Modulation of the Serotonin Reuptake Transporter in RAW264.7 Macrophages

Sienna Marie Arenas Malubay
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd
Part of the Biology Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/706

This Thesis is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
Modulation of the Serotonin Reuptake Transporter in RAW264.7 Macrophages

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By
Sienna Marie Arenas Malubay
B.S. Biology
The College of William and Mary, Williamsburg, Virginia
2003

Director: Jennifer K. Stewart, Ph.D.
Associate Professor, Department of Biology

Virginia Commonwealth University
Richmond, Virginia
May 2006
Acknowledgment

I would like to express my gratitude to the people whose support has made the completion of this project possible. First and foremost, I would like to thank Dr. Jennifer Stewart for being a wonderful mentor and for allowing me to work in her laboratory. During my time in the lab, she has been an unvarying source of knowledge and encouragement. I would also like to thank Dr. Krista Fischer-Stenger for teaching me all I know about Western Blots and for her support and friendship. In addition, I would like to thank my committee members Dr. Michael Fine, Dr. Robert Tombes, and Dr. Joseph Porter for their time and participation in this endeavor.

I would also express my gratitude to the members of the Neuroendocrinology laboratory, both past and present: Meghan Rudd, Kristie Engler, Christopher Waggener, Shauntá Poe, George Georges, Neal Bhatia, and Omar Noor. Your friendship and help in the lab have been invaluable over the past two years. I would also like to thank Amy Senecal of the Stenger Lab at University of Richmond for her help with Western Blots, Lin Zhang of the Zhou Lab (VCU) for his help with MTT assays, and Mohit Kashyap of the Ryan Lab (VCU) for providing CTMC lysates. I also owe my heartfelt appreciation to my friends here at VCU and beyond.

Last, but definitely not least, I would like to thank my family, especially my parents, Oscar and Mila Malubay, and my sister, Hosannah, for their support and love throughout my academic career. I could not have done it without you.
Table of Contents

Acknowledgment...................................................................................................................ii
List of Tables.........................................................................................................................v
List of Figures......................................................................................................................vi
List of Abbreviations..........................................................................................................viii
Abstract...................................................................................................................................xi
Introduction..........................................................................................................................1
  Serotonin and its receptors...............................................................................................2
  Macrophages......................................................................................................................3
  Serotonin and the Immune System..................................................................................5
  Function and regulation of the SERT.............................................................................6
Methods and Materials.........................................................................................................10
  RAW 264.7 Macrophages...............................................................................................10
  Recruited Macrophages.................................................................................................10
  Uptake of $^3$H-5HT Assay..........................................................................................11
  Western Blotting.............................................................................................................12
  MTT Cell Viability Assay...............................................................................................13
  Measurement of TNF-α and IL-1β.............................................................................14
  RNA Isolation..................................................................................................................14
Results

Comparison of $^3$H-5HT uptake in RAW264.7 macrophages and peritoneal macrophages

LPS activation of RAW264.7 macrophages increased SERT activity

LPS modulation of SERT activity was time- and concentration-dependent

IL-10 decreased 5-HT uptake

Mechanism for SERT upregulation by LPS

Discussion

List of References

Appendix: Figures and Tables

Vita
List of Tables

Table.........................................................................................................................Page

1. Summary of serotonin receptor families and their effects........................................34
List of Figures

Figure .................................................................................................................. Page

1. Biosynthesis pathway of serotonin from L-tryptophan ........................................ 33

2. Summary of SERT Regulation ............................................................................... 35

3. Comparison of total, specific, and non-specific uptake of $^3$H-5HT by RAW264.7 macrophages ........................................................................................................ 36

4. LPS-activation increases sodium dependent uptake of $^3$H-5HT in RAW264.7 macrophages ........................................................................................................ 37

5. NaCl dependence of $^3$H-5HT uptake in RAW264.7 macrophages ...................... 38

6A. Effects of fluoxetine on specific uptake of $^3$H-5HT in vehicle-treated RAW264.7 macrophages ........................................................................................................ 39

6B. Kinetics of fluoxetine inhibition of $^3$H-5HT uptake in vehicle-treated RAW264.7 macrophages ........................................................................................................ 39

7A. Effects of fluoxetine on specific uptake of $^3$H-5HT in LPS-treated RAW264.7 macrophages ........................................................................................................ 40

7B. Kinetics of fluoxetine inhibition of $^3$H-5HT uptake in LPS-treated RAW264.7 macrophages ........................................................................................................ 40
8. Time-dependent effects of LPS activation on specific uptake of $^3$H-5HT in RAW264.7 macrophages........................................................................................................................................41

9. Concentration-dependent effects of LPS activation on specific uptake of $^3$H-5HT in RAW264.7 macrophages........................................................................................................................................42

10A. Effects of IL-10 on specific uptake of $^3$H-5HT in vehicle-treated and LPS-treated RAW264.7 macrophages........................................................................................................................................43

10B. Viability of RAW264.7 macrophages after treatment with vehicle (media alone), LPS, and/or IL-10........................................................................................................................................43

11. IL-10 modulation of $^3$H-5HT specific uptake in RAW264.7 macrophages treated with a low (optimal) concentration of LPS........................................................................................................................................44

12. Expression of SERT protein in RAW264.7 macrophages assayed by Western blotting........................................................................................................................................45

13. Inhibition of guanylyl cyclase by LY83583 in RAW264.7 macrophages........................................................................................................................................46

14A. Extracellular concentrations of TNF-α in RAW264.7 macrophages........................................................................................................................................47

