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Chronic 5-HT Causes a Long-Term Blood Pressure Fall in DOCA-Salt Hypertension: Role of Nitric Oxide

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CHRONIC 5-HT CAUSES A LONG-TERM BLOOD PRESSURE FALL IN DOCA-SALT HYPERTENSION; ROLE OF NITRIC OXIDE

By

Jessica Lynn Diaz

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ABSTRACT

CHRONIC 5-HT CAUSES A LONG-TERM BLOOD PRESSURE FALL IN DOCA-SALT HYPERTENSION; ROLE OF NITRIC OXIDE

By

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We have shown that chronic serotonin (5-HT) causes a dramatic fall in mean arterial pressure (MAP) in both DOCA-salt hypertensive rats (deoxycorticosterone acetate) and Sham rats ($Sham_D$). We hypothesized that 5-HT acts to promote the function of nitric oxide synthase (NOS) *in vivo*, and that this hypotensive effect would not be seen in a hypertensive model in which NOS is inhibited using L-NNA ($N\omega$-nitro-$_L$-arginine). 5-HT (25 ug/kg/min) or Vehicle was administered to DOCA-salt and LNNA hypertensive rats and Shams. Within 24 hours, MAP in the DOCA 5-HT infused group fell 53 mmHg while in the $Sham_D$ rats, MAP fell less dramatically (-21 mmHg). This hypotensive response was *not* observed in the L-NNA 5-HT infused group, in which MAP remained unchanged. In contrast, $Sham_L$ 5-HT-infused rats experienced a similar drop in MAP to $Sham_D$ rats (-19 mmHg). Ganglionic blockade using hexamethonium given on Day 4 of 5-HT or vehicle infusion demonstrated marked sympatho-inhibition in the DOCA 5-HT infused rats (peak MAP fall of 40.3±5.9 mmHg), in contrast to DOCA Vehicle infused rats (peak fall MAP 90.6±14.0 mmHg). This sympatho-inhibitory effect of 5-HT was abolished in LNNA hypertension in which there was no difference between 5-HT-infused (peak fall MAP 54.7±4.7 mmHg) and vehicle-infused rats (peak fall MAP 51.1±12.9 mmHg). These data suggest that 5-HT inhibits the sympathetic nervous system in a NOS-dependent fashion.

To my understanding and supportive family:
Mario, Janis, Marisa, Johanna, Marcus,
and all our creatures great and small,
who helped me through.

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LIST OF ABBREVIATIONS

5-HIAA	5-Hydroxyindoacetic acid
5-HT	5-Hydroxytryptamine, serotonin
ACh	Acetylcholine
BCA	Bicinchoninic Acid
BP	Blood pressure
bpm	Beats per minute (heart rate)
BSA	Bovine Serum Albumin
CNS	Central Nervous System
DOCA	Deoxycorticosterone acetate
eNOS	Endothelial nitric oxide synthase
i.p.	Intraperiotoneally
KCl	Potassium chloride
L-NNA	Nw-nitro-L-arginine
MAO	Monoamine oxidase
MAP	Mean arterial pressure
NaCl	Sodium chloride
NO	Nitric oxide
NOS	Nitric oxide synthase
PCPA	Para-chlorophenyalanine
PPP	Platelet-poor plasma

PRP	Platelet-rich plasma
PE	Phenylephrine
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PSS	Physiological salt solution
SERT	Serotonin transporter
SEM	Standard error of the mean
SHR	Spontaneously hypertensive rats
SNS	Sympathetic Nervous System
s.q.	Subcutaneously
SSRIs	Selective Serotonin Reuptake Inhibitors
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline + Tween
TPR	Total peripheral resistance

Introduction

Discovery of 5-HT

Serotonin or 5-HT (5-hydroxytryptamine) is a diverse physiological agent, acting as a neurotransmitter in the central nervous system, a vasoactive agent capable of causing smooth muscle contraction in the cardiovascular system, a regulator of gastrointestinal peristalsis, and as an aggregator of platelets. 5-HT was discovered independently in two laboratories in the mid-20th century. Rapport, Green, and Page discovered a powerful endogenous vasoconstricting substance at low concentrations in clotted blood which they called serotonin: "sero-" for being isolated from serum and "-tonin" for its vasoconstrictor activity (Rapport et al, 1948). Erspamer reported an indole compound obtained from the enterochromaffin cells of the intestine that could cause smooth muscle contraction in isolated tissues, which he called enteramine. In 1949, the chemical structure of serotonin was determined to be 5-hydroxytryptamine. In the 1950s, enteramine and serotonin were found to be the same compound.

The vast majority (95%) of the 5-HT produced in the body is made in the enterochromaffin cells of the gut. A small percentage is produced in the neuroendocrine cells of the lung. Another site of 5-HT production is the central nervous system, largely kept distinct from peripheral 5-HT by the blood-brain-barrier (BBB) (Maurer-Spurej, 2005). 5-HT is produced by a series of enzymatic reactions, beginning with the dietary essential amino acid tryptophan (Côté et al., 2004). As a polar molecule, 5-HT itself cannot cross the BBB in physiologic conditions, in contrast to tryptophan, which is able to transverse the BBB (O'Kane

and Hawkins, 2003). Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in 5-HT production from tryptophan, and 2 isoforms exist: TPH1 (peripheral) and TPH2 (central) (Côté et al., 2004). The action of 5-HT is terminated after its degradation to an inactive metabolite, 5-hydroxyindole acetic acid (5-HIAA), *via* the action of mitochondrial monoamine oxidase (Côté et al., 2004).

While investigations are ongoing, currently there is no published evidence for the enzymatic machinery for 5-HT handling and production being present in peripheral arteries or veins. But it has been reported that there exists a functional serotonin transporter (SERT) on peripheral arteries, whereby smooth muscle cells are able to take up 5-HT from the extracellular to intracellular space (Ni et al., 2004). Thus, the current theory is that 5-HT gets transported from the site of production in the intestine to the 5-HT receptors and/or SERT located on the cellular membranes of the peripheral vasculature *via* the circulating platelets (Ni et al., 2004). Platelets, as anucleate cells, lack the machinery to produce 5-HT themselves, but are able to take up 5-HT *via* SERT localized to the platelet cell membrane, and have the ability to store 5-HT in their dense granules (Ni et al., 2004; Maurer-Spurej, 2005). Platelet storage may be a protective mechanism to keep freely circulating 5-HT levels low in the plasma: 0.1 ng/ml measured by HPLC, while platelet-stored 5-HT levels have been measured at 884±202 ng/10^9 platelets (Maurer-Spurej, 2005).

5-HT receptors

To date, there are currently seven families of 5-HT receptors (5-HT_{1-7}), some of which have subfamilies. 5-HT_3 receptors are ligand-gated ion channels, while the other families are transmembrane-spanning G-protein-coupled receptors. 5-HT exerts its effects in the cardiovascular system either directly on cardiovascular 5-HT receptors or indirectly *via* receptors in the central nervous system. The 5-HT receptors present in the tissues of the cardiovascular system include 5-HT_{1B}, 5-HT_2 family, 5-HT_3, 5-HT_4, and 5-HT_7 receptors.

Cardiac 5-HT receptors:

5-HT affects the heart in a myriad of ways, due to the existence of numerous 5-HT receptors on the various tissue types. There are 5-HT receptors on the cells of the sino-atrial node, the pacemaker of the heart, which influence heart rate. Tachycardia results from activation of the 5-HT_4 receptors (human), 5-HT_{2B} receptors (rat), or 5-HT_7 receptors (cat) (Côté et al., 2004). Increased circulating 5-HT can cause sinus tachycardia and atrial fibrillation (Côté et al., 2004). In contrast, bradycardia can also potentially be induced by activation of the 5-HT_3 receptors located on vagal nerve endings innervating the sino-atrial node (Côté et al., 2004). Cardiac contractility in the myocardial cells is mediated primarily by 5-HT_{2B} receptors (Côté et al., 2004). Expression of 5-HT_{2B} receptors is vitally important to heart development. Lack of the 5-HT_{2B} receptors in knock-out mice results in mid-gestational lethality due to defects in the myocardial architecture, including markedly decreased ventricular wall thickness, and a gross absence of ventricular myocardial trabeculation (Nebigil et al., 2000).

Renal 5-HT receptors:

There are species differences in the receptors mediating effects of 5-HT in the renal artery. In many species, the 5-HT_{2A} receptor is responsible for contraction to 5-HT.

Vascular 5-HT receptors:

In the peripheral vasculature, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_4, and 5-HT_7 receptors are present (Côté et al., 2004; Hoyer et al., 2002; Watts, 2005). 5-HT_{1B} receptors are located in the CNS, but can also mediate contraction in rat caudal arteries and are expressed on cerebral arteries (Hoyer et al., 2002). The 5-HT_{2A} receptor is widely distributed both centrally and peripherally and mediates contraction in a number of tissues containing smooth muscle (Hoyer et al., 2002). The 5-HT_{2B} receptor is present on smooth muscle cells (Kaumann and Levy, 2006; Hoyer et al., 2002) and is upregulated in arteries from DOCA-salt hypertensive rats. The 5-HT_{2B} receptor becomes the 5-HT receptor predominately responsible for vascular smooth muscle contraction in hypertensive rats, as opposed to the 5-HT_{2A} receptor in normotensive rats (Watts et al., 1996; Banes and Watts, 2002; Banes and Watts, 2003). In some tissues, activation of the 5-HT_{2B} receptor results in endothelium- and nitric oxide-dependent vasorelaxation, including the pig pulmonary arteries (Jähnichen et al., 2005) and rat jugular vein (Ellis et al., 1995).

In addition to localization to the heart, 5-HT_4 receptors are expressed in both pig and human coronary artery smooth muscle cells, and weakly expressed

in human endothelial cells (Ullmer et al., 1995). The 5-HT_4 receptor is positively coupled to adenylate cyclase, but its definitive function has yet to be fully uncovered (Hoyer et al., 2002; Ullmer et al., 1995). Finally, 5-HT_7 receptors are expressed extensively in the vasculature and are responsible for mediating endothelium-independent vasorelaxation (Terrón and Martínez-García, 2007; De Vries, 1999).

While 5-HT is a vasoconstrictor on its own, its most probable physiologic contribution to blood pressure regulation is by modulation of other vasoactive agents and hormones. Subcontractile concentrations of 5-HT have the ability to potentiate smooth muscle contraction of isolated tissues to norepinephrine (NE), angiotensin II (AngII), and the potent vasoconstrictor endothelin-1 (ET-1) (Watts, 2000.)

Role of 5-HT in disease states

Because of the physiological diversity of 5-HT receptors and function in the body, dysregulation of 5-HT production, metabolism, or cellular responses are capable of affecting a number of physiological systems. 5-HT has been implicated in a number of gastrointestinal, neurological, hemostatic, as well as cardiovascular diseases. 5-HT in the brain influences a number of behaviors, including compulsion, aggression, anxiety, depression, sleep, cognition, sensory perception, motor activity, appetite, temperature regulation, nociception, sexual behavior and hormone secretion (Goodman and Gilman, 2001). Peripherally, 5-HT plays a role in other disease conditions including migraine headaches and

gastrointestinal disorders, (Goodman and Gilman, 2001) in addition to vascular diseases such as thrombosis and atherosclerosis (Vikenes et al., 1999; Ishida et al., 2001). In the cardiovascular system, 5-HT is a vascular smooth muscle cell mitogen, associated with cellular proliferation and vascular remodeling, which is a hallmark of systemic hypertension. Hypersensitivity to 5-HT has been observed in cardiovascular diseases, including atherosclerosis (Ishida et al., 2001) and systemic hypertension (Watts et al., 1995; Watts, 1998; Banes and Watts, 2001)

Excessive levels of circulating 5-HT have been associated with heart valvular pathology. Gustafsson *et al.* observed that 3 months of daily subcutaneous injection of 5-HT creatinine sulfate complex (50 mg/kg for the first 3 days and 20 mg/kg throughout the duration of the study) resulted in pathology of the aortic and/or pulmonic heart valves (Gustafsson, 2005). In this particular study, no measurements of blood pressure were made with 5-HT administration.

There is a strong connection between increased levels of 5-HT and increased SERT function in patients with pulmonary hypertension (Egermayer et al., 1999). Neuroendocrine cells lining the lung secrete vasoactive mediators, including 5-HT, in response to pulmonary insult, like airway hypoxia and hypercapnia (Egermayer et al., 1999). In fact, 5-HT is identified as the most powerful pulmonary vasoconstrictor known to date (Egermayer et al., 1999). Anorexogenic fluramines act on SERT as 5-HT releasers, thereby increasing 5-HT in circulation (Chapman and Wideman, 2006; Egermayer et al., 1999), and use of these drugs has been associated with development of primary pulmonary

hypertension (Chapman and Wideman, 2006). SERT plays a central role in the pathogenesis of pulmonary-artery smooth muscle cell proliferation, by bringing 5-HT intracellularly to exert physiological effects, ultimately leading to pulmonary hypertension (Guignabert et al., 2006; Nemecek et al., 1986). Patients with platelet-storage pool diseases, in which the platelets have defects in the ability to store substances intracellularly, which results in increased 5-HT levels in plasma, have an increased incidence of developing pulmonary hypertension (Egermayer et al, 1999; Maurer-Spurej, 2005).

As a corollary, the use of SERT-inhibitors, like fluoxetine (Prozac®), is protective against pulmonary hypertension (Maclean et al., 2004). Maurer-Spurej *et al.* were the first to show that the use of fluoxetine inhibits the release of 5-HT from platelets during aggregation, preventing elevations in plasma 5-HT, which is perhaps the mechanism of this protection (2004). While the association between 5-HT and pulmonary hypertension is established currently, the link between 5-HT and systemic hypertension is less so, and more controversial.

5-HT in systemic hypertension

The use of SERT-inhibitors, namely fluoxetine, causes increases in blood pressure in both rodents (Lazartigues et al., 2000) and humans (Amsterdam et al., 1999).

Freely circulating levels of 5-HT in the plasma (platelet poor plasma, PPP) are reported to be increased in hypertensive patients and experimental models of hypertension (Maurer-Spurej, 2005; Brenner et al., 2007), most likely the result of

impaired SERT kinetics and reduced ability of platelets to take up and store 5-HT (Fetkovska et al., 1990; Brenner et al., 2007). Brenner et al. specifically showed that PPP 5-HT levels of hypertensive patients are elevated while the platelet-stored 5-HT (platelet rich plasma, PRP) levels are diminished. They also showed decreased expression of SERT protein on the cellular surface of platelets from hypertensive patients in addition to a decreased rate of uptake (V_{max}) (Brenner et al., 2007).

A strong association between elevated plasma levels of 5-HT and angiographically-confirmed coronary artery disease has been observed in human patients (Vikenes et al., 1999). Platelets are known to become activated and aggregate at sites of endothelial injury or atherosclerosis. Tremendous concentrations of 5-HT are capable of being locally released from platelet granules upon activation and contribute to the decreasing coronary vessel lumen size by vasoconstriction. Additionally, as a smooth muscle cell mitogen, cellular proliferation exacerbates the disease (Vikenes, et al., 1999).

An important hallmark of human hypertension and experimental models of hypertension, such as the mineralocorticoid model of hypertension, the DOCA-salt model (deoxycorticosterone acetate), the L-NNA model (N-ω-L-arginine; an inhibitor of nitric oxide synthase), and the spontaneously hypertensive rat (SHR) is a hyperresponsiveness or increased sensitivity of isolated vessels to 5-HT, demonstrated by a left-ward shift of the cumulative dose response curve as compared to normotensive sham tissues (Watts, 1998; Russell et al., 2002).

This increase in sensitivity begins as early as Day 5 in the DOCA-salt rat (Watts, 1998).

Mineralocorticoid hypertension (i.e. rat DOCA-salt experimental model) is dependent on 5-HT_{2B} receptor expression, specifically causing an increase in the 5-HT_{2B} receptor activation, as 5-HT_{2B} receptor antagonism decreases blood pressure (Banes and Watts, 2003; Watts and Fink, 1999). The DOCA-salt model of hypertension is not the only model in which 5-HT receptor function is altered. In the L-NNA hypertensive model, the 5-HT_{2B} receptor expression level and function were increased compared to shams (Russell et al., 2002).

Actions of acute infusions of 5-HT

The response to exogenous 5-HT acutely varies across species, dose ranges, and time of infusion, and the effect is complex. A triphasic response in blood pressure within minutes was observed in the conscious state in dogs and cattle. This triphasic response is shown in Figure 1 and included 1) an initial transient fall in arterial pressure with concurrent bradycardia, then 2) a pressor response, and finally 3) a prolonged depressor response (Page, 1952; Page and McCubbin, 1953; Comroe et al., 1953; Rudolph and Paul, 1956; Emerson, 1968; Linden et al., 1999). In anesthetized cats and rabbits, an intravenous 5-HT infusion caused bradycardia and hypotension (Page and McCubbin, 1953; Comroe et al., 1953). Blood pressure increases result after intravenous adminstration of 5-HT to conscious sheep (Nelson et al., 1987).

Much is unknown to date as to what 5-HT receptors may be responsible for the triphasic response to acute exogenous 5-HT. However, Terrón helped shed some light on the complex response by using selective agonist and antagonists against the cloned 5- HT_7 receptor to implicate that receptor in the long-lasting hypotensive response to 5-HT (1997).

A gap in the knowledge

5-HT is clearly important in the embryological development of a normal cardiovascular system and plays a role in a myriad of adult body systems, including the CNS, gastrointestinal, and cardiovascular systems. Of particular interest are the effects of 5-HT in the peripheral vasculature and in the cardiovascular system as a whole. The contribution of 5-HT to the regulation of systemic blood pressure is controversial and highly disputed. Chronic exogenous 5-HT administration with concomitant blood pressure measurement has not been published in the literature to date. Elucidating the role of 5-HT in the regulation of systemic blood pressure and the mechanism of action is the focus of this study.

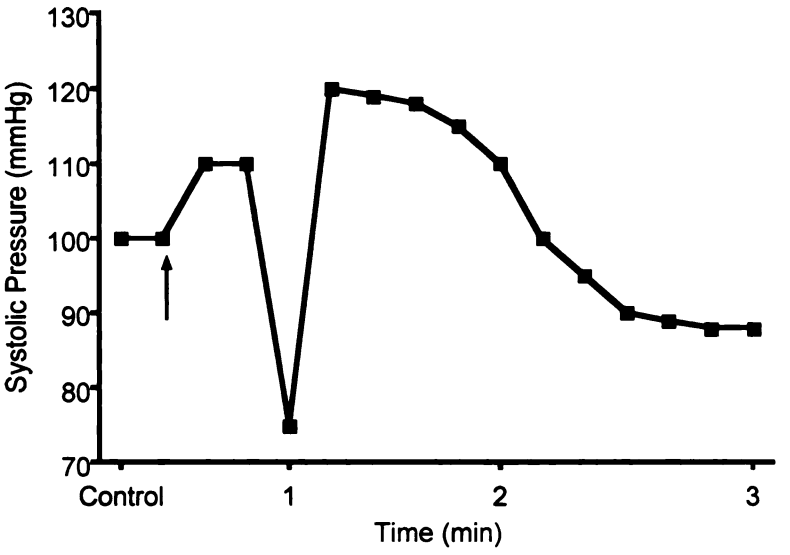


Figure 1

Figure 1.

Effect of 100 μg intravenous 5-HT administered to a conscious dog (arrow indicates time of administration). Systolic blood pressure is shown on the y-axis and time in minutes on the x-axis (adapted from Page, 1952).

Hypotheses:

Hypothesis 1:

Chronic 5-HT infusion will lead to increased blood pressure and arterial 5-HT content in normotensive rats; these responses will be enhanced in DOCA-salt hypertensive rats. Our results led to Hypothesis 2.

Hypothesis 2:

Chronic 5-HT acts to promote the function of nitric oxide synthase (NOS) *in vivo*, and that this effect will not be seen in a model of LNNA hypertension where NOS is inhibited.

Methods:

I. **Animal Use**

All animal procedures were followed in accordance with the institutional guidelines of Michigan State University. Normal male Sprague-Dawley rats (225-250 g) were purchased from Charles River (Portage, MI) for DOCA and $Sham_D$ or Harlan Industries, Inc. (Indianapolis, IN) for L-NNA administration. Rats were kept in clear plastic boxes maintained in dedicated animal rooms kept at 21±2°C and 50±10% humidity under 12:12 hour light/dark cycle with standard rat chow (Teklad®) and tap water provided *ad libitum,* except where stated.

II. **Euthanasia:**

Rats were deeply anesthetized with pentobarbital (60 mg/kg i.p.) prior to inducing bilateral pneumothorax by opening the thoracic cavity and severing aortic arch.

III. Animal Models:

Mineralocorticoid Hypertension:

Male Sprague-Dawley rats (250-300 g; Charles River, Portage, MI) were anesthetized with isoflurane (IsoFlo®) in preparation for left uninephrectomy. The left flank region and upper cervical dorsal region were clipped free of fur and the skin cleaned with povidone iodine solution. Animals were placed in right lateral recumbency, and an incision made perpendicular to the spine and caudal to the last costa. The left kidney was identified, gently exteriorized, and excised after ligation of the left renal artery. A two-layer closure was made, using 6.0 non-absorbable nylon suture to close the abdominal muscle layer, and 4.0 non-absorbable nylon suture to close the skin incision. The animal was then re-positioned to sternal recumbency and a skin incision made on the nape of the neck approximately 1 centimeter caudal to the ears. A Silastic® (Dow Corning, Midland, MI) implant impregnated with DOCA (150 or 200 mg/kg) was placed subcutaneously. The skin was closed with 6.0 non-absorbable nylon suture Post-operatively, the rats were returned to their home cages and examined daily for evidence of redness, swelling, or discharge at the incision sites. Rats were given a solution of 1% NaCl and 0.2% KCl to drink. Sham rats also received a uninephrectomy as described, but received no DOCA implant and drank normal

tap water. Animals were fed standard rat chow and had free access to food and their respective water. The animals remained on this regimen for five weeks. Normotensive sham rats ($Sham_D$) were used as a comparator to DOCA hypertensive rats. At the same time, $Sham_D$ rats underwent uninephrectomy surgery as described for DOCA, but received no DOCA pellet. $Sham_D$ rats were given tap water to drink for the duration of the experiment.

L-NNA Hypertension:

Male Sprague-Dawley rats (250-300 g; Harlan, Indianapolis, IN, USA) were given tap water mixed with Nω-nitro-L-arginine (L-NNA, 0.5 g/L) (Sigma-Aldrich Chemicals, St. Louis, MO, USA). The animals were on this regimen for 17 days. Normotensive sham rats ($Sham_L$) were used as a comparator to DOCA hypertensive rats. $Sham_L$ rats were given tap water to drink for the duration of the experiment.

DOCA plus LNNA Hypertension:

Male Sprague-Dawley rats (Charles River, Portage, MI, USA) underwent uninephrectomy and DOCA pellet implant as described above. On Day 25 post-DOCA surgery, rats were given 1% NaCl, 0.2% KCl, and 0.01 g/L LNNA to drink for an additional 10 days.

