ROLES OF SEROTONIN 2A RECEPTOR IN A SEROTONIN SYNDROME

by

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ABSTRACT

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Serotonin (5-HT) is a neurotransmitter in the central nervous system. Decrease in the brain 5-HT level could induce depression, showing a state of low mood, aversion to motion and feeling of worthlessness. About 12 million adults in the United States have depression. Antidepressants, such as monoamine oxidase inhibitors and selective serotonin reuptake inhibitors, can alleviate the depressive mood by increasing the brain's 5-HT activity, however they can also induce a potentially life-threatening side effect, namely 5-HT syndrome. This syndrome is manifested by neuromuscular hyperactivities, mental disorders and autonomic dysfunctions. Clinical studies have demonstrated that 5-HT_{2A} receptor antagonists could effectively block severe symptoms of patients with the 5-HT syndrome. To understand the underlying mechanisms, in this study we examined the activity of the 5-HT_{2A} receptor in rats with the 5-HT syndrome evoked by a combined injection of

clorgyline, a monoamine oxidase inhibitor, and paroxetine, a selective 5-HT reuptake inhibitor. The major findings from my study were that: (1) Chronic clorgyline treatment significantly exacerbated 5-HT_{2A} receptor-mediated symptoms of the 5-HT syndrome animals; (2) The 5-HT_{2A} receptor-mediated symptoms were also aggravated when the 5-HT syndrome animals were housed in warm (32 °C) ambient temperature; (3) Blocking 5-HT_{2A} receptors in the medial prefrontal cortex alleviated the 5-HT syndrome through a circuit between raphe serotonergic neurons and medial prefrontal cortex glutamatergic neurons. Taken together, my data demonstrate that the activity of 5-HT_{2A} receptors may be enhanced by chronic antidepressant treatment and warm environmental temperature. The sensitized 5-HT_{2A} receptor in the medial prefrontal cortex may exacerbate the syndrome through a positive-feedback circuit between medial prefrontal cortex and raphe nuclei, which would result in excessive 5-HT in the brain. This study casts a new light on the underlying mechanisms of the 5-HT syndrome.

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LIST OF ABBREVIATIONS

aCSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
AH	Anterior hypothalamus
ANOVA	Analysis of variance
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CLG	Clorgyline
DA	Dopamine
DAG	Diacylglycerol
DOI	(+/-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride
DRN	Dorsal raphe (raphé) nucleus
EDTA	Ethylenediamine tetraacetic acid
EEG	Electroencephalogram
EMG	Electromycogram
GPCR	G protein-coupled receptor
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine (serotonin)

5-HTP	5-Hydroxy-L-tryptophan
IP_3	Inositol 1,4,5-triphosphate
mPFC	Medial prefrontal cortex
MAO	Monoamine oxidase
MRN	Median raphe (raphé) nucleus
NE	Norepinephrine
NMS	Neuroleptic malignant syndrome
OCD	Obsessive compulsive disorder
8-OH-DPAT	8-Hydroxy-2-(di-n-propylamino) tetralin
OPA	O-phthaldialdehyde
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
POA	Preoptic area
PRX	Paroxetine
SS	Serotonin syndrome
SSRI	Selective 5-HT reuptake inhibitor
T_{amb}	Ambient temperature
T_{cor}	Core body temperature
TCA	Tricyclic antidepressant
Trp	Tryptophan

CHAPTER ONE: GENERAL INTRODUCTION

The biogenic amine 5-hydroxytryptamine (5-HT) or serotonin was first recognized over one hundred years ago for its effect on the smooth muscle. Since the middle of 19th century, scientists have known a chemical released from blood clots into sera could constrict vascular smooth muscle and increase its tension (Frazer and Hensler, 1999) . Not until 1947 did Rapport et al. (1947) first isolate and name this substance as 5-HT. Serotonin is an alternative name for 5-HT since it exists in sera and induces vascular activities. In 1953, Twarog and Page (1953) reported 5-HT's existing in the brain. At the physiological pH circumstance, 5-HT is a hydrophilic substance that cannot permeate the lipophilic blood-brain barrier readily. Therefore, the discovery from Twarog and Page indicates that brain can synthesize 5-HT by itself. Almost at the same time, Shore et al. (1955) reported that (+) lysergic acid diethylamide (LSD), a psychomimetic, could reverse responses induced by 5-HT. It raised the possibility that 5-HT regulated brain functions. Indeed, 5-HT plays critical roles in maintaining a number of brain functions, and abnormalities of 5-HT activity result in a set of mental disorders and psychiatric diseases, especially depression (for review, see Naughton et al., 2000; Barnes and Sharp, 1999; Frazer and Hensler, 1999; Hensler, 2006).

Depression is a mental disorder associated with a low 5-HT level in the brain. The patients have feeling of sadness, helplessness and worthlessness. A breakthrough of the

modern neurochemistry and neuropharmacology on depression was the development of antidepressants, such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs) and selective 5-HT reuptake inhibitors (SSRIs). These agents can increase the extracellular 5-HT levels in the brain, alleviate depression symptoms and rehabilitate the life quality of the patients. Up to now, millions of patients throughout the world are benefiting from these agents, however long term and inappropriate administration of these compounds may give rise to many side effects, one of which is 5-HT syndrome.

The 5-HT syndrome is an iatrogenic or drug-induced toxic side effect caused by excessive 5-HT in the central nervous system, which can be characterized by autonomic dysfunctions, neuromuscular hyperactivities and mental disorders (Boyer and Shannon, 2005). Although the first case report of the syndrome can be traced back to 50 years ago (Mitchell, 1955), the mechanism underlying this disorder remains unclear. Hence, the aim of my project was to explore the neural mechanisms of the 5-HT syndrome with a focus on serotonin 2A (5-HT_{2A}) receptor. In order to understand this immensely complex syndrome, the general information about the 5-HT system was described below.

1.1 5-HT metabolism and distribution in the central nervous system

The amino acid L-tryptophan (Trp) serves as the precursor for the synthesis of 5-HT in our body. L-Trp is a natural amino acid in daily supplements, such as dairy products and meat. L-Trp can cross the blood-brain barrier with the help of nonspecific amino acid transporters and enters the neurons in the central nervous system (CNS). After hydroxylation by Trp hydroxylase (a rate-limiting enzyme) and decarboxylation by

5-hydroxytryptophan (5-HTP) decarboxylase, 5-HT is produced and stored in the vesicles (Fig.1) located in axon terminals of the serotonergic neurons.

Like other biogenic amine neurotransmitters in the CNS, synthesized 5-HT is stored primarily in the presynaptic vesicles and released by exocytosis. Neurons accumulate 5-HT from cytoplasm and store it in the presynaptic vesicles with the help of vesicular transporters. Some anti-psychiatric drugs, e.g., tetrabenazine and reserpine, block the vesicle transporters and, thus, raise 5-HT level in the cytoplasm and facilitate 5-HT efflux (Gonzalez et al., 1994). Serotonergic neurons release 5-HT by a vesicular, Ca^{2+}- and impulse-dependent exocytotic mechanism (Levi and Raiteri, 1993). After secreted into the synaptic cleft, 5-HT binds presynaptic and postsynaptic receptors. The functional activity of 5-HT in the synaptic cleft is terminated with the assistance of selective 5-HT reuptake transporters (SERTs) distributed in the presynaptic membrane. After reuptake, some of 5-HT recycled is repackaged into vesicles for reuse. The rest is degraded by monoamine oxidase (MAO) at mitochondria. There are two kinds of MAO isoform: MAO-A metabolizes 5-HT, norepinephrine (NE), and dopamine (DA). MAO-B preferentially degrades trace amine (benzylamine, phenylethylamine, tyramine) and DA (Bach et al., 1988) (Fig. 2). Currently, most antidepressants increase the brain extracellular 5-HT level by blocking either MAO or SERT (Horstmann and Binder, 2009) .

Serotonin-containing neurons in the CNS cluster along the midline of the brainstem. Serotonergic neuron cell bodies in the CNS are found exclusively in the midline raphe nuclei ranging from the midbrain to the medulla and classified into nine groups (B1 to B9) (Frazer and Hensler, 1999). Raphe serotonergic neurons have a wide

connection with structures in the brain and spinal cord. The superior clusters (B5 - B9) send projections to cortex and subcortical structures, and the lower groups in the pons and medulla (B1 - B4) send axons to medulla and spinal cord (Fig. 3, Table 1) (Jacobs and Azmitia , 1992; Hesler, 2006). Likewise, raphe nuclei receive afferent fibers. The largest afferent source is from the raphe nuclei themselves. These fibers form a complex of local circuits among the nuclei and within each nucleus (Aghajanian and Wang, 1977). Other brain regions, e.g., hypothalamus, medial preoptic area, lateral hypothalamus and forebrain also send projections to the raphe nuclei (Sakai et al., 1977; Hoover and Vertes, 2007). Dorsal raphe nucleus (DRN, B7 and B6) and median raphe nucleus (MRN, B8 and B5) are the major sources of forebrain 5-HT. DRN lies between cerebral aqueduct and MRN, and consists of medial, lateral and caudal components. MRN is a paramedian and median cluster of cells lying between superior cerebellar decussation and trapezoid body. The dorsal border of this cluster fuses with the ventromedial component of the DRN, and the ventral side lies in paramedian columns extending ventrally from the medial longitudinal fasciculus (Fig. 3) (Jacobs and Azmitia, 1992).

1.2 5-HT receptors

To date, at least fourteen 5-HT receptor subtypes belonging to seven major families have been indentified in the brain. Since the first two distinct types of 5-HT binding sites (5-HT_1 and 5-HT_2 receptors) were reported in 1978, the research in 5-HT receptors has progressed considerately (Leysen et al., 1978; Frazer and Hensler, 1999; Hensler, 2006). Currently, seven distinct 5-HT receptors (5-HT_1 – 5-HT_7 receptors, Table

2) have been identified due to the application of novel molecular biological techniques. Moreover, several 5-HT receptors comprise subtypes. For example, 5-HT_1 receptor consists of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptors. 5-HT receptor protein exists both in the presynaptic and postsynaptic membranes in the CNS. Functionally, except the 5-HT_3 receptor which is a ligand-gated ion channel, all the 5-HT receptors are G protein-coupled receptors (GPCRs) that activate intracellular cascades and produce excitatory or inhibitory responses (Table 2). Detailed information about 5-HT receptors can be found in a number of reviews (Barnes and Sharp, 1999; Jacobs and Azmitia, 1992; Jacobs and Klemfuss, 1975; Naughton et al., 2000; Pritchett et al., 1988; Frazer and Hensler, 1999). Here the studies about 5-HT_{2A} receptor were reviewed for the knowledge served as the background related to this study. In addition, 5-HT_{1A} receptor was also reviewed for its close relation to the 5-HT_{2A} receptor in the development of the 5-HT syndrome (Fig. 4).

5-HT_{2A} receptor

The finding of 5-HT_{2A} receptor can be tracked back to 1978 when Leysen and his colleagues (1978) first indentified a novel receptor that could bind [^{3}H]-spiperone which was named 5-HT_{2A} receptor later (Pritchett et al., 1988). Genetic and molecular properties of the 5-HT_{2A} receptor have been well understood. In human, the locus of the 5-HT_{2A} receptor gene is on chromosome 13q14-q21. A great homogeneity of 5-HT receptor protein sequence exists among species. For example, both human and rat 5-HT_{2A} receptors consist of 471 amino acids with a 91% homology (Fig. 5). 5-HT_{2A} receptor is a member of GPCR family located in the cell membrane. Activation of 5-HT_{2A} receptor

enhances activity of intracellular phospholipase C (PLC) which then hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds its receptor in the membrane of smooth endoplasmic reticula and mitochondria, opens Ca^{2+} channels and increases intracellular Ca^{2+} concentration. Ca^{2+} as well as DAG activates protein kinase C (PKC), increases protein phosphorylation and, thus, leads to a series of cellular cascades (Roth et al., 1986). Other than the IP_3/DAG signaling pathway, the 5-HT_{2A} receptor is also coupled to arachidonic acid and endocannabinoid 2-arachidonoylglycerol pathways in the peripheral tissues (Parrish and Nichols, 2006).

Studies with physiological, pharmacological and molecular biological techniques (e.g., receptor autoradiography, in situ hybridization and immunocytochemistry) have revealed that 5-HT_{2A} receptors are widely distributed in the CNS, such as forebrain regions, cortical areas, caudate nucleus, nucleus accumbens, olfactory tubercle and hippocampus (Lopez-Gimenez et al., 1997; Mengod et al., 1990). Areas with the highest density includes, but not limited to cortex, especially the frontal cortex (Pompeiano et al., 1994; Roth et al., 1991; Wright et al., 1995). Moreover, a number of studies have explored the cellular location of the 5-HT_{2A} receptor in the brain with double immunostaining. 5-HT_{2A} receptor proteins are expressed in the cholinergic neurons of the pontomesencephalic tegmentum (Morilak and Ciaranello, 1993), cortical glutamatergic neurons (de Almeida et al., 2007) and GABAergic interneurons (Willins et al., 1997). Consistently, the distribution of the 5-HT_{2A} receptor expression is deemed to map the distribution of the serotonergic axon terminals arising from the raphe nuclei. For example, the profile of axons from DRN appears to match 5-HT_{2A} receptor distribution in the

prefrontal cortex (Azmitia and Segal, 1978; Conrad et al., 1974; Jacobs and Azmitia, 1992; Steinbusch et al., 1981).

Activation of the 5-HT_{2A} receptor induces neuronal excitation. By using intracellular and whole cell recording in rat brain slices, Aghajanian and Marek (1997) found that bath-applied 5-HT produced an increase in the frequency and amplitude of spontaneous excitatory postsynaptic potentials/currents (EPSPs/EPSCs) in layer V pyramidal cells of neocortex and transitional cortex. The EPSCs were suppressed by selective 5-HT_{2A} receptor antagonists (e.g., MDL 100907 and SR 46349B). Similarly, the 5-HT_{2A} receptor-induced neuronal excitation could also be detected in reticular gigantocellular nucleus, interstitial , the parvicellular and the lateral nuclei (Barresi et al., 2005) as well as locus coeruleus (Szabo and Blier, 2002).

5-HT_{2A} receptor participates in the regulation of a wide range of behaviors and physiological functions (for review, see Bubar and Cunningham, 2008), for example body temperature. 5-HT_{2A} receptor mediates hyperthermia. In the brain, activation of 5-HT_{2A} receptors in the POA increases the body temperature (Chio et al., 2005; Lin et al., 1998). In the peripheral tissue, 5-HT_{2A} receptors lead to vasoconstriction and prevention of heat loss (Ootsuka and Blessing, 2006). Moreover, 5-HT_{2A} receptors in the adipose tissue promote the catabolism of brown adipose tissue (Ootsuka and Blessing, 2006). Given the wide distribution of the 5-HT_{2A} receptor in brain regions (e.g., frontal cortex) that are related in cognitive functions and social activities, it appears that 5-HT_{2A} receptor involves mental regulation and psychiatric disorders. Indeed, 5-HT_{2A} receptor plays an important role in mental activities and related diseases (Jakab and Goldman-Rakic, 1998). Many hallucinogens (e.g., DOI and LSD) are 5-HT_{2A} receptor agonists modulating DA

release and inducing schizophrenia-like symptoms. Currently there is considerable interest in 5-HT_{2A} receptor in the psychiatric disorders for some typical and atypical antipsychotic agents (e.g., clozapine and olanzepine) with a partial 5-HT_{2A} receptor antagonist property (Meltzer, 1999). Chronic treatment with these antipsychotic drugs could decrease 5-HT_{2A} receptor binding capacity (Kuoppamki et al., 1995). Additionally, mood disorders, e.g., depression and anxiety, are thought to be associated with changes in 5-HT_{2A} receptor expression and activity. PET scan research indicated that there was an about 29% reduction of 5-HT_{2A} receptor binding capacity in depressed subjects (Mintun et al. 2004). Long-term antidepressant treatment down-regulated the 5-HT_{2A} receptor density in the cortex (Kuoppamki et al., 1995; Peroutka and Snyder, 1980). Administration of 5-HT_{2A} receptor agonists led to a significant up-regulation of BDNF mRNA, a gene related to depression, in the neocortex (Vaidya et al., 1997). Moreover, the rest studies revealed a connection of the 5-HT_{2A} receptor to the obsessive compulsive disorder (OCD), attention deficit hyperactivity disorder (ADHD), affective disorders, suicide and Alzheimer's disease (Norton and Owen, 2005).

The variation of 5-HT_{2A} receptor activity due to antidepressant treatment has been the focus of many studies in the past decades by testing the level of 5-HT_{2A} receptor protein density, sensitization and mRNA expression. Using a [^{3}H]-spiroperidol binding assay, Peroutka and Snyder (1980) first reported a down-regulation of 5-HT_{2A} receptor density in rats receiving a long-term antidepressant treatment. This finding was later confirmed by other studies with different antidepressants including clomipramine (a TCA) (Todd et al., 1995), desipramine (a TCA) (Goodnough and Baker, 1994; Todd et al., 1995), maprotiline (a TCA) (Todd et al., 1995), citalopram (a SSRI) (Klimek et al., 1994),

5-HTP (Pranzatelli, 2009) and phenelzine (a MAOI) (Todd et al., 1995). Besides antidepressants, 5-HT_{2A} receptor agonists such as DOI (Pranzatelli and Pluchino, 1991; Anji et al., 2000), DOM (Leysen et al., 1989) and LSD (Ferry et al., 1993; Buckholtz et al., 1990) also significantly decreased the density of 5-HT_{2A} receptor. However, some reports showed that chronic uses of fluoxetine (a SSRI) caused little changes (Todd et al., 1995; Goodnough and Baker, 1994) or an increase (Klimek t al., 1994) in the 5-HT_{2A} receptor binding sites. The conflicting results from in vivo studies probably attribute to the differences in experimental techniques or conditions such as the dosage and duration of antidepressant exposure.

Results of in vitro studies also showed that exposure to serotonergic compounds have variable effects on the 5-HT_{2A} receptor density and activity. Both 5-HT_{2A} receptor agonists and antagonists might up-regulate the activity of 5-HT_{2A} receptor transfected into Madin-Darby canine kidney cells, but not into NIH 3T3 cells. The density of 5-HT_{2A} receptor could be decreased and increased, respectively by 5-HT_{2A} receptor agonist and antagonist in the transfected AtT-20 cells (Grotewiel and Sanders-Bush, 1994). The down-regulation of 5-HT_{2A} receptors may attribute to a rapid internalization of 5-HT_{2A} receptor via endosome (Vouret-Craviari et al., 1995; Berry et al., 1996). Additionally, 5-HT or DOI exposure increased the 5-HT_2 receptor mRNA expression (Akiyoshi et al., 1993) and binding sites (Chen et al., 1995) in rat cerebellar granule cells by de novo RNA and protein synthesis in this up-regulation process. Exposure to 5-HT or its agonists increased the level of 5-HT_{2A} receptor mRNA by increasing the half life in Pll cells (Ferry et al., 1994; Ferry and Molinoff, 1996) as well as transfected Madin-Darby canine kidney cells (Grotewiel and Sanders-Bush, 1994).

This discrepancy of the pros and cons suggests the changes of 5-HT_{2A} receptor capacity exposing to it agents depend on the cell milieu where they are expressed (Grotewiel and Sanders-Bush, 1994). In the cerebral neurons, agonist exposure may increase activity of 5-HT_{2A} receptor. In most other cells, the agonist induced a down regulation of the 5-HT_{2A} receptor.

Intracellular mechanisms responsible for the 5-HT_{2A} receptor activation (sensitization) after ligand exposure have also been described in the literature. 5-HT_{2A} receptor agonist induced an acute down-regulation of 5-HT_{2A} receptor-mediated phosphoinositide hydrolysis in smooth muscle cultures (Pauwels et al., 1990) and NIH 3T3 cell line (Bhattacharyya et al., 2002; Grotewiel and Sanders-Bush, 1994). Since chronic agonist exposure did not alter the $G_{a/q}$ level (Roth et al., 1995), it suggests that increased phosphorylation of $G_{q/11}$ proteins (Shi et al., 2007) or the calcium/calmodulin - sensitive kinase (Chen et al., 1995) may account for the change. PKC seems to participate in the 5-HT_{2A} receptor sensitization. The PKC antagonist could attenuate the 5-HT_{2A} receptor agonist-induced desensitization in platelets (Kagaya et al., 1990), but not in HEK-293 cells (Vouret-Craviari et al., 1995). Vouret-Craviari et al. (1995) proposed that it was the PKC activation, not the phosphorylation of the 5-HT_{2A} receptor that was responsible for 5-HT_{2A} receptor desensitization. Roth (1995) reported that the down-regulation of the PKC isozymes alpha and epsilon was responsible for the desensitization induced by agonist exposure. On the contrary, wohlpart and Molinoff (1998) found PKC and calcium could increase 5-HT_{2A} receptor mRNA expression in P11 cell line. Taken together, these results suggest a set of mechanisms involve the 5-HT_{2A} receptor sensitivity when it is exposed to its agents (Table 3).

5-HT_{1A} receptor

The brain 5-HT_{1A} receptor was initially detected in rat diencephalon and lower brain stem with ^{3}H-5-HT binding (Nelson et al., 1981) and was the first 5-HT receptor to be fully sequenced. Genetically, human 5-HT_{1A} receptor gene localizes on chromosome 5q11.2-q13 sharing 89% homology with the rat.

The profile of the 5-HT_{1A} receptor distribution in the brain has been well mapped by receptor autoradiography with a number of ligands, such as [^{3}H]-8-OH-DPAT (Gozlan et al., 1983), [^{3}H]WAY-100635 and [^{11}C] WAY-100636 (Hall et al., 1997). 5-HT_{1A} receptor mRNA and binding sites are high in the hippocampus, septum, amygdala, cortical limbic areas and raphe nuclei, but undetectable in caudate/putamen and cerebellar regions (Wright et al., 1995; Hall et al., 1997; Chalmers and Watson, 1991). At the cellular level, 5-HT_{1A} receptors are expressed in both postsynaptic neurons (e.g., cortical pyramidal neurons) and raphe serotonergic neurons (Chalmers and Watson, 1991).

Like 5-HT_{2A} receptor, 5-HT_{1A} receptor also belongs to the GPCR family with seven transmembrane helices connected by intra- and extracellular loops (Peroutka et al., 1981). Activating 5-HT_{1A} receptor inhibits adenylate cyclase second messenger system, decreases intracellular cAMP concentration, activates G-protein-coupled inwardly rectifying K^{+} channels (GIRKs; kir 3.x) (Tanaka and North, 1993; Andrade and Nicoll, 1987) and, eventually, hyperpolarizes neurons (Fig. 4). 8-OH-DPAT is one of the most selective 5-HT_{1A} receptor agonist and WAY-100635 represents a new class of 5-HT_{1A} receptor antagonists.

Activation of 5-HT_{1A} receptor induces a wide range of behavioral and

physiological responses in rodents, including 5-HT behavioral syndrome, hypothermia, anxiolysis, increase in ACTH and prolactin release (Hedlund et al., 2004; Backus et al., 1990; Hjorth, 1985; Andrade and Nicoll, 1987; Darmani and Zhao, 1998; Tanaka and North, 1993). Both brain and peripheral tissues involve the 5-HT_{1A} receptor-mediated hypothermia. In the central nervous system, activation of 5-HT_{1A} receptors in the POA evokes hypothermia (Lin et al., 1998). In the peripheral tissues, 5-HT_{1A} receptors contribute to cutaneous vasodilation, decreased metabolism and enhanced heat dissipation (Lin et al., 2002). 5-HT behavioral syndrome is a series of behaviors induced by activating post-synaptic 5-HT_{1A} receptor (Lucki et al., 1984) which will be covered in the next section. Besides behavioral regulation, the 5-HT_{1A} receptor also involves the mental regulation and dysfunction of the 5-HT_{1A} receptor leads to many mental disorders, e.g., major depression (Savitz J et al., 2009; Fornal et al., 1996).

1.3 5-HT and brain functions

Serotonin in the brain involves the regulation of a wide range of physiological functions and behaviors. Since Amin first reported brain 5-HT in 1954 (Amin et al., 1954), the role of 5-HT in the CNS have been widely investigated and a great progress has been achieved. The superior rostral end of this raphe serotonergic system sends projection to cortex and subcortical structures, which involves the regulation of sleep-wakefulness cycle, affective behavior, food intake, thermoregulation, migraine, emesis and sexual behavior. The serotonergic neurons in the lower pons and medulla send axons to spinal and medulla, and participate in the regulation of nociception and motor

tone (Saper, 2000; Blundell, 1977; Barnes and Sharp, 1999). Dysfunction of the serotonin system in the brain results in a great array of disorders including depression, irritability, aggression, impatience and anxiety, especially with suicidal depression patients showing a significant decrease in 5-HT levels (for review, see Saper, 2000; Jacobs and Azmitia, 1992; Kriegebaum et al., 2010). Decrease in brain 5-HT level is a major factor associated with the depression, a state of low mood, aversion to motion and feeling of worthlessness. An advance of modern neuropharmacology was the synthesis and application of a set of antidepressants including tricyclics, lithium, MAO inhibitors and SSRIs. These antidpressants can raise brain 5-HT levels, alleviate the depressant syndrome and improve mood (Jacobs and Azmitia, 1992; Saper, 2000; Kriegebaum et al., 2010). Like a double-edged sword, antidepressant exposure could also induce a series of side effects including euphoria, hypomania, restlessness, rapid speech, anxiety, insomnia, aggressiveness, agitation, light headedness and peripheral side effects (e.g., nausea, vomiting, diarrhea, anorexia) (Nonherbal. 1998; van Vliet et al. 1996). Clinically, some of these side effects have been typically classified into the symptoms of the 5-HT syndrome.

1.4 Serotonin syndrome

History

Theoretically, 5-HT syndrome can be defined as an iatrogenic disorder resulted from pharmacological treatment with 5-HT-selective agents that excessively elevate the level of extracellular 5-HT in the brain (Baloh et al., 1982; Darmani and Ahmad , 1999;

Oates and Sleight, 1960). The first case of the 5-HT syndrome could be tracked back to half a century ago when a patient died from the co-administration of a MAOI iproniazid and a synthetic narcotic compound meperidine (Mitchell, 1955). Due to the limited knowledge about 5-HT, this incident was misdiagnosed and received little attention for decades. In the following years, a great number of 5-HT-related behavioral abnormalities were described in animals with elevated brain 5-HT level (Jacobs, 1976). Jacobs (1976) summarized the related animal reports and proposed a specific term "serotonin behavioral syndrome" to define the 5-HT-mediated behaviors. The term "5-HT syndrome" was first introduced by Insel et al. (1982). After the 1980s, more and more antidepressant-related toxicity were reported. Some patients have the similar behavioral symptoms as described early in the 5-HT behavioral syndrome animals. To standardize the diagnosis, Sternbach (1991) reviewed 38 human case reports that had been published in literature up to that time and defined a criterion of the 5-HT syndrome in human. After that, 5-HT syndrome was widely adopted by clinical professionals and lab researchers. Even though 5-HT syndrome is widely used, many researchers employed other terms including 5-HT toxic syndrome, 5-HT toxidrome, 5-HT behavioral syndrome or 5-HT toxicity to substitute 5-HT syndrome in publications.

Epidemiology

To date, the 5-HT syndrome has become a common clinical problem in medicine associated with increasing prescription of antidepressants as well as abusing of serotonergic drugs (Isbister et al., 2007; Bronstein et al., 2008). The American Association of Poison Control Centers-National Poison Data System, which receives case

descriptions from office-based practices, inpatient settings and emergency departments, reported an increasing incident of antidepressant toxicity and death resulted from antidepressant exposure in the past decades (Fig. 6). There were 98,898 incidences reported due to antidepressant exposure and 220 deaths in 2007 (Bronstein et al., 2008).

The incidence of 5-HT syndrome may be underestimated. First, clinicians or patients might misfile the incident reports of the syndrome due to its variable manifestations. For example, they attributed the anxiety and akathisia to the patient's mental disorder (Sampson and Warner, 1999). Second, a strict application of the diagnostic criterion proposed by Sternbach potentially rules out what are now recognized as mild, early, or subacute cases of the disorder (Radomski et al., 2000; Sternbach, 1991; Hegerl et al., 1998). Third, some physicians may be unaware of the 5-HT syndrome as a clinical diagnosis (Mackay et al., 1999). Therefore, performing a rigorous epidemiologic assessment of the 5-HT syndrome is difficult. It is predicated that the syndrome occurs in approximately 14 to 16 percent of persons who overdose on SSRIs (Isbister et al., 2004).

Agents associated with the 5-HT syndrome

In theory, any agents with an ability of increasing 5-HT activity in the brain can induce the 5-HT syndrome. 5-HT syndrome has been reported in patients with a history of taking MAOIs (Kojima et al., 1993), tricyclic antidepressants (TCAs) (Hodgman et al., 1997; Spigset et al., 1993), SSRIs (Bhanji, 2000; Pao and Tipnis, 1997; Graber et al., 1994; Bhanji, 2000; Pao and Tipnis, 1997), opiate analgesics, over-the-counter cough medicines (Skop et al., 1995; Ener et al., 2003), etc. The withdrawal of medications has also been associated with the syndrome (for review, see Ener et al., 2003). Functionally,

these agents can be classified into several groups: (1) Increase the precursor supply (e.g., 5-HTP); (2) Increase 5-HT release (e.g., amphetamines); (3) Block the reuptake transporters (e.g., SSRIs); (4) Inhibit the catabolic enzymes (in the cleft and presynaptic neurons) (e.g., MAOIs); (5) Inhibit the presynaptic 5-HT_{1A} receptor (e.g., sumatriptan); (6) Stimulate the postsynaptic signal transduction (e.g., lithium); (7) Increase the sensitivity of the postsynaptic receptors (e.g., buspirone) (Loza, 1995; Muly et al., 1993; Karle and Bjorndal, 1995). In most circumstances, 5-HT syndrome is evoked by a drug combination. The combination of MAOIs with meperidine, dextromethorphan, SSRIs or amphetamine (MDMA, or "ecstasy") are strongly associated with malignant cases of the syndrome (Ener et al., 2003). A single therapeutic dose of a SSRI, for example sertraline (Kaminski et al., 1994), fluvoxamine (Bastani et al., 1996), venlafaxine (Kolecki, 1997), mirtazapine (Hernandez et al., 2002) and citalopram (Tseng et al., 2005) had also be reported to evoke the 5-HT syndrome.

Clinical manifestations

Clinical manifestations observed in the 5-HT syndrome patients range from tremor and diarrhea in mild cases to coma, muscular rigidity and hyperthermia in life-threatening ones (Ener et al., 2003). Typically, there are mental-status disorders, automatic hyperactivities and neuromuscular abnormalities (Boyer and Shannon, 2005; Hegerl et al., 1998; Sternbach, 1991) (Table 4).

Not all the symptoms listed are present in patients with this disorder. Mild cases may be afebrile but have muscular activities (e.g., limb terminal shivering) and automatic abnormalities (e.g., tachycardia, diaphoresis and mydriasis). The neurologic examination

may reveal intermittent tremor or myoclonus as well as hyperreflexia. In contrast, a patient with a moderate to severe syndrome may have hyperthermia, hypertension and tachycardia that may abruptly deteriorate into frank shock. The onset of symptoms is usually rapid with clinical findings occurring within minutes after a switch in medication, an overdose or self-poisoning. Approximately 60 percent of patients with the 5-HT syndrome present within six hours after medication (Boyer and Shannon, 2005).

Diagnosis and treatments

The diagnostic criterion of 5-HT syndrome was firstly put forward by Sternbach (1991). Based on Sternbach's criterion, patients meeting the following three items will be diagnosed as the 5-HT syndrome. 1. Recent addition or increase known serotonergic agents and induce at least three of the following syndromes: mental status changes (confusion, hypomania), agitation, myoclonus, hyperreflexia, diaphoresis, shivering, tremor, diarrhea, incoordination, fever; 2. Exclude other possible etiologies, such as withdrawal, infection, and so on; 3. There is no addition of a neuroleptic agent recently. During the following years, this criterion was revised by Hegerl (1998), Randomski and Dursun (2000), Kaneda (2000) and Boyer (2005). Recently, Isbister et al. (2007) brought forward a new criterion - the Hunter Serotonin Toxicity Criterion – which are much more specific for screening 5-HT syndrome.

The treatment of the 5-HT syndrome remains controversial and no specific antidotes are available to date. The primary management is supportive therapies. For the mild cases, cessation of any serotonergic agents and addition of supportive therapies consisting of rehydration, external cooling, charcoal lavage and dialysis can alleviate the

syndrome. Most cases resolve in 24 to 36 hours (Ener et al., 2003). For severe cases, the initial treatment is to maintain the function of airway, breathing and circulation. Besides the treatments used in the mild cases, specific pharmacological treatments should be applied. Benzodizaepines, such as diazepam (Fisher and Davis, 2002), are the first line treatment to reduce excessive sympathetic outflow, control seizures, decrease the myoclonus and muscle rigidity, reduce hyperthermia and rhabdomyolysis (Ener et al., 2003). 5-HT_{2A} receptor antagonist cyproheptadine (Lappin and Auchincloss, 1994; Graudins et al., 1998) and DA receptor antagonist chlorpromazine(Gillman, 1996, 1999) could significantly reduce the severity and mortality of the 5-HT syndrome subjects .

Animal research on the 5-HT syndrome

Before 5-HT syndrome was adopted in clinical cases (Insel et al., 1982), researchers have noticed that the increase in brain 5-HT level could produce a series of behaviors in many species (e.g., rats, mice, hamsters, cats and dogs) (Jacobs, 1976; Deakin and Green, 1978; Sloviter et al., 1978). Jacobs (1976) defined a term "5-HT behavioral syndrome" as a simultaneous display of four of six symptoms including hindlimb adduction, forepaw treading, lateral head weaving, resting tremor, rigidity and Straub tail. The 5-HT behavioral syndrome was considered as 5-HT syndrome today. Currently, just several labs explored the mechanisms of the 5-HT syndrome in animals models developed by increasing brain 5-HT level (Izumi et al., 2007; Ma et al., 2008; Nisijima et al., 2001, 2003, 2004). The symptoms of the 5-HT syndrome animals share some characteristics seen in human cases. Table 5 lists the manifestations that can be commonly observed in the 5-HT syndrome animals.

Both brain and spinal cord are involved in the etiology of 5-HT syndrome. For example, the 5-HT syndrome behaviors still existed after lower brainstem transections (Jacobs and Klemfuss, 1975). Infusion of DOI into the CA1 region of the dorsal hippocampus elicited head bobs that were blocked by prior local injection of the 5-HT_{2A} receptor antagonist (Dave et al., 2004). Meanwhile, selective destruction of spinal 5-HT terminals enhanced the syndrome elicited by 5-HT receptor agonists (Deakin and Green, 1978).

Theoretically, all, if not all, 5-HT receptors are activated in the 5-HT syndrome due to excessive 5-HT in the CNS. Of these, 5-HT_{1A} and 5-HT_{2A} receptors are the major targets responsible for the most symptoms of the syndrome. 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist, at high doses (>0.7 mg/kg) induced 5-HT syndrome behaviors, including head weaving, hindlimb abduction, forepaw treading and tremor (Yamada et al., 1988). Those behaviors were also observed in animals treated with non-selective 5-HT receptor agonists or antidepressants (Tricklebank et al., 1984; Deakin and Green, 1978; Ma et al., 2008). In support of involvement of 5-HT_{1A} receptors, selective 5-HT_{1A} receptor antagonists (e.g., WAY-100635, spiperone) could to some extent prevent these behaviors (Tricklebank et al., 1984). Although the majority of 5-HT_{1A} receptor is an autoreceptor located in the presynaptic membrane, the 5-HT_{1A} receptor responsible for the 5-HT syndrome behaviors is likely due to activation of the postsynaptic site. Several studies have revealed that inhibition of 5-HT synthesis with PCPA, destroying 5-HT neurons with 5,7-DHT, or depletion of 5-HT with reserpine failed to inhibit the 8-OH-DPAT-induced behaviors (Tricklebank et al., 1984; Goodwin et al., 1986; Sloviter et al., 1978; Yamada et al., 1988). Besides 5-HT_{1A} receptors, 5-HT_{2A} receptors are

responsible for, if not all, the head shake behavior in the syndrome animals (Lucki et al., 1984). DOI, a 5-HT_{2A} receptor agonist, also evokes head shakes in animals (Darmani and Zhao, 1998). Likewise, selective 5-HT_{2A} receptor antagonists (e.g., ketanserin) or nonselective 5-HT antagonist metergoline or methysergide blocked head shakes in the 5-HT syndrome animals (Lucki et al., 1984; Nisijima et al., 2001).

The change in core body temperature (T_{cor}) is an important manifestation of the 5-HT syndrome animals. Unlike the clinical human cases that display hyperthermia, the 5-HT syndrome animal have tri-phase changes of T_{cor} correlated to the extrasynaptic 5-HT concentration (Zhang et al., 2009). With the increase in the brain 5-HT level, 5-HT syndrome animals display hypothermia, normothermia and hyperthermia subsequently (Zhang et al., 2009; Abdel-Fattah et al., 1997). 5-HT_{1A} and 5-HT_{2A} receptors mediate the hypothermia and hyperthermia, respectively (Ma et al., 2008). It has been further suggested that the neurological balance between 5-HT_{1A} and 5-HT_{2A} receptor activities could result in the normothermia of the body temperature in animals with a mild to moderate syndrome.

5-HT_{2A} receptor and the 5-HT syndrome

5-HT_{2A} receptor plays a critical role in the etiology of the 5-HT syndrome. Animal studies revealed that 5-HT_{2A} receptor antagonists could prevent the fatality, reverse the hyperthermia, alleviate the neuromuscular hyperactivities of animals with a severe 5-HT syndrome (a state of animal having continue tremor, over 60 times/min forepaw treading, All limb out from body, persistent Straub tail, immobility and increase in body temperature) (Nisijima et al., 2001, 2003; Ma et al., 2008). Clinical reports showed that

5-HT_{2A} receptor antagonists (e.g., cyproheptadine and chlorpromazine) could alleviate the symptoms and prevent death of the patients with the 5-HT syndrome (Lappin and Auchincloss, 1994; Graudins A et al., 1998; Gillman, 1996; Graham, 1996). These data suggest that 5-HT_{2A} receptor might work as a key mediator that mediates the 5-HT syndrome from benign to malignant or death.

