THE SYSTEMIC AND REGIONAL HEMODYNAMIC CHANGES RESPONSIBLE FOR 5-HT_7 RECEPTOR MEDIATED HYPOTENSION

By

Bridget Mahon Seitz

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Pharmacology and Toxicology––Doctor of Philosophy

2019

ABSTRACT

THE SYSTEMIC AND REGIONAL HEMODYNAMIC CHANGES RESPONSIBLE FOR 5-HT_7 RECEPTOR MEDIATED HYPOTENSION

By

Bridget Mahon Seitz

Serotonin (5-hydroxytrypamine; 5-HT) is known to cause complex dose-dependent effects within the cardiovascular system by altering vascular resistance and arterial pressure resulting in pressor or depressor outcomes. Increased circulating levels of 5-HT has been reported in chronic cardiovascular diseases. Therefore, it is important to understand the mechanisms by which 5-HT regulates cardiovascular system function under chronic conditions. Previous work in our laboratory found low doses of infused 5-HT causes a sustain fall in arterial pressure. The focus of my work was to understand the hemodynamic mechanisms that cause 5-HT-induced hypotension. A significant finding from my work was determining the activated 5-HT_7 receptor is essential in mediating the chronic fall in arterial pressure with low doses of 5-HT. Rats lacking a functional 5-HT_7 receptor (pharmacologically or genetically removed) resulted in no 5-HT-induced observed depressor response. At the systemic hemodynamic level, a decrease in total peripheral resistance (TPR) during 5-HT infusion was the result of an elevation in skeletal muscle blood flow mediated by the 5-HT_7 receptor, which was measured with Doppler flow probes. Additionally, administration of 5-HT infusion relaxed splanchnic veins, via 5-HT_7 receptor stimulation, when measured using novel imaging methodology. An increase in vascular capacitance from splanchnic venodilation is expected to affect arterial pressure by decreasing stroke volume (SV) and cardiac

output. However, both SV and CO were elevated from the start and throughout the duration of 5-HT infusion, indicating that changes in vascular capacitance were unlikely to contribute to chronic 5-HT-hypotension. Previous evidence suggested chronic (but not acute) 5-HT-induced hypotension was dependent on activation of nitric oxide synthase. However, the magnitude of the pressor response to a nitric oxide synthase inhibitor was not significantly different between the control or 5-HT infused groups, concluding 5-HT-induced nitric oxide synthase may not be contributing. Collectively, my work provides insight into the unique cardiovascular pharmacology of the 5-HT_7 receptor, a member of the 5-HT receptor family whose chronic cardiovascular effects have been little studied up to now. Future work should include: characterizing the impact of 5-HT_7 receptor activation by endogenous 5-HT on chronic cardiovascular diseases such as hypertension and heart failure; and evaluating whether it is possible to capitalize on the unique cardiovascular effects of 5-HT_7 receptor stimulation for therapeutic purposes.

ACKNOWLEDGEMENTS

I am very grateful to the many outstanding scientists, professors and colleagues who have guided and inspired me throughout the pursuit of my doctoral degree. Each one of you have enriched my life and made my years in graduate school so rewarding and fun. There are several that deserve special mention for their contributions to this dissertation work.

To my mentor, Dr. Stephanie Watts, thank you for being a role model. Your passion for science, your work ethic, constant cheerleader for others and generosity have inspired me over the past 13 years. The level of devotion you give to every lab member and your enthusiasm for their science, makes it so rewarding to be a part of your lab. I will always remember singing our hearts out together at Council – to Earth, Wind and Fire.

Thank you to my co-mentor, Dr Greg Fink, for always challenging me to think deeply about my science. I am grateful for your endless patience and enthusiasm for my research, as well as, your willingness to come to the basement, roll up your sleeves and teach challenging in vivo techniques. Your generosity to share your knowledge through countless hours of great cardiovascular banter, at the hallway table, are some of my favorite memories from graduate school. I have learned so much and could not have achieved this degree without your encouragement and guidance.

I would also like to thank my committee members Drs Teresa Krieger-Burke and Jim Luyendyk for their time, support and invaluable input to the pursuit of this degree

Thank you to all the members of the Watts lab for making these past years so much fun. A special thanks to my wonderful in vivo colleague, Hannah Garver. The million hours spent in the surgical suite would have not been the same without you.

My sincere thank you to the Pharmacology and Toxicology Department for granting me the opportunity to finish my Ph.D. You have provided me a tremendous graduate education, which I am so proud of. A heartfelt thank you to Dr. Anne Dorrance, I truly appreciate your dedication to support students through their graduate career. The graduate program is so lucky to have you leading the charge.

Lastly, no one has been more supportive to me in the pursuit of this degree than my family. To my three wonderful children: Mitchell, Natalie and Cameron, thank you for your love, understanding and best of all your laughter. Being your Mom will always be my favorite job. Every day you all continue to inspire me. To my best friend and husband, Ted, I am incredibly grateful for your unwavering support. Your patience and love have been the glue that has kept the pieces together all these years. Thank you!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
Ab A	abdominal aorta
Ab IVC	abdominal inferior vena cava
CO	cardiac output
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated protein 9
DOCA	deoxycorticosterone acetate
EF	ejection fraction
ET-1	endothelin-1
iv	intravenous infusion
KO	knock-out
LNAME	N(G)-Nitro-L-arginine-methyl ester, nitric oxide synthase inhibitor
LNNA	N(omega)-nitro-L-arginine, nitric oxide synthase inhibitor
MAP	mean arterial pressure
NO	nitric oxide
NOS	nitric oxide synthase
PV	portal vein
SB269970	(2*R*)-1-[(3-Hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride, 5-HT_7 receptor antagonist
sc	subcutaneous injection

SEM	standard error of the mean
SMV	superior mesenteric vein
SV	stroke volume
TPR	total peripheral resistance
TVC	thoracic vena cava
WT	wild-type

Chapter 1

Introduction

Administration of acute 5-HT to humans and experimental animals is well known to cause complex, dose-dependent changes in the cardiovascular system function through direct and indirect actions on the heart, blood vessels, brain and autonomic nervous system (Cade et al., 1992; Davis et al., 2012, 2013; Diaz et al., 2008; Kaumann and Levy, 2006; Page and McCubbin, 1953). However, the cardiovascular mechanism underlying the involvement of 5-HT is vague. Through years of work in our laboratory, we have found chronic, low dose 5-HT infusion leads to a significant fall in mean arterial pressure (MAP) not only in normotensive rats, but also in mineralocorticoid-dependent hypertensive rats (> 50 mmHg fall from baseline; Diaz et al., 2008) and in spontaneously hypertensive rats (fall in MAP ~30 mm Hg; Watts et al., 2012). Linking the mechanism(s) that govern chronic 5-HT infusion to the long-term cardiovascular response could help illuminate the possible roles of circulating 5-HT under both normal and pathological condition as well as, provide insight to potential of drug therapies for the treatment of cardiovascular disease, such as hypertension and heart failure. Therefore, the focus of my work is to investigate within the cardiovascular system, the possibly novel mechanism(s) that contribute to chronic 5-HT-induced hypotension,

Cardiovascular disease, with a focus on hypertension

Hypertension is the world's leading risk factor for cardiovascular disease, disability, and death (Ettahad et al., 2016). Most recently, hypertension was redefined as a blood pressure recording of 130/80 mmHg (Whelton et al., 2018) compared to the previous 140/90 mmHg. This change in the definition of high blood pressure has resulted in nearly half of the U.S. population as being hypertensive (McClellan et al.,

2019). This is substantial, as hypertension plays a major etiologic role in the development of other cardiovascular conditions. Even small increments in blood pressure are associated with increased target-organ morbid outcomes, such as stroke, coronary artery disease, renal disease, peripheral vascular disease, and heart failure (Go et al., 2014). Therefore, treatment and control of hypertension is critically important for the prevention of consequent cardiovascular and kidney diseases. Yet, even with the increase in morbidly and morality attributed to elevated blood pressure, cardiovascular drug development has lagged (Frodyce et al., 2015). Reducing systolic blood pressure by 10mmHg slashes the risk of coronary heart disease by 22% and stroke by 41% (Law et al., 2009). Along with diet and physical exercise modification, new and novel therapies for the management of hypertension is a missed opportunity at battling this ever-growing burden.

Determinates of blood pressure

To assist in understanding the possible mechanism(s) 5-HT could employ to decrease MAP, a brief overview of blood pressure regulation and the circulatory system is beneficial. The reason being arterial pressure regulation is complex and encompasses several processes that control circulating blood volume, the vasculature, and the function of the heart.

The time-averaged hydraulic pressure within the arterial system, (MAP), is described as the product of two hemodynamic variables, one directly measured and the other derived from a calculated equation; MAP = cardiac output (CO) times total peripheral resistance (TPR) (Cowley AW, 1992). CO is measured directly as the volume of blood expelled from the left ventricle (heart) during systolic contraction per unit time.

CO is the product of stroke volume and heart rate (HR). TPR is a calculated variable reflecting the amount of force opposing the flow of blood through the circulation. The most important of these forces generating TPR are those that alter the vascular diameter of small arteries and arterioles (Pang, 2000). Together these opposing forces, CO and TPR, apply pressure on the walls of the arteries. Any changes to CO and/or TPR will result in a change in overall MAP. To put these hemodynamic variables into perspective (MAP, CO, and TPR) a quick summary of the cardiovascular system with a focus on the arteries and veins is useful.

The cardiovascular system: structure and function of arteries and veins in determining blood pressure

The peripheral circulation is a semi-closed (excluding the pulmonary circulation), blood-filled circuit made up of a high pressure arterial side and a low pressure venous side arranged in series. The arterial and venous circulations operate in tandem. Both are regulated by humeral and neuronal factors, which assist in generating the overall homeostasis of the circulatory system. The major role of the arteries is to deliver oxygenated blood from the heart to the peripheral organs such as the brain, viscera and muscle. Also, within the arterial circulation resides the control for the regional distribution of blood flow. This occurs in small arteries and arterioles, by altering the diameter of the lumen via changes in the tone of the vascular smooth muscle located in the walls of these vessels (Cowley, 1992). Arterial resistance is 7-10 times greater than venous resistance (Green,1982). Thus, arterial tone determines TPR.

In contrast, the primary role of the venous system is to gather deoxygenated blood and return it to the heart (venous return, VR). Nearly 70% of total blood volume

resides in the venous vasculature (Rothe, 1983). The bulk of this volume is held in the small veins and venules (Pang, 2000). To understand the contribution of differences in venous volume affecting MAP, the venous system can be divided into two compartments: central and peripheral. Importantly, veins within these two compartments are not equal in their compliance or capacitance (Pang, 2000). Compliance is defined as the capability of the vessel to expand and increase volume without increasing transmural pressure i.e. the difference between intraluminal and extramural pressure (Geldman, 2008). Capacitance reflects the total volume of blood contained at a given pressure (Rothe, 1983).

Veins in the peripheral venous compartment are highly compliant and maintain high capacitance, especially, veins within the splanchnic vascular bed (liver, spleen, small and large intestines). Splanchnic veins represent the largest blood volume reservoir within the body (Pang, 2000). This particular venous bed receives approximately 25% of cardiac output and contains approximately 20% of total blood volume (Rothe, 1983; Roswell, 1990). The splanchnic veins are 30 times more compliant than arteries, making them capable of holding relatively large volumes of blood without a change in their intraluminal pressure (Greenway, 1982). Therefore, changes in the capacitance of the veins in this region, by changing their diameter (through constriction or dilation) can easily (increase or decrease) blood volume to the heart; changing the filling pressure; and ultimately affecting MAP.

In contrast, veins found in the central compartment are considerably less compliant. The thoracic vena cava and the great veins in the chest are unable to hold blood volume to any extent. However, it is the veins within the central compartment that

are critical for circulatory dynamics as they determine the level of resistance to venous return (R_{VR}) (Henderson, 2010). R_{VR} is the resistance that needs to be opposed to shift blood from the peripheral to central venous bed on to the heart. The resistance generated in the central venous compartment can be effected by specific events, such as, the passive narrowing of the large veins; by an increase in the viscosity of the blood; passively by circulating vasoactive mediators; or by a receptor-activated contractile response. To appreciate the significance of how changes in R_{VR} can affect and modulate CO, the following are a few examples. An increase in R_{VR} will result in a decrease in the amount of blood returned to the heart (VR). A decrease in R_{VR} will result in an increase in the amount of blood returned to the heart (VR). The heart only pumps out the blood to which it receives from the venous side (VR) into the arterial bed (CO). Therefore, all other things being equal, an increase in R_{VR} will decrease CO and MAP (Gelman, 2008).

The cutaneous veins are categorized as being highly compliant and possess great capacitance capabilities (Pang, 200)). However, cutaneous veins are mainly controlled by changes in body temperature (Rothe, 1983). This suggests their role in blood pressure regulation is mostly limited to times when the ambient temperature is above or below the body temperature. Also, veins that supply blood to the skeletal muscle and extremities are less compliant and maintain little to no capacitance. These veins are filled and emptied by passive forces associated with gravity or external compression by surrounding tissues, such as seen with muscle contraction.

The take away message in this brief overview of the circulatory system is that both arteries and veins affect the regulation of blood pressure. The arteries function to

control both resistance and flow distribution through the circulation. The peripheral veins, specifically the veins and venules in the splanchnic region, function as principle intravascular blood volume reservoirs determined by capacitance and volume. Lastly, the veins within the central compartment function as the primary site of R_{VR} (Gelman, 2008).

The effects of the arteries, heart, brain, kidneys and adrenals in determining blood pressure

Changes in peripheral resistance and cardiac function occur routinely during the course of our normal daily lives. These functions are important for the body to maintain an adequate MAP to allow for sufficient blood flow to the brain and other tissues. Arterial pressure is maintained within a narrow range and is tightly regulated. Various sensors located in the body continuously monitor the pressure in the arterial system. When arterial pressure varies from the "normal" homeostatic set-point, reflexes responses are initiated causing adjustments of TPR and CO needed to return arterial pressure to its normal value. Changes in arterial pressure (P) are determined by flow (Q) and resistance (R) through the arterial system ($\Delta P=Q \times R$). Mechanism that stabilize arterial pressure are different for short term (seconds to minutes) regulation versus long term (minutes to days) regulation. Rapid stabilization of arterial pressure (within seconds), which can occur for example, through changes in posture is coordinated largely by the autonomic nervous system. This occurs by neuronally mediated baroreceptor, located in the arterial walls of the carotid sinus and aortic arch, which sense changes in the arterial blood pressure. When arterial pressure is high, there is an increase in firing of the baroreceptor. Conversely, when arterial pressure is low, the

baroreceptor firing is reduced. The input of the baroreceptor is sent to the vasomotor center located in the medulla and pons of the brain. This afferent input stimulates the autonomic nervous system to activate the sympathetic nervous system (SNS) or the parasympathetic nervous system (PNS) depending on if the arterial pressure is sensed above or below “normal”. In contrast, via the vagus nerve, the PNS functionally innervates the heart to reduce HR, when the arterial pressure is above normal. This results in a reduction in CO and MAP.

The long-term control of arterial pressure (minutes to days) is regulated by the adjustment of blood volume by the kidneys through the renal-pressure natriuresis mechanism. The primary component of this feedback mechanism is to increase or decrease sodium and water excretion by the kidneys based on changes in arterial pressure. When arterial pressure increases above the “normal” homeostatic set point, sodium and water excretion also increase. As long as excretion of sodium and water exceeds intake, the extracellular blood volume will continue to decrease which lowers mean circulatory filling pressure, VR, CO and overall MAP. Conversely, when arterial pressure is low, the kidneys retain fluid until arterial pressure is restored to normal.

The arterial natriuresis mechanism is not involved in the moment-to-moment regulation of arterial pressure as seen with the autonomic nervous system. However, it is extremely powerful for long-term blood pressure control, especially when combined with the renin-angiotensin system (RAS). RAS is hormonally a regulated system, which amplifies the arterial natriuresis system by regulating arterial pressure and volume homeostasis, by releasing hormones from the kidney, liver, and lung. ANG II is the final and major bioactive component of the RAS. It is a potent vasoconstrictor of arterioles.

ANG II influences sodium excretion through several intrarenal actions, as well as, extrarenal actions. The most important intrarenal effects by ANG II is direct stimulation of renal tubular transport of sodium and water excretion. The extrarenal effects of ANG II is to release aldosterone from the adrenal gland to increases the reabsorption of sodium and water; act on the central nervous system to release vasopressin; and activate the SNS.

The effects of the venous circulation on blood pressure

It seems reasonable that changes in the intrinsic cardiac function would be a major determinate of arterial pressure, as outlined above. However, regulation of arterial pressure does not necessarily depend on the steady-state flow of blood through the arterial system (CO), which is generated by the pumping ability of the heart. Instead the product of vascular compliance and steady state changes in arterial blood volume determines pressure in the arterial system. The latter being dependent on transient differences in arterial system inflow (determined by CO regulated by actions on the venous side) and outflow (determined by TPR regulated by action on the arterial side) (Henderson, 2010). Simply put, arterial pressure is determined by factors largely peripheral to the heart. In simplistic terms, the heart functions merely as a “demand” pump, dispensing nearly all the blood it receives back into the arterial system with each contraction. Therefore, vascular factors that regulate the volume of blood the heart receives and then pumps into the arterial compartment, can have a powerful effect on arterial pressure regulation. It is well established differences in blood volume within distinct segments of the circulatory system account for the pulsatile nature of the arterial pressure generated by the cyclic heart contraction (systole) and relaxation (diastole)

(Gelman, 2008). However, differences in blood volume within distinct regional segments of the circulation, such as splanchnic and skeletal, are less often recognized as a major factor in the overall determination of arterial blood pressure.

In this framework, critical determinants of arterial pressure are 1) resistance to blood flow through the circulation and 2) the degree of filling of the circulatory system with blood. One commonly employed measure of circulatory system filling is the mean circulatory filling pressure (MCFP; Guyton, 1968). MCFP reflects the pressure measured when the heart has been stopped and blood rapidly redistributes between the arterial and venous beds creating pressure equilibrium throughout the cardiovascular system (Magder,2016). MCFP is a function of the volume of blood and the capacitance/compliance of the system. MCFP represents the distending pressure in the small veins and venules, where most of the total blood volume is held. Veins are 30–50 times more compliant than arteries (Pang, 2000). When blood volume is assumed to be relatively constant, changes in MCFP provide a useful index of the changes in overall venous smooth muscle tone.

When MCFP is zero, the volume of blood that remains in the cardiovascular system is termed unstressed volume (Magder, 2016). This makes up the blood volume held in the capacitance vessels (in the splanchnic region) in reservoir and cannot be easily measured directly. The bulk of the blood volume is unstressed and held in the small veins and venules. In contrast, the stressed volume is the blood volume generating the distending pressure on the vascular walls and can be calculated from measures of blood volume and pressure. The stressed volume makes up only 25% of total blood volume. (Rothe, 1983). However, it is critical to blood pressure regulation

because it reflects the components of circulating volume in the vasculature that contributes to blood pressure. An increase in stressed blood volume, e.g. via venoconstriction of small veins and venules, will increase MCFP. On the other hand, an increase in vascular capacitance via venodilation will decrease MCFP and stressed blood volume. Changes in venous capacitance are another important way (in addition to altering R_{VR}) that changes in venous tone can modulate CO and MAP.

Background: 5-HT synthesis and metabolism

Serotonin (5-hydroxytrypamine; 5-HT) is predominately synthesized by the enterochromaffin cells (~95%) within the gastrointestinal tract and to a lesser extent in the raphe nucleus (10%) of the central nervous system (Erspamer and Asero, 1952; Dahlstroem and Fuxe, 1964). The biosynthesis of 5-HT starts from the essential amino acid, L-tryptophan. The first step in the 5-HT synthesis pathway is catalyzed by the rate limiting enzyme, tryptophan hydroxylase (TPH). Two genes encode TPH: TPH-1 in the peripheral tissue, which synthesizes 5-HT within the periphery; and TPH-2 in the central nervous system, which synthesizes 5-HT within the brain (Cote et al., 2003; Walther et al., 2003). It is generally thought that 5-HT does not cross the blood-brain barrier, and thus the two pools of this molecule each have their own distinct functions (Berger et al., 2009).

The physiological actions of 5-HT are terminated by removal of 5-HT from the extracellular fluid by the serotonin transporters (SERT), which is in the plasma membrane of serotonergic neurons and platelets (Jedlitschky et al., 2012). 5-HT is also metabolized by the liver and the lung. Monoamine oxidase (MAO), found in the liver and lung convert 5-HT to the metabolite, 5-hydroxy-indoleacetic acid (5-HIAA), via first pass

metabolism prior to entering the general circulation (Ayme-Dietrich et al., 2017). Chemical analysis of urine can reveal 5-HIAA levels in the body. Altered 5-HIAA levels in the urine is a common biomarker in the diagnosis of carcinoid tumors, which are slow-growing neuroendocrine tumors derived from the enterochromaffin cells (Feldman, 1986).

5-HT is also predominately stored, but not synthesized, in the dense granules within the platelets. The platelets maintain the richest source of 5-HT in the body (Berger et al., 2009). These removal (SERT, MAO), and storage (platelets) mechanisms, normally maintain the level of circulating 5-HT to unappreciable amount in the plasma (~2% of total body 5-HT), which is estimated to be in the nanomolar range (3- 20 ng/ml) (El-Merahbi et al., 2015). In the platelets, the estimated free 5-HT concentration is in the millimolar range (Monaghan PJ et al., 2009). Circulating levels of 5-HT is endogenously increased by events that fractures the platelets such as thrombotic events and platelet aggregation. Therefore, it is worth mentioning the difficulty in accurately measuring free circulating 5-HT in the plasma. This is due to the nearly unavoidable contamination from ruptured platelets during blood sampling. (Brand and Anderson, 2011). Techniques such as high-performance liquid chromatography and mass spectrometry have improved this problem and are being implemented for detailed analysis of 5-HT in the blood. (Szeitz and Bandiera, 2017).

The physiological actions of 5-HT are mediated by seven major receptor subtypes ($5\text{-}HT_1$ - $5\text{-}HT_7$). The $5\text{-}HT_3$ receptor is a ligand-gated ion channel, whereas the others are classified as G-protein coupled receptors (GPCR). The GPCR are further classified based on their internal signaling mechanism: Gi for $5\text{-}HT_{1/5}$, Gs for $5\text{-}HT_{4/6/7}$

and Gq for 5-HT_2 (Berger et al., 2009; Noda M et al., 2004). These receptors are located on vascular smooth muscle cells, endothelial cells, autonomic nerve endings, and in the central nervous system. 5-HT regulates many biological processes in several major organ systems including the cardiovascular, pulmonary, gastrointestinal, and endocrine (Roth, 2007). This abundance of physiological actions by 5-HT is supported by the numerous receptors the serotonergic amine can activate.

Cardiovascular pharmacology of exogenous 5-HT in the periphery

Administration of 5-HT into the peripheral circulation of human and experimental animals is well documented to cause both pressor and depressor responses in the cardiovascular system (Cade et al., 1992; Diaz et al., 2008; Kaufmann and Levy, 2006; Page and McCubbin, 1953; Villalon and Centurion, 2007). 5-HT can regulate vascular tone through the contractions and relaxation of blood vessels (De Vries et al., 1999; Saxena and Villalon, 1990, 1991). 5-HT can stimulate HR. This can could occur by activation of the baroreceptor reflex or direct 5-HT receptor activation on the heart, 5-HT_{2A} (rats) and 5-HT_4 (humans) (Cote et al., 2004; Kaumann and Levy, 2006). In the kidney, 5-HT causes contract of the renal artery (Watts and Thompson, 2004) and reduction of sodium excretion (Sole et al.,1986). Both mechanisms support the increase in blood pressure. In the adrenal gland, 5-HT can stimulate the release of epinephrine (Contesse et al., 1999) and aldosterone (Bagdy et al.,1989). 5-HT can act centrally to regulate the cardiovascular reflexes by responding to afferent input to changes in MAP and HR. (Ramage and Villalon, 2008). My focus will be on the cardiovascular actions of 5-HT in the periphery.

These opposing physiological effects of 5-HT are derived by the 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_4 and 5-HT_7 receptors found on the vascular smooth muscle cells and the endothelial cells (Cote et al., 2004; Hoyer et al.,1994, 2002). The overall vascular response is dose dependent and relies on the affinity, signaling mechanism and location of the receptor (Berger et al., 2009; Kaumann and Levy, 2006).

The vasopressor response to 5-HT is mediated primarily by 5-HT_{2A} and 5-HT_{1B} receptors (Ramage and Villalon, 2008). These receptors are found on the smooth muscle of arteries (human; coronary, pulmonary, temporal, mesenteric, internal mammary) and veins (humans; pulmonary, hand, saphenous umbilical) and coronary artery (5-HT_{2A} only) (Hoyer et al., 2002; Kaumann et al., 1993). The contractile response in isolated blood vessels can be blocked by ketanserin (5-HT_{2A} antagonist) and stimulated by the 5-HT_{1B} receptor agonist (sumatriptan) (Yildiz et al., 1998). The vasoconstrictor effects of 5-HT_{2A} receptors are markedly potentiated in experimental hypertensive rats. (Banes and Watts, 2003).

5-HT can also cause vasodepressor responses by dilating peripheral blood vessels. The 5-HT_{1B} and 5-HT_{2B} receptors located on the endothelium and the 5-HT_7 receptor (also 5-HT_{1B}) located on smooth muscle cells have been linked to vascular relaxation (Ulmer et al.,1995; Terron,1997). The 5-HT_{2B} is linked to activation of endothelial nitric oxide synthase (eNOS) contributing to the depressor effects relevant to this receptor (Ramage and Villalon, 2008). However, the exact role of the 5-HT_{2B} receptor to the hypotensive response remains in question for the following reasons: the receptor was upregulated in the smooth muscle cells of hypertensive rats and functioned as a constrictor in isolated blood vessels (Banes and Watts, 2003).

The 5-HT_7 receptor which was the last 5-HT receptor cloned (1993) contributes to 5-HT-induced hypotension, at least acutely (Centurion et al., 2004; Saxena et al., 1998). In anesthetized, pithed rats an acute infusion of 5-HT reduced MAP by a receptor with similar pharmacological profile to the 5-HT_7 receptor (Terron,1997). The cardiovascular effects of chronic (>1 hour) activation of the 5-HT_7 receptor has yet to be investigated. However, the 5-HT_7 receptor is expressed in a variety of human and animal tissues, particularly in the brain, the gastrointestinal tract, and in various blood vessels (Hedlund and Sutcliffe, 2004; Ramage and Villalon, 2008).

The acute cardiovascular effects of endogenous 5-HT on blood pressure

5-HT was first identified in the cardiovascular system (Page and McCubbin, 1953). For decades, it has been recognized that 5-HT lowers blood pressure, when given acutely (< 1 hour duration) (Dalton et al., 1986; Terron, 1997), yet the mechanism is undefined. In determining the acute mechanism for 5-HT-induced hypotension we placed our focus within the splanchnic vascular region testing a subset of arteries, veins, and the sympathetic nervous system, which innervates these vessels. Through a series of in vitro experiments, we discovered: 1) Isolated superior mesenteric artery and mesenteric resistance artery (<200 microns) did not relax, with the addition of 5-HT (Davis et al., 2012). 2) Direct interaction of 5-HT within the splanchnic sympathetic nervous system, at the level of the preganglionic and postganglionic nerve, as well as, at the neuroeffector junction did not alter the depressor response to 5-HT (Darios et al., 2015). 3) The superior mesenteric vein (SMV) however directly relaxed to 5-HT (Watts et al., 2012). This occurred even without having to mask the contractile 5-HT_{2A} receptor. It is important to note, in this study, the expression of relaxant 5-HT receptor subtypes,

(5-HT_{1B}, 5-HT_{2B}, and 5-$HT_{7)}$, using RT-PCR, immunohistochemistry and Western analyses, were all shown to be present in the SMV (Watts et al., 2015). However, it was the 5-HT_7 receptor antagonists (SB 269970 and LY215840) but not the 5-HT_{2B} receptor antagonist (LY272015) that abolished the serotonergic-induced relaxation of the isolated SMV.

In additipon, activation of the 5-HT_7 receptor caused splanchnic venous relaxation, in vitro (Watts et al., 2015). Thus, suggesting an increase in venous capacitance as a possible mechanism for 5-HT_7 receptor mediated fall in MAP. A measurement of splanchnic venous diameter (a surrogate for venous capacitance) and cardiac output would be necessary to confirm if an increase in venous capacitance occurs and contributes to the depressor response.

The chronic cardiovascular effects of exogenous 5-HT on blood pressure

Years of work in our laboratory has focused on understanding the cardiovascular effects of 5-HT under chronic (> 24 hours) conditions in the hope of providing insight to a new and novel cardiovascular disease therapy. Connecting 5-HT to long-term cardiovascular control and perhaps pathophysiological states (e.g. hypertension or heart failure) requires an understanding of the hormone's more chronic effects. It is unclear if the mechanism(s) by which chronic (days to weeks) 5-HT affects cardiovascular hemodynamics is the same as those responsible for the acute responses. Acute hormone actions are likely to be modified under chronic conditions by mechanisms such as receptor down-regulation, receptor desensitization, and physiological compensation (e.g. baroreflex).

In pursuit of a chronic mechanism, we found infusion of 5-HT (25 ug/kg/min) into conscious rats caused a significant fall in MAP, which was dose-dependent (Tan et al., 2011), similar in magnitude in males and females (Davis et al., 2011) and sustained for nearly 30-day infusion timeframe. (Davis et al., 2013). Most importantly, using the same low dose of 5-HT (25 ug/kg/min) over a one-week infusion, dramatically reduced MAP not only in normotensive rats (Davis et al., 2012, 2013; Diaz et al., 2008) but experimentally hypertensive rats (Diaz et al., 2008, Watts et al., 2012). The protocol used in these experiments is a low dose infusion of 5-HT (25 ug/kg/min) via an osmotic mini-pump (ALZET) into conscious, radiotelemetery implanted rats. This has become the established protocol that we will use to investigate the mechanism(s) of chronic 5-HT-induced hypotension. Using this protocol, we have shown the fall in MAP in the presence of 5-HT is associated with an increase CO and decrease TPR (Davis et al., 2012). This unique hemodynamic pattern observed during low dose 5-HT infusion could be reflective of the distinct pharmacology of serotonin. Therefore, possible mechanisms that might contribute to 5-HT-induced hypotension are as follows: 1). Dilation of the arterioles to decrease TPR; 2). An increase in venous capacitance to decrease MCFP; 3). Decrease in the resistance to venous return due to changes in the diameter of the large central “conduit” veins.; and 4). Potential activation of endogenous vasodilator (e.g. nitric oxide).

5-HT and nitric oxide synthase

To address the possibility of nitric oxide contributing to chronic depressor response, we have shown blockade of nitric oxide (NO) formation with the nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine (L-NNA), completely prevented the 5-HT-

induced hypotension (Diaz et al., 2008). In this experiment, a non-selective inhibitor of NOS was given in the drinking water for 10 days to create LNNA hypertensive rats. We conclude from these results the fall in MAP to 5-HT is NO-dependent, because inhibiting NO formation by blocking NOS resulted in an increase in MAP.

It is possible chronic 5-HT infusion increases NO formation. An expected outcome of increase NO release would result in a decrease in TPR and an increase CO (Bolognesi et al., 2014; Colombato et al., 1991; Lee et al., 1993). This is the exact hemodynamic pattern observed during one-week infusion of 5-HT-induced hypotension: a fall in TPR with an increase in CO. A quick overview of nitric oxide formation helps secure this idea.

NO is produced from the amino acid L-arginine and oxygen by the enzymatic action of NOS (Moncada et al., 1991). There are three forms of NOS: inducible NOS (iNSO), neuronal NOS (nNOS), and endothelial NOS (eNOS). The eNOS form is most important to blood pressure regulation, acting as a powerful vasodilator. Vascular endothelial cells contain calcium-dependent constitutive NOS. Under normal basal conditions, NOS synthesizes NO in short bursts to maintain vascular tone to regulate blood pressure. In the cardiovascular system, there are two basic pathways for the stimulation of eNOS, and subsequent release of NO. Both pathways involve the release of calcium ions. One way to activate eNOS is by chemical agonists acting on the endothelial cell including bradykinin, acetylcholine, ATP, adenosine, and platelet-derived serotonin (Andrew and Mayer, 1999). The second way to activate eNOS is flow dependent. Physical agonists such as shear stress and increase in blood flow can mechanically stimulate the release of calcium and activate eNOS (Forstermann and

Sessa, 2012). Once NO is released, regardless of the pathway, it freely diffuses across the blood vessel wall. The diffused NO stimulates guanylyl cyclase found in the vascular smooth muscle to induce the formation of cyclic guanosine monophosphate (cGMP), which leads to relaxation of smooth muscle around the periphery of the blood vessels (Zhao et al., 2015). The blood vessel dilates, allowing greater blood flow to the local vicinity. This response is attributed with a decrease in MAP.

A limitation to our earlier described LNNA data was the experiment was studied in the LNNA hypertensive experimental model. It is possible that the lengthy administration of L-NNA to generate the hypertensive state (10 days) may have completely prevented the chronic depressor action of 5-HT. I will test the contribution of NOS in a more acute (< one hour) timeframe in the presence of 5-HT. A timeframe were vascular remodeling and other long term consequences of blocking NO might yet not occur (Paulis et al. 2008).

The 5-HT_7 receptor mediated 5-HT-induced hypotension: what we know now and what we need to know

Pharmacological evidence supports the acute fall in MAP during 5-HT infusion is mediated by the 5-HT_7 receptors (Centurion et al., 2004; De Vries et al., 1999; Terron, 1997; Villalon and Centurion, 2007). In an earlier study, the acute mechanism for the 5-HT-induced hypotension was attributed to an increase in systemic conductance, which was confined to the skeletal muscle, mesentery/pancreas, adrenals, and carcass vascular bed (De Vries et al.,1999). The increase in systemic conductance was mediated by the 5-HT_7 receptor. It was speculated that dilation of the skeletal muscle arterioles contributed the greatest to the vasodilation of the total systemic vascular,

resulting in the hypotensive response. This idea was considered since the skeletal muscle receives the largest portion of total cardiac output (~30-40%; Pang, 2000).

We took the idea that activation of the 5-HT_7 receptor is responsible for the acute fall in MAP (Centurion et al., 2004; De Vries et al., 1999; Terron, 1997; Villalon and Centurion, 2007) and challenge this idea in the chronic setting (days-week). We asked the question: Is the 5-HT_7 receptor essential beyond the acute (<1 hour) 5-HT-induced hypotension? In addition, what are the hemodynamic mechanism(s) that drive the fall in MAP with chronic 5-HT and are they mediated by the 5-HT_7 receptor? Is the 5-HT_7 receptor constitutively active? Is this receptor essential for basal blood pressure regulation? These are important topics to address considering all the previous work investigating the 5-HT_7 receptor was done in vitro or acutely (< 1 hour) under anesthesia. As well as, the mechanism(s) that drive the depressor response to chronic low dose 5-HT are unknown. These ideas can be addressed by manipulation of the 5-HT_7 receptor in two ways: 1) Pharmacological blockade of the receptor with a 5-HT_7 receptor antagonist. The literature supports, SB269970, as a well-recognized 5-HT_7 receptor antagonist. SB269970 has sub-nM affinity for the 5-HT_7 receptor with 1000X greater affinity for this receptor compared to the other 5-HT receptors (Hagan et al., 2000). 2) Genetic removal of the 5-HT_7 receptor using receptor knockout experimental animals could uncover the potential of this receptor. An established 5-HT_7 receptor knock-out (KO) mouse model already exists (Hedlund et al., 2003). However, the use of a 5-HT_7 receptor KO rat would be more beneficial considering the hemodynamic measurements that need to be accomplished and are better suited for a rat KO model compared to a mouse KO model.

There is one caveat that was observed with 5-HT infusion, however, which needs to be considered. The observed hemodynamic pattern of an increase in CO, in vivo, is not consistent with the splanchnic venous relaxation that was observed with infused 5-HT, in vitro. A possible explanation for this apparent inconsistency, as mentioned previously, is blockade of NO formation by NOS inhibitor, L-NNA, completely prevented the 5-HT-induced hypotension (Diaz et al., 2008). Because there is no evidence linking 5-HT_7 receptors directly with NOS activation, this suggests that 5-HT_7 receptor activation may not be solely responsible for the 5-HT depressor response. An alternative possibility is that activated of the 5-HT_7 receptor in other large vascular beds, such a skeletal muscle could cause direct vasodilation (NOS dependent?) which contributes to the overall hemodynamic response to 5-HT.

Summary

Cardiovascular disease is one of the leading causes of death globally (McClellan et al., 2019). Even though the prevalence of cardiovascular disease is forecasted to increase, drug innovation is lagging (Fordyce et al., 2015). This presents an opportunity for those ready for the challenge. The challenge comes from the complexity of the cardiovascular system and its regulation, as outlined above. To truly understand how 5-HT lowers arterial pressure, knowledge of the primary systemic and regional hemodynamics are needed. The following hypothesis was designed to investigate the mechanism(s) contributing to chronic 5-HT-induced hypotension with an emphasis on the role of the activated 5-HT_7 receptor.

Hypothesis

5-HT lowers blood pressure by inducing a redistribution of blood from the arterial to the splanchnic venous circulation through stimulation of the 5-HT_7 receptor. This leads to a NOS dependent mechanism to sustain 5-HT-induced hypotension.

Specific Aims

Aim 1. Test the hypothesis that 5-HT_7 receptor mediated splanchnic venodilation contribute to 5-HT-induced hypotension.

Aim 2. Test the hypothesis that indirect activation of NOS causes sustained cardiovascular changes during chronic 5-HT infusion.

Aim 3. To determine if the 5-HT_7 receptor contributes to homeostatic cardiovascular regulation.

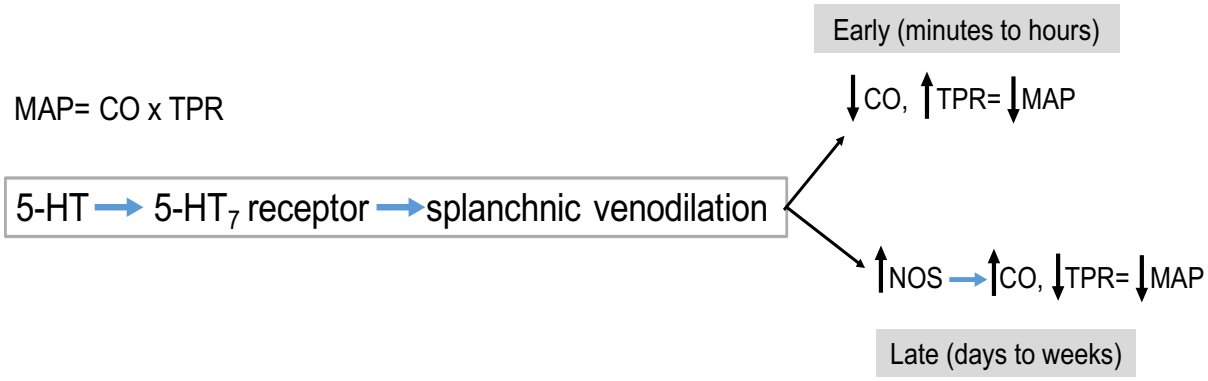


Figure 1.1: Schematic of hypothesis to explain 5-HT induced changes in mean arterial pressure (MAP), cardiac output (CO), and total peripheral resistance (TPR).

REFERENCES

REFERENCES

Andrew PJ, Mayer B. (1999). Enzymatic function of nitric oxide synthase. Cardiovascular Research, 43:521-531.

Ayme-Dietrich E, Aubertin-Kirch G, Maroteaux L, Monassier L. (2017). Cardiovascular remodeling and the peripheral serotonergic system. Archives of Cardiovascular Disease,110:51-59.

Bagdy G, Calogero AE, Murphy DL, and Szemeredi K. (1989). Serotonin agonists cause parallel activation of the sympathoadrenomedullary system and the hypo-thalamo-pituitary-adrenocortical axis in conscious rats. Endocrinology, 125:2664– 2669.

Banes AK, Watts SW. (2003). Arterial expression of 5-HT2B and 5-HT1B receptors during the development of DOCA-salt hypertension. BMC Pharamacol, 3:12.

Berger M., Gray J.A. and Roth B.L. (2009). The expanded biology of serotonin. Annu. Rev. Med, 60:35–366.

Bolognesi M, Di Pascoli M, Verardo A, and Gatta A. (2014). Splanchnic vasodilation and hyperdynamic circulatory syndrome in cirrhosis. World J Gastroenterol., 20(10):2555-63.

Brand T, Anderson GM. (2011). The measurement of platelet-poor plasma serotonin: a systematic review of prior reports and recommendations for improved analysis. Clin Chem, 57:1376–1386.

Brenner B, Harney JT, Ahmed BA, Jeffus BC, Unal R, Mehta JL, Kilic F. (2007). Plasma serotonin levels and the platelet serotonin transporter. J Neurochem, 102:206 –215.

Cade JR, Fregly MJ, Privette M. (1992). Effect of tryptophan and 5-hydroxytryptophan on the blood pressure of patients with mild to moderate hypertension. Amino Acids, 2:133-140.

Centurión D, Glusa E, Sánchez-López A, Valdivia LF, Saxena PR, Villalón CM. (2004). 5-HT7, but not 5-HT2B, receptors mediate hypotension in vagosympathectomized rats. Eur J Pharmacol, 502:239–242.

Colombato LA, Albillos A, Groszmann RJ. (1991). Temporal relationship of peripheral vasodilatation, plasma volume expansion and the hyperdynamic circulatory state in portal-hypertensive rats. Hepatology, 15(2):323-8.

Contesse V, Lenglet S, Grumolato L, Anouar Y, Lihrmann I, Lefebvre H, Delarue C, and Vaudry H. (1999). Pharmacological and molecular characterization of 5-hy-droxytryptamine(7) receptors in the rat adrenal gland. Mol Pharmacol 56:552– 561.

Cote F et al. (2003). Disruption of the nonneuronal tph1 gene demonstrates the importance of peripheral serotonin in cardiac function. Proc Natl Acad Sci, 100:13525-13530.

Cote F, Fligny C, Fromes Y, Mallet J Vodjdani G. (2004). Recent advances in understanding serotonin regulation of cardiovascular function. Trends in Molecular Medicine, 10(5):231238.

Cowley AW. (1997). Long-term control of arterial pressure. Physiol Rev, 72:231-300.

Dahlstroem A, Fuxe K. (1964). Evidence for the existence of monoamine-containing neurons in the central nervous system. Acta Physiol Scand, 232 (Suppl): 1-55.

Dalton DW, Feniuk W, Humphrey PP. (1986). An investigation into the mechanisms of the cardiovascular effects of 5-hydroxytryptamine in conscious normotensive and DOCA-salt hypertensive rats. J Auton Pharmacol, 6:219–228. doi:10.1111/j.1474-8673.1986.tb00648.x.

Darios ES, Barman SM, Orer HS, Morrison SF, Davis RP, Seitz BM, Burnett R and Watts, SW. (2015). 5-hydroxytryptamine does not reduce sympathetic nerve activity or neuroeffector function in the splanchnic circulation. Eur J Pharmacol 754:140-147.

Davis RP, Linder AE, Watts SW. (2011). Lack of the serotonin transporter (SERT) reduces the ability of 5-hydroxytryptamine to lower blood pressure. Naunyn Schmiedebergs Arch Pharmacol, 383:543–546. doi:10.1007/s00210-011-0622-01.

Davis RP, Pattison J, Thompson JM, Tiniakov R, Scrogin KE, Watts, SW. (2012). 5-hydroxytryptamine (5-HT) reduces total peripheral resistance during chronic infusion: direct arterial mesenteric relaxation is not involved. BMC Pharmacol,12:4. doi: 10.11186/1471-2210-12-4.

Davis RP, Szasz T, Garver H, Burnett R, Tykocki NR, Watts SW. (2013). One-month serotonin infusion results in a prolonged fall in blood pressure in the deoxycorticosterone acetate (DOCA) salt hypertensive rat. ACS Chem Neurosci, 4: 141–148. doi:10.1021/cn300114a.

De Vries P, De Visser PA, Heiligers JPC, Villalón CM, Saxena PR. (1999). Changes in systemic and regional haemodynamics during 5-HT_7 receptor mediated depressor responses in rats. Naunyn-Schmied Arch Pharmacol , 359:331-338.

Diaz J, Ni W, Thompson J, King A, Fink GD, Watts SW. (2008). 5-Hydroxytryptamine lowers blood pressure in normotensive and hypertensive rats. J Pharmacol Exp Ther, 325:1031–1038. doi:10.1124/jpet.108.136226.

Ettehad D, Emdin CA, Kiran A, Anderson SG, Callender T, Emberson J, Chalmers J, Rodgers A, Rahimi KL. (2016). Blood pressure lowering for prevention of cardiovascular disease and death: a systematic review and meta-analysis. Lancet, 387(10022):957–67.

El-Merahbi R, Loffler M, Mayer A, Sumara G. (2015). The roles of peripheral serotonin in metabolic homeostasis. FEBS, 589:1728-1734.

Erspamer V, Asero B. (1952). Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. Nature, 169:800-801.

Feldman JM. (1986). Urinary serotonin in the diagnosis of carcinoid tumors. Clin Chem, 32: 840–4.

Forstermann U, Sessa WC. (2012). Nitric oxide synthase: regulation and function. European Heart Journal, 33:829-837.

Fordyce CB, Roe MT, Ahmad T, Libby P, Borer JS, Hiatt WR, Bristow MR, Packer M, Wasserman SM, Braunstein N, Pitt B, DeMets DL, Cooper-Arnold K, Armstrong PW, Berkowitz SD, Scott R, Prats J, Galis ZS, Stockbridge N, Peterson ED, Califf RM. (2015). Cardiovascular drug development: is it dead or just hibernating? J Am Coll Cardiol, Apr 21;65(15):1567-82. doi: 10.1016/j.jacc.2015.03.016.

Gelman S. (2008). Venous function and central venous pressure. Anesthesiology, 108:735-748.

Green JF (1982) Circulatory mechanics in: Fundamental cardiovascular and pulmonary physiology. Philadelphia: Lea& Febiger, 101-10.

Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER 3rd, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. (2014). Heart disease and stroke statistics--2014 update: a report from the American Heart Association. American Heart Association Statistics Committee and Stroke Statistics Subcommittee Circulation, Jan 21;129(3):e28-e292.

Guyton AC. (1968). Regulation of cardiac output. Anesthesiology, 29: 314-326.

Hagan JJ, Price GW, Jeffrey P, Deeks NJ, Stean T, Piper D, Smith MI, Upton N, Medhurst AD, Middlemiss DN, Riley GJ, Lovell PJ, Bromidge SM, Thomas DR. (2000). Characterization of SB-269970-A, a selective 5-HT_7 receptor antagonist. Br J Pharmacol, 130:539-548.

Hedlund PB, Sutcliffe JG. (2004). Functional, molecular and pharmacological advance in 5-HT7 receptor research. Trends Pharmacol Sci, Sept;25:481-486.

Henderson WR, Griesdale DEG, Walley KR and Sheel AW. (2010). Clinical review: Guyton the role of mean circulatory filling pressure and right arteria pressure in controlling cardiac output. Critical Care Medicine, 14:243.

Hoyer D, Clarke DE, Fozard JR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP. (1994). International Union of Pharmacology classification for 5-hydroxytryptamine receptors. Pharmacol Rev, 46:157–203.

Hoyer D, Hannon JP, Martin GR. (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav, 71:533−554.

Jedlitschky G, Greinacher A, Kroemer HK. (2012). Transporters in human platelets: physiologic function and impact for pharmacotherapy. Blood, 119, 3394-3402.

Kaumann AJ, Levy FO. (2006). 5-Hydroxytryptamine in the human cardiovascular system. Pharmacol Ther., 111:674-706.

Law MR, Morris JK, Wald NJ. (2009). Use of blood pressure lowering drugs in the prevention of cardiovascular disease: meta-analysis of 147 randomized trials in the context of expectations from prospective epidemiological studies. BMJ, 338:b1665.

Lee FY, Colombato LA, Albillos A, and Groszmann RJ. (1993). N omega-nitro-L-arginine administration corrects peripheral vasodilation and systemic capillary hypotension and ameliorates plasma volume expansion and sodium retention in portal hypertensive rats. Hepatology, (1):84-90.

Magder S. (2016). Volume and its relationship to cardiac output and venous return. Critical Care, 20:271.

McClellan M, Brown N, Califfe RM, Warner JJ. (2019). Call to action: Urgent challenges in cardiovascular disease. Circulation. 139:e44-e54.

Monaghan PJ, Brown HA, Houghton LA, Keevil BG. (2009). Measurement of serotonin in platelets depleted plasma by liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci, 877:2163-2167.

Noda M, H. Higashida, S. Aoki, K. Wada. (2004). Multiple signal transduction pathways mediated by 5-HT receptors. Mol Neurobiol, 29:31-39.

Page IH, McCubbin JW. (1953). The variable arterial pressure response to serotonin in laboratory animals and man. Circ Res.,1:354–362. doi:10.1161/01.RES.1.4.354.

Pang CCY. (2000). Measurement of body venous tone. Journal of Pharmacological and toxicological Methods, 44:341-360.

Paulis L, Zicha J, Kunes J, Hojna S, Behuliak M, Celec P, Kojsova S, Pechanova O, Simko F. (2008). Regression of L-NAME-induced hypertension: The role of nitric oxide and endothelium-derived constricting factor. Hypertension Research, 31:793-803.

Ramage AG, Villalon CM. (2008). 5-Hydroxytryptamine and cardiovascular regulation. Trends Pharmacol Sci., 29(9):472-481.

Roswell LB. (1990). Editorial: Importance of Scintigraphic measurements of human splanchnic blood volume. Journal of Nuclear Medicine, 31:160-162.

Rothe CF. (1983). Reflex control of the veins and vascular capacitance. Physiol Rev., 63:1281-342.

Saxena PR, Villalon CM. (1990). Cardiovascular effects of serotonin agonists and antagonists. *J Cardiovasc Pharmacol,* 15:S16-S34.

Saxena PR, De Vries P, Villaon CM. (1998). 5-HT1-like receptors: a time to bid goodbye. Trends Pharmacol Sci., 19:311-316.

Sole MJ, Madapallimattam A, Baines AD. (1986). An active pathway for serotonin synthesis by renal proximal tubules. Kidney Int, 29:689–694.

Szeitz A and Bamdiera SM. (2017). Analysis and measurement of serotonin. Biomedical Chromatography, 32:e4135.

Tan T, Watts SW, Davis RP. (2011). Drug delivery, enabling technology for drug discovery and development. IPRECIO micro infusion pump: programmable, refillable and implantable. Front Pharmacol., 2: 44. doi:10.3389/fphar.2011.00044.

Terrón JA. (1997). Role of 5-ht7 receptors in the long-lasting hypotensive response induced by 5-hydroxytryptamine in the rat. Br J Pharmacol.,121:563-571.

Villalon CM, Centurion D. (2007). Cardiovascular responses produced by 5-hydroxytriptamine a pharmacological update on the receptors/mechanisms involved and therapeutic implications. Naunyn Schmiedebergs Arch Pharmacol, 376:45-63.

Walther DJ et al. (2003). Synthesis of serotonin by a second tryptophan hydroxylase isoform. Science, 299:76.

Watts SW, Darios ES, Seitz BM, Thompson JM. (2015). 5-HT is a potent relaxant in rat superior mesenteric veins. Pharmacol Res Perspect., 3:e00103. doi:10.1002/prp2.103.

Watts SW, Morrison SF, Davis RP and Barman SM. (2012). Serotonin and blood pressure regulation. Pharmacol Rev, 64:359-388.

Watts SW and Thompson JM. (2004). Characterization of the contractile 5-hydroxy-tryptamine receptor in the renal artery of the normotensive rat. J Pharmacol Exp Ther, 309:165–172.

Whelton PK, Carey RM, Aronow WS, et al. (2018). ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. Hypertension, 71(6):e13-e115.

Zhao Y, Vanhoutte PM, Leung SW. (2015). Vascular nitric oxide, Beyond eNOS. Journal of Pharm Sci., 129;2:83-94.

Chapter 2

Serotonin-induced Hypotension is Mediated by a Decrease in Intestinal Vascular Resistance

Seitz BM, Watts SW. (2014). Serotonin-induced Hypotension is mediated by a decrease in Intestinal Vascular Resistance. Pharmacolgia, 5(2):50-54.