IV. Blood Pressure Measurements:

Under isoflurane anesthesia, radiotelemeter devices (Data Sciences International, MN, USA) with attached catheters with pressure-sensing tips were implanted subcutaneously through a 1-1.5 cm incision in the left inguinal area. The left femoral artery was gently separated from the femoral nerve and vein, and gentle tension applied with silk suture to occlude blood flow temporarily. After splashing 1% lidocaine to the vessel to prevent vasospasm, catheters were introduced into the femoral artery 3-5 mm distal to the level of the peritoneal wall, and the pressure-sensing tip was advanced approximately 5 cm to the abdominal aorta. The catheter was ligated in place and the subcutaneous and skin layers were closed with 6.0 and 4.0 non-absorbing nylon suture, respectively. The rats were allowed 3-4 days to recover post-operatively in their home cages with daily monitoring for redness, swelling and/or discharge from the incision sites, and then 3-4 days of baseline measurements were made at a sampling schedule of 10 seconds every 10 minutes. Mean arterial pressure, pulse pressure, heart rate, and rat activity levels were recorded at the same sampling schedule throughout the duration of the experiments.

V. Osmotic Mini-pump Implantation:

One week after radiotelemeter placement and under isolfurane anesthesia, osmotic pumps with a release rate of 10.0 μl/hour and release duration of 7 days (Alzet Osmotic Pumps, Model 2ML1, Durect Corporation, Cupertino, CA, USA) were implanted subcutaneously between the scapulae. Two experimental groups were used: 1) Control group (vehicle pump), 2) 5-HT

(25 μg serotonin creatinine sulfate complex/kg/min) (Sigma-Aldrich Chemicals, St. Louis, MO, USA). On the day of pump implantation, the serotonin creatinine sulfate complex and 1% (w/v) ascorbic acid (as an antioxidant) was fully dissolved in 1N HCl using sonication and vortex, and then pH-balanced to between 6-7 with 4 N NaOH, and finally balanced with distilled water. The solution was then passed through a sterile Millex®-GS syringe driven filter 0.22 μM filter (Millipore, Carrigtwohill, Co. Cork, Ireland) to finally load the osmotic pumps with a 25-gauge blunt-tipped needle provided by Alzet with the osmotic pumps. The solution for the vehicle pumps contained 1% ascorbic acid, a proportional volume of 1N HCl as used for the 5-HT pumps, and pH-balanced to between 6-7 with 4N NaOH, and finally balanced with distilled water. Vehicle pumps were loaded as described with a new syringe, syringe filter, and needle.

The pumps were loaded at room temperature immediately before surgical implantation. Since the pumps were not used in combination with a catheter, nor was it imperative that steady state of 5-HT infusion to be reached immediately, it was not essential to prime them prior to use (*i.e.* load the pumps and then place the tubes in sterile isotonic saline at 37°C for at least 4-6 hours prior to surgical implantation). Subsequently, a continuous rate of infusion was not reached for several hours after surgical implantation *in vivo*.

In addition to serotonin creatinine sulfate complex, an alternate form of 5-HT, 5-HT HCl, was used in one small experiment to verify that the response to 5-HT was due to 5-HT and not the creatinine sufate salt (Sigma-Aldrich Chemicals,

St. Louis, MO, USA). 5-HT HCl was dissolved in water with 1% ascorbic acid to load the osmotic pumps.

At the end of selected experimental protocols, remaining fluid from the osmotic pumps was retrieved, volume recorded, and finally 5-HT concentration was quantified using HPLC to look for evidence of pump function and potential degradation of 5-HT after 7 days within the pump *in vivo*.

VI. Tissue Basal 5-HT Level Measurement:

Thoracic aortae, vena cavae, superior mesenteric arteries, jugular veins, and carotid arteries were removed from pentobarbital-anesthetized normtotensive and hypertensive rats, cleaned of fat, connective tissue, and blood, and placed in 75 μL of 0.05 mM sodium phosphate and 0.03 mM citric acid buffer (pH 2.5) containing 15% methanol. Samples were frozen at -80°C at least four hours prior to HPLC quantification.

VII. 5-HIAA and 5-HT Concentration Measurement from Whole Blood:

In anesthetized rats, 5 ml blood was collected from left cardiac ventricle using a heparinized (1000 U/L) 5 ml syringe and 22 gauge needle. The blood was gently transferred into a 7.0 ml EDTA anticoagulant vacutainer tube. Ten μM pargyline and 10 μM ascorbic acid were added. The EDTA tubes were spun at 160 x g (1000 RPM) for 30 minutes at 4°C for platelet-rich-plasma (PRP). Two ml of supernatant containing plasma and buffy coat layer was gently pipetted into EDTA-coated plastic tubes and mixed gently with a 1:1 dilution of 0.5 M EDTA.

Ten μM pargyline and 10 μM ascorbic acid were added. These tubes were centrifuged for 20 minutes at 1350 x g at 4°C for PPP. To the remaining pellet (platelet layer), 1 ml of platelet buffer and 1 μM ADP was added for PRP. Ten μM pargyline and 10 μM ascorbic acid were added. These tubes were vortexed and allowed to sit on ice for 15 minutes for platelets to become activated and degranulate. Then the tubes were centrifuged at 730 x g for 10 minutes at 4°C. Ten percent (TCA) was added to deproteinize both sets of samples and allowed to sit on ice for 10 minutes. These tubes were centrifuged at 4500 x g for 20 minutes at 4°C. The samples were ultracentrifuged at 280,000 x g for 2 hours. The supernatant was collected, placed into new tubes and the samples were ready for quantification analysis. 5-HT and 5-HIAA concentrations were measured using electrochemical detection high-performance liquid chromatography (HPLC) at 0.4 V and 1.25 ml/min flow rate.

VIII. 5-HIAA and 5-HT Measurement from selected vessels:

Samples were thawed, sonicated for 3 seconds and centrifuged for 1 minute (10,000 x g). Supernatant was collected and transferred to new tubes. Tissue pellets were dissolved in 1.0 M NaOH and assayed for protein. The concentration of 5-HIAA and 5-HT in tissue supernatants was determined by isocratic High Performance Liquid Chromatography (HPLC) coupled with electrochemical detection. Twenty microliters of tissue supernatant was injected onto a C18 reverse phase analytical column (ESA, Bedfore, MA, USA). This column was coupled to a coulometric electrode conditional cell in series with dual

electrode analytical cells (ESA, Bedfore, MA, USA) at 0.4 V and 1.25 ml/min flow rate. 5-HIAA and 5-HT content was determined by comparing peak height in samples with a standard curve and from standards run the same day. Values are reported as a concentration normalized to vessel protein content.

IX. Protein Isolation:

Rat thoracic aortae, vena cavae, superior mesenteric arteries, jugular veins, and carotid arteries were removed from the rat and placed in PSS and cleaned as described above. Arteries were snap-frozen and pulverized in a liquid nitrogen-cooled mortar and pestle and solubilized in lysis buffer [0.5 M Tris HCl (pH 6.8), 10% SDS, 10% glycerol] with protease inhibitors [0.5 mM phenylmethylsulfonyl fluoride, 10 μg/μL aprotinin/10 μg/ml leupeptin, and 0.1 M orthovanadate]. Homogenates were centrifuged (11,000 g for 10 min, 4°C). The Bicinchoninic Acid (BCA) method for protein measurement was used (Sigma, St. Louis, MO). Samples were stored at -80°C until use.

X. BCA Protein Assay:

The bovine serum albumin (BSA) protein standard, consisting of a known concentration of BSA, was utilized to make the standard curve to which the protein samples were compared. The working reagent was made by mixing BCA with copper II sulfate (50:1). To determine the protein concentrations of samples, 5 μL protein from each sample, 95 μL H2O and 2 ml working reagent were mixed and incubated in the absence of carbon dioxide for 30 minutes at 37°C. The

samples were analyzed on a spectrophotometer at an absorbance of 562 nm and the protein concentration determined by plotting these values on the standard curve performed on the same day.

XI. Western Blotting:

eNOS

Tissue homogenates (4:1 in denaturing sample buffer, boiled for 5 min) were separated on SDS-polyacrylamide gels and transferred to Immobilin-FL membrane. Membranes were blocked for 3 hours (Odyssey Blocking Buffer, LI-COR Biosciences, Nebraska, USA) at 4°C. Blots were probed overnight with mouse IgG anti-eNOS/NOS Type III primary antibody (BD Transduction Laboratories) at a dilution of 1:1000 at 4°C, rinsed in TBS-Tween (TBS-T, pH 7.6) (20 mM Tris, 137 mM sodium chloride and 0.1% Tween-20), and incubated with fluorescent anti-mouse 800 secondary antibody for 1 hour in the dark at 4°C following with TBS-T washes in the dark. Blots were directly detected on the LI-COR Odyssey Infrared Fluorescent Imaging System (LI-COR Biosciences, Nebraska, USA).

PECAM-1

Tissue homogenates (4:1 in denaturing sample buffer, boiled for 5 min) were separated on SDS-polyacrylamide gels and transferred to Immobilin-FL membrane. Membranes were blocked for 3 hours (Odyssey Blocking Buffer, LI-COR Biosciences, Nebraska, USA) at 4°C. Blots were probed overnight with mouse IgG anti-PECAM-1 (D-11) primary antibody (Santa Cruz Biotechnology,

Inc.) at a dilution of 1:200 at 4°C, rinsed in TBS-Tween (TBS-T, pH 7.6) (20 mM Tris, 137 mM sodium chloride and 0.1% Tween-20), with a final rinse in TBS (20 mM Tris and 137 mM sodium chloride), and incubated with horseradish perioxidase-associated anti-mouse secondary antibody for 1 hour at 4°C following with TBS-T washes. Blots were incubated with ECL® reagents to visualize the bands.

α-actin

Blots were probed for 2 hours with mouse IgG anti-α-actin primary antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1:5000 at 4°C, rinsed in TBS-Tween (TBS-T, pH 7.6) (20 mM Tris, 137 mM sodium chloride and 0.1% Tween-20), with a final rinse in TBS (20 mM Tris and 137 mM sodium chloride), and incubated with horseradish perioxidase-associated anti-mouse secondary antibody for 1 hour at 4°C following with TBS-T washes. Blots were incubated with ECL® reagents to visualize the bands.

XII. Isolated Smooth Muscle Contractility Measurement:

The thoracic aorta and superior mesenteric artery was removed from pentobarbital-anesthetized rats and submerged in PSS. The aorta was cleaned of fat, connective tissue, and blood and cut into helical strips. Aortic strips were then mounted onto stainless steel rods with silk suture and placed into 50 ml tissue baths for isometric tension recordings using a force transducer and Chart® software program (ADInstruments, Colorado Springs, CO, USA). Strips were placed under previously-determined optimum resting tension (1,500 mg for rat

aorta, 600 mg for rat superior mesenteric artery) and allowed to equilibrate for one hour with frequent washes before exposure to pharmacological compounds. To the best of my ability, in experiments testing tissues from hypertensive rats, one aortic strip isolated from a normotensive control and one aortic strip from a hypertensive rat, or one aortic strip from a rat receiving vehicle and one strip from a rat receiving 5-HT were placed in the same bath, thereby controlling for potential experimental variations. Tissue baths contained warmed (37°C), aerated (95% O_2/CO_2) PSS. Administration of an initial concentration of 10 μM PE was used to test arterial smooth muscle strip viability; for rat aorta, the strips must contract to a minimum of 600 mg to be considered viable. For rat superior mesenteric artery, the strips must contract to a minimum of 200 mg to be considered viable. Cumulative concentration curves to selected agonists were performed.

XIII. Data Analysis and Statistics:

For blood pressure data analysis, 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 4). In all cases, a p-value of <0.05 was considered significant.

All results are presented as mean ± SEM. When comparing two groups,

the appropriate Student's t-test was used. In all cases, a *p* value less than or equal to 0.05 is considered statistically significant.

5-HIAA and 5-HT concentrations from vascular tissue were quantified using a standard curve, and also using standards run the same day, and reported as a concentration normalized to protein content.

Isometric contractions are reported as force (milligrams) or as a percentage of response to maximum contraction to PE. ACh response curves are reported as a percentage of response to half-maximal contraction to PE.

Band density quantitation in Western analyses was performed using NIH Image (v.1.61). For each sample, the densities of the tested bands on Western blotting are normalized to the density of the corresponding actin band.

Results:

Hypothesis 1:

Chronic 5-HT infusion will lead to increased blood pressure and arterial 5-HT content in normotensive rats; these responses will be enhanced in DOCA-salt hypertensive rats.

Validation of model: plasma 5-HT measurments

We wanted to prove that 5-HT concentrations in the circulating plasma as well as the peripheral blood vessels increase with osmotic pump implantation loaded with a 5-HT creatinine sulfate solution in both normotensive sham rats and hypertensive rats. To prove that 5-HT was released from the osmotic pump and reached the systemic circulation, we used HPLC analysis to detect and 5-HIAA and 5-HT content first in the plasma, and then in selected vessels. Figure 2 shows a standard chromatogram of a representative of all standard-mix tracings from HPLC (top) and then a representative chromatogram of basal 5-HT and 5-HIAA in rat aorta (bottom).

To prove that 5-HT was released from the osmotic pump reaching the systemic circulation and remained viable throughout the duration of the infusion, whole blood was collected at the time of rat sacrifice after 7 days of 5-HT or vehicle infusion. We used HPLC to quantify 5-HT in both platelet-poor and platelet-rich plasma from uninephrectomized normotensive sham rats, designated $Sham_D$, shown in Figure 3. 5-HT concentrations are increased in free circulating plasma (platelet-poor plasma, PPP) in the $Sham_D$ 5-HT-infused group,

47.1±23.2 ng/ml plasma, as compared to the $Sham_D$ Vehicle-infused group, 2.7±0.3 ng/ml plasma, an increase of 17.4 fold. The 5-HT contained within the granules of platelets (platelet-rich plasma, PRP) increased in the $Sham_D$ 5-HT-infused group, 257.7±40.2 ng/ml plasma, as compared to the $Sham_D$ Vehicle-infused group, 31.9±9.7 ng/ml plasma, an increase of 8 fold.

Figure 4 shows the increased 5-HT levels in both components of plasma in DOCA rats after 7 days of 5-HT as compared to Vehicle infusion. 5-HT concentrations are increased in PPP in the DOCA 5-HT-infused group, 114.5±21.1 ng/ml plasma, as compared to the DOCA Vehicle-infused group, 24.9±5.0 ng/ml plasma, a 4.5 fold increase. The PRP 5-HT concentration increased in the DOCA 5-HT-infused group, 591.3±212.5 ng/ml plasma, as compared to the DOCA Vehicle-infused group, 137.9±35.35 ng/ml plasma, a 4.2 fold increase. Plasma measurements of 5-HT using HPLC indicated that the 5-HT administered *via* osmotic pump was in fact released and reached the systemic circulation.

Validation of model: peripheral vascular tissue 5-HT measurements

The quantification of basal 5-HIAA and 5-HT content levels in selected $Sham_D$ rat vessels harvested on Day 7 of Vehicle or 5-HT infusion are reported in Figure 5. As arterial smooth muscle cells have a functional SERT, we expected an increase in basal 5-HIAA and 5-HT content in those vessels harvested from 5-HT infused $Sham_D$ rats. As 5-HT is rapidly metabolized by the action of monoamine oxidase, and we have not inhibited the metabolism of 5-HT in this

experiment, we cannot forget to examine the metabolite 5-HIAA. In aorta, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused $Sham_D$ (5-HIAA: 1.8±0.8 ng/mg protein; 5-HT: 0.5±0.4 ng/mg protein, n=4) as compared to $Sham_D$ Vehicle-infused (5-HIAA: 0.4±0.2 ng/mg protein; 5-HT: 0.3±0.2 ng/mg protein, n=4). In carotid artery, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused $Sham_D$ (5-HIAA: 3.1±0.8 ng/mg protein; 5-HT: 4.8±1.7 ng/mg protein, n=4) as compared to $Sham_D$ Vehicle-infused (5-HIAA: 1.0±0.2 ng/mg protein; 5-HT: 0.4±0.1 ng/mg protein, n=4). In the superior mesenteric artery, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused $Sham_D$ (5-HIAA: 4.7±2.1 ng/mg protein; 5-HT: 3.6±0.4 ng/mg protein, n=4) as compared to $Sham_D$ Vehicle-infused (5-HIAA: 0.5±0.3 ng/mg protein; 5-HT: 0.9±0.8 ng/mg protein, n=4). In veins, we observe a new phenomenon; the 5-HT measured in veins does not seem to be as rapidly metabolized to 5-HIAA as it is in arteries. Thus, we can measure higher 5-HT content in veins as compared to arteries, even under basal conditions, and less 5-HIAA content in veins as compared to arteries. In the vena cava, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused $Sham_D$ (5-HIAA: 3.5±0.5 ng/mg protein; 5-HT: 27.0±9.9 ng/mg protein, n=4) as compared to $Sham_D$ Vehicle-infused (5-HIAA: 0.6±0.1 ng/mg protein; 5-HT: 13.9±0.4 ng/mg protein, n=4). Similarly, in the jugular vein, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused $Sham_D$ (5-HIAA: 3.1±0.9 ng/mg protein; 5-HT: 39.1±11.6

ng/mg protein, n=4) as compared to $Sham_D$ Vehicle-infused (5-HIAA: 0.4±0.1 ng/mg protein; 5-HT: 8.9±3.1 ng/mg protein, n=4).

5-HIAA and 5-HT values from selected DOCA rat vessels harvested on Day 7 of Vehicle or 5-HT infusion are shown in Figure 6. We again observed a difference in 5-HT uptake in veins versus arteries. In aorta, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused DOCA (5-HIAA: 1.4±0.1 ng/mg protein; 5-HT: 4.2±1.1 ng/mg protein, n=4) as compared to DOCA Vehicle-infused (5-HIAA: 0.1±0.1 ng/mg protein; 5-HT: 0.6±0.1 ng/mg protein, n=4). In carotid artery, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused DOCA (5-HIAA: 2.5±0.9 ng/mg protein; 5-HT: 6.7±2.8 ng/mg protein, n=4) as compared to DOCA Vehicle-infused (5-HIAA: 0.2±0.1 ng/mg protein; 5-HT: 1.8±0.4 ng/mg protein, n=4). In the superior mesenteric artery, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused DOCA (5-HIAA: 2.1±0.3 ng/mg protein; 5-HT: 4.9±2.4 ng/mg protein, n=4) as compared to DOCA Vehicle-infused (5-HIAA: 0.4±0.1 ng/mg protein; 5-HT: 1.1±0.3 ng/mg protein, n=4). Again, in veins, we observe the 5-HT measured in veins does not seem to be as rapidly metabolized to 5-HIAA as it is in arteries. In the vena cava, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused DOCA (5-HIAA: 2.7±0.6 ng/mg protein; 5-HT: 31.7±11.4 ng/mg protein, n=4) as compared to DOCA Vehicle-infused (5-HIAA: 0.2±0.1 ng/mg protein; 5-HT: 10.6±0.9 ng/mg protein, n=4). Similarly, in the jugular vein, we observed an increase in 5-HIAA content as well as 5-HT content in the 5-HT infused DOCA (5-HIAA: 3.6±0.7 ng/mg

protein; 5-HT: 184.4±47.2 ng/mg protein, n=4) as compared to DOCA Vehicle-infused (5-HIAA: 0.2±0.1 ng/mg protein; 5-HT: 6.5±0.5 ng/mg protein, n=4). This increase in 5-HT and 5-HIAA content measured in tissues harvested from rats receiving 5-HT *via* infusion indicates that the 5-HT is transported somehow and does get to the peripheral vasculature, where SERT on the smooth muscle cells transports the 5-HT intracellularly. It is an interesting observation to see a difference in distribution of 5-HT and metabolite content between arteries and veins, as it appears that most of the 5-HT is rapidly metabolized in arteries, but seems to be preserved as 5-HT in the veins.

DOCA-salt hypertension: Effect of 5-HT on telemetric measurements:

Figure 7 shows the 24-hour averaged MAP of both $Sham_D$ and DOCA rats with 2 days of control baseline measurements and 7 days of 5-HT or Vehicle infusion. MAP started to fall within six hours of 5-HT release from the osmotic pumps, and reached a nadir at Day 2 of 5-HT infusion in both normotensive $Sham_D$ and hypertensive DOCA rats. The DOCA rats infused with 5-HT, had a resting baseline MAP of 166.5 ± 7.6 mmHg during the control period, and experienced a maximal MAP fall of -53.7 mmHg at Day 2, with a MAP of 112.8 ± 2.8 mmHg. The normotensive $Sham_D$ rats infused with 5-HT had a resting baseline MAP of 100.8 ± 2.4 mmHg, and experienced a maximal MAP fall of -21.6 mmHg at Day 2, with a MAP of 79.2 ± 1.5 mmHg.

Osmotic pump function and 5-HT viability after 7 days of infusion:

We measured the volume of the fluid remaining in the mini-osmotic pump after 7 days of infusion in the DOCA and the $Sham_D$ rats. Each mini-pump starting volume was 2 ml, and as the top graph in Figure 8 shows, there was no statistical difference in the remaining volume between the four groups, indicating the pumps released virtually equal amounts of either 5-HT or Vehicle at the end of 7 days of infusion. We measured the concentration of the 5-HT from the fluid remaining in the osmotic pumps at the end of 7 days of *in vivo* infusion, and the quantification is shown in the middle graph on Figure 8. The Vehicle pump samples contained 0 ng/ml 5-HT both prior to infusion and after pump removal from the rat as confirmed by HPLC (n=12). A representative chromatogram from a sample retrieved from a Vehicle pump is shown in the top of Figure 9. The HPLC quantification of the 5-HT fluid sampled before implant was 20.7 μg/ml. Post-explant from the rat after 7 days of infusion, a sample of fluid from each of the 5-HT pumps was quantified using HPLC and contained an average of 17.8±0.6 μg/ml (n=18). The 5-HT pump fluid was so concentrated that the sample had to be diluted down $1:10^6$ to be amenable to detection on HPLC. A representative chromatogram from a diluted sample retrieved from a 5-HT pump is shown in the bottom of Figure 9.

We also tested the vasoactivity of the 5-HT in the remaining fluid from the osmotic pump after 7 days of use in a rat. An aliquot of this fluid (5 μl of 17.4 μg/ml) was added to an isometric contractility tissue bath to challenge a ring of aorta. The bottom graph in Figure 8 shows the response of the aortic ring. A

robust tissue contraction of over 6000 mg was observed, indicating the sustained vasoactivity of 5-HT even after 7 days at body temperature.

5-HT HCl infusion:

We repeated this 5-HT infusion with another formulation of 5-HT, 5-HT hydrochloride, in a small experiment to show that this effect on blood pressure is in fact due to 5-HT itself and was not attributable to the creatinine sulfate complex conjugated to the 5-HT molecule. The averaged 24-hour MAP of 5-HT HCl infusion in DOCA rats is plotted with DOCA Vehicle and DOCA 5-HT (serotonin creatinine sulfate complex) and is shown in Figure 10. This indicates that the profound decrease in MAP to 5-HT remains the same, regardless of the formulation of the compound. As a result of this experiment, we utilized the 5-HT creatinine sulfate complex formulation in all of the subsequent experiments.