Even though 5-HT_{2A} receptor is deemed to be involved in the 5-HT syndrome, the neurological mechanisms underlying it are not well investigated. In this study, the role of 5-HT_{2A} receptors in the scenario of 5-HT syndrome would be explored with an animal model developed by clorgyline, a MAOI, and paroxetine, a SSRI, in four aspects. Clinic reports showed that patients susceptible to 5-HT syndrome mostly have a long history of taking antidepressants and measures taken to lower body temperature can alleviate the 5-HT syndrome. Therefore, aim I of this study was to explore the changes of 5-HT_{2A} receptor activity in animals receiving chronic clorgyline treatment. After that, the effects of environmental temperature on the 5-HT_{2A} receptor activity were examined in aim II. The third aim was to characterize the 5-HT syndrome in animals. The last aim of this study was to investigate the effects of 5-HT_{2A} receptor in the mPFC that may aggravate the syndrome by activating a circuit between raphe nuclei and mPFC.

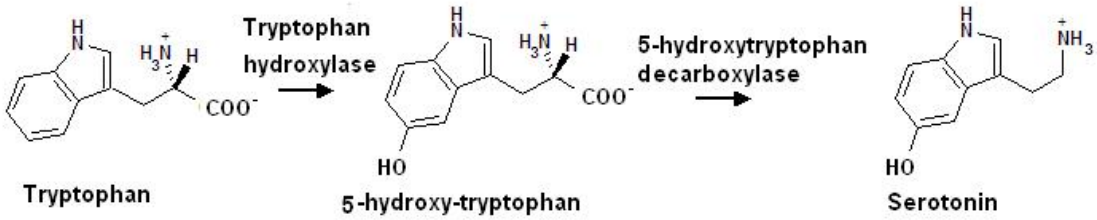

Figure 1. Chemical structures of 5-hydroxytryptophan (5-HT; serotonin) and its precursors

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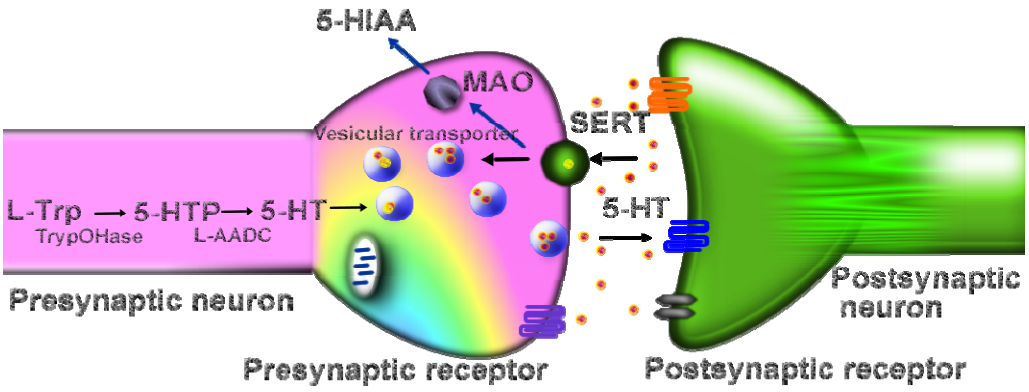

Figure 2. Schematic diagram depicting serotonin transmission in the synapse

Figure 2. Schematic diagram depicting serotonin transmission in the synapse

Serotonergic neurons in the brain synthesize serotonin (5-HT) from L-tryptophan (Trp) and store it in the vesicles before release. 5-HT secreted in the synaptic cleft binds presynaptic and postsynaptic receptors. Most of 5-HT molecules are taken back to the presynaptic neurons by serotonin transporters. After uptake in the cytoplasm, some is repackaged by vesicular transporters and the rest is degraded by A-type monoamine oxidases to 5-hydroxyindoleacetic acid. L-AADC, L-amino acid docarboxylase; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HTP, 5-hydroxytryptamine; MAO, monoamine oxidase; TrypOHase, tryptaphan hydroxidase.

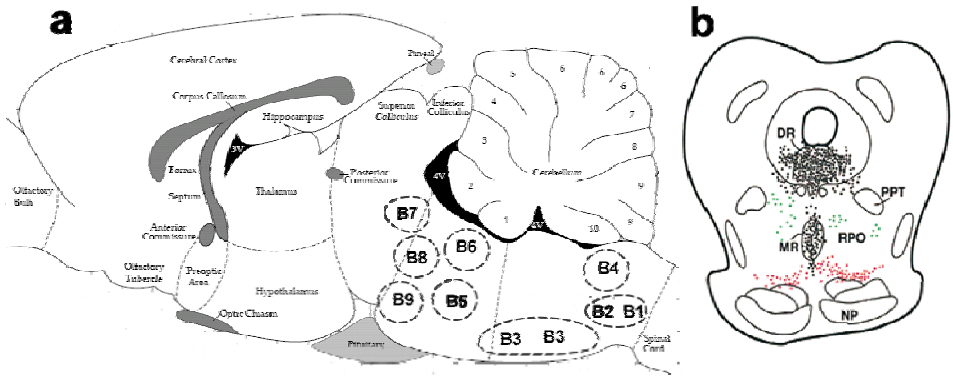

Figure 3. Schematic drawing depicting the location of 5-HT-containing cells in the brainstem

Figure 3. Schematic drawing depicting the location of 5-HT-containing cells in the brainstem

(a) A midsagittal section of the rat brain. The dorsal groups of 5-HT-containing cells (B5 - B9) send projections to thalamus and cortex, and the caudal groups in the medulla and lower pons (B1 - B4) send axons to medulla and spinal cord. (b) Adult rat brain coronal section showing 5-HT-immunoreactive neurons of dorsal and median raphe nuclei (DRN and MRN). PPT, pedunculopontine tegmental nucleus; RPO, nucleus reticularis pontis oralis; NP, nucleus of pons. Fig. b cited from (Vertes and Crane, 1997) with permission from Dr. Vertes.

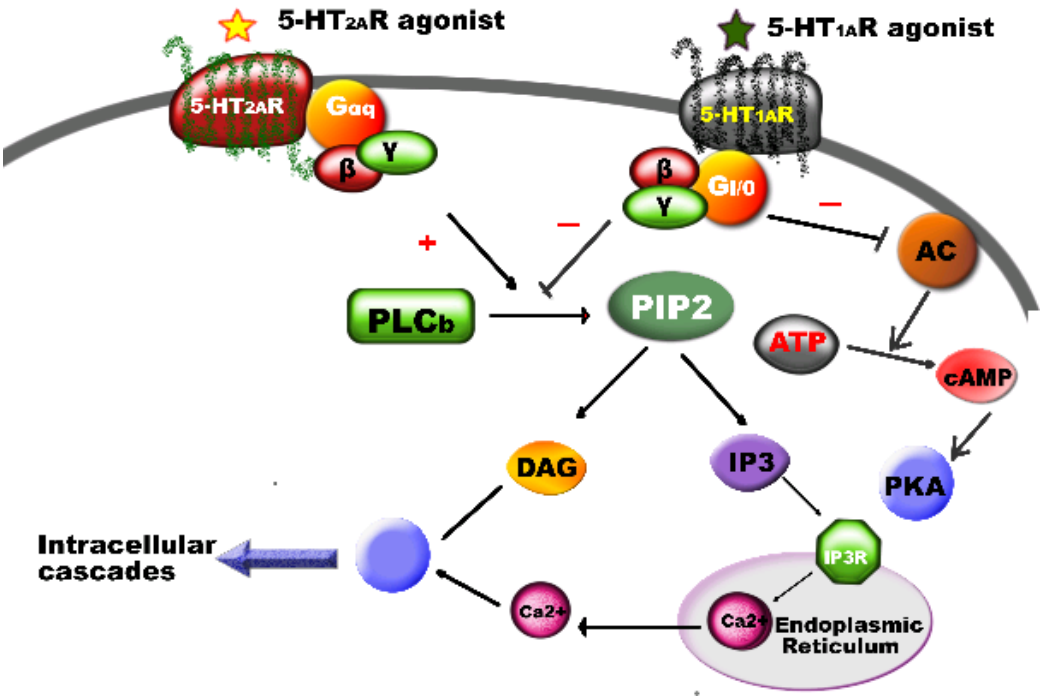

Figure 4. Schematic diagram of signaling pathways of the 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors

Figure 4. Schematic diagram of signaling pathways of the 5-HT_{2A} and 5-HT_{1A} receptors

5-HT_{2A} receptor, a G-protein coupled receptor on cell surface, activates phospholipase C (PLC) in the cytoplasm which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 promotes Ca^{2+} release by activating IP_3 receptors in the membrane of the smooth endoplasmic reticula and mitochondria. Ca^{2+} and DAG activate protein kinase C (PKC), which phosphorylates proteins, changes their catalytic activities, and leads to series of cellular effects. Activation of the 5-HT_{1A} receptor inhibits the activities of adenosine cyclase (AC) and PLC, resulting in opening of potassium channels and hyperpolarization of the host neuron.

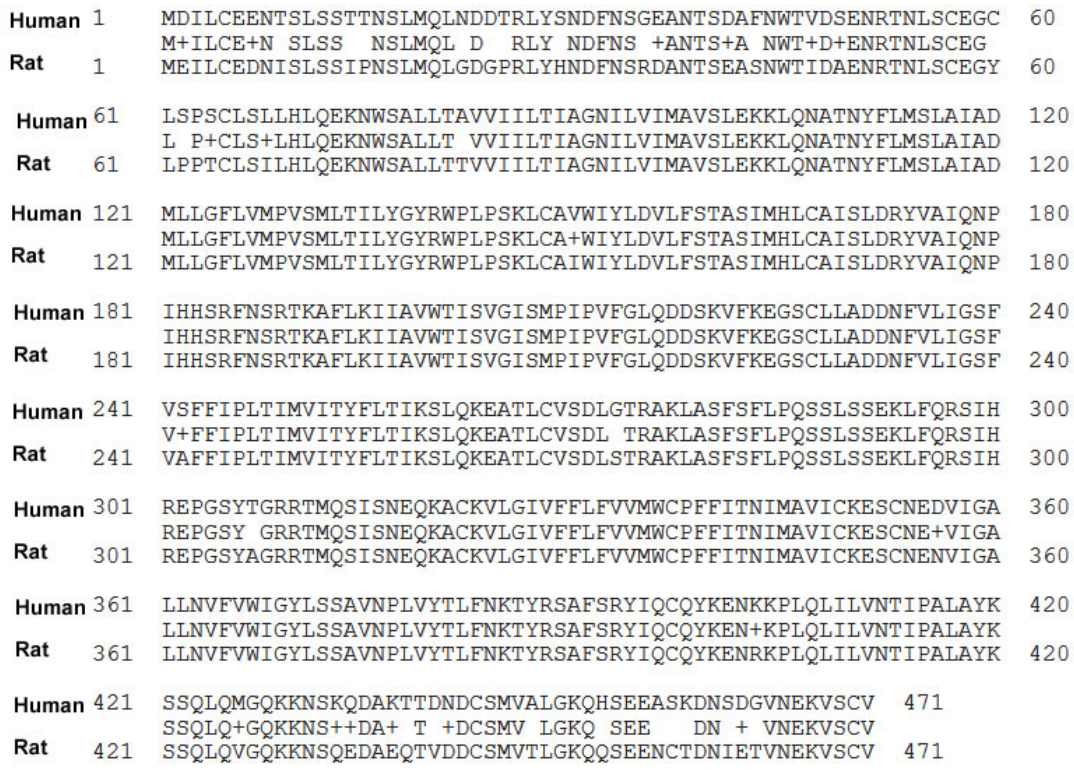

Figure 5. Comparison of amino acid sequences of the human and rat 5-HT_{2A} receptors

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Both human and rat 5-HT_{2A} receptors consist of 471 amino acids and share 91% common sequence. The middle line shows the common sequence shared by both species.

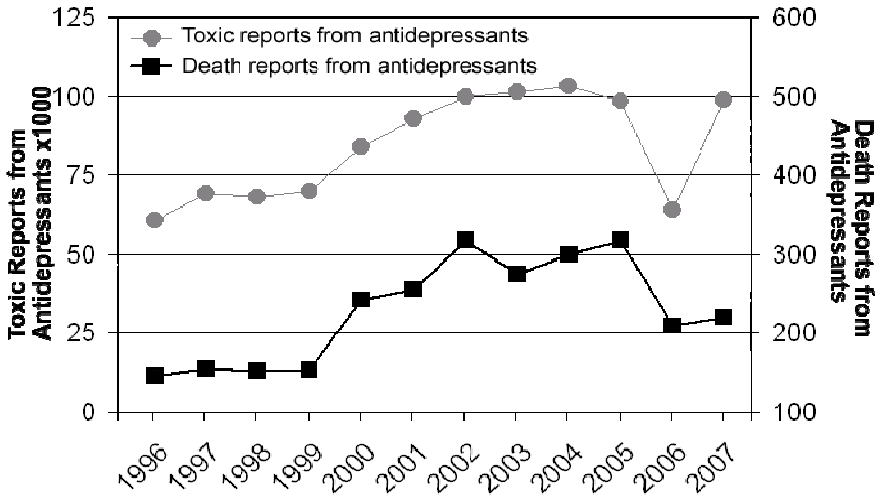


Figure 6. The toxic and death reports in human from antidepressants between 1996-2007

Figure 6. The toxic and death reports in human from antidepressants between 1996-2007

The morbidity and mortality were increasing in the past decades according to the date from the American Association of Poison Control Centers-National Poison Data System (http://www.aapcc.org/dnn/NPDS/AnnualReports/tabid/125/Default.aspx)

Table 1. Classification of serotonergic cell body groups (Hensler, 2006)

Groups of serotonergic neurons	Anatomy position
B1	Raphe pallidus nucleus, caudal ventrolateral medulla
B2	Raphe obscurus nucleus
B3	Raphe mangus nucleus, rostral ventrolateral medulla, lateral paragigantocellular reticular nucleus
B4	Raphe obscurus nucleus, dorsolateral part
B5	Median raphe nucleus, caudal part
B6	Dorsal raphe nucleus, caudal part
B7	Dorsal raphe nucleus principal, rostral part
B8	Median raphe nucleus, rostral main part; caudal linear nucleus; nucleus pontis oralis
B9	Nucleus pontis oralis; supralemniscal region

Table 2. 5-HT receptor superfamily

Family	Type	Mechanism	Potential
5-HT_1	G_i/G_o-protein coupled	Decreasing intracellular level of cAMP	Inhibitory
5-HT_2	G_q/G_{11}-protein coupled	Increasing intracellular level of IP_3 and DAG	Excitatory
5-HT_3	Ligand-gated Na^+ and K^+ cation channel	Depolarizing plasma membrane	Excitatory
5-HT_4	G_s-protein coupled	Increasing intracellular level of cAMP	Excitatory
5-HT_5	G_i/G_o-protein coupled	Decreasing intracellular level of cAMP	Inhibitory
5-HT_6	G_s-protein coupled	Increasing intracellular level of cAMP	Excitatory
5-HT_7	G_s-protein coupled	Increasing intracellular level of cAMP	Excitatory

Table 3. Variation of 5-HT_{2A} receptor mRNA and receptor protein expression to its agonists with in vitro model systems

Model System	mRNA	Receptor
P11 cells (rat pituitary)	Increase (Ferry et al., 1994; Ferry and Molinoff, 1996)	Decrease (Ferry et al., 1993)
C6 glioma cells (rat glioma)	Decrease (Toth and Shenk, 1994)	Decrease (Toth and Shenk, 1994)
NIH-3T3 cells (murine fibroblast)	Decrease (Saucier and Albert, 1997)	Decrease (Saucier and Albert , 1997; Roth et al., 1995)
Cerebellar granule cells (rat cerebellum)	Increase (Akiyoshi et al., 1993; Chen et al., 1995a)	Increase (Akiyoshi et al., 1993)
Myometric smooth muscle cells (rat uterine)	Increase (Rydelek-Fitzgerald et al., 2009)	Decrease (Leysen et al., 1989)
Transfected Madin-Darby canine kidney cells		Increase (Grotewiel and Sanders-Bush, 1994)
AtT-20 cell		Decrease (Grotewiel and Sanders-Bush, 1994)

Table 4. The clinical triad of the 5-HT syndrome in humans

Mental-status disorders	Autonomic hyperactivities	Neuromuscular abnormalities
Agitation, hypervigilance, slightly pressured speech, confusion, hypomania	Tachycardia, hypertension, hyperthermia, mydriasis, diaphoresis, hyperactive bowel sounds, diarrhea	Hyperreflexia, muscle rigidity, myoclonus (seizures), ocular clonus, peripheral hypertonicity shivering, tremor and dizziness

Table 5. Signs of 5-HT syndrome in animals

Mental-status changes	Autonomic hyperactivities	Neuromuscular abnormalities
Agitation, hyperactivity	Temperature dysregulation (hypothermia, normothermia and hyperthermia), Straub tail, salivation, diarrhea.	Tremor, forepaw treading, head weaving and shakes, body shakes, muscle rigidity, clonus, flat body, hind limb abduction.

CHAPTER TWO: THE ENHANCED ACTIVITY OF THE SEROTONIN 2A RECEPTOR IS THE UNDERLYING MECHANISM RESPONSIBLE FOR A MALIGNANT SEROTONIN SYNDROME

2.1 Introduction

Both animal studies and clinical reports have demonstrated that 5-HT_{2A} receptors play a crucial role in the etiology of the 5-HT syndrome (Zhang et al., 2009; Nisijima et al., 2001, 2004). Lab studies revealed that activating 5-HT_{2A} receptor mimicked some symptoms as seen in the syndrome animals (Miller et al., 1996; Koek et al., 1992). Selective 5-HT_{2A} receptor antagonists could suppress the behavioral abnormalities, hyperthermia, brain 5-HT excess and mortality of the severe 5-HT syndrome animals developed by increasing extracellular 5-HT level in the brain (Ma et al., 2008; Zhang et al., 2009; Nisijima et al., 2001, 2004). Consistently, clinical case reports stated that agents with blocking 5-HT_{2A} receptor capacity (e.g., cyproheptadine and chlorpromazine) could alleviate the symptoms and prevent mortality of the 5-HT syndrome patients (Lappin and Auchincloss , 1994; Graudins et al., 1998; Gillman, 1996; Graham, 1996). To date, it is generally accepted that 5-HT_{2A} receptors mediate the severe 5-HT syndrome (Ma et al., 2008).

Both in vivo and in vitro studies have indicated that chronic antidepressant

exposure altered 5-HT_{2A} receptor capacity and sensitivity (Peroutka and Snyder, 1980; Todd et al., 1995; Grotewiel and Sanders-Bush, 1994). However, the 5-HT syndrome animal models employed today were developed mostly with an acute administration of proserotonergic agents (e.g., 5-HTP, MAOI) to drug-naïve animals (Nisijima et al., 2001, 2004; Ma et al., 2008). A discrepancy between the clinical 5-HT syndrome cases and current animal models is that patients have a long history of taking antidepressants. Therefore, the 5-HT_{2A} receptor activity of the patient may changed, which can not be reflected by lab researches with drug-naïve animals.

To illuminate the change of 5-HT_{2A} receptor activity in the 5-HT syndrome patients, a new 5-HT animal model was developed with a MAOI (clorgyline) and SSRI (paroxetine) for this combination was the most common drug regimen reported in human syndrome cases (Boyer and Shannon, 2005). Animals received clorgyline for days before paroxetine challenge to induce the 5-HT syndrome. The aim of this study was to explore the change of 5-HT_{2A} receptor activity to repeated clorgyline treatment in our 5-HT syndrome animals. Since this syndrome occurred in patients with a chronic antidepressant treatment (Boyer and Shannon, 2005), it was hypothesized that chronic clorgyline treatment would increase the activity of 5-HT_{2A} receptor.

2.2 Materials and methods

Animal preparation

Adult male Sprague-Dawley rats (280-350 g, purchased from Charles River Laboratories, Raleigh, NC, USA) were group-housed under a12:12-hour light/dark cycle (lights on at 8:00 a.m.) in a temperature (22.0 ± 1.0 °C) and humidity (60 ± 10%) - controlled facility with free access to food and water. All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local animal study committees. All efforts were made to reduce the number of animals used and their suffering.

Chemicals and schedule for administration

Clorgyline (N-methyl-N-propargyl-3-[2, 4-dichlorophenoxy]-propylamine hydrochloride), (±) DOI-hydrochloride ((+)–1-(4-iodo-2, 5-dimethoxyphenyl)–2-aminopropane hydrochloride) and WAY-100635 ({(N-{2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl}-N- (2-pyridinyl) cyclo-hexanecarboxamide}) were purchased from Sigma Aldrich (St. Louis, MO, USA). Paroxetine was obtained from USP (Rockwille, MD, USA) and ketanserin tartrate was from Tocris Bioscience (Ellisville, MO, USA). Table 6 lists the dose, solvent, volume and route of each chemical used in this chapter.

Preparation of the 5-HT syndrome animal

The 5-HT syndrome animal model used in the study was developed by clorgyline and paroxetine. Animals received clorgyline (2 mg/kg, s.c.) once daily at 12:00 noon for

0, 3 or 6 days according to different protocols. On the following day, animals were challenged with paroxetine and clorgyline. If an antagonist was applied, it was administrated half an hour before paroxetine challenge. In the control groups, vehicle substituted the agents. All the data were collected on the last day.

Core body temperature measurement

Animals habituated individually in transparent Plexiglas bowl in a temperature-controlled environment for at least 2 hours before experiments. A thin, flexible thermoprobe connected to a digital meter (Traceable®, Fisher Science) was inserted 5.0 cm into rectum and stayed at least 30 sec before a stable readout. Two measurements were taken as a baseline before drug injection and six readouts were collected after the drug challenge. The interval was one hour.

Head shake

Head shake (head twitch) is a behavior of shaking head (not the whole body) in a short and firm way. The intensity of 5-HT_{2A} receptor activity was strongly correlated with the frequency of the shakes (Marek, 2003). Based on the preliminary study from our lab as well as other's reports (Speiser et al., 2008), frequency of head shakes was more prominent right after paroxetine challenge and decreasing with time. Therefore, only the first hour of head shakes after paroxetine challenge was collected in this study.

Surgery and microdialysis procedures

Rats were anesthetized by a combination of xylazine (6 mg/kg, i. p.) and ketamine

(80 mg/kg, i. p.), and mounted in a Kopf stereotaxic frame in a flat skull position. Guide cannulae (22 gauge steel tube) were implanted below the skull surface aiming to right mPFC, right POA (Table 7) (Paxinos and Watson, 1998). After surgery, rats were recovered for one week before experiments.

On the day before microdialysis, rats were briefly inhaled with isoflurane for anesthesia. I-shaped microdialysis probes (cut-off 18 kD) were used in this study. Dialysis probe was inserted through the guide cannula targeting at the mPFC or POA and secured in place with dental cement (Fig. 7). The probe inlet was attached to a perfusion line from Raturn™ system (Bioanalytical System Inc., W. Lafayette, IN), and infused with the artificial cerebrospinal fluid (aCSF; containing 140 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.25 mM NaH_2PO_4, and 1.0 mM Na_2HPO_4; pH: 7.4) at a flow rate of 1 μl/min. Next day, two dialysate samples were collected to obtain the basal value before administration of drugs. Samples were collected once per hour starting at 10:00 a.m.

5-HT assay with HPLC-electrochemical detection

5-HT was determined by HPLC-electrochemical detection (HTEC-500; EICOM, Japan) with a CMA/200 refrigerator microsampler (CMA/Microdialysis, Stockholm, Sweden). A reverse-phase column (4.6 × 30 mm, packed with a PP-ODS) was used for 5-HT separation. The composition of mobile phase was 0.1 M phosphate buffer (pH 6.0) containing 1% methanol, 500 mg/L sodium-1-octanesulfonate and 50 mg/L ethylene diamine-tetracetic acid. The flow rate was 350 μl/min and the potential set on the graphite electrode was + 350 mV (relative to Ag/AgCl reference electrode). Extracellular

concentrations of 5-HT was estimated by rationing peak area with its internal standard by Powerchrom V2.2 software. The run time was 7.5 min (Fig. 8).

Histology

Upon completion an experiment, the rats were deeply anesthetized with ketamine (100 mg/kg, i.p.) combined with xylazine (4 mg/kg, i.p.). Probes were infused with a 2% Fast Green for 10 min. The animals were then decapitated and brains were removed, frozen in – 80 °C, and sliced freehand. The probe location was visual inspected by comparison to the rat brain atlas (Paxinos and Watson, 1998). A modification of this procedure was used in making photomicrographs. A 2% solution of Fast Green was perfused through the dialysis probe for 10 min. The rat was then perfused intracardially with 0.9% saline followed by 4% buffered paraformaldehyde solution. The brain was removed and sliced in 40 μm sections using a microtome (Leica CM1850). Sections were mounted on slides and counterstained using a standard Cresyl Violet method. Slides were taken pictures with a microscope (Olympus AX70). Data were excluded from analysis when probes were located outside target structures.

Data analysis

Body temperature was expressed with the absolute values or changes to the baseline. Microdialysis results of 5-HT were expressed as pg per sample (60 μl) or fold increase to the baseline. Data in the Figures were showed as mean ± SEM of the respective time points. Two-way repeated measures analysis of variance (ANOVA) of raw data with time as repeated factor and dose or treatment as dependent factor was used

to evaluate overall response among the control and experimental groups. When significant effects were found, *post hoc* Scheffe's test was used to compare effects of different treatment groups. One-way ANOVA and student *t*-test were also used to assess the statistical differences among groups. The Fisher exact test was used to substitute a Chi Square test to analyze the mortality rate if one or more cells in the data have a value of five or less. The significant level was set at $p < 0.05$.

2.3 Results

Changes in T_{cor} in the 5-HT syndrome animals with acute or chronic clorgyline treatment

The basal (prior challenge) T_{cor} of animals with 0-, 3- and 6-day clorgyline treatment were 37.6 ± 0.1 °C (n = 50), 37.7 ± 0.2 °C (n = 10) and 38.2 ± 0.1 °C (n = 14), respectively. Repeated clorgyline treatment increased the basal T_{cor} as demonstrated by one-way ANOVA ($F_{2, 71} = 11.0$, $p < 0.001$). *Post hoc* comparisons showed that 6-day clorgyline treatment significantly increased the T_{cor} ($p < 0.001$, Scheffe's test), while 3-day clorgyline treatment had no effect on T_{cor} ($p > 0.05$, Scheffe's test) when compared with the T_{cor} of naïve animals.

Since hyperthermia is indicative of activation of the 5-HT_{2A} receptor in the 5-HT syndrome (Nisijima et al., 2001), here the change in T_{cor} of the 5-HT syndrome animals induced by a challenge injection of paroxetine and clorgyline was recorded. Figure 9a showed effect of single administration of paroxetine or clorgyline altered the T_{cor} of the drug-naïve animals. Two-way repeated measures ANOVA revealed a significant effect of treatment ($F_{2, 14} = 7.3$, $p < 0.01$), time ($F_{7, 98} = 7.2$, $p < 0.001$) and treatment × time ($F_{14, 98} = 3.3$, $p < 0.001$). *Post hoc* comparisons showed that clorgyline treatment decrease T_{cor} ($p < 0.01$, Scheffe's test) and paroxetine had no significant effects ($p > 0.05$, Scheffe's test). The maximal decreases in T_{cor} induced by paroxetine and clorgyline were -0.8 ± 0.1 °C and -0.9 ± 0.1 °C, respectively. To further investigate the time course of antidepressant treatment on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that antidepressants decreased the T_{cor} on the 1 to 3 and 4 hours after the drug injection.

Post hoc comparisons indicated that paroxetine decreased the T_{cor} on the first 2 hours, while clorgyline inhibited the T_{cor} on the first 3 hours after treatment. This suggests that antidepressants when used alone can cause a decrease in T_{cor}. The effect may be due to activation of the 5-HT_{1A} receptor in drug-naïve animals.

Figure 9b showed that chronic clorgyline treatment induced a time-dependent increase in $T_{cor.}$ This conclusion was confirmed by two-way repeated measures ANOVA, which revealed a significant effect of treatment ($F_{2, 17} = 16.7$, $p < 0.001$), time ($F_{7, 119} = 21.0$, $p < 0.001$) and treatment × time ($F_{14, 119} = 24.5$, $p < 0.001$). *Post hoc* comparisons showed, compared with CLG + PRX group, both 3- and 6-day CLG pretreatment groups increased T_{cor} ($p < 0.001$, Scheffe's test). There was no difference of T_{cor} between 3-day and 6-day CLG pretreatment groups ($p > 0.05$, Scheffe's test). To further investigate the time course of antidepressant treatment on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that 3- and 6-day clorgyline treatment altered the T_{cor} on the 6 hours after the drug injection to the drug-naïve animals. *Post hoc* comparisons indicated that 3-day clorgyline treatment increased the T_{cor} on the 6 hours after drug. While 6-day clorgyline treatment increased the T_{cor} on the first 3 hours after drug challenge. As shown in Figure 9b of the line with open cycle, the injection decreased the T_{cor}. The lowest T_{cor} is -1.38 ± 0.32 °C. The effect may be due to activation of the 5-HT_{1A} receptor in drug-naïve animals. Paroxetine combined with clorgyline caused a greater decrease in T_{cor} than each drug alone was examined. The effect was relevant to the mild syndrome in drug-naïve animals. This suggests that the mild syndrome is associated with hypothermia. After 3- or 6-day clorgyline treatments, challenge injection of paroxetine combined with clorgyline on day 4 or 7 induced hyperthermia. The maximal increases in

T_{cor} were 2.42 ± 0.41 °C and 2.95 ± 0.26 °C, respectively. The syndrome became severe. These data appears that chronic clorgyline treatment increases the activity of 5-HT_{2A} receptor as demonstrated by the hyperthermia and syndrome severity.

Effects of ketanserin on the hyperthermia and mortality of the 5-HT syndrome animals

To verify 5-HT_{2A} receptors mediated the hyperthermia as well as the fatality, severe 5-HT syndrome animals received a pretreatment of ketanserin, a selective 5-HT_{2A} receptor antagonist. Systemic administration of ketanserin affected the hyperthermia. This conclusion was confirmed by two-way repeated measures ANOVA, which revealed the significant effect of treatment ($F_{2, 17} = 37.1$, $p < 0.001$), time ($F_{8, 136} = 8.2$, $p < 0.001$) and treatment × time ($F_{16, 136} = 22.4$, $p < 0.001$). *Post hoc* analyses showed ketanserin pretreatment blocked the hyperthermia of the 5-HT syndrome animals ($p < 0.001$, Scheffe's test). To further investigate the time course of ketanserin treatment on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered the T_{cor} after its injection. *Post hoc* comparisons indicated that ketanserin decreased the T_{cor} for 7 hours after its administration.

Consistent with the work as described previously (Nisijima et al., 2001), systemic ketanserin blocked the hyperthermia and prevented the fatality ($p < 0.05$, Fisher's exact test, 2-sided) of the severe 5-HT syndrome animals (Fig. 10).

Effects of WAY-100635 and 8-OH-DPAT on the T_{cor} of the 5-HT syndrome animals

Besides the 5-HT_{2A} receptor-mediated hyperthermia, the 5-HT_{1A} receptor-mediated hypothermia has also been reported in the 5-HT syndrome animals (Pranzatelli and Pluchino, 1991; Ma et al., 2008) . Here, we explored the effect of 5-HT_{1A} receptor on the hypothermia observed in the 5-HT syndrome animals. As shown in Figure 11a, 1 mg/kg paroxetine combined with 2 mg/kg clorgyline significantly decreased the T_{cor} on the first hour after the challenge in animals with chronic clorgyline treatment to the vehicle group ($p < 0.05$, Scheffe's test). WAY-100635, a 5-HT_{1A} receptor antagonist, reversed the decrease in T_{cor} in the mild 5-HT syndrome animals, but no statistical difference was found when compared with the control group ($p > 0.05$, Scheffe's test).

The earlier studies have suggested that 5-HT_{1A} receptor may be desensitized after chronic antidepressant treatment. It is very possible that the 5-HT_{1A} receptor-mediated symptoms of the syndrome are to some extent reduced. Figure 11b indicated that chronic clorgyline treatment alleviated the 8-OH-DPAT (a 5-HT_{1A} receptor agonist)-induced hypothermia. This conclusion was confirmed by two-way repeated measures ANOVA, which revealed the significant effect of treatment ($F_{1, 4} = 48.4$, $p < 0.01$), time ($F_{4, 16} = 47.3$, $p < 0.001$) and treatment × time ($F_{4, 16} = 5.575$, $p < 0.01$). To further investigate the time course of chronic clorgyline treatment on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that chronic clorgyline inhibited the hypothermia on the 30, 60 and 90 min after 8-OH-DPAT challenge. The data supports the hypothesis.

5-HT level in the mPFC and POA in the 5-HT syndrome animals with acute or chronic clorgyline treatments

An excessive increase in brain 5-HT efflux would induce the 5-HT syndrome (Zhang et al., 2009). Here extracellular 5-HT in the mPFC and POA in response to clorgyline and paroxetine challenge was tested. The basal concentration of 5-HT from mPFC in drug-naïve, 3- and 6-day clorgyline-treated animals were 1.1 ± 0.1 pg/sample (n = 33), 3.6 ± 0.4 pg/sample (n = 16) and 3.8 ± 0.4 pg/sample (n = 12), respectively. It appears that repeated treatment with clorgyline increases the basal 5-HT concentration as demonstrated by one-way ANOVA ($F_{2, 58} = 39.3$, $p < 0.001$) (Fig.12a). Compared with naïve animal group, there was a significant increase of 5-HT level in CLG chronic treatment groups ($p < 0.001$, Scheffe's test). There were no differences between CLG × 3d group and CLG × 6d group ($p > 0.05$, Scheffe's test). Challenge injection of paroxetine combined with clorgyline did not cause a remarkable difference of 5-HT efflux in the mPFC between the acute and chronic animals. It was confirmed by the insignificant effect of treatment ($F_{2, 20} = 0.76$, $p > 0.05$) and treatment × time ($F_{14, 140} = 1.46$, $p > 0.05$). A significant effect existed in time ($F_{7, 140} = 61.2$, $p < 0.001$). *Post hoc* comparisons did not reveal a significant difference among the three groups ($p > 0.05$, Scheffe's test).

The basal 5-HT level from the POA in drug-naïve, 3- and 6-day clorgyline-treated animals were 0.8 ± 0.1 pg/sample (n = 34), 3.4 ± 0.4 pg/sample (n = 15) and 4.0 ± 0.6 pg/sample (n = 10), respectively. One-way ANOVA demonstrated that chronic clorgyline treatment affected the baseline of 5-HT level in the POA ($F_{2, 56} = 47.7$, $p < 0.001$) (Fig. 12c). Compared with naïve animal group, there was a significant increase of 5-HT level

in CLG chronic treatment groups ($p < 0.001$, Scheffe's test). There were no differences between CLG × 3d group and CLG × 6d group ($p > 0.05$, Scheffe's test). Although caused a remarkable increase, challenge injection of clorgyline and paroxetine had no effect on the maximal 5-HT efflux in the POA of animals treated with 0, 3- and 6-day repeated clorgyline treatment. It was confirmed by the insignificant effect of treatment ($F_{2, 12} = 0.437$, $p > 0.05$), but time ($F_{7, 84} = 61.4$, $p < 0.001$) and treatment × time ($F_{14, 84} = 1.588$, $p > 0.05$) have significances. *Post hoc* comparisons did not reveal a significant difference among the three groups ($p > 0.05$, Scheffe's test) (Fig. 12d). In other words, the increase in 5-HT was similar between acute and chronic clorgyline-treated animals. Altogether, despite previously clorgyline pretreatment, paroxetine and clorgyline challenge had no effect on the maximal 5-HT efflux in the mPFC and POA. This suggests that, in addition to the role of excessive 5-HT in the 5-HT syndrome, other mechanism must be involved in the severity of 5-HT syndrome. For this reason, it suggests that changes in 5-HT_{2A} receptor activity may be the underlying mechanism responsible for exacerbation of the syndrome severity after pretreatment with chronic clorgyline for 3 or 6 days, which will be described further below.

To extend the finding, changes in 5-HT in drug-naïve animals in response to a single acute injection of paroxetine or clorgyline was examined. Similar to those described previously in literature (Hervas et al., 2000; Hervas and Artigas, 1998), clorgyline or paroxetine alone changed 5-HT level in mPFC as demonstrated by the significant effect of treatment ($F_{2,16} = 17.7$, $p < 0.001$), time ($F_{7,122} = 7.9$, $p < 0.001$) and treatment × time ($F_{14,122} = 4.9$, $p < 0.001$). *Post hoc* comparisons showed paroxetine increased 5-HT efflux ($p < 0.01$, Scheffe's test). There was no different between vehicle

group and clorgyline group ($p > 0.05$, Scheffe's test) (Fig. 12e). To further investigate the time course of antidepressant treatment on 5-HT level, one-way ANOVA was run on each time point. These analyses indicated that antidepressants altered 5-HT level on the 6 hours after the drug injection ($p < 0.01$). *Post hoc* comparisons indicated that paroxetine increase 5-HT level on the 6 hours after its injection ($p < 0.05$). Similarly, clorgyline or paroxetine alone could significantly alter 5-HT level in POA as demonstrated by the significant effect of treatment ($F_{2, 14} = 17.3$, $p < 0.001$), time ($F_{7, 98} = 3.9$, $p < 0.001$) and treatment × time ($F_{14, 98} = 4.3$, $p < 0.001$). *Post hoc* comparisons showed both paroxetine and clorgyline increased 5-HT efflux in POA ($p < 0.01$, Scheffe's test) (Fig. 12f). To further investigate the time course of antidepressant treatment on 5-HT level, one-way ANOVA was run on each time point. These analyses indicated that antidepressants altered 5-HT level on the first 5 hours after the drug injection ($p < 0.05$). *Post hoc* comparisons indicated that paroxetine increased 5-HT level 5 hours after its injection ($p < 0.05$) and clorgyline increased 5-HT level on the 4^{th} hour ($p < 0.05$).

In summary, paroxetine or clorgyline alone can only cause a 1-3 fold increase. In contrast, combined injection of paroxetine with clorgyline induces about 80 fold increases in 5-HT efflux. The severity of 5-HT syndrome is determined not only by the excessive 5-HT in the CNS, but also 5-HT_{2A} receptor activity that can be enhanced by chronic antidepressant treatment.