Abstract

Background: Long-term serotonin (5-hydroxytryptamine, 5-HT) infusion produces a sustained reduction in mean arterial pressure (MAP) and a decrease in total peripheral resistance (TPR) through a mechanism dependent on nitric oxide synthase (NOS) in rats. This study sought to determine if the reduction in resistance induced by 5-HT is caused by NOS-dependent relaxation of vessels to all major organs of the body or in a specific vascular bed. *Material and Methods*: Alzet mini-pumps containing vehicle (saline) or 5-HT (25 µg/kg/min) were implanted in male Sprague Dawley rats. A separate group of rats was treated with the NOS inhibitor Nω-nitro-L-arginine (LNNA; 0.5 g/L) prior to 5-HT administration. *Results:* 5-HT reduced MAP after 24 hours (veh = 87$\pm$3, 5-HT = 75$\pm$2 mm Hg; $p<0.05$), a fall prevented by LNNA (LNNA/5-HT= 115$\pm$10 mm Hg).Yellow-green fluorescent microspheres, used to measure blood flow (300,000 spheres; size 15 µm), were introduced into the left ventricle of the heart. Microspheres were recovered in arterial reference blood sample and organs using sedimentation through centrifugation. The intensity of dye extracted from the microspheres was measured spectrophotometrically. 5-HT significantly decreased resistance to the spleen (48%) and small intestine (35%) compared to vehicle-treated animals; this decrease was abolished by LNNA. By contrast, resistance to the ear (175%) was increased by 5-HT. Resistance in brain, liver, lungs, kidneys and muscle did not show a

statistically significant reduction by 5-HT. *Conclusion:* These findings underscore the importance of the splanchnic (particularly intestinal) circulation and NOS in 5-HT-induced reduction in blood pressure.

Introduction

While 5-HT has long been known as a vasoconstrictor, peripheral administration of 5-HT (25 μg/kg/min) to conscious rats over the course of one week and even one month causes a sustained decrease in blood pressure (Diaz et al, 2008; Davis et al, 2012, 2013). This finding could prove markedly important given that the precursor of 5-HT, 5-hydroxytryptophan, diminishes the development of DOCA-salt hypertension (Fregly et al, 1987). The question is how this fall in blood pressure occurs.

The nitric oxide synthase (NOS) inhibitor, LNNA, abolished the ability of 5-HT to lower blood pressure (Diaz et al., 2008; Tan et al., 2011). This suggests that arterial relaxation to 5-HT, likely endothelium- and NOS-dependent, mediates the 5-HT-induced fall in blood pressure. However, studies support that 5-HT does not cause a direct relaxation or endothelium-dependent relaxation when tested in rat aorta, superior mesenteric artery or resistance arteries (Davis et al., 2012). It is possible the arterial bed that mediates the hypotension has not been studied. The present study addresses the identity of the vascular site(s) contributing to the fall in blood pressure; could it be shown that 5-HT increased flow to particular organs, underscoring a fall in blood pressure? Use of fluorescent microspheres to track blood flow allowed for testing, in many different tissues, the ability of 5-HT to change blood flow (Deveci and Egginton, 1999; Glenny et al., 1993). The present study tests the working hypothesis chronic 5-HT would increase blood flow (decrease resistance) in multiple vascular beds, with the

hope of identifying a site of action of 5-HT to decrease blood pressure. Because LNNA abolished 5-HT-induced chronic hypotension, a group of animals treated with 5-HT and LNNA are included.

Materials and Methods

Materials and Animals

Unless otherwise noted, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO USA). Yellow-Green microspheres (15 micron diameter, 10^6 beads/ml) were obtained from Life Technologies (Grand Island, NY USA). All protocols were approved by the MSU Institutional Animal Care and Use Committee, and follow the "Guide for the Care and Use of Laboratory Animals", 8th edition, 2011. Male Sprague Dawley rats (Charles River Laboratories, 225-250 grams) were used. Some rats received LNNA (0.5 g/L; protected from light) in their drinking water one week prior to implantation of pumps.

Surgery

Under isoflurane anesthesia, radiotelemeter transmitters (PA-C40; Data Sciences International, MN) were also implanted subcutaneously through a 1-1.5 cm incision in the left inguinal area as previously described (Diaz et al., 2008). Rats were allowed 3-4 days to recover post-operatively, and then 3-4 days of baseline measurements were made. At this time, osmotic pumps (Model 2ML1, Alzet Osmotic Pumps) were implanted subcutaneously between the scapulae, also under anesthesia. Mean arterial pressure, pulse pressure and heart rate were recorded for 10 seconds every 10 minutes using a commercially available data acquisition system (Dataquest,

DSI). Pumps contained either vehicle (sterile saline with 1% ascorbate, pH balanced to between 6-7) or 5-HT (25 ug/kg/min, s.c.).

Microsphere delivery and quantitation

Yellow-green fluorescent microspheres (sonicated; 300,000 total in 0.3 ml volume; 15 micron in diameter) were delivered in a glass-glass Hamilton syringe over a few seconds, followed by a 0.6 ml flush of saline, into the right carotid artery of the anesthetized rat. This microsphere load does not affect cardiovascular parameters itself (Kobayashi et al., 1994). The left femoral arterial catheter was used for blood pressure determination (Powerlab; ADInstruments, CO USA) and for reference blood withdrawal. A perfusion pump run in withdrawal mode (New Era Pump Systems NE-4000) was begun 30 seconds prior to ventricular injection of the microspheres at a rate of 0.5 ml/min. Two and 1/2 minutes after microsphere injection, tissues were removed and weighed. These included adrenals, heart, spleen, liver, lungs, right kidney, left kidney, small intestine (duodenum), leg muscle, leg skin, visceral fat, brain and ears. Yellow- green spheres were reclaimed through the sedimentation method after 1M KOH digestion (60 ^{o}C, overnight) following Triton Technologies Protocols (sedimentation protocol). Acidified cellusolve acetate [10 ul HCl/100 ml Cellusolve (2-ethoxyethyl acetate); 250 ul] was added to each dried sample, samples vortexed and sat for 15 minutes to dissolve the fluorescent coating. Fluorescent supernatant was recovered through centrifugation. Standard curves were generated for yellow-green beads (0 to 10,000 beads/ml). Fluorescence of samples was read on a white 96-well plate (Packard Optiplate, 6005290, Perkin Elmer, Walther MA, USA) for use in a Labscan

Fluoroskan Ascent FL plate reader using an excitation wavelength of 485 nM and emission wavelength of 510 nM.

Data analyses

All points represent means±SEM for the number of animals (N) reported. For blood pressure data analysis over time, within group differences were assessed by a one-way

repeated measures ANOVA with post-hoc multiple comparisons using Dunnett's procedure (GraphPad Instat 3). Between 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). Blood flow to organ of interest *(i*) = reference blood flow (0.5 ml/min) x [fluorescence organ of interest (i)/ fluorescence of reference blood]. Organ vascular resistance = mean arterial pressure/organ blood flow [mm Hg/(ml min gram)]. In all cases, a p-value of <0.05 was considered significant.

Results

5-HT caused a chronic fall in mean arterial blood pressure in conscious, unrestrained rats (figure 1A). The nadir of blood pressure fall occurred between days 1 and 2 (24 to 48 hours). It was at this time (24 hours) that microspheres were infused to anesthetized rats. Under anesthesia, baseline mean arterial blood pressure was lowered significantly by 24 hours of 5-HT infusion when compared to vehicle-infused rats (75.4±2.0 mm Hg vs 87.3±3.5 mm H, $p< 0.05$). LNNA given one week prior to

infusion with 5-HT abolished the fall in blood pressure (115±10 mm Hg, $p<0.05$). Animals were euthanized for tissue procurement after microsphere administration.

The mass of the tissue samples taken between vehicle- and 5-HT-infused groups were statistically similar (not shown) and the standard curves for yellow-green microspheres were robust with an r value of 0.996 (N=10 separate experiments). The microsphere burden in blood samples was not different between the vehicle, 5-HT and 5-HT/LNNA rats (~500-600 microspheres/0.5 ml). Similar basal flow to the left (2.48±0.42 ml/min/gram) and right kidneys (2.31±0.40 ml/min/gram; $p>0.05$) validated the even circulation of microspheres through the body.

Blood flow was significantly elevated in the spleen and in the small intestine of animals infused with 5-HT compared to vehicle (figure 1B). A group of LNNA treated animals in which vehicle was placed in the pumps was created but majority of these animals died during placement of catheters for microspheres due to the fragility of the blood vessels; we do not have these data to share. The increase in flow to 5-HT observed in both the intestine and spleen was abolished by LNNA. By knowing individual animal blood pressures, resistances [mm Hg/(ml/min/gram] from measured blood flow could be calculated. For the small intestine, vehicle resistance was 61.89±10.64, 5-HT-infused 39.83±4.93 and LNNA + 5-HT-infused 103.31±25.79 ($p<0.05$). Similarly, in the spleen vehicle resistance was 113.19±18.39, 5-HT-infused 58.26±7.71 and LNNA + 5-HT infused 323.4±72.71 ($p<0.05$).

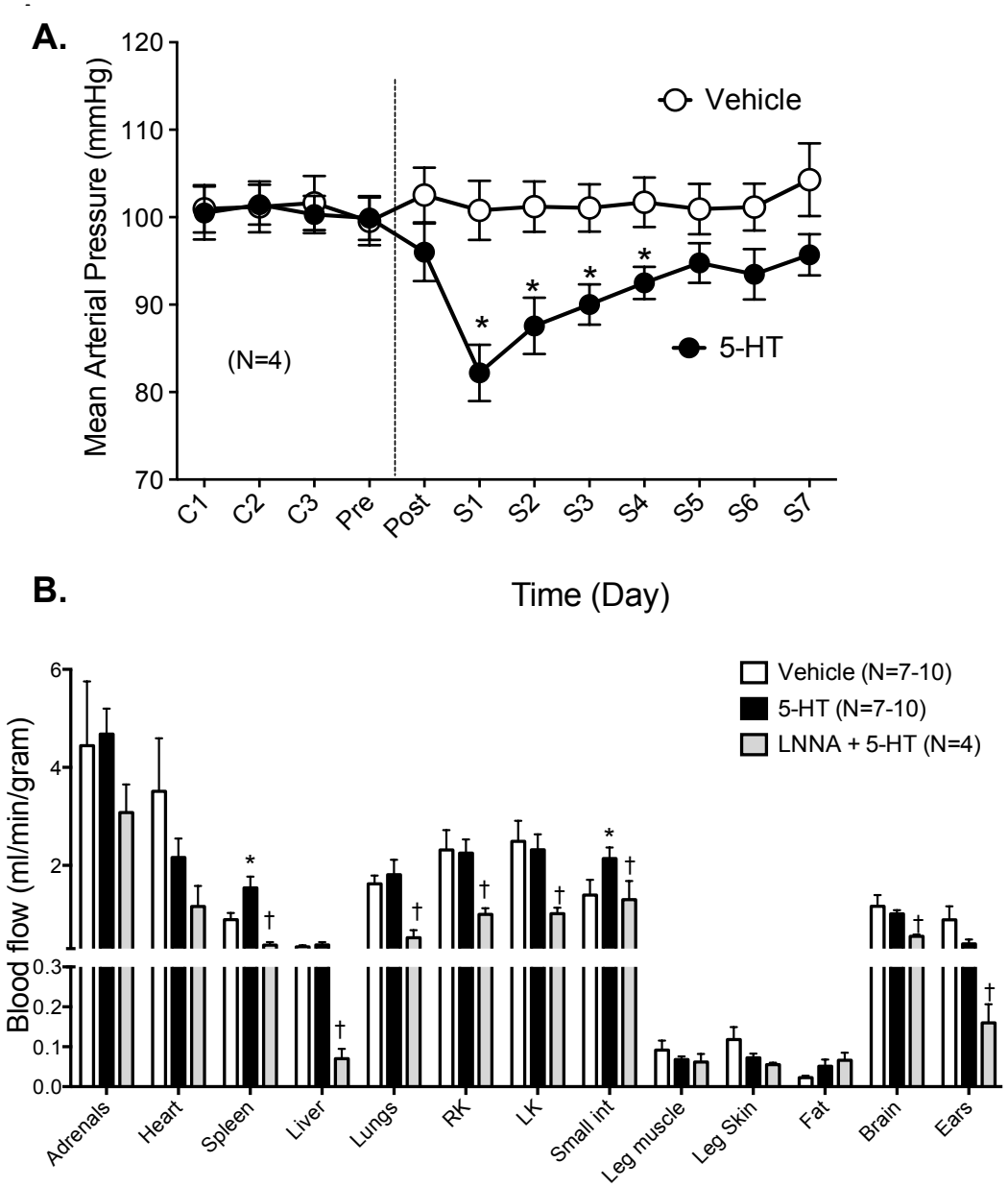


Figure 2.1: Mean arterial pressure measurements in rats treated with 5-HT and blood flow measurements in rats treated with 5-HT and 5-HT + LNNA. (A). **Arterial pressure effect of vehicle or 5-HT (25 ug/kg/min) in normal male Sprague Dawley rats over the course of 10 days. Points represent means±SEM for the number of animals in parentheses. * indicate statistically significant differences from control values prior to pump implantation, p<0.05). (B). Effect of a one week administration of LNNA on collective blood flows in animals receiving vehicle (open bars) or 5-HT (black or gray bars). Bars represent means±SEM for the number of animals in parentheses. * = statistically different from vehicle, † = different from 5-HT-infused.**

Discussion

This study is important because 5-HT caused the mean arterial blood pressure of experimentally hypertensive rats to drop over 50 mmHg, compared to approximately 20 mmHG in the sham group (Diaz et al., 2008). 5-HT virtually normalized blood pressure. Moreover, the administration of 5-HT for a longer, 30-day period continued to depress blood pressure in hypertensive rats (Davis et al., 2013). These findings support the potential use of 5-HT, precursors of 5-HT (5-hydroxytryptophan), or drugs that modify 5-HT concentration in treatment of elevated blood pressure. 5-HT did not cause direct arterial relaxation in large and small resistance mesenteric arteries (Davis et al., 2012). This suggests the *in vivo* mechanism for 5-HT to cause blood pressure to fall is: 1) in a bed different than those previously measured; and/or 2) involves mechanisms not previously considered. Use of microspheres *in vivo* allowed us to encompass both possibilities.

Qualitatively and quantitatively, the blood flows observed in this study are consistent with published results (Gervais et al., 1999; Mishra et al., 2010). In the rats receiving 5-HT chronically, an increase in splenic and small intestinal blood flow was consistently observed. Liver blood flow, also a part of splanchnic circulation, was not modified by 5-HT. LNNA abolished the elevated flow and reduced blood pressure caused by 5-HT, suggesting that the fall in resistance in the intestinal/splenic beds may be a mechanism by which 5-HT lowers blood pressure. LNNA did not lower the flow in all tissues (*e.g.* adrenal, leg muscle) such that one could argue a global and insurmountable vasoconstriction was not established (Takahashi et al., 1995).

How can the 5-HT-induced changes in splenic and intestinal blood flow be understood relative to changes in blood pressure? It would be surprising for the spleen to have large control over blood pressure as a significant but small (5%) of cardiac output is received by the spleen (Mendell and Hollenberg, 1971). By contrast, the activities of 5-HT in the intestine, outside of changing gut motility, are better understood. Biber et al (1973) demonstrated that 5-HT given acutely increased intestinal blood flow in the cat in a tetrodotoxin (TTX)-sensitive manner, and use of TTX converted a typically vasodilatory response into a vasoconstrictor response. Splanchnic blood flow is recognized to modify blood pressure (Mishra et a.l, 2010). 5-HT has been suggested to sensitize β adrenergic receptors (Fozard and Leach, 1968) and to antagonize α adrenergic receptors to promote vascular relaxation. However, interference with either the α or β adrenergic receptors would be observed as a more global (not tissue specific) increase in flow/decrease in resistance, and this was not observed. 5-HT must work in through other mechanisms.

There are two new ideas that emanate from this study. First, we had to consider the ability of 5-HT to inhibit sympathetic neuroeffector junction to decrease sympathetic tone and vascular resistance, an effect observed in several different vascular beds (Engel et al., 1983; Gothert et al., 1986; Molderings et al., 1987). Garcia-Pedraza *et al* recently described that activation of the 5-HT_{1D} and 5-HT_7 receptor inhibited sympathetic neurotransmission in rats treated with the 5-HT_2 receptor antagonist sarpogrelate (2013). Importantly, the 5-$HT_{1B/1D}$ receptor has been linked to NOS (Fujita et al., 2004). Second, the contributions of the venous circulation to the increase in flow caused by 5-HT should be considered. The simplest difference between the present

observation of a 5-HT-induced increase in intestinal flow and lack of direct 5-HT-induced arterial relaxation is 5-HT-induced *venous* relaxation. If veins increase their capacitance, then more flow can occur. Studies of isolated veins support the ability of 5-HT to cause relaxation, and to involve NO (Datte and Offoumou, 2004; Ellis et al., 1995). This does not mean that 5-HT is unable to contract veins, but that 5-HT possess both contractile and relaxant activity in these vessels. This is an exciting possibility given new attention paid to the importance of venous capacitance to blood pressure determination (Fink et al., 2000).

Conclusions

There is now substantial evidence that *in vivo* 5-HT reduces blood pressure. The present findings suggest that 5-HT interacts to increase intestinal blood flow by reducing resistance in these tissues. It is a goal to understand what is presumably a receptor-initiated mechanism set into motion by 5-HT to change intestinal flow and blood pressure.

REFERENCES

REFERENCES

Biber B, Fara J, Lundgren O. (1973). Intestinal vascular responses to 5-hydroxytryptamine. Acta Physiologica Scandinavia, 87:526-534.

Datte JY and Offoumou MA. (2004). Involvement of nitric oxide in the fading of 5-hydroxytryptamine-induced vasocontraction in rat isolated vena portae smooth muscle. Journal of Pharmacy and Pharmaceutical Science, 7:1-74.

Davis RP, Pattison J, Thompson JM, Tiniakov R, Scrogin KE, Watts SW. (2012). 5-hydroxytryptamine (5-HT) reduces total peripheral resistance during chronic infusion: direct arterial mesenteric relaxation is not involved. BioMedCentral Pharmacology, 12:4 doi:10.1186/1471-2210-12-4.

Davis RP, Szasz T, Garver H, Burnett R, Tykocki NR, Watts SW. (2013). One-month serotonin infusion results in a prolonged fall in blood pressure in the deoxycorticosterone acetate (DOCA) salt hypertensive rat. American Chemical Society Chemical Neuroscience, 4:141-148.

Deveci D and Egginton S. (1999). Development of the fluorescent microsphere technique for quantifying regional blood flow in small mammals. Experimental Physiology, 84:615-630.

Diaz J, Ni W, King A, Fink GD, Watts SW. (2008). 5-hydroxytryptamine lowers blood pressure in normotensive and hypertensive rats. Journal of Pharmacology and Experimental Therapeutics, 325:1031-1038.

Ellis ES, Byrne C, Murphy OE, Tilford NS, Baxter GS. (1995). Mediation by 5-hydroxytryptamine$_{2B}$ receptors of endothelium-dependent relaxation in rat jugular vein. British Journal of Pharmacology, 114:400-404.

Engel G, Gothert M, Miller-Schweinitzer E, Schlicker E, Sistonen L, Stadler PA. (1983). Evidence for common pharmacological properties of [^{3}H]5-hydroxytryptamine binding sites, presynaptic 5-hydroxytryptamine autoreceptors in CNS and inhibitor presynaptic 5-hydroxytryptamine receptors on sympathetic nerves. Naunyn-Schmiedebergs Archives of Pharmacology, 324:116-124.

Fink GD, Johnson RJ, Galligan JJ. (2000). Mechanisms of increased venous smooth muscle tone in desoxycorticosterone acetate-salt hypertension. Hypertension, 35:464-469.

Fozard JR and Leach GDH. (1968). The hypotensive action of 5-hydroxytryptamine (5-HT) in anaesthetized and pithed rats. European Journal of Pharmacology, 2:239-249.

Fregly MJ, Lockley OE, Sumners C. (1987). Chronic treatment with L-5-hydroxytryptophan prevents the development of DOCA-salt-induced hypertension in rats. Journal of Hypertension, 5:621-628.

Fujita M, Minamino T, Sanada S, Asanuma H, Hirata A, Ogita H, Okada K, Tsukamoto O, Takashima S, Tomoike H, Node K, Hori M, Kitakaze M. (2004). Selective blockade of serotonin 5-HT_{2A} receptor increases coronary blood flow via augmented cardiac nitric oxide release through 5-HT_{1B} receptor in hypoperfused canine hearts. Journal of Molecular and Cellular Cardiology, 37:1219-1223.

Garcia-Pedraza JA, Garcia M, Martin ML, Gomez-Escudero J, Rodriguez-Barbero A, Roman LS, Benito AM. (2013). Peripheral 5-HT_{1D} and 5-HT_{7} serotonergic receptors modulate sympathetic neurotransmission in chronic sarpogrelate treated rats. Eur J Pharmacol, 714(1-3):65-73.

Gervais M, Demolis P, Domergue V, Lesage M, Richer C, Giudicelli JF. (1999). Systemic and regional hemodynamics assessment in rats with fluorescent microspheres. Journal of Cardiovascular Pharmacology, 33:425-432.

Glenny R W, Bernard S, Brinkley M. (1993). Validation of fluorescent-labeled microspheres for measurement of regional organ perfusion. Journal of Applied Physiology, 74:2585-2597.

Gothert M, Schlicker E, Kollecker P. (1986). Receptor-mediated effects of serotonin and 5-methoxytryptamine on noradrenaline release in the rat vena cava and in the heart of the pithed rat. Naunyn-Schmiedebergs Archives of Pharmacology, 332:124-130.

Kobayashi N, Kobayashi K, Kouno K, Horinaka S, Yagi S. (1994). Effects of intra-atrial injection of colored microspheres on systemic hemodynamics and regional blood flow in rats. American Journal of Physiology, Heart and Circulatory Physiology, 266:H1910-H1917.

Mendell PL and Hollenberg NK. (1971). Cardiac output distribution in the rat: comparison of rubidium and microsphere method. American Journal of Physiology, 221:1617-1620.

Mishra RC, Tripathy S, Gandhi JD, Balsevich J, Akhtar J, Desai KM, Gopalakrishnan V. (2010). Decreases in splanchnic vascular resistance contribute to hypotensive effects of L-Serine in hypertensive rats. American Journal of Physiology, Heart and Circulatory Physiology, 298:H1789-H1796.

Molderings GJ, Fink K, Schlicker E, Gothert M. (1987). Inhibition of noradrenaline release via presynaptic 5-HT_{1B} receptors of the rat vena cava. Naunyn-Schmiedebergs Archives of Pharmacology, 336:245-250.

Takahashi H, Hara K, Komiyama Y, Masuda M, Murakami T, Nishimura M, Nambu A and Yoshimura M. (1995). Mechanism of hypertension induced by chronic inhibition of nitric oxide in rats. Hypertension Research, 18:319-324.
Tan T, Watts SW, Davis RP. (2011). Drug delivery, enabling technology for drug discovery and development. IPRECIO micro infusion pump: programmable, refillable and implantable. Frontiers in Pharmacology, 2:44.

Chapter 3

Serial Measurements of Splanchnic Vein Diameters in Rats

Using High-Frequency Ultrasound

Seitz BM, Krieger-Burke T, Fink GD, Watts SW. (2016).Front Pharmacol, 7:116.

doi: 10.3389/fphar.2016.00116. eCollection 2016.

PMID:27199758

Abstract

The purpose of this study was to investigate serial ultrasound imaging in rats as a fully non-invasive method to 1) quantify the diameters of splanchnic veins in real time as an indirect surrogate for the capacitance function of those veins, and 2) assess the effects of drugs on venous dimensions. A 21 MHz probe was used on anesthetized male Sprague-Dawley rats to collect images containing the portal vein (PV), superior mesenteric vein (SMV), abdominal inferior vena cava (IVC), and splenic vein (SpV; used as a landmark in timed studies) and the abdominal aorta (AA). Stable landmarks were established that allowed reproducible quantification of cross-sectional diameters within an animal. The average diameters of vessels measured every 5 minutes over 45 minutes remained within 0.75±0.15% (PV), 0.2±0.09% (SMV), 0.5±0.12% (IVC) and 0.38±0.06% (AA) of baseline (PV: 2.0±0.12 mm; SMV: 1.7±0.04 mm; IVC: 3.2±0.1 mm; AA: 2.3±0.14 mm). The maximal effects of the vasodilator sodium nitroprusside (SNP; 2 mg/kg, i.v. bolus) on venous diameters were determined 5 minutes post SNP bolus; the diameters of all noted veins were significantly increased by sodium nitroprusside, while mean arterial pressure decreased 29±4 mmHg. By contrast, administration of the venoconstrictor sarafotoxin (S6c; 5 ng/kg, i.v. bolus) significantly decreased PV and SpV, but not IVC, SMV or AA, diameters 5 minutes post S6c bolus; mean arterial

pressure increased by 6±2 mmHg. In order to determine if resting splanchnic vein diameters were stable over much longer periods of time, vessel diameters were measured every two weeks for 8 weeks. Measurements were found to be highly reproducible within animals over this time period. Finally, to evaluate the utility of vein imaging in a chronic condition, images were acquired from 4-week deoxycorticosterone acetate salt (DOCA-salt) hypertensive and normotensive (SHAM) control rats. All vessel diameters increased from baseline while mean arterial pressure increased (67±4 mmHg) in DOCA-salt rats compared to SHAM at 4 weeks after pellet implantation. Vessel diameters remained unchanged in SHAM animals. Together, these results support serial ultrasound imaging as a non-invasive, reliable technique able to measure acute and chronic changes in the diameter of splanchnic veins in intact rats.

Introduction

Dysregulation of venous capacitance (venous volume at a given transmural pressure) is proposed to contribute to the etiology of heart failure, arterial hypertension, hepatic cirrhosis, pre-eclampsia, postural hypotension and shock (Burchell AE et al., 2013; Safar ME, London GM, 1987; Li Y, et al., 2008; Aardenburg R et al., 2005; Stewart JM et al., 2004; Reilly PM at el., 2001). Splanchnic veins, located within the abdominal region, represent the largest blood volume reservoir within the human body (Gelman S, 2009), and also exhibit the largest degree of active capacitance response of all venous beds in the body (Tyberg J, 2002). Therefore, they play an important role in the regulation of the circulation by affecting cardiac preload, and thus, cardiac output and blood pressure. Early methods to study splanchnic venous capacitance and its regulation were highly invasive and could only be readily applied in experimental

animals (Brooksby GA, Donald DE, 1971). Later approaches that were applicable to humans included examining the distribution of blood-sequestered radionuclides (Schmitt M et al., 2002), impedance plethysmography (Leunissen KM et al., 1993), and direct imaging of the large veins (Stewart JM, Montgomery LD, 2004). For example, ultrasonographic assessment of the dimensions of large veins (particularly the inferior vena cava) has been used to estimate venous capacitance as a measure of intravascular volume status in patients with septic shock (Schefold JC et al., 2010) and in patients on hemodialysis (Brennan JM et al., 2006). Recent advancements in ultrasound technology include the development of high-frequency transducers (up to 70 MHz for rodent imaging). The associated enhanced signal processing at rapid frame rates (132 frames/second), with superior resolution (http://www.visualsonics.com/products/vevo-2100), enables quality ultrasound imaging in small animals (Stuart F et al., 2011; Deshpande N et al., 2010). The specific probe used in this study, the 21 MHz MS250 probe, has a resolution of 75 mM axial by 164 mm lateral by 403 m elevational. Importantly, rapid real-time imaging allows longitudinal measurements of the structure and function of small structures without significant impact on the animal or its physiology. While the current literature highlights many uses of high-frequency ultrasound in rodent cardiovascular models (Coatney RW, 2001; Chen JY et al., 2012; Slama M et al., 2003) imaging of the abdominal veins has not been widely described in animal research. While many rat splanchnic veins can be readily visualized using ultrasound (Knipps BS et al., 2003), there is no report of a technique that observes several major splanchnic vessels at once with repeated measures of the same location along a specific splanchnic vessel. The ability to re-

locate and measure a precise section along a vessel is critical to allow the continuous evaluation of diameter changes of that vessel during pharmacological interventions, or in chronic pathological states. We have therefore developed a technique for serial ultrasound imaging and measurement of the splanchnic vessel diameters in the anesthetized rat with the goal of providing a reproducible, longitudinal and non-invasive index of the capacitance function of these vessels. Validation of this technique included determining if imaging could reliably detect acute changes (seconds to minutes) in splanchnic vein diameters caused by venoactive drugs. Another focus was testing whether imaging could detect stable changes in the diameter of splanchnic veins in chronic conditions such as hypertension. Finally, this work was essential in developing a tool that would allow interrogation of the role of the venous circulation in drug-induced blood pressure changes. Specifically, our laboratories are dedicated to understanding how serotonin (5-HT, 5-hydroxytryptamine) lowers blood pressure chronically (Diaz et al., 2008). In vitro work on isolated splanchnic veins strongly supports the ability of 5-HT to cause direct venodilation but not arterial dilation (Watts et al., 2015). As such, a validated mechanism by which to investigate in vivo venous diameter would allow us to connect in vitro and in vivo studies, a powerful approach when investigating cause and effect. This work suggests the feasibility of using serial ultrasound imaging to investigate venous function in rodents.

Materials and Methods

Animals

MSU Institutional Animal Care and Use Committee approved all protocols used in this study. Male Sprague–Dawley rats at 6-weeks of age (300-350 g; Charles River

Laboratories,) were used in all experiments. Rats were housed in a temperature–controlled room (22°C) with 12-hour light/dark cycles and given standard chow and distilled water ad libitum.

Ultrasound Imaging

All animals were anesthetized using isoflurane (1.5-2.5% in oxygen, titrated to maintain stable heart rate, respiration rate and body temperature). The upper abdomen was shaved and depilatory cream (Nair™) applied below the xiphoid process. Animals were positioned supine on a heated platform (Vevo 2100 Imaging Station (integrated rail system); Visualsonics, Toronto, Canada), as shown in Figure 3.1. Coupling gel was applied to all four paws and the paws were taped to conductive pads on the platform to allow continuous collection of heart rate, respiratory rate, body temperature, and electrocardiograms (ECGs) during ultrasound image collection. Body temperature was monitored continuously, via a rectal probe, and maintained at 37°C throughout the experiment using the heated table and when necessary, a supplemental heat lamp. Warmed ultrasound gel was applied to the abdominal skin, just below the xiphoid process to couple the transducer (21 MHz probe; Visualsonics MS250) for imaging. The transducer head was locked in the adjustable arm of the Vevo mechanical rail-system to allow accurate hands-free transducer positioning during image collection. The height of the transducer was set to apply minimal pressure to the abdomen while still allowing adequate views of the abdominal vessels of interest. Once the transducer was positioned, the x-y knobs of the platform were used to move the transducer small increments in the cranial and caudal directions to display the major abdominal vessels significant to this study: abdominal aorta (AA), inferior vena cava (IVC) and portal vein

(PV) in the same view. Transverse B-mode images were collected at 25 frames per second in two locations, as seen in Figure 3.2. In Image 1, we focused on a transverse view at the level of the PV exiting the liver, which provides simultaneous cross-sectional images of the AA, IVC and PV; these structures are labeled as such. Image 2 displays the splenic vein (SpV), the established landmark for locating the noted veins. Finally, image 3 provides a transverse view ~2 mm caudal to Image 1, at a level just below the branching of the SpV (shown in image 2), and shows the structures of the superior mesenteric vein (SMV), SpV and IVC. Figure 3.3 shows a longitudinal image of the PV (as it exits the liver) during systole and expiration. All ultrasound images were saved as cine loops for subsequent measurement and analyses of vessel diameters. This technique was used to collect baseline and post dose images during drug interventions. Ultrasound recordings from individual rats during chronic studies were collected at the same time each day and feeding schedule remained consistent throughout the studies. We assumed venous pressure remained stable during each of these interventions and cardiac function did not change, except in DOCA animals. The AA was used as a control abdominal blood vessel for which minimal dynamic changes in measureable diameter were expected.

Ultrasound Image Analysis

All vessel diameter measurements were made by the same ultrasonographer with considerable experience in the use of the Vevo Imaging system. Cine loops of the images were viewed on a desktop station using Vevo LAB 1.7.1 software (Visualsonic, Toronto, Canada). All vessel diameters were measured using image frames from periods of cardiac systole and during expiration. Both vertical and horizontal diameters

(vessel height and vessel width) of vessel cross-sections were measured and then averaged to obtain final values from each time point.

Time Course Studies

We performed two time course studies: 1) images were collected for 3 animals every 5 minutes over the course of 45 minutes (acute); 2) images were collected for 5 animals every 2 weeks over the course of 8 weeks (chronic).

Blood Pressure Monitoring

Rats used in the sodium nitroprusside and sarafotoxin experiments were anesthetized with isoflurane (2% in oxygen) and a femoral arterial catheter was implanted for blood pressure monitoring. The catheter was placed through a 1-1.5 cm incision in the left inguinal area into the left femoral artery immediately prior to ultrasound imaging. The tip of the catheter was advanced to the abdominal aorta and the catheter was attached to a transducer connected to a data acquisition system (Powerlab, ADinstruments) to record mean arterial pressure (MAP) and heart rate (HR) during imaging. Rats remained anesthetized throughout catheter placement and subsequent imaging.

Vasodilation Study- Sodium Nitroprusside (SNP)

After 3 minutes of baseline recordings of abdominal vessel images and blood pressure, each animal received a 2 mg/kg bolus (0.3 mL, tail vein) of SNP (5054, Sigma Chemical CO., St Louis, MO). Ultrasound images were collected every minute over 30 minutes with 30 minutes being the time at which MAP returned to near baseline values. Data were collected at 5 minutes post-bolus, the point at which the MAP had reach

nadir while vessel diameters initial achieved near maximum dilation during the 30 minutes of the experiment. These maximum changes were reported graphically. MAP was measured continuously

via a femoral arterial catheter connected to data acquisition software on an ADInstruments Powerlab (Chart 7.0, Colorado Springs, CO). The splenic vein was not used as an endpoint in this particular study, but solely as a landmark. Seven animals were used in this study.

Vasoconstriction Study- Sarafotoxin (S6c)

After 3 minutes of baseline recordings of abdominal vessel images and blood pressure, each animal received a 5 ng/kg bolus (0.3 mL, tail vein) of S6c (88-9-35, American Peptide, Sunnyvale, CA). Ultrasound images were collected every minute over 15 minutes at which time all imaged vessels and MAP had returned back to baseline values. Data was graphed at 5 minutes' post-bolus, the point of maximum vessel diameter change. MAP was measured continuously via a femoral arterial catheter connected to data acquisition software on an ADInstruments Powerlab (Chart 7.0, Colorado Springs, CO). The splenic vein was not used as an endpoint in this particular study, but solely as a landmark. Five animals were used in this study.

Deoxycorticosterone acetate-salt Study (DOCA-salt)

All rats used during the DOCA-salt study underwent a left uninephrectomy. A DOCA pellet was implanted subcutaneously in half of the rats to deliver the drug at a dose of 200 mg/kg. The other rats underwent a SHAM pellet surgery (SHAM group). All rats were switched from distilled drinking water, to water containing 1% NaCl plus 0.2%

KCl for 4 weeks. All rats were imaged at baseline (prior to any intervention), and again 4 weeks after surgery/pellet implantation. Systolic arterial pressure was measured 4 weeks after surgery in conscious rats via the tail cuff method (Malkoff, 2005)(Kent Scientific Corp, CODA 6); this was the only time blood pressure was measured. There were four animals in each group: DOCA-salt and SHAM.

Data Analysis

Values were either expressed as mean ± SEM of the number of animals in the experiment, or a percent change from baseline. The baseline was determined by 3 minutes of image collection, prior to injection or during the first day of imaging prior to treatment. Statistical analyses were performed using paired two-tailed t-tests when comparing vessel images to baseline values. A repeated measures ANOVA were performed when comparing vessel diameter values at different time points in timed studies. (GraphPad Prism 6). In all cases, a p value of <0.05 was considered significant

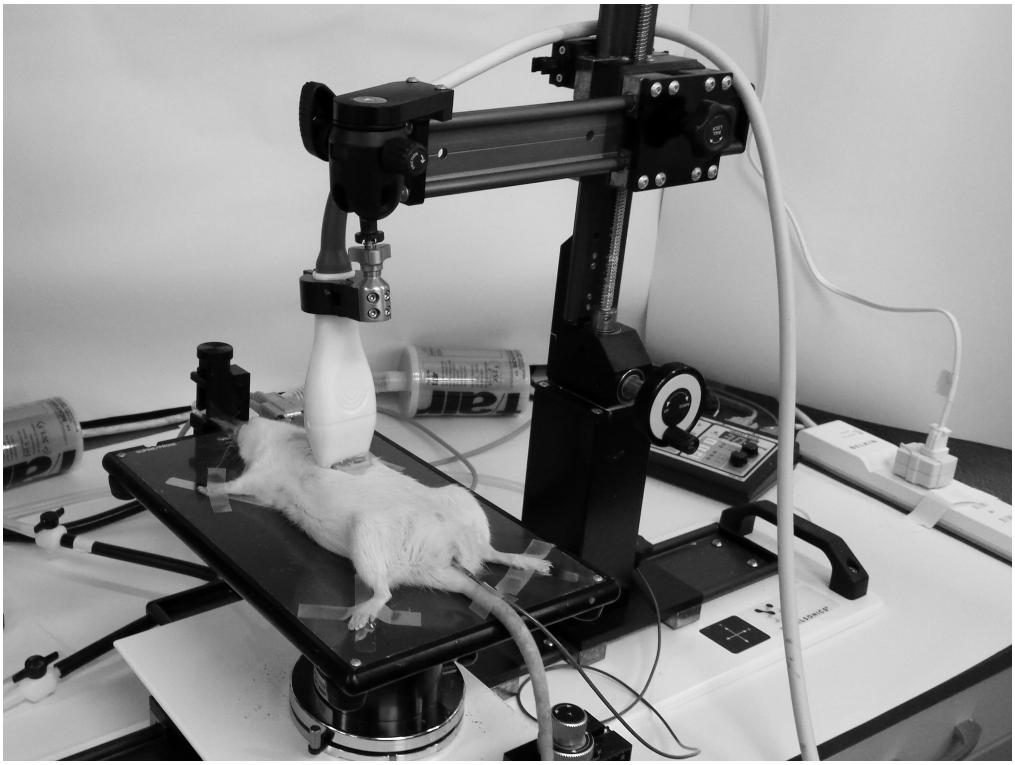
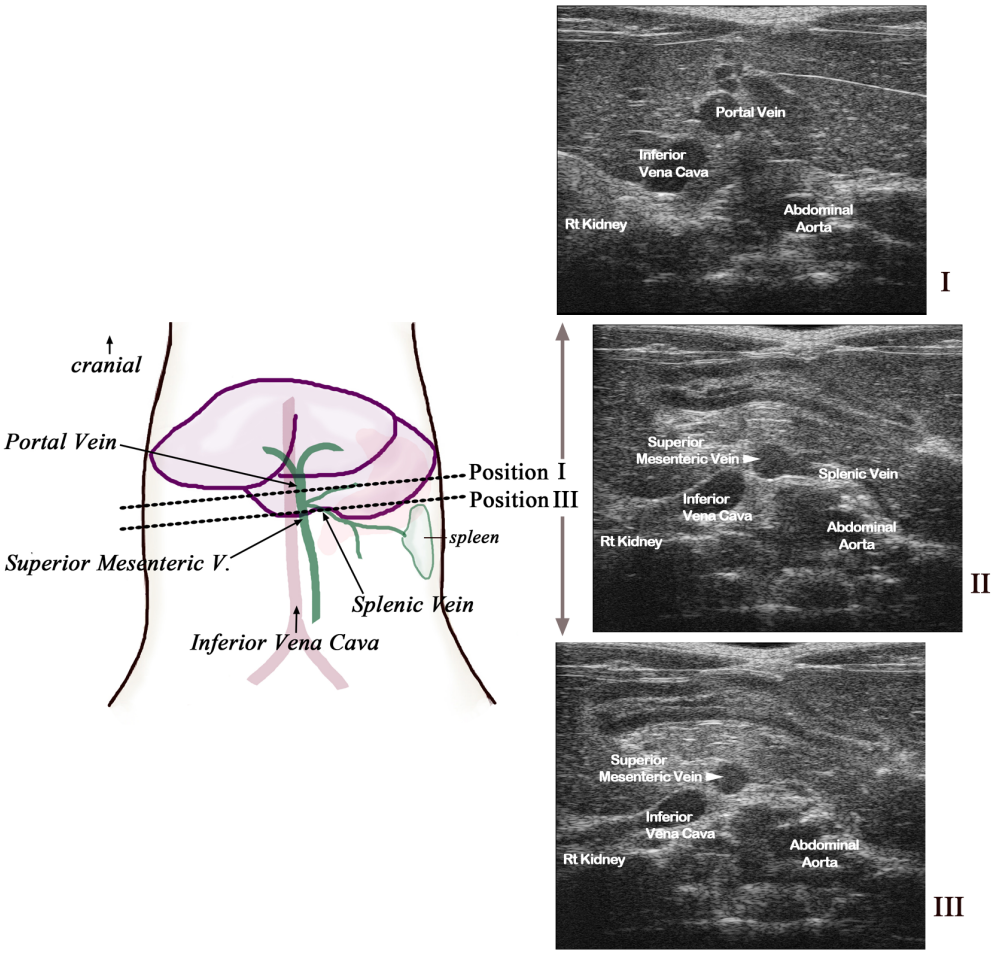


Figure 3.1: Ultrasound imaging of the abdominal vessels. A mechanical rail was used to hold a 21 MHz linear probe perpendicular to the abdomen while collecting transverse images. Body temperature was maintained at 37^{o}C. All measurements were made using images collected during expiration and during cardiac systole.



.

Figure 3.2: Transverse transducer placement (dotted line) **and corresponding images obtained** (Image 1, 2, and 3). The transducer placement to obtain the superior mesenteric vein/splenic vein view is ~2mm caudal to the placement used to obtain the portal vein view.

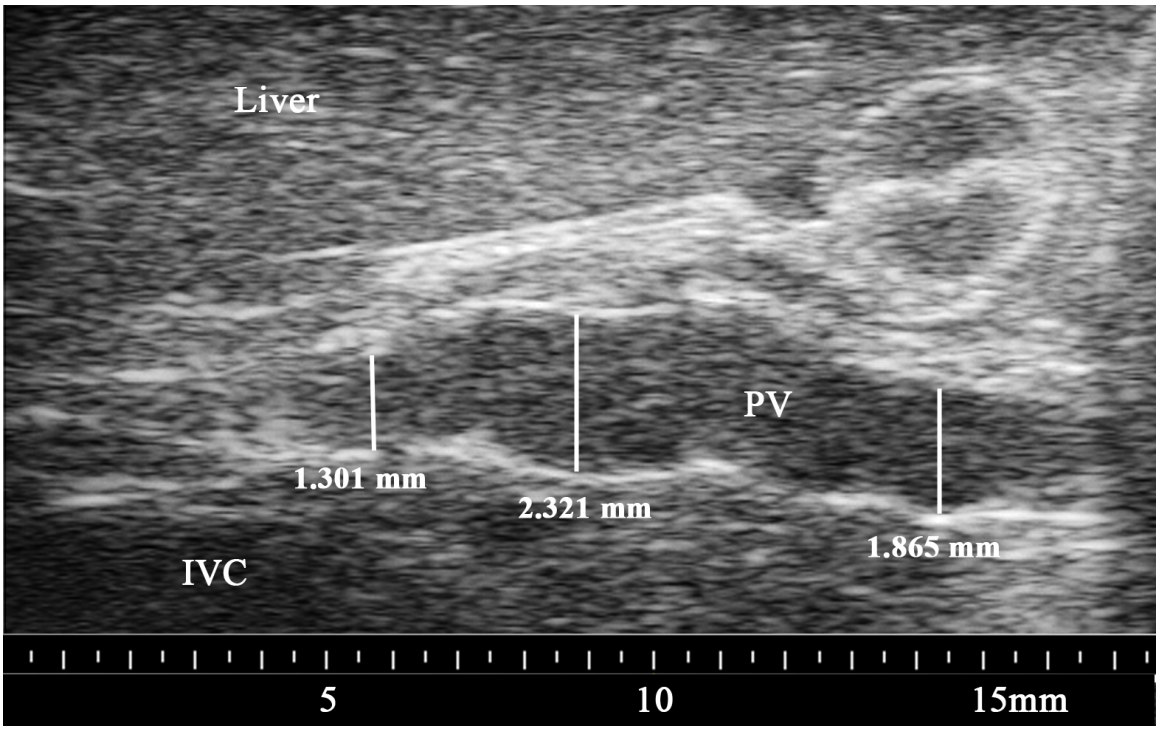


Figure 3.3: A longitudinal image of the portal vein within a naïve rat shows the variability of the diameter of this vessel along its length even when controlled for respiration and cardiac cycle.

Results

Ultrasound Transducer Placement

The splanchnic vein diameters varied considerably along the length of those vessels within the same rat as shown in Figure 3.3 and it was therefore imperative that a landmark be identified to allow repeated placement of the transducer at the exact location along a particular vein. Preliminary work suggested the SpV could be used as a landmark to locate and differentiate the PV and the SMV as shown in Figure 3.2, image 2. Because it was important to visualize the branching of the SpV off the parent vessels and to include multiple vessels of interest within the same view, a transverse positioning of the ultrasound transducer as seen in Figure 3.1 was determined to provide the optimal views for measurements of the PV, SMV, SpV, IVC, and AA diameters. In addition, the use of high-frequency ultrasound allowed the collection of images throughout the cardiac and respiratory cycles despite the rapid heart and respiratory rates of rats. Data could be generated from vessels during systole and exhalation specifically.

Acute Time Course Study

Vessel diameters measured every 5 minutes in anesthetized rats were unchanged with no statistical difference from baseline diameters through at least 45 minutes as seen in Figure 3.4. The diameters remained within 0.75±0.15% (PV), 0.2±0.09% (SMV) 0.5±0.12% (IVC) and 0.38±0.06% (AA) of baseline measurement for each noted vessel. The SpV was not imaged in this particular study.

Acute Pharmacologic Intervention Studies

The venodilator SNP and the venoconstrictor S6c were used in separate experiments to investigate the ability of our imaging method to detect acute drug-induced changes in venous diameter. A single iv bolus of SNP at 2 mg/kg caused peak increases in PV, SMV, SpV, and IVC diameters by 5 minutes post drug administration as seen in Figure 3.5 (A, B); (PV: 34.70±2.2%, SMV: 28±4.1%, SpV: 39.3±4.7% and IVC: 6.6±4.8% change from baseline). Actual diameters of vessels at baseline are reported in the foundation of each bar. In contrast, there was little change in the diameter of the AA to SNP despite MAP being significantly reduced by 29±4 mmHg. In separate rats, a single 5 ng/kg iv bolus of S6c caused a peak decrease in the diameters of the SMV (11.9±2.0%), PV (20.5±1.6%),

and SpV (13.5±2%) 5 minutes after injection, while MAP increased 6±2 mmHg as shown in Figure 3.6 (A, B). Since SMV did not show a statistical significance in the presence of sarafotoxin, the SMV image was not included in Figure 3.6 (B). There was little change in the diameters of the IVC or AA. Baseline diameters are reported in the foundation of each bar.

Chronic Time Course Study

In a longitudinal imaging study, we evaluated the stability of the splanchnic veins and abdominal aorta diameters measurements within an animal when acquired every 2 weeks, over 8 weeks. Vascular diameters measured using our imaging technique were extremely stable over 8 weeks, showing the average of the percent changes from baseline at 8 weeks to be within 2.0±0.5% (PV), 0.95±0.2% (SMV), 11.7±8% (IVC) and 0.9±0.6% (AA), as shown in Figure 3.7 (A). The SpV was not imaged in this particular

study. There was no statistical difference between the time points of the imaged vessels. These findings support the ability to consistently relocate a specific cross-section of abdominal vessel of interest over multiple imaging sessions spanning many weeks within an individual animal. Interestingly, vessel diameters remained stable despite a steady body weight gain (200 grams) over the course of the experiment (Figure 3.7 B). All animals used in these studies were mature rats (10-12 weeks of age at the start of each experiment) with stable body lengths, however.

Deoxycorticosterone acetate-salt Study (DOCA-salt)

We applied our imaging method to an experimental model of hypertension, DOCA-salt hypertension, in which we have previously demonstrated abnormalities of venous capacitance regulation (Fink GD et al., 2007). The current experiment involved ultrasound imaging of splanchnic vessels at baseline and again after 4-weeks of established DOCA-salt hypertension. While vascular diameters were similar in the two groups of rats at baseline prior to treatment (less than a 4% difference in any vessel diameters between groups prior to treatment), we observed an overall increase in the vessel diameters of DOCA-salt treated animals compared with vehicle controls at 4 weeks of treatment as shown in Figure 3.8. Diameters increased significantly from baseline in the DOCA-salt treated group, 32.3±4.6% (PV), 30.8±6.7% (SMV), 20.6±6.4% (IVC), 44.3±4% (SpV), but not in the AA 16.2±6.9% of the DOCA-salt treated group or vehicle group, 0.1±0.4% (PV), 0.7±2.1% (SMV), -6.8±4.7% (IVC), 0.7±11.5% (SpV), and 0.9±1.4% (AA). Tail-cuff blood pressure was 67±4 mmHg higher in DOCA-salt versus SHAM rats.

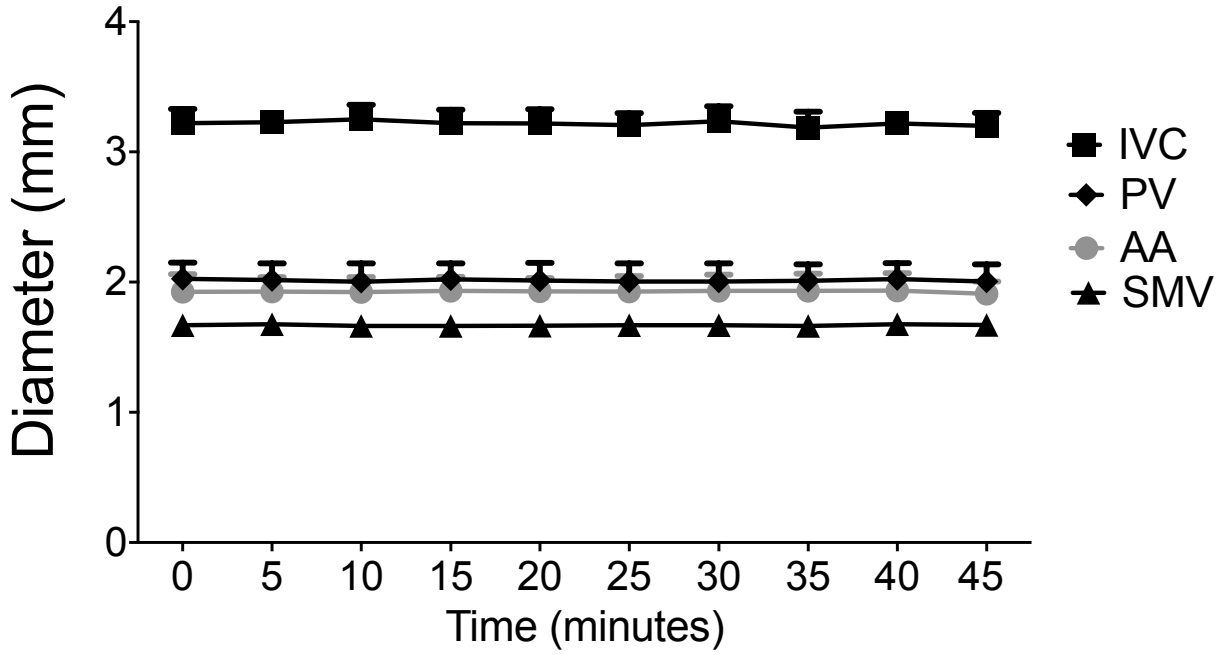
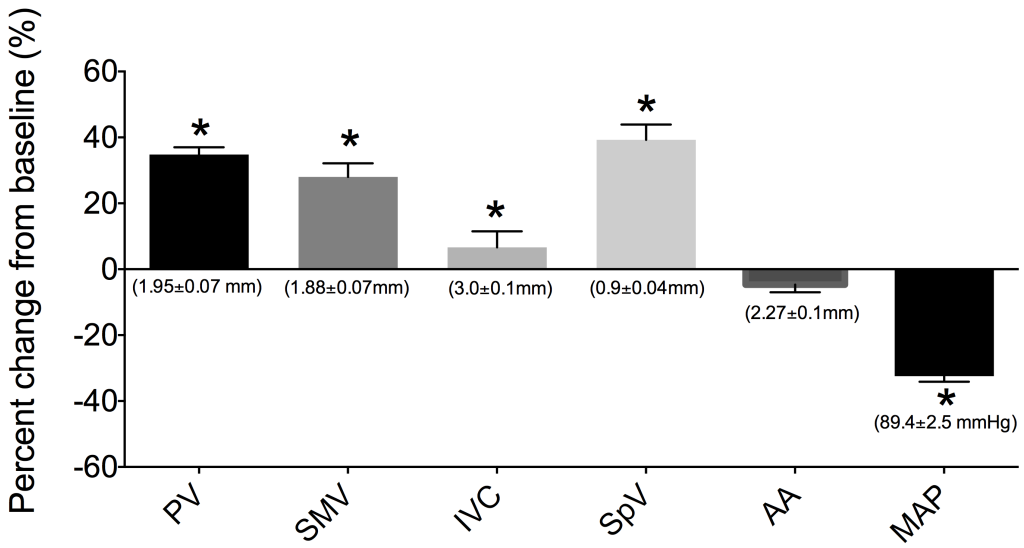


Figure 3.4: Diameter measures of imaged vessels at baseline. Under isoflurane anesthesia, animals were imaged every 5 minutes over 45 minutes to determine the stability and consistency of each vessel diameter. Points on the graph represent vessel diameters (means± SEM of 3 animals).