Effect of 5-HT on DOCA-salt water intake:

We observed that the DOCA rats receiving 5-HT greatly reduced their salt water consumption. We hypothesized that the blood pressure fall may result, at least in part, from a decreased salt intake as compared to DOCA Vehicle rats, whose salt-water intake did not change. The top graph in Figure 11 shows a grouped 7-day average of fluid consumption in both $Sham_D$ and DOCA rats. It is important to point out that the $Sham_D$ rats are consuming tap water, and the DOCA rats are consuming water supplemented with 1% NaCl and 0.2% KCl. There was no statistical difference in the $Sham_D$ rats when 5-HT is administered,

but the salt-water consumption by the DOCA rats receiving 5-HT was decreased to nearly half the consumption of DOCA Vehicle rats. We did one experiment when we restricted the salt-water consumption of the DOCA Vehicle rats based on how much the DOCA 5-HT rats drank. The 24-hour-grouped MAP of the DOCA Vehicles was not significantly altered (a 7 mmHg drop in MAP) when their salt-water consumption was restricted to about half of what they would have consumed *ad libitum*. Thus, reduction of salt-intake, at least beyond Day 28 of DOCA does not decrease blood pressure on its own. The mechanism by which 5-HT alters salt appetite is unknown, and has not been investigated further at this point.

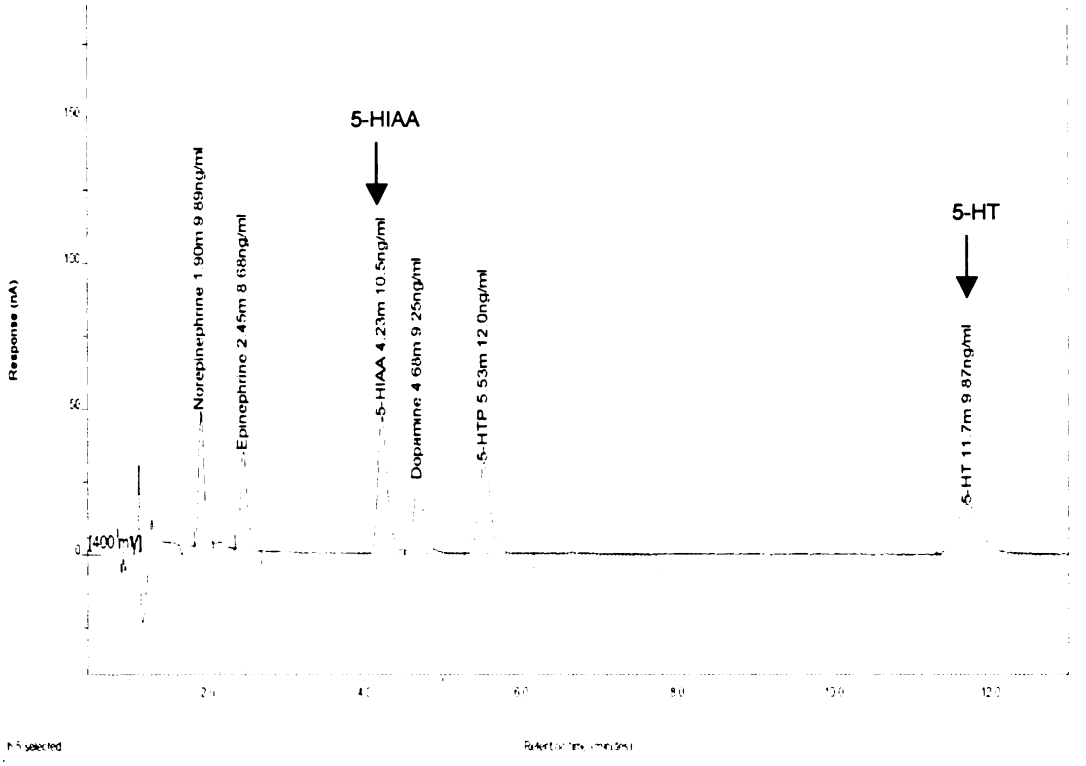
Figure 2.

Top: Chromatogram showing separation of 10 ng standards using HPLC.

Bottom: Detection of basal levels of 5-HIAA of 5-HT in thoracic aorta.

5-HIAA = 5-hydroxyindoleacetic acid. 5-HT = 5-hydroxytryptamine

6-Mix Standard



Rat aorta sample

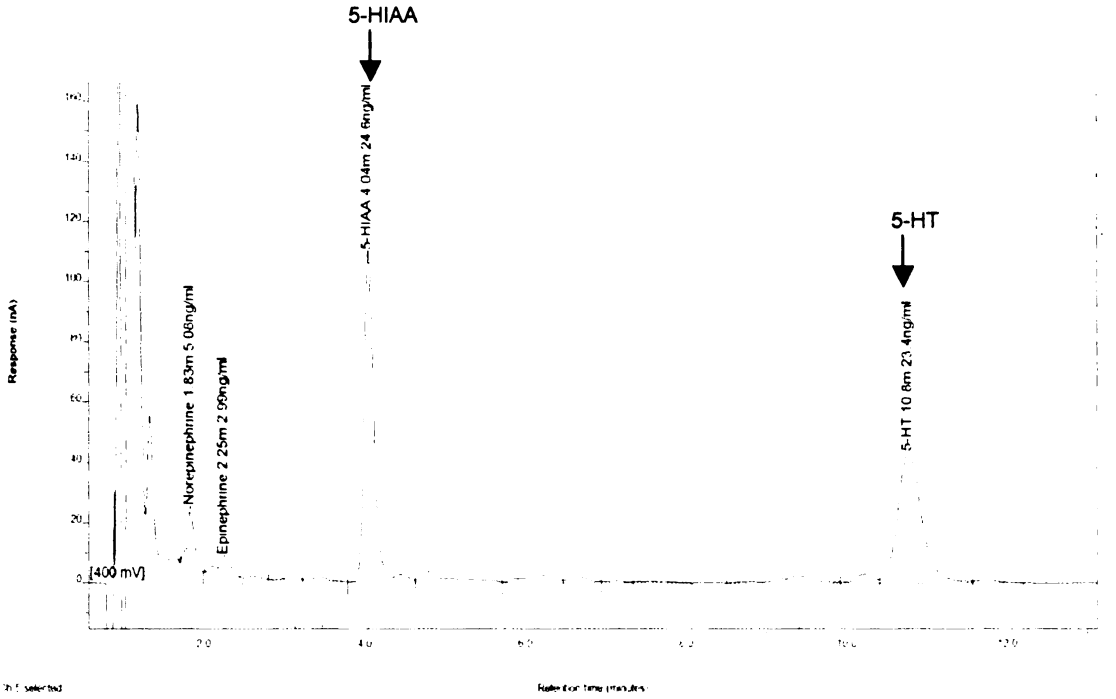


Figure 2

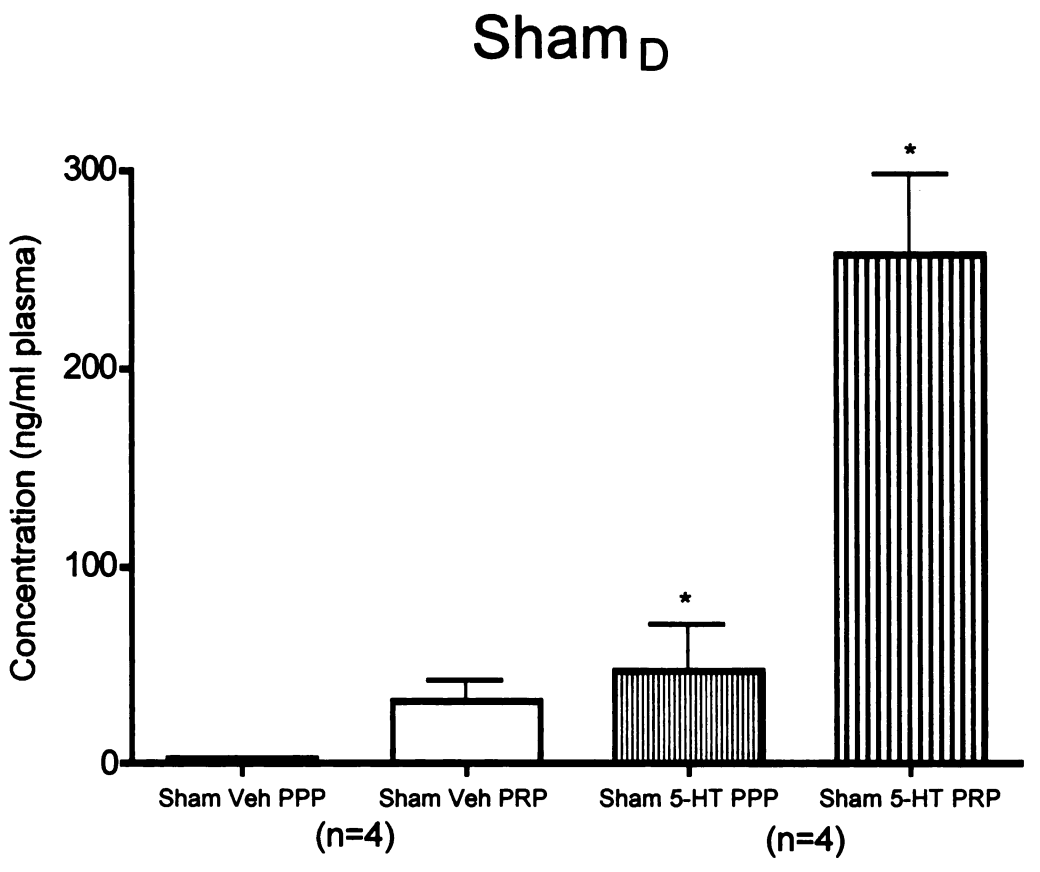


Figure 3

Figure 3.

5-HT quantification from whole blood from $Sham_D$ rats separated into components; that which is freely circulating in the plasma (PPP) and that which is contained within the granules of platelets (PRP). Bars represent means ± SEM for the number of rats in parentheses.

*$p<0.05$ compared to Vehicle.

PPP=Platelet-poor plasma, PRP=Platelet-rich plasma

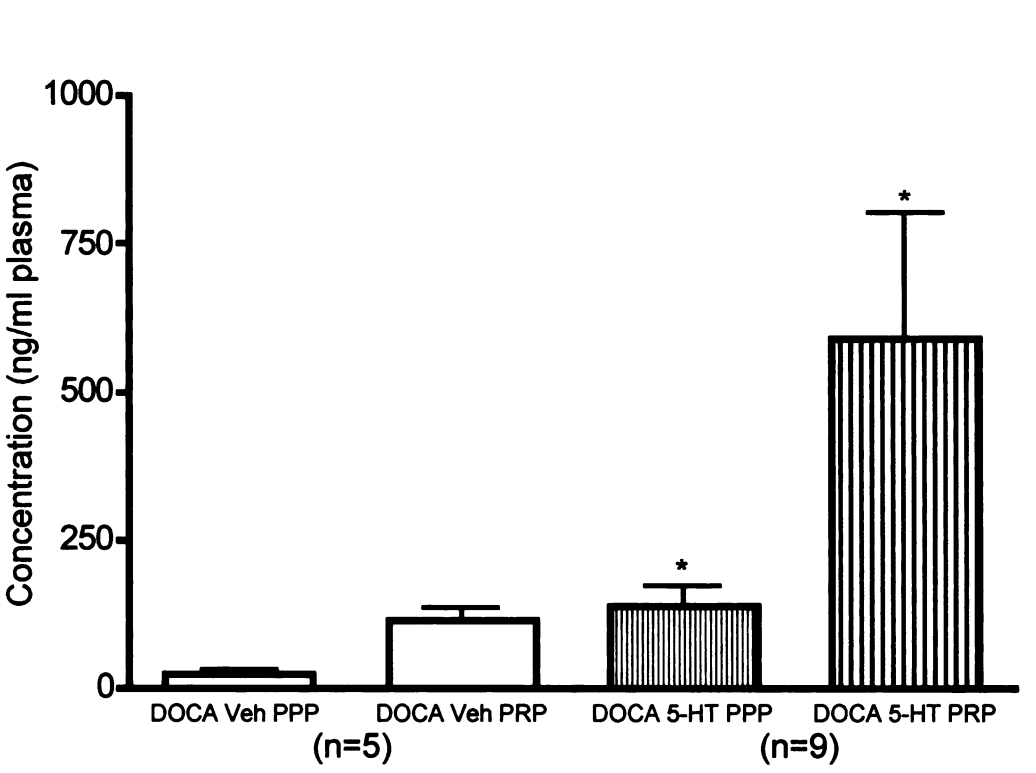


Figure 4

Figure 4.

5-HT quantification from whole blood from DOCA rats separated into components; that which is freely circulating in the plasma (PPP) and that which is contained within the granules of platelets (PRP). Bars represent means ± SEM for the number of rats in parentheses.

*$p<0.05$ compared to Vehicle.

PPP=Platelet-poor plasma, PRP=Platelet-rich plasma.

Figure 5.

Basal 5-HT and 5-HIAA levels in $Sham_D$ Vehicle and $Sham_D$ 5-HT in rat aorta, carotid artery, superior mesenteric artery, vena cava, jugular vein harvested on Day 7 of Vehicle or 5-HT infusion.

*$p<0.05$ compared to Vehicle.

5-HIAA = 5-hydroxyindoleacetic acid. 5-HT = 5-hydroxytryptamine

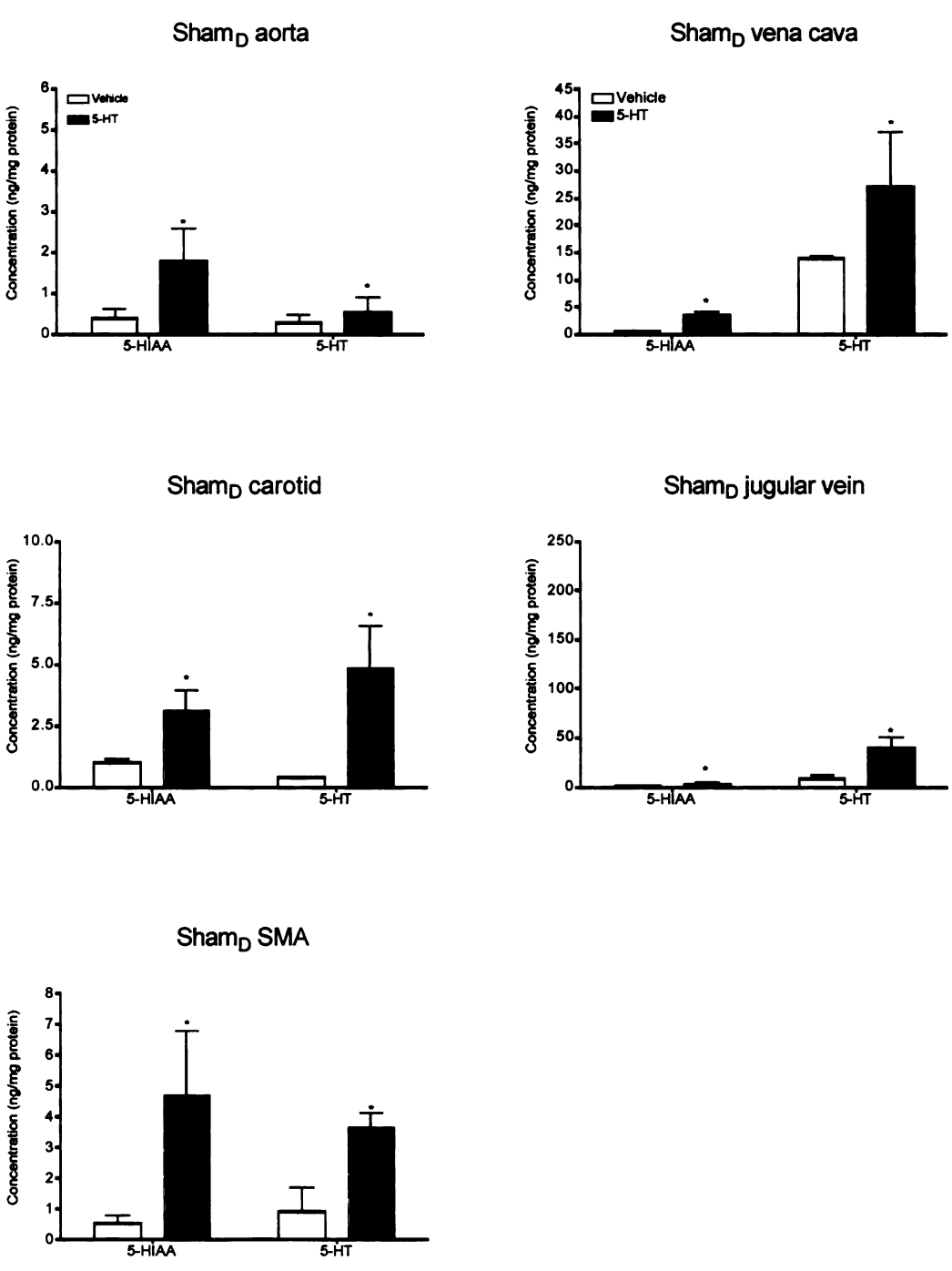

Sham$_D$ aorta
Vehicle
5-HT
Concentration (ng/mg protein)
5-HIAA
5-HT
Sham$_D$ vena cava
Vehicle
5-HT
Concentration (ng/mg protein)
5-HIAA
5-HT
Sham$_D$ carotid
Concentration (ng/mg protein)
5-HIAA
5-HT
Sham$_D$ jugular vein
Concentration (ng/mg protein)
5-HIAA
5-HT
Sham$_D$ SMA
Concentration (ng/mg protein)
5-HIAA
5-HT

Figure 5

Figure 6.

Basal 5-HT and 5-HIAA levels in DOCA Vehicle and DOCA 5-HT in rat aorta, carotid artery, superior mesenteric artery, vena cava, jugular vein harvested on Day 7 of Vehicle or 5-HT infusion.

*$p<0.05$ compared to Vehicle.

5-HIAA = 5-hydroxyindoleacetic acid. 5-HT = 5-hydroxytryptamine

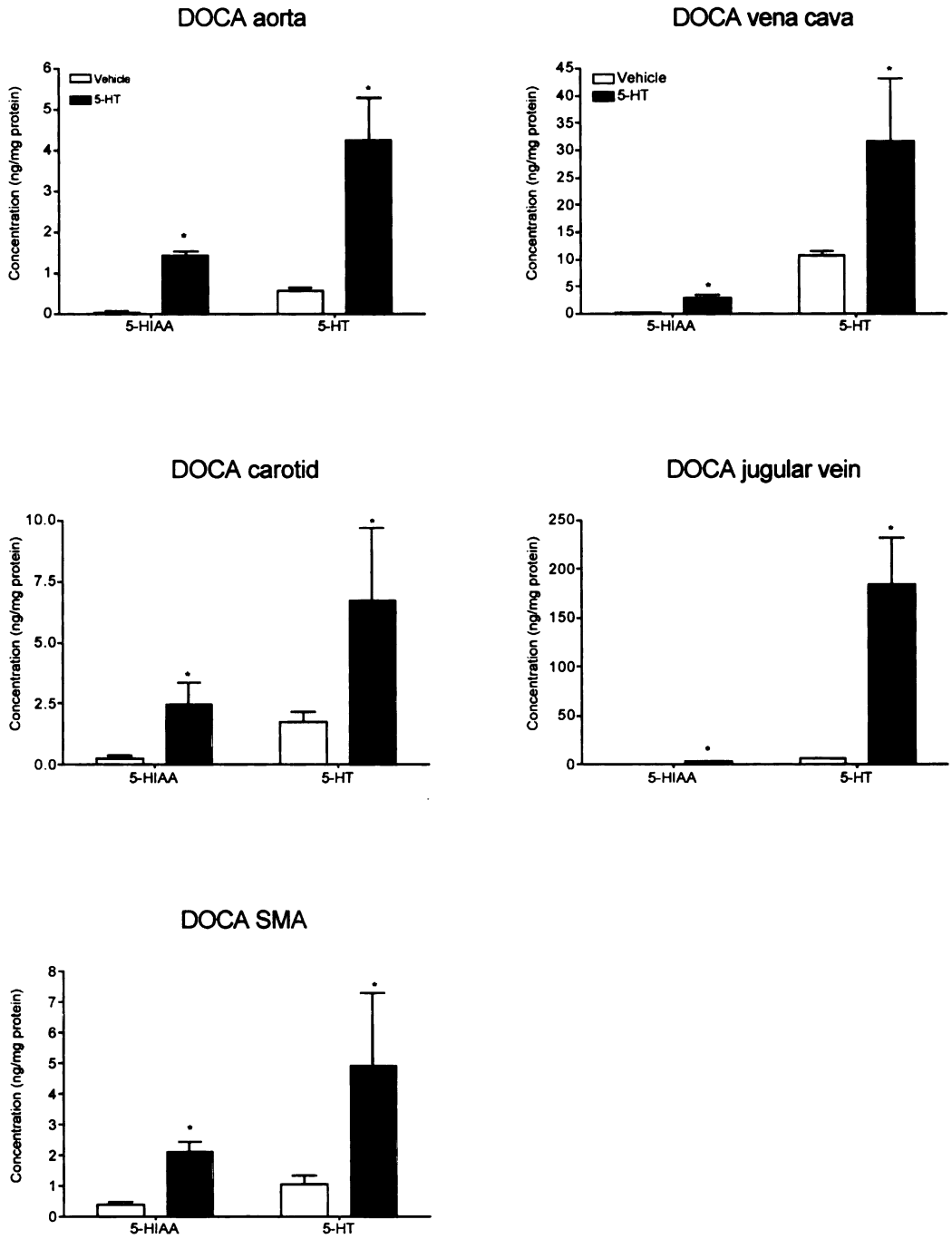

DOCA aorta
Vehicle
5-HT
Concentration (ng/mg protein)
5-HIAA
5-HT
DOCA vena cava
DOCA carotid
DOCA jugular vein
DOCA SMA

Figure 6

Figure 7.

Top: MAP measurements of DOCA hypertensive and normotensive $Sham_D$ rats after 2 days of baseline measurements, then 7 subsequent days of 5-HT or Vehicle infusion.

Middle: Heart rate measurements (bpm) of DOCA hypertensive and normotensive $Sham_D$ rats after 2 days of baseline measurements, then 7 subsequent days of 5-HT or Vehicle infusion.

Bottom: Activity level of DOCA hypertensive and normotensive $Sham_D$ rats after 2 days of baseline measurements, then 7 subsequent days of 5-HT or Vehicle infusion.

Points represent 24-hour averages of the groups ± SEM for the number of rats in parentheses. **$p<0.01$ versus control period average, #$p<0.05$ versus Vehicle.

