IN VITRO AND IN VIVO PHARMACOLOGY OF NOVEL PHENYLAMINOTETRALIN (PAT) ANALOGS AT SEROTONIN 5-HT₂ RECEPTORS: DEVELOPMENT OF DRUGS FOR NEUROPSYCHIATRIC DISORDERS

By

KRISHNAKANTH KONDABOLU

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2013
To my mom, dad, brother, wife
and all those who have inspired and supported me through this journey
ACKNOWLEDGMENTS

I would like to express my most sincere gratitude to my committee chair, Dr. Raymond Booth, for his support and guidance through these five years in graduate school. His constant advice and encouragement were a tremendous source of inspiration for me in this project. I would also like to express my gratitude to our collaborator Dr. Drake Morgan for his time and patience in teaching me the ins and outs of in vivo experiments and disease modeling. I would like to thank Dr. Ken Sloan for his inputs about physical chemistry and also for making our TA sessions as fun as they were. I would like to thank Dr. Margaret James for teaching me all I know about drug metabolism.

I would like to thank all the post docs in my lab for helping me out during the rough patches of graduate school: Dr. Clinton Canal for his constant support and help whenever I had trouble with my experiments, Dr. Tania Cordova-Sintjago for helping me make sense of my experiments, Dr. Rajeev Sakhuja and Dr. Myong Sang Kim for their expertise in organic synthesis. I would also like to thank all my colleagues in the department for their support and encouragement.

Last but not least, I would like to thank my family for their constant support and patience. Special thanks to my wife, Bindu, for putting up with me during these years in graduate school.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>12</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>14</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 G-PROTEIN COUPLED RECEPTORS: MOLECULAR DETERMINANTS AND THERAPEUTIC RELEVANCE OF SEROTONIN 5-HT$<em>{2A}$ AND 5-HT$</em>{2C}$ RECEPTORS IN SCHIZOPHRENIA</td>
<td>16</td>
</tr>
<tr>
<td>Introduction to G-Protein Coupled Receptors</td>
<td>16</td>
</tr>
<tr>
<td>GPCR Structure</td>
<td>16</td>
</tr>
<tr>
<td>GPCR Signal Transduction</td>
<td>17</td>
</tr>
<tr>
<td>Models of Ligand–GPCR Interactions</td>
<td>18</td>
</tr>
<tr>
<td>Ballesteros and Weinstein GPCR Nomenclature</td>
<td>19</td>
</tr>
<tr>
<td>Ligand Stabilized GPCR Conformations</td>
<td>20</td>
</tr>
<tr>
<td>Serotonin and 5-HT$_{2}$ GPCRs</td>
<td>24</td>
</tr>
<tr>
<td>Serotonin Biosynthesis and Metabolism</td>
<td>24</td>
</tr>
<tr>
<td>Serotonin 5-HT$_{2}$ GPCRs</td>
<td>24</td>
</tr>
<tr>
<td>5-HT$_{2}$ GPCR Signaling Pathways</td>
<td>25</td>
</tr>
<tr>
<td>5-HT$_{2C}$ GPCR Post-Translational Modifications</td>
<td>25</td>
</tr>
<tr>
<td>5-HT$_{2}$ GPCR Localization in Brain</td>
<td>27</td>
</tr>
<tr>
<td>Schizophrenia and Serotonin 5-HT$_{2}$ GPCRs</td>
<td>28</td>
</tr>
<tr>
<td>Relevance of 5-HT$_{2}$ GPCRs in Schizophrenia</td>
<td>29</td>
</tr>
<tr>
<td>Serotonin 5-HT$_{2A}$ Receptors and Schizophrenia</td>
<td>29</td>
</tr>
<tr>
<td>Serotonin 5-HT$<em>{2B}$ and 5-HT$</em>{2C}$ Receptors and Schizophrenia</td>
<td>30</td>
</tr>
<tr>
<td>Antipsychotics Drugs and Obesity</td>
<td>31</td>
</tr>
<tr>
<td>Molecular Determinants for Ligand Binding at 5-HT$_{2}$ GPCRs</td>
<td>32</td>
</tr>
<tr>
<td><em>In Vivo</em> Models to Screen for Antipsychotic Drug Efficacy</td>
<td>34</td>
</tr>
<tr>
<td>Central Hypothesis and Goals of this Dissertation</td>
<td>37</td>
</tr>
<tr>
<td>AIM 1: Delineate Molecular Determinants for Binding of 4-phenyl-N,N-dimethyl-1,2,3,4-tetrahydroanphthalene-2-amine (phenylaminotetralin, PAT) Derivatives at Human Recombinant 5-HT$<em>{2A}$ and 5-HT$</em>{2C}$ GPCRs</td>
<td>38</td>
</tr>
<tr>
<td>AIM 2: Characterize the 5-HT$_{2}$ Functional Pharmacology of PATs</td>
<td>38</td>
</tr>
<tr>
<td>AIM 3: Translational Studies: Assessment of PATs Efficacy in rodent models of psychosis</td>
<td>39</td>
</tr>
</tbody>
</table>
2 Delineate Molecular Determinants for Binding of 4-Phenyl-N,N-Dimethyl-1,2,3,4-Tetrahydroanthalene-2-Amine (Phenylaminotetralin; PAT) Derivatives at Human Recombinant 5-HT_{2A} and 5-HT_{2C} GPCRs

Specific Aim 1 .................................................................................................................. 43
Methodology ...................................................................................................................... 44
   Clonal Cell Culture and Transfection ............................................................................. 44
   Radioreceptor Competition Binding Assays ................................................................. 44
Results and Discussion for Competition Binding Assays .............................................. 45
   Affinity of Unsubstituted PATs at the 5-HT_{2A} Receptor ........................................... 45
   Affinity of Unsubstituted PATs at the 5-HT_{2C} Receptor ........................................... 45
   Affinity of 4-(ortho-Substituted)-PATs at the 5-HT_{2A} Receptor ............................... 46
   Affinity of 4-(ortho-Substituted)-PATs at the 5-HT_{2C} Receptor ............................... 47
   Affinity of 4-(meta-Substituted)-PATs at the 5-HT_{2A} Receptor ............................... 48
   Affinity of 4-(meta-Substituted)-PATs at the 5-HT_{2C} Receptor ............................... 52
   Affinity of 4-(para-Substituted)-PATs at the 5-HT_{2A} Receptor ............................... 55
   Affinity of 4-(para-Substituted)-PATs at the 5-HT_{2C} Receptor ............................... 58
   Affinity of 4-(meta-Substituted-Phenyl)- and/or (6, 7-substituted-Tetrahydronaphthalene)-PATs at the 5-HT_{2A} Receptor ........................................ 61
   Affinity of 4-(meta-Substituted-Phenyl)- and/or (6, 7-substituted-Tetrahydronaphthalene)-PATs at the 5-HT_{2C} Receptor ........................................ 64
   Computational Chemistry and Molecular Modeling Studies: In silico Docking of PATs at 5-HT_{2} receptors ............................................................. 67

3 Characterization of the 5-HT_{2} Functional Pharmacology of PAT Analogs

Specific Aim 2 .................................................................................................................. 84
Methodology ...................................................................................................................... 84
Results and Discussion for Functional Assays .............................................................. 86
   Functional Activity of (−)-trans-PAT and (−)-trans-4-(meta-halogenated)-PATs at 5-HT_{2} Receptors ............................................................. 86
   Functional Activity of (+) and (−)-trans-4-(p-Cl)-PATs at 5-HT_{2} Receptors .......... 88
   Functional Activity of (−)-trans-4-(m-Cl)-(6-OMe-Tetrahydronaphthalene)-PAT Analog at 5-HT_{2A} and 5-HT_{2C} Receptors .............................. 88

4 Translational Studies: Assessment of PAT Analogs Efficacy in Rodent Models of Psychosis

Specific Aim 3 .................................................................................................................. 97
Methodology ...................................................................................................................... 97
   In vivo Behavioral Pharmacology .................................................................................. 97
   Psycholocomotor Activity (Head Twitch Response; HTR) Elicited by the Serotonin 5-HT_{2} Agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) ............ 98
   Psycholocomotor Activity Elicited by the Glutamate Antagonist MK-801 ............... 98
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Shows changes in the bond length relative to changes in substituent.</td>
<td>81</td>
</tr>
<tr>
<td>2-2</td>
<td>Binding affinities of parent PAT enantiomers and <em>meta</em>-substituted PAT enantiomers at 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors.</td>
<td>81</td>
</tr>
<tr>
<td>2-3</td>
<td>Binding affinities of <em>para</em>-substituted PAT enantiomers at 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors.</td>
<td>82</td>
</tr>
<tr>
<td>2-4</td>
<td>Binding affinities of tetrahydronaphthalene ring with (or without) pendant phenyl ring substituted PAT analogs.</td>
<td>83</td>
</tr>
<tr>
<td>3-1</td>
<td>Functional activity of PAT analogs at 5-HT\textsubscript{2} receptors.</td>
<td>96</td>
</tr>
<tr>
<td>4-1</td>
<td>Efficacy of PAT analogs, ED\textsubscript{50} (95% ± CL) mg/kg, at <em>in vivo</em> models of schizophrenia.</td>
<td>115</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1-1</td>
<td>Generic 2-dimensional structure of GPCR with amino acids numbered from amino terminal to carboxy terminal.</td>
<td>40</td>
</tr>
<tr>
<td>1-2</td>
<td>3-dimensional representation of GPCR.</td>
<td>40</td>
</tr>
<tr>
<td>1-3</td>
<td>Extended ternary complex model.</td>
<td>41</td>
</tr>
<tr>
<td>1-4</td>
<td>Ligands activating serotonin 5-HT&lt;sub&gt;2&lt;/sub&gt; receptors.</td>
<td>41</td>
</tr>
<tr>
<td>1-5</td>
<td>Structure of hallucinogens (phenylethylamines and tryptamines) acting on 5-HT&lt;sub&gt;2&lt;/sub&gt; receptors.</td>
<td>41</td>
</tr>
<tr>
<td>1-6</td>
<td>Structure of M100,907. A selective antagonist of 5-HT&lt;sub&gt;2A&lt;/sub&gt; receptors.</td>
<td>42</td>
</tr>
<tr>
<td>1-7</td>
<td>Ligands used in in vivo models of psychosis.</td>
<td>42</td>
</tr>
<tr>
<td>2-1</td>
<td>Stereochemical relationship between PAT diastereomers and enantiomers.</td>
<td>72</td>
</tr>
<tr>
<td>2-2</td>
<td>Structure of meta-substituted PAT analogs.</td>
<td>73</td>
</tr>
<tr>
<td>2-3</td>
<td>Change in Ki value at 5-HT&lt;sub&gt;2A&lt;/sub&gt; receptors with increase in the size of substituent at meta-position.</td>
<td>73</td>
</tr>
<tr>
<td>2-4</td>
<td>Structure of para-substituted PAT analogs.</td>
<td>74</td>
</tr>
<tr>
<td>2-5</td>
<td>Effect of steric on the affinity of para-substituted PAT enantiomers at 5-HT&lt;sub&gt;2A&lt;/sub&gt; and 5-HT&lt;sub&gt;2C&lt;/sub&gt; receptors.</td>
<td>74</td>
</tr>
<tr>
<td>2-6</td>
<td>Single X-ray crystallographic structure of (2S,4R)-(-)-trans-p-Cl-PAT. HCl.</td>
<td>75</td>
</tr>
<tr>
<td>2-7</td>
<td>Tetrahydronaphthalene and/or pendant phenyl ring substituted PAT analogs.</td>
<td>75</td>
</tr>
<tr>
<td>2-8</td>
<td>(−)-trans-PAT enantiomer docked at 5-HT&lt;sub&gt;2A&lt;/sub&gt; receptors with the corresponding amino acid interactions in binding pocket.</td>
<td>76</td>
</tr>
<tr>
<td>2-9</td>
<td>(−)-trans-PAT docked at 5-HT&lt;sub&gt;2C&lt;/sub&gt; receptors with the corresponding amino acid interactions in binding pocket.</td>
<td>76</td>
</tr>
<tr>
<td>2-10</td>
<td>(−)-trans-m-Br-PAT enantiomer docked at 5-HT&lt;sub&gt;2C&lt;/sub&gt; receptors.</td>
<td>77</td>
</tr>
<tr>
<td>2-11</td>
<td>(2R,4S)-(+)trans and (2S,4R)-(−)-trans-p-Cl-PAT enantiomer docked at 5-HT&lt;sub&gt;2A&lt;/sub&gt; receptors.</td>
<td>77</td>
</tr>
<tr>
<td>2-12</td>
<td>(2R,4S)-(+)trans and (2S,4R)-(−)-trans-p-Cl-PAT enantiomer docked at 5-HT&lt;sub&gt;2C&lt;/sub&gt; receptors.</td>
<td>78</td>
</tr>
</tbody>
</table>
Effect of highest dose of various ligands on locomotor activity

Attenuation of amphetamine stimulated locomotor activity by PAT analogs

Attenuation of MK 801 induced locomotion.

Clozapine 1 mg/kg dose attenuates both MK-801 and amphetamine (Amp) induced locomotion.

Clozapine decreases DOI induced HTR in a dose dependent manner.

Attenuation of MK-801 induced locomotor activity by (+) and (−)-trans-PAT analogs

Attenuation of amphetamine stimulated locomotor activity by PAT enantiomers

Effect of highest dose of various ligands on locomotor activity
4-6 Attenuation of MK-801 and amphetamine hyperactivity by (−)-trans-m-Br-PAT. .............................................................. 110

4-7 Demonstrates the attenuation of DOI induced HTR by (+) and (−)-trans-m-Cl-PAT. .......................................................................................................................... 110

4-8 Attenuation of MK-801 and amphetamine hyperactivity by (−)-trans-m-Cl-PAT. .......................................................................................................................... 111

4-9 Attenuation of HTR by (+) and (−)-trans-p-Cl-PAT analogs. ......................... 111

4-10 Effect of oral administration of (+) and (−)-trans-p-Cl-PAT analogs 20 minutes prior to DOI. ................................................................. 112

4-11 Effect of (+) and (−)-trans-p-Cl-PAT on MK-801 (0.3 mg/kg) induced locomotor activity ................................................................. 113

4-12 Effect of (+) and (−)-trans-p-Cl-PAT on amphetamine (3 mg/kg) induced locomotor activity ................................................................. 114
LIST OF ABBREVIATIONS

\( \alpha \)-MSH \( \alpha \)-Melanocyte stimulating hormone

5-HT serotonin

ADAR adenosine deaminase acting on ribonucleic acid

CAMs constitutively active mutants

CNS central nervous system

DAG diacylglycerol

DMA 2, 5-dimethoxyphenylisopropylamine

DMEM Dulbecco’s modified Eagle’s medium

DOI 2, 5-dimethoxy-4-idoamphetamine

EPS Extrapyramidal side effects

GAP GTPase accelerating protein

GDP guanine diphosphate

GPCR G-protein coupled receptor

GTP guanine triphosphate

HDL high density lipoproteins

HEK human embryonic kidney

HPLC high-pressure liquid chromatography

HTR head-twitch response

HVD heart valve disease

IP\(_3\) inositol triphosphate

MAO-A monoamino oxidase A

\( m \)-CPP meta-chlorophenylpiperazine

NMDA N-methyl-D-aspartate
PAT phenylaminotetrahydronaphthalene
PIP2 phosphatidylinositol bisphosphate
POMC Pro-opiomelanocortin
RGS regulators of G-protein
RNA ribonucleic acid
SAR structure activity relationship
SN Substantia Nigra
VTA Ventral tegmental area
IN VITRO AND IN VIVO PHARMACOLOGY OF NOVEL PHENYLAMINOTETRALIN (PAT) ANALOGS AT SEROTONIN 5-HT\textsubscript{2} RECEPTORS: DEVELOPMENT OF DRUGS FOR NEUROPSYCHIATRIC DISORDERS

By

Krishnakanth Kondabolu

May 2013

Chair: Raymond G. Booth
Major: Pharmaceutical Sciences – Medicinal Chemistry

Novel 4-phenyl-\textit{N,N}-dimethyl-1,2,3,4-tetrahydroanphthalene-2-amine (phenylaminotetrahydronaphthalin; PAT) analogs synthesized in our laboratories have been shown to specifically activate the human serotonin 5-HT\textsubscript{2C} receptors while acting as antagonists/inverse agonists at 5-HT\textsubscript{2A} and 5-HT\textsubscript{2B} receptors. This unique 5-HT\textsubscript{2} pharmacology was hypothesized to translate to efficacy in animal models of psychosis. The specific aims here were to characterize the molecular determinants for 5-HT\textsubscript{2} affinity of new PAT derivatives synthesized before and during the course of this thesis research (Aim 1), characterize the 5-HT\textsubscript{2} functional pharmacology of PATs meeting minimum affinity criteria (Aim 2), and, determine if PATs that demonstrate 5-HT\textsubscript{2C} specific agonism together with 5-HT\textsubscript{2A/2B} antagonism/inverse agonism translate in preclinical studies to efficacious compounds to treat psychoses (Aim 3).

Some noteworthy results are that \textit{meta}- and \textit{para}-substitution of the 4-phenyl substituent impacted stereoselective high affinity of especially the \textit{trans}-PAT analogs across 5-HT\textsubscript{2} receptors. For example, the (2\textit{S},4\textit{R})\textendash\textendash(\textendash\textendash)enantiomer usually demonstrated highest affinity across 5-HT\textsubscript{2} receptors regarding \textit{meta}-substituted \textit{trans}-PATs, while,
the (2R,4S)-(+) -enantiomer usually demonstrated highest affinity across 5-HT₂ receptors regarding para-substituted trans-PATs. Results from ligand docking and molecular modeling studies conducted by a collaborator suggest that a conserved (across 5-HT₂ receptors) serine S5.43 residue in the fifth transmembrane domain impacts binding of the 4-para-substituted-PATs differently than for the corresponding 4-(meta-substituted)-PATs. The (2S,4R)-(−)-trans-(meta-chloro and meta-bromo)-PAT analogs had the highest affinity across 5-HT₂ receptors and these analogs also demonstrated highest potency and efficacy regarding 5-HT₂C agonism together with 5-HT₂A/2B antagonism/inverse agonism in functional studies using human recombinant 5-HT₂ receptors expressed in HEK clonal cells. Substitutions at the 6- and/or 7-position of tetrahydronaphthyl moiety, with or without concomitant halogen substitution at the 4-meta-phenyl-position, did not provide analogs with enhanced potency or efficacy regarding functional activity. Accordingly, the trans-4-(meta-chloro and meta-bromo)-PATs were selected for study in translational studies that also included the trans-4-(para-chloro)-PATs for comparison.

The PATs were assessed in three different rodent psycholocomotor models of psychosis, which encompass the dopaminergic, glutamatergic and serotonergic neurotransmitter dysfunction currently thought to underlie the pathophysiology of human psychoses. The (2S,4R)-(−)-trans-4-(meta-Cl and meta-Br)-PAT analogs demonstrated superior potency compared to (2S,4R)-(−)-trans- and (2R,4S)-(+) -trans-4-(para-Cl)-PAT analogs, suggesting that the in vitro medicinal chemistry structure-activity relationship accurately translated to predict the preclinical activity of PATs as antipsychotic drugs.
CHAPTER 1
G-PROTEIN COUPLED RECEPTORS: MOLECULAR DETERMINANTS AND THERAPEUTIC RELEVANCE OF SEROTONIN 5-HT$_{2A}$ AND 5-HT$_{2C}$ RECEPTORS IN SCHIZOPHRENIA

Introduction to G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) constitute the largest class of cell surface receptors in the human body, representing, about one percent of the genome, and involved in regulating nearly all known physiological functions$^1$. GPCRs transduce signals from stimuli including neurotransmitters, peptides, small proteins, and even photons of light, intracellularly across the membrane of cell$^2$. Genetic alterations in GPCRs may lead to loss or gain in function at the receptor and account for many diseases like diabetes insipidus, dwarfism and hypothyroidism$^3$-$^5$.

GPCR Structure

All GPCRs share a seven transmembrane structure despite the functional and chemical diversity of their signaling molecules. GPCRs are made up of an extracellular N terminus, seven transmembrane domains and an intracellular C terminus. The seven transmembrane domains are connected to each other by extracellular and intracellular loops (Figure 1-1 and 1-2). The N terminus, extracellular loops and transmembrane domain play a role in ligand selection and prevent non-specific ligands from binding to the receptor$^6$. The $\alpha$ helical transmembrane domains form a binding pocket for the ligand to dock and transmit the stimulus from the ligand to the G-protein via the intracellular loops. This external stimulus is then converted into a chemical messenger and propagated intracellularly$^7$.

Based on the homology of amino acid sequence, GPCRs can be classified into three classes: rhodopsin-like receptors (Class 1), secretin-like receptors (Class 2) and
metabotropic glutamate-like receptors (Class 3). GPCRs within the same class share more than 25% sequence similarity. GPCRs can also be differentiated based on the site of ligand binding: Class 1 receptors bind ligands in their transmembrane region, Class 2 receptors bind ligands in the extracellular loops and Class 3 receptors bind ligands in the N terminus (Venus fly trap).

**GPCR Signal Transduction**

The G-proteins couple to GPCR and mediate the transduction of the external stimuli into intracellular signaling molecules. G-protein is a heterotrimeric protein consisting of α, β and γ sub-units. The α sub-unit is a Ras-like protein and possesses GTPase activity. In its inactivated state the α sub-unit binds to guanosine diphosphate (GDP) and exists as a trimer with the β and γ sub-units. Fatty acylation (palmitoyl group) of α subunit and isoprenylation (farnesyl and geranyl groups) of γ subunit aid in anchoring G-protein at the interface of the plasma membrane. This anchoring of the G-protein is done such that it is in close proximity with both the GPCR and effector systems.

A GPCR system consists of a GPCR, a G-protein and an effector. Activation of G-protein by the GPCR results in the exchange of GTP for a GDP leading to the dissociation of the G-protein trimer into Gα and Gβγ subunits. Both these sub-units can now bind to different effector systems. G-protein mediated activation of effectors produce secondary messengers that propagate the signal by activation or inactivation of a cascade of proteins. Activity of G-protein ceases when the Gα subunit metabolizes the GTP back to GDP. Following this the G-protein collapses back into its inactive trimeric conformation. Some examples of effector systems activated by Gα sub-unit include phospholipase C, phospholipase A2 and adenyl cyclase. Mammals have about 20
different α sub-units that may complex with any of the 5 β sub-units and 12 γ sub-units. These sub-units can be arranged in different permutations and can give rise to a vast signaling diversity for the GPCR. The Gβγ sub-unit is also capable of activating different effector systems. For example, the Gβγ sub-unit regulates the responsiveness of sodium, potassium and calcium channels\textsuperscript{11,14}. Different G-protein sub-units are capable of activating different secondary messengers. For example, activation of Gα\textsubscript{q} leads to activation of phospholipase C that catalyzes the conversion of phosphatidylinositol bisphosphate (PIP\textsubscript{2}) into inositol triphosphate (IP\textsubscript{3}) and diacyl glycerol (DAG). In contrast to this, activation of Gα\textsubscript{s} sub-unit leads to activation of adenyl cyclase enzyme that mediates the cyclization of ATP into cAMP.

