorticosterone acetate salt-sensitive hypertension (Basting et al., 2018). Similar effects have also been observed in salt-sensitive hypertension where PVN microinjections of kynurenate, an excitatory amino acid antagonist, have been shown to significantly decrease both MAP and heart rate in hypertensive Dahl S rats on high salt diets compared to normotensive controls (Gabor & Leenen, 2012a). Additionally, in Ang II-induced hypertension, a 2 week administration of a sub-pressor dose of Ang II results in an increase in plasma membrane NMDAR1 in dendrites of PVN neurons in mice, while

PVN NMDAR1 deletion results in a significant attenuation of Ang II-induced elevations in blood pressure (Glass et al., 2015).

Previous studies have shown that PVN microinjections of AP5 in WKY and SHRs result in a larger decrease in blood pressure in SHRs compared to WKY (Q. H. Chen et al., 2003; D.-P. Li & H.-L. Pan, 2007; Y. F. Li et al., 2006). Meanwhile, a PVN microinjection of L-glutamate or NMDA have typically been found to increase blood pressure, and sympathetic nerve activity in both anesthetized and conscious normotensive rats (Busnardo et al., 2010; Kannan et al., 1989; Y. F. Li et al., 2006; Y. F. Li et al., 2001; Douglas S. Martin & Haywood, 1992). There is some disagreement in the literature, where studies have shown that PVN L-glutamate injections in anesthetized SD rats can lead to no changes in blood pressure and a decrease in heart rate (Darlington, Miyamoto, Keil, & Dallman, 1989), and in anesthetized Wistar rats a decrease in blood pressure with no change in heart rate (Katafuchi, Oomura, & Kurosawa, 1988). There is limited literature on the effect of an NMDA agonist on blood pressure in hypertensive rats. NMDA PVN injections in sedentary heart failure rats has been shown to produce a larger increase in blood pressure compared to sham sedentary rats (Kleiber, Zheng, Schultz, Peuler, & Patel, 2008). There is also a greater increase in renal sympathetic nerve activity in response to NMDA injected into the PVN in heart failure rats compared to sham rats (Y. F. Li et al., 2003). We found that inhibition of NMDA-receptors with AP5 significantly lowered MAP and heart rate in BDNF rats compared to the GFP control group. However, activation of NMDA-receptors did not significantly alter MAP but did significantly lower heart rate responses in the BDNF group compared to the control GFP

group. Our AP5 results agree with the broader literature while our NMDA results agree with parts of the literature that show similar effects on blood pressure and heart rate using L-glutamate injections (Darlington et al., 1989). These discrepancies could be in part attributed to the use of different anesthetics and possibly diffusion of the injected pharmacological agent outside of the PVN or activation of functionally different subpopulations of neurons both within and outside the PVN (Darlington et al., 1989). Activation of different subpopulations of the PVN has been demonstrated in studies where different subregions of the PVN are electrically stimulated leading to opposite responses in blood pressure (Porter & Brody, 1985). This may suggest that there is a potential explanation for why different studies report different effects on blood pressure and heart rate, which may have more to do with the injection site of a pharmacological agent or site of electrical stimulation.

The results from this experiment indicate that an increase in NMDA-mediated signaling within the PVN in response to the overexpression of BDNF may be due to an increase in presynaptic release of glutamate and an increase in glutamatergic innervation from higher order brain areas. Pre-synaptically, BDNF is known to increase glutamate release leading to an increase in the frequency of mEPSCs in the hippocampus (Lessmann & Heumann, 1998; Takei et al., 1998). Post-synaptically, BDNF augments NMDA receptor single channel opening probability (Levine et al., 1998; Levine & Kolb, 2000; Lin et al., 1998; Suen et al., 1997). In SHRs, the basal frequency of mEPSCs of PVN presympathetic neurons is significantly increased compared to normotensive controls (Qiao, Zhou, Li, & Pan, 2017; Ye et al., 2011; Ye, Li, Li, & Pan, 2012).

Furthermore, inhibiting NMDA receptors eliminates the increased frequency of mEPSCs of PVN presympathetic neurons in the SHR only, but not in normotensive rats (D. P. Li, Zhou, Zhang, & Pan, 2017; Shulga et al., 2008). In this study we examined NMDAR1 expression in the PVN by quantifying both protein expression and mRNA expression. NMDAR1 ROI mean, and ROI max was significantly higher in the BDNF group compared to the GFP group. PVN mean was also significantly elevated in the BDNF group compared to the GFP group. However, BDNF overexpression in the PVN did not significantly upregulate NMDAR1 mRNA expression. This discrepancy between the protein results and the mRNA results for the NMDAR1 expression may be due to some key differences between quantifying mRNA and protein expression. Firstly, the RT-PCR PVN punch we used contains the whole PVN and may contain some hypothalamic tissue outside of the PVN, whereas the protein analysis used 1 PVN brain slice to quantify receptor expression where BDNF^{myc} or GFP expression was also high in the PVN. Secondly, the PVN is a very heterogenous structure, known to change morphologically on the caudal-rostral axis and in terms of the different cell types expressed throughout the extent of the PVN (Stern, 2001). Thirdly, post-transcriptional regulation, and differences in mRNA and protein turnover rates could explain some of these differences (N. Fu et al., 2007).

