THE EFFECT OF AMBIENT TEMPERATURE ON SEROTONIN SYNDROME

by

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Serotonin syndrome (SS) is a drug-induced toxicity caused by an excess of serotonin (5-HT) in the central nervous system (CNS). The symptoms of the disorder range from mild to severe, with the severe state evoking life-threatening hyperthermia. Autonomic dysfunction is controlled in part by serotonin receptors, with the 5-HT$_{2A}$ receptor responsible for increasing core body temperature ($T_{cor}$). Our results show that the 5-HT$_{2A}$ receptors on the preoptic/anterior hypothalamus (PO/AH) and prefrontal cortex (PFC), in particular, are sensitive to changes in ambient temperature ($T_{amb}$). The toxic increase of 5-HT is postulated to occur due to the temperature-dependent activation of these receptors that promotes a positive feedback mechanism. Our results suggest that changes in $T_{amb}$ can either exacerbate or alleviate the symptom and that this is mediated by the 5-HT$_{2A}$ receptors. Understanding the mechanism involved in elevating $T_{cor}$ is imperative in treating and preventing the disorder.
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LIST OF ABBREVIATIONS

ADCC  aromatic decarboxylase
BBB   blood brain barrier
CNS   central nervous system
CSF   cerebral spinal fluid
DOI   2, 5-dimethoxy-4-iodophenyl)-2-aminopropane
DRN   dorsal raphe nucleus
G\textsubscript{i}  inhibitory G-coupled protein
G-coupled G-protein coupled
GI    gastrointestinal tract
5-HIAA 5-hydroxyindoleacetic acid
5-HT  5-hydroxytryptamine (serotonin)
5-HTP 5-hydroxy-L-tryptophan
LSD   (+) lysergic acid diethylamide
MAOI  monoamine oxidase inhibitor
MDMA 3, 4-methylendioxymethamphetamine
PLC   phospholipase C
PFC   prefrontal cortex
PO/AH preoptic/anterior hypothalamus
8-OH-DPAT 8-hydroxy-2-(di-\textit{n}-propylamino) tetralin
SSRI  selective serotonin reuptake inhibitor
TRH  tryptophan hydroxylase
T_{amb} ambient temperature
T_{cor} core body temperature
Trp tryptophan
μM micro molar

**List of Drugs**

- clorgyline  monoamine oxidase type A inhibitor
- 5-HTP precursor to 5-HT (serotonin)
- ketanserin  selective 5-HT\textsubscript{2A} receptor antagonist
- MK-801 non-competitive NMDA receptor antagonist

**List of Terminology**

- **clonus**: a series of alternating contractions and partial relaxations of a muscle that in some nervous diseases occurs in the form of convulsive spasms involving complex groups of muscles and is believed to result from alteration of the normal pattern of motor neuron discharge (Webster dictionary)
- **diaphoresis**: sweating or perspiration
- **efflux**: 5-HT release
- **hyperreflexia**: over-activity of physiological reflex (Webster dictionary)
- **myoclonus**: irregular involuntary contraction of a muscle usually resulting from functional disorder of controlling motor neurons (Webster dictionary)
• **tachycardia**: relatively rapid heart action whether physiological or pathological

(Webster dictionary)
1.0 INTRODUCTION

Since the late 1800s, researchers have known that a substance found in the blood serum was responsive for the constriction and tonicity of vascular smooth muscle. But it wasn’t until the 1940s when Rapport and his colleagues were able to isolate and characterize this substance from the serum and name it for its tonic properties as serotonin (Green, 2006, Hensler, 2006). Since serotonin is a hydrophilic molecule and is therefore unable to cross the blood brain barrier (BBB), its discovery in the central nervous system was exciting as it indicated that this agent can also be involved in brain function and signaling. During the same time, it was observed that (+) lysergic acid diethylamide (LSD) was found to prevent the normal function of serotonin, thus suggesting that certain drugs and disorders might be involved in the impairment of the serotonin system. Since then, serotonin was found to play a role in the etiology of a number of psychiatric disorders, like anxiety, depression, and schizophrenia, and serotonin reuptake inhibitors (SSRI) and other drugs that target the serotonin system have been developed (Hensler, 2006). Unfortunately, the use of these drugs in combination with other antidepressants has led to a new problem called serotonin syndrome.

Serotonin syndrome (SS) is an iatrogenic or drug-induced toxicity caused by an excess of serotonin in the central nervous system (CNS). In humans, the toxicity arises due to the combination of monoamine oxidase inhibitors (MAOIs), selective serotonin reuptake...
inhibitors (SSRI), and tricyclic antidepressants (Gillman, 2006). The symptoms, as described by Whyte (2004), include neuromuscular and autonomic hyperactivity and altered mental status. In severe cases, death can occur.

Though serotonin syndrome was first observed over 50 years ago (Gillman, 2006), a clear understanding of this syndrome is still unknown and many questions remain. To better understand this disorder, focus will be on serotonin and its role in depression and the effect ambient temperature has in exacerbating or alleviating the SS-induced symptoms.

1.1 Neurochemistry of Serotonin (5-HT) and their Receptors

1.1.2 Synthetic and Degradative Pathway

In 1948, Rapport, Green, and Page isolated and identified the factor responsible for vascular tonicity from the blood serum as serotonin (5-hydroxytryptamine or 5-HT). Independently, Esparmer isolated a substance from the enterochromaffin cells of the gastrointestinal tract and named it “enteramine.” In 1952, serotonin and enteramine were found to be the same substance. A year later, Twarg and Page were able to extract serotonin from the brain. Thus serotonin was found to be located in three main regions in the body: blood (specifically platelets), gastrointestinal tract (GI), and brain (Hensler, 2006).

Though serotonin is found both in the peripheral nervous system (PNS; e.g. blood, GI) and in the central nervous system (CNS; e.g. brain), the majority of 5-HT is found in the PNS. It is specifically found in the enterochromaffic cells of the gastrointestinal (GI) tract. 
where it is involved in GI regulation or in blood vessel tonicity. The remaining 5-HT is found in the brain where it functions as a neurotransmitter, effecting body functions like appetite, sleep, temperature regulation, gonadotropin secretion, and pain perception (Marczynski, 1976).

The synthesis of serotonin involves the dietary absorption of tryptophan (Trp) in the GI tract. Trp is then hydroxylated to 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase (TRH). Two isoforms of this enzyme have been found to exist, one that is found in the tissue, TRH1, and one that is found in the brain, TRH2. Anxiety and depression has been linked to the genetic polymorphisms of these subtypes, making the individual more susceptible to developing these disorders (Kamrowska, 2007, Porter et al, 2008, Harvey et al, 2007). Under physiological conditions this enzyme can become saturated with 5-HTP and inhibiting TRH by using the drug parachlorophenylalanine (PCPA) results in a marked depletion of 5-HT levels in the brain (Hensler, 2006). PCPA can thereby be used to manipulate levels of cerebral serotonin (Nestler et al, 2001).

Serotonin is a hydrophilic substance and as such, cannot cross the BBB. Therefore oral administration of serotonin cannot pass into the CNS but tryptophan and 5-hydroxytryptophan (5-HTP) can (Bear et al, 2001).

5-HTP is decarboxylated by the enzyme aromatic amino acid decarboxylase (AADC) into serotonin (5-HT). AADC is not only present in serotonergic neurons, but also in
catecholaminergic neurons where it is found to convert 3, 4-dihydroxyphenylalanine (DOPA) to dopamine. However different pH, substrate concentration, or cofactor requirement is needed for optimal activity of the enzyme when using 5-HTP or DOPA as substrates (Hensler, 2006). Serotonin is broken down to 5-hydroxyindoleacetic (5-HIAA) by the enzyme monoamine oxidase (MAO). Two forms of MAO exist, MAO_A and MAO_B. Surprisingly, serotonergic neurons express the low affinity enzyme MAO_B rather than MAO_A. It is speculated that this could be due to the fact that these neurons maintain a pool of cytoplasmic serotonin and the function of MAO_B may be to metabolize other trace amines that may falsely act as transmitter. It is thought that extracellular serotonin is instead oxidized by MAO_A, derived from other sources (Nestler et al, 2001) (see figure 1).

Serotonin (5-HT), once broken down into 5-HIAA, is excreted in the urine. Certain tumors and cancers produce an excess amount of serotonin and 5-HIAA (Joy et al, 2008), while low levels of 5-HIAA in cerebral spinal fluid (CSF) have been reported to correlate with individuals who attempted suicide by a violent means. Hence measuring 5-HIAA levels in the CSF, blood, or urine can be used to test for these tumors or to assess central serotonergic function in neuropsychiatry disorders (Nester et al, 2001).

In the central nervous system, serotonin is an important neurotransmitter that is involved in regulating many biological functions that include appetite, anger, sleep, etc. In the brain, serotonin signaling involves the neurotransmitter binding to its receptor on the pre- or post-synaptic membrane. Depending on the receptor that is bound, an inhibitory or
The excitatory message will be passed to the neuron. The types and function of these receptors will be discussed in the next section.

1.1.2. Receptor Subtypes and Ligands

In 1957, Gaddam and Picarelli proposed that 5-HT receptors possessed two receptor subtypes, D- and M- receptors. This classification was based on experimental observations due to the sensitivity of the receptor to dibenzyline and morphine as blockers. However, it was later found that this was relevant only to peripheral receptors, not the receptors in the brain (Green, 2006). With the advent of ligand-receptor binding techniques in the 70s, it was found that the binding affinities of 5-HT receptors differed. In the next 10-20 years, five other families, designated 5-HT3-7, have been isolated with over 15 sub-families or sub-population cloned to date (Sanders–Bush and Canton, 2000). These receptors either belong to the ligand-gated ion channel superfamily or to the G-protein coupled superfamily of receptors.

Most of the receptors belong to the G-protein coupled superfamily except for the 5-HT3 receptors. These receptors are referred to as ionotropic receptors. They are non-selective cation channels and thereby activation of the receptor results in an excitatory postsynaptic response. 5-HT3 receptors are similar to other ionotropic receptors in that they are composed of five subunits pseudo symmetrically arranged around a central ion conducting pore that is permeable to sodium, potassium, and calcium ions (Thompson and Lummis, 2007). Opening up the pore and allowing permeability of the ions gives rise to fast postsynaptic responses, typically lasting only a few milliseconds. The nature of the
response, whether excitatory or inhibitory, is determined by the type of ion channel bound by the neurotransmitter and by the concentration of permeable ions inside and outside the cell (Purves et al, 2004). The protein subunits are encoded by HTR3A, HTR3B, HTR3C, HTR3D, and HTR3E genes. The functional channels are either compromised of five identical 5-HT\textsubscript{3A} subunits (homopentameric) or a combination of 5-HT3A and one of the other four units, 5-HT3B, 5-HT3C, 5-HT3D, or 5-HT3E (heteropentamerize). The 5-HT3 receptors are targeted by the therapeutic drugs, ondansetron (Zofran\textsuperscript{®}) and granisetron (Kytril\textsuperscript{®}), to prevent postoperative nausea and chemotherapy-induced emesis (Watson, 2000).

The majority of the 5-HT receptors are known as metabotropic or G-coupled receptors. These receptors have been shown to be involved in emotions, circadian rhythms (sleeping), motor behaviors, mental arousal, and thermoregulation. As well, their activation has been shown to mediate satiety and reduce food consumption, making these receptors a target for developing drugs to treat eating disorders. Mental disorders like depression, anxiety, and schizophrenia have also been linked to perturbation in the 5-HT receptor (Terry et al, 2008).

The action of the metabotropic receptors is through a G-coupled second messenger system and activation of the receptor produces slower postsynaptic effects that may last much longer. There are many differences between the receptors. The first is that they act through different secondary messengers. For instance, the 5-HT\textsubscript{1A} receptors are negatively coupled to adenylate cyclase via G\textsubscript{i} family of G proteins and when activated,
function to depress neuronal firing by inducing hyperpolarization. $5\text{-HT}_{2\alpha}$ receptors, on the other hand, are G-coupled to phospholipase C (PLC) and activation of the $5\text{-HT}_{2\alpha}$ receptor causes depolarization (Sanders–Bush and Canton, 2000). Another difference between the receptors are the affinity they have to their natural ligand, 5-HT. For example, 5-HT is found to bind to $5\text{-HT}_{1\alpha}$ receptor at a low concentration while stimulation of $5\text{-HT}_{2\alpha}$ receptor requires a high concentration of 5-HT to become activated (Glennon et al, 1995). In fact, 5-HT has $>$1000 more affinity for $5\text{-HT}_{1\alpha}$ receptor than $5\text{-HT}_{2\alpha}$ (Rusyniak, 2007). A third way in which differential transduction could occur is through “promiscuous coupling.” This happens when receptor couples to a previously unrecognized signal cascade. For example, the $5\text{-HT}_{1\alpha}$ G$_i$-linked receptor is normally negatively coupled to adenylate cyclase but in cell lines, it has been shown to positively couple adenylate cyclase. Furthermore, they were also shown to couple to a different second-messenger, PLC. Cross-talk between the receptors could also influence signal transduction (Sanders-Bush and Canton, 1995). For example, $5\text{-HT}_{2\alpha}$ and $5\text{-HT}_{1\beta}$ receptors were shown to affect the other’s signaling by modulating trafficking (Janoshazi et al, 2007).

The synthesis and storage of serotonin, its release into the synaptic cleft and the binding of the ligand to its receptor on the post-synaptic membrane allows the transmission of chemical signal to the next neuron. The actual process and how this occurs will be elucidated next.
1.1.3 Chemical Transmission in the Mammalian Central Nervous System

Tryptophan, unlike serotonin, can cross the BBB and be converted to 5-HT in the brain by two enzymes tryptophan hydroxylase (TRH) and decarboxylase (AADC). It has been estimated that in the human brain, hundreds of thousands of serotonergic neurons reside. These neurons are found in isolated nuclei in the midline, or raphe of the brain stem, designated B1-B9. The medulla and spinal cord are innervated by the most caudal clusters while the remaining dorsal and median raphe project to the rest of the CNS. The dorsal raphe is found in the midbrain and the median raphe is located ventral to the dorsal raphe. Projections from these and other raphe are found to be so wide-ranging, that many neurons in the brain are innervated by serotonergic fibers (Nester et al, 2001). The raphe is also innervated by other neurotransmitter containing neurons like dopamine, acetylcholine, norepinephrine, and epinephrine. Thus these neurotransmitters can also modulate serotonin signaling (Hensler, 2006, Boyer, 2005).

Serotonin signaling involves the transmission of chemical message through a neuronal pathway. The enzymes that synthesize serotonin are produced in the cell body of the serotonergic neuron and via a slow axon transport, moved to the nerve terminal cytoplasm. The precursor tryptophan is transported from the blood into the brain by a carrier-mediated transport system and then taken up by the serotonergic nerve by serotonin transporters located on the terminal of the neuron (Purves et al, 2004, Owen and Nemeroff, 1994). The neurotransmitter serotonin is synthesized from tryptophan in the cytoplasm of the pre-synaptic terminal and stored into synaptic vesicles. Transduction is initiated when an action potential arrives at the terminal of the pre-synaptic neuron.
This results in a change in the membrane potential and leads to the opening of voltage-gated calcium channels in the pre-synaptic membrane. Due to the substantial $\text{Ca}^{2+}$ concentration gradient between the pre-synaptic neuron and synapse (the external $\text{Ca}^{2+}$ concentration in the synapse is about $10^{-3}$ M, while the internal $\text{Ca}^{2+}$ concentration in the pre-synaptic neuron is about $10^{-7}$ M), a rapid influx of $\text{Ca}^{2+}$ ion enters the pre-synaptic terminal when the ion channels are opened. The increased intracellular $\text{Ca}^{2+}$ levels cause the synaptic vesicles, which contain 5-HT, to fuse with the plasma membrane of the pre-synaptic neuron. This exocytosis causes 5-HT to be released into the synaptic cleft and the neurotransmitter diffuses across the synapse, binding to a specific receptor on the post-synaptic neuron. The binding of the neurotransmitter to the receptors results in the ion channels, located on the postsynaptic membrane to either open or close, depending on the receptor that was bound. The influx (or efflux) of ions into the postsynaptic neuron, changes the membrane potential of the post-synaptic membrane and thereby influences the prospect on whether the post-synaptic neuron will fire an action potential or not (Purves et al, 2004). Serotonin, bound to the receptor for only a finite time, is then released and removed from the synaptic cleft. It re-enters the pre-synaptic neuron via the serotonin transporters (SERT) and is either stored or repackaged into secretory vesicles by the vesicular monoamine transporter (Owen and Nemeroff, 1994) or degraded by monoamine oxidase subtype A (MAO$_A$) into hydroxyindoleacetic acid (HIAA) (Bear et al., 2001, Bartlett, 2006) (see figure 2).

The re-uptake of serotonin by the serotonin transporter, SERT, is the most important step in the termination of the neurotransmitter. The human SERT gene is a 630-amino acid
protein with two putative extracellular N-linked glycosylation sites and eight serine-threonine phosphorylation sites. It is about 48% homologous in its amino acid structure to norepinephrine transporter (NET) and dopamine transporter (DAT) and its proposed protein structure is also similar to them. Although SERT mRNA expression is found exclusively in the raphe nuclei, its protein expression is ubiquitous in the CNS. This is consistent with the fact that serotonergic projections are found throughout the CNS. Drugs that inhibit SERT activity have been shown to extend serotonergic signaling. Many antidepressant drugs have been developed that bind to SERT. In fact, the most widely prescribed antidepressants are selective reuptake inhibitors, SSRIs (Nester et al, 2001).

The development of antidepressant drugs came about by the fact that the impairment of the serotonergic pathway has been shown to contribute too many mental diseases like depression and anxiety. Treatment to restore proper serotonin levels in the brain has had some success in alleviating mental disorders. Although environmental influences can contribute to these diseases, genetic and biological factors play a part and an imbalance in serotonin levels or receptor sensitivity is often seen in such cases. An understanding of the serotonergic function is important in deciphering the etiology and hence effectively treating certain mental illness (Nicholas and Seiden, 2003). Since serotonin is involved in many mood disorders like depression and the current drugs used to treat these disorders target the serotonergic system, an understanding of the what is depression and what role serotonin plays in the mental illness will be discussed next.
1.2 Implication of Serotonin in the Pathophysiology of Major Depressive Disorder

1.2.1 Major Depressive Disorder

The medical dictionary defines depression as “a mental disorder characterized by sustained depression of mood, anhedonia, sleep and appetite disturbances, and feelings of worthlessness, guilt, and hopelessness”. Depression is a disease that affects approximately 121 million people worldwide and an estimated 19 million Americans. World Health Organization (WHO) estimates that the tragic fatality associated with depression results in 850 thousand deaths each year. They also reports that the burden of this disease ranks second in developed countries after cardiovascular disorders and fourth in developing nations (Licinio J and Wong ML, 2005, Halfin, 2007, http://www.who.int/mental_health/management/ depression/ definition/en/).

Depression is a serious disease that affects up to 10% of the American population. It is more prevalent in women than men due to changes in their biological, life cycle, hormonal, and psychosocial factors that are unique to the women (NIH, Mann et al, 2006). Clinically, the symptoms of depression include an abnormal feeling of sadness, despair and bleakness, an unbalanced control of eating and weight, disordered sleeping, an inability to concentrate, a feeling of immense guilt, and a reduced sexual interest (Purves et al, 2000). Abraham Lincoln, who suffered from this ailment, once wrote “I am now the most miserable man living. If what I feel were equally distributed to the whole human family, there would not be one cheerful face on earth. Whether I shall ever be better, I cannot tell; I awfully forebode I shall not. To remain as is impossible. I must die or be better, it appears to me.”
Other eminent figures like Winston Churchill, Kurt Cobain, Edgar Allan Poe, John Keats, Robert Schumann, and Virginia Woolf appeared to have been afflicted by this disorder. Thus, depression is found to affect people of all ages, genders, and backgrounds.

It was once thought that depression arose due to the person’s inability to cope with circumstances. It is now accepted that depression is a neurobiological disorder. Evidence for this comes from studies on twins that show the incidence of the disorder is higher amongst identical or monozygotic twins than fraternal or dizygotic twins. The area(s) of the brain involved in emotional processes are the anterior cingulated cortex, amygdala, hippocampus, ventral striatum, hypothalamus, and several other interconnected structures. The amygdala is the key area where cognition, emotion and learning are processed. Environment clues about potential threats are processed here and the appropriate behavioral and physiological response is communicated to the rest of the brain. Problems with the brain circuitry involving the amygdala have been associated with mood disorders like depression (Lesch, 2007, http://www.upmc.com/Communications/mediaRelations/Research/Articles/SerotoninReceptor.htm). In these individuals, the brain activity in these areas displays an atypical pattern of blood flow (Purves et al, 2000, Mann et al, 2006). There is no single underlying cause of depression but a combination of factors that include genetic, biochemical, environmental, and psychological factors may play a part. The biochemical mechanism is believed to be due to a deficiency of neurotransmitters that include serotonin, dopamine, or/and noradrenalin in the CNS (http://www.brainexplorer.org/brain_disorders/Focus_Depressions.html). Focus will be on the role of serotonin on the etiology of depression.
1.2.2 Monoamine Hypothesis of Mood Disorder

The observation that lysergic acid diethylamide (LSD), a compound similar in structure to serotonin, caused hallucination led researchers to speculate that substances similar to serotonin could cause mental abnormality. Coincidentally, it was also noted that the administration of iproniazid, a drug used to treat tuberculosis, elevated mood. Many years later it was found that the drug was a MAO inhibitor and might therefore be useful in the treatment of depression. Similarly, in the early 1950s, reserpine, a drug used to treat hypertension, was shown to frequently cause depression in patients. Afterward it was discovered that this drug depleted the level of monoamines (e.g. serotonin) in the brain (Green, 2006, Purves, 2000). In the 50s, however, it was unknown whether serotonin was found in the brain. Bioassay and spectrophotofluorometric methods confirmed its presence in enriched areas in the central nervous system (CNS). In the 1960s, Dahlstrom and Fuxe’s groundbreaking histofluorescence studies allowed the visualization of monoamine-containing neurons in the brain. This reinforced the idea that alterations in the 5-HT neurotransmission could be responsible for the pathophysiology of mental illness and could play an important role in the treatment of psychiatric disorders (Owens and Nemeroff, 1994). Henceforward, the idea was put forth that increased levels of monoamine, for instance 5-HT, caused euphoria and decreased levels caused depression (Green, 2006).