**Models of Ligand—GPCR Interactions**

Ligands that bind to GPCRs to elicit a pharmacological response can be endogenous or exogenous in origin. Agonist ligands stabilize an active conformation of the receptor that couples to G-protein, whereas, antagonist ligands stabilize a conformation that does not favor the coupling of GPCR to G-proteins\textsuperscript{15}. However, it was later discovered that GPCRs are capable of activating G-proteins even in the absence of ligands\textsuperscript{16}. This property of the GPCRs is referred to as constitutive activity or basal activity\textsuperscript{17}. Ligands capable of decreasing this basal activity of the receptor are referred to as inverse agonists. The level of constitutive activity depends on the intrinsic property of the receptor and also on the cellular milieu in which the receptor is present\textsuperscript{18}. For example, when both serotonin 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors were expressed to the same density in a heterologous system, 5-HT\textsubscript{2A} receptors showed a lower level of basal activity as compared to 5-HT\textsubscript{2C} receptors\textsuperscript{19}.
The extended ternary complex model is the widely accepted model of GPCR activation\textsuperscript{20}. This theory postulates that a receptor exists in equilibrium between its inactive and active state conformations. GPCRs exist in equilibrium between active conformation, inactive conformation and active G-protein coupled conformation. A ligand may bind with different affinities to any of these receptor conformations (Figure 1-3)\textsuperscript{21}. Agonists have higher affinity for a GPCR in an active conformation as compared to inactive conformation. The coupling of the G-protein to an agonist-stabilized receptor is positively cooperative. This means that binding of a ligand to a GPCR enhances the interaction between GPCR and G-protein. The activation of the G-protein by substituting the GDP for a GTP is negatively cooperative. This is because the binding of GTP induces a conformational change in the G-protein that is 'transmitted' to the GPCR and negatively influences the binding of the agonist. This also initiates the uncoupling of the G-protein from the GPCR. The constitutive activity of the receptor and the affinity of the agonist depend on the amount of GPCR expressed on the membrane relative to the amount of G-protein expressed in the cell system.\textsuperscript{18} Over-expression of the GPCR in a heterologous system results in majority of the GPCRs to be in a G protein-uncoupled state, i.e., a low affinity conformation for binding of agonist ligands.

**Ballesteros and Weinstein GPCR Nomenclature**

Numbering the amino acids starting from the amino terminal and ending at the carboxy terminal does not allow for an efficient comparison of amino acid sequence between GPCRs. Significant diversity between GPCRs present in same class or family hinders such a system of nomenclature. This system of nomenclature also does not give information about the position of the amino acid within the GPCR ternary structure, that is, whether it is present in the extracellular loop, transmembrane domain or
intracellular loop. Ballesteros and Weinstein devised a numbering system for amino acids based on the characteristic conserved amino acid present in GPCRs belonging to the same class. In this system of nomenclature, each amino acid is identified first based on its location in the transmembrane domain. The most conserved residue in the transmembrane domain within the same family is then identified and assigned the number 50. The position of other amino acids is defined relative to this conserved amino acid. For example, asparagine amino acid in transmembrane 1 is the most conserved amino acid across class 1 GPCRs and hence is designated as N1.50, where 1 represents the transmembrane domain. G1.49 represents the glycine amino acid preceding the conserved asparagline when looking down from the extracellular region into the intracellular region. Numbering in this system increases as we move from the extracellular to the intracellular end of transmembrane 1, then, the numbering increases going from the intracellular to extracellular end of transmembrane 2, and so on.

Mutagenesis studies involve mutations of amino acids in the wild type receptor to a different amino acid. Ballesteros nomenclature can be used to identify both the amino acid in the wild type receptor and the amino acid to which it has been mutated. For example, A5.46 represents an alanine amino acid present in the fifth transmembrane of the wild type serotonin 5-HT$_{2A}$ receptors. When this amino acid is mutated to a serine, it is represented as A5.46S.

**Ligand Stabilized GPCR Conformations**

Binding of an agonist to a GPCR stabilizes a conformation of the GPCR that favors the coupling of a G-protein to the receptor. In contrast, an inverse agonist stabilizes a conformation of a GPCR that does not favor the binding of G-proteins resulting in a signaling outcome that is less than basal constitutive signaling. X-ray
crystal structures give a remarkable insight into the conformational changes involved in GPCR activation. Obtaining a crystal structure of a GPCR is difficult, however, due to GPCR inherent thermodynamic flexibility within the phospholipid membrane environment and a heterogeneity of receptor conformations. Purified GPCRs are not stable in detergents which are compatible with crystallography. GPCRs possess structural heterogeneity because of the different post-translational modifications such as phosphorylation, palmitoylation and glycosylation. This structural heterogeneity along with the conformational heterogeneity makes it difficult to standardize a common procedure for GPCR crystallization.

Despite these difficulties a few chimeric GPCRs have been crystallized. Some generalizations can be made about the organization and position of the amino acids in class 1 GPCRs based on the information provided by these crystal structures. For example, for all class 1 GPCRs the amino acids in the alpha helices at the interface between the membrane and the external milieu are comprised mainly of positively charged amino acids that interact with the negatively charged phospholipid heads of the lipid bilayer. The cytoplasmic end of the GPCR has critical residues that interact with G-protein. A conserved DRY (aspartate, arginine, tyrosine) motif at the end of transmembrane domain 3 is surrounded by several hydrophobic residues from helix II (P2.38, L2.39), cytoplasmic loop II (F4.37), helix V (L5.61, V5.65) and helix VI (V6.33, M6.36). These residues together with amino acids from the intracellular loops are hypothesized to constitute the binding site of the G-protein.

The X-ray crystal structures of rhodopsin, β2 adrenergic receptor, adenosine 2A receptor, β1 adrenergic receptor (turkey), dopamine D3, chemokine CXCR-4, and
histamine H₁ class 1 GPCRs have been determined. X-ray crystal structures give an insight about the ‘molecular switches’ involved in the activation of the receptor. Molecular switches are non-covalent interactions between amino acids present in the inactive receptor that are disrupted upon activation of the receptor. Some of the highly conserved motifs that act as molecular switches include the D/ERY motif (ionic lock) at the end of transmembrane 3 interacting with N6.30 at the end of transmembrane 6 and other switches includes C6.47, W6.48 and F6.52 in transmembrane domain 6 (rotamer toggle switch). Conserved amino acid P6.50 introduces a kink in the α helix 6 for a majority of class 1 GPCRs. As a consequence of this kink, the cytoplasmic end of transmembrane 6 is angled towards the cytoplasmic end of transmembrane 3. A cluster of conserved aromatic amino acids F6.44, W6.48, F6.51 and F6.52 surround P6.50 and regulate the angle of the kink (rotamer toggle switch). This cluster of aromatic amino acids faces the binding pocket and agonists interacting with these amino acids induce a conformation change such that the angle of the kink changes resulting in the activation of the receptor.

An agonist when bound to a receptor can activate the receptors in two possible ways. It could disrupt existing amino acid interactions that hold the receptor in the inactive conformation, or it could stabilize a conformation of the receptor that is more active. Partial agonist and agonist differ in the conformation of the receptor they stabilize. For example, catechol, salbutamol and dopamine are partial agonists capable of activating β adrenergic receptors. Despite being partial agonists, these ligands differ in the way they activate the receptor. Catechol stabilizes a conformation of the receptor where the rotamer toggle switch is activated, but the ionic lock is not broken.
Salbutamol stabilizes a conformation where the ionic lock is broken, but the rotamer toggle switch is not activated. In contrast to this, dopamine activates both the rotamer toggle switch and breaks the ionic lock. Taken together, these results indicate that different ligands activate different molecular switches thereby stabilizing different conformations of the receptor. They also show that there are more molecular switches yet to be discovered since dopamine, despite activating both the molecular switches, still behaves as a partial agonist\textsuperscript{27}.

Crystal structures provide an understanding of GPCR–ligand molecular interactions and give inferences about GPCR conformations that lead to agonist or inverse agonist or neutral antagonism functional outcomes. The GPCR molecular determinants involved in ligand recognition and GPCR conformational changes, in turn, provide inferences on how ligands interact with GPCRs to stabilize a particular conformation that leads to agonist, inverse agonist or neutral antagonist function. The few known GPCR crystal structures provide templates for homology modeling of GPCRs that have not yet been crystallized. For example, ligand docking results presented in this thesis were based on the 5-HT\textsubscript{2} receptor homology models developed based on human \(\beta\)-adrenergic GPCR crystal structure\textsuperscript{29}. The 5-HT\textsubscript{2} homology models helped delineate the 3-dimensional arrangement of amino acids involved in binding of novel 4-phenyl-\(N,N\)-dimethyl-1,2,3,4-tetrahydroanphthalene-2-amine (PAT) ligands\textsuperscript{30}. Results were used to design ligands that might better exploit hypothesized PAT–5-HT\textsubscript{2} molecular interactions toward higher affinity ligands. Results were corroborated by mutagenesis studies that helped to validate hypothesized PAT–5-HT\textsubscript{2} molecular interactions, thus, validating the 5-HT\textsubscript{2} homology models.
Serotonin and 5-HT\textsubscript{2} GPCRs

Serotonin Biosynthesis and Metabolism

Serotonin (5-hydroxytryptamine, 5-HT) is synthesized both in the central nervous system and the periphery from the aromatic amino acid tryptophan (Figure 1-4). Synthesis of serotonin involves a two-step process; the first step involves hydroxylation of tryptophan by tryptophan hydroxylase. This hydroxylation step is the rate-limiting step in the synthesis of serotonin. There are two isoforms of tryptophan hydroxylase: tryptophan hydroxylase 1 and 2. Tryptophan hydroxylase 1 is primarily localized in the periphery and tryptophan hydroxylase 2 regulates the synthesis of serotonin in the central nervous system\textsuperscript{31}. 5-hydroxytryptophan, the product of tryptophan hydroxylase, is decarboxylated by 5-hydroxytryptophan decarboxylase to produce serotonin. Serotonin is primarily metabolized by monoamine oxidase A (MAO-A) into 5-hydroxyindoleacetic acid, an inert metabolite that is eliminated from the body. Serotonin can also be metabolized by indole \textit{N}-methyl transferases to generate \textit{N}-methyl serotonin and \textit{N,N}-dimethyl serotonin\textsuperscript{32}.

Serotonin 5-HT\textsubscript{2} GPCRs

Serotonin acts at 7 different families of GPCRs (5-HT\textsubscript{1,2,4-7}), an ion channel family (5-HT\textsubscript{3})\textsuperscript{33}, and the serotonin neurotransporter (SERT), which terminates the action of serotonin released into the synapse and assists in recycling of serotonin\textsuperscript{34}. The work in this thesis focuses on the serotonin 5-HT\textsubscript{2} class 1 GPCRs that are involved in the regulation of diverse physiological processes (e.g., vascular smooth muscle contraction, sleep cycle) and psychological processes (e.g., psychoses, anxiety, mood)\textsuperscript{35}. 


5-HT_2 GPCR Signaling Pathways

The serotonin 5-HT_2 family consists of three GPCRs (5-HT_2A, 5-HT_2B and 5-HT_2C) that share significant structural homology and the same main signaling pathway. For example 5-HT_2A and 5-HT_2C receptors share 80% sequence similarity within their transmembrane region and a 50% overall sequence similarity\(^{36}\). Serotonin 5-HT_2 receptors also share similarity in signaling pathway due to the coupling with the same G-proteins. These receptors couple to Gq G-protein that in turn activates the phospholipase C. Phospholipase C catalyzes the conversion of phosphatidylinositol bisphosphate (PIP_2) into water-soluble inositol triphosphate (IP_3) and lipophilic diacyl glycerol (DAG). Inositol triphosphate binds to IP_3 receptors on the endoplasmic reticulum causing the release of calcium. DAG mediates the activation of protein kinases that play a key role in the phosphorylation of numerous enzymes\(^{37}\). Different ligands dose dependently modulate the activity of effector system to produce secondary messenger. These secondary messengers can be measured to determine the extent of 5-HT_2 receptor activation or inactivation by a test ligand.

The serotonin 5-HT_2 receptors have been shown to also couple to other phospholipases like phospholipase D and phospholipase A_2. Activation of phospholipase A_2 catalyzes the cleavage of PIP_2 to produce arachidonic acid and lysophospholipid\(^{38}\). Phospholipase D catalyzes the hydrolysis of phosphatidylcholine into phosphatidic acid and choline\(^{39}\).

5-HT_2C GPCR Post-Translational Modifications

5-HT_2C receptors are unique in their ability to undergo RNA editing. RNA editing is a type of post-transcriptional modification, like splicing, which alters the primary nucleotide in the pre-mRNA\(^{40}\). RNA editing is characterized by the deamination of
adenosine to form inosine and is catalyzed by adenosine deaminase that acts on RNA (ADAR). The ADAR enzymes can convert adenosine into inosine at 5 different locations on the pre-mRNA of 5-HT₂C receptors. These 5 nucleotides correspond to 3 amino acids located on the second intracellular loop of 5-HT₂C receptors. The unedited human 5-HT₂C receptor has an isoleucine, asparagine and isoleucine (INI) at position 156, 158 and 160 in the amino acid sequence⁴¹. Editing these three amino acids result in 24 isoforms of the 5-HT₂C receptor with different amino acids at these positions⁴². For example, the fully edited isoform replaces the INI amino acids with valine, glycine and valine (VGV) amino acids, and a partially edited isoform has valine, serine and valine (VSV) amino acids at these positions. The mRNA of partially edited VSV isoform is the most abundant isoform in the human brain (33%) where as the VNV isoform mRNA is the most abundant in the rat brain (33%).

The second intracellular loop of GPCR plays an important role in coupling with G-proteins to the GPCR. Therefore alterations of the amino acids at this position due to RNA editing result in reduction in the constitutive activity of the edited isoforms. The unedited INI isoform possesses the highest basal activity while the fully edited isoform VGV had a very low basal activity. The partially edited VSV isoform showed intermediate basal activity⁴³. These isoforms also differ in their functional profile, that is, the potency of an agonist to activate the different isoforms of 5-HT₂C receptors. Agonists like 5-HT, 2,5-dimethoxy-4-iodoamphetamine (DOI) and N,N-dimethyltryptamine (DMT) had a higher affinity and potency to activate the INI isoform relative to the VSV isoform and VGV isoforms. Post-transcriptional RNA editing of the 5-HT₂C receptors contributes to its molecular diversity of signaling. The mRNA level of the edited isoforms were
different in different regions of the brain indicating varying levels of basal activity of 5-HT$_{2C}$ receptors in different brain regions$^{41}$. However, it is difficult to determine the level of 5-HT$_{2C}$ receptor isoform protein expressed on the membrane as it is not proportional to the amount of mRNA detected$^{42}$.

The INI (unedited wild type) isoform of 5-HT$_{2C}$ receptors was used to assess the 5-HT$_{2C}$ affinity and function of the PAT analogs in this thesis. Only the cDNA for the wild type receptor was available commercially. It is noted that the *in vivo* variation of 5-HT$_{2C}$ edited isoform expression in different mammalian brain regions is not accurately reflected by the *in vitro* molecular pharmacology studies here; in fact, as noted, the unedited wild type 5-HT$_{2C}$ receptor is not the major isoform in rodents or humans. Nevertheless, the importance of not accounting for the possibility of 5-HT$_{2C}$ post-translational editing may not be so great in the transient transfection clonal cell system used in the current studies. This is because GPCR’s are greatly overexpressed relative to G-protein, thus, G protein availability and not receptor–G protein interaction may be the limiting factor in functional outcomes. In any event, the molecular pharmacology methods used here were successful to identify and translate PATs with potential antipsychotic activity (see Aim 3 Results).

**5-HT$_2$ GPCR Localization in Brain**

The location of the 5-HT$_2$ receptors in the CNS has been characterized using radioligand binding and immunohistochemical studies$^{33}$. These studies revealed a high localization of the 5-HT$_{2A}$ receptors in forebrain regions (particularly cortical areas like neocortex and pyriform cortex), caudate nucleus, nucleus accumbens and hippocampus. Serotonergic neurons are clustered in the dorsal raphe nucleus and these neurons project from here to other parts of the brain. 5-HT$_{2A}$ receptors are located...
post-synaptically along the distribution of these axons from dorsal raphe nucleus\textsuperscript{44}. The 5-HT\textsubscript{2A} receptors are also widely distributed in the periphery. In contrast, 5-HT\textsubscript{2C} receptors are exclusively present in the CNS. Serotonin 5-HT\textsubscript{2C} receptors are distributed in limbic system (nucleus accumbens), ventral tegmental area (VTA) and basal ganglia (substantia nigra and caudate nucleus).

**Schizophrenia and Serotonin 5-HT\textsubscript{2} GPCRs**

Schizophrenia is a chronic mental disorder that is prevalent across 1-2\% of world population. Clinically, schizophrenia is characterized by “positive” and “negative” perceptual and behavioral symptoms, and, cognitive symptoms. Positive perceptual and behavioral symptoms manifest as hallucinations (mostly auditory) and delusions, whereas, negative symptoms manifest as apathy, social withdrawal and anhedonia. Cognitive symptoms include deficits in attention and memory\textsuperscript{45, 46}. The pathophysiology of schizophrenia is hypothesized to include excessive stimulation of striatal dopamine D\textsubscript{2} receptors, a deficient activation of prefrontal dopamine D\textsubscript{1} receptors (dopamine theory) and alterations in glutamatergic \textit{N}-methyl-D-aspartate (NMDA) receptor connectivity in prefrontal cortex (glutamate theory). Increased activation of mesolimbic dopaminergic pathway is hypothesized to mediate positive symptoms of schizophrenia\textsuperscript{47-49}. Mesocortical projections of dopamine neurons are hypothesized to be hypoactive in schizophrenia. This decreased activity is thought to contribute to negative and cognitive deficits in schizophrenia\textsuperscript{50}. The negative and cognitive deficits can also be attributed to NMDA hypofunction in prefrontal cortex. Multiple lines of evidence implicate the role of glutaminergic neurotransmission in schizophrenia: risk genes for schizophrenia affect functioning of NMDA receptor and administration of NMDA receptor antagonist to normal subjects produce symptoms similar to negative
and cognitive deficits observed in schizophrenia\textsuperscript{51}. In addition to glutamate and dopamine neurotransmission, there is a significant role played by serotonergic neurotransmission in schizophrenia, including, specifically, serotonergic neurotransmission involving 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} GPCRs.

**Relevance of 5-HT\textsubscript{2} GPCRs in Schizophrenia**

Serotonin 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors are localized in brain regions (prefrontal cortex, VTA and accumbens) that regulate both glutamatergic and dopaminergic systems. Numerous clinically approved newer atypical antipsychotics (olanzapine, clozapine, aripiprazole) exert an antagonist action at 5-HT\textsubscript{2A} receptors\textsuperscript{52}. Lack of motor side-effects in the newer antipsychotics has been attributed to their action at serotonin receptors\textsuperscript{53}. Further evidence supporting involvement of 5-HT\textsubscript{2A} receptors in schizophrenia is provided by similarities between hallucinogen induced psychosis and schizophrenia\textsuperscript{54, 55}. Majority of the post-mortem studies on brain tissue of schizophrenic patients reveal decreased levels of 5-HT\textsubscript{2A} receptors. However, it should be acknowledged that not all post-mortem studies found decreased 5-HT\textsubscript{2A} receptor levels in schizophrenic patients. Serotonin 5-HT\textsubscript{2A} receptor T102C and A1438G polymorphism has been reported to increase the risk of schizophrenia\textsuperscript{56, 57}.

**Serotonin 5-HT\textsubscript{2A} Receptors and Schizophrenia**

Serotonin 5-HT\textsubscript{2A} receptors are primarily localized on pyramidal neurons in prefrontal cortex and can regulate the activity of corticotegmental neurons by enhancing glutamate release in VTA. This increased glutamate in turn stimulates glutamate receptors on VTA dopaminergic projections resulting in an increased activity of mesocortical projections\textsuperscript{58}. Administration of serotonin to a midbrain slice increases the firing of a large population of cells in the VTA and this firing was blocked by a selective
antagonist of 5-HT$_{2A}$ receptors$^{59,60}$. This indicates the presence of 5-HT$_{2A}$ receptors in VTA and demonstrates their ability to modulate dopaminergic output from VTA. 5-HT$_{2A}$ receptors are localized on the dopaminergic neurons and are directly involved in their activation$^{61}$. Similar results were obtained in nucleus accumbens where administration of 5-HT$_{2A}$ receptor antagonists decreased 5-HT induced dopamine release in accumbens$^{62}$.

Administration of non-competitive NMDA receptor antagonist to normal subjects results in a behavioral response similar to negative and cognitive deficits of schizophrenia$^{63}$. Both 5-HT$_{2A}$ receptor antagonists and atypical antipsychotic, clozapine, reversed this blockade of NMDA receptor in pyramidal neurons of medial prefrontal cortex. These findings suggest that 5-HT$_{2A}$ antagonists may have a beneficial effect on cognitive deficits in schizophrenia$^{64}$.

**Serotonin 5-HT$_{2B}$ and 5-HT$_{2C}$ Receptors and Schizophrenia**

There is no evidence implicating the 5-HT$_{2B}$ receptors in schizophrenia. However, activation of 5-HT$_{2B}$ receptors has been shown to produce cardiac valvulopathy or heart valve disease (HVD) in the periphery. HVD was found to be a major side effect of the drug combination fenfluramine - phenteramine (Pondimin) (Figure: 1-4). This drug combination was therapeutically useful for weight loss. The mechanism of action of fenfluramine is by indirect activation of serotonergic system by releasing serotonin from their presynaptic vesicles and also reversing the function of serotonin transporter thereby increasing 5-HT concentration in the synapse$^{65}$. Neither of the fenfluramine enantiomers had a good affinity at 5-HT$_{2B}$ receptors. However, norfenfluramine, a metabolite of fenfluramine was found to be an agonist at 5-HT$_{2B}$ receptors. Norfenfluramine mediated activation of 5-HT$_{2B}$ receptors leads to increased mitogenic
activity resulting in matrix remodeling on the heart valves ultimately leading to HVD\textsuperscript{66}. Hence it is important to screen ligands for activity at 5-HT\textsubscript{2B} receptors.