Another important component of neurotransmitter signaling in the CNS involves synapsins which are phosphoproteins found at most synaptic terminals. They regulate the maintenance of the reserve vesicle pool, while also recruiting to the vesicle pool and increasing the release probability of synaptic vesicles to the readily releasable pool

(Khvotchev & Sun, 2009). Deletion of synapsin genes has been shown to reduce the presynaptic response to BDNF, which may indicate that synapsins play a role in BDNF-mediated increase of glutamate release (Jovanovic et al., 2000; Kao et al., 2017). A link between chronic salt loading and increases in synapsin IIa and IIb transcripts in the magnocellular division of the PVN compared to control animals (Nomura et al., 2000) has also been observed. In this study, synapsin 1a/b ROI mean, ROI max, and PVN mean were not significantly different between the GFP and BDNF groups. This may indicate that other synapsins play a greater role in BDNF-mediated hypertensive mechanisms such as synaptophysin, synaptobrevin and synaptotagmin, all of which have also been shown to be upregulated in response to BDNF (Tartaglia et al., 2001).

In terms of the effect of BDNF on inhibitory signaling in the PVN, we found that inhibition of GABAA receptors with gabazine significantly elevated MAP in the GFP group compared to the BDNF group. Conversely, activation of GABAA receptors with muscimol significantly decreased MAP in the GFP group compared to the BDNF group. These results are similar to what has been reported in WKY and SHRs, where PVN injections of the GABAA receptor agonists isoguvacine or muscimol have been found to reduce blood pressure more significantly in WKY and SD rats compared to SHRs (Ding et al., 2015; D. P. Li & H. L. Pan, 2007). Meanwhile PVN microinjections of the GABAA antagonist, gabazine or bicuculline, have been found to increase blood pressure significantly more in WKY and SD rats compared to SHR (Ding et al., 2015; D. P. Li & Pan, 2006; D. P. Li & H. L. Pan, 2007; Y. F. Li et al., 2006), which is also consistent with our results. The results from this experiment suggest that a decrease in GABAA-

mediated signaling within the PVN in response to the overexpression of BDNF may be due to a decrease in both presynaptic and postsynaptic GABA_A signaling mechanisms, such as a decrease in GABA release, a decrease in GABAergic innervation of the PVN, or a decrease in GABA_A receptor number.

The effect of BDNF on GABAergic signaling is well-documented. Acute BDNF application in the hippocampus has been shown to reduce evoked and spontaneous GABAergic currents via the TrkB receptor (Marty et al., 2000; Rutherford, DeWan, Lauer, & Turrigiano, 1997), and facilitate the early phase of LTP (Figurov, Pozzo-Miller, Olafsson, Wang, & Lu, 1996; Kang & Schuman, 1995; Patterson et al., 1996). Hypertensive conditions are also known to impair normal GABAergic inhibition of PVN presympathetic neurons. GABA_A receptor-binding sites are reduced in the PVN in SHR (Kunkler & Hwang, 1995), as is the frequency of GABAergic IPSCs of presympathetic neurons in the PVN (D. P. Li & Pan, 2006; Ye, Li, Byun, Li, & Pan, 2012). Additionally, GABA_A receptor $\alpha 5$ subunit expression within the PVN is reduced in SHR compared to control Wistar rats (Cork et al., 2016). This may be due to reduced presynaptic GABA release, decreased GABA_A receptor number or function, or the loss of GABAergic neurons in hypertension. There is also a reduction in GABA_A-mediated PVN inhibition in rats with congestive heart failure which contributes to increased sympathetic outflow (Carillo et al., 2012; K. Zhang et al., 2002).

In this study we quantified GABA_A- $\alpha 1$ and GABA_A- δ subunit expression in the PVN using protein quantification methods and RT-PCR. GABA_A- $\alpha 1$ ROI max was found to be significantly elevated in the GFP compared to the BDNF group, but ROI

mean was not. The maximum fluorescence measurement is more likely to capture differences in the higher end of fluorescence intensity, while mean fluorescence provides an average of the gradient of fluorescence intensity. This elevation of ROI max in the GFP group compared to the BDNF group could indicate that the difference in fluorescence intensity is such that the GFP group has a greater distribution of fluorescence intensity at the higher end of the scale compared to the BDNF group. GABAA-alpha1 expression was also observed to be consistently lower inside the PVN as compared to outside the PVN, in the peri-PVN area, as can be seen in the representative PVN images from both GFP and BDNFmyc injected animals (**Fig. 16.**). This is consistent with the literature, which shows that GABA interneurons are highly expressed in the peri-PVN area, the anterior hypothalamic area and the perifornical regions, while GABA neurons in the PVN are less common (Alastair V. Ferguson et al., 2008). Thus, the effect of BDNF overexpression may be more pronounced when comparing the peri-PVN vs the PVN both within and between the BDNF and GFP groups. We devised a method to quantify GABAA-alpha1 expression both in the medial parvocellular portion of the PVN and the peri-PVN. We found that GABAA-alpha1 expression in the PVN in the BDNF group compared to the peri-PVN in the BDNF group was significantly lower, GABAA-alpha1 expression was also lower in the peri-PVN of the BDNF group compared to the peri-PVN of the GFP group. BDNF may be acting on neighboring neurons outside the PVN to alter the signaling properties of both PVN and peri-PVN neurons. BDNF injections into the PVN may affect neurons that project into and out of the PVN, which could alter GABAA subunit expression and signaling both inside and outside the PVN. The expression of GABAA-alpha1 subunit has been found to be similar in the PVN of

