MECHANISMS OF 5-HYDROXYTRYPTAMINE-INDUCED HYPOTENSION

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ABSTRACT

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Chronic serotonin (5-hydroxytryptamine, 5-HT) infusion results in a sustained fall in blood pressure in the sham and deoxycorticosterone acetate (DOCA)-salt rat. The exact mechanism(s) underlying a 5-HT-induced fall in blood pressure are not yet known. We hypothesize that 5-HT lowers blood pressure in a nitric oxide (NO)-dependent manner by reducing total peripheral resistance (TPR), either through a reduction in sympathetic nervous system (SNS) tone and/or direct stimulation of vascular 5-HT receptors. 5-HT (25 μg/kg/min; s.c.) or vehicle was infused to male Sprague Dawley or DOCA-salt hypertensive rats. Mean arterial pressure (MAP) was measured *via* radiotelemetry. Cardiac output (CO) was measured *via* aortic flow probes. 5-HT produced a significant reduction in MAP, increase in cardiac output (CO) and stroke volume (SV), and a significant reduction in total peripheral resistance (TPR). Additionally, 5-HT produced a sustained (one-month) fall in blood pressure in the DOCA-salt rat, but did not prevent the development of DOCA-salt hypertension. Isometric contractile force was measured in the sham and DOCA-salt superior mesenteric artery (SMA) to determine whether direct 5-HT receptor activtation was capable of relaxing the SMA. BW 723C86 (5-HT_{2B}), CP 93129 (5-HT_{1B}), and LP-44 (5-HT_{7}) did not relax the SMA from sham or DOCA-salt rats *vs.* vehicle. Electrical field stimulation (EFS) was used to determine whether 5-HT

was acting to inhibit neurogenic contraction in the isolated SMA. EFS-induced contraction was not significantly different in 5-HT incuabted *vs.* vehicle incuabted SMA under several conditions. Finally, several studies implicate the importance of the serotonin transporter (SERT) in mediating the effects of 5-HT. 5-HT produced a significantly greater fall in MAP in the SERT WT rat *vs.* the SERT KO rats suggesting a potentially important role for SERT in producing a 5-HT-induced fall in blood pressure. This research fills a gap in our understanding of how 5-HT functions in the cardiovascular system and validates our hypothesis that 5-HT lowers blood pressure through a reduction in total peripherl resistance. However, this research suggests that 5-HT is not acting through direct receptor mediated vascular relxation or through inhibition of NE release at the nerve terminal in the superior mesenteric artery to lower blood pressure.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HT	5-Hydroxytryptamine, serotonin
ACh	Acetylcholine
BP	Blood pressure
bpm	Beats per minute (heart rate)
CNS	Central nervous system
DOCA	Deoxycorticosterone acetate
eNOS	Endothelial nitric oxide synthase
i.p.	Intraperiotoneal
L-NNA	N-nitro-L-arginine
MAO	Monoamine oxidase
MAP	Mean arterial pressure
NaCl	Sodium chloride
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PE	Phenylephrine
PSS	Physiological salt solution
SERT	Serotonin transporter

SEM	Standard error of the mean
SNS	Sympathetic nervous system
s.c.	Subcutaneously
SSRI	Selective serotonin reuptake inhibitor
TPR	Total peripheral resistance

CHAPTER 1

INTRODUCTION

5-hydroxytryptamine (5-HT, serotonin) is a vasoactive amine synthesized primarily in the enterochromaffin cells of the gastrointestinal tract and the raphe nucleus of the central nervous system (CNS) (Erspamer and Asero, 1952; Dahlstroem and Fuxe, 1964). 5-HT is synthesized from the dietary precursor tryptophan and the rate-limiting enzyme of 5-HT synthesis is tryptophan hydroxylase (TPH). TPH converts the essential amino acid tryptophan, to 5-hydroxytryptophan (5-HTP) (Walther *et. al.* 2003). 5-HTP is then acted upon by a nonspecific decarboxylase to form 5-HT. Two distinct isoforms of TPH exist: 1) tryptophan hydroxylase 1 (TPH1), expressed in the periphery, responsible for 5-HT synthesis primarily in the gut; and 2) tryptophan hydroxylase 2 (TPH2), expressed in the central nervous system (CNS), responsible for 5-HT synthesis within the neuron(s) in the CNS (Zhang *et. al.* 2004).

The physiological actions of 5-HT are terminated by: 1) the serotonin transporter (SERT), which actively takes up free extracellular 5-HT, and 2) the mitochondrial enzyme monoamine oxidase (MAO), which inactivates 5-HT to form the metabolite 5-hydroxyindole acetic acid (5-HIAA) (Marshall *et. al.* 2010). In the periphery, 5-HT is rapidly taken up by the platelet, which stores large amounts (millimolar) of 5-HT. The rapid uptake of 5-HT, its storage within the platelet, and metabolism by MAO results in comparatively low levels of free circulating 5-HT in the periphery (Figueras *et al.* 2005).

The physiological actions of 5-HT are mediated by seven major receptor subtypes (5-HT_1 – 5-HT_7). Each receptor subtype (with the exception of the 5-HT_3 receptor) are heptahelical receptors, coupled to G proteins (Gs, Gi, Gq) with diverse effects. The 5-HT_3 receptor is a ligand gated ion channel (Watts and Davis, 2011). 5-

HT is responsible for a variety of physiological functions in numerous major organ systems including: the cardiovascular, pulmonary, gastrointestinal, genitourinary, and central nervous system(s) (Berger *et al.* 2009). Our focus will be primarily on the actions of 5-HT in the cardiovascular system, paying particular attention to the effects of 5-HT on blood pressure.

Blood Pressure

Blood pressure is defined as the amount of pressure exerted against the arterial wall. Cardiac output (CO) is the amount of blood pumped by the heart (in particular the amount of blood ejected from the left ventricle) during systolic contraction. Total peripheral resistance (TPR) is the amount of force opposing the flow of blood through the circulatory system back to the heart. These opposing forces exert pressure on the arterial wall (recognized as arterial blood pressure) and changes to either CO or TPR result in changes in blood pressure. Thus, blood pressure can be calculated from the following equation: blood pressure (BP) = cardiac output (CO) x total peripheral resistance (TPR). Clinically, blood pressure is reported as the pressure exerted during systole (when the heart contracts) over the pressure exerted during diastole (when the heart is filling). Blood pressure < 120mmHg/80mmHg (millimeter mercury) is considered optimal in an adult (JNC VII; Chobanian *et. al.* 2003).

Changes in blood pressure are achieved by a variety of major organs/systems, including (briefly), the heart, the peripheral vasculature, the kidney, and the sympathetic nervous system (SNS). For example, an increase in the amount of blood pumped from the heart, will result in a change in the force exerted against the arterial wall and a subsequent change in blood pressure. Alternatively, the kidney is capable of changing

total blood volume (through increased/decreased excretion of H_2O, which will change the amount of blood returned to the heart. This change in blood volume will ultimately effect the amount of blood ejected from the left ventricle (CO) and thus will change the force exerted against the arterial wall (i.e. Δ in blood pressure). Finally, a change in SNS activity can change blood pressure at both the level of the heart and the peripheral vasculature. For example, the peripheral vasculature is extensively innervated by SNS fibers and changes in SNS activity, will result in a change in norepinephrine (NE; potent vasoconstrictor) release from the neuroeffector junction. An increase in NE release will increase the tone of the arterial wall, thereby increasing arterial resistance, and blood pressure. Thus, changes in blood pressure can be the result of a single or multiple changes/insults on the cardiovascular system.

Hypertension

Transient changes in blood pressure (i.e. physical activity, increase in blood pressure, or rest, decrease in blood pressure) are not considered clinically significant and exist primarily to ensure adequate delivery/exchange of nutrients/waste when metabolic demand is varied. However, chronic increases in blood pressure result in the clinical condition known as hypertension. Hypertension is defined as a systolic blood pressure > 140mmHg or a diastolic pressure > 90 mmHg (JNC VII; Chobanian *et. al.* 2003). Essential hypertension is a significant risk factor for common causes of morbidity and mortality, including stroke, myocardial infarction, heart failure, and kidney disease (Campese *et. al.* 2011). Unfortunately, the etiology of primary or essential hypertension remains largely unknown.

Historically, 5-HT has been described as a potential contributor to essential hypertension. This distinction is based on decades of research demonstrating that 5-HT is a potent constrictor of isolated blood vessels (Rapport *et. al.* 1948). Additionally, studies demonstrate elevated levels of free plasma 5-HT in both human and animal models of hypertension compared to normotensive controls (Brenner *et. al.* 2007; Fetkovska *et. al.* 1990). 5-HT receptor antagonists lower blood pressure in select animal models of hypertension (Watts and Fink, 1999). Arteries of hypertensive animals are hyperresponsive to the constrictor effects of 5-HT, and selective 5-HT reuptake inhibitors elevate blood pressure (Thompson and Webb, 1987; Amsterdam *et. al.* 1999). This evidence originally led investigators to postulate that elevated levels of free plasma 5-HT might be acting to constrict the vasculature, increasing vascular resistance, and blood pressure.

5-HT in the vasculature

In the peripheral vasculature, the 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{7} receptors are present and active (Bordoff *et. al.* 2002; Calama *et. al.* 2003; Saxena and Villalon, 1990). The literature describes 5-HT as both a constrictor and dilator of peripheral blood vessels.

Under normotensive conditions in the rat, the 5-HT_{2A} receptor is responsible for contraction of isolated blood vessels (Yildiz *et. al*. 1998). Under hypertensive conditions in the rat, the 5-HT_{1B}, and 5-HT_{2B} receptor(s) are linked to arterial hyperresponsiveness

to 5-HT (Banes and Watts, 2003; Banes and Watts, 2001). Thus, if 5-HT were acting primarily as a constrictor in the peripheral vasculature, this would result in an increase in vascular resistance and blood pressure.

5-HT may also function as a dilator of peripheral blood vessels. The 5-HT_{1B}, 5-HT_{2B}, and 5-HT_7 receptors have all been linked to dilation of peripheral blood vessels in the rat. The 5-$HT_{1B/1D}$ and 5-HT_7 receptor(s) are located on vascular smooth muscle and described to mediate relaxation, but they are mechanistically not well-understood (Calama *et. al.* 2003; Saxena and Villalon, 1990). The pharmacological agonists CP 93129 (5-$HT_{1B/1D}$ receptor agonist) and LP-44 (5-HT_7 receptor agonist) are selective agonists that aid in the investigation of these receptors.

The 5-HT_{2B} receptor is linked to falling blood pressure in patients placed on cardiopulmonary bypass (Bordoff *et. al.* 2002). Investigators of this study demonstrated that a bypass-induced (mechanical platelet disruption/5-HT release) fall in blood pressure was nitric oxide (NO) dependent. This finding suggested that activation of the 5-HT_{2B} receptor might mediate NO release, thereby reducing total peripheral resistance, and lower blood pressure.

However, the exact role of the rat 5-HT_{2B} receptor remains controversial as this receptor is found to be upregulated in the smooth muscle of hypertensive rats, and acts as a potent constrictor of isolated blood vessels (Banes and Watts, 2003). The pharmacological agonist, BW 723C86 (5-HT_{2B} receptor agonist) is another tool available for investigation of this particular 5-HT receptor.

5-HT in the SNS

Preganglionic and postganglionic neurons carry/transmit signals from the central nervous system (CNS) to various organ systems (heart, adrenal gland, kidney, blood vessels) that are involved in regulating blood pressure. Preganglionic neurons originate within the CNS and spinal cord (the thoracolumbar region) traveling to sympathetic ganglia where they synapse with postganglionic neurons/nerve fibers. At the synapse, preganglionic neurons release acetylcholine (ACh), which activates nicotinic ACh receptors on the postganglionic neuron, depolarizing the cell, leading to signal propagation along the postganglionic neuron. Postganglionic nerve fibers travel to peripheral target tissues where they synapse and release norepinephrine (NE). NE then traverses the synaptic space and activates adrenergic receptors. These receptors are responsible for the effects of the SNS. For example, NE released along the peripheral vasculature will cause alpha-1 receptor activation and contract the artery and/or vein (depending on innervation and release). Therefore, an increase or decrease in NE release (at the neuroeffector junction) is capable of increasing or decreasing vessel tone, vascular resistance (total peripheral resistance; TPR), and blood pressure. Alternatively, changes in signal initiation/generation (represented by changes in preganglionic activity) or changes in signal propagation (i.e. inhibition of signal transfer at the ganglia, represented by changes in postganglionic activity) are capable of increasing or decreasing blood pressure.

The 5-$HT_{1B/1D}$ receptor is expressed on the nerve terminal (neuroeffector junction) and activation of this receptor results in an inhibition of NE release (Molderings *et. al.* 1990). Thus, a reduction in NE release results in a reduction in peripheral vessel tone, a

fall in TPR, and a reduction in blood pressure. This suggests 5-HT may be capable of acting as a sympatholytic and capable of decreasing blood pressure.

However, alterative reports in the literature suggest 5-HT can be actively taken up at the nerve terminal, resulting in NE displacement and NE release (Villalon and Centurion, 2007). This suggests 5-HT may be acting as an indirect sympathomimetic. Thus, 5-HT may be capable of increasing NE release, increasing TPR, and increasing blood pressure. This suggests 5-HT may be capable of acting as an indirect sympathomimetic and able to increase blood pressure.

5-HT and the serotonin transporter (SERT)

SERT is a bidirectional transporter responsible for the uptake and release of 5-HT (Ni *et. al.* 2008). SERT is member of the sodium chloride-dependent transporter family (SLC6) and while SERT is well known for its uptake of 5-HT into platelets, it is also located on the neuron, the ganglia (rat superior cervical ganglia) (Nishimura M *et. al.* 1999), in the gastrointestinal system (Gershon and Tack, 2007), pulmonary system (Dodson *et. al.* 2004), immune system (Gordon and Barnes, 2003), and on the heart (Pavone *et. al.* 2007). Recently, SERT was also recognized on the systemic artery and vein (Ni *et. al.* 2008). Thus, both the artery and the vein also possess a transporter, which allows local control of intracellular and extracellular 5-HT concentrations. Moreover, SERT was recently identified on the blood brain barrier in the central nervous system (CNS) and thus suggests bidirectional transport of 5-HT across the blood brain barrier is possible (Nakatani *et. al.* 2008; Brust *et. al.* 2000; Wakayama *et. al*. 2002).

Pharmacological manipulation of SERT in the CNS is widely accepted as effective treatment for a variety of psychological disorders, most notably, depression. Antidepressant drugs (selective serotonin reuptake inhibitors; SSRI) target SERT, blocking the reuptake of 5-HT *via* SERT inhibition. These clinically efficacious drugs increase free 5-HT concentrations in the extracellular space and it is believed that this increase is responsible for the antidepressant effects of these drugs. (Keers and Aitchison, 2010). However, changes in blood pressure are noted side effects and therefore suggests changes in SERT function may be capable of modifying systemic blood pressure.

Interestingly, 5-HT uptake *via* SERT has recently been demonstrated to enhance neuronal nitric oxide synthase (nNOS) activity in cells coexpressing both SERT and nNOS (Chanrion *et. al.* 2007). This finding suggests that 5-HT may be taken up by SERT, resulting in nNOS activation and the production of nitric oxide (NO), a powerful vasodilator. This evidence suggests 5-HT uptake *via* SERT may be capable of lowering blood pressure through activation of nNOS and NO production.

The triphasic response

5-HT (delivered over seconds) results in a classic blood pressure response known as the triphasic response**.** 5-HT produces a fast depressor response, (mediated by an abrupt bradycardia, known as the Bezold-Jarisch reflex *via* the 5-HT_3 receptor), followed by a prominent pressor response (mediated by arterial smooth muscle contraction *via* 5-HT_{2A} receptor), and then a long lasting depressor response (likely mediated by activation of the 5-HT_7 receptor producing smooth muscle relaxation).

This suggests 5-HT is capable of increasing and/or decreasing blood pressure when delivered acutely (over seconds).

The triphasic response investigated changes in blood pressure when delivered over seconds to minutes. This is an important distinction, as it requires us to think differently about the mechanism(s) potentially involved in a chronic 5-HT-induced change in blood pressure. Acutely, changes in blood pressure are largely governed by changes mediated by the baroreceptors and baroreceptor reflex. The baroreceptors are stretch receptors located predominately at the carotid sinus and the aortic arch. If for example, blood pressure were to increase, the baroreceptor reflex would respond by slowing the heart, and removing sympathetic nervous system tone to the peripheral vasculature, resulting in a dilation of blood vessels and lower blood pressure. Chronic changes in blood pressure suggest an alternative mechanism, which may potentially be mediated by a change in vessel tone, or SNS activity.

5-HT infusion

5-HT elicits various cardiovascular responses. Thus, our laboratory designed the following experiment to investigate whether elevated levels of free plasma 5-HT would result in increased blood pressure, supporting active 5-HT-induced vasoconstriction or decreased blood pressure, supporting a completely different mechanism, in the sham and deoxycorticosterone acetate (DOCA)-salt rat.

Despite significant literature to support the hypothesis that 5-HT may be contributing to essential hypertension (*via* peripheral artery contraction), the results of this experiment demonstrated that elevated levels of free plasma 5-HT were actually lowering

blood pressure in the sham and DOCA-salt rat (Diaz *et. al.* 2008) (**Figure 2**). This study unequivocally suggested that instead of constricting the peripheral vasculature, contributing to an increase in TPR, and blood pressure, a completely different mechanism is acting when 5-HT is delivered chronically. The exact mechanism(s) of a 5-HT-induced fall in blood pressure are not yet fully understood. However, Diaz *et. al*. demonstrated an absolute dependence on nitric oxide synthase (NOS) to produce a 5-HT-induced fall in blood pressure. The following hypothesis was designed to investigate the mechanism underlying a 5-HT-induced fall in blood pressure.

Hypothesis

5-HT lowers blood pressure, in a nitric oxide dependent manner, by reducing total peripheral resistance, either through a reduction in sympathetic nervous system tone and/or direct stimulation of vascular 5-HT receptors

Specific aim 1: Will test the hypothesis that 5-HT relaxes peripheral blood vessels through activation of peripheral vascular receptor(s).

Specific aim 2: Will test the hypothesis that 5-HT lowers blood pressure through a reduction in SNS tone.

Specific aim 3: Will test the hypothesis that 5-HT uptake *via* SERT is critical to enabling a 5-HT-induced fall in blood pressure.

Figure 1.1

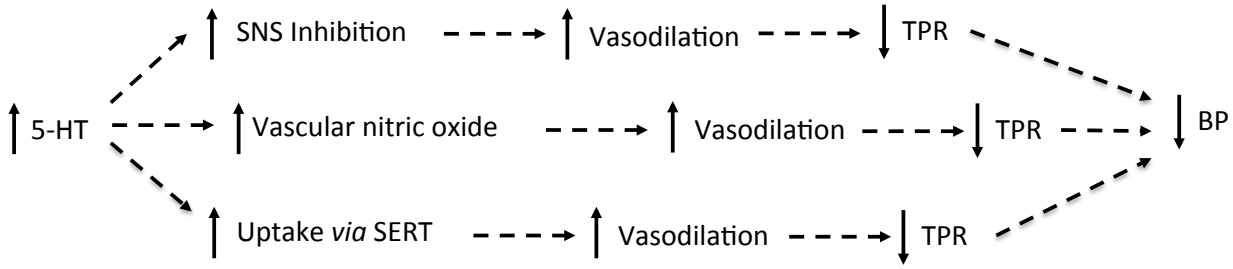
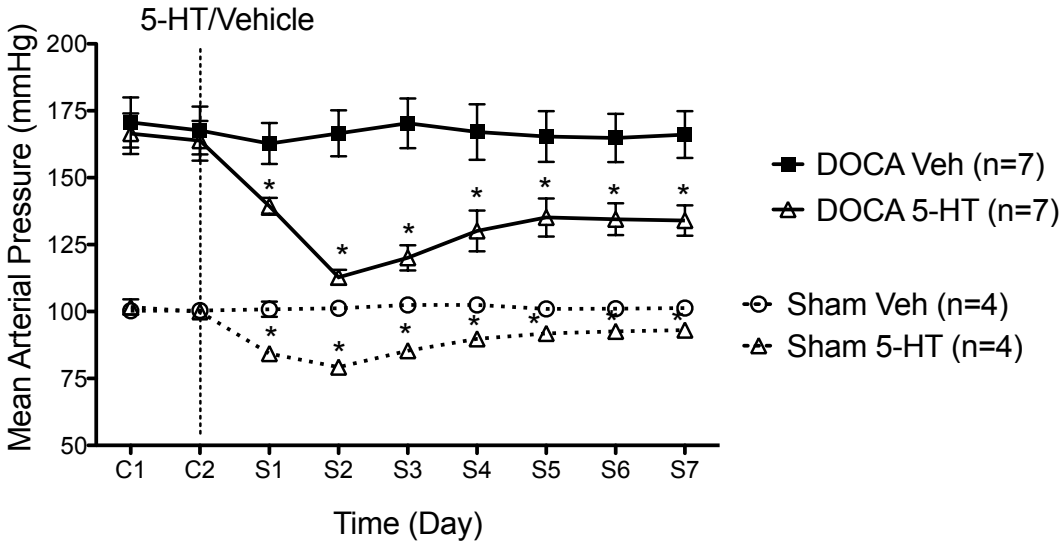


Figure 1.1. Schematic diagram of current hypotheses. (TPR) represents total peripheral resistance. (SNS) represents sympathetic nervous system. (BP) represents blood pressure.

Figure 1.2

A.



B.

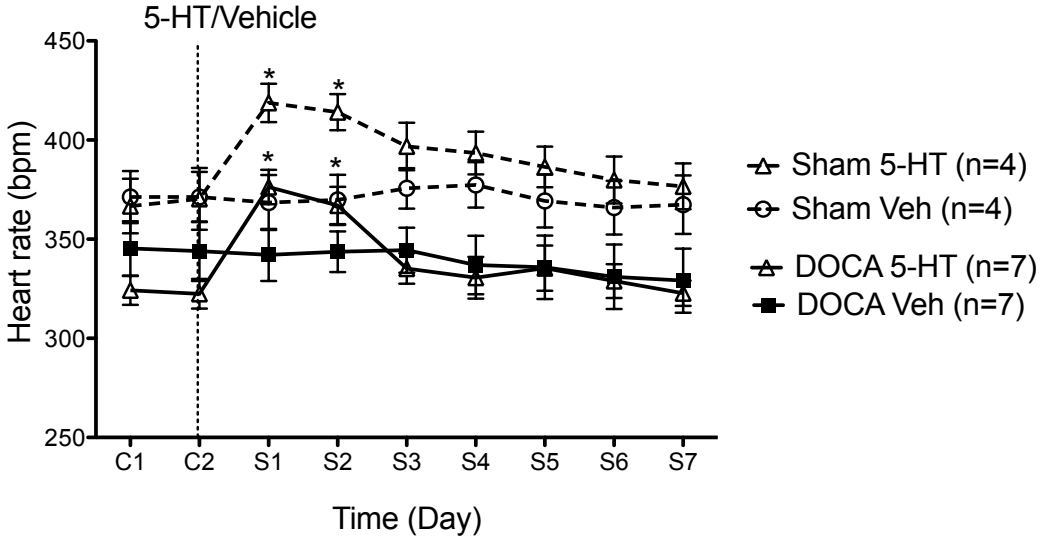


Figure 1.2. **A**. Effect of chronic 5-HT/Vehicle administration on mean arterial blood pressure. Time (days) is represented on the x-axis. C represents control days. S

represents 5-HT administration. **B**. Effect of chronic administration of 5-HT or Vehicle on heart rate (HR). (n) represents the number of animals used. (*) Represents statistically significant difference ($p<0.05$) from control period C2. Vertical dotted line represents start of 5-HT/vehicle. **Reference:** Diaz *et al*. J Pharmacol Exp. Ther. 325:1 031-1038, 2008.

CHAPTER 2

One-month serotonin infusion results in a prolonged fall in blood pressure in the male deoxycorticosterone acetate (DOCA)-salt rat

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ABSTRACT

Chronic serotonin (5-hydroxytryptamine, 5-HT) infusion results in a sustained fall in blood pressure in the deoxycorticosterone acetate (DOCA)-salt rat and is accompanied by improved ACh-induced arterial relaxation. These studies tested the hypothesis that a longer (one-month) and more clinically significant 5-HT infusion could cause a sustained fall in blood pressure in the DOCA-salt rat, and tested the new hypothesis that 5-HT could also attenuate the development of DOCA-salt hypertension. 5-HT or vehicle (Veh) were delivered *via* osmotic pump (25 μg/kg/min; sc) to established male DOCA-salt rats or Sprague Dawley rats prior to DOCA-salt administration for 28 days. Free plasma 5-HT concentrations were significantly higher in 5-HT-infused DOCA-salt rats compared to Veh-infused DOCA-salt rats. On the final day of 5-HT infusion, mean arterial pressure (MAP; radiotelemetry) was significantly lower in 5-HT-infused (135 ± 4 mmHg) *vs.* Veh-infused (151 ± 7 mmHg) established DOCA-salt rats. However, 5-HT-infusion did not prevent the development of DOCA-salt hypertension (144 ± 7 mmHg) *vs.* Veh infusion (156 ± 6 mmHg). Isometric contractile force was measured in aortic strips of 5-HT-infused and Veh-infused DOCA-salt rats. Maximum relaxation to ACh was not significantly greater in the aorta of 5-HT-infused *vs.* Veh-infused established DOCA-salt rats, or in 5-HT *vs.* Veh-infused rats when 5-HT was delivered prior to the onset of DOCA-salt hypertension. This study demonstrates 5-HT is capable of lowering blood pressure in established DOCA-salt hypertensive rats over the course of one-month and suggests a potentially beneficial role for 5-HT in the cardiovascular system.

INTRODUCTION

Hypertension is characterized by an elevation in arterial pressure, increased vascular resistance, cardiac hypertrophy, increased sympathetic nervous system tone, and altered renal function (Campese *et. al.* 2011). Despite the myriad of deleterious changes, the etiology of essential hypertension remains largely unknown, but is thought to be multifactorial, and include both genetic and environmental factors. Interestingly, several lines of evidence suggest an important association between 5-HT and hypertension.

5-HT is a vasoactive amine synthesized primarily in the enterochromaffin cells of the gastrointestinal tract, and the raphe nucleus in the central nervous system (Erspamer and Aerso, 1952; Rapport *et. al.* 1948; Dahlstrom and Fuxe, 1964). Studies demonstrate elevated levels of free plasma 5-HT in both human and animal models of hypertension compared to normotensive human and animal controls (Brenner *et. al.* 2007; Fetkovska *et. al.* 1990). 5-HT receptor antagonists lower blood pressure in select animal models of hypertension (Watts and Fink, 1999). The arteries of hypertensive animals are hyperresponsive to the constrictor effects of 5-HT, and selective 5-HT reuptake inhibitors elevate blood pressure (Thompson and Webb, 1987; Amsterdam *et. al.* 1999). Collectively, this evidence suggests that elevated levels of free plasma 5-HT might be acting to increase vascular resistance and blood pressure, thereby contributing to essential hypertension.

Surprisingly, experimental efforts to elevate free plasma 5-HT actually lowered blood

pressure in the deoxycorticosterone acetate (DOCA)-salt and sham rat over the course of 7 days (Diaz *et. al.* 2008). This suggested a completely different mechanism, that 5-HT might be acting to lower blood pressure in both human and animal models of hypertension. Thus, instead of contributing to essential hypertension, the elevated levels of 5-HT observed in hypertensive patients, may actually represent an adaptation to elevated blood pressure. The exact mechanism(s) of a 5-HT-induced fall in blood pressure are not yet fully understood, but were initially suggested in a study published in 2008 (Diaz *et. al.* 2008).

Diaz *et. al.* demonstrated that inhibition of nitric oxide synthase (NOS) prevented a one-week 5-HT-induced fall in blood pressure in the sham and DOCA-salt hypertensive rat. This study suggested an important role for NOS in enabling a 5-HT-induced fall in blood pressure. Additionally, Diaz *et. al.* demonstrated improved acetylcholine (ACh)-induced relaxation (a measure of endothelial cell health) in the aorta of 5-HT infused *vs*. vehicle infused DOCA-salt rats (10). This suggests a one-week 5-HT infusion may improve endothelial cell function in the DOCA-salt rat, potentially as part of the mechanism for a decrease in blood pressure.

The DOCA-salt model is characterized by subcutaneous delivery of the mineralocorticoid deoxycorticosterone acetate (200 mg/kg/day) and ingestion of salt water (1% NaCl, 0.2% KCl) (Sun and Zhang, 2005; Pinto *et. al.* 1998). Additionally, renal mass is significantly reduced through unilateral nephrectomy. Collectively, this leads to neurohumoral activation and volume expansion in the DOCA-salt rat (Yemane

et. al. 2009). Further, the DOCA-salt model is considered a nitric oxide (NO)-depleted model of hypertension, due to characteristic endothelial cell dysfunction and a reduction in ACh-induced arterial relaxation (Voorde and Leusen, 1986; Cordellini *et. al.* 1988). Lastly, the DOCA-salt rat could also be considered a resistant model of hypertension due to the fact that after approximately 2 months of treatment with DOCA, removal of the pellet and return to normal H_2O does not return blood pressure to pre-hypertensive levels (Beilin and Ziakas, 1972).

The previously studied infusion protocol (7 days) is a relatively short clinical exposure given that most hypertensive patients require lifelong antihypertensive therapy. Thus, part of the present study investigates whether elevated levels of free plasma 5-HT were capable of lowering blood pressure for one-month in the established DOCA-salt hypertensive rat. In addition, this study takes a different tactic to investigate how 5-HT decreases blood pressure by determining if it can *prevent* the development of DOCA-salt hypertension. We hypothesized 5-HT could do so because of support from experiments done decades ago, which demonstrated that chronic treatment with L-5-hydroxytryptophan (5-HTP) prevented the development of DOCA-salt hypertension in the rat (Fregly *et. al.* 1987). 5-HTP is the committed precursor to the synthesis of 5-HT and is synthesized by tryptophan hydroxylase from the dietary precursor tryptophan. Thus, we investigated whether chronically elevated free plasma 5-HT might also impact the development of DOCA-salt hypertension in the rat. This study has not been done before.