Serotonin 5-HT\textsubscript{2C} receptors are localized in both the VTA and substantia nigra (SN). This indicates their ability to modulate both mesolimbic and nigrostriatal dopaminergic pathways\textsuperscript{67}. Administration of a 5-HT\textsubscript{2B} and 5-HT\textsubscript{2C} receptor antagonist increases the firing rate of dopaminergic neurons in both VTA and SN\textsuperscript{68}. However, a 5-HT\textsubscript{2C} receptor agonist selectively decreased the firing of mesolimbic pathway and did not affect basal firing of nigrostriatal pathway\textsuperscript{69}. These data indicate that 5-HT\textsubscript{2C} receptors could tonically inhibit dopaminergic output from nigrostriatal pathway either by constitutive activity or endogenous serotonin mediated activation. These 5-HT\textsubscript{2C} receptors are hypothesized to be located on the GABAergic interneurons of the VTA\textsuperscript{70}.

There is relatively less evidence for the presence of 5-HT\textsubscript{2C} receptor mRNA in prefrontal cortex. Studies demonstrate the presence of 5-HT\textsubscript{2C} receptors in cingulate cortex\textsuperscript{71}. Hence, more studies focus on the effects of 5-HT\textsubscript{2C} receptor ligands on mesocortical dopaminergic pathway (also originating from VTA). Similar to mesolimbic pathway, 5-HT\textsubscript{2C} receptors tonically inhibit the release of dopamine in mesocortical pathway and 5-HT\textsubscript{2C} agonist further decrease the dopamine released in prefrontal cortex\textsuperscript{72}.

\textbf{Antipsychotics Drugs and Obesity}

The major side effect of atypical antipsychotics is weight gain and the hypothesized neurotransmitter receptors implicated in this are 5-HT\textsubscript{2A}, 5-HT\textsubscript{2C}, histamine H\textsubscript{1} receptor, adrenergic $\alpha\textsubscript{1}$, $\alpha\textsubscript{2}$ receptors and muscarinic M\textsubscript{3} receptor\textsuperscript{73}. This study by Kroeze \textit{et al.} showed the correlation between binding affinity of antipsychotics
at these receptors and weight gain. The correlation was found to be highest for the H<sub>1</sub> receptors followed by α<sub>1A</sub>, followed by 5-HT<sub>2C</sub> receptors. Transgenic mice with 5-HT<sub>2C</sub> receptors knocked out showed an increase in weight<sup>74</sup>. Further evidence for the role of 5-HT<sub>2C</sub> receptors in weight gain was provided by FDA approval of 5-HT<sub>2C</sub> receptor agonist, lorcaserin, to treat obesity<sup>75</sup>. Several experimental ligands that act as agonists at 5-HT<sub>2C</sub> receptor also decrease food consumption in animals<sup>76,77</sup>.

The anorexic actions of serotonin 5-HT<sub>2C</sub> receptors are hypothesized to be mediated by pro-opiomelanocortin (POMC) neurons located in arcuate nucleus of hypothalamus. Activation of 5-HT<sub>2C</sub> receptors triggers the conversion of POMC, a peptide precursor, into α-melanocyte stimulating hormone (α-MSH). α-MSH is then released by these activated neurons; this was detected by Fos-like immunoreactivity detected in arcuate nucleus following administration of D-fenfluramine<sup>78</sup>. α-MSH interacts post-synaptically with melanocortin 3 and 4 receptors to alter energy homeostasis and decrease food intake<sup>79,80</sup>. Around 80% of α-MSH neurons were found to have 5-HT<sub>2C</sub> mRNA indicating the modulation of melonocortin pathways by serotonin 5-HT<sub>2C</sub> receptors. This mechanism of action explains the weight gain side effects observed following administration of atypical antipsychotics which act as antagonist at 5-HT<sub>2C</sub> receptors.

**Molecular Determinants for Ligand Binding at 5-HT<sub>2</sub> GPCRs**

The ligand-binding pocket for serotonin 5-HT<sub>2</sub> receptors is well characterized for some ligands. Structural features of ligands binding at 5-HT<sub>2</sub> receptors give insight about the binding pocket based on the ligand-GPCR interactions. Hallucinogens that bind to 5-HT<sub>2</sub> receptors can be classified into tryptamines and phenylethylamines. The
tryptamines include the structurally rigid ergolines like lysergic acid diethylamide (LSD) and the more flexible tryptamines like N,N-dimethyl-tryptamine (DMT). The phenylalkylamines also have structurally flexible ligands like 2,5-dimethoxy-4-iodoamphetamine (DOI) that bind to 5-HT$_2$ receptors (Figure 1-5).

Aspartate amino acid D3.32 is highly conserved in aminergic GPCR’s and interacts with positively charged amines moiety of ligands binding at 5-HT$_2$ receptors. Mutations of this amino acid resulted in a loss of binding at 5-HT$_{2C}$ receptors indicating that this interaction is essential for ligand binding. Aspartate D3.32 anchors the terminal amines of ligands and assists in binding at 5-HT$_2$ receptors. Serine amino acid, S3.36, is present one helical turn below the aspartate and also participates in ligand binding by forming hydrogen bonds with terminal amine of ligand. Mutations of this amino acid to alanine resulted in a decrease in binding affinity for primary amines (5-HT). However, there was no change in affinity for tertiary amines like LSD or DMT at 5-HT$_{2A}$ receptors. Similar results were obtained for 5-HT$_{2C}$ receptors, where a more structurally rigid tertiary amine [(2S, 4R)-trans-4-phenyl-2-N,N-dimethyl-1,2,3,4-tetrahydronaphthalene-2-amine [(–)-trans-PAT] showed only a slight decrease in affinity.

Similar to β$_2$ adrenergic receptor, molecular switches like the ‘ionic lock’ between intracellular end of transmembrane 3 and intracellular end of transmembrane 6 hold 5-HT$_{2A}$ receptors in inactive conformation. Mutation of the serine residue, S5.43A, leads to a decrease in affinity for serotonin at 5-HT$_{2A}$ receptors. The amino acid at 5.46 is a serine at 5-HT$_{2A}$ receptors but is an alanine at 5-HT$_{2C}$ receptors. Interchanging these amino acids switched the binding profiles of ergoline derivatives like mesulergine and
LSD. Their affinities at the corresponding receptors were reversed, for example the affinity at 5-HT$_{2A}$ receptor mutant S5.46A was similar to affinity at 5-HT$_{2C}$ receptors$^{87}$. This position has also been shown to sterically modulate the efficacy of ligands by determining whether the ligand is an agonist or a partial agonist at 5-HT$_{2A}$ receptors. The S5.46A mutation resulted in partial agonists, 1-N-methyl-5-hydroxy tryptamine and 1-N-methyl tryptamine acting as full agonists at 5-HT$_{2A}$ receptor mutants$^{88}$.

Two phenylalanines in transmembrane 5, F5.47 and F5.48, are also important for binding of ligands at 5-HT$_{2A}$ receptors. Mutation of F5.47 to alanine decreased the affinity of ketanserin and ritanserin at 5-HT$_{2A}$ receptors$^{89}$. A number of residues in transmembrane 6 (L6.37, N6.29, K6.32, C6.34 and V6.36) have been shown to be important for 5-HT$_{2A}$ mediated phosphotidyl inositol hydrolysis$^{90}$. Some of the aromatic amino acids (W6.48, F6.51 and F6.52) present in transmembrane 6 have also been shown to be important for activation of 5-HT$_{2A}$ receptors$^{91-93}$. These aromatic amino acids form a $\pi-\pi$ interaction with aromatic systems in ligand$^{89}$. Tyrosine, Y7.43, also had a significant effect in binding of 5-HT and ketanserin at 5-HT$_{2A}$ receptors$^{91}$.

**In Vivo Models to Screen for Antipsychotic Drug Efficacy**

The head-twitch response (HTR) is an easily observable and quantifiable dose-dependent response in rodents that is elicited by hallucinogens and is proposed as a model to screen for antipsychotic drug efficacy$^{55,94}$. Serotonin 5-HT$_2$ agonists that cause hallucinations in humans produce the HTR, however, 5-HT$_2$ agonists that do not produce hallucinations in humans do not exhibit the HTR in rodents. It is noted, however, that the hallucinations produced by 5-HT$_2$ agonists in humans are primarily visual in nature as compared to positive symptoms of schizophrenia that are primarily...
auditory hallucinations\textsuperscript{54}. Nevertheless, 5-HT\textsubscript{2A} receptors are thought to play a role in schizophrenia, in part, because hallucinogens mediate their action by activating 5-HT\textsubscript{2A} receptors in cortical regions of the brain\textsuperscript{95}. Also, as noted, most antipsychotic drugs act as antagonists at 5-HT\textsubscript{2A} receptors, and, correspondingly, these antipsychotic drugs possess the ability to decrease the hallucinogen-induced HTR in rodents\textsuperscript{96, 97}. Furthermore, there is a sensitization to the psychotic effects of amphetamine in schizophrenic patients, and, administration of amphetamine also results in sensitization of HTR in rodents\textsuperscript{98}. These observations argue in favor of HTR as a model to screen compounds for antipsychotic activity. Interestingly, M100907, a selective antagonist of 5-HT\textsubscript{2A} receptors, despite being effective in HTR model demonstrates only limited efficacy as an antipsychotic (Figure 1-6). An important limitation of the HTR is that it does not model the negative and cognitive deficits observed in schizophrenia. The HTR also does not model the hypothesized genetically-linked forms of schizophrenia\textsuperscript{99, 100}. At best, the DOI-induced HTR possesses some validity as a model to screen for drugs that may modulate the positive symptoms of schizophrenia. However, this model does show ‘false positives’ results and hence should be used in conjunction with other behavioral models of schizophrenia. The HTR model in the studies of this thesis involves 2,5-dimethoxy-4-iodoamphetamine (DOI) as the hallucinogen to induce the HTR in mice (Figure 1-7). DOI binds with a high affinity at 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B} and 5-HT\textsubscript{2C} receptors and acts as an agonist or partial agonist at these receptors\textsuperscript{101}. Herein, the HTR is applied as a model to screen for drugs capable of modulating the serotonergic neurotransmission dysfunction thought to be part of the pathophysiology of psychoses in humans.
As mentioned, dysfunction of glutaminergic neurotransmission is also thought to contribute to the pathophysiology of schizophrenia. Psychotomimetic drugs like MK-801 and ketamine are non-competitive antagonist at NMDA receptors, and, in fact, administration of ketamine to schizophrenic patients results in a dose-related increase in both positive and negative symptoms (Figure 1-7)\textsuperscript{102-105}. Hence, the psycholocomotor behavioral responses in rodents that result from acute administration of NMDA antagonists (such as MK-801) have been used to screen for drugs with antipsychotic activity. These behavioral responses to acute NMDA antagonist administration include: hyperlocomotion, disrupted sensorimotor gating, stereotypic behavior and cognitive deficits\textsuperscript{106-108}. Cognitive and sensorimotor gating deficits are some of the well-characterized symptoms of schizophrenia. Several studies have demonstrated the efficacy of clinically approved antipsychotics to attenuated acute MK-801 induced hyperlocomotor activity\textsuperscript{109-112}. The disrupted glutamatergic transmission model appears to have more validity than the HTR as a behavioral model for schizophrenia. For example, genetically-linked forms of schizophrenia are thought to involve altered glutamatergic neurotransmission\textsuperscript{100}. Also, ligands acting on NMDA receptors have been shown to be beneficial in treating negative symptoms of schizophrenia\textsuperscript{113}. Accordingly, in addition to the HTR model, the studies in this thesis utilized MK-801 induced hyperlocomotor activity as model to test the efficacy PAT analogs as potential antipsychotics.

Dysfunction of dopaminergic neurotransmission (mainly hyper-dopaminergic neurotransmission) has long been thought to contribute to the pathophysiology of psychoses in humans. Amphetamine causes release of dopamine from intra-neuronal
storage vesicles and also competitively inhibits dopamine reuptake by dopamine transporter (Figure 1-7). This results in increased brain levels of dopamine, primarily at the synapses in nucleus accumbens which is the terminal input of the mesolimbic dopaminergic pathway. Amphetamine-induced hyperlocomotor activity is hypothesized to model positive symptoms of schizophrenia. Modulation of the hyperlocomotor activity in rodents induced by acute administration of amphetamine has long been used as a model to screen drugs for antipsychotic activity, and, is used in this thesis, along with the serotonergic HTR model and the MK-801 glutamate antagonist model, discussed above. Thus, multiple models, representing the serotonergic, glutaminergic, and dopaminergic neurotransmission dysfunction thought to be involved in the pathophysiology and endophenotypes of psychoses are used in the studies of this thesis to screen PATs for antipsychotic activity.

Central Hypothesis and Goals of this Dissertation

The central hypothesis tested here is that compounds with serotonin 5-HT\textsubscript{2C} agonist activity together with 5-HT\textsubscript{2A/2B} antagonist/inverse agonist activity translate to efficacy in rodent models of psychosis and may suitable for development as novel antipsychotic drugs. The goals of the dissertation are to characterize the molecular determinants for binding and function of novel 4-phenyl-N,N-dimethyl-1,2,3,4-tetrahydroanphthalene-2-amine (phenylaminotetralin; PAT) derivatives synthesized in our laboratories at human serotonin 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B}, and 5-HT\textsubscript{2C}. In silico ligand–5-HT\textsubscript{2} receptor docking studies by Dr. Tania Cordova Sintjago complement the experimental binding studies to help identify molecular determinants for PAT binding and provide inferences of functional interaction at 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors. In vivo antipsychotic efficacy of PAT analogs is evaluated using three different rodent models of psychosis,
the serotonin 5-HT₂ agonist HTR model, the MK-801 glutamate antagonist hyperactivity model, and amphetamine-induced hyperactivity model. The goals are pursued according to the following 3 Specific Aims:

**AIM 1: Delineate Molecular Determinants for Binding of 4-phenyl-\(N,N\)-dimethyl-1,2,3,4-tetrahydroanphthalene-2-amine (phenylaminotetralin, PAT) Derivatives at Human Recombinant 5-HT\(_{2A}\) and 5-HT\(_{2C}\) GPCRs**

PATs possessed a unique attribute of being 5-HT\(_{2C}\)-specific agonists while at the same time functioning as antagonists/inverse agonists at 5-HT\(_{2A}\) and 5-HT\(_{2B}\) receptors. It is well established that there is no therapeutic relevance for compounds that activate 5-HT\(_{2A}\) and/or 5HT\(_{2B}\) receptors, as activation of these receptors is associated with deleterious CNS or cardiovascular events. In order to move forward drug development targeting 5-HT\(_{2}\) receptors by characterizing the molecular determinants for 5-HT\(_{2}\) receptor–PAT binding, this aim will determine affinity \((K_i)\) of PAT analogs with substituents at ortho, meta- or para-position of the 4-phenyl moiety of PATs. Another group of analogs assessed include substituents at the 6,7-position of tetrahydronaphthalene ring, with, and without, concomitant substitution at the meta-position of the 4-phenyl moiety. *In silico* ligand–5-HT\(_{2}\) receptor docking studies by Dr. Tania Cordova Sintjago are used to complement the experimental binding studies to help identify molecular determinants for PAT binding at 5-HT\(_{2A}\) and 5-HT\(_{2C}\) receptors. PATs with \(K_i < 500\) nM across 5HT\(_{2}\) receptors are considered for additional in vitro molecular studies of Aim 2 to characterize their 5HT\(_{2}\) functional pharmacology.

**AIM 2: Characterize the 5-HT\(_{2}\) Functional Pharmacology of PATs**

Selected PATs from Aim 1 that meet 5HT\(_{2}\) affinity criteria, are assessed for their functional potency regarding 5-HT\(_{2}\) receptor-mediated activation of phospholipase C (PLC) signaling at human recombinant 5HT\(_{2A}\), 5HT\(_{2B}\), and 5HT\(_{2C}\) expressed in clonal
HEK cells. PATs that demonstrate agonism at 5-HT$_{2C}$ receptors together with antagonism or inverse agonism at 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors and have EC$_{50}$/IC$_{50}$ potency values <500 nM are considered for translational studies of Aim 3 to assess their preclinical efficacy in psychosis.

**AIM 3: Translational Studies: Assessment of PATs Efficacy in rodent models of psychosis**

The head-twitch response (HTR) is a characteristic response elicited when the hallucinogenic 5-HT$_{2A}$ agonist (±)-2-5-dimethoxy-4-iodoamphetamine (DOI) is administered to mice. MK-801 is a non-competitive antagonist at NMDA receptor and induced hyperlocomotion when administered to mice. Amphetamine targets dopaminergic neurocircuitry and increases dopamine level in synapse resulting in hyperlocomotion when administered to mice. These responses, HTR and hyperlocomotion, can be attenuated by ligands acting on 5-HT$_2$ receptors and other compounds that are known to possess antipsychotic activity. Thus, these assays provide a simple yet powerful *in vivo* model to identify compounds with antipsychotic or 5-HT$_2$ receptors mediated psychotherapeutic activity. Selected PAT analogs identified in Aim 2 as 5HT2C agonists with 5HT2A/2B inverse agonist/antagonist activity are administered orally or by intraperitoneal injection to mice and assessed for efficacy in the three different psychosis models. Results of these studies also provide preliminary information on PAT absorption, distribution, metabolism, elimination, and toxicology (ADMET) *in vivo*. 
Figure 1-1. Generic 2-dimensional structure of GPCR with amino acids numbered from amino terminal to carboxy terminal.

Figure 1-2. 3-dimensional representation of GPCR. A) The extracellular loops are present at the top and the intracellular loops at the bottom of the cartoon. B) 3-dimensional representation of GPCR from a top-down view. The extracellular loops are seen on the top and the binding pocket formed by the transmembrane domain is seen as the space between the helices.
Figure 1-3. Extended ternary complex model. R, R* and R*G: represent the inactive state, active state and active G-protein coupled state of GPCR respectively. $K_a$ and $K_G$ are the equilibrium constants for binding of ligand A and G-protein G to GPCR. $\alpha$, $\beta$ and $\gamma$ are the cooperativity constants that modulate the equilibrium constants. L represents the isomerization constant of GPCR.

Figure 1-4. Ligands activating serotonin 5-HT$_2$ receptors. A) 5-Hydroxytryptamine or serotonin the endogenous ligand for serotonin receptors. B) and C) Fenfluramine and phenteramine ligands, respectively, used therapeutically for weight loss.

Figure 1-5. Structure of hallucinogens (phenylethylamines and tryptamines) acting on 5-HT$_2$ receptors.
Figure 1-6. Structure of M100,907. A selective antagonist of 5-HT$_2A$ receptors.

Figure 1-7. Ligands used in *in vivo* models of psychosis. A) Structure of 2,5-dimethoxy-4-iodoamphetamine (DOI), used in head-twitch response model. B) and C) Structure of MK-801 and amphetamine used in hyperactivity model of psychosis.
Chapter 2
DELINEATE MOLECULAR DETERMINANTS FOR BINDING OF 4-PHENYL-N,N-
DIMETHYL-1,2,3,4-TETRAHYDROANPHTHALENE-2-AMINE
(PHENYLAMINOTETRALIN; PAT) DERIVATIVES AT HUMAN RECOMBINANT 5-HT\textsubscript{2A}
AND 5-HT\textsubscript{2C} GPCRs

Specific Aim 1

The lead compound (−)-\textit{trans}-PAT possesses a high affinity for the serotonin 5-HT\textsubscript{2} family of receptors. Since, 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors are therapeutically relevant in a number of physiological conditions, it is important to determine the molecular determinants that influence PAT analogs binding at these receptors\textsuperscript{120}. Serotonin 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors share a high degree of sequence homology and similarity in their effector systems\textsuperscript{121,122}. The structure activity relationship (SAR) of \textit{meta}- and \textit{para}-substituted PAT analogs assisted in delineating the optimal functional group that achieved the desired selectivity and affinity (PAT \textit{meta}- and \textit{para}-substituents were synthesized by Dr. Rajeev Sakhuja and Dr. Myong Sang Kim). The nature of functional group attached to PAT analogs was determined primarily by their ease of synthesis and also based on the potential interactions of the substituent with the surrounding amino acids in the binding pocket.

Substituents were added to the PAT analogs at the 6 and/or 7 position of the tetrahydronaphthalene ring (synthesized by Dr. Zhuming Sun). These 6 and/or 7 substituted PAT analogs were further modified with the addition of a substituent at the \textit{meta}-position of pendant phenyl ring. \textit{In silico} docking of these substitutions assisted in providing more information about the binding pocket surrounding these substituents. Since these analogs perturb a different region of the binding pocket as compared to the pendant phenyl ring substituents, we investigated the role of these substituents in determining the affinity and selectivity of these substituents for the 5-HT\textsubscript{2A} or 5-HT\textsubscript{2C}
receptors. Evaluating the structure activity relationship helped us glean more information about these substituents and their interactions at the 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors.

**Methodology**

**Clonal Cell Culture and Transfection**

Human embryonic kidney 293 cells (HEK, ATCC CRL-1573) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 5% fetal bovine serum and 1% penicillin – streptomycin. Cells were grown in a humidified incubator at 37 °C with 5% carbon dioxide. The cDNAs encoding the human 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B} and 5-HT\textsubscript{2C-INI} (unedited wild type isoform) wild type receptors were obtained from UMR cDNA Resource Center (Rolla, MO). HEK-293 cells were grown to 90% confluency in DMEM (10-013-CV, Mediatech, Manassas, VA), supplemented with 5% dialyzed fetal bovine serum in 10 cm plates. Cells were washed then transfected with 24 μg of 5-HT\textsubscript{2} receptor subtype cDNA mixed with 40 μL Lipofectamine 2000 reagent in Opti-MEM and placed in an incubator for 24 to 48 hr. Membranes were then collected in 50mM Tris, 10mM MgCl\textsubscript{2}-6H\textsubscript{2}O, and 0.1mM EDTA (assay buffer) using previous methods\textsuperscript{123} and stored at -80 ° Celsius until binding assays were performed.