renal-wrapped and sham-operated rats (Haywood et al., 2001), but BDNF activation of TrkB receptors is known to mediate the sequestration of membrane GABAA alpha-1 subunits via differential phosphorylation pathways in the hippocampus and amygdala (Mou, Heldt, & Ressler, 2011). Additionally, BDNF has been shown to decrease GABAA-alpha1 expression associated with epilepsy (Lund et al., 2008), and in the hippocampus (Roberts et al., 2006).

We also quantified the expression of GABAA-delta subunit and found that BDNF did not significantly alter its expression in the PVN compared to the GFP group. The GABAA-delta subunit is localized exclusively to extrasynaptic or perisynaptic sites, and contributes to tonic inhibition in the CNS (Glykys, Mann, & Mody, 2008; Nusser & Mody, 2002; Nusser, Sieghart, & Somogyi, 1998; Wei, Zhang, Peng, Houser, & Mody, 2003). It is also present in the PVN (Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000), where it tonically inhibits PVN presympathetic neurons (J. B. Park et al., 2009; Jin Bong Park, Skalska, Son, & Stern, 2007). GABAA delta subunit also regulates the activity of CRH neurons in the PVN (V. Lee, Sarkar, & Maguire, 2014; Sarkar, Wakefield, MacKenzie, Moss, & Maguire, 2011), and chronic stress has been shown to significantly reduce the mRNA expression of GABAA delta containing receptors in the PVN (Verkuyl, Hemby, & Joëls, 2004). BDNF's effect on GABAA delta function or expression has only been minimally studied. BDNF has been shown to reduce the buildup of the GABAA delta subunit in the cytoplasmic compartment and increase its surface expression in hippocampal slices (Joshi & Kapur, 2009). Meanwhile infusion of the neurosteroid allopregnanolone, a GABAA receptor-positive allosteric modulator, into the

prefrontal cortex of rats, increased the mRNA expression of the GABAA delta subunit in the prefrontal cortex, while also elevating the expression of BDNF in the hippocampus (Almeida, Gomez, Barros, & Nin, 2019)

To further determine the effect of BDNF on GABAergic neurotransmission in the PVN, we examined the expression of GAD67, which has been found to be elevated in renovascular hypertensive rats compared to normotensive control animals (Biancardi et al., 2010). BDNF release has also been shown to be necessary for activity-dependent upregulation of both GAD65 and GAD67, and further GAD expression is reduced by the application of the TrkB inhibitor, K252a (Hanno-Iijima et al., 2015). We found that the expression of GAD67 in the PVN was not significantly different on any of the parameters measured between the BDNF and GFP groups. This may indicate that GAD65 could play a more significant role in this mechanism since BDNF-TrkB signaling also regulates GAD65 expression (Hanno-Iijima et al., 2015; Sanchez-Huertas & Rico, 2011). Another mechanism might involve GATs, since BDNF is known to elevate presynaptic concentration of GABA via the activation of GAT-1 (Vaz et al., 2011), which also increases GABA transport in astrocytes and neurons. GATs are found in both neurons and astrocytes and are responsible for terminating GABA transmission (Vaz et al., 2011).

BDNF has also been shown to interact with KCC2 expression and signaling. KCC2 serves to establish the chloride ion gradient in neurons via the maintenance of low intracellular chloride concentration (Tang, 2020). BDNF signaling has been shown to increase KCC2 expression in immature neurons and decrease it in adult neurons (Aguado et al., 2003; Boulenguez et al., 2010; A. Ludwig et al., 2011; Claudio Rivera et al., 2002;

Claudio Rivera et al., 2004; Shulga et al., 2008; Wake et al., 2007). The expression of KCC2 is significantly decreased in TrkB double KO mice hippocampi (Carmona et al., 2006), and in adult neurons BDNF has been found to decrease both mRNA and protein KCC2 (Boulenguez et al., 2010; Claudio Rivera et al., 2002; Claudio Rivera et al., 2004; Shulga et al., 2008; Wake et al., 2007). BDNF-TrkB-KCC2-mediated signaling is also implicated in salt-sensitive hypertension whereby this mechanism weakens baroreceptor inhibition of vasopressin magnocellular cells in the SON through a downregulation of KCC2 by BDNF (Choe et al., 2015). KCC2 ROI mean, and ROI max were significantly higher in the BDNF group compared to the GFP group. This elevation of KCC2 in the BDNF group could be a compensatory response to the potential downregulation of KCC2 in the SON reported in the literature in response to elevated blood pressure. Another potential mechanism may involve KCC2 being upregulated in response to elevated mGluR activity, which has been shown to regulate inhibitory synaptic strength in pyramidal neurons in the CA3 area of the hippocampus (Banke & Gegelashvili, 2008; Chorin et al., 2011; H. H. Lee et al., 2007). mGluR activity in the PVN is known to be elevated in SHRs compared to WKY (D. P. Li & Pan, 2010), and further activation of mGluRs increases BDNF mRNA and protein expression in C6 glioma cells (Vivatpinyo & Chongthammakun, 2009).