A.



B.

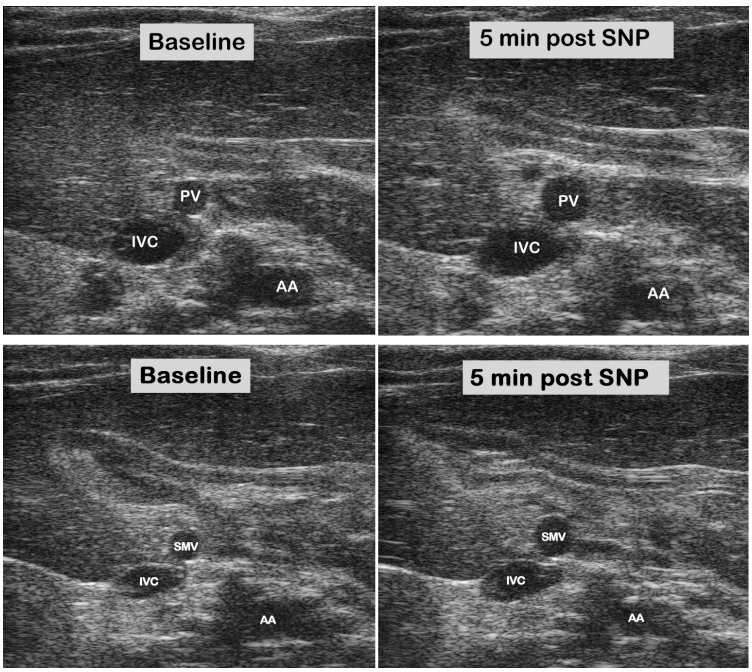
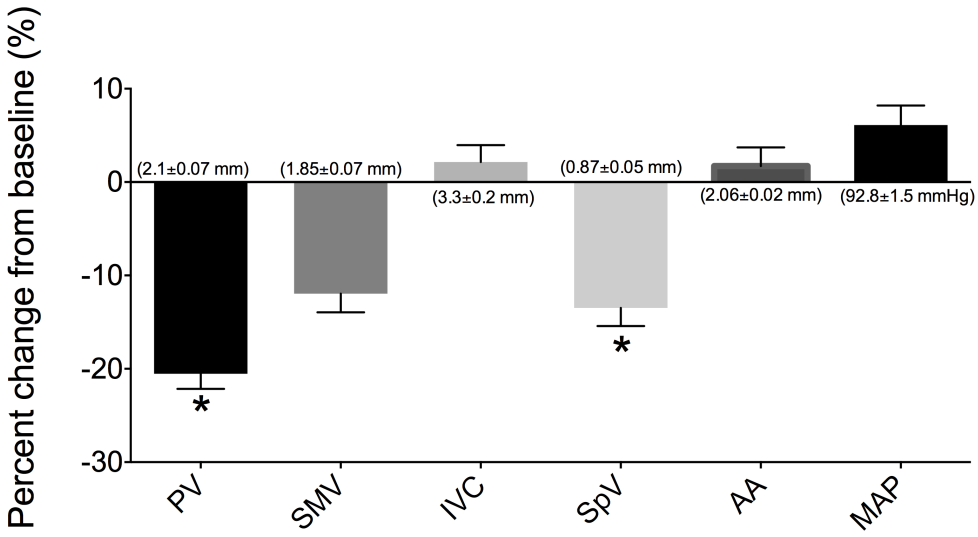


Figure 3.5: Effects of sodium nitroprusside on the diameter of imaged vessels (**A**). Percent change from baseline (baseline measured 3 minutes prior to drug treatment) of vessel diameters and MAP at 5 minutes post SNP i.v bolus. Bars represent means± SEM of 7 animals. Asterisks depicts statistically different from baseline, $P<0.05$. (**B**). Images of PV, IVC, SMV, and AA at baseline and 5 minutes post SNP. Baseline diameters of vessels are shown in the foundation of each bar.

A.



B.

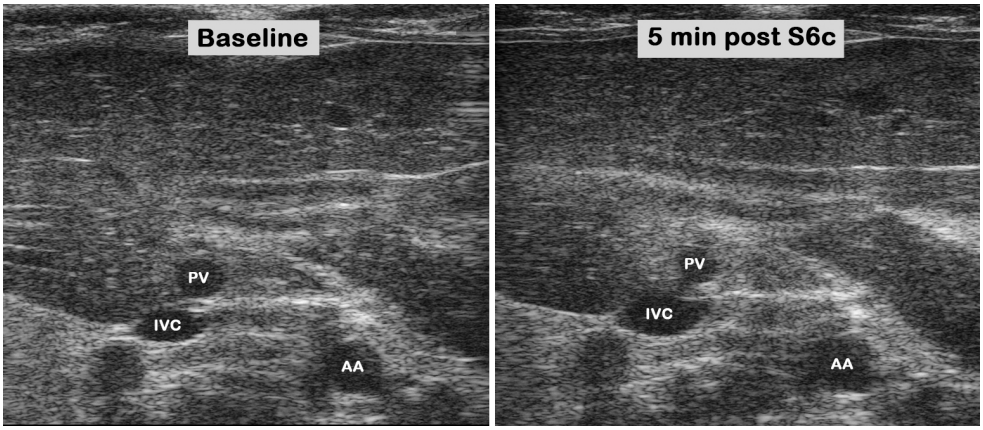


Figure 3.6: Effects of sarafotoxin on the diameter of imaged vessels. (**A**). Graph represents the percent change from baseline (measured 3 minutes prior to drug treatment) of vessel diameters and MAP at 5 minutes post S6c i.v bolus. Bars represent means± SEM of 5 animals. Asterisks depicts statistically difference from baseline, $P<0.05$. (**B**). Images of PV, IVC, and AA at baseline and 5 minutes post S6c. Baseline diameters of vessels are shown in the foundation of each bar.

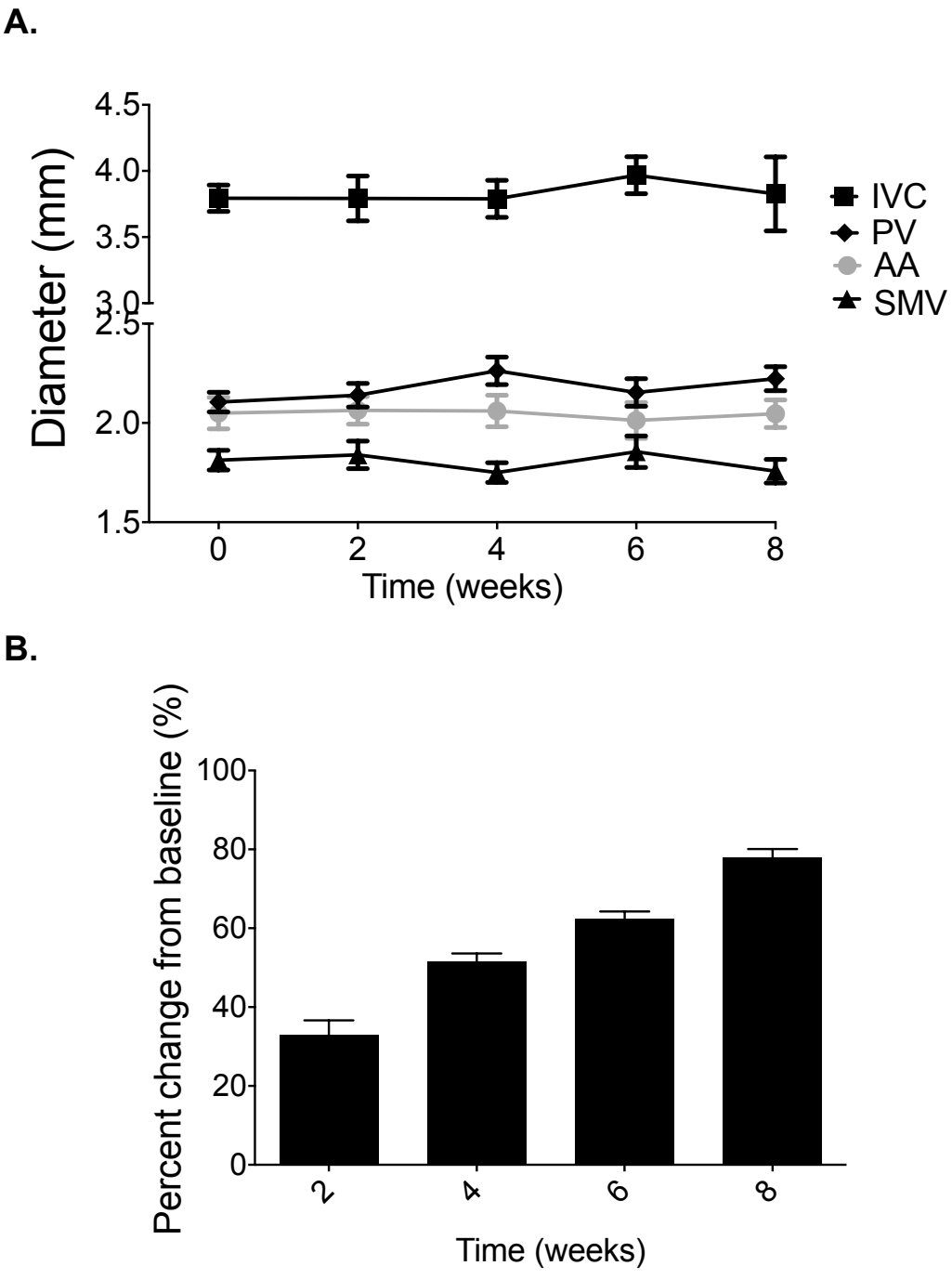


Figure 3.7: Effects of time on the diameter of the images and body weight (**A**). Vessel diameters imaged every 2 weeks through 8 weeks (means± SEM of 5 animals). (**B**). Body weight changes of the same animals over the 8-week time course. Bars represent means± SEM of 5 animals.

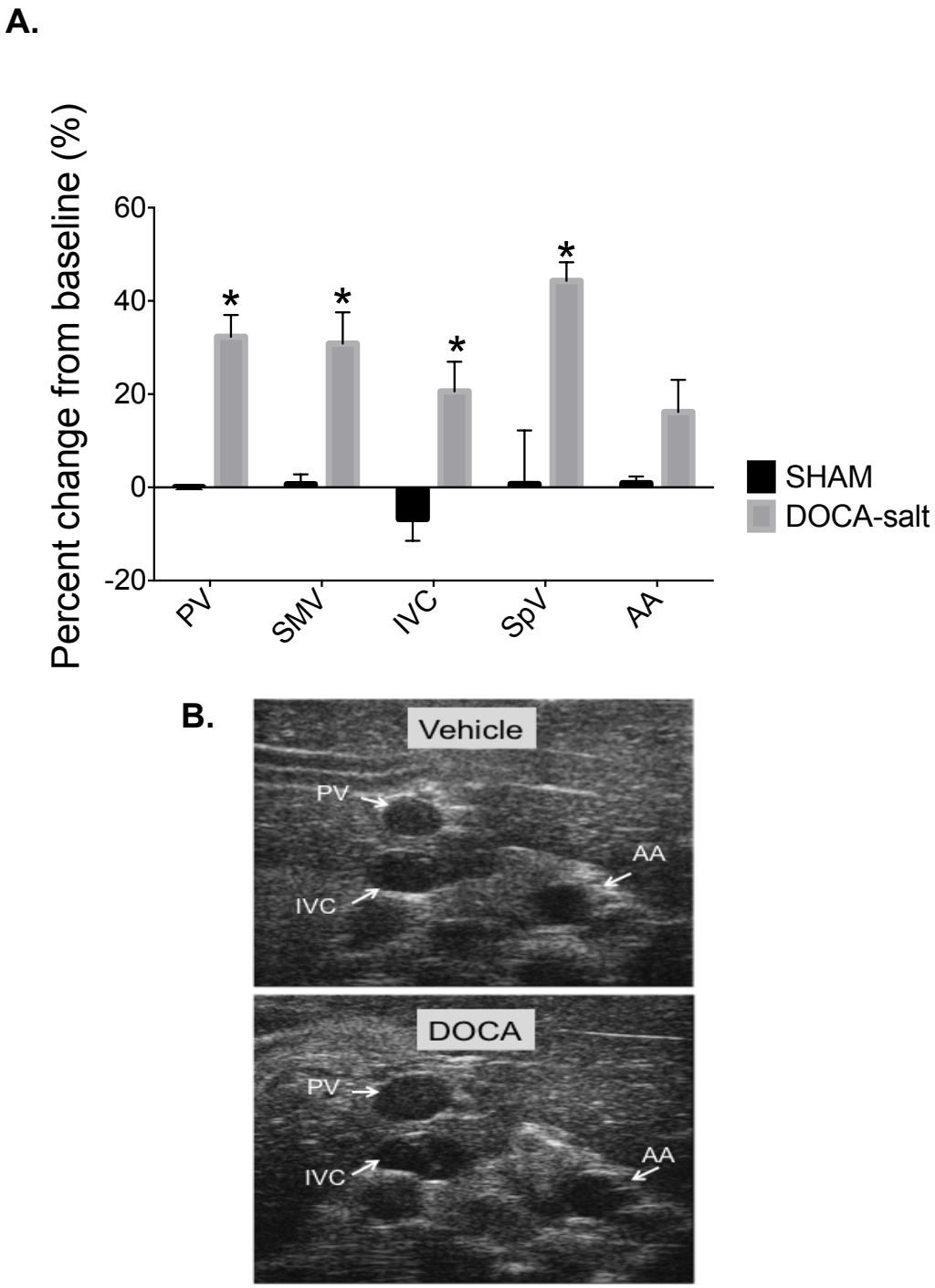


Figure 3.8: Effects of DOCA-salt hypertension on the diameter of the imaged vessels. (**A**). Percent change from baseline (baseline measured prior to DOCA-salt treatment) of vessel diameters compared to 4-weeks post DOCA-salt treatment. Bars represent means± SEM of 4 animals per treatment group. Asterisks depict statistically difference from baseline, P<0.05. (**B**). Images of PV, IVC, and AA at baseline and 4-weeks post DOCA-salt treatment.

Discussion

The goal of the present study was to develop and validate a non-invasive imaging method to make reliable and reproducible serial measurements of splanchnic vein diameters in anesthetized rats as an aid to understanding the role of vascular capacitance in cardiovascular regulation. We investigated several different vein segments (PV, SMV, IVC and SpV) because multiple veins contribute to splanchnic vascular capacitance function, and each segment is unique in terms of structure and contractile regulation (Schmitt M. et al., 2002). Our approach to this goal featured the use of: high frequency ultrasound; an adjustable arm and mechanical rail-system to allow for accurate, stable, hands-free transducer positioning during image collection; identification of reliable anatomical landmarks to facilitate reproducible transducer location; and the measurement of vessels during systole and expiration only (as cardiac and respiratory cycles strongly influence the dimensions of the compliant abdominal veins). The PV, SMV, and SpV all showed marked changes when rats were infused with either S6c or SNP, while the IVC remained relatively stable. This may occur because the effectors of S6c and SNP are differently functional in these compared veins (e.g. is the amount of ETB receptors different, is the expression of guanylate cyclase different, respectively?). Alternatively, diameter changes to the PV, SMV, and SpV may lead to compensatory changes in the IVC that mask effects that might be seen in an isolated IVC. The varying responses of specific vessels *in vivo* highlight the importance of simultaneous and collective diameter measurements.

Our initial study showed that use of this approach produced quite stable measurement of vessel diameters over 45 minutes in anesthetized, temperature-

controlled rats. The stability of the vessel diameters over this time period, in the absence of any intervention, allowed us to conduct subsequent experiments to examine the acute effects of venoactive drugs on splanchnic vessel diameters. Administration of the venodilator SNP and the venoconstrictor S6c resulted in anticipated increases and decreases in splanchnic venous diameters, respectively. These two compounds were chosen because of their relative venoselectivity. This difference in reactivity highlights one of the many differences between veins and arteries, including veins possessing lower smooth muscle content and exposure to lower levels of cyclic strain (Sung et al., 2007).

In isolated rat veins and arteries, S6c causes a venoconstriction but not an arterial constriction (Tykocki et al., 2009). This was the best tool we had available that would permit relatively selective venoconstriction and, used with SNP as a recognized vasodilator, demonstrate that we could observe and quantify both venous constriction and dilation. It is important to note that we did not measure pressure within the abdominal veins during image acquisition; therefore firm conclusions cannot be drawn about whether the observed diameter changes were primarily passive or active in nature. However, in a previous study (Schwarzacher et al., 1992) in rabbits that reported changes in the diameter of the abdominal vena cava in response to venoactive drugs, only very small alterations in venous pressure were observed. Thus the diameter changes we observed were likely due to active alterations in venous smooth muscle tone.

In order to validate this imaging technique for use in more chronic longitudinal studies, in a further experiment we investigated the stability of the splanchnic vein and

abdominal aorta diameters within an animal when acquired every 2 weeks over an 8 week period. The key to this approach was the ability to accurately relocate a specific cross-section of the splanchnic vessel of interest. The consistency of vessel diameter measurements observed in this chronic time course study indicates that these vessels can serve as their own control in experiments examining the potential impact of interventions on long-term changes in vascular diameter. Therefore, we applied our imaging technique to study splanchnic venous diameters in an experimental model of chronic hypertension, i.e. DOCA-salt hypertension. The results showed a significantly increased diameter of all the splanchnic veins in DOCA-salt rats, whereas no increases were observed in SHAM rats. We have previously reported increased venoconstriction in conscious DOCA-salt rats (Fink GD et al., 2000) so anticipated that we would observe reduced splanchnic venous diameters in these animals. However, venomotor tone in splanchnic veins is primarily controlled by sympathetic activity, and isoflurane anesthesia is known to dramatically reduce sympathetic activity (Bencze M et al., 2013). So the increases in venous diameters we observed in DOCA-salt rats under anesthesia are likely due to passive effects (unopposed by sympathetic venoconstriction) of the volume expansion and cardiac hypertrophy that occur in chronic DOCA-salt rats (Titze J et al., 2006; Kobrin I et al., 1990). This experiment highlights the complexities of evaluating moment-to-moment regulation of venous diameter; and indicates the desirability of using multiple, and minimally invasive, approaches to that evaluation. Nevertheless, the results support the ability of our vein imaging technique to detect chronic, physiologically significant changes in venous diameter in intact (albeit anesthetized) rats.

There are some additional limitations to the current study that deserve mention. First, we only studied a relatively small number of male Sprague-Dawley rats between 6-20 weeks of age. Venous diameters measured by ultrasound vary by age and sex in humans (Kutty S et al., 2014; Gui J et al., 2015) and are likely to do so in rats as well. Likewise, it is quite possible that the approach described here may need to be modified if applied to other strains of rats, to female rats, or to sexually immature or old rats. Second, although we found that weight gain of ~200 grams (~80% increase) did not significantly affect the diameters of any abdominal vein, studies is humans suggests that venous diameter correlates modestly with body surface area and height (Gui J et al., 2015). Third, all animals were imaged only in one spatial plane, i.e. supine. It is possible that altering the posture of the rat would result in different findings for splanchnic vein diameters. Finally, a relevant consideration for any measurement technique is the time and effort required to master it (Bowra J et al., 2015) but since all values here were obtained by a single highly experienced rodent ultrasonographer, we cannot comment on how long it would take for a novice to achieve the kind of stable, reproducible measurements we report.

Summary

Although, ultrasound imaging the abdominal vessels, particularly the PV and IVC, is not novel in experimental research or clinical settings, investigation of diameter change in multiple splanchnic vessels is novel as they relate to venous capacitance. There is no current publication that measures vein diameters in the splanchnic vasculature, a highly capacitive and compliant region important in blood pressure regulation. We anticipate that this technique will be useful in a number of different ways.

The goal of the present study was to lay the foundation for repeatable measures, and illustration of changes in one model in which alterations in venous function have been suggested (e.g. the DOCA- salt model). Once we, and hopefully others, accept this type of approach than we can begin to interrogate disease models that would benefit from investigate of the venous circulation. These include other models of experimental and genetic hypertension, hyperlipidemia, obesity and heart failure.

REFERENCES

REFERENCES

Aardenburg R, Spaanderman ME, Courtar DA, Van Eijndhoven HW, De Leeuw PW, Peeters LL. (2005). A subnormal plasma volume in formerly preeclamptic women is associated with a low venous capacitance. J Soc Gynecol Investig**,** 12(2):107-111.

Bencze M, Behuliak M, Zicha J. (2013). The impact of four different classes of anesthetics on the mechanisms of blood pressure regulation in normotensive and spontaneously hypertensive rats. Physiol Res, 62(5):471-478.

Bowra J, Uwagboe V, Goudie A, Reid C, Gillett M. (2015). Interrater agreement between expert and novice in measuring inferior vena cava diameter and collapsibility index. Emerg Med Australas, 27(4):295-299.

Brennan JM, Ronan A, Goonewardena S, Blair JE, Hammes M, Shah D et al. (2006). Handcarried ultrasound measurement of the inferior vena cava for assessment of intravascular volume status in the outpatient hemodialysis clinic. Clin J Am Soc Nephrol, 1:749–753.

Brooksby GA, Donald DE. (1971). Dynamic changes in splanchnic blood flow and blood volume in dogs during activation of sympathetic nerves. Circ Res, 29(3):227-238.

Burchell AE, Sobotka PA, Hart EC. (2013). Chemohypersensitivity and autonomic modulation of venous capacitance in the pathophysiology of acute decompensated heart failure. Curr Heart Fail Rep, 10(2):139-146.

Chen JY, Chen HL, Wu SH, Tsai TC, Lin MF, Yen CC et al. (2012). Application of high-frequency ultrasound for the detection of surgical anatomy in the rodent abdomen. Vet J, (191):246-252.

Coatney RW. (2001). Ultrasound Imaging: Principles and Applications in Rodent Research. National Research Council, Institute of Laboratory Animal Resources, 42(3):233-247.

Deshpande N, Needles A, Willmann JK. (2010). Molecular ultrasound imaging: current status and future directions. Clin Rad, 65:567-581.

Diaz J, Ni W, Thompson J, King A, Fink GD, Watts SW. (2008). 5-Hydoxytryptamine lowers blood pressure in normotensive and hypertensive rats. J Pharmacol Exp Ther, 325**:**1031-1038.

Fink GD, Johnson RJ, Galligan JJ. (2000). Mechanisms of increased venous smooth muscle tone in desoxycorticosterone acetate-salt hypertension. Hypertension, 35(1 Pt 2): 464-469.

Gelman S. (2009). Venous function and central venous pressure. Anesthesiology, 108: 735-748.

Gui J, Guo J, Nong F, Jiang D, Xu A, Yang F et al. (2015). Impact of individual characteristics on sonographic IVC diameter and the IVC diameter/aorta diameter index. Am J Emerg Med, 33(11):1602-1605.

Knipp BS, Ailawadi G, Sullivan VV, Roelofs KJ, Henke PK, Stanley JC et al. (2003). Ultrasound measurement of aortic diameters in rodent models of aneurysm disease. J Surg Res, 112(1):97-101.

Kobrin I, Eimerl J, Mekler J, Ben-Ishay D. (1990). Cardiac hypertrophy developing during DOCA-salt treatment is dissociated from systemic and regional hemodynamics. Am J Hypertens, 3(2):136-139.

Kutty S, Li L, Hasan R, Peng Q, Rangamani S, Danford DA (2014). Systemic venous diameters, collapsibility indices, and right atrial measurements in normal pediatric subjects. J Am Soc Echocardiogr, 27(2):155-162.

Leunissen KM, Kouw P, Kooman JP, Cheriex EC, deVries PM, Donker AJ et al. (1993). New techniques to determine fluid status in hemodialyzed patients. Kidney Int Suppl, 41: S50-56.

Li Y, Liu H, Gaskari SA, Tyberg JV, Lee SS. (2008). Altered mesenteric venous capacitance and volume pooling in cirrhotic rats are mediated by nitric oxide. Am J Physiol Gastrointest Liver Physiol, 295(2):G252-259.

Malkoff J. (2005). Non-invasive blood pressure for mice and rats. Animal Lab News,1-3.

Reilly PM, Wilkins KB, Fuh KC, Haglund U, Bulkley GB. (2001). The mesenteric hemodynamic response to circulatory shock: an overview. Shock, 15:329–343.

Safar ME, London G. (1987). Arterial and venous compliance in sustained essential hypertension. Hypertension, 10(2):133-139.

Schefold JC, Storm C, Bercker S, Pschowski R, Oppert M, Krüger A et al. (2010). Inferior vena cava diameter correlates with invasive hemodynamic measures in mechanically ventilated intensive care unit patients with sepsis. J Emerg Med, 38(5):632-637.

Schmitt M, Blackman DJ, Middleton GW, Cockcroft JR, Frenneaux MP. (2002). Assessment of venous capacitance. Radionuclide plethysmography: methodology and research applications. Br J Clin Pharmacol, 54:565-576.

Schwarzacher S, Weidinger F, Schemper M, Raberger G. (1992). Blockade of endothelium-derived relaxing factor synthesis with NG-nitro-L-arginine methyl ester leads to enhanced venous reactivity in vivo. Eur J Pharmacol, 229:253-258.

Slama M, Susic D, Varagic J, Ahn J, Frohlich ED. (2003). Echocardiographic measurements of cardiac output in rats. Am J Physiol Heart Circ Physiol, 284:H691-697.

Stewart JM, McLeod KJ, Sanyal S, Herzberg G, Montgomery LD. (2004). Relation of postural vasovagal syncope to splanchnic hypervolemia in adolescents. Circulation, 110: 2575–2581.

Stewart JM, Montgomery LD. (2004). Regional blood volume and peripheral blood flow in postural tachycardia syndrome. Am J Physiol Heart Circ Physiol, 287(3):H1319-1327.

Stuart F, Hossack J, Adamson SL. (2011). Micro-ultra sound for preclinical imaging. Interface Focus, 1:576-601.

Sung H-J, Yee, A, Eskin SG, McIntire. (2007) Cyclic strain and motion control produce opposite oxidative responses in two human endothelial cell types. Am J Physiol Cell Physiol, 293:C87-C94.

Titze J, Luft FC, Bauer K, Dietsch P, Lang R, Veelken R et al. (2006). Extrarenal Na+ balance, volume, and blood pressure homeostasis in intact and ovariectomized deoxycorticosterone-acetate salt rats. Hypertension, 47(6):1101-1107.

Tyberg J. (2002). How changes in venous capacitance modulate cardiac output. Eur J Physiol, 445:10-17.

Watts SW, Darios ES, Seitz BM, Thomspon JM (2015) 5-HT is a potent relaxant in rat superior mesenteric veins. Pharmacol Res Perspect, E00103. Doi: 10.1002/prp2.103.

Chapter 4

5-HT causes splanchnic venodilation

Seitz BM, Orer HS, Krieger-Burke T, Darios ES, Thompson JM, Fink GD, Watts SW. (2017). Am J Physiol Heart Circ Physiol, 313(3):H676-H686. doi: 10.1152/ajpheart.00165.2017. Epub 2017 Jun 16.

PMID:28626072

Abstract

Serotonin (5-HT, 5-hydroxytrypamine) causes relaxation of the isolated superior mesenteric vein (SMV), a splanchnic blood vessel, through activation of the 5-HT_7 receptor. As part of studies designed to identify the mechanism(s) through which chronic (≥24 hours) infusion of 5-HT lowers blood pressure, we test the hypothesis that 5-HT causes an *in vitro* and *in vivo* splanchnic venodilation that is 5-HT_7 receptor-dependent. In tissue baths for measurement of isometric contraction, the portal vein (PV) and abdominal inferior vena cava (Ab IVC) relaxed to 5-HT and the 5-$HT_{1/7}$ receptor agonist 5-carboxamidotryptamine (5-CT); relaxation was abolished by the 5-HT_7 receptor antagonist SB269970. Western analyses showed that the Ab IVC and PV express 5-HT_7 receptor protein. By contrast, the thoracic vena cava (TVC), outside of the splanchnic circulation, did not relax to serotonergic agonists and exhibited minimal expression of the 5-HT_7 receptor. Male Sprague Dawley rats with chronically implanted radiotelemetry transmitters underwent repeated ultrasound imaging of abdominal vessels. After baseline imaging, mini-pumps containing vehicle (saline) or 5-HT (25 ug/kg/min) were implanted. Twenty-four hours later, venous diameters were increased in rats with 5-HT-infusion (percent increase from baseline; SMV 17.5±1.9; PV 17.7±1.8; Ab IVC 46.9±8.0) while arterial pressure was decreased (~13 mm Hg).

Measures returned to baseline after infusion termination. In a separate group of animals, treatment with SB269970 (3 mg/kg, i.v.) prevented the splanchnic venodilation and the fall in blood pressure during 24 hours of 5-HT-infusion.Thus, 5-HT causes a 5-HT_7 receptor-dependent splanchnic venous dilation associated with a fall in blood pressure.

Introduction

5-HT was discovered in the cardiovascular system (Greenway, 1983; Page and McCubbin, 1953) but is best known for its actions in the gastrointestinal system and in the central nervous system (Hoyer et al., 2002). Exogenously administered 5-HT causes complex time- and dose-dependent changes in blood pressure and other hemodynamic variables (Hoyer et al., 2002; Watts, 2016; Watts et al., 2012), but it is unclear whether endogenous 5-HT exerts significant effects on overall cardiovascular system function under normal or pathophysiological conditions.

The ability of 5-HT to lower blood pressure, when given *acutely* (< 1 hour duration), has been recognized for decades (Dalton et al., 1986; De Vries et al., 1999; Terron, 1999; Terron et al., 2007). In fact, those that discovered 5-HT were likely the first to demonstrate that acutely infused 5-HT resulted in a fall in blood pressure (dog and human; Page and McCubbin, 1953). In contrast to these studies, we have been interested in identifying the mechanisms by which 5-HT lowers blood pressure when infused *chronically* (24 hours or more). There are two reasons motivating this interest. First, we and others have shown that chronic administration of serotonergic agonists or the 5-HT precursor 5-hydroxytryptophan (5-HTP) can produce a sustained fall in blood pressure in hypertensive animals, potentially suggesting a novel approach to

antihypertensive drug therapy (Diaz, et al., 2008; Dalton et al., 1986; Echizen and Freed, 1981; Itskovitz et al., 1989; Linden et al., 1996). Second, 5-HT is handled dynamically by the body. An elevation in free circulating levels of 5-HT can occur in a number of different ways: greater release from enterochromaffin cells; less uptake or more release from platelets; and less uptake and/or more release from serotonergic neurons (Hoyer et al., 2002). There are thus conditions in which 5-HT could exert a significant effect on regulation of blood pressure and systemic hemodynamics.

In attempting to understand the effects on blood pressure of 5-HT under chronic (≥ 24 hours) conditions, we showed that a one-week infusion of 5-HT (25 ug/kg/min) produced a sustained fall in blood pressure in conscious, unrestrained sham rats. This same infusion significantly reduced the hypertension of the deoxycorticosterone acetate (DOCA)-salt rat (Diaz et al., 2008). The 5-HT-induced fall in blood pressure was dose-dependent (Tan et al., 2011), occurred in males and females (Davis et al., 2011)) and was maintained over a longer 30-day administration (Davis et al., 2013). This chronic fall in blood pressure is qualitatively consistent with the fall in blood pressure that was observed when low doses of 5-HT are given acutely (minutes; De Vries et al., 1999; Terron, 1997; Terron et al., 2007). Use of fluorescent microspheres to measure changes in blood flow supported a focus in splanchnic tissue because 5-HT selectively increased splanchnic blood flow relative to vehicle-infused rats (Seitz and Watts, 2014). We have eliminated two logical targets for reducing blood pressure in the splanchnic vasculature. First, 5-HT does not appear to inhibit the sympathetic nervous system in the splanchnic bed (Darios et al., 2015; Davis et al., 2011). Second, 5-HT does not stimulate a relaxation of isolated arteries greater than 250 microns in diameter,

including the thoracic aorta, abdominal aorta, superior mesenteric artery or mesenteric resistance artery (Davis et al., 2012).

Rather, we discovered that 5-HT directly relaxed the isolated superior mesenteric vein through activation of the 5-HT_7 receptor (Bard et al., 1993; Watts et al., 2015). Others have shown the acute fall in blood pressure seen during 5-HT infusion is due to activation of 5-HT_7 receptors (Terron, 1997). We tested the hypothesis that 5-HT will relax splanchnic veins *in vitro* and cause dilation of splanchnic veins *in vivo* by 5-HT_7 receptor activation. If splanchnic venodilation is one mechanism by which 5-HT causes a chronic fall in blood pressure, then the blood pressure fall and splanchnic venodilation should be attenuated by 5-HT_7 receptor blockade. We use a combination of pharmacology, physiology and a newly developed ultrasound technique to test this hypothesis.

Materials and Methods

Animals

MSU Institutional Animal Care and Use Committee approved all protocols used in this study. Male Sprague Dawley rats (200-350 g; Charles River Laboratories, Portage, MI, USA) were used and were housed in a temperature–controlled room (22°C) with 12-hour light/dark cycles. Animals were given standard chow and distilled water *ad libitum* and housed in standard cages with stainless steel wire lids and containing environmental enrichment (e.g. Bed-r'Nest). The rats used were randomized to *in vitro* and *in vivo* groups, and further randomized within each experimental group (e.g. vehicle, drug-treated). Each n value represents data that came from one (1) animal. When samples were pooled from several animals, this was noted.

Materials

Acetylcholine chloride, forskolin, 5-HT creatinine sulfate, norepinephrine hydrochloride and phenylephrine hydrochloride were obtained from Sigma Chemical Company (St. Louis MO USA). SB269970 and 5-CT maleate were purchased from Tocris (R & D, Minneapolis MN USA) or Abcam (Cambridge MA USA). ET-1 (1-21) was purchased from Bachem (Torrance CA USA).

In Vitro Studies

Tissue preparation for isometric contraction

Naïve rats were anesthetized with pentobarbital (60-80 mg/kg, i.p.) and vessels dissected. The vessels studied were taken from the same rats such that an appropriate intra-animal comparison could be made. All dissections took place under a stereomicroscope and in a Silastic-coated dish filled with physiological salt solution (PSS) containing (mM): NaCl 130; KCl 4.7; KH_2PO_4 1.18; $MgSO_4 \cdot 7H_2O$ 1.17; $NaHCO_3$ 14.8; dextrose 5.5; $CaNa_2EDTA$ 0.03, $CaCl_2$ 1.6 (pH 7.2). The portal vein exiting the liver was dissected away from the liver, the thoracic (above diaphragm) vena cava was removed, and the abdominal inferior vena cava was removed with the abdominal aorta. The abdominal inferior vena cava was dissected away from the abdominal aorta. Once isolated, vessels were guided onto the stabilizing wire, cleaned of fat and used in one of the protocols described below. The endothelium was left intact as validated by a ≤ 50% relaxation to acetylcholine in PGF2α-contracted tissues.

Tissue bath measurement of isometric contraction

Cleaned vessels were cut into rings (~3 mm wide) for measurement of isometric contractile force. Rings were mounted in warmed (37°C) and aerated (95% O_2, 5% CO_2) tissue baths (30 mL PSS) on Grass isometric transducers (FT03; Grass instruments, Quincy, MA, USA), connected to an ADInstruments PowerLab (ADInstruments, Colorado Springs, CO, USA). Tissues were placed under optimal resting tension [portal vein (PV) = 300 mg; thoracic vena cava (TVC) and abdominal inferior vena cava (Ab IVC) = 1000 mg] and equilibrated for 1 hour before an initial challenge with a maximal concentration of norepinephrine (NE; 10^{-5} M). These tensions were determined in experiments for each tissue that allowed us to determine the applied tension that generated a maximum active response. Initial contractions to NE were: PV = 394.6±98.6 mg; Ab IVC = 366.0±41.6 mg; TVC = 53.7±6.1 mg. After this challenge, tissues were washed until tone returned to baseline. Preliminary experiments determined that endothelin-1 (ET-1) caused a stable contraction in vessels. The ET-1 concentration used was submaximal, achieving ~40-50% of maximal ET-1-induced contraction. Once ET-1-induced contraction was stable (~12-15 min after addition), increasing concentrations of the serotonin receptor agonist 5-HT or 5-CT (1) was added cumulatively (10^{-10} – 10^{-5} M). Only one agonist was examined in each tissue. In some experiments, vehicle or the 5-HT_7 receptor antagonist SB269970 was added 45 minutes prior to adding ET-1. Tissues that did not relax to agonist were incubated with the adenylate cyclase stimulator forskolin (10^{-5} M) to determine the relaxing potential of the vessel.

Western Blot Analysis

Vessels were cleaned, frozen, and ground into a powder using a mortar and a pestle. Two to three of the same type of vessels were pooled from multiple rats for each lane upon finding that use of tissue from one animal provided insufficient material to be able to detect the 5-HT_7 receptor. 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 and sonicated for 5 cycles, and centrifuged. Supernatants were collected and protein concentration was determined with the BCA protein kit (catalog #BCA1, Sigma Chemical Co, St. Louis MO USA). SDS-PAGE separation of proteins in tissue homogenates (50 ug) was performed and proteins transferred to PVDF. Positive controls [rat brain for 5-HT_7 receptor (50 ug)] were run in parallel lanes along with molecular weight markers. Blots were incubated overnight at 4°C with 5-HT_7 primary antibody (catalog # ab13898, 1:1000; Abcam, Cambridge MA USA), washed three times with TBS and incubated with secondary antibody (catalog #7074, 1:1000; anti-rabbit HRP linked IgG, Cell Signaling Technology, Danvers MA USA). The same blots were reprobed for smooth muscle α-actin (catalog # 113200, primary antibody = mouse, 1:2000; EMD Chemicals/Calbiochem, Gibbstown NJ USA; secondary antibody = 1:1000, anti-mouse HRP-linked IgG, GE Healthcare Life Sciences, Marlborough MA USA) to compare protein loading. Blots were developed using species-specific HRP-conjugated secondary antibodies and ECL reagents (GE Healthcare Life Sciences Piscataway, NJ USA) on a Kodak X-OMAT Film Processor 2000A. Exposures were 16 seconds for α-actin, 24 minutes for 5-HT_7 receptor. This receptor antibody was

validated previously using brain homogenates as a positive control, and visualization of a band consistent with the expected molecular weight (Watts et al., 2015).

In Vivo Studies

Telemeters

Radiotelemeter transmitters (C40 model; Data Sciences International, MN) with attached catheters with pressure-sensing tips were implanted subcutaneously as described previously (Diaz et al., 2008). Rats recovered 5 days post-operatively, and then 3 days of baseline cardiovascular measurements were made. Mean arterial pressure, pulse pressure and heart rate were recorded for 10 seconds every 10 minutes throughout the duration of the study. On the final baseline day, rats underwent baseline ultrasound imaging of the portal vein, abdominal inferior vena cava, abdominal aorta, and superior mesenteric vein (described below).

Pump Implantation

Osmotic pumps (Model 2ML1; Alzet, Durect, Cupertino, CA, US) containing either 5-HT (at a concentration calculated to deliver 25 ug/kg/min) or vehicle [1% ascorbate (antioxidant) in sterile saline, pH balanced to 7.4-7.6] were implanted subcutaneously between the scapulae in anesthetized rats (isoflurane 1-2%). In some experiments, osmotic pumps were removed under isoflurane anesthesia. Pumps were weighed before and after study as a validation of pump function.

Ultrasound Imaging

More details on this technique are reported in reference 40. Rats (isoflurane-anesthetized, 1-2%) were positioned supine on a warmed platform (Vevo 2100 Imaging

System; Visualsonics, Toronto, Canada). Ultrasound gel was applied to each front and hind paw to obtain measures of heart rate, respiration and electrocardiogram (ECG). Warmed ultrasound gel was applied to the abdominal skin, just below the xiphoid process to couple the transducer (21 MHz probe; MS250) before imaging in B-mode. Two separate real-time images were scanned at 25 frames per second: 1) an image at the location of the portal vein exiting the liver, which provides images of the abdominal aorta, abdominal inferior vena cava and portal vein; 2) an image at the level just below the branching of the portal vein toward the spleen via the splenic vein, which provides images of the superior mesenteric vein, splenic vein and abdominal inferior vena cava and aorta for an arterial comparison. Each session took ~10 minutes per rat, minimizing the amount of time each animal was under anesthesia.

Three separate *in vivo* experiments were performed. All included radiotelemetry implanted male Sprague Dawley rats as described above.

Experiment 1-(*5-HT infusion alone; vessel diameter and hemodynamics*): After baseline imaging, rats were randomized into two treatment groups. They were implanted with osmotic mini-pumps (2ML1, Alzet) containing either 5-HT (25 ug/kg/min; group 1) or vehicle [1% ascorbate (antioxidant); group 2]. Imaging was performed 24-hours post pump implantation, pumps removed and imaging repeated one-week post pump removal. Pressures (mean, systolic and diastolic) and heart rate were continuously recorded in a conscious state throughout this study, except during imaging.

Experiment 2-(5-HT infusion with SB269970 blockade; hemodynamics only*):* All animals were implanted with an indwelling femoral vein catheter and placed in tether jackets. Animals were randomized into two treatment groups. Infusion (iv) of SB269970 (2 mg/kg/hr; group 1); or saline (group 2) was started one hour prior to the implantation of 5-HT osmotic pumps (25 ug/kg/min; 2ML1, Alzet). Infusion was performed because of the short half-life of SB269970 (Hagan et al., 2000). Infusion was continued for the subsequent 24 hours in the presence of 5-HT-containing osmotic pump. SB269970 and saline infusion were stopped while 5-HT infusion continued and blood pressure was measured for an additional 24 hours.

Experiment 3-(5-HT infusion with SB269970 blockade; vessel diameter and hemodynamics)*:* After baseline images of noted vessels, all animals were implanted with an indwelling femoral vein catheter and placed in tether jackets. Animals were randomized and placed into one of three treatment groups: 5-HT + saline (group 1); 5-HT + SB269970 (group 2) and saline + saline (control; group 3). Animals were implanted with either a 5-HT (25 ug/kg/min) or saline osmotic pump and given an iv infusion of either SB269970 or saline. The same surgical protocol and experimental paradigm as in experiment 2 (above) were used. Venous diameters and cardiovascular parameters were collected at baseline and at 24 hours post 5-HT infusion for all three groups.

Data Analyses

Quantitative data are reported as means±SEM for number of animals in parentheses. For isometric contractile measures, relaxation is reported as a percentage

of initial contraction to a half-maximal concentration of ET-1; values of absolute contraction to NE, ET-1 (milligrams) are reported in the methods. Agonist potencies were calculated using a non-linear regression (curve fit) within GraphPad® Prism 6.0 (La Jolla, CA, USA), and are reported as –log EC_{50} values [M]; they were calculated only when maximums could be achieved. Maximums are reported as the maximal effect achieved. Images of Western blots were processed through Photoshop (CC 2014), and were not modified from the original acquisition. Values for Western analyses were densitized in Image J (1.47v, Wayne Rasband, NIH), and data reported as arbitrary densitometry units relative to alpha-actin densitometry units. Data are reported as pooled samples/n values (total number of lanes) for the total number of animals used.

For measurements made on ultrasound images, the same person, who has considerable experience in the use of the Vevo Imaging system and was blinded to the nature of the infusion group, analyzed each vessel diameter (Seitz et al., 2016). All measurements were controlled for respiration and cardiac cycles. Vessel diameters and CV parameters are reported as a maximum percentage (%) change from baseline. Baseline values are reported in Tables 1 and 2.

Statistical Analysis

For *in vitro* measures, t-tests to compare responses at maximum concentration of agonist were used. For densitometry, a Grubs test (extreme studentized deviate) was performed to determine whether the most extreme value was a significant outlier. Multiple tissues went in to making samples for each lane, and we report both the number of total animals and lanes (n) for the Western experiments. For *in vivo* measures, statistical analysis was performed using paired two-tailed t-tests between 2

groups. A repeated measures ANOVA was used when comparing pressure or vessel diameter values from baseline (GraphPad Prism 6). In all cases, a p value of <0.05 was considered significant.

Parameter	Vehicle group (N=9)	5-HT group (N=9)
Mean Arterial Pressure (mm Hg)	101.4±2.1	96.8±2.1
Systolic Pressure (mm Hg)	117.2±3.7	119.5±1.8
Diastolic Pressure (mm Hg)	88.4±2.3	82.5±1.2*
Heart Rate (bpm)	389±22	379±8
	VESSEL DIAMETERS	
SMV (mm)	1.85±0.04	1.81±0.05
PV (mm)	2.13±0.06	2.08±0.03
Ab IVC (mm)	3.53±0.13	3.16±0.12
Ab Aorta (mm)	2.14±0.04	2.20±0.05

Table 4.1:Baseline physiologic parameters and venous diameters of vehicle and control group *prior* to pump implantation for animals in figure 4.5. Values are means±SEM for number of animals in parentheses. *significantly different from vehicle measure ($p<0.05$).5-HT, 5-hydroxytryptamine; SMV, superior mesenteric vein; PV, portal vein; Ab IVC, abdominal inferior vena cava; AB Aorta, abdominal aorta.

	5-HT + saline (N=6)	5-HT + SB 269970 (N=6)	Saline + saline (N=4)
Mean Arterial Pressure (mmHg)	102.0±3.3	102.2±2.9	99.7±0.7
Systolic Pressure (mmHg)	121.5±3.7	126.8±3.4	120.1±0.6
Diastolic Pressure (mmHg)	87.7±3.8	88.8±1.9	84.2±1.0
Heart Rate (bpm)	394±8	394±13	392±12
	VESSEL DIAMETERS		
SMV (mm)	1.80±0.05	1.80±0.07	1.80±0.03
PV (mm)	2.00±0.04	2.10±0.09	2.10±0.05
Ab IVC (mm)	2.80±0.02	3.30±0.25	3.00±0.27
Ab Aorta (mm)	2.30±0.06	2.30±0.03	2.10±0.02

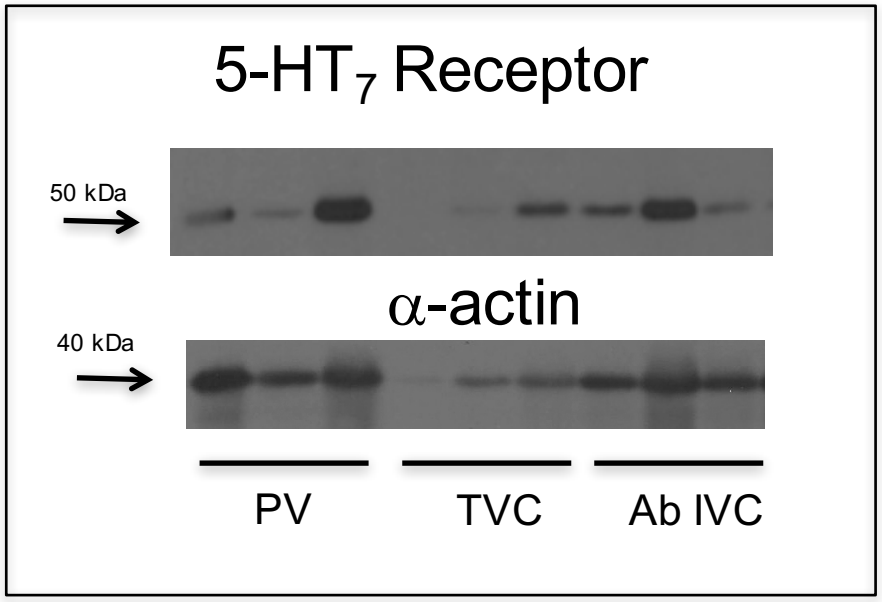
Table 4.2: Baseline physiologic parameters and venous diameters of 5-HT+ saline, 5-HT + SB269970 and saline + saline groups *prior* to pump implantation for animals in figure 4.7. Values are means±SEM for number of animals in parentheses. 5-HT, 5-hydroxytryptamine; SB269970, 5-HT_7 receptor antagonist; SMV, superior mesenteric vein; PV, portal vein; Ab IVC, abdominal inferior vena cava; AB Aorta, abdominal aorta.

Results

5-HT$_7$ receptors are expressed in splanchnic veins

We first investigated 5-HT$_7$ receptor expression in two splanchnic (PV, Ab IVC) and one non-splanchnic vein (TVC), using Western analyses. We have previously used this antibody, validated by the detection of brain protein at the appropriate molecular weight (Watts et al., 2015). Each vessel type was pooled from three animals such that the lanes depicted in figure 1A represent a total of 9 animals (3 lanes x 3 animals from which the three different veins were taken). The 5-HT$_7$ receptor was robustly expressed in the Ab IVC, moderately expressed in the PV and less so in the TVC. Because of the variability in 5-HT$_7$ receptor expression observed in the PV and TVC, this experiment was repeated again for the PV and TVC. Figure 1B combines data from these two separate experiments, with 5-HT$_7$ receptor expression reported as a percentage of α-actin expression. One statistical outlier was observed in both PV and IVC groups. This point for each group was removed to make the bar graphs presented in figure 4.1B. These bars represent a total of 22, 22 and 9 rats for PV, TVC and Ab IVC, respectively. There were no significant differences amongst these values.

A.



B.

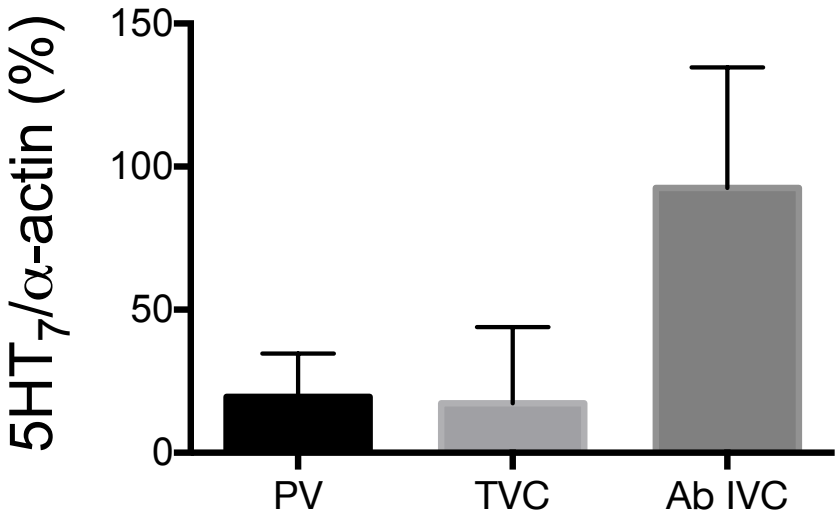


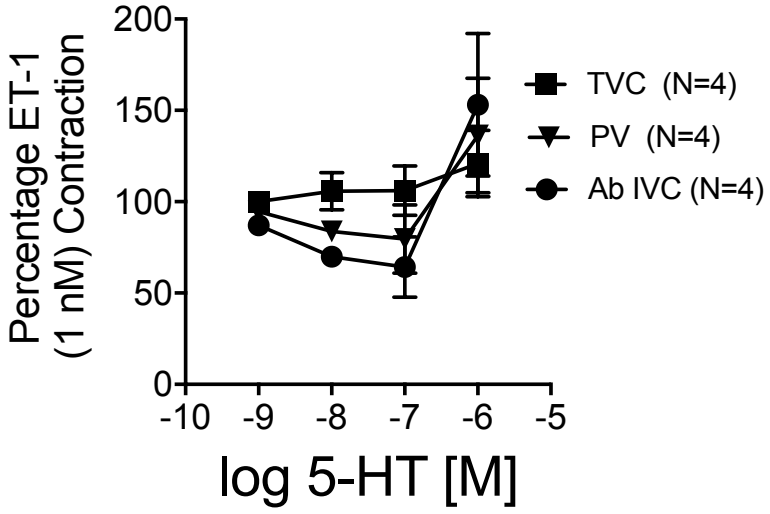
Figure 4.1: **Western analyses and densitometry of the 5-HT$_7$ receptor on splanchnic veins.** (**A**). Western analyses of the 5-HT$_7$ receptor in homogenates of portal vein (PV), thoracic (above diaphragm) vena cava (TVC) and abdominal inferior vena cava (Ab IVC) from the same set of 9 animals (3 animals per lane, 3 lanes). Blot was reprobed with α-actin. (**B**). Densitometry of all non-outlying values for 5-HT$_7$ receptor signal relative to α -actin expression. Bars represent means$\pm$SEM for number of lanes and animals as specified on graph.