14B. Extracellular concentrations of IL-1β in RAW264.7 macrophages........................................................................................................................................47
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H-5HT</td>
<td>tritium-labeled serotonin</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>5-HP</td>
<td>5-hydroxytryptophan</td>
</tr>
<tr>
<td>5-HTR</td>
<td>serotonin receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 5' monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine 5' monophosphate</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund's Adjuvant</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>Epi</td>
<td>epinephrine</td>
</tr>
<tr>
<td>GMP</td>
<td>guanosine 5' monophosphate</td>
</tr>
<tr>
<td>GC</td>
<td>guanylyl cyclase</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
</tbody>
</table>
IFNγ ................................................................. interferon gamma
IgG ............................................................... immunoglobulin G
IL-1 ............................................................... interleukin-1
IL-6 ............................................................... interleukin-6
IL-8 ............................................................... interleukin-8
IL-10 ............................................................. interleukin-10
IL-12 ............................................................. interleukin-12
IL-16 ............................................................. interleukin-16
IP₃ ................................................................. inositol triphosphate
LiCl .............................................................. lithium chloride
LPS ............................................................... lipopolysaccharide
LY83583 ...................................................... 6-anilinoquinoline-5,8-quinone
MAPK .......................................................... mitogen-activated protein kinase
MTT ............................................................. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl ............................................................ sodium chloride
NE ............................................................... norepinephrine
NET ............................................................. norepinephrine transporter
NO ............................................................... nitric oxide
NOS ............................................................. nitric oxide synthetase
OCT 1 .......................................................... organic cation transporter 1
OCT 2 .......................................................... organic cation transporter 2
OCT 3 .......................................................... organic cation transporter 3
PBS ............................................................... phosphate buffered saline
pCPA ............................................................. para-chlorophenylalanine
PCR .............................................................. polymerase chain reaction
PD169316 ........................................ 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole
PDE5 .............................................................. phosphodiesterase 5
PKA .............................................................. protein kinase A
PKC .............................................................. protein kinase C
PKG .............................................................. protein kinase G
PNS .............................................................. peripheral nervous system
PP2A ............................................................ protein phosphatase 2A
RT-PCR ........................................................ reverse transcriptase polymerase chain reaction
SERT ............................................................. serotonin reuptake transporter
SH .............................................................. sucrose hepes
SNARE ........................................................ soluble NSF attachment receptor
SSRI ............................................................. selective serotonin reuptake inhibitor
Syn1A ........................................................... syntaxin 1A
TBST ............................................................ Tris-buffered saline with Tween-20
TCA .............................................................. tricyclic antidepressant
TNF-α ........................................................... tumor necrosis factor alpha
VMAT .......................................................... vesicular monoamine transporter
Abstract

MODULATION OF THE SEROTONIN REUPTAKE TRANSPORTER IN RAW264.7 MACROPHAGES

Sienna Marie Arenas Malubay, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

Major Director: Jennifer K. Stewart, Ph.D., Associate Professor, Department of Biology

Serotonin (5-HT) plays an important role as both a neurotransmitter and an immune modulator. The serotonin reuptake transporter (SERT) clears the extracellular space of 5-HT, which decreases the effects of 5-HT on target cells. This study demonstrated that the RAW264.7 macrophage cell line expresses SERT function, measured by assays of \(^3\text{H}\)-5HT uptake. The 5-HT uptake in RAW264.7 macrophages was more than 10-fold that of peritoneal macrophages, indicating that these cells are an excellent model for studying regulation of the SERT. Activation of macrophages with
lipopolysaccharide (LPS) increased SERT activity in a time- and concentration-dependent manner and Western blots indicate that the increase in activity is partially due to LPS-induced increases in total SERT protein. Both unstimulated and LPS-stimulated activity was inhibited by the specific SERT inhibitor fluoxetine (IC$_{50}$ = 5-8 nM) and was reduced by the anti-inflammatory cytokine interleukin-10. Changes in extracellular concentrations of interleukin-1β and tumor necrosis factor-α did not affect SERT activity.
Monoamines, which include dopamine (DA), epinephrine (Epi), norepinephrine (NE), and serotonin (5-hydroxytryptamine; 5-HT), are found in the central nervous system (CNS) as well as the periphery. These molecules serve as neurotransmitters, hormones, and paracrine regulators and are synthesized at many sites.

Monoamines are transported across the plasma membrane by either Type I neuronal or Type II extra-neuronal transporters. The Type I transporters are sodium-dependent and include the dopamine transporter (DAT), the norepinephrine transporter (NET), and serotonin transporter (SERT). All of these transporters have more than one substrate. For instance, the NET has a two-fold higher affinity for NE than for Epi but transports both across the cell membrane (12). These Type I transporters are generally inhibited by cocaine. Though called the neuronal transporters, the Type I transporters also are found in non-neuronal cells. The Type II extra-neuronal transporters include members of the organic cation transporter family (OCT1, OCT2, and OCT3) (12). The Type II transporters have an affinity for a broad range of substrates. OCT3 also has been called the extra-neuronal monoamine transporter (EMT) (12). These Type II transporters usually are inhibited by catecholamine metabolites, such as metanephrine (2; 12).
Serotonin and its receptors:

Serotonin (5-HT) is an indolamine derived from the amino acid tryptophan. L-tryptophan is converted to 5-hydroxytryptophan (5-HTP) by tryptophan 5-hydroxylase. Next, 5-HTP is converted to 5-HT by 5-HTP decarboxylase (Figure 1) (10).

Serotonin is made primarily by serotonergic neurons found in the midbrain and medulla of the mammalian central nervous system (CNS) and in the enterochromaffin cells of the gastrointestinal tract (GI). In the CNS, vesicles in pre-synaptic neurons store 5-HT until released into the synapse. Once in the synaptic cleft, 5-HT can bind to its receptors on post-synaptic neurons. Similarly, in the periphery, 5-HT is released into the extracellular space in order to bind to its receptors. While synthesized primarily by enterochromaffin cells outside of the CNS, 5-HT also can be picked up and stored by many different non-neuronal cells, such as platelets and mast cells, for later release (29).

Seven families of 5-HT receptors (5-HT₁,7) have been identified to date. Some of these receptors also have subtypes and have been classified by their structure, function, and pharmacology (16). The many subtypes of the 5-HT₁ receptor family are coupled to G₁/G₀ proteins. Generally these receptors mediate a decrease in the concentration of cyclic adenosine 5'-monophosphate (cAMP) in the cytosol through the inhibition of adenylyl cyclase, or the receptors mediate opening of potassium ion (K⁺) channels to hyperpolarize the neuron (13). These receptors affect cerebral vasoconstriction, neuronal inhibition, and behavioral effects, such as sleep, thermoregulation, and anxiety. Whereas the members of the 5-HT₁ receptor family are generally found in the CNS, the three members of the 5-HT₂ family are found in both the CNS and periphery. These G₁/G₁₁-
coupled proteins increase the amount of inositol triphosphate (IP$_3$) and diacylglycerol (DAG) found in the cell and affect neuronal excitation, smooth muscle contraction, and platelet aggregation (13). The 5-HT$_3$ receptor is a serotonin-gated sodium/potassium (Na$^+$/K$^+$) channel that is found in both the CNS and peripheral nervous system (PNS). It stimulates rapid depolarization of the plasma membrane, which in turn leads to neuronal excitation and anxiety (13). Both the 5-HT$_4$ receptor and 5-HT$_7$ receptors are G$_s$-coupled proteins that function in the CNS and GI tract by increasing the levels of cAMP available within the cell. The 5-HT$_4$ receptor affects neuronal excitation and may have a role in peristalsis (16). Not much is known about the effects of the 5-HT$_7$ receptor, though it may have a role in mood, learning, neuroendocrine, and vegetative disorders (16). Although the 5-HT$_5$ and 5-HT$_6$ receptors have been identified, little is known about them (see Table 1 for summary).