Finally, BDNF is known to serve as an important mediator of neuronal plasticity, and functions as an activity-dependent modulator of neuronal structure, function and signaling broadly in the CNS (Bramham & Messaoudi, 2005). BDNF has been reported to increase neuronal soma size of striatal neurons (Yu et al., 2018), cortical and

hippocampal interneurons (Rutherford et al., 1998) and GABAergic neurons in the hippocampus (M. K. Yamada et al., 2002). Thus, we quantified the mean soma size of GFP and BDNFmyc infected neurons in previously injected AAV2-GFP and AAV2-BDNFmyc rats. However, the average neuronal soma size of GFP and BDNFmyc cells was not statistically different.

3.5. Conclusion

In summary, our studies demonstrate that BDNF overexpression in the PVN elevates NMDA-mediated glutamatergic signaling and diminishes GABAA-mediated GABAergic signaling contributing to the chronic hypertensive effects of BDNF. Specifically, we found that inhibition of NMDA-receptors significantly lowered MAP in BDNF rats compared to the control group, while activation of NMDA-receptors did not have a significant effect on MAP but did significantly reduce heart rate in the BDNF group. However, BDNF did not significantly alter NMDAR1 mRNA expression in the PVN but did significantly increase NMDAR1 protein expression in the PVN in the BDNF group.

We also found that inhibition of GABAA receptors significantly elevated MAP in the GFP group compared to the BDNF group, and activation of GABAA receptors significantly decreased MAP in the GFP group compared to the BDNF group. BDNF did not significantly alter GABAA-delta subunit mRNA expression but did significantly decrease GABAA-alpha1 protein expression in the PVN. However, GAD67 and synapsin 1a/b protein expressions were not significantly different between the two groups while KCC2 protein expression was found to be significantly elevated in the BDNF group compared with the GFP group. These BDNF-mediated mechanisms may synergistically

contribute to elevations in blood pressure and play a significant role in the development of related cardiovascular diseases such as hypertension.

CHAPTER 4: SIGNIFICANCE OF FINDINGS

4.1. Hypertension and Sympathetic Cardiovascular Regulation

Hypertension is one of the most significant risk factors for the development of cardiovascular diseases such as heart disease, stroke and heart failure ("Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017," 2018). It is also a very prevalent medical condition affecting approximately 45.6% of US adults. Additionally, of those taking antihypertensive medication, 53.4% have reported blood pressure above treatment goal (Muntner et al., 2018). Lifestyle factors such as high stress (Hamer et al., 2008; McEwen & Gianaros, 2010), high salt intake (Denton et al., 1995; F. J. He & MacGregor, 2002; "Intersalt: an international study of electrolyte excretion and blood pressure. Results for 24 hour urinary sodium and potassium excretion. Intersalt Cooperative Research Group," 1988) and obesity (Kannel et al., 1967) are known to be common risk factors for the development of hypertension. The high rate of uncontrolled hypertension remains a problem, demonstrating the need for more targeted treatments. Several pharmacological and non-pharmacological treatments available today target the heart or vasculature (Aronow, 2018). However, only a few treatments target the underlying mechanisms behind the development of hypertension, most significantly elevated sympathetic activity (Q. Fu et al., 2005), which is a key contributor to most cases of

hypertension (Rahn et al., 1999; Schlaich et al., 2004; Wyss, 1993). Several interventions targeting sympathetic activity have shown limited success in clinical trials, such as catheter-based renal endovascular approaches (Azizi et al., 2018; Desch et al., 2015; Mahfoud et al., 2017), and baroreflex activation therapy (de Leeuw et al., 2017; Wustmann et al., 2009).

Blood pressure regulation can be altered by changes in the neuronal activity in hypothalamic and brainstem nuclei during baseline and stress conditions. RVLM activation increases sympathetic activity and blood pressure while inhibition leads to a decrease in sympathetic activity and blood pressure (R. A. Dampney, 1994). Meanwhile, selective lesioning of NTS catecholaminergic neurons in rats leads to an increase in blood pressure in some cases (Daubert et al., 2012; Duale et al., 2007; Itoh & Bunag, 1993), while other studies have shown no significant effect on baseline blood pressure (Itoh et al., 1992; Itoh & Bunag, 1992; Talman et al., 1980). Activation of PVN projections to other hypothalamic areas and other cardiovascular regulatory nuclei tend to increase blood pressure (R. A. Dampney, 1994; Pyner & Coote, 2000). Specifically lesioning parvocellular PVN neurons has also been shown to reduce renal sympathetic nerve activity (Haselton, Goering, & Patel, 1994; Lovick, Malpas, & Mahony, 1993). Further, an elevation of excitatory signaling and a reduction of inhibitory signaling mechanisms in the PVN are thought to contribute to enhancing sympathetic output in hypertension (Boudaba et al., 1997; Q. H. Chen et al., 2003; Y. F. Li et al., 2006). Hypertensive stimuli such as chronic stress increases the excitability of PVN neurons by elevating the expression of NMDA receptors (Franco et al., 2016) and reducing the expression of GABAA receptors (Cullinan, 2000;

Verkuyt et al., 2004). Studies have also shown that the expression of NMDA receptor subunits can be modified by changes in blood pressure (Coleman et al., 2010; Glass et al., 2015; D. P. Li, H. S. Byan, & H. L. Pan, 2012; Y. F. Li et al., 2003; Marques-Lopes et al., 2014), and further that GABAA-mediated inhibition of the PVN is reduced in hypertension and heart failure (D.-P. Li & H.-L. Pan, 2007; D. S. Martin & Haywood, 1998; K. Zhang et al., 2002).