Isolated splanchnic veins relax to 5-HT and 5-CT

Veins, contracted to ET-1, relaxed to cumulative additions of 5-HT. The maximal relaxant efficacy of 5-HT was as follows (% ET-1-contraction remaining): Ab IVC = 64.3±16.6; PV = 79.5±18.7; no relaxation in TVC (figure 4.2A). All tissues relaxed completely to the adenylate cyclase stimulator forskolin (10 μM). At high concentrations of 5-HT (1 μM), contraction was observed, consistent with the interaction of 5-HT with the 5-HT_{2A} receptor in smooth muscle (Watts et al., 2015). The potency of 5-HT in relaxing the veins was not calculated because of the biphasic nature of these curves. By comparison, 5-CT was more efficacious compared to 5-HT. 5-CT has high affinity for the 5-HT_7 receptor and, compared to 5-HT, considerably lower affinity for the 5-HT_{2A} receptors that mediate contraction in most vessels (1).

The maximum relaxant efficacy of 5-CT was as follows (% ET contraction remaining): Ab IVC (6.6±4.5) >PV (27.8±11.2)>>TVC (74.9±7.2) (figure 2B). The potency of 5-CT (-log EC_{50} [M]) was calculated for the Ab IVC (8.22±0.42), PV (7.88±0.28) and TVC (8.10±0.80). The 5-HT_7 receptor antagonist SB269970 blocked 5-CT-induced relaxation in the isolated Ab IVC and PV (figure 4.3A and B, respectively), and contraction consistent with 5-HT_{2A} receptor activation was observed at high concentrations just as it was for 5-HT (above). The TVC was not tested because of the low efficacy of relaxation to 5-HT and 5-CT. Collectively, these experiments support the ability of serotonergic agonists to directly relax the isolated splanchnic veins through activation of a 5-HT_7 receptor.

A.



B.

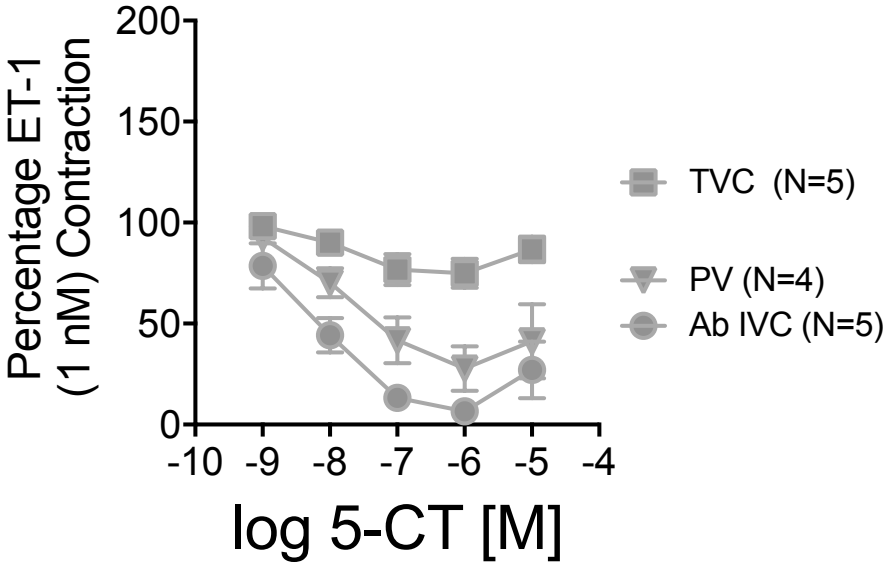
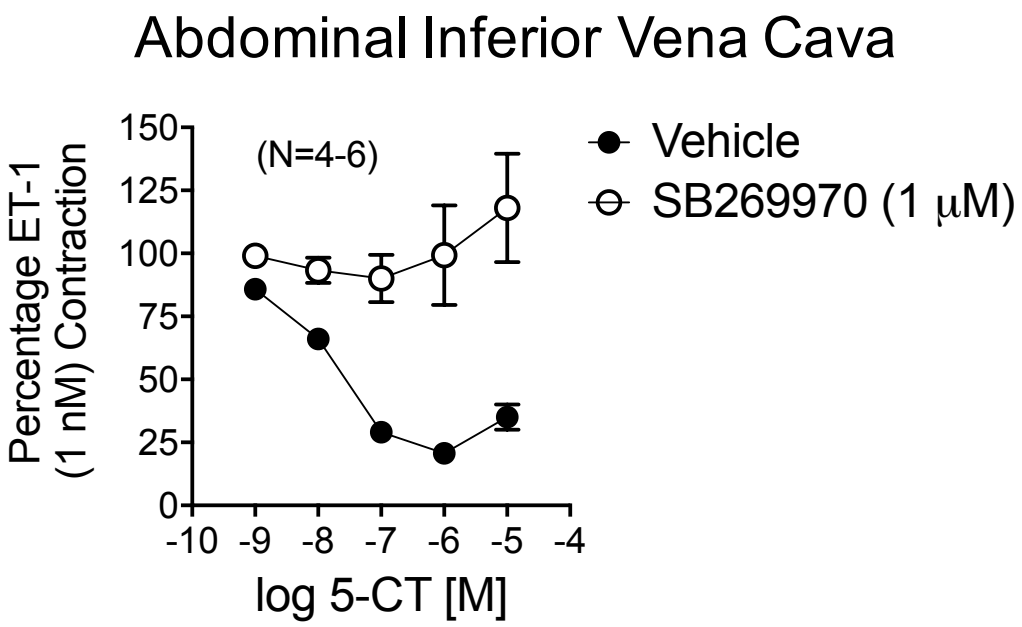


Figure 4.2: **Relaxation of ET-1 contracted splanchnic vein to 5-HT and 5-CT**. (**A**). Relaxation of ET-1-contracted isolated thoracic VC (above diaphragm; TVC), portal vein (PV) and abdominal inferior vena cava (Ab IVC) to 5-HT (A) and 5-CT (**B**). The magnitude of venous contraction to 1 nM ET-1 was: PV = 279.1±44.6 mg; Ab IVC = 206.2±54.7 mg; TVC = 66.1±7.0 mg. Points represent means±SEM for number of animals in parentheses. Veins were taken from the same animals for intra-vessel comparison of sensitivity to each agonist.

A.



B.

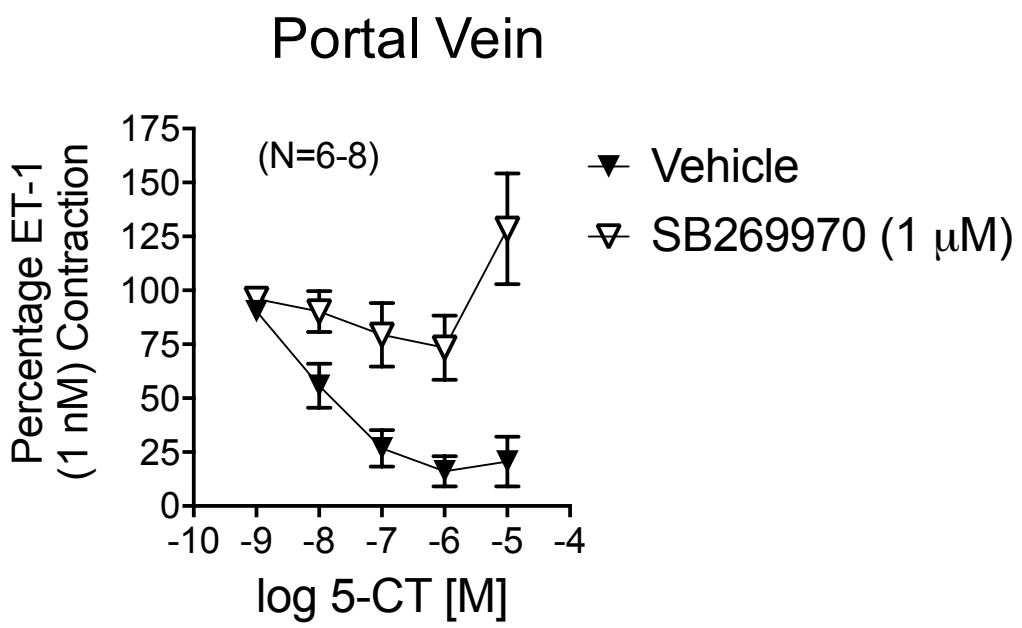
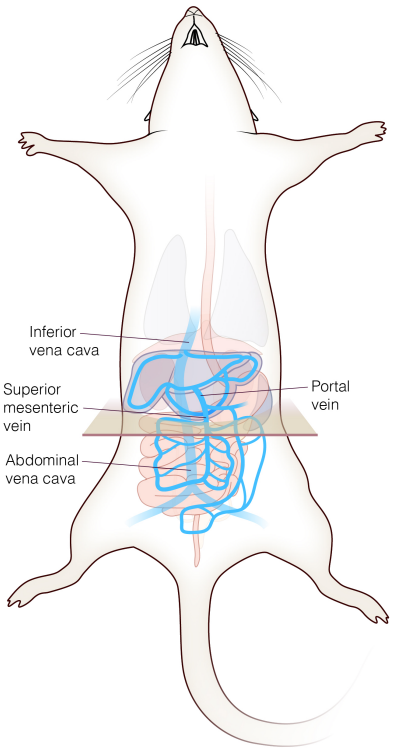


Figure 4.3: Effect of the 5-HT$_7$ receptor antagonist to antagonize 5-CT induced relaxation in Ab IVC and PV. Ability of the 5-HT$_7$ receptor antagonist SB269960 to antagonize 5-CT-induced relaxation in the isolated Ab IVC (**A**) and PV (**B**). Points represent means±SEM for number of animals in parentheses. These veins were taken from the same animals for intra-arterial comparison. * significant difference *vs* vehicle-exposed tissues at maximum agonist concentration tested ($p<0.05$).

5-HT infusion results in splanchnic venodilation in vivo

Radiotelemetery-implanted rats were randomly assigned to one of two groups: one receiving an osmotic pump with vehicle and the other 5-HT. Baseline physiological parameters (venous diameters, mean arterial blood pressure, heart rate, systolic blood pressure, and diastolic blood pressure) prior to pump implantation are reported in table 4.1. Parameters were similar between these two groups, except for diastolic blood pressure, which was lower in the 5-HT group when compared to the vehicle group. Figure 4.4A depicts the first position of ultrasound visualization (tan box); the box was moved 2 mm caudally for imaging the SMV. Figure 4.4B shows representative ultrasound images comparing venous diameter before (left) and after (right) 24 hours of 5-HT administration. The vessel diameters and physiological parameters of rats given vehicle or 5-HT containing pumps are quantified in figure 4.5 at *24-hours post* implantation and one week following pump *removal*. Venous diameter increased with 5-HT infusion by over 17% in all veins, with the Ab IVC being particularly responsive (>40% change; figure 4.5A). Additionally, the SMV, which we have published as having 5-HT_7 receptors that relax to 5-CT (Watts et al., 2015), demonstrated *in vivo* dilation to 5-HT. By contrast, the Ab Aorta demonstrated no change in internal diameter with 5-HT infusion (-1.59±0.86%). This artery was investigated as an arterial comparison to the venous vessels. In parallel, blood pressures (mean, systolic, diastolic) were reduced (-12.58±1.17%) while heart rate was modestly elevated compared to their baseline values and *vs* vehicle infused rats (figure 4.5B). These measures were taken during the morning when the gastrointestinal system exhibited naturally low peristaltic activity, given that 5-HT increases the prokinetic action of the intestine (Hoyer et al., 2002)

A.



B.

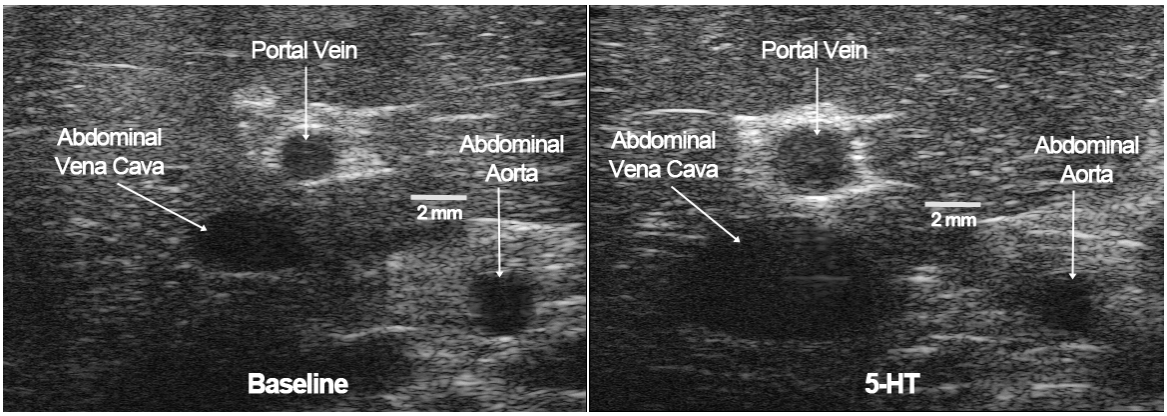
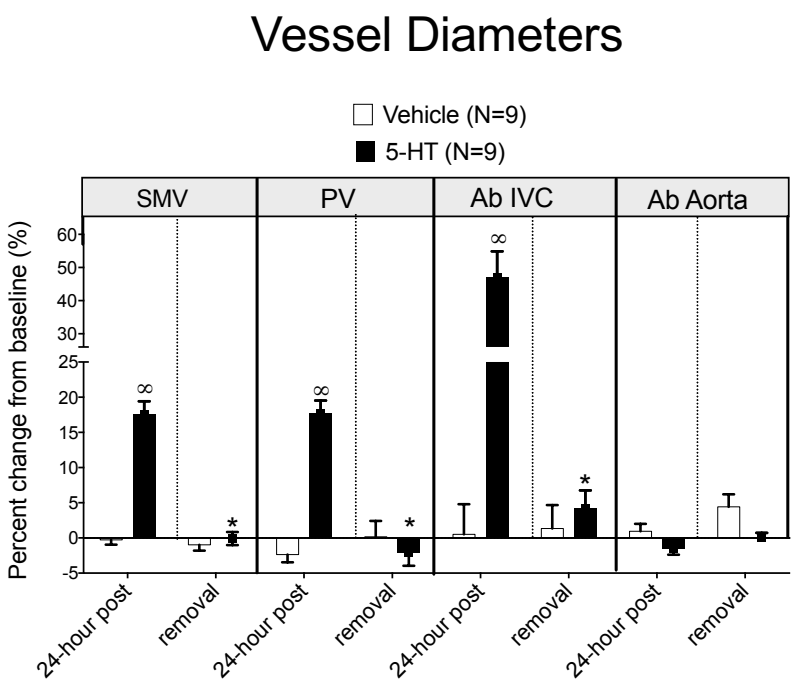


Figure 4.4: Illustration of transducer placement and B-mode image of abdominal vessels. (**A**) Diagram of ultrasound wand/transducer placement for image procurement using the Vevo2100. Tan box shows initial position of the plane imaged by the ultrasound probe. Drawing @ Chris McKee. (**B**) Representative B-mode ultrasound images taken at baseline (left) and 24 hours post 5-HT (right) in the same rat. Horizontal lines quantify size.

A.



B.

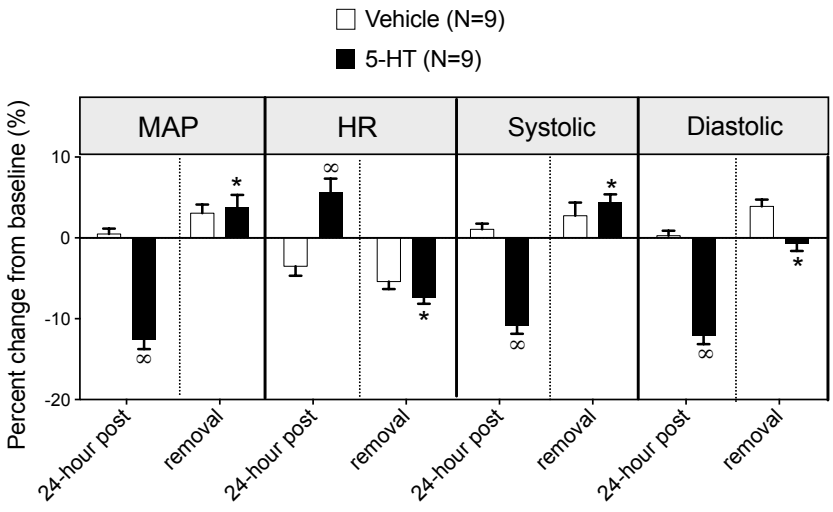


Figure 4.5: Effects of 5-HT on vessel diameter and cardiovascular parameters. Change in vessel diameter (**A**) and physiological parameters (**B**) with infusion of vehicle (open bars) and 5-HT (filled bars). Bars represent means±SEM for number of animals indicated in parentheses (N). * indicate statistical differences between 5-HT 24 hour post and 5-HT reversal. ∞ indicates significant differences between vehicle and 5-HT 24 hours post.

Splanchnic venous dilation is mediated through the 5-HT$_7$ receptor

Our final experiment was to determine if the splanchnic venodilation and fall in blood pressure caused by 5-HT were both mediated by the 5-HT$_7$ receptor. This was done using the 5-HT$_7$ receptor antagonist SB269970. In validation experiments, this dosing protocol for SB269970 abolished the acute hypotension caused by 5-CT administered iv (1 ug/kg 5-CT: vehicle = -17.3±2.2 mm Hg from baseline; SB269970 = -1.1±1.4 mm Hg; $p< 0.05$, n=5-8).

Figure 4.6A shows mean arterial blood pressure, demonstrating a significant fall in blood pressure in those animals receiving 5-HT plus vehicle infusion but not in those receiving 5-HT plus SB269970. Removal of SB269970 infusion from animals still receiving 5-HT resulted in a fall in blood pressure. Figure 4.6B compares the magnitude of fall in blood pressure to 5-HT within 24 hours of infusion in these two groups of rats. SB269970 significantly attenuated the fall in blood pressure to 5-HT compared to vehicle-infused rats (13.3±2.4 to 3.0±1.5 mm Hg).

We performed an additional experiment to investigate the association between 5-HT$_7$ receptor mediated effects on blood pressure and venous dimensions within our infusion model. This was, unlike the above two *in vivo* experiments, done without reversal/removal of infusion. Table 4.2 reports the venous diameters and baseline cardiovascular parameters for the three groups of animals used. Splanchnic venous diameters are reported in figure 4.7A. The control (saline+saline; grey bars) animals demonstrated small, variable changes in vessel diameter in the SMV, PV and Ab IVC with no change in the Ab Aorta. 5-HT infusion alone caused a modest venodilation in the SMV and a significant venodilation in the PV (7.9±2.4%) and Ab IVC (39.9±7.2%),

consistent with data presented in figure 4.2. Infusion of SB269970 along with 5-HT abolished the venodilation in these splanchnic vessels. Figure 4.7B reports the cardiovascular parameters of these same rats. Blood pressure (mean, systolic, diastolic) and HR in the control rats (saline + saline) did not change during the course of this experiment. 5-HT alone (5-HT+ saline) reduced blood pressures (mean = -18.6±2.0%) and elevated heart rate, consistent with data reported in figure 4.2. SB269970 abolished the 5-HT-induced reduction in blood pressure and elevation in HR (figure 4.7B), in line with results of experiments shown in figure 4.6. Thus, 5-HT_7 receptor blockade prevented both 5-HT-induced splanchnic venous dilation and hypotension.

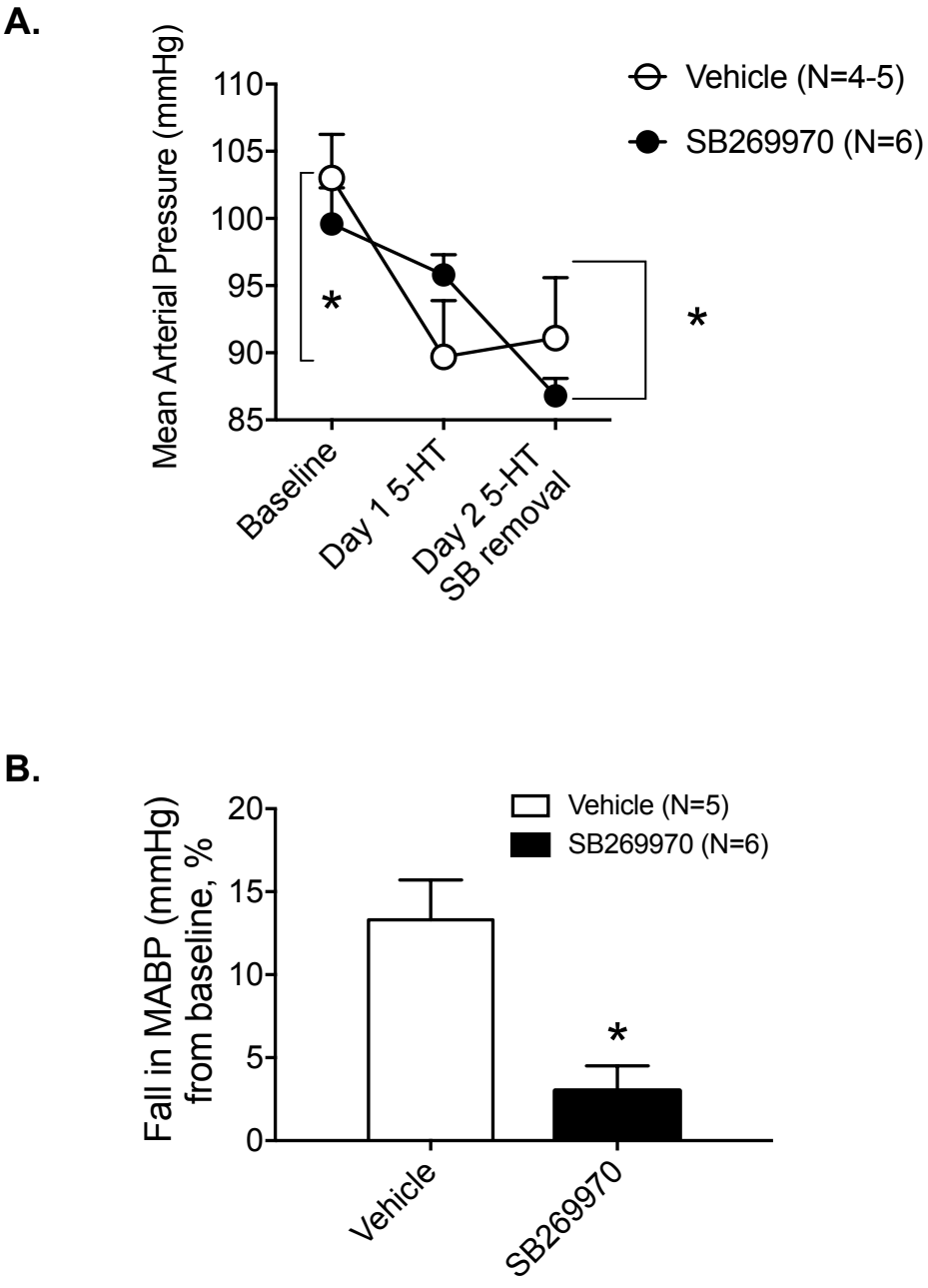


Figure 4.6: **Mean arterial pressure effects of 5-HT mediated by the 5-HT$_7$ receptor.** Mean arterial blood pressure (**A**) and magnitude of fall in blood pressure (% baseline; **(B**) in rats infused with SB269970 or vehicle before a pump implantation with 5-HT (25 μg/kg/min). Measures of blood pressure were done 24 hours after pump implantation and then again after SB removal. Points/bars represent means±SEM for number of animals in parentheses. * indicate statistical differences between the two bracketed bars.

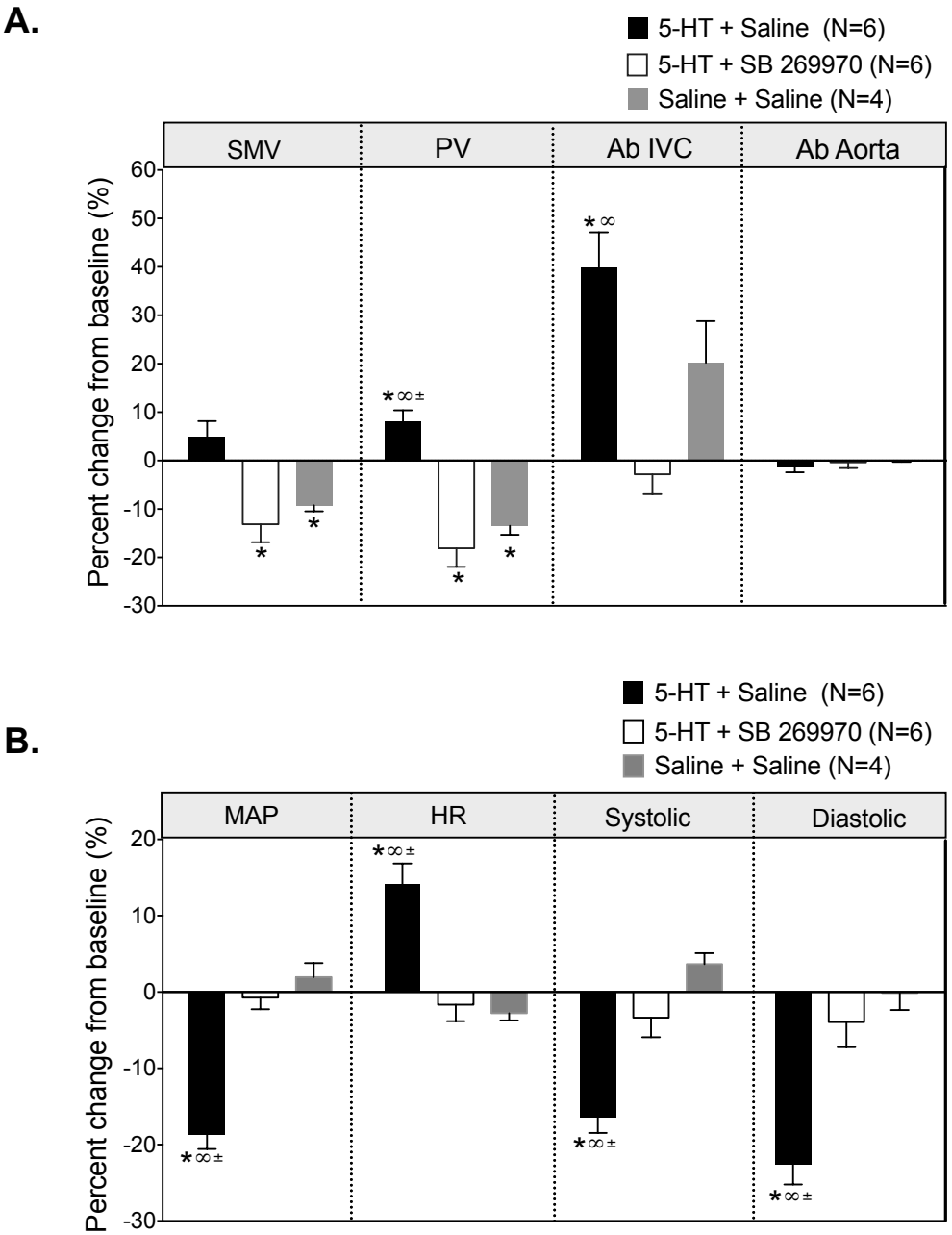


Figure 4.7: **5-HT-induced changes in vessel diameter and cardiovascular parameters and effects of 5-HT7 receptor antagonist.** 5-HT-induced changes in vessels diameters (**A**) and cardiovascular parameters (**B**) in rats infused with SB269970 or vehicle before a pump implantation with 5-HT (25 μg/kg/min). Measures of blood pressure were done 24 hours after pump implantation. Saline + Saline animals were used as controls for infusion of vehicles that carried both 5-HT and SB269970. Bars represent means±SEM for number of animals in parentheses. *=significant from

Discussion

Veins vs Arteries

Splanchnic veins (and abdominal vena cava) relaxed to serotonergic agonists, both *in vitro* and *in vivo*, in a manner dependent on activation of the 5-HT_7 receptor. Our focus on the splanchnic venous system, in attempting to understand the hemodynamic effects of chronic 5-HT infusion, was predicated on several previous findings. First, the superior mesenteric vein relaxed to 5-HT at a lower concentration of 5-HT than arteries contracted to 5-HT (Darios et al., 2015; Watts et al., 2015). A number of different veins relax directly to 5-HT [goat pulmonary vein (Chand, 1981); sheep pulmonary vein (Cocks and Arnold, 1992; Zhang et al., 1995); rat jugular vein (Ellis et al.,1995); guinea pig jugular vein (Gupta, 1992), pig pial veins (Ishine et al., 2000); monkey jugular vein (Leung et al., 1996); pig vena cava (Sumner et al., 1989; Trevethick et al., 1984); and rabbit facial vein (Tsuru et al.,1998)]. By contrast, the human saphenous vein contracted to 5-HT (Schwartz et al., 1992) while cremaster post-capillary venules show significant macromolecular leakage in hypercholesterolemic rats (Scguschke et al., 1991). Thus, a majority of studies support venous relaxation to 5-HT through activation of the 5-HT_7 receptor.

Second, the thoracic vena cava (non-splanchnic, central compartment) had the smallest relaxation (efficacy) to both 5-HT and 5-CT and low 5-HT_7 receptor expression (figure 1, 2). 5-HT_7 receptor protein was, however, detected in some samples but did not mediate agonist-induced relaxation. There is precedent for a 5-HT receptor being present, but not active in modifying vascular tone, in the 5-HT_{2B} receptor expressed in arteries from normotensive rats (Banes and Watts, 2003). This contrasts with the 5-HT_7

receptor expression in splanchnic veins, and the serotonergic agonist-induced relaxation that occurs in the splanchnic veins. At least in the rat, there appears to be a relative selective functioning of the 5-HT_7 receptor in splanchnic (and abdominal) veins. We cannot state that this response is unique to these veins as the only other area we have investigated is the thoracic compartment.

Third and finally, we have been unable to show direct arterial relaxation to 5-HT; relaxant receptors do exist in arteries but in vessels not normally thought of as regulating blood pressure [e.g. 5-HT_{2B} and 5-HT_7 receptor in pig pulmonary artery (Jahnichen et al., 2005); dog coronary artery (Cushing et al., 1996); canine cerebral arteries (Terron and Falcon-Neri, 1999)]. Published work conflicts as to whether 5-HT relaxes small arterioles, vessels clearly important in blood pressure regulation (Altura, 1975; Kurita et al., 1999; Selke and Dai, 1993; Selke et al., 1994; Struyker-Boudler et al., 1990; Takahashi et al., 2000; Wilmoth et al., 1984). Though not addressed in the present study, relaxation of arterioles must remain another possible mechanism by which 5-HT reduces blood pressure and exerts other hemodynamic effects.

Venodilation In Vivo

Given the ability of 5-HT to relax veins *via* the 5-HT_7 receptor, and earlier evidence that acute 5-HT induced hypotension is caused by 5-HT_7 receptor activation (De Vries et al., 1999; Terron, 1997), we hypothesized that venodilation contributes to the chronic depressor response of 5-HT. That idea was strongly supported, but not proven, by the finding reported here that blockade of 5-HT_7 receptors with SB269970 virtually eliminated the fall in blood pressure normally seen during chronic 5-HT infusion, as well as the accompanying splanchnic venodilation. Although 5-HT was shown

previously to increase vascular capacitance during acute administration in dogs (Headberg and Ruten et al., 1990), consistent with a venodilator action *in vivo*, no studies have investigated venous responses during chronic infusion. It should be noted, however, that other agents that are relatively selective venodilators can lower blood pressure chronically (Miller et al., 1982). Furthermore, it is known that directly reducing splanchnic venous capacitance in humans can result in sustained increases in blood pressure (Okamoto et al., 2016). The detection of venodilation through the deeper splanchnic system was limited in that we did not examine small vessels (< 250 microns diameter) in the imaging or *in vitro* studies, and thus we cannot state if all veins of the splanchnic circulation relax to 5-HT. The importance of the splanchnic venous system in man with regards to regulating blood pressure was recently supported by Okamoto *et al* (Okamoto et al., 2016). In this study, the authors demonstrated that a servo-controlled abdominal binder was as effective as the standard of care, midodrine, in treatment of orthostatic hypotension. The mechanism by which this worked is to prevent the splanchnic venous pooling believed to contribute to orthostatic hypotension.

Understanding venous circulation regulation is important because it is a physiological partner of the arterial circulation, and their integrative function assures cardiovascular homeostasis (Fink, 2009; Reddi and Carpenter, 2005). Regulation of arterial pressure is accomplished *via* integrated control of total peripheral resistance and cardiac output; the former is achieved mainly by contraction of small arteries and arterioles, and the latter by regulation of cardiac pumping ability and venous return to the heart. Overall, venous return to the heart is largely dependent on the tone of smooth muscle in the small veins and venules of the splanchnic bed (Greenway, 1983).

Constriction of these vessels reduces vascular capacitance (blood storage) and redistributes “unstressed” blood volume into the central circulation, thereby augmenting cardiac output. These facts suggest that splanchnic venodilation would be expected to lower blood pressure by reducing cardiac output – which is not the hemodynamic pattern that we observed in our model (Brengelmann, 2003; Davis et al., 2012). We cannot resolve this dilemma at this time, but it’s possible that autoregulation or other adaptive responses convert an initial 5-HT induced fall in cardiac output to a reduction in vascular resistance.

There are several limitations to consider in our *in vivo* work. First, all the measures made with the Vevo 2100 ultrasound system were done in anesthetized rats. We may have underestimated the venodilation caused by 5-HT given that blood pressure is lowered by anesthesia. However, in this protocol, 5-HT still caused a fall in blood pressure, meaning there was a signal to measure in the anesthetized rat. Second, we do not know of any specific interaction of 5-HT with isoflurane that could affect the outcome of these studies. Third, our tethered animals in the chronic pharmacology experiments showed a smaller fall (~13 mm Hg) to 5-HT than in non-tethered animals (~22 mm Hg). Tethered animals are stressed, and this was also reflected in the modest venoconstriction observed in the animals shown in figure 4.7A.

5-HT in cardiovascular physiology

An important question is whether endogenous 5-HT could produce active venodilation in the splanchnic and abdominal regions under physiological or pathophysiological circumstances. Circulating levels of 5-HT are at a sufficient concentration to activate the 5-HT_7 receptor, for which 5-HT has sub nM affinity (Bard et

al., 1993). Specifically, we detected 2-10 ng/ml free 5-HT in normal rats (Diaz et al., 2008), levels also within the range observed in human platelet free plasma (Monaghan et al., 2009). Thus, it is possible that venous 5-HT_7 receptors are activated under normal conditions, and this idea will be pursued in future studies. In hypertension, ours and other studies show that basal levels of circulating 5-HT (non-platelet) are elevated in rodent and human (Diaz et al., 2008; Watts et al., 2012). Specifically, in the DOCA-salt model, the basal level of 5-HT was 24.9±5.06 ng/ml, significantly higher than the sham at 2.7±0.29 ng/ml free 5-HT. This raises the possibility that an active 5-HT_7 receptor-mediated venodilator mechanism may abrogate high blood pressure; this is also of interest for future work. A recent finding in human subjects is potentially relevant here: advanced heart failure was found to be associated with elevated circulating levels of 5-HT human heart failure, a chronic disease indisputably influenced by the venous system (Selim et al., 2016).

There are several independent lines of investigation that make wanting to understand the mechanism(s) of a chronic 5-HT-induced hypotension compelling. Page and colleagues identified 5-HT as a molecule that could lower blood pressure in the hypertensive man, but the mechanisms were not uncovered (Page and McCubbin, 1953) and 5-HT given chronically can reduce blood pressure in the experimentally hypertensive rat (Diaz et al., 2008). Importantly, administration of the 5-HT precursor 5-HTP acutely and chronically (12 days) reduced blood pressure of the normal male Sprague-Dawley, spontaneously hypertensive and Dahl salt sensitive rat (Baron et al., 1991; Dalton et al., 1986; Ding et al., 1989; Echizen and Freed, 1981; Itskovitz et al., 1989). Fregly *et al*. (Fregly et al., 1987) demonstrated that chronic treatment with 5-

HTP *prevented* the development of DOCA-salt hypertension, but described no mechanism for how this occurred. While we have not investigated splanchnic venodilation in hypertensive rats, the intent is to determine if a 5-HT_7 receptor-related mechanism contributes to reduce blood pressure (Diaz et al., 2008), and thus could be a potential therapeutic approach. The present study is the first significant positive and directional lead in our understanding of at least one mechanism(s) of 5-HT-induced reduction in blood pressure at a tissue and whole animal level, and it lays the foundation for moving forward into studies in hypertension.

In summary, these findings support the ability of 5-HT infusion to act as a splanchnic venodilator both *in vitro* and *in vivo*. Venodilation was accompanied by a fall in blood pressure and both the 5-HT-induced venodilation and hypotension were blocked by the 5-HT_7 receptor antagonist SB269970.

REFERENCES

REFERENCES

www.pdsp.unc.edu; 5-CT as test ligand.

Altura BM. (1975). Sex and estrogens and responsiveness of terminal arterioles to neurohypophyseal hormones and catecholamines. J Pharmacol Exp Ther, 193:403-412.

Banes AK, Watts SW.(2003). Arterial expression of 5-HT_{2B} and 5-HT_{1B} receptors during development of DOCA-salt hypertension. BMC Pharmacol, 3:12.

Bard JA, Zgombick J, Adham N, Vaysse P, Branchek TA, Weinshank RL.(1993). Cloning of a novel human serotonin receptor (5-HT7) positively linked to adenylate cyclase. J Biol Chem, 268: 23422-23426.

Baron A, Riesselmann A, Fregly MJ. (1991). Reduction in the elevated blood pressure of Dahl Salt sensitive rats treated chronically with L-5-hydroxytryptophan. Pharmacology, 142:15-22.

Brengelmann GL. (2003). A critical analysis of the view that right atrial pressure determines venous return. J Appl Physiol, 94:849-859.

Chand N. (1981). 5-Hydroxytryptamine induces relaxation of goat pulmonary veins: evidence for the noninvolvement of M and D-tryptamine receptors. Br J Pharmacol. 72:233-237.

Cocks TM, Arnold PJ. 5-Hydroxytryptamine (5-HT) mediates potent relaxation in the sheep isolated pulmonary vein via activation of 5-HT_4 receptors. *Br J Pharmacol*. 107:591-596, 1992.

Cushing DJ, Zgombick JM, Nelson DL, Cohen ML.(1996). LY215840, a high affinity 5-HT_7 receptor ligand, blocks serotonin-induced relaxation in canine coronary artery. J Pharmacol Exp Ther, 277:1560-1566.

Dalton DW, Feniuk W, Humphrey PP. (1986). An investigation into the mechanisms of the cardiovascular effects of 5-hydroxytryptamine in conscious normotensive and DOCA-salt hypertensive rats. J Auton Pharmacol, 6:219–228.

Darios ES, Barman SM, Orer HS, Morrison SF, Davis RP, Seitz BM, Burnett R, Watts SW. (2015). 5-hydroxytryptamine does not reduce sympathetic nerve activity or neuroeffector function in the splanchnic circulation. Eur J Pharmacol, 754:140-147.

Davis RP, Linder AE, Watts, SW. (2011). Lack of serotonin transporter (SERT) reduces the ability of 5-hydroxytryptamine to lower blood pressure. Naunyn-Schmied Arch Pharmacol, 383:543-546.

Davis RP, Pattison J, Thompson JM, Tiniakov R, Scrogin KE, Watts SW. (2012). 5-hydroxytryptamine (5-HT) reduces total peripheral resistance during chronic infusion: direct arterial mesenteric relaxation is not involved. BMC Pharmacol, 12:4 doi: 10.11186/1471-2210-12-4, 2012.

Davis RP, Szasz T, Garver H, Burnett R, Tykocki NR, Watts SW. (2013). One-month serotonin infusion results in a prolonged fall in blood pressure in the deoxycorticosterone acetate (DOCA) salt hypertensive rat. ACS Chem Neurosci, 4:141-148.

De Vries P, De Visser PA, Heiligers JPC, Villalón CM, Saxena PR. (1991). Changes in systemic and regional haemodynamics during 5-HT_7 receptor mediated depressor responses in rats. Naunyn-Schmied Arch Pharmacol, 359:331-338.

Ding XR, Stier CT, Itskovitz HD. (1989). Serotonin and 5-hydroxytryptophan on blood pressure and renal blood flow in anesthetized rats. Am J Med Sci, 297:290-293.

Diaz J, Ni W, King A, Fink GD, Watts SW. (2008). 5-hydroxytryptamine lowers blood pressure in normotensive and hypertensive rats. J Pharmacol Exp Ther, 325:1031-1038.

Echizen H, Freed CR. (1981). Long-term infusion of L-5-hydroxytryptophan increases brain serotonin turnover and decreases blood pressure in normotensive rats. J Pharmacol Exp Ther, 220:579-584.

Ellis ES, Byrne C, Murphy OE, Tilford NS, Baxter GS. (1995). Mediation by 5-hydroxytryptamine2B receptors of endothelium-dependent relaxation in rat jugular vein. Br J Pharmacol, 114:400-404.

Fink GD. (2009). Arthur C Corcoran Memorial Lecture: Sympathetic activity, vascular capacitance, and long-term regulation of arterial pressure. Hypertension, 53:307-312.

Fregly MJ, Lockley OE, Sumners C. (1987). Chronic treatment with L-5-hydroxytryptophan prevents the development of DOCA-salt induced hypertension in rats. J Hypertens, 5:621-628.

Gothert M. (2013). Serotonin discovery and stepwise disclosure of 5-HT receptor complexity over four decades. Part I. General background and discovery of serotonin as a basis for 5-HT receptor identification. Pharmacol Rep, 65:771-786.

Greenway CV. (1983). Role of splanchnic venous system in overall cardiovascular homeostasis. Fed Proc, 42:1678-1684.

Gupta P. (1992). An endothelial 5-HT receptor that mediates relaxation in guinea-pig isolated jugular vein resembles the 5-HT_{1D} receptor. Br J Pharmaco, 108:703-709.

Hagan JJ, Price GW, Jeffrey P, Deeks NJ, Stean T, Piper D, Smith MI, Upton N, Medhurst AD, Middlemiss DN, Riley GJ, Lovell PJ, Bromidge SM, Thomas DR. (2000). Characterization of SB-269970-A, a selective 5-HT_7 receptor antagonist. Br J Pharmacol, 130:539-548.

Headberg PA, Ruten DL. (1990). Influence of serotonin on intravascular capacity in the anesthetized dog. Acta Physiol Scan, 139:561-568.

Hoyer D, Clarke DE, Fozard JR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP. (2002). International Union of Pharmacology classification for 5-hydroxytryptamine receptors. Pharmacol Rev, 46:157-203.

Ishine T, Bouchelet I, Hamel E, Lee TJ. (2000). Serotonin 5-HT (7) receptors mediate relaxation of porcine pial veins. Am J Physiol Heart Circ Physiol, 278:H907-H912.

Itskovitz HD, Werber JL, Sheridan AM, Brewer TF, Stier CT. (1989). 5-hydroxytryptophan and carbidopa in spontaneously hypertensive rats. J Hypertens, 7:311-315.

Jahnichen S, Glusa E, Pertz HH. (2005). Evidence for 5-HT_{2B} and 5-HT_7 receptor-mediated relaxation in pulmonary arteries of weaned pigs. Naunyn Schmiedebergs Arch Pharmacol, 371:89-98.

Kurita H, Ernberg M, Tominaga K, Alstergren P, Kopp S. (1999). Effect of 5-hydroxytryptamine-2 and alpha-adrenergic receptor antagonists on the 5-hydroxytryptamine induced decrease in rabbit masseter muscle blood flow. Arch Oral Biol, 44:651-656.

Leung E, Walsh LK, Pulida-Rios MT, Eglen RM. (1996). Characterization of putative 5-ht7 receptors mediating direct relaxation in Cynomolgus monkey isolated jugular vein. Br J Pharmacol, 117:926-930.

Linden AS, Desmecht DJ, Amory H, Beduin JM, Lekeux PM. (1996). Cardiovascular response to exogenous serotonin in healthy calves. Am J Vet Res, 57:731-738.

Miller RR, Fennell WH, Young JB, Palomo AR, Quinones AM. (1982). Differential systemic arterial and venous actions and consequent cardiac effects of vasodilatory drugs. Prog Cardiovasc Dis, XXIV:353-374.

Monaghan PJ, Brown HA, Houghton LE, Keevil BG. (2009). Measurement of serotonin in platelet depleted plasma by liquid chromatography tandem mass spectrometry. J Chromatog B, 877:2163-2167.

Okamoto LE, Diedrich A, Baudenbacher FJ, harder R, Whitfield JS, Iqbal F, Gamboa A, Shibao CA, Black BK, Raj SR, Robertson D, Biaggioni I. (2016). Efficacy of servo-controlled splanchnic venous compression in the treatment of orthostatic hypotension: a randomized comparison with midodrine. Hypertension, 68:418-426.

Page IH, McCubbin JW. (1953). The variable arterial pressure response to serotonin in laboratory animals and man. Circ Res*,* 1:354-362.

Reddi BA, Carpenter RH. (2005). Venous excess: a new approach to cardiovascular control and its teaching. J Appl Physiol, 98:356-364.

Schwartz LB, Radic ZS, O'Donohoe Mek, Mikat EM, McCann RL, Hagen PO. (1992). Saphenous vein endothelium-dependent relaxation in patients with peripheral vascular disease. Ann Vasc Surg, 6:425-523.

Seitz BM, Krieger-Burke T, Fink GD, Watts SW. (2016). Serial measurement of splanchnic vein diameters in rats. Frontiers in Pharmacology; Experimental Pharmacology and Drug Discovery, 7:116; doi: 10.3389/fphar.2016.00116.

Seitz BM, Watts SW. (2014). Serotonin-induced hypotension is mediated by a decrease in intestinal vascular resistance. Pharmacologia, 5(2):50-54.

Selim AM, Sarswat N, Kelesidis I, Iqbal M, Chandra R, Zolty R. (2016). Plasma serotonin in heart failure: possible marker and potential treatment target. Heart, Lung Circ, S1443-9506(16)31583-9. doi: 10.1016/j.hlc.2016.08.003.

Selke FW, Dai HB. (1993). Responses of porcine epicardial venules to neurohumoral substances. Cardiovasc Res, 27:1326-1332.

Selke FW, Wang SY, Friedman M, Harada K, Edelman ER, Grossman W, Simons M. (1994). Basic FGF enhances endothelium-dependent relaxation of the collateral perfused coronary microcirculation. Am J Physiol Heart Circ Physiol, 36:H1303-H1311.

Struyker-Boudier HA, le Noble JL, Le Noble FA, Messing MW, van Essen H.(1990). Hypertension, the microcirculation and serotonin. Clin Physiol Biochem, 8 S 3:28-39.

Sumner MJ, Feniuk W, Humphrey PP. (1989). Further characterization of the 5-HT receptor mediating vascular relaxation and elevation of cyclic AMP in porcine isolated vena cava. Br J Pharmacol. 97:292-300.

Schuschke DA, Joshua IG, Miller FN. (1991). Comparison of early microcirculatory and aortic changes in hypercholesterolemic rats. Arterioscl Thromb Vasc Biol, 11:154-160.

Takahashi R, Sakai T, Furuyama Y, Kondo Y, Inoue CN, Onuma S, Inuma K. (2000). The vasocontractive action of norepinephrine and serotonin in deep arterioles of rat cerebral gray matter. Tohoku J Exp Me, 190:129-142.

Tan T, Watts SW, Davis RP. (2011). Drug delivery, enabling technology for drug discovery and development. IPRECIO micro infusion pump: programmable, refillable and implantable. Front Pharmacol, 2:44.

Terrón JA. (1997). Role of 5-ht7 receptors in the long-lasting hypotensive response induced by 5-hydroxytryptamine in the rat. Br J Pharmacol, 121: 563-571.

Terron JA, Falcon-Neri A. (1999). Pharmacological evidence for the 5-HT_7 receptor mediating smooth muscle relaxation in canine cerebral arteries. Br J Pharmacol, 127:609-616.

Terron JA, Sanchez-Maldonado C, Martinez-Garcia E. (2007). Pharmacological evidence that 5-HT(1B/1D) receptors mediate hypotension in anesthetized rats. Eur J Pharmacol, 576:132-135.

Trevethick MA, Feniuk W, Humphrey PP. (1994). 5-hydroxytryptamine-induced relaxation of neonatal porcine vena cava in vitro. Life Sci, 35:477-486.

Tsuru H, Nakai S, Uchiyama T, Teranishi Y. (1998). Endothelium-independent relaxant effect of 5-hydroxytryptamine (5-HT) on the isolated rabbit facial vein. J Smooth Muscle Res, 34:101-110.

Watts SW. (2016). Oh, the places you'll go! My many colored serotonin (apologies to Dr. Seuss) Am J Physiol Heart Circ Physiol, 311:H1225-H1233.

Watts SW, Darios ES, Seitz BM, Thompson JM. (2015). 5-HT is a potent relaxant in rat superior mesenteric veins. Pharmacol Res Perspect, 3:e00103. Doi: 10.1002/prp2.103, 2015.

Watts SW, Morrison SF, Davis RP, Barman SM. (2012). Serotonin and blood pressures regulation. Pharmacol Rev, 64:359-388.

Wilmoth FR, Harris PD, Miller FN. (1984). Differential serotonin responses in the skeletal muscle microcirculation. Life Sci, 34:1135-1142.

Zhang L, Dyer DC, Fiscus RR, Grujic Z, Wang X. (1995). 5-Hydroxytryptamine induces endothelium-independent relaxations of sheep pulmonary vein: role of cyclic nucleotide. Eur J Pharmacol, 280:335-337.

Chapter 5

Activation of 5-HT_7 receptor but not NOS is necessary for chronic 5-HT-induced hypotension

Abstract

Infusion of low doses of 5-hydroxytryptamine (5-HT) leads to a chronic (days-weeks) fall in arterial pressure. Pharmacological evidence shows the 5-HT_7 receptor mediates the acute (< 24 hours) 5-HT-induced hypotension in a nitric oxide (NO) independent fashion. The contribution of the 5-HT_7 receptor, beyond 24 hours, is unknown. Evidence suggests, however, nitric oxide (NO) may be a critical contributor to the more chronic 5-HT depressor response. In this study, we test the hypothesis that chronic, low dose 5-HT infusion lowers arterial pressure by activating both the 5-HT_7 receptor and nitric oxide synthase (NOS), such that the resulting generation of NO contributes to a sustained reduction in arterial pressure. In this study, mean arterial pressure (MAP) and heart rate (HR) were measured continuously, via radiotelemetry, in conscious rats during 5 days of saline or 5-HT (25 ug/kg/min; osmotic pump) infusion and after infusion was stopped. The selective 5-HT_7 receptor antagonist, SB267790 (33 ug/ kg; iv bolus) or the NOS inhibitor, L-NAME (3 mg/ kg; iv bolus) were given at several distinct time points during chronic 5-HT infusion to quantify the contributions of the 5-HT_7 receptor and NO to 5-HT-induced hypotension. The 5-HT_7 receptor antagonist completely reversed the 5-HT-induced depressor response at each time point the antagonist was given during 5-HT infusion. However, the 5-HT_7 receptor antagonist did not alter MAP when given after the 5-HT-infusion was stopped or in saline treated group. In contrast, L-NAME administration caused similar increases in MAP in both the 5-HT and saline infused rats throughout the experiment. Importantly, the magnitude of

the pressor response to L-NAME was not significantly different between either the saline or 5-HT infused groups. Overall, our findings demonstrate that chronic 5-HT induced hypotension requires continued activation of the 5-HT_7 receptor, and that NOS generated NO appears not to plays a role.