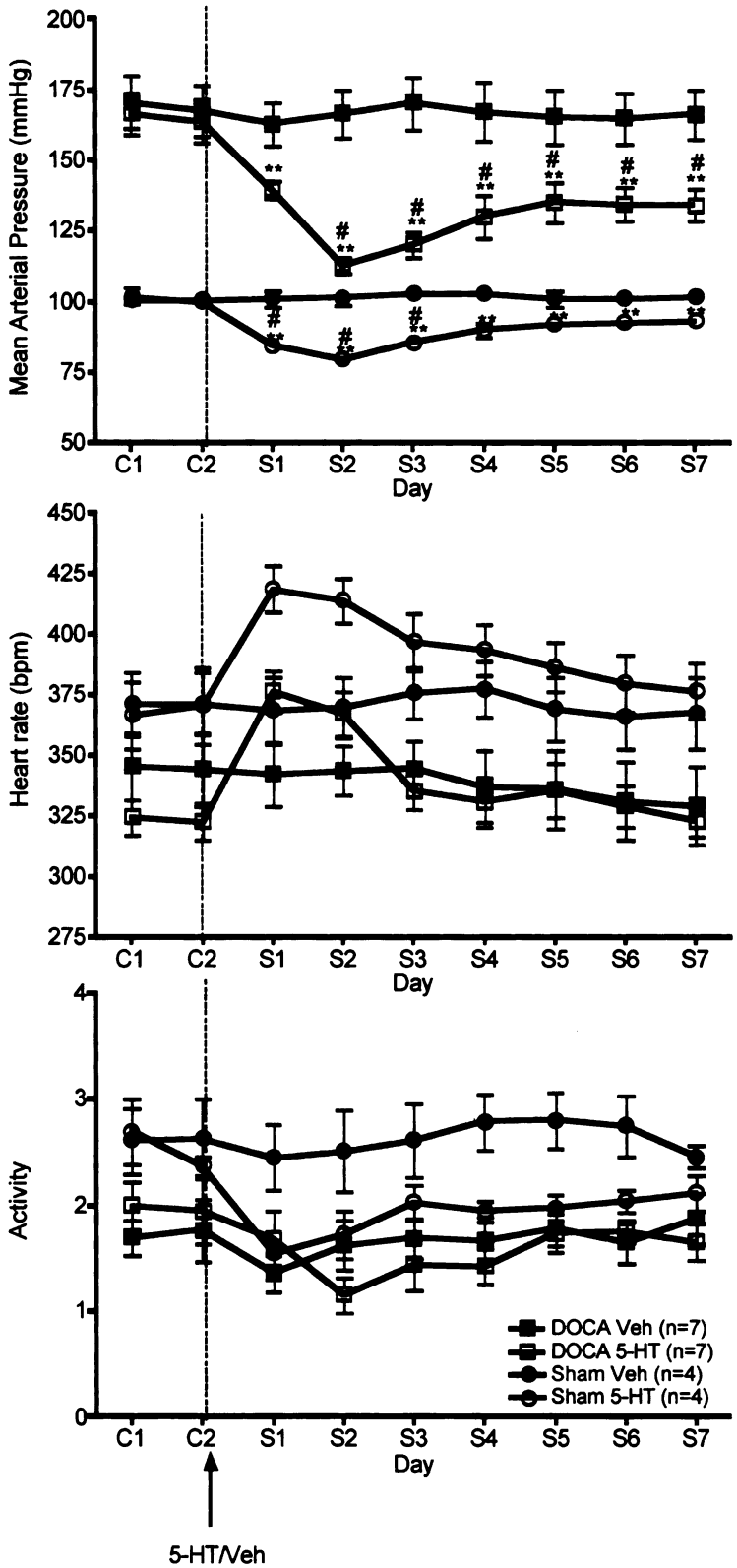

Mean Arterial Pressure (mmHg)
200
175
150
125
100
75
50
C1
C2
S1
S2
S3
S4
S5
S6
S7
Day
Heart rate (bpm)
450
425
400
375
350
325
300
275
Activity
4
3
2
1
0
DOCA Veh (n=7)
DOCA 5-HT (n=7)
Sham Veh (n=4)
Sham 5-HT (n=4)
5-HT/Veh

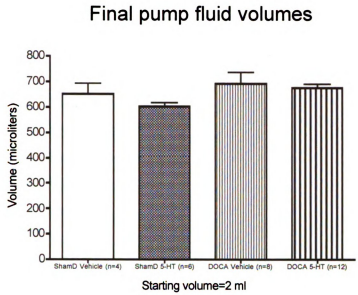
Figure 7

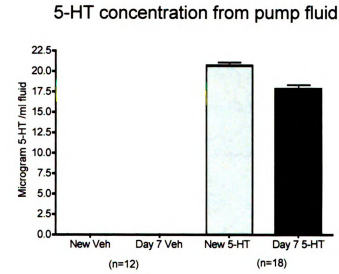
Figure 8.

Top: Group averaged values for fluid volumes extracted from a mini-osmotic pump after 7 days of *in vivo* use in a $Sham_D$ and DOCA rats. Starting volume for all pumps was 2 ml.

Middle: HPLC quantification of 5-HT in the fluid extracted from a mini-osmotic pump after 7 days of *in vivo* use in a DOCA rat.

Bottom: A tracing of the preserved vasoactivity of 5-HT in the fluid extracted from a mini-osmotic pump after 7 days of *in vivo* use in a DOCA rat. Arrow indicates addition of pump fluid to bath.


Final pump fluid volumes
Volume (microliters)
800
700
600
500
400
300
200
100
0
ShamD Vehicle (n=4)
ShamD 5-HT (n=6)
DOCA Vehicle (n=8)
DOCA 5-HT (n=12)
Starting volume=2 ml


5-HT concentration from pump fluid
Microgram 5-HT /ml fluid
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
New Veh
Day 7 Veh
New 5-HT
Day 7 5-HT
(n=12)
(n=18)

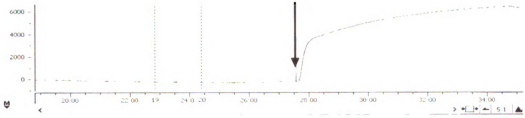

6000
4000
2000
0
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00

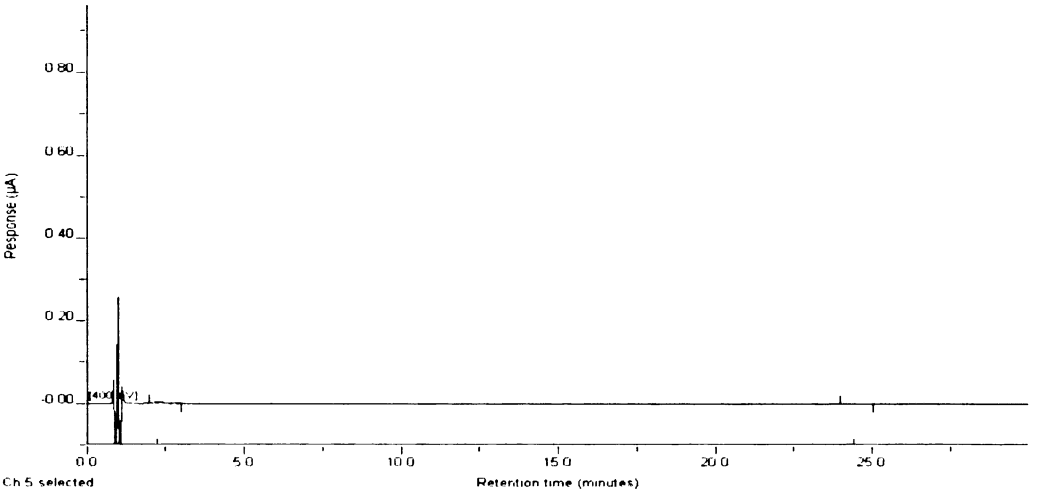
Figure 8

Figure 9.

Top: Representative chromatogram showing a lack of 5-HT in a sample of remaining fluid retrieved from a Vehicle pump after 7 days of *in vivo* infusion.

Bottom: Representative chromatogram showing the ability to detect and quantify 5-HT diluted $1:10^6$ in a sample of remaining fluid retrieved from a 5-HT pump after 7 days of *in vivo* infusion.

Vehicle pump fluid



5-HT pump fluid diluted 1:10^6

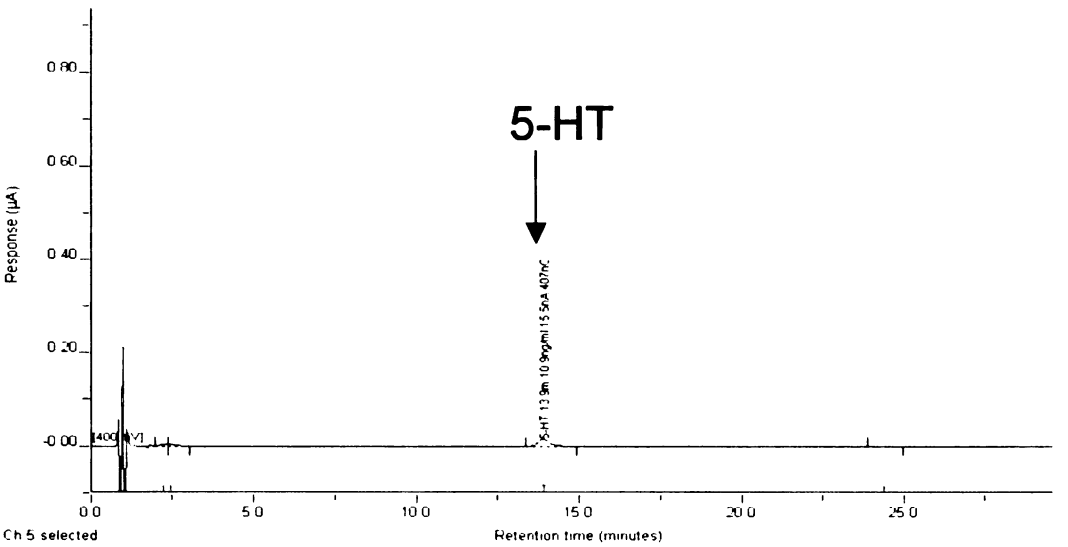


Figure 9

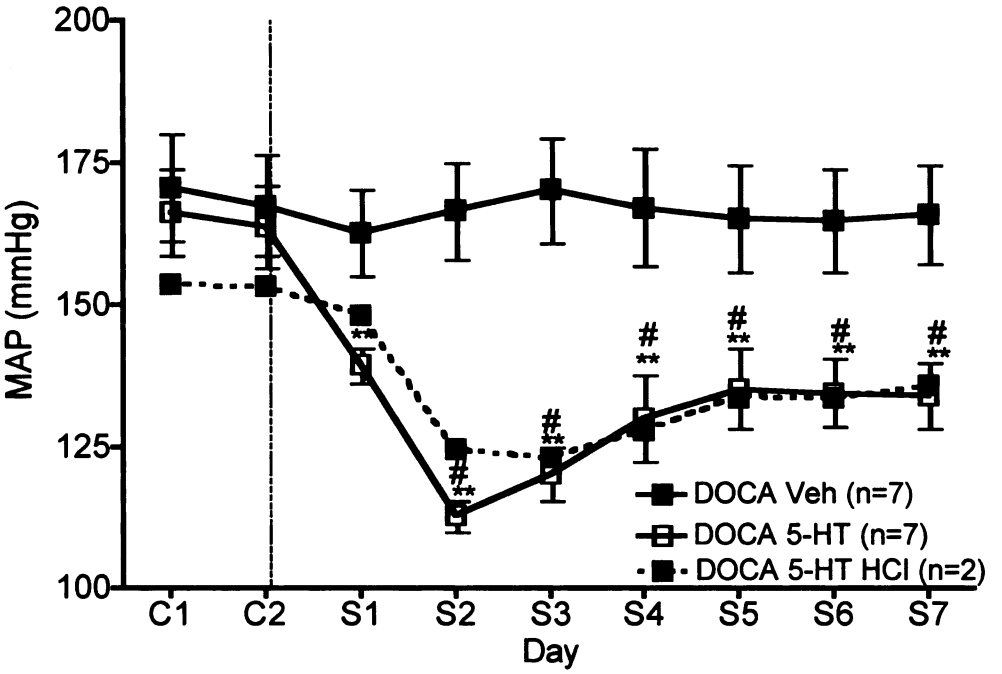


Figure 10

Figure 10.

MAP measurements of DOCA 5-HT HCl infusion, plotted against DOCA Vehicle and DOCA 5-HT creatinine sulfate complex infusion. There are 2 days of baseline recordings, then 7 subsequent days of 5-HT creatinine sulfate complex, 5-HT HCl, or Vehicle infusion. Points represent 24-hour averages of the groups ± SEM for the number of rats in parentheses. **p<0.01 versus control period average, #p<0.05 versus Vehicle.

Figure 11.

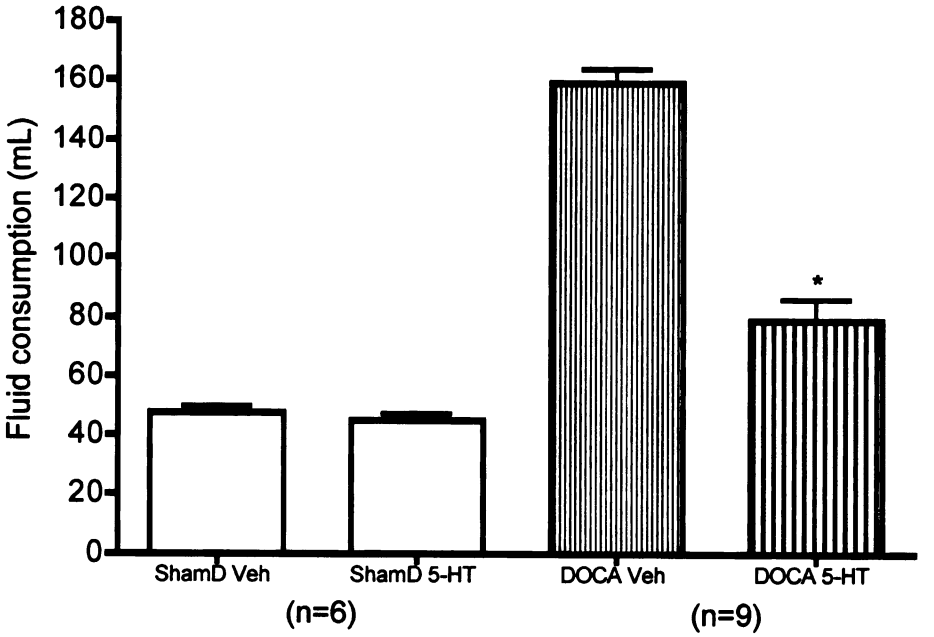
Top: Group-averaged 7-day post-pump fluid consumption comparing both $Sham_D$ and DOCA Vehicle versus 5-HT. It is important to note that $Sham_D$ rats are consuming tap water, while DOCA rats are consuming water supplemented with 1% NaCl + 0.2% KCl.

*$p<0.05$ compared to Vehicle.

Bottom: Effect of restricting DOCA-salt water on the MAP of DOCA Vehicle rats.

WR= DOCA-salt Water Restricted

**$p<0.01$ versus control period average, #$p<0.05$ versus Vehicle.


Fluid consumption (mL)
180
160
140
120
100
80
60
40
20
0
*
ShamD Veh
ShamD 5-HT
DOCA Veh
DOCA 5-HT
(n=6)
(n=9)

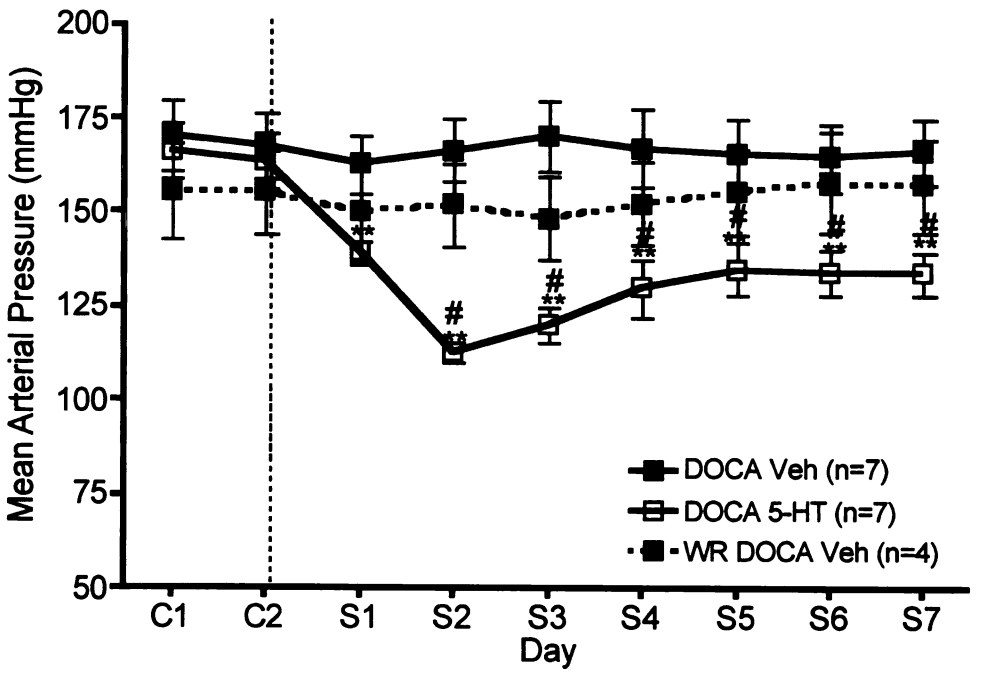

Mean Arterial Pressure (mmHg)
200
175
150
125
100
75
50
C1
C2
S1
S2
S3
S4
S5
S6
S7
Day
DOCA Veh (n=7)
DOCA 5-HT (n=7)
WR DOCA Veh (n=4)

Figure 11

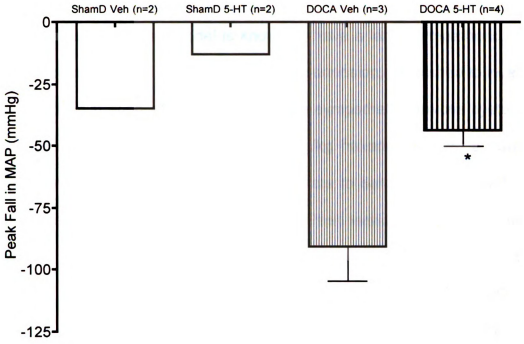


Figure 12

Figure 12.

Peak response in MAP to acute intraperitoneal injections of hexamethonium (30 mg/kg) on Day 4 of 5-HT or Vehicle pump infusion in conscious DOCA or $Sham_D$ rats. $p<0.05$ compared to Vehicle.

Veh=Vehicle pump-infused, 5-HT=5-HT pump-infused.

Effect of hexamethonium:

As the DOCA-salt model is known to have a large sympathetic nervous system component to the development of hypertension, we wished to investigate if 5-HT administration altered the role of the sympathetic nervous system. We accomplished this by administering 30 mg/kg hexamethonium, a ganglionic blocker, intraperitoneally in the conscious state to both the DOCA and $Sham_D$ rats on Day 4 of Vehicle or 5-HT infusion. Peak responses to hexamethonium are shown in Figure 12, with DOCA 5-HT-infused rats experiencing a peak response of -43.4±6.5 mmHg, compared to a peak response of -90.6±14.0 mmHg in the DOCA Vehicle rats. Thus, there is a difference in effect in the sympathetic nervous system between the Vehicle and 5-HT treatment groups in DOCA, and we observe a similar trend in the normotensive $Sham_D$ (n=2).

Isolated tissue contractility:

Aortae and superior mesenteric arteries were harvested from both $Sham_D$ Vehicle-infused, $Sham_D$ 5-HT-infused, as well as DOCA Vehicle-infused and DOCA 5-HT-infused rats on Day 7 of infusion for isometric contractility experiments. Cumulative concentration response curves to PE, ACh, and 5-HT were performed on the isolated vessels. Data from $Sham_D$ rat aorta are shown in Figure 13, and data from $Sham_D$ rat superior mesenteric artery are shown in Figure 14. In both aorta and superior mesenteric artery, we observed no difference in the initial maximal adrenergic stimulation with PE, nor did we observe a difference in the PE concentration response curve, indicating no

appreciable change in the general vasoactivity of the $Sham_D$ vessels with chronic exposure 5-HT as compared to $Sham_D$ Vehicle tissues. It is interesting to note that in my hands there was no evidence of desensitization to 5-HT in the isolated vessels harvested from animals chronically exposed to 5-HT *via* 5-HT infusion. The response to ACh was not statistically different between the 5-HT or Vehicle-infused aorta and superior mesenteric artery from $Sham_D$ rats.

Data from DOCA rat aorta are shown in Figure 15, and data from DOCA rat superior mesenteric artery are shown in Figure 16. As with the $Sham_D$ rats, there was no difference in the initial maximal adrenergic stimulation with PE, nor did we observe a difference in the PE concentration response curve, indicating no change in the general vasoactivity of the DOCA vessels with chronic exposure 5-HT as compared to DOCA Vehicle tissues. There is a statistically significant difference in the response to ACh; we observed a 39.7±14.2% endothelium- and NO-dependent relaxation in the DOCA Vehicle aorta tissue, and the DOCA 5-HT aorta experienced a 69.2±9.1% endothelium- and NO-dependent relaxation. This is a striking difference, as pure DOCA arterial tissues are well known to have pronounced endothelial dysfunction and markedly impaired relaxation to ACh, and suggests that 5-HT is somehow preserving either the endothelial cell function or the endothelial cells themselves. It is surprising that we did not observe the same preservation of relaxation ability to ACh in the DOCA superior mesenteric arteries, and it is unknown why this occurs. In my hands with these particular tissues, the cumulative response curves to PE and 5-HT are not ideal sigmoid response curves as one might expect in an isolated tissue bath, so it is

possible the smooth muscle cells and/or endothelial cells were impaired because of my user error in the preparation and placement of the tissues within the bath. It is important to note that these superior mesenteric arteries were harvested from the same animals as the aorta, placed in the tissue baths at the same time, and exposed to the exact same agonists at the same time.

Role of NO

Based on the finding that aortae isolated from DOCA rats had improved relaxation to ACh after chronic exposure to 5-HT as compared to Vehicle, we wished to investigate the role of NOS as a potential mechanism for the blood pressure fall. We probed for eNOS protein in aortic tissue in a Western blot analysis, and we saw higher expression of eNOS in these DOCA rats receiving 5-HT. The blot is shown in Figure 17.

We also probed for platelet/endothelial cell adhesion molecule-1 (PE-CAM-1), a marker for endothelial cells. The Western blot analysis indicates that likely the endothelial cell numbers are similar based on equal expression of PE-CAM-1 in aortic homogenates between Vehicle-infused and 5-HT-infused.

L-NNA hypertension: plasma 5-HT measurements

To further investigate the role of NOS, we used L-NNA as a NOS inhibitor prior to 5-HT or Vehicle administration. L-NNA was administered *via* the drinking water as a model of hypertension, and those rats receiving the drug are designated as L-NNA, while the normotensive comparator not receiving L-NNA

are designated $Sham_L$. Figure 18 shows that both free and platelet-bound 5-HT levels increased in the plasma with 5-HT administration in the $Sham_L$ rats. 5-HT concentrations are increased in PPP in the $Sham_L$ 5-HT-infused group, 190.7±23.1 ng/ml plasma, as compared to the $Sham_L$ Vehicle-infused group, 67.9±6.3 ng/ml plasma, a 2.8 fold increase. The PRP 5-HT concentration increased in the $Sham_L$ 5-HT-infused group, 801.1±132.8 ng/ml plasma, as compared to the $Sham_L$ Vehicle-infused group, 224.0±78.8 ng/ml plasma, a 3.6 fold increase.