The probable role of serotonin in the pathophysiology of mental illness came from three main factors. The first is the availability of substantial database that supported the view that in depressed patients, the 5-HT containing neurons were altered. For instance in
seminal studies it was shown that depressed patient had significantly lower levels of CSF 5-HIAA concentration. These patients were also found to be more suicidal in nature. Studies on postmortem tissue of depressed patients have also shown a reduction in 5-HT concentration in the whole brain, especially in the hypothalamus and amygdala. Similarly, depressed patients who were taking antidepressants but fed a diet lacking tryptophan were shown to rapidly relapse. Second, due to the progress made in isolating and cloning the 5-HT receptors, potent and selective agonist and antagonist were developed. These compounds were an indispensable tool in the analysis and regulation of 5-HT neural system (Owens and Nemeroff, 1994). Thirdly, drugs that have been effective in treating depression targeted the serotonergic neurotransmission, reinforcing the neurochemical basis of the disorder (Purves et al, 2000). Thus the monoamine hypothesis was developed and credence for this theory was further substantiated when it was found that the highly successful antidepressant drug, tricyclics, worked by inhibiting the 5-HT uptake at the nerve endings and therefore, increasing the concentration of serotonin in the synaptic cleft. Clinically it was also found that zimeldine and fluoxetine, drugs used to treat depression, were potent and selective 5-HT uptake blockers (Green, 2006). Thereby evidence for the connection between serotonin and mental diseases came from studies that showed that changes in serotonin levels can alleviate or exacerbate mental illness.

Thus current therapies used to treat, for example, depressed patients targeted the serotonergic pathway. These antidepressant drugs increased cerebral 5-HT concentrations by either targeting the metabolic pathway, 5-HT receptor, or by preventing the re-uptake of the neurotransmitter from the synaptic cleft. This resulted in increased levels of serotonin in the brain. Unfortunately, high levels of 5-HT induced toxicity or serotonin
syndrome. Serotonin syndrome, also known as serotonin toxicity, is an iatrogenic or drug-induced syndrome. Though it was first observed over 50 year ago, an understanding of the disorder is still unclear. Due to the serious nature of the syndrome caused by the fatal combinations of therapeutic drugs that are prescribed or accidentally combined by patients, this topic will be examine further in the next section.

1.3 Serotonin Syndrome

1.3.1 Overview

For the past 40 years, there has been extensive animal research on the effect of excess CNS 5-HT (Isbister et al, 2005). Initially, animals that were administrated tryptophan were shown to have very few changes in their behavior. In fact, the administration of tryptophan hydroxylase inhibitor, p-chlorophenylalanine (PCPA), which can decrease cerebral 5-HT content by 80%, produced no overt change in animal behavior. However, administration of monoamine oxidase (MAO) inhibitor with tryptophan caused behavior altercations and its symptoms became known as serotonin syndrome. The symptoms, delineated by Grahame-Smith (1971) describe distinct behaviors in animals that include resting tremor, rigidity or hypertonicity, reciprocal forepaw treading, hindlimb abduction, straub tail, and lateral head weaving (Green, 2006; Isbister and Buckley, 2005).

Understanding the serotonin behavioral syndrome, as it become known, was further aided by the discovery of 5-HT receptors and consequently, receptor agonist. The animal serotonin behavioral syndrome was used to give an insight into the mechanism of antidepressants. The different behavior symptoms of SS in animals and in humans do not exactly correlate but it is the best model to understand the serotonin receptor subtypes
involved in serotonin toxicity in humans. The post-synaptic 5-HT$_{1A}$ receptors were found to be involved in reciprocal forepaw treading, hindlimb abduction, and later head weaving. The 5-HT$_{2A}$ receptor was reported to potentiate the syndrome but not induce it. Stimulation of this receptor was shown to cause head twitching, back muscle contractions, and hyperthermia. The advent and use of receptor agonist provided further evidence and support for the fact that the majority of the behavioral syndrome appeared to be mediated by the post-synaptic 5-HT$_{1A}$ receptor. In fact, the syndrome was sometimes referred to as 8-OH-DPAT (8-hydroxy-(di-n-propylamino) tetralin; 5-HT$_{1A}$ selective agonist) behavioral syndrome. More recently, besides head twitching in rodents, 5-HT$_{2A}$ receptor has been found to be associated with back muscle contractions and ear scratch response in rats (Isbister and Buckley, 2005). Some reports also show that no single receptor appears to be responsible for the development of the syndrome, although several lines of support now suggest that the activation of the 5-HT$_{2A}$ receptor may contribute to the condition (Boyer and Shannon, 2005). It should also be noted that it is not possible to isolate this receptor activity completely and in fact, reports have shown that they can actually potentiate the activity of the other. For instances, studies have shown that the 5-HT$_{2A}$ receptor modulates 5-HT$_{1A}$-mediated serotonin behavior for example in reciprocal forepaw threading, while 5-HT$_{1A}$ receptor has been found to have an inhibitory role on 5-HT$_{2A}$-mediated head twitch (Isbister and Buckley, 2005).

The first case of SS in humans was reported in the early 1980s. This toxicity was related to the serotonin behavioral syndrome found in animal literature. Though many cases similar to this syndrome were recognized earlier, it was not reported as serotonin
syndrome. This toxicity in humans was delineated by Sternbach in 1991 when he reviewed 38 human cases from literature. These symptoms, which are often ascribed as a triad, include mental status changes, agitation, myoclonus, hyperreflexia, diaphoresis, shivering, tremor, diarrhea, incoordination, and fever (Green, 2006; Isbister and Buckley, 2005). Not all these symptoms are found in patients and the symptoms range from mild (tremor, diarrhea) to severe (delirium, neuromuscular rigidity, hyperthermia). The increase in extracellular 5-HT observed in more severe SS is a 40-fold increase over baseline, though lethal effects of toxicity involving drug interactions can lead to levels up to 1100-fold over basal concentration (Isbister and Buckley, 2005). Clinicians sometimes overlook the mild symptoms and inadvertently increase the serotonergic dosage, provoking a dramatic deterioration. The incidence of serotonin syndrome has become a problem in the last few years due to the increasing number of antidepressants prescribed (Boyer and Shannon, 2005). That coupled with the fact that the disorder can be missed or not diagnosed in time has made this a serious issue. An understanding of how serotonergic agents contribute to this toxicity is imperative and insight on how these drugs or drug interactions can lead to serotonin syndrome will next be examined.

### 1.3.2 Molecular Mechanism

Certain drugs can cause an increase of serotonin in the synaptic cleft by affecting 5-HT’s release, reuptake, or breakdown. Antidepressant drugs like monoamine oxidase inhibitors (MAOI) prevent the breakdown of serotonin and drugs like selective reuptake inhibitor (SSRI) and tricyclic antidepressants prevent the re-uptake of serotonin from the synaptic cleft. Serotonin synthesis can also be increased by increasing tryptophan concentration in
the brain. Serotonin syndrome in depressed patients occur when there is an excess of serotonin in the synaptic cleft due to a combination of SSRI, MAO inhibitors, and tricyclic antidepressants or a single overdose of one of these drugs (Bartlett, 2006, Birmes et al, 2003). Thus serotonin syndrome is drug-induced disorder and it is found to occur in patients who, as a medication for mental (Boyer and Shannon, 2005; Nelson et al., 2007; Terao and Hikichi, 2007) or physical illness (Mason and Blackburn, 1997), take one or more multiple serotonergic drugs. It is important to understanding and more importantly, recognize the symptoms because SS can become a life-threatening disorder (Boyer and Shannon, 2005) (see figure 3).

Currently the only treatment for serotonin syndrome (SS) is removal of the causative agent and treating the life threatening symptom of SS, elevated core body temperature. The question remains as how elevated levels of cerebral 5-HT can cause hyperthermia. To answer this question, an understanding on how serotonin is involved in autonomic dysfunction will be discussed. Since thermoregulatory impairment is an indicator of autonomic dysfunction, thermoregulation and the role serotonin plays in regulating core body temperature will be looked at first. Then the receptors involved and the affect changes in ambient temperature have on SS will be analyzed.

1.4 The Effect of Elevated Cerebral Serotonin on Autonomic Dysfunction

1.4.1 Homeostasis and Normal Thermoregulation

Experiments done by researchers have shown that humans and animals immersed in cold or hot water and then removed into a neutral environment had their core body
temperature return to the original state or homeostasis. Homeostasis refers to the process of keeping the internal body environment in a steady state when the external environment is changed. This is an important process as enzymes, etc work at an optimal temperature and any internal change can severely impact its efficiency and function. The mechanism of homeostatic control involves a negative feedback mechanism. That is, when there is a change in the system, corrective measures are made to bring the system back to its original normal or set point. This set point is often in a state of constant flux, and homeostatic system that is working efficiently will try to minimize this oscillation (Cooper, 2002).

The thermoregulatory function in many species is regulated by certain areas in the central nervous system, including several brain stem neuronal groups that include the medulla oblongata, pons, midbrain, and the spinal cord, but the area considered to be the most important in initiating autonomic thermal regulating response is the hypothalamus or more specifically, the preoptic area which is located in the anterior hypothalamus, henceforth referred to as the preoptic/anterior hypothalamus (PO/AH). It integrates information from all the regions in the body and sends appropriate heat production or heat conservation signals (Cooper, 2002). The PO/AH is influenced by dopamine, norepinephrine, serotonin, and alpha adrenergic receptors. Drugs that can alter the hypothalamic levels of these neurotransmitters can thereby affect thermoregulation. Besides these neurotransmitters, activation of hypothalamic-pituitary-thyroid and the hypothalamic-pituitary-adrenal (HPA) axis helps maintain body temperature (Rusyniak and Sprague, 2006). In depressed patients, there is a loss of feedback inhibition in the
HPA axis, suggesting that this might also play a key role in the etiology of the disease (McAllister-Williams et al, 2001). For the purpose of this introduction, focus will be made on the involvement of neurotransmitters, particularly serotonin’s, in thermoregulation.

Species have evolved to survive in a wide spectrum of ambient temperatures. In any environment, species have managed to maintain their core body temperature, \( T_{\text{cor}} \), within a certain set point. Temperature changes that deviate from such a set point is often indicative of a pathological condition and monitoring temperature is routine practice in all medical facilities (Romanovsky, 2007). High body temperature, or hyperthermia, is the most severe and life-threatening symptom of serotonin syndrome in humans. Thus the role serotonin plays in normal \( T_{\text{cor}} \) regulation will be elucidated further.

### 1.4.2 Role of Serotonin in Thermoregulation

Serotonin role in thermoregulation has been noted in various studies. For instance, animals given a 5-HT synthesis inhibitor were unable to regulate their body temperature against heat and cold stressor. Likewise, destroying serotonergic nerve terminal in the brain impaired normal thermoregulation. These results suggested that that serotonin was important in regulating core body temperature. To further elucidate the role of serotonin, studies have shown that when 5-HT was microinjected into PO/AH, the core body temperature rose. Furthermore, perfusing tetradotoxin (TX) (sodium channel blocker) into the median raphe nucleus (MRN) caused a decrease in \( T_{\text{cor}} \) under normal and ambient conditions but had no affect under hot ambient temperature, suggesting that
serotonin may be involved in heat production (Ishiwata et al, 2004). Besides serotonin, other neurotransmitters like dopamine, GABA, glutamate and acetylcholine have been shown to be involved in thermoregulation (Bligh, 1973). A model was henceforth proposed in which warm receptors, receiving input from serotonergic synapses, evoked heat loss mechanisms and cold receptors, receiving input from cholinergic synapse, evoked heat production and conservation mechanism. Besides these, cross-over neuron, possibly adrenergic neurons, were arranged between the cold and warm receptors pathways, inhibiting or stimulating heat production or heat loss. In addition, depending on the species, other neurotransmitters could also play a part in modulating or controlling core body temperature. Alternatively, two other molecules involved in thermoregulation and fever production are nitric oxide and prostaglandin E (Cooper, 2002). Focus will be made only on the role of serotonin in thermoregulation.

Although serotonin is implicated in body temperature regulation, there has been some conflicting reports on the extant in which serotonin is involved. For instance, intracranial administration of 5-HT produced an increase in colon temperature (Myers, 1980) and a decrease (Cox et al., 1980, Crawshaw, 1972, Lin et al, 1989). As well, Sheard and Aghajanian (1967) showed that electrical stimulation of the rat midbrain raphe nuclei or intrahypothalamic administration of 5-HT produced a rise in colon temperature but Lin et al (1983) showed the temperature decreased. Similarly, depletion of 5-HT in rat brain by neurotoxins displayed no change in colon temperature when exposed to heat stress. The reason for such discrepancies could be due to experimental technique, location of the probe (PO/AH is a very small area), dosages of 5-HT employed, or even due to the
differential activation of the 5-HT receptors (Li et al, 1998). A further look at to the role of these receptors in regulating body temperature and how their unequal activation could lead to hyperthermia will be analyzed.

1.4.3 Hyperthermia and Serotonin Receptors

The thermoregulatory set point can be raised due to a number of reasons, for instance, by drugs. The mechanism of drug-induced fever can occur through a variety of mechanisms that include increasing the metabolic rate, causing vasoconstriction, or limiting heat dissipation. For instance, antidepressant drugs tricyclics increases $T_{cor}$ by impairing the body’s ability to sweat. Similarly another antidepressant drug, monoamine oxidase inhibitors (MOAI), elevates body temperature by increasing local metabolism. Furthermore, drugs that raise the synaptic concentration of neurotransmitters, like serotonin, can cause life-threatening hyperthermia (Cuddy, 2004).

The affect of administrating 5-HT, 5-HT agonist, or 5-HT releasing agents can have differential effects on the animal’s core body temperature. Depending on the type of agonist used or the route of administration, either hypothermia or hyperthermia developed. For example, 5-HT releasing agents like fenfluramine increased cerebral 5-HT concentration and hyperthermia or hypothermia developed, depending on the ambient temperature (Malberg and Seiden, 1997). Thus 5-HT cerebral levels appeared not be the determining factor. Though some studies speculated the biphasic effect of temperature was due to the dose or the cerebral concentration of 5-HT, other studies suggest that this could be the result of differential stimulation 5-HT receptors. Support for the later theory
came from studies that showed two key findings. The first was that activation of 5-HT$_{1A}$ or 5-HT$_{2A}$ had different effects on core body temperature. It was shown that administrating 5-HT$_{1A}$ agonist, 8-OH DPAT, caused a *decrease* in body temperature that was prevented by 5-HT$_{1A}$ antagonist. Similarly, the activation of 5-HT$_{2A}$ receptors by the agonist DOI (2, 5-dimethoxy-4-iodophenyl)-2-aminopropane led to a dose-dependent *increase* in body temperature. The second support came from studies that showed the affinity the two receptors had for the 5-HT ligand differed. At low cerebral concentration, the high-affinity 5-HT$_{1A}$ receptors were stimulated and *hypothermia* occurred, while at high 5-HT levels, the low affinity 5-HT$_{2A}$ receptors were activated and *hyperthermia* developed (Isbister and Buckley, 2005). Thus the biphasic effect of temperature to increasing 5-HT levels in the brain was attributed to the differential activation of 5-HT receptors.

Serotonin is implicated in many functions in the central nervous system that include neuronal development, pain, sleep, appetite, sexual behavior, anxiety, mood, and *thermoregulation* (Moret and Briley, 2000, Aghajanian and Sanders-Bush, 2002). Its role in the pathophysiology of mental disorders has led to the development of drugs. Unfortunately, the use of these drugs increases cerebral serotonin levels and in some cases, toxicity or serotonin syndrome occurs. Studies have shown that changes in ambient temperature can also affect the severity of the syndrome. Warmer ambient temperature has been found to aggravate the symptoms while cooler ambient temperatures have been shown to alleviate the symptoms (Green et al, 2004). The possible reason and mechanism will be discussed in the next section.
1.5 Effect of Ambient Temperature on Serotonin Syndrome

Serotonin syndrome can occur when there is an acute or chronic administration of antidepressants. With the increase prescription of antidepressants the incident of serotonin syndrome has risen. Although most cases are mild, severe SS can lead to fatality. About one third of the deaths are attributed to abnormally high fevers or hyperpyrexia. The mechanism of SS can be complex and studies have pointed out the interaction between the central catecholamine release, hypothalamic-pituitary-thyroid-adrenal axis, sympathetic nervous system, skeletal muscle, and environment (Rusyniak and Sprague, 2006). Environmental factors like ambient temperature can play a large part in exacerbating or alleviating the disorder, in particular, impairing the body’s ability to regulate core body temperature (Green et al, 2004). This can be explained by the fact that the toxicity of many environmental toxicants and drugs is directly proportional to ambient temperature. In other words, the toxicity of a drug increases as the $T_{amb}$ rises (Gordon, 2008). For this reason it is not surprising that there have been many documented reports on the effect of ambient temperature ($T_{amb}$) on SS. In animal studies it has been shown that as $T_{amb}$ increases, the toxicity and elevated body temperature associated with drugs that cause SS rises. Conversely reducing the $T_{amb}$ has been demonstrated to decrease these factors (Rusyniak and Sprague, 2006). A further examination of this phenomenon will therefore be examined next.

1.5.1 Effect of Ambient Temperature on Serotonergic-Based Drugs

The effect of ambient temperature on serotonin syndrome (SS) came from many studies, in particular, from reports on the usage of illicit drugs. Besides serotonergic-based
antidepressant, drugs of abuse like “ecstasy” or MDMA (3, 4-methylendioxymethamphetamine) has also been shown to cause serotonin syndrome. This has been supported by studies in which the symptoms of 5-HT syndrome have been found to occur when MDMA was administrated. Studies on MDMA suggest that it enters serotonergic neuron via 5-HT transporter and releases 5-HT into the synapse (Malberg and Seiden, 1998). Other illicit drugs like cocaine and methamphetamine have also been linked to serotonin syndrome (Marzuk et al., 1998). All these drugs have been shown to have a greater adverse effect at higher ambient temperatures (Parrott, 2002, Marzuk et al., 1998), whereas lowering the ambient temperature appears to alleviate the symptoms. For example, the administration of the recreational drug MDMA to rats caused an increase in brain 5-HT levels and induced hyperthermia, whereas lowering the ambient temperature decreased these factors. Similarly, MDMA-treated laboratory rats were found to experience hypothermia when they were exposed to cold ambient temperature but experience hyperthermia at higher ambient conditions. Even an increase of 2 °C led to a substantial rise in core body temperature (Parrott, 2002). Given that MDMA is often taken in “rave parties” which are often crowded and warm and the participant are often engaging in prolonged physical activity, it is not surprising that the hyperthermia and in some cases death occurs (Freedman et al, 2005). Furthermore the incident of cocaine-related deaths was shown to increase during the months when the ambient temperature was higher (Marzuk et al., 1998).
1.5.2 Hypothermia Treatment

One critical aspect of serotonin syndrome is the speed and progression of the disorder from mild (tremor, diarrhea) to severe (delirium, neuromuscular rigidity, hyperthermia) symptoms. In the mild cases, cool environment and cessation of the medication or drug should suffice. However in more severe cases, medical intervention is required and the use of 5-HT₂/5-HT₁a antagonist like cyproheptadine or chlorpromazine is necessary to expedite recovery and more important, prevent death (Parrott, 2002). Besides the use of drugs, rapidly cooling the body is essential. Temperatures as high as 43 °C have been reported in hospital emergency rooms and the use of drugs such as dantrolene and the administration of cold intravenous fluids is often required (Dafter and Lynch, 1998).

Although the mechanism of hypothermia protection is not clear, it is known that lowering the body temperature protects tissues from free radicals, lipid peroxidation, chemical metabolites, and tissue edema. Furthermore, lowering the ambient temperature has been shown to alleviate drug-induced toxicity and environmental toxicants and protect against hemorrhaging and hypoglycemia (Gordon, 2001). Therefore inducing hypothermia can prevent cellular, tissue, and even organ damage. Unfortunately, there are also some concerns regarding hypothermia treatment. This issue is discussed next.

The use of hypothermia as a treatment raises some concerns. While the benefit of preventing cellular damage and other deleterious damages are clear, it must be outweighed by the psychological stress and physiological responses like include an altered immune response, lipid hydrolysis, increased heart rate (tachycardia), etc. Furthermore, every 2 °C drop in core body temperature increases the recovery time after
anesthesia and causes haemodynamic instability and depression in cognition function. To achieve hypothermia, clinicians use a procedure known as “forced” hypothermia. This entails the patient being immersed in cold water, washed with cold fluids, or exposed to a cold mattress. The problem with this procedure is that patients can suffer from “rewarming shock” or “post-rescue collapse.” Thus the individual’s condition may degenerate from one stage of hypothermia to another (Giesbrecht and Bristow, 1997). On the other hand, if hypothermia was regulated then these harmful effects can be minimized and hopefully eliminated. The use of antipyretic agents (acetaminophen, aspirin and ibuprofen) has been shown to be effective in lowering the core body temperature (Gordon, 2001, Clark, 1987), but unfortunately, it has not always been successful in lowering $T_{cor}$ in time. The challenge lies in determining what agents could induce regulated hypothermia (Gordon, 2001). In light of the fact that the toxic increase of cerebral serotonin induces hyperthermia, development of drugs to prevent not only SS but also effectively treat high $T_{cor}$ is imperative. Therefore it is necessary to circumvent the toxic level of 5-HT and thereby prevent hyperthermia. To do so, an understanding of how the neural circuitry is involved in increasing 5-HT is essential.

1.5.3 Effect of Ambient Temperature on Receptor Sensitivity

Though studies have shown that impairment in thermoregulation can be due to a modification in 5-HT levels, experiments have also shown that changes in receptor sensitivity may play a role. For example, 5-HT$_{1A}$ antagonist, WAY100635, has been demonstrated to augment MDMA induced hyperthermia in warm environment (Saadat, 2001). Administrating 5-HT$_{1A}$ agonist, 8-OH-DPAT, to rodents caused a decrease in core
body temperature. Nicholas and Seiden (2003) showed that this hypothermic effect was dependent on ambient temperature. That is, at lower ambient temperature, the thermic response to the agonist was greater but as the temperature was increased, the effect was diminished. Conversely, animals pre-treated with 5-HT$_{2A}$ antagonist, ketanserin, were found to protect against heatstroke-induced hyperthermia. Therefore drugs that activate 5-HT$_{1A}$ receptors or antagonize 5-HT$_{2A}$ receptors protect against hyperthermia while drugs that target 5-HT$_{2A}$ receptor or antagonize 5-HT$_{1A}$ receptor exacerbate hyperthermia at higher ambient temperature (Chang et al, 2005). This is especially critical for individuals talking antidepressant drugs or other drugs that target the serotonergic pathway as their thermoregulatory response could be impaired and the treatment could prove to be fatal at higher ambient temperatures (Nicholas and Seiden, 2003). However, the mechanism by which this occurs is unknown and therefore the topic and main research in this thesis.

1.6 Statement of Problem and Hypothesis

Although elevating serotonin (5-HT) in the CNS is effective in treating depression, excess 5-HT may induce a toxicity syndrome that can be mild or severe, even leading to death. The incidence of 5-HT syndrome has recently been found to have risen due to the increased prescription of antidepressant drugs. Under a laboratory condition, the syndrome could be either alleviated by cold ambient temperature or exacerbated when the animals were exposed to hot ambient environment. However little is known about the neural basis for the marked effect of ambient temperature on the severity of the syndrome. It is hypothesized that ambient temperature-dependent activity of 5-HT$_{2A}$
receptors is responsible for the variation in a severity of the syndrome. Insight into the interaction between ambient temperature and the syndrome would provide a neural basis for cold exposure as an alternative non-pharmacological treatment for patients with the syndrome induced by antidepressants.

