ELECTROPHYSIOLOGICAL AND SYNAPTIC PROPERTIES OF RAT SUPERIOR AND INFERIOR MESENTERIC GANGLION NEURONS IN DOCA-SALT HYPERTENSION

By

Timothy Eugene Houchin

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ABSTRACT

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Hypertension (HT) affects one-third of the adult population of the United States, and is a major risk factor for subsequent development of cardiovascular disease. Increased sympathetic outflow precedes the development of HT in humans and is associated with the development of HT in deoxycorticosterone acetate (DOCA)-salt rats. The celiac, along with the superior (SMG) and inferior mesenteric ganglia (IMG) contain the cell bodies of postganglionic sympathetic neurons that innervate the abdomino-pelvic organs and blood vessels. The highly compliant veins of the abdomino-pelvic region represent the largest readily available reserve of blood in the body. It is from these vessels, in response to sympathetic nervous system-mediated venoconstriction, that blood is mobilized during acute sympathetic activation (e.g., during hemorrhage or exercise). It is likely that a chronic increase in the outflow of efferent sympathetic signals from these ganglia contribute to the rise in arterial pressure in HT through increased venoconstriction and the subsequent reduction in capacitance in this region. The overall objective of the studies described in this dissertation was to uncover the changes in cellular and synaptic function that are taking place in DOCA-salt hypertension. I began by

using the rat IMG as a model of synaptic transmission to test the hypothesis that the safety factor (i.e., the tendency for a preganglionic action potential to evoke an action potential in a postganglionic neuron) is increased in DOCA-salt HT. Intracellular recordings of IMG neurons with concurrent stimulation of the preganglionic nerve bundle revealed that in HT, a greater number of neurons receive strong synaptic inputs. Application of high-frequency, low amplitude stimulation of the preganglionic nerve revealed that a greater proportion of neurons from NT rats underwent long-term potentiation of excitatory postsynaptic potential (EPSP) amplitude beyond action potential threshold. In a separate series of experiments, I used pseudorabies-virus retrograde tracing to identify vein- and artery-projecting neurons of the SMG. The properties of these identified neurons were compared between NT and DOCA-salt HT rats using intracellular recording techniques. I found that the amplitude of the depolarization induced by exogenous nicotine is reduced in both vein- and artery-projecting neurons from HT but not NT rats. Application of exogenous glucocorticoid (hydrocortisone 21-hemisuccinate) (CORT) revealed that veinprojecting neurons from HT rats were hyperpolarized to a significantly lower degree than artery neurons from NT and HT rats, as well as vein-projecting neurons from NT rats. The direct hyperpolarizing effect of CORT was blocked by the glucocorticoid receptor antagonist, RU 486. These results suggest that fast synaptic transmission and cellular properties of prevertebral sympathetic ganglion neurons are altered in DOCA-salt HT.

To my wife, Denise, for her unwavering patience, faith, love, and encouragement

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LIST OF ABBREVIATIONS

AP	action potential
AHP	afterhyperpolarization
BP	blood pressure
CAP	compound action potential
CG	celiac ganglion
CNS	central nervous system
CORT	hydrocortisone 21-hemisuccinate
d-TC	d-tubocurarine
DMSO	dimethyl sulfoxide
DOCA	deoxycorticosterone acetate
ECF	extracellular fluid volume
EPSP	excitatory postsynaptic potential
fEPSP	fast excitatory postsynaptic potential
GFP	green fluorescent protein
gLTP	ganglionic long-term potentiation
GR	glucocorticoid receptor
HBSS	Hank's balanced salt solution
НТ	hypertensive
IP3	inositol triphosphate
IMG	inferior mesenteric ganglion
LTP	long-term potentiation
MΩ	megaohms
MR	mineralocorticoid receptor

ms	milliseconds
m/s	meters per second
mV	millivolts
nA	nanoamps
nAchR	nicotinic acetylcholine receptor
NPY	neuropeptide-Y
NT	normotensive
Oxo-M	oxotremorine-methiodide
PBS	phosphate-buffered saline
PRV	pseudorabies virus
RFP	red fluorescent protein
Rin	input resistance
SD	Sprague Dawley
SMG	superior mesenteric ganglion
TH	tyrosine hydroxylase

CHAPTER ONE:

GENERAL INTRODUCTION

Hypertension and the control of blood pressure

Cardiovascular disease accounts for 1 out of 3 deaths in the United States each year, at a rate of 1 individual lost every 39 seconds (Roger et al., 2012a). Hypertension (HT), defined as systolic blood pressure \geq 140 mmHg or diastolic blood pressure \geq 90 mmHg, affects one-third of the adult population of the United States, and is a major risk factor for subsequent development of cardiovascular disease. Essential (primary) hypertension accounts for >90% of the total cases each year, while HT secondary to other causes, including elevated aldosterone or glucocorticoid levels (secondary HT) accounts for the remaining 10% (Gomez-Sanchez et al., 2010). Between 5-30% of hypertensive patients have HT that cannot be controlled (resistant HT) (Laurent et al., 2012). In addition to conventional treatments (angiotensin converting enzyme antagonists, calcium-channel blockers, and diuretics), there is increasing evidence supporting the prescription of aldosterone antagonists for resistant hypertension (Laurent et al., 2012). The main lifestyle-related risk factors (i.e., things that may allow an individual to prevent the development of HT) include body weight, amount of physical activity (and caloric intake), tobacco use, salt consumption, alcohol consumption, psychological and social stress, and sleep apnea (Roger et al., 2012b). Risk factors for HT development that are largely unrelated to lifestyle include age, family and genetic history, ethnicity, and socioeconomic status/education (Roger et al., 2012b).

The precise mechanisms responsible for the development of HT are incompletely understood and involve multiple organ systems including the

cardiovascular, nervous, immune, urinary and endocrine systems. Elevated sympathetic activity (Parati and Esler, 2012, Schlaich et al., 2004, Malpas, 2010), alterations in the function of the renin-angiotensin-aldosterone system (Sinnayah et al., 2006, Mulatero et al., 2007, Ma et al., 2004, Sowers, 2002), and disruptions in mineralocorticoid and glucocorticoid signaling (Martinerie et al., 2012, Hawkins et al., 2012, Gomez-Sanchez and Gomez-Sanchez, 2012, Funder, 2010) are all likely to be involved in the pathogenesis of HT. All of these changes have the net effect of increasing the physiological set-point of arterial pressure.

Regulation of blood pressure is often described in terms of the time scale of involved responses (i.e., in terms of short term and long-term mechanisms that control blood pressure) (Boron and Boulpaep, 2009). Short term regulation of blood pressure occurs within seconds to minutes, and is mediated predominantly by the baroreceptor and Starling reflexes. While these shortterm mechanisms reliably correct acute perturbations in blood pressure (as occur during postural changes)), they do not play a significant role in blood pressure regulation over longer periods of time (Schlaich et al., 2004), and the arterial pressure set-point upon which these reflexes are based is subject to change over time.

Long-term blood pressure regulation occurs over the course of hours to days (Boron and Boulpaep, 2009) (Cowley, 1992) and involves endocrine and paracrine (as well as neural) control over vascular resistance, venous capacitance, cardiac output, and extracellular fluid volume. The regulation of

extracellular fluid volume by the kidney is believed to be a predominant mechanism behind long-term regulation of blood pressure (Cowley, 1992). However, considerable evidence exists in support of a major role for sympathetic nervous system activation in the long-term regulation of blood pressure (Parati and Esler, 2012, Malpas, 2010), through the vasoconstrictor effects on resistance vessels, and (less commonly discussed but at least equally important) through the control of capacitance and compliance of abdominopelvic veins (Fink, 2009).

The highly compliant veins of the abdomino-pelvic region represent the largest readily available reservoir of blood in the body. It is from these vessels, in response to sympathetic nervous system mediated venoconstriction, that blood is mobilized during acute sympathetic activation (e.g., resulting from exercise or hemorrhage) to aid in perfusion of vital structures (Greenway, 1983, Greenway and Lautt, 1986, Greenway et al., 1994, Lundgren, 1983). Pathological increases in sympathetic outflow to these vessels results in increased mean circulatory filling pressure (Fink et al., 2000), which in the short term contributes to increased arterial pressure through an increase in cardiac output (via. the Starling reflex), and in the long term is likely to contribute significantly to the pressure elevation in the systemic arteries as a result of the shift in blood volume to the arteries of the systemic circulation from the abdomino-pelvic veins. Major differences exist regarding the control of mesenteric veins and arteries by postganglionic sympathetic neurons. Stimulation of sympathetic nerves supplying mesenteric blood vessels revealed

that norepinephrine release is greater from vein-projecting vs. artery-projecting neurons (Luo et al., 2004), and that the responsiveness of mesenteric veins to NE is greater than that from arteries (Bobalova and Mutafova-Yambolieva, 2001, Park et al., 2007). Splanchnic/mesenteric veins respond to lower frequencies of sympathetic stimulation than arteries (Kreulen, 1986, Hottenstein and Kreulen, 1987), and mesenteric veins and arteries are innervated by distinct populations of prevertebral sympathetic ganglion neurons (Browning et al., 1999, Zheng et al., 1999, Hsieh et al., 2000). There have been no published reports, however, that have examined directly the electrophysiological properties of neurons of the prevertebral sympathetic ganglia in the context of hypertension. Also, no studies exist in which the properties of vein- and artery-projecting neurons of prevertebral ganglia of HT rats have been compared with those from NT rats. In the studies presented here, my aim was to investigate the involvement of prevertebral sympathetic ganglia as crucial links between central sympathetic outflow and vasoconstriction of splanchnic/mesenteric veins and arteries in HT.

The superior and inferior mesenteric ganglia

The prevertebral sympathetic ganglia contain the cell bodies of postganglionic neurons that innervate abdominal and pelvic structures, including the smooth muscle and glands of the stomach, the small and large intestines, as well as the pancreas, spleen, liver, kidney, bladder, and internal genitalia (Skok, 1973, Szurszewski and Miller, 1994). In addition, neurons of these ganglia provide the sympathetic efferent innervation to the blood vessels

of the abdominal and pelvic cavities. The superior mesenteric ganglion (SMG) lies immediately inferior to the superior mesenteric artery, ventral to the abdominal aorta and is part of the celiac-superior mesenteric ganglion complex. The much smaller inferior mesenteric ganglion (IMG) is located along the inferior mesenteric artery (which exists as a ventral branch from the abdominal aorta, superior to the division of the aorta into the common iliac arteries. The vast majority of anatomical and functional information existing has been obtained from studies of guinea pig IMG and CG/SMG. Although several studies have been performed that have investigated the properties of individual neurons of the superior cervical ganglion in hypertension (Robertson and Schofield, 1999, Magee and Schofield, 1995, Magee and Schofield, 1994, Magee and Schofield, 1992, Jubelin and Kannan, 1990), the prevertebral ganglia have been excluded.

Hypotheses and aims of this work

The primary objective of the studies described herein was to compare the properties of neurons of the prevertebral sympathetic ganglia to determine whether there are any changes in cellular properties or in the mechanisms responsible for synaptic transmission in DOCA-salt HT.

In the studies reported in chapter two, I examined the properties of neurons of the IMG and compared the response of individual neurons to synaptic input evoked through stimulation of a preganglionic nerve bundle with an external electrode (figure 1). I hypothesized that the enhanced sympathetic outflow to blood vessels of the abdomino-pelvic region is due to an

increase in the safety factor of transmission of impulses (i.e., the ability of an action potential in a preganglionic neuron to evoke an action potential in a postganglionic neuron) through the prevertebral ganglia in HT. One mechanism that may be responsible for this would be an increase in the overall excitability of these neurons, which could occur through changes in resting membrane potential, and input resistance. Therefore, I first measured the basic electrophysiological properties of neurons in the rat IMG. I then performed experiments that tested synaptic input strength and the ability of neurons to undergo long-term potentiation in response to repetitive stimulation.

The comparison of properties of neurons of the rat IMG was limited as a result of sampling neurons of the general population of the ganglion. The identity of each recorded neuron was not known. It is possible that functional changes during hypertension are restricted to the population of neurons supplying mesenteric blood vessels. Changes in this population may not be reflected in the general neuronal population of the ganglia (which includes neurons that innervate smooth muscle and glands of the digestive tract). Therefore, in the studies described in chapter three, I employed a novel retrograde labeling technique that resulted in identification of neurons of another prevertebral ganglion, the SMG, that project to mesenteric veins and/or arteries (figure 1). To test the hypothesis that changes in the properties of postganglionic neurons are responsible for increasing synaptic outflow from the SMG in DOCA-salt HT, I compared the basic electrophysiological properties

of identified vein- and artery-projecting neurons from NT and HT animals. As an indirect means of comparing the synaptic function of vein- and arteryprojecting neurons from NT and DOCA-salt HT rats, I tested the ability of exogenous nicotine to depolarize vein- and artery-projecting neurons from the SMG of NT and HT rats.

The involvement of corticosteroid signaling in the development of hypertension is not a novel concept, as evidenced by both the action of aldosterone in long-term blood pressure regulation (through the sodiumretaining effects on the kidney), and the relationship between elevated corticosteroid levels and hypertension. The acute effects of these hormones on neuronal function are becoming known (for neurons of the CNS), with the general pattern of mineralocorticoids exerting excitatory effects on neuronal function and glucocorticoids having inhibitory effects (Joels et al., 2012). It is likely that prolonged exposure to endogenous corticosteroid hormones results in changes in these acute signaling properties, with consequences for neural tissue outside the CNS (especially the prevertebral sympathetic ganglia, which reside in the closest proximity to the adrenal glands and are outside of the blood-brain barrier) (Joels et al., 2012, Ong and Whitworth, 2011, Carey, 2007).

To test the hypothesis that abnormal glucocorticoid signaling is involved in the increased outflow from sympathetic postganglionic neurons, I compared the responses of vein- and artery-projecting neurons of the intact SMG to an

exogenous glucocorticoid, hydrocortisone 21-hemisuccinate (CORT), between NT and HT groups.



Figure 1: Investigation of the superior and inferior mesenteric ganglia in DOCA-salt HT (A) Superior mesenteric ganglion neurons associated with mesenteric blood vessels were labeled *in vivo* prior to intracellular recording *in vitro*; (B) Synaptic transmission was investigated in the inferior mesenteric ganglion neurons through stimulation of the preganglionic nerve bundle with simultaneous blind intracellular recording *in vitro*; Properties of recorded neurons were compared in all cases between normotensive and DOCA-salt hypertensive animals. **SMG**: *superior mesenteric ganglion*; **IMG**: *inferior mesenteric ganglion*.

*For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

CHAPTER TWO:

ENHANCED SYNAPTIC INPUT STRENGTH TO NEURONS OF THE INFERIOR MESENTERIC GANGLION FROM DOCA-SALT HYPERTENSIVE RATS

ABSTRACT

In the United States, 33.5% of adults over 20 years of age are afflicted with some form of hypertension (HT). In many forms of HT, elevated sympathetic outflow from the CNS results in adverse changes in the heart, kidneys, and the abdominal vasculature. The Deoxycorticosterone-salt (DOCAsalt) model of HT is useful because it shares many of the pathological characteristics of human essential HT, including low renin levels, elevated sympathetic nervous system activity, and progressive development. Veins and arteries of the abdomino-pelvic region constitute a large portion of the systemic vascular bed, with the splanchnic and mesenteric veins serving as the largest reservoir of blood in the body. Increases in sympathetic tone may underlie reductions in venous capacitance in this region, resulting in a shift of blood volume from this vascular bed to the arterial side of the circulatory system. The paired celiac, along with the single superior and inferior mesenteric ganglia (IMG) provide the final link between the abdomino-pelvic vasculature and the central nervous system. Although considerable work has been performed at the level of both the CNS and the interface between sympathetic nerve terminals and target blood vessels in HT, functional changes occurring within individual neurons of the prevertebral ganglia in HT are largely unstudied. Our goal in this work was to directly examine the cellular and synaptic properties of neurons of the rat IMG in DOCA-salt HT. Through the use of intracellular recording, nerve stimulation, pharmacological manipulation, and

immunohistochemical techniques, we found that the basic electrophysiological properties of rat IMG neurons are not altered in DOCA-salt HT. By examining several aspects of synaptic transmission through the rat IMG, however, we found that neurons of the rat IMG receive stronger synaptic input from preganglionic nerves in HT and that neurons from HT rats are less susceptible to long-term potentiation of EPSP amplitude beyond action potential threshold following repetitive stimulation of their preganglionic inputs. We believe that the changes in synaptic transmission and plasticity uncovered in these studies may underlie an increase in the safety factor of transmission of impulses through the rat IMG in DOCA-salt hypertension.

INTRODUCTION

In the United States, 33.5% of adults over 20 years of age are afflicted with some form of HT (Roger et al., 2012b). Essential HT (i.e., elevated blood pressure that is not due to a known pre-existing condition) accounts for the majority of these cases (Laine, 2008). While the mechanism of development and progression of HT is still being elucidated, the sympathetic nervous system plays an important role (Joyner et al., 2008, Malpas, 2010). Elevated sympathetic outflow from the CNS results in adverse changes in numerous tissues; including the heart, kidneys, and the abdominal vasculature (DiBona, 2004, Malpas, 2010, Wyss and Carlson, 2001). Increased sympathetic outflow precedes the development of HT in humans (Malpas, 2010), and is associated with the development of HT in DOCA-salt rats (de Champlain et al., 1989). Also, the DOCA-salt experimental model of HT shares many of the pathological characteristics of human essential HT, including low renin levels, elevated sympathetic nervous system activity, and progressive development (Mulatero et al., 2007).