Figure 19 shows plasma data for L-NNA rats and shows that both free and platelet-bound 5-HT levels increased in the plasma with 5-HT administration. 5-HT concentrations are increased in PPP in the L-NNA 5-HT-infused group, 183.3±28.3 ng/ml plasma, as compared to the L-NNA Vehicle-infused group, 20.4±8.5 ng/ml plasma, a 9.1 fold increase. The PRP 5-HT concentration increased in the L-NNA 5-HT-infused group, 754.5±89.6 ng/ml plasma, as compared to the L-NNA Vehicle-infused group, 248.2±78.8 ng/ml plasma, a 3.0 fold increase. A listing of PPP and PRP plasma 5-HT values from the hypertensive models and comparators are shown in Table 1.

L-NNA hypertension: Effect of 5-HT on telemetric measurements:

Figure 20 shows the development of hypertension of rats supplemented with L-NNA for 10 days after 2 days of baseline recordings, and subsequently 7 days of 5-HT or Vehicle infusion. The rats supplemented with L-NNA developed the same degree of hypertension as did the DOCA rats at the time of 5-HT or

Vehicle pump implant, with MAP 24-hour grouped averages between 160 and 170 mmHg in each experimental model. Prior to pump implant, L-NNA Vehicle rats had a 24-hour grouped average MAP of 160.7±7.1 mmHg, while the L-NNA 5-HT rats had an average MAP of 161.4±3.8 mmHg. At Day 2, when we observed the peak fall to 5-HT in the DOCA rats, the L-NNA Vehicle rats had a grouped average MAP of 166.9±6.2 mmHg, while the L-NNA 5-HT rats had a grouped average MAP of 157.7±5.5 mmHg. The important finding in this experiment is that L-NNA hypertensive rats infused with 5-HT experienced *no fall in MAP* as a result of the infusion, and in fact, were not statistically different from L-NNA Vehicle-infused rats. Thus, inhibition of NOS with L-NNA *completely abolished* the ability of 5-HT to effect a drop in MAP in the same way as it could in the DOCA hypertensive rat.

The normotensive comparator, $Sham_L$, did not develop hypertension, but we did observe a similar drop in MAP at Day 2 after 5-HT infusion as compared to Vehicle infusion, with $Sham_L$ rats receiving 5-HT infusion had a 24-hour grouped average MAP of 89.9±1.8 mmHg compared to $Sham_L$ Vehicle rats, with a 24-hour grouped average MAP of 107.4±1.7 mmHg. This drop in MAP occurred with the same profile as observed in the $Sham_D$ rats, with a nadir in MAP at Day 2 of 5-HT infusion. This comparison between the effect of 5-HT on L-NNA and $Sham_L$ rats is critical because the 5-HT solution for the L-NNA and $Sham_L$ pumps were made of the same stock solution and implanted on the same day, so the 5-HT *was* able to effect a drop in MAP in the normotensive rats

without NOS blockade, but 5-HT *was not* able change MAP in those hypertensive rats with NOS blockade with L-NNA.

Figure 21 plots the L-NNA hypertensive rats against the DOCA hypertensive rats and the different responses to 5-HT between the two models. This reinforces the point that 5-HT is not able to effect a drop in MAP in those hypertensive rats with NOS inhibited with supplementation with L-NNA the way 5-HT can cause a drop in MAP in DOCA hypertensive rats.

L-NNA hypertension: Effect of hexamethonium:

As with the DOCA and $Sham_D$ rats, we administered hexamethonium on Day 4 of 5-HT or Vehicle infusion to the L-NNA and $Sham_L$ rats. Figure 22 shows the peak response to 30 mg/kg i.p. hexamethonium in the four groups. The group-average peak effect was not different between the L-NNA Vehicle (peak response of -67.0±12.5 mmHg, n=7) and L-NNA 5-HT (peak response of -52.9±6.2 mmHg, n=7), as a statistical analysis using a paired 1-tailed student's t-test revealed a p value of 0.198. However, there was a statistical difference between $Sham_L$ Vehicle (peak response of -31.6±3.6 mmHg, n=5) and $Sham_L$ 5-HT (peak response of -23.8±2.5 mmHg, n=6) with a p value of 0.034 resulting from a paired 1-tailed student's t-test.

Figure 13.

Cumulative concentration response curves to PE (top), ACh (middle) and 5-HT (bottom) in isolated $Sham_D$ aortae after 7 days of Vehicle or 5-HT infusion. Points represent means ± SEM for the number of rats in parentheses, reported as a percentage of maximal PE contraction.

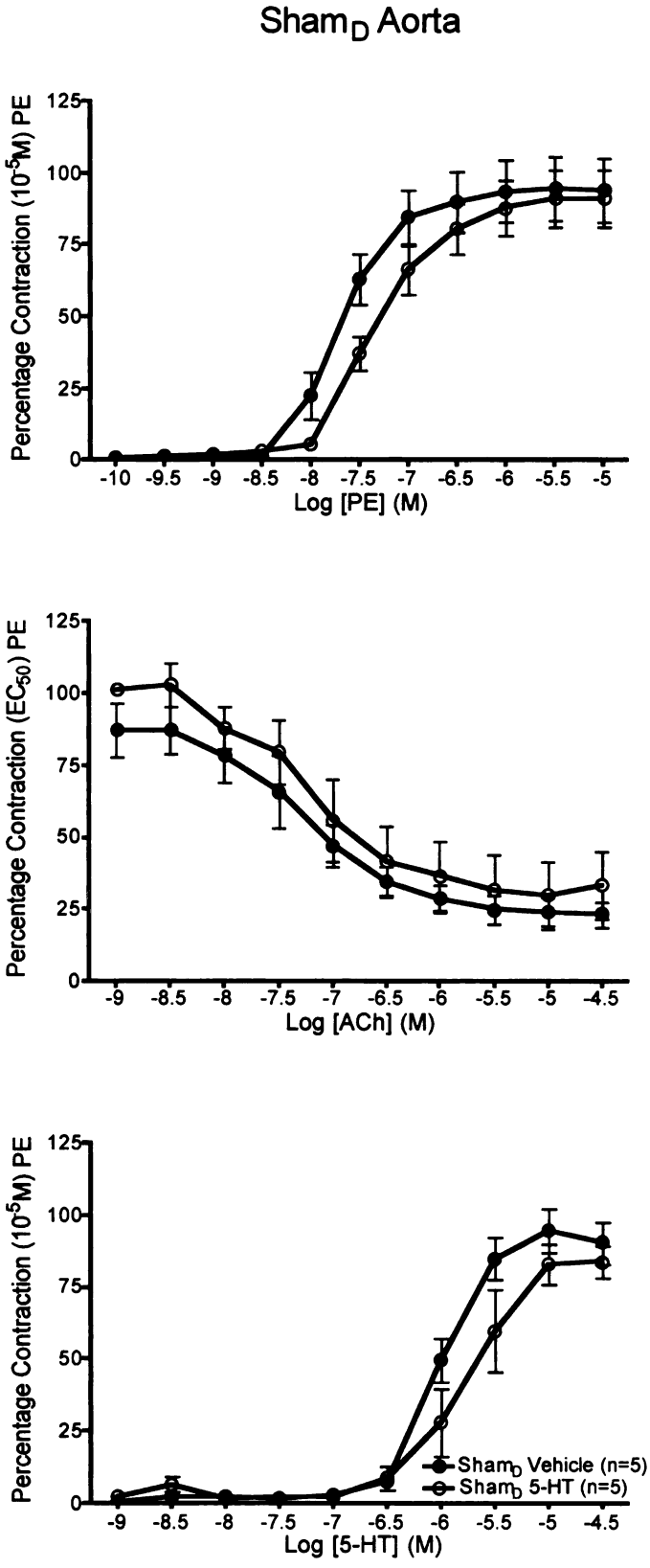
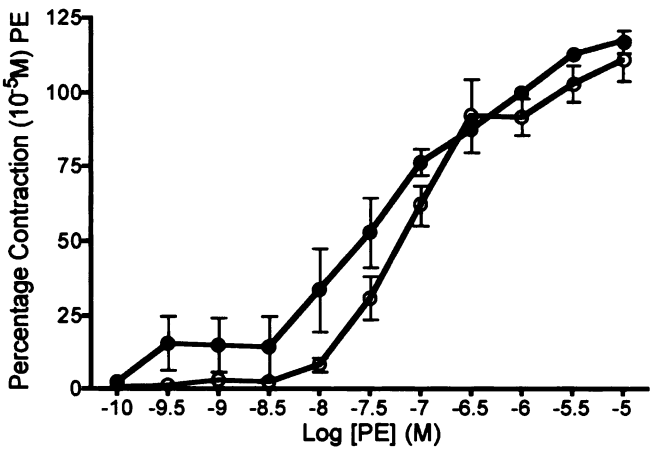

Sham$_D$ Aorta
Percentage Contraction (10^{-5}M) PE
125
100
75
50
25
0
-10 -9.5 -9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
Log [PE] (M)
Percentage Contraction (EC_{50}) PE
125
100
75
50
25
0
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5 -4.5
Log [ACh] (M)
Percentage Contraction (10^{-5}M) PE
125
100
75
50
25
0
Sham$_D$ Vehicle (n=5)
Sham$_D$ 5-HT (n=5)
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5 -4.5
Log [5-HT] (M)

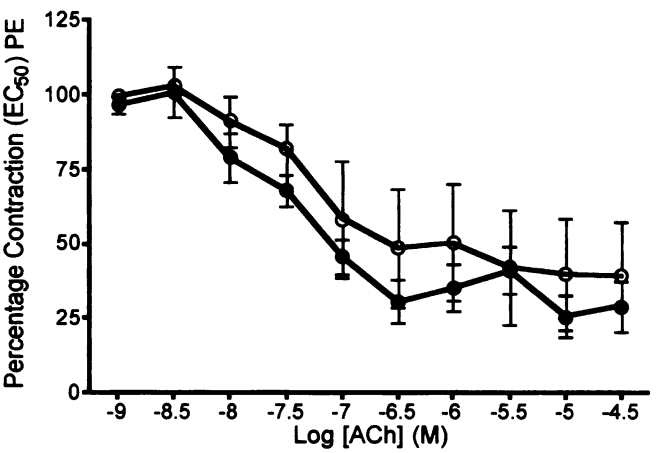
Figure 13

Figure 14.

Cumulative concentration response curves to PE (top), ACh (middle) and 5-HT (bottom) in isolated $Sham_D$ superior mesenteric artery after 7 days of Vehicle or 5-HT infusion. Points represent means ± SEM for the number of rats in parentheses, reported as a percentage of maximal PE contraction.

$Sham_D$ Superior Mesenteric Artery





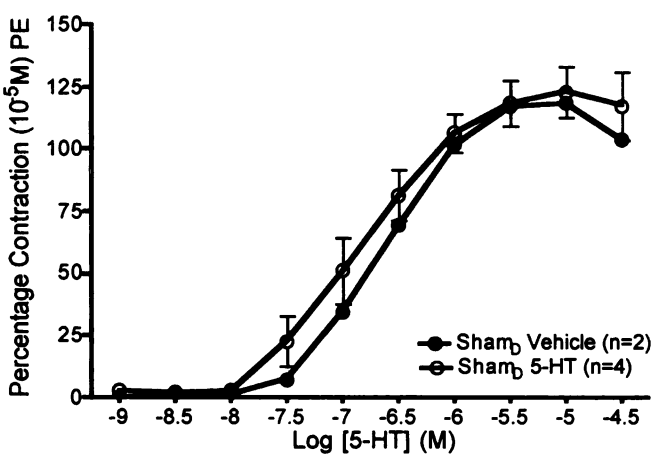


Figure 14

Figure 15.

Cumulative concentration response curves to PE (top), ACh (middle) and 5-HT (bottom) in isolated DOCA aortae after 7 days of Vehicle or 5-HT infusion. Points represent means ± SEM for the number of rats in parentheses, reported as a percentage of maximal PE contraction.

DOCA Aorta

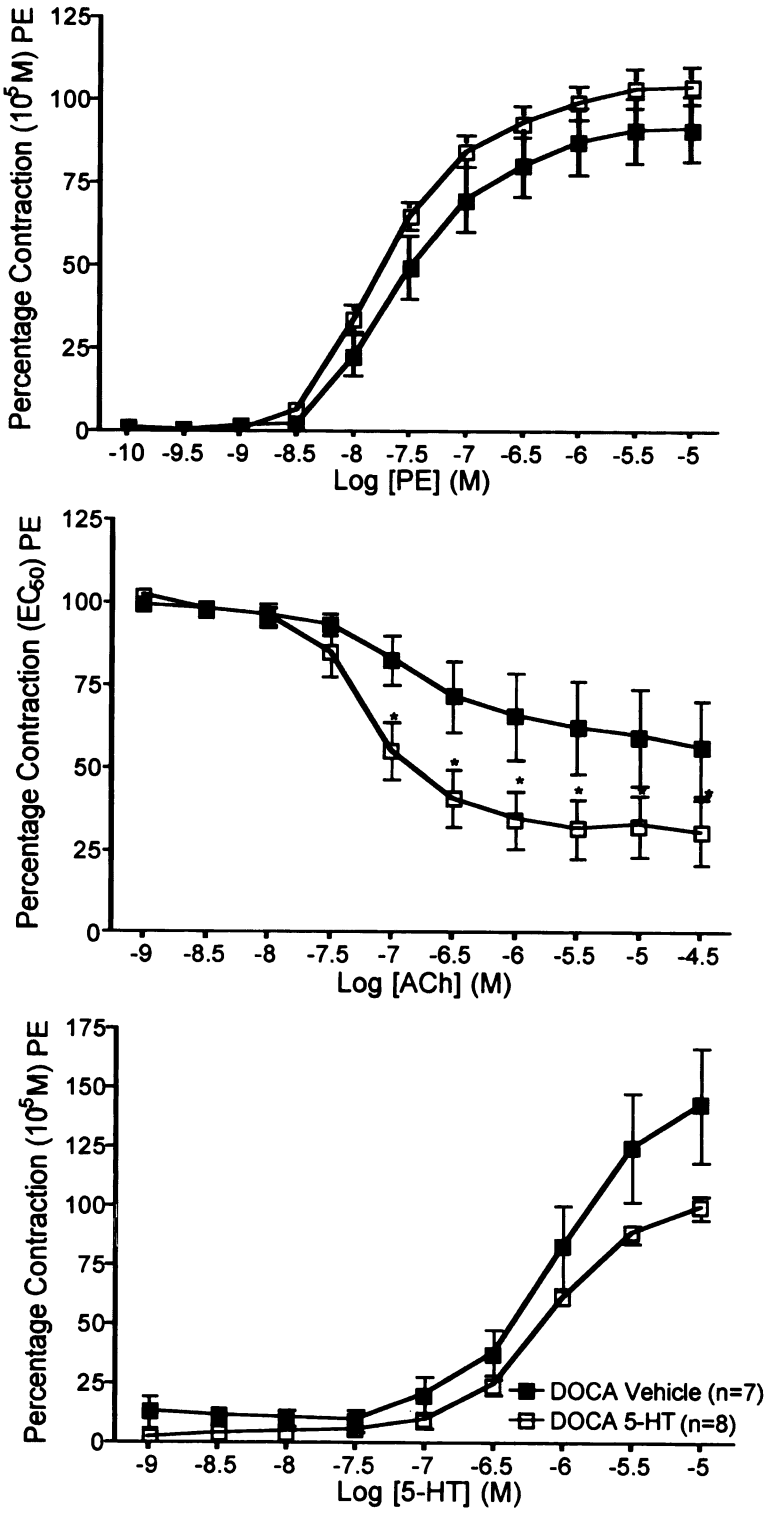


Figure 15

Figure 16.

Cumulative concentration response curves to PE (top), ACh (middle) and 5-HT (bottom) in isolated DOCA superior mesenteric artery after 7 days of Vehicle or 5-HT infusion. Points represent means ± SEM for the number of rats in parentheses, reported as a percentage of maximal PE contraction.

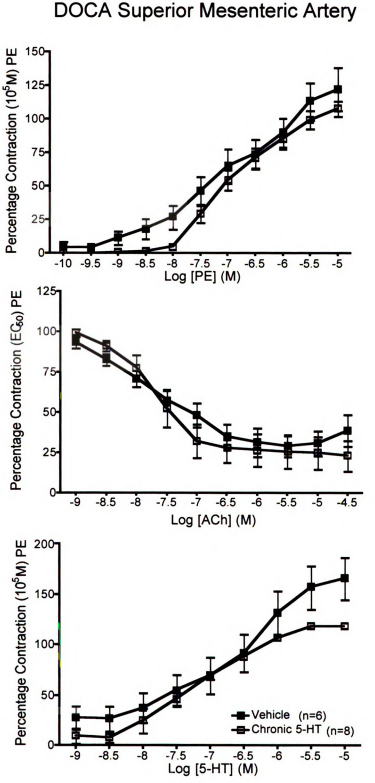

DOCA Superior Mesenteric Artery
Percentage Contraction (10^{-5}M) PE
150
125
100
75
50
25
0
-10 -9.5 -9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
Log [PE] (M)
Percentage Contraction (EC_{60}) PE
125
100
75
50
25
0
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5 -4.5
Log [ACh] (M)
Percentage Contraction (10^{-5}M) PE
200
150
100
50
0
Vehicle (n=6)
Chronic 5-HT (n=8)
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
Log [5-HT] (M)

Figure16

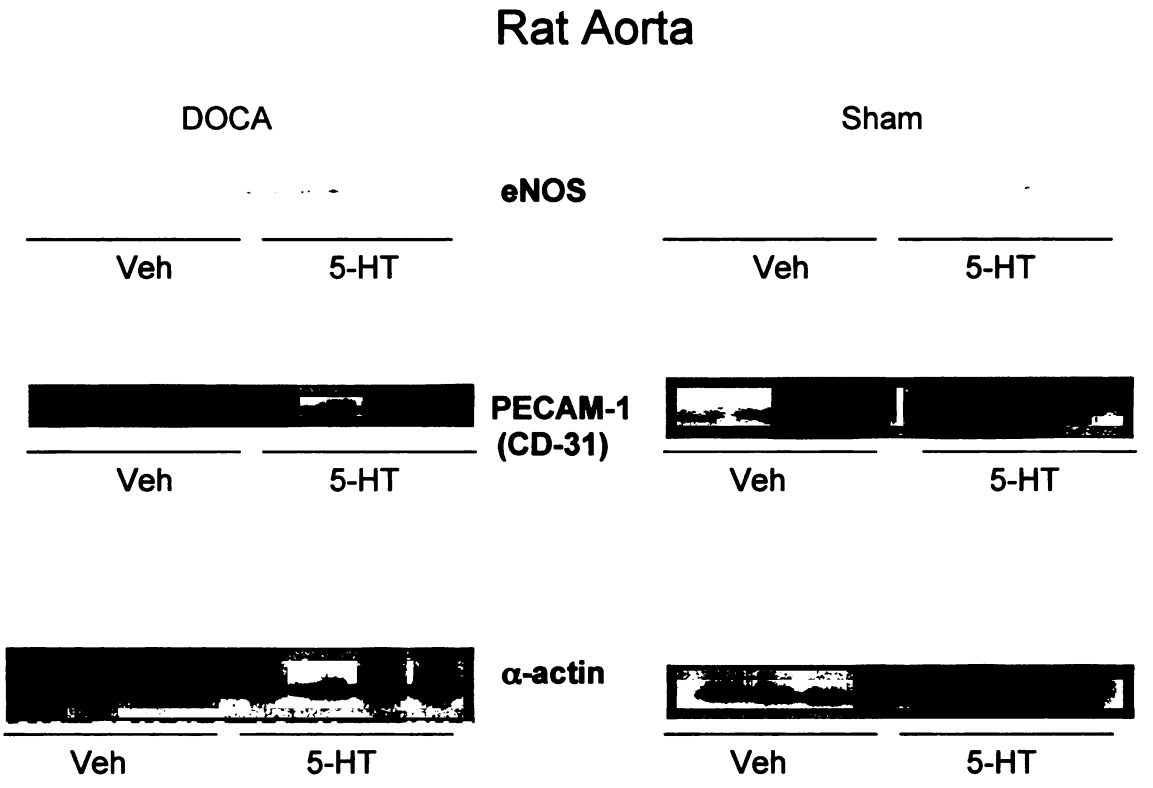


Figure 17

Figure 17.

Top: Western blot comparison between DOCA Vehicle and DOCA 5-HT rat aorta, probing for eNOS.

Middle: Western blot comparison between DOCA Vehicle and DOCA 5-HT rat aorta, probing for PE-CAM.

Bottom: Western blot comparison between DOCA Vehicle and DOCA 5-HT rat aorta, probing for α-actin.

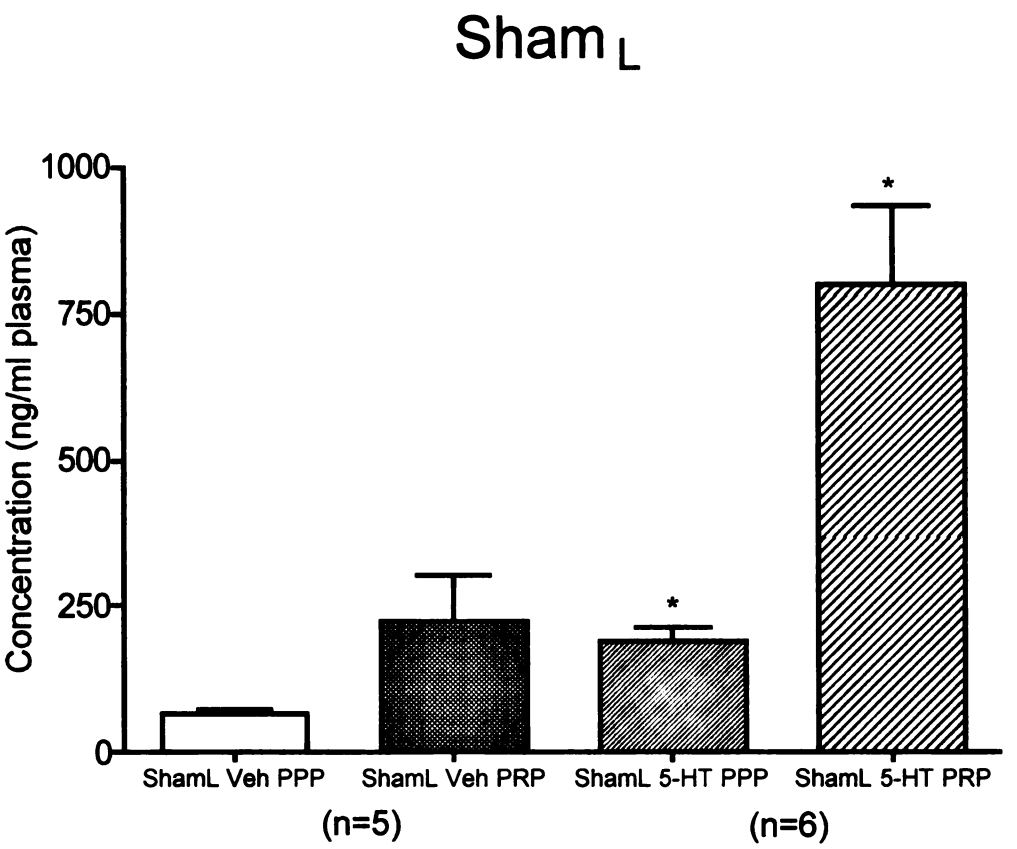


Figure 18

Figure 18.