The main aim of this thesis is to explore the effect of ambient temperature on the severity of serotonin syndrome. The current thesis will examine the hypothesis that the $5-HT_{2A}$ receptors are functionally desensitized by lowering the ambient temperature and hypersensitized by increased the ambient temperature. Evidence regarding the role of ambient temperature is divided into two parts. Section 3.0 (part 1) explores the effect of lowering the ambient temperature on serotonin syndrome and section 4.0 (part 2) focuses on its effect at higher ambient temperature.

1.7 Aim and Objectives

To explore the hypothesis, the effect changes in ambient temperature have on body-core temperature and on extracellular cerebral 5-HT concentration will be determined. Details of each aim and their objectives are given below.

1.7.1. Aim 1: Changes in body-core temperature

Previous work in our lab has characterized the toxic response in pre-clinical models to excess 5-HT by measuring a number of factors that include but are not limited to head shakes, forepaw treading, tremors, and core body temperature. Changes in core body temperature have proven to be accurate in delineating the severity of the serotonin
syndrome (Ma et al, 2008). Hyperthermia is associated with severe toxicity while hypothermia is associated with mild to benign toxicity. The specific goal of Aim I is to understand the effect of core body temperature in serotonin syndrome when animals are housed at different ambient temperatures. Serotonin syndrome is produced by administrating MAO inhibitor (MAOI) clorgyline to the animals 2 hours before 5-HTP is administrated. Supported by preliminary data, it is predicted that changes in ambient temperature will affect the severity of serotonin syndrome, showing that the syndrome becomes mild at low ambient temperature (e.g., 12 °C vs. 22 °C) and severe when being exposed to high ambient temperature (e.g., 32 °C vs. 22 °C). These effects are further examined by selective 5-HT\textsubscript{2A} receptor antagonist ketanserin.

1.7.2 Aim 2: Assessment of the relationship between 5-HT efflux and the severity of a syndrome

Since the syndrome is induced by excess 5-HT in the CNS, it is important to determine the level of efflux relevant to the syndrome in the context of ambient temperature. Previous studies suggest that MAOIs and SSRIs usually produce a 3 – 5 fold increase in 5-HT efflux, the level of which is found to alleviate depression in the CNS. In the current study, the hypothesis that the syndrome is evoked by a 10-fold greater efflux than in the pre-drug level will be tested. Furthermore, the premise that excessive increases in 5-HT efflux is tightly regulated by the central neural circuitry between the dorsal raphe nuclei (DRN) and the prefrontal cortex (PFC) and also the preoptic anterior hypothalamus (PO/AH) will be analyzed. Specifically, using 5-HT microdialysis in the PFC and PO/AH, it is predicted that in MAOI clorgyline-treated animals: a) 5-HTP produces a
dose-dependent increase in 5-HT efflux; b) The increase is also ambient temperature-dependent, the effect of which is dependent on activity of 5-HT$_{2A}$ receptors examined by ketanserin. Thus, excess 5-HT comes from two distinct mechanisms. The first involves 5-HT synthesis and breakdown that cause a threshold release of 5-HT and the second involves a positive feedback mechanism involving neural circuitry between serotonergic neurons in the DRN and glutamatergic neurons in the PFC. The stimulation of the second pathway is predicted to cause toxic levels of CNS 5-HT, leading to fatality. Therefore it is hypothesized that lowering the ambient temperature de-sensitizes 5-HT$_{2A}$ receptor and therefore, the positive feedback circuitry is not stimulated. Conversely, increasing the ambient temperature will result in hyper-sensitization of 5-HT$_{2A}$ receptors and thus will increase 5-HT levels in the brain to toxic levels via the positive feedback pathway.
Figure 1 Synthesis of serotonin
Figure 1 Synthesis of serotonin (figure adapted from Hensler, 2006)

Dietary absorption of the amino acid tryptophan is converted into 5-hydroxytryptophan (5-HTP) by the rate limiting enzyme tryptophan hydroxylase (TRH). This enzyme can become saturated with its substrate, tryptophan, under physiological conditions. The product, 5-HTP, is then converted into 5-hydroxytryptamine (5-HT) by the enzyme aromatic decarboxylase (ADDC). AADC is found not only in serotonergic neurons but also in catecholaminergic neurons where it can convert 3, 4-dihydroxyphenylalanine (DOPA) to dopamine. The enzyme, depending on the pH, substrate concentration, or cofactor requirement, will use either 5-HTP or DOPA as its substrate (Hensler, 2006). 5-HT or serotonin, produced by AADC, is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) by the enzyme monoamine oxidase (MAO) (Hensler, 2006). There are two forms of the enzyme, MAO\textsubscript{A} and MAO\textsubscript{B}. The low affinity form, MAO\textsubscript{B}, is expressed by the serotonergic neurons rather than MAO\textsubscript{A}. It is postulated that serotonergic neurons maintain a pool of cytoplasmic serotonin and the other trace amines are broken down by MAO\textsubscript{B}. The MAO\textsubscript{A} enzyme, in turn, functions to oxidize the extracellular serotonin (Nestler et al, 2001).
Figure 2 Serotonin transduction
Figure 2 Serotonin transduction (figure adapted from Boyer et al, 2003)

Serotonin signaling involves the transmission of chemical message through a neuronal pathway. The enzymes involved in the synthesis of serotonin are produced in the cell body and through a slow axon transport, moved to the nerve terminal cytoplasm. A carrier-mediated transport system carries the precursor tryptophan from the blood into the brain. Serotonin then enters the serotonergic nerve via the serotonin transporter located on the terminal of the neuron (Purves et al, 2004, Owen and Nemeroff, 1994). It is here in the cytoplasm where the precursor is converted into serotonin and then stored into synaptic vesicles. The advent of an action potential (AP) changes the membrane potential of the pre-synaptic neuron, causing the voltage-gated calcium channels to open. The rapid influx of Ca\(^{2+}\) into the pre-synaptic terminal results in the synaptic vesicles, which contain 5-HT, to fuse with the plasma membrane and release 5-HT into the synaptic cleft. 5-HT diffuses across the cleft and binds to the 5-HT receptors on the post-synaptic neuron. The binding of the receptors results in the ions channels, located on the postsynaptic membrane to either open or close, depending on the receptor that was bound. The influx (or efflux) of ions into the postsynaptic neuron, changes the membrane potential of the post-synaptic membrane, influencing the prospect on whether the post-synaptic neuron will fire an action potential or not (Purves et al, 2004). 5-HT, bound a finite time, is released, re-enters the pre-synaptic neuron via the serotonin reuptake transporter (SERT) and is either re-packaged into the synaptic vesicles or broken down to hydroxyindoleacetic acid (5-HIAA) by the enzyme monoamine oxidase (MAO) (Purves et al, 2000, Owen and Nemeroff, 1994, Neuroscience 3rd edition, Bartlett, 2006).
Figure 3 Mechanisms of serotonin syndrome
Figure 3 Mechanisms of serotonin syndrome (figure adapted from Birmes et al., 2003)

Serotonin syndrome is an iatrogenic disorder, mainly occurring in patients who take one or multiple serotonergic drugs as a medication for mental depression (Boyer and Shannon, 2005; Nelson et al., 2007; Terao and Hikichi, 2007) or physical illness (Mason and Blackburn, 1997). Understanding these symptoms is important for recognizing and treating the disorder, which could be mild or life-threatening (Boyer and Shannon, 2005).

The cerebral increase of serotonin (5-HT) is caused by certain drugs the affect the 5-HT synthetic, re-uptake, or breakdown pathway. For instance, increasing the intake of tryptophan raises pre-synaptic 5-HT levels. Similarly, the drug MAOI (monoamine oxidase inhibitors) prevents MAO (monoamine oxidase) form breaking down 5-HT into 5-HIAA (hydroxyindoleacetic acid), thereby also increasing pre-synaptic 5-HT concentration. Furthermore, the antidepressant drugs SSRI (serotonin reuptake inhibitor) and tricyclic antidepressants work by blocking the re-uptake of serotonin from the synaptic cleft, hence augmenting synaptic 5-HT concentration. Thus, patients taking a combination of SSRIs, MAOIs, and tricyclic antidepressants or a single high dose of these drugs can result in toxic levels of 5-HT in their brain and thereby develop serotonin syndrome (Bartlett, 2006, Birmes et al, 2003). Currently the only treatment for serotonin syndrome (SS) is removal of the causative agent and treating the life threatening symptom of SS, elevated core body temperature.
2.0 MATERIAL AND METHOD

2.1 Animals

Sprague Dawley rats are outbred albino rats that are used broadly in medical research. The advantage of using the rat is that it is able to respond to changes in ambient temperature and this can be measured and observed easily. Raised temperature causes the animal to increase vascularization of its tail, which serves as its thermoregulatory organ, while a decrease in ambient temperature causes nonshivering thermogenesis (http://en.wikipedia.org/wiki/%25s, http://aceanimals.com/SpragueDawley.htm). Rats are also considered a better animal model to study human diseases like memory loss, cancer, heart disease, substance abuse, and toxicity (http://www.hsus.org/animals_in_research/species_used_in_research/rat.html).

2.2 Drugs

Animals were subcutaneously injected with 5-HTP and clorgyline to create the serotonin syndrome (SS) model. The model we used was acceptable for two main reasons. The first is that this systemic drug combination has been shown to increase cerebral 5-HT level (Nisijima et al, 2000) throughout the brain, not just in the raphe nucleus. This is validated by reports that show SS patients have high levels of serotonin all through their brain (Boyer and Shannon, 2005), not just in a certain regions. Secondly, the SS model used in our lab had elevated levels of dopamine and glutamate (Zhang and Tao, unpublished).
observation), similarly to what was found by other researchers studying SS (Nisijima et al, 2001, 2003; Shoda et al 2004). This is substantiated by clinical reports that reveal that the cerebrospinal fluids of serotonin syndrome patients have, besides serotonin, elevated levels of norepinephrine and dopamine (Rusyniak and Sprague, 2006), thus making the model we employed feasible.

2.3 Experimental Procedure

The animal model of 5-HT (serotonin) syndrome was created by administrating clorgyline (MAOI inhibitor) and 5-hydroxy-L-tryptophan (5-HTP) (a precursor to 5-HT) to the rats. The administration of MAOI and 5-HTP has been found to elevate 5-HT levels in the brain (Nisijima et al, 2001). Rats were injected with clorgyline subcutaneously two hours before the start of the experiment. Animals were randomly assigned to the test or control group. To habituate the rats to the environment, each animal was kept in an individual clean polycarbonate box for 30 min before the start of the experiment. Then the animals either remained at ambient temperature 22 ºC or were placed in a temperature controlled chamber that was set at ambient 6 ºC, 12 ºC, 32 ºC or 37 ºC. When placed in this environment, the animals were allowed to habituate for an additional 2 hours before the start of the experiment. For the dose dependent experiments, the animals were injected with 0-25 mg/kg of 5-HTP (n = 4-11). Ketanserin, a specific 5-HT2A receptor was administrated 15 minutes before 10 or 25 mg/kg 5-HTP was injected (n = 4-6). For the core body temperature (T_cor) experiment, the animal’s T_cor was recorded every 15 minutes for 2 hours and for the microdialysis experiment, the animal’s core
body temperature was recorded at the start and at the end for the experiment immediately proceeding saline, 5-HT, and/or antagonist injection.

The overview is diagrammed below. The asterisks (*) and the bold lines/font highlight the sections which will be analyzed in more detail in the proceeding sections. These sections include the surgical procedure, how the animals were set-up, details of the temperature controlled chamber, and how high liquid chromatography (HPLC) works.
2.3.1 Surgery

The preoptic anterior hypothalamus (PO/AH) and the prefrontal cortex (PFC) locations were chosen to measure the effect of ambient temperature on 5-HT$_{2A}$ receptor sensitivity. These regions were chosen for the following reasons. The PO/AH is the part of the brain that is said to be responsible for controlling body core temperature. It integrates information from all the regions in the body and sends appropriate heat production or heat conservation signals (Cooper, 2002). One of the symptoms of serotonin syndrome is impairment in thermoregulation and since the PO/AH is the thermoregulatory region in the brain, this area was analyzed. Studying the PFC was important for a few reasons. The first is that the PFC is involved in cognitive and emotional processes. People with brain injuries or lesions to the PFC were found to suffer from depression (Davidson, 2002, Kobayashi et al, 2008), thus postulating that PFC dysfunction can lead to certain mood disorders (Drevets et al, 2008). Secondly, experiments studying the efficacy of antidepressants showed that drugs like fluoxetine work by preventing the re-uptake of neurotransmitters like dopamine and serotonin from the prefrontal cortex (Petty et al, 1996) and drugs like paroxetine increase the density of 5-HT$_{2A}$ receptors on the PFC (Zanardi et al, 2001). This suggests not only the significance of PFC in the pathophysiology of depression, but also the importance of 5-HT$_{2A}$ receptors. The dorsal raphe (DRN), even though it is a major serotonergic nucleus and its neurons project to the PFC (Amargos-Bosch et al, 2006), was not studied. Our research focused on 5-HT$_{2A}$ receptors and DRN, unlike PO/AH and PFC, do not contain 5-HT receptors (Gonzalez et al, 2007, Amargos-Bosch et al, 2006, Stein et al, 2007). Hence for this current study, the efflux at DRN was not analyzed.
The method of surgery is diagramed below. The anesthetic drugs, xylazine and ketamine, were given by an intraperitoneal injection (IP). IP injections are a common type of injection typically given to rodents when introducing anesthetics (Nagy et al, 2006).

AP = anterior posterior; ML = medial lateral; DV = dorsal ventral

2.3.2 Animal Set-up

In vivo microdialysis allows the measurement of the extracellular neurotransmitter concentration in free-moving animals. Its combination with behavioral measurement is a powerful tool to study the action of drugs on 5-HT system in the brain (Green, 2006).

Isoflurane, USP (IsoFlo®, Abbott animal health) was administered to the animals to
anesthetize them. The animals, when the probes were inserted into the desired brain regions, were housed in a Raturn© chamber whose ambient temperature could be controlled. The Raturn© chamber allows free-moving animals to be housed comfortably and water and food can be provided ad libitum. As the animal moves about the chamber, a sensor signal detects the movement and rotates the chamber bowl in the opposite direction, thus preventing connecting tubes and wires from becoming tangled and interrupting the flow of liquids (e.g. cerebral spinal fluid (CSF)). Furthermore, more than one lead can be attached to the animal brain without being concerned that the wiring may become twisted (http://findarticles.com/p/articles/mi_m0EIN/is_1999_Jan_14/ai_53565884). How the animals were set-up the day before the experiment is shown below.

Sprague Dawley Rats (250-350g)

Probes Checked for Leakage

Anesthetized (Isoflurane)

Probes:

- 26-gauge stainless steel tubing
- Length of exchange for PO/AH = 2.5 mm/ for PFC = 10 mm
- To adjust length, 21-gauge stainless steel collar was put on probe
2.3.3 Chamber design

The three sided chamber was made of polyethylene and the dimension of the chamber were 100 cm by 60 cm by 80 cm. To keep the chamber airtight, insulation was placed between the chamber and the wall and the fourth side of the chamber was covered by a “door” to prevent hot or cold air from escaping. The “door” was also made of polyethylene and its dimensions were 100 cm by 80 cm. A 15 cm diameter hole(s) were cut out to allow samples to be taken (see figure 4). A temperature controller (Fisher Scientific) was used to control the temperature and both an air conditioner and a heater were employed to maintain the temperature at the desired temperature. A fan was also utilized to circulate the air and a thermometer was placed inside the chamber to monitor the temperature. A thermometer was placed inside the chamber to ensure that the temperature controller was working and the ambient temperature displayed by the controller was accurate. The $T_{amb}$ was checked periodically throughout the experiment.
2.3.4 HPLC and 5-HT Peak Analysis

In the late 1970s, high liquid performance chromatography (HPLC) was gaining acceptance as a method that was both faster and more sensitive than the previous methods used to analyze 5-HT samples. HPLC is now regarded as the standard approach to analyze monoamines (Green, 2006). The samples were run on a reversed phase high performance liquid chromatograph (HPLC). Reversed phase implies that the mobile phase is polar and the stationary phase in non-polar. This means that the non-polar compounds will be more retained or have longer retention time than polar compounds [In normal HPLC the mobile phase is non-polar and the stationary phase in polar (www.ux1.eiu.edu/~cfdgk/nsfccli/instrumentconcepts/hplc.pdf.)]. To analyze the substance, the retention time of a known substance was compared to the retention time of an unknown substance. The known substance was serotonin (5-HT) and 1 mg/ml 5-HT sample was serially diluted to 1 pg and 10 μl was injected into the Eicon HPLC. The retention time of the 5-HT was then determined. Next 5-10μl of the samples, which were collected every 15 minutes and stored temporarily on ice in a glass vial, were placed inside the SIL-10AD VP Shimadzu auto injector and the area of the peak was calculated by using the software program PowerChrom. Figure 5 and figure 6 shows samples of the chromatograph peak when 5-HTP was administrated to the animal. Figure 7 shows a schematic of the instruments involved in the experiment. The flow rate of the pump was 1 μl/min and in 15 min, 15 μl of the sample should be collected. But due to the animal’s head shakes or other extraneous factors, collecting that volume was not always feasible. Since the samples was collected every 15 min, to account for this loss of volume, appropriate calculation were done. For instance, if 10 μl of the sample was injected into
the HPLC, then the sample’s area was multiplied by 1.5 to obtain 15 μl and if 5 μl of the sample was instead evaluated, then the sample’s area was multiplied by 3. To calculate the pg per sample, the sample’s area was then divided by a known substance’s area, serotonin (standard). The formula used to calculate the pg per sample and an example of a sample calculation is shown in figure 8.

2.4 Probe Location

Probe location was determined by infusing the probe region with 2% Fast Green (Fisher Scientific) for 10 min. The animals were then deeply anesthetized with pentobarbital (100 mg/kg, i.p), decapitated, and the brains were removed, frozen, and sliced using a micro cryostat. The probe location was determined by comparing it to the rat brain atlas (Paxinos and Watson, 1982). Cresyl violet (Harleco, EMD) was used to stain the brain slices and the probes located outside the correct area were excluded from the data. This procedure described above was carried out by Zhiyuan Ma. Figure 9 is a representative sample of brain region frozen and cut by freehand. No crystal violet dye was used and instead the area where the probes were located was photographed. The arrow(s) represents the area of interest.

2.5 Data Analysis

To determine the significance of the data, two-way analysis of variance (ANOVA) followed by post-Scheffe test was conducted. The advantage of using two-way ANOVA is that the effect of two factors can be determined at the same time. For instance, it can be used to determine whether there is a significant difference in the 5-HT efflux based on the drug treatment and time point. If one way-ANOVA was employed, the only
parameter that could have been tested was the effect of 5-HT efflux on the drug treatment or the time course. Furthermore, two-way ANOVA not only allows assessment of time and drug treatment, but also whether there is an interaction between these two factors.

If the values using ANOVA were found to be significant different, post hoc Scheffé test was employed (It should also be noted that this test is usually used when the samples sizes are not equal) (http://people.richland.edu/james/lecture/m170/ch13-def.html). For example, if when ANOVA was used it was found that the dose of 25 mg/kg produced an overall significant change in the animal’s core body temperature (T_{cor}), then post hoc Scheffé test would be employed to determine which specific time point (15 min-120 min) produced the significant change.

The unpaired t-test was used when the significance between two variables was tested. For instance, it was used to determine whether there was any statistical difference between the mean basal T_{cor} or the mean basal 5-HT efflux and the ambient temperatures (T_{amb}). No time course was determined and only two factors were looked at, mean T_{cor} or mean efflux and T_{amb}.

In all the tests, the p-value was 0.05. This means that assuming a normal distribution, the significant difference between two factors is rejected or accepted based on a standard that there is no more than 5% difference in sampling error and if the test was repeated, 95% of the time the same difference would occur (http://people.richland.edu/james/lecture/m170/ch13-def.html).
Figure 4  Measurement and design of chamber
Figure 4 Measurement and design of chamber

The three sided chamber was constructed of polyethylene and the dimensions of each of the three sides were 100 cm by 60 cm by 80 cm. The chamber were kept airtight by placing insulation between the chamber and the wall and a “door” was used in lieu of the fourth side to allow easy access to the animal and when the door was “closed”, it prevented hot or cold air from escaping. The dimensions of this “door” was 100 cm by 80 cm. 15 cm diameter holes were cut out to allow the collection of samples from the animal housed inside. To control the ambient temperature, a temperature controller (Fisher Scientific) was employed and both an air conditioner and a heater were used to maintain the temperature at the desired temperature. Furthermore, to ensure that the warm or cold air was properly circulated in the chamber, a fan was utilized and the thermometer was placed inside the chamber to make certain that the temperature controller was working. The ambient temperature of the chamber was checked at various times throughout the experiment.
Figure 5 Peak analysis of prefrontal cortex
Figure 5 Peak analysis of prefrontal cortex

To analyze the substance, the retention time of a known substance, a serotonin standard, was compared to the retention time of an unknown substance. The standard was created by serially diluting 1 mg/ml 5-HT to a 1 pg concentration. All the peak analysis was done from samples collected at the prefrontal cortex (PFC). 10 µl of each sample, which was collected from the PFC, was injected into the HPLC (high liquid chromatography) machine. The peaks were displayed on a chromatograph and the software program PowerChrom was used to analyze the peaks. Figure 5 shows a sample of the chromatograph peak when 5-HTP was administrated to the animal. The arrows point to the peak. Figure 5A is an example of an animal’s 5-HT peak before any drug was administrated (baseline 5-HT level). Figure 5B is an illustration of the peak when the 5-HT standard was used. Figure 5C is the 5-HT peak when a high dose (25 mg/kg, s.c) of 5-HT was injected into the rat and figure 5D is the 5-HT peak when a lower dose (5 mg/kg 5-HTP, s.c) was given to the animal.
Figure 6 Peak analysis of preoptic/anterior hypothalamus
To analyze the substance, the retention time of a known substance, a serotonin standard, was compared to the retention time of an unknown substance. The standard was created by serially diluting 1 mg/ml 5-HT to a 1 pg concentration. All the peak analysis was done from samples collected at the preoptic/anterior hypothalamus (PO/AH). 10 µl of each sample, which was collected from the PO/AH, was injected into the HPLC (high liquid chromatography) machine. The peaks were displayed on a chromatograph and the software program PowerChrom was used to analyze the peaks. Figure 6 shows a sample of the chromatograph peak when 5-HTP was administrated to the animal. The arrows point to the peak. Figure 6A is an example of an animal’s 5-HT peak before any drug was administrated (baseline 5-HT level). Figure 6B is an illustration of the peak when the 5-HT standard was used. Figure 6C is the 5-HT peak when a high dose (25 mg/kg, s.c) of 5-HT was injected into the rat and figure 6D is the 5-HT peak when a lower dose (5 mg/kg 5-HTP, s.c) was given to the animal.
Figure 7 Flow chart
Following surgery, the animals were allowed to rest for at least one week before microdialysis was done. The day before the experiment, the rats were housed in a free-moving bowl (Ratum) and given food and water ad libitum. They were connected to a machine that pumped 1μl/min cerebral spinal fluid (CSF) into probes inserted in the preoptic/anterior hypothalamus or prefrontal cortex region of their brain. 5 samples were collected before the animals were injected with 5-HTP and/or ketanserin and 8 samples were obtained thereafter. In each case, 5-10μl of the samples were collected every 15 minutes and stored temporarily on ice in a glass vial. The vials were placed inside the SIL-10AD VP Shimadzu auto injector and analyzed by the HPLC (high liquid chromatography). The area of the peak was calculated by using the software program PowerChrom. For each time point, the value obtained was then converted to pg/sample and plotted on a graph.
pg/sample = *(M) (sample area)
(standard area)

* M = multiplication factor necessary to obtain 15

Sample Calculation:

-10 μl of sample was run
-Serotonin standard’s area was 2.7
-Sample’s area was 2.78

pg/sample = (2.78) (1.5) / 2.7 = 3.088

Figure 8 Calculation for determining area of peak
Figure 8 Calculation for determining area of peak

After the animals were administrated the drug, samples were collected every 15 minutes for 2 hours. The vials were temporarily stored on ice and then injected into the HPLC machine. The computer software program PowerChrom calculated the area under the peak. The value obtained was then normalized to the 5-HT standard and converted to pg/sample. This new value took into account the volume of sample obtained in 15 minutes. Ideally 15μl of sample should have been collected since the flow rate of the pump was 1 μl/min. But due to the animal’s head shakes and other uncontrollable factors, this was not always possible. To correct for these factors, the value calculated by PowerChrom was multiplied by a number that gave 15. For instance, if 10 μl of the sample was analyzed, then the sample’s area was multiplied by 1.5 to obtain 15 μl. The corrected value was then normalized by dividing it by the 5-HT standard used in the experiment. This data point was then plotted on a graph.
Figure 9 Probe location
Figure 9 Probe location

To ensure that the probes were placed in the correct region of the brain, the animals were sacrificed after the experiment and their probe location was determined. Fast green dye was infused into each of the probes when the animal was deeply anaesthetized and then the rat was decapitated. The brain was removed, sliced, and the probe location was compared to the location in the rat brain atlas (Paxinos and Watson, 1982). In some cases, the brain slices were then stained with crystal violet to visualize the probe location. In other cases, the sections were cut by freehand and visually observed. No dye was used.

