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THE PRESENCE OF A LOCAL SEROTONERGIC SYSTEM IN
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# THE PRESENCE OF A LOCAL SEROTONERGIC SYSTEM IN PERIPHERAL ARTERIES

By

Wei Ni

A DISSERTATION

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# ABSTRACT

# THE PRESENCE OF A LOCAL SEROTONERGIC SYSTEM IN PERIPHERAL ARTERIES

By

Wei Ni

Serotonin (5-hydroxytryptamine, 5-HT), originally discovered in the intestinal tract and blood, has multiple functions in physiological and pathological conditions. Increases in reactivity to 5-HT have been observed in a number of different forms of hypertension models including DOCA-salt hypertensive rats, spontaneously hypertensive rats and in human patients. However, whether there is a local serotonergic system in peripheral arteries has not been studied. We hypothesized that the existence of a local serotonergic system (including 5-HT synthesis or metabolism, reuptake or release) in peripheral arteries and that this local regulation of 5-HT is altered in hypertension.

We discovered: (1) the presence of essential enzymes for 5-HT synthesis and metabolism in peripheral arteries; (2) peripheral arteries are capable of taking up and releasing 5-HT; (3) the presence of a functional serotonin transporter (SERT), which is the major protein responsible for 5-HT uptake; (4) SERT expression is increased but arterial 5-HT uptake function is decreased in mineralocorticoid hypertension (DOCA-salt hypertensive rats) and nitric oxide synthase inhibited hypertension (LNNA hypertensive rats), but not in spontaneously hypertensive rats compared to their normotensive control.

Our studies showed that a serotonergic system exists in peripheral arteries and may play a role in local control of total peripheral resistance.

# DEDICATION

To my parents, Hengjin Ni and Suli Cai, who gave me life and encouraged me to go after my dream.

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I am appreciated to be in Dr. Watts's lab. I had great time to be with people worked there. To my friend and classmate Dr. Keshari Thakali, thanks her for being my good friend and the person I can always count on. To Janice Thompson, Theo Irina Szasz, Jessica Diaz and Robert Burnett for their friendship and support. I have learned so many things, science and life, from all of you.

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| | |
|---|---|
| 5-HIAA | 5-Hydroxyindoacetic acid |
| 5-HT | 5-Hydroxytryptamine, serotonin |
| 5-HTP | 5-Hydroxytryptophan |
| AADC | Amino acid decarboxylase |
| ACh | Acetylcholine |
| Ang II | Angiotensin II |
| AT 1 | Angiotensin II receptor 1 |
| BCA | Bicinchoninic Acid |
| BH4 | Tetrahydrobiopterin |
| BP | Blood pressure |
| BSA | Bovine serum albumin |
| CNS | Central nervous system |
| DOCA | Deoxycorticosterone acetate |
| DS | Dissociation solution |
| DTT | Dithiothreitol |
| ET-1 | Endothelin- 1 |
| L-NNA | Nω-nitro-L-arginine |
| MAPK | Mitogen-activated protein kinase |
| MAO | Monoamine oxidase |
| mCPP | m-Chlorophenylpiperazine |
| (±)-MDMA | 3,4-methylene-dioxymethamphetamine |
| MI | Myocardial Infarction |

| | |
|---|---|
| NE | Norepinephrine |
| NET | Norepinephrine transporter |
| NOS | Nitric Oxide Synthase |
| NSD 1015 | 3-Hydroxybenzylhydrazine |
| OCT | Organic cation transporters |
| PBS | Phosphate-buffered saline |
| PCPA | Para-chlorophenylalanine |
| PE | Phenylephrine |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PP2A | Protein phosphatase 2A |
| PPH | Primary pulmonary hypertension |
| PSS | Physiological salt solution |
| SBP | Serotonin-binding protein |
| SERT | Serotonin transporter |
| SHR | Spontaneously hypertensive rats |
| SNPs | Single Nucleotide Polymorphisms |
| SNS | Sympathetic Nervous System |
| SSRIs | Selective serotonin reuptake inhibitors |
| TBS | Tris Buffered Saline |
| TBS-T | Tris Buffered Saline + Tween |
| TPH | Tryptophan hydoxylase |
| VMAT | Vesicular monoamine transporter |

| | |
|---|---|
| TGs | Transglutaminases |
| TM | Transmembrane domains |
| TPR | Total peripheral resistance |
| WKY | Wistar-Kyoto rats |
| | |

## Introduction:

Serotonin (5-hydroxytryptamine, 5-HT), as a neurotransmitter in the brain and gastrointestinal tract, is involved in a wide variety of physiological functions such as mood control, urine storage and voiding, the regulation of sleep and body temperature, food intake and intestinal motility. 5-HT was first found as a powerful vasoconstrictor over a century ago and has been recognized as an arterial smooth muscle cell mitogen in the last 20 years (Nemecek et al., 1986). The vascular effects of 5-HT are associated with many vascular diseases including migraine (Buzzi et al., 2005), Raynaud's phenomenon (Cooke JP and Marshall JM, 2005), atherosclerosis (Hara et al., 2004) and were suggested to contribute to liver tissue hypoperfusion following hepatic ischemia-reperfusion (Murata et al., 2003). In the past decade, a strong argument for the role of 5-HT and the serotonin transporter (SERT) in pulmonary hypertension has been built (Marcos et al., 2003; Eddahibi et al., 1999; Eddahibi et al., 2002; Eddahibi et al., 2000; MacLean et al., 2004; Morecroft et al., 1999). However, whether there is a functional serotonergic system present in peripheral arteries and the roles and mechanisms of circulating 5-HT in regulation of non-pulmonary, peripheral arterial function and its contributions to diseases are not clear.

This section begins with an introduction of general background of 5-HT and SERT, followed by a summary of the roles of 5-HT and SERT played in physiological cardiovascular system and cardiovascular diseases, especially in hypertension.

## I. Biosynthesis, storage, and metabolism of 5-HT

### 1. Biosynthesis

**A. Biochemistry:** The essential amino acid tryptophan is the precursor for 5-HT. Serotonin synthesis depends on the specific action and rate-limiting step of the enzyme tryptophan hydroxylase (TPH; EC 1.14.16.4), which transfers a hydroxyl group to the benzyl ring of tryptophan. Subsequent decarboxylation by amino acid decarboxylase (AADC) results in formation of 5-HT (Figure 1). The structure of 5-HT indicates it as a hydrophilic substance, which is preferably protonated at physiological pH. Thus, 5-HT does not pass the lipophilic blood—brain barrier and cell membrane readily.

**B. Location:** Over 95% of 5-HT in the body is synthesized in the enterochromaffin cells of the **intestine**. 5-HT must be synthesized in the brain because 5-HT cannot cross the blood–brain barrier. Those areas responsible for synthesizing 5-HT are the clusters of cells in the **midline/raphe regions** of the pons and upper brainstem. Other sources of 5-HT include neuroendothelial cells that line the **lung**, and a few other discrete sites (Cooper et al., 2003), some of which are in the cardiovascular system. TPH mRNA and protein have been detected in hamster **heart** (Slominski et al., 2002), and 5-HT synthesis has been measured both in an HL-1 cardiomyocyte cell line, which represents adult cardiomyocytes, and in neonatal rat ventricular cardiac myocytes (Ikeda et al., 2005; Cote et al., 2004). Thus, the cardiomyocyte provides a local source of 5-HT in the heart. Both *in vivo* and *in vitro* experiments suggested a **renal** formation of serotonin by decarboxylation of its amino acid precursor L-5-

# 5-HT Biosynthesis and Metabolism Pathway

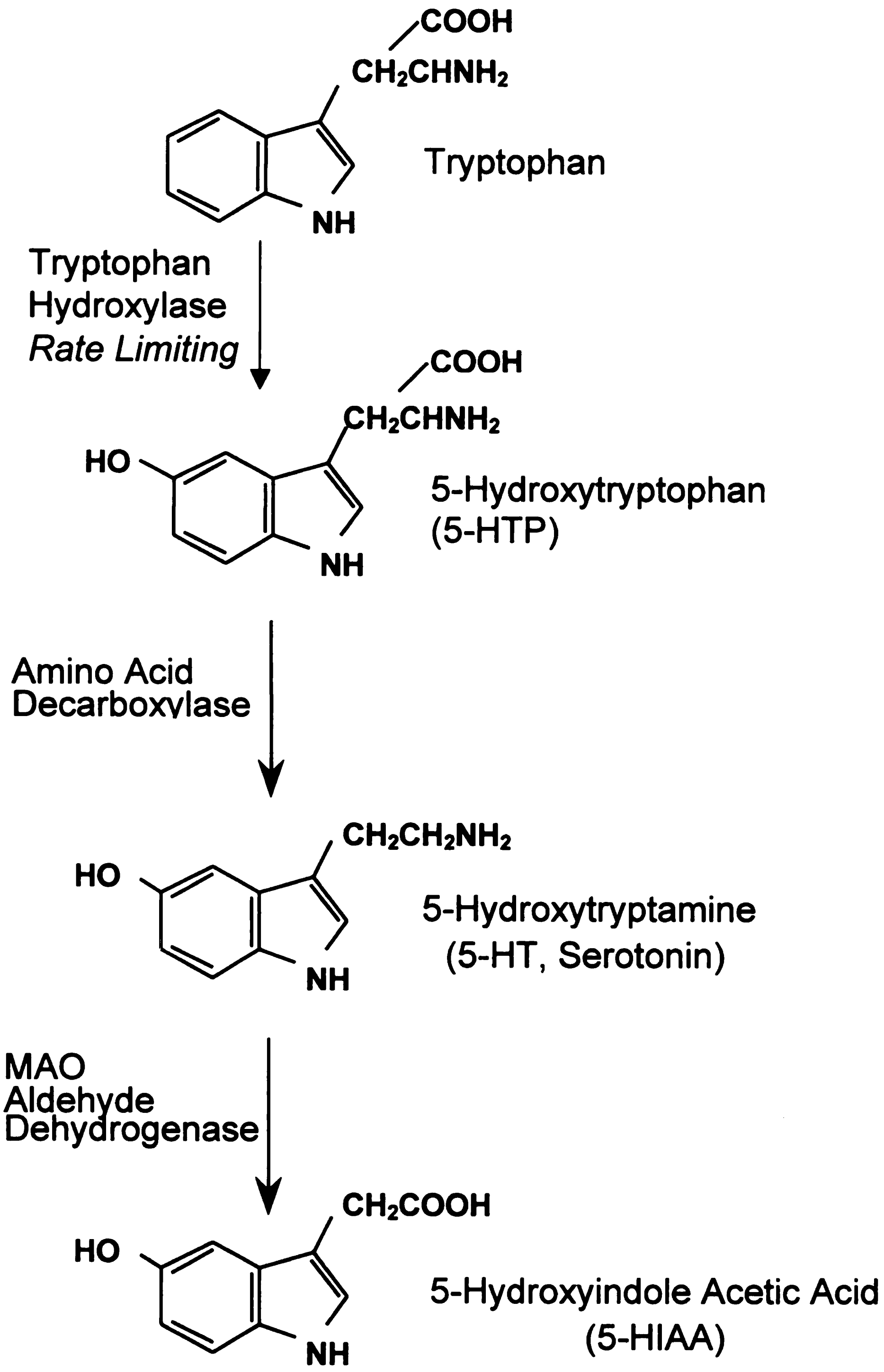


**Figure 1.**

hydroxytryptophan (L-5-HTP) in rat (Stier et al., 1985; Stier et al., 1984). Serotonin synthesis has also been reported in **adrenochromaffin cells** of frogs (Delarue et al., 1992). Recent studies have revealed TPH in new and unexpected places such as the skin (Slominski et al., 2003). Sites of 5-HT synthesis in the non-pulmonary peripheral vasculature have not yet been identified, so our current understanding is that the systemic vasculature is exposed to 5-HT through release of 5-HT by the platelet or to freely circulating 5-HT.

**C. Regulation:** TPH is an extremely labile enzyme, which can be affected by multiple factors. TPH uses **$Fe^{2+}$** as co-factor and **$O_2$** and **tetrahydrobiopterin (BH4)** as co-substrates to hydroxylate tryptophan. Additionally, an *in vitro* study suggested that TPH is an oxygen-inhibitable enzyme. The degree of TPH inactivation is dependent on the partial pressure of **oxygen** to which the enzyme is exposed and the **temperature** at which TPH is preincubated. TPH activity declined in a linear fashion as the temperature increased from 0 to 45 °C (Kuhn et al., 1979). The inactivation of TPH by oxygen could be recovered by anaerobic incubation in the presence of dithiothreitol (DTT) and $Fe^{2+}$ (Kuhn et al., 1979). TPH activity can be irreversibly inactivated by nitric oxide (Kuhn and Arthur, 1997). The phosphorylation of TPH by **protein kinase A (PKA)** and **$Ca^{2+}$/calmodulin-dependent protein kinase II** results in increased TPH activity (Kowlessur and Kaufman 1999). The phosphorylation site for PKA has been reported at serine 58 (Kuhn et al., 1997) and at serine 58 and 260 for $Ca^{2+}$/calmodulin-dependent protein kinase II-induced phosphorylation (Jiang et al., 2000). The 14-3-3 proteins are a group of ubiquitous proteins that were first discovered in the brain (Aitken et al., 1995, review). It has been reported that the **14-3-3 proteins** form a complex with phosphorylated rat brain TPH, thereby increasing TPH enzymatic activity and inhibiting the **protein phosphatase-1** induced dephosphorylation (Banik et al., 1997). It is interesting that, unlike catecholamines, 5-HT synthesis by TPH is not inhibited by the end-product (5-HT) nor by the major metabolite (5-hydoxyindoleacetic acid, 5-HIAA) (Cooper JR et al., 2002). Thus, one way to study 5-HT synthesis is to inhibit AADC to examine the accumulation of 5-HTP.

Recently, Bader and colleagues discovered that tph1 knock-out mice exhibit only a minor reduction of steady state 5-HT levels in brain regions (hippocampus and frontal cortex) with a lack of 5-HT in peripheral organs (gut, blood and pineal gland), suggesting the existence of a second *tph* gene, which they named *tph2*. The expression of *tph1* is predominant in peripheral organs such as the intestine, spleen and pineal gland and *tph2* is present in brain regions (Walther and Bader, 2003; Walther et al., 2003 a), which allows for distinct sources of peripheral and central 5-HT. TPH1 and TPH2 are highly homologous proteins exhibiting 71% of amino acid identity in human (Walther and Bader, 2003). All the residues important for tryptophan, iron, BH4 and 14-3-3 proteins binding are identical in TPH1 and TPH2 (Walther and Bader, 2003). Consistently, both isoforms of TPH can be phosphorylated by $Ca^{2+}$/calmodulin-dependent protein kinase II and PKA. However, the N-terminus of the two isoforms of TPH, which contain the regulatory domains, are quite different. The

$K_M$ values of the purified and recombinant TPH from carcinoid tumors and pineal gland (TPH1) have been reported as 13 and 23 μM, respectively, while the TPH isolated from brain stem (TPH2) exhibits a value of 142 μM (Kowlessur and Kaufman, 1999), which suggests that the substrate of TPH, tryptophan, has higher affinity for TPH1.

**Pharmacology:** Para-chlorophenylalanine (PCPA) is a potent, specific and irreversible inhibitor of TPH activity both *in vivo* and *in vitro*, which drastically reduces the 5-HT concentration in 5-HT neurons and terminals (Pandey et al., 1983). Amphetamine analogs inhibit TPH activity potentially *via* production of nitric oxide, superoxide and peroxynitrite (Kuhn and Geddes, 2000). The interaction of 5-HT and these reactive oxygen species produces tryptamine-4,5-dione, which rapidly and irreversibly inactivates TPH (Wrona and Dryhurst., 2001). 3-Hydroxybenzylhydrazine (NSD 1015) is an AADC inhibitor, which has been used in experiments to block the conversion of 5-HTP to 5-HT (Pandey et al., 1983).

## 2. Storage

**A. Location:** In the periphery, the major 5-HT storage site is platelets. Excluding platelets, the free circulating levels of 5-HT in plasma were reported as 15-120 nM, much lower than the levels of 5-HT in whole blood (μM) (Martin 1994). Other than playing a crucial role in 5-HT synthesis, enterochromaffin cells are also a storage place of 5-HT (Nilsson et al., 1985). In the neuronal system, 5-HT is synthesized in the axon and stored in nerve terminals. Other 5-HT storage sites including mast cells (Pihel et al, 1998), adrenal medullary cells (Brownfield et al., 1985) and pinealocytes (Hayashi et al., 1999).

### B. Mechanisms:

Platelet: Platelets themselves do not synthesize 5-HT. However, they possess a high efficacy SERT, enabling them to take up 5-HT from the gut and lung. As the carrier and storage site of 5-HT, the platelets store 5-HT in dense, electron-opaque granules and release it in a thrombotic event (Vanhoutte, 1991). Storage of 5-HT in platelet granules requires active uptake of 5-HT from the cytoplasm by vesicular monoamine transporter 2 (VMAT 2).

Transglutaminases (TGs) catalyze the calcium-dependent acyl transfer between the $\gamma$-carboxamide of a bound glutamine residue and the $\varepsilon$-amine group of a bound lysine, the primary amine group of a polyamine or possibly monoamine, such as 5-HT (Zhang et al., 1998). Recently, Walther and his colleagues reported that platelet intracellular 5-HT could be transamidated to small GTPases by TGs during activation and aggregation of platelets, rendering these GTPases constitutively active (Walther et al, 2003 b). This observation suggested that 5-HT could be covalently bound to proteins and thus to be stored in platelets.

Enterochromaffin Cells: Study of the subcellular localization of serotonin immunoreactivity in rat enterochromaffin cells showed that 60% of 5-HT was located in the dense cores of the secretory granules following uptake *via* VMAT 1. However, a significant amount of 5-HT (40%) was located in the cytoplasm, outside of the secretory granules (Nilsson et al., 1985), which suggests two different forms of 5-HT storage exist, at least in enterochromaffin cells.

Neuron: After being synthesized in neurons, 5-HT is taken up by VMAT 2 and stored in synaptic vesicles. Different from other non-neuronal 5-HT storing cells such as enterochromoffin cells, mast cells or platelets, soluble serotonin-binding protein (SBP) has been found in serotonergic synaptic vesicles of central and peripheral serotonergic neurons. This difference might come from different origins of the cells. SBP appears to be neuroectoderm-specific, while enterochromaffin cells are from endodermal origin, and mast cells and platelets are derived from mesoderm (Gershon et al., 1983). SBP binds with 5-HT with high affinity in the presence of $Fe^{2+}$. Two SBPs have been identified, with molecular weights of 45 Kd and 56 Kd. Thus, SBP binds 5-HT and functions as a storage protein for 5-HT in neurons (Gershon et al., 1983).

Mast Cells: Mast cells store serotonin and histamine together within large secretory granules (Pihel et al, 1998). Moreover, two serotonin binding proteins were found in mast cells which are different from the SBP found in brain (Tamir et al., 1982).

Thus, from what has been discovered, 5-HT could be stored in vesicles or bound to proteins in cells.

**C. Regulation and Pharmacology:** VMAT functions as a proton-amine exchanger with a stoichiometry of two protons to one amine, which could be 5-HT, dopamine, histamine *etc.* The electrochemical gradient across secretory vesicle membranes (due to the activity of $H^+$-ATPase) provides energy for the transport of monoamines. As indicated above, two types of VMAT have been discovered, VMAT 1 primarily in endocrine cells [enterochromaffin cells, pinealocytes (Hayashi et al., 1999)] and VMAT 2 in neuronal cells and platelets. The two VMATs differ in their ability to transport histamine, and in their sensitivity to certain inhibitors. For example, reserpine blocks both, but tetrabenzine is selective for VMAT2 (Peter et al. 1994). Recently, a study showed that the filling of 5-HT in vesicles initiates an activation of $G\alpha q$ protein (probably on vesicle membrane), which by an unknown 5-HT receptor-independent mechanism downregulates VMAT 2 function in platelets (Holtje et al., 2003). Because VMAT function depends on the electrochemical gradient induced by $H^+$-ATPase, any chemical (such as concanamycin and bafilomycin), which inhibits $H^+$-ATPase will reduce VMAT function and therefore 5-HT storage in vesicles.

TGs transamidate 5-HT to proteins. Cystamine inhibits TGs function and thus potentially reduces 5-HT storage in the form of 5-HT-transamidated proteins.

## 3. Metabolism

### A. Biochemistry:

Metabolism of 5-HT primarily occurs through actions of deamination by monoamine oxidase (MAO) to form 5-hydroxyindole acetaldehyde, which in turn is oxidized by aldehyde dehydrogenase to produce 5-HIAA (Figure 1). Alternatively, 5-hydroxyindole acetaldehyde can be converted to 5-hydroxytryptophol, but this pathway is considered insignificant. Other metabolism pathways include transformation of 5-HT to melatonin. N-acetyl transferase induces acetylation of the amine group of 5-HT to N-acetyl-5-HT. Then, N-acetyl-5-HT is converted to melatonin by adding a methyl group (Cooper JR et al., 2003).

### B. Location:

MAO is an intracellular enzyme, found primarily in mitochondria. 5-HT must be taken up inside a cell prior to being acted upon by MAO and both SERT and norepinephrine (NE) transporter (NET) facilitate this uptake (Kawasaki and Takasaki, 1987). Tissues or cells that contribute significantly to 5-HT metabolism include the lung, intestine and endothelial cells of the arterial system, but any cell that can take up 5-HT and possesses MAO has the potential to metabolize 5-HT. In pineal gland, melatonin synthesis uses 5-HT as a precursor.

### C. Regulation and Pharmacology:

Two isoforms of MAO have been found, MAO A and MAO B. MAO A is more selective and has a much lower $K_M$ value (higher affinity with 5-HT) in metabolizing 5-HT compared to MAO B. MAO A activity increased in rats with low salt diet. This increased activity of MAO A was proved to be related to angiotensin II receptor 1 (AT 1) activation due to increased angiotensin II levels and an increased AT 1 receptor expression induced by low salt diet (De Luca Sarobe et al., 2005). Glucocorticoid increased both MAO A mRNA and protein expression *via* glucocorticoid receptor and Sp1 transcription factor function (Manoli et al., 2005). Pharmacologically, MAO could be inhibited by pargyline.

## II. Uptake and release of 5-HT

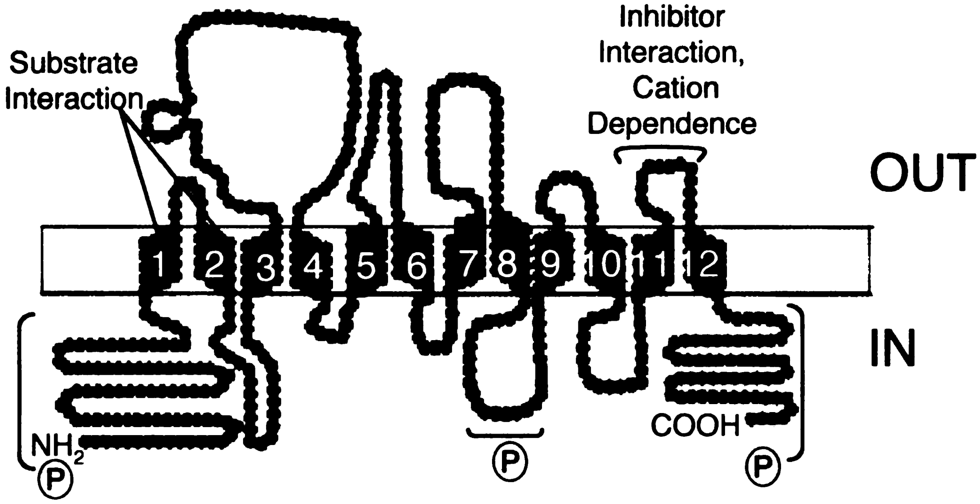
5-HT is a charged molecule (protonated) in physiological condition, which cannot cross lipid bilayer. SERT is the major protein responsible for uptake and release of 5-HT, which transports 5-HT in either direction, depending on the concentration gradient. SERT is important in that it plays a critical role in regulating the function of the 5-HT receptors and the serotonergic system *via* modulation of extracellular and intracellular cytoplasm 5-HT concentrations.

### 1. The major component responsible for 5-HT uptake and release---SERT

#### A. Molecular Biochemistry:

SERT was cloned in 1991 by Hoffman and Blakely in the rat (Hoffman et al., 1991; Blakely et al., 1991). Human SERT was cloned in placental trophoblastic cells in 1993 (Ramamoorthy et al., 1993); the mouse SERT was cloned in 1996 (Chang et al., 1996). Mouse SERT protein has an 88% homology with the human SERT, compared to the rat SERT having 71% homology for the human SERT. SERT proteins are positioned in the plasma membrane. Hydropathy plots of the SERT protein, typically 630 amino acids long, have suggested that the protein spans the bilayer 12 times (12 transmembrane domains; TM) and that both amino and carboxy termini are intracellular (**Figure 2 top**). SERT possesses a large extracellular loop between TM3 and 4. This loop has sites of glycosylation, important for the trafficking and stability of SERT (Chen et al., 2002). In three sites of the protein, the amino terminus, the carboxy terminus and the intracellular loop between TM8 and TM9, consensus sites for

## Proposed Topology of Plasma-membrane SERT



**The 12 membrane-spanning human serotonin transporter**

Modified from http://intramural.nimh.nih.gov/research/lcs/research.html

## Mechanism of 5-HT Transport

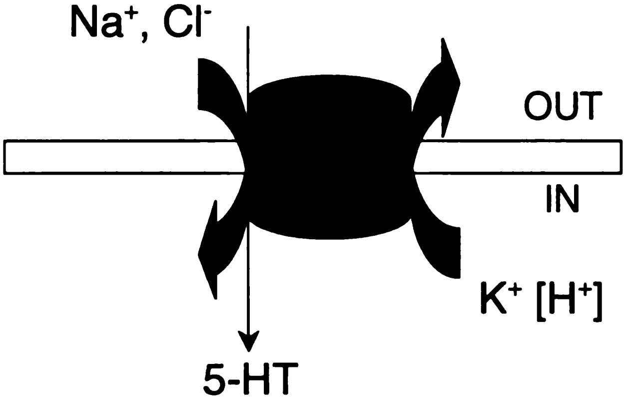


**Figure 2.**

phosphorylation by protein kinase C (PKC) and PKA exist (Ramammoorthy, 2002). TM1-TM3 are important for interactions with substrates such as 5-HT, and TM11 and 12 are crucial for inhibitor interaction and dependence on cations. Translated SERT protein is of a mass anywhere from 60-80 kDa, depending on levels of glycosylation and phosphorylation. In 2000, it was reported that SERT proteins homo-oligomerize (Kilic and Rudnick, 2000). The impact of oligomerization on function is unclear (Schmid et al., 2001 a). Since this time, SERT has been reported to hetero-oligomerize with myosin IIA (Ozaslan et al., 2003), NET (Kocabas et al., 2003), and the GABA transporter 1 (Schmid et al., 2001 b).

### B. Location:

SERT is widely distributed in central nervous system (CNS) and peripheral sympathetic nervous system. SERT was also found in non-neural systems, such as platelets (where 5-HT is stored), cells in gastrointestinal system (where 5-HT is synthesized) and lung (where 5-HT is synthesized and inactivated) (Glilis et al., 1982). Recently, a functional SERT was reported in osteoclast cells (Battaglino et al., 2004).

### C. Function and Physiology:

SERT, a sodium-dependent transporter, diminishes the function of 5-HT at its extracellular cognate receptors by removing 5-HT from outside the cell and bringing it back into a cell for metabolism *via* MAO A or vesicular repackaging. The present model of SERT functioning (**Figure 2 bottom**) begins with extracellular sodium binding to the carrier protein. 5-HT, in its protonated form, then binds to the transporter and is followed by a chloride. This initial complex creates a conformational change in the transporter protein: it revolves from facing extracellularly to an inward-facing position where 5-HT and ions are released in the cytoplasm.

Intracellular potassium binds to SERT to promote reorientation of SERT for another cycle to the outside of the cell, at which time $K^+$ is released. These elements ($Na^+$, $Cl^-$, 5-HT, and $K^+$) are thought to work in a single multifunctional binding site. The driving force of the 5-HT transport across the plasma membrane *via* SERT is dependent on the $Na^+$ concentration gradient. $Na^+$ ions flow down their concentration gradient and 5-HT accumulates in the cells. The energy to build $Na^+$ concentration gradient comes from hydrolysis of ATP by ATPase. ATP is utilized by $Na^+$-$K^+$-ATPase to actively transport 3 $Na^+$ ions out of the cell and pump 2 $K^+$ ions into the cell. Therefore, 5-HT transport across plasma membrane indirectly uses energy released by ATP hydrolysis. Thus, the function of SERT is inhibited by metabolic inhibitors as well as by inhibitors of $Na^+$-$K^+$ ATPase activity.

It is reasonably assumed that the function of 5-HT is terminated once brought inside the cell. However, two laboratories have independently demonstrated the necessity of uptake of 5-HT by SERT in mediating pulmonary arterial smooth muscle proliferation to 5-HT (Marcos et al., 2003; Lee et al., 2001). Additionally, a finding in the platelet supports the idea that 5-HT, once intracellular, exerts physiological effects. Walther *et al.* demonstrated that 5-HT, actively imported into a platelet by SERT, acts as a substrate for an enzyme class of TGs to covalently modify RhoA or Rab 4 with the 5-HT moiety (transamidation or 'serotonylation') (Walther et al., 2003 b). Serotonylation of Rho A renders it constitutively active. Numerous other protein targets of TGs, such as actin, have been identified (Griffin et al., 2002). Thus, intracellular 5-HT has functional effects, which could be regulated by SERT by changing intracellular 5-HT concentrations. While the platelet was the initial cell type in which this was demonstrated, it is currently unknown whether intracellular 5-HT can alter function in cell types of other cardiovascular tissues. It is possible that vasoconstriction could be modulated by Rho A and actin serotonylation.

### D. Pharmacology:

5-HT possesses high affinity (17.4 nM) for the rat and human SERT derived from the brain (Rothman et al., 1999), and recognition of 5-HT occurs within the first three transmembrane domains of the transporter. Drugs that interact with transporter proteins can be divided into two basic classes: **reuptake inhibitors** and **substrate-type releasers**. Reuptake inhibitors bind to transporter proteins, but are not transported into cells themselves. These drugs elevate extracellular 5-HT concentrations by blocking transporter-mediated recapture of extracellular 5-HT. Substrate-type releasers bind to SERT proteins, and subsequently are transported into cytoplasm. In neurons, releasers elevate extracellular transmitter concentrations by a two-step mechanism: first they promote efflux of transmitter by a process of VMAT-mediated exchange, and then they increase cytoplasmic levels of transmitter by disrupting storage of 5-HT in vesicles. The increased 5-HT in the cytoplasm by the second step provides more 5-HT for release by transporter-mediated exchange. Because substrate-type releasing agents must be transported into nerve terminals to promote transmitter release, reuptake inhibitors may block the effects of releasers (Rothman and Baumann, 2002). A variety of serotonin-selective reuptake inhibitors (SSRIs), such as fluoxetine (Prozac®), fluvoxamine (Luvox®), sertraline (Zoloft®) and citalopram (Celexa®) are medications used for the treatment of psychiatric disorders, including depression, panic disorder, and obsessive-compulsive disorder (Gorman and Kent, 1999; Zohar and Westenberg, 2000).

In contrast to the SSRIs, which are commonly used drugs, there is only one 5-HT releaser, (±)-fenfluramine (Pondimin®), that has been used clinically. (±)-Fenfluramine and the more potent stereoisomer (+)-fenfluramine (dexfenfluramine, Redux®) were highly effective anorexigens approved by the Food and Drug Administration in 1996 for use in long-term medical management of obesity. These drugs were widely prescribed until they were withdrawn from the market in September 1997 due to reports of cardiac valvulopathy (Connolly et al., 1997) and primary pulmonary hypertension (PPH) (Abenhaim et al., 1996). (±)-Fenfluramine is metabolized in liver to (+)-norfenfluramine and (-)-norfenfluramine. Most of the studies agree that fenfluramine and norfenfluramine are SERT substrates and potent 5-HT releasing agents (Garattini, 1995). Other 5-HT releasers include the drug 3,4-methylene-dioxymethamphetamine [(±)-MDMA or "ecstasy"] and the non-amphetamine piperazine derivatives m-chloropheylpiperazine (mCPP), which is a major metabolite of the anti-depressant trazodone (Otani et al., 1998).

## E. Regulation:

### Long-term regulation:

The SERT promoter in multiple species contains such basic elements as a TATA-like, CREB, NFκB, P2, and SPI site (Chen and Reith, 2002; Ramammoorthy, 2002; Blakely et al., 1998). Interestingly, an allele in the human SERT promoter has been found, resulting in 14 (short or *s* allele) or 16 (long or *l* allele) repeat elements, a difference of 44 base pairs and expression of an mRNA species either 484 (*s*) or 528 (*l*) base pairs long (Heils et al., 1996). Possession of the *s* allele is associated with a lower expression of SERT (estimated at 1/3 of those possessing two *l* alleles), resulting in a reduced capability to take up and release 5-HT. In the human, the *ss* allele has been associated with, but not proven to cause, an inability to handle stressful life events (Murphy et al., 2004). Thus, a genetically-inheritable difference in alleles suggests that humans, naturally, may express different amounts of SERT throughout the whole body, including the cardiovascular system. Additionally, at least 25 different Single Nucleotide Polymorphisms (SNPs) of SERT have been reported for the human. The phenotypes, cardiovascular or otherwise, of these SNPs are not known.

### Acute regulation:

<ins>Phosphorylation / Dephosphorylation:</ins> When SERT proteins are artificially expressed in cells, SERT activity is regulated in response to alterations in calcium/calmodulin-dependent kinase, PKC, PKA, and protein kinase G (PKG)

activity; SERT bears sites for serine/threonine phosphorylation (Mlinar and Corradetti, 2003; Ramamoorthy et al, 1998; Blakely et al., 1998). Intensive studies were done to demonstrate alteration of SERT activity *via* phosphorylation/dephosphorylation by two pathways, **the PKG/p38 mitogen-activated protein kinase (MAPK)/protein phosphatase 2A (PP2A) pathway** and the **protein kinase C (PKC) pathway**. PKG/p38 MAPK/PP2A activation increases SERT activity by stimulating insertion of intracellular SERT into the cell membrane (Blakely et al., 2005) and increasing total SERT activity (Zhu et al., 2005). By contrast, PKC activation reduces SERT function by phosphorylating SERT protein and causing translocation of SERT to the cytosol (Jayanthi et al., 2005).

Data also support that 5-HT itself may regulate the activation status of the SERT, independent of 5-HT receptors. Ramamoorthy *et al.* found that in HEK293 cells transfected with SERT, which possessed no detectable 5-HT receptors, 5-HT inhibited SERT phosphorylation and decreased the ability of PKC activation-induced SERT activity reduction (Ramamoorthy et al, 1998; Blakely et al., 1998). Preservation of SERT function by 5-HT makes logical sense. Other SERT substrates, such as D-amphetamine and fenfluramine have a similar inhibitory effect on PKC-mediated phosphorylation and internalization (Ramamoorthy and Blakely, 1999).

5-HT$_{2B}$ Receptor-related Regulation: 5-HT receptor-dependent SERT regulation has been revealed. One study using 1C11 cells showed that 5-HT$_{2B}$ receptor regulates SERT function differently in the presence and absence of 5-HT. In the absence of external 5-HT, 5-HT$_{2B}$ receptor coupling to NO production ensures PKG/p38 MAPK induced SERT phosphorylation and maximal 5-HT uptake (Launay et al., 2006; Zhu et al., 2004). In the presence of 5-HT, the 5-HT$_{2B}$ receptor mediates PKC-dependent SERT phosphorylation, which reduces the maximal velocity of 5-HT uptake (Launay et al., 2006). In addition, 5-HT$_{2B}$ receptor activation also reduces $Na^+$-$K^+$-ATPase activity by $Na^+$-$K^+$-ATPase catalytic subunit phosphorylation. Because the basic function of $Na^+$-$K^+$-ATPase is to maintain the high $Na^+$ and $K^+$ gradient across the plasma membrane, which provides energy for 5-HT uptake by SERT, the impaired $Na^+$-$K^+$-ATPase function also reduces SERT activity (Launay et al., 2006). The inhibition of SERT function through 5-HT$_{2B}$ receptor activation is also supported by Dr. Maroteaux group's *in vivo* study. An acute agonist stimulation of 5-HT$_{2B}$ receptor triggers a transient increase in plasma serotonin that is SERT-dependent and blocked by 5-HT$_{2B}$ receptor-selective antagonist or genetic ablation (Callebert et al., 2006). Altered 5-HT$_{2B}$ receptor expression and function have been reported in different diseases, such as hypertension (Banes et al., 2003; Russell et al., 2002) and pulmonary hypertension (Launay et al., 2002). It is possible that this changed 5-HT$_{2B}$ receptor function will modify SERT function in diseases.