Changes in head shakes in the animals with acute or chronic clorgyline treatment

Head shake is a characteristic behavior mediated by 5-HT_{2A} receptor (Backus et al.,

1990). In this study, the number of head shakes was counted on the first hour after paroxetine and clorgyline challenge. The number of head shakes in naïve, 3- and 6-day clorgyline-treated animals were 29 ± 4, 35 ± 7 and 20 ± 5 times per hour to the challenge injection, respectively. Chronic clorgyline treatment did not alter the number of head shakes in rats as demonstrated by a one-way ANOVA ($F_{2, 16} = 2.228$, $p > 0.05$). *Post hoc* comparisons did not reveal any significant difference between groups ($p > 0.05$) (Fig. 13a).

It appears that the head shake data are inconsistent with the 5-HT_{2A} receptor hypothesis, in which it was expected that head shake frequency would be increased in the clorgyline pretreated animals. It has been suggested that muscle rigidity can restrict the head shakes in the 5-HT syndrome animals (Ma et al., 2008). To prevent the interference from muscle activity, DOI, a 5-HT_{2A} receptor agonist, was used to induce the head shakes. As shown in Figure 13b, DOI (1 mg/kg, s.c.) significantly increased the number of head shakes in animals with repeated clorgyline treatment when compared with the control group ($F = 6.669$, $p < 0.05$) (Fig. 13b).

Effects of chronic clorgyline treatment on the mortality of the 5-HT syndrome animals

Although death is not always the endpoint of 5-HT syndrome, it is crucially indicative of the syndrome from benign to malignancy (Nisijima et al., 2001). No animals receiving saline ($n = 7$), clorgyline ($n = 6$) or paroxetine ($n = 6$) died (data not shown). As shown in Table 8, chronic clorgyline treatment increased the mortality rate of the 5-HT syndrome animals with paroxetine and clorgyline challenge. Because some data have

expected count less than five, Fisher's exact test replaced Pearson Chi-Square for statistics here. Both 3-day and 6-day repeated clorgyline treatment increased the mortality to the paroxetine and clorgyline challenge ($p < 0.05$, Fisher's exact test, 2-sided). The increase was blocked by the 5-HT_{2A} receptor antagonist ketanserin in the concordance of the hypothesis that 5-HT_{2A} receptor activity was enhanced in the animals with chronic antidepressant treatment.

2.4 Discussion

The main findings from the present studies were that chronic clorgyline treatment increased the 5-HT_{2A} receptor activity in the 5-HT syndrome animals as demonstrated by the rise in T_{cor}, head shakes and mortality. Additionally, chronic clorgyline treatment attenuated 5-HT_{1A} receptor response, which, on the other hand, potentiated the activity of the 5-HT_{2A} receptor.

To explore the mechanisms underlying the 5-HT syndrome, researchers have developed several animal models by increasing the extracellular 5-HT concentration in the brain (Table 9). The most popular drug paradigm employed is administration of 5-HTP with an antidepressant to drug-naïve animals. This 5-HT syndrome animal model undoubtedly imitates some profiles of the clinical patients, such as behavioral hyperactivities, autonomic dysfunction as well as neurochemistry changes (Finberg, 1995; Turner et al., 2006; Ma et al., 2008; Shioda et al., 2004). Nevertheless, some defects exist. First, clinic cases have a long history of taking antidepressants before suffering from this syndrome (Boyer and Shannon, 2005). Many studies have revealed that chronic antidepressant treatment could increase extracellular 5-HT baseline and change 5-HT receptor responses (Scott and Crews, 1986; Lanteri et al., 2009; Savage et al., 1980), which could not be replicated in the drug-naïve animals. Therefore, the etiological mechanism underlying the patients with a long history of disease and results from acute 5-HT syndrome animal models maybe be different. Second, 5-HTP, a precursor of 5-HT, can increase the brain extracellular 5-HT level up to 1,000 times to the baseline with MAOI. Such a high level of 5-HT was never reported in human cases. Thus, developing a

chronic animal model with appropriate drug regimen is imperative for the 5-HT syndrome research. Indeed, Speiser et al. (2008) developed a chronic 5-HT syndrome model by repeated injection of a MAOI and SSRI. Some mild symptoms of the 5-HT syndrome were observed in the animals, but hyperthermia and death failed to occur even if high dose of the MAOI and SSRI were introduced. It appears that such a model is not a good candidate. To our best knowledge, an acceptable, convenient model for the 5-HT syndrome is unavailable before this study, which has impeded the progress toward better understanding of this disorder. In this study, a novel 5-HT syndrome animal model was developed. Considering co-administration of MAOIs and SSRIs was the most common cause that evoked the 5-HT syndrome in clinic reports showing that 5 of 50 major depressed patients who received the co-prescription of a MAOI and a SSRI developed severe adverse events (Boyer and Shannon, 2005; Hawley et al., 1996), clorgyline, a MAOI, and paroxetine, a SSRI, were used to develop the syndrome. Animals received clorgyline for days to imitate the chronic course of treatment in human. On the last day, rats were challenged with paroxetine at the same time as clorgyline injection. Our preliminary studies have demonstrated that this animal model has similar symptoms and pathophysiological profiles as human cases, which indicates that this model is a suitable candidate for 5-HT syndrome research. With this animal model, we examined the role of the 5-HT_{2A} receptor in the etiology of the syndrome.

Our data indicated that challenge injection of paroxetine induced hyperthermia and other symptoms relevant to severe syndrome in the chronic clorgyline-treated animals (Fig. 9b). It is worthwhile to mention that hyperthermia is a pivotal manifestation of the severe 5-HT syndrome patients and precedent to the fatality (Isbister et al., 2007). In

animal models, however, the severity of syndrome can be easily determined according to T_{cor} responses (Ma et al., 2008; Speiser et al., 2008). Hypothermia is related to a mild syndrome and hyperthermia is seen in severe cases (Ma et al., 2008). It is well-established that 5-HT exerts the dual effects on the T_{cor} by acting on different receptors. Activating 5-HT_{1A} receptors induces hypothermia in rodents (Myers, 1975; Faure et al., 2006; Hjorth, 1985; Lin and Chuang, 2002; Hedlund et al., 2004; Lin et al., 1998). While stimulating 5-HT_{2A} receptor induces hyperthermia (Faure et al., 2006; Hjorth, 1985; Lin and Chuang, 2002; Hedlund et al., 2004; Gudelsky et al., 1986). Therefore, the increase in T_{cor} can be used to evaluate the activity of the 5-HT_{2A} receptor in vivo as well as the severity of the 5-HT syndrome (Gowing et al., 2002; Nisijima et al., 2000, 2004). As shown in Figure 9b, paroxetine and clorgyline challenge induced hypothermia in drug-naïve animals, but provoked hyperthermia in rats with chronic clorgyline treatment. Although the excessive increase in the extracellular 5-HT level is strongly associated with induction of the syndrome, the severity of the syndrome is apparently not determined by the level of 5-HT in the CNS. In support, we measured 5-HT levels in each group and found no difference of the absolute 5-HT level existed between the mild and severe 5-HT syndrome animals (Fig. 12). One corollary of the present study is that chronic clorgyline increased the activity of 5-HT_{2A} receptor. The conclusion was further confirmed by data in Figure 2.4 which stated that ketanserin could blocked the hyperthermia of the syndrome animal.

In a 5-HT syndrome animal model induced by clorgyline and 5-HTP, animals started displaying hyperthermia as the brain 5-HT level beyond 30-fold to the baseline (Zhang et al., 2009). However, co-administration of clorgyline and paroxetine in

drug-naïve animals decreased the T_{cor} with a fold increase in brain 5-HT beyond 60 times greater than the baseline. The conflicting results may attribute to the different agents used because 5-HTP can provide extrinsic 5-HT to any region with 5-HTP docarboxylase, while paroxetine and clorgyline increase 5-HT only in the regions with serotonergic axon terminals.

Interestingly, we noticed that clorgyline combined with paroxetine induced hypothermia in drug-naïve animals (Fig. 9a). In a previous study, we found that less than 10-fold increase in brain extracellular 5-HT could decrease the T_{cor} via activating 5-HT_{1A} receptor (Zhang et al., 2009). It suggests that the 5-HT_{1A} receptor is activated predominately. In agreement with this, our data also verified that WAY-100635, a 5-HT_{1A} receptor antagonist, reduced the hypothermia. Unfortunately, no statistical difference was found (Fig. 11). In a previous study, it was found that WAY-100635 produced the maximal effect around 30 to 45 minutes after injection (Ma et al., 2008), but the data we collected in this study was one hour later after WAY-100635 exposure. The difference of time point may account for the statistical results.

The changes in T_{cor} observed in the 5-HT syndrome animals involve 5-HT_{1A} and 5-HT_{2A} receptors. Under room temperature, the affinity of 5-HT_{1A} receptor to 5-HT is 1000 times higher than 5-HT_{2A} receptor (Dalpiaz et al., 1995). Hence, the mild increase in extracellular 5-HT will induce hypothermia first by acting on 5-HT_{1A} receptors. When 5-HT increases to a certain level, 5-HT_{2A} receptors will be stimulated and hyperthermia will appear. The interaction between the 5-HT_{1A} receptor, if not all, and the 5-HT_{2A} receptor keeps the T_{cor} in a normal range. The neural targets that mediate the thermoregulation by 5-HT was also explored. Lin et al. (1998) found that excitation of

5-HT_{2A} or 5-HT_{1A} receptors in the POA, a pivotal center of thermoregulation (Boulant, 1981), could induce hyperthermia and hypothermia, respectively. Consistently, our data showed that 5-HT level in the POA increased. It suggests that POA may severe as the target that mediates the thermoregulation mediated by the 5-HT_{1A} and 5-HT_{2A} receptors.

Another finding in this study was that chronic clorgyline treatment attenuated 8-OH-DPAT (a 5-HT_{1A} receptor agonist)-induced hypothermia (Fig. 11b). It suggests that the 5-HT_{1A} receptor activity on T_{cor} is desensitized after repeated clorgyline treatment. Paralleled with our study, chronic antidepressant treatment (e.g., tranylcypromine, zimeldine, desipramine) could desensitize the 5-HT_{1A} receptor and attenuate the hypothermia provoked by 8-OH-DPAT (Goodwin et al., 1987; Lanteri et al., 2009). Likewise, chronic buspirone, a partial 5-HT_{1A} receptor agonist, treatment decreased 5-HT_{1A} mRNA level in some brain regions (Chen et al., 1995). The 5-HT_{1A} receptor has an opposite role to the 5-HT_{2A} receptor in regulating the body temperature action. Therefore, the desensitization of the 5-HT_{1A} receptor, on the other hand, may potentiate the hyperthermia and other activities mediated by the 5-HT_{2A} receptor.

Head shake is a characteristic behavior mediated by 5-HT_{2A} receptor (Peroutka et al., 1981). Our study showed that paroxetine and clorgyline challenge could evoke the head shakes in the drug-naïve and chronic clorgyline-treated animals (Fig.13a). It suggests the 5-HT_{2A} receptor is activated in these scenarios. Theoretically, chronic clorgyline treatment could increase the number of head shakes to the challenge injection in term of the sensitized 5-HT_{2A} receptor, however there was no relation between the number of head shakes and duration of clorgyline treatment observed. In a previous study, we found the intensified muscular activity could restrain the head shaking response (Ma

et al., 2008). Indeed, repeated clorgyline treatment increased the muscle rigidity in this study (data not shown), which might account for the response of head shakes. To eliminate the effect of muscle tone on the head shakes, DOI, a 5-HT_{2A} receptor agonist, substituted paroxetine and clorgyline challenge to evoke head shakes in animals with repeated clorgyline treatment. DOI increased the number of head shakes in chronic clorgyline-treated animals (Fig. 13b). A tentative explanation is that chronic clorgyline treatment increases the activity of 5-HT_{2A} receptor, in agreement with the 5-HT_{2A} receptor hypothesis.

Head shake or head twitch is a behavior characterized by the rat's shaking its head (not the whole body) in a short and firm way. Besides as a part of the natural behavioral repertoire in mammalian species, head shakes can be provoked by 5-HT_{2A} receptor agonists (e.g., DOI). Pharmacologically, head shakes are considered to be a stereotyped behavior that provides useful information about the mechanisms of specific drugs, for example hallucinogens, and potential treatments. The relation between the hallucination and head shakes induced by DOI has a close connection (Garcia et al., 2007). It suggests that head shaking behavior is not only a physical behavior but a symptom of mental activity. Indeed, there are mental state changes in the 5-HT syndrome patients (Boyer and Shannon, 2005). Therefore, head shakes may serve as a indicator of the mental disorder.

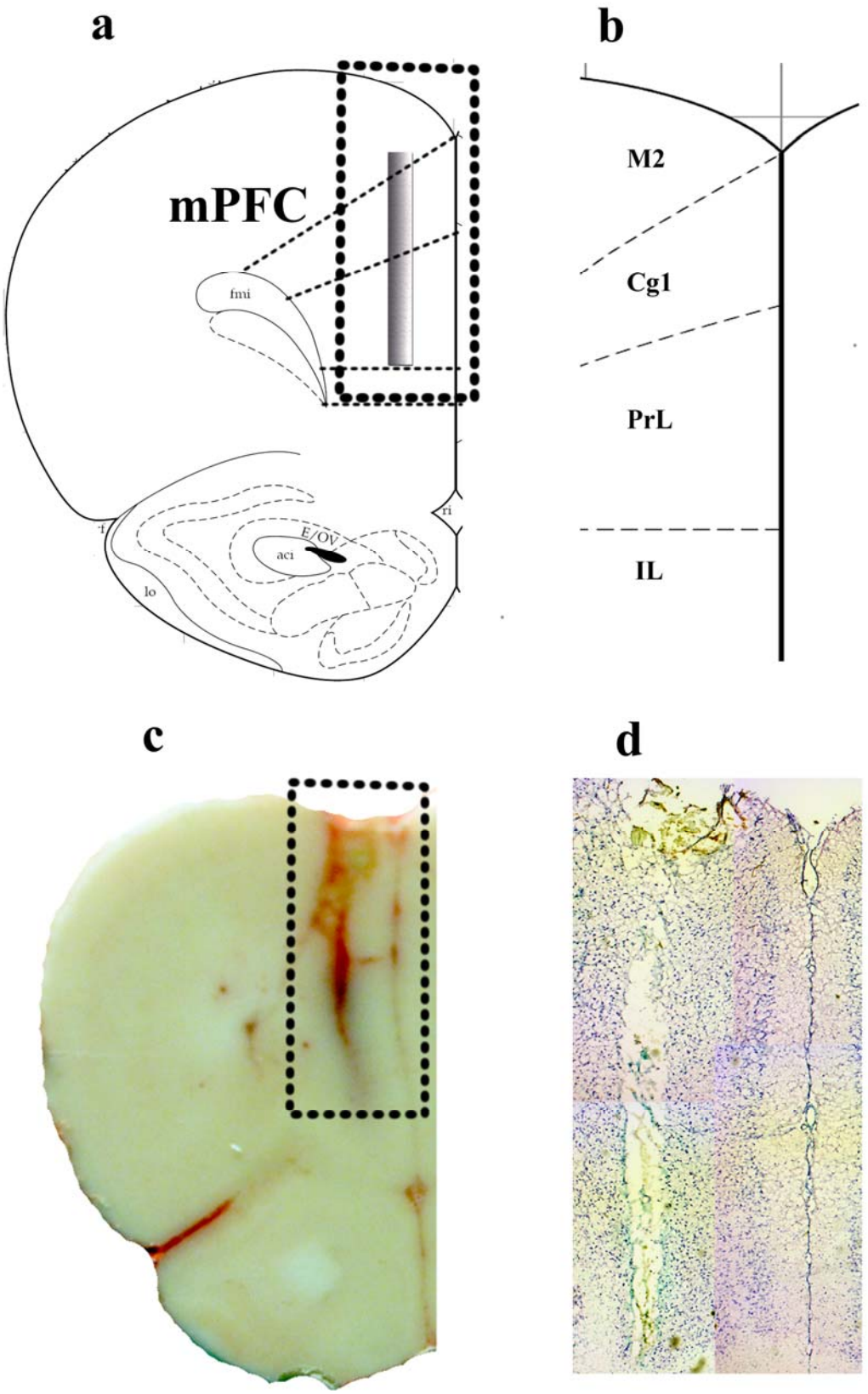
Head shaking behavior is the major behavior mediated by 5-HT_{2A} receptors and other behaviors (e.g., tremor, forepaw treading) are mediated by 5-HT_{1A} receptors (Yamada et al., 1988). It is necessary and relatively easy to monitor head shakes and, also, it is interesting to test the other 5-HT syndrome behaviors. However, the other behaviors were not related to the hypothesis, so they were not included.

Our data in this study revealed that chronic clorgyline treatment increased the mortality rate of the 5-HT syndrome animals (Table 8). 5-HT_{2A} is responsible for the fatality of the syndrome animals (Nisijima et al., 2001; Ma et al., 2008). Both animal studies and clinic case reports have confirmed that blocking 5-HT_{2A} receptors could improve the survival rates of the 5-HT syndrome subjects (Lappin and Auchincloss, 1994; Graudins et al., 1998; Nisijima et al., 2000, 2004). Therefore, the fatality can at least in part serve as a criterion to evaluate the activity of 5-HT_{2A} receptor in the malignant syndrome. In this study, chronic clorgyline treatment increased the mortality significantly (Table 8) without a dramatic increase in brain 5-HT level (Fig. 12). It appears that 5-HT level is a causative but not a decisive factor to the death of the 5-HT syndrome animals. Given the 5-HT_{2A} receptor is responsible for the mortality of the syndrome, a tentative explanation in favor of this result is that the increased activity of 5-HT_{2A} receptors to chronic clorgyline treatment is responsible for the malignant response.

The variation of the 5-HT_{2A} receptor activity to MAOI treatment has been the focus of many studies in the past decades. Some in vivo studies stated the chronic MAOI treatment decreased 5-HT_{2A} receptor density (Todd et al., 1995), however others reported chronic MAOI increased 5-HT_{2A} receptor activity (Lanteri et al., 2009). In vitro studies also revealed that 5-HT_{2A} receptor agonist had inconsistent effects on the receptor binding capacity, and gene expression (Table 3). This discrepancy of the studies suggests that the change of 5-HT_{2A} receptor activity depends on the cell milieu, the agents selected as well as the span of drug treatment (Grotewiel and Sanders-Bush, 1994).

In summary, the data of the present study indicate that chronic clorgyline treatment increases 5-HT_{2A} activity in a 5-HT syndrome animal model developed by clorgyline and

paroxetine. The sensitization of 5-HT_{2A} receptor may account for the occurrence of the 5-HT syndrome in patients with chronic antidepressant treatment. Additionally, our data revealed that repeated clorgyline exposure desensitizes 5-HT_{1A} receptors, which may potentiate the activity of 5-HT_{2A} receptor indirectly.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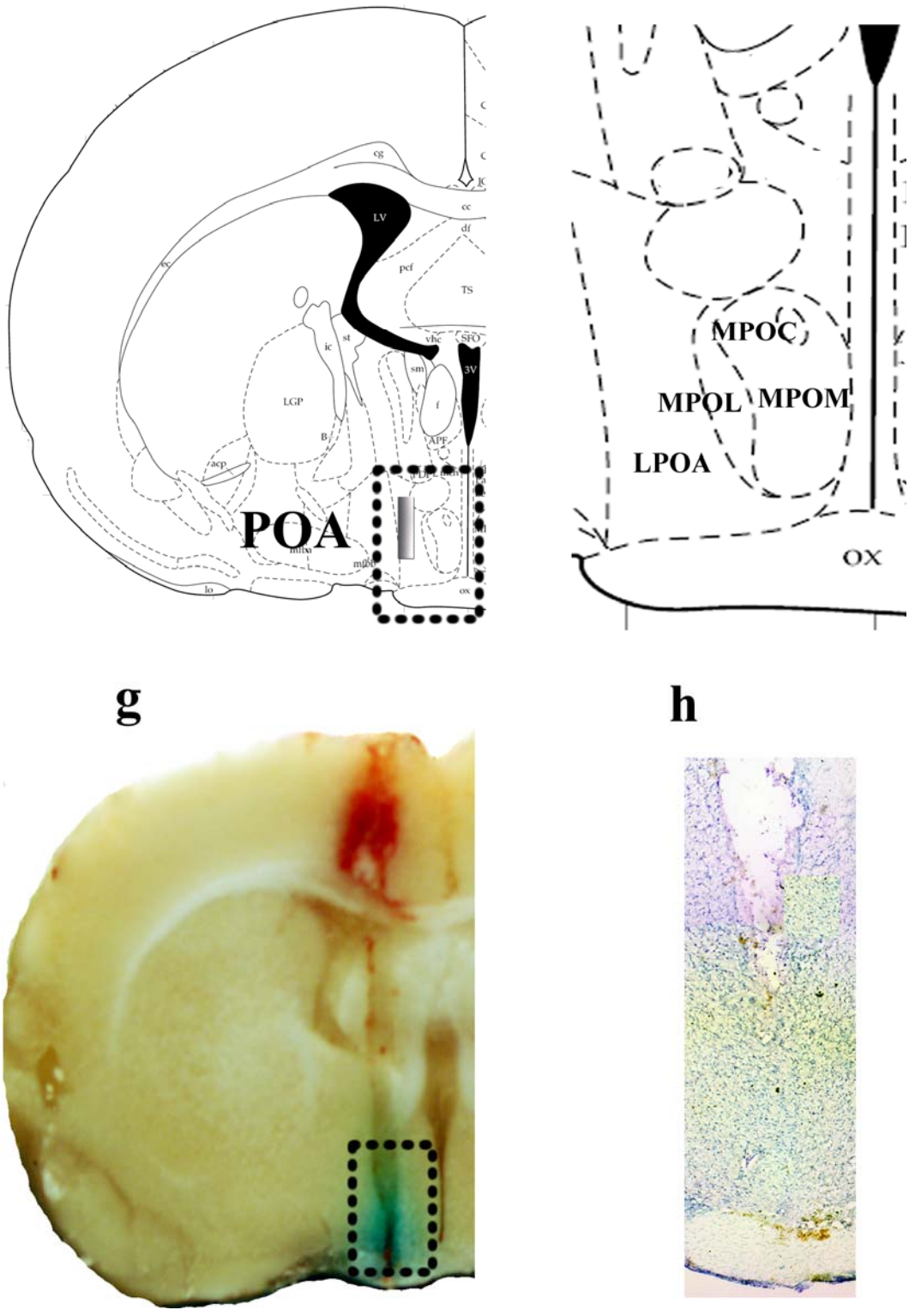


Figure 7. Schematic microdialysis location in the medial prefrontal cortex (mPFC) and preoptic area (POA)

Figure 7. Schematic microdialysis location in the medial prefrontal cortex (mPFC) and preoptic area (POA)

Microdialysis probes were inserted into mPFC (A/P, + 3.3; L/R, ± 0.8; D/R, - 4.5) or POA (A/P, - 1.1; L/R, ± 0.9; D/R, - 9.2). The shade area indicated the microdialysis probe. (a) A diagram of rat brain showing mPFC region. (b) The amplified area as indicated with frame in (a). (c) A representative sample of brain showing mPFC region frozen and cut by microtome (Leica CM1850). Picture was taken by Nikon digital camera (D80). (d) A representative slide (40 μm) showing mPFC region stained by Cresyl Violet. (e) A diagram of rat brain showing POA. (f) The amplified area as indicated with frame in (a). (g) A representative sample of brain showing POA region frozen and cut by microtome (Leica CM1850). Picture was taken by Nikon digital camera (D80). (h) A representative slide (40 μm) showing POA region stained by Cresyl Violet. A/P, anterior/posterior; L/R, left/right; D/V, dorsal/ventral. Cg1,cigulate cortex, area 1; IL, infralimbic cortex; M2, secondary motor area; MPOC, medial preoptic nucleus, center; MPOL, medial preoptic nucleus, lateral; MPOM, medial preoptic area, medial; LPOA, lateral preoptic area; PrL, prelimbic cortex. Unit in mm (Paxinos and Watson, 1998).

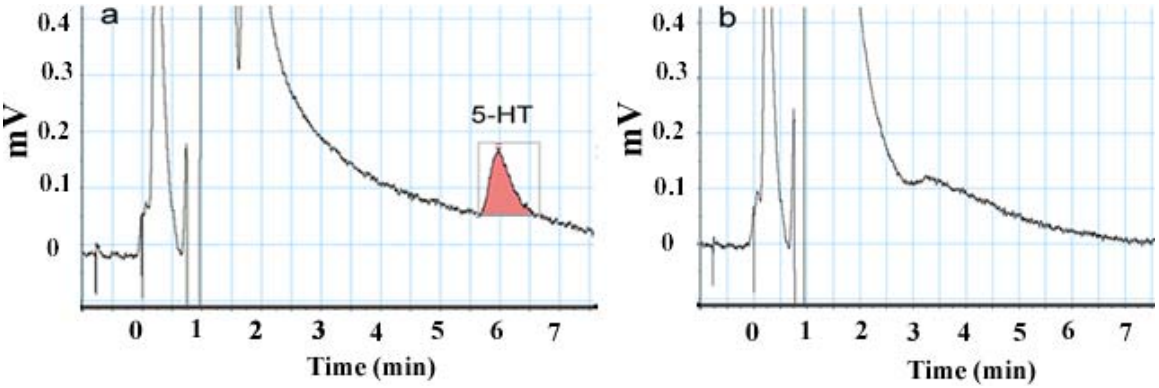


Figure 8. Representative HPLC chromatograms obtained from the analysis of a standard sample (10 pg/10µl, a) and blank control (b)

Figure 8. Representative HPLC chromatograms obtained from the analysis of a standard sample (10 pg/10µl, a) and blank control (b)

Note that retention time for 5-HT peak appeared at 5-7 min after sample injection.

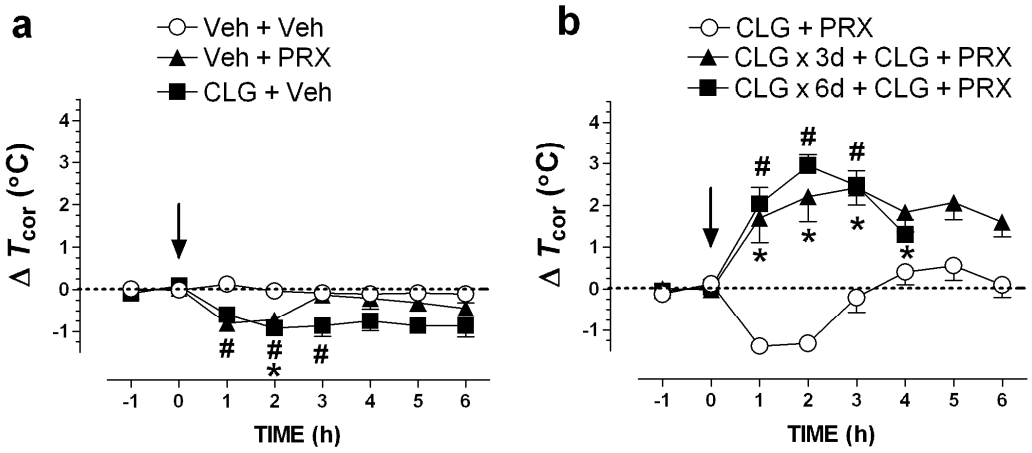
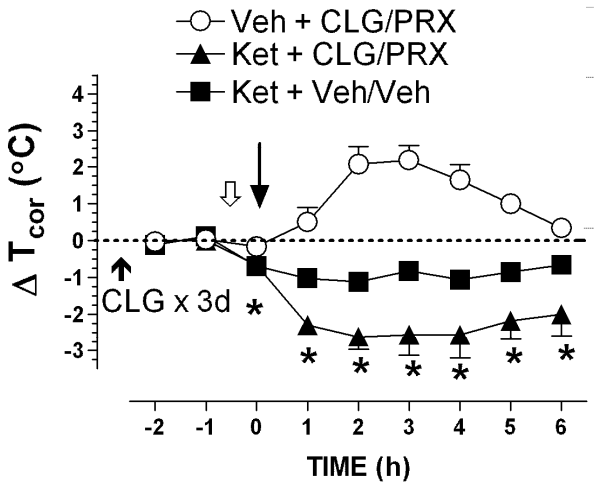


Figure 9. Changes in the core body temperature (ΔT_{cor}) in the 5-HT syndrome animals with acute and chronic clorgyline (CLG) treatment

Figure 9. Changes in the core body temperature (ΔT_{cor}) in the 5-HT syndrome animals with acute and chronic clorgyline (CLG) treatment

Arrow indicates the time of paroxetine (PRX, 15 mg/kg, i.p.) and/or CLG (2 mg/kg, s.c.) injection. (a) A single administration of PRX or CLG decreased T_{cor} in the drug-naïve animals. * $p < 0.05$ CLG vs. vehicle; # $p < 0.05$ PRX vs. vehicle (one-way ANOVA followed by *post hoc* Scheffe's test). (b) PRX and CLG injection increased T_{cor} in animals with 3- and 6-day CLG pre treatment but decreased T_{cor} in the drug-naïve animals. * $p < 0.05$ and # $p < 0.05$ vs. acute group (one-way ANOVA followed by *post hoc* Scheffe's test). In the group of animals received 6-day CLG treatment, PRX and CLG challenge on day 7 caused 6 of 7 animals died within 5 hours after challenge. Therefore, only 4-hour data after challenge were listed in this group. Values are expressed as the change of T_{cor} to the baseline before drug administration on the last day. Each value is the mean ± SEM of 5-8 animals.



Groups CLG x 3d +	Survived	Died	Total
Veh + CLG/PRX	2	4	6
Ket + CLG/PRX	8	0	8
Ket + Veh/Veh	6	0	0

Figure 10. Effects of ketanserin, a 5-HT_{2A} receptor antagonist, on the hyperthermia and fatality of the severe 5-HT syndrome animals

Figure 10. Effects of ketanserin, a 5-HT_{2A} receptor antagonist, on the hyperthermia and fatality of the severe 5-HT syndrome animals

The open arrow indicates the time for ketanserin (Ket, 5 mg/kg, i.p.) injection and the solid line for paroxetine (PRX, 15 mg/kg, i.p.) and clorgyline (CLG, 2 mg/kg, s.c.) challenge. Animals had received CLG for three days. On the fourth day, ketanserin was injected 30 min before CLG and PRX challenge. Ket prevented the hyperthermia. * $p < 0.05$ vs. Veh + CLG/PRX (one-way ANOVA followed by *post hoc* Scheffe's test) and mortality ($p < 0.05$, Fisher's exact test, 2-sided) of the severe syndrome animal. Data are shown as mean ± SEM of 6-8 animals.

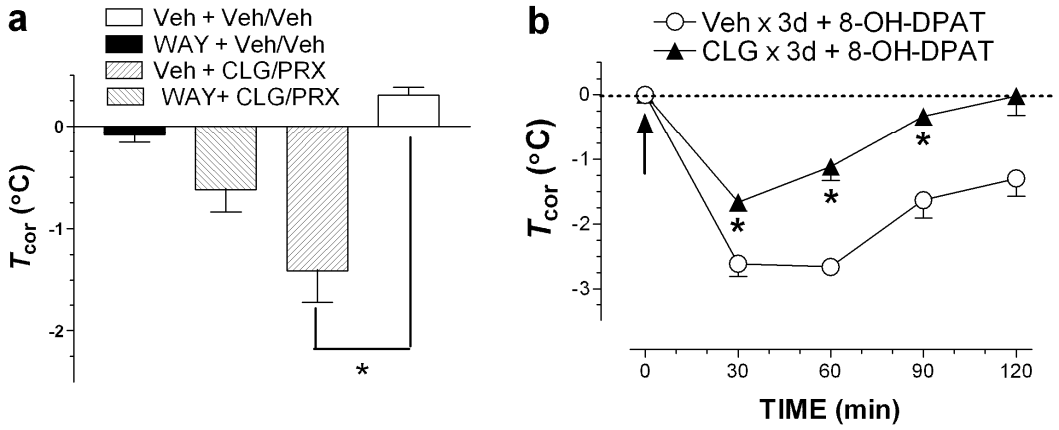


Figure 11. Effects of WAY-100635 (WAY) and 8-OH-DPAT on the T_{cor} of the 5-HT syndrome animals

Figure 11. Effects of WAY-100635 (WAY) and 8-OH-DPAT on the T_{cor} of the 5-HT syndrome animals

(a) Animals were treated with clorgyline (CLG, 2 mg/kg, s.c.) once a day for consecutive three days. On the fourth day, WAY (0.5 mg/kg, i.p.), a 5-HT_{1A} receptor antagonist, was injected 30 min before clorgyline and paroxetine (PRX, 1mg/kg) challenge. T_{cor} was recorded one hour after the challenge. WAY did not block the hypothermia induced by PRX. * $p < 0.05$ (one-way ANOVA followed by *post hoc* Scheffe's test). (b) Rats received clorgyline (2 mg/kg, s.c.) or vehicle once a day for consecutive three days. On the fourth day, 8-OH-DPAT (0.5 mg/kg, s.c.) was administrated at time zero as indicated by the solid arrow. * $p < 0.05$ (independent t-test). Data are shown as mean ± SEM of 3-6 animals.

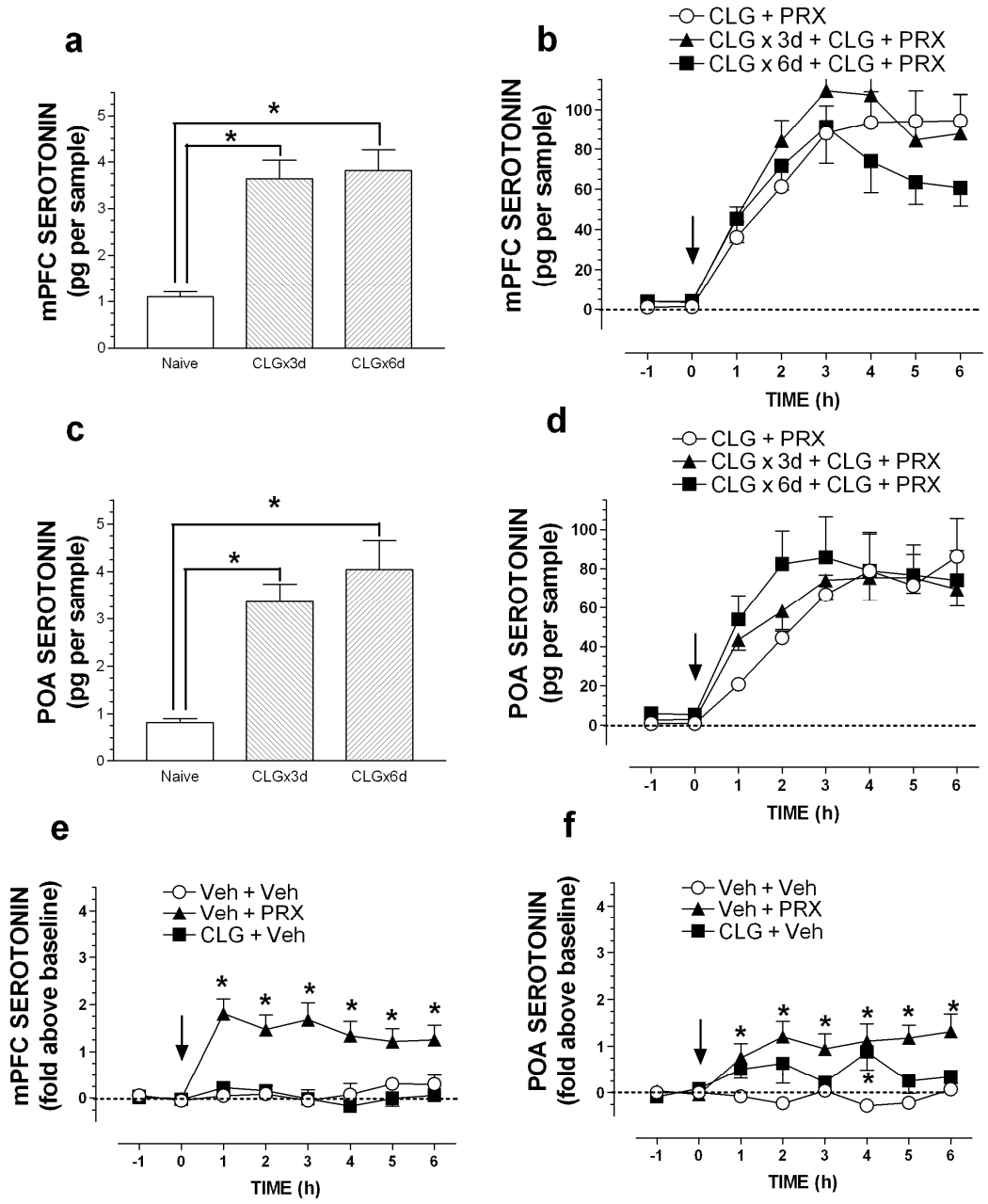


Figure 12. 5-HT level in the medial prefrontal cortex (mPFC) and preoptic area (POA) in the 5-HT syndrome animals with single acute or chronic clorgyline injection in combination with paroxetine

Figure 12. 5-HT level in the medial prefrontal cortex (mPFC) and preoptic area (POA) in the 5-HT syndrome animals with single acute or chronic clorgyline (CLG) injection in combination with paroxetine (PRX)

Arrow indicates the time of CLG (2mg/kg, s.c.) and PRX (15 mg/kg, i.p.) administration. Repeated treatment with clorgyline increased the basal 5-HT level in mPFC (a) and POA (c). * $p < 0.05$ (one-way ANOVA followed by *post hoc* Scheffe's test). Although challenge injection of paroxetine combined with clorgyline caused a remarkable increase, there were no statistical differences of 5-HT level in mPFC (b) and POA (d) between acute and chronic groups ($p > 0.05$, main effect of treatment). (e) PRX increased 5-HT efflux in the mPFC of drug-naïve animals. * $p < 0.05$ vs. vehicle (one-way ANOVA followed by *post hoc* Scheffe's test). (f) Single injection of CLG or PRX increased 5-HT level in POA of drug-naïve animals. * $p < 0.05$ vs. vehicle (one-way ANOVA followed by *post hoc* Scheffe's test). Data are shown as mean ± SEM of 4-9 animals.