**Radioreceptor Competition Binding Assays**

Radioligand competitive displacement binding assays were performed in 96-well plates, using 3-5 μg of protein from membrane samples per well, similar to laboratory methods used previously\textsuperscript{123}. Radioligands were included in assay mixtures at \(\sim K_d\) concentration, i.e., 2.0 nM \[^3\text{H}\]-ketanserin (5-HT\textsubscript{2A} receptors), 1.95 nM \[^3\text{H}\]-mesulergine (5-HT\textsubscript{2B} receptors), or 1.4 nM \[^3\text{H}\]-mesulergine (5-HT\textsubscript{2C} receptors). Non-specific binding was determined in the presence of 10 μM mianserin for all 5-HT\textsubscript{2} receptors. Incubation
of radioreceptor binding assay mixtures was for 1.0 h at 37°C, with termination by rapid filtration through Whatman GF/B filters using a 96-well cell harvester (Tomtec, Hamden, CT) and subsequently washed five times with 50mM Tris-HCl at room temperature. Filters containing bound [$^3$H]-radioligand were dried, placed in vials containing 2mL scintillation cocktail (ScintiVerse), allowed to equilibrate overnight, and then were counted for $^3$H-induced scintillation using a Beckman-Coulter LS6500 counter. Each binding experiment had concentrations of ligands in triplicates, and each experiment was performed a minimum of three times. Data were analyzed using nonlinear regression curve-fitting algorithms in GraphPad Prism, 5.03 for Windows (San Diego, CA). Data points were limited to eight, thus Hill slopes were not calculated$^{124}$; data were fit using the “one site fit-$K_i$” model that constrains the Hill slope to 1.0. Ligand affinity is expressed as $K_i$ values by conversion of the IC50 data using the equation $K_i = IC_{50}/1 + L/K_D$ where L is the concentration of radioligand$^{125}$.

**Results and Discussion for Competition Binding Assays**

**Affinity of Unsubstituted PATs at the 5-HT$_{2A}$ Receptor**

Each PAT analog synthesized in the lab has 4 diastereomers, two pairs of enantiomers for each cis and trans stereoisomer (Figure 2-1). Out of the 4 diastereomers the trans-PAT enantiomers showed the best binding profile at the 5-HT$_{2A}$ receptors$^{131}$. (−)-Trans-PAT had a $K_i$ value of 100 ± 10 nM and (+)-trans PAT showed a binding affinity of 470 ± 46 nM (Table 2-2). (−)-Trans-PAT showed a higher affinity for the 5-HT$_{2A}$ receptors as compared to the (+)-trans PAT enantiomer ($p=0.0098$).

**Affinity of Unsubstituted PATs at the 5-HT$_{2C}$ Receptor**

Binding studies of the PAT stereoisomers followed a similar trend at 5-HT$_{2C}$ receptors like 5-HT$_{2A}$ receptors. The (+)-trans-PAT enantiomer had an affinity of 430 ±
86 nM and the (−)-trans-PAT analog binds at the 5-HT\textsubscript{2C} receptors with a Ki of 30 ± 3 nM (Table 2-2). (−)-trans-PAT had a 13-fold higher affinity at 5-HT\textsubscript{2C} receptor as compared to (+)-trans-PAT and this difference was statistically significant (p=0.0021). 5-HT\textsubscript{2C} receptor affinity of (−)-trans-PAT analog was significantly higher than 5-HT\textsubscript{2A} receptor affinity (100 nM) (p=0.0016). This difference in affinity indicates that (−)-trans-PAT has stronger interactions or interacts with additional amino acids in 5-HT\textsubscript{2C} receptor binding pocket.

**Affinity of 4-(ortho-Substituted)-PATs at the 5-HT\textsubscript{2A} Receptor**

(+) and (−)-Trans-o-Br-PAT enantiomers had an affinity of 760 ± 73 nM and 1100 ± 130nM respectively (p>0.05). Both (+) and (−)-trans-PAT enantiomers had a significantly higher affinity relative to (+) and (−)-trans-o-Br-PAT enantiomers (p=0.009 and P<0.0001) respectively. Introduction of sterically large halogen substituent at the ortho-position could hinder the free rotation of the pendant phenyl ring attached to sp\textsuperscript{3} hybridized carbon of tetrahydronaphthalene ring. Thereby, inducing a conformation of trans-o-Br-PAT that resulted in decreased affinity of both (+) and (−)-trans-o-Br-PAT enantiomers at 5-HT\textsubscript{2A} receptors.

(+) and (−)-Trans-o-Cl-PAT enantiomers had an affinity of 2800 ± 110 nM and 2400 ± 280 nM respectively (p>0.05). The affinity of (+) and (−)-trans-o-Cl-PAT enantiomers was significantly lower than both (+) and (−)-trans-PAT (P<0.0001) and (+) and (−)-trans-o-Br-PAT enantiomers (p<0.005). These data indicate that substitutions at ortho-position of pendant phenyl ring did not favor binding at 5-HT\textsubscript{2A} receptors. Both ortho-substituted analogs demonstrated a loss in stereoselectivity at 5-HT\textsubscript{2A} receptors relative to unsubstituted PAT enantiomers.
Affinity of 4-(ortho-Substituted)-PATs at the 5-HT2C Receptor

(+)-trans-o-Br-PAT enantiomers had an affinity of 78 ± 16 nM and 70 ± 8 nM respectively (p>0.05). Both these enantiomers had 10-fold and 15-fold higher affinity at 5-HT2C receptors relative to 5-HT2A receptors (p<0.0001). (+)-trans-PAT had a 2-fold higher affinity relative to (−)-trans-o-Br-PAT enantiomers at 5-HT2C receptors (p<0.0001). In contrast, (+)-trans-o-Br-PAT enantiomer had a 5-fold higher affinity relative to (+)-trans-PAT enantiomer (p=0.0095). These data indicate that the conformation induced by restricted rotation of the pendant phenyl ring favors selective binding at 5-HT2C receptors without any stereoselectivity.

(+)-trans-o-Cl-PAT enantiomers had an affinity of 50 ± 10 nM and 290 ± 47 nM respectively (p=0.009). These enantiomers demonstrated reversed stereoselectivity relative to unsubstituted PAT enantiomers. There was no significant difference between the affinity of (+)-trans-o-Cl-PAT and (+)-trans-o-Br-PAT enantiomers at 5-HT2C receptors. In contrast, the affinity of (−)-trans-o-Cl-PAT was significantly lower than (−)-trans-o-Br-PAT affinity at 5-HT2C receptors.

4-(Meta-substituted)-PAT analogs. Meta-substituted PATs were synthesized with the intention of perturbing the chemical space surrounding the pendant phenyl ring and also explore the differences in binding pocket interactions between 5-HT2A and 5-HT2C receptors. In silico docking studies of (−)-trans-PAT revealed that the pendant phenyl ring is surrounded by aromatic amino acids (W6.48 and F6.51) that assist in stabilizing the ligand in the binding pocket by forming π-π interactions. Addition of a functional group results in changes in affinity that need to be analyzed keeping in mind the electronic and the steric nature of the substituent. Different substituents like fluorine, chlorine, bromine, nitro and trifluoromethyl were added to the meta-position (Figure 2-
2), and the resulting changes in the binding affinity were analyzed. The steric size of these substituents changed in the order: fluorine < chlorine < bromine = nitro < trifluoromethyl (Courtesy Dr. Cordova-Sintjago Tania). Addition of different halogen atoms to the pendant phenyl ring also results in a change in the bond length between the aromatic carbon and the halogen substituent added. The bond length increases as the substituent changes from a fluorine to a chlorine to a bromine substituent (Table 2-1). Fluorine had the highest electronegativity among the group hence a strong electron withdrawing inductive (-I) effect126. The inductive effect of fluorine is greater than chlorine that in turn is greater than bromine. However halogens, when attached to an aromatic system (in this case, the C(4)-phenyl-moiety of PATs), are unique in their propensity to engage in resonance with the aromatic system by donating a lone pair of electrons (+R). If the halogen is attached at the ortho- or para-position of the C(4)-phenyl moiety, the result is an increase in electron density at these positions. Hence both inductive (-I) and resonance (+R) effects play a role in determining the interactions of the C(4)-phenyl moiety of PATs with the nearby amino acids of the binding pocket of 5-HT2 receptor127.

Affinity of 4-(meta-Substituted)-PATs at the 5-HT2A Receptor

(+)- and (-)-Trans- m-F-PAT were synthesized to examine the effects caused by changing a hydrogen atom to a fluorine at the meta-position on pendant phenyl ring. The Ki value of (-)-trans-m-F-PAT at 5-HT2A was 110 ± 10 nM and the affinity of the (+)-trans-m-F-PAT was 320 ± 26 nM (Table 2-2). (-)-Trans-m-F-PAT had a higher affinity than (+)-trans-m-F-PAT (p=0.0357). (-)-Trans-m-F-PAT affinity was not significantly different from (-)-trans-PAT (100 nM) affinity at the 5-HT2A receptor. Introduction of the fluorine group reduces the electron density of the π system in the
benzene ring because of the high electronegativity of fluorine$^{128}$. Since the pendant phenyl ring of (−)-trans-PAT binds in a region where there is a high density of aromatic amino acids, this could lead to π-π interactions with surrounding aromatic amino acids. Sterically, fluorine substituent is generally considered a bioisoster of hydrogen or methyl group$^{129}$. However, (−)-trans-m-F-PAT has the same affinity as (−)-trans-PAT indicating that there was no significant effect of introduction of fluorine at the meta-position.

(+) and (−)-trans-m-Cl-PAT enantiomers were synthesized with an intent to gain further insight into the milieu of the pendant phenyl ring in the binding pocket. Trans-m-Cl-PAT enantiomers followed a similar trend in stereoselectivity, that is, the (−)-trans-m-Cl-PAT had a higher affinity than (+)-trans-m-Cl-PAT (P=0.0294). The Ki value of (−)-trans-m-Cl-PAT was 40 ± 5 nM and the affinity of (+)-trans-m-Cl-PAT was 130 ± 8.7 nM (Table 2-2). The affinities of (−)-trans-m-F-PAT (110 nM) and (−)-trans-m-Cl-PAT were found to be significantly different (p=0.0223). Addition of a chlorine atom resulted in significant changes in the electronics and sterics of the pendant phenyl ring. Chlorine as compared to fluorine has a larger size and a smaller electronegativity$^{130}$. Hence both -I inductive effect and +R resonance effect are seen to a lower extent with chlorine than fluorine$^{127}$. The decrease in the resonance is because of the disproportionate size of the p orbitals of chlorine compared to carbon. This leads to a decreased propensity of the meta-chloro-PAT analog to form resonance hybrid structures. Introduction of a chlorine at the meta-position also resulted in a significant higher (p=0.0002) affinity of (−)-trans-m-Cl-PAT (40 nM) relative to affinity of (−)-trans-PAT (100 nM) at the 5-HT$_{2A}$ receptors (Figure 2-3).
The series was further expanded with the synthesis of (+) and (−)-trans-m-Br-PAT. The stereoselectivity trend was again confirmed as (+)-trans-m-Br-PAT had a lower affinity of 260 ± 22 nM relative to (−)-trans-m-Br-PAT enantiomer, Ki of 20 ± 3 nM (Table 2-2). This higher affinity of (−)-trans-m-Br-PAT enantiomer was slightly significant (p=0.0459) when compared to (−)-trans-m-Cl-PAT (40 nM) enantiomer. However, when compared to (−)-trans-m-F-PAT (110 nM) the affinity of (−)-trans-m-Br-PAT was significantly higher (p=0.0229). The bromine substituent has the largest size and the lowest electronegativity compared to the fluorine and chlorine atoms. Comparatively, then, displacement of C(4)-phenyl moiety π electrons is expected to be least with the bromine substituent because the probability of resonance effect is low due to the disproportional size of the bromine “p”-orbital as compared to carbon “p”-orbital.

The (−)-trans-m-Br-PAT enantiomer has the highest affinity among the meta-substituted PAT analogs at 5-HT2A receptors. Hence, the characteristics of a substituent at the meta-position that favored high affinity binding at 5-HT2A receptors are: large steric effect and a low electronegativity. The order of affinity of the levorotatory enantiomers is as follows: (−)-trans-m-Br-PAT > (−)-trans-m-Cl-PAT > (−)-trans-m-F-PAT = (−)-trans-PAT (Table 2-2).

Since more bulky atoms at meta-position increased the affinity at 5-HT2A receptors, more analogs with large sterics were synthesized in this series: (+) and (−)-trans-m-NO2-PAT and (+) and (−)-trans-m-CF3-PAT. These derivatives differed from previous meta-halogenated derivatives in being predominantly electron withdrawing and deactivating with respect to the phenyl ring. Trifluoromethyl substituent decreases the electron density in the phenyl ring by inductive effect (-I) and nitro substituent in addition
to having inductive effect (-I) also possessed resonance effect (-R) \textsuperscript{131}. Hence the nitro group was a more potent ring deactivator than the trifluoromethyl substituent. The sterics of the trifluoromethyl substituent were slightly larger than the nitro group by about 20 Å\textsuperscript{3} (Data courtesy Dr. Tania Cordova Sintjago).

The (+)-\textit{trans-m-NO}_2-PAT enantiomer had an affinity of 500 ± 20 nM and the (−)-\textit{trans-m-Nitro-PAT} had an affinity of 74 ± 18 nM (\textit{p}=0.0001) (Table 2-2). The affinity of both (+) and (−)-\textit{trans-m-NO}_2-PAT was not significantly different from the affinity of (+)-\textit{trans-PAT} (470 nM) and (−)-\textit{trans-PAT} (100 nM) enantiomers respectively. Comparing the affinities of other meta-substituted PAT derivatives showed that: (−)-\textit{trans-m-Br-PAT} (20 nM) enantiomer had significantly higher affinity than (−)-\textit{trans-m-NO}_2-PAT enantiomer (\textit{p}=0.0150). The affinity of (−)-\textit{trans-m-F-PAT} (110 nM) and (−)-\textit{trans-m-Cl-PAT} (40 nM) enantiomers were not significantly different relative to (−)-\textit{trans-m-NO}_2-PAT enantiomer affinity at 5-HT\textsubscript{2A} receptors (\textit{p}>0.05).

The (+)-\textit{trans-m-CF}_3-PAT enantiomer had an affinity of 1300 ± 67 nM and the (−)-\textit{trans-m-CF}_3-PAT enantiomer had an affinity of 80 ± 10 nM (\textit{p}=0.0272) (Table 2-2). The affinity of (−)-\textit{trans-m-CF}_3-PAT was not significantly different from (−)-\textit{trans-PAT}, (−)-\textit{trans-m-F-PAT} and (−)-\textit{trans-m-NO}_2-PAT affinity. However, the affinity was significantly lower when compared to (−)-\textit{trans-m-Cl-PAT} (\textit{p}=0.004) and (−)-\textit{trans-m-Br-PAT} (\textit{p}=0.0023) affinity at 5-HT\textsubscript{2A} receptors.

Meta-trifluoromethyl substituent possessed the largest sterics of all the meta-substituted PAT derivatives\textsuperscript{126,132}. The literature values of electronegativity of this substituent varied diversely with some paper reporting the electronegativity to be between fluorine and chlorine and other papers reporting electronegativity similar to
bromine\textsuperscript{133, 134}. Trifluoromethyl substituent is unique; unlike other substituents at meta-position it possesses no resonance effect. Hence, it is only capable of reducing the electron density of the phenyl ring. The newer meta-trifluoromethyl and nitro PAT analogs, possess larger steric than bromine, but resulted in lower affinity at 5-HT\textsubscript{2A} receptors. This drop in affinity when the substituent changes from bromine to nitro or trifluoromethyl substituents could be because the steric effects of these newer substituents are too large to fit into the binding pocket (Figure 2-3). The affinity of meta-substituted derivatives increased steadily as the size of the substituents increases until nitro and the trifluoromethyl substituent were added.

**Summary of 4-(meta-substituted)-PAT analogs at 5-HT\textsubscript{2A} receptor.** Fluorine, chlorine and bromine possessed -I inductive effect and +R resonance effect. Nitro substituent possessed -I inductive and -R resonance effect and trifluoromethyl substituent possessed -I inductive effect only. The order of affinity of the levorotatory meta-substituted PAT analogs is as follows: (--)\textit{trans}-m-Br-PAT > (--)\textit{trans}-m-Cl-PAT = (--)\textit{trans}-m-NO\textsubscript{2}-PAT = (--)\textit{trans}-m-CF\textsubscript{3}-PAT = (--)\textit{trans}-m-F-PAT = (--)\textit{trans}-PAT (Figure 2-3).

**Affinity of 4-(meta-Substituted)-PATs at the 5-HT\textsubscript{2C} Receptor**

(+)\textit{Trans}-m-F-PAT binds to 5-HT\textsubscript{2C} receptors with an affinity of 200 ± 30 nM and (--)\textit{trans}-m-F-PAT has a \textit{Ki} value of 43 ± 14 nM (\(p=0.0076\)) (Table 2-2). The introduction of a fluorine group did not significantly change the affinity of either enantiomer relative to the corresponding trans-PAT enantiomers at 5-HT\textsubscript{2C} receptors. Comparing (--)\textit{trans}-m-F-PAT affinity at 5-HT\textsubscript{2A} with its affinity at 5-HT\textsubscript{2C} receptors revealed no significant difference. Introduction of a fluorine group had no affect on its selectivity between the two receptors.
(+)-Trans-m-Cl-PAT binds to the 5-HT\textsubscript{2C} receptors with an affinity of 170 ± 43 nM and (−)-trans-m-Cl-PAT has a Ki value 8 ± 3 nM (p=0.0098) (Table 2-2). Introduction of chlorine at the meta-position increases the affinity of the (−)-trans enantiomer 3-fold relative to (−)-trans-PAT affinity (p=0.0262). Comparing the affinities of meta-fluorine (43 nM) and chlorine substituted PATs at 5-HT\textsubscript{2C} receptors showed that (−)-trans-m-Cl-PAT has a significantly (p=0.0139) higher affinity. There was no significant difference in the affinity of (+)-trans-PAT (430 nM), (+)-trans-m-F-PAT (200 nM) and (+)-trans-m-Cl-PAT (170 nM). Affinity of (+)-trans-m-Cl-PAT did not significantly differ between the 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors. On the other hand, the affinity of (−)-trans-m-Cl-PAT was significantly higher at 5-HT\textsubscript{2C} receptors relative to its affinity at 5-HT\textsubscript{2A} receptors (p=0.0350).

Enantiomers of trans-m-Br-PAT were tested for their binding at 5-HT\textsubscript{2C} receptors and their affinities compared to other meta-substituted analogs. (+)-Trans-m-Br-PAT had an affinity of 200 ± 30 nM and the (−)-trans-m-Br-PAT enantiomer had a Ki of 4 ± 1 nM (p<0.0001) (Table 2-2). Comparing (−)-trans-m-Br PAT affinity with (−)-trans-PAT (30 nM) affinity showed that (−)-trans-m-Br-PAT bound with a significantly higher affinity at 5-HT\textsubscript{2C} receptors (p=0.0229). The affinity of the (+)-trans-m-Br-PAT enantiomer was not significantly different from (+)-trans-PAT (430 nM). The affinities of (−)-trans-m-F-PAT (43 nM) and (−)-trans-m-Br-PAT were found to be significantly different (p=0.0425). Comparing the affinities of (−)-trans-m-Cl-PAT (8 nM) and (−)-trans-m-Br-PAT revealed that their affinities were not significantly different. This indicates that both chlorine and bromine substituents formed stronger interactions with the amino acids in the binding pocket as compared to fluorine. The (−)-trans-m-Br-PAT analog had 6-fold

53
higher affinity at 5-HT\textsubscript{2C} receptors than 5-HT\textsubscript{2A} receptors (\(p=0.0218\)), similar to trend observed with (\(\text{-}\))\textit{trans}-\textit{m}-\text{Cl}-PAT enantiomer.

The (+)-\textit{trans}-\textit{m}-NO\textsubscript{2}-PAT and (\(\text{-}\))\textit{trans}-\textit{m}-NO\textsubscript{2}-PAT enantiomers had an affinity of 120 ± 13 nM and 10 ± 1 nM respectively (\(p=0.0002\)) (Table 2-2). Comparing these enantiomers with the parent (+)-\textit{trans}-PAT (430 nM) and (\(\text{-}\))\textit{trans}-PAT (30 nM) showed that there was a significant difference between (\(\text{-}\))\textit{trans}-PAT and (\(\text{-}\))\textit{trans}-\textit{m}-NO\textsubscript{2}-PAT affinities at 5-HT\textsubscript{2C} receptor (\(p=0.0159\)). The affinity of (+)-\textit{trans}-\textit{m}-NO\textsubscript{2}-PAT was also higher compared to (+)-\textit{trans}-PAT enantiomer (\(p=0.0291\)). The affinity of (\(\text{-}\))\textit{trans}-\textit{m}-Br-PAT (4 nM) was significantly higher than (\(\text{-}\))\textit{trans}-\textit{m}-NO\textsubscript{2}-PAT (\(p=0.0002\)). Affinities of other \textit{meta}-substituted-PAT analogs (fluorine (43 nM) and chlorine (8 nM)) were not significantly different at the 5-HT\textsubscript{2C} receptors. The affinity of (\(\text{-}\))\textit{trans}-\textit{m}-NO\textsubscript{2}-PAT was significantly higher at 5-HT\textsubscript{2C} receptors compared to 5-HT\textsubscript{2A} receptors (\(p=0.0004\)). The (\(\text{-}\))\textit{trans}-\textit{m}-NO\textsubscript{2}-PAT enantiomer had a 4 fold higher selectivity for 5-HT\textsubscript{2C} receptors compared to 5-HT\textsubscript{2A} receptors.

The next pair of enantiomers assessed at 5-HT\textsubscript{2C} receptors had a trifluoromethyl substituent at the \textit{meta}-position. (+)-\textit{Trans}-\textit{m}-CF\textsubscript{3}-PAT had an affinity of 230 ± 20 nM and (\(\text{-}\))\textit{trans}-\textit{m}-CF\textsubscript{3}-PAT had a \(K_i\) value of 10 ± 2 nM at 5-HT\textsubscript{2C} receptors (\(p<0.0001\)) (Table 2-2). The affinities of both the enantiomers were not significantly different from the corresponding parent unsubstituted PAT analogs. Comparing the affinity with other \textit{meta}-substituted analogs showed that there was no significant difference between (\(\text{-}\))\textit{trans}-\textit{m}-CF\textsubscript{3}-PAT and any of the other \textit{meta}-substituted analogs. The selectivity for 5-HT\textsubscript{2C} receptors versus 5-HT\textsubscript{2A} receptors was the highest for this enantiomer. This
enantiomer is about 9-fold more selective for 5-HT\textsubscript{2C} receptors as compared to 5-HT\textsubscript{2A} receptors \((p=0.0055)\).

