

From THE DEPARTMENT OF CLINICAL NEUROSCIENCE

Karolinska Institutet, Stockholm, Sweden

**THE ROLE OF THE SEROTONERGIC SYSTEM  
AND THE EFFECTS OF ANTIDEPRESSANTS  
DURING BRAIN DEVELOPMENT EXAMINED  
USING *IN VIVO* PET IMAGING AND *IN VITRO*  
RECEPTOR BINDING**

Stal Saurav Shrestha



**Karolinska  
Institutet**

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Cover Illustration:

Voxel-wise analysis of the whole monkey brain using the PET radioligand, [<sup>11</sup>C]DASB showing persistent serotonin transporter upregulation even after more than 1.5 years of fluoxetine discontinuation.

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*To my family*

*Amaze yourself!*





**Karolinska  
Institutet**

The Department of Clinical Neuroscience

**The role of the serotonergic system  
and the effects of antidepressants  
during brain development  
examined using *in vivo* PET imaging  
and *in vitro* receptor binding**

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## THESIS SUMMARY

Serotonin (5-HT) and the serotonergic system, which includes the serotonin transporter (SERT) and the two G protein-coupled 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors, are implicated in the pathophysiology and treatment of several neuropsychiatric disorders including major depressive disorder (MDD) and anxiety. Two classes of antidepressants—selective serotonin reuptake inhibitors (SSRIs), which block SERT, and tricyclic antidepressants (TCAs), which block several monoamine transporters—alter 5-HT levels and modulate the serotonergic system. However, the transient suicidal ideation associated with SSRIs led the U.S. Food and Drug Administration to issue a black-box warning in 2004 on their use in juveniles. In addition, both SSRIs and TCAs exhibit high response variability; roughly only one-third of patients treated with these agents undergo remission. This variability in response is believed to arise from gene-environment interaction; this includes events that unfold during early-life development, and may subsequently influence behavior, treatment outcome, and serotonergic function. My PhD thesis explored several facets of the serotonergic system and its involvement in MDD and anxiety, including: **1.** the long-term effects of fluoxetine (an SSRI) administered during brain development in rhesus monkeys imaged with positron emission tomography (PET) for two serotonergic markers: SERT and 5-HT<sub>1A</sub> receptors (*Paper I*); **2.** the antidepressant effects of escitalopram (an SSRI) or nortriptyline (a TCA) on 5-HT<sub>1A/1B</sub> receptors in the rat brain, explored using a gene-environment model (*Paper II*); **3.** the functionality and selectivity of [<sup>11</sup>C]CUMI-101, a 5-HT<sub>1A</sub> receptor PET radioligand, in rodent and primate brain (*Paper III*); and **4.** the disparate findings regarding 5-HT<sub>1A</sub> receptor PET imaging studies in MDD, and their implications for research studies (*Paper IV*).

*Paper I* (Am J Psychiatry, 2014) examined the long-term effects of fluoxetine administered to juvenile rhesus monkeys who, as young adults, were imaged with two selective PET radioligands: [<sup>11</sup>C]DASB for SERT, and [<sup>11</sup>C]RWAY for 5-HT<sub>1A</sub> receptors. An equal number of monkeys separated from their mothers at birth—an animal model of human childhood stress—were also studied. At birth, 32 male rhesus monkeys were randomly assigned to either maternal separation or normal rearing conditions. At age two, half (N = 8) of each group was randomly assigned to fluoxetine or placebo for one year. To eliminate the confounding effects of residual drug, monkeys were scanned at least 1.5 years after drug discontinuation. Social interactions were assessed both during and after drug administration. Results indicated that fluoxetine persistently upregulated SERT, but not 5-HT<sub>1A</sub> receptors, in both neocortex and hippocampus. Whole-brain, voxel-wise analysis localized SERT upregulation in lateral temporal and cingulate cortices. Neither maternal separation by itself nor the rearing-by-drug interaction was significant for either radioligand. Fluoxetine did not significantly affect behavioral measures.

In order to investigate the issue of response variability, *Paper II* (Neurosci Lett., 2014) examined the antidepressant effects of two agents—escitalopram (an SSRI) and nortriptyline (a TCA)—on 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors in the rat brain using a gene-environment model. The effects of genetic vulnerability were modeled using the Flinders Sensitive Line, a rat model of depression and its control counterpart, the Flinders Resistant Line. The effects of environmental vulnerability were modeled using the maternal separation paradigm. Rats (n=105) were drawn from four groups reflecting experimental crossing of strain and early-life stress to assess the effects of escitalopram or nortriptyline compared to vehicle. Quantitative *in vitro* autoradiography was performed using [<sup>125</sup>I]MPPI (5-HT<sub>1A</sub>) and [<sup>125</sup>I]CYP (5-HT<sub>1B</sub>) in prefrontal cortex (PFC) and hippocampus. Stringent, Bonferroni-corrected statistical analyses showed significant strain-by-rearing-by-treatment interactions in PFC 5-HT<sub>1A</sub> and hippocampal 5-HT<sub>1B</sub> receptors. Either genetic or environmental vulnerability reduced

serotonergic binding. However, the effects of vulnerable genotype and environment were not additive. Antidepressants, in general, increased serotonergic binding.

*Paper III* (J Nucl Med., 2014) assessed the functionality and selectivity of CUMI-101 using *in vivo* PET and *in vitro* receptor binding. Initially, [<sup>11</sup>C]CUMI-101 was reported in 2007 as a putative agonist, selective for the 5-HT<sub>1A</sub> receptor. Based on this, we also scanned 32 monkeys with [<sup>11</sup>C]CUMI-101 to compare the results between an agonist vs. an antagonist PET radioligand for the 5-HT<sub>1A</sub> receptor. Intriguingly, a 2011 study reported that CUMI-101 exhibited potent antagonistic behaviors at 5-HT<sub>1A</sub> receptors in the rat brain. Using [<sup>35</sup>S]GTPγS functional assay, we replicated this finding in rats, and extended the study to also include primates. In primates, CUMI-101, unlike 8-OH-DPAT, which is a 5-HT<sub>1A</sub> receptor agonist, did not stimulate [<sup>35</sup>S]GTPγS binding even up to a concentration of 10 μM. In addition, both *in vivo* PET and *in vitro* receptor binding demonstrated that CUMI-101 exhibited cross-reactivity with α1 adrenoceptors. *In vitro* binding to α1 adrenoceptors was highest in thalamus (>45%) and lowest in neocortex and cerebellum (<10%) in rat, monkey, and human brain. *In vivo* uptake of [<sup>11</sup>C]CUMI-101 was completely blocked only when both WAY-100635 (a 5-HT<sub>1A</sub> receptor antagonist) and prazosin (an α1 adrenoceptor antagonist) were co-administered.

*Paper IV* (NeuroImage, 2012) critically reviewed factors that may have led to the disparate findings surrounding 5-HT<sub>1A</sub> receptor imaging in MDD using the PET radioligand [*carbonyl*-<sup>11</sup>C]WAY-100635. Two key confounding ‘technical’ factors were highlighted: the use of the cerebellum as a reference region, and the imprecision of measuring the concentration of parent radioligand in arterial plasma—the method considered to be the ‘gold standard’. The notion that these technical factors may have confounded results is underscored by the finding that different results were obtained from the same study cohort depending on whether the outcome measure was normalized to cerebellar gray matter or plasma free fraction. The fact that not every study obtained plasma free fraction precluded a meta-analysis. Our study recommends that, in the future, individual centers acquire data using the ‘gold standard’ arterial input to address methodological concerns. This would also allow researchers to meaningfully pool the data, allowing them to reach a consensus regarding putative alterations in 5-HT<sub>1A</sub> receptor function in MDD.

Taken together, the findings of these four papers lead to some important conclusions regarding the role of the serotonergic system in the pathophysiology and treatment of MDD and anxiety. Our finding that fluoxetine leads to persistent SERT upregulation (Paper I) underscores the need for practitioners to exercise caution in prescribing SSRIs to juveniles, though no concrete implications should be drawn regarding whether the persistence of these effects is ultimately ‘good’ or ‘bad’. Paper II sheds light on the manner in which complex gene/environment interactions shape how antidepressants affect 5-HT<sub>1A/1B</sub> receptors in the brain, an issue that contributes directly to research regarding the etiology and pathophysiology of MDD, and the complex interaction between the disorder and the antidepressants used to treat it. Paper III provides important evidence that [<sup>11</sup>C]CUMI-101 is neither an agonist nor selective for the 5-HT<sub>1A</sub> receptor. Finally, the evidence presented in Paper IV makes key recommendations regarding the pooling of data necessary for future human PET studies to enter a more collaborative phase. This would allow researchers to clarify discrepancies and advance psychiatric research, particularly given the planning and labor-intensive costs of achieving these goals.

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## LIST OF SCIENTIFIC PAPERS

- I. STAL SAURAV SHRESTHA, Eric E. Nelson, Jieh-San Liow, Robert Gladding, Chul Hyung Lyoo, Pam L. Noble, Cheryl Morse, Ioline D Henter, Jeremy Kruger, Zhang, Stephen J. Suomi, Per Svenningsson, Victor W. Pike, James T. Winslow, Ellen Leibenluft, Daniel S. Pine, & Robert B. Innis. Fluoxetine Administered to Juvenile Monkeys: Effects on the Serotonin Transporter and Behavior. *The American Journal of Psychiatry*. 2014;171:323-31
- II. STAL SAURAV SHRESTHA, Daniel S. Pine, David A. Luckenbaugh, Katarina Varnäs, Ioline D. Henter, Robert B. Innis, Aleksander A. Mathé, & Per Svenningsson. Antidepressant effects on serotonin 1A/1B receptors in the rat brain using a Gene x Environment model. *Neuroscience Letters*. 2014;559:163-68
- III. STAL SAURAV SHRESTHA, Jieh-San Liow, Shuiyu Lu, Kimberly Jenko, Robert L. Gladding, Per Svenningsson, Cheryl L. Morse, Sami S. Zoghbi, Victor W. Pike, & Robert B. Innis. [<sup>11</sup>C]CUMI-101, a positron emission tomographic radioligand, behaves as a serotonin 1A receptor antagonist and also binds to  $\alpha 1$  adrenoceptors in brain. *The Journal of Nuclear Medicine*. 2014;55:141-46
- IV. STAL SAURAV SHRESTHA, Jussi Hirvonen, Christina S. Hines, Ioline Henter, Per Svenningsson, Victor W. Pike, & Robert B. Innis. Serotonin-1A receptors in major depression quantified using PET: controversies, confounds, and recommendations. *NeuroImage*. 2012;59:3243-251



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## **ABBREVIATIONS**

5-HT	Serotonin (or 5-hydroxytryptamine)
5-HT <sub>1A</sub>	Serotonin subtype 1A
5-HT <sub>1B</sub>	Serotonin subtype 1B
B <sub>max</sub>	Total receptor density
BP <sub>ND</sub>	Binding Potential Non Displaceable
FRL	Flinders Resistant Line
FSL	Flinders Sensitive Line
GPCR	G protein coupled receptor
GTP $\gamma$ S	Guanine Tri Phosphate (non-hydrolyzable)
HPLC	High-Performance Liquid Chromatography
<i>In vitro</i>	“Within the glass”, i.e. in a test tube
<i>In vivo</i>	“Within the living”, i.e. in an intact organism
$k'_2$	Clearance rate constant
K <sub>D</sub>	Dissociation constant
K <sub>i</sub>	Inhibition constant
MAOI	Monoamine Oxidase Inhibitor
MDD	Major Depressive Disorder
MRI	Magnetic Resonance Imaging
MRTM2	Multilinear Reference Tissue Modeling (2 parameters)
MS	Maternally separated
NR	Normally reared
PET	Positron Emission Tomography
SERT	Serotonin Transporter (or 5HTT)
SPM	Statistical Parametric Mapping
SRTM	Simplified Reference Tissue Modeling
SSRI	Selective Serotonin Reuptake Inhibitor
SUV	Standardized Uptake Value

*Stal Saurav Shrestha, 2014*

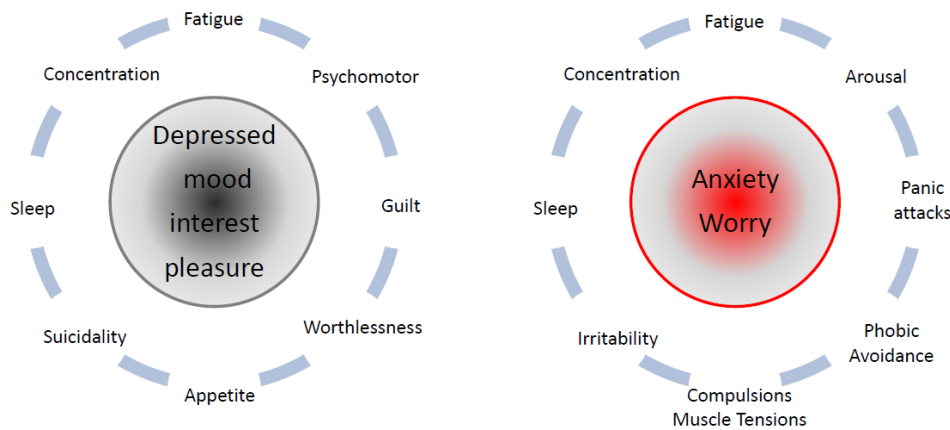
$t_{1/2}$	Half life
TAC	Time Activity Curves
TCA	Tricyclic Antidepressant
ROI	Region of Interest
VOI	Volume of Interest
$V_T$	Volume of Distribution





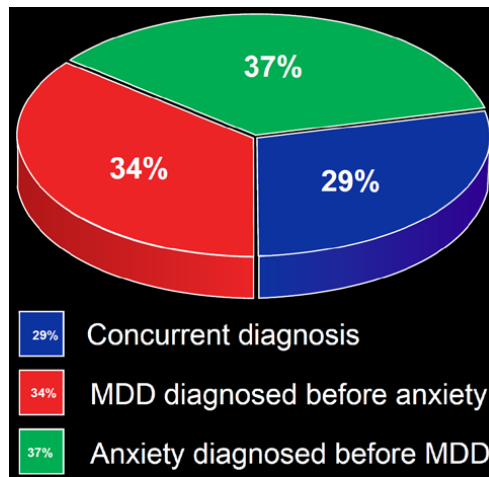
# 1 INTRODUCTION

The serotonergic system is primarily regulated by the versatile neurotransmitter, serotonin—5-hydroxytryptamine (5-HT) and is widely implicated in neuropsychiatric disorders, e.g. major depressive disorder (MDD) and anxiety. The serotonergic system includes the serotonin transporter (SERT or 5HTT) and the two key G protein-coupled receptors (GPCRs)—serotonin 1A (5-HT<sub>1A</sub>) and serotonin 1B (5-HT<sub>1B</sub>). Our current understanding of the role of serotonergic system in mood and anxiety disorders began with a series of serendipitous discoveries in the 1950s with drugs such as iproniazid (*monoamine oxidase inhibitor (MAOI)* used to treat tuberculosis) and imipramine (*a tricyclic antidepressant (TCA)* used to treat psychosis), which demonstrated therapeutic effects and was found to increase monoamine levels. This led to the monoamine theory of depression, which attributes certain psychiatric disorders to lower levels than normal of the three monoamines—serotonin, dopamine, and norepinephrine. However, this hypothesis remains controversial. Because both MAOI and TCA—also referred to as the older classes of antidepressants—act indiscriminately by blocking reuptake transporters of the three monoamines, researchers sought to further refine these classes of drugs for increased selectivity to each monoamine reuptake transporter. Intriguingly, blocking the reuptake property of only the serotonin transporter (SERT), which increases synaptic 5-HT levels, sufficed for antidepressant and anxiolytic effects. As a result of this observation, a new class of psychotropic was developed with a subnanomolar affinity for SERT and was named selective serotonin reuptake inhibitors (SSRIs).



**Figure 1.** Core symptoms of MDD and anxiety differ but several other symptoms overlap.

Although the core symptoms of MDD (e.g. depressed mood, interest, and pleasure) differ from that of anxiety disorders (e.g. anxiety/worry), there is an extensive overlap (Figure 1) between these two conditions (Unick et al., 2009). Both conditions are highly heterogeneous and pose a challenge in diagnoses with the concurrent diagnosis as high as 30% (Figure 2). Some of the similarities shared by the two disorders include clinical



**Figure 2.** Comorbidity between depression and anxiety is as high as 30%. Some patients are diagnosed first with depression prior to having anxiety and *vice versa*.

features (e.g. sleep disturbance, fatigue), neural circuits (e.g. hippocampus), neurotransmitters (e.g. 5-HT), and receptor target (e.g. SERT, 5-HT<sub>1A/1B</sub> receptors). Because of this overlap, effective modeling of anxiety vs. depression especially in animals is challenging. In addition, species (both intra and inter) differences in brain structures (e.g. cortices and limbic) together with high comorbidity prevalent overall in psychiatric conditions significantly constrains the utility of animal models. Because of this high comorbidity, in my thesis, I refer to the serotonergic system as well as animal models in reference to the unipolar mood disorder (i.e. major depressive disorder or MDD) and the anxiety disorder (i.e. general anxiety disorder or GDD).

Although SSRIs revolutionized the field of psychopharmacology in the late 1980s and are preferred for treating MDD and anxiety in pediatric and adult populations, its long-term effects on developing, juvenile brains in primates such as monkeys or humans have not yet been studied. There is also, in general, a high clinical variability in treatment outcome to antidepressants that is not well understood. Additionally, the high comorbidity prevalent in psychiatric disorders and no clinical biomarker constrains the ability of medical practitioners to effectively diagnose and treat depression and anxiety. In this introductory chapter, I divide my thesis into three broad topics (1) SSRIs: Developmental effects on 5-HT neurochemistry and behavior; (2) clinical variability in antidepressant treatment; and (3) the 5-HT<sub>1A</sub> receptor as a potential biomarker candidate for neuropsychiatric disorders.

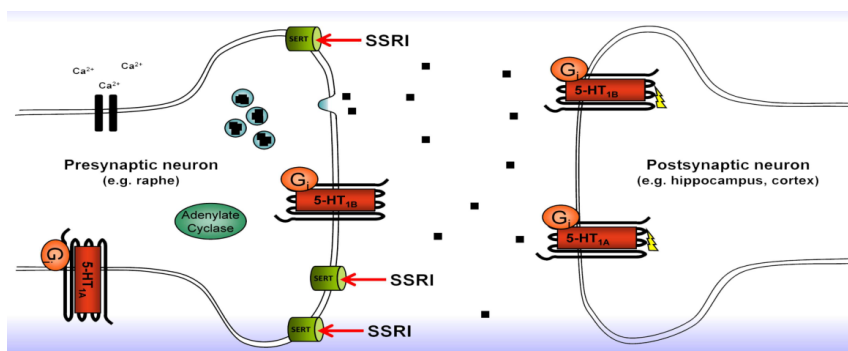
## 1.1 SSRIs: Developmental effects on 5-HT neurochemistry and behavior

SSRIs are prescribed to the juvenile population to treat MDD and anxiety despite lack of understanding of their long-term effects during the critical period of brain development. Although evidence supports the efficacy of SSRIs in the pediatric population, the U.S. Food and Drug Administration issued a black-box warning in 2004 on the use of SSRIs in this pediatric population based on evidence suggesting transient, age-delimited effects on suicidal ideation (FDA, 2004; Leon, 2007; March et al., 2004; Olfson et al., 2006). The black-box warning caused the prescription of SSRIs in the pediatric population to decline, indicating extra caution taken on the part of the medical practitioners (National Center for Health Statistics, 2013). For example, according to the Centers for Disease Control and Prevention, between 1994 and 2010, antidepressant prescriptions quadrupled in the age

group of 18 years and above; in stark contrast antidepressant prescriptions in the age group of < 18 years (or pediatric population) decreased while the incidence of psychiatric disorders, regardless of age, only increased. Below, I will review (1) SSRIs: Mechanism of Action; (2) serotonergic neurotransmission in light of its two key GPCRs— 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub>; and (3) developmental effects of SSRIs in rodents.

### 1.1.1 SSRIs: Mechanism of Action

The mechanism of action of SSRIs can be broadly classified into two types—direct (pharmacological; administered acutely) and indirect (modulatory; administered chronically) (Blier et al., 1998). With regard to the direct effect, SSRI primarily acts on SERT (Figure 3). Briefly, SERT is a 12-transmembrane protein dependent on the Na<sup>+</sup>/Cl<sup>-</sup> pump and a member of the solute carrier neurotransmitter transporter family 6 (SLC6). The SERT gene (*SLC6A4*) synthesizes the SERT protein whose primary role is to remove 5-HT from the synapse into the pre-synaptic neuron and in doing so, maintain homeostatic levels of 5-HT in the extracellular space. SSRIs block SERT with subnanomolar affinity resulting in maximal blockade and several-fold increase in synaptic 5-HT levels within hours. Currently, in the market there are six SSRIs—fluoxetine, escitalopram, citalopram, sertraline, paroxetine, and fluvoxamine. All six SSRIs increase synaptic 5-HT levels indiscriminately throughout the brain. This results from the non-selective blocking action of SSRIs on SERT, which is expressed on both serotonergic and non-serotonergic neurons.

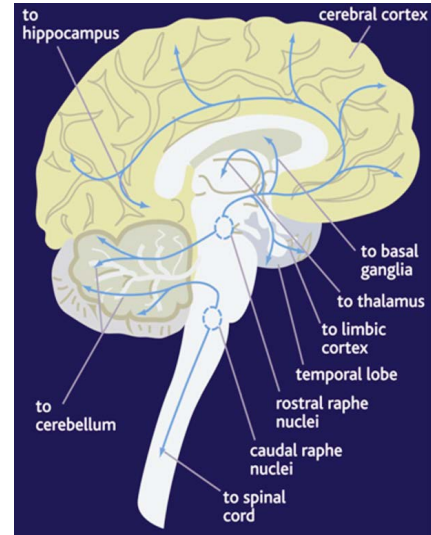


**Figure 3.** SSRIs (e.g. fluoxetine) block the reuptake of 5-HT (■) via the SERT thereby increasing the synaptic 5-HT levels and modulating post-synaptic tone via the key G protein-couple receptors such as 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub>.