5-HT quantification from whole blood from $Sham_L$ rats separated into components; that which is freely circulating in the plasma (PPP) and that which is contained within the granules of platelets (PRP). Bars represent means ± SEM for the number of rats in parentheses.

$Sham_L$=rats not receiving L-NNA in the drinking water, PPP=Platelet-poor plasma, PRP=Platelet-rich plasma, $p<0.05$ compared to Vehicle.

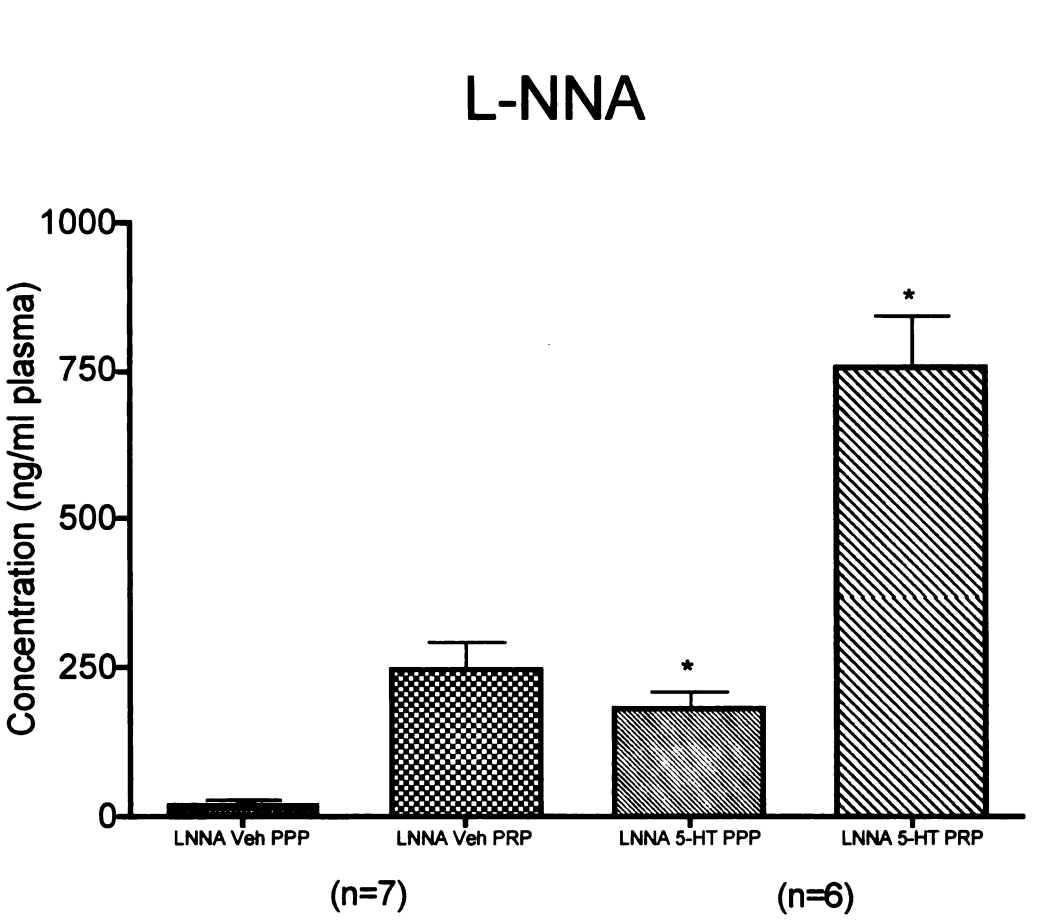


Figure 19

Figure 19.

5-HT quantification from whole blood from L-NNA rats separated into components; that which is freely circulating in the plasma (PPP) and that which is contained within the granules of platelets (PRP). Bars represent means ± SEM for the number of rats in parentheses.

L-NNA=rats receiving L-NNA in the drinking water, PPP=Platelet-poor plasma, PRP=Platelet-rich plasma, $p<0.05$ compared to Vehicle.

	Vehicle	5-HT	Vehicle	5-HT
Model	**PPP**	**PPP**	**PRP**	**PRP**
$Sham_D$ (n=4)	2.7±0.3	47.1±23.2*	31.9±9.7	257.7±40.2*
DOCA (n=5-9)	24.9±5.1	137.9±35.4*	114.5±21.0	591.3±212.5*
$Sham_L$ (n=5-6)	67.9±6.3	190.7±23.1*	224.0±78.8	801.2±132.8*
L-NNA (n=6-7)	20.4±8.5	183.3±28.3*	248.1±47.0	754.5±89.6*
$Sham_D$ + L-NNA (n=4)	5.3±0.4	171.2±33.4*	111.7±9.1	732.8±273.5*
DOCA + L-NNA (n=3)	7.1±3.1	112.8±34.3*	177.9±66.2	255.6±68.1*

Table 1

Table 1.

5-HT quantification from whole blood from all rat models separated into components; that which is freely circulating in the plasma (PPP) and that which is contained within the granules of platelets (PRP). Values are shown as ng 5-HT/ml plasma.

$Sham_D$=uninephrectomized Sham rats, DOCA=rats receiving DOCA pellet, $Sham_L$ =rats not receiving L-NNA in the drinking water, L-NNA=rats receiving L-NNA in the drinking water, PPP=Platelet-poor plasma, PRP=Platelet-rich plasma. *$p<0.05$ compared to Vehicle.

Figure 20.

Top: MAP measurements of L-NNA hypertensive and normotensive $Sham_L$ rats after 2 days of baseline measurements, then 7 subsequent days of 5-HT or Vehicle infusion.

Middle: Heart rate measurements (bpm) of L-NNA hypertensive and normotensive $Sham_L$ rats after 2 days of baseline measurements, then 7 subsequent days of 5-HT or Vehicle infusion.

Bottom: Activity level of L-NNA hypertensive and normotensive $Sham_L$ rats after 2 days of baseline measurements, then 7 subsequent days of 5-HT or Vehicle infusion.

Points represent 24-hour averages of the groups ± SEM for the number of rats in parentheses. *$p<0.05$ compared to Vehicle.

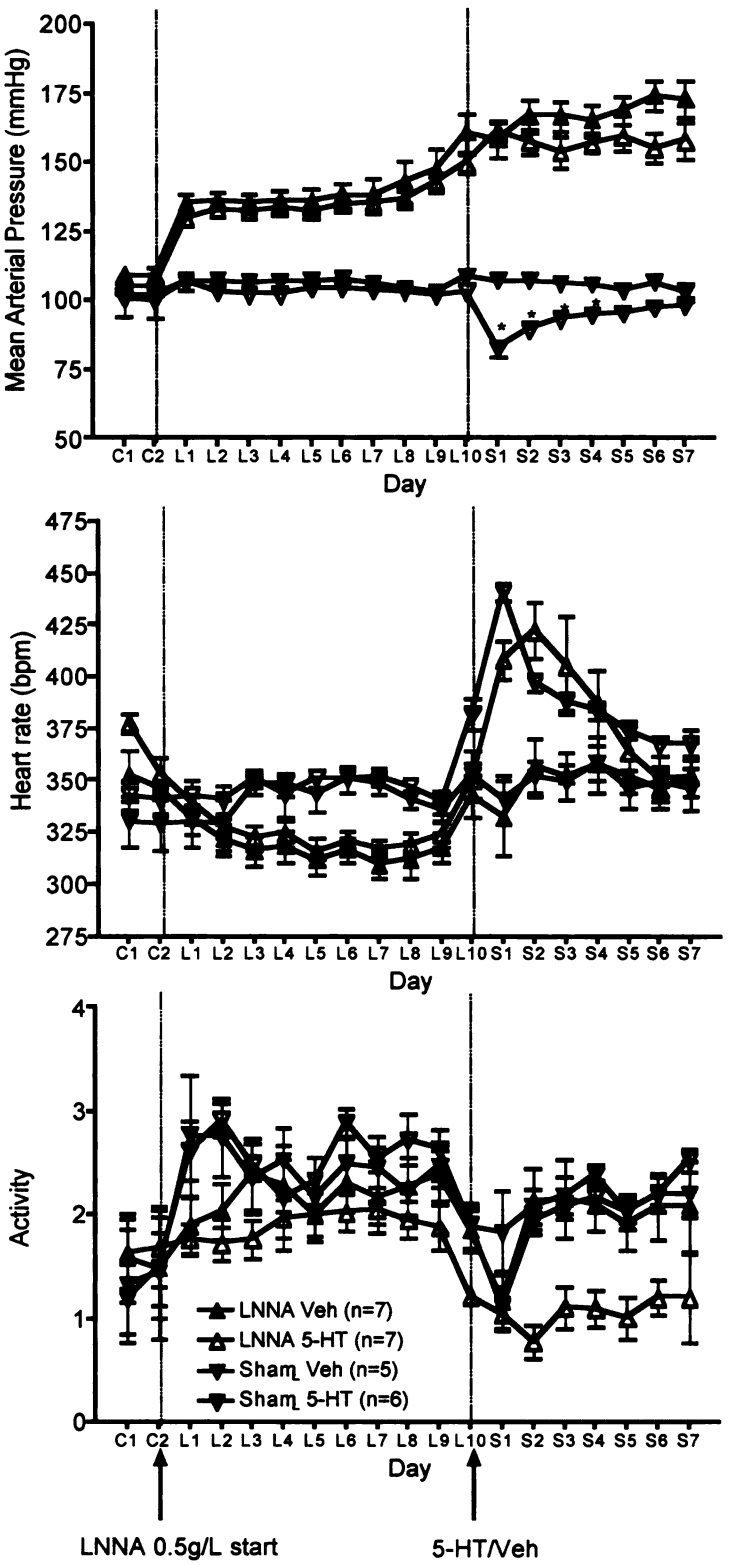

Mean Arterial Pressure (mmHg)
200
175
150
125
100
75
50
C1 C2 L1 L2 L3 L4 L5 L6 L7 L8 L9 L10 S1 S2 S3 S4 S5 S6 S7
Day
Heart rate (bpm)
475
450
425
400
375
350
325
300
275
C1 C2 L1 L2 L3 L4 L5 L6 L7 L8 L9 L10 S1 S2 S3 S4 S5 S6 S7
Day
Activity
4
3
2
1
0
LNNA Veh (n=7)
LNNA 5-HT (n=7)
Sham Veh (n=5)
Sham 5-HT (n=6)
C1 C2 L1 L2 L3 L4 L5 L6 L7 L8 L9 L10 S1 S2 S3 S4 S5 S6 S7
Day
LNNA 0.5g/L start
5-HT/Veh

Figure 20

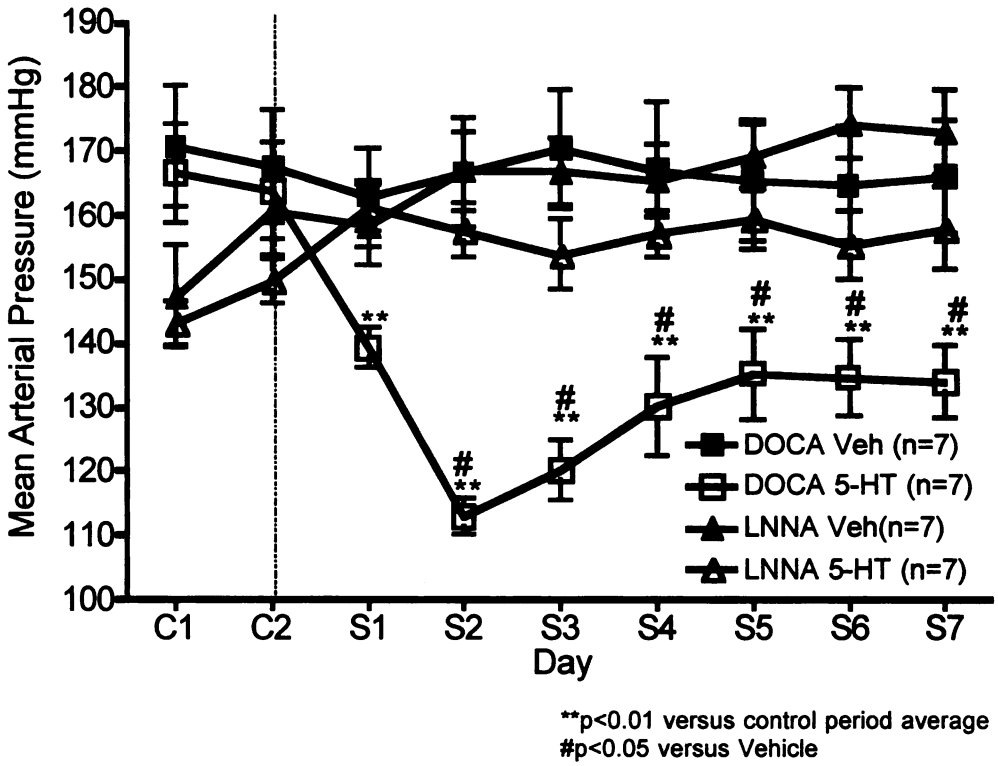


Figure 21

Figure 21.

MAP measurements of both L-NNA and DOCA hypertensive rats after 2 days of baseline measurements, then 7 subsequent days of 5-HT or Vehicle infusion. Points represent 24-hour averages of the groups ± SEM for the number of rats in parentheses. **p<0.01 versus control period average, #p<0.05 versus Vehicle.

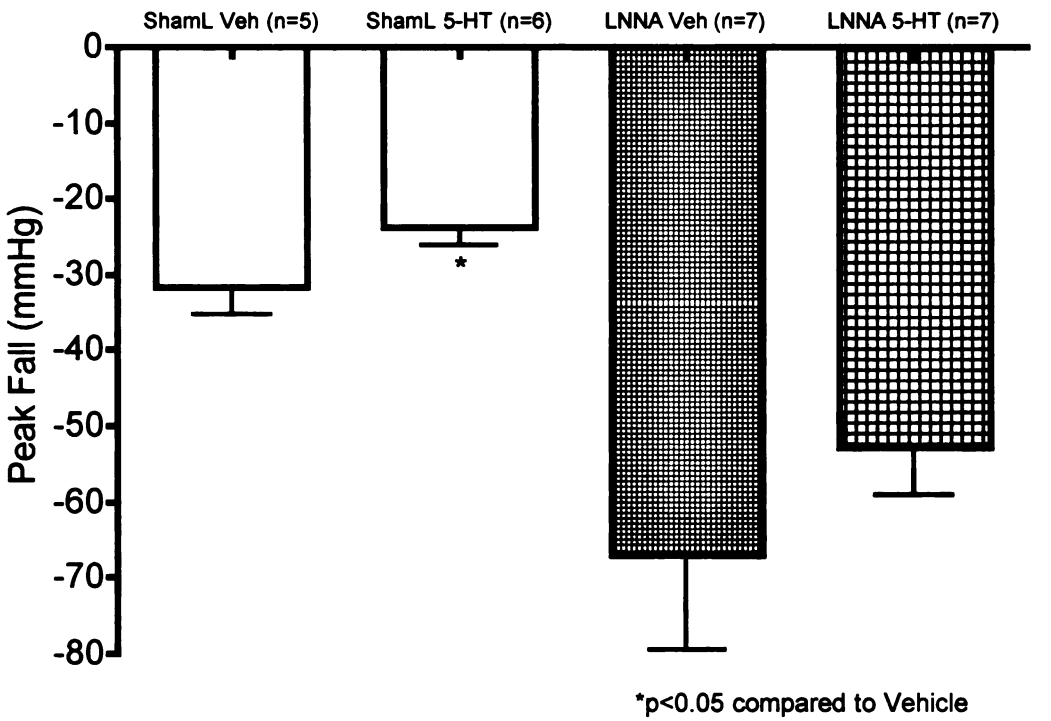


Figure 22

Figure 22.

Peak response in MAP to acute intraperitoneal injections of hexamethonium (30/mg/kg) on Day 4 of 5-HT or Vehicle pump infusion in conscious L-NNA or $Sham_L$ rats. $p<0.05$ compared to Vehicle.

Veh=Vehicle pump-infused

5-HT=5-HT pump-infused

We performed cumulative concentration response curves to PE, ACh, and 5-HT were performed on isolated aortic tissue harvested on Day 7 of infusion from both the Vehicle- and 5-HT-infused L-NNA and $Sham_L$ rats. Isometric contractility data from $Sham_L$ rat aortae are shown in Figure 23. Again, in the aorta harvested in $Sham_L$ Vehicle and $Sham_L$ 5-HT rats, we observed no difference in the initial adrenergic stimulation with PE, nor did we observe a difference in the PE concentration response curve, indicating no appreciable change in the general vasoactivity of the $Sham_L$ vessels with chronic exposure to 5-HT as compared to Vehicle. Since these rats were not exposed to L-NNA, the response to ACh was as expected, with an intact endothelial- and NO-dependent relaxation. The aortae harvested from $Sham_L$ Vehicle experienced a 95.9±13.3% relaxation to ACh (n=5), compared to the aortae harvested from $Sham_L$ 5-HT-infused, who experienced an 82.1±14.4% relaxation (n=6). This response was not statistically significant between the 2 groups, with an unpaired 1-tailed student's t-test p value=0.25. Again, we did not observe desensitization to 5-HT in these vessels after chronic exposure to 5-HT *via* 5-HT infusion.

Data from L-NNA rat aortae are shown in Figure 24. We observed no difference in the initial adrenergic stimulation with PE, nor did we observe a difference in the PE concentration response curve, indicating no appreciable change in the general vasoactivity of the L-NNA vessels with chronic exposure to 5-HT as compared to Vehicle. Since we did administer L-NNA to these rats, which acted to inhibit NOS, we did expect markedly diminished NO-dependent relaxation response to ACh in these vessels. To minimize the possibility that the

L-NNA might wash out in the tissue bath and NOS become un-blocked, an estimated EC_{50} PE contraction was achieved first in the experiment after a truncated equilibration to passive tension with limited washes, and a cumulative concentration response curve to ACh was immediately started. It was only after the ACh curve did I perform the adrenergic stimulation with maximal concentration of PE, followed by the remainder of the cumulative response curves (PE and 5-HT). With this precaution, the aortae from both groups demonstrate impaired NO-dependent relaxation. The aortae from L-NNA Vehicle-infused rats experienced a 54.6±8.1% relaxation (n=7), compared to the aortae harvested from L-NNA 5-HT-infused rats, who experienced a 42.9±5.1% relaxation (n=7). A paired 1-tailed student's t-test produced a p value = 0.10, indicating no difference between the 2 groups in the NO-dependent relaxation to ACh. The cumulative response curves to 5-HT in both groups were virtually identical, indicating no desensitization to 5-HT occurred in those vessels chronically exposed to 5-HT *via* mini-pump infusion as compared to Vehicle.

As the inhibition of NOS abolishes the profound 5-HT-induced blood pressure fall, as well as any effect on the sympathetic nervous system, we decided to inhibit NOS using L-NNA in hypertensive DOCA rats to further investigate the interaction of 5-HT and NOS.

Role of NOS inhibition in DOCA

$Sham_D$ and DOCA rats were prepared as before, except on Day 25 of DOCA or $Sham_D$, their 1% NaCl + 0.2% KCl water was supplemented with L-

NNA. Previous experience with the pure L-NNA rats indicated that each rat consumed approximately 0.01g L-NNA/day. To take into account the known decrease by approximately 50% in salt-water consumption after the start of 5-HT as compared to Vehicle, two stocks of salt-water were made up supplemented with 0.01 g/day/rat of L-NNA. The fluid given to the DOCA+L-NNA+5-HT rats still had 1% NaCl and 0.2% KCl, but contained twice the concentration of L-NNA as that 1% NaCl and 0.2% KCl supplemented L-NNA fluid given to the DOCA+L-NNA+Vehicle rats, so that each rat, regardless of 5-HT or Vehicle infusion, would consume roughly 0.01g L-NNA/day.

DOCA+L-NNA: plasma 5-HT measurements:

Plasma was taken on Day 7 of infusion from DOCA+L-NNA and $Sham_D$+L-NNA rats, and 5-HT concentration determined using HPLC both freely circulating levels of 5-HT (PPP) as well as that which is contained within the platelets (PRP). The 5-HT levels were increased in both components of plasma in $Sham_D$+L-NNA rats after 7 days of 5-HT infusion as compared to Vehicle infusion. 5-HT concentrations are increased in PPP in the $Sham_D$+L-NNA 5-HT-infused group, 171.2±33.4 ng/ml plasma, as compared to the $Sham_D$+L-NNA Vehicle-infused group, 5.5±0.4 ng/ml plasma, a 31.1 fold increase. The PRP 5-HT concentration increased in the $Sham_D$+L-NNA 5-HT-infused group, 732.8±273.5 ng/ml plasma, as compared to the $Sham_D$+L-NNA Vehicle-infused group, 111.7±9.1 ng/ml plasma, a 6.6 fold increase. $Sham_D$+L-NNA plasma data is shown in Figure 25.

Figure 26 shows DOCA+LNNA plasma component concentrations. 5-HT concentrations are increased in PPP in the DOCA+L-NNA 5-HT-infused group, 112.8±34.3 ng/ml plasma, as compared to the DOCA+L-NNA Vehicle-infused group, 7.1±3.1 ng/ml plasma, a 15.9 fold increase. The PRP 5-HT concentration increased in the DOCA+L-NNA 5-HT-infused group, 255.6±68.0 ng/ml plasma, as compared to the DOCA+L-NNA Vehicle-infused group, 177.9±66.2 ng/ml plasma, a 1.4 fold increase. Plasma measurements of 5-HT in the $Sham_D$+L-NNA and DOCA+L-NNA rats using HPLC indicated that the 5-HT administered *via* osmotic pump was in fact released and reached the systemic circulation as previously. For comparison of all plasma 5-HT data from all 3 experiments, please see Table 1.