Finally, in both the reversal (delivery of 5-HT to established DOCA-salt rats) and prevention (delivery of 5-HT prior to the onset of DOCA-salt hypertension) studies, we sought to investigate whether endothelial cell health, measured by ACh-induced relaxation, was preserved and/or improved as has been observed with a shorter 1 week course of 5-HT.

METHODS

Animal

The Michigan State University Institutional Animal Care and Use Committee (IACUC) approved all protocols. Male Sprague-Dawley rats (250–300 g; Charles River Breeding Laboratories, Portage, MI) underwent one of 2 separate experimental protocols: 1) Male Sprague-Dawley rats (250–300 g) underwent left uninephrectomy, and a DOCA pellet was implanted s.c. (200 mg/kg). Water was supplemented with 1% NaCl and 0.2% KCl for the duration of the study. After 4 weeks these rats were considered established DOCA-salt hypertensive rats and were administered either 5-HT or vehicle *via* osmotic pump (s.c.) for one-month. 2) Male Sprague-Dawley rats (250–300 g) underwent left uninephrectomy, and were then administered either vehicle or 5-HT vehicle *via* osmotic pump (s.c.) for one-month. After 5 days of treatment, Sprague Dawley rats received a DOCA pellet (200 mg/kg) and their water was supplemented with 1% NaCl and 0.2% KCl for the remaining duration of the one-month infusion.

Anesthesia and Analgesia

All rats were anesthetized with isoflurane (2% in 100% O_2) and ventilated mechanically. At the time of surgery, the incision site was treated with topical antibiotic containing analgesic to prevent irritation and infection. Incisions were closed with silk suture. Rats were treated with amoxicillin (150 mg/kg/i.m.) following surgery and 3 days thereafter. All rats were treated with rimadyl (5 mg/kg, s.c. for 2 days) for general analgesia.

Surgical Methods: Blood pressure probe

Radiotelemeters (DSI PhysioTel PA series transmitter model PA-C40) were implanted subcutaneously in the lower abdomen and catheters introduced into the left femoral

artery. Pressure sensing tips were advanced into the thoracic aorta. All rats were given 7 days to recover prior to any measure. Mean arterial pressure (MAP) and heart rate (HR) were recorded at 10-minute intervals (10 second recording) for the duration of the study.

Surgical Methods: Alzet osmotic pump

A small incision was made at the base of the neck. Blunt dissection was used to create a small subcutaneous pocket between the scapulae. The pump (Alzet Osmotic Pump, Model 2ML4, Duret Corporation, Cupertino, CA, 2.5 μL/hr 28 days) was inserted and the skin sutured closed. To each pump, a 5-HT creatinine complex (25 μg/kg/min) and 1% ascorbic acid, antioxidant (0.02 g/pump) or vehicle (1% ascorbic acid, antioxidant) was loaded. The solution was dissolved in 1 mol/L HCl, and a pH-balance (~7) was achieved with 4 mol/L NaOH.

Plasma 5-HT measurements

Blood collection and platelet poor plasma (PPP) / platelet rich plasma (PRP) preparation were conducted according to our previously published methods (Diaz *et. al.* 2008). Briefly, five milliliters (ml) of blood was collected from left cardiac ventricle using a heparin coated syringe. The blood was transferred into a EDTA anticoagulant vacutainer tube. Ten μmol/L pargyline and 10 μmol/L ascorbic acid were added. The EDTA tubes were spun at 160 x g for 30 minutes at 4°C for platelet-rich-plasma (PRP). Two ml of supernatant containing plasma and buffy coat were pipetted into EDTA-coated plastic tubes and mixed with a 1:1 dilution of 0.5 mol/L EDTA. Ten μmol/L pargyline and 10 μmol/L ascorbic acid were added. These tubes were centrifuged for 20 minutes at 1350 x g at 4°C for platelet-poor-plasma (PPP). To the remaining pellet (platelet layer), 1 ml of platelet buffer

(145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$ 10 mmol/L D-glucose) and 1 μmol/L ADP was added. 10 μmol/L pargyline and 10 μmol/L ascorbic acid were added. These tubes were then vortexed and allowed to sit on ice for 15 minutes for platelets to become activated and degranulate. The tubes were then centrifuged at 730 x g for 10 minutes at 4°C. Ten percent trichloroacetic acid (TCA) was added to deproteinize both sets of samples and allowed to sit on ice for 10 minutes. These tubes were centrifuged at 4500 x g for 20 minutes at 4°C. Finally, the samples were ultracentrifuged at 280,000-x g for 2 hours. 5-HT concentrations were measured using high-performance liquid chromatography (HPLC) at 0.2 V and 0.6 ml/min flow rate.

Isolated tissue bath

Rats were anesthetized with pentobarbital (60 mg/kg i.p.) and the thoracic aorta was removed and placed in physiological salt solution (PSS) containing (mmol/L): NaCl 130; KCl 4.7; KH_2PO_4 1.8; $MgSO_4 * 7H_2O$ 1.7; $NaHCO_3$ 14.8; dextrose 5.5; $CaNa_2EDTA$ 0.03, $CaCl_2$ 1.6 (pH 7.2). The endothelium-intact thoracic aorta was cleaned and cut into helical strips. It was mounted in a warmed (37°C), and aerated (95% O_2, 5% CO_2) tissue bath connected to Grass isometric transducer (FT03; Grass instruments, Quincy, MA, USA), which was connected to an ADInstruments PowerLab (ADInstruments, Colorado Springs, CO). Tissues were placed under optimal resting tension (1500 mg; previously determined) and allowed to equilibrate for 1 hour. The initial contraction was stimulated by a maximal concentration (10^{-5} mol/L) of phenylephrine (PE). Cumulative concentration response curves to PE (10^{-10} to 10^{-5} mol/L), and 5-HT (10^{-9} to 10^{-5} mol/L) were

generated. The aorta was also half-maximally contracted to PE, followed by generation of an acetylcholine (ACh) concentration response curve (10^{-9} to 10^{-5} mol/L) to determine endothelial cell function. Each concentration response curves was performed in the aorta in the following order, PE, 5-HT, ACh. Potency of an agonist was expressed as = -log EC_{50} calculated by non-linear regression in GraphPad Prism (GraphPad Software Inc., San Diego, CA), where EC_{50} is the effective concentration of the agonist (mol/L) that induces 50% of the maximal response.

Statistical analysis

For *in vivo* data analysis, within-group differences were assessed by a one-way ANOVA with post hoc multiple comparisons using Student Newman-Keuls test (Graphpad Prism 5). An unpaired Students t-test was performed to measure point-to-point differences. In all cases, a *p* value of < 0.05 was considered significant. All results are presented as means ± SEM.

RESULTS

5-HT infusion in *established* DOCA-salt hypertensive rats

In this series of experiments, Sprague Dawley rats underwent DOCA-salt surgery (uninephrectomy, DOCA pellet 200 mg/kg, and water supplemented with 1% NaCl and 0.2% KCl) and were given 4 weeks to become *established* DOCA-salt hypertensive rats. These rats then received either 5-HT (25 μg/kg/min) or vehicle (*via* osmotic pump) for one-month.

Figure 1 shows the concentration of 5-HT, as measured by high performance liquid chromatography (HPLC), in platelet poor plasma (PPP; free 5-HT) and platelet rich plasma (PRP; platelet bound 5-HT). Blood was collected on the 28th day of 5-HT or vehicle infusion in established DOCA-salt rats. PPP was higher in rats that received 5-HT (282.9 ± 48.7 ng/ml) compared to those rats that received vehicle (87.9 ± 43.9 ng/ml) ($p < 0.05$). HPLC analysis revealed no differences between the 5-HT content contained within the osmotic pump and a freshly made 5-HT standard, suggesting a one-month 5-HT infusion did not degrade the 5-HT infused into established DOCA-salt rats.

Figure 2A summarizes the effect of 5-HT or vehicle infusion on MAP in established DOCA-salt hypertensive rats. Following 5-HT infusion, mean arterial pressure (MAP) was significantly reduced (105 ± 5 mmHg) compared to rats receiving vehicle (138 ± 6 mmHg) at S2. Despite modest recovery in 5-HT-infused rats over the one-month study,

MAP never returned to control levels (152 ± 6 mmHg at C2 *vs.* 135 ± 4 mmHg at S27). Blood pressure did not change in rats receiving vehicle (142 ± 5 mmHg at C2 *vs.* 151 ± 7 mmHg at S27) ($p > 0.05$). **Figure 2B** summarizes the effect of 5-HT or vehicle infusion on HR in established DOCA-salt hypertensive rats. Immediately following 5-HT infusion (C4 post-pump), HR was significantly increased (426 ± 10 bpm) compared to rats receiving vehicle (357 ± 3 bpm). However, by the 3rd day of 5-HT/vehicle infusion, HR had returned to normal in those rats receiving 5-HT despite significantly lower MAP compared to rats receiving vehicle.

Isometric contractility in *established* DOCA-salt hypertensive rats infused with 5-HT or vehicle

Aortic strips were mounted in warmed and aerated tissue baths for measurement of isometric contractile force to determine whether a one month infusion of 5-HT or vehicle effected the response to: the α adrenergic receptor agonist, phenylephrine (PE), 5-HT, or ACh. Initial contraction was stimulated by a maximal concentration of PE (10^{-5} M) and the magnitude of this response was not statistically different ($p > 0.05$) in established DOCA-salt 5-HT-infused hypertensive rats (1165 ± 107.8 mg) compared to established DOCA-salt Veh-infused hypertensive rats (1415 ± 126.7 mg). These values were then used to normalize subsequent contractile responses.

A concentration response curve to PE revealed no significant differences in potency between established DOCA-salt rats treated with 5-HT ($-\log EC_{50}$ = 7.45 ± 0.05)

compared to rats treated with vehicle (-logEC_{50} = 7.34 ± 0.1) (**Figure 3A)**. Maximal responses to PE were not different in 5-HT-infused established DOCA-salt rats (143 ± 6.0% of initial PE-induced contraction) compared to Veh-infused established DOCA-salt rats (133 ± 14% of initial PE-induced contraction) (p > 0.05). Similarly, a concentration response curve to 5-HT revealed no significant differences in agonist potency between 5-HT-infused established DOCA-salt rats (-logEC_{50} = 5.96 ± 0.14) compared to Veh-infused established DOCA-salt rats (-logEC_{50} = 5.97 ± 0.09) (p >0.05) (**Figure 3B**). Maximal contraction to 5-HT was not different in 5-HT-infused established DOCA-salt rats (126 ± 20%) compared to Veh-infused DOCA-salt rats (140 ± 11%) (p > 0.05).

Finally, an ACh-induced concentration response curve was performed. Data are reported as a percentage of the contraction elicited by half maximal (EC_{50}) concentration of PE in 5-HT-infused established DOCA-salt rats (972 ± 130.2 mg) and Veh-infused established DOCA-salt rats (1083 ± 102.8 mg) (p > 0.05). 5-HT infusion did not improve aortic sensitivity to ACh in 5-HT-infused established DOCA-salt rats (-logEC_{50} = 7.06 ± 0.17) compared to Veh-infused established DOCA-salt rats (-logEC_{50} = 6.76 ± 0.34) (p > 0.05) (**Figure 3C**). However, ACh-induced relaxation was slightly greater (although not statistically significant) in 5-HT-infused established DOCA-salt rats (33 ± 8.7% PE contraction remaining) *vs.* Veh-infused established DOCA-salt rats (58 ± 13.5% PE contraction remaining) (p > 0.05).

5-HT infusion *prior* to the development of DOCA-salt hypertension

In this series of experiments, Sprague Dawley (SD) rats received either 5-HT (25 μg/kg/min) or vehicle (*via* osmotic pump) for 5 days and then underwent DOCA-salt surgery (uninephrectomy, DOCA pellet 200 mg/kg, and water supplemented with 1% NaCl and 0.2% KCl) to determine whether 5-HT was capable of inhibiting the development of DOCA-salt hypertension.

Figure 4 shows the concentration of 5-HT in platelet poor plasma (PPP) and platelet rich plasma (PRP) in SD rats following a one month infusion with either 5-HT or vehicle and administration of a DOCA-salt pellet. PPP was significantly ($p < 0.05$) higher in rats that received 5-HT (177.1 ± 10.9 ng/ml) compared to those rats that received vehicle (13.3 ± 3.9 ng/ml).

Figure 5 summarizes the effect of 5-HT or vehicle infusion on *developing* DOCA-salt hypertensive rats over the course of one-month. Initially, 5-HT infusion resulted in a significantly ($p < 0.05$) lower MAP compared to Veh-infused rats. Following the DOCA-salt pellet implant, 5-HT did not significantly impact the development of DOCA-salt hypertension in either 5-HT-infused rats or Veh-infused rats. The absolute magnitude of MAP reached in 5-HT-infused rats (144 ± 6.5 mmHg) was not significantly different compared with Veh-infused rats (156 ± 5.9 mmHg). Scheduled sampling was briefly stopped (S12) during one-month infusion; MAP was monitored for the remainder of the study (S13-S28).

Isometric contractility in DOCA-salt hypertensive rats infused with 5-HT or vehicle *prior* to DOCA-salt administration

Aortic strips were mounted in warmed and aerated tissue baths for measurement of isometric contractile force to determine whether 5-HT infusion initiated prior to DOCA-salt administration would affect contractility. Initial contraction was stimulated by a maximal concentration of PE (10^{-5} M) and the magnitude of this response was not statistically different ($p > 0.05$) in DOCA-salt 5-HT-infused hypertensive rats (1094 ± 106.8 mg) compared to DOCA-salt Veh-infused hypertensive rats (939 ± 121.6 mg). These values were then used to normalize subsequent contractile responses.

A concentration response curve to PE revealed no significant differences in potency of PE between 5-HT-infused DOCA-salt rats ($-\log EC_{50}$ = 7.64 ± 0.1) compared to Veh-infused DOCA-salt rats ($-\log EC_{50}$ = 7.65 ± 0.2) (**Figure 6A)**. Maximal responses to PE were also not different in 5-HT-infused DOCA-salt rats (123 ± 5.2% of initial PE-induced contraction) compared to Veh-infused DOCA-salt rats (146 ± 12.1% of initial PE-induced contraction) ($p > 0.05$). Similarly, a concentration response curve to 5-HT was performed. No significant differences existed in 5-HT potency between 5-HT-infused DOCA-salt rats ($-\log EC_{50}$ = 6.39 ± 0.11) compared to Veh-infused DOCA-salt rats ($-\log EC_{50}$ = 6.32 ± 0.16) ($p >0.05$) (**Figure 6B**). Maximal responses to 5-HT were not different in 5-HT-infused DOCA-salt rats (145 ± 13% PE contraction remaining) compared to vehicle-infused DOCA-salt rats (178 ± 23% PE contraction remaining) ($p > 0.05$) when 5-HT was given prior to DOCA-salt administration.

Finally, an ACh-induced concentration response curve was performed. Data are reported as a percentage of the contraction elicited by half maximal (EC_{50}) concentration of PE in 5-HT-infused DOCA-salt rats (748 ± 39.2 mg) and Veh-infused DOCA-salt rats (690 ± 65 mg) ($p > 0.05$). 5-HT infusion did not improve aortic sensitivity to ACh in 5-HT-infused DOCA-salt rats (EC_{50} = 7.09 ± 0.19) compared to Veh-infused established DOCA-salt rats (EC_{50} = 6.85 ± 0.24) ($p > 0.05$) (**Figure 6C**). However, ACh-induced maximum relaxation was also slightly greater (although not statistically significant) in 5-HT-infused DOCA-salt rats (38 ± 9.2%) *vs.* Veh-infused DOCA-salt rats (59 ± 8.4%) ($p > 0.05$) when 5-HT infusion was initiated prior to DOCA-salt administration.

DISCUSSION

In this study, we took 2 independent but related approaches towards examining the ability of chronically elevated free 5-HT to decrease blood pressure asking these questions: 1) Is 5-HT capable of decreasing blood pressure in the *established* DOCA-salt hypertensive rat over the course of one-month; and 2) Is 5-HT capable of inhibiting the *development* of DOCA-salt hypertension over one-month? Endpoints of this study included HPLC analysis to validate elevation of free plasma 5-HT levels, blood pressure measurement *via* radiotelemetry, and isometric contractile studies.

5-HT-induced hypotension in *established* DOCA-salt hypertensive rats

5-HT infusion led to a significant reduction in blood pressure over one-month and a statistically significant increase in free plasma 5-HT in established DOCA-salt hypertensive rats (282.9 ± 48.7 ng/ml) vs. vehicle (87.9 ± 43.9 ng/ml). For comparison, free plasma 5-HT levels in the normotensive sham rat were 2.7 ± 0.29 ng/ml in Veh-infused rats (7 days), and 47.1 ± 23 ng/ml in 5-HT-infused rats (7 days) (Diaz *et. al.* 2008). These values demonstrate an increase in free plasma 5-HT in our hypertensive animal model (the *established* DOCA-salt rat) compared to a normotensive animal control and a further increase in free plasma 5-HT with a one-mouth infusion.

This study suggests elevated levels of free 5-HT (observed in both human and experimental models of hypertension) may be acting to lower blood pressure (rather than contributing to essential hypertension) and demonstrated 5-HT is capable of maintaining lower blood pressure(s) following 2 months of DOCA-salt treatment, a time

at which DOCA-salt hypertension becomes irreversible. Further this protocol also demonstrated that 5-HT infusion (delivered over one month to *established* DOCA-salt rats) does not improve ACh-induced relaxation (despite an observable trend toward improved ACh-induced relaxation). Moreover, the lack of a change in potency to the effects of PE and 5-HT demonstrated the potential of blood vessels to contract to normal stimuli (PE) and that those blood vessels do not lose there contractile response to 5-HT (desensitization) with either infusion protocol.

In the established DOCA-salt rat, elevated blood pressure is characterized by hypervolaemia, high levels of mineralocorticoid, and high salt intake (Yermane *et. al.* 2009). Several factors contribute to the maintenance of established blood pressure and include: 1) enhanced sympathetic nerve activity, 2) increased vasopressin release, 3) increased endothelin expression, 4) attenuation of the baroreceptor reflex, 5) vascular remodeling, and 6) nitric oxide depletion due to endothelial cell damage/dysfunction (Schenk and McNeill, 1992; Crofton *et. al.* 1979; Lariviere *et. al.* 1993). Each of these factors represent a potential target by which 5-HT may be acting to lower blood pressure in the established DOCA-salt rat.

The exact mechanism(s) by which 5-HT is acting to lower blood pressure in this established model have not been identified, but the above factors give some insights. For example, one possibility is that 5-HT might be acting to inhibit sympathetic nerve activity (SNA), thereby acting to reduce total peripheral resistance (TPR) and lower blood pressure. The $5\text{-HT}_{1B/1D}$ receptor is located on the presynaptic nerve terminal and, when stimulated by 5-HT, results in an inhibition of norepinephrine (NE) release

(Molderings *et. al.* 1990; Göthert *et. al.* 1991; Shepard and Vanhoutte, 1985). Moreover, recent evidence suggests that 5-HT may be able to cross the blood brain barrier (Nakatani *et. al.* 2008). Thus, we cannot rule out the possibility that 5-HT might be acting in the central nervous system to lower blood pressure. Future studies will need to be undertaken to elucidate the exact mechanism of 5-HT-induced hypotension in the established DOCA-salt rat.

5-HT on the *development* of DOCA-salt hypertension

5-HT was significantly elevated at the end of the one-month study in 5-HT treated rats *vs.* vehicle treated rats. This measure validated our infusion protocol and suggested that any change or lack of change in the development of DOCA-salt hypertension was not due to insufficient delivery and/or degradation of 5-HT during this study. Despite elevated levels of free 5-HT, there was no significant difference in the development of DOCA-salt hypertension or absolute magnitude of blood pressure reached in 5-HT *vs.* vehicle treated rats. This was a surprising finding given that previous studies demonstrated that 5-HTP, the committed precursor to 5-HT, prevented the development of DOCA-salt hypertension in the rat (Fregly *et. al.* 1987). This suggests that 5-HT cannot prevent DOCA-salt hypertension despite, the previously studied effects of 5-HTP. We further demonstrated that 5-HT infusion was not capable of improving ACh-induced relaxation even if started prior to the development of DOCA-salt hypertension.

Several lines of evidence suggest that enhanced sympathetic nerve activity (SNA) is crucial to the developmental phase of DOCA-salt hypertension (Yemane *et. al.* 2009;

Schenk and McNeill, 1992). While 5-HT was capable of lowering blood pressure in the established DOCA-salt rat, it was unable to prevent the development of DOCA-salt hypertension. Given the importance of enhanced SNA to the development of DOCA-salt hypertension, this finding might suggest another mechanism (rather then SNS inhibition) by which 5-HT lowers blood pressure. Further, while we do not dispute the importance of enhanced SNA in the established DOCA-salt model, an alternate mechanism may help explain why 5-HT was effective in lowering blood pressure in the established DOCA-salt rat but was unable to prevent the development of DOCA-salt hypertension.

Thus, another possibility by which 5-HT may be acting to lower blood pressure is through direct action(s) on the peripheral vasculature. Indirect support for this hypothesis is provided by Diaz *et. al.* who demonstrated improved aortic ACh-induced relaxation in the DOCA-salt rat following 5-HT infusion and the absolute dependence on NOS for enabling a 5-HT-induced fall in blood pressure (Diaz *et. al.* 2008). This suggests that 5-HT might be acting on the endothelial cell to improve overall function and promote nitric oxide (NO) release. Alternatively, the 5-HT_{2B} receptor is located on the endothelial cell and is capable of nitric oxide (NO) release (Ishida *et. al.* 1998). Thus, this receptor may be a potential target for 5-HT, promoting NO release, and a 5-HT-induced fall in blood pressure. However, we cannot rule out the possibility that the improved aortic ACh-induced relaxation is due to lower blood pressure in 5-HT treated DOCA-salt rats compared with vehicle treated DOCA-salt rats. In other words, the improved aortic relaxation may be a consequence of lower blood pressure in DOCA-salt rats rather then 5-HT acting directly on the endothelial cell to improve NOS function and

NO release. In the present study, 5-HT did not improve aortic ACh-induced relaxation when infused over the course of one-month. Despite an observable trend toward an improved maximum relaxation to ACh in 5-HT treated rats compared to vehicle treated rats, this evidence suggests an alternate mechanism by which 5-HT lowers blood pressure.

Finally, several clinical scenarios correlate well with our investigation of elevated levels of free 5-HT and changes to blood pressure. Cardiopulmonary bypass, hemodialysis (Borgdoff *et. al.* 2002), and anaphylactic shock (Meurer *et. al.* 1981) are all associated with release of 5-HT from either the platelet or enterochromaffin cell and are associated with significant falls in blood pressure. Pharmacological inhibition of SERT *via* treatment with an SSRI (selective serotonin reuptake inhibitor) increases extracellular concentrations of 5-HT and changes in blood pressure are noted as side effects of treatment with these drugs (Rodriguez *et. al.* 2001). Further, 5-HTP, the committed substrate to 5-HT synthesis, is taken as a sleep aide, potentially exposing patients to elevated levels of free 5-HT (Das *et. al.* 2004). Taken together, these data suggest, that there are several clinically relevant situations in which patients would have significantly elevated free plasma 5-HT levels. Understanding exactly how 5-HT affects cardiovascular function is critical to understanding and treating both current and future patients populations.

Perspectives and limitations

One potential limitation of this study is the dose of DOCA-salt (200 mg/kg) used in our protocol to test the hypothesis of whether 5-HT was capable of preventing the development of DOCA-salt hypertension. Previously, a dose of 100 mg/kg of DOCA-salt was used to demonstrate 5-HTP (a committed precursor to 5-HT) was capable of preventing the development of DOCA-salt hypertension (Fregly *et. al.* 1987). The higher dose of 200 mg/kg of DOCA-salt (used throughout this study) may have masked the ability of 5-HT to inhibit DOCA-salt hypertension. Another potential limitation of this study is the length of our infusion protocol. Clinically, patients often require antihypertensive medications for a lifetime, thus a one-month infusion remains a relatively short exposure. Newly available infusion pumps may be able to extend this exposure even further (Abe *et. al.* 2009)

In summary, this study reinforces the finding that chronic 5-HT-infusion lowers blood pressure in the established DOCA-salt hypertensive rat, and demonstrates that 5-HT is capable of lowering blood pressure over the course of one-month. 5-HT was unable to prevent the development of DOCA-salt hypertension, but this study suggests a potentially beneficial role for 5-HT in the cardiovascular system.

Figure 2.1

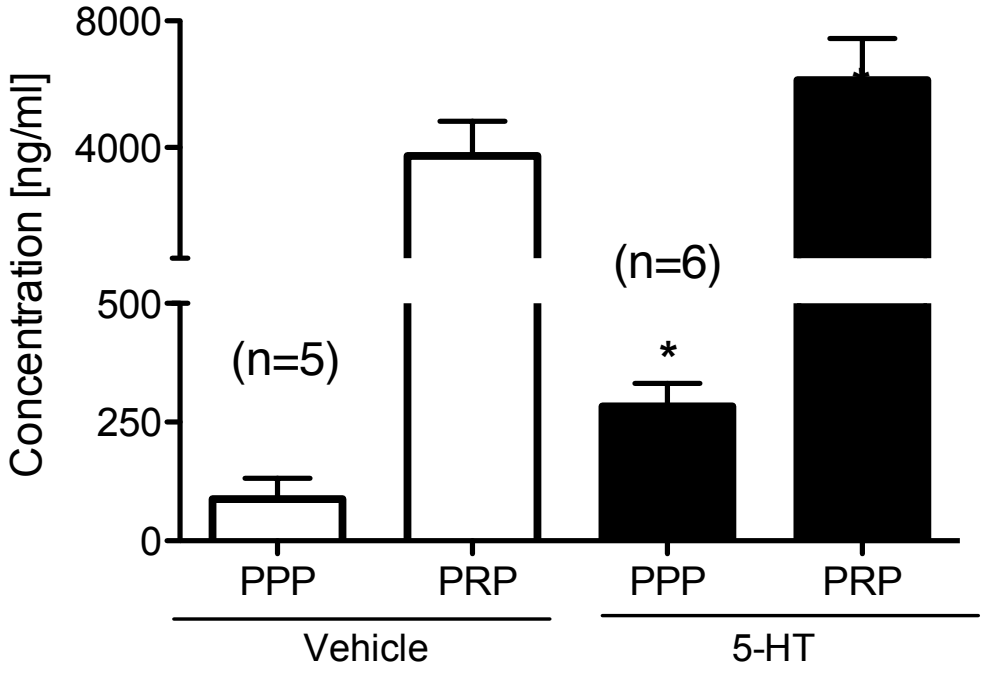


Figure 2.1: Plasma (platelet poor plasma; PPP) and platelet (platelet rich plasma; PRP) 5-HT content in established DOCA-salt hypertensive rats following 28-day infusion of vehicle or 5-HT. Bars represent mean +/- SEM for the number of animals in parentheses. * Statistically significant difference ($p < 0.05$) vs. vehicle.