Introduction

5-HT was first identified in the cardiovascular system (Page and McCubbin, 1953). For decades, it has been recognized that 5-HT lowers arterial pressure when given acutely (<1-hour duration) (Dalton et al.,1986; Terron, 1997) at low doses. Pharmacological evidence supports activation of the 5-HT_7 receptor as the primary mediator of the acute fall in arterial pressure (Centurion et al., 2004; De Vries et al., 1999; Terron, 1997; Villalon and Centurion, 2007). In support of these findings, we have shown blockade of the 5-HT_7 receptor, using the selective 5-HT_7 receptor antagonist SB-269970, completely abolished the fall in arterial pressure during 24 hours of low dose 5-HT infusion (Seitz et al., 2017). Our work here is focused on understanding if activation of the 5-HT_7 receptor is essential for the fall in arterial pressure chronically (beyond 24 hours) to 5-HT administration. Acute hormone actions are apt to be modified under chronic conditions by well-known phenomena such as receptor down-regulation, receptor desensitization, and physiological compensation. Chronic exposure to a hormone also causes time-dependent physiological compensatory adaptions (e.g. baroreflex, pressure natriuresis) either diminishing or reinforcing the initial response. Understanding the mechanisms responsible for chronic actions of 5-HT is essential for determining whether 5-HT_7 receptor agonists have utility in management of chronic cardiovascular diseases, such as hypertension.

A low dose infusion of 5-HT (25 ug/kg/min) into normotensive conscious rats reduced arterial pressure over both one week (Diaz et al., 2008) and one month (Davis et al., 2012). More importantly, the same low dose of 5-HT infused for one week reduced mean arterial pressure (MAP), in hypertensive rat models (> 30-50 mmHg fall from baseline; Diaz et al., 2008; Watts et al., 2012). Although it may seem obvious that the chronic effects of an agonist would be mediated through the same mechanisms as the acute effects, this is not always the case. Changes in receptor expression or function over time, for example, are well known to affect the magnitude of an agonist response. Likewise, gradual engagement of physiological compensatory responses to agonist actions can either oppose or reinforce agonist responses in the long-term. In this study, we evaluated the quantitative importance of the 5-HT_7 receptors to the chronic depressor response during low dose 5-HT infusion. This was achieved by assessing the ability of the 5-HT_7 receptor antagonist SB-269970 to reverse 5-HT induced hypotension at various times during chronic infusion.

One example of a physiological system that can indirectly modify the response to vascular agonists is nitric oxide (NO). NO synthesized by nitric oxide synthase (NOS), in the vascular endothelium, plays a pivotal role in the control of vascular smooth muscle tone and consequently, arterial resistance and blood pressure (Rees et al., 1989). Many vasodepressors work in part by activating NOS and thereby releasing NO from the endothelium. There is no direct evidence that stimulating 5-HT_7 receptors causes NOS activation. For example, the acute depressor response to 5-HT_7 receptor stimulation was not affected by blockade of NOS (Terron, 1997). In marked contrast, we previously demonstrated that blockade of NO formation with NOS inhibitor, LNNA (0.5g L^{-1} in

drinking water for 3-10 days), completely prevented the chronic depressor action of 5-HT during a week-long infusion. (Banes et al., 2002; Diaz et al., 2008; Seitz et al., 2014). This finding suggested a major contribution of NOS activity in the chronic (unlike the acute) 5-HT-induced hypotensive response. Therefore, we hypothesized that 5-HT_7 receptor activation may not be solely responsible for depressor response to chronic 5-HT infusion, and that activation of NOS and generation of NO also could be a key component. In order to test this idea, we measured the pressor response to acute blockade of NOS at different time points during 5-HT infusion as an index of the vascular activity of NO.

Materials and Methods

Animals: MSU Institutional Animal Care and Use Committee approved all protocols used in this study. Male Sprague –Dawley rats (275-300 g; Charles River Laboratories, Portage, MI, USA) were used in all experiments. Rats were housed in a temperature–controlled room 22°C with 12-hour light/dark cycles and given standard chow and distilled water ad libitum.

Surgery: Radiotelemeter, femoral vein catheter and osmotic drug pump: Under isoflurane anesthesia (1.5% in oxygen), radiotelemeter transmitter (HD-S10; Data Sciences International, MN, USA) was implanted subcutaneously through a 1-1.5cm incision in the left inguinal area in all rats. The radiotelemeter catheter tip was introduced into the left femoral artery and advanced into the abdominal aorta below the renal artery. During the same surgery, an additional femoral catheter (polyethylene 50 tubing; Instech Laboratories, PA, USA) was implanted into the left femoral vein and externalized near the scapular region. The rats were placed in a tether jacket and the

external portion of the vein catheter was advanced into a protective spring, which was attached to a swivel cage top for ease of movement around the cage. The femoral vein catheter was flushed each day with heparinized saline (10 units/mL; 250 uL) to maintain patency. The femoral vein line was used for drug infusion of either SB-269970, a selective 5-HT_7 receptor antagonist (33 ug/kg; iv bolus) for one group of rats or N (ω)-nitro-L-arginine methyl ester; LNAME, inhibitor of nitric oxide synthase (3 mg/kg; iv bolus) in another group of rats. All compounds were made up in sterile saline, pH balanced to 6-7.

During surgery, all animals received a dose of enrofloxacin (Baytril®, 2.5 mg kg^{-1}, im) and carprofen (Rimadyl®, 5 mg/kg, sc). After 5 days of post-operative recovery, baseline cardiovascular measurements were recorded for 5 days. Upon completion of baseline measurements, osmotic pumps (Model 2M L1; Alzet osmotic pumps CA, USA; dosing rate of 9.8 uL/hr) containing either sterile saline (with 1% ascorbate) or 5-HT (25 μg/kg in 1% ascorbate in sterile saline, pH balanced to pH 6-7) were implanted subcutaneously, caudal to the scapular area while under isoflurane anesthesia (1.5% in oxygen). Upon completion of the final day of infusion, pumps were removed from the rats (under isoflurane anesthesia) and cardiovascular parameters were monitored for one additional day (termed recovery). MAP and HR were measured every minute (during iv bolus challenge studies) or 10 seconds every 10 minutes throughout the duration of the study. The weights of all osmotic pumps were recorded before implantation and after removal to confirm accuracy of saline or 5-HT delivery.

Time course: Twenty-four hours after (termed 24-hours post) 5-HT or saline pump implantation, rats were separated into two groups. Group 1: rats were challenged with an iv bolus (250 uL) of LNAME (3 mg/kg) during 5-HT or saline mini-pump infusion. Group 2: separate group of rats were challenged with an iv bolus of SB-269970 (33 ug/kg) during 5-HT or saline mini-pump infusion. It is important to note that heparinized saline (10 units/mL; 250 uL) was placed in the vein line following each bolus to ensure drug was washed from line and to fill the catheter dead space. After the bolus is given, MAP and HR were recorded every minute for the following 20 minutes. This same experimental design was conducted at 3 days; 5 days of saline or 5-HT infusion and one day (24 hours) after all osmotic pumps were removed (termed recovery) for both the SB-269970 and LNAME groups.

Data and statistical analyses: Quantitative data are reported as means$\pm$SEM for number of animals in parentheses or figure legend. In some graphs, quantitative data are also presented as a percent change from baseline. The baseline was determined by 5 days prior to the start of either saline or 5-HT administration. Additionally, data are presented as a delta change which is derived by subtracting the MAP value after 20 minutes bolus of either SB-269970 or LNAME from starting baseline value for specific treatment day (24hours, 3 days, 5 days and recovery). Statistical analyses were performed using repeated measures one-way ANOVA when comparing values from baseline or within a group (GraphPad Prism 7) when the F value achieved statistical significance. When comparing between groups, a t-test was used. In all cases, a p value of <0.05 was considered significant.

Results

The MAP response to 5-HT_7 receptor antagonist during one week of 5-HT infusion

Normotensive male rats with statistically similar baseline values for MAP and HR (table 5.1) were implanted with either a 5-HT or saline osmotic drug pump. In the saline-infused rats, there was minimal to no change in MAP during the 5 days of saline infusion compared to baseline. The HR in saline group decreased slightly over time. In contrast, the 5-HT-infused rats had a significant reduction in MAP from day 1 to day 5 during 5-HT infusion. The greatest depressor response occurred 24 hours after the start of 5-HT delivery. This mirrored in time a significant elevation in HR, which only lasted for the first three days from the start of 5-HT infusion, most likely a baroreflex response. Once the 5-HT drug pumps were removed, MAP returned to near baseline values and HR was significantly lower from baseline. The observed cardiovascular response to one week infused saline or 5-HT (25 ug/kg/min) in this study confirms what we have previously reported (Diaz et al., 2008; Davis et al., 2013). This protocol now establishes a basis to examine the contribution of the 5-HT_7 receptor and NOS (second experiment) in 5-HT-induced hypotension.

To determine if 5-HT_7 receptor activation is necessary for the duration of the 5-HT-induced hypotension over one week, the rats from Table 5.1 were challenged with an intravenous bolus of SB-269970 (33 ug/kg), a selective 5-HT_7 receptor antagonist, at 24 hours (figure 5.1A), 3 days (figure 5.1B), 5 days (figure 5.1C) during 5-HT or saline infusion. The response to the 5-HT_7 receptor antagonist was also tested 24 hours after removal of the 5-HT or saline drug pumps, termed recovery (figure 5.1D). In figure 5.1, the left column of graphs represents the conscious MAP recordings in minute averages;

the right column of graphs represents the percent change from baseline for the MAP data over minute averages. It is important to note that the 5-HT-infused rats had a lower baseline MAP (table 5.1) at the start of each day, prior to the SB-269970 bolus, except at recovery (drug pump removed) when compared to their baseline and saline-infused rats.

In the saline-treated rats, there was no change in MAP compared to baseline, when challenged with the 5-HT_7 receptor antagonist at any time point (24 hours, 3 days, 5 days or recovery) (figure 5.1 A-D). In contrast, the 5-HT-induced depressor response was significantly blocked in the 5-HT-treated rats by the 5-HT_7 receptor antagonist at each time point during 5-HT infusion: 24 hours, 3 days, and 5 days (figure 5.1 A-C). When the 5-HT osmotic pump was removed, blocking the 5-HT_7 receptor with the antagonist did not alter MAP (figure 5.1 D). A similar increase in MAP (~20% increase) was observed in the 5-HT-infused rats at each time point the 5-HT_7 receptor antagonist was given except during recovery (figure 5.1, right side graphs).

The change in MAP 20 minutes after the 5-HT_7 receptor antagonist bolus was administered (table 5.2), resulted in a significant increase in MAP which was similar in magnitude at each time point the antagonist was given during 5-HT infusion. Once the 5-HT pumps were removed, 5-HT_7 receptor antagonist had no effect on the MAP response and HR was lowered than baseline. These results suggest activation of the 5-HT_7 receptor is important for the 5-HT-induced hypotensive response. The saline–treated rats showed negligible differences in MAP responses in the presence of the 5-HT_7 receptor antagonist.

		Baseline	24 hours	Day 3	Day 5	Recovery
Saline infused (n=5)	**MAP** (mmHg)	109±1.6	106±1.7	105±3.5	105±2.8	103±2.0
	HR (bpm)	374±8.5	340±12.3*	328±16.1*	352±18.5*	332±16.4*
5-HT infused (n=5)	**MAP** (mmHg)	107±1.5	92±2.8*+	96±2.5*+	93±1.2*+	114±2.9+
	HR (bpm)	370±6.1	410±12.9*+	389±7.1+	356±5.1	344±13.0*

Table 5.1: Mean arterial pressure and heart rate measures in rats infused with saline or 5-HT. MAP and HR values for male rats at baseline and after infusion with either saline or 5-HT (25 mg kg^{-1} min^{-1}) for 24 hours, 3 days, 5 days and 24 hour of recovery. Values are means±SEM for 5 rats per group. Baseline measures were collected for 5 days prior to saline or 5-HT infusion. Recovery is 24 hours after saline and 5-HT drug pumps are removed. These rats were later challenged with SB-269970. Data on MAP responses to SB-269970 are found in Figure 1 and Table 2. * = $p<0.05$ between baseline and determined infusion day within groups; += $p< 0.05$ differences between saline vs 5-HT for same day. BPM=beats per minute.

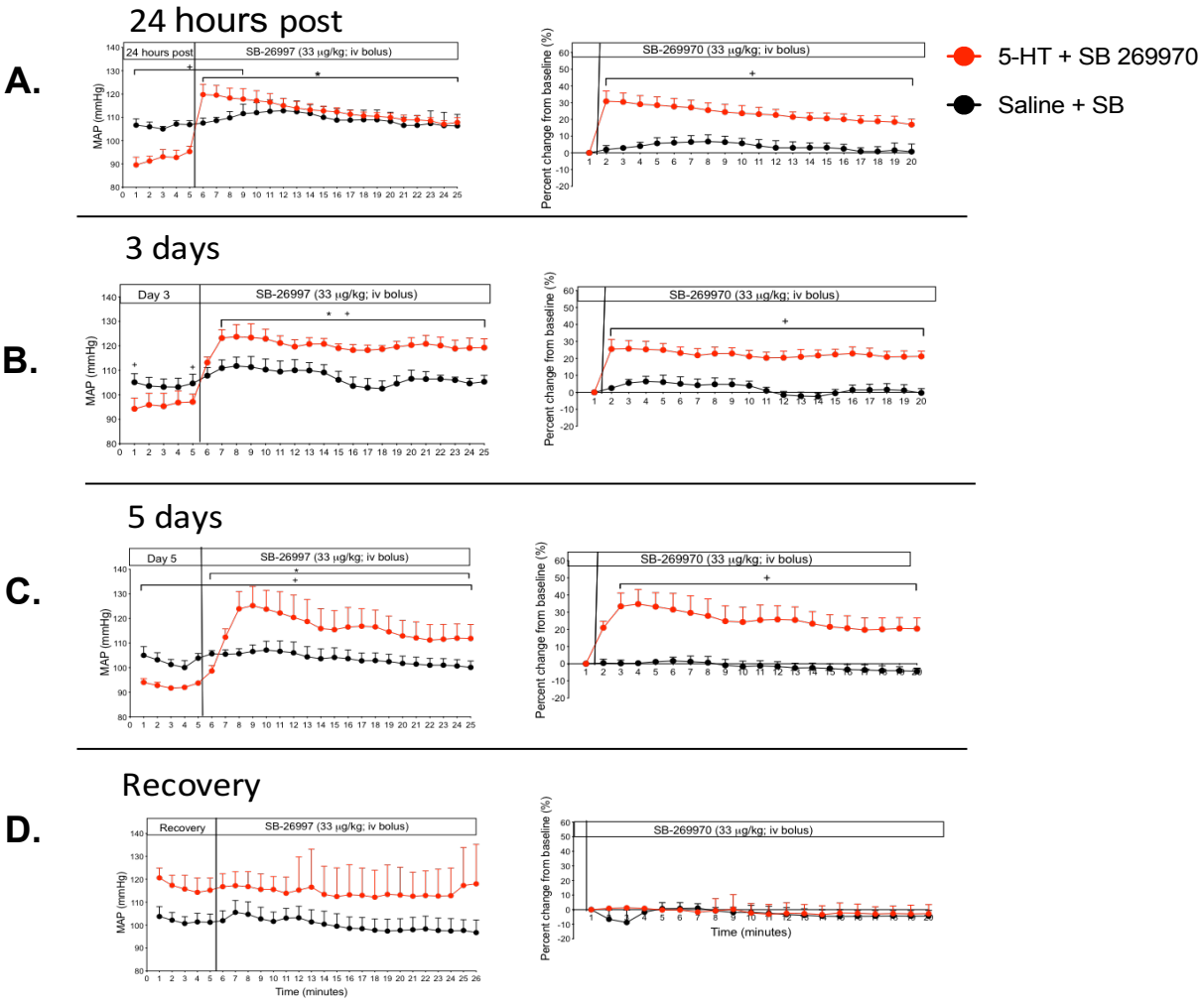


Figure 5.1: Effect of a 5-HT$_7$ receptor antagonist on mean arterial pressure at specific time intervals during saline or 5-HT infusion and recovery. Effect of SB-269970 (a selective 5-HT$_7$ receptor antagonist; 33 mg kg^{-1}; v bolus) on MAP over 20 minutes in normotensive male rats during infusion of either saline or 5-HT (25 mg kg^{-1} min^{-1}) for 24 hours (**A**), 3 days (**B**), 5 days (**C**) and 24 hours of recovery **(D)**. Left side graphs represent raw data and right side graphs represent percent change from baseline. Baseline is determined by 5 minutes prior to SB-269970 bolus. Points represent means±SEM for 5 rats per group. Recovery is determined by 24 hours after the osmotic pump (saline or 5-HT) was removed. * = $p<0.05$ difference between 5-HT-infused rats at baseline and after SB-269970 bolus for same day; += $p< 0.05$ differences between saline-infused vs 5-HT-infused for same day.

	24 hours	Day 3	Day 5	Recovery
Saline infused Δ MAP (mmHg)	-1±3.0	0.5±3.4	-2±2.4	-4±2.4
5-HT infused Δ MAP (mmHg)	17±1.6^{+}	22±3.6^{+}	20±5.0^{+}	4±4.4

Table 5.2: Change in mean arterial pressure in saline or 5-HT infused rats after 5-HT_7 receptor antagonist. Change in MAP 20 minutes after injection of SB-269970 in normotensive male rats after infusion of either saline or 5-HT (25 mg kg^{-1} min^{-1}) for 24 hours (A), 3 days (B), 5 days (C) and recovery (D). Points represent means±SEM difference between baseline and 20 minutes after SB-269970 bolus for 5 rats per group. Recovery is determined by 24 hours after the osmotic pump (saline or 5-HT) was removed. += $p < 0.05$ differences between saline-infused vs 5-HT-infused for same day.

The MAP response to LNAME during one week infusion of 5-HT

A second group of normotensive male rats with similar baseline MAP and HR values (table 5.3) were implanted with either a saline or 5-HT drug pump. This group of animals (table 5.3) had comparable baseline hemodynamic measurements to the group used in the 5-HT_7 receptor study (table 5.1). One exception, the 5-HTY-infused rats in the LNAME study showed restoration of MAP by 5 days. However, a continued vasodepressor action of 5-HT likely was occurring at 5 days since termination of infusion led to a significant rebound increase in MAP.

To determine if activation of NOS is a key component in the chronic 5-HT-induced depressor response, animals (table 5.3) were challenged with an intravenous bolus of inhibitor of nitric oxide synthase LNAME (3 mg/kg) at 24 hours (figure 5.2 A), 3 days (figure 5.2 B), and 5 days (figure 5.2 C), during 5-HT or saline osmotic drug infusion. In addition, the response to the NOS inhibitor was also tested 24 hours after removal of the 5-HT or saline osmotic drug pumps (figure 5.2 D).

The 5-HT-treated group started at a lower baseline MAP after 24-hours of infusion compared to saline-infused animals, as expected. However, after the bolus of LNAME, (figure 5.2 A), both the saline-infused and 5-HT-infused rats showed a similar increase in MAP. At 3 days of infusion (saline or 5-HT), the 5-HT-treated rats showed somewhat of a greater increase in MAP response to LNAME compared to the saline-treated animals although this difference was not significant. At 5 days of infusion and after the drug pumps were removed (recovery), both the saline-treated and 5-HT-treated animals had similar baseline MAP values and nearly similar MAP response to the LNAME bolus.

The change in MAP, 20 minutes after the NOS inhibitor was given, in both the saline and 5-HT infused rats, was nearly the same for each specific day the NOS inhibitor was given, even after the drug pumps (saline or 5-HT) were removed (recovery). after twenty-minutes of a iv bolus of the NOS inhibitor.

		Baseline	24 hours	Day 3	Day 5	Recovery
Saline infused (n=5)	**MAP** (mmHg)	107±1.7	100±1.8*	104±2.1	100±3.4	103±2.5
	HR (bpm)	372±6.4	332±11.2	334±8.8	321±8.7	305±4.6
5-HT infused (n=5)	**MAP** (mmHg)	104±1.0	85±0.8*+	93±1.3*+	102±1.7	110±1.7*
	HR (bpm)	387±8.4	428±11.5+	382±7.8+	380±10.6+	339±9.6

Table 5.3: Mean arterial pressure and heart rate measures in rats infused with saline or 5-HT. MAP and HR values for male rats at baseline and after infusion with either saline or 5-HT (25 mg kg^{-1} min^{-1}) for 24 hours, 3 days, 5 days and 24 hours of recovery. Values are means±SEM for 5 rats per group. Baseline measures were collected for 5 days prior to saline or 5-HT infusion. Recovery is 24 hours after saline and 5-HT drug pumps are removed. These rats were later challenged with LNAME. Data on MAP responses to LNAME are found in Figure 2 and Table 4found in table 4. * = $p<0.05$ between baseline and determined infusion day within groups; += $p< 0.05$ differences between saline vs 5-HT for same day. BPM=beats per minute.

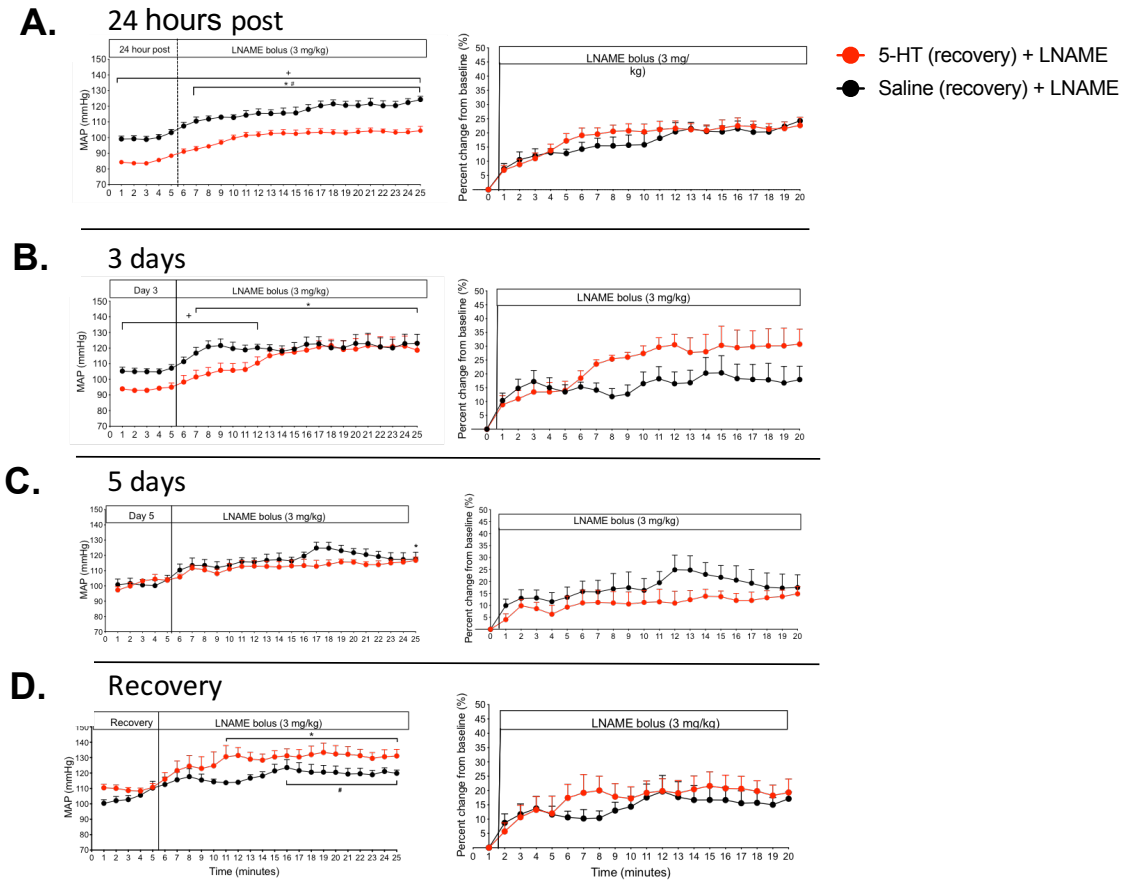


Figure 5.2. Effect of a nitric oxide inhibitor on mean arterial pressure at specific time intervals during saline or 5-HT infusion and recovery. Effect of LNAME (inhibitor of nitric oxide synthase (3 mg kg^{-1}; iv bolus) on MAP over 20 minutes in normotensive male rats after infusion of either saline or 5-HT (25 mg kg^{-1} min^{-1}) for 24 hours (A), 3 days (B), 5 days (C) and recovery (D). Left side graphs represent raw data and right side graphs represent percent change from baseline. Baseline is determined by 5 minutes prior to to LNAME bolus. Points represent means±SEM for 5 rats per group. Recovery is determined by 24 hours after the osmotic pump (saline or 5-HT) was removed. * = $p<0.05$ difference between 5-HT-infused rats at baseline and after LNAME bolus for same day; # = $p<0.05$ difference between saline-infused rats at baseline and after LNAME bolus for same day += $p< 0.05$ differences between saline-infused vs 5-HT-infused for same day.

	24 hours	Day 3	Day 5	Recovery
Saline infused Δ MAP (mmHg)	24±1.6	19±5.4	19±5.1	17±3.7
5-HT infused Δ MAP (mmHg)	20±2.9	25±5.1	19±2.9	21±5.0

Table 5.4: Change in mean arterial pressure in saline or 5-HT infused rats after nitric oxide inhibitor (LNAME). Effect of LNAME (inhibitor of nitric oxide synthase (3 mg kg^{-1}; iv bolus) on MAP after 20 minutes in normotensive male rats after infusion of either saline or 5-HT (25 mg kg^{-1} min^{-1}) for 24 hours (A), 3 days (B), 5 days (C) and recovery (D). Points represent means±SEM difference between baseline and 20 minutes after LNAME bolus for 5 rats per group. Recovery is determined by 24 hours after the osmotic pump (saline or 5-HT) was removed. $p < 0.05$.

Discussion

The focus of this study was to evaluate the quantitative importance of the activated 5-HT_7 receptor and NOS in the chronic depressor response during low dose 5-HT infusion. The importance of this work is based on the findings that a low dose infusion of 5-HT can significantly lower arterial pressure in human and other species (Cade et al., 1992; Diaz et al., 2008; Kaufmann and Levy, 2006; Page and McCubbin, 1953; Villalon and Centurion, 2007). Previous work established that the 5-HT_7 receptor mediates the acute (< hour) 5-HT-induced depressor response (Centurion et al., 2004; De Vries et al., 1999; Terron, 1997; Villalon and Centurion, 2007). For the contribution of NOS in 5-HT-induced hypotension, earlier in vivo studies suggested NOS activation might contribute to the chronic (not acute) 5-HT depressor response. In this study, we demonstrate the continuing activation of the 5-HT_7 receptor is essential for the fall in arterial pressure during chronic (5 days) low dose 5-HT infusion and is independent of NOS activation (either acute or chronic).

Role of the 5-HT_7 receptor in low dose 5-HT-induced depressor response

Infusion of 5-HT into the peripheral circulation can generate either a pressor or depressor response. These outcomes depend on the level of concentration of 5-HT achieved in the circulating plasma and the specific 5-HT receptor subtypes that are activated. In our study, MAP was decreased from day 1 to day 5 (figure 5.1) when low dose 5-HT (25 ug/kg/min) was infused. The selective 5-HT_7 receptor antagonist (SB269970, 33 ug/ kg) completely reversed the fall in MAP at each time point the antagonist was given. The selective antagonist used possesses an affinity (Ki) for the 5-HT_7 receptor that is 50-1,000 times greater than any other serotonergic receptor

(pdsp.unc.edu/databases). Although, from our results, we are confident the low dose of 5-HT infused in our protocol primarily activates the 5-HT_7 receptor, stimulation of other 5-HT receptor subtypes can also cause a depressor response. The vasorelaxant effect of 5-HT can be mediated by activation of 5-$HT_{1B/1D}$, 5-HT_{2B} as well as the 5-HT_7 receptors (Berger et al, 2009; Kaumann and Levy, 2006). Both the 5-$HT_{1B/1D}$ and 5-HT_{2B} receptors are located on vascular endothelium. The 5-$HT_{1B/1D}$ and 5-HT_7 receptors are located on the vascular smooth muscle cells (Kaumann and Levy, 2006). In our studies, the low dose of 5-HT given (25 μg/kg/min) produces a concentration of free circulating 5-HT in the plasma that is in the mid-nanomolar range (Diaz et al, 2008). This circulating plasma level of 5-HT is adequate to activate the 5-HT_7 receptor (affinity; Ki ~7nM) (pdsp.unc.edu/databases); which possesses an affinity, approximately two times greater than the other vasorelaxant serotonergic receptors subtypes (5-HT_{1B} ~14 nM and 5-HT_{2B} ~19 nM). Based on the high affinity of 5-HT for the 5-HT_7 receptor at the given amine dose, we suspect the 5-$HT_{1B/1D}$ or 5-HT_{2B} to have a minimal contribution to the depressor response, if at all. Earlier pharmalogical evidence supports our idea, as the selective 5-HT_{2B} receptor agonist (BW723C86) caused a dose-dependent vasopressor (rather than vasodepressor) response (Centurion et al., 2004). Additionally, sumatriptan, a selective 5-$HT_{1B/1D}$ agonist, evoked contraction, not relaxation, in the saphenous vein of a dog, suggestive of a pressor response to the 5-$HT_{1B/1D}$ agonist (Villalon and Centurion, 2007). In contrast, the selective 5-HT_7 receptor agonist 5-CT significantly reduced arterial pressure in conscious rats when administered for one week (Seitz et al., 2017).

In the absence of 5-HT infusion (in saline infused rats or after 5-HT infusion was terminated) blocking the 5-HT_7 receptor had no influence on the arterial pressure (figure 5.2). In a previous pharmacological study, the antagonist had no effect on the 5-HT_7 receptor induced response until the agonist was co-administered (Albayrak et al., 2013). These findings, suggest the receptor is not active in the basal state. The concentration of free 5-HT in the plasma (platelet poor) in the normal basal state appears to be insufficient to stimulate the vascular smooth muscle 5-HT_7 receptors. However, increasing levels of endogenous 5-HT in the circulating plasma have been linked to chronic diseases effecting the cardiovascular system including carcinoid syndrome, inflammation, shock, systemic and pulmonary hypertension and obesity (Ayme-Dietrich et al., 2017; Biondi et al., 1986; Brenner et al., 2007; Jone and Blackburn, 2002; Kim et al., 2011; Shajib and Khan, 2015). How the 5-HT_7 receptor contributes during these pathological conditions under increased endogenous levels of 5-HT is a fascinating idea to explore.

NOS activity is not involved in 5-HT-induced depressor response

As a powerful vasodilator, NO, is an important cellular signaling molecule. NO functions to regulate blood pressure and flow through its action on vascular smooth muscle tone, primarily through endothelial NOS (eNOS). There is no direct evidence the 5-HT_7 receptor (a G-protein coupled receptor), located on smooth muscle, causes NOS activation. An earlier in vitro experiment suggested NOS may not play a role in early 5-HT induced venodilation, as exposure of the superior mesenteric vein to LNNA did not inhibited 5-CT-induced relaxation (Watts et al., 2015). 5-CT, is a 5-HT_7 receptor selective agonist with a sub-nM affinity for the 5-HT_7 receptor, 1000x greater than for

any other 5-HT receptor (De Vries et al., 1999; Hedlund and Sutcliffe, 2004). However, in a chronic vivo 5-HT infusion study, blockade of NOS with NOS inhibitor completely prevented the chronic depressor action of 5-HT during a week of 5-HT infusion. (Banes et al., 2002; Diaz et al., 2008; Seitz et al., 2014). These findings suggested the chronic 5-HT-induced depressor response maybe dependent on the activation of NOS. In those specific experiments, LNNA was administered for 7-10 prior to 5-HT infusion. This is the classical model of experimental LNNA hypertension and is based on blockade of NOS resulting in endothelial dysfunction and vascular wall remodeling. The physiological alterations however that can occur during lengthy administration of L-NNA (7-10 days) to generate the hypertensive state could have prevented the chronic depressor action of 5-HT. Both structural and functional alterations are known to occur in the vasculature when NO formation is blocked over a long timeframe (Kung et al., 1995). The present experiments different in that it examines the involvement of NOS at select time points during one week of 5-HT-induced hypotension in conscious rats. The idea was that over this shorter exposure to the NOS inhibitor, the vascular remodeling and other long-term consequences of blocking NO are likely to not occur (Paulis et al., 2008)

Our original hypothesis was that NOS inhibition would result in a larger pressor response during chronic 5-HT infusion than in the absence of 5-HT infusion. We found in this study animals that were infused with either 5-HT or saline, both had a similar increase in MAP response to LNAME throughout the duration of the experiment. Even when the 5-HT stimulus was removed. Our work strongly suggest that stimulation of NOS is not contributing during 5-HT-induced hypotension. These findings correlate with previous work, which found LNAME had no effect on the acute 5-HT-induced

hypotensive response (Alsip and Harris, 1992; Terron et al., 1997).

Limitations and future direction

The 5-HT_7 receptor is essential in the long lasting hypotensive effects of 5-HT and is independent of NOS, as supported in this paper. We cannot rule out other endogenous vasodilators such as adenosine, prostacyclin (mediator in cyclooxygenase pathway) and ATP-sensitive K^+ channel mediated hyperpolarization (Brayden, 2002; Triggle et al., 2012) as possible contributors to the chronic depressor effect of 5-HT_7 receptor activation. ATP-sensitive K^+ channels have been implicated in the acute 5-HT_7 mediated vasodilation, as glibenclamide abolished 5-HT_7 agonist induced renal vasodilation, in vitro (Garcia-Pedraza et al., 2016). This lends the possibility that the ATP-sensitive K^+ channel might contribute in chronic 5-HT_7 receptor mediated depressor response. Another limitation, is that we did not block the other known 5-HT vasodilator receptors (5-$HT_{1B/1D}$ and 5-HT_{2B}) to ensure selective activation of 5-HT_7 receptors. It did not seem necessary considering the profound attenuation of the 5-HT depressor response with the selective 5-HT_7 receptor antagonist (figure 5.1). However, to formally rule out these other vasorelaxant receptors requires the use of appropriate antagonists to interpret their involvement in the hypotensive response. Although, we believe the experiment outcome would remain the same. In addition, our laboratory recently created 5-HT_7 receptor knock-out (KO) rats (Demireva et al., 2019). 5-HT_7 receptor KO rats, both male and female, showed no hypotensive response to our standard dose of 5-HT during a week-long infusion. However, a significant hypotensive response was observed in the wild-type littermates (Seitz et al., 2019). These rats clearly support the 5HT-induced hypotension is mediated via the 5-HT_7 receptor. The

use of these rats would be beneficial to explore if other endogenous vasodilator possibly contributes to the 5-HT-induced hypotension.

Conclusion

This study demonstrates the necessity of the activated 5-HT_7 receptor, independent of NOS, in mediating 5-HT-induced hypotension in conscious rats over 5 days of 5-HT infusion. Linking the activated 5-HT_7 receptor to long-term cardiovascular control establishes a potential for the 5-HT_7 receptor as a target to further explore the treatment of cardiovascular diseases, especially when hypertension is involved.

REFERENCES

REFERENCES

Albayrak A, Halici Z, Cadirci, Polat B, Karakus E, Bayir Y, Unal D, Atasoy M, Dogrul A. (2013). Inflammation and peripheral 5-HT7 receptors: The role of 5-HT7 receptors in carrageenan induced inflammation in rats. European Journal of Pharmacology, 715:270-279.

Alsip NL, Harris PD. (1992). Serotonin-induced dilation of small arterioles is not mediated via endothelium-derived relaxing factor in skeletal muscle. European Journal of Pharmacology, 229:117-124.

Ayme-Dietrich E, Aubertin-kirch G, Maroteaux L, Monassier L. (2017). Cardiovascular remodeling and the peripheral serotonergic system. Archives of Cardiovascular Disease, 110:51-59.

Banes AK, Watts SW. (2003). Arterial expression of the 5-HT2B and 5-HT1B receptors during the development of DOCA-salt hypertension. *BMC Pharamacol* 3:12.

Berger M, Gray JA, Roth BL. (2009). The expanded biology of serotonin. Annu Rev Med, 60:355-366.

Berger M., Gray J.A. and Roth B.L. (2009). The expanded biology of serotonin. *Annu. Rev. Med.* 60, 35–366.

Biondi ML, Agostoni A, Marasini B. (1986). Serotonin levels in hypertension. J Hypertens, 4:S39-S41.

Brayden JE. (2002). Functional roles of KATP channels in vascular smooth muscle, Clin Exp. Pharmacol Physiol, 29:312–316.

Brenner B, Harney JT, Ahmed BA, Jeffus BC, Unal R, Mehta JL, Kilic F. (2007). Plasma serotonin levels and the platelet serotonin transporter. J Neurochem, 102:206-215.

Cade JR, Fregly MJ, Privette M. (1992). Effect of tryptophan and 5-hydroxytryptophan on the blood pressure of patients with mild to moderate hypertension. Amino Acids, 2:133-140.

Centurión D, Glusa E, Sánchez-López A, Valdivia LF, Saxena PR, Villalón CM. (2004). 5-HT7, but not 5-HT2B, receptors mediate hypotension in vagosympathectomized rats. *Eur J Pharmacol,* 502:239–242.

Dalton DW, Feniuk W, Humphrey PP. (1986). An investigation into the mechanisms of the cardiovascular effects of 5-hydroytryptamine in the conscious normotensive and DOCA-salt hypertensive rats. J Auton Pharmacol, 6:219-228.

Davis RP, Pattison J, Thompson JM, Tiniakov R, Scrogin KE, Watts, SW. (2012). 5-hydroxytryptamine (5-HT) reduces total peripheral resistance during chronic infusion: direct arterial mesenteric relaxation is not involved. BMC Pharmacol, 12:4 doi: 10.11186/1471-2210-12-4.

Davis RP, Szasz T, Garver H, Burnett R, Tykocki NR, Watts SW. (2013). One-month serotonin infusion results in a prolonged fall in blood pressure in the deoxycorticosterone acetate (DOCA) salt hypertensive rat. ACS Chem Neurosci, 4:141-148.

Demireva EY, Xie H, Flood ED, Thompson JM, Seitz BM, Watts SW. (2019). Creation of the 5-hydroxytryptamine receptor 7 knock-out (5-HT7KO) rat as a tool for cardiovascular research. Physiol Genomics, doi:10.1152/physiolgenommics.0030.2019.

Diaz J, Ni W, King A, Fink GD, and Watts SW. (2008). 5-hydroxytryptamine lowers blood pressure in normotensive and hypertensive rats. J Pharmacol Exp Ther, 325:1031-1038.

De Vries P, De Visser PA, Heiligers JPC, Villalón CM, and Saxena PR. (1999). Changes in systemic and regional haemodynamics during 5-HT_7 receptor mediated depressor responses in rats. Naunyn-Schmied Arch Pharmacol, 359:331-338.

Garcia-Pedraza JA, Garcia M, Martin ML, Rodriguez-Barbero A and Moran A. (2016). 5-HT2 receptor blockade exhibits5-HT vasodilator effects via nitric-oxide, prostacyclin and ATP-sensitive potassium channels in rat renal vasculature. Vascular Pharmacology, 79:51-59.

Hedlund PB, Sutcliffe JG. (2004). Functional, molecular and pharmacological advance in 5-HT7 receptor research. Trends Pharmacol Sci, 481-486.

Jones BJ, Blackburn TP. (2002).The medical benefit of 5-HT research. Pharmacology Biochemistry and Behavior, 71:555-568.

Kaumann AJ, Levy FO. (2006). 5-Hydroxytryptamine receptors in the human cardiovascular system. Pharmacology & Therapeutics, 111:674-706.

Kim HJ, *et al.* (2011). Metabolic analysis of livers and serum from high-fat diet induced obese mice. J Proteome Res, 10:722-731.

Kung CF, Moreau P, Takase H, Luscher TF. (1995). LNAME hypertension alters endothelial and smooth muscle function in rat aorta. Hypertensive, 26:744-751.

Page IH and McCubbin JW. (1953). The variable arterial pressure response to serotonin in laboratory animals and man. Circ Res, 1:354-362.

Paulis L, Zicha J, Kunes J, Hojna S, Behuliak M, Celec P, Kojsova S, Pechanova O, Simko F. (2008). Regression of L-NAME-induced hypertension: The role of nitric oxide and endothelium-derived constricting factor. Hypertension Research, 31:793-803.

Rees D.D., Palmer R.M., Moncada S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc. Natl Acad. Sci. USA, 86:3375–3378.

Saxena PR, Villalon CM. (1990). cardiovascular effects of serotonin agonists and antagonists. J Cardiovasc Pharmacol, 15:S16-S34.

Seitz BM, Watts SW. (2014). Serotonin-induced hypotension is mediated by a decrease in intestinal vascular resistance. Pharmacologia, 5(2):50-54.

Seitz BM, Orer HS, Krieger-Burke T, Darios ES, Thompson J, Fink GD, Watts SW. (2017). 5-HT causes splanchnic venodilation. APJ Heart and Cir, 313:H676-H686.

Seitz BM, Demireva EY, Xie H, Fink GD, Krieger-Burke T, Burke WM, Watts SW. (2019).
5-HT does not lower blood pressure in the 5-HT7 knock-out rat. Physiol Genomics, doi: 10.1152/physiolgenomics.00031.2019.

Shajib MS, Khan WI. (2015). The role of serotonin and its receptors in activation of immune responses and inflammation. Acta Physiol (Oxf), 213(3):561-74.

Terrón JA. (1997). Role of 5-ht7 receptors in the long-lasting hypotensive response induced by 5-hydroxytryptamine in the rat. Br J Pharmacol, 121:563-571.

Villalon CM, Centurion D. (2007). Cardiovascular response produced by 5-hydroxytriptamine: a pharmacological update on the receptors/mechanisms involved and therapeutic implications. Naunyn-Schmied Arch Pharmacol, 376:45-63.

Watts SW, Davis RP. (2010). 5-Hydroxytryptamine Receptors in Systemic Hypertension: an arterial focus. Cardiovasc Ther, 1:54–67.

Watts SW, Morrison SF, Davis RP, Barman SM. (2012). Serotonin and blood pressure regulation. Pharmacol Rev, 64:359-388.

Watts SW, Darios ES, Seitz BM, Thompson JM. (2015). 5-HT is a potent relaxant in rat superior mesenteric veins. Pharmacol Res Perspect 3, e00103. doi: 10.1002/prp2.103.

Chapter 6

5-HT does not lower blood pressure in the 5-HT$_7$ knock-out rat

Seitz BM, Demireva EY, Xie H, Fink GD, Krieger-Burke T, Burke WM, Watts SW. (2019).Physiol Genomic, 51(7):302-310. doi: 10.1152/physiolgenomics.00031.2019. Epub 2019 May 24.

PMID:31125292

Abstract

The fall in mean arterial pressure (MAP) after 24 hours of 5-HT infusion is associated with a dilation of the portal vein (PV) and abdominal inferior vena cava (Ab IVC); all events were blocked by the selective 5-HT$_7$ receptor antagonist SB269970. Few studies have investigated the contribution of the 5-HT$_7$ receptor in long-term cardiovascular control, and this requires an understanding of the chronic activation of the receptor. Using the newly created 5-HT$_7$ receptor knockout (KO) rat, we presently test the hypothesis that continuous activation of the 5-HT$_7$ receptor by 5-HT is necessary for the chronic (one week) depressor response and splanchnic venodilation. We also address if the 5-HT$_7$ receptor contributes to endogenous cardiovascular regulation. Conscious MAP (radiotelemeter), splanchnic vessel diameter (ultrasound) and cardiac function (echocardiogram) were measured in ambulatory rats during multi-day 5-HT infusion (25 ug kg $^{-1}$min^{-1} via mini pump) and after pump removal. 5-HT infusion reduced MAP and caused splanchnic venodilation of wild-type (WT) but not KO rats at any time point. The efficacy of 5-HT-induced contraction was elevated in the isolated abdominal inferior vena cava from the KO compared to WT rats, supporting loss of a relaxant receptor. Similarly, the efficacy of 5-HT causing an acute pressor response to higher doses of 5-HT *in vivo* was also increased in the KO *vs* WT

rat. Our work supports a novel mechanism for the cardiovascular effects of 5-HT — activation of 5-HT_7 receptors mediating venodilation in the splanchnic circulation — which could prove useful in the treatment of cardiovascular disease.

Introduction

Administration of 5-HT is known to cause dose-dependent, depressor and pressor responses within the cardiovascular system. These outcomes depend on the level of free circulating 5-HT in the plasma as well as the location, affinity and signaling mechanisms of the numerous 5-HT receptors found in the heart, blood vessels, brain and autonomic nervous system (Cade et al., 1992; Gamoh et al., 2013; Kaumann and Levy, 2006; Ramage and Villalon, 2008). Early in its discovery, 5-hydroxtryptamine (5-HT, serotonin) was demonstrated to cause an acute reduction in blood pressure when infused in dog and man (Page and McCubbin, 1953). In support of these earlier findings, our laboratory has shown that *chronic*, *low dose* 5-HT (25 ug $kg^{-1}min^{-1}$) infused over one week leads to a fall in mean arterial pressure (MAP) not only in normotensive rats, but also in mineralocorticoid-dependent hypertensive rats (> 50 mmHg fall from baseline; Diaz et al., 2008) and in spontaneously hypertensive rats (fall in MAP ~30 mm Hg; Watts et al., 2012). This fall in MAP was dose-dependent (Tan et al., 2011). Using a selective 5-HT_7 receptor antagonist SB269970, we determined that the fall in MAP observed during the *first 24-hours* of 5-HT infusion is dependent on 5-HT_7 receptor activation (Seitz et al., 2017). Our findings confirmed an earlier experiment where the acute (*minutes*) fall in MAP to low dose 5-HT in rats was also mediated by 5-HT_7 receptor (Terron, 1997).

Moreover, we discovered a mechanism by which 5-HT may lower MAP but have only investigated this at a 24-hour time point. Specifically, the fall in MAP to infused 5-HT was paralleled by a significant dilation of the portal vein (PV) and abdominal inferior vena cava (Ab IVC), but not the abdominal aorta (Ab A). Both, the 5-HT-stimulated venodilation and fall in MAP were blocked by the 5-HT_7 selective receptor antagonist SB269970 (Seitz et al., 2017). This *in vivo* finding was consistent with *in vitro* findings published prior, namely that isolated rat splanchnic veins but not arteries relaxed to serotonergic agonists in a 5-HT_7 receptor-dependent matter (Davis et al., 2012; Watts et al., 2015). This was exemplified by the ability of the 5-$HT_{1A/7}$ receptor agonist 5-carboxamidotryptamine (5-CT) to cause concentration-dependent venorelaxation that was antagonized by SB269970 (Watts et al., 2015).

A caveat, however, to this work is that the mechanisms of 5-HT-induced hypotension and venodilation were defined in only the relatively acute timeframe (minutes to 24 hours). Linking the 5-HT_7 receptor to *long-term* cardiovascular control and perhaps pathophysiological states (e.g. hypertension or heart failure) requires an understanding of the hormone's more chronic effects. We do not know if and cannot assume that the mechanism(s) by which chronic (days to weeks) infusion of 5-HT causes a hypotension are the same as those responsible for acute responses. Acute hormone actions are likely to be modified under chronic conditions by well-known phenomena such as receptor down-regulation, receptor desensitization, physiological compensation (*e.g.* baroreflex), and changes in serotonin plasma concentrations or tissue distribution. Chronic hormone actions can be challenging to explore using pharmacological tools because of concerns about the efficacy, receptor selectivity, and

duration of action and off-target effects of pharmacological antagonists to the hormone. Therefore, we created a 5-HT_7 receptor KO rat to provide a different approach to understanding the acute and chronic cardiovascular actions of 5-HT mediated through the 5-HT_7 receptor. Creation of this rat was reported in the previous paper (Demireva et al., 2019). Validation that the 5-HT_7 receptor was functionally ablated in the KO rat was observed by a total loss of 5-CT-induced venodilation and loss of [^{3}H]SB269970 specific binding.

The newly created 5-HT_7 KO rat allowed us to test the hypothesis, by comparison with WT littermates, that *continuous* activation of the 5-HT_7 receptor is both necessary and sufficient for chronic 5-HT-induced depressor responses and splanchnic venodilation. We extend this hypothesis to test whether endogenous activation of the 5-HT_7 receptor plays a role in basal cardiovascular regulation. Finally, we use vascular tissues from the KO rat to explore *in vitro* the role of the 5-HT_7 receptor in control of splanchnic venous function.

Materials and Methods

Animals

MSU Institutional Animal Care and Use Committee approved all protocols used in this study. MSU is an AAALAC accredited institution (A3955-01). Rats were bred in house or Sprague Dawley rats were purchased (200-350 g; Charles River Laboratories Portage, MI, USA). All rats were housed in a temperature–controlled room (22°C) with 12-hour light/dark cycles. Animals were given standard chow and distilled water *ad libitum.* The rats used were randomized to *in vitro* or *in vivo* studies. Each n value represents data that came from one (1) animal.

Creation, validation, breeding, biological description and genotyping of the 5-HT_7 receptor KO rat are described in the paper published immediately prior to this one (Demeriva et al., 2019). WT and KO littermates from several heterozygous breeding pairs are represented in this paper. Rats were randomized to the experiments performed.

In vivo Protocols

Telemetry and pump implantation

Under isoflurane anesthesia (2% in oxygen), radiotelemeter transmitters (HD-S10; Data Sciences International, MN USA) were implanted subcutaneously through a 1-1.5cm incision in the left inguinal area. The radiotelemeter catheters were introduced into the left femoral artery and advanced into the abdominal aorta below the renal artery. Animals received a dose of enrofloxacin (Baytril®, 2.5 mg kg^{-1}, im) and carprofen (Rimadyl®, 5 mg kg^{-1}, sc). After 5 days of post-operative recovery, baseline cardiovascular measurements were recorded for 5 days. MAP and heart rate (HR) were measured for 10 seconds every 10 minutes throughout the duration of the study. Under isoflurane anesthesia, osmotic pumps (Model 2ML1; Alzet osmotic pumps) containing 5-HT (25 μg kg^{-1} min^{-1} in 1% ascorbate in sterile saline, pH balanced to pH 6-7) were implanted subcutaneously between the scapulae. On the completion of the final day of infusion, pumps were removed from rats under isoflurane anesthesia and cardiovascular parameters were monitored for 6 additional days (termed recovery). The weight of the 5-HT mini pumps was recorded before implantation and after removal to confirm drug delivery.

Ultrasound Imaging of Vessel Diameter and Echocardiograms

This technique was originally reported in Seitz et al., 2016. Both WT and KO, male and female, rats (isoflurane-anesthetized, 1-2%) were positioned supine on a warmed platform (Vevo 2100 Imaging System; Visualsonics, Toronto, Canada). Ultrasound gel was applied to the prepared abdominal skin, just below the xiphoid process to couple the transducer (21 MHz probe; MS250) before imaging in B-mode. Real-time images were scanned at 25 frames per second. Images were taken at the location of the PV exiting the liver, which provides images of the Ab A, Ab IVC and PV. In addition, an echocardiogram was performed to assess cardiac structure and function. A Doppler probe in the parasternal long axis view was used to obtain cardiovascular systemic measurements of stroke volume (SV), ejection fraction (EF) and cardiac output (CO). The Vevo 2100 imaging system software derived each of the above cardiovascular parameters. Each session took ~10 minutes per rat, minimizing the amount of time each animal was under anesthesia. Images for both vessel diameter and echocardiogram were taken at baseline for both sexes in KO and WT animals. Male and female rats showed similar 5-HT-induced depressor responses and analogous outcomes to 5-HT during *in vitro* contractility. Due to the similarities of 5-HT responses between male and females, the vessel diameter image measurements and echocardiogram response to 24 hours of 5-HT infusion, day 4 of 5-HT infusion and recovery (pump removed) data were obtained in *only* female KO and female WT rats.

Acute 5-HT administration and blood pressure measurement

Paired male WT and KO littermates were instrumented with radiotelemetery and intravenous femoral catheters for administration of 5-HT in the conscious state.

Cumulative doses of 5-HT (12.5 – 100 ug kg^{-1}) were given with each dose administered over 20 minutes during which time MAP reached a stable plateau. After several days of washout, these same animals were given a constant infusion of 5-CT (1 ug kg^{-1} min^{-1}).

In vitro Protocol

Isolated tissue bath measurement of isometric contraction

Before terminal tissue removal, rats (both male and female) were given pentobarbital as a deep anesthetic (80 mg $kg^{-1,}$ ip). A bilateral pneumothorax was created prior to vessel dissection. The Ab IVC and paired Ab A were dissected from just above the iliac bifurcation up to the kidneys. Tissue dissection took place under a stereomicroscope and in a Silastic®-coated dish filled with physiological salt solution (PSS) containing [mM: NaCl 130; KCl 4.7; KH_2PO_4 1.18; $MgSO_4 \bullet 7H_2O$ 1.17; $NaHCO_3$ 14.8; dextrose 5.5; $CaNa_2EDTA$ 0.03, $CaCl_2$ 1.6 (pH 7.2)]. The Ab A and the Ab IVC were separated and guided individually onto stabilizing wire, cleaned of fat and used in one of the protocols described below. The endothelium was left intact. Cleaned vessels were cut into rings (~3 mm wide) for measurement of isometric contractile force. Rings were mounted in warmed (37°C) and aerated (95% O_2, 5% CO_2) tissue baths (30 mL PSS) on Grass isometric transducers (FT03; Grass instruments, Quincy, MA, USA) connected to a 4 channel PowerLab (ADInstruments, Colorado Springs, CO, USA). All tissues were randomized in their placement in one of four tissue baths in each experiment (WT Ab IVC, KO Ab IVC, WT Ab A, and KO Ab A). Tissues were placed under optimal resting tension (Ab IVC = 1000 mg; Ab A = 4000 mg) and allowed to equilibrate for 1 hour before an initial challenge with a maximal concentration of

norepinephrine (NE; 10^{-5} M). The magnitude of contractions to NE are reported in the figure legends.