In our first study, we showed how BDNF overexpression in the PVN and lesioning of catecholaminergic NTS neurons with DSAP alters sympathetic cardiovascular regulation. Similar to what has been seen previously in the literature (Daubert et al., 2012), blood pressure was elevated following BDNF overexpression in the PVN and DSAP lesioning of the NTS. Lesioning NTS catecholaminergic neurons was also found to increase blood pressure in GFP control rats but did not further elevate blood pressure following BDNF overexpression in the PVN. Additionally, we found that a PVN microinjection of the general beta-adrenergic agonist isoprenaline decreased blood pressure more significantly in the GFP control group compared to the BDNF overexpressed group, and further that beta-1 adrenergic receptor mRNA in the PVN was downregulated in the BDNF group compared to the GFP group. This indicates that BDNF may be diminishing the sensitivity of the inhibitory catecholaminergic NTS-PVN projections through downregulating beta-1 adrenergic receptors in the PVN.

In our second study, we examined the effect of overexpressing BDNF in the PVN on excitatory NMDA-mediated and inhibitory GABAA-mediated signaling. We found that BDNF augmented NMDA-receptor signaling and reduced GABAA-receptor signaling in

the PVN to elevate blood pressure. Specifically, BDNF overexpression reduced the elevation of blood pressure in response to a PVN injection of the GABAA antagonist gabazine compared to the control group, while also reducing the decrease of blood pressure in response to a PVN injection of the GABAA agonist muscimol. BDNF overexpression in the PVN augmented the reduction in blood pressure in response to the NMDA receptor antagonist AP5 compared to the GFP group but had no effect on blood pressure when NMDA was injected into the PVN. The literature shows that AP5 microinjections into the PVN significantly decrease blood pressure in SHR rats compared to WKY rats (D.-P. Li & H.-L. Pan, 2007; Y. F. Li et al., 2006). GABAA agonist, isoguvacine or muscimol, microinjections into the PVN also reduce blood pressure more significantly in WKY and SD rats compared to SHR rats (Ding et al., 2015; D. P. Li & H. L. Pan, 2007). Meanwhile PVN microinjections of the GABAA antagonist, gabazine or bicuculline, have been found to increase blood pressure significantly more in WKY and SD rats compared to SHR rats (Ding et al., 2015; D. P. Li & Pan, 2006; D. P. Li & H. L. Pan, 2007; Y. F. Li et al., 2006).

In terms of protein and mRNA expression of excitatory and inhibitory signaling components the results were mixed. NMDAR1 ROI max and ROI mean was higher in the BDNF group compared to the GFP group. NMDAR1 subunit mRNA expression and protein level in the PVN have been found to be significantly increased in heart failure compared with sham rats (Y. F. Li et al., 2003), and further administration of a sub-pressor dose of Ang II elevates the plasma membrane expression of NMDAR1 in dendrites of PVN neurons (Glass et al., 2015). Expression of synapsin 1 a/b, a common synaptic marker, was also assessed and was not found to significantly differ between the GFP and BDNF groups.

A link between chronic salt loading and elevations in synapsin IIa and IIb transcripts in the magnocellular division of the PVN compared to control animals has been shown (Nomura et al., 2000), but the role of synapsin 1 a/b has not been investigated in this context.

In terms of inhibitory signaling in the PVN, GABAA-alpha1 ROI max but not ROI mean was elevated in the GFP compared to the BDNF group. Additionally, GABAA-alpha1 expression in the PVN in the BDNF group compared to the peri-PVN was significantly lower while GABAA-alpha1 expression in the peri-PVN of the BDNF group was significantly lower compared to the peri-PVN of the GFP group. In the literature, GABAA-alpha1 expression has been found to be similar in the PVN of renal-wrapped hypertension and sham rats (Haywood et al., 2001), while GABAA-alpha5 subunit has been shown to be significantly decreased in the PVN of SHR rats compared to WKY rats (Cork et al., 2016). Meanwhile protein expression of GAD67, a commonly used marker of GABAergic activity, was found to be similar between the two groups. In renovascular hypertensive rats, GAD67 expression has been reported to be elevated in the PVN compared to normotensive control animals (Biancardi et al., 2010). Finally, the mRNA expression of NMDAR1 and GABAA-delta subunit were unaffected by BDNF overexpression.

4.2. The Importance of BDNF Signaling

BDNF is a neurotrophin crucially involved in mediating neuroplasticity mechanisms such as neuronal growth, differentiation, development and synaptic strengthening during development and in adult neurons (E. J. Huang & Reichardt, 2001). BDNF also plays a key role in learning and memory mechanisms (Alonso et al., 2002; Y.