Figure 2.2

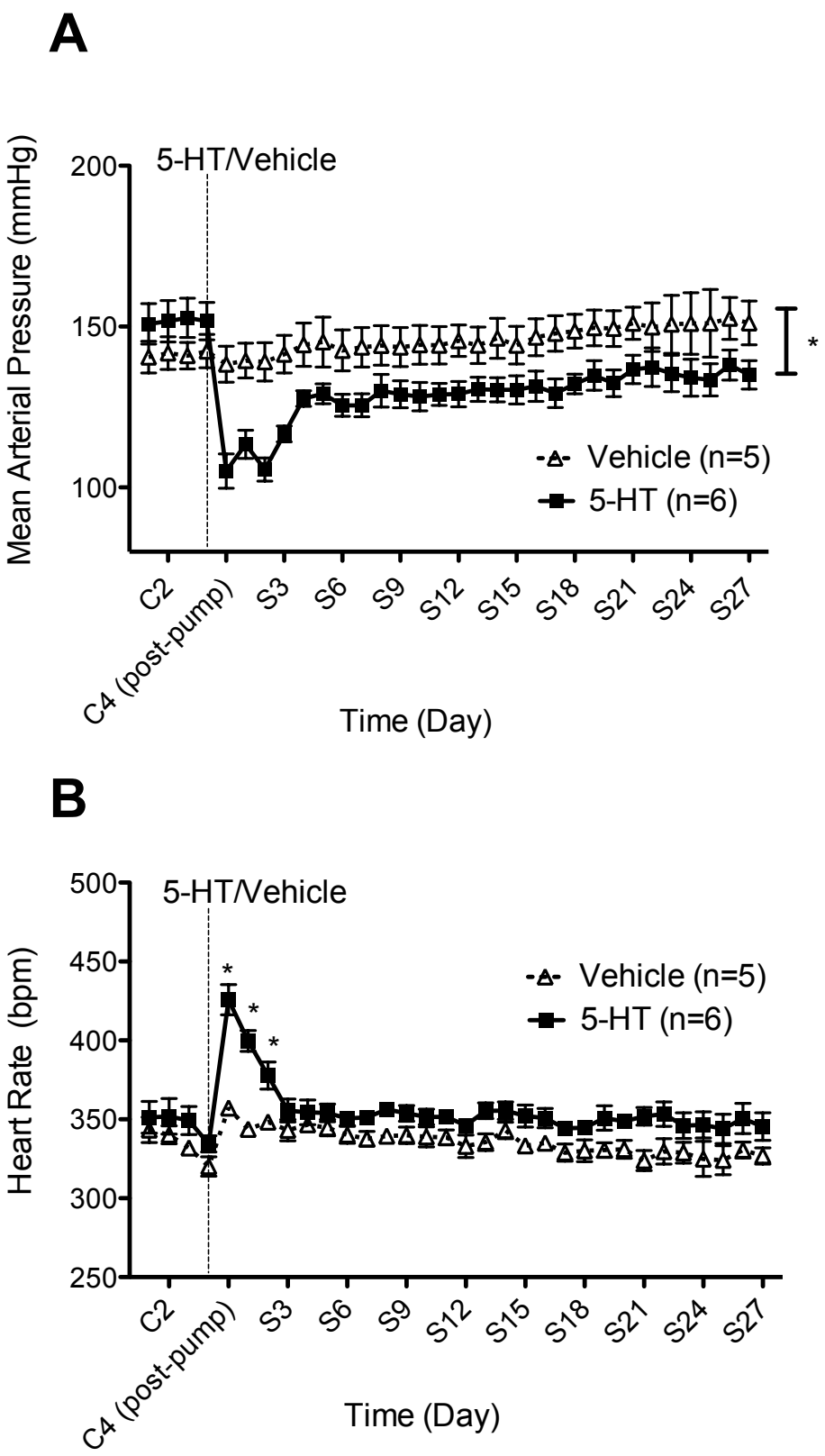
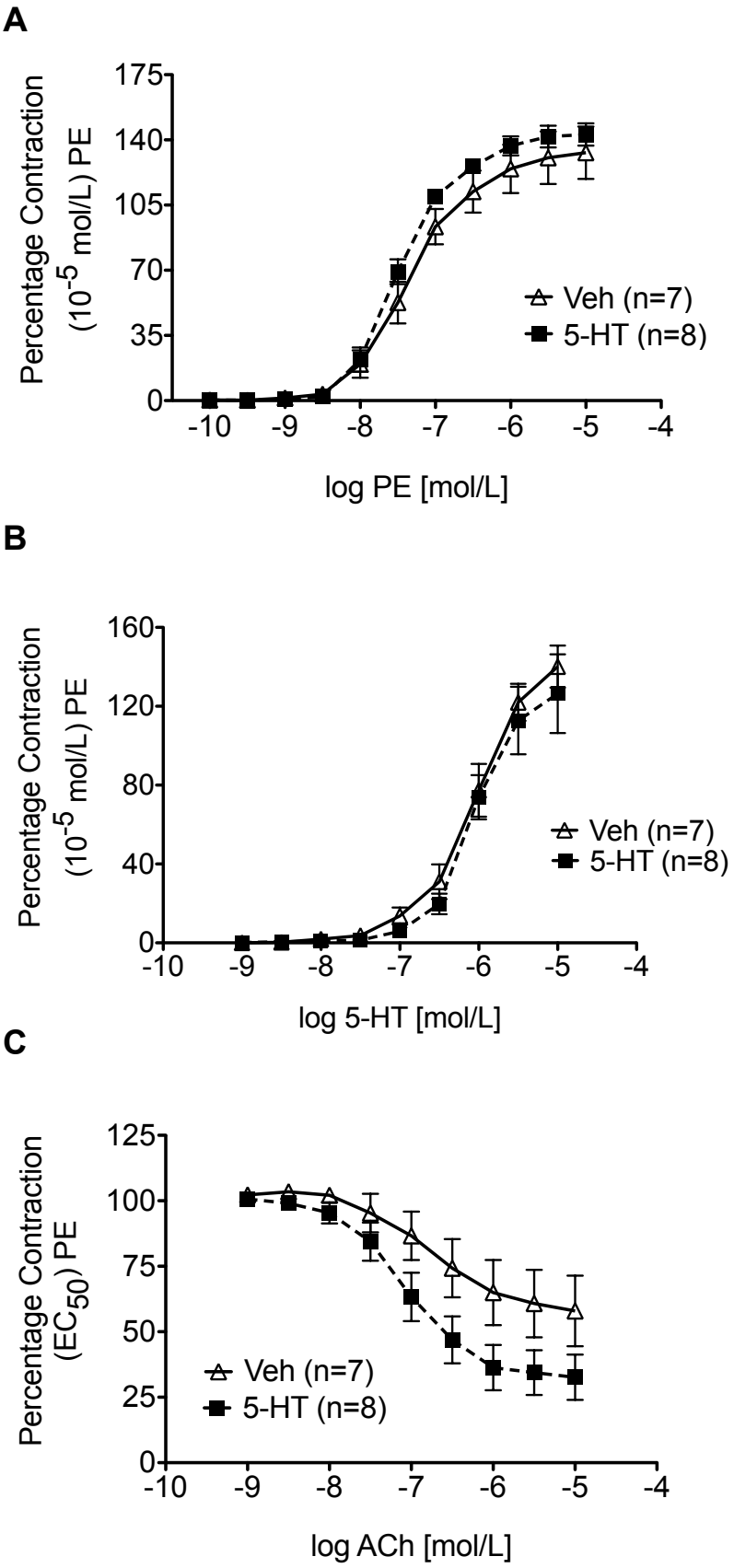

A
5-HT/Vehicle
Mean Arterial Pressure (mmHg)
200
150
100
Vehicle (n=5)
5-HT (n=6)
*
C2
C4 (post-pump)
S3
S6
S9
S12
S15
S18
S21
S24
S27
Time (Day)
B
5-HT/Vehicle
Heart Rate (bpm)
500
450
400
350
300
250
Vehicle (n=5)
5-HT (n=6)
*
*
*
C2
C4 (post-pump)
S3
S6
S9
S12
S15
S18
S21
S24
S27
Time (Day)

Figure 2.2: A. Effect of chronic 5-HT or vehicle infusion on mean arterial pressure (MAP) in established DOCA-salt hypertensive rats. Data points indicate 24-hour averaged MAP +/- SEM for the number of animals in parentheses. The vertical line denotes 5-HT or vehicle osmotic pump implant. Time is represented by days on the x-axis. C represents control recordings. S represents 5-HT/vehicle infusions. **B.** Effect of chronic 5-HT or vehicle infusion on heart rate (HR) in established DOCA-salt hypertensive rats. Data points indicate 24-hour averaged HR (bpm) +/- SEM for the number of animals in parentheses. The vertical line denotes 5-HT or vehicle osmotic pump implant. Time is represented by days on the x-axis. C represents control recordings. S represents 5-HT/vehicle infusions. * Statistically significant difference ($p < 0.05$) vs. vehicle.

Figure 2.3


A
Percentage Contraction
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140
105
70
35
0
-10
-9
-8
-7
-6
-5
-4
log PE [mol/L]
Veh (n=7)
5-HT (n=8)
B
Percentage Contraction
(10^{-5} mol/L) PE
160
120
80
40
0
log 5-HT [mol/L]
Veh (n=7)
5-HT (n=8)
C
Percentage Contraction
(EC_{50}) PE
125
100
75
50
25
0
log ACh [mol/L]
Veh (n=7)
5-HT (n=8)

Figure 2.3: Cumulative concentration response curve to **A.** phenylephrine (PE), **B.** 5-HT, or **C.** acetylcholine (ACh) in the thoracic aorta of established DOCA-salt hypertensive rats. Data points represent means +/- SEM for the number of animals in parentheses.

Figure 2.4

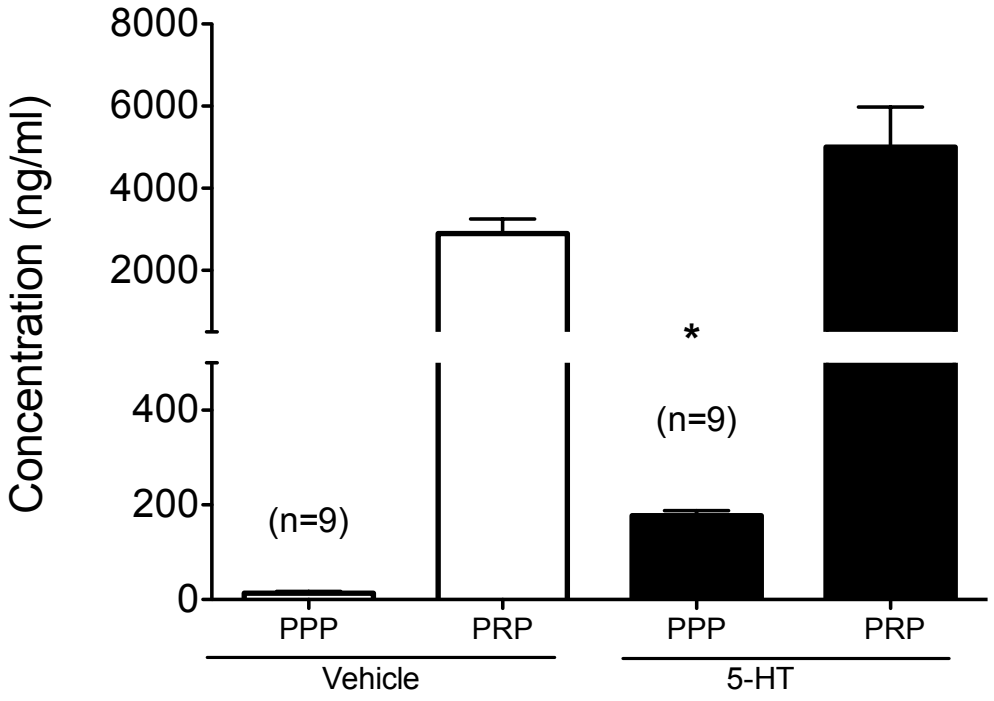

Figure 2.4: Plasma (platelet poor plasma; PPP) and platelet (platelet rich plasma; PRP) 5-HT content in Sprague Dawley rats following a one month infusion with either 5-HT or vehicle, followed by administration of a DOCA-salt pellet. Bars represent mean +/- SEM for the number of animals in parentheses. * Statistically significant difference ($p < 0.05$) vs. vehicle.

Figure 2.5

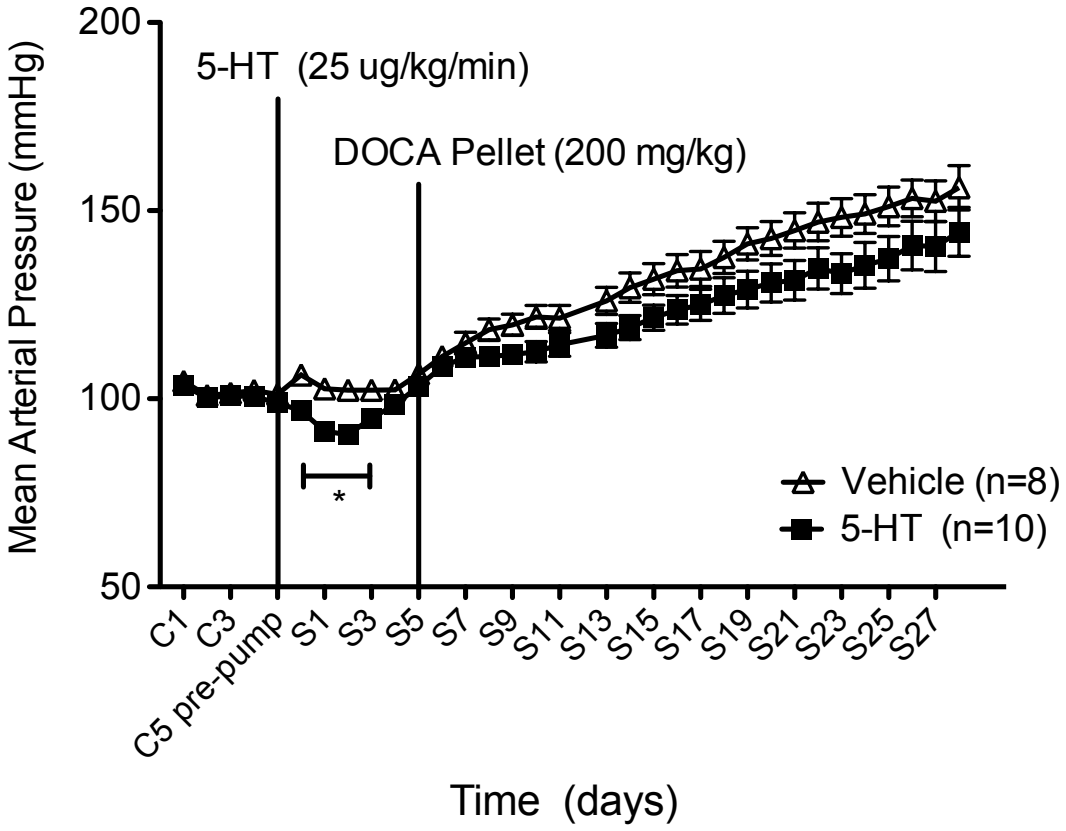

Figure 2.5: Effect of chronic 5-HT or vehicle infusion on mean arterial pressure (MAP) in in Sprague Dawley rats following a one month infusion with either 5-HT or vehicle, followed by administration of a DOCA-salt pellet. Data points indicate 24-hour averaged MAP +/- SEM for the number of animals in parentheses. The vertical line denotes 5-HT or vehicle osmotic pump implant and DOCA pellet implant. Time is represented by days on the x-axis. C represents control recordings. S represents 5-HT/vehicle infusions. * Statistically significant difference ($p < 0.05$) vs. vehicle.

Figure 2.6

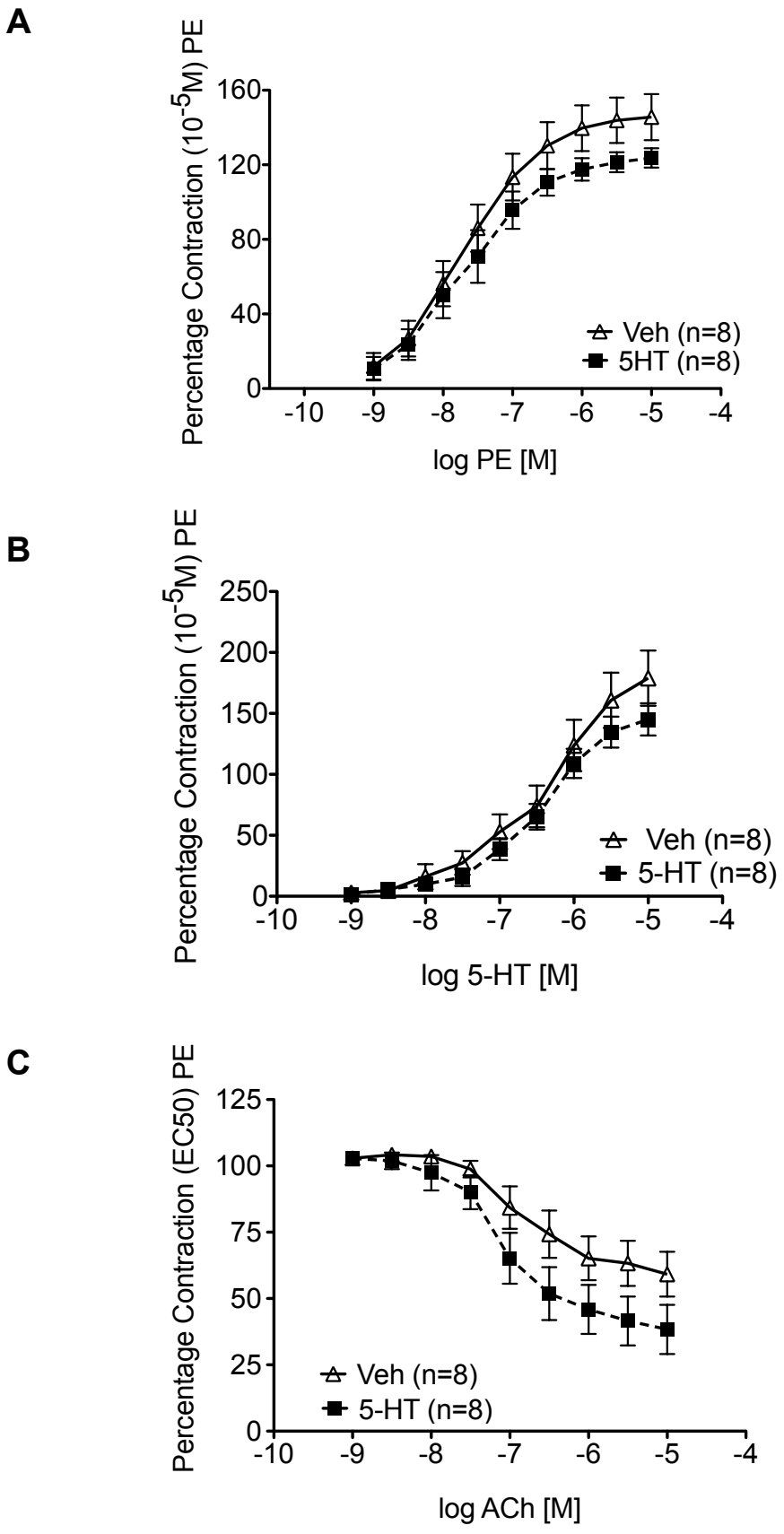
A
Percentage Contraction (10^{-5}M) PE
160
120
80
40
0
-10
-9
-8
-7
-6
-5
-4
log PE [M]
Veh (n=8)
5HT (n=8)
B
Percentage Contraction (10^{-5}M) PE
250
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
log 5-HT [M]
Veh (n=8)
5-HT (n=8)
C
Percentage Contraction (EC50) PE
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
log ACh [M]
Veh (n=8)
5-HT (n=8)

Figure 2.6: Cumulative concentration response curve to **A.** phenylephrine (PE), **B.** 5-HT, or **C.** acetylcholine (ACh) in the thoracic aorta of DOCA-salt rats treated with either 5-HT or vehicle during the development of DOCA-salt hypertension. Data points represent means +/- SEM for the number of animals in parentheses.

Chapter 3

5-hydroxytryptamine (5-HT) does not reduce total peripheral resistance through direct receptor mediated vascular relaxation of the superior mesenteric artery

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ABSTRACT

Serotonin (5-hydroxytryptamine; 5-HT) delivery over 1 week results in a sustained fall in blood pressure in the deoxycorticosterone acetate (DOCA)-salt and sham rat. We hypothesized that 5-HT lowers blood pressure through direct receptor-mediated vascular relaxation. *In vivo*, we show that 5-HT reduced mean arterial pressure (MAP), increased cardiac output (CO), stroke volume (SV), the cardiac index (CI), and reduced total peripheral resistance (TPR) in the normotensive rat when compared to rats receiving vehicle. Real-time RT-PCR demonstrated that mRNA transcripts for the 5-HT_{2B}, 5-HT_{1B}, and 5-HT_7 receptors were present in sham and DOCA-salt superior mesenteric artery (SMA). Immunohistochemistry and western blotting validated the presence of the 5-HT_{2B}, 5-HT_{1B} and 5-HT_7 receptors in sham and DOCA-salt SMA. Isometric contractile force was measured in endothelial-intact sham mesenteric resistance arteries and SMA from sham and DOCA-salt rats. 5-HT did not cause relaxation in the ketanserin-incubated, U46619-contracted resistance artery and superior mesenteric arteries of sham and DOCA-salt rats. Maximum concentrations of BW-723C86 (5-HT_{2B} agonist) and CP 93129 (5-HT_{1B} agonist) did not relax the SMA from DOCA-salt (118 ± 6.7%; 180 ± 15% of half-maximal U46619-induced contraction) or sham (81 ± 7.1%; 88 ± 8.9%) rats *vs.* H_2O vehicle. Further, maximum concentrations of LP-44 (5-HT_7 agonist) did not relax the SMA from DOCA-salt (75 ± 4.7%) or sham (55 ± 6.4%) rats *vs.* vehicle. Thus, although these 5-HT receptors are present in the

SMA, their activation does not play a role in relaxation, and are likely not involved in a 5-HT-induced fall in MAP.

INTRODUCTION

5-HT is a vasoactive amine synthesized in the enterochromaffin cells of the gastrointestinal tract and the raphe nucleus of the central nervous system (Erspamer and Asero, 1952; Dahlstroem and Fuxe, 1964). The physiological actions of 5-HT are mediated by 7 major receptor subtypes (5-HT_1-5-HT_7) (Watts and Davis, 2010). 5-HT is actively taken up by the serotonin transporter (SERT) and metabolized by monoamine oxidase (MAO) to its inactive metabolite 5-hydroxyindole acetic acid (5-HIAA). 5-HT is circulated throughout the peripheral vasculature by platelets, which actively take up and store (millimolar) concentrations of 5-HT. This results in comparatively low levels of circulating free plasma 5-HT under normotensive conditions (Figueras *et. al.* 2005).

5-HT was initially identified as a potent constrictor of isolated blood vessels in the rat (Rapport *et al*. 1948). The primary contractile receptor in the peripheral vasculature of the rat is the 5-HT_{2A} receptor (Yildiz *et. al*. 1998). Activation of 5-HT receptors also mediates contraction of human blood vessels, including: the human coronary arteries (Nilsson *et. al*. 1999), human saphenous vein, internal mammary artery (Gul *et. al.* 2003), human cutaneous hand vein (Bodelsson *et. al.* 1992), and human pulmonary artery (Cortijo *et. al.* 1997).

This led to an initial hypothesis that elevated levels of free plasma 5-HT, present in both human and experimental models of hypertension (Brenner *et al.* 2007; Fetkovska *et al.* 1990) were acting to constrict the peripheral vasculature, resulting in elevated blood

pressure. However, Diaz *et. al.* demonstrated that chronic 5-HT infusion produces a sustained fall in blood pressure in the deoxycorticosterone acetate (DOCA)-salt and sham rat (Diaz *et. al.* 2008). This suggested that elevated levels of free plasma 5-HT might be acting to lower blood pressure. The exact mechanism by which chronic 5-HT infusion lowers blood pressure is not yet known. However, Diaz *et. al.* also demonstrated that inhibition of nitric oxide synthase (NOS) prevented the chronic 5-HT-induced fall in blood pressure in the sham and DOCA-salt rat. This suggested an important role for NOS, and potentially nitric oxide (NO), in enabling a 5-HT-induced fall in blood pressure.

The 5-HT_{2B}, 5-HT_{1B}, and 5-HT_{7} receptors have all been linked to dilation of peripheral arteries (Bordoff *et. al.* 2002; Calama *et. al.* 2003; Saxena and Villalon, 1990). Reports of falling blood pressure in patients placed on cardiopulmonary bypass led Bordoff *et. al.* to postulate that 5-HT released from mechanically disrupted platelets (a result of cardiopulmonary bypass) might be acting to lower blood pressure. Bordoff *et. al.* went on to demonstrate that treatment with either a 5-HT_{2B} receptor antagonist or an inhibitor of NOS abolished a bypass-induced fall in blood pressure in the male normotensive Wistar rat (Bordoff *et. al.* 2002). This suggests 5-HT may be acting at the 5-HT_{2B} receptor to stimulate NOS, and potentially release NO, thereby lowering blood pressure in the rat. Additionally, Calama *et. al.* demonstrated that intra-arterial administration of L-694, 247 (5-$HT_{1B/1D}$ receptor agonist) produced vasodilation in the hindquarter of the anesthetized rat (Calama *et. al.* 2003). This suggests 5-HT may be acting at the 5-

$HT_{1B/1D}$ receptor to lower blood pressure in the rat. Finally, the 5-HT_7 receptor is associated with vascular smooth muscle relaxation, and a long-lasting depressor response to 5-HT in the rat (Saxena and Villalon, 1990). This suggests another potential site (the 5-HT_7 receptor) at which 5-HT may be acting to lower blood pressure in the rat. Collectively, this evidence suggests that the 5-HT_{2B}, 5-HT_{1B}, or the 5-HT_7 receptor(s) may represent potential site(s) of action by which 5-HT acts to lower blood pressure. Thus, we tested the hypothesis that 5-HT lowers blood pressure through direct receptor-mediated vascular relaxation.

To test this hypothesis, we investigated whether 5-HT infusion was lowering blood pressure through a reduction in total peripheral resistance (TPR) or by reducing cardiac output (CO). We then utilized *in vitro* pharmacological techniques to demonstrate the presence of the 5-HT_{2B}, 5-HT_{1B}, and 5-HT_7 receptors in the superior mesenteric artery (SMA) of the sham and DOCA-salt rat. Classical agonists selective for each receptor subtype were used, as well as 5-HT itself. The SMA is a model resistance artery and changes in the tone of this blood vessel reflect changes in blood pressure following 5-HT infusion. Isolated mesenteric arteries were studied at the level of mRNA (real time PCR), protein (western blot and immunohistochemical analysis), and functionally (isolated contractile studies; conducted in the presence of ketanserin, to potentially unmask a relaxant receptor) to determine whether 5-HT caused direct relaxation of the isolated mesenteric artery.

METHODS

Animals

The Michigan State University and Loyola University Chicago, Stritch School of Medicine Institutional Animal Care and Use Committees (IACUC) approved all protocols. Male Sprague Dawley rats (225-250 g, Charles River Laboratories) were used. Hemodynamic studies (measurement of mean arterial pressure (MAP), cardiac output (CO), stroke volume (SV), and calculations of cardiac index (CI), and total peripheral resistance) were completed in conscious freely moving Sprague Dawley rats. Isolated tissue experiments (RT-PCR, western blot, immunohistochemistry, and isolated contractile experiments) were performed in the SMA from sham and DOCA-salt rats.

DOCA-salt hypertension

Sprague-Dawley rats underwent left uninephrectomy, and a DOCA pellet was implanted subcutaneously (200 mg/kg). Sham rats underwent left uninephrectomy, but were not implanted with DOCA pellet. Rats were given standard rat chow ad libitum. DOCA-salt water was supplemented with 1% NaCl and 0.2% KCl for the duration of the study. Sham rats received tap water for the duration of the study. Hypertension was established after 4 weeks of initiation of DOCA. Sham systolic blood pressure was < 140 mmHg, while DOCA-salt systolic blood pressure was > 140 mmHg measured by tail cuff method.

Anesthesia and Analgesia

All rats were anesthetized with isoflurane (2% in 100% O_2) and ventilated mechanically. At the time of surgery, the incision site was treated with topical antibiotic containing

analgesic to prevent irritation and infection. Incisions were closed with silk suture. Rats were treated with amoxicillin (150 mg/kg/i.m.) following surgery and 3 days thereafter. All rats were treated with rimadyl (5 mg/kg, s.c. for 2 days) for general analgesia.

Surgical Methods: Alzet osmotic pump

A small incision was made at the base of the neck. Blunt dissection was used to create a small subcutaneous pocket between the scapulae. The pump (Alzet Osmotic Pump, Model 2ML4, Duret Corporation, Cupertino, CA, 2.5 μL/hr 28 days) was inserted and the skin sutured closed. To each pump, a 5-HT creatinine complex (25 μg/kg/min) and 1% ascorbic acid, antioxidant (0.02 g/pump) or vehicle (1% ascorbic acid, antioxidant) was loaded. The solution was dissolved in 1 mol/L HCl, and a pH-balance (~7) was achieved with 4 mol/L NaOH.

Surgical Methods: Ascending aortic flow probe

Rats were given an injection of antisialogogue, glycopyrrolate (4 mg/kg, s.c.), then anesthetized with isoflurane, intubated and mechanically ventilated with room air. Through an incision in the third intercostal space, an ultrasonic transit-time flow probe (model 3SB; Transonic Systems Inc., Ithaca, NY) was placed around the ascending aorta. The thoracic incision was closed in layers, and the lungs were reinflated with negative pressure. The probe cable was tunneled s.c. and externalized at the nape of the neck using silastic cuff.

Surgical Methods: Blood pressure probe

During the same surgery, radiotelemetric transmitters (PhysioTel C50-PXT series, Data Science International, St. Paul, MN, USA) for blood pressure and ECG recording were implanted subcutaneously. Through the groin incision, left femoral artery was catheterized and the tip of catheter was advanced into the abdominal aorta. All rats were allowed at least 10 days for recuperation prior to beginning data collection.

Mean arterial pressure (MAP), heart rate (HR), and aortic blood flow (i.e. CO) were recorded daily, between 10:00 am and 2:00 pm. Following the connection to recording instruments, animals were allowed at least 60 min for habituation, after which MAP and HR were recorded continuously for 1 hour. Total peripheral resistance (TPR) (mmHg/ml/min/kg) and cardiac Index (CI) are reported as normalized parameters. CI = CO / ($9.1 \times g^{0.66}$), where g = body mass in grams, and ($9.1 \times g^{0.66}$) = body surface area in cm^2).

Tissue preparation

Rats were anesthetized with pentobarbital (60 mg/kg i.p.) and the superior mesenteric artery or the mesenteric resistance artery was removed and placed in physiological salt solution (PSS) containing (mM): NaCl 130; KCl 4.7; KH_2PO_4 1.8; $MgSO_4 * 7H_2O$ 1.7; $NaHCO_3$ 14.8; dextrose 5.5; $CaNa_2EDTA$ 0.03, $CaCl_2$ 1.6 (pH 7.2)

Real-Time RT-PCR

Superior mesenteric artery was removed and placed in sterile water, then cleaned of fat and blood. Total RNA was isolated using the MELT Total Nucleic Acid Isolation System

(Ambion, Austin, TX) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Standard real-time RT-PCR was done using a GeneAMP 7500 Real-Time PCR machine (Applied Biosystems, Carlsbad, CA) and SYBR Green PCR Master Mix (Applied Biosystems). Rat primers were purchased from SABiosciences (Frederick, MD): 5-HT_{1A} (RefSeq Accession #: NM_012585.1; 191 bp amplicon), 5-HT_{1B} (RefSeq Accession #: NM_022225.1; 103 bp amplicon), 5-HT_{1D} (RefSeq Accession #: NM_012852.1; 173 bp amplicon), 5-HT_{2A} (RefSeq Accession #: NM_017254.1; 191 bp amplicon), 5-HT_{2B} (RefSeq Accession #: NM_017250.1; 140 bp amplicon), 5-HT_{3A} (RefSeq Accession #: NM_024394.2; 179 bp amplicon), 5-HT_4 (RefSeq Accession #: NM_01285.31; 83 bp amplicon), 5-HT_{5A} (RefSeq Accession #: NM_013148.1; 154 bp amplicon), 5-HT_6 (RefSeq Accession #: NM_024365.1; 186 bp amplicon), 5-HT_7 (RefSeq Accession #; NM_022938.2; 99 bp amplicon), and calibrator control (beta-2 microglobulin) (RefSeq Accession #: NM_012512, 128 bp amplicon). PCR conditions were: 95^{o}C for 10 minutes followed by 40 cycles of (95^{o}C, 15 sec; 60^{o}C, 60 sec). A standard dissociation curve was run following the above cycle conditions.