Membrane Cholesterol and Lipid Microdomains: Depletion of membrane cholesterol reduces SERT activity by changing the affinity of 5-HT for SERT and a concomitant reduction of the maximal transport rate (Scanlon et al., 2001). The function of SERT could be partially rescued by replacement of cholesterol, but not by other sterols. A further study demonstrated that SERT has to be present in cholesterol rich-lipid rafts (lipid microdomains) on the membrane to be functional. An equilibrium may be found between an active raft-associated state (in cholesterol rich-lipid rafts) and an inactive state residing outside lipid rafts in plasma membrane-bound SERT. The detection of SERT-containing lipid rafts are found in both intracellular and cell surface fractions suggesting that lipid rafts association may be important for trafficking and targeting of SERT (Magnani et al., 2004).

## 2. Other components responsible for 5-HT uptake and release---Non-SERT dependent

NET, dopamine transporter (DAT) and SERT (monoamine transporters) belong to the $Na^+,Cl^-$—dependent gene family and share many similarities in terms of function, mechanism and regulation. In the peripheral cardiovascular system, 5-HT is taken up and released by adrenergic nerves (Kawasaki et al., 1987). DA can be taken up by NET (Gu et al., 1994). It is possible that monoamine transporters have the ability to act promiscuously and thus DAT and NET might take up 5-HT.

Other candidates responsible for non-SERT uptake include the organic cation transporters (OCT). The laboratory of Michael Gershon has demonstrated a gastrointestinal upregulation of OCT-1/3 in SERT-deficient mice. Thus a member of the OCT family is a reasonable candidate for alternative 5-HT uptake (Chen et al, 2001). OCTs are promiscuous transporters, with substrates as diverse as 5-HT, DA, NE, epinephrine, histamine, clonidine, and cimetidine. Pharmacologically, OCTs are difficult to distinguish from one another but can be distinguished from SERT by inhibition by corticosterone, O-methylisoprenaline and levamisole (Martel and Azevedo, 2003; Horvath et al, 2003).

## III. 5-HT Function Mechanisms---- in General

5-HT has a wide variety of effects on almost all systems. Both intracellular 5-HT and extracellular 5-HT have functions in physiological and pathological conditions.

### 1. Extracellular 5-HT functions (5-HT receptors):

Typically, 5-HT exerts its effects by activating 5-HT receptors. There are seven families of 5-HT receptor ($5\text{-HT}_1$-$5\text{-HT}_7$), which have been identified in the last 16 years and 15 subtypes have been cloned (Tierney 2001). Most 5-HT receptors are G-protein coupled receptors, except the $5\text{-HT}_3$ receptor, which is a ligand-gated ion channel. The $5\text{-HT}_{1B}$, $5\text{-HT}_{1D}$, $5\text{-HT}_{2A}$, $5\text{-HT}_{2B}$, $5\text{-HT}_3$, $5\text{-HT}_4$, $5\text{-HT}_7$ receptors are found in the cardiovascular and/or renal systems. The major 5-HT receptors responsible for the 5-HT-induced vasoactive effect are $5\text{-HT}_1$ and $5\text{-HT}_2$ receptors, while $5\text{-HT}_7$ receptor activation results in vessel relaxation.

### 2. Intracellular 5-HT functions:

It has been revealed during the past decade that the function of 5-HT is not terminated once brought inside the cell. Two laboratories have independently demonstrated the necessity of uptake of 5-HT by SERT in mediating pulmonary arterial smooth muscle proliferation to 5-HT (Marcos et al., 2003; Lee et al., 2001). Additionally, as stated above, a finding in the platelet supports the idea that 5-HT, once intracellular, exerts physiological effects by transamidating RhoA or Rab 4 (Walther et al., 2003 b). Moreover, Battaglino *et al.* showed another exciting mechanism by which 5-HT could function intracellularly. They suggested that intracellular 5-HT activates NF-kappa B to enhance osteoclast differentiation and that the SERT inhibitor, fluoxetine could cause bone mass decrease by inhibiting 5-HT uptake into osteoclast cells (Battaglino et al., 2004). These studies showed new signal transduction pathways supporting that intracellular 5-HT could exert its effects independent of 5-HT receptors. Thus, it is possible that in other cell types, such as vascular smooth muscle or endothelial cells, intracellular 5-HT also has function and could regulate vascular smooth muscle tone independent of 5-HT receptors.

## IV. 5-HT and SERT in Physiological Cardiovascular System

### 1. 5-HT in Physiological Cardiovascular System

### A. Blood

Blood elements, particularly platelets, are a rich source of 5-HT. 5-HT is taken up by SERT in platelets (Holmsen and Weiss, 1979) as platelets do not synthesize 5-HT. The function of 5-HT in the blood is to promote platelet aggregation and blood clotting. Although platelets contain 5-HT, they also possess 5-$HT_{2A}$ receptors which, when activated, promote further platelet activation and aggregation (Vanhoutte, 1991). Thus 5-$HT_{2A}$ receptor antagonists such as ketanserin and sarpogrelate have proven effective anti-platelet/antithrombotic agents. Other cells, which contain 5-HT, include mast cells and macrophages.

### B. Brain / Sympathetic Nervous System

Approximately 1–2% of body 5-HT is found in the brain. The central effects of 5-HT on the cardiovascular system are complex. The effects of 5-HT vary according to the site in which 5-HT is administered and the anesthetic status of the animal. In general, in the rat, central injection of 5-HT increases blood pressure (BP), although there are studies that suggest the opposite effect (Callera et al.,1997). Moreover, the effects of central 5-HT in other species, primarily cat and dog, are different from the rat in that 5-HT largely causes a decrease in BP. It is clear that central 5-HT has the capability of altering BP and appears to do so by interacting with both 5-HT$_{1A}$ and 5-HT$_2$ receptors (Watts, 2005).

### C. Heart

5-HT has pluripotent effects in the heart, outside of the well-documented effects in the coronary arteries (Doggrell, 2003). Multiple receptors for 5-HT exist in the heart, including those directly on cardiac myocytes and on the vagus and sympathetic nerves. 5-HT stimulation of 5-HT$_3$ receptors on the vagus nerves accounts for the decrease in heart rate through activation of the Bezold–Jarisch reflex. 5-HT can also act as a sympatholytic through activation of 5-HT$_1$ receptors on sympathetic terminals, inhibiting NE release. However, 5-HT exerts positive chronotropic effect in the rat through activation of 5-HT$_{2A}$ receptors, activation of 5-HT$_4$ receptors in the pig and human and 5-HT$_7$ receptors in the cat

(Cote et al., 2004; Saxena and Villalon, 1991; Villalon et al., 1997). In isolated cardiomyocytes, 5-HT also stimulates mitogenesis *via* the 5-$HT_{2B}$ receptor, which is critical for development of the heart in the mouse (Nebigil and Maroteaux, 2001).

### D. Kidney

Like the brain, the kidney has the ability to synthesize 5-HT from its precursor tryptophan (Stier and Itskovitz; 1984; Stier et al., 1984; Hafdi et al., 1996). 5-HT is also a mitogen in mesangial cells (Mene et al., 1991), but its most profound effect in the kidney is to increase perfusion pressure by increasing renovascular resistance through arterial contraction. This has been demonstrated primarily in isolated perfused kidneys, although 5-HT is vasodilatory in the renal vascular bed of the dog (Tian et al., 2002).

5-HT and dopamine play the opposite role in sodium retention in the kidney. 5-HT causes sodium retention in the kidney, whereas dopamine promotes natriuresis (Berndt et al., 2001). The precursors for 5-HT and dopamine synthesis, L-5-HTP and L-DOPA, use the same transporter to enter the proximal convoluted tubule, thereby compromising synthesis of the other substance. Moreover, 5-HT and 5-$HT_{1B}$ receptor agonists stimulate the sodium/phosphate cotransporter to decrease phosphate retention, potentially contributing to renal failure (Hafdi et al., 1996).

### E. Blood Vessels

Blood vessels, in particular arteries, serve an important function in providing a controlled feed of blood to tissues through creation of a resistance to blood flow or total peripheral resistance (TPR). TPR is defined by the reactivity of small arteries and arterioles (≤200 μm in humans), where resistance to blood flow is determined by the calibre of the artery. This, in turn, is governed by the size of the lumen based on (i) thickness of arterial wall; (ii) reactivity of the smooth muscle and endothelial cells to neuronal neurotransmitters and endogenous hormones; and (iii) concentration of vasoactive substances.

The effect of 5-HT on vasculature is complicated. Under normal conditions, 5-$HT_{2A}$ receptor-mediated 5-HT-induced constriction in the systemic circulation, while the endothelial 5-$HT_{2B}$ receptor relaxes pre-contracted rat aorta (Villazon et al., 2002) and pig pulmonary artery (Glusa and Pertz, 2000). Studies showed that subcontractile concentration of 5-HT interact synergistically with α-adrenergic agonists and other vasoconstrictors including Ang II, histamine and prostaglandin $F_{2\alpha}$ (Yildiz, 1998 for review). 5-HT-induced synergism with a vasoconstrictor presents a more relative physiological role of 5-HT because of the belief that free circulating 5-HT in plasma is low. Moreover, Cohen *et al.* reported that in the rabbit saphenous vein, 5-HT enhances the release of NE by stimulating prejunctional 5-$HT_{1A}$ receptors on the noradrenergic nerves (Cohen et al., 1999), thus 5-HT may also regulate vascular smooth muscle tone by modulating the sympathetic neurotransmission in blood vessels. 5-HT is also a smooth muscle cell and endothelial cell mitogen, and, as in the event of contraction, 5-HT can potentiate the mitogenic effect of other hormones (Nemecek et al., 1986; McDuffie et al., 2000).

### F. Whole body

When 5-HT is administered to a whole animal, the pattern of observed BP changes varies based on species. In the rat, intravenous 5-HT elicits a classical triphasic effect: an initial fast depressor (Bezold–Jarisch reflex *via* 5-$HT_3$ receptor activation), a pressor response (smooth muscle contraction *via* 5-$HT_2$ receptor activation) and a longer depressor response (probably activation of arterial 5-$HT_7$ receptors, Dalton et al., 1996). It should be noted that, in other species, the cardiovascular response to 5-HT is different: 5-HT (administered intravenously) decreased BP in broiler chickens (Chapman and Wideman, 2002), increased BP in healthy calves (Linden et al., 1999) and increased BP in conscious sheep (Nelson et al., 1987). Differences in these responses are probably explained by different receptor expression, metabolism of 5-HT, uptake of 5-HT *etc.*

## 2. SERT in the Cardiovascular System

SERT functions as the major protein responsible for the transport of 5-HT across membranes. It regulates the extracellular and intracellular 5-HT concentrations and thus 5-HT functions. The study of SERT function in cardiovascular system has been done by pharmacologically inhibiting SERT and using genetically-altered mice.

### A. Pulmonary Vasculature:

The physiological importance of SERT function is not well studied in many cardiovascular-related organs. The best studied is the pulmonary vasculature. In pulmonary endothelial and bovine smooth muscle cells, uptake of 5-HT *via*

SERT is necessary for 5-HT to function as a mitogen. Moreover, Morecroft *et al.* demonstrated that citalopram and fluoxetine inhibited 5-HT-induced contraction of pulmonary arteries isolated from the normal Sprague-Dawley rat (Morecroft et al., 2005). In contrast, these agents potentiated contraction to 5-HT in pulmonary arteries from Fawn-Hooded rats. The Fawn-Hooded rat is a strain with genetically-impaired 5-HT storage and reuptake and, interestingly, is susceptible to both pulmonary and systemic hypertension (Gonzalez et al., 1998). The reason(s) for differences in the effects of the inhibitors in arteries from Sprague-Dawley *vs.* Fawn-Hooded rats are not well understood. Citalopram also potentiated contraction to 5-HT in the isolated intralobar pulmonary artery in an endothelium-dependent manner in normoxic rats and endothelium-independent manner in hypoxic rats (Wanstall et al., 2003). The fluramines (SERT substrate and 5-HT releaser) also cause direct pulmonary vasoconstriction (Weir et al., 1996; Desta et al., 1998) or potentiate vasoreactivity to other vasoconstrictors (Barman and Isales, 1999). Collectively, these studies suggest that in the pulmonary vasculature, SERT plays an important role in normal mitogenic and contraction functions.

### B. Platelet Function:

SERT inhibitors decrease platelet 5-HT content, inhibit thrombosis and exacerbate bleeding (Maurer-Spurej, 2005) because of prevention of 5-HT reuptake. However, an alternative argument has been proposed. Immediately upon use of SSRIs, platelet 5-HT would not be depleted but extracellular 5-HT would be higher because of preventing 5-HT reuptake, promoting a thrombotic event (Kurne et al., 2004; Isbister et al., 2004). Thus, SERT inhibition may cause dual effects in thrombosis.

The ability of promoter variants of SERT to influence function of the cardiovascular system is illustrated by studies in platelets. Platelets from humans which possess the *L* variant of the SERT promoter have a higher Vmax than platelets from humans with no *L* variants (Greenberg et al., 1999).

### C. Cardiac Function:

Compared to the tricyclic group of antidepressants, SERT inhibitors have a decreased ability to induce cardiac arrthymias. However, citalopram has been reported to increase the QT interval in humans (Kelly et al., 2004) and Pacher *et al.* have published extensive work demonstrating the arrhythmogenic activities of SERT inhibitors, specifically fluoxetine and citalopram, in the hearts of dogs, rabbits, rats, and guinea pigs (Pacher et al., 1999; Pacher et al., 1998).

### D. Other Cardiovascular Organs:

In the mouse adrenal medulla, SERT is required for stress-evoked increases of catecholamine synthesis and Ang II $AT_2$ receptor expression in the adrenal gland (Armando et al., 2003).

The physiological function of SERT in other cardiovascular organs such as the heart, peripheral vasculature, and kidney has not been thoroughly investigated. One possible way to determine the role of SERT in cardiovascular function is to examine the effect of removal or inhibition of SERT. A mouse, which lacks a functional SERT was created by the group of Dr. Dennis Murphy. Few studies on the physiological cardiovascular parameters in these animals have been performed. Mice overexpressing SERT have recently been created. Systemic arterial pressure was measured in these animals under anesthesia. Wild type animals had a pressure of 76±3.4 mm Hg, while the SERT overexpressing mice had a pressure of 82.9±3.8 mm Hg. Similarly, heart rates were not different between wild-type and SERT transgenic mice. These measures may not reflect true blood pressure and heart rate due to the effects of the anesthetic. Effects in the adrenal, kidney, brain and heart, as they pertain to the cardiovascular system, were not noted, and thus questions as to the function of SERT in these organs remain unanswered.

## V. Serotonergic System in Cardiovascular Diseases

Evidence suggest that 5-HT plays roles in cardiac arrhythmias (5-HT$_4$ receptor activation), coronary artery disease (raised blood 5-HT levels) and vasospastic angina (increased transcardiac levels of 5-HT and supersensitivity of the coronary artery to 5-HT) (Doggrell for review, 2003). The discoveries of anorexigen-induced valvular heart disease (Connolly et al., 1997) and PPH (Abenhaim et al., 1996) have driven the investigation of the association of 5-HT in these diseases. A clinical study showed that the plasma concentration of 5-HT and circulating levels of 5-HIAA are increased in PPH patients (Herve et al., 1995; Kereveur et al., 2000). The loss of *tph1* gene expression, and thus lack of peripheral 5-HT was linked to a cardiac dysfunction phenotype (Cote et al., 2003). This section focuses on 5-HT and SERT related cardiovascular diseases, especially hypertension.

### 1. Serotonergic System and Myocardial Infarction (MI):

5-HT is normally released by activated platelets, causing vascular contraction and enhanced platelet aggregation, which may lead to thrombus formation and contribute to the pathogenesis of acute MI. On the other hand, antidepressants, particularly the SSRIs with high affinity for SERT, attenuate platelet activation by depleting 5-HT storage in normal volunteers and in patients with coronary artery disease (Hergovich et al. 2000; Serebruany et al., 2001). More direct evidence is that the use of SSRIs with high affinity for the SERT is associated with reduced odds of MI, and the extent of SERT inhibition among SSRIs correlates with the degree of reduction in MI risk (Sauer et al., 2003).

The LL genotype of the SERT polymorphism is associated with a higher risk of MI (Fumeron et al., 2002), SS genotype seems to have a protective role against MI, delaying the age of onset of the first episode (Coto et al., 2003). This is in agreement with the evidence that inhibition of SERT reduced the risk for MI (Sauer et al., 2003). Collectively, these studies confirmed that SERT activity (the ability to regulate 5-HT concentration) is important in MI.

### 2. Serotonergic System and PPH:

5-HT and SERT have been reported to play an important role in the pulmonary vascular smooth muscle hyperplasia and vascular remodeling and have been associated with experimental hypoxic pulmonary hypertension and human PPH. Consistently, the SERT inhibitors, citalopram and fluoxetine, protect against hypoxic pulmonary hypertension (Marcos et al., 2003). Mice lacking SERT (5-HTT$^{-/-}$) developed less hypoxia-induced right ventricular systolic pressure increase and vascular remodeling than paired 5-HTT$^{+/+}$ controls (Eddahibi et al., 2000). The LL genotype in SERT promoter was present in a higher level in patients with PPH (Eddahibi et al., 2001) and chronic obstructive pulmonary hypertension (Eddahibi et al., 2003).

### 3. Serotonergic System and Hypertension:

A finding common to experimental and genetic models of hypertension, as well as in the hypertensive patient, is an increase in TPR. Although, 5-HT has been recognized as a powerful vasoconstrictor for many decades, the involvement of 5-HT in the etiology of hypertension is still controversial. The following paragraphs lay out the evidence that support and refute the role of 5-HT and SERT in hypertension.

#### A. Free Circulating 5-HT Concentration and 5-HT Receptors Activation

Evidence Refuting the Involvement of the Serotonergic System in Hypertension: Free circulating plasma levels of 5-HT are relatively low (15–120 nmol/L compared with micromolar levels in whole blood, Martin, 1994). This free circulating 5-HT is the 5-HT that would interact with the blood vessels, and thus it has been argued that this is an insufficient level of 5-HT to activate 5-HT receptors normally expressed (primarily 5-HT$_{2A}$ receptors on smooth muscle and 5-HT$_1$ receptors in the endothelial cell). The role of the relatively newly discovered vascular 5-HT$_7$ receptor is less well understood.

Evidence Supporting the Involvement of the Serotonergic System in Hypertension: Enhanced 5-HT-induced arterial responses in hypertension has been recognized as early as 1970. These effects have been observed as an increased response to 5-HT including decreased threshold concentration of 5-HT to induce contraction, increased potency ($EC_{50}$) and efficacy in experimental hypertension, including the mineralocorticoid (deoxycorticosterone acetate, DOCA)-salt hypertension rats, spontaneously hypertensive rats (SHR), stroke prone SHR, Dahl-salt sensitive, 1 kidney-1 clip, nitric oxide synthase-inhibited model of hypertension and even in coronary arteries from human patients (Cohen et al., 1988; Collis and Vanhoutte, 1977; McGregor and Smirk, 1970; Watts, 1998; Nishimura and Suzuki 1995; Golino et al., 1991). These changes in hypertension have been defined as hyperresponsiveness or hyperreactivity.

The local and/or circulating level of 5-HT can be considered sufficient to activate endogenous 5-HT receptors. The majority of 5-HT is stored in platelets. Thrombotic events in which platelets aggregate can result in a high (micromolar) local concentration of 5-HT (Holmsen and Weiss, 1979). Moreover, subcontractile concentrations of 5-HT amplify arterial contraction to vasoactive agonists such as Ang II, endothelin- 1 (ET-1) and NE, to name a few (Turla and Webb, 1989; MacLennan et al., 1993; Yildiz et al., 1998). This is important as low concentrations of 5-HT (nanomolar) are able to modify arterial contraction to substances which control TPR (e.g. Ang II, ET-1 and NE).

Moreover, 5-HT has a significantly higher affinity for the 5-$HT_{2B}$ receptor ($K_A$ =10 nmol/l), and this receptor is expressed in arterial smooth muscle. Importantly, the arterial 5-$HT_{2B}$ receptor is expressed to a significantly greater level in experimental hypertension and at least three 5-$HT_{2B}$ receptor antagonists have been reported to have antihypertensive effect and reduce vascular reactivity to agonists in experimentally induced hypertension (Watts and Fink, 1999; Shingala and Balaraman, 2004). 5-$HT_{1A}$ and 5-$HT_7$ receptor agonists were suggested to have a role in the treatment of hypertension (Wainscott et al., 1996; Doggrell, 2003) because 5-$HT_{1A}$ and 5-$HT_7$ receptor activation are known to have a vasodilatory effect.

Thus 5-HT has significant effects on the contractile state of vascular smooth muscle directly and indirectly.

### B. Uses of 5-HT Receptor Antagonists in the Treatment of Hypertension

Evidence Refuting the Involvement of the Serotonergic System in Hypertension: There are a number of studies examining the effect of 5-HT receptor antagonists in treating high BP which have had negative outcomes. A majority of this work revolves around the use of ketanserin. This 5-$HT_{2A/2C}$ receptor antagonist was used as an effective antihypertensive strategy over two decades ago. Ketanserin lowered BP of normal and hypertensive subjects, including humans, but BP reduction was largely attributed to $\alpha_1$ adrenergic receptor blockade, not 5-$HT_2$ receptor blockade (Cohen et al., 1983). Presently, ketanserin is used in a population of women with severe pregnancy induced hypertension (van Schie et al., 2002). Additional studies using 5-$HT_{2A}$ receptor antagonists that lacked affinity for the $\alpha$ adrenergic receptors, such as cinanserin, did not lower BP in the anaesthetized cat (McCall and Harris, 1987; Ramage, 1988). Lowering of BP in anaesthetized SHRs by ketanserin or LY53857 seemed to be dissociated from 5-$HT_2$ receptor blockade (Docherty, 1989). In the hypertensive human (Stott et al.,

1988) and in SHRs (Gradin et al., 1985), use of ritanserin did not lower BP (ritanserin lacks affinity for the $\alpha_1$ adrenergic receptor and has a high affinity for the 5-$HT_{2A}$ receptor; http://pdsp.cwru.edu/pdsp.asp). These studies suggest that endogenous activation of 5-HT receptors is not important for maintaining elevated BP.

Evidence Supporting the involvement of the Serotonergic System in Hypertension: Some other studies used 5-HT receptor antagonists as antihypertensive therapy which dissociate the $\alpha$ adrenergic receptor blockade caused by ketanserin from 5-$HT_{2A}$ receptor blockade or which demonstrate the effectiveness of 5-HT receptor antagonists in lowering BP in the absence of appreciable $\alpha$ receptor antagonism. This includes use of ketanserin in the SHR (Balasubramaniam et al., 1993). One study (Wenting et al., 1984), published in 1984, demonstrated a reduction in high BP by ketanserin in humans by 22%. This same concentration of ketanserin was not able to reduce a pressor response to the $\alpha$ receptor agonist phenylephrine (PE), indicating that the reduction in BP caused by ketanserin was independent of $\alpha$ adrenergic receptor blockade. Consistent with the *in vitro* observation that the 5-$HT_{2B}$ receptor antagonist, LY272015, reduces 5-HT-induced vasoconstriction in arteries from DOCA-salt hypertensive rats, but not normotensive rats (Russell et al., 2002; Watts et al., 1996). LY272015 also lowers blood pressure in hypertensive rats but not normotensive rats (Watts and Fink, 1999). There are also specific forms of hypertension that have been described as 5-HT dependent. This includes an erythropoietin-driven model of hypertension (Azzadin et al., 1995) and cyclosporine-induced hypertension (Krygicz et al., 1996).

### C. High L-tryptophan Diet and Blood Pressure

Evidence Refuting the Involvement of the Serotonergic System in Hypertension: Studies using L-tryptophan feeding in SHRs demonstrated a dose-dependent decrease in BP (Sved et al., 1982), as did studies in DOCA-salt rats (Fregly et al., 1987) and in humans (Woittiez et al., 1995). L-Tryptophan is the necessary precursor for 5-HT synthesis and should elevate 5-HT synthesis through law of mass action. Thus, if 5-HT endogenously increases BP, tryptophan should also do so by virtue of increasing endogenous 5-HT. In the later study, salt intake was reduced by tryptophan, suggesting an activity to decrease elevated BP.

Evidence Supporting the Involvement of the Serotonergic System in Hypertension: A number of studies suggest that tryptophan feeding, as a means to increase 5-HT synthesis, may promote higher BP. SHRs given L-tryptophan in their diet had an increased BP within 30 min of administration, and a maximal elevation of BP 60 min post-administration (Howe et al., 2003). In a study published in 1991, Ito *et al.* gave tryptophan to female stroke-prone SHRs prior to mating, and discovered that the offspring of these mothers had a higher BP, were heavier and had a higher CNS load of 5-HT (Ito et al., 1991). These findings suggest, but do not prove, that an increased 5-HT level was associated with a higher systemic BP.

### D. TPH Function Inhibition and Blood Pressure

Evidence Refuting the Involvement of the Serotonergic System in Hypertension: Depletion of 5-HT by PCPA, an irreversible inhibitor of TPH, did not lower the BP of SHRs (Collis and Vanhoutte, 1981).

Evidence Supporting the Involvement of the Serotonergic System in Hypertension: A few studies on removing or destroying 5-HT have demonstrated a concomitant decrease of BP in hypertensive animals. In 1976, Buckingham *et al.* (Buckingham et al., 1976) demonstrated that a single injection of 400 mg/kg of body weight (intraperitoneally) of the TPH inhibitor PCPAME (p-chlorophenylalaninemethyl ester) produced a fall in BP in DOCA-treated rats ranging from 20–43 mmHg within the first day of injection, and kept BP depressed for 8 days. PCPAME competes directly with TPH and binds irreversibly to the enzyme, hence the long-lasting effects of PCPAME. In the normal rat, a fall of 15–20 mmHg was observed only 8 days after injection of PCPAME; there was no immediate fall in BP. No measurements of 5-HT were made in plasma or arteries of the rats receiving PCPAME, and we propose that part of the fall in BP was because 5-HT was depleted in the periphery. This idea is supported by the findings that central 5-HT depletion by intracisternal injections of the serotonergic neurotoxin 5,6-DHT (5,6-dihydroxytryptamine) did not alter the course of development or magnitude of hypertension achieved in the DOCA-salt rat (Myers et al., 1974) or SHRs (Browning et al., 1981), nor did 5,6-DHT (i.c.v) alter the ability of PCPA to cause a reduction in BP (Buckingham et al., 1976).

## E. Other Evidence Supporting the Involvement of Serotonergic System in Hypertension

In hypertension, platelet function and SERT activity are changed. Opposite observations of how platelet function changes in hypertension have been reported. A study in human essential hypertension indicated that platelets in the early stages of essential hypertension display an overall increased aggregation potential with an elevated cytosolic free calcium level (Taylor et al., 1989). More recently, increased platelet aggregability was found in hypertensive patients with carotid artery plaque, suggesting a further promotion of the progression of arteriosclerosis in those patients (Fusegawa et al., 2006).

However, other studies, such as in the stroke-prone SHR, showed that aggregation of platelets from the hypertensive rat is decreased compared to normotensive controls (Tomita et al., 1984). In DOCA-salt hypertensive rats, platelet aggregation is decreased compared to normotensive controls (Umegaki and Ichikawa, 1993). The outcome of a majority of studies is that the content of 5-HT in platelets is decreased. In theory, these platelets, termed 'exhausted' (depleted due to overstimulation), have already been activated early *in vivo* due to hypertension (Umegaki and Ichikawa, 1993). In the human, platelets from hypertensive subjects have a lower 5-HT content (Ding et al., 1994) and take up less 5-HT than do platelets from normotensive patients (Kamal et al., 1984).

There are still some other hints suggesting that SERT function is changed in hypertension. Plasma 5-HT concentration and the Vmax values for the 5-HT uptake process were significantly higher in severely pre-eclamptic women, compared with age and gestation-matched normal pregnant women and plasma 5-HT concentration was directly related to systolic and diastolic BP with severity of the syndrome (Carrasco et al., 1998). Thus, it is conceivable that a changed SERT activity and changed 5-HT concentration could happen in other types of hypertension and related to BP increase or maintain BP at a high level.

### F. An Unanswered Question ----- The Existence of Local Regulation of 5-HT in Peripheral Arteries

The evidence presented suggests the ability of endogenous 5-HT to modulate vascular smooth muscle tone and thereby to alter TPR and BP.

Any process changing 5-HT concentrations—such as synthesis, metabolism, uptake and storage/release will therefore regulate 5-HT functions. It is important to confirm/refute that peripheral arteries have local regulation of 5-HT concentration and functions.

## Hypotheses:

**Hypothesis #1** (Figure 3):

A local serotonergic system, including synthesis, metabolism, storage, uptake and release of 5-HT is present and functional in peripheral vasculature.

**Hypothesis #2**:

Changes in the localized serotonergic system contribute to the increase of blood pressure in hypertension.

**Figure 3.**

Diagram of working hypotheses.

5-HIAA=5-Hydroxyindole acetic acid, 5-HT=5-hydroxytryptamine, 5-HTP=5-hydroxytryptophan, AADC=amino acid decarboxylase, MAO A=monoamine oxidase A, TPH= tryptophan hydroxylase.

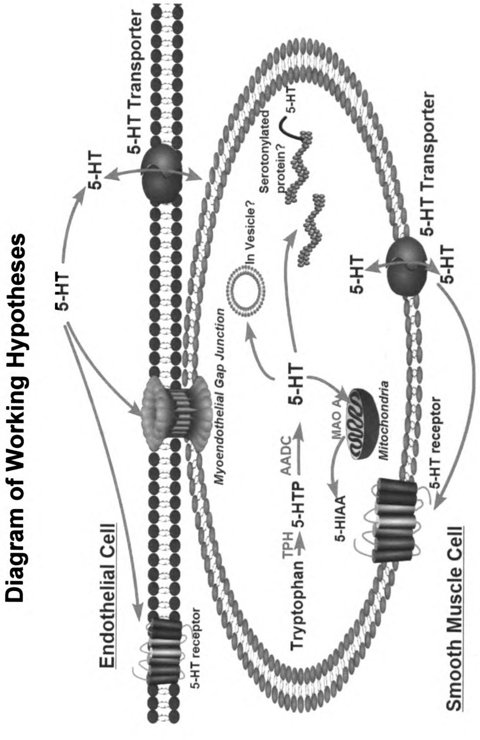


Diagram of Working Hypotheses

Figure 3.

## Methods:

### I. Animal Uses

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) or Harlan Industries, Inc. (Indianapolis, IN). Male SHR and Wistar-Kyoto rats (WKY, 12 weeks) were purchased from Taconic Farms, Inc. (Germantown, NY). Normal male C57BL/6 mice (20-24 g) were purchased from Charles River (Portage, MI). The SERT targeted mutation mice and wild type male mice (C57BL/6; 30-40 g) were received from Dr. Dennis L. Murphy, National Institute of Mental Health. The *Tph1 -/-* mice and wild type mice (C57BL/6, 20-24 g) were received from Dr. Michael Bader, Max Delbrück Center for Molecular Medicine (Germany). Until surgery, the rats were kept in clear plastic boxes with free access to standard rat chow (Teklad ®) and tap water.

### II. Euthanasia:

Rats were anesthetized with pentobarbital (60 mg/kg, i.p.). Mice were sacrificed by asphyxiation with carbon dioxide.

### III. Animal Models:

Mineralocorticoid Hypertension:

Male Sprague-Dawley rats (250-300 g; Charles River, Portage, MI) were anesthetized with isoflurane (IsoFlo®). Animals were uninephrectomized and a

Silastic® (Dow Corning, Midland, MI) implant impregnated with DOCA pellet (200 mg/kg) was placed subcutaneously on the back of the neck. Postoperatively, rats were given a solution of 1% NaCl and 0.2% KCl for drinking. Sham rats also received a uninephrectomy, but received no DOCA implant and drank normal tap water. Animals were fed standard rat chow and had *ad libitum* access to food and water. The animals remained on the regimen for four weeks prior to use.

### L-NNA Hypertension

Male Sprague-Dawley rats (250-300 g; Harlan, Indianapolis, IN) were given tap water mixed with Nω-nitro-L-arginine (L-NNA, 0.5 g/L). The animals were remained on the regimen for two weeks prior to use.

### 6-OHDA Denervation

Sympathetic neuronal denervation was induced by 6-hydroxydopamine (6-OHDA) injections (McCafferty et al., 1997). Male Sprague-Dawley rats were treated with four doses of 6-OHDA over 7 days (50 mg/kg on days 1 and 2 and 100 mg/kg on days 6 and 7; 0.1% ascorbic acid in physiological saline as vehicle i.p.). Rats were euthanized (pentobarbital, 60 mg/kg i.p.), and arteries were removed on day 8. Denervation was validated by glyoxylic acid staining in mesenteric resistance arteries. Glyoxylic acid staining: Mesenteric resistance arteries were removed. After fat was trimmed off, arteries were immersed into glyoxylic acid (2%) for 5 min. Blood vessels were mounted on a microscope slide and placed in an oven (100°C) for 5 min. The slides were removed and blood vessels mounted in mineral oil and cover-slipped. Vessels were viewed using a fluorescence microscope (Nikon LABOPHOT) and UV illumination (G-1A; excitation, 546/10 nm; barrier filter, BA580). The absence of a fluorescent network of staining of catecholamines in arteries confirms the effectiveness of 6-OHDA in causing sympathetic denervation.

## IV. BP Measurements:

Systolic BP of conscious rats were determined by the tail cuff method using a pneumatic transducer. Briefly, the rat was placed in a plastic pail with wood shavings covering the bottom. This pail was then placed on a heating pad and the rat was contained in the bucket by a small metal cage. A warming light was placed over the bucket. The rat was warmed for approximately 6 min. This allowed vasodilatation of the tail artery, which facilitated the measurement of the BP. The rat was placed in a restraint and the blood pressure cuff and balloon transducer was placed on the tail and secured with tape. The BP was measured utilizing a sphygmomanometer in conjunction with the pulse transducer. Three blood pressure measurements were taken to obtain an average measurement.

## V. 5-HT Basal Level Measurement

Aorta from normotensive and hypertensive animals were dissected, cleaned and placed in 75 μL of 0.05 mM sodium phosphate & 0.03 mM citric acid buffer (pH 2.5) containing 15% methanol. Samples were frozen at -80°C at least 4 hours.

## VI. TPH Activity Assay

*In vivo* 5-HT synthesis

Aorta, superior mesenteric artery and hypothalamus from NSD1015 (100 mg/kg, 30 min) or NSD1015 (100 mg/kg) + L-tryptophan methyl ester hydrochloride (300 mg/kg, 60 min)–treated rats were dissected, cleaned in physiological salt solution (PSS, 103 mM NaCl; 4.7 mM KCL; 1.18 mM $KH_2PO_4$; 1.17 mM $MgSO_4$-$7H_2O$; 1.6 mM $CaCl_2$-$2H_2O$; 14.9 mM $NaHCO_3$; 5.5 mM Dextrose, and 0.03 mM $CaNa_2EDTA$) and placed in tissue buffer [0.05 mM sodium phosphate & 0.03 mM citric acid buffer (pH 2.5) containing 15% methanol]. Samples were frozen at –80 °C until assay.