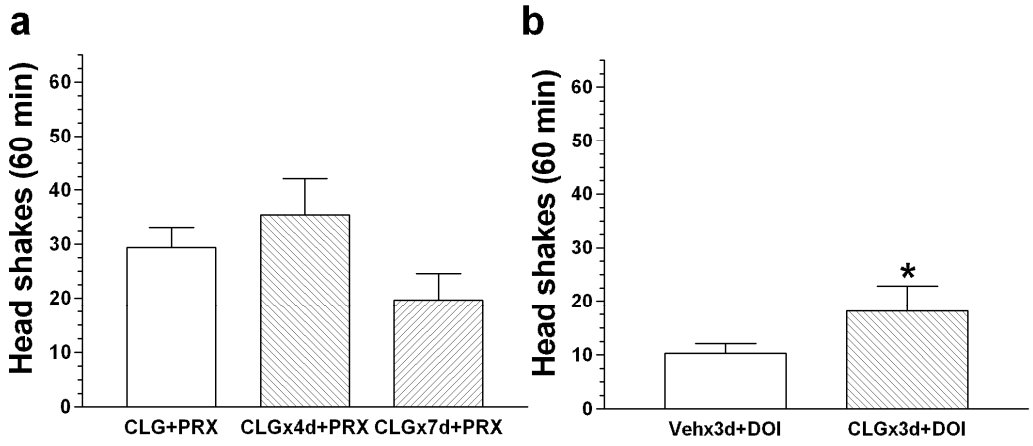


Figure 13. Changes in head shakes in the animals with acute or chronic clorgyline (CLG) treatment

Figure 13. Changes in head shakes in the animals with acute or chronic clorgyline (CLG) treatment

(a) Chronic CLG (2mg/kg, s.c.) treatment did not alter the number the head shakes counted on the first hour after CLG and paroxetine (PRX, 15 mg/kg) challenge. * $p >$ 0.05 (one-way ANOVA followed by *post hoc* Scheffe's test). (b) DOI increased the number of head shakes in animals with 3-day CLG treatment. * $p < 0.05$ (independent t-test). Data are shown as mean ± SEM of 5-10 animals.

Table 6. Dose, solvent, volume and route of each drug used in chapter one

Chemical	Dose (mg/kg)	Solvent	Volume (ml/100g)	Route of administration
Clorgyline	2	Saline	0.1	s.c.
DOI	1	Saline	0.1	s.c.
WAY-100635	0.5	Saline	0.1	i.p.
Paroxetine	1	H_2O	0.1	i.p.
Paroxetine	15	H_2O	0.3	i.p.
Ketanserin	5	H_2O	0.1	i.p.

* i.p., intraperitoneal; s.c., subcutaneous

Table 7. The stereotaxic coordination of medial prefrontal cortex (mPFC) and preoptic area (POA) (unit in mm) (Paxinos and Watson, 1998)

	A/P	L/R	D/V	Depth of guide below skull /length of guide	Length of tip
mPFC	+ 3.3	± 0.8	- 4.5	-2.0/10	2.5
POA	-1.1	±0.9	-9.2	-3.0/11	1.0

* A/P, anterior/posterior; L/R, left/right; D/V, dorsal/ventral

Table 8. Effects of chronic clorgyline (CLG) treatment on the fatality of the 5-HT syndrome animals. CLG (2mg/kg, s.c.) pretreatment for 3 and 6 days significantly increased the fatality to challenge injection of paroxetine (PRX, 15 mg/kg, i.p.) combined with CLG on day 4 and 7.

Groups	Survived	Died	Total
CLG + PRX	17	2	19
CLG x 4d + PRX	8	8	16
CLG x 7d + PRX	6	6	12

Table 9. The 5-HT syndrome animal models used in lab research

Chronic treatment	Acute treatment	References
	Clorgyline + 5-HTP	(Nisijima et al., 2000, 2001, 2003, 2004; Ma et al., 2008; Zhang et al., 2009; Izumi et al., 2006)
	Selegiline or pargyline + 5-HTP	(Izumi et al., 2006)
	Tranylcypromine + L-tryptophan	(Deakin and Green, 1978)
	Tryptophan + pargyline or harmaline	(Abdel-Fattah et al., 1997)
Fluvoxamine × 6d	Selegiline or pargyline or clorgyline + 5-HTP	(Izumi et al., 2006)
Imipramine × 6d	Pargyline or clorgyline or selegiline + 5-HTP	(Izumi et al., 2007)
Milnacipran × 6d	Pargyline or clorgyline or selegiline + 5-HTP	(Izumi et al., 2007)
Rasagiline or selegiline × 7d	Rasagiline or selegiline + fluoxetine × 21	(Speiser et al., 2008)

***** Tranylcypromine and clorgyline, monoamine oxidase (MOA) A inhibitor; Selegiline, pargyline and rasagiline, MAO-B inhibitor; Fluvoxamine, a selective serotonin reuptake

inhibitor (SSRI); Milnacipran, a serotonin norepinephrine reuptake inhibitor (SNRI); Imipramine, a tricyclic antidepressant (TCA)

CHAPTER THREE: EFFECTS OF ENVIRONMENTAL TEMPERATURE ON THE SEVERITY OF SEROTONIN SYNDROME EVOKED BY CHALLENGE INJECTION OF PAROXETINE COMBINED WITH CLORGYLINE

3.1 Introduction

The effects of cold and warm ambient temperature on the toxic responses induced by serotonin-selective psychotropic drugs have been well-documented. Animal studies indicated that cold ambient temperature could decrease methamphetamine (a 5-HT releaser)-induced hyperthermia, neuron damage and death in mice (Ali et al., 1994; Gunn and Thoresen, 2006). In contrast, warm environmental temperature could potentiate the toxicity evoked by methamphetamine and its derivates [e.g., 3,4-methylenedioxy methamphetamine (MDMA, ecstasy)] (Malberg and Seiden, 1998; Miller and O'Callaghan, 2003; Bowyer et al., 1994). Consistently, a retrospective review of Medical Examiners' cases from 1990 through 1995 in New York City revealed that the mean daily number of cocaine or opiate (5-HT releasers) overdose deaths was significantly higher on hot days (Marzuk et al., 1998). These data suggest that warm and cold ambient temperature aggravates and alleviates the toxic responses induced by 5-HT, respectively. Likely, ambient temperature influences the prognosis of the 5-HT syndrome subjects. As described in previous literature (Miller and O'Callaghan, 2003), studies from our lab revealed that warm ambient temperature (32°C) potentiated hyperthermia and mortality

of the 5-HT syndrome animals developed by clorgyline and 5-HTP. On the contrary, cold ambient temperature (12°C) reversed the hyperthermia and prevented fatality. Clinically, cold measures have been employed widely to alleviate 5-HT syndrome patients (Ener et al., 2003). The mechanisms behind the ambient temperature on 5-HT syndrome have not been fully elucidated yet.

In the previous chapter, data verified that 5-HT_{2A} receptor mediated the hyperthermia and mortality of the 5-HT syndrome animals. Interestingly, hyperthermia and death could also be observed in animals taken therapeutic dose of proserotonergic agents and housed in warm ambient temperature. Recently, a pilot study in our lab revealed that ketanserin, a 5-HT_{2A} receptor antagonist, blocked the hyperthermia and prevented death of the 5-HT syndrome animals housed in warm environment. Given that increase in environmental temperature raise the affinity of 5-HT_{2A} receptor (Dalpiaz et al., 1995), it appears that the 5-HT_{2A} receptor serves as a pivotal candidate that bridges the ambient temperature and severity of the 5-HT syndrome, but little evidence is available to date.

The aim of this chapter was to explore the effect of different ambient temperature on the activity of 5-HT_{2A} receptor in the 5-HT syndrome animals developed by paroxetine and clorgyline as well as in naïve animals. Since warm ambient temperature increased the MDMA-induced toxicity (Malberg and Seiden, 1998; Miller and O'Callaghan, 2003; Bowyer et al., 1994), we hypothesized that warm ambient temperature could increase the 5-HT_{2A} receptor activity, resulting in a aggravated 5-HT syndrome. On the contrary, cold ambient temperature could inhibit the activity of 5-HT_{2A} receptor and thereafter alleviate the severe syndrome animals.

3.2 Materials and methods

Animal preparation, chemicals and schedule for injection, preparation of 5-HT syndrome animal model, core body temperature measurement, surgery, microdialysis procedures, histological identification and data analysis were identical with those described in chapter two.

Behavioral score

On the day of testing, animals were transferred to a transparent bowl and habituated it for 2 h before drug administration. Syndrome behaviors examined included tremor, forepaw treading, hindlimb abduction, Straub tail and immobility. Each behavior, rated on a scale of mild (score 1), moderate (score 2) and severe (score 3) (Ma et al., 2008), was assessed once per hour for six consecutive hours after clorgyline and paroxetine administration. The scores from the six one-hour periods were averaged to represent each syndrome intensity. The behavioral score was the sum of the six averaged scores. Thus, the behavioral score ranges from 0 to 15 (Table 10).

Thermostatic chamber

The thermostatic chamber was built as described previously with little modification (Malberg and Seiden, 1998). In brief, the chamber was made of transparent polyethylene with dimension of 100 × 60 ×80 cm. Heat insulation was placed on the 5 sides of the chamber with door left. A polyethylene plate with 6 holes was used a door. A temperature controller (Fisher Scientific) regulated the chamber temperature by control a heater or an air conditioner. The air in the chamber is circulated by two fans (Fig. 14).

3.3 Results

Effects of warm (32 °C) or cold (12 °C) T_{amb} on the T_{cor} of the moderate 5-HT syndrome animals

Our preliminary data indicated that animals previously receiving daily clorgyline (2 mg/kg) for 3 days displayed profiles of a moderate 5-HT syndrome in the standard T_{amb} (22 °C) on day 4 following 2 mg/kg clorgyline and 5 mg/kg paroxetine challenge. This experimental paradigm was exclusively adopted here to examine the effect of other T_{amb} on the 5-HT_{2A} receptor activity. As described in the earlier studies, animals used here have previously received daily 2 mg/kg clorgyline pretreatment for 3 consecutive days at the standard T_{amb} of 22 °C. On the 4th day, animals habituated in chambers with different T_{amb} at least two hours before starting recording. The baseline T_{cor} of rats in cold (12 °C), standard (22 °C) and warm (32 °C) T_{amb} was 38.2 ± 0.1 °C (n = 16), 37.7 ± 0.2°C (n = 10) and 38.3 ± 0.1 °C (n = 12), respectively. One-way ANOVA demonstrated a significant effect of the altered T_{amb} on basal T_{cor} ($F_{2, 35} = 5.032$, $p < 0.05$). *Post hoc* comparisons showed that the warm T_{amb} elevated the baseline T_{cor} ($p < 0.05$, Scheffe's test). Interestingly, basal T_{cor} was not significantly altered at cold T_{amb} ($p > 0.05$, Scheffe's test) (Fig. 15a).

To examine changes in the 5-HT_{2A} receptor activity in the different T_{amb}, the T_{cor} of the 5-HT syndrome animals was measured. Consistent with the previously findings, the T_{cor} of the moderate syndrome animals stayed in a normal range (normothermia) at the standard T_{amb} (22 °C). In contrast, as shown in Figure 15b, cold (12 °C) and warm (32 °C) T_{amb} had significant effect on the T_{cor} as demonstrated by the significant effect of

treatment ($F_{2, 18} = 98.3$, $p < 0.001$), time ($F_{4, 72} = 7.8$, $p < 0.001$) and treatment × time ($F_{8, 72} = 30.8$, $p < 0.05$). *Post hoc* comparisons showed that warm T_{amb} significantly increased the T_{cor} compared with room T_{amb} ($p < 0.01$, Scheffe's test). There was no difference in T_{cor} between the room T_{amb} group and cold T_{amb} group ($p > 0.05$, Scheffe's test). To further examine the time course of T_{amb} on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that T_{amb} altered T_{cor} on the first three hours after drug challenge ($p < 0.05$). *Post hoc* comparisons indicated that only warm T_{amb} increased the T_{cor} to the room T_{amb} after drug challenge. Because all the moderate 5-HT syndrome animals housed in the warm (32 °C) environment without a 5-HT_{2A} receptor antagonist pretreatment died within 6 hours after challenge injection, so only part of the body temperature data were shown.

Same as previous reports that ketanserin could block the 5-HTP-induced hyperthermia of the 5-HT syndrome animals in room T_{amb} (Nisijima et al., 2001), ketanserin had significant effect on the change of T_{cor} in warm T_{amb} (Fig. 15c) here as demonstrated by the significant effect of treatment ($F_{2, 16} = 158.2$, $p < 0.001$), time ($F_{5, 80} = 17.3$, $p < 0.001$) and treatment × time ($F_{10, 80} = 68.9$, $p < 0.001$). *Post hoc* analysis showed ketanserin could significantly block the hyperthermia ($p < 0.001$, Scheffe's test). To further examine the time course of T_{amb} on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that ketanserin pretreatment altered the T_{cor} after clorgyline and paroxetine challenge ($p < 0.001$). *Post hoc* comparisons indicated that ketanserin blocked the hyperthermia of the 5-HT animals housed on warm T_{amb} ($p < 0.001$).

Interestingly, ketanserin potentiated the hypothermic response as the tests were

carried out at cold T_{amb} (Fig. 15d). Two-way repeated ANOVA showed that ketanserin had significant effect on the change of T_{cor} in cold T_{amb} as demonstrated by the significant effect of treatment ($F_{2, 16} = 158.5$, $p < 0.001$), time ($F_{5, 80} = 17.3$, $p < 0.001$) and treatment × time ($F_{10, 80} = 68.9$, $p < 0.001$). *Post hoc* comparison showed ketanserin decreased T_{cor} ($p < 0.001$, Scheffe's test). To further examine the time course of ketanserin on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that ketanserin pretreatment altered the T_{cor} ($p < 0.001$). *Post hoc* comparisons indicated that ketanserin potentiated the hypothermia from 2 to 6 hours after clorgyline and paroxetine challenge. It appears the 5-HT_{2A} receptor mediates the hyperthermia observed.

Effects of T_{amb} on the DOI-induced increase in T_{cor} in drug-naïve animals

The antidepressant data suggest that the effects of 5-HT_{2A} receptor on the 5-HT syndrome depend on the T_{amb}. In support of this hypothesis, DOI, a 5-HT_{2A} receptor agonist, -induced increase in T_{cor} with drug-naïve animals housed in different T_{amb} was examined. As shown in Figure 16, DOI altered the T_{cor} in the standard T_{amb} of 22 °C as demonstrated by the significant effect of treatment ($F_{3, 18} = 32.9$, $p < 0.001$), time ($F_{7, 126} = 11.0$, $p < 0.001$) and treatment × time ($F_{21, 126} = 4.6$, $p < 0.001$). The maximal increase in T_{cor} in response to 0.05, 0.1 and 0.5 mg/kg DOI was 0.29 ± 0.08, 0.64 ± 0.06 and 0.79 ± 0.06 °C. Specifically, *post hoc* comparisons showed that 0.05 mg/kg DOI had no effects on T_{cor} ($p > 0.05$, Scheffe's test). In contrast, 0.1 and 0.5 mg/kg DOI enhanced T_{cor} ($p < 0.01$, Scheffe's test; Fig. 16a). To further examine the time course of DOI on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that DOI altered the T_{cor} on each time point after it injection ($p < 0.001$). *Post hoc* comparisons indicated

that 0.1 mg/kg DOI enhanced T_{cor} on the 30, 45 and 60 min. 0.5 mg/kg DOI increased T_{cor} on the 15, 30, 45, 60 and 90 min. This effect might be due to activation of 5-HT_{2A} receptor, which was supported by the observation that ketanserin completely blocked it (Fig. 16b).

5-HT_{2A} receptor antagonist blocks the increase in T_{cor} induced by DOI in the room T_{amb}. Two-way ANOVA showed a significant effect of treatment ($F_{3, 18} = 15.9$, $p < 0.001$) and treatment × time ($F_{24, 144} = 4.9$, $P < 0.001$), but no difference in time ($F_{8, 144} = 1.5$, $P > 0.05$). *Post hoc* comparisons showed ketanserin inhibited the increase in T_{cor} induced by DOI ($p < 0.001$, Scheffe's test). To further examine the time course of ketanserin on DOI-mediated T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered the T_{cor} on each time point after it injection ($p < 0.05$). *Post hoc* comparisons indicated that ketanserin blocked the DOI enhanced T_{cor} on the 45, 60, 75 and 90 min after DOI injection.

Next, the effects of warm T_{amb} (32 °C) on the DOI-induced increase in T_{cor} were examined. Unsurprisingly, DOI enhanced the T_{cor} as demonstrated by the significant effect of treatment ($F_{3, 18} = 64.9$, $P < 0.001$), time ($F_{7, 126} = 106.6$, $p < 0.001$) and treatment × time ($F_{21, 126} = 30.6$, $p < 0.001$). *Post hoc* comparisons showed that 0.05 mg/kg DOI had no effects on T_{cor} ($p > 0.05$, Scheffe's test), while 0.1 and 0.5 mg/kg DOI both produced a significant increase in T_{cor} ($p < 0.001$, Scheffe's test). To further examine the time course of DOI on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that DOI altered the T_{cor} on each time point after it injection ($p < 0.001$). *Post hoc* comparisons indicated that 0.1 and 0.5 mg/kg DOI enhanced T_{cor} on each time point after DOI injection ($p < 0.05$, Scheffe's test). The maximal increase was

0.50 ± 0.09, 1.70 ± 0.25 and 2.71 ± 0.17 °C, respectively, in response to 0.05, 0.1 and 0.5 mg/kg DOI (Fig. 16c). The increases in T_{cor} at warm T_{amb} were significantly greater than those at standard T_{amb} (22 °C). Ketanserin blocked this effect as demonstrated by the significant effect of treatment ($F_{3, 17} = 28.1$, $p < 0.001$), time ($F_{8, 136} = 7.4$, $p < 0.001$) and treatment × time ($F_{24, 136} = 19.3$, $p < 0.001$). *Post hoc* comparisons showed ketanserin pretreatment blocked DOI-mediated increase in T_{cor} ($p < 0.001$, Scheffe's test) (Fig. 3.3d). To further examine the time course of ketanserin on DOI-mediated hyperthermia, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered the T_{cor} on each time point after it injection ($p < 0.001$). *Post hoc* comparisons indicated that ketanserin blocked the DOI enhanced T_{cor} on the 30, 60 and 90 min after DOI injection ($p < 0.05$, Scheffe's test), suggesting the involvement of 5-HT_{2A} receptor sensitization at warm T_{amb}.

Finally, the effects of different T_{amb} on the 5-HT_{2A} receptor-mediated increase in T_{cor} were recorded. The basal (pre drug injection) T_{cor} was 38.08 ± 0.09 °C ($n = 12$), 37.91 ± 0.04 °C ($n = 98$), 37.33 ± 0.10 °C ($n = 12$), 37.79 ± 0.03 °C ($n = 96$) at the T_{amb} of 12 °C, 22 °C, 27 °C and 32 °C (Fig. 16e). DOI produced a T_{amb}-dependent increase in T_{cor}. It was confirmed by two-way repeated measures ANOVA showing the significant effect of treatment ($F_{3, 18} = 25.2$, $p < 0.001$), time ($F_{7, 126} = 29.6$, $p < 0.001$) and treatment × time ($F_{21, 126} = 8.3$, $p < 0.001$). *Post hoc* comparisons showed that 12 °C ($p < 0.05$) and 32 °C ($p < 0.01$) inhibited and enhanced DOI-induced increase in T_{cor}, respectively. The maximal increase was 0.20 ± 0.07, 0.69 ± 0.06, 0.99 ± 0.13 and 1.45 ± 0.23 °C, respectively (Fig.16f).

Effects on increases in 5-HT efflux of warm (32 °C) or cold (12 °C) T_{amb} in the moderate syndrome animals

Next, the changes in the extracellular 5-HT level in mPFC and POA were tested to reveal the relationship between T_{amb} and the syndrome. The basal 5-HT levels in the mPFC (pg/sample) were 3.0 ± 0.3 (n = 16), 3.4 ± 0.3 (n = 10) and 4.9 ± 0.3 (n = 20) at cold (12 °C), standard (22 °C) and warm (32 °C) T_{amb}. One-way ANOVA demonstrated that T_{amb} had effects on the basal 5-HT level ($F_{2, 41} = 8.5$, $p < 0.01$). Compared with that at standard T_{amb}, the basal 5-HT was increased at warm T_{amb} ($p < 0.05$, Scheffe's test). No difference was found between cold and standard T_{amb} ($p > 0.05$, Scheffe's test). Challenge injection of paroxetine combined with clorgyline at warm T_{amb} caused a greater increase in 5-HT than that observed at the standard T_{amb}. It was confirmed by two-way repeated measures ANOVA showing the significant effect of treatment ($F_{2, 20} = 68.5$, $p < 0.001$), time ($F_{5, 100} = 91.2$, $p < 0.001$) and treatment × time ($F_{10, 100} = 32.995$, $p < 0.001$). *Post hoc* comparisons showed warm T_{amb} enhanced 5-HT release ($p < 0.001$, Scheffe's test). There was no difference between the room T_{amb} group and cold T_{amb} group ($p > 0.05$, Scheffe's test) (Fig. 17a). To further examine the time course of T_{amb} on 5-HT efflux, one-way ANOVA was run on each time point. These analyses indicated that T_{amb} altered 5-HT level on each time point after clorgyline and paroxetine injection ($p < 0.001$). *Post hoc* comparisons indicated that warm T_{amb} enhanced 5-HT release on the first 3 hours after clorgyline and paroxetine challenge ($p < 0.05$, Scheffe's test).

Next step was to examine a T_{amb}-dependent effect on POA 5-HT. Basal 5-HT (pg/sample) was 4.3 ± 0.3 (n = 8) 3.4 ± 0.4 (n = 15) and 5.3 ± 0.6 (n = 8), respectively, at the cold, standard and warm T_{amb}. One-way ANOVA demonstrated that basal 5-HT was

also T_{amb}-related in this region ($F_{2, 27} = 3.7$, $p < 0.05$). Paroxetine and clorgyline challenge produced a much greater 5-HT increase at warm T_{amb} than that at the standard experimental condition. It was confirmed by two-way repeated ANOVA showing the significant effect of treatment ($F_{2, 10} = 38.3$, $p < 0.001$), time ($F_{5, 50} = 44.9$, $p < 0.001$) and treatment × time ($F_{10, 50} = 15.7$, $p < 0.001$). Similar to those happened in the mPFC, *Post hoc* analyses showed warm T_{amb} enhanced 5-HT release ($p < 0.001$, Scheffe's test). There was no significant differences of POA 5-HT between standard and cold T_{amb} ($p > 0.05$, Scheffe's test) (Fig.17b).To further examine the time course of T_{amb} on 5-HT efflux, one-way ANOVA was run on each time point. These analyses indicated that T_{amb} altered 5-HT level on each time point after clorgyline and paroxetine injection ($p < 0.05$). *Post hoc* comparisons indicated that warm T_{amb} enhanced 5-HT release on the 2 and 3 hours after clorgyline and paroxetine challenge ($p < 0.01$, Scheffe's test).

Lastly, a hypothesis that the sensitized 5-HT_{2A} receptor accounted for the enhanced increase in 5-HT at warm T_{amb} was examined. In cold T_{amb}, ketanserin pretreatment altered 5-HT level in mPFC as demonstrated by the significant effect of treatment ($F_{2, 14} = 12.3$, $p < 0.01$), time ($F_{8, 112} = 43.1$, $p < 0.001$) and treatment × time ($F_{16, 112} = 8.9$, $p < 0.001$). *Post hoc* comparisons showed ketanserin had no effect on 5-HT level in the mPFC ($p > 0.05$, Scheffe's test) (Fig. 17c). To further examine the time course of ketanserin on 5-HT efflux, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered 5-HT level on each time point after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin did not block 5-HT efflux ($p > 0.05$, Scheffe's test).

In POA, ketanserin pretreatment also altered 5-HT level as demonstrated by the

significant effect of treatment($F_{2, 9} = 5.0$, $p < 0.05$), time ($F_{8, 72} = 20.6$, $p < 0.001$) and treatment × time ($F_{16, 72} = 4.6$, $p < 0.001$). *Post hoc* comparisons showed ketanserin had no effect on basal 5-HT in the POA ($p > 0.05$, Scheffe's test) (Fig. 17c). To further examine the time course of ketanserin on 5-HT efflux, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered 5-HT level from 2 to 6-hour after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin did not block 5-HT ($p > 0.05$, Scheffe's test) (Fig. 17d). This suggests that chronic antidepressant treatment does not alter the tonic activity of the 5-HT_{2A} receptor.

Meanwhile, ketanserin at the warm T_{amb} significantly blocked the enhanced increase in 5-HT efflux in the mPFC (Fig. 17e). It was confirmed by the significant effect of treatment ($F_{2, 17} = 70.4$, $p < 0.001$), time ($F_{5, 85} = 102.6$, $p < 0.001$) and treatment × time ($F_{10, 86} = 41.5$, $p < 0.001$). *Post hoc* comparisons analyses showed that ketanserin significant inhibited 5-HT release ($p < 0.001$, Scheffe's test). To further examine the time course of ketanserin on 5-HT efflux, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered 5-HT level after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin block increased 5-HT on the first 3 hours after clorgyline and paroxetine challenge ($p < 0.05$, Scheffe's test).

Similarly, ketanserin had significant effect on 5-HT efflux in the POA as demonstrated by the significant effect of treatment ($F_{2, 11} = 51.0$, $p < 0.001$), treatment × time ($F_{10, 55} = 47.0$, $p < 0.001$) and time ($F_{5, 55} = 102.5$, $p < 0.001$). *Post hoc* comparisons analyses showed that ketanserin significantly inhibited 5-HT release ($p < 0.01$, Scheffe's test) (Fig. 17f). To further examine the time course of ketanserin on 5-HT efflux, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered

5-HT level after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin block 5-HT on the third hour after clorgyline and paroxetine injection ($p < 0.001$, Scheffe's test).

Effects on the head shakes and behavioral score of warm (32 °C) or cold (12 °C) T_{amb} in the moderate syndrome animals

Head shake is a characteristic behavior mediated through the 5-HT_{2A} receptor (Ma et al., 2008). Here the changes in T_{amb} on the 5-HT_{2A} receptor-mediated head shaking behavior was examined. As described in the early study, animals had received chronic clorgyline pretreatment at the standard experimental condition for 3 days before the experiment. On day 4, the animals were randomly assigned into warm, standard or cold T_{amb} chambers for head shaking tests. The challenge injection was 5 mg/kg paroxetine combined with 2 mg/kg clorgyline. As shown in Figure 18, the number of head shakes in response to the drug challenge was significantly altered in a T_{amb}-dependent manner as demonstrated by one-way ANOVA ($F_{2, 16} = 4.8$, $p < 0.05$). Specifically, the intensity of head shakes was significantly reduced at warm T_{amb} ($p < 0.01$, Scheffe's test) but not at cold T_{amb} ($p > 0.05$, Scheffe's test; Fig. 18a). It was not very surprising that the head shaking behavior was reduced in the warm condition. As described earlier, such behavioral intensity can be affected by muscular activity in the syndrome. That is to say, the head shaking intensity would be reduced if the syndrome severity were increased.

To test this hypothesis that muscular rigidity restrained the head shaking behaviors, the changes in the syndrome behaviors were recorded by scaling tremor, forepaw treading, hindlimb abduction, immobility and Straub tail. Consistent with the hypothesis, as shown

in Figure 18b, behavioral scores were markedly increased at warm T_{amb} ($p < 0.001$, Scheffe's test). No explanation that the scores were also to small extent increased at cold T_{amb} ($p < 0.01$, Scheffe's test) (Fig. 18b).

Finally, it was hypothesized that the activity of 5-HT_{2A} receptors accounted for the effect of warm T_{amb} on the head shaking behavior and behavioral scores. The 5-HT_{2A} receptor antagonist ketanserin was used to test this possibility. In the cold T_{amb}, the head shakes were significantly reduced in animals pretreated with ketanserin prior to the challenge injection as demonstrated by independent-samples T-test ($F = 7.2$, $p < 0.05$) (Fig. 18c), but ketanserin had no effect on the behavioral score as demonstrated by independent-samples T-test ($F = 0.312$, $p > 0.05$) (Fig. 18d). In the warm T_{amb}, ketanserin significantly decreased the number of head shakes as demonstrated by independent-samples T-test ($F = 7.9$, $p < 0.05$) (Fig. 18e) and behavioral score ($F = 7.193$, $p < 0.05$) (Fig. 18f).

Effects on the syndrome mortality of warm (32 °C) or cold (12 °C) T_{amb} in the moderate syndrome animals

Pathologically, a moderate syndrome at the standard condition should not result in death. Mortality can be altered, depending on the external environmental conditions, particularly ambient temperature. Thus, the hypothesis that mortality rate may be increased at warm T_{amb} was examined. Data were summarized in Table 11. Specifically, no death occurred in the moderate syndrome evoked by 5 mg/kg paroxetine combined with clorgyline at the standard T_{amb}. In contrast, the same dose injection however at warm T_{amb} caused 100% death. This effect was blocked by ketanserin, suggesting that the death

associated with the syndrome at warm T_{amb} was mediated by the enhanced activity of 5-HT_{2A} receptor which in concordance with the principal hypothesis.

Effects on T_{cor} and fatality of cold T_{amb} (12 °C) of the severe syndrome animals

Clinical reports have indicated that cold measures could alleviate the severe syndrome (Ener et al., 2003). It appears that the effect may be ascribed to reduction in 5-HT_{2A} receptor activity, which has not been addressed in the earlier clinical or preclinical investigations. For aforementioned reason, the cooling effect on the severe syndrome animals in my animal study was studied. At the standard lab temperature of 22 °C, 15 mg/kg paroxetine in combination of 2 mg/kg clorgyline was sufficient to cause a severe syndrome on day 4 in animals receiving chronic clorgyline for 3 days. With knowledge in mind, following experiment was designed. After daily clorgyline pretreatment for 3 days, 5 animals were examined on day 4 at standard T_{amb} of 22 °C and the rest of 4 at cold T_{amb} of 12 °C. As shown in Figure 19a, challenge injection of paroxetine combined with clorgyline caused hyperthermia at standard T_{amb}, suggesting that the syndrome was severe. At the cold T_{amb} of 12 °C, the T_{cor} response was virtually absent although the behavioral syndrome was obvious (e.g., tremor, head shaking, forepaw treading, hindlimb abduction, etc). The conclusion was confirmed by the significant effect of treatment ($F_{1, 7} = 16.1$, $p < 0.01$), time ($F_{7, 49} = 4.6$, $p < 0.001$) and treatment × time ($F_{7, 49} = 7.4$, $p < 0.001$). To further examine the time course of cold T_{amb} on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that cold T_{amb} blocked the T_{cor} from 2 to 6 hours after clorgyline and paroxetine injection. This is consistent with the hypothesis that the syndrome could be alleviated at cooling T_{amb}. The

death of these animals was also recorded. Of these, 3 deaths occurred at standard but not cooling T_{amb}. Considering that the 5-HT_{2A} receptor antagonist ketanserin has the same blocking effect, my data clearly support the hypothesis that the activity of 5-HT_{2A} receptor may be reduced at cooling temperature, which at least in part underlies the neural mechanism of clinical application.

3.4 Discussion

The main finding of this study was that the activity of 5-HT_{2A} receptor might be enhanced and reduced, respectively, at warm and cold T_{amb}. Considering that cold T_{amb} could alleviate 5-HT toxicity while warm T_{amb} aggravated it (Malberg and Seiden, 1998; Miller and O'Callaghan, 2003; Bowyer et al., 1994), the present study suggests that changes in 5-HT_{2A} receptor activity may serve as the underlying neural mechanism of this T_{amb} effect.

In the study of T_{amb} effect, the syndrome was moderate at a standard condition, showing normothermia. Paroxetine at 5 mg/kg was sufficient to cause a moderate 5-HT syndrome in combination of 2 mg/kg clorgyline. Thus, this drug regimen was adopted to test a series of symptomatic responses at cold or warm T_{amb}. Under the standard T_{amb}, the moderate syndrome is associated with a 10-fold increase in 5-HT in the mPFC and POA. 5-HT at this level can equal-potently activate 5-HT_{1A} and 5-HT_{2A} receptors for hypothermia and hyperthermia, respectively (Zhang et al., 2008). Because of this, core body temperature was normothermia due to a balanced activity between the 5-HT_{1A} and 5-HT_{2A} receptors in thermoregulation.

Our data indicated that warm T_{amb} potentiated the activity of 5-HT_{2A} receptor resulting in hyperthermia of the syndrome animals. Consistent with this hypothesis, the hyperthermia at warm T_{amb} was blocked by ketanserin treatment (Fig. 15). To further test this hypothesis, changes in extracellular 5-HT were also determined at different T_{amb}. 5-HT was significantly increased at warm T_{amb} as compared to that at standard T_{amb} (Fig. 17). Likewise, ketanserin pretreatment blocked the neurochemical change at the warm

T_{amb}, supporting the hypothesis.

The enhanced activity of the 5-HT_{2A} receptor at the warm T_{amb} was also examined by DOI, a 5-HT_{2A} receptor agonist. In agreement with previous work (Mazzola-Pomietto et al., 1995; Wappler et al., 1997; Salmi P and Ahlenius, 1998), DOI at standard T_{amb} increased T_{cor} by stimulating 5-HT_{2A} receptors. One interesting finding in this study was that DOI-induced increases in T_{cor} were positively correlated with the elevation of T_{amb}. Although neural mechanisms underlying the effect of T_{amb} on the 5-HT_{2A} receptor activity are far from clear, this can be partially explained that the affinity of the 5-HT_{2A} receptor to 5-HT is strengthened at higher experimental temperatures (Dalpiaz et al., 1995).

DOI was also used to test the head shaking behavior to eliminate the possible interference of the muscular activity in the syndrome. However, DOI exhibits in both 5-HT_{2A} and 5-HT_{2C} receptor activities (Halberstadt et al., 2009). DOI-induced hyperthermia and head shaking behavior may be ascribed to the property of 5-HT_{2A} receptor activity since the effects were completely blocked by ketanserin (Fig. 16). Some studies suggested that MDMA has an affinity to 5-HT_{2A} receptors. My data are in line with the findings using MDMA at warm T_{amb} (Malberg and Seiden, 1998).

A second finding of this study was that cold T_{amb} alleviated the syndrome by measuring changes in T_{cor}. It was hypothesized that cold T_{amb} desensitized the 5-HT_{2A} receptor. To test this hypothesis, animals habituated to cold T_{amb}. The T_{cor} was decreased in 5-HT syndrome animals, especially on the first hour. Hypothetically, this effect could be due to a decrease in 5-HT level and/or 5-HT_{2A} receptor activity. To test the involvement of 5-HT efflux at this event, changes in 5-HT in the mPFC and POA were

measured. Interestingly, there was no difference in 5-HT between syndrome animals housed in cold and standard T_{amb} (Fig. 17), ruling out the possibility of involvement of 5-HT changes at cold T_{amb}. Thus, it is likely that reduced activity of the 5-HT_{2A} receptor accounts for the hypothermia and mild syndrome. This conclusion was supported by the results of DOI, the effect of which was also reduced at cold T_{cor} (Fig. 16f). It is well-known that cold T_{amb} is neuroprotective and alleviated its brain damages (Gunn and Thoresen, 2006). It is not very surprising that cold measures alleviates 5-HT syndrome in patients (Ener et al., 2003). My data show that reduced activity of the 5-HT_{2A} receptor may be the mechanism underlying this effect.

Data revealed that changes in T_{amb} increased the basal T_{cor} to some extent (Fig. 15a). Cold exposure may potentiate heat production and reduce the heat loss. On the other hand, the increase in basal T_{cor} at warm T_{amb} may result from the exceeding of thermoregulation (Ishiwata et al., 2004). Interestingly, warm T_{amb} had no effect on basal T_{cor} of drug-naive animals (Fig. 16). It suggests that chronic clorgyline treatment contributes to the effect, likely due to enhanced activity of the 5-HT_{2A} receptor, which is worthwhile investigating.

It was found in this study that ketanserin potentiated the hypothermia at cold T_{amb} (Fig. 15d). This effect may be ascribed to reduced activity of 5-HT_{2A} receptor and/or increased activity of 5-HT_{1A} receptor. This is consistent with a corollary of the observation that two receptors have an opposite role in the regulation of body temperatures.

The effects of T_{amb} on head shakes and syndrome scores were determined in separate experiments. Although the 5-HT_{2A} receptor was considered to be enhanced at

warm T_{amb}, intensity of the head shaking behavior was reduced (Fig. 18a). The reduction may be due to increased muscular clonus and rigidity (Ma et al., 2008). Indeed, the behavioral score was elevated. It suggests that muscular activity of the syndrome animals is increased at the warm T_{amb}. Consistent with this thought, blocking 5-HT_{2A} receptor with ketanserin could significantly reduce the intensity of head shakes at warm T_{amb} (Fig. 18).

Interestingly, the behavioral score was also increased at cold T_{amb}. The effect can be ascribed to the increased activity of 5-HT_{1A} receptor. In support, cold exposure did not alter brain 5-HT and ketanserin had no effect on the behavioral score. Previous study suggested 5-HT_{1A} receptor was sensitized at cold T_{amb}, consistent with this hypothesis (Nicholas and Seiden, 2003).

Mortality is rare but may occur in patients with 5-HT syndrome. Much of evidence suggests that 5-HT_{2A} receptor mediates the mortality (Nisijima et al., 2001). As elucidated in Table 11, the mortality rate was increased at warm T_{amb}. The effect was blocked by ketanserin pretreatment. It supports the hypothesis that the 5-HT_{2A} receptor also mediates warm T_{amb}-mediated death. In addition, the increased toxic responses at warm T_{amb} may also involve other 5-HT subtype receptors since there is an increased release in 5-HT (Malberg and Seiden, 1998; Miller and O'Callaghan, 2003; Marzuk et al., 1998).

Clinic reports have demonstrated that cold exposure could alleviate 5-HT syndrome in patients (Ener et al., 2003). My data demonstrated that cold T_{amb} blocked the hyperthermia and prevented the mortality at a paroxetine dose that normally causes a severe 5-HT syndrome (Fig. 19). As discussed before, the effect of cold exposure may

attribute to the desensitization of the 5-HT_{2A} receptor.