After this challenge, tissues were washed until tone returned to baseline. Cumulative concentration response curves were generated to NE and 5-HT ($1x10^{-9}$ – $3x10^{-5}$ M) in all tissues. Tissues were washed out for at least 90 minutes between curves with buffer exchanges occurring every 5 minutes. In a separate set of experiments, Ab IVC from female KO rats were studied. Tissues were incubated with either vehicle or the $5\text{-HT}_{2A/2C}$ receptor antagonist ketanserin (100 nM) for one hour prior to constructing a cumulative concentration response curve to 5-HT.

Materials

Acetylcholine chloride, forskolin, 5-HT creatinine sulfate, and norepinephrine hydrochloride were obtained from Sigma Chemical Company (St. Louis MO USA). 5-CT maleate was purchased from Tocris (R & D, Minneapolis MN USA) or Abcam (Cambridge MA USA).

Data and statistical analyses

Quantitative data are reported as means±SEM for number of animals in parentheses. WT and KO were littermates. No outliers were removed from any of the data presented. For both ultrasound vessel diameter images and echocardiogram measurements, a trained user/reader of the Vevo Imaging system, blinded to the nature of the rat (KO or WT) or type of measure (baseline, 24-hour, day 4, recovery), analyzed each vessel diameter and heart parameter. All vessel diameter measurements were controlled for respiration and cardiac cycles and are reported in

millimeters (mm). The echocardiogram generated the following cardiovascular measurements: CO (mL min^{-1}), EF (%) and SV (uL). For isometric contractile measures, contraction is reported in milligrams or as a percentage initial NE-induced contraction. Magnitudes of contraction to these agonists for each experimental group are reported in the appropriate figure legend. Agonist potencies were calculated using a non-linear regression (curve fit) within GraphPad® Prism 7.0 (La Jolla, CA, USA), and are reported as –log EC_{50} values [M]. Maximums are reported as the maximal effect achieved. For *in vitro* measures, t-tests to compare agonist-induced maximal responses agonist or potencies were used. For *in vivo* measures, a repeated measures ANOVA was used when comparing values from baseline or within a group (GraphPad Prism 7) when the F value achieved statistical significance and there was no significant variance in homogeneity as tested by Bartlett's. When comparing between groups, a t-test was used. In all cases, a p value of <0.05 was considered significant.

Results

Lack of functional 5-HT_7 receptor does not modify basal MAP

All baseline cardiovascular measurements are reported in table 6.1. 5-HT_7 receptor KO and WT rats were implanted with telemetry for measurement of cardiovascular parameters. Figure 6.1 shows 24-hour average MAP and HR for the males (A) and the females (B). At baseline (prior to first dashed vertical line), MAP was not significantly different between KO and WT controls, nor was there a difference in HR. This was true in both sexes.

One-week infusion of 5-HT did not reduce MAP in the conscious 5-HT_7 receptor KO rat

After five days of baseline cardiovascular data collection, a mini pump containing 5-HT (25 ug kg•min^{-1}) was implanted into all rats. In both WT males and females, infused 5-HT caused a fall in MAP that reached its nadir within the first day (male WT baseline 99.2±3 mmHg vs with 5-HT 80.8±5.1 mmHg; female WT baseline 102.8±1 mm Hg vs with 5-HT 89.7±0.7 mmHg) (figure 6.1, top A-B). The WT male rats maintained significantly reduced MAP during the first 3 days of 5-HT infusion compared to their baseline values. Female WT rats maintained significantly reduced MAP for the entire infusion period, then MAP returned to baseline value once the 5-HT pump was removed. By contrast, neither male nor female KO rats showed a change in MAP during the duration of 5-HT administration. This lack of MAP response to 5-HT infusion was paralleled by no change in HR (figure 6.1, bottom A-B). The WT rats, both male and female, had an increase in HR concurrent with the corresponding fall in MAP, likely a baroreflex response. After removal of the 5-HT pumps, only the WT female rats had a significant overshoot of MAP, which corresponded with a reduction in HR. The weight of the 5-HT pumps upon removal were all similar among groups indicating comparable drug delivery in all groups.

Vessel contractility to NE was not modified in the 5-HT$_7$ receptor KO rat

Paired arteries and veins from WT and KO, male and female, rats were used to investigate contraction to both non-serotonergic and serotonergic stimuli. Figure 6.2 demonstrates that in both the isolated Ab A (figure 6.2 A) and Ab IVC (figure 6.2 B), contraction to the adrenergic agonist NE was not modified in potency or in efficacy (pharmacological parameters in table 6.2). This validates that lack of a 5-HT$_7$ receptor did not change arterial reactivity to NE.

	Baseline values			
	Female (N=6)		Male (N=6)	
	WT	KO 5-HT$_7$ receptor	WT	KO 5-HT$_7$ receptor
Body weight	328±14	285 ±14*	480± 9.2	469±9.2
MAP (mmHg)	102.8±1	103±1	99.2±3	104.2±1
HR (bpm)	390.3±6	400.6±6	384.6±7	381±6
DIA (mmHg)	86.3±0.2	88.7±1	83.4±2	86.8±1
SYS (mmHg)	124.2±0.3	129.9±1	120.6±5	127.8±2
AbIVC (mm)	2.96±0.3 (.009)	2.92±0.1 (.01)	6.3±0.6 (.013)	6.5±0.5 (.013)
PV (mm)	1.7±.09 (.005)	1.8±.04 (.006)	2.9±0.1(.006)	2.5±0.1(.005)
AbA (mm)	2.1±.04 (.006)	1.9±.07 (.006)	2.2±.05 (.004)	2.1±.03* (.004)
SV (uL)	222±7 (.68)	195±15 (.68)	295±12 (.61)	277±9 (.59)
CO (mL/min)	80±4 (.24)	67±6 (.23)	106±7 (.22)	92±2 (.20)
EF (%)	64.3±1 (.20)	63.6±3 (.22)	67.4±2 (.14)	65.8±3 (.14)

Table 6.1. **Baseline cardiovascular measurements for KO and WT rats, both male and female.** Values represent means±SEM for 6 rats per group. Value in the parenthesis represent the individual cardiovascular value indexed to body weight.* =p<0.05 between same sex vs WT use an unpaired t-test.

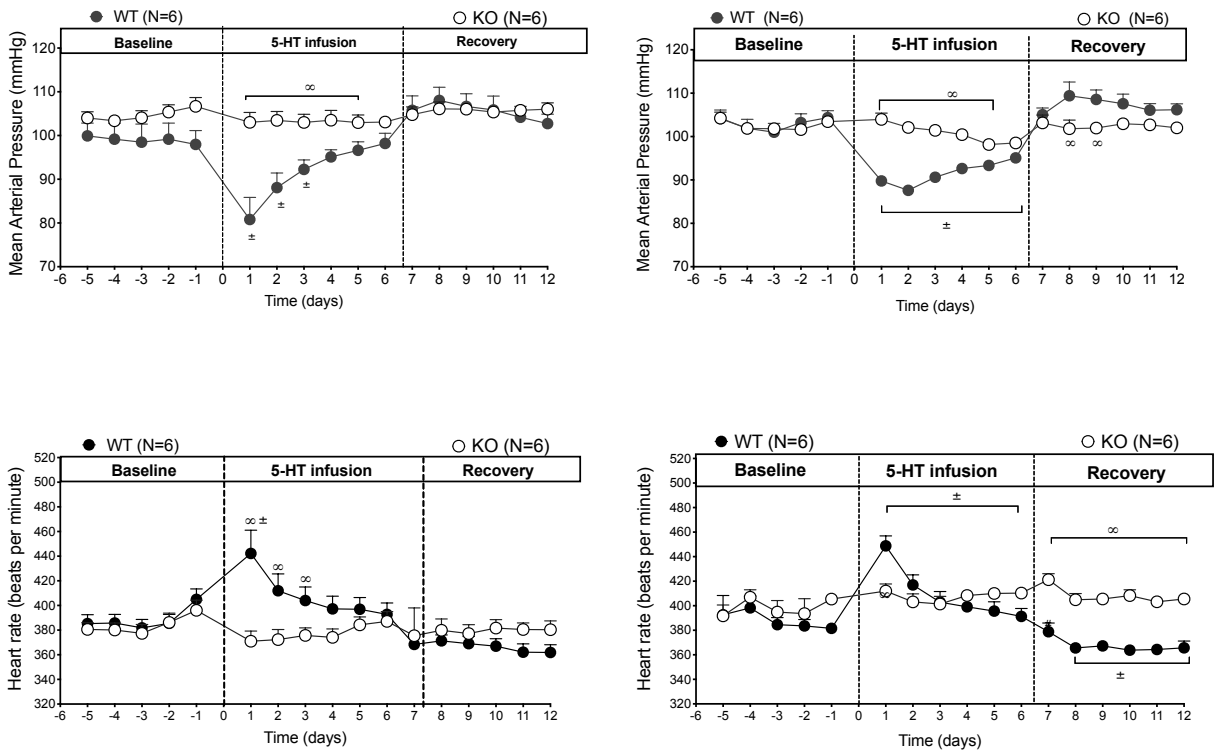


Figure 6.1: **Time course of 5-HT-induced changes in mean arterial pressure and heart rate in 5-HT$_7$ receptor in male and female KO and WT rats**.Time course of 5-HT-induced changes in mean arterial blood pressure (top) and heart rate (bottom) in male (A; N=6) and female (B; N=6) WT and KO rats during baseline, 5-HT-infusion and recovery post pump removal. Points represent means±SEM. +/- indicate significant differences in WT values compared to own baseline (repeated measures ANOVA). Infinity symbol indicate significant differences between WT *vs* KO rat (ANOVA). $p<0.05$. First hatched vertical line = pump implantation, second line = pump removal (recovery).

Venous but not arterial contraction to 5-HT was upregulated in the 5-HT$_7$ receptor KO rat

By contrast to the normal contraction to NE observed in all vessels, 5-HT-induced efficacy of contraction of the Ab IVC from the KO rat (male and female) was markedly increased *vs* the WT male and female (figure 6.3 B). This upregulated contraction was mediated by the 5-HT$_{2A}$ receptor given the ability of the 5-HT$_{2A/2C}$ receptor antagonist ketanserin to cause a rightward shift to the 5-HT-induced contraction in Ab IVC from KO rats (female; figure 6.4**)**. Contraction to 5-HT was not modified in the Ab A of either the WT or KO, reaffirming the lack of a functional 5-HT$_7$ receptor in this tissue (figure 6.3 A).

Lack of splanchnic venodilation or changes in cardiac hemodynamics in female 5-HT$_7$ receptor KO rats after one-week infusion of 5-HT

Thus far*,* data generated *in vivo* and *in vitro* have shown no difference in response to 5-HT between the male and female rats. As such, only female KO and WT rats were used during this next portion of the study. Baseline diameters of the observed vessels and cardiovascular parameters were similar between groups in the female rats as reported in table 6.1. Splanchnic vessel diameters, as well as the cardiac parameters of stroke volume (SV), cardiac output (CO) and ejection fraction (EF), were measured in WT and 5-HT$_7$ receptor KO rats at baseline and then again, in the same animals, 24 hours and 4 days after beginning 5-HT-infusion through mini pump implantation. Recovery measures with pump removal were also made in the same rats.

NE	-log EC_{50} [M]	Efficacy (% NE contraction)
Abdominal Aorta		
Male WT	6.95±0.26	99.4±9.2
Male KO	7.38±0.28	108.9±9.00
Female WT	7.20±0.33	110.6±8.3
Female KO	7.01±0.35	116.1±11.9
Abdominal Vena Cava		
Male WT	6.11±0.07	119.5±9.4
Male KO	6.24±0.07	118.1±7.7
Female WT	6.16±0.08	146.7±14.3
Female KO	6.16±0.13	146.9±21.5

5-HT	-log EC_{50} [M]	Efficacy (% NE contraction)
Abdominal Aorta		
Male WT	5.71±0.19	105.8±11.6
Male KO	5.67±0.12	116.3±9.0
Female WT	5.95±0.15	100.6±13.2
Female KO	5.84±0.09	117.4±9.0
Abdominal Vena Cava		
Male WT	6.01±0.14	19.4±5.4
Male KO	5.83±0.14	80.0±17.2*

Table 6.2. Potency and efficacy of NE and 5-HT in abdominal aorta and vena cava male and female WT and KO rats. Points are means±SEM for number of animals indicated in **figures 2 and 3**. Maximum is the maximum effect achieved reported as a percentage of the maximum NA. * = $p<0.05$ between same sex value vs WT; ** = $p< 0.05$ between different sexes of same genotype.

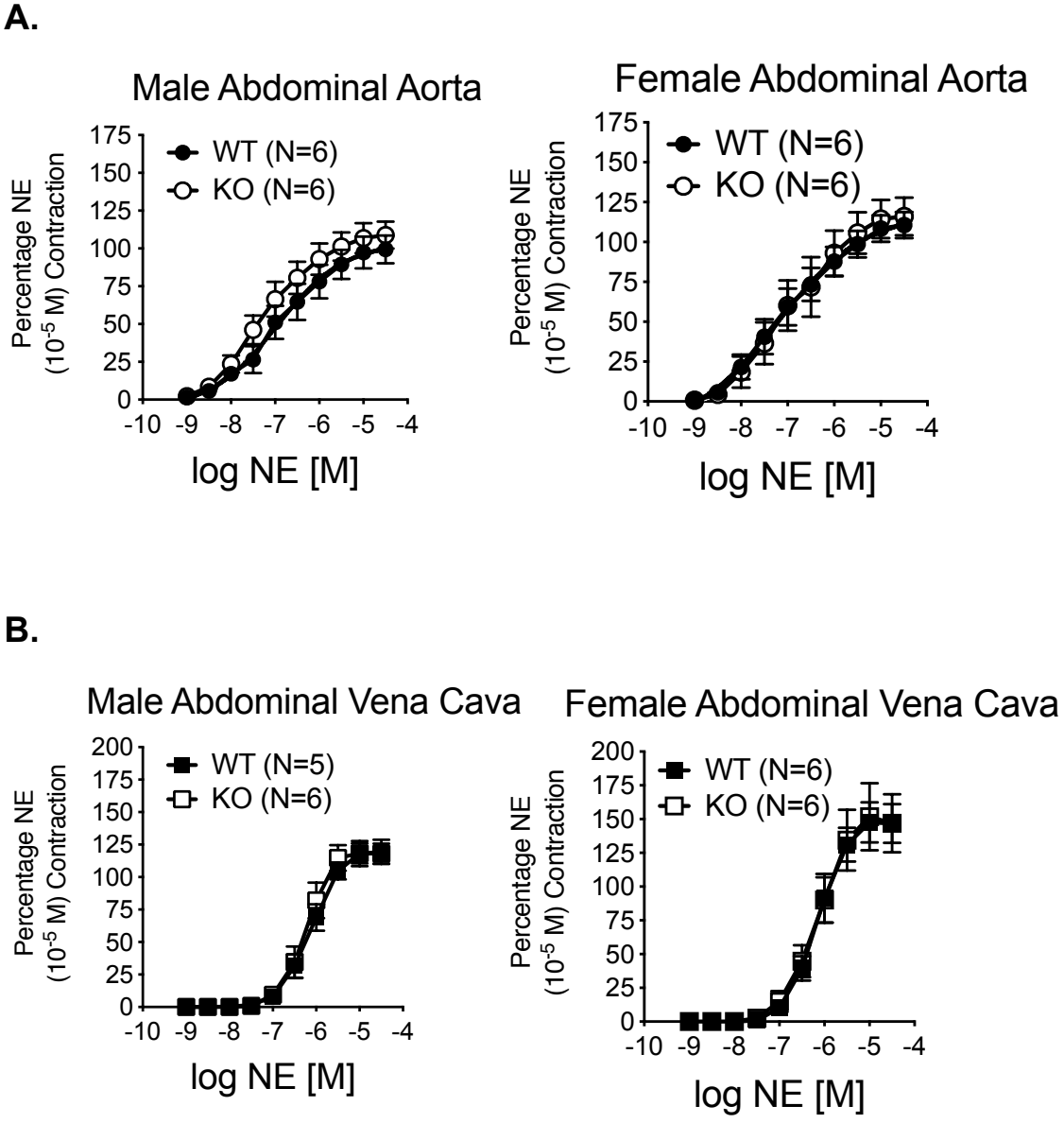
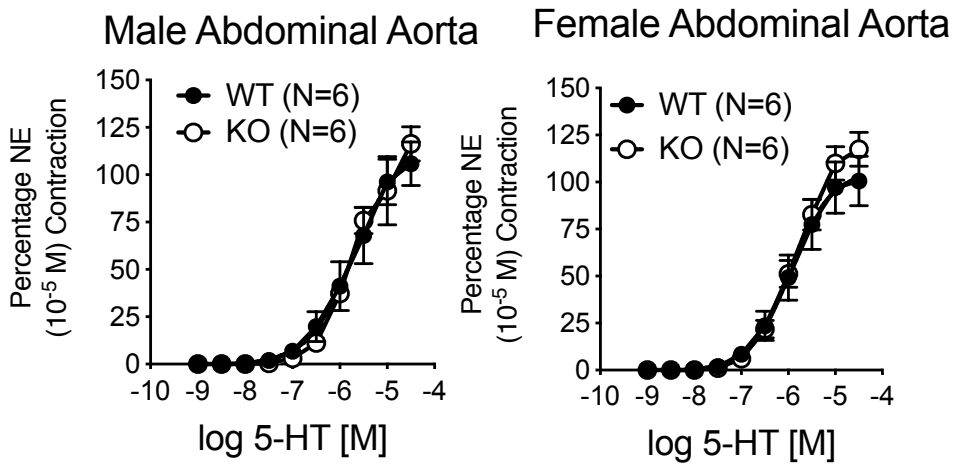


Figure 6.2: **Concentration-dependent contraction to NE of isolated abdominal aorta and abdominal inferior vena cava from male and female WT and 5-HT$_7$ receptor KO receptor.** Concentration-dependent contraction to NE of isolated abdominal aorta (A) and vena cava (B) from the male (left: N=5-6) and female (right; N=6) WT and KO rats. One male WT vena cava ring did not wake up to NE and was not included in analysis. Points represent means±SEM. NE maximum contraction (used for normalization): male abdominal aorta: WT = 1504±193 mg, KO = 1648±153 mg; female abdominal aorta: WT = 1936±129 mg, KO= 1323±100 mg; male abdominal vena cava: WT = 655±189 mg, KO = 608±81 mg; female abdominal vena cava: WT= 381±105 mg, KO = 381±105 mg.

A.



B.

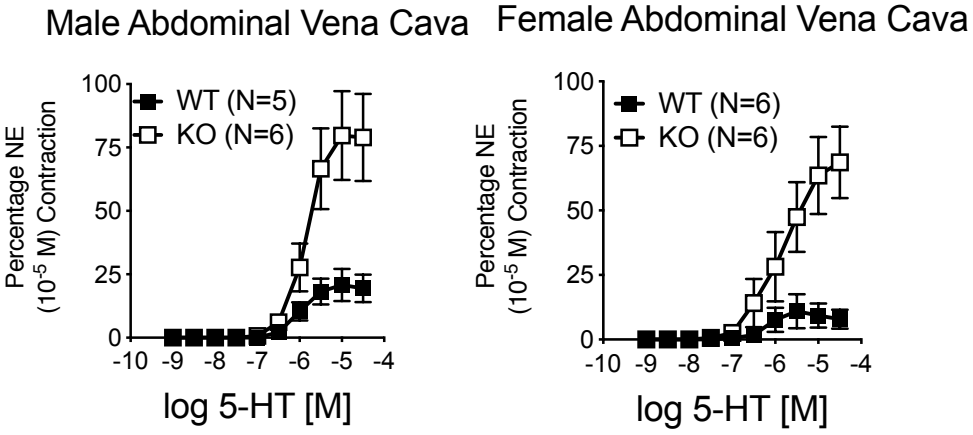


Figure 6.3: Concentration-dependent contraction to 5-HT of isolated abdominal aorta and abdominal inferior vena cava from male and female WT and 5-HT$_7$ receptor KO rats. Concentration-dependent contraction to 5-HT of isolated abdominal aorta (A) and vena cava (B) from the male (left; N=5-6) and female (right; N=6) WT and KO rats. One male WT vena cava ring did not wake up to NE and was not included in analysis Points represent means±SEM. * indicate significant differences ($p<0.05$) in the maximums between WT and KO as determined through an unpaired t test. Absolute magnitudes of NE maximums as reported in legend for figure 6.2.

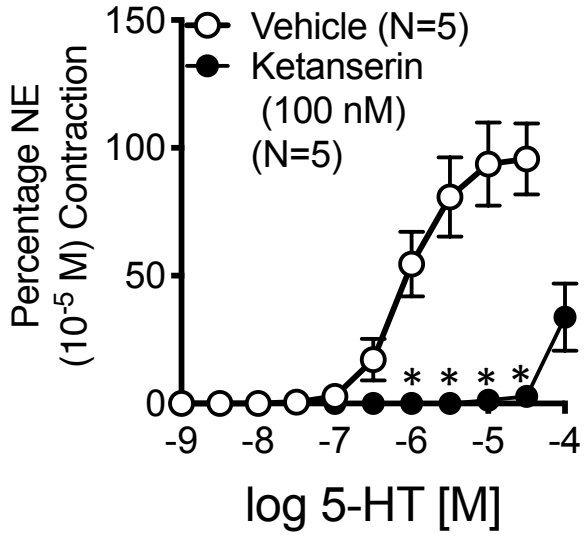


Figure 6.4: Effects of ketanserin on isolated 5-HT-induced contracted vena cava from female 5-HT$_7$ receptor KO rats. Ability of the 5-HT$_{2A/2C}$ receptor antagonist ketanserin (100 nM) to antagonize the 5-HT-induced contraction of the vena cava isolated from the KO female rat (N=5). Points represent means$\pm$SEM for the number of animals indicated in parentheses. * indicate significant differences between vehicle- and ketanserin-incubated values as determined through unpaired t-tests with Bonferroni corrections. NE maximums: vehicle = 394$\pm$79 mg; ketanserin = 508$\pm$81 mg.

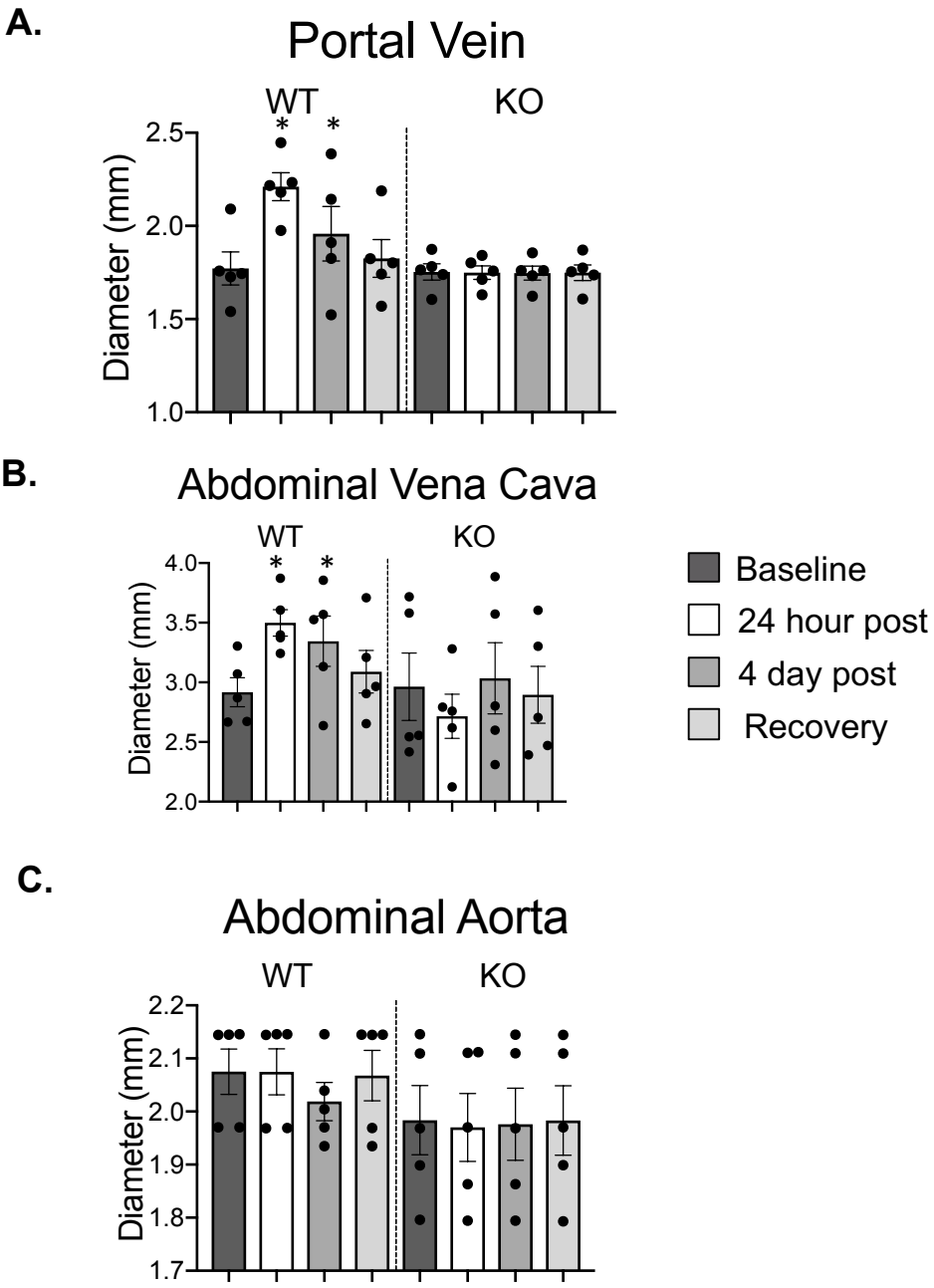


Figure 6.5: **Measures of imaged vessels from female WT and 5-HT$_7$ receptor KO rats at specific time intervals during one week of 5-HT infusion**. Dilation of portal vein (A) and abdominal vena cava (B) but not abdominal aorta (C) in the WT but not the KO female rats (N=5) with 5-HT infusion over a week; measures were made at baseline, 24 post infusion, 4-days post infusion and 5 days after pump removal. Bars represent means±SEM around which points are scattered. * indicate significant differences ($p<0.05$) in WT values compared to baseline using ANOVA.

In WT female rats, both the PV and Ab IVC but not the Ab A dilated in the presence of 5-HT, which was significant during first 24 hours of 5-HT infusion (figure 6.5 A-C). Once the 5-HT stimulus was removed, diameters of the WT PV and Ab IVC were restored to near baseline levels. In contrast, the diameter of neither, the veins (PV or Ab IVC), nor the Ab A of the female KO rat were changed by 5-HT infusion. In this same set of rats, SV was elevated 24 hours post 5-HT infusion in the WT rats only; comparing to its own baseline values and the value of 24 hours post 5-HT KO rat (figure 6.6 A). Surprisingly, CO remained increased in the WT rats during the duration of 5-HT infusion and after pump removal when compared to values in KO rats (figure 6.6 B). EF was similar between KO and WT throughout the duration of the study (figure 6.6 C). None of the cardiac measures changed in the KO rat when 5-HT was infused.

Potency and efficacy of 5-HT as a vasopressor is increased in the KO; KO shows complete loss of response to 5-CT in vivo

We tested whether loss of a functional 5-HT_7 receptor modified the acute pressor response to 5-HT by infusing 5-HT in a dose response manner (0-100 ug kg^{-1}) in conscious male rats. This is an experiment similar in intent to that of the isolated Ab IVC (figure 6.3) in which the efficacy of 5-HT was increased in the KO *vs* the WT. Consistent with findings from this *in vitro* experiment, 5-HT was more efficacious in raising MAP in the KO *vs* WT rat (figure 6.7 A). Finally, the KO rat had no vasodepressor response to acute administration of 5-CT while a reduction in blood pressure was observed in the WT rat (figure 6.7 B).

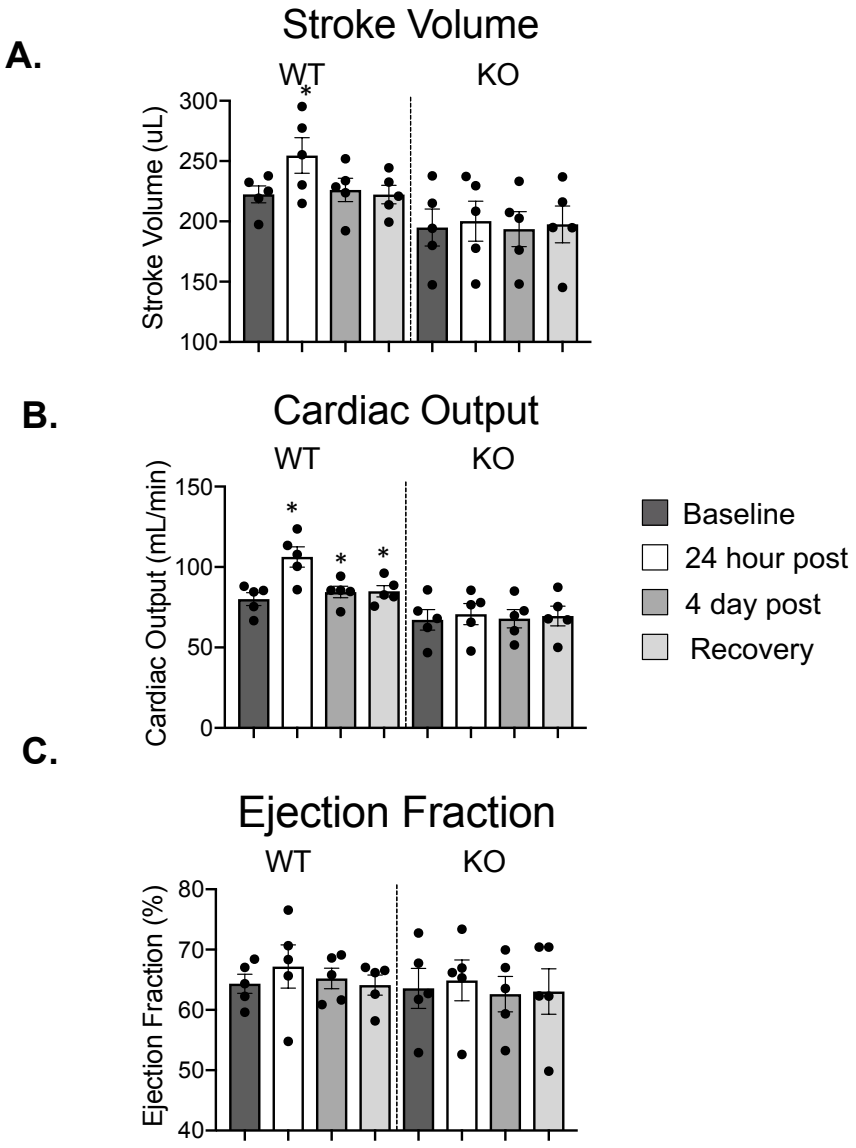
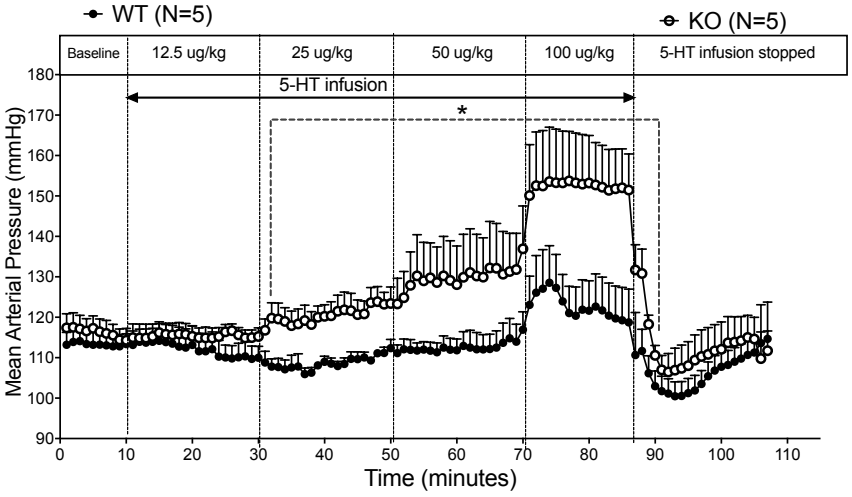


Figure 6.6: Systemic hemodynamic measures in WT and 5-HT$_7$ receptor KO rats at specific time intervals during one week of 5-HT infusion. Doppler echocardiogram measurements of stroke volume (A), cardiac output (B) and ejection fraction (C) in the KO and WT female rats (N=5) with one-week infusion of 5-HT; measures were made at baseline, 24 post infusion, 4-days post infusion and 5 days after pump removal. Bars represent means±SEM for number of animals in parentheses and each dot represents an individual rat. * indicate significant differences ($p<0.05$) in WT values compared to own baseline using ANOVA.

A.



B.

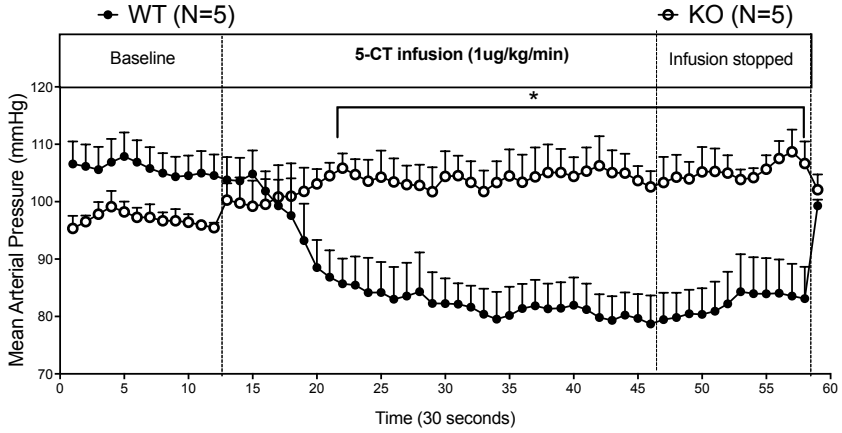


Figure 6.7: **Mean arterial pressure response to 5-HT and 5-CT in male WT and 5-HT_7 receptor KO rats**. Response of conscious male WT and KO rats (N=5) to increasing doses of 5-HT (A) and individual dose of 5-CT (B). Points represent means±SEM for the number of animals indicated in parentheses. * indicate significant differences ($p<0.05$) between WT and KO response using ANOVA.

Discussion

The use of the newly created 5-HT_7 receptor KO rat allowed us new insights into the contributions made by the 5-HT_7 receptor in chronic regulation of blood pressure. Although our previous use of SB269970 provides one approach to demonstrating the importance of the 5-HT_7 receptor in 5-HT-induced hypotension, it is challenging to use for *long-term* studies due to its short half-life and poor parenteral bioavailability. This led us to develop a 5-HT_7 receptor KO rat model using CRISPR-Cas 9 technology (Demireva et al., 2019).

Concentration/dose matters for experimental outcome

It is noteworthy that 5-HT can cause both vasoconstriction and vasodilation, as well as elevation or depression of blood pressure, depending on the concentration achieved in the circulating plasma, and the specific 5-HT receptor(s) activated. The infusion rate of 5-HT used *in vivo* (25 ug kg^{-1} min^{-1}) achieves an elevation of free circulating 5-HT to concentrations that are in the mid-nanomolar range (Diaz et al., 2008). This concentration of free 5-HT in the plasma is insufficient to fully activate the vasoconstrictor 5-HT_{2A} receptors, for which 5-HT possesses affinity (Ki) that is ~300-1000 nM, but is sufficient to nearly maximally activate 5-HT_7 receptors (Ki ~1-10 nM) (pdsp.unc.edu/databases).

If the dose of 5-HT infused is elevated above 25 ug kg^{-1} min^{-1}, then a pressor response, likely mediated by 5-HT_{2A} receptors, is stimulated; data in figure 6.7 of the present paper illustrate this point. Thus, the dose of 5-HT matters significantly when interpreting experimental outcomes. 5-HT_7 receptor KO rats, both male and female, showed *no* hypotensive response to our standard rate of 5-HT (25 ug kg^{-1} min^{-1})

throughout a week-long infusion, whereas a typical hypotensive response was seen in WT rats. A pressor response was not observed in the KO rats, confirming that our typical infusion rate produces plasma concentrations of 5-HT that activate 5-HT_7 but likely not other subtypes of 5-HT receptors. Another important conclusion is that 5-HT_7 receptor activation up to a week can maintain a reduced blood pressure to some degree. The hypotension response wanes in magnitude, likely due to both pharmacological and physiological compensatory responses. This recovery of BP is not because of degradation of 5-HT within the pump (Diaz et al., 2008), and veins from animals infused with 5-HT continue to venodilate (not shown). Males appeared to recover more rapidly than females relative to their own baseline blood pressure; we have no good explanation for this difference. Similarly, the rebound in blood pressure in the females after 5-HT removal, which was accompanied by a sustained fall in HR, is an observation for which we have no mechanistic explanation. Altogether, the 5-HT_7 receptor could function as a long-term regulator of cardiovascular function that could be taken advantage of therapeutically.

Basal function of the 5-HT_7 receptor in blood pressure regulation?

Another question we hoped to answer is whether the 5-HT_7 receptor was important to *basal* blood pressure regulation. If the 5-HT_7 receptor is activated constitutively by endogenous 5-HT, this would lower blood pressure. Thus, removal of the 5-HT_7 receptor would likely be accompanied by an elevated blood pressure. We did not, however, observe an elevated MAP in the KO *vs* WT rat in either males or females. Interestingly, CO and SV were globally lower in the KO rats in both sexes but these measures were not significantly different from WT. Even in isolated blood

vessels with functional 5-HT_7 receptors (splanchnic veins), the loss of the 5-HT_7 receptor did not affect the contractile response to NE. These collective data suggest that 1) it is unlikely endogenous 5-HT is high enough in normal physiology to activate a sufficient cadre of 5-HT_7 receptors peripherally to cause a biological effect; and 2) the 5-HT_7 receptor does not functionally interact with the α adrenergic receptor. This is an important point to make as it divorces the hypotension caused by activation of the 5-HT_7 receptor from the functional control of the alpha-adrenergic receptors, which are undeniably important for elevation of total peripheral resistance and blood pressure.

Impact of 5-HT_7 receptor on venous function

Infusion of 5-HT caused both the PV and Ab IVC to dilate in WT rats but not KO rats. There were no changes in the Ab A diameter during the infusion in either the WT or KO rats. These results confirm earlier pharmacological studies that activation of the 5-HT_7 receptor is more important to venous *vs* arterial function, at least in vessels from the splanchnic circulation. The veins within the splanchnic region have large capacitance capability. They store ~70% of the total blood volume. Constriction of small veins in the splanchnic region causes an increase in mean circulatory filling pressure (MCFP) moving unstressed volume into the circulation. Constriction of larger splanchnic veins cause an increase in the resistance to venous return (R_{VR}). Both variables are critical regulators of cardiac output (Green, 1982). Therefore, changes in the diameter of these splanchnic veins are influential in blood pressure regulation mainly due to changes in SV and CO. In the present and in a previously published work (Davis et al., 2012), CO and SV were increased with a dilation of the Ab IVC during chronic 5-HT infusion. These results suggest that the main function of

splanchnic venous 5-HT_7 receptors is to control R_{VR} rather than MCFP. The lack of venodilation and depressor response observed in the KO rats during 5-HT infusion suggests that venodilation contributes to the chronic depressor response to 5-HT. It is notable that the hypotension stimulated by 5-HT occurred in the absence of any receptor antagonism, and this fact contributes to the importance of the finding. Published studies have used receptor antagonists to uncover a vasodepressor response to 5-HT (Terron, 1997; Villalon et al., 2000) but this was not necessary with our intervention.

Interestingly, there was a markedly increased efficacy of 5-HT in the Ab IVC KO compared to WT. The arterial pair to this vessel, the Ab A, showed no change in 5-HT-induced contraction in KO vs WT. The enhanced 5-HT-induced contraction in the KO Ab IVC was likely mediated by the 5-HT_{2A} receptor because the 5-HT_{2A} receptor antagonist ketanserin antagonized this contraction. This finding suggests an antagonism – physiological or pharmacological – between the 5-HT_{2A} (pressor) and 5-HT_7 (depressor) receptor in the AB IVC. Loss of the 5-HT_7 receptor allowed 5-HT to be a more efficacious venoconstrictor. It has not yet been possible to measure receptor number (e.g. does loss of 5-HT_7 increase 5-HT_{2A}?) because of poor antibody selectivity. By contrast, in the large artery, the 5-HT_7 receptor is not normally functional in modifying smooth muscle tone such that the removal of the receptor does not affect normal contractility. Ultrasound imaging of these same vessels *in vivo* supports these *in vitro* findings.

Conclusion and Clinical Relevance

This study provides insight into the unique cardiovascular pharmacology of the 5-HT_7 receptor, a member of the 5-HT receptor family whose cardiovascular actions have been little studied. Our work here confirms that the 5-HT_7 receptor mediates the hypotension to a low dose of 5-HT infused over days. In addition, this work provides novel insight to the significance of this receptor to controlling the splanchnic veins. The relevance of this work is grounded in intriguing studies in which 5-HT or 5-hydroxytryptophan (precursor to 5-HT) infusion reversed and/or delayed development of multiple forms of hypertension in the experimental animal models (Baron et al., 1991; Dalton et al., 1986; Echizen and Freed, 1981; Fregly et al., 1987; Itskovitz et al., 1989). Collectively, our findings suggest that the 5-HT_7 receptor may be a novel and useful target for creating therapies to manage cardiovascular diseases (Selim et al., 2016).

REFERENCES

REFERENCES

Baron A, Riesselmann A, Fregly MJ. (1991). Reduction in the elevated blood pressure of Dahl Salt sensitive rats treated chronically with L-5-hydroxytryptophan. Pharmacology, 42:15-22.

Cade JR, Fregly MJ, Privette M. (1992). Effect of tryptophan and 5-hydroxytryptophan on the blood pressure of patients with mild to moderate hypertension. Amino Acids, 2:133-140.

Dalton DW, Feniuk W, Humphrey PP. (1986). An investigation into the mechanisms of the cardiovascular effects of 5-hydroxytryptamine in conscious normotensive and DOCA-salt hypertensive rats. J Auton Pharmacol, 6:219–228.

Davis RP, Pattison J, Thompson JM, Tiniakov R, Scrogin KE, Watts SW. (2012). 5-hydroxytryptamine (5-HT) reduces total peripheral resistance during chronic infusion: direct arterial mesenteric relaxation is not involved. BMC Pharmacol, 12:4 doi: 10.11186/1471-2210-12-4.

De Vries P, De Visser PA, Heiligers JPC, Villalón CM, Saxena PR. (1999). Changes in systemic and regional haemodynamics during 5-HT_7 receptor mediated depressor responses in rats. Naunyn-Schmied Arch Pharmacol, 359:331-338.

Demireva EY, Xie H, Darios ES, Thompson JM, Seitz B, Watts SW. (2019). Creation of the 5-Hydroxytryptamine receptor 7 knockout (Htr7 KO) rat as a tool for cardiovascular research. Physiological Genomics.

Diaz J, Ni W, King A, Fink GD, Watts SW. (2008). 5-hydroxytryptamine lowers blood pressure in normotensive and hypertensive rats. J Pharmacol Exp Ther, 325:1031-1038.

Ding XR, Stier CT, Itskovitz HD. (1989). Serotonin and 5-hydroxytryptophan on blood pressure and renal blood flow in anesthetized rats. Am J Med Sci, 297:290-293.

Echizen H, Freed CR. (1981). Long-term infusion of L-5-hydroxytryptophan increases brain serotonin turnover and decreases blood pressure in normotensive rats. J Pharmacol Exp Ther, 220:579-584.

Fregly MJ, Lockley OE, Sumners C. (1987). Chronic treatment with L-5-hydroxytryptophan prevents the development of DOCA-salt induced hypertension in rats. J Hypertens, 5:621-628.

Gamoh S, Hisa H, Yamamoto. (2013). 5-Hydroxytryptamine receptors as targets for drug therapies of vascular-related diseases. Biol. Pharm, 36(9):1410-1415.

Gellynck E, Heyninck K, Andressen KW, Haegeman G, Levy FO, Vanhoenacker P, Van Craenenbroeck. (2013).The serotonin 5-HT_7 receptors: two decades of research. Exp Brain Res, 230:555-568.

Green JF. (1982). Circulatory mechanics. In: Fundamental cardiovascular and pulmonary physiology, Philadelphia: Lea & Febiger, 01-110.

Hagan JJ, Price GW, Jeffrey P, Deeks NJ, Stean T, Piper D, Smith M, Upton N, Medhurst AD, Middlemiss DN, Riley GJ, Lovell PJ, Bromidge SM, Thomas DR. (2000). Characterization of SB-269970-A, a selective 5-HT_7 receptor antagonist. Br J Pharmacol, 130:539-548.

Itskovitz HD, Werber JL, Sheridan AM, Brewer TF, Stier CT. (1989). 5-hydroxytryptophan and carbidopa in spontaneously hypertensive rats. J Hypertens, 7:311-315.

Kaumann AJ, Levy FO. (2006). 5-Hydroxytryptamine receptors in the human cardiovascular system. Pharmacology & Therapeutics, 111:674-706.

Page IH, McCubbin JW. (1953). The variable arterial pressure response to serotonin in laboratory animals and man. Circ Res, 1:354-362.

Ramage AG, Villalon CM. (2008). 5-Hydroxytryptamine and cardiovascular regulation. Trends Pharmacol Sci, (9):472-481.

Saxena PR, Lawang A. (1985). A comparison of cardiovascular and smooth muscle effects of 5-hydroxytryptamine and 5-carboxamidotryptamine, a selective agonist of 5-HT1 receptors. Arch Int Pharmacodyn Ther, 277:235-252.

Seitz BM, Krieger-Burke T, Fink GD, Watts S. (2016). Serial measurement of splanchnic vein diameters in rats. Frontiers in Pharmacology (Experimental Pharmacology and Drug Discovery), 3;7:116. doi: 10.3389/fphar.2016.00116.

Seitz BM, Orer HS, Krieger-Burke T, Darios ES, Thompson JM, Fink GD, Watts SW. (2017). 5-HT causes splanchnic venodilation. Am J Physiol Heart Circ Physiol , 313:H676-H686.

Selim AM, Sarswat N, Kelesidis I, Iqbal M, Chandra R, Zolty R. (2016). Plasma serotonin in heart failure: possible marker and potential treatment target. Heart, Lung and Circulation, S1443-9506(16)31583-9. doi: 10.1016/j.hlc.2016.08.003.

Tan T, Watts SW, Davis RP. (2011). Drug delivery, enabling technology for drug discovery and development. IPRECIO micro infusion pump: programmable, refillable and implantable. Front Pharmacol, 2:44.

Terrón JA. (1997). Role of 5-ht7 receptors in the long-lasting hypotensive response induced by 5-hydroxytryptamine in the rat. Br J Pharmacol, 121:563-571.

Villalon CM, Centurion D, Bravo G, De Vries P, Saxena PR, Ortiz M. (2000). Further pharmacological analysis of the orphan 5-HT receptor mediating feline vasodepressor responses: close resemblance to the 5-HT7 receptor. Naunyn Schmiedebergs Arch Pharmacol, 361:665-671.

Watts SW, Darios ES, Seitz BM, Thompson JM. (2015). 5-HT is a potent relaxant in rat superior mesenteric veins. Pharmacol Res Perspect 3, e00103. doi: 10.1002/prp2.103,

Watts SW, Morrison SF, Davis RP, Barman SM. (2012). Serotonin and blood pressure regulation. Pharmacol Rev, 64:359-388.

Chapter 7

Systemic and regional hemodynamic changes responsible for 5-HT_7 receptor mediated hypotension

Abstract

The 5-HT_7 receptor is the primary mediator of both the *acute* (< hours) and *chronic* (day-week) falls in mean arterial pressure (MAP) during low dose 5-HT infusion in rats. Previous data shows the fall in MAP during chronic infusion of 5-HT is due to a decrease total peripheral resistance (TPR) and specifically splanchnic vascular resistance. We hypothesized that time-dependent changes in vascular resistance in the splanchnic and skeletal muscle vascular beds are critical to the cardiovascular effects mediated by the 5-HT_7 receptor. Hemodynamic data were collected in conscious and anesthetized male rats using radiotelemetry, vascular catheters and transit-time flowmetry. Reversible antagonism of the 5-HT_7 receptor was achieved with the selective antagonist SB269970 (33 ug/kg, iv). From the very beginning and throughout the duration (up to 24 hours) of a low dose (25 ug/kg) infusion of 5-HT, TPR and MAP were decreased while cardiac output (CO) was increased. Surprisingly, acute infusion of 5-HT (20 minutes) caused an increase in splanchnic arterial (saline 3±0.2 vs 5-HT 7±0.5 mmHg/min/ml) and portal venous resistance (saline 0.2±.03 vs 5-HT 0.5±.06 mmHg/min/ml) which paralleled the fall in MAP (saline 88±1 vs 5-HT 63±2 mmHg). Preliminary studies revealed a marked decrease in hindquarters (mainly skeletal muscle) vascular resistance during acute 5-HT infusion, so we measured regional hemodynamics in a separate group of rats subjected to 24 hours 5-HT (or saline) infusion. The decrease in MAP after 24 hour of 5-HT (saline 83±3 vs 5-HT 70±3 mmHg)

was associated with a significantly lower skeletal muscle vascular resistance (saline 6±0.2 vs 5-HT 4±0.4 mmHg/min/mL) while splanchnic arterial resistance was similar in 5-HT and saline-treated rats. When SB269970 was administered, MAP and skeletal muscle vascular resistance rapidly increased, whereas splanchnic resistance was unaffected. Our work suggests time dependent regional hemodynamic effects mediated by the 5-HT_7 receptor during 5-HT-induced hypotension. The most prominent (acute and chronic) regional hemodynamic response to 5-HT_7 receptor activation paralleling the fall in MAP is a decrease in skeletal muscle vascular resistance.

Introduction

A possible role for circulating serotonin (5-HT) in cardiovascular regulation, especially under pathophysiological conditions, is supported by the fact that administration of 5-HT to humans and experimental animals is well-known to affect vascular resistance and arterial pressure (Cade et al.,1992; Kaufmann and Levy, 2006; Saxena and Villalon, 1990). The magnitude and even direction of these cardiovascular responses are dose dependent and mediated through several distinct serotonergic receptor subtypes located on vascular smooth muscle, endothelial cells, autonomic nerve endings and in the central nervous system (Berger et al., 2009; Noda M et al., 2004). High doses of infused 5-HT are well known to cause acute (seconds to minutes) vasoconstriction and pressor responses mediated primarily by 5-HT_{2A} receptors (Calama et al., 2003; Hoyer et al., 2002;). Conversely, a low dose of infused 5-HT (25 ug/kg) reduces mean arterial pressure (MAP) acutely and this effect persists over the course of 1-4 weeks of continuous infusion (Davis et al., 2012, 2013; Diaz et al., 2008,). Previous studies suggested that the acute depressor response is mediated via 5-HT_7

receptors (Centurion et al., 2004; De Vries et al., 1999; Terron, 1997; Villalon and Centurion, 2007). In seeking to confirm that the 5-HT_7 receptor also mediates the more chronic (days to weeks) depressor response during low dose infusion, we have shown that pharmacological blockade with a selective 5-HT_7 receptor antagonist, or genetic knockout of 5-HT_7 receptors, eliminated completely both acute and chronic 5-HT-induced depressor responses in conscious rats (Seitz et al., 2017; 2019). Collectively these finding indicate that the 5-HT_7 receptor could be important in blood pressure regulation in situations where circulating 5-HT levels are modestly increased either acutely or chronically.