*In vitro* 5-HT synthesis

Aorta, superior mesenteric artery and dorsal *raphe* nucleus from normal rats were dissected, cleaned in incubation solution [100 μM $Fe(NH_4)(SO_4)_2$, 1 mM DTT, 50 mM Tris-HCl, pH 7.4]. Tissues were incubated in 1.5 ml plastic centrifuge tubes with incubation solution plus 3250 U/ml catalase and 10 mM NSD 1015 at 37 °C for 30 min. Then 200 μM BH4 and either $H_2O$ or 400 μM tryptophan were added. After 40 min incubation, tissues were rinsed in drug-free PSS, placed in 75 μL tissue buffer and saved at –80 °C until assay.

## VII. 5-HT Storage Assay

At room temperature, naïve or 5-HT-loaded (5-HT 1 µM, 15 min) aortae from untreated or MAO A inhibitor pargyline (100 mg/Kg)-treated rats were incubated with PSS in 1.5 ml plastic centrifuge tubes for 4 or 8 hours. Tissues were then briefly dipped in drug-free PSS and placed in 75 µL tissue buffer. Samples were frozen at –80 °C until assay.

## VIII. 5-HT Uptake Assay:

At room temperature (unless otherwise specified), dissected and cleaned arteries from normal or pargyline (100 mg/Kg)-treated animals were placed in PSS in 1.5 ml plastic centrifuge tubes containing either vehicle or inhibitor for 30 min. 5-HT (1 µM) or vehicle (water) was then added for a varying amount of time (time course studies) or for 15 min in experiments with inhibitor. Tissues were then briefly dipped in drug-free PSS and placed in tissue buffer. Samples were frozen at –80 °C until assay. In some experiments, the endothelium was removed by gently rubbing the luminal face of the artery with a moistened cotton swab.

## IX. 5-HT Release Assay:

At room temperature, dissected and cleaned arteries from normal or pargyline (100 mg/Kg)-treated animals were placed in 100 µL PSS or SERT inhibitor (diluted in PSS) in 250 µL microcentrifuge tubes for 30 min. Water or (+)-norfenfluramine (10 µM), in some experiments (+)-fenfluramine (1, 10 µM), was then added for 20 min. Tissues were taken out of the tubes and the PSS solution in tube was saved on ice (PSS samples) and used to test 5-HT and 5-HIAA release. Tissues were briefly dipped in drug-free PSS and placed in tissue buffer (tissue sample, used to test 5-HT and 5-HIAA left in tissues). PSS samples and tissue samples were frozen in -80 °C until assay.

### X. 5-HIAA and 5-HT Measurement:

Samples were thawed, sonicated for 3 seconds and centrifuged for 30 seconds (10,000 g). Supernatant was collected and transferred to new tubes. Tissue pellets were dissolved in 1.0 M NaOH and assayed for protein. Concentrations of 5-HIAA and 5-HT in tissue supernatants and PSS samples from 5-HT release assay were determined by isocratic High Performance Liquid Chromatography (HPLC) coupled with electrochemical detection. Fifty microliters of tissue supernatant or PSS sample was injected onto a C18 reverse phase analytical column (Biosphere ODS, West Lafayett, IN) protected by a precolumn cartridge filter. This column was coupled to a single coulometric electrode conditional cell in series with dual electrode analytical cells (ESA, Bedfore, MA). The conditioning electrode potential was set at 0.4V, while the analytical electrodes were set at 0.12 and –0.31 V relative to the internal silver reference electrodes. Amounts of 5-HIAA and 5-HT were determined by comparing peaks areas in samples with those obtained from standards run the same day, and reported as a concentration relative to protein content. The lower limit of sensitivity for detection of 5-HIAA and 5-HT was 2-5 picogram/sample.

## XI. Real Time RT-PCR:

Two-step RT-PCR was performed using a GeneAMP 7500 Real Time PCR machine (Applied Biosystems, Foster City, CA). Total RNA from rat aorta and superior mesenteric artery were isolated using the MELT Total RNA isolation System Kit (Ambion, Austin, Taxes). Concentration of RNA was measured spectrophotometrically (A260/A280). One microgram of total RNA was reverse transcribed using a Taqman® reverse transcriptase kit (Applied Biosystems, Foster City, CA, U.S.A.; buffer, $MgCl_2$ 5.5 mM, dNTP 500 µM, of each random hexamer 2.5 µM, RNase inhibitor 0.4 µg/µL and MultiScribe Reverse Transcriptase 1.25 µg/µL; 10 min hold at 25°C, 30 min hold at 48°C, 5 min hold at 95°C). One microliter of this cDNA was taken through PCR using a SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, U.S.A.). PCR conditions were: 95°C 10 min for AmpliTAQ® activation, 40 cycles of PCR (15 seconds 95°C, 60 seconds 60°C). Two sets of rat tph1 primer pairs were used. One of the rat tph1 primer pair, rat β-actin primer pair and β-2-microglobulin (B2m) pair were purchased from Superarray (Frederick, MD). An alternative rat tph1 primer pair was designed based on RefSeq Accession number: XM-341862.1 and was synthesized by the Macromolecular Structures and Synthesis Facility at Michigan State University. The rat *tph1* primer forward= GCC TGC TTT CTT CCA TCA GT. The rat *tph1* primer reverse= AGA CAT CCT GGA AGC TTG TGA. The SERT primer forward= GGC CAG TAC CAC CGA AAC, SERT primer reverse = CGG GGC AGA TCT TCC TCC ATA T.

$C_T$ values were derived as the threshold cycle at which product was first detected and are reported as cycle numbers.

## XII. Immunohistochemistry:

Preparation of frozen sections: Tissues were frozen in OCT compound and stored at -80°C until use. Sections were cut, cold acetone fixed, washed 3 times with phosphate-buffered saline (PBS).

Preparation of paraffin-embedded sections: Formalin-fixed, paraffin-embedded rat thoracic aorta and superior mesenteric artery were washed twice in xylenes and four times in 90% ethanol for 3 min each to dewax. Sections were unmasked by microwaving them twice for 3 min in Vector Antigen Unmasking Solution.

Endogenous peroxidase in fixed frozen sections or dewaxed, unmasked paraffin-embedded section were blocked [0.3% $H_2O_2$ in phosphate-buffered saline, PBS, for 30 min]. Sections were blocked for non-specific binding in PBS containing 1.5% of competing serum. In a humidified chamber, samples were incubated overnight with antibody (10 μg/mL, anti-TPH antibody, Sigma; 5 μg/mL anti-SERT, C-20, Santa Cruz; 5 μg/mL, anti-5-HT YC5/45, Abcam, UK), antibody neutrialized with 5-fold excess of competing peptide or blocking serum with antibody. The remaining steps were carried out in accordance to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Sections were washed 3 times with PBS and incubated with a peroxidase-conjugated secondary antibody (30 min, room temp). Samples were washed and incubated with Vectastain® ABC Elite reagent (30 min, room temp) followed by 3,3'-diaminobenzidine (DAB)/$H_2O_2$. The reaction was stopped with washing, sections were air dried, hematoxylin-stained, mounted and photographed using an inverted Nikon microscope with a Spot digital camera.

### XIII. Immunocytochemistry:

Rat thoracic aorta and superior mesenteric artery were dissected and cut into small rings in chilled dissociation solution [DS, containing (M): NaCl 0.137; KCl, 0.0056; $MgCl_2$, 0.001; $Na_2HPO_4$, 0.00042; $NaH_2PO_4$, 0.00042; $NaHCO_3$, 0.0042; Na nitroprusside 2.6 mg/L and HEPES, 2.383 mg/L; pH=7.4]. Small vessel rings were incubated with enzymatic solution (containing papain, 26 U/ml and DTT, 1 mg/ml) for 35 min and other enzymatic solution (containing, type II collagenase 2.5 U/ml, elastase 0.15 mg/ml and soybean trypsin inhibitor, 1 mg/ml) for 45 min. We then draw off other enzymatic solution and gently added 3 ml of fresh DS and left on ice for 5 min, which was followed by draw off DS without disturbance of the cells at the bottom. The cells were then triturated in DS containing Na nitroprusside (0.01 mM). One hundred uL of titrated cells were put on polylysine coated coverslips and left in incubator (37°C, 5% $CO_2$) for 30-45 min. After the cells attached to coverslips, the DS was carefully removed and the cells were fixed with Zamboni's fixative (20 min). The cells were rinsed with low-salt PBS twice (5 min each time) and cell membrane was permeablized with 1% triton (5 min) followed by blocking serum incubation (5% serum, 20 min). Cells were then incubated with primary antibody for 2 hours (37°C, anti-5-HT antibody, anti-TPH antibody, 1:200, Sigma, anti-SERT antibody 1:200). Cells were washed with low-salt PBS three times, followed by 1 hour incubation with goat-anti-mouse Cy3 antibody or donkey-anti-goat Cy3 antibody (37°C, 1:1000, Jackson ImmunoResearch). The coverslips were then mounted and photographed using an inverted Nikon microscope or a confocal microscope with a digital camera. Images in this dissertation are presented in color.

## XIV. Protein Isolation:

Rat/mouse thoracic aorta and rat superior mesenteric artery were removed from the animal and placed in PSS and cleaned as described above. Arteries were quick 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 and 10 μg/ml leupeptin]. Homogenates were centrifuged (11,000 g for 10 min, 4 °C). We utilized the Bicinchoninic Acid (BCA) method for protein measurement (Sigma, St.Louis, MO). Samples were stored at –80 °C until use.

## XV. 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 $H_2O$ and 2 mL working reagent were mixed and incubated for 30 min at 37 °C, no $CO_2$. 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.

### XVI. Western Blotting:

Tissues homogenates (4:1 in denaturing sample buffer, boiled for 5 min) were separated on SDS-polyacrylamide gels and transferred to Immobilon-P membrane. Membranes were blocked for 3-4 hours (Tris Buffered Saline, TBS, 4% chick egg ovalbumin or 5% dry milk, 0.25% sodium azide). Blots were probed overnight with primary antibody (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 (pH 7.6) (20 mM Tris and 137 mM sodium chloride) and incubated with secondary antibody for 1 hour at 4 °C following with TBS-T and TBS washes. Blots were incubated with ECL® reagents to visualize the bands.

### XVII. Isolated Smooth Muscle Contractility Measurement:

The thoracic aorta was removed and placed in PSS. The aorta was cleaned of fat and connective tissue and cut into helical strips. Aortic strips were then mounted onto stainless steel rod holders and placed into 50 ml tissue baths for isometric tension recordings using Grass polygraphs and transducers. Strips were placed under optimum resting tension (1,500 mg for rat aorta, determined previously) and allowed to equilibrate for one hour before exposure to compounds. In experiments testing tissues from hypertensive animals, one aortic strip isolated from a normotensive control and one aortic strip from a hypertensive rat 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 strip viability; the strips must contract to a minimum of 500 mg for rat aorta to be considered viable. To examine the status of the arterial endothelium, tissues were contracted with a half-maximal concentration of PE (10-100 nM) and once the contraction plateaued, the muscarinic agonist acetylcholine (ACh, 1 μM) was administered. The observation of a relaxation to ACh greater than 60% of the PE (10-100 nM) -induced contraction was considered as endothelium-intact. Cumulative concentration curves were performed to agonists. Antagonists, inhibitors or vehicle were incubated with the vessels for one hour prior to the experimentation.

## XVIII. Data Analysis and Statistics:

Data are presented as means ± standard error of the mean for the number of animals in parentheses.

5-HIAA and 5-HT concentrations were quantified using standards run the same day, and reported as a concentration relative to protein content.

Contractions are reported as force (milligrams) or as a percentage of response to maximum contraction to PE. $EC_{50}$ values (agonist concentration necessary to produce a half-maximal response) were determined using nonlinear regression analysis in Prism version 4.0 (San Diego, CA) and were reported as the mean of the negative logarithm (-log) of the $EC_{50}$ value (M). These values represent the concentration necessary to produce a half-maximal response in each tissue using the tissue's own maximum response.

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.

When comparing two groups, the appropriate Student's t-test was used. When comparing 3 or more groups, an ANOVA followed by Newman Keuls *post hoc* test was performed. In all cases, a *p* value less than or equal to 0.05 was considered statistically significant.

## Results

**Hypothesis #1:**

**A local serotonergic system, including synthesis, metabolism, storage, uptake and release of 5-HT is present and functional in peripheral vasculature.**

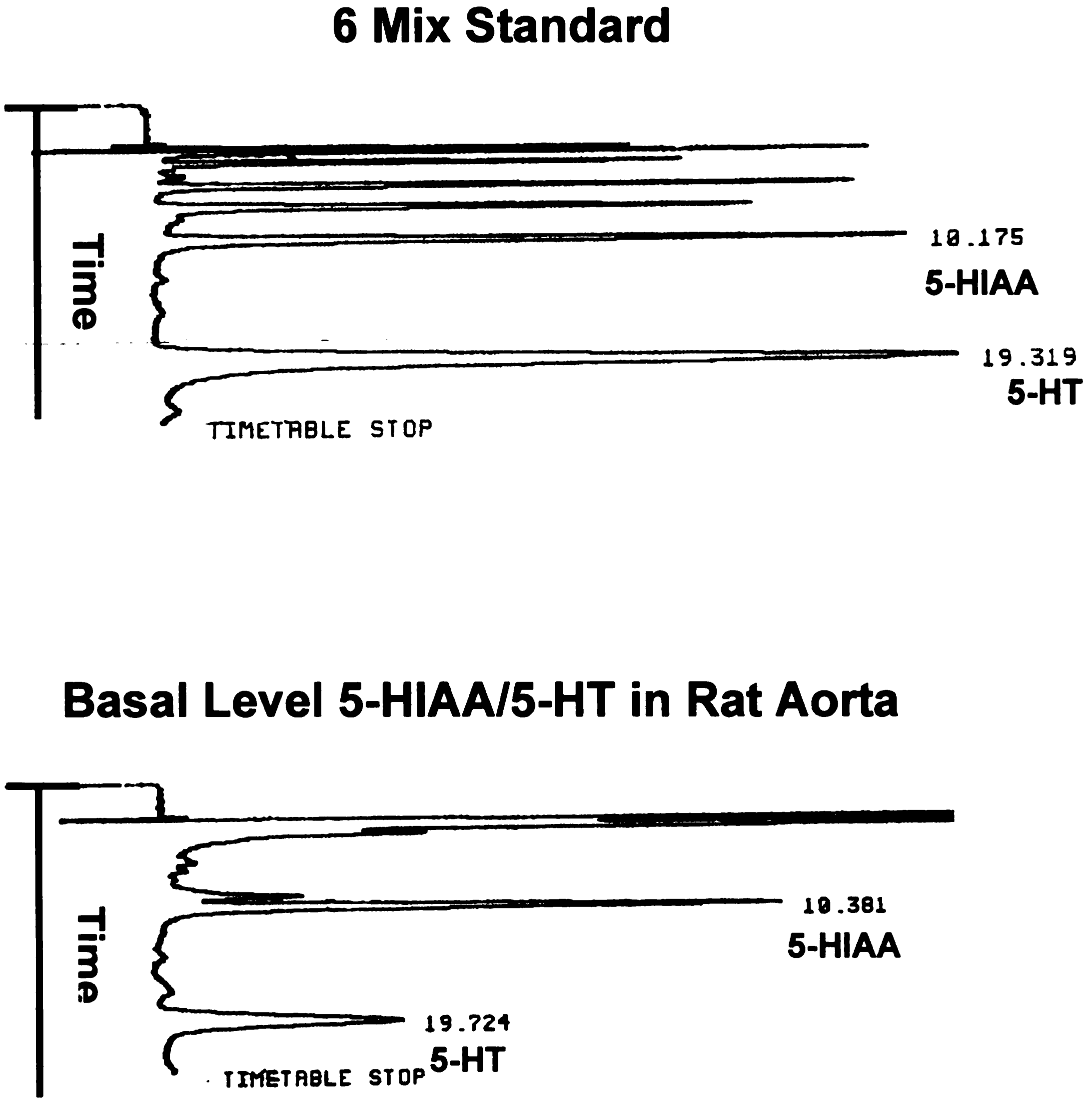
We investigated the serotonergic system in peripheral arteries on the following aspects: (1) presence of 5-HT; (2) 5-HT synthesis and metabolism; (3) 5-HT storage; (4) 5-HT uptake and release.

### I. The Presence of 5-HT in Peripheral Arteries

Whether peripheral arteries contain endogenous 5-HT has not been reported. We used three different experimental techniques, which provided the same results suggesting the presence of 5-HT in peripheral arteries.

By using HPLC analyses, we detected the content of 5-HT and its MAO-A metabolite 5-HIAA in aorta, superior mesenteric artery and carotid artery. Figure 4 shows a standard chromatogram (Top, a representative of all standard tracings in data from HPLC) and a chromatogram of basal 5-HT and 5-HIAA in rat thoracic aorta (bottom). The quantification of basal 5-HIAA and 5-HT levels in rat aorta and superior mesenteric artery and mouse aorta are reported in Figure 5.

Figure 6 depicts the results of IHC experiments using a 5-HT antibody, localizing 5-HT to rat aorta. The presence of 5-HT in peripheral arteries was



6 Mix Standard

Basal Level 5-HIAA/5-HT in Rat Aorta

**Figure 4.**

Top: Chromatogram showing separation of 1 ng standards using HPLC.

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

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

**Figure 5.**

Basal levels of 5-HIAA and 5-HT in rat aorta (top), rat superior mesenteric artery (middle) and mouse aorta (bottom) from normal animals.

5-HIAA=5-hydroxyindoleacetic acid, 5-HT=5-hydroxytryptamine, SMA=superior mesenteric artery.

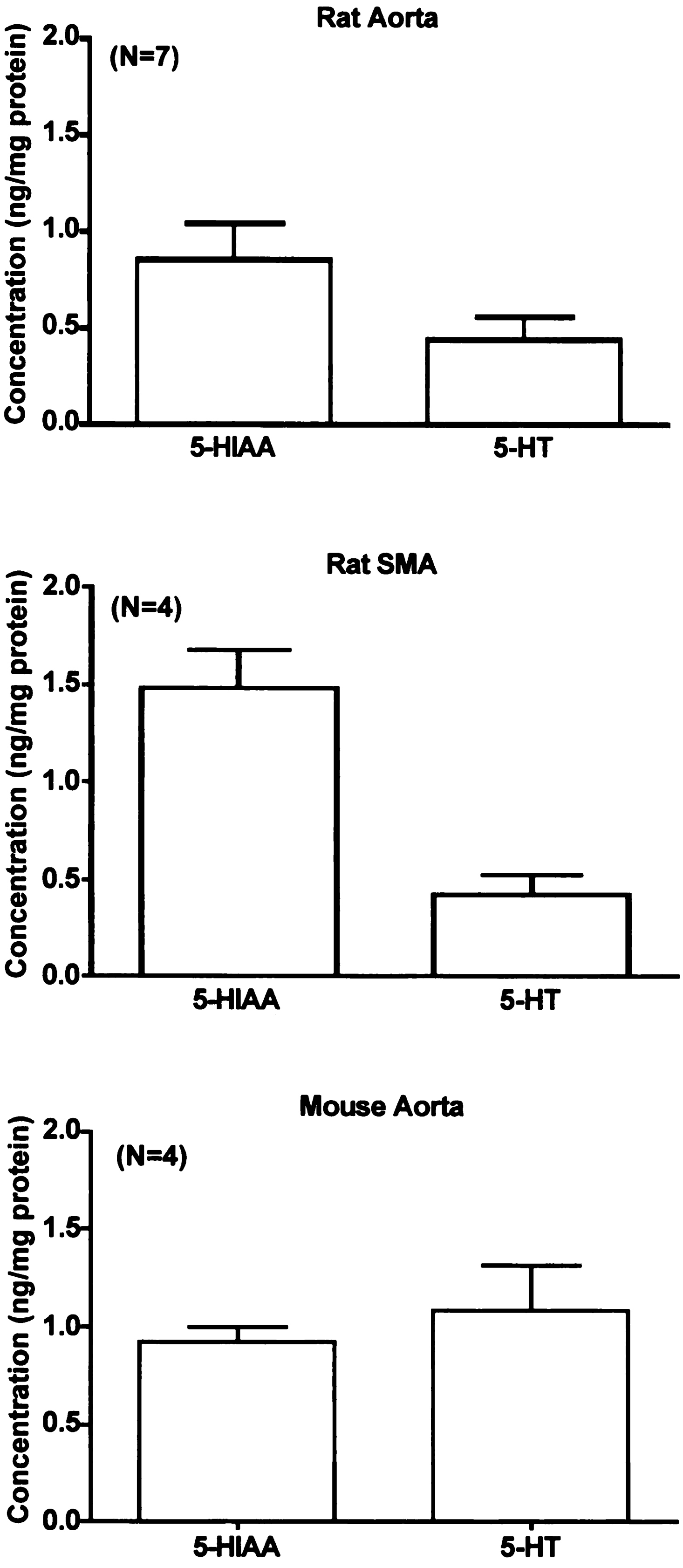

Rat Aorta
(N=7)
Concentration (ng/mg protein)
2.0
1.5
1.0
0.5
0.0
5-HIAA
5-HT
Rat SMA
(N=4)
Concentration (ng/mg protein)
2.0
1.5
1.0
0.5
0.0
5-HIAA
5-HT
Mouse Aorta
(N=4)
Concentration (ng/mg protein)
2.0
1.5
1.0
0.5
0.0
5-HIAA
5-HT

**Figure 5.**

supported by staining of the aorta, throughout the smooth muscle layers and adventitia, with an antibody that recognizes 5-HT (YC5/45, Figure 6, bottom left). Staining was significantly diminished when the antibody was pre-incubated with a 5-fold excess of 5-HT itself (Figure 6, bottom right). The dark precipitate formed by DAB in serotonergic nerves of the guinea pig ileum myenteric plexus (Figure 6, top) was used as a positive control as this tissue contains a rich innervation of serotonergic nerves (Galligan et al., 1986). To eliminate the possibility that the 5-HT we observed in aorta section is actually in mast cells rather than in smooth muscle cells, we used Giemsa's Stain to test the existence of mast cells in rat aorta. The aorta only rarely presented with recognizable mast cells while this staining detected abundant mast cells in a human lymph node (data not shown). Thus, it is unlikely that aortic 5-HT comes from mast cells.

Intracellular staining of 5-HT using the 5-HT antibody (goat anti-rabbit 5-HT, Serotec, Raleigh, NC) and a fluorescent Cy3 secondary antibody in freshly isolated aortic smooth muscle cells further confirmed the existence of endogenous 5-HT in arterial smooth muscle (Figure 7, B). Using the same exposure time, no fluorescence or very low fluorescence was observed in cells with only secondary antibody incubation (Figure 7, C). The staining of $\alpha$-actin verified that the cells were smooth muscle cells (Figure 7, A).

**Figure 6.**

Top: Positive control for use of the 5-HT antibody (YC5/45, Abcam); guinea pig ileum myenteric plexus contains identifiable 5-HT containing neurons marked with the arrow.

Bottom: Immunohistochemical staining for 5-HT in aorta from normal rats. Aortic section incubated with 5-HT primary antibody (left) compared with section incubated with primary antibody prequenched with 5-HT. Arrows indicate the placement of staining. Representative of 4 different experiments/rats.

5-HT = 5-hydroxytryptamine.

## Presence of 5-HT in Rat Aorta

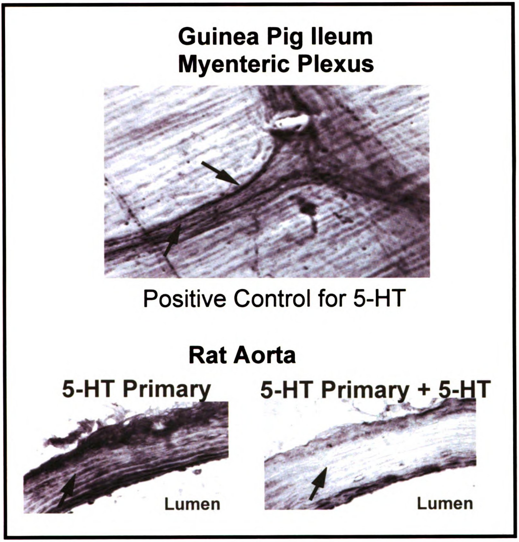


Figure 6.

**Figure 7.**

Freshly dissociated rat aortic smooth muscle cells stained with anti-$\alpha$-actin antibody (A), with anti-5-HT antibody (B) or secondary antibody alone (C). Representatives of four separate experiments, each with a different rat.

5-HT = 5-hydroxytryptamine.

## Presence of 5-HT in Freshly Isolated Rat Aortic Smooth Muscle Cells

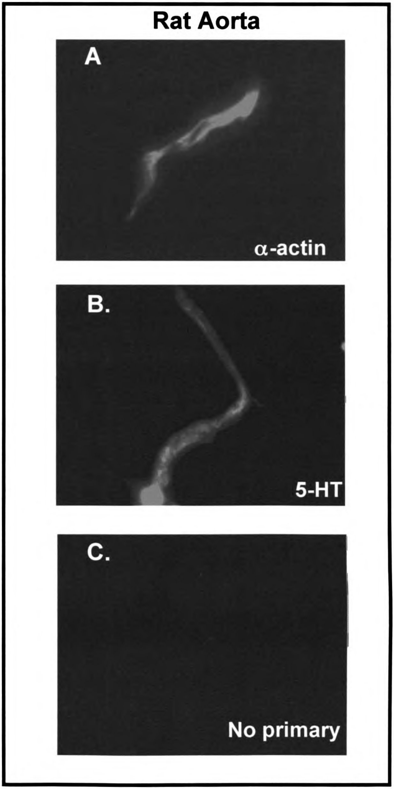


Figure 7.

## II. The Presence of 5-HT Synthesis and Metabolism in Peripheral Arteries

To investigate the synthesis and metabolism system in peripheral arteries, we tested the existence and activity of enzymes that are important for serotonin synthesis and metabolism: TPH, AADC and MAO A.

### 1. The Existence of TPH1 mRNA and Protein in Peripheral Arteries

We first investigated the existence of mRNA in peripheral arteries. Two sets of rat *tph1* primer pairs were used in our experiments. The first rat *tph1* primer pair was purchased from Superarry (Frederick, MD). This primer was designed according to RefSeq Accession #: XM_341862.1. We also designed another rat *tph1* primer. The sequences were rat *tph1*-L: GCCTGCTTTCTTCCATCAGT and rat *tph*1-R: AGACATCCTGGAAGCTTGTGA. Figure 8 A. shows the amplification plots of *tph1* and the housekeeping gene β-2-microglobulin (B2m) in rat aorta and superior mesenteric artery. $C_T$ values were measured as the value at which measurable product was first observed in real time RT-PCR. The $C_T$ values for *tph1* and B2m in rat aorta and superior mesenteric artery are reported in Table 1. Figure 8 B. shows the dissociation curve for *tph1* (both primers) in aorta and superior mesenteric artery. Only one peak was observed in each of the dissociation curve, indicating the single product of *tph1* primer.

The translation of *tph1* mRNA was confirmed by the immunocytochemistry experiments in which we observed an expression of TPH protein in freshly isolated aortic and superior mesenteric smooth muscle cells (Figure 9, top) using

**Figure 8.**

A. Real time RT-PCR amplification curves of *tph1* mRNA expression in normal rat aorta (top) and superior mesenteric artery (bottom) using *tph1* primer purchased from SuperArray® (left, Frederick, MD) or using *tph1* primer designed by us (right).

B. Real time RT-PCR dissociation curves of *tph1* mRNA expression in normal rat aorta and superior mesenteric artery using *tph1* primer purchased from SuperArray® (top, Frederick, MD) or using *tph1* primer designed by us (bottom).

**Table 1.**

The $C_T$ values in real time RT-PCR experiments detecting the expression of *tph1* and β-2-microglobulin mRNA in rat aorta and superior mesenteric artery.

B2m=β-2-microglobulin, *tph*=tryptophan hydroxylase, SMA=superior mesenteric artery.

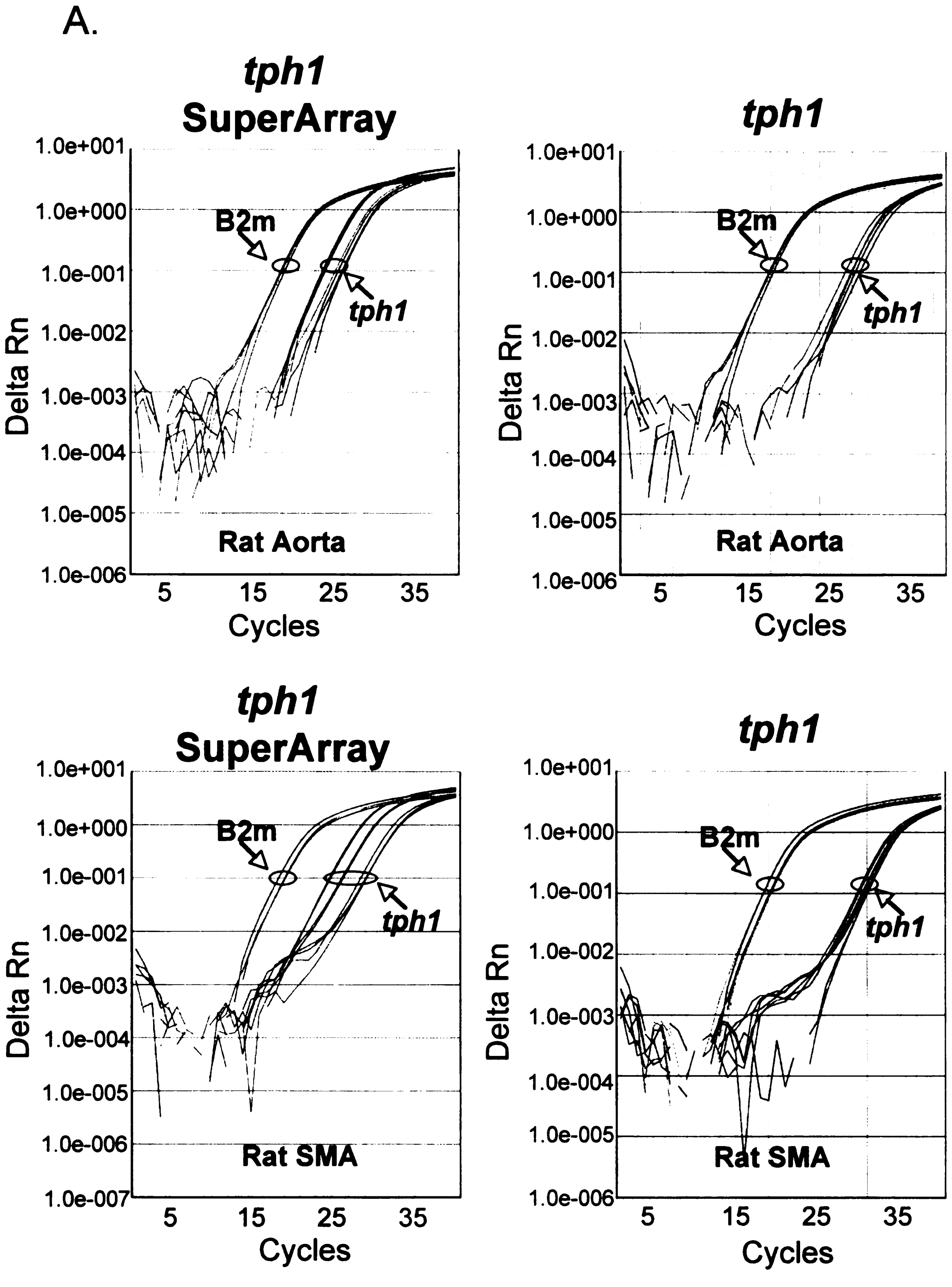

A.
tph1
SuperArray
tph1
1.0e+001
1.0e+000
1.0e-001
1.0e-002
1.0e-003
1.0e-004
1.0e-005
1.0e-006
1.0e-007
Delta Rn
B2m
tph1
Rat Aorta
Rat SMA
5
15
25
35
Cycles

Figure 8.

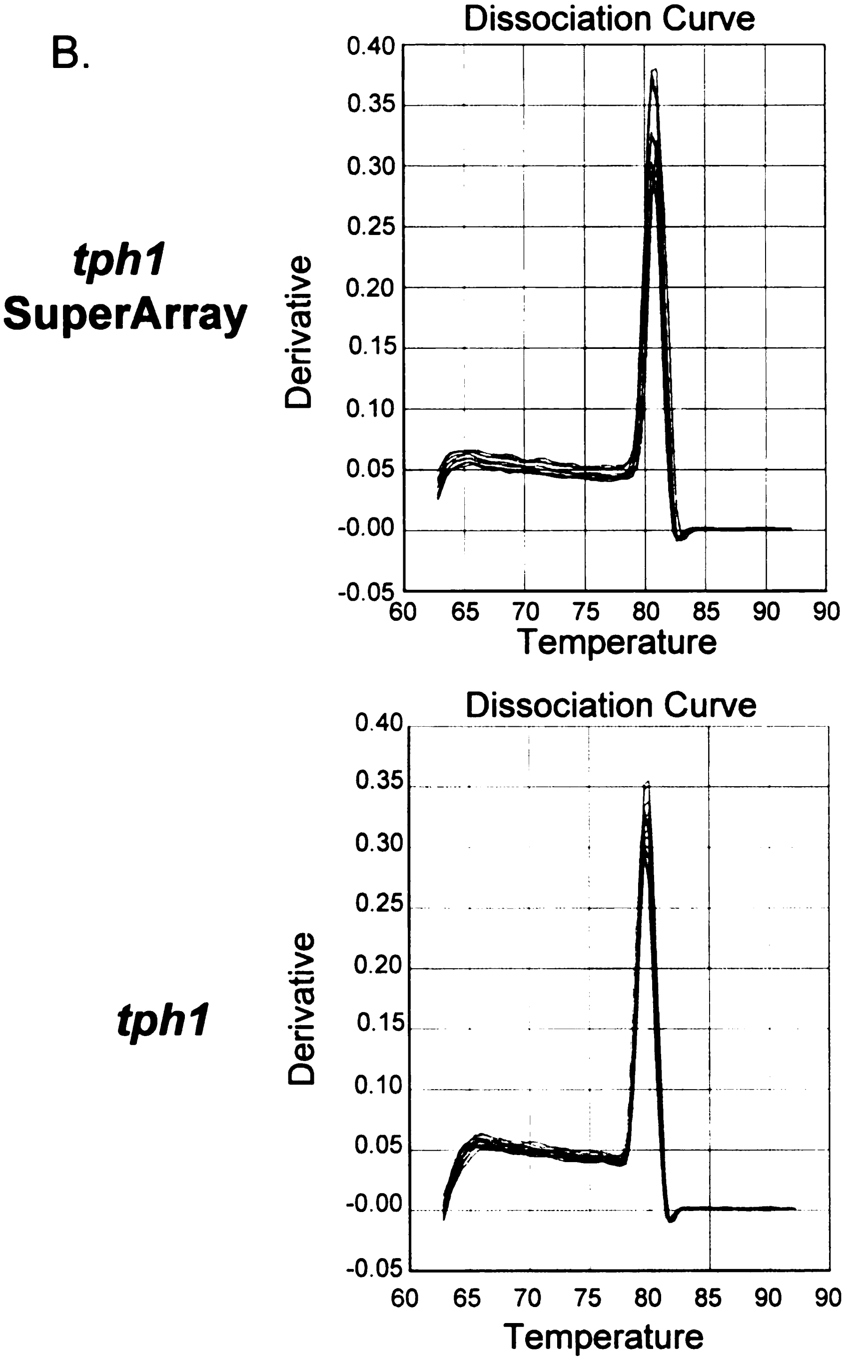


**Figure 8.**

Table 1.

| | *tph1* (SuperArray) | *tph1* | B2m |
|---|---|---|---|
| Rat Aorta | 24.92±0.57 | 28.77±0.33 | 16.81±0.25 |
| SMA | 27.02±1.06 | 30.35±0.21 | 17.70±0.32 |

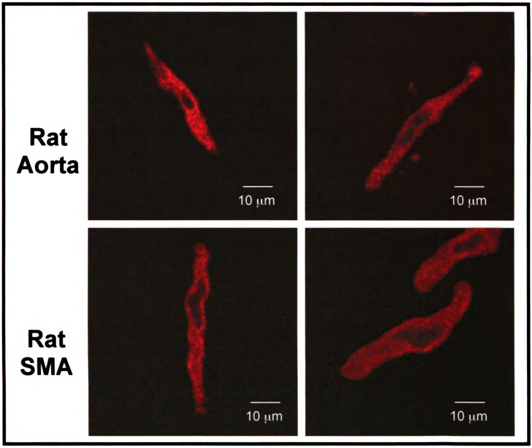
**Figure 9.**

A. Freshly dissociated rat aortic smooth muscle cells (top) and superior mesenteric arterial smooth muscle cells (bottom) stained with anti-TPH antibody. Representatives of four separate experiments, each with a different rat.