**Macrophages:**

Macrophages are mononuclear phagocytes that play an important role in both innate and acquired immunity. Pluripotent hematopoietic stem cells from bone marrow can differentiate into monocytes, the precursor of macrophages. Monocytes travel throughout the body in the circulatory system. Through the process of extravasation, monocytes leave the blood vessels and develop into tissue-resident macrophages.

Macrophages are the first line of defense in the innate immune system. They are one of the first cells to appear at the site of injury or infection and work to clear the area of pathogens and debris through phagocytosis. Bacteria or other pathogens are ingested
by macrophages in a phagosome, which later binds to a lysozome containing digestive enzymes to break down the ingested material (21). In addition, macrophages produce reactive oxygen species, such as nitric oxide. These are released at the site of infection in an oxidative burst and kill invading pathogens (21).

Macrophages synthesize cytokines in response to stimuli, such as lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria. The pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, and IL-12) released by macrophages further develop and coordinate the immune response (21). These cytokines, along with other chemotactic factors secreted by the macrophage, work to further activate the macrophage, as well as other immune cells, and signal additional immune cells to the site of infection (21; 30). The macrophage also produces anti-inflammatory cytokines such as IL-10 that counteract the pro-inflammatory cytokines, and the macrophage functions in the acquired immune response as an antigen-presenting cell. After bacteria have been digested, an antigen fragment is bound to the macrophage’s major histocompatibility (MHC) class II molecule and is brought to the plasma membrane. The helper T cells then bind to the MHC-II+antigen complex with its CD4 protein (44).

Previous studies indicate that macrophages take up monoamines, such as the catecholamine NE (2; 20). Recently in our lab, Meghan Rudd showed that the SERT, which transports DA as well as serotonin, is functional in mouse peritoneal macrophages (40).
Serotonin and the Immune System:

The role of 5-HT as a CNS neurotransmitter has been extensively studied, but its role in the periphery is not as well known. Serotonin has been shown to affect a variety of systems in the body. In the cardiovascular system, for example, 5-HT works as a vasoconstrictor, and in the digestive system, 5-HT stimulates smooth muscle contractions, such as peristalsis (13; 29). Serotonin also affects many of the cells in the immune system.

Different types of 5-HT receptors are found on many immune cells. Recently, mRNA for serotonin receptor subtypes 1E, 2A, 3, 4 and 7 (5-HTR₁E, 5-HTR₂A, 5-HTR₃, 5-HTR₄, and 5-HTR₇) have been found in monocytes (11). Serotonin induces the release of chemotactic factors, such as IL-16, by peripheral blood mononuclear cells (26). In addition, 5-HT has a role in delayed-type hypersensitivity (for review: (29)).

Serotonin also has been shown to affect macrophage phenotype and function. For example, in mice treated with para-chlorophenylalanine (pCPA), a tryptophan hydroxylase inhibitor, the resulting 5-HT depletion decreased splenic macrophage activation of T-cells (29; 46). Moreover, 5-HT, acting through the 5-HT₁A receptor, upregulates the macrophage’s phagocytic activity (14). In addition, 5-HT can modulate cytokine production in macrophages. For example, serotonin inhibits the production of LPS-induced TNF-α in macrophages in a dose-dependent manner (1). Although high levels of 5-HT can reduce IL-6 and TNF-α production, depletion of 5-HT with pCPA also decreases the production of IL-6 and TNF-α (25). Similar effects are observed on the production of pro-inflammatory cytokine IFN-γ and the anti-inflammatory cytokine IL-
10 (24). These findings suggest that low concentrations of 5-HT are needed for production of these cytokines, but high levels of 5-HT are inhibitory (24; 25). In addition, 5-HT-induced increases in the production of IL-6, IL-1β, and IL-8 appear to be mediated by 5-HT4 and 5-HT7 receptors (11).

Furthermore, 5-HT may play a role in lipid storage in monocytes, which are precursors of macrophages. Suguro et al. have found that 5-HT causes an increase in the expression of the acyl-coenzymeA:cholesterol acyltransferase-1 (ACAT-1) protein in monocytes (42). ACAT-1 changes free cholesterol into cholesterol esters for storage. The storage of lipids in macrophages leads to the formation of foam cells, which participate in the formation of atherosclerotic plaque (27).

Function and regulation of the SERT:

The serotonin reuptake transporter (SERT) is a protein with twelve membrane-spanning domains (4). It is found on many different cell types including neurons, platelets, mast cells, and macrophages. The SERT takes up 5-HT, decreasing its concentration in the synapse or extracellular space, which decreases the interaction of 5-HT with 5-HT receptors (18).

Changes in SERT function have been implicated in several neurological diseases including clinical depression and obsessive-compulsive disorders (32; 45). As such, the pharmacology of the SERT has been examined thoroughly. It is the target of many antidepressants, such as the selective serotonin reuptake inhibitors (SSRIs) including
fluoxetine (Prozac®), and is modulated by less specific uptake inhibitors, such as tricyclic antidepressants and cocaine (4; 45).

The SERT gene is a member of a large family made up of transporters genes, such as the NET gene and DAT gene (4; 43). All of the transporters are Na⁺/Cl⁻ dependent and can be blocked by cocaine (2; 4; 37). Specifically, the SERT cotransports 5-HT with Na⁺ and Cl⁻ ions into the cell while transporting K⁺ ions into the extracellular space. Voltage clamp studies indicate that Na⁺, Cl⁻, and 5-HT bind to the SERT inducing a conformational change that allows them to enter the cytosolic space. Once this exchange has been made, K⁺ binds to a site on the SERT causing it to return to the original conformation and allowing K⁺ to exit into the extracellular space (6; 36).

Many recent studies have investigated SERT regulation. SERT has been shown to interact with the SNARE protein syntaxin 1A (Syn1A) (17), and treatment with botulinum C1 toxin, which cleaves Syn1A, causes a decrease in SERT function in thalamocortical neurons (36).