The present study was designed to determine the systemic and regional hemodynamic effects mediated by the 5-HT_7 receptor during 5-HT-induced hypotension. With regards to the systemic hemodynamics, MAP is the product of total peripheral resistance (TPR) and cardiac output (CO). TPR mainly reflects arterial smooth muscle tone within the peripheral circulation (Cowley, 1997), whereas CO is mainly determined by venous smooth muscle tone, circulatory blood volume and heart rate (Gelman, 2008). Knowing how 5-HT_7 receptor activation affects these two distinct hemodynamic measures would help illuminate possible roles of circulating 5-HT under pathophysiological conditions (e.g. Would low circulating levels of 5-HT be expected to worsen or improve cardiac pumping efficiency in heart failure or sepsis?). In addition, from a pharmacological standpoint, any potential therapeutic use of a 5-HT_7 receptor agonist – for example, in hypertension -- would be determined in part by knowledge of the primary systemic hemodynamic target of the drug. In a previous study, chronic (5-day) low dose 5-HT infusion decreased TPR while CO was increased (Davis et al.,

2012). However, these measures were only obtained at 24-hour intervals, so it is unclear if they primarily reflect the direct effect of 5-HT_7 receptor activation or rather time-dependent physiological compensatory responses. Acute hormone actions are apt to be modified under chronic conditions by well-known phenomena such as receptor down-regulation, receptor desensitization, physiological compensation. Chronic exposure to a hormone also causes time-dependent physiological compensatory adaptions (*e.g*. baroreflex, pressure natriuresis) either diminishing or reinforcing the initial response. We suspected the observed increase in CO was likely due to compensatory mechanisms because our previous findings found low dose 5-HT infusion causes dramatic 5-HT_7 receptor dependent dilation of splanchnic veins (but not arteries) *in vivo* and *in vitro* (Seitz et al., 2017). Venodilation generally would be expected to cause a fall in CO rather than an increase. In the current study, CO and TPR were measured both acutely (immediately after starting 5-HT infusion) and chronically (over 5 days) to allow better identification of the direct and indirect hemodynamic responses to 5-HT_7 receptor activation.

How vasoactive substance redistributes blood flow to the various regional vascular beds can greatly affect CO, even without changes in venous compliance or capacitance (Coleman et al., 1974; Ogilvie, 1985), and can also be a key determinant of changes in TPR. However, independent of the systemic hemodynamics (MAP, CO and TPR), knowing the impact of a vasoactive substance on regional blood flow distribution is critical to defining its role in both cardiovascular pathophysiology, and potentially in therapeutics. Therefore, with a focus on understanding CO and TPR responses during 5-HT-induced hypotension, here we investigated regional hemodynamics in the

splanchnic and skeletal muscle vascular beds. A similar study was performed in conscious dogs and provided guidance for this study (Zinner et al., 1983). The two vascular regions were chosen because they receive a large share of cardiac output (Pang et al., 2000) and previous evidence suggested that activated 5-HT_7 receptors in these beds may play a critical role in 5-HT-induced hypotension. The splanchnic and skeletal muscle vascular beds largely make up the “slow time constant” and the “fast time constant” due to their differences in venous compliance. It is well known that changes in venous compliance can produce a dramatic effect on CO. For example, in a microsphere study we showed that chronic infusion of low dose 5-HT in conscious rats caused a relatively selective increase in splanchnic blood flow (Seitz et al., 2014). On the other hand, another study using acute 5-HT infusions and microsphere measurements in anesthetized rats (De Vries et al., 1999) found a large increase in skeletal muscle blood flow that was blocked by a 5-HT_7 receptor antagonist.

Overall our findings indicate that time-dependent changes in regional blood flow do occur during sustained 5-HT_7 receptor activation and that the skeletal muscle vascular bed plays a key role in the associated hypotension and other systemic hemodynamic effects.

Methods

Animals

MSU Institutional Animal Care and Use Committee approved all protocols used in this study. Male Sprague Dawley rats (275-300 g; Charles River Laboratories, Portage, MI, USA) were used in all experiments. Rats were housed in a temperature–

controlled room 22°C with 12-hour light/dark cycles and given standard chow and distilled water ad libitum.

Surgical procedure: Systemic hemodynamics (CO, TPR, SV, MAP and HR) measurements

Under isoflurane anesthesia (2% in oxygen) rats were given pre-operative antibiotics, (Enrofloxacin, 5 mg/kg, im) and analgesic (Carprofen, 5 mg/kg, sq) before being intubated (16-gauge intubation tube) and mechanically ventilated (Kent Scientific Rovent Jr – Rodent Ventilator) with room air. Surgery was performed on a heated surgical platform to maintain body temperature at 36-37^{o}C.The placement of an ascending aorta flow probe to measure CO was done through a median sternotomy with a 3.5 cm incision made to open the pericardium. The flow probe (model 2PSB; Transonic System Inc., Ithaca, NY) was placed around the ascending aorta just above the coronary arteries. Sterile Surgilube® was placed in the window of the flow probe as a coupler to transmit ultrasound signal. Later, fibrous tissue encapsulates the flow probe and provides a good signal transmission during chronic implantation. The sternotomy was closed in layers and the lungs were re-inflated with negative pressure. The rats were given a single dose of sustained release buprenorphine (analgesic;1.0 mg/kg, sc). The flow probe was tunneled subcutaneously and externalized in the mid-scapular region. The flow probe cable was held in placed with a skin button cuff. The rats were placed in a tethered jacket connected to spring top cage lid to allow for movement around the cage.

In the same surgery, a radiotelemeter transmitter (HD-S10; Data Sciences International, MN, USA) for measurement of MAP and heart rate (HR) was implanted

subcutaneously through a 1-1.5 cm incision in the left inguinal area. The radiotelemeter catheter tip was introduced into the left femoral artery and advanced into the abdominal aorta below the renal artery. After 7 days of post-operative recovery, baseline cardiovascular measurements were recorded for 2 days. Osmotic pumps (Model 2ML1; Alzet, Braintree Scientific Inc., Braintree, MA) containing 5-HT (25 μg/kg/min in 1% ascorbate in sterile saline, pH balanced to pH 6-7) were then implanted subcutaneously near the scapular region while under isoflurane anesthesia (1.5%). The pumps delivered either 5-HT or saline for 5 days. On the completion of the final day of drug infusion, pumps were removed under isoflurane anesthesia (1.5%) and cardiovascular parameters were monitored for 2 additional days (termed recovery). The weight of the 5-HT drug pumps was recorded before implantation and after removal to confirm accuracy of drug delivery. The tethered animals were conscious and individually housed during all systemic hemodynamic recordings. MAP and HR were measured for 10 seconds every 10 minutes throughout the duration of the study and averaged in one hour increments (figure 7.1) or 24-hour increments (figure 7.2). CO was measured directly and data was sent through a data acquisition system (Powerlab, ADI instruments). TPR and SV were derived from the following equations.

Total peripheral resistance = mean arterial pressure/cardiac output

Stroke volume = cardiac output/heart rate

The acute study details hour by hour measurements for the first 24 hours from the start of 5-HT infusion (figure 7.2). The chronic study covers 5 days of 5-HT infusion (figure 7.1).

Surgical procedure: Regional hemodynamic measurements (portal, splanchnic and hindquarter resistance)

All animals were anesthetized (2% isoflurane in oxygen) during this portion of the study. One group of rats were studied to measure the acute (20 minutes) regional hemodynamic response to 5-HT. A second group of rats were studied to measure responses 24-hour after the start of 5-HT infusion, followed by acute infusion of the 5-HT_7 receptor antagonist, SB269970. For both groups, all rats were implanted with an indwelling left femoral arterial polyethylene catheter (ID 03. mm, OD 0.5 mm) for continuous MAP recording. An additional pressure measurement polyethylene catheter (ID 03. mm, OD 0.5 mm) was placed in the abdominal inferior vena cava (IVC pressure) by way of the right femoral artery. A final polyethylene catheter (ID 03. mm, OD 0.5 mm) was advanced into the portal vein via the caecal vein for continuous measurement of portal vein pressure. Flow probes (portal, 2 mm; hindquarter, 1.5 mm; Transonic System

inferior vena cava, and portal vein) were attached to a transducer connected to a data acquisition system (Powerlab, ADI instruments). Portal, splanchnic and hindquarters resistance were calculated using the following equations:

Hepatic Portal resistance = (portal vein pressure-inferior vena cava pressure)/portal flow

Splanchnic Resistance = (mean arterial pressure-portal pressure)/portal flow

(portal flow is approximately = splanchnic blood flow)

Hindquarters Resistance= (mean arterial pressure-inferior vena cava)/ hindquarters flow

(hindquarters flow is mostly skeletal muscle/part skin).

Materials

5-HT creatinine sulfate was obtained from Sigma Chemical Company (St. Louis MO USA). SB-269970 was purchased from Torcis (R&D, Minneapolis, MN) or Abcam (Cambridge, MA).

Statistical analyses

Quantitative data are reported as means±SEM for number of animals in parentheses. Statistical analyses were performed using repeated measures ANOVA when comparing values from own baseline or within a group (GraphPad Prism 7) when the F value achieved statistical significance and there was no significant variance in homogeneity as tested by Bartlett's. When comparing between groups, a t-test was used. In all cases, a p value of <0.05 was considered significant.

Results

CO is increased during 5-day infusion of low dose 5-HT

Stable baseline measurements were collected for two days prior to the administration of 5-HT in conscious rats instrumented with a telemeter and chronic ascending aorta flow probe. MAP (figure 7.1 A) was reduced during 5-HT infusion, significantly from day 1 to day 4 when compared to baseline values (baseline day one 100±2.4 vs 5-HT day one 85±1.4 mmHg). In the presence of 5-HT, HR, SV and CO were significantly increased, however TPR was reduced compared to baseline values. The elevation in HR was only significant for the first two days from the start of 5-HT delivery. (figure 7.1 B). In contrast, SV and CO were both increased (figure 7.1 C and D), throughout the duration of 5-HT infusion with CO having the most significant

response. TPR was substantially reduced throughout 5-HT administration. (figure 7.1 E). Once the 5-HT pumps were removed, CO and SV remained elevated, at least for the first day, while MAP was above baseline levels. TPR returned to baseline values during the recovery phase. These findings confirm those of an earlier study (Davis et al., 2012).

CO is elevated during acute 5-HT-induced fall in MAP

In order to gain insight into whether the hemodynamic changes described above were a direct effect of 5-HT infusion or primarily compensatory responses, we analyzed systemic hemodynamic response to 5-HT infusion on an hour-to-hour basis during the chronic experiment shown in figure 7.1. From the start of 5-HT infusion, the fall in MAP (figure 7.2 A) occurred in parallel with decreasing TPR (figure 7.2 B) and increasing SV and CO (figure 7.2 C-D). All variables reached a significant plateau 6-7 hours after the infusion was begun.

Regional blood flow responses to acute 5-HT infusion

Figure 7.3 shows the hemodynamic response within the splanchnic and hepatic-portal circulations during 20 minutes of 5-HT or saline delivery. In the presence of 5-HT, MAP was dramatically reduced both compared to baseline and saline-treated control animals (figure 7.3 A). Portal pressure was reduced in the 5-HT-treated rats (only statistically significant from baseline) and mirrored in time the observed fall in MAP (figure 7.3 B). Additionally, the fall in MAP to exogenous 5-HT nearly paralleled in time the gradual but significant reduction in portal vein flow (figure 7.3 C) (baseline portal pressure saline-treated 7.4±0.95 vs 5-HT-treated 7.6± 0.5 mmHg compared to 20-

minutes of infusion saline-treated 7.3±1.8 vs 5-HT-treated 6.3±0.4 mmHg). After 12 minutes from the start of 5-HT administration, both portal and splanchnic resistance (figure 7.3 D-E) significantly increased while saline-treated showed no change from baseline.

Additional acute regional hemodynamic findings to low dose infused 5-HT are summarized in a Powerlab (™) tracing taken from one rat (figure 7.4). What is different from this experiment compared to the acute regional data in figure 7.3 is that hindquarter blood flow was also measured. This measurement technique captures virtually all hindquarter blood flow, and it is important to note that the hindquarter is made up mainly of skeletal muscle (Pang et al., 2000). The notable finding here was that the hindquarters blood flow increased dramatically in parallel with a fall in MAP during infusion of 5-HT, indicating a major decrease in skeletal muscle vascular resistance at this time.

Regional blood flow changes mediated by the 5-HT$_7$ receptor during 24 hour 5-HT infusion

Based on the observed regional vascular responses to acute 5-HT infusion (figure 7.3), we sought to test resistance changes in the skeletal muscle and splanchnic region regions during a more chronic (24 hours) exposure to 5-HT. There were two goals for this set of *in vivo* experiments: 1) to investigate regional blood flow and vascular resistance in the splanchnic and hindquarter beds after 24 hour of 5-HT infusion; and 2) to determine the role of the 5-HT$_7$ receptor in these responses. Figure 7.5 shows a representative Powerlab tracing of hepatic-portal, splanchnic and hindquarter regional hemodynamic responses in an anesthetized rat at 24 hours of 5-

HT infusion. To determine the contribution of the 5-HT_7 receptor within these vascular beds directly, an acute bolus of the selective 5-HT_7 receptor antagonist (SB269970, 33 ug/kg, iv) was given. Prior to SB269970 administration, MAP was low, hindquarter blood flow was high, and splanchnic (portal) blood flow was within the normal range. Acute blockade of 5-HT_7 receptors was associated with an immediate and parallel rise in MAP, fall in hindquarter blood flow and rise in splanchnic blood flow. Based on this preliminary finding, we went on to make similar measurements in two groups of rats that received either 5-HT or saline infusion for 24 hours while conscious in their home cages.

The first column under saline-treated or 5-HT-treated rats in figure 7.6 summarizes resting hemodynamic measures in anesthetized rats after 24 hours of 5-HT infusion versus saline vehicle infusion only. As expected, MAP was significantly reduced in rats infused with 5-HT compared to saline treated animals (figure 7.6 A). Very surprisingly, splanchnic (portal vein) blood flow (figure 7.6 B) was similar as observed in saline-treated animals. Furthermore, both hepatic-portal and splanchnic resistance were similar after 24 hours of 5-HT infusion when compared to values measured in saline-treated animals (figures 7.6 D-E). This represents a significant difference from what we observed during acute infusion of 5-HT (increase in hepatic-portal and splanchnic resistance and a decrease in splanchnic flow). However, hindquarter blood flow (figure 7.6 C) was higher and hindquarter resistance (figure 7.6 F) was lower in 5-HT infused versus saline infused rats, which parallels the acute 5-HT hindquarter response (figure 7.3)

To understand the contribution of 5-HT_7 receptors to the hemodynamic changes observed during chronic infusion of 5-HT, an acute bolus of the selective 5-HT_7 receptor antagonist SB269970 was given (33 ug/kg, iv). No significant changes in any hemodynamic measure was seen in rats receiving only saline for 24 hours. In rats receiving 5-HT infusions, the low MAP as expected was rapidly restored to near saline–treated levels (figure 7.6 A) after antagonist administration. Splanchnic (portal) blood flow (figure 7.6 B) increased but this was driven mainly by increased MAP, since splanchnic vascular resistance was surprisingly unchanged (figure 7.6 E) by the antagonist. Hepatic-portal resistance was significantly decreased (figure 7.6 D) after antagonist administration. Most notably, in 5-HT infused rats, 5-HT_7 receptor blockade brought about an immediate increase in hindquarter vascular resistance (figure 7.6 F) and decrease in hindquarter flow (figure 7.6 C)

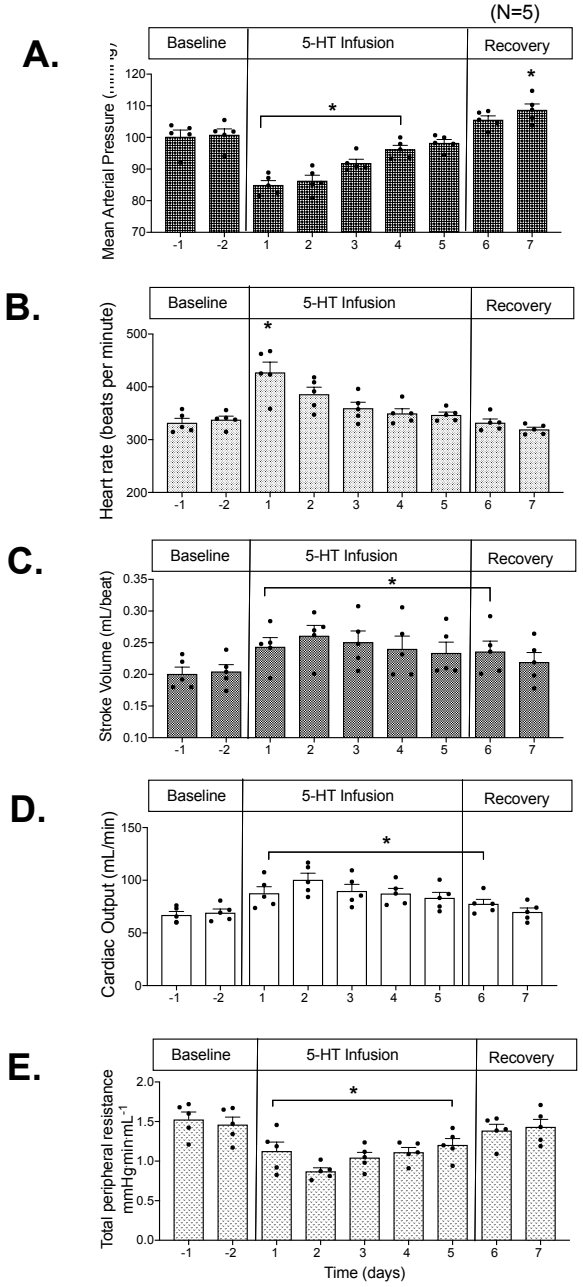


Figure 7.1: Time course systemic hemodynamic measures during 5-days of 5-HT infusion. Time course data of systemic hemodynamics. A). mean arterial pressure, B). heart rate, C). Stroke volume, D). cardiac output E). total peripheral resistance during 5 days of osmotic pump 5-HT infusion and after 5-HT pumps were removed (recovery) in conscious rats. Data points represent 24 -hour averages +/- SEM; n=5. A one-way ANOVA with repeated measures was used to determine significance. * $p<0.05$ compared to baseline. Baseline represents the average of the two days prior to the start of 5-HT infusion.

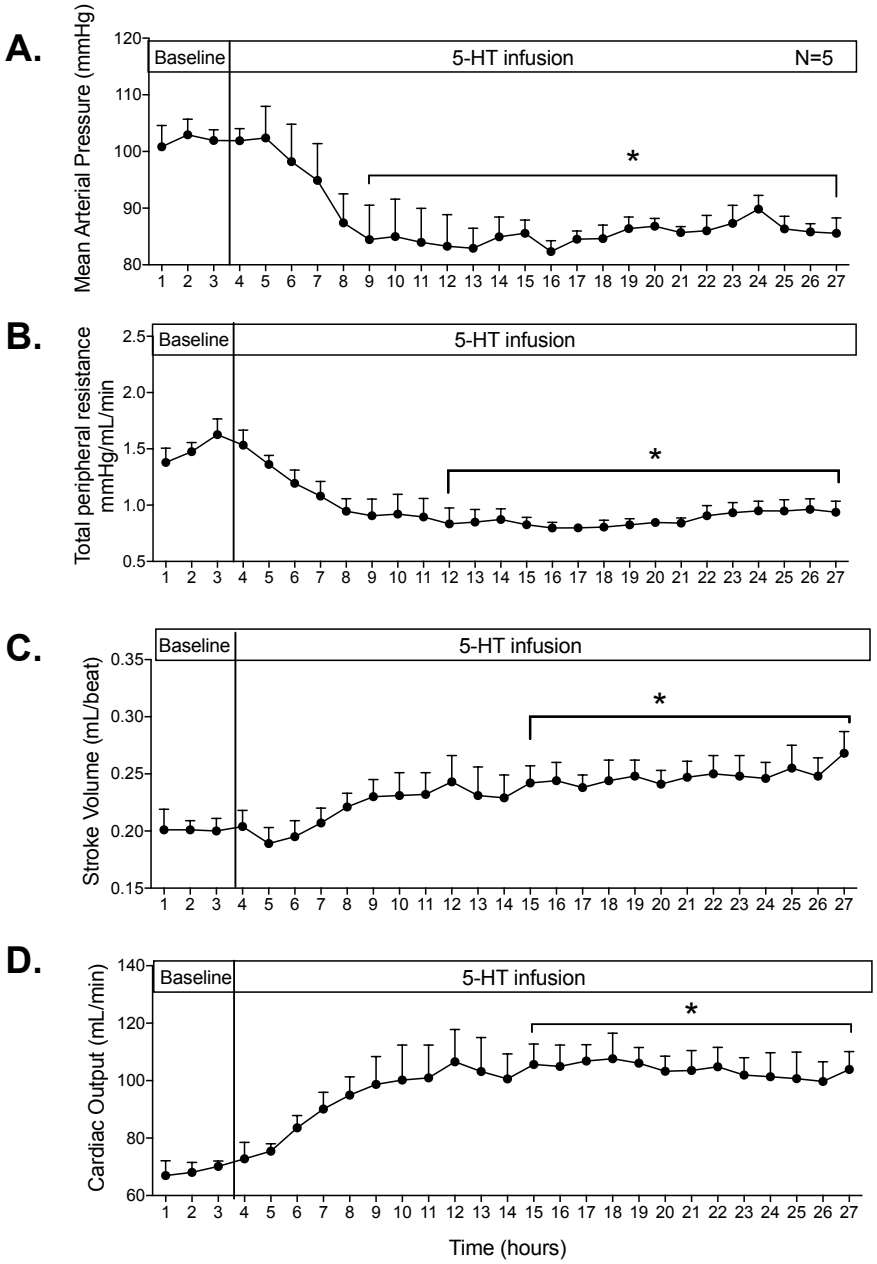


Figure 7.2: Acute systemic hemodynamic measures during 5-HT infusion.
Time course data of systemic hemodynamics A). mean arterial pressure, B). total peripheral resistance, C). stroke volume and D). cardiac output during 24 hours of osmotic pump 5-HT infusion in conscious rats. Data points represent one hour averages +/- SEM; n=5. A one-way ANOVA with repeated measures was used to determine significance. * $p<0.05$ compared to baseline. Baseline represents the average of the three hours prior to the start of 5-HT infusion.

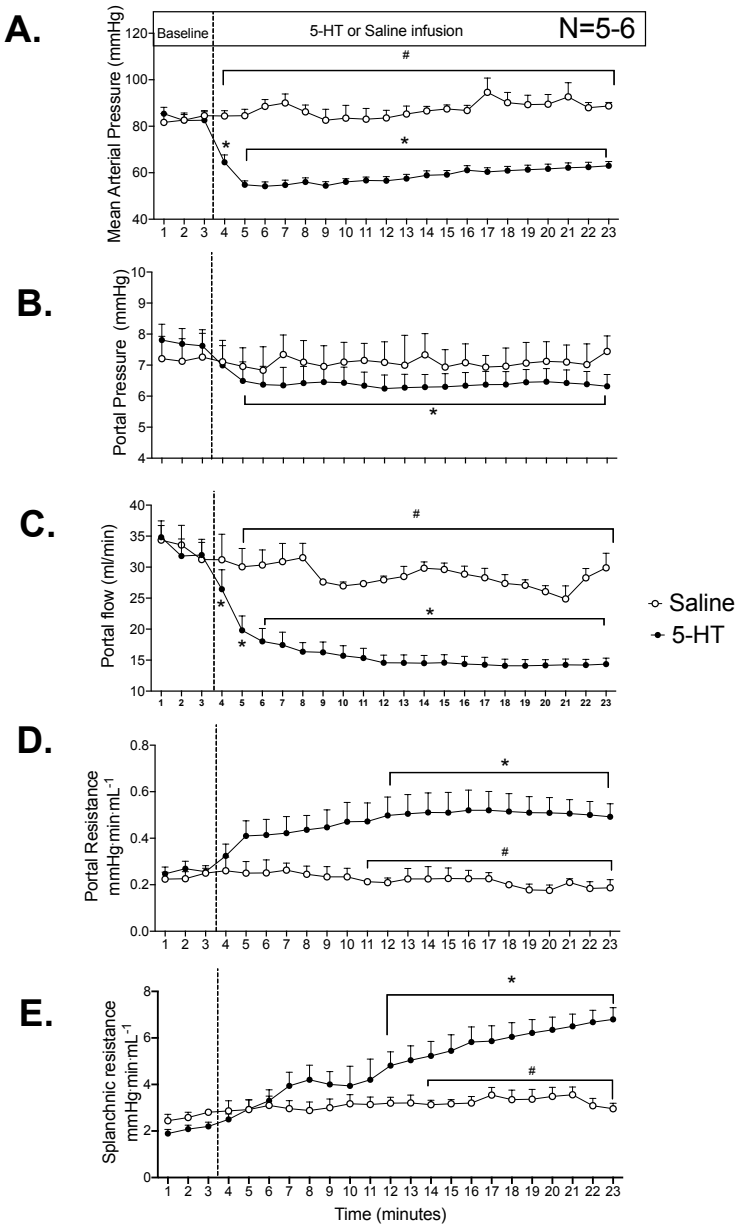


Figure 7.3: Acute regional hemodynamic measures during 5-HT infusion. The hemodynamic measures of A). mean arterial pressure B). Portal vein flow, C). portal vein resistance and D). splanchnic resistance during 20 minute (acute) iv infusion of saline or 5-HT. Data points represent one minute averages +/- SEM for 5-6 animals per group. One-way ANOVA was used to determine significance within groups. * $p<0.05$ baseline vs 5-HT group. Baseline represents the average of 5 minutes prior to the start of saline or 5-HT infusion. T-test was used to determine significance between groups. # $p<0.05$ 5-HT vs saline.

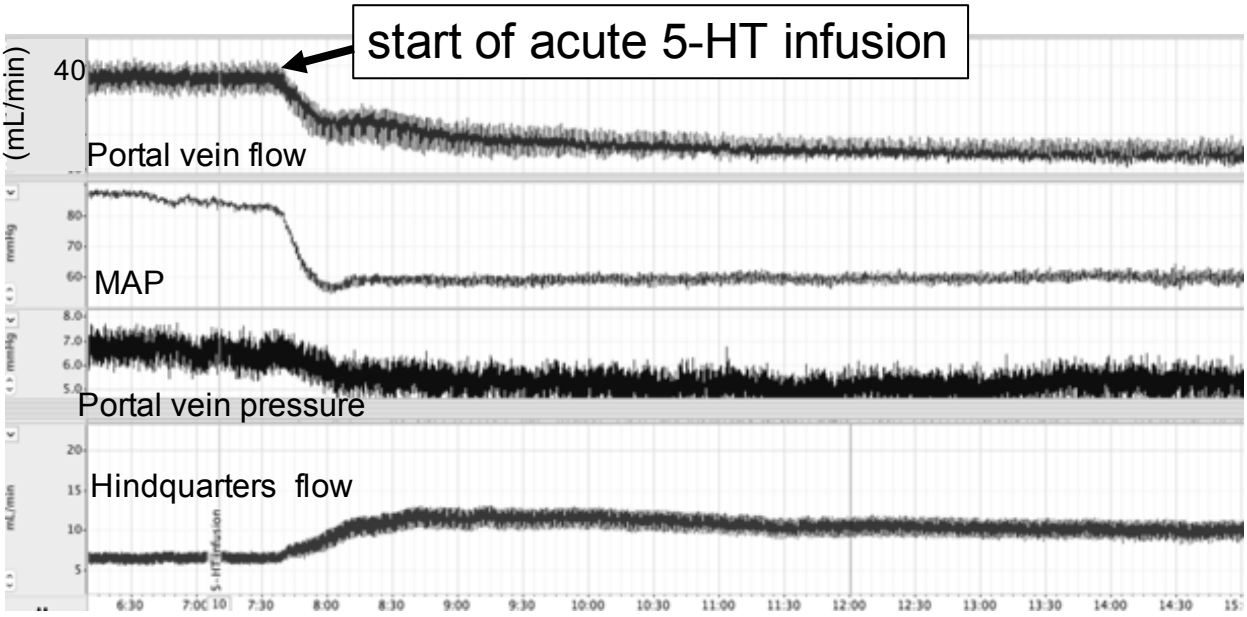


Figure 7.4: Representative recordings of the regional hemodynamic responses in one rat to acute 5-HT (25ug/kg) iv infusion.

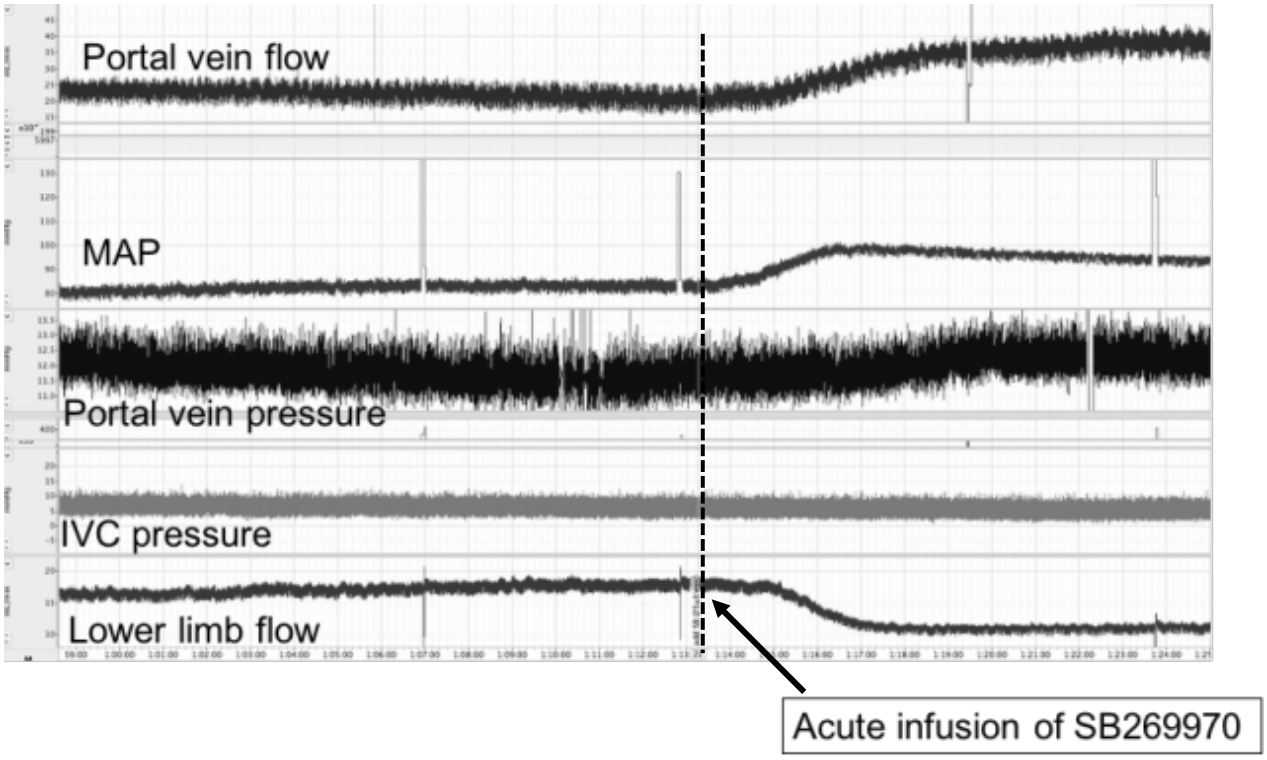


Figure 7.5: Representative recordings of the regional hemodynamic responses in one rat after 24 hours of 5-HT infusion (25 ug/kg/min, osmotic pump) with an acute bolus of 5-HT_7 receptor antagonist (SB269970, iv, dashed line).

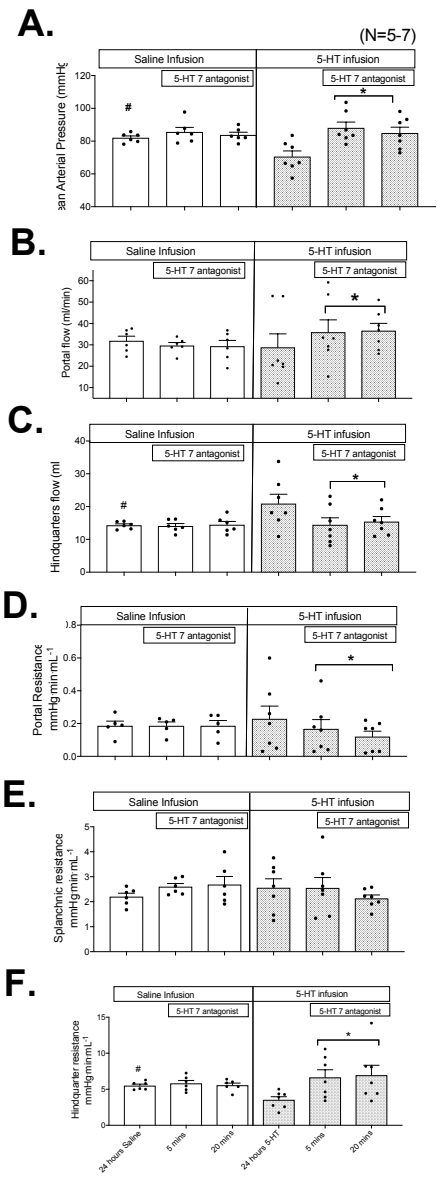


Figure 7.6: Regional hemodynamic effects mediated by the 5-HT$_7$ receptor after 24 hours of 5-HT infusion. The hemodynamic effects of A). Mean arterial pressure, B). Portal vein flow, C). Hindquarters flow, D). Portal vein resistance, E). Splanchnic resistance, F). Hindquarter resistance after 24 hours of infusion of either saline or 5-HT infusion (25 ug/kg/min, osmotic pump) and challenged with the 5-HT$_7$ receptor antagonist SB269970 (33ug/kg, iv bolus). Columns represent distinct time points averaged +/- SEM for the number of animals in the parentheses. One-way ANOVA was used to determine significance within groups. * $p<0.05$ 24 hours of 5-HT infusion vs after 5-HT$_7$ receptor antagonist bolus. T-test was used to determine significance between groups. # $p<0.05$ 5-HT vs saline.

Discussion

In the present study, we evaluated the systemic and regional (splanchnic and skeletal) hemodynamic effects of the 5-HT_7 receptor activation which accompanies acute (minutes) and chronic (days) 5-HT-induced hypotension. Our main findings in conscious rats at the systemic level were a reduction in TPR and increase CO from the very beginning of 5-HT infusion that continued throughout its duration (although all systemic hemodynamic changes tended to return toward control period values by 5 days of infusion). At the regional level, in anesthetized rats, we found an increase in splanchnic and hepatic–portal resistance along with a decrease in skeletal muscle resistance during acute infusion of 5-HT. In a separate group of rats receiving 5-HT infusion for 24 hours, splanchnic and hepatic-portal resistances was similar compared to saline-infused rats. The only difference between the saline and 5-HT-infused rats was skeletal muscle resistance was significant lower in the 5-HT- treated group. The fall in skeletal muscle resistance was depend entirely on 5-HT_7 receptor activation. These results suggest that a major cause of the hypotension observed during acute and chronic low dose 5-HT infusion is reduced vascular resistance in skeletal muscle.

The focus of this work was directed by three earlier findings. First, a low dose of 5-HT (25 ug/kg) deceases MAP both acutely (minutes; Dalton et al.,1986; Page and McCubbin, 1953; Terron, 1997) and chronically (>24 hours –month; Diaz et al., 2008; Davis et al., 2012, 2013) when infused into conscious animals. Second, blocking the 5-HT_7 receptor prevented both the acute (Centurion et al., 2004; De Vries et al., 1999; Villalon and Centurion, 2007) and chronic low dose 5-HT depressor response (Seitz et al., 2017; 2019). Finally, and most noteworthy, the same low dose of 5-HT infused for

one week reduced arterial pressure in several hypertensive rat models (> 30-50 mmHg fall from baseline; Diaz et al., 2008; Watts et al., 2012). This suggests that the 5-HT_7 receptor may be both a critical participant in the cardiovascular effects of endogenously released 5-HT and a potential target for cardiovascular therapeutics.

Concentration of 5-HT to generate depressor response

Infusion of 5-HT can either increase or a decrease arterial pressure depending on the established concentration of circulating 5-HT in the plasma and the specific 5-HT receptor activated. The depressor effect of 5-HT can be mediated by activation of 5-$HT_{1B/1D}$, 5-HT_{2B} as well as the 5-HT_7 receptors (Berger et al., 2009; Kaumann and Levy, 2006). In our established low dose 5-HT infusion protocol used in this study and others, MAP is reduced both acutely (minutes) and chronically (>24 hours-month) (Dalton et al.,1986; Davis et al., 2012, 2013; Diaz et al., 2008; Page and McCubbin, 1953; Terron, 1997). The low dose of 5-HT given produces a concentration of free circulating 5-HT in the plasma that is in the mid-nanomolar range (Diaz et al, 2008). This circulating plasma level of 5-HT is sufficient to activate the 5-HT_7 receptor (affinity; Ki ~7nM), which possesses an affinity approximately two times greater than the other vasorelaxant serotonergic receptors subtypes (5-HT_{1B} ~14 nM and 5-HT_{2B} ~19 nM) (pdsp.unc.edu/databases). Importantly, blockade of the 5-HT_7 receptor with a selective 5-HT_7 receptor antagonist SB269970 completely prevented the acute and chronic low dose 5-HT-induced hypotension (Seitz et al., 2017; Villalon and Centurion, 2007). The selective 5-HT_7 receptor antagonist used has an affinity (Ki) for the 5-HT_7 receptor that is ~50-1,000 times greater than any other serotonergic receptor subtype (pdsp.unc.edu/databases). More recently, 5-HT_7 receptor knock-out (KO) rats which we

created (Demireva et al., 2019) were given the same low dose of 5-HT for one week. These KO rats showed no change in MAP whereas a significant 5-HT-induced depressor response was observed in wild-type littermates (Seitz et al., 2019). These findings indicate that the low dose of 5-HT used in our standard infusion protocol causes a fall in MAP that is solely mediated by the 5-HT_7 receptor under both acute and chronic conditions.

The peripheral hemodynamic mechanisms which underlie the arterial pressure response elicited by low dose 5-HT (5-HT_7 receptor mediated) are not well understood. Our aim in this study was to capture the precise timing (acute < 24 hours; chronic, >24 hours to 5 days) of the cardiovascular hemodynamic events mediated by the 5-HT_7 receptor. Understanding the time-dependent events, not only further characterizes the cardiovascular role of 5-HT_7 receptor but provides potential insight into whether a 5-HT_7 receptor agonist would be beneficial in the therapy of chronic cardiovascular disease especially hypertension.

Systemic hemodynamic responses to low dose 5-HT

Arterial pressure is the product of CO and TPR. The former is primarily determined by heart rate, venous return and stroke volume (Pang, 2000). The latter is determined by the tone of smooth muscle cells in the small arteries and arterioles, which is governed by complex local and central neurohumoral factors (Cowley, 1992). Arterial pressure maintains a circadian rhythm, although it is kept within its habitual range by negative feedback baroreceptor reflexes, volume regulation, and other regulatory systems (Ackermann, 2004). These physiological compensatory mechanisms (*e.g*. baroreflex, sodium and water balance, renin-angiotensin aldosterone system and

others) come into play at different times after an initial perturbation in arterial pressure and exhibit varying degrees of adaptation over time as well. This makes it critically important to investigate both the acute and chronic effects of compounds that alter arterial pressure.

The systemic hemodynamic results to infused low dose 5-HT shown here confirm the findings of our previous study (Davis et al., 2012). The purpose for reinvestigating the systemic response to low dose 5-HT was two-fold. First, it was to capture the initial acute CO and TPR response (<24 hours) to infused 5-HT which was not previously reported. Second, to observe the systemic response when the exogenous amine was removed.

In this study, TPR was reduced and CO was increased from the very start of 5-HT infusion and throughout the duration the amine was given, despite a partial return of MAP towards pre-infusion levels. The decrease in TPR closely mirrored in time the fall in MAP suggesting vasodilation of small arteries and arterioles within the periphery plays a major role in the depressor response to 5-HT. The immediate return of TPR to pre-infusion values after termination of the 5-HT infusion suggests that the change in TPR during infusion is mediated primarily via direct effects of 5-HT_7 receptor activation. In contrast,

the persistent elevations in SV and CO observed after the 5-HT infusion was stopped, suggest the changes are, at least in part, compensatory responses to the initial hemodynamic effects of 5-HT. HR was only elevated during the initial two days of infusion when MAP was at its nadir. The recovery of HR as MAP increased over several days suggests that HR changes during 5-HT infusion are mainly a compensatory

baroreflex response as opposed to a receptor mediated event. It is important to note that 5-HT is readily removed from the circulating plasma by monoamine oxidases found in the liver, or serotonin transporters found in lung and platelets. These processes maintain very low free circulating level of 5-HT in the plasma (~2% of total body 5-HT), which is estimated to be in the nanomolar range (3- 20 ng/ml) (El-Merahbi et al., 2015). Thus, when the 5-HT drug pumps were removed the circulating level of 5-HT would rapidly (plasma $t_{1/2}$ = 1.2 minutes, Zinner et al., 1982) fall below the level necessary to activate the 5-HT_7 receptor.

We had speculated that the acute (< 24 hours) response to infused 5-HT would be a decrease in CO along with a decrease in TPR. Our reasoning was based on previous *in vivo* data which showed a low-dose infusion of 5-HT caused a decrease in MAP that paralleled in time a marked increase in the diameter of the large splanchnic veins (portal vein and superior mesenteric vein). Both events were shown to be mediated via the 5-HT_7 receptor (Seitz et al., 2017). The observed increase in splanchnic vein diameter suggested an increase in venous capacitance and a possible mechanism that could contribute to the 5-HT_7 receptor induced hypotension, i.e. a fall in SV and CO. However, in the current study, when these variables were measured continuously in a conscious state, 5-HT elicited an increase in CO and SV from the very start of infusion. These results suggest that any direct effect of 5-HT_7 receptor activation to increase vascular capacitance -- thereby decreasing SV and CO -- is overridden by other effects that rather serve to increase SV and CO. Such effects could include a reduction in resistance to venous return or a redistribution of blood flow from high compliance to low compliance compartments of the overall circulation, as

described originally by (Krogh, 1912) and supported by (Coleman et al., 1974; Greenway, 1981; Ogilvie, 1985).

Regional hemodynamic response to low dose 5-HT

To effectively interpret the CO and TPR responses produced by low dose 5-HT infusion, we investigated in more detail the hemodynamic response of two regional vascular beds. We focused on the splanchnic, hepatic-portal and skeletal muscle regions, as previous evidence (at least in splanchnic and skeletal) supports involvement of these vascular regions in the overall cardiovascular effects of 5-HT_7 receptor activation (discussed earlier). Another reason for focusing on these regions is they are generally taken to represent the primary high compliance (splanchnic) and low compliance (skeletal muscle) compartments of the Krogh two-compartment model of the circulation (Krogh, 1912). The kidneys might have been logically included in the regional hemodynamic measurements considering the important contribution of this vascular bed to blood pressure regulation. However, earlier microsphere data indicated a minimal involvement of the renal circulation in acute or chronic 5-HT-induced hypotension (Seitz and Watts, 2014).

In the current study, immediately after start of an acute 5-HT infusion in an anesthetized rat, MAP fell abruptly along with a dramatic fall in portal vein flow and reduction in portal venous pressure. It is important to note that portal vein flow represents the sum of all the blood entering and leaving the splanchnic vascular bed (except the small amount entering via the hepatic artery), so this flow divided by MAP is a reasonable (though not perfect) representation of splanchnic vascular resistance. Hepatic-portal resistance gradually increased, with an even slower increase in

splanchnic resistance. These results suggest that 5-HT_7 receptor activation constricts (directly or indirectly) splanchnic arterioles or small arteries and hepatic venules. In support of these findings, earlier *in vitro* studies found 5-HT does not relax the mesenteric artery (~ 250 microns in size) (Davis et al., 2012). Although, 5-HT has been shown to constrict mesenteric arteries in both human and rat, it is by activation of the 5-HT_{2A} receptor (Watts et al., 2012). We believe the low dose of 5-HT given does not stimulate 5-HT_{2A} receptor. Constriction (if direct) is therefore most likely occurring in the splanchnic arterioles, a location we have not yet investigated *in vitro* or *in vivo*.

In contrast, after 24 hours of continuous 5-HT infusion, both hepatic-portal and splanchnic resistances were near baseline values, i.e. comparable to control treated animals. This was the case even though MAP was substantially reduced. This outcome was surprising considering our previous findings with microspheres (Seitz and Watts, 2014), showing a reduction is splanchnic resistance during chronic 5-HT infusion. The reason for the discrepancy is uncertain. Nonetheless, the results suggest some compensatory mechanism must be involved to restore splanchnic and portal venous resistances during the chronic infusion of 5-HT. There are several possible physiological mechanisms such as inhibition of sympathetic nervous system (Esler, 2000), the hepatic arterial buffer response (Eipel et al., 2010), and release of endogenous vasodilators (nitric oxide, adenosine; Lautt, 2007) that could account for the compensation.

Alternatively, to determine if pharmacological tolerance might explain the changes in hemodynamics during chronic infusion of 5-HT, we tested the effects of acute blockade of the 5-HT_7 receptor. This intervention quickly raised MAP in rats

receiving 5-HT infusions, but not in those receiving saline. This showed that the 5-HT_7 receptor was still being activated after 24 hours of continuous 5-HT infusion. Receptor blockade also immediately decreased portal venous resistance suggesting that the compensation occurring during prolong 5-HT infusion in this vascular bed was physiological rather than pharmacological. In contrast, acute blockade of the 5-HT_7 receptor had minimal effects on splanchnic resistance (although it declined slightly). This strongly suggests that the normal splanchnic resistance that was observed in rats after 24 hours of 5-HT infusion is mainly due to pharmacological tolerance (loss of 5-HT_7 receptor activation), perhaps at the level of the splanchnic microcirculation.

The most significant regional vascular response mediated by the 5-HT_7 receptor acutely and chronically was a profound decrease in skeletal muscle resistance, such that an increase in skeletal muscle blood flow occurred even in the face of a fall in MAP. This was not completely unexpected, however, given an earlier study where acute 5-HT_7 receptor-mediated decrease in MAP was associated with a decrease in skeletal muscle vascular resistance determined by microsphere measurements (De Vries et al.,1999). Although the precise location of 5-HT_7 receptors within the skeletal muscle that mediate the fall in resistance is not known. One previous study observed the small (A3 and A4) but not larger (A1) arterioles from rat cremaster muscle dilated to 5-HT, and that the dilation was mediated by a 5-HT_7-like receptor (Alsip and Harris, 1991).

The increase in skeletal muscle flow we observed may help explain the increase in CO observed during 5-HT_7 receptor mediated hypotension. As described earlier, CO can be strongly affected by the distribution of blood flow (determined by arteries and arterioles) within the various regions of the circulation. The systemic vasculature can be

divided into two distinct regions each with a different compliance; low compliance (skeletal muscle and kidney) and high compliance (hepatic-splanchnic) (Coleman et al., 1974; Greenway, 1981; Krogh, 1912; Ogilvie, 1985). *Increasing* blood flow through vascular beds with low compliance, or *decreasing* blood flow through beds with high compliance, will result in an increase in SV and CO even when venous capacitance and compliance are unchanged. Since our results show that chronic low dose 5-HT infusion does both, this may provide the necessary and sufficient mechanism for the systemic hemodynamic effects of 5-HT_7 receptor activation.

Conclusion

The experiments described here characterize and analyze the unique systemic and regional hemodynamic effects mediated by the 5-HT_7 receptor, a member of the 5-HT receptor family whose chronic cardiovascular effects (except for hypotension) have been little studied up to now. Our work reveals a novel, time-dependent set of hemodynamic responses within the splanchnic, hepatic-portal and skeletal muscle vascular beds. The most substantial (acute and chronic) response to activation of the 5-HT_7 receptor paralleling the fall in arterial pressure is an increase in skeletal muscle blood flow by reducing resistance within this region.

REFERENCES

REFERENCES

Ackermann U. (2004). Regulation of arterial blood pressure. Surgery (Oxford) 22(5):120a-120f.

Alsip NL, Harris PD. (1991). Receptor mediation of microvascular responses to serotonin in striated muscle. Am J Physiol, (5 pt 2):H1525-33.

Berger M, Gray JA, Roth BL. (2009). The expanded biology of serotonin. Annu Rev Med, 60:35–366.

Cade JR, Fregly MJ, Privette M. (1992). Effect of tryptophan and 5-hydroxytryptophan on the blood pressure of patients with mild to moderate hypertension. Amino Acids, 2:133-140.

Calama F, Garcia M, Jarque MJ, Moran A, Martin ML, San Roman L. (2003). 5-hydroxytryptamine-induced vasodilator responses in the hindquarters of the anesthetized rat, involve beta 2-adrenoreceptors. J Pharm Pharmacol, 55(10):1371–1378.

Centurión D, Glusa E, Sánchez-López A, Valdivia LF, Saxena PR, Villalón CM. (2004). 5-HT7, but not 5-HT2B, receptors mediate hypotension in vagosympathectomized rats. Eur J Pharmacol, 502:239–242.

Coleman TG, Manning RD, Norman RA and Guyton AC. (1974). Control of cardiac output by regional blood flow distribution. Annals of Biomedical Engineering, 2:149-163.

Cowley AW. (1992). Long-term control of arterial pressure. Physiol Rev, 72:231-300.

Dalton DW, Feniuk W, Humphrey PP. (1986). An investigation into the mechanisms of the cardiovascular effects of 5-hydroxytryptamine in conscious normotensive and DOCA-salt hypertensive rats. J Auton Pharmacol, 6:219–228.

Davis RP, Pattison J, Thompson JM, Tiniakov R, Scrogin KE, Watts SW. (2012). 5-hydroxytryptamine (5-HT) reduces total peripheral resistance during chronic infusion: direct arterial mesenteric relaxation is not involved. BMC Pharmacol, 12:4 doi: 10.11186/1471-2210-12-4.

Davis RP, Szasz T, Garver H, Burnett R, Tykocki NR, Watts SW. (2013). One-month serotonin infusion results in a prolonged fall in blood pressure in the deoxycorticosterone acetate (DOCA) salt hypertensive rat. ACS Chem Neurosci, 4: 141–148. doi:10.1021/cn300114a.

De Vries P, De Visser PA, Heiligers JPC, Villalón CM, Saxena PR. (1999). Changes in systemic and regional haemodynamics during 5-HT_7 receptor mediated depressor responses in rats. Naunyn-Schmied Arch Pharmacol, 359:331-338.

Demireva EY, Xie H, Darios ES, Thompson JM, Seitz B, Watts SW. (2019). Creation of the 5-Hydroxytryptamine receptor 7 knockout (Htr7 KO) rat as a tool for cardiovascular research. Physiological Genomics.

Diaz J, Ni W, King A, Fink GD, Watts SW. (2008). 5-hydroxytryptamine lowers blood pressure in normotensive and hypertensive rats. J Pharmacol Exp Ther, 325:1031-1038.

Eipel C, Abshagen K, Vollmar B. (2010). Regulation of hepatic blood flow: the hepatic arterial buffer response revisited. World J Gastroenterol, 16(48):6046-6057.

El-Merahbi R, Loffler M, Mayer A, Sumara G. (2015). The roles of peripheral serotonin in metabolic homeostasis. FEBS, 589:1728-1734.

Esler M. (2000). The sympathy system and hypertension. Am J Hypertension, 13(6 part 2):99S-105S.

Gelman S. (2009). Venous function and central venous pressure. Anesthesiology, 108: 735-748.

Green JF. (1982). Circulatory mechanics. In: Fundamental cardiovascular and pulmonary physiology. Philadelphia: Lea & Febiger, 01-110.

GREENWAY CV. (1981). Mechanism and quantitative assessment of drug effects on cardiac output with a new model of the circulation. Pharmacol Rev, 33:213-25 1.

Hoyer D, Hannon JP, Martin GR. (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav, 71:533−554.

Kaumann AJ, Levy FO. (2006). 5-Hydroxytryptamine receptors in the human cardiovascular system. Pharmacology & Therapeutics, 111:674-706.

Krogh, A. (1912). The regulation of the supply of blood to the right heart (with a description of a new circulation model). Scandinavian Archives of Physiology, 27:227-248.

Lautt, WW. (2007). Regulatory processes interacting to maintain hepatic blood flow constancy: Vascular compliance, hepatic arterial buffer response, hepatorenal, liver regeneration, escape from vasoconstriction. Hepatol Res, 37:891-903

Noda M, H. Higashida, S. Aoki, K. Wada. (2004). Multiple signal transduction pathways mediated by 5-HT receptors. Mol Neurobiol, 29:31-39.

Ogilvie, RI. (1985). Comparative effects of vasodilator drugs on flow distribution and venous return. Can J Physiol Pharmacol, 63:1345-1355.