B. Immunohistochemical staining of the TPH (Sigma) in smooth muscle between cables of elastin/collagen in the rat aorta frozen section. Parallel sections were incubated with antibody (left) or no primary antibody (right). Arrows indicate the placement of staining.

TPH= tryptophan hydroxylase, SMA=superior mesenteric artery.

A. **Presence of TPH Protein in Freshly Isolated Arterial Smooth Muscle Cells**



B. **Presence of TPH Protein in Rat Aorta**

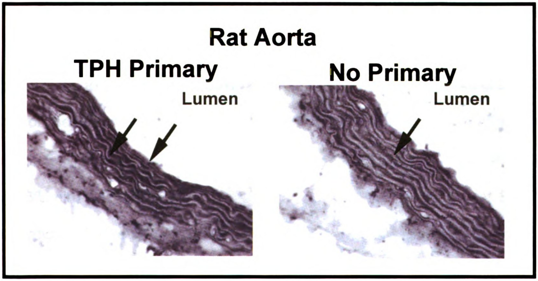


Figure 9.

an anti-TPH monoclonal antibody (Sigma, St Louis, MO, USA) and a fluorescent anti-mouse secondary antibody, which confirmed the existence of TPH protein in arterial smooth muscle. Again, smooth muscle cells were confirmed by $\alpha$-actin staining (data not shown). No fluorescence or very low fluorescence was pictured in cells without primary antibody incubation (data not shown). We also did IHC experiments testing the expression of TPH in artery sections using the same anti-TPH antibody. However, we did not get very convincing results. Relatively darker DAB precipitates appeared in sections with primary antibody incubation (Figure 9, bottom left) compared to control sections (Figure 9, bottom right).

## 2. The Activity of TPH in Peripheral Arteries

To test whether the arterial TPH is functional, we measured 5-HTP levels in peripheral arteries and in hypothalamus from NSD 1015 (100 mg/Kg, 30 min)-treated rats. NSD 1015 is an inhibitor of AADC and blocks the conversion of 5-HTP to 5-HT. Hypothalamic tissues, that contains terminals of central serotonergic neuron terminals, were used as positive control for this experiment. Using HPLC we found non-detectable 5-HTP in aorta, fairly low amount of 5-HTP in superior mesenteric artery ($0.035 \pm 0.021$ ng/mg protein) and $1.61 \pm 0.26$ ng/mg protein of 5-HTP in hypothalamus. In all three tissues from tryptophan (300 mg/Kg, 60 min) and NSD 1015-treated rats, we observed increases of 5-HTP concentrations (Figure 10). We also tested the possibility of peripheral arteries

**Figure 10.**

Quantification of 5-HIAA, 5-HT and 5-HTP in aorta (top), superior mesenteric artery (middle) and hypothalamus (bottom) in rats treated (i.p.) with AADC inhibitor NSD 1015 (100 mg/Kg, 30 min) or NSD 1015 (100 mg/Kg, 30 min) + tryptophan (300 mg/Kg, 60 min). Bars represent means ± SEM for the number of animals in parentheses. * $p<0.05$ compared to NSD1015 (alone)- treated animal. 5-HIAA=5-hydroxyindoleacetic acid, 5-HT=5-hydroxytryptamine, 5-HTP=5-hydroxytryptophan, AADC=amino acid decarboxylase, SMA=superior mesenteric artery.

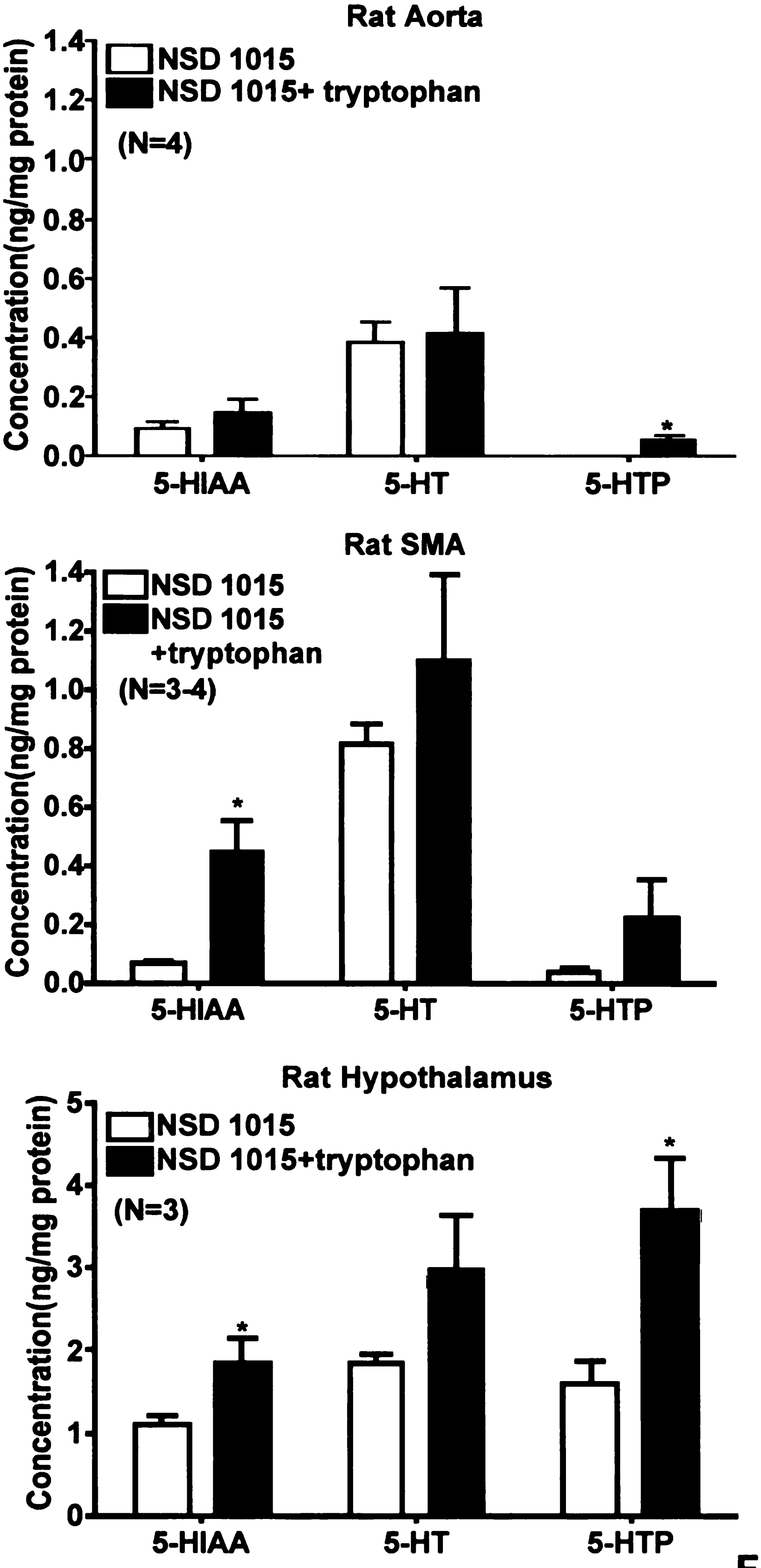

Rat Aorta
Concentration(ng/mg protein)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
☐ NSD 1015
■ NSD 1015+ tryptophan
(N=4)
*
5-HIAA
5-HT
5-HTP
Rat SMA
Concentration(ng/mg protein)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
☐ NSD 1015
■ NSD 1015 +tryptophan
(N=3-4)
*
5-HIAA
5-HT
5-HTP
Rat Hypothalamus
Concentration(ng/mg protein)
5
4
3
2
1
0
☐ NSD 1015
■ NSD 1015+tryptophan
(N=3)
*
*
5-HIAA
5-HT
5-HTP

**Figure 10.**

taking up 5-HTP. The concentration of 5-HTP increased in rat aorta and superior mesenteric artery after incubation with exogenous 5-HTP (1 μM) for 30 minutes (Figure 11).

We also tested TPH activity in *in vitro* experiments. In the presence of AADC inhibitor, substrate and all the co-factors that TPH needs to synthesize 5-HT, no 5-HTP accumulation in arteries (with or without tryptophan Figure 12, A, B), a low level of 5-HTP (without tryptophan) and an increased 5-HTP peak (with tryptophan) in our positive control -- *raphe* nucleus (Figure 12, C, D) were observed.

### 3. The Presence of AADC in Rat Aorta and Superior Mesenteric Artery

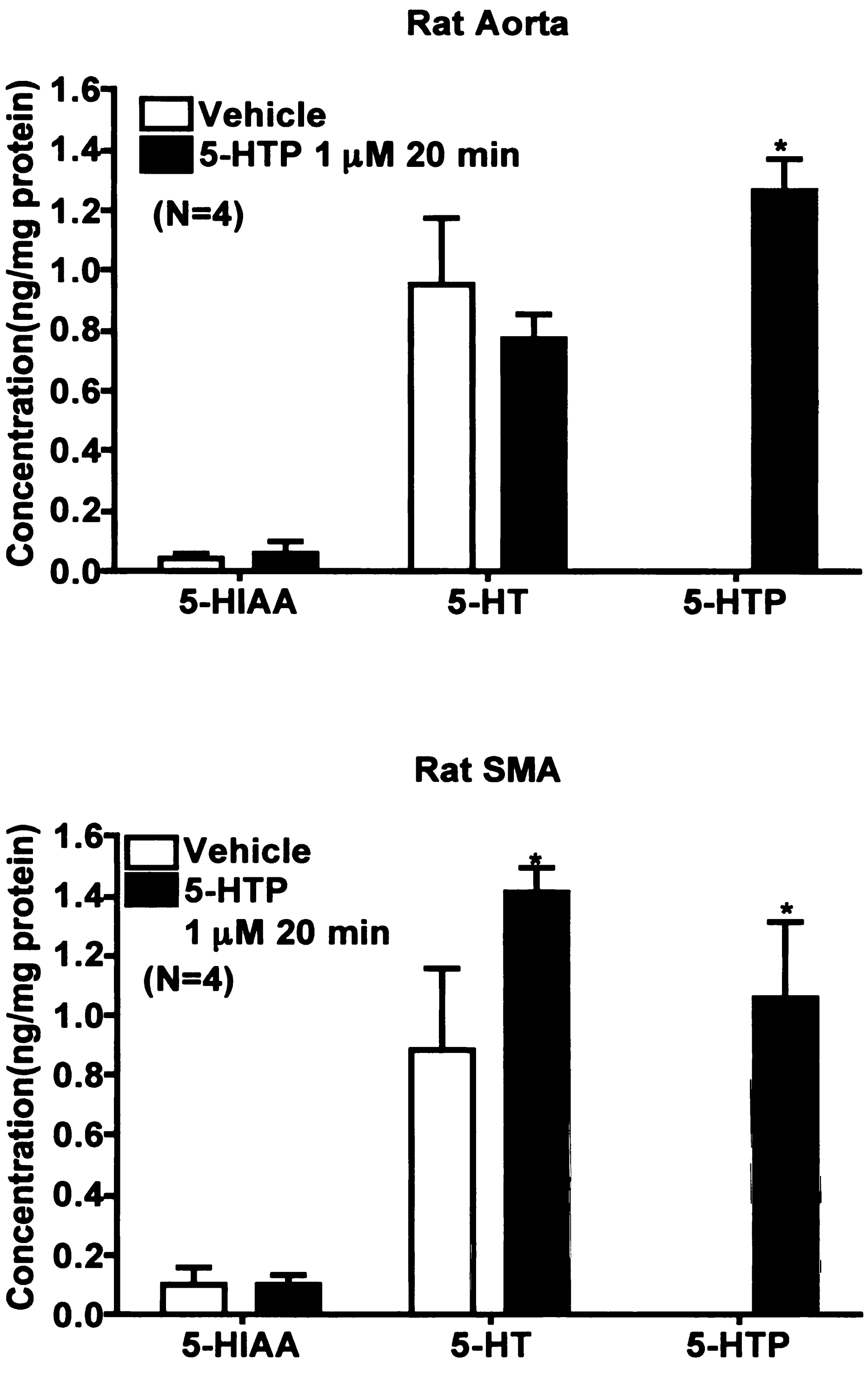
Figure 13 shows the Western analysis of the expression of AADC protein (Abcam, Cambridge, MA) in rat aorta and superior mesenteric artery. We used rat adrenal medulla as a positive control. The bands in rat aorta and superior mesenteric artery samples as well as adrenal medulla migrated at ~55kDa.

### 4. The Presence of MAO A in Rat Aorta and Superior Mesenteric Artery

Western analysis using an antibody specifically recognizing MAO A protein (H-70, Santa Cruz, CA, USA) was performed in rat aorta and superior mesenteric artery whole tissue homogenate supernatant. This MAO A antibody is a rabbit polyclonal antibody raised against amino acids 458-527 of MAO A of human origin. Figure 14, top shows the bands in both rat aorta and superior mesenteric artery samples migrated at ~ 70 kDa, consistent with our positive

**Figure 11.**

Quantification of 5-HIAA, 5-HT and 5-HTP in aorta (top) and superior mesenteric artery (bottom) from MAO A inhibitor pargyline (100 mg/Kg)-treated rats after 20 minutes of vehicle or 5-HTP (1 μM) incubation. Bars represent means ± SEM for the number of animals in parentheses. * $p<0.05$ compared to vehicle incubation. 5-HIAA=5-hydroxyindoleacetic acid, 5-HT=5-hydroxytryptamine, 5-HTP=5-hydroxytryptophan, MAO A=monoamine oxidase A, SMA=superior mesenteric artery.



Rat Aorta

Rat SMA

Figure 11.

**Figure 12.**

Chromatogram showing the measurement of 5-HTP in rat aorta (A: without tryptophan; B: with tryptophan) and *raphe* nucleus (C: without tryptophan; D: with tryptophan) samples incubated with NSD1015 and other cofactors for 5-HT synthesis. Solid line: sample tracing. Dot line: 3 mix standards including 20 μL, 75pg/μL 5-HT, 5-HTP and 5-HIAA.

5-HIAA=5-hydroxyindoleacetic acid, 5-HT=5-hydroxytryptamine, 5-HTP=5-hydroxytryptophan, SMA=superior mesenteric artery.

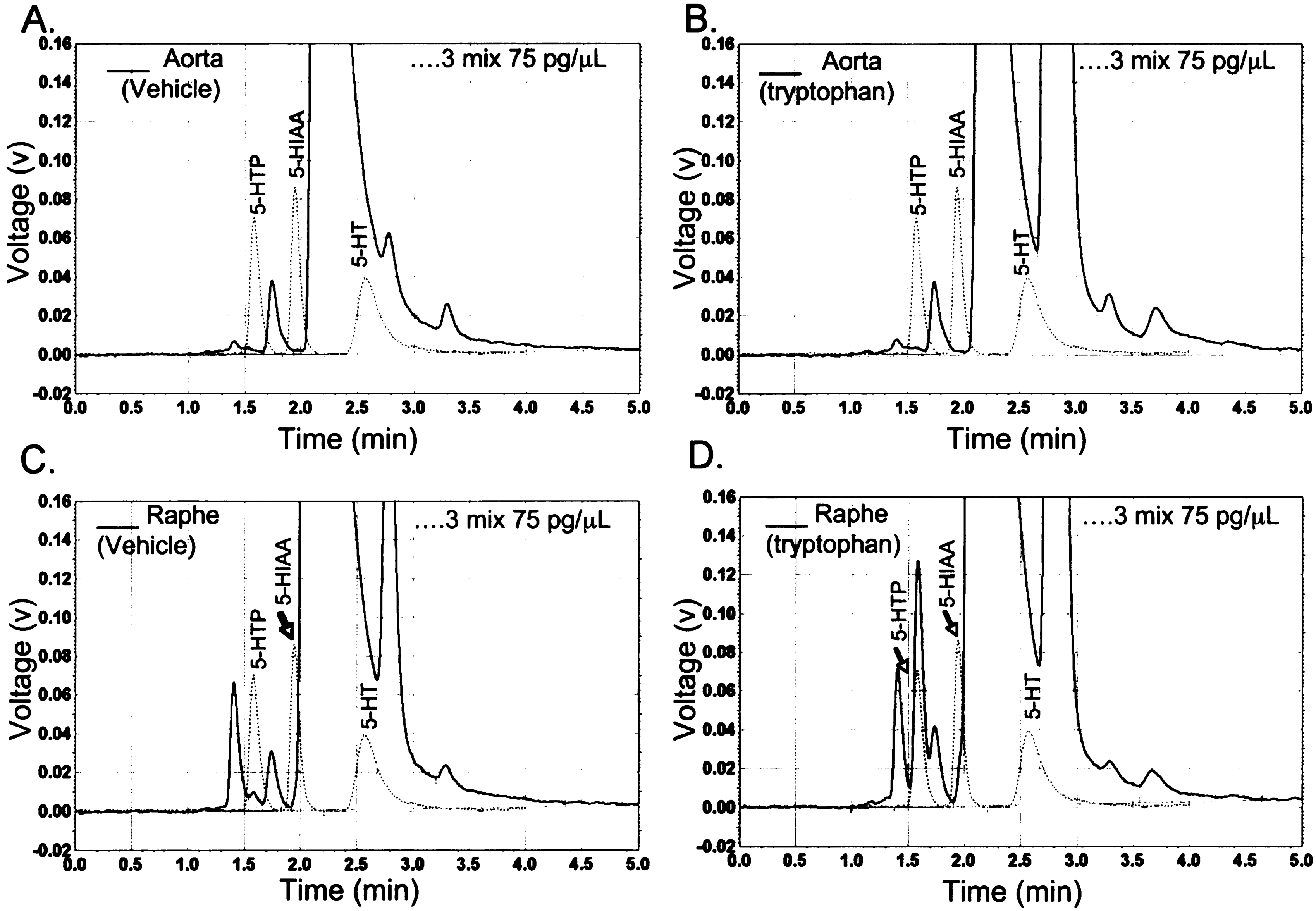

A.
Aorta (Vehicle)
....3 mix 75 pg/µL
5-HTP
5-HIAA
5-HT
B.
Aorta (tryptophan)
....3 mix 75 pg/µL
5-HTP
5-HIAA
5-HT
C.
Raphe (Vehicle)
....3 mix 75 pg/µL
5-HTP
5-HIAA
5-HT
D.
Raphe (tryptophan)
....3 mix 75 pg/µL
5-HTP
5-HIAA
5-HT
Voltage (v)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
Time (min)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0

Figure 12.

**AADC Protein Expression in Arteries**

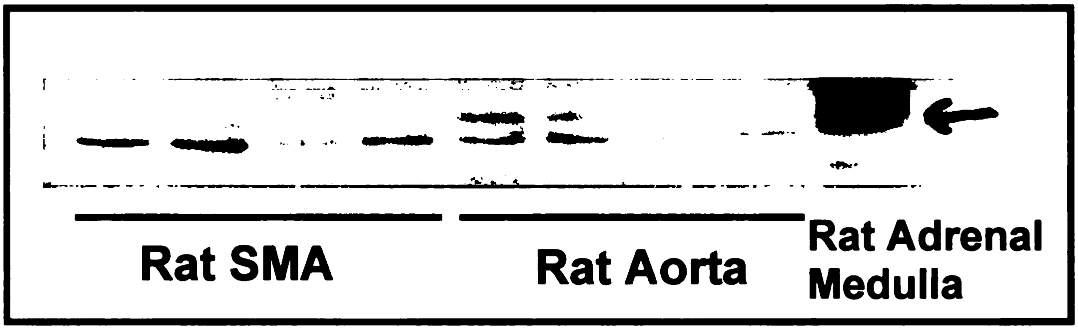


**Figure 13.**

Western blot of AADC in homogenates from rat superior mesenteric artery and aorta, where each lane represents a different rat. Adrenal medulla was used as positive control.

AADC=amino acid decarboxylase. SMA=superior mesenteric artery.

**Figure 14.**

A: Western blot of MAO A in homogenates from rat superior mesenteric artery and aorta, where each lane represents a different rat. Rat gut mucosa was used as positive control.

B: Immunohistochemical staining of the MAO A (Santa Cruz Biotechnology) in smooth muscle between cables of elastin/collagen in the rat aorta and superior mesenteric artery paraffin embeded section. Parallel sections were incubated with antibody (left) or no primary antibody (right). Arrows indicate the placement of staining. Representatives of four separate experiments, each with a different rat.

MAO A = monoamine oxidase A. SMA=superior mesenteric artery.

## MAO A Protein Expression in Arteries

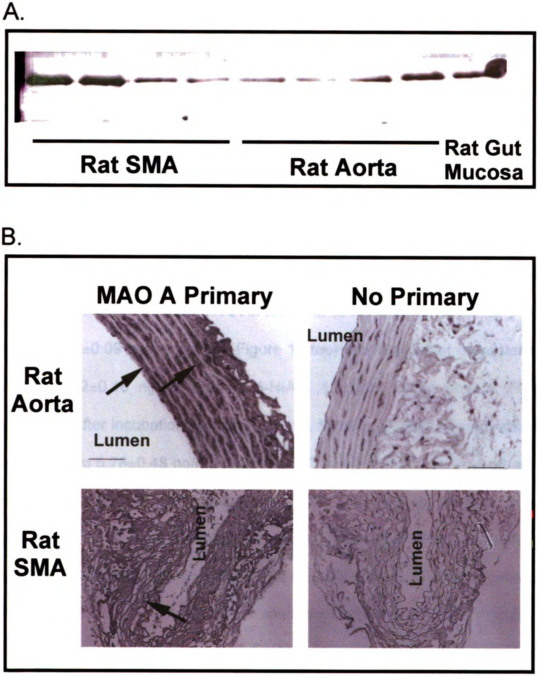


**Figure 14.**

control, rat gut mucosa.

Immunohistochemical experiments using the same antibody localized the MAO A protein to smooth muscle and the endothelial layer of the rat aorta and superior mesenteric artery (Figure 14 B, bottom). The pictures on the right show rat aorta and superior mesenteric artery sections incubated with secondary antibody, but no primary antibody.

### 5. The Activity of MAO A in Rat and Superior Mesenteric Artery

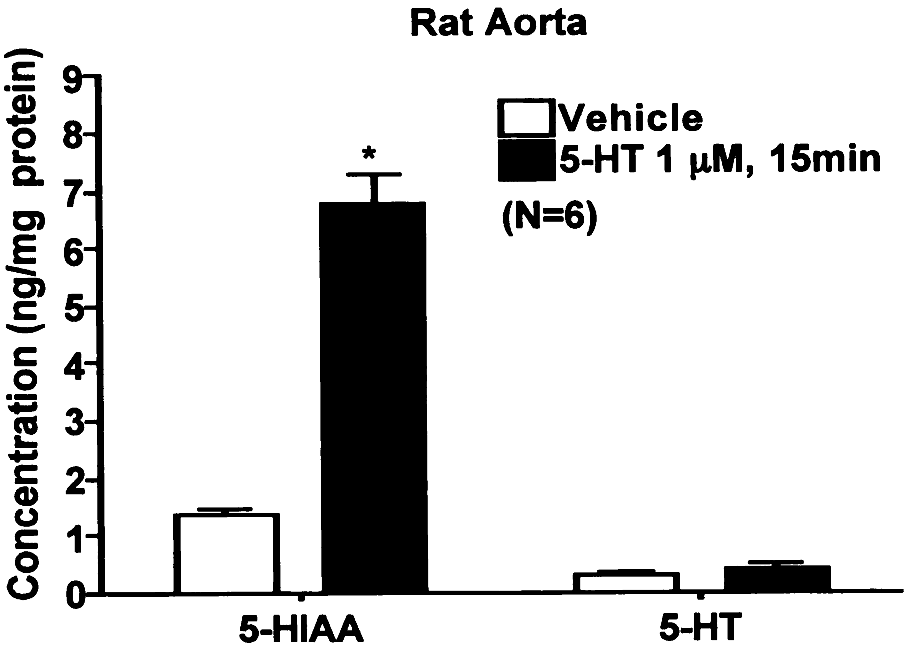
To investigate whether MAO A is functional in peripheral arteries, we compared 5-HT and 5-HIAA concentrations in peripheral arteries with *in vitro* incubation with either exogenous 5-HT (1 μM, 15 min) or vehicle. We measured a basal level of 5-HT and 5-HIAA in rat aorta (5-HT, 0.28±0.05 ng/mg protein; 5-HIAA, 1.38±0.09 ng/mg protein, Figure 15 top) and in superior mesenteric artery (5-HT, 0.32±0.16 ng/mg protein; 5-HIAA, 1.2±0.08 ng/mg protein, Figure 15 bottom). After incubation with 5-HT (1 μM, 15 min), 5-HIAA concentrations were increased to 6.78±0.48 ng/mg protein and 7.9±1.2 ng/mg protein in rat aorta and superior mesenteric artery respectively with minor change of 5-HT levels (rat aorta, 0.43±0.07 ng/mg protein; superior mesenteric artery, 1.2±0.18 ng/mg protein). Moreover, we confirmed that the metabolism of 5-HT in arteries was mediated *via* MAO A by doing the same experiment in arteries from pargyline-treated rats. We observed that 5-HIAA production was abolished and 5-HT levels were increased in arteries from pargyline-treated rats (rat aorta, 5-HT= 0.92±0.07 ng/mg protein, 5-HIAA= 0.0025±0.0025 ng/mg protein; superior

**Figure 15.**

Quantification of 5-HIAA and 5-HT in aorta (top) and superior mesenteric artery (bottom) incubated with vehicle or 1 μM exogenous 5-HT for 15 minutes. Arteries were from normal rats. Bars represent means ± SEM for the number of animals in parentheses. * $p<0.05$ compared to vehicle-incubated tissues.

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

SMA= superior mesenteric artery.


Rat Aorta
Concentration (ng/mg protein)
0
1
2
3
4
5
6
7
8
9
☐ Vehicle
■ 5-HT 1 μM, 15min
(N=6)
*
5-HIAA
5-HT

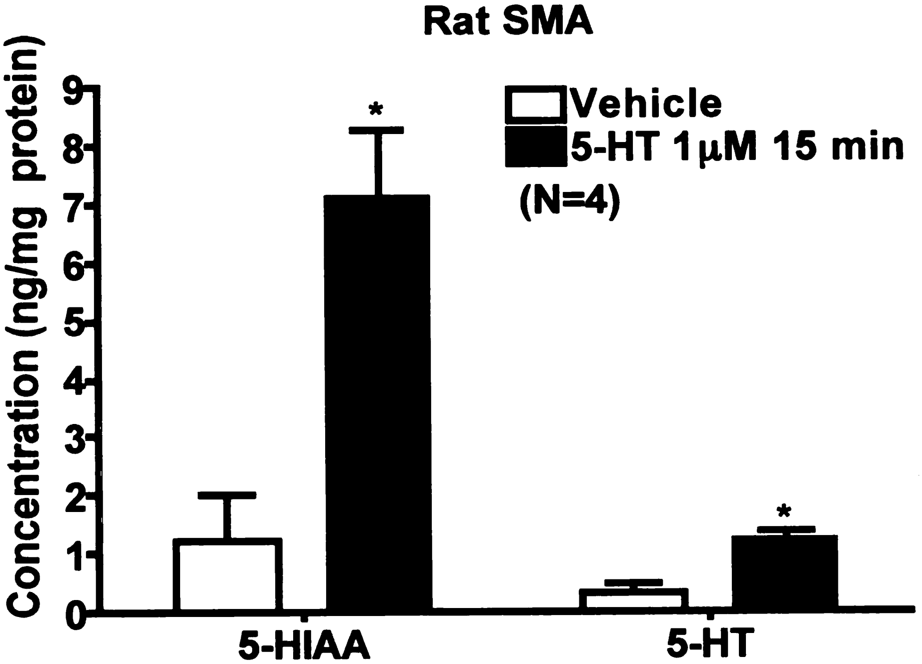

Rat SMA
Concentration (ng/mg protein)
0
1
2
3
4
5
6
7
8
9
☐ Vehicle
■ 5-HT 1 μM 15 min
(N=4)
*
*
5-HIAA
5-HT

**Figure 15.**

mesenteric artery, 5-HT= 2.28±0.24 ng/mg protein, 5-HIAA= 0.025±0.008 ng/mg protein, Figure 16). With exogenous 5-HT incubation, the arterial 5-HT levels significantly increased with minor increase of 5-HIAA (rat aorta, 5-HT= 2.49±0.37 ng/mg protein, 5-HIAA= 0.013±0.013 ng/mg protein; superior mesenteric artery, 5-HT= 4.02±0.54 ng/mg protein, 5-HIAA= 0.13±0.05 ng/mg protein, Figure 16).

## III. The Lack of 5-HT Storage in Peripheral Artery

### 1. Investigation of Whether Rat Aorta Has Ability to Store 5-HT

Basal endogenous levels of 5-HT in rat aorta were reported as above. Here we tested whether a peripheral artery has the ability to store more than basal level of 5-HT. To test this idea, we incubated untreated-aorta and 5-HT-loaded aorta from normal and pargyline-treated rats in PSS and compared 5-HIAA and 5-HT content in rat aorta and PSS after 0, 4 or 8 hours. The 5-HIAA and 5-HT we measured in PSS were released during 4 or 8 hours incubation from rat aorta. The amount of 5-HIAA and 5-HT remaining in rat aorta after incubation was also measured.

First we tested whether aorta would maintain endogenous 5-HT. We observed that in aorta from normal rats, the 5-HT content was maintained in aorta after 4 or 8 hours of PSS incubation (Figure 17 bottom, Table), while 5-HIAA was not and was released to PSS (Figure 17 top, Table 2). We next tested whether aorta could store more than basal level of 5-HT by comparing 5-HIAA and 5-HT levels in aorta after 4 or 8 hours PSS incubation in exogenous 5-HT (1 μM, 15 min) preloaded-aorta with those at time 0 and right after exogenous 5-HT

**Figure 16.**

Quantification of 5-HIAA and 5-HT in aorta (top) and superior mesenteric artery (bottom) incubated with vehicle or 1 μM exogenous 5-HT for 15 min. Arteries were from rats treated with MAO A inhibitor pargyline (100 mg/Kg, i.p., 30 min). Bars represent means ± SEM for the number of animals in parentheses. * $p<0.05$ compared to vehicle-incubated tissues.

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

SMA= superior mesenteric artery. MAO A = monoamine oxidase.

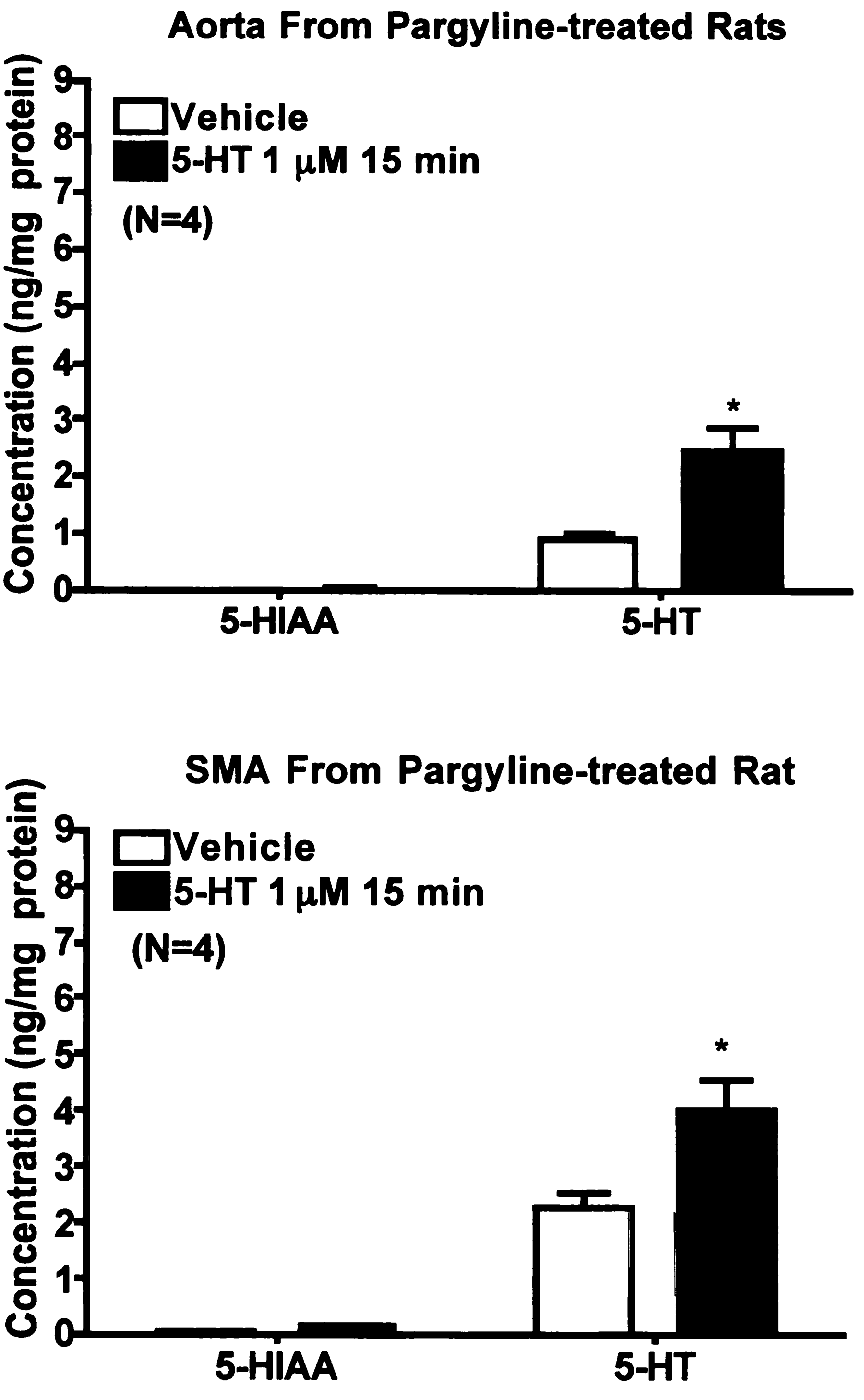

Aorta From Pargyline-treated Rats
Concentration (ng/mg protein)
0
1
2
3
4
5
6
7
8
9
Vehicle
5-HT 1 μM 15 min
(N=4)
*
5-HIAA
5-HT
SMA From Pargyline-treated Rat
Concentration (ng/mg protein)
0
1
2
3
4
5
6
7
8
9
Vehicle
5-HT 1 μM 15 min
(N=4)
*
5-HIAA
5-HT

Figure 16.

**Figure 17.**

Top: Quantification of 5-HIAA and 5-HT released into PSS during 0, 4 or 8 hours incubation.

Bottom: Quantification of 5-HIAA and 5-HT remaining in tissues after 0, 4 or 8 hours incubation in PSS.

Time 0 represents no incubation (basal endogenous 5-HIAA and 5-HT levels).

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to basal level (no incubation, time 0).

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

PSS=physiological salt solution.

**Table 2.**

Quantification of 5-HIAA and 5-HT released into PSS and remained in tissue during 0, 4 or 8 hours incubation. Values reported as ng/mg protein.

* $p<0.05$ compared to basal level (no incubation, time 0).