Western Blot Analysis

Superior mesenteric arteries were cleaned of fat and blood, placed into liquid nitrogen and ground to a powder. Ice-cold homogenation buffer (125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 10 ug/ml aprotinin, 10 ug/ml leupeptin) was added and the homogenates were vortexed briefly

and sonicated for 1 minute (5x each). Samples were centrifuged at 11,000 rpm for 10 minutes at 4°C to pellet debris. Supernatant was collected and placed in clean microtubes, then held at -80°C until protein concentrations could be determined. Protein concentration was determined with the BCA protein kit (Sigma, catalog #BCA1), following standard procedure. Western analysis of rat superior mesenteric artery homogenates (50 μg) was done and proteins were transferred to PVDF (5-HT_{1B} and 5-HT_7) or nitrocellulose (5-HT_{2B}). Blots were then incubated overnight at 4°C with 5-HT_{1B} (1 ug/mL; Abcam, Cambridge, MA; Catalog # ab13896), 5-HT_{2B} (1:1000; BD Pharmingen, San Diego, CA; Catalog # 556334), or 5-HT_7 (1:1000; Abcam, Cambridge, MA; Catalog # ab13898). Following 5-HT receptor antibody incubation, blots were reprobed with smooth muscle α-actin (1:2000; EMD Chemicals/Calbiochem, Gibbstown, NJ) to ensure equal protein loading. All blots were developed using species-specific HRP-conjugated secondary antibodies and ECL reagents (Amersham/GE Healthcare Life Sciences, Piscataway, NJ).

Immunohistochemistry

Slides containing 8 μm sections of paraffin-embedded rat superior mesenteric artery were dewaxed in Histochoice Clearing Agent (2X, 3 minutes each; Amresco, Solon, OH), followed by isopropanol (4X, 3 minutes each) and distilled water (2X, 3 minutes each). Slides were placed in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) and microwaved on high (2X, 30 seconds each), then cooled and dried. Samples were incubated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 minutes to block endogenous peroxidases, followed by blocking for

nonspecific binding with 1.5% blocking serum in PBS for 30 minutes. Slides were incubated overnight in a humidified chamber at 4°C with 5-HT_{1B}, 5-HT_{2B} or 5-HT_7 antibody (5 μg/mL) (same antibodies used for western blots) in 1.5% blocking serum. Slides were washed in PBS (3X) and incubated with a peroxidase-conjugated secondary antibody in 1.5% blocking serum for 30 minutes, followed by 30 minutes with Vectastain Elite ABC Reagent (Vector Laboratories). 3,3-diaminobenzidine/H_2O_2 was applied until staining appeared (1-4 minutes), stopped with PBS washing, and counterstained with hematoxylin (Vector Laboratories, Burlingame, CA). Slides were air dried and coverslipped and pictures were taken using an inverted Nikon microscope with a digital camera (Nikon, Tokyo, Japan). Images were captured using MMI Cellcut software (MMI Inc., Rockledge, FL).

Isolated tissue bath

Endothelium-intact superior mesenteric arteries (SMA) were cleaned and cut into helical strips for measurement of isometric contractile force. SMA strips were mounted in warmed (37°C) and aerated (95% O_2, 5% CO_2) tissue baths (30 mL) on Grass isometric transducers (FT03; Grass instruments, Quincy, MA, USA), which were connected to an ADInstruments PowerLab (ADInstruments, Colorado Springs, CO). Tissues were placed under optimal resting tension (600 mg; previously determined) and allowed to equilibrate for 1 hour before an initial challenge with a maximal concentration of phenylephrine (10^{-5} M). After the initial challenge, tissues were washed until toned returned to baseline. Then, a half-maximal concentration of phenylephrine (10^{-7} M) was added to the bath, followed

by acetylcholine (ACh; 10^{-6} M) to determine the integrity of the endothelial layer. Tissues that relaxed >50% to ACh were considered endothelium-intact. Tissues were washed until they returned to baseline. Strips were incubated with ketanserin (50 nM; 5-HT_{2A} antagonist) for 15 minutes to prevent activation of the 5-HT_{2A} contractile receptor. Tissues were then contracted half-maximally to U46619 (thromboxane A_2 agonist) (~ 30 minute incubation with ketanserin). Cumulative concentration-response curves were generated for each of the following agonists: 5-HT *vs.* vehicle (H_2O), BW-723C86 (5-HT_{2B} agonist) *vs.* vehicle (H_2O), CP 93129 (5-HT_{1B} agonist) *vs.* vehicle (H_2O), and LP-44 (5-HT_7 agonist) *vs.* vehicle (DMSO). All data were captured in the program Chart.

Wire Myograph

Under a stereomicroscope with a calibrating eyepiece and in cold PSS, two tungsten wires were inserted through the lumen of a cleaned mesenteric resistance artery (~200-250 μm diameter). One wire was attached to an isometric force transducer that can detect forces from 0.002 – 10 grams, the other to a micrometer-attached support. Force generation was recorded on a Grass Model 7D polygraph. The dual chamber, kept at 37^{o} C and perfused *via* a peristaltic pump with warmed and oxygenated PSS, allowed mounting of two parallel vessels. A passive tension of ~400 mg (appropriate for generating optimal force in a resistance artery) was applied. Vessels were allowed to equilibrate for 1 hr prior to intial challenge with PE (10^{-5} M). After initial washout, experiments were performed as described above for isolated tissue bath.

Statistical analysis

For *in vivo* data analysis, between group differences were assessed by a two-way ANOVA with repeated measures followed by post hoc testing using Bonferroni's procedure (GraphPad Prism 5). For isometric contractile studies, relaxation is reported as a percentage of initial contraction to a half-maximal concentration of U46619 (thromboxane A_2 agonist). Repeated measures two-way ANOVA followed by the Bonferroni post hoc test was used to compare concentration response curves. An unpaired students *t*-test was used to compare differences in maximal response(s) between agonist *vs.* vehicle. In all cases, $p < 0.05$ was considered significant. All results are presented as the mean ± SEM.

RESULTS

Effect of 5-HT on CO and TPR

There was no between-group difference in blood pressure prior to beginning of 5-HT or vehicle infusion (C1-C2). Continuous 5-HT infusion resulted in a significant fall in MAP on days S1 and S2 (106 ± 5 and 106 ± 3 mmHg) compared to vehicle-infused group (120 ± 2 and 116 ± 3 mmHg) (Figure 1A). Starting on day S3, MAP tended to normalize yet remained significantly below baseline level. Heart rate was significantly elevated 1 day after the start of 5-HT infusion (447 ± 6 bpm vs. 404 ± 9 bpm in vehicle group, $P<0.01$) but then returned to baseline level. Infusion of 5-HT produced a significant increase in cardiac output (CO; 353 ± 24.8 ml/min/kg; S1), stroke volume (SV; 0.25 ± 0.01 ml; S1) and cardiac index (CI; 0.28 ± 0.2 ml/min/cm^2; S1) compared to vehicle infusion (236 ± 4.1 ml/min/kg; 0.18 ± 0.01 ml; 0.18 ± 0.01 ml/min/cm^2, respectively; S1). All these parameters remained significantly elevated throughout the experiment (Figure 1). Rats receiving 5-HT developed a significant reduction in total peripheral resistance (TPR; 0.31 ± 0.03 mmHg/ml/min/kg; S1) compared to vehicle-treated animals (0.51 ± 0.01 mmHg/ml/min/kg). This effect persisted throughout the remainder of the study.

Real Time RT-PCR

5-HT receptor transcripts were quantified using real time RT-PCR ($2^{-\text{delta Ct}}$ x1000) in the SMA of sham and DOCA-salt rats using beta-2 microglobulin as a calibrator (Figure 2A). The 5-HT_{2B} receptor was expressed at high levels in the SMA of both the sham

and DOCA-salt rat (4.46 ± 0.44; 10.41 ± 2.32 respectively). mRNA transcripts for the 5-HT_{1B} (0.83 ± 0.24; 0.81 ± 0.11), and 5-HT_7 (0.78 ± 0.62; 0.21 ± 0.04) were expressed at significantly lower levels *vs.* the 5-HT_{2B} receptor, in the SMA of sham and DOCA-salt rats ($p < 0.05$).

Effect of 5-HT on arterial tone

Endothelial-intact mesenteric resistance vessels (200 μm diameter) were mounted in a wire myograph chamber for measurement of isometric contractile force. 5-HT (10^{-9} M - 10^{-5} M) contracted the isolated mesenteric resistance vessel from baseline and when contracted with prostaglandin F_2 alpha ($PGF_2\alpha$) (Figure 2B). 5-HT (10^{-9} M - 10^{-5} M) also contracted the isolated mesenteric resistance vessel when contracted with $PGF_2\alpha$ and incubated with ketanserin (100 nM; 5-HT_{2A} receptor antagonist) (Figure 2C). These same vessels relaxed to ACh (10^{-6} M) and forskolin (1 μM). Relaxation to 5-HT was not observed under any condition.

Endothelium-intact helical SMA strips from sham and DOCA-salt rats were mounted in an isolated tissue bath for measurement of isometric contractile force. Tissues were contracted half-maximally to U46619 and were incubated with ketanserin to block the contractile effects of the 5-HT_{2A} receptor (these conditions apply to all remaining experiments). 5-HT (10^{-9} M – 10^{-5} M) did not relax the SMA from sham or DOCA-salt

rats compared to vehicle (Figure 2D). Maximum concentrations of 5-HT (10^{-5} M) contracted the sham SMA (125 ± 9.1% of half-maximal U46619-induced contraction) compared to vehicle (78 ± 6.1% of half-maximal U46619-induced contraction). Similarly, a maximum concentration of 5-HT (10^{-5} M) also contracted the DOCA-salt SMA (144 ± 16.2% of half-maximal U46619-induced contraction) compared to vehicle (83 ± 9% of half-maximal U46619-induced contraction). At the end of each experiment, sodium nitroprusside (SNP) was added to each bath to demonstrate the SMA was able to relax under experimental conditions set forth (SNP was also used in all remaining experiments to demonstrate relaxation under various conditions). These findings suggest that 5-HT alone does not relax the SMA in either the sham or DOCA-salt rat.

5-HT_{2B} receptor

Homogenates of SMA from sham and DOCA-salt rats were processed for western blot analysis using antibodies that recognized the 5-HT_{2B} receptor (amino acids 1-58) (Figure 3A). The 5-HT_{2B} receptor antibody recognized protein (~ 45-55 kDa) in lanes loaded with SMA from sham and DOCA-salt homogenates. A band was present in rat stomach fundus (control), thus validating use of this antibody. α-actin was similarly expressed in all SMA homogenates loaded.

Immunohistochemical detection of the 5-HT_{2B} receptor was performed on sections from the SMA of the sham and DOCA-salt rats (Figure 3B). Staining was observed as black/brown precipitate and appeared to be present in the endothelial, smooth muscle,

and adventitial layers of the SMA from sham and DOCA-salt rats. Staining was not observed in those tissues that were not incubated with primary antibody. Black/brown staining was observed in the rat brain (control), thus validating use of this antibody. No staining was observed in rat brain sections that were not incubated with primary antibody. Collectively, western analysis and IHC staining provided qualitative evidence that the 5-HT_{2B} receptor was present in the SMA of the sham and DOCA-salt rat.

The 5-HT_{2B} receptor is located on the endothelial cell of the SMA and is thus a potential target for a 5-HT-induced, nitric oxide dependent, fall in blood pressure. SMA strips that relaxed > 50% to ACh (10^{-6} M) were considered endothelial cell-intact and this test preceded each concentration response curve. BW-723C86 (10^{-9} M – 10^{-5} M; 5-HT_{2B} receptor agonist) did not relax the helical SMA strip from either sham or DOCA-salt rats compared to vehicle (Figure 3C). Maximum concentrations of BW-723C86 (10^{-5} M) did not relax the sham SMA (81 ± 7.1% of half-maximal U46619-induced contraction) compared to vehicle (41 ± 8.3 % of half-maximal U46619-induced contraction). Additionally, maximum concentrations of BW-723C86 (10^{-5} M) contracted the DOCA-salt SMA (118 ± 6.7 of half-maximal U46619-induced contraction) compared to vehicle (60 ± 5.5%of half-maximal U46619-induced contraction). This evidence suggests that 5-HT is not acting at the 5-HT_{2B} receptor in the SMA of either the sham or DOCA rat to lower blood pressure.

5-HT$_7$ receptor

The 5-HT$_7$ receptor antibody (amino acids 13-28) recognized protein (~ 50 kDa) in homogenates of sham and DOCA-salt SMA (Figure 4A). A strong band was also present in rat brain lysate homogenate (control), thus validating use of this antibody. α-actin was similarly expressed in all SMA homogenates loaded and α-actin was not present in rat brain lysate homogenate.

Immunohistochemical detection of the 5-HT$_7$ receptor was similarly performed in sections of SMA from sham and DOCA-salt rats (Figure 4B). Negligible staining was observed as black/brown precipitate in the adventitia of SMA from sham and DOCA-salt rats. In our positive control (rat brain) we observed staining. 5-HT$_7$ receptor proteins was identified by western blot with greater confidence, suggesting this antibody may not perform as well in IHC analysis, but that the 5-HT$_7$ receptor is present in the SMA of both the sham and DOCA-salt rat.

We next determined if the 5-HT$_7$ receptor was functionally active by investigating relaxation to LP-44, a 5-HT$_7$ receptor agonist. LP-44 (10^{-9} M – 10^{-5} M) did not relax the SMA from sham or DOCA-salt rats compared to vehicle (Figure 4C). Maximum concentrations of LP-44 (10^{-5} M) did not relax the SMA from sham rats (55 ± 6.4% of half-maximal U46619-induced contraction) compared to vehicle (51 ± 7.8 % of half-maximal U46619-induced contraction). Similarly, maximum concentrations of LP-44 (10^{-}

^{5} M) did not relax the SMA from DOCA-salt rats (75 ± 4.7 of half-maximal U46619-induced contraction) compared to vehicle (73 ± 7.8%of half-maximal U46619-induced contraction). Collectively, this evidence suggests that 5-HT is not likely acting at the 5-HT_7 receptor to lower blood pressure.

5-HT_{1B} receptor

Homogenates of SMA from sham and DOCA-salt rats were processed for western blot analysis using antibodies that recognized the 5-HT_{1B} receptor (amino acids 8-26). The 5-HT_{1B} receptor antibody recognized multiple proteins in the SMA of sham and DOCA-salt rats (Figure 5A). A band was present in rat brain lysate homogenate (control), which was consistent with one of the bands observed in the SMA from sham and DOCA-salt rats (arrow). α-actin was similarly expressed in all SMA homogenates loaded, and was expressed at low levels in rat brain lysate homogenate.

Immunohistochemical detection of the 5-HT_{1B} receptor was also performed in SMA of sham and DOCA-salt rats (Figure 5B). Staining was observed in the media and adventitia, as black/brown precipitate, in sections of SMA from sham and DOCA-salt rats. Robust staining was observed in rat brain sections, thereby validating use of the 5-HT_{1B} antibody. Taken together, western blot analysis and IHC staining provided evidence that the 5-HT_{1B} receptor was present in the SMA of sham and DOCA-salt rats.

Finally, the same functional protocol was undertaken in endothelium-intact helical SMA strips from sham and DOCA-salt rats to determine if activation of the 5-HT_{1B} receptor was capable of relaxing the SMA. CP 93129 (10^{-9} M – 10^{-5} M; 5-HT_{1B} receptor agonist) did not relax the SMA of sham or DOCA-salt rats compared to vehicle (Figure 5C). A maximum concentration of CP 93129 (10^{-5} M) did not relax the SMA from sham rats (88 ± 8.9% of half-maximal U46619-induced contraction) compared to vehicle (41 ± 8.3 % of half-maximal U46619-induced contraction). In contrast, a maximum concentration of CP 93129 (10^{-5} M) contracted the SMA from DOCA-salt rats (180 ± 15 of half-maximal U46619-induced contraction) compared to vehicle (60 ± 5.5% of half-maximal U46619-induced contraction). This suggests that 5-HT is not acting at the 5-HT_{1B} receptor to lower blood pressure.

DISCUSSION

The main objective(s) of this study were to: 1) identify whether 5-HT was lowering blood pressure through a reduction in total peripheral resistance (TPR) or cardiac output (CO), 2) identify the 5-HT_{2B}, 5-HT_7, and 5-HT_{1B} receptors in the superior mesenteric artery (SMA) of sham and DOCA-salt rats *via* PCR, western, and IHC analysis, 3) determine whether activation of these receptors were capable of relaxing the SMA of sham or DOCA-salt rats.

5-HT reduces total peripheral resistance in sham and DOCA-salt rats

5-HT infusion produced a significant reduction in MAP and TPR, supporting our hypothesis that 5-HT might be acting to lower blood pressure through direct receptor mediated vascular relaxation. This is the first time the effects of 5-HT on TPR in the conscious animal have been reported. Further, our finding that 5-HT infusion increases CO is consistent with previous studies (Diaz *et. al.* 2008), which demonstrated that 5-HT infusion reduces MAP, and elevates heart rate (HR) in sham and DOCA-salt rats. Defining these important hemodynamic parameters (CO, CI, and TPR) allowed us to pursue a careful investigation of the vasculature, the site of TPR, as the primary site of action for a 5-HT-induced fall in blood pressure.

5-HT does not directly relax the mesenteric arterial vasculature

5-HT alone was not capable of relaxing the mesenteric resistance vessel or SMA and thus, suggests 5-HT is not likely acting at the level of the SMA to mediate direct receptor mediated vascular relaxation and lower blood pressure. However, the literature

suggests several 5-HT receptors (5-HT_{2B}, 5-HT_7, and 5-HT_{1B}) are capable of peripheral artery dilation (Bordoff *et. al.* 2002; Calama *et. al.* 2003; Saxena and Villalon, 1990). Therefore, we sought to determine whether these receptors were present in the SMA of the sham or DOCA-salt rat and whether activation of these receptors, in the presence of ketanserin (5-HT_{2A} receptor antagonist), might unmask a potential relaxant role for 5-HT in the SMA.

Moreover, the literature also suggests that the 5-HT_{2B} receptor is located on the endothelial cell and is capable of nitric oxide (NO) release (Ishida *et. al.* 1998). Given the absolute dependence of a 5-HT-induced fall in blood pressure on nitric oxide synthase (NOS) (Diaz *et al*. 2008), the 5-HT_{2B} receptor was a leading candidate in our investigation. However, a significant body of evidence suggests an alternate role for the 5-HT_{2B} receptor, which may depend on the location within the vasculature (endothelial cell; relaxant or smooth muscle; contractile) and/or underlying pathology (presence or absence of hypertension; contractile) (Ishida *et. al.* 1998; Banes and Watts, 2003). In the aorta of the DOCA-salt rat, the functional 5-HT_{2B} receptor is up regulated and mediates contraction of the aorta *vs*. the sham rat (Banes and Watts, 2003). Despite instances in which this receptor has been described as contractile, we determined whether this receptor was present in the SMA of the sham or DOCA-salt rat and whether activation of this receptor in endothelial intact SMA strips might elicit a relaxant response.

The 5-HT_{2B}, 5-HT_7, and 5-HT_{1B} receptor transcripts were identified *via* PCR analysis, thus validating our interest in these receptors as potential targets mediating a fall in resistance, and a 5-HT-induced fall in blood pressure. Prominent expression of the 5-HT_{2B} receptor transcript compared to the 5-HT_7 and 5-HT_{1B} transcripts also validated our interest in this receptor as a potential mediator of a 5-HT-induced fall in blood pressure.

Western blot and IHC analysis identified the 5-HT_{2B}, 5-HT_7, and 5-HT_{1B} receptors in the SMA of sham and DOCA-salt rats. The 5-HT_7 receptor antibody recognized protein (*via* western blot) in the SMA of the sham and DOCA-salt rat, but the same antibody did not elicit a strong immunohistochemical stain in sections of SMA from the sham or DOCA-salt rat. Despite repeated attempts with this antibody, and attempts with a different antibody (ImmunoStar 5-HT_7 receptor antibody; catalog # 24430), we accepted a limited ability to detect the 5-HT_7 receptor *via* IHC analysis. Fortunately, western blot analysis revealed strong bands in the SMA of both sham and DOCA-salt rats, giving us confidence that the 5-HT_7 receptor protein was present in the SMA of the sham and DOCA-salt rat.

Detection of the 5-HT_{2B}, 5-HT_7, and 5-HT_{1B} receptors allowed us to proceed with functional investigation. Despite their presence and literature to support our hypothesis (5-HT lowers blood pressure through receptor mediated vascular relaxation), no significant relaxation was observed in the SMA of the sham or DOCA-salt rat. Instead,

activation of the 5-HT_{2B} receptor (*via* BW723C86) and 5-HT_{1B} (*via* CP 93129) caused contraction of the SMA of the DOCA-salt rat. These responses are consistent with the contractile responses seen in previous studies in the DOCA-salt aorta (Banes and Watts, 2003; Banes and Watts, 2001). Activation of the 5-HT_7 receptor did not elicit a contractile response, but it also did not relax the SMA of the sham or DOCA-salt rat. Therefore, we must conclude that the 5-HT_{2B}, 5-HT_7, and 5-HT_{1B} are not involved in relaxing the SMA and it is unlikely that activation of these receptors in the mesentery is responsible for mediating a 5-HT-induced fall in blood pressure.

Potential mechanism(s) underlying a 5-HT-induced fall in blood pressure

Several other possibilities might explain a 5-HT-induced fall in blood pressure. 5-HT may be acting in a different vascular bed to lower total peripheral resistance (TPR) and blood pressure. Other vascular beds 5-HT might be acting in to lower blood pressure include: the skeletal muscle vascular bed (Proctor and Parker, 2006), or the cutaneous vascular bed (Kellogg *et. al.* 2008). Changes in resistance in either of these vascular beds might be capable of eliciting a 5-HT-induced fall in blood pressure.

Another possibility is that 5-HT is acting to inhibit sympathetic nerve activity (SNA), thereby reducing vascular resistance and lowering blood pressure. The 5-$HT_{1B/1D}$ receptor is located on the presynaptic nerve terminal and when stimulated by 5-HT, results in an inhibition of norepinephrine (NE) release (Molderings *et. al.* 1990). Alternatively, 5-HT may be acting centrally to inhibit SNA, reducing total peripheral resistance and eliciting a 5-HT-induced fall in blood pressure. Recent studies have

demonstrated that 5-HT is capable of crossing the blood brain barrier *via* the serotonin transporter (SERT) (Nakatani *et. al.* 2008), though this remains controversial. Thus, we cannot rule out the possibility that 5-HT may be acting centrally to lower blood pressure.

Perspectives and Limitations

5-HT infusion produced a persistent cyanosis in both the tip of the tail and the left hind limb (instrumented with an arterial catheter) of the sham and DOCA-salt rats. This observation raises the possibility that 5-HT might be acting to constrict the vasculature in the tail and hind limb despite lowering mean arterial pressure in these rats. However, it might also be possible that these beds are hypo-perfused, concurrent with the fall in mean arterial pressure, producing the observed cyanosis. This observation suggests further study is necessary to address exactly how 5-HT is functioning in the cardiovascular system. A limitation of this study is the use of only a single specific 5-HT receptor agonist to investigate each 5-HT receptor (5-HT_{2B}, 5-HT_{1B}, 5-HT_{7}) of interest.

In summary, this study demonstrates the presence of the 5-HT_{2B}, 5-HT_{7}, and 5-HT_{1B} receptors in the SMA of sham and DOCA-salt rats, but activation of these receptors does not participate in relaxation of the SMA and therefore these receptors are not likely involved in a 5-HT-induced fall in blood pressure.

Figure 3.1

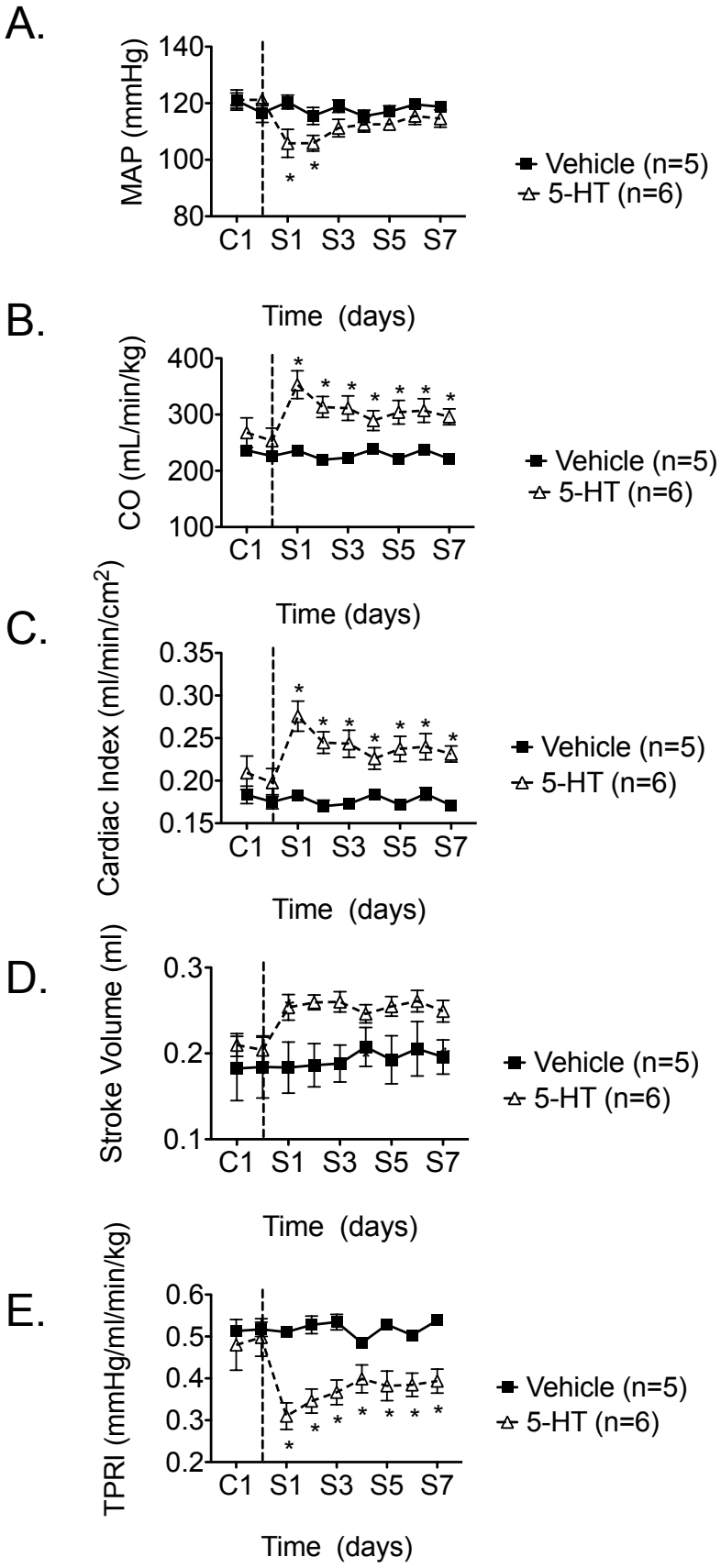
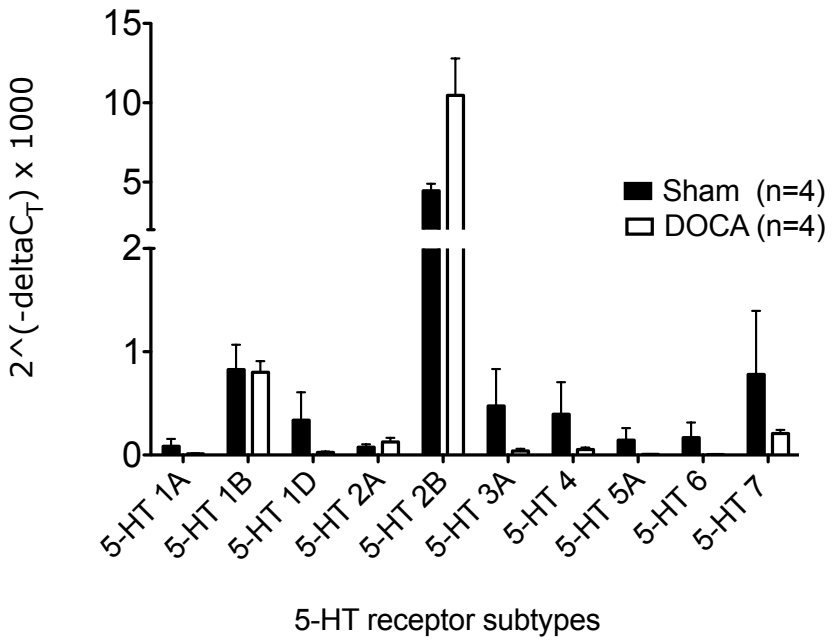

A.
MAP (mmHg)
140
120
100
80
C1 S1 S3 S5 S7
Time (days)
Vehicle (n=5)
5-HT (n=6)
B.
CO (mL/min/kg)
400
300
200
100
C1 S1 S3 S5 S7
Time (days)
Vehicle (n=5)
5-HT (n=6)
C.
Cardiac Index (ml/min/cm2)
0.35
0.30
0.25
0.20
0.15
C1 S1 S3 S5 S7
Time (days)
Vehicle (n=5)
5-HT (n=6)
D.
Stroke Volume (ml)
0.3
0.2
0.1
C1 S1 S3 S5 S7
Time (days)
Vehicle (n=5)
5-HT (n=6)
E.
TPRI (mmHg/ml/min/kg)
0.6
0.5
0.4
0.3
0.2
C1 S1 S3 S5 S7
Time (days)
Vehicle (n=5)
5-HT (n=6)

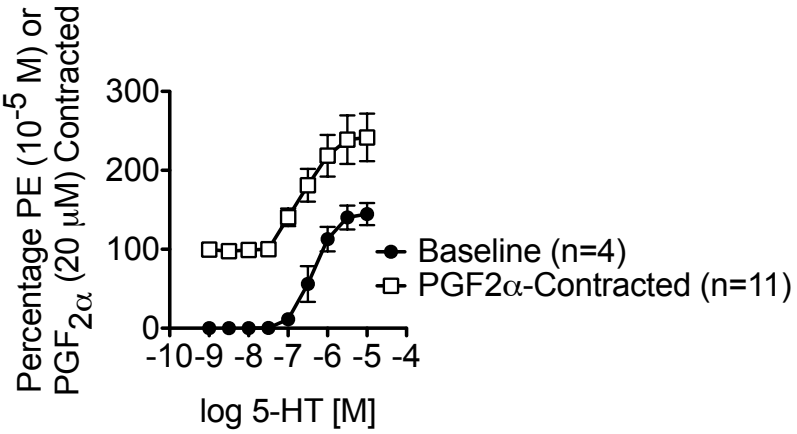
Figure 3.1: Effect of chronic 5-HT or vehicle infusion on A) mean arterial pressure (MAP), B) cardiac output (CO), C) cardiac index (CI), D) stroke volume, and E) total peripheral resistance (TPR). CI = CO/ (9.1 x $g^{0.66}$), where g – body mass in grams, and (9.1 x $g^{0.66}$) – body surface area in cm^2. Data points are 1-hour average +/- SEM. Number of animals per group is shown in parentheses. The vertical line denotes 5-HT or vehicle osmotic pump implant. Time is represented by days on the x-axis. C1 and C2 - control recordings. S1 – S7 – days since beginning of 5-HT/vehicle infusion. * $P < 0.05$ vs. vehicle.