The paired celiac, along with the single superior and inferior mesenteric ganglia comprise the aggregations of postganglionic sympathetic neurons innervating the abdomino-pelvic viscera. In addition to their role in regulating visceral functions (influencing motility and secretion), these neurons provide the final link between the central nervous system and the splanchnic vasculature. While much work has been done to determine the mechanisms of

regulation of sympathetic function at the neuromuscular junction between vascular smooth muscle and the nerve terminals of these neurons in HT (Demel and Galligan, 2008, Luo et al., 2004, Park et al., 2007, Wang et al., 2005), the functional properties of single neurons of the prevertebral ganglia in HT are largely unstudied. Our goal in this work was to directly examine the cellular and synaptic properties of neurons of the rat IMG in DOCA-salt HT. Our results provide clues toward understanding the effects of HT on the function of individual prevertebral sympathetic neurons, and in addition, they introduce the rat IMG as a model for investigating synaptic transmission through the prevertebral sympathetic ganglia in DOCA-salt HT.

Under normal conditions, the prevertebral ganglia function as signal integrators, in that they receive a myriad of synaptic inputs not only from the CNS, but also from visceral afferents, intestinofugal fibers (with cell bodies in the enteric nervous system), other postganglionic neurons, as well as neurohumoral factors (including circulating epinephrine and norepinephrine and pituitary and adrenal hormones) (Gibbins et al., 2003, Myers, 2001). In other words, these ganglia are not mere relay stations that passively transmit signals from the CNS to the effector tissues, but are responsible for serving as a final point of direct regulatory control by the nervous system before affecting target tissue (Myers, 2001). However, it is possible that an increase in the strength of one of these input modalities can reduce the integrative role of these neurons and increase the safety-factor (i.e., the tendency of a preganglionic action potential to evoke a postganglionic action potential) of

transmission of the signal to the effector tissue (Ireland, 1999, McLachlan, 2003). Evidence for this change in synaptic strength has been found in the superior cervical ganglion in several models of HT (Alkadhi and Alzoubi, 2007), and a form of long-term potentiation of the fast excitatory postsynaptic potential (fEPSP) in single sympathetic ganglions has been demonstrated (Briggs, 1992, Briggs and McAfee, 1988). This form of ganglionic LTP involves activation of the phospholipase-C, IP3 intracellular signaling cascade (Heppner and Fiekers, 2003). Also, an early study showed that the excitability of vasoconstrictor neurons can be increased following repetitive stimulation (Blumberg and Jänig, 1983). This suggests that the strength of preganglionic synaptic connections with postganglionic neurons are subject to use-dependent modulation. These alterations may contribute to the abnormal regulation of vascular capacitance and resistance that occurs in hypertension.

Several interesting findings are presented in this report. First, we provide a detailed characterization of the anatomical and electrophysiological properties of the inferior mesenteric ganglion of the rat, followed by a comparison of the properties of rat IMG neurons from NT and HT animals expressing NPY and TH. Since neurons that express TH and NPY are likely to be vasoconstrictor neurons, we hypothesized that the properties of these neurons specifically would be altered in HT (as opposed to the neurons of the general population). However, we found that the density of NPY immunolabeling is greater than TH labeling in the rat IMG, which suggests that NPY is not a specific marker of vasomotor neurons in the IMG of this species.

Finally, through the use of intracellular recording, nerve stimulation, pharmacological manipulation, and immunohistochemical techniques, we found that the basic cellular properties (including resting membrane potential, input resistance, action potential characteristics, and NPY and TH expression) of rat IMG neurons are not altered in DOCA-salt HT. However, after examining several aspects of fast and slow synaptic transmission, we found that the proportion of neurons of the rat IMG that receive strong synaptic inputs is greater in the HT than the NT group, and that excitatory post-synaptic potentials evoked in neurons from HT rats are less susceptible to long-term potentiation beyond action potential threshold following repetitive stimulation of their preganglionic inputs compared to those from NT rats. We believe that the changes in synaptic transmission and plasticity uncovered in these studies may underlie an increase in the safety factor of transmission of impulses through the rat IMG in DOCA-salt hypertension.

METHODS

In vitro rat inferior mesenteric ganglion preparation

All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of Michigan State University. Male Sprague-Dawley rats (Charles River Laboratories, Portage, MI) weighing approximately 250 g were this study. Following intraperitoneal administration of 1-2cc of 6.25% sodium pentobarbital, and demonstration of adequate anesthesia, an incision was made along the ventro-medial surface of the abdomen from the umbilicus to the xiphoid process. Abdominal viscera were reflected to expose the abdominal aorta and distal colon. The distal 5cm of the colon, the common iliac arteries and veins, the abdominal aorta and inferior vena cava along with the adjacent paraspinal musculature were removed and transferred to a petri dish coated with Sylgard (Dow, Inc.) and filled with Krebs' solution (containing in mM: NaCl 118.5, KCl 4.7, KH₂PO₄ 1.2, NaHCO₃ 23.8, dextrose 5.5, and bubbled with $95\%O_2/5\%CO_2$). The colon was pinned to the bottom of the dish and the paraspinal musculature was removed along the aorta. Local lymph nodes, lymphatic ducts, and the hypogastric arteries were carefully removed. Distal blood vessels were sectioned, allowing removal of the colon. The preparation was then transferred to the recording chamber. The visceral peritoneum overlying the IMG was carefully cut and reflected. Small tungsten pins inserted into the Sylgard-coated chamber were used to stabilize the

ganglion. Much care was taken to avoid extensive stretching of the IMG or any of its associated nerve bundles. The pins were inserted into blood vessels or into the mesentery, never into the nerves or lobes of the IMG. Fat and additional connective tissue was removed from a ~5mm section of the intermesenteric/lumbar splanchnic nerves and a bipolar platinum-iridium electrode was placed underneath.

The DOCA-salt model of hypertension

The Deoxycorticosterone-salt model of hypertension was employed in the portions of this study that compared the properties of neurons from normotensive and hypertensive rats. The procedure for preparing DOCA-salt hypertensive rats has been described in detail previously (Demel et al., 2010). Systolic blood pressure was measured on three separate occasions using the tail cuff method four weeks after uninephrectomy and DOCA pellet implantation. The subcutaneous DOCA implant provided a dose equivalent to 200 mg/kg and the animals were given a dilute salt solution (1% NaCl + 0.2% KCl) for their drinking water. Normotensive control rats were also uninephrectomized, but were not given the DOCA implant and received tap water to drink. Normotensive animals had mean blood pressures of 132.4 ± 1.8 mmHg and the mean blood pressure of the DOCA-salt hypertensive rats was 196.8 ± 3.5 mmHg. Rats reached an average of 350g by the time of dissection.

Electrophysiology

The recording chamber was perfused at a rate of ~5mL/min with warm $(35 \pm 2 \text{ °C})$ Krebs' solution. Intracellular recordings were made using sharp microelectrodes (tip resistances 50-80M Ω) filled with 3M KCl or 1M K-acetate. In the experiments in which the recorded neurons were identified, 2% Neurobiotin (VWR) was added to the K-acetate internal solution. An Axoclamp 2A (Axon Instruments) amplifier with an HS-2A headstage, (gain=0.1) was used to collect the signal from the targeted neurons. All recordings were made with the amplifier in "bridge" mode. The bridge balance and pipette capacitance compensation were adjusted when the electrodes first entered the tissue bath and were monitored and adjusted accordingly during the experiments.

After mounting onto the headstage, the electrodes were lowered to approach the tissue under view with a Wild M7 stereomicroscope. An audio monitor was used to confirm contact between the electrode and the tissue. Blind recordings were established by advancing the electrode through the tissue while a -0.05nA, 50 millisecond DC pulse was administered. An increase in the electrode tip resistance (seen as an increase in the amplitude of the voltage response to the -0.05nA current pulse) indicated the presence of a cell. Impalement of neurons resulted after a brief tap on the bench or brief use of the "buzz" function of the amplifier. A membrane potential of at least -35mV that was stable for a minimum of five minutes constituted a successful impalement. Cellular responses were digitized using a Digidata 1440A digitizer (Molecular Devices). For the intracellular labeling experiments, positive current

pulses (1.0nA, 1Hz) were applied for 1-3 minutes to allow sufficient filling of neuronal somata with neurobiotin. One neuron per IMG lobe was filled during each recording session. Orientation of the tissue was maintained by cutting one secondary branch of the IMA shorter than the other and noting the location of each cell accordingly. Data were collected using pClamp 10 (Molecular Devices) and saved on a Dell computer. Analysis was performed offline using Clampfit10 (Molecular Devices). The nerve-stimulation electrode was connected to a Grass SIU5 stimulus isolation unit. Voltage pulses 0.1ms in duration and ranging in amplitude from 5-50V were applied to the nerve to evoke excitatory postsynaptic potentials in the impaled neurons.

In the long-term potentiation experiments, the amplitude of the EPSP evoked in response to the smallest amount of voltage applied to the preganglionic nerve bundle that was necessary to cause a postsynaptic response was measured and considered the initial EPSP amplitude. Neurons were then subjected to the repetitive stimulation protocol (20 Hz stimulation for 20 seconds) of the intermesenteric/lumbar splanchnic nerve. Long term potentiation that remained sub-threshold for action potential generation was quantified by measuring the EPSP amplitude at t=20 minutes and normalizing it to the initial amplitude. Neurons with EPSP amplitudes more than 100% of the initial amplitude at t=20 min. underwent sub-threshold LTP. Potentiation that brought the EPSP amplitude above threshold (resulting in action potential generation following test stimulation of the same magnitude as applied during the initial stimulation) was considered "long-term" if the neuron responded to

the test pulse after 20 minutes with action potentials rather than EPSPs. This type of response was termed "supra-threshold LTP".

Immunohistochemistry

Following the recording session, ganglia were carefully removed from the recording chamber and transferred to a small, Sylgard-coated petri dish filled with Krebs' solution. The tissue was oriented and pinned in the same position as in the recording chamber and subsequently fixed in 4% Paraformaldehyde/ 4% sucrose for 4 hours at room temperature and then cryo-protected in 20% phosphate-buffered sucrose overnight. Ganglia were then embedded in mounting medium and sectioned. Fourteen micrometer thick slices were mounted on Superfrost-Plus (VWR) slides and allowed to dry prior to immunohistochemical processing. Hydrophobic wells were applied to each slide before beginning wash steps. Slides were then washed in phosphatebuffered saline: 0.1M containing 0.2% TritonX-100 (PBS/Tx 0.2%) (5min x 3, $10\min x 1$; then washed $5\min x 2$ in 0.1M phosphate-buffered saline (PBS). Slides were then incubated in Texas Red/Avidin-D, diluted to 1:250 in PBS/Tx 0.1% in a humidified chamber at room temp (RT) for one hour. The slides were then washed again for 5min x 3 and 10min x 1 in PBS/Tx 0.2%. Slides were then blocked in normal goat serum (NGS) consisting of 4% normal goat serum in PBS/Tx 0.2%, for 1 hour at RT. Then, after 2x5min rinses in PBS, slides were incubated at 4°C for 22 hours in a mouse anti-Tyrosine Hydroxylase (1:1000, Millipore) plus rabbit anti-Neuropeptide Y (1:1000 Immunostar) primary antibody cocktail. On the second day of processing, slides were

washed for 5x3min and 1x10min in PBS/Tx 0.2%, blocked in NGS for 20min, rinsed in PBS 5min x 2, and then incubated in Cy5 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit (1:250 in NGS) secondary antibodies for 2 hours at RT. A final rinse in PBS (5min x 3, 10min x 1) preceded coverslipping with Prolong Gold Antifade reagent (Molecular Probes). Images were acquired using an Olympus Fluoview-F1000 Spectral confocal microscope. PMT voltage and gain settings were optimized for each channel prior to collection of sequential Z-stack images. Projections of z-stacks of Texas-Red labeled neurons were used for morphological analysis.

Analysis

Data are reported as mean ± SEM. One-way ANOVA was used as a preliminary test for significant differences. For categorical comparisons, Fisher's exact test was applied. Results were considered significant if the P value was less than 0.05. GraphPad Prism software was used for performing statistical analyses. Recordings suitable for analysis were selected if the resting membrane potential was at least -35mV, the action potential amplitude was at least 50mV, and the recording was maintained for at least 5 minutes.

Projection images (20x for ganglion level analysis and 60x for single neuron analysis) of ganglia and single neurons of the IMG created from z-series optical sections were analyzed using Image J software. For ganglion-level analysis, the perimeter of the ganglion was traced and the area calculated. Cell counts were then taken of NPY and TH positive neurons in each ganglion. To determine the density of immunolabeling for each marker, the total number of

TH and NPY positive neurons was normalized to the ganglion area.

Morphological analysis of 60x images of neurons filled with neurobiotin during intracellular recording consisted of counting the number of primary dendrites and measuring the area of the soma. A somatic process was considered a dendrite if it projected farther than 5 micrometers from the perimeter of the soma and was not the axon. The area was measured using Image J following the creation of an outline of the soma.
RESULTS

Anatomy of the rat inferior mesenteric ganglion

The rat inferior mesenteric ganglion is comprised of two lobes that reside on either side of the inferior mesenteric artery. A large, paired nerve bundle links the IMG to the more rostral superior mesenteric ganglion, this is the intermesenteric nerve. This is a mixed nerve that contains sympathetic preganglionic fibers, but also contains postganglionic fibers from the celiac/superior mesenteric ganglia, sensory fibers from the dorsal root ganglion, and postganglionic fibers from the IMG itself (Quinson et al., 2001, Gabella, 1999 #2191). The lumbar splanchnic nerves travel in the posterio-anterior direction to cradle each side of the aorta and join the intermesenteric nerve. The paired hypogastric nerves exit the IMG and travel caudally, providing sympathetic innervation to the pelvic ganglion and the organs and vasculature of the pelvic cavity (Skok, 1973). The lumbar colonic nerves travel along the distal branches of the inferior mesenteric artery to reach target organs including enteric neurons, smooth muscle, glands, and blood vessels of the descending colon (Szurszewski and Miller, 1994). Figure 2 shows the rat IMG as it would appear during an *in vitro* recording session. The right hypogastric nerve has been reflected in this diagram; in the intact animal, it would be traveling in the same direction as the left hypogastric nerve and the right lobe of the IMG would be on the other side of the inferior mesenteric artery. A layer of visceral peritoneum lies between the two hypogastric nerves

(and over the ganglion), but is removed prior to recording to expose the surface of the ganglion. Neurons of each IMG lobe were targeted for blind microelectrode recordings. The intermesenteric/lumbar splanchnic nerve bundle was used for stimulation of preganglionic inputs to the postganglionic sympathetic neurons being recorded.

We estimated the rat IMG to contain about 3500 neurons. Each lobe of the ganglion is approximately 250 micrometers (um) in diameter and 125 um thick. The average area of the largest frozen section of IMG tissue was 111 square millimeters (mm²). Immunohistochemical analysis of the rat inferior mesenteric ganglion revealed neurons of essentially uniform soma size, but with varying levels of dendritic complexity. The average area of rat IMG neurons was 570.4 \pm 251.3 um^2 in the normotensive (NT) group and 604.2 \pm 240.1 um^2 in the hypertensive (HT) group. Neurons from NT rats had an average of 3.5 ± 1.8 primary dendrites, while neurons from HT animals had 3.7 ± 3. Examples of the morphology of a subset of neurobiotin-filled neurons identified during intracellular recording can be seen in figure 3. When the total number of identified neurons analyzed (from both NT and HT rats) were considered together, 61% had 5 or more primary dendrites (22 of 39), while the remaining 39% (14 of 39) had fewer than 5. There was no correlation between dendritic complexity and firing pattern (phasic vs. tonic -see below), nor was there any relationship between neuron shape and whether the cell was from a NT or a HT rat. Furthermore, as discussed below, no association was seen

between tyrosine hydroxylase (TH) expression, or neuropeptide-Y (NPY) expression and the soma size and/or the dendritic complexity of each recorded neuron (data not shown).

Neuropeptide-Y is presumed to be expressed predominantly by postganglionic sympathetic neurons associated with vasoconstrictor pathways (Macrae et al., 1986, Keast et al., 1993, Matsumoto et al., 1993, Li and Horn, 2006, Lundberg et al., 1982). Since one of the goals of this study was to compare cellular and synaptic properties of rat IMG neurons from NT and HT rats, we expected any changes in these properties to be reflected in neurons that likely project to blood vessels and not necessarily in the general population. The cellular patterns of labeling for TH and NPY were consistent with other reports (Parr and Sharkey, 1996, Anderson et al., 2001, Li and Horn, 2006, Jobling and Gibbins, 1999, Kondo et al., 1988). Tyrosine hydroxylase label was found throughout the cytosol while NPY label was concentrated in the perinuclear regions. An interesting finding that appears to be unique to the rat IMG is that the apparent number of neurons in the rat IMG staining positive for NPY was greater than the number of TH positive neurons (figure 4). Analysis of 20x magnification, confocal z-series projection images from 30 rat IMGs revealed that the density of NPY-positive immunolabeling, expressed as the ratio of the number of NPY-positive neurons divided by the area of the ganglion, was significantly greater than the density of TH labeled neurons (figure 4). NPY positive neurons were present at $3.76 \pm$ 0.22 cells per 10 mm² of ganglion area, TH positive at 2.87 \pm 0.16 cells per 10

mm², and neurons that were positive for both TH and NPY was 2.39 ± 0.17 cells per 10 mm² (figure 4D).