Figure 9A is a schematic representative of a rat brain. Figure 9B-C is a representative sample of brain region frozen and cut by freehand. No crystal violet dye was used and instead the area where the probes were located was photographed. The arrow(s) represents the area of interest. The arrow in figure 9B points to the PO/AH (preoptic/anterior hypothalamus) region and the arrows in figure 9C points to the two PFC (prefrontal cortex) locations.
3.0 PART I: EFFECT OF LOWER AMBIENT TEMPERATURE ON 5-HT INDUCED TOXICITY

Though there are many drugs used to treat the hyperthermic response, an alternative approach to these pharmacological directed therapies is cold exposure. Lowering the body core temperature or inducing hypothermia has been shown to prevent neuronal injuries (Suehiro et al., 1999; Wallace et al., 2001; Kline et al., 2004), thus cold exposure has been considered to be a potential effective management for 5-HT toxicity. However, neural mechanisms underlying the responding changes from hyperthermia to hypothermia are not well understood. This is explored in the following section.

3.1 Effect of lower ambient temperature on 5-HT$_{2A}$R responsivity to excessive 5-hydroxytryptamine in serotonin-toxicity syndrome.

3.2 Abstract

A major concern regarding serotonin-toxicity syndrome (5-HT toxicidrome) is hyperthermia, a sign implicating a severe and life-threatening disorder. Previous studies showed that the hyperthermia was blocked by lowering ambient temperature. However, little was known about the neural mechanisms underlying the effect. In the present study,
ambient temperature lowered from 22 °C to 12 °C or 6 °C was used as a cooling measure while toxidrome-relevant hyperthermia was induced by injection of the 5-HT precursor 5-hydroxy-L-tryptophan (5-HTP) in clorgylinized animals. Our initial results showed that lower ambient temperature exerted an antagonistic effect on hyperthermia similar to that of the 5-HT$_{2A}$R antagonist ketanserin. To extend these findings, 5-HT efflux was determined in the preoptic/anterior hypothalamus (PO/AH) and prefrontal cortex (PFC) using \textit{in vivo} microdialysis. Injection of 25 mg/kg 5-HTP at ambient 22 °C produced an increase in 5-HT efflux by \textasciitilde100 fold in the PO/AH and \textasciitilde240 fold in the PFC. The increase was significantly attenuated by ketanserin, supporting the hypothesis that the excessive 5-HT efflux for hyperthermia was mediated through activation of 5-HT$_{2A}$Rs. The antagonistic effect of ketanserin on 5-HT efflux was reduced at ambient 12 °C and completely suppressed at ambient 6 °C. Moreover, there was only a 25 – 30 fold increase in 5-HT efflux of two regions when 5-HTP was administered at ambient 6 °C. These data suggest that the responsivity of 5-HT$_{2A}$R activation by excessive 5-HT efflux in the CNS is reduced by lowering the ambient temperatures below 22 °C.

3.3 Introduction

Serotonin-toxicity syndrome (5-HT toxidrome) is an iatrogenic disorder, mainly occurring among patients overusing 3,4-methylenedioxymethamphetamine (MDMA) as a recreational drug in the club house (Mueller and Korey, 1998; Rusyniak and Sprague, 2005) or taking multiple serotonergic drugs as a medication for mental depression (Boyer and Shannon, 2005; Nelson et al., 2007; Terao and Hikichi, 2007) or physical illness (Mason and Blackburn, 1997; Dvir and Smallwood, 2008). Neurochemical mechanism
underlying the disease is due to excessive 5-HT in the central nervous system (CNS). However, behavioral signs and neuropathophysiological response appear to be varying clinically, depending on the severity (mild, moderate and severe) of the toxidrome (Mills, 1995; Boyer and Shannon, 2005). Evidence suggests that the severe toxidrome is closely associated with hyperthermia, which can lead to multiple organ failures and death (Mueller and Korey, 1998; Rusyniak and Sprague, 2005). Hence, monitoring body-core temperature and preventing hyperthermia are of utmost concerns in the toxidrome management (Farfel and Seiden, 1995; Isbister and Buckley, 2005).

There are several effective approaches developed for hyperthermia treatment. These include, but are not limited to, muscle relaxants (Nisijima et al., 2001), benzodiazepines (Isbister and Buckley, 2005) and antagonistic drugs targeting for dopamine D1 receptors (Mechan et al., 2002), adrenergic α1/2A receptors (Bexis and Docherty, 2005, 2008), NMDA receptors (Colado et al., 1998; Nisijima et al., 2004) and 5-HT2A Rs (Malberg et al., 1996; Nisijima et al., 2001; Herin et al., 2005). The mortality rate was thereby decreased significantly after pharmacological treatments, particularly after blocking 5-HT2A Rs (Nisijima et al., 2001; Fantegrossi et al., 2003; Gillman, 2005). Thus, 5-HT2A R antagonists are highly recommended as a drug therapy for the 5-HT toxidrome (McDaniel, 2001; Isbister and Buckley, 2005; Nelson et al., 2007).

In addition to pharmacological directed therapies, cooling measures have been suggested as an alternative approach in clinics for the toxidrome treatment (Lane and Baldwin, 1997; Finfgeld, 2004; Isbister and Buckley, 2005). In animals, the hyperthermia would be
reversed to hypothermia if causative drugs were injected at a lower ambient temperature. For instance, MDMA at doses which caused hyperthermia under a normal condition produced a hypothermic response when tested at the ambient temperature below 22 °C (Malberg and Seiden, 1998; Green et al., 2005; Goni-Allo et al., 2007). Since hypothermia is a protective response to neuronal injuries (Suehiro et al., 1999; Wallace et al., 2001; Kline et al., 2004), cold exposure has been considered to be a potential effective management for 5-HT toxicity treatment. However, neural mechanisms underlying the opposing switch from hyperthermia to hypothermia are not understood.

The purpose of this study was to investigate the interaction between ambient temperature and 5-HT toxidrome by measuring changes in core temperature \( T_{cor} \) and 5-HT efflux in the preoptic/anterior hypothalamus (PO/AH) and prefrontal cortex (PFC). The 5-HT toxidrome was induced by injection of a 5-HT precursor, 5-hydroxytryptamine (5-HTP), in rats pretreated with the monoamine oxidase inhibitor (MAOI) clorgyline. Changes in \( T_{cor} \) and 5-HT efflux in response to the 5-HT₂AR antagonist ketanserin at ambient 22 °C were compared with those obtained at ambient 12 °C or 6 °C. The results of our data support the hypothesis that 5-HT₂ARs may be desensitized by lowering ambient temperature, consistent with the finding that cold exposure would alleviate neural injuries (Wallace et al., 2001) and the 5-HT toxidrome (Isbister and Buckley, 2005).
3.4 Material and Method

3.4.1 Animals

Adult male Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC, USA) weighing 300-350g were used in this study. The rats were pair-housed with food and water available at any given time in a temperature- and humidity-controlled room. Housing and the experimental treatment of the animals were in strict accordance with the *NIH Guide for the Care and Use of Laboratory Animals* and *Animal Research Guidelines* at Florida Atlantic University.

3.4.2 Drugs

Clorgyline (N-Methyl-N-propargyl-3-(2, 4-dichlorophenoxy) propylamine hydrochloride) and 5-HTP (5-hydroxy-L-tryptophan), purchased from Sigma (St. Louis MO, USA), were dissolved in saline (NaCl 0.9%) and injected subcutaneously (s.c). Ketanserin tartate obtained from Tocris Bioscience (Ellisville, MO, USA) was dissolved in deionized water for intraperitoneal injection (i.p.).

3.4.3 Induction of 5-HT toxidrome

In animals, 5-HT toxidrome was induced by administration of the MAO inhibitor clorgyline in combination with the 5-HT precursor 5-hydroxy-L-tryptophan (5-HTP) (Nisijima et al, 2001). In this study, clorgyline (2 mg/kg, s.c.) was given 2 h before an experiment. Animals were randomly assigned to the test or control group. To habituate the environment, rats were individually kept in test chambers at least 2 h before clorgyline administration. Toxicity was examined with 5-HTP injection at doses of 5
mg/kg, 15 mg/kg and 25 mg/kg. Ketanserin, a selective 5-HT$_{2A}$R antagonist was administered 15 min before 25 mg/kg 5-HTP.

3.4.4 Measurement of body-core temperature ($T_{cor}$)

$T_{cor}$ was obtained by measuring colon temperature at 15-min intervals. Two measurements were obtained prior to 5-HTP injection as the baseline, and hereafter eight measurements were obtained following 5-HTP injection. For antagonistic studies ketanserin was administered 15 min before 25 mg/kg of 5-HTP, and change in $T_{cor}$ was measured 120 min after 5-HTP injection at the time point when 5-HTP reached the maximum effect. Under gentle restraint, a flexible thermoprobe connected to a digital meter (Traceable®, Fisher Scientific) was inserted into rat colon. The length of the probe was set at 4.6 cm.

3.4.5 5-HT microdialysis

Rats were anesthetized with a combination of xylazine (4 mg/kg, i.p.) and ketamine (80 mg/kg, i.p.) and placed on a stereotaxic frame (Stoelting Co., Wood Dal, Illinois, USA). The skull was surgically exposed and guide cannulae were placed above the preoptic/anterior hypothalamus (PO/AH; coordinates, AP -1.1 mm relative to bregma, ML ±0.9 mm to the midline and DV -3.0 mm below the skull) and prefrontal cortex (PFC, coordinates, AP +3.3, ML ±0.7 mm and DV -2.0 mm). Acrylic dental cement and skull screws were used to secure the guide cannulae into place. One week after the surgery, a hollow probe was inserted through the cannula to the PO/AH or PFC for 5-HT microdialysis. The target coordinates for the tip of the probe were AP –1.1 mm relative to bregma, ML ±0.9 mm to midline and DV -9.0 mm below the skull in the PO/AH or AP
+3.3 mm, ML ±0.7 mm and DV -4.5 mm in the PFC. The probes were secured in place by dental cement. Rats were then placed in the test chamber and attached to a fluid swivel that allowed animals to move freely (Raturn® system, Bioanalytical System Inc., W. Lafayette, IN). Food and water were available ad libitum. The dialysis probes were perfused overnight with phosphate buffered Ringer’s solution (140 mM NaCl, 3.0 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 0.25 mM NaH₂PO₄, 1.0 mM Na₂HPO₄, pH 7.4) at a rate of 1.0 µl/min.

Next day, animals were injected with clorgyline (2 mg/kg, s.c.) 2 h before sample collections. Samples were collected every 15 min with microcentrifuge vials, temporarily stored on ice (0 – 4 °C), and analyzed by high performance liquid chromatography (HPLC) - electrochemical detection (HTEC-500; EICOM, Japan) with a CMA/200 refrigerator microsampler (CMA/Microdialysis, Stockholm, Sweden). Separation of 5-HT was achieved on a 150 mm ×1 mm i.d. column packed with a TSK gel ODS-80 TM, 5 µm particle size. The potential on the graphite was set at + 400 mV (relative to AG/AgCl reference electrode). The mobile phase was made of 0.1 M phosphate buffer (pH 6.0), 1% methanol, 500 mg/L sodium-1-octanesulfonate, and 50 mg/L ethylenediamine-tetracetic acid and pumped at a rate of 500 µl/min. The detection limit for 5-HT was 0.05 pg. Drug injection (ketanserin or/and 5-HTP) was made after four successive baselines were established.

Upon completion of experiments, animals were deeply anesthetized with pentobarbital (100 mg/kg, i.p). Brain location was examined by infusing with 2% Fast Green through the probe for 10 min and then decapitated. Brains were extracted, frozen and sliced. The
dye-tracer location was compared to the rat brain atlas (Paxinos and Watson, 1998). Probes located outside the target area were excluded from the data analysis.

### 3.4.6 Data analysis

Unless otherwise noted, data were expressed as mean ± S.E.M. Changes in $T_{cor}$ in the degree Celsius were expressed on the y-axis (as °C) plotted against the sampling time on the x-axis (in min). The 5-HT efflux expressed as absolute values (pg/sample), were uncorrected from probe recovery. For comparison, microdialysis data were normalized to the relative changes expressed as the fold increases over their respective baselines. Note that the baseline was calculated from the mean of four sequential samples on 5-HT efflux before 5-HTP injection. Statistical analysis was performed by a two-way (drug treatment × time course) factorial ANOVA. If significant interactions of drug treatment × time course were found, further statistical analysis was carried out using the post-hoc Scheffe test in determining the significance of the respective time points. If appropriate, the unpaired t-test was employed for the data analysis. The level of P-value was set at 0.05 for a statistically significant effect.

### 3.5 Results

#### 3.5.1 Changes in $T_{cor}$ in response to 5-HTP injection at ambient 22 °C

The mean basal $T_{cor}$ (pre-5-HTP injection) was 36.7 ± 0.1°C (n = 19) in clorgylinized animals. Figure 10A shows that 5-HTP produced a time- and dose-dependent increase in $T_{cor}$. This conclusion was confirmed by the two-way factorial ANOVA, which revealed a significant main effect of 5-HTP doses ($\text{F}_{(3, 15)} = 5.222$, $P = 0.0114$) and sampling times
(F(7, 21) = 12.97, P<0.001). A 5-HTP × sampling time interaction indicated that 5-HTP increased $T_{cor}$ across times more than vehicle injection (F(21,105) = 4.274, P<0.0001). As shown in fig 10A, vehicle alone (3 ml/kg, s.c.; 0.9% NaCl) had no effect on $T_{cor}$. The post-hoc Scheffe test showed that administration of 5 mg/kg of 5-HTP caused an insignificant change compared to saline-treated rats (P > 0.05). There was a tendency that the $T_{cor}$ was increased following 15 mg/kg of 5-HTP although the effect was not statistically significant (P > 0.05). In contrast, 25 mg/kg of 5-HTP evoked a significant increase (P < 0.0001). Time to reach the maximum increase was in ~60 min after the injection, and the elevation was sustained for at least 1 h. All animals were killed in 2 - 5 h after 25 mg/kg of 5-HTP. Thus, this dose was exclusively used to examine the antagonistic effect of ketanserin.

To determine if changes in $T_{cor}$ were mediated through 5-HT$_{2A}$Rs, ketanserin (5 mg/kg, i.p.) was administered to clorgylinized animals 15 min before 5-HTP injection. Controls were treated with the respective vehicle (H$_2$O, 1 ml/kg, i.p.) followed by 5-HTP or vehicle injection (0.9% NaCl, s.c.) on the same schedule. Figure 10B shows the effect of the 5-HT$_{2A}$R antagonist ketanserin (5 mg/kg, i.p.) on hyperthermia and death induced by a high dose of 5-HTP. Consistent with the previous results, 25 mg/kg of 5-HTP was able to evoke hyperthermia measured at a one time-point of 120 min after the injection. Next, ketanserin was injected 15 min before 5-HTP. As shown in figure 10B, the hyperthermic response was antagonized in rats pretreated with ketanserin (P < 0.001, t-test). Instead, it became hypothermia. In addition, all animals survived from the ketanserin treatment.
3.5.2 \( T_{\text{cor}} \) response to 5-HTP injection at ambient 12 °C and 6 °C

Figure 11A shows that the mean basal \( T_{\text{cor}} \) was 36.7 ± 0.1 °C at ambient 22 °C and 36.9 °C ± 0.1 (n = 23) at ambient 12 °C. Statistical analysis revealed that there was no significant difference in the baselines between two groups (P > 0.05, unpaired t-test). Vehicle administration had no effect on \( T_{\text{cor}} \) at either ambient temperature.

Figure 11B shows changes in \( T_{\text{cor}} \) in clorgylinized rats tested at ambient 12 °C. Compared to the vehicle, 5-HTP produced a dose- and time-dependent decrease in \( T_{\text{cor}} \). Data analysis with the two-way factorial ANOVA revealed a significant difference in doses (\( F_{(3, 19)} = 4.018, P = 0.0227 \)) and sampling times (\( F_{(7, 21)} = 6.678, P < 0.0001 \)) as well as a significant interaction of 5-HTP treatment × sampling times (\( F_{(21, 133)} = 1.997, P = 0.0099 \)). The post-hoc Scheffe analysis showed that there was a small transit but significant reduction in response to 5 mg/kg of 5-HTP (P < 0.05). In contrast, a large and persistent reduction was found in rats administered with 5-HTP at a dose of 15 mg/kg (P < 0.05) and 25 mg/kg (P < 0.001). It should be borne in mind that the maximum reduction reached a plateau 30 min after injection, and it was persistent for at least 90 min during the rest of the experimental time. Most intriguingly, animals survived even though treated with 25 mg/kg of 5-HTP, a lethal dose at normal ambient temperature.

Next, ketanserin was used to test if 5-HT2ARs were involved in changes in \( T_{\text{cor}} \) at ambient 12 °C. In a separate experiment, as shown in figure 11C, 25 mg/kg of 5-HTP was again able to produce hypothermia as measured at one time-point of 120 min. Ketanserin injected 15 min before 5-HTP failed to block this effect (P > 0.05).
One possible interpretation of the findings that 5-HTP produced a hypothermic response at ambient 12 °C in contrast to hyperthermia at ambient 22 °C was that 5-HT2AR responsivity to drug injection was decreased or “desensitized” at low ambient temperature. To test this hypothesis, the hypothermic response was further tested in rats at ambient 6 °C. We predicted that the $T_{cor}$ would be further reduced in response to 5-HTP injection in an ambient-temperature-dependent manner. The mean basal $T_{cor}$ at ambient 6 °C was 37.1 °C ± 0.2 (n = 10). Compared to that at ambient 22 °C, the differences between the two baselines were not significant (P > 0.05). As shown in fig 12A, 25 mg/kg of 5-HTP also produced a decrease in $T_{cor}$ (P < 0.05). As expected, the decrease was significantly greater at ambient 6 °C (P < 0.01) compared to that at ambient 12 °C. Interestingly, the $T_{cor}$ was progressively declining during the 120-min experimental time, and maximum reduction was 8.5 °C ± 1.2 below the pre-injection level. All animals survived after this injection. Again, as shown in figure 12B, injection of ketanserin had no effect on the reduction in $T_{cor}$ (P > 0.05).

3.5.3 Changes in 5-HT efflux at ambient 22 °C

5-HT efflux was measured in the PO/AH. The basal 5-HT at ambient 22 °C was 0.32 pg/sample (± 0.03, n = 36) in clorgylinized rats (2 mg/kg, s.c.). As shown in figure 13A, injections of 5-HTP elicited increases in extracellular 5-HT. The two-way factorial ANOVA revealed a significant effect of 5-HTP treatment ($F_{(3, 30)} = 9.433; P = 0.0002$) and significant effect of sampling times ($F_{(7, 21)} = 7.413; P < 0.0001$). Also, there was a significant interaction between 5-HTP treatment × sampling time ($F_{(21, 210)} = 4.473; P < 0.0001$). Relative to the respective baseline, the increase was 6-fold, 26-fold and 97-fold
in response to 5 mg/kg, 15 mg/kg and 25 mg/kg of 5-HTP, respectively. We next
examined whether the 5-HTP (25 mg/kg, s.c.)-evoked increased efflux was mediated by
5-HT\textsubscript{2A}Rs by administering the 5-HT\textsubscript{2A}R blocker ketanserin (5 mg/kg, i.p.). As shown in
figure 13B, the effect was significantly attenuated, compared to 25 mg/kg of 5-HTP alone
\((F_{(1, 14)} = 17.140, P = 0.001)\). The area under the curve (AUC) analysis reveals that there
was a 40\% reduction compared to the efflux without the ketanserin treatment.