Other laboratories have examined the role of phosphorylation on the expression and function of the SERT. Protein phosphatase 2A (PP2A) interacts with the SERT and down-regulate its activity (38). The SERT also is readily phosphorylated by protein kinase A (PKA) and protein kinase C (PKC). Whereas increased PKA phosphorylation has little effect on activity, increased phosphorylation with PKC causes a down-regulation of 5-HT transport by the SERT (5). In addition, PKC works in a biphasic manner in its regulation of the SERT. Activated PKC phosphorylates the SERT serine residues within 5 min, inhibiting the SERT (23). After 30 min, activated PKC
phosphorylates SERT threonine residues, causing SERT endocytosis and further inhibition of its activity (23). Moreover, amphetamines, cocaine, and a variety of SERT substrates, such as 5-HT, modulate PKC-dependent SERT phosphorylation and modulate surface distribution (22; 37).

Protein kinase G (PKG) and p38 mitogen-activated protein kinase (MAPK) play a role in up-regulating SERT activity (34; 41; 48-50). The inhibition of p38 MAPK by PD169316 (4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole) has shown that p38 MAPK is necessary for maintaining SERT function (41). There is now evidence that p38 MAPK up-regulates the SERT through PP2A. Although p38 MAPK and PP2A activate the SERT, these enzymes do not regulate insertion of the SERT into the plasma membrane (48). In contrast, PKG promotes an increase of SERT surface density (49). Treatment with sildenafil, a phosphodiesterase 5 (PDE5) inhibitor, causes a rise in cGMP, which increases SERT activity and density (50). Adenosine, acting through the adenosine receptor, appears to up-regulate SERT activity via this pathway (49). Figure 2 summarizes major pathways regulating the SERT.

The SERT has been extensively studied in the CNS or in transfected cells, but little studied in other cell types in which this protein is naturally expressed. Nevertheless, Meghan Rudd in our laboratory found that recruited peritoneal macrophages express SERT mRNA as well as functional protein (40). Furthermore, using reverse transcriptase
PCR (RT-PCR) our laboratory has revealed that the RAW264.7 macrophage cell-line also expresses SERT mRNA (39). In the study presented here, we examined the regulation of SERT activity within this cell line.
Methods and Materials

**RAW264.7 Macrophages**

Murine macrophages from the RAW264.7 cell line (American Type Cell Culture, Manassas, VA) were cultured in complete RPMI containing RPMI-1640 (Gibco brand, Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (Cellgro brand, VWR, West Chester, PA), 1% L-glutamine, 1% minimal essential medium vitamins, 1% nonessential amino acids, 100 Units/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES buffer at pH 7.4 (Gibco brand, Invitrogen, Carlsbad, CA). The cell cultures were maintained at 37 °C in 5% CO₂ in 75 cm² tissue culture flasks (Corning brand, Fisher Scientific, Hampton, NH). The cultures were sub-cultured twice weekly.

**Recruited Macrophages**

Female CBA/J mice (6-8 weeks of age) were obtained from Harlan (Indianapolis, IN) and housed in the Virginia Commonwealth University animal facilities under the guidelines of the University Animal Care and Use Committee. Macrophages were recruited via an intraperitoneal injection with 0.5 ml of a 1:1 complete Freund’s Adjuvant (CFA; Sigma-Aldrich, St. Louis, MO) and phosphate buffered saline (PBS; Gibco brand, Invitrogen, Carlsbad, CA). After two weeks, the mice were euthanized with CO₂. The
recruited peritoneal macrophages were harvested, washed with Hank’s Balanced Salt Solution (Gibco brand, Invitrogen, Carlsbad, CA) and allowed to adhere for 4 h in 12-well tissue culture plates (Costar brand, Fisher Scientific, Hampton, NH). Non-adherent cells were discarded, and adherent cells were washed with complete RPMI and treated with vehicle (media alone) or activated with lipopolysaccharide (LPS; E. coli serotype O55:B5; Sigma-Aldrich, St. Louis, MO). The macrophages were maintained in complete RPMI at 37 °C in 5% CO₂ for the times designated in each experiment.

_Uptake of ³H-SHT Assay_

RAW264.7 macrophages were plated in 12-well tissue culture plates (1.5 x 10⁶ cells/well) and were treated as designated in each experiment. The plates were incubated at 37 °C in 5% CO₂ overnight, allowing the cells to grow to a density of approximately 3 x 10⁶ cells/well. The uptake assay is a modification of Ganapathy et al. (15), Horschitz et al. (19), and Rudd et al. (40).

Immediately prior to the assay, the cells were washed twice with 1 ml of room-temperature sucrose Hepes (SH) uptake buffer containing 140 mM NaCl (LiCl or choline chloride for certain experiments), 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 10 mM Hepes (pH 7.4), and 320 mM sucrose. The macrophages were then preincubated for 5 min with SH uptake buffer containing 250 μM ascorbic acid (Sigma-Aldrich, St. Louis, MO), 10 μM pargyline (Sigma-Aldrich, St. Louis, MO), and vehicle or various concentrations of inhibitors. Radiolabeled serotonin (25 nM, 18 Ci/mmol; Vitrax, Placentia, CA) was added, and incubation continued for 10 min (except in time course
experiments). All incubations were conducted at room temperature. The uptake buffer was removed, and cells were washed twice with 2 ml of SH buffer. The cells were lysed with 800 μl of 0.2 N NaOH containing 1% SDS, and the lysate was transferred to 10 ml of Scintisafe scintillation fluid (Fisher Scientific, Hampton, NH). The wells were washed with an additional 600 μl of the NaOH/SDS (Sigma-Aldrich, St. Louis, MO), and the wash was transferred to the scintillation fluid. Radioactivity was measured with a Beckman LS6000IC scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

**Western Blotting**

Cultured cells were lysed with 100-300 μl of ice cold buffer containing 0.05 M Tris (pH 7.5), 0.3 M NaCl, 2 mM EDTA, 1% Triton-X 100, 2 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride. Lysate protein concentrations were determined with the BioRad protein assay (BioRad Laboratories, Hercules, CA).

Proteins (50 μg, or 30 μg for connective-tissue-derived mast cells) were separated by SDS-PAGE on 10% polyacrylamide gels (BioRad Laboratories, Hercules, CA). The separated proteins were transferred via electroblotting to nitrocellulose membranes (BioRad Laboratories, Hercules, CA) and incubated 1 h at room temperature in a blocking solution of 500 ml Tris-buffered saline containing 0.1% Tween-20 (TBST) and 3% dry milk. The membranes were then incubated overnight at 4 °C with either goat anti-mouse SERT (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit anti-rat SERT (Chemicon, Temecula, CA) diluted 1:500. The membranes were washed three times with TBST, and then incubated with either a 1:5000 dilution of donkey anti-goat
IgG conjugated with horseradish peroxidase (HRP) or goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Inc). The membranes were washed four times with TBST, and enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) was used to visualize immunoreactive proteins.