In the next portion of this study, we combined immunohistochemical analysis with sharp electrode electrophysiology to examine the properties of neurons identified with intracellular neurobiotin. When the electrophysiological properties of rat IMG neurons that expressed both NPY and TH were compared between NT and HT groups (12 cells from NT and 13 cells from HT), no differences were found between those neurons and neurons of the general population (i.e., neurons that weren't identified with neurobiotin), and there were no significant differences in any of the active or passive membrane properties nor between the various aspects of synaptic input from the intermesenteric/lumbar splanchnic nerves (data not shown). Therefore the remainder of the analysis was performed on neurons of the general rat IMG population, irrespective of TH/NPY content and morphology.

Electrophysiological properties of rat inferior mesenteric ganglion neurons

Intracellular recordings were obtained from a total of 248 neurons; 122 from the IMG of normotensive rats and 126 from DOCA-Salt Hypertensive rats. Following establishment of a stable recording and collection of basic electrophysiological data, neurons were classified according to their action potential firing response to a maintained depolarizing stimulus (Cassell et al., 1986). Neurons that fired once at the onset of a 2.5x rheobase depolarizing current injection were considered phasic and neurons that fired more than once were considered tonic (figure 5). Adaptation was not seen in neurons that fired more than one action potential (i.e., if a neuron fired more than once during the pulse, it fired throughout). Likewise, phasic neurons never fired more than one action potential during the pulse. As shown in the bottom of table 1, over seventy percent of the neurons of the rat IMG were phasic. In contrast to the proportion of phasic neurons of the guinea pig (Cassell et al., 1986), this suggests that the composition (i.e., based on firing pattern) of the rat IMG more closely resembles that of the dog (King and Szurszewski, 1984), than the guinea pig or the cat (Jule and Szurszewski, 1983).

In a subset of experiments that aimed to determine whether neurons from HT rats exhibited a differential susceptibility to modulation of firing pattern vs. those from NT, the muscarinic agonist oxotremorine-methiodide (oxo-M) (10uM) was introduced to the bathing solution. Oxo-M caused some phasic neurons to begin firing tonically. This was a reversible effect and was seen in 2 out of 8 neurons from NT rats and 3 out of 10 neurons from HT rats examined (figure 6). This result suggests the presence of an M-like current (Adams and Harper, 1995, Brown, 1988, Brown and Selyanko, 1985, Marrion et al., 1989) that may underlie the phasic firing pattern seen in certain rat IMG neurons (Brown and Passmore, 2009, Wang and McKinnon, 1995), but its expression does not appear to be altered in hypertension. A similar effect was seen following application of carbamyl choline (not shown).

A summary of the passive and active membrane properties of phasic and tonic rat IMG neurons from NT and HT animals is shown in table 1. The

resting membrane potentials in the NT group were -49 millivolts (phasic), and -53 millivolts (tonic). Neurons from HT rats had resting membrane potentials of -47mV (phasic), and -50mV (tonic) and were not significantly different from those of the NT group. The input resistance (Rin) of phasic neurons was not significantly different between NT and HT groups: 49 ± 4 megaohms (M Ω) (NT) and 54± 4 M Ω (HT), but the Rin of tonic neurons was significantly higher than phasic in both the NT and the HT groups: $114 \pm 13 \text{ M}\Omega$ (NT) and $102 \pm 12 \text{ M}\Omega$ (HT). Membrane time constants ranged from 4.0 to 6.1 milliseconds (ms) and were not significantly different between phasic and tonic, or between NT and HT. The rheobasic current, which is the minimum amount of depolarizing current necessary to evoke an action potential in the neuron being recorded, ranged from 0.17 nanoamps (nA) to 0.2 nA and was not significantly different between neurons of each electrophysiological class nor between NT and HT groups. The action potential amplitudes of rat IMG neurons ranged from 60 to 64 mV. Action potential afterhyperpolarization (AHP) amplitudes ranged from 13.6 mV to 14.7 mV and showed no change in hypertension. The duration of the AHP ranged from 220 to 394 milliseconds. The AHP duration means were not significantly different between NT and HT as analyzed using Student's ttest (p=0.4675). However, an F test revealed significant differences in the variances of both the NT and the HT groups (figure 9). When the AHP duration of each neuron analyzed was plotted against the cell ID number, there appeared to be two clusters of neurons, one with AHPs lasting >500ms, and one with AHPs of <500ms. While this duration is shorter than that observed in

the guinea pig IMG, it raises the possibility of the existence of an additional electrophysiological class of neuron in the rat IMG (Keast et al., 1993, McLachlan and Meckler, 1989). However, when the properties of these putative "long-afterhyperpolarizing" neurons were compared, their properties were not significantly different than those of phasic neurons with short AHPs (data not shown). Therefore, in the remainder of this report we refer to only two electrophysiological classes of neuron, phasic and tonic. The data from neurons with long afterhyperpolarizations has been pooled with that of phasic neurons.

Synaptic input to the rat inferior mesenteric ganglion

The inferior mesenteric ganglion of the rat is an attractive model for the study of synaptic transmission because the preganglionic nerves (i.e., the lumbar splanchnic and intermesenteric nerves) are readily accessible for stimulation. The nerves have a minimal amount of surrounding connective tissue and can be isolated up to 15+ mm rostral to the ganglion. Thus, the electrophysiological response of the postganglionic neuron, recorded using an intracellular microelectrode, can be obtained following stimulation of the incoming preganglionic nerves (Simmons and Dun, 1985, Schumann and Kreulen, 1985, Jule et al., 1983, Weems and Szurszewski, 1978). In all studies of synaptic input presented here, the intermesenteric nerve, with contributions from the lumbar splanchnic nerves, was stimulated using a bipolar platinum-iridium electrode. Conduction distances were measured from the stimulating electrode to the intracellular electrode at the end of each recording. These

distances ranged from 2mm to 15mm, with a mean of 11mm (n=60); the distribution of distances were constant between NT and HT groups. Preganglionic nerve conduction velocity was calculated by dividing the conduction distance for each recording by the time delay between the nerve stimulating electrode stimulus artifact and the onset of the cellular response. Mean conduction velocity values were similar to those reported previously for unmyelinated, presynaptic autonomic fibers (Simmons, 1985) 1.1 meters per second (m/s) NT and 1.2 m/s HT; these values were not significantly different (table 2).

Fast synaptic input to some neurons of the rat IMG consisted of subthreshold excitatory postsynaptic potentials (EPSPs). When stimuli of increasing amplitude were applied, neurons that initially responded with EPSPs were brought to action potential threshold (figure 7A). In these neurons, the EPSP amplitude increased in a graded fashion up to action potential threshold, suggesting the existence of multiple subthreshold (weak) synaptic inputs (Simmons, 1985). In other neurons, subthreshold EPSPs were not evident; these neurons only fired action potentials in response to nerve stimulation.

Fast synaptic input to neurons of the rat IMG was susceptible to block by reducing the extracellular calcium ion concentration from 2.5 to 0.25 millimolar and also through bath application of the nicotinic cholinergic receptor antagonist d-tubocurarine (100uM) (figure 10). These effects were reversible and reproducible.

The investigation of synaptic input to neurons of the IMG began with determination of the minimum amount of voltage necessary to evoke a response in the postganglionic neuron. With successive stimuli, the voltage dial on the Grass S88 stimulator was increased in 1-2V increments until a response was seen. Neurons that responded to minimal nerve stimulation with an EPSP were considered to receive a weak synaptic input while neurons responding to minimal stimulus amplitude nerve stimulation with action potentials were considered as receiving strong inputs (Jänig and McLachlan, 1987) (figure 7). This strength of synaptic input is of interest because it has been suggested that vasoconstrictor neurons of guinea pigs and rats receive predominantly strong synaptic inputs (Jänig, 2006, Jänig and McLachlan, 1992, McLachlan, 2003). As shown in table 2, a larger proportion of neurons from hypertensive rats received strong inputs (40 out of 73 total neurons) than normotensive (25 out of 67 neurons). Among the weak input neurons, evoked EPSP amplitudes were 3.6 mV for both the NT and HT groups (table 2).

As an additional point of comparison of the synaptic input into the rat IMG between NT and HT animals, the response to suprathreshold, repetitive stimulation was compared. Slow synaptic potentials, mediated by muscarinic actions of acetylcholine or through peptidergic transmission (e.g., from collaterals of visceral afferent neurons) are known to occur in the guinea pig IMG and the rat celiac/superior mesenteric ganglion complex (Simmons, 1985). This slow potential is accompanied by a reduction in input resistance, and is believed to be due to selective inhibition of M-currents (KCNQ or Kv7

potassium channels) in sympathetic ganglion neurons (Brown and Passmore, 2009, Hille, 2001). Therefore, comparison of the frequency of slow EPSP generation and the amplitude of depolarization would give insight as to whether this means of modulation of synaptic transmission is altered in hypertension. For these experiments, the voltage applied to the nerve was increased until the neuron responded with an action potential with each stimulus. Then, the nerve bundle was stimulated at this voltage at a frequency of twenty hertz for four seconds. In many neurons, the cell would respond with a slow depolarization of approximately 4 mV that lasted about 10 seconds (figure 11). The frequencies of generation of this slow EPSP were similar between NT and HT groups (24/29 neurons in the NT group and 19/21 in the HT group), as were the amplitudes (4.5 mV NT and 4.0 mV HT) (Table 2).

Long-term potentiation in neurons of the rat inferior mesenteric ganglion

Several reports have been published recently that suggest a role for potentiation of synaptic transmission through sympathetic ganglia in hypertension (Alkadhi and Alzoubi, 2007, Alzoubi et al., 2008, Alzoubi et al., 2010, Alzoubi and Alkadhi, 2009). Long-term potentiation is defined as a longlasting response to a conditioning stimulus that results in either a change in excitability of the postsynaptic neuron or an increase in the neurotransmitter processing/release at the presynaptic nerve terminal (Heppner and Fiekers, 2003, Kuba and Kumamoto, 1990, Dunant and Dolivo, 1968). Neurons from normotensive animals are expected to show a greater frequency of LTP expression or a greater increase in EPSP amplitude than neurons from

hypertensive animals. This is thought to be a consequence of the increased sympathetic outflow to the neurons from hypertensive animals (Alkadhi and Alzoubi, 2007). Since these neurons are exposed to a greater background level of presynaptic activation, their ability to potentiate will be closer to the saturation point *in vivo* than neurons from NT animals.

Due to the fact that this phenomenon has not been investigated in the IMG of any species to date, and few studies have performed the analysis of long-term potentiation using intracellular recording from postsynaptic neurons in sympathetic ganglia, a series of experiments were performed to determine first, whether long-term potentiation occurs in these ganglia, and second, whether there are any differences in LTP of synaptic input in neurons from HT animals (compared to NT).

In this set of experiments, we defined long-term potentiation as an increase in the measured EPSP amplitude evoked in response to a test stimulus of the same voltage as applied prior to and during the ganglionic LTP (gLTP) induction protocol that is maintained for at least 20 minutes (Heppner and Fiekers, 2003). While the mechanism of long term potentiation in sympathetic ganglia is not known, it is thought to be mediated through presynaptic mechanisms (Briggs, 1995, Briggs and McAfee, 1988) and is dependent upon an increase in the intracellular calcium concentration (Vargas et al., 2007).

Following the determination of the minimal stimulus amplitude necessary to evoke a response, neurons were subjected to the gLTP induction

protocol. By definition, the analysis of LTP could only be performed in those neurons that received a weak synaptic input, as the strong synaptic input neurons will only fire action potentials in response to minimal nerve stimulation. The gLTP induction protocol (figure 8A) consisted of repetitive stimulation (20 Hz) of the intermesenteric/lumbar splanchnic nerves at the same voltage (the minimal amount necessary to evoke an EPSP) as applied during the control protocol, for 20 seconds. As shown in figure 8a, neurons did not fire action potentials during stimulation. One minute after the gLTP induction protocol, and every minute thereafter for 20 minutes, single stimuli were applied to the nerve and the synaptic response of the cell was recorded. Neurons were considered potentiated if the evoked EPSP amplitude increased above 100% of the initial value for at least 20 minutes (figure 8B and C; figure 12), or if the neuron responded to the test stimulus following gLTP induction with action potentials rather than an EPSPs beyond 20 minutes. Potentiation in the form of increased EPSP amplitudes was termed "LTP-subthreshold" and potentiation in the form of action potential firing was termed "LTPsuprathreshold" (figure 8).

In neurons from normotensive animals, 74% (17 out of 23) neurons underwent potentiation of one of the two forms in response to the gLTP induction protocol. A similar proportion of neurons from hypertensive animals potentiated as well: 14 out of 20 neurons tested (70%) had either increased EPSP amplitudes or fired action potentials following repetitive stimulation (table 3). The increase in EPSP amplitude was not accompanied by an

increase in input resistance, since the conditioning stimulus is below the threshold for action potential generation, muscarinic receptor mediated inhibition of M-type potassium currents were not expected to play a role in the potentiation. Also, no neurons responded to the LTP induction protocol with slow EPSPs; no change in resting membrane potential in the postsynaptic neurons was observed.

As shown in the representative plots of EPSP amplitude as a percentage of the control amplitude vs. time following LTP induction, neurons that exhibited potentiation usually had test EPSP amplitudes that were maintained well above the pre-induction value (figure 12). Also, when a neuron underwent LTP, the response was usually maintained for well over 20 minutes, in most cases the duration was limited by the recording itself (most recordings were stable beyond 1 hour, but some were not). Figure 12 also shows representative cells from NT and HT animals that failed to undergo LTP. The EPSP amplitudes remained near the original value following gLTP induction.

Although the proportion of neurons that underwent potentiation was similar, the type of potentiation was different between NT and HT groups. In the NT group, 14 out of 17 neurons responded to gLTP induction with suprathreshold LTP while only 5 out of 14 neurons did so in the HT group. Therefore, a greater percentage of IMG neurons from normotensive rats were potentiated beyond action potential threshold than were neurons from hypertensive rats (table 3).



Figure 2: The Rat Inferior Mesenteric Ganglion

1mm

A drawing of the IMG as it appears during the experimental setup. The right hypogastric nerve has been reflected to the left to improve access to the ganglion for recording. The arrow indicates the position of the nerve stimulating electrode. **HGn.:** *Hypogastric nerves;* **L.Cnn.:** *Lumbar Colonic nerves;* **IMn.:** *Intermesenteric nerve;* **L.Sp. nn.:** *Lumbar Splanchnic nerves;* **IMA:** *Inferior Mesenteric artery;* **IMV:** *Inferior Mesenteric vein*



Figure 3: Examples of the morphology of rat inferior mesenteric ganglion neurons Projection images of confocal Z-stacks (60x objective) of identified rat IMG neurons revealed that 61% (22/39) of neurons had 5 or more primary dendrites and 39% (14/39) had less than 5 primary dendrites. The mean soma area and number of dendrites did not differ between NT and HT groups.



Figure 4: Neuropeptide-Y expression is more prevalent than tyrosine hydroxylase expression in the rat IMG

(A) 20x Image of NPY immunolabeling in rat IMG neurons; (B) TH labeling in the same ganglion; (C) Merged image: yellow neurons contain NPY and TH; (D) Quantification of NPY and TH immunopositive cell density data from 30 ganglia.

**Statistically significant vs. NPY+ (p<0.0001); † vs. TH+ (p<0.05); ‡ vs. NPY+ (p=0.0021)



onset of the current pulse and became quiescent thereafter. (B) A representative trace from a tonic neuron.



Figure 6: Tonic firing evoked in a phasic neuron following application of 10uM oxotremorine-methodide

(A) Phasic response of a rat IMG neuron. (B) Response of the same cell after bathapplication of Oxo-M. (C) Wash, 20 minutes after initial application. The proportion of neurons from NT and HT rats that responded to Oxo-M in this manner was not significantly different.

		Phasic	Tonic
	NT	-49 ± 1	-53 ± 2
Resting Membrane Potential (mV)		(53)	(12)
	υт	-47 ± 1	-50 ± 2
Cell Input Resistance (MΩ)	пі	(63)	(16)
	NT	49 ± 4	*114 ± 13
		(50)	(13)
	нт	54 ± 4	*102 ± 12
		(56)	(15)
Membrane Time Constant (ms)	NT	4.9 ± 0.3	6.1 ± 1.6
		(29)	(3)
	HT	4.0 ± 0.3	5.3 ± 0.6
		(11)	(3)
	NT	0.20 ± 0.02	0.17 ± 0.03
Rheobasic Current (nA)		(15)	(4)
	НТ	0.17 ± 0.02	0.17 ± 0.01
		(8)	(5)
	NT HT	60 ± 1.5	64 ± 5.7
Action Potential Amplitude (mV)		(30)	(3)
		63 ± 2.6	57 ± 6.3
		(22)	(5)
	NT	13.8 ± 0.4	13.6 ± 1.3
Afterhyperpolarization Amplitude (mV)		(66)	(7)
	НТ	13.7 ± 0.4	14.7 ± 1.5
		(64)	(13)
Afterhyperpolarization Duration (ms) Percentage of Neurons in Each Group	NT	387 ± 22	252 ± 72
		(68)	(7)
	НТ	394 ± 30	220 ± 21
		(65)	(13)
	NT	71.1%	28.9%
		59/83	24/83
	υт	72.8%	27.2%
	пі	59/81	22/81

 Table 1: Basic membrane properties of phasic and tonic IMG neurons from NT and HT rats

Number of neurons in parentheses

* Significantly different vs. Phasic, but not between NT and HT; Student's t test (P<0.001)



Figure 7: Strong and weak synaptic input to neurons of the rat inferior mesenteric ganglion.