The serotonergic neurons are located pre-synaptically and found predominantly in dorsal raphe nuclei (DRN) in the brainstem (Figure 4). In DRN, the serotonergic neurons synthesize 5-HT from L-tryptophan and project their axon terminals forming synapses with non-serotonergic neurons, post-synaptically, across several brain regions, including the neocortex and hippocampus. Pre-synaptically, SERT is expressed primarily on the synaptic terminal ends and to a lesser extent on the axons and the cell bodies—soma and dendrites (somatodendritic)—of serotonergic neurons (e.g. DRN). Post-synaptically, SERT is expressed predominantly on the somatodendritic area of non-serotonergic neurons.

While acute administration of SSRIs instantly increases synaptic 5-HT levels by blocking SERT, the chronic administration of SSRIs on SERT is less well known with the literature reporting mixed findings on expression and/or modulation of either its mRNA and/or protein.

With regard to indirect effects, the therapeutic effect of SSRIs is typically delayed by up to 12 weeks, and is postulated to the time necessary for adaptive changes, including altered density and function of 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors. Briefly, both receptor subtypes, similar to SERT, are also located pre- and post-synaptically. Pre-synaptically, they are expressed on serotonergic neurons (e.g. DRN; also referred to as autoreceptors) while post-synaptically, they are expressed on non-serotonergic neurons (e.g. hippocampus; also referred to as heteroreceptors). While the autoreceptors act as brakes and regulate the release and firing of 5-HT, the heteroreceptors modulate signal transduction.



**Figure 4.** The serotonergic neurons originate in the raphe (pre-synaptic site) in the brainstem and project to different areas of the brain, including cortex and hippocampus (postsynaptic sites). Source: Lundbeck Institute's CNSForum.com

Although the 5-HT<sub>1B</sub> autoreceptor is also an indirect target of SSRIs and plays an important role in 5-HT release, the 5-HT<sub>1A</sub> autoreceptor is considered to be the major player in 5-HT release as well as the therapeutic effect as demonstrated by electrophysiology and immunoelectron microscopy studies. Electrophysiology studies in rats demonstrated that the 5-HT<sub>1A</sub> receptor is important for the therapeutic effects of SSRIs as well as the typical delay in response associated with these medications (Lenox and Frazer, 2002). For instance, a study showed that the inhibitory autoreceptor desensitizes only when SSRIs are administered chronically (three weeks), but not acutely (15-60 min) (Blier et al., 1998). In general, desensitization results in receptor internalization, and/or receptor downregulation, and/or G proteins uncoupling. Desensitization blocks the inhibitory action of the 5-HT<sub>1A</sub> autoreceptor, and thereby releases the brakes, which subsequently increases synaptic 5-HT release triggering serotonergic neurotransmission and signal transduction (Blier et al., 1998). Similarly, immuno-electron microscopy studies demonstrated that acute vs. chronic SSRI administration have varying effects on the density and/or function of 5-HT<sub>1A</sub> auto vs. heteroreceptors. For instance, acute but not chronic SSRI administration results in ~35% receptor internalization; only the autoreceptors—but not the heteroreceptors—internalizes suggesting different G protein coupling to auto vs. heteroreceptors. These internalized autoreceptors, detected in cytoplasm and endosomal vesicles, had recovered in the plasma membrane within 24 hours. Furthermore, only chronic SSRI administration leads to a lack of response to DPAT suggesting receptor desensitization, which is said to be critical for the onset of therapeutic response (Descarries and Riad, 2012).



### 1.1.2 Serotonergic neurotransmission

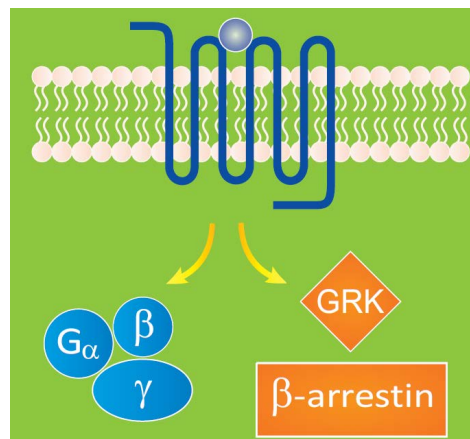
Upon receiving an action potential, the pre-synaptic serotonergic neuron fires releasing 5-HT into the synapse; this neurotransmission (primary action and takes seconds), in turn, affects mood via neuronal plasticity (secondary action and takes up to days) (Lesch and Waider, 2012). In general, synaptic neurotransmission may cause neuronal plasticity via either long-term potentiation (LTP), which results in synaptic strengthening or long-term depression (LTD), which results in synaptic elimination. The interplay between LTP and LTD is also important for mood and anxiety. Furthermore, a spike timing-dependent plasticity (STDP) theory postulates that spiking activities between pre- and post-synaptic neurons require not only sequential but also temporal events to modulate neuronal plasticity (Dan and Poo, 2006).

Within seconds after 5-HT firing and release, the serotonergic neurotransmission is terminated by either SERT or monoamine oxidase (MAO). Unlike the reuptake property of SERT, MAO metabolizes pre-synaptic 5-HT into 5-hydroxyindoleacetic acid (5-HIAA), an inactive metabolite that circulates in the cerebrospinal fluid (CSF). 5-HIAA levels in CSF are commonly used as an indirect marker of brain 5-HT levels (Higley et al., 1992).

Serotonergic neurotransmission occurs primarily via the 16 serotonin receptors, of which all but one (the ionic 5-HT<sub>3</sub>) belongs to the GPCR family. Below, I will discuss (1) GPCRs, their affinity states, and signal transduction and (2) the dual roles of 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors.

### 1.1.3 G protein-coupled receptors (GPCRs)

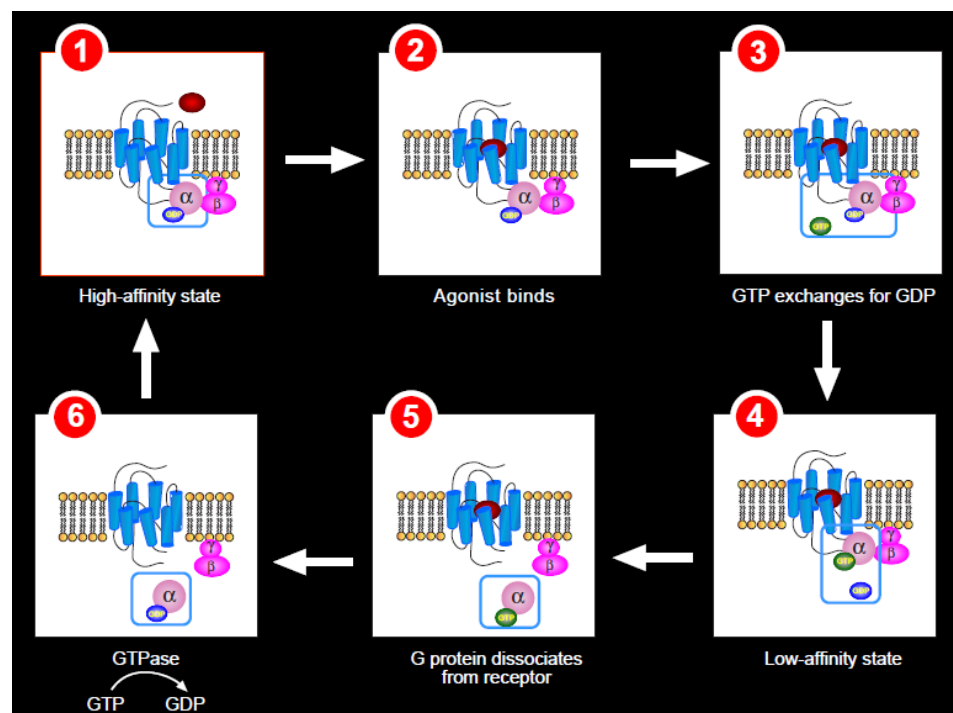
GPCRs or seven-transmembrane (TM1-TM7) receptors constitute 3-4% of the genome and are the largest class of receptors (> 800 GPCR proteins) (Lagerstrom and Schioth, 2008). GPCRs are divided into five families – *Rhodopsin*, *Glutamate*, *Adhesion*, *Frizzled/Taste2*, and *Secretin* (Lagerstrom and Schioth, 2008). In humans, the *Rhodopsin* receptor family is the largest (> 670 receptors) and includes 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors, which bind to the heterotrimeric G proteins—G<sub>α</sub>, G<sub>β</sub>, and G<sub>γ</sub>—via their intracellular C-terminus (Figure 5) (Luttrell and Lefkowitz, 2002). GPCRs exhibit different affinity states and via their G proteins initiate signal transduction pathways affecting a myriad of physiological responses, including behavior.



**Figure 5.** An agonist (●) binds to the seven transmembrane G protein receptor (e.g. 5-HT<sub>1A</sub>) coupled to three guanine proteins—G<sub>α</sub>, G<sub>β</sub>, and G<sub>γ</sub>.

### Affinity states and signal transduction via G proteins

Most pharmacologists believe that GPCRs typically exhibit two affinity states: high and low (Figure 6). The high-affinity state (also referred to as the 'active' state) has guanosine diphosphate (GDP) bound to G proteins, which is coupled to the heptahelical receptor. An agonist binds to the high-affinity state mimicking the effects of an endogenous neurotransmitter (e.g. 5-HT). The low-affinity state (also referred to as the 'inactive' state) has G proteins uncoupled to the receptor to which an agonist does not bind. However, an antagonist indiscriminately binds to either affinity states.



**Figure 6.** A schematic showing a GPCR cycle, their two-affinity states, and signal transduction via G proteins. 1. GPCR is in the high-affinity state that is recognized by an agonist; 2. An agonist binds to the high-affinity GPCR; 3. Agonist triggers conformational change and exchange of GTP for GDP; 4. GPCR is in the low-affinity state; 5. The GTP-bound  $G_{\alpha}$  protein dissociates from the receptor; the  $G_{\beta}$  and  $G_{\gamma}$  also dissociate; these G proteins trigger signal transduction affecting physiology; and 6. An exchange of GDP for GTP occurs at the  $G_{\alpha}$  protein, and the G proteins couple to the receptor switching it back to the high-affinity state.

An agonist's action via GPCRs is similar to an endogenous neurotransmitter and generally consists of four steps: (1) agonist binds to the high-affinity receptor stabilizing the ternary complex (agonist, receptor, and G proteins); (2) conformational changes occur allowing an exchange of GDP for GTP (guanosine triphosphate); (3) G proteins dissociate into  $G_{\alpha}$  and  $G_{\beta\gamma}$  subunits that ultimately regulate cellular and molecular pathways; and (4) GTPase hydrolyses GTP to GDP, and the G proteins reunite and couple to the receptor to undergo another cycle. Unlike an agonist, an antagonist has a neutral effect on GPCRs and may block the actions of agonists.

G proteins regulate both cellular and molecular pathways through secondary messengers, enzymes and effector/adaptor proteins. G proteins activate or inhibit enzymes such as adenylyl cyclase, which regulate cAMP (3'-5'-cyclic--adenosine monophosphate) levels. In turn, cAMP—a key second messenger—modulates the roles of enzymes such as kinases or phosphatases. Kinases such as protein kinase A (PKA), protein kinase C (PKC), or G protein-receptor kinases (GRKs) activate other signaling molecules via phosphorylation; on the contrary, phosphatases, via dephosphorylation, deactivate these signaling molecules.

While the  $G_{\beta\gamma}$  subunits exist as one complex,  $G_{\alpha}$  protein, based on sequence homology and signal transduction pathways, is divided into four subgroups:  $G_{\alpha_{i/o}}$ ,  $G_{\alpha_s}$ ,  $G_{\alpha_q}$ , and  $G_{\alpha_{12/13}}$  (Hewavitharana and Wedegaertner, 2012). In general,  $G_{\alpha_{i/o}}$  decreases intracellular cAMP levels by inhibiting the enzymatic activity of adenylyl cyclase;  $G_{\alpha_s}$  increases intracellular cAMP levels by stimulating adenylyl cyclase;  $G_{\alpha_q}$  increases intracellular inositol triphosphate (IP3) and diacylglycerol (DAG) levels by stimulating the phospholipase C (PLC) pathway; and  $G_{\alpha_{12/13}}$  regulates the Rho guanine exchange factors by stimulating G protein signaling. These varying signaling cascades ultimately affect gene expression and/or synaptogenesis, and in turn, regulate physiological responses.

#### ***The dual role of 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors***

G protein-coupled 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors exhibit dual roles—regulate 5-HT firing and release and aid in serotonergic neurotransmission—which derive, in part, from their presence both pre- and post-synaptically. Pre-synaptically, the receptor subtypes (referred to as inhibitory autoreceptors) emerge from the serotonergic neurons, which regulate 5-HT firing and release via a negative-feedback system. Importantly, while, 5-HT<sub>1A</sub> autoreceptors are located somatodendritically and primarily found in the brainstem area (e.g. DRN), 5-HT<sub>1B</sub> autoreceptors are located on the axon terminals and innervate distant brain regions (e.g. neocortex and hippocampus). Post-synaptically, either receptor subtype (referred to as heteroreceptors) facilitates serotonergic neurotransmission. 5-HT<sub>1A</sub> heteroreceptors are located predominantly in the hippocampus and anterior cingulate, and 5-HT<sub>1B</sub> heteroreceptors are located in striatum and globus pallidus.

As regards signal transduction, both 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> auto/heteroreceptors interact with  $G_{\alpha_{i/o}}$  proteins. Stimulating either receptor subtype induces membrane hyperpolarization via modulating G protein-gated inwardly rectified  $K^+$  (GIRK) or  $Ca^{2+}$  channels. In turn, they reduce excitability of post-synaptic neurons, and subsequently decrease cAMP levels by inhibiting adenylyl cyclase.

#### **4The developmental effects of SSRIs in rodents**

Unlike the adult brain that is more or less fully developed by mid-20's, the brains of children are actively forming new wirings across the brain. Therefore, understanding the effects of long-term SSRI treatment, during early-life development, on both neurochemistry and behavior is imperative. No such study have been investigated in the primate brain primarily because of the high costs of operation, variability in response to SSRIs, and most importantly, ethical reasons that preclude a randomized, placebo-controlled, longitudinal study in the pediatric population. Furthermore, *in vivo*, imaging techniques such as positron

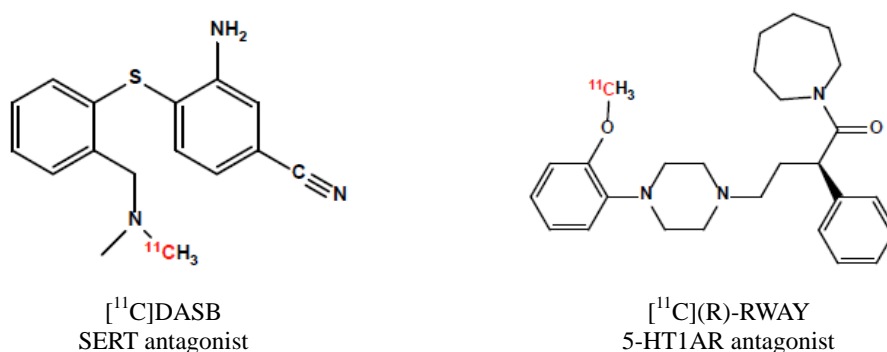
emission tomography (PET), which has successfully been used for research purposes to study brain functionality in adults, is extremely difficult in children due to ethical concerns regarding radiation exposure. Nonetheless, several rodent studies suggest that there is a 'critical' window period where SSRI exposure during development has long-lasting changes in both neurochemistry and behavior into adulthood.

Below I discuss two key neurochemical studies on SERT, followed by three key behavioral studies examining the long-term effects of SSRIs during brain development in rodents. Briefly, the approximated developmental periods in rodents are as follows: juvenile period (*rats* < PND (post-natal day) 40; *mice* < PND21), young adulthood period (PND40 < *rats* < PND90; PND21 < *mice* < PND45), and adulthood period (*rats* > PND90; *mice* > PND45). Adulthood results in the maturity of corticolimbic circuits, which are critical for healthy emotional behaviors.

As regards neurochemistry, two studies found that SSRIs administered during development have long-lasting effects on the SERT density. The first study reported persistent SERT upregulation of up to 20% in cortical regions in Wistar male rats treated with SSRI during the juvenile period (Wegerer et al., 1999). In that study, two groups of rats received fluoxetine (5 mg/kg) for a two-week period; respective controls for each group received placebo. One group was administered fluoxetine starting at PND25 (juvenile period), and the other group at PND50 (early-adulthood period). Both groups were sacrificed at PND90 (adulthood period) with a washout period lasting between 3-7 weeks. Using the *in vitro* homogenate binding technique, the investigators reported persistent SERT upregulation only in the juvenile group that received fluoxetine. In the second study, an independent group replicated this persistent, developmental effect in Wistar male rats. Furthermore, they used *in vivo* pharmacological Magnetic Resonance Imaging (phMRI) technique to demonstrate that rats administered fluoxetine, regardless of age, had lower levels of brain activation in response to 5-HT challenge than controls (Bouet et al., 2012).

As regards behavior, three studies found that SSRIs administered during early life impacts emotional behaviors into adulthood. The first study reported that early-life exposure to SSRIs cause abnormal behaviors into adulthood (Ansorge et al., 2008). Briefly, 129 male mice were administered fluoxetine (10 mg/kg) or other psychotropic drugs (selective for either SERT or norepinephrine transporter, NET) for 2½ weeks starting either at PND4 or PND90. After > 2 months of washout, behavioral tests were done. In the early-life exposure group, abnormal emotional behaviors such as increased anxiety/depression/stress-like behaviors emerged between two to three months of age and persisted up to 16 months of age (late adulthood). Furthermore, this developmentally sensitive effect was specific to selective blockade of SERT and not NET. The second study reported that genetic knockout of SERT (-/-) in mice impairs emotional behaviors, mimicking the effects of early-life (PND4-21) exposure to fluoxetine (10 mg/kg) (Ansorge et al., 2004). The third study reported that male Sprague-Dawley (SD) rats treated with fluoxetine during adolescence exhibits long-lasting abnormalities to both aversive as well as rewarding stimuli (Iniguez et al., 2010). Briefly, adolescent rats (PND35) but not young adult rats (PND65) that received fluoxetine (10 mg/kg) for a two-week period exhibited decreased behavioral reactivity to forced swimming test (FST), increased preference to sucrose, and impaired sexual activity.

In summary, both neurochemical and behavioral studies in rodents demonstrate that SSRI exposure during development has long-lasting effects into adulthood. However, and importantly, such studies have not yet been done in the primate brain. Monkeys (e.g. rhesus macaques), compared to rodents, arguably provide a better model of human brain function related to psychiatric disorders, due to similarities in brain structure, social organization, and most importantly, protracted brain development (Gogtay et al., 2008; Huttenlocher and Dabholkar, 1997; Wise, 2008). Similar to humans, rhesus monkeys exhibit depressive-like phenotypes when exposed to early-life stress (e.g. maternal separation). *Paper 1* examined the long-term effects of fluoxetine administered to juvenile rhesus monkeys who, as young adults, were imaged with two selective PET radioligands: [ $^{11}\text{C}$ ]DASB for SERT, and [ $^{11}\text{C}$ ]RWAY for 5-HT $_{1A}$  receptors (Figure 7). An equal number of monkeys separated from their mothers at birth—an animal model of human childhood stress—were also studied.



**Figure 7.** Structures and labeling of the two PET radioligands—[ $^{11}\text{C}$ ]DASB and [ $^{11}\text{C}$ ](R)-RWAY

## 1.2 Clinical variability in antidepressant response

Remission rates for both SSRIs and TCAs for treating MDD and anxiety are highly variable with only a third of patients attaining remission (Machado et al., 2006). Considerable evidence suggests that variability in antidepressant response arises from a gene-environment (GxE) interaction; this include events that unfold during early-life development, and may subsequently influence behavioral symptoms, antidepressant response, and serotonergic function (Felitti et al., 1998; Gilbertson et al., 2002; Homberg and van den Hove, 2012; Kaufman and Charney, 2000; Kendler, 2001; Shamseddeen et al., 2011). Nevertheless, it is unclear how antidepressants interact with GxE to influence the serotonergic system that may contribute to variability in antidepressant response. Below, I will review (1) remission rates associated with SSRIs, TCAs, and combination treatment and (2) clinical variability in relation to the placebo effect and, most importantly, GxE interaction.

### 1.2.1 Remission rates associated with SSRIs, TCAs, and combination treatment

Depressed patients on SSRIs exhibit high variability in remission rates posing a major challenge for practitioners to successfully treat the disorder. Besides SSRIs, the response

variability is also prevalent among other classes of antidepressants, including TCAs. Although both antidepressants have similar efficacy rates, SSRIs are preferred over TCAs because of their improved safety profile, milder side-effects, better tolerance resulting in lower dropout rates, and less lethality in overdose (von Wolff et al., 2013). A meta-analysis comparing randomized clinical trials of SSRIs and TCAs reported that the remission rates, on average, were 38% for SSRIs and 44% for TCAs (Machado et al., 2006). However, the intra-variability in remission rates within each drug class was also large. For example, of the SSRIs studied, the remission rate, on average, was highest for escitalopram (53%) and lowest for fluoxetine (23%); of the TCAs studied, the remission rate, on average, was highest for amitriptyline (54%) and lowest for imipramine (38%).