It is well known that T_{amb} influences biological functions. For instance, cold T_{amb} is biologically protective and slows down the progress of damages (Chou et al., 2003; Zalis et al., 1966). In contrast, warm T_{amb} aggravates diseases (Marzuk et al., 1998). By measuring changes in body temperature and mortality, my results indicated that warm and cold T_{amb} aggravated and alleviated the severity of 5-HT syndrome, respectively. The affinity of the 5-HT_{2A} receptor to 5-HT at warm milieu was increased (Dalpiaz et al., 1995). Together, it appears that the activity of 5-HT_{2A} receptor may be enhanced at warm T_{amb}, thus aggravating the syndrome. Clinically, the death relevant to 5-HT toxicity is more often at warm T_{amb} (Marzuk et al., 1998). Similarly, recreational drugs such as MDMA at raves are more toxic in hot ballrooms. It was reported that T_{amb} altered activity of sympathetic nerve system (Leppaluoto et al., 1988), hypothalamus-pituitary-adrenal (HPA) axis and hormones, e.g., thyroid hormone (Quintanar-Stephano et al., 1991). Some, if not all, of these changes might relate to the activity of 5-HT_{2A} receptor (Kulikov and Zubkov, 2007). In future, mechanisms underlying the activity of 5-HT_{2A} receptors at different T_{amb} should be determined.

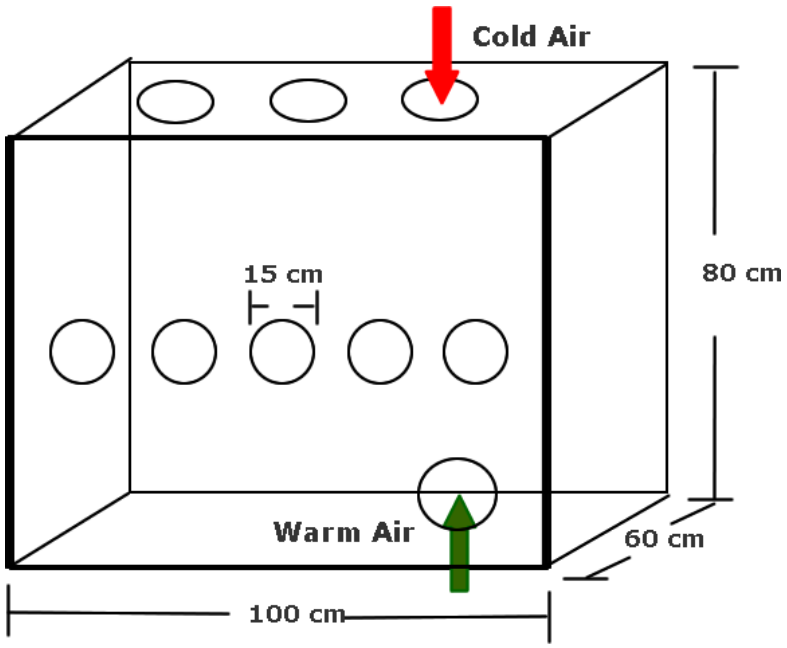


Figure 14. Measurement and design of a thermostatic chamber

Figure 14. Measurement and design of a thermostatic chamber

The chamber was made of transparent polyethylene with dimension of 100 × 60 ×80 cm. To keep temperature of the chamber stable, heat insulation was placed on the 5 sides of the chamber with door left. A 100 × 60 cm transparent polyethylene plate was used as the door with six 15-cm diameter holes. A temperature controller (Fisher Scientific) was used to control the temperature by connecting to a heater or an air conditioner. The air in the chamber was circulated by two fans.

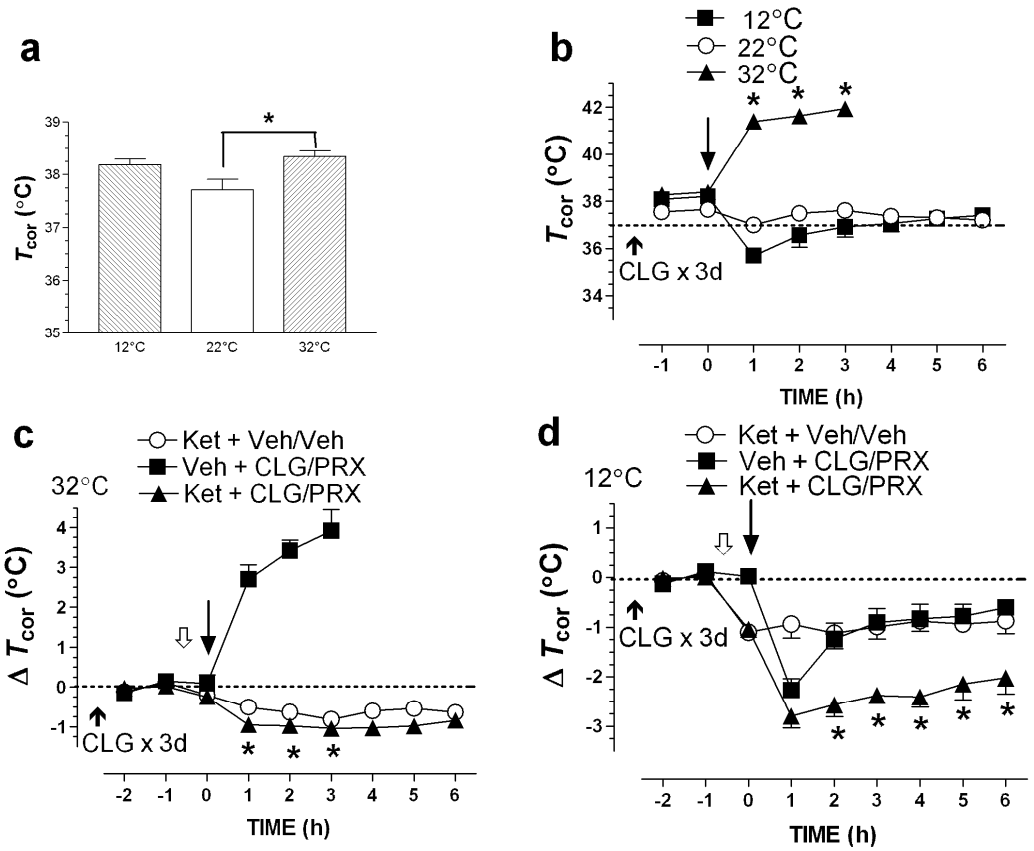


Figure 15. Effects of warm and cold ambient temperature (T_{amb}) on changes in the core body temperature (T_{cor}) of the moderate 5-HT syndrome animals

Figure 15. Effects of warm and cold ambient temperature (T_{amb}) on changes in the core body temperature (T_{cor}) of the moderate 5-HT syndrome animals

In this study, animals had previously received daily clorgyline (CLG, 2 mg/kg, s.c.) for 3 consecutive days. On the 4th day, animals first habituated at warm (32 °C) or cold (12 °C) T_{amb} at least 2 hours before starting experiments. The open arrows indicate the time for ketanserin (Ket, 5 mg/kg, i.p.) injection and the solid line for paroxetine (PRX, 5 mg/kg, i.p.) and CLG challenge. (a), Warm, but not cold, T_{amb} significantly increased the basal $T_{cor.}$ * $p < 0.05$ (one-way ANOVA followed by *post hoc* Scheffe's test). (b) T_{cor} were enhanced when animals were housed in warm T_{amb}. There was a decreasing tendency when animals were housed in warm T_{amb}. * $p < 0.05$ vs. room T_{amb} (one-way ANOVA followed by *post hoc* Scheffe's test). Next, ketanserin was pretreated 30 min before CLG and PRX challenge. Compared to vehicle control, ketanserin blocked the antidepressant-induced hyperthermia of the syndrome animals housed in warm T_{amb} (c) and potentiated the hypothermia in cold T_{amb} (d). * $p < 0.05$ (one-way ANOVA followed by *post hoc* Scheffe's test). Because all the moderate 5-HT syndrome animals without an antagonist died within 6 hours after challenge injection, only part of the body temperature data were shown. Each value is the mean ± SEM of 5-9 animals.

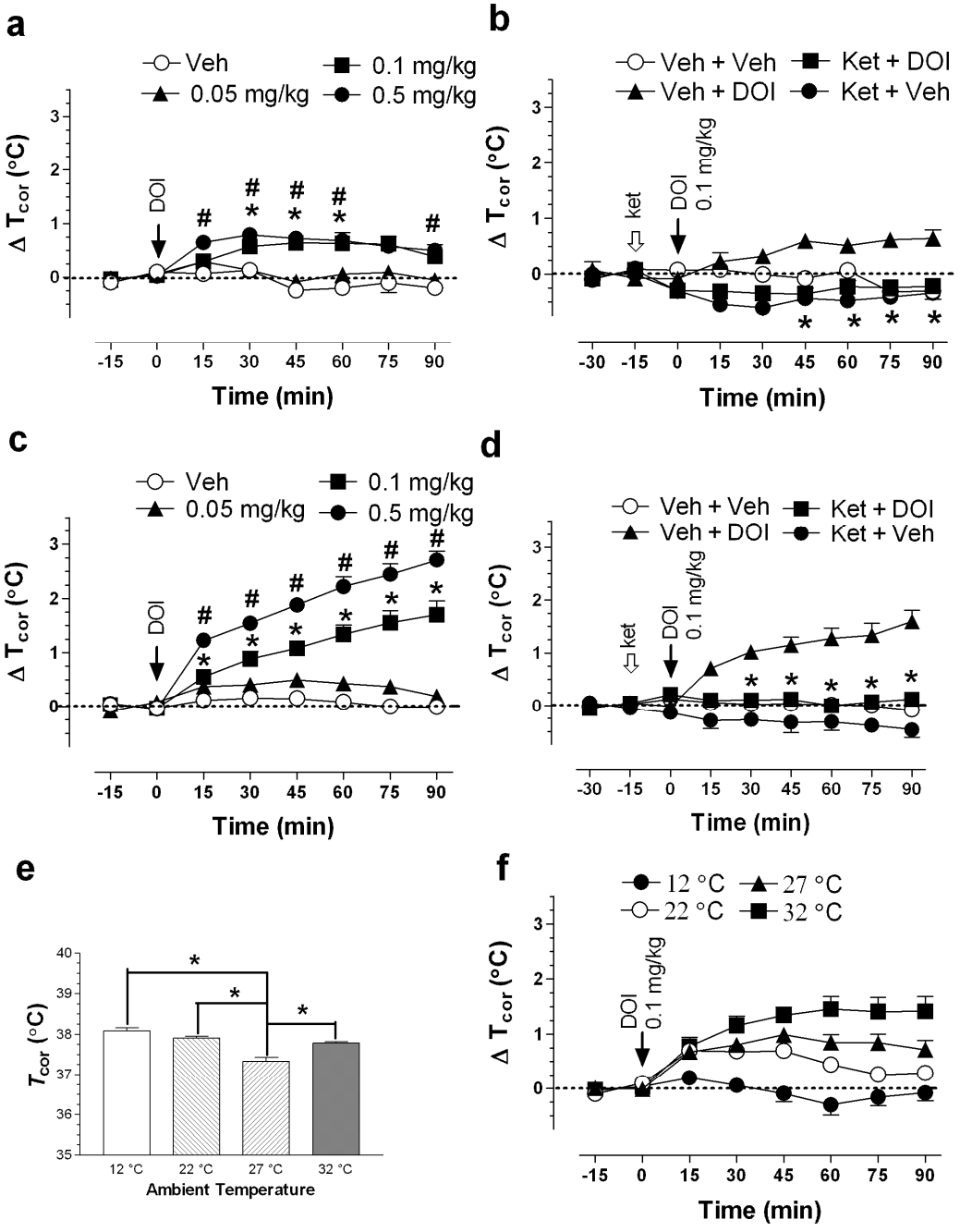


Figure 16. Effects of ambient temperature (T_{amb}) on the DOI-induced increase in core body temperature (ΔT_{cor})

Figure 16. Effects of ambient temperature (T_{amb}) on the DOI-induced increase in core body temperature (ΔT_{cor})

The open arrows indicate the time for ketanserin (Ket, 5 mg/kg, i.p.) injection and the solid line for DOI administration. (a) DOI (0.05, 0.1 and 0.5 mg/kg, s.c.) dose-dependently increased the T_{cor} in the standard T_{amb} of 22 °C. * $p < 0.05$, 0.1 mg/kg vs. vehicle; # $p < 0.05$, 0.5 mg/kg vs. vehicle (one-way ANOVA followed by *post hoc* Scheffe's test). (b) 5-HT_{2A} receptor antagonist Ket blocked the DOI (0.1 mg/kg, s. c.)-induced increase in $T_{cor.}$ * $p < 0.05$ vs. vehicle + DOI (one-way ANOVA followed by *post hoc* Scheffe's test). (c) DOI (0.05, 0.1 and 0.5 mg/kg, s.c.) dose-dependently increased the T_{cor} at warm T_{amb} of 32 °C. * $p < 0.05$, 0.1 mg/kg vs. vehicle; # $p < 0.05$, 0.5 mg/kg vs. vehicle (one-way ANOVA followed by *post hoc* Scheffe's test). (d) Ket blocked the increase in T_{cor} induced by DOI (0.1 mg/kg, s. c.) at warm T_{amb} of 32 °C. * $p < 0.05$ vs. vehicle + DOI (one-way ANOVA followed by *post hoc* Scheffe's test). (e) Effects of T_{amb} on the basal T_{cor} in drug-naïve animals housed in different T_{amb}. There was a T_{amb}-related change in basal $T_{cor.}$ * $p < 0.05$ (one-way ANOVA followed by *post hoc* Scheffe's test). (f) DOI-induced increases in T_{cor} were positively correlated with the level of T_{amb} ($p < 0.001$, main effect, two-way repeated measures ANOVA). Each value is the mean ± SEM of 5-8 animals.

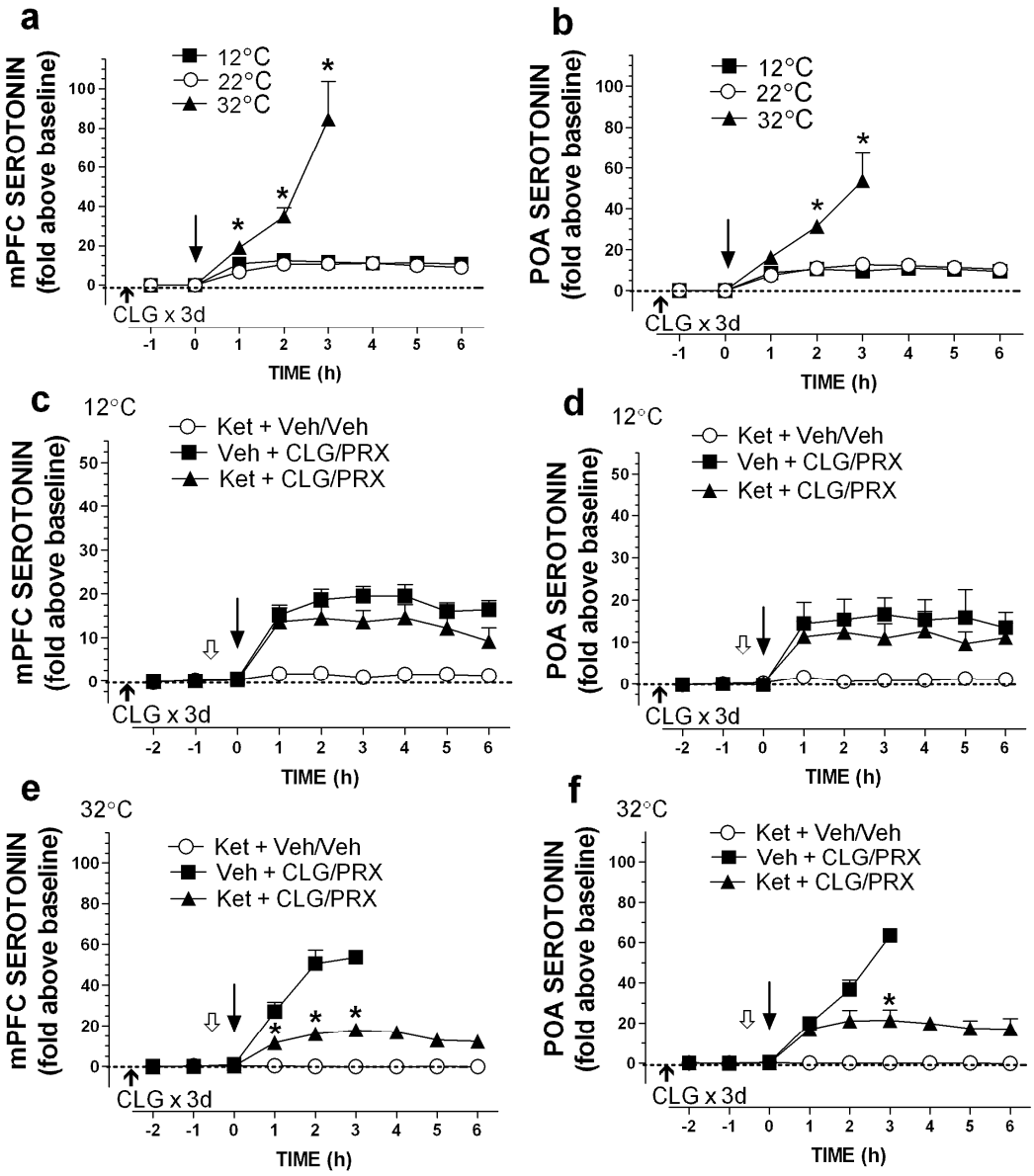


Figure 17. Effects on 5-HT efflux of warm and cold ambient temperature (T_{amb}) in the moderate syndrome animals

Figure 17. Effects on 5-HT efflux of warm and cold ambient temperature (T_{amb}) in the moderate syndrome animals

Animals had previously received daily clorgyline (CLG, 2 mg/kg, s.c.) for consecutive 3 days. On day 4, animals habituated at warm (32 °C) or cold (12 °C) T_{amb} for at least 2 hours before starting experiments. The open arrows indicate the time for ketanserin (Ket, 5 mg/kg, i.p.) injection while the solid arrows for the time of paroxetine (PRX, 5 mg/kg, i.p.) and CLG challenge. Compared to that at the standard condition, paroxetine combined with clorgyline -induced 5-HT increases in the medial prefrontal cortex (mPFC, a) and preoptic area (POA, b) were significantly enhanced at warm but not cold T_{amb}. * $p < 0.05$ vs. room T_{amb} (one-way ANOVA followed by *post hoc* Scheffe's test). Ketanserin (Ket, 5 mg/kg) had no effects on 5-HT level in mPFC (c) and POA (d) when the syndrome animals were housed in cold T_{amb}. The enhanced 5-HT increase in warm T_{amb} was blocked by ketanserin pretreatment in mPFC (e) and POA (f). * $p < 0.05$ vs. vehicle + CLG/PRX group (one-way ANOVA followed by *post hoc* Scheffe's test). Each value is the mean ± SEM of 4-10 animals.

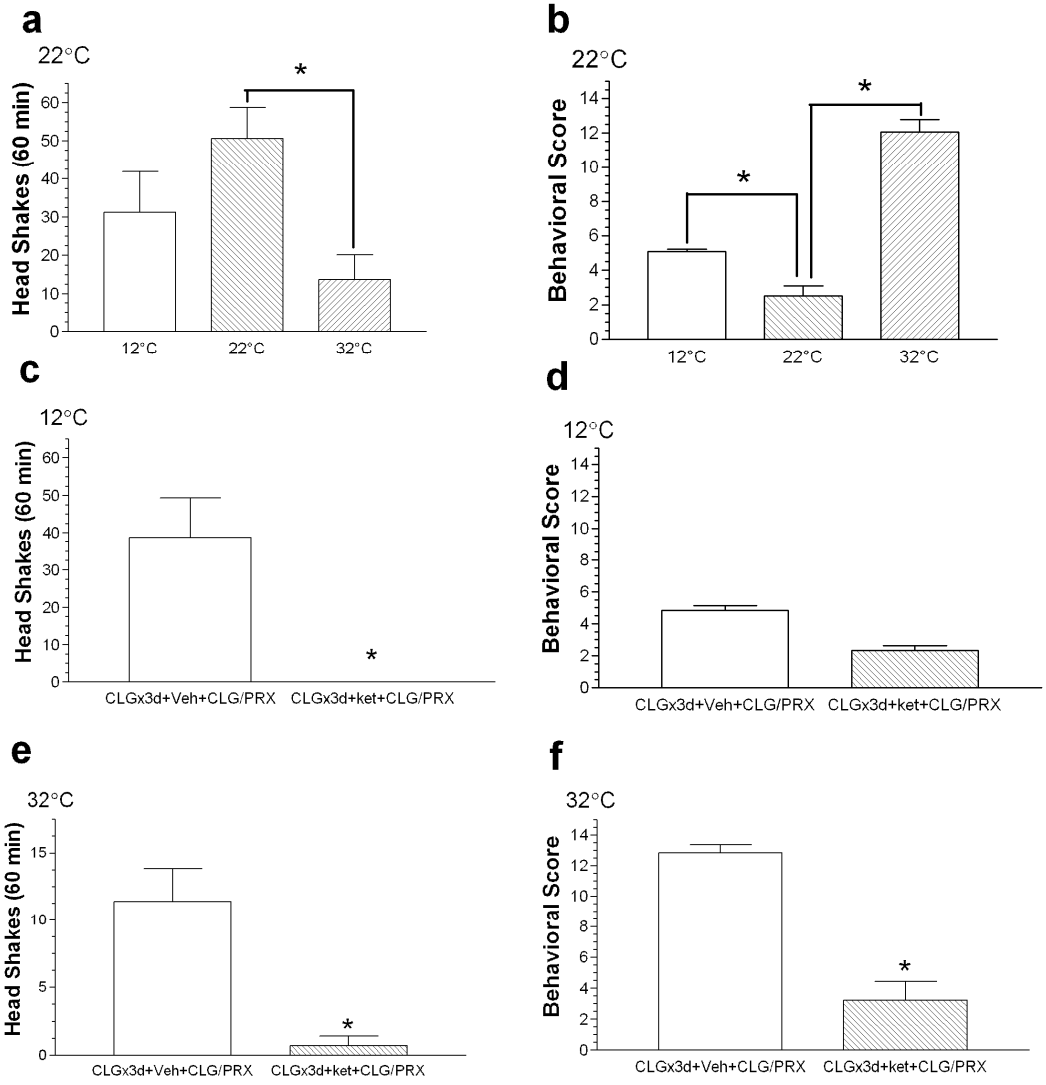
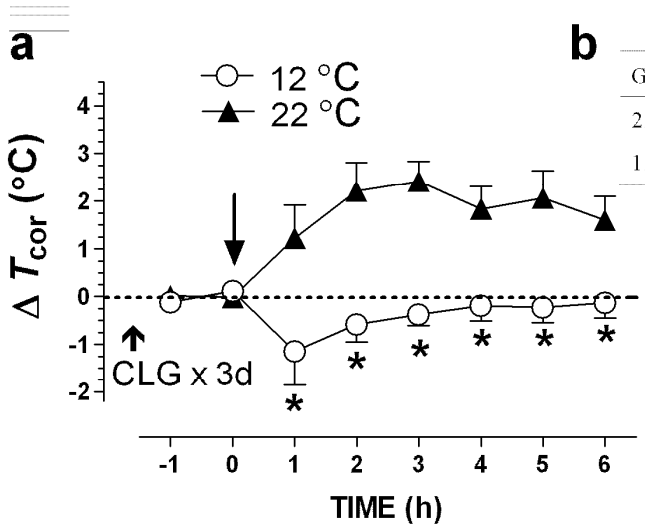


Figure 18. Effects on the head shakes and behavioral score of warm (32 °C) and cold (12 °C) ambient temperature (T_{amb}) in the moderate syndrome animals

Figure 18. Effects on the head shakes and behavioral scores of warm (32 °C) and cold (12 °C) ambient temperature (T_{amb}) in the moderate syndrome animals

Animals had been pretreated with daily clorgyline (CLG, 2 mg/kg, s.c.) for 3 days. On day 4, animals habituated to warm (32 °C) or cold (12 °C) T_{amb} for at least 2 hours before experiments. Ketanserin (Ket, 5 mg/kg, i.p.) was given to rats 30 min before paroxetine (PRX, 5 mg/kg, i.p.) and clorgyline challenge. Head shakes were expressed as the number of shakes counted on one hour immediately after CLG and PRX administration. Behavioral score was the sum of six variables (tremor, forepaw treading, hindlimb abduction, Straub tail and immobility) in the syndrome. Each variable was scaled with 0, no response; 1, mild; 2, moderate; or 3, severe response). (a) Compared to that at the standard condition (T_{amb} setting at 22 °C), warm but not cold T_{amb} reduced the intensity of head shakes. * $p < 0.05$ (one-way ANOVA followed by *post hoc* Scheffe's test). (b) Compared to that at standard T_{amb}, the behavioral scores following paroxetine combined with clorgyline challenge were increased at cold and warm $T_{amb.}$ * $p < 0.05$ (one-way ANOVA followed by *post hoc* Scheffe's test). Ket blocked the head shakes (c) not behavioral score (d) of the syndrome animals housed in cold $T_{amb.}$ * $p < 0.05$ (independent samples T-test). In warm T_{amb}, ketanserin blocked both the head shakes (e) and behavioral score (f). * $p < 0.05$ (independent samples T-test). Each value is the mean ± SEM of 4-8 animals.



b

Groups	Survived	Died	Total
22 °C	2	3	5
12 °C	4	0	4

Figure 19. Effect of cold ambient temperature (12 °C) on the severe symptoms in an animal model of 5-HT syndrome

Figure 19. Effect of cold ambient temperature (12 °C) on the severe symptoms in an animal model of 5-HT syndrome

Note that animals had received clorgyline (CLG, 2 mg/kg, s.c.) for 3 days. On the 4th day, they habituated to testing chambers at either standard or cold T_{amb} for at least 2 hours before experiment. Paroxetine (PRX, 15 mg/kg, i.p.) combined with CLG was given at time zero. Cold T_{amb} prevented the hyperthermia (a) and fatality of the syndrome animals (b). * $p < 0.05$ (independent samples T-test). Each value is the mean ± SEM of 4-5 animals.

Table 10. The behavioral scale used for assessing the 5-HT syndrome animals (Ma, et al. 2008)

Score Behavior	3 = severe	2 = moderate	1 = mild
Tremor	Continue, fast and immobility	Continue, slow and crawling	Occasion
Forepaw Treading	>60 times/min	30-60 times/min	<30 times/min
Hindlimb Abduction	All limb out from body	50% out	Low part of limb
Straub Tail	Induced by drug, persistent	Induced by drugs, occasion	By observer
Immobility	Immobilized	Crawling slowly	Move swiftly

Table 11. Effect on fatality of cold (12 °C) and warm (32 °C) ambient temperature in moderate 5-HT syndrome animals

Animals had received daily 2 mg/kg clorgyline (CLG) for 3 days. Challenge injection occurred on day 4 by administration of 5 mg/kg paroxetine (PRX) combined with 2 mg/kg CLG. In room temperature, this drug regimen evoked a moderate 5-HT syndrome and all animals could survive. Ketanserin (Ket) at the dose of 5 mg/kg was given to rats 30 min before PRX and CLG challenge.

Ambient Temp	Treatment (CLG x 3d)	Survived	Died	Total
12 °C	CLG + PRX	10	0	10
22 °C	CLG + PRX	9	0	9
32 °C	CLG + PRX	0	6	6
12 °C	Ket + CLG + PRX	6	0	6
32 °C	Ket + CLG + PRX	8	0	8

CHAPTER FOUR: BEHAVIORAL AND NEUROCHEMICAL CHARACTERIZATION OF THE MILD, MODERATE AND SEVERE SEROTONIN SYNDROMES EVOKED BY CHALLENGE INJECTION OF PAROXETINE COMBINED WITH CLORGYLINE

4.1 Introduction

Incidence of the 5-HT syndrome is rising in recent years with growing of antidepressant prescription (Isbister and Buckley, 2005; Isbister et al., 2007). Although most of cases are mild that can recover with no needs of medical interference, patients with a severe syndrome are life-threatening. Thus, an understanding of syndrome severity is crucial to identify the severe cases that need immediate medical attention. Some criteria to measure the syndrome severity have been developed for human cases and animal studies. Isbister et al. (2007) suggested three levels of severity: mild, moderate and severe. The severity is usually estimated by changes in neuromuscular responses (Izumi et al., 2006; Speiser et al., 2008) and automatic functions, particularly body temperature (Nisijima et al., 2004; Ma et al., 2008).

In a previous study carried out in our lab, the syndrome severity was determined based on change in body temperature following clorgyline and 5-HTP treatment in animals. It appears that 5-HTP combined with clorgyline causes hypothermia,

normothermia and hyperthermia in the mild, moderate and severe syndromes, respectively (Ma et al., 2008). Changes in body temperature and the syndrome severity were positively correlated with extracellular 5-HT (Zhang et al., 2009).

In this study a new animal model of the 5-HT syndrome was developed, but not systematically characterized such as body temperature, behaviors and brain 5-HT level, which impedes understanding of the 5-HT syndrome. Therefore, the aim of this section was to characterize mild, moderate and severe syndromes evoked by paroxetine combined with clorgyline at the paroxetine doses of 1, 5 and 15 mg/kg, respectively. To characterize this new model, changes in antidepressant dose were correlated to behavior and mortality. Our data indicated that paroxetine combined with clorgyline at the paroxetine doses of 1, 5 and 15 mg/kg evoked mild, moderate and severe syndromes on day 4 in rats receiving clorgyline pretreatment for 3 days.

4.2 Materials and methods

Animal preparation, chemicals and schedule for drug injection, induction of the 5-HT syndrome animals, measurement of core body temperature, head shake and behavioral score recording, surgery, microdialysis procedures and histology were similar to those in chapter two and three.

Data analysis

Changes in body temperature are expressed as mean ± SEM. Microdialysis results of 5-HT are expressed as fold increases above the baseline (mean ± SEM). Two-way repeated measures ANOVA was used to evaluate overall response among the control and experimental groups. If significant effects were found, *post hoc* Scheffe's test was used to compare effects of different treatment groups. One-way ANOVA was also used to assess the statistical differences among groups. The Fisher's exact test was employed to substitute the Chi Square test to analyze the mortality rate if the sample size was five or less. Simple linear regression analysis was used to evaluate the dose-dependent response to paroxetine. The significant level was set at $p < 0.05$.

4.3 Results

Changes in T_{cor} in rats induced by paroxetine combined with clorgyline

Data in the chapter two indicated that the activity of 5-HT_{2A} receptor might be enhanced for inducing the severe 5-HT syndrome in rats receiving clorgyline for 3 days. Therefore, this clorgyline paradigm was also used in this chapter. Animals had received once daily clorgyline (2 mg/kg, s.c.) for 3 days. On day 4, animals were challenged with paroxetine and clorgyline with the paroxetine doses at 0, 1, 5 or 15 mg/kg. The basal (prior challenge) T_{cor} was 37.89 ± 0.08 °C (n = 54). As shown in Figure 20, there were paroxetine dose-dependently increases in T_{cor}. Two-way repeated ANOVA showed that paroxetine had significant effect on the change of T_{cor} as demonstrated by the significant effect of treatment ($F_{3, 27} = 28.3$, $p < 0.001$), time ($F_{7, 189} = 2.7$, $p < 0.05$) and treatment × time ($F_{21, 189} = 10.9$, $p < 0.001$). *Post hoc* analyses showed, compared with vehicle group, 15 mg/kg paroxetine significantly increased the T_{cor} ($p < 0.001$, Scheffe's test). There were no differences of 1mg/kg paroxetine group ($p > 0.05$, Scheffe's test) and 5 mg/kg paroxetine group ($p > 0.05$, Scheffe's test) to vehicle group. To further examine the time course of paroxetine doses on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that paroxetine doses altered the T_{cor} after drug challenge ($p < 0.001$). *Post hoc* comparisons indicated that 1 mg/kg paroxetine decreased T_{cor} on the first hour after paroxetine injection and 15 mg/kg paroxetine increased T_{cor} on the 2 to 6 hours after paroxetine challenge ($p < 0.001$, Scheffe's test). Specifically, 1 and 15 mg/kg paroxetine decreased and increased the T_{cor}, respectively ($p < 0.01$, Scheffe's test), while 5 mg/kg had no significant effect ($p > 0.05$, Scheffe's test). It appears that 1, 5 and 15 mg/kg

paroxetine in combination with clorgyline on day 4 induces mild, moderate and severe 5-HT syndromes, respectively, in animals receiving clorgyline pretreatment for 3 days.

Changes in brain 5-HT in the syndrome induced by paroxetine combined with clorgyline in the chronic clorgyline-pretreated animals

Here changes in brain 5-HT in the mild, moderate and severe syndromes were explored. After 3-day clorgyline pretreatment, basal 5-HT (pg/sample) was 2.40 ± 0.35 (n = 10) in the mPFC and 3.36 ± 0.37 (n = 15) in the POA. As shown in Figure 21, paroxetine challenge injection caused a dose-dependently increase in 5-HT in the mPFC. Two-way repeated ANOVA showed that paroxetine had significant effects on 5-HT efflux in the mPFC as demonstrated by the significant effect of treatment ($F_{3, 20} = 75.3$, $p < 0.001$), time ($F_{7, 140} = 2.7$, $p < 0.05$) and treatment × time ($F_{21, 140} = 28.4$, $p < 0.001$). *Post hoc* analyses showed 5 and 15 mg/kg paroxetine significantly increased the mPFC 5-HT efflux to the vehicle ($p < 0.01$, Scheffe's test). To further examine the time course of paroxetine doses on 5-HT level in the mPFC, one-way ANOVA was run on each time point. These analyses indicated that paroxetine altered 5-HT level after drug challenge ($p < 0.001$). *Post hoc* comparisons indicated that 1 mg/kg paroxetine increased 5-HT on the second hour after paroxetine injection, 5 mg/kg PRX increased 5-HT on the 1, 2, 3 and 6 hours after paroxetine challenge. 15 mg/kg paroxetine increased T_{cor} on the 6 hours after paroxetine challenge ($p < 0.05$, Scheffe's test).

Similarly, 5-HT level in POA was increased after paroxetine injection. Two-way repeated ANOVA showed that paroxetine had significant effect on 5-HT efflux in POA as demonstrated by the significant effect of treatment ($F_{3, 16} = 57.1$, $p < 0.001$), time ($F_{7,}$

$_{112}$ = 2.7, $p < 0.001$) and treatment × time ($F_{21, 112}$ = 28.4, $p < 0.001$). To further examine the time course of paroxetine on 5-HT level in mPFC, one-way ANOVA was run on each time point. These analyses indicated that paroxetine doses altered 5-HT level after drug challenge ($p < 0.001$). *Post hoc* comparisons indicated that 5 and 15 mg/kg PRX enhanced 5-HT release after it injection ($p < 0.05$, Scheffe's test).

In the mPFC, the maximal increase was ~ 5-fold (4.9 ± 0.6) in response to 1 mg/kg paroxetine, 10-fold (10.7 ± 1.2) to 5 mg/kg paroxetine and 30-fold (33.9 ± 3.6) to 15 mg/kg paroxetine. Increased efflux in the POA was similar to those in the mPFC, showing 3.8 ± 0.8, 12.7 ± 2.8 and 24.9 ± 1.5 fold-increase corresponding to 1, 5 and 15 mg/kg paroxetine, respectively (Fig. 20).

Changes in head shakes and behavioral score in the 5-HT syndrome animals

Head shake and behavioral score were measured in the mild, moderate and severe 5-HT syndrome animals. The number of head shakes on one hour was counted immediately after challenge injection of paroxetine combined with clorgyline. In a control study, clorgyline combined with vehicle (0.9% NaCl) had no effect on head shaking behavior. For the sake of clarity, vehicle group was not presented in the graph. Interestingly, paroxetine at three doses evoked the same effect. One-way ANOVA demonstrated that there was no significant differences in head shakes between doses ($F_{2, 18}$ = 1.8, $p > 0.05$).

Behavioral score is the sum of six variables including tremor, forepaw treading, hindlimb abduction, Straub tail and immobility. Each variable was measured with a scale of 1 (mild), 2 (moderate) or 3 (severe) as defined in Table 10. Clorgyline combined with

vehicle had no effect on behavioral score and thus data were not presented in the graph for the sake of clarity. Unlike the head shaking behavior, the behavioral scores were paroxetine dose-dependent ($F_{2, 22} = 41.1$, $p < 0.001$).

Regression analysis of tremor, forepaw treading, hindlimb abduction, Straub tail and immobility in the 5-HT syndrome

Bivariate and regression analysis was used to evaluate the relationship between paroxetine doses and behavioral scores. Each behavioral score was based on the definition as described in Table 10. As a result, paroxetine doses produced a positive correlation with each of behavioral signs. These included tremor (Pearson $r = 0.81$, $F_{1, 31} = 57.834$, $p < 0.001$), forepaw treading (Pearson $r = 0.78$, $F_{1, 31} = 48.648$, $p < 0.001$), hindlimb abduction (Pearson $r = 0.81$, $F_{1, 31} = 57.457$, $p < 0.001$), Straub tail (Pearson $r = 0.87$, $F_{1, 31} = 91.8$, $p < 0.001$), immobility (Pearson $r = 0.86$, $F_{1, 31} = 91.289$, $p < 0.001$) and total behavioral scores (Pearson $r = 0.83$, $F_{1, 30} = 67.959$, $p < 0.001$).

Change in fatality of the 5-HT syndrome animals

Finally, fatality for each group in the syndrome was analyzed. Table 13 showed the number of death in 24 hours after the animals underwent through the mild, moderate or severe syndrome. Some of those injected with a high dose of paroxetine at 15 mg/kg combined with clorgyline resulted in death in the syndrome (Fisher's exact test, $p < 0.05$).

4.4 Discussion

The present results indicated that the 5-HT syndrome severity could be different. After 3-day chronic clorgyline pretreatment, paroxetine combined with clorgyline at the paroxetine doses of 1, 5 and 15 mg/kg evoked mild, moderate and severe syndromes, respectively.