DOCA+L-NNA: Effect of 5-HT on telemetric measurements:

MAP data from the DOCA+L-NNA rats are shown in Figure 27. ·The new salt-water supplemented with L-NNA prepared as described was started on Day 25 of DOCA or Sham to simulate in a truncated way what was done with the pure L-NNA, by having L-NNA on board to inhibit NOS before 5-HT or Vehicle infusion started. It is interesting to note that MAP went up in all groups approximately 20 mmHg in response to the onset of L-NNA administration. DOCA+L-NNA+Vehicle pre-L-NNA 24-hour averaged MAP was 152.5±11.1 mmHg and DOCA+L-NNA+5-HT pre-L-NNA 24-hour averaged MAP was 142.9±3.3 mmHg. On Day 3 of L-NNA water supplementation (*ie* Day 27 DOCA), 24-hour averaged MAP of the DOCA+L-NNA+Vehicle group was 172.0±8.7 mmHg, and the DOCA+L-

NNA+5-HT group was 167.9±2.9 mmHg. While we did observe a change in MAP resulting from the L-NNA administration, there was no significant change in MAP in those DOCA+L-NNA rats receiving 5-HT. At Day 2 of infusion, when we observe the peak fall to 5-HT in the pure DOCA rats, the DOCA+L-NNA+5-HT rats had a group 24-hour average of 153.7±8.4 mmHg, while the DOCA+L-NNA+Vehicle rats had a grouped 24-hour average of 159.1±10.8 mmHg. These experiments further corroborates the pure L-NNA experiment, and indicates it is inhibition of NOS that is responsible for *abolishing* the ability of 5-HT to effect a drop in MAP in DOCA rats.

For unknown reasons, this particular group of DOCA rats had a lower MAP than what we have observed previously in other experiments. In a way, the increase in MAP in response to L-NNA administration was rather fortuitous from an experimental stand-point, as L-NNA supplementation increased MAP to a level comparable to what we have observed in previous experiments in the pure DOCA and pure L-NNA, so pre-infusion MAP values were approximately equal (between 160 and 170 mmHg) in all groups.

DOCA+L-NNA: Effect of hexamethonium:

Hexamethonium was administered on Day 4 of 5-HT or Vehicle infusion, and the data are shown in Figure 28. There appears to be no difference between the DOCA+L-NNA+Vehicle (peak response of -97.3 mmHg, n=2) and DOCA+L-NNA+5-HT (peak response of -103.9 mmHg, n=2). However, there was a difference in response of $Sham_D$+L-NNA+Vehicle (peak response of -90.9

mmHg, n=2) in comparison to $Sham_D$+L-NNA+5-HT (peak response of -41.3 mmHg, n=2).

DOCA+L-NNA: Isolated tissue contractility:

We performed cumulative concentration response curves to PE, ACh, and 5-HT on isolated aortic tissue harvested on Day 7 of infusion from both the Vehicle- and 5-HT-infused DOCA+L-NNA and $Sham_D$+L-NNA rats. Isometric contractility data from $Sham_D$+L-NNA rat aortae are shown in Figure 29. Similar to what we had observed before in the other two models, in the aorta harvested in $Sham_D$+L-NNA Vehicle and $Sham_D$+L-NNA 5-HT rats, we observed no difference in the initial adrenergic stimulation with PE, nor did we observe a difference in the PE concentration response curve, indicating no appreciable change in the general vasoactivity of the $Sham_D$+L-NNA vessels with chronic exposure to 5-HT as compared to Vehicle. These rats were exposed to L-NNA, so we expected the response to ACh to be blunted, with impaired endothelial- and NO-dependent relaxation. To our surprise and for unknown reasons, the aortae harvested from $Sham_D$+L-NNA Vehicle experienced a 74.7±13.7% relaxation to ACh (n=4), compared to the aortae harvested from $Sham_D$+L-NNA 5-HT-infused, who experienced an 78.5±7.8% relaxation (n=4). This response was not statistically significant between the 2 groups, with a paired 1-tailed student's t-test p value=0.34. Again, we did not observe desensitization to 5-HT in these vessels after chronic exposure to 5-HT *via* 5-HT infusion.

Data from DOCA+L-NNA rat aortae are shown in Figure 30. We observed no difference in the initial adrenergic stimulation with PE, nor did we observe a difference in the PE concentration response curve, indicating no appreciable change in the general vasoactivity of the L-NNA vessels with chronic exposure to 5-HT as compared to Vehicle. Since we did administer L-NNA to these rats, which acted to inhibit NOS, we did expect markedly diminished NO-dependent relaxation response to ACh in these vessels. Like with the pure L-NNA rats, we performed an estimated EC_{50} PE contraction first in the experiment after a truncated equilibration to passive tension with limited washes, and a cumulative concentration response curve to ACh was immediately started. This was done to minimize the possibility that L-NNA might wash away and NOS will become un-inhibited. It was only after the ACh curve did I perform the adrenergic stimulation with maximal concentration of PE, followed by the remainder of the cumulative response curves (PE and 5-HT). As expected, the aortae from both groups demonstrate impaired NO-dependent relaxation. The aortae from DOCA+L-NNA Vehicle-infused rats experienced a 46.7±8.4% relaxation (n=4), compared to the aortae harvested from DOCA+L-NNA 5-HT-infused rats, who experienced a 33.4±9.8% relaxation (n=4). A paired 1-tailed student's t-test produced a p value = 0.23, indicating no difference between the 2 groups in the NO-dependent relaxation to ACh. The cumulative response curves to 5-HT in both groups were virtually identical, indicating no desensitization to 5-HT occurred in those vessels chronically exposed to 5-HT *via* mini-pump infusion as compared to Vehicle.

Figure 23.

Cumulative concentration response curves to PE (top), ACh (middle) and 5-HT (bottom) in isolated $Sham_L$ aortae after 7 days of Vehicle or 5-HT infusion. Points represent means ± SEM for the number of rats in parentheses, reported as a percentage of maximal PE contraction.

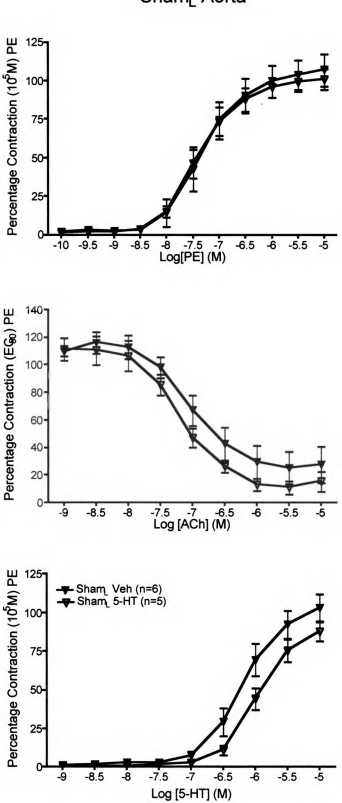

Sham$_L$ Aorta
Percentage Contraction (10^{-5}M) PE
125
100
75
50
25
0
-10 -9.5 -9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
Log[PE] (M)
Percentage Contraction (EC$_{50}$) PE
140
120
100
80
60
40
20
0
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
Log [ACh] (M)
Percentage Contraction (10^{-5}M) PE
Sham$_L$ Veh (n=6)
Sham$_L$ 5-HT (n=5)
125
100
75
50
25
0
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
Log [5-HT] (M)

Figure 23

Figure 24.

Cumulative concentration response curves to PE (top), ACh (middle) and 5-HT (bottom) in isolated L-NNA aortae after 7 days of Vehicle or 5-HT infusion. Points represent means ± SEM for the number of rats in parentheses, reported as a percentage of maximal PE contraction.

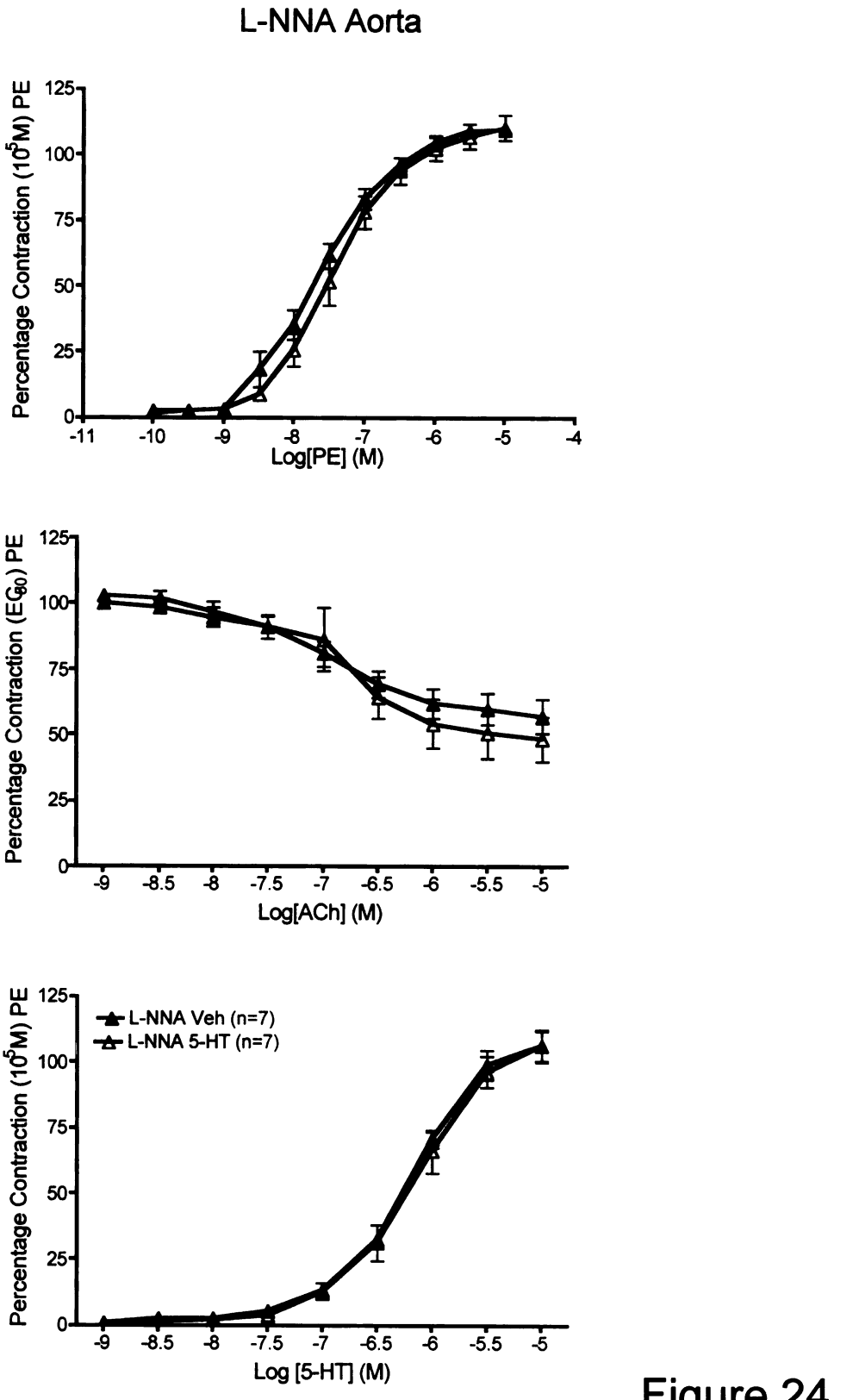

L-NNA Aorta
Percentage Contraction (10⁻⁵M) PE
125
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
-4
Log[PE] (M)
Percentage Contraction (EG80) PE
125
100
75
50
25
0
-9
-8.5
-8
-7.5
-7
-6.5
-6
-5.5
-5
Log[ACh] (M)
Percentage Contraction (10⁻⁵M) PE
L-NNA Veh (n=7)
L-NNA 5-HT (n=7)
125
100
75
50
25
0
-9
-8.5
-8
-7.5
-7
-6.5
-6
-5.5
-5
Log [5-HT] (M)

Figure 24

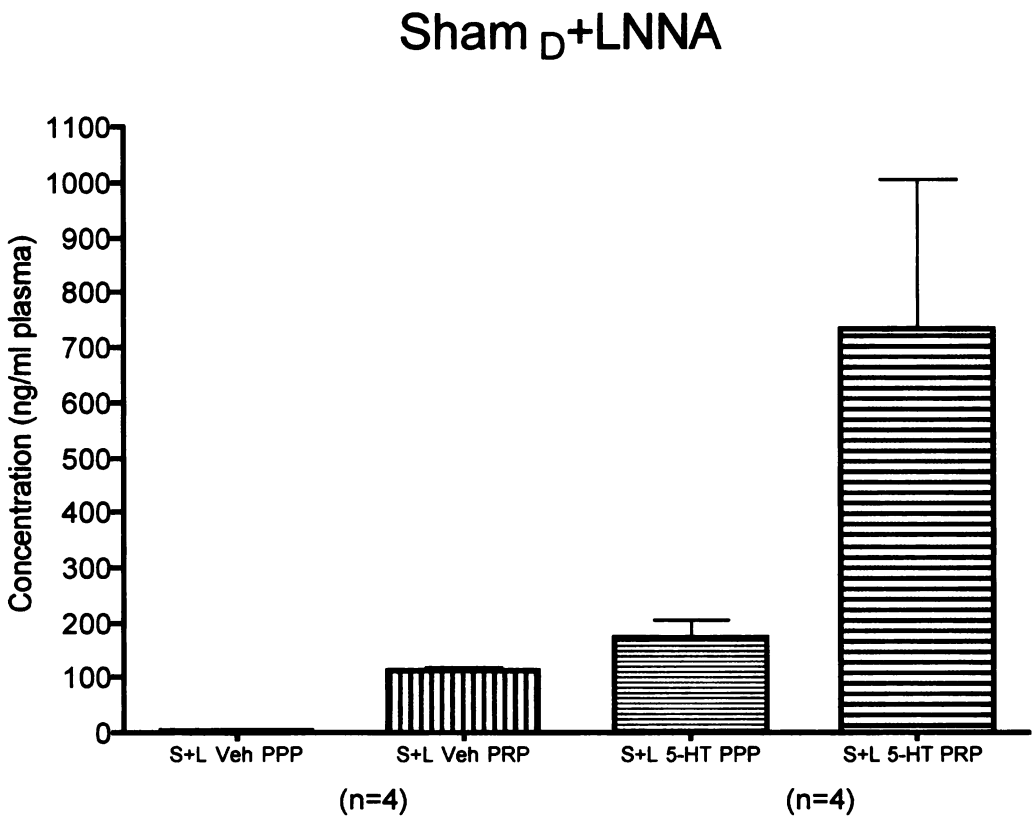


Figure 25

Figure 25.

5-HT quantification from whole blood from $Sham_D$ rats supplemented with L-NNA separated into components; that which is freely circulating in the plasma (PPP) and that which is contained within the granules of platelets (PRP). Bars represent means ± SEM for the number of rats in parentheses. S+L=$Sham_D$ rats receiving L-NNA in the drinking water, PPP=Platelet-poor plasma, PRP=Platelet-rich plasma, $p<0.05$ compared to Vehicle.

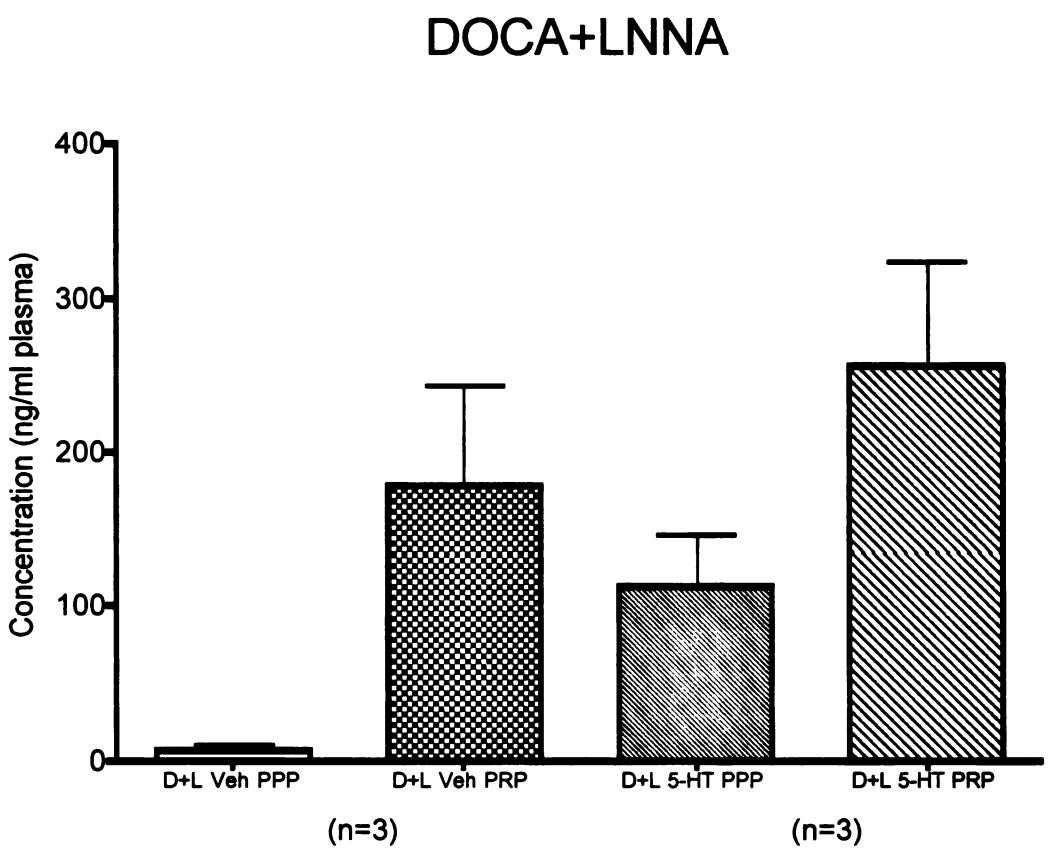


Figure 26

Figure 26.

5-HT quantification from whole blood from DOCA rats supplemented with L-NNA separated into components; that which is freely circulating in the plasma (PPP) and that which is contained within the granules of platelets (PRP). Bars represent means ± SEM for the number of rats in parentheses. D+L=DOCA rats receiving L-NNA in the drinking water, PPP=Platelet-poor plasma, PRP=Platelet-rich plasma, $p<0.05$ compared to Vehicle.

Figure 27.

Top: MAP measurements of hypertensive DOCA and normotensive $Sham_D$ rats after 2 days of baseline measurements, receiving L-NNA in their drinking water for 3 days, then 7 subsequent days of 5-HT or Vehicle infusion.

Middle: Heart rate measurements (bpm) of DOCA+L-NNA and $Sham_D$+L-NNA rats after 2 days of baseline measurements, then 7 subsequent days of 5-HT or Vehicle infusion.

Bottom: Activity level of DOCA+L-NNA and $Sham_D$+L-NNA rats after 2 days of baseline measurements, then 7 subsequent days of 5-HT or Vehicle infusion.

Points represent 24-hour averages of the groups ± SEM for the number of rats in parentheses.

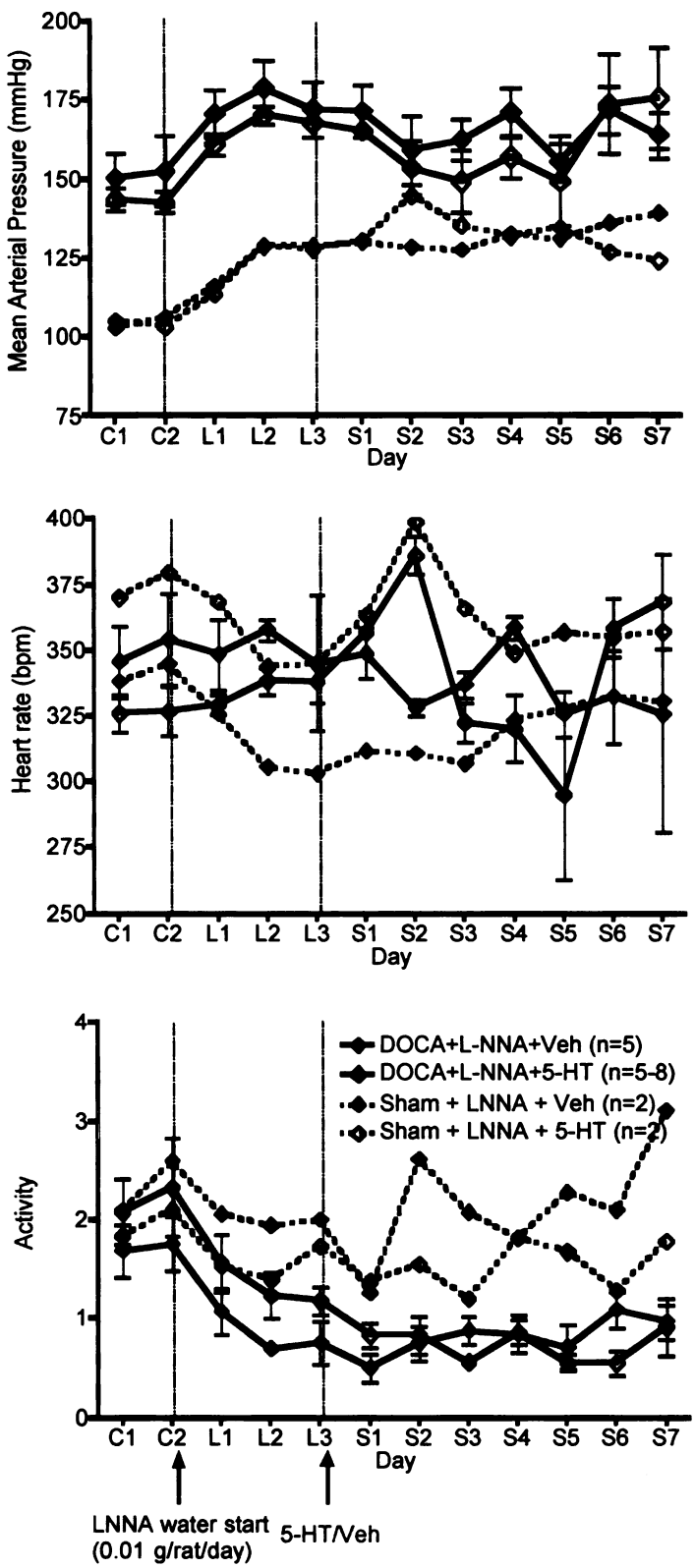

Mean Arterial Pressure (mmHg)
200
175
150
125
100
75
C1 C2 L1 L2 L3 S1 S2 S3 S4 S5 S6 S7
Day
Heart rate (bpm)
400
375
350
325
300
275
250
C1 C2 L1 L2 L3 S1 S2 S3 S4 S5 S6 S7
Day
Activity
4
3
2
1
0
DOCA+L-NNA+Veh (n=5)
DOCA+L-NNA+5-HT (n=5-8)
Sham + LNNA + Veh (n=2)
Sham + LNNA + 5-HT (n=2)
C1 C2 L1 L2 L3 S1 S2 S3 S4 S5 S6 S7
Day
LNNA water start
(0.01 g/rat/day)
5-HT/Veh

Figure 27

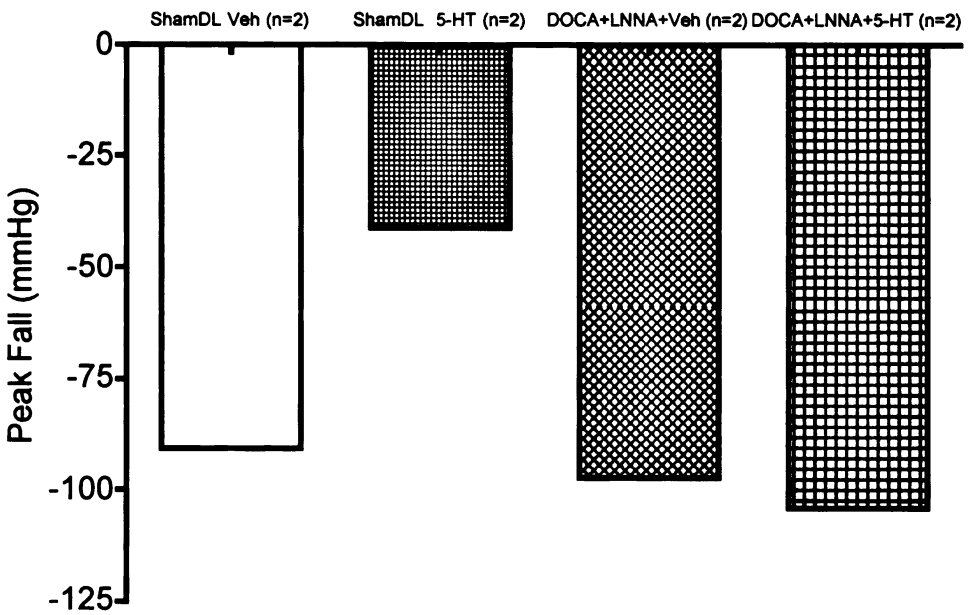


Figure 28

Figure 28

Peak response in MAP to acute intraperitoneal injections of hexamethonium (30/mg/kg) on Day 4 of 5-HT or Vehicle pump infusion in conscious DOCA+L-NNA or $Sham_{DL}$ rats.