3.5.4 Changes in 5-HT efflux at ambient 12 °C and 6 °C

The PO/AH was used to determine 5-HT efflux in response to lower ambient
temperature. First, the experiment was carried out at ambient 12 °C. The basal 5-HT was
0.29 pg/sample (± 0.04, n = 25) in animals pretreated with clorgyline. Injection of 5-HTP
produced a time- and dose-dependent increase in 5-HT efflux (figure 14A – C).
Compared to the baseline, the maximum increase was 2-fold, 12-fold and 86-fold in
response to 5 mg/kg, 15 mg/kg and 25 mg/kg, respectively. There was a tendency that 5-
HT efflux was decreased by lowering the ambient temperature. However, the statistical
analysis failed to reach a significant difference between the ambient 22 °C and 12 °C in
response to 5-HTP at dose of 5 mg/kg \((F_{(1, 13)} = 2.968, P = 0.1106)\), 15 mg/kg \((F_{(1, 9)} =
0.104, P = 0.7534)\) or 25 mg/kg \((F_{(1, 16)} = 2.031, P = 0.1734)\). To further explore the
effect of ambient temperature, changes in 5-HT efflux in response to 25 mg/kg of 5-HTP
were then measured at 6 °C. As shown in figure 14D, there was a significant reduction in
increased efflux while animals were tested at ambient 6 °C \((F_{(1, 16)} = 5.448, P = 0.0396)\).
Compared to that at ambient 22 °C, the time-points for significant reduction began 45 min
after 5-HTP injection.
3.5.5 5-HT efflux in the PFC at ambient 22 °C, 12 °C and 6 °C

The prefrontal cortex (PFC) was examined because it contains a high density of 5-HT$_{2A}$Rs (Ciccocioppo et al., 1999). At ambient 22 °C, baseline 5-HT was 0.29 pg/sample (± 0.04, n = 29). There was a 4-fold, 30-fold and 240-fold increase in 5-HT efflux in response to 5 mg/kg, 15 mg/kg and 25 mg/kg of 5-HTP, respectively (figure 15A – 15C). At ambient 12 °C, the baseline 5-HT was 0.31 pg/sample (± 0.04, n = 25), insignificantly different from that at 22 °C (P > 0.05). Increases in 5-HT efflux were 5-fold, 18-fold and 129-fold following 5 mg/kg, 15 mg/kg and 25 mg/kg of 5-HTP, respectively. Compared to that at ambient 22 °C, there was a tendency that 5-HT efflux was reduced at ambient 12 °C (5 mg/kg F (1, 23) = 0.471, P = 0.4996; 15 mg/kg, F (1, 18) = 0.641, P = 0.4337; 25 mg/kg, F (1, 14) = 20.225, P = 0.0005). Lastly, after ambient temperature was lowered to 6 °C, there was only a 25-fold increase following 25 mg/kg of 5-HTP. The reduction, compared to the level at 12 °C, was significant (F (1, 19) = 12.258, P = 0.0024). The data analysis revealed that there was a significant main effect on ambient temperature (fig 6C; F (2, 14) = 38.303, P < 0.0001), significant effect on sampling time (F (7, 21) = 70.358, P < 0.0001) and significant interactions on ambient temperature × sampling time (F (14, 138) = 23.281, P < 0.0001).

3.5.6 “Desensitization” of 5-HT$_{2A}$Rs at ambient 12 °C and 6 °C

It appears that there exhibits a functional similarity between lowering the ambient temperature and administering 5-HT$_{2A}$R antagonist, suggesting that the 5-HT$_{2A}$Rs may be desensitized in response to reduction in ambient temperature. If the hypothesis is correct, theoretically the antagonistic efficacy of 5-HT$_{2A}$R blockers would be reduced or even
eliminated by lowering the ambient temperature. In this study, ketanserin was thus used to validate the hypothesis. Figure 16 shows the effect of ketanserin on 5-HT efflux in the PO/AH evoked by 5-HTP (25 mg/kg, s.c.) in clorgylinized animals at ambient 12 ºC or 6 ºC. Although the increased efflux had been substantially reduced at ambient 12 ºC, ketanserin was still able to further decrease the level of 5-HT. The reduction was statistically significant (fig 16A; F (1, 12) = 4.643; P = 0.0476) compared to 25 mg/kg alone at the same ambient temperature. However, the antagonistic effect of ketanserin was completely suppressed at ambient 6 ºC (fig 16B; F (1, 9) = 0.511, P = 0.4928).

The “desensitization” hypothesis was also examined in the PFC by comparison of the antagonistic response of ketanserin on ambient temperature of 22 ºC, 12 ºC or 6 ºC. The antagonistic effect of ketanserin was significant on 5-HT efflux at ambient 22 ºC (figure 17A; F (1, 12) = 14.230, P = 0.0023). However, when the ambient temperature was set at 12 or 6 ºC, ketanserin failed to have significant effects on the efflux (figure 17B; F (1, 19) = 2.088, P = 0.1647; fig 17C; F (1, 14) = 0.001, P = 0.9823), supporting the hypothesis.

### 3.6 Discussion

It has been demonstrated previously that hyperthermia was blocked by either 5-HT2AR antagonists (Nisijima et al., 2001) or lower ambient temperature (Bowyer et al., 1994; Malberg and Seiden, 1998; Wallace et al., 2001). However, little was known about the relationship between 5-HT2ARs and ambient temperature. Our pathophysiological results have confirmed that both administering the 5-HT2AR antagonist ketanserin and lowering the ambient temperature (12 ºC or 6 ºC) were effective on blocking hyperthermia induced
by 5-HTP in clorgylinized rats. Likewise, our neurochemical investigations show that excessive 5-HT efflux evoked by 5-HTP in clorgylinized animals was also significantly attenuated by either administering 5-HT$_2A$R antagonist ketanserin or lowering ambient temperature. Interestingly, the effect of lower ambient temperature appeared to have a greater efficacy in blocking hyperthermia and 5-HT efflux compared to that of ketanserin. The finding that the antagonistic effect of ketanserin was reduced and even abolished at lower ambient temperature suggests that 5-HT$_2A$Rs are their convergent target in the treatment of toxidrome. Therefore, it can be concluded that there is an ambient temperature-dependent decrease in responsivity of 5-HT$_2A$Rs to excessive 5-HT in the CNS when the ambient temperature is lowered below 22 ºC.

3.6.1 Hyperthermic response of 5-HT$_2A$Rs to excessive 5-HT efflux

Serotonergic antidepressants produce a small increase in brain 5-HT, usually less than 5-fold from the baseline (Rutter and Auerbach, 1993; Fuller, 1994). However, a massive and widespread increase in 5-HT efflux, which can occur by injection of the recreational drug MDMA (Mechan et al., 2002) or antidepressants combined with other drugs (Nisijima et al., 2003; Shioda et al., 2004), would exceed the tolerant level for normal physiological activity and induce a serotonin-toxicity syndrome or toxidrome. Excessive 5-HT at preoptic/anterior hypothalamus (PO/AH) has long been shown to contribute to the hyperthermic response in the toxidrome. When treated locally in the PO/AH, hyperthermia could be elicited by as low as an ~8-fold increase in 5-HT efflux (Lin et al., 1998). In this study, it was found that 5-HT efflux in the PO/AH associated with hyperthermia required over 30-fold above the baselines, closely agreeing with the
previous results (Nisijima et al., 2004; Shioda et al., 2004). One interpretation for the
difference between local treatment and systemic injection is that a global increase in 5-HT
efflux in other regions during the toxidrome might interfere in the hypothalamic
neuronal activity. Alternatively, the hyperthermic response might be altered by other
types of neurotransmitters which were also elevated in the toxidrome (Shioda et al.,
2004). The relationship between 5-HT efflux and hyperthermic response was also
demonstrated in animals treated with other drugs. For instance, there was an ~15 – 20
fold increase in the hippocampus as the hyperthermia was induced by MDMA (Mechan
et al., 2002) although changes in the hypothalamic area were not available in the
literature. Consistent with this, $T_{cor}$ was not significantly altered by MDMA at the dose
that induced a 4- to 6-fold increase in the striatum (Freezer et al., 2005). In line with this,
the efflux less than a 10-fold increase had no consistent effect on $T_{cor}$ (figure 10A vs.
figure 12A). Altogether, a marked increase in hypothalamic 5-HT is necessary and
predetermined for a hyperthermic response in the toxidrome.

We showed that the hyperthermic effect induced by 5-HTP was completely blocked by
ketanserin pretreatment, consistent with earlier findings (Malberg et al., 1996; Nisijima et
al., 2001; Herin et al., 2005), supporting that 5-HT$_{2A}$Rs are responsible for 5-HT-evoked
hyperthermia. The affinity of 5-HT$_{2A}$Rs to their natural ligand 5-HT is very low, ~ 400 –
1000 times less than that of 5-HT$_{1A}$Rs (Peroutka and Snyder, 1983; Dalpiaz et al., 1995).
It is conceivable that, in response to 5-HTP injection in clorgylinized animals, increased
endogenous ligands might activate 5-HT$_{1A}$Rs which possibly had reached a maximum
response before activation of 5-HT$_{2A}$Rs. This, in addition to the previous finding that
these two receptors had an opposite effect on thermoregulation (Lin et al., 1998; Ootsuka and Blessing, 2006) and our results that hyperthermia was not apparent until 5-HT efflux was increased up to 30-fold above the baseline, suggests that with such a high efflux 5-HT$_{2A}$R-mediated hyperthermia may be able to overcome the opposite effect of 5-HT$_{1A}$Rs.

Intriguingly, a blockade of 5-HT$_{2A}$Rs not only blocked hyperthermia, but also reversed it to hypothermia. A similar observation has been reported in animals co-treated with MDMA and ketanserin (Malberg et al., 1996). Although there are many other receptors involved, accumulative evidence shows that 5-HT$_{1A}$Rs are the primary player responsible for hypothermia (Abdel-Fattah et al., 1995; Rusyniak et al., 2007). Thus, the results of our data support the hypothesis that changes in $T_{cor}$ in the toxidrome are the net effect of 5-HT$_{1A}$Rs and 5-HT$_{2A}$Rs (Abdel-Fattah et al., 1995; Rusyniak et al., 2007), predetermined by 5-HT concentrations in the PO/AH.

3.6.2 Effect of lowering the ambient temperature

The present study showed that changes in $T_{cor}$ depended on not only 5-HT efflux evoked by causative drugs but also the ambient temperature at which the animal experiments were carried out. It should be kept in mind that the thermoneutral zone, which is defined as the optimal ambient temperature at which the subjects have the lowest metabolic rate, is in a range of 23 °C (night) – 30 °C (daytime) for experimental rats (Gordon, 1994). The possible cold-stress response caused by the ambient temperature applied to animals in the current study is beyond the scope of the current objective, thus it will not be discussed further. Studies suggest that ambient 24 °C may be the breakpoint for the net-
zero effect of the 5-HT$_{1A}$R-evoked hypothermia and 5-HT$_{2A}$R-evoked hyperthermia in response to endogenous 5-HT (Malberg and Seiden, 1998). Moreover, a small change in ambient temperature, even with only a 2 °C difference, would have a significant effect. It is therefore concluded that the ambient temperature of 22 °C would theoretically favor a hypothermic response.

To thoroughly understand the role of lower ambient temperature in the toxidrome, the effect on changes in $T_{cor}$ correspondent to 5-HT efflux at ambient 12 °C and 6 °C was analyzed in comparison with the effect at ambient 22 °C. We found that at ambient 12 °C the $T_{cor}$ was reduced about -1 °C, -2 °C and -4 °C in response to 5 mg/kg, 15 mg/kg and 25 mg/kg of 5-HTP, respectively (see figure 11). The characteristic reduction was confirmed by the experiment carried out at ambient 6 °C showing a progressive decrease in $T_{cor}$, maximally a reduction of 8 °C in 120 min observation (see figure 12). Moreover, we observed that when ambient temperature was changed from 22 °C to 12 °C, there was a significant increase in basal 5-HT in the PO/AH from 0.14 pg/sample to 0.22 pg/sample ($n = 8; \ P < 0.05$, paired t-test). This is consistent with other studies which also demonstrated that 5-HT released in this region is indeed associated with thermoregulation (Lin et al., 1998; Ishiwata et al., 2004). Our study showed that lowering the ambient temperature, in conjugation with injection of 5-HTP in the clorgylinized rats, produced the hypothermic response as well as reduction in increased 5-HT efflux. Here raises a question as whether the hypothermia instead resulted from the reduction in 5-HT efflux. Specifically, compared to the efflux induced by 25 mg/kg of 5-HTP at ambient 22 °C, there was a 30% reduction at ambient 12 °C (figure 14C) and a 70% reduction at
ambient 6 °C (figure 14D). However, despite a marked reduction in 5-HT efflux following 5-HTP administration, the remaining was still enormously high, showing an 80-fold and 30-fold increase at ambient 12 °C and 6 °C, respectively. As mentioned earlier, the remaining efflux is high enough to induce hyperthermia at a normal ambient temperature such as 22 °C, suggesting that neither reduction in 5-HT efflux nor the remaining was the primary cause of hypothermia.

If the reduction in 5-HT efflux was not the key cause, what other changes should be the mechanism responsible for switching hyperthermia to hypothermia at lower ambient temperature? Since changes in $T_{\text{cor}}$ are determined by the net effect of 5-HT1AR-induced hypothermia and 5-HT2ARs-induced hyperthermia (Lin et al., 1998), it is reasonable to interpret the data as being that the 5-HT2AR activity relative to 5-HT1ARs was reduced by lowering the ambient temperature. Many studies in previous investigations suggest that the 5-HT2AR activity can be desensitized due to the receptors being uncoupled with G_{a11}-proteins (Shi et al., 2007) and/or internalized (Saucier et al., 1998; Hanley and Hensler, 2002). Although ambient temperature-evoked receptor desensitization under our experimental conditions remains to be further investigated, its antagonistic effect on 5-HT toxicity appears prevail. For instance, the $T_{\text{cor}}$ was reduced more than -4 °C by lowering the ambient temperature to 12 °C (figure 11B) whereas there was only a -1 °C reduction by pharmacological injection with ketanserin (fig 10B). In addition, the ketanserin dose that was increased up to 10 mg/kg (n = 2; data not shown) had no further effect on hypothermia, suggesting that there is a limitation on using the pharmacological approach in toxicity treatment. In contrast, there was a further reduction, – 8 °C below
pre-5-HTP injection in $T_{cor}$ as ambient temperature was set at 6 °C. The hypothesis that 5-HT$_{2A}$Rs were desensitized at cool ambient temperatures was carefully validated by the 5-HT$_{2A}$R antagonist ketanserin examined at ambient 12 °C and 6 °C in comparison with that at the normal experimental condition. Importantly, lethality with 25 mg/kg of 5-HTP injection at ambient 22 °C was completely blocked at ambient 12 °C and 6 °C, similar to the effect of ketanserin (in this study) and cyproheptadine, another antagonist for 5-HT$_{2A}$Rs (Nisijima et al., 2001). It should be kept in mind that it was 5-HT$_{2A}$Rs, not 5-HT$_{1A}$Rs, that were responsible for the lethality in the toxidrome (Nisijima et al., 2001; Isbister and Buckley, 2005; Ma et al., 2008). However, such a large reduction in core temperature at ambient 12 °C and 6 °C should not be exclusively explained by 5-HT$_{2A}$R desensitization alone, and other mechanisms may be involved. Pharmacologically it has been shown that the 5-HT$_{2A}$Rs became desensitized while 5-HT$_{1A}$Rs were activated by their selective ligands (Carrasco et al., 2007; Gajendiran, 2007). Interestingly, one report showed that the responsivity of 5-HT$_{1A}$Rs to 8-OH-DPAT was markedly increased by lower ambient temperature (Nicholas and Seiden, 2003). Thus, the results of our data support the hypothesis that the hypothermic response to excessive 5-HT at lower ambient temperature may be due to a reduced responsivity of 5-HT$_{2A}$Rs, the effect perhaps augmented by an increased activity of 5-HT$_{1A}$Rs.

Pathophysiological changes are complicated by the fact that 5-HT$_{2A}$Rs and 5-HT$_{1A}$Rs have an opposite regulation on not only $T_{cor}$ but other neuronal activities (Nakamura et al., 2005; Ootsuka and Blessing, 2006). Thus, in addition to $T_{cor}$, changes in 5-HT efflux were thoroughly analyzed to compare the antagonistic effects between 5-HT$_{2A}$R
antagonist ketanserin and lower ambient temperatures of 12 °C or 6 °C. As shown in this study, ketanserin significantly attenuated the efflux evoked by 25 mg/kg of 5-HTP in both the PO/AH and PFC, suggesting that the increase in 5-HT efflux during the severe to life-threatening toxidrome was at least in part dependent on activation of 5-HT$_{2A}$Rs. Similarly, 5-HT efflux was reduced at ambient 12 °C relative to that at 22 °C (figure 14), further supporting the 5-HT$_{2A}$R desensitization hypothesis. There was a further reduction at ambient 6 °C compared to 12 °C, implicating that the effect was indeed ambient temperature-dependent. Also, it is of interest to note that the reduction was much larger by lowering the ambient temperature compared to that reduced by the antagonist ketanserin at ambient 22 °C. It can be inferred that the 5-HT$_{2A}$Rs which are sensitive to changes in ambient temperature may have a physiological profile different from those sensitive to ketanserin. Alternatively, if activity of 5-HT$_{1A}$Rs was indeed increased by lower ambient temperature as discussed earlier and agreed upon by others (Nicholas and Seiden, 2003), the pronounced reduction may be interpreted as a self-inhibitory regulation against excessive 5-HT efflux in the CNS. It further suggests that a lower ambient temperature has a greater efficiency in antagonistic effect compared to ketanserin. The finding that ketanserin attenuated the efflux at 12 °C but not 6 °C (figure 15) suggests that 5-HT$_{2A}$Rs was not completely desensitized until 6 °C. Altogether, the results of our data support the hypothesis at a point of neurochemical view that the responsivity of 5-HT$_{2A}$Rs to excessive 5-HT efflux may be reduced by lowering ambient temperature below 22 °C. However, further experiments such as directly measuring phospholipase C and G$_q$ activity (Damjanoska et al., 2003) are required to confirm the findings obtained in this study.
3.6.3 Role of the PFC in 5-HT toxidrome

We demonstrated that there was a larger reduction in 5-HT efflux in the PFC than that of the PO/AH at a lower ambient temperature. The differential effect on two regions was obvious at 25 mg/kg of 5-HTP. For instance, the reduction was 60% and 90% in the PFC in response to ambient 12 °C and 6 °C, respectively, whereas there was only a 30% and 70% reduction in the PO/AH under the same condition. In this context, it is worth mentioning that the PFC expresses a high density of 5-HT$_2$ARs in the CNS (Ciccocioppo et al., 1999). Many studies have demonstrated that 5-HT$_2$ARs played a crucial role in the PFC in mental health and psychotic disorders (Ciccocioppo et al., 1999; Meyer et al., 2001; Zanardi et al., 2001). Moreover, 5-HT$_2$AR expression was high in the PFC among suicide victims (Escriba et al., 2004). This is consistent with clinical observations that some patients with 5-HT toxidrome displayed changes in mental state (e.g., confusion, illusion and hallucination) in addition to physical difficulties (Boyer and Shannon, 2005; Gillman, 2005; Terao and Hikichi, 2007). Thus, it is most likely that, in addition to the PO/AH, the PFC is a crucial region involved in 5-HT toxidrome, particularly in changes of mental states. Our finding that 5-HT efflux evoked by 25 mg/kg of 5-HTP was significantly reduced in the PFC but not PO/AH by lowering ambient temperature to 12 °C suggests that 5-HT efflux is strongly regulated by 5-HT$_2$ARs in the PFC compared to other regions including the PO/AH. Most recently, we found that reverse microdialysis infusion of ketanserin into the PFC significantly blocked hyperthermia in 5-HT toxidrome (Zhang and Tao, unpublished observation), suggesting that, in addition to the PO/AH, the PFC may also involve a thermoregulatory response to excessive 5-HT in the toxidrome.
3.6.4 Conclusion

There is no doubt that ambient temperature exerts a crucial role for reversing hyperthermia to hypothermia in an effort to reduce neural damage caused by excessive 5-HT in the CNS. Importantly, our data suggest that both the antagonistic potency and efficacy appear much greater than those of drugs such as ketanserin (in this study), cyproheptadine, chlorpromazine, diazepam and MK-801 in other studies (Grahame-Smith, 1971; Nisijima et al., 2000; Nisijima et al., 2003, 2004). Our data also support the recommendation in clinics that patients with hyperthermia (> 2 °C) should be treated with cooling measures. Lower ambient temperature (e.g., ambient < 12 °C) prevents hyperthermia and provides substantial protection against the neurotoxic effects of the toxidrome (Goni-Allo et al., 2007). The current study provides evidence that 5-HT2ARs may be desensitized by lowering ambient temperature to achieve the protective effects. However, more research should be done to further explore the mechanisms involving in this effect.
Figure 10 Body-core temperature ($T_{cor}$) response to 5-HTP at ambient 22 °C
Figure 10 Body-core temperature ($T_{cor}$) response to 5-HTP at ambient 22 ºC

Rats were pretreated with clorgyline (2 mg/kg, s.c.) and placed in temperature-controlled chambers at 22 ºC for 2 h before experiments. Data are shown as mean ± S.E.M. **A**, 5-HTP produced a dose-dependent increase in $T_{cor}$: 5 mg/kg, $F_{(1, 8)} = 0.148$, $P = 0.711$; 15 mg/kg, $F_{(1, 7)} = 1.34$, $P = 0.285$; 25 mg/kg, $F_{(1, 6)} = 84.029$, $P < 0.0001$. **P < 0.05, **P < 0.01, and ***P < 0.001 examined by repeated measures ANOVA followed by post-hoc Scheffe test. **B**, The hyperthermic effect of 25 mg/kg 5-HTP was blocked by the 5-HT$_{2A}$R antagonist ketanserin (ket, 5 mg/kg, i.p) injected 15 min before experiments (t = 11.447, $P < 0.001$). ***P < 0.001, unpaired Student t-test.
Figure 11 Body-core temperature ($T_{\text{cor}}$) response to 5-HTP at ambient 12 °C
Figure 11 Body-core temperature ($T_{\text{cor}}$) response to 5-HTP at ambient 12 °C

Rats were pretreated with clorgyline (2 mg/kg, s.c.) and placed in temperature-controlled chambers at ambient 12 °C, 2 h before experiments. Data are shown as mean ± S.E.M. A, Compared to that at ambient 22 °C, vehicle alone did not alter $T_{\text{cor}}$ at 12 °C: $F_{(1,10)} = 3.509$, $P = 0.0905$. B, 5-HTP produced a dose-dependent decrease in $T_{\text{cor}}$: 5 mg/kg, $F_{(1,8)} = 1.113$, $P = 0.3222$; 15 mg/kg, $F_{(1,9)} = 5.045$, $P = 0.0500$; 25 mg/kg, $F_{(1,8)} = 28.144$, $P = 0.0007$. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ examined by repeated measures ANOVA followed by post-hoc Scheffe test. C, The 5-HT$_2$A antagonist ketanserin (ket, 5 mg/kg, i.p) failed to block changes in $T_{\text{cor}}$ at ambient 12 °C ($t = 0.200$, $P = 0.8457$, unpaired Student t-test).
Figure 12 Body-core temperature ($T_{cor}$) response to 5-HTP at ambient 6 °C
Figure 12 Body-core temperature ($T_{cor}$) response to 5-HTP at ambient 6 °C

Clorgylinized rats (2 mg/kg, s.c.) were placed in temperature-controlled chambers at ambient 6 °C. Data are shown as mean ± S.E.M. A, Compared to vehicle, the high dose of 5-HTP (25 mg/kg, s.c.) induced a progressive reduction in $T_{cor}$: $F_{(1, 10)} = 52.828$, $P < 0.0001$. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ examined by repeated measures ANOVA followed by post-hoc Scheffe test. B, Ketanserin injected 15 min before 5-HTP failed to block the reduction induced by 25 mg/kg of 5-HTP ($t = 0.631$, $P = 0.5455$, unpaired Student t-test).
Figure 13 Effect of 5-HTP on 5-HT efflux at ambient 22 °C
5-HT was measured in the preoptic/anterior hypothalamus (PO/AH) of rats pretreated with clorgyline (2 mg/kg, s.c.). The experiments were carried out at ambient 22 °C. Data are expressed as mean ± S.E.M. A, Injection of 5-HTP produced a dose-dependent increase in 5-HT efflux: 5 mg/kg, F (1, 15) = 17.779; P = 0.0006; 15 mg/kg, F (1, 13) = 8.647; P = 0.0101; 25 mg/kg, F (1, 19) = 54.035; P < 0.0001. B, Dashed line represents the data re-plotted from figure 13A. Injection of the 5-HT2AR antagonist ketanserin (open arrow) attenuated the increased efflux evoked by 25 mg/kg of 5-HTP (F (1, 14) = 17.140; P = 0.001).