The relationship between changes in T_{cor} and syndrome severity has been investigated in substantial details in both animal studies and human case reports (Izumi et al., 2006; Speiser et al., 2008). T_{cor} measurement has a great value in terms of understanding of the severity of the syndrome (Isbister et al., 2007; Ma et al., 2008). Hypothermia, normothermia and hyperthermia are indicative of a mild, moderate and severe 5-HT syndrome, respectively, in the animal study (Ma et al., 2008). In agreement with previous studies (Abdel-Fattah et al., 1997; Ma et al., 2008), paroxetine combined with clorgyline also produced three types of T_{cor} response in this new animal model of 5-HT syndrome (Fig. 20). It was paroxetine dose-dependent. Specifically, challenge injection of 1, 5 and 15 mg/kg paroxetine with clorgyline was associated with hypothermia, normothermia and hyperthermia, respectively. It suggests that the animal model developed in this study can be used to reveal the syndrome at different severity levels. Note that neural mechanisms behind T_{cor} in the syndrome have been well established. For instance, post-synaptic 5-HT_{1A} receptor is responsible for the hypothermia (Darmani and Zhao, 1998). In contrast, 5-HT_{2A} receptor mediates the hyperthermia (Nisijima et al., 2001; Abdel-Fattah et al., 1997; Lin et al., 1998). Given that the affinity of the 5-HT_{2A} receptor to 5-HT is 400 – 1000 times lower than that of

5-HT_{1A} receptor (Dalpiaz et al., 1995; Peroutka et al., 1981), it appears that a small increase in extracellular 5-HT would prefer strongly activating 5-HT_{1A} receptors although there might be weakly at 5-HT_{2A} receptors. When extracellular 5-HT reaches a remarkable level, it will activate 5-HT_{2A} receptors and induce hyperthermia. The normothermia may be a functional balance between 5-HT_{1A} and 5-HT_{2A} receptors. The hypothesis was thoroughly tested in this study by measuring extracellular 5-HT at different T_{cor} states.

Figure 21 showed that the levels of extracellular 5-HT in the POA and mPFC had a similar pattern as changes in the core body temperature and severity of the syndrome. POA, especially medial POA is a key region for thermoregulation by sending excitatory signals to control heat loss or gain (Nagashima, 2006). Meanwhile, POA neurons receive the number of serotonergic projection and contain 5-HT_{2A} receptors to regulate the T_{cor} (Pompeiano et al., 1992; Chai and Lin, 1977; Boulant, 1981, 2000, 2006; Lin, 1979). For the aforementioned reason, 5-HT level in the POA was measured. A second brain area investigated in this study was the mPFC, a brain region for consciousness (Miller and Cohen, 2001; Fuster, 2006) and behavioral integration and execution (Duncan, 2001; Frith and Frith, 1999; Miller and Cohen, 2001). Since there was mental abnormality in the syndrome, changes in 5-HT was therefore determined in this cortical region.

It was found that 5-HT efflux in the mPFC was 5-fold, 11-fold and 34-fold in response to paroxetine combined with clorgyline at the paroxetine doses of 1, 5 and 15 mg/kg, respectively. Similarly, 5-HT efflux in the POA was 4-fold, 13-fold and 25-fold, respectively. Considering that the 5-HT_{1A} receptor is responsible for mediating the hypothermia (Darmani and Zhao, 1998) and the 5-HT_{2A} receptor for the hyperthermia

(Nisijima et al., 2001; Abdel-Fattah et al., 1997; Lin et al., 1998; Yamawaki et al., 1986; Goodwin et al., 1987; Gudelsky et al., 1986; O'Connell et al., 1992), it can be extrapolated that the 5-HT_{1A} receptor is predominately active for hypothermia when extracellular 5-HT was less than 5-fold. When 5-HT increases up to 10-fold, the activities of 5-HT_{1A} and 5-HT_{2A} receptors may be equally balanced. This may explain the normothermic response to 5 mg/kg paroxetine combined with clorgyline through which animals have a moderate syndrome. However, when 5-HT exceeds 10-fold, the 5-HT_{2A} receptor may be predominately active, eliciting the hyperthermia. The relationship between extracellular 5-HT and changes in T_{cor} has been reported in the other studies (Ishigooka et al., 1993; Zhang et al., 2009). It suggests that the similar relationship must exist in human patients although 5-HT levels in patients are not available.

The syndrome was associated with a 1100-fold increase in 5-HT in the other study (Nisijima et al., 2004). However, my data revealed only a 35-fold increase. The discrepancy can be ascribed to the difference in agents used. Theoretically, 5-HTP increases extracellular 5-HT in the serotonergic neurons and other cells that contain decarboxylases. In this new animal model of 5-HT syndrome, however, increase in 5-HT mainly takes place in serotonergic neurons. Thus, 5-HT measured in this model more resembles that in the human case.

In this study, it was found that the number of head shakes increased in the syndrome (Fig. 22a). However, it was not correlated with the severity of the syndrome. Head shake is an important indictor for the activity of 5-HT_{2A} receptors (Peroutka et al., 1981; Ma et al., 2008; Goodwin et al., 1987; Van Oekelen et al., 2003). The discrepancy between the severity and the number of head shakes may attribute to the muscle activity.

It has been observed that the head shakes were disrupted by the muscle rigidity (Ma et al., 2008). Indeed, our data showed that the behavioral score increased (Fig. 22b), supporting that the muscle intensity may be the factor that restraints head shaking behavior. Consistently, there was no correlation between the number of head shakes with 5-HT level or mortality.

Except for head shakes, other syndrome behaviors, if not all, are probably mediated by postsynaptic 5-HT_{1A} receptor (Yamada et al., 1988). 8-OH-DPAT at high doses induced tremor, forepaw treading, hind limb abduction and flat body (Darmani and Zhao, 1998; Goodwin et al., 1986, 1987; Darmani and Ahmad, 1999). In this study, we found that paroxetine and clorgyline challenge provoked a paroxetine dose-dependent increase in tremor, forepaw treading, hind limb abduction, Straub tail and flat body posture (Table 12). For comparison, a high dose of 8-OH-DPAT (0.5 mg/kg) was used to elicit the syndrome symptoms (data not shown). It was observed that those behavioral response was alike each other, if not identical. Thus, the 5-HT_{1A} receptor may be one of the mediators of those symptoms of the syndrome.

Several clinical reports suggest that mortality results from the increase in T_{cor} (hyperthermia) in the 5-HT syndrome animals. As body temperature exceeded 41.5 °C, about two-thirds of cases ended in death (Gowing et al., 2002; Nisijima et al., 2001). Similarly, the death in these animals with the syndrome was always preceded hyperthermia.

Immobility and ataxia [due to akathisia and dyskinesia of extrapyramidal disorder (Lane, 1998)] were observed in the syndrome animals. The primary cause for these signs may lie in that the 5-HT neurons directly innervate the basal ganglia, the primary motor

areas that control the axial skeletal muscles. Excessive 5-HT released in the basal ganglia may induce these behaviors (Steinbusch, 1981). Based on the facts that excessive 5-HT increased in the mPFC was similar to that in the POA, it could be assumed that 5-HT level was globally elevated in the syndrome, likely at the same level in other regions including basal ganglia for inducing the immobility and ataxia. Changes in dopamine efflux are associated locomotion disorders (Lambert et al., 1998). Alternative hypothesis is that increased dopaminergic transmission in nigrostriatal pathway involves the muscular hyperactivity in the syndrome, as suggested in the literature (Jacobs and Azmitia, 1992).

In this study, we noticed that diarrhea appeared in the syndrome animals. The receptor mechanism behind this phenomenon may arise from the fact that 5-HT stimulates gastric pepsin secretion, intestinal motility, and conversion of small intestinal mucosa from absorption to secretion of water and electrolytes through 5-HT_3 receptor stimulation (Jaffe, 1979).

In summary, the behavioral results of the present study indicate that challenge injection of paroxetine combined with clorgyline on day 4 with the paroxetine doses at 1, 5 and 15 mg/kg can induce the mild, moderate and severe 5-HT syndrome, respectively, in animals receiving daily clorgyline for 3 days. Microdialysis studies reveal that 5-HT_{1A} receptor may be predominately active in the mild syndrome when extracellular 5-HT is less than 5-fold of basal release for inducing hypothermia. As extracellular 5-HT exceeds 30-fold, the syndrome become severe while 5-HT_{2A} receptor is predominately active for inducing hyperthermia. The syndrome is moderate at a 20-fold (±5 fold) increase in 5-HT.

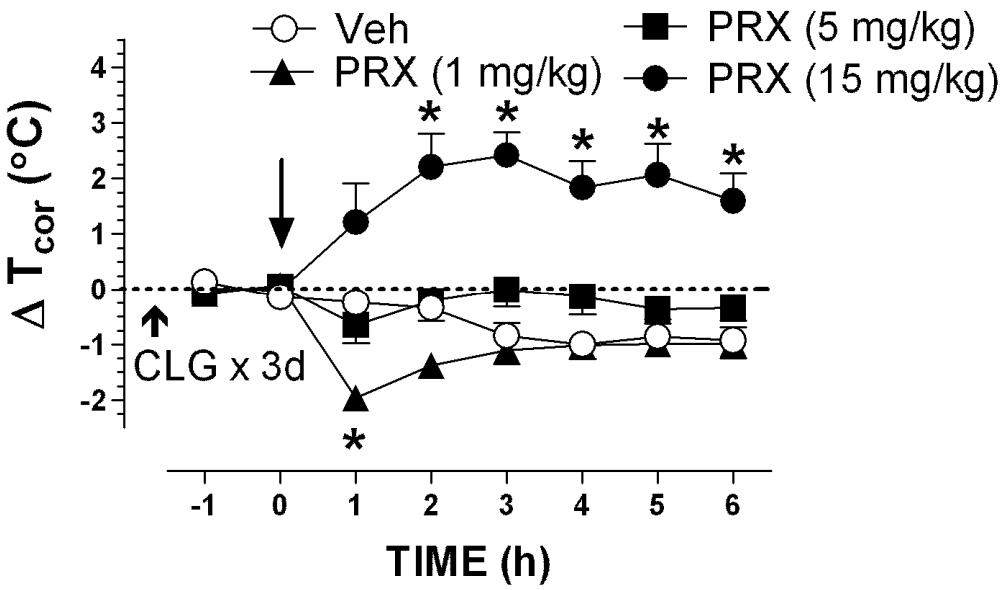


Figure 20. Change in T_{cor} in the 5-HT syndrome induced by paroxetine (PRX) combined with clorgyline (CLG) in rats receiving chronic CLG pretreatment for 3 days

Figure 20. Change in T_{cor} in the 5-HT syndrome induced by paroxetine (PRX) combined with clorgyline (CLG) in rats receiving chronic CLG pretreatment for 3 days

Animals had received clorgyline (CLG, 2 mg/kg, s.c.) for three days. On the fourth day, paroxetine (PRX, 15 mg/kg, i.p.) at the doses of 1, 5 and 15 mg/kg, i.p., was co-administrated with CLG at time 0 indicated by the long arrow. Compared to the vehicle control, 1 mg/kg PRX induced a decrease in T_{cor}, 5 mg/kg PRX had no effect on the T_{cor} and 15 mg/kg PRX induced the hyperthermia. * $p < 0.05$ vs. vehicle (one-way ANOVA followed by *post hoc* Scheffe's test). Each value is the mean ± SEM of 5-11 animals.

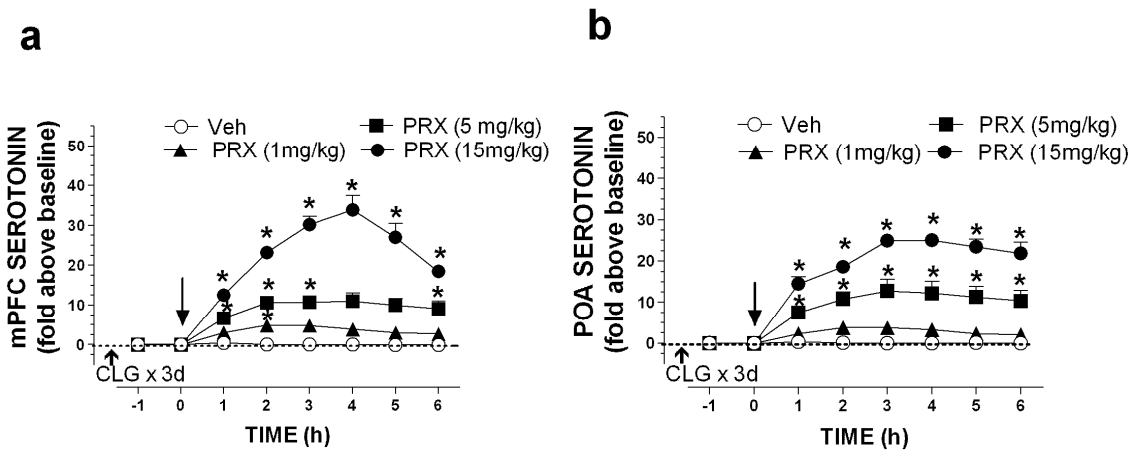


Figure 21. Changes in brain 5-HT in the syndrome induced by paroxetine (PRX) combined with clorgyline (CLG) on day 4 in rats receiving chronic CLG pretreatment for 3 days

Figure 21. Changes in brain 5-HT in the syndrome induced by paroxetine (PRX) combined with clorgyline (CLG) on day 4 in rats receiving chronic CLG pretreatment for 3 days

Animals had received clorgyline (CLG, 2 mg/kg, s.c.) for three days. On the fourth day, paroxetine (PRX, 15 mg/kg, i.p.) at the doses of 1, 5 and 15 mg/kg, i.p., was co-administrated with CLG at time 0 indicated by the long arrow. PRX dose-dependently increased 5-HT efflux in the mPFC (a) and POA (b). * $p < 0.05$ vs. vehicle (one-way ANOVA followed by *post hoc* Scheffe's test). Each value is the mean ± SEM of 4-8 animals.

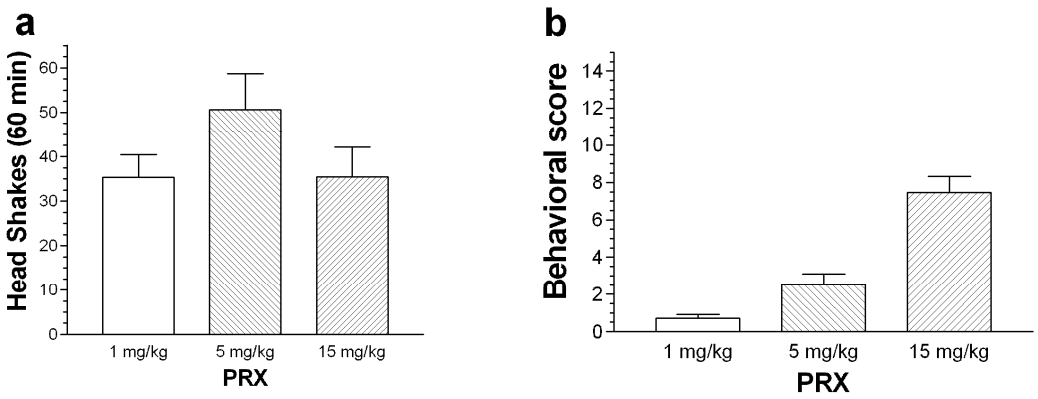


Figure 22. Changes in head shakes and behavioral score in the syndrome induced by paroxetine (PRX) combined with clorgyline (CLG) on day 4 in rats receiving once daily CLG for 3 days

Figure 22. Changes in head shakes and behavioral score in the syndrome induced by paroxetine (PRX) combined with clorgyline (CLG) on day 4 in rats receiving once daily CLG for 3 days

Animals had received clorgyline (CLG, 2 mg/kg, s.c.) for three days. On the fourth day, paroxetine (PRX, 15 mg/kg, i.p.) at the doses of 1, 5 and 15 mg/kg, i.p., was co-administrated with CLG at time 0 indicated by the long arrow. Intensity of head shakes is expressed as the number of shakes on one hour immediately after CLG and PRX challenge. Behavioral score was the sum of 6 variables (tremor, forepaw treading, hindlimb abduction, Straub tail and immobility). (a) Challenge injection of paroxetine combined with clorgyline evoked head shaking behavior. However, there was no difference between PRX doses of 1, 5 and 15 mg/kg ($p > 0.05$, one-way ANOVA). (b) PRX dose-dependently increased the behavioral score ($p < 0.001$, one-way ANOVA). Each value is the mean ± SEM of 5-9 animals.

Table 12. Effect of paroxetine on the 5-HT syndrome behaviors

Each value represents the mean ± SEM, n = 6-10.

Group	Dose of paroxetine (mg/kg)				p value of regression analysis
	0 (control)	1	5	15	
Tremor	0	0.18 ± 0.05	0.45 ± 0.13	1.76 ± 0.23	$p < 0.001$
Forepaw treading	0	0.20 ± 0.04	0.33 ± 0.07	1.01 ± 0.16	$p < 0.001$
Hindlimb abduction	0	0.24 ± 0.08	0.57 ± 0.21	1.73 ± 0.21	$p < 0.001$
Straub tail	0	0.20 ± 0.09	0.60 ± 0.16	1.27 ± 0.06	$p < 0.001$
Immobility	0	0.12 ± 0.07	0.52 ± 0.17	2.03 ± 0.11	$p < 0.001$
Total behaviors	0	0.72 ± 0.20	2.54 ± 0.57	7.47 ± 0.86	$p < 0.001$

Table 13. Fatality of the syndrome induced by paroxetine (PRX) combined with clorgyline (CLG) on day 4 in rats receiving once daily CLG for 3 days

Some of animals challenged with PRX (15 mg/kg, i.p.) and CLG (2 mg/kg, s.c.) after receiving CLG pretreatment for 3 days died in 24 hours due to dysfunction of autonomic system in the severe syndrome. Compared to the vehicle control, this effect was significant ($p < 0.05$, Fisher's exact test).

Group (CLG × 3d)	Survived	Died	Total
CLG + Vehicle	7	0	7
CLG + PRX 1mg/kg	11	0	11
CLG + PRX 5mg/kg	8	0	8
CLG + PRX 15mg/kg	6	7	13

CHAPTER FIVE: INVOLVEMENT OF THE MEDIAL PREFRONTAL CORTEX AND POSITIVE-FEEDBACK CIRCUIT IN THE PROGRESSION OF A BENIGN SYNDROME TOWARD MALIGNANT SYNDROME

5.1 Introduction

Excessive 5-HT in the brain is the initial cause of the 5-HT syndrome. The higher 5-HT level, the more serious the syndrome becomes (Ishigooka et al., 1993; Zhang et al., 2009). Decrease in 5-HT efflux could alleviate the syndrome and prevent the fatality (Zhang et al., 2009). In the brain, 5-HT is derived exclusively from raphe serotonergic neurons, participating in regulating physiological activities as well as involving mental disorders (Jacobs and Azmitia, 1992; Meltzer, 1999). The DRN and MRN are two major resources in the brain for serotonergic projections. They also receive afferents from various brain areas, including hypothalamus, basal ganglia and especially mPFC (Sakai et al., 1977; Hoover and Vertes, 2007; Hajos et al., 1998).

Anatomical and electrophysiological studies have revealed a reciprocal connection between the mPFC and raphe nuclei. Layer V pyramidal neurons in the mPFC receives monoaminergic afferents from DRN and MRN (Azmitia and Segal, 1978) and send glutamatergic efferents back to raphe (Martin-Ruiz et al., 2001; Aghajanian and Wang, 1977; Hajos et al., 1998). The mPFC neurons contain a large density of 5-HT receptors,

especially 5-HT_{2A} and 5-HT_{1A} receptors (Aghajanian and Marek, 1997, 1999). An approximate 60% of postsynaptic neurons in the mPFC that have raphe serotonergic innervations express 5-HT_{2A} receptor (Vazquez-Borsetti et al., 2009). On the other hand, serotonergic neurons in the raphe are regulated by glutamatergic afferents via NMDA receptors (Tao et al., 2007; Tao and Auerbach, 1996). Several studies indicated that 5-HT_{2A} receptor agonists enhanced the EPSC activity of glutamatergic pyramidal neurons in the mPFC (Aghajanian and Marek, 1997, 1999) and cell firing of 5-HT neurons in the DRN (Martin-Ruiz R et al., 2001) and 5-HT release in the mPFC (Amargos-Bosch et al., 2004). Moreover, NMDA increased the 5-HT neuron firing (Gartside et al., 2007) and 5-HT release in the DRN and MRN (Tao and Auerbach, 1996). Thus, it appears that there is a reciprocal connection between glutamatergic pyramidal neurons in the mPFC and serotonergic neurons in the raphe (Arango et al., 2002; Azmitia and Segal, 1978; Conrad et al., 1974). This is a positive-feedback circuit that is of particular importance in psychiatric diseases, e.g., major depression. Part of my study in this section was based on aforementioned circuit and explored its role in the 5-HT syndrome.

It was proposed that excessive 5-HT activated 5-HT_{2A} receptors located on glutamatergic neurons in the mPFC for inducing the syndrome by the circuit between mPFC and raphe. Moreover, 5-HT efflux might consist of primary and secondary components that were responsible for benign and malignant syndromes, respectively. This was because that glutamate was also elicited in the syndrome, causing an additional release of 5-HT associated with this circuit. It was a cycle that would boost 5-HT to exceeding level for inducing more serious syndrome.

5.2 Materials and Methods

Animal preparation, core body temperature and behavioral measurement, histology and data analysis were identical to those described in chapter two.

Chemicals and schedule for injection

Clorgyline, (±) DOI-hydrochloride, O-phthaldialdehyde, mercaptoethanol were purchased from Sigma (St. Louis, MO, USA). Ketanserin and (+)-MK801 were obtained from Tocris (Ellisville MO, USA). U73122 was from Santa Cruz Biotech (Santa Cruz CA, USA). Solvents and routes for drug administration were elucidated in Table 14. Except for paroxetine, drugs were injected at a constant volume of 1 ml/kg of body weight. Paroxetine was dissolved in the water and administered at a volume of 3 ml/kg body weight.

Animal preparation for the syndrome

Clorgyline (2 mg/kg) was injected subcutaneously once daily at 12:00 noon for consecutive 3 days. On day 4, animals were challenged with clorgyline combined with paroxetine for evoking a 5-HT syndrome. Antagonists were administered 30 (systemic injection) or 60 min (local perfusion) before clorgyline and paroxetine challenge. In the control group, vehicle was used to substitute respective agents.

Surgery and microdialysis procedures

Rats were anesthetized by a combination of ketamine (80 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.), and mounted in a Kopf stereotaxic frame in a flat skull position. Guide

cannulae (10 or11 mm in length; 22 gauge stainless steel tube) were in implanted aiming to mPFC, POA, DRN and MRN. The coordination is according to the rat brain atlas (Paxinos and Watson, 1998) and elucidated in Table 15. After surgery, rats were allowed to recover for one week before experiments.

One night before microdialysis, rats were briefly made to inhale with isoflurane for anesthesia. I-shaped microdialysis probes (cut-off 18 kD) were inserted through the guide cannula targeting at the mPFC, POA, DRN or MRN and secured in place with dental cement. The probe inlet was attached to a perfusion line from Raturn™ system (Bioanalytical System Inc., W. Lafayette, IN), and infused with the artificial cerebrospinal fluid (aCSF; containing 140 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.25 mM NaH_2PO_4, and 1.0 mM Na_2HPO_4; pH: 7.4) at a flow rate of 1 μl/min. Next day, two dialysate samples were collected to determine the basal value before administration of drugs. Samples were collected at one-hour interval starting at 10:00 a.m.

5-HT and glutamate analysis

A reverse-phase column (4.6 ×30 mm, packed with PP-ODS) was used for 5-HT separation. The composition of mobile phase was 0.1 M phosphate buffer (pH 6.0) containing 1% methanol, 500 mg/L sodium-1-octanesulfonate, and 50 mg/L EDTA. The flow rate was 350 μl/min. HPLC-electrochemical detection (HTEC-500; EICOM, Japan) with a CMA/200 refrigerator microsampler (CMA/Microdialysis, Stockholm, Sweden) was used to determine 5-HT level in the sample. The potential setting on the graphite electrode was + 350 mV (relative to Ag/AgCl reference electrode) (Fig. 8).

Separate settings of HPLC-electrochemical detection systems were used to determine glutamate concentration in dialysate sample. Samples were first added with derivation reagent (O-phthaldialdehyde/mercaptoethanol). The reagent consisted of 10 µL of 2-mercaptoethanol in 1 ml O-phthaldialdehyde solution (12 mg dissolved in 600 µl of methanol and diluted in 5.4 ml of 0.1 M borate buffer, pH 9.5). Derivation was performed by mixing 10 µl of the sample or standard with 10 µl of the reagent solution. 10 µl derivate solution was injected automatically by a refrigerated autoinjector (SIL 10 AXL; Shimadzu) or manually into a reverse-phase microsphere column (2 × 100 mm, packed with a C18, 3 µm particle size). A working electrode (MF-1000, BAS) was set at + 700 mV (LC-4C) *vs* an Ag/AgCl reference electrode. The mobile phase was 0.1 M phosphate buffer (pH 6.0), containing 5% methanol and 40 mg/L EDTA (Fig. 24). The flow rate was 400 µl /min delivered by a LC -10AT Shimadzu pump. Glutamate was estimated by comparing peak areas between microdialysis samples and known standard using Powerchrom V2.2 software.

5.3 Results

Re-evaluate the circuit between mPFC and raphe using drug-naïve animals

Neurochemical evidence of the circuit between raphe and mPFC was very merger despite a series of electrophysiological investigation (Aghajanian and Marek, 1997, 1999). Therefore, the circuit was re-evaluated by locally activating mPFC or raphe (DRN and MRN) neurons. Local perfusion of 300 μM NMDA (a glutamate NMDA receptor agonist) into the DRN significantly increased 5-HT efflux in the mPFC (Fig. 25a). It was confirmed by two-way repeated ANOVA showed that NMDA enhanced 5-HT efflux in the mPFC as demonstrated by the significant effect of treatment ($F_{1, 12} = 7.6$, $p < 0.05$), time ($F_{6, 72} = 2.3$, $p < 0.05$) and treatment × time ($F_{6, 72} = 2.5$, $p < 0.05$). To further examine the time course of NMDA in DRN on 5-HT level in mPFC, T-test was run on each time point. There was a significant different on the 40 min after NMDA perfusion.

Likewise, NMDA in the MRN also significantly enhanced 5-HT output in the mPFC (Fig. 15b). Two-way repeated ANOVA showed that NMDA enhanced 5-HT efflux in the mPFC as demonstrated by the significant effect of treatment ($F_{1, 11} = 6.8$, $p < 0.05$), but not time ($F_{6, 66} = 1.1$, $p > 0.05$) and treatment × time ($F_{6, 66} = 2.1$, $p > 0.05$). To further examine the time course of NMDA in the MRN on 5-HT level in the mPFC, independent sample T-test was run on each time point. There was a significant difference on the 40 min after NMDA perfusion.

On the other hand, infusion of 300 μM DOI in the mPFC significantly increased glutamate level in the DRN (Fig. 25c). Two-way repeated ANOVA showed that DOI in the mPFC enhanced glutamate efflux in the DRN as demonstrated by the significant

effect of treatment ($F_{1, 10} = 14.9$, $p < 0.01$), time ($F_{6, 60} = 3.0$, $p < 0.05$) and treatment × time ($F_{6, 60} = 4.3$, $p < 0.05$). To further examine the time course of NMDA in the MRN on 5-HT level in mPFC, independent sample T-test was run on each time point. There were significant differences on the 20 and 60 min after NMDA perfusion.

Similarly, infusion of 300 μM DOI in the mPFC significantly increased glutamate level in the DRN (Fig. 25d). Two-way repeated ANOVA showed that DOI in the mPFC enhanced glutamate efflux in DRN as demonstrated by the significant effect of treatment ($F_{1, 10} = 6.8$, $p < 0.05$), time ($F_{6, 60} = 3.3$, $p < 0.01$) and treatment × time ($F_{6, 60} = 3.2$, $p < 0.01$). To further examine the time course of NMDA in MRN on 5-HT level in mPFC, independent sample T-test was run on each time point. There was significant difference on the 20 min after NMDA perfusion.

Effects of ketanserin and MK801 on the antidepressant-evoked hyperthermia in the severe syndrome

To evaluate the role of 5-HT_{2A} receptor-mediated circuit between mPFC and raphe nuclei (DRN and MRN) on the malignant 5-HT syndrome, receptor antagonists were used systemically to disrupt the circuit. My hypothesis predicted that hyperthermia would be blocked in the syndrome. The basal T_{cor} was 37.77 ± 0.08 °C ($n = 55$). As shown in Figure 26a, ketanserin, a 5-HT_{2A} receptor antagonist, blocked the hyperthermia of the severe syndrome ($F_{2, 17} = 37.1$, $p < 0.001$) (Fig. 26a). This conclusion was confirmed by two-way repeated measures ANOVA, which revealed the significant effect of treatment ($F_{2, 17} = 37.1$, $p < 0.001$), time ($F_{8, 136} = 8.2$, $p < 0.001$) and treatment × time ($F_{16, 136} = 22.4$, $p < 0.001$). *Post hoc* analyses showed ketanserin pretreatment blocked the

hyperthermia of the 5-HT syndrome animals ($p < 0.001$, Scheffe's test). To further investigate the time course of ketanserin treatment on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered the T_{cor} on the 6 hours after clorgyline and paroxetine injection. *Post hoc* comparisons indicated that ketanserin decreased the T_{cor} for 7 hours after its administration.

Similarly, systemic administration of the NMDA receptor antagonist MK801 also blocked the hyperthermia (Fig. 26b). Two-way repeated ANOVA showed that MK-801 had a significant effect on the T_{cor} induced by CLG and PRX as demonstrated by the significant effect of treatment ($F_{2, 16} = 7.5$, $p < 0.01$), time ($F_{8, 128} = 3.0$, $p < 0.001$) and treatment × time ($F_{16, 128} = 9.1$, $p < 0.001$). *Post hoc* analyses showed ketanserin pretreatment blocked the hyperthermia of the 5-HT syndrome animals ($p < 0.001$, Scheffe's test). To further investigate the time course of MK801 treatment on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that MK801 altered the T_{cor} 5 hours after clorgyline and paroxetine injection. *Post hoc* comparisons indicated that MK801 decreased the T_{cor} from 1 to 5 hours after clorgyline and paroxetine administration.

Effects of ketanserin and MK801 on 5-HT efflux in the mPFC and POA of the severe syndrome

It was found in the previous study that both 5-HT_{2A} and NMDA receptor antagonists blocked the hyperthermia of the animals with the severe syndrome (Ma, et al. 2008). In this study, effect of the antagonists on 5-HT efflux was examined on the severe 5-HT syndrome animals. As shown in Figure 27a, systemic administration of ketanserin

significantly attenuated the increase in 5-HT of the mPFC in response to the antidepressant challenge for inducing the severe syndrome. Two-way repeated ANOVA showed that ketanserin had significant effect on the change of 5-HT level induced by CLG and PRX as demonstrated by the significant effect of treatment ($F_{2, 12} = 15.3$, $p < 0.01$), time ($F_{8, 96} = 23.8$, $p < 0.001$) and treatment × time ($F_{16, 96} = 8.8$, $p < 0.001$). *Post hoc* comparisons showed ketanserin could significant inhibit the increase of 5-HT efflux ($p < 0.05$, Scheffe's test). To further investigate the time course of ketanserin treatment on mPFC 5-HT, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered 5-HT level 1 to 6 hours after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin decreased 5-HT 3 to 6 hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

Likewise, MK801 at the dose of 0.25 mg/kg also reduced the increase in 5-HT in the mPFC (Fig. 27b). It was confirmed by two-way repeated ANOVA showing that MK-801 had significant effect on the change of 5-HT level induced by CLG and PRX as demonstrated by the significant effect of treatment ($F_{2, 14} = 16.2$, $p < 0.001$), time ($F_{8, 112} = 22.6$, $p < 0.001$) and treatment × time ($F_{16, 112} = 8.9$, $p < 0.001$). *Post hoc* comparisons showed ketanserin could significant inhibit the increase of 5-HT efflux ($p < 0.01$, Scheffe's test). To further investigate the time course of MK801 treatment on mPFC 5-HT, one-way ANOVA was run on each time point. These analyses indicated that MK801 altered 5-HT level on the 1 to 6 hours after clorgyline and paroxetine injection ($p < 0.05$). *Post hoc* comparisons indicated that ketanserin decreased 5-HT 1 to 6 hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

In the POA, both ketanserin and MK801 significantly attenuated the increased

5-HT efflux (Fig. 27c). Two-way repeated ANOVA showed that ketanserin and MK-801 had significant effect on 5-HT level induced by CLG and PRX as demonstrated by the significant effect of treatment ($F_{2, 12} = 16.0$, $p < 0.001$), time ($F_{8, 96} = 80.6$, $p < 0.001$) and treatment × time ($F_{16, 96} = 12.2$, $p < 0.001$). *Post hoc* comparisons showed both ketanserin and MK801 significantly reduced the increase in 5-HT efflux ($p < 0.01$, Scheffe's test). To further investigate the time course of ketanserin and MK801 treatment on POA 5-HT, one-way ANOVA was run on each time point. These analyses indicated that ketanserin and MK801 altered 5-HT level 2 to 6 hours after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin or MK801 decreased 5-HT 2 to 6 hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

To further analyze the data, the maximal effect was re-expressed in Figure 27e and 27d. The maximal 5-HT increase was 35-fold in the mPFC. Ketanserin and MK801 reduced it to 15- and 12-fold, respectively ($p < 0.01$, Scheffe's test; Fig. 27d). Likewise, the maximal increase in POA was 28-fold in the vehicle control, ketanserin and MK801 reduced it to 16- and 10-fold, respectively ($p < 0.05$, Scheffe's test) (Fig. 27e).

Effects of ketanserin and MK801 on glutamate efflux in the DRN and MRN of the severe syndrome animals

To test the circuit hypothesis, glutamate efflux in the DRN and MRN was determined for its role in inducing the syndrome. Baseline glutamate was 0.55 ± 0.09 μM ($n = 30$) in the DRN and 0.47 ± 0.07 μM ($n = 27$) in the MRN. As shown in Figure 28, challenge injection of paroxetine combined with clorgyline for inducing the severe syndrome caused an increase in glutamate in these two raphe nuclei. The increase was

significant. Ketanserin pretreated 30 min before the challenge injection attenuated the Glu increase in the DRN (Fig. 28). Two-way repeated ANOVA showed that ketanserin had a significant effect on the change of Glu level induced by CLG and PRX as demonstrated by the significant effect of treatment ($F_{2, 17} = 6.3$, $p < 0.01$), time ($F_{8, 136} = 4.6$, $p < 0.001$) and treatment × time ($F_{16, 136} = 3.5$, $p < 0.001$). *Post hoc* comparisons showed ketanserin did not significantly reduced the increase in glutamate efflux ($p > 0.05$, Scheffe's test). To further investigate the time course of ketanserin treatment on DRN Glu, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered Glu level 1 to 3 hours after clorgyline and paroxetine injection ($p < 0.05$). *Post hoc* comparisons indicated that ketanserin decreased glutamate on the 2nd hour after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

Similarly, ketanserin pretreated 30 min before clorgyline and paroxetine challenge injection attenuated the Glu increase in the MRN. Two-way repeated ANOVA showed that ketanserin had a significant effect on glutamate level induced by CLG and PRX as demonstrated by the significant effect of time ($F_{8, 96} = 3.0$, $p < 0.01$) and treatment × time ($F_{16, 96} = 2.6$, $p < 0.01$), but not treatment ($F_{2, 12} = 3.5$, $p > 0.05$). *Post hoc* comparisons showed ketanserin did not significantly reduced the increase in glutamate efflux ($p > 0.05$, Scheffe's test). To further investigate the time course of ketanserin treatment on DRN Glu, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered Glu level 1 to 3 hours after clorgyline and paroxetine injection ($p < 0.05$). *Post hoc* comparisons indicated that ketanserin reduced the increased glutamate 1 to 3 hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

Effects of ketanserin and MK801 on head shakes and behavioral score of the severe syndrome

The hypothesis that circuit between mPFC and raphe nuclei was also responsible for the head shaking behavior and other symptoms of the severe 5-HT syndrome which was tested by disrupting the circuit with systemic injection of ketanserin and MK801. Ketanserin pretreatment significantly decreased the number of head shakes as demonstrated by independent-samples T-test ($F = 10.8$, $p < 0.01$) (Fig. 29a), but failed to inhibit behavioral score as demonstrated by independent-samples T-test ($F = 1.086$, $p > 0.05$) (Fig. 29b). MK801 failed to reduce the frequency of the head shakes ($F = 1.9$, $p > 0.05$) (Fig. 29c) or behavioral score ($F = 0.053$, $p > 0.05$) (Fig. 29d).

Effects on hyperthermia of the severe 5-HT syndrome by a local disruption of the circuit between raphe and mPFC

The circuit hypothesis tested in the last section was mainly using systemic approach. However, systemic injection of antagonists has the nonselective effect on many brain areas. To further test the circuit hypothesis, selective disruption techniques were employed by a direct reverse microdialysis infusion of receptor antagonists into the mPFC or raphe nuclei.

Compared to the control, local perfusion of 0.3 mM ketanserin in the mPFC with dual-probe microdialysis significantly blocked the hyperthermia of the severe syndrome animals (Fig. 30a). Two-way repeated ANOVA showed that local perfusion of ketanserin into mPFC had the significant effect on the T_{cor} induced by CLG and PRX as demonstrated by the significant effect of treatment ($F_{2, 20} = 20.7$, $p < 0.001$), time ($F_{8, 160}$

= 6.6, $p < 0.001$) and treatment × time ($F_{16, 160} = 6.0$, $p < 0.001$). *Post hoc* comparisons showed ketanserin significantly blocked the hyperthermia ($p < 0.001$, Scheffe's test). To further investigate the time course of ketanserin treatment on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered T_{cor} 1 to 6 hours after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin decreased T_{cor} 6 hours after clorgyline and paroxetine administration ($p < 0.01$, Scheffe's test).