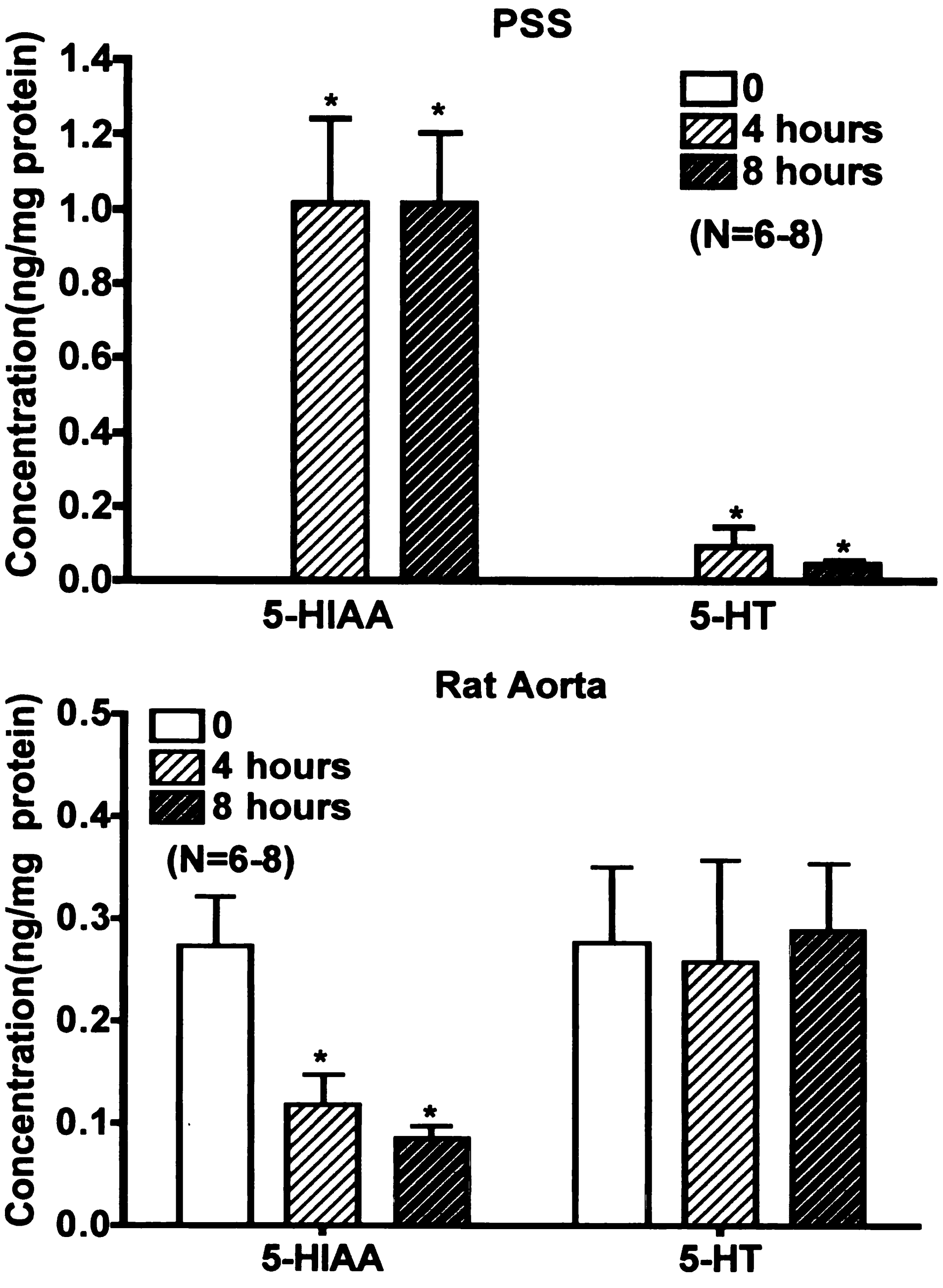


**Figure 17.**

Table 2.

| | | 0 | 4 Hours | 8 Hours |
|---|---|---|---|---|
| PSS | 5-HIAA | 0 | 1.02±0.23* | 1.018±0.19* |
| | 5-HT | 0 | 0.097±0.05* | 0.05±0.006* |
| Rat Aorta | 5-HIAA | 0.27±0.05 | 0.12±0.03* | 0.085±0.01* |
| | 5-HT | 0.28±0.07 | 0.26±0.1 | 0.29±0.065 |

incubation. We rinsed and flushed aorta with fresh drug-free PSS to eliminate exogenous 5-HT attached on the aorta wall. As shown in Figure 18, bottom and Table 3, aortic 5-HIAA level was greatly increased by 15 minutes incubation of 1 μM exogenous 5-HT with a moderate increase of 5-HT in aorta. After 4 or 8 hours incubation with PSS, the majority of 5-HIAA was released to PSS with a slight though significant decrease of 5-HT in aorta with time (Figure 18, top and Table 3). We then took a further step to investigate whether aorta would store 5-HT when 5-HT metabolism was inhibited. We did a similar experiment in aorta from MAO A inhibitor, pargyline (100 mg/Kg, 30 min)-treated rats. After incubation with exogenous 5-HT (1 μM, 15 min), the aortic 5-HT content increased with no change of 5-HIAA (Figure 19, bottom, Table 4). However, this is an acute accumulation of 5-HT, after 4 or 8 hours, 5-HT concentration decreased by releasing both 5-HT and 5-HIAA from aorta to PSS (Figure 19, top, Table 4).

### 2. Investigation of “Serotonylated” 5-HT in Rat Aorta

We tested whether 5-HT covalently bound to protein exists in peripheral arteries by Western analysis. We separated rat aorta protein on a 12% gel by molecular weight and blotted with a 5-HT antibody. Multiple bands were visualized (Figure 20), suggesting the presence of serotonylated proteins in rat aorta.

**Figure 18.**

Top: Quantification of 5-HIAA and 5-HT released into PSS during 4 or 8 hours incubation from 5-HT preloaded rat aorta (5-HT 1 μM, 15 min).

Bottom: Quantification of 5-HIAA and 5-HT in naïve aorta (basal level) and in 5-HT preloaded aorta after 0, 4 or 8 hours incubation in PSS.

Time 0 represents no incubation (basal endogenous 5-HIAA and 5-HT levels).

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to basal level. # $p<0.05$ compared to 5-HT 1 μM incubation.

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

PSS= physiological salt solution.

**Table 3.**

Quantification of 5-HIAA and 5-HT in naïve aorta and 5-HIAA and 5-HT released to PSS or remained in 5-HT preloaded aorta after 0, 4 or 8 hours PSS incubation.

Values are reported as ng/mg protein.

* $p<0.05$ compared to basal level. # $p<0.05$ compared to 5-HT 1 μM incubation.

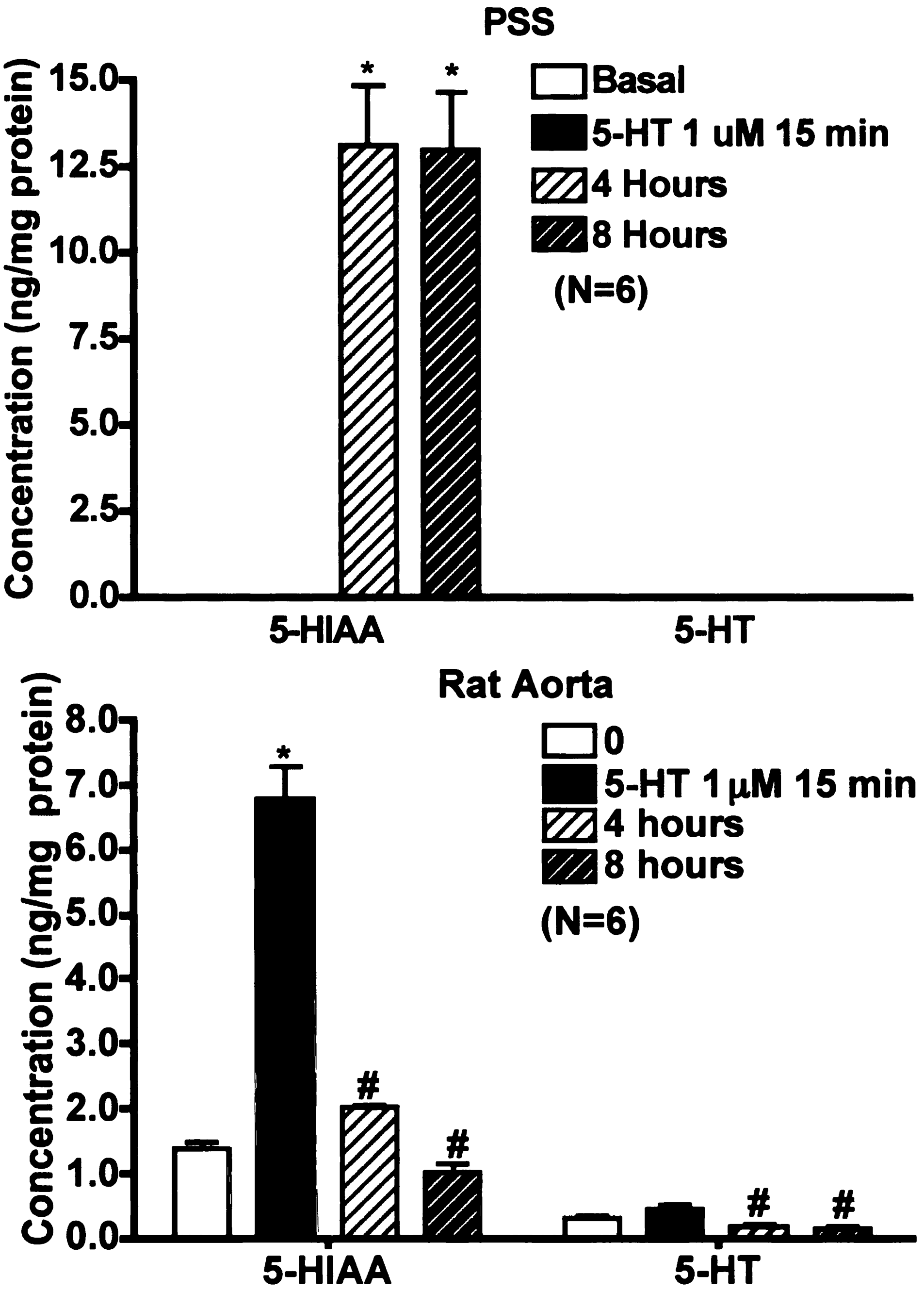


Figure 18.

Table 3.

| | | 0 | 5-HT 1μM 15min | 4 Hours | 8 Hours |
|---|---|---|---|---|---|
| PSS | 5-HIAA | 0 | 0 | 13.08±1.72* | 12.93±1.71* |
| | 5-HT | 0 | 0 | 0 | 0 |
| Rat Aorta | 5-HIAA | 1.38±0.09 | 6.78±0.48* | 2.02±0.03# | 0.99±0.15# |
| | 5-HT | 0.28±0.05 | 0.43±0.07 | 0.16±0.03# | 0.14±0.01# |

**Figure 19.**

Top: Quantification of 5-HIAA and 5-HT released into PSS during 4 or 8 hours incubation from 5-HT preloaded (5-HT 1 μM, 15 min) aorta from pargyline-treated rat.

Bottom: Quantification of 5-HIAA and 5-HT in aorta (basal level) and in 5-HT preloaded aorta from pargyline-treated rats after 0, 4 or 8 hours incubation in PSS.

Time 0 represents no incubation (basal endogenous 5-HIAA and 5-HT levels).

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to basal level. # $p<0.05$ compared to 5-HT 1 μM incubation.

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

PSS= physiological salt solution. Points represent means ± SEM.

**Table 4.**

Quantification of 5-HIAA and 5-HT in aorta and 5-HIAA and 5-HT released to PSS or remained in 5-HT preloaded aorta from pargyline-treated rats after 0, 4 or 8 hours PSS incubation. Values are reported as ng/mg protein.

* $p<0.05$ compared to basal level. # $p<0.05$ compared to 5-HT 1 μM incubation.

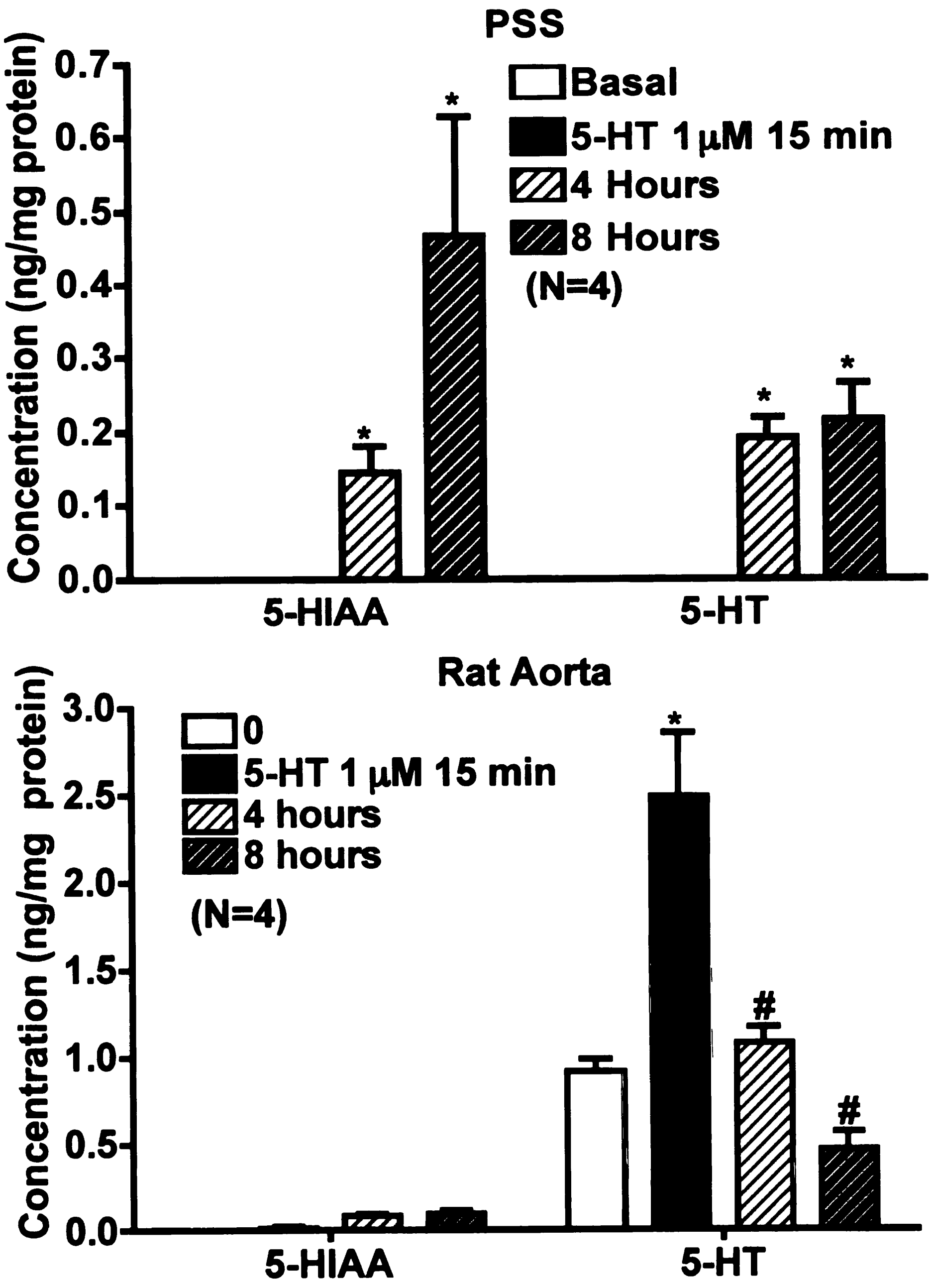


Figure 19.

Table 4.

| | | 0 | 5-HT 1μM 15min | 4 Hours | 8 Hours |
|---|---|---|---|---|---|
| PSS | 5-HIAA | 0 | 0 | 0.15±0.03* | 0.46±0.16* |
| | 5-HT | 0 | 0 | 0.19±0.03* | 0.22±0.05* |
| Rat Aorta | 5-HIAA | 0 | 0.01±0.01 | 0.08±0.01 | 0.09±0.02 |
| | 5-HT | 0.92±0.07 | 2.49±0.37* | 1.08±0.09# | 0.47±0.1# |

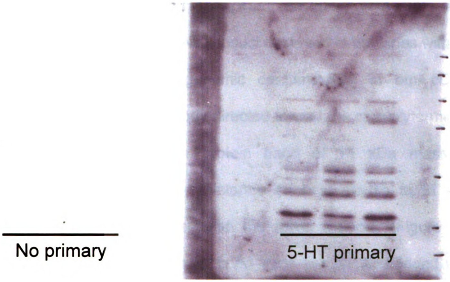


**Figure 20.**

Western blot of 5-HT in homogenates from rat aorta. Representatives of three separate experiments, each with a different rat. Left: Blot incubated with secondary antibody with no exposure to primary antibody. Right: Blot incubated with rabbit anti-5-HT antibody (Serotec, Raleigh, NC).

## IV. The Presence of Uptake and Release of 5-HT in Peripheral Arteries

### 1. The Presence of SERT in Peripheral Arteries

The presence of SERT mRNA was demonstrated by real time RT-PCR. Figure 21 top shows that one PCR product, obtained using primers developed through Primer Express® software for the SERT encoding sequence, was identified in cDNA made from rat aorta. No product observed in the lanes with no reverse transcriptase suggesting no genomic contamination in our cDNA samples. In Western analyses, an antibody directed toward the carboxyterminus of the human SERT recognized one protein band of 74 kDa mass in homogenates of rat aorta, mesenteric resistance arteries, mouse aorta and mouse aorta sample immunoprecipitated with the same antibody (Figure 21, bottom). IHC experiments were performed in rat aorta paraffin embedded and mouse aorta frozen sections using an antibody that recognized the C-terminus (C-20) of the SERT. The SERT was expressed in the smooth muscle and in the endothelial cell layer and the black DAB precipitate did not appear in sections incubated with antibody plus competing peptide (Figure 22, compare top and middle panel). The adventitia of the vessels of rat aorta stained darkly, but this was not competed off by competing peptide; thus this staining is likely nonspecific. The bottom panel showed aorta sections incubated with secondary antibody but no primary antibody.

The presence of SERT in peripheral artery was further validated by immunocytochemistry. We double-stained freshly isolated rat aortic smooth muscle cell using SERT antibody (C-20) and a specific cell plasma membrane

**Figure 21.**

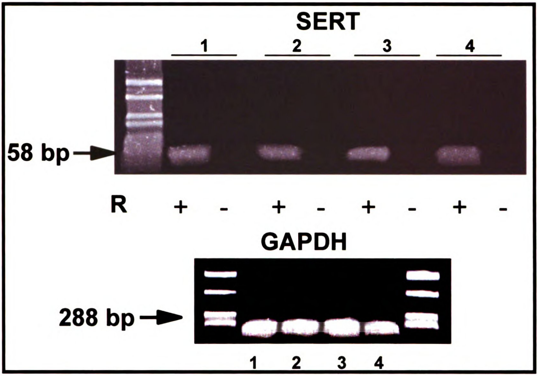
Top: Final product of real time RT-PCR for detection of SERT and GAPDH mRNA in rat aorta. 1-4 are separate rats. RT=reverse transcriptase.

Bottom: Western blot of SERT in homogenates from rat aorta, rat superior mesenteric arteries, mouse aorta homogenates and immunoprecipitated protein (by SERT C-20 antibody, Santa Cruz Biothechnology) from a mouse aorta.

Representatives of at least four separate experiments, each with a different rat/mouse.

SERT= serotonin transporter. SMA= superior mesenteric artery.

SERT mRNA Expression in Rat Aorta



SERT Protein Expression in Arteries

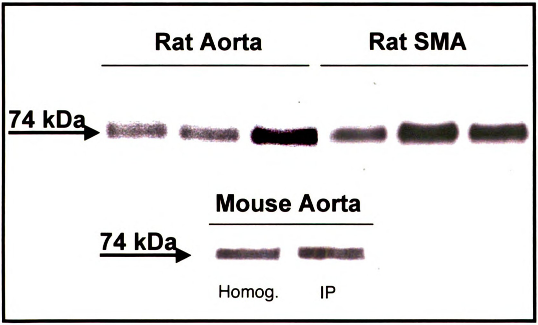


Figure 21.

**Figure 22.**

Immunohistochemical staining of the SERT (Santa Cruz Biotechnology, C-20) in smooth muscle between cables of elastin/collage in the rat aorta (left, paraffin embedded sections) or mouse aorta (right, frozen sections). Parallel sections were incubated with antibody alone (top), quenched with a 5X excess competing peptide (middle) or no primary (bottom). Arrows indicate the placement of staining. Representative of 4-6 different experiments (rats/mice).

SERT= serotonin transporter.

# SERT Protein Expression in Arteries

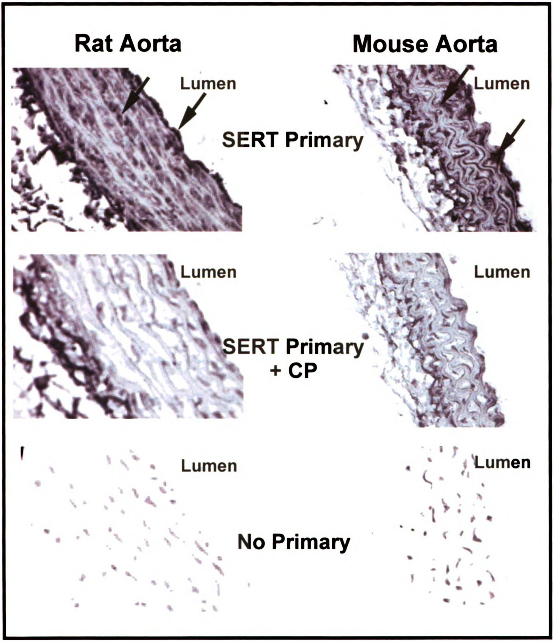


Figure 22.

marker pan-cadherin antibody. By using confocal microscopy, we scanned a single layer of smooth muscle cell. Overlaying the staining of the SERT antibody and the pan-cadherin antibody, we observed that most of the SERT was localized on plasma membrane (Figure 23).

## 2. The Characterization of Active 5-HT Uptake in Peripheral Arteries

### A. 5-HT Uptake Time Course Study

Different tissues (conduit or small vessels) from normal or pargyline-treated rats were used in 5-HT uptake time course studies.

Superior mesenteric arteries from normal rats were incubated in normal PSS with 5-HT (1 μM) for 0, 15, 30 or 45 minutes in room temperature (25 °C). Figure 24 demonstrates that arterial 5-HIAA content increased in a time-dependent manner with a concomitant minor increase of 5-HT.

We also tested 5-HT uptake in a time course study using arteries from pargyline-treated rats. The merits of using pargyline-treated rats are that we can study 5-HT uptake directly and reduce changes in 5-HIAA. Rat aortae were incubated in PSS with 1 μM pargyline and 1 μM 5-HT for 0, 15,30, 45, 60 and 90 minutes at room temperature (25 °C) and 37 °C (Figure 25). At room temperature, aortae took up 5-HT in a time dependent manner and plateaued at 60 minutes with minimal changes of 5-HIAA. The maximal level of 5-HT in aorta was 8.48±0.71 ng/mg protein. However, at 37 °C, uptake plateaued after 30 minutes of exogenous 5-HT (1 μM) incubation with maximal aortic 5-HT level at 5.25±0.27 ng/mg protein. After 15 minutes 5-HT (1 μM) incubation, aortic 5-HT

**Figure 23.**

Confocal image of freshly dissociated rat aortic smooth muscle cells double stained with pan-cadherin antibody (Sigma) and SERT antibody (C-20, Santa Cruz Biotechnology). Representative of cells from 6 different experiments (rats). SERT= serotonin transporter.

# SERT Protein Expression in Freshly Isolated Rat Aortic Smooth Muscle Cells

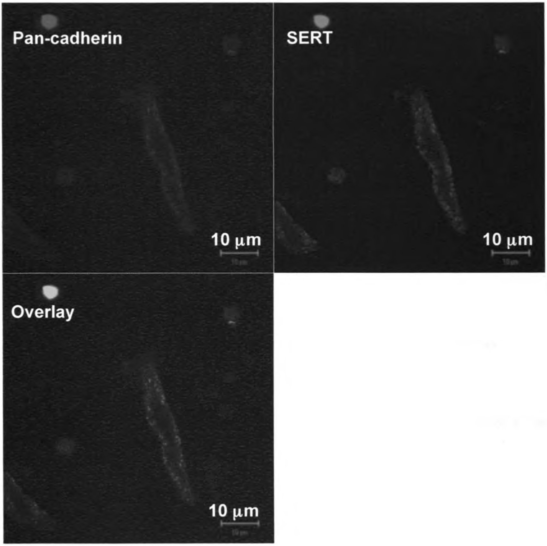


**Figure 23.**

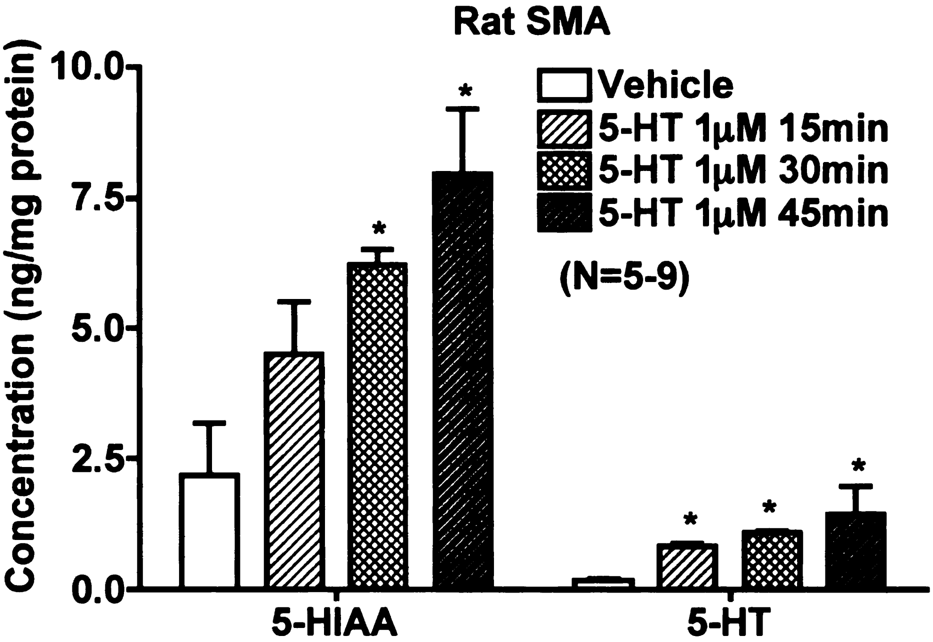


**Figure 24.**

Time course for 5-HT uptake in the rat superior mesenteric artery incubated with 5-HT (1 μM) in room temperature.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared with time 0 (no incubation).

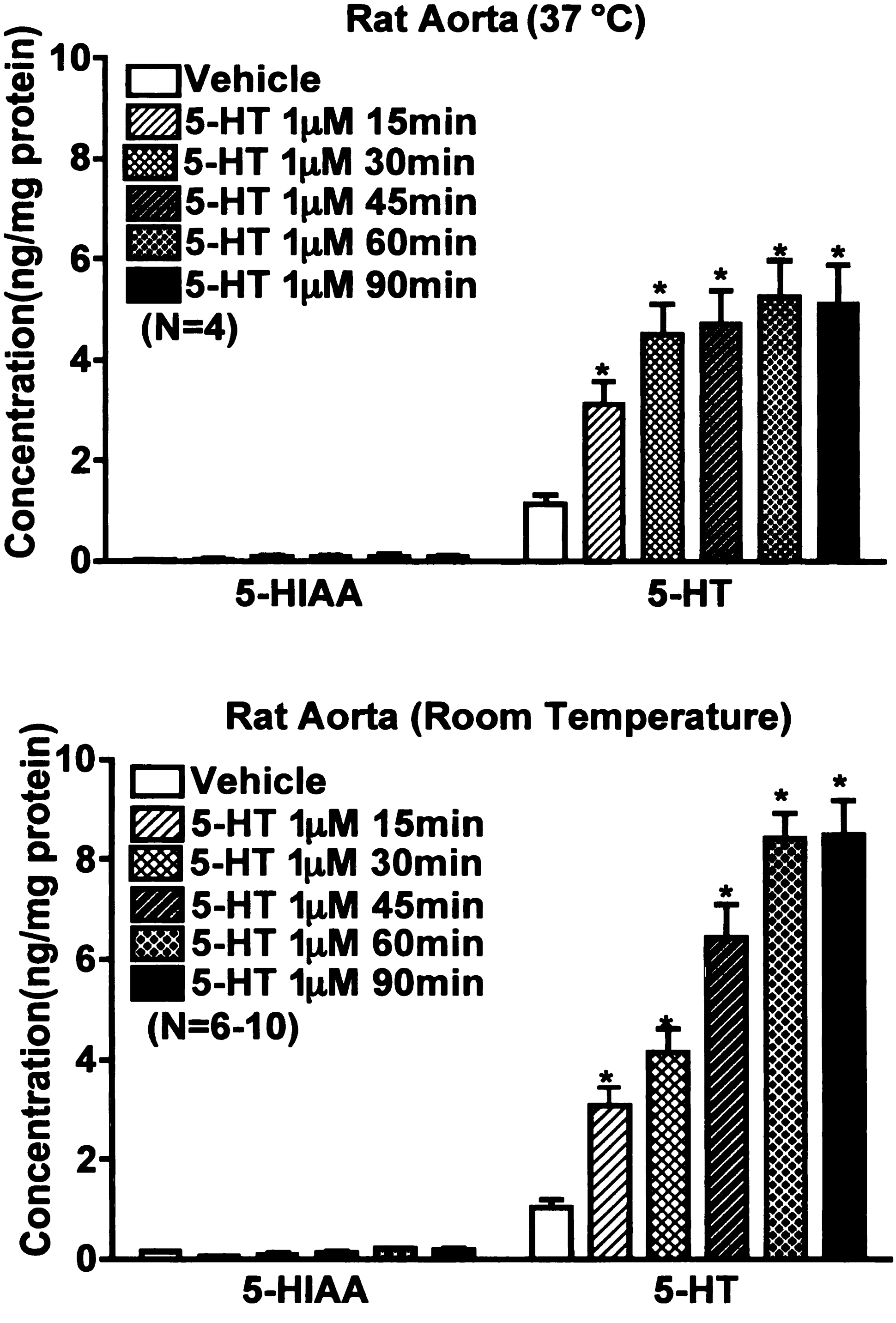
5-HIAA=5-hydroxyindoleacetic acid. 5-HT= 5-hydroxytryptamine. SMA=superior mesenteric artery.

**Figure 25.**

Time course for 5-HT uptake in the aorta from pargyline-treated rats incubated with exogenous 5-HT (1 μM) at 37 °C (Top) or at room temperature (bottom). Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared with time 0 (no incubation).

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


Rat Aorta (37 °C)

Rat Aorta (Room Temperature)

Figure 25.

levels are similar at room temperature and 37 °C incubation groups (room temperate, 3.07±0.35 ng/mg protein; 37 °C, 3.12±0.45 ng/mg protein). Similarly, superior mesenteric artery from pargyline-treated rats time-dependently take up 5-HT, which did not increase further after 60 minutes of 5-HT incubation in room temperature (Figure 26). All of our following 5-HT uptake experiments incubated tissues with 5-HT (1 μM) for 15 minutes at room temperature.

The real uptake of exogenous 5-HT into arteries was confirmed by using *tph1-/-* mice. The *tph1-/-* mice expressed normal amounts of 5-HT in brain but lack of 5-HT in periphery (Walther et al., 2003). The blank background of 5-HT in these mice makes them a good model for studying 5-HT uptake in peripheral arteries. Figure 27, table 5 compares the uptake of exogenous 5-HT in aorta from untreated wild type and *tph1-/-* mice. It is clear that basal 5-HT and 5-HIAA concentrations were significantly lower in aorta from *tph1-/-* mice. Upon incubation with exogenous 5-HT, the level of 5-HIAA increase in mouse aorta is much higher than the 5-HT increase in both wild type and *tph1-/-* mice, which is similar to what we observed in rat arteries.

Thus, we concluded that 5-HT can be taken up by various peripheral arteries and 5-HT metabolized to 5-HIAA quickly after it gets into arteries.

### B. Investigation of SERT-dependent 5-HT Uptake

We next investigated whether the active 5-HT uptake in peripheral arteries was mediated by SERT. We preincubated arteries (rat aorta and superior

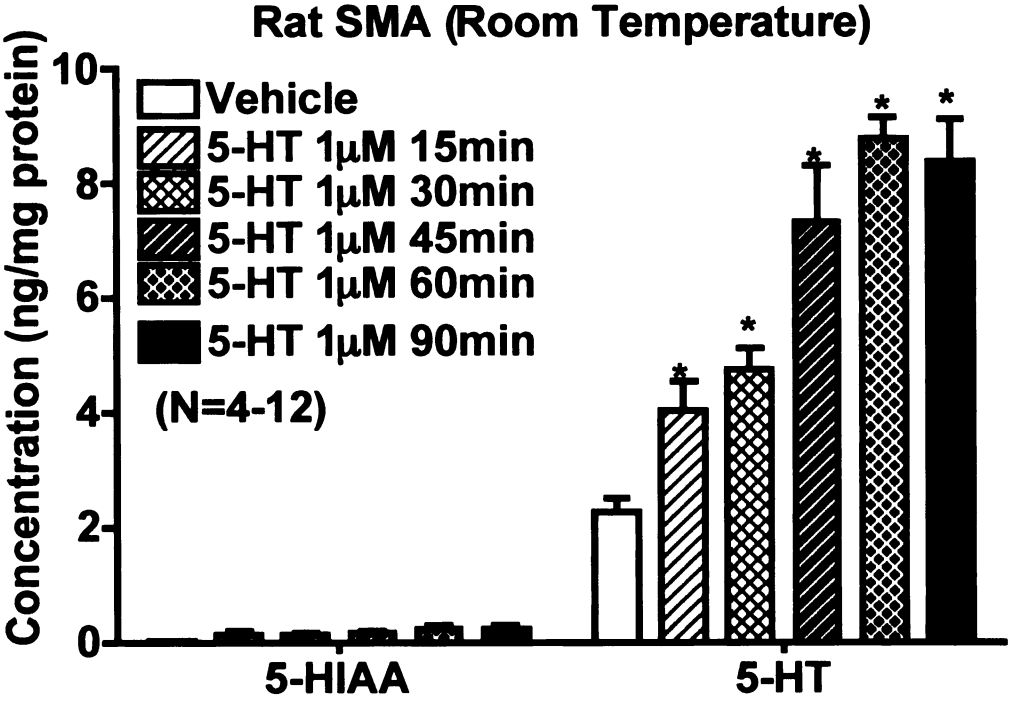


**Figure 26.**

Time course for 5-HT uptake in the superior mesenteric artery from pargyline-treated rats incubated with 5-HT (1 μM) at room temperature.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to time 0 (no incubation).

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

SMA= superior mesenteric artery.

**Figure 27.**

5-HT uptake in the aorta from wild type (top) and *tph1-/-* mice (bottom) incubated with exogenous 5-HT (1 μM, 15 min) at room temperature.

Bars represent means ± SEM for the number of animals in parentheses.

* p<0.05 compared to vehicle.

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

TPH=tryptophan hydroxylase.

**Table 5.**

Quantification of 5-HIAA and 5-HT in aorta from wild type or *tph1-/-* mice incubated with vehicle or exogenous 5-HT (1 μM, 15 min) at room temperature.

Values are reported as ng/mg protein.

Bars represent means ± SEM for the number of animals in parentheses.

* p<0.05 compared to vehicle.