Figure 3.2

A.



B.



C.

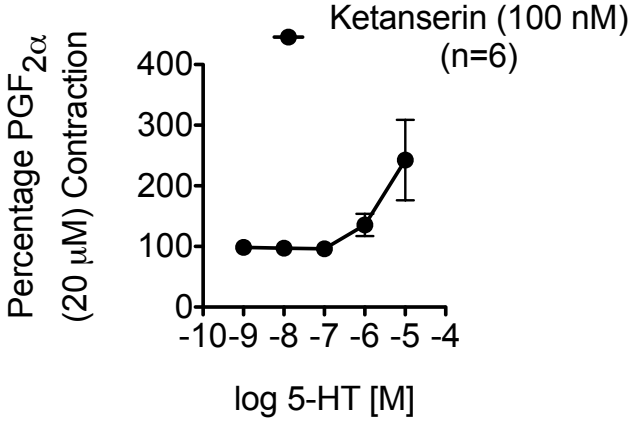


Figure 3.2 (cont'd)

D.

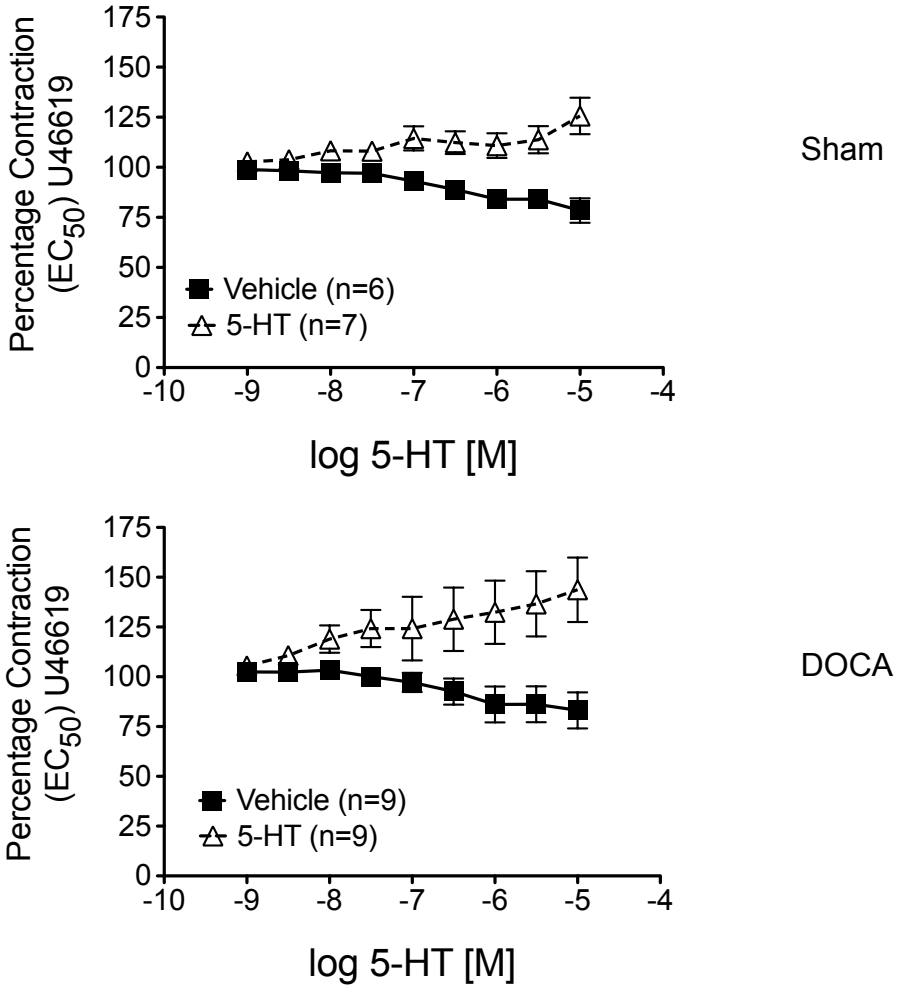
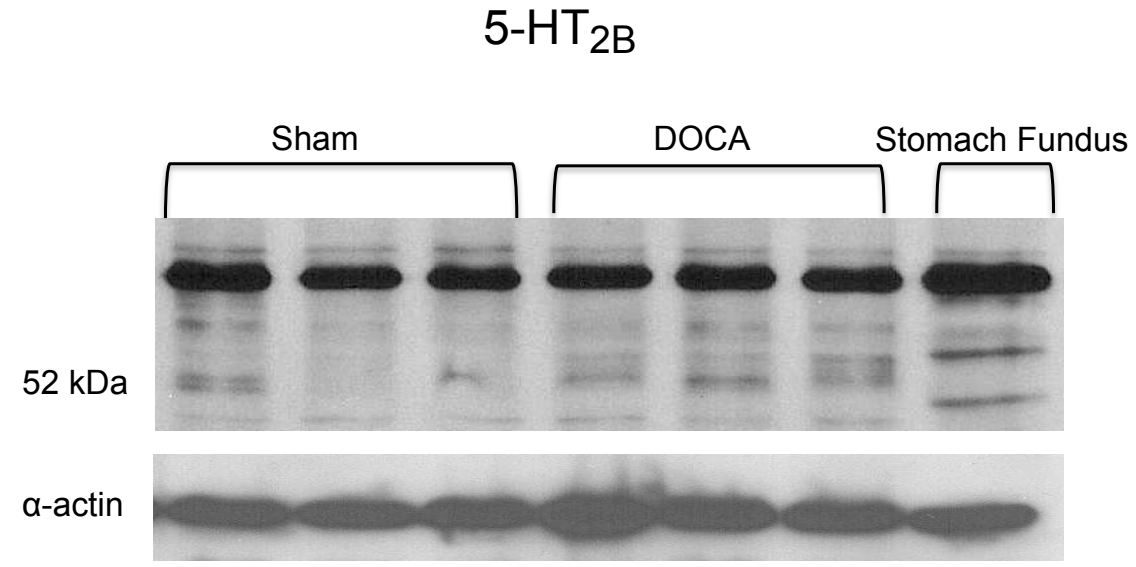


Figure 3.2: **A**. mRNA expression of 5-HT receptors in the superior mesenteric artery (SMA) of sham (n=4) and DOCA-salt (n=4) rats using standard real time PCR analysis. **B**. Cumulative concentration response curve to 5-HT generated from baseline, contracted with prostaglandin F_2 alpha ($PGF_2\alpha$), **C.** and in the presence of ketanserin

in the sham superior mesenteric resistance artery. **D**. Cumulative concentration response curve to 5-HT in SMA contracted with U46619 in sham (left) and DOCA-salt (right) SMA. Data points represent mean ± SEM for the number of animals in parentheses. * $P < 0.05$ *vs.* sham 5-HT_{1B} and 5-HT_7 receptors. † $P < 0.05$ *vs.* DOCA-salt 5-HT_{1B} and 5-HT_7 receptors.

Figure 3.3

A.


5-HT2B
Sham
DOCA
Stomach Fundus
52 kDa
α-actin

B.

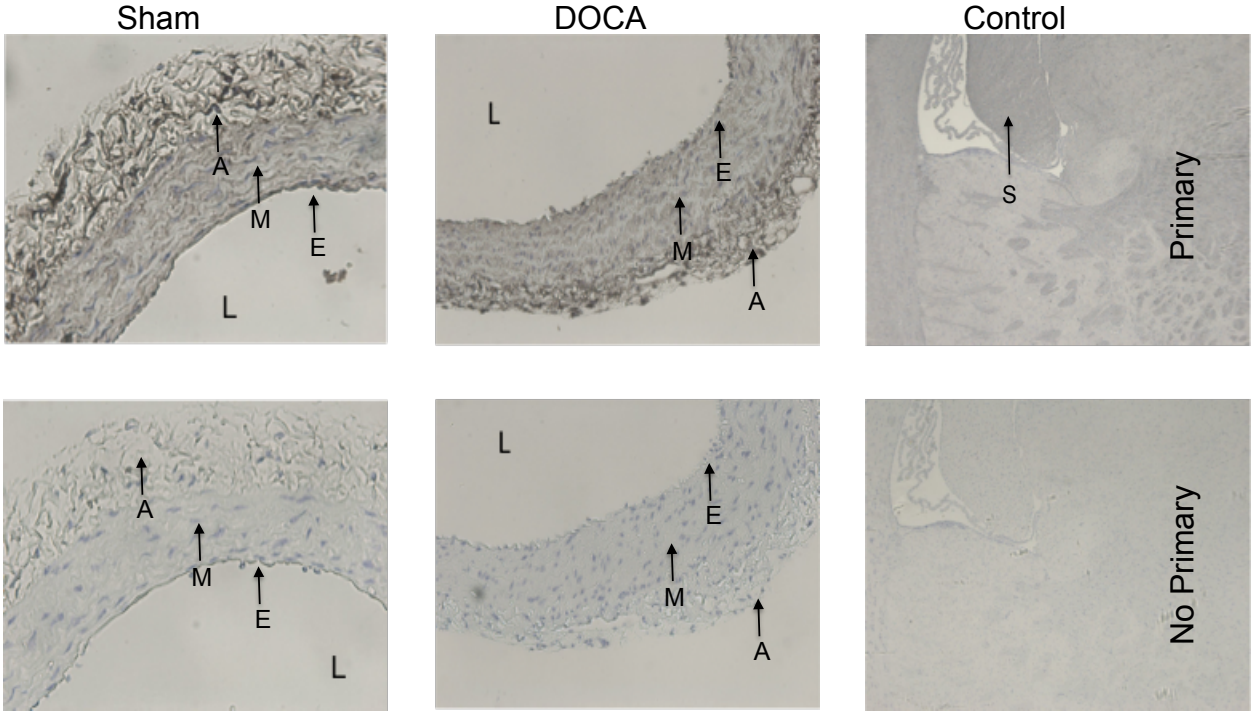

Sham
DOCA
Control
A
M
E
L
S
Primary
No Primary

Figure 3.3 (cont'd)

C.

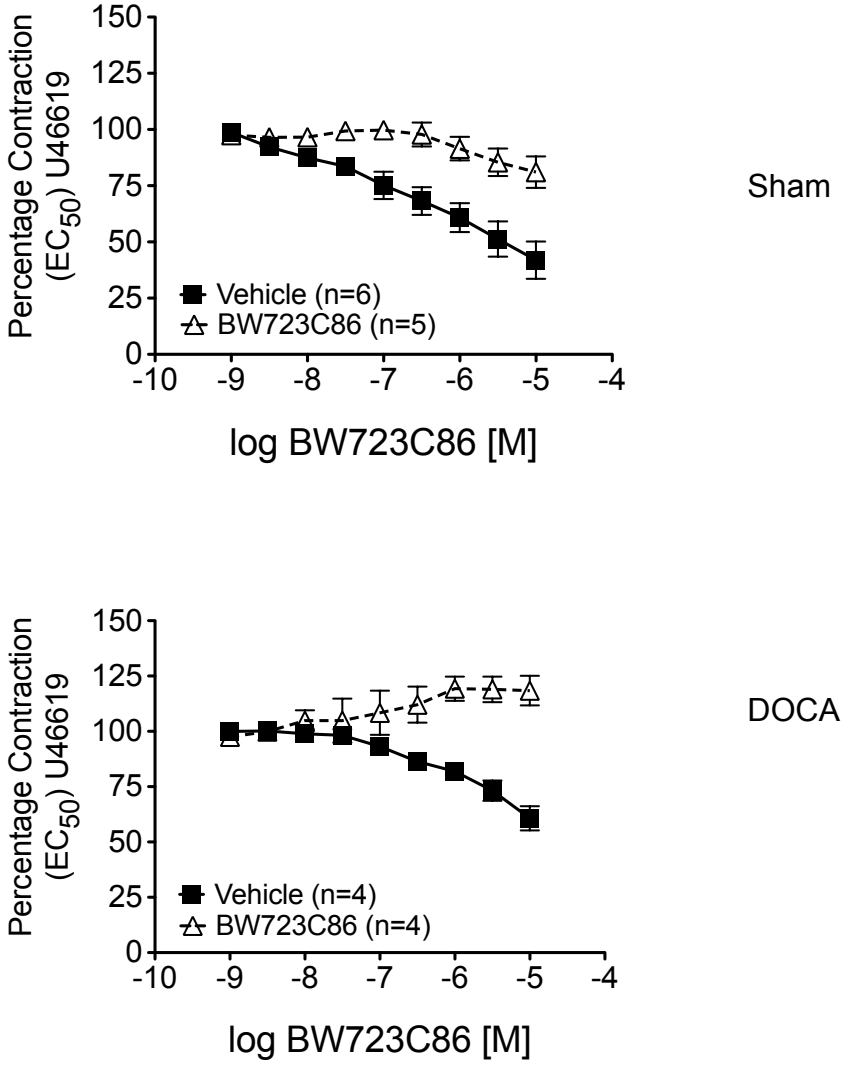


Figure 3.3: **A**. Western blot analyses for identification of the 5-HT_{2B} receptor in homogenates of SMA from sham (n=6) and DOCA-salt (n=6) rats. **B**. Immunohistochemical staining of the 5-HT_{2B} receptor in SMA sections from sham and DOCA-salt rats. No primary sections were exposed only to secondary antibody. Images were taken at 20X objective. E = endothelium, M = media, A = adventitia, S = staining in

rat stomach fundus, L = lumen. **C.** Cumulative concentration response curve to BW723C86 *vs.* vehicle (H_20) in SMA contracted with U46619 in sham and DOCA-salt SMA. Data points represent mean ± SEM for the number of animals in parentheses.

Figure 3.4

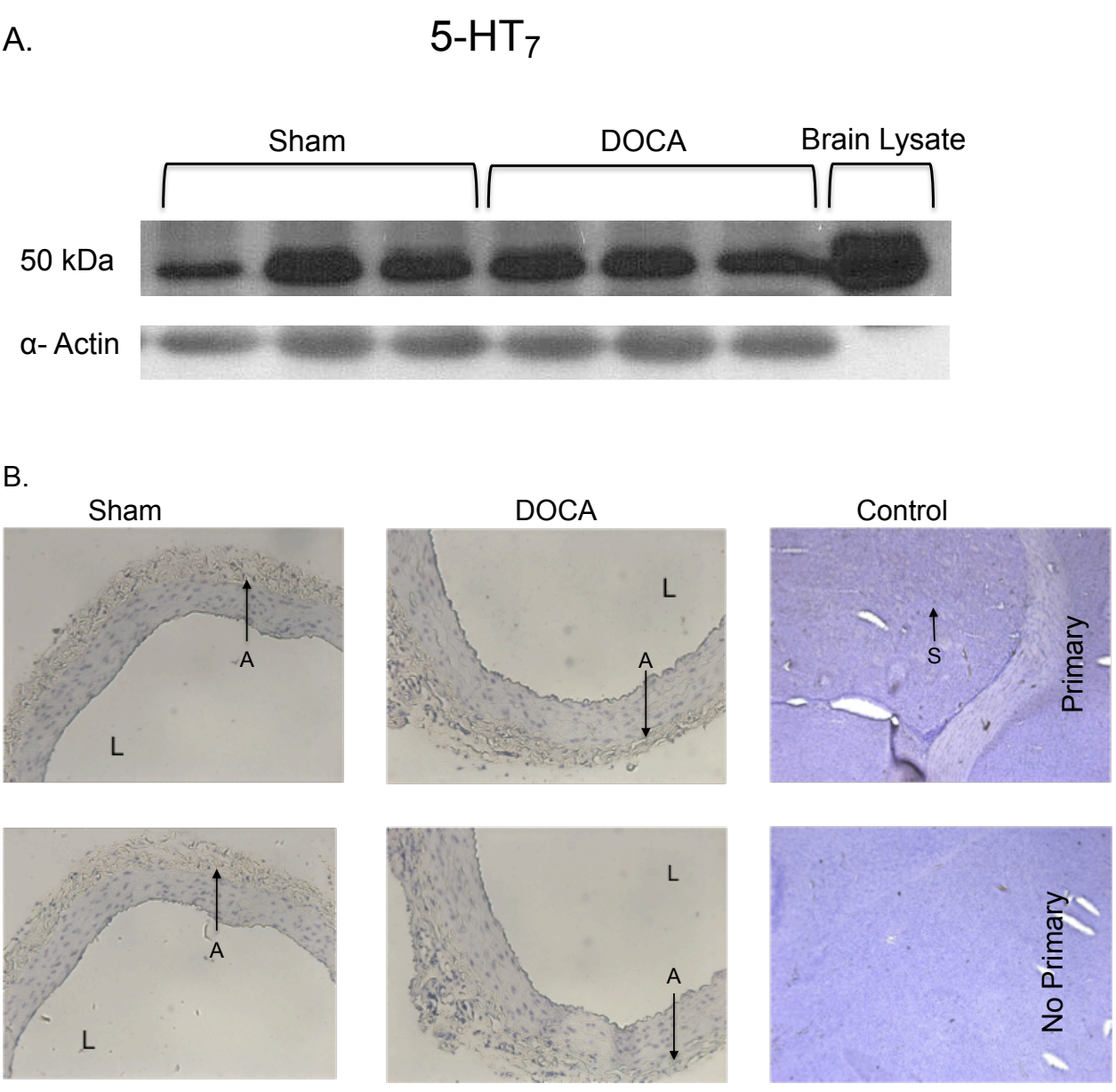

A.
5-HT7
Sham
DOCA
Brain Lysate
50 kDa
α- Actin
B.
Sham
DOCA
Control
A
L
A
L
S
Primary
A
L
A
L
No Primary

Figure 3.4 (cont'd)

C.

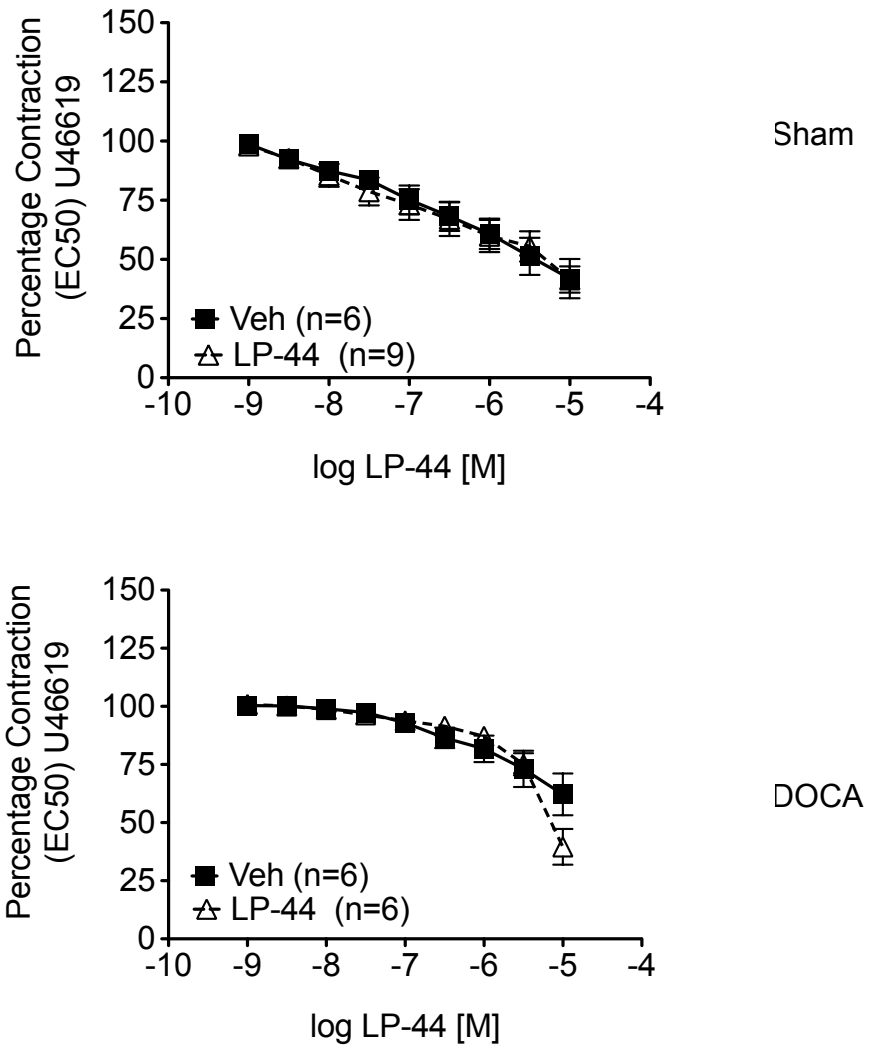
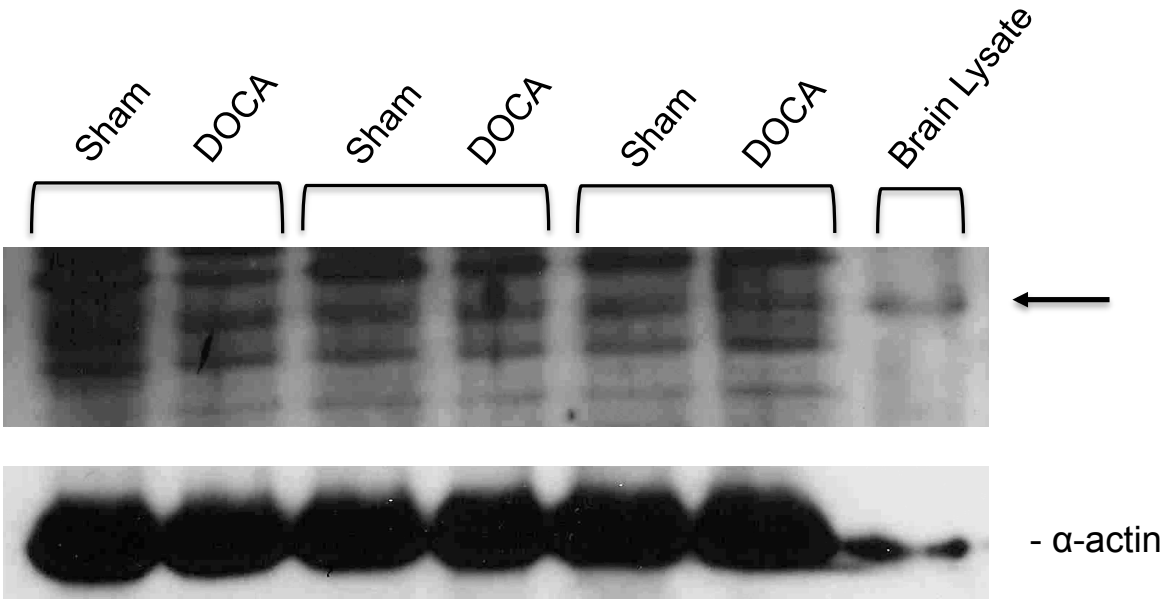


Figure 3.4: **A**. Western blot analyses for identification of the 5-HT_7 receptor in homogenates of SMA from sham (n=6) and DOCA-salt (n=6) rats. **B**. Immunohistochemical staining of the 5-HT_7 receptor in SMA sections from sham and DOCA-salt rats. No primary sections were exposed only to secondary antibody. Images

were taken at 20X objective. A = adventitia, S = staining in rat brain lysate, L = lumen. **C.** Cumulative concentration response curve to LP-44 *vs.* vehicle (DMSO) in SMA contracted with U46619 in sham and DOCA-salt SMA. Data points represent mean ± SEM for the number of animals in parentheses.

Figure 3.5

A. 5-HT_{1B}



B.

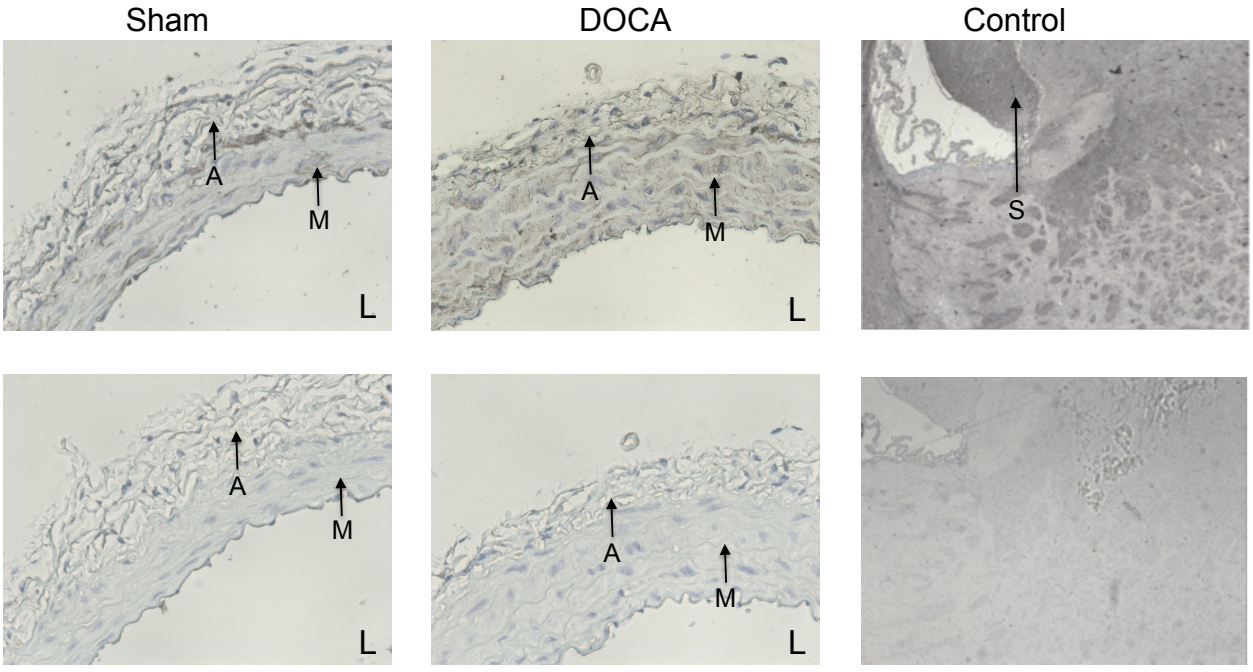


Figure 3.5 (cont'd)

C.