Veh=Vehicle pump-infused

5-HT=5-HT pump-infused

Figure 29.

Cumulative concentration response curves to PE (top), ACh (middle) and 5-HT (bottom) in isolated $Sham_D$+L-NNA aortae after 7 days of Vehicle or 5-HT infusion. Points represent means ± SEM for the number of rats in parentheses, reported as a percentage of maximal PE contraction.

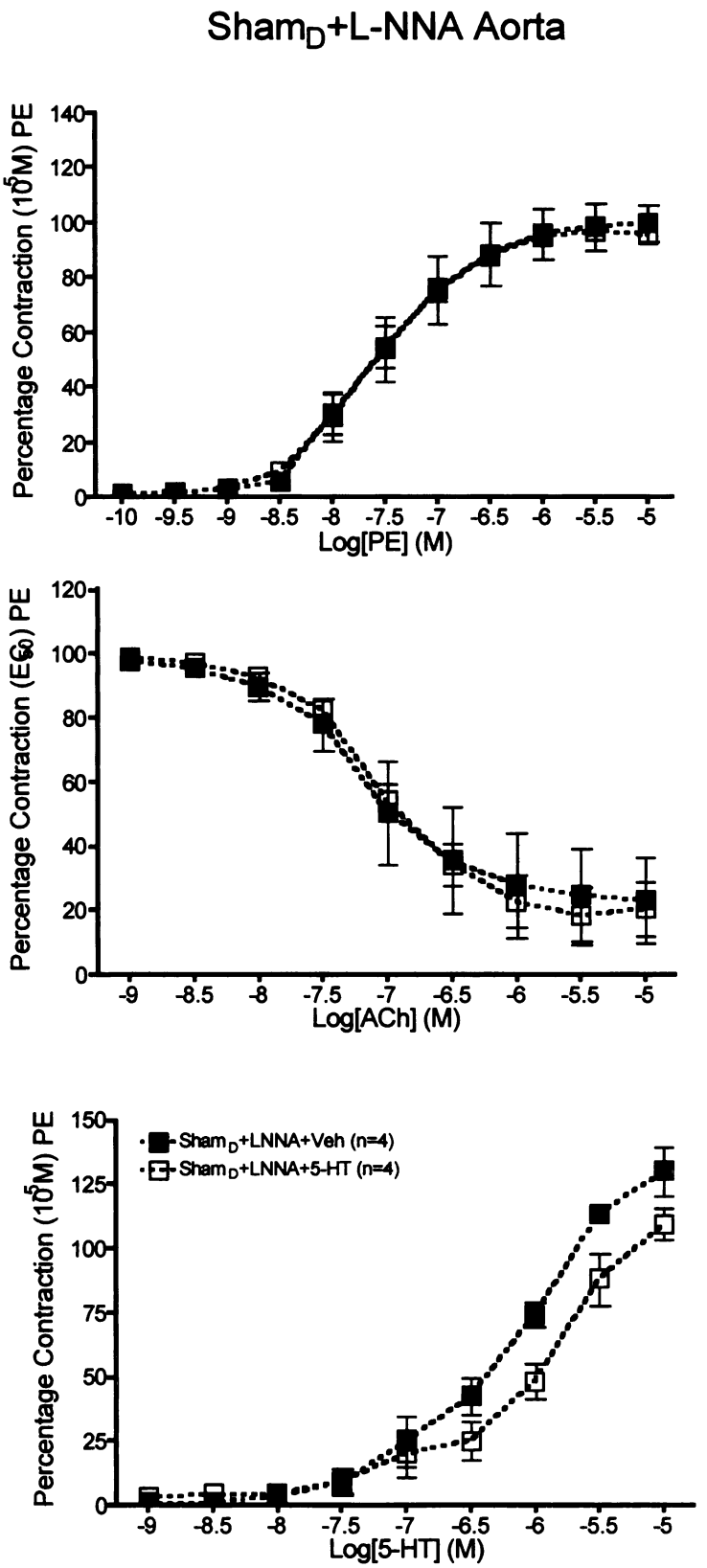
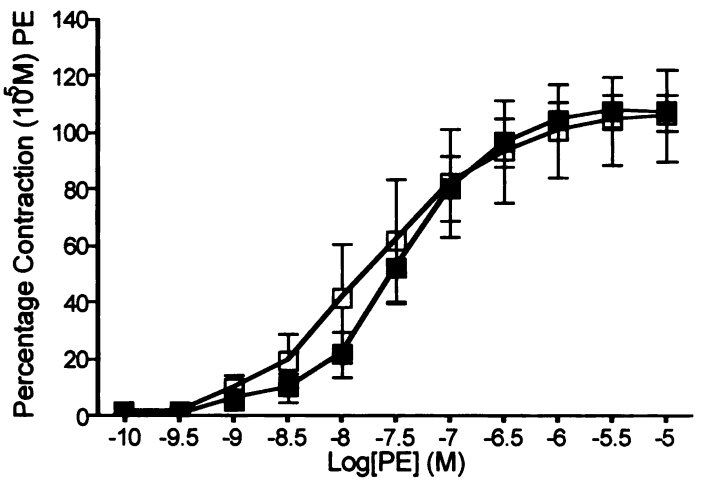

Sham$_D$+L-NNA Aorta
Percentage Contraction (10^{-5}M) PE
Log[PE] (M)
Percentage Contraction (EC$_{50}$) PE
Log[ACh] (M)
Sham$_D$+LNNA+Veh (n=4)
Sham$_D$+LNNA+5-HT (n=4)
Percentage Contraction (10^{-5}M) PE
Log[5-HT] (M)

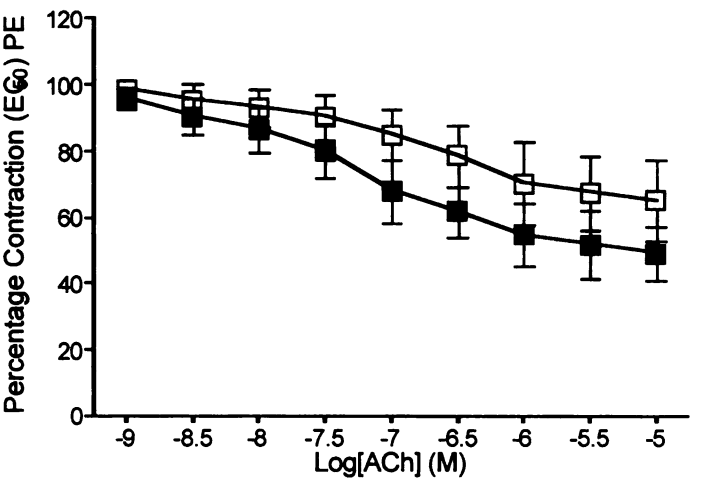
Figure 29

Figure 30.

Cumulative concentration response curves to PE (top), ACh (middle) and 5-HT (bottom) in isolated DOCA+L-NNA aortae after 7 days of Vehicle or 5-HT infusion. Points represent means ± SEM for the number of rats in parentheses, reported as a percentage of maximal PE contraction.

DOCA+L-NNA Aorta





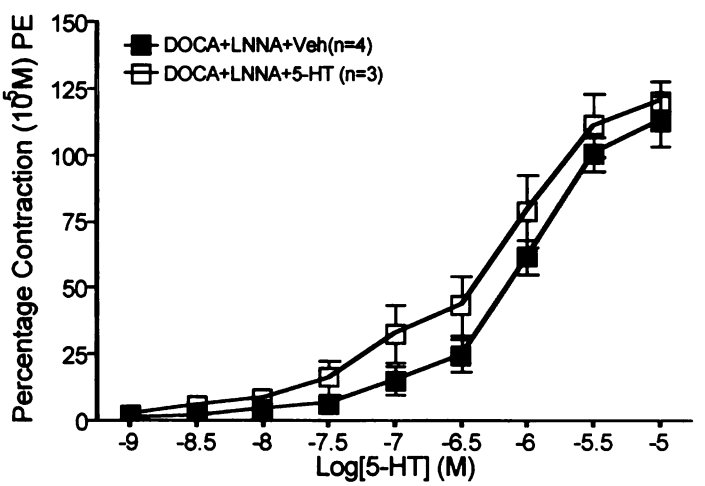


Figure 30

Discussion:

Despite the controversy in the literature as to the role of 5-HT in blood pressure control, administering 5-HT chronically, while monitoring blood pressure using telemetry, has yielded the finding that 5-HT elicits a hypotensive effect in both hypertensive DOCA-salt rats and normotensive $Sham_D$ rats. Chronic infusion using osmotic mini-pumps was quite effective in distributing the exogenous 5-HT to the peripheral circulation and to the peripheral vascular tissues as potential sites of action, as evidenced by our ability to measure increases in 5-HT both in plasma and in peripheral vascular tissue. I have shown for the first time that 5-HT administration causes marked sympatho-inhibition in the hypertensive DOCA and $Sham_D$ as measured by ganglionic blockade using hexamethonium. This effect of 5-HT to cause a drop in MAP and sympatho-inhibition is *completely abolished by blockade of NOS by administration of L-NNA*. These data suggest that 5-HT inhibits the sympathetic nervous system in a NOS-dependent fashion. The interaction between 5-HT and NOS was most striking when considering the improvement in the relaxation response of DOCA aorta to ACh in chronically infused 5-HT rats, and is confirmed by the increased expression of the eNOS protein in 5-HT-infused DOCA aorta. Western blotting for the PE-CAM-1 protein as a measure of endothelial cell number revealed equal amounts of PE-CAM-1 expression in both aortic protein from DOCA Vehicle rats and DOCA 5-HT rats, suggesting that the increase in eNOS expression observed in Western blots is real. This is one potential mechanism by which 5-HT could reduce MAP, however the speed with which blood pressure

falls upon infusion of 5-HT (as early as 6 hours post-pump implantation) suggests that the increased expression of eNOS protein is not the only mechanism at work.

Plasma 5-HT levels

As HPLC measures of PPP and PRP in the various models used here indicate, 5-HT increased in both components of plasma when 5-HT was infused *via* osmotic pumps as compared to Vehicle. The apparent paradox of why we observe increased resting free circulating 5-HT (PPP) in hypertensive DOCA rats as compared to normotensive $Sham_D$ rats, and why there are literature reports of higher free circulating 5-HT in hypertensive patients in comparison to normotensive patients is still unknown. It could be that 5-HT levels increase in the PPP as an adaptive response to hypertension, as opposed to the theory that 5-HT is one of the modulators or a cause of hypertension. A time-course experiment has not been done to evaluate the concentration of 5-HT in the plasma over the development of DOCA-salt hypertension to observe when the concentration begins to increase. In contrast, in my hands, the L-NNA rats had higher 5-HT in the PPP as compared to the $Sham_L$ rats, suggesting an increase in 5-HT is perhaps not a *general* adaptation to increased blood pressure. The L-NNA rats were just as hypertensive as the DOCA rats, so the difference in 5-HT freely circulating in plasma between the L-NNA and DOCA and their respective normotensive shams is quite striking and still unexplained.

SERT is dysfunctional in the DOCA and L-NNA hypertensive models, and as platelet SERT is responsible for taking up 5-HT, we can use PRP 5-HT content as an indicator of platelet SERT function. If the SERT protein on the platelets cannot take up 5-HT as effectively as in the normotensive Shams, we would expect to see lower PRP 5-HT concentrations on the hypertensive rats as compared to Shams. Surprisingly, in my hands, we do not observe evidence to support that expectation in the 5-HT levels in the PRP, and in fact we observe the opposite in all models. We will use the Vehicle-infused data as a representative of basal conditions. The PRP 5-HT concentrations in the DOCA Vehicle (137.9±35.4 ng/ml plasma) are increased compared to the PRP 5-HT concentrations in the $Sham_L$ (47.1±23.2 ng/ml plasma). Likewise, the PRP 5-HT concentrations in the L-NNA Vehicle (248.1.±47.0 ng/ml plasma) are increased compared to the PRP 5-HT concentrations in the $Sham_L$ (224.0±78.8 ng/ml plasma). Additionally, the PRP 5-HT concentrations in the DOCA+L-NNA Vehicle (177.9±66.2 ng/ml plasma) are increased compared to the PRP 5-HT concentrations in the $Sham_D$+L-NNA Vehicle (111.7±9.1 ng/ml plasma). One potential explanation may be that if platelet SERT function really is impaired, it will not be able to take up 5-HT for storage in the platelet granules, but it may not be able to release 5-HT from the granules either. The 5-HT may be effectively stuck in the granules of the platelet for the life of the platelet. Another explanation may be that the platelet numbers are increased in hypertension as opposed to normotension for some reason. In my protocol, platelet numbers are not taken into account, which could be a major limitation to this method of plasma

5-HT measurement. A third explanation might be because the high variability (a high SEM) in this experiment may be obscuring what is really happening, and an increase in the sample size may help elucidate SERT function at basal conditions.

If we examine the PRP data in the context of a challenge with chronic 5-HT infusion, we observe something new. The PRP 5-HT concentrations in the L-NNA 5-HT (754.5.±89.6 ng/ml plasma) are decreased compared to the PRP 5-HT concentrations in the $Sham_L$ (801.2±132.8 ng/ml plasma). Additionally, the PRP 5-HT concentrations in the DOCA+L-NNA 5-HT (255.6±68.1 ng/ml plasma) are decreased compared to the PRP 5-HT concentrations in the $Sham_D$+L-NNA 5-HT (732.8±273.5 ng/ml plasma). In contrast, however, the PRP 5-HT concentrations in the DOCA 5-HT (591.3.±212.5 ng/ml plasma) are still increased compared to the PRP 5-HT concentrations in the $Sham_L$ (257.7±40.2 ng/ml plasma). In the face of a 5-HT challenge, the capacity of the platelet SERT to take up 5-HT seems to be quite large, as they are able to take up anywhere from double the amount of 5-HT, in the case of the DOCA+L-NNA+5-HT PRP, or as as high as an 8-fold increases in the $Sham_D$ PRP. In normal physiologic conditions, 5-HT concentrations may reach the levels achieved by infusion, as platelet aggregation can lead to 5-HT concentrations in the micromolar range, which may explain the great reserve capacity for platelet 5-HT uptake *via* SERT function.

Involvement of 5-HT receptors

Data are not shown here, but an experiment was performed using a non-specific 5-HT receptor antagonist, methiothepin to determine if blockade of 5-HT receptors could abolish the hypotensive effect of 5-HT. A dose of 0.75 mg/kg methiothepin intraperitoneally was chosen because of the ability to cause a rightward shift in the cumulative response curve to 5-HT in an isolated tissue bath experiment using the aorta harvested from a normal rat injected with this dose 30 minutes prior to sacrifice. In the *in vivo* experiment, DOCA rats were prepared as before, with telemeter placement at Day 21 and pump placement at Day 28. Two hours prior to pump placement the DOCA rats received an intraperitoneal injection of 0.75 mg/kg methiothepin in an attempt to allow the antagonist time to equilibrate with the 5-HT receptors before the onset of 5-HT infusion. The half-life of methiothepin is unknown, so once-a-day intraperitoneal dosing was chosen to start. We observed no change in the profile of MAP fall as compared to DOCA rats injected with vehicle (saline) intraperitoneally. We concluded that blockade of 5-HT receptors was ineffective in abolishing the hypotensive effect of 5-HT, at least at the dose and dosing schedule of antagonist used. This experiment does not definitively rule out 5-HT receptors as part of the mechanism of blood pressure fall, though there is evidence to suggest that 5-HT may have actions intracellularly. As an example, 5-HT acts as a mitogen in the pathogenesis of pulmonary hypertension, though it hasn't been shown to have a 5-HT receptor-dependent mechanism. In fact, it may act intracellularly (Marcos et al., 2003). It may be possible that perhaps 5-HT exerts its hypotensive effects seen here for the first time by acting intracellularly to affect cellular signaling.

Effect of 5-HT on MAP

We have observed for the first time here that exogenous 5-HT causes a fall in blood pressure in mineralocorticoid hypertension but not in L-NNA hypertension. It is interesting that 5-HT administration causes a blood pressure fall in the same profile in the normotensive $Sham_D$ and $Sham_L$ rats. Expression of eNOS didn't appear to change, however, in the Sham rats. The sympathetic nervous system is inhibited at some level by some mechanism by 5-HT in the Sham and DOCA, but when NOS is inhibited.

In clinical human literature, it is reported that when a human patient is on cardiopulmonary bypass, they experience profound hypotension. The theory is that platelet activation, such as by shear stress as blood is flowing through pumps in the extracorporeal circulation machine, causes release of 5-HT from the stored granules. The released 5-HT activates $5\text{-}HT_{2B}$ receptors, which are coupled to eNOS, causes NO release and results in vascular dilation. Borgdorff *et al.* showed that in anesthetized autoperfused rats, by using either $5\text{-}HT_2$ antagonists, pizotifen or ritanserin, or the NOS inhibitor L-NNA, the hypotensive effect of platelet-released 5-HT could be abolished, without affecting the activation of the platelets (2002). When we test this *in vitro* using isolated and cleaned vessels from normotensive and hypertensive rats in an isometric tissue bath, vasodilation is not observed; only smooth muscle contraction occurs when 5-HT is applied to the isolated vessels in the tissue bath. Why there is a disjoint between what is observed *in vivo* and *in vitro* is unknown, and it may be that

there is some unknown component present *in vivo* that is not taken into account in the *in vitro* experiments.

There is evidence to suggest that the 5-HT-mediated hypotensive response also may be due to 5-HT_1 receptor activation. Balasubramaniam et al. showed that 5-carboxamidotryptamine (5-CT), a 5-HT_1 receptor agonist, attenuated but did not prevent the development of DOCA-salt hypertension when administered continuously *via* osmotic mini-pump, when on Day 27, the DOCA-salt rats administered 5-CT had a systolic blood pressure 41.7 mmHg lower than Vehicle DOCA-salt rats (1995). 5-HT_1 receptors are reported to be vasodilatory. Ganglionic blockade using hexamethonium was administered on Day 28, and the peak response to hexamethonium in the 5-CT-infused was *smaller* as compared to Vehicle-infused, suggesting a sympatho-inhibitory effect of 5-CT. This attenuated response to DOCA and reduced response to hexamethonium was blocked with methysergide (a 5-HT_1/5-HT_2 receptor antagonist), but not by ketanserin (5-HT_2 receptor antagonist) nor by MDL 72222 (5-HT_3 receptor antagonist).

There is also evidence for the role of 5-HT_7 receptors mediating the long-lasting hypotensive effect of 5-HT seen upon acute intravenous bolus infusion. Terrón *et al.* showed that by using cloned 5-HT_7 receptors and a series of antagonists, the profile of pharmacological data are similar between the cloned 5-HT receptor and that receptor responsible for the hypotensive effect (1997). These data implicate the 5-HT_7 receptor in the functional role of vasodilation mediated by 5-HT.

While strides have been made to elucidate the mechanism by which 5-HT causes a dramatic blood pressure fall in the hypertensive DOCA and normotensive Sham rats, and no change in MAP in the L-NNA nor the DOCA+L-NNA rats, many questions remain. Whether or not 5-HT crosses the blood-brain-barrier or acts though barrier-deficient circumventricular organs to cause centrally-mediated effects has not been investigated. The interaction between 5-HT and NOS and the sympathetic nervous system is yet unclear.

Conclusions

It is clear as a result of these studies described herein that exogenously applied 5-HT causes a drop in MAP in mineralocorticoid hypertension and normotensive sham rats, but does not when NOS is inhibited with L-NNA. What role 5-HT plays in the regulation of blood pressure is not fully elucidated in these studies, but we can draw some conclusions from these data. I have shown that DOCA-salt hypertensive rats have a higher resting PPP 5-HT concentration as compared to $Sham_D$ rats, and that is in agreement with what is currently reported in the literature. While no time course studies have been done here, it appears that there may be a reason for the increase in free circulating 5-HT in established hypertension, and that reason might be the ability of 5-HT to elicit symphatho-inhibition at some level of the sympathetic nervous system and the ability of 5-HT to upregulate or at least preserve eNOS expression and possibly function. These data do not implicate any specific 5-HT receptor to elicit these effects in a mechanistic way, and it is not impossible that 5-HT might be able to exert its effects intracellularly.

Speculation/Future Study

The role of 5-HT in the regulation of blood pressure is not known at this time. The findings of this study are very novel and unexpected. The finding that platelet poor plasma 5-HT levels are increased in the DOCA rats as compared to $Sham_D$ rats was first thought to be a cause or contributor to hypertension may in fact be a result of hypertension, and perhaps may be a physiological attempt to combat hypertension. 5-HT could be protecting the endothelium just by causing a blood pressure fall. Alternatively, if 5-HT upregulates eNOS as Western blots suggest, perhaps this is a physiologic response to increase NO release from the endothelial cells and enhance smooth muscle cell relaxation to dilate vessels to in an attempt to decrease blood pressure. If 5-HT were not present, perhaps blood pressure would have been even higher than is seen in the DOCA.

Vasodilation *via* the action of eNOS to produce NO may only be part of the mechanism. There is evidence that specific gene transfer of neuronal NOS (nNOS) into cardiac noradrenergic cells decreased the sympathetic hyperactivity seen in SHR (Li et al., 2007). Is it possible that other isoforms of NOS, like eNOS, are able to do the same thing?

<u>Other questions for future research</u>

Why are vessels from hypertensive patients and animals hyperresponsive to 5-HT in the isolated tissue bath when the overall net *in vivo* effect of 5-HT is to accomplish a drop in blood pressure? Why does 5-HT potentiate other vasoactive compounds? Why does 5-HT have mitogenic properties that could

contribute to vascular remodeling, one of the cardinal hallmarks of hypertension? The action of 5-HT is quite complex and is still not understood.

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