Lu et al., 2011; Radiske et al., 2015), and the facilitation of LTP (Y. Lu et al., 2011; Patterson et al., 1996). BDNF's role in stress and cardiovascular system regulation in the PVN has also been demonstrated previously. BDNF mRNA and protein levels in the PVN have been shown to be upregulated following acute and chronic stress, hyperosmolality, and repeated amphetamine administration (Aliaga et al., 2002; Hammack et al., 2009; Meredith et al., 2002; Rage et al., 2002; Smith, Makino, Kim, et al., 1995). We (Erdoş, Backes, et al., 2015; Chris L. Schaich et al., 2016) have also shown that both long-term overexpression and acute microinjection of BDNF into the PVN leads to significant increases in blood pressure, and heart rate. Furthermore, we (C. L. Schaich et al., 2018) have found that inhibition of BDNF signaling by blocking the TrkB receptor in the PVN reduces acute stress-induced blood pressure elevations without altering baseline cardiovascular parameters.

BDNF may alter central cardiovascular regulation through catecholaminergic signaling, since BDNF treated rats show a dose-dependent increase in both dopamine content, uptake activity and TH-72 positive neurons (C. Hyman et al., 1994). BDNF could also affect central cardiovascular regulation via altering NMDA and GABA_A-mediated signaling. BDNF can enhance NMDA-mediated signaling by activating the membrane trafficking, expression (Caldeira et al., 2007; Kim et al., 2012; Kolb et al., 2005), and phosphorylation (Black, 1999) of NMDA receptors to increase excitatory synaptic transmission. BDNF has also been shown to decrease the expression of GABA_A receptors, both membrane density and function in hypothalamic neurons (Carreno et al., 2011; Hewitt & Bains, 2006; Lund et al., 2008). In this study, we found that BDNF overexpression in

the PVN resulted in increased DBH and TH expression in the NTS leading to a reduced sensitivity to catecholaminergic projections from the NTS in the PVN. We showed that this occurs at least partly through a downregulation of beta-1 adrenergic receptor mRNA in the PVN by BDNF, which was also supported by a greater hypotensive response to the general beta-adrenergic receptor agonist isoprenaline into the PVN, which was seen in the GFP group but not the BDNF group.

We also found that BDNF upregulated NMDA-mediated signaling and downregulated GABAA-mediated signaling in the PVN. We found that inhibition of NMDA-receptors significantly lowered MAP in BDNF rats compared to the control group, while activation of NMDA-receptors did not have a significant effect on MAP but did significantly reduce heart rate in the BDNF group. However, BDNF did not significantly alter NMDAR1 mRNA expression in the PVN but did significantly increase NMDAR1 protein expression in the PVN in the BDNF group. We also found that inhibition of GABAA receptors significantly elevated MAP in the GFP group compared to the BDNF group, and activation of GABAA receptors significantly decreased MAP in the GFP group compared to the BDNF group. BDNF also reduced the protein expression of GABAA-alpha1 in the BDNF group compared to the GFP control group but did not alter the expression of GABAA-delta mRNA in the PVN.

These studies and others suggest that BDNF overexpression in the PVN could be a potential model for hypertension. Many pro-hypertensive stimuli, which are known risk factors for the development of hypertension and other cardiovascular disorders, are also associated with an upregulation of BDNF expression in the PVN. For example, chronic

stress has been shown to increase BDNF expression in the BNST (Hammack et al., 2009) and in the PVN (Hammack et al., 2009; Rage et al., 2002; Smith, Makino, Kim, et al., 1995). Elevated BDNF expression in the PVN has also been shown to decrease body weight and food intake (C. Wang et al., 2010). Changes in BDNF signaling in the PVN is also a contributing factor to the development of hypertension. We have shown that chronic overexpression of BDNF in the PVN leads to increased baseline blood pressure and heart rate (Erdos, Backes, et al., 2015; Chris L. Schaich et al., 2016), similar to what is seen in hypertension. BDNF-mediated hypertension could serve as a useful model for hypertension since it is easy to replicate in rodent models and is associated with a variety of pro-hypertensive factors. Increased sympathetic activity is a significant contributor to hypertension, and alterations in CNS neurocircuitry could be explained at least partly by an increase in BDNF expression and associated BDNF-mediated neuroplasticity effects on neuronal signaling in the PVN.

4.3. Potential Applications of Findings

BDNF overexpression in the PVN could serve as a useful animal model for BDNF-mediated hypertension, which has been demonstrated by the studies discussed here and by others in the literature (Erdos, Backes, et al., 2015; Chris L. Schaich et al., 2016). BDNF mRNA and protein expression is elevated in response to various hypertensive stimuli such as stress (Givalois et al., 2004; Hammack et al., 2009; Smith & Cizza, 1996; Smith, Makino, Kim, et al., 1995) and hyperosmolality (Aliaga et al., 2002), and thus could mimic certain cases of hypertension in humans. The mechanism behind BDNFs effect on cardiovascular regulation within the PVN remains undetermined. It will be important to

precisely determine this mechanism to better aid in the development of more targeted pharmaceutical or surgical interventions for the treatment of BDNF-mediated hypertension. Hypothalamic levels of BDNF can be increased in gene therapy, and has been shown to be a potential treatment for diabetes, obesity, and metabolic syndrome (Cao et al., 2009; McMurphy et al., 2019; Nagahara & Tuszynski, 2011). However, as seen in these studies, increasing hypothalamic BDNF expression will also increase blood pressure and heart rate, and therefore such a treatment might be associated with harmful cardiovascular side effects.