Combination therapies are often sought, commonly with SSRIs, to increase the standard remission rates. In general, they include either an additional pharmacotherapy or psychotherapy. Of direct relevance, two recent studies compared the effects of combined therapies to pharmacotherapy alone (Sung et al., 2012; von Wolff et al., 2012). Overall, neither study found a statistical difference on efficacy measures between the two therapies. The first study, which was a meta-analysis of eight primary studies, examined the combination of pharmacotherapy + psychotherapy to pharmacotherapy alone (von Wolff et al., 2012). Psychotherapies included were either cognitive behavioral therapy (CBT), interpersonal therapy (IPT), cognitive behavioral analysis system of psychotherapy (CBASP), or brief supportive psychotherapy (BSP). Although no statistical difference was noted, a trend towards higher remission rates was observed for combination therapies, suggesting their potential superiority over pharmacotherapy alone. The second study compared two different combinations of pharmacotherapies to monotherapy—escitalopram + bupropion SR, venlafaxine XR + mirtazapine, or escitalopram + placebo (Sung et al., 2012). This study did not find statistical superiority of the first-step combined treatment over monotherapy.

### **1.2.2 Clinical variability: Placebo Effects and GxE interaction**

The high variability in remission rates in MDD and anxiety is further complicated by the “placebo effect.” Briefly, the placebo effect, in general, either modifies the pharmacologic actions of drugs or results in a potent action attributed to the drug itself. In 1950, a paper reporting several case studies demonstrated the placebo effects of several well-established therapeutic agents, including ipecac and atropine (Wolf, 1950). I will briefly describe the placebo effects for these two drugs. At pharmacological doses, ipecac induces nausea, and atropine inhibits gastric function. As regards ipecac, patients experiencing nausea were cured by ipecac itself; as regards atropine, a patient who displayed gastric hyperfunction was given atropine, which failed to exert its effects. On both cases, the information about the drug remained undisclosed to the patients, who were assured that the ‘treatment’ would relieve them of their symptoms. Over the course of the last 60 years, such placebo effects have only been increasingly reported for several therapeutic drugs, including antidepressants. In fact, a recent meta-analysis examined 35 randomized-controlled trials (RCTs) of antidepressants, which included all but one SSRI, and found that the drug-placebo differences were relatively small in the treatment of MDD (Kirsch et al., 2008). Although the SSRI response was high, so was the placebo response, questioning the efficacy of SSRIs. In contrast, another meta-analysis study that included 20 RCTs reported

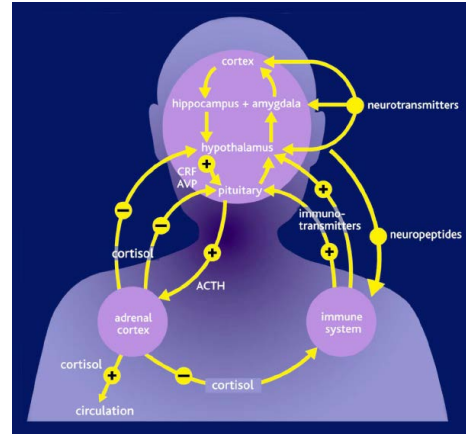
statistical superiority of both SSRIs and TCAs over placebo in treating MDD (von Wolff et al., 2013). Interestingly, in this study, the number needed to treat (NNT) for SSRIs was *six* for response and *seven* for remission and for TCAs was *four* for response and *seven* for remission.

Besides the placebo effect, the variability in remission rates is attributable to several genetic and environmental factors, including socioeconomic disadvantage (e.g. low education, unemployment, and poor quality of life), severity of illness, psychiatric comorbidity, compliance to treatment, drug-drug interactions, and most importantly, interactions of one or more of these factors (e.g. GxE interaction). Currently, the GxE interaction has garnered much interest, especially in demonstrating efficacy of evidence-based antidepressant treatments. As such, GxE interaction is postulated to be a major contributor for response variability.

Though the topic is currently debated, several studies suggest that genetic polymorphisms (e.g. serotonin transporter-linked 5' promoter region (5-HTTLPR)) and early-life stressors (e.g. childhood maltreatment) interact to influence behavioral symptoms and antidepressant response. After all, not everyone who encounters a stressful life situation develops a psychiatric disorder, highlighting the importance of individual genetic make-up; and of those that do, again not everyone responds similarly to treatment (Meaney, 2001). This underscores the importance of understanding the etiology of psychiatric disorders that may better help to understand response variability. Currently, it is unclear how antidepressants such as SSRIs and TCAs interact with GxE to influence treatment outcomes through various neurotransmitter systems, including the serotonergic system.

In this regard, preclinical animal studies have undoubtedly proven helpful for examining either the individual effects of vulnerable genotype or environment or their interactions. Unlike in naturalistic clinical studies, which are invariably impacted by uncontrollable environmental and genetic/ethnic differences, rodents and monkeys pose an excellent and viable alternative in explicitly manipulating genes and/or environment in a well-controlled environmental setting. Rodents have similar genetic background, much shorter life-span, and both genes and environment are easier to manipulate, while monkeys, similar to humans, have the major advantage of similarities in brain structure, social organization, and most importantly, protracted brain development.

As in humans, early environmental events (e.g. early-life stress) in rodents and monkeys also influence the development of maladaptive responses affecting the hypothalamic-pituitary-adrenal (HPA) axis via endocrine hormones (e.g. cortisol) and/or sympathetic neurotransmitters (e.g. adrenaline) that ultimately affect behavior into adulthood (Figure 9). The HPA axis is sensitized due to over secretion of corticotropin-releasing factor (CRF) from the hypothalamus that stimulates the release of adrenocorticotropin hormone (ACTH) from the pituitary that then increases the concentration of cortisol.



**Figure 9.** The hypothalamic-pituitary-adrenal (HPA) axis is dysregulated in MDD and anxiety.

In this context, I will review (1) the effects of environment, in particular early-life stress, focusing on the pioneering works of Michael Meaney in rats and Harry Harlow in monkeys and (2) the effects of genetic vulnerability, in particular the Flinders Sensitive Line (FSL)—a rat model of depression.

### 1.3 Environmental model of depression in rats and monkeys

In rats, differences in maternal care or separation during early-life period alter neuroendocrine responses and ultimately behavior into adulthood. The pioneering works of Michael Meaney demonstrated that rats have distinctive patterns of maternal behaviors such as licking/grooming (LG) and arched-back nursing (ABN), and that the correlation between LG and ABN is > 90 %, i.e. mother rats with high LG behavior also have high ABN behavior. Such behaviors of maternal care not only modulate stress reactivity in offspring but these traits are transmitted across generations. Predictably, offspring from high LG-ABN female rats (i.e. good mothers) have lower levels of plasma ACTH and corticosterone in response to acute stress compared to offspring from low LG-ABN female rats (i.e. bad mothers). Moreover, offspring from good mothers exhibit greater novelty-seeking behaviors. Interestingly, when biological offspring are cross-fostered (i.e. offspring of bad mothers reared by good mothers and *vice versa*), the behavioral phenotype of those biological offspring from bad mothers are affected such that these cross-fostered offspring of bad mothers but reared by good mothers show similar traits (e.g. greater novelty-seeking behaviors) to that of the control group. Intriguingly, the offspring from good mothers exhibit resilience and show similar behavioral reactivity regardless of their caretaker underscoring the importance of ‘good’ genes. Furthermore, as adults, the female offspring of bad mothers that were cross-fostered inherit the high LG-ABN trait and pass it along to



their offspring. Such a phenomenon underscores the importance of epigenetics and also the preferential transmittance of ‘good’ traits across generations that allow for higher fitness.

Although post-handling of pups and maternal separation for up to 15 min increases resilience, longer periods of separation (e.g. 180 min/day) especially from PND2-14 markedly increases susceptibility to maladaptive stress reactivity. In fact, such maternally-separated (MS) rats, regardless of their biological mothers or mother-type, exhibit increased HPA response to stress and decreased novelty-seeking behaviors. Such an environmental manipulation of maternal separation, similar to the offspring raised by bad mothers, induces irreversible, maladaptive changes in the neuroendocrine responses affecting emotional behaviors. Furthermore, early-life stress exacerbates the traits of even good mothers towards their offspring. As such, maternally-separated pups endure sustained maladaptive changes that result in abnormal behaviors into adulthood.

In monkeys, maternal separation as a paradigm for early-life stress induces long-lasting changes in behaviors, which are akin to that seen in depressed humans. Harry Harlow pioneered the science of primate affection in the 1950s. In his seminal paper—*Nature of Love*—he highlighted his lifelong work on the striking behavioral abnormalities resulting from maternal separation to that in humans as well as the importance of affection in rhesus monkeys (Harlow and Suomi, 1970). Harlow’s research, which has been furthered by several others, effectively demonstrates that baby monkeys who are permanently separated, at birth, from their mothers develop persistent signs of depressive behaviors such as non-social, reduced exploration, impulsivity, shyness, alterations in sleep-wake cycles, and even proneness to binge drinking. Such behaviors are negatively correlated with 5-HIAA levels in the CSF suggesting low 5-HT levels in the MS group (Higley et al., 1992; Ichise et al., 2006).

### **1.3.1 Genetic model of depression in rats**

Several genetic models of depression exist in rats. Herein, I use the word ‘genetic’ loosely since the suggestion that these are genetic models is overly simplified. The four commonly used rodent models of depression are the Flinders Sensitive Line (FSL), the Wistar Kyoto (WKY), the fawn-hooded (FH), and the learned helpless (LH). Of these, I’ll only briefly touch on the three of the four models before focusing on the FSL rats. Briefly, the WKY rat was originally bred as the control group for the spontaneous hypertensive rats. Later, the WKY rat was found to exhibit several depressive-like phenotypes, including increased immobility and reduced exploratory behaviors. The FH rat exhibit both exaggerated immobility as well as elevated alcoholic intake and may be a comorbid phenotype of depression and alcoholism. The LH rat was selectively bred over several generations and exhibit enhanced freezing-like behavior when subjected to stress as well as anhedonia-like behavior (e.g. reduced sucrose preference).

The FSL rat is a well-established genetic rat model of depression that meets face-, construct-, and predictive-validity measures (Overstreet and Wegener, 2013). The FSL strain was derived from Sprague-Dawley (SD) rats through a selective breeding program for increased sensitivity to the anticholinesterase compound di-isopropyl fluorophosphates (DFP). Flinders Resistant Line (FRL)—the control counterpart—exhibit low sensitivity to DFP. Similar to FSL rats, human depressives show greater sensitivity to cholinergic agents.

Notably, several neurochemical and behavioral studies comparing FSL and FRL rats have reported differences in 5-HT levels and neurochemistry, antidepressant effects, and depression-like phenotypes (Carboni et al., 2010; El Khoury et al., 2006; Hasegawa et al., 2006; Kovacevic et al., 2010; Petersen et al., 2009; Petersen et al., 2008; Piubelli et al., 2011a; Piubelli et al., 2011b). In particular, the FSL rat exhibit exaggerated immobility, elevated rapid eye moment sleep, and greater behavioral inhibition in response to stressful stimuli.

Despite FSL and other rodent models of depression and/or anxiety meet several key validity measures, several limitations exist in all of these models. The primary limitation is the translational aspect of behavioral measures and treatments in animals to humans stemming from species (intra and inter) differences and the complexity of psychiatric conditions. Therefore, results from these animal models of depression should be cautiously interpreted.

### **1.3.2 Gene-Environment (GxE) interaction**

Several studies in humans, monkeys, and rodents have examined the GxE interaction. In humans, the most influential GxE study suggests that 5-HTTLPR—a polymorphism in the promoter region of the SERT gene—moderates the relationship between environmental stress and mood disorders (Caspi et al., 2010). Briefly, 5-HTTLPR is a functional polymorphism consisting of either the L (long) or the S (short) allele. Compared to the ‘L’ allele, the ‘S’ allele is, overall, less functional with reduced transcriptional activity. Although controversial, the study by Caspi et al., reported a positive relationship between number of self-reported early-life stressors (e.g. childhood maltreatment) and depression risk among individuals who had one or two copies of the ‘S’ allele compared with those homozygous for the ‘L’ allele. However, more than 50 replication attempts, as well as three meta-analyses, have yielded inconsistent findings (Duncan and Keller, 2011). In monkeys, GxE studies have been relatively sparse because of higher costs as well as increased longevity compared to rodents. Similar to humans, rhesus monkeys have L and S alleles orthologous to the human 5HTTLPR. Preliminary studies suggest that, unlike in humans, the effects of rhesus orthologue 5HTTLPR (or rh5HTTLPR) and stressful environment is independent of each other. Each variable, however, has been shown to affect both behaviors as well as neurochemistry (Jedema et al., 2010).

In rodents, behavioral studies, especially in the Flinders strain demonstrate that early-life stress exacerbates depressive-like phenotypes (e.g. immobility) in FSL rats compared to FRL rats (El Khoury et al., 2006; Petersen et al., 2008; Piubelli et al., 2011b). In addition, behavioral studies show complex interactions between genotype, environment, and antidepressant response (El Khoury et al., 2006; Petersen et al., 2009; Petersen et al., 2008; Piubelli et al., 2011a; Piubelli et al., 2011b). For instance, chronic escitalopram decreases immobility only in normally-reared (NR) rats regardless of strain (Piubelli et al., 2011b). On the other hand, chronic nortriptyline decreases immobility only in FSL rats, regardless of rearing (Petersen et al., 2009; Piubelli et al., 2011a). To extrapolate these findings, the development of psychopathology in adult life may be dictated by both genetic and early-life experiences, and antidepressant medications may selectively reverse behavioral abnormalities in the vulnerable group.

To date, few, if any, GxE studies in animal models have examined the effects of different classes of antidepressants (e.g. SSRIs, TCAs) on the serotonergic neurochemistry. Such a study wherein the effects of antidepressants on the serotonergic system (e.g. 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors) are assessed in a GxE model would be of importance to further understand the variability in response to antidepressants. In order to investigate this issue of response variability, *Paper II* examined the antidepressant effects of two agents—escitalopram (an SSRI) and nortriptyline (a TCA)—on 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors in the rat brain using a GxE model.

## 1.4 Serotonergic proteins in MDD and anxiety

Proof-of-concept studies using pharmacology and genetic techniques underscore the importance of the three serotonergic proteins—SERT and 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors—in the pathophysiology and treatment of MDD and anxiety. For instance, drugs selective for each protein as well as gene deletion for each protein affect depressive/anxiety-like behaviors. Because proteins (e.g. receptors) are the ultimate target of chemical neurotransmitters leading to neurotransmission, receptor density techniques such as *in vivo* PET and *in vitro* receptor binding are invaluable in studying both receptor density and function. In the following sections, I will describe (1) receptor density and function using *in vivo* PET and *in vitro* binding and (2) review pharmacological, genetic, and PET studies, which underscore the importance of SERT, 5-HT<sub>1A</sub>, and 5-HT<sub>1B</sub> receptors to the pathophysiology and treatment of MDD and anxiety.

### 1.4.1 Receptor Density and Function

*In vivo* PET and *in vitro* receptor binding—autoradiography and brain homogenate—are two commonly used techniques to study receptor density as well as function. I will first describe PET imaging followed by receptor autoradiography and homogenate binding.

#### ***Positron Emission Tomography (PET) Imaging***

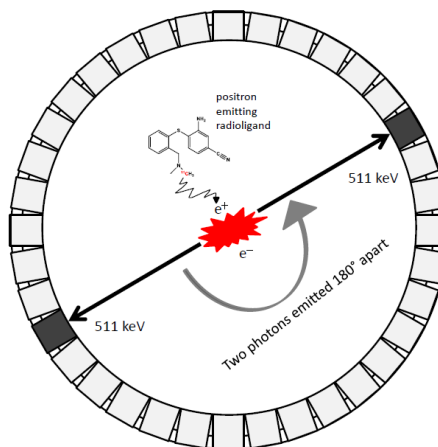
Positron Emission Tomography (PET) imaging provides an unparalleled, state-of-the-art technique to measure physiological functions such as neuroreceptor density and/or function, enzyme activity, and metabolism. Although PET is commonly associated with oncology, its two unique and most important properties—high selectivity (binding to a specific target) and high sensitivity (detecting concentration up to pM)—have appealed to both academia and industry for studying brain diseases as well as facilitating drug development.

PET imaging involves four key steps: (1) a cyclotron produces short half-lived ( $t_{1/2}$ ), positron (an electron with a positive charge) emitting radioisotopes such as <sup>15</sup>O ( $t_{1/2} = 2$  min), <sup>13</sup>N (10 min), <sup>11</sup>C (20 min), and <sup>18</sup>F (110 min); (2) these radioisotopes are rapidly incorporated, with high specific activity and radiochemical purity, into a drug (now referred to as a radioligand) having the desirable affinity, usually in the subnanomolar range, for the target of interest; (3) radioligands are administered intravenously (i.v) at tracer doses as either bolus or bolus plus constant infusion; and (4) a dynamic PET scan is acquired for the duration until optimal kinetic parameters are met, including an identifiable washout.

#### ***PET: Physics, Image Quantification and Outcome Measures***

PET is based on the physics of coincidence events of photon pairs detected by the camera (Figure 11). After injection, a PET radioligand travels to its target of interest through the blood vessels. A positron is emitted and collides with an electron. This collision, in accordance with Einstein's mass-energy conversion, results in two back-to-back photons of 511 keV, 180° opposite to each other. A PET camera's scintillation detectors (e.g. BGO, LYSO) measure random coincidence events of millions of these photon pairs. Localization of the radioligand in the target area can be obtained by reconstructing these coincidence

events along the lines of response. The first PET camera consisted of only a single pair of detectors, and had a spatial resolution of >20 mm (Brownell et al., 1974). Over the past 50 years, significant improvements in both spatial and temporal resolutions have been achieved in human (e.g. HRRT) as well as animal (e.g. microPET) PET cameras (Cherry, 2006). Current PET cameras consist of several thousands of scintillation detectors with a spatial resolution of ~1 mm full width at half maximum (FWHM), and a temporal resolution of <10 sec. Because only a variable fraction of the photon pairs exits the brain, attenuation correction factors derived from a transmission scan is applied during the reconstruction. The two most commonly used reconstruction algorithms to dynamic PET images are filtered backprojection (FBP) and iterative ordered subset expectation-maximization (OSEM).



**Figure 11.** A radioligand (e.g. <sup>11</sup>C-DASB) containing a short half-lived radioisotope emits a positron, which travels a very short distance before colliding with an electron to convert mass into energy in accordance with Einstein’s mass-energy equation ( $E = mc^2$ ) creating a photon pair at an angle of 180° to one another. A PET camera contains scintillation detectors, which capture and measure the random coincidence events of millions of these photon pairs.

After reconstruction, tracer kinetic modeling is applied to the dynamic images for quantification. The tracer kinetic modeling is based on two key assumptions: (1) PET radioligand results in no pharmacological effects because the radioligand occupies < 10% of the target receptor population with an injected mass dose of <1 ng/kg, which is typically < 1% of an endogenous neurotransmitter; and (2) steady state is assumed such that there is no change in either receptor number or blood perfusion.

The two commonly used outcome measures for PET kinetic modeling are binding potential ( $BP$ ) and volume of distribution ( $V_T$ ) (Equations 1&2; Figure 12A) (Innis et al., 2007).  $BP$  is the ratio, at equilibrium, of specific binding to a reference concentration, which contains non-displaceable (or non-specific) binding. Volume of distribution is the ratio, at equilibrium, of the binding in tissue to that in plasma. Mathematically,  $BP$  is obtained from  $V_T$  in regions with and without receptor and is commonly expressed as the product of receptor density and affinity (Equation X). Since affinity is assumed to be constant, any change in  $BP$  reflects change in receptor density and/or function.

$$BP = B_{\max} \times \frac{1}{K_D} = B_{\max} \times \text{affinity} = \frac{B}{F} \quad (\text{Eq. 1})$$

$$V_T = \frac{C_T}{C_P} = V_{FT} + V_{NS} + V_S = V_{ND} + V_S \quad (\text{Eq. 2})$$

**Figure 12A.** The two commonly used outcomes measures are  $BP$  and  $V_T$ .

Binding potential is commonly normalized to one of the three distinct reference concentrations that results in three *BPs* (Equations 3-5; Figure 12B):  $BP_F$  (normalized to free plasma concentration),  $BP_P$  (normalized to total plasma concentration), and  $BP_{ND}$  (normalized to reference region devoid of target receptor in brain).

$$BP_{ND} = f_{ND} B_{avail} \times \frac{1}{K_D} = \frac{(V_T - VND)}{V_{ND}} = \frac{V_S}{V_{ND}} = \frac{k_3}{k_4} \quad (\text{Eq. 3})$$

$$BP_F = B_{avail} \times \frac{1}{K_D} = \frac{(V_T - VND)}{f_P} = \frac{V_S}{f_P} = \frac{K_1 k_3}{f_P k_2 k_4} \quad (\text{Eq. 4})$$

$$BP_P = f_P B_{avail} \times \frac{1}{K_D} = (V_T - VND) = V_S = \frac{K_1 k_3}{k_2 k_4} \quad (\text{Eq. 5})$$

**Figure 12B.** Binding Potential is further divided into three subtypes depending on the reference concentration.

Importantly, accuracy of *BP* depends heavily on the accuracy of the denominator (i.e., the reference concentration). Normalizing to free radioligand in plasma ( $BP_F$ ) is considered the ‘gold standard’ because only the free radioligand in plasma can presumably enter the brain. However, this method requires measuring the parent (non-metabolized) radioligand concentration in plasma as well as protein binding; both measurements are inherently imprecise. In addition, establishing an arterial line is technically challenging and risky. The most convenient outcome measure is specific binding normalized to reference region devoid of target receptor in brain ( $BP_{ND}$ ) because it obviates the need for arterial blood samples. However, inconsistency in measuring reference concentration may lead to discrepant findings as discussed under “*PET imaging of 5-HT<sub>1A</sub> receptor in MDD.*”

Besides the limitation of accuracy in measuring the reference concentration(s), several methodological and technical limitations of PET imaging exist. Some of the methodological limitations include low image resolution, radiometabolite contamination, and partial volume effect. Technical limitations are primarily the scarcity and high cost of suitable radioligands. With regard to the scarcity of selective radioligands, many targets of interest, including the high-affinity state of the 5-HT<sub>1A</sub> receptor, remains to be studied. A suitable brain PET radioligand requires several stringent criteria, including desirable characteristics such as high brain penetrance, high affinity and selectivity to the target, optimal washout, appropriate lipophilicity, low plasma protein binding, and lack of radiometabolite accumulation in brain (Pike, 2009). With regard to its high cost, PET radioligands generally tend to have short half-lived radioisotopes, which necessitate the presence of an on-site medical cyclotron and the orchestration of a multidisciplinary team of experts, including a team of medicinal and radiochemists.