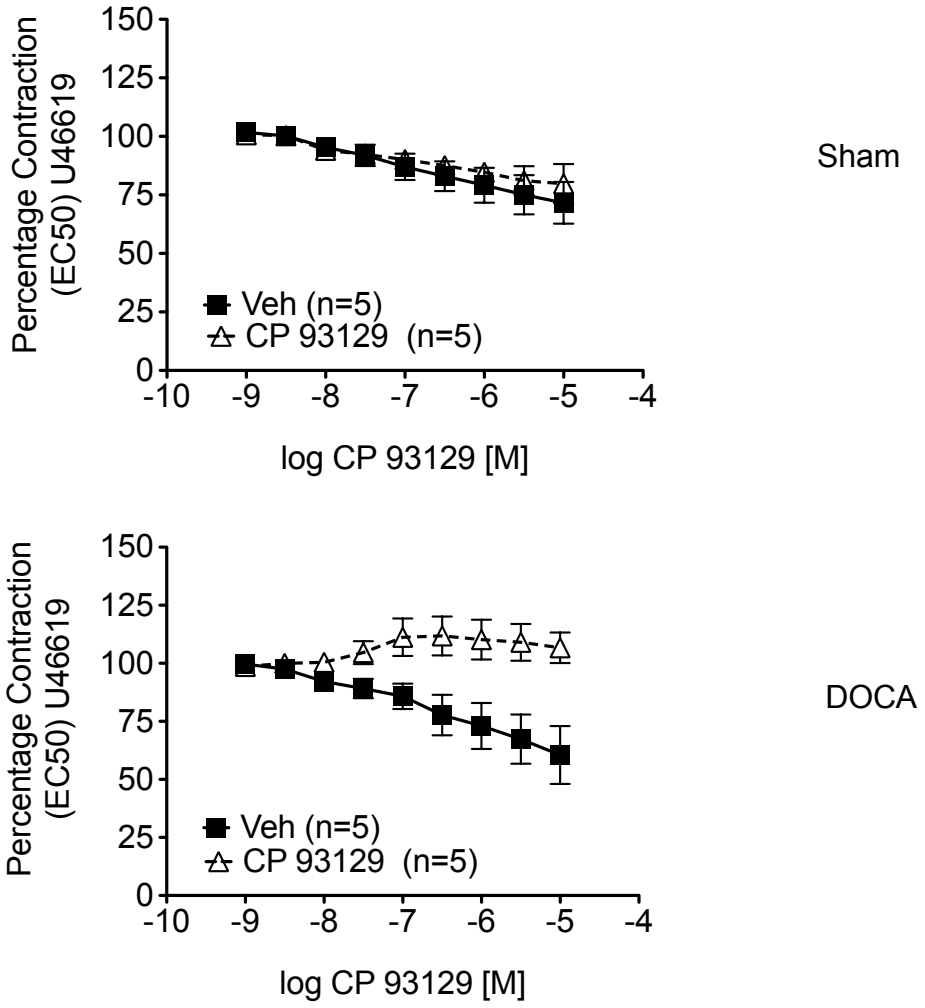


Figure 3.5: **A**. Western blot analyses for identification of the 5-HT_{1B} receptor in homogenates of SMA from sham (n=6) and DOCA-salt (n=6) rats. **B**. Immunohistochemical staining of the 5-HT_{1B} receptor in SMA sections from sham and DOCA-salt rats. No primary sections were exposed only to secondary antibody. Images

were taken at 20X objective. M = media, A = adventitia, S = staining in rat brain lysate, L = lumen. **C.** Cumulative concentration response curve to CP 93129 *vs.* vehicle (H_2O) in SMA contracted with U46619 in sham and DOCA-salt SMA. Data points represent mean ± SEM for the number of animals in parentheses.

CHAPTER 4

5-Hydroxytryptamine (5-HT) does not inhibit sympathetic neurogenic contraction or sympathetic pre-/post-ganglionic sympathetic nerve activity in the rat splanchnic circulation

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ABSTRACT

Serotonin (5-hydroxytryptamine; 5-HT) infusion for 7 days results in a sustained fall in blood pressure (BP) in rodents, and this is accompanied by a robust fall in total peripheral resistance (TPR). Because TPR is significantly maintained by sympathetic nervous system tone, we hypothesized that 5-HT would 1) reduce sympathetic nerve activity (SNA); and/or 2) inhibit sympathetic neuroeffector function. The model was the splanchnic circulation of the rat. In urethane-anesthetized, paralyzed, and artificially respired Sprague-Dawley rats, mean BP was significantly *($p<0.05$)* reduced from 101 ± 4 to 63 ± 3 mm Hg during slow infusion of 5-HT (25 µg/kg/hr, iv). Pre- and postganglionic splanchnic SNA was unaffected during 5-HT infusion. In superior mesenteric arterial rings mounted in isolated tissue bath for electrical field stimulation, 5-HT (10^{-9} – 10^{-5} M; 5 min) did not inhibit neurogenic contraction compared to vehicle (99 ± 11% at 20 Hz; 96 ± 16% at 20 Hz; respectively). Further, 5-HT did not inhibit neurogenic contraction in the presence of fluoxetine (reuptake inhibitor), ketanserin (5-HT_{2A} antagonist), and a longer incubation with 5-HT (30 min) *vs.* vehicle in arteries from normal, sham and DOCA-salt hypertensive rats. The 5-HT_{1B} receptor agonist CP 93129 (5-HT_{1B} agonist) was also ineffective. This study demonstrates that 5-HT does not interact with the peripheral sympathetic nervous system (neuroeffector junction, pre and postganglionic SNA) to remove sympathetic tone from the splanchnic circulation, and suggests an alternate mechanism(s) or vascular bed is mediating a 5-HT-induced fall in blood pressure.

INTRODUCTION

5-hydroxytryptamine (5-HT, serotonin) is synthesized in the enterochromaffin cells of the gastrointestinal tract and the raphe nucleus of the central nervous system (CNS) and is well documented as a potent constrictor of isolated blood vessels (Berger *et al*, 2009; Doggrell, 2003; Erspamer *et al*, 1952; Kaumann and Levy, 2006; Page and McCubbin 1953a, b; Ramage and Villalon, 2008; Rapport *et al*, 1948; Watts 2005, 2009). Historically, 5-HT has been considered a pathogenic factor or potential contributor to the development of essential hypertension due in particular to the knowledge that free plasma 5-HT levels are elevated in both human and experimental models of hypertension compared to normotensive controls (Brenner *et al*., 2007; Diaz *et al*., 2008). If 5-HT is indeed a vasoconstrictor *in vivo*, elevations in free plasma 5-HT would logically promote vasoconstriction, elevation of total peripheral resistance (TPR) and elevation in blood pressure. However, when we experimentally elevate free plasma 5-HT levels in the rodent, mean arterial blood pressure is lowered. Administration of 5-HT has typically been performed through Alzet osmotic pumps, and is done chronically over one week. This is true in multiple strains of rats (Sprague Dawley, Wistar; Diaz et al., 2008), and male and female (Davis *et al*., 2011a). In the mineralocorticoid-dependent, hypertensive deoxycorticosterone acetate (DOCA) salt rat, 5-HT nearly normalized arterial blood pressure. More recently, we have demonstrated that 5-HT causes a robust fall in total peripheral resistance (Davis *et. al*., 2011b). This important piece of information suggests that the vasculature, or control of the vasculature, is the site of action for 5-HT to reduce blood pressure.

Since the sympathetic nervous system is essential for maintaining normal levels of total peripheral resistance, removal of sympathetic tone is a means by which blood pressure could be reduced. 5-HT has an interesting history with respect to sympathetic tone. Several studies suggest that 5-HT inhibits norepinephrine (NE) release at the sympathetic neuroeffector junction (Gothert *et al*., 1991). The $5\text{-HT}_{1B/1D}$ receptor has been implicated in presynaptic inhibition and inhibition of NE release (Molderings et al, 1990). This evidence suggests 5-HT may be acting to reduce sympathetic outflow at the neuroeffector junction, thereby reducing vascular resistance, and potentially lowering blood pressure. Central actions of 5-HT include both increases and decreases in SNA (Barnes and Sharp, 1999; Ramage and Villalon, 2008). Thus, if systemically administered 5-HT can cross the blood-brain barrier, a reduction in sympathetic nerve activity (SNA) could contribute to its vasodepressor response. There are serotonergic receptors on sympathetic ganglia (Hertzler, 1961; Jones et al., 1995; Meehan and Kreulen, 1991; Pickering *et al*., 1994; Ramage and Villalon, 2008; Sheng-Rong *et al*, 1999; Watkins and Newberry, 1996), so this is another potential target for the site of action of 5-HT to reduce BP.

The current study was designed to study the role of sympathoinhibition in mediating the depressor response to a slow intravenous infusion of 5-HT in anesthetized rats. We hypothesized that 5-HT would 1) reduce sympathetic nerve activity (SNA); and/or 2) inhibit sympathetic neuroeffector function.

METHODS

Animal

The Michigan State University Institutional Animal Care and Use Committee (IACUC) approved all protocols. Male Sprague Dawley rats (225-465 g, Charles River Laboratories) were used in this study. DOCA-salt hypertensive and normotensive sham rats were also used.

Methods for studies involving SNA recordings

Anesthesia

Sprague Dawley rats used for recordings of SNA were anesthetized with urethane (1 - 1.1 g/kg ip) and paralyzed with gallamine triethiodide (initial dose of 20 mg/kg iv; supplemental doses administered as needed to maintain paralysis) following induction with isoflurane. The trachea was cannulated for maintenance of artificial respiration using positive pressure ventilation (Harvard Apparatus Inspira ASV ventilator). Rectal temperature was maintained near 37°C with the aid of a heat lamp.

Catheterization

Arterial and venous catheters were introduced into the femoral artery and femoral vein. The arterial catheter was connected to a pressure transducer to monitor changes in blood pressure. Venous catheter(s) was used to delivered drug to the anesthetized rat.

Nerve recording

Potentials were recorded monophasically from the cut central ends of the preganglionic or postganglionic sympathetic sphlanchic nerve placed on platinum bipolar electrodes. The capacity-coupled preamplifier bandpass was set at 1 – 1,000 Hz. Preganglionic or postganglionic sympathetic nerve activity (SNA) was then rectified and integrated (1-volt reset). This signal was then quantified in 1 or 10-min data blocks at baseline and during 5-HT infusion. Hexamethonium (10 mg/kg, iv) was administered at the end of each experiment to verify that recordings were from preganglionic or postganglionic branches of the splanchnic nerve.

Methods for studies involving infusion of 5-HT in conscious animals

Anesthesia

All rats were anesthetized with isoflurane (2% in 100% O_2) and ventilated mechanically. At the time of surgery, the incision site was treated with topical antibiotic containing analgesic to prevent irritation and infection. Incisions were closed with silk suture. Rats were treated with amoxicillin (150 mg/kg/i.m.) following surgery and 3 days thereafter. All rats were treated with rimadyl (5 mg/kg, s.c. for 2 days) for general analgesia.

Blood pressure probe

Radiotelemeters (DSI PhysioTel PA series transmitter model PA-C40) were implanted subcutaneously in the lower abdomen and catheters introduced into the left femoral artery. Pressure sensing tips were advanced into the thoracic aorta. All rats were given 7

days to recover prior to any measure. Mean arterial pressure (MAP) and heart rate (HR) were recorded at 10-minute intervals (10 second recording) for the duration of the study.

Alzet osmotic pump

A small incision was made at the base of the neck. Blunt dissection was used to create a small subcutaneous pocket between the scapulae. The pump (Alzet Osmotic Pump, Model 2ML1, Duret Corporation, Cupertino, CA, 10 μL/hr 7 days) was inserted and the skin sutured closed. To each pump, a 5-HT creatinine complex (25 μg/kg/min) and 1% ascorbic acid, antioxidant (0.02 g/pump) or vehicle (1% ascorbic acid, antioxidant) was loaded. The solution was dissolved in 1 mol/L HCl, and a pH-balance (~7) was achieved with 4 mol/L NaOH.

Methods for electrical field stimulation

DOCA-salt hypertension

Under isoflurane anesthesia, Sprague-Dawley rats underwent left uninephrectomy, and a deoxycorticosterone acetate (DOCA) pellet was implanted subcutaneously (200 mg/kg). Sham rats also underwent left uninephrectomy, but were not implanted with a DOCA pellet. Rats were given standard rat chow ad libitum. DOCA-salt water was supplemented with 1% NaCl and 0.2% KCl for the duration of the study. Hypertension was established after 4 weeks of initiation of DOCA. Sham rats received tap water for the duration of the study. Sham systolic blood pressure was < 140 mmHg, while DOCA-salt systolic blood pressure was > 140 mmHg measured by tail cuff method.

Tissue preparation

Rats were anesthetized with pentobarbital (60 mg/kg i.p.) and the superior mesenteric artery was removed and placed in physiological salt solution (PSS) containing (mM): NaCl 130; KCl 4.7; KH_2PO_4 1.8; $MgSO_4$ * $7H_2O$ 1.7; $NaHCO_3$ 14.8; dextrose 5.5; $CaNa_2EDTA$ 0.03, $CaCl_2$ 1.6 (pH 7.2). Sections of the artery were cut into rings for measurement of isometric contractile force, but the surrounding adventitial fat and connective tissue was not removed.

Isolated tissue bath

Two parallel stainless steel hooks were introduced into the lumen of the artery. One segment was fixed within the warmed (37°C), and aerated (95% O_2, 5% CO_2) tissue bath (30 mL). The other was connected to a Grass isometric force transducer (FT03; Grass instruments, Quincy, MA, USA), which was connected to an ADInstruments PowerLab (ADInstruments, Colorado Springs, CO). Tissues were placed under optimal resting tension (1200 mg; previously determined) and allowed to equilibrate for 1 hour before an initial challenge with a maximal concentration of phenylephrine (10^{-5} M). After the initial PE challenge, tissues were washed until toned returned to baseline.

Electrical field stimulation

Superior mesenteric artery segments were mounted between two platinum electrodes (positioned within the tissue bath) connected to a Grass Instruments stimulator (S88; Quincy, MA) and electrical stimulus was delivered according to the following protocol: 30 stimuli, stimulus duration 0.5 ms, frequency 0.2 – 20 Hz, voltage 120 V). For each

stimulus, isometric contractile force was measured in an isolated tissue bath as outlined above. An initial 20 Hz maximal stimulus was delivered to each vascular segment. All subsequent data (means ± SEM) were expressed as a percentage of the initial 20 Hz maximal contraction. To validate the neural origin of the EFS-induced contractile response, the nerve impulse propagation blocker, tetrodotoxin (TTX; 300 nM) was added to the tissue bath after a brief wash out period following the initial 20 Hz stimulus. A second 20 Hz stimulus was delivered to validate contraction was abolished in the presence of TTX. In preliminary experiments, we validated this EFS-induced contraction was sympathetically mediated as the α_1 adrenergic receptor antagonist prazosin (50 nM) abolished the 20 Hz-induced contraction. The following experimental conditions were used: 1) arterial segments alone, 2) segments incubated with the serotonin transport reuptake inhibitor fluoxetine (10^{-6} M), 3) segments incubated with fluoxetine (50 nM) and the 5-$HT_{2A/2C}$ receptor antagonist ketanserin (50 nM; 15 minutes). EFS-induced contractile studies were conducted in the presence or absence of 5-HT (10^{-8} M), Vehicle (H_2O), or CP 93129 (5-HT_{1B} agonist; 10^{-5} M).

Data analysis:

For SNA data analysis, group differences were assessed by a two-way mixed design ANOVA and post-hoc testing at each time point was performed using Bonferroni's procedure to correct for multiple comparisons (GraphPad Prism 5). In all cases, a p-value of <0.05 was considered significant. A paired *t*-test was used to compare the level of blood pressure changes during control periods versus at the nadir during 5-HT infusion. An unpaired students *t*-test was used to compare differences in maximal EFS

response(s) between groups. In all cases, $p < 0.05$ was considered significant. All results are presented as the means ± SEM.

RESULTS

The effect of 5-HT (chronic and acutely administered) on blood pressure

Normal male Sprague-Dawley rats were our initial model. In **Figure 1A**, we demonstrate the fall in blood pressure caused by 5-HT (25 ug/kg/min, Alzet pump) after one week in conscious rats. Blood pressure was measured through radiotelemetry. **Figure 1B** shows the effects of a slow administration of 5-HT (25 ug/kg/min, iv) over the course of 10 min to 1 hour in anesthetized male Sprague Dawley rats. In both experiments, 5-HT caused a fall in blood pressure. The similarity in qualitative and quantitative response allows for the statement that the mechanisms we observe in the acute situation may apply to that of the chronic situation, our ultimate concern. Having established this, we moved to two separate experimental protocols to investigate the ability of 5-HT to modify sympathetic nervous system tone.

The effect of 5-HT on SNA in the splanchnic bed

One mechanism by which 5-HT could reduce blood pressure is by removal of sympathetic tone at the pre- and/or post-ganglionic level. In urethane-anesthetized rats, we investigated both of these possibilities by recording from the splanchnic sympathetic nerve. **Figures 2A** and **3A** show representative examples of the effect of 5-HT on preganglionic and postganglionic SNA, respectively. While 5-HT caused a robust fall in blood pressure in both experiments (top tracing in **panel A**), there was no change in integrated SNA (**panel B** of each figure). Removal of 5-HT infusion (**figure 3A**, right arrow) resulted in a rebound in blood pressure that, in some cases was accompanied by

an increase in SNA. In some experiments, the alpha adrenergic agonist phenylephrine (PE) was used to elicit a reflex-induced inhibition of. PE robustly inhibited preganglionic activity, suggesting that we would be able to detect a 5-HT-induced inhibition of SNA if it occurred.

Effect of 5-HT on sympathetic synaptic transmission in mesenteric artery

Another potential site of action for 5-HT to remove sympathetic tone from the vasculature is on the presynaptic terminal of the sympathetic nerve that innervates the mesenteric vasculature. In initial trials using a short incubation, 5-HT (5 minutes) did not inhibit neurogenic contraction following EFS frequency response curve (0.2 – 20Hz) *vs.* vehicle (H_2O) (99 ± 11% at 20 Hz; 96 ± 16% at 20 Hz; respectively, reported as percentage of initial 20 Hz contraction) in the superior mesenteric artery of the Sprague Dawley rat (**Figure 4A**). 5-HT (5 minutes) did not inhibit neurogenic contraction in the presence of fluoxetine (10^{-6} M; 5-HT reuptake inhibitor) *vs.* vehicle (H_2O) (140 ± 26% at 20 Hz; 98 ± 6% at 20 Hz; respectively) in tissues from the Sprague-Dawley rat (**Figure 4B**). Fluoxetine was added to minimize uptake of 5-HT and thereby maximize the opportunity of 5-HT to interact with a presynaptic receptor. The addition of fluoxetine appeared to potentiate the neurogenic contractile response (140 ± 26% at 20 Hz, in the presence of fluoxetine *vs.* 99 ± 11% at 20 Hz, the absence of fluoxetine). However, this observation was not statistically significant.

To block the effects of 5-HT on postsynaptic 5-HT_{2A} receptors that are contractile, we next added the $5\text{-HT}_{2A/2C}$ receptor antagonist ketanserin (50 nM) to the buffer. This

concentration of ketanserin is sufficient to block these 5-HT receptors but not the alpha adrenergic receptors that mediate EFS-induced contraction. 5-HT (10^{-8} M) did not inhibit neurogenic contraction in the presence fluoxetine, ketanserin (50 nM; $5\text{-HT}_{2A/2C}$ antagonist) and following a longer incubation with 5-HT (30 minutes) *vs*. vehicle (44 ± 11% at 10 Hz; 53 ± 7% at 10 Hz; respectively) in the artery from the Sprague-Dawley rat (**Figure 5**). We considered this protocol the ideal situation in which to observe 5-HT-induced inhibition of EFS-induced contraction. This was supported by a positive control, use of the α_2 agonist UK 14304 (10^{-5} M; 30 minutes) to virtually abolish neurogenic contraction *vs*. vehicle in the superior mesenteric artery of the Sprague-Dawley rat (4 ± 1% at 10 Hz; 50 ± 7% at 10 Hz; respectively; **Figure 5**). In an attempt to more selectively activate the 5-HT_{1B} receptor, the lead candidate for presynaptic inhibition, we tested the 5-HT_{1B} agonist CP 93129, and it did not inhibit neurogenic contraction *vs.* vehicle (42 ± 9% at 10 Hz vs 40 ± 4% at 10 Hz) in superior mesenteric artery of the Sprague-Dawley rat in the presence of fluoxetine, ketanserin, and following a 30 minute incubation period (**Figure 6A**).

Finally, we took this same protocol [fluoxetine, 10^{-6} M; 5-HT reuptake inhibitor; ketanserin 50 nM; 5-HT_{2A} antagonist; 30-minute incubation period that showed robust inhibition with UK14304) and applied this to superior mesenteric arteries from sham and DOCA-salt hypertensive rats. Under these conditions, 5-HT did not inhibit neurogenic contraction in the superior mesenteric artery of the sham rat *vs.* vehicle (44 ± 11% at 10

Hz; 53 ± 7% at 10 Hz; respectively) or the DOCA-salt rat *vs.* vehicle (66 ± 7% at 10 Hz; 70 ± 4% at 10 Hz) (**Figure 6B**).

DISCUSSION

This study took two different approaches to determining whether 5-HT caused the withdrawal of sympathetic tone, peripherally, from the splanchnic vasculature. Overall, our studies were negative and suggest that a reduction in SNA, at least to the splanchnic bed, does not contribute to the depressor response that accompanies a slow intravenous infusion of 5-HT.

5-HT and SNA

We initiated this series of experiments because of previous findings. Diaz et al (2008) demonstrated that the blood pressure fall induced by hexamethonium, a ganglionic blocker, was significantly reduced in rats infused 5-HT *vs* those infused vehicle. This suggests, but does not prove, that 5-HT may be inhibiting or causing withdrawal of sympathetic tone, such that there is less to be reduced by hexamethonium. We appreciate an alternative conclusion, namely that the effect of hexamethonium may be smaller because blood pressure has already been reduced by 5-HT. Nonetheless, there was also sufficient literature supports that 5-HT may have effects on SNA and/or directly at the ganglion. In the rat, sympathetic preganglionic neurons in the supper thoracic spinal cord responded to 5-HT with largely excitation through what appeared to be a 5-HT_1-like receptor (Lewis and Coote, 1990). Hertzler (1961) demonstrated that 5-HT increased the amplitude of responses of the rat stellate ganglia to preganglionic stimulation, suggesting that 5-HT facilitates transmission, and this was reaffirmed by Pickering *et al* (1994). Sheng-Rong *et al* (1991) similarly demonstrated depolarizations of the guinea pig celiac and inferior mesenteric ganglion (1999). In guinea pig inferior

ganglion, 5-HT was shown to have both excitatory and inhibitory effects on sympathetic transmission (Meehan and Kreulen 1991), while in superior cervical ganglion 5-HT caused a depolarization that promoted transmission (Watkins and Newberry 1996). Jones et al showed 5-HT had a different effect in the cat, where 5-HT_{1D} receptors mediate inhibition of sympathetic ganglionic transmission (1995). Unlike these multiple studies, we did not observe a change in either pre- or post-ganglionic splanchnic SNA during a slow infusion of 5-HT. These nerves were clearly sensitive to stimuli that change blood pressure (*e.g*. PE) and could invoke the appropriate physiological response.

EFS-induced contraction in the superior mesenteric artery was also not reduced by 5-HT, and a number of conditions were tried so as to maximize the opportunity and chances of 5-HT being able to interact with a presynaptic receptor and indirectly cause relaxation through removal of sympathetic activity. While we readily observed robust inhibition of EFS-induced contraction by the α_2 adrenergic agonist, UK14304, this was not observed, in any situation, with 5-HT. Multiple groups have demonstrated the ability of 5-HT, through multiple 5-HT receptors, to cause presynaptic inhibition of sympathetic nerves in blood vessels (Gothert *et al.,* 1991; Kubo and Su, 1983; Molderings *et al,* 1990), and presynaptic inhibition was recently reviewed by Feuerstein (2008). Some of this early work was done in human saphenous veins, but Kubo and Su directly studied the mesentery. They indirectly suggested that 5-HT can inhibit presynaptic receptors because the ability of 5-HT to potentiate EFS-induced contraction was less than that of potentiating norepinephrine-induced contraction. Thus, it is unclear if 5-HT caused presynaptic inhibition of the sympathetic nerve innervating the mesentery. The 5-

$HT_{1B/1D}$ receptor has been most heavily implicated in presynaptic inhibition, hence our use of CP93129. However, both 5-HT and CP93129 were unable to inhibit EFS-induced contraction in the superior mesenteric artery.

Mechanism of 5-HT to decrease blood pressure?

Other groups have demonstrated the acute and long lasting fall in BP in the rat administered 5-HT and/or a 5-HT-induced vasodilator response that one could envision participates in a fall in BP (Calama *et al,* 2003, 2005; Centurion *et al*, 2004, DeVries *et al*, 1999; Garcia *et al*, 2006; Hoffman *et al*., 1990; Terron, 1997; Terron *et al,* 2007). Our studies suggest that if 5-HT does inhibit the sympathetic nervous system, it is not at the level of the splanchnic circulation. We cannot rule out the possibility that this particular bed may be unimportant for the fall in BP caused by 5-HT, and that 5-HT at other sites may have a profound effect on BP. We have not, for example, taken a global measure of SNA such as norepinephrine overflow (Esler *et al.,* 1989). Such a measure might also not be helpful if 5-HT is functioning discretely to influence a particular bed. What beds might those be? The cutaneous circulation is one. When administered to the rodent, 5-HT causes a significant elevation in the temperature of the tail, indicative of heat shedding through vasodilation (Lin *et al.,* 1983). Another is the skeletal muscle, which receives a significant amount (40%) of cardiac output and thus could account for large change in blood pressure. Alsip *et al* demonstrated the ability of 5-HT to dilate the arteriole of the rat skeletal muscle, supporting this possibility (1992).

Another possibility that is more controversial is that 5-HT moves into the central nervous system to reduce sympathetic tone. This is controversial because most state that 5-HT is unable to cross the blood brain barrier; this contrasts with its precursor, 5-hydroxytryptophan, which freely moves across the blood brain barrier and, like 5-HT, can cause a fall in blood pressure (Baron *et al*., 1991; Fregly *et al*, 1987; Itskovitz *et al*., 1989). However, older studies support that 5-HT administration intravenously results in higher levels of 5-HT and its primarily metabolite, 5-hydroxyindole acetic acid (5-HIAA), in the brain (Bulat and Supek, 1968). Expression of the serotonin transporter on the capillary endothelial cells that comprise the barrier has been observed (Brust *et al*, 2000, Wakayama *et al*, 2002), making it possible for 5-HT to move in and out of the CNS, and Nakatani *et al* (2008) recently described the ability of 5-HT to pass from the CNS to the periphery. Westergaard demonstrated nearly 30 years ago that 5-HT itself can modify blood brain barrier function (1978). Activation of the nucleus tractus solitarious is described to inhibit sympathetic activity in rats (Comet *et al.,* 2007), and this would be consistent with our observations. We recently published that the fall in blood pressure to chronically (one week) administered 5-HT was reduced by half in male rats that lack the serotonin transporter (SERT knock out or KO; Davis *et al*, 2011) compared to their wild type control (SERT WT). One explanation for this is that SERT, on the blood brain barrier, is important for 5-HT penetrating the CNS, and thus its genetic loss results in a reduced fall in blood pressure. There is an enormous amount of literature that goes against this idea, but it must be pursued.

In summary, this study suggests 5-HT is not acting to lower blood pressure through inhibition of NE release at the neuroeffector junction or through inhibition of pre- or postganglionic sympathetic neurons innervating the mesentery.

Figure 4.1

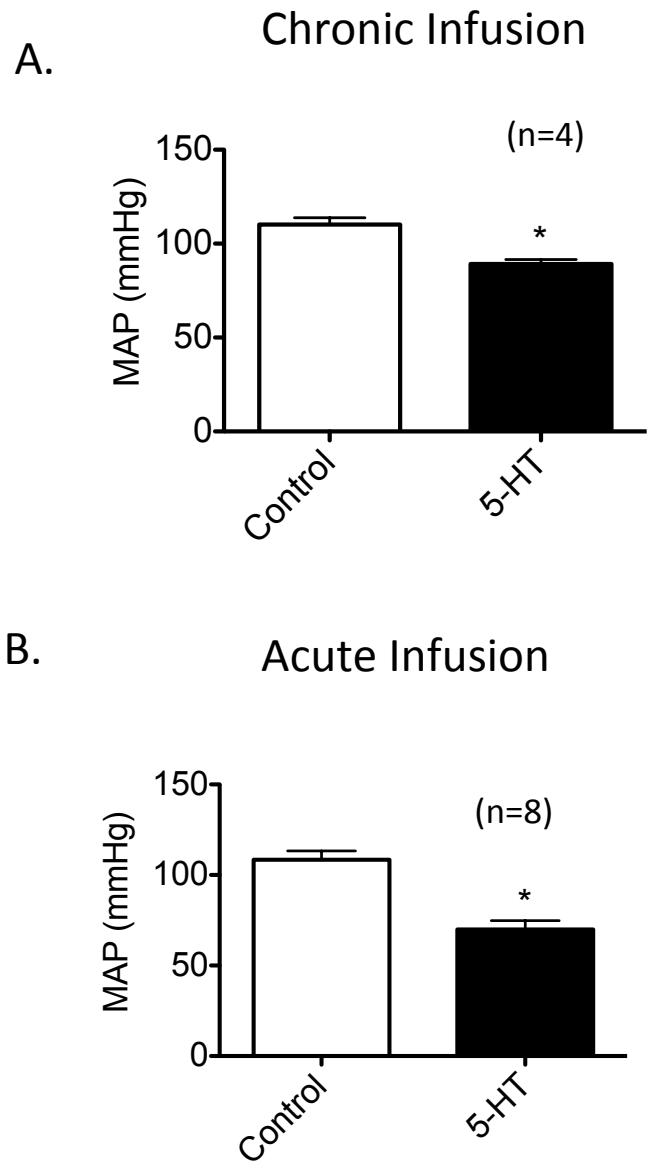

Figure 4.1: 5-HT-induced fall in mean arterial blood pressure of conscious animals receiving 5-HT chronically for one week (5-HT 25 ug/kg/min; **A**) or anesethetized animals receiving 5-HT (25 ug/kg/min) over the course of a 30-60 minute infusion (**B**). Bars represent mean arterial pressure (MAP) in normotensive Sprague Dawley rats before (control) and following 5-HT (reported at nadir of the MAP depressor response) for the

number of animals in parentheses. * statistically significant difference ($p<0.05$) vs. appropriate control.