To control for loading of protein, blots were stripped with Restore Stripping Buffer (Pierce Biotechnology, Inc., Rockford, IL) and reprobed for β-actin by incubating 1 h with a 1:5000 dilution of mouse monoclonal anti-β-actin (Sigma-Aldrich, St. Louis, MO), then washed with TBST. The membrane was incubated with a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) before washing and visualizing with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

**MTT Cell Viability Assay**

The MTT assay protocol is an adaptation of Mosmann (28), Carmichael et al. (7), and Pozzolini et al. (33). RAW264.7 macrophages were plated in 12-well cell culture plates (Costar brand, Fisher Scientific, Hampton, NH) at a density of 1.5 x 10^6 cells/well. The cells were treated with vehicle (media alone) or LPS (30 ng/ml) and incubated at 37 °C in 5% CO₂ for 24 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO) was added to each well at a final concentration of 0.5 mg/ml and incubated for an additional 4 h. All media was then aspirated, and cells were lysed with a buffer containing sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, MO) dissolved in 50% N,N-dimethyl-formamide (DMF; Sigma-Aldrich, St. Louis,
MO) and incubated overnight at 37 °C. The plate was read with the μQuant Universal Microplate Reader (Bio-Tek Instruments, Winooski, VT) at 570 nm.

Measurement of TNF-α and IL-1β

Extracellular TNF-α and IL-6 concentrations were assayed with the OptEIA Multi Component ELISA Set for Mouse TNF-α and Mouse IL-1β, respectively (BD Biosciences, San Diego, CA). The assays were performed in accordance with the manufacturer’s protocols. The plates were read at 450 and 570 nm with a μQuant Universal Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). TNF-α and IL-1β concentrations were calculated with KC4 software (BioTek Instruments, Winooski, VT).

RNA Isolation

RAW264.7 macrophages were cultured in 6-well tissue culture plates (Costar brand, Fisher Scientific, Hampton, NH) and treated with vehicle (media alone) or LPS (30 ng/ml) as designated per experiment. Total RNA was extracted from approximately six million cells in culture with TRI-Reagent (Molecular Research Center, Cincinnati, OH) according to the procedures provided by the manufacturer. RNA samples were stored at -70 °C.
Reagents

Fluoxetine used in $^3$H-5HT uptake assays was a gift from Eli Lilly (Indianapolis, IN). All other reagents, including IL-10 and unlabeled 5-HT, were purchased from Sigma-Aldrich (St. Louis, MO).

Data Analyses

Analysis of variance (ANOVA) and Tukey’s multiple comparison test were used to evaluate effects of transport inhibitors and cytokines on $^3$H-5HT uptake. IC$_{50}$ values were calculated with Prism software (Graphpad, San Diego, CA). For experiments with only two treatments, a t-test was used to compute statistical significance. Differences were considered statistically significant at $p \leq 0.05$. 
Results

Comparison of $^3$H-5HT uptake in RAW264.7 macrophages and peritoneal macrophages

To calculate specific uptake of $^3$H-5HT, the non-specific uptake, measured in the presence of 25 μM non-radioactive 5HT, was subtracted from the total uptake of $^3$H-5HT (Figure 3). The specific uptake of $^3$H-5HT in RAW264.7 macrophages was surprisingly high. Uptake of $^3$H-5HT, measured over 10 min, in these cells was 21-fold that previously measured in mouse peritoneal macrophages (40) and approximately 11 times that measured in primary peritoneal macrophages in this study (Figure 4). In this study, primary cells were incubated with twice the concentration of $^3$H-5HT used in either assays of RAW264.7 cells or previous assays of primary cells.

LPS activation of RAW264.7 macrophages increased 5-HT uptake

Lipopolysaccharide (LPS) activation of RAW264.7 macrophages stimulated a 2.5-fold increase of $^3$H-5HT specific uptake compared to $^3$H-5HT specific uptake in cells treated with media alone (vehicle) (Figure 5). Moreover, specific uptake was sodium dependent in both vehicle- and LPS-treated macrophages (Figure 5). When pre-incubated with SH uptake buffer containing either lithium chloride (LiCl) or choline
chloride, uptake was inhibited more than 90% compared to uptake by macrophages incubated in SH uptake buffer containing NaCl. LPS did not significantly affect uptake measured in LiCl or choline chloride buffer.

In order to verify the role of the SERT in the uptake of 5-HT in RAW264.7 macrophages, \(^3\)H-5HT uptake assays in the presence of the SSRI fluoxetine were performed (Figures 6A and 7A). In both vehicle- and LPS-treated macrophages, uptake of \(^3\)H-5HT was reduced as the concentration of fluoxetine was increased. The IC\(_{50}\) of fluoxetine inhibition was 5.7 nM for 5-HT transport in vehicle-treated macrophages (Figure 6B) and 7.7 nM in LPS-treated cells (Figure 7B). These IC\(_{50}\) values are similar to those reported for the SERT in mouse peritoneal macrophages and in human cells (3; 40).

\textit{LPS modulation of SERT activity was time- and concentration-dependent}

Up-regulation of SERT activity by LPS activation was time-dependent (Figure 8). RAW264.7 macrophages were treated with vehicle (media only) or 30 ng/ml LPS and incubated at 4 h, 12 h, or 24 h prior to assay of \(^3\)H-5HT uptake. At 4 h and 12 h after treatment, there was no significant difference in SERT activity in vehicle-treated and LPS-treated cells. At 24 h after LPS (30 ng/ml), SERT activity increased approximately 2.3-fold compared to SERT activity in vehicle-treated cells.

Although we have routinely used 30 – 50 ng/ml LPS to activate macrophages, and 30 ng/ml LPS was used in initial experiments, a concentration-response curve revealed that \(^3\)H-5HT specific uptake peaked when cells were treated with 1 – 3 ng/ml LPS.
Treatment with 1 ng/ml or 3 ng/ml LPS elevated SERT activity in the macrophages by approximately 3.3 times compared to vehicle-treated cells.