Given that BDNF overexpression in the PVN elevates blood pressure and heart rate chronically, potentially inhibiting the TrkB receptor may lower blood pressure and heart rate. A few TrkB antagonists have been used to study BDNF signaling in hypertension, such as the non-specific TrkB antagonist K252a and the highly selective TrkB antagonist [N2-2-2-oxoazepan-3-yl amino] carbonyl phenyl benzo (b)thiophene-2-carboamide (ANA-12). NTS injections of K252a lead to a significant decrease in blood pressure and heart rate in rats (Clark et al., 2011). Meanwhile, injections of BDNF into the NTS of congestive heart failure rats lead to a depressor effect while ANA-12 injections into the NTS result in a sympathoexcitatory response in sham rats (Becker et al., 2016). Inhibiting the TrkB receptors in the PVN by overexpressing the dominant-negative truncated TrkB receptor (TrkB.T1), which binds to the full-length TrkB receptor and inhibits its activity, in the PVN reduces acute stress-induced blood pressure elevations but does not affect resting blood pressure, food intake and body weight in normotensive animals (C. L. Schaich et al., 2018). Others have found that chemogenetic activation of TrkB-expressing

PVN neurons suppresses food intake, while inhibition of those same neurons increases food intake (An et al., 2020). The effect of a TrkB antagonist in the PVN of hypertensive animals has not been studied.

BDNF does not cross the blood brain barrier (Pardridge, Wu, & Sakane, 1998), but using a magnetically guided nanocarrier targeting BDNF would allow for blood brain barrier transmission. Such a drug carrier could deliver systemic BDNF or TrkB antagonists across the blood brain barrier (Kouhpanji & Stadler, 2020; Pilakka-Kanthikeel, Atluri, Sagar, Saxena, & Nair, 2013), thereby potentially reducing blood pressure in hypertensive patients. However, this technology has only been studied in small animal models so far. Some of the major clinical difficulties associated with moving to a human model would be reductions in magnetic field strength with greater target depth in the body, the necessity for driving a significant number of magnetic nanocarriers to get a significant therapeutic effect (Shapiro et al., 2015), as well as poor retention upon removal of the external magnetic field, leading to a reduction in drug uptake (D'Agata et al., 2017; Estelrich, Escribano, Queralt, & Busquets, 2015).

4.4. Experimental Limitations and Future Directions

In these studies, we examined the effects of BDNF on catecholaminergic, NMDA and GABAA-mediated signaling in the PVN. In agreement with previous papers, we found that MAP and heart rate were increased in response to BDNF overexpression in the PVN (Erdos, Backes, et al., 2015). We also found that BDNF diminished catecholaminergic activity via a downregulation of beta-1 adrenergic receptors in the PVN, which play an inhibitory role on PVN activity under normal physiological conditions (Saphier &

Feldman, 1991; Tsushima et al., 1994; D. Wang et al., 2013). We also found that BDNF overexpression in the PVN elevated NMDA-mediated signaling, while reducing GABAA-mediated signaling without altering mRNA expression of NMDAR1 or GABAA-delta subunit, but with increases in protein expression of NMDAR1 and KCC2 in the PVN and a reduction in protein expression of GABAA-alpha1 subunit. These results mostly agree with prior studies that have shown that BDNF enhances NMDA-mediated glutamatergic transmission (Lessmann, Gottmann, & Heumann, 1994; Lessmann & Heumann, 1998), and where BDNF reduced GABAergic terminals and GABA (Kohara et al., 2007) and GABA release in the visual cortex (Abidin, Eysel, Lessmann, & Mittmann, 2008). PVN overexpression of BDNF as a model for hypertension allows for the investigation of the role of BDNF mediated neuroplasticity mechanisms. However, this model is limited since it leads to a continuously high BDNF expression in the PVN, which is unlike what is thought to occur under acute stress or other pro-hypertensive conditions where BDNF expression changes are believed to be transient (Bland et al., 2005; Y. Lee, Duman, & Marek, 2006). It is also unknown if BDNF is upregulated in the PVN in other hypertension models such as in the SHR. SHRs have been reported to have a higher expression of BDNF in the motor cortex (Banoujaafar, Van Hoecke, Mossiat, & Marie, 2014) and in the NTS (Vermehren-Schmaedick et al., 2013) compared to WKY rats. Conversely, in the hippocampus SHRs exhibit lower levels of BDNF compared to WKY (Pietranera, Lima, Roig, & De Nicola, 2010). Despite this limitation, the BDNF model reveals important information on how PVN neuronal activity is altered under hypertensive conditions.