Figure 4.2

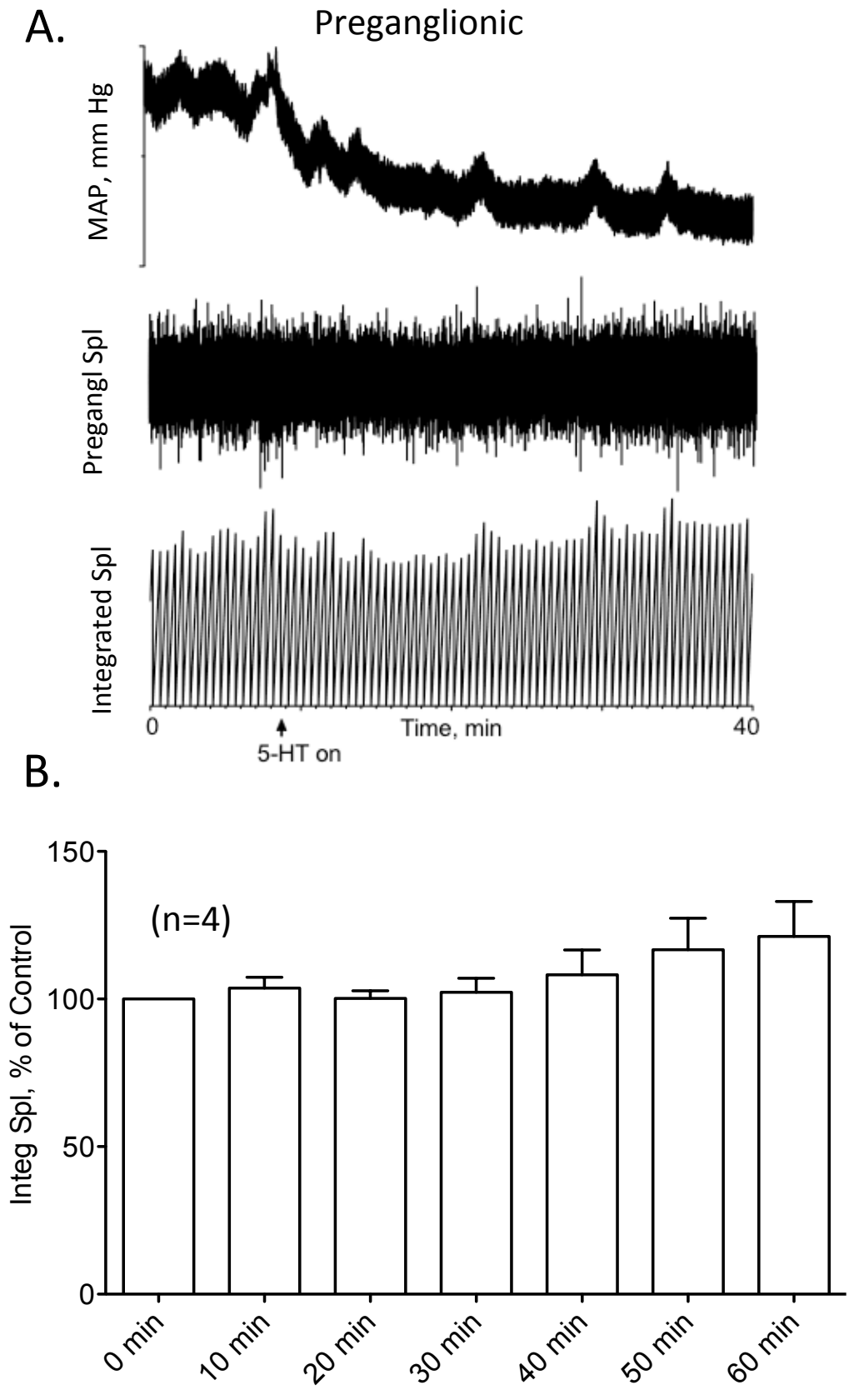
A.
Preganglionic
MAP, mm Hg
Pregangl Spl
Integrated Spl
0
5-HT on
Time, min
40
B.
150
100
50
0
(n=4)
Integ Spl, % of Control
0 min
10 min
20 min
30 min
40 min
50 min
60 min

Figure 4.2: Effect of acute 5-HT infusion on preganglionic sympathetic nerve activity. **A:** Tracing of mean arterial pressure (MAP), preganglionic sympathetic nerve activity (SNA), and integrated (Integ. Spl) preganglionic nerve activity following intravenous 5-HT infusion in the SD rat. Arrow marks the point at which 5-HT was delivered. **B:** Time course of changes in preganglionic sympathetic nerve activity (SNA) based on integrated records in the SD rat. Bars represent integrated preganglionic sympathetic nerve activity (means±SEM) at time(s) indicated following the start of intravenous 5-HT infusion for the number of animals in parentheses.

Figure 4.3

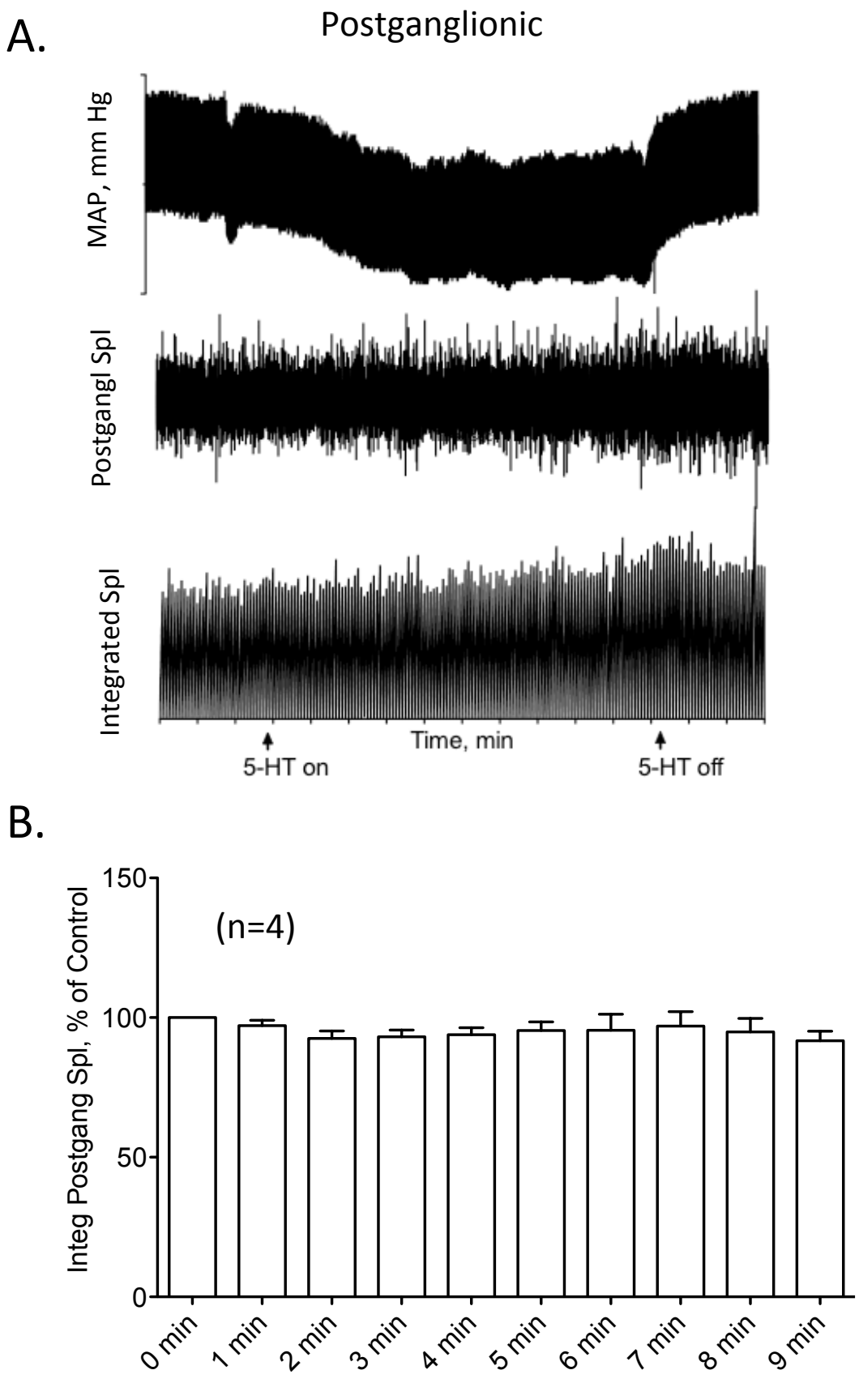
A.
Postganglionic
MAP, mm Hg
Postgangl Spl
Integrated Spl
Time, min
5-HT on
5-HT off
B.
(n=4)
Integ Postgang Spl, % of Control
150
100
50
0
0 min
1 min
2 min
3 min
4 min
5 min
6 min
7 min
8 min
9 min

Figure 4.3: Effect of acute 5-HT infusion on postganglionic sympathetic nerve activity. **A.** Tracing of mean arterial pressure (MAP), postganglionic splanchnic sympathetic nerve activity (SNA), and integrated (Integ. Spl) postganglionic splanchnic nerve activity following intravenous 5-HT infusion in the SD rat. Arrow marks the point at which 5-HT was delivered and when 5-HT infusion was stopped. **B:** Time course of changes in postganglionic sympathetic nerve activity (SNA) based on integrated records in the SD rat. Bars represent integrated postganglionic sympathetic nerve activity (means±SEM) at time(s) indicated following the start of intravenous 5-HT infusion for number of animals indicated in parentheses.

Figure 4.4

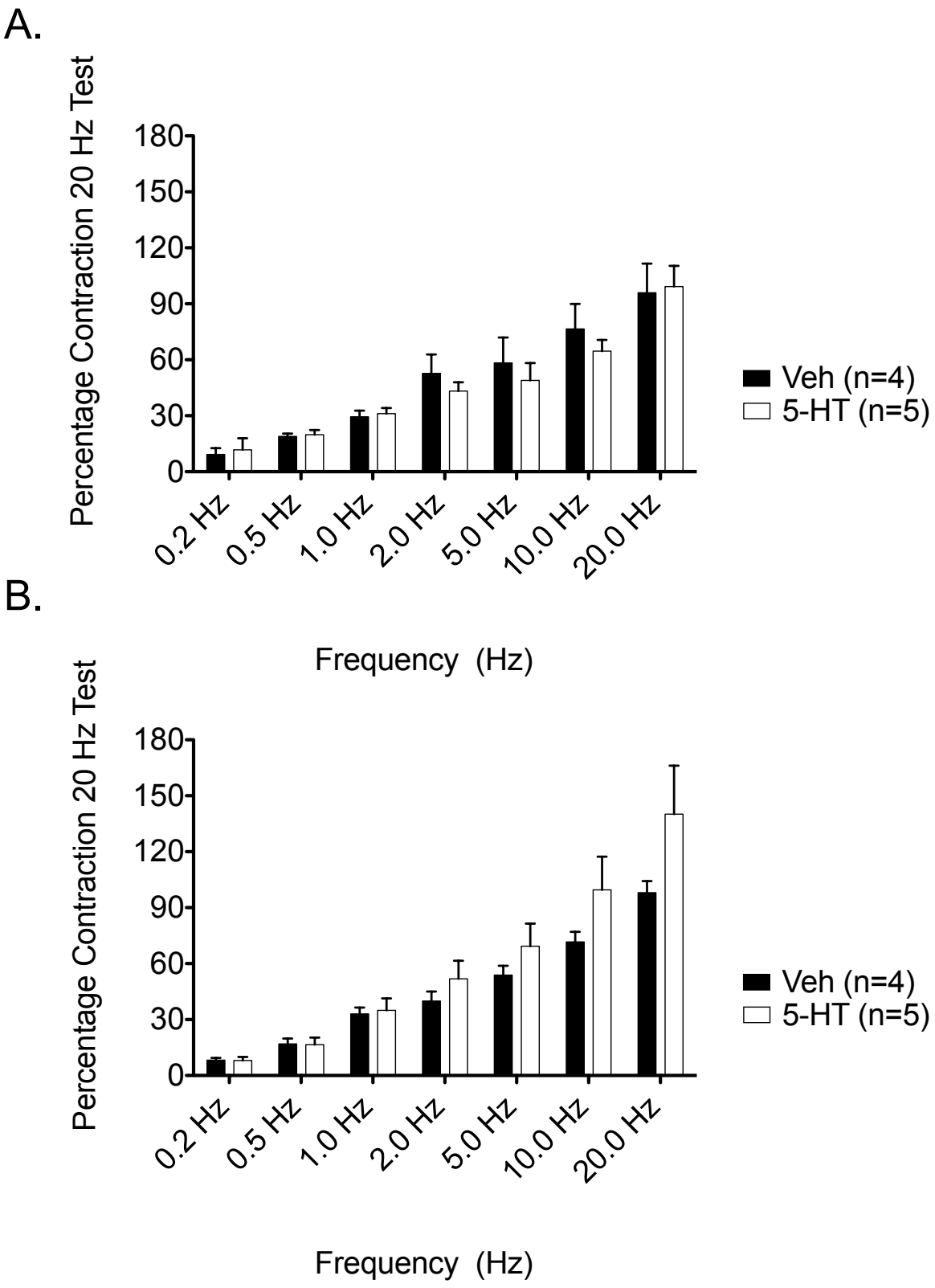
A.
Percentage Contraction 20 Hz Test
180
150
120
90
60
30
0
0.2 Hz
0.5 Hz
1.0 Hz
2.0 Hz
5.0 Hz
10.0 Hz
20.0 Hz
Veh (n=4)
5-HT (n=5)
Frequency (Hz)
B.
Percentage Contraction 20 Hz Test
180
150
120
90
60
30
0
0.2 Hz
0.5 Hz
1.0 Hz
2.0 Hz
5.0 Hz
10.0 Hz
20.0 Hz
Veh (n=4)
5-HT (n=5)
Frequency (Hz)

Figure 4.4: A. Vasoconstrictor response to EFS frequency response curves (0.2 Hz – 20 Hz) in superior mesenteric arteries from Sprague-Dawley rats incubated with either vehicle (H_2O) or 5-HT (10^{-8} M) for 5 min. **B.** Vasoconstrictor response to EFS frequency response curves (0.2 Hz – 20 Hz) in SMA (SD) incubated with either vehicle (H_2O) or 5-HT (10^{-8} M) for 5 min. in the presence of fluoxetine (10^{-6} M). Results (means ± SEM) are expressed as a percentage of the initial 20 Hz maximal contraction for number of animals in parentheses.

Figure 4.5

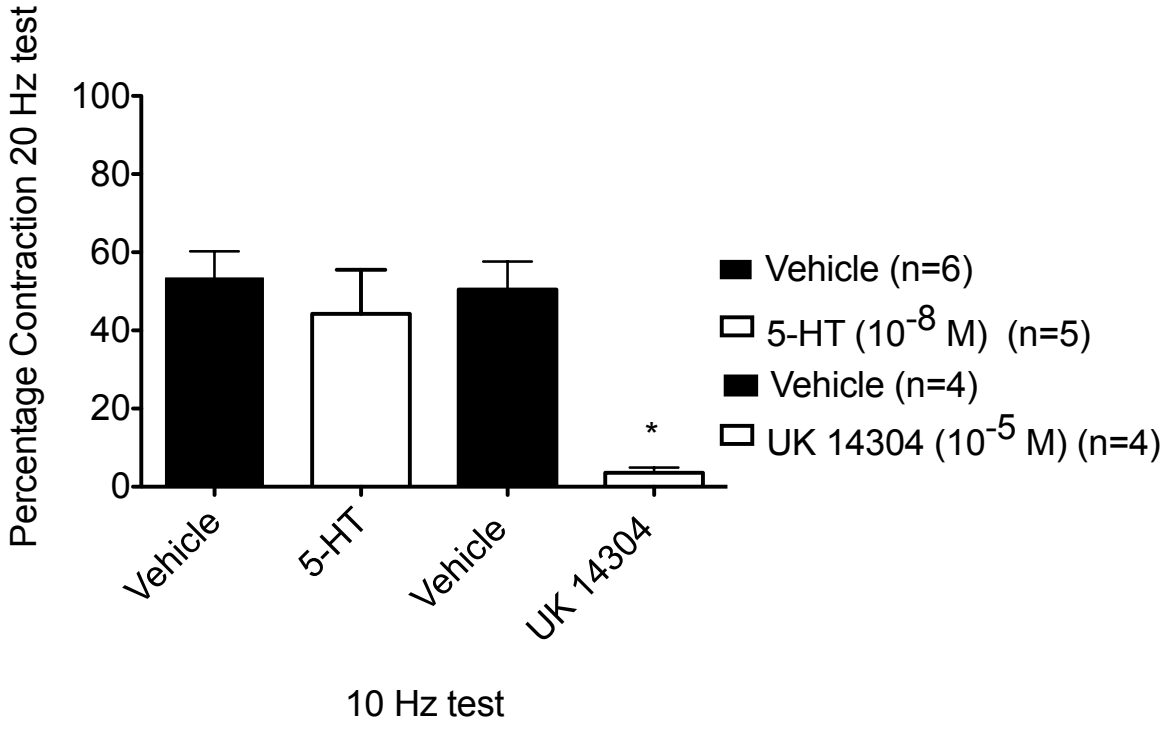

Figure 4.5: Vasoconstrictor response to 10 Hz stimulus in superior mesenteric arteries from Sprague-Dawley rats incubated with either vehicle (H_2O), 5-HT (10^{-8} M), or UK 14304 (10^{-5} M) in the presence of fluoxetine and (10^{-6} M) and ketanserin (50 nM) for 30 min. Results (means ± SEM) are expressed as a percentage of the initial 20 Hz maximal contraction for number of animals in parentheses. * statistically significant difference ($p < 0.05$) vs. vehicle.

Figure 4.6

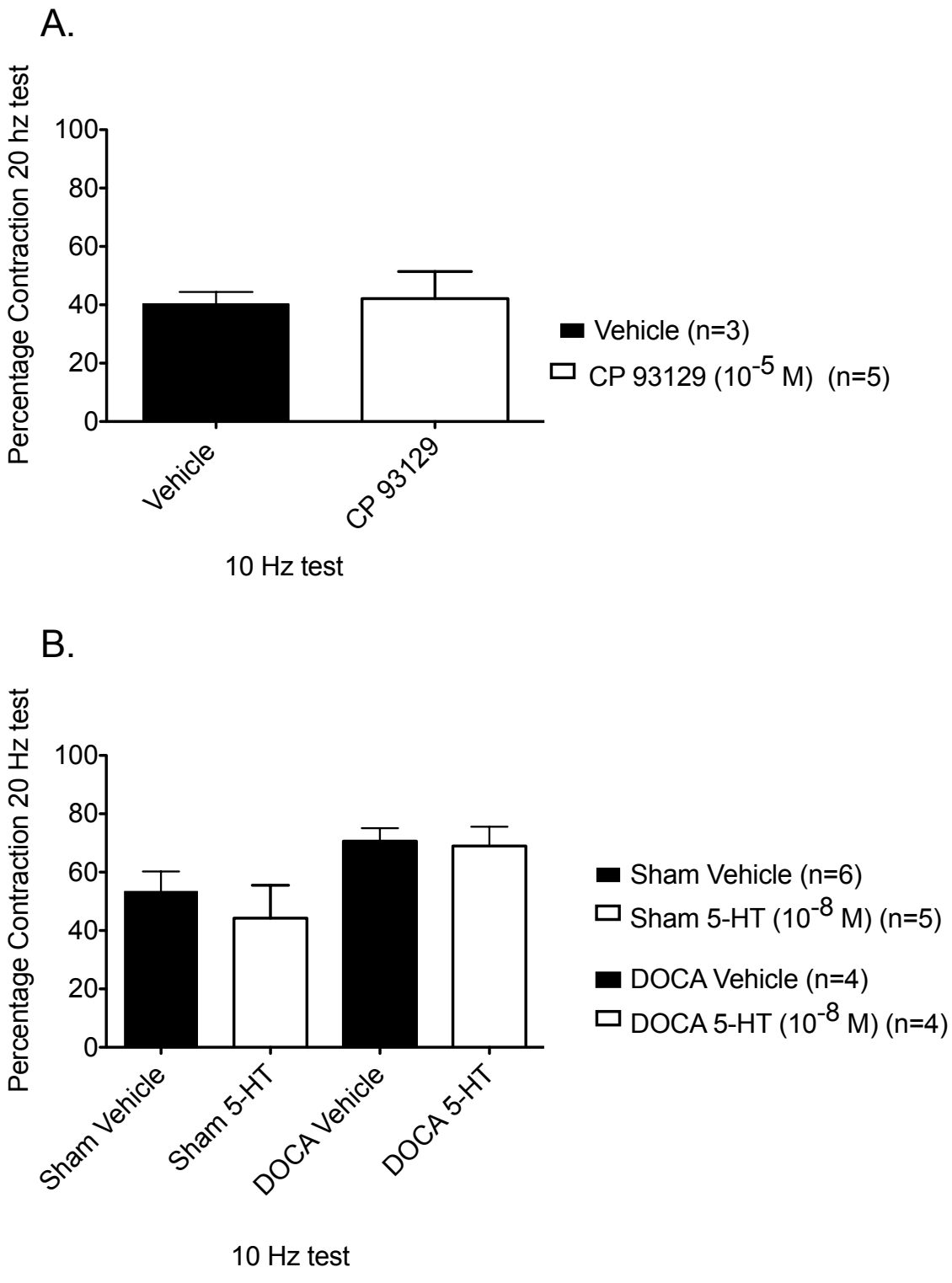
A.
Percentage Contraction 20 hz test
100
80
60
40
20
0
Vehicle
CP 93129
10 Hz test
Vehicle (n=3)
CP 93129 (10-5 M) (n=5)
B.
Percentage Contraction 20 Hz test
100
80
60
40
20
0
Sham Vehicle
Sham 5-HT
DOCA Vehicle
DOCA 5-HT
10 Hz test
Sham Vehicle (n=6)
Sham 5-HT (10-8 M) (n=5)
DOCA Vehicle (n=4)
DOCA 5-HT (10-8 M) (n=4)

Figure 4.6: A. Vasoconstrictor response to 10 Hz stimulus in superior mesenteric arteries from Sprague-Dawley rats incubated with either vehicle (H_2O), or CP 93129 (10^{-5} M) in the presence of fluoxetine and (10^{-6} M) and ketanserin (50 nM) for 30 min. Results (means ± SEM) are expressed as a percentage of the initial 20 Hz maximal contraction. **B.** Vasoconstrictor response to 10 Hz stimulus in superior mesenteric artery from either sham or DOCA-salt rats incubated with either vehicle (H_2O), or 5-HT (10^{-8} M) in the presence of fluoxetine and (10^{-6} M) and ketanserin (50 nM) for 30 min. Results (means ± SEM) are expressed as a percentage of the initial 20 Hz maximal contraction for number of animals indicated in parentheses.

Chapter 5

Lack of the serotonin transporter (SERT) reduces the ability of 5-hydroxytryptamine to lower blood pressure.

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ABSTRACT

Serotonin (5-hydroxytryptamine; 5-HT) is a potent constrictor of isolated blood vessels. However, recent studies demonstrate that chronic 5-HT infusion results in a prolonged fall in blood pressure in the rat. This finding highlights the need for further study of 5-HT in the cardiovascular system. We tested the hypothesis that a functional serotonin transporter (SERT) is critical to enabling a 5-HT-induced fall in blood pressure. Experiments were performed in male and female rats to determine whether gender significantly affected the ability of 5-HT to lower blood pressure and to determine whether SERT dependence was different in male *vs.* female rats. 5-HT (25 μg/kg/min; s.c.) was infused for 7 days to male and female, SERT wildtype (WT), and SERT knockout (KO) rats. Mean arterial pressure (MAP) and heart rate (HR) were monitored *via* radiotelemetry. 5-HT produced a significantly greater fall in MAP (at the nadir) in the male SERT WT rat (-20 ± 1 mmHg) compared to the male SERT KO rat (-10 ± 2 mmHg). *Similarly, 5-HT also produced a significantly greater fall in MAP (at the nadir) in the female SERT WT rat (-19 ± 1 mmHg) compared to the female SERT KO rat (-15 ± 0.4 mmHg).* While the lack of a functional SERT protein did not prevent a 5-HT-induced fall in blood pressure, it did reduce the ability of 5-HT to lower blood pressure in the male and female SERT rat, suggesting a potentially important role for SERT in producing a 5-HT-induced fall in blood pressure.

INTRODUCTION

5-hydroxytryptamine (5-HT) is a vasoactive amine synthesized in the enterochromaffin cells of the gastrointestinal tract and the raphe nucleus in the central nervous system (Erspamer and Asero 1952; Dahlstroem and Fuxe 1964). It is a potent constrictor of isolated blood vessels (Rapport *et al*. 1948) and its actions are terminated by the enzymatic activity of monoamine oxidase.

Elevated levels of free plasma 5-HT are present in both human and experimental models of hypertension (Brenner *et al.* 2007; Fetkovska *et al.* 1990). Initially, this led investigators to postulate that 5-HT might be acting to constrict the peripheral vasculature, resulting in elevated blood pressure. However, chronic 5-HT infusion produced a prolonged fall in blood pressure in the DOCA-salt and sham rat (Diaz *et al.* 2008). This suggested elevated free plasma 5-HT might be acting to lower blood pressure in both human and animal models of hypertension. Thus, 5-HT might represent an adaptation to elevated pressure, rather than a contributing factor to essential hypertension. Interestingly, Diaz *et. al.* demonstrated that inhibition of nitric oxide synthase (NOS) prevented a 5-HT-induced fall in blood pressure, suggesting an important role for NOS and likely nitric oxide (NO) in eliciting a 5-HT-induced fall in blood pressure. However, the exact mechanism underlying a 5-HT-induced fall in blood pressure is not yet fully understood.

Several studies implicate the importance of the serotonin transporter (SERT) as mechanistically important in mediating the effects of 5-HT. Chanrion *et. al.*

demonstrated 5-HT uptake *via* SERT enhanced neuronal nitric oxide synthase (nNOS) activity in cells coexpressing both SERT and nNOS (Chanrion *et. al.* 2007). This led Chanrion *et. al.* to speculate that 5-HT uptake *via* SERT leads to an allosteric change in nNOS, which increases its affinity for calmoudulin, resulting in activation. This suggests elevated levels of free 5-HT may be taken up *via* SERT leading to nNOS activation, NO production, and lower blood pressure.

Moreover, reports also suggest that 5-HT can cross the blood brain barrier (BBB) *via* SERT (Nakatani *et al.* 2008). Thus, elevated levels of peripheral 5-HT might potentially cross the BBB and act centrally to activate nNOS *via* SERT uptake. Collectively, this evidence suggests that SERT may play an important role in eliciting a 5-HT-induced fall in blood pressure. We tested the hypothesis that SERT function and 5-HT uptake is essential to enabling a 5-HT-induced fall in blood pressure.

To test this hypothesis, we utilized the SERT KO rat created by Edwin Cuppen [ethylnitrosurea-induced mutagenesis on a Wistar rat background] (Homberg *et al*. 2007). Previous studies validated the absence of a functional SERT and the presence of a truncated protein in the male SERT KO rat through immunohistochemical (IHC) staining and western blot analysis (Linder *et al.* 2008). Finally, reports suggest SERT may function differently in males vs. females (Maron *et. al.* 2010). Thus, we investigated chronic 5-HT infusion in male and female rats to determine whether gender significantly affected the ability of 5-HT to lower blood pressure.

METHODS

Animal Use

Male and female SERT knockout (SERT-KO), and Wistar-based wild type (WT) rats (Charles River) were used in these studies. The SERT-KO and WT rats were bred at *Michigan State University* under a breeding license obtained from genOway®. All animal use procedures were performed in accordance with the Institutional Animal Care and Use Committee at *Michigan State University*.

Experimental protocol

Animals were anesthetized with isoflurane, and implanted with radiotelemetery devices (Data Sciences International, St. Paul, MN USA: PA-C40 introduced *via* the femoral artery). Rats were allowed 4 days to recover and 3 days of baseline measurements were made. An Alzet® osmotic pump (Model 2ML1, Duret Corporation, Cupertino, CA; 10μL/hr 7 days) containing 5-HT (25 μg/kg/min) was inserted subcutaneously at the base of the neck. Mean arterial pressure (MAP) and heart rate (HR) were monitored for the duration of the study. In table 1, we report MAP and HR on the 2nd control day as well as at the nadir. Hemodynamic measurements were sampled for 10 seconds every 10 minutes for the duration of the experiment. Data are reported as 24-hour averages. Blood was collected at the end of the study and fractionated into platelet poor plasma to validate elevated free plasma 5-HT levels using high performance liquid chromatography.

Plasma 5-HT collection

Blood collection and platelet poor plasma (PPP) preparation were conducted according to our previously published methods (Diaz *et al.* 2008). Briefly, five milliliters (ml) of blood was collected from left cardiac ventricle using a heparin coated syringe. The blood was transferred into an EDTA anticoagulant vacutainer tube. The tubes were spun at 160 x g for 30 minutes for platelet-rich-plasma (PRP). The supernatant containing plasma and buffy coat were pipetted into EDTA-coated plastic tubes. These tubes were centrifuged at 1350 x g for 20 minutes for platelet-poor-plasma (PPP). To the remaining pellet (platelet layer), 1 ml of platelet buffer and 1 μmol/L ADP was added. 10 μmol/L pargyline and 10 μmol/L ascorbic acid were added. The tubes were centrifuged at 730 x g for 10 minutes. Ten percent trichloroacetic acid (TCA) was added to deproteinize both sets of samples. All tubes were centrifuged at 4500 x g for 20 minutes. Finally, samples were ultracentrifuged at 280,000 x g for 2 hours. 5-HT concentrations were measured using HPLC at 0.4 V and 0.9 ml/min flow rate.

Data analysis

For *in vivo* data analysis, within-group differences were assessed by a one-way ANOVA with post hoc comparisons using Student Newman-Keuls test (GraphPad Prism 5). An unpaired Students t-test was performed to measure point-to-point differences. In all cases, a p value of < 0.05 was considered significant. All results are presented as means ± SEM.

RESULTS

In the male rat, chronic 5-HT infusion significantly ($p < 0.05$) elevated free plasma 5-HT levels in the SERT WT rat (61 ± 16.2 ng/ml) compared to baseline measures (8.6 ± 1.3 ng/ml). Similarly, chronic 5-HT infusion also elevated free plasma 5-HT levels in the SERT KO rat (37.3 ± 24.2 ng/ml) compared to baseline measures (1.1 ± 0.5 ng/ml). At baseline, free plasma 5-HT levels were significantly higher in the SERT WT (8.6 ± 1.3 ng/ml) compared to the SERT KO rat (1.1 ± 0.5 ng/ml) rat ($p < 0.05$).