**IL-10 decreased 5-HT uptake**

When the anti-inflammatory cytokine interleukin-10 (IL-10; 20 ng/ml) was added to the cell media 2 h prior to treatment with vehicle or LPS (26 h before measurement of $^3$H-5HT uptake), SERT activity decreased by approximately 45% in vehicle-treated cells and 61% in LPS-treated cells (Figure 10A). An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay demonstrated no significant differences in cell viability that could account for the differences in SERT activity observed 24 h after vehicle, LPS (30 ng/ml), or IL-10 (20 ng/ml) (Figure 10B). Furthermore, IL-10 (20 ng/ml) also inhibited SERT activity roughly 41% in macrophages activated with low levels of LPS (3 ng/ml) (Figure 11).

**Mechanism for SERT upregulation by LPS**

Western blotting was used to determine whether LPS increased SERT protein in RAW264.7 macrophages (Figure 12). Connective tissue mast cells (CTMC), a positive control, produced a distinct band at approximately 70 kDa as expected (8; 35). No immunoreactive SERT was observed in L929 fibroblasts, which served as the negative control. The estimated molecular weight of immunoreactive SERT detected in both vehicle- and LPS-treated RAW264.7 lysates was 75 kDa, whereas the kDa of the SERT in CTMC was 70 kDa. Interestingly, the density of immunoreactive SERT from LPS-
treated RAW264.7 macrophages was greater than that in the vehicle-treated cells (n=3) suggesting that LPS increased total SERT protein; however, the differences were not statistically significant. In order to determine if LPS also increases SERT mRNA, RNA samples have been extracted and frozen for future real-time PCR analysis.

LPS-activated macrophages produce nitric oxide (NO), which activates the SERT through the PKG/p38 MAPK pathway (for review: see Figure 2)(48-50). To investigate whether PKG is involved in the LPS-induced upregulation of SERT activity, the $^3$H-5HT uptake assay was performed in the presence of LY83583, a guanylyl cyclase (GC) inhibitor (Figure 11). As expected, there was a significant increase in 5-HT uptake in cells treated with LPS (30 ng/ml) compared to those treated with media only (vehicle). When vehicle- or LPS-treated cells were incubated with LY83583 (10 μM) or its appropriate vehicle (0.1% ethanol) for 24 h, the inhibitor killed the cells (data not shown). When cells were treated with LPS or vehicle for 24 h and incubated with the inhibitor for 20 min prior to the $^3$H-5HT uptake assay, there were no significant differences between those incubated with LY83583 and those without LY83583. These findings suggest that short-term inhibition of GC does not affect SERT activity in RAW264.7 macrophages, although others have demonstrated short-term effects of this inhibitor on the SERT in other cells (49).

LPS-activated macrophages release pro-inflammatory cytokines such as tumor necrosis-alpha (TNF-α) and interleukin-1β (IL-1β). Preliminary data from enzyme-linked immunosorbent assays (ELISA) indicated that there is more TNF-α (1.3-fold) and IL-1β (6.3-fold) present in the cell media of cells treated with 30 ng/ml LPS than those
treated with 1 ng/ml LPS (Figure 14A and 14B). Yet, the lower concentration of LPS is the optimal stimulus for SERT activity. Despite evidence that TNF-α and IL-1β stimulate increased SERT activity in some cell types (47), these preliminary data suggest that these cytokines are not the only stimulus for 5-HT during macrophage activation.
Discussion

This study demonstrates that RAW264.7 macrophages express high SERT activity, more than 10-fold that observed in peritoneal macrophages in this study or in previous studies in our laboratory (40). Therefore, this cell line is an excellent model for studying regulation of the SERT.

This study shows for the first time that SERT activity is increased by LPS-induced activation of macrophages in a time- and concentration-dependent manner. Because more than 12 h of LPS activation is required to detect increased SERT activity, it is probable this rise in 5-HT uptake is not due simply to a translocation of the SERT protein. Moreover, Zhu et al. suggest that PKG plays a role in the exocytosis of the SERT protein from vesicles in the cytosol to the plasma membrane (49); however, assay of $^3$H-5HT uptake in the presence of a PKG inhibitor, LY83583, failed to significantly decrease SERT activity. Western blotting suggested that there was more SERT protein present in LPS-activated macrophages than in vehicle-treated cells. Conversely, previously we were unable to detect effects of LPS on SERT protein in recruited peritoneal macrophages (40), although recruitment may have maximally stimulated SERT activity prior to LPS treatment.
The mechanism for IL-10-induced reduction of SERT activity in this study is unknown, but it has been shown that IL-10 can work through the STAT3 pathway in order to decrease proliferation of macrophages (31). Using an MTT viability assay, however, we could not find any significant change in cell viability induced by IL-10 (20 ng/ml) and LPS (30 ng/ml). It is conceivable, however, that high concentrations of LPS (90 ng/ml) do not increase SERT activity because cell viability is reduced.

Because the pro-inflammatory cytokines stimulate p38 MAPK activity, it is not surprising that these cytokines also increase SERT activity (9). For example, TNF-α and IL-1β, pro-inflammatory cytokines, have been shown to stimulate 5-HT uptake in a rat embryonic neuronal cell line and in midbrain synaptosomes through the p38 MAPK pathway (47). Data from our lab, however, show that more TNF-α is secreted by RAW264.7 macrophages at higher concentrations of LPS, and the increase in 5-HT uptake peaks at approximately 1 ng/ml LPS. These findings suggest that while pro-inflammatory cytokines play a role in the LPS-stimulation of the SERT, there may be additional stimulation through other pathways.

In summary, this study demonstrates that the RAW264.7 macrophage cell line is a useful model system to study the SERT and its regulation. Moreover, this is the first study to demonstrate that LPS-activation of macrophages increases SERT activity. These findings may have clinical implications in patients taking SSRIs such as fluoxetine (Prozac®), sertraline (Zoloft®), and paroxetine (Paxil®). As these SSRIs inhibit SERT activity, there are higher concentrations of extracellular 5-HT, allowing for increased
binding to 5-HT receptors. This may lead to an increase in the actions caused by the of 5-HT on macrophages such as decreased production of TNF-α (25).
List of References