In these studies, immunofluorescence pixel intensity was used to estimate protein expression which has limitations in terms of antibody specificity, reliability, and expression within the PVN. We investigated excitatory signaling, looking at NMDAR1 and synapsin 1a/b expression, which leaves out the potential role of NMDAR2 and the potential role of other synaptic markers and synapsin II and III. Expression was quantified within a morphologically similar slice of the PVN where a high expression of GFP or BDNF_{myc} was seen. Morphological differences in the PVN in the caudal-rostral axis could still contribute to some variation in the data (Stern, 2001). We also investigated inhibitory signaling, looking at GABAA- α 1, GAD67 and KCC2 expression, leaving out the potential role of other GABAA subunits, GAD65, an enzyme responsible for the conversion of glutamate to GABA, and the sodium potassium chloride co-transporter (NKCC1). The effect of BDNF on GABAA- α 1 subunit expression in the PVN was variable, with some of the analyses showing a significant effect while others did not. The mechanism behind the alteration of excitatory and inhibitory signaling seen in the BDNF overexpressed rats may involve the GABAA- α 1 subunit to some extent but might also involve other subunits of GABAA, as well as other trafficking and phosphorylation mechanisms that have not been studied here.

The main limitations of the RT-PCR protocol could be that the isolated PVN tissue included tissue not affected by the vector injection, and potentially included some tissue outside the boundary of the PVN, such as parts of the peri-PVN. Another potential limitation of this study was the use of only male rats. Sex has been shown to influence the development and maintenance of hypertension (Gillis & Sullivan, 2016; Sandberg & Ji,

2012). However, the extent and impact of sex on hypertension is debated, with a recent meta-analysis showing that there is no significant impact of sex on blood pressure responses to antihypertensive therapy or on cardiovascular outcomes (Doumas, Papademetriou, Faselis, & Kokkinos, 2013). The effect of BDNF signaling in the PVN on hypertension in females has also not been conclusively determined.

This study investigated the effect of BDNF overexpression in the PVN on NMDA-mediated excitatory signaling, which leaves out important roles that might be played by AMPA receptors and mGluRs, both of which have been shown to play a role in elevating blood pressure within the PVN previously in the literature. Microinjection of the GluR2-lacking AMPAR inhibitor 1-naphthyl acetyl spermine into the PVN has been shown to decrease blood pressure and lumbar sympathetic nerve activity in SHR but not in WKY rats (D.-P. Li, H. S. Byan, & H.-L. Pan, 2012). While bilateral PVN microinjection of a selective mGluR1 or mGluR5 receptor antagonist, has been shown to have no significant effect on lumbar sympathetic nerve activity and blood pressure in WKY rats but does decrease lumbar sympathetic nerve activity, blood pressure and heart rate in SHRs (D. P. Li & Pan, 2010). Additionally, a PVN bilateral microinjection of a selective group I mGluR agonist causes an increase in lumbar sympathetic nerve activity, blood pressure and heart rate in both WKY and SHRs (D. P. Li & Pan, 2010). Further, mGluR5 in the PVN is known to be upregulated in hypertension and contribute to the hyperactivity of PVN presympathetic neurons through PKC- and SNAP-25-mediated surface expression of NMDA receptors (D. P. Li, Zhu, Pachuau, Lee, & Pan, 2014). Finally, BDNF is known to increase the surface expression of AMPA receptors in the rat nucleus accumbens core (X.

Li & Wolf, 2011), and activation of group I mGluRs induces BDNF mRNA and protein expression in C6 glioma cells (Viwatpinyo & Chongthammakun, 2009) while chronic treatment of a selective mGluR5 antagonist increases BDNF mRNA levels in the hippocampus (Legutko, Szewczyk, Pomierny-Chamioło, Nowak, & Pilc, 2006).

This study investigated the effect of BDNF overexpression in the PVN on GABA-mediated signaling. However, a potential contributor to the alteration of GABAergic signaling in the PVN in hypertensive conditions are GABAB receptors. A PVN microinjection of the GABAB receptor agonist baclofen has been shown to decrease lumbar sympathetic nerve activity and blood pressure in SHRs but not in WKY and SD rats (D. P. Li & H. L. Pan, 2007). Meanwhile, a microinjection of the GABAB receptor antagonist CGP52432 has been shown to only increase lumbar sympathetic nerve activity and blood pressure in SHR but not in normotensive controls (D. P. Li & H. L. Pan, 2007). Further, stimulation of GABAB receptors has been shown to induce a calcium-dependent release of BDNF via the PLC-PKC signaling cascade and L-type voltage-gated calcium channels (Fiorentino et al., 2009; Kuczewski et al., 2011).

Future studies could focus on delineating the impact of BDNF overexpression on other glutamatergic receptors such as mGluR1 and mGluR5, and the AMPA receptors in the PVN. Additionally, the effect of BDNF overexpression in the PVN on GABAB receptor signaling could be investigated. Other signaling components that would need to be evaluated in the BDNF-model would be GAD65 and NKCC1 expression and signaling in the PVN. Another key element to look at would be if BDNF expression is upregulated in the PVN of SHRs compared to WKY rats and if an injection of a TrkB antagonist into

the PVN lowers blood pressure in the SHRs or not. This would provide a more definitive link between hypertension and an increase in BDNF signaling and expression in the PVN. Furthermore, shifting focus to the development of a central TrkB antagonist to treat hypertension could be an important avenue to pursue.

CHAPTER 5: REFERENCES

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