**Summary of 4-(meta-substituted)-PAT analogs at 5-HT\textsubscript{2C} receptor.**  (\(\sim\))

\(\sim\)-Trans-m-F-PAT did not show selectivity between 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors. However as the sterics of the substituent increased, the selectivity for 5-HT\textsubscript{2C} receptors increased. (\(\sim\)-Trans-m-Cl-PAT had 7-fold higher affinity at the 5-HT\textsubscript{2C} receptors. When the size of substituent further increased in (\(\sim\)-trans-m-Br-PAT this difference was 6-fold. Further increase in sterics with (\(\sim\)-trans-m-NO\textsubscript{2}-PAT revealed a 4-fold increase in affinity for 5-HT\textsubscript{2C} receptors. The largest substituent trifluoromethyl at the meta-position showed the highest selectivity for the 5-HT\textsubscript{2C} receptor, (\(\sim\)-trans-m-CF\textsubscript{3}-PAT has 9-fold higher selectivity for the 5-HT\textsubscript{2C} receptors (Table 2-2). This indicates that 5-HT\textsubscript{2C} receptor is better able to accommodate the sterics of the larger substituents as compared to the 5-HT\textsubscript{2A} receptor leading to higher affinities of these enantiomers at 5-HT\textsubscript{2C} receptor. Similar to the trend observed at 5-HT\textsubscript{2A} receptors, the affinities of meta-substituted PAT at 5-HT\textsubscript{2C} receptors increases with the size of the substituents until the sterics size of nitro and trifluoromethyl substituents. However, the drop in affinities is steeper at 5HT\textsubscript{2A} receptors as compared to 5HT\textsubscript{2C} receptors.

**Affinity of 4-(para-Substituted)-PATs at the 5-HT\textsubscript{2A} Receptor**

(\(+\))-Trans-\(p\)-F-PAT had an affinity of 71 ± 12 nM at 5-HT\textsubscript{2A} receptors and (\(\sim\))-\(\sim\)-trans-\(p\)-F-PAT bound to 5-HT\textsubscript{2A} with a Ki of 140 ± 24 nM \((p=0.0345)\) (Table 2-3).

Comparing the affinity of the para-fluorine enantiomers with the parent unsubstituted PAT shows that (\(+\)-\(\sim\)-trans-\(p\)-F-PAT had 6-fold higher affinity than (\(+\)-\(\sim\)-trans-PAT (470 nM) enantiomer \((p=0.0015)\). In contrast, addition of fluorine at para-position did not change the affinity of (\(\sim\)-\(\sim\)-trans-\(p\)-F-PAT relative to (\(\sim\)-\(\sim\)-trans-PAT (100 nM). Introduction
of a fluorine group at the para-position enhanced the affinity of (+)-trans-p-F-PAT enantiomer, resulting in a reversal of stereoselectivity. This pronounced increase in the affinity (+)-trans-p-F PAT enantiomer probably resulted from the formation of a favorable interaction (hydrogen bonding) with the surrounding amino acids in the binding pocket.

The next pair of enantiomers synthesized were (+) and (−)-trans-p-Cl-PAT (Figure 2-4). Changing the halogen substituent at the para-position from fluorine to chlorine resulted in a more pronounced reversal of stereoselectivity. The affinity of (+)-trans-p-Cl-PAT was 50 ± 8 nM and the other enantiomer, (−)-trans-p-Cl-PAT, binds with a Ki of 250 ± 37 nM (Table 2-3). Similar to para-fluoro-PAT analogs, introduction of the chlorine group at the para-position resulted in reversed stereoselectivity. The affinity of the (+)-trans-p-Cl-PAT enantiomer increased 9-fold as compared to (+)-trans-PAT (470 nM) (p=0.0001) but the affinity of (−)-trans-p-Cl-PAT enantiomer decreased 2-fold as compared to (−)-trans-PAT (100 nM) (p=0.0280). This suggests that the (+)-trans-p-Cl PAT is able to better fit into the binding pocket than the (−)-trans-p-Cl PAT. (+)-trans-p-F-PAT (71 nM) had a 2-fold higher affinity than (−)-trans-p-F-PAT (140 nM), in contrast (+)-trans-p-Cl-PAT shows a 5-fold higher affinity than (−)-trans-p-Cl-PAT. Comparing the affinity of (+)-trans-p-F-PAT and (+)-trans-p-Cl-PAT revealed that these values were not statistically significant.

The reversal in stereoselectivity was further confirmed by the synthesis of (+) and (−)-trans-p-Br-PAT. Introduction of a bulkier bromine atom retained the reversal of stereoselectivity observed in previous analogs. (+)-trans-p-Br-PAT binds with a Ki of 60 ± 10 nM at 5-HT₂A receptors and (−)-trans-p-Br-PAT has an affinity of 220 ± 29 nM (p=0.0025) (Table 2-3). There was no significant difference between the affinity of (+)-
trans-p-Br-PAT when compared to (+)-trans-p-Cl-PAT (50 nM) and (+)-trans-p-F-PAT (71 nM). However, comparing the affinities of (+)-trans-p-Br-PAT and (+)-trans-PAT revealed that there was a 8-fold increased affinity for (+)-trans-p-Br-PAT ($p=0.0003$). This provides further evidence to the hypothesis that (+)-trans- para-substituted PAT enantiomers had higher affinity than parent PAT because they formed favorable interactions with amino acids in the binding pocket.

The next pair of compounds synthesized in this series showed the most pronounced difference in the affinity between (+) and (−)-trans enantiomers relative to any other para-substituted PAT analogs. The affinity of (+)-trans-p-CF$_3$-PAT at 5-HT$_{2A}$ receptors was 210 ± 16 nM and the affinity of (−)-trans-p-CF$_3$-PAT was 2000 ± 200 nM ($p=0.001$) (Table 2-3). (−)-Trans-p-CF$_3$-PAT has 20-fold lower affinity as compared to (−)-trans-PAT (100 nM) ($p=0.0008$) and (+)-trans-p-CF$_3$-PAT has 2-fold higher affinity as compared to (+)-trans-PAT ($p=0.0258$). This loss in affinity could be because the sterics of the trifluoromethyl substituent result in a clash with amino acids in the binding pocket. This is reflected when the statistical analysis was performed to compare the trifluoromethyl substituent affinity with other para-halogenated substituents. (+)-Trans-p-CF$_3$-PAT substituent had significantly lower affinity compared to (+)-trans-p-F-PAT (71 nM) ($p=0.0009$), (+)-trans-p-Cl-PAT (50 nM) ($p=0.0003$) and (+)-trans-p-Br-PAT (60 nM) ($p=0.0002$).

**Summary of 4-(para-substituted)-PATs at the 5-HT$_{2A}$ receptor.** Para-substituted PAT analogs were unique as they demonstrated reversed stereoselectivity of PAT enantiomers (Table 2-3). This reversal in stereoselectivity became more pronounced as the size of the substituents increased. The mean affinities of para-
substituted enantiomers followed a trend where: (+)-\textit{trans-p-}Cl-PAT > (+)-\textit{trans-p-}Br-PAT > (+)-\textit{trans-p-}F-PAT > (+)-\textit{trans-p-}CF3-PAT (Table 2-3), this trend was not statistically significant (Figure 2-5). The absolute conformation of (2\textit{S}, 4\textit{R})-(—)-\textit{trans-4-(4-chloro-phenyl)-\textit{N,N-dimethyl-2-aminotetralin}} or (—)-\textit{trans-p-}Cl-PAT was confirmed (by Dr. Khalil A. Abboud) using X-ray crystallography (Figure 2-6). Affinity of racemate (±)-\textit{trans-p-NO2-PAT} was also determined ($Ki=8100 \pm 180$ nM). This affinity of racemate further confirms the influence of substituent sterics at the \textit{para}-position.

**Affinity of 4-(\textit{para-Substituted})-PATs at the 5-HT\textsubscript{2C} Receptor**

All the \textit{para}-substituted PAT analogs synthesized were also tested at 5-HT\textsubscript{2C} receptors to check whether they show any selectivity between 5-HT\textsubscript{2C} and 5-HT\textsubscript{2A} receptors. These results provided more information about the binding pocket milieu differences between the 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors.

(+)- and (—)-\textit{Trans-p-}F-PAT enantiomers were analyzed for their affinity at 5-HT\textsubscript{2C} receptors. The affinity of these enantiomers revealed an interesting facet; they showed a loss of stereoselectivity instead of a reversal (Table 2-3). The affinity of the (+)-\textit{trans-p-}F-PAT at 5-HT\textsubscript{2C} receptors was $75 \pm 12$ nM and the $Ki$ of (—)-\textit{trans-p-}F-PAT was $68 \pm 11$ nM ($p=0.5665$). Comparing these $Ki$ with the corresponding 5-HT\textsubscript{2A} affinities showed that there was no statistical difference in the affinities of (+)-\textit{trans-p-}F-PAT. However, (—)-\textit{trans-p-}F-PAT had a significantly higher affinity at 5-HT\textsubscript{2C} receptors compared to 5-HT\textsubscript{2A} receptors ($p=0.0339$). This indicated that (—)-\textit{trans-p-}F-PAT binds differently with the two receptors and it was able to make stronger interactions in the binding pocket of 5-HT\textsubscript{2C} receptors. Comparing these affinities with the parent PAT enantiomers showed that (—)-\textit{trans-p-}F-PAT affinity was not significantly different from (—)-\textit{trans-PAT} (30 nM).
In contrast, (+)-trans-p-F-PAT enantiomer had a significantly higher affinity compared to (+)-trans-PAT (430 nM) \(p=0.0045\).

The next pair of enantiomers tested at 5-HT\textsubscript{2C} receptors were (+) and (−)-trans-p-Cl-PAT. (+)-Trans-p-Cl-PAT had a \(K_i\) of 55 ± 17 nM and (−)-trans-p-Cl PAT had an affinity of 120 ± 9.0 nM (Table 2-3). This difference in affinity was statistically significant with a \(p\) value of 0.0035. Substituting a fluorine group with a chlorine group at the \textit{para}-position did not result in statistically significant change in the affinity of the (+) and (−)-trans-p-Cl-PAT relative to (+) and (−)-trans-p-F-PAT respectively. Comparing the affinities of (+) and (−)-trans-p-Cl PAT at 5-HT\textsubscript{2C} receptor with their affinities at 5-HT\textsubscript{2A} receptor showed no significant difference for (+)-trans-p-Cl-PAT and significantly higher affinity for (−)-trans-p-Cl-PAT at 5-HT\textsubscript{2C} receptors \(p=0.0441\). This indicated that (−)-trans-p-Cl-PAT had 2-fold selectively for 5-HT\textsubscript{2C} receptors over 5-HT\textsubscript{2A} receptors.

Introduction of a chlorine group at the \textit{para}-position produced a pronounced reversal in stereoselectivity of (+) and (−)-trans-p-Cl-PAT relative to (+)-trans-PAT enantiomer (430 nM) \(p=0.0011\) and (−)-trans-PAT (30 nM) enantiomer \(p=0.0088\) respectively.

The next pair of enantiomers analyzed at 5-HT\textsubscript{2C} receptors were (+) and (−)-trans-p-Br-PAT. (+)-Trans-p-Br-PAT bound to 5-HT\textsubscript{2C} receptors with a \(K_i\) of 70 ± 8 nM and the other enantiomer (−)-trans-p-Br-PAT had an affinity of 100 ± 3 nM \(p=0.0214\). Comparing the affinities of \textit{para}-bromo enantiomers with other \textit{para}-substituted enantiomers revealed no significant difference in their binding affinities at 5-HT\textsubscript{2C} receptors. In contrast to this, (+)-trans-p-Br-PAT has significantly higher affinity compared to (+)-trans-PAT \(p=0.0038\) and (−)-trans-p-Br-PAT has significantly lower affinity as compared to (−)-trans-PAT \(p=0.0001\). Comparing the affinities of (−)-trans-
$p$-Br-PAT between 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors revealed that its affinity is significantly higher at 5-HT$_{2C}$ receptors ($p=0.0217$). The other enantiomer ($+$)-trans-$p$-Br-PAT did not show any difference in affinities between 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. This 2-fold higher affinity of ($-$)-trans-$p$-Br-PAT at 5-HT$_{2C}$ receptor revealed that 5-HT$_{2C}$ receptor is better at accommodating the large steric of bromine substituent.

The last pair of enantiomers to be tested in this series were ($+$) and ($-$)-trans-$p$-CF$_3$-PAT. Similar to their affinities at 5-HT$_{2A}$ receptors, these enantiomers showed the lowest affinity for para-substituted analogs at 5-HT$_{2C}$ receptors. ($+$)-Trans-$p$-CF$_3$-PAT bound to 5-HT$_{2C}$ receptors with a $K_i$ of 220 ± 27 nM and the ($-$)-trans-$p$-CF$_3$-PAT enantiomers had an affinity of 520 ± 51 nM ($p=0.0009$) (Table 2-3). This difference in affinity between enantiomers was only 2-fold at 5-HT$_{2C}$ receptors as compared to 8-fold difference in affinity between these enantiomers at 5-HT$_{2A}$ receptors (Table 2-3).

Comparing their affinities at 5-HT$_{2A}$ versus 5-HT$_{2C}$ receptors revealed that the ($-$)-trans-$p$-CF$_3$-PAT, bound better at 5-HT$_{2C}$ receptors as compared to 5-HT$_{2A}$ receptors ($p=0.0005$). In contrast, the other enantiomer ($+$)-trans-$p$-CF$_3$-PAT showed no significant difference between its affinities at 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. This lower affinity of the ($-$)-trans-$p$-CF$_3$-PAT at 5-HT$_{2A}$ receptors again indicated that 5-HT$_{2C}$ receptors were better able to accommodate the size and charge on the large trifluoromethyl substituent as compared to 5-HT$_{2A}$ receptors. ($-$)-Trans-$p$-CF$_3$-PAT enantiomer showed 4-fold selectivity for the 5-HT$_{2C}$ receptors as compared to the 5-HT$_{2A}$ receptors, but this selectivity was accompanied by a loss of affinity. Comparing these affinities with parent unsubstituted PAT showed that ($+$)-trans-$p$-CF$_3$-PAT did not
bind significantly different from (+)-trans-PAT (430 nM). (−)-Trans-p-CF₃-PAT, in contrast, had a significantly lower affinity relative to (−)-trans-PAT (p<0.0001).

**Summary of 4-(para-substituted)-PATs at 5-HT₂C receptor.** (+) and (−)-Trans-p-F-PAT showed a loss of stereoselectivity at the 5-HT₂C receptors. However, both (+) and (−)-trans-p-Cl-PAT and (+) and (−)-trans-p-CF₃-PAT showed a 2-fold reversal in stereoselectivity of corresponding enantiomers at 5-HT₂C receptors. (−)-Trans-p-Br-PAT showed a 2-fold selectivity for binding at 5-HT₂C receptors over 5-HT₂A receptors (Table 2-3). This selectivity was further increased when the trifluoromethyl group was introduced at para-position. (−)-Trans-p-CF₃-PAT showed a 4-fold selectivity for 5-HT₂C receptors over 5-HT₂A receptors. These data indicate that 5-HT₂C receptors were better able to accommodate the larger sterics of the substituents as compared to 5-HT₂A receptors (Figure 2-5). Affinity of racemate (±)-trans-p-NO₂-PAT was also determined as was found to be 1000 ± 100 nM, significantly lower than its affinity at 5-HT₂C receptors.

**Affinity of 4-(meta-Substituted-Phenyl)- and/or (6, 7-substituted-Tetrahydronaphthalene)-PATs at the 5-HT₂A Receptor**

(+)- and (−)-Trans-6-OMe-m-Cl-PAT enantiomers were synthesized to evaluate the effects of the methoxy substituent on binding profile at 5-HT₂A receptors. These affinities were compared to (+) and (−)-trans-m-Cl PAT as their structures differed only at 6-methoxy position. The (+)-trans-6-OMe-m-Cl-PAT had a Ki of 350 ± 16 nM and the (−)-trans-6-OMe-m-Cl-PAT bound with an affinity of 35 ± 3 nM (p=0.0015) (Table 2-4). These compounds followed a similar trend in stereoselectivity as the parent PAT and meta-substituted analogs. Comparing these affinities with meta-chlorine substituted PATs revealed that (−)-trans-m-Cl PAT (40nM) and (−)-trans-6-OMe-m-Cl-PAT had no significant difference in their affinities. But, (+)-trans-6-OMe-m-Cl-PAT enantiomer had a
significantly lower affinity relative to (+)-trans-m-Cl-PAT (130 nM) \((p=0.0375)\). This 2-fold reduction in affinity could be because of a steric hindrance caused by the introduction of a methoxy group.

The next pair of enantiomers synthesized in this series had a methoxy substitution at the 6-position and a chlorine substituent at the 7-position of tetrahydronaphthalene ring. Both the cis- and the trans- stereoisomers of this substituent were separated using a chiral HPLC. The affinities of (+) and (−)-trans-6-OMe-7-Cl-PAT were found to be 240 ± 23 nM and 160 ± 24 nM respectively. The affinities of the other pair of enantiomers (+) and (−)-cis-6-OMe-7-Cl-PAT were found to be 2300 ± 130 and 320 ± 42 nM \((p=0.0065)\) respectively (Table 2-4). The cis enantiomers followed the trend of levorotatory enantiomer having a higher affinity than the dextrorotatory enantiomer. The (+)-cis-6-OMe-7-Cl-PAT enantiomer had a 10-fold lower affinity relative to (+)-trans-6-OMe-7-Cl-PAT \((p=0.0141)\). (−)-Trans-6-OMe-m-Cl-PAT had 4-fold higher affinity relative to (−)-trans-6-OMe-7-Cl-PAT (160 nM) enantiomer at 5-HT\(_{2A}\) receptors \((p<0.0001)\). This loss in affinity could be because of either introduction of chloro-substituent at 7-position or removal of the chlorine atom from the meta position.

The next set of compounds synthesized in this series had substituents at 6, 7-position of tetrahydronaphthalene ring and halogen at meta-position of pendant phenyl ring. All the four stereoisomers of 6-OMe-7-Cl-m-Cl-PAT analog were synthesized and isolated. (+) and (−)-trans-6-OMe-7-Cl-m-Cl-PAT bound with an affinity of 95 ± 18 nM and 50 ± 20 nM respectively \((p>0.05)\). (+) and (−)-cis-6-OMe-7-Cl-m-Cl-PAT bound with a \(Ki\) of 230 ± 64 nM and 3500 ± 390 nM \((p=0.0414)\) respectively (Table 2-4). It is
interesting to note that there was a loss of stereoselectivity for the trans-enantiomers and that there was a reversal of stereoselectivity for the cis enantiomers. (+)-Trans-6-OMe-7-Cl-m-Cl-PAT had a 3-fold higher affinity relative to (+)-trans-6-OMe-7-Cl-PAT (p=0.0002). The other enantiomer (−)-trans-6-OMe-7-Cl-m-Cl-PAT also had a 3-fold higher affinity compared to (−)-trans-6-OMe-7-Cl-PAT (160 nM) (p=0.0002). The affinity of (−)-trans-6-OMe-7-Cl-m-Cl-PAT enantiomer was not significantly different from affinity of (−)-trans-m-Cl-PAT (40 nM) (p>0.05). The addition of 6-methoxy and 7-chloro substituents on the naphthalene ring resulted in a significant increase in affinity of (+)-trans-6-OMe-7-Cl-m-Cl-PAT as compared to (+)-trans-m-Cl-PAT (130 nM) (p=0.0091). This higher affinity of (+)-trans-6-OMe-7-Cl-m-Cl-PAT indicates that this enantiomer could bind at a different region in the binding pocket compared to other tetrahydronaphthalene ring substituted analogs.

The next pair of enantiomers synthesized in this series were (+) and (−)-trans-6-OMe-7-Cl-m-Br-PAT. These enantiomers bound to 5-HT2A receptors with affinities similar to trans-6-OMe-7-Cl-m-Cl-PAT enantiomers. The affinity of the (+)-trans-6-OMe-7-Cl-m-Br-PAT was found to be 79 ± 14 nM. The other enantiomer, (−)-trans-6-OMe-7-Cl-m-Br-PAT, bound with a Ki of 61 ± 15 nM (p>0.05). However, the affinities of (+)-trans-m-Br-PAT was significantly lower compared to (+)-trans-6-OMe-7-Cl-m-Br PAT (p=0.0043), indicating again that the presence of the 6-methoxy and 7-chloro groups in the tetrahydronaphthalene ring significantly increased the affinity of only the (+)-trans-enantiomers. Another important point to note is that both (−)-trans-m-Cl-PAT (40 nM) and (−)-trans-m-Br-PAT (20 nM) enantiomers, without the 6,7-substitutions were capable of binding with a similar affinity as (−)-trans-6-OMe-7-Cl-m-Cl-PAT (50 nM) and
(−)-trans-6-OMe-7-Cl-m-Br-PAT (61 nM) respectively. Therefore, (−)-trans-meta-halogen-PAT moiety seems to be the core pharmacophore and the other substituents on the tetrahydronaphthalene ring did not significantly influence the binding of the dextrorotatory enantiomers in the binding pocket.

**Summary of affinity of tetrahydronaphthalene and/or pendant phenyl ring substituted PAT analogs at 5-HT2A receptors.** Comparing the affinities of analogs that have substitutions on both phenyl and tetrahydronaphthalene ring systems with *meta* substituted analogs revealed that both these analogs had similar affinities at 5-HT2A receptors. The tetrahydronaphthalene substituents with halogen at *meta* position of phenyl ring showed a significant increase in affinity for only the (2R,4S)-(+)trans enantiomers. The (2S,4R)(−)-trans enantiomers that have both tetrahydronaphthalene and pendant phenyl ring substituted showed no significant difference in affinity when compared to (−)-trans-meta-halogenated analogs. This indicates that the core pharmacophore required for the activity of the (−)-trans enantiomer was *meta* substitution of (−)-trans-PAT enantiomer with halogen group. This series of compounds are difficult to synthesize and separate, hence these compounds were not selected as candidates for *in vivo* screening in mice.

**Affinity of 4-(meta-Substituted-Phenyl)- and/or (6, 7-substituted-Tetrahydronaphthalene)-PATs at the 5-HT2C Receptor**

The same series of tetrahydronaphthalene and/or pendant phenyl ring substituted compounds were again tested for their affinity at 5-HT2C receptors (Figure 2-7). (+)-Trans-6-OMe-m-Cl-PAT had an affinity of 280 ± 30 nM and (−)-trans-6-OMe-m-Cl-PAT had an affinity of 17 ± 1 nM (*p=0.0155*) (Table 2-4) (Data for (−)-trans-6-OMe-m-Cl-PAT courtesy Dr. Clinton Canal). The affinity of (+)-trans-6-OMe-m-Cl-PAT at 5-
HT$_2$C receptors was not significantly different from their affinity at 5-HT$_2$A receptors. In contrast, (−)-trans-6-OMe-m-Cl-PAT affinity was higher at 5-HT$_2$C receptors compared to their affinity at 5-HT$_2$A receptors ($p=0.0417$). Comparing (−)-trans-6-OMe-m-Cl-PAT and (−)-trans-m-Cl-PAT revealed that (−)-trans-m-Cl-PAT (8 nM) had a 2-fold higher affinity ($p=0.0177$).