Page IH, McCubbin JW. (1953). The variable arterial pressure response to serotonin in laboratory animals and man. Circ Res, 1:354-362.

Pang, CCY. (2000). Measurement of body venous tone. Journal of Pharmacological and toxicological Methods, 44:341-360.

Seitz BM, Watts SW. (2014). Serotonin-induced hypotension is mediated by a decrease in intestinal vascular resistance. Pharmacologia, 5(2):50-54.

Seitz BM, Orer HS, Krieger-Burke T, Darios ES, Thompson JM, Fink GD, Watts SW. (2017). 5-HT causes splanchnic venodilation. Am J Physiol Heart Circ Physiol, 313:H676-H686, 2017.

Seitz BM, Demireva EY, Xie H, Fink GD, Krieger-Burke T, Burke WM, Watts SW. (2019). 5-HT does not lower blood pressure in the 5-HT7 knock-out rat. Physiol Genomics,
doi: 10.1152/physiolgenomics.00031.2019.

Terrón JA. (1997). Role of 5-ht7 receptors in the long-lasting hypotensive response induced by 5-hydroxytryptamine in the rat. Br J Pharmacol, 121:563-571.

Villalon CM, Centurion D, Bravo G, De Vries P, Saxena PR, Ortiz M. (2000). Further pharmacological analysis of the orphan 5-HT receptor mediating feline vasodepressor responses: close resemblance to the 5-HT7 receptor. Naunyn Schmiedebergs Arch Pharmacol, 361:665-671.

Villalon CM, Centurion D. (2007). Cardiovascular responses produced by 5-hydroxytriptamine a pharmacological update on the receptors/mechanisms involved and therapeutic implications. Naunyn Schmiedebergs Arch Pharmacol, 376:45-63.

Watts SW, Darios ES, Seitz BM, Thompson JM. (2015). 5-HT is a potent relaxant in rat superior mesenteric veins. Pharmacol Res Perspect 3: e00103. Doi: 10.1002/prp2. 103.

Watts SW, Morrison SF, Davis RP, Barman SM. (2012). Serotonin and blood pressure regulation. Pharmacol Rev, 64:359-388.

Zinner MJ, Kasher B and Jaffe B. (1983). The Hemodynamic effects of intravenous infusion of serotonin in conscious dog. Journal of Surgical Research, 34:1;171-178

Chapter 8

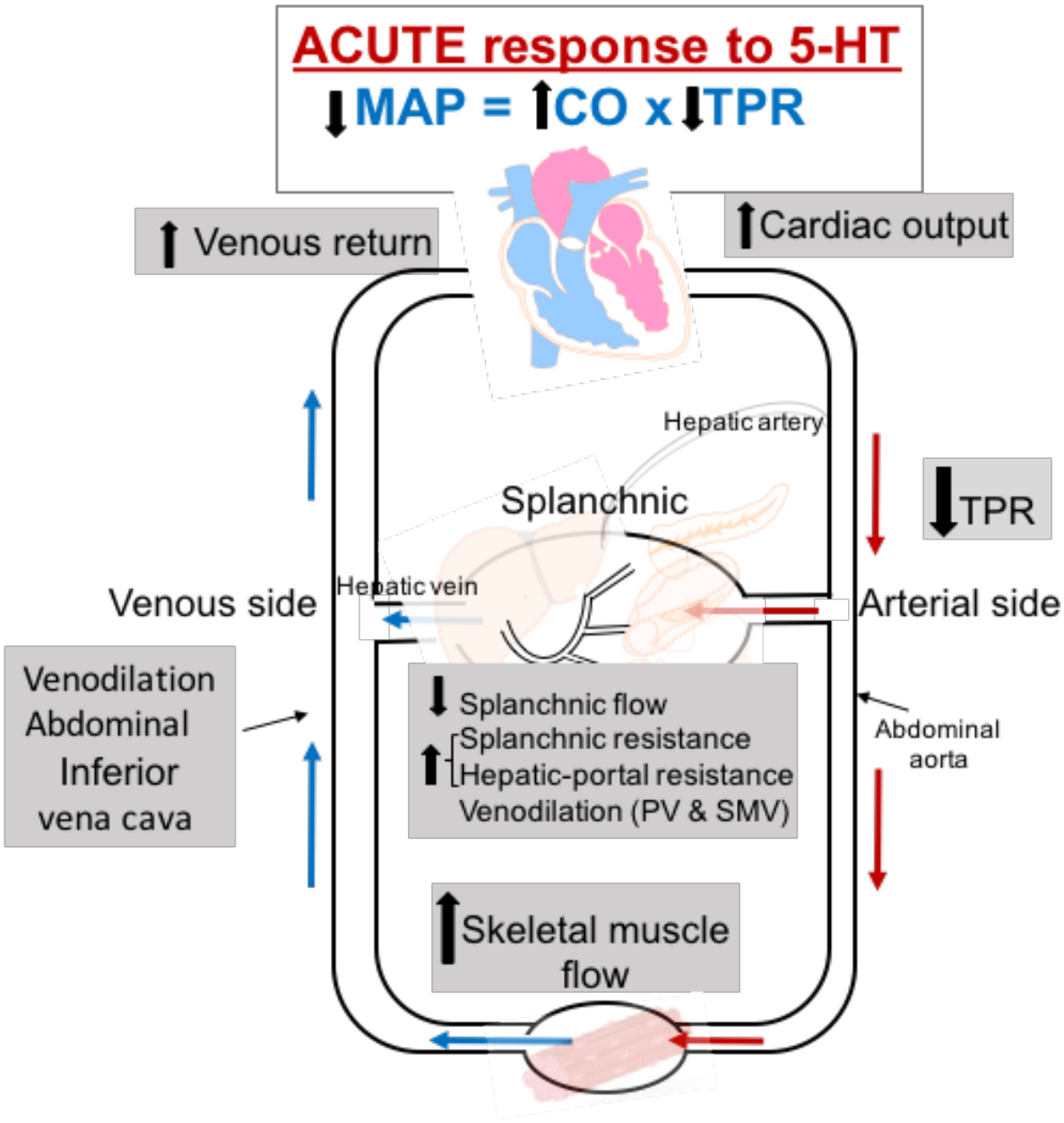


Figure 8.1: Illustrative description of the acute (<1 hour) hemodynamic effects of low dose 5-HT infusion.

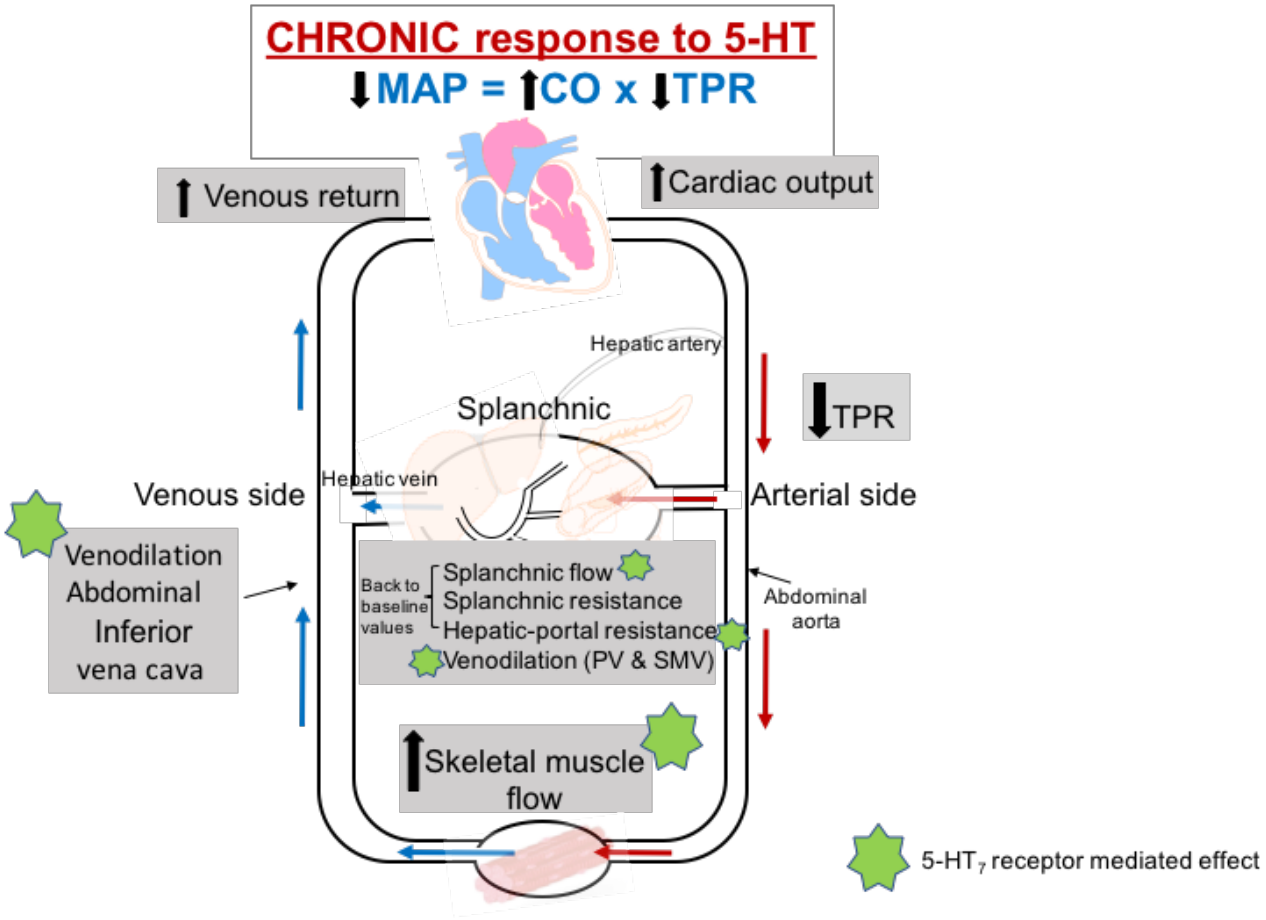


Figure 8.2: Illustrative description of the chronic (24 hours) hemodynamic effects of low dose 5-HT infusion.

Summary of Major Findings

- Activation of the 5-HT_7 receptor is the primary mediator for both the acute (<24 hours) and chronic (> 24 hours) depressor response in conscious, unrestrained male and female rats, tested by pharmacological blockade of the 5-HT_7 receptor and genetic removal of 5-HT_7 receptor in rats.

- Activation of the 5-HT_7 receptor is not endogenously active under basal conditions to alter arterial pressure, evident for the similar MAP in both 5-HT_7 receptor KO and WT rats during baseline and the lack of effect when the 5-HT_7 receptor antagonist was infused alone.

- Under both acute and chronic exposure of low dose 5-HT, the decrease in TPR closely parallels in time the fall in arterial pressure in the presence of exogenous 5-HT supporting arterial dilation, as the primary source for the depressor response.

- Under both acute and chronic exposure of low dose 5-HT, SV and CO are elevated suggesting it might be opposing the fall in arterial pressure with 5-HT.

- Heart rate is only increased for first day during the 5-day infusion of 5-HT. This occurs at a time arterial pressure is low suggestive of compensatory baroreceptor mechanisms (5-HT_7 receptor KO rats had a similar heart rate pattern).

- SV and CO remain elevated (at least for 1 day) after removal of 5-HT infusion suggesting additional non-5-HT_7 receptor mediated mechanism(s) are maintaining the increase in blood flow distribution to the heart. This results in the dramatic increase in arterial pressure when 5-HT stimulus was removed.

- Activation of the 5-HT_7 receptor increases the diameter of large splanchnic veins (PV, SMV) which parallels the fall in arterial pressure, suggesting the increase in venous capacitance might contribute to the fall in arterial pressure by decreasing CO and SV. However, this hemodynamic pattern was never observed. At this time, I am unsure of the 5-HT_7 mediated contribution of splanchnic venodilation to the fall in arterial pressure.

- Activation of NOS does not contribute to the acute or chronic 5-HT-induced hypotension.

- Activation of the 5-HT_7 receptor increases the diameter of abdominal inferior vena cava, a large peripheral vein, which could provide a decrease in resistance to venous return. A reduction in resistance to venous return supports an increase in SV and CO. The hemodynamic pattern is mediated by the 5-HT_7 receptor.

- Acute increase in hepatic-portal resistance due to low dose 5-HT infusion suggest hepatic arterial vasoconstriction, which is reversed after 24 hours of 5-HT infusion. The increase in resistance is mediated by the 5-HT_7 receptor.

- Acute increase in splanchnic resistance and decrease in portal vein (splanchnic) flow suggest constriction in the arteries of the splanchnic vasculature.

- After 24 hour of 5-HT administration, portal vein flow, hepatic-portal resistance and splanchnic resistance reversed to near baseline levels, even when arterial pressure was reduced. This suggest possible pharmacological antagonism or compensatory autoregulation mechanisms are masking the activated 5-HT_7 receptor effect.

- Activation of the 5-HT_7 receptor (acute and chronic) increases skeletal muscle/skin blood flow which actively contributes in the observed decrease in TPR.

- The increase in perfusion into the skeletal muscle compartment, a vascular bed with short transit time, aids in increasing VR and CO.

Discussion

Increased circulating serotonin in the periphery has been reported in chronic diseases that affect the cardiovascular system. The implications of 5-HT in these pathological conditions, gives rise to the question of how does chronic 5-HT influence the cardiovascular function? The aim of my work was to identify the mechanisms responsible for cardiovascular system responses to chronic low dose 5-HT infusion, focusing on the significant fall in arterial pressure.

The rationale for this work was grounded in evidence from our laboratory and others that low dose 5-HT (25 ug/kg) produces a fall in arterial pressure both acutely (< 24 hours) and chronically (>24 hours) in conscious and anesthetized rats (Centurion et al., 2004; De Vries et al., 1999; Terron, 1997; Villalon and Centurion, 2007). Importantly, using the same dose of 5-HT over a one-week infusion dramatically reduced arterial pressure not only in normotensive rats (Davis et al., 2012, 2013; Diaz et al., 2008), but experimentally hypertensive rats (Diaz et al., 2008; Watts et al., 2012). Earlier work

provided evidence that the depressor response to acutely administered 5-HT (<1 hour) was mediated through the 5-HT_7 receptor subtype.

Three major mechanisms were explored: 1) identifying the specific serotonergic receptor involved in the 5-HT-mediated depressor response; 2) investigating systemic and regional hemodynamics effects during low dose 5-HT infusion; with an emphasis in the splanchnic and skeletal vasculature region; and, 3) the possible involvement of activation of nitric oxide synthase (indirect vasodepressor mechanism) during low dose infusion of 5-HT.

The 5-HT_7 receptor: the primary mediator of the 5-HT-induced hypotension

The positive identification of the specific depressor serotonergic receptor subtype was the most important finding in this body of work. My data supports with great confidence that activation of the 5-HT_7 receptor is the primary mediator of 5-HT-induced hypotension. This conclusion is supported by both the use of pharmacological blockade with a selective 5-HT_7 receptor antagonist (SB269970) and by the genetic removal of the 5-HT_7 receptor in the newly created KO rats to support this statement (Seitz et al., 2017; 2019). This is true in both male and female rats, during acute and long-term depressor responses and in the anesthetized and conscious state.

Not all 5-HT experiments in this work used the 5-HT_7 receptor antagonist nor the 5-HT_7 receptor KO rats to test the contribution of the 5-HT_7 receptor. This was due to the high level of certainty that our low dose 5-HT-infusion experimental model achieves a level of 5-HT in the circulating plasma to sufficiently activate the 5-HT_7 receptor. In defense of this idea, the dose of 5-HT given produces a concentration of free circulating 5-HT in the plasma that is in the mid-nanomolar range (Diaz et al., 2008). This

circulating plasma level of 5-HT is adequate to activate the 5-HT_7 receptor (affinity; Ki ~7nM) (pdsp.unc.edu/databases), which possesses an affinity approximately two times greater than the other vasorelaxant serotonergic receptors subtypes (5-HT_{1B} ~14 nM and 5-HT_{2B} ~19 nM). Based on the high affinity of 5-HT for the 5-HT_7 receptor, it was accepted (but not tested), that the 5-$HT_{1B/1D}$ or 5-HT_{2B} had minimal contribution to the depressor response elicited by 5-HT at our dose, if at all. Earlier in vitro pharmacological evidence supports this idea (Centurion et al., 2004; Villalon and Centurion, 2007). With all things considered, the greatest reassurance to defend our low dose model in selectively activating the 5-HT_7 receptor is the data generated from our 5-HT_7 receptor antagonist and 5-HT_7 receptor KO studies.

The selective 5-HT_7 receptor antagonist (SB269970, 33 ug/kg) used to pharmacologically block the 5-HT_7 receptor completely reversed the fall in arterial pressure at each time point the antagonist was given. The selective antagonist used possesses an affinity (Ki) for the 5-HT_7 receptor that is 50-1,000 times greater than any other serotonergic receptor (pdsp.unc.edu/databases). Although,I never tested continuous infusion of the 5-HT_7 receptor antagonist over more than 24 hours, due to its short half-life and poor parenteral bioavailability, the creation of the 5-HT_7 receptor KO rats filled the void. The newly created 5-HT_7 receptor KO rats (Demireva et al., 2019) using Crispr cas-9 technology, infused with the same low dose of 5-HT for one week, showed no change in MAP compared to the significant 5-HT-induced depressor response observed in the WT littermates (Seitz et al., 2019). Together the findings confirm the low dose of 5-HT administered in our experimental model preferential

activates the 5-HT_7 receptor, a receptor that is essential to the 5-HT-induced hypotension.

However, if the dose of 5-HT infused is increased, generating a higher level of circulating 5-HT in the plasma, then a pressor response is observed. This outcome is likely do to stimulation of the contractile 5-HT_{2A} receptors. A similar response was found during the dose response challenge in the 5-HT_7 KO versus WT rats. The KO rats achieved an increase in arterial pressure at a much lower dose of 5-HT compared to the WT rats. It was not until the standard low dose of 5-HT was increased 6-fold higher did the WT rats exhibit a depressor response (Seitz et al., 2019). The activated 5-HT_7 receptor appears necessary for balancing (lowering) the arterial pressure response when the animal is challenged with increased circulating levels of 5-HT.

An outstanding question remains: is the 5-HT_7 receptor endogenously active under normal, basal condition to regulate arterial pressure? The answer to this question is best determined from two of our studies: 1) Rats administrated the 5-HT_7 receptor antagonist alone; and 2) the baseline measurements taken in the 5-HT_7 receptor KO rats prior to 5-HT infusion. In both studies, neither pharmacological blockade nor genetic removal of the receptor resulted in a change in arterial pressure from controls. If the 5-HT_7 receptor was endogenously active, blocking the 5-HT_7, receptor should result in an elevated arterial pressure. However, this was not observed. Therefore, the data suggest the endogenous levels of 5-HT are not high enough under normal physiological conditions to activate the 5-HT_7 receptors peripherally to cause a cardiovascular biological effect. Therefore, it appears the 5-HT_7 receptor remains quiescent under steady state conditions, until the levels of circulating 5-HT are sufficiently elevated to

activated the receptor. Having a more reliable method to measure circulating 5-HT plasma would provide more assurance.

Systemic hemodynamic effects mediated by the activated 5-HT$_7$ receptor

MAP is determined by the coordinated balance of TPR and CO. TPR determines the arterial tone within the peripheral circulation (Cowley, 1992), whereas CO is determined by circulatory blood volume, contractility of the heart and heart rate (Gelman, 2008). Knowing the response of these two distinct hemodynamic measures provides insight into the systemic mechanism that leads to the blood pressure outcome. During 5-HT-induced hypotension, TPR was decreased and CO was increased from the initial start of the 5-HT infusion and throughout the duration the amine was given. This same systemic hemodynamic pattern persisted despite a partial recovery in MAP.

The decrease in TPR closely paralleled the depressor response suggesting vasodilation of small arteries and arterioles within the periphery. The persistent elevations in SV and CO are contributing most likely by opposing the fall in arterial pressure and aiding in the recovery of MAP. Even when the 5-HT pumps were removed, the persistent elevation of SV and CO paralleled a significant elevation of MAP. HR was only elevated during the initial two days when MAP was at the lowest achieved pressure. The recovery of HR suggests a compensatory baroflex response as oppose to receptor mediated event. There are no reports of 5-HT$_7$ receptor actions in the heart.

The systemic hemodynamics were measured in a few 5-HT$_7$ receptor KO rats. Administration of 5-HT did not change MAP response in the KO rat compared to baseline, as excepted. There was also no significant change in CO or TPR from this

preliminary data. However, another interesting finding was observed. In the KO rats, CO and TPR responses oscillated between the circadian cycle (day and night), which was most pronounced during 5-HT administration and did not change MAP. Observed during the daytime (time of rest), CO was decreased and TPR was increased, suggestive of increase flow blood within the splanchnic region. At night when the rats are active, the opposite pattern persisted: an increase in CO with a decrease in TPR, suggestive of more blood flow to the working muscle. This systemic hemodynamic pattern is observed in humans (at night: decrease in CO and increase in TPR; during day: increase in CO and a decrease in TPR) (Veerman et al., 1995). This circadian pattern was not observed in the Sprague-Dawley rats during 5-HT infusion. The 5-HT_7 receptor has been reported to be linked to circadian systems (Ciarleglio et al., 2011). Investigating this circadian systemic pattern in 5-HT_7 receptor KO rats would be interesting in pathological conditions such as heart failure, where the systemic pattern is altered (Ogilvie, 1985).

Our systemic hemodynamic results in Sprague-Dawley rats to infused low dose 5-HT confirms the findings of a previous study (Davis et al., 2012). The purpose for reinvestigating the systemic response was to capture the initial acute CO and TPR response (<24 hours) which was not previously reported. This allowed for comparison of both the acute and chronic effect. It is axiomatic that the physiological regulation of the blood flow distribution under steady state or pathological conditions is never static. Time-dependent events continuously occur to maintain proper perfusion pressure and oxygen demands to the organs and brain. Arterial pressure is kept within its habitual range by mechanisms such as negative feedback baroreceptor reflexes, volume

regulation, and other regulatory system (Ackermann, 2004). Understanding the mechanisms responsible for long-term cardiovascular effects may differ from those responsible for short-term or acute effects. This is because acute (and chronic) exposure of 5-HT may lead to receptor down-regulation; receptor desensitization; changes in serotonin plasma concentrations or tissue distribution. Running time-dependent studies, I found during both the acute and chronic systemic hemodynamic response were the same.

Regional hemodynamic effects mediated by the activated 5-HT$_7$ receptor

The circulation system is considered a semi-closed loop with vascular beds parallel in series, connected by arteries, capillaries and veins. Each vascular bed (i.e. brain, kidney, splanchnic, skeletal muscle and other) contains a finite volume of blood, at a finite pressure and is separated by resistance. The changes in the vascular tone of the arteries or veins within the specific vascular beds causes variations in the flow, pressure and resistance. It is the summation of resistance across all the vascular beds that makes up the measurement of TPR. Vascular factors that regulate the volume of blood the heart receives (VR) and then pumps into the arterial compartment (CO) can also have a powerful effect on arterial pressure regulation. Since the bulk of blood volume within the circulation is held in the small veins and venules, especially within the splanchnic region, changes in venous tone are important in determining arterial pressure. Within the venous circulation, distinct regional segments maintain different degrees of capacitance capabilities (reservoirs) and perfusion times constants (fast and slow). Additionally, each vascular bed maintains a level of autoregulation and compensatory mechanisms to preserve oxygen demand and perfusion of critical

organs. To understand the cardiovascular effects generated by activation of the 5-HT_7 receptor it is critical to measure the resistance, pressure and flow within the vascular beds.

In my initial studies, microspheres were used to globally track blood flow through the circulation during 5-HT infusion. The most significant increase in blood flow was observed within the splanchnic vascular circulation; a circulation essential in blood pressure regulation. This finding lead to the idea that splanchnic arterial relaxation caused the observed decrease in splanchnic resistance. However, from earlier published data, it was observed that 5-HT did not relax isolated splanchnic arteries greater than 250 microns in diameter, including the thoracic aorta, abdominal aorta, superior mesenteric artery or mesenteric resistance artery (Davis et al., 2012). Therefore, dilation of the large splanchnic arteries was not considered in contributing to the arterial response. Another alternative idea was inhibition of sympatric innervation to the splanchnic region. However, removal of the celiac plexus by celiac ganglionectomy did not alter the arterial pressure to 5-HT (Darios et al., 2015). This suggested that the inhibition of sympathetic activity to reduce splanchnic vascular tone is not contributing to 5-HT-induced hypotension. For splanchnic resistance to be decreased during the microsphere studies, somewhere within the splanchnic arterial system, most likely within the small arteries and arterioles, these vessels are relaxing to low dose 5-HT infusion. Additionally, it is possible autoregulation within this vascular bed is causing an increase in splanchnic blood flow that is not 5-HT mediated. Microvasculature experiments would greatly help dispel this idea.

Regional hemodynamics that alter CO mediated by the activated 5-HT$_7$ receptor

Previous work in the laboratory showed 5-HT directly relaxed the isolated superior mesenteric vein via activation of the 5-HT$_7$ receptor (Bard et al., 1993; Watts et al., 2015). This lead to the idea of how does 5-HT effect the splanchnic veins, *in vivo*? Earlier reports found 5-HT increased vascular capacitance during acute administration in dogs (Hedadberg and Ruten, 1990). In clinical practice, relatively selective venodilators, such as nitroglycerin, can chronically lower arterial pressure (Lebendinskiy et al., 2007). Additionally, directly reducing splanchnic venous capacitance in humans, results in sustained increase in arterial pressure. This is observed when elastic abdominal binders deeply compress the splanchnic vasculature resulting in an increase in VR (Fanciulli et al., 2015). From these finding, the following hypothesis was generated: a low dose infusion of 5-HT dilates splanchnic veins *in vivo* by 5-HT$_7$ receptor activation to contribute in 5-HT-induced hypotension. Our newly validated abdominal imaging protocol was used as a surrogate to estimate venous capacitance (Seitz et al., 2017). 5-HT dilated the portal vein, superior mesenteric vein and abdominal inferior vena cava but not the abdominal aorta. The observed venodilation was accompanied by a fall in arterial pressure. Both venodilation and depressor response were blocked by the 5-HT$_7$ receptor antagonist SB269970. In support of these findings, 5-HT caused both the portal vein and abdominal inferior vena cava to dilate in WT but not the 5-HT$_7$ receptor KO rats. The diameter of the abdominal aorta diameter did not change in WT or KO rats during 5-HT infusion. Overall, findings suggested (but could not prove), that splanchnic venodilation by activation of the 5-HT$_7$ receptor contributes to the chronic depressor response.

The best indicators of the capacitance function of veins is by measurement of SV and CO. Dilation of small veins in the splanchnic region cause a decrease in MCFP by sequestering stressed volume within the highly capacitive veins from the circulation. This causes a decrease in SV and CO.

Dilation of medium to large peripheral veins, such as the abdominal inferior vena cava, causes a decrease in the resistance to venous return augmenting VR and CO. This is because large peripheral veins (i.e. abdominal inferior vena cava) are not capacitive vessels and are unable to store blood volume like splanchnic veins. Yet, these veins are critical for circulatory dynamics as they determine the level of resistance to venous return (Henderson, 2010). In the present study, the abdominal inferior vena cava was dilated after 24 hours of 5-HT infusion. This causes a decrease in resistance to venous return, which should promote CO.

Both variables of MCFP and resistance to venous return are critical regulators of cardiac output (Green, 1982). In this study, SV and CO were increased from the initial start of the 5-HT infusion and throughout the duration the amine was given. This captured the acute (<24 hours) timeframe not collected in the previous study. The data from this study supported the notion that the main function of splanchnic venous 5-HT_7 receptors is to reduce resistance to venous return rather than MCFP. In other words, a decrease in resistance to venous return would cause an increase SV and CO, the hemodynamic pattern that was observed with both acute and chronic 5-HT infusion.

Another concept to consider within the venous vasculature is one of transit time constants in regards to blood flow within distinct vascular regions. The vasculature

consists of regional areas with a high compliance (splanchnic) in parallel with regional area with lower compliance (skeletal muscle) (Coleman et al., 1974). The fractional distribution of blood flow between these two regions affects VR and CO. For example, vascular region with larger compliance has a longer time constant to drain and mobilize blood to the heart, decreasing VR and CO. In contrast, vascular region with less compliance can mobilize blood to the heart faster, increasing VR and CO. The splanchnic vasculature has a slow transit time constant of drainage in the range of 20 to 24 seconds whereas the peripheral vasculature bed (skeletal muscle and kidneys) has a fast transit time constant of 4 to 6 seconds (Madger, 2016). In my experiments, administration of 5-HT causes a significant increase in the perfusion in the skeletal muscle bed, a vascular bed with a fast time constant which quickly mobilizes blood toward the heart, augmenting VR and CO.

Regional hemodynamics that alter TPR mediated by activated 5-HT_7 receptor

Distribution of blood flow between the various vascular beds is controlled by regional arterial resistance. Resistance is calculated by the change in inflow pressure minus the outflow pressure divide by the flow within each vascular region of interest. Conceptually, the added total of resistance within each vascular bed makes up total peripheral resistance.

In this study, resistance was measured in the splanchnic, hepatic-portal and skeletal muscle region. Under steady state conditions, the splanchnic bed receives approximately 40% of blood flow and the remaining 60% goes to the peripheral vasculature (skeletal muscle, bone, skin, brain and heart; Cowley, 1992). Choosing the splanchnic vascular bed to measure resistance was an obvious choice (aside from the

high CO demand) considering my previous focus on the splanchnic region, from microsphere data to splanchnic venodilation. The hepatic–portal region was included in the resistance measures, as all splanchnic organs drain into the portal vein moving into the liver. Capturing the resistance within this region, provides complete knowledge of the full splanchnic vasculature effects. Hepatic-portal resistance also is a determinant of resistance to flow out of the splanchnic bed, thus affecting splanchnic volume and thus central blood volume, SV and CO (Zhang et al., 2013). The importance of measuring resistance within the skeletal muscle vascular bed stems from reports that activation of the 5-HT_7 receptor dilates the isolated skeletal muscle small arteriole from the cremaster in rats (Alsip and Harris,1991) and from a microsphere study that reported an increase in skeletal muscle flow during 5-HT infusion (De Vries et al., 1999). Our previous microsphere data did not, however, observe an increase in skeletal muscle blood flow in the presence of 5-HT infusion. To explain this discrepancy, the piece of skeletal muscle that was extracted during our microsphere measurement might have not included the deep skeletal muscle reported to dilated to 5-HT (Alsip and Harris,1991).

The regional resistance was measured to interpret the TPR response mediated by the 5-HT_7 receptor and is discussed in two groups: 1) splanchnic and hepatic-portal; and, 2) skeletal muscle.

Splanchnic and hepatic-portal regional 5-HT_7 mediated effects

The regional hemodynamics by activation of the 5-HT_7 receptor results in an increase in splanchnic and hepatic-portal region resistance from the initial start of 5-HT-infusion. The decrease in MAP was also an important cause of decreased splanchnic

flow. Portal pressure was also reduced. This contrasts with earlier described venous imaging data, where the observed portal vein diameter mediated by the 5-HT_7 receptor was increased. An increase in venous diameter may suggest an increase blood volume within the portal vein with a possible increase in portal pressure. But, this was not observed.

Instead the decrease in portal vein blood flow implies upstream arterial constriction is occurring within the splanchnic vasculature. This finding is supported by the measured increase in splanchnic resistance. The portal veins receive blood from stomach, spleen, pancreas, small intestine and colon. Therefore, arterial vasoconstriction within any one of these vascular beds could play a role in the reduced portal vein flow.

In the hepatic–portal region, the resistance was increased, suggesting a possible constriction within the liver. However, constriction within the liver often manifests an increase in portal pressure, which was not observed. In this study, portal pressure decreased with the initial response to 5-HT. The best estimate of liver resistance is to measure flow from the hepatic vein, which directly drains the liver.

Within 24 hours of 5-HT infusion, all events within the splanchnic and hepatic-portal region, including portal vein blood flow were reversed. Even when arterial pressure was reduced. These time-dependent changes occur even in the presence of 5-HT. It is unclear which exact mechanism generates the reversal of resistance in the splanchnic and hepatic-portal region to return portal blood flow. Pharmacological down regulation of the 5-HT_7 receptor causing a loss of the original effect to 5-HT could be an option. However, this is not likely, because 5-HT_7 receptor antagonist elevated portal

vein blood flow and the hepatic-portal resistance decreased. This indicates activation of the 5-HT_7 receptor is still present and engaged.

The hepatic-portal and splanchnic vascular region undergo continuous autoregulation but not to the significant level seen in the kidney, heart or brain. These mechanisms are occurring simultaneously to counteract the continuous effect of 5-HT during infusion to maintain perfusion. There are several possible compensatory mechanisms such as inhibition of sympathetic nervous system (besides celiac ganglion), hepatic arterial buffer response (Eipel et al., 2010), as well as, indirect mechanisms that release endogenous vasodilators (nitric oxide).

Skeletal muscle regional 5-HT_7 mediated effects

The most prominent (acute and chronic) hemodynamic response mediated by the 5-HT_7 receptor was a decrease in hindquarter resistance. This response paralleled the fall in arterial pressure and TPR and was accompanied by a dramatic increase in hindquarter blood flow. The hindquarter consists of 70% skeletal muscle by mass and receives the largest part of cardiac output (Korthuis et al., 1985), suggesting the increase in observed blood flow is within the small arteries and arterioles of skeletal muscle vascular bed. A previous report found dilation mediated by 5-HT_7 receptor occurred in the fourth-order skeletal muscle arteriole (<40 um) but not the first-order (>40 um) suggesting different regional serotonergic responses within the skeletal bed (Wilmoth et al., 1984).

The precise mechanism of how activated 5-HT_7 receptor mediates the skeletal muscle dilation was not investigated in my research. However, others found the ATP-sensitive potassium channels may be responsible for the vasodilator response by the 5-

HT_7 receptor (Garcia-Pedraza et al., 2015). This seems possible as the ATP-sensitive potassium channels are abundantly expressed on the cellular membrane of smooth muscle cells found around skeletal muscle arteries (Nelson and Quayle et al., 1995). When the ATP-sensitive potassium channel are opened an increase of potassium ions move inside the cell causing a hyperpolarization of the cell membrane this in turn closes voltage-dependent calcium channels, reducing calcium into the cell leading to vasodilation (Ashcroft and Ashcroft, 1990). Protein kinase A has been linked to the opening of the ATP-sensitive potassium channels (Quinn et al., 2004). The cellular signaling mechanism of the 5-HT_7 receptor is via coupling with the G-protein (G_s), which results in increased adenylyl cyclase activity leading to the rise in cAMP levels that in turn activates protein kinase A (Guseva et al., 2014). Therefore, there is a direct link of protein kinase A to phosphorylate the ATP-sensitive potassium channel causing overall skeletal muscle arterial dilation. Earlier reports have found excessive activation of the potassium ATP channel is associated with hypotension (Landry and Oliver et al., 1992), possibly a similar response found with activation of the 5-HT_7 receptors.

The hindquarter blood flow measurement includes skin. This is important to mention because the skin always appears flushed in 5-HT-treated rats. Doppler blood flow measures are also dramatically increased in 5-HT infused rats. Interestingly, the skin was not flushed in rats that were given the 5-HT_7 receptor antagonist or in 5-HT_7 receptor KO rats. Activation of the cutaneous vasculature occurs during changes in ambient temperature. CO to the skin in humans can be as little as 1–2% in the cold, and as much as 60% in acute heat stress (Johnson et al., 2016). The rat has far less thermoregulatory skin which is located only on the tail, paws and ears. Further detailed

experiments are needed to explore the role of cutaneous veins and activated 5-HT_7 receptor.

NOS is not necessary for chronic 5-HT-induced hypotension

We tested the role of NO in our low dose 5-HT experimental model to understand if activation of NOS contributes to the depressor response. Previous data supported LNNA, a non-selective inhibitor of NOS, completely prevented the chronic depressor action of 5-HT (Diaz et al., 2008). However, this published experimental design was different. LNNA was administered for 7-10 prior to 5-HT infusion. This is the classical model of experimental LNNA hypertension and is based on blockade of NOS resulting in endothelial dysfunction and vascular wall remodeling. The physiological alterations occurring during lengthy administration of LNNA (7-10 days) to generate the hypertensive state may have prevented the chronic depressor action of 5-HT. Both structural and functional alterations are known to occur in the vasculature when NO formation is blocked over a long timeframe (Kung et al., 1995).

In the present study, an acute bolus of NOS inhibitor was given at select time points during one week of 5-HT-induced hypotension in conscious rats. 5-HT-treated rats had a similar magnitude of increase in MAP compared to saline-treated rats. The response was observed every time the NOS inhibitor was given during 5 days of 5-HT infusion. One of my original hypothesis was activation of the 5-HT_7 receptors within the splanchnic region generated a later indirect vasodepressor mechanism, such as activation of nitric oxide. My data does not support this portion of the hypothesis as activation of NOS did not contribute to the 5-HT-mediated depressor response.

Major findings

The major findings of this work are shown in figure 8.2 and are briefly summarized here. Activation of the 5-HT_7 receptor lowers arterial pressure (acutely and chronically) by decreasing TPR through dilation of arteries/arterioles. CO is increased. This systemic hemodynamic pattern is observed from the start and throughout the duration the amine is infused. Activation of NOS does not appear to play a role in reducing the resistance during 5-HT-induced hypotension.

The overall cardiovascular responses generated by chronic 5-HT are brought on by changes in resistance to blood flow within the regional vasculature. Blood flow and resistance were measured in the splanchnic, hepatic-portal, and skeletal muscle region; some effects were time-dependent.

Within the splanchnic and hepatic-portal circulation, resistance increased from the start of 5-HT infusion, with a dramatic decrease in portal vein flow, suggesting arterial constriction in the splanchnic circulation. This was an unexpected outcome, because activation of the 5-HT_7 receptor resulted in a substantial increase in the diameter of portal vein and superior mesenteric vein, when imaged under the same acute conditions. The dilation of the observed splanchnic veins mirrored the fall in MAP and gave reason to believe an increase in venous capacitance contributed to the fall in MAP.

Within 24 hours of 5-HT infusion, the hepatic-portal and splanchnic resistance were reversed to near baseline levels along with the portal vein flow. During this same 24-hour time interval, MAP reached its lowest value and splanchnic veins along with the abdominal IVC were still dilated. Activation of autoregulatory or other compensatory

mechanism may be masking the effects of the dilated splanchnic veins, making it appear as if their actions do not contribute to the 5-HT-induced hypotension.

The most prominent regional response to low dose 5-HT infusion mediated by activation of the 5-HT_7 receptor is a decrease in hindquarters resistance (acute and chronic). This response parallels the fall in MAP. Due to the high CO demand and percentage of skeletal muscle mass within the hindquarter, the decrease in hindquarter resistance is most likely attributed to the skeletal muscle vasculature. The significant increase in blood flow to this region indicates the dilation of the skeletal arterial bed.

Therefore, activation of 5-HT_7 receptor lowers MAP by dilating skeletal muscle arterials to decrease TPR. The increase in skeletal muscle perfusion, a vascular bed with a fast time constant, and supports the increase in VR and CO observed. The noted increase in dilation of the large abdominal IVC additionally supports a decrease in resistance to VR further adding to the increase in VR and CO. The decrease in TPR far overshadows the observed increase in CO, which results in the fall in MAP during activation of the 5-HT_7 receptor.

Limitations

There are several limitations within this work which deserves to be noted. First, the measure of reliable circulating level 5-HT in the plasma are challenging. The plasma results can have large variations due to the nearly unavoidable contamination of the plasma with 5-HT from ruptured platelets during blood sampling (Brand and Anderson, 2011). Knowing the precise measures of the circulating 5-HT concentration in the plasma would allow coordination of 5-HT levels with the vascular response (as well as track the plasma levels of 5-HT during steady state and pathological conditions).

Second, the measure of total blood volume during 5-HT infusion was not collected. Resistance measures often assume blood volume is constant. Increasing the volume of blood in the circulation can increase VR and CO. Third, much of the work presented here speculates on the vasculature response within the microcirculation. The vascular location for the greatest degree of resistance resides in the small arteries and arterioles whereas the vascular location for the greatest level of capacitance resides in the small veins and venules. These vascular structures are too small for imaging studies. Microcirculation experiments would greatly extend our knowledge on the cardiovascular response mediated by the 5-HT_7 receptor. Finally, every effort was made to collect all cardiovascular parameters in conscious animals. However, that was not always possible. It is well known that extensive surgical manipulations and anesthesia can alter the regulation of blood flow and modify arterial pressure. Necessary care was given during surgical procedures to maintain proper respiration rate and titrate anesthesia. In addition, control animals were included in all studies when possible.

Future directions

The present work has answered several key questions on the cardiovascular effects mediated by the 5-HT_7 receptor. The knowledge gained provides new opportunities to now use what we have learned regarding the pharmacology of the receptor, as well as, investigating the 5-HT_7 receptor under pathological conditions. For example, investigating the contributions of the ATP sensitive potassium channel with the activated 5-HT_7 receptor would be of interest considering smooth muscle potassium channels have been involved in pathological conditions of the vasculature such as hypotension and ischemia (Landry and Oliver, 1992).

Additionally, investigating the role of 5-HT_7 receptor in an experimental heart failure or shock model. Drugs that are commonly used to manage heart failure operate under similar redistribution of blood volume pattern that are observed during 5-HT_7 receptor activation: decrease in TPR via an increase in skeletal muscle blood flow with an increase in CO. Captopril is a perfect example (Ogilvie,1985). Creating heart failure in the 5-HT_7 receptor KO rats is an interesting idea. It is reasonable to predict the WT rats would have higher survival rates compare to their KO counterparts.

Since the most prominent cardiovascular effect of the activated 5-HT_7 receptor is skeletal muscle vasodilation. Using our low dose of 5-HT or a 5-HT_7 receptor agonist, such as 5-CT, may be beneficial in peripheral vascular disease, such a skin grafts and ulcers.

Another opportunity to explore would be to challenge the 5-HT_7 receptor KO rats via exercise. Activation of the 5-HT_7 receptor increases blood flow and delivers more oxygen needs to the skeletal muscle. Therefore, blocking or removing the 5-HT_7 receptor should cause less skeletal muscle blood flow and unmet oxygen requirement for the exercised muscle, causing the KO rats to fatigue quicker. Thus, the 5-HT_7 receptor rats should be intolerant to exercise demands compared to their WT littermate. The concept of selectively activating the skeletal muscle 5-HT_7 receptor seems beneficial for both the elite athletes to extreme couch potato.

Finally, increasing levels of endogenous 5-HT in the circulating plasma have been linked to chronic diseases effecting the cardiovascular system including carcinoid syndrome, inflammation, shock, systemic and pulmonary hypertension and obesity (Ayme-Dietrich et al., 2017; Biondi et al., 1986; Brenner et al., 2007; Jone and

Blackburn, 2002; Kim et al., 2011; Shajib and Khan, 2015). It would be interesting to investigate how the 5-HT_7 receptor contributes, under increased endogenous levels of 5-HT during these pathological conditions.

Conclusion

The overall conclusion of my research is infused low dose 5-HT decrease arterial pressure mediated by the activated 5-HT_7 receptor. The decrease in TPR is due to decrease in skeletal muscle resistance. The observed increase in blood flow to the skeletal muscle bed is most likely due from dilation of skeletal muscle arterioles. The increase in CO is supported by two events: 1) the increase in perfusion of the fast time constant skeletal muscle vascular bed; and 2) the dilation of the abdominal IVC aiding in the resistance to venous return.

REFERENCES

REFERENCES

Ackermann U. (2004). Regulation of arterial blood pressure. Surgery (Oxford), 22(5):120a-120f.

Alsip NL, Harris PD. (1991). Receptor mediation of microvascular responses to serotonin in striated muscle. Am J Physiol, (5 pt 2):H1525-33.

Ashcroft SJH, Ashcroft FM. (1990) Properties and functions of ATP-sensitive K-channels. *Cell Signaling* 2:197-214.

Ayme-Dietrich E, Aubertin-kirch G, Maroteaux L, Monassier L. (2017). Cardiovascular remodeling and the peripheral serotonergic system. Archives of Cardiovascular Disease, 110:51-59.

Bard JA, Zgombick J, Adham N, Vaysse P, Branchek TA, Weinshank RL. (1993). Cloning of a novel human serotonin receptor (5-HT7) positively linked to adenylate cyclase. J Biol Chem, 268:23422-23426.

Brand T, Anderson GM. (2011). The measurement of platelet-poor plasma serotonin: a systematic review of prior reports and recommendations for improved analysis. Clin Chem, 57:1376–1386.

Biondi ML, Agostoni A, Marasini B. (1986). Serotonin levels in hypertension. J Hypertens, 4:S39-S41.

Brenner B, Harney JT, Ahmed BA, Jeffus BC, Unal R, Mehta JL, Kilic F. (2007). Plasma serotonin levels and the platelet serotonin transporter. J Neurochem, 102:206 –215.

Carleigh CM, Resuehr HES, McMahon DG. (2011). Onteractions of the serotonin and circadian systems: nature and nuture in rhythms and blue. Neuroscience, 1:197:8-16.

Centurión D, Glusa E, Sánchez-López A, Valdivia LF, Saxena PR, Villalón CM. (2004). 5-HT7, but not 5-HT2B, receptors mediate hypotension in vagosympathectomized rats. Eur J Pharmacol, 502:239–242.

Coleman TG, Manning RD, Norman RA and Guyton AC. (1974). Control of cardiac output by regional blood flow distribution. Annals of Biomedical Engineering, 2:149-163.

Cowley AW. (1992). Long-term control of arterial pressure. Physiol Rev, 72:231-300.

Darios ES, Barman SM, Orer HS, Morrison SF, Davis RP, Seitz BM, Burnett R, Watts, SW. (2015). 5-hydroxytryptamine does not reduce sympathetic nerve activity or neuroeffector function in the splanchnic circulation. Eur J Pharmacol, 754.

Davis RP, Pattison J, Thompson JM, Tiniakov R, Scrogin KE, Watts SW. (2012). 5-hydroxytryptamine (5-HT) reduces total peripheral resistance during chronic infusion: direct arterial mesenteric relaxation is not involved. BMC Pharmacol, 12:4 doi: 10.11186/1471-2210-12-4.

Davis RP, Szasz T, Garver H, Burnett R, Tykocki NR, Watts SW. (2013). One-month serotonin infusion results in a prolonged fall in blood pressure in the deoxycorticosterone acetate (DOCA) salt hypertensive rat. ACS Chem Neurosci, 4: 141–148. doi:10.1021/cn300114a.

De Vries P, De Visser PA, Heiligers JPC, Villalón CM, Saxena PR. (1999). Changes in systemic and regional haemodynamics during 5-HT_7 receptor mediated depressor responses in rats. Naunyn-Schmied Arch Pharmacol, 359:331-338.

Demireva EY, Xie H, Darios ES, Thompson JM, Seitz B, Watts SW. (2019). Creation of the 5-Hydroxytryptamine receptor 7 knockout (Htr7 KO) rat as a tool for cardiovascular research. Physiological Genomics.

Diaz J, Ni W, King A, Fink GD, Watts SW. (2008). 5-hydroxytryptamine lowers blood pressure in normotensive and hypertensive rats. J Pharmacol Exp Ther, 325:1031-1038.

Eipel C, Abshagen K, Vollmar B. (2010). Regulation of hepatic blood flow: the hepatic arterial buffer response revisited. World J Gastroenterol, 16(48):6046-6057.

Fanciulli A, Goebel G, Metzler B, Sprenger F, Poewe W, Wenning GK, Seppi K. (2015). Elastic abdominal binders attenuate orthostatic hypotension in Parkinson's disease. Mov Discor Clin Pract, 3(2):156-160.

Garcia-Pedraza JA, Garcia M, Martin ML, Rodriguez-Barbero A, Moran A. (2015). 5-HT2 receptor blockade exhibits 5-HT vasodilator effects via nitric oxide, prostacyclin and ATP-sensitive potassium channels in rat renal vasculature. Vascular Pharmacology, 79:51-59.

Gelman S. (2009). Venous function and central venous pressure. Anesthesiology, 108: 735-748.

Green JF. (1982). Circulatory mechanics. In: Fundamental cardiovascular and pulmonary physiology. Philadelphia: Lea & Febiger, 01-110.

Guseva D, Wirth A and Ponimaskin E. (2014). Cellular mechanisms of 5-HT7 receptor – mediated signaling. Frontiers in Behavioral neuroscience, 8(304)1-8.

Headberg PA, Ruten DL. (1990). Influence of serotonin on intravascular capacity in the anesthetized dog. Acta Physiol Scan, 139:561-568.

Henderson WR, Griesdale DEG, Walley KR and Sheel AW. (2010). Clinical review: Guyton the role of mean circulatory filling pressure and right arterial pressure in controlling cardiac output. Critical Care Medicine, 14:243.

Johnson, RS, Titze, J, Weller R. (2016). Cutaneous control of blood pressure. Current opinion in nephrology and hypertension, 25(1):11–15.

Jones BJ, Blackburn TP. (2002). The medical benefit of 5-HT research. Pharmacology, Biochemistry and Behavior, 71:555-568.

Kaumann AJ, Levy FO. (2006). 5-Hydroxytryptamine receptors in the human cardiovascular system. Pharmacology & Therapeutics, 111:674-706.

Kim HJ, *et al.* (2011). Metabolic analysis of livers and serum from high-fat diet induced obese mice. J Proteome Res, 10:722-731.

Korthuis RJ, Benoit JN, Kvietys PR, Townsley MI, Taylor AE, Granger DN. (1985). Humoral factors may mediate increased rat hindquarter blood flow in portal hypertension. Am J Physiol, 249(4 Pt 2): H827-33.

Kung CF, Moreau P, Takase H, Luscher TF. (1995). LNAME hypertension alters endothelial and smooth muscle function in rat aorta. Hypertensive, 26:744-751.

Landry DW, Oliver JA. (1992). The ATP-sensitive potassium channel mediates hypotension in endotoxemia and hypoxic lactic acidosis in dogs. J Clin Invest, 89:2071-2074.

Lebendinskiy K, Pervanian S and Puzanova I. (2007). Eurpoean Journal of Anesth 9EJA) clinical and experimental circulation, 24:50-51.

Magder S. (2016). Volume and its relationship to cardiac output and venous return. Critical Care, 20:271.

Nelson MT, Quayle JM. (1995). Physiologicla roles of properties of possium channels in arterial smooth muscle. Cell Physiology, 268:4, C799-C822.

Ogilvie, RI. (1985). Comparative effects of vasodilator drugs on flow distribution and venous return. Can J Physiol Pharmacol, 63:1345-1355.

Quinn KV, Giblin JP, Tinker A. (2004). Multisite phosphorylation mechanism for protein kinase A activation of the smooth muscle ATP-sensitive K+ channel. Circ Res, 94(10) 1359-1366.

Seitz BM, Orer HS, Krieger-Burke T, Darios ES, Thompson JM, Fink GD, Watts SW. (2017). 5-HT causes splanchnic venodilation. Am J Physiol Heart Circ Physiol, 313:H676-H686, 2017.

Seitz BM, Demireva EY, Xie H, Fink GD, Krieger-Burke T, Burke WM, Watts SW. (2019). 5-HT does not lower blood pressure in the 5-HT7 knock-out rat. Physiol Genomics,
doi: 10.1152/physiolgenomics.00031.2019.

Shajib MS, Khan WI. (2015). The role of serotonin and its receptors in activation of immune responses and inflammation. Acta Physiol (Oxf*)*, 213(3):561-74.

Terrón JA. (1997). Role of 5-ht7 receptors in the long-lasting hypotensive response induced by 5-hydroxytryptamine in the rat. Br J Pharmacol, 121:563-571.

Villalon CM, Centurion D. (2007). Cardiovascular responses produced by 5-hydroxytriptamine a pharmacological update on the receptors/mechanisms involved and therapeutic implications. Naunyn Schmiedebergs Arch Pharmacol, 376:45-63.

Watts SW, Darios ES, Seitz BM, Thompson JM. (2015). 5-HT is a potent relaxant in rat superior mesenteric veins. Pharmacol Res Perspect 3: e00103. Doi: 10.1002/prp2. 103.

Watts SW, Morrison SF, Davis RP, Barman SM. (2012). Serotonin and blood pressure regulation. Pharmacol Rev, 64:359-388.

Wilmoth FR, Harris PD and Miller FN. (1984). Differential serotonin response in the skeletal muscle microcirculation. Life Science, 34:12, 1135-41.

Zhang W, Shibamoto T, Tanida M, Wnag M, Sun L and Kurata Y. (2013). Rat hepatic and splanchnic vascular responses to anaphylactic shock, compared with hemorrhagic or vasodilator-induced shock. In vivo, 27:485-494.