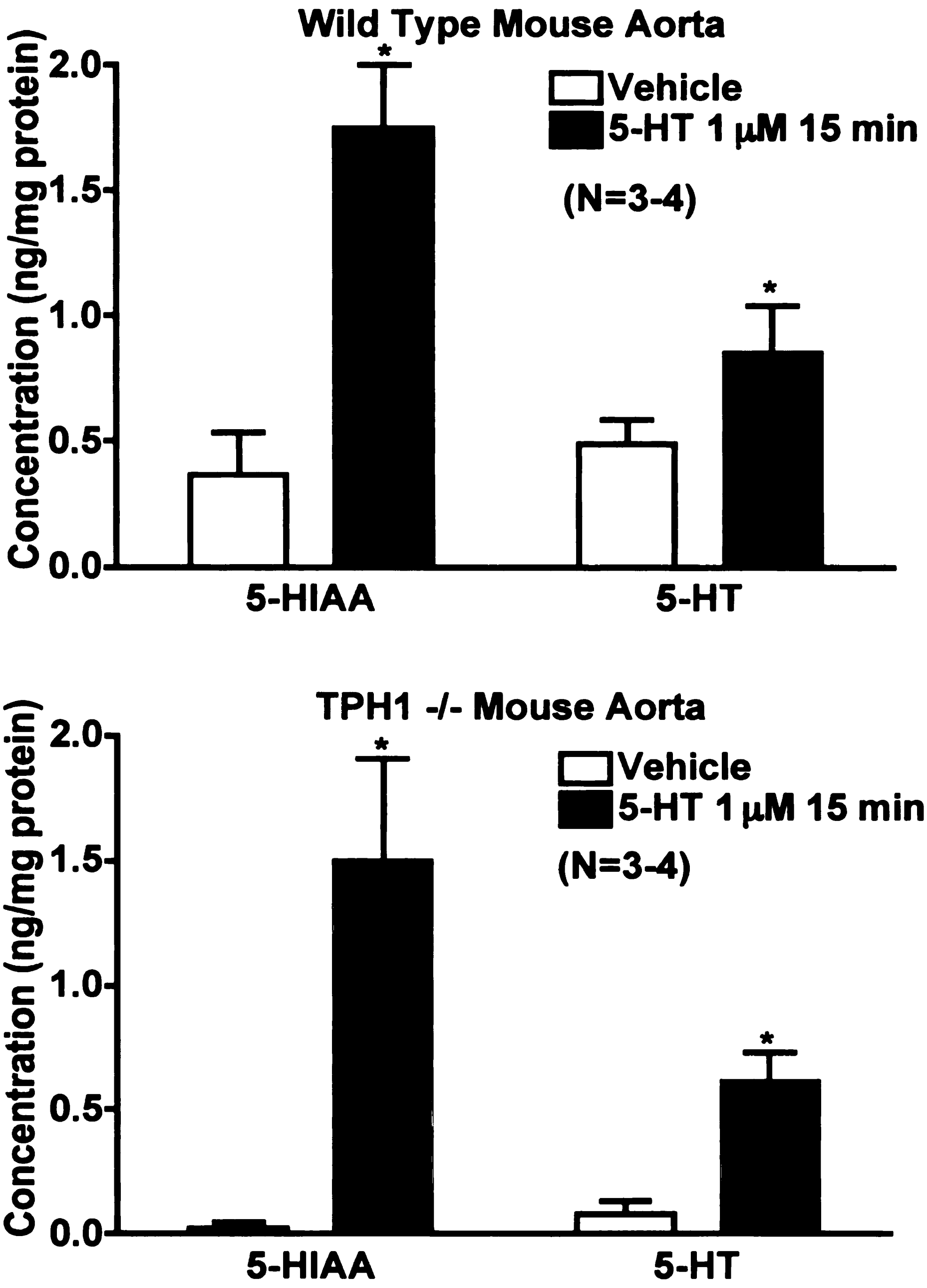


Figure 27.

Table 5.

| | Wild Type Mouse Aorta | | TPH 1-/- Mouse Aorta | |
|---|---|---|---|---|
| | Vehicle | 5-HT 1μM 15min | Vehicle | 5-HT 1μM 15min |
| 5-HIAA | 0.37±0.17 | 1.74±0.25* | 0.023±0.02 | 1.5±0.40* |
| 5-HT | 0.49±0.10 | 0.85±0.19* | 0.083±0.05 | 0.62±0.11* |

mesenteric artery) with either vehicle or one of the SERT inhibitors fluvoxamine (1 μM) or fluoxetine (1 μM) for 30 minutes prior to exposure to 5-HT (1 μM) for 15 minutes. The 5-HT levels in arteries incubated with 5-HT and SERT inhibitors were significantly lower than in arteries only incubated with exogenous 5-HT suggesting a SERT dependent 5-HT uptake in arteries. Neither fluoxetine (1 μM) nor fluvoxamine (1 μM) inhibited the entire uptake of 5-HT (Figure 28).

SERT-targeted mutation mice (SERT KO) were used in our study to test the dependence of SERT in 5-HT uptake in peripheral arteries. Basal level of 5-HIAA in aorta from pargyline-treated SERT KO mice were much lower compared with WT mice with a minor change of basal 5-HT level (Figure 29, vehicle incubation). Fluvoxamine inhibited 5-HT uptake in aorta from WT mice but not in SERT HET or SERT KO mice (Figure 29). Total 5-HT uptake reduced in aorta from SERT KO mice with no further inhibition of fluvoxamine pre-incubation.

### C. Investigation of SERT-independent 5-HT Uptake

We tested whether there is a $Na^+$-independent uptake because monoamine transporters (SERT, NET and DAT) are $Na^+$-$K^+$-$Cl^-$—dependent transporters, which have the potential to take up 5-HT. The result showed that there was a 5-HT uptake in aorta and superior mesenteric artery from pargyline-treated rats in $Na^+$ free ($Na^+$ was replaced isosmotically with N-methylglucamine) - PSS with 1 μM exogenous 5-HT (15 min), though the total uptake was significantly reduced compared to that in regular $Na^+$ containing PSS. SERT inhibitor, fluoxetine or fluvoxamine, did not further reduce 5-HT uptake in aorta

**Figure 28.**

Effects of a 30-minute preincubation with SERT inhibitor fluvoxamine (1 μM) or fluoxetine (1 μM) on 5-HT uptake (1 μM 5-HT incubation for 15 min) in aorta (top) or superior mesenteric artery (bottom) from pargyline treated rats.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle. # $p<0.05$ compared to 5-HT (1 μM).

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

SERT= serotonin transporter. SMA=superior mesenteric artery.

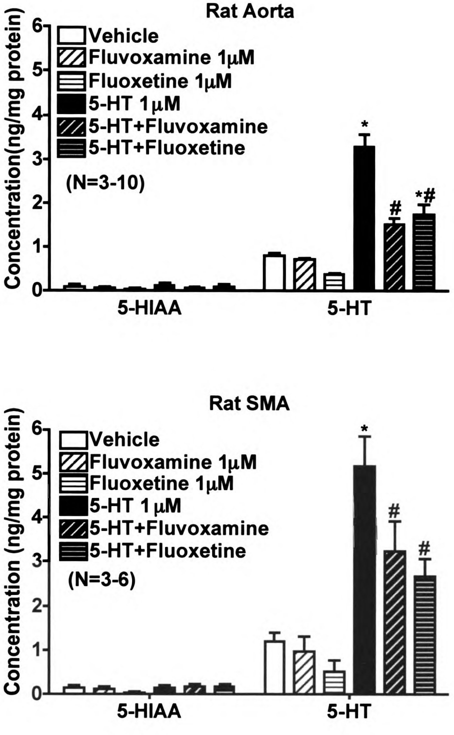

Rat Aorta
Concentration(ng/mg protein)
0
1
2
3
4
5
6
Vehicle
Fluvoxamine 1μM
Fluoxetine 1μM
5-HT 1μM
5-HT+Fluvoxamine
5-HT+Fluoxetine
(N=3-10)
*
#
*#
5-HIAA
5-HT
Rat SMA
Concentration (ng/mg protein)
0
1
2
3
4
5
6
Vehicle
Fluvoxamine 1μM
Fluoxetine 1μM
5-HT 1μM
5-HT+Fluvoxamine
5-HT+Fluoxetine
(N=3-6)
*
#
#
5-HIAA
5-HT

**Figure 28.**

**Figure 29.**

Effects of a 30-minute preincubation with SERT inhibitor fluvoxamine (1 μM) on 5-HT uptake (1 μM 5-HT incubation for 15 min) in aorta from wild type (top), SERT HET (middle) and SERT KO mice (bottom).

Bars represent means ± SEM for the number of animals in parentheses.

* p<0.05 compared with vehicle.

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

SERT= serotonin transporter.

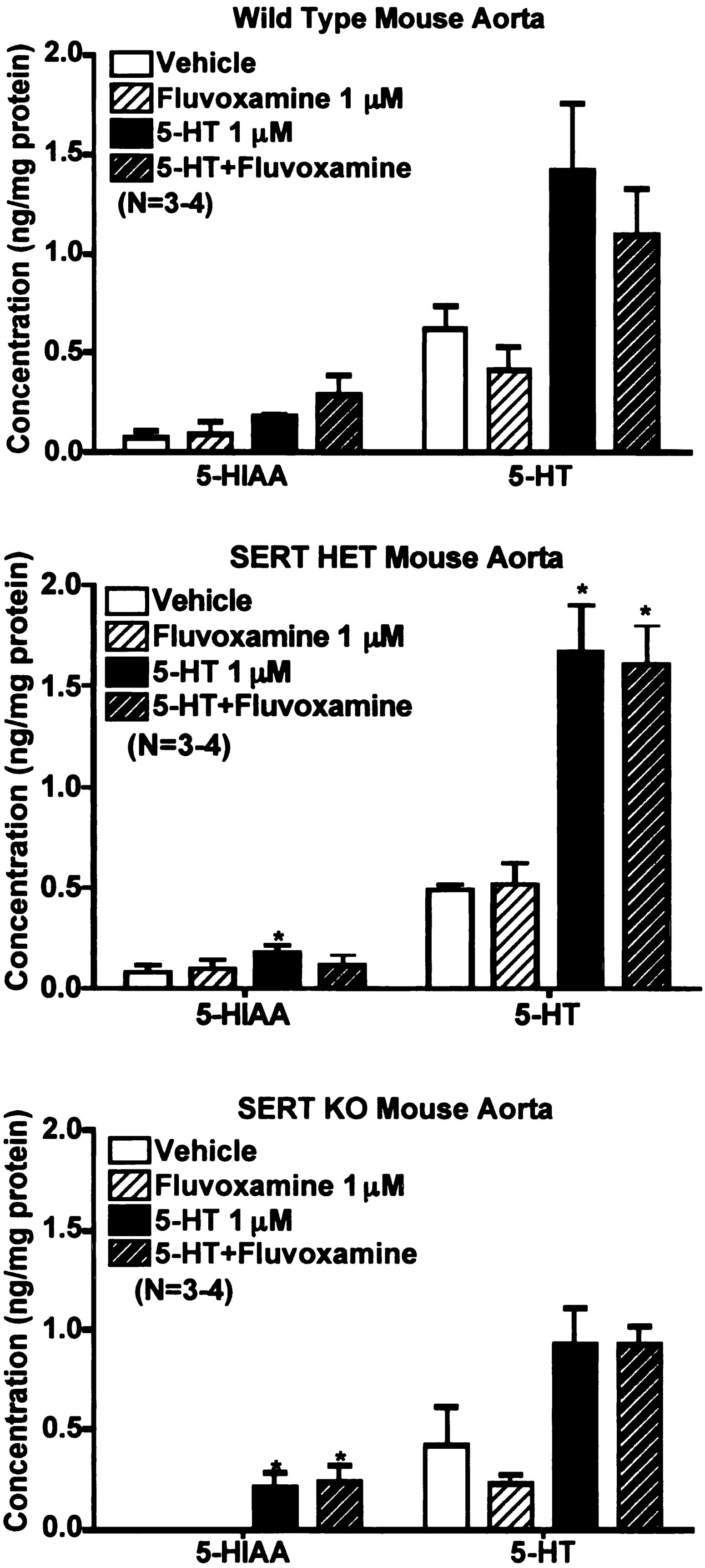

Wild Type Mouse Aorta
Concentration (ng/mg protein)
2.0
1.5
1.0
0.5
0.0
Vehicle
Fluvoxamine 1 μM
5-HT 1 μM
5-HT+Fluvoxamine
(N=3-4)
5-HIAA
5-HT
SERT HET Mouse Aorta
Concentration (ng/mg protein)
2.0
1.5
1.0
0.5
0.0
Vehicle
Fluvoxamine 1 μM
5-HT 1 μM
5-HT+Fluvoxamine
(N=3-4)
*
*
*
5-HIAA
5-HT
SERT KO Mouse Aorta
Concentration (ng/mg protein)
2.0
1.5
1.0
0.5
0.0
Vehicle
Fluvoxamine 1 μM
5-HT 1 μM
5-HT+Fluvoxamine
(N=3-4)
*
*
5-HIAA
5-HT

Figure 29.

**Figure 30.**

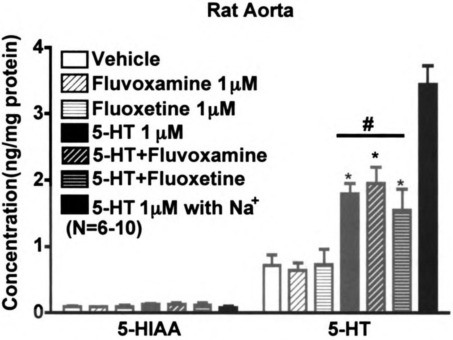
Effects of a 30-minute preincubation with SERT inhibitor fluvoxamine (1 μM) or fluoxetine (1 μM) on 5-HT uptake (1 μM 5-HT incubation for 15 min) in $Na^+$ -free PSS in aorta (top) or superior mesenteric artery (bottom) from pargyline treated rats.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle. # $p<0.05$ compared to 5-HT (1 μM) uptake in normal PSS (containing $Na^+$).

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

SERT= serotonin transporter. SMA=superior mesenteric artery.


Rat Aorta
Concentration(ng/mg protein)
0
1
2
3
4
Vehicle
Fluvoxamine 1μM
Fluoxetine 1μM
5-HT 1 μM
5-HT+Fluvoxamine
5-HT+Fluoxetine
5-HT 1μM with Na+
(N=6-10)
#
*
*
*
5-HIAA
5-HT

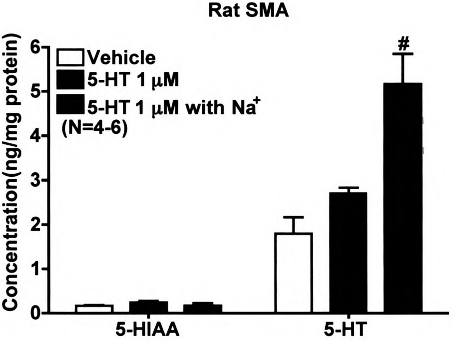

Rat SMA
Concentration(ng/mg protein)
0
1
2
3
4
5
6
Vehicle
5-HT 1 μM
5-HT 1 μM with Na+
(N=4-6)
#
5-HIAA
5-HT

**Figure 30.**

incubated in $Na^+$ free- PSS (Figure 30).

We then tested whether this 5-HT uptake was SNS, NET and OCT-dependent in peripheral arteries. To investigate whether uptake of 5-HT by the artery was dependent on sympathetic nerves, we used 6-OHDA denervated rats. Rats were injected with 6-OHDA during an 8-day protocol, and arteries procured on day 8. Validation of denervation was verified by lack of glyoxylic acid-reacted norepinephrine (Figure 31, top). While basal levels of 5-HT and 5-HIAA were slightly but not significantly lower than in untreated animals, arteries were still capable of taking up and metabolizing 5-HT as the concentrations of 5-HT and 5-HIAA increased in the vessels exposed to 5-HT for 15 minutes. This was true for all 3 arteries examined (Figure 31).

The NET inhibitor nisoxetine (100 nM) was unable to inhibit 5-HT uptake (Figure 32). The OCTs inhibitor, corticosterone (1 μM) did not inhibit 5-HT uptake in aorta but potentially could in superior mesenteric from pargyline-treated rats (Figure 33).

Based on our observation, arterial 5-HT uptake is not dependent on SNS, NET or OCT.

As the barrier between arterial smooth muscle and blood, endothelium could also take up 5-HT. Uptake of 5-HT, at least in the superior mesenteric artery appears to be largely independent of the endothelium as removal of this cell layer did not alter 5-HT uptake or metabolism (Figure 34).

**Figure 31.**

A. Glyoxylic acid fluorescence in small mesenteric resistance arteries from vehicle or 6-OHDA-treated rats. The white network is sympathetic nerve fibers.

B. 5-HT uptake in aorta, carotid artery and superior mesenteric artery from 6-OHDA treated rats.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared with vehicle.

5-HIAA=5-hydroxyindoleacetic acid. 5-HT= 5-hydroxytryptamine. SMA=superior mesenteric artery.

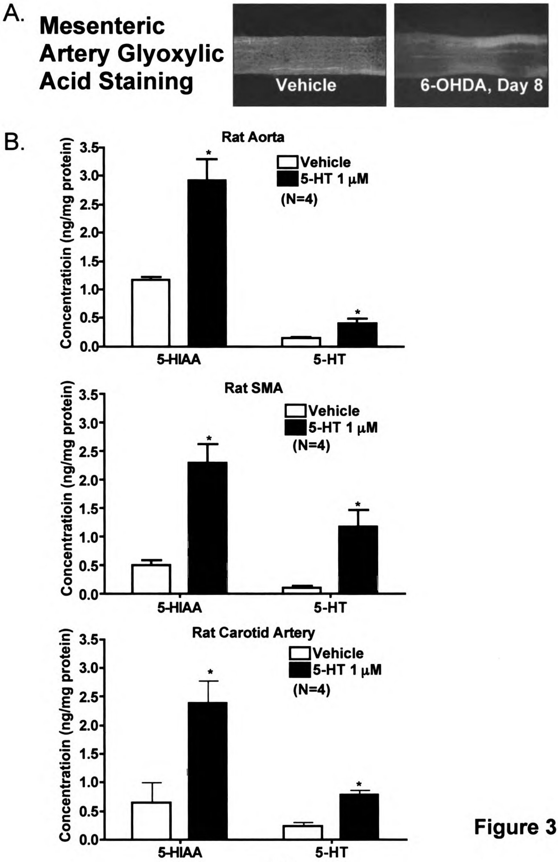

A.
Mesenteric Artery Glyoxylic Acid Staining
Vehicle
6-OHDA, Day 8
B.
Rat Aorta
Concentratioin (ng/mg protein)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
5-HT 1 μM
(N=4)
*
*
5-HIAA
5-HT
Rat SMA
Concentratioin (ng/mg protein)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
5-HT 1 μM
(N=4)
*
*
5-HIAA
5-HT
Rat Carotid Artery
Concentratioin (ng/mg protein)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
5-HT 1 μM
(N=4)
*
*
5-HIAA
5-HT

Figure 31.

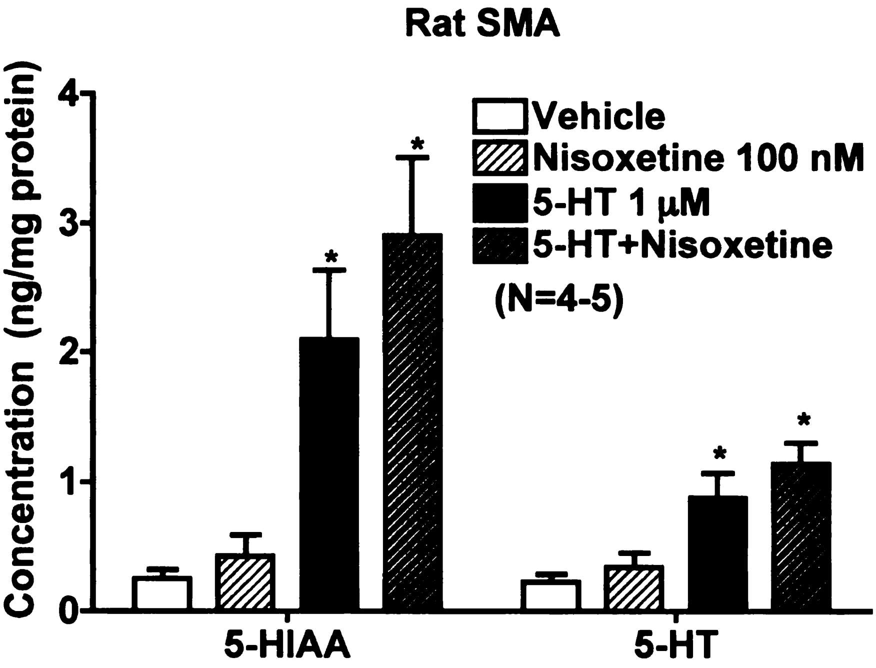


**Figure 32.**

Effects of a 30-minute preincubation with NET inhibitor nisoxetine (100 nM) on 5-HT uptake (1 μM 5-HT incubation for 15 min) in aorta from normal rats.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared with vehicle incubation.

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

NET=norepinepherin transporter. SMA=superior mesenteric artery.

**Figure 33.**

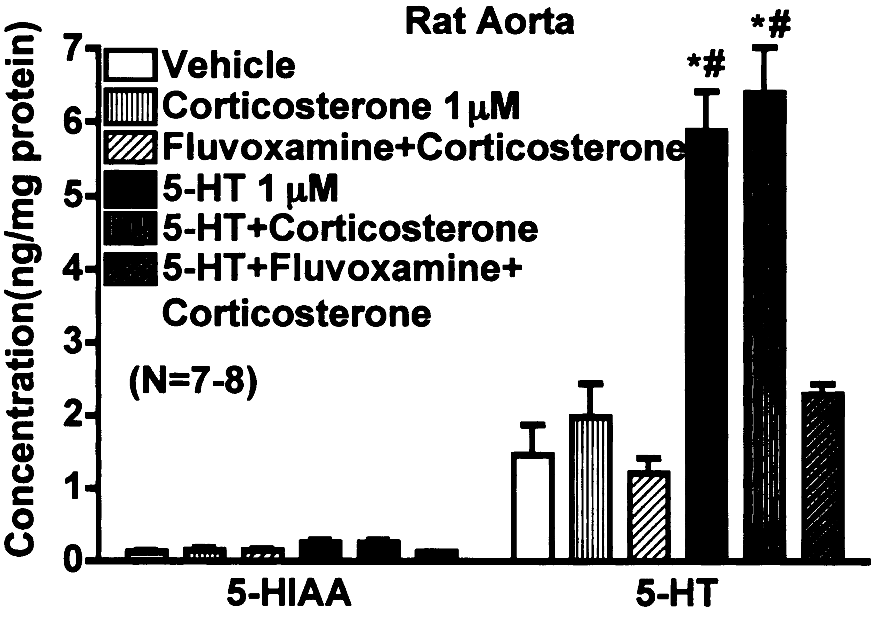
Effects of a 30-minute preincubation with OCT inhibitor corticosterone (1 μM) on 5-HT uptake (1 μM 5-HT incubation for 15 min) in aorta (Top) and superior mesenteric artery (bottom) from pargyline-trated rats.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared with vehicle incubation. #$p<0.05$ compared with 5-HT+fluvoxamine+corticosterone.

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

OCT= organic cation transporter. SMA=superior mesenteric artery.


Rat Aorta
Concentration(ng/mg protein)
0
1
2
3
4
5
6
7
Vehicle
Corticosterone 1 µM
Fluvoxamine+Corticosterone
5-HT 1 µM
5-HT+Corticosterone
5-HT+Fluvoxamine+ Corticosterone
(N=7-8)
*#
*#
5-HIAA
5-HT

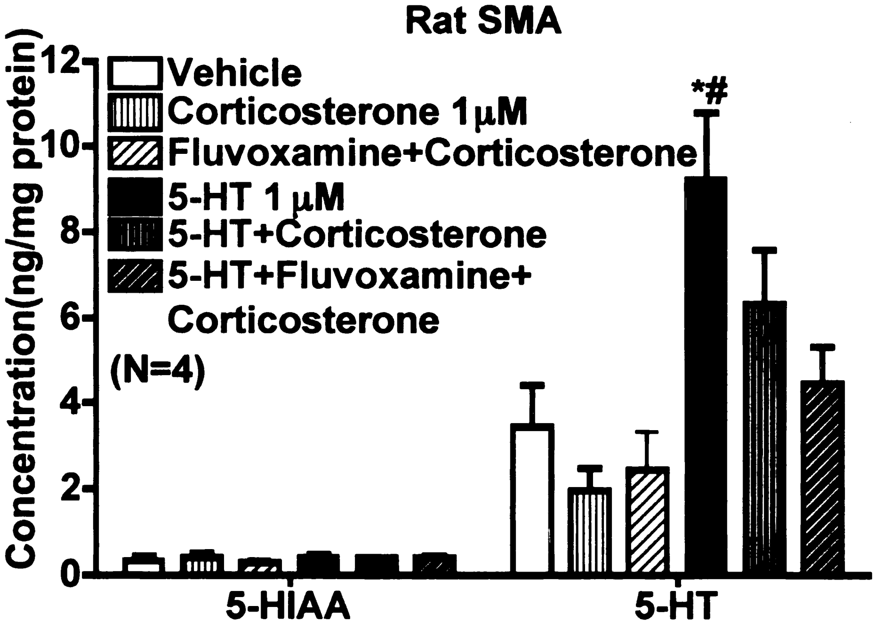

Rat SMA
Concentration(ng/mg protein)
0
2
4
6
8
10
12
Vehicle
Corticosterone 1 µM
Fluvoxamine+Corticosterone
5-HT 1 µM
5-HT+Corticosterone
5-HT+Fluvoxamine+ Corticosterone
(N=4)
*#
5-HIAA
5-HT

Figure 33.

**Figure 34.**

5-HT uptake (1 μM 5-HT incubation for 15 min) in endothelium-intact or endothelium denuded superior mesenteric artery from normal rats.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle incubation.

5-HIAA= 5-hydroxyindole acetic acid. 5-HT= 5-hydroxytryptamine.

SMA=superior mesenteric artery.

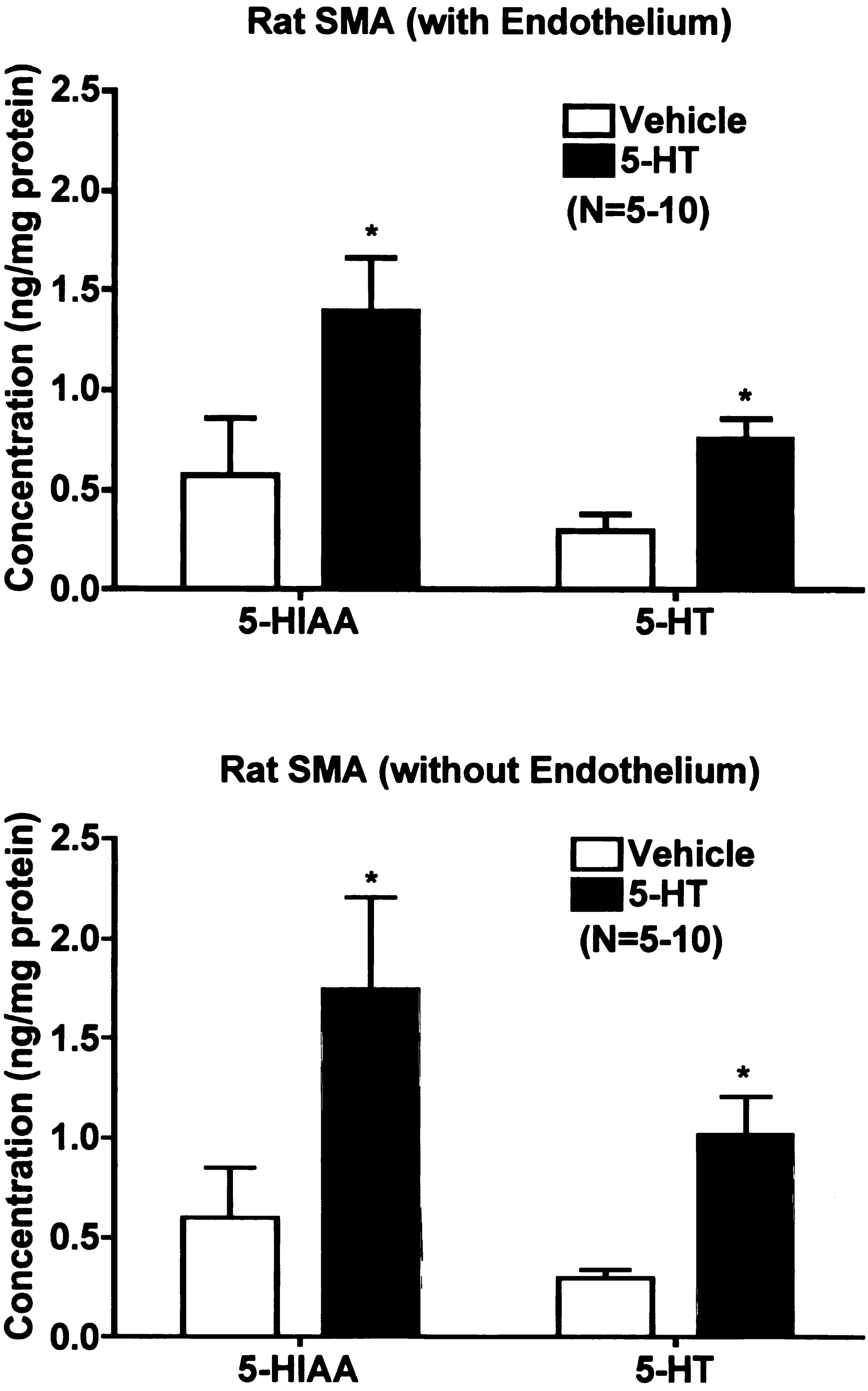

Rat SMA (with Endothelium)
Concentration (ng/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
5-HT
(N=5-10)
*
*
5-HIAA
5-HT
Rat SMA (without Endothelium)
Concentration (ng/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
5-HT
(N=5-10)
*
*
5-HIAA
5-HT

Figure 34.

### 3. The Presence of 5-HT Release in Peripheral Arteries

(+)-Fenfluramine and (+)-norfenfluramine are SERT substrates and 5-HT releasers. We used these two drugs to test the release of 5-HT from arteries. To enhance the signal of 5-HT, we used pargyline-treated animals to study 5-HT release from arteries.

Figure 35 shows that (+)-fenfluramine concentration (1 µM or 10 µM, 20 min) dependently released endogenous 5-HT from rat aorta into PSS. Similar amounts of 5-HT were released from rat aorta by incubation with 10 µM (+)-fenfluramine (0.37±0.07 ng/mg protein) and 10 µM (+)-norfenfluramine (0.35±0.18 ng/mg protein) for 20 minutes. The remaining 5-HT in aorta after fluramines incubation were 0.56±0.12 ng/mg protein and 0.74±0.22 ng/mg protein after (+)-fenfluramine or (+)-norfenfluramine-incubation, respectively. Thus, the released 5-HT during fluramines incubation accounts for about 30% of total endogenous 5-HT in rat aorta (Figure 35). The release of endogenous 5-HT is not a vessel specific effect as we observed fenfluramine (10 µM, 20 min) released endogenous 5-HT in superior mesenteric artery from pargyline-treated rats (Figure 36). Fluoxetine (1 µM, 30 min), which potentiated basal 5-HT release, did not inhibit (+)-fenfluramine (1 µM or 10 µM, 30 min)-induced 5-HT release from rat aorta (Figure 37).

**Figure 35.**

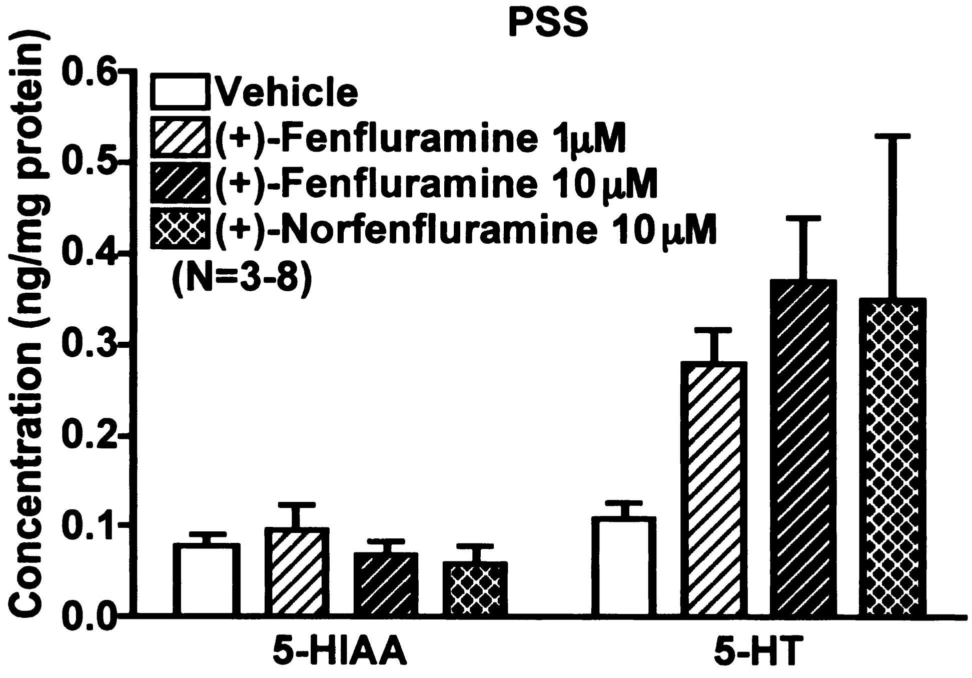
(+)-Fenfluramine (1 μM or 10 μM) or (+)-norfenfluramine (10 μM) induced 5-HT release from aorta obtained from pargyline-treated rats. Top: released 5-HIAA and 5-HT during fluramine incubation. Bottom: remained 5-HIAA and 5-HT after fluramine incubation.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle incubation.

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

PSS= physiological saline solution.


PSS
Concentration (ng/mg protein)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Vehicle
(+)-Fenfluramine 1μM
(+)-Fenfluramine 10μM
(+)-Norfenfluramine 10μM
(N=3-8)
5-HIAA
5-HT

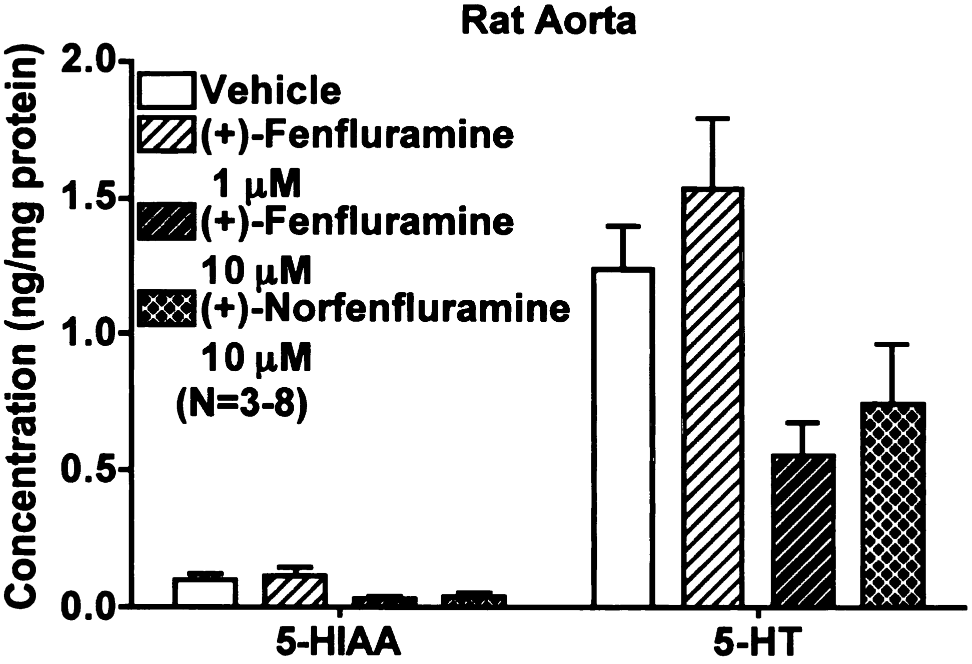

Rat Aorta
Concentration (ng/mg protein)
0.0
0.5
1.0
1.5
2.0
Vehicle
(+)-Fenfluramine 1 μM
(+)-Fenfluramine 10 μM
(+)-Norfenfluramine 10 μM
(N=3-8)
5-HIAA
5-HT

**Figure 36.**

(+)-Fenfluramine (10 μM)-induced 5-HT release in superior mesenteric artery from pargyline-treated rats. Top: released 5-HIAA/5-HT during (+)-fenfluramine incubation. Bottom: remaining 5-HIAA/5-HT after (+)-fenfluramine incubation.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle incubation.

5-HIAA= 5-hydroxyindole acetic acid. 5-HT= 5-hydroxytryptamine.

PSS= physiological saline solution. SMA=superior mesenteric artery.