The next series of analogs that were assayed at the 5-HT$_2$C receptors are (+) and (−)-trans-6-OMe-7-Cl-PAT and (+) and (−)-cis-6-OMe-7-Cl-PAT. (+) and (−)-Trans-6-OMe-7-Cl PAT enantiomers had an affinity of 37 ± 5.1 nM and 24 ± 2.5 nM respectively. The other set of enantiomers, (+) and (−)-cis 6-OMe 7-Cl PAT, had an affinity of 1600 ± 64 nM and 96 ± 18 nM respectively ($p<0.0001$) (Table 2-4). Comparing the affinities of (+)-cis-6-OMe-7-Cl-PAT at 5-HT$_2$A and 5-HT$_2$C receptors revealed no significant difference. The other enantiomer, (−)-cis-6-OMe-7-Cl-PAT, however had higher affinity at 5-HT$_2$C receptors compared to 5-HT$_2$A receptors ($p=0.0194$). Comparing the affinities of (+)-trans-6-OMe-7-Cl-PAT at 5-HT$_2$A receptors with 5-HT$_2$C receptors revealed that it binds with a significantly higher affinity at 5-HT$_2$C receptors ($p=0.0003$). The other enantiomer (−)-trans-6-OMe-7-Cl-PAT also binds with a significantly higher affinity at 5-HT$_2$C receptors compared to 5-HT$_2$A receptors ($p<0.0001$). Comparing the affinities of (−)-trans-6-OMe-m-Cl-PAT with (−)-trans-6-OMe-7-Cl-PAT revealed no significant difference.

The next analog to be assayed at the 5-HT$_2$C receptors was 6-OMe-7-Cl-m-Cl PAT. All the four stereoisomers of this analog were also isolated and tested at this receptor. (+) and (−)-trans-6-OMe-7-Cl-m-Cl-PAT enantiomers had an affinity of 7 ± 3 nM and 9 ± 2 nM respectively. These values indicate a loss of stereoselectivity at 5-
HT₂C receptors similar to 5-HT₂A receptors. The affinity of (+)-trans-6-OMe-7-Cl-m-Cl-PAT was significantly higher at the 5-HT₂C receptors compared to 5-HT₂A receptors \( (p=0.0002) \). (—)-Trans-6-OMe-7-Cl-m-Cl-PAT had a 5-fold higher affinity at 5-HT₂C receptors compared to 5-HT₂A receptors (not significantly different). The cis enantiomers of these compounds followed the same trend at both 5-HT₂A and 5-HT₂C receptors, that is, a reversal in their stereoselectivity. Comparing the affinities of both (+) and (—)-cis-6-OMe-7-Cl-m-Cl-PAT at 5-HT₂A versus 5-HT₂C receptors revealed no significant difference. There was no difference in the affinity of (—)-trans-6-OMe-7-Cl-PAT enantiomer when compared to (—)-trans-6-OMe-7-Cl-m-Cl-PAT. Addition of chlorine substituent at the meta-position of pendant phenyl ring did not significantly change the affinity of \((2S,4R)-(—)-trans\)-enantiomer.

The last pair of enantiomers that were tested at 5-HT₂C receptors were (+) and (—)-trans-6-OMe-7-Cl-m-Br-PAT. (+)-Trans-6-OMe-7-Cl-m-Br-PAT had an affinity of 350 \( \pm \) 130 nM and (—)-trans-6-OMe-7-Cl-m-Br-PAT had a \( K_i \) of 130 \( \pm \) 43 nM at 5-HT₂C receptors (Data courtesy Dr. Clinton Canal) (Table 2-4). These affinities were not significantly different from each other. These enantiomers demonstrated a loss of stereoselectivity at both 5-HT₂A and 5-HT₂C receptors.

**Summary of affinity of tetrahydronaphthalene ring and/or pendant phenyl ring substituted PAT analogs at 5-HT₂C receptors.** The substitution at the 6 and 7 position of tetrahydronaphthalene ring with a concomitant substitution at the meta position revealed a loss in stereoselectivity for the stereoisomers. (+)-Trans-6-OMe-7-Cl-PAT had 7-fold selectivity in binding at 5-HT₂C receptors by exploiting the subtle differences in the binding pocket of 5-HT₂C receptors compared to 5-HT₂A receptors.
Similar to the trend observed at 5-HT$_{2A}$ receptors, comparison of affinities of (−)-trans-meta-halogenated-PAT with analogs that have both tetrahydronaphthalene and pendant phenyl ring substituents revealed no significant difference at 5-HT$_{2C}$ receptors.

Computational Chemistry and Molecular Modeling Studies: In silico Docking of PATs at 5-HT$_{2}$ receptors

The data presented in this section is from work done by our collaborator Dr. Cordova-Sintjago Tania in the Department of Medicinal Chemistry at the University of Florida (2009-2012). The ligand docking studies aim to probe PAT interaction with amino acids in the binding pocket of 5-HT$_{2}$ GPCRs. Delineation of these molecular interactions will assist in explaining the PAT ligand affinity results and also assist in the design of future ligand structure optimized to exploit hypothesized PAT–5-HT$_{2}$ interactions. In silico docking studies revealed that (−)-trans-PAT, protonated at physiological pH, forms an ionic interaction with D3.32 (2Å) at 5-HT$_{2A}$ receptors. The amine group was also able to form a hydrogen bond with para-hydroxy group of Y7.43 (3.3Å). The methyl groups on the amines were in close proximity to F6.51 (4.2Å). The pendant phenyl ring interacts with W6.48 (3.8Å) and F6.52 (4.2Å) amino acids (Figure 2-8). These interactions assist in stabilizing the (−)-trans-PAT molecule in the binding pocket. In contrast, phenyl and tetrahydronaphthalene rings of (+)-trans-PAT did not form stabilizing interaction with aromatic amino acids W6.48, F6.51 and F6.52. This explains the lower affinity of (+)-trans-PAT despite interacting with D3.32 (2Å) of 5-HT$_{2A}$ receptors. In silico docking of (−)-trans-PAT at 5-HT$_{2C}$ receptors revealed that amino acids D3.32 (1.84Å), S3.36 (1.58Å) and Y7.43 (1.56Å) interacted with the protonated amine of (−)-trans-PAT, similar to interactions at 5-HT$_{2A}$ receptors. However, the pendant phenyl ring interacted with W6.48 (5Å), F6.51 (2.2Å) and F6.52 amino acids of
5-HT$_{2C}$ receptors. In contrast to 5-HT$_{2A}$ receptors, tetrahydronaphthalene ring was oriented parallel to Y7.32 (3.7 Å) and in close proximity to S3.36 (3.5 Å) (Figure 2-9). The closer proximity of terminal amine of (−)-trans-PAT to D3.32 and Y7.43 and its added interaction with S3.36 help explain its higher affinity at 5-HT$_{2C}$ receptors in contrast to 5-HT$_{2A}$ receptors.

*In silico* docking studies on (−)-trans-m-Cl-PAT enantiomer at 5-HT$_{2C}$ receptors revealed that some of its amino acid interaction are similar to those of (−)-trans-PAT at the 5-HT$_{2C}$ receptors. The common interactions include D3.32 (3.3 Å), S3.36 (2.9 Å), W6.48 (5 Å), Y7.43 (3 Å) and F6.51 (6 Å). However, different poses of (−)-trans-m-Cl-PAT were possible as the pendant phenyl ring was freely rotatable around the sp$^3$-hybridized carbon of the tetrahydronaphthalene ring. The chlorine at the *meta*-position, when present in one of the poses, is at a distance of 3 Å from asparagine N6.55 amino acid thereby assisting in binding in the binding pocket. This proximity could lead to a stabilizing interaction between the hydrogen on amine of asparagine and chlorine. This additional interaction could explain the significantly higher affinity of (−)-trans-m-Cl-PAT enantiomer at 5-HT$_{2C}$ receptor relative to (−)-trans-PAT enantiomer.

*In silico* docking studies of (−)-trans-m-Br-PAT at 5-HT$_{2C}$ receptors showed that protonated amine interacts with D3.32 (1.7 Å), S3.36 (2.3 Å) and pendant phenyl ring was in close proximity to F6.44 (4.5 Å). The bromine on the pendant phenyl ring was oriented towards transmembrane 6 and was present in close proximity, with in 5 Å, to F6.44, M6.47, W6.48 and C7.45. In an alternate pose, due to rotation of the pendant phenyl ring, the protonated amine of (−)-trans-m-Br-PAT again interacted with D3.32 (1.6 Å), S3.36 (2.8 Å). The tetrahydronaphthalene ring was present parallel to Y7.43 (4 Å).
and bromine was oriented towards transmembrane 2 and 7. Bromine substituent was present in close proximity, with in 5Å, to V2.53, F6.44, C7.45 and S7.46 (Figure 2-10). Presence of these aromatic and polar amino acids in close proximity to the meta- bromine could explain the significantly higher affinity shown by (−)-trans-m-Br-PAT enantiomer at 5-HT2C receptors relative to (−)-trans-PAT.

In silico docking studies revealed that protonated amine of (+)-trans-p-Cl-PAT formed a hydrogen bond with D3.32 (1.75Å) and Y7.43 (1.49Å) at 5-HT2A receptors. In addition to this, the chlorine at para-position could also interact with the hydroxyl group of S5.43 (2.50Å). In contrast, (−)-trans-p-Cl-PAT formed a hydrogen bond with D3.32 (1.83-1.96Å) and indole side chain of W3.28 (1.78Å) at 5-HT2A receptors. The lack of interaction with Y7.43 and S5.43 could explain the decreased affinity of (−)-trans-p-Cl-PAT enantiomer at 5-HT2A receptors (Figure 2-11)134. Docking studies at 5-HT2C receptors revealed that protonated amine of (−)-trans-p-Cl-PAT forms a hydrogen bond with carboxylate moiety of D3.32 (1.78Å), indole side chain of W3.28 (1.78Å) and hydroxyl side chain of Y7.43 (1.36Å). The additional interaction with hydroxyl side chain of Y7.43 could explain the significantly higher affinity of (−)-trans-p-Cl-PAT at 5-HT2C receptors compared to 5-HT2A receptors. The (+)-trans-p-Cl-PAT enantiomer forms similar hydrogen bonds with D3.32 (1.60Å) and Y7.43 (1.48Å). In addition to this, the chlorine on pendant phenyl ring also forms a hydrogen bond with S5.43 (2.50Å), this interaction could explain the higher affinity of (+)-trans-p-Cl-PAT as compared to (−)-trans-p-Cl-PAT (Figure 2-12)30.

Preliminary in silico studies at 5-HT2C receptors revealed the amino acid residues that are present in a 6Å distance from (−)-trans-p-CF3-PAT docked at 5-HT2C receptors.
Similar interactions as were observed in other PAT enantiomers were also observed here (D3.32, S3.36, S5.43 and N6.55). Fluorine atoms in trifluoromethyl substituent at the para-position were found to interact with hydrogen on amide of C7.45 (4Å) and Y7.43 (2Å). This C7.45 amino acid is not conserved at 5-HT<sub>2A</sub> and 5-HT<sub>2B</sub> receptors and this could explain the significantly higher affinity of (−)-trans-p-CF<sub>3</sub>-PAT at 5-HT<sub>2C</sub> receptors. Preliminary in silico studies at 5-HT<sub>2C</sub> receptors revealed the amino acid residues that are present at a 6Å radius around (−)-trans-m-CF<sub>3</sub>-PAT docked at 5-HT<sub>2C</sub> receptors. Interactions that were similar to (−)-trans-PAT include D3.32, S3.36, F6.51, F6.52 and Y7.43. Similar to (−)-trans-p-CF<sub>3</sub>-PAT, C7.45 was present in close proximity with the trifluoromethyl substituent. This could result in hydrogen bonds between fluorine and hydrogen on amide of C7.45.

In silico docking studies explained the higher affinity of (+)-trans-6-OMe-7-Cl-PAT enantiomer at 5-HT<sub>2C</sub> receptors relative to 5-HT<sub>2A</sub> receptors. The 6-methoxy group of (+)-trans-6-OMe-7-Cl-PAT interacted with S5.46 at 5-HT<sub>2A</sub> receptors and the ensuing steric clash explained the 7-fold lower affinity of the enantiomer at 5-HT<sub>2A</sub> receptors. The same position, 5.46, at 5-HT<sub>2C</sub> receptors has an alanine amino acid resulting in less steric hindrance at this position relative to 5-HT<sub>2A</sub> receptors. Hence, the higher affinity of (+)-trans-6-OMe-7-Cl-PAT enantiomer at 5-HT<sub>2C</sub> receptors compared to 5-HT<sub>2A</sub> receptors. This steric clash at 5-HT<sub>2A</sub> receptor between amino acid S5.46 and methoxy substituent of (+)-trans-6-OMe-7-Cl-PAT was further corroborated by the significant higher affinity of (+)-trans-6-OH-7-Cl-PAT at 5-HT<sub>2A</sub> receptors. This high affinity of (+)-trans-6-OH-7-Cl-PAT at 5-HT<sub>2A</sub> receptors was due to favorable hydrogen bond between the 6-hydroxy substituent and S5.46 amino acid (Figure 2-13). The change in amino
acid to alanine at 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors results in a loss of this hydrogen bond seen as a significant drop in affinity of (+)-trans-6-OH-7-Cl-PAT at these receptors (Figure 2-14) (Data for trans-6-OH-7-Cl-PAT courtesy Dr. Clinton Canal).
Figure 2-1. Stereochemical relationship between PAT diastereomers and enantiomers.
Figure 2-2. Structure of meta-substituted PAT analogs. A) Structure of different (+)-trans-m-substituted-PAT analogs. B) Structure of different (−)-trans-m-substituted-PAT analogs.

Figure 2-3. Change in $K_i$ value at 5-HT$_{2A}$ receptors with increase in the size of substituent at meta-position.

Figure 2-5. Effect of sterics on the affinity of para-substituted PAT enantiomers at 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors.
Figure 2-6. Single X-ray crystallographic structure of (2S,4R)-(−)-trans-p-Cl-PAT. HCl.

Figure 2-7. Tetrahydronaphthalene and/or pendant phenyl ring substituted PAT analogs.

\[ X = H, \text{Cl} \& \text{Br} \]
\[ Y = \text{OMe} \]
\[ Z = H, \text{Cl} \]
Figure 2-8. (−)-trans-PAT enantiomer docked at 5-HT₂A receptors with the corresponding amino acid interactions in binding pocket.

Figure 2-9. (−)-trans-PAT docked at 5-HT₂C receptors with the corresponding amino acid interactions in binding pocket.
Figure 2-10. (−)-trans-m-Br-PAT enantiomer docked at 5-HT$_{2C}$ receptors. A) Shows the bromine substituent at the meta-position oriented towards transmembrane 6. B) Shows the bromine substituent at meta-position oriented towards transmembrane 2 and 7.

Figure 2-11. (2R,4S)-(+)-trans and (2S,4R)-(−)-trans-p-Cl-PAT enantiomer docked at 5-HT$_{2A}$ receptors. A) Represents the amino acid interactions of (2R,4S)-(+)-trans-p-Cl-PAT at 5-HT$_{2A}$ receptors. B) Represents the amino acid interactions of (2S,4R)-(−)-trans-p-Cl-PAT at 5-HT$_{2A}$ receptors.
Figure 2-12. (2R,4S)-(+)-trans and (2S,4R)-(−)-trans-p-Cl-PAT enantiomer docked at 5-HT$_{2c}$ receptors. A) Represents the amino acid interactions of (2R,4S)-(+)-trans-p-Cl-PAT at 5-HT$_{2c}$ receptors. B) Represents the amino acid interactions of (2S,4R)-(−)-trans-p-Cl-PAT at 5-HT$_{2c}$ receptors.
Figure 2-13. Binding affinities of (+) and (−)-trans-6-OH-7-Cl-PAT at 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors. The affinity of (+)-trans-6-OH-7-Cl-PAT was significantly higher at 5-HT$_{2A}$ receptors relative to other receptors.
Figure 2-14. (−)-Trans-6-OH-7-Cl-PAT and (+)-trans-6-OH-7-Cl-PAT docked at 5-HT$_{2A}$ receptors. Depicts the view of the binding pocket looking from the extracellular region into the intracellular region. A) (−)-trans-6-OH-7-Cl-PAT enantiomer is docked away from transmembrane 5 in binding pocket. B) The (+)-trans-6-OH-7-Cl-PAT enantiomer is docked closer to transmembrane 5.
Table 2-1. Shows changes in the bond length relative to changes in substituent.

<table>
<thead>
<tr>
<th>Bond type</th>
<th>Bond distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;ar&lt;/sub&gt;-H</td>
<td>1.09766</td>
</tr>
<tr>
<td>C&lt;sub&gt;ar&lt;/sub&gt;-F</td>
<td>1.34015</td>
</tr>
<tr>
<td>C&lt;sub&gt;ar&lt;/sub&gt;-Cl</td>
<td>1.67760</td>
</tr>
<tr>
<td>C&lt;sub&gt;ar&lt;/sub&gt;-Br</td>
<td>1.86251</td>
</tr>
<tr>
<td>C&lt;sub&gt;ar&lt;/sub&gt;-C</td>
<td>1.53114</td>
</tr>
<tr>
<td>C&lt;sub&gt;sp3&lt;/sub&gt;-F</td>
<td>1.35127</td>
</tr>
</tbody>
</table>

Table 2-2. Binding affinities of parent PAT enantiomers and meta-substituted PAT enantiomers at 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors.

<table>
<thead>
<tr>
<th>R</th>
<th>Configuration</th>
<th>5-HT&lt;sub&gt;2A&lt;/sub&gt; Affinity (Ki ± SEM; nM)</th>
<th>5-HT&lt;sub&gt;2C&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>(+) trans</td>
<td>470(46)</td>
<td>430(86)</td>
</tr>
<tr>
<td></td>
<td>(-) trans</td>
<td>100(10)</td>
<td>30(3)</td>
</tr>
<tr>
<td>m-F-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>(+) trans</td>
<td>320(26)</td>
<td>200(30)</td>
</tr>
<tr>
<td></td>
<td>(-) trans</td>
<td>110(26)</td>
<td>43(14)</td>
</tr>
<tr>
<td>m-Cl-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>(+) trans</td>
<td>130(8.7)</td>
<td>170(43)</td>
</tr>
<tr>
<td></td>
<td>(-) trans</td>
<td>40(5)</td>
<td>8(3)</td>
</tr>
<tr>
<td>m-Br-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>(+) trans</td>
<td>260(22)</td>
<td>200(30)</td>
</tr>
<tr>
<td></td>
<td>(-) trans</td>
<td>20(3)</td>
<td>4(1)</td>
</tr>
<tr>
<td>m-CF&lt;sub&gt;3&lt;/sub&gt;-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>(+) trans</td>
<td>1300(67)</td>
<td>230(20)</td>
</tr>
<tr>
<td></td>
<td>(-) trans</td>
<td>80(10)</td>
<td>10(2)</td>
</tr>
<tr>
<td>m-NO&lt;sub&gt;2&lt;/sub&gt;-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>(+) trans</td>
<td>500(20)</td>
<td>120(13)</td>
</tr>
<tr>
<td></td>
<td>(-) trans</td>
<td>74(18)</td>
<td>10(1)</td>
</tr>
</tbody>
</table>
Table 2-3. Binding affinities of *para*-substituted PAT enantiomers at 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors.

<table>
<thead>
<tr>
<th>R</th>
<th>Configuration</th>
<th>5-HT\textsubscript{2A} Affinity (Ki ± SEM; nM)</th>
<th>5-HT\textsubscript{2C}</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-F-\text{C}_6\text{H}_4</td>
<td>(+) <em>trans</em></td>
<td>71(12)</td>
<td>75(12)</td>
</tr>
<tr>
<td></td>
<td>(−) <em>trans</em></td>
<td>140(24)</td>
<td>68(11)</td>
</tr>
<tr>
<td>p-Cl-\text{C}_6\text{H}_4</td>
<td>(+) <em>trans</em></td>
<td>50(8)</td>
<td>55(17)</td>
</tr>
<tr>
<td></td>
<td>(−) <em>trans</em></td>
<td>250(37)</td>
<td>120(9.0)</td>
</tr>
<tr>
<td>p-Br-\text{C}_6\text{H}_4</td>
<td>(+) <em>trans</em></td>
<td>60(10)</td>
<td>70(8)</td>
</tr>
<tr>
<td></td>
<td>(−) <em>trans</em></td>
<td>220(29)</td>
<td>100(3)</td>
</tr>
<tr>
<td>p-CF\textsubscript{3}-\text{C}_6\text{H}_4</td>
<td>(+) <em>trans</em></td>
<td>210(16)</td>
<td>220(27)</td>
</tr>
<tr>
<td></td>
<td>(−) <em>trans</em></td>
<td>2000(200)</td>
<td>520(51)</td>
</tr>
<tr>
<td>p-NO\textsubscript{2}-\text{C}_6\text{H}_4</td>
<td>racemic</td>
<td>8100(180)</td>
<td>1000 (100)</td>
</tr>
</tbody>
</table>
Table 2-4. Binding affinities of tetrahydronaphthalene ring with (or without) pendant phenyl ring substituted PAT analogs.