#### ***In vitro autoradiography and homogenate binding***

Autoradiography and homogenate binding (also referred to as the ‘bind and grind’) use radioisotopes that have much longer half-life than in PET such as <sup>3</sup>H ( $t_{1/2} = 12$  years), <sup>125</sup>I (60 days), and <sup>35</sup>S (87 days). While brain tissues are cut and sections mounted on slides for autoradiography, brain tissues are homogenized for homogenate binding (*detailed descriptions for either technique is given in the Methods section*). A major advantage of

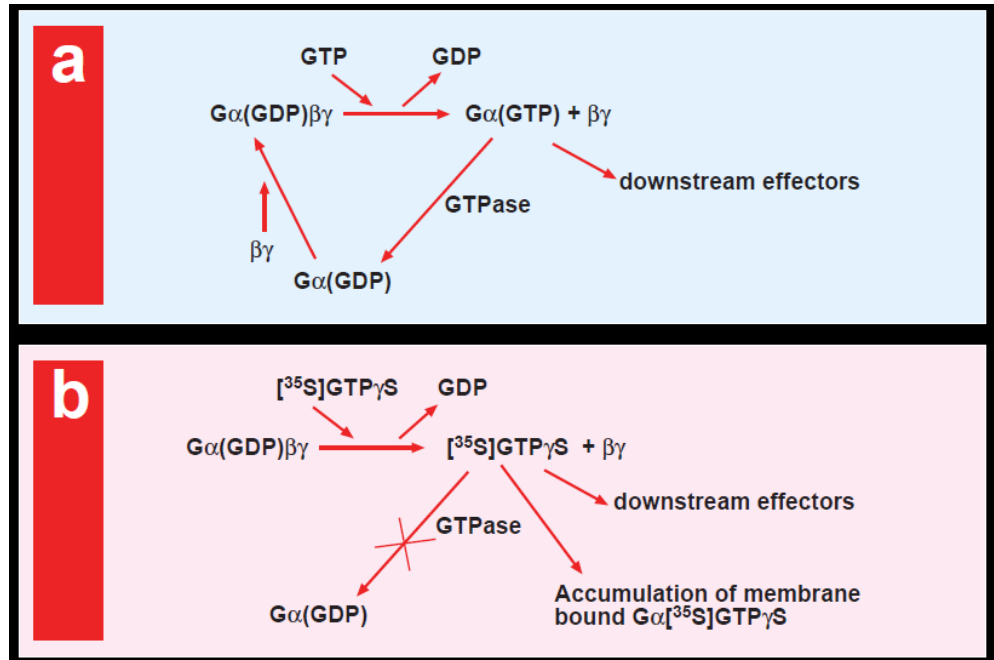
autoradiography is visualizing the distribution of target receptors; homogenate binding may have greater sensitivity because radioligands have access to a larger receptor pool.

$$B = \frac{B_{\max} F}{K_D + F} \quad (\text{Eq. 6})$$

$$BP = \frac{B}{F} = \frac{B_{\max}}{K_D} \quad (\text{Eq. 7})$$

**Figure 13.** The Michaelis-Menten equation 6 describes the receptor bound, *in vitro*, under equilibrium conditions. When the concentration of the free radioligand (F) is low such that  $F \ll K_D$ , the equation reduces to equation 7.  $K_D$  is the dissociation constant using homologous displacement assay.  $K_i$  is the inhibition constant using homologous displacement assay.

Both techniques provide information on receptor density, affinity, and function. Receptor density ( $B_{\max}$ ) is obtained by measuring radioligand binding, at equilibrium, to target receptors at different concentrations of ‘cold’ displacer. Michaelis-Menten equation is typically used to configure density (Equations 6&7; Figure 13). If the displacer is a cold analog of the radioligand (also called homologous displacement) we get dissociation constant ( $K_D$ ) and if not (also called heterologous displacement) then we get inhibition constant ( $K_i$ ). The receptor function is typically obtained by stimulating receptor with an agonist, and measuring per cent stimulation, which is reflected by changes in the radioligand binding. [<sup>35</sup>S]GTPγS, which has a non-hydrolyzable GTP, is commonly used to measure GPCR function. Since a GPCR agonist triggers exchange of GDP for GTP during each stimulation cycle, the [<sup>35</sup>S]GTPγS, which is an analog of non-radiolabeled GTP, accumulates, and provides an indirect measurement on receptor function (Figure 14).



**Figure 14.** Overview of the principles of GTP $\gamma$ S assay. a) Agonist binding triggers exchange of GTP for GDP on  $G_\alpha$  protein subunit;  $G_\alpha$  protein and  $G_{\beta\gamma}$  subunits regulate signal transduction. Normally, GTPase hydrolyzes GTP to GDP, which then allows for the  $G_\alpha$  protein and  $G_{\beta\gamma}$  subunits to reunite and signaling is terminated. b) In the presence of the [ $^{35}$ S]GTP $\gamma$ S, exchange of [ $^{35}$ S]GTP $\gamma$ S for GDP also occurs; however, [ $^{35}$ S]GTP $\gamma$ S is non-hydrolyzable and accumulates during the assay period thereby providing a measure of receptor function



## **1.5 Pharmacological, genetic, and PET studies**

Below, I will review the pharmacological, genetic, and PET proof-of-concept studies highlighting the role of (1) SERT, (2) 5-HT<sub>1A</sub> receptors, and (3) 5-HT<sub>1B</sub> receptors in MDD and anxiety.

### **1.5.1 Serotonin Transporter (SERT)**

As previously described, SERT is the primary target of SSRIs, and its adaptive changes in both density and function is important for normalizing serotonergic neurotransmission.

#### ***Pharmacological drugs targeting SERT***

In addition to SSRIs, other classes of drugs such as the TCAs and SNRIs (serotonin norepinephrine reuptake inhibitors or “dual-action agents”) also block the reuptake function of SERT, thereby increasing synaptic 5-HT levels. TCAs (e.g. nortriptyline, imipramine) and SNRIs (e.g. venlafaxine, duloxetine) also block the reuptake function of either norepinephrine and/or dopamine transporter(s). However, unlike TCAs, SNRIs—the newer generation psychotropic drugs—do not target either cholinergic, histaminergic, or  $\alpha$ -adrenergic system, and avoid some of the adverse side effects such as dry mouth, tiredness, and dizziness. In addition, SNRIs demonstrate similar efficacy to SSRIs in the treatment of mood and anxiety disorders.

#### ***SERT gene: Genetic modulation and polymorphism***

SERT knockout (-/-) mice display abnormal phenotypes such as increased depressive and anxiety traits that persist into adulthood. SSRI treatment fails to reverse these abnormalities demonstrating that SERT is necessary for the therapeutic effect of SSRIs (Fox et al., 2007). Throughout life, SERT<sup>-/-</sup> mice have excess extrasynaptic 5-HT levels whose lifelong exposure may impair synaptic wiring and function, especially during active brain development. This may alter behaviors permanently. The heterozygote (-/+) mice, which may better reflect SERT malfunction, also have similar extrasynaptic 5-HT levels and behavioral abnormalities to that of the homozygotes. However, these two genetically modified mice show altered responses to 5-hydroxytryptamine (5-HTP) (Fox et al., 2008). In humans, several polymorphisms exist in the SERT gene (*SLC6A4*). Although 5HTTLPR is the most investigated genetic variant in mood disorders, several other additional functional variants such as rs25531, rs25532, and Stin2 exist (Murphy et al., 2013). A significant ethnic variability exists in 5HTTLPR such that the frequency of ‘S’ allele is lowest in individuals of African descent and highest in American Indians (Hu et al., 2006). Individuals with the ‘S’ allele are typically more vulnerable to psychiatric-related disorders. This echoes findings in non-human primates where the genetic variant significantly impacts cognitive function as well as gray matter morphology (Jedema et al., 2010; Lesch et al., 1996). A recent meta-analysis reported significant association between 5HTTLPR variants with SSRI remission in Caucasians (Porcelli et al., 2012). Additionally, several studies, to date, have examined 5HTTLPR’s interaction with the environment.

#### ***PET Imaging of SERT in mood disorders***

Several PET studies using the PET radioligand [<sup>11</sup>C]DASB, selective for SERT, have examined serotonergic function in mood-related disorders in monkeys and humans. In monkeys, two studies—both with limited sample sizes—found contrasting effects on SERT neurochemistry. The first study reported that monkeys with a history of early-life stress had lower SERT binding (Ichise et al., 2006). As a model of early-life stress, monkeys were maternally separated (MS, n=9) at birth; their counterparts were normally reared (NR, n=7) with their mothers. This study examined ~ 3.5 years old monkeys (early adolescent period) and reported that the MS group had lower SERT binding in brain regions, including raphe and temporal cortex. Additionally, a positive linear correlation was found between CSF 5-HIAA levels and SERT binding in the MS group, indicating lower synaptic 5-HT levels. In contrast, the second study, which used a similar early-life stress paradigm in rhesus monkeys, did not replicate this finding (Jedema et al., 2010). The study found no difference in SERT binding between the two groups. This discrepancy may be attributable to the older cohort age ~6.5 years (adulthood period) and a much smaller sample size (n=4).

In humans, four studies examining psychiatric disorders found either increased or no change in SERT binding. The first study reported higher SERT binding in striatum and midbrain in patients with depression (Cannon et al., 2007). The second study reported higher SERT binding, globally, in depressed HIV+ patients compared to their non-depressed counterpart; however, compared to healthy controls, HIV+ patients, regardless of depression, had lower binding, especially in insula (Hammoud et al., 2010). The third study reported a group-by-gender interaction; only males with panic disorder had higher SERT binding in anterior cingulate and midbrain (Cannon et al., 2013). In contrast, the fourth study found no difference in SERT binding in aggressive vs. non-aggressive alcoholic patients (Brown et al., 2007).

### **1.5.2 Serotonin 1A (5-HT<sub>1A</sub>) receptor**

Of the 16 different 5-HT receptor subtypes, evidence for the involvement of the 5-HT<sub>1A</sub> receptor in MDD and anxiety is perhaps the most extensive and best characterized (Shrestha et al., 2012 893).

#### ***Pharmacological drugs targeting 5-HT<sub>1A</sub> receptors***

In contrast to SSRIs, which indirectly target and desensitize somatodendritic 5-HT<sub>1A</sub> autoreceptors, several drugs directly target 5-HT<sub>1A</sub> auto- and heteroreceptors. Pindolol, buspirone, gepirone, and tandospirone are four drugs with subnanomolar affinity for 5-HT<sub>1A</sub> receptors and are primarily prescribed as anxiolytics (Celada et al., 2013). These drugs, similar to SSRIs, also exhibit a delayed onset of action, which is postulated to be the time necessary for adaptive changes at the level of neuronal and receptor functions. Pindolol and buspirone are also commonly prescribed as an adjunct for treating SSRI-resistant MDD. Pindolol acts as a partial agonist at both auto- and heteroreceptors; however, it exhibits greater affinity for the autoreceptors. Buspirone, gepirone, and tandospirone belong to the azapirone family, and are full agonists at autoreceptors and partial agonists at heteroreceptors. Gepirone has the advantage of a controlled-release formulation and may allow for increased tolerance.

#### ***5-HT<sub>1A</sub> receptor genetics***

Genetic modulation of 5-HT<sub>1A</sub> receptors affects both receptor function and behavior in age- as well as region-dependent fashion. In mice, 5-HT<sub>1A</sub> receptor knockout (-/-) increases anxiety-like behaviors and exhibit lack of DPAT-mediated hypothermic responses. In contrast, genetic overexpression of 5-HT<sub>1A</sub> receptors during the early-life period reduces anxiety-like behaviors (Kusserow et al., 2004). Furthermore, selective inactivation of only post-synaptic 5-HT<sub>1A</sub> heteroreceptors in PFC, during development, increases anxiety-like behaviors. In mice, altering the 5-HT<sub>1A</sub> autoreceptor density by  $\pm 30\%$  in the dorsal raphe, again only during development, has opposing effects on both behavior and response to SSRIs (Shrestha et al., 2012).

Of the genetic polymorphisms in the 5-HT<sub>1A</sub> gene, *rs6295* (1019C/G), which is a SNP located on the promoter region, is the most well studied. *rs6295* regulates gene expression that ultimately affect the pathophysiology and treatment of MDD (Fabbri et al., 2013). The G allele—the risk variant— is postulated to impair repression of the gene via the transcription factor—nuclear deformed epidermal autoregulatory factor (NUDR/Deaf1). As such, individuals with the G allele have higher density of 5-HT<sub>1A</sub> autoreceptors. In fact, a gene-dose effect was recently demonstrated whereby the 5-HT<sub>1A</sub> autoreceptor density followed the pattern: G/G > C/G > C/C. Because the 5-HT<sub>1A</sub> autoreceptor inhibits 5-HT firing, its upregulation leads to decreased firing and 5-HT release. In addition, the G/G genotype also downregulates post-synaptic 5-HT<sub>1A</sub> heteroreceptors and reduces serotonergic neurotransmission. Studies show that depressed and suicidal patients have higher levels of G(-1019) allele by two- and four-fold, respectively. A recent meta-analysis demonstrated that individuals with the G(-1019) allele have increased susceptibility, although in an ethnic-dependent fashion, to MDD as well as response to SSRIs (Albert and Francois, 2010).

**PET Imaging of 5-HT<sub>1A</sub> receptor in MDD**

Since the initial synthesis and characterization of [*carbonyl*-<sup>11</sup>C]WAY-100635—one of the first PET antagonist radioligands selective for the 5-HT<sub>1A</sub> receptor, several studies have examined this receptor subtype with regard to MDD. Disappointingly, findings are ambiguous showing either increased or decreased or unchanged binding (*Paper IV*). Briefly, five independent groups using eight separate MDD patient cohorts quantified 5-HT<sub>1A</sub> receptors using PET and [*carbonyl*-<sup>11</sup>C]WAY-100635. Drevets and colleagues (1999) were the first to report globally lower 5-HT<sub>1A</sub> binding in patients with MDD compared to healthy subjects (Drevets et al., 1999). Sargent and colleagues (2000) replicated the finding of lower 5-HT<sub>1A</sub> binding in patients with MDD compared to healthy subjects; furthermore, they found that binding was not affected by antidepressant drug treatment (Sargent et al., 2000). Meltzer and colleagues (2004) later extended these findings to elderly patients with late-life depression, and noted that the most prominent decrease (about 40%) was found in the raphe nucleus (Meltzer et al., 2004). Hirvonen and colleagues (2008) reported globally decreased binding in drug-naïve patients with MDD (Hirvonen et al., 2008). Mickey and colleagues (2008) reported no differences in 5-HT<sub>1A</sub> binding between patients with MDD and healthy controls (Mickey et al., 2008). In striking contrast to prior studies showing decreased 5-HT<sub>1A</sub> receptor binding, Parsey and colleagues (2006) reported that medication-naïve patients with MDD had increased 5-HT<sub>1A</sub> receptor binding, although binding in previously medicated patients was the same as in healthy subjects (Kumar et al., 2006). This finding was recently replicated by the same group (Parsey et al., 2010). However, the field needs to enter a more collaborative phase in order to reach a consensus on 5-HT<sub>1A</sub> receptor imaging in MDD as recommended in *Paper IV*.

### **1.5.3 Serotonin 1B (5-HT<sub>1B</sub>) receptor**

The 5-HT<sub>1B</sub> receptor is homologous in structure and function to the 5-HT<sub>1A</sub> receptor and similarly acts as both an auto- and heteroreceptor. Unlike somatodendritic 5-HT<sub>1A</sub> autoreceptors, 5-HT<sub>1B</sub> autoreceptors are located primarily on the axon terminals where they regulate 5-HT release and firing through a negative-feedback system. Unlike 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub> mRNA and protein are not co-expressed. While the 5-HT<sub>1B</sub> mRNA is mostly expressed in the caudate-putamen, the 5-HT<sub>1B</sub> receptor is expressed on both serotonergic (e.g. raphe) and non-serotonergic neurons (e.g. hippocampus, globus pallidus and substantia nigra). Due to less selective radioligands and an ongoing debate about its classification, the 5-HT<sub>1B</sub> receptor is not as extensively studied.

#### ***5-HT<sub>1B</sub> receptor as a target of psychopharmacological drugs***

Pharmacological activation of 5-HT<sub>1B</sub> receptors inhibits 5-HT release and firing. It also modulates activities of other serotonergic receptors (e.g. 5-HT<sub>2B</sub>) and neurotransmitters (e.g. dopamine, GABA ( $\gamma$ -aminobutyric acid)). Similar to 5-HT<sub>1A</sub> autoreceptors, desensitization of 5-HT<sub>1B</sub> receptors is necessary for the delayed therapeutic onset of SSRIs and is a potential pharmacological target in the treatment of MDD and anxiety. However, agonists and antagonists for 5-HT<sub>1B</sub> receptors have yielded mixed results stemming most likely from non-selectivity pharmacological profiles of the currently available drugs.

#### ***5-HT<sub>1B</sub> receptor genetics***

Mice lacking the 5-HT<sub>1B</sub> receptor (-/-) show altered behavioral responses, including increased aggression, anxiety, impulsivity, and addiction (Ruf and Bhagwagar, 2009). In rats, downregulation of 5-HT<sub>1B</sub> mRNA in the raphe increases stress responses and depressive-like phenotype. An adaptor protein, p11, was reported to be co-expressed with 5-HT<sub>1B</sub> receptors and regulate the trafficking of the receptor onto the plasma membrane. In mice, p11 deletion lowered expression of plasma 5-HT<sub>1B</sub> receptors and increase depressive-like behaviors (Svenningsson et al., 2006). Importantly, site-directed deletion of p11 in the nucleus accumbens sufficed to trigger depressive-like phenotype; restoring p11 expression reversed this effect (Alexander et al., 2010). Several nonfunctional SNPs including C291 or G861 have also been reported; however, individuals with these SNPs show lower expression of 5-HT<sub>1B</sub> receptors and increased susceptibility to neuropsychiatric disorders. Postmortem studies in suicides show decreased expression of both 5-HT<sub>1B</sub> mRNA and protein (Anisman et al., 2008).

#### ***PET Imaging of 5-HT<sub>1B</sub> receptor in mood disorders***

The two PET radioligands— [<sup>11</sup>C]P943 and [<sup>11</sup>C]AZ10419369—both antagonists and selective for the 5-HT<sub>1B</sub> receptor, were recently developed. To date, four studies have examined the 5-HT<sub>1B</sub> receptor in neuropsychiatric disorders using [<sup>11</sup>C]P943. Two studies in patients with MDD and early traumatic experiences report decreased 5-HT<sub>1B</sub> receptor binding, although regional differences between the two sub-population groups were noted. In patients with MDD, the decrease was primarily in the ventral striatum/ palladium; in patients with early traumatic experiences, the decrease was primarily in limbic regions. In contrast to these two studies, in patients with alcoholism, the 5-HT<sub>1B</sub> receptor binding was increased in ventral striatum. In healthy subjects, a negative correlation of 5-HT<sub>1B</sub> binding was seen (Murrrough and Neumeister, 2011).

## 1.6 The 5-HT<sub>1A</sub> receptor as a potential biomarker for neuropsychiatric disorders

Currently, no suitable biomarker exists for neuropsychiatric disorders such as MDD and anxiety. The 5-HT<sub>1A</sub> receptor has garnered much attention as a potential biomarker for diagnosis and treatment of MDD. This is largely attributable to the availability of DPAT—a 5-HT<sub>1A</sub> receptor agonist—synthesized in the early 1990s, and underscored this receptor subtype in both pathophysiology as well as treatment of MDD. Since PET allows for *in vivo* imaging of the living brain, several PET radioligands were generated; the five most common are [*carbonyl*-<sup>11</sup>C]WAY-100635, [<sup>11</sup>C]RWAY, [<sup>18</sup>F]FCWAY, [<sup>18</sup>F]mefWAY, and [<sup>18</sup>F]MPPF (Paterson et al., 2013). However, all are antagonists and, as previously mentioned, do not discriminate between the active and inactive states of the receptor. This is particularly relevant to the search for biomarker candidates because the active states to which an agonist binds may be primarily affected in disease conditions including MDD. In addition, an agonist radioligand might be more sensitive to detecting endogenous fluctuations in extrasynaptic 5-HT levels. Thus, an agonist PET radioligand, which may demonstrate greater sensitivity to detecting both receptor density as well as sensitivity to changes in 5-HT levels, is highly desirable.