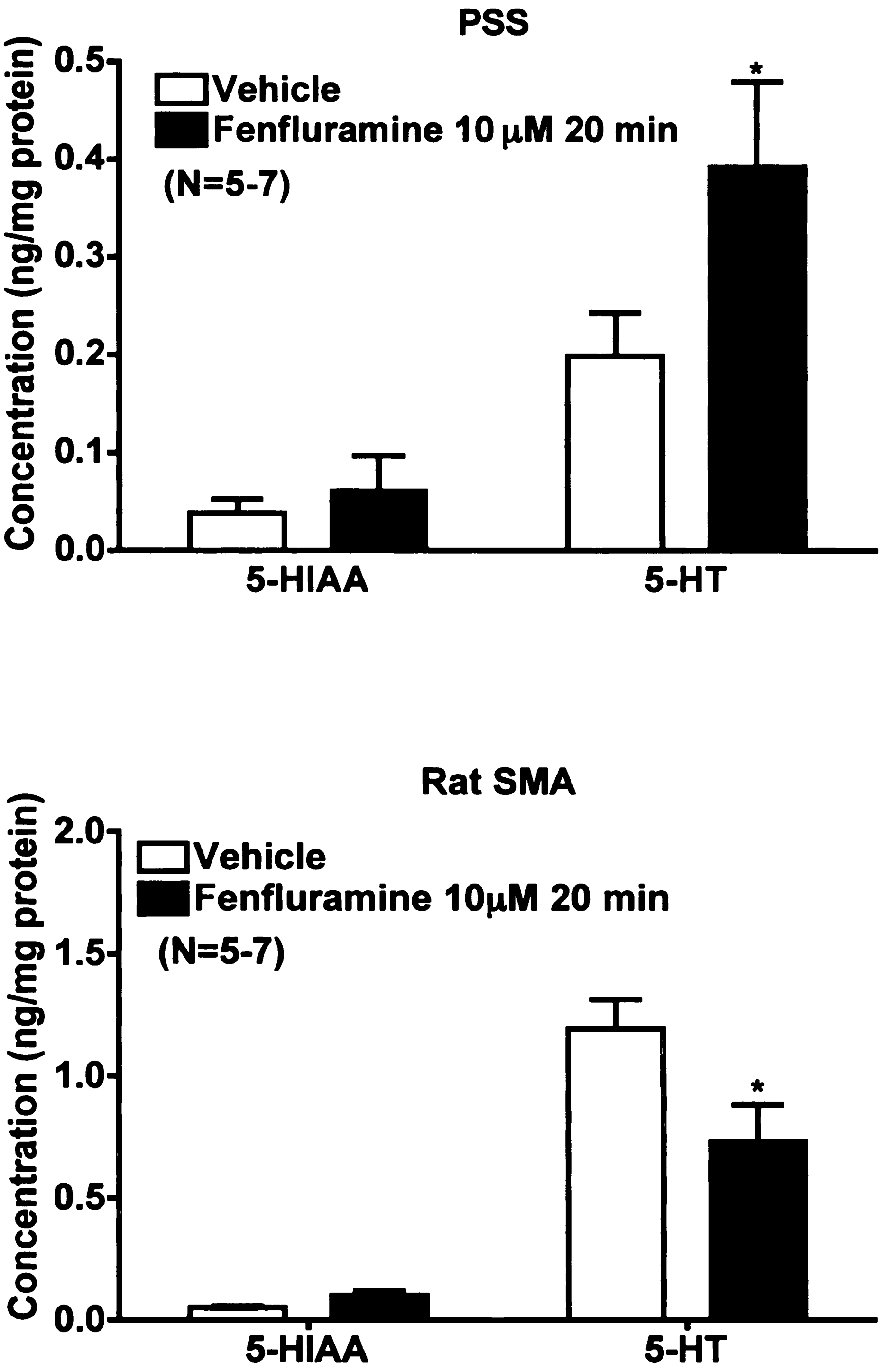

PSS
Concentration (ng/mg protein)
0.0
0.1
0.2
0.3
0.4
0.5
☐ Vehicle
■ Fenfluramine 10 μM 20 min
(N=5-7)
*
5-HIAA
5-HT
Rat SMA
Concentration (ng/mg protein)
0.0
0.5
1.0
1.5
2.0
☐ Vehicle
■ Fenfluramine 10μM 20 min
(N=5-7)
*
5-HIAA
5-HT

Figure 36.

**Figure 37.**

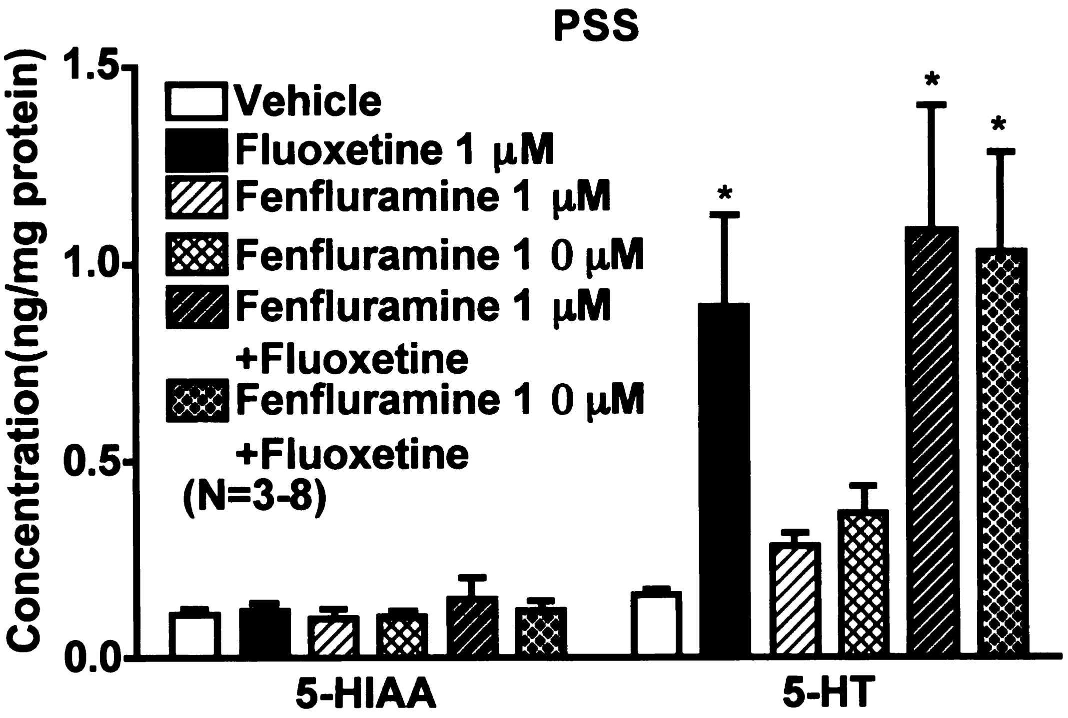
Effects of 30 minutes SERT inhibitor fluoxetine (1 μM) preincubation on (+)-fenfluramine (10 μM) –induced 5-HT release in aorta from pargyline-treated rats. Top: released 5-HIAA and 5-HT during fluramine incubation. Bottom: remaining 5-HIAA and 5-HT after (+)-fenfluramine incubation.

Bars represent means ± SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle incubation.

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

PSS=physiological saline solution. SERT=serotonin transporter.


PSS
Concentration(ng/mg protein)
1.5
1.0
0.5
0.0
Vehicle
Fluoxetine 1 μM
Fenfluramine 1 μM
Fenfluramine 1 0 μM
Fenfluramine 1 μM +Fluoxetine
Fenfluramine 1 0 μM +Fluoxetine
(N=3-8)
*
*
*
5-HIAA
5-HT

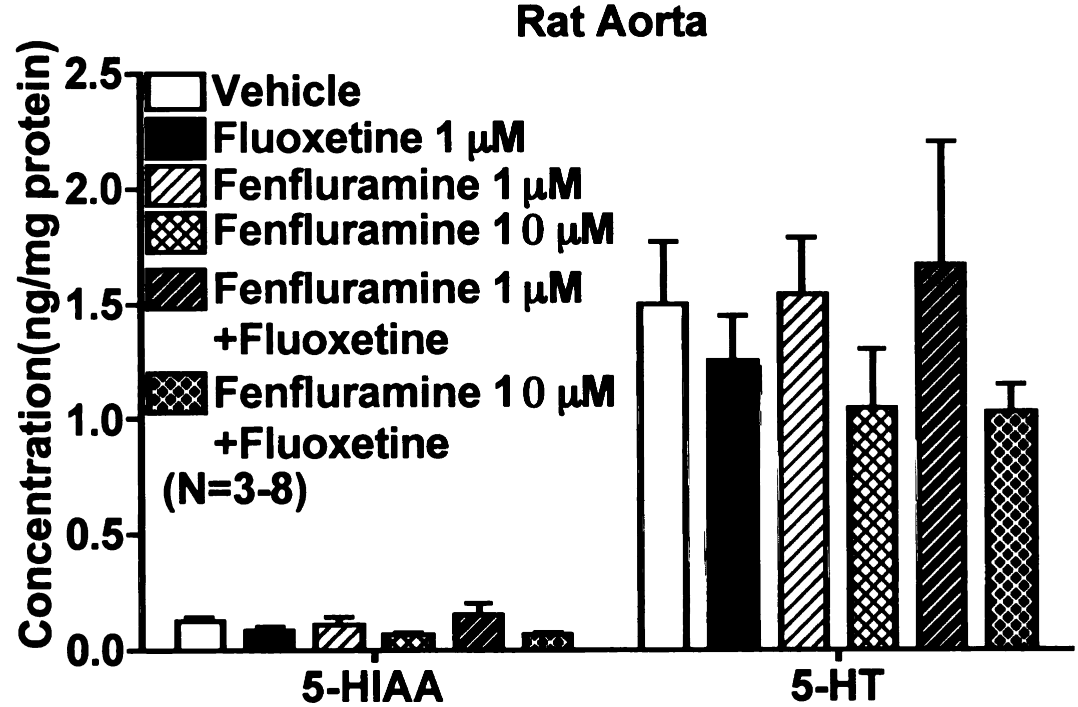

Rat Aorta
Concentration(ng/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
Fluoxetine 1 μM
Fenfluramine 1 μM
Fenfluramine 1 0 μM
Fenfluramine 1 μM +Fluoxetine
Fenfluramine 1 0 μM +Fluoxetine
(N=3-8)
5-HIAA
5-HT

Figure 37.

**Hypothesis #2:**

**Changes in the localized serotonergic system contribute to the increase of blood pressure in hypertension.**

We focused on comparing (1) SERT expression; (2) basal 5-HIAA and 5-HT levels; (3) peripheral arteries 5-HT uptake ability; and (4) function of SERT in modifying 5-HT-induced aortic contraction in normotensive and hypertensive rats.

## I. Measurement of SERT Expression in Aorta from Normotensive and Hypertensive rats

We used two experimental hypertensive rats model (DOCA-salt hypertensive rats and LNNA-hypertensive rats) and one genetic hypertensive model (SHR). The blood pressure of the animals we used in our study were SBP [mmHg], DOCA= 197±6, $SHAM_{(D)}$= 112±4, LNNA= 228±9, $SHAM_{(L)}$= 128±2, SHR= 172±7 and WKY= 121±3. Western blotting analysis using a SERT antibody, which recognizes amino acids (516-630) mapping at the carboxy terminus of the SERT protein (H115 antibody, Santa Cruz, CA) showed a single band slightly lower than 75 kDa (Figure 38). The expression of SERT in aorta from DOCA-salt rats was significantly elevated compared to SHAM rats ($SHAM_{(D)}$, which paired with DOCA-salt hypertensive rats) (arbitrary unit, normalized to $\alpha$-actin expression, $SHAM_{(D)}$= 2774±469, DOCA= 4374±125, $p<0.05$). This upregulation of SERT protein expression was not caused by increased transcription but increased translation of mRNA, as we observed no

**Figure 38.**

Western blotting analysis of SERT in aorta homogenates from SHAM$_{(D)}$, DOCA-salt hypertensive rats, SHAM$_{(L)}$, LNNA hypertensive rats, WKY and SHR. Representative of three experiments/rats.

SERT= serotonin transporter.

SHAM$_{(D)}$ = SHAM rats paired with DOCA-salt hypertensive rats.

SHAM$_{(L)}$ = SHAM rats paired with LNNA hypertensive rats.

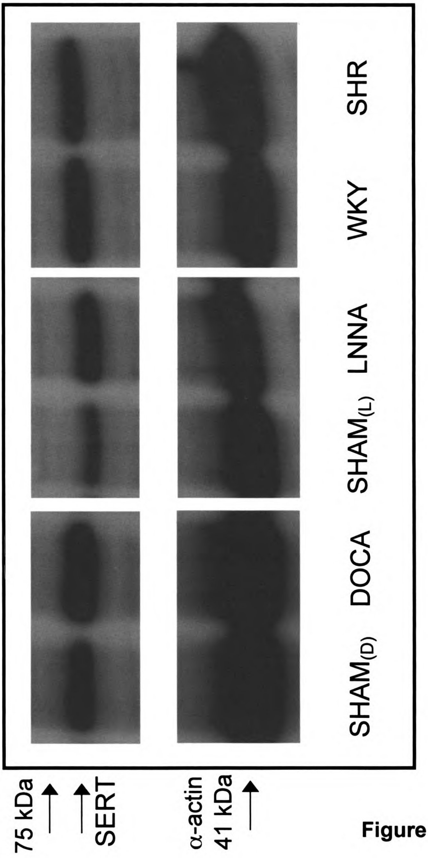


Figure 38.

difference in the delta $C_T$ value of SERT in SHAM and DOCA-salt samples in a real time RT-PCR study [Delta $C_T$(SERT $C_T$– GAPDH $C_T$) $SHAM_{(D)}$= 1.75 ± 0.1, DOCA-salt = 2.45 ± 0.13; $p= 0.064$; $n = 6$]. Similarly, SERT expression was also upregulated in LNNA rat aorta (arbitrary unit, $SHAM_{(L)}$ (normotensive rats paired with LNNA hypertensive rats) = 2074.3±291, LNNA= 3603±169, $p<0.05$). However, we did not observe a significant difference in SERT aortic expression between WKY and SHR. Figure 38 shows the representative picture from three different experiments/rats.

## II. Comparison of the Basal Level of 5-HT in DOCA-salt Rats, LNNA Rats and SHR with Their Normotensive Control Rats

We compared the basal level of 5-HT and its MAO A oxidized metabolite, 5-HIAA, in aorta from untreated (no pargyline treatment) normotensive and hypertensive rats. Figure 39 demonstrates that 5-HT but not 5-HIAA concentrations were significantly lower in aorta from DOCA-salt compared to SHAM rats (5-HT [ng/mg protein], $SHAM_{(D)}$= 0.31±0.07 and DOCA= 0.17±0.03, $p<0.05$). Both 5-HIAA and 5-HT concentrations in aorta were lower in LNNA rats compared to their control normotensive rats (5-HIAA [ng/mg protein], $SHAM_{(L)}$= 0.73±0.16 and LNNA= 0.10±0.02, $p<0.05$; 5-HT [ng/mg protein], SHAM= 0.55±0.16 and LNNA= 0.24±0.03, $p<0.05$). No differences were found in aortic 5-HIAA and 5-HT concentrations when comparing values from SHR to WKY.

**Figure 39.**

Detection of basal concentrations of 5-HIAA and 5-HT in thoracic aorta from $\mathrm{SHAM_{(D)}}$ rats, DOCA-salt hypertensive rats, $\mathrm{SHAM_{(L)}}$ rats, LNNA hypertensive rats, SHR and WKY rats.

Bars represent means±SEM for the number of animals in parentheses. * $p<0.05$ compared to appropriate normotensive control.

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

$\mathrm{SHAM_{(D)}}$ = SHAM rats paired with DOCA-salt hypertensive rats.

$\mathrm{SHAM_{(L)}}$ = SHAM rats paired with LNNA hypertensive rats.

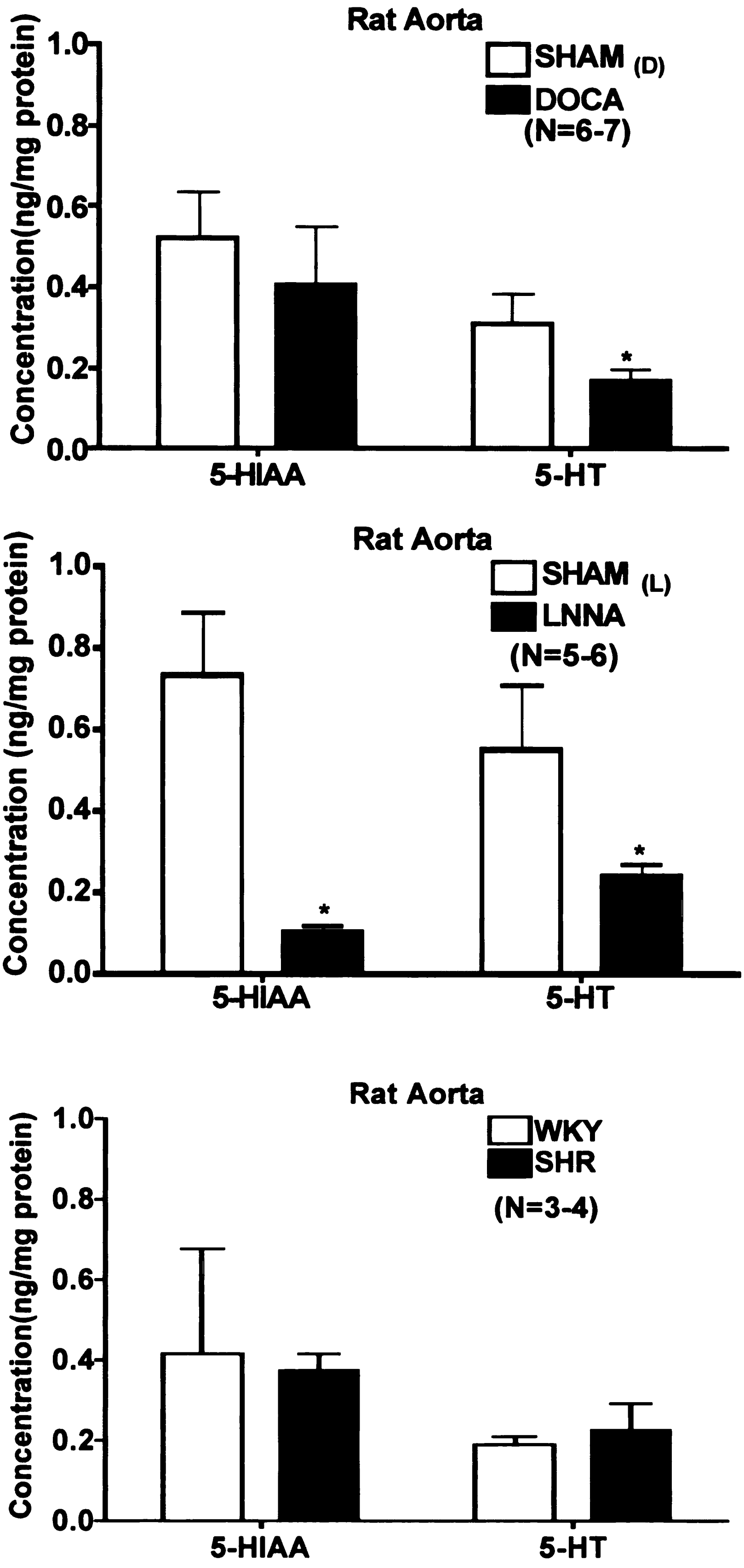

Rat Aorta
SHAM (D)
DOCA
(N=6-7)
Concentration(ng/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
*
5-HIAA
5-HT
Rat Aorta
SHAM (L)
LNNA
(N=5-6)
Concentration (ng/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
5-HIAA
5-HT
Rat Aorta
WKY
SHR
(N=3-4)
Concentration(ng/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
5-HIAA
5-HT

**Figure 39.**

## III. 5-HT Uptake Comparison in DOCA-salt Rats, LNNA Rats and SHR with Their Normotensive Control Rats

Active 5-HT uptake was measured in aorta from pargyline-treated rats. By inhibiting 5-HT metabolism, the observed changes in 5-HT concentrations in aorta after incubation with exogenous 5-HT directly reflect the 5-HT uptake in aorta.

Sections of aorta from the same rat were incubated with vehicle, the SERT inhibitor fluoxetine (1 μM), fluvoxamine (1 μM), 5-HT (1 μM), 5-HT (1 μM) plus fluoxetine (1 μM) or 5-HT (1 μM) plus fluvoxamine (1 μM). Aorta were incubated with inhibitor for 30 minutes before 5-HT was added (15 min incubation). We measured 5-HIAA and 5-HT concentrations in aorta after incubation. Aorta actively took up exogenous 5-HT while both SERT inhibitor fluvoxamine and fluoxetine reduced this 5-HT uptake in aorta from all animals studied (Figure 40,41,42).

To quantify **total 5-HT uptake** after incubation with exogenous 5-HT, we subtracted the 5-HT concentration in aorta incubated with vehicle from that in aorta incubated with exogenous 5-HT (5-HT 1 μM – vehicle, [ng/mg protein], $\text{SHAM}_{(D)}$= 3.14±0.22, DOCA= 2.36±0.31, $p<0.05$; $\text{SHAM}_{(L)}$= 3.50±0.39, LNNA= 2.51±0.25, $p<0.05$; WKY= 2.29±0.25 and SHR= 2.08±0.40, Figures 40,41,42, bottom). These results suggest that aorta from DOCA and LNNA rats took up less 5-HT compared to their normotensive rats while no difference existed between aorta from SHR and WKY. Consistent with our previous experiments, neither the SERT inhibitor fluoxetine (1 μM) nor fluvoxamine (1 μM)

**Figure 40.**

Effect of a 30-minute preincubation with SERT inhibitor fluoxetine (1 μM) or fluvoxamine (1 μM) on 5-HT concentrations in aorta from pargyline-treated $\text{SHAM}_{(D)}$ rats (top) and DOCA-salt hypertensive rats (middle).

Comparison of total 5-HT uptake and SERT dependent 5-HT uptake in aorta from $\text{SHAM}_{(D)}$ and DOCA-salt rats (bottom).

Bars represent means±SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle incubation. $^{\#}$ $p<0.05$ compared to 5-HT (1 μM) incubation.

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

SERT=serotonin transporter.

$\text{SHAM}_{(D)}$=SHAM rats paired with DOCA-salt hypertensive rats.

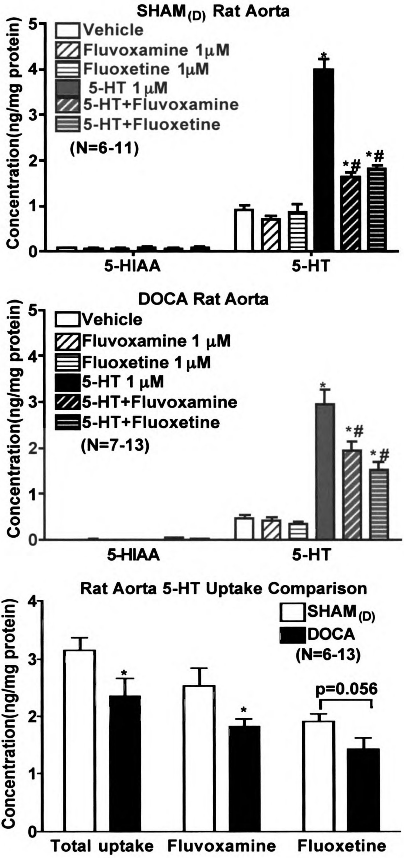

SHAM(D) Rat Aorta
Concentration(ng/mg protein)
0
1
2
3
4
5
Vehicle
Fluvoxamine 1μM
Fluoxetine 1μM
5-HT 1 μM
5-HT+Fluvoxamine
5-HT+Fluoxetine
(N=6-11)
*
*#
*#
5-HIAA
5-HT
DOCA Rat Aorta
Concentration(ng/mg protein)
0
1
2
3
4
5
Vehicle
Fluvoxamine 1 μM
Fluoxetine 1 μM
5-HT 1 μM
5-HT+Fluvoxamine
5-HT+Fluoxetine
(N=7-13)
*
*#
*#
5-HIAA
5-HT
Rat Aorta 5-HT Uptake Comparison
Concentration(ng/mg protein)
0
1
2
3
4
SHAM(D)
DOCA
(N=6-13)
*
*
p=0.056
Total uptake
Fluvoxamine
Fluoxetine

Figure 40.

**Figure 41.**

Effect of a 30-minute preincubation with SERT inhibitor fluoxetine (1 μM) or fluvoxamine (1 μM) on 5-HT concentration in aorta from pargyline-treated SHAM$_{(L)}$ rats (top) and LNNA hypertensive rats (middle).

Comparison of total 5-HT uptake and SERT dependent 5-HT uptake in aorta from SHAM$_{(L)}$ and LNNA rats (bottom).

Bars represent means±SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle incubation. # $p<0.05$ compared to 5-HT (1 μM) incubation.

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

SERT= serotonin transporter.

SHAM$_{(L)}$ = SHAM rats paired with LNNA hypertensive rats.

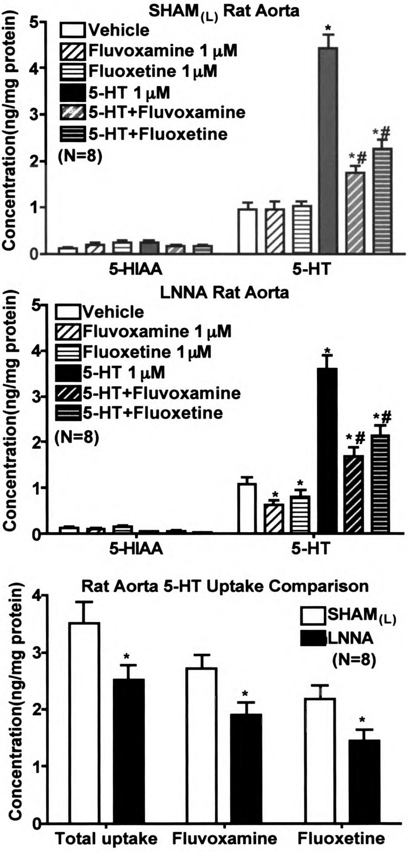

SHAM(L) Rat Aorta
Concentration(ng/mg protein)
0
1
2
3
4
5
Vehicle
Fluvoxamine 1 μM
Fluoxetine 1 μM
5-HT 1 μM
5-HT+Fluvoxamine
5-HT+Fluoxetine
(N=8)
*
*#
*#
5-HIAA
5-HT
LNNA Rat Aorta
Concentration(ng/mg protein)
0
1
2
3
4
5
Vehicle
Fluvoxamine 1 μM
Fluoxetine 1 μM
5-HT 1 μM
5-HT+Fluvoxamine
5-HT+Fluoxetine
(N=8)
*
*
*
*#
*#
5-HIAA
5-HT
Rat Aorta 5-HT Uptake Comparison
Concentration(ng/mg protein)
0
1
2
3
4
SHAM(L)
LNNA
(N=8)
*
*
*
Total uptake
Fluvoxamine
Fluoxetine

**Figure 41.**

**Figure 42.**

Effect of a 30-minute preincubation with SERT inhibitor fluoxetine (1 μM) or fluvoxamine (1 μM) on 5-HT concentration in aorta from pargyline-treated WKY (Top) and SHR (Middle).

Comparison of total 5-HT uptake and SERT dependent 5-HT uptake in aorta from WKY and SHR (Bottom).

Bars represent means±SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle incubation. # $p<0.05$ compared to 5-HT (1 μM) incubation.

5-HIAA= 5-Hydroxyindoleacetic acid, 5-HT= 5-Hydroxytryptamine.

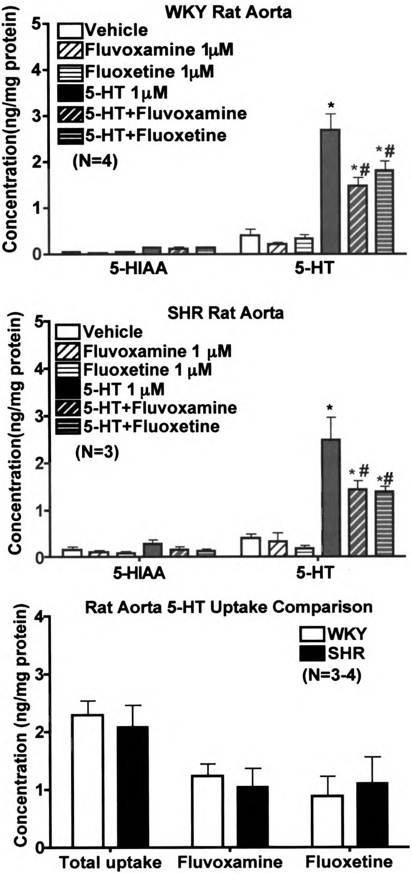

WKY Rat Aorta
Concentration(ng/mg protein)
0
1
2
3
4
5
Vehicle
Fluvoxamine 1μM
Fluoxetine 1μM
5-HT 1μM
5-HT+Fluvoxamine
5-HT+Fluoxetine
(N=4)
*
*#
*#
5-HIAA
5-HT
SHR Rat Aorta
Concentration(ng/mg protein)
0
1
2
3
4
5
Vehicle
Fluvoxamine 1 μM
Fluoxetine 1 μM
5-HT 1 μM
5-HT+Fluvoxamine
5-HT+Fluoxetine
(N=3)
*
*#
*#
5-HIAA
5-HT
Rat Aorta 5-HT Uptake Comparison
Concentration (ng/mg protein)
0
1
2
3
4
WKY
SHR
(N=3-4)
Total uptake
Fluvoxamine
Fluoxetine

Figure 42.

blocked the entire 5-HT uptake stimulated by exogenous 5-HT (Figures 40,41,42).

To quantify the **SERT-mediated 5-HT uptake** in aorta, we compared 5-HT uptake in the presence and absence of fluvoxamine or fluoxetine. We subtracted the 5-HT concentration (ng/mg protein) in aorta after incubation with exogenous 5-HT plus SERT inhibitor (fluvoxamine or fluoxetine) from that of aorta only incubated with exogenous 5-HT [5-HT – (5-HT+fluvoxamine) or 5-HT – (5-HT+fluoxetine)]. This is a value we interpreted to reflect 5-HTT-dependent 5-HT uptake. There was significantly lower SERT-mediated 5-HT uptake (calculated from fluvoxamine inhibition) in DOCA (1.83±0.12 ng/mg protein) compared to $SHAM_{(D)}$ (2.54±0.30 ng/mg protein) and in LNNA (1.45±0.20 ng/mg protein) compared to its normotensive control (2.18±0.23 ng/mg protein) while no difference existed in SHR (1.05±0.32 ng/mg protein) compared to WKY rats (1.23±0.22 ng/mg protein). Similar results were obtained from the calculation of SERT-mediated 5-HT uptake by fluoxetine inhibition (ng/mg protein, $SHAM_{(D)}$= 1.92±0.12, DOCA=1.43±0.18, $p=0.056$; $SHAM_{(L)}$= 2.71±0.25, LNNA= 1.91±0.20, $p<0.05$; WKY= 0.89±0.33, SHR=1.10±0.47)

## IV. SERT Function in Contractility- Compare DOCA-salt Rats with Normal Rats

The next experiments determined whether increased expression and decreased 5-HT uptake ability of the SERT in arteries possessed activity sufficient to modify 5-HT–induced contraction. The model of DOCA-salt hypertensive rats was used, as arteries from these rats are well known for their super-sensitivity to the contraction effect of 5-HT. In aorta isolated from SHAM and DOCA-salt hypertensive rats, cumulative 5-HT concentration response curves were investigated in the presence of vehicle or one of two different SERT inhibitors, fluoxetine or fluvoxamine. We used 1 μM of SERT inhibitors, which is consistent with our 5-HT uptake study. Neither drug at the concentration of 1 μM affected KCl –induced contraction (6-100 mM, data not shown). Figure 43 and Table 6 describes the 5-HT concentration response curves in aorta from SHAM and DOCA rats graphically and using pharmacological parameters, respectively. We confirmed that aorta of DOCA-salt rats were hyperresponsive to 5-HT compared with SHAM, evidenced by a small but significant decrease in the $EC_{50}$ value of 5-HT and a decreased threshold concentration for contraction (Table 6). Fluoxetine and, to a greater measure, fluvoxamine decreased the threshold of 5-HT-induced contraction in aorta from DOCA-salt but not normotensive rats. Similarly, the potency of 5-HT was significantly enhanced by fluvoxamine in aorta from DOCA-salt rats (4.2-fold). Fluvoxamine also potentiated 5-HT-induced contraction in aorta from SHAM rats.

**Figure 43.**

Effect of the SERT inhibitor fluoxetine (1 μM; top) and fluvoxamine (1 μM; bottom) on 5-HT-induced contraction in aorta from SHAM and DOCA-salt rats. Points represent means±SEM for the number of animals in parentheses.

* $p<0.05$ compared to vehicle incubation.

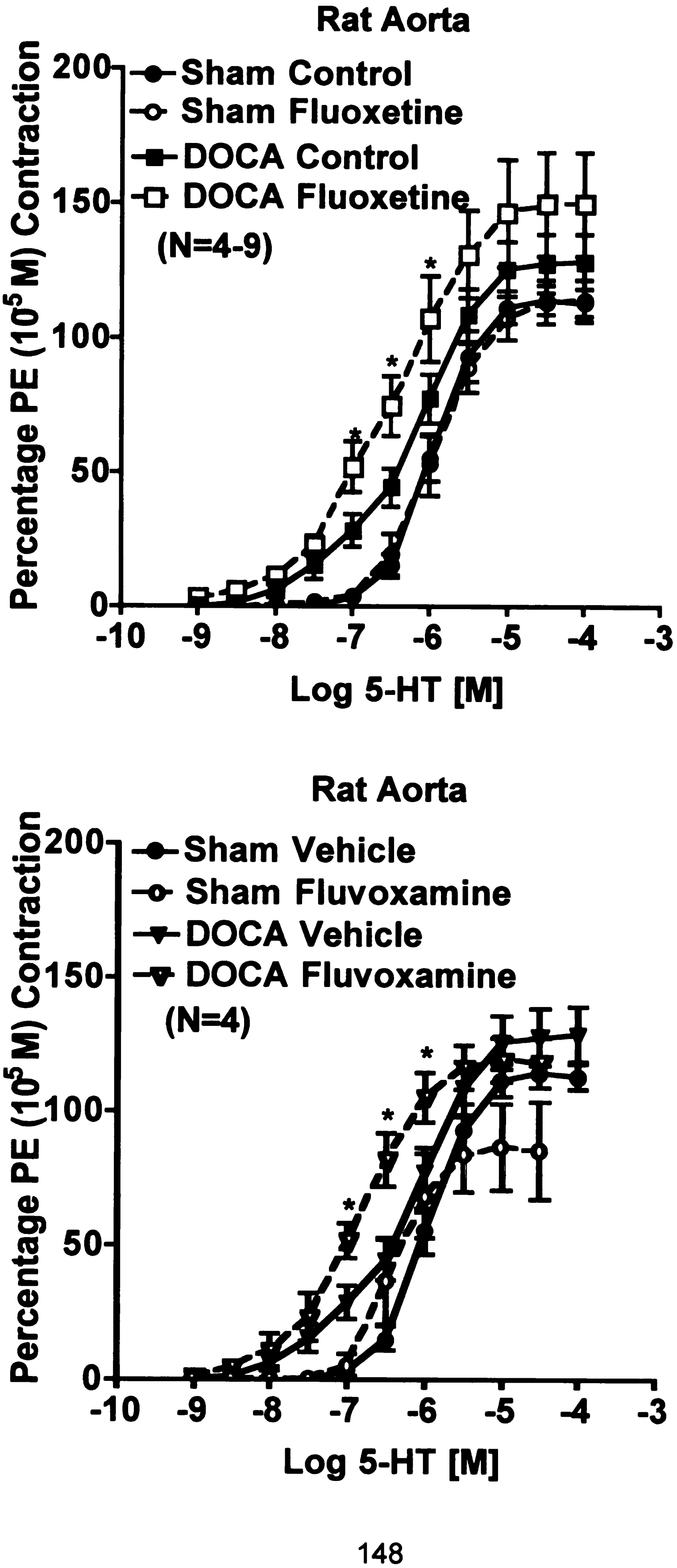

Rat Aorta
Percentage PE (10$^{5}$M) Contraction
200
150
100
50
0
Sham Control
Sham Fluoxetine
DOCA Control
DOCA Fluoxetine
(N=4-9)
*
*
*
*
-10
-9
-8
-7
-6
-5
-4
-3
Log 5-HT [M]
Rat Aorta
Percentage PE (10$^{5}$M) Contraction
200
150
100
50
0
Sham Vehicle
Sham Fluvoxamine
DOCA Vehicle
DOCA Fluvoxamine
(N=4)
*
*
*
-10
-9
-8
-7
-6
-5
-4
-3
Log 5-HT [M]

Figure 43.

| **Control** | **SHAM** | **DOCA-salt** |
|---|---|---|
| Threshold | 6.92±0.07 | 8.00±0.16* |
| $EC_{50}$ | 5.90±0.08 | 6.13±0.09* |
| **Fluoxetine** | **SHAM** | **DOCA-salt** |
| Threshold | 7.06±0.25 | 8.83±0.16* |
| $EC_{50}$ | 5.90±0.10 | 6.44±0.08* |
| **Fluvoxamine** | **SHAM** | **DOCA-salt** |
| Threshold | 7.50±0.20# | 8.50±0.28* |
| $EC_{50}$ | 6.43±0.21# | 6.85±0.08* |

**Table 6.**

Quantification of the threshold and $EC_{50}$ in 5-HT-induced contraction in aorta from normotensive and hypertensive rats. Values reported as the negative logarithm of the value [M].