(A) A weak input neuron, with multiple synaptic inputs, being brought to action potential threshold with increasing stimulus voltage. (B) An example of a strong input neuron, the response of this neuron to minimal stimulus voltage was an action potential (EPSPs could not be evoked in this neuron).

	NT	3.6 ± 0.3
EPSP Amplitude (mV)		(53)
	υт	3.6 ± 0.4
		(49)
Slow EPSP Amplitude (mV)	NT	4.5 ± 0.6
		(22)
		4.0 ± 0.5
		(19)
Nerve Conduction velocity (m/s)	NIT	1.1 ± 0.1
	IN I	(33)
		1.2 ± 0.1
		(17)
Ratio of strong: weak input neurons		0.59
	IN I	25:42
		1.21*
		40:33

Table 2: Synaptic input properties of rat IMG neurons, NT vs. HT

*Statistically significant vs. NT, P=0.0432 (Fisher exact test)



Figure 8: Ganglionic long-term potentiation induction protocol and example responses of rat IMG neurons (A) A trace showing the 20Hz repetitive stimulation protocol; the pulse train lasted 20 seconds; (a) A highzoom view of the first five pulses of the stimulus train given in *A*; notice the lack of action potentials; (B) A control EPSP and (C) An EPSP that has potentiated, but whose amplitude is still sub-threshold (LTP-Sub) for action potential generation; (D) and (E) An example of a neuron that has undergone LTP that has brought the EPSP amplitude above action potential threshold (LTP-Supra). This neuron responded to the test pulse with action potentials rather than EPSPs for the duration of the recording following gLTP induction.

Table 3: A greater proportion of rat IMG neurons from normotensive
animals fire action potentials following low amplitude, high frequency
preganglionic stimulation compared to neurons from hypertensive animals.

	LTP-Subthreshold	LTP-Suprathreshold	no LTP
NT	18%	*82%	26%
	3/17	14/17	6/23
HT	64%	36%	30%
	9/14	5/14	6/20

* Significantly different vs. HT, P=0.012 (Fisher exact test);

"LTP-Subthreshold"=number of neurons that responded with an increase in EPSP amplitude 20 minutes after LTP induction as a percentage of the number of neurons responding with either form of LTP; "LTP-Suprathreshold"= percentage of neurons that potentiated beyond action potential threshold out of the total number of cells that responded with either form of LTP; "no LTP"=neurons that did not undergo long-term potentiation as a percentage of the total number of cells examined (LTP-Subthreshold + LTP-Suprathreshold + no LTP").





(A) A plot of the measured AHP duration in each phasic neuron from the NT and (B) HT groups revealed large variation within groups; *x axis= cell ID number*(C), suggesting the existence of two sub-populations of phasic neuron in the rat IMG; (D) Example traces of phasic (red trace) and "long afterhyperpolarization" neurons (black trace).



Figure 10: Fast synaptic input to rat IMG neurons is blocked by low Ca²⁺ and by d-tubocurarine. (A) Fast synaptic potentials in an IMG neuron evoked via intermesenteric/lumbar splanchnic nerve stimulation before and (a) after bath-application of 100uM curare. (B) Fast synaptic potentials before and (b) during replacement of normal Krebs' with low [Ca²⁺] 0.25mM, and high [Mg²⁺] 3.44mM







Figure 12: Long term potentiation in inferior mesenteric ganglion neurons from normotensive and hypertensive rats

(A) and (B): Examples of neurons that responded to submaximal repetitive stimulation with increases in EPSP amplitude; (C) and (D): Neurons that failed to undergo potentiation.

DISCUSSION

Anatomy of the rat IMG

Anatomical and physiological descriptions of the inferior mesenteric ganglion have been well described for several species including the guinea pig, cat, dog, pig, and rabbit (Majewski and Heym, 1991, Archakova et al., 1982, Crowcroft and Szurszewski, 1971, Lloyd, 1937, Baluk, 1995, Simmons and Dun, 1985). Although only a single report (in abstract form) exists concerning the physiology of the rat IMG (Kreulen, 1982), anatomical descriptions have been published (Gabella, 1999, Quinson et al., 2001). Our estimate of the total number of neurons of the rat IMG (3500 neurons) is consistent with the values reported for rat superior cervical ganglia (25000-45000 neurons) when normalized to total ganglion size, using Hendry's method (Hendry and Campbell, 1976). Also, the average soma diameter and number of primary dendrites is similar to those of other sympathetic ganglia in the rat (Gabella, 1999), with soma diameters of approximately 30 um and 3-5 primary dendrites. That we did not see any significant differences in neuronal morphology between neurons from NT and HT rats is reflective of the conservative nature of these properties.

A major goal of this study was to compare the properties of neurons from NT and DOCA-salt HT rats. Since the principal ganglion neurons of the abdominal prevertebral ganglia innervate multiple visceral targets in addition to the abdomino-pelvic vasculature, we expected to have more success in

revealing these differences if we could selectively compare neurons that were likely to project to blood vessels. Neuropeptide-Y has long been associated with neurons in vasoconstrictor pathways (Macrae et al., 1986, Keast et al., 1993, Gibbins, 1995). Most sympathetic postganglionic neurons innervating cutaneous vascular beds in the guinea pig contain NPY in addition to NE (Gibbins et al., 2003). Putative vasoconstrictor neurons of the rat superior cervical ganglion express NPY (Li and Horn, 2006), and the expression of NPY in cultured guinea-pig prevertebral neurons is enhanced when grown in medium conditioned with dissociated vascular smooth muscle cells (Matsumoto et al., 1993).

Therefore, we combined NPY and TH immunolabeling with intracellular injections of neurobiotin to aid in the classification of rat IMG neurons recorded. This technique did not reveal any differences in the basic membrane electrophysiological properties of neurons from NT and HT rats, and the soma area and average number of primary dendrites was not different in this restricted population of rat IMG neurons. In addition, the number of neurons containing immunolabel for NPY and TH did not differ between NT and HT groups. This series of experiments did reveal that the density of NPY immunolabeling is greater than TH in the rat IMG (figure 3). Compared to the guinea-pig IMG, it appears that NPY expression is more prevalent in the rat IMG (McLachlan and Llewellyn-Smith, 1986). Based on these data and on our finding that the proportion of phasic neurons of the rat IMG is greater than that of the guinea-pig, it is possible that a greater proportion of neurons in the

rat IMG project to the pelvic vasculature. It is also possible, though, that nonvasoconstrictor neurons of the rat IMG express NPY, as it is not an exclusive marker of vasoconstrictor neurons (Gibbins, 1995).

Electrophysiological properties of rat IMG neurons

The values reported here for resting membrane potential, input resistance, membrane time constant and action potential amplitude are comparable to those values reported for guinea pig prevertebral sympathetic ganglia (Cassell et al., 1986), as well as sympathetic ganglia of other species (Adams and Harper, 1995). An interesting finding was that the input resistance of tonic neurons was higher than in phasic neurons (table 1). Since input resistance is inversely related to membrane surface area, we initially thought that this difference may be due to a difference in the size of tonic neurons vs. phasic. However, our morphological findings did not support this hypothesis, as the average soma area and number of primary dendrites was not significantly different between phasic and tonic neurons. Since the presence of M channels (KNCQ) underlies phasic firing (Brown and Passmore, 2009, Marrion et al., 1989, Marrion, 1997), it may be the case that the input resistance of tonic neurons of the rat IMG is higher as a consequence of reduced expression of M channels in these cells.

A finding unique to the rat IMG was the relatively high proportion of phasic neurons present. In IMG recordings from both NT and HT animals, we found that 71.1% and 72.8% of the total number of neurons analyzed had phasic firing properties. In contrast, tonic neurons represent the majority of

IMG neurons in the guinea pig (Cassell et al., 1986), while phasic firing predominates in lumbar paravertebral ganglia. If the discharge properties of neurons of the rat IMG are related to the target tissue innervated, as suggested in previous reports on sympathetic ganglia of other species (Keast et al., 1993, Boyd et al., 1996), then it is possible that a greater number of neurons in the rat IMG are vasoconstrictor neurons.

Synaptic input to the rat IMG

The mean intermesenteric/lumbar splanchnic nerve conduction velocities reported here (1.13 meters per second NT and 1.17 meters per second HT) are within the range reported for prevertebral sympathetic ganglia of other species (Simmons, 1985). Thus, for the input from this nerve bundle, the information is conducted slowly, most likely along unmyelinated fibers. The conduction velocities showed no change in DOCA-salt hypertension.

There are two fundamental ways in which a postganglionic neuron may be stimulated to fire an action potential. Summation of sub-threshold inputs from preganglionic neurons may bring the postganglionic neuron to threshold and cause it to fire, or a single preganglionic input may be sufficient to bring the postganglionic neuron to threshold for AP generation (Jänig, 2006). In the studies presented here, most rat IMG neurons recorded responded to stimulation of the IMN bundle in a graded fashion, indicating the presence of multiple weak synaptic inputs to the postganglionic neurons (figure 6). However, when the proportion of neurons receiving strong vs. weak input were compared between NT and HT groups, we found that 55 percent of the neurons

recorded from hypertensive rats received strong synaptic inputs and only 37 percent of the IMG neurons from NT rats received strong inputs (table 2). This suggests that in hypertension, there is an increase in the safety factor for transmission of the impulse from pre to postganglionic neurons. Studies of guinea-pig prevertebral and guinea-pig and rat paravertebral sympathetic ganglia show that weak (sub-threshold) synaptic inputs predominate in prevertebral ganglia while strong inputs predominate in paravertebral ganglion neurons (Hirst and McLachlan, 1984, Hirst and McLachlan, 1986, Cassell et al., 1986). Postganglionic neurons of vasoconstrictor pathways are believed to receive predominantly strong inputs, while pathways involving gut motility and secretion are thought to function through multiple weak inputs (Jänig, 2006, Jänig and Habler, 2003, Jänig, 1995, McLachlan, 1995, McLachlan, 2007). Although this is not always the case for vasoconstrictor neurons (Bratton et al., 2010), this shift in the proportion of strong inputs is suggestive of a greater involvement of neurons of the rat IMG in vasoconstrictor functions than neurons of the guinea-pig IMG. An alternate explanation is that the strength of synaptic input is elevated in a general manner throughout the ganglion as a result of a widespread increase in central sympathetic outflow from the spinal cord in HT. However, given the evidence for regional and tissue specific activation of sympathetic pathways in hypertension (Esler et al., 1988, Esler, 2000, Victor and Shafiq, 2008, Esler, 2010, Malpas, 2010), this latter explanation is unlikely.

Long-term potentiation in neurons of the rat IMG

In the final series of experiments of this study we compared the response of rat IMG neurons to high frequency, low amplitude stimulation between NT and HT groups. Several recent studies have implicated a form of long term potentiation in certain models of hypertension (Aileru et al., 2001, Alkadhi and Alzoubi, 2007, Alkadhi et al., 2005b, Alzoubi et al., 2008, Alzoubi et al., 2010). While most of these studies measured LTP as an increase in the amplitude of a compound action potential, few studies have examined LTP of the EPSP amplitude measured with intracellular electrodes (Briggs, 1992, Briggs, 1995, Briggs and McAfee, 1988, Briggs et al., 1985), and there have been no studies to date that have investigated LTP in neurons of prevertebral ganglia in hypertension.

We found that approximately 70 percent of rat IMG neurons studied in NT and HT animals underwent a form of gLTP (either sub- or suprathreshold) (table 3). While the proportion of neurons responding to repetitive stimulation with sub-threshold LTP was not significantly different between NT and HT groups (3/17 NT and 9/14 HT), 82 percent (14/17) of IMG neurons from NT rats responded with suprathreshold LTP (in which the neuron responds to minimal stimulus amplitude nerve stimulation with action potentials rather than potentiated EPSPs). Only 36 percent (5/14) of neurons from HT animals did the same; this proportion is significantly less than that seen in the NT group (table 3). This result, when considered with our findings of an increased proportion of strong inputs in HT, suggest that there is a reduction in the

number of neurons available for suprathreshold LTP. In other words, it may be the case that increased preganglionic activation, as a consequence of the increased central sympathetic drive in HT, is contributing to an enhancement of synaptic transmission *in vivo* that renders a proportion of neurons that would otherwise be potentiated beyond AP threshold incapable of doing so (Alkadhi and Alzoubi, 2007, Alkadhi et al., 2005b, Alzoubi et al., 2008). If a neuron has been potentiated *in vivo*, when that same neuron is subjected to a repetitive stimulation protocol, it will fail to show a further enhancement in its response.

Conclusion

This work represents not only the first detailed characterization of neurons of the inferior mesenteric ganglion of the rat, but the first investigation of this ganglion in a disease model. This is also the first demonstration of longterm potentiation in a prevertebral sympathetic ganglion.

We show that several characteristics of the rat IMG are distinct from those of the well-studied guinea pig IMG, although many properties are similar. Interestingly, and distinct from previously published reports of other sympathetic ganglia, we show that the density of neuropeptide-Y immunolabeling is greater than that for tyrosine hydroxylase. Given this and the data showing that a greater proportion of rat IMG neurons have phasic firing properties than those of the guinea-pig, the possibility of a greater proportion of rat IMG neurons with a vasoconstrictor function is raised. Further experiments involving retrograde labeling and identification of rat IMG

neurons projecting to colonic and pelvic blood vessels will need to be performed to test this hypothesis.

When the basic membrane properties of rat IMG neurons from NT and HT rats were compared, no significant differences were found, suggesting that the baseline excitability of these neurons is not altered in DOCA-salt HT. Also, when fast EPSP amplitudes were compared between NT and HT, the mean values were similar. While this result suggests that baseline synaptic transmission is not enhanced in these neurons in HT, when we compared the proportion of neurons receiving strong vs. weak synaptic inputs, we saw that a greater number of neurons from HT animals receive strong synaptic input compared to NT. If this represents an *in vivo* change from weak to strong input with hypertension, the EPSP amplitudes of weak input neurons would not be different. Weak input neurons from HT animals would have been recruited to become strong input neurons and therefore would not be included in the comparison of NT vs. HT EPSP amplitudes.

This is consistent with our results from the LTP experiments. We observed that IMG neurons from NT rats are more susceptible to potentiation beyond action potential threshold compared to those from HT rats. This may be due to the existence of a saturation point for the long-term potentiation of EPSP amplitudes in these neurons (Alkadhi and Alzoubi, 2007, Alkadhi et al., 2005b). Increased synaptic input from the spinal cord in DOCA-salt HT rats is resulting in potentiation beyond action potential threshold *in vivo* and is therefore preventing further increases in potentiation as a result of saturation

of the response. This is similar to the saturation of gLTP as measured with extracellular electrodes (Alkadhi and Alzoubi, 2007). In certain models of hypertension, compound action potentials from NT animals are more susceptible to potentiation than those from HT (Alzoubi et al., 2008, Alzoubi et al., 2010, Gerges et al., 2002). If the amplitude of a compound action potential is a function of the total number of action potentials generated in the nerve recorded, then an increase in the number of neurons receiving strong inputs (as a result of EPSP potentiation beyond AP threshold) would explain the apparent lack of potentiation observed in NT animals.

There are three main factors that influence the strength of nicotinic synapses in autonomic ganglia. These include anatomical considerations (i.e., the proximity of the synapse to the soma and the number of contacts made by the preganglionic neuron), the amount of transmitter (acetylcholine) release from the preganglionic terminal, and finally, the excitability of the postganglionic neuron, including the resting potassium conductance and the membrane potential in the proximity of the synapse (Jänig, 2006). From our anatomical observations, there are no significant differences in the amount of dendritic arborization in IMG neurons from NT vs. HT rats, and we've demonstrated that the basic membrane properties of these neurons are not altered in HT. However, the possibility of increased release of acetylcholine from the preganglionic terminals (Magee and Schofield, 1992, Magee and Schofield, 1994, Magee and Schofield, 1995, McLachlan, 1975) remains plausible given these findings and the results of the experiments involving LTP.

We have presented data that suggest that the strength of synaptic transmission through the rat IMG is elevated in DOCA-salt hypertension, while the general excitability and overall electrophysiological and morphological properties remain unchanged. Although further studies are necessary to determine the precise mechanisms behind these changes in synaptic transmission, it is possible that this increase in synaptic strength contributes to elevated sympathetic tone to the abdomino-pelvic vasculature in HT.
CHAPTER THREE:

REDUCED INHIBITORY EFFECTS OF EXOGENOUS GLUCOCORTICOID IN VEIN-PROJECTING NEURONS OF THE SUPERIOR MESENTERIC GANGLION OF DOCA-SALT HYPERTENSIVE RATS

ABSTRACT

All efferent sympathetic information from preganglionic neurons of the spinal cord must pass through either the paravertebral or the prevertebral sympathetic ganglia before the signal reaches the target tissue. The prevertebral sympathetic ganglia, which include the celiac, superior and inferior mesenteric ganglia, are the last point of signal integration in the final common efferent pathway to the abdomino-pelvic viscera. The veins of the abdomino-pelvic region constitute the largest reservoir of blood in the entire body, containing over 60% of the total blood volume at rest. A significant portion of this blood can be mobilized during systemic cardiovascular responses through increased sympathetic outflow from the prevertebral ganglia to preserve perfusion of vital structures. In chronic diseases like hypertension, it is likely that a general reduction in venous capacitance in the abdominopelvic cavity, due to pathological increases in sympathetic outflow, results in a shift in blood volume to the systemic arteries and thereby contributes to the elevation in mean arterial pressure.