*P < 0.05, **P < 0.01 and *** P < 0.001 examined by repeated measures ANOVA followed by post-hoc Scheffe test.
Figure 14 Reduction of 5-HT efflux in the PO/AH by lowering the ambient temperature to 12 °C or 6 °C
Figure 14 Reduction of 5-HT efflux in the PO/AH by lowering the ambient temperature to 12 °C or 6 °C

5-HT was measured in the preoptic/anterior hypothalamus (PO/AH) of rats pretreated with clorgyline (2 mg/kg, s.c.). The experiments were carried out at ambient 12 °C or 6 °C. Data are shown as mean ± S.E.M. Dashed line represents data re-plotted from the respective dose in figure 13. For the sake of clarity, vehicle injection at ambient 12 °C or 6 °C is omitted from the graphs (n = 4). A, Compared to vehicle, injection of 5 mg/kg 5-HTP produced an increase in 5-HT efflux: (F (1, 7) = 3.323; P = 0.1111). Although there was a tendency that the increase in 5-HT efflux was decreased at ambient 12 °C versus 22 °C, the reduction was not significant: (F (1, 11) = 2.968; P = 0.1106). B, Compared to vehicle, injection of 15 mg/kg 5-HTP produced an increase in 5-HT efflux: (F (1, 7) = 13.550; P = 0.0078). However, there was no significant difference in 5-HT efflux in rats examined at ambient 12 °C compared to ambient 22 °C (F (1, 9) = 0.104; P = 0.7534). C, Compared to vehicle, injection of 25 mg/kg 5-HTP produced an increase in 5-HT efflux at ambient 12 °C, (F (1, 11) = 10.920; P = 0.0057). The increased efflux was reduced insignificantly by lowering the ambient temperature to 12 °C (F (1, 16) = 2.031, P = 0.1734). D, Injection of 25 mg/kg 5-HTP produced an increase in 5-HT efflux at ambient 6 °C (F (1, 8) = 5.121; P = 0.0500). The increased efflux was significantly reduced between 45 – 120 min in contrast to that at ambient 22 °C (F (1, 16) = 5.448, P = 0.0396).

* P < 0.05 examined by repeated measures ANOVA followed by post-hoc Scheffe test.
Figure 15 Reduction of 5-HT efflux in the PFC by lowering ambient temperature to 12 °C or 6 °C
Figure 15 Reduction of 5-HT efflux in the PFC by lowering ambient temperature to 12 °C or 6 °C

5-HT was measured in the prefrontal cortex (PFC) of rat pretreated with clorgyline (2 mg/kg, s.c.). The experiments were carried out at ambient 12 °C or 6 °C. Data are shown as mean ± S.E.M. A, At ambient 12 °C, an increase in 5-HT efflux evoked by injection of 5 mg/kg 5-HTP was not statistically different from that at ambient 22 °C: F (1, 23) = 0.471, P = 0.4996. B, At ambient 12 °C, 5-HT efflux following 15 mg/kg of 5-HTP was insignificantly lower than the efflux at ambient 22 °C: F (1, 18) = 0.641, P = 0.4337, examined by repeated measures ANOVA. C, There was an ambient temperature-dependent change in 5-HT efflux following 25 mg/kg of 5-HTP. Compared to the efflux at 22 °C, there was a significant reduction in the efflux at ambient 12 °C: * P < 0.05, **P < 0.01 and ***P < 0.001 examined by repeated measures ANOVA followed by post-hoc Scheffe test). The efflux was further decreased at ambient 6 °C, compared to that at 12 °C: * Represented for P < 0.05, ** for P < 0.01 and *** for P < 0.001 examined by repeated measures ANOVA followed by post-hoc Scheffe test).
Figure 16 Changes in antagonistic effect of ketanserin (ket) on 5-HT efflux in the PO/AH at ambient 12 °C or 6 °C
Figure 16 Changes in antagonistic effect of ketanserin (ket) on 5-HT efflux in the PO/AH at ambient 12 °C or 6 °C

Rats pretreated with clorgyline (2 mg/kg, s.c.) were placed in temperature-controlled chambers at ambient 12 °C or 6 °C. 5-HT was measured in the preoptic/anterior hypothalamus (PO/AH). Dash lines are represented the data re-plotted from figure 14. Ketanserin was injected 15 min (5 mg/kg, i.p.; open head arrow) before 5-HTP (25 mg/kg, s.c.).

A, At ambient 12 °C, the increase in 5-HT efflux was significantly attenuated by ketanserin injection ($F_{(1,12)} = 4.643; P = 0.0476$).

B, At ambient 6 °C, the increase in efflux was not altered by ketanserin ($F_{(1,9)} = 0.511; P = 0.4928$).

* $P < 0.05$, examined by repeated measures ANOVA followed by Scheffe test.
Figure 17 Changes in antagonistic effect of ketanserin (ket) on 5-HT efflux in the PFC at ambient 12 °C or 6 °C
Figure 17  Changes in antagonistic effect of ketanserin (ket) on 5-HT efflux in the PFC at ambient 12 °C or 6 °C

Animals pretreated with clorgyline (2 mg/kg, s.c.) were placed in controlled temperature chambers at ambient 12 °C or 6 °C. 5-HT efflux in the prefrontal cortex (PFC) was determined using in vivo microdialysis. Dash lines represent the data re-plotted from figure 15. Ketanserin was injected 15 min (5 mg/kg, i.p.; open head arrow) before 25 mg/kg of 5-HTP (s.c.). A, At ambient 22 °C, the increase in 5-HT efflux was significantly attenuated by ketanserin injection (F (1, 12) = 14.230; P = 0.0023). B, At ambient 12 °C, the effect was insignificantly attenuated by ketanserin (F (1, 19) = 2.088; P = 0.1647). C, At ambient 6 °C, the 5-HT efflux was not altered by ketanserin injection (F (1, 14) = 0.001; P = 0.9823).

P < 0.05 and **P < 0.01 examined by repeated measures ANOVA followed by post-hoc Scheffe test.
4.0 PART II: EFFECT OF HIGHER AMBIENT TEMPERATURE ON 5-HT INDUCED TOXICITY

Antidepressants elevate 5-HT in the brain but excess 5-HT caused by administering a combination of drugs or a single high dose can lead to serotonin syndrome. This toxicity can be exacerbated if the ambient temperature is increased (Saadat et al, 2005, Malberg and Seiden, 1998), leading to death. Therefore it is imperative to study the mechanism responsible for worsening the syndrome at higher ambient temperature. This is explored in the following section.

4.1 Effect of ambient temperature on 5-hydroxy-L-tryptophan-induced serotonin-toxicity syndrome in clorgyline-pretreated rats

4.2 Abstract

Cumulative evidence suggests that environmental conditions, particularly ambient temperature ($T_{amb}$), appear to be a critical factor in determining the severity of serotonin-toxicity syndrome (5-HT toxidrome). The goal of this study was to examine the hypothesis that warm $T_{amb}$ enhances 5-HT$_{2A}$R response to excessive 5-HT efflux, the effect responsible for an exacerbation of the toxidrome. In this study, the 5-HT precursor 5-hydroxy-L-tryptaphan (5-HTP) was administered to induce an excessive elevation of 5-HT efflux while the severity of toxidrome was estimated by measuring changes in body-core temperature ($T_{cor}$) of clorgyline-pretreated animals. Experiments were carried
out at $T_{\text{amb}}$ of 22 °C and 32 °C. Injection of 5-HTP produced hypothermia at 22 °C but hyperthermia and death at 32 °C, the alteration blocked by the 5-HT$_2$AR antagonist ketanserin. Microdialysis showed that there was a ~10-fold increase in 5-HT efflux in the preoptic/anterior hypothalamus and prefrontal cortex at $T_{\text{amb}}$ of 22 °C. In contrast, the efflux at $T_{\text{amb}}$ 32 °C was significantly augmented by up to ~20 fold increases. When ketanserin was co-administered with 5-HTP at $T_{\text{amb}}$ of 32 °C, the augmented response was significantly reduced. Data analysis revealed that excessive 5-HT associated with the toxidrome consists of two origins: a primary efflux caused by drug administration and a secondary efflux induced by activation of 5-HT$_2$ARs. Although the primary efflux was critical for initiation of the toxidrome, further data analysis suggests that such efflux was still much lower than the minimum level that usually caused a malignant response. Altogether, our results support the hypothesis that the responsivity of 5-HT$_2$ARs is enhanced at warm $T_{\text{amb}}$, the effect responsible for the augmented efflux of 5-HT and exacerbation of toxidrome under our experimental condition.

4.3 Introduction

Serotonin-toxicity syndrome (toxidrome) is an adverse response to excessive increases in 5-hydroxytryptamine (5-HT; serotonin) in the central nervous system (CNS). The toxidrome is iatrogenic, which occurs among humans who use antidepressants (Boyer and Shannon, 2005; Gillman, 2005) or illicit drugs such as 3,4-methylenedioxymethamphetamine (MDMA) for recreational purposes (Parrott, 2002). Under laboratory conditions, monoamine oxidase inhibitors (MAOIs) in combination with 5-HT precursors is a common approach in producing an animal toxidrome model to
understand the cause-and-effect mechanisms of excessive 5-HT in the CNS (Grahame-Smith, 1971; Jacobs and Klemfuss, 1975; Shioda et al., 2004). Changes in body-core temperature ($T_{cor}$) and mortality in response to drug administration can be used to estimate the severity of the toxidrome (Ma et al., 2008). When hyperthermia occurs, the toxidrome is life-threatening and often ends with death. Blocking 5-HT$_{2A}$Rs are effective in controlling the malignant response (Nisijima et al., 2001; Gillman, 2005; Capela et al., 2006).

By examining the toxic response of MDMA in humans and also in experimental animals, recent studies have led to an interesting suggestion that the severity of neurotoxicity is strongly dependent on environments, particularly ambient temperature ($T_{amb}$) (Gordon et al., 1991; Malberg and Seiden, 1998; Parrott, 2002; O'Shea et al., 2005). Although much of the evidence implicates that 5-HT efflux might be responsible for the $T_{amb}$-dependent effect (Stanley et al., 2007), this notion should be reexamined since MDMA is a multipotent drug which is known to cause an increase in not only the 5-HT levels but also in the levels of dopamine (Baumann et al., 2008). Another issue with using MDMA is that it induces neurodegeneration in culture(Capela et al., 2006), which can complicate the understanding of the $T_{amb}$-dependent response. Thus, the role of 5-HT efflux in the $T_{amb}$-dependent response remains to be validated. To address these complications, a toxidrome, induced in rats pretreated with the MAOI clorgyline followed by administration of the 5-HT precursor 5-hydroxy-L-tryptophan (5-HTP), was adopted in this study. The advantage of this research model was that the drugs alone had no ‘messy’
effect on neurons as caused by MDMA. Hence, it is conceivable that only 5-HT would be involved if a toxicity response was generated.

To date, no precise study of the relationship between $T_{\text{amb}}$ and the toxidrome has been reported. In this study, the drug-induced toxidrome was examined at 22 °C and 32 °C. The degrees of the toxidrome were estimated by muscle reaction, changes in $T_{\text{core}}$ and mortality as described in our previous study (Ma et al., 2008). Using in vivo microdialysis, 5-HT efflux at the preoptic/anterior hypothalamic area (PO/AH) and the prefrontal cortex (PFC) was determined during the toxidrome. The PO/AH was chosen for its implication in thermoregulatory processes (Romanovsky, 2007) and possible involvement of hyperthermia in the toxidrome. The PFC was investigated because it was ascribed crucially for emotional and cognitive dysfunction in the toxidrome (Boyer and Shannon, 2005). Experiments were conducted in temperature-controlled chambers.

4.4 Material and Method

4.4.1 Animals

Adult Male Sprague–Dawley rats were purchased from Charles River Laboratories (Raleigh, NC, USA). The rats were housed in groups of two or three, with food and water available ad libitum, and were kept on a normal 12-h light/dark cycle at a room temperature of 22 ± 0.5 °C and humidity controlled facility. Housing and experimental treatment of the animals were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and Animal Research Guidelines at Florida Atlantic University.
4.4.2 Drugs

N-methy-N-propargyl-3-(2, 4-dichlorophenoxy) propylamine hydrochloride (Clorgyline) and 5-hydroxy-L-tryptophan (5-HTP) were purchased from Sigma (St. Louis MO, USA). Ketanserin tartate was obtained from Tocris Bioscience (Ellisville, MO, USA). Clorgyline and 5-HTP were dissolved in isotonic saline (NaCl 0.9%) and ketanserin (5 mg/kg) was suspended in water (H₂O). Except for ketanserin which was given intraperitoneally (i.p.), all the other drugs were injected subcutaneously (s.c).

4.4.4 Protocol for induction of a toxidrome

Clorgyline is an irreversible inhibitor for monoamine oxidase (MAO) and retains a long-lasting effect by single injection (Fletcher and Yu, 1989) while the effect of 5-HTP is transit due to a rapid metabolism. A toxic response would be variable if 5-HTP was administered before a stable effect of clorgyline on the MAO was established. Thus, an appropriate time period for clorgyline to take effect before 5-HTP injection is crucial for induction of a comparable toxidrome. Our preliminary study showed that the same level of the toxidrome was evoked if clorgyline was administered 2 - 12 h before 5-HTP. In this study, rats weighing 300-350g were pretreated with clorgyline (2 mg/kg) subcutaneously at $T_{amb}$ of 22 °C, 2 h before the start of experiment conducted in temperature-controlled chambers. Clorgyline-pretreated animals were randomly assigned to groups for testing the effect of 5-HTP or vehicle control. Then, animals were habituated for 2 h in $T_{amb}$ of 22 °C, 32 °C and 37 °C chambers and samples were then taken at 15-min intervals. After 2 - 4 baselines, a toxidrome was induced by injection of 5 or 10 mg/kg 5-HTP (or ~4 – 5 h after clorgyline injection). In the case of experiments for
delineating a role of 5-HT$_{2A}$Rs, the selective 5-HT$_{2A}$R antagonist ketanserin was administered 15 min before 5-HTP.

4.4.5 Measurement of body-core temperature ($T_{cor}$)

The temperature in the colon was considered to be $T_{cor}$. Rats were gently restrained and inserted into the colon with a thin, flexible thermoprobe connected to a digital meter (Traceable®, Fisher Scientific). The length of the probe was set at 4.6 cm. Two measurements were obtained as baselines before 5-HTP injection and hereafter eight measurements at intervals of 15 min following 5-HTP injection. In a preliminary study, it was found that a maximum effect on $T_{cor}$ occurred at 120 min. Thus, one measurement was taken at this time particularly for understanding the antagonistic effect of ketanserin.

4.4.6 Brain microdialysis

Rats, which were anesthetized with a combination of xylazine (4 mg/kg, i.p.) and ketamine (80 mg/kg, i.p.), were placed on a stereotaxic frame (Stoelting Co., Wood Dal, Illinois, USA) for implantation of guide cannulae as described elsewhere (Tao and Auerbach, 1995). Briefly, the cannulae, prepared from 21-gauge stainless steel tubing, were surgically implanted. The coordinates for the preoptic/anterior hypothalamic area (PO/AH) were AP -1.1 mm relative to bregma, ML ± 0.9 mm, and DV - 3.0 mm below the surface of the skull and for prefrontal cortex (PFC) were AP +3.3 mm relative to bregma, ML ± 0.7 mm, and DV -2.0 mm (Paxinos and Watson, 1998). Acrylic dental cement and skull screws were used to secure the guide cannulae into the place. Microdialysis was not begun sooner than one week recovery from the surgery.
Dialysis probes were implanted into either the PO/AH or PFC through the guide cannulae the day before the experiment. The exchange surface was 1.0 mm for the PO/AH probe or 2.5 mm for the PFC probe. Coordinates for the probe tip were AP -1.1 mm, ML ±0.9 mm and DV -9.2 mm in the PO/AH and AP +3.3 mm, ML ±0.7 mm and DV -4.5 mm in the PFC. Rats were then placed in the test chamber and attached to a Raturen® system (Bioanalytical System Inc., W. Lafayette, IN, USA). Food and water were available ad libitum. The dialysis probes were perfused overnight with a modified, buffered artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 3.0 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 0.25 mM NaH₂PO₄, 1.0 mM Na₂HPO₄, pH 7.4), which was pumped at a rate of 1.0 µl/min. Chambers were set at T_{amb} of 22 °C for the overnight perfusion.

Next day, after the animals were treated with clorgyline (2 mg/kg, s.c.) for 2 h, chambers were set to desired temperatures (22 °C, 32 °C or 37 °C). 5-HT sampling was typically conducted between 1:00 pm – 6:00 pm after at least 2-h habituation. Samples were collected every 15 min in microcentrifuge vials and analyzed by high performance liquid chromatography (HPLC) - electrochemical detection (HTEC-500; EICOM, Japan) with a CMA/200 refrigerator microsampler (CMA/Microdialysis, Stockholm, Sweden). Separation of 5-HT was achieved on a 150 mm ×1 mm i.d. column packed with a TSK gel ODS-80 TM, 5 µM particle size. The potential on the graphite was set at + 400 mV (relative to AG/AgCl reference electrode). The mobile phase was made of 0.1 M phosphate buffer (pH 6.0), 1% methanol, 500 mg/L sodium-1-octanesulfonate, and 50 mg/L ethylenediamine-tetracetic acid and pumped at a rate of 500 µl/min. Detection limit was 0.05 pg.
After completion of microdialysis, the animals were deeply anesthetized with pentobarbital (100 mg/kg, i.p). Probe locations were determined by infusing with 2% Fast Green for 10 min. The animals were decapitated and brains were then removed, frozen, and sliced using a cryostat. The probe location was determined by comparison to the rat brain atlas (Paxinos and Watson, 1998). Probes located outside the correct area were excluded from the data analysis.

4.4.7 Data analysis

Unless otherwise noted, data were expressed as mean ± standard error. In figure 18 and 19, $\Delta T_{cor}$ on the y-axis ($^\circ$C) was expressed as changes in degree of Celsius from the baseline plotted against the sampling time on the x-axis (min). In figure 20-25, changes in 5-HT efflux were normalized to the relative increases (in fold) over their respective baselines. The 5-HT efflux, expressed as absolute values (pg/sample), was uncorrected from probe recovery. Note that the baseline was calculated from the mean of four sequential effluxes before drug injection. Statistical analysis was performed by a two-way (drug treatment × time) factorial ANOVA. If significant interactions of drug treatment × time course were found, further statistical analysis was carried out using the post-hoc Scheffe test in determining the significance of the respective time points. If appropriated, the Student t-test was employed for the data analysis. The level of P-value was set at 0.05 for a statistically significant effect.
4.5 Results

4.5.1 \(T_{\text{amb}}\)-dependent changes in \(T_{\text{cor}}\) induced by 5-HTP

Clorgyline (2 mg/kg, s.c.) was administered \(~4\ h (2\ h \text{ before and } 2\ h \text{ after chambers were set to desired temperatures})\ before examining the \(T_{\text{amb}}\)-dependent effect on \(T_{\text{cor}}\) in temperature-controlled chambers. The mean basal \(T_{\text{cor}}\) was 36.5 (± 0.2 °C; \(n = 15\)), 37.3 (± 0.2 °C; \(n = 18\)) and 37.8 (± 0.3 °C; \(n = 10\)) measured in \(T_{\text{amb}}\) of 22 °C, 32 °C and 37 °C chambers, respectively. Injection of vehicle (0.9% NaCl) had no effect on \(T_{\text{cor}}\). Figure 18A shows that 5-HTP injection produced a time- and dose-dependent reduction in \(T_{\text{cor}}\) in the clorgyline pretreated rats examined at \(T_{\text{amb}}\) of 22 °C. Data analysis with repeated measures ANOVA revealed that there were significant main effects on doses (\(F(2, 12) = 14.782, P = 0.006\)) and sampling time (\(F(7, 84) = 3.561, P = 0.0021\)). However, an interaction between doses × sampling time was insignificant (\(F(14, 84) = 1.431, P = 0.1572\)). Administration of 5 mg/kg 5-HTP caused no change in \(T_{\text{cor}}\) (\(P > 0.05\)), compared to saline-treated rats. After injection of 10 mg/kg 5-HTP, there was a profound decrease in \(T_{\text{cor}}\) (\(F(1, 8) = 24.958, P = 0.0011\)). The decline in \(T_{\text{cor}}\) was evident with a nadir approximately -2.0 °C at 30 min and gradually returned to baseline in 120 min.

Next groups of animals were tested at \(T_{\text{amb}}\) of 32 °C. As shown in figure 18B, there was a dose- and time-dependent increase in \(T_{\text{cor}}\). Repeated measures ANOVA revealed significant main effects of doses (\(F(2, 15) = 15.722, P = 0.0002\)) and sampling time (\(F(7, 105) = 26.622, P < 0.0001\)) and a significant interaction of dose × time (\(F(14, 105) = 13.103, P < 0.0001\)). Specifically, 5 mg/kg 5-HTP did not produce a significant change (\(P > 0.05\))
in $T_{cor}$, compared to the control group. In contrast, 10 mg/kg caused a significant increase by 5.2 °C ± 0.4 ($F_{(1, 10)} = 25.094, P = 0.001$). It was worthwhile mentioning that animals died 2 h after 5-HTP injection. Thus, considering the fact of hyperthermia and high mortality, the toxicity effect of 5-HTP was exacerbated at warm $T_{amb}$. To confirm the $T_{amb}$-dependent effect, $T_{amb}$ was set at 37 °C to further test the response of clogyline-pretreated animals. We predicted that the toxicity would be more robust if the $T_{amb}$ were the factor for the severity of toxidrome. As shown in figure 18C, $T_{cor}$ rose rapidly after 5-HTP administration and the animals died in 75 min.

4.5.2 Effect of the 5-HT2A antagonist ketanserin on $T_{cor}$

In this set of experiments the mean basal $T_{cor}$ was 36.7 (± 0.2 °C; n = 10), 37.0 (± 0.2 °C; n = 9) and 37.9 (± 0.3 °C; n = 4) at $T_{amb}$ of 22 °C, 32 °C and 37 °C chambers, respectively. Ketanserin (5 mg/kg, i.p.), a 5-HT2A receptor antagonist, was injected 15 min before 5-HTP (10 mg/kg). Figure 19A shows effect of ketanserin on 5-HTP-induced hypothermia at $T_{amb}$ of 22 °C. Ketanserin failed to block the hypothermic response. On the contrary, $T_{cor}$ was further reduced when the antagonist was employed ($F_{(1, 8)} = 5.3336, P = 0.0497$).