Consistently, local perfusion U73122 (50 μM), a PLC inhibitor disrupting the 5-HT_{2A} receptor intracellular singling, also blocked the hyperthermia of the syndrome (Fig. 30b). Two-way repeated ANOVA showed that local perfusion of PLC inhibitor U73122 into mPFC had significant effect on the T_{cor} induced by CLG and PRX as demonstrated by the significant effect of treatment ($F_{2, 14} = 7.5$, $p < 0.01$), time ($F_{6, 84} = 3.1$, $p < 0.01$) and treatment × time ($F_{12, 84} = 4.8$, $p < 0.001$). *Post hoc* analyses showed U73122 significant blocked the hyperthermia ($p < 0.001$, Scheffe's test). To further investigate the time course of U73122 treatment on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that U73122 altered T_{cor} 1 to 3 hours after clorgyline and paroxetine injection ($p < 0.05$). *Post hoc* comparisons indicated that U73122 decreased T_{cor} on the 1 and 2 hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

Next, synapse sites at the DRN and MRN were disrupted by dual-probe microdialysis. Local perfusion of 1 mM MK801 into the DRN and MRN blocked the hyperthermia of the severe syndrome (Fig. 30c). It was demonstrated by the significant effect of treatment ($F_{2, 14} = 6.4$, $p < 0.05$), time ($F_{7, 98} = 3.9$, $p < 0.01$) and treatment ×

time ($F_{14, 98} = 5.5$, $p < 0.001$). *Post hoc* analyses showed MK801 significant inhibited the hyperthermia ($p < 0.05$, Scheffe's test). To further investigate the time course of U73122 treatment on T_{cor}, one-way ANOVA was run on each time point. These analyses indicated that MK801 altered T_{cor} 2 to 5 hours after clorgyline and paroxetine injection ($p < 0.05$). *Post hoc* comparisons indicated that MK801 decreased T_{cor} 2 and 5 hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test). In a control study, blocking either site of the DRN or MRN alone with a single reverse microdialysis probe had no effect on hyperthermia.

Effects on 5-HT efflux of a local disruption of the circuit between raphe and mPFC in animals with the severe syndrome

It was proposed that excessive 5-HT for evoking the severe syndrome would be reduced after a local disruption of the circuit by microdialysis reverse perfusion of receptor antagonists such as ketanserin into the cortex or MK801 into the raphe. As shown in Figure31a, bilateral infusion of 300μM ketanserin into the mPFC significantly reduced 5-HT efflux in the mPFC (Fig. 31a) of the severe 5-HT syndrome animals as demonstrated by the significant effect of treatment ($F_{2, 13} = 18.3$, $p < 0.001$), time ($F_{8, 104} = 25.0$, $p < 0.001$) and treatment × time ($F_{16, 104} = 11.3$, $p < 0.001$). *Post hoc* analyses showed ketanserin significant reduced the increased 5-HT ($p < 0.01$, Scheffe's test). To further investigate the time course of ketanserin perfusion on 5-HT, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered 5-HT level on the 6 hours after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin decreased T_{cor} on the 6 hours after clorgyline and paroxetine

administration ($p < 0.05$, Scheffe's test).

Local perfusion of ketanserin (300 μM) into mPFC reduced increased 5-HT in the POA of the severe 5-HT syndrome animals as demonstrated by the significant effect of treatment ($F_{2, 11} = 35.8$, $p < 0.001$), time ($F_{8, 88} = 51.4$, $p < 0.001$) and treatment × time ($F_{16, 88} = 18.9$, $p < 0.001$). *Post hoc* analyses showed ketanserin significantly reduced increased 5-HT in the POA ($p < 0.01$, Scheffe's test). To further investigate the time course of ketanserin perfusion on 5-HT, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered 5-HT level on the 6 hours after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin decreased T_{cor} on 3 to 6 hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

To verify the involvement of the 5-HT_{2A} receptor in the circuit, U73122, a PLC inhibitor, was bilaterally infused into the mPFC (Fig. 31c). As expected, local perfusion of U73122 (50 μM) into mPFC reduced increased 5-HT in the mPFC of the severe 5-HT syndrome animals as demonstrated by the significant effect of treatment ($F_{2, 19} = 63.1$, $p < 0.001$), time ($F_{8,} 152 = 51.4$, $p < 0.001$) and treatment × time ($F_{16, 152} = 26.6$, $p < 0.001$). *Post hoc* analyses showed U73122 significant reduced increased 5-HT in the mPFC ($p < 0.001$, Scheffe's test). To further investigate the time course of U73122 perfusion on 5-HT efflux, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered 5-HT level 6 hours after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that ketanserin reduced 5-HT 1 to 4 and 6 hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

Similarly, Local perfusion of U73122 (50 μM) into mPFC reduced increased 5-HT in the POA of the severe 5-HT syndrome animals as demonstrated by the significant effect of treatment ($F_{2, 11} = 38.3$, $p < 0.001$), time ($F_{8, 88} = 30.4$, $p < 0.001$) and treatment × time ($F_{16, 88} = 10.2$, $p < 0.001$). *Post hoc* analyses showed U73122 significant reduced increased 5-HT in the POA ($p < 0.05$, Scheffe's test) (Fig. 31d). To further investigate the time course of U73122 perfusion on 5-HT, one-way ANOVA was run on each time point. These analyses indicated that ketanserin altered 5-HT level on the 6 hours after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that U73122 reduced 5-HT on the second and third hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

Next, the synaptic sites of the circuit at the DRN and MRN were disrupted with the noncompetitive NMDA receptor antagonist MK801. Again as predicted, dual-probe infusion of MK801 (1 mM) into the DRN and MRN significantly attenuated the increased 5-HT in the mPFC (Fig. 31e). It was demonstrated by the significant effect of treatment ($F_{2, 17} = 73.8$, $p < 0.001$), time ($F_{8, 136} = 75.3$, $p < 0.001$) and treatment × time ($F_{16, 136} = 28.3$, $p < 0.001$). *Post hoc* analyses showed MK801 significantly reduced increased 5-HT in the POA ($p < 0.01$, Scheffe's test). To further investigate the time course of MK801 perfusion on 5-HT, one-way ANOVA was run on each time point. These analyses indicated that MK801 altered 5-HT level on the 6 hours after clorgyline and paroxetine injection ($p < 0.001$). *Post hoc* comparisons indicated that MK801 reduced 5-HT on the 2 to 4 hours after clorgyline and paroxetine administration ($p < 0.01$, Scheffe's test).

Local perfusion of MK801 (1 mM) into DRN and MRN reduced increased 5-HT in

the POA of the severe 5-HT syndrome animals as demonstrated by the significant effect of treatment ($F_{2, 11} = 63.0$, $p < 0.001$), time ($F_{8, 88} = 75.3$, $p < 0.001$) and treatment × time ($F_{16, 88} = 11.1$, $p < 0.001$). *Post hoc* analyses showed MK801 significant reduced increased 5-HT in the POA ($p < 0.01$, Scheffe's test) (Fig. 31f). To further investigate the time course of MK801 perfusion on 5-HT, one-way ANOVA was run on each time point. These analyses indicated that MK801 altered 5-HT level 6 hours after clorgyline and paroxetine injection ($p < 0.01$). *Post hoc* comparisons indicated that MK801 reduced 5-HT 2 to 6 hours after clorgyline and paroxetine administration ($p < 0.05$, Scheffe's test).

Effect on glutamate of a local disruption of the circuit with ketanserin in the mPFC of animals with the severe syndrome

Glutamate in the DRN and MRN was determined using microdialysis in rats with the severe syndrome induced by paroxetine combined with clorgyline. As shown in Figure 32a, bilateral infusion of ketanserin (0.3 mM) into the mPFC significantly attenuated the increased glutamate in the DRN as demonstrated by the significant effect of treatment ($F_{, 13} = 0.9$, $p > 0.05$), time ($F_{8, 104} = 3.9$, $p < 0.001$) and treatment × time ($F_{8, 104} = 2.0$, $p > 0.05$). *Post hoc* analyses showed ketanserin did not significantly reduced increased glutamate in the DRN ($p < 0.01$, Scheffe's test). To further investigate the time course of ketanserin perfusion on glutamate, one-way ANOVA was run on each time point. These analyses indicated that ketanserin reduced glutamate level on the first hour after clorgyline and paroxetine injection ($p < 0.001$, Scheffe's test).

Although there was a tendency of local ketanserin to attenuate the increased

glutamate in the MRN, the effect failed to reach statistical significance (Fig. 32b). Local perfusion of ketanserin (300 μM) into mPFC reduced increased 5-HT in the POA of the severe 5-HT syndrome animals as demonstrated by the insignificant effect of treatment ($F_{1, 8} = 1.6$, $p > 0.05$) and treatment × time ($F_{8, 64} = 2.2$, $p > 0.05$)), but not time ($F_{8, 64} = 3.0$, $p < 0.01$). *Post hoc* analyses showed ketanserin did not reduced increased glutamate in the MRN ($p < 0.01$, Scheffe's test). To further investigate the time course of ketanserin perfusion on glutamate, one-way ANOVA was run on each time point. These analyses indicated that ketanserin did not reduced glutamate level at any time point ($p > 0.05$, Scheffe's test).

Effects on the head shake and behavioral score of a local disruption of the circuit between raphe and mPFC in rats with the severe syndrome

Bilateral infusion of ketanserin (0.3 mM) into the mPFC had no effect on head shakes (F = 0 .003, $p > 0.05$; Fig. 33a) or behavioral score (F = 3.9, $p > 0.05$; Fig. 33b) evoked by challenge injection of paroxetine combined with clorgyline in the clorgyline-pretreated animals. Similarly, bilateral perfusion of U73122 (50 μM) into the mPFC did not reduce the number of head shakes (F = 0.014, $p > 0.05$; Fig. 33c) or behavioral score (F = 3.9, $p > 0.05$; Fig. 33d). MK801 (1 mM) infusion into the DRN and MRN had no significant effect on the number of the head shaking behavior (F = 3.5, $p > 0.05$; Fig. 33e) or behavioral score (F = 3.6, $p > 0.05$; Fig. 33f).

Effects on the mortality rate of disrupting circuit between the raphe and mPFC in rats with the severe syndrome

As shown in Table 16, the mortality rate occurred in the severe syndrome animals without an antagonist. In contrast, all animals survived after pretreatment with systemic ketanserin (5 mg/kg, i.p.) or MK801 (0.25 mg/kg, i.p.). Next, the circuit between raphe and mPFC was disrupted with microdialysis reverse infusion of receptor antagonists. Similarly, the mortality occurred in the severe syndrome animals without an antagonist local perfusion. The death rate was reduced to zero after infusion of ketanserin (0.3 mM) into the mPFC or MK801 (1mM) into the DRN and MRN. U73122 (50 μM) infusion into the mPFC into the DRN and MRN also reduced the mortality rate (Table 16).

5.4 Discussion

The main result of this study was that disrupting the circuit between the raphe (DRN and MRN) and mPFC alleviated the severe syndrome and prevented the mortality. The observation was deduced from systemic injection of receptor antagonists and verified with local disruption of the circuit between the raphe and mPFC.

The circuit between the raphe and mPFC was re-evaluated with microdialysis approach. The data obtained in Figure 25 confirmed the findings of anatomical and electrophysiological studies, supporting that the circuit consists of 5-HT_{2A} receptors in the mPFC and glutamate receptors in the raphe (Wilson and Molliver, 1991; Lee et al., 2003; Aghajanian and Marek, 1999; Martin-Ruiz et al., 2001; Celada P et al., 2001). DOI, a 5-HT_{2A} receptor agonist, in the mPFC increased glutamate in the DRN and MRN. Consistently, NMDA infusion in the DRN or MRN increased 5-HT in the mPFC (Fig. 25). These results verify the circuit between the mPFC and raphe nuclei, supporting that 5-HT_{2A} receptor in mPFC and NMDA receptor in the DRN and MRN involve the circuit.

With this new animal model of 5-HT syndrome, blocking 5-HT_{2A} receptors with systemic ketanserin reduced the severity of the syndrome, similar to that described in the previous studies (Nisijima et al., 2001; Ma et al., 2008). Systemic ketanserin prevented the hyperthermia and reduced the increased 5-HT efflux in the mPFC in rats with the severe syndrome. In chapter four, it was found that hyperthermia was significantly elicited as the increased 5-HT exceeded 30-fold from the baseline. After pretreatment with ketanserin, however, 5-HT was reduced to 20-fold and animals displayed hypothermia (Fig. 26; 27a). It is well established that activation 5-HT_{2A} and 5-HT_{1A}

receptors induce hyperthermia and hypothermia, respectively (Faure et al., 2006; Hjorth, 1985; Lin and Chuang, 2002; Hedlund et al., 2004). It is likely that ketanserin pretreatment compromises the function of 5-HT_{2A} receptor and, thus, the activity of the 5-HT_{1A} receptor on the decrease in body temperature will be enhanced indirectly (Ma et al., 2008). So the hypothermia was observed in this study.

Present study showed that glutamate efflux was increased in the DRN and MRN in the severe syndrome (Fig. 28). Based on our circuit hypothesis, it can be interpreted that glutamatergic neurons in the mPFC have been excited by activation of the 5-HT_{2A} receptor, releasing glutamate in the axon terminals such as DRN and MRN. Systemic ketanserin pretreatment reduced the increased glutamate in DRN and MRN, supporting our hypothesis that 5-HT_{2A} receptor mediates the increased glutamate.

Head shake is a characteristic behavior induced by activation of the 5-HT_{2A} receptor. In this study, the number of head shakes was significantly reduced by ketanserin pretreatment in rats with the severe syndrome (Fig. 29). This was coincident with the reduced increase in 5-HT and compromised function of the 5-HT_{2A} receptor. Since behaviors as stated in the behavioral score are mediated by the 5-HT_{1A} receptor, it was not surprising that ketanserin did not have the effect on the behavioral score. It suggests that extracellular 5-HT, reduced by ketanserin pretreatment, is sufficient to excite the 5-HT_{1A} receptor.

As described previously in the literature (Nisijima et al., 2004; Ma et al., 2008), systemic injection of MK801 alleviated the severe syndrome. Based on our circuit hypothesis, glutamate released in the DRN and MRN activated NMDA receptors on serotonergic neurons. To test this hypothesis, MK801was given to rats with the severe

syndrome induced by paroxetine combined with clorgyline. Similar to ketanserin, MK801 prevented the hyperthermia (Fig. 26) and reduced the increased 5-HT (Fig. 27). Figure 26 showed that the duration of the hypothermia was very short in the MK801 pretreatment group in contrast to the animals pretreated with ketanserin. The underlying reason may be the slowly elevated 5-HT which activates the 5-HT_{1A} receptor predominately at the initial stage for its high affinity to 5-HT, causing hypothermia. When extracellular 5-HT was beyond 10-fold, 5-HT_{2A} receptors started to be activated for heat gain. In contrast, 5-HT_{2A} receptors in the ketanserin group would not be activated to increase the body temperature and, thus, the 5-HT_{1A} receptor was always predominating for heat loss, resulting in the long lasting hypothermia.

MK801 pretreatment in rats with the severe syndrome also has a tendency of reducing the number of head shakes and behavioral score. However, there was no statistical significance. Two possible reasons account for these results. First, the postsynaptic NMDA receptor is not involved in the head shakes and syndrome signs used for the behavioral score, and the decrease in 5-HT by disrupting the circuit was not sufficient to reduce the head shakes or signs of syndrome caused by 5-HT_{1A} and 5-HT_{2A} receptors. Second, MK801 alone induces locomotion, stereotyped sniffing and ataxia in rats (Andine et al., 1999), which might interfere with the behavioral performances in the 5-HT syndrome animals. Despite this, there was a slight decrease in the number of head shakes and behavioral score. This effect might result from the decrease in 5-HT level relevant to MK801 pretreatment.

To further test the role of the circuit in the severe syndrome, synaptic connections were selectively blocked by local infusion of receptor antagonists. In agreement with

systemic administration, local perfusion of ketanserin into the mPFC blocked hyperthermia (Fig. 30) and reduced the increased 5-HT in the mPFC and POA (Fig. 31). The effect of disruption of 5-HT_{2A} receptors was also tested using U73122 by blocking the 5-HT_{2A} receptor-coupled PLC activity. It was found that U73122 in the mPFC prevented the hyperthermia and increased 5-HT in rats challenged with paroxetine combined with clorgyline. Moreover, ketanserin infusion in the mPFC reduced the increased glutamate in the DRN and MRN (Fig. 30). These results suggests that the 5-HT_{2A} receptor in the mPFC is critical for the progress of the severe syndrome. Interestingly, both ketanserin and U73122 in the mPFC have no effects on the number of head shakes, which suggests that the 5-HT_{2A} receptor in the mPFC is not the target that mediates the head shakes elicited by 5-HT_{2A} receptors.

Based on the circuit hypothesis, DRN and MRN contain serotonergic neurons receiving glutamatergic afferents from the mPFC. MK801 infusion into the DRN and MRN blocked the hyperthermia and the increased 5-HT in the mPFC and POA of rats challenged with paroxetine combined with clorgyline for inducing the severe 5-HT syndrome. Besides NMDA receptor, Glu AMPA and KA receptors also exist in the DRN and MRN neurons (Gartside, et al., 2007). It will be interesting to explore the role of AMPA and KA receptors as well as the receptor-mediated calcium release on the circuit.

Interestingly, ketanserin or MK801 inhibited 50-70% of the increased 5-HT in rats with the severe syndrome (Fig. 31). It suggests that 5-HT efflux relevant to the syndrome consists of two components: primary and secondary. Primary efflux is due to inhibition of 5-HT uptake and degradation at the axon terminals by the antidepressants. When the antidepressant-mediated primary 5-HT release reaches a certain level, it will induce a

secondary 5-HT efflux by the positive-feedback loop between the mPFC and raphe. Ketanserin and MK801 disrupted the circuit between mPFC and raphe, resulting in reducing the secondary component (Figure 34).

The primary component induced by clorgyline and paroxetine could not be blocked by ketanserin and MK801. This component is responsible for benign signs of the syndrome (e.g., tremor, forepaw treading, immobility, hindlimb abduction, upright tail and head shakes). In support, local disruption of the circuit did not have an effect on the aforementioned behavioral signs. The second component is strongly associated with syndrome progression from benign to malignant, manifested by hyperthermia and death.

Altogether, 5-HT efflux in the mPFC of the severe 5-HT syndrome animals results from two distinct mechanisms: (1) the primary efflux results from the manipulation of 5-HT synthesis/metabolic pathway, which represents a direct effect of antidepressants on the axon terminal. The primary efflux is associated with the benign syndrome although the signs of the syndrome can be mild, moderate or severe; (2) the secondary efflux is evoked through the positive feedback loop between mPFC and raphe nuclei (DRN and MRN) as elucidated in Figure 34. It can be concluded that the secondary efflux is associated with the malignant syndrome, showing hyperthermia and death due to activation of 5-HT_{2A} receptor-mediated positive feedback circuit between cortical glutamatergic and raphe serotonergic neurons.

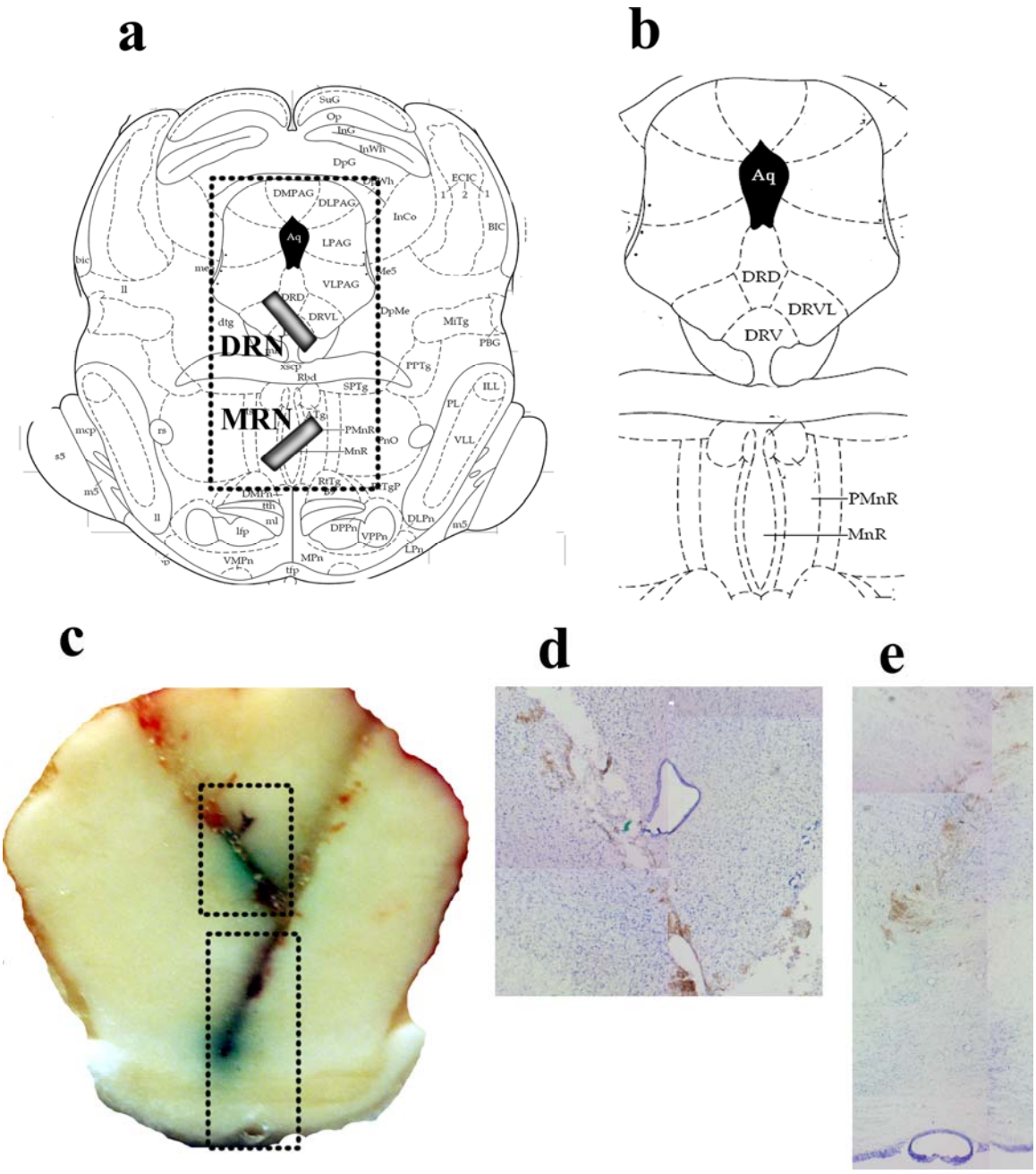


Figure 23. Schematic microdialysis location in the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN)

Figure 23. Schematic microdialysis location in dorsal raphe nucleus (DRN) and median raphe nucleus (MRN)

Microdialysis probes were inserted into DRN [A/P, +1.2 (lambda); L/R, ± 3.4; D/R, - 7.5 (32 ^{o} angle)] and MRN [A/P, +1.2 (lambda); L/R, ± 3.6; D/R, - 9.5 (25 ^{o} angle)]. The shade areas were the probe location. (a) A schematic representative of a rat brain showing DRN and MRN. (b) The amplified area as indicated with frame in (a). (c) A representative sample of brain showing DRN (upper frame) and MRN (lower frame) regions frozen and cut by microtome (Leica CM1850). Picture was taken by Nikon digital camera (D80). (d) A representative slide (40 μm) showing DRN stained by Cresyl Violet. (e) A representative slide (40 μm) showing DRN stained by Cresyl Violet. A/P, anterior/posterior; L/R, left/right; D/V, dorsal/ventral. Unit in mm (Paxinos and Watson, 1998). DRD, dorsal raphe nucleus, dorsal part; DRV, dorsal raphe nucleus, ventral part; DRVL, dorsal raphe nucleus, ventrolateral part; MnR, median raphe nucleus; PMnR, paramedian raphe nucleus.

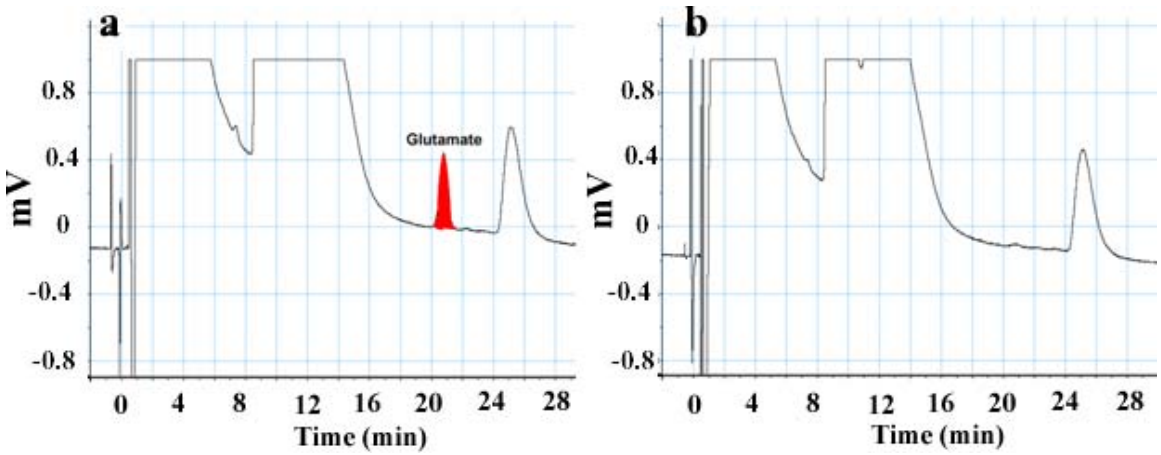


Figure 24. Representative glutamate chromatographs of a 1µM glutamate standard (a) and blank control (b)

Figure 24. Representative glutamate chromatographs of a 1 µM glutamate standard (a) and blank control (b)

Note that retention time for glutamate peak appeared at 20 min after sample injection.

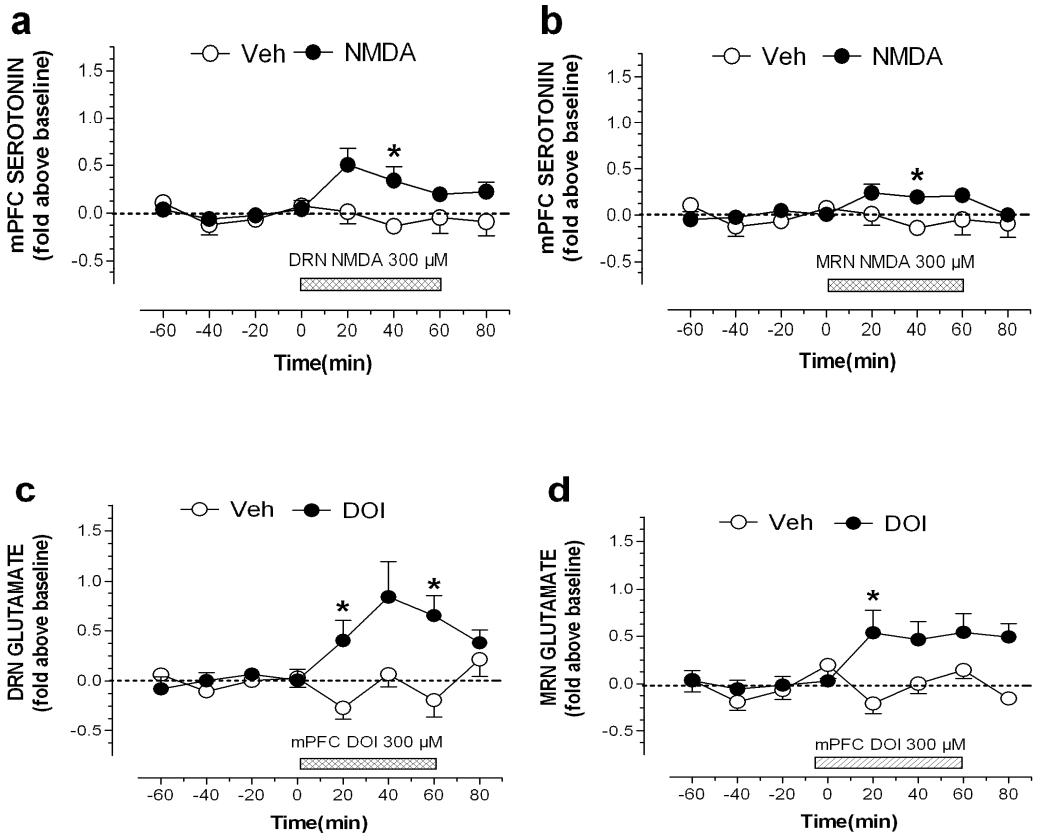


Figure 25. Re-evaluating the circuit between medial prefrontal cortex (mPFC) and dorsal and median raphe nuclei (DRN and MRN) in drug-naïve animals

Figure 25. Re-evaluating the circuit between medial prefrontal cortex (mPFC) and dorsal and median raphe nuclei (DRN and MRN) in drug-naïve animals

The horizontal bar represents the period of perfusion. Local perfusion of NMDA to the DRN (a) or MRN (b) increased 5-HT release in the mPFC. Similarly, DOI infusion into the mPFC enhanced glutamate level in the DRN (c) and MRN (d)**.** * $p < 0.05$ vs. vehicle (independently sample T-test). Data are shown as mean ± SEM of 5-6 animals.

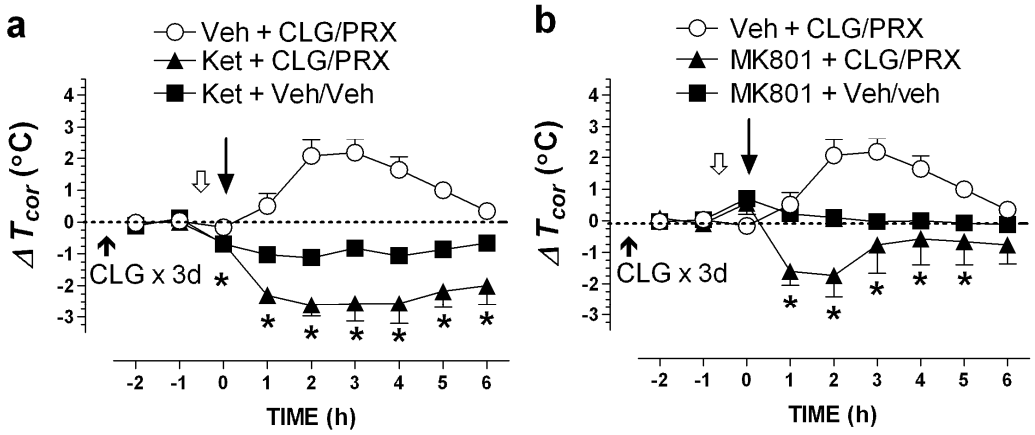


Figure 26. Systemic effects of ketanserin and MK801 on the hyperthermia of the severe syndrome animals

Figure 26. Systemic effects of ketanserin and MK801 on the hyperthermia of the severe syndrome animals

The open arrow indicated the time for the antagonist injection and solid arrow for the time of paroxetine (PRX, 15 mg/kg, i.p.) and clorgyline (CLG, 2 mg/kg, s.c.) challenge. Animals had been pretreated with once daily CLG for consecutive 3 days. On day 4, animals were challenged with PRX combined with CLG. Ketanserin (Ket, 5 mg/kg, i.p.) or MK801 (0.25 mg/kg, i.p.) was administered 30 min before the antidepressant challenge. Both ketanserin and MK801 inhibited the hyperthermia of the severe 5-HT syndrome animals. * $p < 0.05$ vs. Veh + CLG/PRX group (one-way ANOVA followed by *post hoc* Scheffe's test). Values are expressed as the change in T_{cor} relative to the baseline. Each value is the mean ± SEM of 5-8 animals.

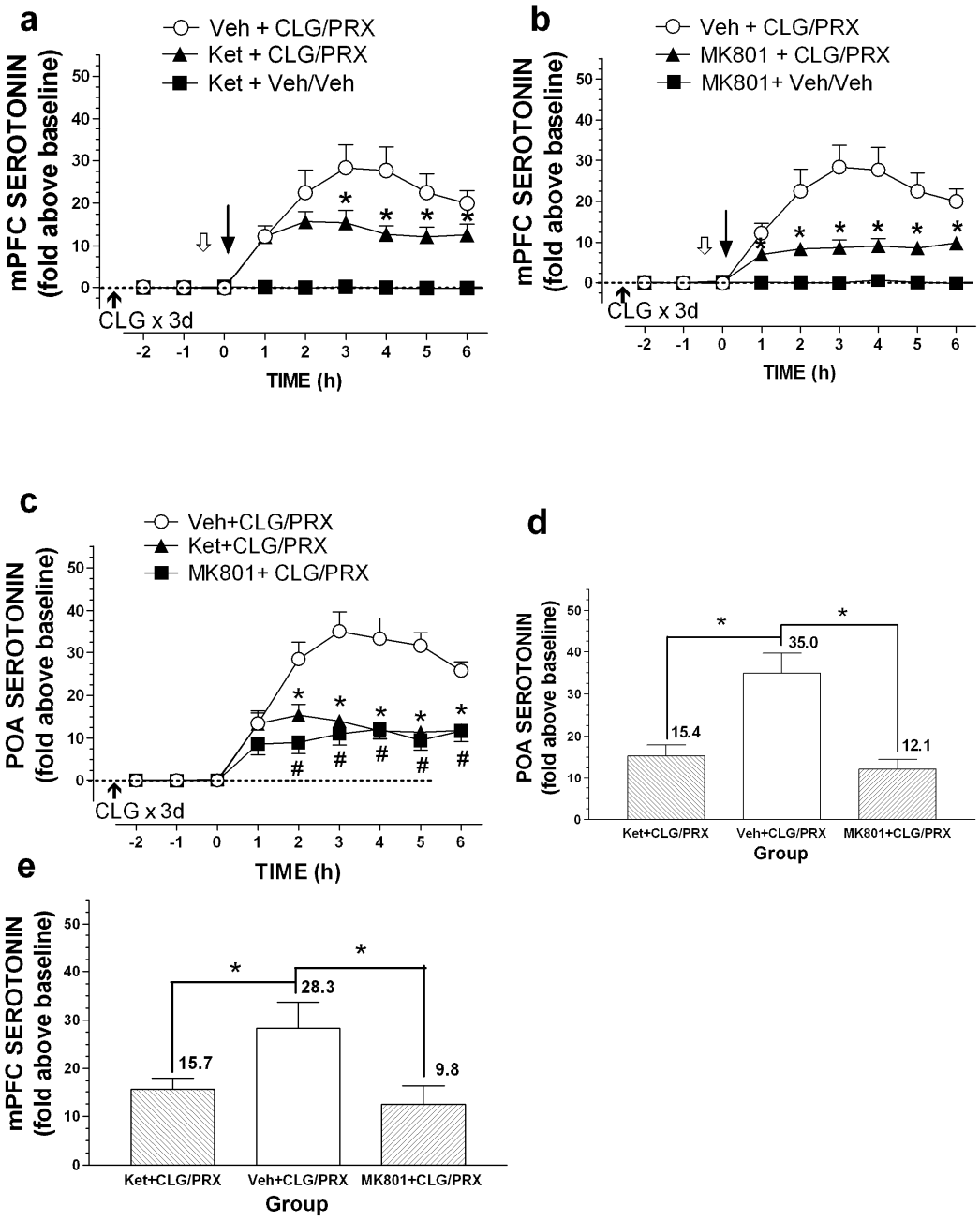


Figure 27. Ketanserin and MK801 attenuated the antidepressant-evoked increase in 5-HT efflux in the medial prefrontal cortex (mPFC) and preoptic area (POA) for inducing the severe 5-HT syndrome

Figure 27. Ketanserin and MK801 attenuated the antidepressant-evoked increase in 5-HT efflux in medial prefrontal cortex (mPFC) and preoptic area (POA) for inducing the severe 5-HT syndrome

Open arrows indicated the time for injection of ketanserin (Ket, 5 mg/kg, i.p.) or MK801 (0.25 mg/kg, i.p.) and the solid arrow for paroxetine (PRX, 15 mg/kg) and clorgyline (CLG, 2 mg/kg, s.c.) challenge. Animals had pretreated with CLG for consecutive 3 days. On day 4, PRX combined with CLG was administered 30 min after ketanserin or MK801 treatment. The antidepressant-evoked increase in 5-HT for inducing the syndrome was attenuated by ketanserin (a) and MK801 (b) in the mPFC. * $p < 0.05$ vs. Veh + CLG/PRX group (one-way ANOVA followed by *post hoc* Scheffe's test). Similarly, ketanserin and MK801 pretreatment also attenuated the evoked 5-HT increase in the POA (c). * $p < 0.05$ Ket + CLG/PRX group vs. Veh + CLG/PRX group; # $p < 0.05$ MK801 + CLG/PRX group vs. Veh + CLG/PRX group (one-way ANOVA followed by *post hoc* Scheffe's test). The data in d were the replot of c by comparison of the maximal responses between groups. * $p < 0.05$ (one-way ANOVA followed by *post hoc* Scheffe's test). Data in e was the replot of a-b at the maximal responses. * $p < 0.05$ (one-way ANOVA followed by *post hoc* Scheffe's test). Each value is the mean ± SEM of 4-6 animals.

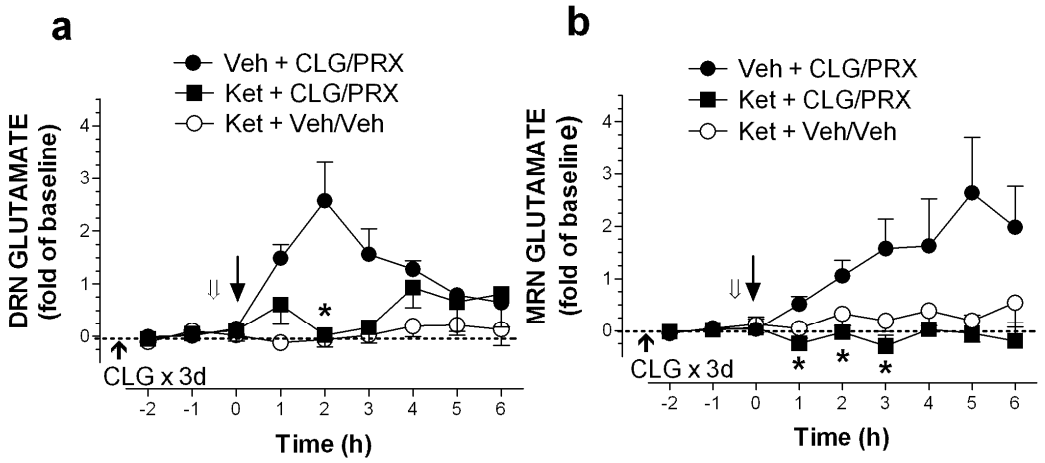


Figure 28. Ketanserin pretreatment attenuated the antidepressant-evoked increase in glutamate efflux in the dorsal raphe nucleus (a, DRN) and median raphe nucleus (b, MRN) of the severe syndrome animals

Figure 28. Ketanserin pretreatment attenuated the antidepressant-evoked increase in glutamate efflux in the dorsal raphe nucleus (a, DRN) and median raphe nucleus (b, MRN) of the severe syndrome animals

Open arrows indicate the time of ketanserin (Ket, 5 mg/kg, i.p.) injection and solid arrow for injection of paroxetine (PRX, 15 mg/kg, i.p.) combined with clorgyline (CLG, 2 mg/kg, s.c.). Animals used in this study had been pretreated with daily CLG for consecutive 3 days. On day 4, animals were challenged with PRX combined with CLG for evoking a severe syndrome. Systemic administration of Ket was given to rat 30 min prior to CLG and PRX challenge. Ketanserin attenuated the glutamate increase in the DRN and MRN of the severe 5-HT syndrome animals. * $p < 0.05$ vs. Veh + CLG/PRX group (one-way ANOVA followed by *post hoc* Scheffe's test). Each value is the mean ± SEM of 4-7 animals.