In 2007, [<sup>11</sup>C]CUMI was reported as the first putative agonist PET radioligand, which was selective for the 5-HT<sub>1A</sub> receptor (Kumar et al., 2007). CUMI-101, similar to DPAT, dose-dependently stimulated [<sup>35</sup>S]GTPγS binding in Chinese Hamster Ovary (CHO) cells expressing the human 5-HT<sub>1A</sub> receptor. CUMI-101 also had subnanomolar affinity for the 5-HT<sub>1A</sub> receptor, and a 45-fold difference in affinity to the next target—the α<sub>1</sub> adrenoceptor. A subsequent study by the same group reported several interesting characteristics of a good PET radioligand such as high brain uptake of [<sup>11</sup>C]CUMI-101, optimal washout, and a plasma free fraction of 60% (Milak et al., 2008). In contrast, a recent study reported that, unlike in recombinant cell lines expressing human 5-HT<sub>1A</sub> receptors, in rat brain tissues, CUMI-101 behaved as a potent 5-HT<sub>1A</sub> receptor antagonist (Hendry et al., 2011). This discrepancy is attributable to species differences and/or methodology—recombinant cell line vs. native tissues, and was investigated in *Paper III*.

The issue of alpha 1 (α<sub>1</sub>) adrenoceptor binding further complicates the picture since CUMI-101 has 0.15 nM (*K<sub>i</sub>*) for 5-HT<sub>1A</sub> receptors and 6.75 nM (*K<sub>i</sub>*) for α<sub>1</sub> adrenoceptors (Kumar et al., 2007). Although a 45-fold difference between these two receptors, this may still not be ‘large’ enough to selectively target only the 5-HT<sub>1A</sub> receptor especially in regions like thalamus, which has moderate 5-HT<sub>1A</sub> receptors but high α<sub>1</sub> adrenoceptors. In addition, studies show that many adrenergic drugs, especially ones targeting α<sub>1</sub> adrenoceptors, have nanomolar affinity for 5-HT<sub>1A</sub> receptors (Chen et al., 2013). Therefore, CUMI-101 may have mild-to-moderate binding, *in vivo*, to α<sub>1</sub> adrenoceptors in the brain.

## 2 AIMS AND HYPOTHESES

My PhD thesis combined both *in vivo* (BrainPET) and *in vitro* binding techniques to examine the serotonergic system and antidepressant effects. My thesis comprised of four interrelated projects (Papers I, II, III, and IV).

1. To assess the effects of long-term SSRI treatment on the developing primate brain, we sought to answer one primary and two secondary questions. First, does long-term, prepubertal fluoxetine treatment have effects on the serotonergic system that are discernible in adulthood? Second, do these effects vary based on rearing conditions, i.e. whether the monkeys were maternally separated or normally reared? Third, does fluoxetine induce any long-lasting behavioral changes into adulthood? To answer these three questions, 32 differentially-reared monkeys received either fluoxetine or placebo from age two to three, which corresponds to the prepubertal period. Monkeys were scanned at ~4.7 years with two serotonergic positron emission tomographic (PET) radioligands: [<sup>11</sup>C]DASB for SERT and [<sup>11</sup>C](R)-RWAY for 5-HT<sub>1A</sub> receptor. (*Paper I*)
2. To examine how antidepressants interact with GxE to influence the serotonergic system that may contribute to variability in antidepressant response, we investigated the neurochemical effects of two antidepressants from different classes—escitalopram (an SSRI) and nortriptyline (a TCA)—in a rat model with genetic (FRL vs. FSL) and/or environmental (normally-reared vs. maternally-separated rats) vulnerabilities. Quantitative *in vitro* autoradiography was done to examine the interactions between 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors in both PFC and hippocampus. The study had three primary hypotheses: 1) both genetic vulnerability (modeled by rat strain) and environmental vulnerability (modeled by maternal separation) affect the serotonergic neurochemistry; 2) a GxE interaction affects serotonergic neurochemistry; and 3) antidepressants would selectively modulate serotonergic neurochemistry in a GxE interaction manner. The three hypotheses were tested within the context of an omnibus statistical model – i.e., a strain-by-stress-by-treatment (three-way) interaction. (*Paper II*)
3. In 2007, [<sup>11</sup>C]CUMI-101 was reported as the first putative agonist PET radioligand selective for the 5-HT<sub>1A</sub> receptor. We sought to answer two key questions regarding the functionality and selectivity of CUMI-101. First, does CUMI-101 behave as an agonist or antagonist? Second, does CUMI-101 demonstrate cross-reactivity with  $\alpha$ 1-adrenoceptors? Herein, cross-reactivity is defined as the non-selective property of CUMI-101 to specifically bind to  $\alpha$ 1-adrenoceptors in addition to its intended target—5-HT<sub>1A</sub> receptors. To address the first question, functional assays were performed using [<sup>35</sup>S]GTP $\gamma$ S in brain homogenates of rat, monkey, and human. To assess whether CUMI-101 binds to  $\alpha$ 1-adrenoceptors, both *in vitro* radioligand binding and *in vivo* PET imaging studies were done in rodent and primate brain. (*Paper III*)

*Stal Saurav Shrestha, 2014*

4. The serotonin-1A receptor is of particular interest in human positron emission tomography studies of major depressive disorder. However, to date, studies report inconsistent findings. We, therefore, sought to explain the disparate findings by examining, in total, eight studies. Since only one of the imaging centers acquired all the data necessary to address methodological concerns, we were only able to do a qualitative review study. (*Paper IV*)



### 3 MATERIALS AND METHODS

#### Drugs and radioligands

[<sup>35</sup>S]GTPγS, [<sup>3</sup>H]CUMI-101, [<sup>3</sup>H](±)-8-OH-DPAT, [<sup>3</sup>H]prazosin, [<sup>125</sup>I]MPPI, and [<sup>125</sup>I](±)cyanopindolol were purchased from PerkinElmer (Waltham, MA, USA). (+)-8-OH-DPAT, 5-HT, WAY-100635, GTPγS, prazosin, and isoprenaline were purchased from Sigma (St. Louis, MO, USA; Stockholm, Sweden). CUMI-101 was purchased from Alpha Biopharmaceuticals (Burlingame, CA, USA). All other reagents were purchased from Quality Biological (Bethesda, MD, USA). All PET radioligands were synthesized in house.

#### 3.1 Study Designs

In *Paper I*, the two-way interaction (2 × 2 matrix) examining the effects of drug and early-life stress is shown in Figure 8. At birth, 32 male rhesus monkeys (*Macaca mulatta*) from four annual birth cohorts were randomly assigned to maternally-separated or normally-reared conditions – i.e., eight newborns from each of four years were equally divided between the two rearing conditions. Maternal separation consisted of the standard protocol as previously described (Shannon et al., 1998).

	Placebo	Fluoxetine (1 year)
Rearing	8 normally reared	8 normally reared
	8 maternally separated	8 maternally separated

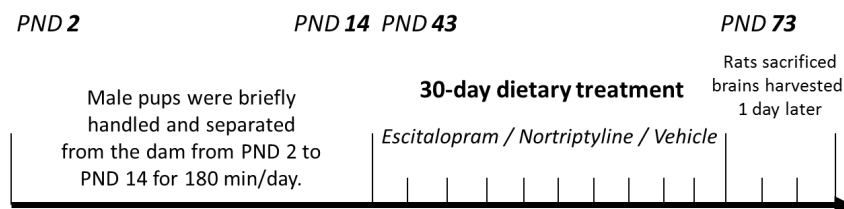
**Figure 8.** A two-by-two matrix study design to examine the interactions of environment and psychotherapeutic drug. In total, 32 monkeys were divided into four groups.

As regards rearing conditions, normally-reared monkeys spent the first six months of life living with their mothers and with other adults and infant pairs from the same birth cohort. In contrast, maternally-separated monkeys were separated under a standard protocol where infants were hand-reared by humans in a nursery within 48 hours of birth until several weeks until they are able to feed themselves (typically four to six weeks) (Nelson and Winslow, 2009; Shannon et al., 1998). Maternally-separated monkeys were then transferred from the nursery and into group housing with three other like-reared, age-matched monkeys from the same birth year cohort. Maternally-separated monkeys remained in this condition until they were approximately six to eight months old, after which all other infants from a given birth year, regardless of rearing, were transferred to a large group housing condition.

Monkeys were then housed in a large indoor (2.5 x 3.0 x 2.5 m) / outdoor (2.5 x 3.0 x 2.5 m) runs that contained one to two males and six to eight females with their current year offspring. As noted above, between six and eight months of age, these monkeys were transferred to a large pen with other maternally-separated and normally-reared monkeys from the same birth year.

At age two, monkeys were removed from group housing and pair-housed with a like-reared cage-mate. One member of each pair received fluoxetine and the other received placebo. The study was approved by the Animal Care and Use Committee of the National Institute of Mental Health.

In *Paper II*, the three-way interaction (2 x 2 x 3 matrix) on the serotonergic system was examined (Figure 10). Maternal separation and antidepressant treatment were performed as previously described (El Khoury et al., 2006; Petersen et al., 2009; Petersen et al., 2008). Briefly, the study used only male rats housed in pairs in an 1800 cm<sup>2</sup> cage under a 12-hour light/dark reverse cycle at 21°C, relative humidity 55%, and food and water *ad libitum*. Post-natal day 0 (PND0) was designated as the day of birth. To model early-life stress, half of the pups were separated from their mothers for 180 min/day for 12 days starting at PND2.



Rats	Flinders Resistant Line (FRL)						Flinders Sensitive Line (FSL)					
Rearing	normally reared			maternally separated			normally reared			maternally separated		
Treatment	VEH	ESC	NTR	VEH	ESC	NTR	VEH	ESC	NTR	VEH	ESC	NTR
# of rats	9	9	9	9	9	9	9	8	8	8	9	9

**Figure 10.** A three-way study design examining strain by stress by treatment (2 x 2 x 3 matrix) interaction on the serotonergic system. In total, 105 rats were divided across 12 experimental groups.

On PND43, equal number of rats in each group was randomly assigned to 30-day dietary supplementation (diet prepared by Lactamin AB, Sweden) with either escitalopram (0.34 g/kg for the first three weeks, 0.41 g/kg during the rest of the experiment) or nortriptyline (0.34 g/kg), or vehicle. The daily administered dose was 25 mg/kg for escitalopram and 20 mg/kg for nortriptyline. Antidepressant serum concentrations were not measured; however, several prior studies found that the selected oral doses provide serum concentrations of 15-35 ng/mL (AAM obtained proprietary results in collaboration with Lundbeck A/S and Pfizer), which are typically used to model therapeutic concentrations in humans. Average food pellet intake during the treatment period was 22 g/rat/day. No difference in food consumption or total brain weight was observed between genotypes. However, a significant weight difference emerged between the strains (FRL = 209±2.4 vs. FSL = 196±2.0).

At the end of the study rats were sacrificed, and brains were immediately harvested and frozen at  $-80\text{ }^{\circ}\text{C}$ . Brain was sectioned ( $14\text{ }\mu\text{m}$ ) using a microtome-cryostat ( $-20\text{ }^{\circ}\text{C}$ ) and mounted on gelatin-coated slides. The Stockholm Ethical Committee for the Protection of Animals approved the study.

## 3.2 PET Radioligands and Imaging

Three PET radioligands: [ $^{11}\text{C}$ ]DASB to label SERT and [ $^{11}\text{C}$ ](R)-RWAY and [ $^{11}\text{C}$ ]CUMI-101 to label 5-HT<sub>1A</sub> receptors were synthesized with high radiochemical purity ( $>99\%$ ), as previously described (Ichise et al., 2006; Kumar et al., 2006; Yasuno et al., 2006). A 90- or 120-minute dynamic PET scan was acquired in rats or monkeys on a Focus 120/220 scanner (Siemens Medical Solutions, Knoxville, TN, USA) as previously described (Liow et al., 2007). For rat imaging, Sprague-Dawley rats or were anesthetized with 1.5% isoflurane and radioligand was injected (i.v.) through a penile vein catheter. Dynamic PET scans were acquired after a bolus injection of radioligand over 30 seconds. For monkey imaging, rhesus monkeys were immobilized with ketamine (10 mg/kg, i.m.) to allow for endotracheal intubation. Monkeys were transported to the PET suite and placed under isoflurane anesthesia (1.5 - 2%) during the entire scan. The head was immobilized in a stereotactic frame, and vital signs were monitored throughout. To minimize the pharmacological effects of ketamine, scans started at least 120 min after ketamine administration. Before radioligand injection, a 10-minute transmission scan was acquired using a  $^{57}\text{Co}$  point source for attenuation correction. Each radioligand was injected as a bolus of 10 mL over one minute via the saphenous or occasionally the cephalic vein. Images were reconstructed using 3D-filtered back-projection and had a resolution of 1.7 mm at full-width half-maximum. Scatter and attenuation corrections were applied. All monkeys also received a T1-weighted spoiled gradient echo (SPGR) magnetic resonance imaging (MRI) scan with a human knee coil on a GE Signa 3T device (GE Healthcare). The dynamic frame sequences for PET imaging were 6 x 20 s, 5 x 60 s, 4 x 120 s, 3 x 300 s, 3 x 600 s, and 2 x 1200 s in rats, and 6 x 30 s, 3 x 60 s, 2 x 120 s, and 16 x 300 s in monkeys. Although PET imaging were performed using all three radioligands in 32 monkeys for our *Paper I*, [ $^{11}\text{C}$ ]CUMI-101 scans were later discarded after our unexpected finding (*Paper III*) that this radioligand was neither an agonist nor selective for the 5-HT<sub>1A</sub> receptor. All PET scans were obtained at the National Institutes of Health and the study was approved by the Animal Care and Use Committee of the National Institute of Mental Health.

In *Paper I*, all 32 monkeys ( $4.7 \pm 0.6$  years and  $7.4 \pm 1.5$  kg; these and subsequent data are expressed as mean  $\pm$  SD) underwent 120-min dynamic scans on a Focus 220. The injected activity, specific activity, and injected mass dose of [ $^{11}\text{C}$ ]DASB and [ $^{11}\text{C}$ ](R)-RWAY were similar for monkeys administered either placebo or fluoxetine (Table X).

For *Paper III*, rodents and monkeys underwent either a 90 or 120-min dynamic scans with [ $^{11}\text{C}$ ]CUMI-101 on a Focus 220/120 camera. For rat imaging, 15 male Sprague-Dawley rats ( $336 \pm 66$  g) were injected (i.v.) through a penile vein catheter ( $29 \pm 6$  MBq; specific activity (SA):  $67 \pm 53$  GBq/ $\mu\text{mol}$ ). Dynamic PET scans were acquired after a bolus injection of [ $^{11}\text{C}$ ]CUMI-101. To determine whether [ $^{11}\text{C}$ ]CUMI-101 binds to 5-HT<sub>1A</sub> and/or  $\alpha 1$ -adrenoceptors, we obtained PET scans under the following five conditions: baseline; pre-

blocking with the 5-HT<sub>1A</sub> receptor antagonist, WAY-100635 (2.0 mg/kg); pre-blocking with the  $\alpha$ 1 adrenoceptor antagonist, prazosin (2.0 mg/kg); pre-blocking with WAY-100635 and prazosin; and self-blocking with CUMI-101 (2.0 mg/kg).

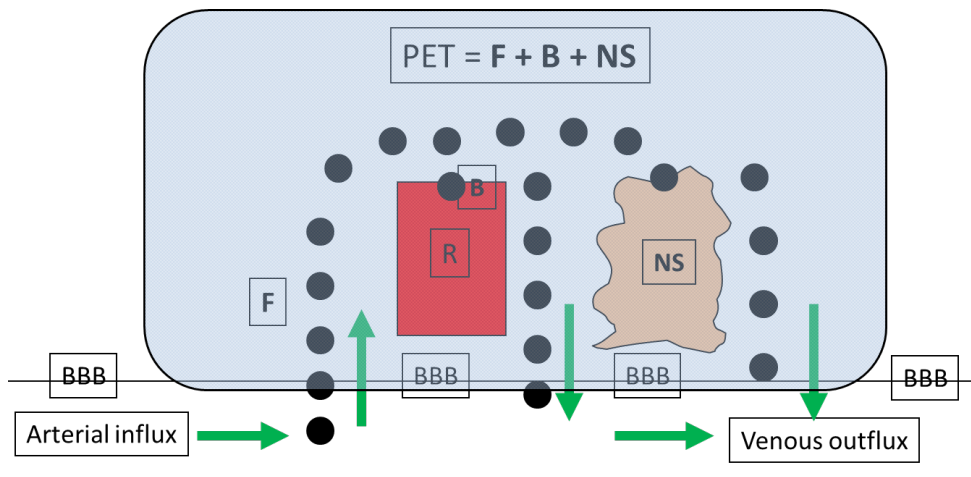
For monkey imaging, five rhesus monkeys ( $8.5 \pm 2.4$  kg) underwent a total of six PET scans with [<sup>11</sup>C]CUMI-101 ( $193 \pm 30$  MBq; SA at time of injection:  $120 \pm 46$  GBq/ $\mu$ mol). Each session consisted of a baseline scan followed by a blocked scan with a three-hour interval to allow for radioactivity decay. The blocked scans were acquired under the following conditions: pre-blocking with WAY-100635 (0.5 mg/kg; n = 3); pre-blocking with prazosin (1.0 mg/kg; n = 2); and pre-blocking with WAY-100635 and prazosin (n = 1). Previously published studies noted that approximately complete receptor occupancy was achieved at these doses (Airaksinen et al., 2013; Kumar et al., 2007). For rats, we used 2.0 mg/kg, which was slightly higher than values reported in the literature to ensure maximum receptor blockade. The estimated baseline occupancy of 5-HT<sub>1A</sub> receptors in hippocampus was ~1.2 % in rats and ~1% in monkeys (Khawaja, 1995; Kohler et al., 1986). Arterial blood samples were obtained in all but pre-blocking with WAY-100635 and prazosin.

Plasma radiometabolites were separated using high-performance liquid chromatography (Zoghbi et al., 2006). Parent plasma concentration was obtained as an input function for compartmental modeling. All pre-blocking agents were administered intravenously 30 min before radioligand injection.

For rats, the brain regions were drawn directly on coronal sections of the summed PET images. For monkeys, dynamic PET images were co-registered using a 12-parameter mutual information algorithm to an MRI template in standardized space where a set of pre-drawn ROIs was applied.

### **3.2.1 PET Pharmacokinetic Modeling and Image Analysis**

PET measures local concentration of radioactivity in tissues. As such specific binding in tissues is affected by blood flow and movement of radioligand between compartments (Figure 15). The basis of modeling assumes that the scan is obtained until equilibrium condition is achieved so that the on-off rate of the radioligand is constant and binding reflects receptor functionality.



**Figure 15.** The radioligand (●) distributes to the brain by passively crossing the blood-brain barrier (BBB). The radioligand in the brain is free (F), specifically bound to the receptor (B), and nonspecifically bound to other binding sites in the brain tissue (NS). These different fractions of the tracer are in equilibrium between each other, and PET measures only the entire activity (F+B+NS) plus a fraction of the blood volume. Modified from Andrea Varrone.

Pharmacokinetic modeling is based on a series of mathematical computation, which divides the radioligand distribution into theoretical compartments. Usually, a one- or two-tissue compartmental (1-TC or 2-TC) modeling is applied for brain quantification. 1-TC modeling requires estimation of two rate constant parameters ( $K_1$ ,  $k_2$ ) while 2-TC modeling requires estimation of four constant parameters ( $K_1$ ,  $k_2$ ,  $k_3$ ,  $k_2$ ). Either 1-TC or 2-TC modeling with arterial input function is regarded as the gold standard for image analysis against which different models including reference tissue are calibrated. Several outcomes measures exist such as binding potential (BP), distribution volume ( $V_T$ ), or distribution volume ratio (DVR) (Figure 12).

In *Paper I*, binding potential ( $BP_{ND}$ ) was calculated with a reference tissue model, using cerebellar white matter excluding the vermis as the reference region for both radioligands. However, the partial volume effect in PET may have resulted in some spillover from adjacent gray matter and vermis.  $BP_{ND}$  was calculated in three steps: (1) co-registering PET and MR images in template space, (2) generating time-activity curves, and (3) performing kinetic analysis. Dynamic PET images were co-registered to averaged MRI templates from six monkeys in standardized space. Time-activity curves were generated using pre-defined regions of interest for both neocortex and hippocampus (Yasuno et al., 2006), and manually for raphe (Kranz et al., 2012). For neocortex, five different cortical regions were combined and weighted for volume: frontal, cingulate, temporal, parietal, and occipital cortices. The raphe (volume = 110 mm<sup>3</sup>) was drawn using a circular region of interest directly on three slices (mid-sagittal and two adjacent) of the summed PET images. The concentration of radioactivity was expressed as standardized uptake value (SUV), a unitless value that is normalized for weight and injected activity. SUV = concentration (kBq/mL) / injected activity (kBq) \* body weight (g). Time-activity curves were obtained and expressed as SUV. Kinetic analyses were performed using the multilinear reference

tissue model with two parameters (MRTM2). MRTM2 requires *a priori* estimation of  $k_2$  of cerebellum ( $k'_2$ ), which is the clearance rate constant from cerebellum relative to a region of specific binding. Using MRTM,  $k'_2$  values were obtained from cerebellum relative to thalamus for [ $^{11}\text{C}$ ]DASB and from cerebellum relative to neocortex for [ $^{11}\text{C}$ ](R)-RWAY. [ $^{11}\text{C}$ ]DASB binding had a good fit with a one-tissue compartment model. [ $^{11}\text{C}$ ](R)-RWAY binding had a good fit with a two-tissue compartment model (Ichise et al., 2003; Yasuno et al., 2006). As such, the start times ( $t^*$ ) were set to 0.25 min for [ $^{11}\text{C}$ ]DASB and 50 min for [ $^{11}\text{C}$ ](R)-RWAY. Parametric images were generated using PMOD 3.0 (PMOD Technologies, Zurich, Switzerland).