Figure 19B shows the effect of ketanserin on hyperthermia engendered by 5-HTP at $T_{amb}$ of 32 °C. Pretreatment with ketanserin blocked the increase in $T_{cor}$. Statistical analysis revealed a significant effect of the antagonistic treatment ($F_{(1, 15)} = 63.617, P < 0.0001$) and time course ($F_{(7, 105)} = 24.491, P < 0.0001$) and a significant interaction of dose × time ($F_{(7, 105)} = 56.525, P < 0.0001$). Since the $T_{amb}$-dependent effect was blocked by ketanserin, this suggests that the responsivity of 5-HT2ARs activation was enhanced at
warm $T_{\text{amb}}$ of 32 °C in contrast to that at 22 °C. To explore this view, animals were tested at $T_{\text{amb}}$ 37 °C. We predicted that exacerbation would be alleviated by ketanserin pretreatment if the $T_{\text{amb}}$-dependent effect was mediated by 5-HT$_{2A}$Rs. As shown in figure 19C, the rising slope was reduced by ketanserin although the hyperthermic response was not completely blocked. A statistical analysis by comparing the difference in $T_{\text{core}}$ between ketanserin-treated and untreated animals revealed that the reduction was still significant ($F_{(1, 6)} = 11.510, P = 0.0146$). Importantly, injection of ketanserin reduced the lethality associated with $T_{\text{amb}}$-dependent response.

4.5.3 Changes in 5-HT efflux in the PO/AH

4.5.3.1 Effect of altered $T_{\text{amb}}$ on basal 5-HT

Samples for measuring 5-HT efflux were first taken 2 h after clorgyline pretreatment (2 mg/kg, s.c.) at $T_{\text{amb}}$ of 22 °C., The basal efflux in the PO/AH was 0.21 pg/sample (± 0.02, n = 12). The animals were then habituated in a 32 °C chamber for another 2 h and tested whether changes in $T_{\text{amb}}$ would alter the basal efflux. At $T_{\text{amb}}$ of 32 °C, the basal efflux became 0.32 ±0.04 pg/sample. The mean increase was 61% (± 17%). The paired t-test revealed the change was significant ($P < 0.05; n = 12$).

4.5.3.2 Dose-dependent effect of 5-HTP at $T_{\text{amb}}$ of 22 °C

In this set of experiments the basal 5-HT was 0.23 pg/sample (± 0.02, n = 21). Figure 20 shows that injection of 5-HTP produced a dose- and time-dependent increase in 5-HT efflux. This conclusion was confirmed by two-way factorial ANOVA, which revealed that there was a significant main effect of doses ($F_{(2, 18)} = 12.865, P = 0.0003$) and
sampling time ($F_{(7, 126)} = 2.606, P = 0.0152$) and a significant interaction of dose × time
($F_{(14,126)} = 2.8, P = 0.012$). Compared to respective baselines, the mean increases were ~3 fold in response to 5 mg/kg and 10-fold following 10 mg/kg 5-HTP.

### 4.5.3.3 $T_{amb}$-dependent effect

The baseline at $T_{amb}$ of 32 °C for this set of experiments was 0.27 pg/sample (± 0.02, n = 19). Vehicle injection had no effect. In contrast, 5 mg/kg 5-HTP produced an increase in 5-HT efflux (figure 21A). Compared to vehicle, the increase was significant: $F_{(1, 10)} = 9.234$, $P = 0.0125$. However, the effect was not different from that observed with the same dose at $T_{amb}$ of 22 °C: $F_{(1, 16)} = 0.381$, $P = 0.5455$. Figure 21B shows the effect of 10 mg/kg 5-HTP on 5-HT efflux at $T_{amb}$ of 32 °C. Compared to vehicle injection, there was a marked increase in 5-HT efflux at $T_{amb}$ of 32 °C following 5-HTP injection: $F_{(1, 9)} = 14.986$, $P = 0.0038$. The maximum increase was ~20-fold above the baseline. Compared to the response at $T_{amb}$ of 22 °C, the increase was greatly augmented. ANOVA test showed a significant main effect of $T_{amb}$ treatment ($F_{(1, 10)} = 6.802$, $P = 0.0261$) and sampling time ($F_{(6, 60)} = 7.431$, $P < 0.0001$) although there was no significant interaction between $T_{amb}$ × time ($F_{(6,60)} = 1.31$, $P = 0.2668$).

### 4.5.3.4 Effect of ketanserin

The 5-HT$_{2A}$R antagonist ketanserin (5 mg/kg, i.p.) was employed to test whether the increases or augmentation evoked by 10 mg/kg 5-HTP were mediated via 5-HT$_{2A}$Rs. Figure 22A shows effect of ketanserin on 5-HT efflux at $T_{amb}$ of 22 °C. Injection of ketanserin attenuated the late phase of efflux between 60 - 120 min ($F_{(1, 10)} = 5.096$, $P = 0.038$).
Figure 22B shows the effect of ketanserin at $T_{\text{amb}}$ of 32 °C. Similarly, ketanserin significantly attenuated the late efflux of 5-HT induced by 5-HTP: ($F_{(1, 9)} = 6.103, P = 0.0355$). After pretreatment with ketanserin, all animals survived from the lethal injection of 5-HTP at warm $T_{\text{amb}}$.

**4.5.4 Changes in 5-HT efflux in the PFC**

**4.5.4.1 $T_{\text{amb}}$-dependent increases in basal 5-HT**

Microdialysis samples were taken from the prefrontal cortex (PFC) of clogyline-pretreated rats at $T_{\text{amb}}$ of 22 °C. The efflux of 5-HT was $0.32 \pm 0.02$ pg/sample ($n = 12$). Rats were then habituated in a 32 °C chamber and samples were taken again 2 h later. There was a 60% increase in 5-HT efflux ($0.50 \pm 0.04$ pg/sample) in response to the switch of $T_{\text{amb}}$ to a warmer condition.

**4.5.4.2 Dose-dependent effect of 5-HTP at $T_{\text{amb}}$ 22 °C**

In this set of experiments, the basal efflux in the PFC was $0.23 \pm 0.02$ pg/sample ($n = 21$). Figure 23 shows a dose- and time-dependent increase in 5-HT efflux. The conclusion was supported by data analysis which revealed that the injection of 5-HTP resulted in a significant main effect of doses ($F_{(2, 17)} = 23.527, P < 0.0001$) and time course of sampling ($F_{(7, 119)} = 4.902, P < 0.0001$) and a significant interaction of dose $\times$ time ($F_{(14, 119)} = 2.646, P = 0.0022$). Subsequent post-hoc analysis of time points indicated that 5-HT efflux in response to either dose of 5-HTP (5 mg/kg or 10 mg/kg) displayed a greater increase throughout 120-min observation than the rats expose to saline. The significant differences in efflux amongst two doses were observed 60 min after 5-HTP injection.
4.5.4.3 $T_{\text{amb}}$-dependent effect of 5-HTP

Mean basal efflux was $0.32 \pm 0.04$ pg/sample at $T_{\text{amb}}$ 32 °C ($n = 15$). Figure 24 shows 5-HT efflux in response to 5 mg/kg and 10 mg/kg 5-HTP at $T_{\text{amb}}$ of 32 °C. Vehicle injection (0.9% NaCl) had no effect on 5-HT efflux. Apparently, the efflux was elevated in response to 5 mg/kg 5-HTP (figure 24A). The effect was significantly greater in the warm $T_{\text{amb}}$ of 32 °C compared to that at 22 °C ($F (1, 14) = 4.658, P = 0.0488$). The post-hoc analysis demonstrated that the significant difference occurred 75-min after drug injection. Injection of 10 mg/kg 5-HTP at $T_{\text{amb}}$ of 32 °C also evoked an increase in 5-HT efflux (figure 24B). The effect was more robust compared to that at $T_{\text{amb}}$ of 22 °C, which was confirmed by ANOVA test ($F (1, 6) = 11.088, P = 0.0158$). Post-hoc comparison yielded a significant difference at time points of 60 and 75 min.

4.5.4.4 Effect of ketanserin

To test whether increases in 5-HT efflux was mediated through 5-HT$_{2A}$Rs, ketanserin (5 mg/kg, i.p.) was administered 15 min before 5-HTP (10 mg/kg, s.c.). As shown in figure 25A, ketanserin pretreatment reduced the increases induced by 5-HTP carried out at $T_{\text{amb}}$ of 22 °C. Post-hoc test showed that the blocking effect of ketanserin occurred in the later phase or 60 min after injection. Next, we tested the effect of ketanserin at $T_{\text{amb}}$ of 32 °C (figure 25B). In a separate experiment, 5-HTP produced robust increases in 5-HT efflux. Pretreatment with ketanserin blocked the efflux. This conclusion was confirmed by two-way factorial ANOVA, showing a significant main effect of the antagonist ($F (1, 8) = 8.505, P = 0.0194$) and sampling time ($F (5, 40) = 5.143, P = 0.001$) and a significant interaction between antagonist and time ($F (5, 40) = 3.427, P = 0.0114$). Scheffe post-hoc
comparison of time points found that 5-HT efflux was significantly lower as early as 45 min after 5-HTP injection in the animal pretreated with the antagonist.

4.6 Discussion

The present study revealed the relationship between ambient temperature (\(T_{\text{amb}}\)) and 5-HT toxicity induced by 5-HTP in the clopyline-pretreated rats. Our results demonstrated that 5-HTP, which evoked an excess of 5-HT efflux in the CNS, caused a \(T_{\text{amb}}\)-dependent response showing hypothermia at \(T_{\text{amb}}\) of 22 °C but hyperthermia and death at warm \(T_{\text{amb}}\) of 32 °C or 37 °C. These results confirmed the previous studies that 5-HT transmission is critical for the \(T_{\text{amb}}\)-dependent changes in severity of the toxidrome (Parrott, 2002; Stanley et al., 2007). The important finding in this current study was the effect was blocked by the 5-HT\(_{2A}\)R antagonist ketanserin, suggesting that the \(T_{\text{amb}}\)-dependent response may be due to an alteration of the 5-HT\(_{2A}\)R activity in response to the environment. Thus, although the relationship between excessive 5-HT and its toxicity has been revealed in the previous reports (Shioda et al., 2004; Baumann et al., 2008), this study extends the finding in demonstrating that 5-HT\(_{2A}\)Rs may serve as a key mediator in transmitting excessive 5-HT to the toxidrome in a \(T_{\text{amb}}\)-dependent manner. If correct, the hypothesis, which is greatly supported by the current observations, provides a new venue to understand the relationship between \(T_{\text{amb}}\) and 5-HT toxicity.

4.6.1 Methodological consideration: were changes in \(T_{\text{cor}}\) relevant to a toxidrome?

The toxidrome, which is induced by either antidepressants (Sternbach, 1991; Gillman, 2005) or MDMA (Parrott, 2002), shows characteristic disorders which can be classified into three aspects: mental state disability, autonomic dysfunction and neuromuscular
hyperactivity. In the animal research, an answered question is to what degree is the toxidrome induced by drugs and how to compare the severity of toxidrome between studies in different laboratories. Although standardized criteria to determine the severity of a toxidrome remains to be established, numerous efforts have been made in the last decades in the literature. For instance, the severity was estimated by scoring the neuromuscular responses including forepaw treading, hindlimb abduction, Staub tail, and tremors (Jacobs and Klemfuss, 1975; Darmani and Zhao, 1998). However, neuromuscular responses were notable to examiners only when the toxidrome became moderate to severe with drug doses closer or even greater than LD50 (Ma et al., 2008). Furthermore, we found that the neuromuscular hyperactivity was significant only when 5-HT efflux was over 30-fold above the baseline (Zhang and Tao, unpublished observation). In contrast, $T_{cor}$ was altered by as low as a 10-fold increase in 5-HT efflux (fig 18A and fig 20). Thus, it appears that measurements of $T_{cor}$ could obtain a subtle dose-response curve under our experimental condition. Based on this, the drug dose required in this study was much less than the previous work in which the toxidrome was typically induced by 50 mg/kg – 100 mg/kg of 5-HTP (Jacobs and Klemfuss, 1975; Nisijima et al., 2004). Also, we demonstrated, in the agreement with our previous finding (Ma et al., 2008), that the toxidrome was mild and benign following injection of 10 mg/kg 5-HTP in clogyline-pretreated rat at $T_{amb}$ 22 °C. This conclusion was supported by measuring the $T_{cor}$ which showed hypothermia. Additionally, although there was a significant head shaking behavior, it did not appear that other neuromuscular activity (forepaw treading, hindlimb abduction, Straub tail or tremor) was apparent. Thus, it is unlikely that such a mild response to the dose of 5-HTP injection could be detected
without an instrumental assistance. As the animals were tested at $T_{\text{amb}}$ 32 °C, a dramatic increase in $T_{\text{cor}}$ was observed (see figure 18B), suggesting the toxidrome became malignant (Rusyniak and Sprague, 2005). This was corroborated by the fact that the test animals died in 2-5 h. Regardless of the high mortality, perhaps even more important was the observation that there was no apparent neuromuscular change associated with the hyperthermia under our experimental condition. Thus, our data support the view that changes in $T_{\text{cor}}$ can be used to estimate the degrees of toxidrome in both normal and warm $T_{\text{amb}}$, particularly under the laboratory condition where the test doses are known to cause only a mild response.

4.6.2 Threshold 5-HT for a toxidrome

It is important to assess the threshold efflux of 5-HT required for induction of a toxidrome. It appears that antidepressants alone such as SSRIs or MAOIs produced a limited scale of 5-HT efflux, in a range of 1 – 5 fold above the baseline (Rutter and Auerbach, 1993; Fuller, 1994; Cremers et al., 2001). Such a small scale of efflux could be therapeutic and thus unlikely cause the 5-HT toxidrome. If this appears to be the case, there should be a critical threshold efflux above which would induce the toxidrome. Our results show that the toxidrome was induced by 10 mg/kg but not 5 mg/kg of 5-HTP, suggesting that the threshold associated with 5-HT toxicity could be estimated by measuring 5-HT efflux induced by two doses. We found that 5-HT efflux at $T_{\text{amb}}$ of 22 °C was ~ 2-3 fold in response to 5 mg/kg and ~10 fold following 10 mg/kg of 5-HTP. There was no difference between the PO/AH and PFC, implicating that the increase was global in the CNS. At warm $T_{\text{amb}}$, the efflux following 5 mg/kg could reach 5 fold. However, the
effect was not contingent with a toxidrome. Therefore, the value of the threshold was estimated between 5 to 10-fold over a basal efflux. The estimation does not conflict with earlier reports that a toxidrome was contingent with a 1000-fold increase of 5-HT efflux in the PO/AH (Nisijima et al., 2004; Shioda et al., 2004). On the contrary, our results suggest that there is a broad spectrum of 5-HT efflux associated with the toxidrome, which may explain the observations why the symptoms are highly variable (Mills, 1995). Similar conclusion was obtained from Baumann and colleagues (Baumann et al., 2007; Baumann et al., 2008) who showed that a MDMA dose, which was likely comparable to the amount for human uses, caused a 10-fold increase in 5-HT efflux in rats. It should be pointed out the fact that a mild toxidrome, if occurred, is hardly recognized by patients or physicians because it is transient and can be self-resolved with no needs of any medical treatment (Boyer and Shannon, 2005). However, as demonstrated in this study, the mild toxidrome would become malignant at warm $T_{\text{amb}}$. Thus, it is critical to understand the mechanism underlying the changes in severity of a toxidrome in a variety of $T_{\text{amb}}$.

4.6.3 Relationship between severity of toxidrome and $T_{\text{amb}}$

Our data strongly indicate that the severity of toxidrome is $T_{\text{amb}}$-dependent, showing that 5-HTP at a dose of 10 mg/kg produced a mild and benign response (hypothermia) at normal $T_{\text{amb}}$ whereas the same dose elicited a malignant reaction (hyperthermia and death) at warm $T_{\text{amb}}$ (figure 18). The malignant effect obtained at $T_{\text{amb}}$ of 37 °C was more robust. However, since animals appeared to be exhausted at 37 °C, our experiments were carried out mainly at $T_{\text{amb}}$ 32 °C, slightly warmer than the thermoneutral zone [ for rats, the thermoneutral zone ranged between 23 – 30 °C (Gordon, 1994) ].

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The question was raised whether the activity of serotonergic neurons was altered in response to changes in $T_{\text{amb}}$. While the data obtained by a single unit recording concluded that serotonergic neuronal activity in the dorsal raphe nuclei was not correspondent to changes in $T_{\text{amb}}$ (Fornal et al., 1987), our findings indicate that there was a marked elevation in basal 5-HT efflux in the PO/AH and PFC in habituation to the altered environment. One interpretation for the difference between the current finding and previous reports was attributable to animal species used in the studies (Von Huben et al., 2007). A more important observation was that 5-HT efflux induced by 5-HTP was augmented at ambient 32 °C. The augmentation was particularly obvious following 10 mg/kg, the dose that caused the toxidrome. However, it did not seem that 5-HT efflux was always augmented by warm $T_{\text{amb}}$. We also observed that although the effect on 5-HT efflux when 10 mg/kg 5-HTP was administrated was markedly augmented by warm $T_{\text{amb}}$, the enhanced increase induced by 5 mg/kg was not consistent in the PO/AH and PFC. One explanation for the observation was that the efflux elicited by 5 mg/kg 5-HTP was too small (< 5 fold above baseline) to activate the $T_{\text{amb}}$-dependent 5-HT$_{2A}$Rs (see discussion further below and figure 27). Thus, it can be concluded that a toxidrome would be exacerbated by warm $T_{\text{amb}}$ if the efflux reached the threshold efflux of 5-HT.

4.6.4 Role of 5-HT$_{2A}$Rs in the toxidrome

We showed that the administration of the 5-HT$_{2A}$R antagonist ketanserin blocked the hyperthermic response at $T_{\text{amb}}$ of 32 °C and attenuated the effect at 37 °C (figure 19). Another important observation was that mortality was aborted at ambient 32 °C and reduced at 37 °C by ketanserin pretreatment. The data supports the hypothesis that the
key factor that perpetuates the benign and malignant toxidrome in a $T_{\text{amb}}$-dependent manner may be the activation of the 5-HT$_{2A}$Rs. It is likely that the activity of 5-HT$_{2A}$Rs was increased at warm $T_{\text{amb}}$ in adaptation to the environment.

It has been demonstrated in many studies that two serotonergic receptors, namely 5-HT$_{1A}$R and 5-HT$_{2A}$Rs, exert crucial roles in the toxidrome (Abdel-Fattah et al., 1995; Darmani and Zhao, 1998; Nisijima et al., 2001; Isbister and Buckley, 2005; Fox et al., 2007). It has been suggested that 5-HT$_{1A}$Rs are responsible for neuromuscular hyperactivity (Darmani and Zhao, 1998; Fox et al., 2007) and hypothermia (Abdel-Fattah et al., 1995; Ma et al., 2008). In contrast, 5-HT$_{2A}$Rs are crucial for hyperthermia and death (Nisijima et al., 2001; Ma et al., 2008). For this reason, we limited our observation to the examination of the 5-HT$_{2A}$Rs.

The mechanism underlying how warm $T_{\text{amb}}$ alters the receptor activity remains to be determined. Several reports, indirectly supporting this study, indicate that the activity of 5-HT$_{2A}$R was indeed subject to changes under a variety of exogenous conditions. For instance, Artigas and his colleagues provided evidence that both densities of 5-HT$_{2A}$Rs and affinities to 5-HT were increased in the PFC following a long term use of antidepressants (Zanardi et al., 2001; Messa et al., 2003). Other investigators found that the intracellular signaling pathways were enhanced and thus responsible for sensitized activity of 5-HT$_{2A}$Rs (Damjanoska et al., 2003; Lee et al., 2007). It would be interesting to examine any molecular processes mentioned above are involved in warm $T_{\text{amb}}$-mediated changes in 5-HT$_{2A}$Rs.
4.6.5 Animal death relevant to 5-HT efflux

We observed that there was a ~20 fold increase in 5-HT efflux coincident with mortality of clorgyline-pretreated animals at warm $T_{\text{amb}}$ following 10 mg/kg of 5-HTP. It appears that the amount of 5-HT caused by lethal injection was much less than the previous reports (Nisijima et al., 2004; Shioda et al., 2004). In a separate investigation, we observed at $T_{\text{amb}}$ of 22 °C that the death occurred when 5-HT efflux was over 50-fold above the baseline (Zhang and Tao, unpublished observation). Thus, although excessive 5-HT in the CNS was responsible for the death, the incident was determined not only by the amount of 5-HT efflux but also by the $T_{\text{amb}}$.

To reveal the triangular relationships between death, 5-HT efflux and $T_{\text{amb}}$, it is necessary to understand the question of how 5-HT efflux is augmented at warm $T_{\text{amb}}$. It has been shown that 5-HT$_2$A Rs have an excitatory effect on neurons (Aghajanian and Marek, 1999) and elicit an increase in 5-HT efflux in the CNS (Martin-Ruiz et al., 2001; Bortolozzi et al., 2003). Thus, the augmented increase in 5-HT efflux was the consequence of the increased responsivity of the 5-HT$_2$A Rs, which were activated by excessive 5-HT. If this were the case, it could be postulated that excessive 5-HT efflux was derived from two distinct origins, as schematically hypothesized in figure 26: 1) a primary ($1^\circ$) efflux, which was strictly related to the causative drugs, was responsible for initiation or induction of the toxic process. In this regard, extracellular accumulation as the result of clorgyline and 5-HTP injection is ascribed to a Ca$^{2+}$-independent and carrier-mediated release via the reverse transportation (Gobbi et al., 1993; Silva et al., 2008); 2) a secondary ($2^\circ$) efflux was added into the 5-HT pools of extracellular space, which was
achieved by 5-HT$_2$AR activation caused by $1^\circ$ efflux. Thus, excessive efflux responsible for the toxidrome contains 5-HT$_2$AR-independent ($1^\circ$) and dependent ($2^\circ$) components. Since activity of 5-HT$_2$ARs was greater in the warm $T_{amb}$, this might explain the notion that the reduction of 5-HT efflux, which was caused by ketanserin pretreatment, was more robust at warm $T_{amb}$ compared to normal $T_{amb}$. Though caution should be made in extrapolating the results from animal studies, our data provide experimental evidence supporting the hypothesis that the responsivity of 5-HT$_2$ARs to 5-HT efflux may be altered significantly for adaptation to environment, the change responsible for increased severity of toxidrome at warm $T_{amb}$ (Parrott, 2002).
Figure 18 $T_{\text{amb}}$-dependent response of $T_{\text{cor}}$ to 5-HTP injection

A, $T_{\text{amb}}$ 22°C

B, $T_{\text{amb}}$ 32°C

C, $T_{\text{amb}}$ 37°C

Figure 18 $T_{\text{amb}}$-dependent response of $T_{\text{cor}}$ to 5-HTP injection
Figure 18 $T_{\text{amb}}$-dependent response of $T_{\text{cor}}$ to 5-HTP injection

Rats were pretreated with clorgyline (2 mg/kg, s.c.) and placed in the temperature-controlled chamber at 22 °C, 32 °C, or 37 °C for 2 h before experiments. Data are mean ± S.E.M. A, Injection of 5 - 10 mg/kg 5-HTP produced a dose- and time-dependent reduction of $T_{\text{cor}}$ in rats tested at $T_{\text{amb}}$ of 22 °C. B, At $T_{\text{amb}}$ of 32 °C, the same doses of 5-HTP evoked a dose- and time-dependent increase in $T_{\text{cor}}$. Additionally, the animals died in 2 h after injection of 10 mg/kg 5-HTP. C, At $T_{\text{amb}}$ of 37 °C, there was a swift response to 5-HTP injection in developing hyperthermia. The animals died within 75 min. *P < 0.05, **P < 0.01, and ***P < 0.001 examined by repeated measures ANOVA followed by post-hoc Scheffé test.
Figure 19 Effect of ketanserin on $T_{cor}$ and mortality evoked by 5-HTP
Figure 19 Effect of ketanserin on $T_{\text{cor}}$ and mortality evoked by 5-HTP

Rats were pretreated with clorgyline (2 mg/kg, s.c.) and placed in the temperature-controlled chamber at 22 °C, 32 °C, or 37 °C 2 h before experiments. The 5-HT$_{2A}$R antagonist ketanserin (5 mg/kg, i.p) was injected 15 min before 10 mg/kg 5-HTP. Data are mean ± S.E.M. A, At $T_{\text{amb}}$ of 22 °C, ketanserin failed to block the hypothermic response. Instead, injection of ketanserin further facilitated reduction in $T_{\text{cor}}$: $F(1, 8) = 5.337, P = 0.0497$. B, At $T_{\text{amb}}$ of 32 °C, ketanserin pretreatment blocked the hyperthermic response induced by 5-HTP. In addition, all animals survived after ketanserin treatment. C, At $T_{\text{amb}}$ of 37 °C, ketanserin pretreatment partially but significantly attenuated the hyperthermic response to 5-HTP injection. The mortality was also reduced.