The prevertebral sympathetic ganglia are in a position unique to the higher centers of the sympathetic nervous system in that the principal ganglion neurons reside outside of the blood brain barrier and are therefore subject to modulation by humoral as well as neural input. In many forms of HT, and in situations of chronic stress, prolonged periods of elevated circulating corticosteroid concentrations occur. Postganglionic sympathetic neurons,

including those of prevertebral ganglia, have been shown to express both mineralocorticoid (MR) and glucocorticoid receptors (GR), and activation of GR have been shown to have rapid inhibitory effects on prevertebral sympathetic ganglion neurons. We hypothesized that prolonged elevations in corticosteroids (and their precursors, including deoxycorticosterone-acetate) during HT results in a change in the function of GR on neurons of the superior mesenteric ganglion that project to blood vessels.

Therefore, in this work, we combined pseudorabies virus retrograde tracing with *in vitro* sharp electrode recording from intact superior mesenteric ganglion neurons from normotensive (NT), and DOCA-salt HT adult rats. Comparison of the majority of basic electrophysiological properties revealed no significant differences between identified vein- and artery-projecting neurons from NT and HT rats. However, an examination of the effects of exogenous glucocorticoid revealed that vein-projecting neurons from HT rats are less susceptible to the inhibitory effects of GR activation when compared to arteryprojecting neurons from HT rats as well as vein- and artery-projecting neurons from NT rats. This reduction in glucocorticoid-mediated inhibition may be a novel mechanism in which vein-projecting neurons of the prevertebral sympathetic ganglia experience a maintained level of excitability in DOCA-salt HT.

INTRODUCTION

All efferent sympathetic information from preganglionic neurons of the spinal cord must pass through either the paravertebral or the prevertebral sympathetic ganglia before the signal reaches the target tissue. The prevertebral sympathetic ganglia, which include the celiac, superior and inferior mesenteric ganglia, are the last point of signal integration in the final common efferent pathway to the abdomino-pelvic viscera (Jänig, 2006). These ganglia are of interest in the context of hypertension (HT) because they contain the postganglionic vasoconstrictor neurons that regulate both the resistance vessels (i.e., mesenteric arteries) and the capacitance vessels (i.e., the mesenteric veins) of the abdomino-pelvic cavity. The veins of the abdominopelvic region constitute the largest reservoir of blood in the body, containing over 60% of the total blood volume at rest (Greenway, 1983). A significant portion of this blood can be mobilized through increased sympathetic outflow from the prevertebral ganglia during systemic cardiovascular responses (e.g. exercise or hemorrhage) to preserve perfusion of vital structures (Greenway et al., 1994). In chronic diseases like hypertension, it is likely that a general reduction in venous capacitance in the abdomino-pelvic cavity, due to pathological increases in sympathetic outflow (Malpas, 2010, Esler, 2010, Joyner et al., 2008), results in a shift in blood volume to the systemic arteries (Fink, 2009, Brengelmann, 2003) and thereby contributes to the elevation in mean arterial pressure.

For a number of years, viral retrograde tracers have been used to map the circuitry of the central sympathetic pathways. In most cases, tracers were introduced to select target tissues (usually visceral organs and occasionally sympathetic ganglia) and anatomical reports of first, second, and subsequent order neurons in the pathway were generated (Smith et al., 1998, Jansen et al., 1995, Strack et al., 1989). The recent development of pseudorabies viral constructs that express enhanced green and red fluorescent proteins have allowed for identification and electrophysiological study of neurons of specific pathways (Loewy, 1998, Banfield et al., 2003, Smith et al., 2000). However, this technique has not been applied to first order neurons of prevertebral ganglia that innervate abdominal blood vessels, and furthermore, it has not been used to compare the properties of individual neurons of a specific functional pathway in HT.

The prevertebral sympathetic ganglia are in a position unique to the higher centers of the sympathetic nervous system in that the principal ganglion neurons reside outside of the blood brain barrier and are therefore subject to humoral as well as neural input. The association between the stress response and hypertension has been well documented (De Kloet and Derijk, 2004, Frey et al., 2004, Gomez Sanchez, 1991, Esler et al., 2008). While both the risk and the incidence of hypertension rise with age in the United States (Roger et al., 2012b), a longitudinal study that compared 144 nuns of a monastic order with 138 laywomen over 20 years showed that isolation from society (and the subsequent reduction in mental stress) prevents this rise in the occurrence of

essential hypertension with age (Timio et al., 1988). Furthermore, over 80 percent of patients with Cushing's syndrome have hypertension (Ong and Whitworth, 2011), and 5-10% of hypertensive patients are afflicted with hypertension secondary to adrenal gland disorders (Young, 2007b, Young, 2007a). In many forms of HT, and in situations of chronic stress, prolonged periods of elevated circulating corticosteroid concentration occur (Mulatero et al., 2007).

The classic understanding of the mechanism of action of corticosteroid hormones involves the activation of mineralocorticoid (MR) and glucocorticoid (GR) receptors (which function as inducible transcription factors) and subsequent regulation of gene transcription (Prager and Johnson, 2009). However, recently it has become widely understood that corticosteroids exert rapid effects on neuronal function in addition to the genomic mechanisms (Groeneweg et al., 2012). Postganglionic sympathetic neurons, including those of prevertebral ganglia, have been shown to express both mineralocorticoid (MR) (McLennan et al., 1984) and glucocorticoid receptors (GR) (Hua and Chen, 1989, Towle and Sze, 1982). In the brain, activation of MR by corticosteroids generally results in excitatory effects while activation of GR usually results in reduced membrane excitability (Joels et al., 2012). Prolonged exposure of cells to corticosteroids (as occurs in chronic stress and in some forms of HT) can alter the balance in expression and function of MR and GR (De Kloet and Derijk, 2004, Zhe et al., 2008).

In this work, we combined retrograde tracing (using pseudorabies virus constructs) with *in vitro* sharp electrode recording from intact superior mesenteric ganglion neurons from normotensive (NT), and DOCA-salt HT adult rats. Comparison of the majority of basic electrophysiological properties revealed no significant differences in SMG neurons between NT and HT groups. Also, the amplitude of the depolarization induced by exogenous nicotine was reduced in both vein- and artery-projecting neurons from HT rats. However, an examination of the effects of exogenous glucocorticoid revealed that veinprojecting neurons from HT rats are less susceptible to the inhibitory effects of GR activation when compared to artery-projecting neurons from HT rats, as well as vein- and artery-projecting neurons from NT rats. This reduction in glucocorticoid-mediated inhibition may be a novel mechanism in which veinprojecting neurons of the prevertebral sympathetic ganglia experience increased excitability in DOCA-salt HT.

METHODS

Animals

All procedures involving the use of live animals were approved by the Institutional Animal Care and Use Committee of Michigan State University and were performed in accordance with the American Physiological Society's "Guiding Principles in the Care and Use of Animals". Male Sprague-Dawley rats, obtained from Charles River Laboratories (Portage, MI), initially weighing approximately 250g were used in all studies. In the initial experiments in which the basic membrane properties of artery- and vein-projecting neurons were compared, untreated rats were used. For the experiments involving the comparison of neurons from normotensive and hypertensive rats, the deoxycorticosterone-acetate/salt (DOCA-salt) model of hypertension was used. The technique for preparing the DOCA-salt animals has been described in detail previously (Demel et al., 2010). Briefly, rats of both the NT and HT groups were subjected to left-kidney uninephrectomy (under isoflurane anesthesia). HT rats were implanted with a subcutaneous DOCA pellet that resulted in an equivalent dose of 200mg/kg and were given salt water (containing 1% NaCl + 0.2% KCl) to drink. NT rats did not receive the DOCA implant and were given tap water. All animals received a diet of standard rat chow ad libitum. Rats weighed approximately 400g at the time of electrophysiological recording. NT animals had an average tail-cuff systolic blood pressure of 130 ± 1.8 mmHg and DOCA-salt HT animals had mean blood

pressures of 197 ± 3.5 mmHg. A total of 28 rats were used in this study (12 untreated, 8 HT, and 8 NT).

Pseudorabies-virus retrograde tracing

Under isoflurane anesthesia, an abdominal incision was made to access the small intestine and mesenteric blood vessels. Adjacently located secondary branches of superior mesenteric artery and vein 5-10 mm in length were pinned onto a glass petri dish coated with Sylgard (Dow Inc.), cleared of surrounding fat, separated from each other, and encased in polyethylene tubing. During this time, the blood vessels and surrounding tissues in the petri dish were constantly bathed with Hanks' balanced salt solution (HBSS) (Gibco). Both ends of the polyethylene tubing were sealed with silicone sealant (Dow Inc.) and retrograde tracers were applied to the blood vessels with micropipettes (~5-10 μ L) and incubated at room temperature for 30 minutes. Then, the tracers were removed from the blood vessels, and the vessels were washed 3X with HBSS. The blood vessels were returned inside the abdominal cavity, the abdominal incision sutured, and the rat was left to recover for 3-4 days prior to electrophysiological recording.

Two isogenic strains of the vaccine PRV Bartha strain were used as retrograde tracers (Banfield et al., 2003): one that expressed the enhanced green fluorescent protein (EGFP; PRV-152) and another that expressed the red fluorescent protein (RFP; PRV-614). These tracers were a kind gift of Dr. Lynn Enquist at Princeton University. Use of these tracers was counterbalanced on arteries and veins and no significant difference was observed in labeled

neurons obtained with PRV-152 or PRV-614 (data not shown). It has been shown that basic electrophysiological properties in PRV infected CNS neurons with short survival times (\leq 4d) are identical to those in uninfected neurons (Smith et al., 2000).

In vitro rat superior mesenteric ganglion preparation

Rats were given a lethal dose of sodium pentobarbital (1-2cc, 6.25% i.p.), and following demonstration of adequate anesthesia (while the animal was still respiring), an incision was made from the umbilicus to the xiphoid process in the skin and then musculature of the ventral surface of the abdomen. The small and large intestines were reflected to expose the abdominal aorta. The left common iliac artery was then transected to allow exsanguination of the animal. While holding the animal upright, the visceral peritoneal reflections that connected the liver to the diaphragm were cut, and the esophagus, stomach, duodenum and small intestine were removed. Care was taken to ensure that a ~15mm section of the superior mesenteric artery was left intact when the small intestine was removed. The abdominal aorta was then transected just below the renal arteries and the abdominal aorta, with the renal, superior mesenteric, inferior mesenteric and hepatic arteries, was removed in a caudo-rostral manner. The paraspinal musculature was removed along with the aorta, to ensure that no ganglionic tissue was accidentally severed.

The aorta, the surrounding muscle and fat, and the CG/SMG complex was then transferred to a petri dish that had been coated with Sylgard (Dow,

Inc.) and filled with Krebs' solution. The Krebs solution was used as the perfusion solution during the subsequent electrophysiological experiments. It consisted (in mM) of NaCl 118.5, KCl 4.7, KH₂PO₄ 1.2, NaHCO₃ 23.8, dextrose 5.5, and was bubbled with $95\%O_2/5\%CO_2$ to bring the pH down to 7.4. Small insect pins were used to stabilize the tissue while the musculature near the dorsal surface of the aorta was removed. The superior mesenteric ganglion was located by following the intermesenteric nerve rostral to the superior mesenteric artery. Adipose tissue and overlying connective tissue was carefully removed from one surface of the SMG and the celiac ganglion. The superior mesenteric artery was transected and removed, along with the aorta and celiac artery, thereby leaving only the CG/SMG complex, some additional visceral peritoneum (mesentery), and the nerve bundles associated with the ganglia. With the preparation illuminated from below, the nerves and mesentery were pinned to the bottom of the petri dish. All remaining adipose tissue and mesentery was then removed from the surface of both the CG and SMG. Finally, under 300x magnification, fine forceps were used to remove the connective tissue/neuropil "capsule" that covered the surface of the SMG and CG. Care was taken to prevent the tip of the forceps from exerting downward pressure onto the ganglion surface. When properly executed, this final step resulted in a very high number of "clean" neurons at the surface of the ganglion that were readily accessible for recording.

Electrophysiology

The recording chamber consisted of a 5mL petri dish with a Sylgardcoated bottom, custom-mounted onto the stage of an Olympus BXW51 microscope. The chamber was perfused at a rate of 5-10 milliliters per minute with Krebs' solution. Intracellular recordings were performed at room temperature (~25 °C) using sharp microelectrodes filled with 1M K-acetate. Electrode tip resistances ranged from 50-80 MΩ. 2% Neurobiotin (VWR) was added to the internal solution during experiments in which recorded neurons were identified. An Axoclamp 2A (Axon Instruments) amplifier with an HS-2A headstage, (gain=0.1) was used to record the signal from the targeted neurons. The membrane voltage signal was digitized with a Digidata 1320A digitizer and saved onto a Dell computer equipped with pClamp9 (Axon Instruments) software. All recordings were made with the amplifier in bridge mode.

The superior mesenteric ganglion was located at low power (10x) and the recording electrode lowered toward the tissue. Neurons that were infected with PRV 152 or 614 were identified through epifluoresence illumination under high power magnification (40x, water immersion objective) with the appropriate filter cubes selected for GFP or RFP.

Successful impalement of identified neurons resulted after a brief tap on the bench or brief use of the "buzz" function of the amplifier. A voltage deflection of at least -35mV that was stable for a minimum of five minutes constituted a successful impalement. The bridge balance was adjusted when

the electrodes first entered the tissue bath and were monitored and adjusted accordingly during the experiments. Initially, a series of depolarizing current pulses were passed, starting from -1nA and progressing up to rheobasic current. This protocol was used to generate I-V plots for calculating neuronal input resistance. Next, a long-pulse protocol involving 1 second, depolarizing pulses (beginning at 0nA and progressing up to 2.5x rheobase) was used to determine whether a neuron had phasic or tonic firing properties. A third protocol was used to investigate afterhyperpolarization properties. A 0.5ms, 10nA pulse was applied through the recording electrode and the membrane potential recorded for 2 seconds thereafter. For the intracellular labeling experiments, positive current pulses (1Hz) were applied for 1-3 minutes to allow sufficient filling of neuronal somata with neurobiotin.

Pharmacological experiments were performed using reagents obtained from Sigma-Aldrich (St. Louis, MO). Nicotine ±, FW 162.23, hydrocortisone 21-hemisuccinate, FW 484.5, and the glucocorticoid receptor antagonist mifepristone (RU 486) FW 429.59, were prepared from stock solutions (nicotine was initially dissolved in 50% ethanol, while hydrocortisone and mifepristone were initially dissolved in 50% DMSO; final concentrations of ethanol and DMSO were <1.0%) on the day of each experiment and were administered directly into the recording chamber with the Krebs' flow stopped. The concentrations reported are the final concentrations in the recording chamber. Perfusion with Krebs' was resumed after application of each reagent (as indicated by the artifacts on the traces in figures 2-6).

Imaging and immunohistochemistry

Following the recording session, ganglia were fixed in 4% Paraformaldehyde/4% sucrose for 4 hours at room temperature and then cryoprotected in 20% phosphate-buffered sucrose overnight at 4°C. The following day, the tissue was processed for whole-mount immunohistochemistry. A 24well cell culture plate was used for all wash and incubation steps. The tissue was transferred from well-to-well with a piece of electron-microscopy grid attached to a steel handle to minimize tissue disruption and to prevent transfer of solutions to each successive well. The tissue was first washed in phosphatebuffered saline (0.1M) containing 0.2% TritonX-100 (PBS/Tx 0.2%) (5min x 3, $10\min x 1$; then washed $5\min x 2$ in 0.1M phosphate-buffered saline (PBS). Slides were then incubated in Texas Red/Avidin-D, diluted to 1:250 in PBS/Tx 0.1% for one hour. The tissue was then washed again for 5min x 3 and 10min x 1 in PBS/Tx 0.2%. A final rinse in PBS (5min x 3, 10min x 1) preceded mounting of the tissue on standard glass slides. Prolong Gold Anti-fade reagent (Molecular Probes) was applied prior to coverslipping. Confocal images were acquired using an Olympus Fluoview-F1000 Spectral confocal microscope. PMT voltage and gain settings were optimized for each channel prior to collection of sequential Z-stack images. During each electrophysiology experiment, images were also captured using the infrared camera attached to the fixed-stage BXW51 microscope. ImagePro software was used for collection and analysis of these images.

Analysis

Values are presented as mean \pm SEM. Means were compared using a two-tailed Student's t-test. P values less than 0.05 were considered significant. Recordings were included in the analysis only if the recording was stable for at least 5 minutes following impalement, the fluorescent signal from the PRVlabeled neuron did not diminish in intensity during the recording, the membrane potential was at least -35mV, and the series resistance did not increase more than 10 M Ω throughout the duration of the recording.