*indicates statistically significant differences ($p<0.05$) when compared to SHAM.

# indicates statistically significant differences ($p<0.05$) when compared to vehicle.

$EC_{50}$ = concentration of 5-HT eliciting a half-maximal contraction.

Threshold = lowest concentration at which contraction is observed.

## Discussion:

Originally discovered in intestinal tract and blood, multiple functions of 5-HT in physiological and pathological conditions have been revealed. The loss of precise serotonergic system regulation (including 5-HT synthesis or metabolism, reuptake or release and 5-HT receptor expression) in the brain leads to mental disease. 5-HT is related to many peripheral vascular diseases but the role of a local serotonergic system has not been studied. In this study, we investigated the existence of a local serotonergic system in peripheral arteries and whether this local regulation of 5-HT was altered in hypertension.

### I. Biochemical Proof of 5-HT and its Metabolite in Peripheral Normal Arteries

It was considered that peripheral arteries are only exposed to 5-HT from blood. Whether peripheral arterial smooth muscle has endogenous 5-HT was not known. The existence of endogenous 5-HT and its metabolite 5-HIAA is the fundamental tenet of our hypothesis -- a local serotonergic system exists in peripheral arteries.

Three different techniques were used in our experiments. All results suggested the existence of 5-HT in peripheral arteries. Use of HPLC (Figure 4), as opposed to a classic assay using measurement of [$^3$H]5-HT uptake, was critical, because we could measure both 5-HIAA and 5-HT in these assays. The existence of 5-HT in smooth muscle is supported by 5-HT immunoreactivity in smooth muscle layers and cytosol of freshly isolated smooth muscle cells that are not exposed to blood, a potential source of extraneous 5-HT through blood platelets. While arteries contain mast cells, it is unlikely that these cells make a significant contribution to the 5-HT concentrations measured as few mast cells could be identified. Mast cell populations have been reported as rich in the coronary adventitia (Wolf et al., 1998), a site where there was in fact strong staining for 5-HT in our study (Figure 6). Additionally, mast cells have been found in femoral arteries (Mathiau et al., 1993). It is important to note that measures of mast cells were not made in the carotid and superior mesenteric arteries, and thus we cannot exclude the possibility that some of the 5-HT and 5-HIAA measured came from mast cells in these particular arteries. The staining of 5-HT antibody in isolated aortic smooth muscle cells (Figure 7) further supported that at least part of the 5-HT we measured using HPLC came from arterial smooth muscle cells.

Thus, we quantified basal arterial endogenous 5-HIAA and 5-HT concentration by HPLC measurements and localized 5-HT in endothelium and peripheral arteries smooth muscle cells by immunostaining.

## II. Investigation of 5-HT Synthesis in Peripheral Normal Arteries

The presence of TPH is considered a hallmark of 5-HT synthesis. In literature, studies investigating the serotonergic innervation of peripheral arterial blood vessels were performed before the advent of PCR and antibody-based IHC and Western analyses.

As depicted in Figure 1, TPH, AADC and MAO A are enzymes responsible for each step of 5-HT synthesis and metabolism. The existence of these enzymes is an essential requirement for synthesizing and metabolizing 5-HT in peripheral arteries. We tried to identify the existence of TPH protein in peripheral artery by Western analysis and IHC. However, due to technique limitations of the antibodies, we were not able to visualize TPH protein in homogenates from rat or mouse aorta in Western analysis. This could be due to degradation of TPH protein in the procedure of protein isolation. The results from IHC experiments were not convincing either. Similar results were observed in both frozen sections (Figure 9) and paraffin embedded sections (pictures not shown) with relatively darker staining in sections incubated with primary antibody compared to no primary incubation. Thus, another experimental technique was necessary to identify the existence of TPH protein. Pictures from immunocytochemistry experiments showed a positive TPH antibody staining in freshly isolated aortic smooth muscle cells. To lend support to this finding, we did real time RT-PCR experiments to investigate the existence of *tph1* mRNA. The results using two different sets of *tph1* primers suggested the presence of *tph1* mRNA in peripheral artery, which reinforced the result of existence of TPH protein in our immunocytochemistry experiment. We followed these measures with an activity assay.

The rationale of our TPH activity assay was that the measurement of the accumulated 5-HTP in arteries, when AADC was inhibited by NSD1015, would indicate that a functional TPH exists. The assumption of this experiment was that peripheral arteries do not take up 5-HTP (the 5-HTP that we measured was synthesized in arteries). Though we did observe a 5-HTP peak in arteries from NSD1015 treated rats and an increased 5-HTP was observed in NSD1015 + 5-HT synthesis substrate, tryptophan-treated rats, we cannot make the conclusion that this 5-HTP was synthesized by the peripheral artery as we also observed that peripheral arteries are capable of taking up 5-HTP (Figure 11). Thus, it is important to do an *in vitro* experiment to test the function of TPH in arteries. As we mentioned in the introduction, TPH is an extremely unstable enzyme, which loses its function quickly due to exposure to oxygen. In our *in vitro* TPH activity assay (Figure 12), we incubated isolated rat arteries with incubation solution, in which we added the reducing agent ferrous ammonium sulfate and DTT to recover oxygen reduced TPH activity, added catalase to stabilize TPH, added 5-HT synthesis cofactor BH4, added NSD1015 to inhibit the conversion of 5-HTP to 5-HT and in some of our experiments we added tryptophan. There was no detectable 5-HTP in artery samples, while very low amount of 5-HTP was observed in rat raphe samples. We have two explanations for our data. The first, **there is TPH expression in peripheral arteries. However, this TPH is not functional, at least in normal animals.** The presence of TPH, AADC and BH4 (Gross et al., 1993) in arteries suggests the possibility of 5-HT synthesis. In disease condition, TPH activity, BH4 production and $Ca^{2+}$/calmodulin-dependent protein kinase could change and TPH might be activated by these changes. Evidence supporting this speculation is increased $Ca^{2+}$/calmodulin-dependent protein kinase activity in SHR (Boknik et al., 2001) and increased vascular gene and protein expression of PKA in two-kidney, one clip (2K-1C) renal hypertensive *vs.* normotensive two kidney (2K) rats (Callera et al., 2004), which might lead to increased TPH activity. However, it was reported that in DOCA-salt hypertensive rats, the arterial BH4 level was decreased. The reduction of BH4 minimized the possibility that a functional TPH exists in peripheral arteries. The second explanation for our data is that **there is 5-HT synthesis in peripheral arteries from normal rats.** However, we are limited by the sensitivity of HPLC to measure this level of 5-HTP.

## III. Investigation of Metabolism of 5-HT in Peripheral Arteries

Uptake study in arteries from normal rats (Figure 15) showed that after 15 minutes of incubation with exogenous 5-HT, the concentration of 5-HIAA in arteries was significantly higher than 5-HT, suggesting that 5-HT was taken up into blood vessel and rapidly metabolized. This observation suggests that **peripheral arteries might play an important role in clearance of free circulating 5-HT in blood**.

## IV. Investigation of Storage of 5-HT in Peripheral Arteries

We do not believe that peripheral arteries function like platelets as a "5-HT sink", which store and release 5-HT, because even in an extreme condition, in which we preload 5-HT in aorta from pargyline-treated rats, we did not observe that 5-HT could be kept in aorta. Peripheral arteries take up and metabolize 5-HT but do not store 5-HT.

We also investigated the localization and the form of endogenous intracellular 5-HT. Western analysis showed serotonin-modified proteins in arteries. Moreover, numerous proteins were reported as the substrates of TGII, such as actin, myosin and troponin (Griffin et al., 2002). Whether this modification is linked to a functional change is not known yet. Other 5-HT could be packed in vesicles, as we observed fluramines-induced 5-HT release, although we do not have direct evidence showing the existing of VMAT in peripheral arteries.

## V. Investigation of functional SERT in Peripheral Normal Arteries

We employed a multi-faceted approach in determining that peripheral smooth muscle has the potential to synthesize SERT. Real time RT-PCR and gel analyses of the final product after 35 cycles indicated the presence of one product. Translation of this message was confirmed by successfully measuring SERT protein product. Importantly, we confirmed immunohistochemically that a majority of the SERT is located in smooth muscle. Endothelial cells were stained positive for the SERT and this is consistent with previous findings (Lee et al., 1986; Small et al., 1977; Brust et al., 2000).

Having demonstrated the presence of the SERT protein in arteries, we determined if peripheral arteries had the ability to take up 5-HT. In a simple assay (Figure 24), we demonstrated that 5-HT was taken up in a time-dependent manner by observing increased 5-HIAA with time in arteries. Because MAO A is an enzyme classically found in mitochondria, the increased arterial 5-HIAA after exogenous 5-HT incubation suggested the ability of peripheral arteries to taken up 5-HT. We proved that this uptake is SERT-dependent by pharmacologically inhibition (Figure 28) and genetic ablation of SERT (Figure 29).

## VI. Investigation of SERT-independent 5-HT Uptake Mechanisms in Peripheral Arteries

We noticed that neither pharmacological inhibition nor genetic ablation of SERT could abolish all the 5-HT uptake in arteries, which suggested that other SERT-independent mechanisms are involved in 5-HT uptake.

One of the vessels we have used is the aorta, which contains sparse innervation by the sympathetic nervous system. Uptake in this minimally innervated tissue lends indirect support that it is the smooth muscle cells rather than nerves, takes up 5-HT (Kawasaki and Takasaki, 1984; Kawasaki et al. 1987). Moreover, in arteries from animals that have been chemically denervated or exposed to the NET inhibitor nisoxetine (Figure 32), uptake in all vessels remained normal. While these experiments do not exclude the possibility that sensory nerves could take up 5-HT, these data strongly support the idea that uptake of 5-HT into the artery is nerve- independent. Smooth muscle is not, however, the only arterial cell type that can take up 5-HT. Cultured endothelial cells take up and metabolize 5-HT (Lee et al., 1986; Small et al., 1977; Brust et al., 2000). By contrast, human fibroblasts appear to have a significantly lower rate of 5-HT uptake and metabolism (Small et al., 1977). In our experiments, the endothelial cell appears to make little contribution to 5-HT uptake in the whole artery, though the SERT was located immunohistochemically to these same cells. The lack of effect of endothelial cell and neuron removal on 5-HT uptake may be because these two cell types do not contribute significantly and/or sufficiently to the total mass and uptake capability of the artery. Thus, the sensitivity of our assay (2–5 pg) may be insufficiently low, such that we were unable to reveal uptake by the endothelial cell and sympathetic neuron. This is a noted limitation of these studies.

NET, DAT and SERT are all $Na^+$-dependent transporters and use the energy provided by ATP. The uptake of 5-HT in $Na^+$-free PSS demonstrated that part of 5-HT that crosses the cell membrane does not depend on $Na^+$ moving downhill of the electrochemical gradient, which was built by $Na^+$-$K^+$ ATPase. We do not know, whether all of the 5-HT that passes the cell membrane is energy dependent, as in our experiment, arteries were incubated in a high concentration (compared to intracellular 5-HT concentration) of exogenous 5-HT (1 μM, 15 minutes) solutions. Experiments depleting ATP would help us answer the question whether the SERT-independent 5-HT uptake is active transport.

OCTs are non-specific cation transporters. Though it has been reported that OCTs are upregulated in the gastrointestinal system in SERT-deficient mice and they might play a role in 5-HT uptake, we did not observe the OCTs inhibitor reducing 5-HT uptake in arteries from normal animals. We did not have a successful experience in identifying OCTs in arteries by Western analysis. We visualized multiple bands in our positive control as well as in our samples by using the antibody and there was no competing peptide available to verify the specificity. OCTs are not involved in 5-HT uptake in normal condition but we could not exclude the possibility that in diseases or situations when the function of SERT lost, OCTs might start to take up 5-HT as a redundant mechanism.

## VII. Investigation of 5-HT Release Mechanisms in Peripheral Arteries

(+)-Fenfluramine and (+)-norfenfluramine (fluramines) are SERT substrates and release 5-HT. The mechanism behind this effect is that fluramines are taken up by SERT into cytoplasm and then act on VMAT to release vesicular 5-HT. With the increased cytoplasmic 5-HT, SERT is reversed and starts to transport 5-HT outside of cells (Rothman and Baumann, 2002). If this is the only mechanism by which fluramines release 5-HT, we may conclude that part of endogenous 5-HT is stored in vesicles in peripheral artery smooth muscle. However, we do not yet have evidence to support the existence of the vesicles. The release of 5-HT from arteries may have significant physiological or pathological effect by changing the local 5-HT concentration.

Because fluramines need to be taken up into smooth muscle cells to cause 5-HT release, SERT inhibition should reduce this effect. In our experiment, fluoxetine itself (1 μM) caused increases of 5-HT in PSS (Figure 37) during incubation, which is much higher than the amount of 5-HT released by fluramines. Thus, the inhibition effect of fluramines-induced 5-HT release by fluoxetine could be masked by fluoxetine action. The observation of increased 5-HT in PSS by fluoxetine suggests that there is a spontaneous release of 5-HT in the peripheral artery. In normal conditions, there could be a balance between

5-HT release and uptake in peripheral arteries. The mechanism of this spontaneous release is not clear, nor is the source of the intracellular 5-HT.

## VIII. Physiological Relevance of 5-HT Uptake in Peripheral Arteries and Speculation

It has been argued that 5-HT plays an insignificant role in the physiological control of blood vessel tone as uptake of 5-HT by platelets and/or adrenergic nerves causes circulating levels of 5-HT to be low [estimated between 15–120 nM, Martin, 1994] and out of a range that can directly cause constriction. One manner by which physiological, autocrine/paracrine concentrations of 5-HT may affect vascular tone is by potentiating arterial contraction and mitogenesis to other vasoactive substances (Szabo et al.,1991; Yildiz et al, 1998; Watanabe et al. 2001). Thus, a local 5-HT system may play a significant role in affecting local contractility and mitogenesis by regulating local 5-HT concentration. This could not be discerned by measuring plasma concentrations of 5-HT.

Because we have observed 5-HT uptake and the physical presence of the SERT in multiple arteries, this finding may be global in the body. Peripheral, non-pulmonary arteries could then be added to the growing list of already recognized sites of 5-HT uptake, including the brain, cerebral arteries (Scatton et al., 1985; Amenta et al., 1985; Brust et al., 1996), pulmonary arteries (Eddahibi, 1999), gut (Chen et al., 2001), pituitary (Johns et al., 1982), airway smooth muscle (Dodson et al., 2004) and cardiac myocytes (Sari and Zhou, 2003). The uptake and metabolism of 5-HT in peripheral arteries could play a role in reducing 5-HT concentrations to maintain a normal physiological 5-HT content in free circulation. One can speculate that, dysfunction of this 5-HT uptake and metabolism system in peripheral arteries would lead to increased free circulating 5-HT and diseases related to that.

## IX. Pathological Relevance of 5-HT Uptake in Peripheral Arteries and Speculation

The role of 5-HT and, more recently, its regulator, SERT, in hypertension is controversial and intriguing. The enhancement of potency for 5-HT in inducing vascular contraction or pressor responses in hypertension is striking. Increases in reactivity to 5-HT have been observed in a number of different forms of hypertension models including DOCA-salt hypertensive rats (Watts, 1998), SHR (Nishimura and Suzuki, 1995), and in human patients (Golino et al., 1991). Many mechanisms may contribute to this hyperresponsiveness such as changes in the 5-HT receptor signaling or changes in the circulating levels of 5-HT. It makes logical sense that a change of the activity of the 5-HT regulator (SERT) would change circulating 5-HT concentrations and thus change the responsiveness of arteries to 5-HT. We compared SERT function by testing 5-HT uptake of aorta from normotensive and hypertensive rats and tested whether changed in SERT expression and function were common to hypertension by using three hypertension models, the DOCA-salt rats, LNNA rats and SHR.

These three models of hypertension are different in terms of mechanisms by which animals became hypertensive and the human hypertension they resemble. DOCA-salt hypertension or mineralocorticoid hypertension resembles the clinical situation of aldosterone excess. DOCA-salt hypertension is caused by a surgical uninephrectomy followed by administration of a DOCA pellet and excess salt (1% NaCl+ 0.2% KCl in water). Blood pressure then rises within a few weeks into the hypertensive range. This is a sodium-dependent, low-renin model. Non-sodium mechanisms have been suggested to play a role in the development of DOCA-hypertension, including activation of sympathetic nervous system and vasopressin activation. LNNA inhibits all isoforms (endothelial, neuronal and inducible) of nitric oxide synthase (NOS), with a moderate selectivity for the endothelial isoform (Takahashi et al., 1995). Established hypertension was developed in two weeks after rats were given water with LNNA (0.5 g/L). The loss of nitric oxide production directly leads to a reduced nitric oxide-mediated vasodilation and superoxide "quenching" ability in blood vessels. Other mechanisms involved in LNNA-induced hypertension include activation of angiotensin converting enzyme (Charpie et al., 1997) and the $AT_1$ receptor (Verhagen et al., 2000) but not vasopressin (Loichot et al., 2000). The model of LNNA hypertensive rats resembles a reduced bioavailability of nitric oxide in hypertensive individuals (Kelm, 2003, review). SHR develops hypertension and target organ damage similar to those seen in human essential hypertension. The blood pressure of SHR is relatively sodium-independent (Griffin et al., 2001).

Therefore, investigation of the serotonergic system in these three models would answer the question of whether there is a change of component of serotonergic system in hypertension and whether this is a general change in all hypertension.

## X. Basal 5-HT Concentration in Aorta of DOCA-salt, LNNA Hypertensive Rats and SHR

As we stated earlier the free circulating plasma 5-HT levels (15-120 nM) (Martin, 1994) could not activate the 5-$HT_{2A}$ receptor (Ki= 100-3000 nM, PDSP Ki Database), which is the major receptor mediating 5-HT-induced arterial contraction in normotensive animals. However, many reports showed that plasma 5-HT concentrations and arterial 5-HT receptors are changed in hypertension. In essential hypertensive humans (Fetkovska et al., 1990) and cyclosporine-induced hypertensive rats (Reis et al., 1999), reduced levels of platelet 5-HT and increased plasma 5-HT concentration were measured. Consistent with these reports, unpublished work from our lab determined that the DOCA-salt rat platelet 5-HT was reduced (ng/ml of whole blood, $SHAM_{(D)}$= 215.6±35.4, DOCA= 150.4±36.7) while free circulating 5-HT concentration was increased (ng/ml whole blood, $SHAM_{(D)}$ =5.82±1.14, DOCA= 28.1±11.1). The current observation of reduced basal 5-HT content in aorta from DOCA-salt rats suggests that arteries function as a metabolism organ for 5-HT with decreased 5-HT uptake ability in DOCA-salt hypertension. We speculate that the increased free circulating 5-HT in DOCA-salt rats may result from decreased 5-HT storage in and uptake by platelets and arteries. The increased extracellular free circulating 5-HT in DOCA-salt hypertensive rats may have significant effects. 5-

HT has high affinity for the 5-HT$_{2B}$ receptor (Ki= 10 nM, Bonhaus et al., 1995), and the expression and function of 5-HT$_{2B}$ receptor is upregulated in arterial smooth muscle in DOCA-salt hypertensive rats (Banes and Watts, 2003; Watts, 2002). This receptor is activated endogenously to maintain the high blood pressure of DOCA rats (Watts and Fink, 1999). Moreover, nanomolar concentrations of 5-HT potentiate arterial contraction to ET-1 and NE (Watts, 2000). Thus, it is possible that increased free circulating 5-HT concentration induces vasoconstriction, increases total peripheral resistance and thus elevates blood pressure in DOCA-salt hypertensive rats.

Unfortunately, there are no literature reports of measurements of platelet 5-HT content or free circulating 5-HT concentration in the LNNA hypertensive rats. Our observation of decreased basal aortic 5-HT and 5-HIAA concentration suggests a changed SERT activity and changed level of circulating 5-HT in LNNA rats. Similar to the DOCA-salt rat, 5-HT$_{2B}$ receptor expression and function is increased in LNNA rats and is necessary for maintaining elevated blood pressure (Russell et al., 2002). It is possible that these changed factors of the serotonergic system in LNNA rats play roles in increasing and maintaining blood pressure. Further studies need to be done to investigate the change of platelet and free circulating 5-HT in LNNA rats.

Only two studies in 1985 investigated platelet 5-HT level in SHR. As opposed to a decreased 5-HT in platelet in DOCA-salt hypertensive rats, SHR have more platelets and similar platelet 5-HT levels in SHR compared to WKY (Guicheney et al., 1985, a, b). Free circulating 5-HT in SHR has not been reported. Our observation of similar basal 5-HT levels in aorta from SHR and WKY was consistent with these reports in platelet. We are aware that the basal level of 5-HT concentration is lower in WKY compared to Sprague-Dawley normotensive rats. This may suggest an inherent difference in these strains. The serotonergic system may have different degree of impact on blood pressure regulation in WKY compared to Sprague-Dawley rats.

It is important to understand arterial function of SERT in hypertension not only as a reflection of circulating 5-HT levels, but also because intracellular 5-HT may have a function. Changing intracellular 5-HT concentration would in turn change arterial contraction. Thus, our observation of an altered basal activity of SERT in DOCA-salt and LNNA hypertension may have consequent pathological results.

### XI. 5-HT Uptake Ability was Reduced in Aorta of DOCA-salt and LNNA Hypertensive Rats, but not in SHR

By normalizing 5-HT content to protein concentration and comparing the change of 5-HT concentrations in ng/mg protein, our results showed a decreased total 5-HT uptake and a decreased SERT-dependent 5-HT uptake (Figure 40, 41) in aorta from DOCA-salt and LNNA hypertensive rats exposed to exogenous 5-HT, compared to that of aorta from their normotensive SHAM rats. We did not observe differences in 5-HT uptake in aorta from SHR and WKY. Our results suggest that SERT function was impaired in DOCA-salt and LNNA hypertensive rats. The decreased total 5-HT uptake observed in DOCA-salt and LNNA hypertensive rats may account for the decreased basal aortic 5-HT content in both models and the increased free circulating 5-HT (DOCA-salt hypertension). Possible explanations for the contradictory findings of an upregulated SERT protein expression and a decreased SERT function in DOCA-salt and LNNA rats includes different membrane/cytosol distribution of SERT in these SHAM and hypertensive animals as well as post translational modification of SERT proteins. Phosphorylation of SERT by p38 MAPK increases SERT function by stimulating the insertion of intracellular SERT into the cell membrane and increasing total SERT activity (Blakely et al., 2005). By contrast, PKC reduces SERT function by phosphorylating SERT protein and causing translocation of SERT to the cytosol (Jayanthi et al., 2005). Thus, it is possible that the total expression of SERT increases in DOCA and LNNA hypertensive animals while the membrane fraction or functional SERT actually decreases.

We did not observe a difference in basal aortic 5-HT content, 5-HT uptake ability or SERT protein expression in aorta from SHR compared to WKY rats. There are several factors that may contribute to the differences we observed. First, as we discussed above, the strain is different (WKY *vs.* Sprague-Dawley). Second, the systolic blood pressures of SHR (172±7 mmHg) were lower than DOCA-salt rats (197±6 mmHg) and LNNA rats (228±9 mmHg). The change of SERT expression and function may only happen in severe hypertension. Third, it is possible that the deoxycorticosterone we used in our DOCA-salt hypertension model causes the different SERT expression and function. However, Kulikov reported that stimulation of mineralocorticoid receptors had no effect on SERT radioligand binding density in rat midbrain (Kulikov et al., 1997). Furthermore, the observation of impaired SERT function was also obtained in LNNA hypertensive rats. Thus, it is unlikely that the change of SERT function in DOCA-salt hypertension is from effects of mineralocorticoid.

More importantly, a correlation between 5-HT$_{2B}$ receptor expression and plasma 5-HT or SERT function was reported recently (Callebert et al., 2006). Callebert *et al.* observed significantly increased plasma serotonin levels in wild type mice with upregulated 5-HT$_{2B}$ receptor after exposure to chronic hypoxia but not in 5-HT$_{2B}$ receptor-/- mice. Acute treatment with 5-HT$_{2B}$ receptor agonist induced a rapid SERT-and 5-HT$_{2B}$ receptor-dependent increase of plasma serotonin levels, which suggests that 5-HT$_{2B}$ receptor activation inhibits SERT function and thus less 5-HT is taken up. Upregulated 5-HT$_{2B}$ receptor expression and function in DOCA and LNNA hypertension have been reported (Watts and Fink, 1999, Watts, 2000; Russell et al., 2002). On the other hand, the 5-HT$_{2B}$ receptor was not involved in 5-HT-induced vasoconstriction in the hindquarters of SHR (Calama et al., 2004), which suggests 5-HT$_{2B}$ receptor was not activated in SHR as it was in DOCA-salt and LNNA hypertensive rats. Our results are consistent with Calleberts' report in that we observed an increased free circulating 5-HT with increased 5-HT$_{2B}$ receptor function while decreased SERT function in DOCA rats; increased 5-HT$_{2B}$ receptor function while decreased SERT function in LNNA rats; no change of 5-HT$_{2B}$ receptor and normal SERT activity in SHR. How the 5-HT$_{2B}$ receptor regulates SERT is not yet clear.

## XII. Other Arterial 5-HT-uptake Mechanism in Hypertensive Animals

Similar to what we observed in normal animals, in arteries from hypertensive rats, a maximal concentration of fluoxetine (1 μM) and fluvoxamine (1 μM) did not block the entire uptake stimulated by exogenous 5-HT. This suggests that there are still other mechanisms by which 5-HT is transported into aorta.

It is important to note that we studied one concentration of exogenous 5-HT at one time point in our active uptake study. Studies that examine 5-HT uptake at various times and with a range of 5-HT concentrations need to be done to compare SERT Km and Vmax values in aorta from DOCA-salt, LNNA hypertensive rats and their SHAM control rats. However, this is difficult without determination of other mechanisms for 5-HT uptake. Thus, kinetic studies have to wait until these alternative mechanisms are discovered.

## XIII. The Effect of SERT Function on Arterial Contractility in Hypertension

Fluoxetine and fluvoxamine differed in their effects in aorta from normal rats, where a potentiation of 5-HT-induced contraction was observed with fluvoxamine but not fluoxetine. Interestingly, Gruetter *et al* (Gruetter et al., 1992) demonstrated nearly 10 years ago that 5-HT-induced contraction in aorta from normal rats could be markedly potentiated by citalopram, another SERT inhibitor, but only modestly by fluoxetine. Moreover, Cohen and Wiley (Cohen and Wiley, 1997) observed similar results in the relative inability of fluoxetine to potentiate aortic contraction to 5-HT.

How SERT modifies arterial contraction is not clear. We may speculate that the active uptake and metabolism of 5-HT in peripheral arteries is a protective mechanism of the body to keep a normal level of free circulating 5-HT and prevent blood pressure increases. When this uptake was inhibited by fluoxetine/fluvoxamine, peripheral arteries lost the ability to clear environmental 5-HT, and were exposed to a relatively higher concentration of 5-HT. In normotensive animals, 5-HT$_{2A}$ receptor-mediated 5-HT-induced contraction would not be changed because of the relatively low affinity of 5-HT for 5-HT$_{2A}$ receptor. The change of the amount of 5-HT by fluoxetine inhibition was not enough to cause change in aortic contraction. In hypertensive animals, the expression and function of 5-HT$_{2B}$ receptors (for which 5-HT has much higher affinity) are upregulated. This change of local 5-HT concentration might be sufficient to activate the 5-HT$_{2B}$ receptors to cause the potentiated contraction as we observed. This speculation can also be applied to explain the potentiated response to 5-HT in aorta from DOCA-salt rats compared to SHAM rats. The impaired SERT function in aorta from DOCA-salt rats suggest a loss of an arterial 5-HT clearance mechanism in these animals, which might lead to elevated free circulating 5-HT, vascular tone and blood pressure.

The alternative explanation of our observation is that fluoxetine and fluvoxamine do not selectively inhibit SERT. Fluoxetine has fairly high affinity for rat 5-HT$_{2A}$ receptor (Ki= 299±31 nM, Rothman et al., 2000) and might cause inhibition of (+)-norfenfluramine-induced contraction. This is not consistent with our observation of potentiation of 5-HT-induced contraction in aorta from DOCA-salt rats. The affinity of fluvoxamine for 5-HT$_{2A}$ receptor is over 12,000 nM (no affinity of fluvoxamine for 5-HT$_{2B}$ receptor has been reported). It is unlikely that the concentration we used in our experiments (1 μM) inhibited 5-HT$_{2A}$ receptor and 5-HT$_{2B}$ receptor.

The important message from these particular findings is that inhibition of the transporter can affect contractility, and likely does so in a local manner. One can also speculate that the change of serotonergic system could also happen in arteries in which 5-HT plays a role in other pathophysiological states such as migraine and coronary vasospasm, and this has certainly been supported by findings in cerebral arteries (Amenta et al., 1985; Brust et al., 1996). With respect to blood pressure regulation, the finding of a functional peripheral SERT is potentially supported by findings that fluoxetine causes a pressor response in conscious rats (Lazarigues et al., 2000).

## XIV. Future Research

The finding of a local serotonergic system in peripheral arteries is exciting and opens a new area of serotonin research. More studies need to be done to further characterize this system.

Western analysis is the best way to quantify and compare protein expression. Showing the direct evidence of the existence of TPH1 protein by Western analysis is very important. A new TPH1 antibody has been reported recently (Sakowski et al., 2006). This is the first antibody that allows the differentiation of TPH1 and TPH2 upon immunoblotting, immunoprecipitation, and immunocytochemical staining of tissue sections from brain (TPH2) and gut (TPH1). Recombinant TPH1 and TPH2 protein should be used as positive control in these future experiments. The activity of tryptophan hydroxylase is enhanced by phosphorylation of Ser58, by cAMP dependent PKA and $Ca^{2+}$/calmodulin kinase II. Tryptophan hydroxylase (phospho S58) antibody is also commercially available and worthy to try.

It is not yet known which proteins could be modified by intracellular 5-HT. With the knowledge of actin and myosin as substrates for TGII and important proteins for vasoconstriction, we could choose these proteins as candidates and perform an immunoprecipitation experiment with 5-HT antibody. This experiment would further elucidate which kind of protein could be modified by 5-HT.

The *in vitro* measurement of TPH activity in peripheral arteries is still an ongoing project in our lab. It is possible that we could detect 5-HTP if we extend the incubation time and/or adjust other conditions, such as increasing tryptophan concentration. It is also worthwhile to test TPH activity in arteries in the presence/absence of PKA or $Ca^{2+}$/calmodulin-dependent protein kinase activator and compare TPH activity in arteries.

Whether a change in the local 5-HT concentration, by the mechanisms that we discussed in our studies, is sufficient to alter vascular tone is not clear. To answer this question, we could use the SERT substrate and 5-HT releaser (+)-fenfluramine to actively release endogenous 5-HT and test whether this is sufficient to cause vasoconstriction or to potentiatie the effect of other vasoconstrictors.

It is necessary to investigate SERT-independent 5-HT uptake mechanisms. After elucidating these mechanisms, we should be able to compare Km and Vmax of peripheral arterial SERT and brain SERT.

## XV. Limitations

The original intention of this study was to determine the presence and potential function of the SERT in peripheral arteries. We have not compared this transporter, either kinetically, biochemically, or pharmacologically, to the classic transporters in the brain and the lung. Such work will be important to determine if these proteins could be potentially separate pharmacological targets. In our studies we used the thoracic aorta in RT-PCR, Western, IHC, and contractility assays. We are aware that the aorta is not a resistance artery and thus we repeated some of these studies in the smaller artery, superior mesenteric artery, which could be more similar to real resistance arteries.

## Conclusions

Unlike previous serotonin research in vasculature, which focused on serotonin receptor functions, we investigated whether peripheral arteries have the ability to regulate 5-HT concentration locally, and possible roles of this regulation in physiological and pathological conditions.

Overall, we demonstrated the presence of a functioning serotonergic system in normal rat peripheral arteries. We proved the presence of the essential components for 5-HT synthesis, such as TPH and AADC, but we were limited by our technique to directly prove or disprove an active local peripheral arterial 5-HT synthesis. The existence of uptake and metabolism of 5-HT was clearly shown by observing increased 5-HIAA in isolated arteries from normal animals incubated with exogenous 5-HT (1 μM, 15 min). Using arteries from pargyline-treated rats, we studied the 5-HT uptake and metabolism mechanisms and eliminated the change of 5-HT concentration caused by metabolism. A basal- and fluramine-induced endogenous 5-HT release was observed, which indicated the possibility of local increases of extracellular and, accordingly, decreases of intracellular 5-HT concentration and as a consequence, changed 5-HT function. The 5-HT uptake of peripheral arteries is partially mediated by SERT, while other mechanisms involved in this process need to be further elucidated. There is no 5-HT storage in peripheral arteries as we observed that 5-HT content reduced back to basal level in 5-HT-loaded aorta after 4 hours incubation in PSS. Studies in arteries from hypertensive animals revealed a decreased basal endogenous 5-HT and increased expression but impaired function of the SERT in peripheral arteries. SERT modifies 5-HT-induced contraction, and inhibition of the transporter is significantly enhanced in mineralocorticoid hypertension.

Understanding the functions of the local serotonergic system in peripheral arteries in physiological and pathological conditions will help us reveal the etiology of 5-HT related peripheral vesicular diseases and find new targets for drug development.

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## BIBLIOGRAPHY

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### Educational Background

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| 1996-2001 | Peking University Health Science Center<br>Beijing, P. R. China<br>B.S. (Pharmacology) |
| 2002-present | Michigan State University, East Lansing, Michigan<br>Doctoral candidate (Pharmacology and Toxicology) |

### Teaching Activities

| | |
|---|---|
| 2005-2006 | Lecturer for Pharmacology (PHM) 450 class |

Research Training

| | |
|---|---|
| 2001-2002 | Hepatoprotective role of Ganoderma lucidum polysaccharide against BCG-induced immune liver injury in rats.<br>Research with Dr. Zhibin Lin and Dr. Guoliang Zhang |
| 2002-present | The presence of a local serotonergic system in peripheral arteries.<br>Doctoral thesis research with Dr. Stephanie W. Watts, Michigan State Unversity |

### Membership in Academic Societies

American Society of Pharmacology and Experimental Therapeutics
American Heart Association
American Physiological Society
Society for Experimental Biology and Medicine

### Awards

American Heart Association Predoctoral Fellowship-July,2004 to June,2006
ASPET Graduate Student Travel Award- Experimental Biology, 2005
ASPET Graduate Student Travel Award- Experimental Biology, 2006
MSU College of Veterinary Medicine Fellowship (Fall,2002; Fall,2003; Spring,2004; Fall,2004)
MSU Graduate School Incentive Fellowship-2004

MSU Phi Zeta Research Day Best Poster Presentation by a Ph.D. Student-2004
MSU the Graduate Office Fellowship- Fall, 2005
MSU the Graduate School Dissertation Completion Fellowship- 2006

**Papers**

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