In *Paper III*, PET image analyses for monkeys were done as described for *Paper I*. For monkeys,  $V_T$ , which is the ratio of brain uptake to parent plasma, was calculated using a one-tissue compartment model (Innis et al., 2007). Although the two-tissue compartment model had a slightly better fit, a small  $k_4$  affected the stability of  $V_T$  in many regions and limited its use.  $BP_{ND}$  was also calculated using a simplified reference tissue modeling with two parameters (SRTM2) and with cerebellar white matter as the reference region. For rats, the brain regions were drawn directly on coronal sections of the summed PET images.

All image analyses and compartmental modeling were performed with the FSL library (FMRIB Software Library; Oxford, UK) and PMOD 3.1 and 3.3 (PMOD Technologies Ltd, Zurich, Switzerland).

### 3.2.2 Statistical Parametric Mapping (SPM)

Parametric images of the brain provide a major advantage of visually comparing the spatial differences in parameter distribution (e.g. radioligand binding to target of interest) between subject groups. As such, a voxel-wise, whole brain image analysis can be done, allowing to visually localize the statistical effect of either disease or drug. Individual images are first converted into parametric images using either a reference tissue or plasma input compartmental modeling. The parametric images are aligned in standardized space and then analyzed using softwares such as the Statistical Parametric Mapping (SPM).

In *Paper I*, a voxel-wise analysis of the whole brain was performed using SPM8 (Wellcome Trust Centre for Neuroimaging, UK). First, parametric images were generated using MRTM2, smoothed to full-width at half maximum of 4 mm, and analyzed using a factorial design in SPM8. Statistical parametric maps were initially thresholded at uncorrected  $p < 0.05$ , and an exploratory stringent Gaussian random field theory cluster level (i.e. family-wise error) correction for multiple comparisons was applied.

## 3.3 Radioligand Binding Assays

Both *in vitro* homogenate and autoradiography binding assays were done as previously described (Johnson et al., 1990; Kindlundh et al., 2003; Vicentic et al., 2006).

### Homogenate binding

Brain tissue homogenates were prepared from rat, monkey, and human brain using a Polytron Homogenizer (Bethesda, Maryland, USA) in 50 mM Tris-HCl (pH 7.4), and

centrifuged at 25,000g for 30 min at 4 °C. The supernatant was discarded and the pellet was resuspended in the same buffer so that the final concentration was ~100 mg wet tissue per mL. Tissues were stored at –80 °C until the day of the experiment. For *Paper III*, we chose brain regions where CUMI's cross-reactivity could be examined using both *in vivo* and *in vitro* techniques. With regard to 5-HT<sub>1A</sub> receptors, we chose two regions with high density—hippocampus and neocortex. Similarly, with regard to  $\alpha$ 1 adrenoceptors, we chose two regions with high density—neocortex and thalamus. We also included cerebellum because it is commonly used as a reference tissue for 5-HT<sub>1A</sub> receptors.

### 3.3.1 Functional assay

#### Agonist-Stimulated [<sup>35</sup>S]GTP $\gamma$ S Binding

[<sup>35</sup>S]GTP $\gamma$ S binding was carried out in brain homogenates, as previously described with minor modifications (Valdizan et al., 2010). Briefly, brain tissues were thawed on ice and resuspended in binding buffer (50 mM Tris-HCl, 1mM MgCl<sub>2</sub>, 100 mM NaCl, 1mM EGTA, 1 mM DTT, 300  $\mu$ M GDP, and 10 mU/mL adenosine deaminase, pH 7.4). Membrane aliquot (50  $\mu$ g protein) and drugs of interest were added to borosilicate vials. The reaction was initiated by adding 100 pM [<sup>35</sup>S]GTP $\gamma$ S, followed by a 30-min incubation in a light-shielded shaker at 30°C. Finally, reactions were terminated by rapid filtration under vacuum in ice-cold buffer (50 mM Tris-HCl, pH 7.4) through Whatman GF/B glass fiber filters. All assays were performed in triplicate.

### 3.3.2 Receptor Density

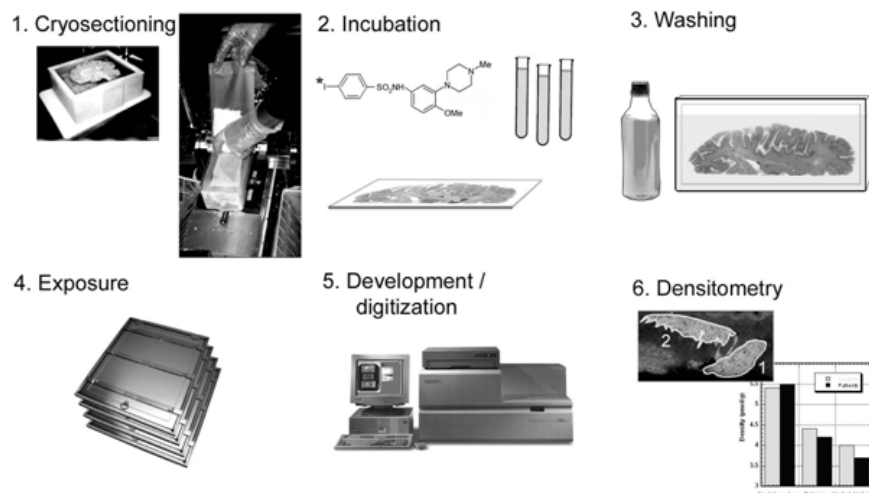
#### [<sup>3</sup>H]Ligand Binding

Radioligand binding assays were done as previously described (Johnson et al., 1990). Briefly, brain tissues were thawed on ice and resuspended in binding buffer (5-HT<sub>1A</sub> receptors: 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA;  $\alpha$ 1 adrenoceptors: 20 mM Tris-HCl, 145 mM NaCl; pH 7.4) to a final concentration of 1 mg wet tissue per milliliter. Radioactivity concentrations for [<sup>3</sup>H]CUMI-101, [<sup>3</sup>H]( $\pm$ )-8-OH-DPAT, and [<sup>3</sup>H]prazosin were in the range of 0.05 – 0.2 nM, so that final concentrations were below their  $K_D$  values (Chamberlain et al., 1993; Daly et al., 1988; Kumar et al., 2007). The following were added sequentially to each borosilicate vial: 100  $\mu$ L of radioligand, 100  $\mu$ L of buffer/displacer (50 nM 8-OH-DPAT or prazosin; this concentration was ~15-150 times the  $K_i$  values for the respective receptor subtype), and 800  $\mu$ L of tissues all mixed in binding buffer solution. This was followed by a 30-min incubation in a light-shielded shaker at either 37°C or 23°C. The affinity of receptors can increase or decrease with temperature. We chose 23°C because it was close to the temperature used for the initial characterization of CUMI-101 (25°C) (Kumar et al., 2007). We chose 37°C because it reflects *in vivo* body temperature. Finally, reactions were terminated by rapid filtration under vacuum in ice-cold binding buffer through Whatman GF/B glass fiber filter (pre-soaked for 30 min in 0.5% polyethyleneimine).

Radioactivity from <sup>35</sup>S and <sup>3</sup>H was measured (five min per vial) in a liquid scintillation Ultima-Gold cocktail (4 mL per vial) in a  $\beta$  counter (PerkinElmer, Illinois, USA). Protein concentration was determined via the Micro BCA™ Protein Assay Kit protocol (Thermo Scientific, California, USA).

### ***In vitro* autoradiography**

[<sup>125</sup>I] autoradiography was performed in *Paper II* as previously described with minor modifications (Figure 16) (Kindlundh-Hogberg et al., 2009; Vicentic et al., 2006). Rat brain sections of the PFC and hippocampus were rinsed (30 min) with 50 mM Tris-HCl at 24 °C. For the 5-HT<sub>1A</sub> receptor, sections were pre-incubated (30 min) in binding buffer (50 mM Tris-HCl and 2 mM MgCl<sub>2</sub>, pH 7.5) followed by two-hour incubation with antagonist [<sup>125</sup>I]MPPI (120 pM; 2200 Ci/mmol) at 24 °C. Non-specific binding was determined in the presence of 10 μM 8-OH-DPAT. Sections were washed 2 x 15 min with ice-cold binding buffer. For the 5-HT<sub>1B</sub> receptor, sections were pre-incubated (30 min) at 24 °C in binding buffer (170 mM Tris-HCl and 150 mM NaCl, pH 7.5) followed by two-hour incubation with antagonist [<sup>125</sup>I]cyanopindolol (12 pM; 2200 Ci/mmol), 30 μM isoprenaline, and 100 nM 8-OH-DPAT to block the β-adrenergic receptor and 5-HT<sub>1A</sub> receptor, respectively. Non-specific binding was determined in the presence of 10 μM 5-HT. Sections were washed 2 x 5 min with ice-cold binding buffer. All sections were quickly dipped in ice-cold distilled water, dried, and exposed, together with <sup>125</sup>I plastic microscale standards (American Radiolabeled Chemicals, Missouri, USA), to Kodak BioMax MR films for 24 hours ([<sup>125</sup>I]MPPI) or 48 hours ([<sup>125</sup>I]cyanopindolol) at 4 °C.



**Figure 16.** Autoradiography is an *in vitro* receptor binding technique commonly used to visualize receptor distribution and measure receptor density. The method typically consists of sectioning tissues using a cryostat followed by incubation with a radioligand. Reactions are terminated by rapid washing in ice-cold buffer. The tissues are exposed to films and then the autoradiograms are developed and quantified. Modified from Katarina Varnäs.

Autoradiograms were quantified using NIH ImageJ (1.44p) (<http://rsbweb.nih.gov/ij/>). Optical densities were normalized per area and converted into fmol/mg tissue based on the microscale standards by using nonlinear regression fit (GraphPAD Prism). Specific binding was determined by subtracting non-specific from total binding. In all graphs, the control group was normally-reared, vehicle-administered FRL rats. All receptor binding data are expressed as percent of control calculated as: receptor density \* 100/average receptor density of control group. All data are presented as % of control ± SEM.



### **3.4 Social Behavior**

Peer social behavior was evaluated in a series of round robin tests that took place between four and eight months after initiation of drug administration, and then again two to six months after drug cessation (See *Paper I* Supplemental Methods for further details of the ethogram). Behaviors from the ethogram were consolidated into nine composite measurements: locomotion; stereotypy; passivity; affiliation (physical proximity or grooming); dominance; submissiveness; coo vocalizations; bark vocalizations; and social (attack or bite) or nonsocial (cage shake) aggression. However, only eight of these were analyzed, due to insufficient variability for one (i.e., aggression) of the nine measures. Intra- and inter-rater reliability was greater than 0.85 on all scored behaviors.

### 3.5 Statistical Analyses

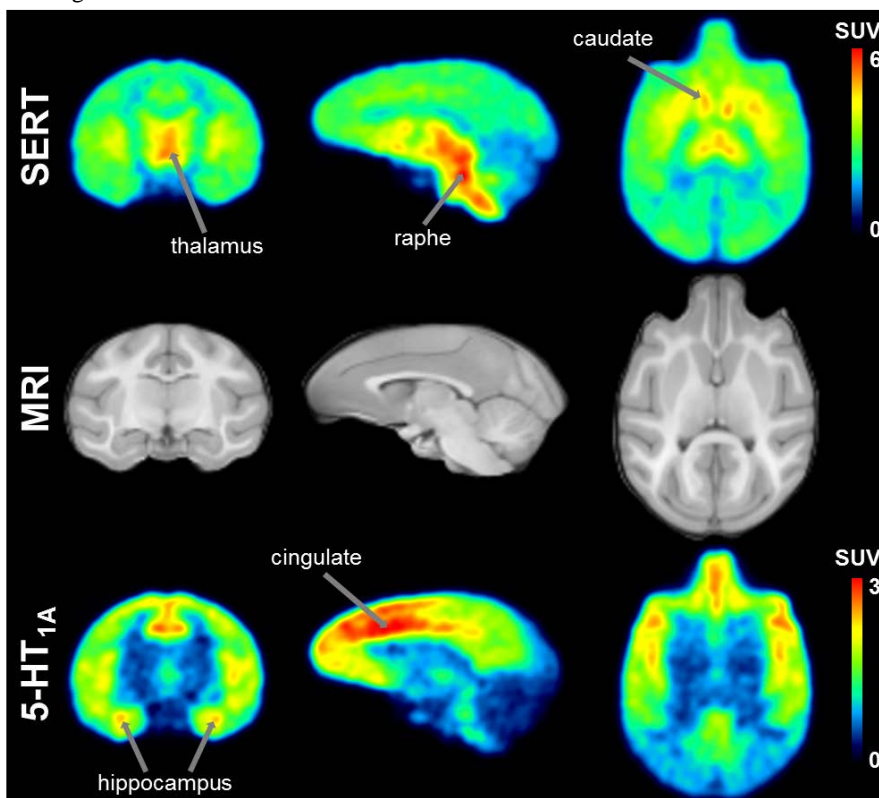
In *Paper I*, PET studies employed rearing-by-drug analyses of variance (ANOVA) to examine both interaction and main effects. Behavioral studies employed a rearing-by-treatment-by-period ANOVA for each of the eight behavioral composite measurements. For both PET and behavioral studies, statistics show the uncorrected  $p$  value, the correction factor, and the corrected  $p$  value.

In *Paper II*, for each of the two regions and two radioligands analyzed, a full factorial ANOVA was used to examine a possible strain-by-stress-by-treatment interaction. Bonferroni post-hoc tests were used to evaluate omnibus main effects and interactions. In addition, *a priori* comparisons were used to examine GxE interactions within the vehicle group before examining treatment effect relative to vehicle. After Bonferroni correction for multiple comparisons, the cutoff  $p$  value was  $< 0.0125$ .

Statistical analyses used IBM SPSS 19.0.0.1 (<http://www-01.ibm.com/software/analytics/spss/>).

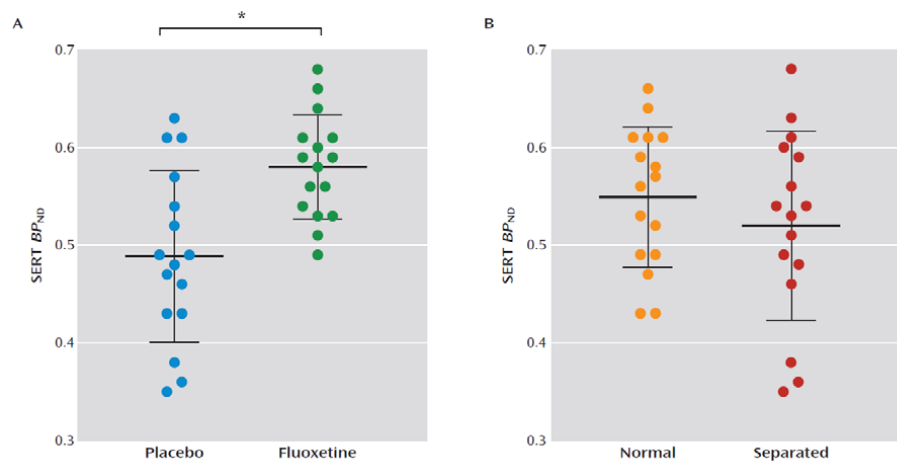
## 4 RESULTS AND DISCUSSION

As regards *Paper I* (Shrestha et al., 2014), [<sup>11</sup>C]DASB and [<sup>11</sup>C](R)-RWAY showed distribution and time course of brain uptake that similar to those observed in previous studies (Ichise et al., 2006; Yasuno et al., 2006). [<sup>11</sup>C]DASB uptake in monkey brain reflected the known distribution of SERT, with high binding in raphe, thalamus, and caudate (Figure 17, upper row). [<sup>11</sup>C](R)-RWAY uptake in monkey brain reflected the known distribution of 5-HT<sub>1A</sub> receptors, with high binding in cingulate cortex and hippocampus (Figure 17, bottom row). With regard to time course of uptake for both radioligands, higher density regions had later times of peak uptake, reflecting the greater amount of radioligand that had to be delivered to achieve equilibrium binding. For both radioligands, uptake in cerebellar white matter (the reference region) peaked early and was similar between groups of animals, the latter fulfilling a requirement to use reference tissue modeling.



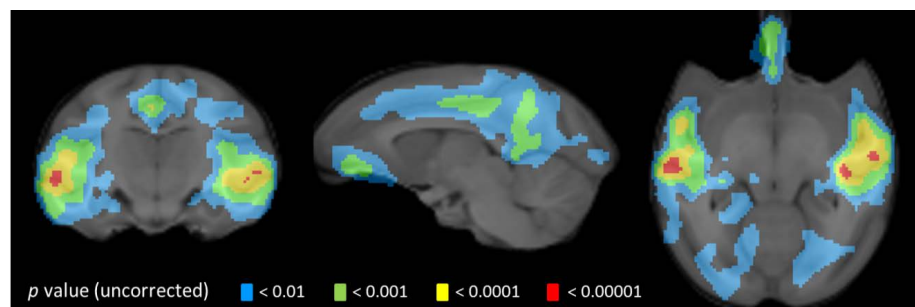
**Figure 17.** Distribution of [<sup>11</sup>C]DASB binding to serotonin transporter (SERT) and [<sup>11</sup>C](R)-RWAY binding to serotonin 1A (5-HT<sub>1A</sub>) receptor in the representative brain of a normally-reared monkey that received placebo. *Upper row:* Summed images from 30 to 60 min show high SERT density in thalamus, raphe, and caudate. *Middle row:* MRI images show the anatomical structures for the co-registered PET images. *Bottom row:* Summed images from 30 to 60 min show high 5-HT<sub>1A</sub> receptor density in hippocampus and cingulate. SERT – Serotonin Transporter; 5-HT<sub>1A</sub> – Serotonin 1A; SUV – Standardized Uptake Value

With regard to SERT, fluoxetine upregulated binding in neocortex (+19%,  $F(1, 31) = 12.8$ ,  $p < 0.001 * 2 = 0.002$ ; Figure 18) and hippocampus (+17%,  $F(1, 31) = 6.6$ ,  $p < 0.016 * 2 = 0.032$ ; Figure 18).



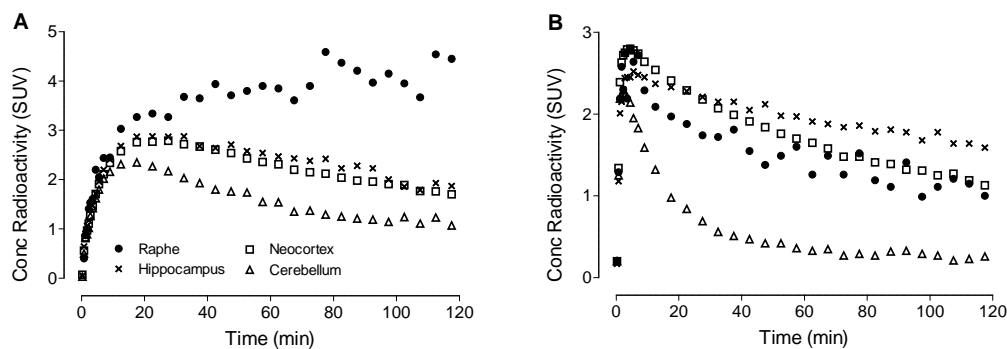
**Figure 18.** Effects of fluoxetine and maternal separation on serotonin transporter (SERT) in neocortex. A) Fluoxetine upregulated SERT by 19% ( $*p < 0.001 * 2 = 0.002$ ). B) Maternal separation had no statistically significant effect on SERT.BP<sub>ND</sub> – Binding potential; Bars represent mean  $\pm$  SD.

Whole brain, voxel-wise analysis showed that fluoxetine’s effects on cortical binding were localized to the lateral temporal, cingulate, and orbito-frontal cortices (Figure 19). However, only lateral temporal and posterior cingulate survived multiple comparisons at the voxel-level using family-wise error correction. Maternal separation had no significant effect on SERT binding. Two-way ANOVA analysis also found no statistically significant rearing-by-treatment interaction in either hippocampus or neocortex.



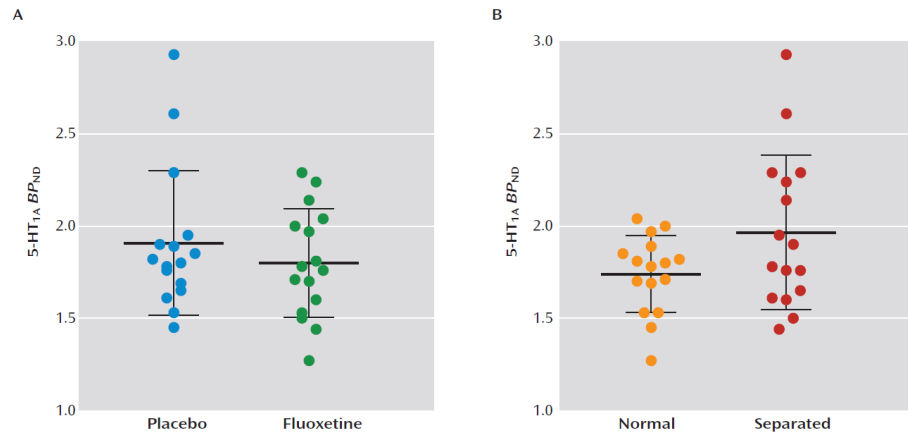
**Figure 19.** Fluoxetine increased serotonin transporter (SERT) binding in the lateral temporal, cingulate, and orbito-frontal cortices as shown by whole-brain, voxel-wise analysis. The four different colors represent uncorrected  $p$  values. The co-registered magnetic resonance imaging (MRI) template is shown in grayscale and is merged with each PET image.