RESULTS

Retrograde identification of vein- and artery-projecting neurons of the SMG

A total of 96 neurons from 28 rats were recorded in vitro, following in vivo pseudorabies-virus (PRV) application to sections of mesenteric vein or artery. Recordings were stable well beyond 1 hour, but were usually terminated once all of the necessary data were collected. Figure 13 shows examples of our application of this technique to neurons of the rat superior mesenteric ganglion (SMG). At the time of recording, numerous PRV-infected neurons could be seen throughout the ganglion. Most of the infected cells were located in the SMG, but many neurons could also be seen in the either lobe of the celiac ganglion in some preparations. The analysis was restricted to SMG neurons largely as a result of the large number of labeled neurons (the blood vessels labeled were tributaries of the superior mesenteric vein and artery), and because more of the surface of the ganglion could be exposed (as it is a singlelobed ganglion unlike the celiac ganglion). Infected neurons were not localized to any particular region of the ganglion, nor did they appear to share any specific morphological properties. Cells were approximately 30 micrometers in diameter and most had several processes projecting from the soma. While infrared and epifluorescence were sufficient to verify that the electrode had impaled an infected neuron, we performed several experiments in which recorded cells were filled with neurobiotin. Figure 13 C-E shows an infected

SMG neuron following intracellular labeling and subsequent immunohistochemical processing.

Basic membrane properties of identified vein- and artery-projecting SMG neurons

Once the ability to reliably label and target the vein- and arteryprojecting neurons of the SMG was confirmed, a series of experiments were performed on untreated Sprague Dawley rats (i.e., that were not uninephrectomized or given a DOCA implant). Our goal was to determine whether the basic properties of artery- and vein-projecting neurons are fundamentally different in untreated SD rats. The results of these experiments are summarized in Table 4. There were no significant differences seen in any of the properties examined between PRV-identified vein- and artery-projecting SMG neurons. Average resting membrane potentials were -43 and -47 millivolts for vein-projecting and artery-projecting neurons, respectively. Cell input resistances ranged from 63 to 76 M Ω . Membrane time constants for both vein- and artery-projecting neurons were approximately 12 milliseconds. Action potential amplitudes were also similar $(62 \pm 3 \text{ for vein-projecting})$ neurons and 71 ± 4 millivolts for artery-projecting neurons); as were the action potential AHP amplitudes (6.8 and 7.7 millivolts) and durations (366 and 559 milliseconds) for vein- and artery-projecting neurons, respectively. These values are consistent with previous descriptions of mammalian prevertebral sympathetic ganglion neurons (Jänig, 2006, Adams and Harper, 1995), and are similar to the values seen in our lab for the rat inferior mesenteric ganglion.

When we compared the firing properties of vein- and artery-projecting neurons, we found that the majority of both neuron types have phasic firing properties in response to a maintained depolarizing stimulus (Table 5). This finding is consistent with findings from pre- and paravertebral ganglion neurons performed in other species, in which phasic firing is one characteristic of neurons in vasoconstrictor pathways (Jänig and Habler, 2003, McLachlan, 1995, McLachlan, 2007).

Properties of vein- and artery-projecting SMG neurons from NT and DOCAsalt HT rats

We then compared the properties of vein- and artery-projecting neurons from NT and DOCA-salt HT rats. As shown in Table 6, the passive and active membrane properties of vein- and artery-projecting neurons were similar between NT and HT groups. The average resting membrane potentials ranged from -41 mV in artery-projecting neurons from HT rats to -50 mV in arteryprojecting neurons from NT rats. Average input resistances ranged from 65 to 96 M Ω . Membrane time constant averages ranged from 8 to 15 milliseconds. Action potential amplitudes were 60 mV in artery-projecting neurons from HT rats and 71 mV in vein-projecting neurons from HT rats. Action potential afterhyperpolarization amplitude averages ranged from 7.0 ± 0.8 mV in arteryprojecting neurons from HT rats to 12 ± 1 mV in artery-projecting neurons from NT rats. Afterhyperpolarization durations were 270 ms in arteryprojecting neurons from HT rats and 600 ms in artery-projecting neurons from NT rats. With the exception of the action potential afterhyperpolarization amplitudes, none of the properties were significantly different between vein-

and artery-projecting neurons. Analysis of firing patterns in response to maintained depolarizing current injections showed, as was the case for the untreated rats, that the majority of vein- and artery-projecting neurons are phasic neurons (table 5). There were no differences in the distribution of phasic and tonic neurons between NT and HT groups, or between artery- and vein-projecting neurons of each group. No additional significant differences were revealed when the properties of vein-projecting neurons were compared between NT and HT groups, nor when artery-projecting neurons were compared between NT and HT groups. These data suggest that in HT, the basic membrane properties of SMG neurons projecting to mesenteric blood vessels are not altered. Also, these results suggest, as shown in previous studies comparing infected vs. non-infected neurons, that infection with PRV-152 or 614 does not adversely affect the functional properties of these neurons (Hofstetter et al., 2005, Banfield et al., 2003, Smith et al., 2000).

Nicotine has reduced efficacy in vein- and artery-projecting SMG neurons from HT rats

Next, we were interested in comparing the response of identified neurons to exogenous nicotine. It is possible that, in hypertension, the transmission of synaptic impulses from pre- to postganglionic neurons is altered (Magee and Schofield, 1994, Alkadhi et al., 2005a). Past studies have suggested a change in membrane properties of rat superior cervical ganglion neurons from spontaneously hypertensive rats (Jubelin and Kannan, 1990, Robertson and Schofield, 1999). As the nicotinic acetylcholine receptor is a major element in the link between central and peripheral sympathetic neurons, we performed a series of experiments to determine whether artery- or vein-projecting SMG neurons from HT rats show any shift in their response characteristics. Nicotine application resulted in depolarization of the neurons in a dose-dependent manner (figure 14A); the final concentration of 10 uM was chosen for the remainder of the experiments in which artery- and vein-projecting neurons were compared between NT and HT groups. Figure 15A shows the response of a SMG vein-projecting neuron from a NT rat to application of 10 uM nicotine. The responses of artery- and vein-projecting neurons from NT rats were similar (~28 mV depolarization peak, with a relatively fast rise and a slow decay). However, the peak amplitude of the depolarization induced by nicotine was significantly less in artery-projecting neurons from HT rats (20.3 \pm 1.6 mV, n=9) compared with NT (27.8 \pm 2.3 mV, n=9) (figure 15C). The peak depolarization amplitude was significantly lower in vein-projecting neurons from HT rats (8.4 \pm 2.6, n=10) vs. NT (28.0 \pm 2.1, n=10) as well (fig 15B).

Reduced inhibitory effect of hydrocortisone on vein-projecting neurons from hypertensive rats

In the final portion of this study, the effects of the glucocorticoid, hydrocortisone 21-hemisuccinate (CORT) on resting membrane potential were investigated in artery- and vein-projecting neurons from NT and HT rats. Therefore, in order to uncover any alterations in the response to glucocorticoids in DOCA salt HT, we used CORT at a concentration high enough to activate both the mineralocorticoid and glucocorticoid receptors in neurons of the SMG. We chose to use hydrocortisone rather than corticosterone because it has been used in previous studies on rat prevertebral ganglia (Towle and Sze, 1982, Hua and Chen, 1989) and because it is relatively easy to prepare in aqueous solutions compared with the more hydrophobic steroid hormones DOCA, corticosterone, and dexamethasone.

CORT (100 uM) introduction resulted a ~5 mV hyperpolarization that lasted about 30 seconds (figure 16A). This effect was seen in artery-projecting neurons from both NT and HT rats (figure 16C), and in vein-projecting neurons from NT rats (figure 16B). The amplitude of the hyperpolarization in veinprojecting neurons from HT rats (-1.11 \pm 0.59 mV, n=9) was significantly less than that of vein-projecting neurons from NT rats (-5.45 \pm 0.78 mV, n=7), and was also significantly less than the amplitude seen in artery-projecting neurons from both NT (-5.32 \pm 1.04 mV, n=5) and HT (-4.39 \pm 0.94 mV, n=7) rats. As seen in figure 14B, the amplitude of the hyperpolarization induced by CORT was dependent upon the concentration applied.

In addition to the receptor mediated effects, glucocorticoids can directly inhibit nicotinic acetylcholine receptors in sympathetic ganglia (Barrantes et al., 2000, Uki et al., 1999). Therefore, we performed a series of experiments in which we tested the ability of CORT to reduce or inhibit the peak amplitude of the depolarization that results from application of exogenous nicotine. In these experiments, we would first apply nicotine and measure the amplitude of the depolarization at its peak; CORT was then applied once the peak nicotineinduced depolarization was reached (figure 17A). In most cases, CORT reduced the amplitude of the nicotinic depolarization by ~50-75% (measured by dividing the amplitude of the reduction in the peak nicotine-induced depolarization

amplitude by the initial peak amplitude) (figure 17). In artery-projecting neurons from NT and HT rats, CORT reduced the peak nicotinic depolarization by $61.39 \pm 10.01\%$, n=6 and $75.72 \pm 5.83\%$, n=7, respectively (figure 17C). Similarly, CORT reduced the peak amplitude of the nicotine-induced depolarization in vein-projecting neurons from NT rats ($52.14 \pm 11.93\%$, n=7). CORT did not have the same inhibitory effect on vein-projecting neurons from HT rats, however. The nicotine-induced depolarization was only reduced by $13.13 \pm 6.89\%$, n=8 in these neurons (figure 17B). This amount was significantly less than the inhibition seen in vein-projecting neurons from NT rats, and in artery-projecting neurons from NT and HT rats (figure 17B and C). These results suggest that vein-projecting neurons from HT rats are less responsive to the inhibitory effects of CORT in DOCA-salt hypertension.

Mifepristone (RU 486) inhibits the hyperpolarization induced by hydrocortisone

If the inhibitory effects of CORT are mediated by the glucocorticoid receptor, then a selective antagonist of that receptor (mifepristone RU 486) should abolish the hyperpolarization response in neurons from NT animals. Therefore, in our final set of experiments, the ability of RU 486 to antagonize the acute effect of hydrocortisone on vein- SMG neurons from NT rats was examined. While RU 486 (1 uM) did not have any effect on the membrane potential of SMG neurons when applied alone (figure 18), when applied prior to CORT, the response of the membrane converted from a -5.0 \pm 1.6 mV, n=3 hyperpolarization to a depolarization of 1.4 \pm 0.9 mV, n=3 mV (figure 18).



Figure 13: Identified neurons of the intact superior mesenteric ganglion

(A) 40x transmitted light image of an artery-projecting neuron infected with PRV-614 during intracellular recording; (B) Epifluorescence illumination showing red fluorescent protein in this neuron; (C) A 60x projection image of a PRV-152 infected neuron expressing green fluorescent protein; (D) The same neuron as labeled during intracellular recording with neurobiotin; (E) merged image.

	Vein	Artery
Resting Membrane Potential (mV)	-43 ± 1.4	-47 ± 1.7
	(19)	(13)
Cell Input Resistance (MΩ)	63 ± 8	76 ± 10
	(20)	(13)
Membrane Time Constant (ms)	12 ± 4	13 ± 2
	(20)	(13)
Action Potential Amplitude (mV)	62 ± 3	71 ± 4
	(20)	(12)
Afterhyperpolarization Amplitude (mV)	6.8 ± 0.6	7.7 ± 1.2
	(18)	(12)
Afterhyperpolarization Duration (ms)	366 ± 73	559 ± 134
	(18)	(12)

Table 4: Properties of vein- and artery-projecting neurons of the SMG from untreated rats

Number of neurons analyzed in parentheses

		Phasic	Tonic
Untreated	Vein	17	3
	Artery	12	1
Normotensive	Vein	10	2
	Artery	6	2
Hypertensive	Vein	9	2
	Artery	4	2

Table 5: Phasic firing is predominant in the rat SMG

Values represent the total number of neurons observed

		Vein	Artery
Resting Membrane Potential (mV)	NIT	-46 ± 2	-50 ± 4
	IN I	(13)	(10)
		-48 ± 2	-41 ± 2
	HI	(12)	(7)
Cell Input Resistance (MΩ)	NT	96 ± 12	74 ± 14
		(12)	(10)
		85 ± 12	65 ± 10
		(12)	(7)
Membrane Time Constant (ms) NT		15 ± 2	10 ± 2
	NI	(12)	(10)
		13 ± 12	8 ± 2
	HI	(12)	(7)
Action Potential Amplitude (mV)	NT	62 ± 5	65 ± 5
		(12)	(9)
	υт	71 ± 5	60 ± 4
	пі	(12)	(6)
Afterhyperpolarization Amplitude (mV)	NT	10 ± 1	12 ± 1
		(9)	(5)
		11 ± 1	*7 ± 0.8
		(9)	(5)
Afterhyperpolarization Duration (ms)	NT	283 ± 100	600 ± 230
		(9)	(5)
	υт	504 ± 125	270 ± 91
	пі	(9)	(5)

Table 6: Properties of artery- and vein-projecting SMG neurons from NT and HT rats

*Statistically significant vs. vein-projecting neurons, P=0.03; number of neurons analyzed in parentheses



Figure 14: Dose-dependent effects of nicotine and hydrocortisone on SMG neurons

(A) The depolarization amplitude evoked by nicotine and (B) The hyperpolarization amplitude evoked by hydrocortisone 21-hemisuccinate were dependent upon the concentration applied.



Figure 15: The peak amplitude of the depolarization induced by nicotine is reduced in vein and artery-projecting neurons from HT rats



***P<0.0001 vs. NT, n=10 NT, n=10 HT; **P<0.02 vs. NT, n=9 NT, n=9 HT





(A) A representative trace from a vein-projecting neuron of a NT animal; 100uM hydrocortisone was applied at the first artifact; the wash began at the second artifact on the trace; (B) and (C) Histograms showing the amplitude of the peak hyperpolarization induced by 100uM hydrocortisone; ***P=0.0005 vs. NT, n=7 NT, n=9 HT



Figure 17: Hydrocortisone failed to reduce the amplitude of the depolarization induced by nicotine in vein-projecting SMG neurons from HT rats

(A) A representative trace from an artery-projecting neuron from a NT animal; 10uM nicotine was applied at the first artifact; 100uM hydrocortisone was applied at the second artifact, when the nicotinic depolarization was at its peak; the third artifact marks the beginning of the Krebs' wash; (B) and (C) Histograms showing the reduction in peak nicotine-induced depolarization by hydrocortisone in vein and artery neurons from NT and HT rats as a percentage of the initial peak nicotinic depolarization amplitude. **P*<0.02 vs. NT, *n*=7 NT *n*=8 HT

Figure 18: The hyperpolarization induced by hydrocortisone is inhibited by the glucocorticoid receptor antagonist RU 486



DISCUSSION

Vein- and artery-projecting neurons of the rat superior mesenteric ganglion

The heterogeneous nature of the neuronal population of prevertebral sympathetic ganglia creates a significant obstacle to the study and comparison of the properties of neurons of target specific pathways (McLachlan, 2007, Keast et al., 1993, Jänig and McLachlan, 1992). In this study, we were able to label neurons of the SMG that projected to mesenteric veins and arteries through infection of these neurons with pseudorabies-viral constructs. This method of neuronal identification provides numerous advantages over other ways of identifying neurons of specific functional pathways. When compared to non-viral tracers such as fluoro-gold and fast blue, it is clear that PRV tracers provide greater specificity (Puigdellivol-Sanchez et al., 2002) as a result of their selective uptake at sites of synaptic transmission (Banfield et al., 2003). Also, the relative ease of use of viral tracers confers significant advantage over the use of concurrent *in vivo* intracellular recording (where cardiovascular rhythmicity is used to determine whether neurons are involved in vasoconstrictor pathways) (Bratton et al., 2010).

Properties of identified vein- and artery-projecting SMG neurons from untreated and NT vs. HT rats

The resting membrane potential, input resistances, membrane time constants and action potential amplitudes that we observed in this study for identified vein- and artery-projecting SMG neurons were similar to those

reported previously for rat prevertebral ganglion neurons (using intracellular recording techniques) (Jänig, 2006, Adams and Harper, 1995, Simmons, 1985). There were no significant differences in any of these properties when vein- and artery-projecting neurons were compared (table 4).

A comparison of the basic electrophysiological properties of vein- and arteryprojecting SMG neurons from uninephrectomized normotensive (NT) and DOCA-salt HT rats revealed no significant differences as well (table 6), with the exception of artery-projecting neurons from HT rats having reduced action potential AHP amplitudes vs. artery-projecting neurons from NT rats. While it is possible that this difference is a result of reduction in the calcium-activated potassium currents that underlie the afterhyperpolarization (Hosseini et al., 2001, Chen et al., 2010, Xu et al., 2005), additional experiments are necessary verification. All other measured properties of vein-projecting neurons were not significantly different from artery-projecting neurons, and they were not altered in DOCA-salt HT. Furthermore, none of these parameters showed any significant differences when compared to the results for untreated rats (not shown). These results demonstrate that SMG neurons that have been infected with PRV tracers retain their normal physiological properties. This has been studied and confirmed other neuronal populations as well (Smith et al., 2000, Banfield et al., 2003) for the use of PRV tracers in electrophysiological studies.