In the female rat, chronic 5-HT infusion also significantly ($p < 0.05$) elevated free plasma 5-HT levels in the SERT WT (62 ± 8.4 ng/ml) compared to baseline measures (3.9 ± 0.6 ng/ml). Similarly, chronic 5-HT infusion also elevated free plasma 5-HT levels in the SERT KO rat (23 ± 7.3 ng/ml) compared to baseline (1.9 ± 1.0 ng/ml). At baseline, free plasma 5-HT levels were not significantly different in the SERT WT (3.9 ± 0.6 ng/ml) compared to the SERT KO (1.9 ± 1.0 ng/ml) rat.

Table 1 summarizes changes in MAP and HR. MAP fell rapidly in all models reaching a nadir after 24-48 hours (Figure 1). HR was elevated (at the nadir) in all models following 5-HT infusion but not statistically different between groups. Both MAP and HR trended toward baseline (C2) throughout the remainder of the study.

In the male rat, 5-HT produced a significantly greater fall in MAP in the SERT WT rat (-20 ± 1 mmHg) compared to the SERT KO rat (-10 ± 2 mmHg) (Figure 1A). MAP remained significantly lower in the SERT WT rat compared to the SERT KO rat during

the 2nd and 3rd day of 5-HT infusion. MAP in the SERT WT also never returned to baseline (C2) at the end of the 7 day infusion whereas it returned to baseline in the SERT KO.

In the female rat, 5-HT produced a significantly greater fall in MAP in the SERT WT (-19 ± 1 mmHg) compared to the SERT KO (-15 ± 0.4 mmHg) rat (Figure 1B). Baseline (C2) MAP was greater in the SERT WT rat (112 ± 1.3 mmHg) compared to the SERT KO rat (106 ± 2.0 mmHg). MAP in both the SERT WT and SERT KO returned to baseline (C2) by the end of the study. There was no significant difference in the fall in MAP between the male and female SERT WT or the male and female SERT KO rats.

DISCUSSION

This study investigated whether a functional SERT protein was essential to enabling a 5-HT-induced fall in blood pressure. The absence of a functional SERT protein significantly reduced the magnitude of the 5-HT-induced fall in blood pressure in the SERT KO rat. This suggests an important role for 5-HT uptake in mediating a 5-HT-induced fall in blood pressure in the Wistar rat.

The mechanisms by which SERT participates in a 5-HT-induced fall in blood pressure are numerous. However, the most compelling evidence for SERT involvement in a 5-HT-induced fall in blood pressure is provided by Chanrion *et. al.* who suggests 5-HT uptake *via* SERT may activate nNOS and stimulate NO release. Interestingly, both arteries and veins also possess SERT and actively take up 5-HT (Ni *et al.* 2004; Linder *et al*. 2008). This suggests elevated free 5-HT may be taken up into the vasculature *via* SERT and lead to activation of endothelial nitric oxide synthase (eNOS), and release of NO.

The present study also sought to determine whether gender affected the ability of 5-HT to lower blood pressure. No statistical differences in MAP were noted between the male SERT WT *vs.* the female SERT WT or the male SERT KO *vs.* the female SERT KO at the nadir. The absence of a functional SERT significantly reduced the ability of 5-HT to lower blood pressure in both male and female Wistar rats. Therefore we conclude gender does not significantly impact a 5-HT-induced fall in blood pressure.

While the absence of a functional SERT significantly reduced the ability of 5-HT to lower

blood pressure, it did not completely abolish a 5-HT-induced fall in blood pressure. Therefore, this raises questions about whether 5-HT might be taken up by another transporter. Alternatively, another mechanism might exist, besides 5-HT uptake, which contributes to a 5-HT-induced fall in blood pressure.

Further, one might expect free 5-HT levels to be higher in 5-HT infused SERT KO rats compared to 5-HT infused SERT WT rats due to the absence of a functional SERT to take up exogenously delivered 5-HT. However, following 5-HT infusion, we observed higher levels of free 5-HT in the SERT WT compared to the SERT KO rat. This raises questions about where exogenously delivered 5-HT might be deposited or whether 5-HT may be rapidly excreted in the SERT KO rat compared to the SERT WT rat.

Finally, we acknowledge that chronic 5-HT infusion will likely result in down regulation of SERT. Thus, we speculate that if 5-HT uptake *via* SERT is critical to enabling a 5-HT-induced fall in blood pressure, then down regulation of SERT may correlate with the observed trend in MAP (Figure 1) back towards baseline in 5-HT infused SERT WT and SERT KO rats.

In summary, this study suggests an important role for 5-HT uptake in enabling a 5-HT-induced fall in blood pressure in the Wistar rat.

Table 1: MAP and HR changes in male and female SERT WT and SERT KO rats. Data are mean ± SEM. * *P* < 0.05 compared with SERT KO.

	Control	
Model	**BP (mmHg)**	**HR (bpm)**
Male SERT WT rat (n=8)	106 ± 1	348 ± 6
Male SERT KO rat (n=7)	101 ± 2	355 ± 7
Female SERT WT rat (n=8)	113 ± 1	366 ± 3
Female SERT KO rat (n=7)	106 ± 2	387 ± 4
	Nadir	
	BP (mmHg)	**HR (bpm)**
Male SERT WT rat (n=8)	85 ± 1	422 ± 6
Male SERT KO rat (n=7)	90 ± 2	412 ± 7
Female SERT WT rat (n=8)	93 ± 2	440 ±4
Female SERT KO rat (n=7)	91 ± 2	456 ± 2
	Change	
	BP (mmHg)	**HR (bpm)**
Male SERT WT rat (n=8)	21 ± 1.0 *****	74 ± 5
Male SERT KO rat (n=7)	11 ± 2.0	57 ± 8
Female SERT WT rat (n=8)	19 ± 1.0 *****	75 ± 5
Female SERT KO rat (n=7)	15 ± 0.4	69 ± 5

Figure 5.1

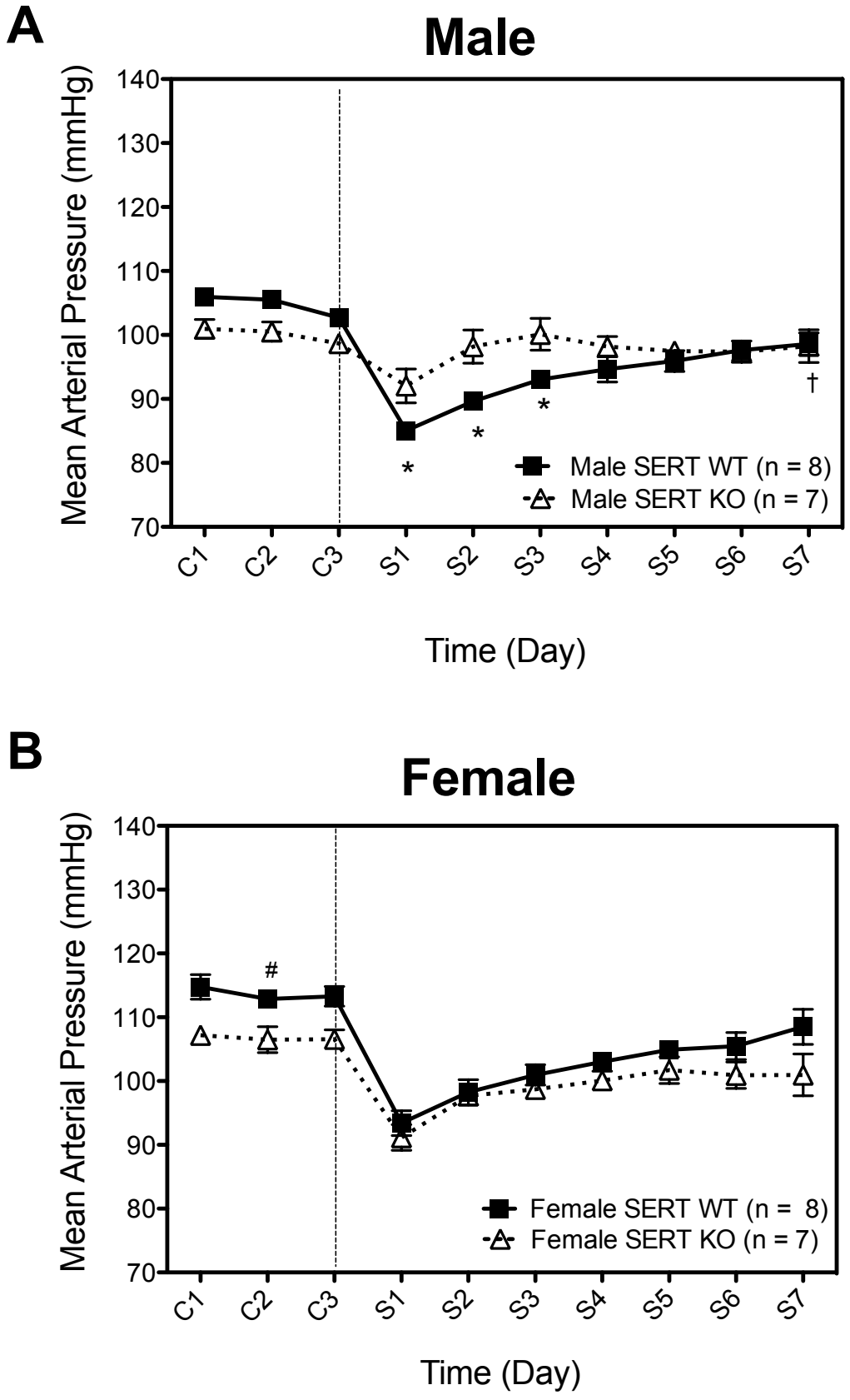
A
Male
Mean Arterial Pressure (mmHg)
140
130
120
110
100
90
80
70
C1
C2
C3
S1
S2
S3
S4
S5
S6
S7
*
*
*
†
Male SERT WT (n = 8)
Male SERT KO (n = 7)
Time (Day)
B
Female
Mean Arterial Pressure (mmHg)
140
130
120
110
100
90
80
70
#
Female SERT WT (n = 8)
Female SERT KO (n = 7)
C1
C2
C3
S1
S2
S3
S4
S5
S6
S7
Time (Day)

Figure 5.1: A. Effect of chronic 5-HT infusion on mean arterial pressure (MAP) in male SERT WT and SERT KO rats. **B.** Effect of chronic 5-HT infusion on mean arterial pressure (MAP) in female SERT WT and SERT KO rats. Data points indicate 24-hour averaged MAP +/- SEM for the number of animals in parentheses. The vertical line denotes 5-HT or vehicle osmotic pump implant. Time is represented by days on the x-axis. C represents control recordings. S represents 5-HT infusion. * statistically significant ($P < 0.05$) difference compared with male SERT KO rat. † statistically significant ($P < 0.05$) difference compared to control (C2) in male SERT WT rat. # statistically significant ($P < 0.05$) difference compared to control (C2) in female SERT KO rat (C2).

DISCUSSION

These studies investigated whether 5-HT lowers blood pressure, in a nitric-oxide dependent manner, by reducing total peripheral resistance, either through a reduction in sympathetic nervous system tone and/or direct stimulation of vascular 5-HT receptors. While the results of these studies were largely negative, significant progress has been made toward elucidating the mechanism underlying a 5-HT-induced fall in blood pressure. Several important findings revealed that: 1. 5-HT lowers blood pressure by reducing peripheral vascular resistance, 2. 5-HT is capable of lowering blood pressure over the course of one-month, 3. 5-HT is not acting in the mesenteric vasculature to lower blood pressure (either *via* direct receptor mediated dilation or by reducing sympathetic nervous system tone), and 4. SERT plays an important role in enabling a 5-HT-induced fall in blood pressure. Despite these important findings, significant questions remain regarding the mechanism underlying a 5-HT-induced fall in blood pressure.

5-HT reduces peripheral vascular resistance

The first major finding of this study was that 5-HT significantly reduces peripheral vascular resistance in the Sprague Dawley rat. This finding resolved the question of exactly how 5-HT is acting to lower blood pressure given that changes in blood pressure are governed by either changes in vascular resistance or changes in cardiac output. However, this study did not reveal exactly where 5-HT is acting to reduce peripheral vascular resistance? Our data strongly suggests against changes in the mesenteric

vasculature. Thus, understanding and elucidating the exact mechanism of 5-HT-induced hypotension will depend upon identifying exactly where 5-HT is acting.

With this in mind, we can speculate about which vascular bed 5-HT may be acting on based indirectly on the distribution of cardiac output in the rat. We hypothesize that changes in vascular resistance in those beds receiving the greatest portion of cardiac output are most likely to exert significant changes on mean arterial pressure following 5-HT infusion. Thus, skeletal muscle, which receives 25-35% of total cardiac output, (depending on conditions) is a reasonable vascular bed to consider when investigating where 5-HT is acting to reduce peripheral resistance and lower blood pressure (Delp *et. al.* 1991). This contrasts with the mesenteric vasculature, and gastrointestinal tract, which receives approximately 14% of cardiac output and was extensively studied throughout this series of experiments (Delp *et. al*. 1991). Therefore, it may be reasonable for us to postulate that the changes in mean arterial pressure following 5-HT infusion are due to changes in vascular resistance in skeletal muscle due to receipt of a greater portion of cardiac output (thus potentially being capable of a greater change in blood pressure).

However, evidence in the literature may suggest against this particular hypothesis. Investigators injected 5-HT (0.1 μmol/L and 1000 μmol/L) into the masseter muscle of 12 healthy patients and measured changes in blood flow using laser Doppler technique (Emberg *et. al.* 2006). These investigators revealed that 5-HT had no effect on local blood flow at either concentration in the microcirculation of the masseter

muscle. The results of this study suggest that 5-HT is not capable of reducing vascular resistance in skeletal muscle, as evidenced by the fact that there was no change in local blood flow. An increase in blood flow in the masseter muscle would have been consistent with the hypothesis that 5-HT is reducing vascular resistance in skeletal muscle. Moreover, investigators also demonstrated that treatment with a mixed 5-HT_{1B}/5-HT_{2A} receptor antagonist improved perfusion in the lower limb following arterial occlusion and ischemia in the obese Zucker rat (Janiak *et. al*. 2002). This study suggests that 5-HT is acting as a constrictor in the collateral vasculature of the lower limb and that receptor blockade improves perfusion in these vessels following arterial occlusion. Thus, this study suggests against 5-HT acting to dilate skeletal muscle vasculature or collateral blood vessels and raises the question about whether another vascular bed may mediate a 5-HT-induced fall in blood pressure.

The skin or cutaneous vasculature receives approximately 6% of cardiac output and thus may be another potential place 5-HT is acting to reduce peripheral resistance and lower blood pressure. Support for this hypothesis is provided by the clinical carcinoid syndrome, characterized by increased release of 5-HT, arising from a carcinoid tumor (Jackson *et. al.* 2009). Skin flushing, tachycardia, and changes in blood pressure are symptoms associated with carcinoid syndrome. Skin flushing can be interpreted as an increase in skin blood flow and therefore it is not unreasonable to hypothesize given the clinical symptoms of the carcinoid syndrome that 5-HT may act at the level of the cutaneous vascular to dilate these vessels causing a reduction in vascular resistance and a reduction in blood pressure.

However, inconsistent with this hypothesis is the observation of marked cyanosis in the lower limb of those rats receiving 5-HT (lower limb instrumented with radiotelemeter). This observation suggests 5-HT infusion decreases perfusion of the lower limb either through direct constriction of the peripheral/cutaneous vasculature or potentially hypo-perfusion of the lower limb due to lower mean arterial pressure. If the cutaneous vasculature were dilated then there would be an increase in perfusion and the skin surface (paw) would appear pink.

The literature also suggests that changes in human cutaneous vascular tone are capable of altering blood pressure and that these changes are linked to neuronal nitric oxide synthase (nNOS) (Kellogg *et. al.* 2008). This suggests that 5-HT may be capable of reducing cutaneous vascular resistance through an nNOS mediated action(s). This hypothesis would be consistent with the finding by Diaz *et. al.* that a 5-HT-induced fall in blood pressure is dependent on nitric oxide synthase (NOS) (Diaz *et. al.* 2008). Collectively, both the skeletal muscle vasculature and the cutaneous vasculature are reasonable and appropriate choices to pursue further investigation toward understanding the exact mechanism underlying a 5-HT-induced fall in blood pressure.

5-HT is capable of lowering blood pressure over the course of one-month

The second major finding of this study was that 5-HT was capable of reducing blood pressure in the DOCA-salt rat over the course of one-month but was unable to prevent the development of DOCA-salt hypertension. The original study that led to the discovery that 5-HT was capable of causing a pronounced fall in blood pressure (Diaz

et. al. 2008) was designed to address the actions of 5-HT on blood pressure, given the clinical observation that hypertensive patients presented with elevated levels of free plasma 5-HT compared to their normotensive counterparts. However, as has been previously discussed, the original study was a relatively short clinical exposure given that the human patients are likely exposed to elevated levels of free 5-HT for many months to years (rather than days). The finding that 5-HT is able to maintain lower blood pressures in the DOCA-salt rat over the course of one-month significantly strengthens the case that elevated levels of free plasma 5-HT are likely acting to lower blood pressure rather then increase or contribute to essential hypertension in human patients.

Despite a significant advance in our understanding of what 5-HT is capable of in the cardiovascular system, a longer 5-HT infusion and/or varied 5-HT infusions would result in better understanding and the design of a more clinically relevant study. We were fortunate to work with the iPrecio® micro infusion pump, a programmable infusion pump that allowed for a longer infusion protocol (moving beyond the previously studied one-month infusion *via the* Alzet® osmotic infusion pump) and variable administration of 5-HT (start, stop, and change the dose of 5-HT delivered over the designated infusion period). We were able to establish that 5-HT is capable of repeated falls in blood pressure in the sham and DOCA-salt rats when the iPrecio® micro infusion pump(s) were paired with animals instrumented with radiotelemetry devices. Moreover, we also undertook a dose response study, which demonstrated that 5-HT is capable of reducing

blood pressure in the Sprague Dawley rat at lower doses then had been previous delivered.

In the future, the iPrecio® micro infusion pump could be employed to study the long-term (> one-month) effects of 5-HT on blood pressure. This device could be employed to study a 3-month infusion of 5-HT in the normotensive and hypertensive rat (when infused continuously at 3.0 μL/hr). Thus, this device will be important in future efforts to elucidate the exact mechanism underlying a 5-HT-induced fall in blood pressure.

If 5-HT is not acting at the mesenteric vasculature then where?

The third major finding of this study was that 5-HT is not likely acting in the mesenteric vasculature (either through direct receptor mediated vascular relaxation or through inhibition of sympathetic nervous system tone) to lower blood pressure. Previous reports in the literature contradict our findings and suggest that direct vascular relaxation of an isolated mesenteric preparation is possible in the presence of ketanserin (Mclennan and Taylor, 1984). Thus, we are forced to ask the question about what differs between our investigation and the studies conducted by Mclennan and Taylor? One possibility is that we investigated the effects of 5-HT in a helical strip (mounted in an isolated tissue bath) as opposed to the perfused mesenteric vascular bed. The act of cutting the vessel into a helical strip may have disrupted the architecture of the superior mesenteric artery and resulted in a vessel that was unable to relax under the conditions we set forth. In the future, we may need to investigate an alternate

technique (rather then the helical strip) to investigate whether 5-HT is acting through direct receptor mediated relaxation to lower blood pressure.

Unfortunately, our present data does not allow us to determine the specific mechanism underlying a 5-HT-induced fall in blood pressure, nor does it provide us with significant insight to determine whether 5-HT is acting through a direct receptor mediated vascular relaxation or through sympathoinhibition. Thus, the question regarding how 5-HT is acting to reduce peripheral vascular resistance remains, as does the question regarding the exact vascular bed in which 5-HT is acting to reduce peripheral vascular resistance and blood pressure (this possibility has been discussed above).

While significant effort has been dedicated throughout this series of experiments to the possibility that 5-HT may have been acting in the periphery to mediate a 5-HT-induced fall in blood pressure, the possibility that 5-HT may be able to cross the blood brain barrier (BBB) and act centrally to mediate a 5-HT-induced fall in blood pressure has largely been ignored. In our laboratory, the crucial experiment that has not yet been undertaken to determine whether 5-HT (delivered subcutaneously) is capable of crossing the blood brain barrier involves radiolabeling exogenously delivered 5-HT to determine whether it is capable traversing the blood brain barrier.

Despite the absence of this critical experiment, investigators demonstrated that intravenous delivery of 5-HT is capable of elevating 5-HT and 5-HIAA levels within the CNS (Bulat and Supek, 1968). Further, the presence of SERT on the endothelial cells

lining the blood brain barrier suggests 5-HT is capable of entering the CNS through a direct transport mediated carrier mechanism (Brust *et. al.* 2000) (Wakaymam *et. al.* 2008). Collectively, these studies suggest that a centrally mediated 5-HT-induced fall in blood pressure is possible and should be explored.

However, we also discovered that 5-HT does not inhibit preganglionic splanchnic sympathetic nerve activity, and this result suggests against a centrally mediated effect given that changes in centrally generated nerve activity are reflected in changes in preganglionic nerve activity. This does not rule out the possibility that 5-HT acts centrally, but without a definite vascular bed to target, identifying another preganglionic nerve to record from will unlikely yield positive results. Thus, it will be crucial to identify whether 5-HT is capable of gaining access to the CNS and which vascular bed 5-HT is targeting to mediate a 5-HT-induced fall in blood pressure. Then important work can begin on elucidating the exact mechanism underlying a 5-HT-induced fall in blood pressure.

SERT plays an important role in a 5-HT-induced fall in blood pressure

The final major finding of this study was that SERT plays an important role in enabling a 5-HT-induced fall in blood pressure. These results suggested that 5-HT uptake is an important component to enabling a 5-HT-induced fall in blood pressure. However, the absence of SERT (SERT KO rats) did not completely abolish a 5-HT-induced fall in blood pressure and raises the question if 5-HT uptake is crucial to the mechanism underlying a 5-HT-induced fall in blood pressure, how is 5-HT taken up in

the absence of SERT? One possibility is that 5-HT is taken up by another monoamine transporter: the dopamine transporter (DAT) and/or the norepinephrine transporter (NET) (Zhou *et. al.* 2002; Suarez-Roca and Cubeddu, 2002). Uptake *via* either of these transporters would allow a 5-HT-induced fall in blood pressure in the absence of SERT.

The importance of SERT in enabling a 5-HT-induced fall in blood pressure, also indirectly supports the hypothesis that elevated levels of free plasma 5-HT are antihypertensive. Our laboratory has previously demonstrated that SERT is dysfunctional in the aorta of the DOCA-salt and L-NNA hypertensive rat (Ni *et. al.* 2006). Thus, impaired 5-HT uptake *via* a dysfunctional SERT protein may contribute to elevated blood pressure in these models of hypertension given that 5-HT uptake *via* SERT lowers blood pressure. However, this study only investigated SERT function in the aorta, and thus we have not yet determined SERT dysfunction globally. Therefore, if SERT enables transport of 5-HT across the blood brain barrier, we cannot state that SERT is impaired in the DOCA-salt and/or L-NNA hypertensive rat without further study of SERT function across the blood brain barrier.

The fact that 5-HT infusion lowered blood pressure in the DOCA-salt rat (when delivered over 7 days) (Diaz *et. al.* 2008) suggests that 5-HT is able to lower blood pressure despite a dysfunctional SERT protein. Thus, it may be possible that excess levels are taken up by alternative monoamine transporters (NET and/or DAT) enabling a nitric oxide-mediated fall in total peripheral resistance and a significant fall in blood pressure.

However, in the L-NNA hypertensive rat, 5-HT did not lower blood pressure. Thus, while a dysfunctional SERT protein did not prevent a 5-HT-induced fall in blood pressure in the DOCA-salt rat, these results suggest that in the absence of nitric oxide synthase (NOS) and NO production that a 5-HT-induced fall in blood pressure is not possible with or without a functional SERT. Collectively these data suggest and important association between SERT and NOS.

5-HT and nitric oxide (NO)

Our laboratory has previously demonstrated an absolute dependence on NOS and likely NO production in enabling a 5-HT-induced fall in blood pressure (Diaz *et. al.* 2008). Additionally, we also demonstrated that SERT is an important component in enabling a 5-HT-induced fall in blood pressure. Thus, the hypothesis put forth regarding SERT and nNOS will need to be explored further given the direct connection between 5-HT uptake *via* SERT and nNOS activation (Chanrion *et. al.* 2007). This hypothesis suggests that 5-HT uptake *via* SERT leads to an allosteric change in nNOS, increasing its affinity for calmoudulin, resulting in its activation, and likely production of NO. This would likely result in a reduction in vascular resistance, and the observed 5-HT-induced fall in blood pressure.

However, another possibility exists. Investigators demonstrated that pretreatment with L-NAME (10 mg/kg) completely abolished a 5-HT_{1A} receptor mediated prejunctional inhibition of an electrically stimulated pressor response in the diabetic

pithed rat (Garcia *et. al.* 2006). This study suggests an important association between 5-HT and nitric oxide (NO) in mediating a prejunctional inhibitory effect in the diabetic pithed rat. Thus, it may still be possible that 5-HT is acting through a prejunctional inhibitory mechanism and that this inhibition is dependent on NO synthesis, despite the results obtained in the superior mesenteric artery following EFS.

This study raises questions about what differs between our study (which concluded 5-HT does not inhibit a prejunctional pressor response in the superior mesenteric artery) *vs.* the study conducted by Garcia *et. al*. One possibility is that the technique of pithing and/or stimulation of the entire sympathetic outflow allowed these investigators to uncover a 5-HT mediated prejunctional inhibition, which we were unable to see in our isolated superior mesenteric artery preparation. Alternatively, these studies utilized a diabetic rat model. Thus, the neurovascular complications of diabetes may have contributed to alternate findings compared to our study.

Free plasma 5-HT levels

Lastly, a table has been constructed comparing free plasma 5-HT levels in various animal models infused with 5-HT (**Table 1**). In the original 7-day study, free plasma 5-HT in the sham rat was greater following 5-HT infusion, compared to plasma 5-HT in the DOCA-salt following vehicle infusion (Diaz *et. al.* 2008). This comparison strongly suggests against the original hypothesis that elevated levels of free 5-HT are acting to elevate blood pressure as elevated levels in the sham rat did not raise blood pressure but rather lowered blood pressure. Moreover, this allows us to hypothesize

that elevated levels of free plasma 5-HT may serve as a brake to increasing blood pressure rather than a driver of elevated pressures.

Table 2: Free plasma 5-HT levels

	Platelet Poor Plasma (PPP)	
Model	**Veh (ng/ml)**	**5-HT (ng/ml)**
DOCA-salt rat (one month infusion in established rats)	87.9 ± 43.9	282.9 ± 48.7
DOCA-salt rat (one month infusion prior to initiating DOCA-salt hypertension)	13.3 ± 3.9	177.1 ± 10.9
	Baseline (ng/ml)	**5-HT (ng/ml)**
Male SERT WT rat (7 day infusion)	8 ± 1.3	61 ± 16.2
Male SERT KO rat (7 day infusion)	1 ± 0.5	37 ± 24.2
Female SERT WT rat (7 day infusion)	4 ± 0.6	62 ± 8.4
Female SERT KO rat (7 day infusion)	2 ± 1.0	23 ± 7.3
	Veh (ng/ml)	**5-HT (ng/ml)**
Sham (7 days infusion)	3 ± 0.3	47 ± 23.2
DOCA-salt (7 day infusion in established rats)	25 ± 5.1	138 ± 35.4

Conclusions

The present studies provide us with several important findings that will aide in unraveling the exact mechanism of a 5-HT-induced fall in blood pressure. These studies demonstrated 5-HT is capable of reducing peripheral vascular resistance (TPR). In the future, it will be critical to identify the exact vascular bed in which resistance falls. This will enable future studies to determine whether 5-HT is acting through direct receptor mediated vascular relaxation or through a sympathoinhibitory mechanism (**Figure 1)**. Additionally, this work defined an important role for SERT in enabling a 5-HT-induced fall in blood pressure and thus understanding how SERT and NOS interact will be another critical component to understanding the exact mechanism of a 5-HT-induced fall in blood pressure.

Figure 6.1

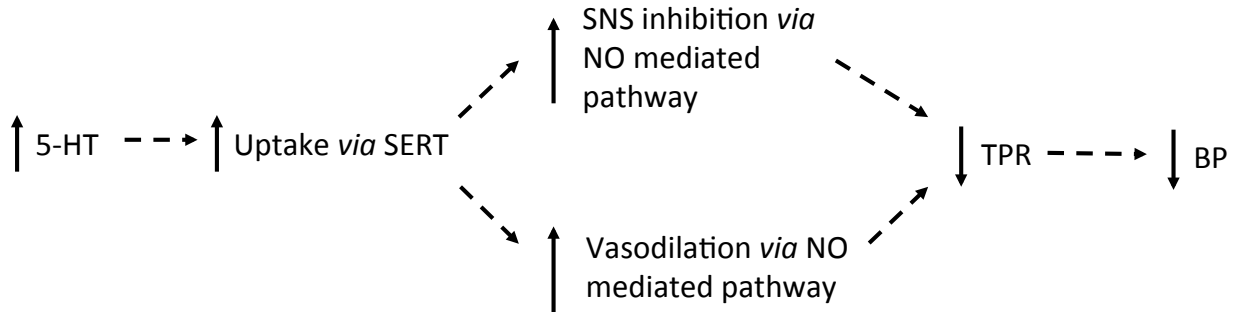

Figure 6.1: Schematic diagram of current hypothesis. (TPR) represents total peripheral resistance. (SNS) represents sympathetic nervous system. (BP) represents blood pressure

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