*P < 0.05, **P < 0.01, and ***P < 0.001 examined by repeated measures ANOVA followed by post-hoc Scheffe test.
Figure 20 Dose- and time-dependent effects on 5-HT efflux in the PO/AH
Figure 20 Dose- and time-dependent effects on 5-HT efflux in the PO/AH

5-HT was measured in the preoptic/anterior hypothalamus (PO/AH) of the rat pretreated with clorgyline (2 mg/kg, s.c.). The experiments were carried out at ambient 22 ºC. Data are normalized and expressed as fold increases above baseline (± S.E.M.). Injection of 5-HTP at $T_{\text{amb}}$ of 22 ºC produced a dose- and time-dependent increase in 5-HT efflux in the PO/AH. *P < 0.05 and **P < 0.01 examined by repeated measures ANOVA followed by post-hoc Scheffe test.
Figure 21  T<sub>amb</sub>-dependent increases in 5-HT efflux in the PO/AH
Figure 21 $T_{\text{amb}}$-dependent increases in 5-HT efflux in the PO/AH

5-HT was measured in the PO/AH of the rat pretreated with clorgyline (2 mg/kg, s.c.). Data are normalized and expressed as fold increases above baseline (± S.E.M.). The dash-lines indicate that the data are re-plotted from figure 20. **A**, Injection of 5 mg/kg at $T_{\text{amb}}$ of 32 °C produced a significant increase in 5-HT. However, there was no statistically significant difference from that at $T_{\text{amb}}$ of 22 °C. **B**, At $T_{\text{amb}}$ of 32 °C, 10 mg/kg 5-HTP produced a significant increase in 5-HT efflux compared to control. The increase was significantly greater than that at $T_{\text{amb}}$ of 22 °C. *P < 0.05 examined by repeated measures ANOVA followed by *post-hoc* Scheffe test.
Figure 22 Effect of ketanserin on 5-HT efflux induced by 10 mg/kg 5-HTP
Figure 22 Effect of ketanserin on 5-HT efflux induced by 10 mg/kg 5-HTP

5-HT was measured in the PO/AH of the rat pretreated with clorgyline (2 mg/kg, s.c.). Data are normalized and expressed as fold increases above baseline (± S.E.M.). Dashed lines represent data re-plotted from figure 21. Ketanserin was administrated 15 min before 5-HTP. A, At $T_{\text{amb}}$ of 22 °C, 5-HT efflux induced by 10 mg/kg 5-HTP was significantly attenuated by ketanserin pretreatment. B, At $T_{\text{amb}}$ of 32 °C, the effect on 5-HT efflux and mortality of 10 mg/kg 5-HTP was significantly attenuated by ketanserin.

* $P < 0.05$ and **$P < 0.01$ examined by repeated measures ANOVA followed by Scheffe test.
Figure 23 Dose- and time-dependent effects on 5-HT efflux in the PFC
Figure 23 Dose- and time-dependent effects on 5-HT efflux in the PFC

5-HT was measured in the prefrontal cortex (PFC) of the rat pretreated with clorgyline (2 mg/kg, s.c.). The experiments were carried out at $T_{\text{amb}}$ of 22 ºC. Data are normalized and expressed as fold increases above baseline (± S.E.M.). Injection of 5-HTP produced an increase in 5-HT efflux in the PFC.

*P < 0.05 and **P < 0.01 examined by repeated measures ANOVA followed by post-hoc Scheffe test.
Figure 24  $T_{amb}$-dependent effects on 5-HT efflux in the PFC
**Figure 24 T\textsubscript{amb}-dependent effects on 5-HT efflux in the PFC**

5-HT was measured in the PFC of the rat pretreated with clorgyline (2 mg/kg, s.c.). Data are normalized and expressed as fold increases above baseline (± S.E.M.). Dash lines represent the data re-plotted from figure 23. **A**, Compared with vehicle, injection of 5 mg/kg 5-HTP at $T\text{amb}$ of 32 ºC produced an increase in 5-HT efflux. The effect was significantly greater than that at $T\text{amb}$ of 22 ºC. **B**, 10 mg/kg 5-HTP at $T\text{amb}$ of 32 ºC evoked a marked increase in 5-HT efflux compared with the vehicle group. Compared with that at $T\text{amb}$ of 22 ºC, the increase was significantly greater. Also, animals died 90 min after 5-HTP injection.

*P < 0.05 and **P < 0.01 examined by one-way repeated measures ANOVA followed by post-hoc Scheffe test.*
Figure 25 Effect of ketanserin on 5-HTP-evoked increases in 5-HT in the PFC
Figure 25 Effect of ketanserin on 5-HTP-evoked increases in 5-HT in the PFC

5-HT was measured in the prefrontal cortex (PO/AH) of the rat pretreated with clorgyline (2 mg/kg, s.c.). Ketanserin (5 mg/kg, i.p.) was injected 15 min before 5-HTP (10 mg/kg, s.c.). Data are mean ± S.E.M. The increases in 5-HT efflux induced by 5-HTP at $T_{\text{amb}}$ of 22 °C (A) and 32 °C (B) were antagonized by ketanserin.

* P < 0.05, **P < 0.01, ***P < 0.001 examined by ANOVA followed by Scheffe test.
Figure 26 Origins of 5-HT effluxes in a toxidrome
Figure 26 Origins of 5-HT effluxes in a toxidrome

Excessive 5-HT responsible for a toxidrome is derived from two origins: primary (I°) and secondary (2°) effluxes. A characteristic role of two effluxes in the toxidrome is hypothetically divided into 3 phases. Phase I°, causative drugs increase the I° efflux, the level of which is typically determined by drug-mediated effect on pathways of 5-HT synthesis and metabolism. Once the I° efflux reaches a threshold (e.g., >10 fold over baseline), toxicity responses are induced by over-activation of 5-HT1ARs and 5-HT2ARs. Phase 2°, although the effect of 5-HT1ARs is inhibitory and causes a decrease in 5-HT efflux (Rutter and Auerbach, 1993), over-activation of 5-HT2ARs increases the 2° efflux of 5-HT (Bortolozzi et al., 2003). However, the effect of 5-HT2ARs is strongly T_amb-dependent. Lastly phase 3°, addition of the 2° efflux in the extracellular space further exacerbates the toxidrome caused by the I° efflux. The present study revealed that over 50% 5-HT in warm T_amb is ascribed to 2° efflux and thus it is critical to control the 2° efflux through 5-HT2AR antagonists in the treatment of toxidrome (Nisijima et al., 2001). Proposed mechanisms are also supporting the hypothesis that the 5-HT2AR-mediated response is life-threatening, which cannot be blocked by 5-HT1AR antagonists (Nisijima et al., 2001).
5.0 GENERAL DISCUSSION

Serotonin syndrome (SS) is an iatrogenic disorder that can lead to life-threatening hyperthermia and death. Understanding the effect ambient temperature has on the syndrome is important in preventing mortality. Although there has been a large amount of animal research on the effect of excess cerebral 5-HT (Isbister and Buckley, 2005), progress still needs to be made in understanding and effectively treating SS in patients. Our results indicate that 5-HT$_{2A}$ receptor, in particular, plays a significant role in contributing to the toxic increase of cerebral 5-HT. Furthermore, the activation of this receptor seems to be sensitive to changes in the ambient temperature. This is a significant finding for many reasons. The first is that there are two pathways postulated to be involved in increasing cerebral 5-HT, one that is dependent on ambient temperature ($T_{amb}$) and one that is independent. It is this “dependent” pathway that contributes to the toxic increase of 5-HT in the brain. Moreover, this $T_{amb}$- dependent effect results in a positive feedback mechanism involving the 5-HT$_{2A}$ receptor on the PFC and on the PO/AH. Furthermore, besides the 5-HT$_{2A}$ receptors, NMDA receptor on the DRN may also contribute to the toxic increase in 5-HT and this receptor also appears to be dependent on ambient temperature (see figure 30 for preliminary data). All these ideas will be explored further below.
5.1 5-HT$_{2A}$ Receptor Activity

There are two pathways postulated to be involved in increasing CNS 5-HT. One involves the metabolic pathway which takes place in the serotonergic (and also noradrenergic) axonal terminal. This pathway involves 5-HT synthesis by the enzyme L-amino acid decarboxylase and deactivation into 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO) (Green and Grahame-Smith, 1974). Thus, excess 5-HT would be induced by combined injection of 5-HTP and MAO inhibitor (MAOI) clorgyline. This pathway is independent of the ambient temperature and the increased level of CNS 5-HT is responsible for serotonin syndrome. This explains why there will always be certain level of 5-HT efflux regardless of the ambient temperature. Excess 5-HT associated with this pathway is responsible for most of signs of the syndrome but not death. Additional increase in 5-HT is determined by the neural circuitry between DRN and PFC. We hypothesis this pathway is dependent on the ambient temperature-dependent activity of the 5-HT$_{2A}$ receptor, which in turn is responsible for fatality (see figure 27). Hence blocking the 5-HT$_{2A}$ receptor is vital in preventing mortality, which is the major reason why our focus has been on this particular subtype among at least 14 5-HT receptors.

The temperature-dependent activation of the 5-HT$_{2A}$ receptors promotes a positive feedback hypothesis. This is explored further below.

5.2 Overview of Positive-Feedback Mechanism

Excess 5-HT evoked by the combination or single high dose of antidepressants can lead to toxicity or serotonin syndrome. Changes in ambient temperature have been shown to
greatly alleviate or augment serotonin syndrome. It is believed that this is due to changes in 5-HT$_{2A}$ receptor sensitivity at different ambient temperatures. Our overall view is shown in figure 28. The DRN serotonergic neurons project to both the PFC and PO/AH. *We hypothesized that the DRN-PFC pathway is responsible for an additional increase in 5-HT, which leads to hyperthermia and death.* We believe that at normal ambient temperature, the DRN-PFC pathway is activated only when a high dose of 5-HTP is administrated since 5-HT$_{2A}$ receptors have a low affinity to 5-HT. The activation of this pathway causes the serotonergic neurons (DRN) to release 5-HT, which, in turn, activates the 5-HT$_{2A}$ receptor in the PFC. The glutamatergic neurons in the PFC then releases glutamate and activates the NMDA receptor in the DRN, causing the serotonergic neurons to release more 5-HT and promote a positive feedback mechanism. At low ambient temperature, however, the 5-HT$_{2A}$ receptors are *desensitized*, and hence the positive feedback mechanism is not activated. At higher ambient temperature, on the other hand, the 5-HT$_{2A}$ receptor becomes *sensitized* thus even a low dose of 5-HT can activate this pathway, leading to a substantial increase in CNS 5-HT. The sensitized 5-HT$_{2A}$ receptor on the PO/AH is also activated causing hyperthermia and death. Thus drugs designed to block the receptor at the PFC or the PO/AH can help prevent or help alleviate this life-threatening symptom.

In further support of this positive-feedback hypothesis, our preliminary results show (discussed in the next section) that the NMDA receptors, found on DRN, appear to be sensitive to changes in $T_{amb}$ and thus glutamate may also be involved in increasing cerebral 5-HT concentration. This view will be explored further.
5.3 Involvement of NMDA receptors

In addition to 5-HT$_{2A}$ receptors, NMDA receptors may be involved in the 5-HT syndrome and the positive feedback pathway between the raphe and the PFC. To lend credence to this view, the effect of pre-treating animals with NMDA antagonist MK-801 (0.25 mg/kg, i.p.) and administering 5-HTP was analyzed at a high ambient temperature. The results show that the core body temperature was prevented from rising and hyperthermia was prevented (see figure 30). Our result suggest that glutamate may plays a role the pathophysiology of SS and this is supported by other researchers that reveal that in SS animal models, there is elevated levels of glutamate in the hypothalamus (Shioda et al, 2004) and that blocking the glutamate receptor, NMDA, prevents SS induced lethality (Nisijima et al, 2004). This is intriguing as glutamate has been reported to be involved in the neurotoxicity associated with brain trauma, hyperthermia, and ischemia. It is possible, that some of the symptoms associated with SS may thereby be attributed to the glutamatergic system (Shioda et al, 2004).

Besides glutamate and serotonin, other neurotransmitters like dopamine and noradrenalin have been shown to be elevated in the severe SS animal model. Their increase was found to occur in the hypothalamus when clorgyline and 5-HTP were employed and microdialysis was used (Nisijima et al, 2001) or when MAO inhibitor and tricyclic antidepressants were utilized and the tissue levels in several brain regions were measured (Marley and Wozniak, 1984). Furthermore, MDMA, a drug that has been shown to cause SS syndrome (Parrott, 2002), was reported to also increase the levels of DA in the brain. These findings are important since SS has many clinical symptoms associated with the
disorder and the possibility that other neurotransmitters, beside serotonin and glutamate, could play a part will further our understanding of the disease and allow the development of better drugs to treat and prevent SS (Shioda et al, 2004). This is imperative as the current therapy to treat depression has some potential concerns. This is delineated below.

5.4 Problem with Current Drug Therapy

The previous accepted notion that a deregulation of monoamine neurotransmission causes depression (i.e. monoamine hypothesis) can no longer be simply used to explain and treat the pathophysiology of the disease effectively (Schechter et al, 2005). In fact, a large portion of patients do not respond adequately to this monoamine pharmacological treatment (Nemeroff, 2008). Studies show that glutamate, GABA, vasopressin, substance P, BDNF (brain-derived neurotropic factor), etc may also contribute to the etiology of the disorder. Hence future generation of successful antidepressant drugs should look into these and other areas (Schechter et al, 2005). Although a lot of literature is available on alternative targets to treat depression (Schechter et al, 2005, Berg et al, 2008, Kvernmo et al, 2008, Malberg and Monteggia, 2008, Martinowich et al, 2007, etc) focus will be made on the problems associated with the current treatment of depression.

There is an increasing change in research to try to determine the pathophysiology of distinct subtypes of syndromes ranging from diabetes to mood disorders. This is essential in not only identifying each disease’s unique cause, but also in the consequential hope of developing novel drug treatment. Currently, the treatment of patients who suffer from major depression is usually treated in a trial-and-error method, using drugs that have been
shown to be effective in the past. Unfortunately, 28-40% patients end up suffering from remission. Although evidence shows that serotonin, norepinephrine, and dopamine play a preeminent role, other factors like corticotrophin-releasing factor (CRF), glutamate, and GABA also play a part. Furthermore, the risk of developing depression is attributed to both genetics (1/3rd) and environment (2/3rd). This adds a multifaceted dimension to the problem since drugs have traditionally targeted the molecular aspect of the disease. Moreover, major advances in genetics and molecular neurobiology have utilized mice where else rats have been used to study psychotic disorders. Thus the lack of animal models to study depression has impeded some advancement in mood disorders (Nemeroff, 2008). Though novel therapies to treat the disorder include targeting excitatory amino acids, GABA, peptidergic system, or neurotrophins, much work still needs to be done to ensure that new drugs target unmet clinical needs and that long-term modification occurs. Thereby, current pharmaceutical therapy that uses a symptomatic or disease modifying approach requires an overhaul if successful antidepressant drugs need to be developed (Schechter et al, 2005). In all cases, adverse serious side-effects like SS should be avoided or greatly minimized to make certain the treatment of patients is not discontinued.

5.5 Side Effect of Antidepressant Treatment: Serotonin Syndrome

Our results give some insight as to how toxicity could arise, therefore addressing a more immediate concern. That is, it underscores the importance the environment, namely ambient temperature, plays in exacerbating the syndrome. Understanding the effect ambient temperature has on the syndrome is crucial in not only preventing mortality, but
also in designing drugs that can treat the elevated rise in core body temperature. Though some reports suggest that the 5-HT₁₅ receptor may contribute to SS, is now considered to play a lesser role in the toxicity, and instead, may augment some of the SS effects but not the life-threatening one (Isbister and Buckley, 2005). Therefore to this end, targeting the 5-HT₂₅ receptor since it is involved in hyperthermia is imperative in preventing fatality. Thus, recognizing the signs and symptoms of SS early is imperative. Serotonin syndrome cannot be attributed to a certain combination of symptoms but more to a continuum of clinical manifestation that differs in number and severity. These range from mild to severe, with hyperthermia attributed to the life-threatening symptom (Isbister and Buckley, 2005).

SS management is often looked at in two-folds. The first is that it is vital that there is good supportive care, especially in severe cases, and the second is to prevent the progression of the disorder to a severe and life-threatening form. Recognizing the moderate to severe form is plausible and the toxicity is predicted based on the drugs (or combination) ingested and clinical symptoms that arise. Patients suffering from SS are simply treated by stopping the suspected drug treatment and treating the symptoms (Isbister and Buckley, 2005). It is recognizing the mild form and preventing its deterioration to a severe form that is critical.

5.6 Conclusion/ Future

A better understanding of the etiology of the disorder will allow the devolvement of successful and safe antidepressant drugs. The current treatment has not proved to be as
effective as previously hoped and worst, serious side-effects like toxicity have arisen. Unfortunately, although our knowledge of depression has increased, we have not been able to develop effective pharmaceutical drugs that not only treat the disorder, but also minimize the side effects. In some cases, these side-effects can be as disruptive to the patient as the disease itself, causing voluntary or mandatory discontinuation of the drug.

It is not enough to learn how the disease occurs but also how to prevent the problems associated with long-term use of the drugs. To this end, our current results shed some light as to how one side-effect, serotonin syndrome, could result and how environment factors like ambient temperature could severely augment the problem. In treating serotonin syndrome, future work still need to be done to determine if and how many other players are involved and whether other receptors like the 5-HT\textsubscript{1A}, etc are implicated in the toxicity. Only when clear understanding of the all the factors involved in SS are discovered, can this disorder be treated and even more importantly, prevented.
Figure 27 Schematic of Proposed Pathway
There are two pathways postulated to be involved in increasing CNS 5-HT. One involves the metabolic pathway and the other involves the neural circuitry or depolarization pathway. The first pathway (1) takes place in the serotonergic (and also noradrenergic) axonal terminal and involves 5-HT synthesis by the enzyme L-amino acid decarboxylase and deactivation into 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO) (Green and Grahame-Smith, 1974). Excess 5-HT is induced by the combined injection of 5-HTP and MAO inhibitor (MAOI) clorgyline. This pathway is independent of the ambient temperature and the increased level of CNS 5-HT is responsible for the symptoms of serotonin syndrome, but not death. Additional increase in 5-HT is determined by a second pathway (2) involving the neural circuitry between DRN and PFC. This pathway is postulated to be dependent on the ambient temperature-dependent activation of the 5-HT$_{2A}$ receptor, which in turn is responsible for fatality.
Figure 28 Proposed Feedback Hypotheses
Figure 28 Proposed Feedback Hypotheses

The DRN serotonergic neurons project to both the PFC and PO/AH. The $T_{amb}$-dependent sensitization of the 5-HT$_{2A}$ receptor promotes a positive feedback mechanism involving DRN’s NMDA receptor. This mechanism involves the temperature-dependent activation of the 5-HT$_{2A}$ receptor on the PFC which causes the glutamatergic neuron to release glutamate and activate the NDMA receptor on the DRN. This in turn causes the DRN serotonergic neuron to release 5-HT and the cycle repeats, further increasing 5-HT into toxic level. The sensitized 5-HT$_{2A}$ receptors on the PO/AH are also activated causing hyperthermia and death. Therefore this pathway is dependent on the ambient temperature. At lower ambient temperature the receptors on the PFC and PO/AH are desensitized and hence the positive feedback mechanism is not activated. At higher ambient temperature, the sensitized receptors become activated and hence, even at a low level of 5-HT efflux can promote the feedback mechanism.
Figure 29 $T_{\text{amb}}$-Dependent Effects on 5-HT$_{2A}$ Receptor Activity
Figure 29 $T_{amb}$-Dependent Effects on 5-HT$_{2A}$ Receptor Activity

At normal ambient temperature (22 ºC), a certain level of 5-HT is required to activate the 5-HT$_{2A}$ receptors, stimulating the positive feedback mechanism. Stimulation of this pathway increases 5-HT to a toxic level, leading to death. At low ambient temperature, the 5-HT$_{2A}$ receptor becomes de-sensitized; hence the positive feedback hypothesis is not activated. As a result, the 5-HT efflux at the PO/AH and the PFC remains low and the animal survives. However, at higher ambient temperature, the sensitization of the 5-HT$_{2A}$ receptor, in particular at the PFC, results in elevated level of 5-HT being released due to a positive feedback mechanism involving the dorsal raphe nucleus (DRN) and PFC. Furthermore, even a lower level of 5-HT can activate the sensitized 5-HT$_{2A}$ receptor on the PFC, leading to fatality. Thus this toxic increase in 5-HT can be due to either high endogenous levels of cerebral 5-HT or due to the temperature dependent activation of the receptors.
Figure 29 Effect of NMDA Antagonist, MK-801, on $T_{cor}$
Figure 30 Effect of NMDA Antagonist, MK-801, on $T_{cor}$

Animals pre-treated with NMDA antagonist MK-801 (0.25 mg/kg, i.p.) and administrated a high dose (10 mg/kg, s.c.) of 5-HTP were housed at a high ambient temperature (32 °C). The results show that compared to animals injected with only 10 mg/kg 5-HTP, pre-treating with MK-801 prevented the core body temperature from rising and hence, hyperthermia and death was prevented. These results lend credence to the view that there is a temperature dependent activation of the 5-HT$_{2A}$ receptors that promotes a positive feedback mechanism and this mechanism involves both the 5-HT$_{2A}$Rs and the NMDA receptors. The result suggest that beside the 5-HT$_{2A}$R, the NMDA receptor might be sensitive to changes in $T_{amb}$. 
6.0 GENERAL REFERENCE


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