An analysis of the firing pattern of identified SMG neurons from NT and HT rats showed that most artery- and vein-projecting neurons have phasic firing properties in response to direct, maintained depolarization of the membrane

(table 5). In untreated, as well as in neurons from NT and HT rats, the proportion of phasic neurons is greater than the proportion of tonic neurons. This observation is consistent with and serves as further support of the concept that most vasoconstrictor neurons have phasic firing properties, as studies from rat and guinea-pig prevertebral and paravertebral ganglia suggest (Boyd et al., 1996, Keast et al., 1993, Jänig and Habler, 2003, Jänig, 1995, Jänig, 2006). However, a comparison of vein- and artery-projecting neurons of the guinea pig IMG identified with Fluoro-gold and/or rhodamine beads (Browning et al., 1999) reported finding only tonic neurons. This likely represents a species-specific difference (as discussed in chapter 2).

Reduced efficacy of exogenous nicotine on vein- and artery-projecting SMG neurons from HT rats

The predominant neurotransmitter involved in conveying preganglionic input into and through sympathetic ganglia is acetylcholine released from terminals of neurons of the intermediolateral cell column of the spinal cord (Jänig, 2006, Simmons, 1985, Szurszewski and Miller, 1994). Nicotinic acetylcholine receptors (nAchR) mediate the fast response of postganglionic neurons to acetylcholine (Skok, 2002). However, long term exposure to nicotine results in upregulation of nAchR in the brain (Buisson and Bertrand, 2001, Buisson and Bertrand, 2002), and the responsiveness of postganglionic sympathetic neurons to acetylcholine may be enhanced in HT (Debinski and Kuchel, 1989). Therefore it is possible that synaptic transmission is augmented in HT as a result of alterations in postsynaptic nAchR expression or function.

Exogenous nicotine (10 uM) was applied to preparations of superior mesenteric ganglia from NT and HT rats during intracellular recording of identified vein- and artery-projecting neurons in order to examine the potential role of alteration in nAchR function in these ganglia in HT. The average peak depolarization amplitudes of vein-projecting and artery-projecting SMG neurons from NT rats were not significantly different (figure 15). However, when we compared the peak nicotine-induced depolarization amplitudes of vein-projecting SMG neurons from NT rats to those of vein-projecting neurons from HT rats, we found that the amplitude was significantly reduced (figure 15B). This effect was also seen when artery-projecting neurons were compared between NT and HT groups (figure 15C), but the effect was more pronounced in vein-projecting neurons. This suggests that the nicotinic response to acetylcholine is reduced in vein- and artery-projecting neurons of the superior mesenteric ganglion in DOCA-salt hypertension. This effect may be due to the increased activity of the preganglionic neurons (and the accompanying increase in acetylcholine release at these synapses) that is likely to be occurring in HT (Magee and Schofield, 1992, Magee and Schofield, 1994, Alkadhi and Alzoubi, 2007, Alzoubi et al., 2008). Nicotinic acetylcholine receptors (nAchR) undergo rapid desensitization following repeated activation (Giniatullin et al., 2005), and with prolonged exposure to nicotine, a long-lasting desensitization can result (Fenster et al., 1999). Alternatively, intracellular reactive oxygen species (ROS) production can inactivate nAchR in peripheral sympathetic neurons (Campanucci et al., 2008), as elevated production of ROS has been reported in

sympathetic ganglia in HT (Briones and Touyz, 2010, Li et al., 2008, Peterson et al., 2006). Finally, downregulation of nAchR in response to nicotine exposure has been reported (Marks et al., 1993) and may involve glucocorticoids (Takita et al., 1999). Therefore, it is possible that the combined effects of increased sympathetic outflow, reactive oxygen species production, and prolonged exposure to DOCA is resulting in inhibitory effects on nAchR function in prevertebral ganglia of DOCA salt HT rats.

Vein-projecting SMG neurons from HT rats are less susceptible to inhibition by hydrocortisone

One of the initial goals of this study was to test the hypothesis that the response to neuroactive steroids (glucocorticoids, in particular) of prevertebral sympathetic neurons innervating blood vessels is altered in DOCA-salt HT. The plasma concentration of glucocorticoids fluctuates in a circadian pattern, with peak concentration occurring early in the morning, and additional increases throughout the day, depending on the level of stress experienced by the organism (De Kloet and Derijk, 2004, Esler et al., 2008, Joels et al., 2012). Evidence exists in support of the ability of both mineralocorticoids and glucocorticoids to exert rapid effects at the level of the plasma membrane of neurons of the CNS ((Joels et al., 2012, Groeneweg et al., 2012) and in peripheral sympathetic neurons (Towle and Sze, 1982, Brown and Fisher, 1986, Hua and Chen, 1989). In sympathetic neurons, glucocorticoid receptor (GR) activation results in hyperpolarization of the membrane (Hua and Chen, 1989), and glucocorticoids can also result in direct inhibition of nAchR (Uki et al., 1999). Furthermore, chronic exposure to corticosteroids is known to affect
expression of both the MR and the GR (Lienhard et al., 2012, Joels et al., 2012, Tamura et al., 2011). We hypothesized, therefore, that the prolonged exposure to DOCA is leading to alterations in the function and/or expression of GR in vasomotor neurons of the prevertebral sympathetic ganglia.

In the PRV-identified vein- and artery-projecting neurons from normotensive rats, as well as the artery-projecting neurons from HT rats (figure 16), application of 100uM CORT to the preparation resulted in a rapid, transient hyperpolarization of the membrane of approximately 5 millivolts. This effect was dose-dependent (figure 14B), and could be repeated in single neurons after a wash of 5-10 minutes with Krebs' solution (not shown). The response seen in these experiments is identical to that reported by Hua et al (Hua and Chen, 1989) in the rat celiac ganglion. When CORT was applied during intracellular recording of vein-projecting SMG neurons from HT rats, the response amplitude was significantly reduced compared to vein-projecting neurons from NT rats (figure 16), and to artery-projecting neurons from NT and HT rats (figure 16C). This suggests that vein-projecting neurons have reduced susceptibility to the inhibitory (hyperpolarizing) effects of CORT in DOCA-salt HT.

Following the experiments in which the effects of CORT were compared between vein- and artery-projecting SMG neurons from NT and HT rats, we performed a series of experiments in which the ability of CORT to inhibit the depolarization induced by nicotine was compared (figure 17). This effect may be due to the same mechanisms responsible for the hyperpolarization that

occurs when CORT is applied alone, or it could be a result of direct inhibition of the nAchR by the steroid (Uki et al., 1999). In artery-projecting and in veinprojecting neurons from NT rats, CORT inhibited the depolarization induced by nicotine by about 50% (figure 17 B and C). This inhibition of the peak nicotine-induced depolarization was not seen in vein-projecting neurons from HT rats (figure 17B), as the inhibition was only about 20% of the initial value of the nicotine-induced depolarization in these neurons. This result, with the previous result showing that CORT failed to have a similar inhibitory effect on the membrane, suggests that vein-projecting neurons undergo alterations that affect their ability to respond to glucocorticoid in DOCA-salt HT. That arteryprojecting neurons apparently do not experience these same changes is a novel finding with interesting implications.

The final experiments performed in this study involved the application of the GR antagonist, mifepristone (RU 486) to the SMG preparation during ICR of vein- or artery-projecting neurons (figure 18). If the effects of CORT on the membranes of SMG neurons are due to activation of GR, then RU 486 should inhibit those effects. Introduction of 1.0 uM RU 486 prior to the application of CORT, during ICR of vein SMG neurons from NT rats, resulted in a reversal of the response (figure 18B). Neurons experienced a depolarization of approximately 2 mV, in contrast to the ~5 mV hyperpolarization that resulted from CORT alone (figure 16A, figure 18A). RU 486 did not have any effect on the membrane when applied alone (figure 18C). It is likely that the resulting depolarization is due to the fact that RU 486 is specific to glucocorticoid and

not mineralocorticoid receptors (MR), and that the MR has high affinity for CORT as well (Hawkins et al., 2012, Gomez-Sanchez, 2011). Also, in a subsequent experiment, RU 486 reduced the inhibitory effect of CORT on the peak depolarization amplitude induced by nicotine, but failed to completely eliminate this inhibition (not shown). These data suggest that the effects of CORT are mediated by membrane-associated GR. When CORT coupled to the membrane-impermeable bovine serum albumin (CORT-BSA) is applied to rat CG neurons, the hyperpolarizing effect is identical to that evoked by CORT, and the ability of RU 486 to inhibit this effect is also unaffected by the presence of the BSA conjugate (Hua and Chen, 1989). That the reduced effect of CORT on the peak nicotine-induced depolarization was specific to vein-projecting neurons supports the conclusion that a receptor mediated mechanism is responsible, although additional experiments are necessary to confirm this.

Summary and conclusions

We have shown that the PRV retrograde tracing technique is applicable to neurons of the peripheral sympathetic nervous system, that it is a reliable and technically straightforward means of identifying neurons that belong to specific functional pathways. Although we were interested in neurons that project to the vasculature, it is likely that other neuronal populations of sympathetic postganglionic neurons can be labeled in a similar manner. The basic electrophysiological properties of identified vein- and artery-projecting neurons of the superior mesenteric ganglion are not significantly different in untreated rats, nor are they altered between DOCA-salt HT and NT controls. Therefore,

we conclude that the fundamental active and passive membrane properties are conserved in this disease model and that PRV infection does not appear to adversely affect these properties. Also, we show that PRV infection is not resulting in any differential effects on the basic membrane properties of SMG neurons from NT or HT rats.

That the ability of exogenous nicotine to depolarize the membrane of veinand artery-projecting neurons from HT rats is reduced was an unexpected finding, given the likelihood of the increased safety factor of nicotinic transmission through sympathetic ganglia in HT. It is possible that either the function or the expression of nAchR is reduced in HT, as a result of increased presynaptic release of acetylcholine, production of ROS, or a combination thereof. Another possibility is that the prolonged exposure of the SMG to DOCA is resulting in both direct and indirect inhibitory effects on postsynaptic nAchRs.

The most surprising result of this study was that vein-projecting neurons from the SMG HT rats have reduced susceptibility to the hyperpolarizing effect of exogenous hydrocortisone. We've shown that CORT application results in a membrane hyperpolarization of significantly reduced amplitude in these neurons when compared to vein-projecting neurons from NT animals (figure 16) and that CORT also has a reduced ability to inhibit the peak depolarization evoked through exogenous nicotine application (figure 17). Together, these results show that specific neuronal populations are differentially affected in DOCA-salt hypertension, and that vein-projecting neurons of the SMG of HT

rats experience changes in the ability to respond to corticosteroids in a manner that is distinct from artery-projecting neurons. Also, it is likely that this differential effect of CORT on vein-projecting neurons is a result of either changes in MR or GR expression or function, although changes in the molecules that regulate corticosteroid bioavailability could produce these effects as well.

Several questions are raised by the results reported here. The first is whether or not the reason for the reduced inhibitory effects of CORT on veinprojecting neurons are due to downregulation of GR or are the result of another effect (e.g., a change in 11 β hydroxysteroid dehydrogenase 2 (11 β HSD2) function or expression). This enzyme is responsible for converting glucocorticoids into the inactive metabolite cortisone. It is possible that increased production of reactive oxygen species in vein-projecting neurons from HT rats is inhibiting the function of 11 β HSD2, resulting in increased availability of CORT. This would contribute to a continuous increase in the exposure of glucocorticoid to the GR (as well as the MR) and subsequent changes in receptor expression. However, since RU 486 was able to inhibit the hyperpolarization evoked by CORT in our experiments, it is likely that the ability of CORT to exert this effect is due to activation of GR.

A second question is why the response of vein and artery-projecting neurons to exogenous nicotine is reduced in HT. In chapter two, I show indirect evidence of increased strength of nicotinic synaptic transmission in IMG neurons from DOCA-salt HT rats, in that a greater number of neurons receive

strong synaptic inputs and the ability evoke supra-threshold long-term potentiation is impinged in HT. The reduced efficacy of nicotine may be a negative-feedback consequence of this, or it may be that the elevated corticosteroid levels in HT are exerting a direct inhibitory tone on these receptors.

Finally, we are curious as to why the changes in responsiveness to CORT are specific to vein-projecting neurons. While no studies have been performed that have compared the properties of target-specific neuronal pathways in sympathetic ganglia from HT rats, there is evidence that differential changes occur at the level of the nerve terminals of vein- and artery-projecting neurons in HT (Park et al., 2007, Perez-Rivera et al., 2005, Luo et al., 2004, Wang et al., 2003, Luo et al., 2003). Furthermore, veins themselves show response characteristics that are markedly different from arteries (in that the frequency of stimulation required to evoke responses in veins is much less than that required for arteries) (Kreulen, 1986). Neurons of the guinea pig IMG are more responsive to mechanical stretch of mesenteric veins than arteries (Keef and Kreulen, 1986).

The results reported here provide some insight into the adaptations that are occurring in neurons that innervate veins or arteries in the rat SMG in DOCAsalt HT. We show evidence of impaired synaptic transmission, in that exogenous nicotine is less able to depolarize these neurons in HT. However, we have also uncovered one possible way in which activity in these neurons may be positively modulated in HT. If glucocorticoids (corticosterone in rodents and

hydrocortisone in humans) are released during times of stress, in addition to their circadian release, one consequence of this increase in the activation of GR is an acute inhibition of sympathetic neurons (e.g., hyperpolarization of the membrane). In prevertebral sympathetic ganglia, this may occur as part of a negative feedback loop that results in attenuation of vasoconstriction following the stressor, a mechanism that would be similar to the rapid inhibitory effect of glucocorticoids on CRH, ACTH, and vasopressin release in the brain (Boron and Boulpaep, 2009, Harris et al., 2012). Therefore, in some forms of HT, it may be the case that this negative-feedback function is disrupted at the level of the prevertebral sympathetic ganglia, which may contribute to the chronic elevation in mean arterial pressure. That this effect appears to be specific to vein-projecting neurons suggests that increased output from these neurons may play a role in the increase in mean circulatory filling pressure (MCFP) and venous capacitance seen in some forms of HT (Gelman, 2008, Simon, 1976, Trippodo et al., 1981, Yamamoto et al., 1981, Fink et al., 2000, King et al., 2007). While additional experiments will be crucial in determining the precise mechanism behind the phenomena reported here, it is clear that the action of corticosteroids on prevertebral sympathetic ganglia should be given careful consideration in the study of hypertension.

CHAPTER FOUR:

GENERAL DISCUSSION

Summary of the major findings of this study

The overall objective of the studies described in this dissertation was to uncover the changes in cellular and synaptic function that are taking place in prevertebral sympathetic ganglia in DOCA-salt hypertension. In the first series of experiments, I used the rat IMG as a model of synaptic transmission *in vitro* to test the hypothesis that the transmission of impulses through the IMG is augmented in HT. Intracellular recordings of principal ganglion neurons with concurrent stimulation of the preganglionic nerve bundle revealed that in DOCA-salt HT, a greater number of neurons of the rat IMG receive strong synaptic inputs (table 2). In a later series of experiments, I applied repetitive, low amplitude stimulation (the LTP protocol) to this nerve bundle and found that although an approximately equal proportion of rat IMG neurons from NT and HT animals underwent long-term potentiation (either sub- or suprathreshold) (figure 8), a greater proportion of neurons from NT rats underwent potentiation beyond action potential threshold (table 3).

Following the experiments in the rat IMG, I proceeded to investigate the properties of neurons of another prevertebral sympathetic ganglion, the superior mesenteric ganglion. Continuing with the same overall objective (uncovering changes in neuronal and synaptic function in HT), and again using sharp microelectrode recording of neurons in intact ganglia, I examined several aspects of the neurophysiology of two specific neuronal populations: the veinand artery-projecting neurons of the SMG. Several interesting differences were discovered when these neuronal populations were compared between NT and

DOCA-salt HT rats. First, I found that the amplitude of the depolarization induced by exogenous nicotine is reduced in both vein and artery-projecting neurons from HT but not NT rats (figure 15). In the next series of experiments in these identified neurons, I examined the acute membrane response to application of the glucocorticoid, hydrocortisone 21-hemisuccinate (CORT) and found that vein-projecting neurons from HT rats were hyperpolarized to a significantly lesser degree than artery-projecting neurons from NT and HT rats, as well as vein-projecting neurons from NT rats (figure 16). In addition, CORT inhibited the peak amplitude of the nicotine-induced depolarization less in vein-projecting neurons from HT rats than artery-projecting neurons from NT and HT rats and vein-projecting neurons from NT rats (figure 17). The hyperpolarizing effect of CORT was blocked by the glucocorticoid receptor antagonist, RU 486 (figure 18).

In addition to the above findings, the work presented here resulted in the development of several novel approaches to the study of neuronal physiology in prevertebral sympathetic ganglia. This was the first detailed electrophysiological and anatomical characterization of the rat IMG (nearly all of our knowledge of prevertebral sympathetic ganglia is from studies in guinea pig). The studies involving LTP represent the first application of this technique (repetitive subthreshold stimulation followed by measurement of fast EPSP amplitude) to the prevertebral sympathetic ganglia, as well as the first report of LTP occurring in these ganglia.

Prior to this work, no reports existed in which pseudorabies viral tracers were used to identify peripheral sympathetic ganglion neurons that project to blood vessels. Furthermore, this was the first time these tracers have been used in the comparison of two distinct neuronal populations in a rodent model of HT. Finally, the experiments involving application of hydrocortisone represent the first enquiry into the role that glucocorticoids play in modulating sympathetic activity in sympathetic ganglia of HT rats. Together, these results have opened up several new possibilities (both technical and theoretical) regarding the role that the prevertebral sympathetic ganglia play in DOCA-salt hypertension.