Appendix

Figures and Tables

Figure 1. Biosynthesis pathway of serotonin (5-hydroxytryptamine) from L-tryptophan.
<table>
<thead>
<tr>
<th>Receptor Family</th>
<th>Distribution</th>
<th>Type</th>
<th>Effector Mechanism</th>
<th>Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₁</td>
<td>CNS, Vascular</td>
<td>Gᵢ/Go-coupled</td>
<td>Inhibition of adenylyl cyclase (decrease cAMP); opening of K⁺ channels</td>
<td>Neuronal inhibition, behavioral effects (e.g. sleep, thermoregulation, appetite control, sexual behavior, aggression), neuronal hyperpolarization, vasoconstriction</td>
</tr>
<tr>
<td>5-HT₂</td>
<td>CNS, GI, Platelets, Choroid Plexus</td>
<td>Gᵢ/G₁₁-coupled</td>
<td>Stimulate phosphoinositide-specific phospholipase C (increase IP₃ and DAG)</td>
<td>Neuronal excitation, smooth muscle contraction (e.g. peristalsis), vasoconstriction, platelet aggregation, cerebrospinal fluid excretion</td>
</tr>
<tr>
<td>5-HT₃</td>
<td>CNS, PNS</td>
<td>Ligand-gated Na⁺/K⁺ cation channel</td>
<td>Rapid membrane depolarization</td>
<td>Anxiety, neuronal excitation, nausea</td>
</tr>
<tr>
<td>5-HT₄</td>
<td>CNS, GI</td>
<td>Gₛ-coupled</td>
<td>Stimulation of adenylyl cyclase (increase cAMP)</td>
<td>Gastrointestinal motility (may be connected to irritable bowel syndrome and gastroesophageal reflux), neuronal excitation, may be involved to memory and learning – decreased in Alzheimer’s patients</td>
</tr>
<tr>
<td>5-HT₅</td>
<td>CNS</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>5-HT₆</td>
<td>CNS</td>
<td>G-protein-coupled</td>
<td>Stimulation of adenylyl cyclase (increase cAMP)</td>
<td>Unknown – may control cholinergic neurotransmission</td>
</tr>
<tr>
<td>5-HT₇</td>
<td>CNS, GI, Vascular</td>
<td>Gₛ-coupled</td>
<td>Stimulation of adenylyl cyclase (increase cAMP)</td>
<td>Largely unknown: may be involved with mood, learning, neuroendocrine, and vegetative behaviors, possibly relaxation of coronary artery</td>
</tr>
</tbody>
</table>
Figure 2. Summary of SERT Regulation. Nitric oxide synthetase (NOS) produces nitric oxide (NO), which activates guanylyl cyclase (GC). GC produces the secondary messenger cGMP, which activates protein kinase G (PKG). PKG then has two actions on the SERT: it causes exocytosis of the SERT in the cytosol (whether active or inactive) to the cell membrane and it activates p38 MAPK, which then activates protein phosphatase 2A (PP2A) causing activation of the SERT. Protein kinase C (PKC) works independently of p38 MAPK. PKC phosphorylates the serine residues at 5 min, down-regulating SERT activity, and phosphorylates threonine residues after 30 min causing endocytosis of the SERT, which further inhibits. (Based on Jayanthi et al. 2005; Samuvel et al. 2005; Zhu et al. 2004a; Zhu et al. 2004b; Zhu et al. 2005)
Figure 3. Comparison of total, specific, and non-specific uptake of $^3$H-5HT by RAW264.7 macrophages. RAW264.7 macrophages were plated at a density of $1.5 \times 10^6$ cells/well and allowed to grow overnight to approximately $3 \times 10^6$ cells/well. LPS (30 ng/ml) or vehicle (media only) was added and incubated for 24 h prior to the assay. Prior to the uptake assay, either vehicle (media only) or unlabeled 5-HT (5-HT; 25 μM) was added and preincubated for 5 min. Macrophages were incubated with 25 nM $^3$H-5HT for time indicated on x-axis. For this and all other experiments, specific uptake was determined to be the non-specific uptake, measured in the presence of 25 μM unlabeled 5-HT, subtracted from the total uptake of $^3$H-5HT. Bars represent mean values ± S.E of 3-6 replicates.
**Figure 4.** Comparison of SERT activity in primary peritoneal macrophages vs. RAW264.7 macrophages. Primary peritoneal macrophages were plated at approximately 3 x 10⁶ cells/well. RAW264.7 macrophages were plated at a density of 1.5 x 10⁶ cells/well and allowed to grow overnight to approximately 3 x 10⁶ cells/well. LPS (30 ng/ml) or vehicle (media only) was added and incubated for 24 h prior to the assay. RAW264.7 macrophages were incubated with 25 nM ³H-5HT for 10 min, whereas peritoneal macrophages were incubated with 50 nM ³H-5HT for 10 min. Bars represent mean values ± S.E of at 4-5 replicates. ** p < 0.001 compared to RAW264.7 macrophages (white bar V).
Figure 5. LPS-activation increases sodium dependent uptake of $^3$H-5HT uptake in RAW264.7 macrophages. RAW264.7 macrophages were plated at a density of 1.5 x $10^6$ cells/well and allowed to grow overnight to approximately $3 \times 10^6$ cells/well. LPS (30 ng/ml) or vehicle (media only) was added and incubated for 24 h prior to the assay. During the uptake assay, SH uptake buffer containing either NaCl (on the left), LiCl (middle), or choline Cl (on the right) were added and preincubated for 5 min. Macrophages were incubated with 25 nM $^3$H-5HT for 10 min. Bars represent mean values ± S.E of at 3-6 replicates. * p < 0.01, ** p < 0.001 compared to uptake measured in the presence of NaCl in vehicle-treated cells (white bar). ♦♦ p < 0.001 compared to uptake measured in the presence of NaCl in LPS-activated cells (black bar). LPS did not significantly alter uptake measured in LiCl or choline Cl buffers.
Figure 6. (A) Effects of fluoxetine on specific uptake of $^3$H-5HT in vehicle-RAW264.7 macrophages. RAW264.7 macrophages were plated at a density of $1.5 \times 10^6$ cells/well and allowed to grow overnight to approximately $3 \times 10^6$ cells/well. Macrophages were preincubated for 5 min with fluoxetine; 25 nM $^3$H-5HT was added, and incubation continued for 10 min. Bars represent mean values ± S.E of at 3-7 replicates. # p < 0.05, ** p < 0.001 compared to vehicle (V) with no inhibitor. (B) Kinetics of fluoxetine inhibition of $^3$H-5HT uptake in vehicle-treated RAW264.7 macrophages. Each data point is the mean ± S.E. of 3-5 replicates. IC$_{50}$ = 5.7 nM.