<table>
<thead>
<tr>
<th>R</th>
<th>Configuration</th>
<th>5-HT$_{2A}$ Affinity ($K_i \pm$ SEM; nM)</th>
<th>5-HT$_{2C}$ Affinity ($K_i \pm$ SEM; nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OMe-7-Cl-PAT</td>
<td>(+)-trans</td>
<td>240(23)</td>
<td>37(5.1)</td>
</tr>
<tr>
<td></td>
<td>(−)-trans</td>
<td>160(24)</td>
<td>24(2.5)</td>
</tr>
<tr>
<td>6-OMe-7-Cl-PAT</td>
<td>(+)-cis</td>
<td>2300(130)</td>
<td>1600(60)</td>
</tr>
<tr>
<td></td>
<td>(−)-cis</td>
<td>320(42)</td>
<td>96(18)</td>
</tr>
<tr>
<td>6-OMe-7-Cl-m-Cl-PAT</td>
<td>(+)-trans</td>
<td>95(18)</td>
<td>7(3)</td>
</tr>
<tr>
<td></td>
<td>(−)-trans</td>
<td>50(20)</td>
<td>9(2)</td>
</tr>
<tr>
<td>6-OMe-7-Cl-m-Cl-PAT</td>
<td>(+)-cis</td>
<td>230(64)</td>
<td>320(41)</td>
</tr>
<tr>
<td></td>
<td>(−)-cis</td>
<td>3500(390)</td>
<td>1560(390)</td>
</tr>
<tr>
<td>6-OMe-7-Cl-m-Br-PAT</td>
<td>(+)-trans</td>
<td>79(14)</td>
<td>350(130)</td>
</tr>
<tr>
<td></td>
<td>(−)-trans</td>
<td>61(15)</td>
<td>130(43)</td>
</tr>
<tr>
<td>6-OMe-m-Cl-PAT</td>
<td>(+)-trans</td>
<td>350(16)</td>
<td>280(30)</td>
</tr>
<tr>
<td></td>
<td>(−)-trans</td>
<td>35(3)</td>
<td>17(1.0)</td>
</tr>
<tr>
<td>6-OH-7-Cl-PAT</td>
<td>(+)-trans</td>
<td>70(2.6)</td>
<td>1000(80)</td>
</tr>
<tr>
<td></td>
<td>(−)-trans</td>
<td>60(5.4)</td>
<td>23(3.0)</td>
</tr>
</tbody>
</table>
CHAPTER 3
CHARACTERIZATION OF THE 5-HT$_2$ FUNCTIONAL PHARMACOLOGY OF PAT ANALOGS

Specific Aim 2

The ideal pharmacological profile of a ligand targeting the 5-HT$_2$ system is: antagonist/inverse agonist at 5-HT$_{2A/2B}$ receptors and potent agonist at 5-HT$_{2C}$ receptors. Analogs that had high affinity at therapeutically relevant 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors were selected from both pendant phenyl ring substituted and tetrahydronaphthalene ring substituted group of PAT analogs. (−)-Trans- m-Cl-PAT and (−)-Trans-m-Br-PAT analogs were selected because these analogs had significantly higher affinity at both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors among all the meta-substituted-PAT analogs tested. (+)-Trans-p-Cl-PAT analog had the highest affinity among all the para-substituted-PAT analogs (not statistically significant) and was chosen from the para substituted group of analogs. (−)-Trans-6-OMe-m-Cl-PAT was chosen from the tetrahydronaphthalene ring series of analogs as this analog had high affinity at both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. Analogs that possessed potency values <500nM are considered for translational studies of AIM 3.

Methodology

Functional assays were performed to measure the ability of the ligands to modulate phospholipase C (PLC) pathway. These assays measured the level of $[^3]$H]-IP formation in the presence of different concentrations of ligand. HEK cells grown to approximately 80% confluency in DMEM containing 10% fetal bovine serum and 1% antibiotic in 10 cm plates at 37°C, 5% CO$_2$ (incubator) were washed one time with PBS, then transfected with 20 µg 5-HT$_2$ pcDNA (5-HT$_{2C-}$, 5-HT$_{2B}$ or 5-HT$_{2A}$), and 30 µl lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in 5 mL DMEM containing 5%
dialyzed fetal bovine serum and 5 mL Opti-MEM (transfection media). Cells were placed in an incubator, and approximately 16 hours later, transfection media was removed and replaced with 16 mL inositol-free DMEM containing 2.5% dialyzed fetal bovine serum. Cells were then detached by vigorous pipetting, 0.1 μCi/mL [³H]-myo-inositol was added to the mixture (labeling media), and cells were seeded 300 µl per well into 48 well CellBind® plates (Corning, Lowell, MA), and placed in an incubator. 24 hr. later, plates were centrifuged at 2500 r.p.m. for 10 min. at room temperature, labeling media was discarded, and 450 µL inositol-free, serum-free DMEM was added to each well. Cells were then incubated for one hour. Cells were then incubated for 30 min. with increasing concentrations of ligands diluted in inositol-free, serum-free DMEM containing a final concentration of 50 mM LiCl and 10µM pargyline per well. Plates were again centrifuged at 2500 r.p.m. for 10 min. at room temperature, drug incubation media was discarded, and 400 µl of 50 mM formic acid was added to each well to lyse cells. One hour later, 200 µl of 150 mM NH₄OH was added to each well to neutralize cells, and plates were stored at −20°C overnight. After thawing, 500 µl of solution from each well was added to individual anion-exchange columns to separate [³H]-inositol phosphates formed from [³H]-myo-inositol. Following a 10 mL wash with dH₂O, bound [³H]-inositol phosphates were eluted with 4 mL 800 mM ammonium formate into vials. 1 mL of eluate was added to 10 mL scintillation fluid (ScintiVerse Cocktail, Fisher). After mixing, ³H-induced scintillations were counted with a Beckman-Coulter LS6500 counter. Each independent experiment was performed a minimum of three times.
Competitive functional antagonism assays were performed using a similar methodology, except the antagonist was incubated in 48-well plates for 15 minutes, prior to addition of serotonin.

Results and Discussion for Functional Assays

Functional Activity of (−)-trans-PAT and (−)-trans-4-(meta-halogenated)-PATs at 5-HT_2 Receptors

(−)-Trans-PAT enantiomer was a partial agonist at 5-HT_2C receptors and an inverse agonist at both 5-HT_2A and 5-HT_2B receptors\(^{123}\). (−)-Trans-PAT had an EC\(_{50}\) of 61.1 ± 18.2 nM at the 5-HT_2C receptors (Table 3-1)\(^{84}\). The IC\(_{50}\) of (−)-trans-PAT at 5-HT_2A and 5-HT_2B receptors was found to be 490 ± 96 nM and 1000 ± 0.5 nM respectively. These data corroborate the \textit{in silico} docking results showing (−)-trans-PAT interacting with differently amino acids in binding pocket at 5-HT_2A versus 5-HT_2C receptors and potentially leading to stabilization of different conformations.

\textit{Meta} substituted analogs of PATs showed the best binding profile at both 5-HT_2A and 5-HT_2C receptors. Similar to (−)-trans-PAT, (−)-trans-m-Cl-PAT enantiomer did not activate both 5-HT_2A and 5-HT_2B receptors (Figure 3-1 and 3-2). (−)-Trans-m-Cl-PAT was a selective agonist at 5-HT_2C receptor and had an EC\(_{50}\) value of 40.1 ± 7.60 nM (Figure 3-3) (Data at 5-HT_2C receptors courtesy Daniel Felsing).

Since (−)-trans-m-Cl-PAT was an antagonist at 5-HT_2A receptor, the ‘potency’ of the ligand at 5-HT_2A receptor can be measured using functional antagonism assays. These assays involved performing functional assays where the ability of (−)-trans-m-Cl-PAT to antagonize 5-HT induced activation of PLC mediated by 5-HT_2A receptor is measured in the presence of different concentrations of antagonist. These assays reveal the nature of the antagonist: competitive or non-competitive antagonist.
Competitive antagonists displace the curve to the right with no change in the maximal response of serotonin. Non-competitive antagonist, in contrast, decrease the maximal response produced by the highest concentration of serotonin. These assays also assist in determining the ‘strength’ of an antagonist by determining the pA$_2$ values, the negative logarithm of the concentration of antagonist required to shift the EC$_{50}$ by 2-fold. The pA$_2$ value for (-)-trans-m-Cl-PAT was calculated to be 6.4 ± 0.092 (Figure 3-4). (-)-Trans-m-Cl-PAT was a competitive antagonist of serotonin at 5-HT$_{2A}$ receptors as there was no decrease in the maximal serotonin response.

Similar to (-)-trans-PAT and (-)-trans-m-Cl-PAT enantiomers, (-)-trans-m-Br-PAT enantiomer was also an antagonist at both 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors. The functional potency of (-)-trans-m-Br-PAT enantiomer at 5-HT$_{2C}$ receptors was 17 ± 3.2 nM (Table 3-1). Functional curves for all 5-HT$_{2}$ receptors are represented in figure 3-5. (Functional characterization of (-)-trans-m-Br-PAT analog was done by Dr. Clinton Canal.)

Functional experiments revealed that (-)-trans-m-CF$_3$-PAT was an inverse agonist at the 5-HT$_{2C}$ receptors with an efficacy of 40 ± 0.7 nM (Figure 3-11). This revealed an interesting shift from agonism to inverse agonism when the substituent at meta-position of pendant phenyl ring was changed from a halogen to a trifluoromethyl substituent. The steric and the electronic nature of the trifluoromethyl substituent are significantly different from other halogen substituents at meta-position. This indicated that the trifluoromethyl substituent bound differently and hence interacted with different amino acids in the binding pocket thereby stabilizing a different conformation of the receptor. This result warrants more investigation to characterize the molecular
determinants that interact (−)-trans-m-CF<sub>3</sub>-PAT and mediate the inverse agonist response.

**Functional Activity of (+) and (−)-trans-4-(p-Cl)-PATs at 5-HT<sub>2</sub> Receptors**

Both (+) and (−)-trans-<i>p</i>-Cl-PAT enantiomers did not activate the PLC pathway at 5-HT<sub>2A</sub> and 5-HT<sub>2B</sub> receptors even at 10μM concentration (Figure 3-6 and 3-7). In contrast, both (+) and (−)-trans-<i>p</i>-Cl-PAT were agonist at 5-HT<sub>2C</sub> receptors. The EC<sub>50</sub> of (+)-trans-<i>p</i>-Cl-PAT was 140 ± 20 nM and EC<sub>50</sub> of (−)-trans-<i>p</i>-Cl-PAT was 1650 ± 149 nM (Table 3-1). (+)-Trans-<i>p</i>-Cl-PAT was 12 times more potent than (−)-trans-<i>p</i>-Cl-PAT at activating PLC pathway (<i>p</i>&lt;0.0001) (Figure 3-8). This reversal in stereoselectivity corroborated the affinity of (+) and (−)-<i>p</i>-Cl-PAT enantiomers observed at 5-HT<sub>2C</sub> receptors. Schild analysis of this enantiomer at 5-HT<sub>2A</sub> receptors revealed a pA<sub>2</sub> value of 6.21 ± 0.545 (Figure 3-9). The pA<sub>2</sub> values of (+)-trans-<i>p</i>-Cl-PAT and (−)-trans-<i>m</i>-Cl-PAT were not significantly different. These values correspond with the observed lack of significant difference in the affinities of (+)-trans-<i>p</i>-Cl-PAT and (−)-trans-<i>m</i>-Cl-PAT at 5-HT<sub>2A</sub> receptors. However, comparing the EC<sub>50</sub> values of (+)-trans-<i>p</i>-Cl-PAT and (−)-<i>m</i>-Cl-PAT at 5-HT<sub>2C</sub> receptors revealed that (−)-trans-<i>m</i>-Cl-PAT enantiomer had significantly higher potency at 5-HT<sub>2C</sub> receptors (<i>p</i>=0.0014).

**Functional Activity of (−)-trans-4-(m-Cl)-(6-OMe-Tetrahydronaphthalene)-PAT Analog at 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> Receptors**

Functional activity of (−)-trans-6-OMe-<i>m</i>-Cl-PAT enantiomer at 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors was assessed. Similar to other PAT analogs it was found to be an antagonist at 5-HT<sub>2A</sub> receptor and an agonist at 5-HT<sub>2C</sub> receptors (Figure 3-10). The EC<sub>50</sub> of (−)-trans-6-OMe-<i>m</i>-Cl-PAT enantiomer at 5-HT<sub>2C</sub> receptors was found to be 43 ± 14nM (Table 3-1). Comparing the EC<sub>50</sub> of (−)-trans-6-OMe-<i>m</i>-Cl-PAT enantiomer with
(−)-*trans-m*-Cl-PAT enantiomer showed no significant difference between their potencies (*p* > 0.05).
Figure 3-1. Functional assay curve representing the action of \((-)-\text{trans-}m\text{-Cl-PAT}\) enantiomer at 5-HT$_{2A}$ receptor.

Figure 3-2. Functional assay curve representing the action of \((-)-\text{trans-}m\text{-Cl-PAT}\) enantiomer at 5-HT$_{2B}$ receptor.
Figure 3-3. Functional assay curve representing the action of (--)trans-m-Cl-PAT enantiomer at 5-HT\textsubscript{2C} receptor.

Figure 3-4. Schild plot of (--)trans-m-Cl-PAT at 5-HT\textsubscript{2A} receptors. (--)Trans-m-Cl-PAT enantiomer was incubated at 100 nM, 1000 nM and 10000 nM concentrations.
Figure 3-5. Functional activity of (−)-trans-m-Br-PAT at 5-HT$_2$A, 5-HT$_2$B and 5-HT$_2$C receptors.

Figure 3-6. PLC functional activity of (+) and (−)-trans-p-Cl-PAT at 5-HT$_2$A receptors.
Figure 3-7. PLC functional activity of (+) and (−)-trans-p-Cl-PAT at 5-HT$_{2B}$ receptors.

Figure 3-8. PLC functional activity of (+) and (−)-trans-p-Cl-PAT demonstrating partial agonism at 5-HT$_{2C}$ receptors.
Figure 3-9. Schild plot of (+)-trans-p-Cl-PAT at 5-HT$_{2A}$ receptors. (+)-trans-p-Cl-PAT enantiomer was incubated at 100 nM, 1000 nM and 10000 nM concentrations.

Figure 3-10. Functional activity of (−)-trans-6-OMe-m-Cl-PAT enantiomer at 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors.
Figure 3-11. Functional response of (−)-trans-m-CF₃-PAT enantiomer at 5-HT₂C receptors.
Table 3-1. Functional activity of PAT analogs at 5-HT<sub>2</sub> receptors.

<table>
<thead>
<tr>
<th>PAT analog</th>
<th>5-HT&lt;sub&gt;2A&lt;/sub&gt;</th>
<th>5-HT&lt;sub&gt;2B&lt;/sub&gt;</th>
<th>5-HT&lt;sub&gt;2C&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-trans-PAT</td>
<td>Inverse agonist; IC&lt;sub&gt;50&lt;/sub&gt; = 490 ± 96 nM</td>
<td>Inverse agonist; IC&lt;sub&gt;50&lt;/sub&gt; = 1000 ± .5 nM</td>
<td>Agonist; EC&lt;sub&gt;50&lt;/sub&gt; = 61.1 ± 18.2 nM</td>
</tr>
<tr>
<td>(-)-trans-m-Br-PAT</td>
<td>Not activated</td>
<td>Not activated</td>
<td>Agonist; EC&lt;sub&gt;50&lt;/sub&gt; of 17 ± 3.2 nM</td>
</tr>
<tr>
<td>(-)-trans-m-Cl-PAT</td>
<td>Not activated; pA&lt;sub&gt;2&lt;/sub&gt; = 6.4 ± 0.092</td>
<td>Not activated</td>
<td>Agonist; EC&lt;sub&gt;50&lt;/sub&gt; of 40.1 ± 7.60 nM</td>
</tr>
<tr>
<td>(+)-trans-p-Cl-PAT</td>
<td>Not activated</td>
<td>Not activated</td>
<td>Agonist; EC&lt;sub&gt;50&lt;/sub&gt; of 140 ± 20 nM</td>
</tr>
<tr>
<td>(-)-trans-p-Cl-PAT</td>
<td>Not activated; pA&lt;sub&gt;2&lt;/sub&gt; = 6.21 ± 0.545</td>
<td>Not activated</td>
<td>Agonist; EC&lt;sub&gt;50&lt;/sub&gt; of 1650 ± 149 nM</td>
</tr>
<tr>
<td>(-)-trans-6-OMe-m-Cl-PAT</td>
<td>Not activated</td>
<td></td>
<td>Agonist; EC&lt;sub&gt;50&lt;/sub&gt; of 43 ± 14 nM</td>
</tr>
</tbody>
</table>
CHAPTER 4
TRANSLATIONAL STUDIES: ASSESSMENT OF PAT ANALOGS EFFICACY IN RODENT MODELS OF PSYCHOSIS

Specific Aim 3

Analogs of \((-\)-\textit{trans}-PAT that had high affinity and potency at \(5\text{-HT}_{2A}\) and \(5\text{-HT}_{2C}\) receptors were screened in different mice models, each manipulating a different neurotransmitter implicated in schizophrenia. \((+)\) and \((-\)-\textit{Trans}-PAT, \((+)\) and \((-\)-\textit{trans}-
\(p\text{-Cl-PAT}, \((+)\) and \((-\)-\textit{trans}-\(m\text{-Cl-PAT and} \((-\)-\textit{trans}-\(m\text{-Br-PAT analogs were selected for} \textit{in vivo} screening. These meta- and para-substituted analogs were chosen because they had the highest affinity and potency at both \(5\text{-HT}_{2A}\) and \(5\text{-HT}_{2C}\) receptors among all the PAT analogs. Another objective was to confirm the effectiveness of these models for screening antipsychotics in our laboratory conditions. This was ascertained by screening clinically approved antipsychotics in these models. These PAT analogs were then screened to test their efficacy in HTR, MK-801 and amphetamine induced hyperlocomotion models. These experiments also provided a preliminary insight into the pharmacokinetics of the analogs being tested.

Methodology

\textit{In vivo Behavioral Pharmacology}

C57Bl/6J male mice were obtained from Jackson Laboratories at approximately 8 weeks of age and allowed to acclimate to the temperature- and humidity-controlled colony room for at least 1 week prior to testing. Mice were housed in pairs, in standard cages and allowed unlimited access to laboratory chow and water. Experiments were conducted at approximately the middle of the light phase (lights on at 6 am, and lights off at 6 pm). All compounds were dissolved in sterile saline prior to behavioral testing, and administered in a volume of 0.01 mL/g body weight. All experimental procedures
were performed in accordance with the Guide for the Care and Use of Laboratory Animals, as promulgated by the National Institutes of Health, and were approved by the University of Florida’s Institutional Animal Care and Use Committee.

**Psycholocomotor Activity (Head Twitch Response; HTR) Elicited by the Serotonin 5-HT$_2$ Agonist 2,5-dimethoxy-4-iodoamphetamine (DOI)**

On the day of testing, mice were habituated to the testing room for approximately 30 minutes. Testing typically consisted of administration (i.p.) of sterile saline or a particular dose of (+) or (−)-trans-PAT analogs. Ten minutes later, mice were administered (−)-DOI or sterile saline. Ten minutes later, mice were individually placed into a clear plexiglas chamber (17” x 17” x 12”). (−)-DOI was administered 20 minutes after oral administration of PAT analogs or saline. Head-twitch responses (HTRs), defined as a clear, rapid, and discrete, back and forth rotation of the head. HTRs during a 10-minute session were counted by a trained observer who was blind to drug treatment conditions. An overhead camera videotaped the session, and activity (distance travelled in cm) was analyzed and calculated by Ethovision software (Noldus Information Technology Inc.).

**Psycholocomotor Activity Elicited by the Glutamate Antagonist MK-801**

Experimentally naive mice were habituated to the testing room for approximately 30 minutes. Locomotor activity testing consisted of administration (i.p.) of saline, clozapine, or PAT analogs (3.0, 5.6, and 10.0 mg/kg), followed 10 minutes later by an injection of the NMDA antagonist MK-801 (0.3 mg/kg). Mice were immediately placed into a plexiglas chamber (43 x 43 cm, Med Associates, Inc.) for a 60 minute session. An overhead camera videotaped the session and activity (distance travelled in cm) was calculated by Ethovision software (Noldus Information Technology Inc.).
**Psycholocomotor Activity Elicited by the Dopamine/Serotonin Agonist Amphetamine**

Experimentally naive mice were habituated to the testing room for approximately 30 minutes. Locomotor activity testing consisted of administration (i.p.) of saline or (+) and (−)-trans-PAT analogs (3.0, 5.6, and 10.0 mg/kg), followed (10 minutes later) by an injection of the indirect dopamine agonist amphetamine (3.0 mg/kg). Locomotor activity was assessed exactly as noted in the MK-801 experiment.

**Results for PAT Efficacy in Rodent Models of Psychosis**

Clozapine is a prototypical atypical antipsychotic used to treat schizophrenia. Clozapine was used as a control to test the efficacy of these models to screen for antipsychotics. Clozapine was administered in 0.1 and 1 mg/kg doses 10 minutes prior to DOI. Both these doses significantly decreased the HTR produced by 1 mg/kg of DOI ($F_{3,31}=138.7; p<0.0001$) (Figure 4-1). The ED$_{50}$ (95% ± CL) for clozapine was found to be 0.23 (0.18-0.28) mg/kg (Table 4-1). The same doses of clozapine were then tested in MK-801 and amphetamine stimulated locomotion models. The 1 mg/kg dose of clozapine was found to significantly lower both MK-801 and amphetamine induced locomotion ($F_{4,61}=33.02$ and $F_{4,49}=35.26$ respectively; $p<0.0001$ in both cases). The ED$_{50}$ (95% ± CL) of clozapine was found to be 0.25 (0.10-0.64) mg/kg for MK-801 induced hyperactivity model and 0.27 (0.16-0.44) mg/kg for amphetamine induced locomotion (Figure 4.2). The 1 mg/kg dose of clozapine alone resulted in significant lower locomotor activity as compared to locomotor activity after saline administration (Figure 4-5). This data is concordant with published literature that show similar effect of clozapine on spontaneous locomotor activity$^{137}$. This data indicates that these models are effective for screening antipsychotics.
(+)-trans-PAT enantiomers significantly attenuated the HTR elicited by 1 mg/kg dose of DOI \( (F_{6,57}=86.03; \ p<0.001) \). (−)-trans-PAT enantiomer was significantly more potent than the other PAT enantiomer \( (p<0.05) \). Locomotor activity was also recorded during these 10 minute observation sessions. There was no significant difference between locomotor activity for DOI-alone dose and PAT enantiomer pretreated DOI dose (HTR data of PAT courtesy Dr. Clinton Canal). These enantiomers were then tested in MK-801 and amphetamine induced hyperlocomotor experiments. Administration of 3, 5.6 and 10 mg/kg doses of (+)-trans-PAT did not result in any attenuation of MK-801 induced locomotor activity. In contrast, both 5.6 mg/kg and 10 mg/kg doses of (−)-trans-PAT resulted in significant reduction in locomotor activity compared to MK-801 induced hyperactivity \( (F_{5,67}=28.77; \ p<0.0001) \) (Figure 4-3). Similar to MK-801 results, (+)-trans-PAT did not modulate amphetamine-induced locomotion. The 10 mg/kg dose of (−)-trans-PAT significantly decreased amphetamine induced hyperlocomotion \( (F_{5,55}=17.73; \ p<0.0001) \) (Figure 4-4). However, administration of 10 mg/kg dose of (+)-trans-PAT alone resulted in a significantly lower locomotor activity relative to saline locomotor activity \( (p<0.05) \) (Figure 4-5). Activity of ligands that modulate both saline and drug-stimulated activity could be attributed to non-specific action on locomotion in general\(^{109}\). However, (+)-trans-PAT did not have any action on NMDA or amphetamine stimulated activity hence these effects on saline activity were not relevant. The ED\(_{50}\) (95% ± CL) of (−)-trans-PAT in MK-801 induced locomotor activity model was found to be 4.02 (3.18-5.08) mg/kg. The ED\(_{50}\) (95% ± CL) of (−)-trans-PAT in amphetamine induced locomotor activity was found to be 6.44 (4.57- 9.08)
mg/kg. There was no significant difference between the ED_{50}’s of (−)-trans-PAT in amphetamine and MK-801 induced hyperlocomotor assays.