The rat IMG as a model of synaptic transmission

Before the *in vitro* rat IMG preparation could be used in any studies involving the comparison of neuronal properties between NT and HT rats, a characterization of the anatomical and basic physiological properties had to be performed. While I found that all of the conventional parameters were similar to those values reported previously in guinea-pig, rabbit, mouse, cat and dog IMG, I did find some characteristics that appear to be unique to the rat. The input resistance of tonic neurons was significantly higher than phasic (table 1), and a greater proportion of the general population of postganglionic neurons of the rat IMG exhibited phasic firing properties (table 1). In the guinea pig IMG, the vast majority of the neurons are tonic and are presumed to innervate the glands and smooth muscle of the colon (rather than the blood vessels) (McLachlan, 2007, McLachlan, 1995, Briggs, 1995). When considered with the

results from the NPY immunolabeling experiments (figure 4), these results provide evidence suggesting that a greater proportion of neurons of the rat IMG serve vasoconstrictor functions than those of the guinea-pig IMG.

During the experiments involving the rat IMG, I attempted to narrow the neuronal populations compared by combining intracellular labeling during intracellular recording with post-hoc immunohistochemical processing for NPY and TH. However, the results of these experiments showed that most neurons of the rat IMG expressed NPY, and that the density of NPY immunolabeling is greater than that for TH. When the electrophysiological properties of the NPY/TH positive neurons were compared between NT and HT, no significant differences were revealed. While NPY is well-known as a co-transmitter of sympathetic neuronal varicosities on both veins and arteries (Dehal et al., 1992, Keast et al., 1993, Macrae et al., 1986, Xiong and Cheung, 1995, Westfall et al., 1996, Westfall et al., 1990, Wahlestedt, 1986, Wahlestedt et al., 1986), there are a significant number of reports that provide evidence that neurons from other efferent pathways also express NPY. These include visceromotor neurons of the colon (Walsh et al., 1993, Messenger and Furness, 1990), neurons that innervate the vas deferens (Milner et al., 1991), and ovaries (Majewski and Heym, 1991), and most other viscera (Ekblad et al., 1985, Ahren et al., 1990, Gibbins, 1995). Therefore, it was concluded that a more specific means of identifying vein and artery-projecting neurons was necessary if a precise comparison of vasoconstrictor neurons of the prevertebral ganglia were to be performed.

Increased strength of synaptic input to neurons of the rat IMG in DOCAsalt hypertension

Previous investigations of synaptic transmission in sympathetic ganglia of HT rats (Magee and Schofield, 1994, Magee and Schofield, 1992) have reported increased EPSP and EPSC amplitudes. When we compared EPSP amplitudes, we did not find them to be significantly elevated in HT. However, if the EPSP amplitude were to be increased beyond action potential threshold, that would exclude such neurons from the analysis of EPSP amplitude (as there would be no EPSP to measure in these neurons). That we did see a greater proportion of neurons that receives strong synaptic inputs in the HT group (table 2), suggests that a general increase in synaptic strength has occurred in IMG neurons from DOCA-salt HT rats.

There are three primary factors that can influence the strength of synaptic input to postganglionic sympathetic neurons (Jänig, 2006). These include the overall excitability of the postsynaptic membrane, the amount and probability of release of acetylcholine from the presynaptic neuron, and the morphology of both the presynaptic and the postsynaptic neurons. The excitability of the postsynaptic membrane is influenced by several factors, including resting conductance and resting membrane potential, as well as the presence and density of certain ion channels at postsynaptic sites. Also, the excitability of postsynaptic neurons may be modulated on a moment-tomoment basis through the closing of potassium channels (e.g., M channels), or through up or down-regulation of nicotinic acetylcholine receptors. Synaptic input strength is also dependent upon the structures of preganglionic

terminals and of postganglionic soma membrane and dendritic architecture. While it is understood that the morphological features of sympathetic neurons are not static, there were no outstanding differences in the number and structure of primary dendrites from rat IMG neurons from NT vs. HT rats (figure 3). Also, we did not observe any significant differences in the membrane parameters that would reflect baseline excitability. Therefore, it is most likely that this increase in synaptic strength is due to an elevation in the quantal content or the probability of release of acetylcholine from presynaptic terminals.

Inferior mesenteric ganglion neurons from NT rats undergo potentiation of EPSP amplitude beyond action potential threshold

That a greater number of neurons from NT rather than from HT rats underwent supra-threshold LTP following repetitive, low amplitude stimulation may initially suggest that synaptic transmission is depressed in ganglia from HT rats. However, the possibility exists as well that augmentation of synaptic function (in association with the development of HT) *in vivo* precludes further measurement of this form of LTP *in vitro* (figure 19). This idea has been raised numerous times regarding *in vivo* expression of LTP in the rat SCG and its role in psychosocial-stress and other forms of hypertension (Alkadhi et al., 2005b, Alzoubi et al., 2010, Alkadhi and Alzoubi, 2007, Alkadhi et al., 2001). In these studies, high-frequency stimulation of the cervical sympathetic trunk (which contains the preganglionic axons of the superior cervical ganglion) results in long-term increases in the amplitude of the compound action potential (measured from the external carotid nerve) in NT control animals, but not in

SCGs from HT animals. Also, in the case of psychosocial-stress induced HT, administration of an inhibitor of gLTP, ondansetron, prevented the development of HT (Alzoubi et al., 2008).

In the study of LTP of single IMG neurons described here, I measured the response of postganglionic neurons with intracellular electrodes. While I did see increases in EPSP amplitudes in IMG neurons from both NT and HT rats, I saw a greater number of instances from NT rats in which EPSPs were potentiated beyond action potential threshold (table 3). This result is consistent with those obtained through measurement of compound action potentials, since the amplitude of a compound action potential is dependent upon the total number of action potentials generated in postganglionic neurons of the ganglion (Magee and Schofield, 1992). Furthermore, since I also observed a greater number of neurons that received strong synaptic inputs in HT (table 2), this suggests that synaptic transmission is augmented in DOCA-salt hypertension.

The precise mechanism involved in this enhancement of synaptic transmission is unclear. However, a substantial amount of work involving LTP in the rat superior cervical ganglion has contributed to a reasonable understanding of this phenomenon. Nearly all of the evidence generated thus far suggests that presynaptic augmentation of acetylcholine release is the primary mechanism behind LTP (Heppner and Fiekers, 2003, Briggs, 1995, Briggs et al., 1985, Magee and Schofield, 1992, Alkadhi et al., 2005a). LTP in sympathetic ganglia is dependent upon extracellular Ca²⁺ (Vargas et al., 2007),

and serotonin for both the induction and maintenance phases (Alkadhi et al., 1996). In addition, increased cAMP production and activation of PKA are involved (Briggs et al., 1988). Activation of PKC may also be involved (Heppner and Fiekers, 2003). These kinases then phosphorylate presynaptic potassium and calcium channels, as well as synaptic machinery including the protein synapsin (Alkadhi et al., 2005a, Briggs, 1995). The net result is an increase in the probability of release of acetylcholine from preganglionic neurons. Further experimentation is necessary to determine whether similar mechanisms are responsible for LTP in neurons of the rat IMG.

Use of pseudorabies-virus retrograde tracers to identify superior mesenteric ganglion neurons projecting to mesenteric vein or artery

Following the experiments on neurons of the rat IMG, the next aim of this study was to develop a reliable method of identifying specific populations of vein- and artery-projecting neurons of prevertebral sympathetic ganglia so that their properties could be compared between NT and DOCA-salt HT animals. Retrograde tracing using genetically engineered viruses, and the pseudorabies viral constructs specifically, have been used to map multisynaptic pathways in the central nervous system (Smith et al., 2000, Banfield et al., 2003, Gonsalvez et al., 2010), as well as in the identification and electrophysiological characterization of spinal cord neurons that innervate specific visceral structures (Derbenev et al., 2010). These tracers feature several key advantages including specific uptake from synaptic varicosities, rapid retrograde transport, self-replication, and expression of fluorescent proteins (Card and Enquist, 2001, Enquist et al., 1998). Due to the receptor-

dependent mechanism of infection, when the tracer is applied to a section of blood vessel, only those axons with synaptic contacts with the vessel should become infected and somata of infected neurons will be easily visualized under epifluorescence microscopy (without further immunohistochemical processing) (Smith et al., 2000, Banfield et al., 2003).

We targeted neurons of the SMG for technical reasons. The blood vessels that were most accessible for *in vivo* virus application were branches of the superior mesenteric vein and artery, therefore, most of the labeled neurons were found in the SMG (although neurons were found in the CG of most preparations). Also, since the SMG is mono-lobular, it was more accessible for intracellular recording.

Vein- and artery-projecting neurons did not show significant differences in their basic electrophysiological properties in DOCA-salt hypertension, with the single exception that the action potential afterhyperpolarization amplitude was greater in artery-projecting neurons from HT rats. That the basic properties of vein- and artery-projecting neurons were not different between one another and between NT and HT rats is consistent with the studies of the rat IMG; there were no changes in any of the basic properties of these neurons in HT. It was only after an extensive study of synaptic transmission that any differences in the rat IMG were revealed. Therefore, a series of pharmacological experiments were subsequently performed in order to reveal any potential changes in cellular or synaptic machinery in these neurons in DOCA-salt HT.

The ability of nicotine to depolarize vein- and artery-projecting neurons of the rat SMG is reduced in DOCA-salt hypertension

In order to gauge any alterations in synaptic transmission in vein- and artery-projecting neurons of the SMG, we examined the response of these cells to exogenous nicotine. Our initial expectation, based on our results from the rat IMG, was that the postsynaptic response to nicotine would be either unchanged, or augmented in HT rats. That we observed the opposite effect in both vein- and artery-projecting neurons was unexpected, but can be explained in terms of the factors that may negatively influence nicotinic acetylcholine receptors in DOCA-salt HT (see Ch. 3 Discussion). Negative-feedback inhibition resulting from continuous acetylcholine release, direct inhibition of nAchR function by intracellular reactive oxygen species, and both the direct inhibitory effects of steroids as well as possible MR and GR-mediated inhibition are all possible causes of the reduction in mean depolarization amplitude in vein- and artery-projecting SMG neurons in DOCA-salt HT. Although additional experiments are necessary to determine whether the changes that favor increased synaptic transmission are sufficient to overcome the inhibitory effects on nicotinic receptors, our results from the rat IMG experiments that a greater number of neurons respond to presynaptic input with action potentials (strong inputs) in HT, as well as the reduction in the inhibitory effects of exogenous glucocorticoids on vein-projecting neurons from HT rats suggest that the transmission of vasomotor impulses through the prevertebral ganglia is augmented in HT.

The inhibitory effect of exogenous glucocorticoid are reduced in veinprojecting superior mesenteric ganglion neurons from DOCA-salt HT rats

The mechanism behind the hyperpolarization that resulted from acute application of 100uM hydrocortisone is unknown. However, based on studies from other tissues, the most likely mechanism is the modulation of calcium and/or potassium currents (Joels et al., 2012). It is likely that the inhibitory effect is mediated through activation of the glucocorticoid receptor, since exposure of the ganglia to RU 486 prior to CORT resulted in slight depolarization rather than hyperpolarization of the membrane (figure 13). The remaining depolarizing response is possibly due to activation of mineralocorticoid receptors, which can result in potassium (IA) channel closure (Joels et al., 2012, Groeneweg et al., 2012, Prager and Johnson, 2009, Heitzer et al., 2007).

CORT also reduced the peak amplitude of the depolarization induced by exogenous nicotine (figure 12). While it is possible that this effect is mediated by the same mechanism responsible for the hyperpolarization that resulted in neurons from CORT application alone, the inhibition was not blocked entirely by prior application of RU 486 (not shown). Glucocorticoids can directly inhibit the conductance of nicotinic acetylcholine receptors (see Ch. 3 Discussion). It is therefore possible that the inhibition is mediated through a similar direct mechanism. However, that the reduction in the effectiveness of CORT to reduce the peak depolarization amplitude induced by nicotine was specific to vein-projecting neurons suggests that this is a receptor-mediated effect (since

nicotinic acetylcholine receptors are found in vein- as well as artery-projecting neurons of sympathetic ganglia. If CORT were exerting its effects directly, it is likely that any differences seen in vein-projecting neurons in HT would also be seen in artery-projecting neurons. Therefore, although further experimentation is necessary to support the conclusion that the selective reduction in the inhibitory effect of CORT on the nicotinic response in vein-projecting neurons is due to a MR or GR-mediated effect, it remains as the most likely explanation for this result.

General conclusions and limitations

The results presented here provide evidence in support of numerous ways in which transmission of vasomotor signals may be augmented in hypertension, including a reduction in the inhibitory effects of glucocorticoids, the increased proportion of strong input neurons of the IMG, and the reduced proportion of neurons susceptible to potentiation beyond action potential threshold in HT (figure 18).

An interesting unifying explanation of these results, based on the prolonged exposure of the prevertebral sympathetic ganglia to DOCA (during HT development) can be raised in light of the possible differential effects on function and expression of the presynaptic mineralocorticoid receptor and the postsynaptic glucocorticoid receptor that are known to occur in the hippocampus in response to chronic stress. In this condition, the continuous elevation in glucocorticoid concentration results in the potentiation of glutamate release from presynaptic terminals. This effect is mediated by an

upregulation of presynaptic mineralocorticoid receptors (Groeneweg et al., 2012, Joels et al., 2012). At the same time, also a consequence of the elevated glucocorticoid concentration, homologous downregulation of postsynaptic glucocorticoid receptors occurs. Postsynaptic glucocorticoid receptors mediate a reduction in excitability as a means of terminating the response to stress (Joels et al., 2012). Thus, it is possible that increased mineralocorticoid receptor expression on preganglionic sympathetic nerve terminals is contributing (or causing) the increase in acetylcholine release in DOCA-salt HT, while postsynaptic down-regulation of glucocorticoid receptors in vein-projecting neurons prevents the normal negative-feedback reduction in excitability that would occur following elevated glucocorticoid concentrations.

There are also results that suggest that many aspects of neuronal and synaptic function are not changed in DOCA-salt HT. The basic membrane properties of IMG and SMG neurons that were measured showed no differences in HT. Both fast and slow excitatory postsynaptic potential amplitudes were not altered in neurons of the IMG, and the proportion of phasic and tonic neurons in the IMG and SMG were not significantly different in HT.

While it has been argued that the safety factor for transmission of impulses through the prevertebral ganglia is increased in HT, there are several limitations to the studies performed here. The main limitation to the studies of the IMG was that recordings were made in neurons of the general population, rather than vein- and artery-projecting neurons. The initial attempt to use NPY and TH as post-hoc markers of vasomotor neurons was inconclusive, as the

properties of these neurons showed no differences between NT and HT, and the density of NPY immunolabeling was greater than that for TH, suggesting that NPY expression is not limited to vasomotor neurons in the IMG. The possibility exists, though, that a greater proportion of neurons of the rat IMG serve vasoconstrictor functions, but further experimentation is necessary to test this hypothesis. Future studies using the IMG, with PRV retrograde identification of vein- and artery-projecting neurons would provide insight into whether the changes seen in vein- and artery-projecting neurons of the SMG occur in similar neurons of the IMG, and it would allow the examination of synaptic input into these neurons. Also, it would be interesting to see if the response of identified neurons of the IMG to exogenous nicotine occurs in a similar manner to that of SMG neurons. The investigation of vein- and artery-projecting SMG neurons was limited by the absence of a means of stimulating the preganglionic nerves (as was performed in the IMG). While it is much easier, technically, to perform studies of synaptic transmission using the IMG, it is possible to do so in the CG/SMG as well. Also, Immunohistochemical identification of mineralocorticoid receptors, glucocorticoid receptors, and the enzyme 11β HSD2 would provide further insight into the role that mineralocorticoids and glucocorticoids play in synaptic transmission and cellular physiology of prevertebral ganglion neurons. Pharmacological investigations in the IMG and SMG using the endogenous glucocorticoid, corticosterone (rather than hydrocortisone, as used in these studies), and the mineralocorticoid antagonist, spironolactone would help in determining the

precise mechanism behind the effects of neuroactive steroids in normal ganglia and in HT.

With these limitations, the novel findings reported here have met the task of introducing both the rat IMG and SMG as models of synaptic and cellular function in DOCA-salt HT. Also, the introduction of the PRV labeling technique in peripheral sympathetic neurons will allow subsequent investigators to study the properties of neurons of specific pathways in a more precise and direct manner than conventional methods allow. While it is understood that hypertension is a complex, multi-faceted disease, these studies have illuminated several ways in which the cellular and synaptic physiology of prevertebral sympathetic ganglion neurons may be altered in DOCA-salt HT.



Figure 19: Transmission of preganglionic impulses through prevertebral sympathetic ganglia is augmented in DOCA-salt hypertension

The ability of an action potential from the spinal cord to evoke an action potential in principal ganglion neurons of the SMG and IMG may be enhanced in HT. Synaptic transmission through vein-specific pathways of the SMG may be augmented as a consequence of elevated circulating corticosteroids. General (or vasomotor neuron-specific) synaptic transmission through the IMG may be enhanced in DOCA-salt HT.

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