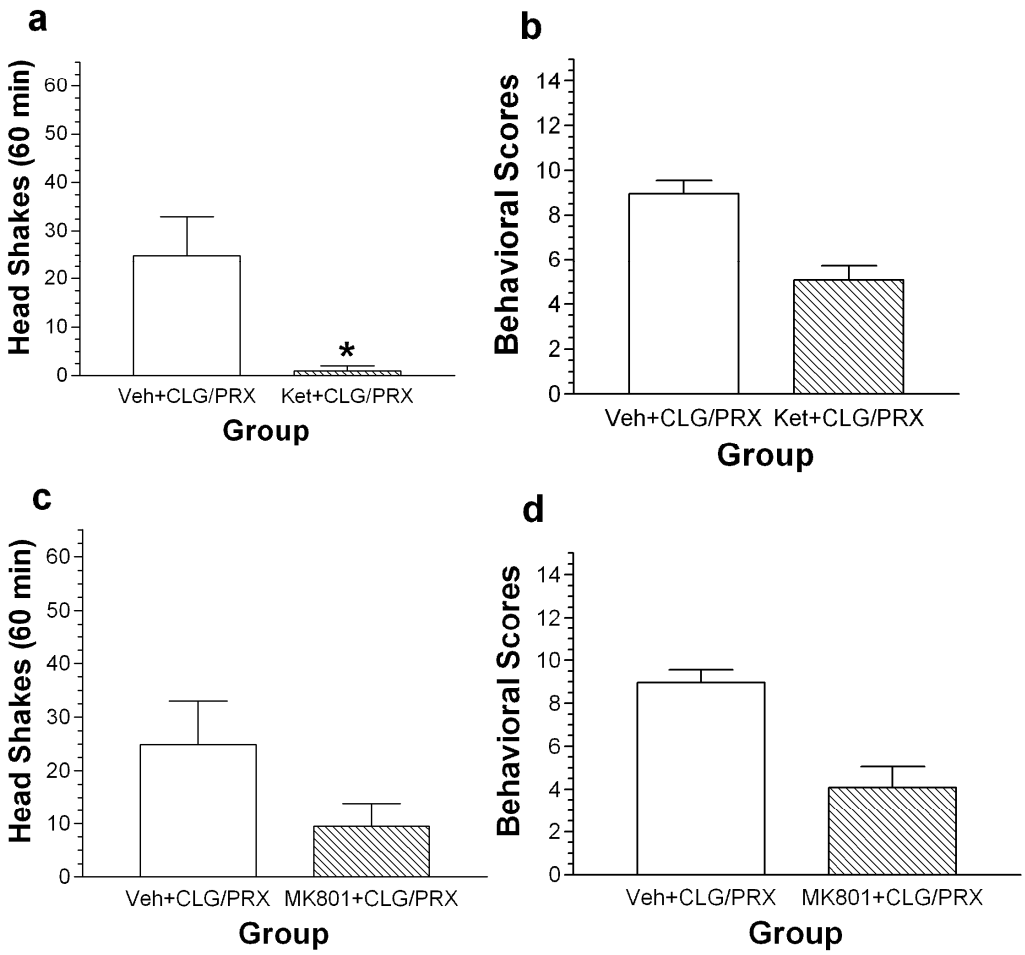


Figure 29. Effects of ketanserin and MK801 on the head shake and behavioral score of the severe syndrome animals

Figure 29. Effects of ketanserin and MK801 on the head shake and behavioral score of the severe syndrome animals

Animals had been pretreated with once daily clorgyline (CLG, 2 mg/kg, s.c.) for 3 days. On day 4, animals were challenged with paroxetine (PRX, 15 mg/kg, i.p.) combined with CLG for inducing the severe syndrome. The number of head shakes was counted for a total of one hour after the CLG and PRX challenge. Behavioral score was the sum of six variables including tremor, forepaw treading, hindlimb abduction, Straub tail and immobility. Each variable was blindly scored with a scale of no response (0), mild (1), moderate (2) or severe response (3). Systemic administration of ketanserin (Ket, 5 mg/kg, i.p.) 30 min prior to CLG and PRX challenge attenuated the number of head shakes (a), but not the behavioral score (b). MK801 had no effect on the head shakes (c) or behavioral score (d). * $p < 0.01$ (independent sample T-test). Data are mean ± SEM of 4-10 animals.

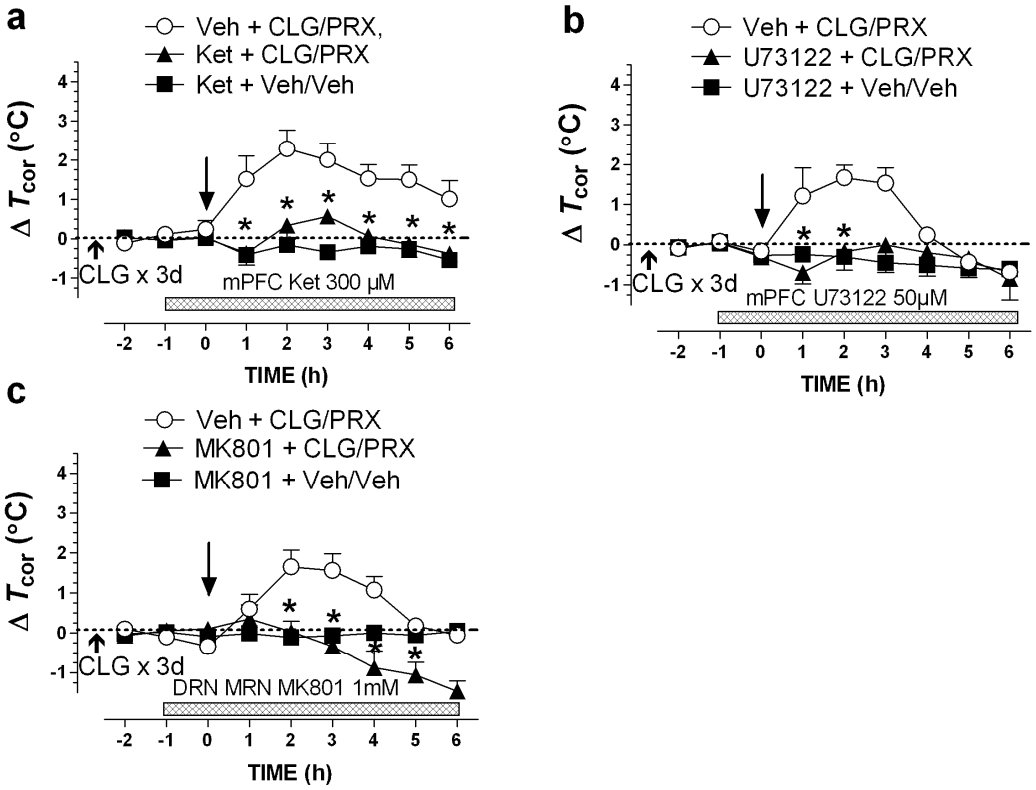


Figure 30. Effects on the hyperthermia of local disruption of the medial prefrontal cortex (mPFC), dorsal and median raphe nuclei (DRN and MRN) in the severe syndrome animals

Figure 30. Effects on the hyperthermia of local disruption of the medial prefrontal cortex (mPFC), dorsal and median raphe nuclei (DRN and MRN) in the severe syndrome animals

Animals had been treated with once daily clorgyline (CLG, 2 mg/kg, s.c.) for consecutive 3 days. On the 4th day, the CLG-pretreated animals were challenged with paroxetine (PRX, 15mg/kg, i.p.) combined with CLG. Arrows indicate the time of drug challenge. The horizontal bar represents the period of local microdialysis reverse perfusion. Compared to the control group, ketanserin (Ket, 0.3 mM) perfusion (a) or U73122 (50 μM) perfusion (b) into the mPFC bilaterally with dual-probe microdialysis blocked the hyperthermia of the severe syndrome. * $p < 0.05$ vs. Veh + CLG/PRX group (one-way ANOVA followed by *post hoc* Scheffe's test). Local perfusion of MK801 (1 mM) (c) into the DRN and MRN blocked the hyperthermia of the severe 5-HT syndrome animals. * $p < 0.05$ vs. Veh + CLG/PRX group (one-way ANOVA followed by *post hoc* Scheffe's test). Each value is the mean ± SEM of 4-10 animals.

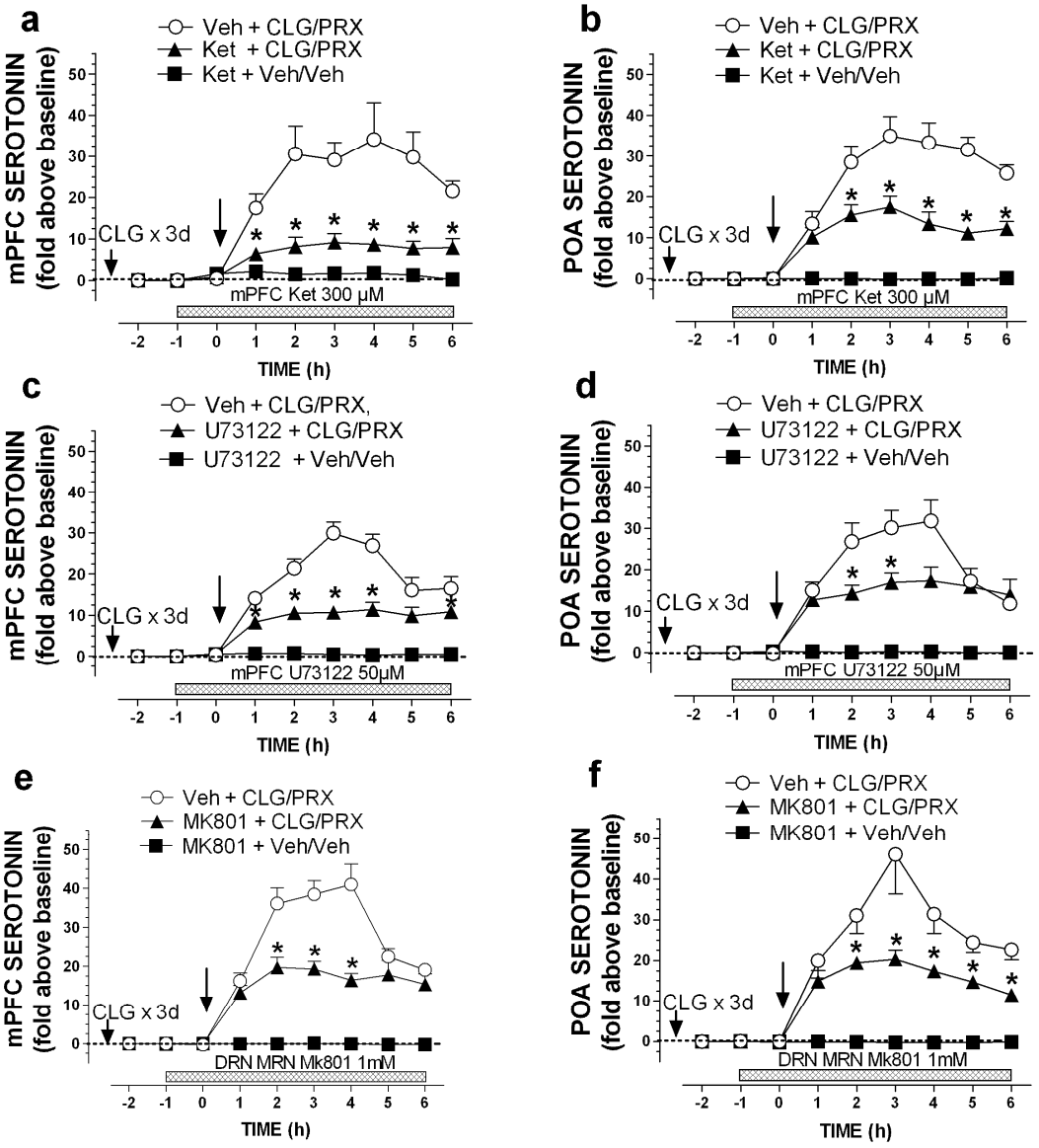


Figure 31. Bilateral infusion of ketanserin into the medial prefrontal cortex (mPFC) with a dual-probe microdialysis technique attenuated the antidepressant-evoked increase in 5-HT in the mPFC and preoptic area (POA) in the severe syndrome animals

Figure 31. Bilateral infusion of ketanserin into the medial prefrontal cortex (mPFC) with a dual-probe microdialysis technique attenuated the antidepressant-evoked increase in 5-HT in the mPFC and preoptic area (POA) in the severe syndrome animals

Animals had pretreated with once daily clorgyline (CLG, 2 mg/kg, s.c.) for 3 days. On day 4, animals were challenged with paroxetine (PRX, 15 mg/kg, i.p.) combined with CLG. Arrows indicate the time of the CLG and PRX challenge. The horizontal bar represents the period of bilateral perfusion. Compared to the control group, bilateral perfusion of ketanserin (Ket, 0.3 mM) or U73122 (50 μM) one hour before the challenge attenuated the antidepressant-evoked increase in 5-HT in the mPFC (a and c) and POA (b and d). * $p < 0.05$ vs. Veh + CLG/PRX group (one-way ANOVA followed by *post hoc* Scheffe's test). Dual- probe perfusion of MK801 into DRN and MRN also attenuated 5-HT increase in the mPFC (e) and POA (f). * $p < 0.05$ vs. Veh + CLG/PRX group (one-way ANOVA followed by *post hoc* Scheffe's test). Each value is the mean ± SEM of 4-10 animals.

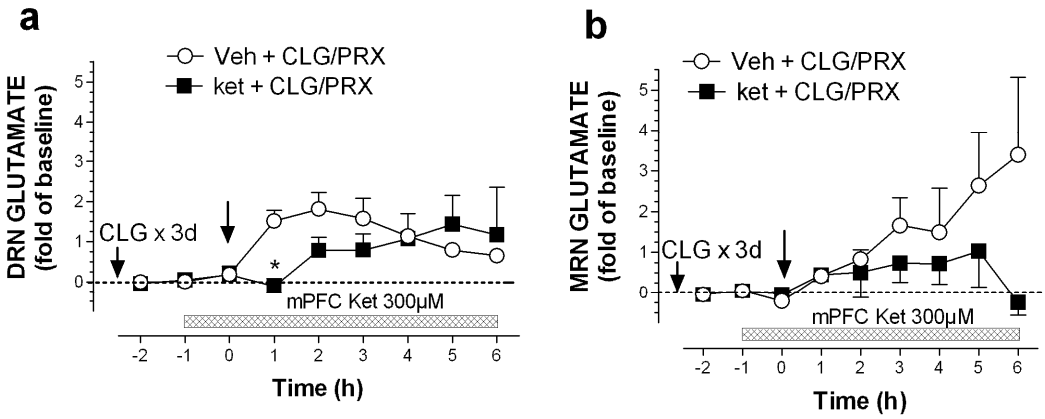


Figure 32. Effects on the increased glutamate of a local disruption of the synaptic site at the medial prefrontal cortex (mPFC) with bilateral infusion of ketanserin using dual-probe microdialysis

Figure 32. Effects on the increased glutamate of a local disruption of the synaptic site at the medial prefrontal cortex (mPFC) with bilateral infusion of ketanserin using dual-probe microdialysis.

Animals had received once daily clorgyline (CLG, 2 mg/kg, s.c.) for 3 days. On day 4, animals were challenged with paroxetine (PRX, 15 mg/kg, i.p.) combined with CLG. Glutamate was sampled in the DRN and MRN. Arrows indicate the time of injection of PRX combined with CLG. The horizontal bar represents the period of ketanserin perfusion. After disrupting the synapse at the mPFC with ketanserin (Ket, 300 μM), the antidepressant-evoked increase in glutamate was significantly reduced in the DRN. * $p <$ 0.01 (independent sample T-test). No difference was found in the MRN. Each value is the mean ± SEM of 4-8 animals.

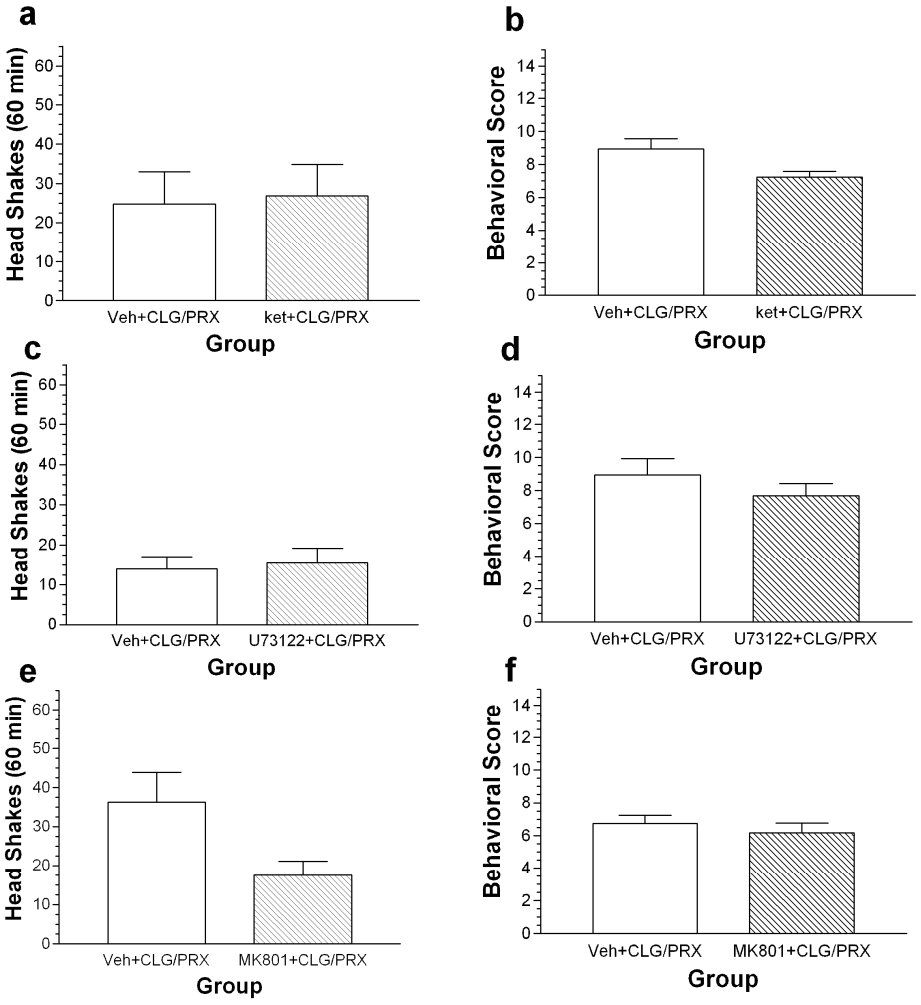


Figure 33. Effects on the number of head shakes and behavioral score of local disruption of the circuit between the medial prefrontal cortex (mPFC) and raphe nuclei in rats with the severe syndrome

Figure 33. Effects on the number of head shakes and behavioral score of local disruption of the circuit between the medial prefrontal cortex (mPFC) and raphe nuclei in rats with the severe syndrome

Animals had received once daily clorgyline (CLG, 2mg/kg, s.c.) for consecutive 3 days. On day 4, rats were challenged with paroxetine (PRX, 15 mg/kg, i.p.) combined with CLG. The number of head shakes was counted for one hour immediately after the CLG and PRX challenge. Behavioral score was the sum of six variables including tremor, forepaw treading, hindlimb abduction, Straub tail and immobility. Each variable was scored with a 3-level scale of 1 (mild), 2 (moderate) or 3 (severe response). Compared to control group, bilateral perfusion of ketanserin or U73122 into the mPFC did not affect the head shakes (a and c) and behavioral scores (b and d) induced by the paroxetine and clorgyline challenge. Similarly, MK801 infusion into the DRN and MRN did not affect the head shakes (e and g) or behavioral scores (f and h). Data are mean ± SEM of 4-10 animals.

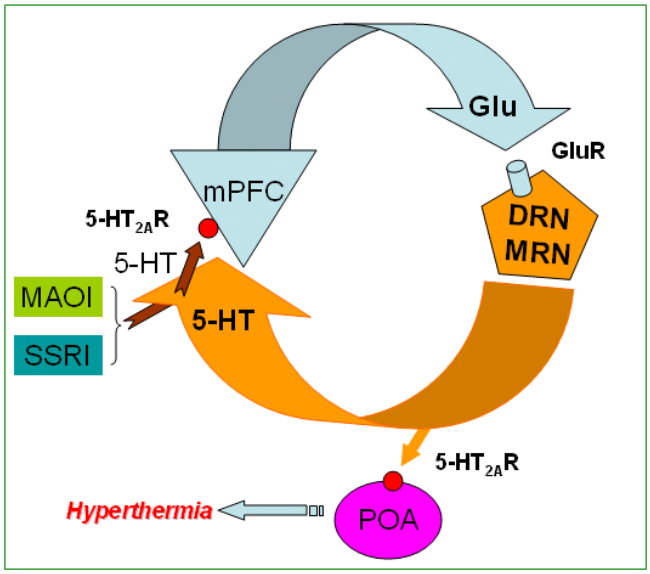


Figure 34. Simplified schematic of a circuit between the medial prefrontal cortex (mPFC) and dorsal and median raphe nuclei (DRN and MRN) responsible for the malignant syndrome induced by paroxetine combined with clorgyline

Figure 34. Simplified schematic of a circuit between the medial prefrontal cortex (mPFC) and dorsal and median raphe nuclei (DRN and MRN) responsible for the malignant syndrome induced by paroxetine combined with clorgyline

Glutamatergic neurons in the mPFC contain the 5-HT_{2A} receptor. Serotonergic neurons in the raphe have glutamate receptors (e.g., NMDA receptor). There are interconnections or circuits between the two areas. One of the circuits is a positive-feedback loop as described in this Figure since both NMDA and 5-HT_{2A} receptors are excitatory. Activation of 5-HT_{2A} receptor in the mPFC increases glutamate release in the DRN and MRN. Glutamate, in turn, excites serotonergic neurons by binding NMDA receptor in the raphe nuclei and then increases a 5-HT release in the mPFC as well as other brain areas such as preoptic area (POA) receiving raphe projection. In a 5-HT syndrome, monoamine oxidase inhibitor (MAOI) and selective serotonin reuptake inhibit (SSRI) induce a primary 5-HT release by inhibiting 5-HT reuptake and degradation. As a result, the primary 5-HT efflux activates 5-HT_{2A} receptors in the mPFC for a secondary 5-HT release by activating this circuit. Activation of the circuit likely results in a malignant 5-HT syndrome by excessive 5-HT efflux in the brain.

Table 14. Drug dose, solvent and route of administration used in chapter five

Drug	Dose	Solvent	Route of administration
Clorgyline	2 mg/kg	0.9% Saline	s.c.
Paroxetine	1; 5; 15 mg/kg	H_2O	i.p.
Ketanserin	5 mg/kg	H_2O	i.p.
MK801	0.25 mg/kg	0.9% Saline	i.p.
DOI	0.05; 0.1; 0.5 mg/kg	0.9% Saline	s.c.
Ketanserin	0.3 mM	aCSF	local perfusion
MK801	1.0 mM	aCSF	local perfusion
DOI	0.3 mM	aCSF	local perfusion
U73122	50 μM	aCSF (2.5% EtOH)	local perfusion

* aCSF, artificial cerebrospinal fluid; i.p.; intraperitoneal; s.c., subcutaneous; U73122 dissolved in 2.5% EtOH.

Table 15. The stereotaxic coordination of the medial prefrontal cortex (mPFC), preoptic area (POA), dorsal and median raphe nuclei (DRN and MRN) (unit in mm)

	A/P	L/R	D/V	Length of guide cannula	Length of microdialysis probe
mPFC	+ 3.3	± 0.8	- 4.5	10	2.5
POA	-1.1	±0.9	-9.2	11	1.0
DRN	+1.2 (lambda)	±3.4	-7.5 (with 32 ^{o} angle to midline)	11	1.0
MRN	+1.2 (lambda)	±3.6	-9.5 (25 ^{o} angle)	11	1.0

* A/P, anterior/posterior relative to bregma (mPFC and POA) or lambda (DRN and MRN); L/R, left/right relative to midline; D/V, dorsal/ventral relative to skull surface.

Table 16. Effects on the fatality of disrupting the circuit between the medial prefrontal cortex (mPFC) and raphe nuclei of rats with the severe syndrome

Groups (CLG ×3d +)	**Survived**	**Died**	**Total**
Vehicle (i.p.) + CLG/PRX	5	7	12
Ketanserin (5 mg/kg, i.p.) + CLG/PRX	8	0	8
MK801 (0.25 mg/kg, i.p.) + CLG/PRX	5	0	5
aCSF (l.p.) + CLG/PRX	5	3	8
Ketanserin (0.3 mM in mPFC) + CLG/PRX	12	0	0
MK801 (1 mM in DRN/MRN) + CLG/PRX	8	0	0
aCSF (containing 2.5% EtOH) + CLG/PRX	5	2	7
U73122 (50 μM in mPFC) + CLG/PRX	5	1	6

* CLG, clorgyline 2 mg/kg, s.c.; PRX, paroxetine, 15 mg/kg, i.p.

CHAPTER SIX: CONCLUSION AND SIGNIFICANCE

Serotonin syndrome has become a clinical problem in medicine over the past decades due to the wide prescription of serotonin-selective psychotropic agents, especially antidepressants (Sternbach, 1991; Gillman, 1999, 2006; Bodner et al., 1995). Given the potential life-threatening nature and high incident rate of the syndrome as well as the myriad of drugs manipulating the 5-HT system (Ener et al., 2003), it is imperative to take a close examination of the syndrome. Both clinical and lab researches have demonstrated that the 5-HT_{2A} receptor mediates some symptoms of the malignant 5-HT syndrome, such as hyperthermia and death (Nisijima et al., 2001; Isbister and Whyte, 2002), but the underlying mechanism remains unavailable. To fill the gap, my study tested the roles of 5-HT_{2A} receptor on the etiology of the malignant 5-HT syndrome. There are two main conclusions from my research: (1) Increase in the 5-HT_{2A} receptor activity or sensitivity is the precondition of inducing a malignant 5-HT syndrome; (2) The sensitized 5-HT_{2A} receptor caused a malignant 5-HT syndrome by initiating a feedback circuit between the mPFC and raphe nuclei (DRN and MRN).

The first aim of my study was to examine the role of 5-HT_{2A} receptor activity in a malignant 5-HT syndrome. It was hypothesized that the 5-HT_{2A} receptor was sensitized in the antidepressant treatment and thereafter excessive increases in 5-HT activated the sensitized receptor for the malignant syndrome. To test this hypothesis, a new animal

model of the syndrome was developed in the study. Interestingly, it was found that the symptoms of the malignant 5-HT syndrome were elicited in the chronic animals receiving clorgyline for 3 or 6 days, but not in the drug naïves. There was no difference in 5-HT level between the acute and chronic groups challenged with clorgyline combined with paroxetine, which excluded the effect of 5-HT level on the 5-HT_{2A} receptor functions. The data suggests that chronic clorgyline treatment increases the 5-HT_{2A} receptor activity and, thereafter, the sensitized 5-HT_{2A} receptor evoke a malignant 5-HT syndrome. Moreover, the conclusion was confirmed by ketanserin, a selective 5-HT_{2A} receptor antagonist, which could alleviate the malignant syndrome to a mild one. Another finding from this study was that chronic clorgyline treatment desensitized the 5-HT_{1A} receptor activity. Since 5-HT_{1A} and 5-HT_{2A} receptors are usually to exert opposite functions, the reduced activity of the 5-HT_{1A} receptor would in turn potentiate the activity of 5-HT_{2A} receptor indirectly.

To the best of our knowledge, this study was the first report in the 5-HT syndrome research with examining the 5-HT receptor activity relevant to antidepressant exposure. Clinical investigation indicated that patients with a 5-HT syndrome have a long history of taking antidepressants (Hegerl et al., 1998). Based on our study, the underlying reason may be the increased sensitivity of the 5-HT_{2A} receptor. Moreover, the results of our study may serve as a safeguard for both the doctor prescribing antidepressants and the patients receiving these compounds that antidepressants could increase the 5-HT_{2A} receptor activity and the susceptibility to this adverse effect. In this present study, my study just tested the role of chronic exposure to clorgyline on the 5-HT_{2A} receptor activity. In fact, a great number of antidepressants with various pharmacological properties are be

using in patients. In the future work, it should clarify the role of each antidepressant on the 5-HT_{2A} receptor activity. If an antidepressant has the ability of increasing 5-HT_{2A} receptor activity, it is better for doctor to switch the compound to another substitute or keep monitoring the patient's responses. In the future, it is also necessary to clarify the mechanisms behind the antidepressant-induced change in the 5-HT_{2A} receptor, for example, receptor protein density, functional activity and intracellular signal transduction.

Besides the chronic antidepressant exposure, the role of ambient temperature on the 5-HT_{2A} receptor activity was also explored. My data stated that warm and cold ambient temperature could potentiate and inhibit the 5-HT_{2A} receptor activity, respectively, in the 5-HT syndrome animals. A great number of studies indicated that warm and cold ambient temperature could potentiate and alleviate 5-HT-related toxic responses (e.g., hyperthermia, death) (Malberg and Seiden, 1998; Miller and O'Callaghan, 2003), respectively, but the mechanisms behind it was unclear before my study. Our data revealed that the change in the HT_{2A} receptor activity might at least in part account for the variation of the 5-HT toxicity due to different environmental temperature. Clinically, it should be cautious for people receiving the serotonergic compounds (e.g., antidepressants, MDMA) to stay in the hot environment. On the other hand, cold measures could be used to alleviate patients with the 5-HT syndrome. In the further work, it is neccessary to clarify the connection between the ambient temperature and the 5-HT_{2A} receptor activity. To date, many studies have stated that ambient temperature could alter the autonomic nerve system activity and hormone secretion (Leppaluoto et al., 1988; Quintanar-Stephano et al., 1991). The thermo sensitive hormones may serve the bridge

that connects the ambient temperature and 5-HT_{2A} receptor activity. However, further studies are needed to elucidate it.

The second major aim of my study was to explore how the sensitized 5-HT_{2A} receptor induced a malignant 5-HT syndrome. Our data showed that the 5-HT_{2A} receptor expressed in the mPFC exacerbated the 5-HT syndrome by initiating circuits between the mPFC and raphe nuclei (DRN and MRN). The circuits between raphe and mPFC have been thoroughly investigated by several laboratories including from Sharp, Artigas and Aghajanian. It has been deemed that the circuits are glutamatergic in the cortex and serotonergic in the raphe, forming feedback loops between these two locations and contributes to the pathophysiology and treatment of the psychiatric diseases, such as the major depression. One of my projects was based on the circuit and explored its role in the malignant 5-HT syndrome. First, our data indicated that disruption of the circuit alleviated the syndrome from malignant to benign. It suggests that the circuit mediates the malignant 5-HT syndrome. Second, glutamate and/or glutamate-mediated Ca^{2+} channels in the DRN and MRN play a crucial role in the circuit. It appears that not only 5-HT, but also glutamate or Ca^{2+} channels involve in the malignant 5-HT syndrome. Third, 5-HT efflux elicited by antidepressants (primary release) and the circuit (secondary release) have different functions. The former involves a benign 5-HT syndrome, and the later mediates a malignant syndrome. In this study, we only tested 5-HT level in the mPFC and POA. Obviously, all the neural structures in the brain receiving projections from the DRN and MRN, if not all, should have two components of 5-HT in the malignant 5-HT syndrome cases. The last and most important finding is that the circuit is triggered primarily by excessive 5-HT on the 5-HT_{2A} receptors in the mPFC.

In the chapter one, it was found that excessive 5-HT could not induce a malignant 5-HT syndrome in the drug-naïve animals. It suggests that in the normal circumstance the 5-HT_{2A} receptor-mediated circuit does not have a sensitized activity as seen in the malignant 5-HT syndrome. Malignant 5-HT syndrome happened in rats receiving chronic clorgyline treatment or housed in warm ambient temperature. It suggests that sensitized 5-HT_{2A} receptor is an important factor for initiating the circuit. Another finding in this chapter is that we indentify the synaptic connection on which MK801 acts. Lines of evidence have proved that glutamatergic NMDA receptor antagonist could alleviate the severe 5-HT syndrome (Nisijima et al., 2004; Ma et al., 2008), however the targets have not been reported. Our study demonstrated that serotonergic neurons in the DRN and MRN contain the receptor targets for the NMDA receptor antagonist to against the malignant 5-HT syndrome. Clinically, based on our study on the circuit, besides 5-HT_{2A} receptor in the mPFC, disrupting glutamatergic NMDA receptor is another way to prevent the exacerbation of the syndrome. Several circuits have been proposed between the mPFC and raphe nuclei (Sharp et al., 2007). The circuit consists of glutamatergic, GABAergic and serotonergic neurons. The receptors in this circuit include, but not limited to, NMDA, 5-HT_{2A}, 5-HT_{1A}, $GABA_A$ and $GABA_B$ receptors (Celada et al., 2001). It is possible that Glu from mPFC enhances GABA release in the raphe by activating GABAergic neurons. GABA would likely inhibit the serotonergic neurons. It is interesting to test GABA level in the raphe as well as in the mPFC of the 5-HT syndrome animals. Unfortunately, no data were reported to date. In the present study, the 5-HT_{2A} receptor on the cortical glutamatergic neurons and the NMDA receptor on the raphe serotonergic neurons were examined. In the future work, it is necessary to examine other

components (e.g., GABAergic interneurons) in the circuit on the etiology of the malignant 5-HT syndrome.

In summary, the underlying mechanism for an excessive 5-HT-evoked 5-HT syndrome was outlined in Figure 35. Chronic clorgyline treatment and increase in environmental temperature could increase the activity of 5-HT_{2A} receptor. The challenge injection of paroxetine combined with clorgyline would dose-dependently elicit an excessive increase in 5-HT, causing mild, moderate and severe 5-HT syndromes. The severe syndrome rarely occurs, resulting from the activation of the sensitized 5-HT_{2A} receptors in the circuit between mPFC and raphe (DRN and MRN). The efflux of 5-HT in the mPFC induced by antidepressants is primary, activating glutamatergic neurons through the 5-HT_{2A} receptor. Then, serotonergic neurons in DRN and MRN receiving mPFC glutamatergic efferent are excited to release glutamate which in turn activate serotonergic neurons for releasing extra 5-HT in mPFC as well as other regions. Therefore, this is a positive feedback circuit between the mPFC and raphe (DRN and MRN) resulting in excessive 5-HT and aggravating the syndrome (e.g., hyperthermia and death). This study, by focusing on 5-HT_{2A} receptor in the circuit and its response to antidepressant treatment as well as environmental temperature, reveals new neuropathological and pharmacological mechanisms underlying the etiology of the malignant 5-HT syndrome and, further, provides a sound basis for future work.

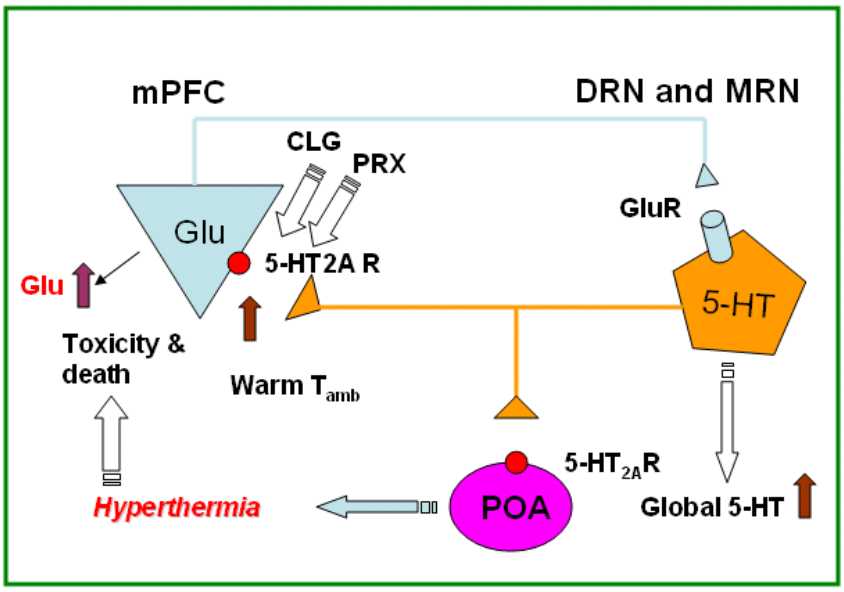


Figure 35. Schematic representative of an overlook of this dissertation

Figure 35. Schematic representative of an overlook of this dissertation

Chronic clorgyline (CLG) treatment and increase in ambient temperature (T_{amb}) enhance the activity of 5-HT_{2A} receptors. Challenge injection of paroxetine (PRX) combined with clorgyline causes an excessive increase in 5-HT efflux in medial prefrontal cortex (mPFC) and induces a severe 5-HT syndrome by initiating a 5-HT_{2A} receptor-mediated circuit between mPFC and dorsal and median raphe nuclei (DRN and MRN). 5-HT accumulated in preoptic area (POA) may induce the hyperthermia. Excitation of mPFC neurons increases the glutamate release in the brain. The excessive 5-HT elicits a severe 5-HT syndrome manifested by hyperthermia and even death.

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