Figure 7. (A) Effects of fluoxetine on specific uptake of $^3$H-5HT in LPS-treated RAW264.7 macrophages. RAW264.7 macrophages were plated at a density of 1.5 x $10^6$ cells/well and allowed to grow overnight to approximately 3 x $10^6$ cells/well. LPS (30 ng/ml) was added and incubated for 24 h prior to the assay. Macrophages were preincubated for 5 min with fluoxetine; 25 nM $^3$H-5HT was added, and incubation continued for 10 min. Bars represent mean values ± S.E. of at 8 replicates. # p < 0.05, ** p < 0.001 compared to vehicle (V) with no inhibitor.  (B) Kinetics of fluoxetine inhibition of $^3$H-5HT uptake in LPS-treated RAW264.7 macrophages. Each data point is the mean ± S.E. of 3-5 replicates. IC$_{50}$= 7.7 nM.
Figure 8. Time-dependent effects of LPS activation on specific uptake of \(^3\)H-5HT in RAW264.7 macrophages. RAW264.7 macrophages were plated at a density of 1.5 x 10^6 cells/well and allowed to grow overnight to approximately 3 x 10^6 cells/well. LPS (30 ng/ml) or vehicle (media only) was added and incubated for times indicated (4 h, 12 h, or 24 h) prior to the assay. Macrophages were incubated with 25 nM \(^3\)H-5HT for 10 min. Bars represent mean values ± S.E of 6 replicates. ** p < 0.001 compared to corresponding vehicle (V).
Figure 9. Concentration-dependent effects of LPS on specific uptake of $^3$H-5HT in RAW264.7 macrophages. Cells were plated at a density of $1.5 \times 10^6$ cells/well and allowed to grow overnight to approximately $3 \times 10^6$ cells/well. LPS (at concentrations indicated above) was added and incubated for 24 h prior to the assay. For 9 ng/ml LPS, only media was added to the well and incubated for 24 h prior to the assay. Macrophages were incubated with 25 nM $^3$H-5HT for 10 min. Bars represent mean values ± S.E. of at 3-7 replicates. ** p < 0.001 compared to vehicle (0 ng/ml LPS).
Figure 10. (A) Effects of IL-10 on specific uptake of \(^3\)H-5HT in vehicle-treated and LPS-treated RAW264.7 macrophages. RAW264.7 macrophages were plated at a density of 1.5 x 10^6 cells/well and allowed to grow overnight to approximately 3 x 10^6 cells/well. IL-10 (20 ng/ml) was added and preincubated for 2 h prior to the addition of LPS (30 ng/ml) or vehicle (media only) and then incubated for an additional 24 h prior to the assay. Macrophages were incubated with 25 nM \(^3\)H-5HT for 10 min. Bars represent mean values ± S.E of at 3-5 replicates. # p <0.05, ** p < 0.001 compared to vehicle (V) without IL-10, †† p<0.001 compared to LPS without IL-10. (B) Viability of RAW264.7 macrophages after treatment with vehicle (media alone), LPS, and/or IL-10. RAW264.7 macrophages were plated and treated as above. MTT was added after a 24 h incubation of LPS (30 ng/ml). After 4 h, media was aspirated, cells were lysed, and incubated again overnight before reading. Bars represent mean values ± S.E of at 3 replicates.
Figure 11. IL-10 modulation of $^3$H-5HT specific uptake in RAW264.7 macrophages treated with a low (optimal) concentration of LPS. RAW264.7 macrophages were plated at a density of $1.5 \times 10^6$ cells/well and allowed to grow overnight to approximately $3 \times 10^6$ cells/well. IL-10 (20 ng/ml) was added and preincubated for 2 h prior to the addition of LPS (3 ng/ml) or vehicle (media only) and then incubated for an additional 24 h prior to the assay. Macrophages were incubated with 25 nM $^3$H-5HT for 10 min. Bars represent mean values ± S.E of at 3 replicates. ** p<0.001 compared to LPS.
Figure 12. Expression of SERT protein in RAW264.7 macrophages assayed by Western blotting. Total protein (50 µg, except for 30 µg CTMC) was separated by SDS-PAGE on 10% polyacrylamide gels. Molecular size standards are shown at the left. Connective tissue mast cells (CTMC) served as positive control. L929 fibroblasts (L929) served as a negative control. Vehicle (V) and LPS-treated macrophages incubated for 24 h are shown. The results are an example of 3 blots. Density of immunoreactive SERT in LPS-treated cells was greater than that in vehicle-treated cells (n=3).
Figure 13. Inhibition of guanylyl cyclase by LY83583 in RAW264.7 macrophages. RAW264.7 macrophages were plated at a density of 1.5 x 10^6 cells/well and allowed to grow overnight to approximately 3 x 10^6 cells/well. LPS (30 ng/ml) or vehicle (media only) was added and preincubated for 24 h prior to the assay. The guanylyl cyclase inhibitor LY83583 (10 μM) or LY vehicle (LY-V; 0.1% ethanol final) was added 20 min prior to the assay. Macrophages were incubated with 25 nM ^3H-5HT for 10 min.
**Figure 14.** (A) Extracellular concentrations of TNF-α of RAW264.7 macrophages. RAW264.7 macrophages were plated at a density of 1.5 x 10^6 cells/well and allowed to grow overnight to approximately 3 x 10^6 cells/well. LPS (1 ng/ml or 30 ng/ml) or vehicle (media only) was added and incubated (4 h) prior to harvest of cell media. Concentration of TNF-α in extracellular media was assayed by ELISA. Bars represent mean values ± S.E of 3 replicates. ♦ ♦ p < 0.001 compared to 1 ng/ml LPS.  
(B) Extracellular concentrations of IL-1β of RAW264.7 macrophages. RAW264.7 macrophages were plated at a density of 1.5 x 10^6 cells/well and allowed to grow overnight to increase to approximately 3 x 10^6 cells/well. LPS (1 ng/ml or 30 ng/ml) or vehicle (media only) was added and incubated for 24 h prior to harvest of cell media. Bars represent mean values ± S.E of 3 replicates. ♦ p < 0.05 compared to 1 ng/ml LPS.
Vita

Sienna Marie Arenas Malubay was born in Naic, Cavite, in the Philippines, on August 7th, 1981. With her family, she immigrated to the United States when she was six years old. She lived briefly in Virginia Beach, Virginia, and Laurel, Maryland, but has grown up primarily in the Washington, D.C. metropolitan area of Northern Virginia. She graduated in 1999 from Thomas Jefferson High School for Science and Technology in Alexandria, Virginia. She then attended The College of William and Mary in Williamsburg, Virginia, where she earned a Bachelor of Science degree in Biology in 2003. Following her undergraduate career, she began her graduate work at Virginia Commonwealth University during the Spring 2004 semester. While at VCU, she has taught BIOL 101, 152, and 300 laboratories.