The next enantiomer to be tested in these models was (−)-trans-m-Br-PAT. This enantiomer when administered prior to DOI demonstrated the ability to attenuate DOI induced HTR. Doses of 3, 5.6 and 10 mg/kg of (−)-trans-m-Br-PAT enantiomers resulted in a statistically significant lowering of DOI induced HTR (F_{6,30}=28.2; p<0.0001). The ED_{50} (95% ± CL) of (−)-trans-m-Br-PAT was found to be 2.67 (1.60-4.20) mg/kg (Data courtesy Dr. Clinton Canal). Higher doses of (−)-trans-m-Br-PAT, 10 mg/kg and 5.6 mg/kg, resulted in a significant decrease in MK-801 induced hyperactivity (F_{5,69}=16.93; p<0.0001). The ED_{50} (95% ± CL) of (−)-trans-m-Br-PAT at MK-801 hyperactivity model was found to be 4.41 (2.96-6.57) mg/kg. In contrast, only the highest dose of 10 mg/kg of (−)-trans-m-Br-PAT resulted in a decrease in amphetamine induced hyperactivity (F_{5,65}= 8.316; p<0.0001). The ED_{50} (95% ± CL) of (−)-trans-m-Br-PAT at amphetamine hyperactivity model was found to be 6.13 (1.94-19.40). There was again no significant difference between the ED_{50} values in MK-801 and amphetamine hyperactivity models (Figure 4-6). There was no significant difference between locomotor activity of 10 mg/kg dose of (−)-trans-m-Br-PAT alone and saline locomotor activity (Figure 4-5). Hence, (−)-trans-m-Br-PAT demonstrated ability to selectively modulate MK-801 and amphetamine hyperactivity and did not affect locomotor activity when administered alone.

Both (+) and (−)-trans-m-Cl-PAT enantiomers were tested for their potency to attenuate DOI induced HTR. Both these enantiomers were effective in attenuating DOI induced HTR. The (−)-trans-m-Cl-PAT enantiomer significantly decreased DOI induced HTR.
HTR at doses of 10, 5.6, 3 and 1 mg/kg ($F_{5.41}=40.62; \ p<0.0001$). In contrast, only the 10 and 5.6 mg/kg doses of (+)-trans-m-Cl-PAT enantiomer attenuated DOI induced HTR ($F_{4.38}=35.31; \ p<0.0001$) (Figure 4-7). The ED$_{50}$ (95% ± CL) of both (+) and (−)-trans-m-Cl-PAT enantiomers was found to be 7.23 (5.51-9.49) mg/kg and 2.19 (1.39-3.42) mg/kg respectively. The ED$_{50}$ value of (−)-trans-m-Cl-PAT was significantly lower than ED$_{50}$ value of (+)-trans-m-Cl-PAT enantiomer in HTR model. Only the (−)-trans-m-Cl-PAT enantiomer was effective at attenuating both MK-801 and amphetamine induced hyperactivity models. The (+)-trans-m-Cl-PAT was not effective at either MK-801 or amphetamine induced hyperactivity models. Both the 10 and 5.6 mg/kg doses of (−)-trans-m-Cl-PAT enantiomer significantly reduced MK-801 induced hyperactivity ($F_{5.73}=18.82; \ p<0.0001$). The ED$_{50}$ (95% ± CL) of (−)-trans-m-Cl-PAT in the MK-801 hyperactivity model was found to be 5.58 (4.43-7.04) mg/kg. In contrast, only the 10 mg/kg dose of (−)-trans-m-Cl-PAT decreased amphetamine induced hyperactivity ($F_{5.55}=13.14; \ p<0.0001$). The ED$_{50}$ (95% ± CL) of (−)-trans-m-Cl-PAT in the amphetamine hyperactivity model was found to be 4.86 (3.50-6.74) mg/kg (Figure 4-8). There was no significant difference between the ED$_{50}$ values of (−)-trans-m-Cl-PAT in amphetamine and MK-801 hyperactivity assays. There was a significant increase in (−)-trans-m-Cl-PAT locomotor activity as compared to saline locomotor activity ($p<0.005$) (Figure 4-5). Hence (−)-trans-m-Cl-PAT was not a selective modulator of amphetamine or MK-801 locomotor activity.

Both (+) and (−)-trans-p-Cl-PAT enantiomers were administered 10 minutes before DOI administration and resulted in a dose dependent attenuation of HTR. Both 10 and 30 mg/kg doses of (+)-trans-p-Cl-PAT resulted in a significantly lower HTR
relative to DOI alone ($F_{6,27}=15.66; p<0.001$). In contrast, only the 30 mg/kg dose of (−)-trans-\textit{p}-Cl-PAT resulted in a significantly lower HTR (Figure 4-9, right panel). The ED$_{50} \pm 95\%$ CL of these enantiomers was found to be 8.2 (5.4-12.4) and 20.1 (14.0-28.8) mg/kg for (+) and (−)-trans-\textit{p}-Cl-PAT respectively. These ED$_{50}$ values for (+) and (−)-trans-\textit{p}-Cl-PAT enantiomers were significantly different. These results were in conformation with the affinity and the potency results of these enantiomers observed in \textit{in vitro} binding and functional studies. A dose of 30 mg/kg of (+) and (−)-trans-\textit{p}-Cl-PAT enantiomer and the racemate of these compounds were administered orally (Figure 4-10). All the three dose combinations resulted in a significantly lower HTR as compared to DOI alone ($F_{3,16}=31.1; p<0.001$). The attenuated HTR response of (+)-trans-\textit{p}-Cl-PAT enantiomer was significantly different relative to (−)-trans-\textit{p}-Cl-PAT enantiomer ($p=0.038$). (Data of \textit{trans}-\textit{p}-Cl-PAT at HTR courtesy Dr. Drake Morgan)

Both (+) and (−)-trans-\textit{p}-Cl-PAT were then screened in MK-801 and amphetamine locomotor models. Both these enantiomers were tested at 3 mg/kg, 5.6 mg/kg and 10 mg/kg doses. None of these doses for both enantiomers resulted in a significant decrease in locomotion induced by administration of 0.3 mg/kg of MK-801 ($p>0.05$) (Figure 4-11). Similar results were obtained for both enantiomers when tested for their ability to attenuate amphetamine-induced (3 mg/kg) locomotion ($p>0.05$) (Figure 4-12). When administered alone 10 mg/kg doses of each enantiomer did not result in any significant difference in locomotor activity from saline (Figure 4-5). This data is in concordance with HTR response observed for (+)-trans-\textit{p}-Cl-PAT enantiomer. Both 5.6 mg/kg and 10 mg/kg doses did not result in attenuation of HTR, MK-801 and amphetamine induced locomotor activity. One explanation for this could be the
significantly lower potency of (+)-trans-p-Cl-PAT enantiomer at serotonin 5-HT$_{2C}$ receptors as compared to parent PAT and (−)-trans-m-Cl-PAT analogs. Alternatively, a lower ‘potency’ of antagonism at 5-HT$_{2A}$ receptors could be a reason for the lack of effect in MK-801 and amphetamine models. But comparison of pA$_2$ valued of (+)-trans-p-Cl-PAT and (−)-trans-m-Cl-PAT did not reveal any significant difference. Hence the lack of response to (+)-trans-p-Cl-PAT enantiomer at both MK-801 and amphetamine hyperactivity assays could be attributed to lower potency at 5-HT$_{2C}$ receptors.

**Summary of in vivo results.** These results indicate that both (+) and (−)-trans-p-Cl-PAT enantiomers are not effective at either MK-801 or amphetamine hyperactivity model (Table 4-1). Both (−)-trans-m-Cl-PAT and (−)-trans-m-Br-PAT enantiomers demonstrated significantly higher potency at HTR model compared to MK-801 and amphetamine hyperlocomotor model. All the (−)-trans enantiomers had a higher potency at MK-801 hyperactivity model than amphetamine hyperactivity model (not significantly different). The enantiomers (−)-trans-PAT, (−)-trans-m-Cl-PAT and (−)-trans-m-Br-PAT demonstrated the highest potency in the in vivo models. All of these enantiomers were also tested for their anorectic potential by our collaborator, Dr. Neil Rowland, in a mice model of compulsive or binge eating. These ligands dose-dependently decreased the amount of treats consumed in a 30-minute session. Hence, these analogs do not exhibit the weight gain side effects showed by clinically approved antipsychotics and also demonstrated a novel mechanism of action for modulating dopaminergic and glutamatergic neurotransmission disrupted in schizophrenia.
Figure 4-1. Clozapine decreases DOI induced HTR in a dose dependent manner. X indicates that the respective doses of clozapine attenuate DOI (1 mg/kg) HTR in a statistically significant manner ($p<0.05$).
Figure 4-2. Clozapine 1 mg/kg dose attenuates both MK-801 and amphetamine (Amp) induced locomotion. A) Shows the effect of clozapine on MK-801 induced locomotion. B) Shows effect of clozapine on amphetamine-induced locomotion. X indicates statistically significant reduction in stimulated locomotion of MK-801 or amphetamine alone compared to pretreated clozapine.
Figure 4-3. Attenuation of MK-801 induced locomotor activity by (+) and (−)-trans-PAT analogs. PAT analogs and their respective doses are represented on the abscissa. X indicates statistically significant difference between pretreated PAT relative to MK-801 alone induced locomotor activity.
Figure 4-4. Attenuation of amphetamine stimulated locomotor activity by PAT enantiomers. X indicates significant difference between amphetamine (3 mg/kg) dose and the corresponding pretreated PAT dose response.
Figure 4-5. Effect of highest dose of various ligands on locomotor activity. X indicates statistically significant changes in locomotor activity of drug alone compared to saline locomotor activity.
Figure 4-6. Attenuation of MK-801 and amphetamine hyperactivity by (−)-trans-m-Br-PAT. X indicates significant difference in locomotor activity at that pretreated dose compared to the corresponding MK-801 or amphetamine induced hyperactivity.

Figure 4-7. Demonstrates the attenuation of DOI induced HTR by (+) and (−)-trans-m-Cl-PAT. X indicates significant decrease in the number of HTR by the respective dose of (+) or (−)-trans-m-Cl-PAT relative to DOI 1mg/kg dose.
Figure 4-8. Attenuation of MK-801 and amphetamine hyperactivity by (−)-trans-m-Cl-PAT. X indicates significant difference in locomotor activity at that dose compared to the corresponding MK-801 or amphetamine induced hyperactivity.

Figure 4-9. Attenuation of HTR by (+) and (−)-trans-p-Cl-PAT analogs. Demonstrates the dose dependent decrease of DOI induced HTR by both (+) and (−)-trans-p-Cl-PAT.
Figure 4-10. Effect of oral administration of (+) and (−)-trans-p-CI-PAT analogs 20 minutes prior to DOI. * Indicates differences from saline treatment and ** indicated differences from saline and each other.
Figure 4-11. Effect of (+) and (−)-trans-p-Cl-PAT on MK-801 (0.3 mg/kg) induced locomotor activity. Both enantiomers did not significantly affect MK-801 induced locomotor activity. MK-801 induced locomotor activity was significantly higher than saline and (+) and (−)-trans-p-Cl-PAT alone locomotor activity.
Figure 4-12. Effect of (+) and (−)-trans-p-Cl-PAT on amphetamine (3 mg/kg) induced locomotor activity. Both enantiomers did not significantly affect MK-801 induced locomotor activity. MK-801 induced locomotor activity was significantly higher than saline and (+) and (−)-trans-p-Cl-PAT alone locomotor activity.
Table 4-1. Efficacy of PAT analogs, ED$_{50}$ (95% ± CL) mg/kg, at *in vivo* models of schizophrenia.

<table>
<thead>
<tr>
<th>PAT analog</th>
<th>HTR</th>
<th>MK-801 induced hyperlocomotion</th>
<th>Amphetamine induced hyperlocomotion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clozapine</td>
<td>0.23 (0.18-0.28)</td>
<td>0.25 (0.1-0.64)</td>
<td>0.27 (0.16-0.44)</td>
</tr>
<tr>
<td>(-)-trans-PAT</td>
<td>4.02 (3.18-5.08)</td>
<td>4.41 (2.96-6.57)</td>
<td>6.44 (4.57-9.08)</td>
</tr>
<tr>
<td>(-)-trans-m-Br-PAT</td>
<td>2.67 (1.6-4.2)</td>
<td>5.58 (4.43-7.04)</td>
<td>6.13 (1.94-19.40)</td>
</tr>
<tr>
<td>(-)-trans-m-Cl-PAT</td>
<td>2.19 (1.39-3.42)</td>
<td>6.13 (1.94-19.40)</td>
<td>4.86 (3.50-6.74)</td>
</tr>
<tr>
<td>(+)-trans-p-Cl-PAT</td>
<td>8.2 (5.4-12.4)</td>
<td>Not active</td>
<td>Not active</td>
</tr>
<tr>
<td>(-)-trans-p-Cl-PAT</td>
<td>20.1 (14.0-28.8)</td>
<td>Not active</td>
<td>Not active</td>
</tr>
</tbody>
</table>
CHAPTER 5
CONCLUSION

In this dissertation, we evaluated the structure activity relationship of various analogs of 4-phenyl-2-N,N-dimethyl-1,2,3,4-tetrahydronaphthalene-2-amines. These analogs targeted the 5-HT\textsubscript{2} receptors, selectively activating 5-HT\textsubscript{2C} receptors and were antagonists/inverse agonists at 5-HT\textsubscript{2A} and 5-HT\textsubscript{2B} receptors. Substitutions were made to this analog to enhance the affinity, potency and selectivity at 5-HT\textsubscript{2} receptors. \textit{In silico} docking studies assisted in identifying the molecular determinants that assisted in binding of these ligands at individual 5-HT\textsubscript{2} receptors.

\textit{N, N-dimethyl-4-(2- or 3- or 4-substituted-phenyl)-1,2,3,4-tetahydro-2-naphthalene-}amines analogs had substitutions at the \textit{ortho-}, \textit{meta-} and \textit{para}-position of the pendant phenyl ring. The general trend that was observed was a relatively higher affinity of sterically larger substituents at 5-HT\textsubscript{2C} receptors compared to 5-HT\textsubscript{2A} receptors. Hence, larger sterics favored preferential binding to 5-HT\textsubscript{2C} receptors.

Reversed stereoselectivity of \textit{para}-substituted enantiomers was due to favorable amino acid interactions in binding pocket (S5.43) assisting the \textit{(2R,4S)-(+)-trans-p-Cl-PAT} configuration. \textit{(2S,4R)-(−)-trans-p-Cl-PAT} configuration lacked this interaction with S5.43 and hence showed decreased affinity at both 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors. \textit{N,N-dimethyl-4-(3-substituted-phenyl)-6,7-substituted-1,2,3,4-tetrahydro-2-naphthalene-}amines had substituents at both tetrahydronaphthalene ring and pendant phenyl ring. These enantiomers did not result in significant enhanced affinity or potency at 5-HT\textsubscript{2} receptors relative to \textit{meta}-substituted-PAT analogs. But the tetrahydronaphthalene substituted analogs identified a key region of the binding pocket that was different between the 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors. There is an alanine amino acid at position
5.46 in 5-HT$_{2A}$ receptors and a serine (S5.46) at the same position in 5-HT$_{2C}$ receptors. This difference resulted in 14-fold selectivity for binding at 5-HT$_{2A}$ receptors over 5-HT$_{2C}$ receptors for (+)-trans-6-OH-7-Cl-PAT enantiomer. The chemical space occupied by the ligand can be exploited to further synthesize ligands that are more selective between these receptors.

*In vivo* studies corroborated the *in vitro* studies and confirmed the stereoselectivity of unsubstituted and *trans*-4-(meta-Cl and meta-Br)-PAT analogs. They demonstrated reversed stereoselectivity when *trans*-4-(para-substituted)-PAT analogs were evaluated. These results demonstrated that *in vitro* pharmacology can predict the preclinical potential of PAT ligands as antipsychotics. The higher affinity and more potent (2S,4R)-(−)-*trans*-4-(meta-Cl and meta-Br)-PAT analogs demonstrated highest potency in these models. These results confirm the efficacy of selective 5-HT$_{2C}$ agonists as potential therapeutic ligands for schizophrenia.
APPENDIX A

AFFINITY OF 4-(META- OR PARA-SUBSTITUTED)-PATs at HISTAMINE H1 GPCRs

Histamine H₁ receptors share a 44% transmembrane domain sequence similarity with 5-HT₂ receptors¹³⁹,¹⁴⁰. This similarity in sequence results in ligands targeting 5-HT₂ receptors also binding to H₁ receptors. Some meta- and para-PAT analogs that were analyzed at 5-HT₂ receptors were also screened for affinity at H₁ receptors. (⁺) and (⁻)-Trans-m-CF₃-PAT enantiomers had an affinity of 4100 ± 160 nM and 230 ± 27 nM respectively at H₁ receptors (p<0.0001). Similar to the trend at 5-HT₂ receptors the (⁻)-trans-m-CF₃-PAT enantiomer had higher affinity at H₁ receptors. The next set of enantiomers (⁺) and (⁻)-trans-m-NO₂-PAT had an affinity of 1000 ± 80 nM and 800 ± 100 nM respectively at H₁ receptors. There was no significant difference between the affinities of these enantiomers. Comparing affinity of (⁺)-trans-m-NO₂-PAT with (⁺)-trans-m-CF₃-PAT revealed that (⁺)-trans-m-NO₂-PAT enantiomer had a 4-fold higher affinity (p<0.0001). In contrast, (⁻)-trans-m-CF₃-PAT enantiomer had a 3-fold higher affinity relative to (⁻)-trans-m-NO₂-PAT (p=0.007). The affinities of both (⁺) and (⁻)-trans-m-NO₂ and CF₃-PAT enantiomers are significantly higher at all the 5-HT₂ receptors. Hence these enantiomers bind preferentially at 5-HT₂ receptors decreasing off target binding.

The next series of enantiomers that were tested at H₁ receptors had substituents at para-position. (⁺) and (⁻)-Trans-p-CF₃-PAT enantiomers had an affinity of 230 ± 35 nM and 5100 ± 360 nM respectively (p<0.0001). These enantiomers had the same trend as other para-substituted enantiomers at 5-HT₂ receptors, in that they demonstrate reversed stereoselectivity. Comparing the meta- versus para-trifluoromethyl substituted analogs revealed significant higher affinity for (⁺)-trans-p-CF₃-PAT and (⁻)-trans-m-CF₃-PAT.
PAT enantiomers relative to (+)-trans-m-CF₃-PAT and (−)-trans-p-CF₃-PAT enantiomers respectively at H₁ receptors (p<0.0001). There was no significant difference in affinity of (+)-trans-p-CF₃-PAT at H₁ receptors relative to its affinity at all other 5-HT₂ receptors. The last analog that was analyzed at H₁ receptors was a racemate of (±)-trans-p-NO₂-PAT. The affinity of this enantiomer at H₁ receptors was found to be 680 ± 31 nM. This racemate had a significantly higher affinity at H₁ receptors relative to other 5-HT₂ receptors (F3,9=93.61; p<0.0001).
APPENDIX B
LIST OF PUBLICATIONS RESULTING FROM THE WORK IN THIS DISSERTATION


Sakhuja R, Kondabolu K, Canal CE, Cordova-Sintjago T, Booth RG. Synthesis and binding affinities of trans-m-substituted phenylaminotetralins (PAT) against 5-HT2A and 5-HT2C serotonin receptors.

Cordova-Sintjago T, Sahuja R, Kondabolu K, Villa N, Canal CE, Booth RG. Molecular determinants for binding and function at serotonin 5-HT$_{2A}$ and 5-HT$_{2C}$ GPCRs: Ligand docking, molecular dynamic and QM studies. 52nd Sanibel symposium, 2012.

LIST OF REFERENCES


64. Wang, R. Y.; Liang, X. M100907 and clozapine, but not haloperidol or raclopride, prevent phencyclidine-induced blockade of NMDA responses in pyramidal neurons of the rat medial prefrontal cortical slice. *Neuropsychopharmacology* 1998, 19, 74-85.


66. Hutcheson, J. D.; Setola, V.; Roth, B. L.; Merryman, W. D. Serotonin receptors and heart valve disease-It was meant 2B. *Pharmacol Ther* 2011.


83. Kristiansen, K.; Kroeze, W. K.; Willins, D. L.; Gelber, E. I.; Savage, J. E.; Glennon, R. A.; Roth, B. L. A highly conserved aspartic acid (Asp-155) anchors the terminal amine moiety of tryptamines and is involved in membrane targeting of the 5-HT(2A) serotonin receptor but does not participate in activation via a "salt-bridge disruption" mechanism. *J Pharmacol Exp Ther* 2000, 293, 735-46.


94. Canal, C. E.; Morgan, D. Head-twitch response in rodents induced by the hallucinogen 2,5-dimethoxy-4-iodoamphetamine: a comprehensive history, a re-evaluation of mechanisms, and its utility as a model. Drug Test Anal 2012, 4, 556-76.


108. Jentsch, J. D.; Roth, R. H. The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology* 1999, 20, 201-25.


125. Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 1973, 22, 3099-108.


BIOGRAPHICAL SKETCH

Krishnakanth Kondabolu was born in 1987, to Harischandra Prasad and Durga Kondabolu, in Andhra Pradesh, India and has a younger brother Srikanth. He attended Smt. SulochanaDevi Singhania School in Mumbai, Maharashtra. After graduation, he was accepted in to one of the premier universities of India, Manipal University. He graduated in 2008 with a Bachelor of Pharmacy degree from Manipal College of Pharmaceutical Sciences. As an undergraduate he took interest in the drug discovery process, which prompted him to pursue doctoral degree in the Department of Medicinal Chemistry at University of Florida in 2008. He was mentored by Dr. Raymond Booth and completed his Doctor of Philosophy from University of Florida in May 2013.