Kinetic modeling essentially calculates the area under the time-activity curves from time zero to infinity. Without a clear identification of the time of peak uptake and rate of washout (slope) prior to the end of the scan, the extrapolated area to infinity is vulnerable to error. Our scan period of 120 min was sufficient to calculate binding potential for both neocortex and hippocampus, because both of these regions had early peak uptake and fast washout (Figure 20A). However, we could not reliably quantify binding potential in raphe because of its late time of peak uptake (consistent with its high SERT density), its slow washout from brain, and its relatively high noise at later scan times. In fact, in some animals, radioactivity continued to rise in raphe for the entire 120-min scan (Figure 20A); that is, we could not clearly identify the time of peak uptake. Such rising time-activity curves seemed randomly distributed in all four groups of monkeys. For these reasons, we excluded raphe from our analysis of [<sup>11</sup>C]DASB binding.



**Figure 20.** Time-activity curves for [<sup>11</sup>C]DASB and [<sup>11</sup>C](R)-RWAY in raphe, hippocampus, neocortex, and cerebellum from a normally-reared monkey that received placebo. (A) For [<sup>11</sup>C]DASB, all regions except raphe had well-defined time of peak uptake (~20 min) and rate of washout during the 120-min scan. In this animal and in about ~20% of all animals, the concentration of radioactivity (standardized uptake value; SUV) in raphe continued to increase for the entire scan; (B) For [<sup>11</sup>C](R)-RWAY, all regions had a well-identified time of peak (~10 min) and rate of washout.

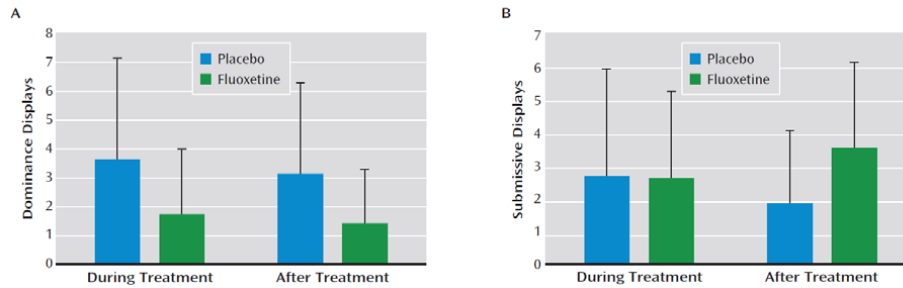
In contrast to [<sup>11</sup>C]DASB, [<sup>11</sup>C](R)-RWAY showed a time-activity curve in raphe with a clearly defined time of peak uptake and rate of washout (Figure 20B). Thus, all three regions (hippocampus, neocortex, and raphe) were analyzed for [<sup>11</sup>C](R)-RWAY. Neither fluoxetine nor maternal separation had a statistically significant effect on 5-HT<sub>1A</sub> receptor binding (Figure 21). Although 5-HT<sub>1A</sub> receptor binding in raphe was increased by 23% in maternally-separated monkeys, this finding did not survive correction for the three regions examined ( $F(1, 29) = 5.1, p < 0.03 * 3 = 0.09$ ). Furthermore, no statistically significant effect was observed for each individual variable or any of the interactions using voxel-wise, whole-brain analysis.



**Figure 21.** Main effects of fluoxetine and maternal separation on serotonin 1A (5-HT<sub>1A</sub>) receptor density in raphe. (A) Fluoxetine had no statistically significant effect on 5-HT<sub>1A</sub> receptor density; (B) Maternal separation increased 5-HT<sub>1A</sub> receptor density by 23%, which was at trend level after correction for multiple comparisons across the three regions ( $p < 0.03 * 3 = 0.09$ ). BP<sub>ND</sub> – Binding potential; Bars represent mean  $\pm$  SD.

With regard to social behavior, eight behaviors expressed in a social context were examined for effects of drug (i.e., fluoxetine vs. placebo), rearing, and period (i.e., whether animals were observed ‘during treatment’ or ‘after treatment’ with either fluoxetine or placebo). None of the behavioral effects of drug or rearing were statistically significant after correction for multiple comparisons. However, overly liberal, uncorrected thresholds generated results that could be pursued in future studies. Namely, fluoxetine reduced dominance displays both ‘during’ and ‘after’ the treatment period in both rearing groups ( $F(1, 28) = 4.75, p < 0.038 * 8 = 0.30$ ). In addition, a drug-by-period interaction was observed for submissive displays ( $F(1, 28) = 4.22, p < 0.049 * 8 = 0.39$ ), reflecting between-group differences after, but not during, fluoxetine treatment (Figure 22). Finally, we observed main effects of both period and rearing (independent of fluoxetine treatment) on other behaviors. Period effects were observed for locomotion ( $F(1, 28) = 5.95, p < 0.02 * 8 = 0.16$ ); passivity ( $F(1, 28) = 18.01, p < 0.0002 * 8 = 0.002$ ); and affiliative behaviors ( $F(1, 28) = 22.35, p < 0.00006 * 8 = 0.0005$ ). Only the period-related increase in passivity and decrease in affiliative behavior survived correction for multiple comparisons. The causes of these period-related effects are unknown but could reflect developmental changes, as the animals were almost one year older ‘after treatment’ than ‘during treatment.’

Rearing effects were observed for locomotion ( $F(1, 28) = 4.26, p < 0.048 * 8 = 0.38$ ), stereotypy ( $F(1, 28) = 7.01, p < 0.013 * 8 = 0.10$ ), and bark frequency ( $F(1, 28) = 5.04, p < 0.03 * 8 = 0.24$ ). These rearing effects were unaffected by fluoxetine and persisted across both testing periods. However, as with the effects of treatment, none of the effects of rearing survived corrected statistical thresholds.



**Figure 22.** Main effects of fluoxetine on social behaviors in monkeys. (A) Fluoxetine reduced dominance displays both in the ‘during treatment’ period (between four and eight months after treatment began) and in the ‘after treatment’ period (two to six months after treatment ceased) but did not survive after correction for eight behavioral measurements ( $p < 0.038 * 8 = 0.30$ ). (B) Fluoxetine increased submissive displays only in the ‘after treatment’ period, which also did not survive the correction for multiple comparisons ( $p < 0.049 * 8 = 0.39$ ). Bars represent mean  $\pm$  SD

The study in *Paper I* was the first in nonhuman primates to demonstrate that an antidepressant administered during development has long-lasting effects in primate brain. The persistent SERT upregulation identified by the present study was a substantial, robust effect—particularly for a PET study conducted with such a limited sample size—and survived stringent statistical analyses both at the regional and voxel level. Specifically, two year-old monkeys receiving fluoxetine, regardless of rearing, had persistently upregulated SERT binding 1.5 years after drug discontinuation. Implications regarding the efficacy or potential adverse effects of SSRIs in patients cannot be directly drawn from this study. Its purpose was to investigate effects of SSRIs on brain development. Non-human primate studies such as this one permit random assignment of SSRI or placebo treatment, whereas human studies are necessarily confounded by the administration of SSRIs only to children who have depression, anxiety, or other mental disorders, which could also have effects on brain development.

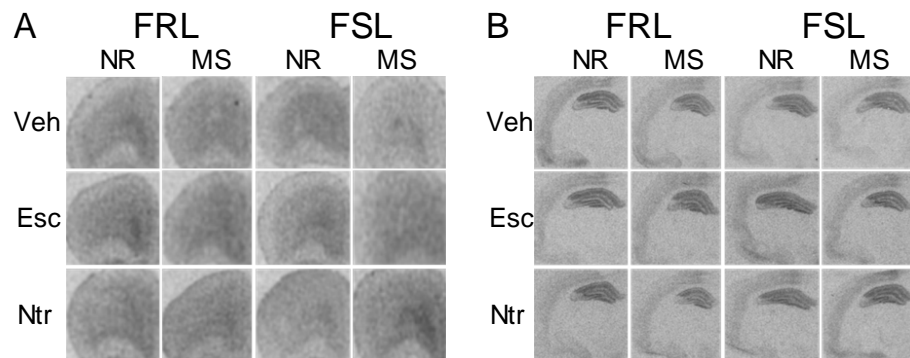
As regards *Paper II* (Shrestha et al., 2014), after stringent corrections for Bonferroni and multiple regions, significant strain-by-rearing-by-treatment (three-way) interactions emerged for only two of the four dependent measures: 5-HT<sub>1A</sub> receptors in the PFC and 5-HT<sub>1B</sub> receptors in the hippocampus (Table 1).

Vulnerability		Antidepressants' normalizing effects	
Genotype	Early-life stress	Escitalopram	Nortriptyline
FRL (low)	normally reared (low)	<i>no change compared to vehicle</i>	
FRL (low)	<b>maternally separated (high)</b>	↑ 5-HT <sub>1B</sub> (HP)*	↑ 5-HT <sub>1B</sub> (HP)*
<b>FSL (high)</b>	normally reared (low)	↑ 5-HT <sub>1B</sub> (HP)*	↑ 5-HT <sub>1B</sub> (HP)*
<b>FSL (high)</b>	<b>maternally separated (high)</b>	↑ 5-HT <sub>1B</sub> (HP)**	↑ 5-HT <sub>1A</sub> (PFC)*

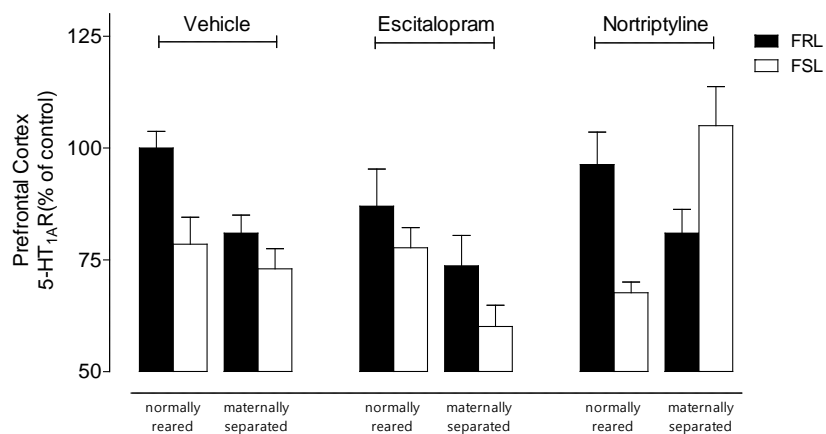
\*  $p < 0.001$ ; \*\*  $p < 0.05$  (after post-hoc bonferroni corrections)

**Table 1.** Effects of genotype, early-life stress, and two classes of antidepressants on the serotonergic system in prefrontal cortex (PFC) and hippocampus (HP).

With regard to the 5-HT<sub>1A</sub> receptor, [<sup>125</sup>I]MPPI autoradiograms showed specific binding in PFC and hippocampus (Figure 23). In the PFC, a significant strain-by-rearing-by-treatment (three-way) interaction emerged (F=5.55, df=2,75, p< 0.01) (Figure 24). Initial post-hoc analyses showed that both vulnerable genotype and environment reduced 5-HT<sub>1A</sub> receptor binding (p< 0.05). However, the effects of these two vulnerabilities were not additive. A second set of post-hoc analyses showed that only nortriptyline selectively increased 5-HT<sub>1A</sub> receptors in the group with both vulnerabilities (p< 0.001). In the hippocampus, significant rearing-by-treatment (F=6.32, df=2,76, p< 0.01) and GxE (F=11.55, df=2,76, p< 0.01) interactions emerged. Both antidepressants increased 5-HT<sub>1A</sub> receptors in the maternally-separated group.



**Figure 23.** Autoradiograms of 5-HT<sub>1A</sub> receptors using [<sup>125</sup>I]MPPI in (A) PFC and (B) dorsal hippocampus.

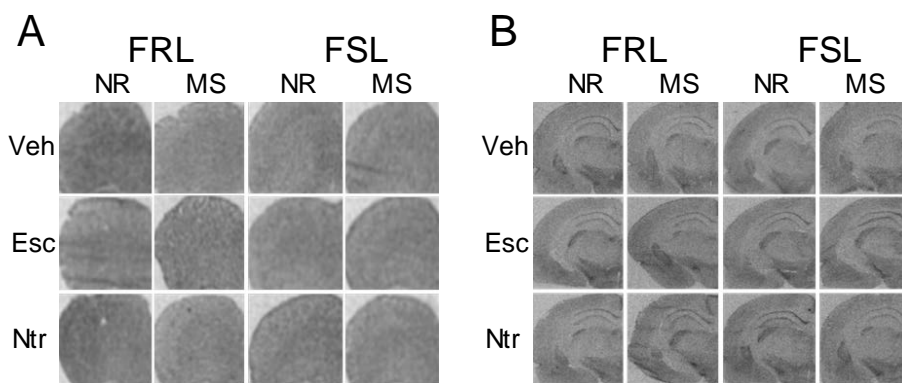


**Figure 24.** With regard to 5-HT<sub>1A</sub> receptors, a three-way interaction emerged in the PFC (F=5.55, df=2,75, p=0.006). Nortriptyline normalized the combined effects of GxE.

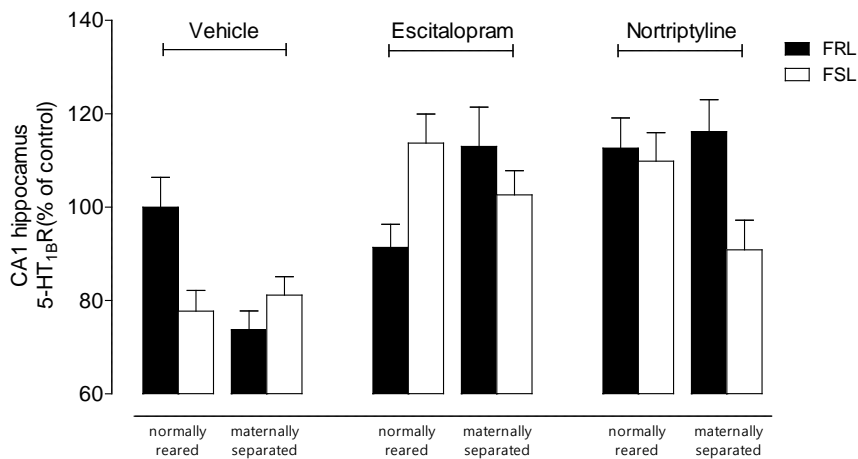
With regard to the 5-HT<sub>1B</sub> receptor, [<sup>125</sup>I]cyanopindolol autoradiograms showed specific binding in both PFC and hippocampus (Figure 25). In the PFC, a significant rearing-by-



treatment interaction emerged ( $F=12.31$ ,  $df = 2,77$ ,  $p < 0.001$ ) (Figure 26). In the maternally-separated group, escitalopram increased 5-HT<sub>1B</sub> receptor binding ( $p < 0.05$ ). In the hippocampus, a significant strain-by-rearing-by-treatment (three-way) interaction emerged ( $F=8.30$ ,  $df=2,75$ ,  $p < 0.001$ ) (Figure 26). Initial post-hoc analyses revealed that both vulnerable genotype and environment reduced hippocampal 5-HT<sub>1B</sub> receptor binding ( $p < 0.05$ ). Similar to 5-HT<sub>1A</sub> receptors, the effects of these two vulnerabilities were not additive. Escitalopram increased binding in the group with either or both vulnerabilities ( $p < 0.01$ ). In contrast, nortriptyline increased binding in the group only with either vulnerability ( $p < 0.001$ ).



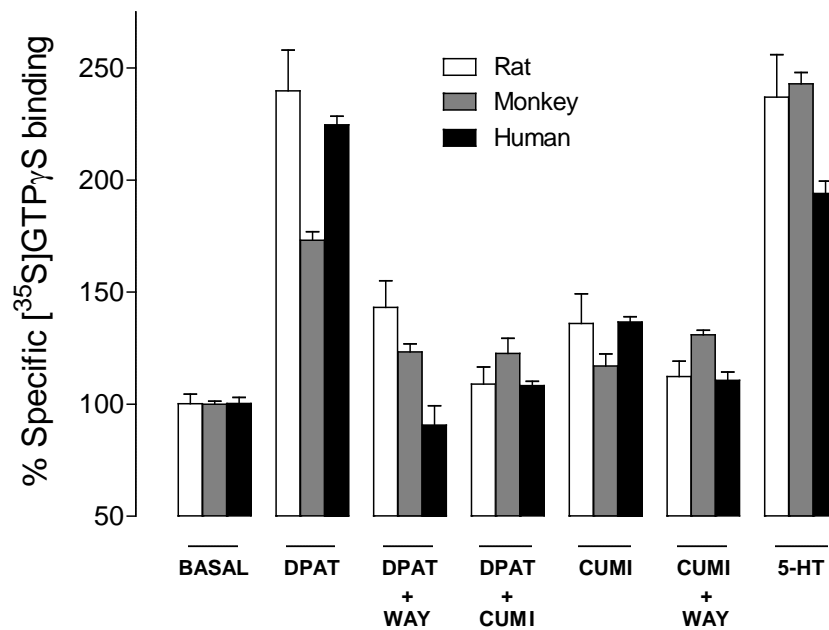
**Figure 25.** Autoradiograms of 5-HT<sub>1A</sub> receptors using [<sup>125</sup>I]MPPI in (A) PFC and (B) dorsal hippocampus.



**Figure 26.** With regard to 5-HT<sub>1B</sub> receptors, A three-way interaction emerged for hippocampus ( $F=8.30$ ,  $df=2,75$ ,  $p < 0.001$ ). Escitalopram normalized the combined effects of GxE, while nortriptyline normalized the effect of either strain or stress.

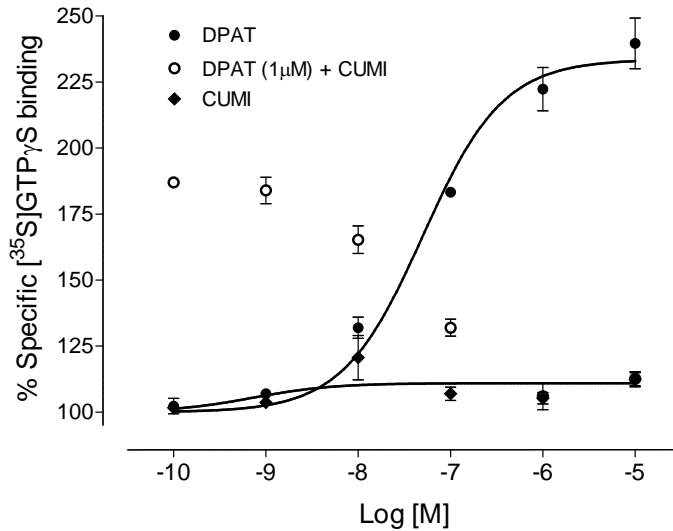
In summary, significant strain-by-rearing-by-treatment (three-way) interactions emerged for 5-HT<sub>1A</sub> receptors in the PFC and 5-HT<sub>1B</sub> receptors in the hippocampus. The findings demonstrate interactions of at least three known variables on the serotonergic neurochemistry where antidepressant class modulates the delicate interplay between genes and environment in a brain region-dependent manner. Our findings provide preliminary validity for using this controlled animal model, albeit with larger sample sizes, to further understand the interaction among these three key factors in MDD: genes, environment, and antidepressant class.

As regards *Paper III* (Shrestha et al., 2014), CUMI-101 did not stimulate [<sup>35</sup>S]GTP<sub>γ</sub>S binding in either monkey or human brain. Unlike the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT, CUMI-101 did not behave as an agonist in either monkey or human hippocampal tissue (Figure 27).



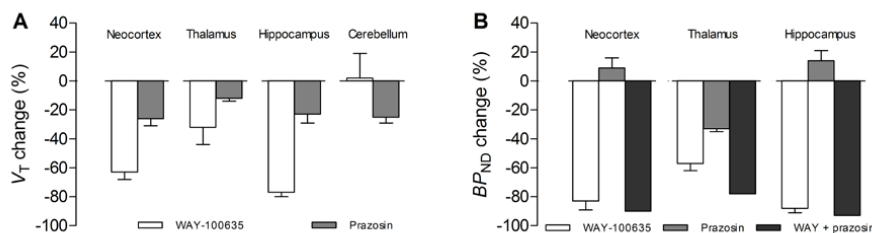
**Figure 27.** Both 8-OH-DPAT (1 μM) and 5-HT (1 μM), but not CUMI-101 (10 μM), stimulated [<sup>35</sup>S]GTP<sub>γ</sub>S binding in rat, monkey, and human hippocampal tissue. Similar to WAY-100635 (10 μM), a potent 5-HT<sub>1A</sub> receptor antagonist, CUMI-101 blocked 8-OH-DPAT-stimulated [<sup>35</sup>S]GTP<sub>γ</sub>S binding in all three species. Bars represent mean ± SEM.

On the contrary, CUMI-101 dose-dependently blocked 8-OH-DPAT-stimulated [<sup>35</sup>S]GTP<sub>γ</sub>S binding in human hippocampal tissue (Figure 28). Similar to WAY-100635, a potent 5-HT<sub>1A</sub> receptor antagonist, CUMI-101 also blocked 8-OH-DPAT-induced [<sup>35</sup>S]GTP<sub>γ</sub>S stimulation in both monkey and human hippocampal tissue.



**Figure 28.** CUMI-101 dose-dependently blocked 8-OH-DPAT-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in human hippocampal tissues. Unlike 8-OH-DPAT, a potent 5-HT<sub>1A</sub> receptor agonist, CUMI-101 did not stimulate [<sup>35</sup>S]GTP $\gamma$ S binding. Bars represent mean  $\pm$  SEM.

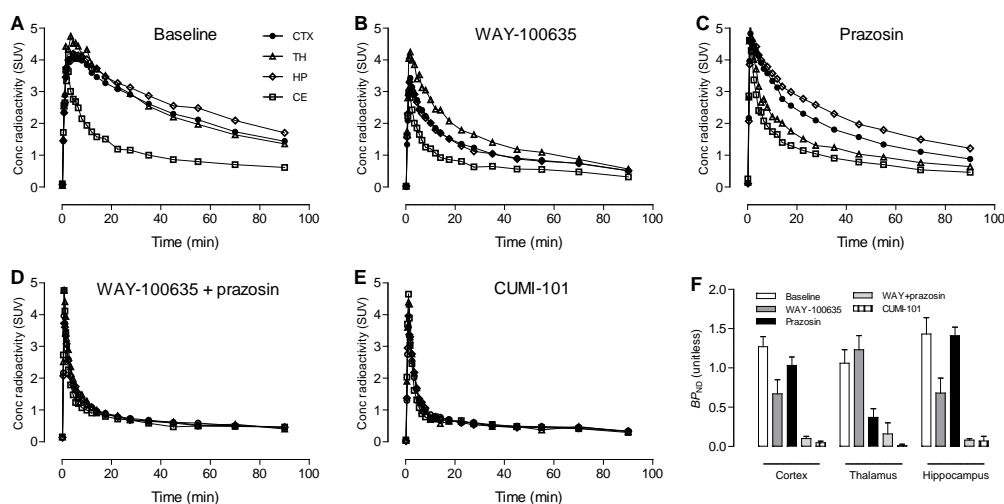
The PET radioligand, [<sup>11</sup>C]CUMI-101, was also displaced by the  $\alpha$ 1 adrenoceptor antagonist, prazosin. In monkeys, pre-blocking with WAY-100635 decreased distribution volume ( $V_T$ ) of [<sup>11</sup>C]CUMI-101 in all regions (Figure 29). Decrease in specific binding as indicated by  $BP_{ND}$  was  $83 \pm 6\%$  in neocortex,  $88 \pm 3\%$  in hippocampus, and  $57 \pm 5\%$  in thalamus. However,  $V_T$  also decreased after pre-blocking with prazosin, although to a lesser extent. The change in  $BP_{ND}$  with prazosin pre-blocking was greatest in thalamus ( $33 \pm 2\%$ ) where  $\alpha$ 1 adrenoceptors are abundant. Pre-blocking with WAY-100635 plus prazosin ( $n = 1$ ) further decreased  $BP_{ND}$  values of [<sup>11</sup>C]CUMI-101 by 78% in thalamus, 90% in neocortex, and 93% in hippocampus. In addition, prazosin also displaced  $V_T$  values of [<sup>11</sup>C]CUMI-101 in cerebellum by  $25 \pm 4\%$ . This decrease in cerebellar  $V_T$  by prazosin pre-blocking may have led to the small increase in  $BP_{ND}$  for both neocortex and hippocampus.



**Figure 29.**  $\alpha$ 1 adrenoceptor cross-reactivity in monkey brain. [<sup>11</sup>C]CUMI-101 displacement after pre-blocking with either WAY-100635 (0.5 mg/kg;  $n=3$ ), prazosin (1 mg/kg;  $n=2$ ), or WAY-100635 plus prazosin ( $n=1$ ). (A) Distribution volume ( $V_T$ ) decreased in all regions after pre-blocking with prazosin. (B) Binding potential

( $BP_{ND}$ ) decreased more after pre-blocking with WAY-100635 plus prazosin than after only pre-blocking with prazosin.

In rats, [ $^{11}\text{C}$ ]CUMI-101 had good uptake in neocortex, thalamus, and hippocampus (Figure 30). Pre-blocking with WAY-100635 decreased brain uptake in all regions, but not to levels obtained with self-block with CUMI-101 alone. Furthermore, WAY-100635 decreased hippocampal uptake by a greater extent than thalamic uptake. In contrast, pre-blocking with prazosin decreased thalamic uptake by a greater extent than hippocampal uptake. Only pre-blocking with WAY-100635 plus prazosin decreased brain uptake in all regions to that of self-block. In mice,  $\alpha_1$ -adrenoceptor cross-reactivity was similar to that of rats, and only pre-blocking with WAY-100635 plus prazosin decreased brain uptake to that of self-block.



**Figure 30.**  $\alpha_1$  adrenoceptor cross-reactivity in rat brain. Time-activity curves for [ $^{11}\text{C}$ ]CUMI-101 under the following five conditions (n=3 for each condition): (A) baseline; (B) pre-blocking with the 5-HT $_{1A}$  receptor antagonist WAY-100635 (2 mg/kg); (C) pre-blocking with the  $\alpha_1$ -adrenoceptor antagonist prazosin (2 mg/kg); (D) pre-blocking with WAY-100635 (2 mg/kg) plus prazosin (2 mg/kg); and (E) self-blocking with CUMI-101 (2 mg/kg). (F)  $BP_{ND}$  values using cerebellum (CE) as the reference region were compared for each condition in the three brain regions: cortex (CTX), thalamus (TH), and hippocampus (HP). Panels A-E show representative time-activity curves from one animal for each condition. Panel F shows the mean  $BP_{ND}$  values  $\pm$  SEM for each condition in panels A-E. Bars represent mean  $\pm$  SEM. SUV = Standardized Uptake Value.

CUMI-101 showed significant cross-reactivity with  $\alpha_1$  adrenoceptors *in vitro* in rat, monkey, and human (Table 2). In rats,  $\alpha_1$  adrenoceptor cross-reactivity was 45% in thalamus, and 42% in neocortex. In monkeys,  $\alpha_1$  adrenoceptor cross-reactivity was 50% in thalamus, and 12% in neocortex. In humans,  $\alpha_1$  adrenoceptor cross-reactivity was 43% in thalamus, and 10% in neocortex.  $\alpha_1$  adrenoceptor cross-reactivity was greater at 23°C than 37°C.

Brain regions	Percent displacement ( $\pm$ SEM) of [ $^3$ H]CUMI-101											
	(+)-8-OH-DPAT (50 nM)						Prazosin (50 nM)					
	Rat		Monkey		Human		Rat		Monkey		Human	
	37°C	23°C	37°C	23°C	37°C	23°C	37°C	23°C	37°C	23°C	37°C	23°C
Cortex	47 $\pm$ 6	43 $\pm$ 4	91 $\pm$ 3	70 $\pm$ 4	87 $\pm$ 3	78 $\pm$ 2	42 $\pm$ 3	45 $\pm$ 2	12 $\pm$ 2	21 $\pm$ 1	10 $\pm$ 1	19 $\pm$ 2
Thalamus	49 $\pm$ 2	39 $\pm$ 3	52 $\pm$ 3	28 $\pm$ 2	66 $\pm$ 2	46 $\pm$ 3	45 $\pm$ 2	51 $\pm$ 5	50 $\pm$ 4	78 $\pm$ 2	43 $\pm$ 1	56 $\pm$ 3
Cerebellum	16 $\pm$ 2	11 $\pm$ 3	33 $\pm$ 3	NA	NA	25 $\pm$ 4	88 $\pm$ 4	91 $\pm$ 5	25 $\pm$ 3	79 $\pm$ 6	NA	89 $\pm$ 5

"NA" refers to three null entries for which we had no data.

**Table 2.** Percent displacement of [ $^3$ H]CUMI-101 by 50 nM DPAT (selective for 5-HT<sub>1A</sub> receptors) and 50 nM prazosin (selective for  $\alpha$ 1 adrenoceptors) in rat, monkey, and human brain at 37°C and 23°C.

In summary, we found that, similar to WAY-100635, CUMI-101 acts as a potent antagonist at the 5-HT<sub>1A</sub> receptor in monkey and human brain. We further found that CUMI-101 had significant cross-reactivity with  $\alpha$ 1-adrenoceptors in rat, monkey, and human brain, primarily in the thalamus (>35%). CUMI-101 had ~10% cross-reactivity with  $\alpha$ 1-adrenoceptors in other regions (e.g., neocortex, cerebellum), which makes quantification problematic, especially using cerebellum as the reference region. These findings suggest that the utility of [ $^{11}$ C]CUMI-101 as a PET radioligand in humans is limited.

As regards *Paper IV* (Shrestha et al., 2012), we qualitatively reviewed PET imaging studies (n = 8) using [*carbonyl*- $^{11}$ C]WAY-100635 to examine the 5-HT<sub>1A</sub> receptor in MDD. This review was an attempt to resolve some of the discrepancies by examining potential methodological confounds. We contend that methodological factors rather than clinical variables most likely explain discrepancies in the eight studies examined showing increased, decreased, or unaltered 5-HT<sub>1A</sub> receptor binding in MDD patients (Table 3). Most studies reported similar diagnostic criteria, illness severity of the patient population, and distribution of demographic variables. The discrepant findings across studies are not random, but rather systematically determined by the outcome measures and reference target.

Although most studies found decreased 5-HT<sub>1A</sub> receptor binding in individuals with MDD, Parsey and colleagues reported increased 5-HT<sub>1A</sub> receptor binding in these patients. Notably, most 5-HT<sub>1A</sub> PET studies reviewed used the reference tissue model, whose main advantage is avoiding the arterial line. This method, however, has the potential to yield inaccurate results because 1) nonspecific uptake in cerebellum (which was used as the reference region) is associated with high variability; 2) cerebellar gray matter contains 5-HT<sub>1A</sub> receptors; and 3) radiometabolites accumulate in cerebellum over time. For these reasons, we propose that using the 'gold standard' of arterial blood sampling (and reporting the free binding potential) is preferable for PET studies of the 5-HT<sub>1A</sub> receptor imaging in individuals with MDD. We further propose that radioligands, in general, undergo more thorough evaluation of the effects of efflux transporters at the blood-brain barrier, which may alter radioligand uptake. Lastly, for receptors that exist in high- and low-affinity states, such as 5-HT<sub>1A</sub> receptors, we recommend using radioligands, if possible, that distinguish

between these states as a way to further our understanding of the pathophysiology of our targets.

Reference	Healthy subjects			Patients with MDD					Arterial blood	Outcome measure	Cerebellar reference region	Result	Correlation with symptoms
	N	Age	Sex (M/F)	N	Age	Sex (M/F)	Drug-naive	HDRS					
Drevets et al. (1999)	8	35.3±13.5	4/4	12	35.8±9.7	5/7	0	22±6	No	BP <sub>MD</sub>	Gray	↓ all regions	No
Sargent et al. (2000)	18	36.4±8.3	17/1	15 <sup>a</sup>	37.7±13.7	15/0	7	22±5	No	BP <sub>MD</sub>	Gray	↓ all regions	No
Meltzer et al. (2004)	17	70.0±6.7	8/9	17	71.4±5.9	4/13	N.R.	18±3	Yes	BP <sub>F</sub>	Gray	↓ raphe	Yes
Parsey et al. (2006; 2010) <sup>b</sup>	51	37.4±14.4	29/22	30 <sup>c</sup>	40.6±13.1	8/22	23	26±7	Yes	BP <sub>F</sub> , BP <sub>MD</sub>	White	↑ (BP <sub>F</sub> ) ↓ (BP <sub>MD</sub> )	No
Drevets et al. (2007)	8	32.4±12.1	4/4	16	32.4±10	6/10	N.R.	18±7	No	BP <sub>MD</sub>	Gray	↓ all regions	No
Hirvonen et al. (2008)	15	32.6±7.7	5/10	21	40.1±9.0	8/13	20	18±3	Yes	BP <sub>F</sub>	White	↓ all regions	No
Mickey et al. (2008)	14	34±12	9/8	17	38±11	5/9	10	18.9±2.6	No	BP <sub>MD</sub>	Gray	↔	N.R.

↓, lower 5-HT<sub>1A</sub> binding in patients with MDD than in healthy subjects; ↑, higher 5-HT<sub>1A</sub> binding in patients with MDD than in healthy subjects; BP, binding potential; HDRS, 17-item Hamilton Depression Rating Scale; N, number; N.R., not reported, wk, week.

<sup>a</sup> Number of untreated first-episode patients.

<sup>b</sup> Reported results are only from the combined 2006–2010 cohort.

<sup>c</sup> Number of not recently medicated patients.

**Table 3.** A summary of PET imaging studies using [*carbonyl*-<sup>11</sup>C]WAY-100635 in patients with MDD vs. healthy subjects. Numbers denote mean ± SD, N, or N/N.

Importantly, while clinical heterogeneity may have contributed to the inconsistent PET imaging results, Parsey and colleagues were able to reproduce discrepant results by analyzing their larger cohort using different reference target. Specifically, they showed that 5-HT<sub>1A</sub> receptor binding was increased when normalized to plasma free fraction and decreased when normalized to cerebellar gray matter. Thus, methodological differences are the plausible cause of the discrepant findings. However, because data from the multiple sites cannot be pooled, we cannot identify subgroups of MDD patients who may have altered 5-HT<sub>1A</sub> receptors. We need larger sample sizes in PET studies in order to reach definitive conclusions about 5-HT<sub>1A</sub> receptor density in MDD. Currently, the clinical relevance of altered 5-HT<sub>1A</sub> receptor binding of ±20% between MDD patients and healthy subjects is uncertain. Nonetheless, achieving definitive answers are important and will require inter-institutional collaborative efforts so as to make data “poolable” and clarify discrepancies. While this is an important goal in its own right, working together now to resolve these issues would, by extension, also have profound implications for studies investigating other protein targets in the brain.

## 5 CONCLUSION AND FUTURE PROSPECTS

Over the last two decades, researchers have studied the interplay between the serotonergic system and the environment in relation to the pathophysiology and treatment of MDD and anxiety. Nevertheless, our knowledge of these psychiatric conditions is only just the beginning. Given the high prevalence of mental disorders and its enormous global burden on public health, studies are warranted, in particular to examine the long-term effects of SSRIs during brain development, the high clinical variability to antidepressant response, and potential biomarkers to diagnose MDD and anxiety.

Taken together, the results of the studies described above lead to some important conclusions regarding the role of the serotonergic system in the pathophysiology and treatment of MDD and anxiety. Notably, one of the key findings was that, in monkeys, fluoxetine administered during the juvenile period had a *persistent* effect on SERT despite the drug having been discontinued for more than 1.5 years. This study allowed us to longitudinally investigate the effects of SSRIs during brain development in a non-human primate model, with or without early-life stress, and importantly with random assignment to SSRI treatment or placebo; such a cross-design study is understandably impossible to conduct in children because of ethical constraints. Given the robust effect on SERT in monkeys, we hypothesize that such changes may be similarly observed in children treated with SSRIs. Although no concrete conclusions can be drawn regarding whether the persistent effects of SSRIs are ‘good’ or ‘bad’, our study nevertheless underscores the need for practitioners to exercise caution in prescribing SSRIs to children.

When we explored clinical variability to antidepressant response, complex gene-environment interactions emerged on serotonergic neurochemistry. These interactions were selectively modified in an antidepressant brain region-dependent as well as antidepressant class-dependent fashion. Although our finding is preliminary, the results underscore the importance of such interaction studies to further our understanding of variability in antidepressant response; key factors that could be explored include the effects of genes, environment, antidepressant class, and ethnicity. In fact, many susceptible genes associated with the serotonergic system appear to be ethnicity-dependent. For example, the frequency of the short allele in the SERT gene is higher in American Indians, and a greater association between SERT polymorphisms and remission in response to SSRI treatment has been identified in Caucasians (Porcelli et al., 2012). Our findings suggest that future studies should adopt a systems biology approach where individual variability is considered in conjunction with other factors such as ethnicity, epigenetics, and genotype in order as to maximize the remission rate associated with antidepressants.

Although the PET radioligand [<sup>11</sup>C]CUMI-101 was initially reported as a selective agonist at the 5-HT<sub>1A</sub> receptor, our *in vivo* and *in vitro* characterizations demonstrated that CUMI-101 exhibits significant, regional dependent  $\alpha 1$  adrenoceptor cross-reactivity as well as is an antagonist at the 5-HT<sub>1A</sub> receptor and further studies are warranted. Of importance, CUMI-101 demonstrated cross-reactivity in both neocortex and cerebellum. In neocortex, cross-reactivity with  $\alpha 1$  adrenoceptors was 10% in humans and 12% in monkeys. Interestingly, in cerebellum, cross-reactivity was 25% in monkeys, both *in vivo* and *in vitro*.

This issue of cross-reactivity is problematic particularly because PET studies, on average, report a difference of 15-30%, including a test-rest variability of 10-15%. Such a modest difference is susceptible to erroneous results when the radioligand lacks specificity. In addition, the displaceable binding to  $\alpha 1$  adrenoceptors in cerebellum necessitates the use of arterial input function, because the use of cerebellum as a reference region will undoubtedly contribute to erroneous results. Furthermore, future studies involving CUMI-101 should carefully assess its cross-reactivity in both regions expressing high density of 5-HT<sub>1A</sub> receptors such as neocortex and hippocampus, which are important targets in MDD and anxiety, as well as regions expressing low density of 5-HT<sub>1A</sub> receptors such as cerebellum. Especially the latter study is critical if cerebellum is to be used as a reference region because small variations in the denominator can lead to erroneous results. Our replication of the prior study in rats, and an extension to monkey and human brain show that CUMI-101 behaves as an antagonist at the 5-HT<sub>1A</sub> receptor.

The 5-HT<sub>1A</sub> receptor is an important target for studying MDD and anxiety, and improved PET radioligands with high selectivity for the receptor are desirable. So far, the WAY analogs, including [*carbonyl*-<sup>11</sup>C]WAY-100635, demonstrate good brain uptake and specificity; however, they are all antagonists and exhibit other undesirable characteristics such as low plasma free fraction and low cerebellum binding making either arterial plasma quantification or reference tissue vulnerable to noise. Because the high-affinity state of the 5-HT<sub>1A</sub> receptor is postulated to be primarily affected in disease conditions—including MDD and anxiety—an agonist radioligand that preferentially binds to the active state of the receptor is desirable. Unfortunately, attempts by several groups to synthesize an agonist radioligand for the 5-HT<sub>1A</sub> receptor have proven unsuccessful. This may arise from intrinsic properties of the dynamic GPCR, which switches conformations between the high- and low-affinity states depending on the availability of an agonist, GTP, etc., which subsequently activates and triggers downstream secondary cascades. As such, synthesizing an agonist radioligand for the 5-HT<sub>1A</sub> receptor—or any other GPCR—remains an intriguing challenge.

With an eye towards future studies, our thorough review of 5-HT<sub>1A</sub> receptor imaging in MDD came to the important conclusion that future PET studies need to be more collaborative, as well as a consensus regarding the modeling parameters that determine outcomes measures for individual radioligands. Given the high cost and multi-disciplinary expertise involved in carrying out PET scans, we recommend a continuous collaborative effort during the study phase so that pooling data among multicenter PET imaging groups is achievable. Such an effort would significantly help researchers and the field reach consensus to answer questions concerning multifactorial brain disorders and their treatments.

To put this work into its larger context, it should be noted that this is a particularly exciting time in neuroscience research. Several promising pharmacological drugs, protein targets, and genetic and viral strategies are currently being explored that have the potential to transform the development of truly useful novel therapeutics for the treatment of MDD and anxiety, and many of these novel therapeutics may overcome the limitations associated with existing drugs (Covington et al., 2010). For example, drugs (e.g. YL-0919) that preferentially activate post-synaptic 5-HT<sub>1A</sub> receptors, together with SERT blockade, may



improve the efficacy of antidepressants. Although the serotonergic system continues to be the major target for the development of newer and improved antidepressants, the glutamatergic system has emerged as the next important target for developing fast-acting antidepressants. For example, a single dose of the non-competitive NMDA receptor antagonist ketamine has rapid (within hours) and long-lasting antidepressant effects (lasting up to 10 days) in treatment-resistant patients with depression. This antidepressant response is thought to be mediated by increased AMPA receptor throughput. A proof-of-concept study of an AMPA receptor antagonist is currently underway with the goal of examining whether this agent can block ketamine's antidepressant effects. Nevertheless, because ketamine is a street drug (known as 'Special K') and has several psychotomimetic side effects as well as addictive properties, the search is underway for agents with a similar mechanism of action but improved pharmacological profiles, including improved selectivity for NMDA or AMPA receptors. New molecular techniques involving genetic and viral strategies also offer significant hope in the treatment of MDD and anxiety. One genetic strategy is the use of adeno-associated viruses as carriers to deliver proteins such as p11. A preliminary study in mice reported the feasibility of such a strategy to target the virus to specific brain regions and induce protein expression that subsequently reverses depressive phenotypes (Alexander et al., 2010). In addition, the use of small interference RNA to silence or knock-down expression of genes such as 5-HT<sub>1A</sub> or SERT had robust antidepressant-like effects in rodents (Bortolozzi et al., 2013). Though promising, this field is in infancy. In particular, it should be noted that such drugs and molecular techniques eventually alter molecular signaling at the intracellular level, which eventually modifies several transcription and neurotrophic factors such as nuclear factor  $\kappa$ B (NF $\kappa$ B) and brain derived neurotrophic factor (BDNF).

Both basic and clinical neuroscience research are integral to our improved understanding of normal and altered brain functioning, as well as to the genetic and environmental interactions that perturb brain circuitry into adulthood. Such knowledge is imperative for understanding the long-term neurochemistry of these devastating brain disorders, as well as for the future development of the improved pharmacological interventions. Fortunately, over the last decade, neuroscience—like much of the rest of medicine—has entered a new and exciting age characterized by vastly improved technologies; these in turn, have brought about both rapid advances in our knowledge and the promise of future gains in our understanding. For instance, optogenetics allows us to activate specific neuronal pathways, CLARITY allows us to simultaneously examine the neuronal networking of different neurotransmitter systems, serial electron microscopy allows us to study connectomics at nanometer resolution, high-throughput sequencing allows us to determine the neuronal expression patterns of genes, and functional imaging techniques such as PET, fMRI, PET/CT, and PET/MRI allow us to study neuronal activity and anatomical structures with high specificity, sensitivity, and anatomical resolution inside the living brain. Such an unprecedented array of experimental advances will directly benefit the field of clinical neuroscience. Most notably, they will help us develop better drugs with fewer side effects—including reduced suicidal ideation—and help us minimize clinical variability; these techniques are also likely to ultimately provide us with biomarkers that will allow visual quantification to effectively diagnose and treat psychiatric conditions, including MDD and anxiety.

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